With the Name of Allah, The Most Beneficent
And The Most Merciful!
Regulation of growth and some key physiological and biochemical attributes in salt stressed plants of rice (Oryza sativa L.) by exogenous application of nitric oxide

By
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MPhil Botany (UAF)

A thesis submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY
IN
BOTANY

DEPARTMENT OF BOTANY
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UNIVERSITY OF AGRICULTURE, FAISALABD
2013
DECLARATION

“I hereby declare that the contents of the thesis, entitled “Regulation of growth and some key physiological and biochemical attributes in salt stressed plants of rice (Oryza sativa L.) by exogenous application of nitric oxide” are product of my own research and no part has been copied from any published source (except the references, standard mathematical or genetic models/equation/formulae etc.). I further declare that this work has not been submitted for award of any other diploma/degree. The University may take action if the information provided is found inaccurate at any stage”.

Noman Habib
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“We, the supervisory committee, certify that the contents and form of this thesis submitted by Noman Habib. Regd. No. 2003-ag-491 have been found satisfactory, and recommend that it be processed for evaluation by the external examiner(s) for the award of the degree”.

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Dedicated to

My Loving Parents
(May they live long)

who always raised their hands for me to achieve highest goals in life
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Noman Habib
Abstract

Keeping in view the newly discovered role of nitric oxide in plant growth, development and salt tolerance, an initial experiment was carried out for optimization of nitric oxide concentrations, which were most effective in improving the seed germination rate and early seedlings growth in rice under saline stress. Pre-sowing seed treatment with varying levels (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM) of nitric oxide was applied to seeds of four rice cultivars (Shaheen Basmati, Basmati PB-95, KS-282 and IRRI-6), which were subjected to two levels (0 and 80 mM) of salt stress. Salt stress markedly inhibited the seed germination attributes and early seedlings growth in all four rice cultivars. Of all nitric oxide levels 0.5 mM was slightly effective, however, 0.1 and 0.2 mM were most effective in improving seed germination attributes and early seedlings growth of salt stressed rice plants. The both levels (0.1 and 0.2 mM), which were found relatively more effective in first experiment, were used in the yield experiment to study the regulatory role of nitric oxide on various growth, physiological and biochemical attributes of salt stressed rice plants. In this experiment both pre-sowing seed treatment and foliar spray modes were adopted for exogenous addition of nitric oxide to salt stressed rice plants. Salt stress caused a marked suppression growth, chlorophyll content, gas exchange attributes, chlorophyll fluorescence, uptake of essential nutrients, total phenolics and yield content while increased tissue Na⁺ and Cl⁻, proline, ascorbic acid, MDA, H₂O₂ and the activity of antioxidant enzymes including CAT, POD and SOD in all four rice cultivars. Of both nitric oxide levels, 0.1 mM was relatively more effective in improving growth and physiological attributes of salt stressed rice plants as compared to 0.2 mM. Of all four rice cultivars, Shaheen Basmati and IRRI-6 performed better for chlorophyll content, gas exchange attributes and activity of enzymatic antioxidants (SOD, POD and CAT), while KS-282 and IRRI-6 performed better for, total phenolics and yield content. Overall, exogenous nitric oxide treatment was effective in improving fresh and dry biomasses (in both shoots and roots), chlorophyll content, photosynthetic rate, water relation attributes, K⁺/Na⁺ ratio, Ca²⁺ content, activity of enzymatic and non-enzymatic antioxidants and proline content, while in decreasing Na⁺ and Cl⁻ ions, MDA and H₂O₂ content.
Chapter 1

Introduction

Vast area of available land on our Earth is affected by salinity which is a major threat to agricultural productivity (Munns and Tester, 2008; Ashraf, 2009). According to an estimate about one third (33%) portion of world irrigated land is affected by salinity (Athar and Ashraf, 2009). Sodium chloride is the most important component of soil salinity affecting more than 800 Mha of land worldwide, especially in the arid and semi arid regions (Munns 2005; Turkan and Demiral 2009). Salinity is one of the major abiotic stresses that substantially reduce the average yield of major crops by more than 50% (Bray, 2000). These losses are of great concern for most countries, like Pakistan, the economy of which relies mainly on agriculture (Athar & Ashraf, 2009). In Pakistan, the salt affected area is about 6.3 million ha which is increasing at the rate of 40,000 ha each year (http://www.pakistaneconomist.com/issue2000/issue19&20/i&e3.htm; Economic Survey of Pakistan, 2012).

Under salt stress, plants show a marked reduction in growth and development due to salt-induced osmotic stress, specific ionic effect, nutrient imbalance, altered hormone level and oxidative damage due to higher levels of reactive oxygen species (ROS) (Ashraf & Foolad, 2007; Ashraf, 2009; Nawaz et al., 2010). Plants often face physiological drought when grown on salt affected lands because accumulation of soluble salts lower the water potential of soil, Higher concentrations of Na+ and Cl– in saline soils are toxic for plants as they cause reduction in chlorophyll synthesis and they also result in impairment of gas exchange and chlorophyll fluorescence attributes (Ashraf, 2004; Moradi & Ismail, 2007; Nawaz et al., 2010). Many potential bio-molecules like chlorophyll are deteriorated under salt stress (Parida & Das, 2005; Shahbaz et al., 2011). Decreased chlorophyll content under salt stress is associated with decline in endogenous 5-aminolevulinic acid content which acts as a precursor for protochlorophyllide, which is further a precursor for chlorophyll biosynthesis (Santos, 2004). Decrease in photosynthetic activity under salt stress also depends on the functioning of non-stomatal and stomatal factors, generation of antioxidants, formation of photosynthetic pigments and various essential metabolites (Ashraf, 2009). ROS such as singlet oxygen (\(^{1}\text{O}_2\)), superoxide anions (\(\text{O}_2^{-}\)), hydroxyl radical (\(\text{OH}^{-}\)), and hydrogen
peroxide (H\textsubscript{2}O\textsubscript{2}) are produced on electron transport chain during electron transfer, so chloroplast is susceptible to these oxidants (Asada, 1999; Ashraf, 2009). Inorganic ions play an important role in various physiological processes such including osmotic regulation and turgor maintenance in plant cells (Greenway and Munns, 1980; Ashraf, 2004). Plant biomass is reduced due to high concentration of Na\textsuperscript{+} and Cl\textsuperscript{-} ions in saline soil these toxic ions cause adverse effects in most of the crop plant species (Munns and Tester, 2008; Ashraf, 2009). The uptake of Ca\textsuperscript{+} and K\textsuperscript{+} decreases under salt stress, because Na\textsuperscript{+} is a competitor with these ions during ion uptake. Salt tolerant plants have the ability to maintain K\textsuperscript{+}/Na\textsuperscript{+} and ROS homeostasis to cope with the adverse effects of salt stress (Munns and Tester, 2008). Cytosolic Na\textsuperscript{+} concentration is lowered through its compartmentalization in vacuole (Apes et al., 1999; Fukuda et al., 2004) or by efflux of Na\textsuperscript{+} ions at plasma membrane by active transport (Shi et al., 2003; Ottow et al. 2005; Wu et al., 2007; Athar et al., 2009; Sun et al., 2009a). Reactive oxygen species (ROS) are produced in plant cells under salt stress. Plant cells posses an antioxidant defense system for ROS quenching (Foyer and Noctor, 2005; Ashraf., 2009; Miller et al., 2010). Antioxidant enzymes help in alleviation of salt-induced oxidative damage by scavenging the ROS (Miller et al., 2010) Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is produced when SOD (superoxide dismutase) reacts with the radical superoxide. Peroxidases such as catalase (CAT) and ascorbate peroxidase (APX) act as the scavenger of H\textsubscript{2}O\textsubscript{2}. Oxidized glutathione (GSSG) is recycled by the activity of glutathione reductase (GR) which forms reduced glutathione (GSH) by using NADPH (Moller 2001; Miller et al., 2010). Plants use a network of multiple signaling pathways for ionic and ROS homeostasis under saline conditions (Zhu, 2003; Munns and Tester, 2008). Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) help plants in rapid adjustments under salt stress (Miller et al., 2010; Qiao and Fan, 2008). Salt induced overproduction of H\textsubscript{2}O\textsubscript{2} is a risk for plants as it causes oxidative damage, but H\textsubscript{2}O\textsubscript{2} mediates defense response under salt stress (Miller et al. 2010). In callus cells of Populus euphratica, NaCl-induced overproduction of H\textsubscript{2}O\textsubscript{2} further increases the PM H\textsuperscript{+}-ATPase activity, which is involved in maintaining the K\textsuperscript{+}/Na\textsuperscript{+} homeostasis (Zhang et al. 2007). H\textsubscript{2}O\textsubscript{2} is involved in activation of enzymatic antioxidants (Tanou et al., 2009; Hernandez et al., 2010). Moreover, H\textsubscript{2}O\textsubscript{2} also facilitates SOS1 mRNA stability and further contributes to Na\textsuperscript{+} detoxification in Arabidopsis (Chung et al., 2008).
Use of plant growth regulators (PGRs) as pre-sowing seed treatment or foliar application is among various shotgun approaches that help crop plants to grow successfully under saline conditions (Ashraf et al., 2008, 2010a). According to some studies, PGRs significantly enhanced NaCl stress tolerance ability in various crop plants such as sunflower, wheat, rice and maize by regulating various physiological processes that have a role in growth and production of yield (Perveen et al., 2010; Iqbal et al., 2011). Various plant growth substances such as salicylic acid (SA), abscisic acid (ABA) gibberelic acid (GA) and triacantanol (Tria) etc. are being used for achieving enhanced crop productivity under stress conditions (Iqbal and Ashraf, 2005, 2010; Ashraf et al., 2010a).

Nitric oxide (NO) is a lipophilic free radical which is highly volatile in nature (Hayat et al., 2010). It is a unique diffusible molecular messenger which was first identified in animals, but recently it has been reported to play a crucial role in various plant physiological processes including growth, development and abiotic stress tolerance (Zheng et al., 2009; Zafrà et al., 2010). It acts as an important signaling molecule in both animal and plant cells (Crawford and Guo, 2005; Besson-Bard et al., 2008). There are many reports about the regulatory role of nitric oxide in plant development such as reduction in seed dormancy and enhanced seed germination (Libourel et al., 2006; Bethke et al., 2007; Zheng et al., 2009, Habib et al., 2010), regulation of senescence and metabolism in plants (Leshem et al., 1998; Pedroso and Durzan, 2000; Guo and Crawford, 2005), regulation of stomatal movement (Guo et al., 2003; Garcia-Mata and Lamattina, 2007), induction of cell death in plants (Pedroso and Durzan, 2000), regulation of photosynthesis (Takahashi and Yamasaki, 2002), maintenance of the functions of mitochondria (Zottini et al., 2002), floral regulation (He et al., 2004), and regulation of gravitropism (Hu, et al., 2005). Nitric oxide (NO) not only plays a regulatory role but it also helps plants to counteract both biotic and abiotic stressES (Crawford and Guo, 2005; Besson-Bard et al., 2008, Siddiqui et al., 2011), thereby leading to enhanced plant stress tolerance ability especially against drought (Garcia-Mata and Lamattina, 2002) and salt stress (Zhao et al., 2007).

Previously, plant biologists considered nitric oxide as an air pollutant and plant growth inhibitor, because higher levels of nitric oxide were reported to cause membrane damage and fragmentation in DNA (Pedroso et al., 2000; Romero-Puertas et al, 2004). High concentrations of NO inhibited seed germination and seedling growth in rice (Habib et al.,
It was also found to impair plant shoot and root growth, leaf expansion, alter thylakoid viscosity and photosynthetic electron transport, damage DNA and induce cell death in plants (Leshem et al., 1997, 1998; Pedroso et al., 2000). But the new status of nitric oxide has been established as an important signaling molecule (Cueto et al., 1996; Yamasaki, 2005).

Moreover, NO is a gaseous signal transmitter that functions as a hormone in plant with activities similar to ethylene (Leshem, 2000; Yamasaki, 2005). In 1992, NO was named “molecule of the year” when its biological significance was first recognized (Koshland, 1992). In 1998, the Nobel Prize in physiology and medicine was awarded jointly to Robert F. Furchgott, Louis J. Ignarro and Ferid Murad for their discoveries concerning the signaling role of nitric oxide molecule (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1998/#). Nitric oxide plays a role in cytoprotection by checking the level of ROS and by regulating hormones (Lamattina et al., 2003; del Rio et al 2004). Nitric oxide induces changes at transcriptional level that permit identification of genes involved in various physiological functions such as metabolism, defense and cell death, transport, ROS production and quenching (Palmieri et al., 2008).

Nitric oxide protects plant cells from oxidative damage by regulating cellular redox homeostasis and promoting transformation from O₂ to H₂O₂ by enhancing the activities of ROS scavenging system (Lamattina et al., 2003; Zhang et al., 2006; Shi et al., 2007; Zheng et al., 2009). Nitric oxide helps in better maintaining the balanced metabolism between nitrogen and carbon by increasing total soluble proteins and the activity of carboxypeptidase and endopeptidase under saline conditions (Zheng et al., 2010). Nitric oxide also regulates K⁺/Na⁺ homeostasis and PM H⁺-ATPase abundance under saline conditions (Zhao et al., 2004, 2007). Moreover, the activities of H⁺-pumps, Na⁺/H⁺ antiporter in the tonoplast, and antioxidant defense mechanism were improved by the application of sodium nitroprusside (SNP), a nitric oxide donor, in salt-stressed plants of maize and citrus (Zhang et al., 2006; Tanou et al., 2009). Increase in endogenous NO levels have been reported in plants subjected to stresses (Zhao et al., 2004; He et al., 2011; Wu et al., 2011). Furthermore, exogenous application of sodium nitroprusside (SNP) also increased the endogenous NO which confers resistance to abiotic stresses (Hao et al., 2008; Tanuo et al., 2009; Zhang et al., 2011). In contrast, decreased endogenous NO by NO scavengers or NO synthase (NOS) inhibitor like
Ng-nitro-L-arginine-methyl ester (L-NAME) increased susceptibility to stressful conditions (Hao et al., 2008; Xu et al., 2010). Similarly, mutant plants with decreased endogenous NO levels due to impaired in vivo NOS activity were less tolerant to abiotic stresses than the wild type (Zhao et al., 2007). As increased activity of nitric oxide synthase AtNOS1 helped in increasing the salt tolerance in Arabidopsis (Zhang et al., 2007).

In aqueous solution the diffusion coefficient of nitric oxide is close to that of O2 (Garcia-Mata and Lamattina, 2007). The concentration of nitric oxide decides the level of its effectiveness. It also depends upon the concentration of target molecule and ambient redox status, proteins which contain metal and thiol group are targets of nitric oxide (Lamattina et al., 2003). Lower concentrations of nitric oxide are reported to be effective for promoting seed germination, and enhancing plant growth and development (Beligs and Lamattina, 2001; Habib et al., 2010). Nitric oxide increases plant abiotic stress tolerance by enhancing antioxidant defense system, both enzymatic and non-enzymatic (Xu et al., 2010).

Nitric oxide also plays a role in the formation of root system architecture, as it mediates auxin-induced root bending due to gravitropism, lateral root formation and growth of adventitious roots and root hairs also (Correa-Aragunde et al., 2004; Hu et al., 2005; Lombardo et al., 2006; Tewari et al., 2008). Nitric oxide up-regulates the expression of pyrroline-5-carboxylate synthetase (P5CS) and gamma-glutamylcysteine synthetase (γ-ECS), the enzymes responsible for proline and glutathione synthesis respectively (Innocenti et al., 2007; Zhang et al., 2008).

World population was 3 billion in 1961, which more than doubled by the year 2007, and it is expected to grow up to 9.1 billion by 2050 from the current 6.8 billion (Nature, editorial “How to feed a hungry World” 2010). In the past, world food production was increased due to Green Revolution which kept a pace with the growing population (Khush, 2001). As the world population is growing rapidly, food prosperity due to Green Revolution seems to reach its limits. Because, growing population still requires 39% more food (FAO STAT, 2002). World food production is required to increase 20% in the developed countries while 60% in the developing countries (Owen, 2001). The threat of food insecurity is becoming more alarming due to various abiotic stresses which severely reduce crop growth. Among various abiotic stresses, drought and salinity are responsible for 80% yield losses (Jenks et al., 2007). Thus, in the present scenario, the soil salinization and salt-induced
decrease in cereal and oil-seed crop plants have become an important global agricultural
issue (Owen, 2001; Ashraf and McNeilley, 2004; Flowers, 2004; Colmer et al., 2005; 2006;
Munns et al., 2006).

Rice (Oryza sativa L.) is one of the most important cereal crops in Asia including
Pakistan, although more than half of the world population uses rice as food (Ma et al., 2007).
Pakistan is world’s fourth largest rice producing country, after China, India and Indonesia
the second major cereal crop after wheat in Pakistan, is grown on 2.57 Mha of land with an
average paddy yield of 2.39 t ha\(^{-1}\) (Economic Survey of Pakistan, 2012). The overall rice
production in Pakistan is 6160 thousand tons that accounts 1.0 percent in GDP and 4.9
percent share of value addition in agriculture (Economic Survey of Pakistan, 2012).

Rice growing area both upland and lowland is spread over from tropical to temperate
climates in a wide range of environmental conditions (Khush, 1984). Farmers adopt both
transplantation and direct seeding methods to establish the rice crop, they grow rice seedlings
in nurseries and then transplant these seedlings in flooded fields so as to overcome the weeds
(Rao et al., 2007). Rice plant is very sensitive to salt stress especially at the early seedling
and reproductive stage (Heenam et al., 1988; Zeng et al., 2003). One million ha of the rice
growing area in Pakistan is affected by salt stress (Qureshi et al., 1991). Na\(^+\) and Cl\(^-\)
accumulation has a limiting effect on rice survival under saline conditions (Flowers and Yeo,
1981; Moradi and Ismail, 2007). Plant survival at the seedling stage, dry matter
accumulation, Na\(^+\)/K\(^+\) ratio and leaf injury are included in physiological parameters that are
used for screening rice cultivars for salt tolerance (Gregorio et al., 1997; Ali et al., 2004; Haq
et al., 2009). Lipid peroxidation, protein and chlorophyll degradation, decreased membrane
stability and rapid occurrence of the leaf senescence are among the salt-induced adverse
effects in rice (Lutts et al., 1996; Misra et al., 1997). As a long-term effect, rice leaf
accumulates considerably high amount of salts when growing on saline soils (Yeo et al.,
1090). Excessive uptake of NaCl by the root system and its transport to leaf is the main cause
of salt-induced damage in rice (Yeo and Flowers, 1986). Salt-induced reduction in
photosynthetic activity is responsible for sensitivity of rice to salt stress up to some extent, as
higher Na\(^+\) concentration in leaf tissue is negatively correlated with the net photosynthetic
rate in rice, which suggests that salt-induced physiological drought in the leaf cells inhibits net photosynthesis due to accumulation of salts in appoplast (Yeo et al., 1985).

Rice leaf becomes more sensitive to salt stress with advancing age. Ultra-structural damage in leaf tissue is caused due to increased salinity which results in swelling of thylakoids and destruction of chloroplast envelop (Mitsuya et al., 2003; Yamane et al., 2003). Electrolyte leakage and lipid peroxidation are symptoms of oxidative damage due to accumulation of higher Na$^+$ concentration under saline conditions, which also causes hyper-osmotic effect and excessive generation of ROS in salt sensitive rice cultivars (Dionisio-Sese and Tobita, 1998).
Chapter 2
Review of literature

Salinity stress
Enhanced soluble salt content in soil and water is an important limiting factor for plant growth (Munns and Tester, 2008; Ashraf et al., 2010a). In Pakistan more than one-third (37%) irrigation area comprising 7.0 Mha land is salt affected (FAO, 2011). Salt stress not only inhibits growth and productivity of crop plants, but can also cause death under severe cases (Munns and Tester, 2008). It has already been established through several reports that electric conductivity (EC) value over 4 dS m\(^{-1}\) which is almost equal to 40 mM NaCl imposes enough stress to reduce yield in crop plants, but the extent of salt-induced yield reduction varies according to stress tolerance ability of various crop plants (Cerda et al., 1982; Maas and Poss, 1989; Sairam et al., 2002; Schleiff, 2005). Electric conductivity (EC) 4 dS/m is considered moderate salinity which generates osmotic pressure of 0.2 MPa approximately, while EC 8 dS/m is considered as high salinity (Rogers et al., 2005; Munns and Tester, 2008). Sodium chloride (NaCl) is highly soluble and most abundant salt which is commonly known as table salt (Munns and Tester, 2008). SO\(_4\), Cl\(^-\), HCO\(_3\)\(^-\) are common soluble salts which cause salinity when their higher concentrations accumulate in soil or water (Khan et al., 2009).

General effects of salinity
Salt stress is one of the most important abiotic stresses which cause considerable reduction in growth and development in different plant species under salt stress (Maathuis, 2006; Lauchli and Grattan, 2007; Naz et al., 2010). Crop plants growing in a medium with high salt content show stunted growth and spotty plant body, which results in poor yield production (Abrol et al., 1988). However, salt-induced adversaries to growth and yield production of crop plants are highly variable and dependant on type and duration of salinity, type of species or cultivars, and on sensitivity and salt tolerability of crop plants at different growth stages of plant development (Ashraf, 1994; Shannon et al., 1994). While, enlisting the damaging effects of NaCl stress on plants, researchers have listed the following; osmotic effect, ionic effect, hormonal imbalance, nutrient imbalance and production of reactive oxygen species (ROS) (Ashraf and Foolad, 2007, Ashraf and Ashraf, 2012). The ROS leads to a gradual lipid peroxidation and inactivation of antioxidant enzymes (Tanou et al., 2009). Decreased water
and solute potential due to high salt concentration, triggers loss of cell turgidity in plants, but this turgor loss is not always permanent. In most cases, plant tissues can regain optimum turgor potential due to osmotic adjustment within a few hours (Flowers, 2004; Arfan et al., 2007). Smaller and thicker leaves due to salt-induced inhibition in size and cell division results in reduction of overall leaf area (Munns and Tester, 2008; Flowers et al., 2010). In cereals, salt stress causes reduction in number of tillers and total leaf area (Munns and Tester, 2008). The primary role of osmotic and ionic effects have been well recognized for worsening salt induced damage in crop plants (Apes and Blumwald, 2002; Munns et al., 2006; Ashraf et al., 2010).

**Ionic effect**

Accumulation of excessive amount of Na⁺ and Cl⁻ in leaves due to root-zone salinity is called ionic effect or ion toxicity (Flowers and Flowers, 2005). In plants, ionic effect accelerates the senescence process (Munns et al., 2006). Excessive uptake of Na⁺ and Cl⁻ by the roots and their transport and accumulation in leaves at higher concentrations cause specific ion toxicity which is lethal for plant survival at later stages, if the emergence and growth of new leaves is slower as compared to leaf senescence. The overall photosynthetic rate decrease, inhibits growth mainly due to limited supply of carbohydrates (Ashraf, 2004: Ashraf et al., 2008; Roshandel and Flowers, 2009). The presence of Na⁺ in both cellular and extra-cellular compartments negatively affects the uptake and accumulation of essential nutrients such as K⁺ and Ca²⁺ (Maathuis, 2006). Despite Na⁺ being a toxic ion for most plant species, Cl⁻ is also considered a toxic ion (Ashraf, 1994). While working with different vegetables such as cabbage, sugar beet, radish, Jamil et al. (2006; 2007) found that high amounts of Na⁺ in the growth medium reduced the K⁺ and Ca²⁺ in the external medium thereby limiting their availability to the vegetable plants and reduced K⁺/Na⁺ and Ca²⁺/Na⁺ ratios. At higher salinity levels, uncontrolled Na⁺ transport in salt sensitive species amplifies the salt-induced ionic effect as compared to osmotic effect (Munns and Tester, 2008).

