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REVIEW

NITRATE ASSIMILATION—ITS REGULATION AND RELATIONSHIP TO REDUCED NITROGEN IN HIGHER PLANTS

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Key Word Index—Nitrate assimilation; enzyme regulation; nitrate reductase; reduced nitrogen; higher plants.

Abstract—The regulation of nitrate assimilation in higher plants is reviewed in relation to the availability and accumulation of reduced nitrogen. The effects of light on these processes are also considered.

INTRODUCTION

Nitrogen plays a key role in high agricultural production. Amelioration of agricultural soils of the world which are predominantly nitrogen-deficient is achieved by application of nitrogenous fertilizers manufactured by high-cost technology. Their utilization is, however, poor[1]. This necessitates the development of an understanding of factors which regulate their uptake and assimilation by plants.

Nitrogen is mostly taken up as nitrate which is reduced to ammonia before being assimilated into amino acids. Reduction of nitrate to ammonia is catalysed by two enzymes, nitrate reductase (NR) and nitrite reductase (NiR). In green tissues, assimilation of nitrate is intimately linked with photosynthetic reactions not only for the reduction of nitrate to ammonia but also for the generation of carbon compounds which are required for the incorporation of ammonia into amino acids. It has been suggested that the inflow of nitrate-nitrogen into amino acids can be controlled by regulating the activity of the enzyme NR because it is (a) the first enzyme in the pathway, (b) substrate inducible, (c) relatively unstable both *in vivo* and *in vitro* and especially when subjected to water stress or high temperature, and (d) its activity relative to other enzymes in the pathway is low and its K_m for nitrate is high[2].

Recently, a considerable amount of work on various aspects of nitrate assimilation, viz. its regulation by light and photosynthesis, and its relationship to reduced nitrogen accumulation in plants, has appeared. In this article, a critical review of the work on these aspects is presented. For previous reviews on this subject see [2–5].

ROLE OF LIGHT IN THE REGULATION OF NITRATE ASSIMILATION IN GREEN LEAVES

A number of heterotrophic organisms such as fungi assimilate nitrate in the dark making use of reducing power generated by the oxidation of glucose. Similarly, in higher plants, non-photosynthetic tissues such as roots are known to assimilate nitrate in the dark making use of photosynthates translocated from the green tissues. On the basis of these observations, it appears that light does not have any direct role in the assimilation of nitrate. However, it has repeatedly been observed that the process of nitrate assimilation in green tissues is considerably accelerated in the presence of light. It is not the purpose of this review to consider exhaustively the various proposals put forward to explain the role of light since this has been amply dealt with elsewhere [2–5]. However, various proposals are enumerated to serve as a background to more recent work. These are: (a) light stimulates the uptake of nitrate, (b) light promotes the transfer of nitrate from the storage to the metabolic pool in the living cells, (c) light is required for the induced synthesis of nitrate reductase, (d) light activates the pre-existing inactive nitrate reductase, and (e) photosynthetic reactions provide the reductant for nitrate assimilation.

Initially, it was not certain whether green tissues can assimilate nitrate even in the dark. More recently, Aslam *et al.* [6] claimed that the role of light is only to supply photosynthates for the assimilation of nitrate. They concluded that light was not obligatory for the reduction of nitrate and nitrite when sufficient metabolites were present in green tissue. However, this conclusion is contrary to innumerable observations that light is essential for the assimilation of nitrate [3] and that the requirement for light cannot be replaced by sucrose in the dark [7, 8]. Lea and Miflin [9] demonstrated that the enzyme responsible for the assimilation of nitrite, viz. NiR, glutamine synthetase (GS) and glutamine oxoglutarate amino transferase (GOGAT) are all localized in the chloroplasts and are directly dependent on the reducing power generated by light in the form of reduced ferredoxin and ATP. It was, however, not clear whether the first step catalysed by nitrate reductase requires light. This

problem was further complicated by the observation that in the *in vivo* assay for NR in leaf discs, nitrate is readily reduced to nitrite under dark anaerobic conditions. However, free nitrite has rarely been detected in plants kept in the dark under physiological conditions. It was, therefore, argued that either nitrate is not reduced to nitrite under dark aeorbic conditions or the nitrite formed does not accumulate because it is rapidly reduced to ammonia under these conditions. A major breakthrough in understanding this problem was achieved by the experimental approach of Canvin and Atkins [10, 11]. By using [^{15}N]-nitrate, they demonstrated that light is absolutely essential for the reduction of nitrate to nitrite and this ceases abruptly when light is extinguished. The instantaneous stoppage of reduction of nitrate when light is turned off argued against the possibility that light functions only through the supply of photosynthates since in that event, the reduction of nitrate would have continued in the dark until the accumulated photosynthates were depleted. These workers have clearly demonstrated that light is essential for the further assimilation of nitrite as well as of ammonia.

