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IMPROVED N_2 FIXATION IN 2,4-D TREATED WHEAT ROOTS ASSOCIATED WITH *AZOSPIRILLUM LIPOFERUM*: STUDIES OF COLONIZATION USING REPORTER GENES

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Summary—Among several *Azospirillum* strains tested for N_2 fixation in association with wheat in a model system, 2 *A. lipoferum* strains (SpBr17 and 596) were found to be more efficient than the 8 other *A. lipoferum* and *A. brasilense* strains tested. Plants treated with low concentrations (0.7 $\mu g\ ml^{-1}$) of 2,4-dichlorophenoxyacetic acid (2,4-D) followed by inoculation supported larger acetylene reduction activity than plants treated only with bacteria. Three broad host range plasmids carrying *lacZ*, as a reporter gene, controlled by *nifA*, *nodG* and *ntrC* promoters were transferred to *A. lipoferum* SpBr17 and strain 596 by conjugation. All the transconjugants showed high β-galactosidase activity in the free-living condition. Studies on colonization of wheat roots using the SpBr17 *nodG–lacZ* gene fusion, which expressed the *lacZ* gene at the highest level, indicated the localization of bacteria on the young parts of roots as a thick coat. Counts of azospirilla on plant roots showed increased association of bacteria in 2,4-D treated plants.

INTRODUCTION

Members of the genus *Azospirillum* associate with plant roots and have been shown in some cases to improve the N nutrition of several agriculturally important crops (Kapulnik *et al.*, 1981; Reynders and Vlassak, 1982). Upon inoculation, azospirilla can proliferate on roots and in some cases can apparently invade the internal parts of roots (Patriquin and Döbereiner, 1978; Umali-Garcia *et al.*, 1980). Although little is known about the *Azospirillum*–host plant specificity, there are indications that some form of specificity of association may exist.

Treatment with concentrations of auxins has been shown to increase colonization and N_2 fixation of azospirilla in wheat (Tchan *et al.*, 1991; Christiansen-Weniger, 1992; Kennedy and Tchan, 1992). A change of root morphology to nodule-like structures (*para*-nodules) has also been reported following the addition of auxin analogues. According to Sriskandarajah *et al.* (1993), these structures are colonized preferentially by *A. brasilense* (Sp7) relative to other parts of the roots and the higher nitrogenase activity associated with auxin treatment is suggested to be partly due to the development of a protected niche.

With the increasing interest of non-legume associations, studies have also focused on the colonization of roots by microorganisms. Techniques such as electron microscopy, use of antibiotic resistance markers and immunofluorescence have been used to study colonization. Recently, fusions of reporter genes associated with N_2 fixation gene promoters (*nifA*, *nifH*, *nodG* and *ntrC*) of *A. brasilense* have been prepared and these have been shown to be expressed under free-living conditions, more or less constitutively (*nifA*, *nodG* and *ntrC*) and under N_2-fixing conditions (Liang *et al.*, 1991, 1993; Vieille and Elmerich, 1992). Such fusions provide a potential tool for studying the colonization. The reporter genes such as *lacZ* (β-galactosidase) (Boivin *et al.*, 1990) with a promoter from rhizobia and *uidA* (β-glucuronidase) (Van de Broek *et al.*, 1992) with promoters derived from *Azospirillum* have been used to study the expression of these promoters in root tissues after inoculation with appropriate fusions.

We have tested the N_2 fixation ability of several *Azospirillum* strains in association with wheat in the presence or absence of 2,4-dichlorophenoxyacetic acid (2,4-D). Root colonization of *A. lipoferum* strain SpBr17 was also studied by using the reporter gene *lacZ* fused with *Azospirillum nodG* promoter.

MATERIALS AND METHODS

Testing of different Azospirillum strains

In our experiment, 7 *A. brasilense* and 3 *A. lipoferum* strains (Table 1) were tested for N_2-fixing ability in association with wheat in the model system described by Zeman *et al.* (1992).

Plants were either treated with 0.7 $\mu g\ ml^{-1}$ of the auxin analogue 2,4-D or did not receive 2,4-D treatment. When the plants were 3 weeks old the acetylene reduction assay (ARA) was performed as described by Sriskandarajah *et al.* (1993) using a

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Shimadzu GC 8F gas chromatograph fitted with a flame ionization detector and a 1 m column of Porapak T.

