3. MATERIAL AND METHODS

3.1. Study Material

The study material used in this present work was plants of *Brassica juncea*. The effects of castasterone (CS) were studied in *B. juncea* plants under copper (Cu) metal stress. *B. juncea* L. is commonly known as Indian mustard. Other names of this plant are rai, brown mustard seeds, mohair, kadugu or avalu.

B. juncea is an ancient oil yielding crop with approximately 20-25% staple oil. This plant is grown all over India, especially in Bengal, Bihar and Uttar Pradesh. It is a perennial herb, but cultivated as an annual or biennial, and can grow upto 1 m or more in height; with long and erect branches. Lower leaves are petioled, green, ovate to obovate, multiple lobed with frilled edges, and lyrate-pinnatisect. Upper leaves are subentire, short petioled, 30-60 mm long and 2-3.5 mm wide. These plants have rooting depth of 90-120 cm. The flowers of plants are arranged in corymbose racemes, stalked and bright yellow in color. The fruits are oblong linear, 2.5-5.0 cm, siliqua. The seeds are tiny and brownish-red, about 5,660–6,000 per 0.01 kg. RLC-1, the selected cultivar of brassica is grown as winter crop (November- March).

3.1.1 Nutritional value

Indian mustard is good source of Vitamin A, C, and iron. A cupful of it provides approximately 60% of Vitamin A, Vitamin C and about one-fifth of iron requirements recommended for an adult. 100 g of fresh leaves contain the minerals as shown in Table 3.1. The seeds were reported to have 24.6 g protein, 28.4 g total carbohydrate, 6.2 g H_2O_2, 8.0 g fiber, 35.5 g fat and 5.3 g ash. Traditionally, this plant is used to cure arthirits, rheumatism, foot ache and lumbago. Indian mustard oil is used for the treatment of ulcers and skin eruptions. It is believed to be mild laxative and tonic. In Java, this plant is used as antisyphilic emmenagogue. Its leaves are used to relieve headache.

Table 3.1 Minerals present in 100 g of leaves of *B. juncea* plants

Mineral	**Content**
Calories	24
Proteins	2.4 g
Fiber	1.0 g
H_2O_2	91.8 g
Fat	0.4 g
Ash	1.1 g
Fe	2.7 g
Ca	160 mg
Na	24 mg
P	48 mg
K	297 mg
Thiamine	0.06 mg
Niacin	0.8 mg
Riboflavin	0.14 mg
Ascorbic acid	21 mg

3.2 Raising of Plant Material

The certified seeds for the present study were purchased from Punjab Agricultural University, Ludhiana, Punjab (India). Healthy and uniform sized seeds were selected for each experiment. These seeds were surface sterilized with cween-20 for five minutes. The seeds were then rinsed thrice with double distilled water to remove the remnants of cween-20. These seeds were soaked in different concentrations of castasterone. These pre-soaked seeds were grown either in pots or in Petri Plates.

3.2.1 In earthen pots

Equal sized earthen pots (25x25 cm) were filled with an equal quantity of soil mixed with farmyard manure in the ratio of 3:1 during the winter season. The experimental period extended from early October to late November. The pots were

organized in simple randomized block design in Botanical Garden of Guru Nanak Dev University, Amritsar, Punjab, India. Well-aerated soil was obtained from the Botanical garden of Guru Nanak Dev University. The soil was analyzed for its physico-chemical parameters (pH and N, P, K content).

3.2.2 *In vitro* conditions:

The surface sterilized and 8 hour CS presoaked seeds were grown on *Whatman* No. 1 filter paper lined in autoclaved Petri dishes (10 cm diameter). 20 seeds per Petri dish were sown. Each Petri Plate was supplied with 3 ml of different concentrations of Cu (0, 0.25 mM, 0.50 mM and 0.75 mM) on first day and 1 ml of solution on every alternate day. Control seedlings were provided DDW only. The seedlings were grown in controlled conditions of 25 ± 0.5°C, 16 h photoperiod, and 175 μmol $m^{-2}s^{-1}$ light intensity for 7 days.

3.2.3 Chemicals

3.2.3.1 Hormones

The selected plant hormone castasterone was purchased from SciTech, Praha. The mother stock of castasterone solution (10^{-3}) M was prepared by dissolving it in HPLC grade methanol and stored at -20°C. The required different working stock solutions of CS were (10^{-11} M, 10^{-9} M, and 10^{-7} M) prepared by serial dilution of mother stock.

3.2.3.2 Heavy metal

The source of copper metal used in the present study was copper sulphate ($CuSO_4.5H_2O$) L.R. (SD Fine-Chem Pvt. Ltd., Mumbai, India). The mother stock of 10 mM was prepared and stored. The IC-50 value of the metal was calculated from the range 0 mM to 5 mM. After calculating the IC-50 value, the working final concentrations were selected. To maintain homogenous concentrations, three concentrations were selected (0.25 mM, 0.50 mM and 0.75 mM).

3.2.4 Treatments

Various concentrations of copper and hormone were selected in combination or alone. Following treatments were used:

1. 0 mM Cu and 0 mM CS (DDW only)
2. 10^{-11} M CS
3. 10^{-9} M CS
4. 10^{-7} M CS
5. 0.25 mM Cu
6. 0.50 mM Cu
7. 0.75 mM Cu
8. 0.25 mM Cu and 10^{-11} M CS
9. 0.25 mM Cu and 10^{-9} M CS
10. 0.25 mM Cu and 10^{-7} M CS
11. 0.50 mM Cu and 10^{-11} M CS
12. 0.50 mM Cu and 10^{-9} M CS
13. 0.50 mM Cu and 10^{-7} M CS
14. 0.75 mM Cu and 10^{-11} M CS
15. 0.75 mM Cu and 10^{-9} M CS
16. 0.75 mM Cu and 10^{-7} M CS

3.2.5 Harvesting of plant samples:

After 7 days of sowing, plants were thinned per pot maintaining 5 replicates of each concentration. These plants were grown under natural climatic conditions with regular water supply. The plants were harvested during three growth phases:

1. Vegetative phase (30 DAS)
2. Flowering phase (60 DAS)
3. Reproductive phase (90 DAS)

The harvested plant material was differentiated into three parts according to analysis:

1. Morphological analysis

2. Biochemical analysis

3. Heavy metal analysis

For biochemical analysis the leaves of *Brassica* was stored in liquid N_2 immediately. For heavy metal analysis the plants were separated into roots, shoots and leaves and oven dried at 80°C for 24 hrs.

