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Nitrogenase activity in wheat seedlings bearing *para*-nodules induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and inoculated with *Azospirillum*

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SUMMARY

Nitrogenase activity (C_2H_2 reduction) was demonstrated in seedlings of wheat roots bearing *para*-nodules induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and inoculated with *Azospirillum brasilense*. Increased nitrogenase activity was observed in inoculated *para*-nodulated seedlings as compared to inoculated roots not treated by 2,4-D under the conditions of assay used. 2,4-D had no stimulating effect on plant ethylene production in the absence of acetylene. When inoculation was performed with a Nif$^-$ mutant of *A. brasilense*, no ethylene production was detected. It was also shown that the energy source required for nitrogenase activity was supplied by the host plant.

Key-words: Nitrogenase, Nitrogen fixation, Nodule, *Azospirillum;* 2,4-D, Wheat, *Para*-nodule, Ethylene production.

INTRODUCTION

Symbiotic nitrogen fixation is restricted to relatively few families of higher plants, mostly associated with rhizobia, *Frankia* and cyanobacteria. In the monocotyledons, including the major crops and economically important plants (such as wheat, rice, maize and pasture grasses), symbiotic nitrogen fixation does not occur naturally. The difficulty involved in introducing functional bacterial nitrogen fixation (*nif*) genes into plants has prevented rapid progress in this direction (Merrick and Dixon, 1984; Tchan, 1988; Steinbiss and Broughton, 1988). Protocols have been developed to induce the formation of bumps, tentatively referred to as nodule-like structures, pseudonodules or *para*-nodules, on the roots of several non-legume plants, including rice, wheat, barley and oil-seed rape (for reviews, see Tchan and Kennedy, 1989; Cocking *et al.*, 1990; de Bruijn and Downie, 1991; Kennedy and Tchan, 1992).

Nie *et al.* (1979) first discovered that "nodule-like" structures, could be induced by 2,4-dichlorophenoxyacetic acid (2,4-D) on plant roots and that they could be colonized by microorganisms including diazotrophs (rhizobia and *Azotobacter*). These experiments have been reproduced (Tchan and Kennedy, 1989; Bender

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et al., 1990; Rolfe *et al.*, 1991a, b). Staining with tetrazolium salts indicated sites of reduction within these structures (Tchan and Kennedy, 1989). Enzyme treatment experiments together with inoculation of rhizobia to induce nodule formation on rice, wheat and other plants, led only to a very low frequency of nodule-like structure formation and failed to demonstrate nitrogenase activity (Al Mallah *et al.*, 1989; Cocking *et al.*, 1990).

This paper reports the combined use of 2,4-D and *Azospirillum*, a rhizospheric and associative diazotroph that normally lacks the ability to form nodules on host plants, to establish a new nitrogen-fixing system using wheat as the host. Significant nitrogenase activity (C_2H_2 reduction/ethylene production) was found in this system. Part of this work has been reported at international conferences (Kennedy *et al.*, 1990, 1991; Tchan *et al.*, 1991; Zeman *et al.*, 1991).

MATERIALS AND METHODS

Plant tubes

A total of 15 ml of nitrogen-free hydroponic solution were added to 15 mm diameter test tubes containing a piece of filter paper to support the wheat seedling. The hydroponic solution contained: KH_2PO_4 40 μM, K_2SO_4 80 μM, $CaCl_2$ 40 μM, $MgSO_4$ 60 μM, $MnSO_4$ 4 μM, and, per liter, Fe-chelate 10 mg, H_3BO_3 120 μg, $ZnSO_4{\cdot}7H_2O$ 46 μg, $Na_2MoO_4{\cdot}2H_2O$ 10 μg, $CuSO_4{\cdot}5H_2O$ 15 μg, $CoCl_2{\cdot}6H_2O$ 4 μg. The pH was adjusted to 6.8 with K_2HPO_4 0.1 M solution. The tube was covered by another test tube of a slightly larger diameter (fig. 1, part 1) and sterilized by autoclaving.

