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Nitrogen Fixation (Acetylene Reduction) Associated with Roots of Winter Wheat and Sorghum in Nebraska†

WAYNE L. PEDERSEN,[1] K. CHAKRABARTY,[1] R. V. KLUCAS,[1]* AND ANNE K. VIDAVER[2]

Laboratory of Agricultural Biochemistry[1] and Department of Plant Pathology,[2] University of Nebraska, Lincoln, Nebraska 68583

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Root segments and root-soil cores (6.5-cm diameter) from fields and nurseries of winter wheat and sorghum were tested for N_2 fixation by using the acetylene reduction assay. Wheat samples (~1,200) from 109 sites generally had low or no activity (0 to 3.1 nmol of C_2H_4 produced per h per g [dry weight] of root segments), even after 24 h of incubation. However, a commercial field of Scout 66, located in western Nebraska, exhibited appreciable activity (290 nmol of C_2H_4 produced per h per g [dry weight] of root segments). Of 400 sorghum lines and crosses, grain sorghums (i.e., CK-60A, Wheatland A, B517, and NP-16) generally exhibited higher nitrogenase activity than forage sorghums or winter wheats. CK-60A, a male sterile grain sorghum, was sampled at four locations and had the most consistent activity of 24 to 1,100 nmol of C_2H_4 produced per h per core. The maximum rate extrapolated to 2.5 g of N per hectare per day. Numerous N_2-fixing bacterial isolates were obtained from wheat and sorghum roots that exhibited high nitrogenase activity. Most isolates were members of the *Enterobacteriaceae*, i.e., *Klebsiella pneumoniae, Enterobacter cloacae,* and *Erwinia herbicola.*

Nitrogen fixation by free-living microorganisms associated with plants has been of interest for many years (4, 5, 16, 20). Recent impetus for this work was the observations in Brazil concerning *Azotobacter paspali* associated with *Paspalum notatum* (8, 11) and *Spirillum lipoferum* with various grasses and cereal crops (10). Nitrogenase activity has been investigated in tropical, subtropical, and temperate regions (e.g., 1, 6, 12, 14, 21, 27, 28, 31, 33). Long-term experiments by research groups at Rothamsted (5) suggested that considerable amounts of nitrogen gain occurred from nonsymbiotic nitrogen fixation and that the nitrogenase activity was highly and positively correlated with soil moisture levels. Recent $^{15}N_2$ studies (7) with soil cores of *Digitaria decumbens* and *P. notatum* indicated that some ^{15}N from $^{15}N_2$ was incorporated into rhizomes, with a lesser amount into leaves. Grasslands in Canada (25, 26, 34), New Zealand (19), and the United States (17, 18, 28, 32) generally exhibited low levels of nitrogen fixation. Nelson et al. (23) surveyed natural grasslands and various grain crops in Oregon and isolated nitrogen-fixing bacteria from wheat roots, including *Bacillus polymyxa, B. macerans, Enterobacter cloacae,* and several unidentified, gram-negative, rod-shaped bacteria. A general conclusion from these studies is that bacterial or algal nitrogenase activity is associated with many plant roots, particularly in tropical and subtropical regions, but whether a significant amount of nitrogen is contributed to the plants, as with legumes, is still questionable.

The objectives of the research in this report were to survey various genotypes and growth sites of winter wheat (*Tricicum aestivum* L. em Thell) and sorghum (*Sorghum bicolor* L. Moench) in Nebraska for nitrogenase activity associated with the roots and to isolate and identify nitrogen-fixing bacteria. Wheat and sorghum were selected because they are major food sources in the world and represent biochemical diversity, and because some information on nitrogen fixation by these crops is available (23, 33). Wheat, which uses the C-3 pathway for photosynthetic CO_2 fixation, is a crop with low or no nitrogen fertilizer added in Nebraska; a wheat-fallow biennial cycle is often followed without crop rotation. Sorghum, which predominantly uses the C-4 pathway for CO_2 fixation, is a moderately fertilized crop and generally is in a crop rotation.

MATERIALS AND METHODS

Winter wheat. Entries from the International Winter Wheat Nursery (29), all winter wheat varieties recommended for Nebraska (13), and 107 commercial winter wheat fields throughout Nebraska were tested

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for nitrogen fixation by using the acetylene reduction assay.

