

Improved Potential for Nitrogen Fixation in *Azospirillum brasilense* Sp7-S Associated with Wheat *nifH* Expression as a Function of Oxygen Pressure

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Summary

The use of a *nifH-lacZ* fusion as an indicator of nitrogen fixing conditions is investigated in relation to two strains of *Azospirillum brasilense* with contrasting patterns of colonization on wheat roots. The degree of expression of *nifH-lacZ* of *Azospirillum brasilense* could be manipulated by controlling the oxygen pressure. A strong correlation between nitrogenase activity and *nifH* expression was found in pure cultures. *nifH* expression was maximal at 0.5% oxygen in pure cultures of both the wild type Sp7 and spontaneous mutant Sp7-S. Differentiation of the maximal expression was observed when the two strains were in association with *para*-nodulated wheat, resulting in greater expression by Sp7-S over a broader range of external oxygen concentrations than by Sp7. This result was observed when expressed as activity per mg of plant protein as well as per bacterium. An increase in *nifH* expression was also noted with *para*-nodulated (2,4-D treated) wheat inoculated with Sp7-S when compared with untreated wheat. No significant difference was found between treated and untreated wheat inoculated with Sp7. The results indicate that the majority of the *Azospirillum brasilense* Sp7-S cells occupy a more protected niche when in association with wheat roots, resulting in conditions that support a greater potential for nitrogen fixation as judged by *nifH* expression.

Introduction

Benefit from nitrogen fixation in associations between *Azospirillum* and wheat may only be achieved if the association sufficiently provides conditions suitable for significant levels of expression of nitrogenase [1].

Oxygen inhibits nitrogen fixation by direct action on proteins [2] and represses the level of DNA transcription [3, 4]. However, as nitrogen fixation is an energy consuming process some oxygen is required for the production of ATP by oxidative phosphorylation. HARTMANN and BURRIS [5] demonstrated via acetylene reduction that the opti-

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imum dissolved oxygen concentration for nitrogenase activity in *A. brasilense* is approximately 0.2%. It is the different way in which nitrogen fixing organisms overcome the environmental limitations of nitrogenase function that differentiate the nitrogen fixing systems.

NifH, which encodes the structural polypeptides of nitrogenase reductase, is only expressed under nitrogen fixing conditions [6]. The gene product of *nifA*, which is directly modulated by oxygen [4], activates it. It follows that a measure of the expression of *nifH* should be an indication of the potential for nitrogen fixation. Using this principle, VANDE BROEK *et al.* [7] demonstrated the value of a *nifH-gusA* fusion as a means of screening different species of plant-associative diazotrophs for relative tolerance to oxygen of their nitrogen fixing apparatus.

Apart from aggregation of cells by clustering and clumping where steep oxygen gradients have been observed [8], *Azospirillum* exhibits some respiratory protection of its nitrogenase. It is capable of a strong aerotactic response [9, 10] allowing migration towards the microaerophilic environment provided by the rhizosphere.

A. brasilense has been observed to colonize wheat roots by a two step process. Adsorption is the initial step and occurs via the polar flagellum and a secondary step involves the production of exopolysaccharides that anchor the bacteria to the plant [11]. *A. brasilense* Sp7 predominantly colonizes the surface of wheat roots [12] whereas Sp7-S, a spontaneous mutant of Sp7, is rarely observed bound to the root surface and does not bind Congo red [13]. PEREG-GERK *et al.* [14] demonstrated that in enriched media there appears to be little differentiation of Sp7 and Sp7-S. The differences occur in specialized growth media where Sp7-S was deficient in flocculation and swarming. The mutation was found to be in a regulatory gene for flocculation (*flcA*) [14]. As a result Sp7-S (*flcA*) does not produce significant exopolysaccharides and shows reduced surface colonization. However, it is capable of colonizing between cortical cells and in crevices around the emergence of lateral roots and *para*-nodules, formed in response to the addition of 2,4-D with its cells remaining in a vegetative form [1]. This mode of colonization exhibits higher rates of nitrogenase activity, at reduced oxygen pressure, than is exhibited by Sp7.

In the work described here, a laboratory model involving *para*-nodulated wheat is used as previously described by several researchers [15–17]. The synthetic auxin has been shown to arrest normal lateral root development [17] and provides numerous modified lateral root structures from a greater number of primordia, able to support colonization by the Sp7-S strain of *A. brasilense*. This enhanced colonization allows higher rates of nitrogenase activity [13] thus simulating a desirable outcome. In this paper, a comparison of the potential for nitrogen fixation as a function of oxygen concentration is investigated using the *nifH-lacZ* fusion to study nitrogenase expression with the two contrasting modes of colonization.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are outlined in Tab. 1.

Tab. 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype or phenotype ^a | Reference |
|--------------------------------|--|-------------------------------|
| <i>Escherichia coli</i> | | |
| S17-1 | pro thi hsdR recA Tra ⁺ IncP | SIMON <i>et al.</i> [28] |
| <i>Azospirillum brasilense</i> | | |
| Sp7 | Wild type | TARRAND <i>et al.</i> [29] |
| Sp7-S | Spontaneous mutant of Sp7, CR ⁻ , Floc ⁻ | KATUPITIYA <i>et al.</i> [13] |
| Plasmids | | |
| pAB358 | pRK290 derivative ^b , <i>nifH-lacZ</i> transcriptional fusion, Tc ^r , Km ^r | LIANG <i>et al.</i> [22] |
| pAB576 | pVK100 derivative ^b , <i>nifA-lacZ</i> transcriptional fusion | LIANG <i>et al.</i> [22] |
| pLA- <i>lacZ</i> | pLA2917 derivative ^b , <i>lacZ-kan</i> constitutive fusion Tc ^r , Km ^r | ARSENE <i>et al.</i> [12] |

^a Tc^r: Tetracycline resistance; Km^r: Kanamycin resistance; CR: Congo red binding; Floc: Flocculation; Lac, *lacZ*.