Recently, Mann *et al.* [12] have confirmed that lack of accumulation of nitrite in dark aerobic conditions is not due to its further reduction to ammonia and amino acids. They, therefore, agreed with the important conclusion of Canvin and Atkins [10] that anaerobic conditions are necessary in the *in vivo* NR assay, more in avoiding inhibition of nitrate reduction by oxygen than through elimination of residual nitrite reduction.

In the *in vivo* assay of NR described by Klepper *et al.* [13], leaf discs are subjected to anaerobic or partially anaerobic conditions during incubation in the dark. Oxygen was generally found to be inhibitory with more precise measurements. Sawhney *et al.* [14] observed that supplying an increasing concentration of oxygen to wheat leaves incubated under dark anaerobic condition resulted in increasing inhibition of nitrite production. They noted that with as little as 1% of the concentration of atmospheric oxygen, reduction of nitrate was completely inhibited. The precise mechanism by which oxygen inhibited nitrate reductase was not clear but it appeared that inhibition of mitochondrial respiration might be involved.

ROLE OF DARK MITOCHONDRIAL RESPIRATION IN THE INHIBITION OF *IN VIVO* NITRATE REDUCTION

Sawhney *et al.* [14] observed that inhibitors of the mitochondrial electron transfer chain, such as amytal and rotenone, could stimulate reduction of nitrate to nitrite under partially aerobic conditions. In fact, leaves exposed to carbon monoxide for a brief period when incubated under aerobic conditions accumulated large quantities of nitrite. It is known that carbon monoxide is an inhibitor of mitochondrial respiration at the cytochrome oxidase step. A brief exposure of carbon monoxide-treated leaves to bright light which dissociates the cytochrome oxidase–carbon monoxide complex was enough to stop the reduction of nitrate completely. These results, therefore, showed that if mitochondrial oxidation of NADH is inhibited, nitrate reduction can take place even under aerobic conditions. This suggested that competition for reducing equivalents from NADH between oxygen and nitrate might be a regulatory mechanism. Thus, when mitochondrial oxidation of NADH was inhibited, the reductant was available for nitrate reduction.

Recently, Canvin and Woo [15] concluded that nitrate reduction does not occur in leaves in the dark in air but does occur under anaerobic conditions. Nitrate reduction in dark aerobic conditions was observed when the respiratory electron transfer chain is inhibited with antimycin A. They concluded that NADH oxidized via the second site of oxidative phosphorylation (antimycin A-sensitive site) is used for nitrate reduction. As regards the effect of amytal and rotenone, they observed almost the same extent of stimulation of nitrate reduction as was reported earlier by Sawhney *et al.* [14]. However, in the opinion of Canvin and Woo [15] this is not significant and the NADH oxidized by the first site of oxidative phosphorylation (rotenone sensitive site) does not have an important role in nitrate reduction. Recently, however, Sherrard and Hageman [16] reported that addition of rotenone, antimycin A or dinitrophenol to the *in vivo* NR assay permitted nitrite accumulation under dark aerobic conditions. Dark respiration was inhibited proportionally by both rotenone and dinitrophenol. They concluded that under dark aerobic conditions, mitochondrial respiration and nitrate reduction compete for reducing equivalents. They also suggested that under dark anaerobic conditions mitochondrial organic acid metabolism generates NADH for nitrate reduction.

From the observations described above, it can be concluded that when mitochondrial oxidation of NADH is inhibited, the accumulated NADH becomes available for the reduction of nitrate.

ROLE OF PHOTOSYNTHESIS AND MITOCHONDRIAL RESPIRATION IN NITRATE ASSIMILATION UNDER PHYSIOLOGICAL CONDITIONS

The two observations that light is essential for reduction of nitrate in green leaves [10] and that reduction of nitrate can take place under dark aerobic conditions, provided mitochondrial respiration is inhibited [14, 15], have to be reconciled in order to understand the regulatory mechanism of nitrate reduction during active photosynthesis. If dark mitochondrial respiration is inhibited during photosynthesis then the requirement of light for nitrate reduction can be explained. A number of workers have observed that triose phosphates, synthesized in chloroplasts during carbon dioxide assimilation, are rapidly transported to the cytoplasm, where they generate ATP by the glyceraldehyde - 3 - phosphate dehydrogenase step of glycolysis [17, 18]. This considerably increases the ATP levels and consequently the cytoplasmic adenylate energy charge in the cytoplasm. This in turn inhibits the mitochondrial electron transport chain, as proposed by Atkinson [19].

