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Effects of plant growth regulators on acetylene-reducing associations between *Azospirillum brasilense* and wheat

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Abstract

Treatment of wheat seedlings with the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), induced nodule-like structures or tumours (termed *para*-nodules) where lateral roots would normally emerge. The formation of these structures promoted increased rates of acetylene reduction at reduced oxygen pressure (0.02–0.04 atm) in seedling inoculated with *Azospirillum brasilense*, compared to seedlings inoculated without auxin treatment. Fluorescent microscopy, laser scanning confocal microscopy and direct bacterial counts all showed that the 2,4-D treatment stimulated internal colonization of the root system with azospirilla, particularly in the basal region of the nodular structures. Both colonization with azospirilla and acetylene-reducing activity were further stimulated by simultaneous treatment with another synthetic auxin, naphthaleneacetic acid (NAA) and, less reliably, with indoleacetic acid (IAA) and indolebutyric acid (IBA). These auxins produced shortening of many initiated lateral roots, although 20 times the concentration of NAA was required to achieve rounded structures similar to those obtained with 2,4-D. Treatment with NAA, IAA or IBA alone also stimulated colonization with azospirilla and acetylene reduction rates at 0.02 atm oxygen, but less effectively than by treatment with 2,4-D. Such exogenous treatments of wheat seedlings with synthetic growth regulators provide an effective laboratory model for studies on the development of a N_2-fixing system in cereals.

Introduction

There has been much interest among researchers in obtaining an effective nitrogen-fixing system for non-leguminous field crops (for review, see Kennedy and Tchan, 1992). In attempting to achieve this, a range of methods has been employed, including the use of chemicals such as 2,4-D pioneered by Nie (Kennedy et al., 1990; Nie, 1983; Ridge et al., 1992; Tchan and Kennedy, 1989; Tchan et al., 1991), the use of genetically engineered or mutated bacteria (Jing et al., 1990; Rolfe and Bender, 1990) and the application of celluloytic and pectolytic enzymes (Al-Mallah et al., 1989, 1990). With the exception of the case of treatment of wheat seedlings with 2,4-D and inoculation with *Azospirillum* (Kennedy et al., 1990; Tchan et al., 1991) nitrogenase activity (acetylene reduction) was always found to be unreliable or near the limit of detection. The ability of 2,4-D to reliably induce nodule-like structures on wheat seedlings and the associated acetylene-reducing activity of seedlings when inoculated with *Azospirill-*

um has opened up a promising avenue for research for achieving nitrogen fixation in non-legumes. Christiansen-Weniger (1992) recently applied similar methods to the study of similar auxin-induced structures on wheat, confirming our general results. However, many of the properties of this system and its potential to be developed into a symbiotic system capable of benefiting the plant remain to be examined.

Plant-growth substances have long been known to modify root morphology in various broad leaf plants and cereals (Allen et al., 1953; Nie, 1983; Rolfe, et al., 1991; Wilde, 1951). Taylor (1946) provided an early report on the morphological effects of synthetic growth-regulating substances, including 2,4-D, on broad-leaf plants including legumes and some cereals. The cereals were more resistant than broad-leaf plants to forming pseudonodules after chemical treatment.

It has been recorded that growth substances are produced by the azospirilla, rhizobia or other soil bacteria and that these, in turn, influence the physiological and morphological responses of plants (Christiansen-Weniger, 1988; Horemans and Vlassak, 1985; Zimmer et al., 1987). Among growth substances, auxins have been found to play a prominant role. Auxins are important in the regulation of root growth and they act in both promotive and inhibitory capacities. The morphogenesis of legume nodules is known to involve the action of endogenous growth regulation (Libbenga and Bogers, 1974). Akao et al. (1991) have reported that 2,4-D treatment of a non-nodulating line of soybean led to the formation of effective nodules when inoculated with *Bradyrhizobium*. All these findings imply that there is a close link between plant growth substances, nodulation and the activity of the bacteria.

In this paper, we extend our previous work (Kennedy et al., 1990; Tchan et al., 1991; Zeman et al., 1992; Yu et al., 1993) with investigations on the effects of some additional plant growth substances on the morphogenesis of wheat roots and the associated acetylene reducing capacity of the *para*-nodulated wheat plants.

Materials and methods

Cultivation of plants and inoculation of seedlings with bacteria

Wheat seeds (*Triticum aestivum* sp., spring cultivar Miskle) were surface sterilized with 0.5% (w/v) $HgCl_2$ for two min at around 20°C, thoroughly washed with sterile water and germinated in the dark at 25°C on standard yeast-mannitol agar (YMA, Vincent, 1970) medium. Uncontaminated seedlings aged about 2–3 days were transferred aseptically for hydroponic (Zeman et al., 1992) growth in 20 mm disposable culture tubes (Corning) under controlled conditions of temperature (18–23°C) and continuous light (ca. 200 μEm^{-2} sec^{-1}). After 1 week of growth, seedlings selected for uniform growth were inoculated with ca 10^6 cells of *Azospirillum brasilense* Sp7 maintained on nitrogen-free medium (Nfb) (Krieg and Döbereiner, 1984) (culture kindly provided by Dr A H Gibson, CSIRO, Canberra), previously grown for 1–2 days in nitrogen-free medium (Nfb) containing malate (5 g L^{-1}) with 0.2% (v/v) potato extract added.

Plant growth regulators

All plant growth regulators except 2,4-dichlorophenoxyacetic acid (2,4-D) were filter sterilized before addition to the growth medium. A stock solution of 2,4-D was made by autoclaving a weighed quantity (5 mg per 100 mL) in water. Naphthaleneacetic acid (NAA, final concentration 0.1–10 mg L^{-1}), indoleacetic acid (IAA, 10–100 mg L^{-1}), indolebutyric acid (IBA, 0.1–10 mg L^{-1}), kinetin and gibberellic acid (GA_3, 0.25–1.5 mg L^{-1}) (Sigma Chemical Co.) were dissolved in dilute NaOH before diluting with sterile water. All plant growth substances were added either singly or in combination to the growth medium at the time of inoculation. Seedlings were examined at intervals for *para*-nodulation and sampled after 12–14 days from inoculation for acetylene reduction assays and microscopy.

