Chapter 2

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Crop plants

The crops selected for the evaluation of effect of the effluent are *Abelmoschus esculentus* (L.) Monech., *Vigna unguiculata* (L.) Walp, *Vigna mungo* (L.) Hepper, *Solanum melongena* L. and *Cucurbita moschata* Duchesne. The certified seeds of the test crops (Plate-2.1; A-E) were collected from the Kottayam District Agricultural Farm, Kozha.

2.1.1.1 Crops selected for both laboratory and field study

A. *Abelmoschus esculentus* (L.) Monech.

*Abelmoschus esculentus* (L.) Monech. is commonly called ladies finger, okra or bhindi which belongs to the family Malvaceae. It is an economically important vegetable crop grown in tropical and sub-tropical parts of the world. This crop is grown commercially in India and is suitable for cultivation as a garden crop as well as on large commercial farms. India ranks first in the world with 3.5 million tons of okra (70% of the total world production) produced from over 0.35 million ha land (FAOSTAT, 2008).

Okra is quite popular in India because of easy cultivation, dependable yield and adaptability to varying moisture conditions. Okra grows in all types of soils and thrives best in a moist, friable soil and needs pre-sowing and post-sowing irrigation for optimum growth (Kochhar, 1986). It is slightly tolerant to acidity and the optimum pH range is between 6 and 8. Okra is mainly propagated by seeds and has duration of 90 - 100 days. Okra plants continue to flower and to fruit for an indefinite time, depending upon the variety, the season and soil moisture and fertility.
Okra is cultivated for its fibrous fruits or pods containing round, white seeds. The fruits are harvested when immature and eaten as a vegetable. The roots and stems of okra are used for cleaning the cane juice from which brown sugar is prepared (Chauhan, 1972). Its ripe seeds are roasted, ground and used as a substitute for coffee in some countries. Mature fruits and stems containing crude fiber are used in the paper industry. Extracts from the seeds of the okra is viewed as alternative source for edible oil. Okra provides an important source of vitamins, calcium, potassium and other mineral matters which are often lacking in the diet of developing countries (IBPGR, 1990). Okra is said to be very useful against genito-urinary disorders, spermatorrhoea and chronic dysentery (Nandkarni, 1927). Its medicinal value has also been reported in curing ulcers and relief from hemorrhoids (Adams, 1975).

B. Vigna unguiculata (L.) Walp

Cowpea (Vigna unguiculata L. Walp.) is a nutritive vegetable and pulse rich in protein which belongs to the family Fabaceae. It grows in tropical, subtropical and temperate regions of Asia, Africa, and Latin America. Cowpea is one of the most ancient pulse crops known to man. Pulses are the basic ingredient in the diet of a vast majority of the Indian population, as they provide a perfect mix of vegetarian protein compound of high biological value when supplemented with cereal (IBPGR, 1983)

Cowpeas can thrive on highly acid to neutral soils but they are less well adapted to alkaline soils. V. unguiculata is a herbaceous, prostrate, climbing or sub erect annual plant, growing 15-80 cm high. Growth habit is either determinate or indeterminate. Cowpea's high protein content, its adaptability to different types of soil and intercropping systems, and its ability to improve soil fertility and prevent erosion makes it an important economic
crop in many developing regions. The sale of the stems and leaves as animal feed during the dry season also provides a vital income for farmers (Duke and James 1990).

The protein found in cowpea is, similar as the one from other legumes, rich in the essential amino acids lysine and tryptophan (Timko and Singh 2008) Minerals and vitamins are the other nutritional important constituents of the cowpea seeds. Beans and seeds of V. unguiculata are eaten fresh as a green vegetable, dried, fried, roasted, boiled and also in the form of snack. Its flour could be used in soup and dhal and to make bread also. Animal feed is another use of these beans in many developing countries like India and makes the soil more fertile when it is grown. It also has medicinal values. The leaves and seeds are applied as a poultice to treat swellings and infections. Leaves are chewed to treat tooth ailments. Powdered and carbonized seeds are applied on insect stings. The root is used as an antidote for snakebites and to treat epilepsy, chest pain, constipation and dysmenorrheal. The unspecified plant parts are used as a sedative in tachycardia and against various pains (Brink and Belay, 2006). The seed is diuretic and used to strengthen the stomach. When boiled and eaten as a food it is considered to destroyed worms in the stomach (Chopra et al., 1986).

