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Soil Inoculation by *Azospirillum* Affects Protein and Carbohydrate of Maize Grain under Nitrogen Deficiency

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMMA wrote the protocol and wrote the first draft of the manuscript. Authors HMA designed and performed data analyses and author MH managed the literature searches. All authors designed the study, managed the experimental process, read and approved the final manuscript.

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ABSTRACT

The use of nitrogen-fixing bacteria as an alternate N resource would play an important role in environmental protection by providing an eco-friendly and cost-effective inputs for farmers. The present investigation was carried out in the experimental field of Agric. Res. Stat. of Fac. of Agric., Cairo Univ., Giza, Egypt in 2014 and 2015 seasons. The primary objective of this investigation was to study the effect of *Azospirillum* bacteria on maize yield as well as grain protein and carbohydrate. Six maize cultivars were evaluated under three N treatments namely, high-N (286 kg N /ha), Low-N (without applying N) and BNF (bacterial nitrogen fixation, 24 kg/ha of bacterial inoculum) using a split-plot design with three replications. The investigation indicated that BNF treatment significantly surpassed Low-N treatment by 7.11% for grain yield/ha and 19.56% for protein yield/ha. The most interesting observation in the study was the superiority of BNF treatment for grain protein

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percentage by 4.0% and 16.91% over High-N and Low-N treatments, respectively. The present investigation concluded that maize yield as well as grain quality could be improved under low soil-N conditions by using *Azospirillum* bacteria not only for N fixation but also by excretion of phytohormones such as auxins and cytokinins and proved that *Azospirillum* bacteria could be used as an alternate N resource to maintain a clean environment as well as maintain soil fertility and sustainability.

Keywords: Maize; biofertilizers; auxin; cytokinin; sustainability.

1. INTRODUCTION

Maize (*Zea mays* L.) is the third important cereal crop after wheat and rice. It is cultivated for several purposes, such as human consumption, livestock and poultry feed, manufacturing starch and cooking oils as well as fermentation industries. Maize is also grown for green fodder and silage. Maize supplies around one-fourth of the world's cereal protein [1]. In Asia and Africa, almost all the maize produced is used for food, and therefore its contribution to dietary calories and proteins is substantial [2]. Grain quality is an important objective in maize breeding [3,4]. Some of the most important traits of interest in the maize market are those related to the nutritional quality of the grain, especially protein and oil content [5]. In a typical hybrid maize cultivar, grain contains approximately 73% starch, 9% protein, 4% oil and 14% other constituents (mostly fibre). The oil is stored mainly in the germ, while starch and protein are found primarily in the endosperm, which makes up the majority of the kernel [6].

Nitrogen is one of the most important factors that determine crop production [7], and it is the most important plant macronutrient because it is an essential component of plant cell compounds such as chlorophyll and proteins [8], that are closely associated with leaf colour, crop growth status and yield [9]. Low nitrogen stress can decrease leaf growth, leaf area and leaf duration and photosynthetic rate per leaf area which can negatively affect crop yields [10]. Increasing maize yield worldwide was accompanied by the increased use of N fertilisation. Nitrogen fertilisation of non-leguminous crops such as maize is one of the most expensive inputs in agriculture. Rejesus and Hornbaker [11] reported that a lot of the applied mineral nitrogen is lost through gaseous emissions, erosion and leaching. They added that there are some negative impacts due to these losses in creating environmental pollution from increased nitrate leaching that may lead to groundwater contamination. In contrast, in many developing countries such as Egypt, the rates of N fertilisers are low because of the limited access to fertilisers and low purchasing power of small farmers.

In recent decades, the use of biofertilizers has gained great importance in sustainable cropping systems and plays an essential role in maintaining long-term soil fertility and sustainability. Rokhzadi et al. [12] defined biofertilizers as products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable forms through biological processes such as nitrogen fixation and solubilization of rock phosphate. In this aspect, Khosro and Yousef [13] suggested that biofertilizers would play a key role in the productivity and sustainability of soil and also protect the environment as ecofriendly and cost-effective inputs for the farmers.

The use of nitrogen-fixing bacteria in agricultural practices is gaining importance. Nitrogen-fixing bacteria that function by transforming atmospheric N_2 into organic compounds [14]. *Azotobacter* and *Azospirillum* are free-living bacteria that fix atmospheric nitrogen in cereal crops without any symbiosis [15]. Although many genera and species of N_2-fixing bacteria are isolated from the rhizosphere of various cereals, mainly members of *Azotobacter* and *Azospirillum* genera have been widely tested for increased yield of cereals and legumes under field conditions [16]. *Azospirillum* is well known for its ability to excrete phytohormones such as gibberellins [17,18], cytokinins [19] and auxins [20-22]. Many studies suggest the involvement of indole-3-acetic acid (IAA), produced by *Azospirillum* in morphological and physiological changes of the inoculated plant roots [19,23,24].