Winter wheat samples were assayed by using either a modification of Döbereiner's washed-root procedure (10) or a modified root-soil core procedure (30). The washed-root procedure, which was adequate for initial screening but probably inadequate for estimations of in situ levels of fixation (14, 33), involved washing the roots thoroughly with distilled water and cutting the stems just above the crown. Roots or segments of roots were placed in 20-ml vials, and the vials were sealed with rubber serum stoppers. The sealed vials were evacuated with a hand vacuum pump to 50 cm of mercury and filled with N_2; this operation was repeated four times. Oxygen and acetylene were injected separately into the vials to 0.01 and 0.1 atm, respectively. Ethylene levels were determined after 18 to 24 h by withdrawing 0.5-ml gas samples from the vials and injecting the sample into a gas chromatograph (Perkin-Elmer model 3920B) equipped with a hydrogen flame detector and a 3-m Porpak N (Waters Associates, Inc.) column. Peaks corresponding to ethylene and acetylene were measured, and average rates of acetylene reduction were calculated, using the total incubation period without regard for the lag observed by us and others (23, 33). This resulted in an underestimation of the rate for 18 to 24 h and represented the average rate for the total incubation period. The root-soil core procedure involved removing tops of the plants just above the crown and driving a steel tube (6.5-cm diameter) 12.5 cm into the ground. Cores containing roots and soil were removed from the tube and placed in 1-quart (ca. 0.946-liter) widemouth jars, and the jars were sealed with a lid previously fitted with a rubber serum stopper. Cores containing only soil were also assayed for ethylene production by this procedure. Methane (42 μmol) and acetylene (about 0.1 atm) were injected into the jars. Gas samples were removed at various times with glass disposable syringes. The gas samples in the syringes were stored for subsequent analyses by inserting the syringe needles into rubber stoppers. Ethylene was measured using the methane as an internal calibration standard, and samples were analyzed as before.

In an attempt to localize sites of nitrogenase activity, roots were cut into small segments: crowns (from crown to 2 cm below the crown), lower roots, and soil; these segments were assayed separately.

Bacterial populations of soil, rhizosphere, and root segments were estimated by using standard dilution series and plating on nutrient agar (Difco Laboratories, Detroit, Mich.) or Döbereiner's N-free malate agar (10) plates. Soil samples were obtained from areas between plants and did not contain wheat roots. For rhizosphere samples, all loose soil was removed from the roots, the entire root system with adhering soil was placed into 100 ml of distilled water and shaken for 10 min, and then samples were removed for dilution. Root segments were treated with 70% ethanol and 0.5% sodium hypochlorite for 3 min and then washed three times in distilled water. The segments were macerated with a glass stirring rod in 0.85% sterile saline. All bacterial populations are reported on a dry-weight basis.

Bacterial isolates were obtained by selecting random colonies from N-free malate agar plates (previously used in determining bacterial populations), restreaking on N-free malate agar plates, and transferring single colonies to nutrient agar slants. A second method of obtaining bacterial isolates involved placing short root segments (2 cm) into semisolid N-free malate agar tubes (0.35% agar), incubating at 25°C for 4 days, and streaking on N-free malate agar plates. Single colonies were then transferred to nutrient agar slants and stored at 5°C. All isolates were tested for nitrogenase activity (acetylene reduction) using semisolid malate agar tubes. The isolates were characterized by Gram staining, by testing for cytochrome oxidase (Pathotec CO; General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.), and by the use of API-20E (Analytical Products Inc.) test kits. Authentic cultures from the American Type Culture Collection (Rockville, Md.) and the National Collection of Plant Pathogenic Bacteria (Harpenden, Herts, England) were used for comparative purposes. The identified isolates and authentic cultures were lyophilized and stored at −20°C for reference throughout this study.

Sorghum. Sorghum samples were obtained from nurseries of the University of Nebraska and commercial fields in eastern Nebraska. Initial screenings were done with root segments as described for wheat. Subsequent samples were obtained by using the previously described root-soil core assay. Bacterial isolation and characterization were performed as described for wheat.

Line CK-60A, a male sterile grain sorghum, was tested for nitrogenase activity in the field under added levels of nitrogen (112, 196, and 280 kg of N per hectare), phosphorus (0, 84, and 168 kg/hectare), and calcium (0, 0.68, and 1.36 metric ton/hectare). The phosphorus and calcium plots contained 112 kg of N per hectare. The plot design was a randomized block with three replications; 9 to 12 root-soil cores were analyzed for nitrogenase activity per replicate.

