Nitric oxide detection using a chemical trap method for applications in bacterial systems

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Abstract: Plant growth-promoting bacteria (PGPB) can be incorporated in biofertilizers formulations, which promote plant growth in different ways, for example, fixing nitrogen, produce phytohormones and nitric oxide (NO). NO is a free radical involved in the growth and defense responses of plants and bacteria. NO detection is vital for further investigation in different agronomically important bacteria. NO production in the presence of KNO_3, was evaluated over 1 to 3 day using 8 bacterial strains, quantified by the usual Griess reaction and monitored by DAN, leading to 2,3-Naphthotriazole (NAT), analyzed by fluorescence spectroscopy, GC-MS and HPLC. The Griess and trapping reaction results showed that *A. brasilense* (HM053 and FP2), *Rhizobium tropici* (Br322) and *Gluconacetobacter diazotrophicus* (Pal 5) produced the highest NO levels 24 h after inoculation, whereas *Nitrospirillum amazonense* (Y2) and *Herbaspirillum seropedicae* (SmR1) showed no NO production. In contrast to the literature, in NFbHP-NH_4Cl-lactate culture medium with KNO_3 the NO trapping led to the recovery of a product with a molecular mass ion of 182 Da, the 1, 2, 3, 4- Naphthotetrazole, (NTT) with one more nitrogen atom instead of the usual NAT product with 169 Da. This strategy allows monitoring and tracking NO production in potential biofertilizing bacteria, providing future work to better understand the mechanisms of bacteria-plant interaction, and also to manipulate the amount of NO that will sustain the PGPB.

Keywords: nitric oxide, Griess reaction, fluorescence, chromatography and N_2-fixing bacteria.

1. Introduction

Rhizobacteria that establish positive interactions with roots are referred to as plant growth-promoting bacteria (PGPB) and are promising for building biofertilizers in sustainable agriculture [1]. PGPBs can directly promote plant growth through biological nitrogen fixation (BNF), scavenging soil nutrients (phosphate solubilization and/or siderophore production), and producing phytohormones including indole-3-acetic acid (IAA), gibberellin and cytokinins that induce morphological and physiological changes in the roots. They can also function indirectly by reducing the negative impact of pathogens [2,3].

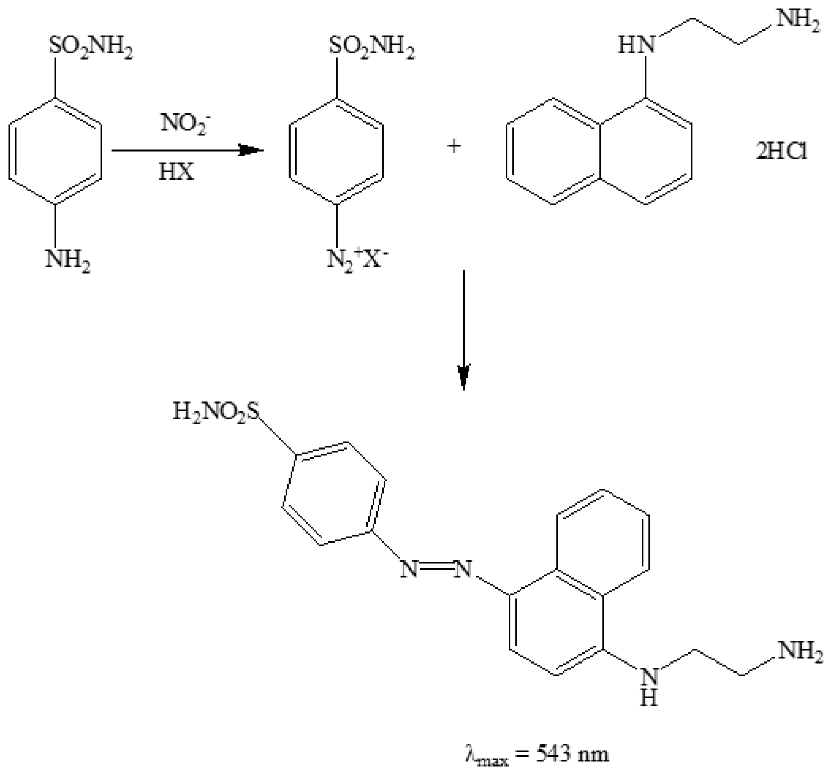
The benefits of biofertilizers arise from the symbiosis of legumes with bacteria, such as the *Bradyrhizobium* genus [4]. Other bacteria, including *Azospirillum, Gluconacetobacter, Azoarcus, Enterobacter, Herbaspirillum, Burkholderia* and *Rhizobium*, have been reported as potential biofertilizers [5-10]. Rhizobacteria, including *Bradyrhizobium* and *Rhizobium*, are responsible for nitrification-denitrification, where nitric oxide (NO) is a key intermediate. During denitrification, nitrate (NO_3^-) is reduced to nitrite (NO_2^-), NO, nitrous oxide (N_2O) and molecular nitrogen (N_2) through the actions of nitrate reductase (reduces NO_3^- and NO_2^-), nitrite reductase (reduces NO_2^- to NO), nitric oxide reductase (reduces NO to N_2O), and nitrous oxide reductase (reduces N_2O to N_2) [11]. Conversely, nitrification involves the nitrogen compound oxidation, primarily ammonia (NH_3) to NO_2^-, with hydroxylamine (NH_2OH) as a key intermediate. Nitrification enzymes include ammonia monooxygenase, (converts NH_3 to NH_2OH) and hydroxylamine oxidoreductase (converts NH_2OH to NO_2^-) along with nitrite and NO reductases [12]. Studies under aerobiosis and anaerobiosis conditions have shown that diazotrophic bacteria, including the *Azospirillum* genus, can use NO_3^-, NO_2^- or nitrous oxide (N_2O) as final electron acceptors [13]

NO is a reactive gaseous molecule with many important biological functions. It is present in all living organisms and can act as a signaling molecule at low concentrations (nM levels). In plants, NO signaling controls growth, development and plant defense responses to pathogens. In bacteria, NO also induces a defense response against pathogens and may protect against oxidative stress [14, 15]. From a plant-bacteria interaction perspective, NO is produced in nodules where it acts as a signaling molecule, regulates gene expression, or acts as an efficient inhibitor of nitrogenase [16]. NO can alleviate oxidative stress caused by antibiosis processes and BNF due to it signaling action [15, 17].