Conjugation of reporter genes to Azospirillum

Fusions of the *lacZ* gene with different *Azospirillum* gene promoters such as *nifA*, *nodG* and *ntrC* were used. *Escherichia coli* strains (S17.1) carrying the plasmids (given in Table 1) were constructed at the Pasteur Institute, Paris. These plasmids were transferred to *A. lipoferum* strains SpBr17 and 596 in order to study colonization.

Conjugation was performed between *E. coli* S17.1 containing the plasmids and *A. lipoferum* as described by Simon *et al.* (1983). The donor strains of *E. coli* S17.1 were grown in Luria–Bertani broth supplemented with 5 μg tetracycline ml^{-1}. The recipients, *A. lipoferum* SpBr17 and 596, were grown in Nfb liquid medium supplemented with ammonium chloride (1 g l^{-1}). Conjugation was allowed to take place after mixing the donor and the recipient on nutrient agar. Transconjugants were selected on minimal lactate medium (Dreyfus *et al.*, 1983), supplemented with ammonium chloride (1 g l^{-1}) and tetracycline (5 μg ml^{-1}).

The expression of the *lacZ* gene in transconjugants was measured using the ability to hydrolyse *o*-nitrophenyl-β-D galactoside (ONPG) (Miller, 1972). Overnight grown liquid cultures of cells containing *lacZ* fusions were used to determine β-galactosidase activity. A sample of 1 ml from the grown culture was centrifuged and resuspended in the same volume of Z buffer (Miller, 1972); 100–200 μl of these suspensions (depending on the density) were brought to the total volume of 1 ml with Z buffer. Sodium dodecylsulphate (1 drop 0.1% SDS in Z buffer) and chloroform (2 drops) were added to the cell suspensions and vortexed thoroughly. Samples were stabilized at 28°C for 15 min and then 100 μl of ONPG (4 μg ml^{-1}) was added to each sample. Tubes were kept at 28°C and the reaction was stopped by adding 250 μl of 1 M sodium carbonate when a yellow colour had developed. The samples were then centrifuged to remove cell debris and the intensity of the yellow colour was measured at 420 nm using a Beckman Du 64 spectrophotometer. The protein content of the samples were determined as described by Gogstad and Krutnes (1982) using Bio Rad protein assay dye reagent.

Table 1. *Azospirillum* strains and plasmids used in this study

Strain/plasmid	Reference
Azospirillum strains	
A. brasilense	
Sp7	Tarrand *et al.* (1978)
Cd	Tarrand *et al.* (1978)
Sp13	Tarrand *et al.* (1978)
SpBr14	Tarrand *et al.* (1978)
Sp245	Baldani *et al.* (1986)
Sp107	Baldani *et al.* (1986)
576	New and Kennedy (1989)
A. lipoferum	
Sp59b	Tarrand *et al.* (1978)
SpBr17	Tarrand *et al.* (1978)
596	New and Kennedy (1989)
Plasmids	
pAB576 (*nifA–lacZ*)	Liang *et al.* (1991)
pAB538 (*nodG–lacZ*)	Vieille and Elmerich (1992)
pAB792 (*ntrC–lacZ*)	Liang *et al.* (1993)

Colonization of wheat roots

Colonization of wheat roots was studied using strain SpBr17 containing *nodG–lacZ* fusion. *In situ* visualization of azospirilla bearing *lacZ* fusions was obtained by staining root pieces and transverse sections of roots with the chromogenic substrate of the β-galactosidase enzyme, 5-bromo-4-chloro-3-indolyl β-galactoside (X-gal).

Two weeks after inoculation, root pieces were taken from seedlings and stained using a modification of the protocol of Boivin *et al.* (1990) as described by Arsène *et al.* (1994). Root segments were fixed in 1% glutaraldehyde solution and the β-galactosidase activity was revealed by staining with X-gal solution. After staining, specimens were washed twice in Z buffer followed by water and examined using a transmission light microscope (Olympus BHA).

Transverse sections (100 μm) of fresh roots embedded in dental wax were made using an Electron Microscopy Services OTS-300-03 oscillating tissue slicer and stained as above to study the internal colonization.

Enumeration of azospirilla

Bacteria associated with 2,4-D treated and untreated roots inoculated with SpBr17–*nodG–lacZ* and 596–*nodG–lacZ* were counted as described by Sriskandarajah *et al.* (1993) using the most probable number (MPN) technique. As the *lacZ* plasmid is tetracycline resistant, the medium used was Nfb supplemented with tetracycline (5 μg ml^{-1}). Numbers of azospirilla were determined using the Niftal MPN enumeration system (MS-Dos Version 1.0) (Bennett *et al.*, 1990).

