Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*

Mercedes Cueto[a], Octavio Hernández-Perera[b], Raquel Martín[a], Maria Luisa Bentura[c], José Rodrigo[c], Santiago Lamas[b], Maria Pilar Golvano[a,*]

[a]*Departamento de Fisiología y Bioquímica Vegetal, Centro de Ciencias Medioambientales, CSIC, Serrano 115 dpdo, 28006 Madrid, Spain*
[b]*Departamento de Estructura y Función de Proteínas, Centro de Investigaciones Biológicas, CSIC, 28006 Madrid, Spain*
[c]*Departamento de Neuroanatomía Comparada, Instituto de Neurobiología Santiago Ramón y Cajal, CSIC, 28002 Madrid, Spain*

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Abstract NO is a widespread messenger molecule in physiology. We were interested in investigating whether an NO-generating system could be present in plants. NO and L-[^{14}C]citrulline were synthesized by roots and nodules of *Lupinus albus* in an L-arginine-dependent manner. L-[^{14}C]Citrulline production was inhibited by N^G-monomethyl-L-arginine, a nitric oxide synthase antagonist, in a competitive way. NADPH-diaphorase activity was localized in the vascular bundles in root and nodules, and also in the nodule infected zone. This staining was significantly reduced in the presence of N^G-monomethyl-L-arginine. These results indicate the presence of a putative nitric oxide synthase in plants.

Key words: Nitric oxide synthase; *Lupinus albus*; *Rhizobium*; Root; Root nodule

1. Introduction

Nitric oxide is now recognized to play an important role in animal physiology acting either as regulator, involved in signal transduction mechanisms or as a cytostatic or cytotoxic effector [1]. These effects are related to the type and origin of the nitric oxide synthase (NOS; EC 1.14.23.39). In mammals two isoforms are constitutively expressed in cells, the neuronal (nNOS or NOS 1) and endothelial (ecNOS or NOS 3). By contrast, the inducible NOS (iNOS or NOS 2) is not normally expressed in cells, but its synthesis can be induced by cytokines or microbial wall products such as lipopolysaccharides. Nitric oxide synthase catalyses NO and L-citrulline formation from one of the guanidine nitrogens of L-arginine requiring NADPH as electron donor and molecular oxygen as a cosubstrate [2,3]. The cofactors FMN, FADH and tetrahydrobiopterin are also required. The constitutive NOS isoforms require Ca^{2+} and calmodulin, while the inducible NOS isoforms are calcium-independent.

Nitric oxide synthase from mammals has been extensively studied [3,4] and characterized and cloned from different tissues [5,6]. In invertebrates, nitric oxide synthases have been described in the hemocytes of an arthropod, *Limulus polyphemus* [7] and in the salivary glands of the insect *Rodnius prolixus* [8]. More recently, a *Drosophila* NOS gene has been cloned [9]. These reports illustrate the conservation of structure and function of nitric oxide synthases between vertebrates and invertebrates, suggesting that NO synthesis is an old biological pathway in eukaryotic evolution. However, in plant tissues there is lack of information concerning NO production.

We have found it interesting to investigate the presence of a nitric oxide generation system in roots and nodules of lupin for several reasons. In a previous work [10], we found an increase in the amino acid arginine in nodules of lupin plants grown in the presence of nitrate, raising the question of possible NO production by the same enzymatic pathway described in mammalians cells. On the other hand, nodule formation in the *Rhizobium*-legume symbiosis involves an exchange of specific signal molecules between the two partners. Furthermore, nitrogen fixation is a tightly regulated process in the microaerobic environmental conditions of the nodule. In leguminous plants, infection with *Rhizobium* leads to the formation of new plant organs, known as root nodules, in which the bacteroids fix atmospheric nitrogen and furnish it as ammonium to the host plant cells. Nitrogen reduction is catalyzed by the nitrogenase, an enzyme complex of two proteins, an MoFe protein and an Fe protein, with marked oxygen sensitivity. However, oxygen is required for respiration of bacteroids and energy production for nitrogen fixation. The hemoprotein leghemoglobin transports the oxygen required for these processes under extremely low free-oxygen concentration.

