Improvement in nitrogen use efficiency: Physiological and molecular approaches

Yash P. Abrol*, Sukumar R. Chatterjee, P. Ananda Kumar and Vanita Jain

Division of Plant Physiology, Nuclear Research Laboratory and National Research Centre for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

Nitrogen deficiency in agricultural systems is a world-wide problem. It is true of the Indian scenario as well. The utilization efficiency of nitrogenous fertilizers under field conditions is poor. This results in loss of a costly input and accentuates the environmental degradation. In this paper, the present status of our knowledge on physiological and molecular approaches to improve nitrogen utilization efficiency at the level of its uptake, assimilation and relationship to photosynthesis, a major factor determining biomass and grain yield, is discussed. The work done in the authors' laboratory for over a decade is summarized. It is hoped that better understanding will help to apply the tools of molecular biology to genetically manipulate the crop plants so as to enhance yields at low inputs of nitrogen.

NITROGEN deficiency in agricultural systems is a world-wide problem[1]. It is true of the Indian scenario as well. Soils of more than two hundred districts have been reported to be poor in nitrogen status[2]. To increase crop productivity per unit area and agricultural production to meet the demand of the ever-increasing population which may touch the 1.3 billion mark by 2025 AD, millions of tonnes of nitrogenous fertilizers are applied to the soil. These are accompanied by other major and minor nutrients depending on the health status of the soils. Their use has increased from 0.7 mt in 1950 to around 13.5 mt in 1996–1997 (ref. 3). Two crops viz. wheat and rice, which form the staple diet of teeming millions, consume 70 per cent of the fertilizers. There is likelihood of change in this pattern of utilization due to diversification of crops, export orientation and emphasis on agro-processing[4]. For wheat and rice, the utilization efficiency of nitrogenous fertilizers under field conditions is around 50 and 25–30 per cent, respectively[5]. This poor efficiency is of great concern for a number of reasons: (i) even if the efficiency of nitrogenous fertilizers remains at the present level, the losses will increase enormously as their consumption is expected to double within the next 25–30 years; (ii) their manufacture involves high-cost technology requiring a whole range of feed stocks, the major one being naphtha, a petroleum product. Its import results in drainage of foreign exchange reserves of the country[6]; (iii) this leads to environmental problems, namely, NO_3^- pollution of the ground water and surface water run off and emission of NO_x (N_2O, NO, NO_2^-) which have positive radiative forcing characteristics. NO_2^- is involved in stratospheric ozone depletion as well[7]. Their excessive and injudicious use, as reported from a number of regions, further accentuates the environmental degradation of these nitrogenous fertilizers besides affecting the quality of crops, human and animal health, and causes lodging in cereals which may affect crop yields and quality[3,8]. In surface water, presence of high N results in growth of algae and plants, thus accelerating eutrophication, and consequently affect water quality and usage. Incidence of stomach cancer in humans, particularly in infants, and of non-Hodgkin's lymphoma due to intake of water contaminated with nitrate have been reported[9,10]. Nitrosamines produced from nitrite are reported to be carcinogenic; and (iv) NH_3 gas is a pollutant because of its corrosive nature and through the formation of ammonium salts[8].

A number of management strategies to improve the efficiency of utilization of nitrogenous fertilizers include application of different types of fertilizers, their mode of application, avoiding runoff, mitigation of losses from soil and plants, use of slow-release fertilizers, nitrification inhibitors; use of organic manures, green manuring; use of legumes in cropping systems; correction in their imbalanced use and integrated nutrient management[3,5,11].

During the recent past, investigations at the physiological and molecular levels, which relate to acquisition and utilization of nitrogen, were conducted. These are related to understanding of the regulation of uptake and the assimilatory processes, redistribution within the cell and balance between storage and current use at the cellular and whole plant level. It is expected that an understanding of these processes will make it possible to apply the tools of molecular biology to genetically manipulate the plant to improve its nitrogen use efficiency (NUE) and thus contribute to precision management[12].

*For correspondence.

In our laboratory, detailed investigations have been conducted over the last two decades on the above-referred aspects. These studies have been primarily confined to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Salient features of the findings along with the present status of our knowledge and molecular approaches to possibly improve nitrogen use efficiency are discussed.

The predominant form of nitrogenous fertilizer applied to the soil is urea[5]. Under the semi-aerobic conditions prevailing in the field, it is converted to nitrate. The nitrate is taken up by the plant and assimilated by a well-documented assimilation pathway which involves the enzymes nitrate reductase and nitrite reductase. The end product, NH_4^+ is incorporated into amino acids via the GS/GOGAT pathway[13] (Figure 1). On senescence, the proteins are degraded and mobilized to the sequentially formed foliage and finally to the grains where the storage proteins serve as a source for human consumption[11,14].

Nitrate uptake

The first step involved in acquisition of nitrogen is the uptake system which is mediated by transporters located on the plasma membrane of the epidermal and cortical cells[15–17]. Two such transport systems; low affinity transport systems (LATS), and high affinity transport systems (HATS) have been postulated on the basis of kinetic studies. Initial studies using fungi and algae have shown that low affinity transporter is constitutive in nature, and possibly has a signalling role rather than a nutritional one. It admits enough nitrate into the cell to induce the expression of transporter and assimilatory genes, and presumably plays a physiological role in the nitrate uptake only above a certain threshold. Both the HATS – constitutive HATS and inducible HATS – become active when the concentration of nitrate in the soil/medium is low, i.e. below 1 mM. Both these high affinity uptake systems are upregulated in response to nitrate. Constitutive HATS provide a high affinity, low capacity pathway for nitrate entry in uninduced plants[17] but their activity become three-fold on exposure to nitrate. Inducible HATS have been extensively studied and are known to be induced by nitrate or nitrite. In all the above-mentioned systems, each ion of nitrate is co-transported with two or more protons. A low affinity nitrate carrier has been identified in *Arabidopsis thaliana*[18,19] and rice[20]. Recent kinetic studies indicate the presence of low affinity constitutive nitrate transporter in wheat, rye and triticale[21] and it has been suggested that in maize roots, two plasma membrane polypeptides of 61 and 39 kDa may be the most likely candidates for a role in the transport system[22].

