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Regional Distribution and pH Sensitivity of *Azospirillum* Associated with Wheat Roots in Eastern Australia

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Abstract. In a survey of *Azospirillum* spp. on the roots and associated soil of wheat grown in eastern Australia, azospirilla were isolated from approximately 40% of samples from areas of soil pH between 5.0 and 6.6. However, azospirilla isolates were rare in soil between pH 4.5 and 5.0 and absent below pH 4.5. Of 25 independent isolates, 17 were *A. brasiliense* and eight were *A. lipoferum*. No selection for *A. brasiliense* Nir^- strains by wheat roots was observed. Only one of six endorhizosphere isolates were *A. brasiliense* Nir^-, compared with three of nine from unsterilized roots plus associated soil, and three of eight from soil. With a medium buffered with 0.05 M malate and 0.05 M phosphate, it was found that all *Azospirillum* isolates had a lower minimum pH for growth when supplied with fixed nitrogen than when grown under nitrogen-fixing conditions. Strains isolated from soils had a minimum pH for growth that was less than the pH of the soil from which they were isolated. However, a significant proportion of strains isolated from roots had a minimum pH for growth that was higher than the pH of the associated soil suggesting that the wheat roots provided an ecological niche protecting against soil acidity.

Introduction

Bacteria of the genus *Azospirillum* are soil organisms commonly found in intimate association with the roots of grasses and other plants [22]. Although known to fix atmospheric nitrogen microaerophilically under laboratory conditions, it is uncertain to what extent *Azospirillum* fixes nitrogen in the field or whether it makes a significant contribution to the nitrogen nutrition of its host plant. A number of authors have reported significant azospirilla-associated improvements in yield of crop plants, particularly of wheat [10, 11, 13, 19, 23], but failures to observe yield improvements in the field are also common [3, 5, 23]. The role of nitrogen fixation in the observed yield increases has also been questioned, with some studies suggesting that stimulation of root growth by bacterial production of plant growth hormones is the major mechanism involved [17, 18].

The reasons for the variable response of plants to inoculation with *Azospirillum* are likely to be complex, involving environmental as well as biological

factors. It is known that there is some specificity in the relationship between plant genotype and bacterial strain. For example, wheat plants are stimulated more strongly by azospirilla isolated from the endorhizosphere of wheat than by isolates from the wheat root surface or from the roots of other host plants [3, 4, 12]. However, there is little information on the influence of edaphic and climatic factors on the bacteria-plant association.

In this preliminary study, we report a survey of azospirilla on roots of wheat plants growing mainly in the agricultural regions of New South Wales. Soil pH emerged as a major factor affecting the occurrence of these bacteria. The results suggest a strategy for future field tests to examine plant responses such as yield increases.

Methods

Bacterial Strains and Identification

Strains of azospirilla were obtained from culture collections (Table 1) or were isolated from roots or root-associated soil of wheat plants grown in New South Wales (Table 2, Fig. 1). Cultures were grown on peptone-yeast-extract (PYE) agar or PYE broth (composition in g/liter: peptone 10, yeast extract 5, NaCl 5, pH 7.2).

Bacteria were identified to species level on the basis of several criteria, including serological characteristics, utilization of certain carbon and energy sources, cell morphology in alkaline media, and cultural characteristics. Utilization of organic acids and carbohydrates was determined in NPb semi-solid medium in which the malate was replaced by 0.5% glucose, sucrose, or α-ketoglutarate (NPb = Nfb medium of Krieg and Döbereiner [16] but pH 6.3). Strains that could utilize a carbon source formed a rising pellicle and raised or lowered the pH, as detected by the bromothymol blue pH indicator in the medium.

The ability to reduce nitrite to N_2O (Nir^+ character) was determined by the method of Baldani and Döbereiner [4] and confirmed by following the reduction of nitrate in nitrate broth (PYE plus 0.1% KNO_3, pH 6.3). An inverted Durham tube trapped bubbles of gas produced by denitrification. Nitrite was detected by reaction with sulphanilic acid (0.8% in 5 M acetic acid) and α-naphthylamine (0.5% in 5 M acetic acid), and residual nitrate was detected following reduction to nitrite by zinc dust.

