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# Towards Integrated Biofertilization Management with Free Living and Associative Dinitrogen Fixers for Enhancing Rice Performance in the Nile Delta

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**Abstract**

Dinitrogen fixers belonging to cyanobacteria, *Azospirillum* and *Azotobacter* were isolated from rice fields in the Nile delta and used as biofertilizer combinations for rice along with different amounts of chemical-N. A combination contained the three $N_2$-fixers along with 1/3 of the chemical-N recommended for the rice variety Giza-172 (108 kg N/ha) produced the highest productive tillering, grain size, grain and straw yields and N-contents, the harvest index (% of grain yield/grain+straw yields) and the agronomic fertilizer N-use efficiency (kg grain yield/kg fertilizer-N).

Keywords: Biofertilization, cyanobacteria, *Azospirillum*, *Azotobacter*, rice, N-use efficiency

## 1. Introduction

Most of chemical N applied to rice fields does not contribute to crop performance, due to low agronomic fertilizer N-use efficiency of less than 40% of the added N (Prasad and De Datta, 1979). Sustainable soil fertility and high potential to enhance rice growth and performance without use of combined-N are objectives in many farming systems (Venkataraman, 1966). In

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Egypt, increases in tillers bearing panicles, nitrogen contents of grain and straw, the harvest index, grain size and in N-use efficiency in addition to a yield of some 9 ton/ha were achieved using nitrogen contributed by cyanobacteria or azospirilla along with 1/3 to 1/2 of the recommended quantities of fertilizer-N (Yanni and Hegazy, 1990). This indicates that 2/3 or at least 1/2 of the N demand of rice is still has to be added as combined-N. Thus, the additive effect of application of two or more of dinitrogen-fixing group of microorganisms when applied together in rice farming system is of potential field studies on more contribution using a consortium of these microorganisms to crop growth and performance. Furthermore, contributions due to inoculation with preparations containing foreign isolates of cyanobacteria or azospirilla were found less than in case of use of indigenous ones isolated from and tested in rice fields of the same area. Normally, local isolates are tolerant/adapted to specific adverse soil conditions like excessive amounts of chemical-N and pesticides (Yanni and Abd El-Rahman, 1993) and their high competitiveness with other microorganisms dominating the same area is expected to be higher than foreign isolates. Such circumstances are considered serious limitations determine whether or not a given isolate can be safely included in an inocula preparation.

The aim of this work is to test the hypothesis of whether or not inoculation by a unique group of microorganisms with relatively higher amounts of chemical nitrogen can be substituted with inoculation by a consortium containing two or three groups of dinitrogen fixers with less or even no amounts of chemical-N, if so, this will decrease rice cropping input in addition to minimize environmental pollution originating from excessive use of chemical nitrogen.

## 2. Materials and Methods

Dinitrogen-fixers belonging to cyanobacteria, *Azospirillum* and *Azotobacter* were isolated from rice fields of the middle and northern Nile delta. Soil and rice rhizosphere samples were collected every 5 to 10 km along three main axis extending all over most of the rice growing area in Egypt. Standard bacteriological procedures using specific media were used for their isolation and identification (El-Nawawy et al., 1958; Döbereiner and Day, 1976; Hegazi and Niemela, 1976). Sixty-two cyanobacterial isolates were examined microscopically and found to be belonging mainly to the genera *Nostoc* and *Anabaena*. Fifty-seven isolates of each of *Azospirillum* and *Azotobacter* were also enumerated. Their dinitrogen fixing efficiencies and tolerance towards some pesticides were tested. Contributions of the most effective dinitrogen-fixing and pesticide-tolerant ones to rice growth and performance were evaluated in green house experiments. The active N-contributors were used for inoculation in rice field plot experiment in the rice growing season 1997. The experimental design comprised: 1) inoculation with a culture of cyanobacteria containing equal numbers of colony forming units (CFU) of the dinitrogen-fixers *Anabaena cylindrica*, *Anabaena oryzae* and *Nostoc muscorum* ($1.7\times10^8$ CFU/ml) at 10 ml/$m^2$ after 5 days from transplanting (dpt); 2) inoculation with a culture containing $1.6\times10^6$ CFU/ml of *Azotobacter* sp. at 10 ml/$m^2$; and 3) inoculation with a culture containing $3.5\times10^5$ CFU/ml of *Azospirillum* sp. at 10 ml/$m^2$. Urea (46% N) was applied at 36, 72 or 108 kg N/ha in equal two split doses at 20 dpt and the early panicle initiation stage (40 dpt). Plant height, productive tillers (tillers bearing panicles)/$m^2$, grain yield, straw, the harvest index (percentage of grain yield/grain + straw), grain N-content, straw N-content, and the agronomic N-use efficiency (kg grain yield/kg fertilizer-N) were estimated at harvest. A representative sample of 100 panicle was taken randomly from each replicate for estimation of the panicle length, number of spiklets/panicle and grain size indexed as 1000-grain weight. The data were computed as a split-plot design experiment with inoculation by one or more dinitrogen-fixer as the main-plot treatments and the different amounts of urea as the split-plot treatments, with four replicates each of 12.6 $m^2$ for each subtreatment. The mean differences were compared to their corresponding least significant differences at the 95 and 99% confidence level.