^b pVK100, pRK290 and pLA2917 are broad-host-range vector derivatives of RK2 that are stable in *Azospirillum* strains.

Production of Transconjugants

Plasmids were transferred from an *E. coli* S17.1 donor to *Azospirillum* recipients by conjugation [12]. Transconjugants were then selected on minimal lactate agar supplemented with tetracycline (5 µg/ml). Pure cultures were then maintained on nitrogen-free malate agar (NFb) containing yeast extract (0.5 g/l), Congo red (0.25%) and tetracycline.

Growth Media and Conditions

Bacteria carrying *lacZ* fusions were shaken at 180 rpm at 30 °C overnight in NFb liquid media supplemented with tetracycline. The following day cultures were spun down in a bench centrifuge, washed twice in nitrogen free minimal medium and the A₆₀₀ was adjusted to 0.1; 10 ml of each culture was placed in 50 ml conical flasks. The cultures were tested for initial β-galactosidase activity to ensure that *nifH* was fully repressed. The flasks were stoppered and flushed with argon and the oxygen concentration adjusted by injection with different volumes of pure oxygen gas.

Correlation between *nifH* and Nitrogenase Activity in *A. brasilense* Sp7 and Sp7-S

A. brasilense Sp7 and Sp7-S carrying *nifH-lacZ* fusions were transferred to 50-ml flasks as described above. The oxygen concentration in the flasks was adjusted to 0.5% and acetylene was added to a final concentration of 10%. The flasks were shaken in a 30 °C incubator at 180 rpm and liquid culture and gas samples were taken at 0, 1.5, 3 and 5 h to test for β-galactosidase activity [18], protein content, BioRad Standard Assay and ethylene production [19]. Bacteria carrying *nifA-lacZ* fusions, which are expressed more constitutively, were used as a control. Correlation coefficients were calculated between acetylene reduction, *nifH* and *nifA* activity using Microsoft Excel®.

nifH Activity of *A. brasilense* Sp7 and Sp7-S as a Function of Oxygen Concentration

A. brasilense Sp7 and Sp7-S carrying *nifH-lacZ* fusions were transferred to 50-ml flasks as described above. The flasks were sealed with rubber serum stoppers, flushed with argon gas and the oxygen adjusted to produce a range of concentrations: 0, 0.5, 1, 1.5, 2, 3, 4, 8, 10 and 20% [v/v]. The flasks were shaken at 180 rpm in a 30 °C incubator for 4 h and tested for β -galactosidase activity and protein content. Bacteria carrying pLA-*lacZ* fusions were treated as a control.

Preparation and Inoculation of Wheat Seedlings

Seeds were sterilized, germinated and transplanted to a test-tube hydroponic system according to ZEMAN *et al.* [20]. The seedlings were then grown under controlled conditions in a Biotron at 20 °C with constant artificial light. Plants were inoculated in the test tubes 6–7 days later with 0.2 ml of *Azospirillum* culture grown overnight, at an A_{600} of approximately 0.8 (10^8 cells/ml). The cultures were grown in NFB liquid media containing yeast extract and shaken at 180 rpm in a 30 °C incubator. Plants that were treated with 2,4-D were inoculated with 0.2 ml of a stock solution of 2,4-D, the final concentration in the tubes being 0.66 ppm.

Transferral to an Aerated Hydroponic System

The plants were grown in the test tubes for a further 5 days until colonization was complete [21] and then transferred to an aerated hydroponic system (Fig. 1). The aerated hydroponic system was designed to maintain sterility of the wheat roots and to allow a longer growth period of the plants. This system consists of a 2-l Nalgene jar filled to the bottom of the thread with N-free hydroponic solution also lacking carbon substrates. Ten holes were press-drilled in the lid for the plants and two further holes were used as a gas inlet and outlet. The fittings to hold the plants were as follows: a 2 cm PVC tubing was tightly fitted into the holes in the lid with another piece of tubing fitted externally to this tubing with a large enough diameter to be closed with a rubber serum stopper (THOMAS SCIENTIFIC, 14 × 18 mm, Cat. No. 1780.J27). The stoppers were slit with a scalpel so that later they would be able to close around the stem of wheat seedlings. Nalgene jars and lids were autoclaved and a sterile syringe barrel was inserted through the slit. The seedlings were placed shoot first through the syringe barrel which was then removed over the shoot (Fig. 1 b). The roots of the seedlings were fed through the holes in the lid with sterile forceps and the lids tightly closed so that the roots were immersed in hydroponic solution with the wheat shoots in the air. The inlet consisted of PVC tubing with a sintered (60 μ m) stainless steel cup inserted at the end. This was fed through the hole in the lid until the cup was submerged in the solution. The outlet was an insert of PVC tubing into the airspace long enough to curl over and avoid contamination from the exterior. Jars, containing 10 plants each, were then placed in the glasshouse, at 25 °C and bubbled vigorously with air from an aquarium air pump (flow rate: 2 cm³/s) for five days.

