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DISCUSSION

5. Introduction

The contamination of agricultural land by Cd is of great concern as it affects plant growth and development worldwide (Anjum et al., 2014; Asgher et al., 2015). Plants regulate oxidative stress with professional scavenging system such as induced activity of CAT, SOD and GSH-AsA cycle turnover (Bowler et al., 1992; Willekens et al., 1997; Noctor and Foyer, 1998). Hydrogen peroxide generated in peroxisomes due to photorespiration is detoxified by CATs (Mittler et al., 2004; Vandenabeele et al., 2004), while $\text{O}_2^-$ is dismutated by SOD (Bowler et al., 1992). It has been found that $\text{H}_2\text{O}_2$ is very stable ROS and has high potential to move easily from one compartment to another, thereby attacking thiol proteins. Thus, an efficient detoxifying antioxidant system is required in every compartment for $\text{H}_2\text{O}_2$ detoxification (Noctor and Foyer, 1998; Cruz de Carvalho, 2008). For the detoxification of $\text{H}_2\text{O}_2$, GSH-AsA cycle plays a key role that operates in every compartment of the cell. In GSH-AsA cycle, the GSSG formed in the last step is recycled back to GSH by GR using NADPH as a reductant (Noctor et al., 2002). In both prokaryotes and eukaryotes, GSH has been reported to be abundant non-protein thiol. The increased ROS production during Cd stress (Nagajyoti et al. 2010; Asgher et al., 2015; Khan et al., 2015) requires more GSH production to detoxify ROS, thus an increased assimilatory flux of S is needed during Cd stress to maintain the higher contents of GSH (Heiss et al., 1999; Anjum et al., 2008; Khan et al., 2009). Similarly, activity of ATP-S enzyme involved in S-assimilation has been found to increase under Cd stress increasing GSH production resulting in protection of photosynthesis and growth of $B. \text{juncea}$ plants (Asgher et al., 2014). One of the fascinating things related to environmental stress is the role of phytohormones (Monteiro et al., 2012). Phytohormones are also associated with regulation of plant metabolism under Cd stress (Asgher et al., 2015). Jasmonates a class of phytohormones regulate various metabolic activities and stress responses in plants (Soares et al., 2010; Wasternack, 2014). Methyl jasmonate treated plants have been reported to show reduction in oxidative stress (MDA and $\text{H}_2\text{O}_2$ content) under Cd stress (Keramat et al., 2009). Exogenous application of MeJA to Cd-stressed $O. \text{sativa}$ plants reduced oxidative stress by stimulating the activity of CAT, SOD, POD.
and GR paralleled with an increased GSH-pools (Singh and Shah, 2014). The research on NO, another emerging phytohormone has shown that exogenous application of NO can provide protection against HMs toxicity such as Al (Sun et al., 2014), As (Singh et al., 2016), Cd (Singh et al., 2008), Cu (Yu et al., 2005) and Mn (Srivastava and Dubey, 2012). Nitric oxide can neutralize HM-induced ROS either directly by reacting with ROS (Laspina et al., 2005) or by stimulating antioxidant system (Lamattina et al., 2003; Laspina et al., 2005). It is, therefore, important to understand the role and response of MeJA and NO in S-mediated Cd stress tolerance in *B. juncea*.

The rationale of work of the present dissertation is to analyze the production and detoxification of ROS together with investigation of the possible role of GSH in detoxification of oxidizing agents (O$_2^-$ and H$_2$O$_2$). Furthermore, the response of plants to exogenous MeJA and NO in modulation of S-assimilation pathway was investigated, which could provide useful information on detoxification mechanism and development of future programme for improved cultivation of plants.

### 5.1 Screening of Mustard Cultivars Differing in Cd Sensitivity

The sensitivity of four cultivars of *B. juncea* to 50 µM Cd was assessed by studying oxidative stress, antioxidant metabolism, S-assimilation, photosynthetic and growth characteristics.

#### 5.1.1 Accumulation of Cd, oxidative stress and antioxidant metabolism

In the present study, plants grown with Cd exhibited an increase in Cd-content in leaf of all the *B. juncea* cultivars. The accumulation of Cd was least in Ro Agro 4001 (Fig. 1). The differential behaviour of the cultivars in Cd accumulation capacity in leaf was due to their different sensitivity to Cd. It is considered that the restriction of HM translocation from root to shoot could be a possible mechanism of plant tolerance to Cd stress. The lower translocation of Cd from root to leaf may, therefore, be a strategy of Ro Agro 4001 to protect its photosynthetic function from Cd-induced oxidative stress. These observations are in close conformity with the results reported by Dixit et al. (2001) and Singh et al. (2008). The accumulation of less Cd in Ro Agro 4001 resulted in lesser production of ROS and oxidative damage. It may be said that
Ro Agro 4001 was equipped with mechanisms that avoided oxidative stress in this cultivar more than the other cultivars.

Cadmium impairs important physiological and biochemical processes by inducing oxidative stress (Masood et al., 2012a; Cuypers et al., 2012; Anjum et al., 2014; Asgher et al., 2015). Treatment of plants with Cd induced least oxidative stress (measured as H$_2$O$_2$ and TBARS content) in Ro Agro 4001 (Fig. 2A-B), while vice versa response was found in antioxidant metabolism (Fig. 3A-C). The maintenance of low oxidative stress and membrane damage in the highest antioxidant metabolism exhibiting Ro Agro 4001 leaves was possible as a result of balanced tuning between GSH pool as well as the activity of SOD, APX and GR enzymes. In Ro Agro 4001, the high activity of APX more efficiently metabolized Cd-induced H$_2$O$_2$ levels and controlled the H$_2$O$_2$-accrued consequences. Moreover, high SOD activity accompanied with greater APX in Ro Agro 4001 was sufficient to detoxify the H$_2$O$_2$ formed as a result of SOD activity. The other way of decreasing oxidative stress in Ro Agro 4001 under Cd stress was possible due to higher activity of GR, a GSH-regenerating enzyme, which maintained the GSH pool.