Serology

Antisera were prepared in New Zealand white rabbits against cells of *A. brasiliense* Sp7 and *A. lipoferum* Sp59b grown in malate broth (Nfb medium [16] plus NH_4Cl, 1 g/liter, minus the agar). Cells were harvested by centrifugation and washed and suspended in normal saline at a concentration of ca. 5×10^8 cells/ml. Immunization was by intradermal injection of 2 ml of a 1:1 (v/v) emulsion of cell suspension in Freund's Complete Adjuvant, followed 1 month later by 1 ml (intradermal injection) without adjuvant. Blood was collected from the marginal ear vein at weekly intervals. Both antisera had an agglutination titer of 80 against heated homologous cells.

Bacterial strains were identified by a slide agglutination test, in which PYE broth cultures of bacteria were diluted 1:10 (v/v) in 0.02 M phosphate-buffered saline (PBS) and mixed with an equal volume of a 1/30 dilution of anti-Sp7 or anti-Sp59b serum or a 1/20 dilution of pre-immune control serum. Drops of the bacteria-serum mixtures were placed on microscope slides, incubated at 37°C for 1–2 hours, and examined for agglutination and/or inhibition of motility under dark-field illumination at $\times 40$ and $\times 100$ magnification.

Table 1. Details of supplied azospirilla strains

Species and strain no.	Source[a]	Minimum pH for growth[b]		Nitrite reduction
		N_2-fixing	With fixed N	
A. brasiliense				
Sp7	UQM 1774[T] *Digitaria decumbens*, Brazil	6.0	5.3	+
Cd	ATCC 29710 *Cynodon dactylon*, USA	6.0	5.3	+
Sp107	BGR Wheat roots, Brazil	5.9	5.3	−
Sp245	BGR Wheat roots, Brazil	6.3	5.3	+
101	DH Wheat straw enriched soil, Australia	6.0	5.3	n.t.
114	DH Wheat straw enriched soil, Australia	6.0	5.9	n.t.
A. lipoferum				
Sp59b	ATCC 29707[T] Wheat roots, Brazil	5.5	5.0	+

[T] Type strain; n.t. = not tested

[a] Suppliers: ATCC = American Type Culture Collection, Rockville, Maryland; BGR = B. G. Rolfe, Genetics Dept, Australian National University, Canberra, A.C.T., obtained from J. Döbereiner, Brazil; DH = D. Halsall, Division of Plant Industry, CSIRO, Canberra, A.C.T. (see ref. 9); UQM = School of Microbiology, University of Queensland, St. Lucia, Queensland, Australia

[b] The lowest tested pH giving growth. Only the following pH values were tested: 4.0, 4.5, 5.0, 5.3, 5.5, 5.7, 5.9, 6.0, 6.3, 6.5, 7.5, 8.0

Sampling and Isolation Methods

Wheat plants were sampled by excavating the roots to a depth of 10–20 cm in a block of soil with surface dimensions of at least 5 × 12 cm. Samples were taken on one occasion only from each farm, the wheat plants ranging from the three leaf to the seed formation stage of growth. In the majority of cases, the plastic bags containing the samples were incubated at 25–30°C for 3–5 days to encourage multiplication of azospirilla, as the wheat had been sampled during winter or spring when soil temperatures were considerably lower than the optimum temperature for these bacteria. Samples were then stored at 4°C until isolation could be attempted.