Na⁺ exclusion and transport within a plant is more emphasized as compared to Cl⁻ because in most plant species, Na⁺ accumulation up to toxic level is more rapid than Cl⁻ (Munns and Tester, 2008). Excessive accumulation of both Na⁺ and Cl⁻ ions in various body parts of salt stressed guava plants, particularly in the leaves, resulted in reduced growth (Ferreira et al., 2001). Qasim and Ashraf (2006) reported that salt tolerance ability among
various canola cultivars differs according to relative accumulation of Na$^+$ concentrations in leaves which is negatively correlated with salt tolerance of canola cultivars. Amtmann and Sanders (1999) suggested that plants try to avoid excessive amount of Na$^+$ in the cytoplasm, because at higher concentrations, Na$^+$ interferes with normal ongoing metabolic processes. Among various salts Na$^+$, Cl$^-$, SO$_4^{2-}$ and HCO$_3^-$ are abundant components of saline soils and waters, and are responsible for ion toxicity in plants which reduce yield (Abrol et al., 1998; Munns and Tester, 2008; Abbas et al., 2010). Ion carrier proteins are integral parts of cell membrane and play a vital role in the transport of ions into the cell by active transport at the expense of adenosine triphosphate (ATP) and pyrophosphate (Flowers and Flowers, 2005).

Water loses as a result of transpiration leaving behind dissolved ions such as Na$^+$. As transpiration is a continuous process on leaf surface, higher concentrations of Na$^+$ accumulate in the leaf, which exceed the salt tolerance limit of plants leading to leaf senescence (Marschner, 1995). Role of Na$^+$ for causing ion toxicity has been well described in several reports. However, higher concentrations of Cl$^-$ are also not desirable as Cl$^-$ is considered comparatively more toxic for grapevine, citrus, and soybean (Lauchli, 1984; Ashraf, 1994; Grattan and Grieve, 1999; Storey and Walker, 1999). In older leaves, drying of leaf tissues especially at extreme tips of leaf and leaf burn are the symptoms of Cl$^-$ toxicity (Marschner, 1995). While explaining Cl$^-$ toxicity in plants, White and Broadley (2001) described that after being taken up through roots, Cl$^-$ is transported to shoots where it interferes in metabolic processes and exert damaging effect on photosynthesis.

**Osmotic stress**

Plants growing on soils with high salt content often face osmotic stress which inhibits growth, induces leaf chlorosis, imbalances the hormone level and reduces the antioxidant activity (Munns, 1993, 2002; Mittler, 2002; Ashraf, 2004; Ashraf et al., 2010a). Root to shoot water conductivity in plants depends upon soil water potential which decreases with increasing root-zone salinity and limits water supply to various plant organs which further results in reduced membrane permeability (Waisel, 1972; Munns, 2002). Adverse effects on growth due to salt-induced osmotic effect are abrupt for most of the plants as compared to ionic effects (Ashraf, 2004; Flowers, 2004). Type of plant tissue and timing of salt stress matters, while, assessing the adverse effects of salt-induced osmotic effect (Ashraf, 1994; Cony and Trion, 1998; Munns et al., 2002 Meloni et al., 2003). Leaf and shoot growth is
inhibited due to salt-induced osmotic effect while roots may continue to grow (Spollen and Sharp, 1991). In plants, number of tillers, lateral buds, branches and lateral shoot formation, emergence of new leaves, growth of developing leaves and leaf area are severely affected due to salt-induced osmotic effect (Taiz and Zeiger, 2006; Munns et al., 2006). Altered functioning of vital biochemical and physiological processes due to salt-induced osmotic phase results in reduced growth (Ashraf and Harris, 2004). Noreen and Ashraf (2009a) reported higher Na\(^+\) and Cl\(^-\) accumulation reduced water potential in salt stressed pea plants. Salt induced decrease in leaf water potential and relative water content proved to be inhibitory for reduced growth in turnip plants (Noreen et al., 2010). NaCl-induced growth reduction due to osmotic stress has been well documented in a number of plants, such as *Brassica* spp. (He and Cramer, 1993), *Brassica napus* (Zheng et al., 1998; Huang and Redman, 1995), maize (Cramer et al., 1994), and wheat (Kingsbury, 1984). Apart from prime importance of ionic and osmotic effects, various other secondary effects of salt stress such as imbalanced hormone level (Anuradha and Rao, 2001; Ashraf, 2004; Yusuf et al., 2008), nutritional imbalance (Gratten and Grieve, 1999), and oxidative stress (Mittler, 2002; Turkan and Demiral, 2009; Ashraf, 2009) also play a major role in worsening the salt-induced damage in plants.

**Nutritional imbalance**

Potassium (K), N, P, and Ca are essential nutrients for plants. Their uptake is greatly affected by the high concentration of Na\(^+\) and Cl\(^-\) in the growth medium, which leads to salinity-induced nutritional imbalance (Marschner et al., 1995; Grattan and Grieve 1999). In *Brassica napus* (canola) increased Na\(^+\) uptake due to enhanced root-zone salinity decreased the K\(^+\) and Ca\(^{2+}\) contents in the leaves and roots (Ali et al., 2006; Qasim and Ashraf, 2006; Ulfat et al., 2007; Ashraf and Ali, 2008). A considerable correlation exists between salt tolerance and inorganic elements in plants, but the information on the mechanism of this correlation is not sufficient (Greenway and Munns, 1980; Ashraf, 1994; Grattan and Grieve, 1999; Ashraf, 2009; Khorshidi et al., 2009). An optimum ratio of essential nutrients is required for normal plant growth which is disturbed due to salt stress. Salt stress inhibits the uptake, accumulation and transport of essential inorganic nutrients within plant tissues (Grattan and Grieve, 1999; Hu et al., 2005; Parida and Das, 2005; Akram et al., 2009a, b; Ashraf, 2009; Akram and Ashraf, 2011a).
A number of previous reports have described reduced accumulation of Ca+, K+ and N in plants under salt stress in different crop plants e.g., sunflower (Akram et al., 2007), green bean (Pessarakli, 1991), radish and cabbage (Jamil et al., 2006; 2007), wheat (Raza et al., 2006) etc. Salinity-induced decrease in the uptake of essential inorganic nutrients and increased ratios of Na+/Ca2+, Cl-/NO3, Na+/K+ and Ca2+/Mg2+ occur due to excessive uptake of Na+ Cl- (Grattan and Grieve, 1999). Reduced Ca2+ content and replacement of K+ by Na+ under salt stress leads to nutritional imbalance as both Ca2+ and K+ play a role in various physiological processes (Marschner, 1995; Kaya et al., 2007; Ashraf et al., 2010a). Ca2+ is known to activate various signaling pathways by acting as a second messenger, thus playing a role in plant growth and development under control and NaCl stress conditions (Sánchez-Barrena et al. 2005). Furthermore, inorganic nutrients have a role in osmotic adjustment in plants exposed to stress conditions (Greenway and Munns, 1980; Flowers, 1985; Moghaieb et al., 2004). Generally, increased cytosolic Ca2+ content is an immediate response to higher Na+ concentration (Munns and Tester, 2008). Higher K+ content and lower Na+/K+ ratio are achieved by Na+ exclusion in salt tolerant cultivars as compared to salt sensitive ones (Chaudhary et al., 1997; Sabir and Ashraf, 2007; Ashraf, 2009; Akram and Ashraf, 2011a). High amounts of Na+ in the growth medium reduce K+ and Ca2+ in the external medium of different vegetables such as cabbage, sugar beet, radish, thereby limiting their availability to the vegetable plants. This was reflected as reduced K+/Na+ and Ca2+/Na+ ratios in these plants (Jamil et al., 2006; 2007).

**Hormonal imbalance**

Phytohormones play a vital role in plant growth, development and biomass production (Brosa, 1999; Ashraf, 2004; Hayat et al., 2007; Ashraf et al., 2010a). High salt content in the growth medium causes a decrease in the phytohormone levels, such as those of gibberellins, auxins and cytokinins, but it increases the level of abscisic acid (ABA) (Moorby and Besford, 1983; Vaidyanathan et al., 1999; Iqbal and Ashraf, 2005, 2006). Generally, phytohormones are produced very low concentrations, but their optimum levels are required for normal plant metabolism and growth (Wang et al., 2001, 2005; Xiong and Zhu, 2002). Imbalanced synthesis of phytohormones under salt stress causes diverse changes in plants, e.g., reduction in seed germination, stomatal movement, ion uptake, permeability of membrane, rate of photosynthesis and yield content (Harper and Balke, 1981; Barkosky and
Roles of some newly discovered hormones such as brassinosteroids (BRs), jasmonic acid (JA), polyamines and salicylic acid (SA) have also been established for plant growth and regulation in addition to already discovered classical hormones, e.g., abscisic acid (ABA), cytokinins (CK), gibberellins (GA), auxins and ethylene (Dietz et al., 1990; Davies, 1995; Ashraf, 2004; Taiz and Zeiger, 2006). ABA contributes to maintain membrane integrity by enhancing Ca$^{2+}$ uptake and thereby regulating ion uptake and transport under salt stress (Chen et al., 2001). ABA is also capable of modulating cell wall extensibility, which contributes beneficially to plant growth under drought or salt stress (Dodd and Davies, 1996; Cramer et al., 1998; Bacon, 1999). ABA protects plants from ion toxicity by delaying the NaCl induced damaging effects (Eberhardt and Wegman, 1989). Similarly, Popova et al. (1995) also reported that ABA helps in increasing plant growth and photosynthesis by reducing the deleterious effects of NaCl. Similarly, jasmonates also have a role in increasing salt tolerance, as Hilda et al., (2003) reported, that higher ABA level was observed in salt tolerant tomato cultivars as compared to salt sensitive cultivars. In *Pisum sativum*, pre-sowing seed treatment with jasmonates counteracted the inhibitory effects of salt stress on growth and photosynthesis (Fedina and Tsonev, 1997; Tsonev et al., 1998). NaCl-induced damaging effects in plants are reduced by jasmonates through signal transduction pathway, but the process of jasmonate-induced signal transduction remains yet to be elucidated (Hilda et al., 2003). Reduced K$^+$ efflux from leaf mesophyll with exogenously application of polyamines in salt stressed pea plants suggested that the activity of plasma membrane ion channels may be modulated by the increase in cellular polyamine content which assisted plants in adapt to salinity by improving ionic relations (Shabala et al., 2007).

Similarly, another group of plant growth regulators named Brassinosteroids reduced the damaging effects of NaCl stress and enhanced growth when applied exogenously on salt stressed wheat plants (Ali et al., 2006). Generally, imbalanced synthesis of phytohormones under salt stress affects yield and growth attributes (Zhang and Zhang, 1994; Ashraf 1994; Aldesuquy, 1999; Debez et al., 2001; Ashraf, 2004; Kim et al., 2009). Cytokinin level in plant tissues declines due to salinity stress (Zhang and Zhang, 1994; Wang et al., 2001). Endogenous levels of ABA, SA, GA and JA were disturbed in soybean under saline
conditions, as endogenous JA and ABA contents were increased while those of SA and GA decreased in salt stressed soybean plants which resulted in reduced growth and yield production (Hamayun et al., 2010). Similarly, Wang et al. (2001) reported increased JA and ABA contents and decreased IAA and SA contents while working with different plant organs of salt stressed Iris hexagona to study the comparative effects of salt stress on IAA, JA, SA and ABA.

**Oxidative stress**

Production of several reactive oxygen species (ROS) as byproducts of aerobic metabolism is a continuous process in plants. However, overproduction of these ROS leads to oxidative stress in plants (Nordberg and Arner, 2001; Apel and Hirt, 2004, Ashraf, 2009). In other words, disturbed balance between production of ROS and antioxidant defense system results in oxidative stress (Betteridge, 2000). As excessive ROS production under salt stress limits the ability of antioxidants defense system to scavenge the ROS (Mittler, 2002). Oxidative stress is a secondary stress due to a number of other abiotic stresses including salinity (Mittler, 2002; Ashraf, 2004; Amor et al., 2005; Ashraf, 2009). Singlet oxygen (¹O₂), hydroxyl radical (·OH), superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) are important ROS, produced under salt stress due to excessive accumulation of Na⁺ and Cl⁻ (Asada, 1994; Ashraf, 2009). Among various cell organelles, chloroplast, mitochondria, cytoplasm, vacuole and microbodies are major sites for ROS generation (Bowler et al., 1992; Ashraf, 1994; Dixon et al., 1998; Van Breusegem et al., 2001; Mittler et al., 2004; Ashraf, 2009). Although oxygen (O₂) is required as an essential gas molecule for life but ROS are produced by reduction of oxygen, and in plants ROS can easily disturb different metabolic processes (Mittler, 2002). Being highly reactive in nature, ROS initiate a series of destructive processes by interacting with different essential cellular molecules and metabolites such as proteins, DNA, lipids, pigments, etc. (Mittler, 2002, Ashraf, 2009). Plant’s ability to minimize the ROS-induced damaging effects on macromolecules is an important stress tolerance trait (Mittova et al., 2004).

Mitochondria are the major ROS production sites in non-photosynthetic plant cells (Fleury et al., 2002). Different types of antioxidant enzymes are produced by plants for detoxification and scavenging of ROS. There are two different types of antioxidants generally known as enzymatic and non-enzymatic antioxidants (Mittler, 2002, Ashraf, 2009).
In plants, antioxidant production is enhanced under salt stress to counteract the increased levels of ROS in the cells. Increased salt tolerance is observed in plants with higher levels of antioxidant production, as antioxidants scavenge increased ROS concentrations (Ashraf, 2009).

Antioxidant defense mechanism comprises two types of antioxidants, enzymatic and non-enzymatic antioxidants. For example, superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POD), monodehydroascorbate reductase (MDHAR), catalase (CAT), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) are enzymatic antioxidants while tocopherols, flavonoids, phenolics, carotenoids, glutathione reductase (GSH), and ascorbate are non-enzymatic antioxidants (Noctor and Foyer, 1998; Asada, 1999; Mittler, 2002; Smirnoff, 2005; Ashraf, 2009). It is now well established through a number of reports that plants with higher levels of antioxidants have better tolerance to salt-induced oxidative stress (Hernandez et al., 1994; Ashraf, 2009). However, the ability of antioxidant defense mechanism to scavenge and detoxify ROS may be perturbed due to various abiotic stresses such as drought, extreme of temperature and salinity (Elstner, 1991; Foyer and Noctor, 2000; Apel and Hirt, 2004; Smirnoff, 2005). Increased antioxidant activity was found to be positively correlated with increased salinity tolerance in cotton (Gosset et al., 1994), and rice (Dionisio-Sese and Tobita, 1998). Similarly, Hernandez et al., (1993; 1995) have reported that higher activity of chloroplastic CuZn-SOD, mitochondrial Mn-SOD and ascorbate peroxidase was observed in salt tolerant cultivars of pea (Pisum sativum) as compared to salt sensitive cultivars. Similarly, increased ascorbate peroxidase (APX) and glutathione reductase (GSH) activity in wheat was found to be positively correlated with the salt tolerance ability of wheat by counteracting the oxidative stress (Sairam et al., 1997; 1998).

Gas exchange characteristics

Among various physiological attributes, gas exchange characteristics are most important in plants (Ashraf, 2009). Salt stress causes considerable perturbation in gas exchange attributes (Raza et al., 2007; Nawaz and Ashraf, 2010). For example, in plants Transpiration rate, photosynthetic rate, water use efficiency and stomatal conductance are among the key gas exchange attributes which are markedly declined under salt stress (Ashraf, 2004; Ashraf, 2009). While working with different canola cultivars Ulfat et al., (2007) found
reduced photosynthetic capacity and stomatal conductance under salt stress which resulted in poor growth. Effect of salt stress on different gas exchange attributes varies not only between plant species but also among cultivars of same species, so gas exchange attributes can also be used as criteria for selective breeding against salt stress in crop plants that have a positive relationship between gas exchange attributes and growth (Ashraf, 2004). For example, yield in wheat and some Brassica species was found to be positively correlated with photosynthetic rate (Nazir et al., 2001). Although growth and yield production is positively correlated with photosynthetic rate in most of crop plants but not necessarily in all crop plants (Ashraf, 2004; Nawaz et al., 2010).

**Photosynthetic Pigments**

Chlorophyll is an important biomolecule which has an utmost importance in plant physiology. Salt stress causes serious damage to chlorophyll molecule (Parida & Das, 2005; Shahbaz et al., 2011). Apart from gas exchange attributes and chlorophyll fluorescence, de novo synthesis and functioning of chlorophyll is also markedly impaired due to increased Na⁺ and Cl⁻ content in plants grown under saline stress (Ashraf, 2004). Decreased 5-aminolevulinic acid and protochlorophyllide content under salt stress are responsible for decreased chlorophyll content as they are precursors for chlorophyll biosynthesis (Santos, 2004). In addition, lower level of glutamic acid was observed in salt stressed plants, as glutamic acid is required for 5-aminolevulinic acid biosynthesis (Beale and Castelfranco, 1974; Santos, 2004). Fang et al. (1998) reported that salt-induced degradation of chlorophyll in plants is due to increased activity of chlorophyllase enzyme which removes phytol group from the chlorophyll molecule. Salt-induced reduction in photosynthetic pigments directly affects the photosynthetic rate, however, degree of reduction in chlorophyll content depends upon salt tolerance ability of crop plants as increased chlorophyll content was observed in salt tolerant species as compared to that in salt sensitive species (Ashraf and McNeilly, 1988). Chlorophyll a, b, and chlorophyll a/b ratio were markedly decreased in some wheat cultivars at different growth stages under salt stress, however, less decrease in chlorophyll content was observed in a salt tolerant wheat cultivar (S-24) under saline conditions as compared to a relatively sensitive cultivar MH-97 (Ashraf and Ashraf, 2012). Velegaleti et al. (1990) also reported that salt-induced reduction in chlorophyll content in salt sensitive species was correlated with increased accumulation of Cl⁻ content. Hernandez et al., (1993;
1995; 1999) reported reduced carotenoids, total chlorophyll, chlorophyll ‘a’, and chlorophyll ‘b’ content in *Pisum sativum* which resulted in poor growth under saline conditions. Reduced chlorophyll content under salt stress has been observed in a number of plants e.g., wheat (Khatkar and Kuhad, 2000), tomato (Doganlar, 2010), canola (Nazarbeygi *et al*., 2011) and sunflower (Santos, 2004). Moreover, Ashraf, (2004) reported that salt-induced decrease in net CO₂ assimilation rate is somewhat attributed to reduced chlorophyll content.

**Chlorophyll fluorescence**

Extent of damage to photosynthetic apparatus can be measured by chlorophyll fluorescence, which is a non-destructive and rapid diagnostic method for estimating damage to photosynthetic apparatus not only in crop plants but also in trees and ornamental plants subjected to various environmental stresses (Palta, 1992; Sestak and Stiffel, 1997; Ashraf, 2004; Percival, 2004; Baker and Rosenqvist, 2004; Athar and Ashraf, 2008; Baker, 2008). Among photosynthetic machinery, photosystem II (PS II) is more sensitive to salt stress as compared to photosystem I (PS I), so salt-induced decrease in photosynthesis is often linked to PSII (Saleem *et al*., 2011). Oxygen evolving potential of PSII is inhibited due to disintegration of thylakoid membranes and also inhibits light energy transfer from antenna complex to PSII (Mehta *et al*., 2010). In plants activity of PSII is down-regulated to improve conversion efficiency of excitation energy under saline conditions (Lu and Vonshak, 2002). Therefore, using chlorophyll fluorescence as an effective tool to measure the damage to PS II is an excellent approach under salt stress (Saleem *et al*., 2011). While studying imbalanced electron transport system and inhibited activities of pigments in salt stressed plants *Brassica juncea*, Alia *et al*., (1993) found that salt stress not only inhibited the electron transport system of thylakoid but also severely affected the activity of PSII. Moradi and Ismail (2007) measured chlorophyll fluorescence in rice at the vegetative and reproductive stages and found that the functioning of PSII was severely affected under saline regime. Similarly, PSII was also reported to be severely damaged in wheat under salt stress (Mehta *et al*., 2010; Kanwal *et al*., 2011; Ashraf and Ashraf, 2012). Furthermore some researchers used chlorophyll fluorescence attributes as a tool for comparing salt tolerance in different wheat cultivars and reported that less damage was observed to PSII in salt tolerant cultivar S-24 as compared to in MH-97, a relatively salt sensitive cultivar (Kanwal *et al*., 2011; Ashraf and Ashraf, 2012). Athar *et al*. (2008) also suggested that chlorophyll fluorescence attributes can
be used as effective criteria for salt tolerance in breeding projects which can help enhance crop salt tolerance.

**Plant responses and mechanism of salinity tolerance**

Naturally plants respond to various environmental conditions including salinity. Plants respond to salt stress not only at molecular or tissue level but also at whole plant level (Munns and Tester, 2008). However, cells, organs and tissues respond independently but their response varies not only at different developmental stages but also on the type and level of stress (Munns, 1993).

**Accumulation of compatible solutes**

Proteins, amino acids, sugars, organic acids, quaternary ammonium compounds (such as glycinebetaine) and polyols etc. are some of the important compatible solutes which accumulate in plants under various environmental stresses including salinity, and they play an important role in enhancing stress tolerance by reducing salinity-induced ion toxicity (Hasegawa et al., 2000; Mansour, 1998; 2000; 2005; Ashraf and Harris, 2004). These compatible solutes play a vital role in osmoregulation by acting as osmoprotectants such as trehalose, proline and glycinebetaine etc, as their concentration increases considerably in various of plant species under various abiotic stresses including NaCl stress (Ashraf and Foolad, 2007; Ashraf, 2009; Abbas et al., 2010; Ali and Ashraf, 2011). For instance, a considerable increase in leaf proline and glycinebetaine (GB) content was observed in okra under salt stress (Habib et al., 2012). While working with different *Brassica spp.* Ashraf and Naqvi (1992) found that total soluble sugar content increased in shoots of *Brassica napus*, *B. campestris* and *B. juncea*, under saline conditions. Generally, these osmolytes increase stress tolerance by helping plants in uptake of water (Flowers, 2004; Ashraf and Foolad, 2007), protecting plants from excessive Na⁺ and Cl⁻ toxicity (Misra and Gupta, 2005), free radicals scavenging (Okuma et al., 2004; Ashraf, 2009; Banu et al., 2010), and by stabilizing the structures in the cells and protecting macromolecules under saline regimes (Hoekstra et al., 2001).

According to a number of reports it is well established that higher concentration of GB accumulates in many plant species under saline conditions (Ashraf and Foolad, 2007; Abbas et al., 2010; Banu et al., 2010). Cha-um and Kirdmanee, (2009) reported that growth of maize plants was enhanced with increase in proline content under salt stress. While
comparing the salt tolerance ability between *Thellungiella halophila* and *Arabidopsis thaliana* Kant *et al.* (2006) found that salt tolerance in *Thellungiella halophila* was increased by lowering the expression of gene (s) responsible for proline dehydrogenase (*PDH*). Similarly, both GB and proline contents were increased under saline conditions in wheat (Carillo *et al*., 2008), barley (Ahmed *et al*., 2008) and okra (Habib *et al*., 2012). Increased salt tolerance in many plant species was positively correlated with both GB and proline content (Ashraf and Harris, 2004; Hassine and Lutts, 2010; Hassine *et al*., 2010; Habib *et al*., 2012). Although osmolytes such as proline, GB, and reducing and non-reducing sugars accumulate in abundance as stress response in many plant species, in some plant species accumulation of GB varies from very low to no GB at all (Maqsood *et al*., 2006; Ahmed *et al*., 2010).