In respect to cytokinin, Kuroha et al. [25] reported the importance of cytokinin as the main hormone in the plant growth and development effects on cell division, chloroplast development, differentiation of bud root, stem meristem initiation, stress tolerance and ageing of plant. In this sense, Fallik and Okon [26] reported that the production of growth regulators such as auxin and cytokinin by *Azospirillum* bacteria is an important mechanism to increase corn yield. Also, Saeedi et al. [27] observed that cytokinin hormone increased grain number and 1000 grain weight in wheat. Kheyrollah et al. [28] observed that corn seeds which treated with *Azospirillum* had more growth and development due to synthesis of more growth hormones. Furthermore, the phytohormone cytokinin plays an important role in the stimulation chloroplast protein and pigment biosyntheses [29-31], and activate the expression of nuclear [30,32,33] and plastid genes encoding chloroplast proteins [34,35].

In order to maintain a clean environment as well as maintain soil fertility and sustainability, an alternate N resource is way. The use of nitrogen fixing bacteria as an alternate N resource would be played important role in the environment protection as ecofriendly and cost effective inputs for the farmers, therefore the objectives of the present investigation were: (1) to study the effect of *Azospirillum* bacteria on maize yield as well as grain protein and carbohydrate, (2) to identify genotypic differences among studied cultivars for their tolerance to low soil-N conditions and (3) to identify characters of the strongest association with grain yield under different N-treatments.

2. MATERIALS AND METHODS

Two field experiments were carried out at the Agricultural Research and Experiment Station, of Faculty of Agriculture, Cairo University, Giza, Egypt (30°02' N and 31°13' E, with an altitude of 30 meter) during the two successive seasons of 2014 and 2015. The climatic variables in the two successive seasons are presented in Table 1. Soil properties of 2014 and 2015 seasons (Table 2) were analyzed at Reclamation and Development Center Desert Soils, Faculty of Agriculture Research Park, Cairo University.

2.1 Plant Material

The genetic materials used in this investigation included six maize cultivars, namely the single cross hybrids SC-128 and SC-130, the three-way cross hybrids TWC-321 and TWC- 352, the open-pollinated composite cultivar Giza-2 and American Early Dent (AED) population. All of the studied genetic materials have white endosperm except TWC-352 which, it has yellow endosperm. Seeds of these cultivars were obtained from Maize Research Section, Field Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

2.2 Experimental Design and Treatments

Taking into consideration the available nitrogen in the soil in 2014 and 2015 seasons as shown in Table 2, the present investigation involved of three N treatments as follows:

(1) High-N: applying 286 kg N/ha (286 kg N/ha + the available N in the soil) by using Urea (46.5% N), added in two equal doses before the first and second irrigations.
(2) Low-N: non-applying any nitrogen fertilisers (*i.e.* 0 kg N/ha in addition to the available N in the soil)
(3) BNF (bacterial nitrogen fixation) by adding 24 kg/ha of bacterial inoculum as recommended by ARC to the soil without adding any nitrogen fertilisers (*i.e.* 24 kg/ha of bacterial inoculum in addition to the available N in the soil). Bacterial inoculum in the present study, *Azospirillum brasilense* carried on vermiculite (Cerealine) was obtained from Production Unit for Biofertilizers, ARC, Giza, Egypt. Each gram of the studied bacterial inoculum contains around of 10^7 bacterial cells of *Azospirillum brasilense*. Soil inoculation with *Azospirillum brasilense* was done by hand at sowing time immediately prior to irrigation as recommended by ARC.

A split-plot design in a randomised complete block arrangement was used with three replications. The main plots were allotted to the three N treatments, and the genotypes were devoted to sub-plot. Each sub-plot consists of four ridges of 0.70 m in width, and 4.0 m in length, i.e. the experimental plot area was 11.2 m^2. Each main plot was surrounded with a wide ridge (1.5 m) to avoid interference of the three N treatments.

2.3 Cultural Practices

The preceding crop was wheat (*Triticum aestivum* L.) in both seasons. Sowing dates were on June 12 and 11 in 2014 and 2015 seasons, respectively. Seeds were sown in hills at 25 cm

Table 1. Some climatic variables recorded at Giza location in 2015 and 2016 seasons

Month	2014		2015	
	Temperature (C)	Relative humidity (%)	Temperature (°C)	Relative humidity (%)
June	28.5	47.4	29.1	44.9
July	29.1	57.5	32.2	46.5
August	29.9	57.9	33.2	46.6
September	28.5	56.2	32.8	46.7

** Data obtained by the Central Laboratory for Agricultural Climate (CLAC), Agricultural Research Center (ARC), Egypt. Precipitation was not detected in both seasons*

apart by hand, thereafter (before the 1st irrigation) were thinned to one plant per hill. Calcium superphosphate fertiliser (15.5% P_2O_5) at the rate of 60 kg P_2O_5/ha was applied uniformly before sowing. Standard agricultural practices were followed throughout the growing seasons. Weed management was carried out during the growing season by hoeing twice, before the 1st and the 2nd irrigations and pest control, if necessary, was done according to practices used at the experimental station. The other cultural practices were applied as recommended by the Agricultural Research Center (ARC), Giza, Egypt.