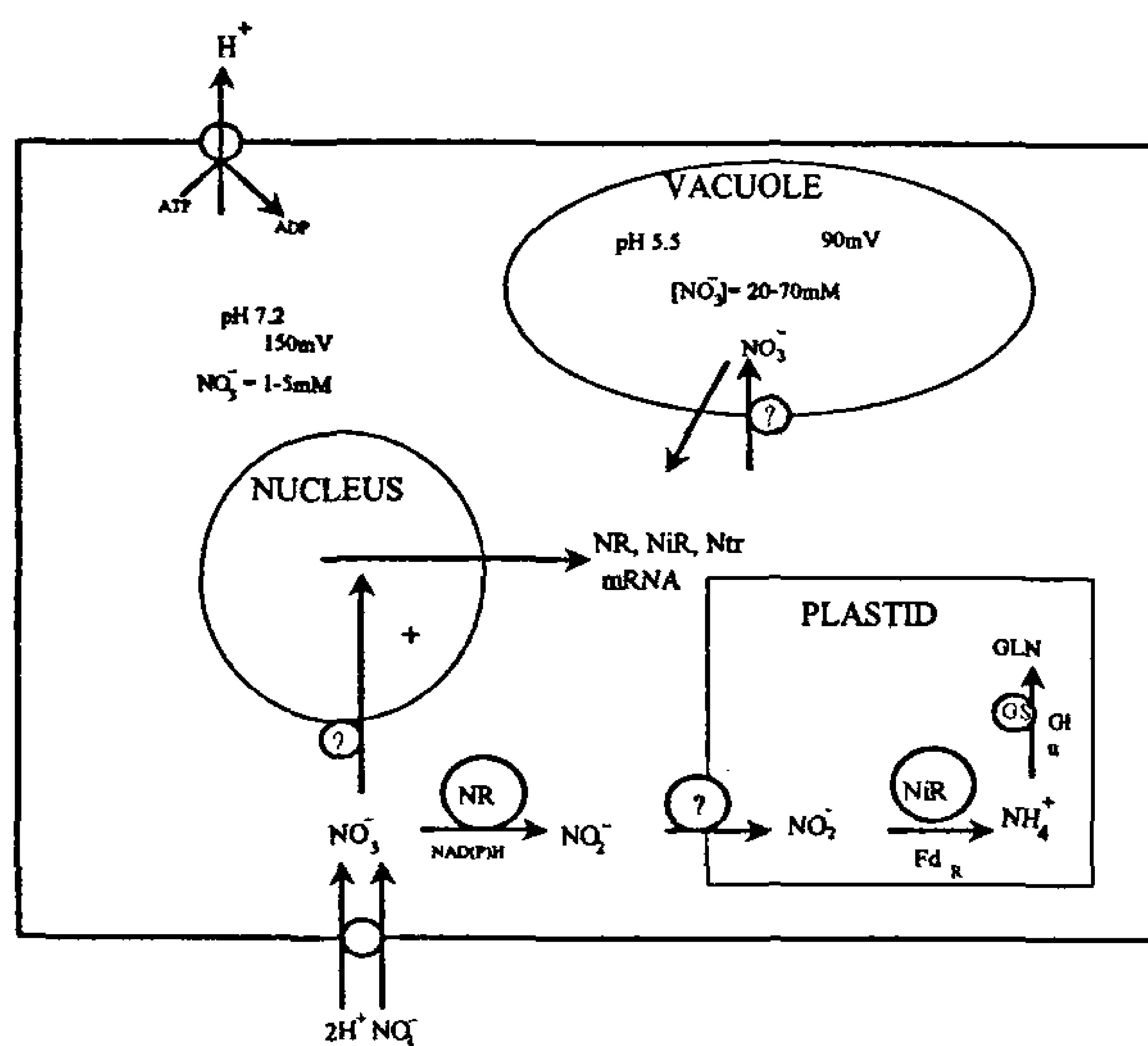

Figure 1. Schematic diagram of nitrate assimilation pathway. The H^+-ATPase in the plasma membrane pumps protons out of the cell, producing pH and electrical ($\Delta\Psi$) gradients. The nitrate transporters (Ntr) cotransports two or more protons per nitrate into the cell. Nitrate can be transported across the tonoplast and stored in the vacuole. Nitrate in the cytosol is reduced to nitrite, which enters the plastid and is reduced to ammonia. Ammonia is fixed into glutamate (Glu) to produce glutamine (GLN) by the action of glutamine synthetase (GS). Nitrate also acts as a signal to increase the expression of nitrate reductase (NR), nitrite reductase (NiR) and *Ntr* genes (Crawford[13]).

