



RESEARCH PAPER

In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, *in vitro* and *in situ*

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Abstract

At oxygen concentrations of $\leq 1\%$, even completely nitrate reductase (NR)-free root tissues reduced added nitrite to NO, indicating that, in roots, NR was not the only source for nitrite-dependent NO formation. By contrast, NR-free leaf slices were not able to reduce nitrite to NO. Root NO formation was blocked by inhibitors of mitochondrial electron transport (Myxothiazol and SHAM), whereas NO formation by NR-containing leaf slices was insensitive to the inhibitors. Consistent with that, mitochondria purified from roots, but not those from leaves, reduced nitrite to NO at the expense of NADH. The inhibitor studies suggest that, in root mitochondria, both terminal oxidases participate in NO formation, and they also suggest that even in NR-containing roots, a large part of the reduction of nitrite to NO was catalysed by mitochondria, and less by NR. The differential capacity of root and leaf mitochondria to reduce nitrite to NO appears to be common among higher plants, since it has been observed with *Arabidopsis*, barley, pea, and tobacco. A specific role for nitrite to NO reduction in roots under anoxia is discussed.

Key words: Alternative oxidase, anoxia, cytochrome c oxidase, nitric oxide, NO, root mitochondria.

Introduction

In the past decade, the biosynthetic origin and physiological significance of NO in plants have gained increasing attention (Beligni and Lamattina, 2001; Neill *et al.*, 2003).

Much of the work on NO in plants dealt with above-ground plant material or cell cultures, whereas knowledge on NO production by roots and its role in root physiology is comparatively limited (but compare Stöhr *et al.*, 2001). This is actually surprising since roots are living in an environment where NO concentrations might be higher than above ground, at least occasionally. Most, if not all of the NO production in the soil has been attributed to the action of nitrifying or denitrifying bacteria, and a potential contribution by the roots themselves has been largely neglected (Ludwig *et al.*, 2001). In plants, several potential sources for NO have been identified, which may be divided into two groups: NOS-like activities use L-arginine as a substrate and require oxygen and a number of cofactors, whereas the other group uses nitrite and NAD(P)H as a substrate and may work in the presence, but also in the complete absence of oxygen as, for example, nitrate reductases (NR). For a recent review see Crawford and Guo (2005).

Roots, quite in contrast to leaves, are often exposed to hypoxic or anoxic conditions during soil flooding periods. Anoxic plant tissues reduce nitrate to nitrite at a high rate, yet are largely unable to reduce nitrite further to ammonia (Stoimenova *et al.*, 2003). In consequence, anoxic roots accumulate and excrete nitrite. Therefore, one may expect a most significant reduction of nitrite to NO by NR or related nitrite-dependent sources in flooded, nitrate-fertilized roots. In that context it seems interesting that nitrate fertilization has long been known to improve the survival of plants in flooding periods, and recently it has been shown that nitrate reduction decreased fermentation (Stoimenova *et al.*, 2003).

Using crude extracts and purified plasma membranes of tobacco roots, Stöhr and colleagues reported a new

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membrane-bound enzyme that also appeared to be involved in NO formation from nitrite. This enzyme required reduced cyt *c* as an electron donor and had a pH optimum of 6.1. It was designated as nitrite-NO oxidoreductase (NI-NOR, Stöhr *et al.*, 2001; Stöhr and Ullrich, 2002; Meyer and Stöhr, 2004).

Mitochondria might be another source for NO. Mammalian mitochondria reduce nitrite to NO (Kozlov *et al.*, 1999), and in the unicellular green alga *Chlorella sorokiniana*, mitochondria contribute to NO production (Tischner *et al.*, 2004). Purified mitochondria from tobacco suspension cells were also able to reduce nitrite to NO at the expense of NADH under anoxic conditions, but not in air (Planchet *et al.*, 2005). In the following, this activity of plant mitochondria is investigated in more detail. It is shown that, probably in all higher plants, only root mitochondria, but not leaf mitochondria are able to reduce nitrite to NO, both *in vitro* (isolated mitochondria) and *in situ*.

Materials and methods

Plant material

Wild-type (WT) plants and a *nia* 30 double mutant of *Nicotiana tabacum* cv. Gatersleben were cultivated in a vermiculite/sand mixture (2 parts vermiculite/1 part sand) for 2–3 weeks and from then on hydroponically for a further 3–4 weeks. Plants of growth stage 5–7 weeks that were selected for similar size were used for the experiments. During the sand/vermiculite phase plants were watered with full-strength nutrient solution twice a week. The full-strength nutrient solution for 'nitrate'-grown plants contained: 5 mM KNO₃, 1 mM CaCl₂, 1 mM MgSO₄, 0.025 mM NaFe-EDTA, 1 mM K₂HPO₄, 2 mM KH₂PO₄, and trace elements according to Johnson *et al.* (1957). The full-strength nutrient solution for 'ammonium' plants was identical to that for nitrate plants except that 5 mM NH₄Cl was used as the sole nitrogen source. For both solutions, pH was adjusted to 6.3 with KOH. Subsequently, the vermiculite/sand plants were transferred into hydroponic tanks, each containing 15 plants in 8.0 l nutrient solution, and placed in growth chambers with artificial illumination (HQI 400 W, Schreder, Winterbach, Germany) at a photon flux density of 300 μmol m⁻² s⁻¹ (PAR) and a day length of 16 h. The day/night temperature regime of the chamber was 25/20 °C, respectively. The root medium was flushed continuously with pre-moisturized air, using four 100 mm long micro-ceramic diffusers per tank (Europet Benelux, Gemert, The Netherlands). On average, fresh nutrient solution was provided every other day for both nitrate- and ammonium-grown plants or daily when it was necessary to avoid acidification of the ammonium-containing medium. During the first four days on hydroponics, plants were gradually adapted (1/10, 1/4, 1/2, and 3/4 of full-strength) to full-strength nutrient solution. If not stated otherwise, experiments were made with plants or roots harvested 6 h into the light phase. Seeds were surface-sterilized with a mixture of Tween detergent (0.05%) and H₂O₂ (0.015%) and washed four times with distilled water. Seeds were watered and kept on wet filter paper for 48 h. Germinated seedlings were transplanted into 2.0 l hydroponic pots and cultivated under the same conditions as described for tobacco. In the case of potato tubers and cauliflower, fresh materials were purchased from a local gardener.

