Materials and methods

3. MATERIALS AND METHODS

The present investigation was undertaken to study the effect of growth-promoting substances on *Crotalaria juncea* L. plants fed with tannery effluent.The growth-promoting substances used are a bio-fertiliser, namely vermiwash and a synthetic growth hormone called gibberellin. Pot experiments with tannery effluent-fed *C. juncea* were carried out by applying different concentrations of vermiwash and plant growth regulators as foliar sprays with deionised water as control, to study the impact on growth parameters in response to their applications. The materials and methods followed to carry out this investigation have been outlined hereunder.

Pilot Study

A pilot study was conducted to standardise the experimental design and the methodology. This study was carried out for a period of 90 days with different concentration of tannery effluent drenching and foliar spray of vermiwash and gibberellin using *C. juncea* to determine the optimal dilution for the present study.

3.1. Setting up of Vermiwash unit

Vermiwash used in organic agriculture both as replacement and supplement for nutrients. Vermiwash units can be setup either in barrels or in buckets or even in small pots. It can be prepared by different ways. The constituents of vermiwash vary not only with the species of earthworms but also with the raw materials used for composting.

In India, where a lot of solid organic waste is available in different sectors with no dearth for manpower, the environmentally acceptable vermicomposting technology using earthworms can very well be adopted for converting waste into

wealth (Hand *et al.,* 1988). In this study, cowdung and wastes such as leaf litter were utilized as the raw material for vermicomposting to extract the vermiwash. The cow dung substrate was used in the form of dry cow dung cakes along with dried leaves.

Selection of earthworm species is a very important factor because only few species are able to survive and adjust to a particular type of environment. Among different species of earthworms, *Eisenia fetida was* found to be the best choice due to its wide temperature and moisture tolerance, strength, ease of handling and competitive nature (Edwards and Bater, 1992; Chaudhuri and Bhattacharjee, 2002; Garg *et al*., 2006; Hatti *et al.,* 2010).

Earthworms *Eisenia fetida* were collected from Mr. Indrakumar, Vermiculturist, Pammal, Chennai, Tamilnadu. The collected earthworms were acclamatised at laboratory conditions, temperature (20 to 30ºC) and moisture were maintained up to 40% to 60% (w/w) and these were utilised in the vermiwash unit.

Systematic position of *Eisenia fetida*

Kingdom	**:**	**Animalia**
Phylum	**:**	**Annelida**
Class	**:**	**Clitellata**
Order	**:**	**Haplotaxida**
Family	**:**	**Lumbricidae**
Genus	**:**	***Eisenia***
Species	**:**	***fetida***

In the present study, Vermiwash was prepared according to the procedure standardized by Ismail (1997) with some modifications. A plastic can of 25 litre capacity fitted with a plastic gate-valve to facilitate drainage of eluates was taken and filled with pebbles, sand in the lower 1/3rd of the can and over this cow dung cakes

and leaf litter were added and above which a layer of coarse sand and garden soil was added. Three litres of water was added to it and the excess water was drained off. The unit was moistened every day. To this, 100 adult earthworms belonging to the species *Eisenia fetida* (Plate 1) were introduced so as to start the vermicomposting process. Water was sprayed to maintain the moisture and the setup was left undisturbed for about 45 days. After 45 days distilled water was poured over the worm bed and the liquid was collected and stored in a tightly stoppered bottle at $4^{o}C$ and a part of it was taken for physicochemical analysis and rest was used for the experiment (Plate 2).

For foliar spray, vermiwash at two different concentrations of 10% and 20% was used along with Gibberellin at 100 µg/ml to compare the growth-promoting effects. Distilled water was used as the control.

3.2. Usage of Plant Growth Regulator

Plant growth regulators can enhance plant growth. Among these, gibberellin a commercially available one used as a foliar spray (Tejada and Gonzalez, 2004). In this study, gibberellin for the experiment was prepared as 1000 µg/ml stock solution. For this one gram gibberellin was dissolved in a litre of water. Gibberellin is insoluble in water and so it was first dissolved in two ml of ethyl alcohol and then made up to 1000 ml by adding distilled water to prepare a 1000 µg/ml stock solution. 100 ml of thus prepared stock solution was made up to one litre by using distilled water to prepare a working solution of GA with a concentration of 100 µg/ml (100 ppm). The above prepared working solution was used for the foliar spray of GA (Plate 3C). The GA foliar spray was given once in a week at early in the morning as fine foliar sprays from hand atomizer to young expanding leaves, so that the top and bottom surfaces of the leaves were thoroughly sprayed. The sprays were given for eight **c**onsecutive weeks. The first treatment started when the plants were 30 days old.

3.3. Collection of Tannery effluent

In India tannery clusters are common in the states of Tamilnadu, West Bengal, Uttar Pradesh and Punjab. In Tamilnadu, tanneries are mainly concentrated in the districts of Vellore, Dindigul, Trichy, Erode and Chennai (Figure 1). Tannery effluent was collected and preserved in accordance with standard methods. For the present study the tannery effluent was collected in clean polythene carboy from the tannery industry that uses chrome tanning process situated at Chromepet near Chennai and stored at 4ºC for the analysis. The effluent was collected at weekly intervals for eight weeks. Every time the effluent was collected at three different spots in the effluent discharge area and then pooled for use in this study. From the effluent, 50% diluted tannery effluent was prepared, and this was used for soil drenched setups along with the raw effluent (Plate 3A and B).

Two different treatments, one with raw tannery effluent without dilution and another with 50% dilution of the tannery effluent were used. 50% dilution of the tannery effluent was prepared by diluting the raw tannery effluent using distilled water. The raw tannery effluent, 50% diluted tannery effluent and tap water were used to irrigate the pots.