RESULTS

ARA of Azospirillum strains

ARA of different *A. brasilense* and *A. lipoferum* strains in association with wheat are given in Fig. 1. The 2,4-D treated plants always showed higher activity than untreated plants. Among the strains tested, 2 *A. lipoferum* strains, SpBr17 and 596, were shown to have the highest nitrogenase activity in association with wheat roots. As shown in Fig. 1, 2,4-D treated plants were up to 10 times more active than the untreated plants.

Conjugation of lacZ plasmids to azospirilla

The plasmids pAB576 (*nifA–lacZ*), pAB538 (*nodG–lacZ*) and pAB792 (*ntrC–lacZ*) were transferred by conjugation to *A. lipoferum* SpBr17 and 596. All the transconjugants showed considerable β-galactosidase activity under aerobic growth, whereas the

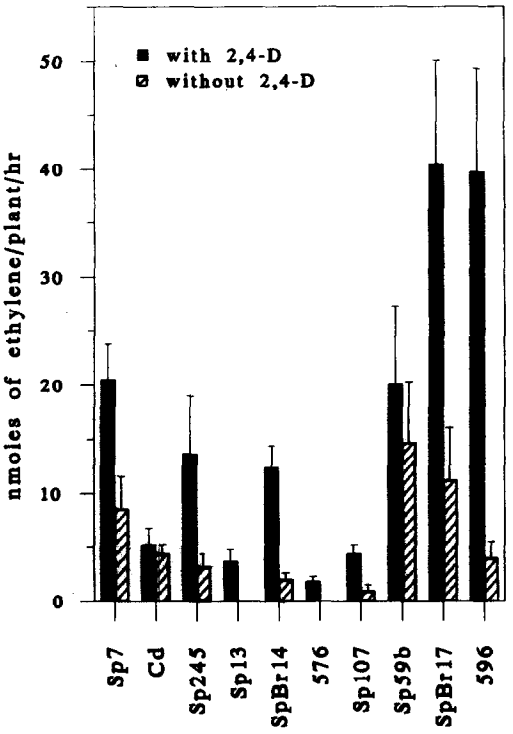

Fig. 1. Acetylene reduction in 2,4-D treated and untreated wheat seedlings inoculated with different *A. brasilense* (Sp7, Cd, Sp245, Sp13, SpBr14, 576, Sp107) and *A. lipoferum* strains (Sp59b, SpBr17, 596). SEs are indicated on the graph.

recipient strains had very little activity. The strains carrying *nodG–lacZ* were found to be the most active (Table 2). Generally, the fusions of strain SpBr17 expressed the *lacZ* gene at a higher level than strain 596.

Colonization of roots

In situ staining with X-gal, a chromogenic substrate of β-galactosidase, clearly indicated the presence of *lacZ* carrying *Azospirillum* cells on the surface of the roots by the blue-coloured staining (Fig. 2). The blue-stained azospirilla were clearly distinguishable from any background colour due to residual plant β-galactosidase. Fixing the root tissues with 1% glutaraldehyde reduced the background staining to a satisfactory level. The 2,4-D treated roots had much denser colonization than untreated plants. Bacteria were found mostly on the young parts of roots and the root surface just behind the tip which was coated with a thick cover (several cells thick) of azospirilla. Transverse sections of young roots clearly showed the presence of *lacZ* carrying azospirilla as a thick coat around the root (Fig. 2). There was no evidence of extensive internal colonization. However, some of the epidermal cells were seen to be filled with bacteria.

Table 2. β-Galactosidase activity of *lacZ* fusions of *A. lipoferum* strains SpBr17 and 596 (cultures were grown overnight in Nfb liquid medium supplemented with ammonia)

Strain	β-Galactosidase activity (Miller units mg^{-1} protein min^{-1})
A. lipoferum	
SpBr17 (wild type)	47 ± 6
SpBr17–*nifA–lacZ*	1737 ± 648
SpBr17–*nodG–lacZ*	3914 ± 1644
SpBr17–*ntrC–lacZ*	2344 ± 845
A. lipoferum	
596 (wild type)	47 ± 8
596–*nifA–lacZ*	1089 ± 90
596–*nodG–lacZ*	3731 ± 188
596–*ntrC–lacZ*	1944 ± 214

Enumeration of azospirilla

The bacterial counts made on inoculated wheat roots indicated higher numbers of bacteria in roots treated with 2,4-D than in untreated roots (Fig. 3). Bacterial numbers associated with the 2,4-D treated plants were several times higher than those associated with untreated plants.

