CHAPTER- 3  MATERIALS AND METHODS

3.1. EXPERIMENTAL ANIMAL

The experimental animal used for the present study was root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949.

3.1.1. Classification

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Nematoda</th>
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<tr>
<td>Class</td>
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<tr>
<td>Species</td>
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3.1.2. Distribution and Host range

*Meloidogyne incognita* is one of the most ubiquitous species in the genus. It occurs over a wider geographic range than any other species, from approximately 40°N to 33°S (Taylor *et al*., 1982). The annual average temperature range for *M. incognita* is between 18 and 30°C, but the majority of population is found in an annual temperature range of 24-30°C.

*M. incognita* has a very broad host range and is able to parasitize more than 750 plant species and varieties. These include fruits, vegetables, pulses, ornamentals, medicinal plants etc. (Sasser, 1979; Dasgupta, 1998; Fourie and McDonald, 2000). *Meloidogyne* spp. are responsible for a large part of the annual 125 billion $ losses attributed to nematode damage (Bird and Kaloshian, 2003). Nationally, Rs 21,068.73 million crop losses have been reported due to nematode parasitism (Khan *et al*., 2010).
Fig. 1. Distribution of plant parasitic nematodes infecting major crops in India (taken from Nematode distribution atlas by Khan et al., 2010)
3.1.3. Life-Cycle

*M. incognita* is a sedentary endoparasite, parthenogenetic and shows sexual dimorphism. The complete life-cycle can be divided into two phases (Taylor and Sasser, 1978)-

A. Preparasitic

B. Parasitic

A. Preparasitic

The life cycle of *Meloidogyne* species starts with an egg, usually in the one-celled stage, deposited by a female which is completely or partially embedded in the root of a host plant. The eggs are deposited into a gelatinous matrix which holds them together in "egg masses" or "egg sacs." Egg development begins within a few hours after deposition, resulting in 2 cells, 4, 8 and so on, until a fully formed juvenile with a visible stylet lies coiled in the egg membrane. This is the first juvenile stage (*J*₁). It can move in the egg but is not very active. The first moult takes place in the egg. Shortly after, the juvenile hatches as second stage juvenile (*J*₂).

B. Parasitic

1. Penetration of Roots

Second-stage infective juvenile usually penetrate roots just above the root cap. They move mostly between undifferentiated root cells, and finally come to rest with heads in the developing stele near the region of cell elongation and bodies in the cortex. Then secretions from the esophageal glands are injected which causes enlargement of cells in the vascular cylinder. This leads to the formation of giant cells (also called syncytia) as a result of the enlargement of cells (hypertrophy), possible dissolution of cell walls, enlargement of nuclei and changes in the composition of cell contents.

2. Development of Parasitic Stages

While the giant cells and galls are forming, the second stage juvenile continues feeding. The body size increases and become flask-shaped. It moult three times without feeding to form the third stage juvenile (*J*₃), fourth stage juvenile (*J*₄) and finally
becomes an adult. The saccate adult female resumes feeding and develops reproductive system which grows into functional gonads. The saccate male juvenile changes to vermiform adult male during the fourth juvenile stage. The adult male does not feed. It leaves the root, remains in the soil and finally dies. First egg-laying females of *M. incognita* are found 19-21 days after penetration (Triantaphyllou and Hirschmann, 1960). The life span of egg-producing females extends from 2 to 3 months, but that of males is shorter. The entire life cycle is completed in 20-25 days at an optimum temperature of 27°C.

### 3.1.4. Host–Parasite Relationship

The primary symptom during nematode pathogenesis is the formation of typical root galls on the root of susceptible host plants. Second-stage juveniles that enter roots, secretes enzymes like cellulase and β-1, 4-endoglucanases that soften the cell wall before penetrating. After penetration, J₂s migrate intercellularly in the cortex to cell differentiation region and begin feeding. The feeding of J₂ on protoxylem and protophloem cells induces these cells to differentiate into specialized nurse cells, which are called giant cells. Once a giant cell is initiated, the nematode becomes sedentary and enlarges greatly to assume a 'sausage' shape. Under favourable conditions, the J₂ moults to the third-stage juvenile, then to the fourth-stage juvenile and finally to the adult stage. The giant cells so formed functions as food transfer cells passing nutrients to the nematode (Jones and Northcote, 1972) and acts as a metabolic sink (McClure, 1977). Photosynthates are mobilized to the giant cells in the roots of the plants; hence, the plant growth and yield are suppressed. Root tissues around the nematode undergo hyperplasia and hypertrophy resulting in the formation of a root gall.
Fig. 2. Life cycle of the root-knot nematode, *M. incognita* on tomato
3.2. PLANT GROWTH REGULATOR

28-Homobrassinolide (22S,23S-Homobrassinolide; HBl) (Sigma-Aldrich, Bangalore, India).