Thus, plants have two sources of ATP, namely dark mitochondrial respiration which provides ATP when the plants are in the dark and the photophosphorylation reaction which occurs in the light. When sufficient ATP is generated by the photophosphorylation reactions mitochondrial generation of ATP is

not necessary and, in fact, inhibited. A direct demonstration that ATP could stimulate nitrate reduction under partially aerobic conditions was made by Sawhney *et al.* [20]. They showed that by increasing the ATP concentration to 8 mM a significant increase in nitrate accumulation could be observed under aerobic conditions which was *ca* 65% of the maximum accumulation of nitrite under completely anaerobic conditions. These results, therefore, suggested that inhibition of mitochondrial oxidation of NADH by ATP levels could bring about reduction of nitrate under aerobic conditions. A switch off–on mechanism was, therefore, proposed which regulates the reduction of nitrate by light. In light, photophosphorylation increases the cytoplasmic ATP levels which inhibits mitochondrial oxidation of NADH. This NADH then becomes available for nitrate reduction. As soon as light is extinguished, mitochondrial respiration takes over and NADH is not available for nitrate reduction. This mechanism explained the observations of Canvin and Atkins [10] that light is essential for the reduction of nitrate and it ceases abruptly when light is extinguished.

SOURCES FOR REDUCTANT FOR NITRITE AND NITRATE REDUCTION IN LIGHT AND DARK

It was shown by Miflin [21] and Wallsgrove *et al.* [22] that reduction of nitrite to ammonia takes place in the chloroplast and reduced ferredoxin generated in the light is the physiological reductant. Canvin and Atkins [10] have also shown that reduction of nitrite is strictly light-dependent. As regards the further incorporation of ammonia into glutamate, it was shown by Miflin [21] that the enzymes GS and GOGAT are present in the chloroplast and make use of ATP and reduced ferredoxin. However, recent reports by Jones and Sheard [23] and Mann *et al.* [12] have shown that reduction of nitrite can take place even in the dark, although at a much slower rate. These workers observed that nitrite allowed to accumulate in the leaves during dark anaerobic incubation was slowly reduced when the leaves came into contact with oxygen. Jones and Sheard [23] reported that the observed rate of nitrite disappearance in the dark is only 7% of that in light while Voskresenskaya and Grishina [24] found the rate of dark nitrite reduction in broad bean and tobacco leaves was 9 and 33% respectively of the rate in light.

Ramarao *et al.* [25] observed a very rapid disappearance of accumulated nitrite in light while in the dark aerobic treatment there was a time lag of 20 min before the disappearance of nitrite commenced. Mann *et al.* [12] have also shown that the rate of nitrite reduction in the dark is much slower in the first 30 min than the next 30 min. Since nitrate is not reduced under dark aerobic conditions [10, 12] nitrite is not expected to be formed in the leaves under these conditions. Hence the physiological significance of dark aerobic nitrite reduction is difficult to understand. It could be a response to unnatural conditions of accumulation of nitrite as a detoxification mechanism, since nitrite is known to be toxic to plants at high concentrations. It is, therefore, safe to conclude that dark aerobic reduction of nitrite may not be making any significant contribution towards nitrate assimilation as compared to the very rapid reduction in light.

It is generally believed that NR is present in the cytoplasm of leaf cells and makes use of cytoplasmic NADH as the reductant. The source of this NADH is not known with certainty in spite of diverse claims. Klepper *et al.* [13] were the first to suggest that triose phosphates synthesized in the chloroplasts are transported to the cytoplasm where NADH generated by the glycolytic triose phosphate dehydrogenase functions as the reductant for nitrate reduction. This conclusion was mainly based on the observed stimulation of dark anaerobic nitrate reduction in leaf discs infiltrated with these and other glycolytic intermediates. Klepper *et al.* [13] did not observe any stimulation of nitrate reduction by infiltration with citric acid cycle intermediates. This was probably because of the fact that the pH of the infiltration medium used by them was 7.5. It has been shown by Beevers [26] that for successful penetration of organic acids into leaf tissues, a more acidic pH (5.0) is required. When Sawhney *et al.* [20] used this pH, they noticed considerable stimulation of *in vivo* NR activity with phosphoenol pyruvate, pyruvate and other organic acids. They, therefore, concluded that NADH generated beyond the triose phosphate dehydrogenase step of glycolysis is also utilized for nitrate reduction. Nicholas *et al.* [27] and Rathnam [28] have proposed that cytoplasmic malate dehydrogenase is a source of reductant for nitrate reduction. The equilibrium of this reaction, however, favours the formation of malate and hence significant accumulation of NADH in the cytoplasm is unlikely to take place unless oxaloacetate, a product of the reaction, is rapidly removed. It is, therefore, doubtful whether NADH generated in the cytoplasm by this reaction is an important source of reductant.

