6. SUMMARY

Increasing levels of heavy metals in soil, water and air are becoming one of the major environmental problem to the present world. In trace amounts, they pose serious problems to the entire organisms. Among various heavy metals, copper (Cu) is an essential micronutrient required for the plant growth and metabolism. It is required in various biochemical processes as these are part of many enzymes and proteins. Cu is a cofactor of various enzymes such as plastocyanin, cytochrome c, and Cu/Zn superoxide dismutase, but the excess of Cu adversely affects the plant growth and metabolism. Level of Cu in soil is increasing by excessive use of Cu laden fertilizers and pesticides, and discharge of industrial effluents. As Cu is a redox active metal, it catalyse the production of reactive oxygen species (ROS) like superoxide, hydrogen peroxide and hydroxyl radicals. ROS formation leads to oxidative stress and it damages the essential cellular components and metabolites like DNA, proteins, lipids, nucleic acids, carbohydrates etc. thus impairing the function of cellular membranes and ultimately affecting the plant growth.

Plants adopt various strategies to survive under stressful conditions. These techniques include activation of antioxidative defence system, enhanced production of osmoprotectant ions, metal chelators, and plant growth regulators. Induction of activities of various enzymes act as first line of defence, which help in removal of oxidative burst occurred under stressful conditions. Non-enzymatic antioxidants like ascorbic acid and glutathione helps in the removal of free radicals and helps in cell integrity. In another mechanism, plants induce sulfur assimilation pathway to satisfy the requirement of glutathione needed for the biosynthesis of phytochelatins. These phytochelatins help in the sequestration of heavy metals. Alongwith it, plants produce and accumulate various organic molecules like proline, sugars, flavonoids, free amino acids, cysteine etc. Proline acts as osmoprotectant and shields enzymes from degradation and stabilizes protein synthesis. Various sugars accumulate under stressful conditions and help in maintaining the osmotic balance. Amino acids are known as building blocks of proteins, but various amino acids like cysteine, proline, and methionine help in improving heavy metal tolerance. Cysteine is final product of sulfur assimilation. It acts as sulfur donor for synthesis of some vitamins, methionine and

protein containing thiols. Accumulation of amino acids increase in stressed cells indicating its role in heavy metal tolerance. Polyphenols along with thiols help in metal sequestration and activates the antioxidative system of plants. Steroidal hormones are commonly found in plants and help in ameliorating the toxicity produced in plants under adverse conditions by enhancing the defence potential of plants. Among various brassinosteroids, castasterone have been reported to show strong biological activities.

The present study was therefore planned to explore the physiological and biochemical responses induced by *Brassica juncea* L. plants grown under Cu stress raised from seeds soaked in castasterone (CS) for possible stress protection. It met with following objectives:

1. To observe the effect of castasterone on growth and oxidative stress in *Brassica juncea* under Cu metal stress.
2. To investigate the effect of castasterone on enzymatic and non-enzymatic antioxidants in metal treated *B. juncea* plants.
3. To study the castasterone mediated changes in the levels of osmoprotectants and other molecular players of stress management in *Brassica* plants.
4. To explore the protective effect of castasterone on photosynthetic parameters in metal treated *Brassica* plants.

To achieve the above laid objectives, certified seeds of *Brassica juncea* L var. RLC-1 were purchased from Punjab Agriculture University, Ludhiana, Punjab, India. Seeds were sterilized with washing in cween-20, followed by repeated rinsing in distilled water. The sterilized seeds were soaked in different concentrations of castasterone (0, 10^{-11} M, 10^{-9} M and 10^{-7} M) for 8 h. These seeds were grown in equal sized earthen pots (25 x 25 cm) organized according to randomized block design. Pots were filled with soil containing farmyard manure in ratio 3:1. Each pot was saturated with water (1.25 L) containing different concentrations of $CuSO_4.H_2O$ (0, 0.25, 0.50 and 0.75 mM of Cu). Plants were allowed to grow under natural growth conditions. Plants were harvested at regular interval of 30, 60 and 90 d from each treatment for analysis. *Brassica juncea* seedlings were also raised in Petri plates under controlled conditions of temperature, light and moisture.

