NORMOXIC AND ANOXIC METABOLISM OF *NICOTIANA TABACUM* TRANSFORMANTS LACKING ROOT NITRATE REDUCTASE

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For my parents – Nikolina and Tsvetan Stoimenov

and my aunt – Dr. Stanka Koleva

1. Introduction

1.1. Higher plant nitrogen metabolism

Nitrogen is present in many forms in the biosphere, for example as atmospheric N_2 (the most abundant form of N), nitrate and ammonium. For the most part N_2 is not available to living organisms. Once fixed into ammonium or nitrate (through biological nitrogen fixation or processes such as lightening and photochemical reactions) nitrogen enters a biogeochemical cycle and passes though several organic or inorganic forms before it eventually returns to molecular nitrogen.

For higher plants nitrate is the most significant source of nitrogen. Plants assimilate it (absorbed by their roots) into organic nitrogen compounds. The first step of this process is the reduction of nitrate to nitrite in the cytosol (Oaks, 1994). The enzyme that catalyzes this reaction (1) is nitrate reductase (E.C. 1.6.6.1):

$$\text{(1)} \qquad NO_3^- + NAD(P)H + H^+ + 2e^- \xrightarrow{NR} NO_2^- + NAD(P)^+ + H_2O$$

The second step in nitrate assimilation is the reduction of nitrite to ammonium. The process (2) is catalyzed by nitrite reductase (NiR, EC. 1.6.6.7) (Wray, 1993).

$$\text{(2)} \qquad NO_2^- + 6\ \text{Ferredoxin}_{red} + 8H^+ \xrightarrow{NiR} NH_4^+ + 6\ \text{Ferredoxin}_{ox} + 2\ H_2O$$

Nitrite is a highly toxic ion. Plant cells immediately transport the nitrite generated during nitrate reduction from the cytosol to chloroplasts in leaves or plastids in roots (Sechley et al., 1992).

As ammonium is toxic on its own, plant cells avoid ammonium toxicity by rapidly converting NH_4^+ into amino acids. The primary pathway for this

conversion involves the sequential actions of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Lea et al., 1992). The following reaction (3) is catalyzed by GS (E.C. 6.3.1.2):

$$(3) \qquad NH_4^+ + \text{glutamate} + ATP \xrightarrow{GS} \text{glutamine} + ADP + P_i$$

Plants contain two classes of GS isoenzymes. The GS1 in shoot chloroplasts reassimilates photorespiratory NH_4^+ and NH_4^+ from NO_3^- reduction. The GS2 in root plastids generates amide nitrogen for local consumption, (Miflin, 1974; Taiz and Zeiger, 1998).

Elevated plastid levels of glutamine stimulate the activity of GOGAT (4). Plants contain two types of GOGAT: One accepts electrons from NADH (E.C. 1.4.1.14), the other accepts electrons from ferredoxin (Fd) (E.C. 1.4.7.1) (Suzuki and Gadal, 1984; Lea et al., 1990; Sechley et al., 1992)

$$(4) \qquad \text{Glutamine} + \text{2-oxoglutarate} + NADH + H^+ \xrightarrow{\text{NADH-GOGAT}} 2\ \text{glutamate} + NAD^+$$

The NADH type of the enzyme (NADH-GOGAT) is located in plastids of non photosynthetic tissues such as roots or vascular bundles of developing leaves. In roots NADH-GOGAT is involved in the assimilation of NH_4^+ absorbed from the rhizosphere (5).

$$(5) \qquad \text{Glutamine} + \text{2-oxoglutarate} + Fd_{red} \xrightarrow{\text{Fd-GOGAT}} 2\ \text{glutamate} + Fd_{ox}$$

The ferredoxin-dependent type of glutamate synthase (Fd-GOGAT) is found in chloroplasts and plastids.

Once assimilated into glutamate and glutamine, nitrogen is incorporated into other amino acids via transamination reactions catalyzed by various aminotransferases.

1.2. Nitrate reductase

Understanding the role of NR, the key enzyme in nitrogen metabolism in higher plants has potential economic importance not only because nitrate is the most common source of nitrogen in crop plants but also because some current studies suggest that the enzyme is the focal point for integration and control of carbon and nitrogen metabolism (Campbell, 1999).

1.2.1. Structural and functional characteristics

NR is a redox system with an internal electron transfer chain localized in the cytosol. The enzyme of higher plants is a homodimer composed of two identical subunits, about 100-115 kDa each (Caboche and Rouze, 1990; Solomonson and Barber, 1990). Both subunits contain one equivalent of flavin adenine dinucleotide (FAD), heme-Fe and Mo-molybdopterin (Mo-MPT). The electron pathway is from NAD(P)H- to FAD-heme-MoCo to nitrate (Hoff et al., 1992). The three structural and functional domains are connected by two hinge regions, hinge-1 and hinge-2. Hinge-1 contains a phoshorylation site (ser-543 in spinach) which plays an important role in the post-translational regulation of NR.

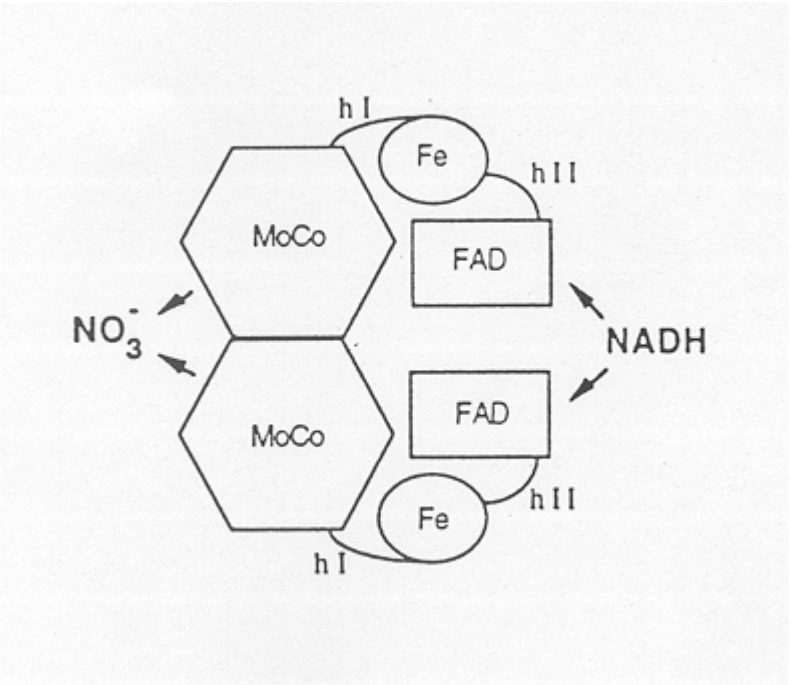


Figure 1. Functional domains of NR. Explanations in the text.

NR's **primary function** is reduction of nitrate. Three isoforms of inducible NR, utilizing different coenzymes as a source of reducing power are known so far:

(i) NADH-NR (E.C. 1.6.6.1) is the most common isoform in higher plants (Solomonson and Barber, 1990)

(ii) NAD(P)H-NR (E.C. 1.6.6.2), typically this isoform plays a role of a 'second isoform' (mainly in roots) in plants which have NADH-NR in the shoots. However, NAD(P)H-NR has been identified as a sole NR isoform in a number of species, for example *Betula pendula* (Friemann et al., 1991)

(iii) NADPH-NR (E.C. 1.6.6.3), this isoform has not been identified in higher plants. It is typical for some fungi.

Constitutive isoforms of NR have also been identified in various plant species (Oaks et al., 1988; Andrews et al., 1990).

A **secondary function** of NR has been discovered recently. NR is found to catalyse production of NO (Dean and Harper, 1988). Both constitutive and inducible NR were found to be involved in the process (Yamasaki and Sakihama, 2000).

The physiological role of NO in higher plants is often connected with growth promotion, stress regulation, plant disease resistance and gene induction (for example genes of phenylamanineammonia lyase – *pal* and chalcone synthase – *chs*; both enzymes catalyze synthesis of defense related products) (Delledonne et al., 1998).

The biological significance of NR as a NO emitter has yet to be established and the induction of NR in response to pathogen attack or other stimuli known to induce NO production is still uncertain.

1.2.2. Regulation

NR is subjected to a very rigid regulation achieved by control on (i) NR expression (ii) NR catalytic activity and (iii) NR protein degradation. In terms of efficiency this is necessary for two reasons. First, reduction of nitrate to ammonium is a process with high-energy consumption (from +5 to –3) and

second, both nitrate and ammonium are highly toxic compounds. Indeed under normal conditions nitrite content of leaves is usually below 15 nmol g^{1} FW (Lang and Kaiser, 1994).

NR expression is inducible, depending on nitrate content (Gowri et al., 1992; Vincentz et al., 1993) and light (Beevers et al., 1965; Galangau et al., 1988; Deng et al., 1990). The response of NR to nitrate depends on a constitutively produced 'nitrate-sensing' protein of yet unknown character, which presumably binds to regulatory regions in the NR gene and turns on transcription of the NR gene (Redinbaugh and Campbell, 1991). The nitrate box regulatory sequences in the promoter of the NR genes have been identified in arabidopsis (Hwang et al., 1997). Induction occurs rapidly in the presence of very low concentrations of external nitrate (less than 10 μM).

Presumably other regulatory boxes for amino acid (Gln/Glu) content (Solomonson and Barber, 1990; Rouze and Caboche, 1992, Scheible et al., 1997), water, carbon metabolites (Cheng et al., 1992), cytokinin (Lu et al., 1990) and photosynthesis are present in the promoters of NR and related nitrate response genes (Redinbaugh and Campbell, 1991; Crawford, 1995).

Post-translational regulation of NR is a complicated multi step mechanism. According to a recent review (Kaiser and Huber, 2001) NR exists basically in three states: free NR (active), phosphorylated NR (pNR, active) and pNR: 14-3-3 complex (inactive). The ratio of these three forms varies and is characteristic for certain external conditions. Often this valuable information is presented as 'activation state' i.e. the percentage of active NR (NR + pNR) to the total NR activity measured in the presence of EDTA. Once NR is phosphorylated, at the phosphorylation site in hinge-1, Ser543 in spinach and Ser534 in arabidopsis (Douglas et al., 1995; Kanamaru et al., 1999), a process catalyzed by protein kinases, the enzyme becomes accessible for binding of an inhibitor protein. The so called 'inhibitor protein' has been identified as a member of 14-3-3 protein family by Bachmann et al., 1996, and Moorhead et al., 1996. The 14-3-3 dimer binds to the enzyme in the presence of divalent cations and thereby converts NR into a completely inactive form which can not transfer electrons from NAD(P)H to nitrate. It is not yet clear if divalent cations are needed as the switch for the active/inactive form or are necessary for the 14-3-3 binding to pNR

(Weiner and Kaiser, 2000) or both. However it is absolutely clear that after complete chelation of divalent cations subsequent to NR phosphorylation, NR remains active, irrespective of its phosphorylation state. In addition, divalent cations fulfil several other purposes in that model: (i) the proteine kinase itself is a Ca^{2+}-dependent enzyme, (ii) the substrate for the kinase is Mg^{2+}-ATP (Figure 2).

Exogenous signals involved in the regulation of phosphorylation process include light (Vincentz and Caboche, 1991), anoxia (Botrel et al, 1997) and CO_2 deprivation (Kaiser and Brendle-Behnisch, 1991).

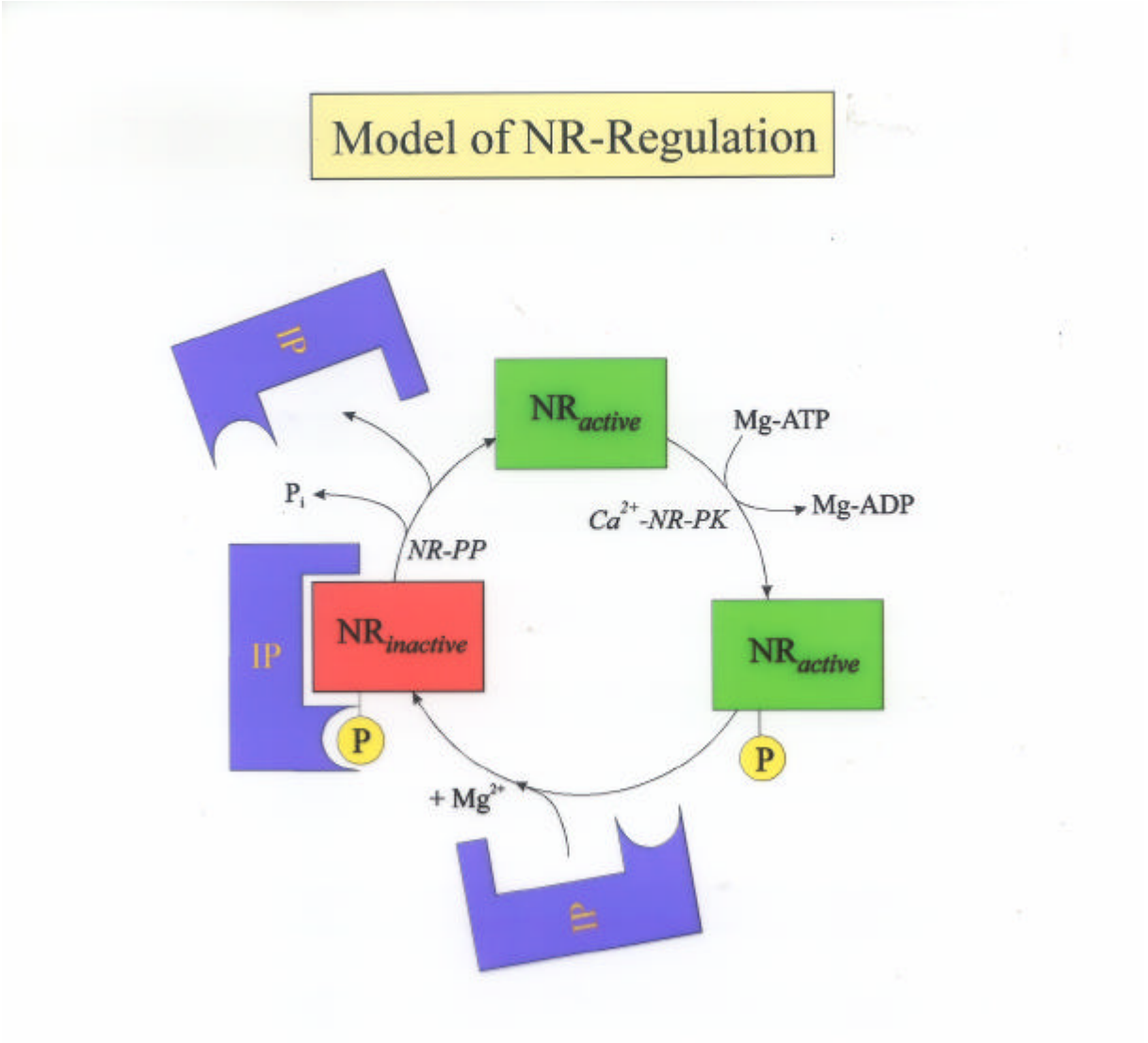


Figure 2. *Post translational regulation of NR. Explanations in the text.*

1.3. Oxygen deprivation – cause, effect and adaptation mechanisms

Anaerobiosis (oxygen deprivation) is a universal biological phenomenon that occurs to some extent in all organisms. Higher plants have an absolute requirement for oxygen to sustain metabolism and growth. That requirement varies among different plant species (Crawford and Braendle, 1996). Most plant tissues will tolerate anoxia for only a short time before a permanent damage occurs (Hook and Crawford, 1978; Drew, 1990). However, as almost ¾ of Earth's surface is covered with water or has submerged soils or sediments that restrict oxygen diffusion (Pannamperuma, 1972), coping with anaerobiosis is common for a wide variety of organisms. In order to adapt, plant aquatic species have evolved features (anatomical and biochemical adaptations) to overcome the fundamental problem during anoxia – the lack of oxygen as a terminal electron acceptor.

(i) Anatomical adaptations. One anatomical adaptation, for example, is development of aerenchyma (Drew et al., 1979; Justin and Armstrong, 1991). Aerenchyma formation is commonly found in the stems and roots of aquatic and flood-tolerant species, developing by cell separation during development or by cell death and dissolution. The process is usually constitutive and does not require external stimuli. Aerenchyma is necessary (for oxygenation) and effective over distances of 80mm. It lowers the resistance to gas diffusion or convection and cuts back on the number of O_2 consuming cells (Drew et al., 1985).

(ii) Biochemical adaptations. The metabolic pathways by which organisms liberate stored energy are referred to as cellular respiration. Under aerobic conditions the main pathway of respiration begins with the breakdown of carbohydrates, continues through Krebs cycle (TCA) and ends with the electron-transport chain (taking place in mitohondria).

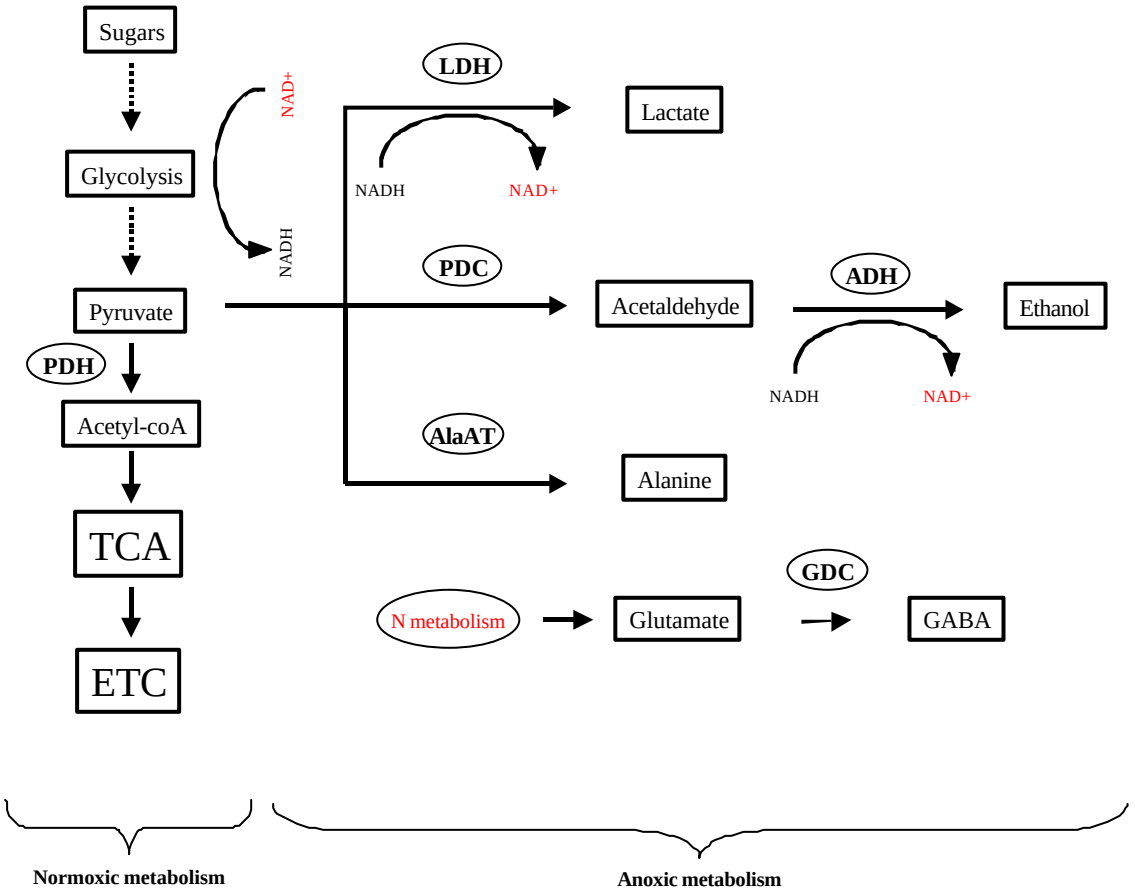
Under strict anaerobic conditions (anoxia), mitochondria have been assumed to be inoperative because the requirement for oxygen as a terminal electron acceptor in higher plants is absolute. At the subcellular level, elongation and swelling of the mitochondria are detectable within few minutes of anoxia (Aldrich et al., 1985; Andreev et al., 1991) but changes in the fine structure are almost as quickly restored in normoxic conditions. Only after 15 h of anoxia

irreversible damage to mitochondrial structure, energy metabolism and cell vitality has been reported (Andreev et al., 1991).

