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REVIEW ARTICLE

Generation and possible roles of NO in plant roots and their apoplastic space

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Abstract

In recent years, three different enzymatic pathways and a few non-enzymatic reactions have been proposed for the generation of NO in plant roots. Two of the enzymatic pathways are located in the cytosol of the plant cells, whereas the third is exclusively located in the root plasma membrane facing the apoplast from where it seems to interact with nitrate metabolism by producing signals. A response of the NO pathways to external nitrate concentrations by preventing excess nitrate nutrition, particularly in the apoplast, as well as a regulatory role in root morphogenesis of NO in interaction with plant hormones is suggested. Other functions of NO, those in stimulating plant defence reactions against pathogens and against abiotic stress are reported. In addition to enzymatic NO formation by the plant, sources of NO in the soil, and hence in the rhizosphere from bacterial nitrification and denitrification, are discussed in view of their possible interaction with the plant roots. A synoptical perspective is given on the assumed roles of apoplastic NO in plant roots, based upon known facts and with some assumptions about the gaps in current knowledge.

Key words: Nitric oxide generation in plant roots, nitric oxide in plant defence, nitric oxide signals in plants, root apoplast.

Introduction

For a long time nitric oxide (NO), like nitrogen dioxide (NO_2), was regarded as being produced mainly by electrical discharges in the atmosphere, whereas more recently it accumulated as a component of pollution gases produced by heavy industry and traffic, first on a local, then on a global scale. Thus in past decades, the investigation in plants was mainly concentrated on damaging effects, for example, effects on the photosynthetic apparatus and on chlorophyll levels in the trees of forests, urban park areas and industrial districts. In medical science, however, the role of nitrogen oxides as physiologically important compounds was recognized much earlier and the indirect use of NO as nitroglycerol in heart medicine has a long tradition.

The biosynthetic pathway of NO and the role of NO as a signal substance in various processes in human and animal physiology, such as smooth muscle relaxation, vessel regulation, tumor stimulation and inhibition, and as a neurotransmitter, have only been elucidated recently (Schmidt and Walter, 1994; Mayer and Hemmens, 1997). These discoveries led to intensive research in plants where stimulatory effects were reported on the delay of senescence and on seed germination. In plants it is a growth regulator (Beligni and Lamattina, 2000) functioning, to some extent, by interaction with ABA (Neill *et al*., 2002; García-Mata and Lamattina, 2002) and as a counteragent of ethylene (Leshem *et al*., 1998). Yet it can also stimulate the induction of defence reactions against plant pathogens (Delledonne *et al*., 1998, 2001; Durner and Klessig, 1999). The search for the origin of NO in plant metabolism resulted in the detection of more than one enzymatic pathway. All of these pathways can produce considerable amounts of NO, sufficient for signal quantities and for the observed NO release from plants. Moreover, the chemical, non-enzymatic formation of NO may also play a certain role in plants.

There is another aspect of research on NO that may be important for plants, the release of the nitrogen oxides NO and N_2O from almost all soils and canopies, at particularly high rates from agricultural soils well supplied with nitrogen and in anaerobic conditions. Most of this release has been attributed to soil bacteria, but there may be an important contribution from the roots, as well as from the

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aerial parts of plants, which has to be considered on the basis of recent knowledge of the biochemistry and physiology of NO.

Chemical properties of NO

NO is an uncharged lipophilic gas with a moderate solubility in water (1.95 mmol l^{-1} at 1 bar (100 kPa) and 20 °C; Henry *et al*., 1997). Its diffusion coefficient in aqueous solution is close to that of O_2, HO_2^* and O_2^-, yet its small Stokes' radius and charge neutrality allow easy intramembrane and transmembrane diffusion (Lancaster, 1996). Compared with most other free radicals it has a relatively long biological half-life, since it does not dimerize nor dismutate. It has third order kinetics for chemical turnover (pH independent), with a second order dependence on NO concentration explaining the highly variable stability and availability in biological systems (Henry *et al*., 1997). At low concentrations (less than 1 µmol l^{-1}) NO can have a half-life of minutes to hours and could thus diffuse over several cell layers or over longer distances in intercellular spaces. At higher concentrations, NO has a much shorter half-life, in the order of seconds (Henry *et al*., 1997). The half-life of NO also depends on the local concentration of its targets, for example, proteins, haemoproteins, bound iron, bound copper, cysteine, ascorbic acid, oxygen, and H_2O_2.