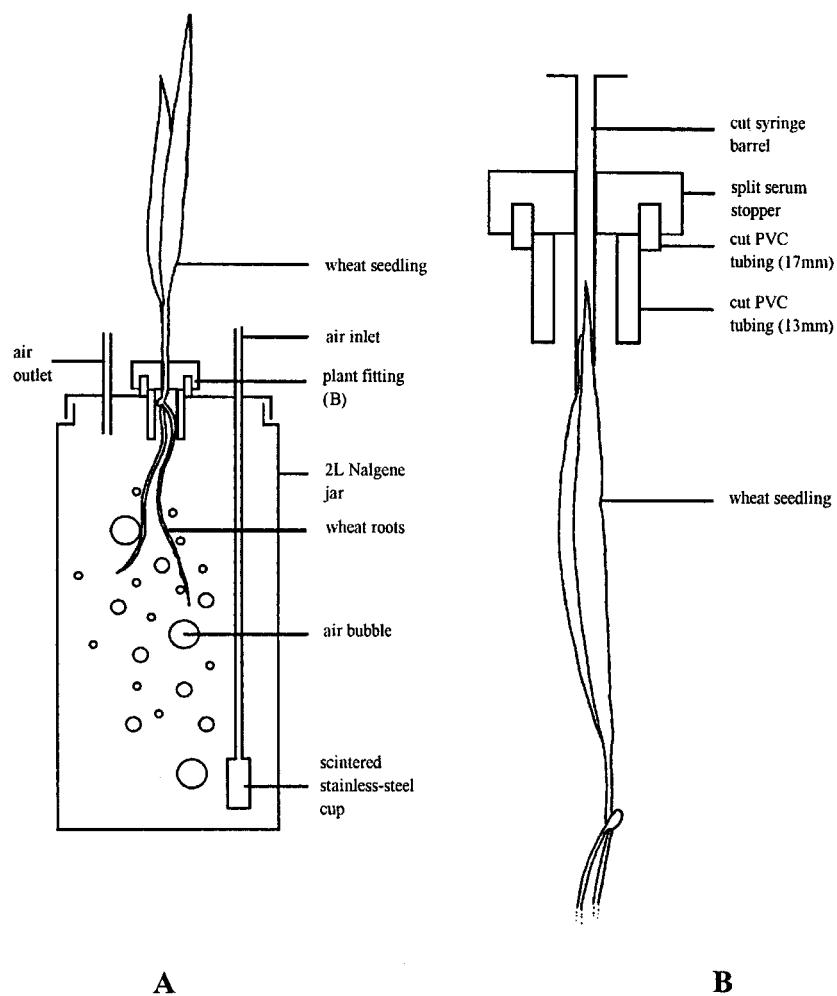
nifH Activity in Wheat Roots Grown in the Aerated Hydroponic System

Plants were inoculated with *A. brasilense* Sp7 and Sp7-S carrying *nifH-lacZ* and constitutive pLA-*lacZ* fusions. Ten plants of each were transferred to the aerated hydroponic system after five days and a replicate set of 10 plants of each was left to grow in the test-tube system. After five days, plants were harvested and assayed quantitatively for β -galactosidase activity [12].

The Effect of 2,4-D and Oxygen Concentration on *nifH* Activity in Inoculated Wheat Seedlings

Plants were inoculated with *A. brasilense* Sp7 and Sp7-S carrying *nifH-lacZ* and pLA-*lacZ* fusions. Plants were treated with 2,4-D and grown in the aerated hydroponic system described above. Plants

without 2,4-D were used as a control. After harvesting, the plants were transferred to sterile MCCARTNEY bottles containing 0.5 ml of WINOGRADSKY's medium to ensure a humid atmosphere. The bottles were stoppered, flushed with nitrogen gas for 20 min and pure oxygen gas was injected to give the final concentrations: 0, 1, 2.5, 5, 10, 20% [v/v]. The bottles were shaken in a 30 °C water bath with constant artificial light for 18 h and the plants were assayed quantitatively for β -galactosidase activity. Plants treated with and without 2,4-D were compared and the results were calculated in terms of *nifH* expression relative to the highest *nifH* expression. A comparison between Sp7 and Sp7-S inoculated *para*-nodulated wheat was also made. The results were calculated as *nifH* expression relative to both the maximum *nifH* expression and average pLA expression of each strain measured at the corresponding oxygen concentration.



A **B**
 Fig. 1. Aerated hydroponic system (A) and plant fitting (B)
 The aerated hydroponic system consists of a 2-l Nalgene jar filled with N-free hydroponic solution. A plant is inserted into the plant fitting as in (B), which is then inserted into the lid of the jar. The solution is aerated by an aquarium air pump through a sintered stainless steel cup at the inlet.

Results and Discussion

Correlation between Nitrogenase Activity and nifH Activity in Cultured Cells

The objective of this experiment was to compare nitrogenase activity and *nifH* expression of the two strains of *A. brasilense*, Sp7 and Sp7-S, at an initial oxygen tension of 0.5%. The cells were resuspended in minimal media thus reducing growth and respiration. The headspace above the liquid culture was 6 times the volume of the culture, and conical flasks were used to ensure a large surface area at the culture/gas interface. Although the flasks were shaken vigorously to ensure rapid equilibrium of the oxygen concentration in the gas and liquid phases, it is likely that an oxygen concentration gradient may have occurred in the liquid phase rendering the dissolved oxygen concentration lower than 0.5%.

The results obtained indicated that there is clearly little or no relationship between nitrogenase activity and *nifA* expression. Nitrogenase activity was induced after a lag phase and then continued to increase, whereas *nifA* activity remained constant throughout the course of the experiment (data not shown), consistent with previous work from this laboratory [17]. *nifA* is constitutively expressed [22] and no induction curve was observed. Nitrogenase is expressed only when oxygen concentration is low [5] and derepression results in the observation of an induction of nitrogenase after a lag phase [23].

In contrast, the *nifH* expression (data not shown) followed the same pattern of induction as the corresponding nitrogenase activity. The correlation coefficient between *nifH* expression and nitrogenase activity was high (0.9 for both strains), whereas the coefficients for *nifA* and nitrogenase activity were low (0.1 in Sp7 and 0.2 in Sp7-S), thus indicating the suitability of *nifH* as a reporter of potential nitrogenase activity.

Expression of nifH over a Range of Oxygen Concentrations

Cultures were washed by centrifugation and diluted in fresh media to a cell concentration of 10^7 cells/ml, in nitrogen-free minimal media. The expression of *nifH* was determined at a range of oxygen pressures after incubation at 30 °C for 4 h.

The profiles of *nifH-lacZ* expression with oxygen are shown in Fig. 2. There was no significant difference found between Sp7 and Sp7-S as indicated by overlapping of the 95% confidence intervals. The maximum oxygen concentration for expression in both strains was 0.5% oxygen. HARTMANN and BURRIS [5] observed an optimum expression of nitrogenase at a dissolved oxygen concentration of 0.2%. As expression was not measured at values between 0 and 0.5% concentration it can only be concluded that the highest expression at 0.5% is a maximum rather than an optimum. *NifH* expression was low at 0 possibly because energy metabolism (oxidative phosphorylation and ATP synthesis) is affected near 0. These results are consistent with results achieved by VANDE BROEK *et al.* [7] at cell concentrations of 10^7 cells/ml. *nifH* expression decreased to zero after 4% oxygen.