Sawhney *et al.* [20] and Kadam *et al.* [29] observed that infiltration of leaf discs with malonate significantly inhibited *in vivo* nitrate reduction and this inhibition was reversed by supplying excess fumarate. This suggested that operation of the citric acid cycle is essential to provide the reductant. If glycolytic dehydrogenase was involved as an important source of NADH as proposed by Klepper *et al.* [13] or, alternatively, cytoplasmic malate dehydrogenase as a source of reductant as proposed by Nicholas *et al.* [27] and Rathnam [28], then the observed inhibition by malonate and the reversal of this effect by excess fumarate cannot be explained. These results rather suggest a mitochondrial origin for NADH. However, since NR functions in the cytoplasm, a mechanism must exist for the transport of reductant generated in the mitochondria. It is known that plant mitochondria are capable of transmembrane hydrogen transfer [30]. Thus, external NADH can be available in the cytoplasm either directly or indirectly via the malate/oxaloacetate shuttle. It has been shown that the dehydrogenase of the citric acid cycle in plant mitochondria is capable of reducing external NAD [30].

Although the above results suggest the probable mitochondrial origin of NADH as a source of reductant for cytoplasmic nitrate reduction, it is still not clear which particular reactions are involved. Sawhney *et al.* [31] observed that during nitrate

reduction succinate accumulates in wheat leaf discs since it cannot be metabolized under anaerobic conditions. This showed that the citric acid cycle is blocked at the succinate dehydrogenase step under dark anaerobic conditions. However, under physiological conditions when leaves are exposed to light in air, succinate is likely to be metabolized in a variety of reactions. One mechanism suggested by Sawhney *et al.* [31] was that succinate could be oxidized to fumarate, under conditions of high adenylate charges produced by photophosphorylation, by the reversal of electron transfer from succinate to NAD in the mitochondria. Another mechanism suggested was the utilization of succinate in the presence of glyoxylate for the synthesis of isocitrate by the reversal of isocitrate lyase. Although the equilibrium of this reaction is towards cleavage of isocitrate, the reaction is known to be reversible, when high concentrations of succinate and glyoxylate, a product of photorespiration, are available [32]. It has been suggested that this enzyme is located in the leaf mitochondria and not in the microbodies [33]. While the function of this enzyme in mitochondria is not clear, it is likely that when higher concentrations of succinate and glyoxylate are available, the enzyme may operate in the reverse direction towards the synthesis of isocitrate.

Sainis *et al.* [34] observed that in wheat leaves exposed to light, malonate inhibited $^{14}CO_2$ evolution from carboxyl-labelled succinate. In malonate-inhibited tissues, addition of nitrate substantially stimulated $^{14}CO_2$ release from succinate, probably via the formation of isocitrate by reaction with glyoxylate. Glycolate and glyoxylate enhanced $^{14}CO_2$ evolution from [^{14}C]succinate. Similarly, succinate stimulated decarboxylation of [1-^{14}C]glycolate in the presence of malonate. Since higher plant NR is NADH-specific, succinate is not a direct electron donor for nitrate reduction. When succinate dehydrogenase is partially inhibited by malonate, nitrate can perhaps stimulate the generation of additional NADH via rapid turnover of the citric acid cycle between isocitrate and succinate.

Recently, Ramarao *et al.* [35] unexpectedly observed that succinate itself significantly inhibited dark anaerobic *in vivo* nitrate reduction. This showed that the earlier reported malonate inhibition cannot be explained on the basis of its action on succinate dehydrogenase. They also found that the inhibition of *in vivo* nitrate reduction by succinate, malonate and D-malate, was relieved by formate and pyruvate. They suggested that competitive inhibition of mitochondrial NAD-malic enzyme by malonate, D-malate [36] and succinate [37] may be responsible for the observed inhibition of nitrate reduction. Thus, NAD-malic enzyme of the mitochondria appears to be an important source of NADH for *in vivo* nitrate reduction.

Palmer [38] has described the uniqueness of plant mitochondria in view of the fact that malate dehydrogenase inside the matrix and NAD-malic enzyme located in the inter-membrane space generate oxaloacetate and pyruvate respectively, which makes it possible for the citric acid cycle to function without the need for pyruvate from glycolysis. Kent [39] has confirmed this mechanism and shown that glycolytic reactions are not an important source of carbon for the citric acid cycle in leaves. Coleman and Palmer [36] have demonstrated that in the inter-membrane mitochondrial compartment NAD-malic enzyme generates NADH which is oxidized by the exogenous NADH dehydrogenase system which bypasses the rotenone-sensitive site (site I) of oxidative phosphorylation.