Plant host

Seeds of wheat (*Triticum aestivum*) cultivar Miskle (Sun 110 C) were first wetted with detergent and rinsed five times with distilled water under a vacuum/air cycle prior to surface sterilization. After two-min treatment with 0.5% $HgCl_2$, the seeds were rinsed as described above and germinated as previously reported (Tchan *et al.*, 1991). Uncontaminated seedlings were grown in the plant tubes in a controlled environment, under constant light (200 $\mu E \cdot m^{-2} \cdot sec^{-1}$, with alternate cycles at 18 and 23°C.

Bacterial strains

Azospirillum brasilense Sp7 ATCC 29145 was a gift from Dr. A.H. Gibson. It was grown in nitrogen-free Nfb malate liquid medium (Krieg and Döbereiner, 1984). *A. brasilense* 7571, is a Nif$^-$ derivative of strain Sp7, impaired in Mo-nitrogenase *nifD* or *nifK* structural gene, which was isolated after ethyl methane sulphonate mutagenesis (Jara *et al.*, 1983); 1% potato extract was added to Nfb medium to grow the strain 7571.

Induction and inoculation of *para*-nodules

Addition of different doses of 2,4-D and inoculation with 0.1 ml of a 24-h culture (routinely 10^6 to 10^7 cells/ml) of *A. brasilense* Sp7 were performed when the root system was approximately 5-7 cm in length. Uninoculated plant tubes containing 2,4-D and inoculated plant tubes devoid of 2,4-D were used as controls.

Nitrogenase assays

Nitrogenase activity was assayed by the acetylene reduction test (ARA) with a "Shimadzu GC8A" gas chromatograph fitted with a flame ionisation detector and a 1-meter column of "Porapak T" (Waters-Millipore).

Plants were grown and inoculated as indicated above. ARA were performed on 12-day old seedlings. Early experiments were carried out with 4 to 8 plants (Tchan *et al.*, 1991); however, for statistical analysis, individual seedlings were tested as follows. Each wheat seedling which had its remaining seed detached aseptically and its roots washed in sterile nitrogen-free Winogradsky mineral solution was transferred to a 30-ml McCartney bottle containing 3 ml of nitrogen-free Winogradsky mineral solution (Tchan and New, 1984). The washing of the roots and detachment of the seeds was done to eliminate soluble substrate that could be carried over by the roots when being transferred. McCartney bottles were stopped with sterile rubber stoppers and incubated at 30°C in a water bath in either a shaken (160 rev/min)

ARA = acetylene reduction (test).
2,4-D = 2,4-dichlorophenoxyacetic acid.
INT = iodonitrotetrazolium.
TPT = 2,3,5-triphenyltetrazolium.

or still position under continuous illumination. The atmosphere in the vials was 87.5% nitrogen, 2.5% oxygen and 10% acetylene. ARA were performed after 24-h incubation. This protocol was applied both to the wild type strain and to mutant 7571.

Nitrogenase activity of free-living bacteria in the presence of different doses of 2,4-D was monitored as follows: 1 ml of a 24-h culture (containing approximately 1.5×10^6 cells) of *A. brasilense* was added to a series of 30-ml vials containing 1 ml of nitrogen-free medium; 2,4-D was added to the vials when required. The vials were evacuated and the atmosphere was replaced by argon. Air was introduced to produce 0.004 atm. oxygen. The vials were then injected with C_2H_2 to a final concentration of 10% and incubated in a Warburg bath at 30°C. ARA were carried out after 6-h incubation.

Tetrazolium salt staining

Para-nodulated seedlings were incubated with a solution of 0.025% of iodonitrotetrazolium (INT) or 2,3,5-triphenyltetrazolium (TPT) to localize the sites of strong reduction. Such sites were detached, crushed and examined by phase-contrast light microscopy (Olympus Series BH). Thin sections of these sites were also made and examined. The sites on the roots that showed no visible reduction were similarly examined.