Without oxygen, pyridine nucleotides are presumed to be largely reduced, and normal cyclic metabolism stops (Ricard et al., 1994). Normally, in anoxia, the ATP pool is sufficient for only 1-2 min in cells that are metabolically very active (Roberts et al., 1984; Roberts et al., 1984a). To overcome this problem, fermentation (a **biochemical adaptation**) takes place in plant tissues.

The main purpose of the fermentation is to maintain glycolysis by reoxidation of NADH to maintain energy supply of the plant cells (aerobic glycolysis and respiration yield 36 ATP molecules per molecule of glucose, anaerobic glycolysis – only 2).

Some of the main fermentative products include ethanol, lactate, alanine



(Ala), (all derived from the end product of glycolysis – pyruvate) and GABA. (Figure 3)

Figure 3. *Carbohydrate degradation in normoxic and anoxic conditions. Explanations in the text.*

Lactate and ethanol production use 1 molecule NADH each and in this way maintain NADH reoxidation. Large amounts of ethanol and lactate are usually found (diffusion) in the hypoxic/anoxic root surroundings. In that way plants minimize cytosolic acidification and toxic side effects of ethanol, the process however, causes severe C losses.

The amino acids alanine and g-aminobutyrate (GABA) accumulate markedly in response to anaerobic stress (Steeter and Thompson, 1972). These amino acids are specifically discussed in relation to anaerobic carbon metabolism, and in intercellular pH relation. It is now recognized that GABA synthetis is catalyzed by a Ca^{2+} -calmodulin –activated enzyme, glutamate decarboxylase (Snedden et al., 1995). And therefore it has a specific role connecting intermediary amino acid metabolism to perturbations of cytoplasmic Ca^{2+} concentrations induced by stress.

The typical and general responses of anoxic cells – decreased ATP levels, cytosolic acidification and accumulation of ethanol, lactate and/or alanine have been included over the years in several hypotheses in an attempted to present a uniform model of anoxia phenomenon.

One theory (the oldest) is **Crawford's Metabolic Theory for Flooding Tolerance** (McManmon and Crawford, 1971). In brief it says that ethanol accumulation would be toxic, therefore, flood tolerance in plants require decreased ethanol production due to low ADH activity, thereby reducing the presumed toxic effects of ethanol. It also takes into consideration the ability of some plants to redirect glycolytic intermediates to alternative end products such as malate, lactate and other organic acids. This theory was proven incorrect in most parts but nevertheless it was a stimulus for research in the general idea of flood tolerance.

Another hypothesis is **Davies-Roberts pH Stat Hypothesis** (Davies, 1980; Roberts, 1989) It involves the tight regulation of pH stat to prevent cytoplasm's acidosis. The later is considered the main reason for cell death under anoxia or hypoxia. Under this hypothesis, the relative rate of lactate versus ethanol fermentation depends upon the cytoplasmic pH. Under anaerobic conditions, pyruvate is initially converted to lactate, but as the cytosolic pH decreases, LDH activity is inhibited (the enzyme has pH optimum around pH 9), PDC activity is stimulated and ethanol synthesis predominates. Although very

elegant as a theory, available data show that the above mentioned cytosolic acidification occurs mainly in flooding sensitive plants (Manegus et all., 1989; Manegus et al., 1991; Kennedy et al., 1992). Flooding tolerant plants seem to possess alternative mechanisms/responses beyond the initial transient changes in pH observed. The Davies-Roberts hypothesis has been further updated. According to additional data obtained mainly by noninvasive ^{31}P and ^{13}C NMR techniques, the pH decrease has two phases. The first phase is a pH drop from pH 7.3-7.4 to pH 6.8. This happens in the first minutes of anoxia. The decrease reaches pH 6.8 within 20 min and it is attributed to initial lactate production. Later on a second pH decrease is monitored (Phase II) which has been assigned to be due to loss of protons from the vacuole. This leakage is passive, as proton-translocating ATP-ases are restricted by the lack of energy (Roberts, 1989).

A different approach to anoxia tolerance and its mechanisms was proposed by **Tadeje** et al. (1999); according to them, the different Km-s of PDH and PDC for pyruvate are the controlling factors that regulate the entry of pyruvate into TCA or the ethanol fermentation pathway. The Km of most plant PDH enzymes is in the µM range whereas that of PDC is in mM range. The internal pyruvate concentration is between 0,1 and 0,4 mM which is too low for PDC to compete with PDH. Thus in aerobic conditions even if PDC remains active pyruvate preferentially enters the TCA cycle. When respiration is blocked by lack of oxygen, the pyruvate concentration increases considerably and pyruvate becomes available for the PDC reaction.

1.4. Nitrate, nitrate reductase and anoxia

As early as 1937 Arnon observed that nitrate fertilization helped flooded plants to overcome some of the negative effects of oxygen deprivation, a direct result of flooding. Ever since a large number of publications covered various aspects of the ‘flooding tolerance’ phenomenon implying, among other things, involvement of nitrogen and carbohydrate metabolism as well as respiration.

Nitrogen availability affects whole plant growth and metabolism as a substrate. In addition, nitrate itself (Crawford, 1995), or products downstream of nitrate reduction may act as signalling compounds which not only control the

expression of genes directly involved in nitrate transport and reduction, but also affect many other aspects of metabolism and plant morphology (Cooper and Clarkson, 1989). In addition, the presence or absence of nitrate reduction in a given plant organ may interact with metabolism based on the fact that reduction of nitrate to ammonia requires reductant and produces OH^-. The latter aspect may be of special importance in environmental situations where reductant may be in excess and/or where cellular acidification may occur, as when roots become anoxic (Roberts et al., 1984, Fox et al., 1995).

The potential impact of nitrate on pH regulation (Roberts et al., 1985; Fan et al., 1988), carbohydrate utilisation (Reggiani et al., 1985b; Fan et al., 1988; Saglio et al., 1988; Müller et al., 1994) and the regeneration of NAD^+ (Reggiani et al., 1985a) during anoxia have been examined in the roots of a range of flooding tolerant and sensitive plants, but these investigations have not provided conclusive evidence for the importance of any particular mechanism.

The assimilation of nitrate during the anaerobic germination of rice seeds, and the subsequent anaerobic growth of the rice coleoptile, has also been investigated (Reggiani et al., 1993a, b; Fan et al., 1997) and again it has been argued that nitrate reduction could have beneficial effects on pH regulation, carbohydrate utilisation and the regeneration of NAD^+, aside of contributing to the supply of reduced nitrogen for the growing plant.

In fact in most plant tissues other than the rice coleoptile, nitrate reduction under anoxia is restricted to the conversion of nitrate to nitrite (Lee 1978, 1979). This step is catalysed by nitrate reductase, and the observation that NR activity often increases in anoxic plant tissues, either through increased gene expression (Mattana et al., 1996) or post-translational activation (Botrel et al., 1996; Botrel and Kaiser, 1997; De la Haba et al., 2001) focuses attention on this step as the key to understanding the beneficial impact of nitrate on plant survival under anoxia.

The usual approach in studies of the impact of nitrate on the response of plants to anoxia has been to compare plants grown or treated in the presence of either ammonium or nitrate (Apostolova and Georgieva 1990; Botrel at al., 1996; Botrel and Kaiser, 1997; Scheible et al., 1997; Walch-Liu et al., 2001). The disadvantage of this approach is that observed responses may be either due to the lack of the nitrate anion, or to a lack of nitrate reduction, or to the presence and uptake of external ammonium as a cation. In addition, nitrate and ammonium

nutrition have opposite effects on the pH of the rooting medium or substrate, which by itself may dramatically effect root growth and cation/anion balance. Therefore, while these comparisons have provided a wealth of information, other approaches have been needed to overcome these complications.

The expression of the NR structural gene *nia2* under the control of a leaf specific promoter in the NR-deficient tobacco mutant Nia30 (Hänsch et al., 2001) has resulted in a plant that offers an alternative way of investigating the contribution of NR activity to anaerobic metabolism in roots. The LNR-H plants, produced by the Mendel's group, have no detectable soluble NR activity in roots but about 70% of normal NR activity in shoots, when grown under sufficient nitrate supply. Thus they seemed well suited, as a new approach, to study the role of root nitrate reduction for root growth and metabolism, largely avoiding the above-mentioned disadvantages of ammonium versus nitrate nutrition.

Growth and physiology of LNR-H plants have already been characterised in part (Hänsch et al., 2001). Briefly, growth of LNR-H was somewhat retarded compared to WT. Root nitrate concentrations were very similar to WT, whereas leaf nitrate concentrations were higher. LNR-H roots had usually higher sugar but lower glutamine concentrations than WT roots.

As an additional control, transgenic WT analogs - 35S-NR plants, have been constructed (Hänsch et al., 2001). Nia30 mutants were transformed with NR-cDNA under the control of the constitutive CaMV-35S promoter resulting in a plant line in which growth rate and basic metabolic parameters were no different of those of the WT. These plants were used to make sure that obzerved differences between WT and LNR-H were not due to some unknown properties of the Nia30 background.

1.5. The aims of this study:

Based on above-mentioned considerations, the final aim of this study was to analyse the role of nitrate reduction for anoxia tolerance of tobacco roots by answering the following questions:

- Does nitrate reduction increase glycolytic flux and ATP-production under anoxia by increasing the recycling of NADH?
- Does nitrate reduction under anoxia facilitate significantly to cytoplasmic pH stabilization, and if yes, how is this achieved?

However, for that purpose the available data on growth and normoxic physiology of LNR-H, and especially on its roots, were not sufficient. Therefore, we first carried out a

(1) Detailed analysis of LNR-H growth and physiology, with emphasis on root architecture and root energy metabolism.

(2) We then characterise fermentation and anoxic metabolism in roots under anoxia.

And last but not least, the secondary function of NR i.e. catalyzing NO production, is a matter of growing interest recently and data are accumulating suggesting a far more complicated role of the enzyme via signaling functions of NO. Therefore, a first attempt was made to measure

(3) NO emission of detached roots of WT and LNR-H tobacco under anoxic and normoxic conditions and evaluate its role in anoxia.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of wild type *Nicotiana tabacum* cv. Gatersleben and tobacco transformants Nia 30/LNR-H and Nia30/35S (Hänsch et al., 2001) were cultivated in vermiculite/sand mixture (2 parts vermiculite/1 part sand) for 3 weeks and from then on hydroponically for additional 5-7 weeks. During the sand/vermiculite phase plants were watered with full strength nutrient solution twice a week.

The full strength nutrient solution (pH 6.3) contained: 5 mM KNO_3, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.025 mM NaFe-EDTA, 1 mM K_2HPO_4, 2 mM KH_2PO_4 and trace elements according to (Johnson at all., 1957).

Subsequently plants were kept in pots, each containing one plant in 1,8 L nutrient solution, in growth cambers with artificial illumination (HQI 400W, Schreder, Winterbach, Germany) at a photon flux density (PAR) of 300 µmol $m^{-2}s^{-1}$ and a day length of 12 hours. The day/night temperature regime of the chamber was 25°C/20°C respectively.

Root systems were continuously supplied with pre-moisturized air. Fresh nutrient solution was provided three times a week. During the first two weeks the hydroponic plants were gradually adapted to full strength nutrient solution (1/10, ¼, ½ and ¾ of full strength).

If not stated otherwise, roots were harvested 6h into the light period. Careful attention was paid to achieving an equal distribution of the different root tissues among the single samples and time points. The plant material (1 g FW per tube) was placed in glass tubes each containing 5 mL full strength nutrient solution or 20 mM MES, pH 6.3 where indicated. Anoxia was achieved by intensive flushing with nitrogen gas for 10 min. Tubes were then sealed with Parafilm. Normoxic controls were flushed constantly with pre-moisturized air. Incubation time was up to 6h. Samples were taken at the indicated times, and the root material was then shock-frozen with liquid nitrogen and either analyzed immediately or stored at –80°C. In some cases the root medium was also analyzed.

The plants were grown under similar conditions for the NMR measurements in Oxford. Seedlings, in groups of 15, were transferred from vermiculite to hydroponic culture after 2 weeks germination. The growth medium, at 10% in the first week and full strength thereafter, was changed twice a week. The growth medium contained 4 mM KNO_3, 1 mM NH_4Cl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 25 µM FeNa-EDTA and 0.3 mL L^{-1} trace elements from a stock solution containing 92.5 mM H_3BO_3, 18.3 mM $MnCl_2$, 1.5 mM $ZnSO_4$, 0.64 mM $CuSO_4$ and 0.24 mM Na_2MoO_4. The pH was adjusted to 6.3 using KOH.

After two weeks, the plants were transferred to single pots and the growth medium, now containing 5 mM KNO_3 and no NH_4Cl, was replaced every three days, and one week before the experiments started, the iron and trace elements were omitted from the medium to avoid the line-broadening effects of paramagnetic ions on the NMR spectra.

NMR measurements were carried out between the sixth and 12th weeks, and during this period the growth medium was replaced every other day. The largest available plants were selected for each experiment, making sure that the growth medium for that plant had been replaced the day before.

2.2. Feeding experiments

2.2.1. Tungstate feeding

Seven to nine week old WT and LNR-H plants were fed daily for 8 to 10 days with full strength nutrient solution containing 20 µM tungstate (to inhibit NR activity) and 2 mM NH_4Cl (to avoid N starvation) in addition. Other conditions as described above.

2.2.2. Sucrose feeding

Eight to ten week old WT and LNR-H plants were fed with 50mM sucrose in addition to the standard nutrient solution. To avoid a possible contamination antibiotic-antimycotic solution had been added following the manufacturer's instructions.

2.3. In vitro assays of enzyme activities

2.3.1. In vitro assay of nitrate reductase activity and activation state

Root samples were ground in liquid nitrogen and 2 mL of extraction buffer containing 100 mM Hepes-KOH, pH 7.6, 10 µM flavine-adenine-dinucleotide (FAD), 1 mM dithiothreitol (DTT), 15 mM $MgCl_2$, 0.5% BSA, 1% poly-vinyl-pyrolidone (PVP), and 50 µM leupeptin, 50 µM cantharadine and 2 mM pefabloc were added per g FW. The suspensions were centrifuged for 10 min (16000g at 4°C) and the supernatant was desalted on Sephadex G-25 equilibrated with buffer containing 100 mM Hepes-KOH, pH7.6, 10 µM FAD, 1 mM DTT and 15 mM $MgCl_2$ (Mg^{2+} reaction buffer) or 5 mM EDTA (EDTA reaction buffer). The activity of NR was measured with an aliquot of the extract, plus 5 mM KNO_3 and 0.2 mM NADH and reaction buffer to give final volume of 1 mL. The reaction was started by adding an aliquot from the extract (0.3 mL) and after 5 min the reaction was terminated by adding 75 mM (final concentration) Zn Acetate. Excess NADH was removed by phenazine metho-sulfate (PMS) treatment and nitrite was measured colorimetrically (Hageman and Reed, 1980). The activation state of NR is defined as the ratio of NRA with Mg^{2+}/ NRA with EDTA.

2.3.2. In vitro assay of lactate dehydrogenase and alcohol dehydrogenase activities

Root samples were ground as described above and extracted with buffer containing 0.1 M Tris-HCl, pH 8.5, 10 mM Na borate (10 mM Na ascorbate for ADH), 10 mM DTT, 0.5% BSA and 15% (v/v) glycerol. The suspensions were then centrifuged for 10 min (16000g at 4°C), and the supernatant was used directly.

a) LDH was assayed in the pyruvate - lactate direction, according to equation (6), by monitoring pyruvate dependent NADH oxidation at 340 nm in the presence of 4-metyl pyrasole to inhibit ADH activity. The assay mixture contained 81.3 mM Tris-HCl, pH 8.0, 203.3 mM NaCl, 0.2 mM NADH, 10 µM 4-methylpyrazole, 10mM pyruvate and 0.1 mL extract. Final volume was 1 mL. Adding the extract started the reaction. A Sigma ZFP22 (Berlin, Germany) spectrometer and a SE120 (BBC, Goerz Metrawatt) recorder (1V, 1cm min^{-1}) were

used for monitoring the reaction. A RM6 Lauda water bath (Lauda Dr. R. Wobser GMBH & Co., Lauda – Königshofen, Germany) was connected to the spectrometer sample holder in order to achieve a constant temperature during the reaction (30^{o}C). Recording time was up to 15 min.

$$\text{(6) Pyruvate + NADH + H}^{+} \xrightarrow{\text{LDH}} \text{Lactate + NAD}^{+}$$

b) ADH was assayed as NADH oxidation during acetaldehyde reduction at 340nm, as shown in equation (7). The reaction mixture (final volume of 1 mL) contained 85 mM MES-KOH pH 6.5, 0.15 mM NADH, 5 mM acetaldehyde and 20 µL extract. Reaction was started by adding the extract. Recording conditions and equipment as described above.

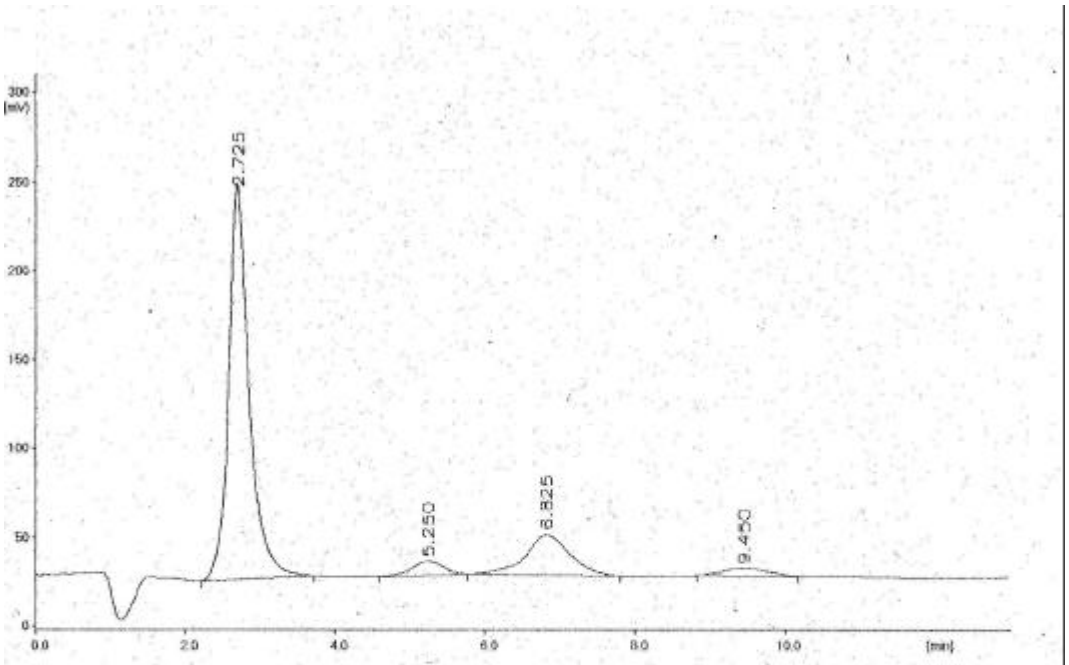
$$\text{(7) Acetaldehyde + NADH + H}^{+} \xrightarrow{\text{ADH}} \text{Ethanol + NAD}^{+}$$

2.4. Solute analysis

2.4.1. Anion determination

Anion content was determined in the root medium and in the roots. After suitable dilution, aliquots (1 mL from each sample) from the medium (total volume 5 mL) were immediately used for determination. Root segments (1 g FW) were removed from the medium, briefly washed in deionised water, blotted and ground in pre-cooled porcelain mortars with liquid nitrogen and the fine powder was suspended in 2 mL deionised water. After thawing they were boiled at 100^{o}C for 5 min in a heating block and insoluble materials were removed by centrifugation (10 000 g, 20 min), selected anions in the tissue (in the supernatant) and in the incubation medium were determined by using isocratic anion chromatography and suppressed conductivity detection (Biotronic, Maintal, Germany). The extraction procedure usually removed 95% or more of the total contents, as indicated by repeated extraction of the pellets. A mixture of 0.1 mM anions (chloride, nitrate, malate, phosphate, sulfate and oxalate) was used as a

standard. Chromatograms were processed (see below) with Winpeak-software (Chromatography Data System, Biotronic, Maintal, Germany). Detection limit was about 1 µM. Anion content is given in µmol g^{-1} FW.