3.3 Analysis of *Brassica juncea* plants/seedlings

Harvested plants and seedlings were analyzed for various morphological, physiological, biochemical and molecular parameters. Morphological studies include root, shoot length, fresh, dry weight and dry matter content. Physiological analysis included Cu uptake, gas exchange parameters, cell death, GSH accumulation, NO production, H_2O_2 production and stomatal studies. Biochemical study included studies of antioxidative enzymes, polyphenols, sugars, elemental analysis, cell peroxidation, photosynthetic pigments etc. (Fig. 3.1)

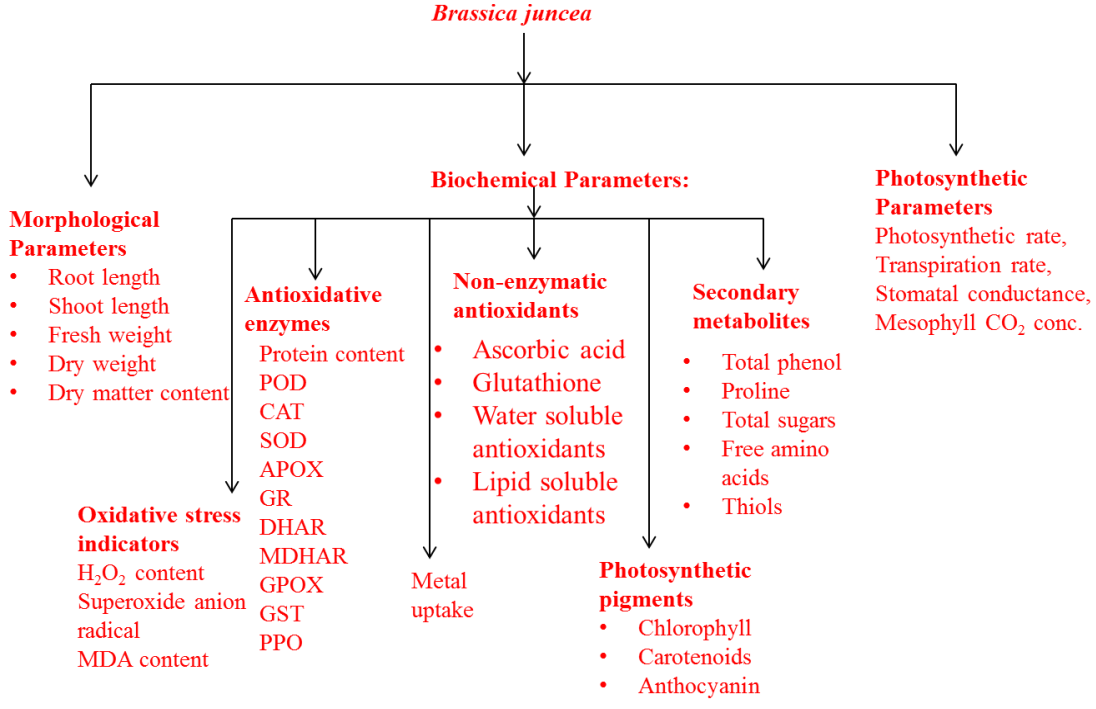

Fig. 3.1 Analysis of *Brassica juncea* plants

3.3.1 Morphological parameters

Various morphological parameters were assessed at various stages of plant growth as specified.

3.3.1.1 Seedlings studies:

7 days old seedlings were removed carefully from the Petri dish and growth of seedlings was observed towards root and shoot length. The fresh and dry biomass of seedlings was also observed. After noting the fresh biomass, the seedlings were oven dried for 24 hours at 80°C to observe the dry biomass. To determine the root, shoot length and fresh, dry biomass twenty seedlings per Petri plate were selected. The experimentation was repeated two times with three replicates.

3.3.1.2 Field grown plants

The plants were harvested on 30^{th}, 60^{th} and 90^{th} days after sowing. 3 plants per pot were selected for analysis of morphological parameters. The plants were removed along with soil from each pot and dipped in bucket full of water to remove the soil particles and to get intact roots. The plants were washed under running tap water. The root length and shoot length was measured using meter scale. The plants were dried on blotting paper and separated into root, shoot and leaves. The fresh biomass of a plant was observed by noting the fresh biomass of root, shoot and leaves separately. Plants were oven dried at 80°C for 48 hours. The root, shoot and leaves were again weighed to observe the dry biomass.

3.3.2 Photosynthesis measurements

Photosynthesis of field grown *Brassica* plants was measured in intact leaves using infrared gas analyzer as a function of carbon dioxide gas exchange. The data file obtained from instrument was converted to Microsoft Excel format and the values were plotted in graph. The same leaf was used for the estimation of photosynthetic pigments.

3.3.2.1 Photosynthetic parameters

Various photosynthetic parameters like transpiration rate, internal CO_2 concentration, stomatal conductance and net photosynthetic rate was measured at selected stages of 30, 60 and 90 DAS. For the analysis and estimation of various photosynthetic parameters portable infra-red gas analyzer (IRGA LI-COR-6400XT,

Lincoln, NE, USA) was used. It is an open gas exchange system in which the CO_2 fixation rate is estimated by measuring the change in concentration of CO_2 of air passing through the chamber.

Second fully expanded leaf of plant was selected in each concentration. The relative humidity, CO_2 concentration, air temperature and photosynthetic photon flux density were maintained at 85%, 600 µmol^{-1}, 25°C and 800 µmol mol^{-2}s^{-1} respectively. The measurements were taken during 10:00 to 12:00 hours under clear sun light.

3.3.2.2 Chlorophyll content

Chlorophyll is a natural pigment present in plants which absorbs solar energy for photosynthesis. Chlorophyll content was estimated using method of Arnon (1949). Chlorophyll has porphyrin ring co-ordinated with central atom. Chlorophyll a and chlorophyll b are two major chlorophylls which differ due to slight change structural composition (-CH_3 in side chain of chl A, while CHO in side chain of chl B). Chlorophyll is soluble in acetone. Visible light is absorbed by chlorophyll a at 663 nm while chlorophyll b absorbs at 645 nm.

1 g of fresh plant tissue was homogenized in 4 ml of 80% acetone and incubated for 24 hours in dark with intermittent shaking. The centrifugation of homogenate was done at 13,000 rpm for 20 min at 4°C. The supernatant was collected and absorbance was noted at 643 and 663 nm.

Calculations:

Total chlorophyll content (µg/ml)

$$= (\text{Absorbance}_{645} \times 20.2) + (\text{Absorbance}_{663} \times 8.3) \times (V/ 1000 \times W)$$

Chlorophyll A content (µg/ml)

$$= (\text{Absorbance}_{663} \times 12.7) - (\text{Absorbance}_{645} \times 2.29) \times (V/ 1000 \times W)$$

Total chlorophyll content (µg/ml)

$$= (\text{Absorbance}_{645} \times 22.9) - (\text{Absorbance}_{663} \times 4.68) \times (V/ 1000 \times W)$$

Where,

V = Volume of plant extract

W = fresh weight

3.3.2.3 Carotenoids content

Carotenoids are pigments found in chloroplast and chromoplast of plants. Carotenoids are tetraterpenoids, produced from 8 isoprene molecules and have 40 carbon atoms. Carotenoids absorb visible light at 400-550 nm and are soluble in acetone. They help in photosynthesis as well prevent damage of chlorophyll. Carotenoids content were estimated by following the method of Maclachlan and Zalik (1963).