In this study, the activity on nitric oxide synthase in cytosolic preparations of uninoculated roots and nodules was assayed in three ways: (1) measuring the synthesis of NO, by monitoring methemoglobin formation; (2) quantifying the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline; (3) using a histochemical method for the detection of NADPH-diaphorase activity, commonly employed as a marker for nitric oxide synthase. Our results provide functional evidence for the existence of a NOS like enzyme in the roots and nodules of *Lupinus albus*.

2. Materials and methods

2.1. Chemicals

L-[U-^{14}C]Arginine monomethylchloride was from Amersham (Amersham, UK); hemoglobin and anion-exchange resin, Dowex 1-X8, were purchased from Fluka (Dow Chemical Co., USA). Sephadex G-25 was obtained from Pharmacia Biotech AB (Sweden). Cation-exchange resin, AG 50W-X8, was from Bio-Rad Laboratories (Richmond, USA). L-Arginine, L-NMMA and L-NAME were from Sigma (Sigma Chemical Co., St Louis, MO, USA).

2.2. Plant culture

Lupin plants (*L. albus* L. cv Multolupa) uninoculated or inoculated at sowing with *Bradyrhizobium* sp. (*Lupinus*) ISLU 16, were grown in

*Corresponding author. Fax: (34) (1) 564 08 00.

Abbreviations: L-NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; LPS, lipopolysaccharide

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autoclaved Leonard jars [11]. Plants were supplemented with a nutrient solution containing 2.45 mM nitrate and maintained in a greenhouse until harvest 35–45 days after planting.

2.3. Preparation of enzymatic extracts

Fresh samples of roots or nodules were homogenized using a mortar and pestle in 10 mM HEPES pH 7.4 buffer containing 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, the protease inhibitors E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane) (10 μM), phenyl methylsulphonyl fluoride (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}), pepstatin (5 μg ml^{-1}) and PVP (1 g tissue, 0.3 g PVP (polyvinylpolypyrrolidone) and 5 ml of buffer). The homogenate was successively centrifuged at $10000\times g$ for 10 min and at $15000\times g$ for 20 min at 4°C. The supernatant was passed through a 2 ml column with AG 50 WX-8 resin Na^+ form to remove endogenous L-arginine and the extract was divided into aliquots and frozen until use. Protein concentration was determined by the Bradford method using bovine serum albumin as protein standards.

2.4. Detection of methemoglobin formation

Nitric oxide formation from L-arginine was measured by monitoring methemoglobin (MetHb) formation. This method is based on the rapid and stoichiometric reaction of NO with oxyhemoglobin (HbO_2) to yield methemoglobin [12]. NO formation was followed spectrophotometrically for 15 min by measuring the increase in absorbance at 401 nm, using for calculation an extinction coefficient of 60000 M^{-1} cm^{-1} [A_{401}(metHb)−A_{401}(HbO_2)] [13]. Oxyhemoglobin was prepared freshly every day. A solution of 2 mM hemoglobin in 50 mM Tris-Cl buffer pH 7.0 was reduced with sodium dithionite (2 mg ml^{-1}) under N_2 at 4°C. This solution (0.5 ml) was desalted by passing it through a Sephadex G-25 column (2 ml), eluted with 50 mM Tris-0.1 M NaCl buffer [14] and quickly frozen in small aliquots. For the assay [15], the reaction mixture (3 ml) contained, except where indicated, 10 mM potassium phosphate buffer pH 7.2, 0.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 μM oxyhemoglobin and 1 ml of extract. After 5 min incubation the reaction started by addition of L-arginine (1 mM) and NADPH (0.3 mM). The blank cuvette contained all reagents with the exception of L-arginine and NADPH.

Measurement of methemoglobin formation was only used to assay NO generation in roots from 20–30-day-old plants. In roots from older plants, NO formation was difficult to detect, possibly due to the appearance of some compounds which interfere with the hemoglobin assay. For nitric oxide synthase activity in nodules, exclusively the radiolabelled assay with L-[^{14}C]arginine was used, since nodule leghemoglobin could also bind the NO released in the reaction.