Azospirillum spp. were isolated from roots and soil by inoculating tubes of NPb or NFS semi-solid media. (NFS: the same as NPb, but with malate and bromothymol blue replaced by 0.5% sucrose and 0.001% bromocresol purple, respectively). Particles of soil from around the roots were picked up by a sterile wire loop and stab-inoculated into the media. A sample of 0.2–1.0 g of wheat roots was washed for 7 min in running tap water, then placed in sterile distilled water and divided into two equal portions, one of which was surface sterilized by immersion in 70% ethanol for 2 min followed by 1% NaOCl for 5 min. After thorough rinsing in sterile distilled water, the roots were cut into pieces 1–2 mm long and the pieces (approx. 4/tube) were used to inoculate four tubes of each medium. The roots in the other portion were placed in sterile 1/5-strength Fåhraeus solution [8], and ground with a mortar and pestle for 1 min. Dilutions of 1/10 and 1/100 in Fåhraeus solution and the undiluted macerated roots were each used to inoculate two tubes of each medium (0.1 ml per tube).

Tubes producing a typical rising pellicle [16] were used to inoculate plates of Congo Red agar [20] in the case of NPb tubes, or NBS or NMS agar in the case of NPS tubes (NBS agar (per liter): $(NH_4)_2SO_4$, 0.5 g; DL-malic acid, 5.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; Nfb trace elements [16], 2.0 ml; Nfb vitamin solution [16], 1.0 ml; $FeCl_3 \cdot 6H_2O$, 0.047 g; KOH, 4.0 g; agar, 18.0 g; pH 6.3. NMS agar was the same as Nfb medium with the addition of 5.0 g sucrose and 0.02 g yeast extract per liter, with 15 g/liter agar). Single colonies from these plates were further purified by streaking on plates of PYE or NBS agar, and were retested for pellicle formation in nitrogen-free, semi-solid media before storage of cultures.

The soil associated with the roots was then examined to determine pH (1:5, w/v, slurry in 0.01 M $CaCl_2$ with 24-hour equilibration).

Table 2. Isolation details, acid tolerance, and nitrite-reducing ability of wheat root isolates

Species and location[a]	Isolation details		Minimum pH for growth[b]		Nitrite reduction	Strain no.
	Soil pH	Root vs. soil[c]	N_2-fixing	With fixed N		
A. brasiliense						
Cowra (1)	4.5	RS	5.7	5.5	−	585
Cowra (1)	4.7	R	6.3[d]	5.3	−	586
Cowra (2)	5.1	R	6.3[d]	5.7	+	587
Griffith (1)	5.4	S	6.3[d]	5.5	+	593
Griffith (2)	5.4	S	n.t.[e]	n.t.	−	594
Griffith (2)	5.7	S	6.3[d]	5.5	−	592
Griffith (2)	5.8	R	5.5	5.0	+	597
Gunnedah (1)	5.5	RS	6.3[d]	5.9	+	583
Gunnedah (2)	6.4	RS	6.3[d]	5.9	+	576
Moree (1)	5.8	RS	6.3[d]	5.7	+	579
Moree (1)	5.8	S	n.t.	n.t.	+	578
Tamworth (1)	6.5	S	6.0	5.9	+	581
Tamworth (1)	6.5	RS	n.t.	n.t.	+	582
Tamworth (2)	n.t.	RS	6.3[d]	5.7	−	575
Tamworth (3)	n.t.	RS	n.t.	n.t.	−	572
Narrabri (1)	n.t.	R	6.0	5.7	+	562
Narrabri (2)	n.t.	S	5.7	5.5	−	564
A. lipoferum						
Griffith (1)	5.0	RS	n.t.	n.t.	+	596
Griffith (2)	5.2	R	5.3	5.3	+	591
Griffith (2)	5.4	S	5.9	5.3	+	598
Griffith (2)	5.6	R	5.7	5.3	+	600
Griffith (2)	6.2	RS	6.3[d]	5.9	−	595
Tamworth (3)	n.t.	RS	6.0	5.3	−	571
Narrabri (2)	n.t.	S	5.9	5.3	−	565
Narrabri (2)	n.t.	RS	6.0	5.3	−	566