1 g of fresh plant tissue was homogenized in 4 ml of 80% acetone and incubated for 24 hours at 4°C. The homogenate was centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was used to note absorbance for carotenoids at 480 and 510 nm.

Calculations:

Total carotenoid content (mg/g)

$= 7.6 (O.D_{480}) – 1.49 (O.D_{510}) \times (V/ d \times W \times 1000)$

Where,

V = Volume of plant extract

W = fresh weight

d = path length of cuvette (1cm)

3.3.2.4 Anthocyanins content

Anthocyanins are water soluble pigments, belonging to flavonoids class synthesized through phenylpropanoid pathway. Anthocyanins behaves as sunscreen, absorb blue-green and UV light and thus protect cells from high light damage. For the estimation of total anthocyanin content method provided by Mancinelli (1984) was followed 530 nm is peak absorption of anthocyanin while 657 nm represents the peak absorbance wavelength for chlorophyll derivatives. The formula A_{530}- 0.25 A_{657} helps in compensating the contribution of chlorophyll derivatives in absorption at 530 nm.

1 g of fresh plant tissue was homogenized in 3 ml of acidified methanol and incubated for 24 hours. The homogenate obtained was centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was used to record absorbance 530 and 657 nm.

Calculations:

Anthocyanin content was calculated using following formula:

$A = \text{Absorbance}_{530} - 0.25\ \text{Absorbance}_{657}$

Total anthocyanin content (mg g^{-1} FW) = $(A \times MW \times DF \times 1000)/\varepsilon$

Where

MW= Molecular weight of cyanidin-3-glucoside)

DF= Dilution factor

ε = Molar absorptivity of cyanidin-3-glucoside (26,900)

3.3.2.5 Stomatal studies

Stomata in leaves of 7-day old *B. juncea* seedlings were studied using scanning electron microscope (SEM, Zeiss, EVO LS10).

Leaves of *Brassica* seedlings were cut from petioles. Then these sections of leaves were mounted on the specimen stub covered with aluminum tape. These leaf sections were studied with the help of SEM under low vacuum, pressure varying from 90-120 Pa and under magnification 1.50 KX.

3.3.3 Elemental analysis

Elemental analysis was done using the atomic absorption spectrophotometry (AAS) and energy dispersive methods (EDS).

3.3.3.1 Acid digestion of plant samples

After harvesting, plants were separated into root, shoot and leaves. The plant parts were washed with deionized water and followed by washing in 0.5 M EDTA. Then, they were dried in hot air oven at 80°C for 24 hours. 1 g of dried plant parts were digested in solution of HNO_3 and $HClO_4$ (2:1 ratio). The samples were digested over hot plate at the temperature 90-95°C until the clear solution (approximately 2 ml) was left. This solution was cooled and diluted with DDW to make the final volume of 100 ml. The samples were then analyzed in triplicate for copper content using atomic absorption spectroscopy (AAS).

3.3.3.2 Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) was used for quantitative estimation of elements of interest (Shimadzu, Japan). DDW in which sample was digested used as reference material. The concentration of metals was noted from the computer interfaced to the instrument. The copper content was calculated from the absorbance using dilution factor and represented as Cu µg g^{-1} DW.

3.3.3.3 Elemental analysis

On the basis of AAS analysis the most effective Cu concentration and CS was used for elemental analysis using energy dispersive X-ray spectroscopy (EDS). It is an analytical technique used for elemental analysis, which relies on interaction of source of X-ray excitation and a sample. Each element has unique atomic structure which allows unique set of peaks on its X-ray emission spectrum. To initiate the emission of characteristic X-rays from a specimen, a high energy beam of charged particles (electron, proton or X-ray beam) is focused on the sample to be studied. Within sample, atom is at ground state which gets excited by energy beams and come out of its shell creating electron hole and an electron from outer shell fills this hole. The difference in energy between higher-energy shell and lower energy shell may release as X-ray. Number and energy of X-ray emitted by sample can be estimated by energy-dispersive spectrometer. The energies of X-ray are due to difference in energy between two shells and atomic structure of emitting element, EDS allows the elemental composition of the sample measured.

Dry leaf samples were examined for elemental analysis by using environmental scanning electron microscope (ESEM) equipped with EDX system. Plant samples were mounted on an aluminium stub and coated with silver (Ag) for 10 min using vacuum system. Elemental composition was recorded in EDS spectrum collected in spot mode (~1 µm) for collection time of 60 s. Elemental composition was quantified using EDX software (INCA) coupled with scanning electron microscope (ESEM). Analysis was done using working distance of 10 mm and operating voltage of 30 KV. Each sample was analyzed in three replicates.

3.3.4 Reactive Oxygen Species (ROS)

ROS like superoxide and hydrogen peroxide were estimated. Localisation of hydrogen peroxide production and superoxide production was done using confocal laser scanning microscopy and digital camera respectively.

3.3.4.1 Hydrogen peroxide estimation

The hydrogen peroxide content was estimated using method proposed by Jana and Choudhri (1981). When an acidic solution of titanium sulphate reacts with hydrogen peroxide, it leads to production of intense yellow color due to the production of pertitanic acid. The intensity of this yellow color can be noted at 410 nm to calculate H_2O_2 content.

$Ti^{4+} + H_2O_2 + 2H_2O = H_2TiO_4 + 4H^+$

Fresh leaves (1g) were homogenized in 3 ml of phosphate buffer (50 mM and pH 6.5) and centrifugation was done at 6,000 g for 20 min at 4°C. Then, 3 ml of extract was mixed with 1 ml of 0.1% titanium sulphate in 20% (v/v) H_2SO_4 and centrifuged at 6,000 g for 15 min. The absorbance of yellow colored supernatant was observed at 410 nm. H_2O_2 content was calculated using extinction coefficient 0.28 μmol^{-1} cm^{-1} and represented as µMol/g FW.

3.3.4.2 Localization of hydrogen peroxide production

A fluorescent dye 2'7' dichlorofluorescein diacetate (2'7' DCF-DA) was used to monitor the production rate of H_2O_2 in 7 days old seedlings of *Brassica juncea* (Murata *et al.,* 2001). 2'7' DCF-DA is cell permeable non-fluorescent probe. 2'7' DCF-DA is de esterified (by esterase) and oxidized (primarily by H_2O_2) intercellularly and become fluorescent 2'7' dichlorofluorescein.

Intact roots of *Brassica* seedlings were stained by submerging the roots in 10 µM fluorescent dye for 15 min and water mounted sections were observed under confocal laser scanning microscope (Nikon A1R confocal laser scanning microscope (CLSM), Japan,) at excitation wavelength of 495 nm and emission wavelength of 530 nm. Intensity of green color in roots represents the rate of H_2O_2 production, thus more green color means more H_2O_2 production.