Preparation of root segments and leaf slices

Root segments: After a quick rinse with deionized water, root systems were carefully spread on a glass plate and gently blotted with tissue paper. One gram of the lowest 2–3 cm of the secondary and tertiary roots (including the root tips) were set aside, cut into 5 mm segments and submerged in a glass vessel containing 10 ml 20 mM HEPES-KOH, pH 7.0, 5 mM KNO₃ (or 5 mM NH₄Cl, respectively) and 50 mM sucrose. Sucrose was added to make sure that no carbohydrate starvation took place.

Leaf slices: Freshly harvested leaves were double rinsed with deionized water and cut into 1 cm long and 1–3 mm wide segments, avoiding the mid-rib portion. The leaf segments (1 g FW) were vacuum infiltrated for 2–3 min in 10 ml 25 mM HEPES-KOH pH 7.4 and 0.5 mM CaSO₄. After infiltration, segments were washed once and subsequently suspended in the same buffer for the NO measurements.

Purification of mitochondria from different tissues

The isolation procedure followed previously published methods (Nishimura *et al.*, 1982; Vanlerberghe *et al.*, 1995), with slight modifications. All procedures were carried out at 4 °C. Roots (25–30 g FW) were sliced. In the case of leaves, 30–40 g of leaves were cut into slices avoiding the mid-rib portion. In the case of potato, 100–150 g of potato tubers were chopped after removing the corky peel. With cauliflower, the outer 1 cm tissue from several florets were sliced. The tissues were equally homogenized with an Ultra Turrax (IKA, Janke and Kunkel, Germany) in 250 ml of homogenization medium containing 0.3 M sucrose, 100 mM HEPES pH 7.6, 0.1% (w/v) defatted milk powder, 0.6% PVP (w/v), 1 mM EDTA, 2 mM MgCl₂, 4 mM cysteine, 5 mM KH₂PO₄, and a ready-to-use protease cocktail ('Complete', Roche, Mannheim, Germany, 1 tablet in 100 ml medium). The homogenate was filtered through several layers of cheesecloth. The filtered cell extract was centrifuged at 1000 g for 15 min, and the supernatant was centrifuged again at 10 000 g for 20 min. The resulting pellet was suspended in 4 ml of suspension medium containing 20 mM HEPES pH 7.6, 0.3 M sucrose, 0.1% (w/v) defatted milk powder, 2 mM MgCl₂, 1 mM EDTA, and 0.5 mM KH₂PO₄. The mitochondria were further purified on a discontinuous Percoll gradient composed of the following steps (bottom to top): 3 ml of 60% (v/v), 4 ml of 45% (v/v), 4 ml of 28% (v/v), 4 ml of 5% (v/v), all containing 250 mM sucrose, 20 mM HEPES pH 7.6, and 0.1% defatted milk powder. The mitochondria (4 ml) were layered on top, and the gradient was centrifuged at 30 000 g for 30 min. The mitochondrial fraction appeared at the interface between the 45% and 28% (v/v) layer and was collected, diluted in 8 ml suspension medium, centrifuged (17 000 g, 10 min), and resuspended twice in 8 ml, in order to remove Percoll. The final mitochondrial pellet was suspended in 8 ml medium. Mitochondrial respiration was followed polarographically at 25 °C in 2 ml suspension medium containing 0.5 mg protein and in addition 0.5 mM ADP and 0.5 mM Pi. Oxygen uptake was started by addition of 0.5 mM NADH (10 mM), if not mentioned otherwise. Protein content of the resuspended mitochondrial pellet (free of milk powder) was determined using the BCA protein assay reagent (Pierce, Rockford, Illinois, USA).

Catalase activity in different fractions of the Percoll gradient was determined by following the oxygen evolution after addition of 20 mM H₂O₂. A blank (medium without fractions) was always performed but gave usually no measurable oxygen evolution.

NR activity was measured in aliquots (0.1–1 ml) of the different fractions of the Percoll gradient and in the supernatant of the 10 000 g fraction following standard procedures described previously (Planchet *et al.*, 2005).

Measurement of NO by chemiluminescence

The vials containing root segments or leaf slices (1 g FW in 10 ml buffer), or mitochondrial suspensions (1.5–2.5 mg protein in 8 ml buffer) were immediately placed in a glass cuvette (1.0 l air volume) which was mounted on a rotary shaker (150 U min⁻¹). A constant flow of measuring gas (purified air or nitrogen) of 1.6 l min⁻¹ was pulled through the cuvette, through a cold moisture trap and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt; 20 s time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The measuring gas (air or nitrogen) was made NO free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). Calibration was routinely carried out with NO free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griesheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260, Tylan General, Eching, Germany) were used to adjust all gas flows. Air temperature in the cuvette was 25 °C. The cuvette's lid was custom-made to allow the injection of various solutions through a serum stopper directly into the sample without opening the cuvette or interrupting shaking cycles. The same system was used with leaf slices and mitochondrial suspensions. NO analyser data were logged into a computer and processed using customized 'Visual designer' based software (Intelligent Instrumentation Inc., Tucson, USA).