Calculation Method : External Standard
Calibration Method : One Point
Weight: 100.00

Peak-No.	Win-No.	Ret.Time (min)	Area	Resp.-Fact.	Absolute (mM)	Weight (%)	Name
1	1	2.725	2229046	1.096E-007	2.444E-001	0.244	Chlorid
2	2	5.250	115225	1.755E-007	2.022E-002	0.020	Phosphat
3	3	6.825	496413	1.593E-007	7.906E-002	0.079	Nitrat
4	4	9.450	90845	8.537E-008	7.755E-003	0.008	Sulfat
No Peak found in time-window 9.6 to 10.8 (min)							
Totals:			2931529		3.514E-001	0.351	

2.4.2. Nitrite determination

Nitrite was determined colorimetrically in root extracts (prepared as described above) or in the bathing medium. After suitable dilution to a final volume of 1 mL the reagents according to (Hageman and Reed, 1980) were added. Nitrite content is given in µmol g^{-1} FW.

2.4.3. Cation determination

Root samples and medium were prepared as described in 'Anion determination' and after suitable dilution selected cations were analyzed by ICP

Spectrometer (Jobin Yvon JY 70 Plus, ISA Division d'instruments S.A.). Various cations at the stock concentration of 1000 mg L^{-1} and final concentration between 1 and 100 ppm (depending on natural occurrence of the particular cation in the plant tissue) were used as standards. Cation content is given in µmol g^{-1} FW.

2.5. Metabolite measurements

2.5.1. Sugars

Root samples and medium were prepared as described in 'Anion determination' and after suitable dilution sugars (glucose, fructose and sucrose) were analyzed by using isocratic ion chromatography with pulsed amperometric detection (4500i, Dionex, Idstein, Germany). A mixture of 0.1 mM glucose, fructose, sucrose, arabinose and manitol was used as a standard. In most cases arabinose and manitol contents of the samples were below detection limit. Occasionally, samples were pretreated with mixed-bed ion exchanger (Serdolit MB -1, Boehringer, Ingelheim, Germany) prior to sugar analysis in order to remove charged molecules like amino acids, which also gave some amperometric signals and co-migrated with certain sugars. In any case, standards were either subjected to the same treatments as the samples, or were added as internal standards. Here again chromatograms were processed with Winpeak-software (Chromatography Data System, Biotronic, Maintal, Germany). Detection limit was below 1 µM. Sugar contents are given as µmol g^{-1} FW.

2.5.2. Sugar phosphates

Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) levels were determined enzymatically in extracts from roots subjected to normoxia or anoxia as described above. The principle is to oxidise G6P in the presence of glucose-6-phosphate dehydrogenase (G6P-DH) and $NADP^+$ (8) and subsequently to convert F6P into G6P by adding G6P isomerase (P-glucose isomerase, PGI), continuously following NADPH formation at 366 nm (9).

$$\text{(8) G6P + NADPH}^+ \xrightarrow{\text{G6P-DH}} \text{gluconolactone-6-P + NADPH + H}^+$$

$$\text{(9) F6P} \xleftrightarrow{\text{PGI}} \text{G6P, catalyzed by PGI}$$

Frozen root samples were ground in mortars with liquid nitrogen. 2 mL 4.5% $HClO_4$ were added to each gram root FW. After centrifugation at 10 000g for 10 min, 60 mM Tris and 250 mM K_2CO_3 (final concentrations) were added to 1 mL of the supernatant in order to neutralize the samples to pH 6.5. The actual reaction mixture (1 mL total volume) contained 100 mM Hepes-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ and 50 µL extract. The G6P determination was started by adding 1U G6P-DH. After 10 to 15 min F6P was determined by adding 0.7U phospho-glucose isomerase to the same mixture.

2.5.3. Starch assay

One g frozen root material (per sample) was ground in liquid nitrogen and 2 mL of deionised H_2O were added. The resulting suspensions were centrifuged at 16 000 g for 10 min. The pellet was then frozen and, after thawing, washed twice with cold deionised H_2O. 950 µL of deionised H_2O and 1.5U amyloglucosidase (total volume 1 mL) were added to the pellet. Samples were incubated for 2h at 37°C and subsequently boiled at 105°C for 3 min, then centrifuged at 16 000g for 10 min. Glucose was analyzed as in 2.5.1.

2.5.4. Adenine nucleotides

Root samples (1g FW) were ground in precooled porcelain mortars and 2 mL 5% TCA was added to the deep-frozen tissue powder. After 30 min on ice the suspension was cleared by centrifugation (10 min, 4°C, 10 000 g). The supernatant was then washed 3 times with diethylether and after removal of the ether phase the water phase was adjusted to pH 6.5-7.5 with 1 M NaOH. Adenine nucleotides (ATP, ADP and AMP) in the aqueous phase were determined luminometrically with the 'firefly' luciferin-luciferase assay (Boehringer, Mannheim, Germany) in a Lumat LB 9501 (Berthold, Wildbad, Germany) (equations 10,11,12).

$$\text{(10) Luciferin + ATP} \rightarrow \text{Luciferyl adenylate + PPi}$$

$$\text{(11) Luciferyl adenylate} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + CO_2 + \text{AMP} + h\nu$$

$$\text{(12) Oxyluciferin} \rightarrow\rightarrow\rightarrow \text{Luciferin (regenerating reactions)}$$

ATP was measured directly or after conversion of ADP and AMP to ATP using pyruvate kinase or pyruvate kinase plus myokinase. The conversion assay was carried out in a heating block at 37°C for 30 min. The conversion buffer was 50 mM Hepes-KOH pH 7.6 plus 20 mM $MgCl_2$, 250 µM PEP diluted in 133 mM $MgCl_2$ and 266 mM KCl. Pyruvate kinase (200U/mL), myokinase (500U/mL) and 0.2 mL of root extract per sample (total volume 1 mL) were used. For the reaction assay an aliquot of 50 µL of conversion assay was added to 0.6 mL of reaction buffer (50 mM Hepes-KOH pH 7.6 plus 5 mM $MgCl_2$) in the presence of 0.02 mL firefly.

2.5.5. L-Lactate

Samples from the medium and root extracts were prepared as described in 'Anion determination', except that 5% PVPP was added to the extracts. A commercial kit (Boehringer, Mannheim, Germany) was used for the enzymatic determination of the L-lactate content, according to the manufacturer's protocol. The principle of the method is as follows:

L-lactic acid (L-lactate) is oxydased by nicotinamide-adenine-dinucleotide (NAD) in the presence of L-lactate dehydrogenase (L-LDH) to pyruvate (13).

$$\text{(13) L-Lactate} + NAD^+ \xleftrightarrow{\text{L-LDH}} \text{pyruvate} + NADH + H^+$$

The equilibrium of this reaction lies almost completely on the side of L-lactate. However, by trapping pyruvate in a subsequent reaction catalyzed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate, the equilibrium can be displaced in favor of pyruvate and NADH (14).

$$\text{(14)}\quad \text{Pyruvate + L-glutamate} \xleftrightarrow{\text{GPT}} \text{L-alanine + 2-oxoglutarate}$$

The amount of NADH formed in the above reaction is stoichiometric to the amount of L-lactic acid. The increase in NADH is determined by means of its light absorbance at 340 nm.

2.5.6. Ethanol

Samples from the root medium (where more than 90% of the ethanol was found) were prepared as described in 'Anion determination' except that 5% PVPP was added to the extracts. A commercial kit (Boehringer, Mannheim, Germany) was used for the enzymatic determination of the ethanol content, according to the manufacturer's protocol. The principle of the method is as follows:

Ethanol is oxidised to acetaldehyde in the presence of the enzyme alcohol dehidrogenase (ADH) by nicotinamide-adenine dinucleotide (NAD^+) (15)

$$\text{(15) Ethanol + } NAD^+ \xleftrightarrow{\text{ADH}} \text{acetaldehyde + NADH + } H^+$$

The equilibrium of this reaction lies on the side of ethanol and NAD^+. It can, however, be completely displaced to the right at alkaline conditions and by trapping of the acetaldehyde formed. Acetaldehyde is quantitatively oxidized to acetic acid in the presence of aldehyde dehydrognenase (Al-DH) (16).

$$\text{(16)}\quad \text{Acetaldehyde + } NAD^+ + H_2O \xleftrightarrow{\text{Al-DH}} \text{acetic acid + NADH + } H^+$$

NADH accumulation is determined by means of its light absorbance at 340 nm.

2.5.7. Amino acids

Samples from root and medium were prepared as described in 'Anion determination' and after suitable dilution amino acid content was analyzed by

using a LC 501 analyzer (Biotronic, Maintal, Germany). Root extracts were diluted with lithium citrate buffer (100 mM lithiumcitrate, 68.5 mM citric acid, 0.5% thiodiethanol, pH 2.2). Levels of eluted amino acids were determined photometrically at 570 nm (post column reaction with ninhydrine). For the standard, a mixture of amino acids (200 µM each) was used. The amino acid content is given in µmol g^{-1} FW.

2.6. Total protein

The protein content of root extracts was determined with the BCA reagent (bicinchoninic acid, Pierce, Rockford, Illinois, USA). Bovine serum albumin was used as a standard.

2.7. Oxygen consumption of roots

O_2 consumption was measured in solution with a commercial oxygen electrode (Hansatech, England). Calibration of the electrode was made with $Na_2S_2O_4$ as 0 point and with air saturated water (21% O_2). All measurements were performed at 25°C. Two cm long root parts, from tip section of the root (four to six per root system) were submerged in 10 mM $CaSO_4$ and O_2 consumption was recorded for up to 20 min. The data corresponding to a single root system (on a FW basis) were pooled before statistical analysis. Up to eight plants from different generations at growth stage 8 to 10 weeks were used.

2.8. Root morphology

Image analysis was employed for comparing the root architecture of LNR and WT tobacco plants. Five replicate plants of each type were used. From each plant four to six representative secondary roots were removed at the point of origin, blotted with tissue paper and weighed. Each root portion was submerged in deionised water (in a transparent tray) and carefully spread in order to avoid any overlapping. The sample was then scanned on a desktop scanner (AGFA SNAP

SCAN 1236), the images were recorded and analyzed using WinRHIZO 4.1 software (Regent Instrument Inc., Blain St, Quebec, Canada). Data from root portions corresponding to a single plant were pooled before statistical analysis.

2.9. ^{31}P NMR spectroscopy

^{31}P NMR spectroscopy on WT, 35S-NR and LNR-H tobacco plants has been carried out under supervision of Prof. G R Ratcliffe, at Department of Plant Physiology, University of Oxford, Oxford, England. Approximately 1 g FW of root tissue, in the form of 5 mm root segments taken from a single plant, were transferred to a 10 mm diameter NMR tube containing 50 mM sucrose, 10 mM MES, 0.1 mM $CaCl_2$ at pH 6.3. Immediately prior to this, the excised root segments were vacuum-infiltrated for 5 min in the same medium to eliminate intercellular air spaces that would otherwise have caused a line broadening effect on the NMR signals. A combined air-lift and circulation system was used to circulate the suspending medium through the NMR tube and to supply the tissue with either oxygen or nitrogen (Fox et al., 1995). In a typical experiment, NMR spectra were recorded for 3 h under normoxia, followed by 3 h under anoxia and a further 3 h under normoxia. The sample preparation was timed so that the anoxic period coincided with the expected maximum in the NR activity in the early afternoon. *In vivo* ^{1}H-decoupled ^{31}P NMR spectra were acquired at 121.49 MHz using a double tuned ^{13}C/^{31}P 10 mm diameter probehead on a Bruker CXP 300 NMR spectrometer equipped with an Oxford Instruments 7.05 T superconducting magnet. The spectra were recorded in blocks of 3600 scans using a 45º pulse angle and a recylce time of 0.5 s. The chemical shift of the cytoplasmic inorganic phosphate (P_i) signal was measured relative to the signal at 22.49 ppm from a capillary containing a 2% (v/v) solution of the tetraethyl ester of methylene diphosphonic acid and the corresponding cytoplasmic pH was estimated using a calibration curve described elsewhere (Spickett et al., 1993). It was usually necessary to add three consecutive 30 min spectra to give a reliable estimate of the chemical shift at a particular time point.

2.10. NO measurements

NO gas emission of WT and LNR-H tobacco plants (leaves and detached roots) were measured with a chemiluminescence analyzer ECO PHYSICS's CLD 770 AL ppt, Durnten, Switzerland, detection limit 20 ppt, 1 min resolution. According to U.S. Environmental Protection Agency, the reference measurement principle for nitrogen dioxide (NO_2) is the gas phase chemiluminescent reaction of nitrite oxide (NO) with ozone. NO is measured directly, NO_2 indirectly. The NO_2 to NO reduction is achieved by the use of a converter. The reactions of NO with an excess amount of O_3 are as follows:

(17) $NO + O_3 \rightarrow NO_2 + O_2$

(18) $NO + O_3 \rightarrow NO_2^* + O_2$

(19) $NO_2^* \rightarrow NO_2 + h\nu$

(20) $NO_2^* + M \rightarrow NO_2 + M$

NO2* - the excited nitrogen gas

M – deactivating colliding partners (i.e. N_2, O_2, H_2O)

The spontaneous deactivation of NO_2 occurs with emission of light (19). By far the larger fraction of NO_2^* loses its extraction energy without light emission by colliding with other molecules (M) (20). In order to achieve a high yield of light the reaction of NO with O_3 needs to take place under low pressure. The light intensity produced by chemiluminescent reactions (18) and (19) is proportional to the mixing ratio of NO. A photo amplifier tube is used to convert the light energy emitted from (19) into electrical impulses. A counter counts the electrical impulses over a chosen time interval and a microprocessor calculates the signal in ppbv. A customer made software based on Visual Designer (PCI-20901S, Ver. 4.0, Tuscon, Arisona, USA) was used to process the data.

When NO emission of tobacco leaves (WT and LNR-H) was measured, the leaves were harvested 6h into the light period and placed in a beaker containing 50 ml deionised water. The beaker was put into a transparent lid chamber connected to the CLD 770 AL ppt. The flow rate was 1.75 L min^{-1}. NO

emission was recorded in light or dark conditions in air or in nitrogen atmosphere for up to 4 hours.

In the case of determining root NO emission, tobacco roots of WT and LNR-H plants were harvested 6h into the light phase. After a quick double rinse with deionised water and subsequent blotting, a number of secondary roots from 4 plants (usually up to 3 g FW), detached at the point of origin, were placed into a chamber connected to the CLD 770 AL ppt in a Petry dish containing moistened (with deionised H_2O) tissue paper (to prevent roots from drying). NO emission was monitored in dark in air or nitrogen atmosphere. Records were made for up to 4 hours.

2.11. Porometry

A cycling AP4 Porometer (Delta –T devices Ltd., Cambridge, UK) was used to determine transpiration of LNR and WT tobacco leaves. Two to four repeated measurements for each leaf were carried out. First and second fully developed leaves were used. Control measurements were taken at the beginning of the light period after witch eight intact hydroponic plants (four WT and four LNR) were subjected to anoxia via intensive flushing of their root system with nitrogen. Leaf transpiration was measured at the times indicated. All measurements were performed under standard growth conditions.

2.12. List of chemicals

Chemicals	Company
Adenosine 5’ diphosphate (ADP), potassium salt	Sigma (Deisenhofen)
Adenosine 5’ monophosphate (AMP), disodium salt	Boehringer (Mannheim)
Adenosine 5’ triphosphate (ATP), disodium salt	Sigma (Deisenhofen)
Amiloglucosidase	Boehringer (Mannheim)
Amino acids as standard for LC 501 analyzer	Alfa (Karlsruhe)
Antibiotic, antimycotic solution	Sigma (Steinheim)
Bovine serum albumin (BSA)	Biomol (Hamburg)
Cantharidin	Biomol (Hamburg)
Citric acid	Merck (Darmstadt)
Di-ethyl-ether	AppliChem (Darmstadt)
DMSO	Sigma (Hamburg)
DTT (1, 4-ditiotritol)	Biomol (Hamburg)
EDTA	Merck (Darmstadt)
Firefly	Sigma (Steinheim)
Flavine adenine dinucleotide	Sigma (Deisenhofen)
Fructose	Merck (Darmstadt)
Fructose-6-phosphate	Merck (Darmstadt)
Glucose	Merck (Darmstadt)
Glucose-6-phosphate	Boehringer (Mannheim)
Glutamine	Sigma (Deisenhofen)
Glycerol	Sigma (Steinheim)
Glycine	Sigma (Deisenhofen)
Hepes	Roth (Karlsruhe)
Leupeptin	Biomol (Hamburg)
L-Glutamate dehydrogenase	Sigma (Steinheim)

L-Glutamic acid	Sigma (Deisenhofen)
Lithium citrate	Merck (Darmstadt)
L-lactate dehydrogenase (LDH)	Boehringer (Mannheim)
Mes	Biomol (Hamburg)
MP (4-methyl-pyrasole)	Aldrich Chem Co.
Myokinase	Boehringer (Mannheim)
N-(1-naphthyl)-ethylene-dihydrochloride	Merck (Darmstadt)
$Na_2S_2O_4$	Merck (Darmstadt)
Na-borate	Sigma (Deisenhofen)
NADH	Biomol (Hamburg)
NADH	Biomol (Hamburg)
NADP	Biomol (Hamburg)
Pefablock	Biomol (Hamburg)
Perchloric acid	Merck (Darmstadt)
Phenacin-meta-sulfonate (PMS)	Sigma (Deisenhofen)
Phosphoenolpyruvate (PEP)	Sigma (Deisenhofen)
Phospho-glucose-isomerase	Boehringer (Mannheim)
Poly-vinyl-poly-pyrolidone (PVPP)	Sigma (Deisenhofen)
Poly-vinyl-pyrolidone (PVP)	Serva (Ingelheim)
Pyruvate kinase (PK)	Boehringer (Mannheim)
Pyruvic acid, sodium salt	Sigma (St.Louis)
Sephadex-G25	Sigma (Deisenhofen)
Serdolit MB-1	Boehringer (Ingolheim)
Sucrose	Merck (Darmstadt)
Sulfanilamide	Serva (Heidelberg)
TCA (3-chloro-acetic acid)	Merck (Darmstadt)
Thio-di-ethanol	Merck (Darmstadt)
Tris	Biomol (Hamburg)
Tungstate, Sodium salt	Sigma (Steinheim)

Urea	Roth (Karlsruhe)
Zn-acetate	Merck (Darmstadt)

3. Results

3.1. Characterization of normoxic metabolism of LNR-H tobacco plants in comparison to WT.

3.1.1. Root morphology and root:shoot ratio

LNR-H root systems were usually shorter than roots of WT of similar age (Figure 4).

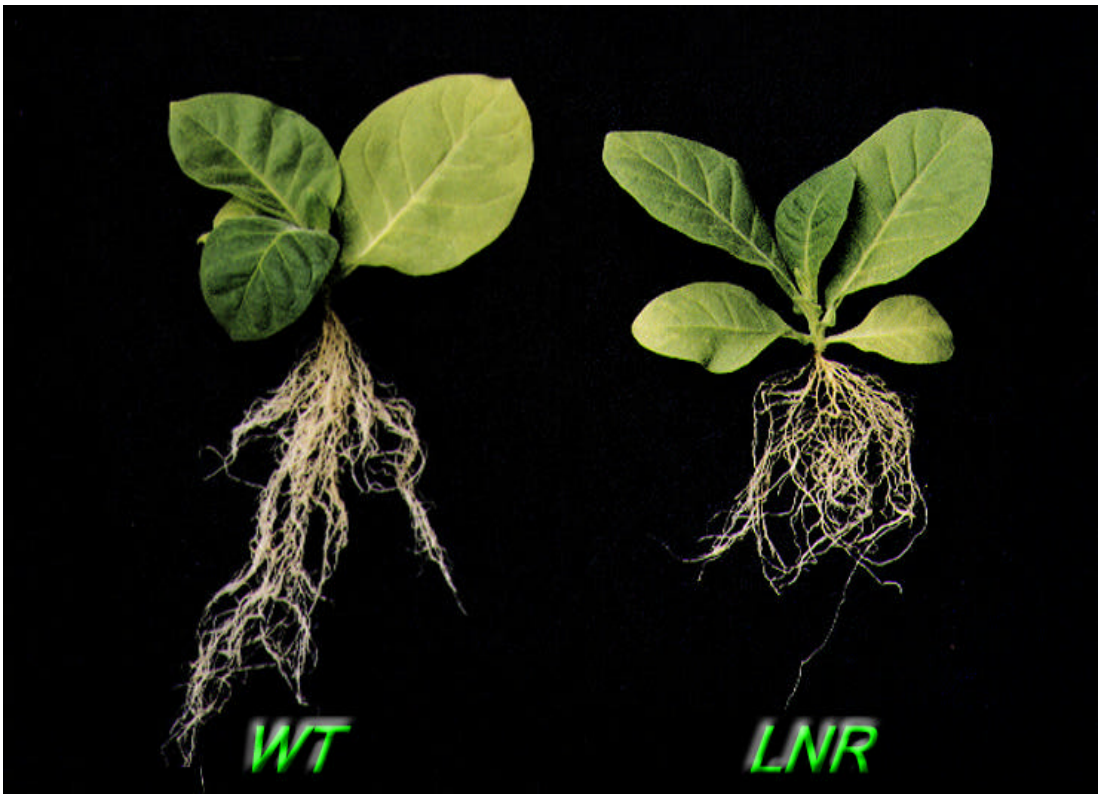


***Figure 4**: Size and root system of 5 week old WT plant and 6 week old LNR-H tobacco plants.*

Mean shoot FW of LNR-H plants was about 58% of the FW of WT (Table 1). Root FW per LNR-H plant was only one third of WT. Accordingly, the root/shoot FW-ratio of LNR-H was only 0.2 as compared to 0.3 for WT. Surprisingly, the fresh weight:dry weight ratio was also different (higher in WT) (Table 1).