3.3.4.3 Superoxide anion content

The superoxide anion content was determined following the method of Wu *et al.,* (2010) with some modifications. The production of nitrites from hydroxylamine is observed as diazo compound formed with 3-aminobenzenesulphonic acid and α-naphthylamine in the presence of $O^{2\bullet-}$.

Leaf extract for the estimation of superoxide content was prepared by homogenizing 1 g of fresh leaves in 3 ml of phosphate buffer (50 mM and pH 7.8) and centrifuged for 15 min at 4°C (12,000 g). 0.5 ml of supernatant was mixed with 0.5 ml of phosphate buffer and 0.1 ml of Hydroxylamine hydrochloride and reaction mixture was incubated at 25°C for 30 min. After incubation, 1 ml of incubated solution was mixed with 1 ml of 3-aminobenzene and 1 ml of 1-napthylamine. The reaction mixture was again incubated at 25°C for 30 min. The absorbance was noted at 530 nm and superoxide anion content was calculated from standard curve prepared using $NaNO_2$.

3.3.4.4 Localization of superoxide anion content

The imaging of superoxide anion content was done on 30, 60 and 90 DAS old plants of *B. juncea* using the NBT staining method followed by imaging with Nikon digital camera. For imaging of the $O^{2\ -\bullet}$, the method suggested by Jabs *et al.,* (1996) with slight modifications was used. Leaves of *B. juncea* were vacuum infiltrated with sodium azide solution for 2 min. Then the leaves were vacuum infiltrated with NBT for 2 minutes and incubated for 2 hours in dark. Leaves were decolorized for imaging using solution of boiling glycerol, acetic acid and ethanol (1:1:3). The imaging of leaves was done using Nikon digital camera.

3.3.5 Membrane damage

Membrane damage was represented by malondialdehyde content. Imaging of membrane damage was done using staining of roots with propidium iodide and imaged under confocal laser scanning microscope.

3.3.5.1 Malondiadehyde content

Thiobarbituric acid reactive substances (TBARS) are produced as by product of lipid peroxidation. TBARS assays the malondialdehyde (MDA) present in sample and produced from lipid hydroperoxides by hydrolytic conditions of the reaction. The level

of lipid peroxidation products produced in fresh leaves was estimated by measuring the malondialdehyde (MDA) content following the method of Heath and Pecker (1968). The lipid peroxidation starts with the subtraction of hydrogen atom from unsaturated fatty acid, leading to the formation of lipid radical. This lipid radical reacts with thiobarbituric acid to form pink color chromagen.

Fresh leaves (1g) were homogenized in 3 ml of 0.1% TCA and centrifugation was done at 10,000 rpm for 10 min at 4°C. Supernatant obtained was mixed with 3 ml of TBA and heated in water bath at 95°C for 30 min. The reaction mixture was cooled quickly by placing test tubes in ice bath to stop reaction. The absorbance was noted at 532 and 600 nm. The absorbance was corrected by subtracting the O.D. at 600 nm from O.D. at 532 nm. The MDA content was calculated using the extinction coefficient of 155 $mM^{-1}cm^{-1}$.

3.3.5.2 Cell viability studies

Propidium iodide (PI) binds to DNA of dead cells. PI is membrane impermeable and is excluded from viable cells. It reaches the nuclei of cells by passing through dead cell membranes, and intercalates with DNA double helix of cell. PI is red fluorescent stain. The stock solution of PI is made by dissolving PI in water in concentration of 1 mg/ml (1.5 mM) as stored at -20°C until use. The working solution of 50 µM PI was prepared fresh by diluting the stock solution.

The intact roots of 7 day old seedlings were dipped in 50 µM propidium iodide for 10 min. After incubation, the root fragments were placed on microscopic glass slides and covered with coverslips and was observed/ imaged under confocal laser scanning microscopy at excitation and emission wavelengths of 543 nm and 617 nm respectively. The intensity of red color shows the extent of damage of plasma membrane of cells.

3.3.6 Activity of antioxidant enzymes

The activity of antioxidative enzymes was assessed through spectrophotometer. Some of the antioxidative enzymes were also visualized using native gel electrophoresis. 7 days old seedlings were harvested and separated from roots. To estimate protein content and antioxidative enzyme activity, 1 g of seedlings were homogenized in 3 ml of phosphate buffer (50 mM, pH 7.0) containing 1 mM PMSF,

0.5% triton X-100, 1 mM EDTA, and 2% PVP-30 using pre-chilled pestle and mortar. For APOX and DHAR activity estimation, 0.5 mm ascorbate was added to extraction buffer. In case of superoxide dismutase analysis, the extraction of 1g seedlings was done in 3 ml of sodium carbonate buffer (50 mM, pH-8.2). For polyphenol oxidase, 1 g of plant material was homogenized in 3 ml of phosphate buffer (100 mM, pH-6.0). The centrifugation of homogenates was done at 12,000 g for 20 min at 4°C. The supernatant was poured in new eppendorf tube and used for analysis of antioxidative enzymes and proteins. In case of pot grown plants, third leaf from apex of plants was removed. 1 g of leaf tissue was homogenized in 3 ml of phosphate buffer (50 mM, pH-7.0) containing 1 mM PMSF, 1 mM EDTA, 2% PVP-30 and 0.5% triton X-100 in pre-chilled pestle and mortar. Further, the extracts were prepared using method described above. After quantification of proteins, enzyme activity was expressed as specific activity.

3.3.6.1 Catalase *(CAT, EC 1.11.1.6)*

Catalase activity was measured using the method provided by Aebi (1984). The catalase activity was assayed by disappearance of H_2O_2 and/or liberation of O_2. The decrease in extinction per unit time at 240 nm gives the measure about the decomposition of H_2O_2 in solution. CAT activity is measured by decrease in extinction per unit time.

Reaction mixture (3.0 ml) prepared containing 50 mM phosphate buffer (50 mM, pH 7.0), H_2O_2 (15 mM) and 100 µl plant extract. The decrease in absorbance was noticed at 240 nm for 1 min with 3 sec interval at 25°C. The amount of enzyme requisite to release half the peroxide oxygen from H_2O_2 represents one unit of enzyme and calculated from the following equation.

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 39.4 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g-1 FW)}}$$

3.3.6.2 Studies on isoenzymes of catalase

For catalase profiling, the enzyme was extracted using the phosphate buffer (50 mM, pH-7.0) containing 1 mM PMSF. Equal protein content (50 µg) was loaded in each well and subjected to native discontinuous PAGE omitting SDS (Laemmli, 1970). The gel was run at 4% stacking gel and 12.5% resolving gel for 3 hours at 4°C under constant current of 25 mA per gel. After pre-electrophoretic separation, gel was stained for catalase enzyme activity using the method provided by Woodburry *et al.*, (1971). Gel was incubated in 5 mM H_2O_2 for 10 min. Then the gel was 2 times washed with double distilled water and stained with reaction mixture containing 1% (w/v) ferrocyanide and 1% (w/v) ferric chloride until the transparent bands were visualized. Transparent bands were illuminated against green background. Reaction was stopped using tap water (Woodburry *et al.*, 1971).