## 3. Results

Data of plant height, productive tillers/$m^2$, panicle length, number of spiklets/panicle, grain size (Table 1) grain yield, straw and the harvest index (Table 2), N contents of grain and straw and the agronomic N-use efficiency (Table 3) showed increases with biofertilization by the cyanobacteria, *Azotobacter*, *Azospirillum* or their combinations even under application of combined N at 108 kg N/ha. Data of the main-plot treatments inoculated with cyanobacteria alone or in combination with *Azotobacter* and/or *Azospirillum* came significantly higher than those of their corresponding counterparts confirming the results of Roger and Kulassoria (1980) who reported cyanobacteria as the main N contributors to the flooded rice ecosystem. In most cases, data of growth parameters registered with biofertilization along with application of combined N at 108 kg N/ha did not show significant changes comparing to those at 72 kg N/ha, specially when the cyanobacteria had been included in the fertilization management. However, increases in grain yield amounted to 23.6, 29.8 and 28.7% were obtained with inoculation by the cyanobacteria along with fertilizer N at 36, 72 and 108 kg N/ha, respectively. The corresponding increases in case of inoculation with the *Azospirillum* were 45.7, 24.3 and 22.5%, in the same respective order. It seems that inoculation

Table 1. Effect of integrated biofertilization management with cyanobacteria, *Azotobacter* and *Azospirillum* vs. combined-N on growth parameters of the Japonica rice Giza-172

