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FINAL REPORT

PROJECT NO. I-254-80

Biological Nitrogen Fixation in Grass-Azospirillum Association

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Title of Proposed Research

Biological Nitrogen Fixation in Grass-Azospirillum Association.

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C. ABSTRACT

The aerotactic properties of Azospirillum were studied. It was found that azospirilla either growing on N_2 or NH_3 responded in a matter of seconds to self-created oxygen gradients by forming aerotactic bands moving towards a zone with low dissolved oxygen tension. Azospirillum responded chemotactically to a wide variety of amino acids, sugars and organic acids which have been reported to be present in root exudates. A. brasilense cells, grown under high or low dissolved oxygen tensions possess a branched electron transport system with oxidases with high and low affinities to O_2 . Azospirillum brasilense formed poly- β -hydroxybutyrate (PHB) which comprised 50% of the cell dry weight. In cells grown under low dissolved oxygen tension and under high C/N ratio. During starvation, the survival and respiration rates of bacteria containing 40% PHB, were higher than those of cells containing 5 % PHB. Polymer-rich cells fixed atmospheric nitrogen in the absence of exogenous carbon and combined nitrogen. In the presence of stress factors such as dessication and osmotic pressure, PHB-poor cells died more rapidly than PHB-rich cells. The properties outlined above make Azospirillum a more likely candidate to be used to colonize, proliferate and survive in the rhizosphere of plants.

The surface distribution of Azospirillum on inoculated roots of maize and wheat was generally similar to that of other rhizoplane microflora. During the first three days, colonization took place mainly on the root elongation zone, on the base of root hairs and, to a lesser extent, on the surface of young root hairs. Inoculation of several cultivars of wheat, corn sorghum and setaria with several strains of Azospirillum caused morphological changes in roots starting immediately after germination. Root length and surface area were differentially affected according to bacterial age and inoculum level. During the first three weeks after ger-

mination, the number of root hairs, root hair branches and lateral roots was increased by inoculation, but there was no change in root weight. Root biomass increased at later stages. Cross sections of inoculated corn and wheat roots showed an irregular arrangement of cells in the outer layers of the cortex. These effects on plant morphology may be due to the production of plant growth-promoting substances by the colonizing bacteria or by the plant as a reaction to colonization. The rate of NO_3^- , K^+ and H_2PO_4^- uptake was greater in inoculated seedlings. In the field, dry matter N, P and K accumulated at faster rates, and water content was higher in Azospirillum-inoculated corn, sorghum, wheat and setaria. The above improvements in root development and function lead in many cases to higher crop yield.

D. WORK CARRIED OUT DURING SUPPORT BY BARD NO I-254-80 IN ISRAEL 1981-1984

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I. Studies on Physiology of Azospirillum

1. Aerotactic Response of Azospirillum brasilense
(BARAK, NUR, OKON and HENIS, Journal of Bacteriology,
152:643-649 (1982).

Five strains of Azospirillum brasilense and two of Azospirillum spp. from Israel, responded to self-created and preformed oxygen gradients by forming aerotactic bands in capillary tubes and actively moving toward a specific zone with low dissolved oxygen. Increasing the oxygen concentration in capillaries containing phosphate buffer increased the number of attracted bacteria and decreased band velocity. High O_2 concentrations and H_2O_2 temporarily repulsed the bacteria, causing the formation of a bacterial arc around the capillary mouth. There was no band formation under anaerobic conditions, although the bacteria remained highly motile. Exogenous energy sources were unnecessary for aerotaxis in Azospirillum spp. The addition of oxidizable substrates to the capillary slightly enhanced aerotaxis, possibly by accelerating O_2 consumption. Aerotactic band formation was affected by pH, bacterial concentration and age, incubation time, and respiratory inhibitors, but not by the lack of combined nitrogen in the growth medium. It is proposed that aerotaxis plays a role in the capacity of Azospirillum spp. to reach an environment suitable for N_2 fixation.

2. Detection of Chemotaxis in Azospirillum brasilense
(BARAK, NUR and OKON, Journal of Applied Bacteriology
53:399-403 (1983).

Azospirillum brasilense strain Cd responded chemotactically to amino acids, sugars and organic acids. Chemotactic rings were observed in semisolid agar plates containing oxidizable substrates. Increasing sodium succinate concentration decreased the velocity of ring expansion. Chemotactic activity of Azospirillum was also examined by a newly developed technique using a channelled chamber. Varying the concentrations of aspartic and glutamic acids affected the chemotactic response of the bacteria. In both assays chemotaxis was obtained under conditions that prevented aerotaxis.

3. Effect of Oxygen Concentration on Electron Transport Components and Microaerobic Properties of Azospirillum brasilense.
(OKON, NUR and HENIS, Experimentia Supplementum 48:115-126, 1983).

Azospirillum brasilense - Cd ATCC 29729 was grown in a malate ammonia mineral salts medium in a chemostat under low d.o.t. 0.007 atm and under high d.o.t. 0.20 atm. Centrifugation of cell free extracts from high d.o.t. cultures resulted in a heavier membrane layer and a lighter diffuse layer that could be separated in a sucrose gradient. Cell free extracts from low d.o.t. cultures did not form the diffuse layer. The membranes from both high and low d.o.t. possessed cytochrome b_{560} and similar amounts of cytochromes C_{551} . Addition of 5×10^{-5} M KCN to high d.o.t. membranes did not affect their succinate oxidase activity but it sharply inhibited O_2 succinate-dependent uptake in low d.o.t. membranes. Oxygen uptake by cells, cell free extracts and membranes of low d.o.t. malate grown cultures was not inhibited at all by CO ,

whereas high d.o.t. preparations were completely inhibited. Antimycin A (2×10^{-5} mol per mg protein) inhibited 82% of NADH oxidase activity in high d.o.t. cell free extracts, but 10^{-4} mol per mg protein Antimycin did not inhibit succinate oxidase. It was concluded that A. brasilense possesses a branched electron transport system that differs in high and low d.o.t. grown cells. The adaptability of A. brasilense to different d.o.t.'s makes it a likely candidate to colonize, proliferate and survive in the rhizosphere.

