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Nitrogen assimilation by legumes - processes and ecological limitations

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Abstract

Legumes may feed on three different sources of nitrogen: nitrate, ammonium, and, due to symbiotic N_2 fixation, atmospheric dinitrogen. In all three cases ammonium is finally assimilated by the glutamine synthetase (GS) / glutamate synthase (GOGAT) system. NH_4^+ produced by nitrogenase in symbiosomes of legume nodules is released into the host cell cytosol where it is incorporated into amino acids and amides. The release of NH_4^+ into the cytosol appears to occur purely by diffusion. Therefore, the activity of the GS / GOGAT enzymes is decicive to avoid product inhibition of nitrogenase by NH_4^+. No information is available on the mechanism of xylem loading with amides or ureides, a process that may play a key role in avoiding accumulation of amino acids in infected nodule cells. The same applies to phloem unloading of sucrose. Both transport processes, however, may determine the efficiency of N_2 fixation by legumes.

There is no convincing evidence that N_2 fixation by legumes is generally limited by energy supply to nodules. On the other hand, N_2 fixation is often restricted by environmental constraints. Environmental stresses may limit N_2 fixation of legumes at four different levels: *Rhizobium (Bradyrhizobium)* multiplication in soil, rhizobial infection of roots, nodulation, and N_2 fixation. There is increasing evidence that, sufficient infection by effective rhizobial strains provided, N demand of the host plant determines the potential of N_2 fixation. Various environmental stresses and supply of mineral N reduce nodulation and nitrogenase activity without affecting total N concentration of the plant tissue. Stress-induced reduction of plant growth, however, results in an accumulation of free amino acids, amides, or ureides in shoots, roots, and nodules which may be responsible for the regulation of nodulation and nitrogenase activity via a feedback system. This implies that enhancement of N_2 fixation by legumes can be realized in two different ways: either by improvement of stress resistance and dry matter accumulation or by uncoupling of the feedback control.

Introduction

Legumes may feed on three different sources of nitrogen: NO_3^-, NH_4^+, and, due to symbiotic N_2 fixation, atmospheric N_2. In terms of metabolic processes, NH_4^+ -N nutrition is the simplest case. Although NH_4^+ may be readily oxidized to NO_3^- in soil, there is evidence that, particularly in tropical soils, available NH_4^+ concentrations may be comparable with NO_3^- concentrations [81]. Nitrification may be inhibited by high temperature and low pH [8]. Consequently, NH_4^+ nutrition may play a major role in tropical soils whereas in temperate soils N is predominantly taken up as NO_3^- [5, 2, 43].

The incorporation of N from the three sources into plant constituents involves metabolic differences that largely affect plant growth. This is especially the case when N_2 is symbiotically fixed by legumes. The purpose of this paper is to describe the major metabolic and transport processes that play a role in N assimilation by legumes. In a second part, the effect of soil acidity, drought, and mineral N on N_2 fixation will be treated. Emphasis will be laid on processes in those legumes that are sufficiently infected (and nodulated) by effective rhizobial bacteria. The effect of soil acidity, drought, and mineral N on rhizobial population, root infection, and nodulation is recognized but will not be discussed here.

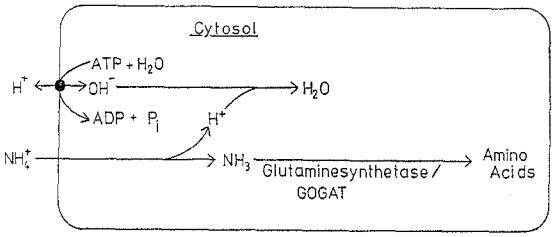

Fig. 1. Model of ammonium uptake and assimilation by root cells. Depolarization of the membrane potential and dissociation of ammonium to ammonia and proton stimulates the plasmalemma ATPase that is responsible for rhizosphere acidification.