Table 1: *Fresh and dry weight of root and shoot systems of WT and LNR-H plants (g/plant), root/shoot ratio and FW/DW ratio. Data are means of six to twelve plants each ± SD.*

	WT	**LNR-H**
Shoot FW	57.1 ± 27.1	33.4 ± 12.9
Root FW	14.7 ± 6.20	5.1 ± 2.1
Root:Shoot ratio	0.3 ± 0.02	0.2 ± 0.03
FW:Dry W ratio	27.5 ± 1.60	17.1 ± 3.14

Data from a closer analysis of root morphology of the two tobacco lines are summarized in Figure 5. Relative surface area, projected area, length, average diameter, root tips per plant and root surface: g leaf FW were measured of roots from 8 to 10 weeks old LNR-H and WT plants. On average, the root surface area and projected area per plant were 2.5 fold higher for WT than for LNR-H (Figure 5A, B). The average diameter was 0.424 mm for the LNR-H roots and 0.367 for the WT roots (Figure 5C). The length per root system was 1924 cm for LNR-H and 4815 cm for WT (Figure 5D). WT showed higher number of root tips per plant (Figure 5E).

Importantly, root surface per g leaf FW was 10.6 in WT but only 7.8 in LNR-H (Figure 5F).

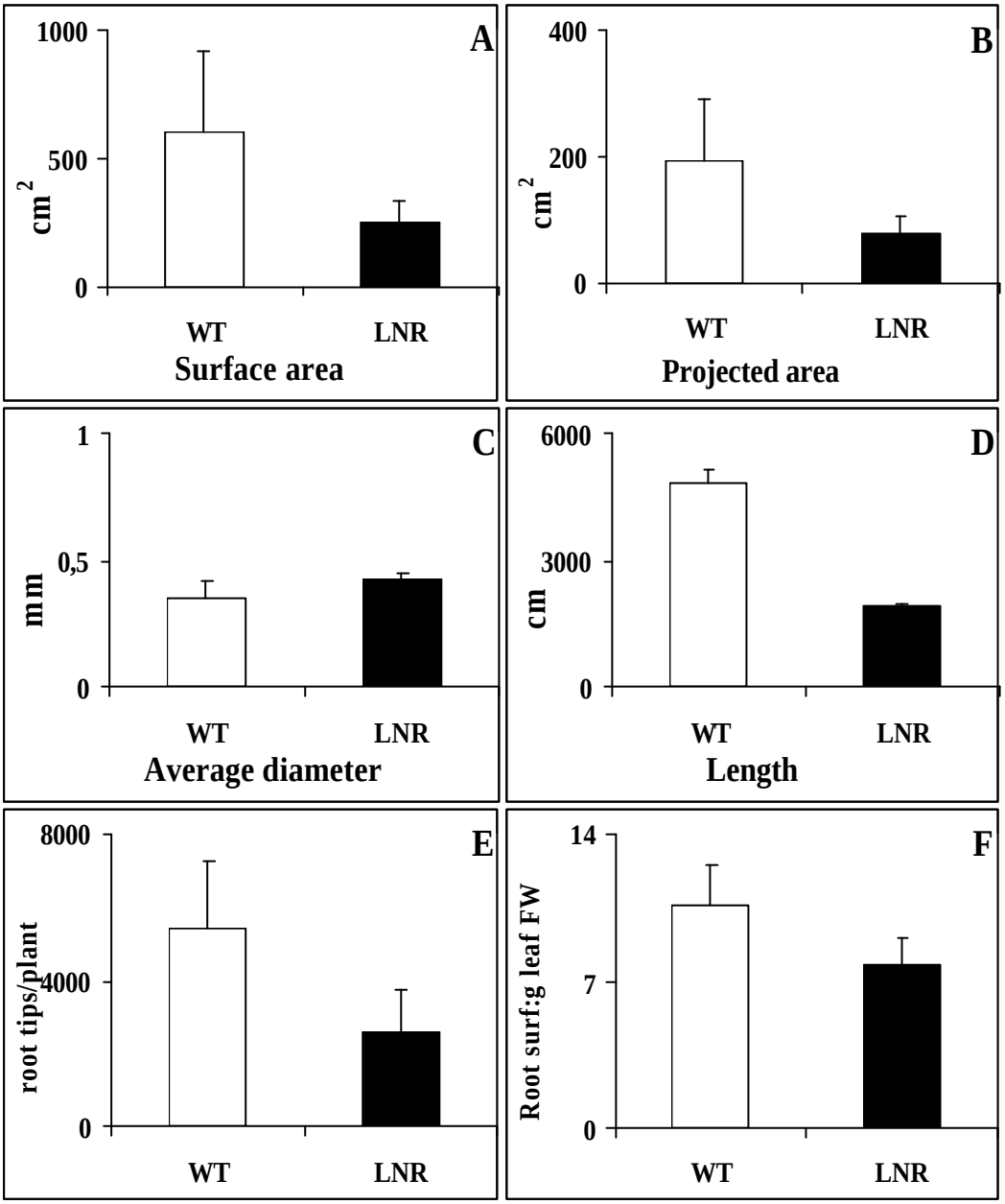


Figure 5: *Root architecture of eight to ten week old WT and LNR-H plants. Results are means of four to six root systems each.* ***A.*** *Root surface area per plant (cm^2).* ***B.*** *Root projected area per plant (cm^2).* ***C.*** *Root average diameter (mm).* ***D.*** *Root length per plant (cm).* ***E.*** *Root tips per plant.* ***F.*** *Ratio of root surface: g leaf FW.*

3.1.2. Porometry

When measured 2h into the light period, WT leaves generally transpired more (per leaf area) than LNR-H leaves (Figure 6).

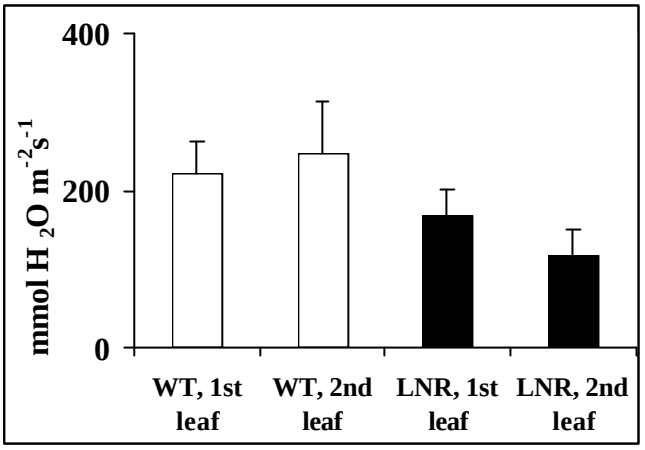


Figure 5: *Specific transpiration of the first and second fully developed leaf of WT and LNR-H (mmol H_2O $m^{-2}s^{-1}$). Data are means of four subsequent measurements per leaf of up to leaves six each. All measurements were carried out under standard growth conditions.*

3.1.3. Anion and cation content

In previous investigations where ammonium grown plants were compared with nitrate grown plants, the different nitrogen sources often caused drastic changes in anion and cation contents (Botrel et al., 1996; Botrel and Kaiser, 1997; Lang and Kaiser, 1994). One reason for comparing the anoxic response of WT and LNR-H tobacco plants was that the ion contents and cation/anion balance might be less different than with ammonium versus nitrate grown plants. Indeed, in spite of the different root morphology, no significant differences were found in the chloride, phosphate, nitrate, Mg, Ca, Na, K, S, B, Zn, P, Mn, Fe, Cu and Al content of root systems from both types of tobacco. Only the sulfate content was somewhat higher, and the malate content somewhat lower in WT roots than in LNR-H roots (Table 2).

Table 2: *Anion and cation contents in roots of WT and LNR-H plants. Data (µmol g^{-1} FW ± SD) are means of eight plants each. All samples were collected 6 h into light phase and immediately frozen in liquid nitrogen as described in Material and Methods.*

Anions	**WT**	**LNR-H**
Chloride	24.8 ± 6.90	23.7 ± 4.60
Phosphate	4.98 ± 1.22	4.23 ± 1.36
Nitrate	68.07 ± 15.40	66.42 ± 12.99
Sulfate	6.17 ± 1.66	1.68 ± 0.69
Malate	2.51 ± 1.58	5.52 ± 2.36
S (-)	**106.48**	**101.41**
Cations		
Mg	6.17 ± 1.68	4.92 ± 1.82
Ca	0.09 ± 0.02	0.11 ± 0.09
Na	1.15 ± 0.59	0.89 ± 0.31
K	81.89 ± 19.88	94.61 ± 21.69
S	5.53 ± 1.99	8.04 ± 2.21
B	0.02 ± 0.01	0.02 ± 0.01
Zn	0.13 ± 0.03	0.14 ± 0.04
P	11.54 ± 3.97	10.91 ± 3.15
Mn	0.72 ± 0.16	1.11 ± 0.30
Fe	0.35 ± 0.23	0.30 ± 0.11
Cu	0.05 ± 0.01	0.08 ± 0.02
Al	0.03 ± 0.01	0.01 ± 0.01
S (+)	**107.7**	**121.15**

3.1.4. Respiration

Respiration was measured with small (2 cm long) root segments in solution and under continuous stirring. In that system, LNR-H plants consumed oxygen at almost twice the rate compared to WT (Table 3).

Table 3: *Root oxygen consumption of WT and LNR-H plants. Data of oxygen consumption (µmol O_2 g^{-1} FW h^{-1} ± SD) are means of up to eight plants each. Other conditions as in Table 2.*

	WT	**LNR-H**
Oxygen consumption	28.11 ± 9.49	54.67 ± 7.45

3.1.5. Nucleotide levels

Higher rates of oxygen uptake of LNR-H roots as compared to WT roots are not necessarily indicative of a higher ATP production, since it is not known to what extent alternative respiration (AOX) might contribute to total oxygen uptake nor how tight the coupling is. Therefore ATP, ADP and AMP levels in LNR-H and WT tobacco roots harvested freshly 6h into the light phase were also measured. Generally, ATP levels were by far higher than levels of ADP and AMP. In LNR-H roots (on a FW basis) all adenine nucleotide contents where higher than in WT, but ATP/ADP ratios were practically identical (Table 4), suggesting a similar energy status.

Table 4: *Adenine nucleotide levels in WT and LNR-H plants. Nucleotide levels (µmol g^{-1} FW± SD) are means of eight plants each. Other conditions as in Table 2.*

Nucleotide levels	**WT**	**LNR-H**
ATP	0.099 ± 0.068	0.238 ± 0.064
ADP	0.011 ± 0.013	0.025 ± 0.030
AMP	0.003 ± 0.030	0.037 ± 0.066
? ATP+ADP+AMP	0.112	0.299
ATP:ADP ratio	9.4	9.5

3.1.6. Metabolite content in WT and LNR-H roots

Total sugars (glucose, fructose and sucrose content combined) were not significantly different in freshly harvested roots from the two lines. However, hexose monophosphate (G6P and F6P) and starch content in LNR-H roots were twice as high as in WT (Table 4a).

Table 4a: *Total sugars (glucose, fructose and sucrose), glucose-6-phosphate, fructose-6-phosphate and starch content in roots of WT and LNR-H (µmol g^{-1} FW ± SD). Data shown are means of eight to twelve plants each. Other conditions as in Table 2.*

	WT	**LNR-H**
Total sugars	9.66 ± 2.32	12.85 ± 3.53
G6P	0.114 ± 0.028	0.285 ± 0.064
F6P	0.062 ± 0.027	0.112 ± 0.022
Starch	0.18 ± 0.05	0.38 ± 0.05

3.1.7. Amino acids and total protein content

As nitrate reduction in LNR-H roots was lacking (Hänsch et al., 2001), these root systems depend on translocation of amino-N from the shoots. It was therefore of interest to compare the total protein and amino acid content of the root systems. A similar analysis has already been carried out (Hänsch et al. 2001). However, due to the different growth conditions applied it seemed necessary to repeat a comprehensive analysis. Our previous experiments had shown that LNR-H roots had significantly lower contents of soluble amino-N on a FW basis, and lower contents of the major amino acids Glu and Gln. No differences in total protein content of roots from LNR-H or WT were observed (Hänsch et al. 2001). Surprisingly, under our conditions the total amino acid content (sum of all measured single amino acids) of LNR-H roots was almost twice as high as in WT (Table 5). That difference between both lines was mainly due to higher levels of Thr, Ser, Asn, Met, Ile, Tyr, Gaba, His and Arg. Confirming our previous

investigation, however, total soluble protein contents were almost identical in both lines. Overall the data indicate that LNR-H roots certainly did not suffer from N-deficiency, in spite of lacking nitrate reduction.

Table 5: *Amino N (µmol g^{-1} FW ± SD) and total protein content (mg g^{-1} FW ± SD) in roots of WT and LNR-H plants. The first group represents those amino acids where contents were similar in both tobacco lines. The second group summarizes those, which were significantly different in WT and LNR-H. The 'total amino acid content' is the sum of all measured single amino acids. Data shown are means of four to eight plants each. n.d. - not detectable*

Amino acids	**WT**	**LNR-H**
Pro	0.06 ± 0.01	0.10 ± 0.12
Pser	0.03 ± 0.01	0.06 ± 0.03
Tau	0.01 ± 0.01	0.04 ± 0.02
Asp	0.32 ± 0.05	0.39 ± 0.21
Glu	0.27 ± 0.04	0.34 ± 0.13
Gln	1.03 ± 0.17	0.67 ± 0.36
Gly	0.04 ± 0.01	0.09 ± 0.04
Ala	0.1 ± 0.03	0.18 ± 0.09
Citr	0.01 ± 0.01	0.01 ± 0.01
Val	0.02 ± 0.01	0.03 ± 0.01
Phe	0.05 ± 0.03	0.14 ± 0.06
Orn	n.d.	n.d.
Lys	0.03 ± 0.01	0.09 ± 0.05
Ammonium	0.41 ± 0.09	0.43 ± 0.01
Thr	0.07 ± 0.01	0.31 ± 0.02
Ser	0.20 ± 0.04	0.67 ± 0.21
Asn	0.04 ± 0.01	0.11 ± 0.05
Met	0.01 ± 0.01	0.08 ± 0.04
Ile	0.010 ± 0.002	0.04 ± 0.02
Leu	0.21 ± 0.10	0.06 ± 0.02
Tyr	n.d.	0.03 ± 0.02
Gaba	0.730 ± 0.170	1.66 ± 0.53
His	0.020 ± 0.003	0.06 ± 0.02
Arg	0.020 ± 0.001	0.05 ± 0.02
Total amino acids	3.47 ± 0.73	6.31 ± 3.05
Total Protein	16.91 ± 0.52	16.12 ± 0.88

3.2. Anoxic metabolism of LNR-H and WT tobacco plants

3.2.1. Whole plant responses to short term anoxia in the root system

At the whole plant level there was an obvious difference between the response of the WT and LNR-H plants to the imposition of anoxia on the root system: LNR-H plants displayed symptoms of wilting just 2 h after the onset of anoxia (Figure 7) whereas WT plants developed the first wilting symptoms much later.

Figure 7: *Hydroponic WT (white pot) and LNR-H (black pot) plants after 2 hours in anoxic nutrient solution. Picture was taken 2h into the light phase.*

Measurements of transpiration rates showed that the LNR-H leaves continued to transpire at a lower rate than the WT leaves in plants with anoxic root systems and that the imposition of anoxia caused an initial increase in transpiration, followed by a sharp decline after 2 h and then a slow return to the initial value (Figure 8).

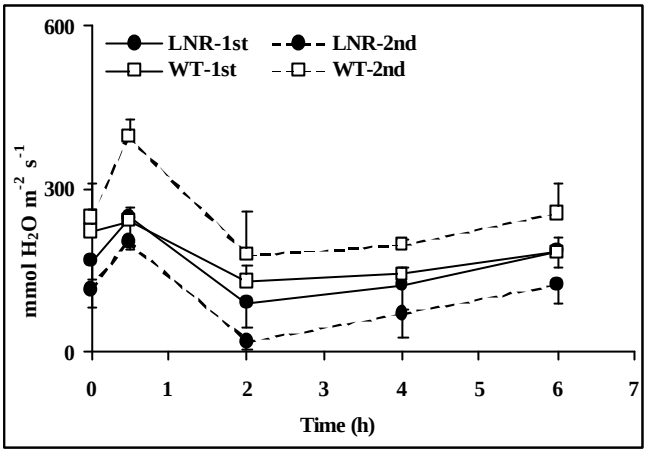


***Figure 8**: Specific transpiration (mmol H_2O m^{-2} s^{-1} ± SD) of the first two fully developed leaves of intact WT and LNR-H tobacco plants in anoxic nutrient solution. Time 0 corresponds to the beginning of the light phase. At the same time the root medium was made anoxic by intensive flushing with pre-moisturized nitrogen. Data are the means of four plants.*

3.2.2. Nitrate reductase activity and nitrite production in vivo

As expected (Hänsch et al., 2001) LNR-H root segments had no detectable soluble NR activity. In contrast, NR was present in extracts prepared from WT root segments, allowing measurements of NR_{act}, NR_{max} and the NR activation state (Figure 9). Under normoxia, NR_{act} in detached WT root segments decreased slightly during incubation, whereas NR_{max}, which indicates total NR protein, increased. Accordingly, the NR activation state decreased during normoxic incubation (Figure 9C). In contrast, NR_{act} and NR_{max} both increased under anoxia. The NR activation state in the excised root segments also increased under these conditions, in agreement

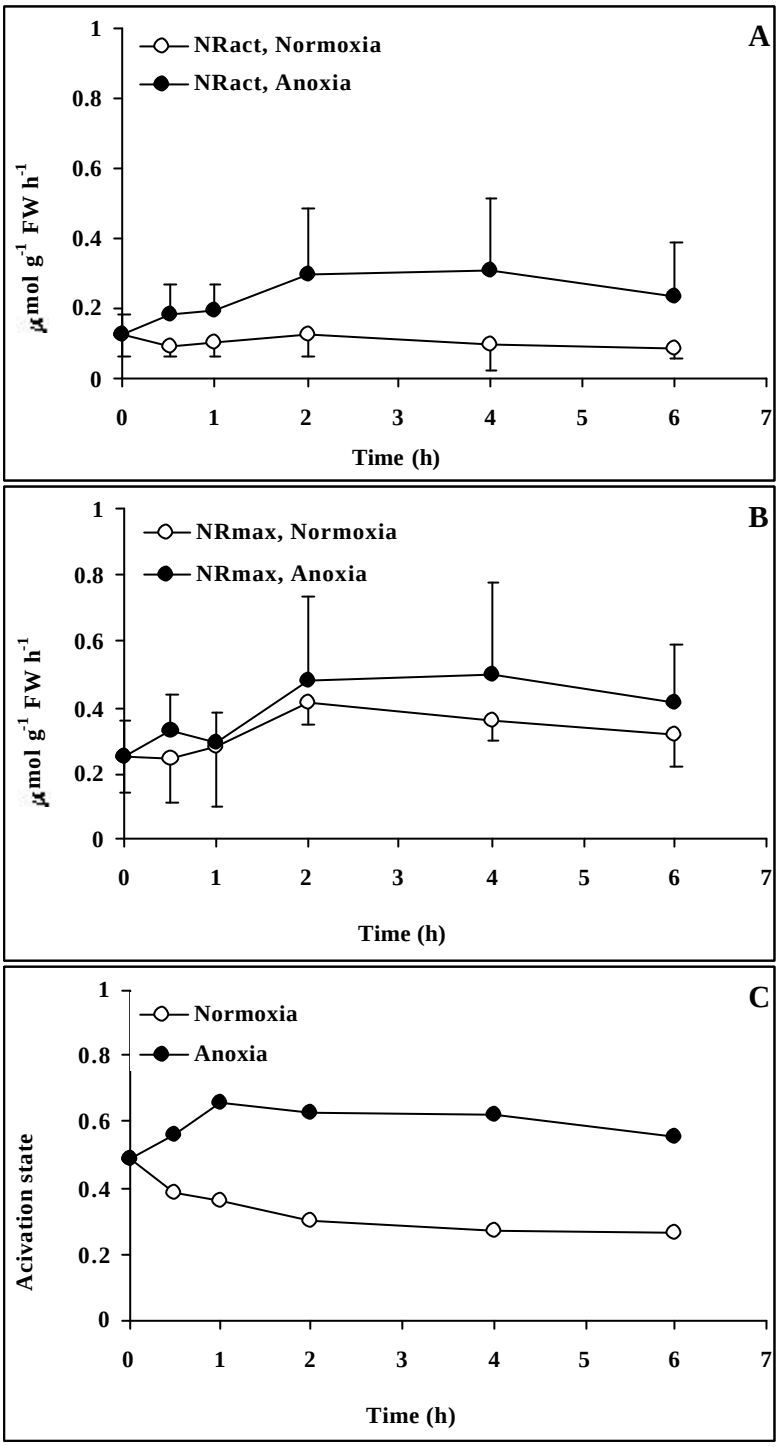


Figure 9: *(A) NR_{act} and (B) NR_{max} in extracts from normoxic (?) and anoxic (?) WT roots. Time zero was 6 h into the light phase, corresponding to the highest activity of the enzyme in the roots. Data (µmol g^{-1} FW h^{-1} ± SD) are means of samples from 10 plants. (C) Activation state (NR_{act}/NR_{max}) of NR in WT and LNR-H roots during the normoxic and anoxic incubations.*

with observations on anoxic barley roots (Botrel et al. 1996, Botrel and Kaiser, 1997).

