DISCUSSION
## 5.1 Screening of Mustard Cultivars Treated with Different Levels of Cadmium
- Growth characteristics 86
- Photosynthetic characteristics 88
- Cadmium accumulation 92
- Yield characteristics 93

## 5.2 Alleviation of Cadmium-toxicity by Sulfur and Nitrogen in Alankar (Cd tolerant) and RH30 (Cd non-tolerant) Cultivars of Mustard
- Growth characteristics 96
- Photosynthetic characteristics 97
- Sulfur and nitrogen metabolism 100
- Cadmium accumulation 103
- Cadmium-induced oxidative stress 104
- Components of antioxidant system 105
- Enzymatic antioxidants 105
- Non-enzymatic antioxidants 110
- Yield characteristics 113

## 5.3 New Report in the Thesis and Future Prospects 115
Every year India loses hundreds of millions of rupees from reductions in crop productivity caused by abiotic stresses (Mahajan and Tuteja 2005). According to Bray et al. (2000), the relative decreases in potential maximum yields associated with abiotic stress factors vary between 50-80%. Among abiotic stresses, heavy metal contamination is a serious environmental problem that limits plant productivity and threatens human health (Wagner 1993, Sanita di Toppi and Gabbrielli 1999). The agricultural soil may have toxic levels of heavy metals due to agricultural and industrial practices such as application of pesticides and chemical fertilizers, waste water irrigation, precipitation from heavy coal combustion, and smelter wastes and residues from metalliferous mining (Lombi et al. 2000, Vassilev et al. 2006, Xie et al. 2006, Verma et al. 2007).

For sustainable crop production, it is important to develop methods or techniques for alleviating the Cd-induced growth inhibition and reducing its accumulation in plants. One option is the use of balanced S and N fertilizers for alleviating Cd stress and maintaining productivity of cultivated soils. As Cd induces essential nutrient deficiency and even decreased concentration of several macronutrients in plants (Jiang et al. 2004), it seems possible to reverse or at least partly reduce the Cd-induced growth inhibition by optimization of S and N. Increasing evidence suggests that mineral-nutrient status of plants plays a critical role in increasing plant resistance to environmental stresses (Marschner 1995, Vassilev et al. 2005, Hassan et al. 2005a,b, 2008a,b, Anjum et al. 2008a,b, Khan et al. 2008a). Sulfur and N are essential nutrients for normal growth and development of plants. Their assimilation pathways are well coordinated (Brunold 1993, Takahashi and Saito 1996, Abdin et al. 2003, Hawkesford et al. 2006). Deficiency of one element was shown to repress the other pathway (Neuenschwander et al. 1991, Kim et al. 1999, Koprivova et al. 2000, Migge et al. 2000, Prosser et al. 2001, Hesse et al. 2004, Scherer 2008). S is a structural constituent of several coenzymes and prosthetic groups, such as ferredoxin, which are also important for N assimilation. Thus, S plays an important role in plant growth and in the regulation of plant development. It has also been found that S nutrition is a critical factor for the alleviation of Cd toxicity (Popovic et al. 1996, Chen and Huerta 1997, Hassan et al. 2005b, 2006, Vassilev et al. 2005, Anjum et al. 2008a,b). A positive effect of S nutrition on Cd detoxification
in *Beta vulgaris* plants has also been established (Popovic et al. 1996). It has been found that at suboptimal S nutrition Cd exposed plants preferably allocate S to phytochelatin synthesis (McMahon and Anderson 1998). Anjum et al. (2008a) also reported that S supplementation increased the production of glutathione content under low level of Cd which protects the *Brassica campestris* plants by improving the growth and photosynthesis. Pankovic et al. (2000) have shown that optimal N supply decreased the inhibitory effects of Cd on photosynthesis of *Helianthus annuus* plants. A proper N supply has been shown to have a positive effect in overcoming the adverse effects caused by Cd toxicity in various crop species (Hassan et al. 2005a). Therefore, coordination of S and N may alleviate the Cd-induced toxicity in crop plants.

The present study was undertaken to understand the response of *Brassica juncea* cultivars to Cd stress and the use of S nutrition alone and in combination with N in the amelioration of Cd toxicity in *Brassica juncea* cultivars.

5.1 Screening of Mustard Cultivars Treated with Different Levels of Cadmium

The order of decrease in growth, photosynthetic and yield characteristics for all the cultivars was RH30 >Sakha >Pusa Bold >Varuna >Alankar. RH30 accumulated greatest amount of Cd and Alankar accumulated lowest Cd in root and leaf (Figures 2-21).

5.1.1 Growth characteristics

Plant growth and development are an outcome of coordination of the main biological processes in plants (Vassilev et al. 1998). Plant growth and development are susceptible to stresses of all kinds including those of heavy metals. Cd adversely affects plant growth and metabolism. The most common effect of Cd toxicity in plants is stunted growth, leaf chlorosis and alteration in the activity of many key enzymes of various metabolic pathways (Godbold and Hutterman 1985, Arduini et al. 1994, Wu and Zhang 2002b, Chen et al. 2003, Wu et al. 2003, Rai et al. 2005). Bachir et al. (2004) found decrease in root length, plant height and fruiting branch number in cotton plants with increasing Cd concentration in a pot experiment. Growth reduction in Cd-treated plants has been described (Ouzounidou et al. 1997, Wu and Zhang 2002b, 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 (Jalil et al. 1994, Wu et al. 2006). In my studies shoot length, root length, leaf area and plant dry mass of all the *Brassica juncea* cultivars were
decreased with different concentrations of Cd (0, 25, 50, 100 and 150mg Cd kg\(^{-1}\) soil). Root growth was found more susceptible to Cd toxicity compared to the shoot. Metwally et al. (2005) also reported that the roots of Pisum sativum plants were more sensitive to Cd toxicity than shoot. Increasing concentration of Cd in liquid culture and pot experiment decreased the germination and root growth of Raphanus sativus and Daucus carota plants (Chen et al. 2003). In the present study, greater impact of Cd observed on root growth as compared to shoot in all Brassica juncea cultivars, could be due to higher accumulation of Cd in root, leading to impaired growth metabolism. A greater reduction in the root length could also be an adaptation of the cultivars studied in response to high external Cd concentration, so as to reduce the uptake of Cd along with water and their subsequent transport to the shoot. Parameters such as root length, shoot length, leaf area as well as plant dry mass have been used as indicators of metal toxicity in plants (Ouzounidou et al. 1997). In my study, Alankar cultivar showed minimum decrease in shoot length, root length, leaf area and plant dry mass, while RH30 showed maximum decrease in these parameters (Figures 2-7). The reduction in the growth of these cultivars could also be due to suppression of the elongation growth rate of cells, because of an irreversible inhibition exerted by Cd on the proton pump responsible for the process (Aidid and Okamotono 1993). It has also been reported previously that species and cultivars display marked differences for Cd tolerance in Triticum aestivum (Zhang et al. 2002, Khan et al. 2006), Hordeum vulgare (Wu et al. 2003), Gossypium hirsutum (Wu et al. 2004), Brassica juncea (Qadir et al. 2004), Pisum sativum (Metwally et al. 2005), Oryza sativa (Wu et al. 2006, He et al. 2006), Vigna radiata (Wahid and Ghani 2007, Anjum et al. 2008c) and Brassica campestris (Anjum et al. 2008d). Cadmium treatments inhibited shoot and root growth and dry weight of two Triticum aestivum cultivars, C-1252 and Balcali-85. The decrease was more distinct in cultivar C-1252 (Ozturk et al. 2003). The presence of Cd decreased the seedling growth of Zea mays, Triticum aestivum, Cucumis sativus and Sorghum bicolor in terms of root and shoot growth (An 2004). Gnaya et al. (2005) reported that Cd severely inhibited Mesembryanthemum crystallinum growth even at low concentration. The process of tolerance in plants is exhibited at different structural and functional levels, viz., molecular, biochemical, cellular and the whole plant. At the whole plant level it is possible that the tolerance mechanism is connected to the
pattern of Cd distribution as well as the response of components of antioxidant system.

Analyzing the effect of Cd on many plant species, Vassilev and Yordanov (1997) concluded that Cd diminishes relative growth rate through inhibiting mainly net assimilation rate, and to a lesser extent, by restricting leaf area ratio. Inhibition of net assimilation rate is caused by disturbances of dark respiration and photosynthesis. Decreased leaf area ratio in Cd-treated plants is a consequence of the decrease of turgor potential and cell wall elasticity, resulting in smaller size of leaf cells formed with smaller intercellular space area.

5.1.2 Photosynthetic characteristics
Cd induces changes in the physiological processes such as respiration, photosynthesis and gas exchange of the plants (Lagriffoul et al. 1998, Hegedus et al. 2001, Mobin and Khan 2007). Chloroplasts comprise only about one percent of the total Cd accumulated by a plant. Despite its very low relative concentration in chloroplast, it seriously blocks the activity of photosynthetic processes at different routes (Ghoshrony and Nadakavukaren 1990, Siedlecka and Krupa 1999). As a visible symptom the preferential degradation of chlorophyll and carotenoids content can be used to monitor the metal-induced damage in leaves (Horvath et al. 1996, Sanita di Toppi et al. 1998, Oncel et al. 2000).