Root and para-nodule morphology

The number of *para*-nodules per plant and their dimensions were measured using an Olympus stereo dissecting microscope after incubating the roots with iodonitrotetrazolium (INT) (0.25% w/v aqueous solution). Roots and hand-sections of the stained *para*-nodules were examined by phase/light microscopy to detect sites of reduction and bacterial infection. Roots were also stained with 0.1% acridine orange and examined for bacterial colonization using laser scanning confocal (Biorad MRC600) and fluorescence microscopy (Olympus BHA).

Bacterial counts in wheat roots

Inoculated wheat seedlings were assayed for acetylene reduction rates before determining the viable bacterial population in the root tissue (endorhizosphere). The entire roots system of each seedling was separated and surface sterilized by immersion in 95% (v/v) ethanol for 3 sec and then in 0.01% (w/v) $HgCl_2$ for exactly 15 sec. The roots were immediately washed seven times with sterile water. (NaOCl was found to give less consistent results than the use of $HgCl_2$). Surface-sterilized roots were then dipped in tubes containing Npb medium (New and Kennedy, 1989) for 30 sec to test for residual viable surface bacteria. These roots were then ground in 1 mL one-fifth strength sterile hydroponic solution in a sterile mortar and pestle. The volume was made up to 10.0 mL and 0.1 mL dilutions of $10^1, 10^2, 10^3, 10^4, 10^5$ and 10^6 in sterile one-fifth strength hydroponic solution were inoculated into triplicate tubes containing Npb medium.

After incubating the Npb tubes for four days at 30°C, the number of tubes with growth containing a typical rising pellicle were counted for each dilution. A spread-plate method was used to identify azospiralla and to check for contamination of inoculated plants. A volume of 0.1 mL of the first three dilutions was immediately spread on BMS agar plates. The plates were incubated for six days and then exposed to light. The characteristic change in colony colour from white to pink on BMS medium was used to confirm their identity as *Azospirillum*. For dilution tubes, 3–10 plants from each treatment from an experiment were analyzed with three replicates of the Npb tubes at each dilution. The numbers of *Azospirillum* in the root tissue were calculated using the Niftal Most Probable Number (MPN) Enumeration System (MS-DOS Version 1.0, Bennett et al., 1990).

Acetylene reduction assays

The acetylene reduction assay was performed using 1 plant per sample in glass reaction flasks (28 mL McCartney bottles, 30 mL internal volume) containing 3 mL of mineral Winogradsky's medium free of carbon and nitrogen and sealed with rubber serum stoppers (Thomas, U.S.A.). Each treatment consisted of 5 to 10 replicate exposure tubes. The flasks normally had an atmosphere of 2.5% O_2, 10% acetylene and the remainder argon or nitrogen and assays were conducted under artificial light with the temperature raised to 30°C.

Acetylene reduction in wheat seedlings was demonstrated using an assay designed to measure the nitrogenase activity of azospirilla located within the root tissue of the seedlings (i.e. in the endorhizosphere) rather than at the root surface in the rhizosphere (Tchan et al., 1991). This assay employs oxygen pressure set at a value (2% or greater) inhibiting the nitrogenase activity of superficial bacteria, allowing activity only by bacteria located in root zones depleted of oxygen by respiratory consumption and limitation by diffusion. The root system was immersed in a liquid medium containing no nutrient carbon and nitrogen compounds and shaken at a rate adequate to ensure equilibration with oxygen for *Azospirillum* cells in the exorhizosphere or possibly growing in the liquid medium during the assays. Thus, the activities recorded are less than the maximum possible by adjusting the oxygen pressure to an optimum level (see Kennedy and Tchan, 1992), being a compromise between the need to eliminate a contribution from external bacteria and the selective expression of activity by bacteria in protected niches within the root.

Sterile flasks containing seedlings transferred aseptically were evacuated and flushed four

times with argon and the required concentration of other gases introduced by syringe. A Shimadzu GC8F gas chromatograph fitted with a flame ionization detector and a 1 meter column of Porapak T (Walters-Millipore) was used for measurement of ethylene production, using 0.2 or 0.5 mL samples taken by syringe. Oxygen concentration in the flasks was monitored by gas chromatography of 0.2 mL samples, using a Shimadzu GC8T gas chromatograph fitted with a thermal conductivity detector and a 1 meter column of molecular sieve (13 × 60–80 mesh, Alltech Pty. Ltd.) using helium as a carrier gas.

In some exposures, tubes were either re-evacuated and re-gassed with nitrogen and oxygen plus acetylene as before or exposed to air with occasional shaking over 10–60 min and re-evacuated and re-gassed to determine the effects of these treatments on the acetylene reduction rate.

Statistical analysis

Data in Table 1 was analyzed as a completely randomized design analysis of variance using the MINITAB Version 8.2 program. These were log-transformed prior to analysis of variance and least significant differences between treatments means were calculated. The data in Table 1 are presented in a non-transformed format. Data in Figures 1 and 2 was subjected to analysis of variance as split plots with time. In other cases, standard error or deviation values are shown.

Results

The time course of acetylene reduction in 2,4-D *treated wheat seedlings*

The time course of acetylene reduction in this system has been examined and is shown in Figure 1, for assays using 2.5% and 4% oxygen. Typically, a lag of at least 5 h occurred before ethylene formation was observed, followed by an extended period of approximately linear activity. Tests using gas chromatography showed that during the developed of this activity and up to 24 h, the oxygen concentration in the reaction flasks remained at least at the level initially set (data not shown).

