

## Response of Winter Wheat to Co-Inoculation with Azotobacter and Arbuscular Mycorrhizal Fungi (AMF) under Different Sources of Nitrogen Fertilizer

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**Abstract:** In order to evaluate the effects of inoculants and chemical fertilizer on quantitative and qualitative yield, a bread wheat cultivar treated with Azotobacter, arbuscular mycorrhiza fungi (AMF) and nitrogen sources by using split plot on the basis of randomized complete block design with three replications in Fars Agricultural Research Station during 2007-2008. Main plots consisted of nitrogen fertilizer sources, which were ammonium nitrate, ammonium sulfate, urea and SCU (Sulfur Coated Urea). Sub plots consisted of four treatments i.e. control, inoculation of arbuscular mycorrhiza fungi (AMF), *Azotobacter chroococcum* and dual inoculation of AMF + *Azotobacter chroococcum* (AMF + Azc). Results showed that the highest plant height was related to SCU fertilizer, resulting to lodging in some plots. The most spike per square meter was obtained by ammonium nitrate and urea fertilizers. Single application of Azotobacter and Mycorrhiza inoculation and in combination to each other increased significantly spike per square meter compared to without inoculation treatment. Ammonium nitrate and ammonium sulfate fertilizers produced more grain per spike than urea and SCU fertilizers. Also, interaction effects of biofertilizers and N sources were significant at 5% probability level in this trait. The highest value of kernel weight was obtained with urea and ammonium nitrate fertilizers and the lowest value was belonged to SCU fertilizer. Maximum kernel weight was found in Azotobacter and Azotobacter+Mycorrhiza and minimum in control and Mycorrhiza treatments. Ammonium nitrate and Azotobacter + Mycorrhiza treatments gave significantly higher grain yield than the other N sources and biofertilizers. Biologic yield and harvest index were only affected by N sources treatments. As, ammonium nitrate and urea fertilizer treatments were higher than two other N sources. Grain protein percent increased up to 19 % in ammonium nitrate than urea and SCU fertilizers. Azotobacter + Mycorrhiza treatment increased grain protein by 13 % than control. In general, results from the present study indicated that grain yield and yield components of wheat have been affected significantly by the inoculation with Azotobacter and Mycorrhiza. Also, ammonium nitrate and ammonium sulfate resulted in increasing grain yield and nitrogen fertilizer efficiency compared with urea and SCU.

**Key words:** Wheat • Nitrogen sources • Azotobacter • Mycorrhiza • Yield and yield components  
• Grain protein

### INTRODUCTION

Integrated nutrient management strategies involving chemical fertilizers and bio-fertilizers have been suggested to enhance the sustainability of crop production [1].

The excessive use of chemical fertilizers has generated several environmental problems. Some of these problems can be tackled by use of biofertilizers, which are natural, beneficial and ecologically friendly.

Rhizobial inoculation of legume seed is well studied and exploitation of this beneficial N<sub>2</sub>-fixing root-nodule symbiosis represents a hallmark of successfully applied agricultural microbiology. However, much less information is available regarding the association of rhizobia with nonlegumes. The estimated contribution of free-living N fixing prokaryotes to the N input of soil ranges from 0-60 kg ha<sup>-1</sup> year<sup>-1</sup> [2]. Azotobacter and Azospirillum are used as biofertilizers in the cultivation of most crops. Azotobacter and Azospirillum are obligate aerobic