RESULTS AND DISCUSSION

Effect of 2,4-D on the root system

The plant tube system used in our experiments (fig. 1, part 1) has several advantages. Firstly, the shadow effect created by the traditional cotton wool plug has been eliminated, thus providing better illumination. Secondly, the outer tube can easily be lifted up to accommodate the increase in the size of the plants during growth without the need to remove it completely, thus reducing the risk of contamination.

The concentration of 2,4-D influenced the growth of the wheat seedlings and *para*-nodule formation, as reported in table I. There was no significant difference in terms of root development and *para*-nodule formation between inoculated and uninoculated plants with *Azospirillum* (data not shown). At 10 ppm of 2,4-D, all seedlings died in less than two weeks. At 5 ppm, symptoms of disorder were apparent. The variations in number of *para*-nodules (per plant) within the range of 0.50-2.50 ppm were too wide to precisely determine the best concentration of 2,4-D. The concentration of 1.0 ppm was adopted for general use, a concentration recommended by Nie *et al.* (see also Tchan and Kennedy, 1989). It was also noted that better results were obtained by adding 2,4-D after the root system had reached a length of 5-7 cm, since more space was available for nodulation and more *para*-nodules were formed.

Site of infection

For general experimentation, 0.1 ml of a young culture containing 10^6-10^7 cells/ml was used. The whole *para*-nodule structure was stained red with tetrazolium salts indicating a site of strong reduction. INT gave faster reduction than TPT. This observation suggested that *Azospirillum* colonized the structure and found a site of low redox potential, possibly better suited for nitrogen fixation (fig. 1, parts 2 and 3). Using light microscopy, large numbers of bacteria were found associated with the *para*-nodules (Tchan *et al.*, 1991). These observations were substantiated by using resin embedding, thin and ultrathin sections of *para*-nodulated tissues. Substantial numbers of azospirilla were located intercellularly (fig. 1, part 4; fig. 2A, B, C) at the junction of the *para*-nodule with the root tissue. Bacteria were also observed in the main parts of the root *para*-nodule (fig. 2C). By contrast, root tissue of plants not treated with 2,4-D showed only a few bacteria located mainly in the cortex zone (fig. 2D, E). *Azospirillum* cells have also been observed on the surface of the *para*-nodules. Further experiments are required to decide whether the bacteria multiply within the *para*-nodules or simply at the junction between the *para*-nodules and the roots.

Azospirillum Nif$^-$ mutant 7571 cells have been observed in *para*-nodulated seedlings, although the actual number of bacteria was lower than with the wild-type. This was expected, due to the inability of this strain to multiply effectively in an environment limited in combined nitrogen. Details of cytological studies will be reported elsewhere.

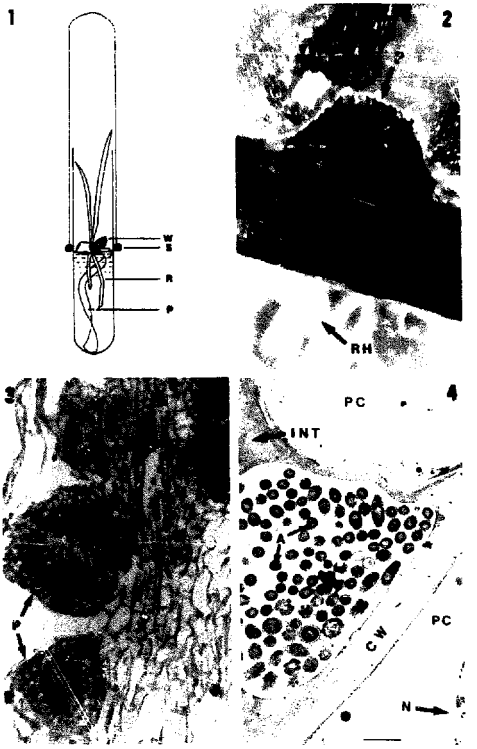

Fig. 1. Representation of the hydroponic system and examination of *para*-nodules.

1) Schematic representation of the hydroponic system used for plant growth, p = filter paper strip to support seed; r = roots; s = support for outer tube; w = wheat seed.

2) *Para*-nodule (P) of wheat root stained with tetrazolium. RH = root hair. Total magnification: × 14.