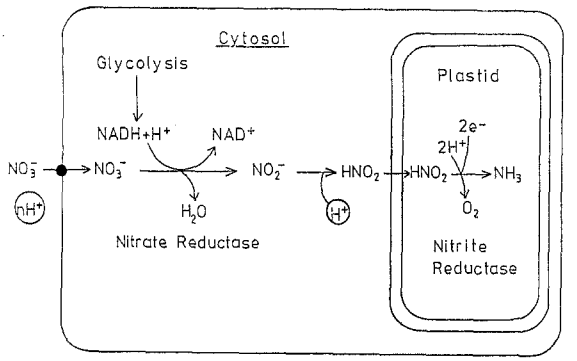

Fig. 2. Model of nitrate uptake and reduction by root cells. Nitrate/proton co-transport and nitrate reduction are proton-consuming processes that are responsible for rhizosphere alkalinization.

Nitrogen assimilation

Uptake and assimilation of ammonium and nitrate

A strong driving force for the uptake of NH_4^+ by root cells is established by a plasmalemma-bound H^+ ATPase (Fig. 1) which generates both an electrical and a pH gradient [31, 45, 67, 77]. As a cation NH_4^+ is attracted by the negative charge and enters the cell passively via a specific transport system [9, 36]. Both the partial depolarization of the plasmamembrane and the liberation of protons upon incorporation of NH_3 into glutamate stimulates net proton release by ATPase [22, 45]. Therefore, legumes (and other plants) that depend on NH_4^+ nutrition will strongly acidify their rhizosphere [40, 61].

In the cytosol, NH_4^+ is incorporated into glutamate by glutamine synthetase (GS) forming glutamine. A transferase reaction catalyzed by glutamine oxoglutarate aminotransferase (GOGAT, glutamate synthase) synthesizes two molecules of glutamate by transferring the amino group from glutamine to oxoglutarate [64]. This is the major pathway of NH_4^+ incorporation into amino acids for all three sources of nitrogen. Assimilation of NH_4^+ into oxoglutarate by glutamate dehydrogenase is of minor importance due to the high k_m of this enzyme [12]. According to other results [76], however, the activity of glutamate dehydrogenase may increase under water stress conditions.

In contrast to NH_4^+, NO_3^- has to be transported actively across the plasmalemma of root cells, i.e. against an electro-chemical gradient (Fig. 2). Transport is assumed to be a proton/nitrate symport [78, 80]. Symport of protons with NO_3^- consumes protons in the rhizosphere and therefore increases the pH [49]. This is in contrast to uptake of NH_4^+ or N_2 fixation where generally acidification is observed [6, 29, 35, 46, 59].

Nitrate reduction, catalyzed by nitrate reductase occurs in the cytosol and generates NO_2^-. Because NO_2^- is reduced to NH_4^+ in plastids, NO_2^- has to overcome two membranes of the organelle envelope. This is realized by protonation of NO_2^- to HNO_2 which, as a weak acid, may readily diffuse through membranes in its undissociated form [30]. The consumption of protons in the cytosol adds up to alkalinization and therefore inhibits proton release by ATPase. This is a second reason why there is an increase in rhizosphere pH with NO_3^- nutrition [2, 49].

Dinitrogen assimilation

The third source of N is atmospheric N_2 which can only be made available to legumes by a symbiosis with rhizobia. Within the family of Rhizobiaceae four different genera are distinguished today [19] with *Rhizobizium* and *Bradyrhizobium* being the most important ones from an agricultural point of view. Rhizobial infection by effective strains leads to the formation of nodules which are the specialized organs for N_2 fixation. They derive from a secondary meristem in the root cortex tissue [7, 84].

