Chapter-5

Discussion
DISCUSSION

In the first instance plants maintain a homeostatic balance with the environment therefore, even a slight deviation from normalcy may impose plants under stress. Thus under a narrow range of environmental fluctuations some plants sustain growth and reproduce successfully whereas others may show growth flexibility under a wide range of fluctuations, like variations in light, temperature, water and/or nutrients. The crop species either show a diminished capacity to adapt to sub-optimal conditions or may perform better because of slight molecular changes incorporated due to long term selection and cultivation. There are varied external factors that regulate the repression and/or de-repression of genes out of which light (Thompson and White, 1991), pollutants/elicitors/phytotoxins (Royals et al., 1992), phytohormones (Cleland, 1999) and metabolites such as proline (Ashraf and Foolad, 2007) are noteworthy. Out of these major factors, salinity, brassinosteroids (BRs) and proline were selected for the present study with an aim to elucidate whether exogenous brassinosteroids and/or proline application could confer tolerance against salt stress? And if so which BR analogue and proline concentration would be most effective in overcoming the stress.

The enzyme carbonic anhydrase (CA) catalyzes the reversible inter-conversion of CO$_2$ and HCO$_3^-$, whose activity is largely determined by photon flux density, concentration of CO$_2$, Zn availability (Tiwari et al., 2005) and/or genetic expression (Kim et al., 1994). The enzyme ensures a constant supply of CO$_2$ to Rubisco, at the level of grana of the chloroplast (Majeau and Coleman, 1994; Price et al., 1994), otherwise Rubisco activity seizes at the ambient concentration of inorganic carbon (Majeau and Coleman, 1994). In the present study, the NaCl-induced stress could have caused a decrease in the activity of CA (Tables 9, 45, 57 and 69) a reason to inactivate Rubisco (Soussi et al., 1998) which could have sequentially reduced photosynthetic carbon metabolism, leaf chlorophyll content and photosynthetic efficiency (Seeman and Critihley, 1985). Moreover, the negative impact of the salinity on gene expression of CA (Liu et al., 2012) could be another reason for the observed decrease in its activity. Similarly Ali et al. (2007a), Hayat et al. (2010c, 2011a), Idrees et al. (2012), Liu et al. (2012) have reported the loss in CA activity, under stress. Proline is a well-known enzyme protectant (Krall et al., 1989) which is due to the fact that 3-D structure of proteins (enzymes) is governed by hydrophobic/hydrophilic interactions between side chains of the constituent amino
acids. Proline interferes with these side chains and thus plays a protective role (Paleg et al., 1981) thereby increases the activity of enzymes. A similar type of interaction might be occurring between CA and proline to enhance the activity of the enzyme. Moreover, foliar application of BRs (HBL or EBL) increases the activity of CA by elevating the rate of CO₂ assimilation (Yu et al., 2004) may be because of the enhanced expression of genes that encode other enzymes of the calvin cycle which also play an important role in the regeneration of RUBP, thereby maximizing the carboxylation rate of Rubisco (Xia et al., 2009). A similar concept has also been floated by Hayat et al. (2010b) in tomato and Swamy and Rao (2009) in Pelargonium graveoleus. Therefore, application of proline and EBL to NaCl-stressed or stress-free plants might have imparted additive effects on CA (Table 69).

