Nitric Oxide Emissions from Soybean Leaves during in Vivo Nitrate Reductase Assays

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ABSTRACT

Recent work identified acetaldehyde oxime as the predominant product purged by inert gases from anaerobic in vivo nitrate reductase (NR) assays of soybean (Glycine max [L.] Merr.) leaves. Another recent study supported earlier research findings which identified the primary product evolved from soybean leaves as nitric oxide (NO). This paper provides evidence that eliminates acetaldehyde oxime and confirms that NO is the primary nitrogenous product purged from the in vivo NR assay system. A portion of the evidence is based on the high water solubility of acetaldehyde oxime. Other evidence presented is the failure by chemical and spectrophotometric means to detect oximes in gases emitted in the purging of the reaction medium or in the leaf tissues. The gaseous product from the in vivo NR assay system reacted identically to NO standards and did not resemble acetaldehyde oxime standards. It was concluded that the predominant N product within the leaves was nitrite and that the predominant gaseous N product evolved from the assay was NO.

Evolution of NO, was first reported from herbicide-treated soybean leaves (4, 5). This research also showed that nitrite accumulated within leaf tissue prior to gaseous emissions. It was postulated that reactions between plant metabolites and nitric acid formed the NO. Nitric oxide (NO) was identified as the primary product, although traces of nitrogen dioxide (NO2) were detected. Nitrogen dioxide was also thought to be formed within the leaf but, due to its high water solubility, was not freely emitted from the leaves.

Harper (3) attempted to quantify the evolution of nitrogen oxides from soybean leaves during in vivo NR assays with aerobic and anaerobic gas purging. He found that NO, was derived from nitrate and was dependent upon the formation of 'threshold' levels of nitrite, but a stoichiometric relation between NO, and nitrite was not found. Also, it was later shown that NO, evolution appears to be associated with the constitutive NR activity in young soybean leaves (8, 9).

Mulvaney and Hageman (7), studying volatile nitrogenous products of the in vivo NR assay could not detect NO as a product but identified acetaldehyde oxime, a highly water soluble but volatile liquid, as the predominant N compound evolved from the in vivo NR assay medium. However, Dean and Harper (1) using gas chromatography and mass spectrometry recently identified NO as the dominant gaseous product purged from the in vivo NR assay.

The primary objective of this study was to examine further the differences in conclusions of Mulvaney and Hageman (7) and Dean and Harper (1) as to the identity of the predominant nitrogenous product formed and evolved as a gas from soybean leaf tissues under the in vivo NR assay system.

MATERIALS AND METHODS

Plant Culture. Soybean (Glycine max [L.] Merr. cv Williams) plants were grown in a growth chamber (16 h, 350 μE m⁻² s⁻¹ fluorescent and incandescent light, 30°C day, 15°C night) in a vermiculite base and irrigated daily with a nutrient solution. The nutrient solution contained in mm concentrations: MgSO₄, 3; KH₂PO₄, 1; Ca(NO₃)₂, 0.05 (Sequestrene 330 CIBA-GEIGY Corp., Greensboro, NC 27409); in μm concentrations: H₂BO₃, 23; MnSO₄, 46; ZnSO₄, 15; CuSO₄, 1.6; ammonium molybdate, 0.7. The pH was adjusted to 5.5 with H₂SO₄. Unifoliate (10- to 12-d-old) and trifoliate (16- to 18-d-old) leaves were used in experiments. Leaves were harvested for daily tests 4 to 6 h after the beginning of illumination.

Assays and Analyses. The basic in vivo NR assay system was conducted as described previously (3) except that n-propanol was not used. All samples were vacuum-infiltrated into leaves prior to testing. Gas purging of the system was always done with nitrogen.

From 1 to 2 g of leaf discs (1 cm diameter) were vacuum infiltrated (750 mm Hg) in a solution (50 ml) of 50 mm KNO₃ and 50 mm KH₂PO₄, adjusted to pH 7.5 with 3 N KOH. No surfactant was used. Leaf discs were vacuum infiltrated twice. The leaf discs were visibly wetted and did not float on the medium surface. The discs were transferred to a 250 ml gas washing bottle containing 150 ml of infiltration solution. The bottle and contents were placed in a water bath (33°C) and covered with a black cloth, and N₂ was bubbled through the medium (200 ml/min). The exiting gas from the in vivo NR system was passed through a dichromate-H₂SO₄ solid oxidizer and then analyzed for NO₃ in an Aerom NO₂ analyzer as previously described (5). In trapping procedures, small water traps (25 ml midget impingers) were inserted into the system before the oxidizing column (H₂SO₄-dichromate) as noted in the text and figures. Glass tubing was used for the gas flow system of NO₃ analysis except for several tygon tubing connections between traps, reaction vessel, and the oxidizing column. The ends of the glass tubes were butted together closely to expose as little tygon tubing to the system as possible. In experiments in which known gases were injected into the system, the tygon connections served as injection ports. It is estimated that the tygon tubing connections provided less than 1% of the total tubing surface area of the analysis system.

