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The role of root-associated *Klebsiella pneumoniae* in the nitrogen nutrition of *Poa pratensis* and *Triticum aestivum* as estimated by the method of ^{15}N isotope dilution

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Key words Blue grass *Klebsiella pneumoniae* *Poa pratensis* *Triticum aestivum* Nitrogen fixation Nitrogen nutrition ^{15}N isotope dilution Spring wheat

Summary The technique of ^{15}N isotope dilution was used to verify that nitrogen was fixed and transferred to the plant by *Klebsiella pneumoniae* strain Pp in association with *Poa pratensis* or *Triticum aestivum.* Surface sterilized, sprouting seeds were inoculated with *K. pneumoniae* and grown in sand in modified Leonard jars. Potassium nitrate enriched with ^{15}N was used to provide N concentrations ranging from 10–40 mg N l^{-1} nutrient solution. After 10–18 weeks the shoots and roots were analyzed separately for dry matter, N content, total N, and atom % ^{15}N excess. The acetylene reduction technique was used to test for the presence of N_2-fixing organisms on the roots. The data from ^{15}N isotope dilution demonstrated that up to 33.8% of N in the shoots of *P. pratensis* and 15.9% in those of *T. aestivum* were derived from associative N_2 fixation by *K. pneumoniae.* In most experiments the dry matter yield, N content, and total N yield of the shoots of *P. pratensis* were increased by *K. pneumoniae* inoculation, whereas inoculation had no significant effect on the dry matter yield, N content or total N of the shoots of *T. aestivum.*

Introduction

Among N_2-fixing Enterobacteriaceae, organisms of the genus *Klebsiella* have been isolated from the roots of several grasses growing in temperate zones[3,6,11,14,17,28]. Tjepkema and Burris[26] tested Wisconsin prairie grasses for nitrogenase activity and found that such activity, although very weak, was associated with the bluegrass *Poa pratensis* among others. Shearman *et al.*[25] investigated associative nitrogen fixation in 'Park' Kentucky bluegrass (*Poa pratensis* L.) with various N_2-fixing isolates. Undisturbed turfs of a bluegrass inoculated with *Klebsiella pneumoniae* (W6) originating from winter wheat[17] expressed significant nitrogenase activity, and there was a greater accumulation of nitrogen in aerial tissues of the test grasses than in those of control grasses. Wood *et al.*[29] confirmed that observation by demonstrating an association between *K. pneumoniae* (W6) and 'Park' Kentucky bluegrass that appeared to have some degree of specificity.

Conclusive proof that grasses derive some of their nitrogen from the atmosphere must be based on ^{15}N isotopic measurements of N_2, the true substrate. $^{15}N_2$ gas has been used successfully to demonstrate N_2

fixation in association with tropical grasses[4], sugarcane[15,22,23,24], and rice[5,10,30]. These studies showed that the level of N_2 fixation in such crops was lower than in legumes. The use of $^{15}N_2$ gas allows short term kinetic measurement, thus avoiding the risk of recording artefacts by growing plants in an enclosed atmosphere, as well as the need for complicated equipment[20]. ^{15}N isotope dilution was used to measure N_2 fixation in maize and sorghum inoculated with *Azospirillum lipoferum*[16], but no N_2 fixation was observed. Lethbridge and Davison [13] reported that N_2 fixation in wheat inoculated with several N_2-fixers was negligible unless carbohydrate was added. Rennie[18] used isotope dilution, and showed that when sufficient C substrate was available to the bacteria 12.6–38.0% of maize plant N was derived from associated N_2 fixation by *Azospirillum brasilense.*

In the work discribed in this paper our objective was to use ^{15}N isotope dilution in a simple system to determine whether root-associated *K. pneumoniae* enables *Poa pratensis* or *Triticum aestivum* to derive significant quantities of nitrogen from atmospheric N_2.

Materials and methods

Bacterial strain

N_2-fixing *Klebsiella pneumoniae* strain Pp was isolated from the roots of *Poa pratensis* growing in an abandoned, previously cultivated field[6]. The bacteria were grown in static malate broth for 48 h at 28°C[7,12]. The suspension obtained, containing *ca* 10^9 colony forming units per millilitre, was used for plant inoculation. Part of the inoculum was autoclaved to kill bacteria inoculation of control plants. For acetylene reduction assay bacterial samples were grown in 22-ml serum bottles in 5 ml of N-free semisolid medium[8] containing 0.25% glucose and 0.25% malate.