Nodules consist of three major tissues: peripheral cortex, vascular bundles, and central infection zone [33, 74, 75]. Surrounded by the cortex and infected cells, vascular bundles are connected with root phloem and xylem strands. Energy and C skeletons are delivered as sucrose into the infection zone via the phloem. There is uncertainty about the route of sucrose transport into infected cells. Noninfected cells may play a role in the translocation of C and metabolites to infected cells as judged from the high frequency of plasmodes-

matal connections between infected and non-infected cells [74]. Low concentration of sucrose and absence of fructose in the nodule apoplast favor the concept of symplastic unloading of sucrose from phloem into infected cells [74]. For root tissue there is evidence that phloem unloading may occur via a symplastic pathway [24, 50]. Alternatively, phloem unloading of sucrose into the apoplast and hydrolysis by invertase may represent a possibility of selective uptake of hexoses into infected cells (Fig. 3). However, experimental evidence for the nature of sugar import from phloem into infected nodule cells is still missing.

Hexoses are degraded in the glycolytic pathway and organic acids are imported into the bacteroids. Like mitochondria and chloroplasts bacteroids are surrounded by a second membrane which originates from the host cell plasmalemma. Thus bacteroids, surrounded by the peribacteroid membrane, form an organelle similar to chloroplasts and mitochondria. This organelle is called 'symbiosome' [84]. The symbiosome is responsible for N_2 fixation by catalytic activity of the nitrogenase. The product of N_2 fixation, NH_4^+, is released into the cytosol by passive diffusion across the symbiosome membranes [36, 73].

In the host cell cytosol NH_4^+ is assimilated by the GS/GOGAT enzymes [25, 44, 76]. Amides (or in many tropical legumes ureides) are exported to the shoot via the xylem [64]. Again nothing is known about the transport processes at the plasmalemma of infected cells which are responsible for amide or ureide export into the xylem. Active transport mechanisms may contribute to low cytosolic amino acid concentrations in infected cells which are required for efficient NH_4^+ assimilation. This in turn generates the diffusion gradient for NH_4^+ between symbiosome and cytosol. A steep gradient enhances the release of NH_4^+ into the cytosol and avoids product inhibition of nitrogenase by NH_4^+ [76].

In many agro-ecosystems of the tropics or subtropics N_2 fixation by legumes makes an important contribution to the N economy of soils [56]. Therefore, breeding programs have been initiated that intend to increase the potential for N_2 fixation [e.g. 10, 14]. Identifying the rate-limiting steps in the supply with sucrose and/or the export of reduced N may represent a key to further success in these efforts. Transport processes at the plasmalemma of infected cells may be an important factor in the efficiency of N_2 fixation in legumes. However, there are deep gaps in our knowledge of membrane processes of carbon import into and nitrogen export out of infected cells.

Soil acidity

Several ecological constraints may limit the N_2 fixation potential of legumes [11, 28, 79]. The identification of the process that is most sensitive to the stress situation promises the greatest success in breeding programs or in an improvement of agronomical practices [1]. In humid areas legume growth may be adversely affected by soil acidity [3]. Soil acidity is a complex of soil factors that may all limit growth to varying degree depending on the conditions.

High proton activity (low pH) may directly affect plant growth. [34, 39]. However, low pH also promotes the solubilization of toxic ions e.g. Al and Mn. In addition, in soils of low pH deficiencies of Ca, Mg, P, Mo, and S are likely to occur [20]. For an N_2-fixing legume the situation becomes even more complicated because all these constraints may not only become apparent at the host plant level but also at the level of rhizobial multiplication, infection, and nodulation [25, 51]. Improvement of N_2 fixation first requires an understanding of which of the four different levels is most sensitive to soil acidity. The best parameter to decide this question is N concentration of the plant tissue [52]. If N_2 fixation is directly impaired by low pH, N deficiency will occur. On the other hand, if growth of the host plant is more sensitive, total N concentration will either increase or will remain unchanged.

Field bean (*Vicia faba*) is extremely sensitive to low pH. In a series of experiments it has been demonstrated that this sensitivity is independent of Al or Mn toxicity and nutrient deficiency, although these factors may come into play when pH values of less than 5 are considered [63, 65, 85]. However, plant growth may be significantly reduced at moderately low pH (6.2) [65]. This is of particular importance in soils of poor buffer capacity because N_2 fixing beans strongly decrease their rhizosphere pH [65].