DISCUSSION

Of the 10 *Azospirillum* strains tested, 2 strains of *A. lipoferum* (SpBr17 and 596) were found to be most effective for N_2 fixation in association with wheat. The differences of the nitrogenase activity associated with plants did not correlate with the nitrogenase activity of the strains alone *in vitro* (only some of the strains were tested). This indicated that there were differences in the ability of *Azospirillum* strains to associate with the roots of plant hosts and fix N. Although, little work has been done on azospirilla–host plant specificity, the basis for any such specificity by azospirilla in associating with the host plant is unknown.

The bacterial counts using the MPN technique provide clear evidence that the 2,4-D treated plants carried more associated azospirilla than the plants without 2,4-D. It has previously been reported by Sriskandarajah *et al.* (1993) that synthetic auxins such as 2,4-D and naphthaleneacetic acid at low concentrations enhance the colonization and N_2-fixing ability of *A. brasilense* Sp7 in association with wheat.

In contrast to the findings of Sriskandarajah *et al.* (1993) with *A. brasilense* (Sp7), we did not observe large numbers of *A. lipoferum* strain SpBr17 associated with 2,4-D induced nodule-like structures (*para*-nodules). A dense layer of bacteria was observed covering the surface of the young parts of 2,4-D treated roots. Although the nitrogenase in the outermost cells of the bacterial layer would probably have been inactivated by the O_2 (2.5%) present in the assay vessel, the azospirilla near the rhizoplane may have been protected and active in reducing acetylene. The higher acetylene reduction in 2,4-D treated plants (Fig. 1) could be partly attributed to the higher numbers of azospirilla associated with the root systems of the treated plants, the exact mechanisms for the enhanced colonization and N_2 fixation resulting from the 2,4-D treatment are not yet clear. This effect could also be due to structural and metabolic changes occurring

Staining with X-gal, which becomes a blue colour when hydrolysed by the enzyme β-galactosidase, readily enabled visualization of *Azospirillum* cells bearing *lacZ* plasmid in the root system. This technique provides a simple and efficient means of recognition of bacteria *in situ* compared with several other techniques used to study the colonization. According to our observations, *A. lipoferum* strain SpBr17 mainly colonizes the surface of roots forming a dense layer. This effect was more pronounced at the young parts of the root just above the tip, indicating that a large proportion of the N_2 fixation may be associated with the young root tissues. There was no evidence to support the idea of extensive internal colonization of root tissues as observed with some other strains of *Azospirillum* (Patriquin and Döbereiner, 1978; Bashan and Levanony, 1988), although epidermal cells were occasionally colonized internally by azospirilla. Thus, our experiments suggest that different strains of azospirilla have different patterns of colonization, which may have different consequences for the N_2-fixing ability of the association.

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N_2 FIXATION (C_2H_2 REDUCTION) IN 2,4-DICHLORO-PHENOXYACETIC ACID (2,4-D) TREATED WHEAT INOCULATED WITH FREE-LIVING DIAZOTROPHS

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Summary—N_2 fixation associated with 2,4-D-treated wheat seedlings was investigated using a number of free-living, non-associative, O_2- and acid-tolerant diazotrophs. Not all diazotrophs tested formed an N_2-fixing association. Even strains within one species behaved differently. *Azospirillum* was found to have a narrow pH and pO_2 range for nitrogenase activity (C_2H_2 reduction) in 2,4-D-treated wheat seedlings, whereas *Derxia gummosa* was capable of forming an effective N_2-fixing association under a wide range of pH and pO_2.

INTRODUCTION

Nitrogenase activity (C_2H_2 reduction) was found to be enhanced in 2,4-dichlorophenoxyacetic acid (2,4-D) treated wheat seedlings inoculated with *Azospirillum* over seedlings not treated with 2,4-D under carefully controlled reduced O_2 tension (Tchan *et al.*, 1991; Zeman *et al.*, 1992; Yu *et al.*, 1993). In a review paper, the term *para*-nodule induced by the 2,4-D treatment was introduced to distinguish it from the naturally-occurring legume nodule (Tchan and Kennedy, 1990; see Kennedy *et al.*, 1990). This model system (Zeman *et al.*, 1991), however, only shows very low to negligible N_2 fixation (C_2H_2 reduction) under an O_2 tension > 0.04 atm (A. M. M. Zeman, unpubl. data). This restricts the usefulness of *Azospirillum* as a contributor to the N economy of the system under well-aerated soil.