| Treatment | Plant height (cm) | | | Productive tillers ($m^2$) | | | Panicle length (cm) | | | Spiklets/spike | | | 1000-grain wgt. (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N |
| Uninoculated | 87.3 | 88.0 | 94.5 | 379 | 461 | 500 | 17.4 | 18.4 | 18.4 | 7.3 | 8.3 | 7.8 | 25.3 | 25.3 | 24.7 |
| Cyanobacteria (cyan.) | 88.8 | 92.8 | 102.8 | 425 | 498 | 480 | 17.7 | 18.6 | 19.6 | 7.6 | 8.3 | 8.0 | 26.0 | 24.4 | 25.2 |
| *Azotobacter* (Azot.) | 87.5 | 98.8 | 98.3 | 485 | 429 | 481 | 17.4 | 19.3 | 19.0 | 7.7 | 7.6 | 7.8 | 25.6 | 25.9 | 25.7 |
| *Azospirillum* (Azos.) | 93.8 | 101.5 | 100.3 | 439 | 476 | 466 | 18.4 | 20.5 | 20.2 | 7.7 | 8.0 | 8.1 | 25.2 | 25.2 | 25.9 |
| Cyan. + Azot. | 91.3 | 95.3 | 98.0 | 458 | 479 | 469 | 18.8 | 19.4 | 19.4 | 7.3 | 7.8 | 8.1 | 25.9 | 25.2 | 25.2 |
| Cyan. + Azos. | 91.8 | 94.0 | 97.3 | 490 | 490 | 529 | 18.6 | 19.4 | 19.5 | 8.1 | 7.9 | 7.8 | 25.7 | 26.4 | 25.6 |
| Azot. + Azos | 91.3 | 93.3 | 105.0 | 471 | 508 | 534 | 17.6 | 20.2 | 19.1 | 7.6 | 7.9 | 8.2 | 25.1 | 25.2 | 25.6 |
| Cyano. + Azot. + Azos. | 88.3 | 93.0 | 102.0 | 534 | 575 | 603 | 18.3 | 19.9 | 20.3 | 7.9 | 8.1 | 8.5 | 25.1 | 25.3 | 24.6 |
| LSD | 0.05 | 0.01 | | 0.05 | 0.01 | | 0.05 | 0.01 | | 0.05 | 0.01 | | 0.05 | 0.01 | |
| Biofertilizers (Biof.) | 1.7 | 2.3 | | 24 | 32 | | 0.4 | 0.6 | | 0.2 | 0.3 | | 0.2 | 0.3 | |
| N-fertilizer (N) | 1.3 | 1.8 | | 14 | 18 | | 0.3 | 0.4 | | 0.1 | 0.2 | | 0.2 | n.s. | |
| Biof. × N | 3.7 | 5.0 | | 38 | 51 | | 0.9 | n.s. | | 0.3 | 0.4 | | 0.4 | 0.6 | |

N: kg N/ha applied in two equal doses: 20 days after transplanting and at the early panicle initiation stage.

Table 2. Effect of integrated biofertilization management with cyanobacteria, *Azotobacter* and *Azospirillum* vs. combined-N on crop performance of the Japonica rice Giza-172

| Treatment | Grain yield (ton/ha) | | | Straw (ton/ha) | | | Harvest index* | | |
|---|---|---|---|---|---|---|---|---|---|
| | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N |
| Uninoculated | 3.81 | 4.36 | 4.94 | 6.57 | 7.07 | 9.34 | 36.7 | 38.2 | 37.2 |
| Cyanobacteria (cyan.) | 4.71 | 5.66 | 6.36 | 6.97 | 8.36 | 9.84 | 40.3 | 40.4 | 39.5 |
| *Azotobacter* (Azot.) | 4.38 | 5.23 | 5.30 | 6.85 | 8.23 | 8.72 | 39.0 | 38.9 | 37.9 |
| *Azospirillum* (Azos.) | 5.55 | 5.42 | 6.05 | 9.45 | 8.64 | 12.06 | 37.1 | 38.5 | 38.7 |
| Cyan. + Azot. | 5.18 | 4.92 | 5.78 | 9.13 | 8.64 | 9.41 | 36.2 | 36.3 | 38.0 |
| Cyan. + Azos. | 5.49 | 5.57 | 6.34 | 8.58 | 8.68 | 9.19 | 39.0 | 39.1 | 40.8 |
| Azot. + Azos. | 5.55 | 5.56 | 5.61 | 8.52 | 8.94 | 9.39 | 39.5 | 38.3 | 37.5 |
| Cyano. + Azot. + Azos. | 5.21 | 6.22 | 6.69 | 9.33 | 9.65 | 8.79 | 35.8 | 39.3 | 43.3 |
| LSD | 0.05 | 0.01 | | 0.05 | 0.01 | | 0.05 | 0.01 | |
| Biofertilizers (Biof.) | 0.25 | 0.34 | | 0.53 | 0.72 | | 1.7 | 2.3 | |
| N-fertilizer (N) | 0.18 | 0.24 | | 0.35 | 0.47 | | n.s. | n.s. | |
| Biof. × N | 0.52 | 0.69 | | 1.00 | 1.33 | | 3.5 | n.s. | |

N: kg N/ha applied in two equal doses: 20 days after transplanting and at the early panicle initiation stage. *The harvest index: grain yield/grain + straw × 100.

