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Review of Literature
Global population growth is increasingly putting pressure on agricultural production, leading to demands for higher yields from arable land. Higher yields of both food and energy crops usually demand increased fertilizer inputs; however, current application levels often have negative environmental impacts. The importance of improving crop fertilizer use efficiency and increasing grain yield and quality is very clear (Vitousek et al., 1997; Cassman et al., 2003). The challenge of maintaining sustainability in agricultural systems is certain to be one of the leading social scientific problems of the 21st century (Tilman, 1999; Tilman et al., 2002). N fertilizer application is directly linked to crop yield and quality (protein content) (Good et al., 2004; Barneix, 2007). Sulphur (S) nutrition is more specifically linked to improved nitrogen utilization efficiency of crop plants. Because plants use most assimilated sulphate and nitrate for protein synthesis at a 25:1 N to S molar ratio, that interaction has been proposed as the mechanism that coordinates the flow of these two essential elements (Reuveny and Filner, 1977; Reuveny et al., 1980). Studies have suggested that joint action of assimilatory processes of N and S is critical for the plant in the synthesis of some important amino acids like cysteine and methionine, which in turn are fundamental for protein synthesis (Leustek et al., 2000). Since most of the organic S in plants is bound in amino acids and proteins, it is apparent that a crop's requirement for S is closely related to N nutrition (McGrath and Zhao, 1996). Thus, there seems to be a reciprocal regulatory coupling between NO\textsuperscript{3-} and SO\textsubscript{4}\textsuperscript{2-} assimilation pathways. However, little is known about the interaction of N metabolism with S metabolism.

3.1 NITROGEN AND SULPHUR AS PLANT NUTRIENTS

Nitrogen has traditionally been considered as one of the most important plant nutrients. It is found in rocks, soils, sediment, oceans, and living matter in various forms. Plants, animals, and microbial populations need a continuous
source of N for growth. It is an essential component of proteins that build cell material and plant tissue. In addition, it is necessary for the function of other essential biochemical agents, including chlorophyll, many enzymes, and nucleic acids. Most microorganisms and plants obtain N from the surrounding soil and water. Animals get N from the food they eat. A few organisms obtain N directly from the pool of atmospheric nitrogen gas ($N_2$) but this requires the organism to expend energy. N is also important for plant growth and crop yield. N fertilizers application and crop yield have been considered as synonymous to each other. Plant growth and crop yield usually increase when N is added, despite the presence of N in soils. Sulphur is recognized as an essential nutrient in crop nutrition cycles all over the globe. The primary importance of S in crop nutrition arises from its being an essential component of amino acids, which act as building blocks in the synthesis of proteins. Further, S is a constituent of oil in oilseed crops. It has a role to play in increasing chlorophyll formation and aiding photosynthesis. S also plays a role in the activation of enzymes, nucleic acids and forms a part of Biotin and Thiamine (vitamins). Specifically in fruit crops, it increases the ascorbic acid content in the fruit as well as the total soluble solids. S is important not only for yields, but also for quality. Consumption of S as a fertilizer has increased three-fold since the 1970s and currently stands at over one million tons (TSL, 2009).

3.2 SOURCES OF NITROGEN AND SULPHUR

In well-aerated soil of natural and agricultural ecosystems, most of the N is present typically as $NO_3^-$ (Crawford and Glass, 1998). It is a major nitrogen source for plants (Abrol et al., 1999). In some soils, for example, those of late-successional temperate forests, $NH_4^+$ becomes the major or sole inorganic N source (Bijlsma et al., 2000). $NH_4^+$ is commonly found at low soil pH and in soils of cold climates and irrigated rice fields of the world (Kronzucker et al., 1997,
Usually these two main sources coexist in the soil at various ratios and therefore, most of the plants are able to absorb both NO$_3^-$ and NH$_4^+$. The threshold concentration at which toxic effects of NH$_4^+$ become pronounced tends to be much lower than that of NO$_3^-$, although the value varies with the plant species involved and other nutritional factors (Britto and Kornzucker, 2002). Among all sources of S, plants generally utilize sulphate as the source of S for growth (Durenkamp and De Kok, 2004). A number of S-containing fertilizers are available in India. These consist of mined as well as manufactured products. Major sources of S are single superphosphate (SPS), ammonium sulphate (AS), ammonium phosphate sulphate (APS), gypsum and pyrite. As plants take up S in SO$_4^{2-}$ form, (NH$_4$)$_2$SO$_4$, K$_2$SO$_4$ and Na$_2$SO$_4$ have been found more effective sources of S for oilseed crops grown on neutral to slightly alkaline soils (Nad and Goswami, 1983). Use of gypsum as a sulphur fertilizer has become popular with the detection of S-deficiency. It is a dehydrated form of calcium sulphate (CaSO$_4$.2H$_2$O). Pure gypsum contains 18.6 per cent S, while the commercial agricultural grade gypsum of 70-80 per cent purity contains 13-15 per cent S (Singh, 2001). Large deposits of gypsum are present in India. Elemental S and H$_2$SO$_4$ are also used as S sources in calcareous soils. The biochemical oxidation of elemental S may decrease soil pH and solubilize CaCO$_3$ to make soil conditions more favorable for plant growth. Aulakh and Dev (1978) reported H$_2$SO$_4$ as a good source of S when applied in calcareous and slightly alkaline soils, because it lowers soil pH and improves the availability of other plant nutrients.

3.3 FORMS OF NITROGEN AND SULPHUR

Many forms of N are present in any environment at any point in time, mainly because N readily shifts from one form to another. NO$_3^-$, NH$_4^+$, and N$_2$ can be converted to organic N (ON) by plants and microorganisms (Miller et al.,
ON can be converted back to inorganic forms as the organic compounds decompose. N can also shift between inorganic forms (for example, NH$_3$ to NH$_4^+$, NO$_3^-$ or to N$_2$). These shifts occur as nature attempts to establish equilibrium among the various forms as environmental conditions change. N in the soil interacts with the atmosphere, soil particles, soil solution (the water that surrounds or moves through the soil), microorganisms, and plants. If a new source of N is added to alter the balance or if environmental conditions (such as temperature and moisture) change, N transformations take place. Thus, new N transformations are on with the constantly changing environmental conditions. This continuous movement of N from one form to another is known as the "nitrogen cycle."

Sulphur occurs in the soil in two basic forms, viz., organic and inorganic. In the surface horizon of most well drained acid soils, nearly all of the S is present in organic forms and only a small amount of S is present as sulphate (SO$_4^{2-}$). In the sub-soil horizons, however, appreciable amount of SO$_4^{2-}$ may accumulate and inorganic S compound may constitute a major proportion of the total S. The occurrence of S as soluble salts of sodium, magnesium and calcium in the soil is well known. However, except under arid or poorly drained conditions, the presence of appreciable amounts of soluble SO$_4^{2-}$ is likely to occur only in sub-soil horizons. Insoluble sulphate, like barium sulphate, may also occur, although precise knowledge of their true chemical nature is limited. Except under anaerobic conditions, most of the inorganic S in the soil occurred as sulphates. In anaerobic conditions, reduced forms of S, particularly sulphides, might dominate, compared with the inorganic S.
3.4 TRANSFORMATION OF NATIVE AND APPLIED NITROGEN AND SULPHUR IN SOIL

3.4.1 Nitrogen mineralization and immobilization

Mineralization of N occurs when the organic matter decomposes. The soil microorganisms break organic bonds to obtain energy. When the organic matter is completely broken down (oxidized), carbon dioxide (CO$_2$), water (H$_2$O), and minerals are left. The inorganic (available) form of N resulting from decomposition is NH$_4^+$. Decomposition is not complete in one year. It continues for several years at progressively slower rates. Some of the N is retained in stable organic matter (such as humus), which continues to mineralize very slowly.

Often N released from organic matter may be taken up by soil microorganisms and converted back to organic forms. This process is called "immobilization". Immobilization generally occurs in nutrient-poor soils, in soils with a lot of matter that is low in N and high in carbon (such as woody material or straw), or in soils where organic amendments (such as sawdust or low-N compost) have been added. The carbon-to-nitrogen (C:N) ratio is often used as an indication of whether mineralization or immobilization will occur. When surface soil layers have a C:N ratio greater than 30:1, then immobilization is highly likely to occur. This is because microorganisms need N to assimilate the available C. When the C:N ratio is below 20:1, N mineralization is likely to occur. When the C:N ratio is between 20-30:1, both mineralization and immobilization may occur but they will generally balance.

3.4.2 Ammonia volatilization

The potential for loss of ammonia gas (NH$_3$) to the air depends on several equilibrium relationships in the soil. Ammonium (NH$_4^+$) plays an important role in these relationships. Most of the NH$_4^+$ is bound to soil surface exchange
sites; the remainder is dissolved in soil solution. If some \( \text{NH}_4^+ \) is removed from the soil solution through plant uptake or other means, \( \text{NH}_4^+ \) will move from the soil surface sites into the soil solution to re-establish equilibrium. The \( \text{NH}_4^+ \) in the soil solution also tries to maintain the equilibrium with the \( \text{NH}_3 \) in the soil solution. If more \( \text{NH}_3 \) is needed to maintain the equilibrium with the \( \text{NH}_3 \) in the soil solution, \( \text{NH}_4^+ \) will transform to \( \text{NH}_3 \). If more \( \text{NH}_4^+ \) is needed in the soil solution, \( \text{NH}_4^+ \) will move from the soil exchange sites. This equilibrium depends on the soil pH. At pH 6.0, only 0.1 percent of the total of these two is \( \text{NH}_3 \); at pH 7.0, 1 percent is \( \text{NH}_3 \); and at pH 9.0, the ratio is one to one. When a significant amount of \( \text{NH}_3 \) is present in the soil solution, some of it will "volatilize" into the air. When this happens, all relationships tend to re-establish. The more \( \text{NH}_4^+ \) transforms to \( \text{NH}_3 \), the more \( \text{NH}_4^+ \) comes off the soil exchange sites. When the soil solution evaporates, the concentration of \( \text{NH}_3 \) increases and so does the potential for loss.