Oxygen profiles were also determined for the pLA-*lacZ* fusion (Fig. 3). The results indicate that there is no significant change in expression of this gene over the range of oxygen concentrations. As pLA-*lacZ* expression correlates highly with bacterial cell number [12] there appears to be little or no growth in the experimental flasks.

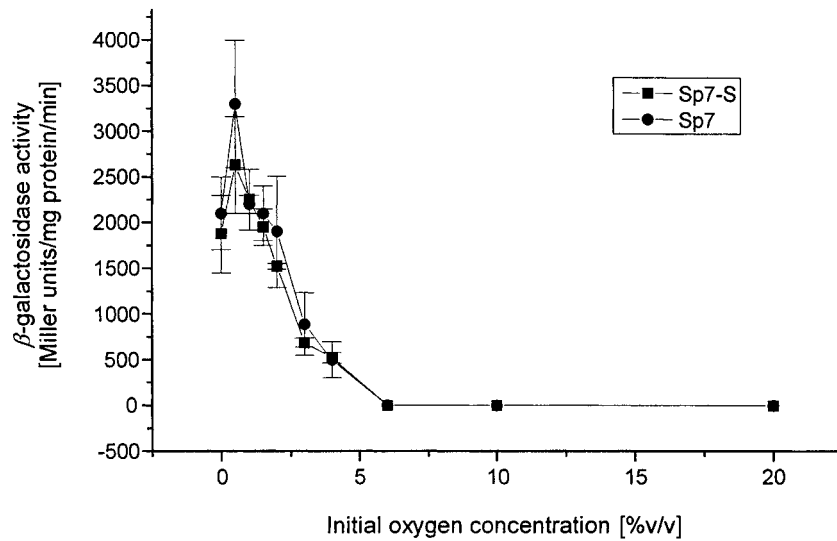


Fig. 2a. Expression of *lacZ* fusions in pure cultures of *A. brasilense* Sp7 and Sp7-S as a function of oxygen concentration (*nifH-lacZ* fusion)
The cells were incubated for 20 hours in nitrogen-free minimal media at different initial oxygen tensions. The results are averages of six samples, 95% confidence intervals are shown.

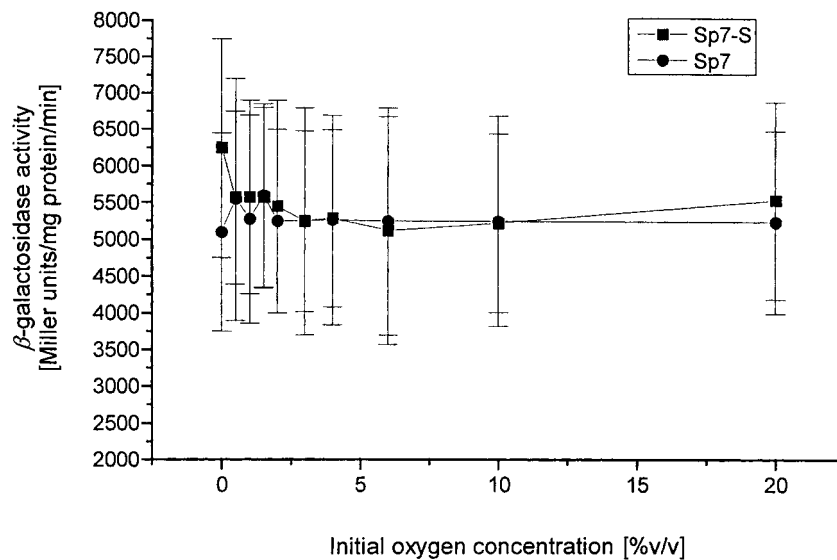


Fig. 2b. Expression of *lacZ* fusions in pure cultures of *A. brasilense* Sp7 and Sp7-S as a function of oxygen concentration (pLA-*lacZ* fusion)
The cells were incubated for 20 h in nitrogen-free minimal media at different initial oxygen tensions as described in Materials and Methods section. The results are averages of six samples; 95% confidence intervals are shown.

Thus, at a constant cell density *nifH* expression varies as a function of oxygen concentration.

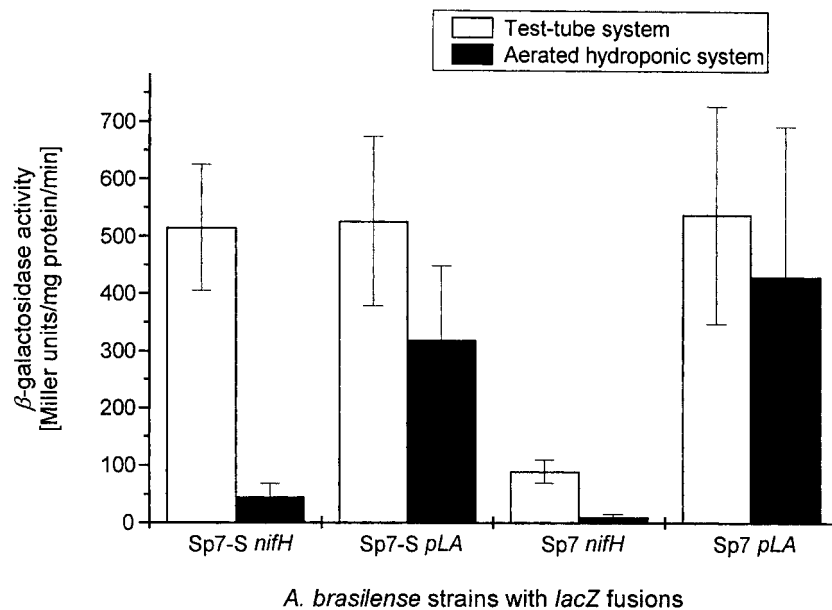


Fig. 3. β -Galactosidase activity of *para*-nodulated wheat inoculated with *A. brasilense* and grown in different hydroponic systems. The data are averages of two independent experiments with five plants; 95% confidence intervals are shown.