Table 3. Effect of integrated biofertilization management with cyanobacteria, *Azotobacter* and *Azospirillum* vs. combined-N on N-attributes of the Japonica rice Giza-172

| Treatment | Grain N (kg N ha) | | | Straw N (kg N/ha) | | | N-use efficiency* | | |
|---|---|---|---|---|---|---|---|---|---|
| | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N | 36 N | 72 N | 108 N |
| Uninoculated | 28.3 | 44.8 | 45.9 | 23.1 | 28.3 | 32.4 | 106 | 61 | 46 |
| Cyanobacteria (cyan.) | 42.0 | 53.0 | 65.5 | 27.7 | 34.1 | 48.2 | 131 | 79 | 59 |
| *Azotobacter* (Azot.) | 38.6 | 50.1 | 55.2 | 27.9 | 34.2 | 38.0 | 122 | 73 | 49 |
| *Azospirillum* (Azos.) | 52.1 | 57.3 | 65.3 | 43.1 | 40.9 | 57.5 | 154 | 75 | 56 |
| Cyan. + Azot. | 52.4 | 42.5 | 52.8 | 39.1 | 34.4 | 43.6 | 144 | 68 | 54 |
| Cyan. + Azos. | 53.6 | 56.2 | 56.5 | 33.4 | 36.0 | 43.0 | 153 | 77 | 59 |
| Azot. + Azos. | 48.7 | 52.6 | 57.0 | 38.9 | 36.0 | 38.0 | 154 | 77 | 52 |
| Cyano. + Azot. + Azos. | 49.8 | 62.9 | 65.7 | 40.7 | 37.6 | 30.4 | 145 | 86 | 62 |
| LSD | 0.05 | 0.01 | | 0.05 | 0.01 | | 0.05 | 0.01 | |
| Biofertilizers (Biof.) | 3.1 | 4.2 | | 2.6 | 3.5 | | 5 | 6 | |
| N-fertilizer (N) | 1.9 | 2.5 | | 2.3 | 3.0 | | 3 | 5 | |
| Biof. × N | 5.3 | 7.1 | | 6.4 | 8.6 | | 9 | 13 | |

N: kg N/ha applied in two equal doses: 20 days after transplanting and at the early panicle initiation stage. *The agronomic N-use efficiency: kg grain yield/kg fertilizer-N.

with the cyanobacteria or *Azospirillum* was effective than with the *Azotobacter* in respect of contributing to grain yield even under the high doses of fertilizer N. Data of grain yield obtained with biofertilization by the combination cyanobacteria plus *Azospirillum* came notably higher than in case of the other combinations, and comparable to the figure obtained using biofertilization with the three factors which came slightly higher by only 5.5%. Data of N-contents of grain and straw followed the same pattern of changes, the only exception is that in those cases of biofertilization along with low amounts of chemical-N. This may be due to slow release of N contributed by the dinitrogen-fixers all over longer periods within the crop growth stage than in case of chemical fertilization which is highly effective within relatively shorter period. However, data of the agronomic fertilizer N-use efficiency (Table 3) indicating better chemical-N performance in case of biofertilization with each of the $N_2$-fixers or their combinations.

## 4. Discussion

Inoculation of rice fields with dinitrogen-fixing cyanobacteria was found capable of meeting a considerable proportion of rice nitrogen demand (Yanni et al., 1988b). Asymbiotic dinitrogen-fixation is not the only contribution of cyanobacteria. Suppression of growth of aquatic macrophytes (Subramanyan et al., 1965; Yanni et al., 1988a), aiding soil particles aggregation (Roychoudhury et al., 1980), increasing P-availability (Arora, 1969; Yanni and Abd El-Rahman, 1993) and decrease of sulphide toxicity (Jacq and Roger, 1977) were also reported. Ability of the cyanobacterial $N_2$-fixing enzymatic system to be inhibited by high amounts of combined N and restimulated with N-absorption or loss due to agronomic circumstances may be the key factor interpreting incidence of natural infection and infestation with some fungal and insect pests in inoculated rice fields which are normally affected by N status of the rice plant (Yanni and Abdallah, 1990; Yanni and Osman, 1990; Yanni et al., 1996; Yanni, 1998). However, field observations in this experiment revealed less infection of rice plants with *Pyricularia oryzae*, *Helminthosporium oryzae*, *Alternaria* sp. and *Sclerotium oryzae*, the causative agents of the rice blast, brown spot, leaf spot and stem rot diseases, respectively, and also infestation with the rice stem borer *Chilo agamemnon* (Bles.) and the leaf miner *Hydrellia prosternales* (Deeming) when inoculation with the cyanobacteria was included in the fertilization schedule especially in case of inoculation with the *Azospirillum* along with 36 or 72 rather than 108 kg N/ha. However, this is a potential research field of studies needs to be explored through more experimentation.