Low rhizosphere pH prevents cell elongation but not cell division [70, 85]. Recent evidence suggests that poor growth of field bean at low pH is caused by a lack of net proton release by ATPase activity ([85], Fig. 4). An insufficient net proton release by root cells apparently results in a decrease of cytoplasmic pH [23, 85]. This in turn may not only have serious consequences for root cell metabolism due to changed enzyme activities [17] but may specifically change gene activation [22].

Despite strong inhibition of nitrogenase activity, N concentrations in the plant tissue were optimal, indicating that inhibition of N_2 fixation was not limiting

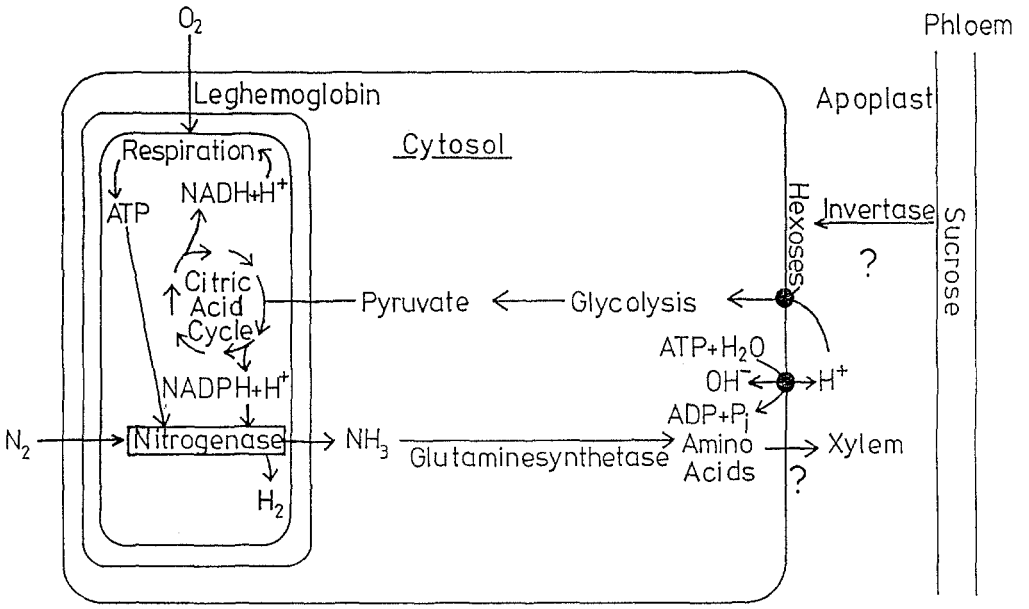

Fig. 3. Model of metabolic and transport pathways in infected cells of legume nodules.

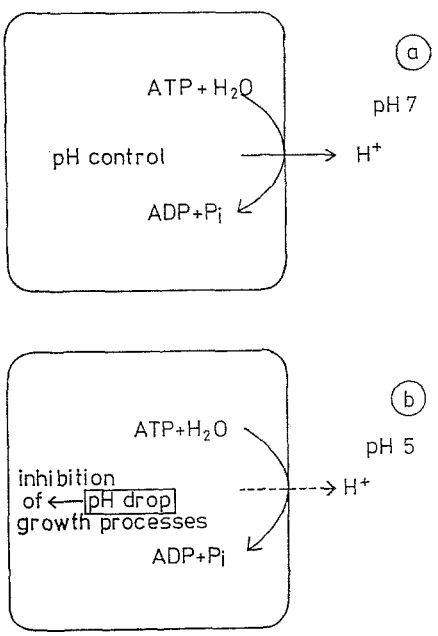

Fig. 4. Model of inhibition of root cell elongation by low medium pH. a: sufficient net proton release by ATPase activity, b: inhibited net proton release.

plant growth at low pH [63]. This example not only shows that growth of field beans is more pH-sensitive than the establishment and functioning of the symbiosis, it also infers that there must be a feedback control of N_2 fixation by plant growth.

