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Field inoculation of rice with *in vitro* selected plant-growth promoting-rhizobacteria

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Summary — In a previous study of two Egyptian soils a great diversity of N_2-fixing bacteria was found in the dominant microflora of the rhizosphere of rice. Under gnotobiotic conditions, strain NO40 of *Azospirillum brasilense* was more efficient than most isolates. NO40 was used under field conditions in 2 locations in the Nile delta for inoculating rice at sowing and at transplanting. In both locations, inoculation significantly increased yield ($0.01 < P < 0.05$). These increases were higher at higher rates of nitrogen fertilization, and reached 15 and 20% at the Sakha and Gemmeiza experimental stations, respectively. Late components of yield were more affected.

PGPR – *Azospirillum brasilense* – spermosphere model – yield – inoculation

Résumé — Inoculation du riz, au champ, par des PGPR sélectionnées *in vitro*. *Dans 2 sols égyptiens précédemment étudiés, une grande variété des bactéries fixatrices d'azote avait été mise en évidence dans la rhizosphère du riz, ainsi que de grandes différences quant à l'efficience de leur fixation d'azote en conditions gnotobiotiques. La souche bactérienne la plus efficiente en modèle spermosphère était la souche NO40 d'*Azospirillum brasilense*, qui apparaissait significativement plus efficiente que la souche NO13 d'* Enterobacter cloacae*, utilisée comme référence et dont l'efficience était représentative de la plupart des isolats étudiés. Cette souche a été inoculée au riz, au semis et au repiquage dans 2 stations expérimentales du delta du Nil. L'inoculation de la souche NO40, dans les deux cas, s'est traduite par un accroissement significatif de la récolte (0,01< *P*< 0,05). L'effet était d'autant plus marqué que la fertilisation azotée était plus élevée et atteignait 15% à Sakha et 20% à Gemmeiza. L'effet semble concerner surtout les composantes tardives du rendement.*

PGPR* – Azospirillum brasilense *– modèle spermosphère – récolte – inoculation

INTRODUCTION

Studies on plant-growth-promoting rhizobacteria (PGPR) have proliferated over recent years (Davison, 1988), but the concept of PGPR is still vague. Occasionally it has been applied to bacteria which have been studied for positive effects on plant growth such as phosphate solubilization (Mishustin and Naumova, 1962), nitrogen fixation or hormone production (Gaskins *et al.*, 1985). More recently, it has been used for other phenomena such as the antagonism against plant pathogens (Burr and Caesar, 1984), the ability to increase plant physiological processes, or plant tolerance to a great variety of stresses. The only common feature is the beneficial effect observed on crops following inoculation.

Common difficulties in all studies of PGPR responses are : the necessity to screen large numbers of plants; the lack of correlation between *in vitro* and *in situ* results; and the inconsistency of observed effects on crops. Nitrogen-fixing bacteria associated with cereals provide a good example of the problems involved. Their positive effects on plant growth were established long ago (see for instance, Truffaut and Bezssonnof, 1925) and their use on a large scale was attempted, then abandoned, in the Soviet Union in the forties (Rubenchik, 1963). Following the discovery (Day and Döbereiner, 1975) that *Azospirillum* was a common inhabitant of grass rhizospheres, their use again became fashionable in the late seventies. This enthusiasm abated when it became evident that the ability of a bacterium to positively affect plant growth could not be reliably predicted.

During a study of the dominant N_2-fixing bacteria associated with the roots of rice, Thomas-Bauzon *et al.* (1982) designed a gnotobiotic experimental model which they named the spermosphere model. Among other purposes this model showed large differences among strains in their ability to fix nitrogen in association with their host plants under gnotobiotic conditions *in vitro*. Inoculation with the most efficient strain (*Azospirillum lipoferum* 4B), increased yields of rice under field conditions (Charyulu *et al.*, 1985).

To further evaluate the predictive value of the *in vitro* behaviour of strains associated with sterile plantlets, a study was conducted on two Egyptian soils. A great diversity was observed amongst the most abundant N_2-fixing bacteria (Omar *et al.*, 1988) which included *Enterobacter cloacae, E. agglomerans, Citrobacter freundii, Klebsiella planticola* and *Azospirillum brasilense.* The efficiency of each of these isolates was measured in spermosphere models (Heulin *et al.*, 1989) and *A. brasilense* NO40 was found to be the most efficient. This article describes the use of this strain to inoculate rice *in situ* in Egypt.