Although the effectiveness of N_2 fixation in relation to pH has been reported (Döbereiner *et al.*, 1976; Gibson *et al.*, 1988), information regarding the performance of azospirilla and other free-living diazotrophs in an acid environment with 2,4-D-treated wheat seedlings is still lacking. The survival of the diazotrophs in the rhizosphere would determine the initial formation of an of the association with the host plant as well as the ability to fix N. There are several aerobic diazotrophs capable of fixing N under acid environments in the soil or in association with plants, such as *Azotobacter beijerinkii* (Tchan, 1953), *Azotobacter paspali* (Döbereiner, 1966; Döbereiner *et al.*, 1972), *Beijerinkia* spp (Tchan, 1968; Becking, 1984) and *Azospirillum amazonense* (Magalhaes *et al.*, 1983). Furthermore, under reduced O_2 tensions the N_2 fixation of some of these organisms was improved (Becking, 1971; Spiff and Odu, 1973). *Derxia* is a soil diazotroph but has been isolated from roots of grasses (Campêlo and Döbereiner, 1970). In pure culture, it fixes N at a range of pO_2 from atmospheric pressure to <0.02 atm. Its pH range extends from slightly less than 5.0 up to 9.0.

Also, Akao *et al.* (1991) showed that a non-nodulating soybean will form nodules with rhizobia when treated with 2,4-D. Some of these diazotrophs were used in an attempt to establish an N_2-fixing association with non-legumes treated with 2,4-D. Hence, we investigated the use of the free-living diazotrophs mentioned as well as the associative diazotroph, *Azospirillum brasilense* for their ability to form an effective N_2-fixing association using 2,4-D-treated and untreated wheat as the host plant under a wide range of O_2 tensions and pH. We also investigated whether 2,4-D treatment could break the barrier of specificity between *A. paspali* and *Paspalum notatum* (Döbereiner *et al.*, 1972) and form an association with other species of *Paspalum*.

MATERIALS AND METHODS

Cultivation of seedlings

The method of Zeman *et al.* (1992) was adopted. Each treatment was replicated five times unless otherwise stated. Plant tubes were rotated three times a week in a controlled environment light cabinet with continuous lighting (200 $\mu E\ m^{-2}\ s^{-1}$) and 18 and 23°C temperature cycles every 24 h.

Paspalum dilatatum seeds were surface sterilized by wetting with a 0.05% Tween 80 solution and vortexing for 30 s. The flask containing the seeds was placed under a vacuum and refilled with air three times. The seeds were then washed with water and sterilized as with wheat. Wheat (*Triticum aestivum* cv. Miskle) was surface sterilized according to Zeman *et al.* (1992).

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Preparation of hydroponic solution at different pH

Hydroponic solution (Zeman *et al.*, 1992) was adjusted to the desired pH using diluted HCl. The pH was not adjusted during the experiment.

Acetylene reduction assay (ARA)

The method described in Zeman *et al.* (1992) was adopted with modifications. ARA flasks (30 ml McCartney bottles) containing 3 ml of Winogradsky's mineral solution were stoppered with sterile rubber stoppers. The gas phase of the bottles was exchanged by evacuation and replaced with N. C_2H_2 (0.1 atm) and O_2 were introduced to produce a range of different pO_2 tensions, this is indicated in Tables 1 and 2. For host plants grown at different pH, the roots of the seedlings were aseptically washed in sterile neutral Winogradsky's mineral solution prior to placement into the ARA flasks. This was to minimize any unforeseen effect from pH on the C_2H_2 reduction of the association during the assay and minimize the carryover of soluble C that could be used during the ARA. The ARA flasks were placed in a shaking water bath at 30°C at 160 oscillations min^{-1}. Routinely, 500 μl of the gas phase was sampled at the times indicated in Fig. 1 or Tables 1 and 2 following injection of C_2H_2.

Diazotrophs

A. chroococcum SU was recently isolated from a soil sample from the University of Sydney and *D. gummosa* SUT1 from tropical soil. The following cultures [listed in Thompson and Skerman (1979)] were obtained: *A. beijerinckii* WR 40 and *A. bejerinckii* WR 50 from Queensland University; *A. vinelandii* AVO_2, *A. paspali* AX 52, *Beijerinckia indica* var. alba, *B. derxii* Q13 from the culture collection of Y. T. Tchan. All cultures were grown in N-free glucose mineral liquid medium (Tchan and Jensen, 1963). *A. brasilense* Sp7 ATCC 29145 (Sydney strain: isolated by Tchan from the original culture supplied by Dr A.H. Gibson of CSIRO, Canberra, Australia) was grown in N-free Nfb malate liquid medium (Krieg and Döbereiner, 1984).