Effect of the Aerated Hydroponic System on *nifH* Activity

The aerated hydroponic system successfully repressed *nifH* activity (Fig. 4). Although the *nifH* expression in test-tube grown plants inoculated with *A. brasilense* Sp7 was relatively low, there was a significant reduction of this activity in plants grown in the aerated hydroponic system. A similar ratio between *nifH* expression in test-tube grown plants and aerated plants is observed in both strains. The *nifH* expression of Sp7-S in the test tubes was relatively high (590% of that of Sp7), consistent with recent data of PEREG-GERK *et al.* [21], indicating that the colonization pattern on *para*-nodulated wheat in a protected niche affords some protection from oxygen (in pure culture, *nifH* expression in both Sp7 and Sp7-S was not significantly different). There was a significant difference between this activity and that observed after growth in the aerated hydroponic system. pLA-*lacZ* expression did not appear to be significantly affected by altering pO_2 in either system. As the pLA-*lacZ* fusion is expressed constitutively, this is an indication that bacterial numbers on the roots are sustained after transfer to the aerated hydroponic system and during forced aeration.

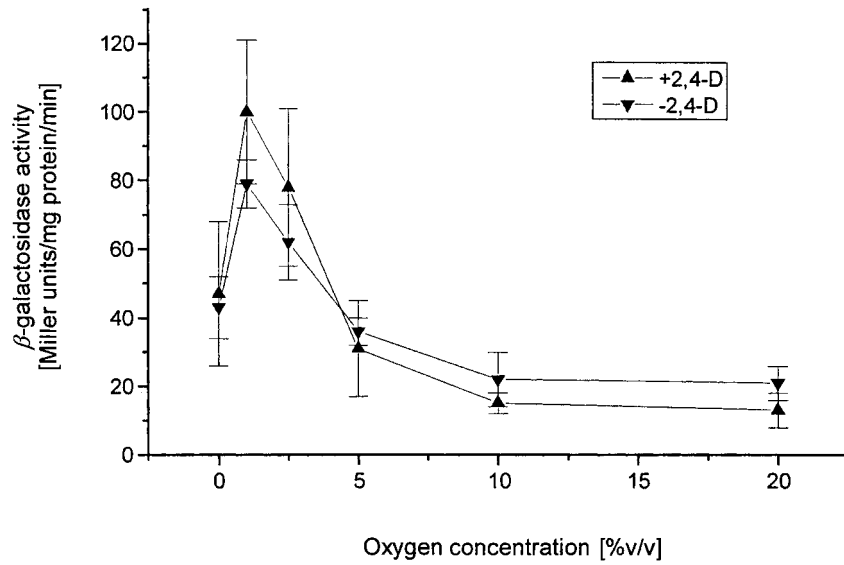


Fig.4a. Relative *nifH* expression as a function of oxygen concentration in wheat inoculated with *A. brasilense* Sp7 with and without 2,4-D
The results are relative to the highest *nifH* expression indicated as 100%. The data are averages of five plants; 95% confidence intervals are shown.

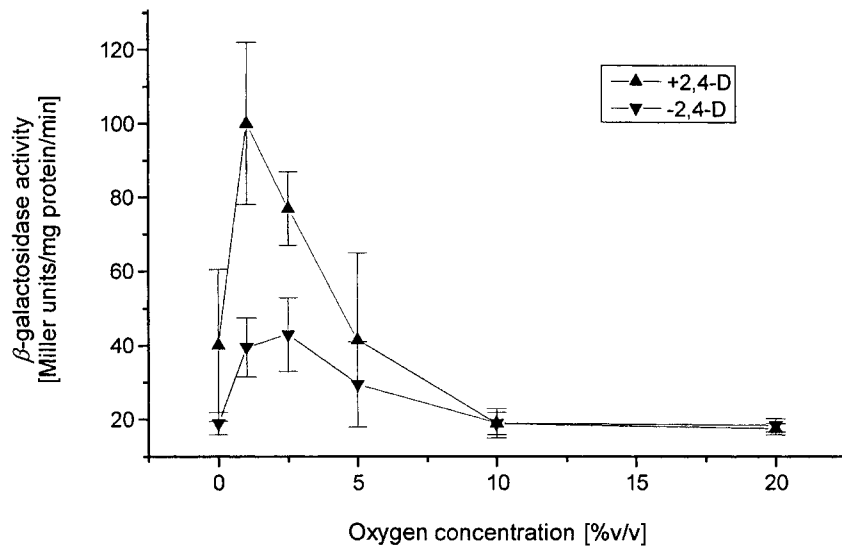


Fig.4b. Relative *nifH* expression as a function of oxygen concentration in wheat inoculated with *A. brasilense* Sp7-S with and without 2,4-D
The results are relative to the highest *nifH* expression indicated as 100%. The data are averages of five plants; 95% confidence intervals are shown.

Effect of 2,4-D on nifH Expression on Plants Inoculated with A. brasilense

Figs. 4a and b demonstrate the effect of 2,4-D treatment on *nifH* expression of *A. brasilense* over a range of oxygen concentrations. There was no significant effect of 2,4-D treatment on the relative expression of Sp7 *nifH* (Fig. 4a). In each case *nifH* expression was low at 0% oxygen, demonstrating the need for some oxygen for the synthesis of nitrogenase. It then increased to reach an optimum between 1–2.5% oxygen, before decreasing rapidly between 2.5–5% after which expression remained relatively constant. However, there was a significant effect of 2,4-D treatment on plants inoculated with Sp7-S (Fig. 4b), where maximum expression is 1.5 times greater than on untreated plants. Higher average expression is also observed at higher oxygen concentrations. Clearly, addition of 2,4-D causes an increase in the maximum *nifH* activity in Sp7-S and allows for a greater average expression at higher oxygen pressures.