2.5. Conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline

NO synthase activity was measured by monitoring the conversion of L-[U-^{14}C]arginine into L-[^{14}C]citrulline [16]. The reaction was carried out in incubates containing, except where indicated, the following: 50 mM HEPES buffer pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol, 1.250 mM $CaCl_2$, 1 mM NADPH, 2.6 μM L-[U-^{14}C]arginine (spec. act. 317 mCi $mmol^{-1}$), and 42 μl of the nodule extract or 82 μl of the root extract in a total volume of 200 μl. L-[U-^{14}C]Arginine was purified by passing 0.250 ml (12.5 μCi) through a 1 ml Dowex 1-X8 column (100–200 mesh, HO^- form). Concentrations higher than 3 μM were prepared using unlabelled arginine. After 30 min incubation at 37°C, the reaction was stopped by addition of 1.8 ml of 20 mM HEPES buffer containing 2.5 mM EDTA (pH 5.5). The sample was applied to a 2 ml column with AG 50W-X8 resin (Li^+ form) to bind L-[U-^{14}C]arginine and eluted with 2 ml water. The column effluent was recovered in 12 ml scintillation fluid and radioactivity quantified by liquid scintillation counting. When extracts were boiled for 10 min no significant conversion into L-[^{14}C]citrulline was detected. These values were considered background and their amount of radioactivity subtracted from samples containing intact extracts.

Table 1
L-Arginine-dependent formation of NO by extracts of 22-day-old uninoculated roots

L-Arginine (mM)	NO formation (pmol mg^{-1} protein min^{-1})
0.05	68 ± 21 [3]
0.1	124 ± 24 [6]
1.0	203 ± 10 [3]
5.0	204 ± 42 [3]

NO synthesis was measured based on methemoglobin formation, by the increase in absorbance at 401 nm for 15 min. The values are means ± S.E.M. of *n* determinations (in parentheses) in different extracts.

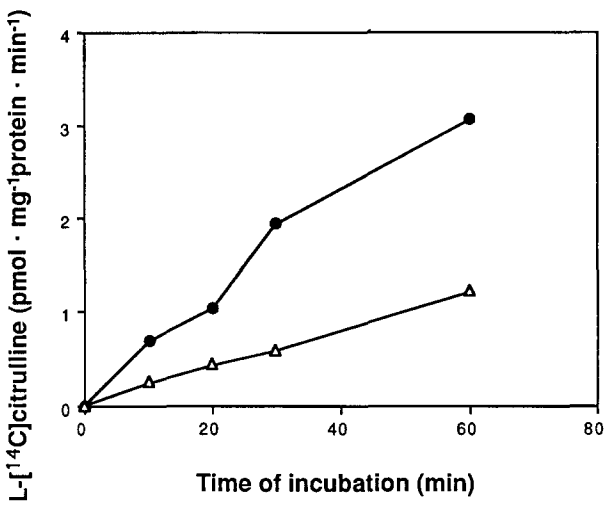

Fig. 1. Time-dependent formation of L-[^{14}C]citrulline from L-[^{14}C]arginine by cytosol of 35-day-old uninoculated roots (△) and 37 day-old root nodules (●) of lupine. The assay mixtures contained in 200 μl: 82 μl of root (17 μg of protein) or 42 μl of nodule (90 μg of protein) extract and 2.6 μM L-[^{14}C]arginine. After incubation as indicated in the abscissa the reaction was stopped. Results from a representative experiment are shown; the experiments were performed twice with similar results.

2.6. NADPH-diaphorase staining

Histochemical localization of NADPH-diaphorase is based on colored formazan salt formed by reduction of nitroblue tetrazolium. Staining was carried out as described [17]. Fresh samples of nodules and uninoculated main roots were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 for 3 h and transferred to 30% sucrose in 0.1 M phosphate-buffered saline overnight. The following day, tissue samples were embedded in optimum cutting temperature compound (Miles, Elkhart, IN), frozen and stored at −20°C. Cryosections (20–25 μm), were mounted on gelatin-coated slides and air dried for 30–60 min. Sections were then washed with 0.1 M Tris-HCl, pH 7.4 and incubated in 0.1 mM Tris-HCl, 1 mM NADPH, 2 mM nitroblue tetrazolium (in 70% dimethyl formamide) for 30–45 min at 37°C, dehydrated in alcohol and xylene mounted and examined by light microscopy. Control slides incubated without the addition of NADPH to the stained solution, revealed no reaction product.