2.1.1.2 Crop seeds selected for only laboratory study

C. Vigna mungo (L.) Hepper

Vigna mungo (L.) Hepper is commonly called black gram, which belongs to the family Fabaceae. Black gram originated in India, where it has been in cultivation from ancient times and is one of the most highly prized pulses of India. It is an erect, sub erect or trailing, densely hairy, annual herb. The tap root produces a branched root system with smooth, rounded nodules.
Chapter II

The pods are narrow, cylindrical and up to six cm long. It is nutritious and is recommended for diabetics, *V. mungo* is used in traditional Indian Ayurveda medicine (Kochhar, 1986). Pharmacologically, extracts have demonstrated immuno stimulatory activity. Black gram is an annual food legume. It shows both erect and crawling growth habit.

D. *Solanum melongena* L.

*Solanum melongena* L. is commonly called brinjal, which is a native of India. It belongs to the family Solanaceae. Brinjal requires a long warm growing season and is one of the most common vegetables grown thought the country. Being a long standing crop it needs good dose of manure and timely irrigation. Different varieties of the plant produce fruit of different size, shape and color, though typically purple. The brinjal has got ayurvedic medicinal properties and white brinjal is said to be good for diabetic patients (Kochhar, 1986).

E. *Cucurbita moschata* Duchesne

Pumpkin is the fruit of the species *Cucurbita moschata* which belongs to the family Cucurbitaceae. Pumpkins are grown all around the world for a variety of reasons ranging from agricultural purposes to commercial and ornamental sales. Pumpkins are a warm-weather crop and may suffer if there is a lack of water or because of cold temperatures pumpkins are widely grown for commercial use, and are used both in food and recreation. They are monoecious having both male and female flowers on the same plant. The female flower is distinguished by the small ovary at the base of the petals. These bright and colorful flowers have extremely short life spans and may only open for as short a time as one day. The medicinal properties of pumpkin
include anti-diabetic, antioxidant, anti-carcinogenic, and anti-inflammatory (Kochhar, 1986).

Plate – 2.1: Surface view of seeds used in the present study for evaluating the toxicity of effluent

A. Seeds of *Vigna unguiculata* (L.) Walp
B. Seeds of *Vigna mungo* (L.) Hepper
C. Seeds of *Cucurbita moschata* Duchesne
D. Seeds of *Abelmoschus esculentus* (L.) Moncheh
E. Seeds of *Solanum melongena* L.
Chapter II

2.1.2 Effluent

2.1.2.1 Site of the collection of effluent

The rubber industry is one of the most important industries of Kottayam district. A field survey conducted in Kottayam revealed that the number of centrifuged latex factories established in this district was about 12, which are registered at present in this district making use of centrifugation process using rubber latex (http://rubberboard.org.in/Processor.asp).

The effluent for the present study was collected from centrifuged latex concentration unit Neerackal Latex Pvt. Ltd. situated in Kaduthuruthy panchayath, Kottayam district (Plate-2.2; A-C). At present two centrifuged latex units are established in Kaduthuruthy panchayath itself and another unit in the nearby panchayath Kuravilangad. The panchayath is located in latitude 9.7500°N, longitude 76.5000°E. The area of the panchayath is 36.31sq.km with a population of 30720 (Plate-2.2; A-C). The centrifugal latex factory in the panchayath, Neerackal Latex Pvt. Ltd having an effluent output of 45000 lit./day.

The meteorological data associated to the area in which the factory situated, is obtained from Regional Rubber Research Institute of India. The maximum temperature during the period of collection of effluent was an average of 32\(^0\)C and minimum temperature was within 22-23\(^0\)C. The relative humidity was between 77-79 percentage and rain fall was varied between 218-298mm (Table.2.1).
Plate – 2.2: Location of collection of effluent.

A. State of Kerala showing 14 districts. The collection site of effluent is located in Kottayam district marked in the red square.

B. Map of Kaduthuruthy panchayath showing exact location of Neerackal Latexd Pvt. Ltd.

C. An enlarged view of Kottayam district where location of Neerackal Latexd Pvt. Ltd. is marked with star (Kaduthuruthy).
Table 2.1: Meteorological data of Kottayam district

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<th>May</th>
<th>Jun</th>
<th>Jul</th>
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<th>Oct</th>
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<td>24.2</td>
<td>23.3</td>
<td>22.9</td>
<td>23.4</td>
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<td>89.0</td>
<td>255.6</td>
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<td>76</td>
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2.1.2.2 Profile of Neerackal Latex Pvt. Ltd.

