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HISTOCHEMICAL ESTIMATION OF H_2O_2 AND ANTIOXIDANT RESPONSE OF DIFFERENT BRASSICA VARIETIES UNDER CR STRESS SOIL

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ABSTRACT

Four varieties of Brassica were used as heavy metal accumulator to clean up the chromium (Cr) contaminated soil. A pot experiment was conducted with completely randomized design (CRD) containing four treatments and three replicates. Different concentrations of Cr contents, i.e. 50%, 30% and 10% contaminated soil were used. Plant cells continuously produce Reactive Oxygen Species (ROS). However, increased accumulation of ROS occurred under abiotic stress. Qualitative detection of H_2O_2 was done by using 3, 3′-Diaminobenzidine (DAB) that produced reddish brown spots on the surface and veins of leaves. Quantitative estimation of H_2O_2 (%) was measured by DPPH radical scavenging assay in four varieties of Brassica. Among four treatments, maximum DPPH radical scavenging activity by antioxidants occurred in T_0 with 0% Cr. Amount of Chlorophyll was assessed by standard procedure. By using standard protocols enzymes antioxidants (superoxide dismutase, catalase, ascorbate peroxidase and lipid peroxidation) activity was determined. This study aimed to investigate the histochemical estimation of H_2O_2 and response of different enzymatic antioxidants as a defense against ROS in mechanism of the Cr stress tolerance in Brassica.

Keywords: Antioxidant enzymes, H_2O_2, ROS, Cr stress, Phytoremediation.

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INTRODUCTION

Cr is found naturally in earth crust, animals and plants bodies (Jaishankar *et al*., 2014). Electroplating and other man-made industries have played a major role in Cr entrance to our surroundings (Shahid *et al*., 2017). Reduction in the plant growth and development occurs in Cr contaminated soil, that lead to accumulation of several antioxidant in cell that activates plant defense mechanism (Panda, 2003).

Leather processing is an important industry in Pakistan, where more than 600 tanneries are concentrated in three major cities (Kasur, Karachi, and Sialkot). Leather industries discharge tons of waste Cr resulting in production of large area of Cr polluted soil which are dangerous for human health, as human use brassica plants grown in Cr contaminated soil as food source. Chromium (VI) compounds are well-known human carcinogens and high levels of Cr are major reasons of nose problems and breathing issues as shortness of breath, asthma, cough (Sabine and Griswold, 2009).

Phytoremediation is a technique used to clean up the pollutants from environment by using green plants (Sumiahadi and Acar, 2018). Brassica has the potential to accumulate heavy metals including Cr and is widely used to clean up the Cr contaminated soil in Pakistan. *Brassica napus* and *Brassica juncea* have gained much importance toward the tolerance of stress induced by heavy metals (Mourato *et al*., 2015), therefore a number of studies were made on these species to estimate its phytoremediation potential (Bhuiyan *et al*., 2011).

Related to antioxidant enzymes, three types of metabolic changes were reported in the plants: metabolites synthesis is increased (Shanker at al., 2005), the mechanism of production of pigments is affected (Boonyapookana *et al*., 2002) and synthesis of different molecules such as amino acids, proteins and antioxidative enzymes increased (Schmfger, 2001). Upon exposure to Cr stress, plants respond through two types of mechanisms, one contains the non-enzymatic components flavonoids, phenols etc. and other consists of enzymatic part including catalase, superoxide dismutase, ascorbate peroxidase (Waszczak *et al*., 2018). The synthesis of ROS lead to increase in the oxidative damage to Cr stressed plants thus resulting in reduced growth (Al-Mahmud *et al*., 2017).

Catalase, superoxide dismutase and peroxidase help plants to defend themselves from toxic effects of reactive oxygen molecules (Kachout *et al*., 2009). The studies on oxidative stress and function of antioxidant enzyme mechanism in plants under Cr stress are very negligible (Diwan *et al*., 2010). The main purpose of this study is to investigate the histochemical estimation of H_2O_2 in brassica upon exposure to Cr stress, assessment of antioxidant enzymatic activity and chlorophyll content measurement.

MATERIALS AND METHODS

Seeds of four varieties of Brassica genus i.e, *Brassica juncea* (NIFA-Raya) and *Brassica napus* (Abasin-95, Durr-e-NIFA, NIFA-Gold) were obtained from NIFA (www.nifa.org.pk). An experiment containing 48 pots was conducted in experimental area of Botanical Garden of University of the Punjab Lahore during mid of March, 2019 (www.pu.edu.pk). Ten (10) seeds per replicate per treatment were sown in chromium contaminated soils brought from tannery soil polluted area of Kasur, Punjab, Pakistan. The experiment was laid out in Completely Randomized Design (CRD) and there were three replications of each treatment. The experiment was comprised of four treatments as T_0:100% garden soil, T_1:50% garden soil and 50% Cr contaminated soil, T_2:70% garden soil and 30% Cr contaminated soil, T_3:90% garden soil and 10% Cr contaminated soil. Four groups of pots containing seeds were placed in the wire house with temperature of ±28°C. Irrigation was performed by tap water after regular intervals of 2 days. Soil digging was done at 7 days regular intervals to provide aerated soil for growth. Seeds started to germinate after few days.