3.4.3 Nitrification and denitrification

Microorganisms oxidize available \( \text{NH}_4^+ \) to \( \text{NO}_3^- \) in order to get energy. This process is called "nitrification." During the process, the microorganism \( \text{Nitrosomonas} \) oxidizes \( \text{NH}_4^+ \) to \( \text{NO}_2^- \), and the microorganism \( \text{Nitrobacter} \) oxidizes the \( \text{NO}_2^- \) to \( \text{NO}_3^- \). Nitrification rates are highest when soils are warm and moist and the pH is neither strongly acidic nor alkaline. Over-application of inorganic N in arid climates will promote build-up of excess of \( \text{NO}_3^- \) in the soil. If leaching losses are insignificant, this build-up provides a bank of available nutrients for the next crop. However, in moist climates (where rainfall exceeds evapotranspiration), \( \text{NO}_3^- \) leaching may occur. Like higher plants and animals, many microorganisms use \( \text{O}_2 \) as an essential part of their metabolism.

If the soil becomes depleted of \( \text{O}_2 \), some microorganisms can use the oxygen in \( \text{NO}_3^- \) as an alternative by reducing the \( \text{NO}_3^- \) to nitrogen gases (\( \text{N}_2 \) or
N$_2$O). This process is called "denitrification". The amount of denitrification depends on the availability of NO$_3^-$, the soil saturation, soil temperature, and availability of easily decomposable organic matter. When a site within the soil becomes saturated with water (either an entire soil layer or pockets within a layer), O$_2$ can no longer diffuse readily into that site. If microorganisms are active within that site, they will deplete the O$_2$ and then begin using the NO$_3^-$.

If a soil is too cold for microbial activity or if there is too little food (organic matter) available for the microorganisms, the O$_2$ will not be depleted and denitrification will not occur.

3.4.4 Sulphur mineralization and immobilization

The mineralization of S in soil refers to the breakdown of large organic-S molecules in soil to smaller units and finally to inorganic-sulphate. Generally, it is believed that as N and S are closely associated in soil organic matters, the two elements are mineralized in related amounts. The S mineralization has been reported to occur at ratio narrower than the N:S ratio in soil organic matter (Tabatabai and Al-Khafaji, 2000). Immobilization is the reverse of mineralization. In this case, microorganisms convert simple inorganic S-molecules to organic compounds and eventual incorporation into humus. Under conditions when organic matter is accumulating rapidly, considerable amount of sulphate-sulphur may be transformed to organic forms.

3.4.5 Sulphur oxidation and reduction

The most thoroughly studied group of S-oxidizing organism belongs to the family Thio bacteriaceae. These autotrophic organisms are ubiquitous and capable of very rapid oxidation rate in \textit{in vitro}. Hydrogen sulphide, elemental sulphur, thiosulphate and polythionates are oxidized to sulphate by various members of the thiobacilli, while oxidation of reduced-S compounds in flooded
soil can be brought about by the photosynthetic bacteria such as *Chromatium* and *Chlorobium*. In agricultural soils, the heterotrophic sulphur organisms greatly outnumber the autotrophs.

Dissimilatory sulphate reduction in the soil is brought about exclusively by certain bacteria that use sulphate as the terminal electron acceptor in their respiratory processes. The predominant microorganisms in this reaction are obligate anaerobes belonging to sulphur reducing bacteria, *Desulphotomaculum* and *Desulphovibrio* (Roy and Trudinger, 1970). These all sulphur-reducing bacteria contain cytochrome, the respiratory pigments which act as electron donor in the reducing process. They reduce sulphate to sulphide ion through the potential inorganic intermediate- thiosulphate, tetrathionate and colloidal S.

### 3.5 NITROGEN IN PLANTS

Nitrogen is available in the form of molecular N₂, volatile ammonia/NOx, mineral (NO₃⁻ and NH₄⁺) and organic N (urea and amino acids) (von Wiren et al., 1997). However, nitrate and ammonium are the preferred source of N by most plant species (Leleu and Vuylsteker, 2004). In a typical aerobic agricultural soil, both nitrate and ammonium are present but nitrate is the major form. It is the most abundant source of N in many soils, especially those in cultivation with annual crops. In general, most crop plants prefer a mixture of ammonium and nitrate and usually take up a higher proportion of ammonium to nitrate than is present in the soil solution. In a study of 35 agricultural soil samples, the mean soil solution concentration of nitrate was 6.0 mM, compared with 0.77 mM for ammonium (Wolt, 1994). Uptake and assimilation of N are described in the following sections.

#### 3.5.1 Nitrogen Uptake

The first step of N acquisition and utilization in plants is the transport of nitrate from soil through plasmalemma of epidermal and cortical cells of the root
According to Forde (2002), nitrate appears to be a signal that can directly affect the expression of genes related to nitrate uptake, transport and assimilation. It thus acts as both a nutrient and a signal. Although most of the higher plants are capable of reducing nitrate in both roots and shoots, the nitrate is reduced more efficiently in leaves than in roots because of the readily available reductants, energy and carbon skeletons produced by photosynthesis (Chen et al., 2004). Nitrate uptake is an active process driven by the proton gradient or the proton motive force maintained by H*-ATPase. Two or more protons are co-transported along with every nitrate ion (Crawford, 1995; Santi et al., 1995). In many higher plants, there exists a biphasic relationship between nitrate uptake rate and external nitrate concentration. It is suggested that there are two different types of transporter systems in higher plants, namely, High Affinity Transporter System (HATS) and Low Affinity Transporter System (LATS). HATS are further categorized into Inducible High Affinity Transporter System (iHATS) and Constitutive High affinity transporters system (cHATS) (Okamoto et al., 2006).

3.5.2 Nitrogen assimilation

Once the nitrate has been taken up by the plant cell, the next step in the N assimilation pathway is the reduction of nitrate. The nitrate entering the plant cell is assimilated in a series of steps involving the action of a number of different enzymes (Crawford et al., 2000). This reduction is catalyzed by nitrate reductase (NR) and nitrite reductase (NiR). NR reduces the nitrate into nitrite, which is then reduced to ammonium by NiR.

\[
\text{NO}_3^- + \text{NAD(P)H} + \text{H}^+ \xrightarrow{\text{NR}} \text{NO}_2^- + \text{NAD(P)}^+ + \text{H}_2\text{O} \\
\text{NO}_2^- + 6 \text{Fd}_{\text{red}} + 8\text{H}_2\text{O}^+ \xrightarrow{\text{NiR}} (6e^-) \text{NH}_4^+ + 6\text{Fd}_{\text{ox}} + 10\text{H}_2\text{O}
\]
Ammonium, either produced by plants from nitrate or directly taken up through root, is rapidly assimilated into non-toxic organic compounds. In most plants, NH$_4^+$ is assimilated into amino acids through the co-operative activity of two enzymes: glutamine synthetase (GS) and glutamate synthase (GOGAT). GS catalyses the first step of NH$_4^+$ incorporation into glutamate to yield glutamine (Suzuki and Knaff, 2005). The reaction is as follows:

$$\text{GS} \quad \text{Glutamate} + \text{ATP} + \text{NH}_4^+ \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$$

GOGAT catalyses the formation of two molecules of glutamate. The reaction is as follows:

$$\text{GOGAT} \quad \text{Glutamine} + 2\text{-oxoglutarate} \rightarrow 2\text{Glutamate}$$

GS/GOGAT pathway is of crucial importance since the glutamine and glutamate produced are donors of amino groups for the biosynthesis of major N-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines and alkaloids (Lea and Ireland, 1999; Hirel and Lea, 2001). An alternative pathway for the formation of glutamate involves reductive amination of 2-oxoglutarate by NH$_4^+$, catalysed by mitochondrial glutamate dehydrogenase (GDH). The reaction is as follows:

$$\text{GDH} \quad \text{2-oxoglutarate} + \text{NH}_4^+ + \text{NAD(P)H} \rightarrow \text{Glutamate} + \text{H}_2\text{O} + \text{NAD(P)}^+$$

3.6 MOLECULAR PHYSIOLOGY OF NITRATE UPTAKE

3.6.1 Biochemical characterization

Inducible High Affinity Transporter System (iHATS) is substrate-inducible and is responsible for uptake at low concentrations of NO$_3^-$ (below ~1mM) that are characterized by low $K_m$ values (5-200 µM). Constitutive High affinity
transporters (cHATS) is responsible for uptake at low concentrations of NO$_3^-$ and provides a low capacity pathway in un-induced plants but operates simultaneously with iHATS in the induced state. Their activity becomes three-fold on exposure to nitrate (Crawford and Glass, 1998). It is characterized by low values of both $K_m$ (6-20 $\mu$M) and $V_{max}$. Low affinity transporters form a constitutive transport system which is responsible for uptake at high external nitrate concentrations (>1 mM). Despite of showing linear kinetics, it appears to be an active H$^+$-dependent transport system (Kronzucker et al., 1995). LATS are characterized by high $K_m$ values (>0.5 mM). It allows enough nitrate into the cell which is sufficient to induce expression of transporter and assimilatory genes and presumably plays a physiological role in the nitrate uptake only above a certain threshold. Molecular studies of nitrate transporters in plant suggest that nitrate transporters belong to two different families, NNP and PTR (Forde, 2000).