The nitrate assimilation that represents a very small pool of total leaf protein (Larcher, 1995) involves the enzyme, nitrate reductase (NR) whose activity declines significantly with increasing level of NaCl in the soil (Tables 8, 44, 56 and 68). The reason could be the stress-induced enzyme inhibition and/or its metabolic dysfunction (Hopkins, 1995). The enzyme catalyzes the conversion of nitrate to nitrite which is a rate limiting step in the process of nitrate reduction (Salisbury and Ross, 1992). Soil-salinity is known to retard the nitrate uptake by the plants (Aslam et al., 1984) which is substrate cum the inducer of NR (Solomonson and Barber, 1990), therefore causes a decline in the level of NR. However, proline application improved the NR activity both in stressed and stress free plants possibly because of proline induced increase in the total phenolic contents (Kwok and Shetty, 1998) which in turn prevent auxin degradation (Schneider and Whitman, 1974). Higher auxin levels could have increased the NR activity as proposed by Ahmad and Hayat (1999) and Hayat et al. (2009). Moreover, HRs alone or as a follow-up treatment to NaCl-stressed plants, improved their NR activity that could be an expression of BRs impact on translation and/or transcription machinery (Khripach et al., 2003). The additional possible reason could be the involvement of BRs in increasing the substrate (NO₃⁻) level by acting at the level of cell membrane (Mai et al., 1989) as BRI I peptide has basic residues at P-3, P-4, P-6 and a hydrophobic residue at P-5, related to phosphorylated Ser which is similar to regulatory phosphorylation sites of sucrose-phosphate synthase (SPS), NR and HMGCoA reductase (HMR) in their sequence of amino acids (Man-Ho et al., 2000). A decrease in NR activity under salt stress and also the anti-stress effects of
BRs is in conformity with other studies (Anuradha and Rao, 2003; Shahid et al., 2011; Hayat et al., 2010e). Moreover, both proline (Schneider and Whitman, 1974) and BRs (Nemhauser et al., 2004) prevent auxin degradation under stress therefore, its elevated level increases the activity of NR (Ahmad and Hayat, 1999; Hayat et al., 2009). Therefore, it looked quite obvious that a cumulative effect of proline and brassinosteroids, in our studies (Table 68) could have enhanced the activity of NR.

The plants, under salt-stress lose a significant level of leaf chlorophyll (SPAD value) (Zhao et al., 2007; Hayat et al., 2010e; Hayat et al., 2011a; Ahmad et al., 2012; Akbari ghogdi et al., 2012; Heidari, 2012 and Tables 4, 40, 52 and 64), in a concentration and variety dependent manner, possibly salinity either inhibits its synthesis or accelerates the degradation of chlorophyll molecules (Iyengar and Reddy, 1996). However, this harmful effect of NaCl was overcome in the plants sprayed with BRs (HBL or EBL) and/or proline (Tables 40, 52 and 64). Being membrane bound, the stability of chlorophyll molecules highly depends on the membrane integrity which has been possibly maintained, in our study by proline application as it acts as a membrane stabilizer (Ashraf and Foolad, 2007). These studies are in conformity with other crops (Wani et al., 2012; Ahmed et al., 2010; Ahmed et al., 2011b; Aggarwal et al., 2011). Moreover, BRs also elevate the level of chlorophyll in various crops (Bhatia and Kaur, 1997; Hayat et al., 2000, 2001, 2011b; Yu et al., 2004, Ali et al., 2006, 2007a; Yusuf et al., 2011) because of their involvement in improving the related transcription and/or translation machinery (Bajguz, 2000 and Bajguz and Asami, 2005). Brassinosteroids also retard the rate of degradation of chlorophyll molecules and that of the proteins associated with them, in particular the proteins of light-harvesting complexes located in thylakoid membranes (Ihola, 2011).