SOURCES OF CARBON FOR THE GENERATION OF CARBON COMPOUNDS FOR AMINO ACID SYNTHESIS DURING ACTIVE PHOTOSYNTHESIS

As explained earlier, mitochondrial oxidation of NADH is inhibited by ATP levels in light [17]. However, according to Chapman and Graham [40], the dehydrogenases of the citric acid cycle do function even in light to supply carbon compounds for amino acid synthesis. Palmer [38] has suggested that a modified citric acid cycle could operate in light, with the help of internally located malic enzyme and malate dehydrogenase to generate pyruvate and oxaloacetate at the expense of malate and citrate known to be present in large amounts in leaves. This would allow the citric acid cycle to function even at high levels of ATP provided the NADH generated is oxidized by alternate pathways such as the malate–oxaloacetate shuttle or cytoplasmic nitrate reductase. Kent [41] has shown that export of triose phosphates from chloroplasts may not at all be a significant source of carbon for the citric acid cycle during photosynthesis, since they are mostly utilized for sucrose synthesis in the cytoplasm. Thus the problem of sources of carbon for the generation of keto acids for amino acid synthesis during active photosynthesis is still unresolved. Kent [39] recently suggested that a malate pool may be the source by generating oxaloacetate and pyruvate, as explained by Palmer [38].

Assimilation of carbon dioxide by PEP carboxylase and subsequent reduction is suggested as a source of malate. Another important source is the chloroplastic NADP-malic enzyme. Both these reactions depend on phosphoenol pyruvate or pyruvate. However, various reports by Kent [39, 41] have shown that pyruvate is not at all a significant source of carbon for the citric acid cycle in light. Naik and Singh [42] suggested that the reaction between succinate and glyoxylate could be a possible source of carbon dioxide during photorespiration due to the formation of isocitrate by the reversal of isocitrate lyase as explained above. Subsequently, carbon dioxide is released during the oxidation of isocitrate in the citric acid cycle and it was suggested that this could be a source of photorespiratory carbon dioxide. It is, therefore, possible that glyoxylate serves as a source of carbon for the citric acid cycle during active photosynthesis via the formation of isocitrate. This pathway could be significant as a substitute for the anaplerotic carboxylation reactions required to replenish the 2-oxo acids which are rapidly consumed for the synthesis of amino acids in photosynthetic tissues.

It was demonstrated that when wheat leaves were incubated in the presence of [^{14}C]glycollate under conditions of photorespiration, viz. high light intensity, high temperature and low ambient carbon diox-

ide concentrations, incorporation of ^{14}C was detected in citric acid cycle intermediates [42].

It is perhaps possible that glyoxylate also enters the citric acid cycle by formation of malate as a result of reaction between glyoxylate and acetyl CoA catalysed by malate synthetase. However, the presence of malate synthetase in leaf mitochondria is not known.

STORAGE AND METABOLIC POOLS OF NITRATE

Ferrari *et al.* [43] suggested that there are two distinct pools of nitrate in plant cells, a large storage pool, not accessible to reduction, and a small metabolic pool which is readily reduced by NR. This concentration was based on the observation that only a small fraction of the total quantity of nitrate present in the leaf cell is reduced to nitrite over a prolonged period of incubation. It was suggested that light stimulates reduction by bringing about transport of nitrate from the storage to the metabolic pool [44]. Ferrari *et al.* [43] also suggested that monohydroxy alcohols such as propanol stimulate nitrite production by leakage of nitrate from the storage pool to the metabolic pool. In their experiments, it was likely that a strictly anaerobic condition was not obtained. It has been estimated that the concentration of dissolved oxygen in solution is about 240 μm [45]. This is about 4 times higher than the concentration of oxygen required for complete inhibition of nitrate reduction [14]. Hence, it is essential to remove traces of dissolved air in order to obtain maximum nitrite production. Subbalakshmi *et al.* [46] showed that under strictly anaerobic conditions a major part of the endogenous nitrate was reduced to nitrite in wheat and rice leaves. They also suggested that a supply of metabolites such as sugars of pyruvate, which generate NADH bring about an almost complete reduction of endogenous nitrate. It, therefore, appears that generation of NADH and prevention of its oxidation by oxygen are the factors that regulate nitrate reduction in leaves. Over a prolonged period of incubation of isolated leaf discs in the dark, the limiting factor for nitrate reduction is likely to be the supply of NADH. Even if two distinct pools of nitrate do exist [47, 48], it appears that the storage pool is also readily available for reduction provided sufficient reductant energy is supplied, which is quite feasible under physiological conditions in actively photosynthesizing plants.

IN VITRO VERSUS *IN VIVO* NITRATE REDUCTASE ASSAY (ACTIVITY) IN RELATION TO REDUCED NITROGEN ACCUMULATION

Since the inflow of nitrate-N into amino acids is controlled by the activity of NR, a quantitative measurement of its activity could provide a quantitative estimate of reduced nitrogen input. This can be done by calculating the daily input of reduced nitrogen and integrating the values obtained over the life of the plant or crop [49], consideration being given to the length of photoperiod since nitrate reduction does not take place in the dark [10]. However, a close correlation between the two has not usually been obtained in practice largely due to the failure of the methods available at present to measure the *in situ* activity. In addition, complications arise in long-term field experiments from the necessity of having to measure the enzyme activity and total mass of the plant throughout growth and development, inability to obtain complete root system, diurnal and day-to-day variation in the activity and continuous changes in the field temperature in contrast to the fixed temperature usually used for the assay.

