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24-epibrassinolide mediated regulation of endogenous contents of auxins, abscisic acid, lipids and sugars in *Brassica juncea* L. under copper stress

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Abstract: *Brassinosteroids (BRs) role in increasing plant tolerance to various abiotic stresses has been a well established fact. In the present investigation, we evaluated the effect of seed pre-soaking treatment of 24-epibrassinolide (24-EpiBR) on the soil Cu(II) stress tolerance of 30-day old Brassica juncea L. plants. It increases the ability of plants to survive under stress conditions by modulating the endogenous content of various plant growth regulators and other biomolecules. We observed that the application of 24-EpiBR improved the shoot weight and root weight of the plants under Cu(II) stress. Improvement in the content of various parameters when Cu(II) was supplemented with 24-EpiBR such as sugars (glucose and sucrose); lipids (phospholipids, total sterols and esterified sterols) and plant growth regulators (abscisic acid and auxins), further proved the role of 24-EpiBR in stress amelioration. It reduced the cellular damage by reactive oxygen species (ROS) generated by Cu(II) as evident by the reduction in malondialdehyde (MDA) content with its application under Cu(II) stress. Our study has given a further insight into the functions of 24-EpiBR in increasing plant survival under heavy metal stress in a more elaborate way.*
Keywords: *24-epibrassinolide, copper, auxins, abscisic acid, lipids, sugars*

I. Introduction

Cu is an essential micronutrient required for the normal growth of plants as it is a component of various enzymes and proteins such as superoxide dismutase, cytochrome oxidase, polyphenol oxidase, amino oxidase and plastocyanin etc. But, at high concentrations, it disturbs the metabolic processes and inhibits the growth processes of plants. Cu concentration has increased in the environment due to industrial processes such as mining and smelting; its use as a pesticide (Bordeaux mixture); municipal, industrial and agricultural waste disposal etc. [1,2]. Excess Cu in the environment leads to the adverse effects on plant growth; alters the activities of various important enzymes which are involved in the normal plant metabolic pathways; inhibits photosynthetic electron transport; affects chloroplast integrity and composition of plastid membrane [3]. The adverse effects of Cu when present in excess have led the scientists to focus their research on its toxicity to various living systems. Excess Cu disturbs the electron movement in photosystem I and II and leads to the generation of superoxide radicals that further give rise to the hydroxyl radicals. Peroxidative chain reactions started by these ROS affect membrane lipids and disintegrate cell membranes [4].

Auxins influence many processes in plants during a plant life cycle such as lateral root formation, tropism, pattern formation at the time of embryogenesis and vascular pattering. At the cellular level they control the division of cells, their expansion and differentiation [5]. For optimum growth of plants, indole acetic acid level is highly controlled by regulating its biosynthesis, transport and interconversion of its inactive and active forms [6]. Auxin homeostasis is affected by various types of stresses such as mechanical damage, metal stress, drought, salinity, wounds, cold etc., which further affects the plant growth and development [7,8].

Abscisic acid is a phytohormone and plays important role during seed development and seed dormancy. Along with it, it is a stress hormone and acts as signalling molecule in various environmental stresses such as drought stress, chilling stress, salt stress, heavy metal stress etc. [9,10]. Many studies have reported an increase in abscisic acid levels under the stress of various heavy metals such as Cu, Ni, Cd, Hg etc. [11,12]. An increase in the endogenous content of abscisic acid increased tolerance to Cd in *Oryza sativa* L. seedlings [13]. BRs regulate a number of physiological processes in plants such as differentiation of tracheary elements, cell division, cell elongation, seed germination, cell membrane polarization, senescence etc. In the recent past more research has been focussed on BRs as they are also involved in increasing plant tolerance to various abiotic and biotic stresses. They provide resistance to plants against heavy metal stress by reducing the uptake of metals by roots; increasing the activity of antioxidants and antioxidant enzymes for detoxification of ROS; regulating the endogenous levels of other plant hormones such as auxins, abscisic acid, polyamines etc. [14].

In the present study we investigated the effects of Cu(II) and exogenous 24-EpiBR, alone and in combination, on various parameters such as shoot weight; root weight; the endogenous levels of abscisic acid and auxins such as indole acetic acid (IAA), indole butyric acid (IBA), phenylacetic acid (PAA); contents of lipids such as phospholipids, total sterols and esterified sterols and the contents of sugars such as glucose and sucrose in the 30-day old *B. juncea* plants.

II. Materials and methods

Certified seeds of *B. juncea* were procured from Punjab Agricultural University, Punjab, India. A field was prepared according to randomized block design and different blocks were given the treatment of different concentrations of Cu(II) solutions (0, 0.25, 0.50 and 0.75 mM). Seeds of *B. juncea* were surface sterilized and pre-soaked in different concentrations of 24-EpiBR (0, 0.01, 1 and 100 nM). The seeds were then sown in the field and the plants were harvested after 30 days of the sowing.

2.1 Morphological parameters

Shoot and root weights of the harvested plants were measured.

2.2 Estimation of lipid peroxidation (MDA content)

Lipid peroxidation was determined following the method of Heath and Packer [15]. Homogenised 1 g of the plant sample in 3 ml of 0.1% trichloroacetic acid. Then centrifuged the homogenised samples for 5 min at 10,000 r.p.m. The resulting supernatant was removed and 3 ml of thiobarbituric acid was added into it. The resulting solution was heated in a water bath at 95^{o}C. After 30 min it was removed from the water bath and was cooled immediately for the reaction to stop. Its absorbance was taken at 532 nm and 600 nm.

Lipid peroxidation was calculated in terms of MDA content [µmol g/fresh weight (FW)] formed. It was calculated by the following formula:

$$\text{MDA} = \frac{\text{Absorbance} \times \text{Total volume (ml)} \times 1000}{\text{Extinction coefficient} \times \text{sample volume (ml)} \times \text{weight of plant material}}$$

Here, Absorbance = absorbance at 532nm – absorbance at 600 nm and extinction coefficient = 155 mM^{-1} cm^{-1}.

2.3 Estimation of lipids

Extraction: For the extraction of lipids, method of Kates [16] was used. Leaves of *B. juncea* were homogenised with methanol-chloroform (2:1). After the filtration of the homogenate, the filter residue was washed using 30 ml of methanol-chloroform (2:1). All the filtrates were combined and were put in a separatory funnel. 58 ml of water and 50 ml of chloroform were added into the mixture. Chloroform layer was taken out and dried. The residual lipids were then dissolved in chloroform.

2.3.1 Phospholipids

Phospholipids were estimated using the method of Ames and Dubin [17]. Chloroform was evaporated from the test extract taken in a test tube. Then, added 10% of magnesium nitrate into it. The solution was heated first on low flame and after that on strong flame. 0.5 N HCl was added to it, followed by heating in boiling water bath. After that tubes were cooled. A reagent was prepared by mixing ammonium molybdate (0.42 w/v in 1 N H_2SO_4) and ascorbic acid (10% w/v). The 4.2 ml of this reagent was added to the sample solution obtained above and was incubated at 45^{o}C. Absorbance was noted at 820 nm against a blank. A standard curve was prepared using potassium dihydrogen orthophosphate and was used to estimate the phosphorous amount in the samples.

2.3.2 Total sterols

We used the method of Sperry and Webb [18]. Test extract was taken in a test tube. 1 ml of acetic anhydride, 5 ml of chloroform and 0.1 ml of conc. H_2SO_4 were added into the test tube. Absorbance of the resulting solution was noted down at 625 nm against blank. A standard curve prepared using ergosterol was used to calculate the content of total sterols.

2.3.3 Esterified sterols

To the lipid sample taken in a test tube, 1% solution of digitonin was added. The mixture was kept till evaporation. It was followed by the addition of 3 ml of petroleum ether. Tubes were heated in a water bath until the evaporation of half of the solvent. The method used for the estimation of total sterols was then followed for the estimation of esterified sterols.

2.4 Estimation of sugars

Extraction: Sugars were extracted using the method of Singh and Luthra [19]. Ethanol was used for the extraction of lipids from the leaves of *B. juncea* plants in a boiling water bath. Extracts were dried to aqueous syrup using rotary vacuum evaporator. Final volume was raised to 100 ml with distilled water.

