# Nitrogen fixation, hydrogen production and $N_2O$ emissions

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Flynn, B., Scott, N. and Dong, Z. 2014. **Nitrogen fixation, hydrogen production and $N_2O$ emissions**. Can. J. Plant Sci. **94**: 1037–1041. $H_2$ is a by-product of the nitrogenase reaction. Exposure to $H_2$ is linked to increased $N_2O$ production, increased $CO_2$ fixation and plant growth promotion in soil. The effects of $H_2$ exposure on soil were observed using controlled $H_2$ gas treatments and field trials with legumes. In field trials, increased $N_2O$ production was observed in soil adjacent to legume nodules and inoculation of $H_2$-oxidizing isolates led to increased $N_2O$ emissions in corn fields. Many $H_2$-oxidizing isolates tested positive for key denitrification genes, indicating a connection between $H_2$ uptake and $N_2O$ emissions. $H_2$ treatment significantly increased copy number of the nitrite reductase (nirK) gene suggesting increased denitrification as the source of $N_2O$. There was also a significant increase in copy number and expression of the RubisCO (cbbL) gene in soil. $H_2$-oxidizing bacterial isolates (JM63 and JM162a) were found to promote plant growth, increasing tiller number and yield in spring wheat and barley. Combined results of T-RFLP and 16S rDNA clone libraries analysis revealed bacterial community structure changes in response to $H_2$ treatment, primarily with increases to the Gammaproteobacteria and Betaproteobacteria groups. The results of these studies help provide a better understanding of the soil bacterial community's responses to $H_2$ exposure and may lead to the development of a commercially viable plant growth promoting inoculant.

**Key words:** Soil, $H_2$ exposure, denitrification, $CO_2$ fixation, plant growth promoting rhizobacteria, rhizosphere

Flynn, B., Scott, N. et Dong, Z. 2014. **Fixation de l'azote, production d'hydrogène et émissions de $N_2O$**. Can. J. Plant Sci. **94**: 1037–1041. L'hydrogène est un sous-produit de la réaction commandée par la nitrogénase. On associe l'exposition à $H_2$ à une production accrue de $N_2O$, à une plus grande fixation du $CO_2$ et à une croissance accélérée de la plante dans le sol. Les auteurs ont observé les effets de l'exposition du sol à l'hydrogène en recourant à divers taux d'application de $H_2$ et en effectuant des essais sur le terrain avec des légumineuses. Lors des essais sur le terrain, on a relevé une production plus importante de $N_2O$ dans le sol adjacent aux nodules des légumineuses et l'inoculation d'isolats oxydant l'hydrogène engendre de plus forts dégagements de $N_2O$ dans les champs de maïs. Beaucoup d'isolats oxydant l'hydrogène ont réagi positivement lors des essais visant à identifier les principaux gènes de dénitrification, signe qu'il y a un lien entre l'absorption de $H_2$ et les émissions de $N_2O$. Le traitement au $H_2$ a augmenté sensiblement le nombre de copies du gène codant la nitrite réductase (nirK), ce qui laisse croire que la dénitrification est la source des émissions de $N_2O$. On a aussi noté une augmentation du nombre de copies et de l'expression du gène RubisCO (cbbL) dans le sol. Les isolats bactériens qui oxydent $H_2$ (JM63 et JM162a) accélèrent la croissance des plantes, augmentant le nombre de talles et le rendement du blé de printemps et de l'orge. Les résultats combinés de l'analyse des banques de clones par T-RFLP et ADNr 16S révèlent que la structure de la population bactérienne change avec le traitement au $H_2$, principalement avec la prolifération des gammaprotéobactéries et des bêtaprotéobactéries. Les résultats de ces études nous aideront à mieux comprendre la réaction de la microflore du sol à l'exposition au $H_2$ et pourraient déboucher sur l'élaboration d'une inoculant commercialement rentable, capable d'accélérer la croissance des plantes.