RESULTS

Winter wheat. Root samples (~1,200) from throughout Nebraska, representing 109 sites and at least 44 different varieties, were examined for nitrogenase activity during a 2-year period. Nitrogenase activity ranged from 0 to 3.1 nmol of C_2H_4 produced per h per g (dry weight) of roots; a mean of 0.4 nmol of C_2H_4 per h per g (dry weight) was observed with most of the root samples, even after 18 to 24 h of incubation (Table 1). However, a commercial winter wheat field of Scout 66 located near Chappell, Neb., had appreciable activity. This field was sampled four times from early heading through the hard dough stage, and a range of 1.0 to 9.1 nmol of C_2H_4 produced per h per g (dry weight) of root was obtained, based on total root systems. When roots were cut into segments to localize activity, a maximum of 290 nmol of C_2H_4 produced per h per g (dry weight) was obtained from 2-cm segments (Table 2) collected directly below the

crown. Other segments of the roots exhibited substantially less activity. Analysis of soil samples from this field for nitrogen, potassium, phosphorus, zinc, pH, and organic matter revealed very low amounts of nitrogen (<1 μg of N per g of soil), with levels of the other components being typical of that area (soil pH, 7.7). Scout 66 was tested in at least 10 other locations and did not exhibit high nitrogenase activity (>5 nmol of C_2H_4 produced per h per g [dry weight]) in incubated roots. Bacterial counts on soil, rhizosphere, and root segments (Table 3) revealed that 4.8 to 7.4% of the total bacteria (estimated by growth on nutrient agar) grew on nitrogen-free malate agar. Both nonfermentative and fermentative bacteria were obtained from the N-free malate medium, but none of the former was able to fix nitrogen in tubes of semisolid N-free malate agar as measured by the acetylene reduction assay. The predominant nitrogen-fixing bacteria isolated from root segments exhibiting high acetylene reduction activity were identified primarily as members of the *Enterobacteriaceae*. Biochemical properties

TABLE 1. *Acetylene reduction by winter wheat roots and root segments from several locations in Nebraska*

Location	Varieties tested	Times sampled[b]	Total samples	Activity[a]	
				Mean	Range
Lincoln	33	12	418	0.4	0.0–1.8
	Scout 66	4	28	0.2	0.0–1.0
	Centurk	5	73	0.5	0.0–1.8
	Gage	2	25	0.3	0.0–1.0
Mead	6	4	76	0.2	0.0–3.1
	Scout 66	3	21	0.5	0.0–2.0
	Centurk	2	16	0.2	0.0–3.1
	Gage	2	16	0.2	0.0–0.4
Eastern[c]		1-2	125	0.4	0.0–2.1
Western[d]		1-2	288	0.3	0.0–1.0
Chappell	5	4	126	4.9	0.0–292.5
	Scout 66	4	106	5.2	0.0–292.5
	Centurk	1	4	0.1	0.0–0.6
	Gage	2	4	0.2	0.0–0.6

[a] Nanomoles of C_2H_4 per hour per gram (dry weight).
[b] All samples were obtained from April through July of 1975 and 1976.
[c] Samples from 45 commercial fields in eastern Nebraska.
[d] Samples from 62 commercial fields in western Nebraska.

TABLE 3. *Bacterial populations from isolations of winter wheat roots, rhizosphere, and soil from Chappell, Nebr.*

Source	Growth medium	Population (no. per g [dry weight])
Root segments	Nutrient agar	1.4×10^5
	N-free agar	8.4×10^3
Rhizosphere	Nutrient agar	7.8×10^8
	N-free agar	5.7×10^7
Soil	Nutrient agar	2.5×10^6
	N-free agar	1.2×10^5

TABLE 2. *Rates of acetylene reduction for roots, root segments, and root-soil cores for Scout 66 from Chappell, Nebr.*

Date	Stage of development	Sample[a]	Activity (nmol of C_2H_4 per h per g [dry weight])
5-17-76	Early heading[b]	Whole roots	5.2
5-30-76	Flowering	Whole roots	7.0
		Crowns	3.6
		2 cm below crown	111.3
		Lower roots	2.4
		Soil	0.2
6-7-76	Soft dough	Whole roots	9.1
		Crowns	20.5
		2 cm below crown	292.5
		Lower roots	16.4
		Soil	0.2
		Root-soil core[c]	1.5 (per core)
6-21-76	Hard dough	Whole roots	0.2
		2 cm below crown	0.3
		Root-soil core	8.9 (per core)

[a] Roots were divided into three parts: crowns, from crown to 2 cm below the crown, and roots below 2 cm.
[b] At this stage of development, most of the heads had partially emerged from the boot.
[c] Samples assayed for ethylene production in the absence of acetylene were all below 0.1 nmol of C_2H_4 per h per g (dry weight), and no significant oxidation of methane was detected.

and probable identification of these isolates are shown in Table 4. Nitrogen fixation was obtained with *Erwinia herbicola* only on N-free malate agar plates and not in liquid cultures or in semisolid agar tubes. Most of the *E. herbicola* colonies were distinguished from *E. cloacae* and *K. pneumoniae* colonies because of the presence of a yellow pigment on nutrient agar in the former.