Plant material, inoculation, and conditions of growth

Modified Leonard jar assemblies[27] each consisting of a bottomless 1-litre wine bottle inverted on a 1-litre jar, were used. Each bottle was filled with 500 g of sand and the jar with Hoagland's nutrient solution[9] (1/4 concentration) containing KNO_3 to give concentrations of 10, 20, 30, and 40 mg KNO_3-N l^{-1} enriched with 0.5 or 1.0% $K^{15}NO_3$. Total amounts of nutrient solutions used were 1.6 l at N level of 20 mg N l^{-1} and 2.0 l at N level of 40 mg N l^{-1} in the first experiment. In the second experiment with *P. pratensis* the amounts were 1.0, 1.2 and 1.2 l, and with *T. aestivum* 1.8, 2.0, and 2.0 l at N levels of 10, 20, and 30 mg N l^{-1}, respectively. Sterile solution was added aseptically with an injection syringe, as required. The dry matter yields and total N yields in all tables were calculated per litre of nutrient solution used in the experiment to facilitate comparison.

Seeds of bluegrass (*Poa pratensis* cultivar Arina Dasas) and spring wheat (*Triticum aestivum* cultivar Ruso) were surface sterilized with 5% sodium hypochlorite and germinated on water agar plates as previously described[12]. They were then inoculated by incubating them in a suspension of *K. pneumoniae* Pp for 1 h at room temperature with occasional shaking, a procedure that allows the bacteria to become attached to the roots[12]. After inoculation 30 seeds of *P. pratensis* or 20 seeds of *T. aestivum* were planted in each autoclaved Leonard jar. Also, to ensure association of roots and bacteria, 50 ml of *K. penumoniae* Pp culture were added to the jars. Six jars were treated with living bacteria and six control jars with autoclaved bacteria. The seedlings were first grown under cover of a Petri dish lid. Gravel was then added to prevent

contamination, when seedlings were taller than 2–3 cm. The jars were wrapped in aluminium foil to prevent growth of photosynthetic micro-organisms. The plants were grown under greenhouse conditions with an 18-h photoperiod (mercury vapour lamps) from October 1982 to February 1983 or in sunlight from July to October 1983. *Poa pratensis* was grown for 16–18 weeks and *T. aestivum* for 10 weeks before harvesting.

Plant yield and nitrogen analysis

Shoots and roots were harvested and analyzed separately. Samples were dried overnight at 70°C and then weighed. The samples were ground and total nitrogen was determined as NH_4-N following Kjeldahl digestion[1]. After titration, excess H_2SO_4 was added and the samples evaporated to dryness. Atom % ^{15}N excess was determined with a Micromass 622 (VG-Isotopes Limited, England) mass spectrometer[2]. Seeds and inoculum were also analyzed for nitrogen to calculate the dilution of nitrogen caused by their addition to the system. In experiments with *P. pratensis* and in those with *T. aestivum* 14.3 mg ^{14}N per jar came from the inoculum growth medium, which consisted of mineral nitrogen, 0.4 mg from the seeds of *P. pratensis*, and 8.3 mg from seeds of *T. aestivum*, respectively. The amount of nitrogen in bacterial cells was negligible.

Acetylene reduction assay

Samples from the roots were incubated with acetylene in N-free semisolid medium in serum bottles. After incubation the production of ethylene was determined by gas chromatography as previously described[6,8].