4. Production of the Reserve Material Poly- β -hydroxybutyrate and its Function in Azospirillum brasilense-Cd. (SARA TAL and OKON, Canadian Journal of Microbiology (1985) in press).

Azospirillum brasilense-Cd was grown in ammonium-malate mineral salts media in batch culture and in chemostat continuous culture. It was found that poly- β -hydroxybutyrate (PHB) synthesis was favoured under oxygen limitation in chemostat and under high C/N ratio towards the end of exponential growth in batch. The degradation and synthesis of PHB under starvation conditions occurred in a biphasic pattern and was affected by the PHB content of the cells. During a 7-day incubation period in phosphate buffer, the survival and respiration rate of bacteria containing about 40% PHB per dry weight, were higher than those of cells containing about 5% PHB. Polymer-rich cells fixed atmospheric nitrogen in the absence of exogenous carbon and combined nitrogen. Biphasic nitrogenase activity was observed during starvation. When nitrogenase activity was high, hydroxybutyrate dehydrogenase activity was low and vice-versa. Aerotactic response was higher in polymer-rich cells. In the presence of stress factors such as U.V.

irradiation, dessication and osmotic pressure PHB-poor cells died more rapidly than PHB-rich cells.

II. Interactions Between Azospirillum and Roots of Plants

1. Adsorption of Azospirillum brasilense to corn roots (GAFNY, OKON, KAPULNIK and FISCHER, Soil Biology and Biochemistry (1985) in press).

The adsorption of Azospirillum brasilense strain Cd to corn roots was measured by using ^{32}P -labelled cells. Binding kinetics showed that adherence of bacteria to roots increased during the first 90 minutes and attained a maximum level within 4.5 hours of incubation. Maximum binding occurred at pH 6.1. The presence of Ca^{+2} , Mg^{+2} , Na^{+} and poly-L-lysine (a polycation) reduced binding. Bacterial adherence to roots increased with increasing bacterial concentration up to 10^9 cells/ml of binding mixture. At higher concentrations, binding approached saturation. The bacteria attached to the roots and the bacteria in the solution around the roots were found to be in a dynamic equilibrium. Killing bacteria or roots with heat or glutaraldehyde inhibited the attachment. High concentrations of bacteria in the binding mixture (10^9 - 10^{11} cells/ml) resulted in binding of bacteria to the roots in aggregates. The presence of several sugars, amino acids and organic acids inhibited adsorption. Chemotaxis of Azospirillum towards corn root exudates was observed. Treating the extract with trypsin eliminated this effect. At pH 6.1, Azospirillum brasilense strain Cd adsorbed in higher numbers than Klebsiella pneumoniae, Pseudomonas fluorescens and Escherichia coli. Differences in adsorption levels were also found among several

strains and mutants of Azospirillum.

2. Changes in Root Morphology of Wheat Caused by Azospirillum inoculation (KAPULNIK and OKON, Experientia Supplementum 48:163-170 (1983); KAPULNIK, OKON and HENIS, Canadian Journal of Microbiology (1985) in press).

Wheat seeds (Triticum aestivum L.) were inoculated with Azospirillum brasilense Cd, Sp7, the local isolate Cd-1 and with other types of bacteria. Inoculation with 10^5 to 10^6 colony forming units (cfu) of Azospirillum caused the largest root elongation and total surface of seedlings whereas 10^8 to 10^9 cfu of Azospirillum caused inhibition of root development. Similar effects were obtained in ten different cultivars of wheat inoculated with Azospirillum. Scanning electron micrographs of inoculated wheat root segments showed denser and longer root hairs as compared to the control inoculated with dead cells. This effect was less apparent in more mature roots. In inoculated roots bacteria were located mainly on the cell elongation area and on the basis of root hairs, but much less bacterial cells were present on the root cap or adsorbed to root hairs. Cross sections of Azospirillum inoculated roots showed prominent alterations of the cell arrangement in the layers of the cortex. The results suggest the existence of critical numbers of sites for Azospirillum colonization on the roots, to such an extent that root growth is affected.

3. Effect of Azospirillum spp. Inoculation on Root Development and NO_3^- Uptake in Wheat (Triticum aestivum cv. Miriam) in hydroponic systems (KAPULNIK, GAFNY and OKON, Canadian Journal of Botany 63: (1985) in press).

Wheat (Triticum aestivum cv. Miriam) was grown in petri dishes and in vermiculite filled pots. Inoculation with a mixture of Azospirillum brasilense strains Cd, Sp 7 and a local strain Cd-1,

significantly enhanced above non-inoculated controls the root length and branching. In a hydroponic system containing NO_3^- the surface area of roots and the uptake of NO_3^- from the mineral solution during plant growth was increased by inoculation. However, no significant changes were obtained in the NO_3^- uptake/root surface area ratio. Heading and flowering occurred earlier in the inoculated plants as compared to the non-inoculated ones. Total shoot and root weights, plant height, the number of fertile tillers and grain yield were significantly increased by inoculation. The inoculated roots did not show any nitrogen fixation activity. It was concluded that under these experimental conditions the benefits obtained in wheat inoculated with Azospirillum are mainly derived from an enhancement of root development and the amount of NO_3^- taken up by the roots.