3.3.6.3 Guaiacol peroxidase *(POD,* EC 1.11.1.7*)*

Guaiacol peroxidase activity was measured following the method of Sànchez (1995). The dehydrogenation of various organic molecules like aromatic amines, phenols etc. and particularly guaiacol, pyrogallol etc. is catalyzed by peroxidases. POD activity can be ascertained by decrease of hydrogen donor, H_2O_2 or formation of oxidized compounds (this can be achieved using number of compounds). The end product formed is known as guaiacol dehydrogenation product (GDHP). The rate of formation of (GDHP) represents the measure of POD activity and noted at 436 nm.

Reaction mixture (3.0 ml) prepared contained 50 mM phosphate buffer (50 mM, pH 7.0), H_2O_2 (12.3 mM), guaiacol solution (20 mM) and 100 µl plants extract. The increase in optical density was noticed at 436 nm for 1 min with 3 second interval at 25°C. The amount of enzyme required to catalyze the formation of 1 µM of GDHP $min^{-1}g^{-1}$ FW represents one unit of enzyme and calculated as follows:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 26.6 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{}$$

Protein content (mg g-1 FW)

3.3.6.4 Studies on isoenzymes of Guaiacol peroxidase

To prepare enzyme extract for POD, 1 g of plant tissue was homogenized in sodium acetate buffer (50 mM, pH 4.0) containing 1 mM PMSF (phenyl methyl sulfonyl fluride). The homogenized plant material was centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was mixed in equal volume with Laemmli sample buffer. Equal content of protein (50 µg) was loaded in each well and run under discontinuous PAGE with non-denaturing and non-reducing conditions described by Laemmli (1970). Electrophoretic separation of samples was done at 4°C for 3 hours at constant current of 25 mA per gel. After pre-electrophoretic separation, gels were stained for POD activity using the method given by Fielding and Hall (1978). Gel was pre incubated for 15 min in 25 mM phosphate buffer (pH-7.0) to lower the pH. The gel was incubated in freshly prepared solution of phosphate buffer (25 mM, pH 7.0) containing 18 mM guaiacol and 25 mM H_2O_2. The gels were incubated in this solution till the brown color bands of enzyme were visualized.

3.3.6.5 Ascorbate peroxidase *(APOX, EC 1.11.1.11)*

APOX activity was estimated according to the method provided by Nakano and Asada (1981). This enzyme is highly specific to plants and is present in cytosol, chloroplast, vacuoles and apoplastic space of leaf cell. It scavenges H_2O_2 using substrate ascorbate. Decrease in absorbance at 290 nm is noted which shows the rate of oxidation of ascorbate.

Reaction mixture (3.0 ml) was prepared containing 50 mM phosphate buffer (50 mM, pH 7.0), H_2O_2 (1.0 mM), ascorbate (0.5 mM) and 100 µl enzyme extract. The decrease in optical density was noticed at 290 nm for 3 min at 6 sec interval at 25°C. The amount of enzyme required to oxidize 1 µM of ascorbate $min^{-1}g^{-1}$ FW represents one unit of enzyme and calculated from the following equation:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 2.8 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{}$$

Protein content (mg g^{-1} FW)

3.3.6.6 Dehydroascorbate reductase *(DHAR, EC 1.8.5.1)*

Dehydroascorbate activity was assayed using the procedure provided by Dalton et al. (1986). The reduction of dehydroascorbate involves oxidation of GSH to form ascorbate and GSSG is catalyzed by DHAR. DHAR activity can be measured by tracking the increase in absorbance at 265 nm.

Reaction mixture (3.0 ml) to assay the activity of enzyme was comprised of 50 mM phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM), GSH (2.5 mM), Dehydroascorbate (0.2 mM) and 100 µl enzyme extract. The increase in optical density was noticed at 265 nm for 1 min at 3 sec interval at 25°C. The amount of enzyme required to catalyze the formation of 1 M of ascorbate $min^{-1}g^{-1}$ FW represents one unit of enzyme and calculated as follows:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 14 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g-1 FW)}}$$

3.3.6.7 Superoxide dismutase *(SOD, EC 1.15.1.1)*

Superoxide dismutase activity was measured using the method given by Kono *et al.,* (1978). Autoxidation of hydroxylamine occurs at pH 10.2 in the presence of EDTA accompanied by reduction of nitroblue tetrazolium (NBT) leading to the production of nitrite (NO_2^-). The reduction of NBT can be seen by increase in absorbance at 560 nm. The SOD activity can be assayed by measuring the potential of enzyme to reduce the reduction of NBT.

Reaction mixture (3.0 ml) contained sodium carbonate buffer (50 mM, pH 10.2), EDTA (0.1 mM), hydroxylamine hydrochloride (1 mM), NBT (24 µM), triton X-100 (0.03%) and 100 µl enzyme extract. The increase in absorbance was noticed at 560 nm for 2 min at 6 sec interval at 25°C. The amount of enzyme required to cause 50% of NBT reduction is defined as one unit of enzyme and calculated as follows:

% reduction of NBT was calculated as given:

$$\frac{\text{Change in Abs/min (Blank) - Change in Abs/min (Sample)}}{\text{Change in Abs/min (Blank)}} \times 100 = y$$

Where, y (%) of inhibition is produced by 70 µl of sample. Thus, 50% inhibition will be produced by:

$$\frac{50\times70}{Y} = z \text{ µl of sample}$$

One unit of enzyme activity is represented by the enzyme concentration required for 50% reduction of absorbance at 560 nm of chromogen production in one minute under the assay conditions. Thus, activity and specific activity is calculated as:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Total volume (ml)}}{z \times \text{FW of sample}}$$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g-1 FW)}}$$

3.3.6.8 Studies on isoenzymes of SOD

For superoxide dismutase isoenzyme profiling, the enzyme was extracted in Tris-HCl. 1 g of fresh plant material was homogenized in 1 ml of Tris-HCl buffer (50 mM, pH-7.0) containing 50 mM NaCl, 0.05% Tween-80 and 1 mM PMSF. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and used for electrophoretic separation of SOD. 50 µg of total soluble protein with non-reducing Laemmli sample buffer was loaded on 12.5% discontinuous gel. Vertical gel electrophoresis was performed for 3 hours at 4°C. After electrophoresis, the gel was submerged and incubated in 2.5 mM NBT for 25 min. Then, the gel was soaked in 50 mM phosphate buffer (pH-7.8) containing 28 µM riboflavin and 28 mM TEMED for 20 min. After incubation, gel was exposed to light for 10-15 min at room temperature. The gel became uniformly blue during light exposure except the places of SOD activity. When maximum contrast between achromatic zone and blue color was

achieved illumination was stopped. The imaging of gels was done using the gel documentation system.