**Scientific name:** [(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-24-etyl-o-B-homo-7-oxa-5α-cholestan-6-one]

**Molecular formula:** C_{29}H_{50}O_{6}

**Molecular weight:** 494.7

**Melting point:** 193-194°C

**Appearance:** Crystalline Solid

![Fig.3. Structure of 28-Homobrassinolide](image)

28-Homobrassinolide (HBl) is one of the most potent and an active member of brassinosteroid family (Khripach *et al.*, 1999). It is a steroidal lactone with potent plant growth-promoting property and stimulated protein synthesis. It was isolated for the first time from *Brassica campestris* var. pekinensis L. by Ikekawa *et al.* (1984). Brassinosteroids play an essential role in plant growth and development (Khripach *et al.*, 2000). They are also reported to confer resistance to plants responding to abiotic stresses such as heat, cold, drought, heavy metals, salinity etc. (Khripach *et al.*, 2000; Anuradha and Rao, 2001; Nakashita *et al.*, 2003; Yu *et al.*, 2004; Arora *et al.*, 2010) and to plants interacting with different pathogens- bacterial, fungal and viral (Wachsman *et al.*, 2000; 2002; Upreti and Murty, 2004).

The stock solution of HBl was prepared in methanol (HPLC grade; 10^{-4}M). The stock solution was diluted further with double distilled water to make the required concentrations: 10^{-11}M, 10^{-9}M and 10^{-7}M.
3.3. **EXPERIMENTAL PLANT: *Lycopersicon esculentum* Mill. (syn. *Solanum lycopersicon*)

Tomato (*Lycopersicon esculentum* L.) is one of the most popular and widely consumed fruit in the world (Norman, 1992). Tomatoes are widely known for their outstanding antioxidant content, including rich concentration of lycopene (a dietary carotenoid; antioxidant) which is found in high concentrations in processed tomato products.

3.3.1. **Classification**

<table>
<thead>
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<tr>
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<tr>
<td>Species</td>
<td><em>esculentum</em></td>
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Fig.4. Tomato plant

Fig.5. Tomato flower

In the present study, two varieties of tomato were selected: Pusa Ruby (susceptible) and PNR-7 (resistant). The seeds of PusaRuby and PNR-7 were procured from the local seed market, Amritsar and Punjab Agricultural University, Ludhiana respectively.

3.4. **MAINTENANCE OF NEMATODE CULTURE**

Heavily infected roots of tomato and brinjal were collected from Guru Nanak Dev University Campus, Amritsar. These were brought to the lab in plastic bags. The
collections were used for identification of *Meloidogyne* populations and for maintaining the cultures.

3.4.1. **Identification of *Meloidogyne incognita***

The species was identified on the basis of characteristics of the perineal pattern, as proposed by Chitwood (1949). Perineal patterns are formed by expansion and alteration of the juvenile body and retain the lateral lines, the tail tip, and the phasmids. The lateral lines point to the tail tip (end). The perineal patterns were studied by using the method described by Taylor and Netscher (1974).

Infected roots were washed under running tap water. The mature females were removed from galls and placed in petri dish containing 0.9% saline solution. The females were then transferred to a drop of 45% lactic acid on a microscopic slide under stereoscopic binocular microscope to facilitate the removal of body tissues adhering to the cuticle. The cuticle was ruptured near the neck with the help of surgical blade. Body tissues were removed by lightly brushing with a fine brush. The cuticle with the perineal pattern was trimmed to a square. Finally, the cuticle with the perineal pattern was transferred to a drop of glycerine on a clean microscopic slide. The coverslip was gently placed on the glycerine drop and sealed with a sealant. The excess glycerine was absorbed by a piece of filter paper.

For setting up of nematode culture, soil was sterilized in autoclave for 15-20 minutes. Soil was also sterilized by spreading on a large, black and tough polythene sheet and exposing it to intense sunrays for 4-5 days and turning it upside down in between. Six inches cleaned earthen pots were used to fill the sterilized soil. Two to
three weeks old tomato cv. Pusa Ruby seedlings and Brinjal cv. F1 Hybrid Navkiran seedlings grown separately in sterilized soil, were transplanted singly in these pots. After one week of transplantation, seedlings were inoculated with concentrated suspension of second-stage juveniles, egg-masses or galled root-cuttings. For optimal egg production, seedlings were grown for 45-60 days under glasshouse conditions. The nematodes were then transferred periodically to new seedlings. Pure cultures maintained through *M. incognita* J2s and egg-masses were used for experimentation.

3.5. EXPERIMENTATION

The present study was envisaged to evaluate the effect of HBl on plant morphological parameters (percentage germination of seeds, total plant height, root length, shoot length, root weight, shoot weight and number of galls) and biochemical changes in the activities of antioxidative enzymes (catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase, glutathione peroxidase and superoxide dismutase) as well as changes in the content of non-enzymatic antioxidants (total phenolic content, total flavonoid content, ascorbic acid content and total glutathione content) in both susceptible and resistant cultivars of tomato during nematode infection.