Common methods for NO detection in bacteria include the Griess method, electron paramagnetic resonance (EPR), electrochemical methods and fluorescent probes. These methods have been used for NO detection in plants inoculated with PGPB [18]. Studies with *A. brasilense*, using fluorescence probes as 4,5-diamino fluorescein diacetate (DAF-2D) have been developed for NO detection, quantification and mechanism elucidation [19]. Inoculants containing *A. brasilense* show evidence of NO mediation of the indole-3-acetic acid (IAA) signaling pathway to increase lateral and adventitious root formation in tomato plants [19, 20].

Nitrogen oxide species are produced because NO reacts rapidly with O_2 and H_2O, mainly forming NO_3^- and NO_2^-. An indirect method for NO determination involves the spectrophotometric measurement of its decomposition products, NO_3^- and NO_2^-. This method is known as the Greiss reaction and involves the reduction of NO_3^- to NO_2^-. NO_2^- subsequently reacts with sulfanilamide in an acidic medium to produce a diazonium ion that reacts with *N*-(1-naphthyl) ethylenediamine to form an azo-chromophore compound that exhibits strong absorbance at 543 nm (Scheme 1) [21].

The Greiss method is a simple colorimetric indirect method for detecting NO in biological systems, requiring only a UV-visible spectrophotometer, but is limited to sensitivities of 0.1–1.0 µmol/L [21].



Scheme 1. Reaction steps involved in the Greiss method (Nagano, 1999).

Another method was developed to improve NO detection sensitivity based on the diamino aromatic compound 2,3-diaminonaphthalene (DAN) as an indicator of NO formation [22]. DAN is relatively non-fluorescent and reacts rapidly with N_2O_3 generated by nitrite under acidic conditions or the interaction of NO with oxygen to produce a strongly fluorescent product, 2,3-naphthotriazole (NAT). DAN detection exhibits NO detection limits in the nanomolar range from 10 nM to 10 μM [21-23], and can be equal to or 10-fold more sensitive than the Griess method. Therefore, both methods were used as a strategy to improve the determination of NO production in bacteria.

Reports have shown an improved understanding of NO production in biological systems based on the DAN method. Wada et al. (2002) [24] showed that NO can be detected in Agave Pacifica plant cells using DAN with HPLC analysis and fluorescence detection. Furthermore, the DAN method is efficient for use in fungal and bacterial cells, although research has mainly focused on plant growth-promoting symbionts including mycorrhizae, nutrient solubilizers and diazotrophs [25-29].

Fewer studies have investigated NO detection in diazotrophic biological systems, including bacteria and cyanobacteria [20, 30, 31]. Thus, it is important to improve the methods that are suitable for these systems. Rapid detection of NO synthesis in these bacteria is essential to better understand the effects of NO on plant-bacteria interactions and BNF [32]. Therefore, the hypothesis that N_2-fixing bacteria can synthesize different amounts of NO, in the presence of KNO_3, at different cultivation times was tested herein along that NO can be detected by fluorescent probes. Bacteria that form associations with grasses and soybean were selected, Greiss reaction and DAN chemical trap was applied to quantify and monitoring the synthesized NO levels, respectively. Fluorescent probes have provided much information regarding the location and mechanism of reactive species produced in biological systems due to their detection sensitivity. Herein, an improved DAN chemical trap and Griess methods were applied as facile and inexpensive methods to identify PGPB that produce NO. This is an important advance because few species and strains have been evaluated for NO production to date. The developed method will allow for the identification of strains with the potential for use as biofertilizers for plants of agronomic interest. In addition, the methods can be applied in future studies to monitor and manipulate the NO prodution on plant-bacteria interactions.

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2. Materials and Methods

2.1. Bacterial growth conditions

Eight strains of bacteria were used: *Azospirillum brasilense* (FP2); *A. brasilense* (HM053); *Herbaspirillum seropedicae* (SmR1); *Nitrospirillum amazonense* (Y2); *Rhizobium tropici* (BR322); *Agrobacterium fabrum* (L40); *Gluconacetobacter diazotrophicus* (Pal 5) and *Agrobacterium fabrum* (R5). The bacteria were grown in 96-well plates containing liquid NFbHP-NH_4Cl-lactate [25] supplemented with 10 mM KNO_3 at 30 °C with shaking (140 rpm). After 24, 48 and 72 h of growth, the bacteria were collected and centrifuged, then supernatant was used for NO measurements.

2.2. Determining NO production in bacteria via the Greiss reaction and fluorescence.

Bacterial NO production was quantified by Griess method [33] and also monitored by fluorescence based on the classical reactions involving NO and DAN.

The Griess method was performed according to the procedures described by [34], and samples were analyzed after 24, 48 and 72 h of growth in liquid media. The observed NO values were standardized for the bacterial growth using the total protein concentration by the Bradford method [35]. These values were expressed as μg mL^{-1} of NO/μg mL^{-1} of total protein.