The NaCl-stress causes closure of stomata due to salt-induced ABA accumulation (Yang and Lu, 2005), thereby decreases partial pressure of CO\textsubscript{2} in the stroma (Iyengar and Reddy, 1996) that becomes the main reason for the observed loss of stomatal conductance ($g_{s}$), internal CO\textsubscript{2} concentration ($C_{i}$) and transpiration rate ($E$) in the present study (Tables 6, 7, 42, 43, 54, 55, 66 and 67). Cumulative response of all these ill effects could have led to the observed decrease in net photosynthetic rate ($P_{n}$) (Tables 6, 42, 54 and 66). The net photosynthetic rate has already positively correlated with $g_{s}$ and $C_{i}$ (Lu et al., 2009). Moreover, stress-induced activation of the process of senescence and a shift in the activities of related enzymes as a result of the
changes in cytoplasmic structure and negative feedback of reduced sink activity (Iyengar and Reddy, 1996) and slow pace of transport of photosynthates, under potassium deficiency (Cakmak, 2005) cause a significant loss in the rate of photosynthesis. The decrease in SPAD chlorophyll values (Tables 4, 40, 52 and 64) and CA activity (Tables 9, 45, 57 and 69) are the other reasons to justify the lowering of $P_N$ in NaCl-stressed plants. A positive correlation between $P_N$ and chlorophyll content (SPAD value) (Figs. 1, 2 and 3) as well as between $P_N$ and CA (Figs. 4, 5 and 6) further corroborate the present observations. The support is also gained from others (Norren et al., 2010; Akram and Ashraf, 2011; Salcem et al., 2011; Wu et al., 2012; Wang et al., 2012; Eisa et al., 2012; Ahmad et al., 2012). The recovery in $P_N$ and the related attributes ($g_t$, $C_t$ and $E$) in the salt-stressed plants could be attained by exposing them to BRs and/or proline as a follow-up treatment (Tables 18, 19, 30, 31, 42, 43, 54, 55, 66 and 67). Since photosynthesis is mainly dependent on the stomatal movement and the metabolism of mesophyll cells (proteins associated with PSI, PSII and chlorophyll) (Lawlor and Cornic, 2002; Athar and Ashraf, 2005), therefore it can be inferred from the present study that the exogenous application of proline to stressed plants causes an increase in stomatal conductance by maintaining appropriate cellular turgor (Kamran et al., 2009) thereby facilitating sub-stomatal accumulation and assimilation of CO$_2$ at a higher pace. The observations suggest that the photosynthetic enhancement primarily corresponds to the increased stomatal conductance with higher CO$_2$ diffusion rate within the leaves to activate $P_N$. Ahmed et al. (2010) in young Olea europaea plants proposed similar inferences. Moreover, higher chlorophyll contents (Tables 28, 52 and 64) and CA activity (Tables 33, 57 and 69) under exogenous proline application would also expectedly result in higher $P_N$. The two important enzymes that initiate the process of photosynthesis i.e., CA and Rubisco are activated by BRs (Yu et al., 2004; Anuradha and Rao, 2009; Hayat et al., 2011b, 2012b; Yusuf et al., 2011). The higher CA activity increases the carboxylation state of Rubisco (Bajguz and Asami, 2005), thereby improves $P_N$. These observations are further corroborated by the observed positive correlation between CA and $P_N$ (Figs. 4, 5 and 6). It may be derived from the present observations that BRs improved the CO$_2$ concentration (Tables 19, 43 and 67) by increasing $g_t$ (Tables 18, 42 and 66) and also the efficiency of light harvesting system by elevating the level of chlorophyll (Tables 16, 40 and 64). These in a cumulative action speeded up the net
Figure 1 Correlation coefficient values between net photosynthetic rate and chlorophyll content (SPAD level) in (A) Varuna and (B) RH-30 (Experiment 4).
Figure 2 Correlation coefficient values between net photosynthetic rate and chlorophyll content (SPAD level) in (A) Varuna and (B) RH-30 (Experiment 5).
Figure 3 Correlation coefficient values between net photosynthetic rate and chlorophyll content (SPAD level) in (A) Varuna and (B) RH-30 (Experiment 6).
Figure 4 Correlation coefficient values between net photosynthetic rate and carbonic anhydrase activity in (A) Varuna and (B) RH-30 (Experiment 4).
Figure 5 Correlation coefficient values between net photosynthetic rate and carbonic anhydrase activity in (A) Varuna and (B) RH-30 (Experiment 5).
Figure 6 Correlation coefficient values between net photosynthetic rate and carbonic anhydrase activity in (A) Varuna and (B) RH-30 (Experiment 6).
photosynthetic rate of the plants (Hola, 2011 and Tables 18, 42 and 66). Similar reasons have also been given by others to explain the increase in \( P_\text{N} \) values in the crops treated with BRs under various abiotic stresses (Alam et al., 2007; Ali et al., 2008a,b; Hasan et al., 2008; Hayat et al., 2010a,b, 2012b; Fariduddin et al., 2009a, 2011, Yusuf et al., 2011). Besides this, BRs and/or proline also improved the cell water relations i.e. leaf water potential (Tables 17, 29, 41, 53, 65) and membrane structure and its stability (Wang and Zeng, 1993; Slathia et al., 2012; Yan et al., 2011) so as to decrease the electrolyte leakage (Tables 17, 29, 41, 53, 65) which could have been helpful in maintaining normal cellular metabolism.