Seeds of tomato (Pusa Ruby and PNR-7) were sterilized separately with 1% sodium hypochlorite for 10 minutes followed by washing with distilled water 3-4 times. They were further sterilized with 0.01% HgCl₂ for 1 minute followed by repeated rinsing with distilled water. The sterilized seeds were then dipped in 3ml of different concentrations (10⁻¹¹M, 10⁻⁹M, 10⁻⁷M) of 28-Homobrassinolide for 4 hrs. Seeds dipped in distilled water served as controls. Treated and untreated seeds were then germinated in sterilized glass petri dishes. Thirty seeds were germinated per petri dish both for morphological studies and for estimations of non-enzymatic antioxidants whereas sixty seeds were germinated per petri for estimations of antioxidative enzymes. Seeds were allowed to germinate in BOD incubator at 24±2°C. Six-seven days old seedlings were then inoculated with second-stage juveniles (@ 5J₂s/seed). Untreated and uninoculated plants served as control I whereas untreated and inoculated plants served as control II. Three replicates of each concentration and controls were made. Time course experiments were set in which morphological and biochemical estimations were done separately in shoots and roots of both the cultivars at different stages of pathogenesis.
Fig. 8: Maintenance of nematode culture in glass house
Materials and Methods

Fig. 9. Pre-sowing seed treatment of sterilized tomato seeds with HBl

Fig. 10. Tomato seeds (before germination) in petri-dish

Fig. 11. Tomato seeds (after germination) in petri-dish

Fig. 12. Single petri-dish showing germinated tomato seeds
3.5.1. Morphological Studies

Observations regarding morphological changes were carried after 24, 72, 120 and 168 hrs of nematode inoculation in roots and shoots of both the cultivars. Percentage germination of seeds, root and shoot length, root and shoot weight as well as number of galls were recorded. To confirm the nematode penetration in plant tissue, roots were stained with the help of acid fuchsin (Byrd et al., 1983). Roots were immersed for 2-3 minutes in a boiling solution of equal parts of lactic acid, glycerol, distilled water and 0.05% acid fuchsin. Then the roots were allowed to cool in the stain and washed under running tap water to remove excess stain. Further, the roots were placed in a clear glycerol solution (equal parts of glycerol and water) and were left for 48-72 hrs. The stain was removed from most of the plant tissue leaving nematodes stained deeply pink. The plant material was preserved in the clearing solution by adding 2-3 drops of formalin.

3.5.2. Biochemical Studies

3.5.2.1. Antioxidative Enzymes

For the preparation of root and shoot extract, the roots and shoots were separately weighed. Each was crushed in a pre-chilled pestle and mortar using 0.1 M phosphate buffer (pH 7). The extract was collected in 1.5 ml eppendorf and then centrifuged at 10000 rpm for 25 minutes in a cooling centrifuge. The supernatant so collected was used for enzymatic estimations while the pellet was discarded.

3.5.2.2. Protein Content

Protein content was estimated in shoots and roots separately using Thermo Scientific Spectrophotometer at a wavelength of 550 nm. The methodology followed for the estimation of protein was as given by Lowry et al. (1951).

Reagents
1) Reagent A- 2% Sodium carbonate in 0.1 N Sodium hydroxide
2) Reagent B- 0.5% Copper sulphate in 1% Sodium potassium tartarate
3) Reagent C- Reagent A: Reagent B:: 50:1
4) Reagent D- 3 times dilution of Folin Ciocalteau reagent
Estimation

The assay mixture consisted of 900 μl distilled water, 100 μl extract and 5 ml reagent C. This was allowed to stand for 10 minutes and then 0.5 ml of reagent D was added. The mixture was well shaken and was kept in dark for 30 minutes. After 30 minutes, the optical density (O.D.) was recorded at 550 nm on Spectrophotometer against blank. The blank consisted of all constituents except the extract. For each sample three concordant readings were taken. The standard curve was prepared by using Bovine Serum Albumen (BSA) as standard. The protein content of the sample was calculated by using the following formula:

\[
\text{Concentration of protein content (mg/g)} = \frac{\text{O.D. of test} \times \text{Conc. of standard (μg/ml)} \times \text{Total volume made (ml)} \times 1}{\text{O.D. of standard} \times \text{Volume of test sample taken (ml)} \times \text{Weight of sample} \times 1000}
\]

3.5.2.3. Catalase

Catalase (CAT; EC 1.11.1.6) is a heme–containing enzyme present in the tissues of animals, plants and micro-organisms. It is responsible for dismutation of hydrogen peroxide into oxygen and water in the peroxisomes, protecting the cell from deleterious effects of hydrogen peroxide accumulation. The methodology used for catalase estimation was as given by Aebi (1983).