Various morphological, physiological and biochemical parameters were studied on the harvested plant samples. Morphological studies include plant growth in terms of root and shoot length, fresh and dry weight and dry matter content at regular intervals. Various physiological parameters included metal uptake, photosynthetic parameters, stomatal studies, cell death, H_2O_2 production, NO production, superoxide anion radical production, glutathione accumulation, and nutrient elemental studies. Copper accumulation in roots, shoots and leaves was determined using AAS (atomic absorption spectrophotometer). Photosynthetic parameters like photosynthetic rate, transpiration rate, intercellular CO_2 concentrations and stomatal conductance were studied in intact leaves of *Brassica* using IRGA (infra-red gas analyser). Stomatal studies were done with SEM (scanning electron microscope). Cell death, H_2O_2 production, NO production and glutathione accumulation was determined in the roots of 7-day old *Brassica* seedlings. Superoxide anion radical accumulation was done by NBT staining of *Brassica* leaves at each harvesting. Elemental nutrients were studied through SEM-EDX.

For biochemical parameters, production of free radicals (H_2O_2 and superoxide anion radical content) and lipid peroxidation (MDA content) were studied spectrophotometerically. Various antioxidative enzymes (SOD, POD, CAT, APOX, DHAR, GR, GST, GPOX and PPO) and antioxidants (ascorbic acid and glutathione) were studied using spectrophotometer. Zymography of enzymes (SOD, POD and CAT) was done by native PAGE. For carbohydrate metabolism, total sugars content (Anthrone method) was estimated spectrophotometerically along with profiling of various sugars by HPLC (high performance liquid chromatography). Total free amino acids (Hamilton and VanSlyke, 1943) and cysteine content (Gaitonde, 1967) was estimated by spectrophotometer. Other amino acids were estimated and quantified through amino acid analyser. Polyphenols were analysed by UHPLC (ultra-high performance liquid chromatography). Total water soluble and total lipid soluble antioxidants were quantified by antioxidant analyser. Thiol content (Total thiols, protein thiol and non-protein thiols) was estimated by following the Sedlak and Lindsey (1968) using spectrophotometer. Osmoprotectants (proline, flavonoids) were also quantified spectrophotometerically. Chlorophyll (chlorophyll a, b and total chlorophyll), carotenoids and anthocyanin content was also analysed spectrophotometrically.

The observations made from the various studies on 7-days old *Brassica juncea* seedlings and field grown 30, 60 and 90 d old plants are as follows:

Physiological studies

1. Metal uptake

Copper uptake in various parts of plants (root, shoot and leaves) increased with the increasing concentration of Cu in the soil. Maximum uptake was observed in the plants grown in 0.75 mM treated soil. Same results were obtained for the seedlings grown with Cu in growth media. Pre-sowing soaking of seeds in the castasterone had increased the metal content in pot raised plants. While in seedlings, application of CS reduced the Cu uptake and accumulation.

2. Growth characteristics

Copper toxicity lead to decrease in root, shoot length, fresh and dry weight of plants and seedlings. Seed soaking in castasterone enhanced the growth of plants seedlings raised in Cu containing growth media.

3. Gas exchange parameters

Gradual decrease in the gas exchange parameters (net photosynthetic rate, stomatal conductance, transpiration rate and intercellular CO_2 concentration) was observed under various levels of copper, where maximum reduction was reported in 0.75 mM of Cu toxicity. The application of castasterone improved the gas exchange parameters.

Biochemical studies

4. ROS production

ROS (H_2O_2 and superoxide anion content) content was increased with the increasing concentration of Cu in growth media. The seeds given pre-sowing soaking in CS decreased the ROS content. Leaves of 30, 60 and 90 d old plants were stained for superoxide anion radical accumulation using NBT. It was observed that accumulation of superoxide anion radical increased with the Cu treatment. While with the application of CS as seed soaking under Cu stress, reduction in accumulation of superoxide anion has been observed.

5. Lipid peroxidation

Membrane damage shown by lipid peroxidation in the leaves of plants at different intervals and in seedlings enhanced with the Cu application. Maximum lipid peroxidation was recorded in the plants grown in 0.75 mM of Cu containing growth media. However, decrease in lipid peroxidation was observed in the plants raised from seeds presoaked with CS.

6. Antioxidative Enzymes

Activity of antioxidative enzymes like SOD, POD, CAT, APOX, DHAR, GR, GST, GPOX and PPO increased in the plants grown in soil amended with various levels of Cu. The presoaking of seeds in CS further enhanced the activities of these enzymes. Native PAGE analysis for isoenzymes of SOD, POD and CAT showed that band intensities increased under Cu as well with application of CS.