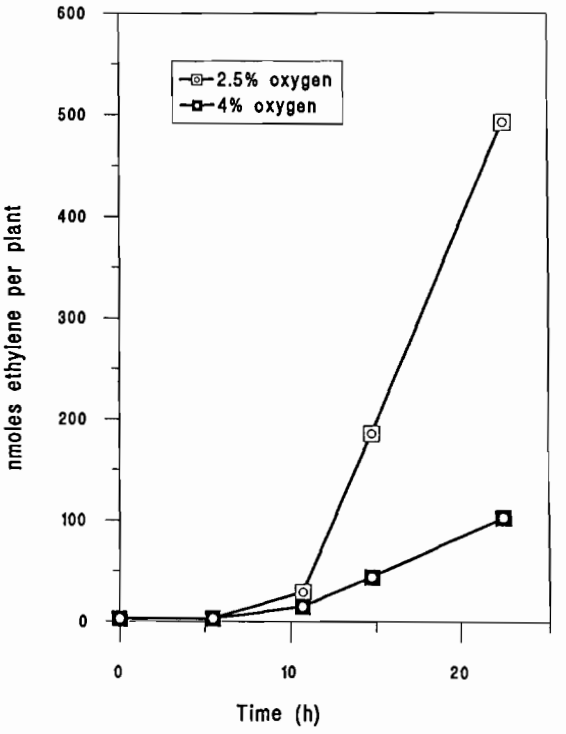

Fig. 1. Time course of acetylene reduction in 2,4-D treated wheat seedlings inoculated with *Azospirillum brasilense*. Increasing inhibition of nitrogenase activity by increasing oxygen (2.5% and 4%) is shown, as well as a lag period before ethylene is formed. Analysis of variance (split plot in time) showed that both the effect of oxygen pressure and the trends with time were statistically significant ($p < 0.01$).

Tests were made to determine whether exposure of seedlings to air during preparation for acetylene reduction assays affected the nitrogenase activity. As shown in Figure 2, if seedlings were aseptically re-exposed to air and the oxygen pressure subsequently reset to 2.5% after re-evacuation and introduction of nitrogen, or the gas phase simply replaced with 2.5% oxygen in nitrogen without exposure to air in a control, a similar though shorter lag period occurred before a significant rate of acetylene reduction was regained. Thus, the disturbance of change of gas phase and a period of anaerobiosis was sufficient to cause a new lag in the nitrogenase activity of these seedlings.

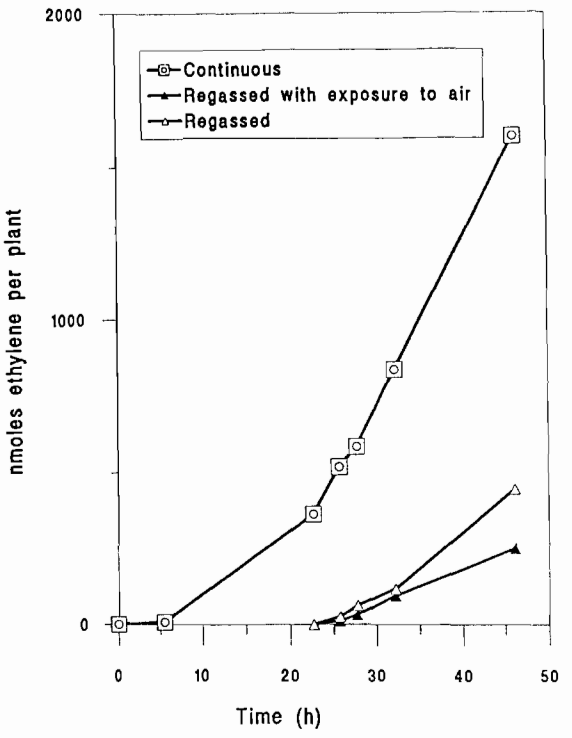

Fig. 2. Effect of re-evacuation and re-gassing of acetylene reduction assays in 2,4-D treated wheat inoculated with *Azospirillum brasilense*. A similar though briefer lag is shown, compared to the continuous control assays (all assayed with 2.5% oxygen pressure). Analysis of variance showed that there were significant differences in activity ($p < 0.01$) between continuous exposure and the re-exposed treatments up to 9.5 h after re-exposure, although no significant difference between exposure of seedlings in the flasks to air for one h and anaerobic incubation was observed, despite the appearance that full activity was not regained on re-exposure.

Effect of growth regulators on rates of acetylene reduction

The nitrogenase activity of wheat seedlings inoculated with *Azospirillum brasilense* is enhanced at least five-fold if these seedlings are treated with 2,4-D at the time of inoculation. Seedlings without bacterial inoculation, either with or without 2,4-D, failed to produce ethylene (Table 1). Thus there was no evidence of ethylene production by 2,4-D induced stress. Tests (data not shown) also proved that added ethylene was not removed by metabolism, such as by oxidation.

While the rates of acetylene reduction were variable between replicate seedlings and separate experiments, analysis of variance of the data from individual experiments indicated highly significant effects ($p < 0.001$) of added 2,4-D or NAA (Table 1). In most experiments, the standard deviation of the rates of acetylene reduction by individual seedlings was 50–60% of the mean value of sets of replicates. However, the variance of the data was reduced to a satisfactory level by a log transformation. In order to obtain statistically significant effects, at least four replicate seedlings for each treatment were required. The possible causes of variation between replicates and experiments are not yet defined. However, the extent of colonization with azospirilla (see below) would be expected to influence the nitrogenase activity strongly, providing a possible cause for variation between replicate seedlings.