Principle

The reaction catalyzed by catalase is given as under:

\[
\begin{align*}
2 \text{H}_2\text{O}_2 & \xrightarrow{\text{Catalase}} 2 \text{H}_2\text{O} + \text{O}_2 \\
\end{align*}
\]

The change in absorbance due to decomposition of \( \text{H}_2\text{O}_2 \) was recorded spectrophotometrically.

Reagents

1) 0.1 M Phosphate buffer (pH 7.0)
2) 150 mM Hydrogen peroxide (\( \text{H}_2\text{O}_2 \))

Estimation

The assay mixture consisted of following reagents which were prepared separately and mixed in the given order at the time of taking readings-1.5 ml phosphate
Materials and Methods

buffer, 1.2 ml H$_2$O$_2$ and 300 µl enzyme extract. The decrease in absorbance was recorded on the UV–VIS Spectrophotometer at 240 nm for a total of 1 minute at an interval of 6 seconds against phosphate buffer as blank.

**Calculations**

One unit of enzyme activity is defined as the amount of enzyme required to liberate half of the peroxidase oxygen from H$_2$O$_2$ at 25ºC and is calculated from the following equation:

$$\text{Units/minute/g tissue} = \frac{\text{Change in absorbance/min} \times \text{Total volume of reaction mixture}}{\text{Extinction coefficient} \times \text{Amount of sample} \times \text{dilution factor}}$$

where

Extinction coefficient = $6.93 \times 10^{-3}$ mM$^{-1}$ cm$^{-1}$

3.5.2.4. Peroxidase

Peroxidase (POD) is a ubiquitous enzyme which is found in plants, animals, fungi and even prokaryotes. It includes in its widest sense a group of specific enzymes as NAD- peroxidase, NADP- peroxidase, guaiacol peroxidase, ascorbate peroxidase, fatty acid peroxidase etc. as well as a group of very non-specific enzymes from different sources which are simply known as POD (donor:H$_2$O$_2$-oxidoreductase 1.11.1.7.). Peroxidase catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc.

3.5.3.4.1. Ascorbate Peroxidase

Ascorbate peroxidase (APOX; EC 1.11.1.11) catalyses the reduction of H$_2$O$_2$ involving the oxidation of ascorbate. It uses two molecules of ascorbate to reduce H$_2$O$_2$ to water, with the concomitant generation of two molecules of monodehydroascorbate (MDHA). The methodology followed for ascorbate peroxidase estimation was as given by Nakano and Asada (1981).

**Principle**

$$2\text{Ascorbate} + H_2O_2 \xrightarrow{\text{POD}} 2\text{MDHA} + 2H_2O$$
Reagents
1) 0.1 M Phosphate buffer (pH 7.0)
2) 5 mM Ascorbate
3) 0.5 mM Hydrogen peroxide (H$_2$O$_2$)

Estimation
For the estimation of enzyme, following constituents were added in a test tube in given order – 1.5 ml phosphate buffer, 300 µl ascorbate, 600 µl H$_2$O$_2$ and 600 µl enzyme extract. The readings were taken on UV–VIS Spectrophotometer at 290 nm. The decrease in absorbance was recorded at an interval of 6 seconds for a total time period of 1 minute.

Calculations
One unit of enzyme activity is defined as the amount of enzyme required to liberate half of the peroxidase oxygen from H$_2$O$_2$ at 25°C and is calculated from the following equation:

\[
\text{Units/minute/g tissue} = \frac{\text{Change in absorbance/min} \times \text{Total volume of reaction mixture}}{\text{Extinction coefficient} \times \text{Amount of sample}} \times \text{dilution factor}
\]

where

Extinction coefficient = 2.8 mM$^{-1}$ cm$^{-1}$

3.5.2.4.2. Guaiacol Peroxidase

Guaiacol (GPOX; EC 1.11.1.7) is used as a substrate for the assay of peroxidase. The methodology used for guaiacol estimation was as given by Putter (1974).

Principle

\[
\text{Guaiacol + 4H}_2\text{O}_2 \xrightarrow{\text{POD}} \text{Oxidised guaiacol} + 2\text{H}_2\text{O}
\]

The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the POD activity and can be assayed spectrophotometrically at 436 nm.
**Reagents**
1) 0.1 M Phosphate buffer (pH 7.0)
2) 20 mM Guaiacol
3) 12.3 mM Hydrogen peroxide (H₂O₂)

**Estimation**

The assay mixture consisted of following reagents which were prepared separately and mixed at the time of taking readings in given order- 3 ml phosphate buffer, 50 µl guaiacol, 30 µl H₂O₂, and 100 µl enzyme extract. The increase in absorbance was recorded on the UV–VIS Spectrophotometer at 436 nm at an interval of 6 seconds for a total of 1 minute.