In the reported study, chlorophyll and carotenoids content decreased with the increase in Cd levels at all growth stages in all Brassica juncea cultivars. The extent of decrease in chlorophyll and carotenoids content was maximum in RH30 while it was minimum in Alankar (Figures 9-10,14). It has been reported earlier that Cd decreased the chlorophyll and carotenoids content in Helianthus annuus (Di Cagno et al. 2001), Hordeum vulgare (Wu et al. 2003, Vassilev et al. 2004), Oryza sativa (Kuo and Kao 2004), Glycine max (Drazic et al. 2004), Sedum alfredii (Zhou and Qiu 2005), Matricaria chamomilla (Kovacik et al. 2006), Brassica juncea (Mobin and Khan 2007), Triticum aestivum (Khan et al. 2007a), Brassica campestris (Anjum et al. 2008a) and Vigna mungo (Singh et al. 2008a). Decreased photosynthesis can be caused by the decrease in the level of photosynthetic pigments, breakdown or the inhibition of its synthesis (Padmaja et al. 1990, Mobin and Khan 2007). Cadmium interferes with chlorophyll formation, with functional SH-group of sulfhydryl requiring enzymes such as ALA synthetase, ALA dehydratase and protochlorophyllide reductase. Apart from inhibition of biosynthetic enzymes of
chlorophyll formation, the increased level of free radicals of fatty acids produced from polyunsaturated fatty acids due to the higher activity of lipooxygenase may also contribute to the decreased level of chlorophyll with Cd treatments (Klein et al. 1984, Somashekararaiiah et al. 1992). Cd might have replaced the central Mg from chlorophyll molecules and thereby reduced the photosynthetic light harvesting ability of plants (Kupper et al. 1996). The decrease in chlorophyll content may also be correlated with the adverse effect of Cd on the uptake and accumulation of other essential nutrients in plants viz. Fe, Mg, Ca and K (Greger and Oger 1991, Greger and Lindberg 1987, Ouzounidou et al. 1997), since both Fe and Mg are essential elements required for the formation of chlorophyll. Earlier reports also suggest that the change in Fe:Zn may be responsible for the reduced chlorophyll content in plants (Root et al. 1975).

Carotenoids are synthesized in the chloroplast of plants. Carotenoids are important as they absorb light at wavelength between 400 and 550nm and transfer it to the chlorophylls (an accessory light-harvesting role) (Siefermann-Harms 1987) and protect the photosynthetic apparatus by quenching a triplet sensitizer (Chl$^3$), singlet oxygen and other harmful free radicals which are naturally formed during photosynthesis (an antioxidant function) (Oelmuller 1989, Thiele et al. 1996, Havaux et al. 2000). Moreover, they are important for the photosystem (PS1) assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (a structural role) (Mayfield and Taylor 1984, Siefermann-Harms 1987, Wrischer et al. 1998, Niyogi et al. 2001, Kim et al. 2004). The decrease of carotenoids content in seedlings of Raphanus sativus, Ulva lactuca, Triticum aestivum and Oryza sativa growing in the presence of Cd was reported by Naguib et al. (1982). Panda and Khan (2003) reported decrease in carotenoids content due to heavy metal treatments. In my experiments, carotenoids content decreased with the increase in Cd concentration in all the cultivars at all growth stages. When the level of carotenoids was decreased by Cd treatments (Khudsar et al. 2001, Khan et al. 2007a, Singh et al. 2008a), the protective functions of carotenoids could not be maintained. Consequently, the oxidative degradation of chlorophyll and rapid destruction of photosynthetic membranes occurred.

Photosynthesis is an integral process with a high degree of self regulation. Inhibition of photosynthetic rate could be due to structural and functional disorders. Cadmium may interact with the photosynthetic apparatus at various levels of organization and architecture (Mysliwa-Kurdziel and Strzalka 2002). Besides
affecting the functions of chloroplast membranes, Cd can also alter the components of photosynthetic electron transport chain, particularly the PSI and PSII (Siedlecka and Krupa 1996).

In the present study, increasing concentration of Cd in the soil leads to reduced photosynthetic characteristics in all *Brassica juncea* cultivars at all growth stages. Maximum decrease in these characteristics was noted with 150 mg Cd kg$^{-1}$ soil. The decrease in net photosynthetic rate, stomatal conductance and carbonic anhydrase activity varied among cultivars and RH30 exhibited maximum decrease while Alankar showed minimum decrease (Figures 11-13, 15-16). Earlier investigations have also demonstrated a marked reduction in the overall rate of photosynthesis by Cd in different plant species (Arduini *et al.* 2004, Wojcik and Tukiendorf 2005, Jing *et al.* 2005, Khan *et al.* 2006, 2007, Hayat *et al.* 2007, Mobin and Khan 2007, Anjum *et al.* 2008a, Singh *et al.* 2008a). Deleterious effects of Cd on various facets of photosynthesis such as, gas exchange, biosynthesis of chlorophyll, functioning of photochemical reactions and the activities of the enzymes of Calvin cycle have been studied (Stobart *et al.* 1985, Weigel 1985, Padmaja *et al.* 1990, de Fillippis and Zeigler 1993, Chugh and Sawhney 1999, Verma and Dubey 2002, Vassilev *et al.* 2005, Mobin and Khan 2007). In addition, conductance and index of stomata, 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). Both the PSI and PSII have been shown to be affected by Cd. Photosystem II is highly sensitive to deleterious effect of Cd and its functioning is inhibited to a greater extent than that of PSI (Chugh and Sawhney 1999). Inhibition of PSII might be due to Cd-induced alterations in the level of magno-protein (Mg-protein) in the water splitting system. Cd restricts the PSII-related electron transport (Baszinsky *et al.* 1980) probably as a result of the structural and functional changes in thylakoid membranes, the reduced ferredoxin-NADP$^+$ oxidoreductase activity, and the arrested plastoquinine synthesis (Krupa and Baszynski 1995). The Cd stress mostly causes metal inhibition of Rubisco, an important enzyme of the Calvin cycle (Vassilev *et al.* 2005, Mobin and Khan 2007). As observed in the study, the decrease in net photosynthetic rate has also been attributed to the reduction in carbonic anhydrase activity under Cd stress (Siedlecka and Krupa 1999, Mobin and Khan 2007, Singh *et al.* 2008a). The inhibitory effects of Cd might be either due to its interaction with $-\text{SH}$ group, as two molecules of $-\text{SH}$ group are found in enzyme center (on cys 173 and cys 456).
essential for normal activity of Rubisco (Lorimer 1981), and/or metal substitution property of Cd leading to the decrease in ratio of combined activity of carboxylation/oxygenation.

In *Brassica juncea* cultivars, 150mg Cd kg$^{-1}$ soil maximally reduced the stomatal conductance and the maximum decrease was noted in RH30 and minimum in Alankar (Figure 16). The loss of water from crop plants is controlled mainly by the stomata on leaves that have been shown sensitive to Cd (Barcelo et al. 1986a,b). To minimize the water loss the process of stomatal closure is adopted by plants exposed to various stresses affecting plant-water status. It has been identified as an early event in plant response to Cd-induced water deficiency leading to limitations to C uptake by leaves (Barcelo et al. 1986a,b, Poschenreider et al. 1989). Cd-induced alteration in mineral nutrients may also contribute to the reductions in stomatal conductance which in turn lead to suppression of major physiological processes and metabolic activities as well.

Carbonic anhydrase activity in all *Brassica juncea* cultivars also decreased in the similar manner as photosynthesis and stomatal conductance. Maximum decrease was noted with 150mg Cd kg$^{-1}$ soil. Among cultivars the decrease in its activity was maximum in RH30, whereas, Alankar experienced minimum decrease (Figure 15). As observed in this study, the decrease in carbonic anhydrase activity was also reported previously in other Cd treated plants (Aravind and Prasad 2004, Hasan et al. 2007, Khan et al. 2007a, Mobin and Khan 2007, Singh et al. 2008a). Siedlecka and Krupa (1999) also reported Cd-induced decrease in carbonic anhydrase activity and other elements of Rubisco activation system. Cadmium (like several other heavy metals) is known to affect the structure and functioning of enzymes (Van Assche and Clijsters 1990) through peroxidation and production of ROS (Dietz et al. 1999) which would affect the functioning of the enzymes and proteins. ROS induce fragmentation of protein and impose oxidative modification, rendering cells susceptible to enzymatic proteolysis and hydrolysis. Carbonic anhydrase is in fact a Zn metalloprotein catalyzing reversible inter-conversion of HCO$_3^-$ and CO$_2$ (Xin Bin et al. 2001, Khan et al. 2004); represents 1-2% of total soluble protein in leaf (Okabe et al. 1984) and is related to photosynthesis in higher plants (Khan 1994, Henry 1996). Cd can readily inhibit most of the Zn-dependent processes, either by displacement or by occupying the active sites of the Zn metalloproteins (Nieboer and Richardson 1980, Siedlecka 1995). Aravind and Prasad (2004) noted toxic effect of Cd on carbonic anhydrase
activity in *Ceratophyllum demersum* which proved the concept that Cd occupies the active sites of important Zn-metalloproteins. The study also showed a marked reduction in Zn content and its substitution by Cd impaired the structure as well as the activity of carbonic anhydrase in *Ceratophyllum demersum*. Consequently, this leads to nonfunctioning of carbonic anhydrase and hence the associated photosynthetic processes were impaired.

5.1.3 Cadmium accumulation

In the present study, plants grown with increasing levels of Cd exhibited an increase in Cd-concentrations in root and leaf of all *Brassica juncea* cultivars at all growth stages. Among cultivars, RH30 accumulated maximum Cd in root and leaf, whereas, Alankar accumulated less (Figures 17-19). The results obtained in this study suggest that roots are efficient barriers to Cd translocation to the aboveground plant parts. Accumulation of heavy metals by plants and their restriction at root level is a widespread phenomenon. The retention of Cd in roots may be due to cross linking of Cd to carboxyl groups of cell wall proteins (Barcelo and Poschenreider 1990) and/or an interaction of Cd ions with the thiol groups of soluble proteins and non-protein thiols operating as a tolerance mechanism in root cells (Chaoui et al. 1997a). In some cases, Cd ions may also be bound by pectic sites and histidyl groups of cell wall (Leita et al. 1995). Ranieri et al. (2005) suggested that retention/immobilization of high amount of Cd in root tissues typical of several plants can be regarded as an important protection mechanism against the diffusion of the metal in plants. For this reason, Cd-concentration in roots can reach to an average of 75-80% of the total metal taken up (Wojcik and Tukiendorf 1999). The movement of Cd from roots to shoots is also likely to occur via the xylem and is driven by the transpiration from the leaves. In this regard the closure of stomata may reduce the accumulation or retention of Cd in root (Salt et al. 1995). In addition, reduced movement of Cd from roots to shoots in plants is believed to result from barrier function of root endodermis and mechanism involving sequestration and decreased xylem loading of Cd (Hart et al. 1998). However, the immobilization of Cd at root level is primarily dependent on the concentration of Cd supplied and the plant species (Sanita di Toppi and Gabbrielli 1999). Due to disturbed root function(s) and/or the reduction in the selectivity of plasma membrane root-Cd may be translocated to the aboveground parts (Vassilev et al. 1998). In the present study, the translocation of Cd from root to leaf was found Cd-dose dependent and higher in cultivar RH30 than in Alankar (Figures 17-18). In fact,
restriction of heavy-metal transport from root to shoot has been thought as a mechanism of plant tolerance to Cd. The lower translocation of Cd from root to leaf may, therefore, be a strategy of Alankar to protect its photosynthetic function from Cd-induced oxidative stress which is in close conformity with Dixit et al. (2001), Anjum et al. (2008a) and Singh et al. (2008a).