5.1.2 Sulphur-assimilation under Cd stress

Cadmium stress resulted in the highest increase in ATP-S activity and content of Cys and GSH in Ro Agro 4001 than the other cultivars (Fig. 4A; Fig. 5A-B). However, content of S decreased under Cd stress (Fig. 4B). ATP-sulphurylase, the first and rate-limiting enzyme of S-assimilation pathway regulates the synthesis of S-compounds. Thus, increase in ATP-S activity can provide tolerance to plants through increase in thiol compounds formation under Cd stress. Haroda et al. (2002) reported induction of enzyme transcripts of S-assimilation pathway in A. thaliana under Cd stress. Studies have shown that thiol metabolism and antioxidant defense system increased in H. vulgare subjected to metals stress (Astolfi et al., 2012). It has been reported that increase of ATP-S activity is necessary for the maintenance of optimal GSH levels required for the proper functioning of AsA-GSH cycle (Khan et al., 2009; Asgher et al., 2014). The results reported here suggested that higher activity of ATP-S plays a crucial role in maintaining Cys and GSH content required for Cd stress tolerance. In fact, Cys is the final product of S-assimilation and is the major limiting
substrate for GSH synthesis (Noctor et al., 1996) and GSH is the substrate for the biosynthesis of PCs (Cobbett and Goldsbrough, 2002). Exposure to higher concentrations of Cd has been shown to maintain higher contents of Cys and GSH by stimulating the activity S metabolism enzymes for detoxification of metals in B. juncea (Heiss et al., 1999; Khan et al., 2009; Masood et al., 2012a; Asgher et al., 2014; Per et al., 2016a). Studies have shown that ATP-S played important role in limiting Cd accumulation and enhancing Cd tolerance in A. thaliana (Howarth et al., 2003), B. juncea (Wangeline et al., 2004; Masood et al., 2012a; Asgher et al., 2014), T. aestivum (Khan et al., 2007; Khan et al., 2015) and Sedum alfredii (Guo et al., 2009). It has been reported that S-assimilation was up-regulated leading to alleviation of Cd toxicity and thereby improved potential of plants to survive under Cd stress (Wangeline et al., 2004; Khan et al., 2009; Bashir et al., 2013). In A. thaliana, exposure to Cd led to the activation of S-assimilation pathway by increasing transcription of specific genes that enhanced the supply of GSH for PCs synthesis (Jobe et al., 2012; Matthewman et al., 2012). Therefore, the higher increase in ATP-S activity and Cys and GSH synthesis showed increase in Cd stress tolerance in Ro Agro 4001. The increase in GR activity under Cd stress also implies an important role of GR in maintaining GSH pool and ROS detoxification.

5.1.3 Photosynthesis and growth under Cd stress

Cadmium-induced oxidative stress inhibits enzyme activity, photosynthetic capacity and growth of plants (Masood et al., 2012a; Dias et al., 2013; Kapoor et al., 2014; Liu et al., 2014; Khan et al., 2015; Asgher et al., 2015). In the present study, it is evident that Cd significantly reduced gas exchange parameters, Chl content and Rubisco activity (Fig. 6A-C; Fig. 7A-B). However, lesser reduction was observed in Ro Agro 4001 in comparison to other cultivars. The increase in Cd accumulation and corresponding increase in oxidative stress resulted in reduction in photosynthetic and growth characteristics. The reduction in photosynthesis by Cd could be attributed to the decrease in gas exchange parameters, Chl content and Rubisco activity. Shi and Cai (2008) observed an inhibition in net photosynthetic rate due to the decreased stomatal conductance and content of photosynthetic pigment in A. hypogaea. Liu et al. (2011) also observed decrease in gas exchange parameters including net photosynthesis, stomatal conductance, intercellular CO₂ concentration and
transpiration rate in *Ricinus communis* due to Cd stress. The decrease in Chl content with Cd treatment possibly decreased the absorption of light by the chloroplast and thus indirectly impaired photosynthesis. Parmar et al. (2013) reported that decrease in Chl content in Cd exposed plants could have been due to disorganisation in Chl biosynthesis. Earlier finding of Myśliwa-Kurdziel and Strzalka (2002) has shown that Cd inhibited Chl biosynthesis by means of a reaction with the thiol groups of the enzymes of δ-aminolevulinic acid (ALA) synthesis and protoChlide reductase complex. Substitution of Mg$^{2+}$ ion of the Chl molecule by Cd might be a reason of arrested photosynthesis (Küpper et al., 1996). Earlier investigations have also demonstrated a marked reduction in the overall rate of photosynthesis by Cd in different plant species (Arduini et al., 2004; Wójcik and Tukiendorf, 2005; Jing et al., 2005; Khan et al., 2006, 2007; Mobin and Khan, 2007; Asgher et al., 2014). Deleterious effects of Cd on various facets of photosynthesis such as gas exchange, biosynthesis of Chl, functioning of photochemical reactions and the activities of the enzymes of the Calvin cycle have been studied (Stobart et al., 1985; Weigel, 1985; De Filippis and Zeigler, 1993; Chugh and Sawhney, 1999; Vassilev et al., 2005). In addition, conductance, transpiration and net CO$_2$ uptake are greatly reduced with elevated Cd levels in the growth media (Bindhu and Bera, 2001; Balakhnina et al., 2005). Siedlecka and Krupa (1999) also reported Cd-induced decrease in carbonic anhydrase activity and other elements of Rubisco activation system.

The cumulative adverse effect of Cd on metabolism resulted in decrease in leaf area and plant dry mass in the cultivars of *B. juncea* but less decrease occurred in Ro Agro 4001 (Fig. 8A-B). Ro Agro 4001 with more increase in leaf area efficiently utilized solar radiation resulting in higher photosynthesis and plant dry mass. It has been found that Cd contamination resulted in visible symptoms of chlorosis, stunted growth and growth inhibition in plants (Baryla et al., 2001; Dai et al., 2006; Gallego et al., 2012). The increasing concentration of Cd significantly decreased the morphological parameters of plants. The reduction in number of leaves as well as leaf area negatively affected dry matter accumulation in plant (Sharma et al., 2010; Asgher et al., 2013). Growth reduction in Cd-treated plants has been described (Wu et al., 2003; Demirevska-Kepova et al., 2006) due to the higher accumulation of Cd and reductions in the availability of other nutrients resulting in disturbed metabolism (Wu
et al. 2006). It has also been reported previously that species and cultivars display marked differences for Cd tolerance in *T. aestivum* (Zhang et al., 2002; Khan et al., 2006), *H. vulgare* (Wu et al., 2003), *G. hirsutum* (Wu et al., 2004), *B. juncea* (Qadir et al., 2004), *P. sativum* (Metwally et al., 2005), *O. sativa* (Wu et al., 2006; He et al., 2006), *V. radiata* (Anjum et al., 2008) and *B. campestris* (Anjum et al., 2008).

5.2 Role of MeJA in S-Mediated Alleviation of Cd Stress

An approach leading to the decreased toxicity of HMs is beneficial to plants. One of the best methods rendering the plants resistant to metals is the exogenous application of phytohormones. The present study demonstrated the ability of MeJA in S-mediated alleviation of Cd stress.