For fluorescence detection, the bacteria were grown under the same conditions described above, except that the NO trap DAN was added. The following incubation conditions were used: 1) NFbHP-NH_4Cl-lactate with 10 mM KNO_3 and 80 μg mL^{-1} DAN with bacteria; 2) NFbHP-NH_4Cl-lactate medium with 10 mM KNO_3 and 80 μg mL^{-1} DAN without bacteria; and 3) NFbHP-NH_4Cl-lactate with 10 mM KNO_3 without DAN and bacteria. Conditions 2 and 3 were used as controls. After incubation, the bacteria were centrifuged, the supernatant was deposited in a 96-well ELISA plate (suitable for fluorescence), and analyses were performed using ExpectraMax Paradigm (Molecular Devices) equipment with excitation and emission at 360 and 465 nm, respectively.

2.3. Monitoring NO production in the NFbHP-NH_4Cl-lactate medium

DAN fluorescence analyses were performed using Diethylamine NONOate diethylammonium salt (DEA NONOate), a NO generator. The following conditions were used: 1) no sample; 2) water and NFbHP-NH_4Cl-lactate medium; 3) DAN 0.005 mg mL^{-1}; 4) DAN 0.005 mg mL^{-1} with DEA NONOate 0.005 mg mL^{-1}; 5) DAN 0.005 mg mL^{-1} with DEA NONOate 0.0125 mg mL^{-1}; and 6) DAN 0.005 mg mL^{-1} with DEA NONOate 0.025 mg mL^{-1}. Conditions 1 and 2 were used as controls. The analyses were performed using a 96-well ELISA plate and an ExpectraMax Paradigm (Molecular Devices) equipment with excitation and emission wavelengths of 365 and 465 nm, respectively.

2.4. Monitoring NO production in bacteria by chromatography

This procedure is based on the detection of NAT complex formed in bacteria using GC-MS and Varian HPLC.

NAT was extracted with toluene, after addition of 1 mL toluene in a 1 mL bacterial sample. The phases were separated and the toluene phase was inserted in a vial to GC-MS injection.

For GC-MS NAT detection, a Perkin Helmer Claris SQ 8T instrument with an Elite-5 ms Perkin Elmer column (30 m x 0.25 mm I.D. x 0.25 μm) was used. The oven was programmed from an initial temperature of 60 °C to 240 °C at a rate of 3 °C min^{-1} and to 280 °C at a rate of 15 °C min^{-1}. He was used as a carrier gas. The injector, interface and source temperatures were 240 °C, 250 °C, and 250 °C, respectively. The injection volume was 1 μL, with a split of 20 mL min^{-1}.

HPLC was used to evaluate the products formed in the DEA NONOate experiment with method development featuring an acetonitrile/water elution system with 30–90% acetonitrile gradient for the first 10 min, following of 90–30% acetonitrile in more 2 min and remaining 30% acetonitrile until 15 min, with flux rate of 1 mL/min. A C18 Luna Phenomenex column (250 x 4.6 mm, 5 μm i. d.) was used with an injection volume of 20 μL sample and standard injection volume.

2.5. Statistical analysis

NO production by bacterial strains was evaluated in a 10 × 3 factorial scheme, with 8 bacterial strains at 3 timepoints after inoculation (24, 48 and 72 h). The fluorescence data obtained from NAT production by the different bacterial strains were also evaluated in a 10 × 3 factorial scheme, including the 8 bacterial strains and 2 controls at 3 timepoints after inoculation (24, 48, and 72 h). Data were submitted to analysis of variance, and means were compared using Tukey's test at a 5% probability.

3. Results

3.1. Greiss and 2,3-diaminonaphthalene (DAN) assay

NO is an important messenger in biological systems and our aim was to determine its production in different bacterial strains using a Greiss assay and a fluorescent probe to quantify and monitored NO generation processes, in the presence of KNO_3.

Generally, colorimetric quantification results obtained by the Griess assay corroborated the semi-quantitative fluorescence results. The bacterial strains synthesized different NO amounts depending on the incubation time, as shown by the Griess method and fluorescence results (Figures 1 and 2). Furthermore, after 24 h of inoculation, *A. brasilense* HM053 and FP2 synthesized the highest amounts of NO (0.1435 and 0.1259 μg mL^{-1}, respectively), followed by *R. tropici* Br 322 (0.0521 μg mL^{-1}) and *G. diazotrophicus* Pal 5 (0.0215 μg mL^{-1}; Figure 1). The same strains showed the highest NO production in the fluorescent assay at 24 h (Figure 2). During the second evaluation period, FP2, HM053, Br 322 and Pal 5 strains showed decreased NO production, whereas FP2 and HM053 showed the highest NO levels (0.0594 and 0.0458 μg mL^{-1}, respectively; Figure 1). At 72 h after inoculation, a significant decrease in NO production was observed for the FP2, Br 322 and Pal 5 strains when compared to HM053, which maintained a higher NO production (0.0240 μg mL^{-1}; Figure 1). These results were similar to the fluorescence data (Figure 2). *N. amazonense* (Y2) and *H. seropedicae* (SmR1) did not produce NO at any timepoint, as indicated by the Griess and fluorescent assays (Figures 1 and 2). *A. fabrum* (R5) showed a low (0.0036 μg mL^{-1} NO) production at 24 h of growth and no NO production at other times, similar to the fluorescence results (Figures 1 and 2).