The genes coding for LATS and HATS have been identified in fungi, algae and more recently, in higher plants[16]. The genes in higher plants appear to be root specific. Two different but homologus genes were isolated from a root hair-specific tomato cDNA library. Of these, while *Ntr1:1Le* is expressed in root hairs as well as other root tissues under all nitrogen treatments and may correspond to a constitutively expressed LATS, *Ntr1:2Le* mRNA accumulation is restricted to root hairs that had been exposed to nitrate. Recently, a full length cDNA, *Ntr2:1Np*, was isolated from a dicot, *Nicotiana plumbaginifolia*[16]. The expression of this gene is root specific, nitrate inducible, and is negatively regulated by nitrogen metabolites. The physiological and biochemical characterization of plants, transgenic or otherwise, that specifically overexpress or underexpress these genes will be of great interest.

Nitrogenous fertilizers (along with other nutrients), as per the management package, are invariably applied at early growth stages of the crop. Accordingly, the nitrogen concentration in the soil at this stage is 100 mM which declines subsequently because of its utilization and losses due to leaching and other processes[23,24]. The concentration may become as low as 10 μM during flag laminae emergence, flowering, and grain development. Thus, it is likely that while both the low and high affinity transporters are functional during the early stages of growth, the high affinity systems are operative at later stages too, when the soil N concentration is low. These

systems offer great potential for future detailed studies on the mechanisms of transport function, particularly when combined with specific site-directed mutagenesis.

It seems, however, that excessive influxes are usually accompanied by rising effluxes. Taking analogy from the studies with Pi and SO_4^{2-}, where an excessive uptake of ions across the plasma membrane may on its own do little to improve the nutrition, the improvement would have to be co-ordinated at the very least with corresponding increase in influxes across the tonoplast where ions can be safely stored. The storage of vacuolar nitrate however seems to be for a short period. For example, it has been shown in barley and tomato that stocks of nitrate in vacuoles of both roots and leaves were virtually eliminated in 48 h, while growth continued for ten days by remobilization of protein N (ref. 25).

A few approaches to improve NUE, however, may be worthy of investigation. (i) It is likely that over-expression of high affinity transporter operative at later stages of growth, when soil nitrogen concentrations are low and the potential nitrate assimilatory activity is not fully utilized due to lack of availability of nitrate (see later section), will be beneficial to the plant. Nitrogen acquired at this stage seems to help in improving the grain protein content and the number of fully developed grains[23]. (ii) Constitutive expression of nitrate transporter in the root cells of transgenic plants will circumvent the problem of repression and may result in continuous uptake (see Figure 2). Expression of low affinity transporter gene in *Arabidopsis* and rice resulted in enhanced nitrate uptake[18,20]. Such an enhancement can be coupled to an increase in xylem loading of nitrate by constitutively expressing another gene involved in nitrate translocation[26]. (iii) A considerable amount of variation in C_{min} (lowest concentration at which roots can extract ions from the soil solution) at species level has been reported. Screening of cultivars for low C_{min} may be helpful in identifying the ones which have higher influx of nitrate as compared to efflux[27].

At the whole plant level, the rate of nitrate uptake matches the growth of the plant. Its demand increases when there is an internal deficiency. It seems to be regulated by specific amino acids[28]. When plants are adequately supplied with nitrogen, transport processes are down-regulated so that only a small fraction of the potential for transport is expressed. Over-expression of the transporter genes for increased nitrate transport may be feasible if (i) the feedback inhibition of the transport system by amino acids is relaxed, and (ii) adequate storage of nitrate in organs like the shoot were possible. It appears doubtful whether over-expression for increased transport will be a feasible approach. However, manipulating storage capacity of organs may be possible. In wheat and barley, nitrate, whose concentration is high in the soil, is taken up by the plant and stored in the internodes and assimilated subsequently by the upper laminae which show sub-optimal activity (Table 1) (ref. 29). Similar observations have been reported in maize[30]. In spinach, it was shown by Steingrover *et al.*[31] that the petioles contain 3–4-fold higher concentration of nitrate than the laminae.

Nitrate assimilation

A series of investigations are being conducted to understand the structure, function and regulation of the enzymes involved in nitrate assimilation. Particular emphasis is on the enzyme nitrate reductase (NR), which is the key enzyme involved in nitrate assimilation showing low affinity for nitrate, is succeptible to stress, and is unstable under *in vitro* conditions[32]. The studies relate to regulation at transcriptional and post-transcriptional levels, interaction with environmental factors e.g. light, substrate availability, moisture stress, carbohydrate supply, phytohormones, phytochrome and covalent modifications[13,14,23,33–40]. Studies to elucidate the molecular structure of the enzyme have led to the identification of gene products that regulate its expression. Three different isoforms of NR: inducible NADH:NR; constitutive NADH:NR and NAD(P)H: NR have been purified from different plant sources. The predominant form, inducible NADH:NR in higher plants, is mostly present as a homodimer with molecular weight ranging from 200 to 230 kDa and sub-unit molecular weight ranging from 110 to 115 kDa. The three prosthetic groups, namely, FAD, cytochrome b_{557} and molybdenum cofactor (MoCo) present in 1:1:1 stoichiomètry are 23, 10, and 90 kDa, respectively. Three functional domains of NR are joined to each other by the hinge regions. On these functional domains are also the active sites where the enzyme's partial and main reactions take place. Cloning and sequencing of higher plant NRs show that amino acid sequences present at the active site are well-conserved. Two cysteine residues corresponding to cys-191 and cys-245 in *Arabidopsis* are found conserved in higher plant NRs studied so far. The residues are proposed to be involved in forming an interchain disulphide bridge during sub-unit association. Histidine residues act as ligands for binding of heme prosthetic group of higher plant NRs. A lys-731 and a

Table 1. Total nitrate content (µmol/plant part) in stem, sheath and laminae after ear emergence in barley (*Hordeum vulgare* L.) cv. Jyoti S

