PROTEIN OXIDATION IN RESPONSE TO NITROGEN STRESS IN WHEAT SEEDLINGS

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PROTEIN OXIDATION IN RESPONSE TO NITROGEN STRESS IN WHEAT SEEDLINGS

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CERTIFICATE

This is to certify that the thesis entitled “Protein Oxidation in Response to Nitrogen Stress in Wheat Seedlings” submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi in partial fulfillment of MASTER of SCIENCE in PLANT PHYSIOLOGY, embodies the results of bonafide research work carried out by DEEPIKA KUMAR UMESH, Roll No. 4907 under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

This assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by her.

Date: June 30, 2011
Place: New Delhi-12

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Advisory committee
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INTRODUCTION
1. INTRODUCTION

All animals need oxygen for efficient production of energy in mitochondria. Aerobes survive only because they have evolved well developed antioxidant defenses during evolution. Plants do not always grow in optimum conditions during their life cycle, but suffer many adverse situations that cause different types of stresses, and prevent them from reaching maximum development. Stress is considered as a change in any environmental factor that has an impact on the plant by affecting its biochemical and physiological response to such changes, and may on occasions lead to damage or injury (Tuteja et al., 2010).

Plant faces both abiotic and biotic stress. Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. Whereas, a biotic stress would include such living disturbances as fungi or harmful insects. Abiotic stress is essentially unavoidable and the most harmful factor concerning the growth and productivity of crops worldwide. All kinds of stress increases production of reactive oxygen species (ROS) which can damage the cell (Cakmak et al., 2005).

ROS are therefore the unwelcome companions of aerobic life. In contrast to O$_2$ these are partially reduced or activated derivatives of O$_2$ [singlet oxygen ($^{1}$O$_2$), superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicle (HO')]. These are highly reactive and toxic and lead to oxidative destruction in the cell. Reactive oxygen species (ROS) are produced continuously in chloroplasts, peroxisomes and mitochondria as by products of respiration and photosynthesis and can also be produced as the end product of certain enzymes such as NADPH oxidase. In presence of metals, even mild oxidants such as H$_2$O$_2$, can result in modification of amino acid side chains of proteins to form reactive carbonyls via the metal catalyzed protein oxidation pathway (Cadenzas et al., 2000).

During stress there is an increase in the level of oxidized proteins due to an increased production of reactive oxygen species (ROS) and decline in the capacity to remove these ROS. Thus, there is an increased susceptibility of the proteins to oxidative attack and the oxidized proteins can be carbonylated. The carbonyl derivatives so formed by oxidative attack on amino acid side chains are irreversible and unrepairable. It inhibits or alters the activity of the protein or increases its susceptibility to proteolytic attack. It is an important factor in protein function and removal. The mechanism of degradation of these proteins under various stresses is unclear. Many studies have indicated that highly selective 26S proteasomal complex may be involved in the
degradation of oxidatively modified proteins and this is ATP and ubiquitin dependent (Grune et al., 1995; Velrich et al., 1999; Dunlop et al., 2002; Shringarpure et al., 2003). The direct role of proteasome only in protein degradation has been shown only in few studies. Another system that could degrade carbonylated/oxidized proteins in bulk is autophagy, either macro, micro or chaperone mediated (Cuvervo et al., 2004).

Developmental studies with Arabidopsis indicated that carbonylated proteins can be degraded in vivo. The protein carbonyls were during vegetative phase and content decreased dramatically as plants began reproductive development (Johansson et al. 2004). Interestingly, different pathways may operate/participate in plants for degradation of protein carbonyls under different conditions. In wounded tobacco leaves the ubiquitin-proteasome system has been shown to be involved in wound signaling (Ito et al., 1999).

In detached Arabidopsis leaves carbonylated proteins were rapidly and selectively degraded when kept in the dark (Jain et al., 2008). Under oxidative stress, proteins were oxidized extensively by highly reactive and toxic reactive oxidative species, and these damaged, oxidized proteins were degraded by autophagy. Application of hydrogen peroxide or the reactive oxidative species inducer methyl viologen also induced macroautophagy in Arabidopsis thaliana (Xiong et al. 2006, 2007).

Nitrogen is an important component of many important structural, genetic and metabolic compounds in plant cells. It is a major component of chlorophyll and amino acids. Nitrogen is also a component of energy-transfer compounds, such as ATP (adenosine triphosphate) which allow cells to conserve and use the energy released in metabolism, as well as of nucleic acids. It is also one of the most abundant elements on earth but nitrogen deficiency is probably the most common nutritional problem affecting plants worldwide (Abrol et al., 1999). Almost 60% of the soils in India are deficient in nitrogen. This deficiency of nitrogen (stress) seriously affects plant growth, yield and quality. In terms of quality the protein content goes down. When plant faces nitrogen stress, it is possible that due to stress inspite of low protein content of tissues, they might be oxidised and carbonylated leading to increased degradation in such plants facing nutrient stress (Jain et al., 2008).

Cytokinins, a group of mobile phytohormones, play an important role in plant growth and development (Hirose et al., 2007). Inorganic nitrogen sources such as nitrate are major factor regulating gene expression of adenosine phosphate-isopentenyl transferase (IPT), a key enzyme of cytokinin biosynthesis (Hirose et al., 2007). N-assimilation pathway genes
expression (Jain et al., 2005; Yu et al., 1998 and Frenshi et al., 2009; Lu et al., 2009) indicate the critical role played by cytokinins in balancing acquisition and distribution of the nutrient-N within the plants. The trans zeatin (TZ) type cytokinins in xylem and N6-(A2-isopentenyl) adenine (ip)-type cytokinins in phloem sap suggests that in addition to acting as local signals, cytokinins communicate long distance signals. Recent studies have indicated the role of cytokinins in preventing degradation of protein carbonyls. Disruption of cytokinin flux to detached leaves probably triggers the selective degradation of carbonylated proteins via the proteasome pathway (Jain et al., 2008). There is also a very close relationship between availability of nitrogen, its assimilation and cytokinin levels in plants. So, there may be role of nitrogen nutrition in protein degradation and turnover. The results may have implications for the control of protein mobilization in response to changes in N availability.

In view of the above we hypothesize the low level of cytokinins, in vivo may lead to protein degradation. Studies were conducted in wheat genotypes with PBW-343 (Triticum aestivum, 2n=42, AABBDD) was studied with the following objectives:

1) To study protein carbonylation in wheat seedlings in response to nitrogen stress.

2) To correlate the level of cytokinins expression and its effect on protein oxidation.
BACKGROUND
2. BACKGROUND

The response to the level and forms of nitrogen applied and the nutrition stress experienced by the wheat plants has not been investigated extensively. It is a wide spread notion that plant growing under any kind of abiotic or biotic stresses have increased generation of ROS leading to increased oxidation of macromolecules including proteins. These proteins can be irreversibly oxidized (carbonylated), if the plant faces stress or if they are in excess, and are marked for degradation. Our earlier studies have indicated that cytokinins play an important role in preventing the degradation of proteins under specific kind of abiotic stress (Jain et al., 2008).

This chapter briefly reviews the information on various aspects mentioned above viz, generation of ROS during stress, fate of oxidized proteins, their pathways of degradation and the effect of N supply on the level of proteins.

2.1 Generation of Reactive oxygen species in plants

If the external conditions like salinity, cold, heat, nutritional stress occurs the entire chloroplastic, peroxisomal and mitochondrial superoxide radical and H$_2$O$_2$- scavenging system of the plant may collapse, leading to increased ROS generation, and oxidation of the macromolecules. Over the last few decades, achieving sustainability in agriculture has emerged as a major goal to fulfil the requirements of enough food to feed a rapidly increasing world population in changing environmental conditions. Various biotic and abiotic stress factors continue to negatively affect various aspects of plant growth, development and productivity (Anjum et al., 2010). In fact, the relative decrease in potential maximum yields caused by biotic and abiotic stress factors varies between 54 and 82% (Bray et al., 2000; Cakmak, 2005).

Reactive oxygen species (ROS) are partially reduced or excited forms of atmospheric oxygen (O$_2$) continuously produced in cells during aerobic metabolism (Halliwell and Gutteridge, 1989). They can cause extensive cell injury or death, but they play a central role in many signaling pathways in plants involved in stress perception, photosynthesis regulation, pathogen response, programmed cell death, hormonal action, and plant growth and development (Dat et al., 2000; Mittler, 2002; Mullineaux and Karpinski, 2002; Neill et al., 2002; Apel and Hirt, 2004). In Arabidopsis thaliana, a network of at least 152 genes controls ROS metabolism (Mittler et al., 2004). The network is thought to regulate the rates of ROS production and ROS scavenging in the different cellular compartments and to modulate the steady state level of ROS for signaling as well as defense purposes. In leaf cells, an intricate balance exists between H$_2$O$_2$ and O$_2^-$ production in the chloroplast and peroxisome during photosynthesis and the activities of the ROS-scavenging enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (Asada, 1999; Mittler, 2002; Apel and Hirt, 2004). The chloroplast is well equipped to scavenge the H$_2$O$_2$ produced during photosynthesis in
plants grown under controlled conditions or subjected to light stress (Asada and Takahashi, 1987; Asada, 1999; Polle, 2001).

Formation of ROS, therefore, in the cell is an unavoidable consequence of aerobic metabolism. Low concentration of ROS functions in signal transduction leading to activation of defense responses (Mittler 2002). When the concentration of these reactive molecules increase oxidative damage is caused in the cells (Ghezzi and Bonetto 2003). Hence, any kind of stress adversely affect growth and productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Drought, temperature extremes, and saline soils are the most common abiotic stresses that plants encounter. Globally, approximately 22% of the agricultural land is saline (FAO, 2004), and areas under drought are already expanding and this is expected to increase further (Burke et al., 2006).

Overproduction of ROS generated in the plants as a result of various stresses lead to the toxicity and cause damage to proteins, lipids, carbohydrates and DNA. The ROS comprises both free radical (O$_2^-$, superoxide radicals; OH$, $ hydroxyl radical; HO$_2^-$, perhydroxy radical and RO$, $ alkoxy radicals) and non-radical (molecular) forms (H$_2$O$_2$, hydrogen peroxide and O$_2^-$, singlet oxygen). In chloroplasts, photosystem I and II (PSI and PSII) are the major sites for the production of O$_2$ and O$_2^-$.

In mitochondria, complex I, ubiquinone and complex III of electron transport chain (ETC) are the major sites for the generation of O$_2^-$ (Halliwell, 2006). Under steady state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms (Foyer et al., 2005).

The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks. These disturbances in equilibrium lead to sudden increase in intracellular levels of ROS which can cause significant damage to cell structures and it has been estimated that 1-2% of O$_2$ consumption leads to the formation of ROS in plant tissues (Bhattararjee, 2005). Through a variety of reactions, O$_2$ leads to the formation of H$_2$O$_2$, OH$^-$ and other ROS. The ROS comprising O$_2^-$, H$_2$O$_2$, 'OH', HO$_2^-$, OH$^-$, ROOH, ROO$, $ and RO$^-$, are highly reactive and toxic and causes damage to proteins, lipids, carbohydrates, DNA which ultimately results in cell death. Accumulation of ROS as a result of various environmental stresses is a major cause of loss of crop productivity worldwide. ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation (LPO); Foyer et al., 2005.

**2.2 ROS (reactive oxygen species) chemistry**

It is well established that organelles such as chloroplast, mitochondria or peroxisomes with a highly oxidizing metabolic activity or with intense rate of electron flow are a major source of ROS in plant cells. The ability of phototrophs to convert light into biological energy is critical for life on earth and therefore, photosynthesizing organisms are especially at the risk of oxidative damage, because of their bioenergetic lifestyle and the abundance of the photosensitizers and polyunsaturated fatty acids (PUFA) in the chloroplast envelope. This situation leads to oxidative stress. The appearance of O$_2$ in
the atmosphere enabled respiratory metabolism and efficient energy generation systems which use O₂ as final electron acceptor, lead to the formation of ROS in cells (Temple et al., 2005). Although, atmospheric oxygen is relatively non-reactive, it can give rise to ROS which include O₂⁻, H₂O₂, OH⁻ and ¹O₂ (Scandalios et al., 2005). It has been estimated that 1 to 2% of O₂ consumed by plants is sidetracked to produce ROS in various subcellular loci. The single electron reduction of O₂ results in the generation of the O₂⁻. At low pH, dismutation of O₂⁻ is unavoidable, with one O₂⁻ giving up its added electron to another O₂⁻, and then with protonation resulting in the generation of H₂O₂. Furthermore, O₂⁻ can be protonated to form the HO₂⁻. Additionally, in the presence of transition metals such as copper and iron, further reactions take place, e.g. through the Haber Weiss mechanism or the Fenton reaction to give up OH⁻, which is the most reactive chemical species in the biological world. O₂⁻ can also react with another very influential signalling free radical species, NO⁻ to give up peroxynitrite (OONO⁻). ¹O₂ is another form of ROS, but here there is no addition of an extra electron to O₂; rather an electron is elevated to a higher energy orbital, thereby freeing oxygen from its spin-restricted state. ¹O₂ can be formed by photoexcitation of chlorophyll and its reaction with O₂ (Navrot et al., 2007).

2.2.1 Superoxide radicals

The major site of O₂⁻ production is the thylakoid membrane-bound primary electron acceptor of PSI. The production of ROS is an inevitable consequence of aerobic respiration. When the terminal oxidases-cytochrome-c oxidase and the alternative oxidases react with O₂, four electrons are transferred and H₂O is released. However, occasionally O₂ can react with other ETC components. Here, only one electron is transferred, and the result is the O⁻, a moderately reactive ROS with approximately 2e⁻⁴ ms of half-life. It has been noted that O₂⁻ is usually the first ROS to be generated. In plant tissues about 1 to 2% of O₂ consumption leads to the generation of O₂⁻ (Elstner et al., 1987). The O₂⁻ is produced upon reduction of O₂ during electron transport along the non-cyclic pathway in the ETC of chloroplasts and other compartments of the plant cell. Reduction of O₂ to the O₂⁻ can occur in the ETC at the level of PS I. The generation of O₂⁻ may trigger the formation of more reactive ROS like OH⁻, and more possibly ¹O₂ (Beliski et al., 1983), each of which may cause peroxidation to membrane lipids and cellular weakening.

2.2.2 Hydrogen peroxide (H₂O₂)

The univalent reduction of O₂⁻ produces H₂O₂. H₂O₂ is moderately reactive and has relatively long half-life (1 ms) whereas, other ROS such as O₂⁻, OH⁻ and ¹O₂, have much shorter half-life (Quan et al., 2008). It has been established that excess of H₂O₂ in the plant cells leads to the occurrence of oxidative stress. H₂O₂ may inactivate enzymes by oxidizing their thiol groups. H₂O₂ plays a dual role in plants: at low concentrations, it acts as a signal molecule involved in acclimatory signalling triggering tolerance to various biotic and abiotic stresses and, at high concentrations, it leads to PCD. H₂O₂ has also been shown to act as a key regulator in a broad range of physiological
processes, such as senescence, photorespiration and photosynthesis, stomatal movement, cell cycle and growth and development (Noctor et al., 1998). $\text{H}_2\text{O}_2$ is starting to be accepted as a second messenger for signals generated by means of ROS because of its relatively long life and high permeability across membranes. In an interesting study the response of pre-treated citrus roots with $\text{H}_2\text{O}_2$ (10 mM for 8 h) or sodium nitroprusside (SNP; 100 mM for 48 h) was investigated to know the antioxidant defence responses in citrus leaves grown in the absence or presence of 150 mM NaCl for 16 d. It was noted that $\text{H}_2\text{O}_2$ and SNP increased the activities of leaf SOD, CAT, APX and GR along with the induction of related-isoform(s) under non-NaCl-stress conditions. Salinity induced reduction in the ASH redox state was partially prevented by $\text{H}_2\text{O}_2$ and SNP pre-treatments, on the other side the GSH redox state was increased by SNP under normal and NaCl-stress conditions. Moreover, NaCl-dependent protein oxidation was totally reversed by pre-treatments with $\text{H}_2\text{O}_2$ and SNP (Bright et al., 2006).

**2.3 Damage to cells due to ROS production**

**2.3.1 Lipid peroxidation (LPO)**

The peroxidation of lipids is considered as the most damaging process known to occur in every living organism. Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction under various stresses. Now, it has been recognized that during LPO, products are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, MDA, etc and compounds related to them. Some of these compounds react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS). LPO, in both cellular and organelle membranes, takes place when above-threshold ROS levels are reached, thereby, not only directly affecting normal cellular functioning, but also aggravating the oxidative stress through production of lipid-derived radicals (Montilett et al., 2005).

The overall process of LPO involved three distinct stages: initiation, progression and termination steps. Initiation step involves transition metal complexes, especially those of Fe and Cu. However, $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2$ are capable of initiating the reactions but as $\text{OH}^\cdot$ is sufficiently reactive, the initiation of LPO in a membrane is initiated by the abstraction of a hydrogen atom, in an unsaturated fatty acyl chain of a polyunsaturated fatty acid (PUFA) residue, mainly by OH$^\cdot$. In an aerobic environment, oxygen will add to the fatty acid at the carbon-centred lipid radical to give rise to a ROO$^\cdot$. Once initiated, ROO$^\cdot$ can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from adjacent PUFA side chains. The resulting lipid hydroperoxide can easily decompose into several reactive species including: lipid alkoxyl radicals, aldehydes (malonyldialdehyde), alkanes, lipid epoxides, and alcohols (Davies et al., 2001; Fam et al., 2003). A single initiation event thus has the potential to generate multiple peroxide molecules by a chain reaction. The overall effects of LPO are to decrease membrane fluidity; make it easier for phospholipids to exchange between the two halves of the bilayer, increase the leakiness of the membrane to substances that do not normally cross it other
than through specific channels and damage membrane proteins, inactivating receptors, enzymes, and ion channels.

2.3.2 Damage to deoxyribonucleic acid (DNA)

Plant genome is very stable but its DNA might get damaged due to the exposure to biotic and abiotic stress factors which might damage the DNA, and thereby exerts genotoxic stress (Valko et al., 2006)). Endogenously generated damage to DNA is known as “spontaneous DNA damage” which is produced by reactive metabolites (OH−, O2− and NO−). High levels of ROS can cause damage to cell structures, nucleic acids, lipids and proteins. It has been reported that OH− is the most reactive and cause damage to all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone. 1O2 primarily attacks guanine, and H2O2 and O2− don’t react at all. ROS is capable of inducing damage to almost all cellular macromolecules including DNA which includes base deletion, pyrimidine dimers, cross-links, strand breaks and base modification, such as alkylation and oxidation (Tuteja et al., 2009). DNA damage results in various physiological effects, such as reduced protein synthesis, cell membrane destruction and damage to photosynthetic proteins, which affects growth and development of the whole organism. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability.

2.3.3 Protein oxidation

Proteins comprise approximately 68% of the dry weight of cells and tissues and are therefore, potentially major targets for oxidative damage. Two major types of processes can occur during the exposure of proteins to UV or visible light. The first of these involves direct photooxidation arising from the absorption of UV radiation by the protein, or bound chromophore groups, thereby generating excited states (singlet or triplets) or radicals via photo-ionisation. The second major process involves indirect oxidation of the protein via the formation and subsequent reactions of singlet oxygen generated by the transfer of energy to ground state (triplet) molecular oxygen by either protein-bound, or other, chromophores (Davies, 2003).

2.3.3.1 Oxidation reaction of various amino acids

2.3.3.1.1 Reaction with tryptophan (Trp) residues: Initial reaction of 1O2 with Trp is believed to yield either a dioxetane across the C2–C3 double bond or a hydroperoxide at C3. Subsequent decomposition of these intermediates via C2–C3 bond cleavage has been reported to give N-formylkynurenine, whereas ring closure yields 3a-hydroperoxypyrroloindole and 3a-hydroxypyrroloindole. Decomposition of 3a-hydroperoxypyrroloindole and 3a-hydroxypyrroloindole also yields N-formylkynurenine (Saito et al., 1977; Langlois et al., 1986).

2.3.3.1.2 Reaction with tyrosine residues: For the free amino acid it has been shown that the cyclised product 3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indol-2-carboxylic acid (HOHICA)
is formed on reaction with $^1\text{O}_2$. This product is believed to arise via the initial formation of an unstable endoperoxide, which subsequently undergoes ring opening to give a C-1 hydroperoxide, and cyclised products via the involvement of the a-aminogroup (Katsuia et al., 1988; Jin et al., 1995; Criado et al., 1998; Wright et al., 2000, 2002).

### 2.3.3.1.3 Reaction with histidine (His) residues:

Oxidation of free His by $^1\text{O}_2$ occurs via the initial formation of one or more endoperoxides and the consumption of a single molecule of O$_2$ per mole His. These reactions, which involve ring opening, consume further molecules of O$_2$, possibly via radical reactions. These materials have been shown to undergo a range of further reactions, including the formation of His–His and His–Lys cross-links via the reaction of nucleophilic nitrogen on one His ring, or a Lys side-chain, with a keto group on a second oxidised His (Kai et al., 1996; Chang et al., 1997).

### 2.3.3.1.4 Reaction with methionine (Met) residues:

Oxidation of free Met occurs via the formation of a zwitterionic species that undergoes subsequent reaction with a second molecule of Met to give two moles of the sulfoxide. The stoichiometry of O$_2$ to Met consumption varies with pH, with this being 1:1 above pH 7 and 1:2 below pH 5. The overall stoichiometry, including subsequent dark reactions, is 1 mol of Met and molecular O$_2$ consumed to give 1 mol each of sulfoxide and H$_2$O$_2$. With some sensitisers, other intermediates including a nitrogen–sulphur cyclic intermediate have been reported. Subsequent hydrolysis of this species gives the same products and ratios (Ando et al., 1985).

### 2.3.3.1.5 Reaction with cysteine (Cys) and cystine residues:

Free Cys reacts rapidly with $^1\text{O}_2$ to give the disulphide, though in a non-quantitative manner. The remaining products have not been fully elucidated, though these are likely to include cysteic acid. The yield of these products would be expected to vary dramatically on going from the free amino acid. Qualitative studies have suggested that reaction of $^1\text{O}_2$ with free cystine also occurs via a similar zwitter ions. This species is then believed to react with a further molecule of cystine to give rise to two molecules of RSS(=O)R (Shen et al., 1996).

### 2.3.4 Reversible oxidation:

Proteins are modified in a variety of ways by ROS, some direct, others indirect e.g. by conjugation with breakdown products of fatty acid peroxidation. Reversible oxidation of cysteine and methionine side chains is an important mechanism for regulating enzyme activity (Moller et al., 2003). Perception of H$_2$O$_2$ could involve chemical modification of amino acid side chains in cellular proteins. Cysteine residues are generally considered to be the archetypal redox-regulatory amino acid as reversible oxidation of the thiol group to form disulfides, or in some cases sulfenic acid (–SOH) or sulfinic acid (–SO$_2$H), is recognized to operate in redox signalling of a variety of proteins (Fourquet et al., 2008). However, Met residues in proteins can also be highly susceptible to modification by mild oxidants (Vogt et al., 1995). It has been demonstrated that Met oxidation occurs primarily on the surface of proteins and correlates closely with the solvent-exposed sulfur
surface area (Griffith et al., 2002). Consequently, small changes in protein conformation can influence the susceptibility of specific Met residues to oxidation, and static structural analysis can often not accurately predict the degree to which specific residues are solvent exposed (Yin et al., 2000). Interestingly, oxidation of Met to the sulfoxide MetSO converts the side chain of this amino acid from hydrophobic to polar and hydrophilic (Cho et al., 2004). This dramatic change in the chemical nature of the residue, coupled with the fact that oxidation is reversible (Kim et al., 2007; Tarrago et al., 2009), makes this modification of Met of potential regulatory significance. However, whether the rate of Met oxidation is rapid enough to function in redox signalling has been questioned (Autreaux et al., 2007). Although, there is some debate about whether the rate of Met oxidation is rapid enough to function in redox signalling, it is clear that MetSO exists in vivo. Thus, MetSO is likely to play a role in ROS responses, but perhaps other mechanisms also play a role in the early and rapid responses. It is important to understand the impact of MetSO on protein function to develop a fuller understanding of ROS responses. Much of what is known about the significance of Met oxidation has come from animals (Black et al., 1991). Structural changes can be induced as a result of Met oxidation that paradoxically result in an overall increase in the surface hydrophobicity of the protein (Chao et al., 1997). It can have a large impact on protein functionality, including interactions with other proteins. For example, the oxidation of specific Met residues attenuates binding of calmodulin to clients and is thought to play a role in downregulation of energy metabolism during stress and in altered calcium metabolism in aging animals (Bigelow et al., 2008). Furthermore, a link between Met oxidation and post-translational modification of proteins involving phosphorylation has recently emerged with the demonstration that Met oxidation can alter the activities of protein phosphatases and protein kinases. The activity of calcineurin, a calcium/calmodulin activated Ser/Thr protein phosphatase, is reduced by oxidation of Met residues in the calmodulin-binding domain of the enzyme (Carruthers et al., 2008), whereas CaMKII (Ca\(^{2+}\)/calmodulin-dependent protein kinase II), a Ser/Thr protein kinase, is activated by oxidation of Met residues in the autoinhibitory domain of the enzyme (Erikson et al., 2008).

2.3.5 Irreversible oxidation: Reactive oxygen species (ROS) may also cause irreversible carbonylation of proteins, resulting in structural and/or functional modifications. Presence of free metal cations, redox active or inactive, in mitochondria may significantly contribute to the initiation and perpetuation of oxidative stress. One of the best described mechanisms for metal linked damage is metal-catalyzed oxidation (MCO) of proteins, which involves the oxidation of susceptible amino acids such as Arg, Lys, Pro, and His (Stadtman, 1990). One of the major consequences of MCO is the irreversible formation of reactive carbonyls on amino acid side chains (Stadtman, 1990). Such carbonyls are known to accumulate in the wheat mitochondrial proteome during environmental stress, even more so than in other ROS-producing subcellular organelles of plants (Bartoli et al., 2004).

In the presence of metals, even mild oxidants such as H\(_2\)O\(_2\) can result in modification of amino acid side chains to form reactive carbonyls via the metal-catalyzed protein oxidation pathway.
(Adams et al. 2001). There are also non-oxidative pathways for production of reactive carbonyls in proteins, but protein carbonylation is generally recognized as a marker of oxidative stress and damage (Adams et al. 2001, Dalle-Donne et al. 2003, Ghezzi and Bonetto 2003). In plants, protein carbonylation is readily observed even under normal, non-stress conditions, indicating that some proteins are very susceptible to oxidative modification in vivo (Johansson et al. 2004, Kristensen et al. 2004, Job et al. 2005). Protein oxidation is often considered as a valuable marker of the oxidative stress (Shacter, 2000; Shulaev and Oliver, 2006; Møller et al., 2007) since the formation of the carbonyl groups (ketones and aldehydes) requires more stringent oxidation conditions than, for example, the reversible oxidation of thiols (Berlett and Stadtman, 1997; Møller and Kristensen, 2004).