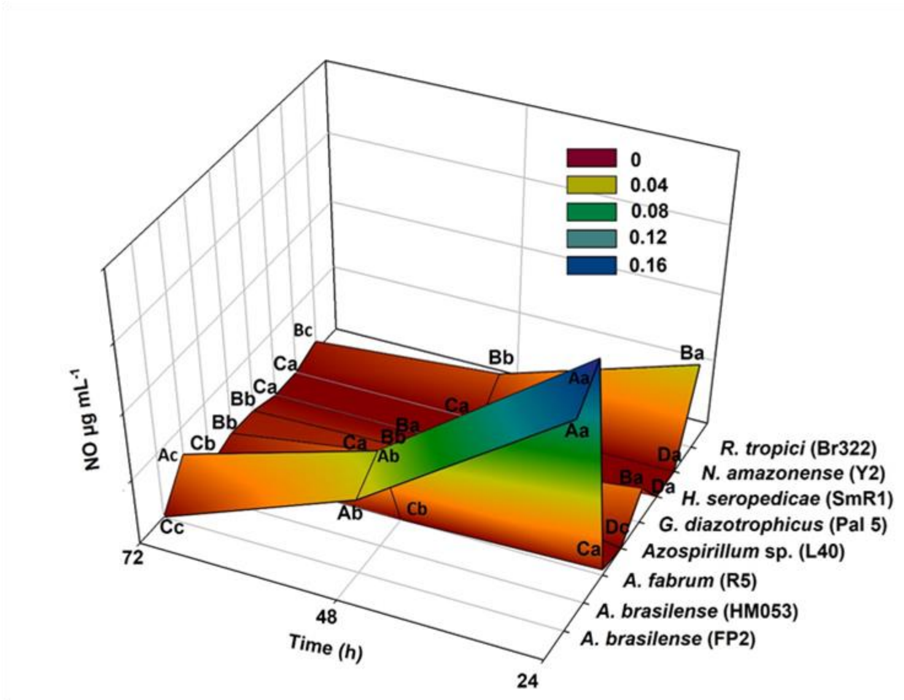

Figure 1. Nitric oxide (NO) quantification for 8 strains of bacteria grown for 24, 48 and 72 h by Griess assay. The capital letters compare the bacteria within the same time and the lower-case letters between different timepoints for the same bacterium. Means followed by the same letter do not significantly differ from each other by the Tukey test at 5% probability.

In summary, NO synthesis decreased with cultivation time for all strains, except for the *Azospirillum* sp. (L40) strain which did not produce NO after 24 h. However, after 48 and 72 h, L40 showed NO concentrations of 0.0239 and 0.0058 µg mL^{-1}, respectively (Figure 1), differing from the fluorescence results, which did not show significant NAT formation at any timepoint (Figure 2). Thus, the Griess reaction is more efficient for capturing NO than DAN, but the DAN method is more sensitive for measuring low NO concentrations (Figure 2), explaining divergence in the results.

The fluorescence results confirmed fluorescent complex formation from the reaction of NO with DAN to yield the NAT complex. After 24 h of incubation in DAN-containing media, the highest average fluorescence rates were observed for *R. tropici* Br 322 (3.9 x 10^9 a.u.) and *G. diazotrophicus* Pal 5 (2.9 x 10^9 a.u.), followed by those of *A. brasilense* FP2 (6.9 x 10^8 a.u.) and HM053 (8.8 x 10^8 a.u.; Figure 2).

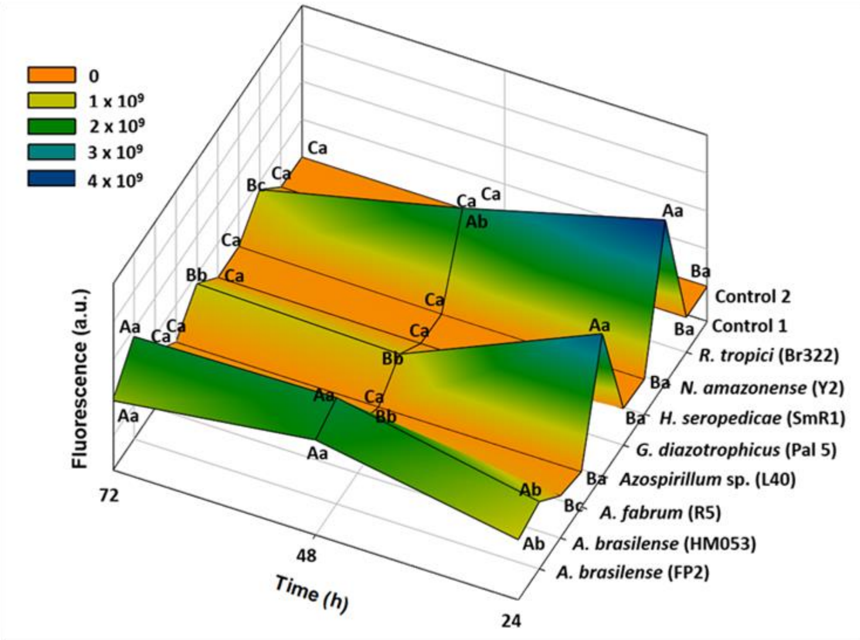

Figure 2. Fluorescence analysis to semi-quantification for 2,3-Naphtotriazole (NAT) complexes for 8 bacteria strains after 24, 48 and 72 h of incubation. **Control 1 -** Medium supplemented with 10 mM KNO_3 and 80 µg mL^{-1} of 2,3-Diaminonaphthalene (DAN). **Control 2 -** Medium with 10 mM KNO_3. The capital letters compare bacteria at the same timepoints and the lower-case letters different timepoints for the same bacteria.

A similar pattern was observed in the second evaluation, when the averages for the fluorescence index were 2.0×10^9 a.u. for *R. tropici* Br 322, 1.6×10^9 a.u. for *A. brasilense* FP2 and 1.9×10^9 a.u. for HM053, with a significant increase in fluorescence for *A. brasilense* (Figure 2). After 72 h of incubation, high fluorescence was observed only for *A. brasilense* strains FP2 and HM053 (9.3×10^8 and 1.8×10^9 a.u., respectively; Figure 2).

Azospirillum sp. (L40), *N. amazonense* (Y2), *H. seropedicae* (SmR1) and *A. fabrum* (R5) did not show significant NAT formation over time, except for *A. fabrum* (R5) which showed low fluorescence 24 h after inoculation (3.7×10^8 and 5.9×10^8 a.u., respectively for 24 and 48 hours; Figure 2).

