NITROGEN FIXATION ASSOCIATED WITH WHEAT ROOTS

Ruby I. Larson

Wheat Cytogeneticist (Retired)
Formerly of Canada Agriculture Research Station
Lethbridge, Alberta

Available nitrogen is a necessary plant nutrient in the soil because N is a component of protoplasm and plants cannot directly use N_2, which constitutes about 80% of the atmosphere. The N present in field soils comes from various sources: decaying plants, animals, and microorganisms; physical and chemical interactions; and dinitrogen fixation by certain bacteria and blue-green algae. Since crops remove N from the soil, it is the practice to replenish it by application of organic fertilizers such as barnyard manure and to incorporate legumes as green manure, and especially by the use of inorganic N fertilizers, which are increasing in cost every year as the price of feedstocks and energy increases.

An attractive alternative is to use biological N_2-fixation, such as occurs in the well-known symbiotic relationship of *Rhizobium spp.* with legumes, or the associative symbiotic relationship of various bacteria with tropical grasses (Döbereiner *et al.* 1972). Unfertilized wheat on the Rothamstead Broadbalk plots gained up to 34 kg N/ha per year (Jenkinson, 1976) of which 2-3 kg/ha was attributable to bacterial fixation and the remainder to blue-green algae (Day *et al.* 1975).

This paper will deal with the historical development of research at the Canada Agriculture Research Station, Lethbridge into the potential for using associative symbiosis between N_2-fixing bacteria and the roots of wheat. It was carried out in steps to answer the following questions:

a) Can bacteria potentially capable of N_2-fixation be isolated from the rhizosphere of wheat growth in our soils?
b) If so, are there genetic differences between wheat strains with respect to the occurrence of such bacteria around their roots?
c) What is the nature of the association of these bacteria with the wheat root?
d) Do these bacteria actually produce nitrogenase, the enzyme responsible for biological N_2-fixation, in association with wheat roots?
e) Do the potential N_2-fixers actually fix N_2, and, if so, is the N so fixed taken up by the plant and incorporated into it?

These questions have been answered to the extent that it is considered feasible to start a breeding program to produce adapted strains of wheat capable of utilizing a biological N_2-fixation technology, which is also being developed.

ORIGINS

The research had its beginnings in the demonstration by means of whole-chromosome substitution lines that chromosome 5B had a major gene for reaction to common root rot (Larson and Atkinson, 1970), that changing the genetics of the plant by chromosome substitution altered the rhizosphere populations as to total bacteria, nutritional groups, and root rot antagonists (Neal et al. 1970) and physiological groups (Neal et al. 1973). Some of these relationships (Table 1) showed that the rhizosphere

Table 1. Rhizosphere phenomena in selected lines of common wheat

Line	Root rot reaction	Bacterial populations[1]			C_2H_2 reduction by rhizosphere soil[2]
		Total (x 10^6)	Cellulolytic (x 10^3)	NO_3^- reductase (x 10^5)	(n moles C_2H_4)
Cadet	Res.	165.4b[4]	4.7b	1.2b	0
Rescue	Susc.	335.2a	131.2a	3.8b	0
C-R 5B[3]	Susc.	325.9a	146.9a	2.6b	0
C-R 5D[3]	Res.	180.4b	3.2b	14.6a	2100
Non-rhizosphere soil	-	32.1c	0.2b	1.2b	30

[1]Adapted from Neal et al. 1973.

[2]Adapted from Neal and Larson 1976.

[3]Chromosome substitution lines, identical to Cadet except for substitution of Rescue chromosome pairs 5B and 5D respectively.

[4]Data in each column followed by the same letter do not differ statistically ($P = 0.01$).

of root rot susceptible lines had about twice the total number of bacteria of the resistant lines, probably because of a greater amount of root exudate in the susceptible lines. They also had about 30 times as many cellulolytic bacteria as the rhizospheres of the resistant lines, which did not differ from non-rhizosphere soils. On the other hand, C-R 5D, like Cadet except for the substitution of Rescue chromosome 5D, a homoeologue of 5B, had a relatively high proportion of nitrate-reducing bacteria, unlike either parent. Consequently, it was concluded that "... the genotype of the host governs the magnitude and composition of bacterial populations in the rhizosphere with surprising specificity" (Atkinson et al. 1975). The corollary followed that "Rhizosphere microbial characteristics can be changed by genetically altering the plant".