[a] General region surrounding a designated town. Different farms are distinguished by different numbers in brackets
[b] The lowest tested pH giving growth. Only the following pH values were tested: 5.0, 5.3, 5.5, 5.7, 5.9, 6.0, 6.3, 6.5, 7.5, 8.0
[c] Isolated from soil (S) or from surface-sterilized roots (R) or from roots plus associated soil (RS)
[d] pH 6.0 not tested
[e] n.t. = not tested

pH Tolerance of Strains

The ability of *Azospirillum* strains to grow at different pH values was measured in semisolid media based on NPb, but having increased buffering capacity. Several buffers were tested with malate or fructose as the carbon and energy source. Medium NPH3, which is the same as NPb except for increased concentrations of malate (6.7 g) and K_2HPO_4 (8.7 g), gave the best buffering capacity and best growth of all media tested, and was used to test all the bacterial strains in the absence of fixed nitrogen (NPH3−U) or the presence of 0.002% urea (NPH3+U). A decrease in the firmness of the semisolid media was noticed at the lowest and highest pH values, and in some tests this was overcome by doubling the concentration of agar at all pH values.

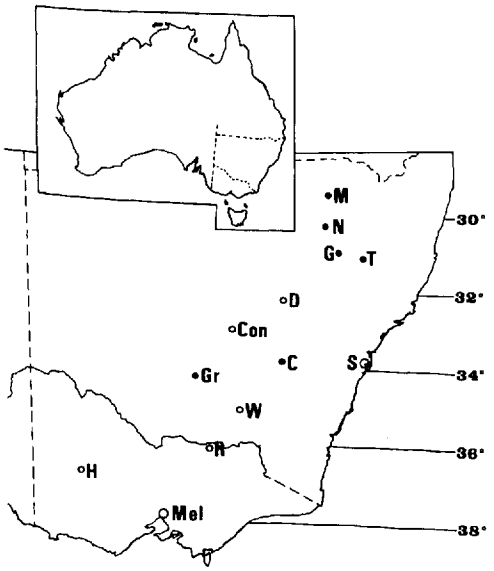

Fig. 1. Map indicating field sites from which samples were obtained (● = *Azospirillum* isolated from wheat root samples; ○ = *Azospirillum* not isolated). State capital cities Sydney (S) and Melbourne (Mel) as well as the regional centers (North to South) Moree (M), Narrabri (N), Gunnedah (G), Tamworth (T), Dubbo (D), Condobolin (Con), Cowra (C), Griffith (Gr), Wagga Wagga (W), Rutherglen (R), and Horsham (H) are shown.

Each strain was grown in NPb medium until a good pellicle was produced. The pellicle was then harvested with a minimal volume of medium using a Pasteur pipette, which was then used to thoroughly mix the pellicle and medium by resuspension using repeated suction. One or two drops of mixture were used to inoculate 5 ml of NPH3 buffer at each pH being tested (NPH3 buffer: as for NPH3−U, but without agar), and 0.1 ml of each inoculated buffer was then used to inoculate one tube each of NPH3−U and NPH3+U at the same pH and one tube of NPb. The inoculum contained 10^6–10^7 cells/ml (total count). Growth (the presence of a typical pellicle) was assessed after incubation at 35°C for 2–9 days, and purity of the culture was checked by streaking from one of the positive tubes onto a PYE plate.

Results

Isolations

Azospirillum was isolated from wheat roots and associated soil from 22 of 100 samples, representing 13 of the 32 farms that were sampled (see Fig. 1). Isolation data on all independently isolated strains are given in Table 2.

Of the 88 wheat root samples for which soil pH was measured, azospirilla were not isolated from any of the 31 with soil pH below 4.5 and were infrequently isolated between pH 4.5 and 5.0. Approximately 40% of wheat root samples from soil between pH 5.0 and 6.6 contained *Azospirillum* (Table 3). No azospirilla were isolated from two regions of very low soil pH, i.e., Wagga Wagga (three sites, 21 samples, pH 3.3–5.1) and Rutherglen, Victoria (four sites, four samples, pH 3.5–5.0). A third region of low soil pH (Cowra, four sites, 20 samples, pH 3.7–5.5) yielded azospirilla in only three of 20 samples.