The signature of carbonyl groups can be readily detected by the conjugation of oxidized proteins with 2,4-dinitrophenylhydrazine (DNPH) and their quantitative spectrophotometric measurement (Levine et al., 1994) or by combining the anti-DNP antibody with one or two-dimensional gel electrophoresis which gives more qualitative data (Shacter, 2000).

**Approaches for using DNPH to test for protein carbonyls: immunoassay and spectrophotometric assay (Shacter, 2000).**

ROS-induced protein carbonylation in mammalian cells has been studied extensively because of the serious consequences of protein damage by oxidation in several human diseases (Shacter, 2000). For plants, application of this approach is still very limited because of the natural difficulties in optimization of the separation procedures of intact organelles (chloroplasts, mitochondria, peroxisomes) due to the barrier of the cell wall. Several carbonylated proteins have been identified by mass spectrometry in the soluble mitochondrial matrix fraction of rice leaf mitochondria in vivo or under the controlled oxidative stress in vitro (Kristensen et al., 2004). In response to H$_2$O$_2$ titration of Arabidopsis cell cultures, carbonylation of several proteins, among them mitochondrial, has been confirmed (Sweetlove et al.,
The carbonyl concentration increased in wheat leaf mitochondria due to the oxidative damage under drought stress conditions (Bartoli et al., 2004).

Increased cellular levels of reactive oxygen species are known to occur during seed development and germination. Specific changes in protein carbonylation patterns are probably required for counteracting and/or utilizing the production of reactive oxygen species caused by recovery of metabolic activity in the germinating seeds of Arabidopsis (Job et al., 2005). Mitochondria are the main target for oxidative damage to proteins under well-irrigated and drought conditions (Bartoli et al., 2004). Environmental stresses lead to damage of specific mitochondrial targets through the direct action of reactive oxygen species and indirect action of lipid peroxidation products. Both TCA cycle and photorespiratory capacity of mitochondria are sensitive sites for damage (Taylor et al., 2003).

2.3.6 Pathways for protein degradation: The widely accepted assumption is that protein carbonylation leads to loss of function, and hence those proteins must ultimately be degraded, as there are no repair mechanisms known to replace damaged side chains. There are two major pathways for degradation of damaged proteins.

2.3.6.1 Proteasomal Pathway: Proteins can be degraded via 26S /20S proteasomal pathway. 26S proteasome is a ATP dependent self compartmentalized protease. It has two subunits: 20S catalytic protein which consists of seven heptameric rings having α & β subunits. The other subunit is a 19S regulatory protein which gives the structure its ATP dependence and substrate specificity. This subunit target unfolding and transport (Genschik et al., 1998; Lee et al., 1998).

Proposed scheme for oxidation-induced protein degradation by the 20S proteasome, or fragmentation and aggregation (Davies, 2001).

The ubiquitin/26S proteasome pathway has been implicated in diverse aspects of eukaryotic cell regulation through its ability to rapidly remove intracellular proteins (Hershko and Ciechanover,
In this pathway, proteins destined for degradation first become modified by the covalent attachment of polymeric ubiquitin chains. These chains are assembled on one or more Lys residues within the target via an ATP-dependent reaction cascade involving the sequential action of activating (E1), conjugating (E2), and ligating (E3) enzyme families (Hershko and Ciechanover, 1998). The resulting polyubiquitinated proteins then are recognized by the 26S proteasome and degraded, with the concomitant release of the ubiquitin moieties for reuse. Through this conjugation/degradation cycle, the ubiquitin/26S proteasome pathway controls numerous physiological and developmental events by selectively removing key cell regulators (Hershko and Ciechanover, 1998). Although few of the targets are known in plants, genetic analyses have implicated the pathway in hormone regulation, embryogenesis, photomorphogenesis, circadian rhythms, floral homeosis, stress responses, senescence, and pathogen defense (Callis and Vierstra, 2000; Hellmann and Estelle, 2002). The 26S proteasome is an ATP-dependent, self-compartmentalized protease (Voges et al., 1999; Glickman, 2000). Although most work on this 2-MD complex is derived from yeast and animals, evidence is accumulating that the higher plant version is similar in structure and function (Fu et al., 1998a, 1999a, 1999b; Shibahara et al., 2002). The 26S proteasome can be divided further into two particles, the 20S core protease (CP) and the 19S regulatory particle (RP). The CP is a broadspectrum ATP- and ubiquitin-independent peptidase created by the assembly of four stacked heptameric rings of related subunits in an configuration. The protease active sites within and 5 polypeptides are sequestered in a central chamber. Access to this chamber is restricted by a narrow gated channel created by the subunit rings that allows only unfolded proteins to enter (Glickman, 2000).

2.3.6.2 Vacuolar Autophagy: Carbonylated proteins can also be degraded by vacuolar autophagy by various acid proteases present in the vacuole (Takatsuka et al., 2008). Developmental studies with Arabidopsis indicated that carbonylated proteins can be degraded in vivo. The protein carbonyls were during vegetative phase and content decreased dramatically as plants began reproductive development (Johansson et al. 2004). Interestingly, different pathways may operate/participate in plants for degradation of protein carbonyls under different conditions. Wounding and dark stress in Arabidopsis leaves enhanced degradation of carbonylated proteins via proteasomal pathway (Ito et al., 1999; Jain et al., 2008). In the leaves of the plants exposed to oxidative stress the protein carbonyl were degraded by autophagy (Xiong et al. 2006, 2007).

2.3.7 Inhibitors of these pathways: MG 132 (carbobenzoyl leucinyl leucinyl leucinol) is the known proteasomal inhibitor. Whereas, 3 Methyl adenine (3-MA) is the potent inhibitor of autophagy. It inhibits autophagy by blocking the formation of autophagosomes in tobacco culture cells (Takatsuka et al., 2004).

2.4 Nitrogen level and protein oxidation
Although concerted efforts so far have led to the development of a number of improved cultivars, yield has been virtually static owing to susceptibility of these cultivars to biotic and abiotic stresses, and a limited genetic variability in the cultivar germplasm. Nutrient management may be an important aspect for better plant growth and development and can help to achieve optimum yield of major crop plants growing in varied environmental conditions (Cakmak 2005). The use of plant-mineral nutrients and/or plant hormones and growth regulators are, in fact, potential options which can be used to alleviate a number of abiotic stress-induced effects in plants and to achieve enhanced crop productivity as well in a sustainable way (Marschner 1995; Cakmak 2005).

Nitrogen is an important nutrient. It is component of many important structural, genetic and metabolic compounds in plant cells. It is a major component of chlorophyll, the compound by which plants use sunlight energy to produce sugars from water and carbon dioxide (i.e. photosynthesis). It is also a major component of amino acids, the building blocks of proteins. Some proteins act as structural units in plant cells while others act as enzymes, making possible many of the biochemical reactions on which life is based. Nitrogen is a component of energy-transfer compounds, such as ATP (adenosine triphosphate) which allows cells to conserve and use the energy released in metabolism. Finally, nitrogen is a significant component of nucleic acids such as DNA, the genetic material that allows cells (and eventually whole plants) to grow and reproduce. Despite nitrogen being one of the most abundant elements on earth, nitrogen deficiency is probably the most common nutritional problem affecting plants worldwide.

Nutritional stress caused by the deficiency of certain macro and micronutrient in the soils, or presence of certain forms of nutrient which are toxic to the plants. In our country 60 % of the soils are deficient in nitrogen (Abrol et al., 1999) and the use efficiency of the nitrogen is between 33-50 % depending on the crops. For much of the human history, N supply is the single major cause limiting productivity. A complex chain of events determines the uptake of N, its use in plant production and the efficiency with which it is used.

2.4.1 N levels, forms and ROS generation

All major nutrients, nitrogen, phosphorous and sulphur deprivation trigger distinct redox changes and induce oxidative stress with a rather defined pattern in the context of nutrient-specific alterations in metabolism. (Kandibinder et al., 2004). Deficiency of nitrogen results in nutrient imbalance affecting several metabolic pathways (Abrol et al., 1999) including increased production of ROS (Dominguez-Valdivia et al., 2007). In plants low concentration of ROS functions in signal transduction leading to activation of defense responses (Mittler, 2002), while higher level causes serious oxidative damage to membrane lipids, DNA and proteins (Ghezzi and Bonetto, 2003). Abiotic stress increases the generation of ROS in the cells. The rise of ROS imposes oxidative stress on the plants and to prevent their excessive accumulation plants contain protective enzymes such as superoxide dismutase (SOD), peroxidases (POX) and catalase (CAT) (Agarwal et al., 2005). Decline in the \( \text{H}_2\text{O}_2 \) scavenging
capacity in the flag leaves of kalyansona might be responsible for the oxidative membrane damage and overall metabolic reduction in the leaves of the N stressed plants (Jain et al., unpublished). Elevated carbohydrate levels have been reported for N- limited plants and it has been suggested (Paul and Driscoll 1997) that the primary targets of N limitation are the growing meristems of plants, resulting in severely decreased sink strength and, in turn, leading to the well- characterized downregulation of photosynthetic activity in source leaves (Krapp and Stitt 1995; Koch 1996). This repression of photosynthetic genes affects a host of photosynthetic proteins, including, for example, D1 (Kilb et al. 1996), the light-harvesting chlorophyll a/b- binding protein of photosystem II (LHCII), and Rubisco. This downregulation results in a concerted decrease of various chloroplast functions, including light collection, photosynthetic electron transport, and CO$_2$ fxation. Nevertheless, sudden induction of sink limitation typically resulted in a more rapid decline of mRNA levels for, for example, Rubisco and thylakoid ATP synthase compared with that for LHCII. This might lead to at least a temporary imbalance between light absorption and, for example, Rubisco-based processes. This possibility is consistent with reports that the ratio of Rubisco activity to electron transport activity declines under N limitation (Evans and Terashima 1987). The resultant surplus electron flow could lead to enhanced oxygen photoreduction in the chloroplast (via the Mehler reaction; see Asada 1994) leading to the production of elevated levels of reduced reactive oxygen species. In this case one might also expect chloroplasts of N-limited plants to possess elevated activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR), as well as higher ascorbate contents, since all of these constituents are involved in the detoxification of reduced reactive oxygen species. Increases in the activities/contents of foliar antioxidants in response to other environmental stresses such as low temperatures or high light have been reported (Anderson et al. 1992; Asada 1994; Grace and Logan 1996; Logan et al. 1998a,b). However, it also seems plausible that strong downregulation of the capacity for photosynthetic electron flow could be associated with a lower overall chloroplast antioxidant capacity (irrespective of the partitioning of electrons to oxygen photoreduction). Furthermore, the repression of photosynthetic activity under sink-limiting conditions is not accompanied by decreases in respiratory activity; respiration rates typically remain unchanged or increase (Koch 1996). Therefore, the need for reactive-oxygen scavenging in the mitochondria is likely to be unchanged or increase as a result of sink limitation. Lastly, when considering the effect of N limitation on foliar antioxidant enzymes one must also consider that they are proteinaceous and their production might be constrained like that of other N- based macromolecules in N-limited plants.

2.5 Ammonium (NH$_4^+$) - Nutrition: Ammonium nutrition is of interest as an alternative to that of nitrate. Nitrate salts are highly susceptible to leaching causing a reduction in the N available to plants, detrimental ecological effects such as eutrophication of surface waters and human health problems derived from high nitrate consumption. To minimize leaching of nutrients from soil, it is considered that the use of ammonium together with nitrification inhibitors may reduce the N impact
on aquifers. However, ammonium nutrition turns out to be stressful to many plants including some important crops, leading to a reduced growth (Britto and Kronzucker 2002, Cruz et al. 2006, Gerendas et al. 1997, Lasa et al. 2001, 2002, Mehrer and Mohr 1989). Besides growth reduction, several characteristics of their metabolism – lower content of mineral cations and organic anions and levels of amino acids are altered, resulting in the so-called ‘ammonium syndrome’. After many years of research in ammonium nutrition, several hypotheses have been proposed to explain the toxicity of this nutrient form (Britto and Kronzucker 2002), but none have yet been conclusively confirmed. Endogenous levels of ammonium accumulation and ammonium assimilating enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) have been measured during stress, but it is not possible to establish a relation among them and ammonium tolerance (Cruz et al. 2006, Lasa et al. 2001, 2002).

2.5.1 \(\text{NH}_4^+\) and ROS generation: The implication of ROS during ammonium stress has not yet been well characterized. Studies that combine ammonium and salt stress in sunflower and corn suggest that ammonium or its assimilation molecules, glutamate or glutamine, may serve as a stress signal to activate antioxidant enzymes, which play a key role in adaptation to stress situation (Rios-Gonzalez et al. 2002). Polesskaya et al. (2004) indicated that \(\text{NH}_4^+\) induced antioxidant enzyme activities, and that N deficiency implies an oxidative stress because of an increased Mehler reaction and because of an energetic imbalance related to a reduction in N assimilation. However, they did not observe low-molecular weight antioxidant status or markers of oxidative damage to demonstrate that an oxidative stress was taking place. More recently, Skopelitis et al. (2006) have shown that ammonium ions induce generation of ROS in a study with cell suspensions of Vitis vinifera. However, the cell suspension model may behave differently to the whole plant model, and the analysis of ROS production and its effects in the whole plant deserve attention. Understanding antioxidant metabolism is important because ROS production is increased in many adverse environmental conditions such as drought, high salinity, heat, cold, UV-B radiation and ozone, which may cause a reduction in antioxidant and an increase in oxidative damage detected within the plant cell (Hernandez et al. 1995, Moran et al. 1994, Perl-Treves and Perl 2002). Plants have evolved various protective mechanisms to eliminate or reduce ROS, which includes enzymes such as ascorbate peroxidise (APX), superoxide dismutase (SOD), glutathione reductase (GR), guaiacol peroxidase (GPX) and catalase (CAT). In biological systems, reduced glutathione (GSH) and ascorbate (ASC) appear to be the most important soluble low molecular antioxidants, which can react directly with superoxide and hydroxyl radicals or participate in enzymatic reactions that scavenge ROS (Foyer et al. 2005, Smirnoff 2005). Plant phenolic compounds may also act as antioxidants or prooxidants in different reactions (Ka‘hko‘nen et al. 1999, Moran et al. 1997).

2.6 Effect of N supply and forms on growth of plants
Plants adjust to variations in resource availabilities by variable partitioning to root and shoot growth (Marschner H., 1995; Hodge A., 2009). Among various edaphic factors that affect plant biomass partitioning, nitrogen (N) is particularly important; higher rates of available N shift partitioning from roots to shoots (Hermans C., 2006; Brouwer R., 1962). Studies of partitioning responses to shifts in N supply have revealed a positive linear relationship between shoot:root ratios and the internal N status of plants (Agren et al., 1987; Tan et al., 1998). Plants growing on infertile, low N soils are also reported to have a higher root mass fraction than plants growing on more fertile and N rich soils (Shaver et al., 1991; Tilman et al., 1991). Thus, in natural ecosystems, soil N availability and plant biomass partitioning exhibit strong co-variation.

Biomass accumulation and shoot moisture contents of wheat and maize were lower with NO$_3^-$ than with NH$_4^+$ nutrition. Shoot root ratios of ammonium fed plants were more compared to NO$_3^-$ fed plants in the case of wheat but in the case of maize it was unaffected. Photosynthetic ratios also varied interspecifically in relation to nitrogen nutrition. Organic nitrogen content in the xylem was more in NH$_4^+$ fed plants (Gerner et al., 1993). Differences exist between the responses of wheat and maize growth to NO$_3^-$ and NH$_4^+$ nutrition. Biomass accumulation in wheat is smaller in plants grown on NH$_4^+$ than on NO$_3^-$ nutrition (Haynes et al., 1989).

Nitrogen is important to plant roots because it can be used for producing and altering root biomass (Vogt et al., 1993). Nitrogen usually encourages the production of roots used for absorption (Kolesnikov, 1971). Roots normally abound in areas of higher N concentration. The form of N taken up by a plant may determine the growth pattern and lifespan of fine roots (Vogt et al., 1993). Application of N in the form of ammonium resulted in smaller root biomass and less turnover of roots compared to N in the form of nitrate; the latter form of N supported more root biomass and more rapid root turnover (Smucker, 1984; Aber et al., 1985). Nitrogen fertilizer may be used mainly for aboveground herbage production. This idea is supported by a study done by Holt and Fisher (1960), using Coastal Bermudagrass. Coastal Bermudagrass was treated with annual N applications ranging from 0 to 1,600 pounds per acre. Total plant mass for those plants subjected to the highest N rate was significantly higher than plants subjected to the lowest N rates. When plants were fertilized at the highest N rate, roots accounted for only 26% of the plant mass, and the root to shoot ratio was 1:2.78. For plants that received no N, roots contributed 76% of the dry weight, and the root to shoot ratio was 1:0.32. The highest N rate did not retard root growth, but it increased root growth and development only slightly.

The ability to respond to localized nitrate supplies by proliferating lateral roots within the nitrate-rich zone is a property common to many species of plants (Robinson, 1994; Hodge, 2004). In barley, this ability is due to a combination of increased numbers of lateral roots and increased rates of lateral root elongation (Drew and Saker, 1975). In Arabidopsis, the primary effect of a localized nitrate treatment was to stimulate lateral root elongation (Zhang and Forde, 1998; Linkohr et al., 2002), with one report indicating a small localized increase in lateral root numbers (Linkohr et al.,
This stimulation of lateral root elongation appears to be attributable to a signalling effect from the NO$_3$ ion itself rather than to a downstream metabolite (Zhang and Forde, 1998; Zhang et al., 1999). Nitrate stimulates lateral root elongation by increasing rates of cell production in the root tips directly exposed to the signal (rather than through any effect on cell elongation) (Zhang et al., 1999).

$\text{NH}_4^+$-$\text{N}$ nutrition stimulated cluster root formation and $\text{H}^+$ extrusion in P-deficient lupine (Sas et al., 2002). Nitrate is required to maintain biosynthesis and/or root to shoot transfer of cytokinins at a level that is sufficient to mediate normal leaf morphogenesis (Walch-Liu et al., 1999). Development of leaf area is a major determinant of canopy photosynthesis and plant productivity (Monteith 1977). With an increase in total leaf area per unit ground area (referred to as leaf area index [LAI]), more photons are absorbed by the canopy and the canopy photosynthesis would increase. However, leaf area development is strongly constrained by the availability of nitrogen (e.g., Hirose and Kitajima 1986; Kuers and Steinbeck 1998). Anten et al. (1995) derived the optimal LAI that maximizes canopy photosynthesis for a given nitrogen availability in the stand. They showed that the optimal LAI is indeed smaller at lower N availabilities even though leaves in the lowest layer receive PPFD much higher than the light compensation point ( Hirose et al. 1997; Anten 2002, 2005). Nitrogen availability in the soil may influence the balance between production and loss of leaf area (Ackerly and Bazzaz 1995; Hikosaka 2003). Leaf-loss rate may also be influenced by the nitrogen availability. Several studies have demonstrated that leaf senescence is accelerated in leaves subjected to nitrogen deficiency (Makino et al. 1984; Crafts-Brandner and Egli 1987; Guitman et al. 1991; Hikosaka et al. 1994). This is because limited nitrogen absorption from soil does not meet nitrogen demand for new leaf growth at the tip and the rest must be supplied with the nitrogen retranslocated from older leaves (Hirose 1971; Jonasson and Chapin 1985; Aerts 1989; Ono et al. 1996).

Winter et al. (1982) studied about the leaf anatomy and photosynthetic properties of nitrate and ammonia grown plants of Moricandia arvensis a C$_3$-C$_4$, intermediate plant. Nitrate grown plants had high levels of malate as well as high activity of phosphoenol pyruvate carboxylase than in ammonia grown plants. Plants with nitrate as sole source of nitrogen had lower leaf mass ratio but higher specific leaf area compared to plants supplied with ammonium (Gloser et al., 2002). Ammonium nutrition increases the biomass of leaves per unit leaf area and cause significant decrease in allocation to roots (Cramer and Lewis, 1993; Troelstra et al., 1995).

### 2.7 Effect of N supply and forms on N-Metabolism

Nitrate absorbed by the roots from soil is transported to the shoot through transpiration stream via xylem (Shaner and Boyer, 1976). No correlation was observed by Deckard et al. (1977) between the level of nitrate in the leaves and NR activity. Although a number of workers like, Ferrari et al. (1973) and Martinova et al. (1981) suggested that it could be due to the presence of two nitrate pools viz. storage and metabolic pool in leaf tissue. Grandstedt and Huffaker (1982) opined that nitrate in metabolic pool regulates induction and activity of nitrate reductase and nitrate in vacuole serves as storage pool. According to Rao and Rains (1976b), the existence of two nitrate transport systems in
barley (cv. Arivat), one operating during dark, with little nitrate reductase activity detectable and one closely correlated with nitrate reductase activity. Behl et al. (1988) found that nitrate reduction in barley seedlings proceed at rates which were roughly 10 per cent of the rates of the respective, nitrate uptake processes. But, Skoczek (1998) reported that an increase of NR in leaves treated only with 10 mM KNO₃ and about a 6-fold increase of the enzyme activity in comparison of leaves treated with 0.5 mM nitrate reductase activity was determined by the rate of nitrate uptake and not vice versa. But, the studies of Warner and Huffaker (1989) also did not support the hypothesis that nitrate reductase had direct or indirect role in nitrate uptake and concluded that in barley neither of the NR isozymes had a direct role in nitrate uptake. Aslam et al, (2001) used some amino acids as inhibitors of nitrate reductase to see their effect on nitrate uptake process. At low nitrate supply (0.1 mM nitrate concentration in root zone), the inhibition of NR activity by the amino acids was a result of the lack of substrate availability due to inhibition of nitrate uptake system. But, Skoczek (1998) reported that an increase of nitrate concentration in the root medium of barley genotypes to 10 mM caused a significant increase of NR activity in all genotypes. In the leaves of maize and barley nitrate induction of NR activity evolved de novo synthesis of nitrate reductase enzyme protein (Somers et al., 1983; Remmler and Campbell, 1986). Using a nitrate reductase cDNA from maize as a probe, the nitrate induction of maize nitrate reductase activity and mRNA were analyzed over a 24 h period. In the light, etiolated maize leaves, which initially had no nitrate reductase activity and very low levels of NR-mRNA, were induced by nitrate to 50 % of their maximum level of NR transcript within 2 hours, while NR activity did not reach 50 % of maximum until after 12 h of incubation (Gowri and Campbell, 1989). Thus, Campbell (1990) concluded that exposure of maize plants to nitrate initiates a sequence of events in leaves, in which the steady state levels of nitrate reductase mRNA increase, followed by an increase in nitrate reductase protein levels and finally by the appearance of nitrate reductase activity. Similar observations on nitrate reductase mRNA and activity were recorded in barley and rice (Hamat et al., 1989; Melzer et al., 1989). Campbell (1988) observed that nitrate ion does not interact directly with the NR promoter, rather through some other protein. Nitrate induction system in maize is constitutively expressed and protein synthesis is not a prerequisite for nitrate induction of nitrate reductase. When barley plants were grown in 0.14 to 5 mM nitrate containing solution, there was a rapid accumulation of NADH-specific NR-mRNA in both the leaves and the roots and the xylem sap nitrate concentration ranged from 15-50 mM (Kawachi et al., 2002).

2.7.1 Nitrate reductase enzyme

The enzyme nitrate reductase (NR) is present in green tissues as well as root tissues of the plant. The reduction of nitrate by nitrate reductase in root tissues is important in the very early stages of plant growth (Ouhmidou et al., 1990). In mature plant major part of nitrate reduction takes place in leaves. NR is cytosolic in nature (Solomonson and Barber, 1990). There is also a report which shows that the enzyme may be membrane associated (Ward, 1989) Three types of nitrate reductases are present in the plant system. These are NADH specific NR (EC 1.6.6.1), NAD(P)H bispecific NR (EC 1.6.6.2)
and NADPH specific NR (EC 1.6.6.3) (Guerrero et al., 1981). The NADH specific NR uses NADH as electron donor, the NAD(P)H bispecific NR uses NADH or NADPH as electron donor while NADPH specific NR uses NADPH as electron donor. The enzyme uses nitrate as substrate and NADH or NADPH as pseudo substrate. Some plants have only one type of NR encoded by a single gene. Other plants contain more than one form of the enzyme. According to Sueyoshi et al. (1995) barley has two, differentially regulated, nitrate reductase genes, one encoding the NADH specific NR (Nar1) and the other encoding the NAD(P)H bispecific NR (Nar7). The two isoforms of NR in corn leaves have also been reported by Nakagawa et al. (1984). According to Poulle et al. (1987), NR isoforms had different molecular weight and also differed in their sensitivity towards corn root proteinase. Nitrate reductase is an inducible enzyme in higher plants although constitutive form has also been reported (Abrol et al., 1984; Solomon and Barber, 1990; Cathala et al., 1992).

Nathawath (2005) observed activity of nitrate reductase and nitrate reductase was maximum with nitrate fertilizer compared to ammonical form.

2.8 Effects of different forms of N supply

Ota and Yamamoto (1989) found that compared to NH$_4^+$ or NO$_3^-$ fed plants. Plants grown with mixed NH$_4^+$ NO$_3^-$ nutrition grew well and accumulated more total nitrogen, whereas, Bao (2003) studied effect of ammonium nutrition on photosynthesis, nitrate reductase and glutamine synthetase activities of winter wheat and found that there was enhanced chlorophyll content, net photosynthetic rate and soluble sugar content in NH$_4^+$ NO$_3^-$ fed plants. Soluble protein content in leaves and nitrate reductase activity in leaves also increased, whereas, no effect was found on GS activity. A subcellular flux analysis was conducted by Krozucker et al. (1999) to demonstrate the nitrate ammonium synergism with the aid of $^{15}$N tracer and found that plasma membrane fluxes for, cytosole NH$_4^+$ accumulation and ammonium metabolism are enhanced in presence of NO$_3^-$ whereas, NO$_3^-$ fluxes, accumulation and metabolism are strongly repressed by NH$_4^+$. But net N acquisition and translocation to shoot with dual N source was substantially larger than single N source. GS, GOGAT and GDH showed highest activity when dual nitrogen source was given. Trith (1972) observed that presence of ammonium ions markedly reduce the activity of nitrate reductase in roots of apple seedlings. Gansel et al. (2001) concluded that Arabidopsis thaliana ammonia transporter gene AtAmt1 is upregulated only under severe N deficiency and respond to local N status of roots whereas AtNRT2.1 (gene for high affinity nitrate transporter) is strongly upregulated under moderate N limitation and act as a target for long distance signaling. Beck and Renner (1989) demonstrated that ammonia triggers uptake of NO$_3^-$ as well as its remobilization from vacuole nitrate pool in Chenopodium rubrum suspension culture cells.