Days after sowing	Stem	Sheath	Laminae
98	23.1 ± 0.44	8.5 ± 0.21	5.4 ± 0.40
105	25.4 ± 0.19	9.2 ± 0.23	3.9 ± 0.21
111	20.5 ± 0.32	6.4 ± 0.17	3.6 ± 0.11

From: Chatterjee *et al.*[29].

cys-889 are found to be conserved in all higher plant NRs. The amino acids may be essential for NADH binding. However, six amino acids, gly-pro-pro-pro-met-ile, upstream of cys-889 determine the relative affinity for either NADH or NADPH. In birch NR, two proline residues are replaced by alanine and serine which perhaps allows it to bind either NADH or NADPH equally well[41]. Arginine residues are also implicated in binding of NADH (ref. 42). On the other hand, histidine residues may be involved in electron transfer from the reduced FAD to the heme-iron protein of the cytochrome domain of NR (ref. 43). Furthermore, ser-543 present at hinge 1 region, preceding the MoCo domain appears to be involved in phosphorylation–dephosphorylation[44]. Site-directed mutation of ser-543 to asp in *Arabidopsis* NR prevents phosphorylation, modification, and inactivation of NR (refs 38, 45). In brief, once various roles of the different amino acids in the NR molecule have been elucidated, site-directed mutagenesis could be used to modify the activity of the enzyme at will.

Nia gene, which encodes NR in higher plants, is conserved among plants and is present in multiple copies per haploid genome. The stimulatory effect of nitrate on NR gene transcription is mediated by a constitutively expressed nitrate-sensor protein. This protein either binds to nitrate-responsive promoter of the *nia* gene in higher plants or activates *nit*-2-like transacting DNA-binding protein in the presence of nitrate and allows enhanced transcription of NR mRNA. The exact mechanism is worth investigation. Light induces *nia* gene transcription by some still unknown mechanism. Light induction of *nia* mRNA is also mediated via phytochrome. Over-expression of NR is found in transgenic tobacco plants transformed with oat *phyA* gene[38].

In one of the earlier hypotheses put forward by Hageman and his group[32], a relationship between activity of the enzyme, NR, and grain yield and various other characteristics was suggested. It was postulated that since the enzyme was rate limiting, any increase in its content/activity will be beneficial and thus improve nitrogen utilization. Recent molecular studies with mutants and transgenics of *Arabidopsis* and *Nicotiana*, however, tell a different story[35]. Transgenic plants expressing NR activity at a level five- to six-times of the wild type obtained by transformation of the E23 *nia* mutant with tobacco *nia* 2 cDNA under the control of the 35S promoter, showed no differences in chlorophyll, proteins or amino acid accumulation. Plants do not seem to benefit from excess NR activity. It needs to be investigated if *in vitro* NR activity reflects the *in vivo* situation[35,46]. *A. thaliana* mutants affected in *nia* 2 gene, and barley mutants which express only 10 per cent of the wild type NR activity, did not show any decline in the nitrogen content and biomass under greenhouse growth conditions[35]. Interestingly enough, it was observed that higher NR activity in *Nicotiana* resulted in lower NO_3^- content compared to the wild ones[47]. Similar relationship between the NR activity and nitrate content, which have been reported to show two- to three-fold variations in wheat cultivars was observed. There was 30 to 56 per cent reduction in nitrate content of high NR cultivars as compared to low NR ones[48] (Table 2). The findings have a bearing on the improvement in the nutritional quality of the leafy vegetables, particularly the ones grown under low light conditions. It may be mentioned here that over-expression of the enzymes of ammonia assimilation (GS and GOGAT) in transgenic tobacco resulted in an enhancement in total protein content under nitrogen deprivation. The plants also had higher carbon dioxide assimilation capacity[49].

An interesting approach, which has implications to improve the NR enzyme potential and thus possibly improve NUE, was postulated. According to this, the reconstitution of chloroplasts of higher plants with cyanobacterial type NR would make the organelle self-containing and independent in terms of N metabolism. Cyanobacterial-NR derives its energy from reduced ferredoxin through photosynthetic electron transport (Figure 2). The enzyme is small (80 kDa) and the gene

Table 2. Nitrate concentration (µmol g^{-1} dry wt) in the laminae at various growth stages in high (cv. Shera) and low (cv. Pusa Lerma) nitrate reductase (NR) activity wheat cv

	Days after sowing		
	16	23	30
High NR	267.7	151.5	159.7
Low NR	380.3	218.2	245.8

From: Ramraj *et al.*[48].