3.3.6.9 Polyphenol oxidase *(PPO, EC 1.10.3.1)*

PPO are copper containing enzymes which catalyze oxidation of phenolic substances to quinines under aerobic conditions, which further auto-oxidize to dark brown substance known as melanins and estimated spectrophotometrically at 495 nm. PPO was measured by using the method provided by Kumar and Khan (1982).

Reaction mixture containing 1 ml of phosphate buffer (100 mM, pH-6.0), 0.5 ml of 100 mM catechol and 0.25 ml enzyme extract was incubated for 2 min. Reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The increase in absorbance was recorded at 495 nm for 1 min at 3 sec interval at 25°C. The amount of enzyme required to is defined as the one unit of enzyme and calculated as follows:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 2.9 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g-1 FW)}}$$

3.3.7 Glutathione dependent enzymes

Activity of few enzymes associated with the cellular glutathione homeostasis like glutathione s-transferase and glutathione peroxidase detoxify electrophiles by conjugating with the GSH and glutathione peroxidase which catalyze synthesis of GSH from GSSG were analyzed. Enzymes were extracted using phosphate buffer (50 mM, pH-7.5)

3.3.7.1 Glutathione reductase (*GR, EC 1.6.4.2)*

Glutathione reductase activity was measured by following the method proposed by Carlberg and Mannervik (1975). GR reduces GSSG through oxidation of NADPH and GR activity can be noticed by decrease in absorbance at 340 nm.

Reaction mixture (3.0 ml) contained 50 mM phosphate buffer (50 mM, pH 7.6), GSSG (1 mM), EDTA (0.5 mM), NADPH (0.1 mM) and 100 µl enzyme extract. The decrease in optical density was noticed at 340 nm for 1 min at 3 sec interval at 25°C. The amount of enzyme required to oxidize the 1 µM of NADPH $min^{-1}g^{-1}$ FW represents one unit of enzyme and calculated as follows:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 6.22 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g}^{-1}\text{ FW)}}$$

3.3.7.2. Glutathione peroxidase (*GPOX, EC 1.11.1.7)*

Glutathione peroxidase activity was estimated using the method given by Flohe and Gunzber, 1985. GPOX catalyzes the reduction of H_2O_2 and a large variety of organic peroxides to water and stable alcohols consuming cellular glutathione as reducing reagent. In the method used, formation of glutathione disulfide (GSSG) was continuously monitored. The GSSG formed during GPOX reaction is reduced by GR activity which provides constant level of GSH. The simultaneous oxidation of NADPH can be noticed by decrease in absorbance at 340 nm.

Reaction mixture (3.0 ml) contained phosphate buffer (50 mM, pH 7.5), EDTA (0. mM), GSH (1 mM), sodium azide (1 mM), NADPH (0.15 mM), H_2O_2 (0.15 mM) and 100 µl enzyme extract. The decrease in absorbance was recorded at 340 nm for 2 min at 6 sec interval at 25°C. The amount of enzyme required is defined as one unit of enzyme and calculated as follows:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 6.22 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g}^{-1}\text{ FW)}}$$

3.3.7.3 Glutathione-S-transferase (*GST, EC 2.5.1.13)*

Glutathione–S-transferase was estimated using the method given by Habig and Jacoby (1981).

Reaction mixture (3.0 ml) contained phosphate buffer (100 mM, pH 7.5), GSH (1 mM), cDNB (1 mM), and 100 µl enzyme extract. The decrease in absorbance was recorded at 340 nm for 2 min at 6 sec interval at 25°C. The amount of enzyme required is defined as one unit of enzyme and calculated as follows:

$$\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\text{Change in Abs/min} \times \text{Total volume (ml)}}{\varepsilon \times \text{Volume of sample used (ml)} \times \text{FW of sample}}$$

where, ε represents extinction coefficient= 9.6 $mM^{-1}cm^{-1}$

$$\text{Specific activity (UA mg}^{-1}\text{ protein)} = \frac{\text{Unit activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g}^{-1}\text{ FW)}}$$

3.3.8 Estimation of antioxidants

Major antioxidant molecules ascorbic acid and glutathione were estimated spectrophotometrically. For the estimation of antioxidants ascorbic acid and glutathione, 1 g of fresh plant tissue was homogenized in 3 ml of Tris Buffer (50 mM, pH- 10.0) and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant obtained was used for the further analysis.

3.3.8.1 Ascorbic acid

The method given by Roe and Kuether (1943) was followed to estimate the content of ascorbic acid. 2,4-dinitrophenyl hydrazine under strong acidic conditions forms hydrazone. The absorbance of this product is measured at 520 nm using spectrophotometer.

100 mg activated charcoal was added to the 4 ml DDW, 0.5 ml plant extract and 0.5 ml 50% TCA. This reaction mixture was mixed well and filtered through Whatman no.1. To 1 ml filtrate, 0.4 ml DNPH reagent was added. This reaction mixture was incubated at 37°C for 3 hours. After incubation, 1.6 ml of cold 65% H_2SO_4 was added and incubated at room temperature for 30 min. The absorbance of reaction mixture was taken at 520 nm.

Calculations:

$$\text{mg vitamin C/g tissue} = \frac{\text{Absorbance of test} \times \text{Conc. of standard} \times \text{Total Volume}}{\text{Absorbance of standard} \times \text{Volume of sample taken}}$$

3.3.8.2 Glutathione

For the estimation of glutathione content method given by Sedlak and Lindsay (1968) was followed. Ellman's reagent is used for the spectrophotometric estimation of glutathione. In this DTNB is reduced by –SH group to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of GSH. This product has intense yellow colour and is used to measure the thiol groups.

100 µl of plant extract was mixed with 1 ml of Tris buffer and 50 µl of DTNB. Then, 4 ml of absolute methanol was mixed to this and kept at room temperature for 15 min. Then this reaction mixture was centrifuged at 3, 000 g for 15 min at 4°C. The absorbance of mixture was noted at 412 nm.

Calculations:

$$\textbf{mg GSH/g tissue} = \frac{\textbf{Absorbance of test} \times \textbf{Conc. of standard} \times \textbf{Total Volume}}{\textbf{Absorbance of standard} \times \textbf{Volume of sample taken}}$$

3.3.8.3 Localization of glutathione content

Glutathione content was visualized in the roots of 7 days old seedlings of *Brassica juncea* by *in vivo* conjugation with MCB containing 5 mM sodium azide (Hartmann et al., 2003) to form a fluorescent GSB (glutathione-s-bimane) adduct. Approximately 5 mm apical root fragments were incubated in 100 µM MCB for 1 hour. After incubation, root fragments were placed in DDW on a glass slide and covered with cover slip. The water mounted slides were observed under confocal laser scanning microscope with excitation at 405 nm and emission at 475 nm. The intensity of blue color represents the quantity of glutathione in roots.