**Calculations**

One unit of enzyme activity is defined as the amount of enzyme required to liberate half of the peroxidase oxygen from H₂O₂ at 25°C, the enzyme activity per litre of extract is calculated as below:

\[
\text{Units/minute/g tissue} = \frac{\text{Change in absorbance/min} \times \text{Total volume of reaction mixture}}{\text{Extinction coefficient} \times \text{Amount of sample} \times \text{dilution factor}}
\]

where

Extinction coefficient = 25.5 mM⁻¹ cm⁻¹

**3.5.2.5. Glutathione Reductase**

Glutathione reductase (GR; EC 1.6.4.2) is a flavoprotein that is required for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). At the same time, it oxidizes nicotinamide adenine dinucleotide phosphate (NADPH). Its activity was determined by using the method of Carlberg and Mannervik (1975).

**Principle**

\[
\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The oxidation of NADPH to NADP⁺ is accompanied by decrease in absorbance at 340 nm, thus providing a spectrophotometric means of detection which is directly proportional to GR activity in the sample.
Reagents
1) 50 mM Phosphate buffer (pH 7.6)
2) 3 mM Ethylenediaminetetraacetic acid (EDTA)
3) 0.1mM NADPH
4) 1mM GSSG

Estimation
The assay mixture consisted of 2 ml sodium phosphate buffer, 300 µl GSSG, 300 µl EDTA, 300 µl NADPH and 100 µl enzyme extract. The reaction was initiated by the addition of 300 µl of NADPH at 25°C. The GR activity was determined spectrophotometrically by the oxidation of NADPH at 340 nm with extinction coefficient of 6.22 mM⁻¹ cm⁻¹ at an interval of 10 seconds for two minutes.

Calculations
One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μM of NADPH min⁻¹ g⁻¹ tissue at 25°C. The enzyme activity was calculated from the equation below:

\[
\text{Units/minute/g tissue} = \frac{\text{Change in absorbance/min} \times \text{Total volume of reaction mixture}}{\text{Extinction coefficient} \times \text{Amount of sample} \times \text{dilution factor}}
\]

where

Extinction coefficient = 6.22 mM⁻¹ cm⁻¹

3.5.2.6. Glutathione Peroxidase
Glutathione peroxidase (GPOD; EC 1.11.1.9) is found in cytoplasmic and mitochondrial fractions of cells. It catalyzes the reduction of hydroperoxides, including hydrogen peroxide by reduced glutathione and helps to protect the cell from oxidative damage. Enzyme activity was estimated by following the methodology of Flohe and Gunzler, 1984.

Principle
The activity of GPOX is coupled to glutathione reductase (GSSG-R), which maintains reduced glutathione (GSH) levels. Using glutathione (GSH) as a reducing reagent, GPOX catalyze the reduction of H₂O₂ and organic peroxides (R-O-O-H) to water and the corresponding stable alcohol thus inhibiting the formation of free radicals.
Materials and Methods

Reagents
1) 50 mM Potassium phosphate buffer (pH 7.0)
2) 0.5 mM Ethylenediaminetetraacetic acid (EDTA)
3) 1mM Glutathione reduced (GSH)
4) 0.15 mM Sodium azide (NaN₃)
5) 0.15 mM NADPH
6) 0.15 mM Hydrogen peroxide (H₂O₂)

Estimation
The reaction mixture contained 500 µl sodium phosphate buffer, 100 µl EDTA, 100 µl of GSH, 100 µl NaN₃, 100 µl NADPH, 100 µl H₂O₂ and 50 µl enzyme extract. The enzyme activity was monitored by the oxidation of NADPH at 340 nm at an interval of 10 seconds for a total of one minute.

Calculations
One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μM of NADPH min⁻¹ g⁻¹ tissue at 25°C and is calculated from the equation below:

\[
\text{Units/minute/g tissue} = \frac{\text{Change in absorbance/min} \times \text{Total volume of reaction mixture}}{\text{Extinction coefficient} \times \text{Amount of sample} \times \text{dilution factor}}
\]

where
Extinction coefficient = 6.22 mM⁻¹ cm⁻¹

3.5.2.7. Superoxide Dismutase
Superoxide dismutase catalyzes the dismutation of superoxide radical in a broad range of organisms, including plants. The dismutation of superoxide into hydrogen peroxide and oxygen constitute the first line of cellular defence to prevent undesirable biological oxidation by oxygen radical generated during cellular metabolism.
Superoxide dismutase (SOD; EC 1.15.1.1) was estimated as per the methodology given by Kono (1978).