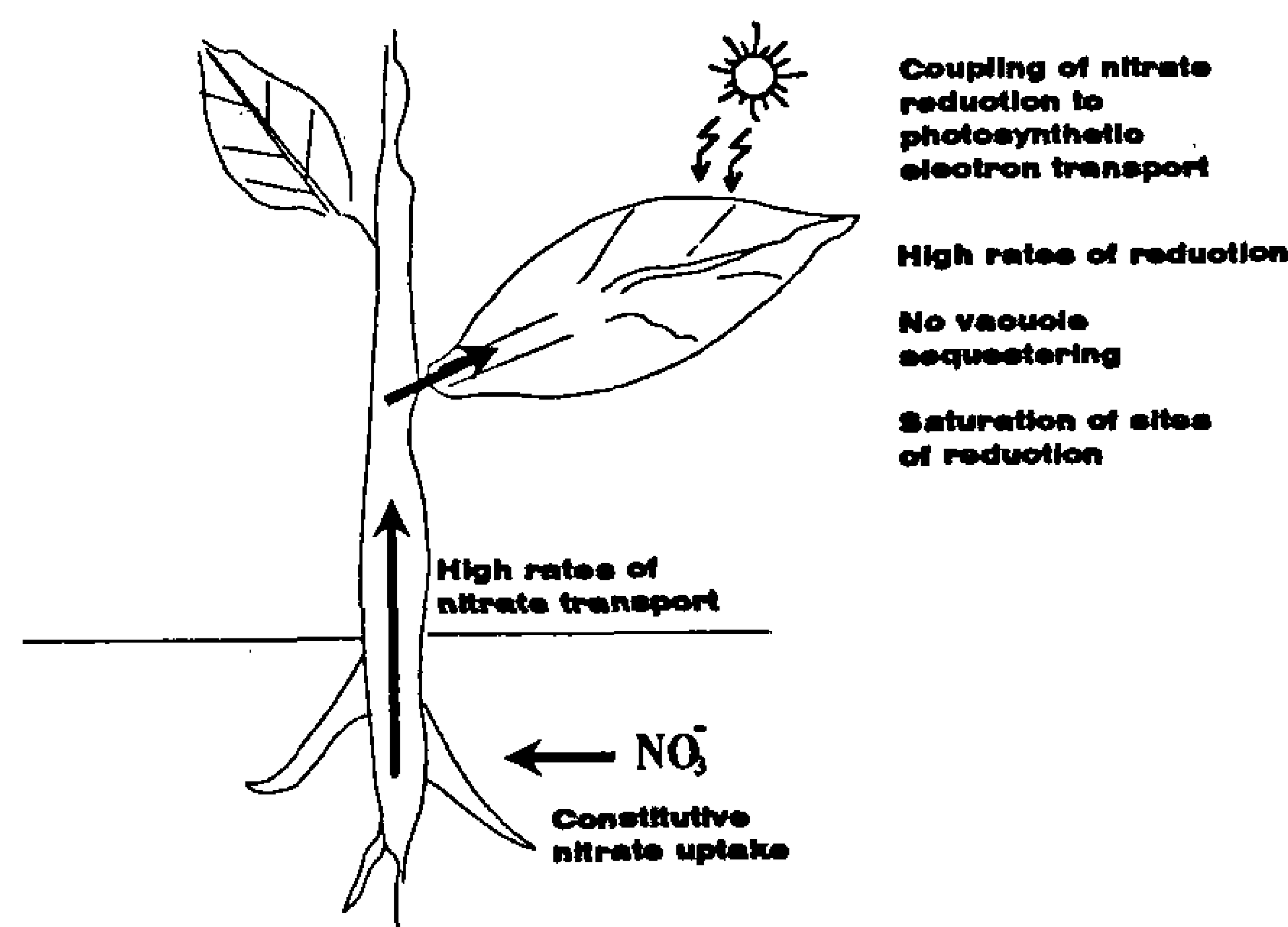

Figure 2. Diagrammatic representation of the possible approaches to improve nitrate uptake and its utilization by the plants.

encoding for it was isolated and characterized from *Synechococcus*[50]. There are two possible approaches to introduce *Synechococcus* NR in higher plant chloroplasts: (i) Engineer the chloroplast genome by inserting the NR gene into it and, (ii) Synthesize the enzyme in cytosol and transport it to the organelle with the aid of transit peptide sequences. Nucleotide sequence encoding pea Rubisco small subunit transit peptide was fused translationally to the *Synechococcus* NR gene[51]. The chimeric gene was used to synthesize fusion protein *in vitro* using rabbit reticulocyte system. The translation product with the transit peptide was translocated into the isolated pea chloroplasts and kinetics of transport were studied. The fusion protein was processed completely and NR protein was found to be localized in the stromal compartment of the chloroplast. The chimeric gene was cloned in a binary *Agrobacterium* vector carrying a CaMV 35S promoter to express the foreign gene constitutively. Transgenic tobacco plants have been developed and the analysis of cyanobacterial-NR expression is in progress in our laboratory.