Plants Sample Collection: Harvesting of plants was done in the start of May, 2019. The leaves were collected and washed with distilled water. Qualitative and quantitative measurement of H_2O_2 in different varieties of Brassica was done.

Qualitative Estimation of H_2O_2: Qualitative determination of H_2O_2 was performed by following the already reported method (Kumar *et al*., 2014). Total 16 leaf samples (one sample per treatment) were taken. Immersed the detached leaves from each treatment separately in petri plates containing 1% DAB (250 µl), 0.3% H_2O_2 (250 µl) and PBS (Phosphate Buffered Saline, 5ml). The solution was mixed well and kept for overnight at room temperature. Infiltrate the leaves by vacuum infiltration at 100-150mbar for 1 minute and then release the vacuum gently. The protocol was performed three times to make leaves infiltrated.

Immersed the leaves in absolute ethanol to remove the chlorophyll and leaves were placed in a water-bath for 10 minutes. Leaves were transferred on a paper towel pre-dipped in glycerol. Reddish brown spots were formed on the veins and surface of leaves.

Quantitative Estimation of Antioxidant Activity: Leaf samples were taken (one from each treatment), dried in oven and pulverized to fine powder. Total 16 tubes were prepared for experiment each contained 1g of leaf powder and 200 ml of distilled water.

Preparation of Leaf Extracts: Leaf powder was mixed in 200ml of distilled water and heated to make volume 100ml. Shaked well at 25℃ and then centrifuged at 10000 × g for 14 minutes as reported in previous study (Bhanu *et al*., 2013).

Determination of DPPH Activity (2,2-diphenyl-1-picrylhydrazyl): Total 2mg of DPPH was dissolved to 70ml of ethanol and kept on laboratory shaker (HY-4) for 24 hours at 80-100rpm until DPPH was completely dissolved (Cervato *et al*., 2000). Then 1ml of leaf extract and 3ml of DPPH was mixed in falcon tube (10ml) and O.D (517nm) was taken by spectrophotometer (UV-1800: SHIMADZU) with water taken as control. Same procedure was used for all sixteen samples. Following formula was used for determination of DPPH activity (Amin and Jamei, 2014).

Inhibition (%) = [(Control Absorbance - Sample absorbance) / Control Absorbance] x100

Chlorophyll Contents Estimation: Total chlorophyll contents were determined by using Dimethyl Sulfoxide (DMSO) and its value was calculated by using Arnon and Kennedy (2008) formula.

Extraction of Antioxidant Enzymes: Total 0.1g plant material (leaves) was taken and crushed to fine powder with pistil and mortar using liquid nitrogen. The tissue was homogenized with 4ml of 150mM ice-cold phosphate buffer (pH 7.0) and then transferred to falcon tube (15ml). All samples were centrifuged at 12000 × g (4℃) for 20 minutes. The supernatant was placed at 20℃ until further processing. SOD activity was determined by modified method of Giannopolitis and Ries (1977). Catalase activity was measured by Chance and Maehly (1954) with minor modifications. APX activity was determined by standard procedure (Nakano and Assada, 1981). Malondialdehyde (MDA) was estimated to assess the level of lipid peroxidation by using Heath and Packer (1968) and Dhindsa and Wandekayimatowe (1981) techniques.

Statistical Analysis: The experiment was executed with two factor (variety × treatment) completely randomized design (CRD). Statistical Package for Social Sciences (SPSS) version 21 was used for ANOVA and means were separated using tukey test at 5% probability level. The graphs were plotted using MS-Excel with mean values obtained from data analysis.

RESULTS

Qualitative and Quantitative Estimation of H_2O_2: A histochemical determination of H_2O_2 resulted in formation of reddish-brown spots on surface of leaves. In *Brassica juncea* (NIFA-Raya) leaves from T_1 showed the maximum, T_2 and T_3 moderate, while T_0 showed minimum reddish-brown coloration. Therefore, H_2O_2 was in higher concentration in leaves of the plants grown under Cr stress than control plants (Fig.1). Leaves from

Brassica napus (Abasin-95) T_1 showed maximum, while T_0 showed minimum formation of reddish-brown spots. In *Brassica napus* (Durr-e-NIFA) histochemical detection of H_2O_2 was maximum in T_1 and minimum in T_0. In *Brassica napus* (NIFA-Gold) maximum reddish-brown spots was appeared on the leaves of plants in highly Cr contaminated soil (T_1). While plants in T_0 produced fewer brown spots (Fig. 2).