It is suggested that both the high and the low affinity transporters are functional during the early stages of growth but the high affinity systems are functional at later stages too, when the soil N concentration is low. Studies on oilseed rape showed that HATS accounted for about 89% of the total nitrate uptake (18% and 79% for cHATS and iHATS, respectively) when no fertilizer was applied (Malagoli et al., 2004). They also found that LATS accounted for a minor proportion of the total nitrate uptake. It is proposed that NO$_3^-$-inducible part of HATS functions chiefly as a sensor for root NO$_3^-$ availability (Miller et al., 2007a).

3.6.2 Genomic organization

*Nrt1* and *Nrt2* gene families define two classes of membrane proteins, probably involved in low and high affinity nitrate transport, respectively (Williams and Miller, 2001; Okamato et al., 2003). The first eukaryotic NO$_3^-$ transporter gene was isolated over 16 years ago from the fungus, *Aspergillus nidulans* (Unkles et
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al., 1991). A few years later, a nitrate transporter gene (AtNrt1:1) was identified in *Arabidopsis thaliana* (Tsay et al., 1993). Later, this gene was used as a probe to isolate two more genes (LeNrt1:1) and (LeNrt1:2) in tomato (Lauter et al., 1996). Here, Nrt1:2 is shown to be nitrate-inducible and its expression restricted to roots but not in the stem or leaves but Nrt1:1 is not restricted to roots and is constitutively expressed. Nitrate transporter genes have been cloned from wide range of plants. BnNrt1:2 is identified in *Brassica napus* also (Crawford and Glass, 1998). AtNrt1:4 has a very specific pattern of expression in leaf petiole where it plays a role during nitrate accumulation within these tissues (Chiu et al., 2004). AtNrt1:3 expression was nitrate induced in the leaf, but in roots it was not found to be a significant contributor to LATS (Okamato et al., 2003). According to Li et al. (2007), AtNrt2:1 was the major contributor to iHATS and cHATS.

Ammonium (NH$_4^+$) transport shows a normal homeostatic tendency but the range of the concentration at which absorption occurs is very limited due to the potential toxicity at elevated NH$_4^+$ concentrations. Like nitrate, ammonium is also transported by transporter protein located in the plasma membrane. AMT-type transporters handle NH$_4^+$ influx and mediate the uniport of this ion. First NH$_4^+$ transporter AtAMT1;1 was isolated from *Arabidopsis thaliana* (Ninnemann et al., 1994) and then another five homologous sequences, AtAMT1;1 to AtAMT1;5 were discovered.

3.6.3 Regulation of transporters

For the regulation of nitrate uptake, signals are derived from nitrate, which are involved in triggering widespread changes in gene expression; resulting in reprogramming of N metabolism to facilitate the uptake and assimilation of nitrate and its incorporation into amino acids. The nitrate assimilatory pathway is under tight regulation by the available nitrate and reduced N. In strawberry,
increasing external nitrate concentration from 0mM to 4mM markedly increased the cumulative nitrate uptake (Taghavi and Babalar, 2007). Several of the LATS- and HATS-related genes, apart from being root specific, are also inducible by nitrate and there is evidence that at least one HATS-related gene, *NpNrt2*:1 is also repressible by reduced nitrogen (Quesada et al., 1997). In barley and white spruce, cHATS provides a high affinity, low capacity pathway for nitrate entry in uninduced plants. Nevertheless, cHATS activity is up regulated (approximately three folds) by exposure to nitrate (Trueman et al., 1996). In barley, the fully induced iHATS flux was approximately 30 times higher than that resulting from the cHATS (Quesada et al., 1997). The increase in transcript is accompanied by increased rates of nitrate uptake (Imsande and Tourine, 1994). The results on citrus seedlings suggest that LATS is under feedback control by the N status of plant. A decline in uptake rate by the addition of amino acids (Glu, Asp, Asn, Gln) to the external solution has been reported (Cerezo et al., 2000). The use of chemical inhibitors in physiological studies has suggested that protein synthesis is important for nitrate uptake (Aguera et al., 1990) and the transporters may turn over relatively slow. A degradation mechanism for transporter protein in *Arabidopsis* (*AtNrt2*:1) has been suggested (Cerezo et al., 2001). The presence of a number of conserved protein kinase C recognition motifs in the N and C domains of *HvNRT2*:1 (Forde, 2000) suggests that phosphorylation events are involved in regulating *AtNrt2*:1 activity in response to environmental cues. Remans et al. (2006) found that under N-limited conditions, *AtNrt2*:1 played a key role as a major NO₃⁻ uptake system and coordinated lateral root initiation and development with external NO₃⁻ availability.
3.7 MOLECULAR PHYSIOLOGY OF N-ASSIMILATORY ENZYMES

3.7.1 Nitrate reductase (EC 1.6.6.1)

3.7.1.1 Biochemical characterization and localization

Nitrate reductase (NR) is a key enzyme involved in the first step of nitrate assimilation in plants (Leleu and Vuylsteker, 2004). It catalyzes the reduction of nitrate to nitrite with pyrimidine nucleotide in higher plants. NR in plants is a soluble enzyme, located primarily in the cytosols of root epidermal and cortical cells and shoot mesophyll cells; it exists as a homodimer metalloprotein of 110-kD subunits. It catalyzes reduction of nitrate to nitrite by transferring two electrons from NAD(P)H to nitrate via three redox centres composed of two prosthetic groups (flavin adenine dinucleotide [FAD] and heme) and a MoCo cofactor in a 1:1:1 stoichiometry per subunit. Each redox centre is associated with a functional domain of the enzyme that has activity independent of the other domains. NR is a substrate inducible enzyme and is thought to be the most limiting step in N assimilation. For this reason, NR activity is considered as a selection criterion for grain yield and N assimilation potential. There are three main forms of NR in plants that are defined by their electron donor (reductant) source, either NADH, NADPH or both (Miller and Cramer, 2004).

3.7.1.2 Genomic organization

Nitrate reductase is regulated in both shoots and roots. Most plants have two or more genes for NR. Clones of both genes have been isolated and mapped (Sivasankar and Oaks, 1996). Molecular and genetic analyses have revealed that most plants have two or more structural genes for NR, the only known exception being Nicotiana plumbaginifolia, which has a single NR gene that encodes an NADH-dependent NR (Caboche and Rouze, 1990). In barley, the NADH-specific NR is encoded by nar1 gene, while the NADPH bispecific NR is
encoded by *nar7* gene. Clones of both genes have been isolated and mapped (Miyazaki et al., 1991) and their induction properties have been compared (Sueyoshi et al., 1995). Although, the two proteins are distinct, the genes respond similarly to NO$_3$.

### 3.7.1.3 Regulation

Control of NR activity can be achieved either by altering the activity level of existing enzyme or by controlling the amount of enzyme by synthesizing new enzyme and degrading the old one. Many factors regulate this enzyme. Nitrate triggers transcription of inducible genes (NIA) encoding NR. *De novo* synthesis of new NR, stimulated by NO$_3$, is one of the mechanisms for controlling enzyme level when combined with NR protein degradation (Stitt, 1999). NO$_3$ induced increase in the NR activity and NR protein is due to the enhanced steady state level of NR-mRNA (Miyazaki et al., 1991; Sueyoshi et al., 1995) and this induction is shown to be repressed by downstream N assimilation products like glutamine and asparagine (Vincentz et al., 1993; Sivasankar et al., 1997). Sucrose addition (Sivasankar and Oaks, 1996) and light also enhances NR protein and mRNA induction. It appears that NR expression is regulated by light *via* phytochrome after it is triggered by NO$_3$. Role of light in induction is probably more related to the activity of the enzyme rather than to the activation of the NR gene. In another study, NR protein kinase was used to identify the apparent key serine residue in spinach NR (Bachmann et al., 1996).

Many environmental (stress) factors trigger the modulation and post-translational regulation of NR. The existing amount of NR protein depends not only on the rate of synthesis, but also on the rate of degradation. Activity level can be controlled by mechanisms involving phosphorylation of the NR protein and binding of Mg$^{2+}$ or another divalent cation and an inhibitor protein (Stitt, 1999). Light has a very important effect though it is not a direct signal to
activate NR as photosynthesis is required for NR activation. It has been shown that light and oxygen availability are the major external triggers for the rapid and reversible modulation of NR activity. Indeed, NR can be activated in the dark by feeding sugars to the leaves. Sugar and/or sugar phosphates are the internal signals regulating the protein kinase(s) and phosphatase. Roots usually do not change their reduction rate as rapidly as shoots. However, during sudden anoxia the enzyme is rapidly modulated, being activated within minutes (Kaiser and Huber, 2001).