2.4.1 Glucose

Method of Gascon and Lampen [20] was used for the estimation of glucose. For it three reagents were prepared. Reagent 'A': 50 mg of glucose oxidase was added to 0.1 M potassium phosphate buffer of pH 7.0. To this solution, 2.5 mg of peroxidise was added. Reagent 'B': o-dianisidine (30 mg) was added to methanol. Reagent 'C': 45% of glycerol, 6 ml of reagent A and 3 ml of reagent 'B' were mixed together for the preparation of reagent 'C'. Procedure: To the test extract taken in a test tube, 1 ml of reagent 'C' was added. It was followed by the addition of 2 ml of 2N HCl. Took absorbance at 540 nm. A standard curve was prepared using glucose and was used to calculate the amount of glucose in the test sample.

2.4.2 Sucrose

Sucrose estimation was done using the method of Roe [21], with some changes. To the test extract, we added 0.5 ml of 6% KOH. The mixture was heated in a boiling water bath. Cooled the mixture, followed by the addition of 1 ml of resorcinol (0.1%) and 3 ml of HCl (30%). The resulting solution was incubated for 10 min at 80^{o}C. Absorbance was taken at 490 nm. A standard curve prepared using sucrose was used to calculate the amount of sucrose present in the sample.

2.5 Estimation of endogenous contents of auxins and abscisic acid

Sample preparation: 0.50 g of leaves of *B. juncea* were subjected to homogenisation in 5 ml of 80% methanol. The homogenate obtained was centrifuged and the resulting supernatant was filtered through 0.22 micron pore size nylon filter membrane. The filtrate was used to measure the endogenous contents of auxins and abscisic acid.LCMS analysis: The conditions employed by Banerjee and Kulkarni [22] were followed for LCMS analysis. 2 µL volume of the filtrate obtained above was injected for LCMS analysis. Mobile phase 'A' was constituted of water (0.5% formic acid) and mobile phase 'B' was constituted of methanol. Column temperature was set at 40^{o}C. Flow rate was 200 µL/min. 16 min run time was set in the positive mode and 6 min run time was set in the negative mode.

2.6 Statistical analysis

The data obtained was analysed statistically using self coding software and was presented as mean ± standard deviation. Two way analysis of variance (ANOVA) was performed. Tukey's multiple comparison test was applied to determine honestly significant difference (HSD). The data was considered significant at $p \leq 0.05$. The data was also analysed using multiple regression with interaction and % variability explained was calculated.

III. Results

3.1 Effect of 24-EpiBR on the plant growth under Cu(II) stress

3.1.1 Shoot weight

Shoot weight of the plants decreased with increase in the Cu(II) treatment in the soil. When compared with the control (0.38 g), maximum reduction (-71.05%) in shoot weight (0.11g) was caused by 0.75 mM Cu(II) treatment to soil. Seed pre-soaking treatment with 24-EpiBR improved shoot weight in the plants grown under Cu(II) stress. Seed pre-soaking treatment with 100 nM 24-EpiBR was most effective in improving the shoot weight in plants grown in soil applied with various Cu(II) concentrations, in comparison to the other two 24-EpiBR concentrations (1 and 0.01 nM). Binary combination of 100 nM 24-EpiBR with 0.75 mM Cu(II) caused maximum improvement (0.20 g, 81.8%) in the shoot weight as compared to the respective Cu(II) (0.75 mM) alone treatment (0.11 g) (Table 1). Cu(II) and 24-EpiBR had F-ratios significant at $P<0.01$, whereas the F-ratios for Cu(II) and 24-EpiBR was not significant (Table 1). HSD was 0.06 g. Plants grown in the soil treated with various concentrations of Cu(II) (0.25, 0.50 and 0.75 mM) showed significant decline in the shoot weight as compared to the control. Binary combination of 100 nM 24-EpiBR and 0.75 mM Cu(II) resulted in maximum improvement in the shoot weight (Table 1). The data was analysed with multiple regression with interaction. There was no interaction between Cu(II) and 24-EpiBR as evident from the β-regression (0.01) for Cu(II) x 24-EpiBR (Table 1). Cu(II), 24-EpiBR and their interaction explained 91.07% of variability (Table 1).

3.1.2 Root weight

Cu(II) application to soil decreased the root weight in 30-day old *B. juncea* plants as compared to control. 24-EpiBR seed pre-soaking improved root weight in plants grown in Cu(II) amended soils. Plants raised

from the seeds given pre-soaking treatment with 100 nM 24-EpiBR and grown in 0.50 mM Cu(II) applied soil, showed maximum improvement (0.03 g, 200%) in the root weight in comparison to the respective Cu(II) (0.50 mM) alone treatment (Table 2). Cu(II) and 24-EpiBR had F-ratios significant at $P<0.01$, whereas the F-ratio for Cu(II) x 24-EpiBR was not significant (Table 2). HSD was 0.01 g. As compared to the control, plants grown in soil treated with Cu(II) showed significant decrease in the root weight. Maximum improvement was caused by the binary combination of 100 nM 24-EpiBR and 0.50 mM Cu(II) in comparison to the respective Cu(II) (0.50 mM) alone treatment (Table 2). Multiple regression with interaction was applied on the data. The value of β-regression (-0.21) for Cu(II) x 24-EpiBR showed negative interaction between Cu(II) and 24-EpiBR (Table 2). Cu(II), 24-EpiBR and their interaction explained 83.48% variability (Table 2).

3.2 Effect of 24-EpiBR on the relative abundance of auxins under Cu(II) stress

3.2.1 IAA

Content of IAA (relative abundance: 104.50) declined (-64.1%) in the leaves of plants when compared with the control (relative abundance: 291.30). IAA content (relative abundance: 188.30) improved (80.2%) with the seed pre-soaking treatment with 100 nM 24-EpiBR in the leaves of plants under the stress of 0.50 mM Cu(II) (Table 3, Fig. 1). Cu(II), 24-EpiBR and their interaction (Cu(II) x 24-EpiBR) had F-ratios significant at $P<0.01$ (Table 3). HSD for relative abundance was 7.85. As compared to the control, treatment of soil with 0.50 mM Cu(II) significantly declined the IAA content in the leaves of plants. The plants given seed pre-soaking treatment with 100 nM 24-EpiBR and grown in soil applied with 0.50 mM Cu(II) maximally improved the IAA content in comparison to 0.50 mM Cu(II) alone treatment (Table 3). The data was subjected to analysis with multiple regression with interaction. β-regression value (0.20) for Cu(II) x 24-EpiBR indicated positive interaction between Cu(II) and 24-EpiBR (Table 3). 99.90% variability was explained by Cu(II), 24-EpiBR and their interaction (Table 3).

3.2.2 IBA

The leaves of *B. juncea* plants grown in the soil treated with 0.50 mM Cu(II) showed decline (-45.3%) in the IBA content (relative abundance: 910.90) in comparison to the control (relative abundance: 1666.50). Seed pre-soaking with 100 nM 24-EpiBR improved (42.6%) the content of IBA (relative abundance: 1298.80) in the leaves of plants grown under 0.50 mM Cu(II) stress (Table 4, Fig. 2). Cu(II), 24-EpiBR and Cu(II) x 24-EPiBR had F-ratios significant at $P<0.01$ (Table 4). HSD for relative abundance was 19.73. As compared to control, a significant decline was observed in the IBA content in leaves of plants grown in soil applied with 0.50 mM Cu(II). Plants raised from the seeds pre-soaked with 100 nM 24-EpiBR and grown in soil treated with 0.50 mM Cu(II) showed maximum improvement in the IBA content as compared to the respective Cu(II) (0.50 mM) alone treatment (Table 4). The data was also analysed with multiple regression with interaction. β-regression (0.53) for Cu(II) x 24-EpiBR indicated positive interaction between Cu(II) and 24-EpiBR (Table 4). Cu(II), 24-EpiBR and their interaction explained 99.94% variability (Table 4).