2.9 Interaction between N and Cytokinins (Cks)

Root development is known to be remarkably sensitive to variations in the supply and distribution of inorganic nutrients in the soil and morphology of the roots is closely involved with nutrient acquisition. Growth of roots/shots is inhibited in the presence of ammonium (Zhou et al.
The changes in plant growth and development in response to N supply or forms are linked to the alterations in hormonal balance. (Britto and Kronzucker 2002). Of the hormonal factors CTKs and auxins are particularly involved. Cytokinins are a group of mobile phytohormones that play a critical role in plant growth and development and nutritional signalling.

When plant faces nitrogen stress there is an increase in production of ROS leading to protein oxidation. Earlier studies on protein carbonylation in Arabidopsis indicated the role of cytokinins in prevention of protein carbonyl degradation in detached leaves. The disruption of cytokinin flow to the leaf may be the trigger (Jain et al., 2008).

Cytokinin content in higher plants is also correlated with nitrogen availability and this hormone may influence several enzymes involved in the nitrogen metabolism, including NR (Jain and Abrol, 2005; Sakakibara et al., 2006, Takei et al., 2002). External application of these hormones can induce NR in variety of plants. Reports indicated the involvement of cytokinins as possible mediators in the induction of NR expression by nitrate (Samuelson et al., 1995). Synergisms between cytokinins and light in the induction of NRA has been proposed (Suty et al, 1993).

A comparison of genes regulated by cytokinins and nitrate provide some insight into the potential interactions of the regulatory pathways. Given that nitrate up-regulates cytokinin biosynthesis via IPT3 and CYP735. The apparent response to the assimilatory genes by nitrate is due to cytokinins. Cytokinin accumulated in response to nitrate in roots is generally isopentenyladenine (iP) type. This converts to trans zeatin riboside (tZR) in the xylem and to free zeatin in leaves (Hirose et al., 2007).

CKs produced locally within the root, or translocated from the shoot may signal that there is sufficient nitrogen present. In this regard, one of the proposed roles of CKs is negative regulation of nitrogen uptake-related genes. Microarray analyses have shown that exogenous application of CK represses two AtNRT2 genes (AtNRT2.1 and AtNRT2.3), three ammonium transporter genes, three amino acid transporter genes, and a urea transporter gene in Arabidopsis (Brenner et al., 2005; Kiba et al., 2005; Sakakibara et al., 2006; Yokoyama et al., 2007). The expression levels of AtNRT1 and AtNRT2 correlate well with low- and high-affinity nitrate transport activity, respectively (Forde, 2000; Okamoto et al., 2003), it is therefore, conceivable that CK repression of the AtNRT genes results in a reduction in nitrate uptake activity.

Nitrate reductase (NR) activity increased up to 14-fold in response to treatment of Arabidopsis thaliana seedlings with the cytokinin, benzyladenine. Induction of NR has been observed in seedlings germinated directly on cytokinin-containing medium, seedlings transferred to cytokinin medium, and seedlings grown in soil in which cytokinin was applied directly to the leaves. The level of induction was similar in wild-type as well as Nia2-deletion mutants, indicating that increased NR activity is related to the expression of the minor NR gene, Nia1. The steady-state Nia1 mRNA level was increased severalfold in both wild-type and mutant seedlings after benzyladenine treatment. In Barley, transcript levels of the Nia2 gene, which is responsible for 90% of the NR activity in developing wild-
type seedlings, did not show any changes upon cytokinin treatment. Nuclear run-on assays demonstrated that Nia1 gene transcription increased dramatically after cytokinin treatment (Yu et al., 1998 Lu et al., 1992, 1998; Jain et al. 2005).

Diurnal analysis of endogenous cytokinins in Pineapple indicated that transitory increases in the levels of zeatin, zeatin riboside and isopentenyladenine riboside coincided with the accumulation of NR transcripts and preceded the rise of NR activity in the shoot during the day and in the root at night, suggesting these hormones as mediators of the temperature-induced modifications of the NR cycle. Thermoperiodism can modify the diurnal cycle of NR expression and activity in pineapple both temporally and spatially, possibly by modulating the day/night changes in the cytokinin levels (Freschi et al., 2009). In tomato the nitrate application increased the levels of zeatin riboside in xylem (Lu et al., 2009). Nitrogen deprivation in *Pisum sativum* (rms2) led to decrease in xylem flow of cytokinins (Dodd et al., 2004).

Studies have also shown that coordination of both nitrate-specific and cytokinin-mediated nitrogen signaling pathways is important for the integration of nitrogen signals at the whole plant level (Sakakibara, 2003 & 2005). Long distance nitrogen signaling takes place via cytokinin in Poplar. Nitrate applied to the roots of herbaceous plants can be transduced via the *ipt* enzyme to cytokinin which can then be transported up the xylem to the shoot with subsequent binding to a CRE1-type receptor and promotion of leaf outgrowth. Nitrogen fertilization of the roots with 5mM NH₄NO₃, NH₄Cl, or KNO₃ strongly enhanced sylleptic branching of the Poplar clone (Cline et al., 2002). Nitrogen plays a central role in rice growth and development because it modulates a wide variety of processes, including CK metabolism. CK-mediated signaling is also related to nitrogen metabolism (Ding et al., 2011).

In addition to nitrate ion, plant growth regulators such as cytokinin function to communicate nitrogen availability between organs. The cytokinin-mediated nitrogen signal is transmitted by a His-Asp phosphorelay system in the target cells. ABA also participates in cytokinin-mediated nitrogen signaling by means of negative regulation of (*ZmRRs*) *Zea mays* Response Regulators (Gowronska et al., 2003).

### 2.9.1 Regulation of CK biosynthesis by nitrogen:

Until the identification of genes encoding adenosine phosphate-isopentenyl transferase (IPT), which catalyses the initial step of CK biosynthesis, it was believed that CKs are synthesized in roots (Letham, 1994). In *Arabidopsis*, IPT is encoded by seven genes that are differentially expressed in various tissues, indicating that CK production is not confined to roots (Miyawaki et al., 2004; Takei et al., 2004). Among these seven genes, *AtIPT3* is nitrate inducible. Accumulation of CKs was greatly attenuated in an *AtIPT3* mutant, indicating that *AtIPT3* is a key determinant of nitrate-dependent CK biosynthesis (Miyawaki et al., 2004; Takei et al., 2004). Interestingly, nitrate-inducible expression of *AtIPT3* was also observed in detached shoots (Miyawaki et al., 2004). Similarly, nitrogen supplementation induces CK accumulation in detached sunflower and tobacco leaves (Salama and...
Wareing, 1979; Singh et al., 1992). Microarray analyses have shown that the nitrate inducible expression of AtIPT3 is partly mediated by NRT1.1/CHL1 (NRT1.1), a protein which functions as a dual-affinity nitrate transporter and nitrate sensor (Liu et al., 1999; Ho et al., 2009; Wang et al., 2009). AtIPT3 is expressed in phloem throughout the plant (Miyawaki et al., 2004; Takei et al., 2004), and this expression pattern overlaps with that of NRT1.1 in roots (Guo et al., 2001). However, in shoots, NRT1.1 expression is detected only in young leaves (Guo et al., 2001). Therefore, whether or not NRT1.1 mediates nitrate-inducible AtIPT3 expression in shoots is an open question. Given that AtIPT3 is expressed in phloem, it is likely that CKs synthesized by AtIPT3 in shoots function as a shoot-to-root long-distance signal of shoot nitrate availability. In this context, it has been shown that xylem sap predominantly contains trans-zeatin (tZ)-type CKs, and phloem sap mostly contains N6-(D2- isopentenyl)adenine (iP)-type and cis-zeatin (cZ)-type CKs (Hirose et al., 2008). Thus, either the iP- or cZ-type CKs, or possibly both, could be the shoot-to-root long-distance signal in Arabidopsis.

Grafting experiments using a higher order atipt mutant (atipt1;3;5;7) have provided unequivocal evidence that iP-type CKs are translocated from the shoot to the root (Matsumoto-Kitano et al., 2008). The atipt1;3;5;7 mutant is characterized by extremely low iP and tZ-type cytokinin levels, retarded shoot growth, and enhanced lateral root outgrowth. When a wild-type shoot was grafted onto the atipt1;3;5;7 mutant root, the normal growth phenotype and levels of iP-type CKs were restored in the mutant root, indicating that iP-type CKs translocated from the shoot are biologically functional (Matsumoto-Kitano et al., 2008). Notably, the expression of AtIPT3 is also regulated by iron, phosphate, and sulphate availability, both in shoots and in roots (Hirose et al., 2008; Seguela et al., 2008). It could be that AtIPT3 functions as an integrator of nutrient availability signals.

The work of Smalle et al. (2002), on 26S proteasomal mutant (mutation rpn10 SU) indicates decreased cytokinin sensitivity of rpn10-1 superficially linking RPN10 to cytokinin signaling. The seeds have reduced hypocotyls and root length. Slower rate of leaf initiation and expansion, chlorosis on leaf edges and slower growth of rosette. The mutant also responds to external cytokinins, indicating close relation between cytokinins and proteasomal pathway.

This prompted us to investigate the effect of different N levels and form of protein carboxylation and as there is close relation between cytokinins and nitrate levels its role in preventing degradation of protein carbonyls.
MATERIALS & METHODS
(GENERAL)
3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant material

Wheat variety i.e. PBW-343 (*Triticum aestivum*, 2n=42, AABBDD) was procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi. All the studies were conducted using 15 days old seedlings and were grown in National Phytotron Facility.

3.1.2. Chemicals, kits and other materials

All the chemicals used in this study were of analytical grade procured from SD Fine Chemicals and Qualigens. In the work related to molecular biology, the kits used for RNA extraction were RNeasy® Plant Minikit (Qiagen), RT-PCR kit from ABM (Applied Biological Materials) and Protoscript (New England Biolabs). Oligos for wheat actin gene and *At IPT3* were synthesized from MWG-Biotech AG. Antibodies and inhibitors of protein degradation pathways (MG132 and 3MA) were from Sigma.

To raise the plants in hydroponics, thermocol (Styrofoam) sheets of 2 inch thickness were used to support the plants. Plastic tubs of 4 L capacity, inner surface of plastic tubs were coated with black paints and aquarium pumps (Tropi) with PVC tubings were used to grow the plants and for continuous aeration of the nutrient solution, respectively (Plate 1).

3.1.3. Growing media

The growing media was nitrogen free Hoagland solution as described by Hoagland and Arnon (1950).

Composition of nitrogen free Hoagland solution (-N) was as given below:

(A) Macronutrient solution

<table>
<thead>
<tr>
<th></th>
<th>Molarity (M)</th>
<th>Quantity (ml/l of solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂SO₄</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1.0</td>
<td>2</td>
</tr>
<tr>
<td>Micronutrient</td>
<td>Quantity (g/l of solution)</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------------</td>
<td></td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>2.86</td>
<td></td>
</tr>
<tr>
<td>MnCl$_2$ .4H$_2$O</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>ZnSO$_4$ .7H$_2$O</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>CuSO$_4$ .5H$_2$O</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

The solution 'B' and 0.5% ferric citrate solution (1 ml each) were added to solution 'A' and pH was adjusted to 6.5 and total volume made up to 1 L. The N was added to the above N free Hoagland solution at various concentrations before use.

Four treatments were taken:

1. Zero N
2. Nitrate (KN0$_3$) : 50 μM
3. Nitrate (KN0$_3$) : 5 mM
4. Ammonium Sulphate (NH$_4$SO$_4$) : 5 mM

### 3.1.4. Growth conditions

The plants were raised in controlled environment chambers at National Phytotron Facility, IARI, New Delhi. In these chambers, except CO$_2$, the growth conditions were maintained as follows:

- Temperature : 22°C/12°C day/night,
- Photoperiod : 10 h
- Photon flux density : 450 μmol m$^{-2}$ s$^{-1}$ (PAR)
- Relative humidity (RH) : 90%

### 3.2. Methodologies
3.2.1. Seed treatment and seedling growth

Seeds of wheat sp. PBW 343 were washed with double distilled water and then surface sterilized with 0.1% HgCl₂ for 5 min. To remove the traces of HgCl₂, seeds were thoroughly washed for 5-6 times with double distilled water. The surface sterilized seeds were germinated in trays on germination paper and kept inside growth chamber.

3.2.2. Growth of plants in nutrient solution

After emergence of coleoptiles (5-6 days after sowing), seedlings were transferred into Hoagland solution N were supplied as nitrate, ammonium and ammonium nitrate. The plants were supported on Styrofoam (2” thickness) and grown in plastic containers having 10 L of nutrient solution each (Plate 1.). The solution was aerated continuously through aquarium pumps. The solution was changed every alternate day. Growth chamber had 3 trays for nitrate, ammonium and ammonium nitrate. Staggered sowings were done to get continuous supply of 15 D old seedlings. Each tray had about 40 seedlings and for changing the solution, whole sheet could be lifted with minimal disturbance to roots and then keep on tray with new solution of same compositions.

For Morpho-physiological, biochemical and molecular work, 15 D old plants were used.

3.2.3. Morpho-physiological observations

3.2.3.1. Root characteristics

Root morphological studies were undertaken in plants grown in growth chambers. To study the root morphology and other growth parameters, 15d old plants raised in growth chamber with different N levels were taken. Following observations were recorded to study changes in root characteristics:

(i) Root length

Root length of plants from each treatment was measured by using a ruler and expressed as cm plant⁻¹. Three replications were taken and each replication had 5 plants.

(ii) Root shoot ratio

The roots and shoots were dried at 65⁰C till the constant dry weight and dry weight were recorded. Root shoot ratio was calculated from the dry weight of shoots and roots as:

Root to shoot ratio = Root dry weight / Shoot dry weight

Root:shoot=Root dw/ Shoot dw

(iii) Root surface area
Root surface area was estimated following the method of Ansari et al. (1995), which is based on negative adsorption of NO$_2^-$, when a dilute solution of nitrite comes into contact with the negatively charged root surface (Brett and Woldron, 1990), it forms a diffusive layer (i.e. a thin layer of water saturated with nitrite was held stoichiometrically by secondary adsorption). The distribution of negative charge in the double diffusive layer is uniform and does not depend on the nature of the surface (Schofield, 1947).

The method of Ansari et al. (1995) utilizes very dilute solution of nitrite, which spreads on an aqueous thin layer on root surface. Afterwards, this nitrite is desorbed in distilled water and estimated calorimetrically after diazotization reaction (Wray and Fido, 1990). The method was calibrated with wheat root sections of known surface area. The correlation coefficient was found to be 0.99. From standardization protocol, the factor for calculating surface area was worked out to be 40.36 μmol NO$_2^-$ for 100 cm$^2$ root surface area (Kalita, 1999).

A loose bundle of roots of 5 seedlings from each N treatment constituted one replicate. Tied with a nylon thread, it was immersed in 0.05 M of NaNO$_2$ solution for 10 seconds, and then hung to drain out excess solution present on the surface of root. Care was taken to ensure that only roots were in contact with the solution. The roots were then transferred to an Erlenmeyer flask containing 200 ml of distilled water and shaken for 15 min. The nitrite desorbed in the solution was measured by pipetting suitable aliquots into test tubes and adding 1 ml of 1% sulphanilamide in 1N HCl. Then, to the colourless diazo compound so formed, 1 ml of 0.01% N (1-naphthyl) ethylene diammonium dichloride (NEDD) was added. The color was allowed to develop for 20 min and the absorbance was read at 540 nm using double beam UV Visible Spectrophotometer (ECIL, Hyderabad, India). The amount of nitrite was calculated from a standard curve prepared with NaNO$_2$ concentrations ranging from 10 to 100 nmoles (1 O.D. = 0.1525 μmoles). The root surface area was expressed as cm$^2$ plant$^{-1}$.

3.2.3.2 Measurement of plant height
Plant height from each treatment was measured by using a ruler and expressed as cm plant$^{-1}$. Three replications were taken and each replication has 5 plants.

3.2.3.3 Measurement of fresh and dry weight of the plant tissue
The plants were separated into roots and shoots. Excess water was removed from the roots and fresh weight was recorded. To obtain the dry mass, the root and shoot tissues were dried in hot air oven at
65°C till a constant weight was recorded. Three replications were taken and each replication has 5 plants.

3.2.3.4. Total leaf area and specific leaf weight
The total leaf area was recorded on three replicates from each genotype and treatment using leaf area meter (LiCOR 3000, Lincon Nebraska, USA). Three replications were taken and each with 5 plants. To compute the SLW the leaf area was measured and then their dry weight was recorded after drying in an oven at 65°C till constant dry weight was recorded. The SLW was calculated and expressed as mg cm\(^{-2}\) (Gardner et al., 1985).

Specific leaf weight (SLW) = Leaf dw / Leaf area

3.2.4. Biochemical parameters
3.2.4.1. Nitrogen estimation in plant tissue
Total N in roots and shoots was measured separately using N\(_2\) analyzer (Gerhardt, Turbotherm digestion unit and Gerhardt Vapodest distillation unit).

Reagents used:

i. NaOH 40%: 400g NaOH was dissolved in distilled water and volume was made upto 1 L.

ii. Boric acid solution (4%): 40 g of pure H\(_3\)BO\(_3\) was dissolved in 1 L distilled water.

iii. Mixed indicator: Dissolved 0.07 g methyl red with 0.1 g bromocresol green in 100 ml of 95% ethanol (alcohol).

iv. Concentrated sulphuric acid.

v. Kjeltab or catalyst mixture of potassium sulphate and copper sulphate in the ratio of 10:1.

Method:
50 mg of finely powdered homogenous sample was weighed and put in a clear and dry digestion tube. To this 5.5 g of K\(_2\)SO\(_4\) : CuSO\(_4\) digestion mixture and 10 ml H\(_2\)SO\(_4\) was added. The sample was digested at 700°C for 15 min and then at 450°C for 1 h. When the samples were colorless tubes were taken out from the digestion unit.

100 ml conical flask containing 4% solution of boric acid (10 ml) was taken. To this one or two drop of indicator solution was added and the color of the solution turned pink. This solution was used to trap the ammonia liberated during distillation.
For distilling the sample, the digestion tube was placed in distillation unit and 50 ml water and 70 ml NaOH (40%) was added. After stream distillation, color of the indicator dye changed to green as (NH₄)₂SO₄ is trapped by boric acid. This was titrated against 0.01 N HCl until the pink color reappeared. Each digestion set had two control tubes. N per cent in the tissues and the N content per plant was calculated using the formula:

\[
\frac{(\text{ml HCl in sample}) - (\text{ml HCl in blank}) \times \text{Normality of acid} \times 14.01 \times 100}{\text{Weight of sample (mg)}}
\]

3.2.4.2. Estimation of nitrate reductase activity (NR; EC 1.6.6.1)

Estimation of \textit{in vivo} nitrate reductase activity was done by estimating nitrite formed by the enzyme present in cells and nitrite formed was then diazotized using sulphanilamide in acidic medium and NEDD using the method of Klepper \textit{et al.} (1971) and modified by Nair and Abrol (1973). Nitrite was estimated by the method of Evans and Nason (1953). The leaves were cut into 2 mm pieces and after thorough mixing of leaf sample, 0.3 g was weighed and was added to ice cold incubation medium containing 3 ml each of phosphate buffer (0.2 M, pH 7.5) and potassium nitrate solution (0.4 M). To it, 0.2 ml of n-propanol was added. The leaf samples were infiltrated with the solution using a vacuum pump and then incubated in water bath at 33°C for an hour under dark. At the end of incubation period, tubes were placed in water bath (70-80°C) for 3-4 min to stop the enzyme activity and for the complete leaching of the nitrite into the medium. The nitrite was then estimated by taking adequate amount of aliquot in a test tube, to it one ml of sulphanilamide (1% in 1 N HCl) was added. After mixing, 1 ml NEDD (0.02%) was added and again mixed well. Pink colour was formed immediately and after 20 min the total volume was made up to 4 ml with double distilled water. Absorbance was measured using a double beam UV Visible Spectrophotometer (UV57045S) at 540 nm. The calibration curve was prepared using standard sodium nitrite solution (1 O.D. =152.46 µmoles). The enzyme activity was expressed as µmol nitrite formed g⁻¹ dw h⁻¹.

3.2.4.3. Measurement of nitrate in plant tissues

The method adopted for the estimation of nitrate was based on hydrazine sulphate reduction method as described by Downes (1978).

3.2.4.3.1. Reagents used
(a) **Catalyst solution**: 0.0319 g CuSO$_4$.5H$_2$O + 0.99 g ZnSO$_4$.7H$_2$O in one litre of double distilled water.

(b) **NaOH solution (0.1 M)**: 4.09 g in one litre double distilled water.

(c) **Hydrazine sulphate solution**: 1.529 g in one litre double distilled water.

(d) **Acetone (10% w/v)**: 100 ml of acetone in one litre double distilled water.

**3.2.4.3.2. Extraction of nitrate from plant material (roots and shoots)**

About 100 mg of oven dried ground plant tissue (20 mesh) was used for extraction. Plant material and equivalent amount of activated charcoal (free from nitrate) were added to 100 ml conical flask having 15 ml of double distilled water. The material was then boiled for 3-4 min and the extract was filtered through Whatman filter paper (No. 42). The residue was re-extracted and the volume was made up to 50 ml with double distilled water.

**3.2.4.3.3. Estimation of nitrate**

Adequate amount of aliquot either from the incubation solutions of various concentrations of nitrate (to measure depletion) or from plant extracts was taken in a test tube. To it, 0.5 ml catalyst solution, 0.25 ml 0.1 N NaOH solution, 0.25 ml hydrazine sulphate were added and the volume was made to 3.5 ml. The tubes were incubated for 10 min at 33°C. After that the tubes were kept in ice and 0.5 ml acetone was added. One ml of 1% sulphanilamide in 1 N HCl and 1 ml of 0.01 % NEDD in water were added subsequently to develop pink colour. Absorbance was measured at 540 nm using double beam UV Visible spectrophotometer (UV 57045 S) after 20 min. The calibration curve was prepared using nitrate solutions with varying nitrate concentration from 10 to100 nmol/ml. The data obtained from the above experiment was used to calculate the rate of uptake, kinetics and mechanisms of nitrate uptake.

**3.2.4.4. Superoxide radicals**

The spectrophotometric assay of total superoxide radical content in the fresh leaf tissues is based on the principle of formation of blue coloured formazone by nitroblue tetrazolium chloride with superoxide radicals (O$_{2}^{-}$) by inhibiting total superoxide dismutase (SOD) activity, as described by Chaitanya and Naithani (1994).
3.2.4.4.1. REAGENTS USED

(i) Methionine (200 mM): L-methionine 0.298 g was dissolved in water and the volume was made up to 10 ml with double distilled water.

(ii) Nitroblue tetrazolium chloride (NBT) (2.25 mM): NBT 0.0184 g was dissolved in water and the volume was made up to 10 ml with double distilled water.

(iii) EDTA (3.0 mM): EDTA 0.0558 g was dissolved in water and the volume was made up to 50 ml with double distilled water.

(iv) Sodium carbonate (1.5 M): Sodium carbonate 7.95 g was dissolved in double distilled water and the volume was made up to 50 ml with double distilled water.

(v) Grinding media: (0.2 M phosphate buffer, pH 7.2, containing 1 mM diethyl dithio carbamate)

Solution A: Potassium dihydrogen phosphate 13.60 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Solution B: Di-potassium hydrogen phosphate 17.42 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

28 ml of Solution A + 72 ml of solution B and 0.017 g sodium diethyl dithio carbamate were mixed and final pH was adjusted with the help of pH meter.

3.2.4.4.2. Extraction

Superoxide radical content was quantified by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorption of end product measured at 540 nm. One gram leaf tissue was homogenized in 10 ml of pre-cooled phosphate buffer (0.2 M, pH 7.2). The homogenate was centrifuged in Sorvall refrigerated centrifuge, model-RC 6, rotor SS-34 at 10000g For 10 min and supernatant was immediately used for the estimation of superoxide radical.

3.2.4.4.3. Estimation of O$_2^-$

The reaction mixture contained 0.25 ml supernatant, 0.075 mM NBT, 25 mM Na$_2$CO$_3$, 0.1 mM EDTA, 13.33 mM L-methionine and water to make the volume 3 ml. Reaction mixture was incubated at 30 °C for 10 min and absorbance was recorded at 540 nm. Superoxide radical content was calculated according to its extinction coefficient, i.e., 12.8 mM$^{-1}$ cm$^{-1}$.

3.2.4.5. Hydrogen Peroxide
3.2.4.5.1. Reagents

Titanium reagent: One gram titanium dioxide and 10 g potassium sulphate were digested in 150 ml conc. sulphuric acid over a hot plate for 4 h. The digested mixture was diluted to 500-600 ml and stirred with a magnetic stirrer cum heater at 70 – 80 °C till a clear transparent solution was obtained. It is diluted to 1.5 L and stored in dark brown bottle.

3.2.4.5.2. Estimation

Hydrogen peroxide was estimated by forming titanium-hydro peroxide complex (Rao et al., 1997). One gram leaf material was ground with liquid nitrogen and the fine powdered material was mixed with 10 ml cooled acetone in a cold room (10 °C). Mixture was filtered with Whatman No.1 filter paper followed by the addition of 4 ml titanium reagent and 5 ml ammonium solution to precipitate the titanium-hydro peroxide complex. Reaction mixture was centrifuged at 10,000 g for 10 min in the Sorvall refrigerated centrifuge, model-RC 6, rotor SS-34. Precipitate was dissolved in 10 ml of 2 M H₂SO₄ and then recentrifuged. Supernatant was read at 415 nm against blank in UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). Hydrogen peroxide contents were calculated by comparing with a standard curve drawn with known hydrogen peroxide concentrations (Rao et al., 1997).

3.2.4.6. Lipid peroxidation

Lipid peroxidation was estimated as the thiobarbuturic acid reactive substances, according to the method of Heath and Packer (1968).

3.2.4.6.1. Reagents

(i) Trichloro acetic acid solution: Trichloro acetic acid (TCA) solution (0.1%) was obtained by dissolving 0.1 g TCA in double distilled water and volume was made up to 100 ml with double distilled water.

(ii) Thiobarbituric acid reagent: Thiobarbituric acid (TBA) 0.5 g was dissolved in 20% trichloro acetic acid (TCA) (obtained by dissolving 20 g TCA in 100 ml distilled water) and volume was made up to 100 ml with 20% TCA solution.

3.2.4.6.2. Estimation

Leaf sample (0.5 g) was homogenized in 10 ml 0.1% trichloro-acetic acid (TCA). The homogenate was centrifuged at 15,000 g for 15 min. To 1.0 ml aliquot of the supernatant 4.0 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95
³C for 30 min in the laboratory electric oven (Scientific, India) and then cooled in an ice bath. After centrifugation at 10,000 g for 10 min in Sorvall refrigerated centrifuge, model-RC 6, rotor SS-34, the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated according to its extinction coefficient, i.e., 155 mM⁻¹ cm⁻¹. The values for non-specific absorbance at 600 nm were subtracted (Heath and Packer, 1968).