3.7.2 Nitrite reductase (EC 1.7.7.1)

3.7.2.1 Biochemical characterization and localization

The second enzyme in the sequence, nitrite reductase (NiR, ferredoxin nitrite oxidoreductase) catalyses the six-electron transfer reaction from reduced ferredoxin to NO$_2^-$ leading to the synthesis of NH$_4^+$. It is localized within chloroplasts in leaf and in plastids in root tissues (Sechley et al., 1992). It is a monomeric protein of about 63 kDa containing sirohaem and a 4Fe4S centre as prosthetic groups (Faure et al., 2001). Reduced ferredoxin serves as the electron donor in both leaves and roots. This enzyme obtains its reducing power from NADPH, generated by the oxidative pentose phosphate pathway located within the root plastids (Bowsher et al., 1989). Levels of both the ferredoxin and the NADPH-dependent ferredoxin:NADP-oxidoreductase in isolated pea root plastids increase in response to NO$_2^-$ additions.

3.7.2.2 Genomic organization

The gene for the NiR apoprotein has been cloned in at least six different plant species. There is one NiR apoprotein gene per haploid genome in barley and spinach, two in maize and four in *N. tabaccum* (Duncanson et al., 1993; Kronenberger et al., 1993). The four NiR apoprotein genes in tobacco are known
to encode two distinct isoforms in shoots and a further two in roots, as indicated by the gene expression studies of Kronenberger et al. (1993). The promoter region of NiR gene has been fused to β-glucoronidase (GUS) and trans-gene has then been successfully introduced into tobacco (Rastogi et al., 1993).

3.7.2.3 Regulation

This gene appears to be very responsive to NO₃⁻ additions and to the additions of sucrose, glutamine or asparagine. The experiments of Rastogi et al., (1993) provide evidence that induction of this gene by NO₃⁻ is a transcriptional event. However, addition of asparagine or glutamine results in a repression of induction, whereas sucrose enhances the induction (Vincentz et al., 1993; Sivasankar and Oaks, 1995). Light is also an important environmental cue in the NiR induction (Wray, 1993).

3.7.3 Glutamine synthetase (EC 6.3.1.2)

3.7.3.1 Biochemical characterization and localization

Glutamine synthetase (GS) catalyses the ATP-dependent conversion of inorganic N (NH₄⁺) to an organic form (glutamine). This enzyme along with GOGAT represents the major pathway for incorporation of ammonia (toxic to plant function) into amino acids (Hirel and Lea, 2001; Fei et al., 2006). There are two types of GS: type I-GS, which is dodecameric with subunits of about 52 kDa and type II-GS that is octameric and composed of about 40 kDa subunits (Nogueira et al, 2005). Type I is found mainly in bacteria and type II is best characterized in higher plants. It exists in plants as two major isoforms, a chloroplastic (GS2) and a cytosolic (GS1) enzyme (Scarpeci et al., 2007). During the plant development, N is moved into and out of proteins in different organs, and transported through a limited number of transport compounds. Major
portion of N is released as NH$_3$ and reassimilated via GS (Miflin and Habash, 2002).

3.7.3.2 Genomic organisation

Molecular analysis of GS genes reveals a multigene family whose individual members encode several distinct cytosolic GS (GS1) polypeptides and a single chloroplastic GS (GS2) polypeptide. Li et al. (1993) have identified five distinct cDNA clones of GS in maize. Six distinct genes encoding for GS in maize (Li et al., 1993) and five in sugarcane (Nogueira et al., 2005) have been identified. It has been demonstrated that GS occurs in an organ-specific manner; roots and nodules generally contain proportionally more cytosolic GS, while leaves contain more chloroplastic GS (Becker et al., 1992). Genetic study of GS has helped in explicating the role of each isoform. Chloroplastic GS is considered to be involved in the reassimilation of photorespiratory NH$_4^+$.

3.7.3.3 Regulation

Both GS2 and GS1 genes are regulated by external N application, but the extent of this regulation depends on the plant species, N source and plant organ/tissue (Cren and Hirel, 1999). Regulatory effects of N assimilation, NH$_4^+$ and/or NO$_3^-$, on gene expression has been reported in many plants including rice, maize, tobacco, tomato, sunflower and mustard (Zozaya-Hinchliffe et al., 2005). N and C metabolites may also control the expression of GS in the leaf of Arabidopsis (Oliveira and Coruzzi, 1999), and tobacco (Masclaux-Daubresse et al., 2005). According to Zozaya-Hinchliffe et al. (2005) light and metabolic factors associated with light (sucrose and carbon substrates) also regulate the expression of this enzyme. Detailed studies on *Pinus sylvestris* by Elmlinger et al. (1994) have shown that light regulation of GS2 expression occurs crudely at transcriptional level but fine regulation occurs at post-transcriptional level.
Similar observations were made in tomato seedlings (Migge et al., 1998). Some mechanisms controlling the stability and activity of GS have been discovered. Finnermann and Schjoerring (2000) presented a tentative model for the reversible control of GS1 by phosphorylation and dephosphorylation incorporating the roles of ATP, Mg$^{2+}$ and 14-3-3 binding. The model is based on the central role of ATP/AMP ratio under light. In that model, it is proposed that in dark, ATP/AMP levels are high, so GS1 is phosphorylated and binds 14-3-3 proteins, which protects it against degradation. Conversely, in the light, GS1 unphosphorylates and becomes susceptible to damage. Riedel et al. (2001) have also demonstrated that GS2 is phosphorylated in tobacco. Many workers have shown that important factors affecting GS activity are light, carbon status and N nutrition. Experiments with white, red, far-red or blue light by Becker et al. (1992) and Migge et al. (1998) have shown that the phytochrome and the blue light photoreceptors are involved in the positive response to light. C-compounds, important in stimulating GS1 and GS2 synthesis, include sucrose and 2-oxoglutarate (Oliveira et al., 2002). Studies on dark-adapted Arabidopsis seedlings have shown that sucrose enhances the expression of GS2, thus mimicking the effect of light. Temperature is also an important environmental factor controlling the GS expression, as shown by the studies of Woodall et al. (1996) on pea and barley plants. Within 2 days of keeping the plants in 15°C instead of 25°C, they observed 50% reduction in GS2 activity while GS1 activity remained unaffected. There are indications that substrate availability (Ortega et al., 1999) or phosphorylation (Moorhead et al., 1999) may be an important factor controlling the enzyme turnover and activity respectively.
3.7.4 Glutamate synthase (E.C. 1.4.1.13)

3.7.4.1 Biochemical characterization and localization

Glutamate synthase (Glutamine (amide): 2-oxoglutarate aminotransferase, GOGAT) catalyses the reductive transfer of the amide group of glutamine (produced by GS) to 2-oxoglutarate (α-keto glutarate) to form two glutamate molecules (Ireland and Lea, 1999). The discovery of NAD(P)H-dependent GOGAT in bacteria (Tempest et al., 1970), ferredoxin (Fd)-dependent GOGAT in pea chloroplast (Lea and Miflin, 1974) and NAD(P)H-dependent GOGAT in carrot cell cultures (Dougall, 1974) established a route; GS-GOGAT cycle, for the incorporation of NH$_3$ into organic compounds. The synthesized glutamate can be used either to replenish the glutamate pool for subsequent GS catalysis or to donate its amino group to form other N-containing compounds. One important fate of glutamate and glutamine is the synthesis of aspartate and asparagine. These amino acids are important N-transport compounds in many plants (Temple et al., 1998). In higher plants, GOGAT occurs as two distinct isoforms, NADH-GOGAT (EC 1.4.1.14) and Fd-GOGAT (EC 1.2.7.1) and these differ in molecular mass, subunit composition, enzyme kinetics and metabolic functions (Gregerson et al., 1993; Sakikabara et al., 1991). Fd-GOGAT, an iron-sulphur flavoprotein, generally functions as a monomer with subunit molecular mass of 130-180 kD. Maize roots contain a Fd-GOGAT isoform that is immunologically distinct from the enzyme found in leaves, suggesting that the two forms are encoded by distinct genes. The root isoform has been implicated in assimilation of NH$_4^+$ derived from soil NO$_3^-$ (Redinbaugh and Campbell, 1993). NADH-GOGAT is also an iron-sulphur flavoprotein and is found primarily in non-green tissues. In higher plants, it occurs as a monomer with a native subunit mass of 225-230 kDa and has a pH-optimum range from 7.5 to 8.5 (Lea et al., 1990).
3.7.4.2 Genomic organisation

GOGAT is found in all types of organism and its amino acid sequence is remarkably well conserved (Temple et al., 1998). The expression pattern of the genes encoding cytosolic GS and NADH-GOGAT appears to be coordinated in non-legumes, where the proteins function together in processes such as primary assimilation of NH$_4^+$ derived from soil NO$_3^-$ and reassimilation of NH$_4^+$ released by amino acid catabolism. cDNA clones for Fd-GOGAT have been isolated from a number of species including barley (Avila et al., 1993); maize (Sakakibara et al., 1991) and A. thaliana (Coschigano et al., 1998). Full-length cDNA and genomic clones of NADH-GOGAT have been isolated from alfalfa (Trepp et al., 1999) and rice (Goto et al., 1998).