In vivo nitrite production was also followed by measuring nitrite contents of root extracts and of the root medium. Under normoxia, WT roots and medium contained very little nitrite, and in LNR roots nitrite was hardly detectable. Under anoxia, nitrite production of WT roots increased strongly, as indicated by nitrite accumulation in root extracts and in the medium. Anoxic LNR roots also produced some nitrite (mainly found in the medium), though much less than WT roots (Table 6).

Table 6: *Nitrite content in roots and medium of WT and LNR-H tobacco plants (mmol g^{-1} FW). Data are the means of four plants. Control samples (Time 0) were taken six hours into the light period. Normoxic and anoxic samples were incubated for one and four hours. 'n.d.' – not detectable.*

Nitrite	**Medium**		**Roots**	
	WT	**LNR-H**	**WT**	**LNR-H**
Normoxia				
Time 0	n.d.	n.d.	0.021 ± 0.015	0.003 ± 0.007
1h	0.054 ± 0.023	0.020 ± 0.007	0.007 ± 0.005	0.002 ± 0.004
4h	0.054 ± 0.040	0.021 ± 0.005	0.012 ± 0.011	0.003 ± 0.003
Anoxia				
Time 0	n.d.	n.d.	0.021 ± 0.015	0.003 ± 0.007
1h	0.427 ± 0.048	0.059 ± 0.004	0.092 ± 0.015	0.005 ± 0.008
4h	0.293 ± 0.038	0.095 ± 0.051	0.123 ± 0.082	0.004 ± 0.007

3.2.3. Ethanol production

Ethanol was measured in the medium (Figure 10), where more than 90% of the total ethanol was usually found. LNR-H root segments produced slightly more ethanol than WT root segments under normoxic conditions (Figure 10A) and substantially more under anoxia (Figure 10B).

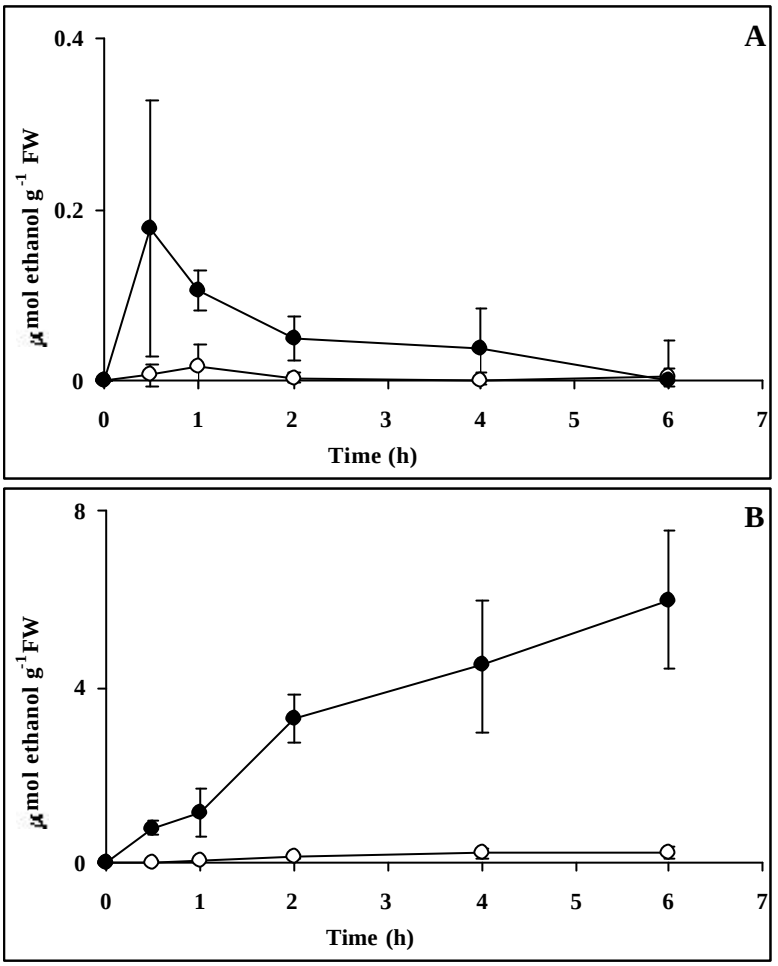


Figure 10: *Ethanol concentration (µmol g^{-1} FW ± SD) in the root medium of (A) normoxic and (B) anoxic WT (?) and LNR-H (?) root segments. The medium volume was 5 mL g^{-1} FW and the data are the means of eight plants.*

3.2.4. Lactate production

Similar results as for ethanol were obtained for the lactate in the medium (Figure 11), with the LNR-H roots again showing a much greater capacity to produce lactate than the WT roots. The lactate content of the roots themselves remained approximately constant under normoxia (Figure 12A), with a higher level in the LNR-H roots than the WT roots, but with the onset of anoxia there was an immediate and substantial increase in the lactate content of the LNR-H roots (Figure 12B), which leveled off with time. After the first two hours of anoxia newly formed lactate was mainly found in the root medium (Figure 11B).

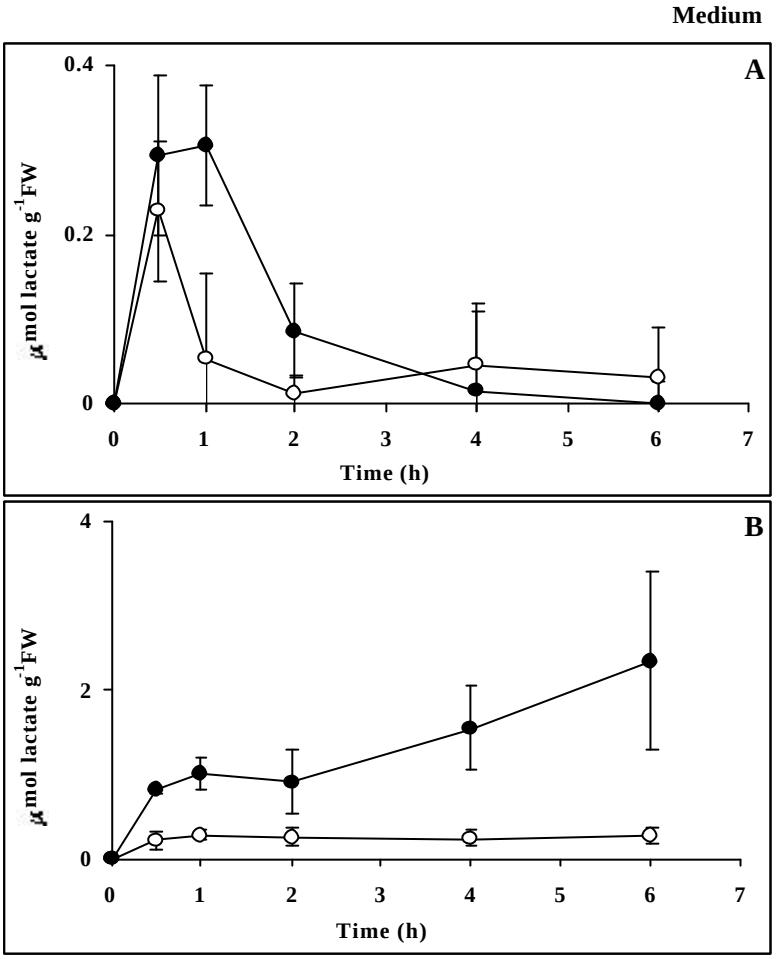


Figure 11: *Lactate concentration (µmol g^{-1} FW ± SD) in the root medium of (A) normoxic and (B) anoxic WT (?) and LNR-H (?) root segments. Data are the means of eight to twelve plants.*

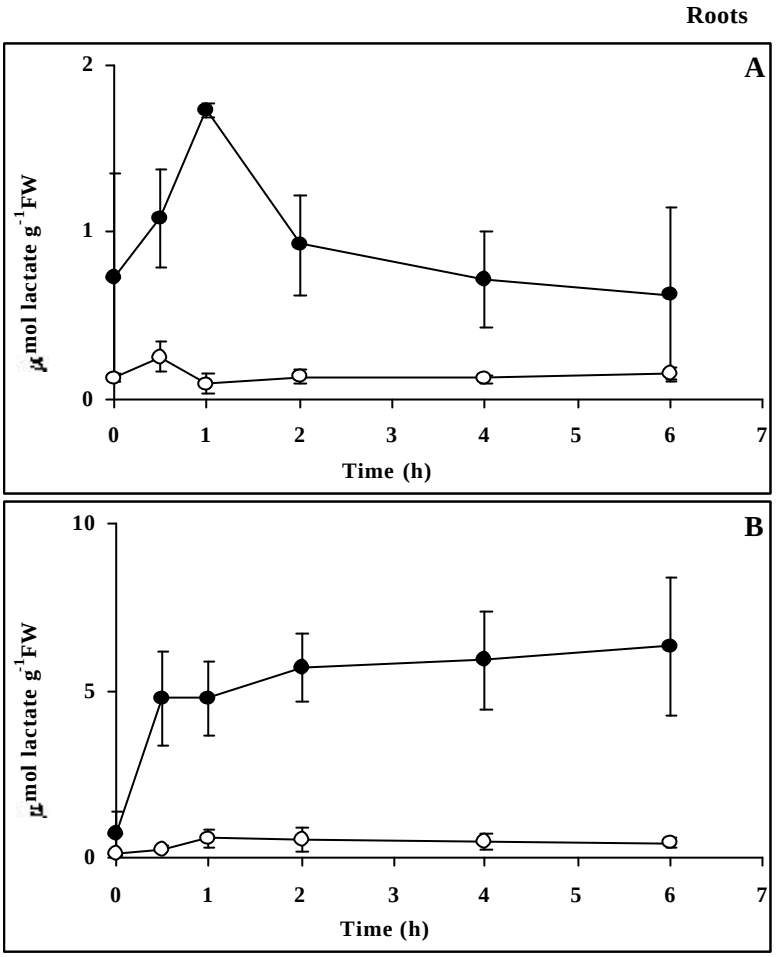


Figure 12: *Lactate concentration (µmol ḡ[1] FW ± SD) in (A) normoxic and (B) anoxic WT (?) and LNR-H (?) root segments. Data are the means of eight to twelve plants.*

3.2.5. Alcohol dehydrogenase activity

The ADH activity was the same in the WT and LNR-H extracts (Figure 13A), and a 4 h incubation under anoxia did not increase the activity. The measured activities were sufficient to support the observed production of ethanol.

3.2.6. Lactate dehydrogenase activity

Similarly, there was no increase in LDH activity over a 4 h incubation, although in this case the LDH activity was significantly higher in the LNR-H

roots than the WT roots (Figure 13B). In all cases the measured activities were sufficient to support the observed production of lactate.

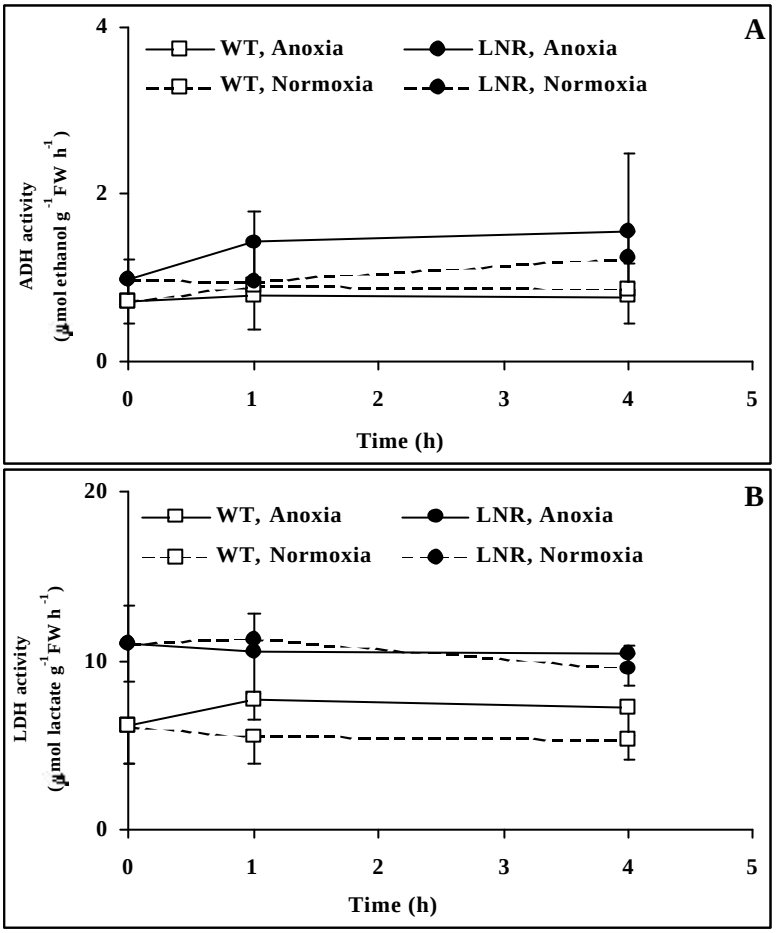


Figure 13: *(A) Alcohol dehydrogenase activity (µmol ethanol g^{-1} $FW h^{-1}$ ± SD), and (B) lactate dehydrogenase activity (µmol lactate g^{-1} $FW h^{-1}$ ± SD), in WT (?) and LNR-H (?) root segments under normoxic and anoxic conditions. Data are the means of eight plants.*

3.2.7. Nitrate reductase, ethanol and lactate production in tungstate treated WT plants

Tungstate treatment (10 days) of WT plants, with a supply of ammonium to avoid N-deficiency, was used to decrease the amount of functional NR without the morphological changes observed in the LNR-H plants. This treatment eliminated the NR activity in the roots and reduced the NR activity in the leaves

to about 10% of the activity of leaves from plants grown to a similar stage under normal conditions (Table 7; also compare Hänsch et al., 2001).

Table 7: *NR activity of leaves and roots of control and tungstate treated WT tobacco plants (mmol NO_2^- g^{-1} FW h^{-1} ± SD). Data are the means of four plants.*

NR activity	**WT**		**WT+tungstate**	
	NR_{act}	**NR_{max}**	**NR_{act}**	**NR_{max}**
Leaves	4.019 ± 0.210	6.584 ± 0.864	0.364 ± 0.297	0.657 ± 0.529
Roots	0.117 ± 0.045	0.242 ± 0.096	n.d.	n.d.

As in the NR-free LNR-H roots, ethanol production of tungstate treated WT roots were well above the values for control plants and it was comparable to the LNR-H plants under both normoxic and anoxic conditions (Table 8).

Table 8: *Ethanol content of the medium of control and tungstate treated roots of WT tobacco plants (mmol g^{-1} FW ± SD). Numbers in brackets give ethanol content as a % of the value obtained with LNR-H roots. Data are means of four plants. Other conditions as in Table 2.*

Ethanol	**Medium**	
Normoxia	**WT**	**WT + tungstate (% of LNR-H)**
Time 0	n.d.	n.d.
1h	0.016 ± 0.026	0.11 ± 0.009 (102.2)
4h	0.002 ± 0.006	0.05 ± 0.070 (130.2)
Anoxia		
Time 0	n.d.	n.d.
1h	0.049 ± 0.029	1.66 ± 1.10 (143.5)
4h	0.217 ± 0.103	3.00 ± 1.11 (67.10)

Similarly the tungstate treatment increased lactate production in WT anoxic roots, although the values were not as high as those recorded for the LNR-H plants (Table 9).

Table 9: *Lactate content of the roots and medium of control and tungstate treated WT tobacco plants (mmol g^{-1} FW ± SD). Numbers in brackets give the lactate content as % of the value obtained with LNR-H roots. Data are the means of four plants. Other conditions as in Table 2.*

Lactate	**Medium**		**Roots**	
Normoxia	**WT**	**WT+tungstate (% of LNR-H)**	**WT**	**WT+tungstate (% of LNR-H)**
Time 0	n.d.	n.d.	0.128 ± 0.022	0.093 ± 0.092 (12.7)
1h	0.052 ± 0.100	n.d.	0.094 ± 0.061	0.039 ± 0.017 (2.20)
4h	0.045 ± 0.062	n.d.	0.123 ± 0.024	n.d.
Anoxia				
Time 0	n.d.	n.d.	0.128 ± 0.022	0.093 ± 0.092 (12.7)
1h	0.280 ± 0.063	0.362 ± 0.042 (35.7)	0.593 ± 0.268	2.543 ± 1.023 (53.4)
4h	0.241 ± 0.102	0.549 ± 0.058 (35.5)	0.506 ± 0.243	1.786 ± 0.982 (30.2)

3.2.8. Free sugars and hexose monophosphates

As expected, sugar levels declined rapidly when the detached root segments were incubated under both normoxic and anoxic conditions (Table 15,16). For free hexoses (glucose and fructose) the decrease was partly due to leakage to the medium (Figure 14), whereas for sucrose there was almost no leakage.