3.2.3 PAA

PAA content (relative abundance: 1486.40) decreased (-46.2%) in the leaves of plants grown under the stress of 0.50 mM Cu(II) when compared with the control (relative abundance: 2761.90). The leaves of plants grown in the soil treated with 0.50 mM Cu(II) but raised from the seeds given pre-soaking treatment with 100 nM 24-EpiBR, showed an improvement (17.8%) in the content of PAA (relative abundance: 1751.20) (Table 5, Fig. 3). The F-ratios for Cu(II), 24-EpiBR and Cu(II) x 24-EpiBR were significant at $P<0.01$ (Table 5). HSD for relative abundance was 15.09. In comparison to the control, 0.50 mM Cu(II) solution given to the soil caused significant decrease in the PAA content in the leaves of plants. Binary combination of 100 nM 24-EpiBR and 0.50 mM Cu(II) resulted in significant improvement in PAA content in the leaves of plants as compared to the plants treated with 0.50 mM Cu(II) solution alone (Table 5). The data was analysed with multiple regression with interaction. Since, β-regression for Cu(II) x 24-EpiBR was 0.13, there was positive interaction between soil Cu(II) treatment and 24-EpiBR seed pre-soaking treatment (Table 5). Cu(II), 24-EpiBR and their interaction explained 1% variability (Table 5).

3.3 Effect of 24-EpiBR on the relative abundance of abscisic acid under Cu(II) stress

Abscisic acid (relative abundance: 1901.10) enhanced (443.9%) in leaves of the plants grown in Cu(II) treated soil in comparison to the control (relative abundance: 349.50). Abscisic acid content (relative abundance: 3041.80) enhanced (60%) further in the leaves of plants raised from the seeds pre-soaked with 24-EpiBR and grown in 0.50 mM Cu(II) treated soil as compared to the plants given no 24-EpiBR seed pre-soaking but grown in the soil treated with 0.50 mM Cu(II) solution (Table 6, Fig. 4). F-ratios for separate treatments of Cu(II) and 24-EpiBR and also for their interaction (Cu(II) x 24-EpiBR) were significant at $P<0.01$ (Table 6). HSD for relative abundance was 13.56. The leaves of plants grown in the soil applied with 0.50 mM Cu(II) solution

showed significant enhancement in the abscisic acid content in comparison to the control. Plants grown in soil applied with 0.50 mM Cu(II) which were given seed pre-soaking treatment with 100 nM 24-EpiBR showed significant enhancement in the abscisic acid content in their leaves as compared to the soil 0.50 mM Cu(II) alone treatment (Table 6). The data was subjected to analysis with multiple regression with interaction. Cu(II) and 24-EpiBR interacted negatively as evident from the β-regression value (-0.16) for Cu(II) x 24-EpiBR (Table 6). 1% variability was explained by Cu(II), 24-EpiBR and their interaction (Table 6).

3.4 Effect of 24-EpiBR on lipid peroxidation and lipids content under Cu(II) stress

3.4.1 Lipid peroxidation

Plants grown in soil applied with different concentrations of Cu(II) solutions (0.25, 0.50 and 0.75 mM) showed elevation in MDA content in their leaves as compared to the control (0.98 µmol g^{-1} FW). Maximum enhancement (40.8%) in the MDA content (1.38 µmol g^{-1} FW) was observed in the leaves of plants grown in 0.75 mM Cu(II) treated soil. 24-EpiBR seed pre-soaking declined the MDA content in the leaves of plants grown in Cu(II) treated soil. Plants raised from the seeds given pre-soaking treatment with 100 nM 24-EpiBR and grown in 0.75 mM Cu(II) applied soil showed maximum decline in the content of MDA (1.17 µmol g^{-1} FW, -15.2%) in their leaves in comparison to the respective Cu(II) (0.75 mM) alone treatment (1.38 µmol g^{-1} FW) (Table 7). F-ratio values for Cu(II) and 24-EpiBR had F-ratios significant at $P<0.01$, whereas F-ratio value for Cu(II) x 24-EpiBR was not significant (Table 7). HSD was 0.09 µmol g^{-1} FW. Cu(II) treatments to soil led to significant enhancement in the lipid peroxidation in comparison to the control. Lipid peroxidation declined to the maximum in the binary combination of 100 nM 24-EpiBR and 0.75 mM Cu(II) when compared with the respective Cu(II) (0.75 mM) alone treatment (Table 7). Data was analysed with multiple regression with interaction. Since, β-regression value for Cu(II) x 24-EpiBR was -0.22, there was negative interaction between Cu(II) and 24-EpiBR (Table 7). Cu(II), 24-EpiBR and their interaction explained 83.50% of variability (Table 7).

3.5 Lipids

3.5.1 Phospholipids

A decline was observed in the content of phospholipids in the leaves of plants grown in soils treated with Cu(II) as compared to the control (0.10 mg g^{-1} FW). 0.75 mM Cu(II) soil treatment (0.02 mg g^{-1} FW) caused the maximum decline (-80%). 24-EpiBR seed pre-soaking treatment improved the phospholipids content in the leaves of plants grown in soil applied with Cu(II) solutions. Plants raised from the seeds given pre-soaking treatment with 100 nM 24-EpiBR and grown in soil applied with 0.75 mM Cu(II), showed maximum improvement (0.06 mg g^{-1} FW, 200%) in the phospholipids content in their leaves in comparison to the plants without seed pre-soaking treatment of 24-EpiBR but grown in the respective Cu(II) (0.75 mM) alone treatment (0.02 mg g^{-1} FW) (Table 8). F-ratios for Cu(II) and 24-EpiBR were significant at $P<0.01$ but F-ratio for Cu(II) x 24-EpiBR was not significant (Table 8). HSD was 0.02 mg g^{-1} FW. Content of phospholipids decreased significantly in comparison to the control by treatment of soil with Cu(II) solutions of different concentrations (0.25, 0.50 and 0.75 mM). Binary combination of 100 nM 24-EpiBR and 0.75 mM Cu(II) improved the phospholipids content to the maximum when compared with the respective Cu(II) (0.75 mM) alone treatment (Table 8). Multiple regression with interaction analysis was performed on the data. β-regression for Cu(II) x 24-EpiBR was 0.17. It showed that Cu(II) and 24-EpiBR interacted positively (Table 8). Cu(II), 24-EpiBR and their interaction explained 83.56% of variability (Table 8).

3.5.2 Total sterols

Application of various concentrations (0.25, 0.50 and 0.75 mM) of Cu(II) solution in soil caused decline in the content of total sterols in the leaves of plants. Maximum reduction (-56%) was observed in the plants grown in soil applied with 0.75 mM Cu(II) solution (1.85 mg g^{-1} FW) in comparison to the control (4.20 mg g^{-1} FW). An improvement was observed in the content of total sterols in leaves of the plants raised from the seeds given 24-EpiBR seed pre-soaking and grown in soil treated with Cu(II). Binary combination of 100 nM 24-EpiBR and 0.75 mM Cu(II) caused maximum elevation (4.05 mg g^{-1} FW, 118.9%) in the content of total sterols in the plant leaves as compared to the plants given 0.75 mM Cu(II) alone treatment (1.85 mg g^{-1} FW) (Table 9). Cu(II), 24-EpiBR and Cu(II) x 24-EpiBR had F-ratio values significant at $P<0.01$ (Table 9). HSD was 0.27 mg g^{-1} FW. Various concentrations of Cu(II) solutions applied in soil resulted in significant decline in the content of total sterols in comparison to the control. The content of total sterols improved maximally by the binary combination of 100 nM 24-EpiBR and 0.75 mM Cu(II) when compared with the respective (0.75 mM) Cu(II) alone treatment (Table 9). Multiple regression with interaction analysis was performed on the data. Cu(II) and 24-EpiBR interacted positively to increase the total sterols as evident from the β-regression (0.14) for Cu(II) x 24-EpiBR (Table 9). Cu(II), 24-EpiBR and their interaction explained 60.79% of variability (Table 9).

