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Effect of Inoculation with N_2-Fixing Spirilla and *Azotobacter* on Nitrogenase Activity on Roots of Maize Grown Under Subtropical Conditions

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Inoculated and non-inoculated seedlings of maize were grown in fertile clay-loam soils of Egypt and Belgium under subtropical conditions provided in a greenhouse. Acetylene-reducing activity and microbial counts were determined during a period ranging from 6 to 12 weeks after sowing. Irrespective of soil origin, N_2-fixing spirilla and *Azotobacter* were common under maize cultivation. Inoculation resulted in a transitional increase in their numbers at early stages of growth. Nitrogenase activity was not detected in the rhizosphere of young plants. The maximum activities measured (81 to 1,436 nmol of C_2H_4 g^{-1} h^{-1}) occurred close to the 50 to 70% silking stage. Inoculation with N_2-fixing spirilla, particularly in Nile Delta soil, doubled the amount of N_2 fixed in a late period of growth (12 weeks), whereas inoculation with *Azotobacter* had no noticeable effect.

Recent discovery of the associative growth of particular asymbiotic N_2-fixing bacteria in the rhizosphere of certain C_4 plants, mainly in the tropics, has received the attention of many investigators all over the world (10). *Spirillum lipoferum* Beijerinck (recently placed in the separate genus *Azospirillum*), in particular, was found to be responsible for nitrogenase activity on roots of field-grown tropical grain and forage grasses (13). The present study is one of a number of investigations carried out to determine the occurrence of such bacteria in the soils and rhizospheres of various plants in Egypt, to characterize local isolates, to test methods of enumeration and to assess their possible contribution to nitrogenase activity on roots of major crops (1, 7, 8; H. A. Amer, M.S. thesis, Cairo University, Giza, Egypt, 1978; M. Eid, M.S. thesis, Cairo University, Giza, Egypt, 1978; N. A. Hegazi, H. A. Amer, and M. Monib, Soil Biol. Biochem., in press). We report here on the acetylene-reducing activity in the rhizospheres of maize plants grown in fertile clay-loam soils of Egypt and Belgium under subtropical conditions in a greenhouse. The effects of the inoculation of seedlings with N_2-fixing spirilla and *Azotobacter* on such activity were also investigated.

MATERIALS AND METHODS

Inoculation. The roots of germinated seedlings of maize (open pollinated, cultivar Shandwil) were dipped in Ashby's N-deficient medium minus carbon source or in a liquid mixed culture of *Azotobacter chroococcum* and *A. vinelandii* (Amer, M.S. thesis, 1978; Eid, M.S. thesis, 1978) or *S. lipoferum* (ATCC 29145). The inoculation was carried out with a heavy bacterial suspension containing $>10^9$ cells per ml. Colony counts recovered from *Azotobacter*-inoculated seedlings were in the range of 1×10^8 to 10×10^8 per root. Seedlings were transferred to pots containing 1 kg of fertile clay-loam soil from Egypt (pH 7.9; total nitrogen, 0.13%) or from Belgium (pH 7.2; total nitrogen, 0.25%). The plants were grown in the greenhouse with supplementary lighting and heating to give a 14-h day, with the light at a mean intensity of 12,000 lx and the temperature at 28 to 30°C, and a 10-h night, with the temperature at 28°C.

Nitrogenase activity. The nitrogenase activity of roots, rhizosphere soil, and soil away from the roots as well as in bare pots was studied by the acetylene reduction technique during a growth period of 6 to 12 weeks. Whole-root systems and soil of three to eight plants from each treatment were sampled late in the afternoon, washed in distilled water, and placed in vessels stopped with Suba-Seals. The vessels were evacuated to 40 mmHg (ca. 5.3 kPa) and flushed four times with argon containing 2% oxygen; 10% of this gas mixture was then removed and replaced with acetylene. The vessels were incubated at 28 to 30°C, and 1.0-ml samples were assayed after 24 to 72 h for ethylene by injection into a Hewlett-Packard gas chromatograph with a Poropak R column. Ethylene production per hour was finally related to the dry weight of the soil or root. Checks for C_2H_2-independent C_2H_4 production were made. The results were always negative after 48 h of incubation.