4. Contribution of Nitrogen Fixed by Azospirillum to the N Nutrition of Spring Wheat in Israel (KAPULNIK, FELDMAN, OKON and HENIS, Soil Biology and Biochemistry (1985) in press).

Acetylene reduction activity (ARA) was measured in cores containing roots of various Israeli wild and cultivated wheat lines colonized by Azospirillum. The inoculated plants were grown under greenhouse or field conditions. Although no measurable ARA was detected during earlier stages of wheat development, 50 to 600 nmol $\text{C}_2\text{H}_4 \text{ g}^{-1} \text{ dry root h}^{-1}$ was measured during heading and flowering stages. By using N yield balance and ^{15}N dilution techniques, it was found that Triticum aestivum cv. Miriam inoculated with Azospirillum accumulated 20% more N (^{14}N and ^{15}N) at the booting stage than did the uninoculated control. This difference in N content became less apparent in grains. No significant ^{15}N

dilution could be found and the contribution of atmospheric N_2 to the N content of grains of inoculated plants was negligible. It was concluded that the potential contribution of biological N_2 fixation to spring wheat cultivation in Israel is very low.

III. Field Inoculation Studies

1. Yield Response of Spring Wheat Cultivars to Inoculation with Azospirillum brasilense Under Field Conditions (KAPULNIK, SARIG NUR and OKON, Canadian Journal of Microbiology (1983); KAPULNIK, OKON and HENIS, Crop Science (1985) submitted).

Agronomic benefits of applying rhizosphere bacteria (Azo-
spirillum brasilense) to several crops in Israel have been de-
monstrated. This study is part of a series designed to assess
the benefits of inoculation to wheat plants under field conditions.
Eight commercial Israeli spring wheat cultivars (six Triticum
aestivum and two T. turgidum) grown under varying nitrogen levels
were inoculated with Azospirillum. At low levels of nitrogen, five
of those cultivars showed a significant increase in plant dry weight
as measured at the milky ripe stage. However, by maturation, only
one cultivar (cv. Miriam) showed a significant increase in plant
grain yield, while three other cultivars showed a significant
decrease in grain yield. No effect was found at the high nitrogen
level. To confirm these results, five wheat (T. aestivum) cultivars
were tested separately during 1980-1983 in different locations
under varying nitrogen levels and environmental conditions. Only
cv. Miriam was found to have been consistently positively affected
by Azospirillum inoculation under different levels of nitrogen fer-
tilizer. The striking effect of inoculation on cv 'Miriam' plant
development was the enhancement in the number of roots per plant

compared to unionculated plants. This effect was found at all nitrogen levels. Nutrient (N, P and K) accumulation and number of fertile tillers per area unit were also enhanced by Azospirillum, but these parameters were greatly affected by the applied nitrogen level. These changes during the growth season lead to a significant increase in the grain yield of inoculated plants under field conditions.

2. Response of Non-Irrigated Sorghum bicolor to Azospirillum Inoculation (SARIG, KAPULNIK, NUR and OKON, Experimental Agriculture 20:59-66 (1984)).

The objectives of this field study were to determine the benefits of Azospirillum inoculation in non-irrigated sorghum growing under semi-arid conditions on stored soil moisture (ca. 200-280 mm), after the rainy season. Unirrigated sorghum is known in Israel as a crop that does not respond to N fertilization. In an experiment carried out in the same location (1980) with S. bicolor cv. 6078 for forage, inoculation significantly increased dry matter yield by 19% and the total N yield by 90% as compared to non-inoculated controls (Sarig et al., 1984). With S. bicolor cv. 610 (in 1981) increases in grain yield (17%), significant increases in plant dry weight, mineral content (N, P and K) and panicle number were obtained by inoculation with Azospirillum (Sarig et al., 1984).

An inoculation experiment with Sorghum bicolor cv 610 was carried out near Sede Yoav in 1983. Azospirillum increased total dry weight accumulation and N and K accumulation in plant parts. The plant water status was improved as indicated by relatively higher leaf water potentials, at two growth stages. There were

differences in some morphological parameters of the inoculated plants as compared to controls, such as increased stem diameter and panicle length. There were indications that root systems were more developed in inoculated plants as measured by the average force needed for pulling out the plant from the ground.

In a field experiment with Sorghum bicolor cv. 610 carried out in 1984 at Bet Dagan, inoculation with Azospirillum significantly enhanced top dry matter accumulation. There was a clear improvement in plant-water status as measured by the leaf water potential, osmotic potential at full turgor, stomatal conductance and canopy temperature. Grain yield was significantly increased by 14%. It was concluded that Azospirillum inoculation benefits plant growth and yield of sorghum by mechanisms involved with root development, mineral uptake and plant water relationships. Sorghum grown under semi-arid conditions was an excellent plant model to study Azospirillum effects.

3. Response of Setaria italica to Inoculation with Azospirillum brasilense as compared to Azotobacter chroococcum (YAHALOM, KAPULNIK and OKON, Plant and Soil 82:77-85 (1984)).

Under controlled conditions in pots filled with sand, vermiculite and field soil, inoculation with A. brasilense-Cd ATCC 29729 or with Azotobacter chroococcum caused increases above controls in the weight and N content of panicles of Setaria italica. In no case, however, did N increases in test plants exceed the initial total N content. High acetylene reduction activities (1,000-2,000 nmole/h/pot) could be found only in plants inoculated with Azospirillum. Inoculation with Azospirillum (strain-Cd) in the field caused a significant increase above noninoculated controls

of 18.5% in shoot dry weight, of Setaria italica. Azotobacter caused a non significant increase of 8%. No significant differences were found between yields of Setaria italica grown in soil inoculated with A. chroococcum, and of those plants grown in the presence of A. vinelandii. A. brasilense-Cd was more effective in the field than A. brasilense Sp-7 ATCC 29145. The results suggest that Azospirillum may increased yields of S. italica more efficiently than Azotobacter under local field conditions.