**Mots clés:** Sol, exposition au $H_2$, dénitrification, fixation du $CO_2$, RFCP, rhizosphère

Crop rotation and intercropping with legumes are long standing agricultural practices. The benefits of crop rotation include avoiding the diminishing yield of continuous mono-cropping, the control of pests and the occurrence of certain crop diseases (Emmond and Ledingham 1972; Roush et al. 1990; Peters et al. 2003). Legumes form symbiotic relationships with rhizobial bacteria in soils to form root nodules; these nodules are the site of biological nitrogen fixation. It is this nitrogen fixation that explains much of the benefits of legume rotation and the fixed nitrogen can reduce the need for synthetic nitrogen-based fertilizers, which contribute to $N_2O$ emissions (Kelner et al. 1997; Verge et al. 1997). However, studies suggest that significant amounts of $N_2O$ are released from legume fields, and little has been done to understand these emissions (Rochette and Janzen 2005). Preliminary studies (MacKinnon, Drury and Layzell, unpublished data) have found no evidence that growing legumes are able to produce $N_2O$ under a wide range of treatments to produce hypoxic or anaerobic conditions within nodules,

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**Abbreviations:** **HUP**, uptake hydrogenase; **PGPR**, plant growth promoting rhizobacteria

despite the fact that rhizobia extracted from legume nodules have displayed denitrification of nitrate to $N_2O$ (O'Hara and Daniel 1985).

$H_2$ gas is an obligate by-product of the nitrogen fixation pathway that occurs within legume nodules and is produced in large quantities in legume crops (Conrad and Seiler 1980). In nodules that lack an uptake hydrogenase enzyme (HUP) this $H_2$ is released from the nodule into the soil where it induces bacterial $H_2$ oxidation (La Favre and Focht 1985). The majority of agricultural legume crops are Hup- (lacking the HUP enzyme) and release $H_2$ into the soil (Ruiz-Argueso et al. 1979; Uratsu et al. 1982). It is possible that this $H_2$ plays a role in the $N_2O$ emissions that are observed in legume fields. The increase in $H_2$ oxidation has also been shown to be closely linked with both $O_2$ consumption and $CO_2$ fixation in soils. When $H_2$ is oxidized 60% of the electrons produced are used in the consumption of $O_2$ and 40% in the fixation of $CO_2$ (Dong and Layzell 2001). The enhanced $O_2$ uptake in soils exposed to $H_2$ may create the hypoxic or anaerobic conditions around $H_2$-releasing nodules which promote the denitrification process, leading to $N_2O$ production and emission (Sahrawat and Keeney 1986). As previously mentioned, little work has been done to investigate the $N_2O$ emissions from legume fields. An understanding of the mechanism through which $N_2O$ emissions occur may aid in their mitigation.

Legume crops have also been reported to increase soil carbon stocks (Hussain et al. 1988), providing the potential to offset fossil fuel emissions. This may be achieved in part through the noted $CO_2$ fixation observed in soils exposed to $H_2$ evolved from legume nodules (Dong and Layzell 2001). Again, a better understanding of the mechanism through which $CO_2$ is fixed in leguminous soils may allow for the better control this carbon-sequestering potential.

## OBJECTIVES

Many legume symbioses allow this $H_2$ to diffuse into soil where it stimulates the growth of soil microbes, some of which have been implicated in stimulating plant growth (plant-growth-promoting rhizobacteria, PGPR), building soil carbon pools, and increasing $N_2O$ (a potent greenhouse gas) emissions. Over the past few years the research interest of this team has focused on three main areas. First, to investigate the $N_2O$ emission from legume fields, particularly the impacts of $H_2$ released from legume nodules on denitrification in soil adjacent to nodules. Second, to understand the $CO_2$ fixation in soil linked with $H_2$ oxidation to explain the role of legumes in enhancing soil C stocks, and to provide a scientific basis for claiming emission reduction credits for legume cultivation. Third, to study the plant-growth-promoting effects of bacteria isolated from $H_2$-treated soils and soils exposed $H_2$-releasing legumes, to develop commercially viable $H_2$-oxidizing bacterial inoculants.