The activity of NR can be measured both *in vitro* and *in vivo*. The *in vitro* assay is conducted under optimum conditions which are rarely obtained *in situ*. In addition, inhibitors or inactivating enzymes present in the tissue [50–58], which are otherwise spatially separated, are likely to be mixed up during extraction and can cause inhibition of the activity of NR. Therefore, care has to be taken during extraction to keep the enzyme free from such inhibitors. In spite of these drawbacks, the *in vitro* method has been found to estimate several times the actual amount of reduced nitrogen [49, 59–62] probably because the enzyme is assayed under non-limiting conditions of substrate and reductant availability. If the modification in extraction procedure suggested by Schrader *et al.* [53] is incorporated, the estimate of input of reduced nitrogen in the above studies would further increase. It can, therefore, be concluded that the *in vitro* method is far from satisfactory for measurement of the *in situ* activity of the enzyme or reduced nitrogen accumulation, even though a significant correlation is often obtained with the latter [61, 62].

The other method available is the *in vivo* assay. The initial observation of Kumada [63] that embryos could reduce nitrate and release nitrite into the incubation medium in darkness under anaerobic conditions, has been developed into a good method for the *in vivo* assay of NR in plant tissues. The assay is now widely used in various laboratories [13, 20, 64–71] especially because of its two advantages over the *in vitro* assay: (a) it can be used to assay the activity of plant tissues where the extraction of active enzyme is a problem; and (b) it can be used to study the link between nitrate reduction and other concurrent physiological processes operating in the tissue [13]. It is presumed that *in vivo* activity is a function of the enzyme level [68, 70, 73]. The method has been used to measure induction of the enzyme [65, 70, 74, 75]. The *in vivo* assay also varies, as expected, with the nitrate concentration in the soil or nutrient solution used as a growth medium, and also with the age of the individual organ and the whole plant and other environmental factors which are expected to affect the activity of the enzyme.

The *in vivo* assay has been the subject of various studies [60, 77–80]. According to Brunetti and Hageman [60], the *in vivo* assay is subject to several factors which may influence nitrate reduction e.g. membrane permeability, recycling of NAD, substrate and enzyme level. Higher concentrations of nitrate have to be used when the enzyme is assayed in intact tissue compared with the *in vitro* assay [80, 81]. The requirement of a high concentration was attributed to changes in cell permeability rather than to the supply of substrate [77]. Other factors, which control *in vivo* activity include the movement of nitrate to enzyme catalytic sites, the generation and supply of reductant

[13], the movement of nitrate at a rate sufficient to avoid possible feedback inhibition [82] and further metabolism of nitrite and anaerobiosis in the assay system [14, 69]. Nevertheless, present evidence shows that the *in vivo* assay provides a close approximation to the accumulation of reduced nitrogen [62, 83].

Even though many laboratories use the *in vivo* technique, the procedure followed varies [13, 68, 69, 84–86], in substrate concentration, in the concentration of the incubation medium, pH of the incubation medium and concentration and type of chemicals used to increase membrane permeability, thickness of tissue slices, condition of incubation, partially or completely anaerobic conditions and incubation temperature. While most of these factors can vary with the material under study, standardization of technique not only to provide optimal condition to get maximum activity but also to obtain a correspondence with actual accumulation of reduced nitrogen is needed. Outward diffusion of nitrite from the tissue and its release into the incubation medium can vary with the type of the tissue under study while such movement of nitrite may not be a problem with young tissue, older ones, especially if the size of the slice is large, may offer greater resistance and hence care has to be taken to extract the accumulated nitrite in the tissue. A good method to stop the reaction and get nitrite out of the tissue is to heat-kill the tissue in a hot water bath [71].

Nevertheless, it has been possible to obtain a good correlation between the calculated value for *in vivo* activity of the enzyme and actual accumulation of reduced nitrogen in cotton seedlings [87], wheat seedlings [60] and in chickpea [85]. However, under field conditions the *in vivo* assay again overestimated the amount of reduced nitrogen accumulated [62, 72, 88]. Since the magnitude of overestimation is not large, one of the reasons for this may be due to the movement of reduced nitrogen to the roots from the shoot, which is largely dependent upon nitrate assimilation in the shoot.

