

## Nitrogen fixation in *para*-nodules of wheat roots by introduced free-living diazotrophs

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### Abstract

Nitrogen-fixation ( $C_2H_2$ -reduction) was demonstrated in wheat root nodules (*p*-nodules) induced by 2,4-dichlorophenoxyacetate (2,4-D) and inoculated with *A. brasilense*. By lowering the  $O_2$  tension it was possible to distinguish the nitrogenase activity of bacteria located within the *p*-nodule of the wheat root system from that in the rhizosphere. Using cytological evidence, nitrogenase activity was attributed mainly to be coming from the bacteria within the *p*-nodule. It was also shown that the host plant was able to supply the necessary substrate required for the bacterial  $N_2$ -fixation ( $C_2H_2$ -reduction) within the *p*-nodules.

### Introduction

The role of free living diazotrophs in the nitrogen economy has been discussed recently (Tchan, 1988). The difficulties in obtaining a genetically active *nif* gene in non-legumes prevented fast progress in this direction. Alternatively, introducing a free-living diazotroph with its fully equipped *nif* gene into non-legumes would avoid the difficulties involved in genetic manipulation. Giles and Whitehead (1977) reported the introduction of *Azotobacter* into the mycorrhizal system as a means of fixing  $N_2$  for a woody plant (*Pinus*). Its validity, however, has been challenged by Terzachi and Christensen (1986).

Nie et al. (1980) reported that nodule like structures induced by 2,4-D can be inhabited by diazotrophs. However, using rhizobia and *Azotobacter*, Nie et al. did not provide convincing evidence of nitrogen fixation in the living nodule. This work was recently reviewed by Tchan and Kennedy (1989).

The term *p*-nodule (*para*-nodule) was intro-

duced by Tchan and Kennedy to describe the chemically induced nodule since it differs from the naturally occurring legume nodule (Kennedy et al., 1990).

Although the formation of *p*-nodules using 2,4-D could be reproduced (Bender et al., 1990; Tchan and Kennedy, 1989) nitrogenase activity in non-legumes using rhizobia was not detected (Bender et al., 1990).

Similarly, Al-Mallah et al. (1989), Benson et al. (1990), and Cocking et al. (1990) were able to induce nodule formation at a low frequency using enzyme treatment in non-legumes, but like Jing et al. (1990) who used a mutant of *R. sesbania* to induce nodulation on rice roots, obtained only low nitrogenase activity in their systems.

Cellular manipulation to introduce a complete  $N_2$ -fixing system into non-legumes is of little value to the nitrogen economy (Patriquin, 1982) unless such introduced organisms can express their nitrogenase activity. Significant  $N_2$ -fixation had not yet been demonstrated in any previous work on induced nodules.

This paper describes positive N<sub>2</sub>-fixation with *A. brasilense* in the *p*-nodules of the wheat root system.

## Materials and methods

### *Bacterial culture, plant host and induction of p-nodules*

Wheat seeds (cultivar Miskle) were surface sterilized using 0.5% HgCl<sub>2</sub> for 2.5 min. The seeds were germinated on potato-malate agar at 25°C for 2–5 days. Uncontaminated seedlings were transferred and grown in sterile hydroponic solution at 25°–30°C under continuous lighting. When the roots of the wheat plant were 5–7 cm in length, 0.1 mL of a 24 hour culture of *Azospirillum brasilense* containing 10<sup>6</sup> to 10<sup>7</sup> cells mL<sup>-1</sup> and a sufficient quantity of a sterile 2,4-D solution was added to a final concentration of 0.5–1 ppm (2,4-D). After 7 to 10 days, *p*-nodules were well formed on the wheat root system and ready for experimentation (see Fig. 1a).

### *Nitrogen fixation (C<sub>2</sub>H<sub>2</sub> reduction)*

The acetylene reduction assay (ARA) was used to test for the N<sub>2</sub>-fixing capacity of the *p*-nodules using 4–8 plants per flask. The flasks contained 10 ml of a 0.1% glucose solution or when the sugar was omitted, they were exposed to a light source. The O<sub>2</sub> tension in the flasks was reduced to that of 10% of air and they were then either shaken or not. A Shimadzu GC8A gas chromatograph fitted with a flame ionisation detector and a 1 metre column of Porapak T (Walters Millipore) was used for measurement of ethylene production.