3.7.4.3 Regulation

Light and a variety of metabolites exert major regulatory controls over metabolic pathways. Evidence by Suzuki and Rothstein (1997) indicates that light exerts a positive regulatory effect on the expression of Fd-GOGAT (GLU1). GLU2 expression is also induced by light but the induction of this gene by sucrose in dark indicates that light-induced expression may in part be caused by increased concentration of C metabolites. During the development and expansion of a new leaf, Fd-GOGAT activity increases with the onset of photosynthesis and photorespiration (Emes and Tobin, 1993). In Barley, enzyme activity, protein and mRNA increased as the leaf emerged and expanded, and decreased as the leaf aged (Pajuelo et al., 1997). Nitrate also acts as a signal resulting in widespread changes in the expression of key genes in N metabolism pathway, including Fd-GOGAT (Scheible et al., 1997). Gene expression study in developing alfalfa nodules suggests that NADH-GOGAT is uniquely regulated, as compared with other genes of N metabolism (Vance et al., 1994). The maximum expression of NADH-GOGAT occurred in effective

3.7.5 Glutamate dehydrogenase (EC 1.4.1.2)

3.7.5.1 Biochemical characterization and localization

Glutamate dehydrogenase (GDH) is one of few enzymes capable of releasing amino nitrogen from amino acids to give keto-acid and NH$_3$ that can be separately recycled and used in respiration and amide formation, respectively. It is thought to be an alternative pathway for the formation of glutamate involving reductive amination of 2-oxoglutarate by NH$_4^+$. Its role in plant cells remains controversial (Miflin and Habash, 2002). It is yet to be clearly demonstrated that the enzyme plays a significant role either in NH$_3$ assimilation or in carbon (C) recycling (Dubois et al., 2003). Studies have shown that it has a role in the deamination of glutamate to provide energy and return C from amino acids into the reactions of C metabolism during C or energy shortage (Miflin and Habash, 2002). However, Dubois et al. (2003) have still argued that the physiological function of GDH in plants remains speculative. GDH is capable of synthesizing or de-aminating glutamate but the direction of activity depends on specific environmental cues (Pahlisch, 1996). One isoform of
enzyme, localized in mitochondria in roots and leaves, uses NADH as the electron donor (Sechley et al., 1992). Another isoform, having specific requirement for NADPH is present in chloroplasts of photosynthetic tissues. The primary role of GDH could be replenishment of TCA cycle intermediates via its oxidation to 2-oxoglutarate. Glutamate is deaminated to 2-oxoglutarate in isolated mitochondria; however in the presence of aminooxyacetate, glutamate no longer contributes to mitochondrial respiration (Sechley et al., 1992). This observation indicates that GDH does not oxidize glutamate.

3.8 REGULATION OF NITROGEN METABOLISM

Competition for N resources by crops leads to nutrient deficiencies in the root medium, mainly during the interval between fertilizations. In agriculture, situations of sub- versus supra- optimal N supply are usually assessed using N nutrition index (NNI) (Richard-Molard et al., 2008). Understanding these processes helps in the production of crops more environmentally sound, which sustainable agriculture requires. Now-a-days, detailed study of nitrate response in plants is possible using new bioinformatic tools (Sungear software) to perform comparative studies of multiple transcriptome responses. These analyses have identified many genes and pathways (Gutierrez et al., 2007). One might think that NO$_3^-$ is utilized in a linear pathway involving uptake and transport, followed by its assimilation, amino acid biosynthesis and protein synthesis. But, there are complex interactions with many other aspects of N metabolism like storage and remobilization of NO$_3^-$, de novo NH$_4^+$ assimilation, distribution of N between highly branched pathway of amino acid biosynthesis. N metabolism is regulated by many factors. NO$_3^-$ assimilation in tobacco leaves is characterized by dramatic diurnal changes in gene expression, enzyme activities, metabolite levels and fluxes. When leaves were grown in high nitrate and favorable light regime, leaves contained high levels of NIA transcript at the
end of night (Kaiser et al., 1999), leading to around 3-fold increase during early hours of light period (Scheible et al., 1997). The rate of NO$_3^-$ assimilation also exceeds net flux through GOGAT pathway by around 25%, leading to accumulation of reduced N in immediate downstream products such as NH$_4^+$ and glutamine, and also in the photorespiratory metabolites, glycine and serine. Usually, NO$_3^-$ and NH$_4^+$ assimilation are regulated transcriptionally, in response to the balance between NO$_3^-$ influx and its assimilation, and post-translationally in response to downstream signals from N metabolism. Feeding glutamate led to increase of NH$_4^+$ and glutamine. This observation indicates that glutamate plays a major role in the sensitive feedback mechanism that regulates NH$_4^+$ assimilation. Internal pools of amino acids in plants may indicate N status by providing a signal, which regulates NO$_3^-$ uptake by plants (Miller et al., 2007b). The results are reviewed in terms of feedback regulation and putative cell sensing systems for N status. A systematic search for further feedback mechanisms indicates that specific minor acids, including cysteine and asparagine, exert feedback regulation on NO$_3^-$ assimilation. The three amino acids identified to date as feedback inhibitors of NO$_3^-$ reduction; glutamate, cysteine and asparagine, occupy strategic positions in amino acid metabolism.

3.9 SULPHUR IN PLANTS

Sulphur (S), because of its many oxidation states, represents one of the most versatile elements in biology. These states range from +6 (SO$_4^{2-}$) to -2 (H$_2$S). The most oxidative and thus, the most stable of them is SO$_4^{2-}$. So, for the plants, SO$_4^{2-}$ is the major source of S from soil. S metabolism in plants includes uptake of ion from the environment, assimilation into organic compounds, and their channeling into proteins and secondary substances. According to Droux (2004), assimilation of SO$_4^{2-}$ could be summarized in four steps: uptake of SO$_4^{2-}$; activation of SO$_4^{2-}$; reduction of SO$_4^{2-}$ and synthesis of cysteine.
Sulphate uptake from soil is almost exclusively via roots and it is an energy independent process by proton/sulphate co-transporters (Leustek et al., 2000; Saito, 2000). After transport of SO$_4^{2-}$ across the plasma membrane, intracellular transport from roots to shoots occurs by unload/download mechanism via xylem and finally transfers between tissues via the phloem.

Inorganic SO$_4^{2-}$ is chemically very stable and therefore, has to be activated prior to reduction to sulphite. Reduction of SO$_4^{2-}$ requires eight electrons and about twice as much energy as NO$_3^-$ reduction. In plants, the high potential needed for SO$_4^{2-}$ reduction is surmounted by the activation step i.e. the formation of adenosine 5'-phosphosulphate (APS) from SO$_4^{2-}$ and this step is catalyzed by ATP sulphurylase (ATPS). APS is an energy-rich mixed anhydride of phosphate and SO$_4^{2-}$. Subsequently, APS is reduced to sulphite by APS reductase (APR), and the sulphite formed is reduced to sulphide by sulphite reductase (SiR). Sulphide is incorporated into cysteine, catalyzed by O-acetylserine(thiol)lyase (OASTL) using O-acetylserine as a substrate (Droux, 2004; Saito, 2004).

3.10 MOLECULAR PHYSIOLOGY OF SULPHATE UPTAKE

3.10.1 Biochemical characterization and localization

Sulphate transport is driven by a proton-gradient force and is a 3H$^+$/SO$_4^{2-}$ co-transport mechanism. Multiple transport steps through many membranes are involved. Many workers have worked for identification and functional characterization of SO$_4^{2-}$ transporters. Plasma membrane SO$_4^{2-}$ transporters are classified as proton/sulphate co-transporters. Thus, uptake mediated by this transporter is pH-dependent, and the proton gradient is generated by plasma membrane proton ATPase. In yeast, Takahashi et al. (1997) and Yoshimoto et al. (2002) have identified SO$_4^{2-}$ transporters with high and low affinity for SO$_4^{2-}$. These observations were similar to those reported by Vidmar et al. (2000). The
requirement for a pH gradient to drive uptake was also shown in higher plants using cultured tobacco cells and in an isolated vesicle system (Hawkesford et al., 1993). A complex array of transporters differing with regard to localization, transport kinetics, and inducible expression contribute to facilitate effective transportation of SO$_4^{2-}$.