Drought stress

Drought stress severely inhibits nitrogenase activity [26], N_2 fixation [54], and nodulation [54]. Various hypotheses have been proposed to explain the effect of water stress on N_2 fixation. During the seventies it was suggested that water stress inhibits photosynthesis by stomatal closure and thereby decreases the supply with sucrose to nodules. This would result in an energy shortage of N_2 fixation. Although at severe stress primary inhibition of photosynthesis cannot be ruled out [18], there is convincing evidence that drought stress does not generally limit the supply of nodules with carbohydrates [13, 32, 57, 58].

In the eighties an alternative hypothesis was advanced that explains reduced nitrogenase activity in terms of an increased resistance of the variable O_2 diffusion barrier in the nodule inner cortex [32, 33, 48, 68]. According to this concept various stresses alter the diffusion of O_2 in the inner cortex tissue thus inhibiting carbon metabolism and nitrogenase activity. Alternatively, it was proposed that the increased gas resistance may also lead to an accumulation of H_2 which may inhibit nitrogenase activity [32].

There is a need for efficient control of the O_2 supply of infected cells because of the extreme O_2 sensitivity of the nitrogenase enzyme [25, 71]. However, the question arises whether O_2 supply by variation of the gas diffusion barrier is the immediate point of metabolic

control of nitrogenase activity. The fact that O_2 concentration in the infection zone limits nitrogenase activity and that increasing O_2 concentrations stimulate nitrogenase activity [32] does not necessarily imply that nitrogenase activity is under primary control of the variable O_2 diffusion barrier. Furthermore, increasing O_2 concentrations did not completely abolish the inhibition of nitrogenase by water stress [26].

Some authors have reported on an accumulation of reduced N in the form of NH_4^+, amino acids, and ureides in shoots, roots, and even nodules under stress conditions [57, 83]. An example is shown in Figure 5. Drought stress (−0.5 MPa), induced by PEG in nutrient solution, reduced the leaf water potential of alfalfa from −0.5 MPa to −1.2 MPa. This significantly inhibited shoot growth as indicated by a significant difference in shoot dry weight between control and stress treatments after 5 days of stress (not shown). Total amino acid concentrations in shoots, roots, and nodules were significantly increased by the stress (Fig. 5) whereas total N concentrations remained unchanged (not shown) in agreement with other findings [54].

The accumulation of amino acids indicates that growth reduction was not due to N deficiency but probably to a drop of turgor [4] or cell wall extensibility [82]. The accumulation of amino acids suggests that O_2 supply did not primarily limit nitrogenase activity. In contrast, this suggests that nitrogenase activity was limited by product inhibition in the form of NH_4^+ which may accumulate when its incorporation into amino acids and/or amides is inhibited. Because growth of young vegetative parts (meristems) requires the supply of amino acids for growth processes, the inhibition of cell extension and especially cell division will result in an accumulation of amino acids [44]. The reduced demand for amino acids will then in turn result in an accumulation in roots and nodules. The decrease of glutamine synthetase and increase in glutamate dehydrogenase activity during water stress applied to soybean [76] indicates that under water stress conditions ammonium may indeed accumulate in infected cells.

The proposed model of the regulation of nitrogenase activity during reduced nitrogen demand under water stress conditions suggests that nitrogenase activity is under control of the NH_4^+ concentration of the host cell cytosol. According to this concept the increase in resistance of the variable O_2 diffusion barrier is a regulation mechanism superimposed on the control of O_2 supply to the infected zone. The latter may respond to the lower O_2 demand of infected cells when nitrogenase activity is reduced. Thus the superimposed regulation mechanism of O_2 diffusion avoids inhibitory oxygen concentrations when O_2 utilization is reduced under stress conditions. To test this model, comparative data of O_2 concentrations and substrate concentrations in infected cells under stress conditions is needed.