The uptake of Cd by plants varies not only among plant species but also among cultivars (Salt et al. 1995, Arao et al. 2003, Metwally et al. 2005, Khan et al. 2006, Anjum et al. 2008c). The difference in the ability of cultivars to accumulate Cd may also be related to differences in their root morphology (An 2004). Das et al. (1997) suggested that plant with numerous thin roots would accumulate more metals than one with few thick roots. The transport of Cd in plants has been found highly species dependent. Cucumis sativus retained greatest amount of Cd in roots followed by Triticum aestivum and Zea mays (An 2004). Differences in the net uptake of Cd in these cultivars may be the result of differences in the electrochemical gradient that is created by ion exchange reactions in the membrane structures as reported in the foliar uptake of metals by Martin and Juniper (1970). A lower electrochemical gradient in Alankar (tolerant cultivar) than in the RH30 (non-tolerant) could, therefore, be one of the reasons for lesser uptake of Cd in Alankar.

5.1.4 Yield characteristics

Cadmium stress affects growth and yield through disturbances in several morphophysiological processes and nutrient uptake. Reduction in growth and yield with increased levels of Cd in growth media arises because of increased leaf rolling and chlorosis of leaf and stem (Ghani and Wahid 2007) and reduced photosynthetic rate (Chugh and Sawhney 1999, Khan et al. 2006, 2007a). Like growth and photosynthetic characteristics, yield and its attributes were decreased in mustard cultivars with Cd treatments. The maximum reduction was noted with 150mg kg\(^{-1}\) soil in all the cultivars. The tolerant cultivar, Alankar exhibited minimum decrease in yield characteristics (number of siliqua per plant, number of seeds per siliqua, seed yield per plant and 1000 seed weight) while maximum decrease in these characteristics was observed in non-tolerant cultivar RH30 (Figures 20-21). In the present study, decrease in yield and its attributes in Brassica juncea cultivars treated with Cd is possibly the fall out of the poor growth and photosynthesis. The decrease in photosynthesis may be considered as one of the important factors responsible for reduced plant growth and productivity under Cd stress (Khan et al. 2007a). Inhibition of photosynthetic pigment
and its biosynthesis is one of the primary events in plants under Cd stress condition. As a consequence of delay in the assembly of the photosynthetic apparatus, lower photosynthetic efficiency, slower plant growth and decreased biomass production occurs that leads to the reduced yield.

Wahid and Ghani (2007) reported significant reduction in number of pods per plant and seeds per pod, 100 seed weight, seed yield and harvest index of *Vigna radiata* genotypes as a result of Cd toxicity. It has been suggested that although varietal difference exists, the accumulated Cd is mainly toxic to the mesophyll tissue, most probably by interfering with the uptake of essential nutrients, thereby reducing growth and yield at various stages. Cadmium significantly reduced number of ear, ear weight, ear length, spikelet number, grains per ear, 1000 grain weight and grain yield of *Triticum aestivum* cultivars and the decrease was correlated with photosynthetic capacity of the cultivars (Khan *et al.* 2006, 2007a). Genotypic differences on the basis of biomass production, yield and yield components were observed in *Triticum aestivum* and it was noted that Cd significantly reduced the root and stem biomass and spikes per plant but grains per ear and grain weight were not significantly reduced (Zhang *et al.* 2002). Liu *et al.* (2007) noted variation among *Oryza sativa* cultivars in their tolerance to soil Cd stress with respect to tillering, plant height, leaf area, dry matter accumulation and grain yield. The relative change in the number of grains per panicle showed a strong positive correlation with relative change in grain yield and, of the grain yield components measured (panicles per pot, grains per panicle, filled grain percentage, weight per grain) and the reduction of grains per panicle was noticed as the main cause of grain yield loss under soil Cd stress. Wu *et al.* (2004) reported reduction in yield of three *Gossypium hirsutum* genotypes under Cd stress and found that the reduction in yield was proportional to Cd accumulation. Among the *Gossypium hirsutum* cultivars tested, the cultivar Simian 3 showed higher Cd concentration and greater decrease in lint yield than the other two genotypes (Zhongmian 16 and Zhozmian 16-2).

Taken together, the present study revealed that all the *Brassica juncea* cultivars responded differentially to Cd stress and the severity of Cd stress in terms of decrease in the growth, photosynthetic and yield characteristics was minimum in cultivar Alankar followed by Varuna, Pusa Bold, Sakha and RH30. Contrarily, in terms of the accumulation of Cd in root and leaf, RH30 accumulated maximum
followed by Sakha, Pusa Bold, Varuna and Alankar. On the basis of overall performance of all *Brassica juncea* cultivars under Cd stress, Alankar proved as Cd-tolerant and showed lesser decrease in the characteristics, whereas, RH30 emerged as Cd non-tolerant and suffered maximum decrease.

5.2 Alleviation of Cadmium-toxicity by Sulfur and Nitrogen in Alankar (Cd tolerant) and RH30 (Cd non-tolerant) Cultivars of Mustard

Increasing evidences suggest that mineral nutrient status of plants plays significant role in increasing plant resistance to heavy metal stress (Marschner 1995, Astolfi *et al.* 2004, Ahmad *et al.* 2005, Vassilev *et al.* 2006, Hassan *et al.* 2005a,b, 2008a,b, Bouranis *et al.* 2008, Anjum *et al.* 2008a,b). Among the plant nutrients, S and N are of great importance and are required by plants for maintaining normal growth and development (Marschner 1995). Several studies have established regulatory interactions between assimilatory sulfate and nitrate reduction in plants (Takahashi and Saito 1996, Ahmad *et al.* 1999). The two pathways are very well coordinated (Brunold 1993, Koprivova *et al.* 2000) and the deprivation of one leads to reduction in the metabolism of the other (Reuveny *et al.* 1980, Prosser *et al.* 2001).

Positive interaction between S and N results in increased crop productivity (Abdin *et al.* 2003). *Brassica* genotypes require higher amounts of S in addition to N for optimum growth and yield (Abdin *et al.* 2003, Aulakh 2003, Khan *et al.* 2005). Application of S along with N leads to enhanced biomass production and increased leaf area as both nutrients are involved in biosynthesis of proteins and several other molecules. Nitrogen is a basic constituent of proteins and with the increase in the rate of N application, the N availability increases. Similarly, increased S supply increases seed yield with higher protein content. Combined application of S and N promotes the uptake of S and N, which leads to significant enhancement in seed protein and oil content in *Brassica juncea* and *Brassica campestris* (McGrath and Zhao 1996, Kachroo and Kumar 1997, Ahmad and Abdin 2000a,b, Abdin *et al.* 2003). Sulfur and N relationship in terms of crop productivity has also been established in many studies (Singh and Bairathi 1980, Sachdev and Deb 1990, Lakkineni and Abrol 1992, McGrath and Zhao 1996, Ahmad *et al.* 1998, Fismes *et al.* 2000).

Experiments 2 and 3 were conducted where two cultivars of *Brassica juncea* namely, Alankar (Cd tolerant) and RH30 (Cd non-tolerant) (screened out from the Experiment 1) were given S alone and in combination with N to ameliorate Cd-
induced toxicity. In Experiment 2 both the cultivars of *Brassica juncea* were treated with 0, 50 and 150mg Cd kg\(^{-1}\) soil and supplemented with 0, 50 and 100mg S kg\(^{-1}\) soil. Application of 50mg S kg\(^{-1}\) soil to 50mg Cd kg\(^{-1}\) soil-treated Alankar plants overcome the Cd-induced toxicity whereas, in RH30, S-supplementation only lowered the severity of Cd toxicity. Therefore, in Experiment 3, 50mg Cd kg\(^{-1}\) soil-treated cultivars were given 50mg S kg\(^{-1}\) soil along with different levels of N (0, 40, 80 or 120mg N kg\(^{-1}\) soil) to strengthen the S induced tolerance. In Experiment 3, it was found that combined application of 50mg S kg\(^{-1}\) soil and 80mg N kg\(^{-1}\) soil completely overcome the ill effects of 50mg Cd kg\(^{-1}\) soil in Alankar (tolerant) whereas, in RH30 (non-tolerant), this combination only lowered the Cd-induced toxicity. The following section discuses major results of the Experiments 2 and 3 with relevant supporting citations. In addition, the participation of S and N in plant tolerance mechanisms has also been discussed.