NO is not an electrophilic nitrosating agent, unless it is converted to NO^-, NO_2, N_2O_3 or peroxynitrite. The most pronounced chemical property of NO is an unpaired electron, leading to a high reactivity with O_2 and O_2^- and with several N compounds. Autoxidation of NO yields nitrite but not nitrate. In addition, dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2) are formed and represent the reactive N- and S-nitrosating species produced during autoxidation of NO, which is a much slower reaction in the gas phase than in an aqueous phase. Another important reaction under aerobic conditions is that of NO with O_2^- to give peroxynitrite ($ONOO^-$) (Huie and Padmaja, 1993). Peroxynitrite is a strongly oxidizing molecule and effects attributed to NO are, in part, due to this compound. In the absence of air or another oxidant, NO can react with thiols in an alkaline environment to yield disulphide and N_2O. The reaction of NO with secondary amines leads to the formation of nitrosamines, that, with aromatic amines, leads to deamination, for example, the irreversible deamination of deoxynucleotides in DNA and causes point mutations. The toxicity of NO is a consequence of its reactivity with transition metal proteins and oxygen and of its ability to form adducts with amines and thiols of varying stability (Van der Vliet *et al*., 1996). Thus, NO in excess is toxic to bacteria, fungi, microbial parasites, tumour cells, and viruses (Zumft, 1997) as well as to higher organisms.

NO generation in plants

The inorganic forms of oxidized nitrogen are sources of NO formation in plants much more than in animals, since they are components of the nitrogen assimilation pathway. In certain conditions, enzymatic and non-enzymatic reduction of nitrate and nitrite can lead to the production of NO. The non-enzymatic formation of NO is favoured at acidic pH values, when nitrite may dismutate to NO and nitrate. Between pH 3 and 6 nitrite can also be chemically reduced by ascorbic acid to produce NO and the semi-hydroascorbyl radical and dehydroascorbate (Henry *et al*., 1997). This reaction may occur in the chloroplasts and also in the apoplastic space for which the presence of ascorbic acid has been reported (Horemans *et al*., 2000). In the leaf apoplast ascorbate may amount to 5% of the total ascorbate content (C Foyer, personal communication), whereas data for the root apoplast are not available so far. Chemical apoplastic formation of NO, therefore, strongly depends on an acidic pH and on the extracellular accumulation of nitrite, which may occur under anaerobic conditions. The light-mediated reduction of NO_2 to NO by carotenoids may be another mechanism for the non-enzymatic formation of NO in plants (Cooney *et al*., 1994).

It has been discussed whether NO could be regularly formed *in vivo* during nitrate assimilation in plants (Fig. 1).

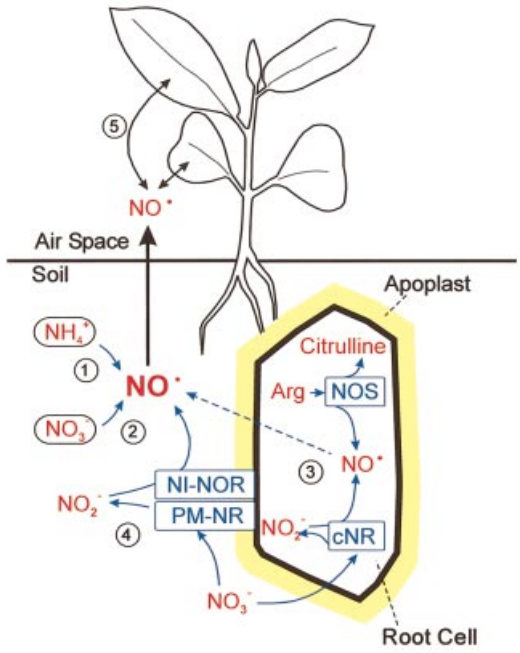

Fig. 1. Generation and distribution of NO as proposed for the roots and rhizosphere of higher plants. (1) Bacterial nitrification; (2) bacterial denitrification; (3) NO generation in root cells by cytosolic nitrate reductase (cNR) and/or NO synthase (NOS); (4) NO generation in the root apoplast from nitrate by plasma membrane-bound nitrate reductase (PM-NR) and nitrite:NO reductase (NI-NOR); (5) exchange of NO (probably mainly release from the soil) between the atmosphere and aerial plant organs.

Wildt *et al*. (1997) presented data proving NO emission from different plant species such as sunflower (*Helianthus annuus*), sugar cane (*Saccharum officinarum*), rape (*Brassica napus*), spruce (*Picea abies*), spinach (*Spinacia oleracea*), and tobacco (*Nicotiana tabacum*), as a side-reaction of the nitrate assimilation process. Accordingly, the two enzymes catalysing nitrate reduction in plants, nitrate reductase in the cytosol (cNR) and nitrite reductase (NIR) located in the plastids, were studied for their NO-forming capacity.