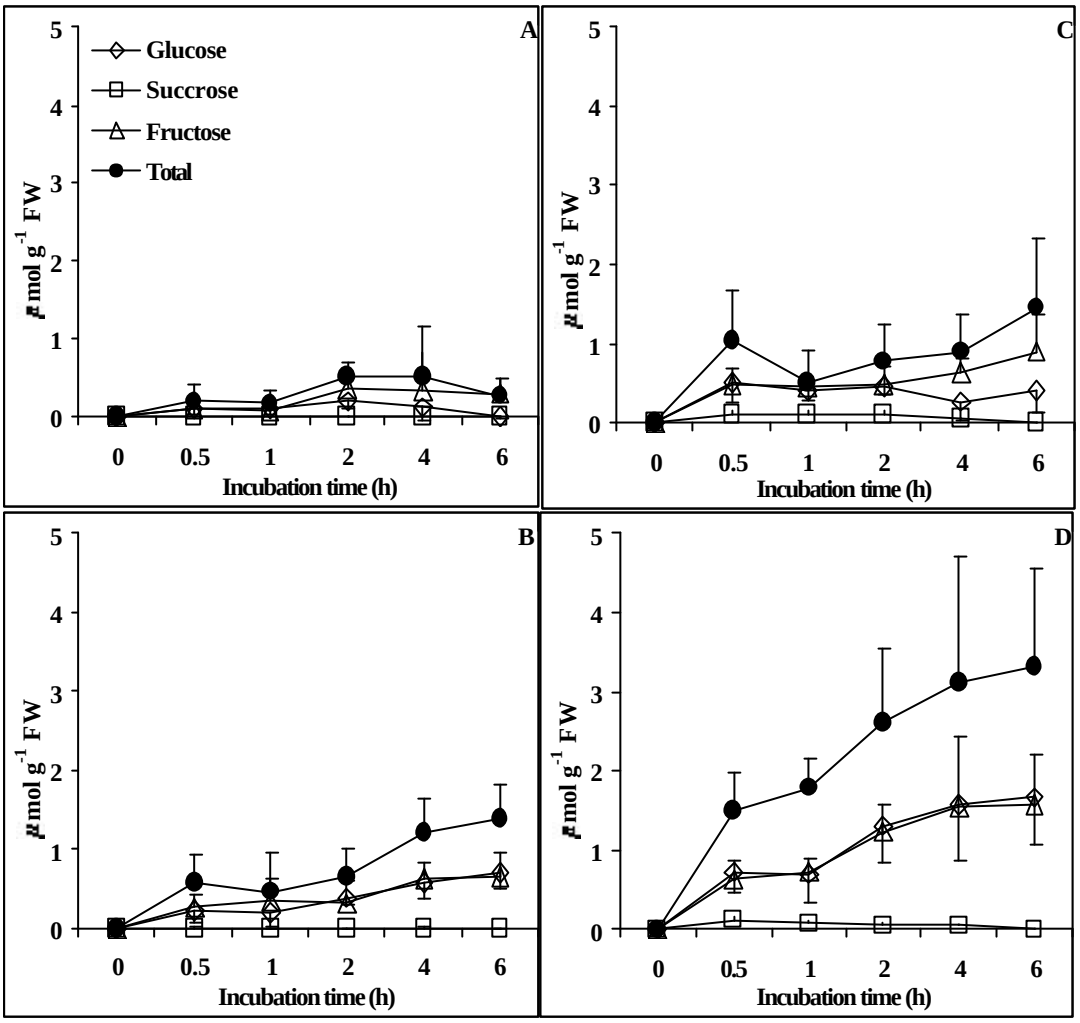


Figure 14: *Glucose, fructose, sucrose and 'total' sugar content of the medium of WT and LNR-H roots.* ***(A)*** *WT, normoxia.* ***(B)*** *WT, anoxia.* ***(C)*** *LNR-H, normoxia.* ***(D)*** *LNR-H, anoxia. One g root segments were incubated in 5 mL medium. Time 0 corresponds to 6 h into the light phase. Data (mmol g^{-1} FW ± SD) are means of ten plants each. Other conditions as described in 'Material and methods'.*

Although the respiration rate of freshly excised root segments was higher for LNR-H plants than WT plants, the sugar consumption was similar over a 6 h incubation under normoxic conditions. Total sugar consumption of both root types was somewhat lower under anoxic conditions, but the leakage to the medium was higher. In LNR-H roots, hexose leakage was also higher under anoxia, yet the sum of hexoses in roots plus medium remained constant. However, in anoxic LNR-H

roots sucrose was still consumed and the rate was sufficient to account for the markedly increased production of ethanol and lactate formation under anoxia.

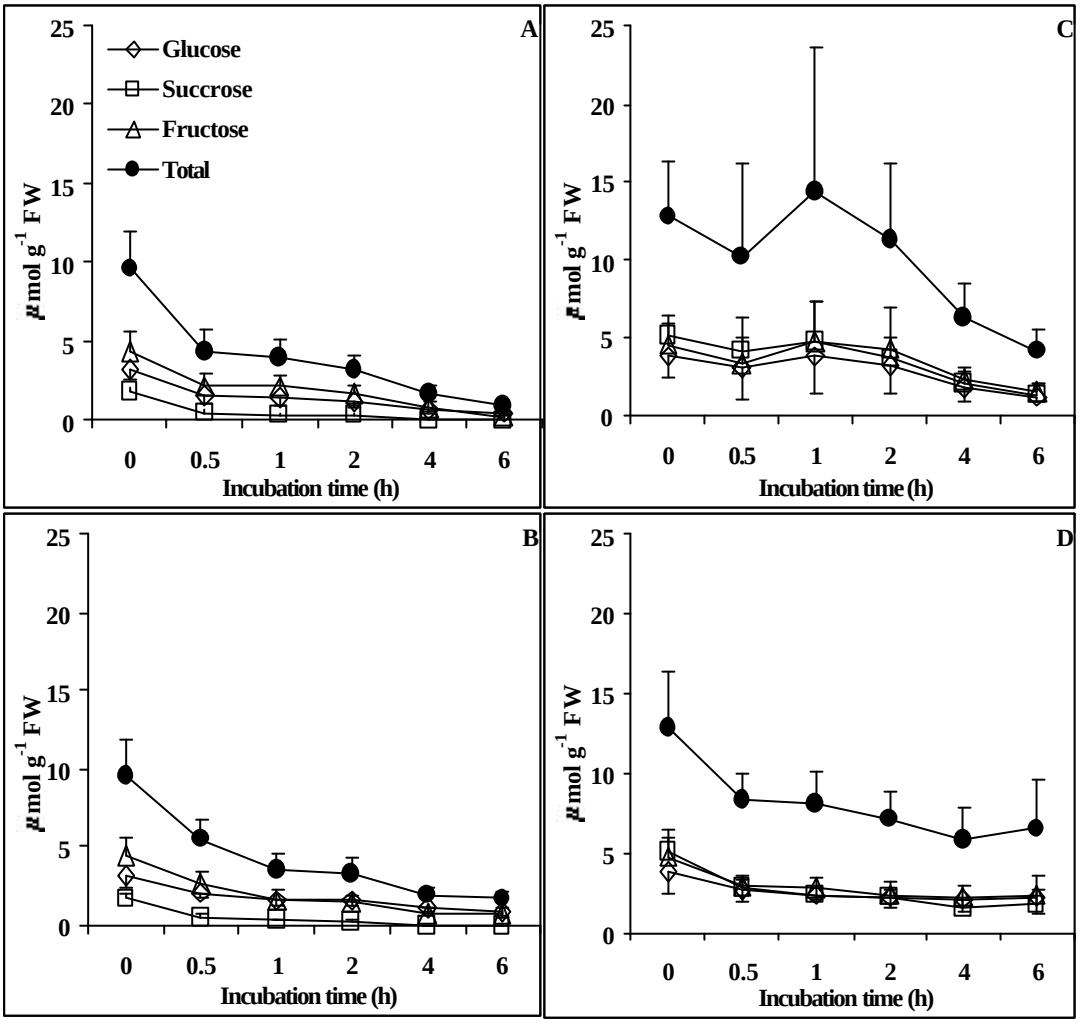


Figure 15: *Glucose, fructose, sucrose and 'total' sugar content of WT and LNR-H roots.* ***(A)*** *WT, normoxia.* ***(B)*** *WT, anoxia.* ***(C)*** *LNR-H, normoxia.* ***(D)*** *LNR-H, anoxia. Time 0 corresponds to 6 h into the light phase. Data (mmol g^{-1} FW ± SD) are means of 10 plants each. Other conditions as described in Material and methods.*

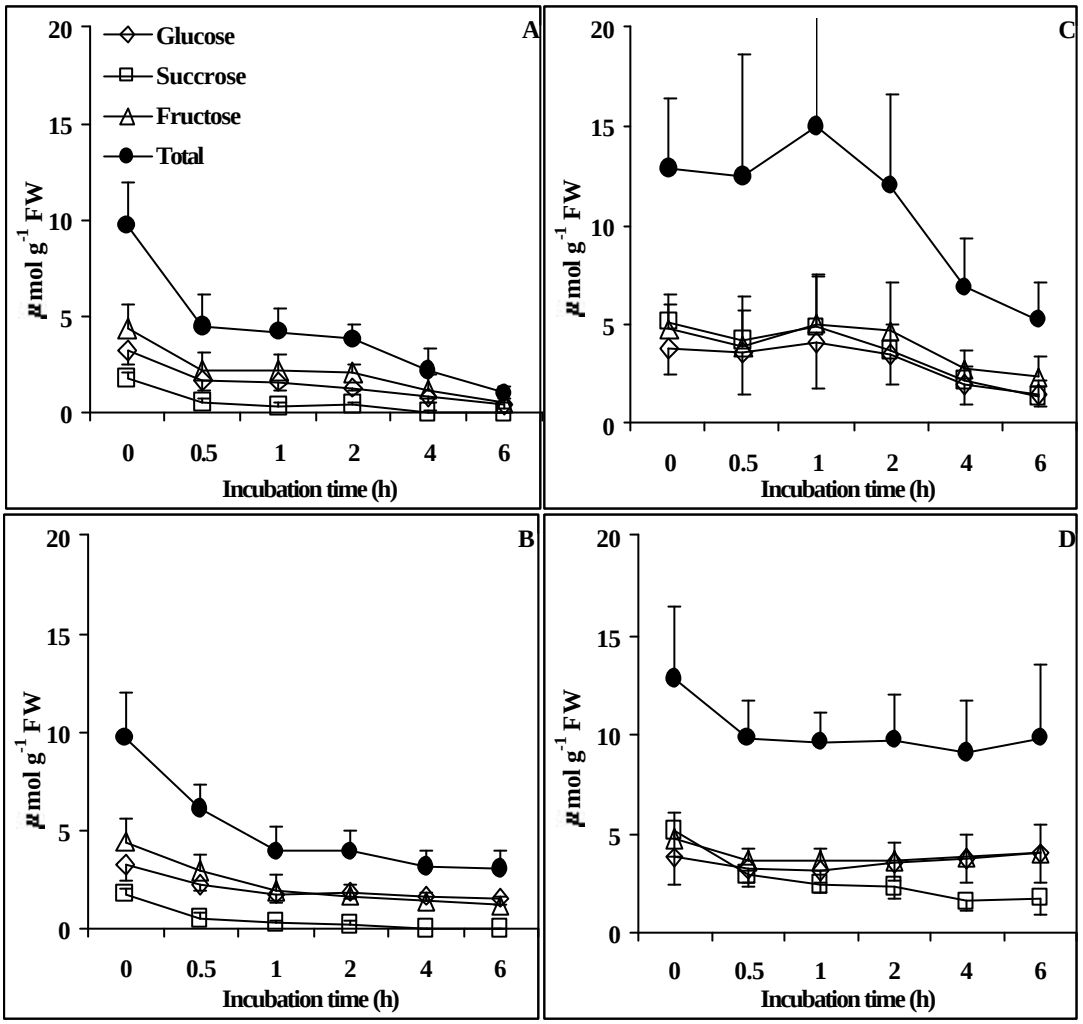


Figure 16: *Medium and root content (combined) of glucose, fructose, sucrose and 'total' sugar content of WT and LNR-H roots.* ***(A)*** *WT, normoxia.* ***(B)*** *WT, anoxia.* ***(C)*** *LNR-H, normoxia.* ***(D)*** *LNR-H, anoxia. One g root segments were incubated in 5 mL medium. Time 0 corresponds to 6 h into the light phase. Data (mmol g^{-1} FW ± SD) are means of 10 plants each. Other conditions as described in Material and methods.*

The hexose monophosphate content was higher in LNR-H roots than WT roots, as expected and the levels of G6P and F6P remained constant during a normoxic incubation (Table 10). In contrast, the levels fell during an anoxic incubation, although they remained higher in the LNR-H roots.

Table 10: *Hexose monophosphates in the roots of WT and LNR-H tobacco plants (mmol g^{-1} FW ± SD). Data are the means of eight plants. Other conditions as in Table 2.*

	WT			LNR-H		
	Time 0	**1h Normoxia**	**1h Anoxia**	**Time 0**	**1h Normoxia**	**1h Anoxia**
G6P	0.124 ± 0.020	0.140 ± 0.053	0.013 ± 0.023	0.297 ± 0.035	0.241 ± 0.044	0.084 ± 0.002
F6P	0.060 ± 0.018	0.053 ± 0.028	0.013 ± 0.022	0.166 ± 0.037	0.148 ± 0.027	0.060 ± 0.004

3.2.9. Sucrose feeding experiments

In order to investigate if differences in WT and LNR-H sugar content affect fermentation rates, root medium of plants fed with 50mM sucrose was tested for ethanol and lactate production. WT and LNR-H hydroponic plants were incubated for 24h with aerated, full strength nutrient solution which contained 50 mM sucrose and Sigma antibiotic, antimycotic solution in addition.

In the first experiments 25 mM KCl was added instead of 50 mM sucrose as an additional control. As osmotic pressure proved not to be an issue in this set of experiments KCl control was abandoned in the later experiments.
No significant difference in ethanol or lactate production was observed among the different time points and treatments both in WT and LNR-H plants (Figure 17 and figure 18)

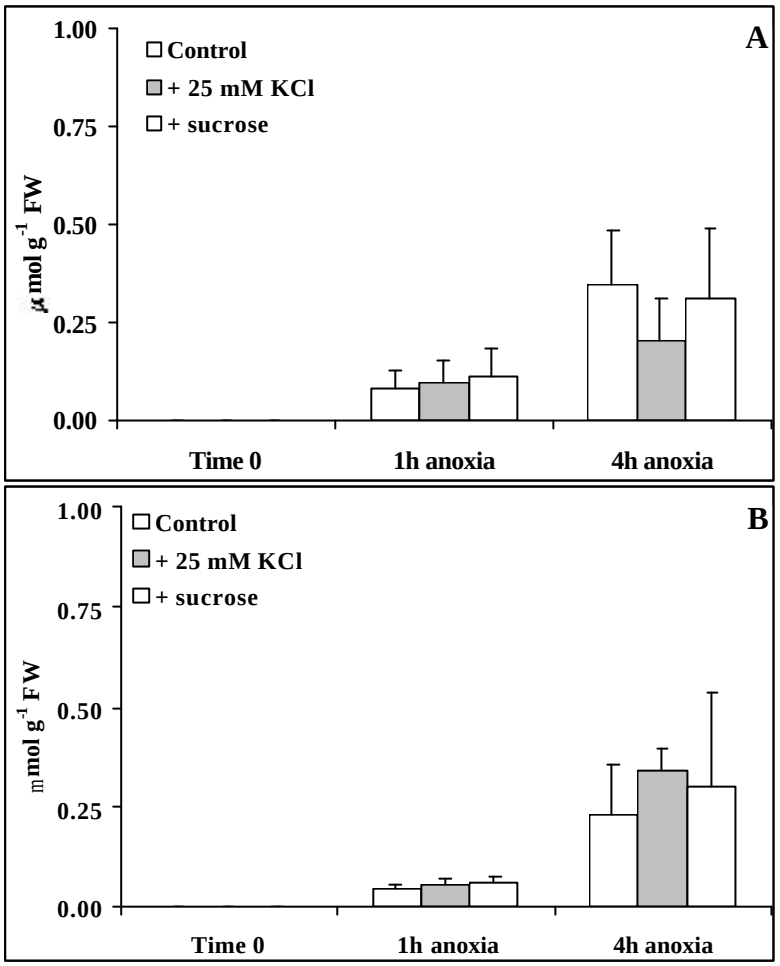


Figure 17: *(A) Ethanol content (in the medium) and (B) lactate content (in the medium) of anoxic WT-sucrose fed plants. Data (mmol g^{-1} FW ± SD) are means of 4 plants each. Other experiments as described in 'Material and methods'.*

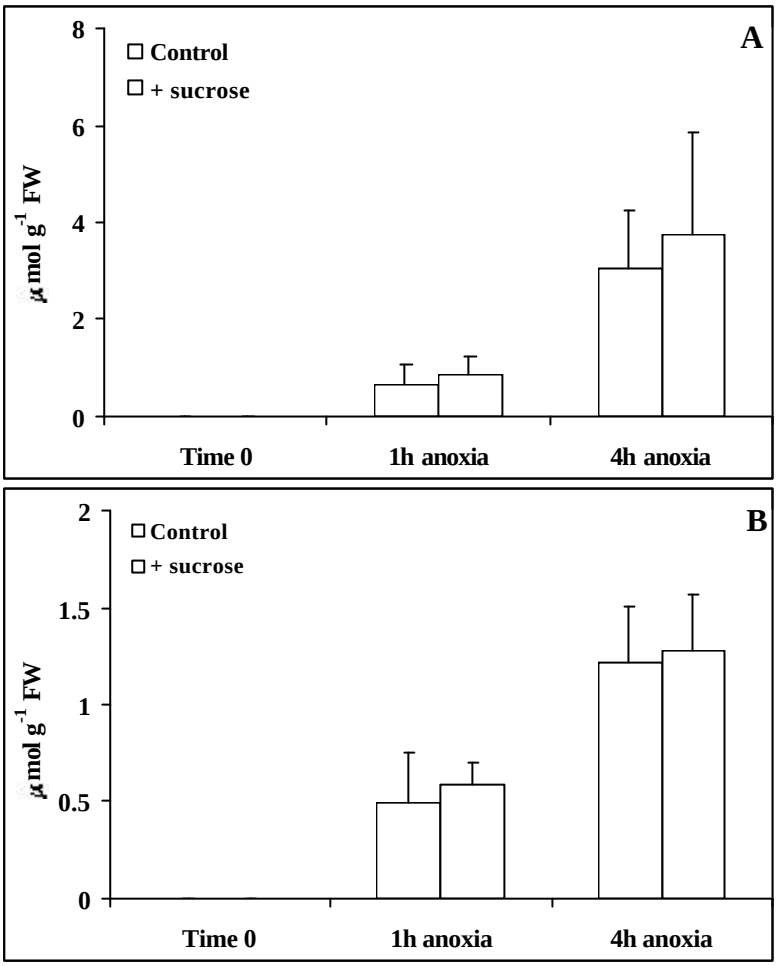


Figure 18: *(A) Ethanol content (in the medium) and (B) lactate content (in the medium) of anoxic LNR-H-sucrose fed plants. Data (mmol g^{-1} FW ± SD) are means of 4 plants each. Other experiments as described in 'Material and methods'.*

3.2.10. Adenine nucleotides

LNR-H roots also contained higher initial concentrations of ATP than WT roots, but these levels fell by approximately 50% during the first hour of a normoxic incubation (Table 11). The decrease was more marked under anoxia, but the LNR-H roots still contained more ATP than WT (36 nmoles g^{-1} FW for LNR-H and 9 nmoles g^{-1} FW for WT). The decrease in ATP did not cause an increase in ADP and AMP, and consequently that the total adenine nucleotide

pool became smaller in the detached roots during incubation under both normoxia and anoxia.

Table 11: *Adenine nucleotides in the roots of WT and LNR-H tobacco plants (mmol g^{-1} FW ± SD). Data are the means of eight plants. Other conditions as in Table 2.*

	WT			LNR-H		
	Time 0	**1h Normoxia**	**1h Anoxia**	**Time 0**	**1h Normoxia**	**1h Anoxia**
ATP	**0.114**	**0.047**	**0.009**	**0.304**	**0.154**	**0.036**
	± 0.058	± 0.033	± 0.008	± 0.097	± 0.079	± 0.046
ADP	**0.011**	**0.008**	**0.004**	**0.030**	**0.007**	**0.011**
	± 0.010	± 0.005	± 0.003	± 0.015	± 0.002	± 0.008
AMP	**0.005**	**0.009**	**0.005**	**0.017**	**0.010**	**0.016**
	± 0.004	± 0.008	± 0.006	± 0.020	± 0.008	± 0.011
Total	**0.130**	**0.064**	**0.018**	**0.351**	**0.171**	**0.063**

3.2.11. ^{31}P NMR measurements of cytoplasmic pH

The ^{31}P NMR spectra of WT and LNR-H root segments showed the expected features (Ratcliffe, 1994), including signals from the cytoplasmic and vacuolar P_i pools, ATP and various phosphomonoesters (Figure 19).

The highly vacuolated nature of the tissue meant that the cytoplasmic signals were difficult to detect and the minimum useful acquisition time for defining the pH-dependent chemical shift of the cytoplasmic P_i signal was 90 min. The intensity of the cytoplasmic signals was generally too low to provide accurate estimates of the root content of the corresponding metabolites, but spectra from the WT and LNR-H root segments were not noticeably different under normoxic conditions (data not shown). In contrast it was possible to measure the position of the cytoplasmic P_i signal accurately and Table 12 summarises the results of a set of experiments in which a series of spectra were recorded from WT and LNR-H root segments before, during and after a period of anoxia. The initial value of the cytoplasmic pH was the same in the normoxic WT and LNR-H roots and as expected it fell following the onset of anoxia. However the acidification was greater in the LNR-H roots, where the pH fell to around 6.3, than in the WT roots, where the pH fell

to around 6.5. After the return to normoxia, the cytoplasmic pH recovered towards its initial value and the difference observed between the WT and LNR-H roots under anoxic conditions disappeared (Table 12).