3.2. GC-MS analyses

After different incubation times in the presence of DAN, the samples were analyzed by GC-MS to confirm NAT complex formation. According to the mechanism of NO trapping by DAN, NAT is the dominant product formed, but another product with an additional nitrogen was detected by GC-MS (Figure 3). The same product was observed for *A. brasilense* FP2 and HM053, *R. tropici* Br 322 and *G. diazotrophicus* Pal5, which showed fluorescence after 24 and 48 h of growth (Figures 3d and e). Therefore, an alternative metabolic pathway stimulated by supplying KNO_3 as a nitrogen source for N_2 fixing bacteria can be proposed. In this pathway, DAN is converted into a compound with a molecular mass ion of 182 Da, the 1, 2, 3, 4-Naphthotetrazole (NTT) instead NAT, given the presence of RNS in the medium(Figures 3a, d and e).

In the total ion chromatogram of DAN, a peak was observed at 9.31 min and the mass spectrum showed the molecular mass ion of DAN, m/z = 158 Da (Figures 3b and c). After extraction of the bacterial supernatant with toluene, the peak at 12.28 min arose from the product formed in the reaction of DAN with

NO (Figures 3d). The ion *m*/*z* = 182 Da corresponds to the molecular mass ion of the product, but to obtain *m*/*z* = 182 Da, two additional nitrogen atoms in the DAN structure would be necessary to form the 1, 2, 3, 4-Naphthotetrazole (NTT). In addition, the mass spectrum shows the loss of two nitrogen atoms with *m*/*z* = 155 Da, a third nitrogen with *m*/*z* = 127 Da, and lastly another nitrogen with *m*/*z* = 140 Da as fragment ions (Figure 3e). The other peak at 5.91 min corresponds to impurities present in the toluene solvent (Figure 3d).

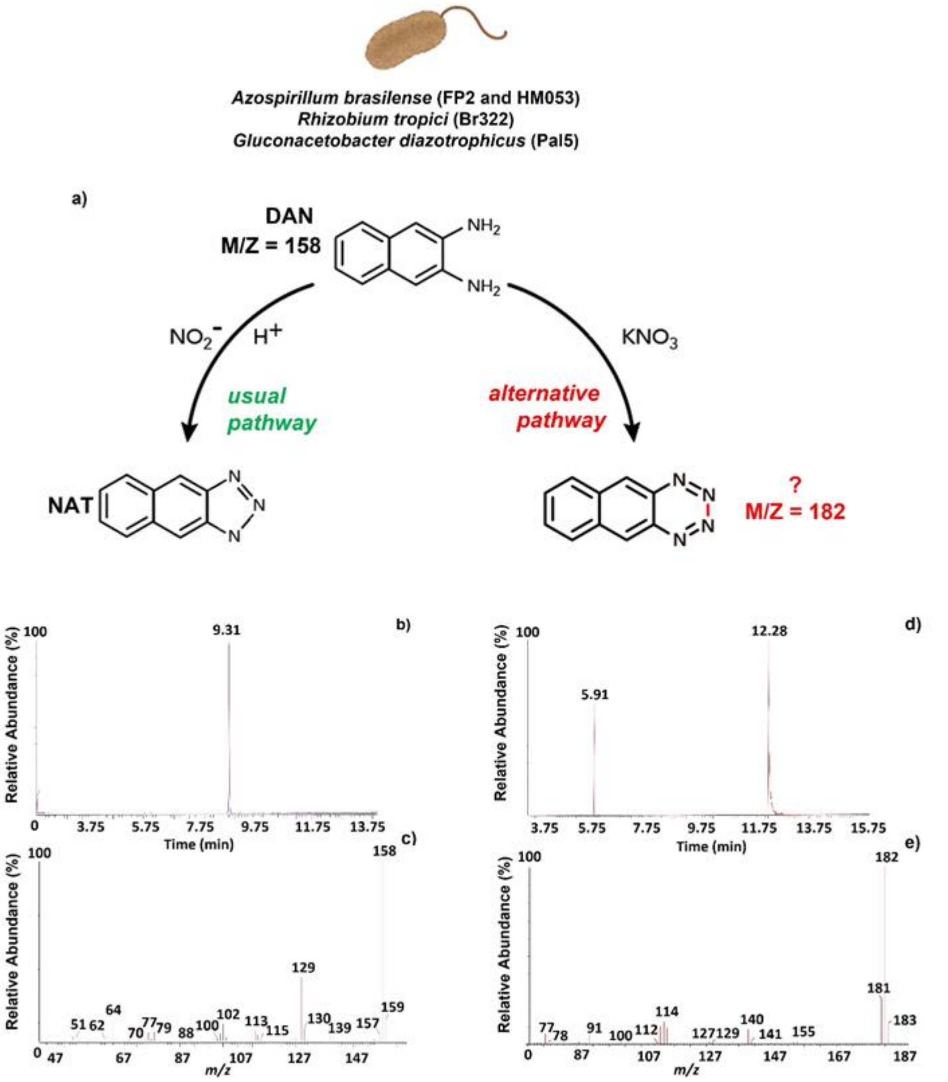

Figure 3. GC-MS analyses for 2,3-Naphtotriazole (NAT) complex detection. **a)** Typical and alternative metabolic pathway stimulated by supplying KNO_3 as a nitrogen source for N_2 fixing bacteria. **b and c)** Total ion chromatogram and mass spectrum obtained for 2,3-Diaminonaphthalene (DAN). **d and e)** Total ion chromatogram and mass spectrum obtained by DAN trapping of nitric oxide (NO) produced by bacteria *A. brasilense* (FP2 and HM053), *R. tropici* (Br 322) and *G. diazotrophicus* (Pal5).