NO scavenging by mitochondria

To check scavenging of NO by mitochondria, a solution containing a defined amount of NO was prepared by flushing 10 ml distilled water with gas containing 95 ppm NO in nitrogen. According to the solubility of NO in water at atmospheric pressure (1.9 μmol ml⁻¹), the NO concentration in the solution was 180 pmol ml⁻¹. Aliquots (2 ml) of this NO solution were injected into 10 ml of water, mitochondrial buffer alone or buffer with mitochondria (1.5–2.5 mg protein in 10 ml buffer). All solutions were stirred in a head space cuvette flushed continuously with purified air or nitrogen, as indicated. Immediately after injection the NO starts to escape from the stirred solution into the gas phase, where its concentration was continuously measured for 20 min until NO emission came to an end. Integration of the emission curve revealed the total amount of NO recovered. In order to determine whether NO scavenging by mitochondria (in air or nitrogen) would be different under respiratory or non-respiratory conditions, scavenging was also compared with or without the addition of 1 mM NADH.

Results

Root segments of WT tobacco (grown hydroponically on nitrate as the N-source) usually released very little or no NO in air (Fig. 1A). After switching to nitrogen, however, the NO concentration in the gas stream increased, reaching a steady-state after approximately 30 min, where the rate of the anoxic NO release was about 9 nmol g⁻¹ FW h⁻¹ (Fig. 1A). The mean NR activity in corresponding root extracts was about 1 μmol g⁻¹ FW h⁻¹ (not shown). Thus, NR could produce nitrite at about a 100-fold higher rate than required for the measured NO release. Nitrate reductase (NR)-free root segments (either from a *nia* 30 double mutant (Fig. 1A, B) or from WT plants grown on ammonia as the N source plus tungstate (Fig. 1B), did not emit NO,

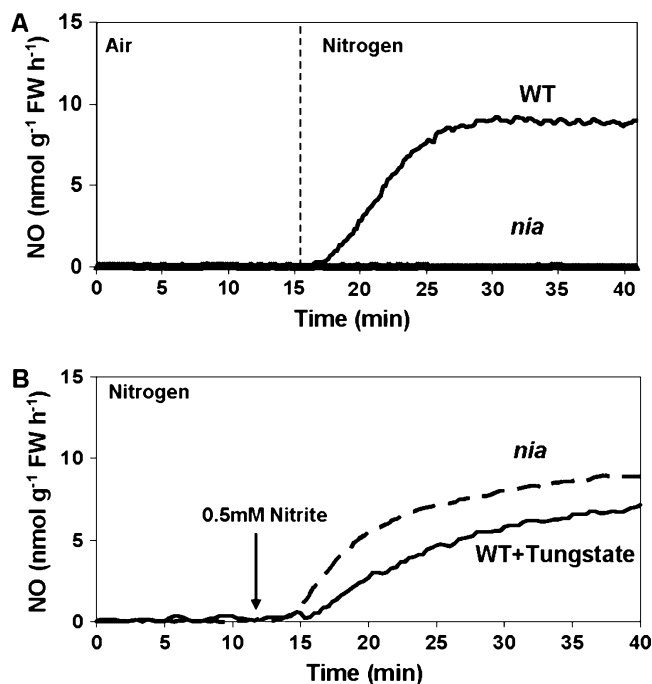


Fig. 1. (A) NO emission from endogenous nitrite by root segments of WT and of the NR-free *nia* 30 double mutant (both grown on nitrate). (B) NO emission from root segments of either WT grown on ammonium plus tungstate, or from the *nia* mutant, after the addition of nitrite. The root segments were suspended in buffer solution as described in the Materials and methods, and were flushed with air or nitrogen as indicated. The curves show representative experiments out of 3–8 independent experiments.

either in air or under anoxia, which was originally interpreted to indicate that NR was the only NO source (Rockel *et al.*, 2002). However, importantly, when supplied with nitrite, NO emission in anoxia was restored even in NR-free tissues (Fig. 1B), consistent with previous observations with NR-free mutants of the unicellular green alga *Chlorella sorokiniana* (Tischner *et al.*, 2004). It should be noted, that NO emission from intact root systems attached to the plant was also measured in a specific cuvette humidified with water-saturated air. The kinetics and rates of NO emission from such intact root systems were very similar to those obtained with root segments (not shown). However, as intact root systems were much more difficult to handle than suspensions of root segments, all experiments shown here were carried out with root segments, as in Fig. 1.

Mitochondria were isolated from roots of hydroponically grown WT tobacco (Fig. 2B) and also from roots of a *nia* double mutant (Fig. 2C). Like root segments, mitochondrial suspensions supplied with NADH and nitrite emitted NO under anoxia, but not in air (Fig. 2). Consistent with our previous observations with heterotrophic tobacco cell suspensions (Planchet *et al.*, 2005) and *Chlorella* (Tischner *et al.*, 2004), NO emission was strongly blocked by Myxothiazol, a complex III inhibitor, and the inhibition became even stronger when the AOX-inhibitor SHAM was

present in addition to Myxothiazol (Fig. 2). The figure also shows the distribution of catalase activity (as a peroxisomal marker enzyme) and of NO-emission of the four major fractions of the Percoll gradient used for purification of mitochondria (Fig. 2A). The mitochondrial fraction was practically free of peroxisomes, and it was the only fraction able to consume oxygen (numbers are given in the legends of various figures) and to emit NO. NR activity of the different fractions was also determined using the standard