Acetaldehyde oxime was subjected to mild acidic hydrolysis (10) and determined as hydroxylamine by the 8-hydroxyquinoline method (6). Nitrite content was determined as described previously (5).

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2 Abbreviations: NO₃, nitrogen oxide gases; NR, nitrate reductase.
RESULTS AND DISCUSSION

In initial experiments, the in vivo NR assay system was tested
to determine patterns of NO\textsubscript{3} evolution versus nitrite content of
the assay. These experiments were repeated 15 to 20 times over
a period of several weeks. The data presented in Figure 1 are
typical of the results. After a 10- to 15-min lag, nitrite accumu-
lated linearly in the medium, reaching 20 \textmu mol nitrite g\textsuperscript{-1} fresh
weight in 2 h. NO\textsubscript{3} evolution increased rapidly during the first
30 min and reached a steady state of emission of approximately
7 \textmu mol nitrite equivalents g\textsuperscript{-1} fresh weight h\textsuperscript{-1}. From h 1 to h 2,
after NO\textsubscript{3} emission had reached steady state, nitrite content of
the medium increased 10 \textmu mol, while 7 \textmu mol of nitrite equiva-
lents were evolved as NO\textsubscript{2} (a total of 17 \textmu mol of nitrite formation
g\textsuperscript{-1} fresh weight). This demonstrates a loss of more than 40% of
the total nitrite formed if an inert gas is used in the assay to
purge the gaseous product. These data again demonstrate that
nitrite accumulation occurs during NO\textsubscript{3} evolution. The rate of
NO\textsubscript{3} evolution, after a relatively low level of nitrite had ac-
cumulated in the total assay, no longer appeared to be closely
related to nitrite content. Certainly, after 30 min of nitrite
accumulation, nitrite content of the medium was not the limiting
factor of NO\textsubscript{3} evolution.

Water solubility of the gaseous product of the in vivo NR assay
was first tested by installing a small water trap (a 25 ml midget
impinger) between the emission source (in vivo NR assay) and
the solid H\textsubscript{2}SO\textsubscript{4}-dichromate oxidizer. If the compound purged
by the N\textsubscript{2} from the in vivo NR assay was highly water soluble, it
would be dissolved in the water trap and would not pass through
the oxidizer and be converted to NO\textsubscript{2}. Data presented in Figure
2 illustrate that virtually 100% of the gaseous compound purged
from the in vivo NR assay passed through the water trap and
was registered as NO\textsubscript{2} by the analyzer. The small negative deflection
(Fig. 2) could have been due to at least two factors. First, the
manual insertion of the trap into the system required discon-
nection and reconnection of tubing (performed as quickly as possible
but could account for a small loss) and second, the time required
to saturate the small water trap with the evolving gas. If the water
trap was rapped sharply on the lab table after the 'saturation
period,' a small positive deflection of gaseous sample was de-
tected (data not shown). This suggests a sudden release of gas
dissolved in water. This negative deflection was characteristic of
each of these tests involving the gas from the in vivo NR assay
medium.

Over several months of repeated testing, with and without
water traps, 95 to 100% of the gas purged from the in vivo NR
system passed through an unbuffered water or dilute phos-
phate buffered (0.01 M, pH 7.5) trap. The water traps, the in vivo
NR assay medium, and the leaf discs were analyzed separately
for oximes repeatedly throughout this research period and no
oxime-type compound was ever detected. On the basis of these
data, it was concluded that the gas formed and purged from the
in vivo NR system was relatively insoluble in water and appar-
ently was not altered by passing through water. These results
agree closely with results from similar experiments by Dean and
Harper (1).

In the next series of experiments, the in vivo NR assay system
was replaced as a source of NO\textsubscript{3}. Instead, a known amount of
acetaldehyde oxime was added to aqueous solutions (300 \textmu mol/
150 ml). These solutions were purged with N\textsubscript{2} at the rate of 400
ml/min. This relatively high level of acetaldehyde oxime was
required so that sufficient quantities of the oxime could escape
and be detected by NO\textsubscript{3} analysis.