3.3. Validation method

The use of DAN, as a probe in bacteria, was validated using DEA NONOate in water and NFbHP-NH_4Cl-lactate media. DEA NONOate was used to monitor NO production in the culture medium. This compound can also produce NO in 0.1 mol L^{-1} phosphate buffer pH 7.4 ($t_{1/2}$ = 16 min at 22–25 °C, $t_{1/2}$ = 2–4 min at 37 °C), where its decomposition is nearly instantaneous at pH 5.0. Fluorescence analysis was performed with excitation and emission at 365 and 465 nm, respectively, showing product formation in water and culture medium (Figures 4 and 5).

The fluorescence intensity increased with increasing NO generator concentrations in stirred water for 30 min. In the culture medium, the fluorescence intensity remained unchanged, except after 24 h of stirring where increased fluorescence was observed due to complex formation. The observed fluorescence increased as a function of NO generator concentration (Figure 4a and b). This validation test provided confidence for incubation in NFbHP-NH_4Cl-lactate medium as a method for detecting NO in bacterial systems, since no NO release by the medium was observed in the absence of the generator.

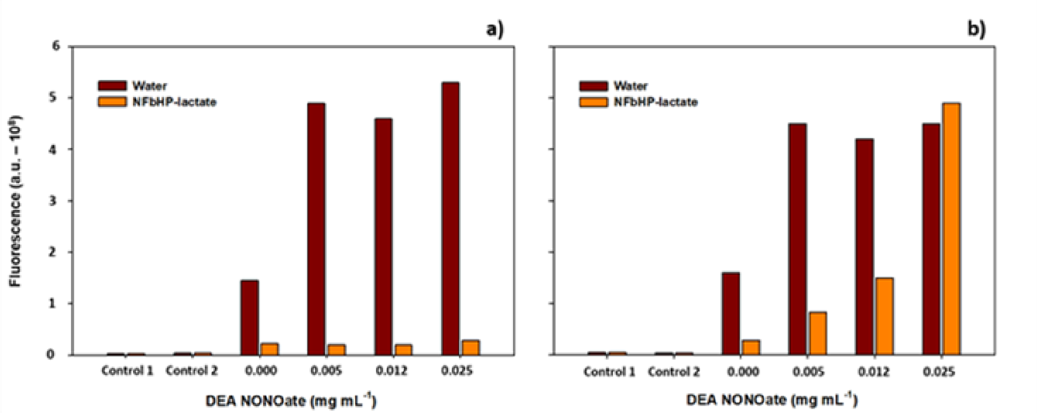

Figure 4. Fluorescence analyses for nitric Oxide (NO) production by Diethylamine NONOate diethylammonium salt (DEA NONOate) generator using 2,3-Diaminonaphthalene (DAN) as a probe in water and NFbHP-NH_4Cl-lactate. **a)** Data after 30 min of incubation. **b)** Data after 24 h of incubation. Wavelength excitation and emission at 365 and 465 nm. **Control 1** - No sample. **Control 2 -** Water and NFbHP-NH_4Cl-lactate;

Thus, NO release occurs when bacteria are stressed in the presence of KNO_3, as shown by the DAN analysis in the medium without NO generator such as DEA NONOate or KNO_3, where there is no product formation. The product was observed when the DEA NONOate concentration was increased.

In the sample with only DAN in the concentration 0.005 mg mL^{-1}, it is observed an intensity of fluorescence, this can be due to the compound show fluorescence in the wavelength used to obtain the results with excitation and emission at 365 and 465 nm. In the culture medium the complexity of components quenches the DAN fluorescence.

The samples in the NFbHP-NH_4Cl-lactate medium used in the experiment of fluorescence were also analyzed by HPLC, confirming that a different product was formed during NO trapping (figure 5b). As show the chromatogram of the Figure 5 the NAT complex formed in the acid medium has a retention time of 11.8 min (Fig. 5a) and the complex obtained in the culture medium has a retention time of 8.1 min (Fig. 5b). These results also showed that all DEA NONOate was consumed and all DAN reacted with NO in the system, since the DAN presented the retention time of 10.1 min initially (Fig. 5a e b) and disappear completely after 30 min and 24 h of reaction (Fig. 5a e b). For this reason, the samples was also analyzed by GC-MS, and was constated that the same product with *m*/*z* 182 Da was formed (figures 3 and 5). Considering a 1:2 stoichiometric reaction ratio (DAN:NO), a concentration of 0.025 mg mL^{-1} NO was determined (figure 5) to react completely with DAN, showing that two nitrogen is add to the DAN molecule, in contrast to the usually NAT complex formed.

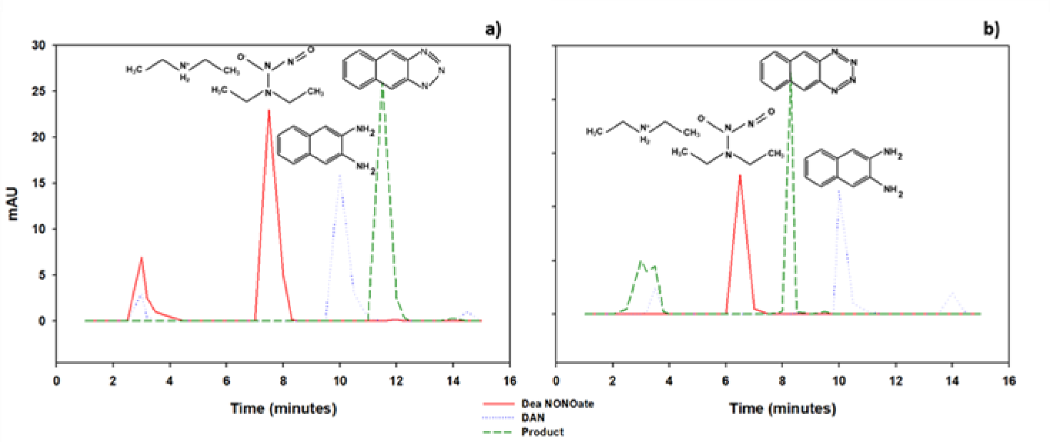

Figure 5. HPLC chromatogram obtained from reaction of 0.025 mg mL^{-1} Diethylamine NONOate diethylammonium salt (DEA NONOate) with 0.005 mg mL^{-1} 2,3-Diaminonaphthalene (DAN). **a)** in acid medium after 30 min incubation.**b)** in NFbHP-NH_4Cl-lactate medium after 24 h incubation.