### 5.2.1 Growth characteristics

In Experiment 2, application of 50mg S kg\(^{-1}\) soil ameliorated the Cd-induced toxicity and improved the growth characteristics of tolerant cultivar Alankar but lowered the reductions in non-tolerant cultivar RH30. The application of 50mg S kg\(^{-1}\) soil nullified the effects of Cd, to a lesser extent, in the plants fed with higher concentrations of Cd (150mg Cd kg\(^{-1}\) soil) but completely, if given low level of Cd (50mg Cd kg\(^{-1}\) ). In particular, the application of 50mg S kg\(^{-1}\) soil proved most effective in improving growth characteristics of both the *Brassica juncea* cultivars treated with 50mg Cd kg\(^{-1}\) soil. A significant increase in root length, shoot length, plant dry mass and leaf area was observed with 50mg S kg\(^{-1}\) soil which varied greatly between cultivars grown with 50mg Cd kg\(^{-1}\) soil (Figures 22-25). Thus, application of S was instrumental in mitigating to some extent the toxic effects of Cd on plant growth characteristics. This indicates that S application reduces the toxic effects of Cd on plant through improvement in the tolerance capacity of the plant. S supplementation to plants has been shown to result in greater biomass production under normal (Ahmad *et al.* 2005) and Cd stress conditions (Hassan *et al.* 2005b, Anjum *et al.* 2008a). However, the mitigating effect by S nutrition was found dependent on Cd dose and on *Brassica juncea* cultivar. Between the cultivars, the ameliorative effect of S nutrition was found maximum in Alankar while minimum effect was noted in RH30. Our findings on ameliorative effect of S in Cd-induced growth reductions are in agreement with Anjum *et al.* (2008a). Anjana *et al.* (2006) reported that S could alleviate the Cd
induced impairment of biochemical and anatomical features of the *Brassica campestris* plant. Hassan *et al.* (2005b) also showed that S application ameliorated the Cd-induced inhibition of growth parameters in two *Oryza sativa* cultivars and the effect of S treatment on these characteristics varied greatly with Cd level and between cultivars.

In the present study, combined application of S and N further increased the growth characteristics of Cd-treated *Brassica juncea* cultivars Alankar (tolerant) and RH30 (non-tolerant) (Figures 48-51). A combination of 50mg S kg$^{-1}$ soil plus 80mg N kg$^{-1}$ soil proved superior in enhancing the growth characteristics of both the cultivars treated with 50mg Cd kg$^{-1}$ soil (Figures 50-51). S and N are essential macro nutrients required for normal growth and development of plants (Marschner 1995) and have been found to play a pivotal role in the protection of plants against environmental stresses, including Cd toxicity (Pankovic *et al.* 2000, Hassan *et al.* 2005a,b, 2006, 2008a,b, Anjum *et al.* 2008a,b). It has also been found in previous studies that the coordination of S and N results in enhanced crop yield and quality of S-requiring *Brassica* (Aulakh *et al.* 1980, Lakkineni and Abrol 1992, 1994, Abdin *et al.* 2003, Aulakh 2003). In the present study, the application of S along with N led to enhanced biomass production and increased leaf area of Alankar than RH30 under Cd stress. It is suggested that combined application of S and N promotes the uptake of S and N, which leads to significant enhancement in growth of plants (Ahmad and Abdin 2000a,b, Abdin *et al.* 2003). Another explanation is that both nutrients are involved in the synthesis of amino acids, proteins and various other cellular components, including thiol compounds and the so-called secondary S compounds which have a significant role in the protection of plants under stressed conditions. Moreover, N is a basic constituent of proteins and the N availability increases with the increase in the rate of N application. It has been shown in *Hordeum vulgare* that high levels of nitrate and ammonium can induce a high affinity sulfate transporter gene and hence sulfate uptake in N-fed plants suggesting that N metabolite may affect sulfate transporter gene expression (Vidmar *et al.* 1999) and hence leads to enhancement in growth characteristics.

**5.2.2 Photosynthetic characteristics**

All the Cd levels used in the present study significantly reduced the photosynthetic characteristics. The effect of Cd stress on these characteristics also varied between cultivars. Non-tolerant cultivar RH30 suffered maximum decrease in photosynthetic characteristics.
characteristics, whereas, tolerant cultivar Alankar showed minimum decrease (Figures 26-31). More severe decreases of photosynthetic characteristics in RH30 than in Alankar under Cd stress may partially account for the differential responses of growth parameters between these two cultivars grown under Cd stress condition. Earlier studies have also showed that photosynthetic functions are highly sensitive to heavy metals including Cd (Zhang et al. 2003). Cd inhibits the enzymes of the Calvin cycle, biosynthesis of chlorophyll and accessory pigments and declines the activity of Rubisco (Siedlecka et al. 1997, Mobin and Khan 2007). In the present study, Cd treatment declined the net photosynthetic rate, stomatal conductance and carbonic anhydrase activity with the increase in the concentrations of Cd in the soil (Figures 27-28, 30-31). Moreover, Cd-induced reductions in photosynthetic responses have been found to involve both stomatal and non-stomatal limitations (Mobin and Khan 2007). Contrarily, Di Cagno et al. (2001) observed no change in stomatal conductance but noted significant reductions in CO2 assimilation rate and Rubisco activity in Cd-exposed Helianthus annuus. Ali et al. (2000) reported that the decrease in the rate of photosynthesis was associated with decreased stomatal conductance and transpiration in in vitro grown Bacopa monniera plants treated with Cd. In the present investigation, Cd significantly decreased both the net photosynthetic rate and stomatal conductance in tolerant (Alankar) and non-tolerant (RH30) cultivars; suggests that stomatal functions limit the photosynthesis.

Application of 50mg S kg⁻¹ soil ameliorated the Cd-induced toxicity and improved the photosynthetic characteristics of both the tolerant (Alankar) and non-tolerant (RH30) cultivars (Figures 26-28). In comparison to 100mg S kg⁻¹ soil, application of 50mg S kg⁻¹ soil ameliorated the Cd-induced (50mg Cd kg⁻¹ soil) reductions in photosynthetic characteristics of both the cultivars (Figures 29-31). In Triticum aestivum, an efficient S assimilation and antioxidative system was helpful in protecting the photosynthetic ability and maintaining high yield potential under Cd stress (Khan et al. 2007a). Resurreccion et al. (2001) reported that S application increased the chlorophyll and Rubisco content which led to increased photosynthesis of Oryza sativa. Khan et al. (2005) has also reported that S application increased the relative growth rate, plant growth rate, net assimilation rate and carbon dioxide exchange rate of Brassica juncea plants. Anjana et al. (2006) reported that the reductions caused by lower level of Cd in leaf dry weight, chlorophyll, sugar and protein content were reversed by S treatment in Brassica campestris. At the cellular
level S starvation reduces the mesophyll cell number per cm² and the chlorophyll content per chloroplast. The photosynthetic CO₂ uptake by intact leaves, photoreduction of ferricyanide, cyclic and non-cyclic photophosphorylation of isolated chloroplast and the rate of CO₂ assimilation by Rubisco may decrease with the decreased total S content (Terry 1976), showing the importance of S in plant metabolism. Chloroplasts contain proteins rich in S (Hanson et al. 1941), and chloroplast morphology is considerably affected by S (Repka et al. 1971, Whatley 1971, Hall et al. 1972). The decreased sink strength in turn could lead to a feedback inhibition of photosynthetic rate and a decrease in Rubisco synthesis (Krapp and Stitt 1995, Sexton et al. 1997). Further, the stresses usually were shown to enhance the process of degradation of cellular proteins, also reduce Rubisco (Sheoran et al. 1990a,b, Stiborova et al. 1986), the major source of S-concentration in the chloroplast (Ferreira and Teixeira 1992). Thus, Cd usually interacts with -SH group of Rubisco and degrades them (Majumdar et al. 1991). Ferreira and Teixeira (1992) have shown lesser number of -SH groups, the major form of reduced S in Rubisco, isolated from S-deprived plants. Further, Cd-induced reductions in the rate of photosynthesis in S-deprived plants might also be due to restricted use of ATP and NADPH by the Calvin cycle enzymes (Dietz and Heilos 1990) and decrease in phosphorylation capacity (Becerril et al. 1989). Nitrogen is one of the essential nutrient elements with a remarkable effect on crop growth and productivity (Marshner 1995). Sugiharto et al. (1990) found a significant positive correlation between the photosynthetic capacity of leaves and their leaf N concentration suggesting that most of the N is used for the synthesis of components of the photosynthetic apparatus. In particular, Rubisco, the leaf protein playing the major role in carbon assimilation, was strongly affected by N deficiency (Seemann et al. 1987).

The decrease in stomatal conductance was possibly due to the fact that cell growth depends primarily on turgor potential as the driving force of expansion, and Cd affects both turgor potential and cell wall elasticity (Barcelo et al. 1986b). On the other hand, S may help in maintaining the water status of the plant by enhancing the root growth and thereby reducing the stress condition imposed by Cd. S availability regulates N utilization efficiency of plants and, thus photosynthesis, growth and dry mass accumulation of crops since photosynthates accumulation has a close relationship with N and S assimilation (Tandon 1995, Kopriva et al. 2002, Khan et al. 2005).
5.2.3 Sulfur and nitrogen metabolism

In the present study the parameters of S and N assimilation responded differentially to Cd stress. Cd stress increased the ATP-sulfurylase activity and S content, whereas, NR activity and N content were decreased in both tolerant (Alankar) and non-tolerant (RH30) cultivars at all growth stages (Figures 32-35). ATP-sulfurylase activity and S content were higher in Alankar than in RH30. Cd-induced increase in ATP-sulfurylase could be a possible consequence of an increased requirement of glutathione for the biosynthesis of phytochelatins. The capacity of plants to survive in a polluted environment is partially linked to the efficiency of their reductive sulfate assimilation pathway and its induction has been reported in several plants exposed to heavy metals (Tukiendorf and Rauser 1990). Expression of genes involved in reductive sulfate assimilation pathway and enzyme activities are stimulated by Cd (Ernst et al. 2008). Cd-induced induction of enzymes of sulfate assimilation pathway has been observed in Arabidopsis thaliana (Harada et al. 2002, Herbette et al. 2006), Chlamydomonas reinhardtii (Dominguez et al. 2003) and Triticum aestivum (Khan et al. 2007a). Nussbaum et al. (1988) reported that the accumulation of Cd increased ATP-sulfurylase activity in Zea mays seedlings. Ruegsegger et al. (1990) also showed that APS reductase activity is induced coordinately with glutathione synthetase in Cd-treated Pisum sativum plants. Cd-induced decrease in the NR activity of both the cultivars is in conformity with Gouia et al. (2000), Chaffei et al. (2003), Anjana et al. (2006), Ghnaya et al. (2007), Wahid et al. (2007) and Wang et al. (2008). Cd reduces absorption of nitrate and its transport from root to shoot by inhibiting NR activity in shoots (Hernandez et al. 1996). Wahid et al. (2007) reported that Cd-induced inhibition of growth was mainly due to the damaged photosynthetic apparatus and disruption of the coordination between C and N metabolism in Vigna radiata. In the present study a consistent relationship between photosynthesis and NR activity was observed in both the cultivars. Since deprivation of CO2 causes inactivation of NR (Kaiser and Behnisch 1991), it is not surprising that Cd decreased the NR activation state. On the other hand, Gouia et al. (2000) have reported that Cd not only decrease the uptake and transport of water and nitrate but also cause a decrease in NR activation state and 80% decrease in NR protein level in short term exposure (24h) in Phaseolus vulgaris plants. Barcelo and Poschenrieder (1990) provided direct reasons for Cd-induced changes in N-metabolism showing that Cd inhibits the transport and uptake of water. They proved that the accumulation of Cd in roots alters the process
of uptake and transport of water and nitrate to the shoot. Furthermore, the uptake of nitrate declined under Cd stress (Hernandez et al. 1996, 1997, Ouariti et al. 1997) that possibly lowered NR activity and disturbed N metabolism.