diazotrophic soli-dwelling organisms with a wide variety of metabolic capabilities, which include the ability to fix atmospheric nitrogen by converting it to ammonia. Treatment of seeds with *Azotobacter* and *Azospirillum* can help to control disease incidence and severity [3], improve nutrient uptake efficiency [4], produce thiamin, riboflavin, indole acetic acid and gibberellins and promote growth leading to enhanced yield [5]. Inoculation with arbuscular mycorrhiza fungi (AMF) has been found to increase the availability of phosphorous and other nutrients in crop plants because of its symbiotic associations with plant roots, colonizing cortical tissues and extending hyphae into the rhizosphere [6]. Vesicular-Arbuscular Mycorrhiza (VAM) was able to alter water relation of its host plants and effects of VAM on morphology, metabolism and protective adaptation of host plants in the drought stress condition. P concentrations themselves may affect host water balance, but it is often fixed in soil and not available to plant. Phosphatase produced by VAM fungi play an important role in changing fixed or insoluble into soluble P, which can be used by plant freely. Increased yield and nutrient uptake by the use of biofertilizers in many crops has been documented. Higher nutrient uptake and seed yield in canola (*Brassica napus L.*) was reported by Yasari *et al.* [7]. Likewise, higher biological yields in wheat and barley after inoculation the seeds with *Azotobacter* and *Azospirillum* was found by Ali *et al.* [8]. Recently, Volpin and Phillips [9] reported that inoculated rhizobia influence the physiological status of inoculated plants by increasing root respiration. Biswas *et al.* [10] reported that rhizobial inoculation significantly increased uptake of N, P, K and Fe by rice plants compared with the uninoculated control. In a subtropical environment in India, *Azotobacter* and *Azospirillum* increased maize yields by 1.15 folds over the control [11]. Yield improvements of more than 20% have been observed for wheat as a result of application of *Azotobacter* and *Azospirillum* inoculums in controlled field trials in Iran [12].

A variety of nitrogen fertilizer types can be utilized by wheat and barley. Ammonium forms of nitrogen result in a higher stem nitrate concentration initially than urea or nitrate since ammonium will not leach past the limited root system of the young plant. Nitrate or urea can usually correct a nitrogen deficiency during the season faster than ammonium forms of nitrogen. Urea has been a major N source used on great parts of Iran. Because of its relative lower costs of production and transport and represents a more economical alternative to supply pastures with N than AN. Burton and Hanna [13] reported

that Coastal bermudagrass responded similarly to anhydrous ammonia, AS and AN in terms of percentage crude protein, N recovery and yields, while urea was less effective. Sloan and Anderson [14] observed that urea effectiveness depended on soil characteristics. These latter authors indicated that urea was as effective as AN on a calcareous Ships clay (Chromic Hapludert), but on an acidic Lufkin fine sandy loam, yield of lands fertilized with urea were lower than when fertilized with AN. In a greenhouse study, Picchioni and Quiroga-Garza [15] observed no significant differences from urea, AS and AN on DM production. When surface-applied, urea can release significant amounts of N by volatilization of NH<sub>3</sub>. Increased temperature and soil CaCO<sub>3</sub> concentrations have been shown to increase NH<sub>3</sub> volatilization [16]. Several compounds have been proposed to inhibit NH<sub>3</sub> volatilization. For instance, ammonium thiosulfate (ATS) and calcium chloride may reduce N loss from urea fertilizers [14]. Fenn *et al.* [17] suggested that Ca-N fertilizer mixtures may reduce ammonia volatilization due to precipitation of CaCO<sub>3</sub> and subsequent retention of ammonium by the soil cation exchange sites. Take-all severity in wheat production was influenced by the N source; with more severe root damage in plots fertilized with nitrate (NO<sub>3</sub><sup>-</sup>) compared with ammonium (NH<sub>4</sub><sup>+</sup>) forms of N [18-22]. When comparing the effects of fertilization with urea-containing sources and AN on no-till corn (*Zea mays L.*) Howard and Essington [23] speculated that lower yields for the urea-containing N sources resulted from N volatilization losses. Howard [24] reported that urea-containing N sources were less efficient than AN in promoting wheat yields. When comparing broadcast urea and UAN with broad-cast AN, Howard *et al.* [25] found that wheat yield reductions of about 12% probably resulted from N volatilization losses for the urea-containing N sources.

At present, the government in Iran is heavily subsidizing mineral fertilizers for wheat and offers guarantee prices to achieve the national policy on self sufficiency for wheat. Besides environmental concerns of the use of high rates of chemical fertilizers, agricultural subsidies put a high burden on Iran's economy. There is now a shift in that policy towards more market-orientation and there are plans to reduce subsidies on fertilizers. Hence, any technology that could at least partly substitute fertilizer applications would be both helpful for farmers and Iran's economy [26, 12, 27]. This experiment designed to evaluate the effects of co-inoculation of *Azotobacter* and or Mycorrhiza and also effectiveness of various N sources on yield and yield components and grain protein percent of winter wheat.