5.2.1 Effect of MeJA on Cd-induced oxidative stress

The increased Cd content in leaf grown with Cd resulted in the production of ROS and oxidative stress as found in Experiment 1. The increase in oxidative stress in plants stimulates synthesis of antioxidant metabolites and enhances antioxidant enzyme activities that protect plant tissues. In this study, the application of MeJA or S in Cd stressed plant increased the activity of antioxidant enzymes (SOD, APX and GR) in leaves and substantially decreased H$_2$O$_2$ and TBARS content (Fig. 10A-B; Fig. 11A-C) and the levels of ROS as revealed by lower level of synthesis of O$_2$•– and H$_2$O$_2$ (histochemical staining of leaves with NBT and DAB) (Fig. 12; Fig. 13) determined in leaf. The effect was maximal when 10 µM MeJA and 1.0 mM SO$_4^{2–}$ was applied together. It has been shown that the increase in activity of antioxidants has potential to balance the ROS generation and protect plants against Cd toxicity (Khan et al., 2009; Anjum et al., 2012; Asgher et al., 2014). Recently, it has been shown that S nutrition was directly linked to tolerance via increasing antioxidant system (Gomes et al., 2014). The increase in the activity of antioxidant enzymes with the application of S has been reported in *O. sativa* (Hassan et al., 2005), *B. campestris* (Anjum et al., 2008), *T. aestivum* (Gaafer et al., 2012; Khan et al., 2015), *A. thaliana* (Bashir et al., 2013) and *B. juncea* (Asgher et al., 2014) under Cd stress.

In this study it was found that 10 µM MeJA significantly reduced Cd-induced increase in H$_2$O$_2$ and TBARS content and also level of generation of O$_2$•– and H$_2$O$_2$ in
leaves by increasing antioxidant enzymes activity. Application of MeJA has been found to either increase or decrease the oxidative stress and antioxidant system in plants depending on the concentration applied and plant species used. The higher concentration (100 μM) of JA has been reported to increase accumulation of Pb, whereas 0.1 μM application of JA inhibited Pb accumulation in *Wolffia arrhiza* (Piotrowska et al., 2009). Methyl jasmonate at 5 μM concentration has been shown to increase activity of CAT, SOD, POD and GR and ameliorated the Cd-induced oxidative stress in *O. sativa* seedlings (Singh and Shah, 2014). Similarly, the treatment of 0.1-1.0 μM MeJA enhanced Cd tolerance in *C. frutescens* (Yan et al., 2013) and 0.1 μM MeJA in *S. nigrum* (Yan et al., 2015b). Chen et al. (2014) also found that 0.1-1.0 μM MeJA alleviated Cd-induced oxidative damage and enhanced antioxidant enzyme activity in *K. obovata* leaves. Furthermore, application 0.1-10 μM MeJA minimized oxidative stress in As exposed *B. napus* plants enhancing enzymatic activities and gene expression of antioxidant enzymes (CAT, SOD, APX, POD) and the regulation of multiple transcriptional pathways which were involved in oxidative stress responses (Farooq et al., 2016). It has been found by Yan et al. (2013) that MeJA limited lipid peroxidation under Cd stress in *C. frutescens* as evidenced by reduced content of MDA. Our results provide a supportive indication that MeJA reduced TBARS content (Fig. 11B). The application of MeJA showed protective effects on the cell membrane lipid and mitigated the Cd-induced lipid peroxidation in *B. juncea*. The results on MeJA capacity to reduce lipid peroxidation in the stressed plants are consistent with earlier findings about MeJA-induced alleviation of stress due to HMs (Singh and Shah, 2014; Hanaka et al., 2016; Farooq et al., 2016).

The present study, however, shows more promising results in enhancing the antioxidant system and lowering the oxidative stress when S and MeJA were applied together. It is likely that MeJA grown plants reduced the oxidative stress more efficiently when plants received S through increased antioxidant metabolism. This was apparently because of higher S-assimilation capacity of plants due to S and MeJA treatment under Cd stress. The present study therefore, suggests a tight correlation between MeJA and S in plants for alleviating Cd stress as greatest alleviation was found with the combined treatment of MeJA and S.


### 5.2.2 MeJA enhances S-assimilation for Cd tolerance

Application of MeJA and/or S increased activity of ATP-S and content of S, Cys and GSH under Cd stress (Fig. 15A-B; Fig. 16A-B). Studies have shown that thiol metabolism and antioxidant defense system increased in *H. vulgare* plants subjected to metals stress (Astolfi et al., 2012). The results reported here suggested that ATP-S plays a key role in maintaining Cys and GSH pool required for Cd stress tolerance. Addition of S to Cd-stressed plants increased ATP-S activity with increased Cys and GSH contents. Masood et al. (2012a) and Asgher et al. (2014) have reported that increased GSH content as a result of S treatment inhibits the Cd-induced oxidative stress with lesser damage to photosynthesis in *B. juncea*. The potential of plants to survive in Cd contaminated conditions has been found with improved S-assimilation pathway (Wangelin et al., 2004; Khan et al., 2009; Bashir et al., 2013). It has been reported that Cd induces activation of S-assimilatory enzymes as well as thiol pools in order to tackle Cd imposed stress (Harada et al., 2002; Rother et al., 2006). Nazar et al. (2008) and Fatma et al. (2014) have reported that higher ATP-S activity and sulphate transport index in *B. juncea* indicated its higher sulphate accumulation capacity with increased photosynthesis and plant dry mass.

Under normal conditions, a steady state GSH level is maintained in plant cells via a low level of transcription, translation and optimal enzyme activity. However, the homeostasis is perturbed and GSH pool is consumed to combat stress. Elevated GSH pools in presence of MeJA as observed herein in Cd-stressed mustard plant is similar to the results of Sing and Shah (2014) in *O. sativa* under Cd stress and Farooq et al. (2016) in *B. napus* under As stress. This is in confirmation to the earlier findings that plants with improved capacity for GSH synthesis display higher Cd-tolerance (Schützendubel and Polle, 2002; Yan et al., 2015b). The results have shown that MeJA could regulate the GSH pathway by inducing the activities and transcript levels of GR and GSH under As stress (Farooq et al., 2016). It has been reported that JA treatment increased the transcript levels of *γ-ECS (GHS1)* and *GSH2*, as well as GR (Xiang and Oliver, 1998) and several GSH-S-transferase (GST; EC 2.5.1.13) encoding genes (Schenk et al., 2000; Sasaki et al., 2001). Harada et al. (2000) reported a transient up-regulation of four genes of sulphate reduction in 10 day old seedlings. Farooq et al. (2016) also concluded that MeJA could regulate the GSH pathway by inducing the
activities and transcript levels of GR and GSH under As stress. Consequently, more GR enzyme is required which is supplemented via de novo protein synthesis. Our study is further similar to the observations showing that MeJA application resulted in enhancement in tolerance to As, Cu, Cd and Pb stress by promoting GSH accumulation (Maksymiec et al., 2007; Piotrowska et al., 2009; Farooq et al., 2016). The genes for S metabolism have been found to be up-regulated by MeJA. Genes encoding key reactions of sulphate reduction as well as of Cys, Met and GSH synthesis were rapidly up-regulated, but none of the known S-deficiency induced sulphate transporter genes (Jost et al., 2005).