Of 258 lines and crosses in a forage sorghum and Sudan grass breeding nursery that were assayed for acetylene reduction using root segments, only 12 reduced acetylene. Activities of these 12 ranged from 0.2 to 17 nmol of C_2H_4 produced per h per g (dry weight). Only one line, Coleman, reduced acetylene when assayed 20 days later. Of the 12 lines and crosses that reduced acetylene, 8 were tested in a subsequent year with the root-soil core assay. Low activities ranging from 5 to 75 nmol of C_2H_4 produced per h per core were observed, and the Coleman line had only 6 nmol per h per core of activity. Acetylene reduction activities with root segments or root-soil cores of lines and crosses obtained from forage sorghum and Sudan grass breeding nurseries were generally very low.

Lines and crosses in grain sorghum nurseries were tested for nitrogenase activity initially by the washed-root assay. Of the 29 entries tested, 26 reduced acetylene with values ranging from 2 to 102 nmol of C_2H_2 reduced per h per g (dry weight). Line B517 had the highest activity, but subsequent samplings indicated a great deal of variation. Seven randomly selected commercial grain sorghum fields yielded three fields with acetylene reduction rates between 1.3 and 8.6 nmol of C_2H_4 produced per h per g (dry weight), whereas the rates from the four other fields were near or at zero. In the second year, 145 lines and crosses from four nurseries were tested for acetylene reduction by using the root-soil core technique (Table 5). Root cores from sorghum lines CK-60A, Wheatland A, B517, NP-16, and a few breeding lines exhibited highest nitrogenase activity. CK-60A, a cytoplasmic male sterile, was the only line that exhibited activity at all nurseries, with activities ranging from 24 to 1,100 nmol of C_2H_4 produced per h per core and a mean of 300 nmol of C_2H_4 produced per h per core. No detectable ethylene (>0.1 nmol of C_2H_4 produced per h per g [dry weight]) was produced in any of the 12 soil cores tested.

An experiment was done to determine the effect of increasing levels of N, P, and Ca on acetylene reduction by soil-root cores of CK-60A and to determine variations of activity in sampling within a plot (Table 6). High levels of nitrogen and phosphorus significantly depressed nitrogenase activity, while high levels of calcium had no significant effect. Due to large differences among the means from N, P, and Ca control, which cannot be explained, the three treatments were analyzed independently. However, the

TABLE 4. *Differentiation of nitrogen-fixing bacterial isolates*[a] *on the basis of biochemical reactions*

Determinant	% Positive reactions					
	Klebsiella pneumoniae		*Enterobacter cloacae*		*Erwinia herbicola*	
	Isolates (50)[b]	ATCC 15574 (1)	Isolates (6)	ATCC 13047 (1)	Isolates (2)	NCPPB 548 & 663 (2)
Decarboxylase for:						
Arginine	6	−	0	+	0	0
Lysine	100	+	100	−	0	0
Ornithine	0	−	100	+	0	0
Urease	48	+	17	−	0	0
Indole formation	20	−	0	−	0	50
Voges-Proskauer reaction	98	+	100	+	100	50
H_2S formation	0	−	0	−	0	0
Citrate utilization	92	+	100	+	50	50
Gelatin liquefaction	14	−	17	−	0	0
Nitrate reduction	100	+	100	+	100	100
Fermentation of:						
Glucose	100	+	100	+	100	100
Mannitol	100	+	83	+	100	100
Inositol	100	+	83	−	0	0
Sorbitol	100	+	100	+	0	50
Melibiose	100	+	100	+	50	50

[a] All isolates were gram-negative, oxidase-negative, facultative anaerobes, which hydrolyzed *o*-nitrophenol-galactose and fermented rhamnose, sucrose, amygdalin, and arabinose.

[b] Number of isolates tested.

TABLE 5. *Acetylene reduction by root-soil core samples of grain sorghum from locations in Nebraska*

Location	Lines tested	Times sampled[b]	Total samples	Activity[a]	
				Mean	Range
Mead Nursery	85	6	215	22.1	0–660
	CK-60A	3	30	241.3	24–660
Lincoln Nursery	60	12	850	214.1	0–1,100
	CK-60A	10	560	412.1	31–1,100
	B517	2	4	34.0	6–104
	Wheatland A	1	3	82.1	45–398
	NP-16	1	3	124.6	24–566
	R-8191	2	8	242.2	5–880
	R-8192	2	8	82.9	3–295
	R-8193	2	7	128.3	19–453
Random fields		1–3	140	21.6	0–80

[a] Nanomoles of C_2H_4 per hour per core.
[b] All samples were obtained from June through September of 1976.