3) Section of wheat root treated with 2,4-D and inoculated with *A. brasilense* Sp7. p = *para*-nodules. Total magnification: × 14.

4) Transmission electron micrograph of *para*-nodule showing *Azospirillum* cells in intercellular space. A = *Azospirillum* cells; CW = plant cell wall; INT = intercellular space; N = nucleus of plant cell; PC = plant cell. Bar = 2.5 μm.

Table I. Influence of 2,4-D concentration on the development of wheat seedlings and *para*-nodule formation.

2,4-D concentration (ppm)	Length of main root (L) (cm)	No. of *para*-nodules (n) (main root)	n/L	Length of shoot (cm)
0	32.6	–	–	18.7
0.25	6.5	11	1.7	17.0
0.5	10.0	22	2.2	22.0
1.0	9.3	15	1.6	16.9
2.0	6.5	20	3.0	14.5
2.5	8.0	21	2.6	16.5
5.0	3.0	14	4.6	13.0
10.0	–	–	–	–

Measurements are made after 14 days of growth. Data represented here were obtained with uninoculated seedlings using only the main root for the measurements. When lateral roots were included, the total number of *para*-nodules per seedling was approximately 50. Very similar results were obtained with inoculated seedlings.

Oxygen tension and energy supply for nitrogen fixation

As azospirilla were found inside as well as outside the roots and *para*-nodules, it was essential to differentiate the ethylene produced by the bacteria located on the surface of the root and *para*-nodules from the ethylene produced within the plant. It was previously reported that *Azospirillum* in the free-living state can only fix nitrogen under microaerobiosis. Indeed, nitrogenase activity, which was maximal at 0.004 atm., was totally abolished under shaking conditions at 0.02 atm. O_2 (Tchan *et al.*, 1991). This oxygen tension was increased to 0.025 atm. to perform acetylene reduction determinations with the *para*-nodulated plantlets. This was chosen to ensure that nitrogenase activity from outside the plant system was excluded. In early experiments using shaken and unshaken vials, it was repeatedly observed that acetylene reduction was greater with unshaken vials (Tchan *et al.*, 1991). It was assumed that ethylene produced in the shaken vials containing wheat seedlings arose mainly from the activity of the bacteria inside the plant. The unshaken vials provided the total acetylene reduction value produced by azospirilla situated inside and outside the plant roots. Moreover, it was also found that exposure to continuous lighting under shaking conditions led to a substantial increase in the nmoles of ethylene produced per hour per plant (Tchan *et al.*, 1991).

These results were substantiated using a single plant in each reaction vessel for each treatment, and statistically analysed. Table II shows that plants inoculated with *Azospirillum* and treated with 2,4-D gave a mean value of 89.80 nmoles/plant/hour of ethylene compared to those inoculated with *Azospirillum* but lacking 2,4-D, which gave a mean value of 7.7 nmoles/plant/hour. In the absence of azospirilla, acetylene reduction was very low. It is worth mentioning that the oxygen concentration in the vial at the time of the assay varied between 2.5 and 5% due to possible infiltration of oxygen into the vial during the incubation period and to the photosynthesis of the seedling. Therefore, the increase in acetylene reduction of inoculated seedlings treated with 2,4-D could not be attributed to the reduction of oxygen tension during incubation. It is tempting to speculate that *para*-nodulated seedlings provide better oxygen protection, which might also be compatible with better proliferation of the bacteria. Since acetylene reduction is conducted without any added carbon source, the necessary energy supply for nitrogen fixation must come from the photosynthetic system of the host plant.