5.2.3 MeJA and S improve photosynthesis under Cd stress

Oxidative stress due to Cd disturbs the structural and functional integrity of photosynthetic system including chloroplast and reduce the efficiency of PSII and activity of Rubisco (Mobin and Khan, 2007; Afef et al., 2011; Dias et al., 2013; Kapoor et al., 2014; Liu et al., 2014). The present study showed that Cd significantly reduced gas exchange parameters, Chl content and Rubisco activity. It was however, found that supply of MeJA or/and S improved these characteristics (Fig. 17A-C; Fig. 18A-B). The individual or combined application of S and MeJA favoured S-assimilation and GSH synthesis. This cumulatively protected chloroplast and enzymes of the C3 cycle. The relationship between S allocation in leaves and Rubisco activity has been shown (Iqbal et al., 2012). The decreased Chl content in response to Cd supply may be due to disturbance in Chl biosynthesis (Parmar et al., 2013).

Application of MeJA with S significantly increased the activity of Rubisco, suggesting a restoration of photosynthetic apparatus by MeJA in Cd-stressed plants. However, the role of MeJA in protecting plants from various abiotic stresses is controversial. At a concentration of 100 μM or higher MeJA has been found to repress germination and plant growth (Jubany-Mari et al., 2010; Kobayashi et al., 2010) and Rubisco genes, chlorophyll-binding proteins and light harvesting complex II (Jung et al., 2007). It has been suggested that in Pb-treated fronds of W. arrhiza, 100 μM JA enhanced the inhibitory effect on the Chl formation by binding with -SH group of ALA dehydratase (EC 4.2.1.24) and through Mg2+ which is an essential co-factor for this enzyme (Piotrowska et al., 2009). The additive phytotoxic effect of JA and HMs
(Cd, Cu) on the chloroplast pigments in *A. thaliana* has also been found by Maksymiec and Krupa (2002a). On the other hand, at a concentration of 50 µM or lower MeJA has been reported to stimulate plant growth, plant dry mass, photosynthetic pigment levels, and increased photosynthesis (Tung et al., 1996; Mabood et al., 2006; Piotrowska et al., 2010; Wu et al., 2012). The present study showed that 10 µM MeJA improved chlorophyll content, gas exchange parameters and Rubisco activity under Cd stress. It seems that the improved gas exchange parameters, Chl content and Rubisco activity observed in our study was due to the activation of antioxidant enzymes and recovery of photosynthetic efficiency resulted with MeJA application. Keramat et al. (2010) reported that MeJA at 0.01 mM concentration was more effective in reducing the damages of Cd stress to shoot dry weight and total Chl content in *G. max*.

It has been suggested that Cd irreversibly binds to -SH groups on the active site of Rubisco, thereby lowering its activity (StiborovÁ et al., 1988). SDS-PAGE profile of Rubisco protein followed the similar pattern in band width of proteins as Rubisco activity on the application of different treatments (Fig. 19). Application of MeJA or/and S promoted synthesis of protein band as the increased demand of reduced S under Cd stress was utilized for Cys and GSH synthesis, consequently the increased assembly of new proteins and increased tolerance to Cd stress. Recently, it has been shown that S and Se increased photosynthesis via increase in the allocation of N and S to Rubisco in *B. juncea* and *T. aestivum* (Fatma et al., 2014; Khan et al., 2015). However, Cd plus S or/and MeJA increased the intensity of the original protein band and showed broader intensity band compared to their respective control.

The efficiency of photochemical processes is provided by measuring Chl fluorescence in leaves. In the present study, Chl fluorescence parameters were reduced under Cd stress which contributed to the decrease in the photosynthesis except non-photochemical quenching. Cadmium stress decreased PSII efficiency (Fig. 20A-F). This observation is in good agreement with the increased rate of lipid peroxides formation expressed as TBARS content in leaves under the influence of Cd. TBARS is the decomposition product of PUFAs of biomembranes and its increase shows plants are under high level oxidative stress (Singh et al., 2006). It has been reported that the decrease in Chl fluorescence under HMs stress results from
destruction of antenna pigments by the partial block of electron transport from PSII to PSI (Krupa et al., 1993; Khan and Khan, 2014; Liu et al., 2014). Application of S in plants invests a larger fraction of leaf S into photosynthetic apparatus under Cd stress conditions. A substantial increase in antioxidants with S application under Cd stress helps in reversing the effects of Cd-induced ROS on photosynthesis.

Methyl jasmonate did exert effect on Cd exposed plants on Fv/Fm ratio. Previously, Zou et al. (2011) and Hristova and Popova (2002) reported similar phenomena about the promoting effect of MeJA on Fv/Fm, when subjected to stress conditions. In the present study, the increase in Chl fluorescence parameters in the Cd + MeJA and Cd + S + MeJA indicated less damage to chloroplasts, especially thylakoid membranes. A higher value of Chl fluorescence is an indicator of thylakoid membrane stability and relative efficiency of electron transport, resulting in more photosynthesis and growth (Bhardwaj and Singhal, 1981; Bidabadi et al., 2013). Sharifi et al. (2012) showed a correlation between enzyme activity, Chl, and membrane stability. Their result indicates a positive correlation between POD activity with crop production and Chl stability. The unchanged Fv/Fm ratio in control plants with MeJA supplementation is in agreement with the results obtained in the initial growth stage in other research papers (Krupa et al., 1993; Maksymiec and Baszyński, 1996; Ananieva et al., 2007; Fedina et al., 2009; Hanaka et al., 2016).

5.2.4 MeJA and S improve growth under Cd stress

Cadmium stress reduced development of leaf area and plant dry mass (Fig. 21A-B). In the present study, the growth inhibition was the consequence of Cd interference with the vital metabolic processes such as photosynthesis (Iqbal et al., 2010; Nazar et al., 2012). The increase in growth with MeJA or/and S is attributed to GSH-mediated changes in photosynthesis. The maximum alleviation of Cd stress or Cd induced-inhibition in growth was observed with the combined treatment of MeJA plus S apparently because of more efficient S-assimilation and GSH production that resulted in maximum protection of cell. Studies have shown that S was instrumental in mitigating the toxic effects of Cd and increasing growth due to the synthesis of Cys and GSH (Hassan et al., 2005; Anjum et al., 2008; Masood et al., 2012a; Gaafar et al., 2012; Bashir et al., 2013; Asgher et al., 2014; Khan et al., 2015). Methyl jasmonate
treatment has also been reported to reduce toxic effects of Cd and restore plant growth (Keramat et al., 2009; Piotrowska et al., 2009; Kováčik et al., 2011; Yan et al., 2015b). Exogenously applied JA at 0.1 µM concentration has been found to show protective function against HM stress leading to restoration of fresh weight. Exogenously applied MeJA has been found to improve morphological responses and photochemical efficiency in Iranian grapevine (Bidabadi et al., 2013). Similar result has been reported in *A. thaliana* that showed applying MeJA at 0.001 and 0.01 mM concentrations alleviated the toxicity of Cu and Cd ions (Maksymiec and Krupa, 2002a; Yan et al., 2013). It has been reported that MeJA has dual roles, promoting and inhibiting effects on growth, Chl contents and plant performance depending on the concentrations used and crop species. Model 5 explains how the supplementation of S and MeJA reduced Cd-induced oxidative stress via increased production of GSH and improved photosynthesis and growth.