3.4. Physico-chemical Analysis of Vermiwash and Tannery Effluent

To assess the physico-chemical properties of vermiwash and tannery effluent, the methods mentioned in the guidelines of American Public Health Association (Standard Methods for the examination of water and waste water, 23rd Edition (2017), APHA-AWWA-WEF) were followed. Deionised distilled water was used in the preparation of all reagents and calibration standards and also as dilution water.

3.4.1. Physico-chemical Properties of Vermiwash

The primary nutrients found in the vermiwash are Nitrogen (N), Phosphorous (P), and Potassium (K) (Shakhashiri, 2018) which can be easily absorbed by plants (Sundaravadivelan *et al*., 2011; Nath and Singh, 2012) and improves the quality of seed and fruit (Kidder, 1997; Quaik *et al*., 2012; Shakhashiri, 2018). For the analysis of vermiwash the above mentioned guidelines of American Public Health Association (Standard Methods for the examination of water and waste water, 23rd Edition (2017), APHA-AWWA-WEF) were followed.

3.4.2. Physico-chemical Properties of Tannery effluent

The different physico-chemical properties of tannery effluent were analysed by following the APHA method (Standard Methods for the examination of water and waste water, 23rd Edition (2017), APHA-AWWA-WEF).

pH

The intensity of the acidic or basic character of a water or solution is indicated by pH or hydrogen ion activity at a given temperature. The pH of the sample was measured by using pH meter and calibrated the electrode system against standard buffer solution of known pH. (Part 4500-H^+ of APHA-AWWA-WEF 2017). The pH of the solution was measured in Eutech pH Meter (Eutech Instrument pH 1500).

Electrical Conductivity

Electrical conductivity is the measure of the ability of an aqueous solution to carry an electric current. Conductivity was measured in Conductivity Meter using standard Potassium chloride solution as a standard which has a conductivity of 1413 µmhos/cm at 25º C (Part 2510 of APHA-AWWA-WEF 2017). The conductivity of the solution is measured in Hach HQ 430D conductivity Meter.

Total Suspended Solids (TSS)

Measurement of total suspended solids in water is used for control of various treatment processes and for examination of water / wastewater quality. Total suspended solids are measured by gravimetric method. A well-mixed sample is filtered through a weighed standard glass fibre filter and the residue retained on the filter is dried to a constant weight at 103ºC to 105ºC. The increase in weight of the filter represents the total suspended solids (Part 2540 D of APHA-AWWA-WEF 2017).

Total Dissolved Solids (TDS)

Total dissolved solids are the total amount of charged ions, including salts, minerals, metals dissolved in water, expressed in units of mg per unit volume of water (mg/L). Total dissolved solids are measured by gravimetric method. A well-mixed sample is filtered through a standard glass fibre, and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 180º C. The increase in dish weight represents the total dissolved solids (Part 2540 C of APHA-AWWA-WEF 2017).

Chlorides

Chloride is one of the major inorganic anions present in water and wastewater. Chloride estimation was done by Argentometric method. In a natural or slightly alkaline solution, potassium chromate can indicate the end point of the silver nitrate titration of chloride. The pH of the sample was adjusted to seven and one ml of potassium chromate was added to the solution as indicator and titrated with standard silver nitrate solution to a pinkish yellow end point. After precipitation of silver chloride is completed, a red silver chromate is formed. The titrant silver nitrate was standardised against sodium chloride (Part 4500-Cl of APHA-AWWA-WEF 2017).

Sulphate

Sulphate ion is precipitated in an acetic medium with barium chloride so as to form barium sulphate crystals of uniform size. A suitable portion of the sample was taken and mixed with buffer solution and the solution was stirred constantly. Sulphate in the sample was determined by Turbidimetric method by a spectrophotometer at 420 nm (Part 4500-SO_4 of APHA-AWWA-WEF 2017). Sulphate absorbance was measured in Shimadzu UV 2100 Spectrophotometer.

Chemical Oxygen Demand

The chemical oxygen demand (COD) is used as a measure of the oxygen equivalent of the organic matter of a sample that is susceptible to oxidation by a strong chemical oxidant. The open reflux method was adopted to estimate the COD of the sample. Most types of organic matter are oxidized by a boiling mixture of chromic and sulfuric acid. A sample is refluxed in strongly acid solution with the known excess of potassium dichromate. After digestion the remaining unreduced potassium dichromate is titrated with ferrous ammonium sulphate to determine the amount of potassium dichromate consumed and the oxidisable organic matter is calculated in terms of oxygen equivalent. A suitable portion of sample was taken and Mercuric sulphate was added into it. Then sulfuric acid was added very slowly. Potassium dichromate solution was added and attached the flask to condenser and turned on cooling water. The remaining sulfuric acid was added through open end of condenser and reflux for two hours. Cool and diluted the mixture with distilled water. Then titrated excess Potassium dichromate solution with ferrous ammonium sulphate using ferroin indicator. The end point of the titration is first sharp colour change from blue green to reddish brown. A blank containing the reagents and volume of distilled water equal to that of sample was also refluxed and titrated against ferrous ammonium sulphate for blank correction (Part 5220 of APHA-AWWA-WEF 2017).

Biochemical Oxygen Demand (3 days at 27º C)

The test measures the oxygen utilized during a specified incubation period for the biochemical degradation of organic material and the oxygen used to oxidize inorganic material (Part 5210 of APHA-AWWA-WEF 2017). The Biochemical oxygen demand (BOD) of the sample was determined by using Hach DO Meter by estimating the initial dissolved oxygen (DO) and final DO after the incubation period of three days at 27º C.