ISOLATION AND CHARACTERIZATION OF A NITROGENASE POSITIVE BACTERIUM

On the basis that there might be differences in compatibility of wheat strains with N_2-fixing bacteria, chromosome substitution lines and their parents were grown for seven weeks in soil from experimental plots at the Lethbridge Research Station that had been cropped to wheat since 1911 without N fertilizer. Presumably, such soil would contain bacteria compatible with the wheat rhizosphere, and the low N content would be favorable to N_2-fixing bacteria. Selective isolation of anaerobic N_2-fixing bacteria by inoculating Hungate roll tubes containing Hino-Wilson medium under anaerobic conditions with a dilution series of slurries from the wheat rhizospheres. Nitrogenase activity was tested by reduction of C_2H_2 to C_2H_4. Of the four lines tested, only the rhizosphere of C-R 5D was nitrogenase positive. At a dilution of 1×10^{-6} there was still some activity, but not at 1×10^{-7}. (Neal and Larson, 1976). A comparison with other rhizosphere characteristics (Table 1) shows that the high number of N_2-ase positive bacteria in the rhizosphere of C-R 5D was not related to high total number of bacteria.

Of 28 N_2-ase positive cultures isolated from the roll tubes, 20 were pure. All were bacilli, alike in staining and cultural characteristics, with high but not equal N_2-ase activity. Isolate C-11-25, later shown to be Bacillus polymyxa, was chosen for further study. It was used in all subsequent experiments. It is a spore-forming faculative anaerobe. In vitro tests showed that the reduction of C_2H_2 to C_2H_4 by the cultures was highly sensitive to O_2, so it was due to nitrogenase (Neal and Larson, 1976).

Further in vitro tests by Neal when on transfer of work at the Macaulay Institute for Soil Research, Aberdeen, Scotland, demonstrated that N_2-ase production stopped with the addition of inorganic N, but growth increased. Growth occurred between 10-40 C, was most rapid at 30 C, but reached highest cell production at 20 C, showing its compatibility with summer soil temperatures at Lethbridge. The most favorable pH was 6.8 to 7.2, also compatible with our soils.

ASSOCIATION OF BACILLUS C-11-25 WITH WHEAT ROOTS

The association of the bacillus with the wheat roots was studied in monoxenic cultures by growing C-R 5D wheat aseptically in liquid culture for 5 weeks, when seed nutrients and the N in the culture were depleted (Larson and Neal, 1978). Six plants were inoculated with C-11-25 and NH_4NO_3 was added to the culture medium of the six checks. Observations were made ten days later. Phase contrast micrographs showed that the bacteria proliferated on the roots near the point of emergence of lateral roots. They were imbedded in a polysaccharide slime. Scanning electron micrographs confirmed these observations. Transmission electron micrographs showed bacteria in the intercellular spaces of the cortex of the roots of inoculated plants but not inside the cells. No bacteria were found in or on roots of the uninoculated checks.

Tests of nitrogenase activity (C_2H_2 reduction) of inoculated roots were made on surface sterilized and unsterilized root portions from each of the six inoculated plants. The sterilized roots showed a little less than half the activity of unsterilized roots, indicating that the invading bacteria produce a functional N_2-ase (Larson and Neal, 1978).

NITROGEN FIXATION

Dr. J. L. Neal left the Lethbridge Research Station in 1977 and the microbiological investigations on dinitrogen fixation were resumed by Dr. R. J. Rennie in 1978. Rennie undertook to determine whether the N_2-ase positive bacteria that are associated with wheat roots actually fix N_2 and supply N to the plants.

The first experiments were done in Leonard jar assemblies with cultivars Cadet and Rescue and some of their reciprocal chromosome substitution lines: C-R (Cadet with Rescue chromosomes substituted) and R-C (Rescue with Cadet chromosomes substituted) for chromosomes 2A, 2D, 5B, and 5D (Rennie and Larson, 1979). The liquid growth medium was carbon free and contained 100 mg/ℓ KNO_3. After two weeks, one third of the plants were inoculated with the bacillus C-11-25, one third with Azospirillum brasilense (ATCC 29145) isolated from wheat by Döbereiner in Brazil, and one third were left uninoculated as checks. Three weeks later, plants were harvested and dry weight and N content were determined for shoot, root, and total for each pot of three plants. Except for R-C 5B, which had an extremely high root weight when inoculated with C-11-25, shoot weight was indicative of total weight. Only shoot weight and shoot N are given in Table 2. In all the Cadet lines except C-R 5B shoot weight and shoot N were increased by both bacterial inoculations. C-11-25 was detrimental to shoot weight of C-R 5B and 29145 increased its N content. The Rescue lines reacted more selectively. C-11-25 was detrimental to Rescue and R-C 2A, did not affect R-C 2D, and greatly enhanced both shoot weight and shoot N in R-C 5B and R-C 5D. Inoculation with 2915 produced the greatest increases in R-C 2D and lesser increases in R-C 5B and R-C 5D. It was dileterious to R-C 2A.