The application of S further increased the ATP-sulfurylase activity and S content of Cd-treated plants but the extent of increase was greater in the tolerant cultivar Alankar than non-tolerant cultivar RH30. Maximum significant increase was noted in plants supplemented with 50mg S kg\(^{-1}\) soil in 50mg Cd kg\(^{-1}\) soil treated plants (Figures 32-35). Application of 50mg S kg\(^{-1}\) soil with different levels of N (40, 80 and 120mg N kg\(^{-1}\) soil) again increased the ATP-sulfurylase activity and S content in both the cultivars treated with 50mg Cd kg\(^{-1}\) soil at all growth stages (Figures 58,60). Our results suggest that maximum activity of ATP-sulfurylase can only be achieved at a suitable S and N supply (50mg S kg\(^{-1}\) soil + 80mg N kg\(^{-1}\) soil) to the plants. The involvement of S in Cd-tolerance mechanism was reported in Arabidopsis thaliana (Dominguez-Solis et al. 2001, Harada et al. 2002), Brassica juncea (Zhu et al. 1999a,b), Nicotiana tabacum (Harada et al. 2001), Triticum aestivum (Khan et al. 2007a) and Brassica campestris (Anjana et al. 2006, Anjum et al. 2008a). A good part of S incorporated into organic molecules in plants is located in thiol (-SH) groups in proteins (cys-residues) or non-protein thiols, glutathione (Noji and Saito 2003, Tausz et al. 2003, De Kok et al. 2005, Anjum et al. 2008a). Chen and Huerta (1997) showed that S is a critical nutritional factor for reduction of Cd toxicity. Popovic et al. (1996) reported a positive effect of S nutrition on Cd-detoxification in Beta vulgaris. In fact Cd stress provokes plants to enhance the biosynthesis of glutathione for the synthesis of phytochelatins (Herbette et al. 2006). Besides, it has been previously studied that good S nutrition diminished the toxicity of Cd by restoring a new steady state of the glutathione level earlier than in plants grown at low S supply (McMahon and Anderson 1998). Hence, the improved S nutrition allows a more adequate plant defense response to Cd toxicity and also prevents S deficiency. It has been found that at sub-optimal S nutrition, Cd exposed plants preferably allocate S to phytochelatins synthesis. Sulfur being a component of phytochelatins may play an important role in their synthesis and ultimately in detoxification of Cd through the formation of Cd-binding peptides (CdBP) (Cobbett 2000a,b, Harada et al. 2002, Cobbett and Goldsbrorough 2002). Exposing the heavy metal-accumulator Brassica juncea to Cd was reported to induce a rapid synthesis of phytochelatins, which appeared to be sufficient to chelate all the Cd taken up, presumably resulting in a complete

The reductions in NR activity and N content were reversed by S application in Cd treated Alankar (tolerant) and RH30 (non-tolerant) cultivars (Figures 33-35). Lesser decrease in NR activity and N content was observed when plants were given 50mg S kg\(^{-1}\) soil plus 50mg Cd kg\(^{-1}\) soil in comparison to 50mg Cd kg\(^{-1}\) soil alone. The combined application of 80mg N kg\(^{-1}\) soil plus 50mg S kg\(^{-1}\) soil to 50mg Cd kg\(^{-1}\) soil-treated plants maximally lowered the reductions in NR activity and N content in both the cultivars at all growth stages (Figures 59,61). The negative effects of heavy metals on NR activity may be due to interaction of metals with thiol/histidyl groups of proteins, thereby slowing down their catalytic activity and/or completely inhibiting their functions (Wang and Evangelou 1995). As a result, enzymatic reactions may be blocked, leading to accumulation of nitrate in plants and disturbing the normal plant metabolism. Also, it may be due to interference with the uptake of nutrients and/or induction of leakage of nutrients by damaging plasma membrane and protein alterations due to heavy metal toxicity in leaves (Brune et al. 1994). However, the beneficial effect of S on NR activity is primarily due to its being a component of various enzyme proteins, cofactors, and metabolites (Sairam et al. 1995). S application results in adequate N uptake and in high protein yields in *Brassica juncea* and *Triticum aestivum* (Purakayastha and Nad 1997). Therefore, plants supplied with S showed an increased NR activity and improved N assimilation as a result of balanced supply of both N and S, ultimately leading to increased protein formation. It may also be due to the role of S in increasing S-amino acids (Met and Cys) (Bapet et al. 1986). N metabolism, therefore, may act as a marker for the study of the response of plants to Cd toxicity. Upon exposure to Cd, plants often synthesize a set of N-containing metabolites through N metabolism, such as proline, glutathione and phytochelatins, which are well known for their significant roles in Cd tolerance of plants (Sharma and Dietz 2006). Accordingly, plants might exhibit a higher Cd tolerance by the maintaining the normal N metabolism levels under Cd stress (Gussarsson et al. 1996). The ameliorative effect of S in recovering the NR activity as
obtained in our results, are in agreement with the findings of Friedrich and Schrader (1978), Prosser et al. (2001) and Astolfi et al. (2004).

Combined application of S and N increased the activity of ATP-sulfurylase while improved the NR activity of Cd treated plants.

5.2.4 Cadmium accumulation

In the present study, Cd content in the root and leaf of both the cultivars increased with the increasing Cd concentration in soil. Non-tolerant cultivar RH30 accumulated more Cd in root and leaf than tolerant cultivar Alankar. In both the cultivars, increased accumulation of Cd in root and leaf due to 50mg Cd kg\(^{-1}\) soil was lowered with the application of 50mg S kg\(^{-1}\) soil alone and in combination with 40, 80 and 120mg N kg\(^{-1}\) soil at all growth stages. However, combined application of 50mg S kg\(^{-1}\) soil and 80mg N kg\(^{-1}\) soil to 50mg Cd kg\(^{-1}\) soil-treated plants maximally lowered the Cd content in root and leaf of both the tolerant (Alankar) and non-tolerant (RH30) cultivars at all growth stages (Figures 36-37, 62-63).

The differences in Cd accumulation among cultivars have previously been reported (Salt et al. 1995, Arao et al. 2003, Metwally et al. 2005, Khan et al. 2006, Anjum et al. 2008c). In the present study, roots of both the cultivars accumulated more Cd than leaves. The retention of high amount of Cd in the root tissues typical of several plants can be regarded as an important protection mechanism against the diffusion of the metal (Ranieri et al. 2005). For this reason, Cd-concentration in roots can reach an average of 75-80% of the total metal taken up (Wojcik and Tukiendorf 1999). Ranieri et al. (2005) have reported that the highest level of Cd was retained in roots. In the present study, S application reduced the uptake and accumulation of Cd in both the plant parts. A combined application of S and N also reduced the concentration of Cd in root and leaf of both the cultivars. Pankovic et al. (2000) found the lowest inhibition of photosynthetic activity by Cd at optimal N supply, when an investment in soluble proteins and Rubisco were at their maximum and that higher N supplies did not alleviate the toxic Cd effects. A proper N supply has positive effects in overcoming the adverse effects caused by Cd toxicity in *Oryza sativa* (Hassan et al. 2005a, Pankovic et al. 2000). In addition, S may reduce Cd availability in nutrient solution through binding with Cd and detoxify Cd in plant cells through enhancing synthesis of glutathione, which is considered as a first line of defense against Cd toxicity (Hassan et al. 2005b, Anjum et al. 2008a).
5.2.5 Cadmium-induced oxidative stress

Heavy metals, in general, cause toxicity and cellular disruption in plant species through induction of oxidative stress via increased generation of ROS, like \( ^1\text{O}_2 \), \( \text{O}_2^- \), \( \text{OH}^- \) and \( \text{H}_2\text{O}_2 \) (Gratao et al. 2005, Singh et al. 2008b). In the present study, Cd-induced oxidative stress in *Brassica juncea* cultivars is evident from the significant increases in TBARS and \( \text{H}_2\text{O}_2 \) contents with increasing Cd concentration. However, compared to the non-tolerant cultivar RH30, tolerant cultivar Alankar showed a much lower \( \text{H}_2\text{O}_2 \) content in leaves and TBARS production, which are indicative of lower oxidative stress in Alankar. Less Cd accumulation and lower level of \( \text{H}_2\text{O}_2 \) content, together with the reduced formation of TBARS, thus explain the higher potential of Alankar than RH30 (Figures 36-39). Malondialdehyde, as the decomposition product of polyunsaturated fatty acids of bio-membranes showed greater accumulation under Cd stress in other studies also (Qadir et al. 2004, Metwally et al. 2005, Liu et al. 2007, Anjum et al. 2008a, Singh et al. 2008a). Cell membrane stability has been widely used to differentiate stress tolerant and susceptible cultivars of some crops and that higher membrane stability can be correlated with Cd stress tolerance (Mobin and Khan 2007). The enhanced cellular damage in RH30 seems to reflect the deterioration on the equilibrium between generation of ROS and defense mechanisms towards removal of ROS. Increase in TBARS with increasing Cd concentration has also been reported in germinating *Phaseolus vulgaris* seedlings (Somashekaraiah et al. 1992). This was related to restriction of electron flow in PSII by metal ions that led to the formation of excited chlorophyll which in turn caused the production of free radicals (Kato and Shimizu 1985). Cd-induced increase in lipid peroxidation has also been found earlier in different plants by Dixit et al. (2001), Chien et al. (2001), Groppa et al. (2001), Wu et al. (2003), Balestrasse et al. (2004), Metwally et al. (2005), Guo et al. (2007), Ahsan et al. (2007), Razinger et al. (2007), Mobin and Khan (2007), Noriega et al. (2007), Liu et al. (2007), Ammar et al. (2007), Filek et al. (2007), Anjum et al. (2008a) and Singh et al. (2008a).