3.5.3 Esterified sterols

Cu(II) stress declined the esterified sterols content in the leaves of *B. juncea* plants. 0.75 mM Cu(II) soil treatment caused maximum decrease (-54.7%) in the esterified sterols (0.58 mg g^{-1} FW) in comparison to the control (1.28 mg g^{-1} FW). As compared to the individual Cu(II) alone treatments, the leaves of plants raised from the seeds pre-soaked in 1 nM 24-EpiBR and grown in 0.50 mM Cu(II) applied soil, showed maximum increase (1.12 mg g^{-1} FW, 51.4%) in the content of esterified sterols (Table 10). When Cu(II) and 24-EpiBR were applied alone, their F-ratios were significant at $P<0.01$. Their interaction (Cu(II) x 24-EpiBR) had F-ratio significant at $P<0.05$ (Table 10). HSD was 0.08 mg g^{-1} FW. In comparison to the control, leaves of the plants grown in soil amended with different concentrations of Cu(II) solutions (0.25, 0.50 and 0.75 mM), showed significant decrease in the content of esterified sterols. Esterified sterols content improved maximally by the binary combination of 1 nM 24-EpiBR and 0.50 mM Cu(II) as compared to the respective Cu(II) (0.50 mM) alone treatment (Table 10). The data was analysed with multiple regression with interaction. β-regression for Cu(II) x 24-EpiBR (0.11) revealed positive interaction between Cu(II) and 24-EpiBR (Table 10). Cu(II), 24-EpiBR and their interaction explained 85.78% variability (Table 10).

3.6 Effect of 24-EpiBR on sugars under Cu(II) stress

3.6.1 Glucose

Plants grown in Cu(II) amended soils showed decrease in the glucose content in their leaves. In comparison to the control (0.81 mg g^{-1} FW), leaves of the plants grown in the soil treated with 0.75 mM Cu(II), showed maximum decrease (-40.7%) in the content of glucose (0.48 mg g^{-1} FW). Plants raised from the seeds given pre-soaking treatment with 24-EpiBR and then grown in the soil amended with solutions of various Cu(II) concentrations, showed improvement in the glucose content in their leaves in comparison to the respective Cu(II) alone treatments. In such comparison, maximum improvement (0.84 mg g^{-1} FW; 52.7%) was observed in the binary combination of 100 nM 24-EpiBR and 0.50 mM Cu(II) when compared with the Cu(II) (0.50 mM) alone treatment (0.55 mg g^{-1} FW) (Table 11). F-ratio values for Cu(II) and 24-EpiBR were significant at $P<0.01$ whereas F-ratio value for Cu(II) x 24-EpiBR was not significant (Table 11). HSD was 0.05 mg g^{-1} FW. The leaves of plants grown in the soil applied with Cu(II) showed significant decline in glucose amount as compared to the control. Maximum improvement was noted in the binary combination of 100 nM 24-EpiBR and 0.50 mM Cu(II) in comparison to the respective Cu(II) (0.50 mM) alone treatment (Table 11). Multiple regression with interaction analysis was performed on the data. Since, the β-regression value for Cu(II) x 24-EpiBR was 0.15, there was positive interaction between Cu(II) and 24-EpiBR (Table 11). Cu(II), 24-EpiBR and their interaction explained 82.92% of variability (Table 11).

3.6.2 Fructose

An increase was reported in the fructose in the leaves of plants grown in the soil applied with Cu(II) solution in comparison to the control (0.37 mg g^{-1} FW), with maximum increase (94.6%) noted in the leaves of plants grown in 0.75 mM Cu(II) treated soil (0.72 mg g^{-1} FW). A further elevation was observed under Cu(II) stress in the leaves of plants raised from seeds given pre-soaking treatment of 24-EpiBR. The leaves of plants raised from the seeds given pre-soaking treatment with 1 nM 24-EpiBR and grown in the soil amended with 0.50 mM Cu(II), showed maximum enhancement (0.80 mg g^{-1} FW, 29%) in the fructose content as compared to the respective Cu(II) (0.50 mM) alone treatment (0.62 mg g^{-1} FW) (Table 12). Cu(II) and 24-EpiBR had F-ratio values significant at $P<0.01$. F-ratio value for the interaction between Cu(II) and 24-EpiBR was not significant (Table 12). HSD was 0.05 mg g^{-1} FW. When compared with the control, fructose content decreased significantly in the leaves of plants grown in the soils amended with various concentrations (0.25, 0.50 and 0.75 mM) of Cu(II) solutions. When comparison was done with the Cu(II) alone treatments, the content of fructose showed maximum enhancement in the leaves of plants raised from the seeds given pre-soaking treatment with 1 nM 24-EpiBR and grown in the soil applied with 0.50 mM Cu(II) (Table 12). The data was subjected to analysis with multiple regression with interaction. There was very little interaction between Cu(II) and 24-EpiBR as evident from the β-regression value (0.02) for Cu(II) x 24-EpiBR (Table 12). Cu(II), 24-EpiBR and their interaction explained 86.16% of variability (Table 12).

IV. Discussion

As the concentration of Cu(II) application in soil increased, the more reduction was observed in the shoot weight. The reduction in the shoot and root weights of plants under Cu(II) application shows that the Cu(II) when present in excess adversely affects the plant growth. The reduction in the plant growth under Cu(II) stress might be due to the reduction in cell wall elasticity due to the over accumulation of Cu in cell walls. Cu declined the shoot and root FW in four genotypes of *Solanum melongena* L. [23]. Negative effects of Cu stress on shoot and root weight of *Hordeum vulgare* and *Sinapis alba* were observed by Gvozdenac et al. [24], and on shoot weight of *Phaseolus vulgaris* were observed by Ashagre et al. [25]. We observed an improvement in the

shoot and root weights when Cu(II) treatment was supplemented with 24-EpiBR. The improvement might be due to the enhancement in the content of auxins with 24-EpiBR application as compared to the auxins content in Cu(II) alone treatment. Similar ameliorative effects of 24-EpiBR under Cu stress were observed by Choudhary et al. [26] in the seedlings of *Raphanus sativus*.

Cu adversely affects the plant growth which subsequently reduces the plant biomass in terms of reduced shoot and root weights. To test our hypothesis that whether this reduction in the shoot and root weights was due to the effect of Cu(II) on the auxin content of *B. juncea* plants, we determined their relative abundance through LCMS. A correlation in the reduction in the auxins content and the resulting decrease in the shoot and root biomass under Cu(II) stress has been found in our investigation.

Decline in the content of auxin in *Populus canescens* plants under Cd treatment was observed by Elobeid et al. [27]. The effect of Cu and Cd on auxin degradation was evaluated by Chaoui and Ferjani [28] in pea leaves by determining the activity of IAA oxidase, the enzyme responsible for the auxin breakdown. They observed that the activity of IAA oxidase enhanced with increase in the concentrations of Cu and Cd which caused the oxidation of auxin. Cu at high concentrations affects the auxin content in the cotyledons and apices of primary roots of *Arabidopsis* [29]. Cu led decline in the auxins levels was reported by Kolbert et al. [30] in *Arabidopsis thaliana* L. plants, which was responsible for the decrease in the growth of plants. IAA application enhanced the total FW of *Musa* sp. as compared to the untreated plants. IBA enhanced the shoot and root FW of the seedlings grown under the stress of alpha endosulfan and lindane contaminated soils [31]. In the present study FW of shoots and roots and auxin content increased by the seed pre-soaking treatment of 24-EpiBR. It has been proposed by Nassar [32] that BRs might not act directly on plant growth but might be the enhancer of plant growth by interacting with endogenous or exogenous growth regulators such as auxins. Interdependent and synergistic interaction of BRs and auxins to increase the plant growth was shown by Nemhauser et al. [33] in *Arabidopsis* plants. They reported that there was strong interaction between auxin and BRs controlled gene regulation and tissue elongation. Mandava [34] proposed that BRs influence plant growth by elevating the auxin action.