Previous work has shown that there is a significant difference between the number of Sp7-S cells on plant roots treated with and without 2,4-D, whereas the difference in numbers of Sp7 was not significant [16, 21]. The main reason for this is that Sp7-S does not colonize the root surface well, but prefers points of emergence of lateral roots. It may, therefore, be suggested that this increase in *nifH* activity is due to increased numbers reducing the pO_2 . The increased numbers of Sp7-S, and not Sp7, may be related to the greater number of colonization sites provided by the increased number of lateral root initials observed in 2,4-D treated plants [17], while the surface area of the epidermal tissue may remain unchanged.

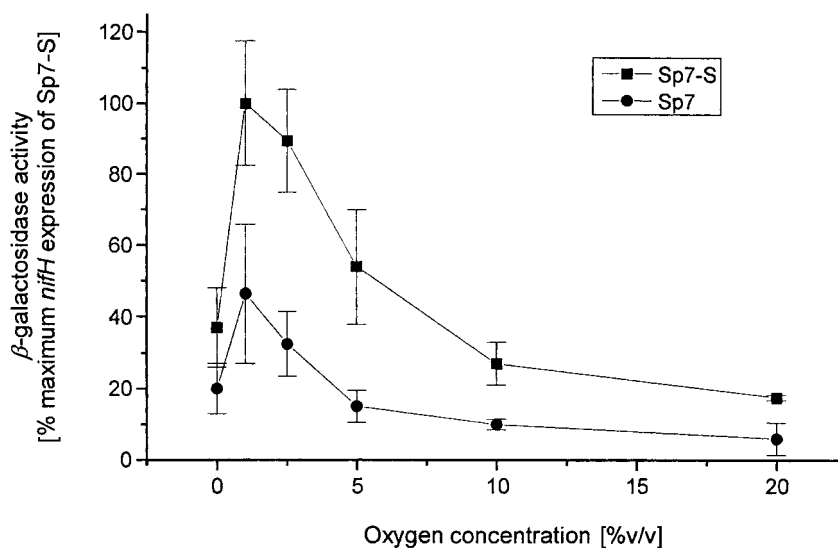


Fig. 5. *nifH* expression in *para*-nodulated wheat inoculated with *A. brasilense* as a function of oxygen concentration

Comparison of Sp7 and Sp7-S; expression is relative to the highest average *nifH* expression given by Sp7-S.

The results are averages of five plants from four experiments; 95% confidence intervals are shown.

Comparison of nifH Expression in Para-Nodulated Wheat Inoculated with A. brasilense Sp7 and Sp7-S

The relative *nifH* expression of *para*-nodulated wheat inoculated with *A. brasilense* Sp7 and Sp7-S as a function of oxygen concentration is presented in Fig. 5. Relative expression of *nifH* was calculated as a percentage of the maximum *nifH* expression, which was exhibited by Sp7-S in each experiment. The expression of Sp7 *nifH* decreased rapidly to almost a minimum value at 5% oxygen, whereas Sp7-S *nifH* was still expressed at a relatively high rate at 5% and did not reach a minimum until the oxygen concentration was between 10–20%. At its optimum expression, the *nifH* of Sp7-S was more than twice as high as Sp7 *nifH*. This higher expression over a broader range of oxygen concentrations with Sp7-S is consistent with the protected mode of colonization [13, 24].

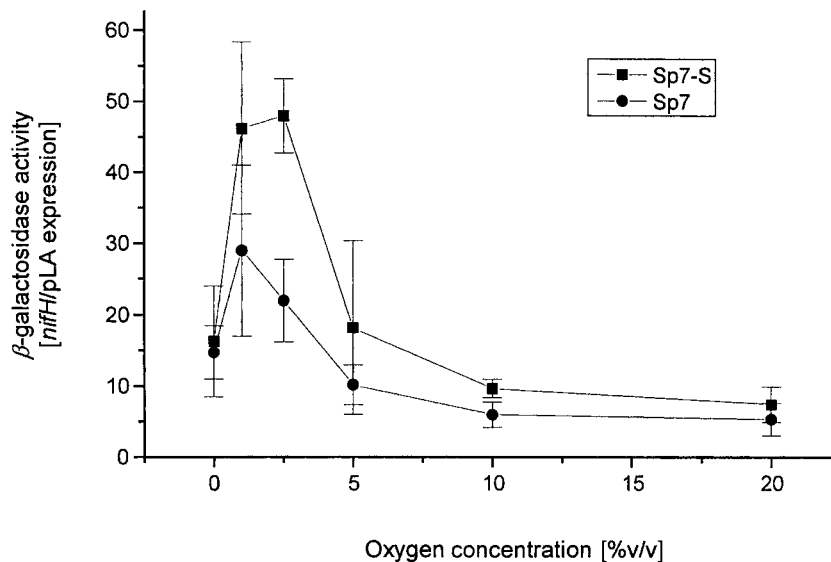


Fig. 6. Relative *nifH* expression of *A. brasilense* Sp7 and Sp7-S on *para*-nodulated wheat relative to pLA-*lacZ* expression for each strain. The results are averages of five plants from four experiments (*nifH*) and five plants from two experiments (pLA); 95% confidence intervals are indicated by error bars.

Expression of nifH-lacZ Relative to Bacterial Number

As bacterial number will clearly affect *nifH* expression by creating localized areas of low oxygen pressure through greater respiratory activity, it is necessary to express *nifH* activity relative to a measure of bacterial number. pLA-*lacZ* expression, in this system, does not change significantly over the range of oxygen concentrations (data not shown). As pLA-*lacZ* is constitutively expressed, it is an indication of bacterial numbers. A high correlation was illustrated by ARSÈNE *et al.* [12]. Although the average

number of Sp7-S appeared to be higher than that of Sp7, this difference was not statistically significant. Sp7-S exhibits significantly higher relative expression of *nifH* than Sp7 at 2.5% oxygen and although 95% confidence intervals do not always indicate significance, the average expression is consistently higher at other oxygen concentrations. Each result is expressed as a ratio of *nifH* expression to the average pLA-*lacZ* expression of each strain. The average pLA-*lacZ* expression for Sp7 was lower (602 MILLER units/mg protein/min) than that of Sp7-S (852 MILLER units/mg protein/min) in this experiment.