3. Results

Incubation of root and nodule cytosol extracts with radiolabelled arginine resulted in the conversion of L-[U-^{14}C]arginine into L-[^{14}C]citrulline. Fig. 1 shows the time course of L-[^{14}C]citrulline production over a 1 h incubation period. L-[^{14}C]Citrulline production in nodules was 3 pmol/min per mg protein after 1 h and in roots about 1 pmol/min per mg protein. Formation of L-[^{14}C]citrulline also increased proportionally to the amount of extract in the reaction mixture (data not shown). Addition of increasing concentrations of L-arginine (0.3 to 1000 μM) to nodule reaction mixtures, resulted in a concentration-dependent increase in the rate of L-[^{14}C]citrulline formation to a maximum of 150 pmol/min per mg protein at 1 mM arginine (Fig. 2), consistent with the expected kinetics of an enzymatic system.

When the synthesis of NO was measured spectrophoto-

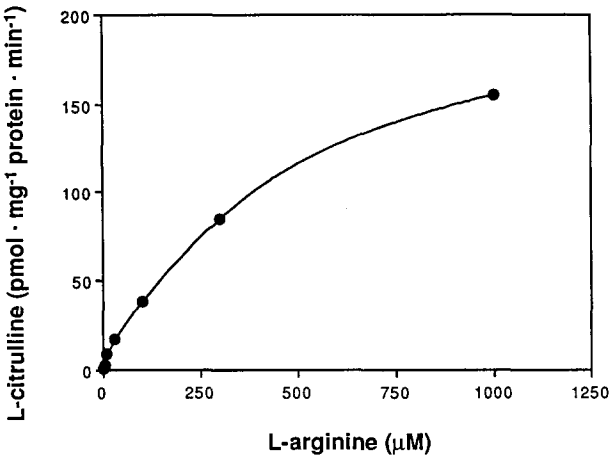

Fig. 2. L-Arginine-dependent formation of L-citrulline by the cytosol of lupine root nodules. Assay mixtures containing nodule extract (93 μg of protein) were incubated for 30 min. Substrate concentrations varied from 0.3 to 1000 μM. Concentrations higher than 3 μM were attained with unlabeled L-arginine. Results from a representative experiment are shown; the experiments were performed twice with similar results.

metrically in incubates of root preparations, addition of increasing concentrations of L-arginine (0.05 to 5 mM) also resulted in a concentration-dependent increase in the rate of NO synthesis (Table 1). A maximum of 203 pmol of NO/min per mg protein of 1 mM L-arginine was reached, which did not increase with 5 mM L-arginine.

A Ca^{2+} dependence has been observed in the NOS-like enzymatic activity of root preparations. As quantified with the hemoglobin assay, the NO produced in an experiment performed in the absence of $CaCl_2$ was only 19% compared to control. The formation of L-[^{14}C]citrulline by roots decreased by 50% in the absence of $CaCl_2$. With nodule preparations L-[^{14}C]citrulline formation was not found to be significantly Ca^{2+} dependent. These experiments were carried out in presence of 1 mM EGTA as chelator.

In an effort to define the potential similarities between this enzymatic system and those described in higher order organisms the inhibitory effect of L-NMMA, an L-arginine analog, was studied. It is well known that L-NMMA is an inhibitor of constitutive and inducible nitric oxide synthase in animal cells. When the reaction mixture was incubated with L-NMMA, L-[^{14}C]citrulline formation was inhibited in a concentration-dependent manner (Fig. 3). At concentrations of 0.1 and 1 mM L-NMMA, L-[^{14}C]citrulline formation was markedly inhibited, compared to control, by 39 and 56% in nodule extracts and