Total Hardness

Water hardness is a measure of the capacity of that particular water to precipitate soap and hardness of water due to soluble salts like bicarbonates, chlorides and sulphates of calcium and magnesium. The hardness of the samples was determined by EDTA (Ethylene diamine tetra acetic acid) titrimetric method. Ethylene diamine tetra acetic acid and its sodium salts when added to a solution of certain cations of metal form a chelated soluble complex. When a pinch of Eriochrome Black T is added to an aqueous solution which contains calcium and magnesium ions at a pH of 10.0 ± 0.1, the solution becomes wine red. The calcium and magnesium will be complexed with the EDTA titrant and after magnesium and calcium has been completely complexed the solution become turns from wine red to blue, which is the end point of the titration. Magnesium ion must be present to yield a sharp end point. To insure this, a small amount of complexometrically neutral magnesium salt of EDTA is added to the buffer, this automatically introduces sufficient magnesium and obviates the need for a blank correction. The sharpness of the end point increases with increasing pH (Part 2340 of APHA-AWWA-WEF 2017).

Calcium Hardness

The calcium hardness of the samples were determined by EDTA Titrimetric method. If EDTA is added as a titrant to water containing calcium and magnesium, first it combines with the calcium. Calcium can be determined directly with EDTA, the pH of the solution is made sufficiently high that the magnesium is precipitated as the hydroxide and an indicator is used that combines with calcium only. The sample is mixed with sufficient volume of sodium hydroxide solution to produce a pH of 12 or 13 and add a drop of Murexide indicator (ammonium purpurate). The calcium at a pH of 12 to 13 has been complexed by the titrant EDTA. The Murexide indicator changes from pink to purple at the end point (Part 3500-Ca of APHA-AWWA-WEF 2017).

Calcium

Calcium is estimated by calculation from Calcium hardness as $CaCO_3$.

Ca mg/L = Calcium hardness (as mg $CaCO_3$/L) x 0.4008.

Magnesium Hardness

Magnesium hardness is estimated as the difference between the total hardness and calcium hardness (Part 3500-Mg of APHA-AWWA-WEF 2017).

Magnesium

Magnesium is estimated as the difference between hardness and calcium as $CaCO_3$.

Mg mg/L = Total hardness (as mg $CaCO_3$/L) – Calcium hardness (as mg $CaCO_3$/L) x 0.243.

Sodium

Sodium is determined by Flame Photometer in flame emission mode. The sample was sprayed into a gas flame and excitation is carried out under carefully controlled and reproducible condition. The desired spectral line is isolated by the use of interference filters or by a suitable slit arrangement in light dispersing device such as prism or gratings. The phototube potentiometer measures the intensity of light. Standard sodium solution prepared from sodium chloride was used to prepare a calibration curve (Part 3500-Na of APHA-AWWA-WEF 2017). Sodium and Potassium were determined in Elico Flame photometer CL 378.

Potassium

Potassium is determined by Flame photometer in flame emission mode. The sample was sprayed into a gas flame and excitation is carried out under carefully controlled and reproducible condition. The desired spectral line is isolated by the use of interference filters or by a suitable slit arrangement in light dispersing devise such as prism or gratings. Phototube potentiometer measures the intensity of light. Standard potassium solution prepared from potassium chloride was used to prepare a calibration curve (Part 3500-K of APHA-AWWA-WEF 2017).

Phosphorous

Phosphorous content occur in combination with organic matter. To determine total phosphorous, the nitric acid and sulfuric acid method is adopted to digest the sample to oxidise the organic matter effectively and release phosphorous as orthophosphate. After digestion the liberated orthophosphate is determined colorimetrically by Ascorbic acid method. Ammonium molybdate and potassium antimonyl tartrate react in acid medium with orthophosphate to form a coloured molybdenum blue by ascorbic acid. The phosphate of the solution is calculated from

the calibration curve prepared by using anhydrous Potassium dihydrogen phosphate (KH_2PO_4) as standard (Part 4500-Pof APHA-AWWA-WEF 2017).

Total Nitrogen

A measured volume of sample in kjeldahl flask was taken and neutralised to pH 7.0. Then digestion reagent was added and mixed. After mixing heated under a hood until the volume is greatly reduced and copious white fumes are observed. The digestion was continued for an additionally 30 minutes till turbid samples turn in to clear solution. Hydroxide thio sulphate reagent was added to form an alkaline layer. The mixture was distilled and the distillate was collected in Boric acid and titrate ammonia in distillate with standard sulfuric acid titrant until indicator turns a pale lavender. The ammoniacal and kjeldahl nitrogen was determined by using Kel plus nitrogen analyser (Part 4500-Nof APHA-AWWA-WEF 2017).

Chromium

The chromium present in the effluent sample was measured by colorimetric method, in which only hexavalent chromium can be measured. Therefore, to determine total chromium all the chromium was converted into the hexavalent state by oxidation with potassium permanganate. The Cr(VI) is determined colorimetrically by reaction with 1,5-diphenyl carbazide solution in acidic medium. A red violet colour solution is produced. To determine the total chromium, the sample was digested with a nitric acid-sulfuric acid mixture and then oxidized with potassium permanganate before reacting with the diphenylcarbazide. Cr(VI) concentration was measured by colorimetric method, based on the purple complex formed by Cr(VI) in the presence of 1,5-diphenylcarbazide. The colour was fully developed after 15 min and the absorbance was measured at 540 nm in a one cm long glass cell using Shimadzu UV 2100 spectrophotometer. The calibration curve was prepared by using

standard potassium dichromate solution (Part 3500-Cr of APHA-AWWA-WEF 2017).