Table 2. Some physical and chemical properties of soil at the experimental site in 2014 and 2015 seasons

Soil analysis	2014	2015
Physical properties		
Sand (%)	33.7	33.2
Silt (%)	30.0	31.5
Clay (%)	36.3	35.3
Texture class	Clay loam	Clay loam
Chemical properties		
pH $_{(1:1)}$	7.73	7.80
$Ec_{(1:1)}$ (dS m^{-1})	1.9	1.9
Organic matter (%)	2.1	2.2
Total Ca Co_3 (%)	3.4	3.5
Available N (mg kg^{-1})	44.0	47.0
Available P (mg kg^{-1})	8.9	9.0
Available K (mg kg^{-1})	240.0	230.0
Irrigation water analysis		
Ec of Irrigation water (ds/m)	0.78	0.86
pH of Irrigation water	7.02	7.50
Irrigation system	Flooding	Flooding

2.4 Data Collection

At harvest the following data were recorded:

1- Grain yield per hectare in ton/ha was calculated by weighing grain yield in kg from the whole area of each experimental unit (sub-plot, each sub-plot consists of 4 ridges) and then adjusted into ton per hectare (ton/ha). The grain yield per hectare was adjusted on the basis of 15.5% grain moisture content.
2- Grain protein percentage (GP %) according to A.O.A.C. [36].
3- Grain carbohydrate percentage (GC %) according to Minhas et al. [37].
4- Protein yield per hectare in ton/ha, calculated by multiplying GP % by grain yield per hectare.
5- Carbohydrate yield per hectare in ton/ha, calculated by multiplying GC % by grain yield per hectare.

Analyses of GP % and GC % were done at Faculty of Agriculture Research Park - Faculty of Agriculture - Cairo University.

2.5 Statistical Analysis

Tests of normality of distributions were carried out according to Shapiro and Wilk, [38], by using SPSS v. 17.0 [39] computer package. Combined analysis of variance of a RCBD across the two seasons was computed after carrying out Bartelet's test according to Snedecor and Cochran [40]. Estimates of LSD were calculated to test the significance of differences between means according to Snedecor and Cochran [40]. Simple correlation coefficients were calculated between grain yield and each of the other studied traits under each N treatment across the two seasons according to Steel et al. [41].

Change percentage was calculated as follows Change % = 100 × [(High-N - Low-N or BFN)/High-N]. Stress tolerance index (STI), was calculated according to Fernandez [42] as follows: STI = (Y_S) (Y_N) / ($\bar{Y}_N$)^2, where: Y_S = grain yield of a given hybrid under N stress. Y_N = grain yield of a given hybrid under non-stress.

$\overline{Y}_N$ = average grain yield of all hybrids under non-stress. When STI is ≥ 1 it indicates that genotype is tolerant (T) to stress, if STI is ≥ 0.5 to < 1 it indicates that genotype is moderately tolerant (M) and if STI is < 0.5 it indicates that genotype is sensitive (S).

3. RESULTS

3.1 Analysis of Variance

Combined analysis of variance for all studied traits is presented in Table 3. Mean squares due to years were highly significant for only three traits, namely grain yield/ha, protein yield/ha and carbohydrate yield/ha. Highly significant mean squares were also detected among N-treatments as well as among studied genotypes for all traits. Significant or highly significant mean squares due to years × genotypes and N-treatments × genotypes interactions were also observed for all studied traits, except grain protein and grain carbohydrate percentages for years × genotypes interaction. In contrast, mean squares due to years × N-treatments and years × N-treatments × genotypes interactions were insignificant.

3.2 Effect of N-treatments

Means of all studied traits across all studied genotypes under all N-treatments are presented in Fig. 1. The highest mean values were observed under High-N treatment for grain yield (7.17 ton/ha), protein yield (0.92 ton/ha) and carbohydrate yield (5.0 ton/ha). On the other hand, BNF treatment had the highest mean value for grain protein percentage (13.53%). In addition, BNF was the second best treatment after High-N for grain yield (6.06 ton/ha), protein yield (0.81 ton/ha) and carbohydrate yield (4.19 ton/ha). In contrast, Low-N treatment showed the lowest mean values for all studied traits with the exception of grain carbohydrate percentage, where it had the highest mean value (70.96%) for that trait.

The percentage of change of means by Low-N and BNF compared with High-N is presented in Table 4. Low-N treatment caused significant reductions by 22.59% for grain yield/ha, 12.91% for grain protein percentage, 31.52% for protein yield/ha and 20.8% for carbohydrate yield/ha compared to High-N. In contrast, a significant increase of 2.59% for grain carbohydrate percentage was observed due to Low-N treatment compared to High-N. On the other hand, BNF treatment caused significant reductions by 15.48% for grain yield/ha, 11.96% for protein yield/ha, 0.64% for grain carbohydrate percentage and 16.2% for carbohydrate yield/ha. In contrast, BNF treatment significantly exceeded High-N treatment by 4.0% for grain protein percentage.