The higher average relative expression of Sp7-S at 2.5%, 5% and 10% oxygen suggests that this strain exhibits greater *nifH* expression per bacterium than Sp7, indicating better nitrogen fixing conditions in the niche that it occupies. This improved potential for nitrogen fixation may be due to a number of factors: (I) physical protection from oxygen by a more endophytic mode of colonization; (II) better access to carbon substrates resulting in higher respiratory rates (thus reducing oxygen concentration further and increasing ATP production to drive nitrogen fixation); and (III) when Sp7 and Sp7-S are in association with plants they differ in cell morphology. The encysted form of Sp7 may have a decreased metabolism, thereby restricting ATP synthesis which is less likely in the vegetative form of Sp7-S [21]. Encystation was suggested to be a physiological device to assist in survival [25, 26]. It is likely that the improved plant-associated *nifH* expression in Sp7-S is due to a combination of these factors rather than any one in particular.

Conclusions

The primary level of control of nitrogen fixation is the expression of the structural genes encoded in *nifHDK*. If these are not activated, then nitrogenase activity will not occur. Oxygen limits nitrogen fixation by repressing the genetic expression of *nifH*, as well as inactivating the nitrogenase enzyme.

The results presented here are consistent with the pattern of nitrogenase inhibition demonstrated by HARTMANN and BURRIS [5] using ARA and analysis of the nitrogenase enzyme. It was shown that inactivation by covalent modification occurred in anaerobiosis and when the oxygen concentration was increased from 1% to 10% in pure culture. This is consistent with the fact that although oxygen inhibits nitrogen fixation, a small amount is necessary in order to produce enough ATP for the nitrogenase reaction.

The relationship of *nifH* expression to nitrogenase activity is demonstrated and the oxygen dependence of *nifH* is confirmed. A high correlation between *nifH* and nitrogenase activity (ARA) was found. Hence, *nifH* expression should be a good indication of nitrogenase activity under these conditions. VANDE BROEK *et al.* [7] made use of a *nifH-gusA* fusion as an indication of oxygen tolerance of various diazotrophs and *A. brasilense* was classified in a group of organisms with a relatively low degree of oxygen tolerance. Supporting this, the results presented here indicate a maximum oxygen concentration for expression of *nifH* in axenic cultures of *A. brasilense* Sp7 and Sp7-S of 0.5%.

There was no significant difference in oxygen tolerance between *A. brasilense* Sp7 and Sp7-S in pure culture. This result was expected, as phenotypic differences in the two strains are not apparent unless specialized medium is used [14]. As phenotypic differences occur in the mode of colonization of wheat roots by the two strains [13], differences in oxygen tolerance are expected when *A. brasilense* Sp7 and Sp7-S are in association with the plant.

A system was developed that was capable of growing plants in a sterile aerated environment sufficient to repress initial *nifH* expression (as judged by β -galactosidase activity) and allow a relationship between oxygen concentration and plant-associated *nifH* expression to be determined. A constitutive pLA-*lacZ* fusion, not affected by oxygen pressure, was sufficient to show that bacterial numbers are sustained in this system. A high correlation between pLA-*lacZ* expression and bacterial numbers was demonstrated by ARSÈNE *et al.* [12].

Comparison of the two strains, contrasting in their mode of colonization of wheat roots, has resulted in different patterns of *nifH* expression over a range of oxygen concentrations. VANDE BROEK *et al.* [27] demonstrated that *nifH* activity could be observed in bacteria colonizing the surface of wheat roots and these results were consistent with a low oxygen concentration. An oxygen concentration gradient in a static hydroponic system is clearly sufficient to derepress *nifH* activity. Clearly, a protected mode of colonization results in even steeper oxygen gradients, allowing for greater expression of *nifH* over a wider range of external oxygen concentrations such as that exhibited by Sp7-S.

Finally, the oxygen sensitivity of the *nifH-lacZ* fusion is clearly useful in studying the potential for nitrogen fixation in associations between *A. brasilense* and wheat. This biotechnology may be useful to probe various media, including soil, for conditions that may support nitrogen fixation.

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References

- [1] KENNEDY, I. R., TCHAN, Y. T.: Biological nitrogen fixation in non-leguminous field crops: Recent advances. *Plant Soil* **141** (1992), 93–118.
- [2] FÜ, A. H., HARTMANN, A., LOWERY, R. G., FITZMAURICE, W. P., ROBERTS, G. P., BURRIS, R. H.: Posttranslational regulatory system for nitrogenase activity in *Azospirillum* spp. *J. Bacteriol.* **171** (1989), 4679–4689.