**Intracellular Na⁺ Compartmentalization**

Plants have developed various mechanisms to maintain lower cytosolic Na⁺ concentration however, the efficiency of these mechanisms varies in different plant species or even in cultivars which helps in categorizing plants from sensitive to moderately tolerant or even highly tolerant against salt stress. Balanced cytosolic Na⁺ concentration in plants is almost 10-30 mM. Plants try to maintain these lower Na⁺ concentrations by partitioning Na⁺ within cells exposed to salt stress (Carden *et al*., 2003). Although the activities of most of enzymes are inhibited with increase in Na⁺ concentration however, the toxic concentrations of Na⁺ and Cl⁻ are not well defined, (Flowers and Dalmond, 1992; Munns and Tester, 2008). Compartmentalization of excessive Na⁺ and Cl⁻ ions in vacuole of the plant cells helps in proper functioning of enzymes and various other metabolic and physiological processes in leaves (Tester and Davenport, 2003; Munns and Tester, 2008). In vitro salt stress tolerance ability of important enzymes is almost similar in halophytes and non-halophytes which suggests that Na⁺ compartmentalization in plant cells is an important mechanism for salt stress tolerance (Munns and Tester, 2008). In plants, expression of *AtNHX1* or *AtAVP1* affects the compartmentalization of Na⁺ in vacuole, and a number of reports are also available in the literature that increased salt stress tolerance in various plant species is found to be positively correlated with overexpression of *NHX* genes (Apse *et al*., 1999; Zhang and Blumwald, 2001; Zhang *et al*., 2001; Xue *et al*., 2004; He *et al*., 2005; Brini *et al*., 2007; Chen *et al*., 2007; Munns and Tester, 2008) or *AtAVP1* (Gaxiola *et al*., 2001). Stress
tolerance varies between closely related species which can be explained on the basis of increased intracellular compartmentation efficiency, as activity of salt-induced Na\(^+/\)H\(^+\) antiporter is greater in *Plantago maritima* (a salt tolerant species) as compared to that in *Plantago media* (a salt sensitive species) (Staal et al., 1991).

**Biochemical response**

Salt-induced oxidative stress causes damage not only to proteins and DNA, but also causes peroxidation of lipid structures (Neill et al., 2002; Ashraf & Foolad 2007; Ashraf, 2009). To minimize the salt-induced oxidative damages due to production of ROS, plants have antioxidant defense mechanism which comprises antioxidant enzymes and secondary metabolites of low molecular weight such as carotenoids, ascorbate, phenolics, tocopherols and glutathione (Posmyk et al., 2009). To elaborate the antioxidative properties of these metabolites lot of research work is being conducted. Phenolics are an important group of plant secondary metabolites with biological and antioxidant properties (Tsai et al., 2002; Wang & Lin, 2000; Posmyk et al., 2009). Greater radical stabilization and H-donating ability enhance the activity of phenolics as antioxidant metabolites (Rice-Evans et al., 1996). Different abiotic stresses including salinity affect plant phenolic content (Parida et al., 2004). As in *Cynara cardunculus*, leaf phenolic content was decreased under saline conditions (Falleh et al., 2008). Ashraf et al. (2010b) reported that a positive correlation exists between growth and phenolic content in wheat under salts stress which suggests that phenolic compounds have a role in enhancing salt stress tolerance in wheat. Malondialdehyde (MDA) provides an effective means for assessing extent of membrane damage due to oxidative stress, as accumulation of MDA takes place in plants due to lipid peroxidation of membranes (Shao et al., 2005). Salt induced increase in MDA content in wheat cultivars was found to be negatively correlated with plant growth under salt stress, and less increase in MDA content was observed in salt tolerant cultivar S-24 as compared to relatively salt sensitive cultivar MH-97 (Ashraf et al., 2010b). To establish the degree of salt tolerance among crop cultivars, cell membrane stability can be used as an effective criterion (Meloni et al., 2003; Sairam et al., 2005). Some earlier studies have shown that reduced MDA content is an important indicator of salinity tolerance in salt tolerant cultivars of tobacco (Ruiz et al., 2005), sorghum (Brankova et al., 2005), and barley (Liang et al., 2003). Similarly, salt induced increase in MDA content resulted in reduced plant growth in sesame (Koca et al., 2007), and tomato (Li,
2009), and less increase in MDA content was observed in salt tolerant cultivars/lines as compared to salt sensitive cultivars (Koca et al., 2007; Li et al., 2009). While working with contrasting wheat cultivars for salt tolerance Ashraf et al., (2012) found that total soluble proteins, H$_2$O$_2$ and proline content were increased but ascorbic acid content decreased under salt stress.

**Molecular response (gene expression)**

At molecular level, plant abiotic stress tolerance depends upon the expression of genes responsible for stress-related traits (Wang et al., 2003; Ashraf, 2004; Yamaguchi and Blumwald, 2005). As scientists have already discovered some stress-related genes in plants which are responsible for late embryogenesis abundant proteins, heat-shock proteins, osmoprotectants and antioxidants, activation of ion transporters, etc. (Zhu, 2001; Ashraf et al., 2008; Ashraf, 2009). The products of these genes are involved in protection of membranes and proteins, regulation of signal transduction, transcriptional control and overcoming free-radicals under salt stress (Akram, 2011). Newly developed molecular tools can be helpful in enhancing crop salt tolerance as production of transgenic plants and use of biotechnological approaches show promising results for enhancing stress tolerance in crop plants (Ashraf and Akram, 2009). Plants readily sense excessive Na$^+$ accumulation and transducer stress-related signals which activate various mechanisms involved in defensive response against salt stress (Yoshida, 2002; Yamaguchi and Blumwald, 2005). A salt stress signaling cascade comprising Salt Overly Sensitive proteins (SOS) have a considerable role in salt tolerance (Ashraf and Akram, 2009).

**Cellular signaling**

Plants directly respond to excess of Na$^+$ and this response is very rapid and specific for Na$^+$ (Knight et al., 1997; Tracy et al., 2008). However, the mechanism by which plants sense excessive Na$^+$ and altered osmotic pressure, yet remains obscure (Munns and Tester, 2008). Increased cytosolic free Ca$^{2+}$ ($[\text{Ca}^{2+}]_{\text{cyt}}$) is the first response to excessive accumulation of Na$^+$ around roots. Extracellular Na$^+$ addition activates the Ca$^{2+}$ flux into the cytosol across the plasma membrane and tonoplast (Kiegle et al., 2000; Knight et al., 1997; Moore et al., 2002; Tracy et al., 2008). Salt-induced increase in $([\text{Ca}^{2+}]_{\text{cyt}}$) is involved in signaling pathways specific to salinity (Zhu, 2002). In this signaling pathway, Na$^+$-induced increase in cytosolic Ca$^{2+}$ is sensed by a calcineurin B-like protein (CBL4) which was originally
identified as SOS3 (Halfter et al., 2000; Munns and Tester, 2008). Dimerization of CBL4 and its subsequent interaction with CBL-interacting protein kinase (CIPK24), which was originally identified as (SOS2) is facilitated by increased cytosolic Ca^{2+} (Halfter et al., 2000). SOS3/SOS2 complex is targeted to the plasma membrane by a fatty acid chain which is covalently bound to SOS3 (Ishitani et al., 2000). Thus, phosphorylating and subsequently activating membrane bound Na^{+}/H^{+} antiporter is SOS1 (Qiu et al., 2002; Quintero et al., 2002; Shi et al., 2002). This pathway is important for salinity tolerance as Arabidopsis thaliana plant mutants for SOS were less tolerant to salt stress as compared to wild type plants (Zhu et al., 1998). However, various components of signaling pathway have been identified in plant response to salt stress inferred by a variety of other approaches such as reverse genetics and transcriptomics (Xiong et al., 2002; Zhu, 2002; Cheong and Yun 2007).

**Plant response at whole plant level**

At whole plant level, primary response to salt stress is reduced growth and yield production (Ashraf and Akram, 2009; Noreen and Ashraf, 2010). A number of vital processes such as protein synthesis, photosynthesis, membrane permeability, nutrient uptake, lipid metabolism, oxidative defense system, and osmotic and energy balance are severely perturbed when plants are exposed to salt stress (Ashraf, 2009; Flowers et al., 2010). Reduced growth and yield production is mainly due to reduced leaf expansion (Apse and Blumwald, 2002; Parida and Das, 2005; Ashraf, 2010). As reduced photosynthesis, stomatal regulation and substomatal CO\_2 due to salt stress results in reduced growth and yield production as observed in Solanum melongena (Abbas et al., 2010). Similarly, excessive Na\(^+\) and Cl\(^-\) accumulation in both leaves and roots results in reduced yield and biomass production as observed recently in okra (Habib et al., 2012). Poor growth and yield production in salt stressed sunflower plants can be due to salt-induced decrease in chlorophyll content and quantum yield, \(F_{v}/F_{m}\) (Akram and Ashraf, 2011c). Response to salt stress varies not only between species and cultivars of a same species but also at different growth stages of plant development (Maas and Poss, 1989; Zheng et al., 2001; Lutz et al., 2005; Ashraf et al., 2010b). As salt-induced yield loss was more severe in wheat when salinity was applied at the booting stage as compared to that at the reproductive stage (Maas and Poss, 1989). Similarly, while studying gas exchange parameters and chlorophyll fluorescence in wheat cultivars at different growth stages, Ashraf and Ashraf (2012) found...
that adverse effects of salt stress in wheat plants were more severe at early growth stages as compared to those at later stages.

**Strategies for enhancing crop salt tolerance**

Although some plants have inherent ability to tolerate high saline conditions, this is not the case with all plants (Greenway and Munns, 1980; Ashraf, 1994; 2004; Flowers, 2004). Genetic diversity exists among species and cultivars which controls salt tolerance level of plants, as dicotyledonous species are generally more salt tolerant than monocotyledonous. Among cereals, barley (*Hordeum vulgare*) is most tolerant, bread wheat (*Triticum aestivum*) moderately tolerant and rice (*Oryza sativa L*) most sensitive to salt stress (Ashraf, 1994; Munns and Tester, 2008). Various biotic and genetic approaches can be practiced for improving salt tolerance in crop plants (Epstein *et al*., 1980; Ashraf, 1994; Ashraf *et al*., 2008). Biological strategies include exogenous applications of osmoprotectants, marker-assisted selection, conventional breeding, generation of transgenic plants, exogenous application of antioxidant compounds, growth promoters and inorganic salts (Ashraf, 1994; 2004; Munns, 2005; Flowers, 2004; Munns and Tester, 2008).

**Screening and selection for salt tolerance**

Genetic diversity among individuals of a plant population at species or even at cultivar level provides a basis for screening and selection procedures which can be used for improving salt tolerance in crop plants. For instance, differential salt tolerance in bean cultivars (*Phaseolus vulgaris* L.) at seedling stage is due to genetic variability which results in improved root growth and higher accumulation of mineral nutrition in salt tolerant cultivars as compared to relatively sensitive cultivars (Moreno *et al*., 2000). While screening swede rape (*B. napus*) for salt tolerance, Pokrovskii (1990) screened 165 varieties and reported three of them as salt tolerant. Although seed germination rate of *Brassic napus* was adversely affected under salt stress in some cultivars, yield reduction was not significant (Sharma and Manchanda, 1997). While screening 100 genotypes of sorghum, 46 were identified as comparatively salt tolerant at the seedling stage (Krishnamurthy *et al*., 2007). According to Dasgan *et al*. (2002) screening at seedling stage is less laborious and time consuming. However, salt tolerance should be evaluated at germination, seedling and maturity stages (Ashraf, 2004). For example, a well known salt tolerant wheat cultivar S-24 which was developed using screening and breeding techniques was found to be salt tolerant
at all growth stages on the basis of various physiological and biochemical attributes (Ashraf, 2010; Ashraf and Ashraf, 2012; Ashraf et al., 2012). These physiological or biochemical processes which are more sensitive to salt stress can be used as selection criteria for salt tolerance (Ashraf, 2004; Ashraf and Harris, 2004).

A number of factors influence screening process. Soil heterogeneity is a great constraint for screening under natural field conditions (Munns et al., 2002). Furthermore salt tolerance ability of crop plants is considerably influenced by Ca$^{2+}$ content in the growth medium (Ashraf, 1994). According to Munns et al. (2002) a specific trait associated with screening for salt tolerance is more preferable, as working with large number of lines for studying the effect of salt stress on yield and biomass production is not an easy task.

**Conventional breeding for improving salt tolerance**

Breeding for salt tolerance using key traits can be carried out by evaluating inter-cultivar genetic variation. Hybridization of high yielding genotypes with selected salt tolerant genotypes can be helpful in enhancing crop productivity under saline conditions (Munns et al., 2006). Using the conventional breeding method some salt tolerant lines have been developed for different crop plants such as in maize (Ashraf and McNeilly, 1990), brassica (Ashraf and McNeilly, 2004; Purty et al., 2008) and wheat (Ashraf, 2010). It has already been reported that many physiological processes can be set as criteria for breeding for salt tolerance (Ashraf, 2004; Ashraf and Harris, 2004). For example Na$^+$ exclusion in some crop species including *Trifolium* is a premier criterion (Rogers and Noble, 1992; Rogers et al., 1997). Many researchers have suggested that complex nature of salt tolerance mechanism and non-significant intra-specific genetic variation for various physiological traits are the major obstacles in developing crop plants with increased salt tolerance (Flowers, 2004; Colmer et al., 2005; Cuartero et al., 2006; Munns et al., 2006; Munns, 2007). Furthermore, intra-specific genetic diversity still needs to be fully exploited for improving crop salt tolerance (Munns, 2008; Munns and Tester, 2008). Ashraf et al. (2008) have pointed out some reasons for limited success in enhancing crop salt tolerance through conventional breeding such as transfer of undesirable genes along with desirable ones. Conventional breeding requires intensive labor and time as well as restricted transfer of favorable alleles due to reproductive barriers. Although conventional breeding methods have made
considerable progress in enhancing crop salt tolerance, this progress could not keep pace with the growing demand of increased crop productivity on saline soils (Flowers, 2004).

**Biological approaches at molecular level**

As discussed earlier various physiological and biochemical traits determine salt tolerance in plants (Ashraf, 2004; Ashraf and Harris, 2004), and expression of multiple genes related to salt stress make salt tolerance a complex mechanism (Hasegawa *et al*., 2000; Bartels and Sunker, 2005; Munns, 2005; Munns and Tester, 2008). Furthermore, it is also well evident that various environmental factors have significant influence upon screening and selection procedures through conventional breeding for improving crop salt tolerance (Ashraf *et al*., 2008). As molecular markers are unaffected from environmental factors, so the identification of molecular markers highly specific for genes controlling salt response in plants could be used for selection in a segregating plant population (Athar, 2008). According to Flowers (2004), use of QTLs (Quantitative Trait Loci) has improved selection efficiency for traits which are controlled by the expression of multiple genes and are highly dependent on environmental factors. As discussed earlier that some plants show differential salt tolerance at different growth stages which is a obstacle in selection for enhanced salt tolerance. Salt tolerance related QTLs at germination stage in tomato (Foolad *et al*., 1999), barley (Mano and Takeda, 1997), and *Arabidopsis* (Quesada *et al*., 2002) were found to be different from early growth stage related QTLs. Therefore, plants which have higher germination rate under saline conditions do not necessarily exhibit similar salt tolerance level during vegetative stage (Yamaguchi and Blumwald, 2005). Although salt tolerance related QTLs have been identified in various cereal crops such as in barley, wheat and rice, very few robust markers have been found yet, which are found in a range of germplasm (Munns, 2008). According to a number of reports it is well evident that salt tolerance ability in crop plants can be improved by over-expression of genes which control traits related to salt tolerance (Flowers, 2004; Bartels and Sunker, 2005; Munns, 2005; Cuartero *et al*., 2006; Ashraf *et al*., 2008). For instance, transgenic canola cultivar with over-expression of *Arabidopsis AtNHX1* gene responsible for Na$^+$/H$^+$ antiport showed increased salt tolerance (Zhang *et al*., 2001). Flowers (2004) reported that some transgenic plants were rather accumulators of Na$^+$ so there are also some doubts about gene functionality and success in transgenic plants. Munns (2005) arranged the genes associated with salt tolerance into three
categories. According to her first type of genes are responsible for uptake, regulation and transport of salts, products expressed by the second type of genes actively participate in osmo-protection, while expression of third type of genes accelerates plant growth under saline conditions. Large numbers of reports are available about the successful transformation experiments which show increased salt tolerance in transgenic plants with over-expression of genes responsible for Na\(^+\) exclusion and for tolerance against Na\(^+\) at tissue level (Munns and Tester, 2008). However inappropriate methodology and poor experimental designs to evaluate salt tolerance led these claims of improved salt tolerance to face heavy criticism (Flowers, 2004; Munns, 2005; Cuartero et al., 2006; Ashraf et al., 2008).

### Shotgun approaches to improve crop salt tolerance

Although traditional plant breeding, genetic engineering and molecular biology techniques helped in enhancing crop salt tolerance, the success rate of developing salt tolerant cultivars is limited due to certain problems like differential salt tolerance at cellular, tissue or whole plant level, involvement of environmental factors, complex mechanism of salt tolerance and lack of efficient selection criteria (Flowers, 2004; Ashraf et al., 2008; Munns and Tester, 2008). Due to limitations in earlier-mentioned techniques researchers have proposed various shotgun approaches as an alternate for enhancing crop salt tolerance such as foliar spray and seed priming with antioxidants, plant growth regulators, osmoprotectants, and inorganic salts Ashraf and Foolad (2005; 2007). Seed priming with all the above mentioned chemicals has been reported to enhance salt tolerance at seed germination stage, early seedling growth stage and also at later growth stages including the reproductive one (Ismaeil et al., 1993; Rehman et al., 1998; Al-Hakimi and Hamada, 2001; Ashraf and Rauf, 2001; Iqbal and Ashraf, 2005; 2006; Habib et al., 2010). According to a number of reports, NaCl-induced growth inhibition was counteracted with foliar application of compatible solutes, proline and glycinebetaine, in different crops including tomato (Makela et al., 1998a), wheat (Diaz-Zorita et al., 2001; Raza et al., 2006), and swede rape (Makela et al., 1999). Similarly, promoted growth and improved yield have been reported in a number of studies by foliar spray or pre-sowing seed treatment with salicylic acid under saline regimes (Shakirova et al., 2003; El-Tayyeb, 2005; Arfan et al., 2007). However, the ameliorative effect of these compounds through seed priming or foliar spray in plants under salt stress depends upon developmental stage at which they are applied, concentration and
nature of a compound used, amount absorbed by the plant and type of plant species (Ashraf and Foolad, 2005; 2007).

**Pre-sowing seed treatment**

Various chemical compounds can be used for seed priming to enhance salt tolerance in crop plants. These priming compounds include sugars, glycerol, sorbitol, mannitol, different plant growth regulators, inorganic salts and water etc. (Ashraf and Foolad, 2005). Various means for exogenous supplementation of these compounds are being used which include pre-sowing seed treatment, foliar application and supplementation through root growth medium. Pre-sowing seed treatment with water (Hydropriming) has been shown to improve salt tolerance in pigeon pea (Jyotsna and Srivastava, 1998), and maize seeds (Ashraf and Rauf, 2001). In another study pre-sowing seed treatment with salicylic acid improved growth and yield in salt stressed wheat plants (Arfan et al., 2007). Similarly, seed priming with polyethylene glycol (PEG) improved salt tolerance in tomato plants as compared to plants developed from non-primed seeds (Bennett et al., 1992; Balibrea et al., 1999; McDonald, 2000). In our initial experiment related to this study, we observed increased seed germination rate and improved early seedling growth in rice, that developed from seeds pre-treated with nitric oxide under salt stress (Habib et al., 2010). Different plant growth regulators are also effective in enhancing seed germination rate and improving growth and yield production under both saline and no-saline regimes (Iqbal and Ashraf, 2005; 2006). As pre-sowing seed soaking with different plant growth regulators like GA, IAA and CK improved seed germination rate in wheat under salt stress (Balki and Padole, 1982; Iqbal and Ashraf, 2005; 2006). Similarly, GA was also reported to be effective in counteracting salinity-induced adverse effects in okra (Vijayaraghavan, 1999), and tomato (Kang et al., 1996).

**Foliar application**

Being a complex mechanism, salt tolerance is attributed to single or multi-factors in plants which include altered nutrient status, overproduction of various compatible solutes, changed hormonal balance and increased activity of different antioxidants (Ashraf and Harris, 2004; Ashraf and Foolad, 2007; Munns and Tester, 2008). In order to exist and produce optimum yield, crop plants have to adjust their metabolism according to deficiency or overproduction of these compounds under saline conditions. However, as plants often face deficiency of one
or more of these compounds while growing under saline conditions, so researchers suggest exogenous application of these compounds including inorganic salts, plant growth regulators, osmoprotectants and compatible solutes (Ashraf and Foolad, 2007; Ashraf et al., 2008). For instance, foliar application of glycinebetaine and glycinebetaine containing sugarbeet extract helped in improving growth, biomass production and yield in okra (Habib et al., 2012) and Solanum melongena (Abbas et al., 2010) by increasing rate of photosynthesis, stomatal regulation and substomatal CO2 under saline conditions. While working with different wheat cultivars Ashraf et al., (2002) reported improved vegetative growth with exogenous application of gibberellic acid (GA3) under saline conditions however grain yield decreased slightly. Likewise, foliar application of brassinosteroids helped in mitigating the adverse effects of salt stress in wheat by improving growth, however, grain yield was not improved (Shahbaz et al., 2008). In another study, Ali et al. (2008) reported increased growth and grain yield production in wheat with brassinosteroid application under both control and saline conditions. Foliar application of ascorbic acid, a potential antioxidant compound helped in reducing the adverse effects of salt stress on Brassica napus (Dolatabadian et al., 2008), and wheat (Athar et al., 2008; 2009) by enhancing antioxidant activity and regulating nutrient status in plants. As mineral nutrients also have a role in increasing plant salt tolerance, and these nutrients become deficient under saline conditions so foliar application of these mineral nutrients can help in improving salt tolerance by maintaining endogenous nutrient balance (Grattan and Grieve, 1999). Enhanced growth was observed in salt stressed maize plants due to regulation of minerals uptake by the exogenous supplementation of urea (Irshad et al., 2002). However, among various inorganic mineral nutrients, potassium salt has been used extensively as foliar application to improve crop salt tolerance ability (Ashraf et al., 2008). For instance, ion homeostasis and photosynthetic activity were found to be enhanced in sunflower with foliar spray of different inorganic potassium salts under saline conditions (Akram et al., 2007). Similarly, in Legenaria spp. foliar application of KNO3 increased growth and K content under saline conditions (Ahmad and Jabeen, 2005).

Exogenous supplementation of nitric oxide through foliar spray of sodium nitroprusside (nitric oxide donor) helped in counteracting NaCl-induced oxidative stress in chickpea plants by increasing antioxidant activity and reducing membrane lipid peroxidation (Sheokand et al., 2010). Similarly, nitric oxide also helped in reducing Cd toxicity in chickpea plants when
sodium nitroprusside (nitric oxide donor) applied through foliar spray by regulating antioxidant defense mechanism (Kumari et al., 2010). Foliar-applied sodium nitroprusside helped in alleviating drought induced water loss and ion leakage by reducing transpiration rate in rice leaves (Xiong et al., 2012). In Arabidopsis thaliana, foliar-applied nitric oxide (sodium nitroprusside) modulated ozone-induced cell death, gene expression and hormone biosynthesis (Ahlfors et al., 2008). From the above-discussed reports, it is well evident that like various other compatible solutes, plant growth regulators, inorganic salts and antioxidants, exogenous application of nitric oxide either as pre-sowing seed treatment or foliar application can be beneficial for plants subjected to a variety of abiotic stresses including salinity.

**Nitric oxide**

Nitric oxide is a small redox-active molecule which plays a key role in various physiological and developmental processes in plants (Lin et al., 2012). It is electro-neutral, water soluble and lipophilic gaseous molecule (Krasylenko et al., 2010), which acts as a signaling molecule both in plants and animals (Crawford & Guo, 2005; Besson-Bard et al., 2008). In higher plants, nitric oxide has also been emerged as an important regulator of leaf cell death and senescence process (Guo and Crawford, 2005; Zago et al., 2006). Nitric oxide also has a regulatory role in leaf expansion, flowering, germination, stomatal closure, lateral root development and both biotic and abiotic stress tolerance in plants (Neill et al., 2002; He et al., 2004; Zhang et al., 2006; Hong et al., 2008; Wilson et al., 2008; Ashraf, 2009; Leitner et al., 2009; Habib et al., 2010), ultimately enhancing plant stress tolerance ability especially against drought (Garcia-Mata and Lamattina, 2002) and salt stress (Zhao et al., 2007).