Azospirillum sp. was not isolated from the Dubbo region (three sites, five samples, pH 4.6–6.5), Condobolin (four sites, four samples, pH 4.6–6.0) or Horsham, Victoria (one site, three samples, pH 6.5–6.7) (Fig. 1).

Table 3. Isolation of *Azospirillum* from soils of different pH

pH	No. of sites[a]	No. samples tested	*Azospirillum* isolated		
			No. sites	No. samples	% samples
3.3–4.49	9	31	0	0	0
4.5–4.99	10	19	2	3	16
5.0–5.49	8	13	3	5	38
5.5–5.99	9	14	4	6	43
6.0–6.59	7	8	3	3	38
6.6–7.2	2	3	0	0	0
3.3–7.2	30	88	9	17	19

[a] Each site is one farm, but may contain soils in more than one pH range

Identification of Isolates

All strains utilized malate, producing a typical *Azospirillum* pellicle in semi-solid nitrogen-free medium. Of 25 separate isolates associated with wheat roots, 17 were identified as *A. brasiliense* and eight as *A. lipoferum*, on the basis of cell morphology and cultural characteristics. Strains of *A. lipoferum* were isolated only from sites at Griffith (two farms), Tamworth (one farm), and Narrabri (one farm), whereas *A. brasiliense* was present at every positive site.

A. lipoferum tended to be pleomorphic producing many larger cells of variable shape in NPb medium which had become alkaline during incubation; *A. brasiliense* cultures did not exhibit such pleomorphism, although older cultures contained enlarged roughly spherical cells (C-cells) which were refractile under phase contrast microscopy [16].

Half of the strains of both species were nitrite reductase negative (Nir^-) (Table 2). Omitting rhizosphere isolates 579 and 582, which appear to be identical to the soil isolates 578 and 581, respectively, the proportion of Nir^- isolates from surface-sterilized roots, from roots plus associated soil, and from soil were 1/4, 3/5, and 3/6, respectively, for *A. brasiliense*, and 0/2, 3/4, and 1/2, respectively, for *A. lipoferum*.

A number of local isolates (seven *A. brasiliense* and four *A. lipoferum*) did not agglutinate with the antisera against strains Sp7 and Sp59b. Most of the remaining *A. brasiliense* isolates were agglutinated to some extent by both antisera; only five isolates gave a stronger reaction with the anti-*A. brasiliense* serum. Serology was more useful in identifying *A. lipoferum*, because half the local isolates of this species reacted strongly with anti-*A. lipoferum* serum and not at all with anti-*A. brasiliense* serum.

When tested for their ability to utilize various carbon and energy sources, 16 of the *A. brasiliense* strains failed to grow on glucose, sucrose, or α-ketoglutarate as expected [16], but one strain (597) was unusual in utilizing glucose. The expected pattern of utilization of glucose and α-ketoglutarate but not sucrose was observed in only four of the *A. lipoferum* strains, whereas two more strains (591, 595) could utilize only glucose, and one strain (596) used all three carbon sources. Strain 571 was allocated to *A. lipoferum* on the basis of cultural,

morphological, and serological properties, but resembled *A. brasiliense* in its failure to grow on all three carbon sources. Nir^- and Nir^+ strains were found in all groupings based on utilization of carbon sources or on serological reaction.

Design of a Buffered Medium

It was necessary to find a buffer system that would stabilize pH over the whole of the range pH 4.0–8.0, particularly in the lower half of the range. Most buffers used for microbiological work operate near neutrality and are effective only within one pH unit of the pK of the buffer salt. Pyrophosphate was tested for suitability as a single buffer compound, because it has four pK values (0.85, 1.5, 5.8, 8.2), but it was found toxic to some strains even at a concentration of 0.0125 M.

Potassium hydrogen phthalate (buffers in the pH range 2.0–6.0, on the basis of its pK values), citrate (pH 2.5–7.0), and malate (pH 3.0–5.5) were assessed for use in conjuction with 0.05 M phosphate (pH 5.8–8.0). Potassium hydrogen phthalate (0.05 M) was unsuitable because its inclusion led to gradual discoloration of the indicator in the medium following autoclaving, and citrate was toxic to some strains at concentrations above 0.025 M.