The generation of NO and N_2O by cNR from nitrite as the substrate was observed in soybean (Dean and Harper, 1988; Klepper, 1990), in maize and spinach (Rockel *et al*., 2002), and in tobacco roots (Fig. 1). Only recently was this reaction biochemically characterized by Yamasaki *et al*. (1999) using purified cNR from maize. It was shown that a side-reaction of cNR is the reduction of nitrite to NO with NADH as an electron donor, probably catalysed by the same molybdenum cofactor-containing domain as in nitrate reduction. NO formation by cNR requires high nitrite concentrations (K_m of cNR for nitrite is about 300 μM; Yamasaki and Sakihama, 2000). Yet the accumulation of nitrite to high concentrations within the cell seems unlikely under natural conditions, since nitrite as well as its acid form, nitrous acid, are highly toxic (Sinclair, 1987), and nitrite is rapidly reduced by NIR. Only under anaerobic conditions does nitrite accumulate *in vivo* (Botrel *et al*., 1996). Interestingly, the presence of oxygen favours another side-reaction of cNR, the production of superoxide anions (Barber and Kay, 1996). It has been demonstrated that superoxide anions react with NO to form the highly toxic compound peroxynitrite (Huie and Padmaja, 1993). Altogether this may suggest that NO can be formed by cNR and accumulates only when the cells are in transition to the unfavourable anaerobic conditions. This will occur more often in roots than in leaves. Yet, the distribution of cNR in plant organs depends on nitrate supply. It was shown for tobacco plants (Stöhr, 1999) that, with a limiting external nitrate concentration, cNR activity in roots is induced but declines with higher external nitrate. The cNR in leaves is regulated inversely to the root enzyme under those conditions. This behaviour has also been demonstrated for other species (e.g. *Phleum arenarium*, *Dactylis glomerata*, M Fersche, unpublished data). This also indicates a differential role of cNR in NO formation in shoots and roots that is dependent on the physiological conditions. Whereas cNR might play an important role for NO formation in leaves at sufficient nitrate (Rockel *et al*., 2002), its role in roots is probably restricted to conditions when nitrate is limiting and/or under anaerobic conditions when cNR is highly activated in roots and nitrite is accumulating.

The involvement of NIR in NO_x formation was studied in transgenic tobacco plants that express an antisense NIR construct (Vaucheret *et al*., 1992) and have very low NIR activities and hence accumulate nitrite (Goshima *et al*., 1999). In these plants, emission of N_2O occurred, but not in the wild type or in transgenic plants grown on ammonium. When NR activity was blocked, no evolution of N_2O was found (Goshima *et al*., 1999). In any case, *in vivo* NO production by NIR seems to be negligible. However, other reactions could account for the NO production when nitrite concentrations are high. For instance, the respiratory chain of mammalian mitochondria seems to be able to reduce nitrite to NO (Kozlov *et al*., 1999). Similarly, xanthine oxidase, a ubiquitous molybdo-enzyme, was found to catalyse the reduction of nitrite to NO under hypoxia and in the presence of NADH (Zhang *et al*., 1998; Godber *et al*., 2000).

Recently, a new plasma membrane-bound enzyme, nitrite:NO reductase (NI-NOR), was discovered as being involved in NO formation from nitrite by plant roots. Almost all NO forming activity with nitrite at pH 6.1 found in the crude extract of tobacco roots was recovered in the microsomal fraction. Further purification of the membrane fraction resulted in a high enrichment of NO forming activity (40-fold) in the plasma membrane (PM) fraction (Stöhr *et al*., 2001) with reduced cytochrome *c* as the electron donor, not with NADH. The optimum activity was reached at pH 6.1 and the reaction was completely independent of oxygen and unaffected by NR inhibitors. Most important for this pathway, increased nitrite accumulation was not a precondition as for cNR, since V_{max} was attained at approximately 100 μM nitrite. The reduction of nitrite to ammonia was not found in PM vesicles, indicating that neither cNR nor NIR were involved. Size exclusion chromatography demonstrated that NO formation in roots was not catalysed by PM-NR either, but by the hitherto unknown enzyme, NI-NOR.

Some denitrifying prokaryotes are able to reduce nitrite to NO with a respiratory nitrite reductase (reviewed by Zumft, 1997). Beside a copper-containing NIR most bacterial strains from aerobic soils possess a membrane-bound periplasmic cytochrome *cd1* NIR. This is a tetrahaem protein containing two different types of haem, the covalently bound haem C and the non-covalently bound haem D_1. Electron donors are the cytochromes c_{551} and c_{552} present in the plasma membrane, but also the externally added cytochrome *c* of mitochondria. The homodimer with a subunit mass of about 60 kDa appears to be considerably smaller than the NI-NOR protein of plant roots. However, the native plant NI-NOR with an apparent molecular mass of about 300 kDa could also be a protein complex and/or a polymer of the subunits. Until now an enzyme similar to that in bacteria has not been detected in plants.