3.2.4.7. Total Free Amino Acids

Estimated by method given by Moore S and Stein WH. (1954). 200 mg of sample was grinded with 10 ml of 80% hot ethanol. Extraction was allowed for 30 min with intermittent vortexing. Sample was centrifuged at 5000 rpm for 10 min and the supernatant was collected in a 25 ml volumetric flask. Pellets were extracted with 80% hot ethanol as described above once again. The volume of the supernatant was made to 25ml. One ml of this sample was taken in a test tube and 1 ml of ninhydrin solution was added. It was heated in an water bath at 100°C for 15 min. Removed from water bath and 5ml of diluents was added, then it was vortexed and cooled to the room temperature. Absorbance was measured at 570 nm using a spectrophotometer. Blank (minus sample extract) was run simultaneously and its value was subtracted from sample absorbance and standard graph was prepared by using L-Glycine as a standard.

3.2.4.8. Total protease activity

Method given by Niere et al., 1996 was used and estimation was done by method given by Lee and Takahashi (1966).

Exraction of protease

1.0 gm of leaf tissue was homogenized with 10 ml of extraction buffer into the homogenate. Homogenate was passed through 4 layers of muslin cloth and centrifuged at 25,000 g for 20 min at 4°C. Supernatant was used for the enzyme assay. To 1.0 ml of the enzyme extract, 1.0 ml of 1 % casein in 0.2 M diphosphate buffer (pH=7) and 1.0 ml of 0.2 M of phosphate buffer (pH=7) and were added. Solution was incubated at 40°C for 90 min. The reaction after the end of incubation period was stopped by adding 1.0 ml of 20 % TCA. Reaction vials were kept in refrigerator for 30 min. The reaction mixture was centrifuged at 3000 g for 15 min. Supernatant was used for the amino acid analysis. A control was run along with sample.

Estimation of protease activity

0.5ml of supernatant was taken from the reaction mixture. 0.5ml of 55% glycerol and 0.5ml of ninhydrin solution were added. Volume was made to 6.0 ml by adding double
distilled water, followed by boiling for 20 min. Spectrophotometer reading was recorded at 570 nm. To calculate amount of amino acid formed due to protease activity, a standard curve was prepared by using 0.1 to 1.0 µM of glycine. The protease activity was expressed as µM of amino acid produced per hour per gram F.W or per hour per gram per dry weight.

**Reagents used**

(A) Extraction medium consisted of 2.72 gm of potassium Phosphate (Mr. 136.1) in 400 ml of DDW (0.05 M); 280 µL mercaptoethanol; 5.24 gm of EDTA and final volume was made to 500 ml

(B) Assay Mixture

1.0 gm casein in 100 ml of diphosphate buffer (pH = 7)

0.2 ml of phosphate buffer (pH=7)

1.0 ml of 20% TCA

(C) Reaction mixture consisted of 55 ml of 55% glycerol in 45 ml of DDW and 1.5 gm of ninhydrin in 0.5 M of citric buffer

(D) Citric buffer was made by dissolving 1.05 gm of citric acid in 100 ml DDW, 1.47 gm of sodium citrate in 100 ml DDW (0.5 M) and 13.7 ml of citric acid and 36.3 ml of sodium citrate (pH = 5.6)

(E) Diphosphate buffer was prepared by dissolving 2.72 gm of monobasic KH₂PO₄ in 100 ml DDW; 3.48 gm of dibasic in 100 ml DDW; 39 ml of monobasic solution were added and volume made up to 100 ml with DDW(pH=7).

**3.2.5. Estimation of total proteins and quantification of oxidized proteins**

*Extraction of protein and derivatization of the proteins with DNPH:*

The leaf samples were taken and were immediately wiped thoroughly and plunged in liquid nitrogen. The leaf tissue (100 mg) was powdered in liquid nitrogen and added to prewarmed extraction buffer (1: 5). These were centrifuged at 16,000 rpm for 10 min and supernatant was derivatized by the method of (Jain et al., 2008). Protein extracts were mixed with two volumes of DNPH solution at room temperature for 1 h with gentle agitation. A control sample was mixed with two volumes of 2 N HCl. Five volumes of ice-cold phenol (Tris-buffered, pH 7.9) was added to each tube. After vortexing for 1 min, the mixture was centrifuged for 10 min at 10,000 rpm. The upper phase was removed and discarded leaving the interface intact, and the phenol phase was re-extracted twice with ice cold Tris-HCl buffer (50 mM, pH 8.0). Five volumes of cold 0.1 M ammonium acetate in methanol was added to the lower phase and incubated at -20 °C overnight. The mixture was subsequently centrifuged
at 16000 rpm for 20 min and the resulting pellets were washed three times with 1 ml 0.1 M ammonium acetate in methanol and once with 1 ml cold ethanol. Each pellet was dissolved in 100 μL SDS buffer [containing 0.1% bromophenol]. The protein concentration was estimated by Bradford (1976) method. 1 μl of the extract was taken and 799 μl of water was added to it. Subsequently 200 μl of Bradford reagent was added and absorbance was recorded at 590 nm. The concentration of the proteins was calculated as µg protein per µl or per 100 mg sample. 10 µg protein was loaded for the western blotting on SDS PAGE.

**SDS – Polyacrylamide gel electrophoresis for protein profile**

Electrophoresis of total soluble proteins was performed using denaturing gels (Laemmli 1970). Polyacrylamide gels were formed by polymerizing acrylamide with a cross linking agent (bis acrylamide) in the presence of a catalyst (per sulphate ion) and chain initiator (TEMED; N, N, N', N, '- tetramethylene diamine).

**Stock solutions:**

Running gel buffer (8xStock) (3M Tris HCl, pH 8.8): The buffer was prepared by dissolving 36.34 g of Tris HCl in 50 ml of distilled water and pH was adjusted to 8.8 with concentrated HCl and final volume was adjusted to 100 ml.

Stacking gel buffer (4xStock) (0.5M Tris HCl, pH 6.8): For the preparation of stacking gel buffer, 6.052 Tris HCl was dissolved in 60 ml of distilled water and pH was adjusted to 6.8 with concentrated HCl and final volume made up to 100 ml.

SDS (10 %) (w/v): 10 g of sodium dodecyl sulphate was dissolved in 100 ml of water and stored at room temperature.

Acrylamide stock (30%): Stock solution of acrylamide was prepared by dissolving 58.4 g of acrylamide and 1.6 g of bisacrylamide in distilled water and final volume was made up to 200 ml.

Ammonium persulphate(10%): Ammonium persulphate (0.5 g) was dissolved in 10 ml distilled water and stored at 4°C. (Prepare fresh).

Reservoir buffer (5X; Electrode buffer) (pH 8.3): Tris base : 15.1 g, glycine : 94 g 50 ml SDS (10%) and final volume made up to 1l with distilled water.

Sample loading buffer: The buffer was prepared by dissolving 0.3 g Tris HCl in 4 ml SDS(20%), 2ml mercaptoethanol (10%) and 4ml glycerol (20%). Bromophenol blue (40 mg) mixed with this solution. Final volume was made up to 20 ml with distilled water.
Staining solution: The staining solution was prepared using 1.25 g coomassie brilliant blue (R-250) dissolved in 500ml methanol and 100ml glacial acetic acid. Final volume was adjusted to one litre with distilled water.

Destaining solution: 30% methanol and 10% glacial acetic acid.

Preparation of the gel

SDS-PAGE was performed with 12 % running gels and 5 % stacking gels.

**Working solutions:**

Resolving Gel (12%): 30% acrylamide(3.3ml) , 1.5M Tris-HCl pH 8.8 (2.5ml), 10% SDS (0.1ml), Distilled water (4ml), 10% ammonium persulfate (0.0ml), TEMED (10:1).

Stacking gel (5%): 30% acrylamide (0.67ml), 1 M Tris- HCl pH 6.8 ( 0.5ml), distilled water(2.7 ml), 10 % SDS ( 0.04ml), 10 % ammonium persulfate (0.04 ml), TEMED (0.004 ml).

The solutions for the required concentration of the running and stacking gels were mixed and degassed for 10 min. Just before casting the gel, freshly prepared 10 % APS and TEMED were added and mixed thoroughly. The solution was poured into the gel mould and thin layer of water was laid over the surface and was allowed to polymerize at room temperature. For pouring stacking gel the water was poured off and stacking gel was poured and comb was placed in the position.

Total soluble proteins from the grains the protein samples 10 μg each were mixed with sample loading buffer and heated for 3-4 min to denature the proteins. After short spin these were loaded on to the gel.

**The composition of extraction buffer was**

1M Tris  (pH 8.0 ) 7.5ml ;β-Mercaptoethanol 6ml;Sodium dodecyl sulphate 2.4g; Urea 7.2g ;Glycerol 12ml and Bromophenol blue 0.006g.

**3.2.6. Western blotting for the DNPH derivatized proteins:** Resolved proteins were electrophoretically transferred to nitro cellulose membrane (8.3x5.4cm). The cassette was kept in transfer buffer over night at 16 volt. Next day the membrane with transferred proteins was washed with 30 ml Blocking solution (Fish Gelatin in 30 ml PBST) for 1h to fix the proteins and block the remaining hydrophobic sites on membrane with continuous shaking at 70 rpm on a platform shaker at room temperature. The solution was discarded and the membrane was washed three times in 30ml PBST solution for 30 min. with continuous shaking. The membrane was then probed with 1.5μl primary antibody (anti-DNPH antibody,Alex Fluor, Sigma, USA,1:20000) in 30ml PBST for shaking for 1h. The solution was discarded and washed three times with 30 ml PBST. Again the membrane was probed with 5μl secondary antibody (Goat anti-rabbit HRP antibody, dilution 1:15000) in 30ml
PBST for 1h. The membrane washed three times with PBST and then PBS and image of the membranes were taken by infra-red imager LiCor, Odyssey where the membranes are exposed to Infra-red radiation at 700 channel and density of bands recorded.

**5X Phosphate buffer saline pH 7.5(1000ml) was prepared as follows:**

\[ \text{NaCl (8g);KCl (0.2g); NaH}_2\text{PO}_4 \text{ (0.2g) and Na}_2\text{HPO}_4 \text{(2.9g)} \]

**PBST was prepared by dissolving**

200 ml of 1X PBS and 2 ml of tween 20 in water and volume was made to 2000ml.

**3.2.7. Quantification of protein carbonyl by incubating the seedlings in various solutions**

Seedlings were incubated in four different solutions and control was kept in water for 6 hrs in light. Treatments were as follows: 10µM BA, 10µM Kinetin, 1mM 3MA and 50µM MG132. Similar treatments were also given to detached leaves from the seedlings and were kept in both dark and light for 6 h.

**3.2.8. Expression studies**

To confirm the data obtained from biochemical analysis, gene expression studies were conducted. 15d old plants grown with different N sources in phytotron were taken to study the expression of cytokinin in them.

RT-PCR was done using primer for *AtIPT3* gene

**PRIMER:**

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**3.2.8.1. Isolation of total RNA**

Total RNA isolation from plant material was done using the RNeasy® Plant Mini Kit (Qiagen). Approximately 100 mg shoot samples stored previously at -80°C was ground to a
fine powder using liquid nitrogen and pre-chilled mortar and pestle. Ground tissue was added into RNase free, liquid nitrogen cooled, 2 ml eppendorf tube. The liquid nitrogen was allowed to evaporate and 450 µl buffer RLT was added immediately. This was vortexed vigorously. The lysate was pipetted directly onto a QIA shredder spin column placed in 2 ml collection tube, and centrifuged for 2 min at maximum speed. Supernatant of the flow-through fraction was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. This supernatant was used in subsequent steps.

0.5 volume of ethanol (96 – 100%) was added to the cleared lysate and mixed immediately by pipetting. The sample (usually 650 µl) was applied to an RNeasy mini column (pink) placed in a 2 ml collection tube. The tube was closed gently and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm). The flow-through was discarded.

700 µl buffer RW1 was added to the RNeasy column. The tube was closed gently, and centrifuged for 15 s at ≥ 8000 x g (≥10,000 rpm) to wash the column. The flow-through and collection tube were discarded.

RNeasy column was transferred into a new 2 ml collection tube. 500 µl Buffer RPE was added to the RNeasy column and centrifuged for 15 s at ≥ 8000 x g (≥10,000 rpm) to wash the column. The flow through was discarded.

Another 500 µl Buffer RPE was added to the RNeasy column and centrifuged for 2 min at ≥ 8000 x g (≥10,000 rpm) to dry the RNeasy silica-gel membrane. The RNeasy column was transferred to a new 1.5 ml collection tube. 40 µl RNase-free water was added directly onto the RNeasy silica-gel membrane. The tube was gently closed and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

### 3.2.8.2. RNAeasy kit – buffers and reagents

Buffer RLT and RLC : Lysis buffers containing GTC or guanidine hydrochloride.

Centa shredder homogenizer: Removes insoluble material and selectively binds RNA in presence of ethanol.

RNAeasy mini column: Binds total RNA which is diluted with high quality RNA free water.

### 3.2.8.3. RNA quantification

Agarose gel 0.8% was prepared using 100 ml 0.5x TAE (Tris-acetate EDTA) buffer prepared in sterile water. Two µL of RNA samples were diluted with DEPC water to 10 µl and mixed
with RNA loading buffer. After adding loading buffer, the sample was incubated at 65°C for 10 min in heating block and then placed immediately on ice for 5 min. After brief spin (2-3 s), the samples were loaded along with 5 µl of RNA ladder for 30 min at 70 V on horizontal gel electrophoresis. The RNA band was viewed using UV transilluminator and gel was photographed using gel documentation system (Alphaimager). An approximate estimate of RNA concentration was obtained by comparing the band intensities of the marker fragment and sample RNA.

Simultaneously, the RNA was quantified spectrophotometrically using Nanodrop1000. The purity of extracted RNA was checked on gel that showed two distinct bands of 18s and 23s without any DNA contamination which was further confirmed by 260/280nm ratio on Nanodrop.

### 3.2.8.4. REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Two step RT-PCR kits (ABM and Protoscript®, NEB) were used to synthesize first strand cDNA from RNA. The components of cDNA synthesis kit were oligo dT primer, RT buffer, dNTP mix, RT enzyme (MuLV), RNaseH, and RNase-free water.

A master mix was prepared as described below. The master mix contained all the components required for RT-PCR except the template RNA. Volume of master mix prepared was 10% more than that required for the total number of reactions to be performed. A negative control (without template RNA) was included.

#### 3.2.8.5. First strand cDNA synthesis

For synthesizing the first strand of cDNA, the components of reaction mixture i.e., template RNA, oligo dT primer solution and dNTPs mix were thawed on ice. These reaction components were mixed with following components in sterile RNase-free water in 0.2 ml PCR tubes.

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<th>Volume</th>
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</tbody>
</table>
This reaction mixture was incubated at 65°C and 5 min the tube was immediately chilled on ice for 2 min and then given a short spin. The following components were added to the 16 µl RNA/primer/dNTP solution and mixed well.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1µl</td>
</tr>
<tr>
<td>M-MuLv Reverse transcriptase</td>
<td>1µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>-</td>
</tr>
<tr>
<td>Final volume</td>
<td>20µl</td>
</tr>
</tbody>
</table>

The 20 µl cDNA synthesis reaction was incubated at 42°C for 50 min. The reaction was terminated by inactivating the enzyme at 70°C and 10 min. The final volume was made to 50 µl with sterile water and cDNA product stored at -20°C.

### 3.2.8.6. Polymerase chain reaction with gene specific primers of *AtIPT3* and *TaActin*

A master mix was prepared as described below. Volume of master mix prepared was 10% more than that required for the total number of reactions. A negative control without template was also included.

- **Reaction components for RT-PCR per reaction**
  - 10x RT buffer: 2µl
  - dNTPs: 2µl
  - MgCl₂: 1µl
  - Forward primer (10µM): 1µl
  - Reverse primer (10µM): 1µl
  - Diluted cDNA: 3µl
  - Taq DNA polymerase: 1µl

The RT-PCR mix was mixed thoroughly and dispensed into PCR tubes in appropriate volume. Template cDNA was added (3µl/reaction) to the individual PCR tubes. Thermal cycler was programmed according to the program outlined below. RT-PCR program was started while PCR tubes were still on ice. The tubes were kept inside when 50°C temperature was attained.
3.2.8.7. Agarose gel electrophoresis

Preparation of 0.8% agarose gel: Agarose gel was prepared by melting 0.8 g agarose in 100 ml 1x TAE buffer. Upon sufficient cooling GelRed™ (Nucleic Acid Gel Stain, Biotium, non-carcinogenic) was added at the rate of 25 µl/50 ml of gel and contents were gently swirled and poured in gel casting tray with a properly placed comb. After the gel was set, comb was carefully removed without any damage to the wells. 20 µl of PCR product was loaded along with 8 µl of DNA 1kb Marker and electrophoresed for 1h at 70V and photographed using Alpha imager.

3.2.9. Statistical analysis

The experiment was laid out in completely randomized design. The data was subjected to analysis of variance. For variance analysis, the ANOVA procedure of the MSTAT C programme was used. Statistical significance was determined at 5% and 1% probability level. Means were compared by the critical difference (CD at P = 0.05, 0.01) following a significant F-test (Chandel, 1997).
RESEARCH PAPER-I
Response of wheat plants to different levels and forms of nitrogen

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4.1. Abstract

Indian soils are deficient in nitrogen and nitrogen use efficiency is between 33-50% depending on the crops. For much of the human history, N supply is the single major cause limiting productivity. A complex chain of events determines the uptake of N, its use in plant production and the efficiency with which it is used. The present experiment was conducted to find out the response of heat to different N supply and forms. It was noticed that wheat is sensitive to ammonia and N limiting conditions. Wheat seedlings (var. PBW 343) were grown under four different N treatments and various parameters of growth and N-assimilation were studied. Growth and nitrogen metabolism was enhanced in seedlings under high NO$_3^-$-N when compared to those grown with low and without N. The growth of the wheat seedlings was severely inhibited in terms of biomass accumulation, leaf area in NH$_4^+$-N and zero –N treatments. Root : shoot ratio was enhanced in zero- N and ammonium grown plants as compared to the nitrate N fed wheat seedlings. There was almost 5 fold increase in shoot dry weight of NO$_3^-$-N fed seedlings when compared to those grown without N. Shoot dry weight of NH$_4^+$-N fed seedlings declined by 63% as compared to the wheat seedlings grown in high NO$_3^-$-N. There was a relative increase in shoot length by 20% to 40% in the seedlings grown in solution having N salts as compared to those grown without N. In zero-N and ammonium fed plants the nitrate levels, total reduced N and total soluble protein content was significantly lower as compared to both low and high NO$_3^-$-N grown wheat seedlings. These parameters directly correlated with low NR activity in zero-N and NH$_4^+$-$-N treatments.

4.2. Introduction

Nitrogen is one of the most abundant elements on earth but nitrogen deficiency is probably the most common nutritional problem affecting plants worldwide (Abrol et al., 1999). Almost 60% of the soils in India are deficient in nitrogen. This deficiency of nitrogen (stress) seriously affects plant growth, yield and quality. Nitrogen is an important component of many important structural, genetic and metabolic compounds in plant cells. It is a major
component of chlorophyll, amino acids and energy-transfer compounds, such as ATP (adenosine triphosphate) which allow cells to conserve and use the energy released in metabolism, as well as of nucleic acids.

Soil nitrogen availability can fluctuate greatly in both space and time due to factors such as precipitation, temperature, wind, soil type and pH. Therefore, the preferred form in which N is taken up depends on plant adaptation to soil conditions. Generally, plants adapted to low pH and reducing soils as found in mature forests or arctic tundra tend to take up ammonium or amino acids, whereas plants adapted to higher pH and more aerobic soils prefer nitrate (Maathuis, 2009).

Nitrate uptake occurs at the root level and two nitrate transport systems have been shown to coexist in plants and to act co-ordinately to take up nitrate from the soil solution and distribute it within the whole plant (Daniel-Vedele et al., 1998; Tsay et al., 2007). Nitrate reduction takes place in both roots and shoots but is spatially separated between the cytoplasm where the reduction takes place and plastids/chloroplasts where nitrite reduction occurs. Nitrate reduction into nitrite is catalysed in the cytosol by the enzyme nitrate reductase (NR) (Meyer and Stitt, 2001). After nitrate reduction, nitrite is translocated to the chloroplast where it is reduced to ammonium by the second enzyme of the pathway, the nitrite reductase (NiR). The NII genes encoding the NiR enzyme have been cloned from various species, the number of genes varying from one to two copies (Meyer and Stitt, 2001). Ammonium, originating from nitrate reduction, and also from photorespiration or amino acid recycling, is mainly assimilated in the plastid/chloroplast by the so-called GS/GOGAT cycle (Lea and Miflin, 1974; Lea and Forde, 1994).

Plants can absorb and use various forms of nitrogen (N) from soils, most importantly the inorganic ions ammonium (NH$_4^+$) and nitrate (NO$_3^-$), and in legumes, N can be obtained by N$_2$ fixation by means of a symbiotic relationship of rhizobial species (Munoz and Weaver, 1999). The N source affects numerous physiological processes including not only N assimilation, but also other processes such as root respiration (Matsumoto and Tamura, 1981), water relations (Ragab, 1980), photosynthesis (Shelp and Taylor, 1990), and secondary metabolism (Wang and Below, 1996). Although most plants use either or both forms as a source of N, NO$_3^-$ is generally the preferred source for crop growth (Britto and Kronzucker, 2002).

Excessive NO$_3^-$ application can have detrimental effects such as contamination of ground water via NO$_3^-$ leaching and gaseous losses of N as N$_2$O, a factor leading to deterioration of ozone layer (Barker and Mills, 1980). Considering the high ability to
accumulate NO$_3^-$ in leaves (Santamaria et al., 1998) and the high toxicity of NO$_3^-$ to human (Gangolli et al., 1994) and animal health (Bruning-Fun and Kaneene, 1993), NH$_4^+$ fertilization can be a desirable source of N nutrition under certain conditions (Britto and Kronzucker, 2002). NH$_4^+$ application would seem to be a factor in establishing best management practices since the NH$_4^+$ ion is not readily subject to leaching and denitrification losses (Xiaoyang and Jinfeng, 2007).

Despite the fact that NO$_3^-$ assimilation consumes more energy than NH$_4^+$ assimilation, only a few species perform well when NH$_4^+$ is the sole N source (Marschner, 1995). Indeed, many plant species develop symptoms of toxicity when subjected to high concentrations of NH$_4^+$, which are not detected when plants are grown with the same concentration of NO$_3^-$ or in mixed N nutrition (Britto and Kronzucker, 2002). Although N assimilation is associated with reduction of nitrate to ammonia, growth inhibition has been observed when NH$_4^+$ is supplied as the exclusive N source (Gerendas et al., 1997).

The response to the level of nitrogen applied and forms of nitrogen applied to wheat plants and the nutrition stress experienced by the plants has not been investigated extensively. This prompted us to investigate the effect of altered N levels and form of N supplied on wheat growth and nitrogen metabolism.

4.3. Materials and Method

4.3.1. Plant material and growth conditions

Wheat variety i.e. PBW-343 (Triticum aestivum, 2n=42, AABBDD) was procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi. Seeds were grown on germination paper after surface sterilization with 0.1% HgCl$_2$. After emergence of coleoptiles (5-6 days after sowing), seedlings were transferred into Hoagland solution with (low N, 50 μM KNO$_3$, control N, 5 mM KNO$_3$ and 5 mM ammonium sulphate, AS) and without N. The composition of nutrient solution used was macronutrient in mM: Ca(NO$_3$)$_2$ 1.5, KNO$_3$ 5.0, NH$_4$(NO$_3$)$_2$ 1.0, MgSO$_4$ 2.0 and micronutrient in μM: H$_3$BO$_3$ 1.0, MnCl$_2$.4H$_2$O 0.5, ZnSO$_4$.7H$_2$O 1.0, CuSO$_4$.5H$_2$O 0.2, (NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O 0.075 and FeCl$_3$+EDTA.

The plants were supported on styrofoam (2” thickness) and grown in plastic containers of 4 l capacity. The solution was aerated continuously through aquarium pumps and PVC tubings. The solution was changed every alternate day. The whole set up was maintained in controlled environment chambers at National Phytotron Facility, IARI, New Delhi. In these chambers, the growth conditions were maintained as: 22°C/12°C day/night
temperature, 10 h photoperiod with photon flux density of 450 µmol m$^{-2}$ s$^{-1}$ (PAR) and the relative humidity (RH) was 90%.

4.3.2. Morpho-physiological parameters

To study the various morpho-physiological parameters, 15 d old plants raised in growth chamber with different N levels were taken. Three replications, each having 5 plants were taken for all the studies. Root length of plants from each treatment was measured by using a ruler and expressed as cm plant$^{-1}$. The root surface area was estimated by the method of Ansari et al (1995) modified by Kalita (1999) for wheat. In this method, the roots of 5 plants were tied together, and dipped in 0.05 M sodium nitrite solution and then desorbed in known volume of double distilled water. An aliquot was taken from the desorbed solution and by diazotization reaction; the concentration of nitrite was estimated. The surface area was then calculated from the factor 40.36 µmol NO$_2^-$ for 100 cm$^2$ root surface area and was expressed as cm$^2$ plant$^{-1}$. Plant height from each treatment was measured by using a ruler and expressed as cm plant$^{-1}$. The plants were separated into root and shoot. Excess water was removed from the roots and fresh weight was recorded. To obtain the dry mass, the root and shoot tissues were dried in hot air oven at 65°C till constant weight was recorded. The total leaf area was recorded using leaf area meter (LiCOR 3000, Lincon Nebraska, USA).

4.3.3. Biochemical parameters

Total N in roots and shoots was measured separately using N$_2$ analyzer (Gerhardt, Turbotherm digestion unit and Gerhardt Vapodest distillation unit). The whole experiment in this method was carried in three steps viz. digestion, distillation and titration (Kjheldal, 1970). N per cent in the tissues and the N content per plant was calculated using the formula:

\[
\% \text{N} = \frac{(\text{ml HCl in sample}) - (\text{ml HCl in blank}) \times \text{Normality of acid} \times 14.01 \times 100}{\text{Weight of sample (mg)}}
\]

In vivo nitrate reductase activity was assayed by estimating nitrite formed and measured by diazotization reaction using sulphanilamide and NEDD following the method of Klepper et al. (1971) and modified by Nair and Abrol (1973). Nitrite was estimated by the method of Evans and Nason (1953). Absorbance was measured using a double beam UV Visible Spectrophotometer (UV57045S) at 540 nm. The calibration curve was prepared using standard sodium nitrite solution (1 O.D. = 152.46 µmoles). The enzyme activity was expressed as µmol nitrite formed g$^{-1}$ DW h$^{-1}$.

The method adopted for the estimation of nitrate was based on hydrazine sulphate reduction method as described by Downes (1978). Absorbance was measured at 540 nm using double beam UV
Visible spectrophotometer (UV 57045 S) after 20 min. The calibration curve was prepared using nitrate solutions with varying nitrate concentration ranging from 10 to 100 nmol/ml.