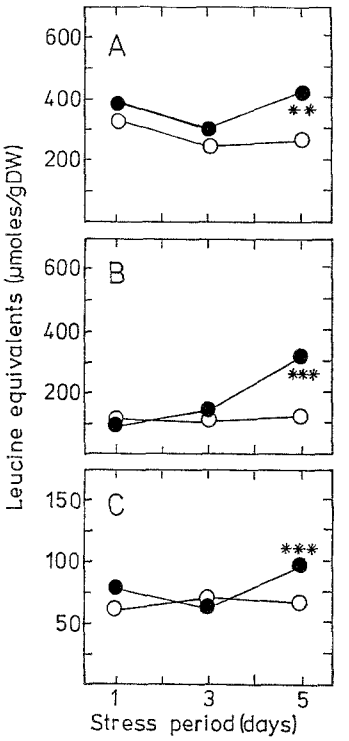

Fig. 5. Effect of water stress (−0.5 MPa) induced by PEG on the concentrations of amino acids in nodules (**A**), roots (**B**), and shoots (**C**) of alfalfa in nutrient solution. o control, • stress treatment. Significant differences at $^{**}p$ = 1% and 0.1% level, respectively. (Schubert, Serraj, Plies-Balzer, and Mengel, unpublished results)

Two alternative hypotheses have recently been proposed to explain the accumulation of nitrogenous compounds in the nodule infection zone. According to Streeter [75] the morphological structure of legume nodules makes it appear unlikely that reduced N is pulled into the xylem by the transpiration stream. The vascular bundles come to a dead end in the nodule. Therefore water leaves the nodule via the xylem, whereas water import occurs via phloem (and cortex). Since water import and export are in an equilibrium, it is possible that disturbance of this equilibrium results in a build-up of reduced N.

Available water within the nodule would then be a key factor for the potential of N_2 fixation. In this con-

text, it is important to understand the mechanism that drives water through the nodule. Because water movement in plants generally follows a potential gradient established by solute transport across membranes, the identification of the responsible active transport mechanism is of crucial importance in the understanding of N_2 fixation limitation under water stress conditions. In principle, two major possibilities of active transport are conceivable: active phloem unloading of sugars into infected cells and active xylem loading of reduced N (amides or ureides). In any case, the plasmalemma of cells in the infection zone is predestined for either of the two alternatives.

A second hypothesis advanced by Parsons *et al.* [53] proceeds on the assumption that reduced N that is not consumed in the shoot tissue will be recycled through phloem. If plant growth is restricted by a stress condition the accumulation of amino acids will decrease the C/N ratio in the phloem sap. Thus amino acids in phloem sap that arrive at the nodule may regulate N_2 fixation and possibly also nodule growth. In amide exporters glutamate, serine and proline may be of particular importance in this respect. Interestingly, proline accumulated severalfold in field bean exposed to water stress [57].

In ureide exporters glutamine and asparagine may regulate nodule activity in a feedback control. Possibly these metabolites are also involved in changes of the gas diffusion barrier and thereby regulate the oxygen availability in the infection zone [53]. An important implication of this hypothesis appears to be a lack of selectivity of phloem unloading. Symplastic unloading of sugars from phloem should prevent cells of the infection zone from discriminating between sugars and amino acids. On the other hand, apoplastic unloading would allow the exclusion of amino acids at the plasmalemma of infected cells.

An exact understanding of the inhibition of nitrogenase activity requires a profound understanding of the membrane transport processes that are responsible for phloem unloading of sucrose and xylem loading with amides and ureides. In this context two aspects are of particular importance. First, which are the active transport steps at the plasmalemma of infected cells? Second, does selectivity of phloem unloading and/or xylem loading explain the feedback control of the regulation of nitrogenase activity?