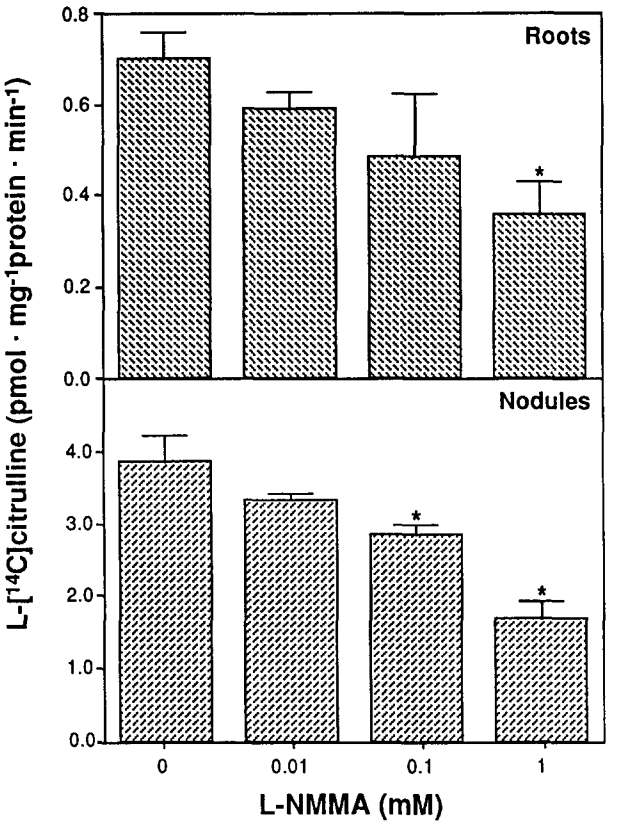

Fig. 3. Inhibition by L-NMMA of L-[^{14}C]citrulline formation in extracts of lupin roots (52 μg of protein) and nodules (69 μg of protein). Reaction mixtures were incubated in the presence of L-NMMA (0.01, 0.1, 1 mM). Values are the means ± S.E.M. (bar) of three independent experiments with two replicates. *Significant ($P < 0.05$, Student's t-test, $n = 3$).

49% in roots. L-N^G-Nitroarginine methyl ester (L-NAME) was also used with similar results. We performed experiments to demonstrate that the inhibitory effect of L-NMMA could be reversed by increasing concentrations of substrate. Table 2 shows the effect of increasing concentrations of L-arginine (3–1000 μM L-arginine) on the inhibition by L-NMMA of L-citrulline formation in nodule extracts. This inhibitory effect was completely reversed in the presence of 1 mM L-arginine.

In nodules, three sites of NADPH-diaphorase staining were observed; a very intense staining in the tissues of the vascular bundles, a second one in the cells from the periphery of the infected zone and a third lighter staining inside the infected zone (Fig. 4A). In roots, NADPH-diaphorase staining was

Table 2
Effect of increasing concentrations of L-arginine on the inhibition by L-NMMA of L-citrulline formation in extracts of lupine nodules (82 μg of protein)

L-Arginine (μM)	L-NMMA (1 mM)	L-Citrulline (pmol mg^{-1} protein min^{-1})	Inhibition (%)
3	–	4.91 ± 0.31	
3	+	2.78 ± 0.33	43
10	–	11.70 ± 0.81	
10	+	9.22 ± 1.17	21
100	–	73.70 ± 6.88	
100	+	67.00 ± 8.70	9
1000	–	248.00 ± 28.00	
1000	+	296.00 ± 69.00	–

Reaction mixtures were incubated in presence of 1 mM L-NMMA and increasing concentrations of L-arginine (3–1000 μM). Values are the means ± S.E.M. of three independent experiments.