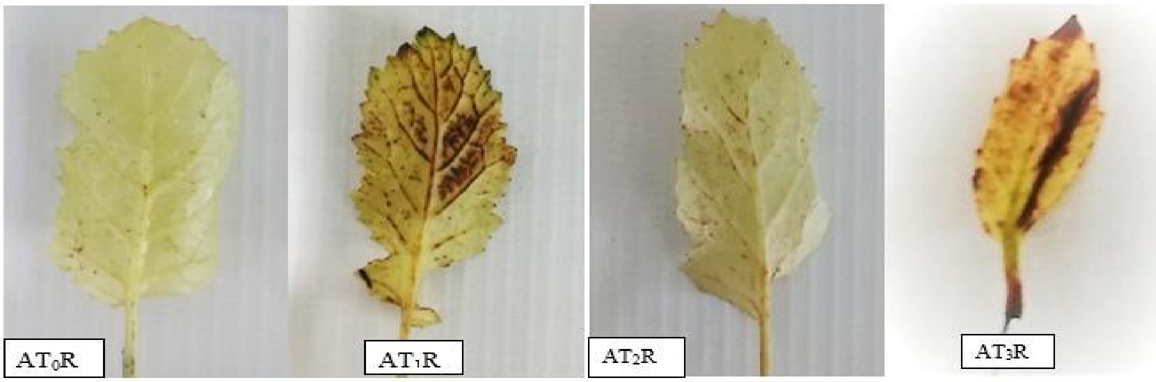

Fig. 1. Qualititative estimation of H_2O_2 in *Brassica juncea* (NIFA-Raya) leaves. Brown spots show the deposits of H_2O_2. 'A' indicates *Brassica juncea* (NIFA-Raya).T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil). R shows the different replicates.

Among *Brassica juncea* (NIFA-Raya) and *Brassica napus* (Abasin-95, Durr-e-NIFA, NIFA-Gold) degree of accumulation had significant difference. However, in *Brassica napus* (NIFA-Gold) even under least Cr stress more Cr was accumulated than other varieties (Fig. 2).

Fig. 2. Qualititative estimation of H_2O_2 in *Brassica napus* (NIFA-Gold) leaves. Brown spots show the depositions of H_2O_2. 'D' indicates *Brassica napus* (NIFA-Gold). T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil). R indicates the different replicates.

DPPH Activity: DPPH activity of leaves from four verities of Brassica ((*Brassica juncea* (NIFA-Raya), *Brassica napus* (Abasin-95), *Brassica napus* (Durr-e-NIFA) and *Brassica napus* (NIFA-Gold) at different Cr concentrations was measured with water taken as control by using DPPH assay.

In *Brassica juncea* (NIFA-Raya) maximum % inhibition of DPPH by antioxidants occurred as maximum antioxidants were produced by plant at 0% Cr (T_0). However, with 50% increase in concentration of Cr % inhibition decreased significantly. It indicates that Cr treatment extracts showed lowest DPPH activity as compared to T_0 (Fig. 3). In *Brassica napus* (Abasin-95) the % inhibition of DPPH was maximum in T_0, because at normal environmental conditions production of antioxidants was maximum. So less the exposure of plants to Cr, more the production of antioxidants. Percentage inhibition (%) of DPPH was observed minimum under the highly contaminated Cr soil. However, to some extent the antioxidants were in Cr contaminated soil which showed species tolerance against heavy metal stress (Fig. 3). Similarly in *Brassica napus* (Dure-e-NIFA) % inhibition was maximum in T_0 as compared to Cr treated plants in which antioxidants production was reduced, so % inhibition also reduced. In *Brassica napus* (NIFA-Gold) free radical scavenging activity was maximum in T_0. While minimum percentage observed in plants treated with Cr (Fig. 3).

Maximum DPPH radical scavenging activity by antioxidants was observed in T_0 i.e., in Cr free soil. Because of the production of large amount of antioxidants maximum % inhibition was observed. If the comparison of varieties of Cr contaminated soil was made than at 50% and 10% Cr treatment *Brassica napus* (Durr-e-NIFA) showed the resistance toward Cr by producing antioxidants. So, in the present research work it was confirmed that under Cr stress all the four varieties were able to tolerate and accumulate the Cr however, *Brassica napus* (Durr-e-NIFA) showed maximum resistance against Cr stress (Fig. 3).