Rice rhizosphere harbours a diverse nitrogen-fixing microflora including the genera *Azospirillum, Pseudomonas, Enterobacter* and *Bacillus* (Rajaramamohan Rao et al., 1978; Roger and Watanabe, 1986). These bacteria can utilize sugars, organic acids and amino acids from root exudates (Sadhu and Dhas, 1971). *Azospirillum* bacteria can colonize the root cortex and proliferate in the intracellular spaces (Patriquin and Döbereiner, 1978). Since their discovery in association with the roots of grasses, *Azospirillum* bacteria have attracted considerable attention as potential fertilizer (Elmerich, 1993). Some reports (Gupta et al., 1989) compared between rice field inoculation with cyanobacteria vs. inoculation with *Azospirillum* or *Azotobacter*. The results came in accordance with those which presented out through this work, as the cyanobacteria-treated plots showed greater yields than the bacteria-inoculated ones, but the differences were statistically not significant. Omar et al. (1993) reported that inoculation of rice with *Azospirillum brasilense* improved grain quality as indexed by 14% increase in total protein comparing to rice of the noninoculated control, albumins increased non significantly by 12.8% and globulins by 11.4% over the control. However, they suggested the need for more detailed understanding of how *Azospirillum* affects protein quality of rice. The results of our study here indicate increases in grain nitrogen percentage from 0.74% for the uninoculated control to 0.89, 0.88 and 0.94% with inoculation by the cyanobacteria, *Azotobacter* or *Azospirillum*, respectively, along with combined N at 36 kg N/ha. The corresponding figures with fertilizer-N at 72 kg N/ha were 1.03, 0.94, 0.96 and 1.06% while with 108 kg N/ha they were 0.93 for the noninoculated plants and 1.03, 1.04 and 1.08% for the cyanobacteria, *Azotobacter* and *Azospirillum* biofertilized counterparts, respectively. Similar trend was found to be due to application of combined inocula containing two of the three biofactors or all of them together, the effects of combined inoculation on grain quality in some cases seems then to be additive. However, additive effect was reported by Yanni and Mohamed (1985) who recorded enhancement of nodulation and symbiotic dinitrogen-fixation by *Bradyrhizobium japonicum* in soybean plant as contribution of the asymbiotic $N_2$-fixer *Azotobacter chroococcum*. Also, Okon et al. (1995) related corn growth promotion to inoculation by *Azospirillum* and production of indole-3-acetic acid (IAA) via multiple IAA biosynthetic pathways: amino transferase, indole-3-acetamide and by a tryptophan-independent pathway(s), besides, they reported production of gibberellin GA, GA3 and iso-GA in cultures of *Azospirillum lipoferum*. However, they evaluated the worldwide data accumulated over the period 1975–1995 on field inoculation experiments with *Azospirillum* and concluded that these bacteria are capable of promoting the yield of agriculturally important crops in different soils and climatic regions, while, it is difficult to accurately estimate the percentage of success due to *Azospirillum* inoculation, as the data indicate 60–70% of positive response with significant increases in yield ranged from 5–30%. However, these data are in accordance with those which presented in this work as increases amounted to 45.7, 24.3 and 22.5 were obtained with inoculation by *Azospirillum* under fertilization with combined-N at 36, 72 and 108 kg N/ha, respectively. Burdman et al. (1996) introduced a mechanism comprising promotion of flavonoids signals, nodulation and growth of *Phaseolus vulgaris* when inoculated by *Azospirillum brasilense* as attributed to improved root development with subsequent increase in the rate of water and mineral uptake, increase in number of nodules and higher $N_2$-fixation.