**Biosynthesis of nitric oxide**

many potential sources for nitric oxide generation have been reported in plants (Gupta et al., 2010), including nitrate reductase (Stohr and Stremlau, 2006; Zhang et al., 2006; Stoimenova et al., 2007; Seligman et al., 2008; Zheng et al., 2009), NOS (nitric oxide synthase) like activity in plants (Delledonne et al., 1998; Besson-Bard et al., 2008; Zheng et al., 2009), and hydroxylamine/ polyamine-mediated nitric oxide generation (Rumer et al., 2009). One of the sources for nitric oxide generation in plants is from the activity of nitrate reductase which catalyze in vivo and in vitro reduction of nitrite to nitric oxide. Nitrite reductase also has a minor contribution in nitric oxide production (Besson-Bard et al., 2009). Nitrate reductase
catalyzes nitric oxide production from NO₂ by using NAD(P)H (Kaiser et al., 2002). Molybdenum is involved as a cofactor in nitric oxide generation from NO₂. This nitric oxide generation depends upon the quantity of nitrate or nitrite in a tissue (Rockel et al., 2002; Kaiser and Huber 2001; Yamasaki et al., 1999). Nitrate reductase dependent nitric oxide production is also reported in many plant species such as in Arabidopsis (Desikan et al., 2002), cucumber (de la Haba et al., 2001), tobacco (Planchet et al., 2005, 2006), wheat (Xu and Zhao et al., 2003), and maize (Rockel et al., 2002).

Identification of Arabidopsis nitric oxide synthase (AtNOS1) protein was a notable advancement in studies of enzymatic nitric oxide generation in plants. Calmodulin, Ca²⁺, and NADPH are cofactors for this enzyme and it requires L-arginine as a substrate (Guo et al., 2003; Arasimowicz, et al., 2007). Archetypal NOS enzymes found in green algae, Ostrococcus tauri (Foresi et al., 2010), and in animals also (Palmer et al., 1988) Properties of NO-synthases are tissue specific as their production is localized in particular organelles or compartments, e.g., mitochondria, nuclei or peroxisomes in various plant species (Leitner et al., 2009). Expression of atnos1 gene is required for in vivo nitric oxide production and for NO involvement in lipopolysaccharides and ABA activated signaling pathways (Guo et al., 2003).

**Physio-biochemical roles of nitric oxide in plants**

As discussed earlier, nitric oxide regulates diverse physio-biochemical and developmental processes at different growth stages of plant life cycle. These include enhancement of seed germination (Beligni and Lamattina, 2000; Habib et al., 2010), maturation and senescence in plants (Leshem et al., 1998; García-Mata and Lamattina, 2001; Guo and Crawford, 2005), regulation of growth and development (Durner and Klessig, 1999), suppressed floral transition (He et al., 2004), regulation of movements of stomata (García-Mata and Lamattina, 2001; Neill et al., 2002; Desikan et al., 2004; Guo and Crawford, 2005; Bright et al., 2006), and greening mediated by light (Zhang et al., 2006a). Nitric oxide induced regulation of various physiological processes are not only affected by gene transcription in plants (Huang et al., 2002; Polverari et al., 2003; Ashraf, 2004; Parani et al., 2004; Shoulars et al., 2008), but also through interplay with other small bio-molecules. For instance, nitric oxide helps protect plant cells from damage by scavenging H₂O₂ (Beligni et al., 2002). It has also been reported that nitric oxide and H₂O₂ have combined effect in controlling cell death.
associated with the hypersensitive response due to plant pathogen interaction (Delledonne et al., 2001). According to Lin et al. (2012) nitric oxide is an important mediator of H₂O₂ induced leaf cell death in rice.

Due to its role as an endogenous gaseous plant growth regulator, nitric oxide was considered as a phytohormone (Leshem, 2000), as well as a non-traditional plant growth regulator (Beligni and Lamattina, 2001b). Nitric oxide is itself a highly reactive nitrogen species and its effectiveness on different types of cells has been proved either to be protective or toxic, depending on dose applied, the plant species, and the stages of growth at which it is applied (Zhao et al., 2007). Nitric oxide acts as a signal that controls the timing of flowering at the genetic level (He et al., 2004). It was also reported that nitric oxide donors inhibits the expression of the genes constans and gigantea while increases the expression of the flowering locus C gene (Arasimowicz et al., 2007).

Nitric oxide has an ability to reduce seed dormancy in different plants species such as Arabidopsis (Batak et al., 2002, 2006), lettuce (Beligni and Lamattina, 2000), and barley (Bethke et al., 2004). It also been shown to improve the seedling growth in pea (Leshem and Haramaty, 1996) and rice (Habib et al., 2010). The regulatory role of nitric oxide in root formation has also been reported as its treatment improved root growth in maize comparable to that by indole acetic acid (Gouvêa et al., 1997). Similarly, nitric oxide-mediated responses stimulate adventitours root formation in cucumber (Pagnussat et al., 2002). Nitric oxide is an important molecule operating downstream of auxin through a linear signaling pathway during root growth and development (Correa-Aragunde et al., 2007). For example, in pea leaves decreased ethylene content was observed with exogenous application of nitric oxide under senescence promoting conditions due to NO-induced inhibition of ethylene biosynthesis (Leshem et al., 1998; Leshem 2000; Leshem and Haramaty, 1996). However, in Arabidopsis, nitric oxide treatment increased ethylene level (Magalhaes et al., 2000).

Nitric oxide also improved stomatal conductance, chlorophyll content, photosynthesis, and transpiration rate in cucumber seedlings (Fan et al., 2007). Nitric oxide has been found to increase chlorophyll content in lettuce, potato and Arabidopsis (Beligni and Lamattina, 2000). Besides plant developmental processes, nitric oxide can also be involved in the regulation of plant defense responses to biotic and abiotic stresses (Wang et al., 2011).

**Role of nitric oxide in abiotic stress tolerance**
Nitric oxide was found to be involved in both abiotic and biotic stress responses, such as water deficit, salinity, and heat stresses, apoptosis and pathogen resistance (Delledonne et al., 1998; Durner and Klessig, 1999; García-Mata and Lamattina, 2002; Neill et al., 2003; Zhao et al., 2004; Zhang et al., 2006b). For example nitric oxide treatment was reported to increase height, dry weight and activities of peroxidase, catalase, and superoxide dismutase, and decreased MDA content and ion leakage ratio in maize plants under waterlogging stress (Wang et al., 2011). It has also been reported that nitric oxide can ameliorate UV-induced damage by lowering H₂O₂ content and ion leakage and enhancing the activities of key scavenging enzymes (Shi et al., 2005). Pretreatment with nitric oxide helped in mitigating the deleterious effects of UV-A and UV-B irradiation in Solanum tuberosum tubers and Zea mays seedlings and activated the nitric oxide synthase in cytosol and microsomes (An et al., 2005).

Possible involvement of nitric oxide in tolerating heavy metal stress is an adaptive response in plants (Corpas et al., 2007). Nitric oxide decreased heavy metal-induced oxidative damage in many plants (Yu et al., 2005; Singh et al., 2008; Rodríguez-Serrano et al., 2009). For instance, increased endogenous nitric oxide generation was observed in the roots of Brassica juncea and Pisum sativum in response to copper, cadmium and zinc stress (Groppa et al., 2008). Nitric oxide application reduced auxin degradation by inhibiting the activity of IAA oxidase and also enhanced the uptake of K⁺ and Ca²⁺ in Medicago truncatula under cadmium stress (Xu et al., 2010). The protective role of nitric oxide against cadmium toxicity has also been observed in sunflower leaves (Laspinas et al. 2005), rice leaves (Hsu and Kao 2004), and wheat roots (Singh et al. 2008). Nitric oxide is considered to reduce cadmium toxicity by maintaining the auxin equilibrium, enhancing ion absorption and reducing oxidative damage (Xu et al., 2010). Furthermore, exogenous nitric oxide supplementation protects plants from membrane damage due to peroxidation of lipids and enhances the activity of membrane transporters which help in excluding excess or toxic ions of heavy metals from root cells (Beligni and Lamattina 2002; Singh et al. 2008). Mechanical wounding is another type of abiotic stress in plants. Nitric oxide and H₂O₂ generation increase in plants after mechanical wounding (Delledonne et al., 1998), as in epidermal cells of T. cuspidata leaves, nitric oxide concentration was increased in response to mechanical stress (Cheng et al., 2009).
In wheat plants, increased nitric oxide synthesizing activity was observed under water deficit conditions (Zhao et al., 2008). Nitric oxide also promotes the germination of wheat seeds under osmotic stress by improving antioxidant capacity (Zhang et al., 2005). Nitric oxide and polyethylene glycol (PEG) treated suspension culture of *P. communis* attributed to decreased ion leakage, reduced H$_2$O$_2$ and superoxide anion content and activation of antioxidant defense system (Zhao et al., 2008). ABA-induced stomatal closure is somewhat regulated by nitric oxide together with H$_2$O$_2$ in various plant species (Neill et al., 2008). This regulatory role of nitric oxide is possibly mediated by change in Cl$^-$ and K$^+$ channel activity in the guard cells due to S-nitrosylation of respected proteins (Zhao et al., 2008). Independent pathways are involved in either nitric oxide or ABA-induced stomatal closure, though NO and ABA can act synergistically (Neill et al., 2008). Under water deficit conditions, H$_2$O$_2$ and nitric oxide are involved in regulation of signaling cascade leading to ABA-induced stomatal closure due to its hyperproduction in maize mesophyll cells (Neill et al., 2008). It was also reported that in the presence of ROS and NO donors, ABA synthesis in wheat roots was enhanced under water deficit conditions which indicates the synergism of nitric oxide and ROS action (Zhao et al., 2008). ABA impact on guard and non-guard cells of epidermis was ascribed to increased endogenous nitric oxide generation in *Arabidopsis*, *Pissum sativum* and *Vicia faba* (Neill et al., 2008). Although, subsequent stages of this signaling cascade remain to be elucidated; however, exogenous nitric oxide supplementation can participate in the process of stomatal closure through the Ca$^{2+}$ dependent pathway (Neill et al., 2008).

Another important property of nitric oxide in plants is its cryoprotective role. Exogenous supplementation of nitric oxide donor can improve low temperature stress tolerance in plants (Neill et al., 2008). Nitric oxide generation was enhanced in response to high temperature in plants, whereas exogenous nitric oxide supplementation improved cold resistance in *Zea mays*, *S. lycopersicum* and *Triticum aestivum* (Neill et al., 2008; Song et al., 2008). In *P. communis* calluses ABA-induced thermotolerance was believed to be mediated by nitric oxide (Song et al., 2008). Treatment with truncated hemoglobin (trHb) and isoprene, the nitric oxide scavengers, improved germination of *Arabidopsis* seeds at increased temperature due to elimination of excessive nitric oxide produced as a result of high temperature stress (Hossain et al., 2006). However, in another study it was reported that exogenous nitric oxide supplementation during heat shock in *Phaseolus radiatus* improved stability of chlorophyll a
fluorescence parameters, H$_2$O$_2$ content, membrane stability and activity of antioxidant defense system at a level comparable to those in the non-stressed conditions (Song et al., 2008).

**Role of nitric oxide in salt stress tolerance**

As discussed earlier that salinity imposes both osmotic stress and ion toxicity in plants, leading to various other secondary stresses such as nutritional imbalance and oxidative stress, which results in decelerating of growth and developmental processes ultimately affecting yield attributes in crop plants. Therefore, salt tolerance in plants is a multifaceted trait involving a network of different signaling networks and a cross-talk among different sensors, biological molecules and signal transduction (Wang et al., 2003; Zhu et al., 2003). Nitric oxide has been widely recognized as an important endogenous signaling molecule that regulates multiple defense responses to both abiotic and biotic stress (Neill et al., 2003), including salinity (Zhao et al., 2007). There are a number of reports which demonstrate that exogenous supplementation of nitric oxide in various plant systems, possibly act by interplaying with other signals to enhance salt tolerance ability (Uchida et al., 2002; Zhao et al., 2004; Zhang et al., 2006; Liu et al., 2007; Zhao et al., 2007; Tanou et al., 2009a; Tanou et al., 2009b; Wang et al., 2009). Moreover, it has also been reported that exogenous nitric oxide supplementation can improve salinity tolerance in not only in treated tissues but also in distant non-treated tissues, which indicates that nitric oxide triggers systemic biological responses during salinity stress (Uchida et al., 2002; Tanou et al., 2009a; Tanou et al., 2009a).

Pre-sowing seed treatment with sodium nitroprusside (nitric oxide donor) enhanced seed germination and early seedling growth in different rice cultivars under saline conditions (Habib et al., 2010). Exogenous nitric oxide treatment through sodium nitroprusside supplementation also helped in alleviating the adverse effects of salt-induced oxidative damage in seedlings of lupin (Kopyra and Gwozdz, 2003), rice (Uchida et al., 2002, Habib et al., 2010) and also in cucumber (Fan et al., 2007; Yu-qing et al., 2007). It also improved seedling growth (Song et al., 2009) and dry matter accumulation in Kodetzkya virginica and maize seedlings under salt stress (Zhang et al., 2007b; Guo et al., 2009). Nitric oxide decreases ROS production rate, permeability of membranes, malondialdehyde (MDA) content, intracellular CO$_2$ concentration and H$_2$O$_2$ content under saline conditions by
inducing excessive ROS scavenging through increased activity of enzymatic antioxidants such as peroxidises (POD), CAT, and ascorbate peroxidise (APX), and increased accumulation of proline (Kopyra and Gwozdz, 2003; Yu-quing et al., 2007; Shi et al., 2007; Zheng et al., 2009; Guo et al., 2009).

Furthermore, nitric oxide has also been reported to participate in improving photosynthetic rate by inducing and protecting photosynthetic pigments content under saline conditions (Ruan et al., 2002; Fan et al., 2007; Habib et al., 2010), and also in synthesis of ATP and two respiratory electron transport systems in mitochondria of plant cells (Yamasaki et al., 2001; Zottini et al., 2002), wherein it is involved in modulating the amount of ROS generation and also in inducing antioxidant defense system in plants under saline conditions (Zheng et al., 2009). According to Zeng et al. (2011) exogenous application of nitric oxide markedly enhanced the POD, SOD and APX activity, while limited the MDA production and free proline content in Brassica juncea seedlings under NaCl stress. Similarly, Chang-li et al. (2011) also reported that nitric oxide could regulate the activity of antioxidant enzymes thereby improving salt stress tolerance by eliminating ROS in the seedlings of Brassica campestris. Nitric oxide also mediates brassinosteroid-Induced ABA production which is believed to be involved in oxidative stress tolerance in maize leaves (Zhang et al., 2011). Nitric oxide treatment has also been reported in maintaining K+/Na+ ratio by regulating H2O2 dependant increase in plasma membrane H+-ATPase activity under saline conditions in calluses of Populus euphratica (Zhang et al., 2007) and Phragmites communis (Zhao et al., 2004), which ultimately helped to improve salt tolerance ability.

Rice

Among cereals, rice (Oryza sativa L.) contributes highest share to human caloric intake across the world. Rice is generally cultivated as an annual crop in semi-aquatic conditions. However, in tropical areas it can also survive as a perennial plant and can produce ratoon crop for almost up to 30 years (http://en.wikipedia.org/wiki/Rice, International Rice Research Institute The Rice Plant and How it Grows).

Rice production in Pakistan and worldwide
Maize, wheat and rice are considered most important cereal grains as they are major staple foods in most parts of the world. Among these cereals, rice is the most important crop in Asia, because more than half of the world population consumes rice as food (Ma et al., 2007). According to FAO (2012) estimate, world rice production was 729 million tones. In Pakistan, rice is the second major cereal crop after wheat, with 6160 thousand tons total production and 2.39 t ha\(^{-1}\) average paddy yield (Economic Survey of Pakistan, 2012). Rice growing area in Pakistan comprises 2.57 Mha of land (Economic Survey of Pakistan, 2012).

**Rice and salt stress**

Salt stress not only inhibits growth and productivity of crop plants (Ashraf, 2004; Ashraf and Foolad, 2007; Ashraf, 2009) but in severe cases it can also lead to partial or even complete mortality (Munns and Tester 2008), especially in salt sensitive crop plants. Rice is generally considered a salt sensitive plant especially during seedling and reproductive stages (Pearson and Ayers 1960). Although considerable progress has been made to understand the physiological and biochemical bases of salt tolerance in rice and in developing salt tolerant rice cultivars (Ismail et al. 2007; Ashraf, 2009; Thomson et al. 2010), improvement in salt tolerance ability in rice without compromising grain quality is still a challenge for plant biologists.

Salt-induced ionic effect appears in rice within 24 h of NaCl treatment which results in excessive accumulation of Na\(^+\) and Cl\(^-\) in leaf tissues and also affects dry weight (Roshandel and Flowers, 2009). A marked inhibition was observed in seed germination index, fresh and dry seedling weights in four different rice cultivars under saline conditions (Habib et al., 2010). Salt-induced injury in rice is mostly due to excessive accumulation of Na\(^+\) as compared to that of Cl\(^-\) unless excessive Cl\(^-\) accumulation reaches to very high concentration in plant tissues (Clarkson and Hanson 1980; Munns and Tester 2008). Higher Na\(^+\) concentrations in soil solution alter the uptake of other essential nutrients particularly K\(^+\) leading to ion toxicity (Loupassaki et al., 2002). Comparatively salt tolerant rice cultivars have been shown to maintain high Ca\(^{2+}\)/Na\(^+\) and K\(^+\)/Na\(^+\) ratios, and differential Na\(^+\) uptake and accumulation was also observed among different rice genotypes (Fageria, 2001; Loupassaki et al. 2002; Ismail et al. 2007; Munns and Tester 2008). Considerable variation in the uptake of these inorganic elements (Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\)) is substantial in determining salt tolerant and sensitive cultivars (Maas and Grieve, 1987; Hussain et al.,
2008) becomes salt tolerant genotypes tend to maintain balanced ratios of these essential nutrients in plant tissues (Fageria, 2001; Munns and Tester, 2008). Salt stress causes considerable alterations in various physiological and biochemical processes in rice. Deivanai et al. (2011) reported reduced shoot and root length, and decreased chlorophyll and protein content in salt stressed rice plants. Relative water content (RWC) decreased while proline and flavonoids content increased in different rice varieties under salt stress (Chutipaijit et al., 2009). Salt stress also affected the chlorophyll fluorescence parameters in rice, as electron transport rate decreased while non-photochemical quenching increased (Moradi and Ismail, 2007). Gas exchange attributes including photosynthetic rate and stomatal conductance also decreased in different rice cultivars under saline conditions (Moradi and Ismail, 2007; Naheed et al., 2007). According to Moradi and Ismail (2007) lipid oxidation increased in different rice cultivars under saline conditions, however, less increase in lipid peroxidation was observed in relatively salt tolerant rice cultivars. Activity of different enzymatic antioxidants has also been reported to increase in response to salt-induced oxidative stress in rice (Lin and Kao, 2000; Moradi and Ismail, 2007; Nounjan et al., 2012), including catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), and ascorbate peroxidase (APX) (Nounjan et al., 2012). Activity of GR has been reported to increase in rice leaves (Lee et al., 2001) and shoot culture of rice (Fadzilla et al., 1997) under salt stress. Increased H$_2$O$_2$ accumulation has also been reported in rice under saline conditions (Tsai et al., 2005; Nounjan et al., 2012). Lee et al. (2001) suggested that increased H$_2$O$_2$ accumulation in rice leaves under NaCl stress was due to altered activity of enzymatic antioxidants. However, according to Tsai et al. (2005) NaCl-induced increased accumulation of H$_2$O$_2$ in rice roots is somewhat attributed to activation of NADPH oxidase. Salt stress affects the growth of rice at different growth stages starting from germination up to maturity however, extent of this salt induced damage varies at different growth stages (Alam et al., 2000). Some researchers have reported that rice is relatively salt tolerant at tillering and grain filling stages (Pearson and Ayers, 1960; Yeo and Flowers, 1990). For example before panicle emergence, salinity reduced the number of tillers. It also affected panicle weight and number of spikelets in rice (Zeng et al., 2003).
**Chapter 3**

**Material and methods**

Two independent experiments were conducted to study different growth, physiological and biochemical attributes in salt stressed rice (*Oryza sativa* L.) plants by exogenous application of nitric oxide. These experiments were conducted in the net-house of the Botanical Garden of the University of Agriculture, Faisalabad. Four rice cultivars were used in these experiments. Of these four cultivars seeds of two fine rice cultivars (Shaheen Basmati and Basmati PB-95) were obtained from the Soil Salinity Research Institute, Pindi Bhatian, and of two coarse rice cultivars (KS-282 and IRRI-6) from the Rice Research Institute Kala Shah Kaku, Pakistan.

**Experiment No: 1**

**Optimization for effective nitric oxide concentrations**

It is now evident that salt tolerance varies with the change in plant developmental stages (Foolad and Lin, 1997; Cuartero *et al*., 2006; Munns and Tester, 2008) and the effective role of nitric oxide in enhancing plant stress tolerance depends upon its concentration. As the higher levels of NO are toxic for plants and have been reported to reduce seed germination and seedling growth (Zhang *et al*., 2003; Kopyra and Gwozdz, 2003), and also cause membrane damage and fragmentation of DNA (Yamasaki, 2000), so a pilot experiment was aimed to optimize effective nitric oxide concentrations and to study the effect of various nitric oxide levels on seed germination and early seedling growth in salt stressed rice plants. Seeds of all four rice cultivars were surface-sterilized with 0.1% mercuric chloride solution for one minute and soaked in varying levels (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM) of sodium nitroprusside (nitric oxide donor) solutions for 16 h as pre-sowing seed treatment. The control seeds were soaked in distilled water only. Ten seeds of uniform size from each cultivar were sown in small plastic pots containing 0.5 kg sand. Two levels of NaCl (0 and 80 mM) were prepared in Hoagland’s nutrient solution and 200 ml of each treatment solution were applied to each pot before sowing. The experiment was arranged in a completely randomized design with four replicates.

The pots were placed in a net-house under natural conditions (*PPFD* 1275 μmol m$^{-2}$ s$^{-1}$; average day and night temperatures 36±3°C and 27±2°C, respectively; relative humidity
44.1%, and day-length 13.7 h). The seeds were allowed to germinate under these conditions and number of seeds germinated counted every-day until five days. A seed was considered germinated when both plumule and radical had emerged ≥ 0.5 cm. The data so obtained was used for the calculation of seed germination percentage, germination index and time to achieve 50% germination (in days). Seedlings were harvested after 12 days of sowing, washed with distilled water and fresh weight of plumule recorded. The seedlings were wrapped in a paper bag, dried in an oven at 65°C to a constant dry weight, and dry weights recorded. Data of various seed germination and seedling growth parameters were analyzed statistically by analysis of variance technique (Steel & Torrie, 1986) based on a two-factor factorial design using the Costat computer package (CoHort Software, 2003, Monterey, California).

**Experiment No: 2**

**Exogenous application of effective nitric oxide levels**

It is now well evident that effectiveness of exogenously applied compatible solutes, osmoprotectants and plant growth regulators in ameliorating the adverse effects of salt stress on growth of crop plants not only depends upon concentration and plant developmental stage at which applied, but also on the mode of application (Agboma et al., 1997; Heuer, 2003; Ashraf and Foolad, 2007). The effective levels of nitric oxide optimized in the 1st experiment were applied exogenously on all four rice cultivars under salt stress. Different growth, physiological, biochemical and yield attributes were recorded during the course of experiments. These experiments were also conducted in the nethouse of Botanical Garden of the University of Agriculture, Faisalabad. Two different modes were adopted for exogenous supplementation of nitric oxide including pre-sowing seed treatment and foliar application of nitric oxide. These experiments were repeated twice in two consecutive years (summer 2010 and 2011). During experimentation, average temperature, relative humidity and day-length recorded are presented in the following figure:
Foliar spray of effective nitric oxide levels

For exogenous application of nitric oxide on salt stressed rice plants as foliar spray, a total of 96 plastic tubs (45×66×23 cm) were used, each filled with equal weight of sandy clay and loam soil. Rice seedlings were first grown in a nursery. For growing nursery of rice seedlings, seeds of all four rice cultivars (Shaheen Basmati, Basmati PB-95, KS-282 and IRRI-6) were hand sown in plastic tubs. Thirty-day old seedlings were transplanted in 96 plastic tubs. After establishment of these rice seedlings (10 days after transplantation) two salt treatments, control and 80 mM of NaCl, were applied with a gradual increase in salt level. Sodium chloride was maintained at 80 mM. After imposition of salt stress, three different levels (0, 0.1, 0.2 mM) of sodium nitroprusside (nitric oxide donor) were applied. Two weeks after the nitric oxide treatment, two plants per replicate from each treatment were harvested and different growth, physiological and biochemical attributes were determined.