Abscisic acid participates in the plant responses to various environmental stresses. Monni et al. [12] reported increase in abscisic acid content in plants exposed to heavy metals such as Ni and Cu. Abscisic acid helps the plants in adapting to environmental stress. For e.g. under the conditions of water deficit the endogenous amount of abscisic acid enhances which prevents the water loss through transpiration by decreasing the size of stomatal aperture. Increase in the content of abscisic acid under Zn and Cu stresses increased the metalothionenins content in *Prosopis juliflora*, thereby helped in the managing the heavy metal stress in the plants [35]. In the present study the seed pre-soaking treatment of 24-EpiBR increased the relative abundance of abscisic acid in *B. juncea* plants as compared to the control. The supplementation of Cu(II) treatment with 24-EpiBR treatment further increased the content of abscisic acid when compared with the control. Brassinolide induced increase in the levels of abscisic acid has been reported under various abiotic stress conditions. 24-EpiBR role in enhancing the chilling tolerance of *Chorispora bungeana* plants by triggering the production of abscisic acid has been reported by Liu et al. [9]. Exogenously applied brassinolide enhanced the content of abscisic acid in *Chlorella vulgaris* under heat stress, which increased the thermo-tolerance of the plants [36]. Plant hormones provide stress tolerance not through linear pathways, but through complex interactions with other molecules [37]. There occurs crosstalk between BRs and abscisic acid which is helpful in stress alleviation. Our results are supported by the results of Choudhary et al. [26]. They observed an enhancement in the endogenous content of abscisic acid in *Raphanus sativus* under Cu stress with the application of EpiBR.

Heavy metals cause cellular destruction in plants by inducing the oxidative stress due to increased ROS production [38]. ROS damage biomembranes and convert unsaturated fatty acids into hydrocarbon fragments for e.g. MDA [39]. In the present investigation, the increase in the MDA content with increase in the concentrations of Cu(II) treatments showed increase in the oxidative damage because of increasing metal toxicity. Metals increase lipoxygenase activity which subsequently leads to lipid peroxidation [40]. It was observed in the present study that the MDA content reduced with the supplementation of Cu(II) treatment with 24-EpiBR. BRs increase the scavenging of ROS by enhancing the amount of various antioxidants and antioxidant enzymes [41]. Reduction in ROS reduces lipid peroxidation and the subsequent damaging effects on the biomembranes organisation. Reduction in the MDA content and so the lipid peroxidation with the co-application of 24-EpiBR with NaCl was reported in *Oryza sativa* L. [42] and also with its co-appication with Cu in *Raphanus sativus* seedlings [26].

Lipid peroxidation and then the destruction of the cellular membranes is one of the main effects of heavy metal toxicity. In the present study, lipids such as phospholipids, total sterols and esterified sterols decreased under the Cu(II) stress. Decrease in phospholipids influences their interactions with membrane intrinsic proteins and negatively affects the membrane integrity. Decline in the phospholipids was observed by Al-Hakimi and Hamada [43] in seedlings of *Triticum aestivum* under Cu stress. The results of sterols content were consistent with the results of Hernandez and Cooke [44] who observed a decline in the content of total

sterols in *Pisum sativum* under the Cd stress in comparison to the control. Total sterols and phospholipids content reduced in *Zygophyllum album* and *Zygophyllum coccineum* grown in the polluted soil as compared to the same plants grown in the unpolluted soil [45]. The decrease in the lipids could be linked with the increased lipid peroxidation under the metal stress. Both the free and esterified sterols decreased under water deficit conditions in the seedlings of *Brassica napus* L. [46]. It was observed in the present study that the supplementation of Cu(II) treatment with 24-EpiBR improved the contents all of the lipids. It might be due to the decrease in the lipid peroxidation by 24-EpiBR application.

In the present investigation, the contents of glucose and sucrose decreased in plants grown in soil given Cu(II) treatments. Azmat and Riaz [47] showed decrease in glucose and sucrose under Cu stress by their experiments on *Vigna radiata*. They proposed that this might be due to the damage to the chlorophyll structure due to increased content of Cu, which affected the light absorption by the chlorophyll molecule. Decrease in the sucrose content has been observed under various abiotic stress conditions. Decrease in the content of non-reducing sugars was observed by Verma and Dubey [48] in the rice seedlings with the increase of $Cd(NO_3)_2$ amount. They proposed that this was due to increase in the activity of sucrose degrading enzymes like sucrose synthase and acid invertase and the decreased activity of sucrose phosphate synthase under Cd stress. Non-reducing sugars declined in two rice cultivars, Ambemohar and Indrayani, due to salt stress [49]. In the present investigation it was observed that in the binary combination of 24-EpiBR and Cu(II), the sucrose content increased in comparison to the Cu(II) alone treatments. It was shown by Yu *et al.* [50] that the spray of 24-EpiBR on cucumber seedlings enhanced the content of sucrose after 6 h of the treatment. It occurred because of increased activity of sucrose phosphate synthase due to 24-EpiBR application.

V. Figures and tables

Table 1. Effect of seed pre-soaking with 24-EpiBR on shoot weight (g) in 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.25 mM Cu(II)**	**0.50 mM Cu(II)**	**0.75 mM Cu(II)**
Control	0.38 ± 0.046	0.26 ± 0.046	0.18 ± 0.044	0.11 ± 0.026
0.01 nM 24-EpiBR	0.43 ± 0.026	0.31 ± 0.070	0.25 ± 0.026	0.16 ± 0.046
1 nM 24-EpiBR	0.46 ± 0.020	0.34 ± 0.061	0.27 ± 0.026	0.18 ± 0.046
100 nM 24-EpiBR	0.48 ± 0.056	0.35 ± 0.036	0.31 ± 0.036	0.20 ± 0.017
Two way ANOVA				
F-ratio (3, 32) (Cu) = 91.17**			F-ratio (3, 32) (24-EpiBR) = 13.28**	
F-ratio (9, 32) (Cu x 24-EpiBR) = 0.12			HSD = 0.06	
Multiple regression with interaction				
Y = 0.41 – 0.36 (Cu, mM) + 0.001 (24-EpiBR, nM) + 4E-05 (Cu x 24-EpiBR)				
β-regression (Cu) = -0.93			β-regression (24-EpiBR) = 0.23	
β-regression (Cu x 24-EpiBR) = 0.01			Multiple correlation; % variability explained = 0.9543***; 91.07	
Significant at: ** P < 0.01, *** P < 0.001, Y = shoot weight (g)				

Table 2. Effect of seed pre-soaking with 24-EpiBR on root weight (g) in 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.25 mM Cu(II)**	**0.50 mM Cu(II)**	**0.75 mM Cu(II)**
Control	0.04 ± 0.010	0.02 ± 0.010	0.01 ± 0.006	0.01 ± 0.006
0.01 nM 24-EpiBR	0.05 ± 0.017	0.03 ± 0.010	0.02 ± 0.012	0.01 ± 0.006
1 nM 24-EpiBR	0.06 ± 0.017	0.03 ± 0.010	0.02 ± 0.012	0.02 ± 0.010
100 nM 24-EpiBR	0.06 ± 0.010	0.04 ± 0.017	0.03 ± 0.010	0.01 ± 0.006
Two way ANOVA				
F-ratio (3, 32) (Cu) = 26.07**			F-ratio (3, 32) (24-EpiBR) = 3.84*	
F-ratio (9, 32) (Cu x 24-EpiBR) = 0.51			HSD = 0.01	
Multiple regression with interaction				
Y = 0.04 – 0.05 (Cu, mM) + 0.0001 (24-EpiBR, nM) - 2E-04 (Cu x 24-EpiBR)				
β-regression (Cu) = -0.81			β-regression (24-EpiBR) = 0.38	
β-regression (Cu x 24-EpiBR) = -0.21			Multiple correlation; % variability explained = 0.9137***; 83.48	
Significant at: ** P < 0.01, *** P < 0.001, Y = root weight (g)				

Table 3. Effect of seed pre-soaking with 24-EpiBR on IAA (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.50 mM Cu(II)**
Control	291.30 ± 4.55	104.50 ± 3.52
100 nM 24-EpiBR	334.60 ± 2.42	188.30 ± 3.01
Two way ANOVA		
F-ratio (1, 11) (Cu) = 6930.39**		F-ratio (1, 11) (24-EpiBR) = 1009.02**
F-ratio (1, 11) (Cu x 24-EpiBR) = 102.45**		HSD = 7.85
Multiple regression with interaction		