The factory was established in 1999 and started the manufacturing of the centrifuged latex in 2000. They are India’s 1’st centrifuging unit to use the decanted centrifugal method effluent treatment plant to maintain complete pollution free environment. The major product of the company 60% centrifugal latex HA, 52% Centrifugal latex MS barrels, flexi bags and ISO tanks. The output of the factory is 60000liters of latex per day and the effluent generation is 45000lit./day and the treated latex effluent were used for irrigation purposes and the rest part discharged into nearby water bodies. The effluent production process is as shown in the Chart.2.1. The various processing units in the factory are shown in Plate 2.3(A-F) and Plate 2.4(A-F).

Chart.2.1. The effluent production process of Neerackal Latex Pvt. Ltd
Plate – 2.3: The various processing units in the factory

A. Latex centrifuging unit
B. Latex collection tank
C. Centrifuging machine
D. Skim serum storage tank
E. Rubber trap and equalization tank
F. Chemical mixing channel (lime+electrolyte)
Plate – 2.4: The various processing units in the factory

A. Primary settling tank  
B. Sludge drying bed  
C. Aeration tank.  
D. Secondary settling tank  
E. Final collection tank  
F. Final outlet
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Ammoniated field latex containing 30-33% rubber is concentrated by centrifuging to 60% rubber and skim latex containing 3.5-6% rubber. High speed centrifuging machines used for the concentration of field latex is Alpha Laval Make machine. In the centrifuging process of latex the centrifugal force brings about the separation of rubber particles. The rotating mass of the ammoniated field latex is broken up into a number of thin conical shells within the bowl rotating at a speed of 6000rpm whereby individual rubber particles tend to separate into a layer surrounding the axis of rotation leaving behind an outer layer (skim) having a comparatively lower rubber content. The dry rubber content of the centrifuged latex is around 60%. About 85%–93% of the total rubber in the field latex is separated into the concentrated fraction. The concentrated latex is stored in drums and marketed. To recover the rubber in the skim latex containing 0.8% ammonia is coagulated with 98% sulphuric acid to bring down the pH to 4.5 and the serum left out is drained off and is known as skim serum effluent. This is collected in skim rubber traps for rubber recovery before the waste water treatment. Apart from the skin serum the water requirement is for cleaning the latex storage tanks once a week, washing the barrels and floor, washing the bowls of the centrifuging machine twice in a shift and coagulation of skim latex. The washings are collected in separate rubber trap for rubber recovery and is called composite effluent

2.1.2.3 Process of effluent treatment

The effluent treatment process of Neerackal latex Private Ltd. Kottayam, involves different stages (Chart.2.2) of treatments including physicochemical treatment and biological treatment. The plant is custom designed based on the specific application requirement of the effluent of the industry. The physical treatment process is rubber trap and is the first unit of treatment system to remove suspended rubber in the equalization tank and
send for neutralization and chemical treatment by alum and iron salt. From this, effluent sent to primary settling tank in which sludge is removed with the aid of a decanter. Following this primary treatment, effluent sent to aeration activated sludge type biological treatment. After this, effluent reached in the effluent collection tank through secondary settling tank and finally disposed.

**Chart.2.2. Effluent treatment process of Neerackal latex Private Ltd.**
2.1.2.4 Collection of effluent

Effluent was collected in 35 litter polythene cans at an interval of 3 months. Untreated effluent was collected from Untreated Skim serum effluent tank and treated effluent was collected from the effluent outlet of the factory. The effluents were placed under refrigeration till use.

2.2 Methods of analysis of effluent

The effluent collected were analysed for physical, chemical and biological parameters. The analysis was carried out in regular intervals of 3 months throughout the study period. For various parameters analyzed the mean and standard deviation were computed and presented.

2.2.1 Physico-chemical parameters analysed

2.2.1.1 Analysis of effluent

The physico-chemical characteristics of the effluents analysed are pH, Oil and grease, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Electrical conductivity (EC), Dissolved solids, Suspended solids, Ammoniacal nitrogen, Total nitrogen, Chloride, Sulphate, Sulphide, Potassium, Phosphate, Sodium, Copper, Iron, Calcium, Magnesium, Zinc, Boron, Chromium, Cadmium, Nickel and Lead. The analysis was carried out in the Central Lab of Rubber Research Institute of India.