NR activity values not only help to estimate the total accumulation of nitrogen in the plant as a whole but also help to estimate the role of various organs in nitrate reduction if assays are conducted separately with each organ throughout its growth and development and the total activity is integrated over the life of the organ. The *in vivo* measurement is most useful for this, since a large number of samples can be assayed with ease and because it is less likely to overestimate the actual input of reduced nitrogen. This could not be undertaken earlier by chemical estimation since nitrogen is highly mobile within the plant. An alternative approach to this problem was the removal of the organ to study its role [89, 90] but this may result in a compensatory role being played by other organs. In addition, the role of certain organs such as the stem in nitrate reduction can never be ascertained. A more reliable method is, therefore, to have an estimate of total *in vivo* NR activity. Using this method, Abrol *et al.* [72] estimated that in field-grown wheat plants, 72.4% of the total nitrate reduced by the shoot occurred in the leaf laminae, 21.0% in sheaths and stem, and 6.6% in the ears. 74% of the total assimilation of nitrate occurred by ear emergence. The technique thus also served as the most reliable proof that wheat plants do assimilate nitrate during the reproductive phase. On the basis of the total NR activity of each organ, the amount of nitrate reduced by individual organs was quantized. This showed that the major amount of nitrate is reduced by the upper leaf blades, their percentage contribution increasing with increase in soil application [72, 88, 91, 92] while the contribution of the laminae and to a certain extent leaf sheaths, increased with increasing soil nitrogen level, the share of the internodes and reproductive parts decreased which showed a preferential flow of nitrate towards being reduced in the laminae [88].

NITRATE REDUCTASE ACTIVITY LEVEL AND ITS POTENTIAL

In the sequentially developed foliage of the main shoot of field-grown wheat and barley plants, *in vivo* NR activity is high in first-formed leaf blades and then gradually declines. The upper three to four leaf blades which are metabolically active during the reproductive phase had low NR activity [72, 93, 94]. Similar observations have been made on the basis of analysis of pooled leaf blades sampled at frequent intervals [49, 95, 96]. This ontogenetical pattern of NR activity corresponds to the nitrate content with which it is significantly correlated [96]. This is expected since the concentration of nitrate in the tissue is the primary factor controlling activity of the enzyme [97–99]. The tissue pattern in turn had a parallel in the known pattern of soil nitrate [96]. Placement of sequentially developed leaf blades, at their fully expanded stage, on nitrate revealed that while there was little or no enhancement in the activity of the lower leaf blades, the activity of the upper four or five leaf blades was enhanced considerably [76, 96]. An observation of interest was that the magnitude of enhancement was greater in a 'high' NR as compared to a 'low' NR cultivar [76]. Addition of sucrose to the incubation medium did not increase the activity any further. Considering the increased weight and the time for which the upper leaf blades remained green, compared to the lower ones, these investigations suggest that there is vast unrealized potential for nitrate assimilation at later stages of growth [100]. Development of suitable methodology whereby nitrate is made available during the later stages of growth could result in higher reduced nitrogen accumulation [100, 101].

Based on the above observations, attempts to harvest a higher reduced nitrogen content by suitable modification of management technology whereby nitrate is made available during the later stages of wheat were successful [102]. Application of nitrogen when the upper three or four leaf blades were at the fully expanded stage resulted in an enhancement of the assimilatory activity. Cultivar differences in this response were observed.

NITRATE REDUCTASE ACTIVITY IN RELATION TO GRAIN NITROGEN AND GRAIN YIELD

Crops respond to applied nitrogenous fertilizer markedly in growth, grain yield and grain nitrogen production. In view of the key role played by NR in nitrate assimilation, it is natural to expect it to be related to these characters. However, since crop yield

is the ultimate result of innumerable factors operating at both the genetic and environmental levels, a correlation with any single factor is too much to expect. Establishing a relationship with grain nitrogen is complicated. Besides the difficulties in currently known methods to measure the *in situ* activity of the enzyme (see above), there are genotypic differences in enzyme efficiency in accumulating reduced nitrogen and genotypic variation in nitrogen translocation efficiency [61, 103]. The latter variation is attributed to differences in proteolytic enzymes [104–110].

Nevertheless, a significant relationship between seasonal mean *in vitro* NR activity or its conversion to total activity per day or per season with grain yield and grain protein production was reported in maize and wheat cultivars [49, 111–120]. In some studies, however, the relationship did not hold good among genotypes. Dalling *et al.* [59] reported a relationship between total seasonal *in vitro* activity and total plant nitrogen at maturity and to grain nitrogen only if allowances are made for significant differences between cultivars in efficiency of translocation.

Seasonal mean activity or total seasonal activity is difficult to estimate. In this context, the finding of Deckard *et al.* [111] that the *in vitro* enzyme activity/unit fr. wt or total activity/day estimated during the reproductive phase was more closely related to grain yield and grain nitrogen in maize hybrids is noteworthy in that it reduces the number of samplings needed. *In vitro* NR activity determined at the boot stage of wheat maturity showed a highly significant positive correlation with wheat grain yield and grain protein yield among genotypes at varied nitrogen levels [112]. Abrol and Nair [119, 120] reported that the mean *in vivo* NR activity of the flag leaf and the penultimate leaf, but not of the leaf below, of wheat genotypes under variable nitrogen levels was correlated with percentage grain protein, and protein yield and grain yield. This further simplifies the procedure as *in vivo* NR activity is easy to assay and gives a better estimate of reduced nitrogen [83].