\( \text{H}_2\text{O}_2 \) is produced at high flux rates by two processes associated with photosynthesis, the Mehler reaction and the Glycolate oxidase reaction of photorespiration (Foyer and Noctor 2000). In addition, there are number of other enzymes in leaves that are capable of producing significant amounts of \( \text{H}_2\text{O}_2 \), including peroxidases, NADPH oxidases and oxalate oxidase (Berna and Bernier 1999, Bolwell 1999, Sagi and Fluhr 2001). \( \text{H}_2\text{O}_2 \) is a relatively stable molecule that
can pass freely across the membranes and is capable of spreading damage (Azpilicueta et al. 2007). Metabolic modeling has also suggested that the loss in antioxidative defense was due to H$_2$O$_2$ accumulation (Polle 2001, Schutzendubel and Polle 2002). H$_2$O$_2$ generation is induced in plants following exposure to a wide variety of stresses including Cd (Dixit et al. 2001, Schutzendubel et al. 2002, Romero-Puertas et al. 2004, Cho and Seo 2005, Balestrasse et al. 2006b, Zawoznik et al. 2007, Yang et al. 2007, Filek et al. 2007, Mobin and Khan 2007, Singh et al. 2008a). Also a pronounced increase in the activity of superoxide dismutase in RH30 with the increase in Cd levels possibly generated higher level of superoxide radicals and resulted in higher cellular damage in comparison to Alankar. Gossett et al. (1994) reported that higher superoxide dismutase activity without complementary increase in the ability to scavenge the formed H$_2$O$_2$ can result in the increased cellular damage.

In the present study, application of 50mg S kg$^{-1}$ soil maximally lowered the TBARS and H$_2$O$_2$ content of 50mg Cd kg$^{-1}$ soil-treated plants. Application of 50mg S kg$^{-1}$ soil with different levels of N (40, 80 and 120mg N kg$^{-1}$ soil) further lowered the contents of TBARS and H$_2$O$_2$ of both the cultivars grown with 50mg Cd kg$^{-1}$ soil and enhanced the effectiveness of S application (Figures 38-39, 64-65). These results on the significant reduction in TBARS content with S application is in conformity with the findings of Anjum et al. (2008a) in Brassica campestris leaves. In addition, net photosynthetic rate and plant dry mass were strongly and positively correlated with the contents of ascorbate and glutathione. It was concluded that S may ameliorate Cd toxicity and protects growth and photosynthesis of mustard involving ascorbate and glutathione. Vassilev et al. (2005) also reported that supply of (NH$_4$)$_2$SO$_4$, a well known fertilizer consisting both S and N nutrients, could reduce Cd toxicity in Hordeum vulgare plants by lowering the TBARS content.

5.2.6 Components of antioxidant system

5.2.6.1 Enzymatic antioxidants

Oxidative stress is a central factor in abiotic stress phenomena that occurs when there is a serious imbalance in any cell compartment between the production of ROS and antioxidant defense, leading to physiological changes (Foyer and Noctor 2000, Agrawal and Mishra 2008). The presence of high concentration of ROS causes oxidative damage to photosynthetic pigments, bio-molecules such as lipids, proteins and nucleic acids, leakage of electrolytes via lipid peroxidation resulting in the disruption of cellular metabolism (Asada 1994, Mobin and Khan 2007). Thus, it is
important for cells to have tight control on the concentration of ROS (Schutzendubel and Polle 2002). Plants are in fact endowed with a wide array of defense strategies to protect the photosynthetic apparatus and cellular functions from ROS (Foyer et al. 1994). Plant antioxidant defense system comprises antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and low molecular weight antioxidants (ascorbate; glutathione; proline; carotenoids, α-tocopherols and phenolics etc.) (Foyer and Noctor 2003). These components help plants to cope with the menace of ROS, thus minimizing the oxidative damages, during exposure to stress factors including Cd (Foyer et al. 1994, Mobin and Khan 2007, Anjum et al. 2008a, Singh et al. 2008a,b). Changes in antioxidant enzyme activities play an important role in metal tolerance. Though, the expression for antioxidant enzymes is altered under stress conditions, their upregulation has a key role in combating the abiotic stress-induced oxidative stress.

In my study, Cd treatments increased superoxide dismutase activity in both cultivars, the extent of increase was higher in RH30 than Alankar (Figures 40,42). Mobin and Khan (2007) also reported that higher superoxide dismutase activity in Brassica juncea cv. RH30 was responsible for higher cellular damage due to excessive accumulation of H$_2$O$_2$. It has been suggested that high SOD activity may be harmful for plants due to high H$_2$O$_2$ production, which in turn might inhibit other enzymes such as ascorbate peroxidase and catalase (Asada 1994, Agrawal and Mishra 2008). Cd-dependent induction of superoxide dismutase activity has also been observed in earlier studies in other plant species (Dixit et al. 2001, Schutzendubel et al. 2001, Vitoria et al. 2001, Qadir et al. 2004, Cho and Seo 2005, Metwally et al. 2005, Wu et al. 2006, Krantev et al. 2007, Ekmekci et al. 2008, Hasan et al. 2008, Agrawal and Mishra 2008, Singh et al. 2008a). Increased superoxide dismutase activity has also been shown to confer increased protection from oxidative damage in transgenic plants (Allen et al. 1997). The activity of superoxide dismutase is of great relevance in metal stress studies for the maintenance of overall defense system of plants subjected to oxidative damage (Slooten et al. 1995).

Sulfur supplementation to Alankar (tolerant) and RH30 (non-tolerant) treated with Cd lowered the superoxide dismutase activity (Figures 40,42). The application of 50mg S kg$^{-1}$ soil plus 80mg N kg$^{-1}$ soil further lowered the Cd-induced (50mg Cd kg$^{-1}$ soil) increase in superoxide dismutase activity (Figures 66-68). The decrease in 50mg Cd kg$^{-1}$ soil activity due to S and N application conferred to less formation of ROS.
The function of S in alleviating the stress may be attributed to its participation in the synthesis of glutathione, an important ROS scavenger. Sulfur helped the plants to maintain CO₂ (whose absence is the major cause of O₂⁻⁻ production in the process of photosynthesis) and major reductants (ascorbate, glutathione) in chloroplasts, maintenance of the sulf-hydryl status of proteins; and hence diminished the chances of O₂⁻⁻ formation and ultimately provided protection from O₂⁻⁻. In a study conducted on Oryza sativa cvs. Bing 97252 and Xiushui 63, Hassan et al. (2005b) found that higher S levels caused significant reduction in superoxide dismutase activity in Cd treated plants. They found that the reduction in superoxide dismutase activity was more pronounced in Bing 97252 than in Xiushui 63. They concluded that high Cd and MDA content were consistently accompanied by higher superoxide dismutase activity and higher S levels caused a marked increase in GSH content and a reduction in SOD activity, indicating a positive effect of S in alleviating Cd-induced oxidative stress. Ajnum et al. (2008a) also reported that S supplementation alleviates the Cd-induced oxidative stress in Brassica campestris plants. Hassan et al. (2008a) suggested that the oxidative stress of Oryza sativa plants exposed to Cd toxicity could be alleviated when (NH₄)₂SO₄ was supplied as N fertilizer. Moreover, it was also found that (NH₄)₂SO₄-fed plants had higher GSH content under Cd stress.

Catalase activity increased with the increasing Cd concentration at all growth stages in Alankar, whereas, in RH30, the increase was noted only at 30DAS (Figures 40-42). At later stages (60 and 90DAS), Cd significantly decreased the catalase activity. In the present study the inhibition of catalase activity in non-tolerant cultivar RH30 may be due to its inactivation by excess H₂O₂ produced by superoxide dismutase under Cd stress. Agrawal and Rathore (2007) also reported that the decline in catalase activity was due to more consumption of catalase to detoxify H₂O₂ or its inactivation. The variable response of catalase activity has been observed under Cd stress in different plant species. Catalase activity increased in Glycine max nodules (Balestrasse et al. 2001), Oryza sativa leaves (Hsu and Kao 2004), Brassica campestris leaves (Ajnum et al. 2008a), in tolerant varieties of Solanum tuberosum (Stroinski and Kozlowska 1997), in roots of Raphanus sativus seedlings (Vitoria et al. 2001), Brassica juncea (Mobin and Khan 2007), Triticum aestivum (Khan et al. 2007a) and Cicer arietinum (Hasan et al. 2008). However, catalase activity decreased in Amaranthus lividus (Bhattacharjee 1998), Glycine max roots (Balestrasse et al. 2001), Phragmites australis (Iannelli et al. 2002), Capsicum annuum (Leon et al.
2002) and Arabidopsis thaliana (Cho and Seo 2005) under Cd stress conditions. Furthermore, catalase activity has also been found to remain unaltered under Cd stress in Glycine max leaves (Ferreira et al. 2002).

Sulfur supplementation further increased the Cd-induced increase in catalase activity in Alankar. In RH30, addition of S lowered the decrease in catalase activity only at 60 and 90DAS, whereas, slight increase in its activity was noted at 30DAS. In Alankar, the increase in catalase activity due to 50mg Cd kg\(^{-1}\) soil was further increased with the application of 50mg S kg\(^{-1}\) soil alone and in combination with 80mg N kg\(^{-1}\) soil at all growth stages (Figures 40,42,66,68). A combined application of S and N increased the Cd-induced catalase activity in Alankar which is in close conformity with the findings of Hassan et al. (2008a). They noted that (NH\(_4\))\(_2\)SO\(_4\) the source of both S and N also increased the catalase activity of Cd treated Oryza sativa plants.