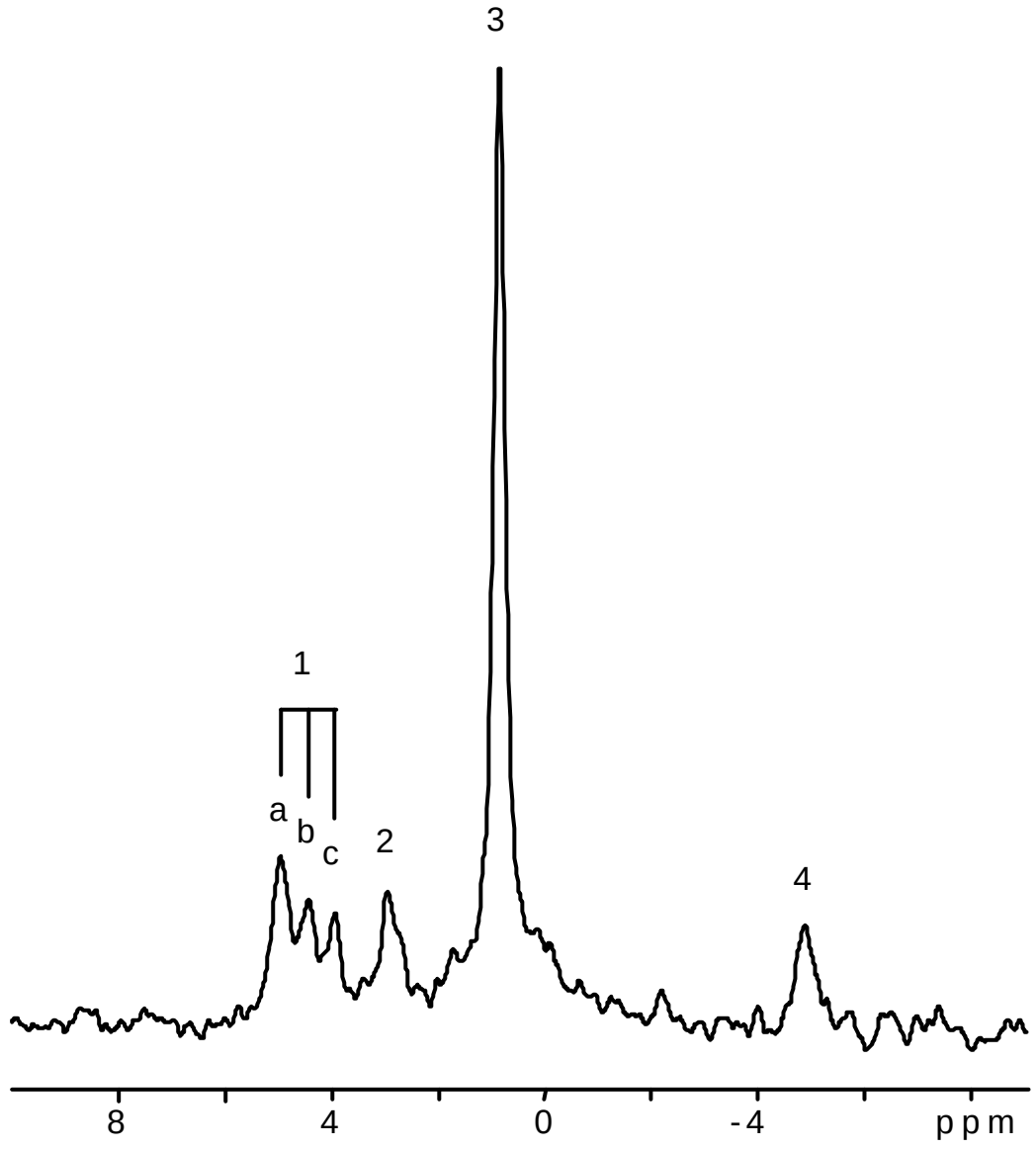


Figure 19*: A region of the* ^{31}*P NMR spectrum of normoxic LNR-H root segments recorded after a 3 h period of anoxia. The spectrum was accumulated in 90 min, and the labelled signals can be assigned to: 1, several phosphomonesters, including (a) glucose 6-phosphate and (c) phosphocholine; 2, cytoplasmic* P_i*; 3, vacuolar* P_i*; and 4, the ?-phosphate of nucleoside triphosphate, which is mainly*

ATP. The position of peak 2 is sensitive to the cytoplasmic pH and it shifts to lower chemical shift values as the cytoplasm acidifies.

Table 12: *Chemical shifts (± SD; n=5) for the cytoplasmic P_i signal observed in the ^{31}P NMR spectra of WT and LNR root segments, and the corresponding cytoplasmic pH values. A series of spectra were recorded before, during, and after a period of oxygen deprivation and the time refers to the midpoint of each 90 min spectrum. The switch to anoxia took place at time zero and the return to normoxia occurred after 180 min.*

Time (min)	WT		LNR-H	
	Chemical shift (ppm)	pH	Chemical shift (ppm)	pH
-45	2.91 ± 0.12	7.50	2.95 ± 0.03	7.57
45	2.12 ± 0.21	6.74	1.81 ± 0.22	6.51
75	2.01 ± 0.23	6.66	1.56 ± 0.10	6.31
105	1.77 ± 0.07	6.48	1.51 ± 0.08	6.27
135	1.81 ± 0.22	6.51	1.55 ± 0.19	6.30
225	2.81 ± 0.10	7.36	2.78 ± 0.11	7.31

3.2.12. NO measurements

NO emission of detached roots of WT and LNR-H in normoxic and anoxic conditions has been measured (Table 13). Surprisingly LNR-H roots produced a significant amount of NO. It was, however, lower on average than that produced of WT.

Table 13: *NO emission of WT and LNR-H detached roots. Data (nmol NO g^{-1} FW) are means of 4 plants each. Time 0 corresponds to 6 to 8 h into the light phase. Normoxic treatment lasted up to 30 min. Anoxia values correspond to 1h anoxia.*

NO	WT	LNR-H
Normoxia	1.02 ± 1.21	1.78 ± 0.76
Anoxia	4.68 ± 1.53	2.16 ± 2.0

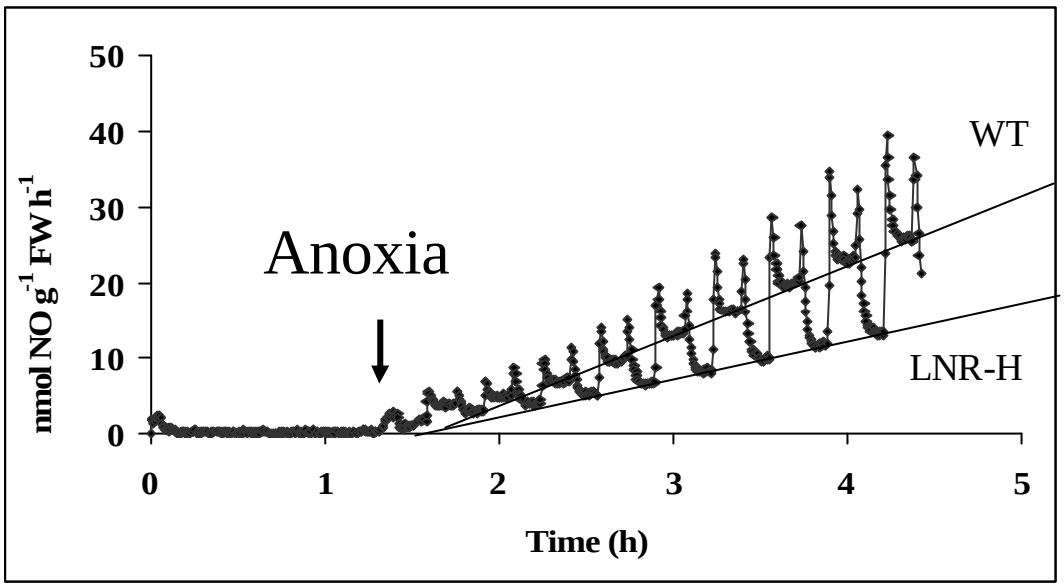


Figure 20. *An example of a simultaneous measurement of NO emission of detached WT and LNR-H roots. 0.5 g FW WT or LNR-H roots were placed in two measuring cuvettes respectively and NO emission was recorded consecutively at 10 min intervals.*

3.3. Anoxic metabolism of 35S-NR tobacco plants

3.3.1. Nitrate reductase activity

As expected (see also Hänsch et al., 2001 for additional information), 35S-NR root segments NRA activity was similar to that of WT plants (Figure 21). Under normoxia, NR_{act} and NR_{max} in detached 35S-NR root segments decreased slightly, but not significantly. In contrast, NR_{act} and NR_{max} both increased under anoxia in agreement with observations on anoxic barley roots (Botrel et al. 1996, Botrel and Kaiser, 1997).

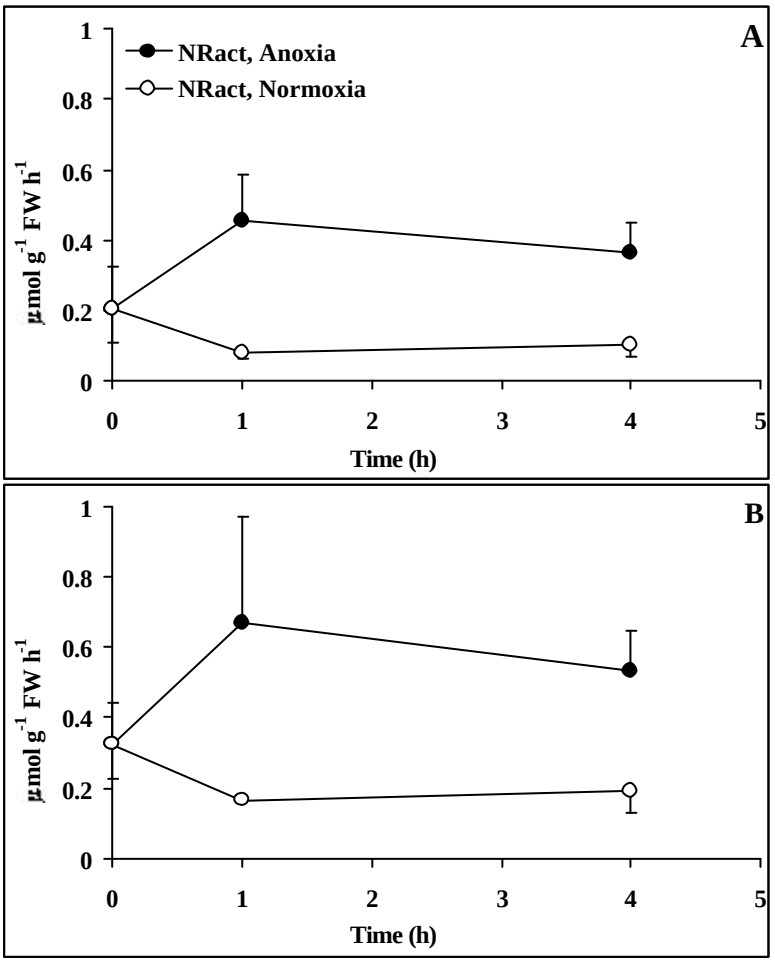


Figure 21: *(A) NR_{act} and (B) NR_{max} in extracts from normoxic (?) and anoxic (?) 35S-NR root segments. Time zero was 6 h into the light phase. Data (µmol g^{-1} FW h^{-1} ± SD) are means of samples from three plants.*

3.3.2. Ethanol production

Ethanol was measured in the medium (Figure 22), where more than 90% of the total ethanol was usually found. 35S-NR root segments ethanol production was similar to that of WT roots (Figure 10) and substantially lower than that of LNR-H root segments (Figure 10).

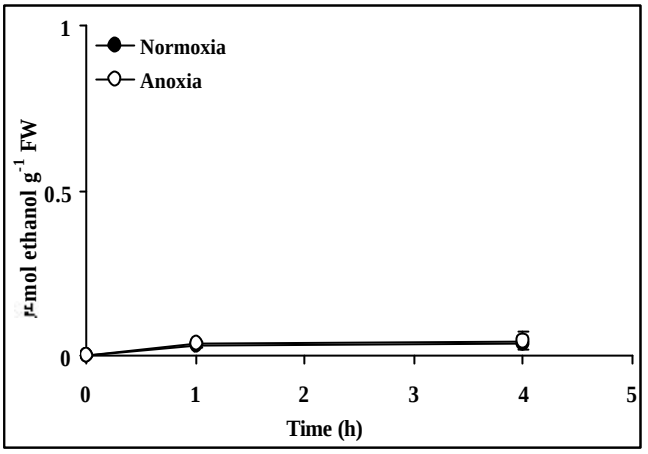


Figure 22: *Ethanol concentration (µmol g^{-1} FW ± SD) in the root medium of normoxic (?) and anoxic (?) 35S-NR root segments. The medium volume was 5 mL g^{-1} FW and the data are the means of three plants.*

3.3.3. Lactate production

Similar results as for ethanol were obtained for the lactate in the medium (Figure 23A), with the 35S-NR roots again showing a much lower capacity to produce lactate than the LNR-H roots. The lactate content of the roots themselves remained approximately constant under normoxia (Figure 23B), but with the onset of anoxia there was an immediate and substantial increase in the lactate content of the 35S-NR roots, comparable with that of WT roots in similar conditions (Figure 12).

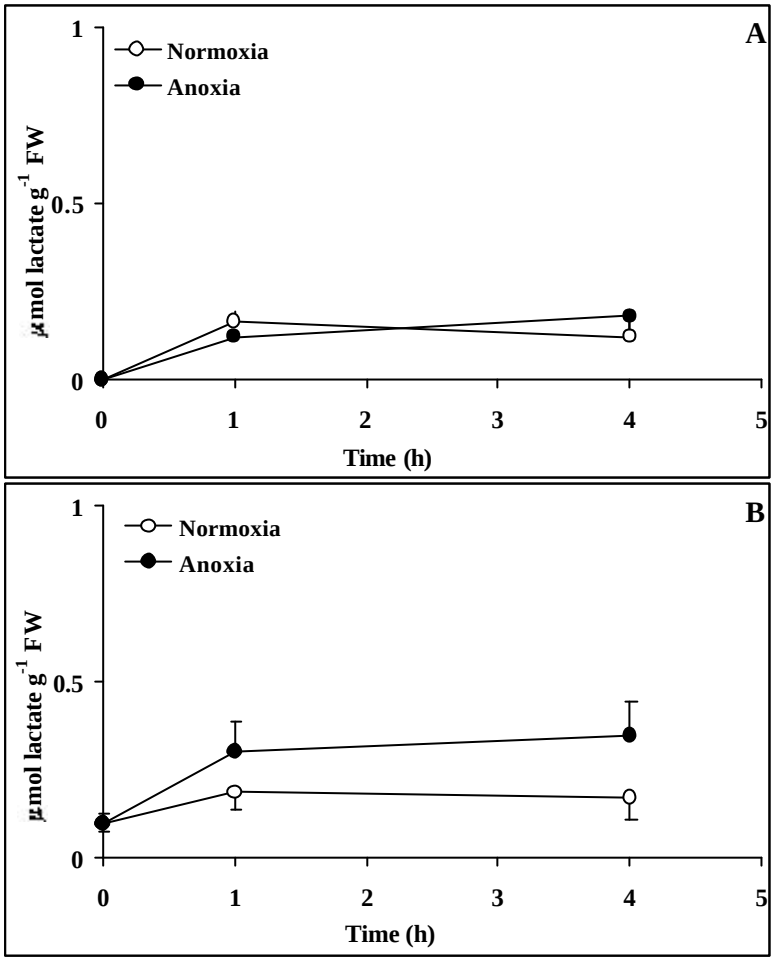


Figure 23: *Lactate concentration (µmol g^{-1} FW ± SD) in (A) medium and and root segments (B) in normoxic (?) and anoxic (?) 35S-NR plants. Data are the means of three plants.*

4. Discussion

4.1. Normoxic metabolism and root architecture of WT and LNR-H plants

4.1.1. Nitrate reduction and root morphology and architecture

Nitrate assimilation in wild type tobacco roots represents only about 10% of the total nitrate assimilation capacity of the plant and so it would not be expected to have a profound effect on growth and metabolism (Foyer et al., 1994; Gojon et al., 1998). However, current data show that root nitrate reduction has a far more complicated role on plant development and metabolism than just supplying reduced nitrate.

Developmental processes in roots are subjected to both localized and/or systemic responses exerted by nutrients (Forde and Lorenzo, 2001).

Localized developmental responses of roots are correlated with localized proliferation within nutrient-rich soil patches (Drew et al., 1973; Drew and Saker, 1975; Drew, 1975). For example lateral root elongation and/or lateral root initiation could be provoked in such conditions (i.e. high nitrate concentrations, Zhang et al., 1999). However, the degree of the response varies among species and is dependent on the overall nutrient status of the plant (Friend et al., 1990). In (Hänsch et al., 2001) the two tobacco lines (WT and LNR-H) had been grown in sand culture, where localized nutrient supply could not be excluded. In the experiments presented here plants were grown on hydroponics where localized nutrient supply was avoided.

Root morphology and architecture however are also subject to some form of **systemic control** i.e. they are not only dependent on the prevailing external nutrient concentration but also on the nutrient status of the plant as a whole (Drew et al., 1973, Friend et al., 1990). It has been proposed that root/shoot biomass partitioning could be connected to N and/or C partitioning (Cheeseman, 1993; Kronzucker et al., 1998), phytohormone partitioning (Back, 1996) or nitrate accumulation in the shoot (Scheible et al., 1997). Also, developmental responses

appear to be modulated by the rapid circulation of amino acids between root and shoot (Cooper and Clarkson 1989; Imsande and Touraine, 1994).

LNR-H plants exhibit slightly retarded growth combined with changes in root morphology in comparison to WT (Figure 5). The observed differences are somehow surprising as total protein levels (Table 5) and nitrate content (Table 2) in WT and LNR-H roots were not significantly different. Also, in contrast to previous data (Hänsch et al. 2001), sugar levels in roots of hydroponically grown LNR-H plants were only slightly, but not significantly, higher than in WT (Table 4a). However LNR-H roots had a higher amino acid content (Table 2) and negative correlation between internal N status (amino acids) and root growth has been reported before (Scheible et al., 1997).

Certainly, the above described differences in root morphology and root/shoot ratios of our LNR-H and WT lines are not due to N- or C-deficiency, nor to differences in the major anion- and cation contents. Levels of abscicic acid in roots of both lines were also not significantly different (W. Hartung, personal communication). Other hormones have not been measured.

Many plant species exhibit plasticity in root diameter according to environmental conditions. For example fine roots allow the root system to explore the soil efficiently when at the same time the investment needed for constructing and maintaining the root system is kept at a minimum. Thicker roots a costly to produce but they have a greater transport capacity and are less prone to physical damage and pathogens. The finesse of a root is often expressed as specific root length (length per unit weight of root) and roots with high specific root length are often found in nutrient deficient plants (Fitter, 1985). In barley and wheat abundance of nitrate provoked an increase in root diameter (Hackett 1972, Drew et al., 1973, Cruz et al., 1997). Few studies have examined the cytological basis of the plasticity in the specific root length. Drew et al, 1978 observed that the increased diameter of lateral roots in their localized nitrate treatment was due to 2-fold increase in the diameter of the stele and in the numbers of cells in both stele and cortex.

Our finding that LNR-H roots were in fact thicker and their length, surface area, projected area and number of root tips were significantly decreased (Figure 5) compared to WT roots suggests, that developmental control of roots extends beyond external and internal root nitrate concentration, total protein levels or

sugar content. Rather it appears that the presence or absence of nitrate (and nitrite) reduction itself may be relevant, eventually via changing physical parameters.

While the reason for the different root size and morphology of LNR-H versus WT plants is still not fully understood, the resulting differences in root/shoot ratio, and specifically the lower root surface area per leaf FW of LNR-H versus WT may be one reason for the observed differences in leaf transpiration.

4.1.2. Nitrate reduction and energy status

WT and LNR-H plants showed marked differences in root respiration (Table 3) and nucleotide levels (Table 4). The higher root respiration of LNR-H plants as compared to WT may indicate that nitrate reduction can compete with respiration for reductant. However, respiration rates of roots (WT) exceeded extractable NR activity almost 100-fold. Therefore, a simple competition for reductant cannot be the reason for the different respiration rates between roots with and without nitrate reduction.

The higher respiration rates of LNR-H roots may be causally connected with the higher ATP contents. LNR-H plants did not exhibit a large difference in total sugar content in comparison to WT, but they had higher starch and hexose-monophosphate levels (Table 4a). Therefore a possible involvement of the carbohydrate status in determining root respiration rates cannot be excluded.

While the above analysis has elucidated some metabolic and morphological differences between the root systems of WT and LNR-H, the two lines seemed similar enough in other basic features such as cation and anion contents or total protein contents to be used for a comparison of their response to anoxia, in order to reexamine the role of nitrate reduction for survival of anoxia.