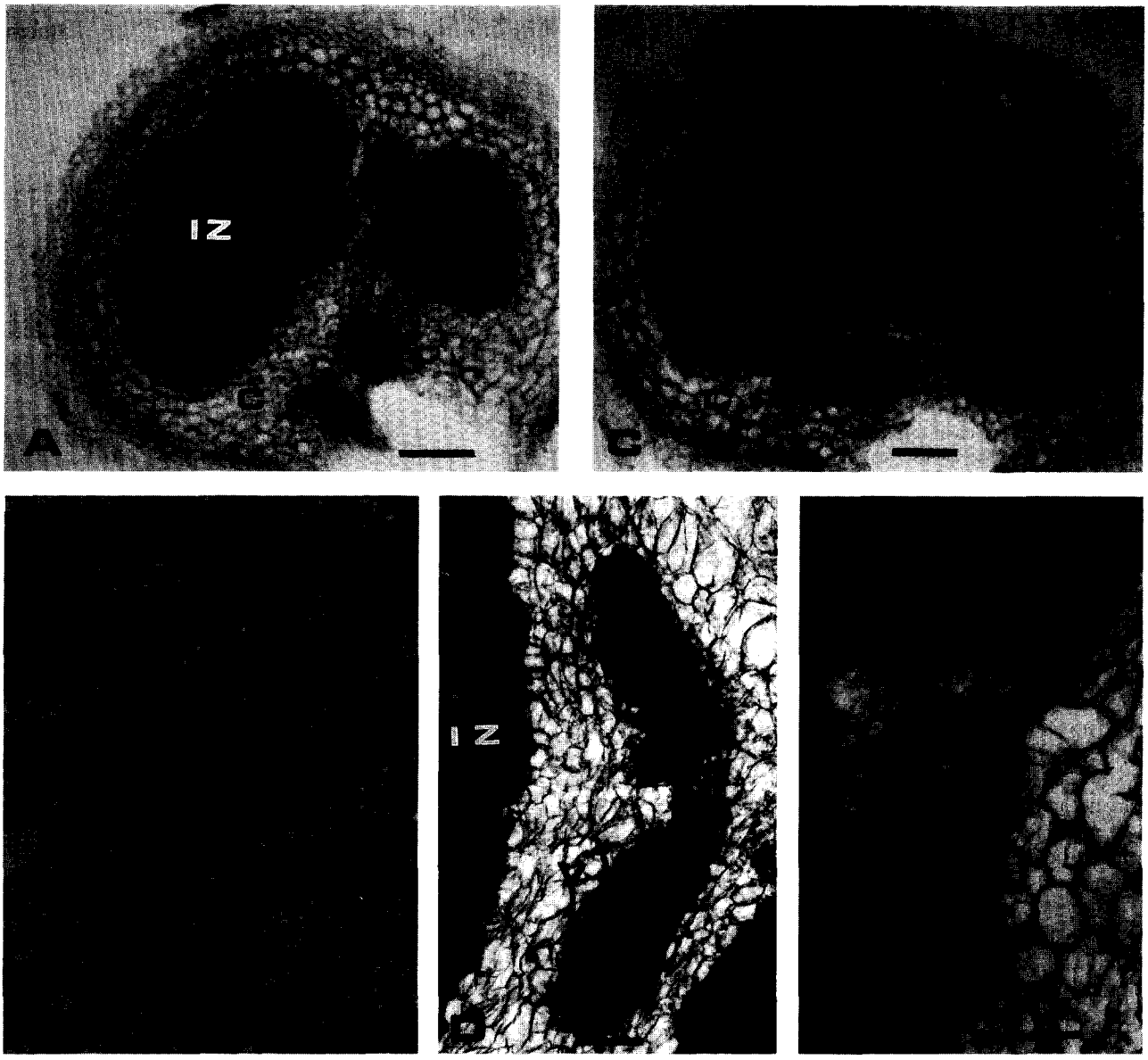

Fig. 4. Light micrographs of NADPH-diaphorase histochemical localization in nodules and uninoculated roots of lupine. (A) Overview of a nodule cross-section showing NADPH-diaphorase staining in vascular bundles (V) and in the infected zone (IZ). (B) Root cross-section showing staining in the phloem and parenchyma cells (Pa) of inner stele. (C) Overview of a nodule cross-section after L-NMMA treatment showing a significant decrease in NADPH-diaphorase staining in vascular bundles (V) and inside the infected zone. Sections were first incubated for 10 min in 10 mM L-NMMA in 0.1 Tris-HCl, pH 7.4 and then stained for 45 min in the continuous presence of 10 mM L-NMMA. (D,E) Comparative staining of vascular bundles in the absence (D) and presence (E) of L-NMMA. C, cortex; X, xylem. Bar markers represent 250 μm in A,C and 50 μm in B,D,E.

observed in phloem cells and parenchyma cells (Fig. 4B). We studied the effect of L-NMMA on nodule NADPH-diaphorase staining. The intensity of staining decreased mainly in cells inside the infected zone, remaining in the cells of the periphery (Fig. 4C). Staining also decreased in the vascular bundles, which show small unstained zones (Fig. 4D,E).