**Principle**

The principle involves the inhibitory effects of SOD on reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are generated by the auto-oxidation of hydroxylamine hydrochloride. It catalyzes the destruction (dismutation) of superoxide free radical ions as follows:

$$2O^2- + 2H_3O^+ \xrightarrow{SOD} O_2 + H_2O_2 + 2H_2O$$

The superoxide ion ($O^2-$) is believed to be responsible for lipid peroxidation and peroxidative hemolysis of erythrocytes.

**Reagents**

1) 50 mM Sodium carbonate buffer (pH 10.0)
2) 96 µM Nitroblue tetrazolium (NBT)
3) 0.6 % Triton X-100
4) 20 mM Hydroxylamine hydrochloride (NH$_2$OH.HCl) (pH 6.0)

**Estimation**

The assay mixture consisted of 1.3 ml sodium carbonate buffer, 500 µl NBT, 100 µl triton X-100, 100 µl hydroxylamine hydrochloride and 70 µl enzyme extract. The increase in absorbance was recorded on UV-VIS Spectrophotometer at 540 nm for 2 minutes at an interval of 6 seconds.

**Calculations**

Hydroxylamine hydrochloride is auto-oxidized by superoxide radicals to nitrite in the form of blue formazone. The addition of NBT to the above reaction mixture induces an increase in absorbance, stimulating oxidation of hydroxylamine and reduction of NBT. With the addition of enzyme, SOD, superoxide radicals get trapped and hence there is an inhibition of reduction of NBT to blue formazon formation. The percent inhibition of NBT reduction is calculated as below:

$$X = \frac{\text{Change in absorbance/min(Blank)} - \text{Change in absorbance/min(Test)}}{\text{Change in absorbance/min(Blank)}} \times 100$$
where

\[ X \text{ is inhibition induced by } 70 \mu l \text{ of sample} \]

Hence, 50% inhibition is produced by

\[ \frac{50 \times 70}{X} = Y \mu l \text{ of sample} \]

3.5.3. Non-Enzymatic Antioxidants

For estimation of phenols and flavonoids, roots and shoots were separately weighed. Each was crushed in a pre-chilled pestle and mortar using 80% methanol. The extract was collected in 1.5 ml centrifuge tubes and then centrifuged at 10000 rpm for 20 minutes at 4°C. The supernatant so collected was used for estimations of antioxidants while the pellet was discarded.

3.5.3.1. Total Phenolic Content

Phenols, aromatic compounds with hydroxyl groups, are widespread in plant kingdom. They are said to offer resistance to diseases and pests in plants. Total phenolic content (TPC) was estimated following the methodology of Malik and Singh (1980) with slight modifications.

**Principle**

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

**Reagents**

1) Folin-Ciocalteau Reagent
2) 20% Sodium carbonate

The assay mixture consisted of following reagents which were prepared separately and mixed in the given order– 100 µl extract, 2.9 ml distilled water, 0.5 ml folin-ciocalteau reagent (1:1 with water) and then 2 ml sodium carbonate in a test tube. The contents of test tube were vortexed and were warmed for one minute using warm water and then cooled. Blue colour developed in each tube. The O.D. was recorded at 760 nm on UV-VIS Spectrophotometer. The blank consisted of all constituents except the extract. A standard calibration plot was generated at 760 nm using known concentration of catechol as standard.
Calculations
Total phenolic content of sample was calculated using the given equation:

\[
\text{mgTPC/g tissue} = \frac{\text{Absorbance of test} \times \text{Conc. of standard} \times \text{Total volume}}{\text{Absorbance of standard} \times \text{Volume of sample taken}} \times \text{dilution factor}
\]

3.5.4.2. Total Flavonoid Content
Flavonoids are a large group of compounds having a central (flavone) structure. They are widely distributed in the plant kingdom, especially among the woody species. Flavonoid derivatives constitute many of the vivid colours seen in flowers, fruits, and autumn leaves. Flavonoids play a role in protecting plants from both insect and mammalian herbivory. Total flavonoid content (TFC) was estimated using AlCl₃ method given by Lamison and Carnet in 1990.

Reagents
1) Methanol
2) 10% Aluminium chloride (AlCl₃.6H₂O; 2 gm in 100 ml methanol)
3) 5% Sodium potassium tartarate

Aliquots of extract (100 µl) were taken and made the volume to 3 ml with methanol (2.9 ml). Then 100 µl AlCl₃.6H₂O, 100 µl Sodium potassium tartarate and 0.5 ml distilled water were added sequentially. The mixture was vigorously shaken and absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using known concentration of rutin.

Calculations
The concentrations of flavonoid content in the test samples were calculated as given below:

\[
\text{mgTFC/g tissue} = \frac{\text{Absorbance of test} \times \text{Conc. of standard} \times \text{Total volume}}{\text{Absorbance of standard} \times \text{Volume of sample taken}} \times \text{dilution factor}
\]

3.5.3.3. Ascorbic Acid Content (Vitamin C)
Ascorbic acid content (AsC) was estimated using the methodology of Chinoy, 1962.
Principle

The hydrogen atoms of the two enol groups of ascorbic acid can be readily oxidized making ascorbic acid a strong reducing agent. The dye 2,6 dichlorophenol-indophenol (DCIP) is blue in alkali and can be reduced by ascorbic acid to a colourless “leuco” form. If a drop of the blue dye is added to an acidified extract, it will turn pink, then colourless.