MATERIALS AND METHODS

Bacterial strains

Strain NO40 of *Azospirillum brasilense* was isolated from a 10^{-3} dilution of a rice rhizosphere soil, originally taken from the Moshtohoor experimental station, 50 km North of Cairo. The soil sample was used to grow rice cv. Giza 171 in a phytotron for 8 d until nitrogenase activity was high (Omar *et al.*, 1988). Among 23 strains of N_2-fixing bacteria compared *in vitro* in spermosphere model, strain NO40 is 2 to 5 times more active than most strains isolated from this soil (Heulin *et al.*, 1989).

Stations

Inoculation trials were performed at 2 different experimental stations in the Nile delta, *viz.* (1) Gemmeiza, 120 and (2) Sakha, 150 km north of Cairo, respectively. Table I summarizes some properties of the soils in the above areas.

Experimental design

In the 2 experiments, the effect of inoculation was compared to the effect of a regular nitrogen fertilization. The maximum rate of N fertilizer (96 kg N/ha in Sakha and 76 kg N/ha in Gemmeiza) was the rate recommended by the Ministry of Agriculture for the location and in consideration of the previous crop. A zero N treatment and a medium N treatment (48 and 38 kg N/ha, respectively) were included. Control plots were inoculated with a heat-killed inoculum. Plots were randomized inside blocks.

Inoculation

Bacteria were grown to maximum density (10^9 cells/ml) in Difco nutrient broth. Bags filled with seeds were soaked in water for 24 h then kept warm for another 24 h under a layer of decomposing manure, following traditional practice. Germinated seeds were placed in large basins overnight in contact with the bacterial suspension or its autoclaved counterpart. Each inoculated seed received approximately 10^6 bacteria. Nurseries were sown the next morning. Care was taken to avoid cross-contamination : plots were separated by mud bands, 50 cm wide inside blocks and 1 m wide between blocks, and water was prevented from flowing between plots. At transplanting, young plantlets were inoculated again by soaking their roots overnight in a similar bacterial suspension.

Fertilizers

A trace amount of N was applied to the nursery beds, along with 11 kg $ZnSO_4$/ha to avoid Zn deficiency.

Before transplanting, the plots received a PK fertilization equivalent to 35.7 kg P_2O_5 and 57.1 kg K_2O/ha. Nitrogen fertilization (ammonium sulfate) was split : half the rate was applied before tillering between 16 and 23 d after transplanting (July 13, 1985, in Gemmeiza; July 27 in Sakha), the other half was applied 17 d later.

Cultivation

Nursery beds ware sown on May 16 in Gemmeiza and May 28 in Sakha. At the same date zinc sulfate was

Table I. Soil characteristics of the stations in which inoculation trials were conducted (Sakha and Gemmeiza) and of the station where NO40 was isolated (Moshtohoor).

Station	*Clay (%)*	*C/N*	*Total N (%)*	*pH*	*$CaCO_3$ (%)*
Sakha	59.8	13.6	0.088	8.5	3.17
Gemmeiza	59.9	13.1	0.137	8.4	2.91
Moshtohoor	58.6	11.8	0.136	8.3	3.08

applied. Trace amounts of N were applied on June 5 and 16, respectively. Transplanting was done on June 28 in Gemmeiza and July 4 in Sakha, 2 weeks after the soil had been waterlogged. Flowering occurred on August 30 and September 7. No pesticides were used; nevertheless, development of algae was extremely low. Plots were weeded by hand, and hoed after water removal at each fertilizer application. Birds were repelled.

Harvest

Plots were harvested on October 26 and 22, 120 and 110 d after transplanting, at Gemmeiza and Sakha, respectively. Harvest was done by hand, using a sickle. Determinations were made of grain and shoot dry matter, nitrogen content, 1 000 grains dry weight, panicle dry matter.

Statistics

Classical variance analyses were performed. Studied factors were : N fertilizer, inoculation and blocks. Each experimental station was treated separately.

RESULTS AND DISCUSSION

At both locations, a clear-cut effect of nitrogen fertilizer (*P*=1%) was found, suggesting that nitrogen was actually the limiting factor of yield and N exported with harvest (Table II and III). This effect of fertilizer was also found for the N-content of the grain (Table IV), but not for the weight of 1 000 grains (Table V). As grain N- content mainly depends upon remobilization of foliar proteins, whereas the weight of 1 000 grains is a late component of yield, we assume that the effect of N fertilizer is an early effect on plant N nutrition. At the time of tiller initiation, this effect was still observable in Sakha (Table VI), but it was absent in Gemmeiza (no effect of N on this parameter; Table VI), perhaps due to the lower supply of N fertilizer.