### *Cytological examination*

Nodules formed on wheat roots by the 2,4-D/*Azospirillum* system were incubated with iodinitrotetrazolium (INT) or 2,3,5 triphenyltetrazolium (TPT) to locate sites of strong reduction. Such sites were detached, crushed or sectioned and examined using light phase micro-

scopy. Sites on the root that showed no visible reduction were similarly examined.

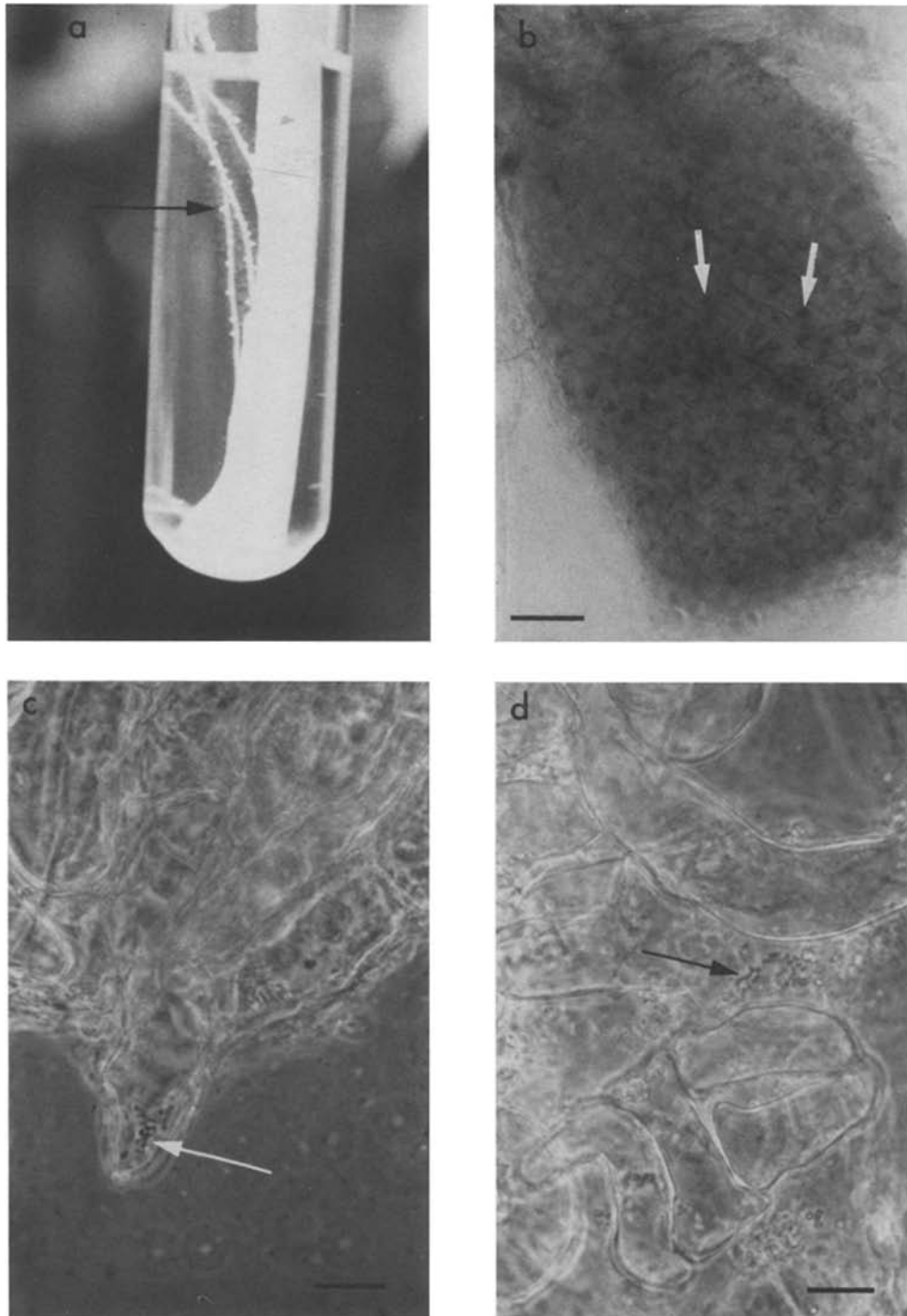
## Results and discussion

When the 2,4-D/*Azospirillum* wheat root system was first tested for ethylene production, only one plant per flask was used and no change in the flask's atmosphere was made, producing negative or erratic results. This led to two proposals: (1) that the normal O<sub>2</sub> tension of the air was too high for the nitrogenase of *A. brasilense* to operate efficiently and (2) that a single seedling may not provide adequate ethylene production for analysis. It was also necessary to distinguish ethylene produced by the azospirilla inside the plant root system including the *p*-nodule from the azospirilla located in the rhizosphere.

By shaking the flasks during the ARA incubation period and reducing the O<sub>2</sub> tension to 10% of air, the nitrogenase activity attributable to the azospirilla in the rhizosphere was greatly reduced. Yet under these conditions, the expression of nitrogenase inside the *p*-nodules was protected