It is hypothesized (Meyer and Stöhr, 2002) that succinate-dependent PM-NR and NI-NOR might be tightly associated with each other at the apoplastic surface of root plasma membranes (Figs 1, 2). The specific NO formation

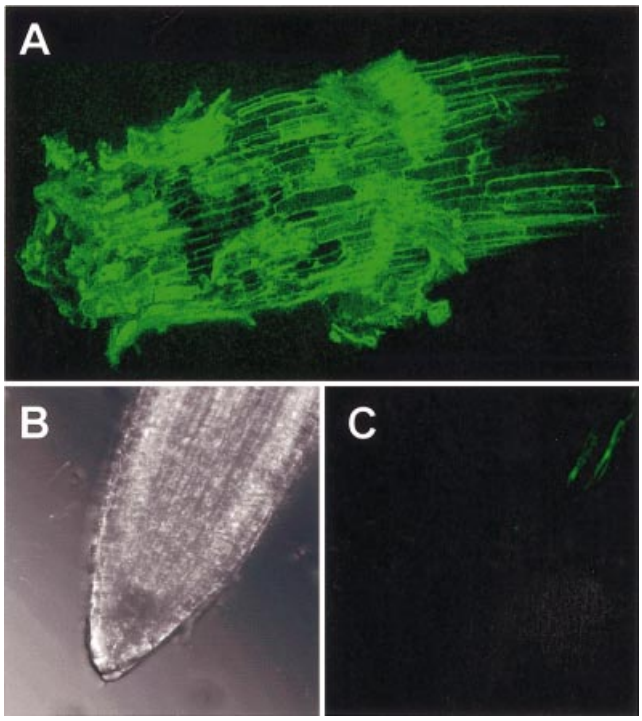

Fig. 2. NO formation by tobacco root tips. NO captured and visualized by reaction with 10 μM 4,5-diaminofluorescein (DAF-2) (Kojima *et al*., 1998; Foissner *et al*., 2000) at pH 6.0 in the presence of 100 μM nitrite. (A) Fluorescence in the apoplast of root cells detected by CLSM after 5 min incubation. The display is a projection of 62 confocal images. Cells of the root tip, mainly root cap cells and rhizodermis cells, apparently produce much NO, older and inner tissues are barely labelled by DAF-2. The strongest NO staining is in detached root cap cells. (B) Overview of a root tip presented as a transmission micrograph in white light. (C) In the presence of the NO scavenger methylene blue (1 μM) no fluorescence was detected indicating the specificity of the reaction of DAF-2 with NO; same root tip as in (B).

activity found at the root plasma membrane with about 300 nmol h^{-1} mg^{-1} PM protein would be sufficient to reduce all nitrite produced by PM-NR at pH 6.0, the widely determined apoplastic pH (Felle, 2001). Considering losses in activity during plasma membrane preparation, the *in vivo* activity could be even 10–20-fold higher.

In animals, NO is enzymatically produced by NO synthase with NADH, arginine and O_2 as substrates in the presence of the cofactors FAD, FMN and tetrahydro-L-biopterin (Mayer and Hemmens, 1997). Although in various plants NO synthase-like enzymatic activity was detected (Cueto *et al*., 1996; Durner and Klessig, 1999; Wendehenne *et al*., 2001; Corpas *et al*., 2001), the occurrence of the NOS protein has not been finally proved, neither biochemically nor genetically. But recently a partial clone of NOS in pea leaves was reported with high homology to mammalian NOS (Corpas *et al*., 2001). Cueto *et al*. (1996) and Ribeiro *et al*. (1999) presented data showing the existence of a NOS-like enzyme in roots of lupin (*Lupinus* sp.) and maize. A 166 kDa protein cross-reacting with the antibody against NOS protein from macrophages and with the antibody against the NADPH-binding region of mammalian NOS protein was located preferentially in the cytosol of the division zone and in the nuclei of the elongation zone of roots.

Effects of NO in metabolism and development of plant roots

In plants, as mainly reported from shoots, NO is involved in various metabolic and developmental processes as recently reviewed by Beligni and Lamattina (2001a, b). NO delays leaf senescence (Leshem and Haramaty, 1996). Thereby, an inverse relationship between the release of ethylene and NO seems to exist (Leshem *et al*., 1998; Magalhães *et al*., 2000). Such an inverse relationship was also observed during heat stress in *Medicago* (Leshem *et al*., 1998). Moreover, NO seems to play an inducing role in seed germination (Grubišić and Konjević, 1990), in de-etiolation and in hypocotyl elongation (Beligni and Lamattina, 2001b).

In a similar way to the effect on leaves (Leshem and Haramaty, 1996), an increase in tissue expansion was observed in the roots, but only with low concentrations, whereas high concentrations of NO were inhibitory (Gouvêa *et al*., 1997). There are different pathways for the production of NO in roots and it may also be involved in developmental processes. Depending on the developmental zone of the root, a NOS-like protein has been localized in the cytosol or in the nucleus (Ribeiro *et al*., 1999), indicating that NO could activate a nuclear transcription factor necessary for rapid growth in the root elongation zone.