Comparing with High-N treatment, it was interesting to note that the reductions due to BNF treatment were less than that due to Low-N treatment for grain yield/ha (15.48 *vs*. 22.59%), protein yield/ha (11.96 *vs*. 31.52%) and carbohydrate yield/ha (16.20 *vs*. 20.8%), meaning that BNF treatment significantly surpassed Low-N treatment by 7.11% for grain yield/ha, 19.56% for protein yield/ha and insignificantly increasing by 4.6% for carbohydrate yield/ha. The most interesting observation in the present study was the superiority of BNF treatment for grain protein percentage over High-N treatment by 4.0% and consequently over Low-N treatment by16.91% (Table 4).

Table 3. Combined analysis of variance of a split plot design for all studied traits of six maize cultivars evaluated under three N-treatments across 2014 and 2015 seasons

S.O.V	d.f	Grain yield/ha	Grain protein %	Protein yield/ha	Grain Carbohydrate%	Carbohydrate yield/ha
Years (Y)	1	5.34**	0.006	0.079**	0.12	2.51**
R(Y)	4	0.45	0.167	0.007	0.19	0.23
N-treatments (A)	2	24.67**	47.64**	0.765**	50.20**	10.85**
YA	2	1.37	0.053	0.022	0.25	0.62
$Error_{(a)}$	8	0.41	0.222	0.006	0.13	0.20
Genotypes (B)	5	52.60**	13.77**	0.565**	72.93**	29.83**
YB	5	1.16**	0.079	0.018*	0.27	0.56**
AB	10	1.20**	5.52**	0.019*	11.69**	0.84**
YAB	10	0.07	0.044	0.001	0.14	0.03
$Error_{(b)}$	60	0.32	0.242	0.007	0.42	0.15

and indicate significant at 0.05 and 0.01 levels of probability, respectively*

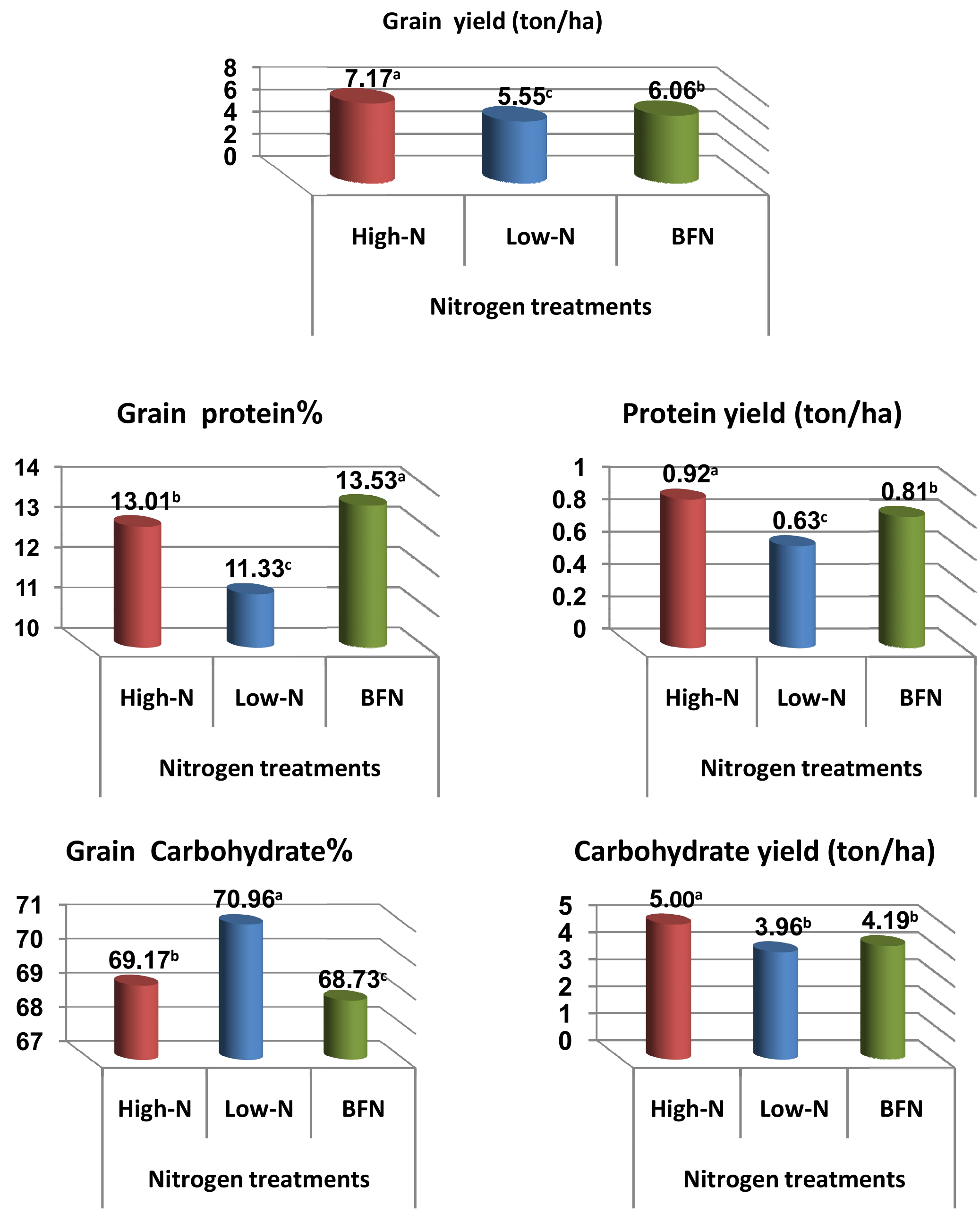

Fig. 1. Means of the studied traits across all cultivars under all N-Treatments (data are combined across 2014 and 2015 seasons). Means followed by the same letter are not statistically different at P< 0.05