Inoculation had a positive significant effect on yield and N exported with harvest. This effect was observed mainly on late components of yield, such as the weight of 1 000 grains (Table V) which is not affected by N fertilizers.

Thus, there seems to be a balance between : an effect of fertilizers on early components of yield (N content of grains, number of fertile tillers in Sakha); an effect of inoculation on the filling of grain, a late component of yield, as if the period of active photosynthesis was extended by inoculation. At the same time, it must be emphasized (data not shown) that, in both stations, inoculation had a significant effect on the panicle length

Table II. Design of the field experiments. Effect of inoculation and of 3 rates of nitrogen fertilizer on grain yield (kg/10m^2 which can be extrapolated to T/ha).

Station		*Gemmeiza*			*Sakha*	
Previous crop		Clover			Barley	
Plot size (m^2)		20			14	
Replicates		6			8	
N,kg/ha	0	38	76	0	48	96
Killed inoculum	9.33	9.87	11.09	5.91	6.83	7.55
Live inoculum	9.95	11.49	13.44	6.22	7.10	8.75
Fertilizer effect		s (*P*=1%)			s (*P*=1%)	
Inoculum effect		s (*P*=1%)			s (*P*=5%)	

Grain weights refer to a 14% water content. Variation coefficients are 13.3% in Gemmeiza and 11.6% in Sakha. Respective SDs are 14.5 and 8.2.

Table III. Effect of inoculation and of 3 rates of nitrogen fertilizer on the total nitrogen exported with harvest (kg N/ha).

Station		*Gemmeiza*			*Sakha*	
N, kg/ha	0	38	76	0	48	96
Killed inoculum	95.5	108.0	126.0	56.4	85.3	80.7
Live inoculum	101.3	117.5	156.1	67.5	79.7	122.3
Fertilizer effect		s (*P*=1%)			s (*P*=1%)	
Inoculum effect		s (*P*=5%)			s (*P*=1%)	

Variation coefficients are 17.0% and 10.4% in Gemmeiza and Sakha, respectively. Respective SDs are 19.96 and 8.51.

Table IV. Effect of inoculation and of 3 rates of nitrogen fertilizer on the nitrogen content of grain (%).

Station	*Gemmeiza*			*Sakha*		
N, kg/ha	0	38	76	0	48	96
Killed inoculum	1.02	1.09	1.13	0.96	1.25	1.07
Live inoculum	1.02	1.01	1.16	1.08	1.13	1.41
Fertilizer effect		s (*P*=1%)			s (*P*=1%)	
Inoculum effect		ns (*P*=5%)			s (*P*=1%)	

Variation coefficients are 5.1%, and 2.3% in Gemmeiza and Sakha, respectively. Respective SDs are 0.06 and 0.03.

Table V. Effect of inoculation and of the 3 rates of nitrogen fertilizer on the weight of 1 000 grains (in g).

Station	*Gemmeiza*			*Sfakha*		
N, kg/ha	0	38	76	0	48	96
Killed inoculum	25.3	25.3	23.5	25.9	26.1	25.6
Live inoculum	26.1	25.6	27.1	26.1	25.8	28.9
Fertilizer effect		ns (*P*=5%)			ns (*P*=5%)	
Inoculum effect		s (*P*=5%)			s (*P*=5%)	

Variation coefficients are 6.6% and 6.9% in Gemmeiza and Sakha, respectively. Respective SDs are 1.69 and 1.83.

Table VI. Effect of inoculation and of the 3 rates of nitrogen fertilizer on the number of fertile tillers per hill.

Station	*Gemmeiza*			*Sakha*		
N, kg/ha	0	38	76	0	48	96
Killed inoculum	23	17	23	15	19	18
Live inoculum	21	23	25	17	19	24
Fertilizer effect		ns (*P*=5%)			s (*P*=1%)	
Inoculum effect		ns (*P*=5%)			s (*P*=5%)	

Variation coefficients are 29.0% and 21.0% in Gemmeiza and Sakha, respectively. Respective SDs are 6.42 and 3.94.

(1%<*P*<5%), whereas N fertilizer only stimulated length in Sakha.

As confirmed in 3 subsequent field experiments (reported elsewhere), seed inoculation of rice by *Azospirillum* NO40 thus appears to mimic the effect of N fertilizers beyond their probable time of exhaustion.