As described before, one source of NO in roots is the reaction of NI-NOR in the apoplast, the reduction of nitrite to NO, the nitrite being generated from nitrate by the apoplastic PM-NR. For this reason, the concentrations of apoplastically formed NO are dependent on the nitrate concentration in the immediate environment around the plant root, usually the soil. The influence of nitrate, however, is complex. The initiation and development of additional lateral roots were observed as a response to nitrate (Forde and Lorenzo, 2001). The authors distinguish two different mechanisms with different signal systems. A local effect of nitrate implies a 2- to 3-fold elongation of already formed lateral roots, but in some cases their initiation as well (Zhang *et al*., 1999). Very high nitrate concentrations, however, do not stimulate the initiation of lateral roots, but delay their development and elongation in the state of emergence from the parent root. Zhang and Forde (2000) and Forde and Lorenzo (2001) suggest that nitrate itself may function as the inducer molecule, since mutations with defects in the *nia* genes show the same reaction as the wild type. Zhang and Forde (2000) demonstrated that a nitrate-inducible, root specific,

MADS-box protein is required for the nitrate stimulation of root development. They also assume the involvement of a plasma membrane-bound protein in nitrate-triggered signal transduction, finally leading to the activation of transcription factors. However, so far it has not been taken into account that, even in *nia* mutants, PM-NR activity is always observed (C Stöhr *et al*., unpublished results). It appears the NO produced by PM-NR and NI-NOR in the plant roots is a good candidate for regulating root growth as a consequence of external nitrate supply. Due to its apolarity, NO will easily enter the cells via diffusion across the plasma membrane and may induce secondary reactions in the cytosol, such as the reported activation of guanylyl cyclase and hence increased cGMP concentrations (Kröncke *et al*., 1997; Durner *et al*., 1998). Calcium and calmodulin also seem to be involved in NO-mediated signal transduction (Stamler, 1994).

A proposed model (Meyer and Stöhr, 2002) suggests that with limited nitrate availability NO might be one of the primary signals to announce the presence of nitrate. Higher concentrations of nitrate in the apoplast would lead to high rates of NO formation. This might result in a loss of N to the soil and to the air space as gaseous NO, but, equally, might result in higher intracellular NO concentrations, which might either be consumed by assimilation to organic N-compounds, particularly during the night (Stöhr and Mäck, 2001) or lead to the detrimental effects on growth as observed with tobacco plants grown at high nitrate concentrations (Stöhr, 1999).

As mentioned before, short-distance transport of NO across membranes with lipophilic layers seems to be easy. Long-distance transport has been shown indirectly by experiments with NO and N_2O release from soil and plants, where in plants, at least in those with a more or less coherent intercellular space system, NO (like N_2O) could migrate to the atmosphere and from root to shoot at a sufficiently high speed and with an apparently sufficient half-life (Skiba *et al*., 1997; Hereid and Monson, 2001).

Thus, NO released or internally transported from the root system, like that from the soil, may even function as a gaseous signal to send information to the shoots. This may be the case for nitrate-triggered signal transduction, but also for physiological reactions independent of the nitrogen assimilation pathway, such as the observed closure of stomata in response to drought (García-Mata and Lamattina, 2001, 2002). It was demonstrated that endogenous as well as exogenous NO contributes to the ABA-dependent stomatal closure (Neill *et al*., 2002). Thus it could act in addition to the well-known abscisic acid signal via the xylem water conduct (Hartung and Davies, 1991; Sauter *et al*., 2001). Obviously NO and ABA could induce stomatal closure independently, but have a synergistic effect (Neill *et al*., 2002; García-Mata and Lamattina, 2002). Drought often affects the root system first, which may respond with increased NO release. NO could then be released to the atmosphere where it may reach the shoot and induce stomatal closure, in the same way as, but less than, ethylene does (Taylor and Gunderson, 1986). When the transport of gaseous NO through the plant organs is very limited, other transport forms in solution, for example, as *S*-nitrosoglutathione, may replace it, as described for animal systems (Hogg *et al*., 1996; Durner and Klessig, 1999).

In some respects, the distribution of NO could be similar to that of ethylene, another long-distance messenger in the regulation of plant development, which can migrate as a gas and as a dissolved precursor (ACC). An inverse correlation between NO and ethylene formation has been described for various plant species (Leshem *et al*., 1998; Magalhães *et al*., 2000), but this mainly concerns the development of plant organs between anthesis and senescence, when NO is usually formed at much higher rates than ethylene (Magalhães *et al*., 2000). Young plants and tissues produce much more NO, and not only in stress conditions, whereas senescence is characterized by an increasing production of ethylene (Leshem *et al*., 1998). From these data NO is suggested to be a senescence-delaying agent for the plants. Yet both messengers are very much increased in a burst upon abiotic or biotic stress (Garcês *et al*., 2001).

NO in plant defence reactions

According to the chemical properties and to the biochemical reactions and enzymes so far investigated, contrasting roles of NO were observed under biotic stress dependent on its concentration (see review by Durner and Klessig, 1999; Beligni and Lamattina, 1999a, b). At low concentrations NO can interrupt the radical-mediated lipid oxidation and thus play a protective role, whereas at higher concentrations it can have a synergistic effect with reactive oxygen leading to toxic products (Beligni and Lamattina, 1999a, b). Direct effects on plant pathogens have been reported and postulated as well as indirect effects attributable to the signal role of NO.