Although biofertilization increased N percentage of paddy yield, the results suggest an inverse relationship between quantity of the crop dry matter and N-content. However, some reports, for instance, Shahaby et al. (1993) indicated that inoculation of rice with *Azotobacter choroococcum* or *Azospirillum brasilense* significantly increased grain yield and N uptake regardless of combined N application. The increases here were registered only with inoculation by viable cells and could not be detected with application of cell-free filtrate of growth cultures. The results here confirm those of Rovira (1963) on more establishment in plant root system with inoculation by various free-living nitrogen fixing bacteria including *Acetobacter*, *Azotobacter* and *Azospirillum* with enhancement of growth and performance of maize, tomato and wheat.

It is interesting here to monitor to what extent each of the three main sources of variation in this experiment namely: biofertilization with dinitrogen-fixing organism(s), increasing chemical-N amounts or their first order interaction, affected variations appeared in the statistical analysis of the data collected for each determined parameter. Table 4 shows percentage of variation proportional to total variation in data of each parameter which, in this experiment, originated from the three abovementioned sources, as appeared in the analysis of variances. Biofertilization scored the highest variation percentage, it seems to be the most effective factor in enhancement of productive tillering, grain yield, straw and their nitrogen contents comparing to N-fertilization or the first order interaction between the two factors. In the contrary, N-fertilization seems the most effective factor controlling plant height, panicle length and the agronomic fertilizer N-use efficiency, while data of the number of spiklets/panicle, grain size and the harvest index registered their greatest variations to be due to the first order interaction between biofertilization and N-fertilization. The results here suggest the superior contribution of biofertilization to rice growth and performance over those which were originated from increasing chemical N-fertilizer doses from 36 up to 108 kg N/ha. However, it is extensively recorded that the N-use efficiency in rice fields normally do not exceed 35–40% of the total chemical-N applied to rice field.

Ultimately, the results presented here indicate that contribution from bio-

Table 4. Percentage variations due to biofertilization, N-fertilization and their first order interaction compared to the corresponding total variations driven from the analysis of variances

| Parameter | Biofertilizers | N-fertilizer | Biofertilizers × N-fertilizers |
|---|---|---|---|
| Plant height | 18.9** | 44.8** | 22.0** |
| Productive tillers | 52.9** | 7.5** | 21.9** |
| Panicle length | 22.9** | 42.7** | 11.2* |
| Spiklets/panicle | 15.8** | 25.8** | 30.0** |
| 1000-grain weight | 30.6** | 2.7* | 43.1** |
| Grain yield | 46.2** | 25.7** | 11.7** |
| Straw | 37.9** | 18.4** | 21.0** |
| Harvest index | 20.5** | 1.0 | 24.9* |
| Grain-N | 41.6** | 29.5** | 17.1** |
| Straw-N | 39.7** | 15.5** | 25.1** |
| Agronomic N-use efficiency | 5.2** | 89.8** | 2.9** |

*, **: statistically significant or highly significant on the 95 or 99% confidence level, respectively.

fertilizer combinations are more than in case of application of a unique biofertilizer, while a clear inverse relationship is exists between chemical N amounts and benefit from this N. This necessitates reconsideration of amounts of chemical fertilizer when biofertilization is included in fertilization schedules for rice. However, it seems that biofertilization can not completely compensate for chemical fertilization with combined-N when maximum rice growth and performance are targeted. It must be considered here that the most N-hunger period in the rice growth stage is the maximum tillering one which normally exist during the first 40 days after transplanting. During this stage a biofertilizer agent is expected to continue establishment of its association with rice roots as for *Azospirillum* or in the rice ecosystem as for the *Azotobacter* or cyanobacteria, and start to contribute considerable amounts of biologically fixed N.

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