A relationship between enzyme activity and various economically useful harvest products will be more useful to plant breeders if a relationship between the two can be established at the seedling stage. Croy and Hageman [113] compared seedling NR activity (*in vitro*) and the grain nitrogen yield of 32 wheat cultivars under field conditions. Even though no close relationship was found between the two, they were able to select 9 of 13 cultivars which gave the highest grain nitrogen yield on the basis of seedling assay. Deckard *et al.* [114] found a good correlation with a simple *in vitro* assay value obtained 28 days after sowing and grain yield and grain protein yield in wheat. Similarly, Johnson *et al.* [115] obtained a good correlation between seedling *in vitro* activity and grain yield in wheat and barley, Dalling and Loyn [103] with grain protein yield in wheat, Singh *et al.* [116] with grain yield and grain protein yield in triticales, and Blackwood and Hallam [117] between seedling *in vivo* activity and grain yield in wheat.

Under the present nitrogen management practice where nitrogen is applied at and around the time of sowing, the synthesis of the enzyme and/or its activity may be limiting in the assimilation of nitrogen during the early phase of plant development. At this stage, cultivars possessing a higher activity of the enzyme are likely to be advantageous especially since the important yield-determining factors such as the numbers of tillers and number of spikelets are determined quite early in the life of the plant. Thus, the correlation obtained by many workers [103, 112, 114, 115, 117] between NR activity in the seedling and grain yield need not be fortuitous. However, this is not true in the later stages of ontogeny where nitrate supply rather than the enzyme *per se* is the most limiting factor. Assimilation of nitrogen during these later stages may control the grain protein content and realization of the unutilized potential at this stage is most likely to improve the storage protein content in the grains as a large amount of mobilizable nitrogen would then be available. It is, therefore, reasonable to expect a relationship between the nitrogen assimilated by the upper organs, especially laminae, and grain protein content and protein yield. That the nitrate assimilated by the upper leaf blades largely controls the grain nitrogen content has been reported [109, 121] and many workers have obtained a correlation between NR activity at this stage and grain protein content [111, 112]. That the unutilized capacity if realized can increase the protein content is also revealed by many studies with late application of nitrogen [62, 102], while increase in grain yield from such studies is only marginal largely because of the increase in grain size and number/spike. However, high nitrogen at later stages can also decrease the grain size because of the consumption of carbon compounds to meet the energy requirements of higher protein synthesis.

DIFFERENCES IN NITRATE REDUCTASE ACTIVITY IN CULTIVARS

Genotypic differences in NR levels have been reported in maize [2, 61, 111, 122], wheat [4, 49, 61, 103, 114, 119, 123–128] and in barley [129–133]. Genetic studies also revealed that the level of enzyme is highly heritable with the result that a hybrid could be bred with a predictable enzyme level by selecting parents with known NR activity [133–136]. Genotypic differences in the capacity to accumulate reduced nitrogen have also been found to be present and the reason for the same has been ascribed to differences in NR activity [111, 113]. However, since total nitrogen accumulation is a function of both biomass and nitrogen concentration, specific activity of the enzyme can at best be only a component and total activity of the enzyme may be more related to reduced nitrogen accumulation. Such a relationship has been found [49, 59, 127]. However, no such relationship between NR activity and total reduced nitrogen was found in wheat by Rao *et al.* [137] and in barley and in barley mutants deficient in apparent NR activity [138].

Another observation of interest is the occurrence of genotypic differences in enzyme efficiency. Cultivars having the same cumulative seasonal input of reduced nitrogen (total NR activity) have been found to possess different amounts of reduced nitrogen. The plausible reasons attributed to such differences are basic biochemical differences in the enzyme itself,

differences in induction and substrate pool of nitrate, competition for cofactor NADH *in situ* and, since in these studies the *in vitro* assay method has been used, the decay of the enzyme and presence of inhibitors of NR, released on homogenization [59, 61]. The biochemical and physiological basis of genetic variation in NR activity have been studied [2, 122, 139]. However, these studies are far from complete and no definite conclusion has been reached. The level of NR often varied with the method employed [53, 140]. It is also known that NR is under a complex regulatory mechanism [139]. It may be controlled by nitrate partition [43, 141], nitrate flux [142, 143], end-product inhibition by ammonia and amino acids [144, 145], availability of protein precursors such as free amino acids and *m*RNA [146] and various other factors [5]. Hence, the observed genetic variation in the activity of NR may be due to any or a combination of the aforesaid regulatory mechanisms operating *in situ*.

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