Pre-sowing seed treatment with effective nitric oxide levels

For pre-sowing seed treatment with sodium nitroprusside (nitric oxide donor), seeds of all four rice cultivars were surface-sterilized with 0.1% mercuric chloride for one minute. After sterilization, the seeds were soaked in three different levels (0, 0.1, 0.2 mM) of sodium nitroprusside for 16 h. After priming, these seeds were hand-sown in plastic tubs for growing a nursery of rice seedlings. Thirty-day old seedlings were transplanted in plastic tubs. After

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**Figure:** Average temperature (°C), relative humidity (%) and average day length during June to September, 2011.
Figure: General overview of the experiment No. 2 at seedling stage
Figure: Influence of different nitric oxide levels on salt stressed and non-stressed rice plants (of Shaheen Basmati) when applied as pre-sowing seed treatment with sodium nitroprusside (a nitric oxide donor).
establishment of these rice seedlings (10 days after transplantation) two salt treatments (control and 80 mM) of NaCl were applied with a gradual increase in salt level. Sodium chloride was maintained at 80 mM. Two weeks after the salt stress treatment, two plants per replicate from each treatment were harvested and different growth, physiological and biochemical attributes were determined.

**Soil analysis**

Analysis of a sample of soil used in this experiment was carried out in the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad. For this analysis, standard soil analysis protocols were followed and data were recorded which is presented below.

**Physical properties of the soil used in the experiment**

<table>
<thead>
<tr>
<th>Physical property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>71%</td>
</tr>
<tr>
<td>Clay</td>
<td>19%</td>
</tr>
<tr>
<td>Silt</td>
<td>10%</td>
</tr>
<tr>
<td>Textural classes</td>
<td>Sandy clay loam</td>
</tr>
</tbody>
</table>

**Chemical properties of the soil used in the experiment**

<table>
<thead>
<tr>
<th>Chemical property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available P</td>
<td>8.7 ppm</td>
</tr>
<tr>
<td>CEC (Cation exchange capacity)</td>
<td>17.2 meq 100 g⁻¹</td>
</tr>
<tr>
<td>Organic matter</td>
<td>0.93%</td>
</tr>
<tr>
<td>Calcium carbonate (CaCO₃)</td>
<td>2.69%</td>
</tr>
<tr>
<td>Total N</td>
<td>0.71%</td>
</tr>
<tr>
<td>pH</td>
<td>7.80</td>
</tr>
<tr>
<td>ECe (Electrical conductivity of the soil extract)</td>
<td>2.51 dS.m⁻¹</td>
</tr>
<tr>
<td>SP (Saturation percentage)</td>
<td>33%</td>
</tr>
<tr>
<td>Soluble CO₃⁻</td>
<td>Traces</td>
</tr>
<tr>
<td>Soluble Ca²⁺ + Mg²⁺</td>
<td>13.90 meq L⁻¹</td>
</tr>
<tr>
<td>Soluble Cl⁻</td>
<td>8.48 meq L⁻¹</td>
</tr>
<tr>
<td>Soluble SO₄²⁻</td>
<td>1.98 meq L⁻¹</td>
</tr>
<tr>
<td>Soluble Na⁺</td>
<td>2.45 meq L⁻¹</td>
</tr>
<tr>
<td>Soluble HCO₃⁻</td>
<td>4.89 meq L⁻¹</td>
</tr>
<tr>
<td>SAR (Sodium adsorption ratio)</td>
<td>0.090 meq L⁻¹</td>
</tr>
</tbody>
</table>
Morphological Characters
Per replicate, two plants were collected (after 15 days in case of foliar nitric oxide addition) at the vegetative phase and data for shoot fresh weigh, root fresh weight and number of tillers per plant was recorded. The rice plants were then dried in an oven at 65°C for 72 h after which dry weights of both shoots and roots were measured. At the stage of maturity, different yield related attributes, such as total yield of grain per plant and weight of 100 grain were recorded.

Physiological Attributes
Water relation parameters
Water potential (Ψw) of leaf
At vegetative stage, a fully grown top second leaf was removed for estimating its water potential using a pressure chamber (between 6 a.m. to 8.30 a.m.) of Scholander type. And the leaf was frozen at -20°C in a freezer for determining Ψs from it after seven days of freezing.

Osmotic potential (Ψs) of leaf
After seven days the cell sap was extracted by thoroughly thawing the frozen leaf material and sap was separated by using a disposable syringe. An osmometer (Wescor 5500) was used for measuring Ψs from cell sap.

Turgor potential (Ψp) of leaf
was determined by subtracting the value of Ψs from Ψw:
Ψp = Ψw – Ψs

Relative water contents (RWC) of the leaf
From each replicate, a fully grown young leaf was taken and its weight was recorded immediately. Distilled H2O was used for 8 h long immersion of all leaf samples in it, so the leaf became turgid due to excess amount of water absorption on their surface and the weight of each leaf was again recorded. After that all leaf samples were dried at 70 °C in an oven and their dry weights were recorded. The RWC was figured out using the equation following:
Relative water content of leaf (%) = (Fresh weight of leaf - dry weight of leaf / leaf turgid weight-dry weight) × 100

Gas exchange attributes
Stomatal conductance \((g_s)\), sub-stomatal CO\(_2\) concentration \((C_i)\), \(A, E\) from the top 3rd leaf of each plant were recorded by using IRGA (infrared gas analyzer) (LCA-4 was its model; and manufacturer by Analytical Development Company, Hoddesdon, England). All these measurements were noted between 13.00-14.00 h. In the leaf chamber the rate of molar gas flow was 248 μmol s\(^{-1}\), and 352 μmol mol\(^{-1}\) ambient concentration of CO\(_2\), the variation in leaf chamber temperature was between 36.1 to 40.4 °C, 98.01 kPa was ambient pressure \((P)\), 221.06 mol m\(^{-2}\) s\(^{-1}\) was flow of air (molar)/area of leaf, 380 mL/min was volume gas flow rate in leaf chamber and 1050 μmol m\(^{-2}\) s\(^{-1}\) was maximum PAR rate.

**Chlorophyll Fluorescence**

Light exclusion clips were attached on the surface of the light adapted leaves, so they were adapted to darkness in the presence of sunlight for 30 min. A chlorophyll fluorometer (Model, OSSP Optisciences, Inc. Winn Avenue Hudson, USA) which was portable with multimode properties was used in this study following Strasser *et al.* (1995). After 30 minutes period of dark adaptation, various attribute related to the activity of reaction centres at PSII were measured, minimum fluorescence \((F_0)\) was measured through a weak red light, which was not able to cause any significant variable fluorescence because of its sufficiently low strength \((< 0.1 \text{ μmol m}^{-2} \text{ s}^{-1})\).

The 0.8 s saturating pulse \((8000 \text{ μmol m}^{-2} \text{ s}^{-1})\) was used to measure the maximum fluorescence \((F_m)\) of leaves adapted to dark, with all closed reaction centers at PSII. The measurements of \(F_0\) were recorded by adjusting the frequency of measuring beam at 6000 Hz, while, all the values of \(F_m\) were measured with a measuring beam frequency switching to 20 kHz automatically,during saturating flash. The other attributes were also determined in the same range as mentioned above.

**Biochemical Attributes**

**Leaf chlorophyll contents**

For determining chlorophyll content (both “\(a\)’’ and “\(b\)’’”) the method of Arnon (1949) was followed. For chlorophyll extraction 0.1 g fresh leaf was put in acetone (80%) over-night at -4.0 °C. After centrifuging (at 10,000 x g for 5 min) the extract, the supernatant was used for measuring the absorbance through spectrophotometer (Hitachi-U2001, Tokyo, Japan) at 645, 663 and 480 nm. The formulas used for calculating the both (“\(a\)’’ and “\(b\)’’) chlorophyll contents are following:
Chl. \( a \) = \( \frac{V}{1000} \times W \times [12.7 \text{ (OD 663)} - 2.69 \text{ (OD 645)}] \)
Chl. \( b \) = \( \frac{V}{1000} \times W \times [22.9 \text{ (OD 645)} - 4.68 \text{ (OD 663)}] \)

\( W \) = gram weight of leaf tissue (fresh)
\( V \) = volume (mL) of extract

**Determination of antioxidant enzymes**

**Enzyme Extraction**

For the determining the activities of antioxidant enzymes, the fresh rice leaves (0.5 g) were ground using a mortar and pestle containing 5 mL of 50 mM of phosphate buffer (pH 7.8) cooled. Then the homogenate was filtered and centrifuged for 20 min at 15000 g with 4°C temperature. The enzymes assays were determined from obtained supernatant.

**Estimation of superoxide dismutase (SOD) activity**

The method of Giannopolitis and Ries (1977) was followed to measure the SOD activity by monitoring the decrease in photochemical reduction of NBT (nitroblue tetrazolium) at 560 nm.

To determine the SOD activity, 50 μl of the enzyme extract was added to a solution (total volume of solution including enzyme extract was 1 mL) containing 1.3 μM riboflavin, 75 nM EDTA, 50 μM NBT (NBT dissolved in ethanol), 13 mM methionine, 50 μl enzyme extract and 50 mM phosphate buffer (pH 7.8).

A chamber with aluminum coat inside was used to keep the reaction solution and a fluorescent lamp (of 30 W) was used for illumination. The reaction took place under fluorescent lamps which was turned off after 5 min. the photoreduction of NBT produced blue formazane which was measured at 560 nm through an increase in absorbance. A control was set by reaction mixture without leaf extract and was kept in light. A UV-visible spectrophotometer (IRMECO U2020) was used read the absorbance of the irradiated solution at 560 nm.

One SOD unit was equal to the quantity of enzyme required for 50% inhibition in NBT reduction rate at 560 nm as compared to tubes without plant extract.

**Estimation of the activities of Catalase (CAT) and Peroxidase (POD)**

Catalase (CAT) and POD activities were assayed with some modifications in Chance and Maehly (1955) method. The total 3 mL volume of CAT reaction mixture was containing 50 mM of phosphate buffer (pH 7.8), and 0.1 mL enzyme extract and 5.9 mM H2O2. Once the
leaf extract was added to the mixture, the reaction was initiated. H2O2 decomposition resulted in changes in absorbance which was read at 240 nm every 20 second. The unit (μmol of H2O2 decomposed per min) of CAT activity was shown as per mg of protein and it was defined as change in absorbance at 0.01 units/min. The method used for determining the POD activity was based upon oxidation of guaiacol. The final 3 mL volume of POD mixture contained 40 mM H2O2, 20 mM guaiacol, 50 mM phosphate buffer (pH 7.0), and 0.1 mL enzyme extract. At every 20 s interval the absorbance of the mixture (in which reaction occurs) was measured at 470 nm. The activity of POD was determined as 0.01 change in absorbance and the unit was shown as per mg of protein/min.

**Leaf total phenolics**

Leaf total phenolics content was measured according to the method described by Julkenen-Titto (1985). 0.05 g Leaf material was homogenized in acetone solution (80%), and it was centrifuged (at 10,000x g) for 10 min and supernatant was obtained. From the supernatant 100 μl of aliquot was mixed with 2.0 ml of distilled water and the 1 ml reagent of Folin–Ciocalteau’s phenol, then the mixture was added with 20% Na2CO3 solution (5 ml) and the final volume of 10 ml was achieved by adding H2O (distilled). After vigorous mixing, a UV-Visible spectrophotometer (model: IRMEOCO U2020; manufacturer: G mbH, Germany) was used for measuring OD at 750 nm.

**Leaf ascorbic acid contents**

10 ml of 6% TCA solution was used to homogenize the 0.25 g fresh leaf material in it for measuring ascorbic acid content following the procedure reported by Mukherjee and Choudhuri (1983). From this homogenized solution 4 ml of extract was separated and added with 2 ml solution of 2% dinitrophenyl hydrazine (medium was acidic in nature). Then the mixture was added with a drop of 10% solution of thiourea (prepared in 70% ethanol), and boiled in a water bath for 20 min. At 0 °C, the 5 ml of H2SO4 (80%) was added after cooling the solution at 25 °C. The absorbance at 530 nm was read, and varying standards of AsA were used to prepare the standard curve for measuring the AsA content in the solution containing leaf extract.

**Hydrogen peroxide**

Pre-chilled mortar was used for grinding 0.5 g leaf material added with 5 ml of 0.1% TCA (tri-chloroacetic acid). Then the centrifugation of the homogenate was carried out for 15 min
at the rate of 12,000 x g. Then 0.5 ml of supernatant was added with 1ml of KI and 0.5 ml buffer (pH 7.0) of potassium phosphate. The OD of the solution was noted at 390 nm after vortexing it and H2O2 was estimated following the method reported by Velikova *et al.* (2000).

**Malondialdehyde (MDA)**

Minor modifications were made to the method described by Carmak and Horst (1991) for determining MDA content. The 3 ml of TCA (trichloroacetic acid) solution (0.1%) was used for homogenizing 1 g leaf material in it. The centrifugation of the homogenate was carried out for 15 min at 20000 x g. and then 0.5 ml of separated supernatant was added with 3 ml of 0.5% TBA (thiobarbituric acid) prepared in 20% TCA. Then the mixture was heated at shaking water bath for 50 min at 95 °C. Then the tubes containing the mixture were cooled in a chilled water bath and the mixture was again centrifuged for 10 min at 10,000 x g. then the absorbance was measured from separated supernatant at 532 and 600 nm. The content of MDA was figured out as absorbance difference between 600 and 532 nm using the formula following:

\[ \Delta (A_{532 \text{ nm}} - A_{600 \text{ nm}})/1.56 \times 10^5 = \text{MDA level (nmol)} \]

And 156 mmol⁻¹cm⁻¹ was the coefficient of Absorption for MDA calculation.

**Proline**

0.1 g fresh leaf material was added with 5 ml of 5-sulfosalicylic acid (3%) and mixture was homogenized to determine the leaf proline content in it following Bates *et al.* (1973). glacial acetic acid and acid ninhydrin (2 ml of both) were added to the 100 μl aliquot from leaf mixture at 100 °C for 1 h, then the mixture was shifted to an ice bath to terminate the reaction. 1 ml of toluene (containing chromophore) was added to the reaction solution, and optical density (OD) was measured at 520 nm. A standard curve was used for determining the proline concentration which was calculated as following:

Proline μmole g⁻¹ fresh leaf material = (mL of toluene/115.5 x μg proline mL⁻¹)/sample (g)

**Mineral nutrients determination**

**Digestion mixture**
A chemical mixture was prepared for digestion of dried plant material, the solution was prepared by adding Se and LiSO₄·2H₂O as 0.42 g and 14 g by weight respectively to H₂O₂ (350 ml) and mixed well then 420 ml of sulphuric acid was added slowly by keeping it in an ice bath. And the digestion reagent was stored at 2°C.

**Digestion**

Ground and dried material (0.1 g) was taken in each of digestion tubes, added 1.5 mL of digestion mixture and incubated it overnight at room temperature. Then 0.1 mL of perchloric acid was poured down the sides of the digestion tube, heated for 30 minutes. The colorless digested material was obtained by continuous repeating of above mentioned step and 50 mL volume was maintained in a volumetric flask. After that of Na⁺, K⁺ and Ca²⁺ was determined from the filtrate obtained from the filtration of digestion mixture.

**Determination of Cl⁻:**

Chloride determination was carried out by a simple method of its extraction in water. In a digestion tube, dried and ground leaf material (0.1 g) was taken and 10 mL of distilled H₂O were added to it and left for overnight incubation at room temperature. Then the volume was reduced to half by continuously heating it at 80°C. Then the digestion tubes were cooled and added with distilled water to regain the original 10 ml volume. Then a chloride analyzer (with Model No. 926; manufactured by Sherwood Scientific Ltd., Cambridge, UK) was used to determine the Cl⁻ concentration in the extract.

**Determination of cations (K⁺ Na⁺ Ca²⁺)**

The concentration of Na⁺, K⁺ and Ca²⁺ was estimated using a Jenway PFP-7 flame spectrophotometer.

**Yield attributes**

At maturity, panicles were harvested by hand, and data for total grain yield per plant and 100 grain weight were recorded.

**Statistical analysis**
A computer software named CoSTAT V 6.3 (developed by Cohort software, Berkeley, California) was used to calculate the ANOVA (analysis of variance) for each attribute, in a completely randomized design (Steel and Torrie, 1986).

**Chapter 4**

**Results**

**1st germination experiment**

Imposition of salt stress significantly decreased the seed germination percentage in all four rice cultivars. Pre-sowing seed treatment with nitric oxide significantly improved the germination percentage of seeds of all four rice cultivars under control and saline conditions (Table 1, Fig 1). Of varying levels (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM) of nitric oxide, lower levels (0.05, 0.1 and 0.2 mM) were effective in improving germination percentage, particularly 0.1 and 0.2 mM were more effective. A non-significant effect was observed at 0.3 mM level of nitric oxide, while higher levels (0.4 and 0.5 mM) markedly inhibited the seed germination percentage in seeds of all four rice cultivars under saline conditions. Among rice cultivars Basmati PB-95 showed less germination percentage particularly under salt stress while all other cultivars remained indifferent.

Nitric oxide addition as pre sowing seed treatment also improved also increased the germination index significantly, under control and saline conditions (Table 1, Fig 1). Although, the lowest nitric oxide level (0.05 mM) used in this experiment showed a slight increase in seed germination index, however, the improvement was more prominent at 0.1 and 0.2 mM of nitric oxide in all four cultivars under both saline stressed and non-stressed conditions. A non-significant difference was observed in seed germination index with further increase in nitric oxide level (0.3 mM), while germination index was markedly decreased at higher levels (0.4 and 0.5 mM) of nitric oxide under both control and saline conditions. Of rice cultivars Shaheen Basmati and IRRI-6 showed a maximum increase in germination index with exogenous nitric oxide treatment under both control and saline conditions.

Time to achieve 50% seed germination was increased significantly due to salt induced inhibition of seed germination (Table 1, Fig 1). However, a marked decrease was observed in time to achieve 50% seed germination due to pre-sowing seed treatment with lower levels of nitric oxide (0.05, 0.1 and 0.2 mM) under both control and saline conditions. There was a
significant increasing trend in time to achieve 50% germination with further increase in nitric oxide levels 0.3, 0.4 and 0.5 mM respectively, under both saline and non-saline conditions. Of rice cultivars IRRI-6 and KS-282 performed better under control and saline conditions.

Imposition of salt stress significantly decreased the plumule fresh and dry weight of all four rice cultivars (Table 1-2, Fig 2). Pre-sowing seed treatment with lower level (0.05 mM) of nitric oxide improved both fresh and dry plumule weights in cultivar IRRI-6 and KS-282, however, with further increase in nitric oxide levels (0.1 and 0.2 mM), a marked increase was observed in plumule fresh and dry weights of all four rice cultivars under both control and saline conditions. A non-significant effect was observed in plumule weights (both fresh and dry) at 0.3 mM levels of nitric oxide, whereas higher levels (0.4 and 0.5 mM) of nitric oxide decreased the fresh and dry plumule weights in all four rice cultivars under control and saline conditions. Of rice cultivars, IRRI-6 and KS-282 showed higher plumule fresh and dry weights under both control and saline conditions.
Pre-Sowing seed treatment with nitric oxide

Imposition of NaCl stress markedly inhibited the shoot fresh and dry weights of all four rice cultivars (Table 3, Fig 3). Exogenous application of nitric oxide as pre–sowing seed treatment considerably increased the shoot fresh and dry weights in all four rice cultivars under both control and saline conditions. However, this increasing effect was more prominent at 0.1 mM of nitric oxide both under control and saline conditions. Of the cultivars, Shaheen Basmati and IRRI-6 had higher shoot fresh and dry weights under saline conditions, however under control conditions, Basmati PB-95 showed higher shoot fresh and dry weights. As with shoot biomass, root biomass (both fresh and dry weights) also decreased significantly in all four rice cultivars under saline conditions (Table 3, Fig 4). Pre-sowing seed treatment with nitric oxide had a significant increasing effect on root fresh and dry weights in all four rice cultivars under both control and saline conditions. Cvs. IRRI-6 and KS-282 showed higher root fresh and dry biomass and both nitric oxide levels (0.1, and 0.2 mM) were equally effective for improving the biomass of these two cultivars under saline conditions. Shoot length decreased significantly in all four rice cultivars due to imposition of NaCl stress (Table 4, Fig 5). Pre-sowing seed treatment with nitric oxide considerably alleviated to reduce the inhibitory effect of salt stress on shoot length in all four rice cultivars. Nitric oxide @ 0.1 mM was more effective for increasing shoot length under both control and saline conditions in all four rice cultivars.

Of the cultivars, cv Shaheen Basmati showed the maximum shoot length under saline conditions, however, maximum shoot length under control conditions was recorded in cv. Basmati PB-95. Number of tillers also decreased significantly under salt stress in all four rice cultivars (Table 4, Fig 5). Pre-sowing seed treatment with nitric oxide increased the number of tillers in all four rice cultivars under salt stress. Nitric oxide @ 0.2 mM was more effective in increasing the number of tillers under saline conditions. Of rice cultivars, the maximum number of tillers was observed in fine rice cultivars (Shaheen Basmati and Basmati PB-95) under control conditions, while in coarse rice cultivars (KS-282 and IRRI-6) under saline conditions. Excessive amount of NaCl in the growth medium significantly decreased chlorophyll ‘a’ and chlorophyll ‘b’ contents in all four rice cultivars (Table 5, Fig 6). Pre-
sowing seed treatment with nitric oxide significantly improved the both chlorophyll ‘a’ and chlorophyll ‘b’ contents in all four rice cultivars under both control and saline conditions. Among nitric oxide levels 0.1 mM was more effective in increasing both chlorophyll ‘a’ and chlorophyll ‘b’ contents under salt stress, however, both nitric oxide levels (0.1, and 0.2 mM) were equally effective under control conditions.
Similarly, total chlorophyll content was also decreased significantly in all four rice cultivars under NaCl stress (Table 5, Fig 7). Pre-sowing seed treatment with nitric oxide significantly improved total chlorophyll content in all four rice cultivars under both control and saline conditions. Of nitric oxide levels, 0.1 mM was more effective under saline conditions for all four rice cultivars, however, the cultivars remained almost indifferent with respect to chlorophyll ‘a’ and chlorophyll ‘b’ and total chlorophyll.

Of different gas exchange attributes, net CO₂ assimilation rate ($A$) (Table 6, Fig 7) and transpiration rate ($E$) (Table 6, Fig 8) of all four rice cultivars decreased significantly due to the addition of NaCl to the growth medium. Exogenous application of nitric oxide as pre-sowing seed treatment markedly increased the transpiration rate ($E$) and photosynthetic rate ($A$) of all four rice cultivars. The maximum increase in leaf $A$ and $E$ in all four rice cultivars under saline and non-saline conditions was observed by the application of 0.1 mM of nitric oxide, however, for leaf $E$ both nitric oxide levels were equally effective under non-saline conditions. Salt stress caused a marked decrease in $g_s$ (stomatal conductance) (Table 6, Fig 8) and sub-stomatal CO₂ concentration ($C_i$) (Table 6, Fig 9) in all rice cultivars. Pre-sowing seed treatment with nitric oxide significantly enhanced the intrinsic CO₂ concentration ($C_i$) and stomatal conductance in the stressed and non-stressed plants of all four rice cultivars. Both nitric oxide levels (0.1, and 0.2 mM) were equally effective in increasing $C_i$ and $g_s$ under saline condition, however, the cultivar difference was non-significant for these gas exchange attributes.