Y = 291.30 – 373.60 (Cu, mM) + 0.43 (24-EpiBR, nM) + 0.81 (Cu x 24-EpiBR)	
β-regression (Cu) = -1.04	β-regression (24-EpiBR) = 0.24
β-regression (Cu x 24-EpiBR) = 0.20	Multiple correlation; % variability explained = 0.9995***; 99.90
Significant at: ** P < 0.01, *** P < 0.001, Y = IAA (relative abundance)	

Table 4. Effect of seed pre-soaking with 24-EpiBR on IBA (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.50 mM Cu(II)**
Control	1666.50 ± 13.68	910.90 ± 5.98
100 nM 24-EpiBR	1671.30 ± 6.50	1298.80 ± 6.19
Two way ANOVA		
F-ratio (1, 11) (Cu) = 12550.09**		F-ratio (1, 11) (24-EpiBR) = 1521.14**
F-ratio (1, 11) (Cu x 24-EpiBR) = 1447.69**		HSD = 19.73
Multiple regression with interaction		
Y = 1666.50 – 1511 (Cu, mM) + 0.05 (24-EpiBR, nM) + 7.66 (Cu x 24-EpiBR)		
β-regression (Cu) = -1.20		β-regression (24-EpiBR) = 0.01
β-regression (Cu x 24-EpiBR) = 0.53		Multiple correlation % variability explained = 0.9997***; 99.94
Significant at: ** P < 0.01, *** P < 0.001, Y = IBA (relative abundance)		

Table 5. Effect of seed pre-soaking with 24-EpiBR on PAA (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.50 mM Cu(II)**
Control	2761.90 ± 7.81	1486.40 ± 4.81
100 nM 24-EpiBR	2843.10 ± 4.74	1751.20 ± 8.43
Two way ANOVA		
F-ratio (1, 11) (Cu) = 94656.02**		F-ratio (1, 11) (24-EpiBR) = 2021.89**
F-ratio (1, 11) (Cu x 24-EpiBR) = 569.31**		HSD = 15.09
Multiple regression with interaction		
Y = 2761.90 – 2551 (Cu, mM) + 0.81 (24-EpiBR, nM) + 3.67 (Cu x 24-EpiBR)		
β-regression (Cu) = -1.06		β-regression (24-EpiBR) = 0.07
β-regression (Cu x 24-EpiBR) = 0.13		Multiple correlation; % variability explained = 1***; 1
Significant at: ** P < 0.01, *** P < 0.001, Y = PAA (relative abundance)		

Table 6. Effect of seed pre-soaking with 24-EpiBR on abscisic acid (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.50 mM Cu(II)**
Control	349.50 ± 6.66	1901.10 ± 6.56
100 nM 24-EpiBR	1843.70 ± 2.65	3041.80 ± 7.00
Two way ANOVA		
F-ratio (1, 11) (Cu) = 160698.20**		F-ratio (1, 11) (24-EpiBR) = 147560**
F-ratio (1, 11) (Cu x 24-EpiBR) = 2655.95**		HSD = 13.56
Multiple regression with interaction		
Y = 349.50 + 3103 (Cu, mM) + 14.95 (24-EpiBR, nM) – 7.07 (Cu x 24-EpiBR)		
β-regression (Cu) = 0.81		β-regression (24-EpiBR) = 0.78
β-regression (Cu x 24-EpiBR) = -0.16		Multiple correlation; % variability explained = 1***; 1
Significant at: ** P < 0.01, *** P < 0.001, Y = abscisic acid (relative abundance)		

Table 7. Effect of seed pre-soaking with 24-EpiBR on lipid peroxidation (MDA content, µmol g^{-1} FW) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	**Control**	**0.25 mM Cu(II)**	**0.50 mM Cu(II)**	**0.75 mM Cu(II)**
Control	0.98 ± 0.105	1.19 ± 0.101	1.27 ± 0.017	1.38 ± 0.082
0.01 nM 24-EpiBR	0.86 ± 0.020	1.13 ± 0.026	1.24 ± 0.046	1.29 ± 0.082
1 nM 24-EpiBR	0.88 ± 0.026	1.11 ± 0.030	1.18 ± 0.062	1.22 ± 0.125
100 nM 24-EpiBR	0.90 ± 0.026	1.07 ± 0.046	1.14 ± 0.098	1.17 ± 0.044
Two way ANOVA				
F-ratio (3, 32) (Cu) = 64.87**			F-ratio (3, 32) (24-EpiBR) = 8.85**	
F-ratio (9, 32) (Cu x 24-EpiBR) = 0.73			HSD = 0.09	
Multiple regression with interaction				
Y = 0.96 + 0.50 (Cu, mM) – 2E-04 (24-EpiBR, nM) – 0.002 (Cu x 24-EpiBR)				
β-regression (Cu) = 0.95			β-regression (24-EpiBR) = -0.05	
β-regression (Cu x 24-EpiBR) = -0.22			Multiple correlation; % variability explained = 0.9138***; 83.50	
Significant at: ** P < 0.01, *** P < 0.001, Y = lipid peroxidation (µmol g^{-1} FW)				

Table 8. Effect of seed pre-soaking with 24-EpiBR on phospholipids (mg g^{-1} FW) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	Control	0.25 mM Cu(II)	0.50 mM Cu(II)	0.75 mM Cu(II)
Control	0.1 ± 0.010	0.05 ± 0.010	0.04 ± 0.010	0.02 ± 0.010
0.01 nM 24-EpiBR	0.11 ± 0.020	0.07 ± 0.017	0.05 ± 0.010	0.03 ± 0.010
1 nM 24-EpiBR	0.12 ± 0.026	0.09 ± 0.010	0.06 ± 0.026	0.05 ± 0.010
100 nM 24-EpiBR	0.12 ± 0.017	0.08 ± 0.010	0.07 ± 0.010	0.06 ± 0.017
Two way ANOVA				
F-ratio (3, 32) (Cu) = 50.81**			F-ratio (3, 32) (24-EpiBR) = 10.16**	
F-ratio (9, 32) (Cu x 24-EpiBR) = 0.43			HSD = 0.02	
Multiple regression with interaction				
Y = 0.10 – 0.10 (Cu, mM) + 8E-05 (24-EpiBR, nM) + 0.0002 (Cu x 24-EpiBR)				
β-regression (Cu) = -0.93			β-regression (24-EpiBR) = 0.11	
β-regression (Cu x 24-EpiBR) = 0.17			Multiple correlation; % variability explained = 0.9141***; 83.56	
Significant at: ** P < 0.01, *** P < 0.001, Y = phospholipids (mg g^{-1} FW)				

Table 9. Effect of seed pre-soaking with 24-EpiBR on total sterols (mg g^{-1} FW) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	Control	0.25 mM Cu(II)	0.50 mM Cu(II)	0.75 mM Cu(II)
Control	4.20 ± 0.420	3.40 ± 0.106	2.52 ± 0.130	1.85 ± 0.078
0.01 nM 24-EpiBR	5.00 ± 0.210	4.27 ± 0.166	3.80 ± 0.123	3.30 ± 0.095
1 nM 24-EpiBR	5.25 ± 0.296	4.43 ± 0.062	4.37 ± 0.212	3.85 ± 0.115
100 nM 24-EpiBR	5.46 ± 0.353	4.63 ± 0.128	4.42 ± 0.066	4.05 ± 0.311
Two way ANOVA				
F-ratio (3, 32) (Cu) = 144.01**			F-ratio (3, 32) (24-EpiBR) = 151.03**	
F-ratio (9, 32) (Cu x 24-EpiBR) = 3.86**			HSD = 0.27	
Multiple regression with interaction				
Y = 4.74 – 2.37 (Cu, mM) + 0.01 (24-EpiBR, nM) + 0.01 (Cu x 24-EpiBR)				
β-regression (Cu) = -0.72			β-regression (24-EpiBR) = 0.27	
β-regression (Cu x 24-EpiBR) = 0.14			Multiple correlation; % variability explained = 0.7797**; 60.79	
Significant at: ** P < 0.01, *** P < 0.001, Y = total sterols (mg g^{-1} FW)				