## RESULTS AND DISCUSSION

### $H_2$ Oxidation and $N_2O$ Emission

In repeated laboratory experiments with field soils that have not been exposed to legume crops for at least 20 yr, long-term (weeks) exposure of soil to elevated concentrations of $H_2$ (similar to that experienced by soils adjacent to legume nodules) results in a major (8–10 times) increase in the emissions of $N_2O$. This result clearly shows the connection between the $H_2$ gas and soil $N_2O$ production, and provides a foundation for the hypothesis that the elevated levels of $N_2O$ production in leguminous crops is linked to the evolution of $H_2$ from the legume nodules.

$H_2$ oxidation increases soil oxygen uptake (Dong and Layzell 2001) and may cause hypoxic conditions favored by denitrifying bacteria. Dose-response of $N_2O$ emissions with $H_2$ exposure rates was investigated to test this hypothesis. Soil samples from fields that have not seen legume crops for at least 20 yr received three $H_2$ treatments. The high $H_2$ treatment soil received $H_2$ at a rate of 200 nmol $H_2$ $cm^{-3}$ $h^{-1}$, an exposure rate calculated to be representative of that measured in soil within a few centimeters of a legume nodule (Dong and Layzell 2001). The medium $H_2$ treatment received $H_2$ at a rate of 20 nmol $H_2$ $cm^{-3}$ $h^{-1}$. The air-treated soil received $H_2$ at a rate of 0.1 nmol $H_2$ $cm^{-3}$ $h^{-1}$. The soil $N_2O$ emissions rate increased sharply for the first phase of soil exposed to either high or medium $H_2$ levels. The soil $N_2O$ emissions rate reached saturation first under a high $H_2$ level. For the soil treated at a medium $H_2$ level, the $N_2O$ emissions rate continued to increase for several weeks and eventually reached the level similar to that under the high $H_2$ level. The air-treated soil showed a constant low $N_2O$ emissions rate throughout treatment. These results suggest that a medium level of $H_2$ oxidation is enough to induce soil $N_2O$ production. The medium level of $H_2$ is capable of triggering a full level of soil $N_2O$ emission, suggesting that the $N_2O$ production is not only limited to the surface of the nodule, but spreads to soil exposed to a low level of $H_2$ gas such as that distant from Hup − nodules, or around Hup + nodules.

The similar $N_2O$ emission rates under medium and high $H_2$ levels suggest that there are other factors in the system besides the oxygen level changes. Studies were carried out to look more closely at the bacteria responsible for the $H_2$ oxidation in soils for genes involved in nitrification and denitrification. A real-time PCR study showed that gene copy numbers of nirK, a gene that codes for nitrite reductase in the denitrification process, was significantly higher in $H_2$-treated soil samples than in air-treated soil. This suggests that the increased $N_2O$ production in $H_2$-treated soil samples may come from the denitrification process. It is possible that $H_2$ gas exposure promoted certain denitrifying bacterial populations; the activity of these bacteria caused an increase in $N_2O$ emissions. This result provides evidence to support the

linkage between the $H_2$ oxidation in soil and $N_2O$ production in legume fields.