Microbial counts and enrichments. After assay, the roots of control and *Azotobacter*-inoculated plants were shaken in Ashby's liquid medium minus carbon source, and suitable dilutions were plated on N-deficient medium to give a count of *Azotobacter* (6).

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Washed roots were dried and weighed, and the soil suspension was evaporated to dryness. The numbers of *Azotobacter* were related to dry weight of roots or soil. In addition, root segments of plants from all treatments were partially surface sterilized in absolute ethanol for 2 min and washed twice in sterilized distilled water. They were transferred to 10 ml of semisolid malate medium (5). The resulting enriched cultures were examined for nitrogenase activity and for the presence of a characteristic pellicle and active motile cells of spirilla. Unfortunately, numbers of *Azospirillum* were not followed due to lack of a proper method of enumeration by the time of analysis.

The nonparametric method of Friedman rank sum was used for the statistical analysis of microbial and nitrogenase data, and the Fisher sign test was used for comparing rates of nitrogenase obtained at 24 and 48 h of incubation of roots (9).

RESULTS

Changes in microbial counts. Examination of noninoculated pots, irrespective of soil origin, revealed that *Azotobacter* and N_2-fixing spirilla commonly occurred under maize cultivation. They were encountered in appreciable densities in soil, rhizosphere, and roots throughout the growing period.

The lowest counts of *Azotobacter* (Table 1) were found in the first period of analysis (6-week-old plants). They were particularly abundant in the rhizosphere soil and roots of 9-week-old plants, as their densities in the roots of such plants were 200- to 900- and 6- to 20-fold those reported at 6 and 12 weeks, respectively. A similar development of N_2-fixing spirilla was reported. Enrichment cultures prepared for the roots of 9-week-old plants, in particular, exhibited rather high acetylene-reducing activity.

Inoculation with heavy suspensions of *Azotobacter* led to a rather pronounced increase in counts reported for 6-week-old plants. The effect diminished with the prolongation of plant growth, particularly at the age of 9 weeks, when the roots of noninoculated plants harbored double the densities reported for those of inoculated ones. The persistence of the bacterial inoculum was traced by following the development of *A. vinelandii* isolates, which were absent in the soil and roots in noninoculated pots. Their numbers constituted 18 to 47% of the total *Azotobacter* occurring in the rhizosphere and roots of inoculated plants. Their appearance in soil away from roots was delayed until week 9 of growth, when they represented 3 to 16% of total *Azotobacter*.

Patterns of nitrogenase activity (Tables 2 and 3). With both inoculated and noninoculated pots, the amount of acetylene reduced by rhizosphere soil freed of roots, as well as by nonrhizosphere-free soil, did not follow a particular trend and was relatively low and irregular. Rates obtained were in the range of 0.05 to 0.61 nmol of C_2H_4 g^{-1} h^{-1}.

The high rates of acetylene reduction obtained for detached roots indicated that nitrogenase activity is mainly associated with root systems and the very closely surrounding soil. Washing the roots in distilled water relatively enhanced acetylene-reducing activity, which was also increased by the prolongation of the incubation period.

The roots of 6-week-old plants exhibited rather low acetylene-reducing activity (0.01 to 0.4 nmol of C_2H_4 g^{-1} h^{-1}) in both types of soil;

TABLE 1. *Colony counts of Azotobacter and percentage of A. vinelandii under maize cultivation*[a]