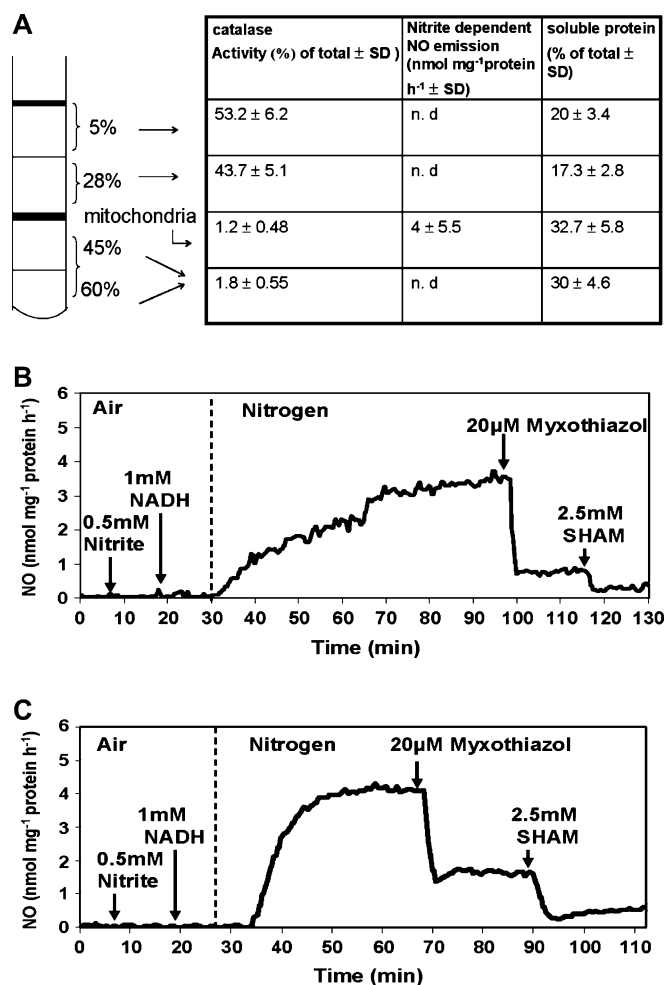


Fig. 2. (A) Characterization of Percoll fractions obtained during purification of mitochondria from tobacco roots. Catalase activity was measured as peroxisomal marker, and NO emission from nitrite plus NADH was measured as described above. NR-activity was not detectable in any of the fractions (measured with a 1 ml aliquot and 15 min incubation time), whereas NR activity was detected in the crude extract and in the supernatant of the 10 000 g fraction. Only the mitochondria fraction was able to reduce oxygen at the expense of NADH (not shown separately). It should be noted that the mitochondrial fraction was washed twice to remove Percoll, as in the standard purification procedure, whereas all other fractions were taken directly from the gradient as indicated. (B) NO emission from mitochondria purified from tobacco WT roots, and (C) mitochondria from *nia* roots. The complex III inhibitor Myxothiazol and the AOX-inhibitor SHAM was added where indicated. Curves represent typical recordings from 6 or more separate experiments. Oxygen uptake rates were 6–7 μ mol mg⁻¹ protein h⁻¹.

assay, but absolutely no NR activity could be detected in any of the Percoll fractions (see legend of Fig. 2).

The K_m for nitrite and the I_{50} for oxygen for mitochondrial NO emission were also determined. Under anoxia, the K_m for nitrite of purified mitochondria was about 175 μ M (Fig. 3A) and for root segments it was 210 μ M (Fig. 3B), which is within the range of the K_m for nitrite of NO production by purified NR (100–300 μ M; Rockel *et al.*, 2002). A 50% inhibition of NO production by oxygen was achieved at about 0.05% O₂ (Fig. 4).

While quantitative data on NO emission by roots are rare, NO production by leaves has been frequently demonstrated (see Introduction). Specifically, it has been shown that NR-free *nia* mutant leaves or NR-free tobacco cell suspensions do not emit NO (Planchet *et al.*, 2005), just like WT- and *nia* mutant cell suspensions of the unicellular green alga

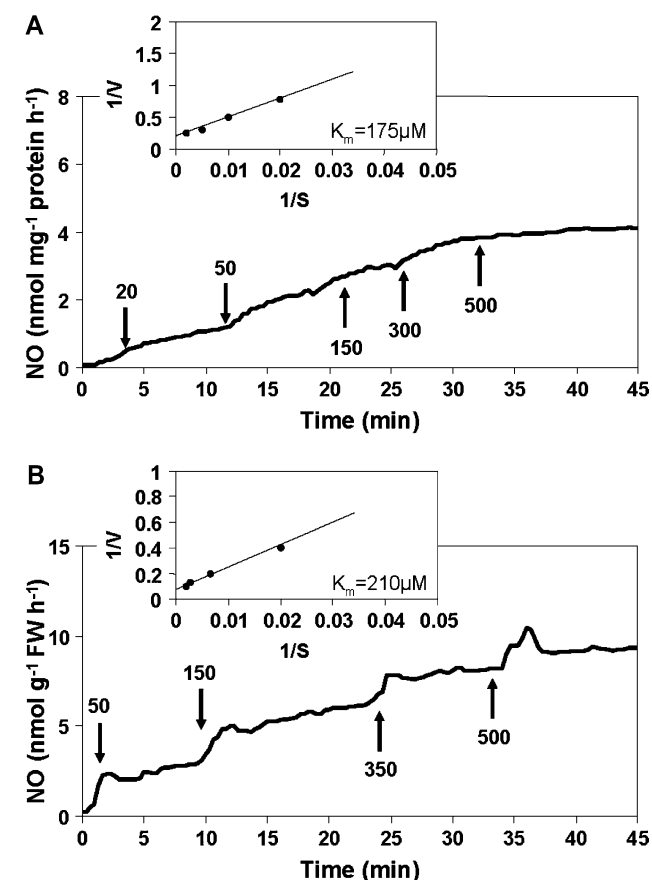


Fig. 3. Nitrite affinity of NO production by isolated root mitochondria (A) or root segments (B) (tobacco). Nitrite (100 μ l) was injected consecutively (arrows) into the reaction mixture containing either 8 ml of a mitochondrial suspension (1.5–2.5 mg protein) or 1 g root segments (FW) under the stream of nitrogen. Numbers at the arrows give final (additive) concentrations of nitrite, assuming that only a small part of the preceding addition had been consumed. Oxygen uptake of mitochondria was 6–7 μ mol mg⁻¹ protein h⁻¹. Inset gives K_m values determined by Lineweaver–Burk plots and linear regression analysis. Each NO emission curve was a representative curve out of four independent experiments, whereas mean values from three independent experiments were used to calculate the K_m .