To understand the situation in the field, soil samples were collected adjacent to $N_2$-fixing legume nodules. The soil $H_2$ uptake rate, bacterial community structure, and the $N_2O$ emissions rates from soil around different nodules were studied. The results show that soil adjacent to active Hup– nodules has a greater $H_2$ uptake ability and increased $H_2$-oxidizing microbial population (Zhang et al. 2009). Data also showed much higher rates of $N_2O$ emissions from soil adjacent to nodules compared with soil collected further away from nodules. However, similar $N_2O$ emission rates were detected from soil samples around Hup − and Hup+ legume nodules, although the $H_2$ release rate of the Hup+ nodule was only about 20% of the Hup − nodule. This result may be explained by the similar soil $N_2O$ emission rates after medium and high $H_2$ level treatment in the laboratory. To further study the effects of Hup status on soil $N_2O$ production and persistence of the elevated $N_2O$ emission, corn plants were used to rotate with soybeans that had received different inoculations. The results show that corn fields whose soils were exposed to nodulated legume roots the previous year had higher rates of $N_2O$ production than soils exposed to non-nodulating legume roots and control bulk soil. Again, no significant differences were observed between the Hup+ and Hup − symbioses. These results indicate that the effect of soybean nodule activity on soil $N_2O$ emissions is not limited to the time when soybeans are grown, but also occurs for crops grown in subsequent years following the soybeans. The similar $N_2O$ emissions seen in fields treated with different Hup statuses may be due to the fact that Hup+ nodules still release some $H_2$ into the soil, although at lower rates than the Hup − nodules. This overwintering effect shows that elevated $N_2O$ production continues in the absence of $H_2$ gas. This suggests that $H_2$-oxidizing microbes isolated from legume soils may also enhance $N_2O$ emissions when added to bulk soil that has not seen legumes. For this, two previously isolated $H_2$-oxidizing microbes, JM63 (*Variovorax paradoxus*) and JM162a (*Flavobacterium johnsoniae*) (Maimaiti et al. 2007) were used to inoculate corn seeds. Soil samples collected from corn fields inoculated with JM63 and JM162a showed a trend of having higher $N_2O$ emission rates than the non-treated control soils when measured in the laboratory; however, there was no significant difference between the two inoculants. The corn plots inoculated with JM63 and JM162a showed a trend of higher soil $N_2O$ emissions in 8 of the 11 field measurements. Again, no statistically significant differences were observed between the JM63 and JM162a. These results again show a trend of elevated $N_2O$ emissions from soil, related to legumes, in the absence of $H_2$ gas. The soil had not been exposed to $H_2$ gas in this experiment. It is possible that $H_2$ is having an indirect effect on $N_2O$ emissions through the $H_2$-oxidizing bacteria. Previous studies have shown that there is an increase in the population of $H_2$-oxidizing bacteria surrounding legume nodules (La Favre and Focht 1985). We have also demonstrated that several $H_2$-oxidizing isolates contain nitrifying and denitrifying genes. It seems the $H_2$-oxidizing microbial populations play a crucial role in soil $N_2O$ production with growth of $N_2$-fixing legume crops.

In order to understand the effects of $H_2$ exposure on the soil bacterial community, four separate 16S rRNA gene clone libraries were constructed from air- and $H_2$-treated soil in the laboratory, and soil adjacent to Hup+ and Hup − nodules, respectively. From each of the four bacterial clone libraries, 350 clones were randomly picked and sequenced.

The sequence data show that the largest bacterial group is Proteobacteria, followed by Bacteroidetes and Actinobacteria. The Gammaproteobacteria population increased most after laboratory $H_2$ treatment, while β and γ subdivisions increased significantly in the soil, supporting Hup– nodules. Flavobacteria contribute a little to the increase in Bacteroidetes in soil supporting Hup– nodules.

The results show that there are detectable changes occurring in the soil bacterial community as a result of $H_2$ exposure. Similar results were also found in a German study looking at $H_2$-treated soils with net $CO_2$ fixation; the study found that there were increases in β and γ subclasses of Proteobacteria, as well as bacteria of the Cytophaga–Flavobacterium–Bacteroides phylum (Stien et al. 2005). This may explain the changes that are seen in soil $H_2$, $CO_2$ and $O_2$ exchange that occur gradually with $H_2$ exposure (Dong and Layzell 2001). It may also help to explain the persistence of $N_2O$ emissions in fields that have contained legumes in the absence of $H_2$ gas.

The previously mentioned studies should aid in the understanding of the elevated $N_2O$ emissions seen in legume fields, and may aid in finding methods for the reduction of these emissions. Some previous work has shown that there is a strong growth response to $H_2$-treated soils and soils inoculated with $H_2$-oxidizing bacteria (Maimaiti et al. 2007). Further studies may allow for the reduction of legume-related $N_2O$ emissions through the use of PGPR inoculants isolated from $H_2$-treated soils. Such an inoculant is one that would promote plant growth, reduce $N_2O$ emission and also enhance the $CO_2$-fixing ability of $H_2$-treated soils.