Table 10. Effect of seed pre-soaking with 24-EpiBR on esterified sterols (mg g^{-1} FW) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	Control	0.25 mM Cu(II)	0.50 mM Cu(II)	0.75 mM Cu(II)
Control	1.28 ± 0.066	1.02 ± 0.026	0.74 ± 0.017	0.58 ± 0.017
0.01 nM 24-EpiBR	1.36 ± 0.035	1.13 ± 0.072	0.89 ± 0.095	0.72 ± 0.044
1 nM 24-EpiBR	1.41 ± 0.056	1.20 ± 0.066	1.12 ± 0.056	0.86 ± 0.036
100 nM 24-EpiBR	1.41 ± 0.036	1.18 ± 0.062	1.02 ± 0.066	0.87 ± 0.095
Two way ANOVA				
F-ratio (3, 32) (Cu) = 243.47**			F-ratio (3, 32) (24-EpiBR) = 43.21**	
F-ratio (9, 32) (Cu x 24-EpiBR) = 2.39*			HSD = 0.08	
Multiple regression with interaction				
Y = 1.34 – 0.84 (Cu, mM) + 0.001 (24-EpiBR, nM) + 0.001 (Cu x 24-EpiBR)				
β-regression (Cu) = -0.94			β-regression (24-EpiBR) = 0.08	
β-regression (Cu x 24-EpiBR) = 0.11			Multiple correlation; % variability explained = 0.9262***; 85.78	
Significant at: * P < 0.05, ** P < 0.01, *** P < 0.001, Y = esterified sterols (mg g^{-1} FW)				

Table 11. Effect of seed pre-soaking with 24-EpiBR on glucose (mg g^{-1} FW) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	Control	0.25 mM Cu(II)	0.50 mM Cu(II)	0.75 mM Cu(II)
Control	0.81 ± 0.020	0.71 ± 0.066	0.55 ± 0.036	0.48 ± 0.010
0.01 nM 24-EpiBR	0.86 ± 0.010	0.74 ± 0.040	0.71 ± 0.026	0.61 ± 0.070
1 nM 24-EpiBR	0.89 ± 0.017	0.80 ± 0.090	0.73 ± 0.017	0.63 ± 0.020
100 nM 24-EpiBR	0.94 ± 0.026	0.86 ± 0.026	0.84 ± 0.010	0.71 ± 0.044
Two way ANOVA				
F-ratio (3, 32) (Cu) = 94.47**			F-ratio (3, 32) (24-EpiBR) = 50.92**	
F-ratio (9, 32) (Cu x 24-EpiBR) = 2.04			HSD = 0.05	
Multiple regression with interaction				
Y = 0.85 – 0.37 (Cu, mM) + 0.001 (24-EpiBR, nM) + 0.001 (Cu x 24-EpiBR)				
β-regression (Cu) = -0.84			β-regression (24-EpiBR) = 0.33	
β-regression (Cu x 24-EpiBR) = 0.15			Multiple correlation; % variability explained = 0.9106***; 82.92	
Significant at: ** P < 0.01, *** P < 0.001, Y = glucose (mg g^{-1} FW)				

Table 12. Effect of seed pre-soaking with 24-EpiBR on fructose (mg g^{-1} FW) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

Treatments	Control	0.25 mM Cu(II)	0.50 mM Cu(II)	0.75 mM Cu(II)
Control	0.37 ± 0.026	0.53 ± 0.053	0.62 ± 0.036	0.72 ± 0.046
0.01 nM 24-EpiBR	0.45 ± 0.030	0.59 ± 0.036	0.7 ± 0.026	0.76 ± 0.066
1 nM 24-EpiBR	0.47 ± 0.010	0.64 ± 0.036	0.8 ± 0.035	0.84 ± 0.036
100 nM 24-EpiBR	0.48 ± 0.036	0.65 ± 0.036	0.78 ± 0.062	0.83 ± 0.036
Two way ANOVA				
F-ratio (3, 32) (Cu) = 172.14**		F-ratio (3, 32) (24-EpiBR) = 27.05**		
F-ratio (9, 32) (Cu x 24-EpiBR) = 0.64		HSD = 0.05		
Multiple regression with interaction				
Y = 0.45 + 0.46 (Cu, mM) + 0.001 (24-EpiBR, nM) + 0.0001 (Cu x 24-EpiBR)				
β-regression (Cu) = 0.90		β-regression (24-EpiBR) = 0.17		
β-regression (Cu x 24-EpiBR) = 0.02		Multiple correlation; % variability explained = 0.9282***; 86.16		
Significant at: ** P < 0.01, *** P < 0.001, Y = fructose (mg g^{-1} FW)				

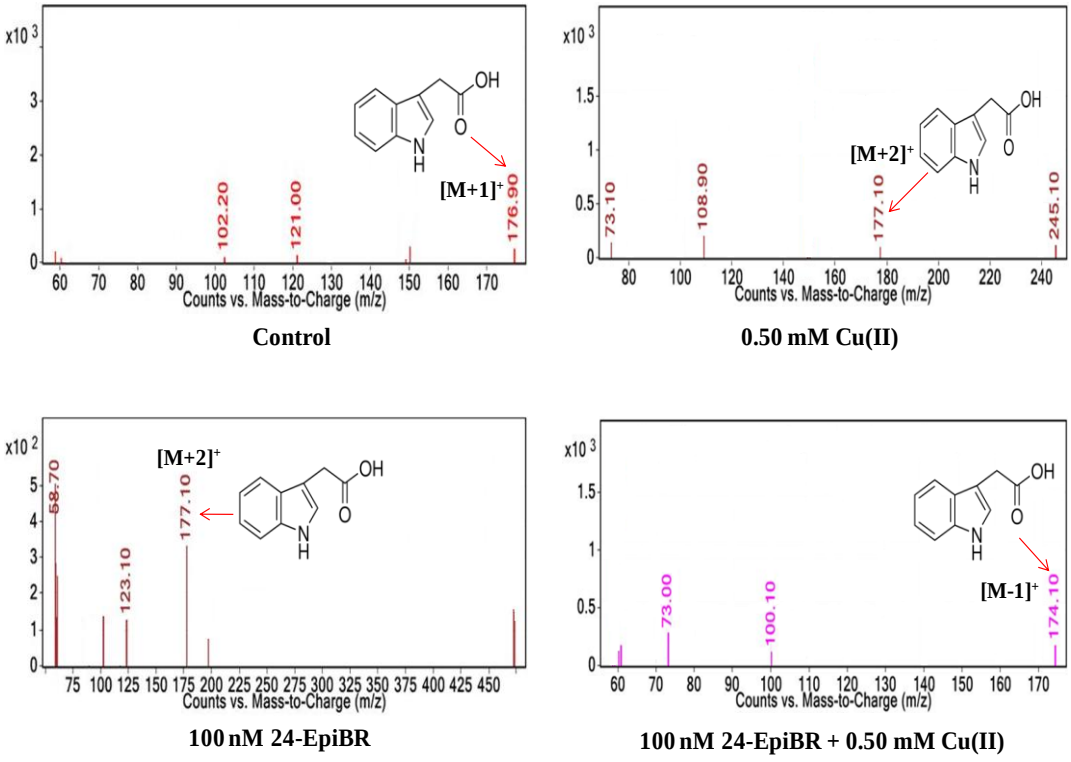

Fig. 1. Effect of seed pre-soaking with 24-EpiBR on IAA (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