![Fig.13. Ascorbic acid and its oxidation to ascorbate and dehydroascorbic acid](image)

![Fig.14. Reduction of DCIP to DCIPH₂](image)

Preparation of Homogenate

Roots and shoots were weighed and crushed separately in a pestle-mortar in 2% Meta phosphoric acid. These were then centrifuged at low speed (2500 rpm) for fifteen minutes. The residues were discarded and the supernatants were used for the estimation of ascorbic acid.

Reagents

1) 2% Meta phosphoric acid
2) 5% Meta phosphoric acid
3) n-Amyl alcohol
4) 2, 6-dichlorophenol-indophenol (5mg/100ml)

Each of the 100 µl test solutions were mixed with 200 µl of 5% Meta phosphoric acid and kept for 30 minutes without stirring at room temperature. 500 µl of n-amyl
alcohol and 320 µl of dye (2, 6-dichlorophenol-indophenol) were added. Each test tube mixture was shaken vigorously and upper layer was used for the estimation of ascorbic acid at a wavelength of 546 nm. Blank consisted of all reagents except dye. A standard calibration plot was generated at 546 nm using known concentration of ascorbic acid as standard.

**Calculations**

Total ascorbic acid content of the sample was calculated from the given equation

\[
\text{mgAsC/g tissue} = \frac{\text{Absorbance of test} \times \text{Conc. of standard} \times \text{Total volume}}{\text{Absorbance of standard} \times \text{Volume of sample taken}} \times \text{dilution factor}
\]

**3.5.3.4. Total Glutathione Content**

The glutathione content (GSH) was determined by the method given by Sedlak and Lindsay (1968).

**Principle**

The spectrophotometric procedure is based upon the use of Ellman’s reagent. According to the principle, 5,5’- dithiobis-(2-nitrobenzoic acid) (DTNB) is reduced by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of GSH. 2-nitro-5-mercaptobenzoic acid, so produced, has an intense yellow colour and can be used to measure –SH groups.

![Fig.15. Reduction of 5,5’- dithiobis-(2-nitrobenzoic acid) to 2-nitro-5-mercaptobenzoic acid (Colourless-Yellow)](image)

**Preparation of Homogenate**

The plant extract for glutathione content was prepared by homogenising 1 gm of fresh plant tissue (both root and shoot) in pestle and mortar under ice cold conditions in 10 ml of 0.02M disodium salt of Ethylenediaminetetraacetic acid. The homogenate was
centrifuged at 3000 g for 15 minutes at 4°C. The pellet was discarded while supernatants were used for the assay and were kept ice cold until used.

**Reagents**

1) 0.2M Tris buffer containing 0.2M disodium salt of Ethylenediaminetetraacetic acid (pH8.2)
2) 0.1M 5,5'- dithiobis-(2-nitrobenzoic acid) (DTNB)
3) Absolute methanol

For total glutathione estimation, 500 µl of extract was added in a test tube followed by 750 µl Tris buffer (pH 8.2), 50 µl DTNB and 3.95 ml absolute methanol. The test tubes were shaken and were kept at room temperature for 15 minutes. The absorbance was recorded at 412 nm against blank which consisted of all the reagents excluding extract. The standard curve was prepared from stock solution of glutathione (GSH; 1mg/ml) dissolved in 0.02M disodium salt of Ethylenediaminetetraacetic acid.

**Calculations**

The total glutathione content of the sample was calculated from the equation given below:

\[
\text{mgGSH/g tissue} = \frac{\text{Absorbance of test} \times \text{Conc. of standard} \times \text{Total volume}}{\text{Absorbance of standard} \times \text{Volume of sample taken}} \times \text{dilution factor}
\]

**3.6. QUALITATIVE ANALYSIS OF PHYTOHORMONES**

Phytohormones are the compounds (natural/synthetic) that regulate plant growth and development. These hormones also play their role in cellular responses during stress. Some of the hormones actively involved includes: auxins, abscisic acid, salicylic acid, jasmonic acid, putrescine etc. The presence of indigenous phytohormones were observed by using Agilent 1100 LC coupled with Bruker make mass spectrometer model Esquire 3000. LC/MS analysis was carried out at the Indian Institute of Integrative Medicine (IIIM), Jammu (Council of Scientific and Industrial Research, Govt. of India).

**3.6.1. Principle**

LC/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry. The principle of LC/MS is based on the
Materials and Methods

Fragmentation of charged ions and detection of the resulting fragments. The major advantage of LC/MS is its very high sensitivity and selectivity and hence, makes it possible to elucidate metabolite structures (Ardrey, 2003).