Metals (Copper, Zinc, Iron and Manganese)

Metals can be determined satisfactorily by atomic absorption method. After sampling the samples were preserved by acidifying with concentrated nitric acid to pH <2. To reduce organic matter and to convert metal associated with particulates to a free metal form that can be determined by atomic absorption spectrometry. Nitric acid-sulfuric acid digestion was carried out and set aside for determination of metals. From the digested sample a portion of solution was used for metal determination in Atomic absorption spectrophotometry (Part 3110 of APHA-AWWA-WEF 2017). Standard solutions of known metal concentration in water similar to the sample from stock standard solution were prepared for each metal. The metal analysis was carried out in PerkinElmer Atomic absorption spectrophotometer 800.

3.5. Germination Studies of *Crotalaria juncea* L. as Effected by Tannery Effluent and Growth-promoting Substances

3.5.1. Experimental Plant material

Crotalaria juncea L. plant was selected for this study. It is commonly known as Sunnhemp or Indian hemp (Kirtikar and Basu, 1999) (Plate 4).

Systematic position of *C. juncea*

Kingdom : Plantae

Family : Fabaceae

Tribe : Crotalarieae

Genus : *Crotalaria*

Species : *juncea*

The plant is native to Asia widely cultivated in the drier areas of the tropics and subtropics and in many temperate areas with hot summers. It has an annual lifecycle and has been used for several purposes, including paper production and as green manure in rotation culture systems due to its high nitrogen fixation capacity when in association with rhizobium. *C. juncea* also has been documented to increase nematode-antagonistic and bacterivorous nematodes involved in soil nutrient cycling (Wang *et al.*, 2001) and improve soil nutrient levels (Reeves *et al.*, 1996). Therefore it is considered a good rotation crop for organic and other sustainable agricultural systems and it has been proven to meet Nitrogen (N) demand for various crops (Reeves *et al.*, 1996; Marshall, 2002). There is increasing interest in using amendments of *C. juncea* hay as a means of achieving fertilizer needs in organic agricultural production (Marshall, 2002).

C. juncea was identified and authenticated by Dr. P. Jeyaraman, Father of Indian Plant Anatomy and Senior Plant Taxonomist at Plant Anatomy Research Centre (PARC), Chennai, Tamilnadu, India (Annexure and Plate 4).

3.5.2. Procurement of Seeds

The seeds of *C. juncea* were procured from farmers near Thiruvallur district and treated with 0.1% Mercuric chloride for 2 minutes and washed with running water to remove contamination of seed coat, prior to germination studies.

3.5.3. Seed Germination study

The seeds of *C. juncea* were surface sterilized with 0.1% $HgCl_2$ (Mercuric chloride) for the prevention of surface fungal/bacterial contamination. Sterilized petriplates prepared with cotton bed and a known volume of different concentration of tannery effluent (50 % and 100%) was poured into different petriplate in the cotton bed and the seeds were sprayed with gibberellin (100 ppm) and vermiwash (10% and

20%) as mentioned in the treatment group. All the seeds, germinated and grown in distilled water served as control. Each treatment including control was performed in triplicate and ten seeds were evenly placed in each petriplate for all treatment groups. Five ml solution was used to moisten the cotton bed. The seeds were allowed to germinate and grow at room temperature (28 ± 2ºC).

Growth of radicle and plumule was measured with the help of scale. The rate of germination and seedling growth were observed daily (1-7 days) at 24 hours interval. The germination percentage was calculated by the formula given by Asfaw (2012).

$$\text{Germination (Percentage)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \text{ x } 100$$

3.5.4. Seedling Vigour Index

The process of seed germination starts with imbibition and it is affected by various external factors including water, oxygen, temperature, light and internal factors like seed dormancy. Thus, the seedling vigour index (SVI) is required to compare the relative phytotoxicity in plants. Recently, the seedling vigour index (SVI) has been used as a phytotoxicity index to evaluate the effect of heavy metal on seedling growth (Srivastava and Thakur (2006); Kabir *et al*. (2008)). Seedling vigour is a measure of the extent of damage that accumulates as viability declines, and the damage accumulates in seeds until the seeds are unable to germinate and eventually die (Copeland and Mc Donald, 2012).

In this seed germination study the radicle and plumule length of germinated seeds were measured from zero day to 7th day (168 h) after calculating the germination percentage. The total length of the seedling (radicle + plumule) is multiplied with the germination percentage calculated as above to arrive at the

seedling vigour index. The seedling vigour index (SVI) was calculated by using the formula given by Abdul-Baki and Anderson, (1973).

Seedling vigour index = (Root length + Shoot length) x % Seed germination

3.5.5. Analysis of Chromium in Seedlings and Substrate

To assess whether there was any accumulation of chromium at the early growth stage of *C. juncea*, the seedlings subjected to various treatments after the seventh day of the experiment were analysed for the presence of chromium. These seedlings were thoroughly washed with distilled water, and naturally dried in room temperature. After drying, two gram of seedlings were weighed and taken for acid digestion with nitric acid and sulphuric acid. From the digested sample a portion of solution was used for chromium determination by eluting in Atomic absorption spectrophotometry (Part 3110 of APHA-AWWA-WEF 2017). The estimation of chromium was done for all the treatments in the seed germination study. Standard solutions of known chromium concentration in water from stock solution were prepared and the standard graph was plotted. The metal analysis was carried out in Perkin Elmer Atomic absorption spectrophotometer 800.

The substrate, cotton and filter paper which were used for seed germination were also analysed for presence of chromium on the seventh day. The cotton and the filter paper drenched with water and different dilution of tannery effluent were digested as per the standard procedure (Part 3110 of APHA-AWWA-WEF 2017). The digested sample was aspirated in Atomic Absorption Spectrophotometer for chromium determination.