3.3 Effect of Genotypes

Mean performance of all studied genotypes across all N-treatments is presented in Table 5. The single cross hybrids SC-128 and SC-130 ranked as the first best genotypes for grain yield/ha, protein yield/ha, grain carbohydrate percentage and carbohydrate yield/ha. The three-way cross hybrid TWC-321 ranked the second best genotype for all studied traits, except grain protein % and grain carbohydrate %, where it ranked the third best genotype. Regarding the open-pollinated composite cultivar Giza-2 it ranked the third best genotype for grain yield/ha, protein yield/ha and carbohydrate yield/ha. On the other hand, TWC-352 and the population AED were the lowest genotypes for all studied traits, except grain protein % where AED and TWC-352 ranked the first and second best genotypes, respectively.

3.4 Effect of Genotypes × N-treatments

The maize cultivars studied showed significant differences in their absolute mean values under Low-N and BNF compared to High-N for all studied traits. Therefore, ranks of all studied cultivars under Low-N and BNF were different from that under High-N. The percentage of change of means for Low-N and BNF compared to High-N for each cultivar is presented in Table 6.

Reductions in grain yield/ha ranged from 17.42% (TWC-352) to 34.24% (SC-128) under Low-N and from 5.81% (TWC-352) to 22.43% (SC-128) under BNF compared to High-N. Grain protein percentage reduced under Low-N treatment from 2.08% (Giza-2) to 32.47% (TWC-352) under Low-N. In contrast, the most interesting observation was the superiority of BNF treatment over High-N for grain protein percentage in all studied cultivars, except TWC-352 where it reduced by 8.33%. Increases in grain protein percentage under BNF ranged from 1.88% (SC-128) to 12.34% (AED). On the other hand, reductions in protein yield/ha ranged from 20.22% (Giza-2) to 44.71% (TWC-352) under Low-N and from 1.85% (AED) to 20.51% (SC-128) under BNF treatment. Regarding grain carbohydrate percentage, mean performance under Low-N exceeded High-N treatment for the most studied cultivars, except SC-128 where it was reduced by 0.71%. Increases in grain carbohydrate percentage ranged from 0.6% (Giza-2) to 7.06% (AED). In contrast, reductions in performance for all studied cultivars were observed for grain carbohydrate percentage under BNF treatment compared to High-N treatment except for TWC-352 where it increased by 4.12% under BNF treatment. Reductions in grain carbohydrate percentage for the other cultivars under BNF treatment ranged from 0.19% (Giza-2) to 3.15% (SC-128). In respect to carbohydrate yield/ha, reductions ranged from 15.32% (TWC-352) to 34.79% (SC-128) under Low-N and from 1.88% (TWC-352) to 24.96% (SC-128) under BNF treatment (Table 6).

Comparing performance of the studied cultivars under High-N with their performance under Low-N and BNF treatments (Table 6), it is worth noting that the reductions due to BNF were less

Table 4. Change % due to Low-N and BNF compared to High-N for all studied traits across all studied cultivars (data are combined across 2014 and 2015 seasons)

Trait	Change %	
	High-N vs. Low-N	**High-N vs. BFN**
Grain yield (ton/ha)	22.59**	15.48**
Grain protein %	12.91**	-4.00**
Protein yield (ton/ha)	31.52**	11.96**
Grain Carbohydrate %	-2.59**	0.64**
Carbohydrate yield (ton/ha)	20.80**	16.20**

and indicate significant at 0.05 and 0.01 levels of probability, respectively. Change % =100 x [(High-N – Low-N or BFN) / High-N]*

Table 5. Mean performance of all studied cultivars for all traits across N-treatments (data are combined across 2014 and 2015 seasons)