Results

Effect of K. pneumoniae *Pp on growth of* P. pratensis

The first experiment was carried out in October 1982 to February 1983 at 20 and 40 mg N l^{-1} with an atom % ^{15}N excess of 1.0%. Greenhouse temperature was 20 to 24°C in daytime and 15–20°C at night. Plants were harvested after 18 weeks. The plants grown at the lower N level (20 mg N l^{-1}) and inoculated with viable *K. pneumoniae* Pp had significantly higher dry matter yields, total N yields and N contents than had controls treated with autoclaved bacteria (Table 1). Total N yield increased by 48%. The plants grown at the higher N level (40 mg N l^{-1}) and inoculated with viable *K. pneumoniae* Pp had significantly higher dry matter yields but not significantly different total N yields or N contents than had plants inoculated with autoclaved bacteria (Table 1). Incorporation of atmospheric N_2 into *P. pratensis* was detected with the plants that had been inoculated with viable *K. pneumoniae* Pp and grown at the lower N level. Atom % ^{15}N excess in the shoots was decreased significantly (Table 1), as compared with the controls. The roots also showed ^{15}N dilution, though the effect was not significant (data not shown). The percentage of N derived from atmosphere (% Ndfa) was 7.3 in the shoots and 4.0 in the roots. % Ndfa has been calculated according to Rennie and Rennie[20]:

Table 1. Effect of inoculation with *K. pneumoniae* Pp on growth of *P. pratensis* grown under greenhouse conditions from October 1982 to February 1983.

Inoculum	N level ($mg\ l^{-1}$)	Dry matter yield ($g\ l^{-1}$)[b]	Total N yield ($mg\ l^{-1}$)[b]	N content $mg\ g^{-1}$	Atom % ^{15}N excess	% Ndfa[a]
Viable	20	0.841 ± 0.163*	11.12 ± 3.18*	13.09 ± 1.52**	0.593 ± 0.043*	7.3
Deal	20	0.705 ± 0.036	7.51 ± 0.69	10.66 ± 0.77	0.640 ± 0.028	
Viable	40	1.406 ± 0.083**	24.15 ± 0.67	17.21 ± 1.23	0.862 ± 0.012	−8.7
Dead	40	1.228 ± 0.167	18.85 ± 3.09	13.69 ± 2.71	0.793 ± 0.090	

[a]
$$\%\ Ndfa = \left[1 - \frac{\text{Atom}\ \%\ ^{15}N\ \text{excess (fs)}}{\text{Atom}\ \%\ ^{15}N\ \text{excess (nfs)}}\right] \times 100$$

where fs = fixing system = inoculated with viable bacteria

nfs = nonfixing system = inoculated with heat-killed bacteria

[b] Calculated per litre of nutrient solution. Total amounts used were 1.6 l at 20 $mg\ N\ l^{-1}$ and 2.0 l at 40 $mg\ N\ l^{-1}$.

* Significantly different ($P < 0.05$) from heat-killed control by *t*-test.

** Significantly different ($P < 0.02$) from heat-killed control by *t-test*.

Figures refer to shoots and are means ± standard deviations for 4 to 6 replicates.

$$\% \text{ Ndfa} = \text{percentage of } N_2 \text{ fixed} = \left[1 - \frac{\text{Atom } \% \ ^{15}N \text{ excess (fs)}}{\text{Atom } \% \ ^{15}N \text{ excess (nfs)}}\right] \times 100$$

where fs = fixing system (plants inoculated with viable bacteria)

nfs = nonfixing system (plants inoculated with heat-killed bacteria)

The plants grown at the higher N level showed no incorporation of atmospheric N_2 (Table 1).

In order to verify the results of the first experiment and to find the most favourable N level for N_2 fixation the second experiment was carried out in July to October 1983 at 10, 20, and 30 mg N l^{-1} with an atom % ^{15}N excess of 0.5%. Greenhouse temperature was 20–28°C in daytime and 15–22°C at night. The plants were harvested after 16 weeks. Results are summarized in Table 2. The shoot dry matter yield had increased in the plants inoculated with viable *K. penumoniae*, as compared to controls (Table 2). The difference was significant at the level of 30 mg Nl^{-1}. There were no increases in the dry matter yields of the roots or in the total N yields and the N contents of the shoots or the roots (Table 2). At each N level the atom % ^{15}N excess had decreased in shoots and roots of the plants inoculated with viable bacteria (Table 2). In shoots of the plants grown at the level of 20 mg Nl^{-1} the difference was significant, as compared with controls. %Ndfa reached 33.8 in the shoots and 20.0 in the roots.