4. Effect of Azospirillum Inoculation on Nitrogen Fixation and Growth of Several Winter Legumes (SARIG, KAPULNIK and OKON, Plant and Soil (1985) in press).

Inoculation of naturally nodulated Pisum sativum L. (garden pea), with Azospirillum in the greenhouse caused a significant increase in nodule numbers above controls. Field inoculation of garden peas in the winter 1981-1982 and Cicer arietinum-L (chick pea), winter 1982-1983, with Azospirillum one week after plant emergence produced a significant increase in seed yield, but did not affect plant dry matter yield. For Vicia sativa-L (vetch) grown in soil in the greenhouse and in the field for forage, winter 1980-1981, inoculation significantly increased dry matter yield, % N, N-content, and acetylene reduction (nitrogen fixation) activity. In Hedysarum coronarium-L (sulla clover), winter 1981-1982 inoculated with both its specific Rhizobium (by the slurry method) and Azospirillum, 7 days after emergence, there was as increase in acetylene reduction above controls inoculated with Rhizobium alone.

These results suggest that it is possible, under conditions tested in this work, to increase nodulation, nitrogen fixation, and crop yields of winter legumes by inoculation with Azospirillum.

5. Summary, table, years, location and results (since 1981 BARD I-254-80)

Table 1 summarizes the field experiments that were carried out between 1979 and 1984 by the staff of the Department of Plant Pathology and Microbiology in cooperation with personnel of the extension service of the Israel Ministry of Agriculture and with growers. Inoculation of the C_4 plants corn, sorghum, Panicum and Setaria resulted in greater yield increases than did inoculation of spring wheat, a C_3 plant. In the summer crops, 75% of the experiments showed significant yield increases, where in spring wheat, the rate of success was 50%. The optimal temperature for growth of Azospirillum is 32-35°C, and therefore it is feasible that bacterial activity, including BNF, on/in the roots, was greater in the summer, particularly in irrigated crops. In comparison, soil temperatures during the vegetative phase of wheat growth are 10-15°C in Israel. Nevertheless, wheat fields inoculated with Azospirillum also showed significant increases in foliage and grain yield (Table 1).

Peat inoculants were found to give more consistent results than inoculation with bacterial suspensions.

Field experiments were carried out under different fertilization levels and diverse environmental and soil conditions. In most cases, the effect of Azospirillum was influenced by the season and year in which the experiment was conducted. In general, the most marked effects of inoculation were obtained at intermediate levels of initial N-fertilizer. "Intermediate levels" is an arbitrary concept because it depends on the level of available combined N present in soil before fertilization and on rates of mineralization and nitrification in the particular soil. Therefore, when comparing inoculated to non-inoculated plots, the largest differences in yield were obtained when the soil was

properly but not excessively fertilized. In soil with a low level of fertilization, there was a positive but generally less marked influence. The same held true for soil fertilized in excess.

Effect on vegetative yield

Inoculation with Azospirillum affected vegetative yield. In sweet corn, there was an increase in top fresh weight, and in maize for forage an increase was seen in fresh and dry weight of leaves, stems and percentage of leaves. Inoculation of Sorghum led to an increase in fresh and dry weight of tops. The improved final dry weight of inoculated plants was due to a 48% larger rate of accumulation during the period 40 to 70 days from emergence, which was due in turn to larger stem weights. In Setaria and Panicum, there were significant increases in weight of leaves and fresh and dry weight of tops. Plant height was also significantly greater in inoculated plots. In wheat both plant height and the weight of all cultivars tested was favoured.

Effect on reproductive yields

In sweet corn the average number of marketable ears per plant was significantly increased by inoculation with Azospirillum in 4 experiments. This was the major contributing factor to increases of ear yield in sweet corn. In sorghum, yield increases resulted from a significant increase in the number of panicles per plant, their weight and weight per 1000 seeds. In other experiments with sorghum, more panicles were obtained in inoculated plots. Significant yield increases in sorghum derived from Azospirillum inoculation were attributed to a significant increase in the number of panicles, since the weight per 1000 seeds was unaffected. In all experiments with wheat (Table 1), increases in grain yield obtained were derived

from increases in the number of fertile tillers per area unit, but not from an increase in seed size.

Other effects in the field

Root development. The most obvious effect exerted by Azospirillum inoculation was the enhancement of root development and branching in the field. The effect on root development started immediately after Azospirillum colonization of roots. Enhancement of root elongation and root exploration of soils is a property especially important for sorghum grown under semi-arid conditions, such as in the Northern-Negev, where, during the dry summer, soil moisture is available for a very short period. The same is true for wheat in years that the rainy season is short.

Mineral accumulation. By analyzing the N, P, K content (yield) of inoculated plants in the field at different times during plant development, we have clearly shown that minerals are accumulated in significantly higher rates in inoculated sorghum, corn and wheat. The minerals are then transferred to the developing panicles and ears, resulting in higher yields.