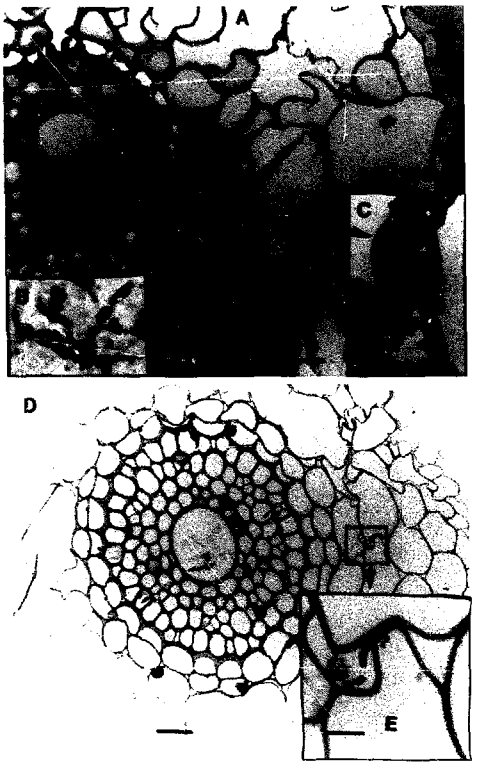

Fig. 2. (A to E). Light microscopy of roots of wheat seedlings grown under reduced oxygen tension.

A) Seedling treated with 2,4-D showing azospirilla in the intercellular spaces of plant cells between the root and *para*-nodule (PN) as well as in the main part of the *para*-nodule in (B) and (C). bar = 32 μm (A), 5 μm (B) and (C).

D) Root of wheat seedling not treated with 2,4-D showing azospirilla in the intercellular spaces of cortical cells. bar = 33 μm (D), 5 μm (E).

Correlation between ethylene production and nitrogenase activity and the effect of 2,4-D on acetylene reduction by *Azospirillum*

Tittle *et al.* (1990) demonstrated that ethylene was produced in a very small quantity (in the absence of added acetylene) when soybean and barley seedlings were treated with 1 mM 2,4-D (221 ppm). There are also reports of nitrogenase-independent ethylene production by some aerobic bacteria (Nagahama *et al.*, 1991). It was therefore important to ascertain whether the ethylene produced by the *para*-nodulated wheat system was the result of the activity of nitrogenase and was not due to an abnormal reaction of the plant in the presence of 2,4-D and azospirilla, or to nitrogenase-independent ethylene production by the bacteria. This was checked by comparing the ethylene production of *para*-nodulated wheat seedlings inoculated either with the wild type Nif^+ Sp7 strain or with the 7571 Nif^- mutant. Plants inoculated with strain 7571 did not show ethylene production (< 0.05 nmoles ethylene/h/plant), in contrast with those inoculated with the wild type strain (see table II). This constituted evidence that ethylene production was related to nitrogenase activity. In addition, ethylene production could be inhibited by NH_4^+ and NO_2^-, and ^{15}N could be detected in plant tissues after a relatively short exposure (Yu, personal communication).

Table II. Table of means of nitrogen fixation (C_2H_2 reduction) by the wheat-root system under different treatments.

Plant treatment	Ethylene formation n.moles/h/plant under shaking conditions
None	< 0.05
2,4-D	< 0.05
Sp7 only	7.70
Sp7 and 2,4-D	89.30

Measurements were made with 12-day old plants incubated under 0.025 atm. oxygen as described in Materials and Methods. Six replicate seedlings were used for each treatment. Analysis of variance was performed on log-transformed values. Differences between treatments of controls and each other were significant [$p < 0.05$].

Another control sought to determine the effect of 2,4-D on acetylene reduction in *Azospirillum* grown under free-living conditions. Martinez-Toledo *et al.* (1990) showed that 2,4-D at a concentration of 100 ppm had no effect on ethylene production by azospirilla. In contrast, Christiansen-Weniger (1988) indicated, that amongst plant growth substances, 2,4-D at a concentration of 2.5 ppm was capable of increasing ethylene production in *A. brasilense* by nearly 3-fold. The mean values obtained from several determination of acetylene reduction in the presence of different concentrations of 2,4-D are reported in figure 3. Up to 10 ppm, in spite of some dispersion in individual measurements, the line of best fit did not indicate any stimulation of ethylene production. On the contrary, a slight tendency to a decrease in acetylene reduc-