- [3] MERRICK, M. J.: Regulation of nitrogen fixation genes in free-living and symbiotic bacteria. In: Biological Nitrogen Fixation. (STACEY, G., BURRIS, R. H., EVANS, H. J., eds.). New York: Chapman and Hall, 1992, 835–876.
- [4] ELMERICH, C., DE ZAMAROCZY, M., ARSÈNE, F., PEREG, L., PAQUELIN, A., KAMINSKI, A.: Regulation of *nif* gene expression and nitrogen metabolism in *Azospirillum*. *Soil Biol. Biochem.* **29** (1997), 847–852.
- [5] HARTMANN, A., BURRIS, R. H.: Regulation of nitrogenase activity by oxygen in *Azospirillum brasilense* and *Azospirillum lipoferum*. *J. Bacteriol.* **169** (1987), 944–948.
- [6] LIANG, Y. Y., DE ZAMAROCZY, M., KAMINSKI, P. A., ELMERICH, C.: Regulation of *nif* gene expression in *Azospirillum brasilense* Sp7. *Symbiosis* **13** (1992), 307–315.
- [7] VANDE BROEK, A., KEIJERS, V., VANDERLEYDEN, J.: Effect of oxygen on the free-living nitrogen fixation activity and expression of the *Azospirillum brasilense nifH* gene in various plant-associated diazotrophs. *Symbiosis* **21** (1996), 25–40.
- [8] BERGERSEN, F. J.: Oxygen and the physiology of diazotrophic microorganisms. In: Advances in Nitrogen Fixation Research. (VEEGER, C., NEWTON, W., eds). The Hague: Nijhoff/Junk, 1984, 171–180.
- [9] ZHULIN, I. B., ARMITAGE, J. P.: Motility, chemokinesis, and methylation-independent chemotaxis in *Azospirillum brasilense*. *J. Bacteriol.* **175** (1993), 952–958.
- [10] GRISHANIN, R. N., CHALMINA, I. I., ZHULIN, I. B.: Behaviour of *Azospirillum brasilense* in a spatial gradient of oxygen and “redox” gradient of an alternative electron acceptor. *J. Gen. Microbiol.* **137** (1991), 2781–2785.
- [11] MICHIELS, K., CROES, C. L., VANDERLEYDEN, J.: Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. *J. Gen. Microbiol.* **137** (1991), 2241–2246.
- [12] ARSÈNE, F., KATUPITIYA, S., KENNEDY, I. R., ELMERICH, C.: Use of *lacZ* fusions to study the expression of *nif* genes of *Azospirillum brasilense* in association with plants. *Mol. Plant-Microbe Interact.* **7** (1994), 748–757.
- [13] KATUPITIYA, S., MILLET, J., VESK, M., VICCARS, L., ZEMAN, A., LIDONG, Z., ELMERICH, C., KENNEDY, I. R.: A mutant of *Azospirillum brasilense* Sp7 impaired in flocculation with a modified colonization pattern and superior nitrogen fixation in association with wheat. *Appl. Environ. Microb.* **61** (1995), 1987–1995.
- [14] PEREG-GERK, L., PAQUELIN, A., GOUNON, P., KENNEDY, I. R., ELMERICH, C.: A transcriptional regulator of the *LuxR-UhpA* family, FlcA, controls flocculation and wheat root surface colonization by *A. brasilense* Sp7. *Mol. Plant-Microbe Interact.* **VII** (1997), 177–187.
- [15] TCHAN, Y. T., KENNEDY, I. R.: Possible N₂-fixing root nodules induced in non-legumes. *Agric. Sci. (AIAS Melbourne)* **2** (1989), 57–59.
- [16] KATUPITIYA, S., NEW, P. B., ELMERICH, C., KENNEDY, I. R.: Improved N₂ fixation in 2,4-D treated wheat roots associated with *Azospirillum lipoferum*: Studies of colonization using reporter genes. *Soil Biol. Biochem.* **27** (1995), 447–452.
- [17] SRISKANDARAJAH, S., KENNEDY, I. R., YU, D., TCHAN, Y. T.: Effects of plant growth regulators on acetylene-reducing associations between *Azospirillum brasilense* and wheat. *Plant Soil* **153** (1993), 165–178.
- [18] MILLER, J. H.: Assay of β -galactosidase. In: Experiments in Molecular Genetics. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press, 1972, 352–355.
- [19] TURNER, G. L., GIBSON, A. H.: Measurement of nitrogen fixation by indirect means. In: Methods for Evaluating Biological Nitrogen Fixation (BERGERSEN, F. J., ed.). John Wiley and Sons, 1980, 111–138.
- [20] ZEMAN, A. M. M., TCHAN, Y. T., ELMERICH, C., KENNEDY, I. R.: Nitrogenase activity in wheat seedlings bearing *para*-nodules induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and inoculated with *Azospirillum*. *Microbiol Res.* **143** (1992), 847–855.
- [21] PEREG-GERK, L., GILCHRIST, K., KENNEDY, I. R.: Mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense*-wheat associations. *Appl. Environ. Microb.* **66** (2000), 2175–2184.

- [22] LIANG, Y. Y., KAMINSKI, P. A., ELMERICH, C.: Identification of a *nifA*-like regulatory gene of *Azospirillum brasilense* Sp7 expressed under conditions of nitrogen fixation and in the presence of air and ammonia. *Mol. Microbiol.* **5** (1991), 2735–2744.
- [23] DÖBEREINER, J., DAY, J. M.: Associate symbioses in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites. In: Proceedings of the 1st International Symposium on Nitrogen Fixation. (NEWTON, W. E., NYMAN, C. J., eds.). Washington: Washington State University Press, Pullman. **II** (1976), 518–538.
- [24] KENNEDY, I. R., PEREG-GERK, L. L., WOOD, C., DEAKER, R., GILCHRIST, K., KATUPITTIYA, S.: Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat. *Plant Soil* **194** (1997), 65–79.
- [25] SADASIVAN, L., NEYRA, C. A.: Cyst production and brown pigment formation in aging cultures of *Azospirillum brasilense* ATCC 29145. *J. Bacteriol.* **169** (1987), 1670–1677.
- [26] TAL, S., OKON, Y.: Production of the reserve material poly- β -hydroxybutarate and its function in *Azospirillum brasilense* Cd. *Can. J. Microbiol.* **31** (1985), 608.
- [27] VANDE BROEK, A., MICHIELS, J., VAN GOOL, A., VANDERLEYDEN, J.: Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacterial *nifH* gene during association. *Molecular Plant Microbe In.* **6** (1993), 592–600.
- [28] SIMON, R., PRIEFER, U., PÜHLER, A.: A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in GRAM-negative bacteria. *Bio/Technology* **1** (1983), 784–791.
- [29] TARRAND, J. J., KRIEG, N. R., DÖBEREINER, J.: A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov., and two species, *Azospirillum lipoferum* (BEIJERINCK) comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.* **24** (1978), 967–980.