### $H_2$ Fertilization and $CO_2$ Fixation

Hydrogen oxidation in soil has been studied using simultaneous measurements of $H_2$, $O_2$ and $CO_2$ exchange to quantify the rate of $H_2$ oxidation, $O_2$ uptake and $CO_2$ fixation of soils treated with $H_2$ in the laboratory (Dong and Layzell 2001; Stien et al. 2005). The results show that 60% of the reducing power from $H_2$ oxidation was coupled to $O_2$ uptake, and the remaining 40% was coupled to $CO_2$ uptake. If this finding is typical of legume soils, it could help to explain the role of legumes in enhancing soil C stocks, and may provide a scientific

basis for claiming emission reduction credits for legume cultivation.

It was, therefore, believed that soil near Hup − legume nodules will have high and predictable rates of $H_2$ uptake-coupled $CO_2$ fixation. To test this, soils exposed to either air or $H_2$ at 200 nmol$H_2$ $cm^{-3}$ $h^{-1}$ for 3 mo were incubated with 1% $^{13}CO_2$ at room temperature in the presence or absence of $H_2$. Results show a significantly higher $^{13}CO_2$ uptake into the $H_2$-treated soil, in the presence of $H_2$. This result demonstrates a clear link between $H_2$ oxidation and $CO_2$ uptake in the soil. This shows that even $H_2$-treated soils in the absence of $H_2$ lose their $CO_2$-fixing ability. It suggests that the reducing power for $CO_2$ fixation comes directly from $H_2$.

$H_2$-treated soil demonstrated strong rates of $^{13}CO_2$, particularly in the presence of $H_2$ gas, while oven-dried and autoclaved soil showed no changes in isotopic composition in the presence of $^{13}CO_2$ with or without the addition of $H_2$, suggesting that the uptake of $^{13}CO_2$ in the presence of $H_2$ results from biotic processes.

Since the $H_2$-coupled $CO_2$ fixation is a biotic process, it narrows the possibilities for the mechanism through which it acts. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is involved in the first step of the Calvin Benson Bassham cycle, and its abundance and importance has been shown in agricultural soils (Selesi et al. 2007). To investigate the possible role of RubisCO in $H_2$ related $CO_2$ fixation, bacterial RubisCO gene copies and expression were quantitatively compared between soils with and without $H_2$ exposure. A significant increase in *cbbL* (coding for the large subunit of RubisCO) gene copies and expression was found in $H_2$-treated soils compared with air-treated soils. A trend was noted, that higher gene copies and expression were found in soil adjacent to Hup − nodules compared with Hup+ nodules; however, there was no significant difference between the two. These results show that there is a link between the $H_2$-induced $CO_2$ fixation and bacterial RubisCO activity of the soil. This suggests that the $CO_2$ fixation is achieved, at least in part, through bacterial RubisCO.

In order for the $H_2$-coupled $CO_2$ fixation to be a viable option for $CO_2$ mitigation, the carbon fixed in the soil should be in a stable form. Soils were labelled with $H_2$-coupled $^{13}C_2$ and fractionated in various ways to test the stability of fixed C in soil. Results indicate significant incorporation of $^{13}C$ into the microbial biomass following treatment with $H_2$ and $^{13}CO_2$. Measurements of $^{13}C$ in the light fraction material were highly variable and difficult to reproduce. Interestingly, even during this short-term incubation, some of the $^{13}CO_2$ was incorporated into the more resistant acid-stable fraction of soil organic matter. Future work is needed to calculate the exact proportion of the label that was incorporated into different SOM fractions, and estimate the long-term release of this material based on estimates of the mean residence time of carbon in these different soil organic matter pools.

## Plant Growth Response and Inoculant Production

As mentioned, previous work has shown a strong plant growth response to $H_2$-treated soils and soils inoculated with $H_2$-oxidizing bacteria (Dong et al. 2003; Maimaiti et al. 2007). It is possible to develop commercially viable inoculants from $H_2$-oxidizing bacteria isolated from soil adjacent to legume nodules. The ideal commercial inoculant would be a PGPR that promotes plant growth, while having minimal effect on the $N_2O$ emission of the soil.