7. Antioxidants content

Ascorbic acid and glutathione content increased with the increasing concentration of Cu. The application of CS as seed soaking method enhanced the content of antioxidants.

8. Carbohydrate metabolism

Total sugar content was analysed using spectrophotometer. Increase in the total sugar content was observed in the Cu treated plants and seedlings. CS treatment as seed soaking method increased the total sugar content in leaves of plants and seedlings.

9. Pigment analysis

Plant pigments like chlorophyll (total chlorophyll, chlorophyll a and chlorophyll b), carotenoids and anthocyanin were estimated spectrophotometrically. Chlorophyll and carotenoids content decreased under Cu, maximum decrease was observed in 0.75 mM. The application of CS as seed soaking method improved chlorophyll and carotenoids content. Anthocyanin content was increased in the plants and seedlings treated with Cu alone and further increased in plants raised from seeds soaked in CS.

10. Phenols analysis

Total phenolic content was estimated spectrophotometrically. Cu treatment in the growth media increased the total phenolic content of leaves and seedlings of *Brassica.* CS seed soaking treatment increased the contents of total phenols in plants grown under Cu stress. Polyphenols like catechin, chlorogenic acid, ellagic acid etc. were analysed qualitatively and quantitatively by UPLC. The number of polyphenols and their quantity was increased under Cu stress. Further enhancement in the quantity of polyphenols increased in plants grown from seeds soaked in CS and grown under metal stress.

11. Amino acid analysis

Free amino acids and cysteine content was quantified by spectrophotometer. The content of free amino acids and cysteine was observed to enhance under Cu treatment. The application of CS as seed soaking further enhanced the content of free amino acid and cysteine when grown under Cu stress.

12. Osmoprotectants analysis

Various osmoprotectants like proline, flavonoids were estimated by spectrophotometer. The levels of osmoprotectants increased with the increasing concentrations of Cu. The application of CS as seed soaking further enhanced the levels of osmoprotectants when grown under Cu stress.

13. Thiols analysis

Thiols (totals, protein and non-protein thiols) were estimated spectrophotometrically. The content of thiols increased under various concentrations of Cu. Maximum increase was observed in 0.75 mM Cu grown plants. Further enhancement in the thiol content has been observed in the plants raised from seeds soaked in CS and grown in Cu amended soil.

Following conclusions were made from the studies on the 7-days old *Brassica juncea* seedlings:

Physiological studies

1. Cell death:

Cu stress resulted in increased cell death in the roots of 7 d old seedlings of *Brassica juncea* as observed in confocal microscopy. The co-application of CS as seed soaking decreased the cell death.

2. H_2O_2 production:

It had been found that presence of Cu in growth media of 7 day old *Brassica* seedlings led to enhanced production of H_2O_2 as seen by confocal microscopy. The application of CS reduced the H_2O_2 content in seedlings.

3. NO production:

NO production was enhanced under Cu stress in the roots of 7 d old seedlings of *Brassica.* Further increase in the content of NO was seen in seedlings raised from CS treated seeds and grown under Cu stress.

4. Glutathione content:

The level of glutathione enhanced both under Cu treatment as well with co-application of CS in the roots of 7 d old seedlings of *B. juncea* as observed by confocal microscopy.

5. Stomatal studies:

Stomatal studies were done by SEM. It has been observed that Cu stress in leaves of 7 d old *Brassica* led to closing of stomata. The seedlings raised from seeds soaked in CS and grown in Cu showed open stomata.

Biochemical studies

1. Amino acid analyser:

Level of amino acids like cysteine, methionine, proline etc. which play role in the synthesis of phytochelatins increased in seedlings grown under Cu stress. Further

enhancement in the contents of these amino acids was observed in the seedlings raised from seeds soaked in CS and grown in growth media containing Cu.

2. HPLC analysis:

Carbohydrates like trehalose, xylose, mannitol, sorbitol, fructose etc. were analysed qualitatively by HPLC. Increase in the number of carbohydrates was observed in the Cu treated seedlings and it further enhanced with the application of CS as seed soaking.

3. Antioxidant analyser analysis:

Total water soluble and total lipid soluble antioxidants content was found to increase under various levels of Cu. The plants grown from seeds soaked in CS and raised in Cu amended soil had maximum antioxidants.

It is thus concluded that treatment of seeds with castasterone is an effective method to remove toxicity produced by Cu in *Brassica juncea* plants by activating the antioxidative defence system and elevating the levels of various stress managers.