Imposition of salt stress significantly increased water use efficiency $A/E$, WUE of all four rice cultivars (Table 7, Fig 9). Pre-sowing seed treatment with nitric oxide showed a non-significant effect on the WUE of all four rice cultivars under control and NaCl conditions. Leaf $C_i/C_a$ ratio of all four rice cultivars decreased markedly due to addition of salt stress to the rooting medium (Table 7, Fig 10). Pre-sowing seed treatment with nitric oxide increased the leaf $C_i/C_a$ ratio of rice cultivars both under stress and non-stress conditions, and this increase was more prominent at 0.1 mM level of nitric oxide.

Leaf water (Table 7, Fig 10) and osmotic potential (Table 7, Fig 11) increased markedly due to imposition of NaCl stress in all four rice cultivars. A non-significant
difference was observed among all four rice cultivars with respect to $\Psi_w$ and $\Psi_s$. Pre-sowing seed treatment with nitric oxide decreased the values of water and osmotic potential in all four rice cultivars, under both control and saline conditions, and this decreasing trend was more prominent in the plants developed from seeds pre-treated with 0.1 mM of nitric oxide.
Leaf turgor potential (Table 8, Fig 11) and relative water content (Table 8, Fig 12) decreased significantly in plants of all four rice cultivars due to root-zone salinity. Pre-sowing seed treatment with nitric oxide significantly increased the leaf turgor potential and relative water content in all four rice cultivars under both control and saline conditions. Of rice cultivars, Shaheen basmati and IRRI-6 showed the maximum increase in leaf turgor potential and relative water content at 0.1 mM level of nitric oxide applied as pre-sowing seed treatment.

A significant change in different chlorophyll fluorescence parameters in all four rice cultivars was observed under salt stress. Salt stress caused a significant decrease in leaf $F_t$ (minimum fluorescence in light) of all four rice cultivars under saline conditions (Table 9, Fig. 13). Exogenously applied of nitric oxide as pre-sowing seed treatment significantly increased $F_t$ in all cultivars both under control and saline conditions. The maximum increase in $F_t$ value was observed at 0.2 mM of nitric oxide under NaCl stress. Minimum fluorescence in dark ($F_o$) also decreased due to root-zone salinity in all four rice cultivars (Table 9, Fig. 13). Nitric oxide application as pre-sowing seed treatment increased the $F_o$ value of salt stressed plants of all four rice cultivars. Of rice cultivars, Basmati PB-95 and Shaheen basmati showed higher increase in $F_o$, especially at 0.1 mM of nitric oxide under salt stress.

The values of maximum chlorophyll fluorescence ($F_m$), maximum fluorescence at steady state in dark adapted leaves ($F_{ms}$) (Table 9, Fig. 14), leaf fluorescence at steady state in light adapted leaf ($F_s$), and the maximum quantum yield of primary photochemical reaction in dark adapted leaves ($F_{m}/F_{v}$) also decreased significantly in all four rice cultivars under saline conditions (Table 10, Fig. 15). Nitric oxide application as pre-sowing seed treatment caused a significant increase in the values of $F_m$, $F_s$, $F_{ms}$ and $F_{v}/F_{m}$ in all four rice cultivars under both control and salt stressed conditions however, the maximum values for these parameters were observed at 0.2 mM of nitric oxide. Salt stress caused an increase in $ETR$ value (electron transport rate) of all four rice cultivars under saline conditions (Table 10, Fig. 16). Pre-sowing seed treatment with nitric oxide decreased the value of $ETR$ under both saline and non-saline conditions. Salt stress caused a significant decrease in the values of $Y$ (quantum yield of electron transport) (Table 10, Fig. 16) and $Q_p$ (leaf photochemical
fluorescence) in all rice cultivars (Table 11, Fig. 17). Seed priming with nitric oxide increased the values of both $Y$ and $QP$ in all four rice cultivars under NaCl stress.
Of cultivars, Basmati PB-95 and Shaheen basmati showed a decrease in $QP$ at 0.2 mM of nitric oxide under non-saline conditions. A significant increase in the values of non-photochemical quenching ($Q_n$) (Table 11, Fig. 17) and non-photochemical chlorophyll fluorescence quenching ($NPQ$) was observed due to root-zone salinity in all four rice cultivars (Table 11, Fig. 18). Nitric oxide application as pre-sowing seed treatment significantly decreased the values of both $Q_n$ and $NPQ$ under saline conditions, the maximum decrease in $NPQ$ being at 0.2 mM of nitric oxide in Shaheen basmati and basmati PB-95 under both saline and non-saline conditions, however, in cvs. KS-282 and IRRI-6, the minimum value of $NPQ$ was observed at 0.1 mM of nitric oxide under both control and NaCl stress, but for $NPQ$ the effect of both nitric oxide levels was more prominent under saline conditions.

Na$^+$ accumulation increased significantly in both shoots and roots of all four rice cultivars due to increased salinity in the root-zone (Table 12, Fig. 19). Pre-sowing seed treatment with nitric oxide decreased Na$^+$ accumulation in the shoots and roots of all four rice cultivars under saline conditions, and lower concentration (0.1 mM) of nitric oxide was more effective in this regard. However, under non-stress conditions the effect of nitric oxide was non-significant for both shoot and root Na$^+$ accumulation. Of rice cultivars, Shaheen basmati and IRRI-6 performed better (less Na$^+$ accumulation) then other cultivars under saline conditions. Like Na$^+$ content, the accumulation of Cl$^-$ also markedly increased in both shoots and roots of all rice cultivars under NaCl stress (Table 12, Fig. 20). Exogenous application of nitric oxide as pre-sowing seed treatment significantly reduced the Cl$^-$ accumulation in both shoots and roots of all four rice cultivars under saline conditions. Although lower (0.1 mM) concentration of nitric oxide was more effective in reducing Cl$^-$ accumulation, however, for cultivar Basmati PB-95, both levels (0.1 and 0.2 mM) of NO were equally effective particularly under salt stress.

Potassium (K$^+$) concentration in the roots and leaves of all four rice cultivars also decreased significantly due to salt stress (Table 13, Fig. 21). However, seed priming with nitric oxide improved the K$^+$ content in the shoot and root tissues of all four rice cultivars under saline conditions. Of rice cultivars Shaheen basmati and IRRI-6 showed higher
increase in shoot and root K⁺ content as compared to Bsmati PB-95 and KS-282 under both control and saline conditions. Both nitric oxide concentrations (0.1 and 0.2 mM) were effective in increasing K⁺ content in shoot and root tissues of all four rice cultivars under salt stress. However, this increasing effect was more prominent at 0.1 mM of nitric oxide under both control and saline conditions.
While pre-sowing seed treatment with 0.2 mM nitric oxide showed a non-significant effect on shoot and root K⁺ content in all four rice cultivars under non-stressed conditions.

Calcium (Ca²⁺) content in both shoots and roots decreased significantly when plants of all four rice cultivars were subjected to salt stress (Table 13, Fig. 22). However, the cultivars remained indifferent for Ca²⁺ accumulation in shoot and root tissues under both control and salt stressed conditions. Pre-sowing seed treatment with nitric oxide significantly increased the shoot and root Ca²⁺ content in all four rice cultivars under both NaCl stress and control conditions. Of nitric oxide levels, 0.1 mM was more effective than the other levels used, when applied as pre-sowing seed treatment.

The activities of superoxide dismutase (SOD) and peroxidase (POD) increased significantly in plants of all four rice cultivars under saline stress (Table 14, Fig. 23). Exogenous application of nitric oxide as pre-sowing seed treatment further increased the activity of both enzymes (SOD and POD) in all four rice cultivars under control and salt stress conditions, and a maximal increase was observed at 0.1 mM nitric oxide. Both nitric oxide levels (0.1 and 0.2 mM) were equally effective in increasing SOD activity in IRRI-6 under control and salt stress conditions. Of rice cultivars, Shaheen basmati showed a maximal increase in leaf SOD and POD activity under saline conditions. Like SOD and POD, the activity of leaf catalase (CAT) also increased markedly in all four rice cultivars under saline stress (Table 14, Fig. 24). A further increase in leaf CAT activity was observed due to nitric oxide addition as pre-sowing seed treatment in all four rice cultivars under both control and salt stress conditions. Both nitric oxide levels (0.1 and 0.2 mM) were equally effective in increasing the CAT activity under salt stress and non-stress conditions. Cultivars Shaheen basmati and IRRI-6 showed higher increase in leaf CAT activity under saline conditions.

A significant increase in leaf proline content was observed due to imposition of NaCl stress in all four rice cultivars (Table 15, Fig. 24). Seed priming with nitric oxide resulted in increased proline content under saline conditions and 0.1 mM nitric oxide was found more effective in this regard. Cultivar Shaheen Basmati showed higher increase in leaf proline
content under saline conditions, however, inter-cultivar difference was non-significant under control conditions.

Leaf malondialdehyde (MDA) content increased significantly in all four rice cultivars due to root zone salinity (Table 15, Fig. 25). Less increase in leaf MDA content was observed in cultivars Shaheen basmati and IRRI-6 under saline stress. Pre-sowing seed treatment with 0.1 mM nitric oxide showed a significant decrease in leaf MDA content under saline conditions, however, 0.2 mM nitric oxide showed a non-significant effect on leaf MDA content under both under control and NaCl stress conditions.

Leaf H$_2$O$_2$ content increased significantly in all four rice cultivars under saline regimes (Table 15, Fig. 25). Exogenous application of nitric oxide as pre-sowing seed treatment caused a marked decrease in leaf H$_2$O$_2$ content under saline conditions, and both nitric oxide levels (0.1 and 0.2 mM) were equally effective to this end. However, 0.2 mM nitric oxide showed a non-significant effect on leaf H$_2$O$_2$ content under control conditions. Inter-cultivar difference was non-significant with respect to leaf H$_2$O$_2$ content under control and salt stress stressed conditions.

A marked increase was observed in leaf ascorbic acid content of all four rice cultivars under saline conditions. Pre-sowing seed treatment with nitric oxide further increased the leaf ascorbic acid content under both control and saline conditions (Table 15, Fig. 26). Of nitric oxide levels, 0.2 mM was more effective in improving leaf ascorbic acid content under control conditions, however, both nitric oxide levels (0.1 and 0.2 mM) were equally effective under saline stress. Rice cultivars Shaheen Basmati and IRRI-6 showed higher leaf ascorbic acid content under both control and saline conditions.

Leaf total phenolics content was markedly decreased in all four rice cultivars under salt stress. Nitric oxide addition as pre-sowing seed treatment markedly improved the total phenolics content under both salt stressed and non-stressed conditions (Table 15, Fig. 26). Of nitric oxide levels 0.1 mM was more effective in improving total phenolic content as compared to 0.2 mM concentration, however, both levels were equally effective in cultivar IRRI-6 under control conditions. Higher total phenolic content was observed in cultivar IRRI-6 and KS-282 under both control and saline conditions.

Hundred grain weight was decreased significantly due to salt stress in all four rice cultivars (Table 16, Fig. 27). Pre-sowing seed treatment with 0.1mM nitric oxide increased
hundred grain weight in all four rice cultivars under saline conditions, however, the effect of 0.2 mM nitric oxide was not prominent. Of rice cultivars, KS-282 and IRRI-6 showed higher hundred grain weight under both control and salt stress conditions. Like hundred grain weight, grain weight per plant also decreased markedly, due to increased salinity in the root-zone of all four rice cultivars (Table 16, Fig. 27).
Pre-sowing seed treatment with nitric oxide increased the total grain weight per plant in all four rice cultivars under saline conditions. The nitric oxide level, 0.1mM was more effective than the other levels used in increasing the total grain weight per plant under NaCl stress, however, both NO concentrations (0.1 and 0.2 mM) showed a non-significant effect on total grain weight per plant under non-stress conditions. Of rice cultivars KS-282 and IRRI-6 showed higher total grain weight per plant than the other cultivars both under control and salt stressed conditions.

**Nitric oxide application as foliar spray**

Shoot and root biomasse, both fresh and dry, decreased significantly due to salt stress in all four rice cultivars (Table 17, Fig. 28-29). Exogenous application of nitric oxide as foliar spray increased the dry and fresh weights of both shoots and roots under control and salt stress conditions in plants of all four rice cultivars. Both nitric oxide levels were equally effective under non-stressed conditions, however, 0.1 mM was more effective than the other NO levels under saline conditions. Rice cultivar KS-282 showed higher shoot fresh and dry weights under non-stress conditions, however, under salt stress conditions cv. Shaheen Basmati performed better. In contrast, for root fresh and dry weights the cultivars remained indifferent under both control and stress conditions.

Shoot length significantly decreased due to increased salt content in the root-zone of all four rice cultivars (Table 18, Fig. 30). Nitric oxide treatment increased the shoot lengths when applied as foliar spray in all four rice cultivars both under NaCl stress and control conditions. Of nitric oxide levels, 0.2 mM was more effective in increasing the shoot length under non-saline conditions, however 0.1 mM being more effective under NaCl stress. Rice cultivar Basmati PB-95 showed maximum increase in shoot length under control and salt stress conditions. Salt stress also decreased the number of tillers per plant in all four rice cultivars (Table 18, Fig. 30). Foliar applied nitric oxide as showed a non-significant effect on number of tillers in all four rice cultivars under salt stress and non-stress conditions. Of rice cultivars, KS-282 and IRRI-6 produced higher number of tillers per plant under control and salt stress conditions.

Chlorophyll a, chlorophyll b and total chlorophyll contents decreased significantly in all four rice cultivars under salt stress (Table 18-19, Fig. 31-32). A significant increase was
observed in chlorophyll a, chlorophyll b and total chlorophyll contents due to foliar application of nitric oxide under both control and salt stress conditions. Both levels (0.1 and 0.2 mM) of nitric oxide were equally effective in increasing the chlorophyll content under non-stress conditions, however, 0.1 mM was more effective under salt stress.
The response of all four rice cultivars was almost similar with respect to chlorophyll $a$, chlorophyll $b$ and total chlorophyll contents under control conditions, however, cultivars Shaheen Basmati and IRRI-6 performed better under NaCl stress.

Addition of excessive amount (80 mM) of NaCl to the rooting medium (soil) significantly reduced the values of $A$ (net CO$_2$ assimilation rate), $g_s$ (stomatal conductance), $E$ (transpiration rate), and $C_i$ (sub-stomatal CO$_2$ concentration) of all four rice cultivars (Table 20, Fig. 33-34). Exogenous application of nitric oxide as foliar spray increased the values of all four gas exchange parameters in all four rice cultivars under salt stress. The maximal increase in the values of $A$, $g_s$, $E$ and $C_i$ was observed at 0.2 mM of nitric oxide under non-stress conditions, however, 0.1 mM was found to be more effective under saline stress. Although cultivar Basmati PB-95 showed a maximal increase in $A$ and $E$ under non-stress conditions, cultivar Shaheen Basmati and IRRI-6 performed better in terms of $A$, $E$ and $g_s$ under saline conditions. For $C_i$, the cultivars remained indifferent under both control and saline conditions. Leaf water use efficiency (WUE, $A/E$) also decreased in all four rice cultivars in response to root-zone salinity (Table 21, Fig. 35). Foliar application of nitric oxide markedly altered the WUE, as an increase was observed under saline conditions, while a decrease observed at 0.1 mM nitric oxide under non-saline conditions in all four rice cultivars. A marked reduction in $C_i/C_a$ ratio was observed in all four rice cultivars under salt stress (Table 21, Fig. 35). Exogenously applied nitric oxide treatment as foliar spray increased the $C_i/C_a$ ratio in all four rice cultivars under control and saline conditions. Of nitric oxide levels, 0.2 mM was more effective under non-stress conditions, whereas both 0.1 and 0.2 mM were equally effective for all four rice cultivars under salt stress.

Imposition of salt stress to the root growth medium decreased the values of leaf water and osmotic potential in all four rice cultivars (Table 21, Fig. 36). Exogenous foliar application of nitric oxide increased the leaf water and osmotic potential values in both salt stressed and non-stressed plants of all four rice cultivars. Both nitric oxide levels were equally effective in regulating leaf $\Psi_w$ and $\Psi_s$ under control conditions, however, 0.1 mM being more effective under salt stress. Cultivar difference was non-significant for leaf water and osmotic potential. Leaf turgor potential and relative water content (RWC) decreased due to salt stress in plants of all four rice cultivars (Table 22, Fig. 37). Foliar-applied nitric oxide
increased the turgor potential of leaf and also the relative water content in all four rice cultivars under both NaCl stress and non-stress conditions.
Rice cultivars Shaheen basmati and IRRI-6 performed better in leaf turgor potential and relative water content and a maximal increase in these two parameters was observed at 0.1 mM nitric oxide under salt stress.

Sodium (Na⁺) and Cl⁻ contents was increased significantly in both leaves and roots of all four rice cultivars under saline conditions (Table 23, Fig. 38-39). Foliar nitric oxide treatment caused reduction in both Na⁺ and Cl⁻ accumulation in the leaves and roots of salt stressed plants of all four rice cultivars, and in his regard 0.1 mM NO was found more effective. However, foliar-applied nitric oxide yielded no significant effect on leaf or root Na⁺ content under non-stress conditions. Rice cultivars KS-282 and Basmati PB-95 showed higher Na⁺ and Cl⁻ accumulation under saline conditions.

A marked decrease was observed in K⁺ content of both leaves and roots of all four rice cultivars due to root-zone salinity (Table 24, Fig. 40). Exogenously-applied nitric oxide resulted in increased K⁺ accumulation under saline conditions. Of nitric oxide levels, 0.2 mM was effective under non-stress conditions however, 0.1 mM was more effective in increasing leaf and root K⁺ content in salt stressed plants of all rice cultivars. Cultivars Shaheen Basmati and IRRI-6 showed higher leaf and root K⁺ content then the other cultivars under salt stress.

Like K⁺, calcium (Ca²⁺) accumulation also decreased in leaves and roots of all four rice cultivars due to NaCl stress (Table 24, Fig. 41). Foliar applied nitric oxide resulted in increased Ca²⁺ accumulation under salt stress. Of nitric oxide levels, 0.2 mM was more effective under both control and salt stress conditions. Rice cultivar Shaheen Basmati and IRRI-6 showed higher Ca²⁺ accumulation in both shoots and roots under saline conditions, however, the cultivars remained indifferent under non-stress conditions. Salt stress markedly increased the activities of leaf superoxide dismutase (SOD) and peroxidase (POD) in all four rice cultivars (Table 25, Fig. 42). A further increase in the activities of these antioxidant enzymes was observed due to foliar application of nitric oxide under control and saline conditions. Both nitric oxide levels (0.1 and 0.2 mM) were equally effective in increasing SOD and POD activities under non-stress conditions, however, 0.1 mM was more effective under saline conditions. Of rice cultivars, Shaheen Basmati and IRRI-6 performed better for SOD and POD activities under saline conditions. Similarly, a marked increase was also observed in leaf catalase (CAT) activity in all four rice cultivars due to root-zone salinity.
(Table 25, Fig. 43). Foliar application of nitric oxide further increased the leaf catalase activity under control and salt stress conditions.
Of nitric oxide levels, 0.2 mM was more effective in increasing the catalase activity under non-stress conditions, however, both levels (0.1 and 0.2 mM) were equally effective under saline conditions in terms of improving CAT activity. Rice cultivars Shaheen Basmati and KS-282 showed higher catalase activity under salt stress conditions and a maximal increase in catalase activity was observed in cultivar KS-282.

Leaf proline content increased significantly in all four rice cultivars due to root-zone salinity (Table 26, Fig. 44). Foliar-applied nitric oxide was found to be effective in increasing the proline content under saline conditions, however, no such effect was observed due to foliar addition of nitric oxide in all four rice cultivars under control conditions. Of nitric oxide levels, 0.2 mM was more effective in increasing proline content in the rice cultivars under saline conditions. Rice cultivars KS-282 and IRRI-6 showed higher increase in proline content than Shaheen Basmati and Basmati PB-95 under saline conditions.

Salt stress increased the leaf malondialdehyde (MDA) content in all four rice cultivars (Table 26, Fig. 44). Foliar-applied nitric oxide decreased the leaf MDA content in all rice cultivars under saline conditions, however, such effect was not observed under control conditions. Both nitric oxide levels (0.1 and 0.2 mM) were equally effective in decreasing leaf MDA content in the rice cultivars under saline conditions. Rice cultivars KS-282 and IRRI-6 showed less increase in leaf MDA content as compared to that in Shaheen Basmati and Basmati PB-95 under saline conditions.

Leaf H$_2$O$_2$ content increased significantly in all four rice cultivars due to salt stress of the growth medium (Table 26, Fig. 45). Exogenously applied nitric oxide decreased the leaf H$_2$O$_2$ content in all rice cultivars under saline conditions. Of nitric oxide levels, only 0.1 mM was effective for cultivars Shaheen basmati and IRRI-6, and 0.2 mM for in cultivar KS-282 for reducing the leaf H$_2$O$_2$ content under saline conditions.

Imposition of salt stress markedly increased the leaf ascorbic acid content in all four rice cultivars. Exogenous application of nitric oxide as foliar spray further increased the ascorbic acid content under both control and saline conditions (Table 26, Fig. 45). Both nitric oxide levels were equally effective in improving ascorbic acid content under non-stressed conditions, however, 0.1 mM was more effective under salt stress. Rice cultivars KS-282 and IRRI-6 showed higher ascorbic acid content under both control and saline conditions, while Shaheen Basmati performed better as compared to Basmati PB-95.
Total phenolics content was decreased significantly in all four rice cultivars due to root zone salinity. A marked increase in total phenolic content was observed with exogenous foliar application of nitric oxide under both control and salt stress conditions (Table 27, Fig. 46). Of nitric oxide levels 0.1 mM was more effective in increasing total phenolic content under both control and saline conditions as compared to 0.2 mM, however, 0.2 mM was also equally effective in increasing total phenolics content in cultivar IRRI-6 under control conditions. Rice cultivars KS-282 and IRRI-6 showed higher total phenolic content under both control and saline conditions.

Hundred grain weight decreased in all four rice cultivars due to root-zone salinity (Table 27, Fig. 46). Foliar-applied nitric oxide showed a non-significant effect on hundred grain weight in plants grown under control and saline conditions. Among rice cultivars KS-282 and IRRI-6 showed higher hundred grain weight than the other two cultivars under both control and salt stress conditions. Grain yield per plant decreased significantly in all four rice cultivars due salt stress. A significant increase was observed in total grain yield per plant due to foliar application of nitric oxide under both control and saline conditions (Table 27, Fig. 47). Of nitric oxide levels, 0.1 mM was more effective in increasing grain yield under salt stress, however, both levels (0.1 and 0.2 mM) were equally effective under control conditions. Rice cultivars KS-282 and IRRI-6 showed higher total grain yield per plant than the other cultivars under both control and saline conditions.
Chapter 5
Discussion
Discussion 1st experiment
Salt sensitivity of rice plants at early growth stages have been reported due to salt induced specific ion toxicity and osmotic stress, particularly at seed germination and early seedling growth stages (Mohammad & Sen 1990; Duan et al., 2004). In this initial study, various seed germination attributes such as germination index, germination percentage and time to achieve 50% germination were severely affected due to saline stress in all four rice cultivars (Basmati PB-95, Shaheen Basmati, KS-282 and IRRI-6). In addition, plumule weights (both fresh and dry) were also decreased significantly in all four rice cultivars under salt stress conditions. Pre-sowing seed treatment with lower levels (0.1 and 0.2 mM) of nitric oxide markedly improved the germination percentage in all four rice cultivars under both control and saline conditions. Nitric oxide has been reported as an important signaling molecule that has a regulatory role in variety of physiological processes not only in both plants and animals (Crawford & Guo, 2005; Besson-Bard et al., 2008). Therefore, due its regulatory properties, nitric oxide can increase seed germination of various crop plants including rice in response to stressed conditions (Bethke et al., 2007; Libourel et al., 2006). Nitric oxide induced improvement in dry matter accumulation of salt stressed maize seedlings has already been reported (Zhang et al., 2006). Similarly, Zheng et al., (2009) have reported that exogenous nitric oxide treatment had resulted in increased seed germination rate and improvement in coleoptiles and radical weights of wheat seedlings under saline conditions. From all these reports it is clear that pre-sowing seed treatment with nitric oxide markedly improved the salt tolerance in different crop plants at early growth stages. Pre-sowing seed treatment with higher nitric oxide (0.4 and 0.5 mM) concentrations severely inhibited the seed germination attributes under both control and saline conditions. The higher concentrations of nitric oxide have been reported to inhibit the seed germination and early seedlings growth in various crop plants (Zhang et al., 2003; Kopyra & Gwozdz, 2003).