3.6. Study of Exo-morphological Variations as Effected by Tannery Effluent and Growth-promoting Substances

3.6.1. Experimental Design

In this experiment a set of 12 treatments by drenching the soil with raw and diluted tannery effluent combined with foliar spray of growth-promoting substances of different dilution were given to the experimental plants grown in pots. The plants grown in water with foliar spray of deionised water served as control. All these 12 treatment set ups have been duplicated for replication of results (Table 1). Four plants were grown in each pot and two pots were maintained for each treatment including control. The following treatments were given in the present study.

Treatment 1

Pots were drenched with water, and foliar sprays were given with deionised water (C).

Treatment 2

Pots were drenched with water, and foliar sprays were given with 10% Vermiwash (V1).

Treatment 3

Pots were drenched with water, and foliar sprays were given with 20% Vermiwash (V2).

Treatment 4

Pots were drenched with 50% tannery effluent, and foliar sprays were given with deionised water (T1).

Treatment 5

Pots were drenched with 50% tannery effluent, and foliar sprays were given with 10% vermiwash (T1V1).

Treatment 6

Pots were drenched with 50% tannery effluent, and foliar sprays were given with 20% vermiwash (T1V2).

Treatment 7

Pots were drenched with 100% tannery effluent, and foliar sprays were given with deionised water (T2).

Treatment 8

Pots were drenched with 100% tannery effluent, and foliar sprays were given with 10% vermiwash (T2V1).

Treatment 9

Pots were drenched with 100% tannery effluent, and foliar sprays were given with 20% vermiwash (T2V2).

Treatment 10

Pots were drenched with water, and foliar sprays were given with 100 ppm gibberellin (G).

Treatment 11

Pots were drenched with 50% tannery effluent and foliar sprays were given with 100 ppm gibberellin (T1G).

Treatment 12

Pots were drenched with 100% tannery effluent and foliar sprays were given with 100 ppm gibberellin (T2G).

3.6.2. Preparation of Pots and Raising of Seedlings

3.6.2.1. Preparation of Nursery pots

The selected healthy seeds were sown in a nursery pot of 60 cm diameter. The *C. juncea* seeds were sown at ¾ inch depth and seedlings emerged after three days. The seedlings were allowed to grow for fifteen days (Plate 5).

3.6.2.2. Preparation of Pots and Transplantation of Seedlings

The pots for the experiment were filled with 15 kg of sand, red soil free from pebbles and farmyard manure in the ratio of 1:1:1 and maintained under garden land conditions. The seedlings after fifteen days were transplanted to the experimental pot at a distance of 5-7 cm between plants. The experiment was conducted in an open terrace with natural light, temperature and humidity, to keep the plants under conditions similar to those on the field. The experiment was carried out at ambient temperatures (25 ± 5ºC).

Pot experiments have been conducted to know the growth of plant in tannery effluent and compared with plants subjected to foliar spray of plant growth-promoting substances. Foliar feeding is a technique of applying liquid fertilizer directly to their leaves of a plant. Foliar spray of vermiwash of vermicomposting is a method of fertilizing plants as the plants absorb all the nutrients applied more rapidly than root application (Marschner, 1995). The plants were treated with various dilutions of tannery effluents along with foliar spray of deioised water / vermiwash / gibberellins

and the observation was made for 90 days since reproductive growth is attained in 90 days.

Experiments were started when the plants were well established in the experimental pot after transplantation.The treatment started when the plants were 30 days old. Plants were fed by drenching the soil uniformly throughout the period of experiment with respective dilution of tannery effluent / water approximately 500 ml daily at 24 hourly intervals. Various combinations of different concentrations of plant growth regulators like GA and vermiwash were applied as foliar sprays. Foliar spray of vermiwash and gibberellin was given when the air was cool by using hand spray every week, early in the morning between 6.00 and 7.00 am for eight weeks. To the foliar spray solutions, 0.01% of liquid detergent was added to act as a surfactant which enhances adherence of the spray solution to the leaves. The spraying was done until there was run-off of the excess spray solutions. The foliar spray was done at the end of each week. The plants were harvested on 90^{th} day (Table 2).

The observation of exo-morphological parameters were carried out in control and treated plants. The morphological data like plant height, intermodal diameter, number of leaves and leaf area of the plants were recorded at zero hour and then taken once a week and other data like root length, root nodules, and biomass were obtained after harvesting of the plant. The biochemical analysis such as Chlorophyll, Protein, Carbohydrates and Lipids were also determined in the leaves before the flowering stage was attained by using respective methodology. The anatomical study was done by taking cross section of the stem and leaves of 90-day-old plants.

3.6.3. Measurement of Plant Growth Parameters

The experiment was started in 30-day-old plants. At the end of every week, just before giving the foliar spray, the exo-morphological characters of the plants were recorded. The following exo-morphological characters were measured at

intervals of seven days in control and as well as in treated plants throughout the experimental period from the day of starting the treatment (Table 3).

1. Height of the plant
2. Internodal diameter
3. Number of leaves
4. Leaf area
5. First flowering
6. Number of inflorescence
7. Survival percentage

The following growth parameters were recorded at harvest.

1. Root length
2. Root nodules
3. Shoot biomass
4. Root biomass

3.6.3.1. Height of the Plant

Plant height was recorded using a measuring tape. The shoot length of the plant was recorded every week for 90 days.

3.6.3.2. Internodal Diameter

The diameter of internode was measured every week for 90 days using a screw gauge.