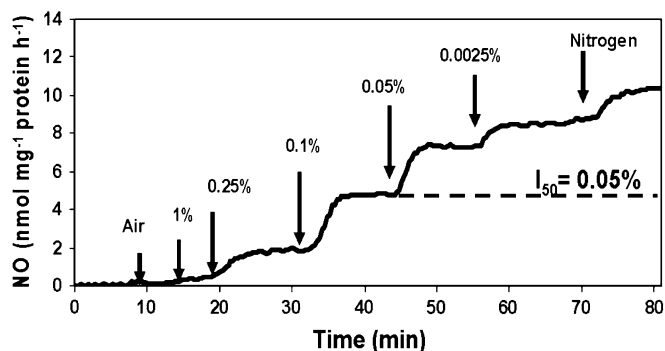


Fig. 4. Oxygen response curve of NO emission from purified root mitochondria (tobacco) in the presence of nitrite and NADH. The reaction was started in air. Where indicated by arrows, mixtures of air and nitrogen were produced with two mass flow controllers to give a constant total gas flow of 1.6 l min^{-1} . The resulting oxygen concentration is given as %. Nitrite was 0.5 mM , NADH was 0.2 mM . The curve is a representative experiment out of three separate experiments which produced almost identical curves.

Chlorella (Tischner *et al.*, 2004). When such NR-free tobacco or *Chlorella* cell suspensions were supplied with nitrite, NO production was restored. However, whether nitrite feeding to *nia* mutant leaves would also restore NO production was not examined at that time because nitrite feeding through the petiole of detached leaves is difficult to control with respect to local nitrite concentrations. Therefore thin leaf slices in solution were used here. Under anoxia, leaf slices from nitrate-grown WT plants produced NO at similar rates (on a FW basis) as root segments. But, in contrast to roots, this NO emission was insensitive to Myxothiazol (Fig. 5). Leaf slices from *nia* mutants did not emit NO even when fed with 0.5 mM nitrite under anoxia (Fig. 5). Both findings strongly suggest that in leaf tissues, all NO is derived from reduction of nitrite by NR, and conclusively, leaf mitochondria are not able to produce NO, both *in vitro* and *in situ*.

In order to check whether the above-described differential function of leaf and root mitochondria was unique for tobacco, nitrite-dependent NO production of other species was examined, both with leaf and root slices as well as with mitochondria purified from leaves and roots. In Fig. 6, leaf slices from nitrate-grown barley (Fig. 6A) and pea (Fig. 6B) produced even higher amounts of NO under anoxia than tobacco leaf tissues. Leaf slices from ammonium-grown plants, which do not express NR in leaves, emitted almost no NO even when fed with nitrite (Fig. 6A, B). Consistent with that, mitochondria purified from young barley (Fig. 6C) or pea (Fig. 6D) leaves emitted only very little NO when fed with nitrite plus NADH. Similar results were obtained with leaf slices and mitochondria from *Arabidopsis* (not shown separately). In contrast to leaves, root segments from barley (Fig. 7A) and pea (Fig. 7B) grown on ammonium produced NO at rates comparable with those of tobacco root segments (Fig. 1), with slightly different

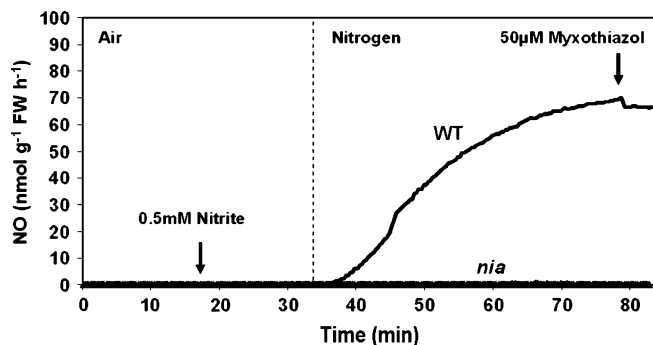


Fig. 5. Comparison of NO emission from tobacco leaf slices from WT and from the NR-free *nia* mutant. Myxothiazol was added were indicated by an arrow. Curves are representative recordings out of at least three similar independent experiments.

kinetics. Importantly, unlike leaf mitochondria, root mitochondria purified from barley (Fig. 7C) and pea (Fig. 7D) also emitted NO when fed with nitrite and NADH, just like root mitochondria from tobacco. Again, root segments and mitochondria purified from *Arabidopsis* roots gave similar data as those from the three other species (not shown separately).

The above results indicate that leaf mitochondria either lack a nitrite reductase-like activity, or they might have a very strong capacity to scavenge NO. In order to test for the latter possibility, the following experiment was carried out: mitochondria were isolated from barley leaves and roots. First, both preparations were tested separately for their ability to reduce nitrite to NO. Expectedly, NO emission by root mitochondria was $15 \text{ nmol mg}^{-1} \text{ protein h}^{-1}$, and by leaf mitochondria it was zero. In a 1:1 mixture of root and leaf mitochondria, it was 7.5 (not shown). Thus, there was no scavenging by leaf mitochondria of NO produced by root mitochondria.