Cultivar	Grain yield (ton/ha)	Grain protein %	Protein yield (ton/ha)	Grain Carbohydrate %	Carbohydrate yield (ton/ha)
SC-128	7.48	12.58	0.95	70.67	5.29
SC-130	7.78	11.39	0.89	72.34	5.62
TWC-321	7.25	12.37	0.89	70.05	5.07
TWC-352	5.08	13.28	0.68	69.03	3.51
Giza-2	6.61	12.22	0.81	69.32	4.58
AED	3.38	13.90	0.47	66.28	2.23
LSD $_{0.05}$	0.38	0.33	0.06	0.43	0.26

than that observed under Low-N treatment for grain yield/ha, protein yield/ha and carbohydrate yield/ha. It could be concluded their performance of the studied cultivars under BNF treatment surpassed that performance under Low-N treatment for the previous traits. Superiority of the studied cultivars for grain yield/ha under BNF treatment compared to Low-N treatment were 12.63% (AED), 11.81% (SC-128), 11.61% (TWC-352), 6.1% (Giza-2), 3.1% (SC-130) and 1.45% (TWC-321). In addition, superiority for protein yield/ha were 33.34% (AED), 30.59% (TWC-352), 17.31% (SC-130), 17.1% (SC-128), 13.86% (TWC-321) and 13.48% (Giza-2). Furthermore, superiority for carbohydrate yield/ha were 13.44% (TWC-352), 9.83% (SC-128), 5.95% (AED) and 5.29% (Giza-2). It is worthy to observe that SC-130 and TWC-321 slightly reduced by 0.48 and 0.28 % due to BNF compared to Low-N treatment for carbohydrate yield/ha.

As stated earlier, it is interesting to remember that the most interesting observation in the present study was the superiority of BNF treatment over High-N for grain protein percentage in all studied cultivars except for TWC-352, where it reduced by 8.33%. In addition, BNF treatment surpassed Low-N treatment for grain protein percentage. Such increases in grain protein percentage under BNF compared to Low-N treatment were 25.34% (AED), 24.14% (TWC-352), 17.67% (SC-130), 16.2% (TWC-321), 8.38% (Giza-2) and 7.28% (SC-128). In contrast, Low-N treatment showed superiority over BNF treatment for grain carbohydrate percentage for all studied cultivars except for SC-128, where it reduced by 2.44% under Low-N.

Increases in grain carbohydrate percentage under Low-N compared to BNF were 8.42% (AED), 6.58% (TWC-352), 5.15% (TWC-321), 4.4% (SC-130) and 0.8% (Giza-2).

3.5 Stress Tolerance Index

Values of stress tolerance index (STI) are presented in Table 7. The most tolerant cultivars (STI ≥ 1) were SC-128, SC-130 and TWC-321

Table 6a. Effect of cultivars × N-treatments Interaction and change% of each treatment compared to High-N for all studied traits (data are combined across 2014 and 2015 seasons)

Cultivar	N-treatments			Change %	
	High-N	Low-N	BNF	High-N vs. Low-N	High-N vs. BFN
			Grain yield (ton/ha)		
SC-128	9.23	6.07	7.16	34.24**	22.43**
SC-130	8.81	7.13	7.40	19.07**	16.00**
TWC-321	8.25	6.68	6.80	19.03**	17.58**
TWC-352	5.51	4.55	5.19	17.42**	5.81
Giza-2	7.37	6.01	6.46	18.45**	12.35**
AED	3.88	2.88	3.37	25.77**	13.14
$LSD_{0.05}$		**Cultivars = 0.38**		**Cultivars X N-treatments = 0.65**	
Grain protein %					
SC-128	12.71	12.07	12.95	5.04*	-1.88
SC-130	11.80	10.14	12.22	14.07**	-3.60
TWC-321	12.22	11.46	13.44	6.22**	-9.98**
TWC-352	15.37	10.38	14.09	32.47**	8.33**
Giza-2	12.05	11.80	12.81	2.08	-6.30**
AED	13.94	12.11	15.66	13.13**	-12.34**
$LSD_{0.05}$		**Cultivars = 0.33**		**Cultivars X N-treatments = 0.57**	
			Protein yield (ton/ha)		
SC-128	1.17	0.73	0.93	37.60**	20.51**
SC-130	1.04	0.73	0.91	29.81**	12.50**
TWC-321	1.01	0.77	0.91	23.76**	9.90*
TWC-352	0.85	0.47	0.73	44.71**	14.12**
Giza-2	0.89	0.71	0.83	20.22**	6.74
AED	0.54	0.35	0.53	35.19**	1.85
$LSD_{0.05}$		**Cultivars = 0.06**		**Cultivars X N-treatments = 0.10**	

and indicate significant at 0.05 and 0.01 levels of probability, respectively. Change % =100 x [(High-N – Low-N or BFN) / High-N]*

Table 6b. Effect of cultivars × N-treatments Interaction and change% of each treatment compared to High-N for all studied traits (data are combined across 2014 and 2015 seasons)

Cultivar	N-treatments			Change %	
	High-N	Low-N	BNF	High-N vs. Low-N	High-N vs. BFN
Grain carbohydrate %					
SC-128	71.59	71.08	69.33	0.71	3.15**
SC-130	71.69	74.23	71.10	-3.54**	0.82
TWC-321	69.90	71.93	68.33	-2.90**	2.24**
TWC-352	67.55	69.21	70.33	-2.46**	-4.12**
Giza-2	69.23	69.64	69.10	-0.60	0.19
AED	65.05	69.64	64.16	-7.06**	1.37**
$LSD_{0.05}$		**Cultivars = 0.43**	**Cultivars X N-treatments = 0.75**		
Carbohydrate yield (ton/ha)					
SC-128	6.61	4.31	4.96	34.79**	24.96**
SC-130	6.32	5.29	5.26	16.29**	16.77**
TWC-321	5.77	4.81	4.65	16.64**	19.41**
TWC-352	3.72	3.15	3.65	15.32**	1.88
Giza-2	5.10	4.19	4.46	17.84**	12.55**
AED	2.52	2.01	2.16	20.24*	14.29
$LSD_{0.05}$		**Cultivars = 0.26**	**Cultivars X N-treatments = 0.45**		