3.6.3.3. Number of Leaves

Number of leaves per plant was counted and recorded every week for 90 days.

3.6.3.4. Leaf area

The leaf surface area was estimated graphically by drawing the outline of the leaf on a graphpaper and counting the number of squares (cm^2) and recorded every week for 90 days.

3.6.3.5. First Flowering

The time of appearance of the first inflorescence for each treatment group was noted.

3.6.3.6. Number of Inflorescences

The total number of inflorescence every week and on 90th day in all treatment groups was recorded.

3.6.3.7. Survival Percentage

The number of plants that survived in each treatment throughout the experimental period was noted.

3.6.3.8. Root Length

After harvesting the plants, root length was measured using a standard scale.

3.6.3.9. Root Nodules

The nature of root nodules was visually compared among the treatments.

3.6.3.10. Shoot Biomass

Biomass is the amount of biological matter, usually described in terms of net loss or net gain for a specific amount of time. This value is expressed in terms of dry weight, or in terms of a single element such as carbon or nitrogen. Plants were

harvested and the samples were collected after 90 days from each treatment and the vegetative parts were separated and washed with tap water followed by distilled water. Leaves, stems were separated from the root system and sliced into smaller pieces and then weighed immediately to determine the wet weight of the shoot. Then, samples were dried in the oven at 80°C for 24 hours and after that they were cooled in a desiccator and weighed. The weighed plant materials were then subjected to drying in an oven at 80°C for further four hours. After four hours, the plant materials were again cooled in a desiccator and weighed. Thus the plant materials was reweighed at an interval of four hours after drying in oven and cooling in a desiccator till a constant weight of the plant materials were recorded. The constant weight of the plant materials were observed after the complete drying of the plant.

3.6.3.11. Root Biomass

Plant samples were collected from each treatment and the root parts were separated and washed with tap water followed by distilled water to remove adsorbed soil particulates. The root biomass was determined in a method similar to that of shoot biomass.

3.7. Study of Biochemical Variations as Effected by Tannery Effluent and Growth-promoting Substances

The 90-day-old plant samples of the different treatments were utilised for the estimation of the biochemical constituents like chlorophyll-a, chlorophyll-b, total chlorophyll, protein, carbohydrates and lipids.

3.7.1. Estimation of Chlorophyll

Chlorophyll content was estimated in fresh leaf samples by the method of Arnon (1949) using 80% acetone. Three gram of fresh *C. juncea* leaves were blended

and extracted with 10ml of 80% acetone. The extraction was left aside for 15 minutes. The liquid extraction was decanted into a test tube and the same was centrifuged for three minutes at 2,500 rpm. The supernatant of the solution was then collected and the absorbance of the solution was taken at 645nm and 663nm using a spectrophotometer.

3.7.2. Estimation of Protein

Protein content in *C. juncea* leaf tissues was estimated by the method of Lowry *et al*. (1951) using egg albumin as standard. The peptide nitrogen in the protein reacts with copper [II] ions under alkaline conditions and subsequent reduction of Folin-Ciocalteay phosphor molybdic phosphor tungstic acid to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic acids. This change in colour can be determined spectrophotometrically at 660 nm. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5. The Lowry method is sensitive even to low concentrations of protein.

A standard protein solution of Bovine serum albumen (1 mg) was dissolved in one mL of distilled water. Various aliquots of the standard solution (20, 40, 60, 80, 100 µL containing 20, 40, 60, 80, 100µg) were added in test tubes. 100 µL of samples dissolved in 10% DMSO (containing 1000 µg sample) were added in test tubes. A test tube containing one mL of distilled water served as blank solution. All tubes were made up to one mL with distilled water. The test tubes were added with five mL of Reagent I (2% sodium carbonate in 0.1 N sodium hydroxide, 1% sodium potassium tartrate in distilled water and 0.5% copper sulphate in distilled water; 48:1:1) and incubated for ten minutes at room temperature. 0.5 mL of Folin-Phenol: Water (1:1) was added and the contents were mixed well. The tubes were incubated at room temperature for 30 minutes and the colour developed was read at 660 nm. A standard calibration curve was constructed by plotting the absorbance of standard solution

(20-100 µg) and the concentration of the protein in the unknown sample was determined after the colour development and recording the reading at 660 nm and comparing the reading of the standard graph developed.

3.7.3. Estimation of Carbohydrates

Carbohydrates were determined by anthrone method (Loewus 1952). Carbohydrates are dehydrated with concentrated sulphuric acid to form furfural which condenses with anthrone to form a green coloured complex and the concentration of carbohydrates can be determined with spectrophotometrically at 620 nm.

Anthrone reagent was prepared by dissolving 20mg of anthrone in 10 mL of concentrated sulphuric acid followed by 90 mL of distilled water. Glucose solution prepared by dissolving 10 mg of D-Glucose in one mL of distilled water was used as a standard carbohydrate solution. Various concentration of the standard glucose solution (20, 40, 60, 80 & 100µL containing 200, 400, 600, 800 and 1000 µg glucose) was added in five test tubes. Samples were powdered and dissolved in 10% DMSO. 100 µL of the solution containing 1000 µg of each sample were added in test tubes. A test tube containing 0.5 mL distilled water was taken as control. Distilled water was added and made up to one mL. Anthrone reagent (4ml) was added into each tube and the contents were mixed well and placed in a boiling water bath for 10 minutes. It was allowed to cool to room temperature and the optical density was measured at 620 nm. A calibration curve was constructed by plotting the absorbance of standard glucose (200 to 1000 µg). The concentration of carbohydrates in the samples was calculated from the standard graph prepared.