In many stress reactions, particularly those induced by pathogens or their avirulent strains, plants respond with an oxidative burst (Murphy *et al*., 1998), detectable as a rapid overproduction of reactive oxygen species (ROS), i.e. of the superoxide anion (O_2^-) and, some hours later, of hydrogen peroxide (Goodman and Novacky, 1994; Fritz *et al*., 1996; Lamb and Dixon, 1997; Ferrer and Ros Barceló, 1999; Foissner *et al*., 2000). O_2^- itself can function as an antimicrobial agent in the apoplastic space of plant cells or at the surface of tissues, but it can also react with NO, which is produced at higher rates under those conditions, to form peroxynitrite, an even more reactive agent and lethal for many pathogens. O_2^- and peroxynitrite can act synergistically and be used as primary chemical weapons in the defence against invaders,

when the invasion has been recognized and if the invading pathogens are not sufficiently protected against these agents. As a direct effect of triggering the oxidative burst, NO has been reported to stimulate lignification in the cell walls of infected tissues (Ferrer and Ros Barceló, 1999).

However, there are also contrasting results, in which NO, instead of stimulating peroxide formation, caused a decrease in ROS generation (Caro and Puntarulo, 1998) or even protected potato plants against the deleterious effects of *Phytophthora infestans* when added via chemical NO donors (Laxalt *et al*., 1997; Beligni and Lamattina, 1999a). This seems to depend on the reaction of NO with endogenous organic radicals (Beligni and Lamattina, 1999b).

Apart from these rather direct effects, especially when its concentration in the cells and in the apoplast increases, NO is known to induce a higher expression of various enzymes involved in defence reactions. The most rapid of the inducible overall reactions is the hypersensitive response (HR) (Heath, 2000; Delledonne *et al*., 2001). It is suggested that HR might be a combination of direct local attacks by the oxidants on the host cells causing lipid layer decay and permeabilization of the plasma membranes (Goodman and Novacky, 1994; Heath, 2000), as visualized by a general decay of the electrical membrane potential (Pavlovkin *et al*., 1986), in addition to the activation or induction of superoxide dismutase and other enzymes related to programmed cell death (Durner *et al*., 1998; Wendehenne *et al*., 2001). In haploid callus cultures of *Taxus brevifolia* NO accumulation, upon the application of a NO donor, could be shown histochemically and was followed by nuclear DNA fragmentation and cell death. This cell death could be prevented by a NOS inhibitor (Pedroso *et al*., 2000). *Arabidopsis* cell suspension cultures treated with the avirulent strain *Pseudomonas syringae* pv. *maculicola* responded with NO release showing a first maximum after 1 h and a further increase some hours later, while the maximum level of H_2O_2 was reached after 5 h and hypersensitive cell death was almost complete after 24 h, thus indicating an early place in the signal chain for NO (Clarke *et al*., 2000; Delledonne *et al*., 2001). However, Clarke *et al.* could show that the programmed cell death was independent of the oxidative burst with ROS and also of the formation and concentration of peroxynitrite, which suggests a separate regulatory role of NO. As one of the mechanisms leading to cell death, it is suggested that NO converts cytosolic aconitase to a mRNA binding protein (Navarre *et al*., 2001), which inhibits the accumulation of ferritin and causes the accumulation of free iron, which then catalyses ROS formation by the Fenton reaction (Wendehenne *et al*., 2001; Murgia *et al*., 2002).

Other, even more indirect, effects of NO are induced resistance (IR) and systemic acquired resistance (SAR). For details and the many, partly controversial data refer to some recent reviews (Durner and Klessig, 1999; Heath, 2000; Shirasu and Schulze-Lefert, 2000; Beligni and Lamattina, 2001a). Pathogenesis-related protein 1 (PR1) and phenylalanine ammonia lyase (PAL) were found to be increased by external NO or NO donors and also by the secondary messengers cGMP or cyclic ADP-ribose (Durner *et al*., 1998). An increase in the formation of cGMP apparently is one of the earliest effects of NO, followed by increased concentrations of salicylic acid and, in contrast to the induction by H_2O_2, by higher activities of PAL (Wendehenne *et al*., 2001). This suggests that both NO and ROS can lead to the final result of acquired resistance, but not completely via the same pathway. Both agents together will act synergistically, as often found during the development of resistance. For systemic resistance it would be important to know how far NO can be regarded as a transmitter of signals over shorter and long distances, a role so far mainly attributed to ethylene, salicylic acid and jasmonic acid or the methylated forms of the latter two (Van Loon *et al*., 1998; Kuć, 2001).

Finally, some data also report the increased formation of phytoalexins upon the application of NO or NO donors. In a particular case, potato plants (*Solanum tuberosum*) produced high concentrations of rishitin (Noritake *et al*., 1996).