Adding 2,4-D and NAA significantly increased the rate of acetylene reduction by the wheat-*Azospirillum* association (Table 1). The optimum concentration of 2,4-D, in terms of 24 h values of acetylene reduction, was 0.5 mg L^{-1} added to the growth medium at the same time as inoculation with *Azospirillum* (data not shown). A further stimulation of acetylene reduction rate was found with added NAA (1.0 mg L^{-1}).

IAA without 2,4-D can also stimulate the rate of acetylene reduction (Table 1, Exp. 3 and 4) but there is insufficient evidence to suggest that IAA significantly increases the activity of seedlings treated with 2,4-D (0.5 mg L^{-1}). NAA, on the other hand, usually increased the activity obtained with 2,4-D (Table 1, Exp. 1, 2 and 4) and, more reliably than IAA, could sometimes be effective when used alone (Table 1, Exp. 1 and 2). Available evidence (Table 1, Exp. 4) suggests that the activity stimulated by IBA in combination with 2,4-D was comparable to activity obtained by IAA. In this experiment, NAA together with 2,4-D produced the highest activity.

Treatment with kinetin (0.5–1.5 mg L^{-1}) reduced the size of the *para*-nodules. GA_3 (0.25-1.0 mg L^{-1}) inhibited lateral root development and when added together with 2,4-D, also re-

Table 1. Effect of plant growth substances on acetylene reduction by wheat seedlings

Treatment											
Azospirillum brasilense Sp 7			−	−	+	+	+	+	+	+	+	+
2,4-D (mg L^{-1})			−	0.5	−	0.5	0.5	−	0.5	0.5		0.5
NAA (mg L^{-1})			−	−	−	−	1.0	1	−	−	−	−
IAA (mg L^{-1})			−	−	−	−	−	−	5.0	10.0	−	−
IBA (mg L^{-1})			−	−	−	−	−	−	−	−	5.0	1.0
Exp	Incubation time (h)	Replicates (n)	Acetylene reduction (nmoles $plant^{-1}$)									
1	21	7–9	0	0	4.2[a]	157.5[b]	846.3[c]	168.0[b]	−	−	−	−
2	9	7	0	0	11.7[a]	27.0[b]	30.6[c]	26.1[b]	−	−	−	
2	22.5	7	0	0	15.8[a]	119.3[b]	279.0[c]	162.0[b]		−	−	
3	24	7–8	0	0	21.6[a]	799.2[b]	−	−	508.8[b]	−	268.8[a]	
4	24	5	0	0	−	232.8[a]	878.4[c]	−		398.4[b]	−	604.8[b]

Analysis of variance was performed on log transformed data as indicated in Methods for each experiment. Activities are not significantly different ($p < 0.05$) when followed by the same letter within each experiment. Analysis of variance indicated statistical significance of treatments as follows: Exp. 1, $p = <0.001$; Exp. 2, $p = 0.001$; Exp. 3, $p = 0.003$; Exp. 4, $p = <0.001$.

duced the size of the induced structures. Consistent with these effects on morphology, acetylene reduction activity was also inhibited in these plants treated with cytokinins or GA_3 (data not shown).

Root and para-nodule morphology

At the time of treatment with plant growth substances and azospirilla, the week-old seedlings had produced only one or two main roots, without any branch or lateral roots. Synthetic auxin treatments stunted the main roots and also arrested lateral root development (Plate 1). These artificially modified laterals were found to be a response to the application of synthetic auxin alone, although nearly all the experiments described on the effects of hormones on nodule morphology were obtained using simultaneous inoculation with *Azospirillum*.

Treatment with 2,4-D (0.5 mg L^{-1}) consistently induced structures that were round in shape. The outgrowths were distributed at regular intervals along the primary roots (Plate 1). Those formed near the proximal end of the roots were found at intervals averaging 4 mm, but were much more crowded and smaller near the distal end. In an experiment to determine the effect of 2,4-D concentration, 0.5 mg L^{-1} was found to produce the largest structures and concentrations higher than 1.0 mg L^{-1} inhibited their development. This optimum concentration of 2,4-D also corresponded to the 2,4-D concentration giving seedlings with the greatest rate of acetylene reduction (data not shown).

Without 2,4-D, NAA, IAA and IBA at higher concentrations also induced many strongly shortened lateral roots, but these structures lacked the well-rounded appearance of the structures induced by 2,4-D (Plate 2). Addition of 2,4-D (0.5 mg L^{-1}) invariably altered the morphology of the nodular structure towards roundness, even when NAA or IAA were also added. NAA was more consistent in producing structures similar to those characteristic of 2,4-D (Fig. 3) than IAA. Higher concentrations of NAA (up to 10 mg L^{-1}) showed an even more inhibitory effect on the extension of the lateral roots, resulting in roundness similar to that produced by treatment with 2,4-D. Alternatively, the laterals showed developed into root-like structures when treated with only low levels of IAA or NAA (less than 0.1 mg L^{-1}).

Occasionally, the modified root structures became multi-lobed. This effect was particularly noted when either IAA or NAA was used in conjunction with 2,4-D. In one experiment, NAA (1 mg L^{-1}) together with 2,4-D (0.5 mg L^{-1}) produced structures averaging 0.056 (SD = 0.025) mm^3 in volume in comparison to those induced by 2,4-D alone which averaged only 0.030 mm^3. These increases in