When the experiment was conducted in this fashion, vapors
of the oxime were purged from the aqueous source by N\textsubscript{2}, passed
into the solid oxidizer column, and were oxidized to NO\textsubscript{2} for
detection by the analyzer. With such a source of N vapors, steady
state NO\textsubscript{3} detection of approximately 2 \textmu mol NO\textsubscript{3} equivalents/
h could continue for hours (Fig. 3). This illustrates that, without
a water trap, no major factor interfered with the transfer, the
oxidation, or the detection of oxime vapors.

When a midget impinger (25 ml water) was placed between
the source of aqueous oxime and the oxidizer column, the rate
of NO\textsubscript{3} detected quickly dropped to zero (Fig. 3). After the 25
ml water trap had been in place for 1 h with no evidence of the
oxime passing through, the trap was removed. Immediately, NO\textsubscript{3}
was detected again, and the rate reached was the same as that
before placement of the trap. This rate continued for several
hours.

Chemical analysis (6, 10) of the solution in the water trap from
Figure 3 revealed that 1.87 \textmu mol oxime was present. This rep-
represents a 93% recovery of the oxime in the water trap, considering that NO oxidation analysis indicated that 2 \( \mu \)mol NO\(_2\) equivalents/h were being detected. These data demonstrate reasonable agreement between NO\(_2\) analysis and simple chemical analysis. This type of experiment was accomplished at least 10 times, with essentially identical results in both the pattern illustrated by Figure 3 and in the quantity of oxime trapped and recovered. The oxime content of the water traps as determined by chemical analysis (6, 10) yielded from 92 to 102% recovery as compared with oxime content as determined by NO\(_2\) analysis.

Results from this simple experiment (Fig. 3) illustrate that the vapors of acetaldehyde oxime do not easily leave an aqueous environment, even by purging the reaction vessel with 400 ml \( N_2 \)/min. Also, 300 \( \mu \)mol of the oxime were required to provide a NO\(_2\) detection rate of 2 \( \mu \)mol NO\(_2\) equivalents/h. With the \textit{in vivo} NR assay system as source of NO\(_x\), only 10 to 20 \( \mu \)mol nitrite in the same volume were required to evolve NO\(_2\) at the rate of 7 \( \mu \)mol NO\(_2\) equivalents/h. Chemical analysis of the \textit{in vivo} NR assay medium did not reveal the presence of oxime compounds. The primary product of the \textit{in vivo} NR assay was nitrite. Chemical analysis of the aqueous acetaldehyde oxime solution revealed that less than 1% of the oxime had been purged from the solution. The oxime that was purged from the solution, passed through the oxidizer, and was accurately detected by NO\(_2\) analysis.

In a different experiment, samples of \textit{in vivo} NR ‘gas’, NO gas standards, and vapors of acetaldehyde oxime were compared. These samples (2–10 ml) were injected through the tygon tubing connections into a stream of \( N_2 \) gas (300 ml/min) which carried the samples through the oxidizer and the NO\(_2\) analyzer. With this manner of introducing a limited volume, output of the samples appears as peaks with readings as millivolts (Fig. 4) instead of steady state emission patterns as shown in the previous figures.

In the case of each sample, the arrow marks the point of injection, while A marks the injection with a water trap (25 ml) in place before the oxidizer and B signifies no trap. In the case of the collected \textit{in vivo} NR gas, both peaks appeared to be nearly identical. This illustrates that the gas evolved from soybean leaf discs under anaerobic conditions is not very water soluble and passes through the water trap easily. The NO standard behaved similarly to the \textit{in vivo} NR gas sample, with both peaks nearly the same height whether they passed through a water trap or not. Acetaldehyde oxime, when injected before the water trap failed to pass through the trap, as shown by the absence of a peak. The sample of acetaldehyde oxime vapor injected without a water trap appeared as a peak similar to those of the \textit{in vivo} NR gas and the NO standard. The results presented in Figure 4 do not permit identification of different nitrogenous compounds by the amount of time required for oxidation and detection as in gas chromatography. However, with the simple use of water traps, it is shown that the \textit{in vivo} NR gas and the NO gas behave similarly, while the vapors of acetaldehyde oxime have different solubility characteristics.