4. Discussion

The Griess assay, EPR, fluorescent probes and electrochemical sensors are established NO detection methods in bacteria [18]. In addition, fluorescent probes can be used to monitor the real-time NO production in root tissues of inoculated plants by microscope analyses. NO production by *Mesorhizobium loti* in *Lotus japonicus* and *Medicago sativa* roots has been suggested as a specific recognition signal between plants and bacteria [36, 37]. In *M. truncatula* - *Sinorhizobium meliloti* symbiosis, NO production was detected with DAF probe by confocal microscopy, at different root sites and the nodule fixation zone during the infection process [38, 39]. For *Azospirillum* inoculated in tomato, DAF probe was also used to NO detection, and was confirmed that NO accumulation mainly occurred in vascular tissues and subepidermal root cells [20].

NO synthesis in bacteria is a mechanism associated with denitrification, which is an important part of the nitrogen cycle wherein bacteria reduce NO_3^- to N_2O and N_2 to obtain energy or achieve redox balance during anaerobic respiration [40].

The DAN assay has already been demonstrated as a plausible model for NO production monitoring in *E. coli* [41, 42], but has not been previously tested in rhizobacteria. *E. coli* RF1005 grown in LB medium supplement with 12 mM KNO_3 showed initial rates of NO production of 10 nmol min^{-1} per mg of cell protein and a maximum concentration of 200–300 µmol L^{-1} [41, 42].

In our work the main goal has showed that the Griess and DAN assays were efficient for NO detection, and confirmed the ability of *A. brasilense* HM053 and FP2, *R. tropici* Br322 and *G. diazotrophicus* Pal5 to synthesize NO in NFbHP-NH_4Cl-lactate medium supplemented with 10 mM KNO_3. This study is the first to establish DAN fluorescent probes as an inexpensive and reliable NO detection method that can be applied in different species of rhizobacteria and applied in studies that aim to understand the mechanisms associated with plant-bacteria interactions.

Denitrification can also occur under fully aerobic conditions [43], as shown for *A. brasilense* Sp245, which can produce NO by aerobic denitrification in OAB medium containing NO_3. The NO concentration was approximately 25-fold higher in NO_3^-containing media (120 nmol NO per gram of bacteria) than OAB with NH_4^+ (4.2 nmol NO per gram of bacteria) detected by EPR spectroscopy [19]. This suggests that NO_3^- is the main source of NO production by *A. brasilense*. In our work, NFbHP-NH_4Cl medium supplemented with

KNO_3 - an extra N source - was used to grow the bacteria strains; after 24 h to 72 h, NO quantification was performed using the Griess assay. NO concentrations ranged from 0.1435 for *A. brasilense* HM053) to 0.0036 for *A. fabrum* R5, normalized to total protein content.

Moreover, genes associated with denitrifying activity have been identified in *A. brasilense* plasmids (i.e., the *nap, nir, nor*, and *nos* genes) [44, 45]. Also, in these bacteria, multidomain metalloprotein-NO synthase- might be present and produce NO aerobically by oxidizing arginine. Studies on these bacterial NO synthases revealed new roles for NO, as the participation in toxin biosynthesis, in the protection against oxidative stress and in radiation damage recovery regulation [46].

Important functions have been attributed to NO in plant growth-promoting bacteria. Molina-Faveiro et al. (2008) [19] and Creus et al. (2005) [20] observed NO production in *A. brasilense* and concluded that the bacterially-derived NO is involved in the induction of lateral roots in tomato plants.

NO produced by *A. brasilense* is likely involved in biofilm formation, which is important for root colonization and growth, also considerably quantities of endogenous NO can be formed by *A. brasilense* by different metabolism using two N sources, such as NH_4Cl and KNO_3. They concluded that there are a correlation of the production of NO_3^- and the two N sources used [47]. However, studies have suggested that changes in root architecture induced by *A. brasilense* are associated with NO-promoted activation of IAA signaling pathways [48-50]. Previous studies have demonstrated a cross-talk between IAA and NO in the rhizosphere, where IAA-producing, and NO-producing bacteria such as *A. brasilense*, *R. tropici*, and *G. diazotrophicus* may take part [51-52]. These species may have genes/proteins that are responsive to NO stimulation, which directly impacts the quorum sensing system and biofilm formation [53], which are processes key to the plant-bacteria interaction [54].

For the *Rhizobium* genus, NO production occurs during the symbiosis process between *Rhizobium* and legumes, from the initial interaction to nitrogen fixation nodule formation [55]. Signorelli et al. (2020) [56] studied the role of NO in legume-rhizobia symbiosis and inferred that although this reactive species can reduce nitrogenase activity, NO exerted positive effects on BNF. In fact, the negative effects of NO require direct interaction with nitrogenase, whereas the positive effects are related to signaling functions which can amplify beneficial processes. Thus, detection of NO synthesis by nitrogen fixing bacteria can be used to detect the ability of the bacteria to amplify BNF, which would be particularly interesting for strain choice in agriculture.