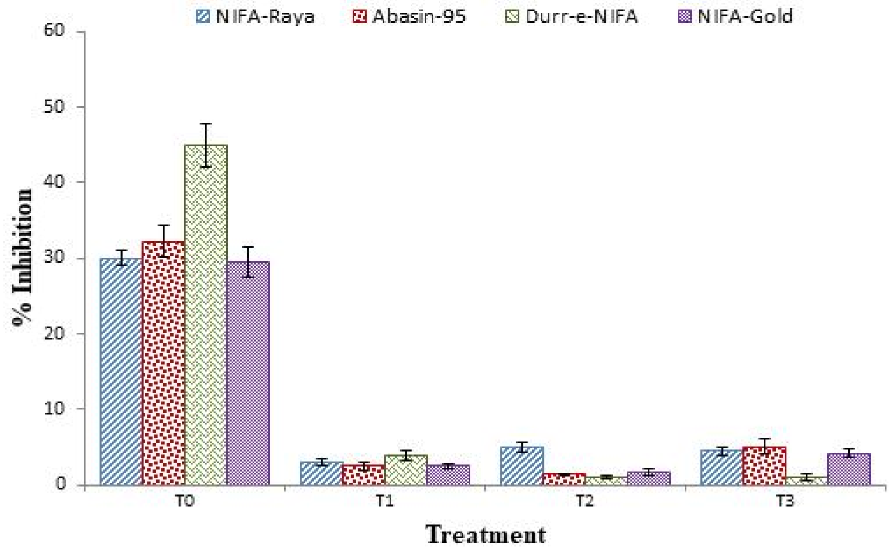

Fig. 3. % Inhibition in four treatments of Cr in Brassica varieties. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Chlorophyll Content (mg/g F. wt): The findings of our study depict that those plants grown in 100% garden soil (T_0) have high chlorophyll content compared to T_1, T_2 and T_3 (Fig. 4). NIFA-Gold T_3 plants showed maximum chlorophyll content, whereas Abasin-95 T_1 showed minimum chlorophyll content under Cr stress. As Cr stress increases, chlorophyll content decreases. Abasin-95 plants grown in Cr stress soil (T_1, T_2 and T_3) exhibit lesser chlorophyll content compared to other varieties. NIFA-Raya and Durr-e-NIFA plants showed maximum values of 0.92 and 1.43 under Cr stress (Table 1-3).

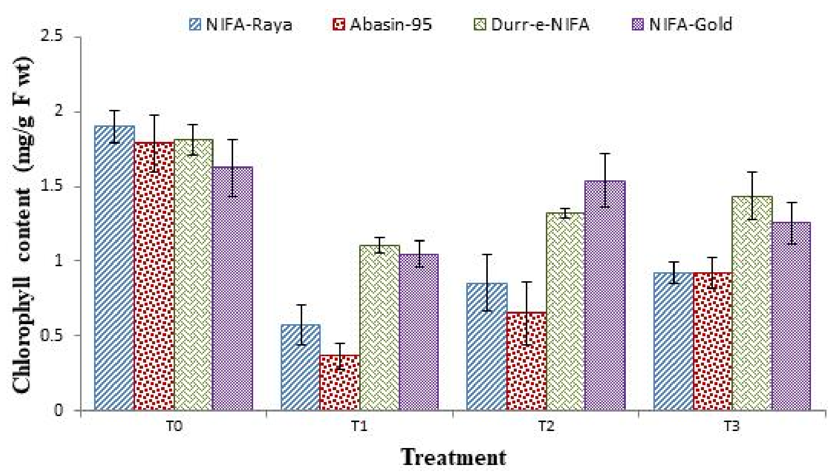

Fig. 4. Chlorophyll content in four treatments of Cr in leaves of Brassica varieties. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Table 1. Different parameters of NIFA-Raya (*Brassica juncea*) in four treatments of Cr. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Parameter	T_0	T_1	T_2	T_3
Chlorophyll conent (leaf tissue) (mg/gF.wt)	1.9	0.57	0.85	0.92
Cat activity (leaf tissue) (Units/g.FW)	0.67	1.54	1.08	0.84
APX activity (leaf tissue) (Units/g.FW)	0.803	2.13	1.45	1.32
SOD activity (leaf tissue) (Units/g.FW)	0.24	1.08	0.73	0.58
MDA content (leaf tissue) (Um/g FW)	2.76	12.04	7.27	4.66