3.10.2 Genomic organization

The plant sulphate transporter gene was first isolated from the tropic legume *Stylosanthes hamata*, using a yeast complementation system (Smith et al., 1995). Since then, a large number of genes and cDNAs encoding sulphate transporters have been isolated from different plant species. About 12 sulphate co-transporters like genes were identified and were divided into four groups in *Arabidopsis thaliana* (Takahashi et al., 2000; Yoshimoto et al., 2003) and *Brassica oleracea* (Buchner et al., 2004). In *Arabidopsis*, 14 genes encoding for SO$_4^{2-}$-transporters have been reported (Yoshimoto et al., 2002; Hawkesford, 2003). These are classified into five subfamilies (*AtSULTR* 1-5) based on their deduced amino acid sequences. These subgroups suggest specialized function and catalytic properties for the transport of SO$_4^{2-}$ between compartments and tissues. Members in *SULTR1* are high-affinity transporters. *SULTR1;1* and *SULTR1;2* of *Arabidopsis* are localized to root epidermal cells and are inducible by SO$_4^{2-}$ depletion, thus are responsible for initial SO$_4^{2-}$ uptake from rhizosphere (Shibagaki et al., 2002). Low-affinity transporters of *SULTR2* and *SULTR3* are localized to vascular tissues and are thought to be involved in the uptake from plant apoplast into vascular cells. Transporters of *SULTR4* are responsible for efflux of SO$_4^{2-}$ from vacuole to the cytoplasm (Kataoka et al., 2004). Phylogenetic analysis shows subdivision of transporters into 4 distinct groups. Group 1 is the best characterised and its analysis particularly in *A. thaliana* and *Hordeum vulgare*, suggests that these sulphate transporters are
mainly responsible for sulphate uptake by the root (Smith et al., 1997; Takahashi et al., 2000; Vidmar et al., 2000; Yoshimoto et al., 2002). Sulphate transporters have been described in *S. hamata* and *A. thaliana*, which have a low affinity for sulphate, with $K_m$ values of 0.41 mM and more (Takahashi et al., 1997, 2000). These sulphate transporters have been placed in group 2 because of phylogenetic analysis and are thought to be responsible for vascular sulphate transport. Group 3 contains 5 *Arabidopsis* $SO_4^{2-}$ transporter genes but these are less well characterized (Takahashi et al., 1997). The sulphate transporters of the group 4 are characterised by a C-terminal plastidal transit peptide.

3.10.3 Regulation

Many studies have taken the impact of S-nutritional status on $SO_4^{2-}$ influx capacity as a model for studying the regulation of S nutrition. Earlier studies indicated an increase in $SO_4^{2-}$ uptake following a period of S limitation (Clarkson et al, 1989). Indirect evidence using inhibitors showed a rapid turnover of $SO_4^{2-}$ transporter proteins and thus, the importance of transcriptional regulation (Clarkson et al., 1992). Cytokinin-mediated regulation of gene expression is also indicated as cytokinins down-regulate the iHATS of $SO_4^{2-}$ i.e. SULTR1;1 and SULTR1;2 in *Arabidopsis* (Maruyama-Nakashita et al., 2004). There is clear evidence that transcription of the genes encoding the transporters involved in initial uptake at the soil-root interface, cell-to-cell transfer, vascular transportation and vacuolar efflux transporter is controlled by plant S nutritional status (Buchner et al., 2004). The coordinated expression of this gene family helps in the optimum management of plant $SO_4^{2-}$ under varying conditions of supply and demand. Smith et al. (1997) studied the impact of S status on the transcription of HATS of $SO_4^{2-}$ in roots. Their observations were that following the removal of S, an increased abundance of mRNAs for high-affinity transporters was in parallel with decreasing tissue
contents of $\text{SO}_4^{2-}$, cysteine and glutathione. Upon re-supply of S, a de-repression of activity and decrease in mRNA abundance occurred within hours. During the regulation of expression of $\text{SO}_4^{2-}$ transporters, de-repression is mediated by feedback loops involving products of S-assimilation (Hawkesford et al., 2003). In addition to the quick responses to S-nutritional status in terms of transcriptional regulation and protein turnover, there are some additional levels of post-translational regulation acting on $\text{SO}_4^{2-}$ transporters. The carboxy-terminal region contains a $\text{SO}_4^{2-}$ transporter and anti-sigma antagonist (STAS) domain (Shibagaki and Grossman, 2004; Rouached et al., 2005). Mutations or deletions in this region affect function and plasma membrane targeting. This region contains a phosphorylation site and may be involved in protein:protein interactions, both of which contribute to regulation.

3.11 MOLECULAR PHYSIOLOGY OF S-ASSIMILATORY ENZYMES

3.11.1 ATP sulphurylase (EC 2.7.7.4)

3.11.1.1 Biochemical characterization and localization

ATP sulphurylase (ATPS) catalyzes the first step in sulphate assimilation, the adenylation of sulphate to APS. This is the sole entry step for the metabolism of $\text{SO}_4^{2-}$. The formation of APS is an energetically unfavorable process, which is driven forward by the consumption of APS by subsequent reactions, reduction to sulphite by APS reductase or phosphorylation to PAPS by APS kinase (APK). In this reaction, $\text{SO}_4^{2-}$ is linked to a phosphate residue by an anhydride bond using ATP. The formation of APS is an energetically unfavorable reaction, which is driven by the consumption of pyrophosphate (Leustek et al., 2000). ATPS activity occurs in chloroplasts and cytosol. It was detected in chloroplasts and in the cytosol of spinach leaves (Renosto et al., 1993). There are different functions of ATPS in the two compartments: sulphate reduction in the plastids
and activation of sulphate for synthesis of sulphonated compounds in the cytosol (Rotte and Leustek, 2000).

3.11.1.2 Genomic organization

The initial step of SO$_4^{2-}$ utilization has received considerable attention, since entry reactions are usually good candidates for the control of the pathway. ATPS is represented by a small multigene family (four genes) in A. thaliana with cDNA products encoding proteins targeted to the plastids and one of the cytosolic localization (Hatzfeld et al., 2000; Rotte and Leustek, 2000). ATPS is encoded by small multigene families in all plant species. cDNAs encoding chloroplastic and cytosolic isoforms of ATPS have been isolated from potato (Klous et al., 1994). On the other hand, four isoforms of ATPS were isolated from Arabidopsis, all of them containing a chloroplast transit peptide (Logan et al., 1996; Hatzfeld et al., 2000). All four ATPS genes contain 5 exons and 4 introns and are localized on different chromosomes. In Brassica juncea, two isoforms of ATPS were cloned both containing an organelle-targeting peptide (Heiss et al., 1999).

3.11.1.3 Regulation

Regulation of S assimilation by C metabolites has received very little attention. It has long been known that cysteine production from SO$_4^{2-}$ is stimulated by light and the activities and mRNA levels of enzymes of SO$_4^{2-}$ assimilation are higher in green leaves than in etiolated tissues (Hell et al, 1997). Furthermore, activity of ATPS was shown to be light induced in maize. As it is known that several products and intermediates of SO$_4^{2-}$ assimilation are toxic, the pathway undergoes strict regulation by the S status of the plant (Brunold, 1990; Leustek et al., 2000). SO$_4^{2-}$ assimilation seems to be controlled by demand (Herschbach et al., 2000; Westerman et al., 2001). The pathway is repressed under normal levels of external SO$_4^{2-}$ and de-repressed by SO$_4^{2-}$ limitation. Attention was first
paid to ATPS during the investigations on the molecular mechanisms of feedback regulation of $\text{SO}_4^{2-}$ assimilation by thiols. In *A. thaliana*, ATPS activity and mRNA levels of the APS1 isoform were decreased by GSH treatment (Lappi-ertt et al., 1999). As external GSH supply increases the accumulation of cysteine, both GSH and cysteine might be responsible for the control of ATPS. Phloem sap analysis in *Brassica napus* (Lapparteint and Touraine, 1996) and poplar (Herschbach et al., 2000) indicated that GSH rather than cysteine was the acting signal. Studies by Lapparteint et al. (1999) in *Brassica* showed that blocking GSH synthesis relieved the repression of ATPS. By contrast, in maize, cysteine was able to regulate the levels of ATPS mRNA without the need for conversion to GSH (Bolchi et al., 1999).

3.11.2 APS reductase (EC 1.8.99.2)

3.11.2.1 Biochemical characterization and localization

APS formed by the action of ATPS is further reduced to sulphite ($\text{SO}_3^{2-}$) by APS reductase (APR) by a thiol-dependent two-electron reduction (Bick and Leustek, 1998; Bick et al., 1998). This enzyme was identified by chance while searching for cDNAs encoding plant PAPS reductase (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Since the reaction equilibrium of ATPS favors the reverse direction, i.e. the formation of ATP and $\text{SO}_4^{2-}$, the products of the forward reaction, i.e. APS and Pi, must be further metabolized immediately by APR (Saito, 2004). It is localized in chloroplasts. A key feature of this enzyme is that they are able to use a variety of reduced thiol compounds such as dithiothreitol and glutathione as a sole source of electrons. *In vivo* APR is present as a homodimer probably linked by a disulphide bond of conserved Cys residue (Kopriva and Koprivova, 2004). The APR enzymes are composed of three domains. At the amino terminus is a region that resembles a transit peptide that allows translocation of the mature protein to plastids and is
cleaved from the protein once it has been imported into the chloroplast. Adjacent to it is the amino-terminal domain of the mature protein that is homologous with PAPS reductase from a variety of organisms. At the carboxyl end is a domain that resembles thioredoxin. Thioredoxin is redox-active protein that functions with a number of different reductases. This fusion of reductase and cofactor into a single protein implies that thioredoxin-like domain may act as an exclusive electron donor for the reductase domain. Sulphite is generated by this enzyme through the addition of two electrons, through the reduced glutaredoxin/thioredoxin-like domain (Suter et al., 2000; Kopriva et al., 2001). APR is encoded by a small multigene family of 2-3 isoforms in most plant species (Kopriva et al., 2004). Feeding experiments with $^{35}$SO$_4^{2-}$ for control flux analysis (Vauclare et al., 2002) to quantify the role of APR in control of SO$_4^{2-}$ assimilation showed that flux control coefficient of APR was between 0.7 and 0.9 (equivalent to 70% and 90% of the total control). These observations indicated strong control of pathway by APR and thus it could be concluded that APR is indeed a key enzyme of the SO$_4^{2-}$ reduction pathway.