Effect of K. pneumoniae *Pp on growth of* T. aestivum

The experiment with *T. aestivum* was carried out in July to September 1983. Nitrogen levels and growth conditions were similar to those in the experiment with *P. pratensis*. The plants were harvested after 10 weeks. Results are summarized in Table 3. There were no differences in dry matter yield, total N yield or N content for either shoots or roots (data not shown) of the plants inoculated with viable *K. pneumoniae* Pp, as compared with the control plants. On the other hand atom % ^{15}N excess in the shoots had decreased at every N level (Table 3) and % Ndfa reached 15.9. There were scarcely any differences in the atom % ^{15}N excess for roots of the plants inoculated with viable or autoclaved bacteria (data not shown).

Nitrogenase activity

Roots of the plants inoculated with viable *K. pneumoniae* Pp showed high nitrogenase activity, whereas those of the control plants inoculated with autoclaved bacteria showed none. *Klebsiella pneumoniae* Pp was

Table 2. Effect of inoculation with *K. pneumoniae* Pp on growth of *P. pratensis* grown under greenhouse conditions from July to September 1983.

Inoculum	N level ($mg\ l^{-1}$)	Dry matter yield ($g\ l^{-1}$)[b]	Total N yield ($mg\ l^{-1}$)[b]	N content ($mg\ g^{-1}$)	Atom % ^{15}N excess	% Ndfa[a]
Shoots						
Viable	10	0.822 ± 0.120	10.46 ± 1.59	11.90 ± 0.27	0.182 ± 0.035	12.0
Dead	10	0.778 ± 0.008	9.76 ± 1.20	13.43 ± 1.91	0.205 ± 0.023	
Viable	20	0.986 ± 0.313	12.14 ± 3.93	12.81 ± 1.73	0.197 ± 0.072*	33.8
Dead	20	0.945 ± 0.240	12.73 ± 4.74	12.60 ± 1.23	0.295 ± 0.053	
Viable	30	1.258 ± 0.246*	17.63 ± 2.77	14.20 ± 1.55	0.315 ± 0.065	16.9
Dead	30	0.903 ± 0.172	18.11 ± 5.18	16.78 ± 1.76	0.379 ± 0.031	
Roots						
Viable	10	1.441 ± 0.148	6.85 ± 0.00	4.94 ± 0.44	0.156 ± 0.006	20.0
Dead	10	1.199 ± 0.168	7.00 ± 1.41	5.81 ± 0.37	0.195 ± 0.011	
Viable	20	1.381 ± 0.509	7.37 ± 1.76	5.61 ± 1.03	0.206 ± 0.095	19.5
Dead	20	1.708 ± 0.666	7.61 ± 1.49	5.28 ± 0.69	0.257 ± 0.048	
Viable	30	1.475 ± 0.263	8.09 ± 0.93	5.54 ± 0.43	0.277 ± 0.054	18.0
Dead	30	1.449 ± 0.330	8.26 ± 1.53	5.76 ± 0.54	0.338 ± 0.031	

[a] See footnote a in Table 1.

[b] Calculated per litre of nutrient solution. Total amounts used were 1.0 l at 10 mg N l^{-1} and 1.2 l at 20 or 30 mg N l^{-1}.

* Significantly different ($P < 0.05$) from heat-killed control by *t*-test.

Figures refer to shoots and roots and are means ± standard deviations for 3 to 6 replicates.

Table 3. Effect of inoculation with *K. pneumoniae* Pp on growth of *T. aestivum* grown under greenhouse conditions from July to September 1983

Inoculum	N level ($mg\ l^{-1}$)	Dry matter yield ($g\ l^{-1}$)[b]	Total N yield ($mg\ l^{-1}$)[b]	N content ($mg\ g^{-1}$)	Atom % ^{15}N excess	% Ndfa[a]
Viable	10	2.020 ± 0.073	18.41 ± 0.03	9.20 ± 0.25	0.195 ± 0.027	15.9
Dead	10	2.020 ± 0.099	18.86 ± 2.75	9.43 ± 1.77	0.232 ± 0.047	
Viable	20	2.022 ± 0.237	22.64 ± 4.56	9.26 ± 4.68	0.272 ± 0.070	12.3
Dead	20	2.252 ± 0.191	21.85 ± 1.85	10.25 ± 0.83	0.310 ± 0.021	
Viable	30	2.244 ± 0.105	29.81 ± 5.16	13.32 ± 2.14	0.334 ± 0.036	10.0
Dead	30	2.019 ± 1.069	22.23 ± 11.44	11.13 ± 0.58	0.371 ± 0.095	

[a] See footnote a in Table 1.