Most of the literature reported here was focused on shoot and leaf material and on cell cultures. Roots, the main subject of this article, are not easily accessible to phytopathological experiments and, hence, have been much less investigated with respect to the formation and effects of NO. Systemic resistance against the typical root pathogens of the genus *Pythium* induced by *Pseudomonas* spp. has been reported for cucumber (*Cucumis sativus*) (Chen *et al*., 1998). The presence of NOS has been indicated for roots and root nodules of *Lupinus albus* with ^{14}C-labelled arginine and NOS antagonists in tissue extracts (Cueto *et al*., 1996).

NO in soils and possible interactions with plants

In recent years various papers have dealt with fluxes of NO and N_2O from the soil to the atmosphere, mostly on a regional or even a global scale and in connection with atmospheric fall-out, with fertilization and with the environmental problems of the so-called greenhouse gases (Matson *et al*., 1998; Stange *et al*., 2000; Venterea and Rolston, 2000; Parton *et al*., 2001; Butterbach-Bahl *et al*., 2001). They report on data from various ecosystems such as temperate and tropical forests and plantations, fertilized and unfertilized grasslands and agricultural field crops. For general information about NO and N_2O exchange between soils and the atmosphere and its modelling, the reader is referred to this literature because the present article is restricted to plant roots and their close environment in a mainly physiological context. However,

some of the data show that there is a strong influence by ecological parameters on NO and N_2O release rates from the soil and this probably reflects strong variations in their synthesis rates within the soil. High temperatures, in temperate climates during the summer season, stimulate NO fluxes, as well as heavy rainfall with subsequent temporary or general anoxia, as N fertilization or P limitation and low pH. In this respect, the soil with its micro-organisms (and the plant roots) respond in the same way as the plants in the physiological experiments (see above). The composition of the soils, pore volume, clay content, and size and distribution of 'anaerobic balloons' together with microbial activity are other important parameters (Stange *et al*., 2000; Parton *et al*., 2001; Butterbach-Bahl *et al*., 2001). Usually, though not generally, N_2O is released at higher rates than NO, both of them in the order of a few mg N m^{-2} h^{-1} (Matson *et al*., 1998; Maggiotto *et al*., 2000; Venterea and Rolston, 2000). There are less data available for particular soil samples in laboratory experiments which could be more directly representative of root environments (Ambus, 1998). In such soil samples NO release rates of less than 1 μg N cm^{-3} h^{-1} could usually be determined. The contributions of nitrification and denitrification by soil bacteria to such rates vary a great deal. The variability even of the overall data is high and does not allow more quantitative information on NO production and its distribution in the closer rhizosphere and whether there are NO concentration gradients due to microbial activity as well as to diffusion from and to the plant roots. Disturbance of the root systems by establishing the NO-measuring equipment in the soil has been shown to cause remarkable increases in NO release for several weeks in tropical forest soils, and hence to distort the data recorded (Keller *et al*., 2000); the increase was explained by NO release from dying roots. In addition, the limitation of the exchange between soil and atmosphere can cause serious errors in the quantitative estimation of real NO concentrations in the soil. For instance, low gas exchange rates, combined with the relative stability of NO, were demonstrated by Skiba *et al*. (1997) in comparative experiments with soil cylinders flushed either only over the surface or with a vertical air current passing through the soil: surface ventilation resulted in only about 13% of the NO obtained with the vertical flow. Flushing with N_2 instead of air caused a more than 4-fold NO release rate compared with air (about 300 ng NO-N g^{-1} wet soil h^{-1} (Skiba *et al*., 1997). Relatively high but quite variable concentrations must exist in the soil and particularly in the pores, where NO may accumulate over several hours. As a consequence, the present article can only try to give qualitative ideas for NO in the relationship between roots and their close environment. A much more detailed analysis of this relationship will be necessary in the future.

Apart from the soils, release of both gases was also found for the roots and shoots of several plant species. Thus, Rusch and Rennenberg (1998) reported experiments with young alder trees (*Alnus glutinosa*) which apparently 'mediated' emission of methane and N_2O from wet soil to the atmosphere within the bark of the trees, attaining up to 350 μmol N_2O m^{-2} of bark surface h^{-1} within the aerenchyma of the root cortex. Similar performances of the aerenchyma had been reported already previously for rice (*Oryza sativa*) (Mosier *et al*., 1990), for *Juncus effusus* and *Pontederia cordata* (Reddy *et al*., 1989). Apparently all plants release and take up NO with their roots and aerial parts as reported, for example, for maize (*Zea mays*), for which a compensation point of about 1 ppbv NO was found (Hereid and Monson, 2001), a very low value, since Gasche and Papen (1999) report up to 72 ppbv for compensation points in forest soils. The differences between measurements with soil chambers and with 'micro-meteorological' methods (Laville *et al*., 1999), in this case of N_2O, could partly be explained by the release of the gas from maize plants in addition to that from the soil. These and other data show the dynamics and the participation of the whole plants, as also found for NO effects on stomatal closure in leaves (Neill *et al*., 2002; García-Mata and Lamattina, 2002), where NO has special physiological functions not applicable to roots.