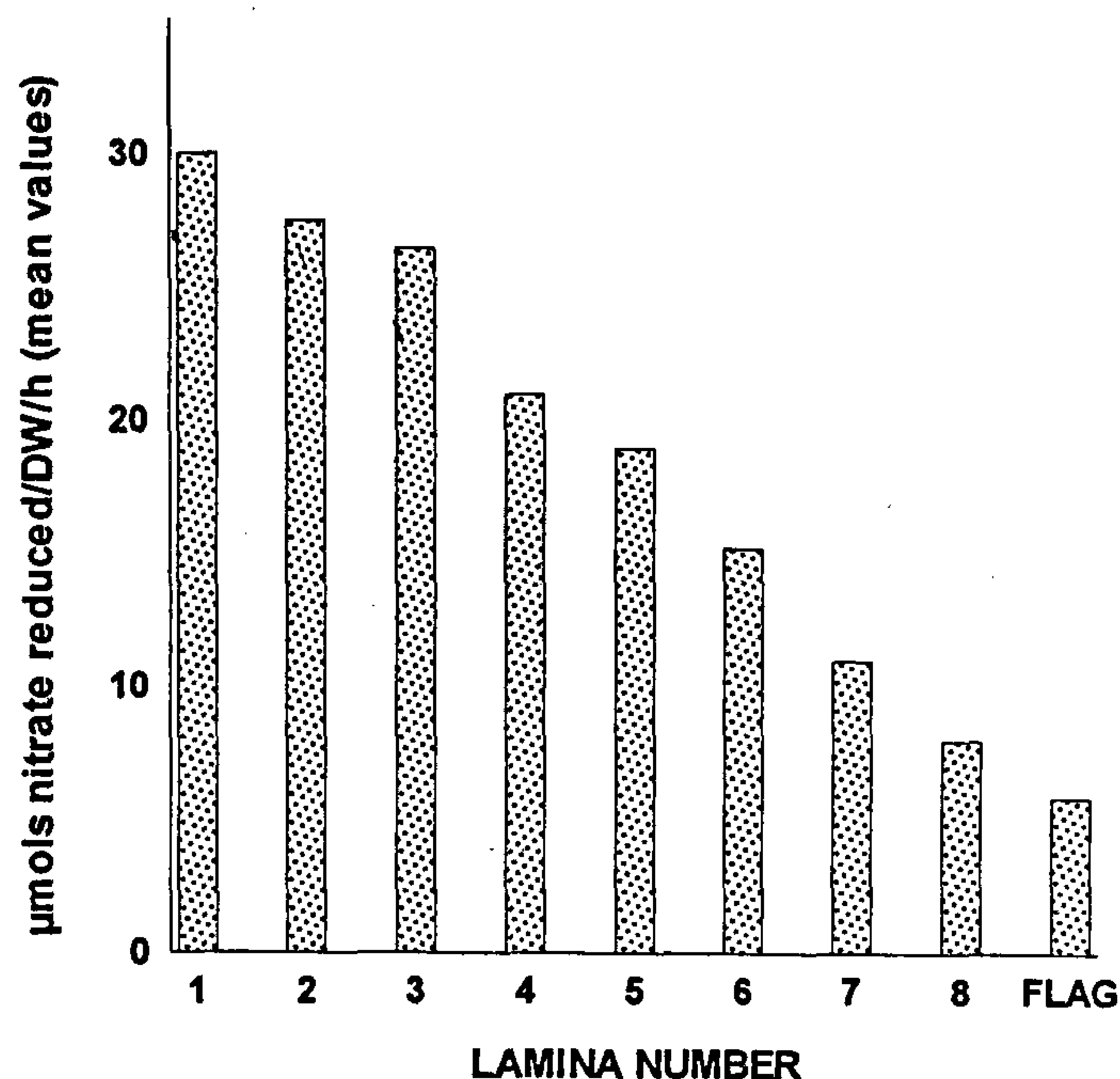

Figure 3. Mean *in vivo* NR activity of the laminae on the main shoot. Each value is a mean of 3 values at full expansion (Abrol[23]).

Field studies

To identify the genotypes and management strategies by which efficient use of nitrogenous fertilizer can be made, a series of field-based experiments with the various cultivars were conducted. These involved a complete analysis of the nitrogen uptake, assimilation of nitrate, storage and, mobilization of nitrogen throughout growth and development[52–58]. A brief summary of major findings is given below.

Pattern of nitrate assimilation

Following the prevalent management practices, nitrate assimilation is high in the first-formed laminae and declines in the subsequently formed ones (Figure 3). This suggests either a lack of availability of substrate at later stages of growth[52,57] (refer to earlier section) or reduction in the capacity to take up and/or assimilate nitrate. Measurements of soil nitrate concentrations throughout the growth period and feeding nitrate to the excised laminae from field-grown plants showed that it was the lack of availability of substrate viz. nitrate at the later stages[55]. At early stages of growth, however, the laminae are saturated and one can say that the nitrate assimilatory pathway, as per the present management practices, is rate limiting. In fact, it was observed that at the optimal level, as determined by agronomic experiments, there is reduction in NR activity compared to the activity at sub-optimal levels. This may possibly be related to toxicity[23,56].

Nitrate assimilation by different plant parts

In wheat and barley, while the laminae contribute approximately 65 to 70 per cent of the total nitrate assimilated, roots, stem (leaf sheaths + internodes), and reproductive parts reduce the rest of the amount equally[52]. Maximum nitrate reduction takes place during the flag laminae emergence. During the post-flowering phase, ear components make significant contribution[29] (Figure 4). Amongst the laminae, the upper ones, despite their low NR activity, reduce major amount of the nitrate taken up by the plant. This supports the observation mentioned earlier that nitrate acquired at earlier stages is stored in the internodes (see section Nitrate uptake).

Differential response of the various cultivars

Response of the wheat cultivars which differed two- to three-fold in NR activity, revealed that (i) there was more than 100 per cent increase in activity in upper laminae of the high NR cv. when they were placed in Hoagland's solution containing nitrate. The magnitude of increase was much less in the upper laminae of low NR cultivars, (ii) major amount of the nitrate was reduced by the upper laminae, which is because of their larger size as compared to the lower ones, and (iii) at optimal doses of nitrogenous fertilizer application, as recommended by the agronomic studies, high as well as