and indicate significant at 0.05 and 0.01 levels of probability, respectively. Change % =100 x [(High-N – Low-N or BFN) / High-N]*

for grain yield/ha under both Low-N and BNF treatments. On the other hand, Giza-2 was moderately tolerant (STI ≥ 0.5 to < 1) under both Low-N and High-N and TWC-352 was moderately tolerant only under BNF treatment. In contrast, TWC-352 was sensitive (STI < 0.5) only under Low-N. In addition, AED was sensitive under both Low-N and BNF treatments for grain yield/ha.

Regarding grain protein percentage, all the studied cultivars were moderately tolerant under Low-N and BFN, except TWC-352 was tolerant only under BNF and AED was tolerant under both Low-N and BNF treatments. In respect to protein yield/ha the single cross hybrid SC-128 was tolerant under Low-N and High-N treatments. In addition, SC-130 and TWC-321 were moderately tolerant only under Low-N treatment and tolerant under BNF treatment. Giza-2 was moderately tolerant under Low-N and BNF treatments and TWC-352 was moderately tolerant only under BNF treatment. In contrast, AED under Low-N and BNF along with TWC-352 only under Low-N were sensitive.

In respect to grain carbohydrate percentage, the hybrids SC-128, SC-130 and TWC-321 along with the open-pollinated composite Giza-2 were tolerant under Low-N and BNF treatments. In addition, the hybrid TWC-352 and the population AED were moderately tolerant under both Low-N and BFN. Regarding carbohydrate yield/ha the hybrids SC-128, SC-130 and TWC321 were tolerant under low-N and BFN. In addition, Giza-2 under Low-N and BNF and the Hybrid TWC-352 only under BNF were moderately tolerant. In contrast, TWC-352 under Low-N and AED under both Low-N and BNF treatments were sensitive (Table 7).

3.6 Interrelationships among Traits

Significant and positive correlation coefficients were observed among grain yield/ha and each of protein yield/ha, grain carbohydrate percentage and carbohydrate yield/ha (Table 8). On the contrary,the significant and negative correlation coefficient was observed among grain yield/ha and grain protein percentage. Correlation coefficients under BNF treatment were higher than that under High-N and Low-N treatments for grain protein percentage and protein yield/ha. On the other hand, correlation coefficients under High-N treatment were higher than that under Low-N and BNF treatments for grain carbohydrate percentage and carbohydrate yield/ha.

4. DISCUSSION

The present investigation showed that genotypes and N-treatments had significant effects on all studied characters. Genotypes × N-treatments

Table 7. Stress tolerance index (STI) for all studied genotypes and studied traits (data are combined across 2014 and 2015 seasons)

Cultivar	Grain yield (ton/ha)		Grain protein %		Protein yield (ton/ha)	
	High-N vs. Low-N	High-N vs. BFN	High-N vs. Low-N	High-N vs. BFN	High-N vs. Low-N	High-N vs. BFN
SC-128	1.09 (T)	1.28 (T)	0.91 (MT)	0.97 (MT)	1.02 (T)	1.29 (T)
SC-130	1.22 (T)	1.27 (T)	0.71 (MT)	0.85 (MT)	0.90 (MT)	1.13 (T)
TWC-321	1.07 (T)	1.09 (T)	0.83 (MT)	0.97 (MT)	0.93 (MT)	1.09 (T)
TWC-352	0.49 (S)	0.56 (MT)	0.94 (MT)	1.28 (T)	0.48 (S)	0.74 (MT)
Giza-2	0.86 (MT)	0.92 (MT)	0.84 (MT)	0.91 (MT)	0.75 (MT)	0.88 (MT)
AED	0.22 (S)	0.25 (S)	1.00 (T)	1.29 (T)	0.22 (S)	0.34 (S)

Cultivar	Grain carbohydrate %		Carbohydrate yield (ton/ha)	
	High-N vs. Low-N	High-N vs. BFN	High-N vs. Low-N	High-N vs. BFN
SC-128	1.06 (T)	1.04 (T)	1.14 (T)	1.31 (T)
SC-130	1.11 (T)	1.07 (T)	1.33 (T)	1.33 (T)
TWC-321	1.059 (T)	1.00 (T)	1.11 (T)	1.07 (T)
TWC-352	0.98 (MT)	0.99 (MT)	0.47 (S)	0.54 (MT)
Giza-2	1.01 (T)	1.00 (T)	0.85 (MT)	0.91 (MT)
AED	0.95 (MT)	0.87 (MT)	0.20 (S)	0.22 (S)

T= tolerant, MT= moderately tolerant and S= sensitive.

were significant or highly significant for all studied traits, concluding that performance of the studied cultivars varies with N-treatments. It was indicated that selection of suitable genotypes could be identified under each N-treatment. Data of the present investigation are in harmony with that reported by El-Moselhy [43], Omoigui et al. [44], Atta [45], Atta and Amein [46] and Kheyrollah et al. [28].