3.7.4. Estimation of Lipids

Estimation of lipids was done by Folch method (Folch *et al*. 1957). One mL of concentrated sulphuric acid was added to 1000 mg of crushed sample powder for the

digestion of fats into lipids and kept for 15 minutes. Six mL of chloroform and methanol (3:1) was added to the mixture and it was mixed well. The contents were centrifuged at 5000 rpm for 10 minutes and maintained at a temperature of 20°C. The aqueous layer was transferred into a fresh tube and this step was repeated again. Hexane: Isopropyl alcohol (2:1) was added to the tubes and centrifuged at 4000 rpm for five minutes. The organic layer of the content was transferred to a fresh tube. Five mL of distilled water was added to separate the lipids. The organic layer was pipetted out carefully and the tubes were placed on a hot lid maintained at 40°C for 20 minutes to evaporate the residual hexane and alcohol. The lipids extracted were weighed and the yield was calculated.

3.8. Study of Anatomical Variations as Effected by Tannery Effluent and Growth-promoting Substances

Histological studies are promising field to understand the anatomical variations in structural organization that occur due to the impact of pollutants in the environment. These structural changes vary with the complexity of tissue, nature of the pollutant, medium and duration of exposure (Vijaymadhavan and Iwai, 1975). The microscopic evaluation is one of the simplest and cheapest methods for the correct identification of the structural changes in the plant materials. The present anatomical study was undertaken with a view to explore the changes in tissue level which could be useful to detect the impact of tannery effluent on *C. juncea* treated with foliar spray of vermiwash and gibberellin.

3.8.1. Specimen preparation for Anatomical study

When the plants were 90 days old they were harvested and subjected to anatomical study. The fifth internode of the plant in control and treated plants was chosen uniformly. The excised internodal segments used for anatomical studies, were

fixed in FAA (5 ml of Formalin + 5 ml of Acetic acid + 90 ml of 70% Ethyl alcohol) a common fixative. The specimens were dehydrated with graded series of Tertiary-Butyl alcohol (TBA) after 24 hours of fixation (Sass, 1940). The infiltration of the specimens were done by addition of paraffin wax at melting point 58-60°C until tertiary butyl alcohol solution attained super saturation. The specimens were cast into paraffin blocks. About 100 cross-sections of stem and leaves of control and treated plants from each treatment were analysed for anatomical variations and the best five transverse sections were selected for study of anatomical characteristics (Sass, 1940).

3.8.2. Sectioning

The sectioning of paraffin embedded specimens were taken with the help of rotary microtome. The thickness of the section was 10-12 μm. Dewaxing of the section was by customary procedure (Johansen 1940). The sections were stained with Toluidine blue as per the method of O'Brien *et al.*, (1964).

3.8.3. Photomicrographs

Nikon Lab photo II research microscope was used for taking photomicrographs pertaining to histological studies. Magnifications of the figures are indicated by scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy books (Cutter, 1978; Easu, 1964; Easu, 1979).

3.9. Randomly Amplified Polymorphic DNA (RAPD) Analysis to study the genetic variations caused by Tannery effluent and Growth-promoting substances

Plant DNA isolation was done by following the method of Doyle and Doyle (1987). RAPD technique was used to evaluate the genotoxic effect of tannery effluent on the seven day old seedlings during seed germination study and leaves of 90-day

old plants of *C. juncea*. The plant material was weighed (0.5g) and frozen in liquid nitrogen. The frozen tissue was homogenised using mortar and pestle. To this extract 1.5ml (w/v) of preheated 4X CTAB buffer was added and incubated at 65°C for about 60 minutes and mixed well by inversion for every ten minutes. Equal volumes of chloroform and isoamylalcohol (24:1) was added and mixed gently by inverting the tubes until the phase was completely mixed. It was centrifuged at 10,000 rpm for 15 minutes at 4° C and the upper aqueous layer was transferred to new sterile microfuge tube using wide bore tip. One-tenth in the volume of 3M sodium acetate and 0.6 volume of cold isopropanol was added and thoroughly mixed to precipitate the nucleic acids. It was stored at -20°C for about one hour and the tube was spun for 15 min at 10,000 rpm at 4°C to recover the nucleic acid pellets. The pellet was washed with 0.5 ml of 70% ethanol twice and dried at room temperature. The DNA pellet was then suspended in TE buffer and stored at -20°C for further use.

3.9.1. Agarose Gel Electrophoresis

Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups. Separation on agarose gel is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficult in moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated such as for plasmid molecules-1%; genomic DNA-0.8% and RNA-1.5%; mitochondrial DNA-0.8% and amplified samples at 1.5% was used.

Agarose gel was weighed and transferred to a conical flask. 50 ml of 1X TAE was added and agarose gel was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 2.5µl of Ethidium bromide stock

solution was added. A gel casting tray was placed on a levelling table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged. DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading buffer reached 2/3rd of the gel. This gel was then viewed under UV Trans-illuminator.

3.9.2. Qualitative and Quantitative determination of DNA by Spectrophotometric method

A solution of nucleic acids absorbs UV at 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution. A ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. But values less than 1.8 signify the presence of proteins as impurities. The values greater than 1.8 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation.

3.9.3. Random Amplification of Polymorphic DNA (RAPD)

The principle is that, a single short oligonucleotide primer binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by Polymerase Chain Reaction (PCR) depends on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualized by Ethidium bromide staining. The standard RAPD utilizes short synthetic oligonucleotides (10 bases long)

of random sequences as primers to amplify nano gram amounts of total genomic DNA under low annealing temperatures by PCR. Primers are commercially available from various sources. In RAPD analysis, the target sequence(s) (to be amplified) is unknown. A primer is designed with an arbitrary sequence. In order for PCR to occur: i) the primers must anneal in a particular orientation (such that they point towards each other) ii) they must anneal within a reasonable distance of one another.