The methodologies adopted for the analysis of various parameters were presented in Table 2.2.
Table–2.2: The methodologies adopted for the analysis of various parameters

<table>
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<td>Chemical Oxygen Demand (COD)</td>
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<td>Suspended solids</td>
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<td>26</td>
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2.2.1.2 Analysis of soil

The soil analysis was carried out before and after harvesting of the crop plants. The soil was bulked, mixed thoroughly, air dried, and sieved through a two mm sieve to remove debris. The parameters analysed include pH, Conductivity (ds/m), Organic carbon (%), Phosphorous (kg/ha), Potassium (kg/ha), Calcium (mg/kg), Magnesium (mg/kg), Sulphur (mg/kg), Copper (ppm), Iron (ppm), Zinc (ppm) Manganese (ppm) and Boron (mg/g). The analysis was carried out in the Radiotracer Laboratory at Kerala Agricultural University. Detailed procedure of analysis is presented below.

A. Soil pH

The pH of the soil was determined in a 1:2.5 (10g soil in 25 ml distilled water) soil water suspension, potentiometrically using a pH meter (Jackson, 1958).

B. Electric conductivity

EC. was estimated in the supernatant liquid of the soil water suspension (1:2.5) used for pH estimation after 24 hours of settling, with the help of a conductivity meter (Jackson, 1958).

C. Organic carbon

Organic carbon of the soil was estimated by wet digestion method (Walkley and Black, 1934).

D. Available phosphorus

Available phosphorus in the soil samples were extracted using Bray No.1 reagent (Bray and Kurtz, 1945) and estimated colorimetrically by reduced Molybdate Ascorbic acid blue colour method (Watanabe and Olsen, 1965) using a spectrophotometer (Model: Genesys 20).
E. **Available potassium**

Available potassium in the soil samples were extrac ted using neutral normal ammonium acetate and its content in the extract was estimated by flame photometry (Jackson, 1958).

F. **Available micronutrients (Fe, Cu, Mn, and Zn) in the soil**

Available micronutrients (Fe, Cu, Mn, and Zn) in the soil samples were extracted using 0.1M HCl (Sims and Johnson, 1991). 4g soil with 40 ml of 0.1M HCl was shaken for 5 minutes. It was filtered through Whatman No. 1 filter paper and the filtrate was collected and analysed for Fe, Cu, Mn, and Zn using Parkin Elmer Atomic Absorption Spectrophotometer (Model: Analyst 400).

2.2.2 **Biological parameters - Phytoplankton**

Algal samples were collected from the effluent tank. The samples were collected along with the effluent sample and brought to the laboratory and are preserved using an algal preservative (John, 2008). From temporary micro preparation, algal flora is observed under research microscope photographs taken (Camera model: E4500 JPEG image) and identified using standard text books. (Fritsch, 1965; Desikachary, 1959; Desikachary *et al.*, 1990; Krishnamurthy, 2000; Sarode and Kamat, 1984).

2.2.3 **Cytological parameters analysed**

2.2.3.1 **Root growth inhibition test**

Approximately equal sized onion bulbs were bought commercially. Before initiating the test, both thin outer skins of the bulbs and the dry bottom portion were removed without destroying the primordial roots. To account for a number of bulbs in the population that would be naturally slow or poor growing, four replicate bulbs were used for each test sample and control
(distilled water) and the best bulbs were chosen for examination (Rank and Nielsen, 1993).

The growth inhibition assay was performed by exposing the root primordia to different concentrations of effluent (Rank, 2003). *Allium cepa* was exposed for 96 hours to different dilutions of the industrial effluents as follows:

- **Treated effluent:** 0, 5, 10, 20, 30, 40, 60, 80 and 100%
- **Non-Treated effluent:** 0, 2, 5, 10, 15, 20, 25 and 30%

Each concentration was set-up in 3 replicates. The base of each of the bulbs was suspended on the effluents inside 20mL test tube. The test solutions were replaced every 24 h with fresh solutions. At the termination of exposure period, the roots of 3 onion bulbs with the best growth at each concentration were removed with a forceps and their lengths measured (in cm) with a scale.