Total soluble protein (Bradford 1976) was also measured using the dye Coomassive Blue G250. Absorbance was measured using a double beam UV Visible Spectrophotometer (UV57045S) at 590 nm. The calibration curve was prepared using standard BSA (Bovine Serum Albumin) solution (1 O.D. =16.8). Protein content was expressed as mg/g fresh weight.

4.3.4. Statistical analysis

The experiment was laid out in completely randomized design. The data was subjected to analysis of variance. For variance analysis, the ANOVA procedure of the MSTAT C programme was used. Statistical significance was determined at 5% and 1% probability level. Means were compared by the critical difference (CD at \( P = 0.05, 0.01 \)) following a significant \( F \)-test (Chandel, 1997).

4.4. Results

4.4.1. Morphological characters

4.4.1.1. Shoot dry weight

Shoot dry weight (Table 1, Fig. 1a) of all the treatments were significantly different. There was significant increase in shoot dry weight of the seedlings grown in solution containing N salts. Nitrate fed plants had greater biomass than ammonium fed plants. The seedlings grown in Hoagland solution containing nitrate (\( \text{NO}_3^- \)) or ammonium (\( \text{NH}_4^+ \)) salts had shoot dry weight varying from 0.022 to 0.036 g plant\(^{-1}\) compared to those raised without N (0.013 g plant\(^{-1}\)). There was a relative increase in shoot dry weight (Fig. 1b) by 2 folds and 3 folds in the seedlings grown in solution having 50 \( \mu \text{M} \) and 5 mM \( \text{NaNO}_3 \) respectively, as compared to those grown at zero N. But the dry weight of the wheat seedlings was significantly reduced when \( \text{NH}_4^+ \) instead of \( \text{NO}_3^- \) was supplied as safe N source. Seedling grown with 5mM \( \text{NH}_4^+ \) -N recorded a 63% decrease in shoot dry weight as compared to those grown in 5mM \( \text{KNO}_3 \). Seedlings grown under \( \text{NH}_4^+ \) nutrition also showed increase in shoot dry weight as compared to the seedlings grown without N. Maximum increase was seen in the seedlings grown with 5 mM \( \text{NO}_3^- \) -N.

4.4.1.2. Shoot length

Shoot length (Table 2, Fig. 2a) of all the treatments varied significantly. There was significant increase in shoot length of the seedlings grown in solution containing N salts. The seedlings grown in Hogland solution containing nitrate (\( \text{NO}_3^- \)) or ammonium (\( \text{NH}_4^+ \)) salts had
shoot length varying from 13.6 to 16.1 cm plant\(^{-1}\). Whereas, seedling grown without N had shoot length of only 11.5 cm plant\(^{-1}\). There was a relative increase in shoot length (Fig. 2b) by 20\% to 40\% in the seedlings grown in solution having N salts as compared to those grown without N. Seedlings grown in NH\(_4^+\)-N also showed increase in shoot length, but there was a 18\% decrease in its shoot length as compared to seedlings grown with same concentration of NO\(_3^-\)-N salt. Maximum increase was seen in the seedlings grown with 5mM NO\(_3^-\)-N.

4.4.1.3. Root length

Root (Table 3, Fig. 3a) lengths of all the treatments were significantly different. Maximum root length was seen in the seedlings grown without N. There was significant decrease in root length of the seedlings grown in solution containing N salts. The seedlings grown in Hogland solution containing NO\(_3^-\) or NH\(_4^+\) salts had root length varying from 15.4 to 11.3 cm plant\(^{-1}\). Whereas seedling grown without N had root length 16.5 cm plant\(^{-1}\). There was a relative decrease in root length (Fig. 3b) by 10\% and 30\% in the seedlings grown in solution having 50 μM and 5mM NO\(_3^-\)-N respectively as compared to those grown at zero N. Wheat seedlings grown in solution having NH\(_4^+\) salt had minimum root length followed by seedling grown under 50 μM NO\(_3^-\) N salt. Seedling grown with NH\(_4^+\) salt showed 36\% decrease in root length as compared to seedlings grown with low nitrate salt (50µM).

4.4.1.4. Root surface area

Root Surface Area (Table 4, Fig. 4a) of all the treatments varied significantly. There was significant increase in root surface area of the seedlings grown in solution containing NO\(_3^-\) salts. The seedlings grown in Hoagland solution containing NO\(_3^-\) salts had root surface area varying from 0.69 to 0.79 cm\(^2\) plant\(^{-1}\). Whereas, seedling grown without N had root surface area of only 0.39 cm\(^2\) plant\(^{-1}\). There was a relative increase in root surface area (Fig. 4b) by 2 folds in the seedlings grown in solution having NO\(_3^-\) salts as compared to those grown without N. Seedlings grown in solution having ammonium salt showed least root surface area as compared to all other treatments. The decrease in surface area of roots of ammonium fed plants was more than1 fold as compared to NO\(_3^-\) -N grown plants at similar concentrations. Maximum increase was seen in the seedlings grown with 5mM NO\(_3^-\)-N.

4.4.1.5 Root : shoot ratio

Root:Shoot ratio (Table 5, Fig.5a ) of all the treatments varied significantly. There was significant decrease in Root:Shoot ratio of the seedlings grown in solution containing N salts as compared to seedlings grown without N salts. The seedlings grown in Hogland solution
containing nitrate (NO$_3^-$) salts had Root:Shoot ratio varying from 0.65 to 0.71. Whereas, seedling grown without N had maximum Root:Shoot ratio of 1.27. There was a relative decrease in Root:Shoot ratio (Fig. 5b) by almost 67% in the seedlings grown in solution having N salts as compared to those grown without N.

4.4.1.6. Leaf area

Leaf Area (Table 6, Fig. 6a) of all the treatments varied significantly There was significant increase in leaf area of the seedlings grown in solution containing N salts as compared to seedlings grown without N salts. The seedlings grown in Hogland solution containing NO$_3^-$ or NH$_4^+$ salts had leaf area varying from 28.4 to 37 cm$^2$ plant$^{-1}$. Whereas, seedling grown without N had leaf area of only 9.7 cm$^2$ plant$^{-1}$. There was a relative increase in leaf area (Fig. 6b) by 3 folds to 4 folds in the seedlings grown in solution having N salts as compared to the seedlings grown without N. Seedlings grown in solution with NH$_4^+$ had least leaf area. Increasing concentration of NO$_3^-$-N resulted in increased leaf area of 15d old seedlings. But ammonium fed seedlings had higher leaf area (10%) as compared to low nitrate (50µM) grown seedlings. Maximum increase was seen in the seedlings grown with 5mM NO$_3^-$-N.

4.4.1.7. Specific leaf weight (SLW)

SLW (Table 7, Fig. 7a) of all the treatments varied significantly There was significant increase in SLW of the seedlings grown in solution containing N salts as compared to seedlings grown without N salts. The seedlings grown in Hogland solution containing nitrate (NO$_3^-$) salts had SLW varying from 0.008 to 0.014g cm$^{-2}$. Whereas, seedling grown without N had SLW of only 0.003 g cm$^{-2}$. There was a relative increase in SLW (Fig. 7b) in the seedlings grown in solution having NH$_4^+$-N as compared to those grown with low NO$_3^-$-N.

4.4.2. Biochemical parameters

4.4.2.1. Activity of enzyme nitrate reductase

Nitrate reductase (NR) activity (Table 8, Fig. 8a) of all the treatments was significantly different. There was significant increase in NR activity of the wheat seedlings fed NO$_3^-$ N salts. The seedlings fed with NO$_3^-$-N salts had NR activity varying from 7.26 to 25.73 µmol NO$_2^-$ formed g$^{-1}$ FW h$^{-1}$. Whereas, seedling grown without N had NR activity of only 3.6 µmol NO$_2^-$ formed g$^{-1}$ FW h$^{-1}$. There was a relative increase in NR activity (Fig. 8b) by 2 folds and 7 folds in the seedlings grown in solution having 50µM and 5mM KNO$_3$.
respectively, as compared to those grown without N. Seedlings grown with NH$_4^+$-N had low NR activity and the activity of these seedlings was similar to the NR activity of the seedlings grown at zero N.

4.4.2.2. Shoot nitrate content

There was significant increase in nitrate content (Table 9, Fig. 9a) of the seedlings grown in solution containing N salts. The seedlings grown in Hogland solution containing NO$_3^-$ salts had higher nitrate content varying from 5.69 to 2.129 µ mol g$^{-1}$ DW. Whereas, seedling grown without N had nitrate content of only 2.24 µ mol g$^{-1}$ DW. There was a relative increase in nitrate content (Fig. 9b) by 9 folds in the seedlings grown in 5mM NO$_3^-$-N as compared to those grown at zero N. Seedlings grown in solution with NH$_4^+$–N had least nitrate content. It was not significantly different from the nitrate content of the seedlings grown without N.

4.4.2.3. Root nitrate content

Root nitrate content followed pattern similar to the shoot nitrate levels. Although the levels of nitrate in roots was much lower than the shoot. Maximum root nitrate was observed in 5mM nitrate fed plants followed by 50 µM nitrate fed plants (Table 10, Fig. 10a). The NH$_4^+$-N fed seedlings and seedlings grown without N had similar levels of nitrate in their roots which was not significantly different. The nitrate levels of nitrate fed plants (low and high) differed by 63%.

4.4.2.4. Shoot nitrogen content

Nitrogen (Table 11, Fig. 11a) content of all the treatments was significantly different. The N content of the shoots of nitrate N fed seedlings was significantly higher as compared to the N content of the seedlings grown without N. the increase of N content of shoots of seedling grown in 50 µM N was 2.07% and 5mM was 3.3% as compared to the seedlings grown without N. There was a relative increase in N content (Fig. 11b) by 2 folds in the seedlings grown in solution having 5mM NO$_3^-$ N respectively as compared to those grown without N. Seedlings grown with NH$_4^+$ -N had higher N content as compared to the shoot N content of the seedlings grown without N. 5mM NO$_3^-$-N grown seedlings had maximum level of N in the shoots.

4.4.2.5. Root nitrogen content
Nitrogen (Table 12, Fig. 12a) content of all the treatments was significantly different. The N content of the roots of NO$_3^-$-N fed seedlings was significantly higher as compared to the N content of the seedlings grown without N. The increase of N content of roots of seedling grown in 50 µM N was 1.7% and 5mM was 2.3% as compared to the seedlings grown without N. There was a relative increase in nitrate content (Fig. 12b) by 2 folds in the seedlings grown in solution having 5mM NO$_3^-$-N respectively, as compared to those grown without N. Seedlings grown in solution having ammonium salt also showed increase in nitrogen content as compared to the root N content of the seedlings grown without N. 5mM NO$_3^-$-N grown seedlings had maximum level of N in the roots.

4.4.2.6. Total soluble protein

Protein (Table 13, Fig. 13a) content of all the treatments was significantly different. Changes in protein content followed the changes in the shoot and root N content. The NO$_3^-$-N grown seedlings had higher protein content as compared to NH$_4^+$ fed seedlings. The protein content was higher even in 50 µM NO$_3^-$-N grown seedlings as compared to NH$_4^+$-N grown seedlings. The seedlings grown in Hogland solution containing NO$_3^-$ or NH$_4^+$ salts had protein content varying from 2.1 to 10.3 mg g$^{-1}$ FW. Whereas, seedling grown without N had protein content of only 1.8 mg g$^{-1}$ FW. There was a relative increase in protein content (Fig. 13b) by 2 folds and 5 folds in the seedlings grown in solution having 50µM and 5mM NO$_3^-$-N respectively, as compared to those grown without N. Seedlings grown with having NH$_4^+$-N, recorded increase in protein content as compared to protein content of seedlings grown without N. The maximum total soluble proteins were observed in the seedlings grown with 5 mM NO$_3^-$-N.

4.5. Discussion

4.5.1. Morpho-physiological responses of the wheat plants to the N form and supply

4.5.1.1. Shoot growth in response to N supply

Plants have remarkable capability to adapt and grow effectively in wide range of environments. They do so by changing their morphological and physiological characteristics in response to environmental conditions of growth (Lambers et al 1990).

In this study, three N concentrations and nitrate and ammonium N forms were used. The results were compared among the seedlings grown without N with nitrate and ammonium fed seedlings. The nitrate N concentrations (50 µM, low; and 5 mM, adequate) and ammonium–N (5mM, adequate doses); and therefore compatible with physiological studies were used.
Concentrations below 1mM are considered low and can cause secondary effects arising from N limitations. At the studied doses, wheat seedlings under ammonium nutrition grew dramatically less (~40%) than those fed nitrate (Fig. 1 and Table 1). Characterizing and defining ammonium toxicity is still a controversial subject in plant nutrition and physiology (Britto and Kronzucker 2002). The form and level of N supply has profound effect on the growth of the wheat plants as ammonium fed plants had 40% reduction in dry weight as compared to the plants grown under 5mM nitrate salt. Hence, ammonium can be considered toxic for the wheat plants. Similarly, there was reduction of about 24% in biomass of low nitrate grown plants as compared to the high N grown plants. Ammonium is considered as having toxic effects if the biomass accumulation of ammonium-fed plants is significantly inhibited when compared with nitrate-fed plants at the same N concentration. Similar criteria have been used in several works (Cruz et al. 2006). Peuke and Jeschke (1993) have showed that ammonium as sole N source resulted in stress for most plants, although they may show differential toxicity thresholds. We observed that wheat is very sensitive to ammonium nutrition at the range of N doses applied. Similar results were also reported by Zhang et al. (2000), who showed that ammonium induced cell senescence.

Biomass partitioning between roots and shoots is often affected by ammonium, especially in the more sensitive species (Scheurwater et al. 1999). We observed that in this study the biomass production was mainly affected by the inhibition of root growth. The leaf area was much less affected but, specific leaf weight of the ammonium fed wheat seedlings was significantly lower as compared to the wheat seedlings grown at 5 mM NO$_3^-$-N.

Ammonium (NH$_4^+$) is one of the major nutrients for plants, and a ubiquitous intermediate in plant metabolism. However, this ion is notorious for its toxic effects on many, if not most, plant species. The reasons for this toxicity have been the subject of much speculation, and have included proton extrusion associated with NH$_4^+$ uptake, cytosolic pH disturbances, displacement of crucial cations such as K$^+$ and Mg$^{2+}$, shifts in plant carbohydrate status, and the uncoupling of photophosphorylation (Sas et al., 2002). However, a comprehensive explanation of NH$_4^+$ toxicity has remained elusive. Study by Britto et al., (2001) proposes a new hypothesis, that NH$_4^+$ toxicity is the result of the high energetic cost of pumping NH$_4^+$ back out of cells, after entering at unusually high rates in NH$_4^+$-sensitive species. Influx increases at unusually high rates in the sensitive spp. as these cannot control the intake of ammonium. As most of the energy of the plants is spent on ammonium uptake and then pumping it back out of the cells the growth is affected.
This toxicity may be because of several other factors: hormonal, nutrient or anion–cation balances (Andrews et al. 1999), and probably the relative strength of the carbon and N sinks, among others. Ammonium-fed plants and low N did exhibit a significant changes in their root to shoot ratios. Root:shoot ratio declined in response to NH₄⁺-N. In some studies the ratio increased in response to ammonium nutrition (Liu et al., 2006). The ammonium fed plants were also shorter, and less green as compared to the nitrate fed plants. (Plate 2). In our earlier study (Lekshmy 2006) it was shown that NH₄⁺ grown plant had higher leaf area when the concentration of the ammonium was lower (upto 1mM). In this study the concentration of the ammonium was 5mM and the ammonium grown plants had lower leaf area as compared to the nitrate grown plants at high and low concentration. Probably due the toxic effect and unlimited uptake by the wheat plants as shown in barley by Britto et al (2001), and other toxic effects (Liu et al 200). Ammonium induced inhibition of leaf growth has been reported from other plant species such as Phaseolus vulgaris, Lycopersicon esculentum, and Zea mays (Rabb and Terry, 1994; Chaillon et al., 1986; Cramer and Lewis, 1993). Earlier study by (Dai et al 2003) indicated enhanced leaf chlorophyll content, net photosynthetic rate and increased soluble sugars in rice plants. But in this study, the wheat plants receiving ammonium as the sole source of N leaf area declined and leaves were less green indicating lower chlorophyll content.

**4.5.1.2. Root morphological changes in response to level and form of N nutrition**

Elliot et al (1997) observed that root dry matter yield was not affected by nutrient deficiency until 42 days after sowing, whereas, shoot yield depressed from 21d onwards. In the wheat plants grown without N the root growth was maintained at the expense of the shoot growth. The root growth was maximum in plants grown without N followed by low N fed wheat plants. Root surface area was affected more in the ammonium fed plants and it was minimum as compared to the other three treatments. The importance of root characteristics in plant productivity stems from the fact that many soil resources are unevenly distributed or are subject to localized depletion. The spatial deployment of the root system largely determines the ability of the plant to exploit these resources (Lynch, 1995). Larger surface area is highly desirable character for plants to absorb N from the soils. these roots allow plants to explore large volumes of soils for N uptake. The increased root length of the plants grown without N indicates increased efficiency of the plants in terms of nutrient acquisition.
Modification of root morphology has been found from the results of experiments conducted in the localized nutrient supply or forms of nutrient supplied (Granato and Raper, 1989). The root length and surface area was minimum in ammonium grown plants. It has been shown in the earlier studies that acidification of the root environment can lead to inhibition of root growth and even to destruction of root tissue (Claussen and Lenz, 1995). It has been reported earlier that moderate concentrations (3 mM) \( \text{NH}_4^+ \) had no negative effects on intercellular pH regulation (Bligny et al., 1997). Troelstra et al. (1995) also suggested ammonium nutrition caused significant decrease in allocation to roots.

At the physiological level, the pathway of \( \text{NO}_3^- - \text{N} \) uptake and assimilation in roots is induced by \( \text{NO}_3^- - \text{N} \) and feedback regulated by downstream N metabolites, while the \( \text{NH}_4^+ \) uptake system is rapidly up-regulated under conditions of low N availability (Rawat et al. 1999). Plants also respond to changing N supply by adjusting the balance between shoot or root growth: when \( \text{NO}_3^- \) but not \( \text{NH}_4^+ \) is detected by the root it stimulates leaf expansion (McDonald and Davies 1996; Walch-Liu et al. 2001), while the accumulation of \( \text{NO}_3^- \) in the shoot has a negative effect on root growth (Scheible et al. 1997). Developmental consequences of variations in the N supply include effects on flowering time, root and shoot branching and (in legumes) nodulation (Crawford 1995; Forde 2002). From these examples one can appreciate that mechanisms must exist for communicating information about external N availability and internal N status between the above-ground and below-ground parts of the plant. While much about the ways that the plant integrates the shoot and root responses to changing N supply remains mysterious.

**4.5.2. Biochemical parameters influenced by level and from of N supply**

\( \text{NR} \) catalyses the reduction of nitrate to nitrite, and is regarded as the rate-limiting and regulatory step in the nitrate-assimilation process. Regulation of \( \text{NR} \) is complex and involves a hierarchy of transcriptional and posttranscriptional controls (Crawford and Campbell, 1990). The activity of enzyme nitrate reductase was minimum in the plants grown without N followed by \( \text{NH}_4^+ \) grown plants. The maximum activity was in the 5mM nitrate fed plants. Studies of Matt et al., 2001 have indicated that there are two separate effects on N uptake in plants growing on ammonium nitrate. Firstly, the plants growing on nitrate, N uptake is stimulated at night. Secondly, even ammonium nitrate or ammonium leads to decrease in NIA (NR) genes expression in contrast to plants growing with nitrate (Geiger et al., 1999). Gene expression for nitrate reductase is induced by nitrate and decreased by glutamine or \( \text{NH}_4^+ \) (Stitt and Krapp, 1999). In the presence of ammonium there is absence of
Nitrate in the plant tissues (Rabb and Terry 1994), resulting in low NR activity as nitrate is the signal to induce NR gene expression. Hydrologically mediated N supply contributes to the NR activity has been shown in the study by (Koyama and Kielland, 2011).

Nitrate utilization may also be partially inhibited in presence of ammonium, resulting in some accumulation of nitrate derived from the seeds. Ammonium nutrition may also involve coordinated changes in nitrate uptake, reduction, and ammonium assimilation in roots and leaves of plants. Plant tissue concentrations of nitrate N declined over the experimental period in both treatments i.e NH$_4^+$ and zero N. In each case these reductions were much lower in these treatments than those in the well nourished control treatment, where nitrate –N was maintained in the plants. The decline in tissue nitrate N, and total N concentrations were high in the Zero-N treatments and ammonium N treatments. As no more N entered the zero-N plants from external sources, this could be due largely to the dilution effects of growth. However, the rate of decline varied between the different N forms. The most rapid changes were in the concentration of nitrate which was very low after 15d. This was caused by its conversion into organic forms of N, reflecting a reallocation of N from storage to metabolic pools within the plant. Once this nitrate had been completely assimilated, the change in total N concentration per unit change in dry weight decreased, much as had been observed earlier (Burns, 1994). By comparison, the much smaller decline in tissue N concentrations in the control (+N) plants was not caused by increasing N deficiency, but rather by an increase in the amount of structural material (with an inherently lower N content) needed to support the plants as they became larger (Caloin and Yu, 1984).

One of the most frequent responses of plants to ammonium nutrition is a considerable increase in GS and GDH activities (Cruz et al. 2006, Lasa et al. 2002, Skopelitis et al. 2006). Protein metabolism (synthesis and degradation) is therefore expected to respond differentially to ammonium nutrition depending on plant ammonium tolerance. Accordingly, at lower N supply the protein declined at low levels of N supply and also in the shoots of the ammonium fed plants. In our earlier studies it was observed at the lower level of ammonium (1 mM) the reduction in the protein content was lower approximately 40% (unpublished data) as compared to when the plants were grown at 5mM.

Ammonium nutrition results in the acidification of the root environment. Changes in the ammonium, wherein the reduction was 2.5 fold, proportion of plant dry mass found in the shoot and root components can only be attributed to altered partitioning of carbon within the plant. As a result of the assimilation of ammonium in the roots demands may be placed upon root carbohydrate supply which diverts carbon away from root extension (Cramer and Lewis,
1993). Ammonium nutrition has been reported to result in susceptibility to water stress and reduced water uptake rates (Salsac et al, 1987). Studies have indicated reduction in photosynthesis might be responsible for the reduced growth in ammonium fed plants. The growth of the wheat plant was affected by the form and level of nitrogen supply. The plants grown without N had longest roots resulting in unusually high root : shoot ratio, followed by the plants grown under low N. The plants grown under ammonium nutrition had lower root: shoot ratio as compared to the plants grown under similar level of nitrate N. The reduction in growth was apparent in the plants grown under ammonium nutrition. The toxic effect of the ammonium nutrition were apparent from the low N, nitrate and protein content of the seedlings when compared with the seedlings grown at similar level of N supply.
5. RESEARCH PAPER-II

Protein carbonyl degradation and role of Cytokinin in relation to N-nutrition in wheat seedlings

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5.1. Abstract

Ammonium as the sole source of N is stressful for the wheat plants. Wheat seedlings were grown at varied N forms and supply. Increased ROS generation, increased lipid peroxidation, free amino acid levels and protease activities were observed in wheat seedlings grown under zero-N and NH$_4^+$-N. Presence of carbonylated proteins, in plants is indicative of oxidative stress damage. Conditions that promote formation of reactive oxygen species (ROS) enhance protein carbonylation, and protein degradation is required to reverse the damage. In spite of high ROS ammonium and zero-N grown wheat plants had low levels of protein carbonyls indicating increased degradation as total soluble proteins in these treatments loss of carbonylated proteins corresponded to a loss of soluble protein and accumulation of free amino acids. However, it is not clear how the degradation of carbonylated proteins is controlled in planta. In this report, we show that detached wheat leaves rapidly and selectively degrade carbonylated proteins when kept in the dark as compared to the detached leaves incubated in light or in the intact seedlings. Degradation of carbonylated proteins was blocked by carbobenzoxy-leucinyl-leucinyl-leucinal (MG132) in dark detached leaves, but not by 3-methyladenine, suggesting that the 26S proteasome pathway rather than the autophagic pathway was involved in degrading protein carbonyls in dark detached leaves. During the incubation in light the use of inhibitors revealed that both autophagic and proteasomal pathways were involved in degrading protein carbonyls. The study also indicated the role of cytokinins in preventing the the protein carbonyl degradation. The expression of $IPT3$ gene of cytokinin biosynthetic pathway was almost absent in the seedlings grown at zero N and NH$_4^+$-N, and in these treatments the degradation of protein carbonyls was not prevented. Exogenous application of cytokinins led to the accumulation of
protein carbonyls and in all treatments and maximum inhibition of degradation was in ammonium salts wherein the IPT3 gene expression was not detected.

5.2. Introduction

Nitrogen is the major mineral nutrient required by the plant for its proper growth and development. Maintenance of nitrogen status of plants plays a critical role in increasing both crop productivity and resistance to environmental stress (Cakmak et al., 2005). Nitrogen deprivation triggers distinct redox changes and induce oxidative stress with a rather defined pattern in the context of nutrient-specific alterations in metabolism. (Kandibinder et al., 2004). Deficiency of nitrogen results in nutrient imbalance affecting several metabolic pathways (Abrol et al., 1999) including increased production of ROS (Dominguez-Valdivia et al., 2007). N deficiency implies an oxidative stress because of an increased Mehler reaction and because of an energetic imbalance related to a reduction in N assimilation (Polesskaya et al., 2004).

ROS are produced continuously as by-products of various metabolic pathways that are localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes (del Rio et al., 2006; Navrot et al., 2007). Under steady state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms (Foyer et al., 2005). The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks.

These disturbances in equilibrium lead to sudden increase in intracellular levels of ROS which can cause significant damage to cell structures and it has been estimated that 1-2% of O₂ consumption leads to the formation of ROS in plant tissues (Bhattacharjee et al., 2005). Through a variety of reactions, O₂ leads to the formation of H₂O₂, OH⁻ and other ROS. The ROS comprising O₂⁻, H₂O₂, O₂⁻, HO₂⁻, OH⁻, ROOH, ROO⁻ and RO⁻ are highly reactive and toxic and causes damage to proteins, lipids, carbohydrates, DNA which ultimately results in cell death. Accumulation of ROS as a result of various environmental stresses is a major cause of loss of crop productivity worldwide. ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid per oxidation (LPO). It is
important to note that whether ROS will act as damaging, protective or signalling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (Gratao et al., 2005).

Reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide and hydroxyl radical can cause lipid peroxidation and consequently membrane injury which leads to leakage of cellular content, protein degrading, enzyme inactivation, pigment bleaching and disruption of DNA strands and thus cell death (Scandalios, 1993). Enhanced production of oxygen free radicals is responsible for peroxidation of membrane lipids and the degree of peroxides damage of cell was controlled by the potency of peroxidase enzyme system (Sairam and Tyagi, 2004). Plants have developed a series of both enzymatic and non-enzymatic detoxification systems to counteract AOS, thereby protecting cells from oxidative damage (Sairam and Tyagi, 2004). Tolerance to wide varieties of environmental stress conditions has been correlated with increased activity of antioxidant enzymes and levels of antioxidant metabolites (Davis, 1987). Plants protect the cellular and sub-cellular systems from the cytotoxic effects of these ROS in the form of enzymes such as superoxide dismutase, ascorbate peroxidase, peroxidase, glutathione reductase and catalase and metabolites such as glutathione, ascorbic acid, α-tocopherol and carotenoids (Liebler et al., 1986). Modulation of the activities of these enzymes may be important in the resistance of plant to environmental stresses (Allen, 1995).

The carbonylation of proteins is a well recognized marker of oxidative stress and damage. Assumption is that protein carbonylation leads to loss of function, and hence those proteins must be degraded, as there are no repair mechanisms known to replace the damaged side chains.

Protein oxidation is defined as covalent modification of a protein induced by ROS or by-products of oxidative stress. Most types of protein oxidations are essentially irreversible, whereas, a few involving sulphur-containing amino acids are reversible. Protein carbonylation is widely used marker of protein oxidation (Job et al., 2005). The oxidation of a number of protein amino acids particularly Arg, His, Lys, Pro, Thr and Trp give free carbonyl groups which may inhibit or alter their activities and increase susceptibility towards proteolytic attack. Protein carbonylation may occur due to direct oxidation of amino acid side chains (e.g. proline and arginine to g-glutamyl semialdehyde, lysine to amino adipic semialdehyde, and threonine to aminoketobutyrate). Whatever the location of ROS synthesis and action, ROS are likely to target proteins that contain sulfur-containing amino acids and thiol groups. Cys and Met are quite reactive especially with $O_2^-$ and OH$. Activated oxygen
can extract an H atom from cysteine residues to form a thiol radical that will cross-link to a second thiol radical to form disulphide bridges (Shringarpure et al., 2002). Alternatively, oxygen can add on to a methionine residue to form methionine sulfoxide derivatives. It has been found that various stresses lead to the carbonylation of proteins in tissues. Carbonylation of storage proteins has been noted in dry Arabidopsis seeds but carbonylation of a number of other proteins increased strongly during seed germination (Job et al., 2005). Bartoli et al. found that protein carbonylation was higher in the mitochondria than in chloroplasts and peroxisomes in wheat leaves which suggest that the mitochondria are more susceptible to oxidative damage.

Protein carbonylation is a widely used marker of protein oxidation and sensitive methods for its detection have been developed (Levine 2002). Direct oxidative attack on Lys, Pro and Thr by secondary reactions on a reactive carbohydrate and lipid on Cys, His and Lys residues can lead to formation of protein carbonyl derivatives (Nystrass, 2005). Carbonylation of protein inhibits or alters the activities and may increase the susceptibility of the protein to the proteolytic attack (Berlett and Stadman 1997). In some animals like in rats and in yeast and bacteria nearing senescence, the oxidation has been found to be specific for the respiratory and the stress proteins. (Dan et al., 2001). The quantitative and qualitative pattern of protein carbonylation has been studied in Arabidopsis thaliana during the progression of the life cycle of the plants. The carbonylation increases with the age of the plants similar to that of the animals/microbes but then drops steeply prior to translation of reproductive phase and again increases sharply during the senescence (Johansson et al. 2004).

When plant faces nitrogen stress there is an increase in production of ROS leading to protein oxidation. Earlier studies on protein carbonylation in Arabidopsis indicated the role of cytokinins in prevention of protein carbonyl degradation. The disruption of cytokinin flow to the leaf may be the trigger (Jain et al., 2008). Plants have evolved a system for nitrogen detection to constantly monitor nitrogen status, and continuously adapt to its changes by modulating enzyme activity, gene expression, and metabolite contents (Scheible et al., 2005). To integrate the actions of the nitrogen signal at the whole plant level, plants use multiple signalling routes that communicate internal and external nitrogen status. One route depends on nitrate itself, and many studies indicate that part of the nitrogen signal is replaced by CK (Takei et al., 2004). CK supply also changes nitrate reductase activity and protein concentration. Previous studies have highlighted the close correlation between nitrogen and CK in several plant species (Takei et al., 2001). A comparison of the genes regulated by CK
and nitrate provides some insights into the potential interaction of the regulatory pathways (Sakakibara et al., 2006). Despite some quantitative differences in relative expression levels, several genes have the same regulatory patterns in response to CK and nitrate applied individually. Slower rate of leaf initiation and expansion, chlorosis on leaf edges and slower growth of rosette. Response to external cytokinins indicates close relation between cytokinins and proteasomal pathway (Smalle et al., 2002).

Hence an attempt was made to investigate the effect of varied N supply and form on protein carbonyl formation, role of cytokinin and pathway of degradation of protein carbonyls.

During the course of our earlier studies we (Jain et al. 2006) observed that cytokinin prevented the degradation of protein carbonyls in dark stressed plants. So we initiated feeding of N stressed / non stressed leaves and ammonium treated leaves with various exogenous compounds to check the level of protein carbonyls. In these experiments we demonstrate that proteasome is involved and cytokinins may play an important role in preventing the triggering of degradation.

5.3. Materials and Method

5.3.1. Plant material and growth conditions

Wheat variety i.e. PBW-343 (Triticum aestivum, 2n=42, AABBDD) was procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi. Seeds were grown on germination paper after surface sterilization with 0.1% HgCl₂. After emergence of coleoptiles (5-6 days after sowing), seedlings were transferred into Hoagland solution (low N-50 μM KNO₃, control N-5 mM KNO₃ and 5 mM NH₄(SO₄)₂, AS and without N-zero N). The composition of nutrient solution used was macronutrient in mM: Ca(NO₃)₂ 1.5, KNO₃ 5.0, NH₄(NO₃)₂ 1.0, MgSO₄ 2.0 and micronutrient in μM: H₃BO₃ 1.0, MnCl₂ .4H₂O 0.5, ZnSO₄ .7H₂O 1.0, CuSO₄ .5H₂O 0.2, (NH₄)₆Mo₇O₂₄ .4H₂O 0.075 and FeCl₃ + EDTA.

The plants were supported on Styrofoam (2” thickness) and grown in plastic containers of 4 L capacity. The solution was aerated continuously through aquarium pumps and PVC tubings. The solution was changed every alternate day. The whole set up was maintained in controlled environment chambers at National Phytotron Facility, IARI, New Delhi. In these chambers, the growth conditions were maintained as: 22°C/12°C day/night temperature, 10 h photoperiod with photon flux density of 450 μmol m⁻² s⁻¹ (PAR) and the relative humidity (RH) was 90%.
5.3.2. ROS levels in wheat seedlings in response to N

The spectrophotometric assay of total superoxide radical content in the fresh leaf tissues is based on the principle of formation of blue coloured formazone by nitroblue tetrazolium chloride with superoxide radicals (O$_2^-$) by inhibiting total superoxide dismutase (SOD) activity, as described by Chaitanya and Naithani (1994). Hydrogen peroxide was estimated by forming titanium-hydro peroxide complex (Rao et al., 1997). Supernatant was read at 415 nm against blank in UV-visible spectrophotometer (model Specord Bio-200, AnalytikJena, Germany). Hydrogen peroxide contents were calculated by comparing with a standard curve drawn with known hydrogen peroxide concentrations (Rao et al., 1997). Lipid peroxidation was estimated as the thiobarbuturic acid reactive substances, according to the method of Heath and Packer (1968). Protease activity was measured by the method of Moore and Stein (1954) and total free amino acids were also measured by the method of Nearrie et al. (1960). Estimation for both the above parameters was done by the method of Lee and Takahashi.

5.3.3. Estimation of protein carbonyls

Total soluble proteins were extracted and derivatized by DNPH (Jain et al., 2008). SDS-PAGE was performed (Lammelli et al., 1980) and resolved proteins were transferred to PVDF membranes and protein carbonyls were detected using anti-DNP antibodies and area of bands was analyzed using infra red imager (LICOR Odyssey).

5.3.4. Expression analysis of IPT3 gene

To confirm the data obtained from exogenous application of cytokinin, gene expression of IPT3 was estimated. RT-PCR was done using primer for IPT3 gene which codes for cytokinin biosynthesis in Arabidopsis.

5.4. Results

Wheat seedlings responded differently to the type of the N nutrition as well as to the level of N supply.

5.4.1. Free radicals levels in the wheat seedlings in response to different levels and forms of nitrogen supply

5.4.1.1 Level of superoxide radicals in wheat

There was significant difference of free radical (Table 14) concentration among various N treatments. The maximum levels of superoxide radicals among the three levels of nitrogen supply were in the wheat seedlings grown without N followed by seedlings grown in 50 µM
nitrate N, where decline of 20% in the superoxide radical concentration was observed. Increase of NO3⁻-N dose had a positive effect as the level of free radicals in the wheat leaves as minimum levels of free superoxide radicals were observed in the wheat seedlings grown under 5mM NO3⁻-N. The level of superoxide radicals was 25% lower in 5 mM nitrate fed plants as compared to the plants grown without N (Fig. 14a).

It was observed that maximum concentration of the superoxide radicals was in the wheat seedlings grown in NH4⁺-N. The relative increase in these seedlings was 8% as compared to the seedlings grown without N and it was approximately 33% more as compared to the seedlings grown in 5 mM NO3⁻-N.

5.4.1.2. Level of hydrogen peroxide (H2O2) in response to different levels and forms of nitrogen supply

Maximum level of H2O2 (Table 15) was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO3⁻-N. Increase in the N dose resulted in the decline in the content of H2O2 in the wheat seedlings. Percent decrease (Fig. 15b) in the level of H2O2 in the seedlings grown in 50 µM N was 2% and in the seedlings grown under 5mM N was 17% as compared to the seedlings grown without N. The maximum concentration of the H2O2 was observed in ammonium grown 15d old wheat seedlings.

5.4.1.3. TBARS levels

There was significant difference of TBARS (Table 16) concentration among various N treatments. Maximum level of TBARS 509.64 nmol/g DW was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO3⁻-N. Percent decrease in the level of TBARS in the seedlings grown in 50 µM NO3⁻-N was 64.19% and in the seedlings grown under 5mM NO3⁻-N was 62.4% as compared to the seedlings grown without N. Percent decrease in the level of TBARS in the seedlings grown in NH4⁺-N was 34% as compared to seedling grown without N (Fig. 16b).

5.4.1.4. Protease activity

Maximum Protease activity (Table 17) was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO3⁻-N. Increase in the N dose resulted in the decline in the level of protease activity in the wheat seedlings. Percent decrease in the activity of protease in the seedlings grown in 50 µM NO3⁻-N was 34% and in the seedlings grown under 5mM NO3⁻-N was 148% as compared to the seedlings grown without N. Percent decrease in the level of protease activity in the seedlings grown in NH4⁺-N was 6.3% as compared to seedling grown without N (Fig. 17b).

5.4.1.5. Total free amino acids
There was significant difference of total free amino acids (Table 18) concentration among various N treatments. Maximum level of total free amino acids was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO$_3^-$-N. Percent decrease in the level of total free amino acids in the seedlings grown in 50 µM NO$_3^-$-N was 36 % and in the seedlings grown under 5mM N was 87% as compared to the seedlings grown without N. Percent decrease in the level of total free amino acids in the seedlings grown in NH$_4^+$-N was 13% as compared to seedling grown without N (Fig. 18b).

5.4.2. Protein carbonyls in the leaves of wheat seedlings grown at different levels and forms of N

The 15d old wheat seedlings grown under various levels of N supply and nitrate and ammonium salts were analyzed for the level of protein carbonyls. It was observed that wheat seedlings grown without N and ammonium salts had minimum levels of carbonyls followed by 5 mM NO$_3^-$-N. The seedlings from 50 µM NO$_3^-$-N treatment had maximum levels of protein carbonyls. The level of protein carbonyls was 4 fold more in low N grown wheat seedlings as compared to the level of protein carbonyls in wheat seedlings grown without N. The minimum level of protein carbonyls was in ammonium fed plants wherein the content was 17 % lower as compared to the wheat plants grown without N. The protein carbonyls in the low N grown wheat seedlings were 2 fold higher as compared to the level of protein carbonyls in the high N grown wheat plants. In the wheat plants grown in 5 mM NH$_4^+$-N the level of carbonyls was 60 % less as compared to the level of carbonyls in the seedlings from 5 mM NO$_3^-$-N fed plants.

Subsequent experiments were designed to determine the basis for the loss of carbonylated proteins and the nature of the signals that may be transmitted in the presence and absence of nitrogen slats. For this, the seedlings were incubated in solutions of hormones BA and Kin as there is close interaction between cytokinins and nitrogen nutrition. We wanted to determine if cytokinins prevented the loss of protein carbonyls. The seedlings were also incubated in solutions of 3 MA, inhibitor of autophagy and MG132, proteasomal inhibitor to determine which proteolytic system was involved in degradation of protein carbonyls. The approach adopted was to incubate the whole seedlings in light for 6h in various solutions and compare them with control (water) and also compare the control with the initial level of protein carbonyls i.e. prior to incubation.

5.4.2.1 Incubation of seedlings for 6h in various solutions under growth light conditions.

The 15d old wheat seedlings were removed from their respective, treatments and incubated in light for 6h in various solutions. When the seedlings grown without N were analyzed for the
protein carbonyl levels 6h after incubation it was observed that the levels of protein carbonyls increased in all the treatments as compared to the control by 20 % to 1 fold. The increase of protein carbonyls was more in 3 MA and MG132 treatments as compared to the BA and kin treatments. In control (water incubated) seedlings the level of protein carbonyls was similar to the initial level, i.e. before incubation. Maximum increase of ~1.2 fold as compared to control was observed in 3MA treated seedlings (Fig. 20).

The seedlings incubated in nitrate solution low N (50 µM) were analyzed for the level of protein carbonyls. The levels of protein carbonyls were high as compared to the seedlings grown without N. In the seedlings incubated in various solutions the level of protein carbonyls differed from control (water) by 19-37 % and from the initial level of carbonyls prior to incubation by 10-20% only (Fig. 21).

The seedlings from 5 mM NO3^- -N were incubated in various solutions of hormones and inhibitors. It was observed that the levels of protein carbonyls increased by 50 % to 1 fold, as compared to the control. In control the levels declined by 20% as compared to initial levels before incubation. Both 3MA and MG 132 in the incubation medium prevented the degradation of the protein carbonyls to the similar extent (Fig. 22).

When the seedlings from the ammonium treatment were incubated for 6h in light, the levels of protein carbonyls were increased in control (90%) as well as in the seedlings incubated in various solutions by 2-3 folds. Although the presence of cytokinin in the medium prevented the degradation of protein carbonyls in this treatment but we could not differentiate the involvement of the specific pathway in light treatment (Fig. 23).

We could not identify the pathway of degradation in the intact seedlings, so the next approach we followed was to detach the leaves of the wheat seedlings from the various treatments and incubate them in various mediums as described before for 6h in light. Secondly, in attached seedlings there was not much loss of protein carbonyls, so we wanted to confirm the notion that something provided from the root may control metabolism of the carbonylated proteins. The aim was to determine if detaching the leaves would affect the extent of protein carbonyl loss when the leaves were detached and incubated in hormone and inhibitor solutions in light.

5.4.2.1 Incubation of the detached leaves in hormone/inhibitor solutions in light

The leaves from 15d old wheat seedlings were cut under water and then detached leaves were incubated in various solutions for 6h in light. The decline in the protein carbonyl content was
20% in the control (water) incubated leaves from the seedlings grown without N. In all the other treatments the increase of 4-5 folds was observed as compared to the control. The detached leaves from low N grown plants were incubated in control i.e. water the carbonyl content declined by 27% and in the detached leaves incubated in various solutions of hormones and inhibitors an increase of 40-60% in protein carbonyl content was observed (Fig. 25). The detached leaves from wheat seedlings fed 5 mM NO₃⁻-N surprisingly showed high levels of carbonylation (Fig. 26) 6h after incubation in light in various solutions which probably was due to high soluble protein in this treatment and stress due to detaching increased the level of protein carbonyls. In control the level of protein carbonyls was only 12% less as compared to the initial value. But, after 6h of incubation in various hormone/inhibitor solutions, 1.5-2.5 fold increase in the level of carbonylated proteins was observed.

In the detached leaves of 15d old NH₄⁺-N (Fig. 27) grown wheat seedlings the protein carbonyls declined by 40% as compared to the initial value prior to incubation. In the detached leaves incubated in hormones/inhibitor solutions an increase of 20-70% was observed. We observed larger loss of proteins in the detached leaves incubated in light as compared to the attached seedlings. Hence, the leaves were detached from the seedlings and incubated in dark for 6h and light and dark treatments were compared.

5.4.2.2. Incubation of the detached leaves in hormone/inhibitor solutions in dark

The leaves from the 15d old wheat seedlings grown without N (zero N) were detached and these were incubated in various solutions as stated in methods and materials and incubated in dark for 6h. The loss of carbonylated proteins was high in control leaves up to 50% (Fig. 28) as compared to the initial value, before incubation. In other treatments after 6h incubation large increase ranging from 50% in 3MA to 3.8 fold in MG 132 was observed. In dark treated leaves both Kin and BA as well as MG 132 prevented the degradation of the carbonylated proteins. 3-MA could not prevent the degradation of the protein carbonyls.

In the detached leaves from the low N (Fig. 29) grown wheat seedlings 60% loss of carbonylated proteins was observed incubation of the detached leaves in 3-MA reduced this to 40%. Incubation in the solutions of BA, Kin and MG 132 prevented the degradation of the protein carbonyls and increase of 26-44% was observed in these treatments.

As cytokinins prevented the degradation of protein carbonyls, the expression of **IPT3** gene was analyzed in wheat seedlings incubated in various N solutions as well as from
the detached leaves of control treatments of light and dark. The relation between expression of IPT3 gene and prevention of degradation by cytokinins was analyzed.

The results (Plate 3) clearly indicated that in the absence of IPT3 gene expression as in NH\textsubscript{4}\textsuperscript{+} fed wheat seedlings or as in plants with low expression as in zero N grown plants, the turnover of proteins via degradation of protein carbonyls was high.

5.4.3. Discussion

Reactive oxygen species (ROS) are produced continuously as byproducts of various metabolic pathways. Under steady state conditions these are scavenged by various antioxidative defense mechanisms (Gill and Tuteja 2010). The equilibrium between the production and scavenging of ROS may be perturbed by various biotic and abiotic stress factors, such as salinity, drought, UV radiation, nutrient deficiency, leading to sudden increase in ROS, causing significant damage to cell structures. ROS are highly reactive and causes damage to the macromolecules of the cells, such as lipids, proteins, DNA etc. the changes in the concentration of ROS can also lead to changes in cell signaling by modulating gene expression.

The implication of reactive oxygen species (ROS) during nitrogen and specifically ammonium stress has not yet been well characterized. Studies that combine ammonium and salt stress in sunflower and corn suggest that ammonium or its assimilation molecules, glutamate or glutamine, may serve as a stress signal to activate antioxidant enzymes, which play a key role in adaptation to stress situation (Rios-González et al. 2002). Polesskaya et al. (2004) indicated that NH\textsubscript{4}\textsuperscript{+} induced antioxidant enzyme activities, and during N deficiency implies an oxidative stress because of an increased Mehler reaction and because of an energetic imbalance related to a reduction in N assimilation. However, they did not observe low-molecular weight antioxidant status or markers of oxidative damage to demonstrate that an oxidative stress was taking place. More recently, Skopelitis et al. (2006) have shown that ammonium ions induce generation of ROS in a study with cell suspensions of Vitis vinifera. However, the cell suspension model may behave differently to the whole plant model, and the analysis of ROS production and its effects in the whole plant deserve attention. Plants have evolved various protective mechanisms to eliminate or reduce ROS, which includes enzymes such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), guaiacol peroxidase (GPX) and catalase (CAT). In biological systems, reduced glutathione (GSH) and ascorbate (ASC) appear to be the most important soluble low molecular antioxidants, which can react directly with superoxide and hydroxyl radicals or participate in enzymatic reactions that scavenge ROS (Foyer et al. 2005, Smirnoff,
Plant phenolic compounds may also act as antioxidants or prooxidants in different reactions (Kahkonen et al. 1999, Moran et al. 1997). The production of superoxide radicals and $\text{H}_2\text{O}_2$ was maximum in ammonium fed wheat seedlings followed by zero N seedlings. Parallel to this, we observed that lipid peroxidation was enhanced in nutrient stressed and ammonium fed wheat seedlings. Enhanced production of oxygen free radicals is responsible for peroxidation of membrane lipids and the degree of peroxides damage of cell is controlled by the potency of peroxidase enzyme system (Gomathi and Rakkiyappan, 2011). Very low levels of protein carbonyls in low N and ammonium grown wheat seedlings indicate degradation. This correlates well with the low content of the total soluble proteins and high levels of total free amino acids in these treatments (Fig. 24). Ammonium being toxic for the wheat plants as seen in this study led to high levels of ROS in the medium. But surprisingly the levels of protein carbonyls were low in wheat seedlings grown in $\text{NH}_4^+$-N and zero-N grown plants. This indicated high turnover rates of protein in $\text{NH}_4^+$-N fed wheat seedlings as the level of free amino acids was high, protease activity was high and level of soluble proteins was low. This also correlates with low expression of $IPT3$ gene of cytokinins, thereby, indicating lower level of cytokinins in these treatments. Cytokinins are known to play an important role in prevention of protein degradation and $IPT3$ gene which is the major gene for the cKs and expressed throughout the plant in phloem is down regulated in absence of $\text{NO}_3^-$-N and in the presence of $\text{NH}_4^+$-N.

Among the nitrate fed wheat seedlings, we observed high levels of protein carbonyls in the low N grown seedlings. Low N grown seedlings had lower levels of free amino acids and protease activity thereby indicating lower degradation of protein carbonyls. In the presence of nitrate the $IPT3$ gene expression was also observed. Hence, this again confirms the involvement of cytokinins in the prevention of protein degradation. We speculate that probably their degradation was prevented due to the presence of Cks, as the expression of the Ck gene in these seedlings was observed. In high N grown seedlings the level of carbonyls was low, this indicates low production of protein carbonyls and not their degradation as the level of free amino acids in these plants was lower, and the total soluble proteins were low. This has been observed in our earlier studies that when the level of protein carbonyls is high under stress conditions when levels of free amino acids is low (Jain et al 2008). Excessive degradation of the proteins in high $\text{NO}_3^-$-N grown plants seems unlikely as cytokinin gene expression was high in these and levels of total soluble protein was high and free amino acid level was low.
When the whole seedlings were incubated in light, less stress was experienced by them as compared to the detached leaves. The detached leaves may experience more stress as detaching the leaves may lead to closure of stomata which may be equivalent to dark stress. In the intact seedlings the level of protein carbonyls was similar to that of initial level and no significant differences were apparent visually of densitometry scanning in the level of the protein carbonyls either in control or in the presence of hormones/ inhibitors.

It is generally assumed that carbonylation results in loss of function and the oxidatively damaged protein must be degraded. In animal systems the carbonylated proteins can be degraded by the 20S proteasome (Grune et al 2003) or by chaperon mediated autophagy (Kiffin et al, 2004). In plants, developmental regulation of carbonylated proteins degradation was inferred because there was a dramatic reduction in leaf protein carbonyl content when Arabidopsis plants began reproductive development (Johansson et al, 2004). As the total soluble protein remained constant degradation of the oxidized proteins followed by re-synthesis was postulated but the pathway of degradation was not elucidated. However, two plant studies have examined the pathway involved in degradation of oxidatively modified proteins. In maize (Basset et al., 2002), reported that dark treatment of maize plants for 24 h stimulated 20S proteasome activity possibly as a result of oxidative modification of the proteasome itself, and the authors suggested that carbonylated 20S proteasomes may function to degrade oxidatively modified proteins during carbon starvation. More recently, the pathway of carbonylated protein degradation was carefully explored in Arabidopsis under severe oxidative stress imposed by application of H₂O₂ or methyl viologen (Xiong et al. 2007). Under these conditions, oxidized proteins were degraded by the autophagy pathway. During normal senescence, plants also use autophagy pathways to degrade the organellar and cytoplasmic proteins indiscriminately (Doelling et al. 2002).

In the present study, involving proteins oxidatively modified due to N supply, we found that the proteasome was involved rather than the autophagic pathway when the detached leaves were incubated in dark. When the seedlings or leaves were kept in light involvement of specific pathway could not be elucidated. Thus, it appears that different pathways may be involved under different conditions and in response to varying levels of protein oxidation.

In the whole seedlings incubated in light it was clear that both autophagic and proteasomal pathways were involved in the degradation of protein carbonyls, as both 3 MA and MG 132 prevented the protein carbonyl loss. External cytokinin or their presence in the incubation medium had less effect in the seedlings from the nitrate solutions as probably
these seedlings maintained the Ck levels as evident from the expression $IPT3$ gene in the seedlings incubated in light. Exogenous Ck in the incubation prevented the degradation of the protein carbonyls in detached leaves incubated in light and dark and maintained the levels of soluble proteins, as the expression of $IPT3$ was not observed in zero-N grown, NH$_4^+$-N grown plants. The expression of the $IPT3$ was also very low in low N plants (50 µM N). This also explains the low effect of external Cks on the protein carbonyl levels in the attached seedlings as in these the level of Cks may have been high during the 6h incubation period in light.

Our results therefore, suggest that the trigger for degradation of carbonylated proteins in darkened detached leaves may be disrupted Ck flux to the leaf. This is based primarily on the observation that exogenous kinetin / BA almost completely prevented the loss of protein carbonyl content as well as the loss of soluble protein. When leaves are attached, xylem flux of hormones (and other constituents) is maintained and protein carbonyl content remains high and stable even for extended dark periods (up to 6h). This could explain why leaf detachment was required for rapid degradation of carbonylated proteins. It also appears that degradation of the carbonylated proteins was prevented in light especially in NO$_3^-$-N leaves as it is possible that light influences the metabolism of endogenous Ck (such that a deficiency does not occur within 6 h), we observed the expression of Ck related genes in both low N and high N grown plants even after 6h of incubation in light. It is also possible that light signals somehow attenuate the impact of restricted Ck flux to the leaf. Darkened leaves also experience carbon starvation (Brouquisse et al. 1998), which could be another important factor; however, sucrose feeding to darkened, detached leaves had no effect despite the fact that sucrose is readily taken up into darkened detached leaves (Fujiki et al. 2001).

Previous studies have highlighted the close correlation between N and Cks in several plant species (Cline et al 2006). Comparison of genes regulated by Ck and nitrate provides some insights into the potential interaction of the regulatory pathways (Ding et al 2011). The coordination and interaction of nitrogen and Ck regulatory pathways is important for normal plant growth under variable N supply conditions. There may be some quantitative differences in relative expression levels, but several genes have same regulatory pattern in response to Cks and nitrate applied individually.