**Model 5.** Model representing the the potential mechanism of Cd stress mitigation by exogenous MeJA in plants. Arrows indicate the effect of JA on different metabolites under abiotic stress condition. Metabolite abbreviations; Cd, cadmium; Cys, cysteine; GSH, glutathione; H₂O, water; H₂O₂, hydrogen peroxide; H₂S, hydrogen sulphide; MeJA, methyl jasmonate; O₂, superoxide; O₂⁻, superoxide ion; PC, phytochelatin; PS, photosystem; ROS, reactive oxygen species: SO₄²⁻, sulphate; SO₃²⁻, sulphite; Enzyme abbreviations: APX, ascorbate peroxidase; SOD, superoxide dismutase.
5.2.5 MeJA and S influence stomatal behaviour

Compound and scanning electron microscopy study revealed the potential of MeJA and S on stomatal responses of plants. In our study comparatively closed stomata were found in Cd treated plants than the control plants (Fig. 22A-P; Fig. 23A-F). Treatment with MeJA or/and S reduced the closing effect of Cd stress on stomata (Fig. 22A-P). It was interesting to note that MeJA showed no effect on stomatal behaviour in absence of Cd stress. However, combined treatment of MeJA and S prominently reduced the effect of Cd on stomatal width aperture. The results were further confirmed by the scanning electron microscopy study as shown in the Fig. 23. The treatment with MeJA and S highly influenced GSH content by modulating the production of GSH that resulted in stomatal opening. Generally, it has been found that MeJA induces stomatal closure (Suhita et al., 2004; Jahan et al., 2008; Islam et al., 2010), but it has been found that increase in intracellular GSH suppresses stomatal closure (Akter et al., 2013). It is interesting to note that the stomatal closure occurs by decreasing intracellular GSH and that intracellular GSH regulates stomatal movement (Jahan et al., 2008). Another interesting thing to be noted is that ROS production in guard cells is required for stomatal closure (Munemasa et al. 2007; Jahan et al. 2008). This study therefore, indicates the involvement of intracellular GSH in S and MeJA-mediated regulation of Cd-induced ROS production and stomatal closure. This is the first report on the response of stomatal movement to MeJA and S supplementation to Cd stressed plants.

5.2.6 Effect of MeJA and S on chloroplast ultrastructure under Cd stress

A coordinated impact of MeJA and S on the ultrastructural changes of chloroplasts under Cd stress has not earlier been described. The application of MeJA and S prevented the chloroplast structural damage under Cd stress (Fig. 24A-F). Chloroplast structure showed a well developed and regular shape with well arranged thylakoid systems. The plants exhibited more chloroplasts per cell or more thylakoid membranes per chloroplast than the control or Cd treated plants with MeJA plus S due to the presence of higher Chl content and lower level of lipid peroxidation. It has been suggested that the lipid-to-Chl ratio is a good estimate for the protein-packing density in thylakoid, and the high lipid-to-Chl ratio reflects a low protein-packing density (Kirchhoff et al., 2013). Chloroplasts are unique organelles that contain a relatively
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high level of unsaturated fatty acids in its membranes, therefore are especially
sensitive to stress factors generating oxidative burst and lipid peroxidation (Apel and
Hirt, 2004). In the present study, plants receiving S and MeJA exhibited lower level of
lipid peroxidation and showed higher Chl contents than control or Cd treated plants as
shown in the Fig. 24. Thus, it is possible that the lipid-to-Chl ratio is lower or the
plants have more chloroplasts per cell or more thylakoid membranes per chloroplast
than the control or Cd treated plants.

5.3 NO Involvement in S-Mediated Alleviation of Cd Stress

5.3.1 NO-mediated reduction in oxidative stress

In the present study, SNP was used as the efficient NO donor because it gives
rise to a persistent pattern of NO generation and delivers NO for longer duration than
the other NO donors (Bethke et al., 2006; Mur et al., 2013). However, SNP also
releases cyanide and/or there is production of complexes of cyanide with Fe. It has
been suggested that these compounds of cyanide may have separate or overlapping
effects with the effects of NO alone on biological tissues (Bethke et al., 2006). To
counter this problem, we ensured the effect of NO from SNP in photosynthetic
protection of plants under Cd stress using cPTIO, as NO scavenger.

Application of SNP decreased Cd accumulation in leaves, however, slight
increase in Cd content in roots was noted which did not differ significantly with Cd
treated plants. This reduction in leaf Cd accumulation may be due to reduction in
translocation of Cd. Studies on *Matricaria chamomilla* and *Scenedesmus quadricauda*
have shown that NO stimulated Cd accumulation in shoots and roots (Štork et al.,
2013; Kováčik et al., 2014). Nitric oxide has been shown to be a crucial ingredient in
plant Fe metabolism because of the metal nitrosylation of protein Fe (He et al., 2014).
Further, NO has been shown to favour accumulation of Cd in roots by counteracting
the Cd-induced suppression of the Fe starvation response genes (Colangelo and
Guerinot, 2004; Besson-Bard et al., 2009). The confinement of Cd in roots might be
the result of efficient association or sequestration of Cd with GSH and phytochelatins
(PCs) in vacuoles (Mishra et al., 2006). It has been confirmed that exogenous NO
mitigated Cd toxicity in *O. sativa* by improving Cd content in the root cell walls and
reducing its accumulation in the leaves (Xiong et al., 2009). Spraying of SNP together
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with S supplementation maximally minimized the contents of $\text{H}_2\text{O}_2$ and TBARS in Cd-treated plants because of the enhanced S-assimilation capacity and antioxidant metabolism. Many reports have indicated that exogenous application of SNP improved tolerance of plants to HM stress (Singh et al., 2009, 2016; Chen et al., 2010b; Xu et al., 2010; Wang et al., 2013; Xu et al., 2015). These studies ascertained that the protective function of NO includes the regulation of ROS and antioxidants, induction of gene expression, and absorption and distribution of elements (Hsu and Kao, 2004; Xiong et al., 2009; Xu et al., 2010).