Table 1: Summary of *Azospirillum* inoculation experiments in Israel, 1979-1984
(Since 1981 BARD I-254-80)

Crop	Year	Location	Cultivar	% increase of yield from non- inoculated controls	Statistically significant P = 0.05	Level of N ferti- zation (% recom- mended)
Forage corn	1979	Sede Yoav*1	H. Nanasi	18.4	+	0
	1980	Kefar Harub*2	851	17.0	+	100
	1979	Sede Yoav	Jubilee	13.7	+	50
Sweet corn (ears)	1980	Sede Yoav	Jubilee	29.6	+	50
	1980	Beth Kama*3	Rinat	26.5	+	50
	1981	Ramat Mag- shimim ⁴	Jubilee	0	-	65
	1981	Yodfat ⁵	Jubilee	13.5	-	100
	1981	Yodfat	Jubilee	38.0	+	25
	1981	Eden Station ⁶	Jubilee	4.9	-	50
	1981	Eden Station	Jubilee	6.8	-	50
	1979	Sede Eliahu ⁷	851	10.5	-	organic field
Grain corn	1980	Sede Eliahu	851	16.5	-	organic field
	1982	Sede Yoav	H 224	15.2	+	0
Forage wheat	1980	Sede Yoav	Barkai	19.5	+	0
Grain wheat	1980	Sede Yoav	Miriam	5.2	+	33
	1980	Sede Eliahu	Barkai	7.9	+	100
	1981	Sede Yoav	Lackhish	9.0	-	66
	1981	Gevulot ⁸	H-895	2.6	-	33
	1982	Sede Yoav	Miriam	13.4	+	100
	1982	Gevulot	Barkai	6.0	-	75
	1983	Gat ⁹	Barkai	3.1	-	0
	1983	Moledet ¹⁰	Miriam	12.0	+	0
Hard wheat	1979	Sede Eliahu	Inbar	10.9	-	organic field
Forage sorghum	1980	Sede Yoav	6078	18.8	+	0
Grain sorghum	1979	Sede Yoav	H-226	12.1	+	0
	1981	Sede Yoav	H-610	17.6	+	0
	1983	Sede Yoav	H-610	19.9	+	0
	1984	Beth Dagan ¹¹	H-610	15.0	+	0
<i>Setaria italica</i> forage	1979	Sede Yoav	-	47.5	+	0
	1982	Beth Kama	-	38.5	+	0
<i>Panicum miliaceum</i> seeds	1979	Sede Eliahu	-	13.2	+	organic field

1,3,9: - Northern Negev; 2-4: - Golan Heights; 8: - Negev; 5,10: - Lower Galilee;
6,7: Beth Shean Valley; 11: - Coastal plain.

E. DESCRIPTION OF COOPERATION

During the execution of the project, the participants of both Israel and the USA exchanged information constantly. Dr. Henis (July 1983) and Dr. Okon (February 1982, May 1983 and June 1984) visited Dr. Burris' laboratory, reported to him on the current research and discussed future work. They supplied Dr. Burris with samples of wheat and corn plants grown on ^{15}N -enriched fertilizers with and without Azospirillum. Dr. Burris analysed the ^{15}N - ^{14}N ratio for the Israeli group. Dr. Burris kindly helped in the preparation of manuscripts related to this work.

Dr. Burris' laboratory continued the investigation on the physiology of azospirilla and conducted rather extensive field experiments on the effect of Azospirillum sp. Dr. Okon visited the field experiments in Wisconsin in 1983, and worked ten days with the group working on physiology of Azospirillum.

F. BENEFIT TO AGRICULTURE

The research has demonstrated that inoculation with Azospirillum sp. can benefit several crops under Israeli conditions. The benefit may be derived from a variety of factors: biological N_2 fixation, enhanced root growth, improved utilization of water or elaboration of growth hormones by the microorganisms. The benefit may appear as increased plant growth, increased crop yield or higher nitrogen content of plant and crop. Of primary economic importance is enhanced yield of harvestable crop, and this occurred in approximately 65% of cases reported on wheat, corn, sorghum, millet and other cereals and forages tested. Results of many of the experiments have been published in reviewed journals.

In 1982 and 1983 we conducted rather extensive field experiments in Wisconsin on the effect of Azospirillum sp. on the productivity of maize. The 1982 experiment included 50 plots with 5 replicates for each treatment. In addition to plus and minus inoculation with the azospirilla, we varied the level of nitrogen fertilizer and the plant cultivar. Replication was good, but the yield of corn was not benefited beyond experimental error by inoculation. The experiment was conducted on a rather heavy and productive soil, because the experiment was designed to ascertain whether there would be any benefit to maize in the U.S.

The 1983 experiment embraced 100 plots 30' x 50' on two soil types; both soils were sandy, but one had considerable organic matter. Fertilizer level and level of inoculum were varied, but only one maize cultivar was used. Again there was no significant benefit to crop yield from inoculation with the azospirilla. Hence, there seems to be little commercial opportunity to promote inoculation of maize with the azospirilla in the U.S. corn belt. This is not to detract from its promise under other soil and climatic conditions. Plant culture conditions in Israel differ considerably from those in the U.S. and appear conducive to benefit from an association between the azospirilla and several cereal and forage crops.

It should also be emphasized that a rather narrow spectrum of non-leguminous plants and bacterial strains have been tested to date. It is quite possible that more effective associations will be discovered if projects such as that of Okon and Henis are continued. The limiting factor in N_2 fixation commonly is an adequate energy supply. The azospirilla are capable of vigorous N_2 fixation if they are supplied a suitable energy source. Increased attention should be paid to dis-

covering plant cultivars that excrete organic acids or carbohydrates from their roots in amounts adequate to support vigorous N₂ fixation by associative bacteria.

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2. Description of research results.