Nitric oxide is a highly reactive molecule, and the toxicity of higher nitric oxide concentrations have been reported due to its reactivity which results in DNA fragmentation and membrane damage (Pedroso et al., 2000). Furthermore, higher nitric oxide
concentrations can also result in inhibited cell division, apoptosis and senescence at whole plant level, in addition inhibited activity of some potential antioxidant enzymes has also been reported due to application of higher nitric oxide concentrations which could lead to higher H2O2 content other reactive oxygen species (ROS) (Bethke et al., 2007). From above reports it is evident that low concentrations of nitric oxide are effective in improving seed germination rate and early seedling growth of rice, however its higher concentrations can inhibit seed germination and early seedling growth.

**Discussion 2nd experiment**

Enhanced soluble salt content in soil or water is an important limiting factor for growth and yield production of most crops (Munns and Tester, 2008; Chen et al., 2009; Kaya et al., 2009; Shi et al., 2009; Ashraf et al., 2010a; Flowers et al., 2010). Salt stress not only inhibits growth and productivity in crop plants, but can also cause death under severe cases (Munns and Tester, 2008). In the present study, salt stress significantly suppressed the fresh and dry biomass (both of shoots and roots) in all four rice cultivars. However, exogenous nitric oxide application improved the root and shoot weights of salt stressed plants of all four cultivars. Such type of nitric oxide-induced growth improvements have already been reported in wheat (Zheng et al., 2009), cucumber (Shi et al., 2007), maize (Zhang et al., 2006b), and yellow lupine seedlings (Kopyra and Gwozdz, 2003) under saline conditions. Bai et al. (2011) reported that nitric oxide mitigates inhibitory effect of salt on shoot and root growth of plants either by accelerating cell division or by remodeling cytoskeleton.

It is also well evident from a number of reports that salt stress causes a substantial decrease in the amount of photosynthetic pigments in various plants such as in, tomato (Doganlar et al., 2010), sunflower (Akram et al., 2009a), *Ricinus communis* (castor bean) (Pinheiro et al., 2008), and wheat (Arfan et al., 2007). Salt stress-induced breakdown of chlorophyll molecule is generally attributed to increased Na⁺ accumulation as higher Na⁺ accumulation is toxic for many bio-molecules (Athar and Ashraf, 2008; Ashraf et al., 2010a). This salt-induced reduction in leaf chlorophyll pigments could be due to increased degradation and/or impaired biosynthesis of chlorophyll molecules (Akram et al., 2011). However, according to some reports, chlorophyll biosynthesis is more seriously affected by salt stress as compared to the breakdown of chlorophyll (Santos and Caldeira, 1999; Santos et al., 2001). In the present investigation, chlorophyll content decreased significantly in all
four rice cultivars under salt stress. Again exogenous nitric oxide treatment (both as pre-sowing seed treatment and foliar spray) markedly improved the chlorophyll content in salt stressed rice plants. Nitric oxide induced increase in chlorophyll content under salt stress has already been reported in tomato (Wu et al., 2010) and wheat (Ruan et al., 2002), suggesting that nitric oxide protects the photosynthetic apparatus from the damaging effects of salt stress (Wu et al., 2010). These results are in agreement with a number of other studies showing increased photosynthetic pigments in salt stressed plants by exogenous application of nitric oxide (Laxalt et al., 1997; Shi et al., 2005; Pahwa et al., 2009).

Inhibitory effect of NaCl stress on photosynthetic capacity of plants is not only attributed to breakdown of chlorophyll pigments, but also to gaseous exchange (CO2/O2), alteration in chloroplast structure, and type of species or cultivar (Liu and Shi, 2010). Limitations to the photosynthetic performance under salt stress may be due to stomatal or non-stomatal factors (Debez et al., 2008). Ashraf (2009) has suggested that reduced photosynthetic rate in salt stressed plants may be due to stomatal closure. Decreased stomatal conductance further leads to a marked reduction in other gas exchange parameters such as transpiration rate (E), internal CO2 concentration (Ci) and photosynthetic rate (A) (Ashraf, 2004). In the present study a considerable decrease was observed in stomatal conductance (gs), photosynthetic rate (A), transpiration rate (E) and sub-stomatal CO2 concentration (Ci) in all four rice cultivars under saline growth medium. However, exogenous nitric oxide treatment improved A, E, gs and Ci in salt stressed plants of all four rice cultivars. It has already been reported that endogenous nitric oxide level is important for stomatal closure (Kolla and Raghavendra, 2007; Niel et al., 2008; Riberio et al, 2009), because nitric oxide plays an important role in regulation of stomatal movement (Desikan, 2004). Similarly, nitric oxide-induced increase in A, E, gs and Ci has been reported in salt stressed tomato plants (Wu et al., 2010).

Plants growing on soils with high salt content often face osmotic stress which could inhibit growth, induce leaf chlorosis, imbalance the hormone level and reduce the antioxidant activity (Munns, 1993, 2002; Mittler, 2002; Ashraf, 2004; Ashraf et al., 2010a). Root to shoot water conductivity in plants has been reported to be dependent upon soil water potential which decreases with increasing root-zone salinity and could limit the water supply to various plant organs which could further result in reduced membrane permeability
Damaging effects on growth due to salt-induced osmotic effect have been reported to be abrupt for most of the plants as compared to ionic effects (Ashraf, 2004; Flowers, 2004). Type of plant tissue and timing of salt stress matters, while, assessing the adverse effects of salt-induced osmotic effect (Ashraf, 1994; Cony and Trion, 1998; Munns et al., 2002 Meloni et al., 2003). Inhibited leaf and shoot growth has been reported due to salt-induced osmotic effect while roots could continue to grow (Spollen and Sharp, 1991). In plants, number of tillers, lateral buds, branches and lateral shoot formation, emergence of new leaves, growth of developing leaves and leaf area have been reported to be severely affected due to salt-induced osmotic effect (Taiz and Zeiger, 2006; Munns et al., 2006). Altered functioning of vital biochemical and physiological processes due to salt-induced osmotic phase could be responsible for reduced growth (Ashraf and Harris, 2004). Higher Na$^+$ and Cl$^-$ accumulation reduced water potential in salt stressed pea plants Noreen and Ashraf (2009a). In turnip plants, salt induced decrease in leaf water potential and relative water content proved to be inhibitory for normal growth (Noreen et al., 2010). NaCl-induced growth reduction due to osmotic stress has been well documented in a number of plants, such as Brassica spp. (He and Cramer, 1993), wheat (Kingsbury, 1984). Brassica napus (Zheng et al., 1998; Huang and Redman, 1995), and maize (Cramer et al., 1994),

In the present study, water relation attributes (leaf water potential, osmotic potential, turgor potential and relative water content) were also adversely affected in all four rice cultivars under saline conditions. However, exogenous application of nitric oxide considerably improved these water relation attributes. Decreased leaf water potential under salt stress is mainly due to higher accumulation of Cl$^-$ and Na$^+$ ions (Hasegawa et al., 2000; Flowers et al., 2010), leading to decreased osmotic potential thereby reducing plant cell’s turgidity due to water loss (Munns, 2002; Zhu, 2002; Ali and Ashraf, 2010; Siddiqi and Ashraf, 2008). But little information is available in the literature regarding the effect of nitric oxide on water relation parameters, however according to one report nitric oxide treatment increased water potential while decreased solute potential in tobacco plants under osmotic stress (Ke et al., 2013).

Chlorophyll fluorescence is used to measure the activity of photosystem II (PS II) (Saleem et al., 2011). Of photosynthetic machinery, photosystem II (PS II) is more sensitive to salt stress as compared to photosystem I (PS I) so salt-induced decrease in photosynthesis
is often linked to PSII (Saleem et al., 2011). In the present study, salt stress markedly altered various chlorophyll fluorescence attributes in all four rice cultivars. As the values of minimum fluorescence in dark ($F_o$), minimum fluorescence in light ($F_t$), maximum chlorophyll fluorescence ($F_m$), maximum fluorescence at steady state in dark adapted leaves ($F_{ms}$), leaf fluorescence at steady state in light adapted leaf ($F_s$), quantum yield of electron transport ($\eta$), leaf photochemical fluorescence ($Q_p$) and maximum quantum yield of primary photochemical reaction in dark adapted leaves ($F_{v}/F_{m}$) decreased in all four rice cultivars under salt stress. Salt-induced decrease in the value of $F_o$ indicates the loss of energy transfer to reaction centers from antenna complex (Lutts et al., 1996; Baker, 2008). Decreased $F_o$ value also indicates the impairment of plant ability to repair the photosystem II, damaged by salt stress (Allakhverdiev et al., 2002; Amirjani, 2010). Decreased $F_{v}/F_{m}$ value is related to the salt-induced decrease in $F_m$ value which indicates the destruction of reaction center and disruption of antenna complex at photosystem II which results in increased dissipated energy (Lutts et al., 1996; Santos et al., 2001). Decreased $F_{v}/F_{m}$ value also indicates that RUBP regeneration ability has also been impaired due to salt stress (Kafi, 2009). $Q_p$ value indicates the inactivated proportion of reaction centre at PS II (Moradi and Ismail, 2007; Abdeshahian et al., 2010). Salt-induced decrease in $Q_p$ value could also be due to the separation of PS II reaction center from the light harvesting complex II (Wu et al., 2010). In the present study, electron transport rate ($ETR$) and non-photochemical chlorophyll fluorescence quenching ($NPQ$) values increased in all four rice cultivars under saline conditions. Increased electron transport rate in C$_3$ plants like wheat, could be due to salt-induced increase in photorespiration (Megdiche et al., 2008). Similarly, increased $NPQ$ value exhibits the adaptive energy dissipation process which protects the photosynthetic apparatus against photo-damage under salinity stress (Netondo, et al., 2004). While studying the unbalanced electron transport system and inhibited activities of pigments in salt stressed plants Brassica juncea, Alia et al., (1993) found that salt stress not only inhibited the electron transport system of thylakoid but also severely affected the activity of PSII. Moradi and Ismail (2007) measured chlorophyll fluorescence in rice at the vegetative and reproductive stages and found that the functioning of PSII was severely affected under saline regimes. Similarly, PSII was also reported to be severely damaged in wheat under salt stress (Mehta et al., 2010; Kanwal et al., 2011; Ashraf and Ashraf, 2012). In the present study, exogenous nitric oxide
treatment markedly improved various chlorophyll fluorescence parameters in salt stressed rice plants. As exogenous nitric oxide could keep more proportion of PSII reaction centers in an open state so that more excitation energy can be used for electron transport (Wu et al., 2010). Nitric oxide treated tomato plants showed a less decrease in \( Fv/Fm \) value and less increase in \( Qn \) value under salt stress, suggesting that nitric oxide treatment resulted in less dissipation of excitation energy as heat from the PSII antennae (Wu et al., 2010). Nitric oxide treatment slowed down the electron transport rate and inhibited the steady-state photochemical and nonphotochemical quenching processes. It also appears to modulate reaction center-associated nonphotochemical quenching (Wodala et al., 2008). Wu et al. (2010) suggested that improved \( Qp \) value in salt stressed tomato plants could be due to nitric oxide induced improvement photochemical efficiency. However, some contrasting reports are also available in the literature as Yang et al. (2004) reported decreased photochemical activity of PSII in \textit{Solanum tuberosum} leaves with nitric oxide treatment. According to some reports, nitric oxide treatment also did not affect the \( Fv/Fm \) value and in some studies even a decrease was observed in \( Fv/Fm \) value with nitric oxide treatment (Takahashi and Yamasaki, 2002; Yang et al., 2004).

Ion accumulation pattern is important in determining the plant salt tolerance level (Ashraf, 2004; Munns and Tester, 2008). Amtmann and Sanders (1999) suggested that plants try to avoid excessive amount of \( \text{Na}^+ \) in the cytoplasm, because at higher concentrations, \( \text{Na}^+ \) interferes with normal ongoing metabolic processes. A number of reports have described \( \text{Na}^+ \) and \( \text{Cl}^- \), as abundant components of saline soils and waters, which are responsible for ion toxicity in plants which reduce yield (Abrol et al., 1998; Munns and Tester, 2008; Abbas et al., 2010). Ion carrier proteins have been reported as an integral parts of cell membrane that play a vital role in the transport of ions into the cell by active transport at the expense of adenosine triphosphate (ATP) and pyrophosphate (Flowers and Flowers, 2005). Water loses as a result of transpiration leaving behind dissolved ions such as \( \text{Cl}^- \) and \( \text{Na}^+ \). As transpiration is a continuous process on leaf surface so it could lead to higher \( \text{Na}^+ \) concentrations in the leaf, which could exceed the salt tolerance limit of plants leading to leaf senescence and limited growth (Marschner, 1995). Role of \( \text{Na}^+ \) for causing ion toxicity has been well described in several reports. However, higher concentrations of \( \text{Cl}^- \) are also not desirable as
Cl⁻ is considered comparatively more toxic for grapevine, citrus, and soybean (Lauchli, 1984; Ashraf, 1994; Grattan and Grieve, 1999; Storey and Walker, 1999).

In older leaves, Cl⁻ has also been reported to cause drying of leaf tissues especially at extreme tips of leaf and leaf burn (Marschner, 1995). While explaining Cl⁻ toxicity in plants, White and Broadley (2001) have described that after being taken up through roots, Cl⁻ is transported to shoots where it interferes in metabolic processes and exert damaging effect on photosynthesis. Na⁺ and Cl⁻ exclusion and maintenance of higher K⁺/Na⁺ ratio in plant organs and cells as they are the key responses of salt tolerance in crop plants (Zheng et al., 2009). In the present study, increased accumulation of Cl⁻ and Na⁺ while, decreased K⁺ and Ca²⁺ were observed in leaf and root tissues of salt stressed rice plants. Various reports have described increased Na⁺ uptake, while decreased accumulation of Ca²⁺ and K⁺ under salt stress in different crop plants, e.g., sunflower (Akram et al., 2007), green bean (Pessarakli, 1991) radish and cabbage (Jamil et al., 2006; 2007), wheat (Raza et al., 2006) and okra (Habib et al., 2012), and Functioning of various metabolic processes and activity of different antioxidant enzymes could be impaired due to NaCl induced decrease in K⁺/Na⁺ ratio (Tester and Davenport, 2003; Sudhir and Murthy, 2004; Munns and Tester, 2008). Salt-induced decrease in K⁺/Na⁺ ratio was found to be related to decreased antioxidant activity and high malondialdehyde content in wheat (Zheng et al., 2009; Ashraf et al., 2011, 2012). Balanced K⁺/Na⁺ ratio also has a role in turgor maintenance, cell osmoregulation and stomatal function. It also facilitates proper functioning of photosynthetic process and protein synthesis (Shabala et al., 2003). In the present study, exogenous nitric oxide treatment decreased the Na⁺ accumulation while increased that of K⁺ and maintained high K⁺/Na⁺ ratio in salt stressed rice plants. Our results are also in agreement with some previous studies in which exogenous nitric oxide treatment decreased Na⁺ uptake while increased K⁺ accumulation and maintained high Na⁺/K⁺ ratio in salt stressed wheat (Zheng et al., 2009) and Arabidopsis plants (Zhao et al., 2007). In another study, Zhang et al. (2006b) reported that nitric oxide helped in alleviating the salt-induced toxicity in maize plants by increasing the activities of H⁺-ATPase, H⁺-PPase, and Na⁺/H⁺ antiport in the tonoplast. It has already been established that enzymatic antioxidants such as CAT, POD and SOD play an important role in scavenging salt-induced over-production of reactive oxygen species (ROS) (Ashraf and Ali, 2008; Tuna et al., 2008; Ashraf, 2009). In the present investigation, the activities of different
antioxidant enzymes such as CAT, SOD and POD increased in salt stressed rice plants of all four cultivars. Analogue to our findings, increased activities of CAT, POD and SOD enzymes were also observed in salt stressed plants of pea (Noreen and Ashraf, 2009a), wheat (Ashraf et al., 2012), and radish (Noreen and Ashraf, 2009b).

Exogenous nitric oxide treatment is believed to be an effective practice to protect plants against salt-induced oxidative damage (Zheng et al., 2009). In the present investigation, exogenous nitric oxide treatment markedly enhanced the activities of different antioxidant enzymes such as POD, SOD and CAT. Nitric oxide-induced increase in the activity of different enzymatic and non-enzymatic antioxidants have already been reported in salt stressed plants of cucumber (Shi et al., 2007), barley (Li et al., 2008), wheat (Hai-Hua et al., 2005), and chickpea (Sheokand et al., 2010). The protective role of nitric oxide against salt-induced oxidative damage can be explained by evaluating its properties as a signaling molecule, which activates enzymatic antioxidants defense system (Huang et al., 2002; Shi et al., 2005). Several reports have already described the positive effect of nitric oxide on SOD activity under salt stress (Kopyra and Gwozdz, 2003; Shi et al., 2007; Li et al., 2008). A similar enhancing effect of nitric oxide on CAT activity has also been reported in earlier studies under saline stress (Shi et al., 2007; Li et al., 2008), osmotic stress (Zhao et al., 2008), and heavy metal stress (Kopyra and Gwozdz, 2003; Singh et al., 2008).

In plants, increased accumulation of different compatible solutes like proline and GB is an adaptive response of plants to salt stress (Ashraf and Foolad, 2007; Park et al., 2007; Habib et al., 2012). Under salt stress, increased proline and GB accumulation plays a vital role in protection of membranes, enzymes, proteins and plant cells or tissues by scavenging salt-induced generated ROS (Banu et al., 2010; Ashraf et al., 2012; Habib et al., 2012). In the present study, increased proline accumulation was observed in all four rice cultivars due to salt stress. Salt-induced increase in proline accumulation has already been reported in a number of plants including wheat (Ashraf et al., 2012), and okra (Habib et al., 2012). In the present investigation, exogenous nitric oxide treatment also increased proline content in salt stressed plants of all four rice cultivars. Nitric oxide-induced increase in proline content has already been reported in salt stressed plants of Kosteletzkya virginica (Guo et al., 2008).

Increased malondialdehyde (MDA) content in salt stressed plants of most species occur due to membrane lipid peroxidation (Ashraf et al., 2010). Generally, lipid peroxidation
takes place due to salt-induced over-generation of ROS (Liang et al., 2003; Zhang et al., 2006; Shi et al., 2007; Ashraf and Ali, 2008; Li et al., 2008), which results in membrane damage by making it more leaky (Ashraf and Ali, 2008; Sheokand et al., 2010). In this investigation, leaf MDA content increased significantly in all four rice cultivars due to salt stress. NaCl-induced increase in MDA content has already been reported in a number of plants, e.g., wheat (Ashraf et al., 2010), sunflower (Akram et al., 2009a; Akram and Ashraf, 2011c), canola (Ashraf and Ali, 2008), and Kosteletzkya virginica (Guo et al., 2008). However, exogenous nitric oxide treatment markedly decreased the leaf MDA content in salt stressed rice plants. Sheokand et al., (2008, 2010) reported that exogenous nitric oxide treatment decreases salt-induced increase in lipid peroxidation which results in protecting the membranes. The authors were of the view that nitric oxide readily reacts with ROS which help prevent ROS-induced membrane damage. Nitric oxide reaction with lipid peroxy (LOO\(^{-}\)) and alcoxyl (LO) radicals is very rapid which could stop further generation of lipid peroxidation (Sheokand et al., 2010).

A protective role of nitric oxide against membrane lipid peroxidation in most plants has already been reported under saline conditions (Zhao et al., 2004; Zhang et al., 2006; Shi et al., 2007; Li et al., 2008), heavy metal stress (Hsu and Kao, 2004; Singh et al., 2008), drought stress (Garcia-Mata and Lamattina, 2001; Wang et al., 2004; Zhao et al., 2008), wounding (Grun et al., 2006), and UV stress (Shi et al., 2005).

Increased hydrogen peroxide (H\(_{2}\)O\(_{2}\)) content is correlated with salt-induced oxidative damage (Sheokand et al., 2010). In the present investigation, increased H\(_{2}\)O\(_{2}\) content was observed in salt stressed rice plants of all four cultivars. Increase in H\(_{2}\)O\(_{2}\) content has already been reported in salt stressed plants of wheat (Ashraf et al., 2012), rice (Uchida et al., 2002), chickpea (Sheokand et al., 2010), barley (Li et al., 2008), and cucumber (Shi et al., 2007). In the present investigation, exogenous nitric oxide treatment markedly decreased the H\(_{2}\)O\(_{2}\) content in salt stressed plants of all four rice cultivars. Our results are in accordance with some previous reports showing nitric oxide induced decrease in H\(_{2}\)O\(_{2}\) content of salt stressed plants of Arabidopsis (Zhao et al., 2007) and chickpea (Sheokand et al., 2010). Nitric oxide-induced decrease in H\(_{2}\)O\(_{2}\) content and its protective role against oxidative damage have already been reported under salt stress (Shi et al., 2007; Li et al., 2008), heavy metal stress (Singh et al., 2008) and water stress (Zhao et al., 2008).
Leaf phenolics are important compounds which help in protecting plant from salt induced oxidative damage by acting as antioxidants (Ashraf et al., 2010b). The antioxidative role of phenolics is mainly due to their capacity to donate hydrogen, scavenging singlet O2 and act as reducing agents (Rice-Evans et al., 1997). Phenolics synthesis is generally altered under different abiotic stresses including salinity (Parida et al., 2004). In present study leaf total phenolics content was decreased in salt stressed plants of all four rice cultivars. Salt induced decrease in total leaf phenolics content has already been reported in wheat (Ashraf et al., 2010), pepper (Navvaro et al., 2006), radish (Yuan et al., 2010), sunflower (Akram et al., 2011) and Cynara cardunculus plants (Falleh et al., 2008). However, in present study, exogenous application of nitric oxide markedly improved the total leaf phenolic content under both control and saline conditions. In plants, increased total leaf phenolic content due exogenous application of nitric oxide has already been reported in response to pathogen attack (Buss et al., 2011) and osmotic stress (Shehab et al., 2010).

Ascorbic acid is an important non-enzymatic antioxidant which is also required for many metabolic processes in plants (Smirnoff and Wheeler, 2000). In plants, ascorbic acid plays a protective role against salt induced oxidative damage by eliminating ROS including hydroxyl radical, superoxide and singlet oxygen (Foyer, 2001). In present study, leaf ascorbic acid content was increased in plants of all four rice cultivars due to root zone salinity. Increased ascorbic acid content in response to salt stress has already been reported in wheat (Sairam et al., 2005; Athar et al., 2008; Ashraf et al., 2012). In present investigation, exogenous nitric oxide treatment further increased the ascorbic acid content in rice plants under both salt stressed and non-stressed conditions. Nitric oxide induced increase in ascorbic acid content could be due to signaling properties of nitric oxide which is involved in activation of antioxidant defense system. Increased ascorbic acid content due to exogenous nitric oxide treatment has already been reported in rice plants under drought stress (Shehab et al., 2010).

Crop yield is the final output of various interrelated growth and physiological attributes which is severely reduced due to salinity stress. It is also an important criterion for evaluating salinity tolerance in crop plants (Katerji et al., 2003; Ashraf, 2004; Flowers, 2004; Munns et al., 2006; Ashraf, 2009; Flowers et al., 2010; Ashraf et al., 2010a, b). In this investigation, yield attributes were negatively affected due to salt stress in all four rice cultivars. Salt-
induced decrease in yield production has already been reported in different plants such as in sunflower (Akram and Ashraf, 2011c), okra (Habib et al., 2012), wheat (Shahbaz et al., 2008), mungbean (Ahmed, 2009), and barley (Endris and Mohammed, 2007). Saline stress is an important limiting factor for plant growth and yield production (Munns and Tester, 2008; Kaya et al., 2009; Ashraf, 2009; Shi et al., 2009; Ashraf et al., 2010a; Flowers et al., 2010).