3.11.2.2 Genomic organization

APS reductase cDNA was cloned from several plant species: A. thaliana (Gutierrez-Marcos et al., 1996; Setya et al., 1996), Brassica juncea (Heiss et al., 1999), Lemna minor (Suter et al., 2000), Allium cepa, Zea mays and Populus tremula x P. Alba. APR is encoded by multigene families in most species. Three different cDNAs were cloned from Arabidopsis thaliana (APR1, 2 and 3) that were able to complement the cysteine auxotrophy of an E. coli PAPS reductase mutant strain. The APR cDNAs encode individual members of a small, highly conserved gene family. At the level of nucleotide homology, the coding sequence of APR1 is more closely related to APR3 (78 %) identity than to APR2 (68 % identity). On the other hand, the genes of APR2 and APR3 have the same exon/intron
organization with 3 introns, APR1 gene lacks the intron separating exon 2 and 3 in APR2 and APR3. APR was cloned recently from a cDNA library in soybean plant (Phartiyal et al., 2008). They observed abundant expression of the gene and activity of encoded protein in early developmental stages of soybean seed, which declined with seed maturity. Two cDNAs were obtained from B. juncea (Heiss et al., 1999) and southern analysis revealed at least two APR genes in L. minor (Suter et al., 2000), poplar, and several species of the genus Flaveria (Koprivova et al., 2001). In A. thaliana the corresponding genes were cloned and sequenced (Chen and Leustek, 1998). In the APR1 gene sequence duplication is found at the 5' end of intron 2 and just before the thioredoxin active site in exon 3, which might possibly represent a remnant of an original intron separating the two domain-coding exons (Chen and Leustek, 1998).

3.11.2.3 Regulation

Many experiments based on the study of S assimilation have concentrated on APR, as this enzyme has long been known to be regulated strongly by various environmental factors, nutrient availability and stress. Kopriva et al. (1999) during the investigation of the control of SO₄²⁻ assimilation by light, have shown that APR activity undergoes a diurnal rhythm with the maximum activity at 4 h after light onset and the minimum activity at the beginning of night. Furthermore, the same study also revealed that sucrose was able to imitate the effect of light. It has been observed by many workers that carbohydrates induce APR mRNA accumulation and activity in the dark (Kopriva et al., 2002; Hesse et al., 2003). Nutritional stress also effect APR regulation at translational level. Withdrawal of N for 3 d in A. thaliana led to specific decrease of APR activity and these changes corresponded to changes in mRNA levels of all three isoforms and APR protein accumulation (Koprivova et al., 2000), showing that APR is primarily regulated at the level of transcription. S-deficiency is one of the major problems connected with S metabolism and this aspect has been
studied by many workers. Hirai et al. (2005) and Nikiforova et al. (2005), while working on Arabidopsis, observed that more than 2700 genes were affected by S coding for SO₄⁻ transporters and APR; other genes of SO₄⁻ assimilation were not significantly and/or consistently affected. Bick et al. (2001) revealed a post-transaltional level of APR regulation by redox processes. Not much is known about the role of phytoharmones in the control of S assimilation, but some experimental results indicate that this group of compounds is very important for regulation of S nutrition (Ohkama et al., 2002; Maruyama-Nakashita et al., 2004, 2005). Ohkama et al. (2002), using zeatin treatment on transgenic Arabidopsis plant, showed that it resulted in increased APR mRNA accumulation. The hypothesis that SO₄⁻ assimilation is regulated by carbohydrates and not by light was confirmed by a finding in which Lemna plant cultivated in CO₂-free atmosphere showed rapid decrease in APR activity and mRNA level (Kopriva et al., 2002). This reduction in APR activity, but not in mRNA level, was attenuated by supplying sucrose to the nutrient solution.

3.11.3 Sulphite reductase (EC 1.8.7.1)

3.11.3.1 Biochemical characterization and localization

Hydroden-sulphide:Fd oxidoreductase, commonly known as sulphite reductase (SiR) catalyzes the transfer of six electrons from ferrodoxin to SO₃⁻ producing sulphide (S²⁻) in the presence of light (Nakayama, 2000). This enzyme in plant cells consists of a homodimer of around 65 kDa and contains a siroheme and an iron-sulphur cluster [4Fe-4S] per subunit as co-factors. It is localized in plastids in both photosynthetic (electrons supplied from PSI) and non photosynthetic tissues (electrons supplied from NAD(P)H) [Saito, 2004]. SiR is represented by a single gene, and is localized exclusively in the chloroplast (Hawkesford and De Kok, 2006). It has an N-terminal cleavable extension peptide necessary for its plastid import. A proper combination of different isoforms of ferredoxin,
ferredoxin-NADP⁺ reductase and SiR is critical for efficient SO₂⁻ reduction (Yonekura-Sakakibara et al., 2000). Fd and Fd-SiR form an electrostatically stabilized 1:1 protein-protein complex, with Fd supplying the negatively charged groups; this specific interaction is crucial for efficient electron transfer between the two proteins (Akashi et al., 1999). Fd seems to have both common and unique electrostatic interaction sites to SiR and FNR.

3.11.3.2 Genomic organization
cDNAs have been cloned and complete amino acid sequences, deduced from the corresponding cDNAs, are available for Fd-SiRs from three higher plants, maize (Ideuchi et al., 1995), Arabidopsis (Bruhl et al., 1996) and tobacco (Yonekura-Sakakibara, 1998). The extent of amino acid sequence homologies among plant SiRs is very high (77% to 82%) (Nakayama, 2000). A cDNA and a single copy gene encoding SiR were isolated from Arabidopsis (Bruhl et al., 1996; Bork et al., 1998). The gene consists of 8 exons, with the first intron localized behind the first amino acid of the mature protein, and is localized on chromosome 5. Two SiR isoforms are present in leaves and non-photosynthetic organs of Brassica rapa and tobacco (Takahashi et al., 1997; Yonekura-Sakakibara et al., 1998). One cDNA and corresponding gene encoding SiR were isolated from tobacco; the gene possessed the same exon/intron organization as the gene from Arabidopsis (Yonekura-Sakakibara et al., 1998). Additional full-length cDNA clones for SiR were obtained from Glycine max and maize. SiR contains 19 % identical amino acids with nitrite reductase, indicating that these genes may have the same evolutionary origin.
3.11.4 Serine acetyltransferase (EC 2.3.1.30)

3.11.4.1 Biochemical characterization and localization

Biosynthesis of cysteine (first thiol-containing amino-acid) is a two-step process. The first step is the activation of L-serine by acetyl-coenzyme A, catalysed by serine acetyl transferase (SAT) to form O-acetylserine (OAS) (Hass et al., 2008). This OAS formed is used further in the second step. By contrast, to many amino acids whose synthesis takes place in plastids, this enzyme is associated with cytosol and mitochondria (Droux, 2003). It has a molecular mass of 300-350 kDa. The association of SAT with OASTL is necessary for SAT stability and activity. They form a multi-enzyme complex in plants (Bogdanova and Hell, 1997; Droux et al., 1998; Wirtz et al., 2001). Interaction with OAS-TL is a prerequisite for SAT activity, whereas OAS-TL is active as a free dimer (Droux et al., 1998; Wirtz et al., 2001).

3.11.4.2 Genomic organization

Serine acetyltransferase is encoded by a multigene family. cDNA clones encoding Ser acetyltransferase have been isolated from watermelon (Saito et al., 1995), spinach (Noji et al., 1998), Arabidopsis thaliana (Howarth et al., 1997) and Allium tuberosum (Urano et al., 2000). In particular, cDNAs of three Ser acetyltransferase isoforms that exhibit different subcellular localization, SAT-c (cytosolic isoform), SAT-p (plastidic isoform) and SAT-m (mitochondrial isoform) have been cloned from A. thaliana (Noji et al., 1998). In Arabidopsis, SAT gene family consists of five members (Kawashima et al., 2005). The organization of the SAT family is still not very clear. SATc1 is a cytosolic isoform located on chromosome 1 (Murillo et al., 1995; Gutierrez-Alcala et al., 2000). Another cytosolic isoform, SATc2, is associated with chromosome 5 and a mitochondrial SATm gene is located on chromosome 3 (Hell et al., 2002). Biochemical analyses of these SATs are still scarce as this enzyme is very instable (Droux et al., 1998).
3.11.4.3 Regulation

S-starvation can induce the mRNA levels for the chloroplastic isoform of SAT. The level of cysteine in plant compartments controls SAT activity through feedback inhibition (Noji and Saito, 2002). In soybean, regulation of cytosolic SAT in response to Cys levels, has been studied through phosphorylation, involving a large family of calcium-regulated protein kinases (Yoo et al., 1997). Expression of SAT genes is modulated to some extent by nutritional conditions. In *A. thaliana*, short-term S-deficiency increased OAS level but cysteine level remained unchanged so SAT activity did not change much but during a long-term deficiency, cysteine level was strongly affected, thus SAT activity was also de-repressed (Nikifovora et al., 2003). H\textsubscript{2}S has an effect on SAT, since it stabilizes the complex with OASTL and, thus increases the activity (Droux et al., 1998). Another potential signal in the regulation of S assimilation is OAS, which most probably acts as a transcriptional regulator because its addition increased the mRNA levels of cytosolic SAT (Koprivova et al., 2000). During the search for genes undergoing a circadian control of expression (Harmer et al., 2000), it was observed that mRNA levels of SAT started rising at the beginning of light period.