The NaCl decreased the photochemical efficiency which has been ascribed with the suppression of PSII activity (Mehta et al., 2010 and Tables 8, 44, 56 and 68). This suggests that the salt stress caused damage to PSII electron transport (Megdiche et al., 2008) where it blocks the electron transfer from the primary acceptor plastoquinone (QA) to the secondary acceptor plastoquinone (QB) at the acceptor side of PSII which lead to the decrease in Fv/Fm values (Mehta et al., 2010; Shu et al., 2012). Similar observations have been reported in Triticum aestivum (Shahbaz et al., 2008; Kanwal et al., 2011), Vigna radiata (Hayat et al., 2010e), Brassica napus (Naeem et al., 2010), Solanum melongena (Wu et al., 2012), Cucumis sativus (Shu et al., 2012), Brassica juncea (Ahmad et al., 2012) under salt stress. However, the spray of BRs and/or proline to the stressed/stress-free plants improved the values of Fv/Fm (Tables 20, 32, 44, 56 and 68). Similarly observations have also been reported in Triticum aestivum (Shahbaz et al., 2008), Brassica juncea (Hayat et al., 2012a) and Solanum melongena (Wu et al., 2012). Brassinosteroids protect PSII against over-excitation, under salt stress that otherwise could have caused the loss of integrity of thylakoid membranes (Ogweno et al., 2008). Moreover, PSII machinery gets similar type of protection from applied proline (Tables 56 and 68) which may be supported by Oukarroum et al. (2012), Moustakas et al. (2011) and Yan et al. (2011) who cultured the plants under various types of stresses.

Plants possess complex antioxidative defense system comprising of non-enzymatic (such as proline) and enzymatic components (such as CAT, POX, SOD) to scavenge reactive oxygen species (ROS), produced during their exposure to stress. Various cell organelles (chloroplasts, mitochondria, and peroxisomes) are the seat for the synthesis and scavenging of ROS, the pathways are recognized and well-
coordinated (Pang and Wang, 2008). Under normal conditions, ROS are generated at a very slow pace and an appropriate balance is maintained between their productions and quenching. However, various environmental stresses disturb this balance as they give rise to rapid increases in the intra and inter-cellular ROS levels (Noctor et al., 2002; Sharma et al., 2010) which may induce oxidative damage to lipids, proteins and nucleic acids (Sharma et al., 2012). In order to avoid this oxidative damage, plants raise the level of endogenous enzymatic and non-enzymatic scavenging components (Sharma et al., 2010 and Tables 9-11, 45-47, 57-59 and 69-71). Different other crop plants such as *Helianthus annuus* (Noreen et al., 2009), *Panicum miliaceum* (Sabir et al., 2011), *Triticum aestivum* (Ashraf et al., 2010a), and *Carthamus tinctorius* (Siddiqi, 2010), *Solanum lycopersicum* (Hayat et al., 2010c), *Brassica juncea* (Hayat et al., 2011a; Ahmad et al., 2012) are also reported to behave similarly. Salt-induced increase in endogenous proline content (Tables 11, 47, 59 and 71) could have been due to the increased rate of hydrolysis of proteins (Irigoyen et al., 1992) as protein synthetic machinery is diverted towards the proline accumulation (Claussen, 2005). Secondly, an enhanced level of proline could be due to its slower rate of degradation (Kiyosue et al., 1996). Similar observations have also been reported earlier in *Brassica juncea* (Ahmad et al., 2012; Hayat et al., 2007b, 2011a; Yusuf et al., 2008), *Vigna radiata* (Hayat et al., 2010c), *Solanum lycopersicum* (Hayat et al., 2010c) and *Beta vulgaris* (Farkhondeh et al., 2012) grown under salt stress. Out of the two cultivars tested, Varuna possessed higher proline content and the activity of CAT, POX and SOD enzymes than RH-30. Such types of responses differing in salt tolerance have been reported earlier in which salt tolerant varieties possessed better antioxidative defense system (both enzymatic and non-enzymatic components) than the salt sensitive varieties (Sabir et al., 2011; Hayat et al., 2011a). In the present study, we noted that the treatment of stress-free and stressed plants with BRs and/or proline improved their antioxidant enzymes activity and the proline content (Tables 21-23, 33-35, 45-47, 57-59 and 69-71). Being a membrane stabilizer, proline application results in its rapid uptake coupled with its *de novo* synthesis (Zhu et al., 1990; Santos et al., 1996), thereby increasing the endogenous level of proline (Tables 35, 59 and 71). Proline action is carried over through its involvement at transcription and/or translation level (Cuin and Shabala, 2007; Ashraf and Foolad, 2007). Furthermore, higher endogenous proline content improves water uptake (Jain et al.,
Discussion