The effect of inoculation on yield in the absence of added N is low : +5% in Sakha, and +6% in Gemmeiza. At the highest fertilization rate it is surprisingly high : +15% in Sakha, and +21% in Gemmeiza (Table II and Fig. 1). Obviously, in this experiment there is no antagonism between nitrogen fertilizers and the effect of inoculation by bacteria. On the contrary, a synergistic effect does exist in many cases, as shown by the presence of significant interactions between the inoculation and fertilizer factors. For instance, on the weight of 1000 grains, this interaction is significant (*P*=2% in Sakha and *ca.* 5% in Gemmeiza), the corresponding *F* values being 4.60 and 3.34, respectively.

This effect of inoculation was observed under conditions when N was the actual limiting factor of growth, as shown by the yield of controls increasing with the rate of N fertilization. In another experiment (data not shown), where the controls did not respond to N application, no effect of inoculation was observed. This supports the hypothesis that *Azospirillum* acts through the nitrogen nutrition of rice.

At the end of the vegetative growth phase, photosynthesis and exudation are maximum and nitrogen fertilizer usually exhausted. These

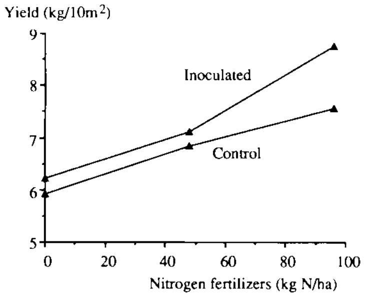

Fig. 1. Sakha experimental station; effect on yield of *Azospirillum brasilense* NO40 inoculation, compared to the effect of nitrogen fertilizers.

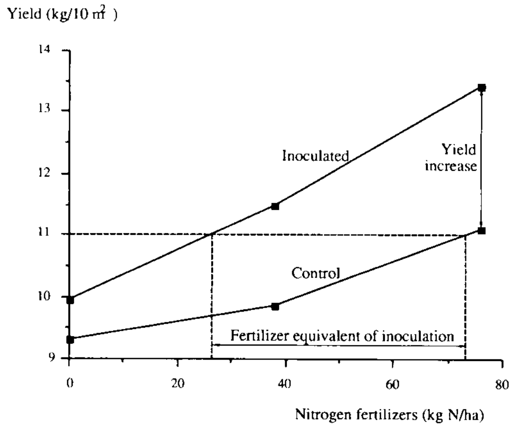

Fig. 2. Gemmeiza experimental station; effect on yield of *Azospirillum brasilense* NO40 inoculation, compared to the effect of nitrogen fertilizers. A same level of yield, *e.g.* 11 T/ha can be obtained by applying 70 kg N/ha of nitrogen fertilizers, or alternatively by inoculating the plant and using only 25 kg N/ha. The effect of inoculation is thus equivalent to the use of 45 kg N/ha at this level of yield.

conditions are clearly conducive to a high nitrogenase activity (Balandreau and Ducerf, 1980) which, in turn, could supply inoculated plants with extra nitrogen, enabling photosynthesis to extend longer into the grain-filling period. Other explanations are also possible : if *Azospirillum* acts through hormone production rather than nitrogen fixation, extended root development may open access to an extended pool of soil nitrogen, so improving the fertilizer use. In the absence of labelled nitrogen fertilizers, the 2 mechanims cannot be distinguished, for both of them would similarly affect the apparent increase in fertilizer use. Table III shows that there is such an increase in the apparent coefficient of fertilizer use (increase in exported N, in percentage of the corresponding increase in N fertilizer) : increases in N exported with harvest are approximately 40% of increases in N fertilizer in Gemmeiza and 30% in Sakha for controls, whereas for inoculated plots they apparently reach 70% and 60% respectively. Moreover, it must be stressed that nitrogen fixation and hormonal effects are not exclusive mechanisms and could work synergistically.

Whatever the underlying mechanism, the practice of cereal inoculation should not be regarded as a substitute for nitrogen fertilizers, but as, a simple means of extending their effect beyond economically feasible rates. Nor is inoculation a practice for inefficient agricultures : the yield levels of rice reported here are among the highest in the world. Figure 2 emphasizes the 2 extreme types of situation in which inoculation can be considered : in developing countries in which the cost of nitrogen fertilizers is a limiting factor, the use of seed inoculation would allow for an increase in yield without increasing fertilizer consumption. In developed countries where very high rates of nitrogen fertilizers are used, pollution is often a concern : in this situation, seed inoculation can be used to decrease the fertilizer application while keeping yield constant.

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