## MATERIALS AND METHODS

This experiment was conducted in Fars Agricultural Researches Station, Iran (52° 36' E, 29°33' N, 1510 m height from sea level) during 2008-2009. The experiment was randomized block, split plot design with four replications. Main plots consisted of nitrogen fertilizer sources treatments which were ammonium nitrate, ammonium sulfate, SCU and Urea. Sub plots consisted of four treatments i.e. control, inoculation of arbuscular mycorrhiza fungi (AMF), *Azotobacter chroococcum* and dual inoculation of AMF + *Azotobacter chroococcum* (AMF + Azc). For AMF inoculation, pearl millet roots infected with arbuscular mycorrhiza fungi *Glomus fasciculatum* were chopped to small pieces and mixed with soil in furrows at the time of sowing. *Azotobacter chroococcum* mutant Mac27 (methyl ammonium chloride resistant) was grown on nitrogen free Jensen medium [28] containing 2 per cent sucrose at 30°C for 72 hrs. For Azc inoculation wheat seeds were first treated with traditional jaggery or molasses solution prior to treatment with charcoal based *Azotobacter chroococcum* Mac27 in a beaker and shaken thoroughly to facilitate uniform coating of seeds with the inoculum using colony forming units (CFU) 109 cells/ml. CFU was determined by plate count method [29]. Azc treated seeds were kept under shade for about one hour for drying before sowing so that Azc inoculum could adhere to seeds nicely. For dual inoculation pre-inoculated wheat seeds with Azc were co-inoculated with AMF. To determine the soil characteristics 15 samples from 30 cm depth were collected and analyzed by Shiraz Soil Testing Laboratory for basic soil physical and chemical properties (Table 1). All SCU fertilizer was added in planting time. Urea, ammonium nitrate and ammonium sulfate fertilizers were added half at planting and half at booting stage. Plots were sown on 14 November 2008 with a cone seeder and were 8 m long and 1.5 m wide, with 6 rows 0.2 m apart. Plots were plowed and disked after winter wheat harvest in July. The plots were disked again before seeding in November. Apirus was applied in early April to the crop to control both broad and narrow leaved weeds. Above-ground dry matter production at heading during both years was measured by making cutting at ground level in 0.3 m<sup>2</sup> quadrants per plot. Immediately prior to harvest, number of spikes per m<sup>2</sup> was determined by averaging three counts of 1-m sections of rows with in each plot. The number of kernel per spikes was determined from 20 spikes taken at random from a 1 m section of each plot and counted with an electronic seed counter. And average kernel weight was determined by weighing 250 kernels

Table 1: Soil physical and chemical characteristics of the experimental site.

Variable	
Texture	Si-C
PH	7.96
EC dS m <sup>-1</sup>	1.88
Organic matter (%)	0.25
N(%)	0.023
P mg Kg <sup>-1</sup>	5.4
K mg Kg <sup>-1</sup>	340
Fe	3.7
Zn	0.64
Mn	5.8
Cu	0.48

randomly drawn from the bulk grain sample from each plot. The central four rows (of 6 rows) of each plot were harvested for grain yield and converted to grain yield per hectares. Harvest indexes (HI) [wt. of grain/ (wt. of grain +straw)] were calculated using yield from the square meter samples. Total nitrogen concentration was determined by standard macro-Kjeldhal procedure. Grain protein concentration was calculated by multiplying grain N concentration by 5.7 [30]. Data were analyzed by analysis of variance [31]. When significant differences were found ( $P=0.05$ ) among means, Duncan's multiple range test (DMRT) were applied.

## RESULTS

**Plant Height:** There was only significant difference observed in N sources treatments (Table 2). The highest plant height was related to SCU fertilizer, resulting to lodging in some plots. With applying of SCU, plants are expected to have a continuous supply of N during the post anthesis stage. This condition is probably led to more plant height, thus increasing in lodging.