application of higher proline concentrations impose a check on its biosynthesis through feedback inhibition thereby decreasing the endogenous proline content (Zhang et al., 1993; Garcia-Rios et al., 1997 and Table 35).

BRs regulate the antioxidant enzymes activity in the tissues where free radical accumulation occurs (Ashraf et al., 2010b). This peculiarity of BRs of managing cells in dual state i.e. to provide defense and to promote growth, places them in the list of novel regulators in plant growth (Sun et al., 2010). BRs confer the tolerance through the increased activity of NADPH oxidase enzyme and elevate the level of H$_2$O$_2$ in the apoplast (Xia et al., 2009). Brassinosteroid perception by receptors activates the plasma membrane-bound NADPH oxidase (RBOH) which results in the elevation of the level of H$_2$O$_2$ to initiate protein phosphorylation cascade (Xia et al., 2009). The H$_2$O$_2$ mediates the transcriptional induction of defense or antioxidant genes. Transcription factors may be activated via a phosphorylation cascade by MAPKs (Mitogen-activated protein kinases). Finally, the products of target genes participate directly in cellular protection against the stress (Xia et al., 2009). In addition to this, on the basis of molecular, physiological and genetic studies it is reported that the enhanced expression of del-2 genes results in an increase in the activity of antioxidant enzymes that provides resistance to oxidative stress in Arabidopsis (Cao et al., 2005). Moreover, BRs induce the expression of proline biosynthetic genes (Ozdemir et al., 2004) which results in the accumulation of proline in the stressed plants (Tables 47 and 71). Similarly proline accumulation has been reported in Sorghum bicolor, under osmotic stress (Vardhini and Rao, 2003), Cicer arietinum, under cadmium stress (Hasan et al., 2008), Brassica juncea, under NaCl stress (Hayat et al., 2012a), Zn stress (Arora et al., 2010) and copper stress (Fariduddin et al., 2009a), Raphanus sativus, under copper stress (Choudhary et al., 2012), Arachis hypogaea, under in vitro conditions (Verna et al., 2012), Pisum sativum, under salt stress (Shahid et al., 2011). Proline is designated as a natural cytosolic osmoticum and scavenges free radicals, interacts with macromolecules of the cells such as enzymes, DNA and membranes to stabilize their structure and function (Anjum et al., 2000; Kavi Kishor et al., 2005). Among various compatible solutes, only proline has the property to protect plants from singlet oxygen and free radical damages, that results from the stress (Alia et al., 1997). Therefore, a combination of BRs and proline elevated the
plant proline content and the level of antioxidant enzymes (CAT, POX, SOD) possibly through its action at transcription and/or translation, as discussed (pp. 87-88) earlier, thereby prevented the mustard plants against the damage caused by the salinity stress.