### 2.2.3.2 Genotoxicity assay

For the evaluation of induction of chromosomal aberration, four onion bulbs were suspended on 20, 40, 60, 80, 100% concentrations (v/v) of each of the effluents and the control for 48 hours at the end of which root tips from these bulbs were cut and fixed in ethanol: glacial acetic acid (3:1, v/v). These were hydrolyzed in 1N HCl at 60°C for five minutes after which they were washed in distilled water. Two root tips were then squashed on each slide, stained with aceticarmine for 10 min and cover slips carefully pressed on it to exclude air bubbles. The cover slips were sealed on the slides with clear fingernail polish as suggested by Grant (1982). This is to prevent drying out of the preparation by the heat of the microscope (Sharma, 1983). Six slides were prepared for each concentration and the control out of which five were
analyzed at ×100 magnification (Trinocular Research Microscope) for induction of chromosomal aberrations.

The mitotic index was calculated as the number of dividing cells per 1000 observed cells (Fiskesjo, 1985, 1997).

\[
\text{Mitotic Index (\%)} = \frac{\text{No. of dividing cells}}{\text{Total No. of cells}} \times 100
\]

The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total dividing cells scored at each concentration (Bakare et al., 2000).

2.2.4 Effect of effluent on plant species

Effect of effluent was tested on crop plants under laboratory conditions and field conditions. Laboratory experiments were carried out by treating the seed materials using different concentrations of untreated and treated effluent and field evaluation was conducted by irrigating the crops using different concentrations of the treated effluent.

2.2.4.1 Laboratory studies

Laboratory studies were carried out at the research laboratory, Department of Botany, St. Thomas College, Pala, Kottayam, Kerala. The concentration of the untreated effluent used for the evaluation was 5, 10, 20 and 25 percent. The concentration of the treated effluent used for the evaluation was 25, 50, 75 and 100 percent. The dilutions were made with distilled water of required quantity.

The seeds of the crop plants, *Abelmoschus esculentus* (L.) Monch., *Vigna unguiculata* (L.) Walp, *Vigna mungo* (L.) Hepper, *Solanum melongena* L. and *Cucurbita moschata* Duchesne were treated with the respective
concentrations of the effluent for 24 hours and placed in moist filter paper lined petridishes. Ten seeds were inoculated in each petridish. The seeds were allowed to germinate. In studies with untreated effluent, root growth in *A. cepa* and germination percentage of crop seeds was evaluated. The observations on germination and seedling morphology were carried out with respect to different concentrations of treated effluent. Observations were taken from 10 day old seedlings.

### 2.2.4.1.1 Parameters of crop plants studied for evaluation.

**A. Germination percentage**

Protrusion of radicle as well as emergence of cotyledons through the seed coat was taken as the criteria for germination. Percentage of germination was expressed on the basis of the total number seeds tried for germination (Vilasini, 1978).

Seeds showing the signs of germination were counted in each treatment and control and percentage was estimated as

\[
\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds placed}} \times 100.
\]

**B. Root length**

Root length was measured from 10 randomly selected seedlings of each concentration of treatment and control. It was measured from the tip of primary root to base of hypocotyle and mean root length was expressed in centimeters.

**C. Shoot length**

Shoot lengths were measured from 10 randomly selected seedlings of each concentration of treatment and control. The shoot length was measured
from the base of primary leaf to base of hypocotyle and mean shoot length was expressed in centimetres.

D. Total length

By adding the shoot and root lengths of already selected ten seedlings the seedling length was calculated and expressed as mean seedling length in centimetre.

E. Root shoot ratio

Root/Shoot ratio in terms of length was calculated using the data of already selected ten seedlings.

F. Phytotoxicity

The percent phytotoxicity was calculated following Chou and Muller (1972).

Percent Phytotoxicity = \{(Radicle length of the control – Radicle length of the treated) / Radicle length of the control\} x 100.

G. Vigour index

Vigour index was computed by adopting the following formula as suggested by Abdual Baki and Anderson (1973) and expressed in number.

\[
SVI = \text{Germination} \% \times \text{Seedling length (cm)}
\]

H. Germination Index

Germination index was computed by adopting the following formula as suggested by Zucconi et al., 1981

Germination index = % seed germination x % root length elongation / 100
% root elongation = (Average root length of the treated – root length of control) x 100
I. **Fresh weight and dry weight measurements**

For fresh weight and dry weight measurement the plant parts were blotted and wrapped separately in pre weighed labeled aluminium foils. Fresh weight of the sample was determined by weighing in a digital balance. For dry weight determination, the samples after fresh weight measurement is kept in an oven maintained at 80°C. After 48h the samples were transferred to desiccators, allowed to cool and weighed. The samples were again kept back in an oven reweighed as described above at regular intervals until the weights become constant (Evans and Bhatt, 1977).