4. Discussion

To our knowledge, this is the first documented report providing evidence for a NOS-like enzyme in plants, suggesting the conservation of NOS function across the animal and plant kingdoms. Nitric oxide and L-[^{14}C]citrulline were synthesized in lupin roots and nodules in an L-arginine-dependent manner. Furthermore, L-NMMA was found to be an effective inhibitor of L-[^{14}C]citrulline formation by roots and nodules. This inhibition was overcome when increasing concentrations of the substrate L-arginine were added (Table 2), consistent with the expected kinetics of substrate-antagonist competition for the enzyme. Studies carried out to examine the potential inhibitory effect of various substrate analogs on other arginine-utilizing enzymes, like arginine decarboxylase, arginase [18–20] and arginine dihydrolase [21], have demonstrated that L-NMMA only inhibits nitric oxide synthase activity.

Preliminary observations have shown that a Ca^{2+} dependence in the synthesis of NO and L-[^{14}C]citrulline is present in root preparations. This effect was not observed in nodules. These data suggest the potential presence of a constitutive

form of NOS in roots, while in nodules, an inducible NOS could be the predominant isoform, a concept which needs further confirmation. The presence of an inducible Ca^{2+}-independent NO synthase has been described in several mammalian cells, after activation with cytokines or lipopolysaccharide [15,22]. It is attractive to speculate that the nodule Ca^{2+}-independent NO synthase may be induced by the *Rhizobium* LPS. Cell wall rhizobia LPS are essential in the early interaction processes between plant host and bacteria, initially avoiding host cell defense reactions and also in later stages during nodule development. Mutants of *Rhizobium leguminosarum* with modifications in the structure of LPS induce ineffective pea root nodules, showing little or no capacity for nitrogen fixation [23].

The first question arising from the presence of an NO synthase in nodules concerns its biological significance. Nitric oxide is an inhibitor of nitrogenase from nitrogen-fixing bacteria, through interaction with Fe-S clusters [24]. NO binds tightly to leghemoglobin, forming nitrosylleghemoglobin complexes [25]. It has been reported that accumulation of nitrosylleghemoglobin in nodules of leguminous plants supplied with nitrate inhibits nitrogenase activity [26]. Recently, a new class of heme proteins has been described. These are heme-based sensors, distinct from oxygen carriers or electron transporters. The first member is the FixL protein kinase, an oxygen-regulated protein from symbiotic nitrogen-fixing rhizobia [27]. The active form, the deoxy-FixL, induces the regulatory cascade of nitrogen fixation gene expression in *R. meliloti* [28]. In response to oxygen, FixL proteins shut down nitrogen fixation. FixL may sense nitric oxide and carbon monoxide in addition to oxygen [27]. The synthesis of NO could be related to the presence of heme protein sensors of nitric oxide capable of mediating certain physiological activities of NO.

In view of the co-localization of NO synthase and NADPH-diaphorase activity in mammals [29] we examined the distribution of NADPH-diaphorase in root and nodule tissues. NADPH-diaphorase activity was located in the vascular bundles of both roots and nodules, and in the infected cells of nodule. To demonstrate that NOS-associated NADPH-diaphorase staining could be inhibited by an L-arginine analog, nodule tissues were stained in the presence or absence of L-NMMA. A significant disappearance of diaphorase activity was observed inside the cells of the infected zone and in the vascular bundles. Although the degree of inhibition is not complete, one should bear in mind that other enzymes might have NADPH-diaphorase activity and hence the NOS-associated activity is only a fraction of the total. These data indicate two sites of localization for this putative plant NOS, one in the vascular bundles, common for root and nodule, since nodule vascular bundles are connected to the vascular system of the roots [30] and a second one in the inner cells of the nodule infected zone, which may be induced during symbiosis. Many kinds of proteins are reported to be localized in the vascular bundles, most of them in the phloem vessels and in the adjacent parenchyma cells. Some of these enzymes are dehydrogenases [31] or enzymes implicated in amino acid metabolism [32], the major components together with sucrose which are transported in the phloem sap to the sink tissues. It has been suggested that the amino acids coming from the plant through the phloem into the nodules may regulate their growth and nitrogenase activity [33]. Other phloem specific proteins, such as thioredoxins [34] phosphorylating enzymes [35] and lipoxygenases [36] are involved in the transport of signaling molecules. In plants, as in animal cells, the NO released by NO synthase might be involved in signal transduction mechanisms, since phloem vessels are now recognized as a signal transport system. We believe that the symbiosis legume-*Rhizobium* may provide a useful model system to analyze potential physiological roles for these putative NOS isoforms in plants.

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