The addition of SNP inhibited the level of generation of $\text{O}_2^{-}$ and $\text{H}_2\text{O}_2$ under excess Cd, and protected cell membrane from oxidative injury and alteration in cell structure and decreased the accumulation of TBARS. Huaifu et al. (2007) have suggested that NO mitigates the damage to the cell membrane system by reducing the membrane permeability and lipid peroxidation, thereby preventing electrolyte leakage. Some authors believe that NO is a stress-inducing agent, whereas others firmly believe that the protective effect of NO depends on the supplied dose, treatment time, and the selected species (Kováčik et al., 2014). Sodium nitroprusside increased the activity of antioxidant enzymes, SOD, APX and GR in the present study (Fig. 29A-C). Addition of SNP improved the activity of SOD, APX, POD, CAT and GR when plants were exposed to Cd stress (Wang et al., 2013; Xu et al., 2015; Liu et al., 2015; Dong et al., 2016) and increased the activity of SOD, CAT, APX and GR when plants were subjected to salt stress (Ahmad et al., 2016a; Fatma et al., 2016a). Therefore, SNP-mediated increase in activity of antioxidant enzymes might be important mechanism on the improvement of antioxidative ability under Cd stress. However, the mechanism of NO-mediated alteration of antioxidative enzyme activities is still not clear. It is widely accepted that NO protects plant cells against oxidative stress by scavenging Fenton-reaction and regulating antioxidant enzymes (Laspina et al., 2005). Lamattina et al. (2003) reported that NO might regulate the expression of antioxidant genes to stimulate the relative enzyme activities. Chen et al. (2010b) have shown that the protective effect of NO in relation to Cd toxicity is partly related to its role in up-regulating the expression of genes encoding antioxidative enzymes under Cd stress. The positive effect of NO on oxidative stress was found after plant exposure to salt (Zhao et al., 2007; Zheng et al., 2009; Ahmad et al., 2016a;
Fatma et al., 2016a), heat (Song et al., 2009), drought (García-Mata and Lamattina, 2001), and ion deficiency (Graziano et al., 2002).

5.3.2 NO increases Cd stress tolerance by enhancing S-assimilation

The substantial increase in S-assimilation capacity of plants under Cd stress with supplementation of S is in accordance with the earlier studies (Masood et al., 2012a; Asgher et al., 2014). The effect of SNP spraying plus S supplementation, however maximally increased the activity of ATP-S and content of Cys and GSH under Cd stress; and the higher demand was met with the increased ATP-S activity by exogenously applied S (Fig. 31A-B; Fig. 32A-B). It has been reported that the accumulation of Cd in plants competes for the transmembrane carriers of mineral nutrients, such as K, Ca, Mg, Fe, Mn and Zn (Di Toppi and Gabbrielli, 1999; Xu et al., 2015), which might account for the reduction of Ca, Mg, Fe and Cu in leaves (Xu et al., 2015). Supplementation of SNP however, has been found to stimulate the uptake of nutrient elements such as Ca, Mg, Fe and Cu (Wang et al., 2013; Xu et al., 2015). It is well known that H⁺-ATPase in plasma membrane plays an important role in transport of multiple ions (Palmgren and Harper, 1999), and there are investigations indicating that NO could induce H⁺-ATPase activity thereby increasing the absorption of Ca, Mg, Fe and Cu under Cd stress (Zhang et al., 2009; Cui et al., 2010; Xu et al., 2015). An increasing number of research articles have focussed on the effects of exogenous NO in ameliorating Cd-induced oxidative stress in plants (Wang et al., 2013; Xu et al., 2015; Bai et al., 2015; Liu et al., 2015; Dong et al., 2016). However, this is the first report discussing the involvement of NO in enhancing S-assimilation in plants supplemented with S under Cd stress. Here, in this study NO influenced S-assimilation leading to the synthesis of GSH. It has been shown that NO affects S-assimilation and enhanced GSH production (Arasimowicz-Jelonek et al., 2011). Innocenti et al. (2007) have provided the evidence that GSH synthesis pathway was regulated by NO in M. truncatula. They have shown the up-regulation of γ-ECS (γ-ecs) and GSH synthetase (gshs) genes expression after treatment with SNP and GSNO. Likewise, exogenous NO improved γ-ECS activity in S. lycopersicum roots and facilitated the synthesis of GSH and PCs and thereby enhanced peroxide removal under Cu stress (Wang et al., 2015). Xu et al. (2010) also reported that Cd treatment decreased both NO and GSH content in M. trunculata roots and exogenous NO
alleviated Cd toxicity partly by recovering Cd-induced decreases of NO and GSH content. Several studies have shown increase in GSH content in different plants under different stress conditions such as salt stress in T. aestivum, Z. mays and B. juncea (Hasanuzzaman et al., 2011; Keyster et al., 2012; Fatma et al., 2016a), As in T. aestivum (Hasanuzzaman and Fujita, 2013), Cu in Panax ginseng (Tewari et al., 2008), Cd in H. annuus and O. sativa (Laspina et al., 2005; Hsu and Kao, 2004) and UV-B in P. vulgaris (Shi et al., 2005). Although these researches require further elaboration, it is possible that exogenously applied NO enhanced HM tolerance by regulating the expression of GSH synthesis-related genes and increasing GSH content in plants. Therefore, the relationships between NO-regulated GSH synthesis and NO-enhanced HM tolerance could be an important potential direction for future research. However, GSH may also play an important role in regulating NO bioactivity. Nevertheless, NO has been proposed to regulate plant gene expression (Begara-Morales et al., 2014). The S-nitrosylation reaction of NO with GSH to form GSNO has an important physiological function in plants, as GSNO is regarded as a mobile reservoir of NO bioactivity. The enzyme GSNOR modulates the content of GSNO in plants by catalyzing the NADH-dependent reduction of GSNO to GSSG and NH3 (Wang et al., 2006). Thus, a rapid recycling or synthesis of GSH may be responsible for the role of NO in maintaining the GSH pools as well as the ratio of GSH to GSSG. The activity and expression of GSNOR have been reported to be induced by various stimuli, including pathogen infection, wounding, low and high temperature and HMs (Delledonne et al., 1998; Pagnussat et al., 2002; Desikan et al., 2002; Neill et al., 2003). Ruan et al. (2004) reported an increased GSH/GSSG ratio with exogenous application of NO in T. aestivum seedlings subjected to salt stress. Exogenous NO increased GSH content in S. lycopersicum roots and leaves under Cu stress, adjusting the GSH/GSSG ratio, and mitigated the effects of ROS (Wang et al., 2015). Similarly, heat treatment resulted in decrease in GSH/GSSG ratio which was significantly reversed by the supplementation of NO (Hasanuzzaman et al., 2012). Thus, elevation in GSH content, redox state could strengthen antioxidant system, such as improving efficiency of AsA-GSH cycle (Drążkiewicz et al., 2003) and thereby promoting S-assimilation capacity. Seth (2012) has shown that exogenous NO accelerated GSH transport to the underground parts under Cu stress.
5.3.3 NO amplifies S-induced increase in photosynthesis under Cd stress