Table 3. Different parameters of Durr-e-NIFA (*Brassica napus*) in four treatments of Cr. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Parameter	T_0	T_1	T_2	T_3
Chlorophyll conent (leaf tissue) (mg/gF.wt)	1.81	1.11	1.32	1.43
Cat activity (leaf tissue) (Units/g.FW)	0.48	1.65	1.28	0.91
APX activity (leaf tissue) (Units/g.FW)	0.67	2.003	1.63	1.15
SOD activity (leaf tissue) (Units/g.FW)	0.33	0.93	0.78	0.69
MDA content (leaf tissue) (Um/g FW)	2.97	12.9	9.53	6.19

Catalase (CAT) Activity (Units/g.FW): From the data it is clear that maximum CAT activity (1.65) was recorded in T_1 leaves of Durr-e-NIFA (0.48), whereas minimum was recorded in T_0 plants of Durr-e-NIFA (Fig. 4). Plants growing in chromium stress (T_1, T_2 and T_3) show significantly higher CAT activity as compared to garden soil (T_0). NIFA-Gold leaves show a maximum CAT activity of 1.63 (T_1) and minimum in T_0 (0.59). NIFA-Raya and Abasin-95 (T_1) also exhibits increased activity in higher Cr stress (Table 4).

Ascorbate Peroxidase (APX) Activity (Units/g.FW): From data it is evident that APX activity in leaf tissue was highest in NIFA-Gold T_1 (2.24), and lowest in T_0 (Table 4). The plants grown in Cr contaminated soil (T_1, T_2 and T_3) show an increased trend for APX activity comparatively to plants grown in garden soil (T_0) in all four varieties (Fig. 6). NIFA Raya shows maximum value (2.13) for APX activity in Cr stress soil (T_1). Durr-e-NIFA also exhibits an increased value in T_1 (2.003) as compared to T_0 (0.67). Abasin-95 leaves show values of 0.56 and 2.08 in T_0 and T_1 plants respectively (Table 2).

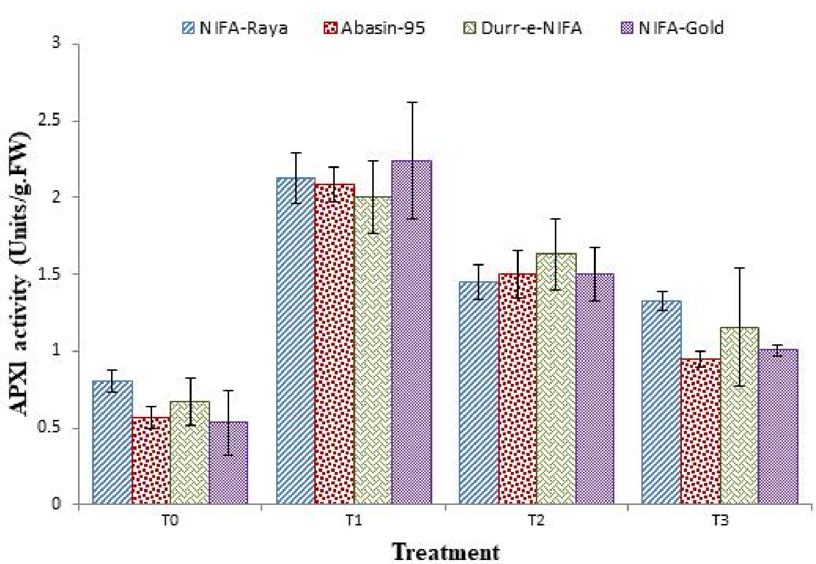

Fig. 6. Ascorbate peroxidase (APX) activity in four treatments of Cr in leaves of Brassica varieties. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Superoxide Dismutase (SOD) Activity (Units/g.FW): The findings of our study reflect that there is a decrease in SOD activity in all four varieties grown in 100% garden soil (T_0) comparative to Cr stress soil (T_1, T_2 and T_3). NIFA-Raya T_1 shows maximum SOD activity in leaf tissue in Cr contaminated soil (Fig. 7). Abasin-95 showed 0.26 and 0.93 in T_0 and T_1 respectively (Table 2). NIFA-Gold T_1 leaves showed an increased trend (0.84) in Cr contaminated soil as compared to T_0 (0.24) plants grown in 100% garden soil for SOD activity (Table 4). Durr-e-NIFA exhibits 0.78 and 0.69 in T_2 and T_3 leaves.

Lipid peroxidation (MDA) in Leaf Tissue (Um/g FW): Results of the present study reflect that MDA content was maximum in Durr-e-NIFA T_1 leaves (Fig. 8). Abasin-95 showed MDA content 2.95 and 9.44 in T_0 and T_1 respectively (Table 2). NIFA-Raya leaves show 2.76 and 12.04 of lipid peroxidation in T_0 and T_1 respectively (Table 1). NIFA-Gold shows 3.46 and 5.55 MDA content in T_0 and T_3 respectively.