In opposition to NO-producing bacteria as *A. brasilense*, the *H. seropedicae* (SmR1) genome was completely sequenced and no genes encoding denitrification enzymes, such as nitrous oxide reductase, [57] were identified. Baldani et al. (1986) [6] also found no evidence of denitrification in *H. seropedicae*.

In regards to *N. amazonense*, Kloos et al. (2001) [58] evaluated the Y1 strain and found no active genes for denitrification, a finding consistent with the results reported herein.

Generally, the synthesis of RNS in NO-producing bacteria decreased or did not occur after 24 h of incubation as indicated by the detection of the reaction product with DAN. In fact, NO synthesis as a signaling molecule appears to be a transient process rapid, as it is rapidly converted into important biological derivatives including nitrogen dioxide (NO_2), NO^{3-}, NO^{2-} and others [59]. NO signaling can activate metabolic stress response pathways, including those that protect from oxidative stress and convert NO into metabolic molecules by detoxification, DNA deamination, thiol S-nitrosation and Fe-S nitrosylation [60]. Thus, when N_2-fixing bacteria were cultivated in NFbHP-NH_4Cl-lactate medium supplemented with KNO_3 under aerobic conditions, it was the transient NO formation that occurs during denitrification through the action of nitrite reductases was observed [61].

Herein the NO produced in presence of KNO_3 in the bacterial growth in NFbHP-NH_4Cl-lactate medium was studied, wherein reaction with DAN formed a fluorescent compound with a molecular ion of 182 Da, 1, 2, 3, 4-Naphthotetrazole (NTT). When the NO formed by bacteria was replaced with that obtained from DEA NONOate after 24 h was shown to be efficient for detecting increases in NO concentration. This mechanism underpins an important tool for monitoring NO production in bacterial culture supplemented with KNO_3.

In fact, N-nitrosation of DAN, which occurs through the action of strong agents such as N_2O_3 and N_2O_4 produced from NO, can form a highly fluorescent compound-NAT-that offers specific, sensitive and versatile detection [62]. Ji and Hollocher (1988) [41, 42] demonstrated that *E. coli* can catalyze the nitrosation of DAN by NO^{2-} in the required presence of NO. Thus, KNO_3 likely acts as the substrate for the nitrosating agent formation, which directly promotes DAN nitrosation in the presence of NO. In contrast, Brew and Forsythe (1990) [63] demonstrated that the rate of bacterial nitrosation observed for *Neisseria subflava* is optimal with glucose as an electron donor for NO^{2-} reduction to a nitrosating species. Herein, NFbHP-NH_4Cl-lactate medium was used with lactate as the carbon source. Thus, it is plausible that lactate decarboxylation provides the electrons necessary for reduction of the nitrogen by products from the KNO_3 and NH_4Cl reactions.

Through bacterial metabolism and the generation of NO via DEA NONOate in NFbHP-NH_4Cl-lactate medium and the same medium with KNO_3, DAN was converted into a product with a molecular mass of 182 Da, 1, 2, 3, 4-Naphthotetrazole (NTT). Future work will focus on structural elucidation of this product to help further understand the mechanism by which DAN acts as a NO probe in bacterial systems and its application for monitoring processes related to KNO_3 metabolism by N_2-fixing bacteria.

In the course of this work we present results that prove the production of NO metabolized by Bacteria, which were treated with a source of KNO_3, by an indirect detection (Greiss reaction and DAN chemical trap). It is important to mention that in the absence of KNO_3, NO was not detected.

This work is composed of important data for the elucidation of a NO production mechanism in bacterias, since this molecule is a very important signal, mainly in the agribusiness sector, as it promotes plant growth through the BNF mechanism. However, more refined future experiments, such as isotopic labeling and elucidation of the product formed in chemical trapping are needed.

5. Conclusion

The results presented herein are consistent with our hypothesis that *A. brasilense* HM053 and FP2, *R. tropici* Br322 and *G. diazotrophicus* Pal5 can produce detectable NO concentrations in NFbHP-NH_4Cl-lactate medium containing KNO_3. At 24 h of growth, NO concentrations ranged from 0.1435 (*A. brasilense* HM053) to 0.0036 $\mu g\ mL^{-1}$ (*A. fabrum* R5), as determined using the Griess assay. At 48 and 72 h of growth, these strains showed decreased or no production of NO, similar to R5. *Azospirillum* sp. L40 differed from the other strains, as it did not produce detectable amounts of NO at 24 h of growth, but started forming NO after 48 and 72 h. Overall, the Griess and DAN results are mutually consistent, confirming the applicability and reliability of DAN for NO detection. Furthermore, plant-microorganism studies require suitable NO detection methodologies in conjunction with the Griess method, with the DAN fluorescent method meeting these criteria.

The ability to synthesize NO is linked to oxidative stress resistance, which can amplify BNF and promote plant growth. DAN probing of NO is an efficient method for detecting NO production in bacterial systems. The use of NFbHP-NH_4Cl-lactate as a culture medium containing KNO_3 results in the recovery of a product different from NAT (naturally produced by the reaction of DAN and NO) with or without bacterial metabolism. This product has a molecular mass of 182 Da (1, 2, 3, 4-Naphthotetrazole (NTT)) and may assist in the understanding of mechanisms associated with the action of DAN and in the elucidation of KNO_3 metabolic pathways as a nitrogen source for NO generation in diazotrophic bacteria. The approaches used herein may contribute to further understanding processes associated with NO production by bacteria and be applied in future projects to better understand the role of NO in cross-talk between plants and PGPB. These advances will spur the development of biotechnologies with potential agronomic applications and selection of more efficient PGPB. Future studies should focus on verifying the balance between NO levels necessary for nodulation and root growth, and those that are limiting for nitrogenase activity, which will elucidate the processes underlying plant-rhizobacteria interactions.

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