J. **Percentage of moisture content**

Percentage moisture content was calculated on the basis of fresh weight basis \(\frac{(\text{fresh weight} - \text{dry weight})}{\text{fresh weight}}\) (Johnson et al., 1997).

2.2.4.2 **Field studies**

The crops for field studies were selected based on the significance and by evaluating the preliminary laboratory studies with the effluent on the five crop seeds. Field studies were conducted to find out the effect of different concentration of treated effluent on the two selected crops *A. esculentus* (L.) Monech. and *V. unguiculata* (L.) Walp. The top soil (loam), sand and cow dung mixture in the ratio of 1:1:1 was used as potting medium. 5kg of the potting mixture was weighed into individual polythene bags measuring 32x24cm in size. The experiment was laid out in a completely randomized design (CRD), using five (5) treatments namely 0% (control) 25%, 50 %, 75% and 100% of effluent. Each treatment was replicated six times using six polythene bags per treatment – giving a total of 30 polythene bags for each crop. Each bag was labeled with the required treatments. Four seeds of each crop were planted per bag. The seedlings were thinned to one plant per bag at
two weeks after planting. Weeds were eliminated by hand picking. Studies were carried out under natural atmospheric conditions to simulate actual field conditions. The soils were irrigated daily with 500ml of 25%, 50%, 75% and 100% treated effluent from centrifugal rubber latex factory. Control pots were similarly irrigated with water only. Spacing of the pots and other filed management were according to the Package of Practices Recommendations (Crops), KAU (2002). Experiment lasted till the harvest of the crop. Observations on the various morphological parameters were carried out and the data for field evaluation were collected pertaining the effect of different concentration of effluent on morphological, anatomical, physiological and biochemical level on the crop plant.

### 2.2.4.2.1 Morphological

Morphological parameters were measured at two growth stages in the life cycle, vegetative and flowering.

**A. Vegetative**

During the vegetative growth phase, plant height and number of leaves are noted.

- **a) Plant height**
  
  The height of randomly selected 6 plants was measured in cm from each treatment and control on 30th day for *V. unguiculata* and on 30th and 90th day after sowing for *A. esculentus*. Mean and standard deviation are computed and tabulated.

- **b) Number of leaves**
  
  The number of leaves on 50th day for *V. unguiculata* and 40th day after sowing for *A. esculentus* were noted from 6 plants from control and treated population.
c) **Leaf area**

Area of leaves was determined by graph paper method. 5 individual leaves from each treatment and control were placed on a graph paper and outlines were drawn carefully just along the margins. Compute the area by counting the area in the graph paper within the traced margin.

**B. Flowering**

When the plants began to flower the following parameters were observed up to the harvest period.

a) **Days to flower**

Days taken to flower were noted in each of the plants with respect to the date of sowing. Mean was computed for presentation

b) **Floral characteristics**

Floral characteristics like size of the flower; Pollen sterility and germination were noted from samples collected from the treated and control population.

c) **Flower size**

Flower size was documented by measuring the length of 5 flowers collected at random from control and treated plants.

d) **Pollen sterility**

Pollens were collected from the flower before anthesis. Pollen sterility was assessed by smearing mature pollen in 0.5 percent acetocarmine solution. (Alexander, 1969 & 1980). The slides were scored for (i) fertile for stained and (ii) sterile as non-stained or lightly stained.
Materials and Methods

e) **Pollen Germination**

Flowers are harvested between 9.30h and 10.30h from the plants (Cowpea and Bhendi) and placed in ziplock plastic bags for transport into the laboratory. Pollen collected by gently tapping the inverted flower.

Pollen culture media were prepared according to standard medium of Brewbaker and Kwack (1963). Pollen grain was considered germinated when the length of the pollen tube is more than the diameter of the pollen grain. Pollen germination was determined microscopically. The percentage germination was determined for 5 replicate experiments for control and treated plants.

f) **Fruits per plant**

The number of fruits per plant was noted from 6 plants from each treatment and control.

g) **Fruit length**

Fruit length was taken from ten fruits from each treatment and control.

h) **Fresh weight**

The weight of 10 fruits was taken from each treatment and control

i) **Fruit wall weight**

The fruit wall weight of 10 fruits was taken from control and treated plants

j) **Seeds per fruit**

Number of seeds per fruits was noted from randomly selected 10 fruits from each treatment and control.

k) **Weight of 10 seeds**

Weight of randomly selected 10 seeds was noted from each treatment and control.
2.2.4.2.2 Phytomass

Plants were cleaned carefully and plant parts were separated and kept in paper packets. They were dried thoroughly in an oven at 80°C till the weight became constant. The weight of each plant part was found separately and from this the total phytomass was calculated. The values were compared with those of control.