The NO scavenging by isolated mitochondria in nitrogen and air was also compared using a method similar to one previously published by Shiva *et al.* (2001). Distilled water was brought into equilibrium by flushing with NO gas (usually 500 ppb) in nitrogen. An aliquot of that freshly prepared solution was injected using a gas-tight syringe through a serum stopper into a suspension of mitochondria shaking under nitrogen in the headspace cuvette. When NO was injected into pure water, NO emission started immediately, reached a peak value after a few minutes and then declined to zero. The total amount of NO emitted was more than 90% of the added NO. By comparison, recovery of NO from a mitochondrial preparation was much less, about 17%; but at least part of the total scavenging was due to a reaction of NO with the buffer used for the mitochondria (Fig. 8). In nitrogen, NO scavenging by all systems was only slightly less than in air. Also, when the mitochondria were supported with NADH as electron donor, NO scavenging was only slightly increased (Fig. 8).

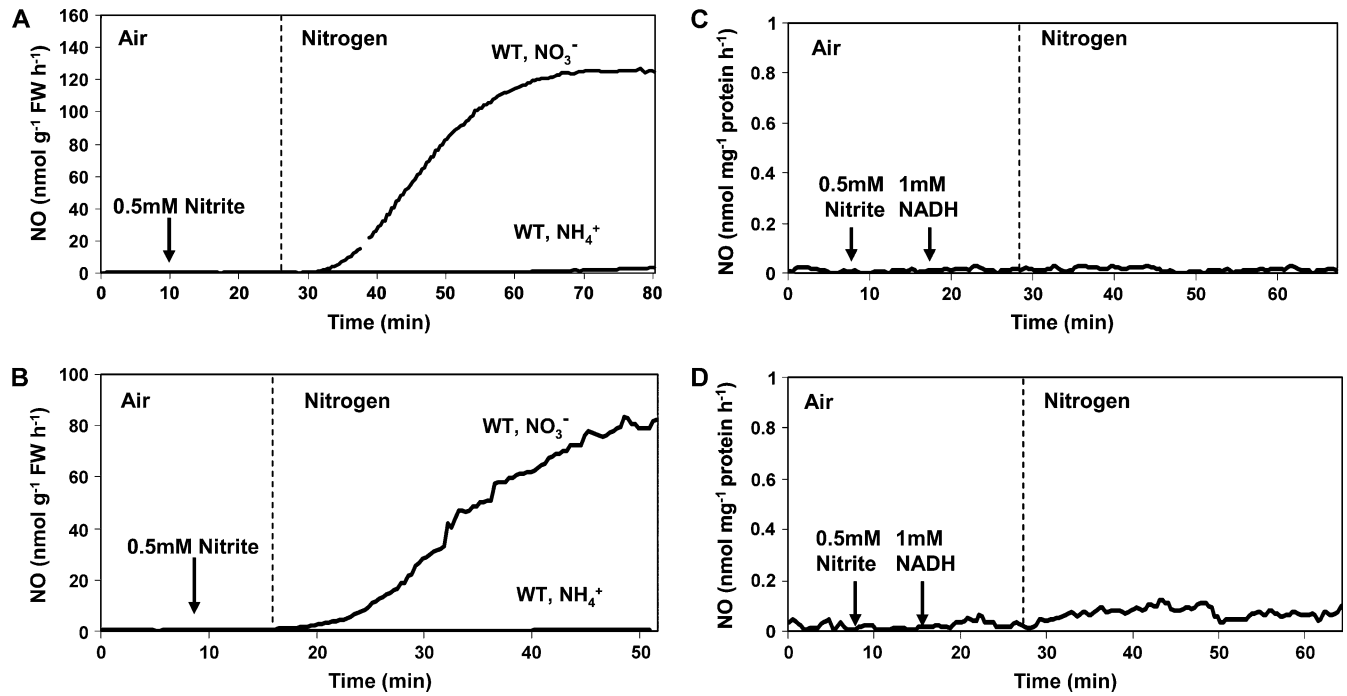


Fig. 6. NO-emission from barley (A) or pea (B) leaf slices or isolated leaf mitochondria of barley (C) and pea (D). WT plants were grown hydroponically on nitrate (=with NR activity); or on ammonium plus tungstate (=without NR activity). Mean oxygen uptake for barley leaf mitochondria was 4.5 ± 0.6 ($n=3$) $\mu\text{mol mg}^{-1}$ protein h^{-1} and oxygen uptake of pea leaf mitochondria was 5.5 ± 0.8 ($n=3$) $\mu\text{mol mg}^{-1}$ protein h^{-1} . Very similar data have been obtained with *Arabidopsis*, which are not shown separately. Curves are representative recordings from two or three typical experiments.