In our study, Cd-dose dependent increase in ascorbate peroxidase activity was observed in both the cultivars but its activity was greater in Alankar (tolerant) than RH30 (non-tolerant) at all growth stages (Figures 41,43). Ascorbate peroxidase has an important role in the scavenging of H\(_2\)O\(_2\) under stressed conditions but its activity depends on the Cd concentration applied (Mobin and Khan 2007, Singh et al. 2008a,b). Increased leaf ascorbate peroxidase activity under Cd stress has been reported in Phaseolus aureus (Shaw 1995b), Phaseolus vulgaris (Chaoui et al. 1997b), Pisum sativum (Romero-Puertas et al. 1999), Ceratophyllum demersum (Aravind and Prasad 2003), Brassica juncea (Mobin and Khan 2007), Zea mays (Krantev et al. 2007), Triticum aestivum (Khan et al. 2007a), Brassica campestris (Anjum et al. 2008a) and Vigna mungo (Singh et al. 2008a). The action of ascorbate peroxidase may be correlated with the reductions in the total ascorbate content i.e., greater ascorbate peroxidase activity needed more substrate and hence consumed more ascorbate as electron donor, thus causing a decline in the ascorbate concentration in the present study. Similar variations in the ascorbate content were observed by Schutzendubel et al. (2001) and Qadir et al. (2004) in Cd-exposed Pinus sylvestris and Brassica genotypes, respectively.

Sulfur supplementation further increased the ascorbate peroxidase activity of both the cultivars and maximum significant increase in its activity was noted in plants treated with 50mg Cd kg\(^{-1}\) soil. Combination of 50mg S kg\(^{-1}\) soil and 50mg Cd kg\(^{-1}\) soil proved best in enhancing the ascorbate peroxidase activity. The increase in
ascorbate peroxidase activity due to 50mg Cd kg⁻¹ soil was further increased by the application of 50mg S kg⁻¹ soil alone and in combination with 80mg N kg⁻¹ soil in both the cultivars at all growth stages (Figures 41-42, 67-68). The demand-driven increase in the ascorbate peroxidase activity implies the existence of stress sensors and signal transduction cascades that elicit the response of ascorbate peroxidase activity using ascorbate as reductant. In fact, S supplementation to the Cd-exposed plants helped to maintain the synthesis of electron providers (ascorbate and glutathione), that improved the regeneration of ascorbate (Anjum et al. 2008a), an electron source for ascorbate peroxidase. Thus, S alleviated the Cd stress alterations and helped plants to continue the ascorbate peroxidase activity to maintain the conversion of H₂O₂ into H₂O and O₂ by regenerating ascorbate and glutathione efficiently. Role of glutathione as a signal intermediate in increasing ascorbate peroxidase expression under metal stress has also been reported by Pekker et al. (2002). The addition of S and N to Cd-fed plants increased the ascorbate peroxidase activity in both the cultivars which may be correlated with the improvement in ascorbate content by S and N.

Glutathione reductase activity of both the cultivars was increased under Cd stress. Maximal significant increase in glutathione reductase activity was noted in both the cultivars treated with 50mg Cd kg⁻¹ soil. Treatment of 150mg Cd kg⁻¹ soil significantly decreased the glutathione reductase activity in non-tolerant cultivar RH30 (Figures 41-42). The majority of the studies determining the response of glutathione reductase to Cd exposure have shown that glutathione reductase activity increases as part of the defense against the Cd-exposure, an alteration that has often been dose-dependent and variable over time (Fornazier et al. 2002a,b).

The up regulation of glutathione reductase activity may result in the improvement of abiotic stress tolerance by reducing oxidized glutathione (GSSG) produced in Ascorbate-Glutathione cycle. Besides, S plays a crucial role in the synthesis of Cys, a precursor molecule for the production of glutathione (Suter et al. 2000). Addition of 50mg S kg⁻¹ soil plus 80mg N kg⁻¹ soil further increased the glutathione reductase activity of both the cultivars at all growth stages and extent of increase was higher in Alankar than RH30 (Figures 41-42, 67-68). The enhancement in glutathione reductase activity might be due to enhancement in the overexpression of enzymes (γ-ECS and GSH synthetase) in the chloroplasts (Noctor et al. 1998a,b). In almost all the biological functions, glutathione is oxidized to GSSG which should
be converted back to glutathione in plant cell to perform normal physiological functions. Hence, rapid recycling of glutathione is more essential rather than synthesis of glutathione, which is a highly regulated and ATP requiring process. Glutathione is regenerated by glutathione reductase in a NADPH-dependent reaction. In this way glutathione reductase activity maintains cellular GSH:GSSG ratio which is essential for the control of gene expression and normal protein functions (Foyer and Noctor 2001, Noctor et al. 2002). Hence, the S-induced upregulation of glutathione reductase activity maintained one of the important hydrophilic antioxidants, the glutathione. Another explanation of the higher glutathione reductase activity in the present work may be that as a component of Ascorbate-Glutathione cycle, glutathione maintains the normal operation of Ascorbate-Glutathione cycle at a high rate in order to detoxify the ROS; it is also essential to keep glutathione in a reduced form prior to its incorporation into the synthesis of phytochelatins (Cobbett 2000a,b).

5.2.6.2 Non-enzymatic antioxidants

All plants can synthesize ascorbate, which can accumulate to millimolar concentrations in both photosynthetic and non-photosynthetic tissues (Foyer et al. 1983). Ascorbate is one of the most powerful antioxidants (Noctor and Foyer 1998, Smirnoff et al. 2001), reacts directly with OH•, O2•− and ¹O₂ and reduces H₂O₂ to H₂O via ascorbate peroxidase (Noctor and Foyer 1998, Chen and Gallie 2004). Ascorbate is the major, probably the only, antioxidant buffer in the apoplast and is an essential metabolite involved with vital cell functions. The ascorbate content of plant tissues is modulated by a large number of factors internal or external to plants.

In the present study, Cd treatments significantly decreased the ascorbate content at all growth stages and the decrease was greater in non-tolerant cultivar RH30 than tolerant cultivar Alankar (Figures 44-45). Ascorbate together with glutathione affects plant tolerance to ROS by participation in the detoxification of ROS in plant cells (Noctor and Foyer 1998, Wu and Zhang 2002a). The ascorbate peroxidase activity, in the present investigation, was found enhanced in both the cultivars. Maximum utilization of ascorbate by up-regulated ascorbate peroxidase activity, might be one of the major reasons of reduction in ascorbate content. Secondly, the reduction of MDHA/or DHA into the reduced ascorbate (regeneration) form might be delayed by Cd. Other workers have also observed that Cd toxicity reduces ascorbate content (Ozturk et al. 2003, Hsu and Kao 2004, Kuo and Kao 2004,
Sulfur supplementation lowered the Cd-induced reduction in ascorbate content of both the cultivars at all growth stages. The decrease in ascorbate content due to 50mg Cd kg\(^{-1}\) soil was lowered by the application of 50mg S kg\(^{-1}\) soil alone and in combination with 80mg N kg\(^{-1}\) soil in both the cultivars at all growth stages (Figures 44-45, 70-71). Sulfur application under Cd stress has shown important role in the alleviation of Cd-induced oxidative stress (Hassan et al. 2005b, Anjum et al. 2008a, Khan et al. 2008a). In the present study, S application improved the ascorbate content which probably reacted with \(O_2^-\), \(^1\)\(^{O_2}\) (directly), \(H_2O_2\) (enzymatically through ascorbate peroxidase) and thereby assisted in maintaining these potential toxicants. The Cd-induced changes in the levels of cellular antioxidants severely affected and impaired the functioning of the Ascorbate-Glutathione cycle (Zhang and Kirkham 1996). The Ascorbate-Glutathione cycle is a major \(H_2O_2\) scavenging antioxidant pathway that operates both in chloroplasts as well in cytosol (Zhang and Kirkham 1996).

The tripeptide (\(\gamma\)-GluCysGly) glutathione (GSH) plays a central role in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics (Xiang et al. 2001) and the expression of stress-responsive genes (Mullineaux and Rausch 2005). The reduced form of glutathione, GSH, is an abundant compound in plant tissue that exists interchangeably with the oxidized form, GSSG. GSH has been associated with several growth and development related events in plants, including cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation (Ogawa 2005, Rausch and Wachter 2005) and its content is affected by S nutrition (Blake-Kalff et al. 2000, Anjum et al. 2008a,b, Khan et al. 2008a). In addition, GSH is a substrate for glutathione peroxidase (GPX) and glutathione-S-transferases (GST), which are also involved in the removal of ROS (Noctor et al. 2002). GSH is a precursor of phytochelatins, which are crucial in controlling cellular heavy metal concentrations in plants (Wojcik et al. 2005, Herbette et al. 2006, Zhang et al. 2008, Anjum et al. 2008a,b).

Cd dose-dependent increase in GSH content was observed in both the cultivars but its content was greater in Alankar (tolerant) than RH30 (non-tolerant) at all the growth stages (Figures 44-45). Environmental stresses trigger an increase in ROS.
levels in plants and the response of GSH can be crucial for adaptive responses. Antioxidant activity in the leaves and chloroplast of *Phragmites australis* Trin. (cav.) ex Steudel was associated with a large pool of GSH, protecting the activity of many photosynthetic enzymes against the thiophilic bursting of Cd and exerting a direct important protective role in the presence of Cd (Pietrini *et al.* 2003). Increased concentration of GSH has been observed with the increasing Cd concentration in *Pisum sativum* (Gupta *et al.* 2002), *Lactuca sativa* (Maier *et al.* 2003), *Phragmites australis* (Pietrini *et al.* 2003), *Brassica juncea* (Qadir *et al.* 2004), *Pisum sativum* (Metwally *et al.* 2005), *Sedum alfredii* (Sun *et al.* 2007), *Oryza sativa* (Hassan *et al.* 2008a).