Malate (0.05 M) was chosen as the low pH component of the buffer, as all components of the medium could be autoclaved together and this concentration was completely noninhibitory to most strains of azospirilla, being only slightly greater (0.67%) than the concentration normally used in NPb medium (0.5%). Malate at the proposed concentration had considerably greater buffering capacity than 0.02 M citrate (2.5 mmol malate required approx. 1.65 mmol NaOH per pH unit change between pH 3.5 and 5.8, whereas 1.0 mmol citrate required 0.68 mmol NaOH per pH unit change) but is utilized as a carbon source by all species of *Azospirillum.*

Fructose was tried, but was found to be unsuitable as a carbon source in the buffered medium. Although it was utilized preferentially to malate when both were present in the medium, growth on fructose was much weaker than on malate. Therefore, malate was used as the sole carbon source as well as one component of the pH buffering system, even though the pH must inevitably rise as malate is utilized. This is not a problem at low pH, as the organism must be capable of growing at the initial pH in order to alter the pH to a higher (more favorable) value, but it could be a problem at pH levels near the maximum for a strain, as slight growth could raise the pH and prevent further growth. Thus, the figure obtained for the upper pH limit will be an underestimate of the true value.

Acid Tolerance of Strains

Twenty-seven strains, including 20 local isolates from wheat roots, were tested for growth at different pH values. *A. lipoferum* was generally more sensitive to alkaline pH, with only one (571) of eight strains able to produce visible growth in NPH3+U tubes of initial pH 8.0, and three more (Sp59b, 566, and 591)

able to grow at pH 7.5. In contrast, all 19 strains of *A. brasiliense* grew under nonnitrogen-fixing conditions in tubes of initial pH 7.5, and all but two (564 and 593) grew in medium of initial pH 8.0.

All strains could tolerate more acidic conditions when grown in the presence of fixed nitrogen than in its absence. Only seven out of 20 local wheat root strains could grow at pH 5.9 under nitrogen-fixing conditions, whereas all could do so when supplied with urea (Table 2). There was a higher proportion of acid-tolerant strains among the isolates of *A. lipoferum*. When supplied with fixed nitrogen, six of seven strains of *A. lipoferum* grew at pH 5.3, compared with two of 13 strains of *A. brasiliense*.

It is noteworthy that a significant proportion of strains were isolated from wheat growing in soils of a lower pH than the minimum pH for growth of those strains. Under nitrogen-fixing conditions, 57% of strains could not grow within 0.1 pH unit of the pH of the soil from which they were isolated, and 29% could not do so even when supplied with fixed nitrogen. There was no significant correlation between soil pH and minimum pH for growth under nitrogen-fixing or nonnitrogen-fixing conditions for the strains as a whole or for the strains isolated from the root interior or root surface. However, there was a trend for soil isolates from soils of lower pH to be more acid tolerant in medium containing fixed nitrogen (correlation coefficient = 0.943, $0.05 < P < 0.10$).

Discussion

Strain Identification

A. brasiliense Nir^+ strains were as common as Nir^- strains in the present study, with no particular selectivity noted in the rhizosphere, where proportions of both groups were similar to those in the soil. Although *A. lipoferum* was isolated from only a few farms, it was as likely to be closely associated with wheat roots as with soil at those sites. It appears that the observation made in Brazil [4], that *A. brasiliense* Nir^- strains comprise almost 100% of isolates from the endorhizosphere of wheat, with increasing proportions of *A. brasiliense* Nir^+ and *A. lipoferum* on the root surface and in the surrounding soil, does not apply in all ecological situations.

Based on cell morphology and utilization of three carbon substrates, the strains of *A. brasiliense* on wheat roots in eastern Australia conform reasonably well to the properties of that species described in other countries [16]. One strain could utilize glucose, a property reported in 9% of strains of this species [22]. However, four of eight *A. lipoferum* strains were atypical in their utilization of carbon sources.