3.3.9 Estimation of total water soluble and total lipid soluble antioxidants

For the estimation of total water soluble and total lipid soluble antioxidants in 7 days old seedlings, automated photo-chemiluminescent system antioxidant analyzer

(Photochem BU, Analytic Jena Model AG, USA) was used. The superoxide anion free radicals are produced through irradiation of a photosensitizer substance. These radicals are partially removed from the sample by reaction with antioxidants present in sample. The remaining radicals produce luminescence and thus antioxidant capacity of the sample is determined.

3.3.9.1. Total water soluble antioxidants

Total water soluble antioxidative capacity of sample is calculated by comparing with the standard of ascorbic acid (by constructing the calibration curve) and given as ascorbic acid equivalents (nmol AA/ g FW). For the estimation of total water soluble antioxidants 1 g of plant sample was homogenized in 3 ml of Tris-buffer (50 mM, pH-10.2) and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was diluted and 20 µl of diluted samples were mixed with the reagent kits supplied by manufacturer. This reaction mixture was subjected to automated PCL system at ACW mode which measured the total antioxidant capacity of the water.

3.3.9.2. Total lipid soluble antioxidants

The total lipid soluble antioxidative capacity of samples was calculated by comparison with the calibration curve constructed using Trolox as standard and represented as trolox equivalents (nmol TE/ g FW). For the estimation of total lipid soluble antioxidants 1 g of plant sample was homogenized in 5 ml of methanol and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was collected and 100 µl of this supernatant was mixed with the reagent kits supplied by the manufacturer. The reaction mixture was subjected to automated PCL system and run at ACL mode for measuring the total lipid soluble antioxidants.

3.3.10 Metabolites estimation

Various metabolites like sugars, proteins, phenols, flavonoids, and glycine betaine were also studied:

3.3.10.1 Quantitative estimation of sugars

Total soluble sugar content was analysed using anthrone method. In this method, the carbohydrates were first hydrolysed to simple sugars by HCl. The hot acidic medium dehydrates sugars to hydroxymethyl furfural. This compound condenses

with anthrone reagent to form green colored product whose absorption can be measured at 630 nm.

25 mg dried plant sample was soaked in 2.5 N HCl and boiled for 3 hours to make the extract. This extract was neutralized with Na_2CO_3 and final volume was made up to 25 ml with DDW. The centrifugation was done for 10 min at 4°C. 1 ml from supernatant was taken and mixed with 4 ml of anthrone reagent. Reaction mixture was heated for 8 minutes in boiling water bath and then cooled rapidly to stop the reaction. The absorbance was taken at 630 nm. 1 ml distilled water mixed in 4 ml of anthrone reagent served as blank. Total soluble sugar content was calculated from the graph plotted using the standard solution of glucose and represented as mg/g DW.

3.3.10.2 Qualitative estimation of sugars

For the analysis of sugars by HPLC, dried plant sample was ground to fine powder and 1 g of sample was extracted using 10 ml of DDW for 12 hours at room temperature. After incubation the extract was centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant obtained was filtered through micro membrane filter prior to injection to the HPLC. 20 µL of sample was injected to the HPLC. Different carbohydrate solutions used were sorbitol, raffinose, mannitol, sucrose, rahmnose, lactose, galactose, maltose, glucose, dextrose, fructose, xylose and inositol served as standrds. For the HPLC analysis, Refractive Index detector and mobile phase were MilliQ water while other conditions maintained were flow rate: 0.5 ml/ min, oven temperature 80°C and 25 min sample run. The chromatograms were obtained for each sample.

3.3.10.3 Protein content

Protein content was estimated using the method given by Bradford (1976). The protein reacts with Coomassie Brilliant Blue G-250 dye to form protein dye complex of greenish blue color.

Preparation of Bradford reagent: 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% (v/v) ethanol and 100 ml of 85% (v/v) orthophosphoric acid. Final volume of solution was made up to 1 liter when dye was completely dissolved.

The solution was kept overnight in dark and filtered next day using *Whatman* no. 1 filter paper next day.

Total soluble proteins were extracted using phosphate buffer (50 mM, pH-7.0), centrifuged at 13,000 rpm for 20 min at 4°C and supernatant obtained was used to calculate the protein content by microassay method given by Bradford (1976). 3 ml Bradford reagent was added to 50 µl supernatant and mixed thoroughly for 2 min and incubated in dark. After 20 min incubation, the absorbance of blue color of each sample was measured at 595 nm. Total soluble protein content was calculated from the graph plotted using standard solution of bovine serum albumin (BSA).

3.3.10.4 Quantitative estimation of phenols

The reaction of Folin-Ciocalteu (FC) reagent with phenols is basis of colorimetric estimation of phenolic compounds in the sample. The phenols present in sample reacts with the phosphomolybdic acid present in FC reagent and produce blue colored complex in alkaline medium. This can be quantified spectrophotometrically at 650 nm.

Total phenolics content was estimated using the method of Singleton and Rossi (1965). Total phenolic content was extracted from dry plant samples using 60% ethanol. 2.5 ml of this extract was filtered through Whatman filter and diluted to 5 ml. 2 ml of this aliquot was mixed with FC reagent (10 fold diluted with DW). After 5 min 7.5% (w/v) sodium carbonate solution was added. Reaction mixture was incubated for 2 h at room temperature and absorbance was measured at 765 nm against blank prepared with DW. Total phenolic content was calculated from standard curve prepared by using gallic acid as standard.

3.3.10.5 Qualitative estimation of phenols:

Qualitative determination of phenolic compounds was done using UHPLC (ultra-high performance liquid chromatography). For profiling, extracts were prepared by homogenizing 1 g of plant samples in 4 ml methanol. The extracts were allowed to incubate for 24 hours at 4°C. Then the homogenate was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and filtered through micro filters. About 10 µL sample was injected in the UHPLC (Shimadzu Nexera system, USA) coupled

with detector photodiode array. The sample was run under conditions 25° C temperature with flow rate of 1 ml/min at 280 nm and C18 column (150 mm × 4.6 mm) with pore size of 5 µM. Two solvents, 0.01% acetic acid in water as solvent A and methanol as solvent B were used. Analysis of peaks was done using software provided with system. Standards of various polyphenols (gallic acid, epicatechin, caffeic acid, coumaric acid, ellagic acid, quercetin, and kaempferol) were run to obtain calibration curves and obtained by plotting concentration versus peak area. Each compound was detected based on combination of retention time and spectral similarity.

3.3.10.6 Total flavonoids content

Aluminium chloride colorimetric method was used to quantify the total flavonoids content. Aluminium chloride reacts with sample and form acid stable complexes with C-4 keto group and either C-3 or C-5 hydroxyl group of flavones and flavonols. Acid labile complexes with orthohydroxyoxyl groups in A- or B- ring of flavonoids are also formed.