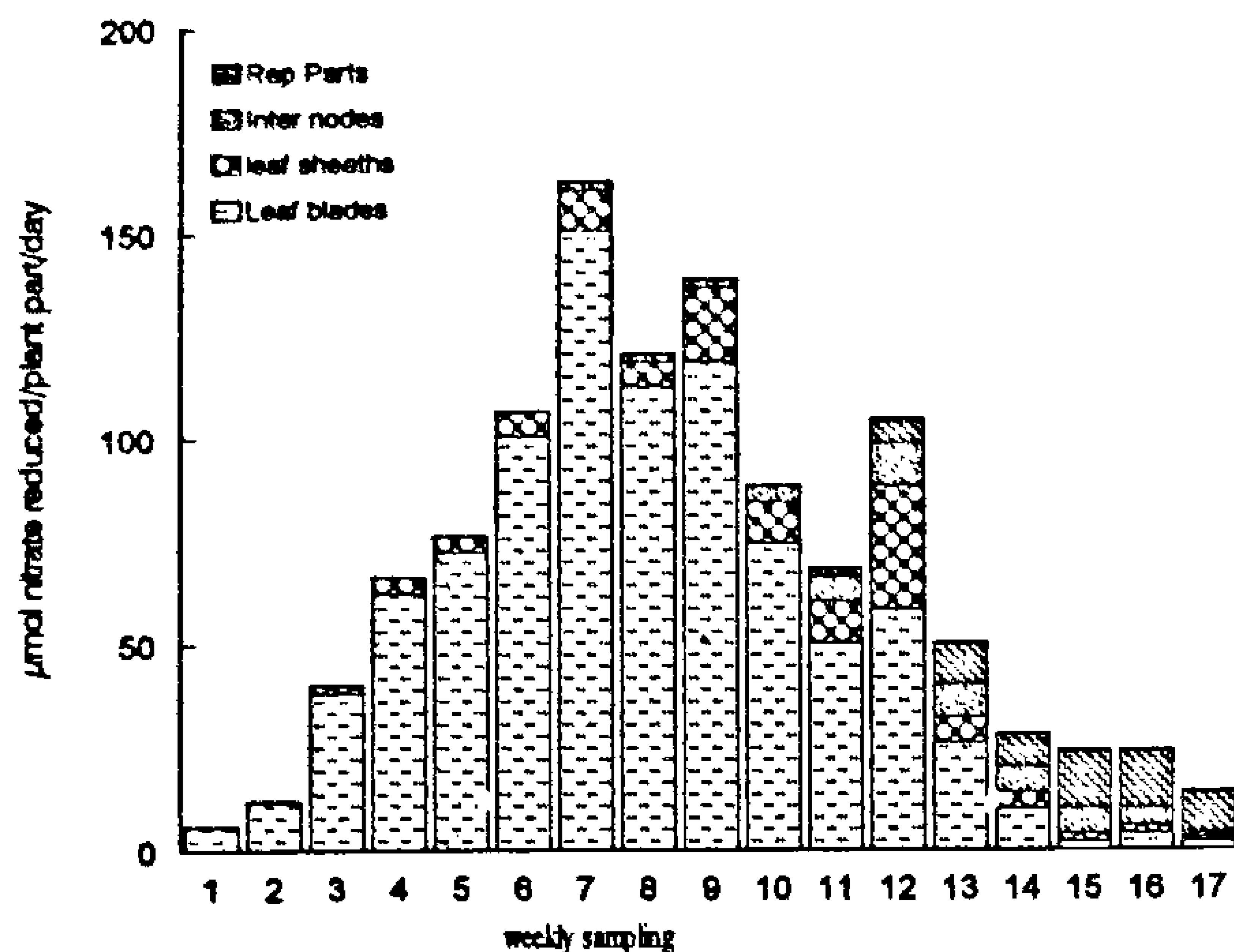

Figure 4. Nitrate reductase activity (*in vivo*) in individual plant parts expressed as µmol nitrate reduced plant part^{-1} day^{-1} (Chatterjee *et al.*[24]).