[b] Calculated per litre of nutrient solution. Total amounts used were 1.8 l at 10 mg $N\ l^{-1}$ and 2.0 l at 20 or 30 mg $N\ l^{-1}$.

repeatedly re-isolated from roots of the plants inoculated with viable bacteria.

Discussion

The results from the two experiments with *P. pratensis* showed that the plants benefited from inoculation with viable N_2-fixing *K. pneumoniae* Pp. Shoot dry matter yield, total N yield and N content increased in the first experiment (Table 1). In the second experiment there was an increase in the dry matter yield, but results for total N yield and N content were variable (Table 2). Several yield-dependent techniques have been used to assess N_2 fixation. Dry matter yield and N content may or may not be related to fixed nitrogen[20]. Total N yield, a product of dry matter and N content, could be used to estimate N_2 fixation. Rennie and Kemp[19] found that in experiments *in vitro* dry matter and total N yield correlated with each other to a high degree, so dry matter yield can presumably be used to estimate N_2 fixation.

In the soilless system used, nitrogen was derived from nutrient solution (fertilizer), the atmosphere (N_2 fixation), seeds, and inocula which contained mineral nitrogen from the bacterial growth medium. Both the N_2-fixing and the non-N_2-fixing system received the same extra amount of nitrogen, which became mixed with the fertilizer solution, although because inoculum was added to the top of the vessel, a vertical $^{14}N/^{15}N$ gradient might have developed and caused differences between the systems, if the roots were growing at different rates. In the sand culture, however, such a gradient would not persist. Moreover, there were no significant differences between yields of root dry matter, which indicates that the roots were not growing at different rates. The nitrogen derived from seeds of *P. pratensis* was negligible, but was rather large from seeds of *T. aestivum.* This means that we cannot know exactly how much seed nitrogen is retained in the plants or taken up by the bacteria in the case of the plants inoculated with viable cells. Thus, calculations of N_2 fixation were less accurate in the case of *T. aestivum.* There was also a possibility of denitrification by the viable bacterial cells. This error, though theoretical, may indicate that the amounts of nitrogen fixed are too small.

With *P. pratensis*, the % Ndfa was 7.3–33.8 in the shoots and *ca* 20.0 in the roots (Tables 1 and 2). In *T. aestivum*, the % Ndfa was 10.0–15.9 in the shoots (Table 3). Rennie[18] used isotope dilution to determine N_2 fixation of maize *in vitro* and calculated that 12.6% of maize nitrogen was derived from the atmosphere, and that the addition of a suitable carbon source increased the amount to 38%. Lethbridge

and Davison[13] used ^{15}N dilution to study N_2 fixation in wheat inoculated with several diazotrophic bacteria. Small amounts of mineral N in sand cultures were used. Root-associated N_2 fixation was negligible unless carbohydrate was added to the rooting medium[13]. In our research we did not add carbon source, but only mineral N fertilizer so that the plants could become established well and support bacterial N_2 fixation by exuding carbon compounds. Rennie *et al.*[21] also used ^{15}N isotope dilution to quantify N_2 fixation associated with Canadian and Brazilian wheat grown in soil under greenhouse conditions. Inoculation with *Bacillus polymyxa* or *Azospirillum brasilense* resulted in Ndfa of up to 32.3 % in some varieties. Inoculation had no significant effect on total N yield. A high degree of plant-bacterial specificity existed. In our study inoculation by *K. pneumoniae* Pp produced a much better effect on *P. pratensis* than on *T. aestivum.* Shearman *et al.*[25] and Wood *et al.*[29] studied as a model system the association of *P. pratensis* and *K. pneumoniae* and showed by the acetylene reduction method that N_2 was being fixed in the association and that the association had at least some degree of specificity.

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