TABLE 6. *Effect of three levels of nitrogen, phosphorus, and calcium on acetylene reduction activity in cores of sorghum CK-60A*[a]

Treatment (per hectare)[b]	Mean activity[c]	Coefficient of variation (%)
Nitrogen (kg)		
112	589 (A)	
196	345 (AB)	96.6
280	120 (B)	
Phosphorus (kg)		
0	3,885 (A)	
84	1,027 (B)	99.8
168	1,670 (B)	
Calcium (metric ton)		
0	4,318 (A)	
0.68	3,563 (A)	136.6
1.36	4,247 (A)	

[a] All samples were obtained during July and August 1976.
[b] All phosphorus and calcium treatments had 112 kg of N per hectare applied as a base level.
[c] Nanomoles of C_2H_4 per 18 h per core. These values represent a mean of 9 to 12 samples per replication. Numbers followed by the same letter (A) or (B), are not significantly different ($P = 0.05$), according to Duncan's multiple range test.

coefficient of variability was still very high and indicated the extreme variability among samples.

DISCUSSION

Fields and nurseries of winter wheat and sorghum in Nebraska were tested for N_2 fixation by using the acetylene reduction assay. Root samples or root-soil cores having high levels of acetylene reduction activity were obtained from a commercial winter wheat field of Scout 66 near Chappell, Nebraska, and several grain sorghum lines in eastern Nebraska. However, the sorghum samples generally had higher activity than the wheat samples in both assays. The activity in the wheat roots appeared to be localized, with 85% of the activity being found in 2-cm segments located directly below the crown. Very little activity was found in the lower roots or in the soil. The root systems from the Chappell field were extremely fibrous, and the root systems directly below the crown were unusually large in diameter. None of the other wheat fields sampled during this study had roots that were as large or fibrous as those from the Chappell field. This appears to be similar to the results of von Bülow and Döbereiner (35) on maize. Root-soil cores of wheat exhibited very low activity throughout this study. However, the soil was extremely dry and sandy and tended to break apart in the sample jars. Thus, the environment in the cores would be quite different for the small vials used in the washed-root assay. Tjepkema and Van Berkum (33) and Eskew and Ting (14) found very poor correlation between washed roots (excised roots) and root-soil cores. Barber et al. (3) and Okon et al. (24) reported increases in bacterial populations during the washed-root assay. In the present study, 8 to 12 h of incubation was required before acetylene reduction could be detected in washed roots or cores. It is probable that bacterial populations would increase during incubation, but the differences among wheat fields and among genotypes of sorghum at the same location are not readily explained. If the following assumptions are made: (i) the conversion factor for converting C_2H_2 reduced to N_2 fixed is 3; (ii) a rate of 1,100 nmol of C_2H_4 produced per h per core (maximum rate for sorghum line CK-60A) is linear for 24

h; (iii) a core represents a single plant at a population of 100,000 plants per hectare; and (iv) all of the fixed N_2 is available to the plant; then 2.5 kg of N per hectare would be available over a 100-day growing season. This value is about 10-fold greater than that estimated by Nelson et al. (23) for inoculated sorghum lines in Oregon. Estimations of nitrogen fixation rates using surface area of the core would not be valid, since activity was associated only with the root system. No corrections for the lag in activity were made in the present study. The nitrogen effect on acetylene reduction observed with sorghum root-soil cores is similar to previous observations by others (2, 9). The decrease of nitrogenase activity as nitrogen levels increase is consistent with the general observation that fixed nitrogen depresses nitrogen fixation and indirectly supports the use of this assay as an indication for nitrogen fixation. Also, the findings that high N and P levels appear to depress nitrogen fixation suggest that with high fertility conditions significantly less nitrogenase activity would be expected.

It is recognized that our procedures for estimating total and nitrogen-fixing bacteria underestimate actual values. However, the estimated percentage of 4.8 to 7.4% for nitrogen-fixing bacteria, as measured by growth on N-free malate agar, is a valid range for our restrictive procedures. Most of the nitrogen-fixing isolates were *K. pneumoniae,* which is commonly found associated with roots of plants grown in temperate zones (15, 17, 19, 34). *E. cloacae,* previously reported as being associated with corn (23), was found in both wheat and sorghum. *E. herbicola* was isolated only from the wheat field at Chappell. Although *E. herbicola* is generally not recognized as a nitrogen fixer, such isolates from paper pulp water are reported by Neilson and Sparell (22).

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