In our study, we observed that expression of $IPT3$ gene of the Ck biosynthetic pathway was up regulated by nitrate supply. Its expression increased with increasing N supply. The $IPT3$ expression was very low when nitrate was absent from the medium or in
the presence of ammoniacal N. Hirose et al (2008) conclusively showed that IPT3 was upregulated in response to nitrate form of N nutrition.

In the recent study by Ding et al (2011), it has been shown that three proteins related to protein degradation identified as highly specific regulators of plant development are regulated in response to Cks. Also a protein involved protein degradation in 26S proteasome regulatory particle (RPN 10) was down regulated both by N and BA treatment (Ding et al 2011). Hence in the presence of BA and Kin (Jain et al 2008), RPN 10 might be under the direct regulatory control of N and Cks and hence preventing degradation carbonylated proteins.

This confirms the results obtained in our study that Ck increases protein concentration by decreasing protein degradation (Criado et al 2009). Cks accumulate in plants in response to NO$_3^-$ -N supply (Takei et al 2001). Our studies also show that ammonium-N does not show similar response as nitrate N. The Cks levels might be low in presence of ammonium as expression of IPT3 was low. Only one gene is upregulated in response to both nitrate and ammonium, IPT5 which is expressed in lateral roots and is not the major gene. So, level of Cks in the plants under ammonium nutrition may be one of the major factors for the oxidative stress and reduced growth and high protein degradation. From our study it also appears that expression of IPT3 gene prevents ROS generation and hence excess protein oxidation in plant grown under ample N supply. Earlier studies have indicated that Cks such as zeatin and BA can increase the activity of some antioxidant enzymes (SOD, catalase) and reduce ROS (Liu et al 1996). This could be the reason for low ROS generation in NO$_3^-$ -N plants as they had higher levels of Cks in plant tissues.

Important point that emerged from the study was that in intact seedlings incubated in light the level of protein carbonyls did not decline more than 5-10% in controls from their initial value from the respective treatments. The decrease in carbonylated proteins in detached leaves incubated in light varied between 20-25 % from their initial values. But when detached leaves were incubated in dark the carbonyl levels declined by 50-60%. In light both the pathways i.e. lysosomal pathway and autophagy was involved in degrading protein carbonyls. The exogenous application of cytokinins prevented the degradation of protein carbonyls more so in dark detached leaves wherein the expression declined to very low levels even in 5mM NO$_3^-$ –N treated plants. Interaction among N supply and cytokinins may play an important role in maintaining the protein homeostasis in plants.
6. DISCUSSION

Nitrogen (N) is often the most essential and most deficient of all the plant nutrients. Wheat is very sensitive to N limitation and very responsive to nitrogen fertilization. Plants contain more nitrogen than any other essential elements derived from the soil, and absorb and use various forms of N most importantly the inorganic ions ammonium (NH$_4^+$) and nitrate (NO$_3^-$). The largest amounts are taken up during early stages of growth, held for later use and translocated within the plant where needed, for example, to the kernels from the leaves and stem during maturation. The most important role of N in the plant is its presences in the structure of protein, which are the building substances from which the living material or protoplasm of every cell is made. In addition, nitrogen is also found in all most all the macromolecules of plants. The nitrogen supply to the plant will influence the amount of protein, protoplasm and chlorophyll formed. In turn, this influences cell size and leaf area, and photosynthetic activity.

Plants grown with adequate supply of nitrogen make rapid and thrifty growth and are dark green in color. Leaf and stem development is stimulated. Insufficient nitrogen results in lighter green color, reduced tillering, and disturbance of normal cell growth division, and a decrease in rate, and extent of protein synthesis. Because of this, crop yields may also be greatly reduced. Nitrate assimilation consumes more energy than ammonium assimilation only a few species perform well when NH$_4^+$ is the sole source of nitrogen. Many species develop symptoms of toxicity when subjected to high concentrations of NH$_4^+$ which are not detected when plants are grown with the same concentration of NO$_3^-$ or mixed N nutrition (Hochani et al., 2011). High concentrations of ammonium in the soil or in the nutrient solution may lead to an “NH$_4^+$ syndrome”, which may include leaf chlorosis, lower plant yield and root/shoot ratio, lower cation content, acidification of the rhizosphere, and changes on several metabolite levels such as amino acids or organic acids (Britto and Kronzucker, 2002). Since NH$_4^+$ is a photophosphorylation uncoupler (Peltier and Thibault, 1983), its accumulation can decrease net photosynthesis and therefore reduces plant growth (Goyal et al., 1982; Britto et al., 2001).

In the present study, we investigated the effect of varied N supply and form (ammonium N) on growth and N assimilation in wheat. The mechanism of reduced growth was related to increased stress, protein oxidation, reduced cytokinin production in N deprived and ammonium fed wheat plants. Effects of nitrogen on growth might result from effects on root development and/or on leaf area. The effects of NO$_3^-$ and NH$_4^+$ nutrition in wheat were compared. Wheat was found to be sensitive to ammonium nutrition and nitrogen stress. The wheat plants grown under NH$_4^+$-N had decreased shoot growth, lower root:shoot ratio. Whereas, plants grown without N had higher root:shoot ratio. Rate of leaf area production was higher in high N grown plants than at low N, especially in wheat plants grown with nitrate N. Leaf area of high N plants increased due to the larger leaf area of the individual
leaves. In our earlier study studies we clearly showed that increasing N supply increases leaf area by increasing cell size as well as cell numbers (Sivasankar et al. 1998). Some studies have indicated that reduction in leaf area at low N supply may be due to reduced cell number only (Liu et al. 2000). Leaf number often remained the same in low and high N grown plants.

There were significant differences in dry matter accumulation, root: shoot ratio with increasing N supply as well as between NH$_4^+$ and NO$_3^-$ fed plants. Inhibition of growth caused by N deprivation as well as ammonium nutrition has been reported for several plant species (Raab and Terry, 1994; Liu et al 2000). Inhibition of the shoot growth of NH$_4^+$ fed plants when compared with similar level of NO$_3^-$–N grown plants could be attributed to low SLW, reduced leaf area and increased root growth as indicated by decreased root:shoot ratio. Studies have indicated that dry matter partitioning to shoots and roots was strongly related to plant N concentration. Dry matter partitioning is affected by plant N concentration (Pinheiro et al 2000). These two parameters are tightly coupled. Form of nitrogen strongly affected the partitioning of absorbed nitrogen between shoots and roots. Shoot root ratios were more in ammonium fed plant. There is also a possibility of involvement of Cks in the regulation of root architecture in response to nitrogen. In nitrate fed plants IPT3 expression was observed and it increased with increasing NO$_3^-$-N supply and is known to act at both initiation and organization of lateral root primordium, and therefore, high N supply had lesser root growth (Laplaze et al 2007, Kiba et al 2011).

The responses of plants have been examined using non-steady-state techniques by measuring the patterns of growth and plant N concentration zero or low external nitrate supply (Burns, 1994a,b). These results indicate the relationship between RGR and plant N concentration can be curvilinear, implying that, the rate of recycling of N within the plant declines as the reserves become depleted. These conclusions are in contrast with those from the steady-state approach. Although the interpretation of these data was questioned recently (Wikström and Ågren, 1995), subsequent investigations have confirmed the original observations (Burns et al., 1997; Walker et al., 1997). These results suggest that the main cause of the difference in the form of the relationship between growth rate and plant N concentration lies in the way in which N is partitioned or reallocated between tissues when plants are forced to rely on either limited external or internal sources of N. In the presence of NH$_4^+$ the root respiration is enhanced and the root : shoot ratio decreases when compared with NO$_3^-$ fed plants. It has therefore, been suggested that excessively high sink strength for carbon in the roots occurs in the presence of NH$_4^+$ as the sole N source and this may be responsible for ammonium toxicity. High NH$_4^+$-N also leads to the reduction of sugars/starch content of the plant tissues. The nitrate content in the shoots and roots of zero N and ammonium grown plants is highly reduced. This might further lead to reduction in growth of the plants caused by the lack of nitrate as an osmoticum, as it is an important osmotic anion for leaf cell expansion.

The studies also indicate that when N supply is limiting N is used with priority for maintaining photosynthesis. Ammonium nutrition increases rates of dark respiration as compared to
nitrate nutrition. The zero-N plants developed paler green leaves and grew at an increasingly slower rate than the control (+N) plants during the course of the experiment. The roots on the zero-N plants also tended to be proportionately larger with less branching than those from the well-nourished control treatment (data not shown).

Activity of enzyme nitrate reductase increased with increasing N supply and very low NR activity was detected in low N and ammonium grown plants. Our previous results indicated that ammonium nutrition failed to enhance the GS / GOGAT activities (unpublished data). Toxicity may be due to the inability of the sensitive plants to control the uptake of ammonium, leading to intercellular pH disturbances (Kosegarten et al 1997, Britto and Kronzucker, 2002). The mechanism of low affinity transport for ammonium is not yet resolved. Many research effort have been directed towards unraveling the causes and mechanisms of NH$_4^+$ toxicity. Chemical changes in the plants induced by NH$_4^+$ exposure include tissue depression, essential cations decline, and anions increase (Gloser and Gloser, 2000), organic acids decline (Leport et al 1996) and free amino acids increases. The decline in total N was due to the reduction of activity and expression of ammonia assimilating enzymes (unpublished data) in zero N and NH$_4^+$ grown plants. Hence the growth was limited by the N availability, unlike the results obtained by Liu et al (2000). The reduction in N content of the seedlings grown in zero N and NH$_4^+$ grown plants as compared to NO$_3^-$ grown plants, may be due to very low activity of nitrate reductase enzyme and even the GS activity. It is known that NIA gene is upregulated by nitrate and not by ammonium (Jain et al 2005). NIA gene is also upregulated by cytokinin and in zero N plants as well as in NH$_4^+$ –N plants the expression of IPT3 was very low. This probably resulted in lower N and lower total soluble proteins.

As stated earlier the ROS may cause irreversible carbonylation of proteins, resulting in structural and functional modifications. Low protein content, increased free amino acids, increased generation of ROS, and high protease activity in zero–N and NH$_4^+$-N fed plants indicate higher levels of protein carbonyl formation and high rates of protein degradation. Higher ROS generation is generally accompanied by increased antioxidant capacity. The studies have shown that increase in N dose in terms of nitrate –N significantly increases oxidized ascorbate pool, whereas, in plants with limiting N supply and NH$_4^+$ N the Asc pool was more reduced (Dominguez et al 2008). Our earlier studies indicated that ammonium nutrition effects on antioxidant enzymes are also variable (unpublished data). APX responded to high dose of ammonium when compared with similar dose of nitrate. But, the activity of SOD and catalase was not affected leading to high ROS in the NH$_4^+$-N fed plants. In NH$_4^+$ fed spinach plants SOD and catalase increased (Dominguez et al 2008).

Higher ROS and increased free amino acids as well as lower total soluble protein content of zero N and NH$_4^+$- N fed plants as compared to NO$_3^-$-N grown wheat plants indicated that there might be enhanced protein oxidation along with membrane damage, as peroxidation in terms of MDA (full form) content was enhanced in these treatments.
Protein carbonyl content is a valuable oxidative stress biomarker resulting from oxidation of amino acids residues in proteins by ROS. Carbonyl content was low in NH$_4^+$-N and zero-N grown 15 d old wheat seedlings, as compared to low and high nitrate –N grown plants. Protein carbonylation is a non enzymatic process that originated exclusively from ROS attack upon specific side amino groups in proteins (Requena et al 2001). Wheat plants showed considerable effect on protein carbonyl content in response to N supply.

In contrast to high ROS generation, increase in carbonyls was expected in plants grown at zero-N and NH$_4^+$-N. But, the level of protein carbonyl were low in these plants. There can be two explanations for this, either there was increased turnover of protein carbonyls or secondly abiotic stress may not always cause damage to biomolecules (Domínguez et al., 2005). Here we speculate that protein carbonyls were lost from the leaves of these treatments.

We observed the loss of protein carbonyl from the control leaves of whole seedlings incubated in light from various N treatments ranged from 10-20% when compared with their respective controls. In detached leaves incubated in light in H$_2$O (control) the loss of protein carbonyls was 30-35%, when compared with their initial values, prior to incubation. When the leaves were detached and incubated in dark, the loss of protein carbonyls in control leaves ranged from 50-60%. As the loss of protein carbonyls increased further in dark we hypothesise that light somehow prevents the extensive turnover of the proteins even in detached leaves.

Based on the speculation that ammonium N and zero N limitation caused increased protein turnover led to the experiments to identify the pathway of protein degradation. This mechanism is not yet fully understood, but it has been thought that the mechanism can be divided in to two pathways: (i) Lysosomal pathway and (ii) Non-lysosomal pathway (Takatsuka et al., 2004). Lysosomal pathway is mainly performed by autophagy and 3MA is potent inhibitor of autophagy, it blocks the formation of autophagosomes and inhibits protein degradation without affecting protein synthesis (Seglen and Gordon, 1982). Under starvation conditions autophagy is used (Vierstra, 2003). Carbobenzoyl-leucinyl-leucinal (MG132) is a potent inhibitor of proteasomal activity as it inhibits lysosomal cysteine proteases, and binds reversibly to N terminal through residue of β$_1$ subunit with 26S proteasome. Using these two inhibitors we could identify the pathways of protein carbonyl degradation in light and dark.

Our experiments with inhibitors showed that during incubation in light (whole seedlings as well as detached leaves) both autophagic and lysosomal pathways operated. In detached leaves incubated in dark, 3MA had little effect in preventing protein carbonyl degradation. MG132 prevented the loss of protein carbonyls, thereby indicating specifically the role of proteasome, especially in dark. There are still many questions that remain unanswered that needs further studies. There are no reports on how autophagy is prevented in light and not in dark. But earlier study in maize by Basset et al. (2002) reported dark treatment stimulated proteasome activity. Otherwise, during normal light
conditions plants use extensively, the autophagic pathway to degrade oxidized proteins (Xiong et al., 2007).

As protein carbonyls were lost more rapidly from detached leaves as compared to whole seedlings incubated in either light or dark, we speculated that some signal from root may prevent degradation. Hence, we tested the effect of Kin (synthetic Ck) and BA (endogenous Ck) on the loss of protein carbonyl content. Our results indicate the prevention of protein carbonyl degradation in the presence of exogenous Cks.

Cytokinin content in higher plants is correlated with N availability and influences several enzymes involved in N-metabolism (Freschi et al., 2009). Some reports indicate that Cks produced locally within root or translocated from shoot may signal that there is sufficient N present. Application of Cks exogenously represses the two ATNRT2 and NRT1 genes (high affinity and low affinity transporter genes) for nitrate (Bunner et al., 2005) resulting in low uptake of nitrate and effect was Cks specific. Ck also affects root growth (Laplaze et al., 2007). Apart from interaction between nitrate and cytokinin signaling, N-metabolism is also linked with C-metabolism.

Our results suggests that trigger for degradation of carbonylated proteins in light incubated detached leaves may be disrupted Ck flux to leaves. As exogenous Ck prevented the loss of protein carbonyls. In attached plants probably its leaves are maintained and effect was not apparent. Therefore, in attached seedlings, this could explain the requirement of leaf detachment for Ck effect. IPT3 gene expression also declined in detached leaves incubated in water in light for 6h and it was almost absent in detached control (water) leaves incubated in dark. We therefore identified the apparent linkage between Ck and carbonylated protein degraded and N supply may have relevance to protein homeostasis in response to changes in N nutrition. Understanding the mechanism would have important implications during N limitation when rate of protein turnovers are high and proteins are preferentially mobilized towards roots.
In the present investigation wheat variety PBW 343 was examined to find out the effect of different N levels and forms on growth and N-metabolism as well as to elucidate the pathway of degradation of carbonylated proteins and role of cytokinins in relation to N-nutrition. The seedlings were grown in Hoagland solution under controlled condition in growth chambers. Four N-treatments were given to the seedlings (zero N, low NO$_3^-$-N, high NO$_3^-$-N and NH$_4^+$-N) and difference in their growth, N-metabolism, ROS production and protein carbonyl content was observed along with total free amino acids, protease activity and TBARS. Level of cytokinin expression was also analysed in all the treatments.

Wheat is sensitive to N supply. The biomass reduction was observed in ammonium grown plants and at 5mM ammonium dose the biomass accumulation was more as compared to the reduction in low NO$_3^-$-N fed plants when these both treatments were compared with NO$_3^-$-N fed plants. Biomass partitioning between root and shoot was affected leading to very high root: shoot ratio in zero-N plants. The root: shoot ratio of NH$_4^+$-N grown plants also increased. There was a large significant reduction in the activity of enzyme nitrate reductase in the zero-N and NH$_4^+$-N treatments, and NR activity increased with increasing N dose. We speculate that this might be due to the regulation of NIA gene by Cks and nitrate levels. The expression of IPT3 gene declined in the treatments with low NR activity and the nitrate content of the wheat seedlings in these two treatments was 200-350 % lower as compared to NO$_3^-$-N fed plants. As a result of these the total soluble protein content was low under ammonium and zero N fed wheat seedlings. Increase in NO$_3^-$-N dose had a positive effect on the protein content of the wheat plants.

Ammonium fed and zero N plants had higher free amino acid content in shoots. Along with increase in amino acids, the ROS generation in terms of O$_2^.$ and H$_2$O$_2$ and TBARS increased in ammonium fed and zero N plants as compared to NO$_3^-$-N plants. The generation of ROS was more in 5mM NH$_4^+$-N fed plants as compared to low 50µM NO$_3^-$-N fed plants also. The increased H$_2$O$_2$ levels caused parallel increase in lipid peroxidation and protein oxidation. Lipid peroxidation measured in terms of MDA content was high in zero and ammonium N grown plants, 166% and 90 % increase as compared to the NO$_3^-$-N grown plants was observed. It was maximum in 5mM NH$_4^+$-N grown plants as compared to even low N i.e. just 50µM grown NO$_3^-$-N plants, thereby again indicating the toxicity of ammonium nutrition to wheat, as well as the damage caused by limiting N supply.

To ascertain whether increased production of ROS is affecting plant cellular components under ammonium nutrition and limiting N supply, carbonylation of proteins was
quantified as oxidative stress biomarker. Protein carbonylation was low in zero-N and ammonium fed wheat plants, thereby indicating high turnover of damaged proteins consistent with high free amino acid levels in these plants as compared to NO$_3$-N fed plants. Content of the proteins in a plant depends not only on the rate at which it is synthesised, but also on the rate at which its degradation occurs. Lower soluble protein content of ammonium fed and zero N plants together with higher amino acids contents along with high protease activity as compared to the NO$_3$-N fed wheat seedlings may be interpreted as an indicator of higher protein turnover rates. The increase in amino acid synthesis and the high rates of protein turnover would require extra energy, that may explain overall reduced growth in these treatments as compared to the NO$_3$-N treatments. Increased root respiration may also implicate carbon deficiency in N limited and NH$_4^+$-N fed plants.

Increase protein turnover implies that proteins are being degraded in plants. These can occur by two pathways, either autophagic or proteasomal pathway. Hence, we used the inhibitors of both the pathways to understand the mechanism being used in degradation because it is an important event in plant regulatory process. 3MA and MG132 are potent inhibitors of lysosomal and non-lysosomal pathways, respectively. We observed that in intact/seedlings/or detached leaves incubated in light from all the four treatments of N supply and form (described earlier), the carbonyl content of the control treatment was similar to initial value of the respective treatment. But both 3MA and MG132 prevented the degradation of protein carbonyls as the accumulation of protein carbonyls was observed. The exogenous application of hormones prevented the degradation of the protein carbonyls further and the increase in their contents was observed in these treatments. We therefore, speculated the involvement of root signal in preventing the degradation of the carbonylated proteins.

As in intact seedlings in control (water incubated seedlings) treatment the level of protein carbonyls remained more or less the same when compared with their initial values from prior to incubation, although an overall increase was detected in the level of protein carbonyls in the treated plants, indicating prevention of degradation. In the subsequent experiments, therefore, the leaves were detached under water and incubated in solutions of hormones (BA and Kin) and inhibitors (3 MA and MG132) and control (water) for 6h in light.

Detaching the leaves and incubating them in hormone and inhibitor solutions enhanced the level of the carbonyls. The decline of protein carbonyls in control plants more as compared to that observed for attached seedlings. The decline of protein levels in control
plants ranged from 10-15 % in detached leaves from various treatments. Again both 3MA as well as MG132 prevevted the degradation of protein carbonyls thereby implying the involvement of both pathways. Maximum levels of protein carbonyls were observed in NH$_4^+$-N fed plants.

Very low decline of protein carbonyls in attached and detached leaves incubated in light, led us to compare detached leaves in light and dark in the subsequent experiments. The loss of carbonylated proteins was enhanced in detached leaves incubated in dark. It was observed that loss of protein carbonyls in the controls from various treatments ranged from 40-63 % which was significantly higher as compared to that observed in light incubation. Hence we conclude that dark stress accelerates the degradation of protein carbonyls.

Secondly an interesting result was obtained in dark seedlings, wherein the protein degradation was prevented only in the leaves incubated in MG 132 and not in 3 MA. This clearly shows the involvement of proteasomal pathway for protein carbonyl removal during dark stress. It appears under light mechanism for protein carbonyls is different which allows the degradation via both autophagy and proteasomal pathways. Hence, we conclude that different pathways may operate under different conditions and dark proteasomal pathway is predominant.

As then differences existed in loss of protein carbonyls in attached/detached leaves from control treatments, we speculated that some root signal from root may play an important role. In our earlier study with Arabidopsis, in dark detached leaves we observed that cytokinins prevented the loss of protein carbonyls. In the present investigation too, exogenous application of cytokinins enhanced the levels of protein carbonyls. We examined the effect of both Kin (synthetic Ck) and BA (endogenous Ck) and concluded that presence of cytokinin like molecules prevented protein turnover and both the Cks used, enhanced the levels of protein carbonyls.

In the series of experiments, we checked the expression of IPT3 gene in the wheat seedlings grown under various levels and forms of N supply. The expression of the IPT3 gene was enhanced with increasing N concentrations in the medium. The expression of this gene was almost absent in ammonium treated plants and low in zero-N plants. The degradation or the prevention of protein carbonyl degradation correlated with the levels of IPT3 gene expression in the wheat plants from various treatments. It is possible that cytokinins interact with proteasomal pathway, especially 26S proteasome (Smalle et al 2002), or as shown recently in regulating the RPN10 SU of the proteasome in proteomic approach to assess protein oxidation in rice.
8. CONCLUSION

Our results suggest that the wheat plants are sensitive to limiting N supply and form of N provided in the medium. Trigger for degradation of carbonylated proteins in darkened detached leaves, may be disrupted cytokinin flux to the leaf. In detached leaves incubated in light, the effect of exogenous application of Cks was more as compared to that observed in intact seedlings. Cks almost completely prevented the loss of protein carbonyl content as well as the loss of soluble protein. When leaves are attached, xylem flux of hormones (and other constituents) is maintained and protein carbonyl content remains high. This could explain why leaf detachment was required for rapid degradation of carbonylated proteins, but it is not immediately clear why light prevents the degradation of carbonylated proteins in detached leaves. It is possible that light influences the metabolism of endogenous cytokinins (such that a deficiency does not occur within (6 h), or that light signals somehow attenuate the impact of restricted cytokinin flux to the leaf. Another unresolved but important question is how carbonylated proteins are selectively targeted for degradation by the 26S proteasome, especially in dark. Clearly much remains to be done to elucidate details of the mechanism, but it is apparent that carbonylated proteins can be selectively and rapidly degraded under certain conditions. The apparent linkage identified between cytokinins and carbonylated protein degradation may have relevance to protein homeostasis in response to changes in N nutrition. It is well known that N availability is a major factor controlling cytokinin biosynthesis in roots for transport to shoots (Takei et al. 2001, Takei et al. 2004, Sakakibara et al. 2006). Thus, cytokinins, along with organic and inorganic forms of N, are signals to the shoot regarding soil N status. One response to a decrease in N availability is increased protein mobilization from old leaves to sustain life and support new growth such as root elongation to explore new soil for resources. Indeed, careful studies of wheat leaf senescence in response to a rapid removal of nitrate identified a rapid decrease in the cytokinin, isopentenyl adenosine, and suggested that this was the trigger to induce protein degradation in response to N deficiency (Criado et al. 2007). Our results suggest that carbonylated proteins may be specifically targeted for degradation under these conditions. How this selectivity is achieved, and the mechanism(s) by which cytokinins control this process, are important questions for future study. Our study reveals the unidentified link between 26S proteasome and cytokinin responses.
ABSTRACT

Nitrogen is one of the most important nutrients required by plants and its deficiency is also widespread. Sixty percent of the soils in India are deficient in nitrogen (N). This deficiency of N seriously affects plant growth, yield and quality. Lot of work in diverse areas has been done but many questions regarding N nutrition remain unanswered. The nitrogen in wheat is taken up mainly in the form of nitrate, and occasionally in the form of ammonium. But, wheat plants although capable of taking up ammonium are sensitive to ammonium nutrition. In terms of quality the protein content goes down. When plant faces nitrogen stress, it is possible that due to stress in spite of low protein content of tissues, they might be oxidised and carbonylated leading to increased degradation in such plants facing nutrient stress. In the present study the, the effect of different levels and forms of N has been investigated in wheat seedlings grown under controlled conditions. The interaction between cytokinins gene expression and N was also investigated. The extent of protein carbonylation was analyzed and an attempt was made to elucidate the pathway of protein degradation. Wheat seedlings (var. PBW 343) were grown under four different N treatments and various parameters of growth, N-assimilation and stress was studied. Result indicated that growth and nitrogen metabolism was enhanced in seedlings under high NO$_3^-$-N when compared to those grown with low and without N. The growth of the wheat seedlings was severely inhibited in terms of biomass accumulation, leaf area and root: shoot ratio was enhanced in zero N and ammonium grown plants as compared to the nitrate N fed wheat seedlings. There was almost 5 fold increase in shoot dry weight of NO$_3^-$-N fed seedlings when compared to those grown without N. In seedling grown with 5mM NH$_4^+$-N shoot dry weight declined by 63% as compared to the wheat seedlings grown in 5 mM NO$_3^-$-N. There was a relative increase in shoot length by 20% to 40% in the seedlings grown in solution having N salts as compared to those grown without N. In zero-N and ammonium fed plants the nitrate levels, total reduced N and total soluble protein content was significantly lower as compared to both low and high NO$_3^-$-N grown wheat seedlings. These parameters directly correlated with low NR activity in zero-N and NH$_4^+$-N treatments. There was increased production of ROS in terms of increase in superoxide radicals and H$_2$O$_2$ in NH$_4^+$-N fed wheat seedlings followed by those grown without N. Increase in protease activity, total free amino acids and TBARS was also observed in these treatments. Under normal conditions, plants contain numerous carbonylated proteins, which are thought to be indicative of oxidative stress damage. Conditions that promote formation of reactive oxygen species (ROS) enhance protein
carbonylation, and protein degradation is required to reverse the damage. However, it is not clear how the degradation of carbonylated proteins is controlled \textit{in planta}. In this report, we show that detached wheat leaves rapidly and selectively degrade carbonylated proteins when kept in the dark as compared to the detached leaves incubated in light or in the intact seedlings. The loss of carbonylated proteins corresponded to a loss of soluble protein and accumulation of free amino acids. Degradation of carbonylated proteins was blocked by carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) in dark detached leaves, but not by 3-methyladenine, suggesting that the 26S proteasome pathway rather than the autophagic pathway was involved in degrading protein carbonyls in dark detached leaves. In light incubation pathway the use of inhibitors revealed that both autophagic and proteasomal pathways were involved in degrading protein carbonyls. The study also indicated the role of cytokinins in preventing the the protein carbonyl degradation. The expression of IPT3 gene of cytokinin biosynthetic pathway was almost absent in the seedlings grown at zero N and NH4+-N, and in these treatments the degradation of protein carbonyls was high as supported by low level of total soluble proteins and high levels of free amino acids. Exogenous application of cytokinins led to the accumulation of protein carbonyls and in these treatments.
गेहूँ की पौधे में नाइट्रोजन प्रतिबल की अनुक्रिया में प्रोटीन–आक्सीकरण सार