Stage of plant growth	No. of *Azotobacter* (× 10^4)/g of dry matter							
	Egyptian soil				Belgian soil			
	Inoculated		Non-inoculated		Inoculated		Non-inoculated	
	Total	%[b]	Total	%	Total	%	Total	%
6 wk								
Soil	26.4	0	0.4	0	24.7	0	0.2	0
Rhizosphere	58.5	30.7	0.2	0	130.0	30.7	0.4	0
Roots	262.2	30.5	1.0	0	602.6	38.5	1.9	0
9 wk								
Soil	10.3	4.9	8.3	0	9.7	15.5	5.9	0
Rhizosphere	19.7	25.0	46.8	0	33.0	34.5	35.7	0
Roots	348.6	22.0	842.2	0	109.1	46.5	371.2	0
12 wk								
Soil	1.2	13.3	0.1	0	1.9	3.1	0.1	0
Rhizosphere	13.3	18.8	1.2	0	28.4	35.2	7.1	0
Roots	52.5	18.2	41.1	0	65.3	35.2	60.5	0

[a] Statistical analysis indicated: (i) a significant ($P < 0.05$) effect of inoculation in Belgian soils; (ii) significant effects due to age of plants. The highest densities were reported for 6- and 9-week-old plants.

[b] Percentage of *A. vinelandii*.

TABLE 2. *Acetylene-reducing activity on roots of maize planted in Belgian soil*

Incubation period (h)	Accumulated nmol of C_2H_4 g^{-1} h^{-1}					
	Non-inoculated		*Azotobacter* inoculated		Spirilla inoculated	
	Nonwashed	Washed	Non-washed	Washed	Non-washed	Washed
6-wk-old plants						
24	0.1	0.05	0.8	3.4	0.4	0.03
48	0.1	0.4	0.9	6.8	0.4	0.02
9-wk-old plants						
24	43.3	62.7	11.9	48.3	38.2	106.9
48	145.3	61.7	20.0	80.4	53.2	166.7
72	—[a]	238.9	—	—	—	294.5
12-wk-old plants						
24	33.3	53.0	67.8	353.4	38.5	99.5
48	34.3	53.0	50.9	366.5	37.3	123.1
72	64.9	—	68.4	—	58.7	—

[a] —, Not determined.

TABLE 3. *Acetylene-reducing activity on roots of maize planted in Egyptian soil*[a]

Incubation period (h)	Accumulated nmol of C_2H_4 g^{-1} h^{-1}					
	Non-inoculated		*Azotobacter* inoculated		Spirilla inoculated	
	Non-washed	Washed	Non-washed	Washed	Non-washed	Washed
6-wk-old plants						
24	0.05	0.02	0.3	3.2	0.8	0.5
48	0.04	0.02	0.3	3.3	0.8	3.2
9-wk-old plants						
24	62.7	229.9	50.4	167.3	49.9	797.3
48	81.3	826.6	98.4	320.6	82.9	2,027.1
72	—[b]	1436.6	—	346.6	—	2,380.9
12-wk-old plants						
24	6.1	28.4	27.7	210.9	109.9	2,109.6
48	7.7	45.5	26.6	213.0	134.0	2,193.5
72	9.8	—	30.0	—	141.0	—

[a] Irrespective of soil type, i.e., Egyptian or Belgian, statistical analysis indicated: (i) no significant difference attributed to inoculation or washing of roots; (ii) significant effects of plant age, with the highest rates reported for 9- and 12-week-old plants; (iii) rates after 48 h were significantly higher than after 24 h of incubation.

[b] —, Not determined.

inoculation slightly increased such activity. Further growth of maize plants encouraged asymbiotic N_2 fixation, as appreciable rates of acetylene reduction were reported for 9- and 12-week-old plants, i.e., during the stage of 50 to 75% silking. Non-inoculated plants yielded highest activities at the age of 9 weeks; Egyptian soil supported relatively higher (62 to 1,440 nmol of C_2H_4 g^{-1} h^{-1}) activities than Belgian soil (43 to 240 nmol of C_2H_4 g^{-1} h^{-1}). Inoculation exerted no noticeable effect at this stage in general, except for a slight increase reported for washed roots of spirilla-inoculated plants grown in Egyptian soil. Later on, a decrease in acetylene-reducing activity was reported for the roots of 12-week-old noninoculated plants. During this particular stage of plant growth marked effects of inoculation with spirilla in Egyptian soil, and to a lesser extent with *Azotobacter* in Belgian soils, were demonstrated.