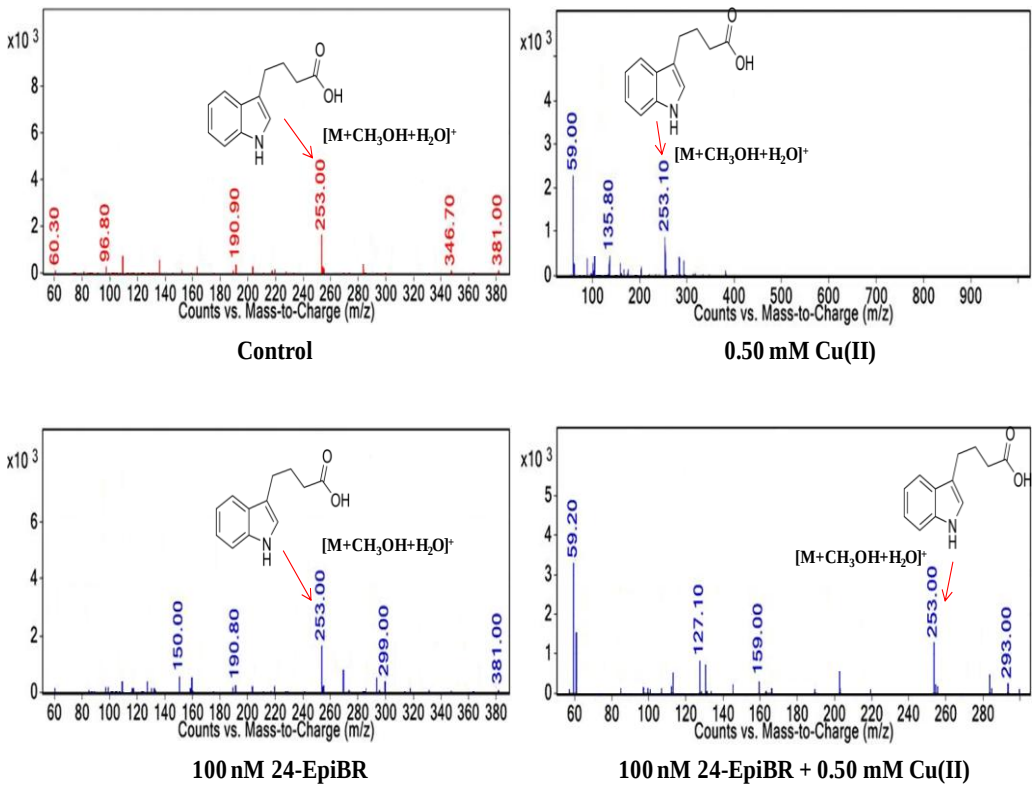

Fig. 2. Effect of seed pre-soaking with 24-EpiBR on IBA (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

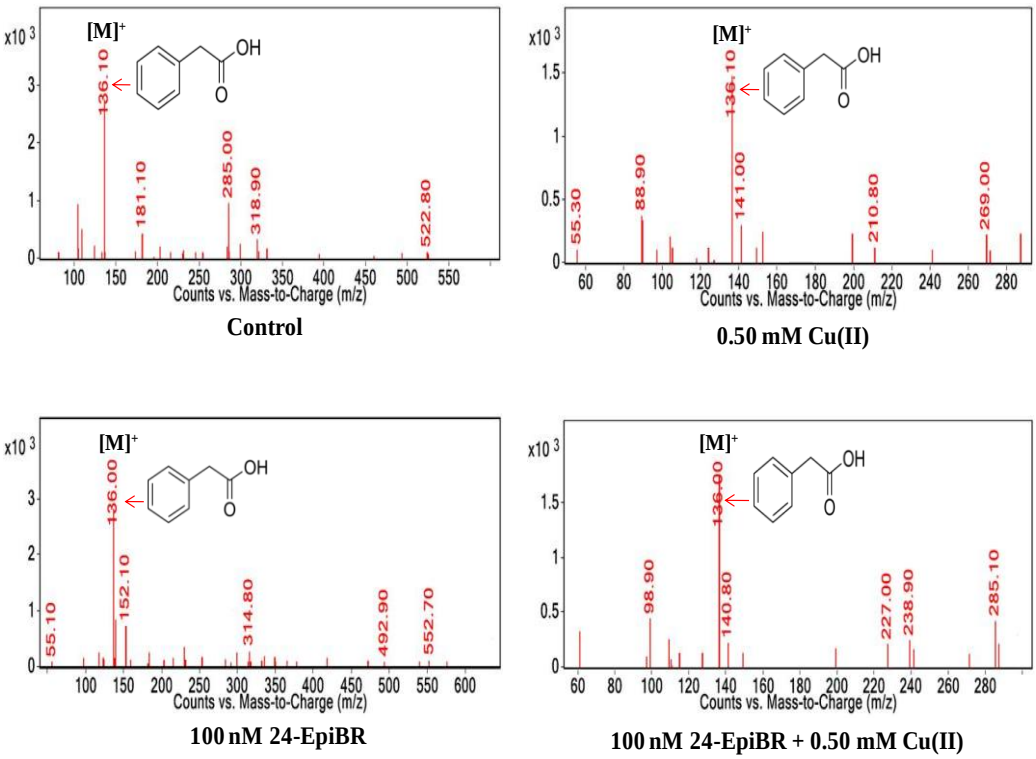

Fig. 3. Effect of seed pre-soaking with 24-EpiBR on PAA (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

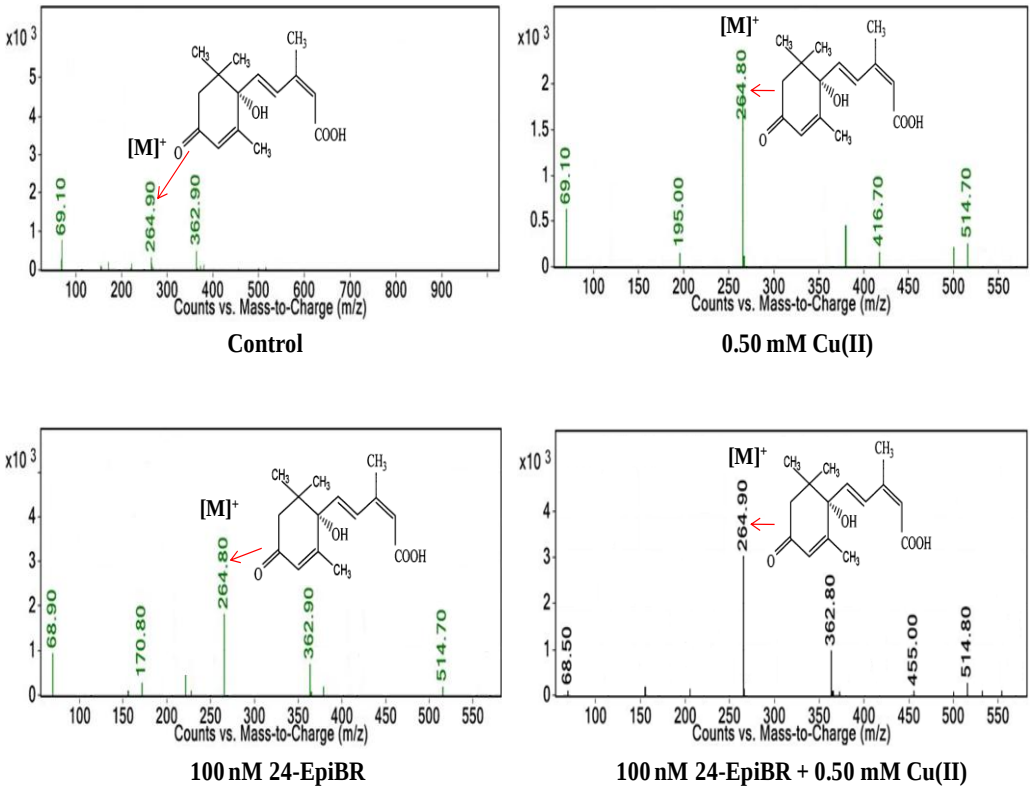

Fig. 4. Effect of seed pre-soaking with 24-EpiBR on abscisic acid (relative abundance) in the leaves of 30-day old plants of *B. juncea* grown in soil amended with Cu(II) solution before sowing.

VI. Conclusion

Cu stress affects the plant growth and metabolism. We noticed a reduction in plant biomass and various biomolecules such as sugars (glucose and sucrose) and lipids (phospholipids, total sterols and esterified sterols) and an enhancement in MDA content under Cu(II) stress. However, the supplementation of soil Cu(II) treatment with 24-EpiBR seed pre-soaking treatment increased the plant biomass by increasing the endogenous contents of auxins (IAA, IBA and PAA) and also improved the contents of the sugars and the lipids and reduced the content of MDA. 24-EpiBR crosstalk with abscisic acid was also evident as its co-application with Cu(II) enhanced abscisic acid content and increased the plants ability to cope with the Cu(II) stress.

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