नाइट्रोजन पौधे में नाइट्रोजन प्रतिबल के लिए आवश्यक सर्वाधिक महत्वपूर्ण पौधें में से एक है और इसकी कमी भी सर्वाधिक प्रदर्शित है। भारत की स्रोत विश्वास गृहों में नाइट्रोजन (एन) की कमी है। इने इस कमी की पादर वृद्धि, उपर और गुणवत्ता को गंभीर रूप से प्रभावित करती है। विविध क्षेत्रों में काफी कार्य हुआ है किन्तु ऐसे शिकार और अनेक प्रश्न अनुसरित है। गेहूँ में नाइट्रोजन मुख्यतः नाइट्रोजन के रूप में प्रभाव की जाती है और कमी-कमी अभियोगितम के रूप में ली जाती है। यहाँ गेहूँ के पौधे अभियोगितम तो नहीं है किन्तु अभियोगितम पौधे के प्रति संरचनाशील होते हैं। गुणवत्ता की दृष्टि से, प्रोटीन अंश कम हो जाता है। जब पौधा नाइट्रोजन प्रतिबल सहायता करता है तो यह सम्बन्ध है कि प्रतिबल के कारण उत्तरों में कम प्रोटीन अंश के स्थान पर उनका आक्सीकरण तथा कार्बनामीनलच्छन् हो सकता है जिसके परिणाम स्वरूप पौधा-प्रतिबल सहायता वाले ऐसे पौधों का निमीकरण बढ़ सकता है। प्रस्तुत अध्ययन में नियामक परिशिष्टों में उगर गए गेहूँ के नोर्दिमनों (पौधे) पर ऐसे विभिन्न स्तरों और अवस्थाओं के भ्रम को ज्ञात किया गया है।

सायटोकाइनस्स जीन आक्सीकरण एवं एन के बीच अभियोगितम को भी देखा गया है। प्रोटीन कार्बनामीनलच्छन् की सीमा का विशेषज्ञ पौधे के प्रति-प्रोटीन-निमीकरण के पक्षवाले की वापसी करने का प्रयास किया गया है। गेहूँ (किसम पी की ड्वील 343) की पौधा को दर निहित उपायकों के अन्तर्गत उगाया गया तथा उद्देश्य, एन-स्वीकरण एवं प्रतिबल का अभ्यास किया गया। परिणामों से दर्शाया कि कम एवं एन के निहित उगाई गई पौधा की तुलना में उच्च ऐसे ऑ 3 — एन की उगाई गई पौधा की वृद्धि एवं नाइट्रोजन उपजायन में बढ़ोतरी पायी गई। नाइट्रोजन एवं अभियोगितम के अन्तर्गत उगाई पौधा में जैव मात्रा संचयन की दृष्टि से गेहूँ की पौधा की वृद्धि का गंभीर रूप से संदर्भ हुआ तथा एवं जड़ प्रोट्रो अनुपात बढ़ गया। एन के निहित उगाई गई पौधा की तुलना में एन अत्यधिक उगाई गई पौधा की तुलना में एन उगाई युक्त घोल में उगाई गई पौधा की प्रोट्रो-व्यायाम में 20% से 40% की आपूर्ति बढ़ोतरी देखी गई। कम एवं उच्च ऐसे ऑ 3 — एन के साथ उगाई गई गेहूँ की पौधा की तुलना में एन एवं एन 4 + — एन के साथ उगाई गई पौधा की प्रोट्रो-शुक्र भार 63% कम हो गया। जिस एन के निहित उगाई गई पौधा की तुलना में एन उगाई गई पौधा की प्रोट्रो-व्यायाम में 20% से 40% की आपूर्ति बढ़ोतरी देखी गई। कम एवं उच्च ऐसे ऑ 3 — एन के साथ उगाई गई गेहूँ की पौधा, जैव मात्रा की तुलना में, दूधी एवं अभियोगितम के अन्तर्गत उगाई पौधा में जैव मात्रा संचयन की दृष्टि से गेहूँ की पौधा की वृद्धि का गंभीर रूप से कम हो गया। इन प्रदर्शनों का जैवी — एवं एन के अवधिक एवं एक कुल घुनश्रील प्रोटीन अंश महत्वपूर्ण रूप से कम हो गया। इन प्रदर्शनों का जैवी — एन के अवधिक एवं एक कुल घुनश्रील प्रोटीन अंश महत्वपूर्ण रूप से कम हो गया। इन प्रदर्शनों का जैवी — एवं एन के अवधिक एवं एक कुल घुनश्रील प्रोटीन अंश महत्वपूर्ण रूप से कम हो गया। इन प्रदर्शनों का जैवी — एवं एन के अवधिक एवं एक कुल घुनश्रील प्रोटीन अंश महत्वपूर्ण रूप से कम हो गया।
प्रोटियसमत पाथवे दोनों का योगदान होता है। इस अध्ययन ने प्रोटीन कार्बोनाइल्स निमीण करण का रोकने में सायटोकाइनिन्स के योगदान को भी दर्शाया। जीरो एन एवं एन एच 4 + - एन के अन्तर्गत उपाई बीच मे सायटोकाइनिन बायोसिंथेसिस पाथवे की आई थी टी3 जीन की अभिव्यक्ति लगभग अनुपस्थित थी तथा इन उपचारों में प्रोटीन कार्बोनाइल्स का अधिक निमीण करण था जैसा कि कूल धूमकेतूल सूर्योदय के मूर्त सतर तथा स्वतंत्र अन्नों अम्लों के उच्च स्तरों से पता चलता है इस उपचारों में सायटोकाइनिन्स के बहिष्कार अनुप्रयोग से प्रोटीन कार्बोनाइल्स का संचयन होता है।
BIBLIOGRAPHY


Drew M. C. and Saker L. R. (1975). Nutrient supply and the growth of the seminal root system of barley. II. Localized, compensatory increases in lateral root growth and rates of nitrate uptake when nitrate supply is restricted to only part of the root system. *Journal of Experimental Botany*, 26: 79–90.


Table 1: Change in shoot dry weight (g plant\textsuperscript{-1}) of wheat seedlings grown without N, with NO\textsubscript{3}\textsuperscript{-}N (50 µM and 5 mM) and NH\textsubscript{4}\textsuperscript{+}-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot dry weight</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>0.013</td>
<td>±0.000491</td>
</tr>
<tr>
<td>LN</td>
<td>0.024</td>
<td>±0.001713</td>
</tr>
<tr>
<td>HN</td>
<td>0.036</td>
<td>±0.001033</td>
</tr>
<tr>
<td>AHN</td>
<td>0.022</td>
<td>±0.000549</td>
</tr>
</tbody>
</table>

CD at 5% = 0.004  
SE(m) ±0.001

Figure 1a. Change in shoot dry weight (g plant\textsuperscript{-1}) of wheat seedlings grown without N, with NO\textsubscript{3}\textsuperscript{-}N (50 µM and 5 mM) and NH\textsubscript{4}\textsuperscript{+}-N (5 mM)

Figure 1b. Relative change in shoot dry weight (g plant\textsuperscript{-1}) of wheat seedlings grown without N, with NO\textsubscript{3}\textsuperscript{-}N (50 µM and 5 mM) and NH\textsubscript{4}\textsuperscript{+}-N (5 mM)
Table 2: Change in shoot length (g plant⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot length</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>11.536</td>
<td>±0.320963</td>
</tr>
<tr>
<td>LN</td>
<td>14.906</td>
<td>±0.214819</td>
</tr>
<tr>
<td>HN</td>
<td>16.12</td>
<td>±0.340206</td>
</tr>
<tr>
<td>AHN</td>
<td>13.613</td>
<td>±0.216519</td>
</tr>
</tbody>
</table>

CD at 5% = 0.925
SE(m) ± 0.279

Figure 2a. Change in shoot length (g plant⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

Figure 2a. Relative change in shoot length (g plant⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)
Table 3: Change in root length (cm plant⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root length</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>16.56</td>
<td>± 0.336065</td>
</tr>
<tr>
<td>LN</td>
<td>15.46</td>
<td>± 0.34802</td>
</tr>
<tr>
<td>HN</td>
<td>12</td>
<td>± 0.577367</td>
</tr>
<tr>
<td>AHN</td>
<td>11.36</td>
<td>± 0.497787</td>
</tr>
</tbody>
</table>

CD at 5% = 1.495
SE(m) ± 0.451

Figure 3a. Change in root length (cm plant⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

Figure 3b. Relative change in root length (cm plant⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)
Table 4: Change in root surface area (cm$^2$ plant$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root surface area</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>0.391</td>
<td>±0.039857</td>
</tr>
<tr>
<td>LN</td>
<td>0.697</td>
<td>±0.019233</td>
</tr>
<tr>
<td>HN</td>
<td>0.798</td>
<td>±0.039857</td>
</tr>
<tr>
<td>AHN</td>
<td>0.361</td>
<td>±0.026092</td>
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</table>

CD at 5% = 0.108
SE(m) ±0.033

Figure 4a. Change in root surface area (cm$^2$ plant$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 4b. Relative change in root surface area (cm$^2$ plant$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 5: Change in root : shoot ratio of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root : shoot Ratio</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>1.27</td>
<td>0.021</td>
</tr>
<tr>
<td>LN</td>
<td>0.76</td>
<td>0.008</td>
</tr>
<tr>
<td>HN</td>
<td>0.65</td>
<td>0.017</td>
</tr>
<tr>
<td>AHN</td>
<td>0.61</td>
<td>0.005</td>
</tr>
</tbody>
</table>

CD at 5% = 0.0213
SE(m) ±0.0071

Figure 5a. Change in root : shoot ratio of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 5b. Relative change in root : shoot ratio of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 6: Change in leaf area (cm$^2$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Leaf area</th>
<th>SE</th>
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<tbody>
<tr>
<td>Zero-N</td>
<td>9.786</td>
<td>±0.324054</td>
</tr>
<tr>
<td>LN</td>
<td>28.433</td>
<td>±1.131862</td>
</tr>
<tr>
<td>HN</td>
<td>37.033</td>
<td>±0.31798</td>
</tr>
<tr>
<td>AHN</td>
<td>31.533</td>
<td>±0.808977</td>
</tr>
</tbody>
</table>

CD at 5% = 2.423
SE(m) ± 0.732

Figure 6a. Change in leaf area (cm$^2$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 6b. Relative change in leaf area (cm$^2$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 7: Change in SLW (g cm\(^{-2}\)) of wheat seedlings grown without N, with NO\(_3^-\)-N (50 µM and 5 mM) and NH\(_4^+\)-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SLW</th>
<th>SE</th>
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<tbody>
<tr>
<td>Zero-N</td>
<td>0.003</td>
<td>±0.0011</td>
</tr>
<tr>
<td>LN</td>
<td>0.008</td>
<td>±0.0008</td>
</tr>
<tr>
<td>HN</td>
<td>0.014</td>
<td>±0.0018</td>
</tr>
<tr>
<td>AHN</td>
<td>0.0103</td>
<td>±0.0008</td>
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</tbody>
</table>

CD at 5% =0.0042
SE(m) ± 0.0014

Figure 7a. Change in SLW (g cm\(^{-2}\)) of wheat seedlings grown without N, with NO\(_3^-\)-N (50 µM and 5 mM) and NH\(_4^+\)-N (5 mM)

Figure 7b. Relative change in SLW (g cm\(^{-2}\)) of wheat seedlings grown without N, with NO\(_3^-\)-N (50 µM and 5 mM) and NH\(_4^+\)-N (5 mM)
Table 8: Change in NR activity (µmol NO$_2^-$ formed g$^{-1}$ FW h$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
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<th>SE</th>
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<tr>
<td>Zero-N</td>
<td>3.633</td>
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<tr>
<td>LN</td>
<td>7.256</td>
<td>±0.644058</td>
</tr>
<tr>
<td>HN</td>
<td>25.733</td>
<td>±0.47219</td>
</tr>
<tr>
<td>AHN</td>
<td>4.74</td>
<td>±0.302141</td>
</tr>
</tbody>
</table>

CD at 5% = 1.884
SE(m) ±0.569

Figure 8a. Change in NR activity (µmol NO$_2^-$ formed g$^{-1}$ FW h$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 8b. Relative change in NR activity (µmol NO$_2^-$ formed g$^{-1}$ FW h$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 9: Change in Shoot NO\textsubscript{3} content (µM g\textsuperscript{-1} DW) of wheat seedlings grown without N, with NO\textsubscript{3}\textsuperscript{-}N (50 µM and 5 mM) and NH\textsubscript{4}\textsuperscript{+}-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot NO\textsubscript{3} content</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>2.24</td>
<td>±0.516</td>
</tr>
<tr>
<td>LN</td>
<td>5.69</td>
<td>±0.089</td>
</tr>
<tr>
<td>HN</td>
<td>21.29</td>
<td>±0.569</td>
</tr>
<tr>
<td>AHN</td>
<td>1.84</td>
<td>±0.181</td>
</tr>
</tbody>
</table>

CD at 5% = 1.316
SE(m) ±0.397

Figure 9a. Change in Shoot NO\textsubscript{3} content (µM g\textsuperscript{-1} DW) of wheat seedlings grown without N, with NO\textsubscript{3}\textsuperscript{-}N (50 µM and 5 mM) and NH\textsubscript{4}\textsuperscript{+}-N (5 mM)

Figure 9b. Relative change in Shoot NO\textsubscript{3} Content (µM g\textsuperscript{-1} DW) of wheat seedlings grown without N, with NO\textsubscript{3}\textsuperscript{-}N (50 µM and 5 mM) and NH\textsubscript{4}\textsuperscript{+}-N (5 mM)
Table 10: Change in root NO$_3$ Content (µM g$^{-1}$ DW) of wheat seedlings grown without N, with NO$_3$$^-$-N (50 µM and 5 mM) and NH$_4$$^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root NO$_3$ content</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>0.933</td>
<td>±0.066</td>
</tr>
<tr>
<td>LN</td>
<td>1.467</td>
<td>±0.026</td>
</tr>
<tr>
<td>HN</td>
<td>1.387</td>
<td>±0.0905</td>
</tr>
<tr>
<td>AHN</td>
<td>1.033</td>
<td>±0.088</td>
</tr>
</tbody>
</table>

CD at 5% = 1.571
SE(m) ±0.474

Figure 10a. Change in root NO$_3$ Content (µM g$^{-1}$ DW) of wheat seedlings grown without N, with NO$_3$$^-$-N (50 µM and 5 mM) and NH$_4$$^+$-N (5 mM)

![Figure 10a](image)

Figure 10b. Relative change in root NO$_3$ Content (µM g$^{-1}$ DW) of wheat seedlings grown without N, with NO$_3$$^-$-N (50 µM and 5 mM) and NH$_4$$^+$-N (5 mM)

![Figure 10b](image)
Table 11: Change in shoot N content (%) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot N content</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>1.488</td>
<td>±0.255841</td>
</tr>
<tr>
<td>LN</td>
<td>2.07</td>
<td>±0.040416</td>
</tr>
<tr>
<td>HN</td>
<td>3.375</td>
<td>±0.062338</td>
</tr>
<tr>
<td>AHN</td>
<td>1.775</td>
<td>±0.137838</td>
</tr>
</tbody>
</table>

CD at 5% = 0.0497
SE(m) ± 0.150

Figure 11a. Change in shoot N content (%) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 11b. Relative change in shoot N content (%) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 12: Change in root N content (%) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root N Content</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>0.919</td>
<td>±0.079602</td>
</tr>
<tr>
<td>LN</td>
<td>1.795</td>
<td>±0.12801</td>
</tr>
<tr>
<td>HN</td>
<td>2.354</td>
<td>±0.322974</td>
</tr>
<tr>
<td>AHN</td>
<td>1.238</td>
<td>±0.129865</td>
</tr>
</tbody>
</table>

CD at 5% = 0.628  
SE(m) ± 0.190

Figure 12a. Change in root N content (%) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 12b. Relative change in root N content (%) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 13: Change in total soluble proteins (mg g⁻¹ FW) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root N Content</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>1.875</td>
<td>±0.103937</td>
</tr>
<tr>
<td>LN</td>
<td>4.803</td>
<td>±0.341744</td>
</tr>
<tr>
<td>HN</td>
<td>10.36</td>
<td>±0.534206</td>
</tr>
<tr>
<td>AHN</td>
<td>2.136</td>
<td>±0.175021</td>
</tr>
</tbody>
</table>

CD at 5% = 1.103

SE(m) ±0.333

Figure 13a. Change in total soluble proteins (mg g⁻¹ FW) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

Figure 13b. Relative change in total soluble proteins (mg g⁻¹ FW) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)
Table 14: Change in total superoxide radicals (n mol min\(^{-1}\) g\(^{-1}\) FW\(^{-1}\)) of wheat seedlings grown without N, with NO\(_3^{-}\)-N (50 µM and 5 mM) and NH\(_4^{+}\)-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>(\text{O}_2^{-})</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>16.64806</td>
<td>±0.910364</td>
</tr>
<tr>
<td>LN</td>
<td>13.47696</td>
<td>±1.107043</td>
</tr>
<tr>
<td>HN</td>
<td>12.74683</td>
<td>±0.436438</td>
</tr>
<tr>
<td>AHN</td>
<td>18.01695</td>
<td>±0.523695</td>
</tr>
</tbody>
</table>

CD at 5% = 2.629

SE(m) ± 0.794

Figure 14a. Change in superoxide radicals (n mol min\(^{-1}\) g\(^{-1}\) FW\(^{-1}\)) of wheat seedlings grown without N, with NO\(_3^{-}\)-N (50 µM and 5 mM) and NH\(_4^{+}\)-N (5 mM)

Figure 14b. Relative change in superoxide radicals (n mol min\(^{-1}\) g\(^{-1}\) FW\(^{-1}\)) of wheat seedlings grown without N, with NO\(_3^{-}\)-N (50 µM and 5 mM) and NH\(_4^{+}\)-N (5 mM)
Table 15: Change in H$_2$O$_2$ level (µ mol g$^{-1}$ FW$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>H$_2$O$_2$</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>0.113</td>
<td>±0.003489</td>
</tr>
<tr>
<td>LN</td>
<td>0.111</td>
<td>±0.002946</td>
</tr>
<tr>
<td>HN</td>
<td>0.068</td>
<td>±0.001092</td>
</tr>
<tr>
<td>AHN</td>
<td>0.132</td>
<td>±0.001387</td>
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</table>

CD at 5% = 0.008

SE(m) ± 0.003

Figure 15a. Change in H$_2$O$_2$ level (µ mol g$^{-1}$ FW$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)

Figure 15b. Relative change in H$_2$O$_2$ level (µ mol g$^{-1}$ FW$^{-1}$) of wheat seedlings grown without N, with NO$_3^-$-N (50 µM and 5 mM) and NH$_4^+$-N (5 mM)
Table 16: Change in TBARS (n mol g\(^{-1}\) DW\(^{-1}\)) of wheat seedlings grown without N, with NO\(_3\)\(-\)N (50 µM and 5 mM) and NH\(_4\)\(+\)N (5 mM)

<table>
<thead>
<tr>
<th></th>
<th>TBARS</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td>509.64</td>
<td>±5.902306</td>
</tr>
<tr>
<td>HN</td>
<td>310.63</td>
<td>±10.74369</td>
</tr>
<tr>
<td>AHN</td>
<td>191.71</td>
<td>±4.572007</td>
</tr>
<tr>
<td>Zero-N</td>
<td>381.96</td>
<td>±13.28439</td>
</tr>
</tbody>
</table>

CD at 5% = 30.847
SE(m) ± 9.324

Figure 16a. Change in TBARS (n mol g\(^{-1}\) DW\(^{-1}\)) of wheat seedlings grown without N, with NO\(_3\)\(-\)N (50 µM and 5 mM) and NH\(_4\)\(+\)N (5 mM)

![Figure 16a](image1.png)

Figure 16b. Relative change in TBARS (n mol g\(^{-1}\) DW\(^{-1}\)) of wheat seedlings grown without N, with NO\(_3\)\(-\)N (50 µM and 5 mM) and NH\(_4\)\(+\)N (5 mM)

![Figure 16b](image2.png)
Table 17: Change in protease activity (µ mol g⁻¹ FW⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Protease activity</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>67.62</td>
<td>4.528</td>
</tr>
<tr>
<td>LN</td>
<td>50.20</td>
<td>3.460</td>
</tr>
<tr>
<td>HN</td>
<td>27.27</td>
<td>3.216</td>
</tr>
<tr>
<td>AHN</td>
<td>63.62</td>
<td>1.290</td>
</tr>
</tbody>
</table>

CD at 5% = 11.044
SE(m) ± 3.335

Figure 17a. Change in protease activity (µ mol g⁻¹ FW⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

Figure 17b. Relative change in protease activity (µ mol g⁻¹ FW⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)
Table 18: Change in free amino acids (µ mol g⁻¹ FW⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Free amino acids</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-N</td>
<td>45.3</td>
<td>1.75</td>
</tr>
<tr>
<td>LN</td>
<td>33.34</td>
<td>0.604</td>
</tr>
<tr>
<td>HN</td>
<td>24.16</td>
<td>0.898</td>
</tr>
<tr>
<td>AHN</td>
<td>40.63</td>
<td>1.12</td>
</tr>
</tbody>
</table>

CD at 5% = 3.894
SE(m) ± 1.176

Figure 18a. Change in free amino Acids (µ mol g⁻¹ FW⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)

Figure 18b. Relative Change in Protease activity (µ mol g⁻¹ FW⁻¹) of wheat seedlings grown without N, with NO₃⁻-N (50 µM and 5 mM) and NH₄⁺-N (5 mM)
Figure 19a. Protein oxidation in different N treated seedlings

Lane 1: Marker
Lane 2: -N
Lane 3: 50 μM KNO₃
Lane 4: 5 mM KNO₃

Figure 19b. CBB Stain

Figure 19c. Protein carbonyl levels in various N-treated seedlings
Figure 20a. Seedlings grown without N and whole seedlings incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 20b. Protein carbonyl level

Figure 20c. Relative change in protein carbonyl level
Figure 21a. Seedlings grown with 50 μM-NO₃ and whole seedlings incubated in light in various solutions for 6 hrs

Lane 1: Control (water)
Lane 2: BA (10 μM)
Lane 3: Kinetin (10 μM)
Lane 4: 3-Methyl adenine (1 mM)
Lane 5: MG132 (50 μM)

Figure 21b. Protein carbonyl level

Figure 21c. Relative change in protein carbonyl level
Figure 22a. Seedlings grown with 5 mM-NO3 and whole seedlings incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 22b. Protein carbonyl level

Figure 22c. Relative change in protein carbonyl level
Figure 23a. Seedlings grown with 5 mM-AS and whole seedlings incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 23b. Protein carbonyl level

Figure 23c. Relative change in protein carbonyl level
Figure 24a. Seedlings grown without N and detached leaves incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 24b. Protein carbonyl level

Figure 24c. Relative change in protein carbonyl level
Figure 25a. Seedlings grown with 50 µM NO₃ and detached leaves incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 25b. Protein carbonyl level

Figure 25c. Relative change in protein carbonyl level
Figure 26a. Seedlings grown with 5 mM NO₃ and detached leaves incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 26b. Protein carbonyl level

Figure 26c. Relative change in protein carbonyl level
Figure 27a. Seedlings grown with 5 mM AS and detached leaves incubated in light in various solutions for 6 hrs.

Lane 1: Control (water)
Lane 2: BA (10 µM)
Lane 3: Kinetin (10 µM)
Lane 4: 3-Methyl adenine (1 mM)
Lane 5: MG132 (50 µM)

Figure 27b. Protein carbonyl level

Figure 27c. Relative change in protein carbonyl level
Figure 28a. Seedlings grown without N and detached leaves incubated in dark in various solutions for 6 hrs

Lane 1: Control (water)
Lane 2: BA (10 µM)
Lane 3: Kinetin (10 µM)
Lane 4: 3-Methyl adenine (1 mM)
Lane 5: MG132 (50 µM)

Figure 28b. Protein carbonyl level

Figure 28c. Relative change in protein carbonyl level
Figure 29a. Seedlings grown with 50 µM- NO$_3$ and detached leaves incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 29b. Protein carbonyl level

Figure 29c. Relative change in protein carbonyl level
Figure 30a. Seedlings grown with 5 mM-NO₃ and detached leaves incubated in light in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 30b. Protein carbonyl level

Figure 30c. Relative change in protein carbonyl level
Figure 31a. Seedlings grown with 5 mM AS and detached leaves incubated in dark in various solutions for 6 hrs

Lane1: Control (water)
Lane2: BA (10 µM)
Lane3: Kinetin (10 µM)
Lane4: 3-Methyl adenine (1 mM)
Lane5: MG132 (50 µM)

Figure 31b. Protein carbonyl level

Figure 31c. Relative change in protein carbonyl level
Plate 1: Wheat seedlings raised in Hoagland solution under controlled conditions with different levels and forms of N supply (15 d). (A) Without N (Zero-N), (B) Low (50 µM) NO₃⁻-N; LN, (C) High (5 mM) NO₃⁻-N; HN, (D) 5 mM NH₄⁺-N; AHN
Plate 2: Changes in growth in terms of shoot and root growth. (A) Without N (Zero-N), (B) NO$_3^-$-N; (LN, 50 µM), (C) NO$_3^-$-N; (HN, 5 mM), (D) NH$_4^+$-N; (AHN, 5 mM)
Plate 3: Changes in expression of IPT3 gene in wheat seedlings in response to various treatments

Lane 1: Zero –N
Lane 2: 50 µM NO$_3$-N
Lane 3: 5 mM NO$_3$-N
Lane 4: 5 mM NH$_4$-N