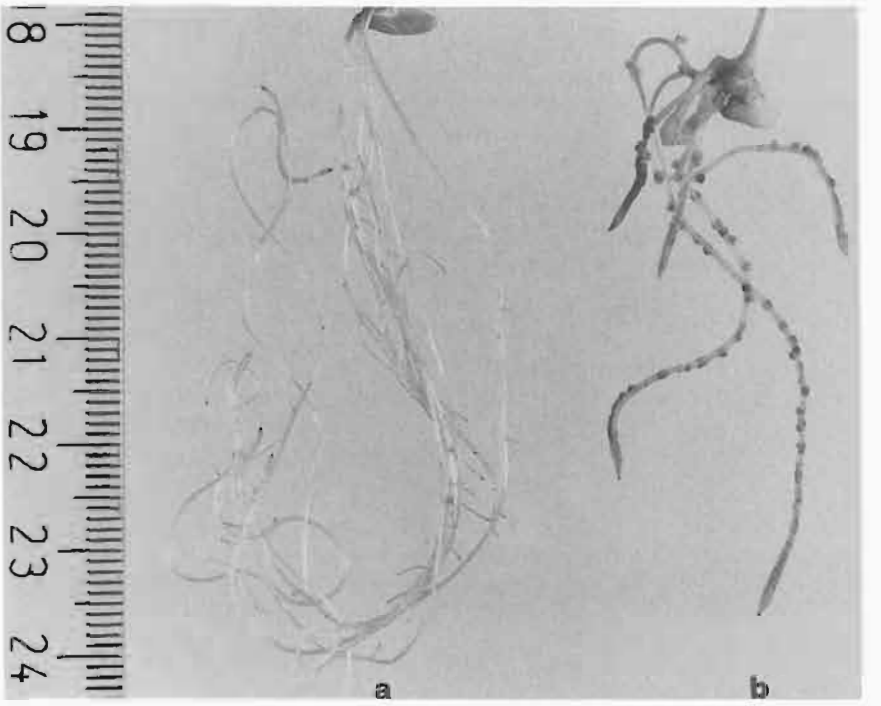

Plate 1. Iodonitrotetrazolium stained roots of wheat seedlings. **a**. treated with *Azospirillum* alone **b**. treated with *Azospirillum* plus 2,4-D (0.5 mg L^{-1}).

volume on NAA treatment in this experiment were highly significant ($p < 0.001$), and the structures gave a more intense colour with INT than those formed by treatment with 2,4-D alone, consistent with the greater colonization observed by microscopy. NAA was also found to encourage root-hair growth at rates about three times the normal density.

Microscopy

INT-stained bacteria were found to be particularly associated with the enlarged outer cortical cells (basal cells) underlying the meristematic region of the *para*-nodules. Direct observation of bacteria in other meristematic regions of the structures was difficult because of the large quantity of reduced dye. Treatment with NAA as well as 2,4-D caused more intense staining, probably due to the greater colonization with azospirilla noted below.

More reliable observations were obtained by fluorescent and confocal microscopy. Bacterial cells were stained with acridine orange, and produced an intense greenish-yellow fluorescence at low magnification of the basal region not observed in controls lacking *Azospirillum* (Plates 3a, 3b). *Azospirillum* in the tissue appeared to brighten the fluorescence of nearby plant nuclei (Plates 3a, 3b). Intercellular spaces and other cavities created in the zone of the emergence of laterals were found to harbour most of the azospirilla. The identity of these bacteria as azospirilla was confirmed by their appearance in confocal and electron (Zeman et al., 1992) micrography, typical pellicle formation in dilution tubes on reisolation and the occurrence of a single type of colony on spread plates.

A more prolonged growth of plants after auxin treatment (up to 3–4 weeks) at 18–24°C or incubation at 30°C for 1–2 days following growth for 2 weeks at 18–24°C increased the extent of colonization. Examination of these roots by confocal microscopy showed the occasional cell in the basal region apparently filled with bacteria. The adjacent meristematic zone (see Plate 3) inside the *para*-nodule was also 'sectioned' optically by the confocal microscope, and it was

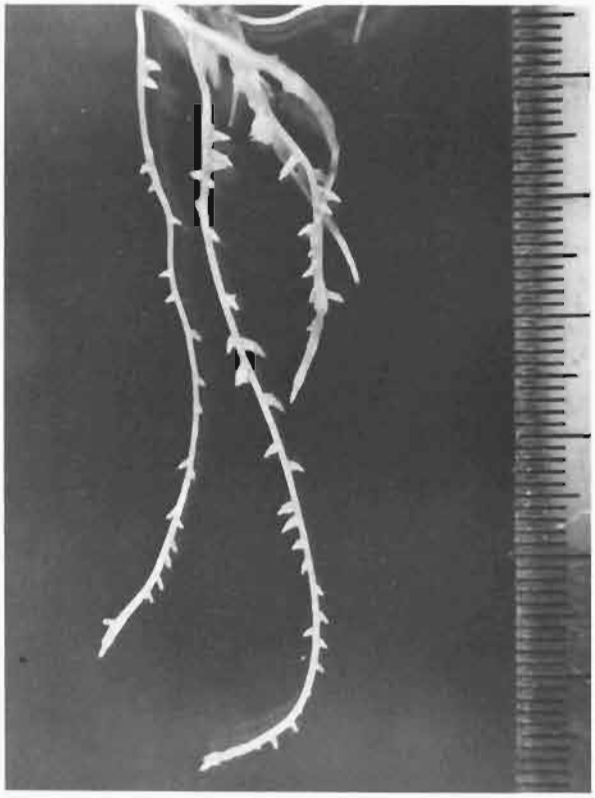

Plate 2. *Para*-nodules induced by NAA (1 mg L^{-1}).

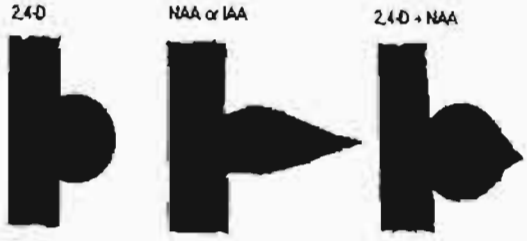

Fig. 3. The shape of *para*-nodules with different auxin treatments.

found that azospirilla were consistently present between the plant cells (Plate 4), although relatively fewer than in the basal region of cortical cells underlying the modified laterals (Plate 5), and were not obviously influenced by treatment with NAA or IAA in addition to 2,4-D. Plate 5 shows bacteria in the large intercellular spaces between the cortical cells in the basal zone underlying the modified lateral root structures induced by 2,4-D or NAA treatment, the plane of focus only partly indicating the longitudinal shape of the basal plant cells that is indicated more clearly in Plates 3a and 3b. Some of the more rounded plant cells in the outermost layer of cells of the *para*-nodules also had associated bacteria but relatively few. Bacteria associated more readily with the root surface of 2,4-D treated plants, but the relative numbers depended on the treatment. NAA in particular caused colonization in larger numbers in the basal region and also along the root surface. Aggregates of bacteria were sometimes observed among the root hairs, found in higher density in NAA-treated plants. The possible contribution that these aggregates could make to the enhanced rates of acetylene reduction requires further investigation.