by the plant tissue. The number of plants tested in the ARA was also increased from one to 4 to 8 seedlings per flask, providing a more accurate estimation of ethylene production. The 0.1% glucose solution used in the flasks was to ensure that nitrogenase activity was not limited by the possible lack of energy supply during our assay. The suppression of nitrogenase activity in the shaken flask compared to the unshaken flask was demonstrated using a culture of *A. brasilense* (Fig. 2).

The shaken flask gave a value of 1.9 nmoles h<sup>-1</sup> of ethylene compared to the unshaken flask with 24.3 nmoles h<sup>-1</sup>.

By comparing the ARA results of plant roots not inoculated with 2,4-D under shaken and unshaken conditions, a small amount of ethylene was produced by the azospirilla residing within the root system. In the presence of 2,4-D, however, the N<sub>2</sub>-fixation capacity of the shaken flasks increased five fold indicating that only the azospirilla protected from oxygen within the plant cells were able to express their nitrogenase activity. Furthermore, the presence of 2,4-D



*Fig. 1. a, p-nodules on wheat root induced by 2,4-D; b, p-nodule stained with INT showing reduction sites (arrow) within nodule cells (bar = 250  $\mu$ m); c, Azospirilla within cells of nodule (bar = 20  $\mu$ m) and d, Azospirilla located intercellularly (bar = 20  $\mu$ m).*