A. lipoferum strain 596 could utilize sucrose as sole carbon source, a property normally found only in *Azospirillum amazonense* [2], from which it differs in cultural, morphological, and serological properties and in ability to utilize α-ketoglutarate. *A. amazonense* was not isolated, despite the use of media containing sucrose (NPS, NMS) at the favorable pH of 6.3. If present at all in

eastern Australia, *A. amazonense* must have a very limited distribution on wheat roots.

Soil pH Values and the Occurrence of Azospirilla

From the results, a general picture is clear in which the organism is plentiful in soils above pH 5.0 but virtually absent from soils below pH 4.5 (0.01 M $CaCl_2$). From a very small number of samples, some indication of sensitivity to alkaline pH in soils was obtained, suggesting an optimum range of soil pH values of 5.0–7.0 for the occurrence of this organism in the rhizosphere of wheat plants. The technique used here of employing wheat seedlings as a "trap" to indicate the occurrence of azospirilla in soil is a selective one. The actual distribution of the genus may therefore have been wider than is apparent in this work.

The data indicate that azospirilla survive poorly in acidic soils; the cause of this (e.g., Al toxicity) remains to be determined. Intolerance of acidic conditions is by no means rare in other soil bacteria such as *Rhizobium* [15] or *Nitrosomonas* [1]. However, our data indicate the existence of tolerant strains of azospirilla that might have practical significance. Also, a relationship between acid tolerance of soil isolates in laboratory media and pH of the soils is suggested by the data, although the number of strains is too low for certainty. If a real effect, this suggests that the number of acid-tolerant strains could be expanded by further isolations from fairly acidic soils.

The effect of pH on azospirilla isolated from wheat has not been intensively studied. Some workers have examined the effect of pH on nitrogen fixation rates in association with other grasses [21] or in pure culture in the laboratory [6]. The pH ranges for optimum growth of *A. amazonense, A. lipoferum,* and *A. brasiliense* strains from a variety of habitats were found to be 5.7–6.5, 5.7–6.8, and 6.0–7.3, respectively [2]. *A. brasiliense* strain Sp7 is reported to fix nitrogen at the extremes of pH 4.5 and 9.2 [6], but this result may be an artefact produced by high initial cell densities ($>10^8$ cells/ml, compared with approximately 2×10^4–2×10^5/ml in this work) and by working outside the effective pH range of the phosphate buffer used. There is one report [7] that strains of *A. brasiliense* and *A. lipoferum* isolated from roots are more tolerant of acid pH values (down to pH 5.7) in reducing acetylene than strains isolated from soil (optima nearer pH 7). However, we have not found any simple correlation between site of isolation (roots vs. soil) and pH minima for growth.

One practical consequence of the results is that the absence of azospirilla from some areas presents an excellent opportunity for demonstration of inoculation effects. Although the capacity of the organism to survive from one season to the next may be small in some soils, infection of the rhizosphere and roots may be substantial if the bacteria are directly inoculated onto wheat at planting. The wheat plant itself would be expected to exert an alkaline effect in the rhizosphere when absorbing nitrate ions, and the activity of azospirilla or other organisms in oxidizing any organic acid anions excreted from the wheat plant could be expected to exert a further alkaline effect [14]. Thus, the

survival of inoculated azospirilla could be favored. Once roots have been colonized, acidic conditions in the soil may have little effect. This is supported by the observation that almost half the isolates from roots came from plants growing in soil with pH values below the minima for growth of the particular bacterial strains (Table 2). There was no pH protection seen in the soil itself, all soil isolates having a minimum growth pH essentially the same as or lower than that of the soil in which they were found.