In field studies (Table 3), inoculations with C-11-25 increased shoot weight and shoot N in all Cadet substitution lines but decreased them in Cadet. The grain yield, however, was increased for Cadet and C-R 5D. In the Rescue lines, shoot N was increased in R-C 5B and grain yield in Rescue and R-C 5D. In general, the effects in the field, where plants had access to soil N and, presumably, the interactions with soil microflora prevented deleterious effects on susceptible lines, were less extreme than in monoxenic cultures.

Table 2. Effect of inoculation with the bacillus C-11-25 or *Azospirillum brasilense* (ATCC 29145) on dry matter weight and N content of chromosome substitution lines of spring wheat grown monoxenically *in vitro*[1]

	Shoot weight (mg)			Shoot N (mg)		
Line	0	C-11-25	29145	0	C-11-25	29145
Cadet	287	351+[4]	436+	12.64	17.20+	20.30+
C-R 2A[2]	256	348+	543+	11.45	15.81+	25.06+
C-R 2D[2]	408	673+	748+	17.30	27.45+	30.56+
C-R 5B[2]	389	314-[4]	413	14.12	14.18	18.06+
C-R 5D[2]	320	461+	405+	13.64	25.08+	19.00+
Rescue	372	243-	349	18.01	10.32-	16.21
R-C 2A[3]	509	440-	429-	21.16	16.13-	19.02
R-C 2D[3]	359	382	603+	15.18	16.07	24.41+
R-C 5B[3]	411	939+	521+	17.03	32.43+	24.28+
R-C 5D[3]	270	806+	377+	12.46	31.84+	16.68+
LSD (P = 0.05)						
Inoculation		56			2.46	

[1]Adapted from Rennie and Larson, 1979.

[2]Identical to Cadet except for substitution of Rescue chromosome pairs 2A, 2D, 5B, and 5D respectively.

[3]Identical to Rescue except for substitution of Cadet chromosome pairs 2A, 2D, 5B, and 5D respectively.

[4]+ = higher than uninoculated; - = lower than uninoculated.

Table 3. Effect of inoculation with the bacillus C-11-25 of yield and N content of chromosome substitution lines of spring wheat in the field[1]

	Shoot yield (g)		Shoot N (mg)		Grain yield (kg x 10^{-2}/ha)	
Line	O	C-11-25	O	C-11-25	O	C-11-25
Cadet	288	261-[4]	4.20	3.77-	23.8	24.9+
C-R 2A[2]	263	288+[4]	4.19	4.67+	22.0	22.0
C-R 2D[2]	259	290+	4.24	4.83+	23.3	23.4
C-R 5B[2]	217	254+	3.68	4.06+	17.4	17.6
C-R 5D[2]	291	324+	4.11	4.74+	20.0	22.6+
Rescue	201	200	3.20	3.20	17.6	18.7+
R-C 2A[3]	198	187	3.02	2.94	16.2	15.6
R-C 2D[3]	200	202	3.09	3.17	16.5	16.6
R-C 5B[3]	163	177	2.28	2.66+	19.4	18.4
R-C 5D[3]	230	222	3.74	3.48	18.2	19.3+
LSD (P = 0.05)						
Inoculation	20		0.38		1.1	

[1]Adapted from Rennie and Larson, 1981.

[2]Identical to Cadet except for substitution of Rescue chromosome pairs 2A, 2D, 5B, and 5D respectively.

[3]Identical to Rescue except for substitution of Cadet chromosome pairs 2A, 2D, 5B, and 5D respectively.

[4]+ = higher than uninoculated; - = lower than uninoculated.

CONCLUSIONS

Bacteria capable of fixing N_2 can associate with wheat roots and fix N_2 which is taken up by the wheat. The amount of N incorporated into the plant is not enhanced by high amounts of root exudate, since Cadet and C-R 5D have lower exudation rates than Rescue and C-R 5B yet are benefitted more by inoculation. The genotype of the wheat determines the interaction, which is specific for each bacterium. This provides a rationale for a plant breeding program, a related search for N_2-fixing bacteria compatible with the prairie soils and climate, and development of inoculation technology and soil management.

These experiments were done with only one isolate from our soils and one from wheat in Brazil. Because of the method of isolation and the narrow range of wheat genotypes and soil, only the bacillus C-11-25 was isolated. Since then, Rennie (1980) has identified N_2-ase positive bacteria from eight sources representing three ecosystems in southern Alberta using API microtube systems and a computer assisted analysis which he developed. Of the seven species isolated, Erwinia herbicola was most prevalent (Table 4), occurring in all ecosystems and in association with all but one species. Clostridium pasteurianum was isolated only from fallow. Wheat harbored the largest number of bacterial species, including Bacillus polymyxa to which C-11-25 belongs. The bacterial populations were strongly influenced by their hosts.