Due to the incompatibilities of the two techniques, an interface (i.e. a particle beam type) is required that separates sample from solvent and allows introduction of sample in the form of dry particles into high vacuum region. It then eliminates the solvent and generates gas phase ions, thereby transferring them to the optics of mass spectrometer.

![Graphical representation of LC/MS](image)

Fig.16. Graphical representation of LC/MS

3.6.2. Sample Preparation

Qualitative analysis of phytohormones was carried out using the methodology of Banerjee and Kulkarni (2011). Solvents used were of HPLC grade and the samples were prepared as followed:
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Fig. 17. Schematic representation of steps followed for sample preparation

1g fresh plant material

Homogenization in 8 ml of extraction solvent (methanol: water; 80:20)

Vortex and Centrifugation at 10,000 rpm for 15 min at 4°C

Supernatant collection

Repeat extraction three times

Combine supernatants

Dilute 0.4 ml with 1 ml methanol

Inject 20 μl for analysis

Sample analyzed by LC/MS
3.6.3. **Methodology**

The specifications used for hormone estimation were as followed:

<table>
<thead>
<tr>
<th>Specifications</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>C18</td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>0.2 µl/min</td>
</tr>
<tr>
<td><strong>Total Run time</strong></td>
<td>20 min</td>
</tr>
<tr>
<td><strong>Column temperature</strong></td>
<td>40 °C</td>
</tr>
<tr>
<td><strong>Mobile phase A</strong></td>
<td>Water (0.5% Formic acid)</td>
</tr>
<tr>
<td><strong>Mobile phase B</strong></td>
<td>Methanol</td>
</tr>
<tr>
<td><strong>Operating Mode</strong></td>
<td>+ve and -ve</td>
</tr>
</tbody>
</table>

**Table1. Specifications for LC/MS analysis**

3.7. **STATISTICAL ANALYSIS**

To access the significance of differences in various parameters of treated and control plants, the data was subjected to statistical analysis. The following statistical methods were employed:

3.7.1. **Standard Deviation**

The standard deviation measures how widely spread the values are in a data set. If the data points are close to the mean, then the standard deviation is close to zero. If many data points are far from the mean, then the standard deviation is far from zero and was calculated by applying the following formula:

\[ S.D. = \sqrt{\frac{\sum (X_i - X)^2}{N}} \]

where

- S.D. = Standard deviation
- \( X_i \) = Reading in different replications
- \( X \) = Mean value
- \( N \) = Number of readings
3.7.2. **Standard Error (S.E)**

Standard error (S.E.) provides simple measures of uncertainty in a value and was calculated as follows:

\[
S.E = \frac{S.D}{\sqrt{N}}
\]

where

\(N\) = Number of replications

\(S.D\) = Standard deviation

3.7.3. **Analysis of Variance (ANOVA)**

Analysis of variance is used to test the significance of regression lines and F-test is used to find out whether two independent variables differ significantly and is described by Bailey (1995). The value of variance - ratio of ‘F’ is computed as follows.

(a) The sum of square deviation (total sum of squares) is calculated using the formula:

\[
\frac{\sum X^2 - (\sum X)^2}{n}
\]

For calculation of f-ratio, the sum of squares is split into two parts.

(i) Sum of squares due to variance between the samples. This can be obtained by using the formula:

\[
\sum \left( \frac{X^2}{V} \right) - \frac{\sum X^2}{n}
\]

where

\(X^2\) = Squares of observations in each sample

\(V\) = Sum of observations in each sample.

(ii) Sum of squares due to variation within samples that is sum of squares within sample.

\[
= \text{Total sum of squares} - \text{sum of squares between samples}
\]
(b) Variance of each part can be calculated by dividing the sum of squares by the respective degrees of freedom (df)

Total number of df = total number of items - 1 = N-1

(i) df for ‘between’ samples = number of samples - 1 = n-1

(ii) df for ‘within’ samples = total df - df for between samples = (N-1)-(N-1) = n-1

3.7.4. Tukey’s HSD test (Honestly significant difference)

Tukey’s test is a single-step multiple comparison procedure and statistical test generally used in conjunction with ANOVA to find which means are significantly different from one another. It compares all possible pairs of means and if the differences between these means are larger than the range statistic, then the means are reliably different from each other. It is based on a studentized range distribution (q). Tukey’s HSD was designed for a situation with equal sample sizes per group, but can be adapted to unequal sample sizes as well (the simplest adaptation uses the harmonic mean of n-sizes as n). The formula for Tukey’s is:

\[ HSD = q \frac{\sqrt{MSE}}{n} \]

where

q = the relevant critical value of the studentized range statistic

n = is the number of replicates used in calculating the group means