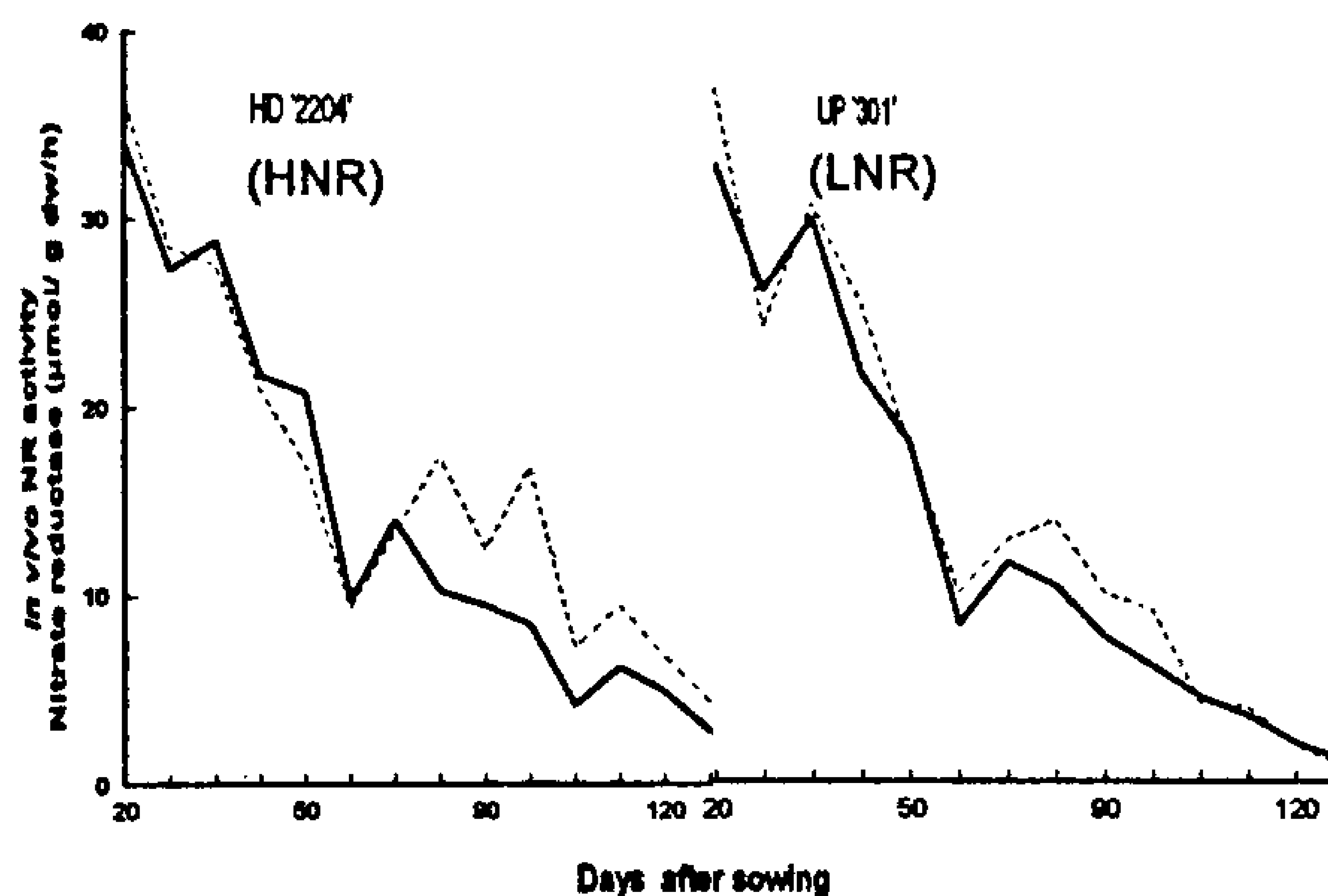

Figure 5. *In vivo* nitrate reductase activity (µmol g^{-1}dw h^{-1}) in the fully expanded laminae (pooled) at different intervals during the ontogeny of the field grown wheat [High (HD 2204) and low NR (UP 301) cv.] The nitrogen was applied in two (solid lines) splits, i.e. 60 kg N each at 0 and 28 days after sowing, and four (dotted lines) splits, viz. 30 kg N each at 0, 28, 54 and 76 days after sowing (Abrol[23]).

low NR cultivars, reduce much less nitrate compared to their total reduction potential[23,54].

Split application of nitrogenous fertilizer

Application of the same amount of nitrogen in more than two splits under field conditions increases the nitrogen availability at later stages of growth so that the suboptimal activity of the upper laminae can be exploited. It was observed that there was a significant improvement in the nitrate assimilatory activity of the upper laminae[23] (Figure 5) and enhancement in the total N harvest and grain protein content (Table 3). The magnitude of enhancement was higher in the high NR cv. compared to the low NR ones. Application of the additional nitrogen at later stages of growth was also useful, as has been demonstrated by a number of studies. It needs to be mentioned that high NR cv. show better response than the low NR cv. at low soil N levels as well[59].

Table 3. Additional nitrogen accumulated as a result of increasing the splitting frequency of the nitrogen application

Cultivar	mg additional N in Whole plant	Grains	% additional N in grains
Low NR			
Pusa Lerma	32.7	29.7	90.8
UP 301	17.8	15.1	84.8
High NR			
HD 2177	57.4	42.4	73.8
HD 2204	43.8	40.0	91.3

From: Abrol[23].

Nitrogen and photosynthesis

Physiologically speaking, efficiency of nitrogen use is evaluated in terms of development of efficient photosynthetic machinery involving biosynthesis of proteins which mediate the various metabolic steps in the chloroplast and especially the enzyme Rubisco, which is involved in the primary step of carbon dioxide fixation. The other aspects of NUE are the overall leaf growth, canopy development, light interception and contribution to total photosynthesis. All these eventually determine the biomass.

In this context, few approaches which are being followed and need further investigations are: (i) why much more Rubisco protein is present in the laminae than is actually needed for fixing carbon dioxide? The studies have shown that at ambient irradiance and with good supply of nitrogen, there is excess investment in Rubisco. Lawlor *et al.*[60] observed that there is a linear relationship between the photosynthetic rates and Rubisco amount up to a certain level. Thereafter, the relationship does not hold good. This has been confirmed by lowering the Rubisco content in tobacco plants expressing Rubisco genes in anti-sense orientation. As much as 43 per cent of the wild-type Rubisco could be lost without any negative effect on photosynthesis. It was calculated that there is about 15 per cent more investment in Rubisco in wild type, which is absent in transformed tobacco, resulting thereby in corresponding gain in nitrogen use efficiency[61]. In this context, some of the observations of interest are that at elevated carbon dioxide levels there is sparing effect on Rubisco content[62]. It is

postulated that in future, under carbon dioxide-enriched scenario, the NR overexpressors may perform much better than the wild types[63], especially under N-limiting conditions, (ii) how to enhance the storage of N in Rubisco and in other vegetative storage proteins at early stages, when soil N is high? This would decrease NUE in the shorter term, but there could be long-term benefits. These storage proteins can be mobilized when deficiency occurs at later stages. Our study with sequentially developed foliage shows that maximum Rubisco content is accumulated in the upper laminae[64]. Is Rubisco already serving as a storage protein by being subsequently mobilized to the grains? and (iii) why various wheat cvs differ in nitrogen responsiveness to photosynthesis and laminae growth[64–66]. An understanding of the mechanism may help in evolving cultivars which are able to maintain the high photosynthetic activity and lamina area at limiting soil-N levels.

This paper has briefly summarized the present status of our knowledge pertaining to nitrogen uptake and its utilization by crop plants. Better understanding at the cellular level and regulation at the whole plant level, coupled with various agronomic management practices specific to agroecological regions may help to improve the utilization of nitrogenous fertilizers, which are one of the major inputs in Indian agriculture.

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