Table 4. Percentages of N_2-fixing (acetylene reducing) isolates from southern Alberta sources belonging to certain species of bacteria[1]

Source	Bacterium					
	Azospiril. brasilense	*Azotobact. vinlandii*	*Bacillus polymyxa*	*Enterobac. cloacae*	*Erwinia herbicola*	*Klebsiella pneumoniae*
Triticum aestivum[2]	12	4	19	12	46	8
Fallow[3,5]	--	--	40	--	20	30
Agropyron trichophorum[4]	50	50	--	--	--	--
A. dasystachyum[4]	--	--	--	--	100	--
A. elongatum[4]	--	--	--	--	100	--
Elymus junceus[4]	--	--	--	10	90	--
Melilotus officinales[4]	11	44	--	11	33	--
Astragalus cicer[4]	--	--	--	33	66	--

[1]Adapted from Rennie, 1980.

[2]From a cultivated field.

[3]From ADA Experimental Station, Burdett.

[4]From a coal strip-mine reclamation site.

[5]Plus 10% *Clostridium pasteurianum*.

The question is, when, where, and to what extent we can expect biological N_2-fixation to replace part or all of the N fertilizer needed to produce a good crop of wheat. That can be answered only with further research. There are, however, a few guide posts. The development of a successful wheat-bacterium combination requires a wheat breeding program, which usually takes about ten years to produce a variety. If modification of a currently licensed variety by backcrossing will suffice, the time may be shortened. A search for more efficient strains of bacteria, perhaps mutated to make them less sensitive to repression of N_2-ase synthesis by inorganic N, should continue. The cost-benefit relations between cost of fertilizer and added yield and cost of bacterial inoculation and added yield, both related to world wheat prices will determine when and where the bacterial N_2-fixation in wheat will become part of agricultural practice. The potential is there.

LITERATURE CITED

Atkinson, T. G., Neal, J. L. Jr., and Larson, R. I. 1975. Genetic control of the rhizosphere microflora of wheat. In Biology and Control of Soil-borne Plant Pathogens. G. W. Bruehl, ed., American Phytopathological Society, St. Paul, pp. 116-122.

Day, J. M., Harris, D., Dart, P. J., and Van Berkum, P. 1975. The Broadbalk Experiment. An investigation of nitrogen gains from non-symbiotic nitrogen fixation. In Nitrogen Fixation by Free-living Microorganisms. Vol. 6. W. D. P. Stewart, ed., Cambridge University Press, London. pp. 71-84.

Döbereiner, J., Day, J. M., and Dart, P. J. 1972. Nitrogenase activity in the rhizosphere of sugar cane and some other tropical grasses. Plant Soil 37: 191-196.

Jenkinson, D. S. 1976. The nitrogen economy of the Broadbalk experiments. I. Nitrogen balance in the experiments. Rothamsted Report for 1976. Vol. II pp. 103-109.

Larson, Ruby I. and Atkinson, T. 1970. A cytogenetic analysis of reaction to common root rot in some hard red spring wheats. Can. J. Bot. 48: 2059-2067.

Larson, R. I. and Neal, J. L. Jr. 1978. Selective colonization of the rhizosphere of wheat by nitrogen-fixing bacteria. In Environmental Role of Nitrogen-fixing Blue-green Algae and Asymbiotic Bacteria. U. Granhall ed. Ecol. Bull. (Stockholm) Vol. 26, pp. 331-342.

Neal, J. L. Jr., Atkinson, T. G., and Larson, Ruby I. 1970. Changes in the rhizosphere microflora of spring wheat induced by disomic substitution of a chromosome. Can. J. Microbiol. 16: 153-158.

Neal, J. L., Jr., Larson, R. I., and Atkinson, T. G. 1973. Changes in rhizosphere populations of selected physiological groups of bacteria related to substitution of specific pairs of chromosomes in spring wheat. Plant Soil 39: 209-212.

Neal, J. L., Jr., and Larson, R. I. 1976. Acetylene reduction by bacteria isolated from the rhizosphere of wheat. Soil Biol. Biochem. 8: 151-155.

Rennie, R. J. 1980. Denitrogen-fixing bacteria: computer-assisted identification of soil isolates. Can. J. Microbiol. 26: 1275-1283.

Rennie, R. J. and Larson. 1979. Dinitrogen fixation associated with disomic chromosome substitution lines of spring wheat. Can. J. Bot. 57: 2771-2775.

Rennie, R. J. and Larson, R. I. 1981. Dinitrogen fixation associated with disomic chromosome substitution lines of wheat in the phytotron and in the field. In P. B. Vose and A. P. Ruschel, eds., Associative Dinitrogen Fixation. CRC Chemical Press (in press).