Flavonoids content were estimated using the method of Zhishen *et al.,* (1999). Th**e** flavonoids content was extracted from dry samples using absolute methanol. 0.1 g of dry sample was extracted in 4 ml methanol for 24 hours. In 1 ml extract 4 ml distilled water, 0.3 ml $NaNO_2$ and 0.3 ml $AlCl_3$ was added. It was incubated for 5 min. and then added 2 ml of NaOH. Absorbance was taken at 510 nm. Rutin was used to draw standard curve for the calculation of flavonoid content.

3.3.11 Analysis of metal chelators

Metal chelators (thiols) were estimated using Ellman's reagent i.e. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Free thiols react with 5,5'-dithiobis-(2-nitrobenzoic acid) and reduce it to form 2-nitro-5-thiobenzoic acid. This compound has intense yellow color and is quantified by noting the absorbance at 412 nm. Same procedure is also used for estimation of disulfides present in sample after blocking the free thiols and reducing the disulfides before reaction with reagent.

Metal chelators like thiols were estimated using spectrophotometer. For the estimation of thiols plants samples were extracted in EDTA buffer. Total thiols, non-protein thiols (NPT) and protein thiols (PT) were estimated using the method of Sedlak

and Lindsay (1968). For extraction of thiols, 1 g of leaf samples was homogenized in 3 ml EDTA. Extracts were centrifuged at 13,000 rpm for 15 min. at 4°C. Supernatant was used for estimations.

3.3.11.1 Total thiols

For total thiols estimation, 0.5 ml sample was mixed with 0.2M Tris buffer, 6 mM DTNB and methanol to make final volume 10 ml. The reaction mixture was incubated at room temperature for 15 min and then absorbance was noted at 412 nm.

3.3.11.2 Non-protein and protein thiols

For non-protein thiols, 2 ml sample was mixed with 1 ml DW, 3.06 M TCA and incubated for 15 min at room temperature. Then the centrifugation of reaction mixture was done at 12,000 g for 15 min at 4° C. 2 ml of above supernatant was reacted with 0.4 M Tris buffer (pH 8.9) and 6 mM DTNB. The reaction mixture was incubated at 30°C for 30 min. Absorbance was noted at 412 nm. Calculations were done using extinction co-efficient of 13100 $mM^{-1}cm^{-1}$. Protein thiols were calculated by subtracting the non-protein thiols from total thiols.

3.3.12 Analysis of amino acids

Amino acids such as total free amino acids, proline, and cysteine were estimated by spectrophotometer. Free amino acids were quantified using ninhydrin method. Ninhydrin which is yellow in color reacts with free alpha-amino group (NH_2-C-COOH) present in sample and turns to deep blue color. The profiling of amino acids was also done using amino acid analyzer.

3.3.12.1 Total free amino acids

Total free amino acids were estimated following the method of Hamilton and VanSlyke, 1943. Briefly, after homogenizing leaf samples in phosphate buffer (50 mM, pH 7.0), it was centrifuged at 13,000g for 15 min at 4° C. In 1 ml extract 1ml aqueous pyridine (10%), and 2% ninhydrin was mixed and heated in boiling water bath for 30 min. After heating reaction mixture was diluted to 50 ml with distilled water. Optical density was measured at 570 nm. Amino acid content was calculated using standard curve prepared using leucine.

3.3.12.2 Proline content

Proline content was estimated following the method suggested by Bates *et al.*, 1973. For this 1 g of fresh plant samples was homogenized in 3% sulphosalicylic acid and centrifuged at 13000 g for 15 min at 4° C. In 2 ml supernatant 2 ml of glacial acetic acid and 2 ml of acid ninhydrin was mixed. This reaction mixture was incubated for 1 hour in boiling water bath. Reaction was terminated by placing the test tubes in ice bath. After cooling, 4 ml of toluene was added to the reaction mixture and stirred for 20-30 seconds. Toluene layer was separated and warmed to room temperature. Intensity of red color was absorbed at 520 nm. Standard curve of proline was run for calculation the proline content.

3.3.12.3 Cysteine content

Cysteine content was analysed using the method given by Gaitonde (1967). Cysteine was extracted from plant sample using 5% perchloric acid. 1 g of fresh plant sample was homogenized in 3 ml of perchloric acid and centrifuged for 20 minutes at 4°C. The supernatant was collected and used for the analysis. 1 ml of extract was mixed with 0.5 ml of acetic acid and 0.5 ml of acid ninhydrin. This reaction mixture was kept in boiling water bath for 10 minutes. It was cooled rapidly under tap water and diluted to 5 ml with 95% ethanol. Blank was prepared under same conditions without cysteine. The absorbance was measured at 560 nm and content was calculated from the standard curve prepared using cysteine.

3.3.12.4 Qualitative estimation of amino acids

For amino acid profiling 1 g of 7 days old seedlings were homogenized in 5 ml of HPLC methanol. This mixture was incubated for 24 hours at 4°C in dark. Then homogenate was centrifuged at 13,000 rpm for 20 minutes at 4°C. The supernatant was digested with 5% sulphosalicylic acid for 12 hours. The digested sample was filtered through milipore syringe filters and 3 µl of sample was injected in the amino acid analyzer (Shimadzu, Nexera system, USA). C18 column was used with pore size of 5 µM. The solvent system includes solvent A (0.01% trifluoroacetic acid) and solvent B (acetonitrile). The sample was run at 30°C with flow rate of 1 ml/min. The obtained results were processed for quantitative analysis using the software provided with Shimadzu Amino Acid Analyzer system. Various amino acids like proline, methionine, valine, cysteine, glutamine, were run to plot the standard or calibration curve. Every

amino acid was detected on the basis of combination of retention time and spectral similarity.

3.3.13 Localization of NO

DAF-DA is reagent which is used to detect and quantify low concentrations of NO. It is cell permeable and passively diffuse through cellular membrane. After entering into the cells it became impermeable. It is non-fluorescent, became fluorescent after reacting with NO to form benzotriazole.

Nitric oxide was detected using specific fluorescent probe 4, 5 diaminofluorescein diacetate (DAF-DA; Sigma Aldrich). The roots of *Brassica* were incubated for 1 h at room temperature in darkness in 10 µM DAF- DA solution prepared in tris-HCl (10 mM, pH-7.4) by diluting the 5 mM stock prepared in DMSO (Serrano *et al.*, 2009). The roots were examined using confocal laser scanning microscope using standard filters for DAF- DA (excitation-495 nm; emission 515 nm). Intensity of green color in roots represents labeling of NO, thus more green color means more NO accumulation.

3.3.1 Statistical analysis

Self-coded software was used for statistical analysis of data. Mean and standard deviation was calculated and used for the presentation of data as mean±SD. In order to signify the null hypothesis (H_0), that no two sample means were similar, two way ANOVA was performed on the data. To affirm that different means were significantly different from each other or not, honestly significant difference (HSD) was calculated using Tukey's multiple comparison test.

Multiple regression analysis with interaction was used to determine the type of effect induced by independent variables Cu, CS and their interaction (Cu and CS). β-regression values give relative effects of independent variables: X_1 – Cu, X_2 – CS and X_1X_2 – Cu x CS.