Bacterial counts

Direct counts in dilution tubes following the procedure described in Materials and Methods provided quantitative evidence that the treatment with 2,4-D and 2,4-D plus NAA clearly stimulated internal colonization of the roots with azospirilla. The use of NAA provided a statistically significant ($p < 0.05$) stimulation of the effect of 2,4-D on internal colonization. Surface bacteria were effectively killed by treatment with $HgCl_2$, as shown by the absence of either colour change or development of a pellicle in Npb medium. However, significant numbers of viable bacteria remained in the interior of the roots after mercury treatment (ca 10^6 per seedling treated with auxins). In the experiment shown in Figure 4, no bacteria were observed after surface sterilization in the roots of seedlings inoculated with *Azospirillum* without 2,4-D or NAA treatment.

Despite the good general correlation between treatment with synthetic auxins, greater colonization and greater acetylene reduction rates, the bacterial counts and rates of ethylene formation were not closely correlated for individual seedlings. The seedlings with the highest nitrogenase activity did not necessarily give the largest numbers of viable azospirilla, possibly indicating the death of many bacteria in the endorhizosphere during the sterilization procedure.

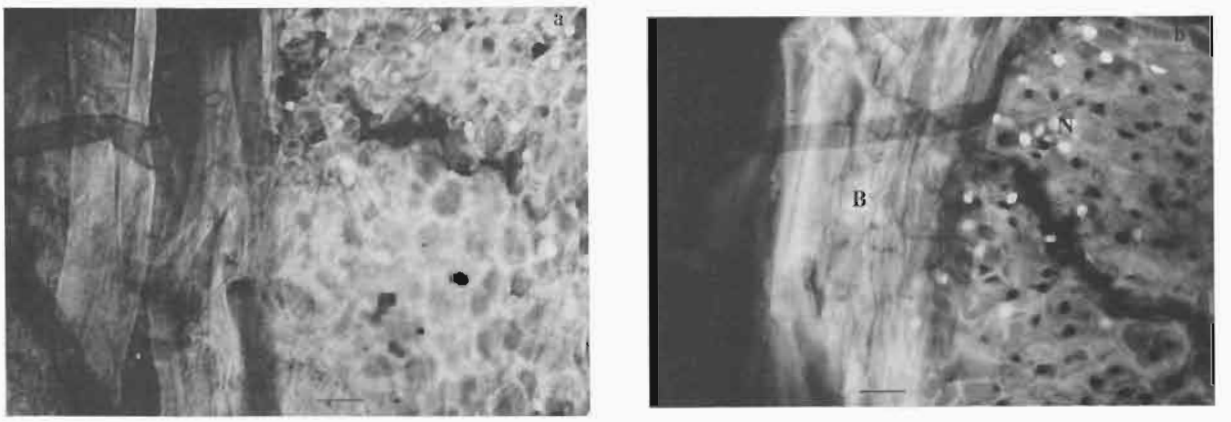

Plate 3. Confocal micrographs of basal region of *para*-nodules. a: control treated with 2,4-D (0.5 mg L^{-1}) alone. b: berated with *Azospirillum* and 2,4-D. A heavily colonized fluorescing basal region (B) containing cortical cells with an orientation parallel to the vertical root axis, root hair (R) and bright plant nuclei (N) within cells of the meristematic zone of the *para*-nodule are shown: note that individual bacteria cannot be distinguished at this magnification. Magnification, *bar* = 40 um.