The increase in photosynthesis with SNP or/and S involved both stomatal and non-stomatal limitations as the treatments increased stomatal conductance allowing more exchange of intercellular CO\(_2\) concentration on one hand and increased Rubisco activity and expression on the other. The combined effect of SNP plus S was found more promising in increasing gas exchange parameters, Chl content and Rubisco activity in both absence and presence of Cd (Fig. 33A-C; Fig. 34A-B). The improvement of Chl content might be related to alleviating effects of S and NO, thus promoting photosynthesis in plants. The mechanisms of beneficial effects of NO on photosynthesis under Cd stress may be related to the protection of Chl (Fig. 34A), decreased ROS and increased activity of antioxidant enzymes. Nitric oxide influenced photosynthesis under Cd stress by favouring S-assimilation, and GSH synthesis. Moreover, significant effect of NO on stomatal movement was also noticed. The increased production of GSH by NO provided an important regulatory loop for NO bioactivity leading to increased reduced environment of cells under Cd stress. Several reports have shown the enhancement in photosynthetic pigments and protection of chlorophyll damage in NO treated *T. aestivum* (Ruan et al., 2004), *L. esculentum* (Wu et al., 2011) and *B. juncea* (Fatma et al., 2016a) under salt stress, *H. annuus* (Laspina et al., 2005) under Cd stress, *L. perenne* (Dong et al., 2016) under Cu stress. Chen et al. (2010b) found that NO increased stability and integrity of the subcellular structure under Cd stress and prevented Cd-induced leaf chlorosis and inhibition of photosynthesis in *H. vulgare*. Moreover, NO increased the uptake of Fe and Mg and improved Chl synthesis accompanied by the recovery of the rate of photosynthesis and transpiration (Wang et al., 2013). Similarly, Kong et al. (2014) found that foliar application of NO and SA improved photosynthesis and uptake and translocation of Fe in *A. hypogaea*. Moreover, exogenous NO promoted the uptake and translocation of K, Fe and Zn mineral elements under Cu stress in *L. perenne* (Dong et al., 2016).

The present study also showed the effect of SNP or/and S on activity and SDS-PAGE profile of Rubisco and Chl fluorescence characteristics (Fig. 35; Fig. 36A-F). Supplementation of NO with S was beneficial in increasing the activity and band intensity of Rubisco and PSII efficiency. These results were mainly attributed to the prevention of growth inhibition and Chl degradation, the recovery of GSH levels, and
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the enhancement of antioxidant machinery allowing the plant to cope better with Cd stress. The reduction in Chl fluorescence parameters in Cd exposed plants might be attributed either to the alterations in the levels of Chl biosynthetic pathway or to an alteration in stoichiometry between PSI and PSII (Balakrishna et al., 2005). On the contrary, application of SNP kept Fv/Fm, Fv/Fo, and ΦPSII at similar level with control, which indicated that Cd stressed plants could keep higher activity of reaction centre by NO application. Moreover, Zhang et al. (2006) reported that application of NO increased the quantum yield of H. vulgare. Vladkova et al. (2011) found that the SNP is the only NO donor which stimulates electron transport through PSII. They hypothesized that NO interacts with the tyrosine residue of the D2 protein, YD and the formation of YD•–NO complex decreased redox potential to a level which is low enough to become a more efficient electron donor in isolated thylakoid membranes than the redox-active tyrosine residue, YZ, located on the D1 protein. The stimulating effect of NO on PSII photochemistry has been correlated with an increase in the proportion of the open PSII reaction centres. Enhancement in oxygen consumption with application of NO has been suggested as another possible explanation of its reported protective effect (Santisree et al., 2015).

5.3.4 NO cooperates with S to improve growth under Cd stress

Application of SNP or S to the Cd-treated plants alleviated Cd-stress effect on plant growth. Previous studies have reported that exogenous NO provides protection against Cd toxicity in O. sativa (Hsu and Kao, 2004), H. annuus (Laspina et al., 2005), T. aestivum (Singh et al., 2008), and L. perenne (Wang et al., 2013; Bai et al., 2015; Dong et al., 2016). Singh et al. (2016) have shown that NO enhanced plant growth in terms of root, shoot length, and biomass in As stressed O. sativa and Tian and Lei (2006) have found that low concentration of NO promoted the growth of T. aestivum. Application of NO has been found to enhance plant dry mass and leaf area under salinity stress in B. juncea (Fatma et al., 2016a). The study of Bai et al. (2015) showed that the inhibitory effect of leaf growth was alleviated by the application of NO by counteracting the oxidative damage and by increasing the mineral nutrient under Pb stress in L. perenne. Model 6 explains how the application of SNP and S reduced Cd-induced oxidative stress via increased GSH production and improved photosynthesis and growth.
Model 6. Model representing the potential mechanism of Cd stress mitigation by exogenous SNP. Metabolite abbreviations; APS, adenosine-5-phosphosulphate; BSO, buthionine sulphoximine; Cys, cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; GSNO, S-nitrosoglutathione; γ-GC, γ-glutamyl cysteine; H₂O₂, hydrogen peroxide; NO, nitric oxide; O₂⁻, superoxide ion; ROS, reactive oxygen species; S, sulphur; SNP, sodium nitroprusside; Enzyme abbreviations; APX, ascorbate peroxidase; GR, glutathione reductase; GSNOR, GSNO reductase; SOD, superoxide dismutase. Arrows (→) show promoting effect and symbol (↔) shows inhibition.

5.3.5 NO and S influence stomatal behaviour

The stomatal frequency was higher in SNP and S supplied plants than control or Cd treated leaves as shown in the compound microscope and SEM images (Fig. 38A-P; Fig. 39A-F). Various studies, however, have shown that SNP induces stomatal closure both in stress and no stress conditions (Bright et al., 2005; Neill et al., 2008; Fan and Liu, 2012). Here, SNP induces stomatal opening contrary to the earlier studies. Sakihama et al. (2003) showed that NO was involved in the signal transduction mechanisms for stomatal opening in V. faba. Moreover, the study of Frey et al. (1996) reported the effects of ozone and mineral nutrients supply on stomatal response in Betula pendula and suggested that mineral nutrients not only determine the structural components of guard cells but also participate in photosynthetic
reactions. The opening of stomata under Cd stress in the present study can be ascribed to the increase in antioxidant metabolism and also increase in the synthesis of S-assimilatory enzymes and the higher S-assimilation capacity resulting in more production of GSH that delayed the transpirational water loss and the stomata were observed to be normal with stomatal aperture open.

### 5.3.6 Effect of NO and S on Ultrastructure of chloroplast

A coordinated effect of SNP and S on the ultrastructural changes of chloroplasts under Cd stress revealed that thylakoid showed similar pattern with the content of Chl under Cd stress. Chloroplast ultrastructure grown under control conditions showed well-developed thylakoid membrane system, while Cd treatment resulted in misshaped thylakoids. Similar results were obtained in our previous study that showed the influence of salt stress on chloroplast structure (Fatma et al., 2016a). Application of SNP or/and S prevented the effect of Cd stress on thylakoid structure as also found in salt treated plants by Fatma et al. (2016a). The present study also showed that plants receiving SNP and S exhibited lower level of lipid peroxidation and showed higher Chl contents than control or Cd treated plants. Thus, it is possible that the lipid-to-Chl ratio is lower or the plants have more chloroplasts per cell or more thylakoid membranes per chloroplast than the control or Cd treated plants.