In the present study mustard plants, exposed to three levels of soil amended NaCl, had reduction in growth traits, reflected in the form of loss in length, fresh and dry mass of shoot and root and leaf area (Tables 1-4, 37-40, 49-52 and 61-64). The salt stress is known to cause reduction in cell division and elongation (Yasseen et al., 1987; Pitann et al., 2009) which is mainly due to salt induced alterations in the nutrient uptake, reactive oxygen species accumulation (Ashraf, 2009), inhibition of the activity of cytoplasmic enzymes, turgor loss (Pitann et al., 2009) and hormonal imbalance (Ashraf et al., 2010b; Iqbal and Ashraf, 2010) which in turn impair plant growth and biomass production. Moreover, the growth inhibition, under salinity stress could be partly due to the shortage of energy (observed slower rate of photosynthesis) as the processes involved in the transport of salts and repair of salt damage on membranes or proteins is energy consuming (Kleinkopf and Wallace, 1974). Similar impact of salt stress on the growth of Beta vulgaris (Ghoulam et al., 2002), Brassica juncea (Hayat et al., 2006, Hayat et al., 2011a), Cicer arietinum (Ali et al., 2007a), Solanum lycopersicum (Zribi et al., 2009; Hayat et al., 2010c), Helianthus annuus (Akram and Ashraf, 2011), Capsicum annuum (Chartzoulakis and Klapaki, 2000), Populous alba (Imada and Tamai, 2009), Fragaria ananassa (Keutgen and Pawelzik, 2009), Morus alba (Ahmad and Sharma, 2010), Abelmoschus esculentus (Saleem et al., 2011) and Panicum miliaceum (Sabir et al., 2011), has been reported earlier. The ill effects generated by the salt stress can, however, be overcome by the application of BRs and/or proline alone or as a follow-up treatment to salt-stressed plants. The increase in the endogenous proline content (Table 35, 59 and 71) by its application to the plant foliage protects the enzymes (Khedr et al., 2003) and 3-D structure of proteins (Paleg et al., 1981), cell organelles and membranes by checking lipid peroxidation (Okuma et al., 2004) and facilitates the supplies of energy for plant growth and survival, thereby helps to overcome stress (Hoque et al., 2007; Ashraf and Foolad, 2007). Therefore, higher proline content acts as an osmoregulator to overcome the impact of salt stress and improves plant growth (Csonka and Hanson,
1991 and Yancey, 1994 and Tables 49-52 and 61-64). Similarly, Deivani et al. (2011) reported higher proline content in rice plants associated with improved growth.

The foliar spray of BRs (IBL or EBL) to plants under stress or non-stress conditions enhanced all the growth traits in both the mustard varieties (Tables 13-16, 37-40 and 61-64). BRs are involved to modulate a number of metabolic phenomena leading to the plant tolerance against the stress (Ashraf et al., 2010b). The amelioration of salt stress by BR application is well documented in plants of *Oryza sativa* (Anuradha and Rao, 2003; Ozdemir et al., 2004), *Phaseolus vulgaris* and *Hordeum vulgare* (Akrarn and Abdel-Fattah, 2006), *Triticum aestivum* (Qayyum et al., 2007; Elciwa et al., 2011), *Cicer arietinum* (Ali et al., 2007a), *Capsicum annuum* (Houmili et al., 2008), *Cucumis sativus* (Xia et al., 2009), *Pisum sativum* (Shahid et al., 2011), *Fragaria ananassa* (Karlidad et al., 2011), *Cajanus cajan* (Dalio et al., 2011), *Phaseolus vulgaris* (Rady, 2011), *Brassica juncea* (Hayat et al., 2012a), *Solanum melongena* (Wu et al., 2012), *Vigna sinensis* (El-Mashad and Mohamed, 2012). BRs have a positive impact on cell division and cell elongation (Catteroul et al., 2001), regulation of genes encoding XTHs (xyloglucan endo-transglycosylase/hydrolase) i.e., the enzymes responsible for the modification of cell wall activity and cell enlargement, cellulose synthase and sucrose synthase (Ashraf et al., 2010b) that play a vital role in growth and development of plants. Besides this, BRs also act along with auxins to stimulate cell elongation (Katsumi, 1991). Exogenous application of BRs accelerates plant growth and development, however, the extent of their effects may vary with plant species and the concentration applied (Ashraf et al., 2010b). *Brassica* plants sprayed with BRs possessed larger leaves (Table 16, 40 and 64) which could have been an expression of activated cell division and cellular enlargement (Clouse and Sasse, 1998; Bajguz and Tretyn, 2003). Similarly BRs have improved the leaf area in *Vigna radiata*, under aluminum stress (Ali et al., 2008a), *Brassica juncea*, under copper stress (Fariduddin et al., 2009a), and *Triticum aestivum*, under stress-free conditions (Shahbaz et al., 2008). Moreover, a combination of proline and BRs had an additive effect on growth and development (Tables 61-64). It looked quite obvious in the light of the above discussion as both of them individually generated beneficial effects.