Superiority of BNF treatment *via Azospirillum* bacteria over Low-N treatment for grain yield/ha, protein yield/ha and carbohydrate yield/ha indicates to the ability of *Azospirillum* bacteria to increase yield under Low-N conditions. In addition, the superiority of BNF treatment (*via Azospirillum* bacteria) over High-N and Low-N treatments for grain protein percentage was the most interesting observation in the present study. It seems likely that yield as well as grain quality of maize can be improved by using *Azospirillum* bacteria under low soil-N conditions not only via N fixation but also because the bacteria secrete of phytohormones such as auxins and cytokinins. Results proved that *Azospirillum* bacteria can be used as an alternate N resource which could help to maintain a clean environment as well as maintain soil fertility and sustainability. The production of growth regulators such as auxins and cytokinins by *Azospirillum* bacteria is an important mechanism to increase maize yield as reported by several authors [17-19,21-26,28,46]. Many studies suggest the involvement of indole-3-acetic acid (IAA), produced by *Azospirillum* in morphological and physiological changes of inoculated plant roots [19,23,24]. Furthermore, the phytohormone cytokinin plays an important role in the stimulation of chloroplast protein and pigment biosyntheses [29-31], and activates the expression of nuclear [30,32,33] and plastid genes encoding chloroplast proteins [34,35].

Table 8. Simple correlation coefficients (r) between grain yield/ha and each of other studied traits under each N-treatment (data are combined across 2014 and 2015 seasons)

Trait	N-treatments		
	High-N	Low-N	BFN
Grain protein %	-0.738**	-0.334*	-0.961**
Protein yield/ha	0.960**	0.963**	0.989**
Grain carbohydrate %	0.987**	0.732**	0.782**
Carbohydrate yield/ha	0.999**	0.997**	0.998**

and indicate significant at 0.05 and 0.01 levels of probability, respectively*

Maximizing maize grain yield depending on BNF conditions could be achieved by using natural supplement such as cytokinin. In this aspect, Kheyrollah et al. [28] observed that increasing in grain yield was achieved by applying *Azospirillum* mixed with the soil and spraying 100 mg/ liter cytokinin hormone on plants before flowering. Furthermore, natural growth promoters could be applied to increase maize yield. In this sense, Atta et al. [47] studied the effect of the growth promoter VIUSID agro (VIUSID agro acts as a natural bioregulator and composed of amino acids, vitamins and minerals, [48] on maize to determine the optimal dosage of VIUSID agro which increase maize grain yield. They concluded that increasing maize grain yield was obvious for most studied cultivars by applying the dosage of 0.96 L/ha of VIUSID agro than other dosages, it where yield has significantly exceeded the control by 26.0%.

Regarding the most tolerant genotypes to Low-N conditions in the present study, it is worth mentioning that the tolerant genotype to stress should have the highest absolute mean yield under stress and the lowest reduction in yield under stress compared to non-stress [49]. From this point of view, the cross hybrids SC-128 and SC-130 followed by TWC-321 could be regarded the most tolerant genotypes under both Low-N and BNF treatments in the present study for grain yield/ha, protein yield/ha and carbohydrate yield/ha. On the other hand, the open-pollinated composite Giza-2 could be regarded moderately tolerant under Low-N and BNF treatments in the present study for the same traits. On the contrary, the open-pollinated population AED could be regarded the most sensitive genotype under both Low-N and BNF treatments for grain yield/ha, protein yield/ha and carbohydrate yield/ha. In respect to TWC-352, it could be regarded as moderately tolerant under BNF treatment but under Low-N treatment it could be regarded as sensitive genotype for the same traits.

Regarding to the positive association between grain yield/ha and each of protein yield/ha and carbohydrate yield/ha that might be due to the calculation of these traits, where grain yield/ha is a common component in all these traits. In addition, the highly positive association between grain yield/ha and grain carbohydrate percentage might be due to that starch is the main component of grain yield as reported by Tan and Morrison [6]. On the contrary, the negative correlation between grain yield and grain protein percentage in the present investigation was reported by several investigators [50-53]. Therefore, breeding progress for increasing grain protein percentage has been limited by such clear inverse relationship between maize grain yield and grain protein content [52-55].

5. CONCLUSION

The most interesting observation in the present study was the superiority of BNF treatment via *Azospirillum* bacteria for grain protein percentage over High-N treatment by 4.0% and consequently over Low-N treatment by 16.91%. The present investigation concluded that maize yield as well as grain quality could be improved under low soil-N conditions by using *Azospirillum* bacteria not only by N fixation but also by excretion of phytohormones such as auxins and cytokinins and proved that *Azospirillum* bacteria would be used as an alternate N resource to maintain a clean environment as well as maintain soil fertility and sustainability.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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