Table 2. Different parameters of Abasin-95 (*Brassica napus*) in four treatments of Cr. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Parameter	T_0	T_1	T_2	T_3
Chlorophyll conent (leaf tissue) (mg/gF.wt)	1.78	0.37	0.65	0.92
Cat activity (leaf tissue) (Units/g.FW)	0.5	1.52	1.12	0.77
APX activity (leaf tissue) (Units/g.FW)	0.56	2.08	1.503	0.94
SOD activity (leaf tissue) (Units/g.FW)	0.26	0.93	0.86	0.48
MDA content (leaf tissue) (Um/g FW)	2.95	9.44	8.39	5.17

Table 4. Different parameters of NIFA-Gold (*Brassica napus*) in four treatments of Cr. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Parameter	T_0	T_1	T_2	T_3
Chlorophyll conent (leaf tissue) (mg/gF.wt)	1.62	1.04	1.53	1.25
Cat activity (leaf tissue) (Units/g.FW)	0.59	1.63	1.02	0.99
APX activity (leaf tissue) (Units/g.FW)	0.53	2.24	1.49	1.003
SOD activity (leaf tissue) (Units/g.FW)	0.24	0.84	0.65	0.6
MDA content (leaf tissue) (Um/g FW)	3.46	9.14	5.57	5.55

DISCUSSION

Cr is hazardous to plants and it is a serious environmental issue (Afshan *et al*., 2015). Cr enters through plant roots and accumulated at different trophic levels of food chain and ultimately reached to human body. Reactive oxygen molecules are generated in cells causing oxidative damage which results in reduction in plant growth. Cr present in the cells of plants causes the generation of reactive oxygen peroxides such as H_2O_2 (Shanker *et al*., 2005). There is a thirty-fold increase in the production of reactive oxygen peroxides in Cr stress soil (Mittler, 2002). Various environmental stresses like low or high temperature, toxicity of heavy metals, deficiency of nutrients, pathogen attack, drought and salinity induce the synthesis of oxygen containing peroxides in cells (Tripathi *et al*., 2012). Reactive oxygen molecules are chemically more reactive than molecular oxygen and the important examples of ROS in plants are H_2O_2, O_2^- and HO radicals (Waszczak *et al*., 2018). There is an increase in the lipid peroxidation and H_2O_2 with subsequent increase in Cr level alone in the soil (Hussain *et al*., 2018). This increase in ROS is one of the results of oxidative damage due to Cr (Shahid *et al*., 2017). Our study reflects that overproduction of ROS and oxidative damage is suppressed by the scavenging potential of various enzymatic antioxidants (SOD and CAT).

Our findings of present research work were confirmed from previous studies (Kumar *et al*., 2014) in which 3,3′-Diaminobenzidine (DAB) was used for the histochemical analysis of H_2O_2 as ROS in the seedling of *Brassica juncea*. Reddish brown precipitates are produced when the oxidation of DAB takes place by H_2O_2 in the occurrence of peroxidases (Kumar *et al*., 2014). Histochemical assay with DAB depends on the formation of reddish-brown spots on the surface of leaves of pea plants grown under the treatment of Cd as reported earlier (Romero-Puertas *et al*., 2004). *Arabidopsis thaliana* seedlings were treated with Cr (VI) exhibiting decreased growth of roots but increased production of H_2O_2 and dead cells (Eleftheriou *et al*., 2015). In the pods of *Pisum sativum* and *Brassica chinensis* DAB reacts with H_2O_2 and brown compounds were localized histochemical (Liu *et al*., 2014). As the concentration of arsenic (Ar) increases the accumulation of H_2O_2 increases which was observed in *Brassica napus* leaves by the formation of brown spots based on DAB (Farooq *et al*., 2016). Mercury (Hg) induces the production of reactive oxygen species and caused the oxidative damage to plants. The dark brown coloration (by reaction of DAB with H_2O_2) was clearly observed and photographed in leaf tissue of Brassica. According to Romero-Puertas *et al*. (2004) leaves from pea plants grown under the cadmium stress have six-time higher concentration of H_2O_2 as compared to control plants.

It was clearly investigated that by exposure to heavy metal as Cr, the DPPH activity reduced significantly. DPPH is applied for the estimation of antioxidant activity because its radical form is stable. The colour of DPPH solution changes from purple to yellow due to inhibition by ROS (Bozin *et al*., 2008). In plants antioxidative defense system included the antioxidative enzymes and growth regulators play role in protection against stress. Many antioxidative enzymes such as CAT, SOD, POD and GR help to scavenge the free radicals. When plants are exposed to heavy metals the antioxidative defense system becomes active. Which then help in the scavenging of free radical as DPPH (Izbianska *et al*., 2014).