Sulfur supplementation further increased the glutathione content of both the cultivars and maximum significant increase in its content was noted in plants grown with 50mg S kg⁻¹ soil at all growth stages. The increase in glutathione content due to 50mg Cd kg⁻¹ soil was further increased by the application of 50mg S kg⁻¹ soil alone and in combination with 80mg N kg⁻¹ soil in both the cultivars at all growth stages (Figures 44-45, 70-71). S is required for the synthesis of various compounds, such as thiols (GSH), sulpholipids and secondary S compounds, which play an important role in the metabolism of plants, and in the protection and adaptation of plants against stresses. The content of the secondary S compounds is strongly dependent on the stage of development of the plant, temperature, water availability, and the level of S and N nutrition (Randle *et al.* 1993, 1995, Randle 2000, Randle and Lancaster 2002, Coolong and Randle 2003a,b). Fitzgerald *et al.* (1999) reported that when plants receive adequate S and N, they develop substantial reserves of sulfate in the root and glutathione in the leaves. Glutathione besides performing critical functions in regulating plant growth and adaptation to abiotic stresses, acts as an important S sink in the plant system (Leustek *et al.* 2000, Maughan and Foyer 2006). The synthesis of glutathione is mainly regulated by the availability of its constituent amino acids Cys, Glu and Gly along with transcriptional regulation of enzymes of glutathione biosynthesis, γ-glutamylcysteine synthase and GSH synthetase (Tomaszewska 2002). Further, the S-supplementation might help plants to improve the content of GSH by enhancing γ-ECS enzymes as shown by Schneider and Bergmann (1995) and Strohm *et al.* (1995). Cadmium is known to rapidly induce the synthesis of phytochelatins, thiol-based complexing substances through the up-regulation of glutathione biosynthesis (Xiang and Oliver 1998, Rauser 2000). As phytochelatins are Glu- and
Cys-rich peptides, S and N assimilation pathways are most likely involved in their synthesis: enzymes of the S metabolism are required for the synthesis of Cys, enzymes of N metabolism are required for the synthesis of Glu. The glutathione synthesis in plant cells is dependent on and regulated by the S-supply of the plants. In addition, glutathione contains three moles of N per mole of S and glutathione biosynthesis may depend on the availability of N precursors and thus N nutrition of plants. However, the sink strength of GSH biosynthesis for N may be low compared with the other major N sinks such as the synthesis of proteins or nucleotides (Kopriva and Rennenberg 2004). Our results are in close conformity with the observations of Hassan et al. (2005a,b, 2008a) and Anjum et al. (2008a). Hassan et al. (2005b) reported that the higher S level in the growing medium alleviate the oxidative stress of the *Oryza sativa* plants exposed to Cd. Anjum et al. (2008a) found strong and positive correlation between net photosynthetic rate and plant dry mass with the contents of ascorbate and glutathione. In addition, application of S to Cd-treated plants showed increase in the contents of ascorbate and glutathione which reduced the Cd and TBARS content in leaves and restored the growth and photosynthesis of *Brassica campestris*. Hassan et al. (2008a) reported that addition of (NH₄)₂SO₄ to Cd-fed plants showed increase in glutathione content which reduces the oxidative stress in *Oryza sativa* plants that was related to more S supply for this N form. It has also been suggested that (NH₄)₂SO₄ is a better fertilizer because of the presence of both S and N for use in Cd-contaminated soil (Hassan et al. 2008a,b).

### 5.2.7 Yield characteristics

Yield is the final manifestation of growth, photosynthesis and biochemical traits of a plant which are strongly regulated by several environmental factors. In our study, Cd treatments alone significantly decreased the yield characteristics and the extent of decrease was greater in RH30 than Alankar. The reductions in yield characteristics due to 50mg Cd kg⁻¹ soil were lowered with the application of 50mg S kg⁻¹ soil alone and in combination with 40, 80 and 120mg N kg⁻¹ soil in both the cultivars. The application of 50mg S kg⁻¹ soil plus 80mg N kg⁻¹ soil maximally lowered the Cd-induced (50mg Cd kg⁻¹ soil) reduction in yield characteristics of both the cultivars (Figures 46-47,72-73). The improvement in yield characteristics due to S and N application to Cd-treated plants can be correlated with the enhancement in growth, photosynthesis, and biochemical characteristics. Furthermore, S and N application reduced the Cd uptake and H₂O₂ and TBARS content along with significant increase
in the enzymatic and non-enzymatic components of antioxidant defense system which in turn reduced the Cd-induced oxidative stress and hence provided tolerance to plants and maintained the yield. S and N supplementation to the Cd-stressed plants ameliorated the ill effects of Cd on yield and its components by greater increase in siliqua per plant, seeds per siliqua, 1000 seed weight and seed yield per plant of Alankar (tolerant) than RH30 (non-tolerant) cultivars. Sulfur supplementation to plants has also been shown as a result of greater biomass production under normal (Abdin et al. 2003, Ahmad et al. 2005, Bouranis et al. 2008, Bimbraw 2008) and Cd stress conditions (Hassan et al. 2005b, Anjum et al. 2008a).

Studies have shown a marked influence of applied S on the yield of pulses, oilseeds and other crops (Pasricha and Aulakh 1991, Tandon 1991, Ahmad et al. 1998, Aulakh and Pasricha 1986, 1998, Singh 2001, Abdin et al. 2003, Bimbraw 2008). Sulfur fertilization is useful not only for increasing crop production, oil content, and protein content, but also in improving soil conditions for crop growth (Abdin et al. 2003). Besides it was suggested that an adequate S fertilization to Brassica is a feasible technique to suppress the uptake of Cd by plants, and to enhance the uptake efficiency of several essential elements which resulted in increased crop production and improved quality. Nitrogen is critical in plant growth and development and is an essential component of amino acids, proteins, nucleic acids, and many enzymes. Plants grown under limited N levels have reduced Chl a and Chl b pigments, resulting in stunted plants and characteristic leaf chlorosis (Marschner 1995). As observed also in the present study, increased additions of N usually result in increased yield of crop plants (Mills and Jones 1996, Hochmuth et al. 1999). N increases yield by influencing a number of growth parameters such as branches and flowers per plant and by producing more vigorous growth and development (Allen and Morgan 1972, Taylor et al. 1991). Wright et al. (1988) reported that N prolongs the life of leaves, improves leaf area duration after flowering and increases overall crop assimilation, thus contributing to increased seed yield. Therefore, the combined application of S and N will improve the yield of the crop.

Hassan et al. (2008a) reported that the addition of NH$_4$(SO$_4$)$_2$ to Cd-fed plants improved the yield characteristics of Oryza sativa. They suggested that the improvement in yield characteristics was due to the ameliorative action of N and S in the NH$_4$(SO$_4$)$_2$ on growth and components of antioxidant defense system of Cd-treated plants.
Conclusively, nutrients are known to decipher essential role in plant metabolism and augmenting growth and productivity of crops. However, a nutrient package is required for sustainable agriculture under varied environmental conditions. Crop cultivars display their inherent potential. The cultivar Alankar surpassed other cultivars tested and tolerated Cd stress to a significant degree. The cultivar RH30 was weak in performance and least tolerant to Cd among the cultivars tested. Therefore, Alankar exhibited lesser decreases in growth, photosynthetic and yield characteristics under Cd stress. Correspondingly, Alankar also showed lesser oxidative stress and increased antioxidant system than RH30 to protect photosynthetic machinery and consequent effects on other attributes. Sulfur proved significant potential in the alleviation of Cd stress in both the cultivars. The decreases in the characteristics observed due to Cd stress were lowered by S application and 50mg S kg\textsuperscript{-1} soil proved effective in alleviation of Cd stress than the high doses of S. The coordination of S and N proved most effective in nullifying the Cd stress effects. The potential of 50mg S kg\textsuperscript{-1} soil in the alleviation of Cd stress was substantially enhanced by the simultaneous application of 80mg N kg\textsuperscript{-1} soil. The combined application of 50mg S kg\textsuperscript{-1} soil plus 80mg N kg\textsuperscript{-1} soil not only resulted in restricting the decrease caused by Cd but also nullified the Cd effects and even enhanced the characteristics values over the control. A package of 50mg S kg\textsuperscript{-1} soil plus 80mg N kg\textsuperscript{-1} soil appears to be most effective in the cultivation of mustard under Cd stress. The effect of this combination was due to coordination of S and N in maintaining plant metabolism and alleviating Cd stress.

5.3 New Report in the Thesis and Future Prospects
Mineral nutrient status of plants plays a critical role in increasing plant resistance to environmental stress factors. Of the mineral nutrients, S and N are major macronutrients necessary for the plant life cycle. The uptake and assimilation of S and N in higher plants are the crucial factors determining plant growth and vigor and crop yield. The present study showed that a balanced S and N supply can mitigate the inhibitory effects of Cd on mustard metabolism. The individual effects of S and N and other nutrients have been studied. However, no effort has been made to study combined application of S and N in tolerance of plants, although coordinated functions of these nutrients are well known. For sustainable agriculture a nutrient protocol with balanced S and N is necessary in the growth and development of crops under Cd stress. The results reported in this thesis have confirmed this. The effect of
combined application of S and N on oxidative stress and antioxidants under Cd stress has been reported for the first time. Efforts have been made for the first time to develop S and N protocol for alleviation of Cd stress in mustard.

Further, the efforts should be focused to dissect the mechanism(s) of regulation of sulfate and nitrate assimilation at the molecular level in crop plants under varied environmental conditions. Strategies should also aim to manipulate steps of pathways leading to the production of thiols and their products in plants through overexpressing Ser acetyl transferase (SAT), γ-ECS and GSHS enzymes of S and N nutrition under Cd stress. More detailed studies are required to understand the Cd-induced stress response modulated by S and N metabolism at molecular and mechanistic levels. This would help to develop an effective strategy to raise transgenic species for stress resistance.