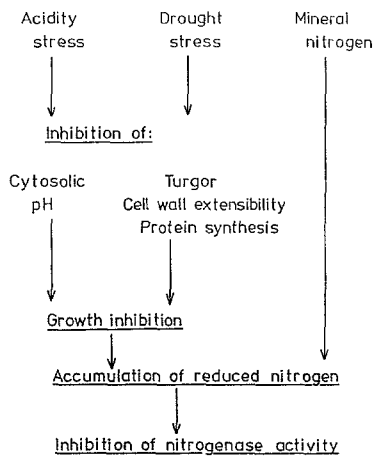

Fig. 6. Model of inhibition of N_2 fixation by various environmental constraints.

Mineral nitrogen

There are large differences in N_2 fixation efficiency among the various legume – rhizobium symbioses [15, 37, 56]. Generally, forage legumes harvested in the vegetative state are more efficient than grain legumes. Whereas the former meet 80 to 90% of their N demand by N_2 fixation, the latter show large differences in efficiency [28]. This has been explained in terms of source-sink relationships. In contrast to field bean (*Vicia faba*) and pigeon pea (*Cajanus cajan*), soybean, pea, and white lupin show poor N_2 fixation capabilities after flowering. The latter three species develop strong sink activity in pods thereby drawing away N from photosynthesizing leaves [28, 38, 41, 47, 69]. This ends up with a lack of sucrose supply to nodules (or alternatively: a low C/N ratio in phloem sap [53]) which then decreases nodule activity. Therefore, mineral N application to legumes with poor N_2 fixation capacity may increase yield performance [41, 47], particularly if applied via the leaves [16]. Legumes with high N_2 fixation capacity (e.g. *Vicia faba*) will take up available mineral N from soil and correspondingly reduce N_2 fixation [41, 60, 62].

On the other hand, there are many reports on an inhibition of nitrogenase activity after mineral N fertilization [25, 66, 72]. Although the reduced nodule activity is related to the increase of the variable O_2 diffusion resistance [48] the mechanism is still under debate [32]. One possible explanation for the reduction of nitrogenase activity with NO_3^- -N supply may be the feedback mechanism described above [5]. After uptake, NO_3^- may be reduced in roots or shoots and

may then meet the N demand of plant growth. Amino acids from N_2 fixation not consumed for plant growth will then be recycled to nodules via phloem where they suppress nitrogenase activity [5].

Higher N_2 fixation capabilities of legumes in the presence of NO_3^- (e.g. supernodulating mutants [27]) may be explained in terms of uncoupling of N_2 fixation from the feedback control. Mechanistically this may be realized by inclusion of NO_3^- or reduced N compounds in vacuoles or other cell compartments where these metabolites are hindered from participation in the recycling and feedback control. This may also play a role in genotypes with higher leaf weight where a higher percentage of N may be transiently stored in proteins which can be mobilized during pod filling [55].

Conclusions

There is increasing evidence that, in effectively nodulated legumes, N_2 fixation is under tight metabolic control of the host plant. Soil acidity, water stress, and other environmental constraints (e.g. low light intensity or potassium deficiency [21]) may decrease N_2 fixation regulated by a feedback control system which is triggered by disturbed plant growth and accumulated reduced N (Fig. 6).

Two possibilities may be envisaged to increase N_2 fixation by plant breeding. Because N_2 fixation appears to be controlled by feedback inhibition, inefficient fixation in some legume species may be caused by inefficient membrane transport processes for phloem unloading of sugars and/or xylem loading of ureides or amides. In efficiently fixing legumes, it is possible to further increase N_2 fixation by uncoupling it from the feedback control. From an ecological point of view this approach appears to be questionable because it increases N concentrations in the tissue. After harvest N losses due to fast mineralization of the N-rich material provokes environmental hazards. For this reason it appears to be more appropriate to improve the stress tolerance of legumes. This not only improves yield and yield stability but, at the same time, increases the amount of N fixed.

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