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References

1. Alexander M (1980) Effects of acidity on microorganisms and microbial processes in soils. In: Hutchinson T, Havas M (eds) Effects of acid precipitation on terrestrial ecosystems. Plenum Publishing Corp, New York, pp 363–364
2. Baldani JI, Baldani VLD, Seldin L, Döbereiner J (1986) Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated N_2-fixing bacterium. Int J Syst Bacteriol 36: 86–93
3. Baldani VLD, Baldani JI, Döbereiner J (1983) Effects of *Azospirillum* inoculation on root infection and nitrogen incorporation in wheat. Can J Microbiol 29:924–929
4. Baldani VLD, Döbereiner J (1980) Host-plant specificity in the infection of cereals with *Azospirillum* spp. Soil Biol Biochem 12:433–439
5. Boddey RM, Baldani VLD, Baldani JI, Döbereiner J (1986) Effect of inoculation of *Azospirillum* spp. on the nitrogen assimilation of field grown wheat. Pl Soil 95:109–121
6. Das A, Mishra AK (1983) Utilization of fructose by *Azospirillum brasilense.* Can J Microbiol 29:1213–1217
7. Döbereiner J (1983) Ten years *Azospirillum.* In: Klingmüller W (ed) Azospirillum II. Genetics, physiology and ecology. Birkhäuser Verlag, Basel, pp 9–23
8. Fåhraeus G (1957) The infection of clover root hairs by nodule bacteria, studied by a simple glass slide technique. J Gen Microbiol 16:374–381
9. Halsall DM, Turner GL, Gibson AH (1985) Straw and xylan utilization by pure cultures of nitrogen-fixing *Azospirillum* spp. Appl Environ Microbiol 49:423–428
10. Hegazi NA, Khawas H, Monib M (1981) Inoculation of wheat with *Azospirillum* under Egyptian conditions. In: Gibson AH, Newton WE (eds) Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra, p 493
11. Hegazi NA, Saleh H (1985) Possible contribution of *Azospirillum* spp. to the nutritional status of wheat plants grown in sandy soil. In: Klingmüller W (ed) Azospirillum III, genetics, physiology, ecology. Springer-Verlag, Berlin, pp 189–202
12. Jain DK, Patriquin DG (1984) Root hair deformation, bacterial attachment, and plant growth in wheat-*Azospirillum* associations. Appl Environ Microbiol 48:1208–1213
13. Kapulnik Y, Sarig S, Nur I, Okon Y (1983) Effect of *Azospirillum* inoculum on yield of field-grown wheat. Can J Microbiol 29:895–899
14. Kennedy IR (1986) Acid soil and acid rain: The impact on the environment of nitrogen and sulphur cycling. Research Studies Press/John Wiley and Sons, Chichester
15. Keyser HH, Munns DN (1979) Tolerance of rhizobia to acidity, aluminum and phosphate. Soil Sci Soc Am J 43:519–523
16. Krieg NR, Döbereiner J (1984). Genus *Azospirillum* Tarrand, Krieg and Döbereiner 1979, 79^{AL} (effective publication: Tarrand, Krieg and Döbereiner 1978, 978). In: Krieg NR (ed) Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore, pp 94–104

17. Kucey RMN (1988) Plant growth-altering effects of *Azospirillum brasilense* and *Bacillus* C-11-25 on two wheat cultivars. J Appl Bacteriol 64:187–196
18. Millet E, Avivi Y, Feldman M (1984) Yield response of various wheat genotypes to inoculation with *Azospirillum brasilense*. Pl Soil 80:261–266
19. Rai SN, Gaur AC (1982) Nitrogen fixation by *Azospirillum* spp. and effect of *Azospirillum lipoferum* on the yield and N-uptake of wheat crop. Pl Soil 69:233–238
20. Rodríguez Caceres FA (1982) An improved medium for the isolation of *Azospirillum* spp. Appl Environ Microbiol 44:990–991
21. Smith RL, Schank SC (1981) Factors affecting nitrogenase activity in grass field plots. In: Gibson AH, Newton WE (eds) Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra, p 495
22. Tarrand JJ, Krieg NR, Döbereiner J (1978) A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can J Microbiol 24: 967–980
23. Vlassak K, Reynders L (1981) *Azospirillum* rhizocoenoses in agricultural practice. In: Gibson AH, Newton WE (eds) Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra, p 494