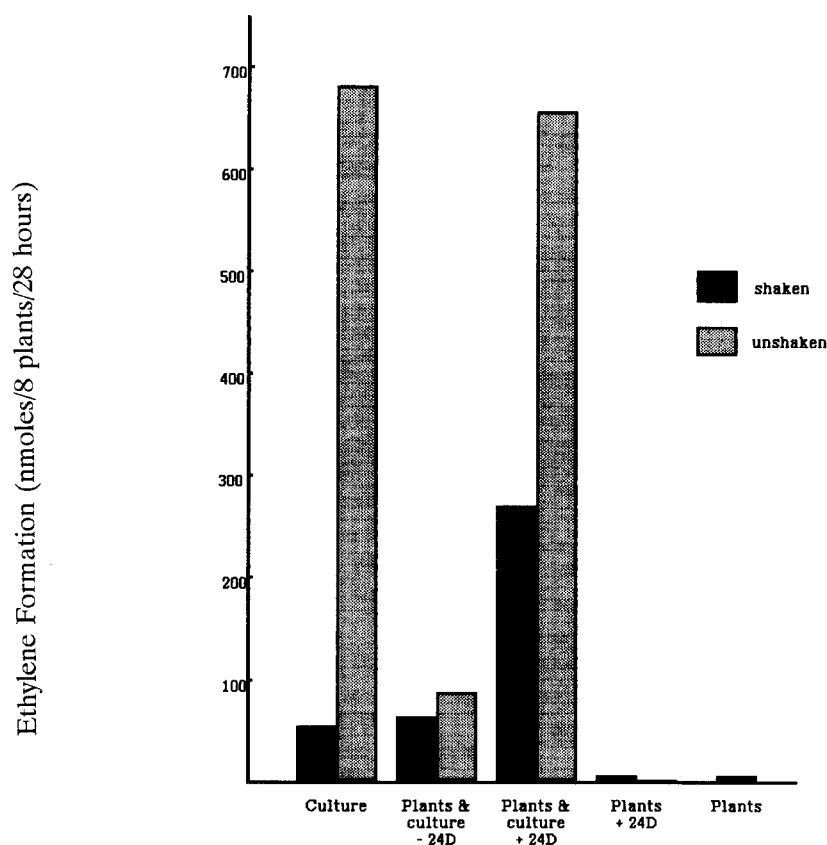


Fig. 2.  $N_2$  fixation ( $C_2H_2$ -reduction) by *Azospirillum brasilense* in *p*-nodules of wheat plant roots.

considerably enhanced the nitrogenase activity of azospirilla within the rhizosphere.

Cytological investigations revealed that there were more azospirilla present within the *p*-nodule than in the root system. This indicates that the major part of ethylene production came from the azospirilla activity within the *p*-nodules (Fig. 1 b,c,d).

The data strongly suggested that *A. brasilense* in the *p*-nodule was potentially capable of fixing  $N_2$  provided that adequate requirements and environmental conditions were fulfilled. Such potential for  $N_2$ -fixation from the 2,4-D treatment would have a greater significance if the required energy could be supplied by the host plant. This was tested by keeping the wheat seedlings in the dark for 18 hours prior to the ARA and detaching their seeds to reduce the

supply of substrate to a minimum. The glucose in the solution was eliminated and the shaken flasks were exposed to a light source. Flasks containing plants not treated with 2,4-D were used as controls (Fig. 3).

The results showed that wheat plants with *p*-nodules (+2,4-D) produced 9.3 nmoles of ethylene per plant per hour compared to 2.3 nmoles of ethylene obtained in plants not treated with 2,4-D. This indicated that the host plant was capable of supplying the necessary substrate for  $N_2$ -fixation ( $C_2H_2$ -reduction) of the bacteria residing mainly in the *p*-nodule.

The demonstration of significant  $N_2$ -fixation in the wheat plant using the 2,4-D/*Azospirillum* system is an important development which may open new fields for both basic and applied aspects of  $N_2$ -fixation.

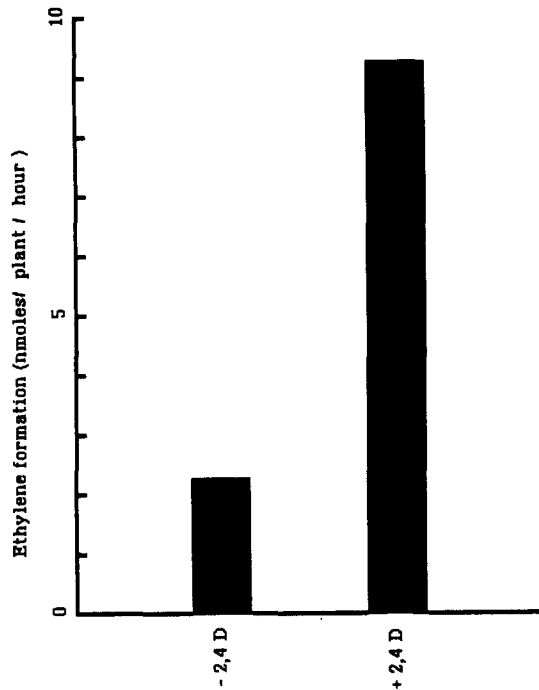


Fig. 3. *N*<sub>2</sub>-fixation (*C*<sub>2</sub>*H*<sub>2</sub>-reduction) by *Azospirillum brasilense* in *p*-nodules of wheat plants using energy supplied by the host plant.

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