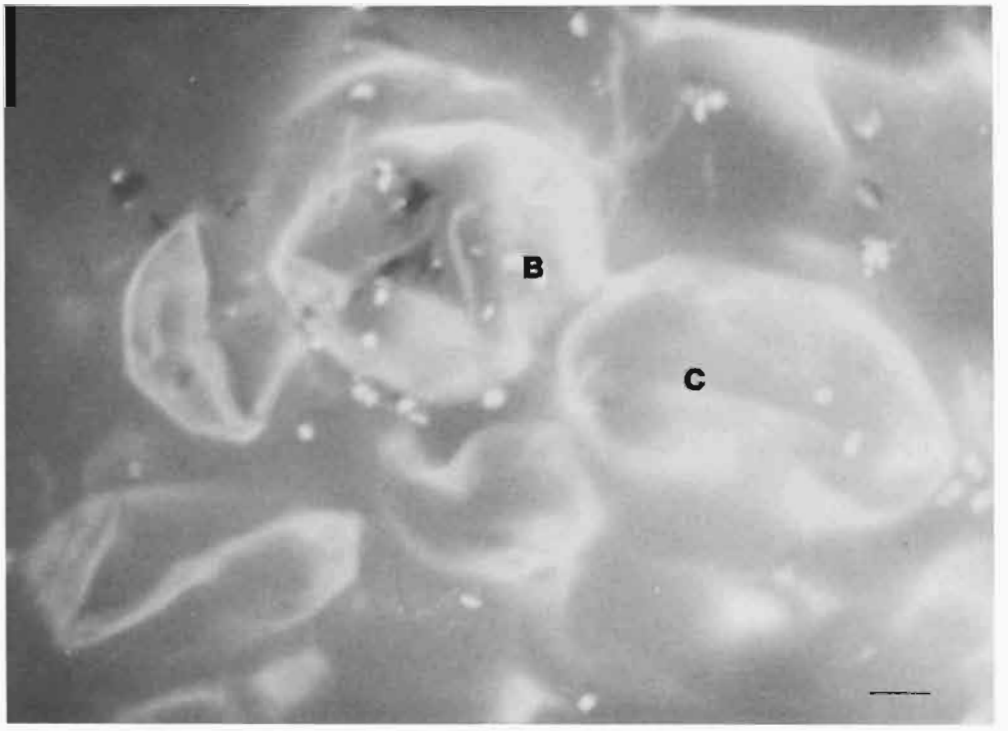

Plate 4. Confocal micrograph of central region of *para*-nodule. Bacteria (B) are shown interspersed with enlarged plant cells (C) in the central region of the *para*-nodule. Magnification, *bar* = 8 µm.

Discussion

Observations made either by microscopy (Plates 3 and 5) or by direct bacterial counts (Fig. 4) indicate that synthetic auxins such as 2,4-D increase the degree of colonization of the interior of the root system of wheat seedlings with *Azospirillum*. The sterilization with $HgCl_2$ probably led to the death of many azospirilla in the endorhizosphere, in addition to all those external

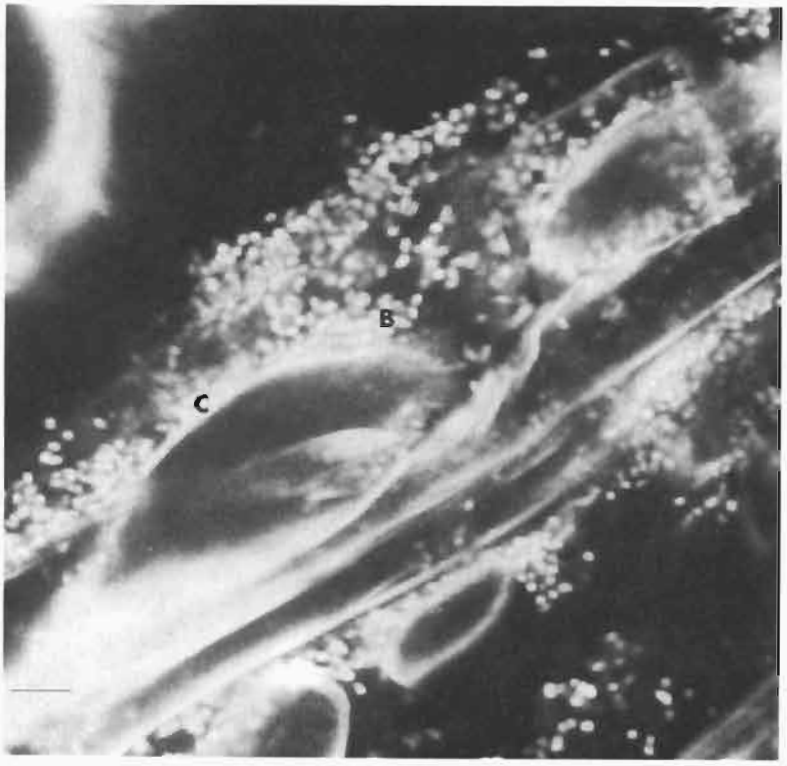

Plate 5. Confocal micrograph of basal cells of *para*-nodule. Azospirilla, confirmed as intercellular in the basal region of *para*-nodules by changing the plane of focus, are shown. Bacteria (B), cell wall (C). Magnification = 20 μm.

to the root. More informative means of estimating internal colonization and of attributing nitrogenase activity to particular colonies of bacteria would be valuable. However, the data presented in this paper indicate a strong general correlation between the stimulating by 2,4-D of internal colonization by *Azospirillum brasilense* SP7 and increased rates of acetylene reduction.

In the natural associative system with wheat, *Azospirillum* is known to colonize the root surface (rhizoplane) and intercellular spaces in the root cortex (endorhizosphere) (Bashan and Levanony, 1988; Christiansen-Weniger, 1988; Döbereiner and Pedrosa, 1987). The relative proportion of viable azospirilla located in the endorhizosphere compared to the rhizoplane is a controversial topic and may be low (Jain and Patriquin, 1984; New et al., 1991), particularly with some strains, although other work indicates significant endorhizosphere colonization (Baldani et al., 1986). If internal colonization is too limited, this could limit the potential for nitrogen fixation by such associative systems because of poor access to carbon substrates in the rhizosphere (Whipps and Lynch, 1983), even without competition, and the effect of inactivation of nitrogenase in azospirilla at the rhizoplane by oxygen.

It has been suggested that the sites of emergence of lateral roots are normally the ports for infection of *Azospirillum* entering the endorhizosphere (Okon, 1982; Umali-Garcia et al., 1981). Possibly, the colonization of the basal region of *para*-nodules observed with 2,4-D treatment involves the same point of entry, resulting from the formation of 'wound' tissue following induction of these structures. Initiation of the characteristic round-shaped *para*-nodules and the associated basal cell enlargement are mainly a re-