We have not done field experiments with Azospirillum spp. this year, as in the two previous summers we found no positive response to inoculation in maize in our midwestern USA soils. Rather we have concentrated on studies of the physiology and biochemistry of the azospirilla. The research has been done by Anton Hartmann, Seung-Dal Song, and Haian Fu.

Purification of dinitrogenase and dinitrogenase reductase from *A. amazonense*.

The nitrogenase of the free-living, microaerobic N_2 -fixing bacterium *Azospirillum amazonense* (strain Y1) was purified by chromatography on DEAE-52 cellulose, heat treatment and by preparative polyacrylamide gel electrophoresis. The specific nitrogenase activities were 2,400 nmoles C_2H_4 formed $min^{-1}mg$ protein $^{-1}$ for dinitrogenase (Aa_1 , MoFe-protein) and 1,800 nmoles C_2H_4 formed $min^{-1}mg$ protein $^{-1}$ for dinitrogenase reductase (Aa_2 , Fe-protein). The MoFe-protein was composed of a minimum 1,852 amino acid residues, had an isoelectric point of 5.2 and contained 2 atoms of Mo, 24 atoms of Fe and 28 atoms of acid-labile sulfide per molecule. The Fe-protein had 624 amino acid residues, an isoelectric point of 4.6 and contained 4 atoms of Fe and 6 atoms of acid-labile sulfide per molecule. The purified MoFe-protein showed two types of subunits with molecular weights of 55,000 and 50,000. Also, the Fe-protein revealed two non-identical subunits on SDS-PAGE with apparent molecular weights of 35,000 and 31,000.

The two types of Fe-protein subunits were demonstrated in the purified, highly active enzyme, as well as in extracts, with immunological techniques. In the high resolving SDS-PAGE system used, *Azotobacter vinelandii* Fe-protein also showed two closely migrating subunits that migrated differently from the Fe-protein subunits of *Azospirillum brasilense* or *Rhodospirillum rubrum*. The nitrogenase activity of *A. amazonense* Y1 was independent of Mn^{2+} and the addition of activating enzyme had no effect. No activating

enzyme could be found in A. amazonense. Obviously, the nitrogenase system of A. amazonense Y1 is different from Azospirillum brasilense Sp7 and resembles the Azotobacter system.

The nitrogenase components of A. amazonense Y1 were purified to high specific activities. The nitrogenase of A. amazonense Y1 is a typical two protein system, consisting of dinitrogenase (MoFe protein) with a molecular weight of 204,000 and dinitrogenase reductase (Fe protein) with a molecular weight of 67,000. Unlike R. rubrum or in A. brasilense, no evidence for an inactive Fe protein species or an activating enzyme could be found in A. amazonense. Only a weak short-term inhibition of nitrogenase activity and no inhibition of in vitro nitrogenase activity by ammonium chloride has been found in A. amazonense Y1. Extracts of A. brasilense had low nitrogenase activities, and a high portion of their Fe protein was modified. In A. amazonense extracts, high nitrogenase activities ($30 - 40 \text{ nmoles C}_2\text{H}_4 \text{ formed min}^{-1} \text{mg protein}^{-1}$) were obtained; these activities were comparable to those of extracts from Azotobacter vinelandii. Apparently, A. amazonense does not possess the inactivating/activating enzyme system for Fe protein that regulates the Fe protein of R. rubrum and A. brasilense.

The nitrogenase of A. amazonense again exhibits the high degree of conservation among these enzymes. The amino acid composition of the MoFe protein and Fe protein of A. amazonense was compared with other nitrogenases. The difference index $\sum |\Delta D| \times 50$ showed closest relatedness between A. amazonense and Rhizobium lupini and R. japonicum. The least related Fe protein was that of R. rubrum.

As A. amazonense is acid tolerant, the pH profile of its nitrogenase is of interest. As reported for other nitrogenases, the optimum pH range was from pH 6.0 - 7.5. During short exposure times at low pH, about 50% activity was observed at pH 5.0.

Whereas MoFe protein was relatively stable at room temperature, if oxygen was excluded, the Fe protein broke down in a few hours. This higher lability of Fe protein is common among Fe proteins of nitrogenases, and may reflect easily damaged Fe-S centers in the Fe protein. The Fe and S content of the purified, highly active Fe protein is in agreement with the values for other Fe proteins and is compatible with the report that there is a correlation between the Fe and S content of Azotobacter vinelandii Fe protein preparations and their activities.

A high resolving SDS-PAGE system, which was developed by Kanemoto and Ludden to resolve minor differences in the active and modified Fe protein of R. rubrum, consistently revealed two non-identical migrating subunit types of the Fe proteins of A. amazonense and Azotobacter vinelandii. The crossreaction of proteins of these two bands with antiserum against Fe-protein in immunoblotting experiments clearly identified them as Fe protein species. Because the crude extracts and the purified Fe protein of A. amazonense had very high nitrogenase activities, and because the slower migrating subunit did not comigrate with the modified Fe protein subunit from R. rubrum and A. brasilense, it appears that none of the Fe protein subunits represent inactivated species. In addition, there was no spectrophotometric evidence for a bound

adenine residue in the purified Fe protein from A. amazonense.

A separation of the Fe protein of A. vinelandii into two non-identically migrating subunits on SDS-PAGE also has been reported, and the same effect had been described for the Fe protein of Klebsiella pneumoniae. As there is convincing evidence (e.g., DNA- and protein-sequence data) that the Fe protein subunits are identical, the different behavior on SDS-PAGE could be caused by posttranscriptional alterations (processing) of the Fe-protein subunit.

Control of nitrogenase in Azospirillum spp.