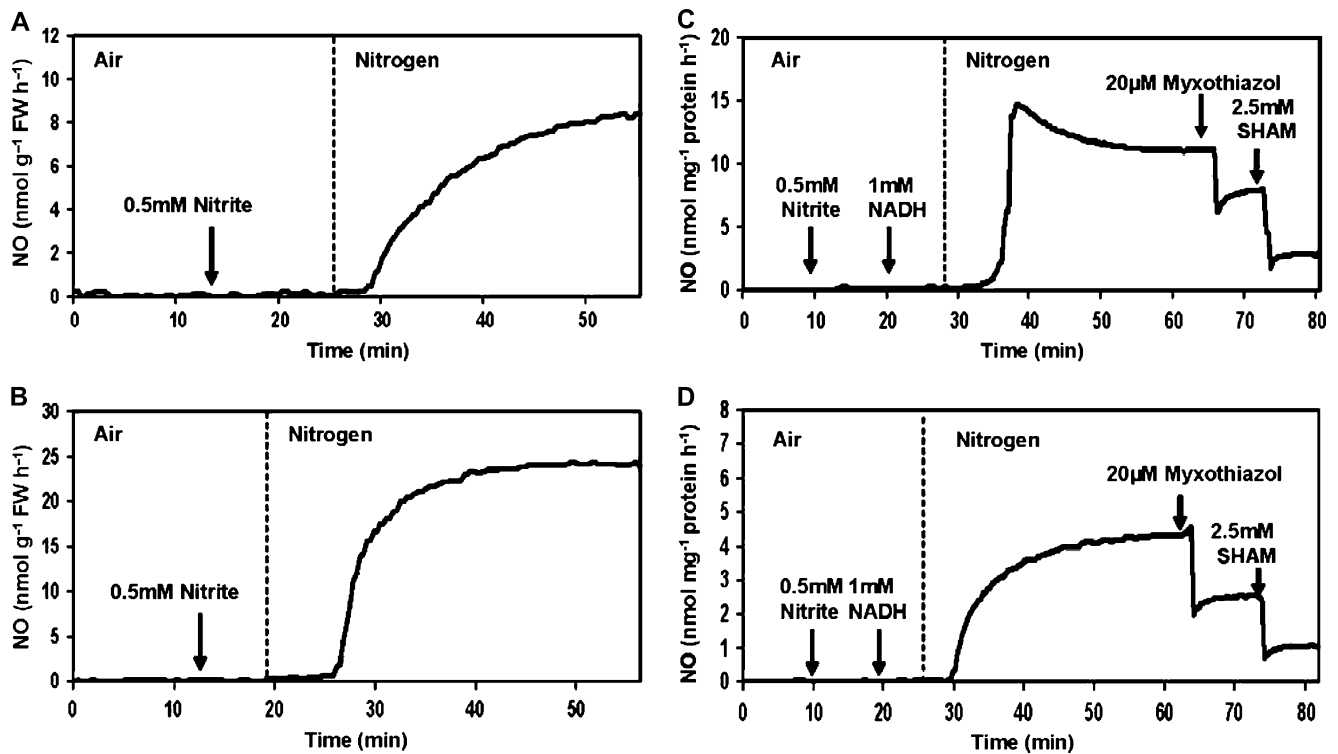


Fig. 7. NO emission from root segments of barley (A) and pea (B) and from purified mitochondria of barley (C) and pea (D) roots (both grown on ammonium as N-source). Inhibitors were added as indicated. The very rapid initial drop of the NO emission after inhibitor addition is an artefact of the injection. Mean oxygen uptake for barley root mitochondria was 6.2 ± 0.8 ($n=3$) $\mu\text{mol mg}^{-1}$ protein h^{-1} and oxygen uptake of pea root mitochondria 6.6 ± 1.2 ($n=3$) $\mu\text{mol mg}^{-1}$ protein h^{-1} . Similar results were obtained with *Arabidopsis* (not shown separately). Curves are representative recordings out of two or three separate preparations.

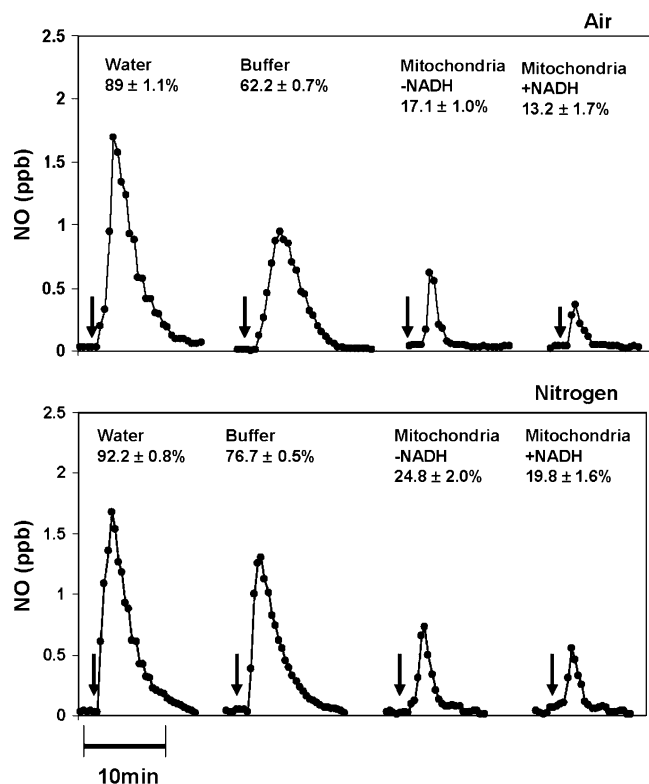


Fig. 8. Recovery of NO (180 pmol) injected into 10 ml of either distilled water, or of mitochondrial suspension buffer, or of a suspension of mitochondria (isolated from *nia* mutant roots) without or with NADH, under a stream of air or nitrogen. Arrows indicate the time of injection. As all solutions were agitated on a rotary shaker (140 r.p.m.), added NO was rapidly released. The area under the curve (integral) is a measure of the total amount of NO released. Numbers at the curve give the released NO as a percentage of the total NO added.

In order to accomplish the above comparison of non-green root tissues and green leaf tissues, mitochondria were also isolated from non-green cauliflower and from potato tubers. In both cases, oxygen uptake rates (on a protein basis) were in the range of the above experiments ($5\text{--}7 \mu\text{mol O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$), yet their rates of NO emission were below $0.25 \text{ nmol g}^{-1} \text{ FW h}^{-1}$ (data not shown).