Again, noting the high solubility of acetaldehyde oxime in water and that large amounts of the oxime were needed to permit low levels of vapors to be purged from the solution into the oxidizer and NO\(_2\) analyzer, attempts were made to duplicate the transfer of a quantity of acetaldehyde oxime similar to that shown by Mulvany and Hageman (7). They reported that 48.6 \( \mu \)mol of acetaldehyde oxime was evolved and purged in 1 h from an \textit{in vivo} NR assay (3 g leaf tissue/150 ml medium) and 20.1 \( \mu \)mol remained in the tissue and reaction medium. For this experiment, a total of 68.7 (48.6 + 20.1) \( \mu \)mol acetaldehyde oxime was placed in 150 ml reaction mixture and purged by 250 ml of \( N_2 \)/min. This system was connected to the solid oxidizer and NO\(_2\) analyzer. In accordance with the data of Mulvany and Hageman, at the end of 1 h, 48.6 \( \mu \)mol should have been detected by the NO\(_2\) analyzer, and 20.1 \( \mu \)mol should have remained in the original solution. At the end of 1 h, a total of 0.87 \( \mu \)mol was purged from the aqueous source. This quantity is less than 2% of the amount reported by Mulvany and Hageman (7) but generally agrees with other data presented in this paper (Figs. 3 and 4). This experiment was repeated five times with similar small quantities transferred, which again demonstrates the high water solubility of acetaldehyde oxime and its reluctance to leave an aqueous solution even by purging with an inert gas. During initial experiments, it was found that acetaldehyde oxime was quickly absorbed by tygon tubing while NO or the \textit{in vivo} NR gas passed through freely (data not shown). For this reason, it is noted in “Materials and Methods” that the flow system was composed of primarily glass tubing with only several small tygon tubing connections.

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**FIG. 3.** NO\(_2\) detected by purging an aqueous solution of acetaldehyde oxime with and without a water trap. To 150 ml of 0.01 M phosphate buffer (pH 7.5), 300 \( \mu \)mol of oxime was added and purged with 400 ml \( N_2 \)/min. The water trap was inserted into the system for a 1-h period and later analyzed for oxime content.

**Fig. 4.** Peaks of NO\(_2\) detected by the NO\(_2\) analyzer after injection of three different gas samples with and without water traps. The arrows note injection points. A denotes a water trap in the system; B denotes no water trap. Injection volumes and known quantities were 10 ml for the \textit{in vivo} NR gas, 10 ml for the NO standard (19 nmol), and 2 ml for acetaldehyde oxime (25 nmol). The \( N_2 \) flow rate was 300 ml/min.
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The absorption spectra as illustrated by Mulvaney and Hageman (7) were not duplicated. They report peaks at 196 nm for the oxime and 195 for acetamide (although the peaks are illustrated at 205 and 206 nm, respectively). The CRC Handbook (2) lists λ_{max} for acetaldehyde oxime at 190 nm and acetamide at 185 nm. The broad peak reported (7) at 274 nm for the oxime was not detected in any sample of medium, tissue, trapping solution, or aqueous acetaldehyde oxime solution.

Mulvaney and Hageman (7) reported that the N compound evolved during the in vivo NR assay isomerized during sample manipulation. They proposed that samples of collected acetaldehyde oxime completely isomerized (possibly by Beckmann rearrangement) to acetamide at room temperature within 1 week. Experiments in my laboratory showed that a standard solution of acetaldehyde oxime in the same dilute buffer they used (7) for collection sustained a 10% loss in 1 week and a 24% loss after 56 d. While acetaldehyde oxime is not completely stable in aqueous solutions, in my experiments it appeared to be more stable than reported earlier (7). Also, organic chemistry texts commonly describe the use of concentrated sulfuric acid or phosphorus pentachloride in ether for the Beckmann rearrangement to effect the conversion of an oxime into an amide. This reaction is not thought likely to occur to any great extent within a dilute phosphate buffer solution at pH 7.5.

CONCLUSIONS

Results of this study support the earlier findings (1, 4, 5) which concluded that nitric oxide is the predominant gaseous product evolved from soybean leaves when under anaerobic environments such as the N_{2}-purged in vivo NR assays described by Harper (3). Dean and Harper (1) based similar conclusions on results using techniques of NO_{x} analyses, gas chromatography and mass spectrometry. This paper confirms their NO_{x} analyses, and in addition, offers evidence by spectrophotometric and chemical analyses. In more than 6 months of testing, involving hundreds of analyses, no vapors of acetaldehyde oxime were detected evolving from soybean leaves. This observation is consistent with the high water solubility of acetaldehyde oxime. If formed, this liquid should remain within the aqueous interior of the leaf. However, chemical analyses made throughout this study never detected the presence of acetaldehyde oxime in the in vivo NR assay medium or in the leaf tissue at any time.

It was not the purpose of this study to eliminate the possibility of any compounds formed in trace amounts within the leaf. Considering the high reactivity of nitrite (as nitrous acid) with metabolites found within the living cell, the formation of a myriad of secondary stable and unstable products should be possible. The data presented in this paper illustrate that nitrite is the predominant product which accumulates during the in vivo NR assay, and that further conversion of nitrite, whether by chemical or enzymic reaction, produces the predominant evolving gas, nitric oxide.

LITERATURE CITED

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