OPN10 and OPN11are the primers used for the present study

Reaction set up for RAPD

Components	Stock concentration	Final concentration	Volume for 20 µl setup
Milli Q water			11.63µl
dNTP mix	2 mM	0.2 mM	2 µl
$MgCl_2$	25 mM	0.25 mM	0.2 µl
Taq buffer	10X	1X	2 µl
RAPD PRIMERs	3µm	0.4µm	2.67 µl
DNA Template	100ng/µl	100ng	1 µl(1 in 10 Dilution)
Taq DNA polymerase	5U/µl	2.5U	0.5 µl

PCR Reaction conditions

Initial denaturation : 94°C – 3 min

Denaturation : 94°C – 45sec

Annealing : 37°C – 1 min

Extension : 72°C – 1 min 20sec

Final extension : 72°C – 7 min

Hold : 4°C

Total number of cycles 40

The samples were run on a 2% Agarose gel.

Statistical Analysis and Documentation

The experimental data was expressed as mean ± S.E. The IBM SPSS statistics analysis program was used for the statistical analysis. The data were analysed with one-way analysis of variance (ANOVA) to determine the effect of the treatments, and Duncan's multiple range test was applied to test the statistical significance of the differences in the averages of the treatments. Standard Deviation (SD) was done for Biochemical parameters.

Documentation was done using Nikon Lab photo II research microscope was used for taking photomicrographs pertaining to histological studies. Magnifications of the figures are indicated by scale-bars. Descriptive terms of the anatomical features are as given in the standard anatomy books (Cutter, 1978).

Table 1: Various Experimental Setups used in the study

S.No.	Group code	Treatment group	Pot A	Pot B (Replicate of Pot A)
1	C	Water (sd) + Deionised water (fs)	4 Plants	4 Plants
2	V1	Water (sd) + 10% Vermiwash (fs)	4 Plants	4 Plants
3	V2	Water (sd) + 20% Vermiwash (fs)	4 Plants	4 Plants
4	T1	50% Tannery effluent (sd) + Deionised water (fs)	4 Plants	4 Plants
5	T1V1	50% Tannery effluent (sd) + 10% Vermiwash (fs)	4 Plants	4 Plants
6	T1V2	50% Tannery effluent (sd) + 20% Vermiwash (fs)	4 Plants	4 Plants
7	T2	100% Tannery Effluent (sd) + Deionised water(fs)	4 Plants	4 Plants
8	T2V1	100% Tannery effluent (sd) + 10% Vermiwash (fs)	4 Plants	4 Plants
9	T2V2	100% Tannery effluent (sd) + 20% Vermiwash (fs)	4 Plants	4 Plants
10	G	Water (sd) + 100 ppm Gibberellin (fs)	4 Plants	4 Plants
11	T1G	50% Tannery effluent(sd) + 100 ppm Gibberellin (fs)	4 Plants	4 Plants
12	T2G	100% Tannery effluent(sd) +100 ppm Gibberellin(fs)	4 Plants	4 Plants

(sd) – soil drench
(fs) - foliar spray

Table 2: Details of different treatments used in the experimental setups

Treatment group	Pot soil used	Treatment after 30 DAS		
		Soil drenching* with water/ day	Soil drenching* with Tannery Effluent / day	Foliar spray**
C	Red soil + Sand + Manure 1:1:1 (15 kg)	500 ml	-----	Deionised water
V1	Red soil + Sand + Manure 1:1:1 (15 kg)	500 ml	-----	10% Vermiwash
V2	Red soil + Sand + Manure 1:1:1 (15 kg)	500 ml	-----	20% Vermiwash
T1	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 50% tannery effluent	Deionised water
T1V1	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 50% tannery effluent	10% Vermiwash
T1V2	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 50% tannery effluent	20% Vermiwash
T2	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 100% tannery effluent	Deionised water
T2V1	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 100% tannery effluent	10% Vermiwash
T2V2	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 100% tannery effluent	20% Vermiwash
G	Red soil + Sand + Manure 1:1:1 (15 kg)	500 ml	-----	100 ppm Gibberellin
T1G	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 50% tannery effluent	100 ppm Gibberellin
T2G	Red soil + Sand + Manure 1:1:1 (15 kg)	-----	500 ml of 100% tannery effluent	100 ppm Gibberellin

DAS – days after sowing

*Evening at 6.00 pm

**Morning at 6.00 am (once in a week up to eight weeks)

Table 3: Measurement of different Plant Growth Parameters

From Planting	Plant growth	Parameter	Procedure
Seed	Germination	% of germination	Percentage of seeds that germinated
		Radical and plumule length	Measuring the length by scale
Young Plant	Growth rate	Plant Height	From base to tip of the plant stem using scale
		Diameter of the plant	Using a screw gauge
		No. of Leaves	Counting the number of leaves (Visible leaf)
		Leaf area	Trace the leaf on graph paper and count the squares covered
Flowering Plant	Reproductive period	1^{st} Flowering	Number of days from planting to the first flower
		No. of Inflorescence	Counting the number of Inflorescence on each plant
Matured Plant	Root system	Root length	From base of the stem to tip of the root using scale
		Root nodules	Nature of nodules
	Growth by weight (Bio mass)	Fresh weight	Remove the plant from pot, wash off the soil, blot plants gently, pat it dry and weigh before the plant lose its moisture
		Dry weight	Put the plant in 80°C in an oven and heat for 24 hours, then allow it to cool, and weigh until constant weight