In order to proceed with the use of $H_2$-oxidizing inoculants, it must first be possible to isolate $H_2$-oxidizing bacteria from $H_2$-treated or leguminous soils. A novel gas flow-through incubation system has allowed isolation of $H_2$-oxidizing bacteria from $H_2$-treated soil and soil adjacent to Hup − soybean nodules grown in greenhouse and field conditions (Maimaiti et al. 2007). These isolates either had apparent activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase or rhizobitoxine activity. Both are known to decrease the concentration of ACC, an immediate precursor of ethylene, and are therefore inhibitors of ethylene biosynthesis (Maimaiti et al. 2007). The reduction of ethylene biosynthesis makes isolates capable of being excellent candidates for plant growth promotion inoculants (Glick et al. 1998).

To test the plant growth promotion ability of these isolates, corn seeds were inoculated with strains of $H_2$ oxidating microbes: JM63 (*Variovorax paradoxus*) and JM162a (*Flavobacterium johnsoniae*) in a field trial. The inoculated corn was taller and had greater leaf area and plant dry weight than the control plants. Cob weight was also greater in the inoculated plants. Yield was greater for inoculated plants compared with the control, but the differences were not statistically significant. Similar tests were performed on spring wheat and barley. Significantly higher number of tillers per plant and higher head density were observed with inoculated plants.

These results suggest that plant growth promotion through the use of $H_2$-oxidizing isolates is possible, but clearly more work is needed in this area. Although the preliminary results do look promising there may be other factors involved in the inoculation of these isolates. As mentioned previously, our studies have shown that corn plots inoculated with JM63 and Jm162a have both been found to have higher soil $N_2O$ emissions compared with non-inoculated controls. In order for these isolates to be viable options for promoting plant growth and reducing $N_2O$ emissions from agricultural fields, the denitrification ability of these isolates has to be reduced or eliminated.

It is the belief of this group that it should be possible to develop an inoculant that will be useful for promoting crop growth and reducing $N_2O$ production in legume fields. Hydrogen-oxidizing bacterial isolates with less or no $N_2O$ production can be used as a co-inoculant with rhizobia for legume crops. The rhizobial bacteria

will form nitrogen fixation symbiosis with legume plants inside nodules, while the hydrogen-oxidizing bacteria will have a better chance to propagate in the soil around the nodules. This kind of inoculant could potentially reduce the $N_2O$ production from legume fields and promote $CO_2$ fixation without losing the plant-growth-promoting ability.

The stimulation of plant growth in corn, spring wheat and barley is very promising. The detection and re-isolation of inoculants at the end of the growing season suggests persistent and possible propagation of inoculants in the rhizosphere. Once hydrogen-oxidizing PGPR isolates without denitrification genes (or with low expression) are identified, they can be tested as inoculants for soybean crops and cereals.

## FUTURE GOALS

Our original proposal set as a major goal to assess whether it would be possible to produce a bacterial inoculant that would lead to a more positive greenhouse gas balance for soybean cropping systems. While we have made significant advances in understanding both the biogeochemical processes and microbial genetics that influence this balance, there is still more work needed to quantify the benefits adequately to justify an investment in this technology. We have shown a clear link between soil $H_2$ oxidation and both production of $N_2O$ and uptake of $CO_2$, and demonstrated a molecular basis for both of these processes. In terms of the biophysical controls, we need further studies on field-based estimates of $N_2O$ production to reconcile the discrepancy between field and laboratory-based flux measurements. To get a better understanding of the impact that $CO_2$ fixation can have on this balance, we need further experiments on the impact of longer labelling periods on both rates of $CO_2$ uptake and the turnover time of that fixed carbon. On the genetic side, further work on soil microbial activities associated with hydrogen oxidization, particularly on $N_2O$ production, $CO_2$ fixation, and plant growth promotion is needed. We are working on the soil microbial meta-transcriptome analysis. Once we have better insights into these key controlling factors we can begin testing the impact of inoculating a soybean field with a $H_2$-oxidizing bacteria that no longer has the genes for $N_2O$ production. If successful, these bacteria could revolutionize the production of soybeans by enhancing soybean production while at the same time reducing net greenhouse gas emissions (perhaps even leading to soybeans becoming net greenhouse gas sinks) from an expanding (globally) cropping system.

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