Discussion

Root tissues, as well as mitochondria isolated from roots, were obviously able to reduce nitrite to NO via mitochondrial electron transport. This also indicates that nitrite at the concentrations used was able to enter cells and mitochondria *in situ*. The apparent NO production rate was extremely low in air, and much higher under hypoxia/anoxia. This behaviour is in marked contrast to that of purified NR, where nitrite to NO reduction was only slightly increased by anoxia (Planchet *et al.*, 2005). NO scavenging by respiring mitochondria was not sufficient to explain the large difference in aerobic and anoxic NO emission. Thus,

although competition experiments have not been carried out yet, there is speculation that oxygen and nitrite compete for the same reduction site in the mitochondrial electron transport chain. The I_{50} was in the range of 0.05% oxygen, corresponding to an oxygen concentration in water of about $0.63 \mu\text{M}$, at a nitrite concentration of $500 \mu\text{M}$. Thus, without any further investigation of the reaction kinetics it appears that the oxygen affinity of the reaction site is several orders of magnitude higher than the affinity for nitrite.

The sensitivity of NO production to the complex III inhibitor, Myxothiazol, and to the AOX inhibitor, SHAM, suggests that both terminal oxidases (CytOx and AOX) may participate in NO production, in confirmation of previous data obtained with a green alga (Tischner *et al.*, 2004) or mitochondria purified from tobacco suspension cells (Planchet *et al.*, 2005). Whether an additional nitrite:NO reductase protein is involved, or whether this activity is inherent to the terminal oxidases themselves is not clear yet.

According to the above data and to literature, a mitochondrial 'nitrite:NO reductase activity' now appears to exist in bacteria, green algae, higher plants, and animals (Kozlov *et al.*, 1999; Tischner *et al.*, 2004; Planchet *et al.*, 2005). Considering the fact that this activity covers such a wide range of organisms, including photoautotrophic algal cells, it is even more surprising that leaf mitochondria were not able to produce NO from nitrite. Whether that is due to a different property of leaf and root terminal oxidases, or to the absence from leaf mitochondria of an additional nitrite:NO reductase protein associated with the oxidases (see above), is not yet known. However, an absence of NO emission from leaf mitochondria is certainly not due to a specifically high scavenging capacity, as shown.

An obvious question, then, is why do root mitochondria do it but leaf mitochondria do not. Anoxic mammalian mitochondria also produced NO from nitrite (Kozlov *et al.*, 1999) in a Myxothiazol-sensitive reaction. As mammalian mitochondria synthesize NO primarily via NOS (Giulivi, 1998), the reduction of nitrite to NO was considered secondary and was suggested to serve for a recycling of NO oxidation products (Kozlov *et al.*, 1999). This cannot be the purpose of nitrite to NO reduction in plants, where nitrite is a normal intermediate of N-metabolism.

Roots, in contrast to leaves, may frequently experience oxygen deficiency in water-logged soils. Under those conditions, nitrate and nitrite may serve as alternative electron acceptors and may partly replace fermentation for NADH reoxidation during glycolysis, even though electron flow through NR was comparatively low (Stoimenova *et al.*, 2003). However, the above-described rates for the reduction of nitrite to NO are probably too low to be relevant as an electron sink for the maintenance of a minimum energy metabolism. It was recently suggested (Dordas *et al.*, 2004; Igamberdiev *et al.*, 2004) that

a 'haemoglobin cycle' would exist in plants that would oxidize NO to nitrate. In that case, a 'hidden' nitrite/NO cycle would maintain a much higher electron flow than suggested by NO emission. This role of plant haemoglobins would correspond to the role played by bacterial flavohaemoglobins (Pool and Hughes, 2000). However, the haemoglobin cycle is not functional under anoxia. Also, NO has been suggested to be involved in signalling of oxygen concentrations by binding to haemoglobin, and to regulate the citric acid cycle (by inhibiting aconitase) and respiration (by inhibiting CytOX and activating AOX) (Millar *et al.*, 2004).

A specific property of roots with respect to nitrite-dependent NO production was also suggested by the detection of Stöhr and colleagues (Stöhr *et al.*, 2001; Stöhr and Ullrich, 2002; Meyer and Stöhr, 2004), of a nitrite:NO reductase associated with the PM-bound NR in roots. Thus roots, in contrast to leaves, would even have two separate membrane-bound systems to reduce nitrite to NO. Such redundancy suggests that nitrite-dependent NO formation in roots serves a specific and important purpose. As a secondary messenger, NO appears to trigger cell death in plant-pathogen interactions (Delledonne *et al.*, 1998; Wendehenne *et al.*, 2001, 2004) or in differentiating xylem elements (Gabaldon *et al.*, 2005). In roots and stems of flooding-tolerant plants, hypoxia leads to the differentiation of air-conducting 'aerenchyma', which may involve the induction of lysigenous cell death. Ethylene biosynthesis also triggers aerenchyma formation. However, the conversion of ACC into ethylene requires oxygen and thus does not work under anoxia. On the other hand, NO production is optimal under anoxia, and thus NO might take over some of the signalling for lysigenous cell death when oxygen is rapidly and completely consumed. While this is speculative at this point, the fact that root and leaf mitochondria are different suggests at least some connection of NO production with specific aspects of root metabolism in relation to hypoxic/anoxic conditions.

The lack of ability of leaf mitochondria to produce NO might somehow be related to photosynthesis. However, nitrite-dependent NO production was also lacking in mitochondria from other non-green tissues like potato tubers and cauliflower inflorescences (not shown). On the other hand, as mentioned above, mitochondria from non-green tobacco cell suspensions reduced nitrite to NO, although with a lower capacity than root mitochondria (on a protein basis). Thus, more work is required to find out what the molecular basis for the observed differences is and which physiological purpose they might fulfil.

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