Perspectives concerning the role of NO in roots

As mentioned before, there is apparently a complicated network of NO effects in plants, in various cases the shoot being regulated differently from the roots (Gouvêa *et al*., 1997; Beligni and Lamattina, 2001a, b). Thus, shoot elongation has been reported to be inhibited by NO, whereas the formation and elongation of the main roots as well as of the lateral roots are enhanced. The latter effect has been extensively described by Forde and Lorenzo (2001) as a result of nitrate supply. Root growth regulation can also vary due to the local nutrient supply (Robinson, 1994, 2001). As discussed before, Forde and Lorenzo (2001) and Forde (2002) distinguished two different regulation systems for root growth by nitrate, a direct nutrient effect mediated by a nitrate sensor at the root cell surface and an indirect regulation system, effective after nitrate transport into the whole plant.

It is proposed here that the local nitrate response system could use NO as a signal substance instead of nitrate. The reaction would start with apoplastic nitrate reduction by PM-NR and the formation of NO at the same site of the plasma membrane by NI-NOR, then continuing with the same regulatory signal chain as that assumed by Forde and Lorenzo (2001). In support of this assumption, these facts are put forward: 1. Nitrate uptake can be regulated by the external nitrate concentration separately from internal nitrate reduction. 2. There are the plasma membrane-

bound enzymes PM-NR and NI-NOR that are regulated independently of cNR. 3. Over-optimum concentrations of nitrate retard plant growth and nitrate (net) uptake at the same time, not only the later steps of nitrate assimilation, in tobacco and several other species (Stöhr, 1999; Stöhr *et al*., 2001; C Stöhr, unpublished results; for algae see Ullrich, 1983). In tobacco this is accompanied by strongly increased activities of PM-NR and NI-NOR. At low external nitrate the low PM-NR activity is sufficient for signalling, at excess nitrate a large proportion is reduced in the apoplast and could be released as volatile NO to the rhizosphere, i.e. to the soil, or some NO could even be translocated to the above-ground parts of the plant. Interestingly in this respect, neither succinate-dependent PM-NR with its high activities nor NI-NOR have been found in plant shoots as yet.

Little appears to be known about the regulation of root hair development apart from the well-studied effects of auxin (Reed *et al*., 1998; Casimiro *et al*., 2001) and jasmonate (Feussner and Wasternack, 2002; Wasternack and Hause, 2002). Since it seems to be correlated with the development of lateral roots (except in the case of mycorrhiza), it may be implicated in the same response system. Another aspect of this hypothesis could be the general observation that in hydroponics the root system greatly differs from that in soil. Root hair formation is usually almost completely suppressed and root branching less pronounced in water culture. From what is known about NO production by soil bacteria and by the plant roots themselves it may be speculated that concentration and distribution of NO could play an important role in these phenomena. In the soil the narrow pores and the restricted pore volume will prevent NO from being diluted and removed at high rates, apart from generation by bacterial sources, whereas in a nutrient solution, especially when it is constantly stirred and aerated, the NO which could accumulate will be withdrawn from the apoplast, mainly from the peripheral layers of the roots. Perhaps not enough is known about the half-life of NO in soils, but at least in the experiments described by Skiba *et al*. (1997) and by others, sufficient NO was released from thick soil layers to the air space to account for many signalling effects.

For a verification of the hypotheses presented here several facts can be claimed, but a more detailed knowledge about the conditions for accumulation and stability of NO in the rhizosphere is still missing. Different soil qualities, sand or clay with different pore volumes, different composition of organic components or pure silicates, aerobic or anaerobic conditions, are known to play a role in the regulation of root growth and these effects could partly be explained by the concentration and distribution of NO. Local or systemic resistance of roots and whole plants against pathogen attack may, among other factors, be enhanced by NO, comparable to the increased resistance against pathogens in the presence of mycorrhiza or by nodulation with symbiotic bacteria. With respect to these observations it may be assumed that, under conditions of low NO production by the plants, their ability to develop hypersensitive reactions or acquired resistance could be weaker. Actually, in cases of fertilization with ammonium or urea alone, without nitrate, which means when the plants themselves produce very little NO, they seem to be more easily and more heavily attacked by biotrophic and by many root rot pathogens (Huber and Watson, 1974). Of course, this may, in part, be due to a generally increased nitrogen supply with ammonium and to the related changes in intermediate metabolism and also to the concentration of NO available in the root apoplast. In soils with sufficient microbial activities, nitrifying soil bacteria may compensate for a deficiency in NO synthesis in the plant roots. Altogether, imbalance in soil ecology and low NO could contribute to the complex effects of 'over-fertilization' or otherwise of deficiency of nitrogen in crop cultures.

As a consequence, it is certainly worthwhile and important to focus further research on the interaction of the soil and its micro-organisms with the plant roots and their development, rhizosphere and apoplastic space, when studying the effects of nutrient supply with respect to concentrations and formation rates of NO and other active agents such as plant hormones in combination with the involved signal chain in the plant roots.

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