The DPPH activity of plant extracts decreased expressively with a two-fold increase in Cd and Cu. The treatment extracts showed the lowest DPPH activity comparatively to plants grown in normal soil (Ibrahim *et al*., 2017). Free radical scavenging activity percentage was reduced to 61% and 74% in leaves of *Brassica juncea* and *Sorghum vulgare* when exposed to Cr contaminated soil. However, in both plants under control soil antioxidant activity was maximum i.e., 90% (Revathi *et al*., 2013). The activity of catalase and ascorbate peroxidase decreased after Cr application (Bhaduri and Fulekar, 2012). Decrease in Cd accumulation causes increase in catalase activity (Islam *et al*., 2009). In rice seedlings grown under Cd stress, the antioxidant activity reduced as quantified by DPPH radical scavenging assay (Tripathi *et al*., 2012). As the concentration of Cd increases in Sugar cane, the activity of CAT also decreased (Fornazier *et al*., 2002).

Results of our findings suggest that there is a decrease in chlorophyll content of Cr stressed plants comparative to plants grown in 100% garden soil (Fig. 4). There is a reduction in chlorophyll and other accessory pigments with subsequent increase in Cr concentration in rice plant (leaf tissue) comparative to plants grown in 100% garden soil (Hussain *et al*., 2018). When *Brassica juncea* was grown in Cr contaminated soil, there is an alteration in the amount of chlorophyll and carotenoids (Pandey *et al*., 2005). Ali *et al*. (2015b) suggested that Cr stress damages the photosynthetic machinery of the plant leading towards the reduction in the chlorophyll contents. The findings of our research suggest that catalase activity decreases in plants grown in 100% garden soil as compared to plants grown in Cr contaminated soil in all four varieties (Fig. 5). Earlier research suggests that Cr treatments decreased the catalase activity in both Brassica species in comparison to control, but catalase activity in *Brassica juncea* (leaf tissue) does not show any significant difference between the treatments after a sharp decrease (Zeynep *et al*., 2011). With the increase in Cr accumulation, CAT and SOD activity generally decrease in roots and leaves but maintain higher concentration compared to control (Pandey *et al*., 2005). Catalase acts as a scavenger to eradicate H_2O_2 in plants (Anjum *et al*., 2016).

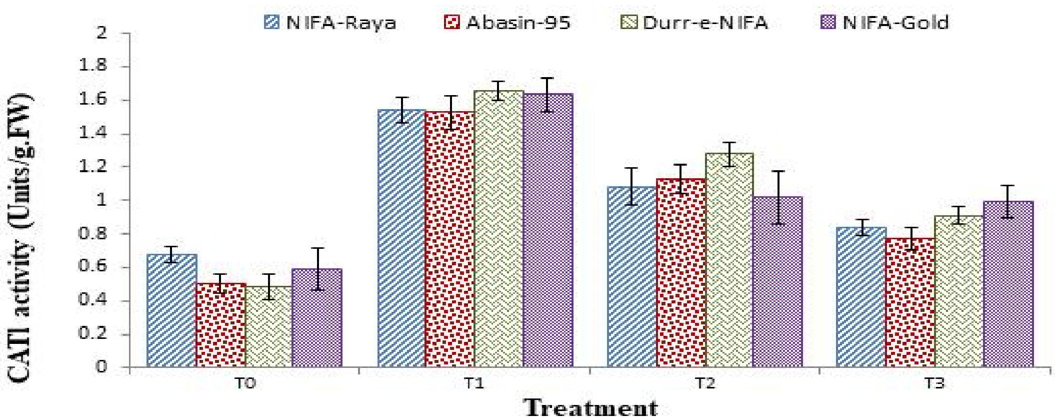

Fig. 5. Catalase (CAT) activity in four treatments of Cr in leaves of Brassica varieties. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