2.2.4.2.3 Anatomical

A. Stomatal index

Epidermal peelings were taken from the ventral side of the fresh leaves. These peelings were stained in 50 percent safranin and mounted on slides and observed under microscope. The stomatal index was calculated using the formula

\[
\text{Stomatal index} = \frac{(\text{Number of stomata (S)})}{\text{Number of epidermal cells (E)} + \text{Number of stomata (S)}} \times 100 \quad (\text{Salisbury, 1927}).
\]

B. Microphotography

Microphotographs of the relevant slides were taken using Nikon D.X.S.W.M.

2.2.4.2.4 Physiological

A. Chlorophyll

The chlorophyll and carotenoid contents were quantitatively estimated by Arnon’s (1949) method. The results thus obtained were compared with the control.

A preweighed (250mg) quantity of fresh leaf material was ground into a fine paste. 10 ml of 80% acetone was added in to it. The extract was centrifuged and the green supernatant was obtained. Using small quantities of
acetone the extract was centrifuged repeatedly till the leachate became colourless. The supernatant was taken together and was made up to 25 ml with 80% acetone. The extract was kept away from direct sunlight. The optical density of the extract was read at 480, 510, 645, 652 and 663 wave lengths using spectrophotometer (UV spectrophotometer, Shimadzu Corp. 01477, Model 10800 ENG. 240V). The samples were analysed in duplicates.

From the optical densities, the chlorophyll and carotenoid contents were calculated using the formula:

\[
\text{Chlorophyll a (mg/g)} = 12.7 \times \text{OD}_{663} - 2.69 \times \text{OD}_{645} \times \frac{V}{1000} \times \frac{W}{1000}
\]

\[
\text{Chlorophyll b (mg/g)} = 22.9 \times \text{OD}_{645} - 4.68 \times \text{OD}_{663} \times \frac{V}{1000} \times \frac{W}{1000}
\]

\[
\text{Total Chlorophyll (mg/g)} = \frac{\text{OD}_{652}}{34.5} \times \frac{V}{1000} \times \frac{W}{1000}
\]

\[
\text{Carotenoid mg/g} = 7.6 \times \text{OD}_{480} - 1.49 \times \text{OD}_{510} \times \frac{V}{1000} \times \frac{W}{1000}
\]

Where OD = Optical density

\[V = \text{Final vol. of 80% acetone (25ml)}\]

\[W = \text{Wt. of sample taken (0.25g)}\]

**B. Oxygen evolution**

Clark oxygen electrode was used for the estimation of oxygen evolution. The collected leaves were put in labeled zip lock polythene covers containing moisture. Leaf discs having 10cm² leaf area was immediately transferred to the leaf disc chamber of oxygen electrode. (LD/3, Hansatech, UK). The leaf disc were acclimatized to dark for 5 minutes and then exposed to a light intensity of 600µmolm⁻²S⁻¹ using LED source (LH36, Hansatech, UK) and the photosynthetic oxygen evolution was measured at 25°C. To
avoid any CO$_2$ limitation for photosynthesis 100µml of 0.5M bicarbonate buffer was added to the spongy capillary mating of the electrode chamber.

2.2.4.2.5 Biochemical

A. Protein estimation by Lowry’s method.

For estimating the buffer soluble proteins, extracts were prepared by homogenizing pre-weighed fresh leaf tissue (250 mg) in ice cold 5-10 ml of phosphate buffer (pH –7.2) using a chilled pestle and mortar. The homogenate was centrifuged at 18000 rpm for 15 minutes at 4°C. The supernatant was precipitated with trichloroacetic acid and was dissolved in 0.1N Sodium hydroxide and estimated using the procedure of Lowry et al., (1951).