It is quite evident (Aldesuquy and Ibrahim, 2001; Afroz et al., 2005; Ali et al., 2007a and Asgari et al., 2012 and Tables 12, 48, 60 and 72) that the observed yield
characteristics, at harvest, decreased significantly under salt-stress in a concentration dependent manner. The cultivar, Varuna expressed slight resistance, compared with RH-30. The most prominent reason that could have contributed most to this loss is the poor vegetative plant growth (Tables 1-4, 37-40, 49-52 and 61-64). The other related phenomenon may be limited supply of photosynthates under lower pace of CO₂-reduction (Tables 6, 42, 54 and 66 and Chen et al., 2009) and unfavorable nature of conducting pathway (Aldeasquy and Ibrahim, 2001) where the leaves start behaving as sinks rather than source (Arbona et al., 2005). This causes inhibition of assimilate movement towards the developing reproductive organs, to make them weak and less productive (Tables 12, 48, 60 and 42). However, BRs and/or proline applied to the foliage improved the values for yield characteristics both in stressed and stress-free plants (Tables 24, 36, 48, 60, and 72). The extended life span of vegetative and reproductive organs under the impact of proline (Balestrasse et al., 2004) and/or BRs (Iwahori et al., 1990) could be the reason other than referred earlier where an improvement in any one of the parameters could have a favorable impact on the other. Therefore, increased seed yield, under BRs (Tables 24, 48 and 72) may be an expression of higher rate of photosynthesis (Tables 18, 42 and 66) that facilitated the availability of more carbohydrates for metabolism and export to the sink (Bajguz and Asami, 2005) for healthy growth. Similarly, higher biological yield in passion fruit, correlating with higher photosynthetic CO₂ assimilation, under BRs (Gomes et al., 2003) was reported. To strengthen the above statement a positive correlation was observed between PN and seed yield (Figs. 7, 8 and 9). BRs are also reported to favor the yield characteristics in Vigna radiata and Brassica juncea (Fariduddin, 2002) and also in Cicer arietinum, under saline stress (Ali et al., 2007a) and cadmium stress (Hasan et al., 2008) and Lycopersicon esculentum under cadmium stress (Hayat et al., 2010a). From the above discussion it is quite evident that both proline and BRs individually have a positive (additive) effect on most of the characteristics determining growth and yield. Therefore, it may not be a surprise if the combination of these two increased the values further (Table 72) to give better yield both under stress and stress-free conditions.

In the light of the presentations, it can be inferred that the concentration (NaCl) dependent variation appeared almost in all the parameters investigated. Moreover, out of the two BR analogues (HBL or EBL), EBL proved more effective in
Figure 7 Correlation coefficient values between seed yield per plant and net photosynthetic rate in (A) Varuna and (B) RH-30 (Experiment 4).
Figure 8 Correlation coefficient values between seed yield and net photosynthetic rate in (A) Varuna and (B) RH-30 (Experiment 5).
Figure 9 Correlation coefficient values between seed yield and net photosynthetic rate in (A) Varuna and (B) RH-30 (Experiment 6).
improving the values of most of the morphological, physiological and biochemical parameters in the presence as well as in the absence of NaCl-induced stress. This superiority of EBL over HBL may be because of differences in the structure and stability of these two analogues (Khripach et al., 2000; 2003). S-oriented alkyl (methyl or ethyl) group at C-24 of side chain is present in almost all BRs while EBL and CS (another BR analogue) being exceptions carry R-oriented alkyl group on the side chain of the steroid nucleus (Plate IV). It is, therefore, inferred that the attachment of EBL at its receptor on the plasma membrane leads to more distorted three dimensional structure conformational state as compared to HBL. This thermodynamically acquired new stable state of EBL which seems to be more actively involved in triggering wide array of cascades, more efficiently involved than HBL. However, further study is warranted to know about the transcription factors that are involved in BKI-1 dissociation with BAK-1 to avail the binding in BRI-1 at membrane (Swaczynova et al., 2007; Hategan et al., 2010; Codreanu and Russinova, 2010).