From the results it becomes clear that APX activity increases in all four varieties cultivated in Cr stress soil in contrast to T_0 (Fig. 6). These results are similar with the results of previous study that there is a gradual increase in APX activity in *Brassica oleracea* and *Brassica juncea* after Cr application respectively. APX enzyme showed the maximum activity in Brassica species after Cr treatment (Zeynep *et al*., 2011). There is a decrease in the activity of APX in rice with increasing Cr in the soil (Hussain *et al*., 2018). Previous results showed that when Cr content in cell raises, APX activity decreases, consequently catalase acts as to reduce loss created by Cr (Pandey *et al*., 2005). The findings of our study suggest that Cr stress enhanced SOD activity in contrast to plants grown in garden soil (Fig. 7). According to previous study, there is an advanced mechanism in plants to cope up with the harmful effects caused by reactive oxygen peroxides. This system comprises of enzymatic antioxidants (Ali *et al*., 2015a). SOD enzyme constitutes the central defense mechanism in scavenging ROS (Gill *et al*., 2015). Activities of enzymes (SOD and CAT) were reduced in roots at 20μM Cr where cellular Cr showed maximum accumulation (Pandey *et al*., 2005). The findings of our work are in coincidence (Fig. 8) with previous work that there is an increased level of MDA content and enhanced H_2O_2 concentration in plants which proves that Cr is the source of oxidative stress through production of ROS (Pandey *et al*., 2005). According to another study, MDA content raises in *Brassica napus* with citric acid application under Cr stress may be due to increase in antioxidant enzyme activity (Afshan *et al*., 2015).

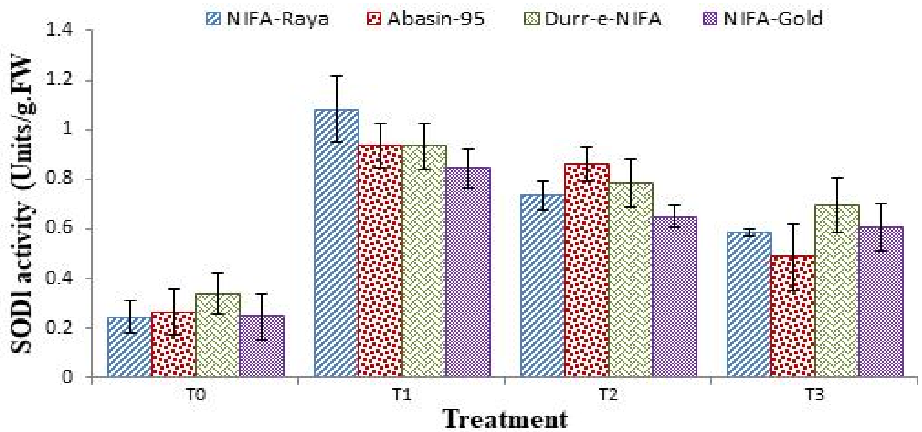

Fig. 7. Superoxide dismutase (SOD) activity in four treatments of Cr in leaves of Brassica varieties. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

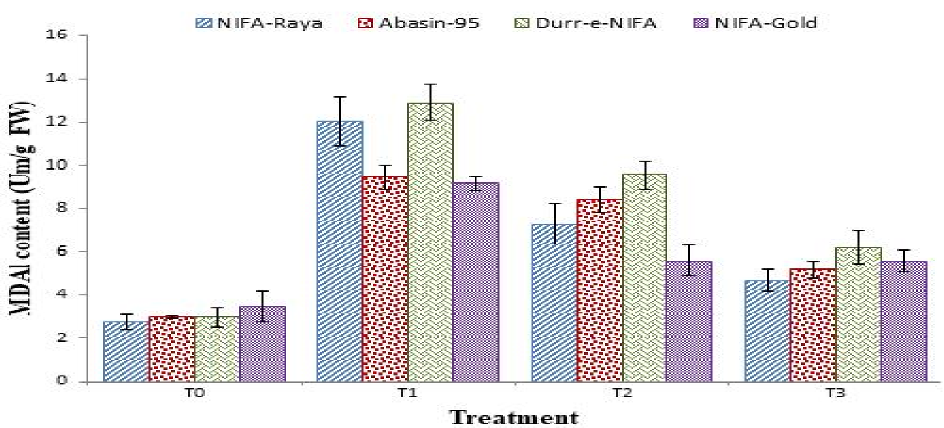

Fig. 8. Lipid Peroxidation (MDA) in four treatments of Cr in leaves of Brassica varieties. T_0 (100% Garden soil), T_1 (50% Garden soil and 50% Cr stress soil), T_2 (70% Garden soil and 30% Cr stress soil), T_3 (90% Garden soil and 10% Cr stress soil).

Conclusion: Histochemical estimation of H_2O_2 and antioxidant activity (%) of four varieties of Brassica depicts that under Cr stress, increased production of ROS takes place and ultimately the production of H_2O_2 were also increased. In all varieties accumulation of H_2O_2 was visualized by the formation of reddish-brown spots on the surface of leaves. Under increased concentration of Cr, brown coloration was also increased. Activation of antioxidants even at higher concentrations of Cr indicates the tolerance of the species by detoxifying the ROS. In order to tolerate the Cr stress, plants produce the enzymatic antioxidant defense system which inhibits the production of free radical formation. So, for the purpose of phytoremediation, brassica can be widely used to clean up the contaminated soil and make positive use of Cr rich soil.

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