Reagent for Lowry Method

**Reagent A** 2% Sodium Carbonate in 0.1 N Sodium hydroxide.

**Reagent B** 0.5% Copper Sulphate (CuSO$_4$.5H$_2$O) in 1% Potassium Sodium Tartrate.

**Reagent C** Mixed 50 ml of Reagent A with 1 ml of Reagent B prior to use.

**Reagent D** 1 part Folin phenol reagent + 1 part water.

Two tubes containing 0.05 ml and 0.1 ml of protein extracts were prepared for protein estimation. The volume of each tube was made up to 1.0 ml with phosphate buffer (pH - 7.2). The blank contained only 1.0 ml of phosphate buffer. The tubes were contained with 5.0 ml of reagent C and mixed well. It was allowed to stand for 10 minutes at room temperature. Then 0.5 ml of reagent D was rapidly added and mixed immediately and allowed to stand for room temperature in the dark for 30 minutes. The optical density was
measured at 660nm. Bovine Serum Albumin was used as the standard and the amount of protein in the sample was calculated.

**B. Phenol estimation**

For estimating the total phenols the method adopted that of (Bray & Thorpe, 1954) was followed. Extracts were prepared by homogenizing preweighed fresh leaf tissue (250 mg) in 10- time volume of 80% ethanol with a pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 minutes saved the supernatant. The residue should be re-extracted with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. The supernatant was evaporated to dryness. The residue was dissolved in 5 ml of distilled water.

Different aliquotes (Co.2 to 2 ml) were pipetted out into test tubes. The volume in each tube was made up to 3 ml with distilled water. 0.5ml of Folin-Ciocalteau reagent was added and after 3 min, 2 ml of 20% Sodium Carbonate solution into each tube. Thoroughly mixed the tubes and placed in boiling water for exactly one minute. After cooling the absorbance was measured at 650nm against reagent blank.

A standard curve was prepared using different concentrations of phenol and calculated the concentration of phenols in the test sample and expressed as mg phenols / 100 g material.

**C. Proline estimation**

Proline is a basic amino acid found in high percentage in basic proteins. Free proline is said to play a role in plants under stress conditions. Though the molecular mechanism has not yet been established for the increased level of proline, one of the hypothesis refers to breakdown of proteins into amino acids and conversion to proline for storage. Many workers
have reported a several fold increase in the proline content under physiological and pathological stress conditions. Hence the analysis of proline in plants has become routine in pathology and physiology divisions of agricultural sciences. The proline was estimated by using Bates et al., (1973) method.

**Principle**

During selective extraction with aqueous sulphosalicilic acid, proteins are precipitated as complex. Other interfering materials are also presumably removed by the absorption to the protein-sulphosalicilic acid complex.

The extracted proline is made to react with ninhydrin in acid conditions (pH 1.0) to form cromophore (red color) and read at 520nm.

**Materials**

1. Acid Ninhydrin: - Warm 1.25g ninhydrin in 30ml glacial acetic acid 20ml 6M phosphoric acid with agitation until dissolved. Store at 4°C and use within 24 hours.
2. 3% aqueous sulphosalicilic acid.
3. Glacial acetic acid.
4. Toluene.
5. Proline from plant tissue.

**Procedure**

1. Extract 0.5g of plant materials by homogenizing in 10ml of 3% aqueous sulphosalicilic acid.
2. Filter the homogenate through Whatman No.2 filter paper.
3. Take 2ml of filtrate in a test tube and add 2ml of glacial acetic acid and 2ml acid ninhydrin.
4. Heat it in the boiling water bath for 1 hour.
5. Terminate the reaction by placing the tube in ice bath.
6. Add 4 ml toluene to the reaction mixture and stir well for 20-30 seconds.
7. Separate the toluene layer and warm to room temperature.
8. Measure the red color intensity at 520 nm.
9. Run a series of standards with pure proline in a similar way and prepare a standard value.
10. Find out the amount of proline in the test sample from the standard curve.

**Calculation**

Express the proline content on fresh weight basis as follows:

$$\text{Moles per g tissue} = \left( \text{g proline/mL} \times \text{mL toluene/115.5} \right) \times \left( \frac{5}{\text{g sample}} \right)$$

Where 115.5 is the molecular weight of proline.

**2.2.5 Scanning Electron Microscopic Study (SEM Study)**

In order to study the responses brought about by the effluent on root surface and seeds, SEM study was adopted. The specimens were mounted on specimen stubs using double sided sticky tape, coated with platinum in Vacuum coater and viewed with JEOL JSM-6390 Stereo Scan and photographed.

**2.2.6 Statistical analysis**

The statistical analysis was carried out by using SPSS software (Version. 16). Analysis of variance (ANOVA) was done at 0.05 level to find out the significant difference between different percentages of effluent and different parameters of growth and development of the crops.