Out of the three concentrations of proline used, the medium concentration (20 mM) proved most effective. The possible reason for this impact is attributed to the fact that proline activates a cycle of cytosolic proline synthesis from glutamate and mitochondrial proline degradation which simultaneously provided NADP⁺ to drive cytosolic purine biosynthesis (Hare, 1998). An induction of Arabidopsis gene encoding proline dehydrogenase (PDH), a mitochondrial enzyme, by exogenous proline (Kiyosue et al., 1996; Nakashima et al., 1998) is consistent with this hypothesis. However, at higher proline levels, feedback inhibition of δ-1-pyroline-5-carboxylate synthetase (P5CS) (Garcia-Rios et al., 1997; Zhang et al., 1995) blocks the biosynthetic portion of this cycle and thereby inhibits organogenesis, as in Arabidopsis (Hare et al., 2001).

The foliar spray of a combination of EBL and proline improved almost all the growth parameters both in the presence or absence of the salt stress. A diagrammatic summary of the effect of proline and/or BRs on the salinity induced changes in plants is shown in Plate V. However, more study is needed at molecular level to disclose the crosstalk between BRs and proline and with other phytohormones in providing tolerance against the stress. Out of the two cultivars, Varuna was found more tolerant to salt stress. This varied growth response of the two varieties of mustard could
Plate IV: Structural difference between (A) Brassinolide, (B) 24-epibrassinolide and (C) 28-homobrassinolide

✓ The structures of other BRs differ from brassinolide (A) within the boxed areas I and II.

✓ The two important BRs, 24-EBL (B) and 28-HBL (C), differ from BL by the substituent in the side chain at C-24 or by its configuration (R or S orientation) at C-24, respectively.
Plate V: Diagrammatic representation of the effects of proline and/or brassinosteroids on the salinity induced changes in plants.
possibly be due to differential regulation of the processes related to growth at their genetic, biochemical and physiological levels.

**Conclusions**

The present study revealed:

1. Out of the three levels (2.8, 4.2 or 5.6 dsm$^{-1}$) of NaCl applied through the soil, 5.6 dsm$^{-1}$ generated maximum toxicity and damage in the plants.

2. Out of the two varieties (Varuna and RH-30), the variety RH-30 was more susceptible to the stress than Varuna.

3. Out of the two BR analogues (HBL/EBL) used in this study, EBL excelled over HBL to generate favorable responses both in stressed and stress-free plants.

4. Out of the various concentrations (10, 20 or 30 mM) of proline, 20 mM proved the best in inducing resistance to the salt stress.

5. All the morphological, photosynthetic and various biochemical parameters decreased significantly with the increasing level of NaCl, amended into the soil.

6. All the morphological biomarkers and photosynthetic traits along with various biochemical parameters increased significantly in the plants treated with either of the BR analogues (HBL/EBL), over the control (water sprayed) plants.

7. Toxic effects generated by the lower concentration of NaCl were completely neutralized by the follow up treatment with either of the BR analogues where EBL excelled in its effect over HBL.

8. Activity of the enzymes (nitrate reductase and carbonic anhydrase) and the values for all the photosynthetic attributes increased by the application of either of the BR analogues, alone or as a follow up treatment to plants, exposed to NaCl.

9. Antioxidant enzymes (catalase, peroxidase and superoxide dismutase) and proline accumulation in the plants increased in response to BRs and/or proline alone or as a follow-up treatment to NaCl stressed plants.

10. The foliar application of proline (20 mM) and EBL ($10^{-8}$ M) in combination or alone as a follow-up treatment to stressed plants showed an additive effect thereby maintained healthy growth and productivity.

11. The yield of the plants decreased significantly in the plants grown in the soil supplemented with different levels of NaCl in a concentration dependent manner.
12. The seed yield of the plants was significantly increased by the application of BRs (HBL/EBL) and/or proline application. Combination of EBL with proline was most effective.

13. The foliar spray of EBL (10^{-8} M) with proline (20 mM) as a follow-up treatment proved most potent salt stress alleviator by enhancing the level of antioxidant system and osmolyte (proline), manifested as rich growth, higher rate of photosynthesis, and biological yield of plants.