Salt-induced decrease in growth and yield production is generally attributed to decreased chlorophyll content (Khan et al., 2009), high accumulation of Na⁺ in leaf and root tissues (Akram et al., 2009a; Khan et al., 2009), stomatal and nonstomatal limitations for attributes of gas exchange (Dubey, 2005) and over-generation of ROS (Munns and Tester, 2008; Ashraf, 2009). In the present study, exogenous nitric oxide treatment improved the yield attributes in salt stressed rice plants of all four cultivars. Nitric oxide induced improvement in grain yield of water stressed wheat plants has already been reported (Wang et al., 2011). Improved grain yield in salt stressed rice plants could be due to interplay between different growth physiological and biochemical attributes such as improved seedling growth, increased number of tillers, improved chlorophyll content, and gas exchange attributes, and increased activities of different antioxidant enzymes.

**General Discussion**

Low agricultural productivity and limited food supply due to different abiotic stresses like salinity has become a serious global challenge for plant researchers (Munns, 2007; Ashraf et al., 2008; Athar and Ashraf, 2008). Although salts are the natural content of soil and water, increased salt concentration in both soil and water is mainly due to increased human interference and overexploitation of natural resources (Ashraf, 1994; Ashraf and Foolad, 2007). Plants face dual (both ionic and osmotic) stress due to increased salt content in the growth medium (Gregorio et al., 2002; Zhu, 2003; Ashraf, 2004; Munns and Tester, 2008), which inhibits plant growth due to salt-induced nutrient imbalance, altered hormone level and oxidative damage due to over-generation of reactive oxygen species (ROS) (Ashraf, 2004; Ashraf & Foolad, 2007; Ashraf, 2009; Nawaz et al., 2010). During the last three decades, a large number of studies have been conducted on various plant species in order to better understand the mechanism of salt tolerance and plant response to salt stress at whole plant and/or at cellular level (Greenway and Munns, 1980; Ashraf, 1994; 2004; Tester and Davenport, 2003; Flowers, 2004; Munns, 2005; 2007).
To overcome the salt-induced losses to crop plants, a number of strategies have been devised and practiced such as shotgun approaches, selection and breeding for developing salt tolerant lines, identification and mapping of QTL (quantitative trait loci), marker assisted selection (MAS) and development of transgenic plants with over-expression of genes responsible for salt tolerance (Epstein, 1977; Ashraf and Foolad, 2011). Economically feasible approaches are required to be practiced on salt affected lands to minimize the salt induced losses on yield and productivity of crop plants (Flowers, 2004; Athar and Ashraf, 2009).

Hybridization of high yielding genotypes with selected salt tolerant genotypes could be helpful in enhancing crop productivity under saline conditions (Munns et al., 2006). Using conventional breeding methods some salt tolerant lines of different crop plants have been developed such as in Maize (Ashraf and McNeilly, 1990), brassica (Ashraf and McNeilly, 2004; Purty et al., 2008) and wheat (Ashraf, 2010). Conventional breeding has a limited potential in developing salt tolerant cultivars/lines due to low intra-specific genetic variation (Athar and Ashraf, 2009; Noreen, 2010; Siddiqi, 2010). During the recent years the main emphasis is focused on developing salt tolerant lines either through marker-assisted selection (MAS) or genetic engineering (Shi et al., 2003; Ashraf et al., 2010a). Development of transgenic plants for salt tolerance is still at an early stage, effective progress would be possible after getting sufficient information about complex mechanism of salt tolerance (Yamaguchi and Blumwald, 2005). Particularly sufficient knowledge in the area of genomics and proteomics will help in increasing plant salt tolerance using approaches of molecular breeding (Bhattacharya et al., 2004). As salt tolerance is a complex trait of multiple genes, limited progress has so far been made in developing transgenic salt tolerant lines/cultivars of different crops (Zhu, 2002; Ashraf and Akram, 2009).

Various shotgun approaches are also equally effective in increasing plant salt tolerance, these which include exogenous application of various organic and inorganic compounds, osmoprotectants and plant growth regulators, either as pre-sowing seed treatment or foliar application (Debez et al., 2001; Iqbal and Ashraf, 2005; Ashraf et al., 2008, 2010a). A number of reports are available in the literature, describing the protective role of exogenously applied plant growth regulators against salt stress (Raza et al., 2006; Arfan et al., 2007; Athar and Ashraf, 2009; Habib et al., 2010, 2012). Proteins, amino acids, sugars, organic acids, quaternary ammonium compounds and polyols etc. are some of
important compatible solutes which accumulate in plants under various environmental stresses including salinity, and play important role in enhancing stress tolerance by reducing salinity-induced ions toxicity (Hasegawa et al., 2000; Mansour, 2000; 2005; Ashraf and Harris, 2004). These compatible solutes play a vital role in osmoregulation by acting as osmoprotectants such as trehalose, proline and glycinebetaine etc, as their concentration increases considerably in number of plant species under various abiotic stresses including salinity (Ali and Ashraf, 2011, Habib et al., 2012). Under saline stress, these osmolytes play various important roles such as, protecting plants from excessive Na⁺ and Cl⁻ ions toxicity (Misra and Gupta, 2005), and stabilizing cellular structures and protecting macromolecules under saline regimes (Hoekstra et al., 2001).

Nitric oxide is an important signaling molecule in animals as well as plants cells (Crawford and Guo, 2005; Besson-Bard et al., 2008). It plays an important role in various plant physiological processes including growth, development and abiotic stress tolerance (Zheng et al., 2009; Zafra et al., 2010), ultimately enhancing plant stress tolerance ability especially against drought (Garcia-Mata and Lamattina, 2002) and salt stress (Zhao et al., 2007). During plant life cycle, nitric oxide regulates various developmental and physio-biochemical processes such as improvement of seed germination rate (Beligni and Lamattina, 2000; Habib et al., 2010), senescence and maturation in plants (Leshem et al., 1998; Guo and Crawford, 2005), suppression of floral transition (He et al., 2004), regulation of stomatal movement (García-Mata and Lamattina, 2001; Neill et al., 2002; Guo et al., 2003; Desikan et al., 2004; Bright et al., 2006), regulation of growth and development (Durner and Klessig, 1999), and light-mediated greening (Zhang et al., 2006a). Nitric oxide have been reported to perform its regulatory roles in various physiological processes not only by interacting with other biomolecules but also by affecting gene transcription (Huang et al., 2002; Wang et al., 2002; Polverari et al., 2003; Parani et al., 2004; Shoulars et al., 2008). Although nitric oxide is an important mediator of H₂O₂ induced leaf cell death (Lin et al., 2012), however, it also helps in protecting plant cells from damage by scavenge H₂O₂ (Beligni et al., 2002; Crawford and Guo, 2005). Nitric oxide controls the time of flowering by effecting the gene expression through its signaling properties (He et al., 2004), as nitric oxide have been reported to inhibit the expression of constans and gigantea genes, while enhanced the expression of the gene *flowering locus C* (Arasimowicz et al., 2007). The
regulatory role of nitric oxide has also been reported in the formation of root system architecture. As effectivity of nitric oxide for improving root growth in maize was equal to that of indole acetic acid (Gouvêa et al., 1997). According to Pagnussat et al., (2002) adventitious root formation was stimulated due to nitric oxide mediated responses in cucumber. Nitric oxide is an important signaling molecule which operates downstream of auxin during root system formation and development (Correa-Aragunde et al., 2007). Under senescence promoting conditions, exogenous nitric oxide treatment decreased ethylene content in pea leaves due to nitric oxide-induced decrease in ethylene biogeneration (Leshem et al., 1998; Leshem 2000; Leshem and Haramaty, 1996).

Likewise in present investigation exogenous nitric oxide treatment markedly improved the seed germination rate, seedling growth, chlorophyll pigments and gas exchange attributes of salt stressed rice plants. Improved seed germination rate due to pre-sowing seed treatment with nitric oxide could be due to increased activity of α and β amylases (Zheng et al., 2009). Nitric oxide induced increase in amylases activity have been reported in germinating seeds of wheat (Zhang et al., 2005), under salt (Zheng et al., 2009) and copper stress (Hu et al., 2007). Increased amylases activity could enhance the starch metabolism which could accelerate the germination process. In addition, total soluble sugar content has also been reported to be increased in germinating wheat seeds under salt stress due to pre-sowing treatment with nitric oxide (Zheng et al., 2009). Improved seedling growth has already been reported due to exogenous nitric oxide treatment in salt stressed wheat (Zheng et al., 2009), rice (Habib et al., 2010) and maize seedlings (Zheng et al., 2006). Nitric oxide induced improvement in seedling growth could help in better establishment of crop plants at later growth stages under saline conditions. In present study, improved growth of salt stressed rice plants could also be due to nitric oxide induced increase in chlorophyll pigment and photosynthetic rate, as various other reports have shown the increased photosynthetic pigment content in salt stressed plants by nitric oxide treatment (Laxalt et al., 1997; Shi et al., 2005; Pahwa et al., 2009). Improved growth due to exogenous application of nitric oxide has been reported in a number of crop plants including Kodetzkya virginica, maize under salt stress (Zhang et al., 2007b; Guo et al., 2009). Furthermore, Nitric oxide induced improvement in gas exchange attributes of salt stressed plants could be due to its role in stomatal movement (Kolla and Rayhavndra, 2007; Niel et al., 2008), because nitric oxide
have been reported to plays an important role in ABA induced stomatal closure (Desikan, 2004). From present study it is clear that salt stress markedly altered the chlorophyll fluorescence parameters in salt stressed rice plants however, exogenous nitric oxide treatment improved the chlorophyll fluorescence attributes. The protective role of nitric oxide on chlorophyll fluorescence attributes of salt stressed plants could be due to nitric oxide induced improvement of photochemical efficiency and protection of reaction centre at photosystem II (Wodala et al., 2008; Wu et al., 2010). Exogenous nitric oxide treatment protects the photosynthetic machinery from salt induced oxidative damage which results in improved photosynthetic rate (Ruan et al., 2002; Fan et al., 2007), and also in mitochondria, the synthesis of ATP and two respiratory electron transport systems (Yamasaki et al., 2001; Zottini et al., 2002).

Nutrient accumulation pattern is important not only for proper functioning of various metabolic processes but also for osmotic adjustment and turgor maintenance in plant cells (Flowers, 1985; Moghaieb et al., 2004; Ashraf et al., 2010a). Under saline stress, higher accumulation of Na$^+$ and Cl$^-$ ions results in stunted growth and reduced biomass production. Excessive accumulation of Na$^+$ ions negatively affects the uptake other essential nutrients such as Ca$^{2+}$ and K$^+$, as Na$^+$ is their competitor for being uptake. K$^+$ is essentially required for maintaining proper activity of various enzymes while Ca$^{2+}$ is an integral part of membrane thus both of these cations have important and essential role in plant growth and development (Bhandal and Malik, 1988; Lauchli, 1990; Alam, 1999). In present investigation nitric oxide treatment reduced the uptake of Na$^+$ and Cl$^-$ ions while increased that of Ca$^{2+}$ and K$^+$. The signaling role of nitric oxide could be a cross talk between nitric oxide and Ca$^{2+}$, as nitric oxide can act as Ca$^{2+}$ mobilizing messenger (Besson-Bard et al., 2008). Number of reports have described that improved salt tolerance due to exogenous nitric oxide treatment could be due to its interplaying with other signals in various plant systems (Uchida et al., 2002; Zhao et al., 2004; Zhang et al., 2006; Liu et al., 2007; Zhao et al., 2007; Tanou et al., 2009a; Tanou et al., 2009b; Wang et al., 2009). The regulatory role of nitric oxide has been reported in maintaining K$^+$/Na$^+$ homeostasis and PM H$^+$-ATPase abundance under NaCl stress (Zhao et al., 2004, 2007). Furthermore the activity of H$^+$-pumps, Na$^+$/H$^+$ antiporter in the tonoplast, and the activities of antioxidants system were also reported to be enhanced by
the application of sodium nitroprusside (SNP) a nitric oxide donor, in salt-stressed plants of citrus and maize (Zhang et al., 2006; Tanou et al., 2009).

Nitric oxide has been reported to protect plant cell from oxidative damage not only by regulating cellular redox homeostasis by enhancing the activities of ROS scavenging enzymes (Lamattina et al., 2003; Ashraf, 2004; Shi et al., 2007; Zheng et al., 2009), but could itself acts as an antioxidant when applied at lower concentrations (Zhang et al., 2006; Hasanuzzaman et al., 2011) due to its nature as a radical, which could react with ROS. Exogenous nitric oxide treatment has been reported to alleviate the adverse effects of salt-induced oxidative damage in seedlings of lupin (Kopyra and Gwozdz, 2003), and cucumber (Fan et al., 2007; Yu-qing et al., 2007). In contrast, higher nitric oxide concentrations have been reported to inhibit growth and development in various crop plants (Zhang et al., 2003; Kopyra & Gwozdz, 2003). Exogenous application of higher nitric oxide levels are toxic for plants as it has been reported to cause fragmentation of DNA and membrane damage (Pedroso et al., 2000; Yamasaki, 2000; Romero-Puertas et al., 2004). Furthermore, it has also been reported that higher concentrations of nitric oxide could result in senescence at whole plant level, inhibited cell division and apoptosis, in addition it could inhibit the activities of various enzymatic and non enzymatic antioxidants which could lead to higher production of reactive oxygen species (ROS) including H\textsubscript{2}O\textsubscript{2} (Bethke et al., 2007).

To minimize the oxidative damage, various protective roles of nitric oxide have been described such as decreased ROS production rate, membrane permeability, intracellular CO\textsubscript{2} concentration, malondialdehyde (MDA) and H\textsubscript{2}O\textsubscript{2} content under salt stress by inducing excessive ROS scavenging through increased activity of enzymatic antioxidants such as peroxidises (POD), CAT, ascorbate peroxidase (APX) and increased proline accumulation (Kopyra and Gwozdz, 2003; Fan et al., 2007; Yu-quing et al., 2007; Shi et al., 2007; Sheokand et al., 2008; Lopez-Carrion et al., 2008; Guo et al., 2009). In present study the activities of different antioxidant enzymes were increased in salt stressed rice plants with exogenous application of nitric oxide. Chang-li et al., (2011) have reported that nitric oxide could regulate the activity of antioxidant enzymes thus could improve the salt stress tolerance by eliminating ROS in seedlings of Brassica campestris. Similarly according to Zeng et al., (2011) exogenous application of nitric oxide treatment markedy increased the activities of POD, SOD and APX, while decreased the MDA content in Brassica juncea seedlings under
NaCl stress. Increased activities of various enzymatic and non-enzymatic antioxidants have been reported due to exogenous nitric oxide treatment in salt stressed plants of barley (Li et al., 2008), wheat (Hai-Hua et al., 2005), cucumber (Shi et al., 2007) and chickpea (Sheokand et al., 2010). The protective role of nitric oxide against salt induced oxidative damage has been reported due to its nature as a signaling molecule, which activates the defense system comprising of antioxidants enzymes (Huang et al., 2002; Shi et al., 2005). In present study exogenous nitric oxide treatment decreased the malondialdehyde (MDA) and H$_2$O$_2$ content in salt stressed rice plants. This could be due to the ability of nitric oxide to act as an antioxidant, which readily reacts with ROS, reducing lipid peroxidation and help in protecting plants from the damaging effect of salt induced oxidative damage (Sheokand et al., 2010; Hasanuzzaman et al., 2011).

Overall, in present study exogenous nitric oxide treatment improved the growth and yield of rice plants by improving photosynthetic pigments content, gas exchange attributes, K$^+$/Na$^+$ ratio, activity of different antioxidant enzymes and by reducing the membrane lipid peroxidation and H$_2$O$_2$ content under saline conditions. Finally it is concluded that exogenous application of nitric oxide can be used as an effective mean to alleviate the salt induced adverse effects on rice plants.
Chapter 5
Summary

Rice is one of the most important cereal crops and rice grain is the largest source of human caloric uptake, consumed by more than half of the World population as food. Improved growth and yield of this important crop plant is required for assuring food security for the growing world population. However, like many other abiotic stresses, salt stress is a serious threat to crop productivity and rice plant is highly sensitive to salt stress. Nitric oxide is an important signaling molecule which is known to have a role in growth, development and stress tolerance in a number of crop plants. Therefore the aim of present study was to assess, whether exogenously applied nitric oxide have role in alleviating the adverse effects of salt stress on growth of rice plants. For this purpose, pot experiments were conducted in the net-house of botanical garden of the University of Agriculture, Faisalabad, Pakistan during 2010-2011. Total four rice cultivars were used in this experiment, two of them were fine rice cultivars (Shaheen Basmati and Basmati PB-95) and two were coarse rice cultivars (KS-282 and IRRI-6). Two salt treatments, control (0 mM) and 80 mM of NaCl were used in this experiment. Three different levels (0, 0.1, 0.2 mM) of sodium nitroprusside (nitric oxide donor) were applied both as pre-sowing seed treatment and foliar spray. And data for different growth, biochemical and physiological attributes of salt stressed rice plants were collected. From the results of the present experiment it is clear that salt stress markedly reduced the fresh and dry biomasses (both of shoot and root), chlorophyll content, gas exchange attributes \((A, E, g, \text{ and } C_i)\), chlorophyll fluorescence attributes, root and shoot \(K^+\) and \(Ca^{2+}\) uptake, hundred grain weight and total grains weight per plant in salt stressed plants of all four rice cultivars. While NaCl stress significantly increased the \(Cl^-\) and \(Na^+\) uptake (both in root and leaf), free proline, malondialdehyde (MDA), \(H_2O_2\) content and activities of POD, CAT, and SOD. Exogenous nitric oxide treatment was effective in improving shoot and root biomasses (both fresh and dry), chlorophyll content, gas exchange attributes, chlorophyll fluorescence attributes. Exogenous nitric oxide treatment further increased the activities of different antioxidant enzymes (POD, CAT and SOD), while decreased the MDA and \(H_2O_2\) content in salt stressed rice plants. Of nitric oxide levels, 0.1 mM was relatively more effective in improving fresh and dry biomasses, chlorophyll content, activity of
antioxidant enzymes and gas exchange attributes under saline conditions, however both levels (0.1 and 0.2 mM) were equally effective under control conditions. Of rice cultivars Shaheen Basmati and IRRI-6 performed better for growth, chlorophyll content, gas exchange attributes and for activity of antioxidant enzymes, while cultivars KS-282 and IRRI-6 showed higher hundred grains weight and total grains weight per plant.

**Conclusions**

1. Salt stress markedly inhibited the seed germination rate, growth and physiological attributes in plants of all four rice cultivars.
2. Exogenous nitric oxide application as pre-sowing seed treatment significantly improved the seed germination parameters, growth, chlorophyll content and gas exchange attributes in salt stressed plants of all four rice cultivars.
3. Of various levels (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM) of nitric oxide, 0.1 and 0.2 mM were most effective in improving seed germination rate and early seedlings growth of rice plants under salt stress.
4. The protective effect of nitric oxide on chlorophyll pigment and photosynthetic rate was positively correlated with improved vegetative growth of salt stressed rice plants.
5. Decreased MDA and H₂O₂ content indicates that exogenous nitric oxide treatment protected rice plants from salt induced oxidative damage due to nitric oxide-induced activation of antioxidant enzymes, and also due to its rapid reaction with excessively generated ROS.
6. Exogenous nitric oxide treatment increased the uptake of essential nutrients (Ca²⁺ and K⁺) and maintained higher K⁺/Na⁺ ratio, while decreased the Cl⁻ and Na⁺ uptake and helped rice plants in reducing the Na⁺ toxicity.
7. Of rice cultivars, Shaheen Basmati and IRRI-6 performed better for various growth, physiological and biochemical attributes, while KS-282 and IRRI-6 showed higher grain yield.
8. Of nitric oxide levels (0.1 and 0.2 mM), 0.1 mM was relatively more effective under saline conditions.
9. Overall, nitric oxide induced growth improvement in salt stressed rice plants was found to be positively correlated with increase in chlorophyll content, photosynthetic
rate, K+/Na+ ratio, stability of reaction centre at photosystem II and activity of antioxidant enzymes, while decrease in MDA and H2O2 content.

Future Prospects

Low agricultural productivity due to increased soil salinity is a serious threat for global food security. Although considerable achievement has been made in enhancing crop salt tolerance by practicing various strategies, however, salt induced loses to crop productivity is still a major limitation for sufficient food supply to rapidly growing world population. It is now well established through number of reports that exogenous application of different compatible solutes and plant growth regulators (PGRs) is an effective shotgun approach in improving salt tolerance in various crop plants. Nitric oxide, which is an important signaling molecule, is also considered a non-traditional plant growth regulator. That has a regulatory role in improving plant growth and development. It is evident from present study that salt induced inhibitory effect on growth and yield of rice plants can be counteracted by exogenous application of nitric oxide. Since very low concentrations of nitric oxide are effective in improving growth, the commercial use of nitric oxide on crop plants would also be cost effective and economically feasible. However, nitric oxide is a highly reactive gaseous molecule and its higher concentrations are toxic for plants, so the effective lower levels of nitric oxide are still required to be optimized for different crop plants. Apart from dose optimization the affectivity of nitric oxide would also depend upon its mode of application, growth stage at which it is applied and duration of application. In addition the efficacy of exogenous nitric oxide application is still required to be tested under field conditions to confirm its agronomic use for enhancing salt tolerance in specific crop plants. Salt stress is a major constraint for optimum growth and crop productivity due to salt induced oxidative damage to photosynthetic machinery. Improved growth in crop plants is attributed to increased photosynthetic rate which mainly depends upon chlorophyll content. Nitric oxide induced improvement in growth of salt stressed rice plants was found to be positively correlated with increased chlorophyll content and photosynthetic rate. So the mechanism of nitric oxide involvement in increased chlorophyll biosynthesis is still remained to be elucidated. Both pre-sowing seed treatment and foliar application of nitric oxide were used in this experiment, however, its addition through rooting medium could also be equally effective in improving growth and physiological attributes of salt stressed rice plants, the
mechanism of which needs to be appraised. Although the exogenous nitric oxide application (both pre-sowing seed treatment and foliar spray) was effective in improving growth and physiological parameters of rice plants, but the factors and conditions that effect the uptake of exogenously applied nitric oxide were not studied in this experiment. Thus further studies are recommended to understand the mechanism of nitric oxide uptake under various environmental factors and conditions. Apart from its role as a growth regulator, nitric oxide is also involved in regulating various physio-biochemical processes due to its signaling properties such as nutrient uptake, ABA induced stomatal movement and activation of antioxidant defense system. The signaling role of nitric oxide has been described in many reports, its signaling properties could be a cross talk between nitric oxide and Ca$^{2+}$, as nitric oxide can act as Ca$^{2+}$ mobilizing messenger but the mechanism involved in nitric oxide signaling, still remains to be elucidated. The regulatory role of nitric oxide has also been described in controlling the process of senescence and maturation, suppression of floral transition and lateral root formation in plants. There are many reports in literature regarding cross talks or interplay between nitric oxide and other phyto-hormones, but further studies are recommended for getting sufficient knowledge of interactions between nitric oxide and other phyto-hormones, especially under salt stress.

Development of transgenic plants through genetic engineering is an important strategy that could help in improving stress tolerance in crop plants. A number of transgenic plants have been developed with increased salt tolerance in different crop plants. Due to its importance as potential growth regulator and its role in salt tolerance, over-production of nitric oxide through manipulation of responsible gene (s) could improve salt tolerance in various crop plants. Thus, genetic manipulation in different crop plants with the goal to increase the endogenous nitric oxide generation is an important physio-genetic issue, which needs further research for improving crop salt tolerance.

Although nitric oxide has been found effective in improving plant growth and development, it is still required to bridge the gaps in information of nitric oxide perception, its physio-biochemical responses and mechanism of its signaling and its role in other signal transduction pathways under salt stressed conditions. Therefore, detailed biochemical, genetic and molecular level studies are still need to be carried out to explore different steps involved, including nitric oxide signaling in plants subjected to NaCl stress.
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