Ammonium chloride concentrations as low as 0.05 mM effectively and reversibly inhibited the nitrogenase activity of A. brasilense, A. lipoferum and A. amazonense. The glutamine synthetase inhibitor methionine sulfoximine (MSX) abolished this switch off in A. lipoferum and A. brasilense, but not in A. amazonense. Azaserine, an inhibitor of glutamate synthase, inhibited nitrogenase activity itself. This provides further evidence for glutamine as a metabolite of regulatory importance in the NH_4^+ -switch off phenomenon.

With optimal concentrations of dissolved oxygen (0.001 atm) the addition of 1 mM ammonium chloride to nitrogen-fixing A. brasilense and A. lipoferum cultures caused a gradual decline of nitrogenase activity until it was almost zero after twenty minutes. The in vitro nitrogenase activity also was drastically decreased upon addition of ammonium. On SDS-PAGE, a second Fe protein subunit increased, and this migrated in coincidence with the modified

subunit of inactive Fe-protein of the nitrogenase of Rhodospirillum rubrum. After ammonium addition, ^{32}P was incorporated into the modified subunit of the Fe-protein of A. brasilense.

In A. amazonense, the inhibition of nitrogenase activity by ammonium was only partial, and no transition period could be observed. The in vitro nitrogenase activity of ammonium-treated cells was not decreased, and no evidence for a modified Fe protein subunit was found. Nitrogenase extracts of A. amazonense were active and had a dinitrogenase reductase that migrated as a close double band on SDS-PAGE.

The nitrogenase reaction - reduction of dinitrogen to ammonium - is an extremely energy demanding process and therefore is subject to sophisticated regulatory controls. Besides the regulation of nitrogenase synthesis at the level of nif-gene transcription by combined nitrogen, oxygen and temperature, a posttranslational regulation of nitrogenase activity itself has been described in several species of nitrogen-fixing bacteria.

The rapid and reversible inhibition of nitrogenase activity by small extracellular concentrations of ammonium was found in Rhodopseudomonas palustris and was termed " NH_4^+ -switch off" by Zumft and Castillo. The underlying mechanism has been most intensively studied in members of the family Rhodospirillaceae, including Rhodospirillum rubrum, Rhodopseudomonas capsulata, and Rhodopseudomonas viridis. A covalent modification of the dinitrogenase reductase (Fe protein) was reported to inactivate nitrogenase in response to the addition of ammonium. The modifying

group of the inactive Fe protein of R. rubrum was recently identified as ADP-ribose. An activating enzyme (AE) for inactive Fe-protein from R. rubrum has been purified to homogeneity; AE catalyzes the activation of Fe-protein by the removal of the modifying group. The sensitivity to " NH_4^+ -switch off" and the extent of Fe protein inactivation was dependent on cultural conditions and exhibits strain differences.

In several other nitrogen fixing species and in blue green algae, a short-term inhibition of nitrogenase activity by ammonium has been observed, but the underlying mechanisms were not completely characterized. In Rhodopseudomonas sphaeroides the inhibition of nitrogenase by ammonium chloride was not accompanied by a decrease of the in vitro-nitrogenase activity, and therefore a covalent modification mechanism could not be involved in the " NH_4^+ -switch off". It was suggested that glutamine inhibited the electron flow to nitrogenase in R. sphaeroides. In Azotobacter vinelandii, ammonium chloride inhibited the nitrogenase activity to various extents, depending on the conditions of culture and assay. Changes in the membrane potential could be correlated with the nitrogenase inhibition.

By the use of analogues of amino acids as inhibitors of glutamine synthetase (e.g., MSX) and glutamate synthase (e.g., azaserine), and with mutants in these ammonium assimilatory enzymes, glutamine was found to be the most probable candidate as mediator of the ammonium inhibition of nitrogenase. The glutamine synthetase, producing glutamine from ammonium and glutamate, may play a major

role in the " NH_4^+ -switch off". However, ammonium per se might regulate nitrogenase activity in some Anabaena species.

In the ten years since the rediscovery of Spirillum lipoferum, renamed Azospirillum lipoferum, considerable research has focussed on the azospirilla because they are able to fix nitrogen and to colonize the root surface or invade the roots of important forage and grain crops. They fix nitrogen only under microaerobic conditions. All Azospirillum spp. can use organic acids readily, and some use sugars as carbon and energy sources to support nitrogen fixation. A. lipoferum can fix nitrogen with glucose as its source of energy. Azospirillum amazonense has similar properties but a considerably lower pH-optimum (pH 6); it has the ability to grow and fix nitrogen with sucrose as its carbon source.

Little is known about the biochemistry of nitrogenase regulation. Ludden et al. studied the nitrogenase system of Spirillum lipoferum (renamed Azospirillum brasilense Sp7). The nitrogenase system of A. brasilense is a three component system (dinitrogenase, dinitrogenase reductase, and activating enzyme) and the dinitrogenase reductase (Fe protein) normally is isolated in an inactive form. It can be activated by activating enzyme in a manganese-dependent reaction. The fact, that activating enzyme of R. rubrum can activate the inactive Fe-protein of A. brasilense indicated the close relationship of their nitrogenase systems.

We have found a short-term inhibition by ammonium of nitrogenase in A. brasilense Sp7, A. lipoferum SpRG20a and SpBr17 and A. amazonense Y1. The nitrogenase of A. brasilense and A. lipoferum

was regulated by covalent modification of the Fe protein, whereas in A. amazonense a different, non-covalently inhibitory mechanism caused only a partial inhibition of nitrogenase activity by ammonium.