DISCUSSION

Nitrogenase activity reported for noninoculated plants was associated with the presence of appreciable densities of *Azotobacter* and with the consistent isolation of N_2-fixing spirilla. Other organisms, e.g., *Enterobacter cloacae*, *Klebsiella*, and *Bacillus*, are possibly involved (12; Amer, M.S. thesis, 1978).

Acetylene-reducing activity in roots results from a complementary interaction between plants and bacteria, and many factors are involved, such as photosynthetic efficiency, trans-

location, root anatomy and physiology, and bacterial characteristics (2, 13). Similar to the legume-bacterial symbiosis, the growth stage of maize plants under investigation played an important role. In agreement with results of von Bulow and Dobereiner (13), maximal acetylene-reducing activity was reported during the flowering stage of maize plants. The rather low activity found around the roots of 6-week-old plants, including inoculated ones, which contained densities of *Azotobacter* comparable to those reported at other stages of growth, might be attributed to insufficient photosynthates available for bacterial growth. In addition, such activity might be inhibited by the amount of nitrogen available in soil and only developed when plant growth removed this nitrogen and a condition of nitrogen stress prevailed (2, 3).

The values of nitrogenase activity in the nonwashed roots of 9- to 12-week-old plants described in this paper were much lower (maximum 145 nmol of C_2H_4 g^{-1} h^{-1}) than those reported for washed roots (maximum, 2,380 nmol of C_2H_4 g^{-1} h^{-1}), particularly with prolongation of the incubation period. An explanation recently presented by Okon et al. (11) was that an anaerobic metabolism is established in the wet maize roots during the extended preincubation period which probably produces organic acids (lactic acid, etc.) that support the vigorous growth of asymbiotic N_2 fixers, mainly spirilla. Therefore, to avoid the overestimation of N_2 fixation based on measurements of C_2H_2 produced, the values obtained after the total 24-h incubation of nonwashed roots are used for further discussion. Converting C_2H_2 reduction into kilograms of N_2 fixed is subject to criticism, and the assay using detached roots set further limitations. However, it is helpful in understanding the potential of N_2 fixation in maize as well as in other plants. Generally, an amount close to 10 g of N_2 h^{-1} day^{-1} was calculated under the subtropical conditions provided in the greenhouse irrespective of soil origin (Table 4). At a late phase of growth, inoculation with spirilla doubled the amount of N_2 fixed in Nile Delta soil. This encourages further studies, including $^{15}N_2$ analysis, under natural conditions where higher estimates of fixation are expected, since the light intensity provided in the greenhouse, as well as other possible factors which may be important for N_2 fixation in plant rhizospheres (4), never reached those of the natural habitat. Delayed positive effects of inoculation with members of asymbiotic N_2-fixing bacteria have been reported in the literature, e.g., *A. paspali* and *Paspalum notatum* (2). However, the effect was attributed to a stimulatory factor, without N_2 fixation necessarily occurring, produced in the rhizospheres of young plants and its effect was manifested later.

TABLE 4. *Amounts of nitrogen fixed under maize cultivation*[a]

Stage of plant growth (weeks)	Amt (g) of N_2 fixed h^{-1} day^{-1}		
	Non-inoculated	*Azotobacter* inoculated	Spirilla inoculated
Egyptian soil			
6	0.02	0.10	0.26
9	21.07	16.93	16.77
12	3.05	9.31	36.93
Mean	8.05	8.78	17.99
Belgian soil			
6	0.04	0.27	0.13
9	14.55	4.00	13.84
12	11.19	22.78	12.94
Mean	8.59	9.02	8.97

[a] Calculations based on acetylene-reducing activity reported for nonwashed roots after total 24-h incubation period: theoretical, 3:1 (C_2H_4/N); mean dry weight of maize roots, 1,500 kg/ha (13; E. A. Mahmoud, M. S. thesis, Cairo University, Giza, Egypt, 1967).

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