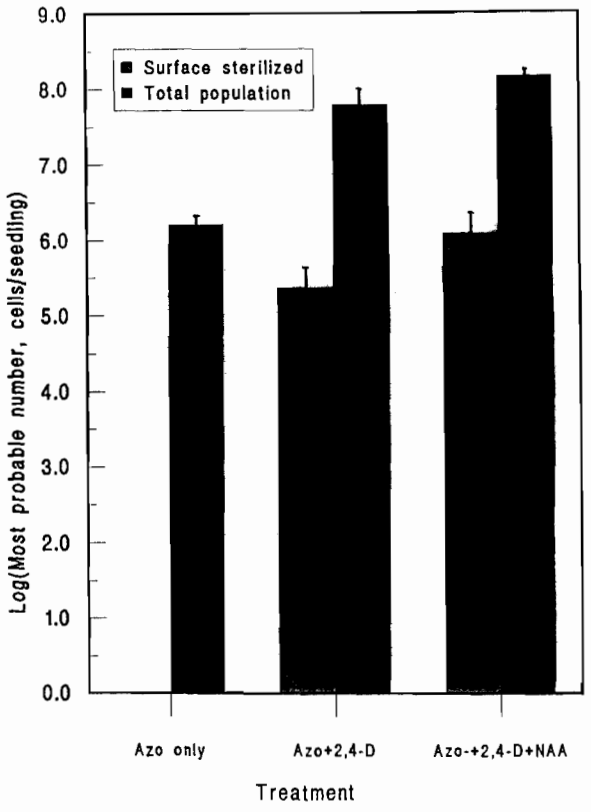

Fig. 4. Dilution tube counts of azospirilla in surface-sterilized and unsterilized wheat roots per seedling. A significant stimulation by auxin treatment of internal colonization ($\log_{10}$MPN) with both 2,4-D ($p < 0.01$) and 2,4-D plus NAA ($p < 0.05$) was shown by analysis of variance and computation of least significant differences for particular effects. The figure shows mean values for single seedlings analysed in triplicate (standard errors indicated on the graph).

sponse to 2,4-D, since this occurs in the absence of bacterial inoculation.

The ethylene production observed here to be stimulated by auxin treatment (Table 1) is acetylene dependent, requires inoculation with nitrogen-fixing azospirilla and corresponds with the extent of colonization. This suggests that the activity observed is indeed nitrogenase, supported by the negative result obtained when a *Nif*- mutant was employed for inoculation (Zeman et al., 1992) and our work employing ammonium and nitrite to inhibit all the ethylene formation, the observation of H_2 evolution associated with the system and the use of $^{15}N_2$ (Yu et al., 1992).

The increase in the rate of acetylene reduction observed here with auxin treatments needs to be interpreted carefully. Assays were performed under conditions of oxygen pressure reduced from that in which the seedlings were grown, but with oxygen pressure sufficient to inhibit nitrogenase activity by azospirilla at the root surface. Thus, primarily the activity of azospirilla protected from oxygen within the root system was measured. The initial absence of acetylene-reducing activity (Figs. 1 and 2) is not understood, but it could have resulted from an inactivated state of the nitrogenase in seedlings cultured in hydroponic solution exposed to air, or by the disturbance of their transfer for assay. A lag may be required for depression of nitrogenase in azospirilla maintained under N_2-fixing conditions (low O_2 and low fixed nitrogen) in the assay tubes. Such lags in the expression of nitrogenase activity are characteristic of diazotrophs associated with grasses, apparently sometimes a result of a need to deplete fixed nitrogen (Van Berkum, 1978, quoted in Giller and Wilson, 1991).

However, the data in Figure 2 indicate that the disturbance caused by evacuation and re-gassing without exposure to air also caused a reversible lag in acetylene reduction, of shorter duration. Thus the initial lag is possibly also partly an outcome of the metabolic regulation of nitrogenase activity, previously described for free-living *A. brasilense* (Hartmann and Burris, 1987). With a generation time of approximately 6 h in free-living N_2-fixing conditions, the numbers of *Azospirillum* could potentially quadruple in 12 h. From our observations with the microscope, there may be some increase in internal bacterial cell numbers during assays, but the time course could only partly explained by such new growth. Nor can the appearance of acetylene-reducing activity be attributed to the external growth of azospirilla. The oxygen levels of 2.5% or 4% used in this study completely prevent the expression of nitrogenase activity by unprotected azospirilla under these conditions of assay (Tchan et al., 1991) and we have confirmed by direct measurement that respiratory consumption of oxygen is insufficient to reduce the oxygen pressure relieving the inhibition.

Examination of the roots of seedlings treated with 2,4-D giving high rates of acetylene-reducing activity by fluorescent and confocal microscopy confirms the presence of large numbers of azospirilla associated with the nodular structures. From this association we conclude that the effect of auxins such as 2,4-D for wheat seedlings grown hydroponically is to create a niche in which azospirilla can grow. Most auxins have the ability to increase the number of lateral or adventious roots and among these NAA is very effective (Blakely et al., 1972). In our results, although IAA applied at higher concentration gave responses morphologically similar to NAA, the resultant acetylene reduction rates were usually lower and variable. This might be attributed to the reduced biological or chemical stability of IAA compared to both NAA and 2,4-D. In pea root segments, it has been demonstrated that IAA and NAA taken up were metabolically conjugated to other molecules but 2,4-D was not metabolized (Andrae, 1967). 2,4-D is also known to be transported less specifically and more slowly than IAA. Therefore, the effect of 2,4-D in a particular location is much stronger than any other auxins (Penny and Penny, 1978), consistent with our results. While auxins play a major role in root formation in many plant species, cytokinins and GAs were found to be inhibitory (Sriskandarajah, 1984). In agreement with these findings, GA_3 and the cytokinin used in our study not only inhibited lateral root development but also had a deleterious effect on the whole root system.

While 2,4-D exerted a dominant effect in controlling the shape of the induced structures, it did not cause the initiation of greater numbers of lateral structures than NAA. The distinctive effect of 2,4-D could also result from its different pattern of uptake and metabolism in plant tissue (Allen et al., 1953; Kim and Bidwell, 1967). With low levels of IAA, IBA and NAA (less than $0.1\,mg\,L^{-1}$), the laterals developed into normal root structures, although still shortened. Therefore, these structures formed with synthetic auxins are modified lateral roots and not the callus tissue that auxins have been known to induce on the roots of some plant species (Schaeffer et al., 1984; Witham, 1968).

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