Results

The " NH_4^+ -switch off" of nitrogen fixation in A. brasilense Sp7, A. lipoferum SpBR17 and SpRG20a and A. amazonense Y1 is concentration dependent. The addition of 50 or 100 μM ammonium chloride caused a transient inhibition of nitrogenase activity. Higher ammonium concentrations extended the inhibition period. With 0.25 and 1 mM NH_4Cl a very rapid and total inhibition of nitrogenase activity occurred in A. lipoferum and A. brasilense Sp7, whereas in A. amazonense Y1 the inhibition was only partial (even with 20 mM NH_4Cl the " NH_4^+ -switch off" was incomplete).

The addition of MSX, an inhibitor of glutamine synthetase, abolished the inhibition of nitrogenase by ammonium chloride in A. lipoferum SpBR17 and A. brasilense Sp7. In A. amazonense Y1, however, MSX could not abolish the " NH_4^+ -switch off". When the inhibitory effect of MSX on glutamine synthetase was tested in vitro, both the glutamine synthetase of A. brasilense and A. amazonense were equally sensitive. The ammonium uptake, measured as ^{14}C -methylammonium uptake, by A. amazonense cells was less inhibited by MSX than in A. brasilense. A. amazonense could grow readily in the presence of MSX (0.1 mg/ml), whereas the growth of A. brasilense and A. lipoferum was totally inhibited by MSX.

The time course of " NH_4^+ -switch off" in A. brasilense Sp7 was

measured at different constant oxygen concentrations in solution. At low oxygen concentrations (0.001 and 0.004 atm O_2), nitrogenase activity slowed down gradually within 10-15 minutes until the inhibition was almost complete. At higher oxygen concentrations (0.006 - 0.015 atm O_2), which already drastically inhibited nitrogenase activities, the inhibition by ammonium was complete within 5 - 10 minutes. Similar results were obtained in A. lipoferum. In contrast, the time course of " NH_4^+ -switch off" in A. amazonense Y1 was different. Immediately after the addition of ammonium chloride, a slower rate of nitrogenase activity was observed without a transition period as in A. brasilense Sp7 and A. lipoferum SpBR17. At low oxygen concentrations (0.0004-0.001 atm) the remaining activity after ammonium addition was 55-60%, whereas at higher oxygen concentrations (0.010 - 0.040 atm O_2) only about 30% activity remained.

To elucidate the mechanism involved in the short-term inhibition of nitrogenase activity by ammonium chloride, the in vitro nitrogenase activities and the status of Fe protein of nitrogenase before and after ammonium addition were examined.

In A. brasilense and A. lipoferum whole cell nitrogenase activities had dropped to about 1%, 15 minutes after the addition of 1 mM ammonium chloride. The in vitro nitrogenase activities also decreased drastically in extracts prepared from culture samples after ammonium addition. However, in A. amazonense, addition of 1 or even 10 mM ammonium chloride reduced the whole cell nitrogenase activity only partially, and the in vitro nitrogenase activity of

extracts was not inhibited upon ammonium addition to the culture. In response to the addition of 1 mM ammonium chloride, the oxygen uptake rates in A. brasilense and A. lipoferum were increased, whereas in A. amazonense the oxygen uptake was unaltered.

The nitrogenase activities of extracts of A. brasilense and A. lipoferum were less than one-tenth of those of A. amazonense. In accordance with this, extracts of N_2 -fixing A. brasilense and A. lipoferum cultures showed an inactive subunit type of Fe protein on SDS-PAGE, but A. amazonense did not. To test the involvement of phosphate incorporation in inactivation of the Fe protein, the ammonium switch-off was performed with a ^{32}P -labelled, N_2 -fixing culture of A. brasilense Sp7. Ten minutes after the addition of 1 mM ammonium chloride, the culture sample (10 ml) was rapidly filtered, freeze quenched, and extracts were prepared. After immunoprecipitation, an autoradiogram of the SDS-PAGE clearly demonstrated an incorporation of ^{32}P into the slower migrating Fe-protein subunit of A. brasilense Sp7 upon " NH_4^+ -switch-off".

Although a short-term inhibition of nitrogenase by ammonium chloride could be demonstrated in all three Azospirillum species, only in A. brasilense and A. lipoferum was covalent modification of the Fe-protein involved in NH_4^+ -switch-off. The observed weak inhibition of nitrogen fixation by ammonium in A. amazonense Y1 is interesting and may prove important for a root-colonizing bacterium fixing N_2 in the soil.

3. Evaluation of research achievement with respect to the original research proposal and its objectives.

As two years of field experiments on the nutrient-rich soils of midwestern USA showed no statistically significant increase in crop yield from inoculation with Azospirillum spp., we turned to a re-examination of the nitrogenase system of the organisms. It is hoped that the information on the physiology and biochemistry of the organisms will be helpful eventually in the practical use of the azospirilla. The observation that N_2 fixation by A. amazonense, in contrast to the other species, is not "turned off" by added ammonium suggests that serious field trials should be performed with this organism.

4. Description and results of cooperation.

We maintained regular communication with Dr. Okon, although there was less interchange of personnel between laboratories than in earlier years.

5. Conclusions and plans.

R. H. Burris has retired and as a result will not continue the collaboration. Instead, collaboration has been initiated with Dr. D. Phillips of the University of California-Davis.

6. Published papers and manuscripts.

Martinez-Drets, Gloria, Del Gallo, Maddalena, Burpee, Cheryl and Burris, Robert H. Catabolism of carbohydrates and organic acids and expression of nitrogenase by Azospirilla. J. Bacteriol. 159, 80-85 (1984).

Two papers are in manuscript form describing: (a) purification of nitrogenase, and (b) control of nitrogenase in the azospirilla.