Table 1. Influence of pH on nitrogenase activity of wheat seedlings inoculated with *A. brasilense* or *D. gummosa*

Diazotroph	pH	C_2H_4 formation nmol $plant^{-1}$ 21 h^{-1} − 2,4-D	+ 2,4-D
A. brasilense Sp7	6.5	Trace	93 (±7.02)
	5.5	Trace	62.4 (±8.37)
	4.5	Trace	176* (±28.6)
D. gummosa	6.5	16.6 (±5.0)	175.8 (±42.5)
	5.5	22 (±7.40)	229 (±52.1)
	4.5	20.38 (±6.85)	395 (±179)

Each value represents the mean of 5 replicates.
*This value was recorded with only 4 replicates. Standard error of the mean is shown (±SE).
ARA was carried out in 12-day-old seedlings under N_2 at a pO_2 of 0.025 atm (*A. brasilense*) and *D. gummosa* (0.08 atm) under shaken conditions.

Table 2. Time course of nitrogenase activity of 2,4-D-treated wheat seedlings inoculated with *Derxia* under varying O_2 tensions

pO_2	C_2H_4 accumulation (nmol $plant^{-1}$) at various incubation times 2 h	5 h	21 h
0.2	8.5 (±2.9)	9.6 (±1.8)	11.3 (±1.8)
0.1	ND	31.6 (±7.7)	196.2 (±48.9)
0.01	8.5 (±3.3)	19.5 (±3.4)	320.0 (±194.0)
0.002	5.5 (±3.3)	20.0 (±10.2)	439.0 (±310.0)

Assay time started at the time of C_2H_2 injection. Each value represents the mean of 4 replicates. Standard error of the mean is shown (±SE). The effect of pO_2 and time was significant. ARA was carried out under N_2 at the O_2 indicated in 12-day-old seedlings.

Cytology

Wheat roots bearing modified lateral roots were cut into pieces of 5–7 mm and fixed in 2% gluteraldehyde in 50 mM phosphate buffer, pH 7.2 overnight at 4°C. Root samples were washed three times for 10 min each in 50 mM phosphate buffer and post-fixed with 1% osmium tetroxide in the same buffer for 2 h 15 min at room temperature. After washing with five changes of distilled water in 15 min, the roots were dehydrated in an ethanol series (30%, 50%, 70%, 90% and two changes of 100%) for 10 min each and infiltrated overnight in 50–50 ethanol (100%)–Spurr's (low viscosity) resin (Spurr, 1969) on a rotator. The root samples were then infiltrated with 100% resin overnight on a rotator, embedded in fresh Spurr's resin in flat embedding moulds and polymerized at 65°C in an oven overnight. Sections (0.7 μm) for light microscopy were cut and stained with 0.5% Toluidine

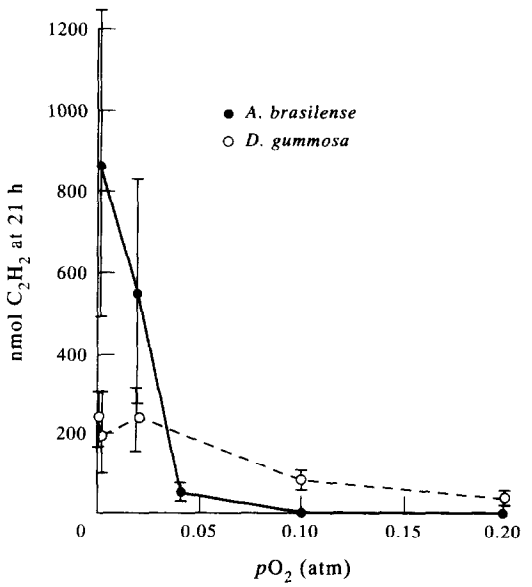

Fig. 1. C_2H_4 formation of 2,4-D-treated wheat seedlings under a range of O_2 tensions. Seedlings were inoculated with 0.1 ml of an overnight culture of either *A. brasilense* or *D. gummosa*. ARA was carried out on 14-day-old seedlings under N_2 at the O_2 tension indicated. C_2H_2 was added (0.1 atm) and C_2H_2 reduction was assayed after 21 h incubation at 30°C under shaken conditions. Each point represents the mean value of 4 replicates. SEMs are shown.