### 5.4 Involvement of GSH in MeJA/NO Mediated Alleviation of Cd Stress

To consolidate the findings of the present study that GSH was involved in MeJA/NO mediated Cd tolerance, GSH biosynthesis inhibitor BSO in the present experiment was used. Buthionine sulfoximine inhibits the synthesis of GSH from Cys by inhibiting the activity of enzymes GSH1 (γ-ECS). In the present experiment BSO was given along with S and MeJA/NO to Cd treated plants and assessment was made for oxidative stress, GSH content, photosynthetic and growth characteristics.

Treatment with MeJA/SNP resulted in a significantly increased production of GSH in the leaves of mustard (Table 9). Pre-treatment with BSO substantially blocked the increase in the production of GSH induced by MeJA/SNP both in the presence and absence of S in Cd treated plants. It was observed in the present study that BSO inhibited GSH synthesis in MeJA/SNP plus Cd-treated plants in the presence and
absence of S and aggravated oxidative damage by significantly increasing the production of $O_2{^{\cdot-}}$ and $H_2O_2$ in the leaves which were, however, decreased by the application of MeJA/SNP in presence and absence of S in Cd treated plants (Fig. 41; Fig. 42). The resulted increase in oxidative stress in presence of BSO resulted in decreased photosynthetic (gas exchange parameters, Chl content, Rubisco activity and PS II efficiency) and growth (leaf area and plant dry mass) characteristics in Cd stressed plants. The effect of MeJA/SNP on S-mediated responses of stomatal behaviour was also reversed in BSO treated plants (Fig. 43A-L; Fig. 44A-J). Moreover, ultrastructural studies of chloroplast were similar to the effect of BSO + Cd on Chl content showing more damaged thylakoids and misshaped chloroplasts (Fig. 45A-J). However, in the presence S, MeJA stimulated GSH production via increased S-assimilation under Cd even with the supplementation of BSO. It has been suggested that MeJA either up-regulated the expression of genes involved in biosynthesis of GSH or facilitated the synthesis of GSH (Xiang and Oliver, 1998; Farooq et al., 2016) and enhanced peroxide removal and eventually photosynthetic efficiency under Cd stress. The increase in GSH production by SNP application provided an important regulatory sphere for NO bioactivity due to better reduced environment under Cd stress. Likewise, exogenous NO improved γ–ECS and GSH synthetase activities in *Solanum lycopersicum* roots and facilitated the synthesis of GSH and PCs and enhanced peroxide removal under Cu stress (Wang et al., 2015). During stress, excess GSH has been shown to readily react with NO and form GSNO, which serves as NO reservoir in plants (Chaki et al., 2009, Wang et al., 2015). GSNO is formed by the covalent attachment of NO to the Cys thiol within the GSH, and is thought to constitute a relatively stable store of NO bioactivity (Corpas et al., 2013). The GSNO formed is catalyzed by GSNOR producing GSSG and $NH_3$. The GSSG produced is reduced again to form GSH by GR. Thus, a rapid recycling of GSH is related to the role of NO in maintaining the GSH pools. Therefore, MeJA/SNP-mediated GSH production is important potential mechanism in Cd tolerance in our study. Taken together, our results clearly suggest that MeJA/SNP-induced GSH production results in the enhancement of tolerance to Cd-induced oxidative damage and maintenance of photosynthesis. Model 6 and 7 explains how the application of S and MeJA/SNP reduced Cd-induced oxidative stress via increased GSH production and improved photosynthesis and growth.
Model 7. Model representing the potential mechanism of Cd stress mitigation by exogenous MeJA. Metabolite abbreviations; APS, adenosine-5-phosphosulphate; BSO, buthionine sulfoximine; Cys, cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; γ-GC, γ-glutamyl cysteine; H$_2$O$_2$, hydrogen peroxide; MeJA, methyl jasmonate; O$_2^-$, superoxide ion; ROS, reactive oxygen species; S, sulphur; Enzyme abbreviations; APX, ascorbate peroxidase; GR, glutathione reductase; SOD, superoxide dismutase. Arrows (→) show promoting effect and symbol (→↓↑) shows inhibition.

5.5 Conclusion and Future Research Prospects

In conclusion, the results reported in the present study clearly demonstrated that cultivars differ in their sensitivity to Cd stress. Ro Agro 4001 showed high photosynthetic potential under Cd stress mainly because of high antioxidant metabolism and S-assimilation capacity, which increased GSH production and improved photosynthesis and growth of plants.

Methyl jasmonate and NO played important role in S-mediated regulation of photosynthesis and growth under Cd stress. It was evident that MeJA and NO were involved in protecting and improving photosynthesis and growth of plants grown under Cd stress. The Cd stress caused the induction of oxidative stress in the *B. juncea* plants due to overproduction of ROS contents. Application of MeJA/SNP induced the tolerance in *B. juncea* plants against Cd stress by maintaining antioxidant
system which played a key role in scavenging the $\text{H}_2\text{O}_2$ and $\text{O}_2^{\cdot-}$ contents. The GSH production is an important defense response to oxidative stress and MeJA and SNP application substantially enhanced GSH contents that helped in removing the excess ROS. In the present study, our data on BSO application along with the other treatments clearly showed that exogenous MeJA/SNP induced an increase in the synthesis of GSH and enhanced tolerance to Cd-induced oxidative stress.

The toxicity of HMs in plants depends on absorption and bioaccumulation which is associated with the availability, uptake, storage, degradation, excretion and also avoidance/tolerance mechanisms. The employment of biological approaches or techniques concerning the molecular mechanisms of MeJA and NO activity will allow for better and more detailed understanding of anti-stress activities of these phytohormones and assist the development of suitable strategies for MeJA and NO-induced protection in plants from metal stress. Owing to the importance of MeJA and NO in the crop improvement, further investigations are needed to identify the key regulatory elements in MeJA and NO signalling pathway and the underlying mechanism of MeJA and NO-modulated growth and developmental responses in plants to design optimal strategies to enhance crop yield and improve their performance under stress conditions. There appears existence of elegant regulatory controls to switch ‘on or off’ the GSH homeostasis mechanisms in accordance with the changing status of the stress. There are no reports on involvement of both MeJA/NO and S in Cd tolerance. Therefore, the response of plants to MeJA or NO in enhancing/strengthening Cd tolerance involving mineral nutrients at biochemical and molecular level needs to be investigated. Future strategies should be focussed on to study the interaction of MeJA with NO in modulating the S assimilatory enzymes under Cd stress and molecular regulation of enzyme activities in different plant tissues. Moreover, analyses of some more S-containing compounds should be done in addition to Cys and GSH to gain insight into the involvement of thiols in Cd stress alleviation. Studies should also be focussed on to unravel the cross-talk between MeJA and NO and their interaction with other phytohormones in the regulation of metabolism under Cd stress that will give more insight under optimal or stress conditions on photosynthetic capacity of plants. The integrated approach from other disciplines of plant sciences such as molecular biology, biochemistry and genetic engineering towards better understanding of the mechanisms involved in Cd tolerance induced by S and phytohormones would help in solving the problem.