3.11.5 O-acetyl serine(thiol)lyase (EC 4.2.99.8)

3.11.5.1 Biochemical characterization and localization

This is the final step of cysteine biosynthesis where sulphide is incorporated into amino acid skeleton of O-acetyl serine (OAS) to form cysteine and acetate by the action of OAS (thiol) lyase (Hass et al., 2008). It is a β-replacement reaction as OASTL belongs to a large family of enzymes catalyzing the reaction of β-substitution of amino acids (Saito, 2004). This step, i.e. the formation of cysteine, marks the almost exclusive entry of reduced S into organic compounds in plants (Hell, 1997). It exists as a homodimer of 60-70 kDa and is a
pyridoxal-dependent enzyme. The cellular activity of OASTL is 100-300 fold in excess over SAT (Saito, 2000; Hell et al., 2002; Droux, 2003). OASTL seems to be required in all cellular compartments that carry out protein biosynthesis. Like SAT, this enzyme is found associated with not only with plastids but also with cytosol and mitochondria of plant cells (Hell et al., 2002; Droux, 2003). The OASTL bound to SAT is inactive in the synthesis of Cys, but triggers SAT stabilization. Free OASTL, as an auxiliary enzyme, consumes OAS in the presence of S\textsuperscript{2-} to achieve full capacity for cys synthesis (Droux et al., 1998, 2001).

3.11.5.2 Genomic organization

This enzyme is encoded by multigene family with up to 10 members (Howarth et al, 1997). OAS-TL cDNA clones have been isolated from A. thaliana (Hell et al., 1994), spinach (Rolland et al., 1993; Saito et al., 1994), wheat (Youssefian et al., 1993), bell pepper (Römer et al., 1992), and Chinese chive (Urano et al., 2000). The binding site of PLP cofactor was determined by site-directed mutagenesis of conserved lysine residues (Saito et al., 1993) and by identification of the cofactor-binding partial peptide fragment (Rolland et al., 1996). It was first cloned by Romer et al. (1992) and Saito et al. (1992), but meanwhile cDNAs encoding OASTL have been isolated from spinach (Rolland et al., 1996) and watermelon (Noji et al, 1994).

3.11.5.3 Regulation

S-starvation induced the mRNA levels for the cytosolic isoform of OASTL (Barroso et al, 1995). Contrary to the above study, Warrilow and Hawkesford (1998) observed reduction and Takahashi and Saito (1996) observed no changes in the activity of OASTL by S deficiency. The activity of this enzyme is strongly affected by the nutritional status of N. An upregulation of mitochondrial
OASTL in spinach has been reported in response to N-deprivation (Takahashi and Saito, 1996).

### 3.11.6 Cysteine synthase

SAT occurs in tight association with OASTL to form a high molecular weight multienzyme complex, designated as cysteine synthase (CS). This complex is composed of a homotetrameric SAT and two dimers of OASTL (Wirtz et al., 2001; Hell et al., 2002). OASTL, upon binding to SAT, becomes silent in activity and behaves like a ‘chaperone-like’ subunit. CS could be defined as a sensor whose activity is balanced between $S^2-$ availability and cysteine consumption. Results of *in vitro* experiments showed that the stability of CS complex is controlled by the level of metabolites. The product of SAT i.e. OAS, triggers the dissociation of the complex in the absence of $S^2-$. By contrast to this situation, $S^2-$ counteracts the dissociation (Berkowitz et al., 2002). Studies by Droux (2003) have shown that cysteine level also controls the dissociation of this complex. CS complex detached in the presence of physiological cellular concentration of cysteine. The function of this complex is highly dependent on the reduced S input in the system, accumulation of OAS and cysteine consumption (Droux, 2003).

### 3.12 INTERACTION OF NITROGEN AND SULPHUR IN PLANTS

Several studies have documented that assimilatory pathways of N and S are well coordinated in such a way that deficiency of any one seriously affects the metabolism of the other (Reuveny et al., 1980; Kopriva and Rennenberg, 2004; Kopriva and Koprivova, 2005). S deficiency causes variations in the concentration of N in plant tissue. Accumulation of NO$_3$-N in the leaves of S-deprived plants is a strong indication of disruption in N-metabolism during S deficiency. According to Hesse et al. (2004), there is accumulation of organic
and inorganic nitrogenous compounds under S-deficient conditions. Amino-N concentration increased considerably in sugar beet, with very low S supply (Hoffmann et al., 2004). Plants under such starvation accumulate arginine and asparagine with reduced levels of cysteine and methionine (Nikiforova et al., 2005). Thomas et al. (2000), while working on sugar beet, observed marked changes in free amino acids in response to S-deficiency. In shoot tissue, arginine increased from 2% to more than 36% of the total amino acids, while in roots glutamine was the major amino acid accounting for 50% of the total pool. N-rich amino acids like asparagine and glutamine are increased in amount, possibly an approach by the plant tissue to buffer the excess of reduced N (Nikiforova et al., 2003). NR activity declined with a decline in NR transcripts and this decline was considered as a negative regulation of nitrate reduction by accumulated amino acids. The accumulation of free amino acids could result from protein breakdown or from de novo synthesis. To decide between the two alternatives, Amancio et al. (1997) incorporated $^{15}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ in maize cells and observed that when cells were S-limited, there was a large accumulation of amide-N. Karmoker et al. (1991), in barley plant, concluded that accumulation of free amino acids due to S deficiency not only affects protein synthesis but also contributes to a slower incorporation and/or transport of recently absorbed N through the root amide pool, so that the export of reduced N is diminished. Similarly, Scherer and Lange (1996) postulated that accumulated amino acids could have some feedback repression on N$_2$ fixation of legumes. Ruiz et al. (2005), while working on bean, demonstrated that S deficiency translated as decreased assimilation of NO$_3^-$ by drastically declining the activities of all the key enzymes of N assimilation, namely, NR, NiR, GS and GOGAT. A notable reduction was also observed in the biomass production, which decreased by as much as 70%, as compared with the control.

Seed yield is an important aspect to consider during the production of oilseeds. The interaction of N and S has been shown to affect the yield and
McGrath and Zhao (1996), while working on oilseed rape, observed a 42-267% increase in seed yield in response to the application of 40 kg S/ha with 180 and 230 kg N/ha treatments. In contrast, when no S was applied and N application was increased from 180 to 230 kg N/ha, seed yield decreased. Janzen and Bettany (1984) obtained similar results in oilseed rape where increasing N fertilizer decreased the seed yield under S-deficient conditions. Results of Ahmad et al. (1998) indicated significant favorable effects of N and S, when applied together. Maximum response, in terms of seed yield, biological yield and harvest index; was observed by applying 40 kg S/ha along with 100 Kg N/ha, but the percentage of oil content of seeds was maximal at 60 kg S/ha with the same amount of N fertilizer. They also found a constructive response of N and S interaction on leaf area index, rate of photosynthesis and biomass production. In wheat and canola, highest dry matter yields were achieved where the ratios of available N:S in soil and plant tissue ranged between 5 and 13 (Qian and Schoenau, 2007).

Fertilizer use efficiency is also affected by the interaction of N and S. While working on grasses, Tallec et al. (2008) found an increase in production and S-use-efficiency along the N gradient and similarly S gradient enhanced the N-use-efficiency. N$_2$ fixation appears to be affected by S fertilization in faba bean (Scherer and Lange, 1996) and pea (Scherer and Lange, 1996). Varin et al. (2009) studied functional plasticity in Trifolium repens and reported that fertilizer S addition enhanced N$_2$ fixation, photosynthesis and potential vegetative reproduction. Their findings were consistent with the data obtained for other legumes (DeBoer and Duke, 1982; Zhao et al., 1999). Effect of various N and S supply on glucosinolate concentration in turnip root (Brassica rapa ssp. rapifera L.) was studied by Li et al. (2007).

Protein quality of seeds is regarded as an important character, mainly in plants like soybean and legumes. Higher quantity of proteins is a desirable trait but a greater amount of S-containing amino acids (methionine and cysteine)
enhances the protein quality and hence, the nutritive value of soybean. Thus, quality of the protein is much more important than the quantity. The N:S ratio of the plant is a good indicator of protein quality and thus N and S balance is a very important factor. Although the ratio of total N to total S [(N/S)] varied widely, yet the ratio of organic N to organic S remained rather constant at about 17.5 in legumes and 13.8 in graminaceous plants. N to S ratios have been used widely to diagnose the S status of the plant (Zhao et al., 1997). Thomas et al. (2000) found significant reductions in the yield with N:S ratio in the shoot below 20, whereas Haneklaus et al. (1998) found a lower N:S ratio of 14 as limiting value in field-grown sugar beet. When S assimilation is impaired due to S deficiency, N assimilation continues but the relative ratio of N to S is shifted towards an excess of N. Photosynthesis and growth increase with N supply but it is mainly dependent on leaf N:S ratio. In plants without visible N or S deficiency, a N : S ratio of ~9 is considered to be near optimum for the maximum growth. Under S-deficiency conditions, N application increased the N:S ratio due to N accumulation in the leaf.