4.2. Anoxic metabolism of WT and LNR-H plants

The above mentioned detailed comparisons of WT and LNR-H tobacco plants grown under normal aerobic conditions have shown that the loss of the root NR activity results in a distinct phenotype for the transformant. The distinctive physiological and metabolic phenotype of the LNR-H plants was also evident when the roots were deprived of oxygen.

At a whole plant level, the LNR-H plants were more prone to wilting when the root system was deprived of oxygen (Figure 7) and this commonly observed anoxic response, which may have its origin in a reduction in root hydraulic conductivity (Vartapetian and Jackson, 1997), can perhaps be attributed to the altered morphology of the LNR-H root system. Moreover there were substantial differences between the response of the WT and LNR-H roots to anoxia at the metabolic level, and this was particularly striking in the production of fermentation end products (Figures 10, 11, 12).

The generalized biochemical basis for anoxia tolerance must involve maintenance of glycolysis for generation of ATP, regeneration of NAD from NADH so that glycolysis does not stall and metabolic end products that are innocuous or are readily transported to the external solution. The net effect of the many differences between the WT and LNR-H plants under aerobic and anaerobic conditions is to complicate the apparently simple task of using the transformant to establish the metabolic significance of root NR activity in the anoxic response. However by focusing on the main differences in metabolism, in particular the higher fermentation rate in the LNR-H roots, it is possible to assess the impact of root nitrate reduction on the recycling of NADH and the acidification of the cytoplasm. To do this, Table 14 summarizes the production of ethanol, lactate and nitrite by the roots of WT and LNR-H tobacco plants during a 4 h anoxic incubation, using data from Table 1 and Figures 10, 11, 12.

Table 14: *Summary of the production of fermentation products by the roots of WT and LNR-H tobacco plants during a 4 h incubation under anoxia (µmol g^{-1} FW). The summary is based on the data presented in Table 6 and Figures 10, 11, 12. For each metabolite, the table shows the quantity produced, the amount of NADH reoxidised, and the associated H^+ production. The final set of columns shows the total quantity of NADH reoxidised, the total H^+ production and the ratio of the two.*

	Ethanol			Lactate			Nitrite			Total		
	Ethanol	NADH	H^+	Lactate	NADH	H^+	Nitrite	NADH	H^+	NADH	H^+	H^+/NADH
WT	**0.22**	**0.22**	**0**	**0.75**	**0.75**	**0.75**	**0.42**	**0.42**	**0.42**	**1.38**	**1.16**	**0.84**
LNR-H	**4.47**	**4.47**	**0**	**7.46**	**7.46**	**7.46**	**0.10**	**0.10**	**0.10**	**12.0**	**7.56**	**0.63**

Table 14 also summarizes the quantity of NADH recycled by the synthesis of these metabolites – the molar ratio is 1 in each case - and the H^+ production associated with their accumulation. The latter was calculated from the stoichiometric equations for ethanol production:

(21) $C_6H_{12}O_6$? $2C_2H_6O + 2CO_2$

for lactate production:

(22) $C_6H_{12}O_6$? $2C_3H_5O_3^- + 2H^+$

and for nitrate reduction to nitrite:

(23) $C_6H_{12}O_6 + 2NO_3^-$? $2C_3H_3O_3^- + 2\,NO_2^- + 2H_2O + 2H^+$

in each case assuming glycolysis to be the source of the NADH.

The first conclusion to be drawn from Table 14 is that there was substantially less recycling of NADH in the WT roots than the LNR-H roots, indicating that the metabolic rate of the LNR-H roots was not limited by the absence of NR, and that NR did not act as an unregulated sink for NADH in the WT plants. Thus, in agreement with some other studies (eg Saglio et al., 1988), the frequently discussed proposal that NR should be able to promote carbohydrate metabolism under anoxia by providing an extra sink for NADH needs to be treated with caution. The validity of this conclusion hinges on the comparability of the WT and LNR-H plants, and ultimately on the explanation for the lower metabolic rate of the WT roots under anoxia (Table 14).

There appear to be two possible explanations for the reduced rate: (i) down regulation of metabolism mediated by a metabolite downstream from NR, for example nitrite itself; or (ii) a difference in the physiology of the LNR-H roots, such as the higher respiration rate of the LNR-H plants under aerobic conditions. However some support for the comparison between the WT and LNR-H roots is provided by the observation that the tungstate treated WT plants produced substantially more lactate and ethanol than untreated plants under anoxia (Figures 17, 18) and in principle further support could be obtained by conducting experiments on WT tissues exposed to tungstate for much shorter periods than the 8-10 days used here.

The second conclusion to be drawn from Table 14 is that anaerobic metabolism in the WT roots was more acidifying than in the LNR-H roots. Thus the recycling of NADH for glycolysis generated 0.84 H^+ per molecule of NADH in the WT roots and 0.63 H^+ in the LNR-H roots. However this did not lead to a greater acidification of the cytoplasm in the WT roots under anoxia (Table 12) because of the much greater metabolic rate in the LNR-H roots (Table 14). So while nitrate reduction and the fermentation of glucose to lactate are equally acidifying under anoxia, the potentially beneficial effect of the absence of nitrate reduction on the cytoplasmic pH in the LNR-H roots was overwhelmed by the much greater metabolic rate and especially by production of lactate. These arguments assume that the observed acidification of the cytoplasm was dominated by the contribution of the three reactions shown above, and that other biochemical and biophysical processes affecting the cytoplasmic pH were sufficiently similar in the WT and LNR-H roots to be put to one side.

Some data indicate (eg Saint-Ges et al., 1991) that patterns of response to anoxia should not only be connected to lactate production as the main cause of cytoplasmic acidification but also to concurrent energy metabolism and the availability of ATP to energize tonoplast H+ pumps. To support this point an observation was made that cytoplasmic pH decreased more rapidly than the production of lactic acid (Ratcliffe, 1995; Menegus et al., 1991). The decrease was parallel to the decrease in nucleotide triphosphates. In the WT:LNR-H model H^+ production associated with ATP hydrolysis must have been greater in the LNR-H roots than the WT roots (Table 11), however, the contribution of this process would have been small in comparison with the much greater H^+ production associated with lactate formation.

So, while the comparison of the anaerobic metabolism of the WT and LNR-H plants is less straightforward than might have been expected, the data show that the absence of NR activity does not necessarily limit NADH recycling and that the presence of NR activity can result in a more acidifying metabolism. The latter point is of particular interest because it has usually been expected that NR activity under anoxia would counteract cytoplasmic acidosis (Roberts et al., 1985; Reggiani et al., 1993a; Ratcliffe, 1999) and that the activation of NR at acidic pH values could form the basis of a pH regulatory mechanism (Botrel et al., 1996). However since the reduction of nitrate to nitrite is an acidifying process under anoxia, it seems likely that the well documented beneficial effect of nitrate on cytoplasmic pH regulation under anoxia (Roberts et al., 1985), as well as the less pronounced acidification of the WT roots observed here (Table 7), must be caused by a reduction in metabolic rate.

One intriguing possibility is that this might be achieved through a side reaction of NR, the reduction of nitrite to nitric oxide (NO), that has recently become the focus of attention (Yamasaki and Sakihama, 1999; Rockel et al., 2002,). Under anoxia, NR is usually activated, nitrite accumulates and NO emission increases (Rockel et al., 2002). NO is known to inhibit respiratory electron transport and to induce the alternative electron transport pathway (Leshem, 2000). NO also inhibits many enzymes with prosthetic heme groups, and it may also interact with ethylene production (Leshem, 2000). Thus it appears that NO may exert hitherto unknown regulatory functions in plant growth and metabolism, and future experiments on NO production and its function under

anoxia could bring new insights into the old problem of the relationship between anoxia tolerance and nitrate reduction.

5. Summary

The aim of this work was to find out whether and how nitrate reduction in roots would facilitate survival of hypoxic and anoxic (flooding)-phases.

For that purpose, we compared the response of roots of hydroponically grown tobacco wildtype (*Nicotiana tabacum* cv. Gatersleben) and of a transformant (LNR-H) with no nitrate reductase (NR) in the roots but almost normal NR in leaves (based on a nia2-double mutant). As an additional control we used occasionally a 35S-transformant of the same *nia2*-double mutant, which on the same genetic background constitutively expressed NR in all organs. In some cases, we also compared the response of roots from WT plants, which had been grown on tungstate for some time in order to completely suppress NR activity.

The following root parameters were examined:

1) Growth and morphology

2) Root respiration rates and leaf transpiration

3) Metabolite contents in roots (ATP, hexosemonophosphates, free sugars, starch, amino acids, total protein)

4) Inorganic cation and anion contents

5) Lactate and ethanol production

6) Extractable LDH-and ADH-activities

7) Cytosolic pH values (by 31P-NMR)

8) NO

Cation and anion contents of roots from WT and LNR-H were only slightly different, confirming that these plants would be better suited for our purposes than the widely used comparison of nitrate-versus ammonium-grown plants, which usually show up with dramatic differences in their ion contents.

Normoxia: LNR-H-plants had shorter and thicker roots than WT with a lower roots surface area per leaf FW. This was probably the major cause for the significantly lower specific leaf transpiration of LNR-H. WT-roots had lower respiration rates, lower ATP-and HMP-contents, slightly lower sugar- and starch

contents and somewhat lower amino acid contents than LNR-H roots. However, total protein/FW was almost identical. Obviously the LNR-H transformants did not suffer from N-defciency, and their energy status appeared even better than that of WT-roots. Data from the 35S-transformant were similar to those of WT. This indicates that the observed differences between WT and LNR-H were not due to unknown factors of the genetic *nia2*-background, but that they could be really traced back to the presence resp. absence of nitrate reduction.

Anoxia: Under short-term anoxia (2h) LNR-H plants, but not WT-plants exhibited clear symptoms of wilting, although leaf transpiration was lower with LNR-H. Reasons are not known yet. LNR-H roots produced much more ethanol (which was excreted) and lactate compared to WT, but extractable ADH and LDH activities, were not induced by anoxia. However, the LDH activity background was twice as high as that of the WT troughout the time period studied. Tungstate-treated WT-roots also gave higher fermentation rates than normal WT roots. Sugar- and HMP-contents remained higher in LNR-H roots than in WT. NR in WT roots was activated under anoxia and roots accumulated nitrite, which was also released to the medium. ^{31}P-NMR spectroscopy showed that LNR-H- roots, in spite of their better energy status, acidified their cytosol more than WT roots.

Conclusions: Obviously nitrate reduction affects - by as yet unknown mechanisms - root growth and morphology. The much lower anoxic fermentation rates of WT-roots compared to LNR-H roots could not be traced back to an alternative NADH consumption by nitrate reduction, since NR activity was too low for that. An overall estimation of H^+-production by glycolysis, fermentation and nitrate reduction (without nitrite reduction, which was absent under anoxia) indicated that the stronger cytosolic acidification of anoxic LNR-H roots was based on their higher fermentation rates. Thus, nitrate reduction under anoxia appears advantageous because of lower fermentation rates and concomitantly lower cytosolic acidification. However, it remained unclear why fermentation rates were so different.

Perspective: Preliminary experiments had indicated that WT-roots produced more nitric oxide (NO) under anoxia than LNR-H-roots. Accordingly,

we suggest that nitrate reduction, beyond a merely increased NADH-consumption, would lead to advantageous changes in metabolism, eventually via NO-production, which is increasingly recognized as an important signaling compound regulating many plant functions.

6. Zusammenfassung

Ziel der Arbeit war es herauszufinden, ob und wie Nitratreduktion in der Wurzel das Überleben von hypoxischen und anoxischen (Überflutungs)-Phasen erleichtert.

Hierzu wurden Wurzeln eines hydroponisch angezogenen Tabak-Wildtyps (Nicotiana tabacum cv. Gatersleben), sowie einer Tabaktransformante auf der Basis der nia Doppelmutante, welche Nitratreduktase nur noch in den Blättern exprimierte (LNR-H), im Hinblick auf verschiedene Parameter verglichen. Als zusätzliche Kontrolle wurde eine 35S-Transformante der nia-Doppelmutante gelegentlich in die Vergleiche mit einbezogen, da diese auf dem genetischen Hintergrund der nia Doppelmutante NR in Blättern und Wurzeln konstitutiv exprimierte mit Aktivitäten, die in etwa denen des Wildtyps entsprachen. In einigen Fällen wurde die Nitratreduktase des WT durch Aufzucht auf Wolframat (an Stelle von Molybdat) gehemmt, und diese Pflanzen wurden ebenfalls mit normalen WT-Wurzeln verglichen.

Folgende Parameter wurden untersucht:

1) Wachstum und Wurzelmorphologie

2) Atmungsraten, Transpirationsraten

3) Metabolitgehalte (ATP, Hexosemonophosphate, freie Zucker, Aminosäuren)

4) Gehalte anorganischer Kationen und Anionen

5) Lactat- und Ethanolproduktion

6) LDH und ADH-Aktivitäten in Wurzelextrakten

7) Cytosolische pH-Werte mittels 31P-NMR

8) NO

Die Analyse des Kationen- und Anionengehaltes der Wurzeln bestätigte zunächst, das die LNR-H-Transformante und der WT sich in dieser Hinsicht nur unwesentlich unterschieden und von daher zum weiteren Vergleich besser geeignet waren als die vielfach verwendete Paarung von nitrat-bzw-ammoniumernährten Pflanzen.

Normoxia: LNR-H-Pflanzen hatten kürzere und dickere Wurzeln mit einer niedrigeren Wurzeloberfläche pro Blattfrischgewicht als WT. Dies war vermutlich

die Hauptursache für die deutlich niedrigeren Transpirationsraten von LNR-H. WT-Wurzeln hatten unter normoxischen Bedingungen niedrigere Atmungsraten, niedrigere ATP und HMP-Gehalte, etwas niedrigere Zucker und Stärkegehalte und etwas niedrigere Gesamt-Aminosäuregehalte als LNR-H-Wurzeln. Andererseits waren die Gesamt-Proteingehalte (pro FG) praktisch identisch. Offensichtlich litt die LNR-H-Transformante nicht unter N-Mangel, und ihr energetischer Zustand war unter Normalbedingungen eher besser war als der des WT. Die Daten der 35S-Transformante entsprachen weitgehend denen des WT. Dies zeigt, dass die beobachteten Unterschiede nicht auf unbekannten Faktoren des nia2-Hintergrunds beruhten, sondern definitiv auf dem Vorhandensein (bzw. der Abwesenheit) von Nitratreduktion.

Anoxia: Unter Anoxia (4h) traten bei LNR-H deutliches Welken der Blätter auf, bei WT dagegen nicht. Die Ursachen sind unklar. Unter Anoxia produzierten LNR-H-Wurzeln sehr viel mehr Ethanol und Lactat als WT, obwohl weder ADH-noch LDH Aktivitäten in Wurzelextrakten unter Anoxia erhöht wurden. Allerdings besaß die LNR-H Transformante permanent doppelt so hohe LDH Aktivitäten wie der WT.h. Auch Wolframat-versorgte WT-Wurzeln produzierten unter Anoxia mehr Lactat und Ethanol als der normale WT. Zucker und HMP-Gehalte blieben in LNR-H höher als in WT. Die NR von WT-Wurzeln wurde unter Anoxia aktiviert und die Wurzeln akkumulierten Nitrit, das großteils an die Nährlösung abgegeben wurde. 31P-NMR-Messungen zeigten, dass LNR-H-Wurzeln trotz ihres besseren Energiezustandes unter Anoxia das Cytosol stärker ansäuerten als WT-Wurzeln.

Schlussfolgerungen: Offensichtlich beeinflusst Nitratreduktion auf noch unbekannte Weise Wachstum und Morphologie der Wurzeln unter Normoxia. Die viel niedrigeren Gärungsraten der WT-Wurzeln unter Anoxia konnten nicht auf einen alternativen NADH-Verbrauch der Nitratreduktion zurückgeführt werden, weil dazu die NR-Aktivitäten zu niedrig waren. Bilanzierung der H+-Produktion durch Glycolyse, Gärung und Nitratreduktion zeigte, dass die stärkere cytosolische Ansäuerung der anoxischen LNR-H Wurzeln auf den insgesamt höheren Gärungsraten der LNR-H-Wurzeln beruhen muss. Nitratreduktion ist unter Anoxia also vorteilhaft, weil sehr viel weniger Gärung abläuft und damit cytosolische Ansäuerung abgeschwächt wird. Warum allerdings die Gärungsraten so unterschiedlich waren, blieb unklar.

Ausblick: Vorversuche hatten ergeben, dass WT-Wurzeln unter Anoxia mehr Stickstoffmonoxid (NO) produzierten als LNR-H-Wurzeln. Es wird deshalb hypothetisch vorgeschlagen, dass die Nitratreduktion über den bloßen NADH-Verbrauch hinaus durch eine anoxische NO-Produktion ein Signal erzeugt, das vorteilhaft regulierend in Stoffwechsel und Wachstum eingreift.

7. Abbreviations

ADP	adenosine 5’ diphosphate
AMP	adenosine 5’ monophosphate
ATP	adenosine 5’ triphosphate
BSA	bovin serum albumin
DTT	1,4-dithiothreitol
EDTA	ethylene diamine tetra acetic acid
FAD	flavine adenine nucleotide
Fd	ferredoxin
FW	fresh weight
G6P	glucose-6-phosphate
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthase
h	hour
Hepes	N-2-hydroxylpiperazine N-2-ethanesulfonic acid
Mes	2-N-morphilino ethane sulfonic acid
Min	minute
MK	miokinase
NAD^+	nicotine adenine dinucleotide (oxidized)
NADH	nicotine adenine dinucleotide (reduced)
$NADP^+$	nicotine adenine dinucleotide phosphate (oxidized)
NADPH	nicotine adenine dinucleotide phosphate (reduced)
NiR	nitrite reductase
NMR	nuclear magnetic resonance
NR	nitrate reductase
NRA	nitrate reductase activity

NR-PK	protein kinase of nitrate reductase
NR-PP	protein phosphatase of nitrate reductase
PEP	phospho enol piruvate
pH	hydrogen ion concentration, negative logarithm
phospho-NR	phosphorilated nitrate reductase
PK	pyruvate kinase
PMS	phenasine methosulphate
PVP	polyvinyl pirrolidone
Tris	tris hydroxymethyl amino methane

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10. Curriculum Vitae

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Research project: Normoxic and anoxic metabolism of *Nicotiana tabacum* transformants lacking nitrate reductase. Superviser: Prof. Dr. Werner M. Kaiser

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M.Sc. in Biochemistry and Microbiology, University of Sofia, Sofia, Bulgaria
Research project: AHE modulation by GABA and bicuculin. Supervisor: Dr. N. Muleshkova

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Research Experience:

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Research scientist, Laboratory of Immunology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria. Advisor: Prof. H. Taskov M.D., Ph.D.

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Research scientist, Institute of Physiology, Bulgarian Academy of Science, Sofia, Bulgaria. Advisor: Prof. R. Radomirov, Ph.D.

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11. List of publications

- Stoimenova M, Hänsch R, Mendel R, Gimmler H, Kaiser WM (2002) The role of root nitrate reduction in the anoxic metabolism of roots
  I. Characterization of root morphology and normoxic metabolism of wild type tobacco and a transformant lacking root nitrate reductase. Submitted for publication to Plant and Soil on 27.03.2002

- Stoimenova M, Libourel I G L, Ratcliffe R G and Kaiser W M (2002) The role of root nitrate reduction in the anoxic metabolism of roots
  II. Anoxic metabolism of tobacco roots with or without nitrate reductase activity. Submitted for publication to Plant and Soil on 27.03.2002

- Werner M. Kaiser, Maria Stoimenova, Hui-Min Man (2001, in press) What limits nitrate reduction in leaves? In: Advances in photosynthesis series: photosynthetic nitrogen assimilation and associated carbon metabolism. Kluwer Academic, UK.

- Kaiser WM, Kandlbinder A, Stoimenova M, Glaab J (2000) Discrepancy between nitrate reduction rates in intact leaves and nitrate reductase activity in leaf extracts: What limits nitrate reduction in situ? Planta, 210 (5): 801-807.

Poster presentations:

- July 8-12, 2001, 6th international Symposium on Inorganic Nitrogen Assimilation, Reims, France

- September 22-24, 2000, IGP on Plant Responses to Physical, Chemical and Biotic Constraints, Retzbach, Germany

12. Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation in allen Teilen selbst angefertigt and keine anderen als angegebenen Quellen und Hilfsmittel verwendet habe.

Ich habe die Dissertation werder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt.

Ich erkläre weiterhin, dass ich bislang noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Wuerzburg, Juni 2002