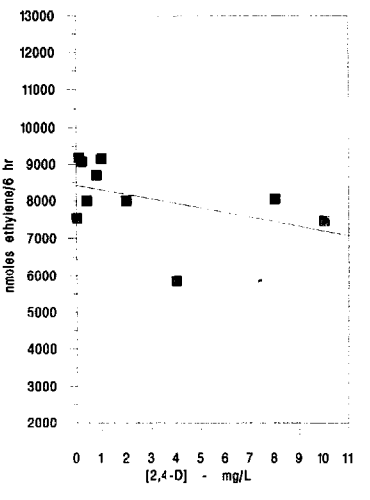

Fig. 3. Influence of 2,4-D on nitrogenase (ethylene formation) of a culture of *A. brasilense*.

Points are the mean values of three of five replicates for each concentration. The line of best fit is indicated.

tion with increasing 2,4-D concentrations was observed. This effect was stronger at higher concentrations (data not shown). Thus, the 3-fold increase in acetylene reduction reported by Christiansen-Weniger was not reproduced with the strain of *A. brasilense* used in our experiments.

Conclusion

A substantial increase in C_2H_2 reduction in plants treated with 2,4-D compared to those which were not was demonstrated. The following observations justify the conclusion that ethylene production is correlated with nitrogenase activity of bacteria located in *para*-nodulated seedlings: (1) *para*-nodulated plants inoculated with the wild-type strain displayed nitrogenase activity that was relatively insensitive to the inhibitory effect of oxygen; (2) *para*-nodulated non-inoculated plants, or plants inoculated with a Nif$^-$ mutant did not display nitrogenase activity; (3) the plant itself did not produce ethylene under our experimental conditions; (4) at the concentrations used, 2,4-D had practically no effect on *Azospirillum* nitrogenase activity. In addition, as the assay was carried out without any external carbon source, this suggests that the energy needed for nitrogenase activity must come from the host plant.

One of the perequisites for symbiosis is the establishment of nitrogen-fixing bacteria which accumulate in a protected niche provided by the plant. The experiments reported here provide an experimental model to identify limiting factors and the improve the association. Another major requirement is the beneficial transfer to the host plant of nitrogen fixed by the bacteria. Such a requirement could be realised by constructing genetically modified *Azospirillum* strains capable of secreting higher amounts of ammonia than the wild-type.

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Activité nitrogénase de structures induites sur les racines du blé par l'acide 2,4-dichlorophénoxyacétique et colonisées par *Azospirillum*

Des racines de blé portant des structures, appelées *para*-nodules, induites sur les racines du blé par l'acide 2,4-dichlorophénoxyacétique (2,4-D) peuvent être colonisées par *Azospirillum brasilense* Sp7, qui s'établit dans les espaces intercellulaires. Dans ces conditions, la plante exprime une activité nitrogénase mesurée par la réduction de l'acétylène. En jouant sur la tension partielle de l'oxygène ambiant qui, au-delà de 0.02 atm., inhibe totalement l'activité nitrogénase, on a pu montrer que cette activité est due majoritairement aux bactéries localisées dans les plantules *para*-nodulées, et minoritairement aux bactéries localisées à la surface des racines. Cette activité est très fortement stimulée par illumination de la plante, ce qui suggère que le système photosynthétique végétal est capable de fournir aux bactéries la source d'énergie nécessaire au fonctionnement de la nitrogénase. Différents contrôles ont permis d'exclure la possibilité que l'éthylène formé soit le produit d'un métabolisme particulier de la plante ou des bactéries, indépendant de la présence d'acétylène comme substrat. D'autre part, aux concentrations utilisées, le 2,4-D n'a pratiquement aucun effet sur l'activité nitrogénase de la souche Sp7. Ce système, où des bactéries diazotrophes s'établissent dans une niche protégée de l'oxygène, fournie par la plante, et y expriment une activité nitrogénase, pourrait constituer un premier pas vers la construction de symbioses artificielles fixatrices d'azote chez les céréales.

Mots-clés: Nitrogénase, Fixation de l'azote, Nodule, *Azospirillum*; 2,4-D, Production d'éthylène, Blé, *Para*-nodule.

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