**Grain Yield and Yield Components:** To better understanding the large variability in grain yield due to different environments, grain yields were partitioned into yield components such as kernel per spike, spike per square meter and kernel weight.

**Spike Number per Square Meter:** Both N sources and bacteria inoculation were significant in this trait (Table 2). The data (Table 3) indicated that the highest spike per square meter obtained by ammonium nitrate and urea fertilizers. SCU and ammonium sulfate, with the same statistical group, produced the lowest values. Single application of *Azotobacter* and Mycorrhiza inoculation and in combination to each other significantly increased spike per square meter about 3% and 9.1 % compared to control.

Table 2: Results of analysis of variance combined across, N sources and bacteria inoculation in winter wheat.

Sources	df	Plant height(cm)	Spikesno. m <sup>2</sup>	Kernel Spike <sup>-1</sup> no.	Kernel weightg	Grain yield	Straw yield	Harvest index(%)	Grain protein(%)
Replication	3	NS	NS	NS	NS	NS	NS	NS	NS
Nitrogen sources	3	*	*	*	**	*	*	*	*
Ea	9								
Bacteria inoculation	3	NS	*	*	*	*	NS	NS	*
Nitrogen sources									
×									
Bacteria inoculation	9	NS	NS	*	NS	NS	NS	NS	NS
Eb	24								
Total	47								
CV, %		8	17	13	6	15	18	19	7

Table 3: Mean values of plant height, number of spikes per m<sup>2</sup>, kernel per spike, kernel weight, grain yield, straw yield, harvest index and grain protein percent under four N sources and four bacteria inoculation.

Treatments	Plant height(cm)	Spikesno. m <sup>2</sup>	Kernel Spike <sup>-1</sup> no.	Kernel weightg	Grain yield	Straw yield	Harvest index(%)	Grain protein(%)
N sources								
Urea	77 c	790 b	44 b	38 a	11.2 b	27 a	48 a	9.6 c
AS†	79 b	774 c	47 a	34 b	10.3 b	25 b	42 b	10.7 b
AN‡	79 b	848 a	48 a	37 a	12.1 a	28 a	48 a	11.3 a
SCU¶	87 a	760 c	36 c	31 c	8.4 c	21 c	39 c	9.3 c
Avg.	80	793	44	35	10.5	25	43	10.2
Bacteria inoculation								
Control	78 a	512 b	42a	41 b	8.8 ab	13.1 b	49 ab	10.6 b
AMF §	78 a	525 a	34 b	46 a	7.9 b	15.7 a	40 c	10.2 ab
Azc§	81 a	504 b	35 b	45 a	8.1 a	14.1 ab	50 ab	12.0 a
AMF+Azc	81 a	559 a	40 a	43 b	9.4 a	14.9 a	53 a	10.8 b
Avg.	80	525	38	44	8.5	14.5	48	10.9

†:AS=ammonium sulfate ‡:AN=ammonium nitrate and ¶:SCU= sulfur coated urea.

§:AMF= arbuscular mycorrhiza fungi and §:Azc= *Azotobacter chroococcum*

Same letters are not significantly different at P0.05.

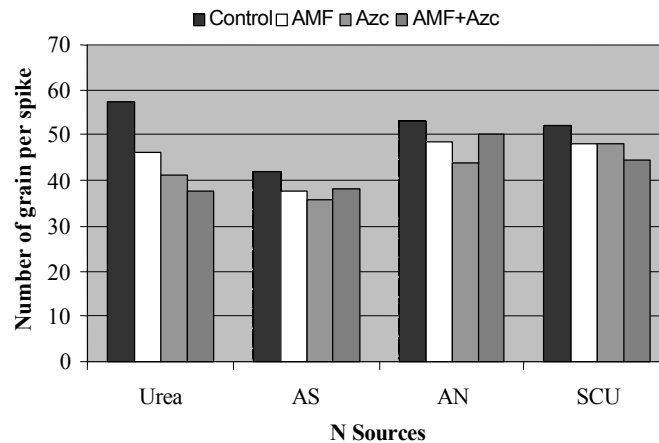


Fig. 1: Number of grain per spike for wheat fertilized with various N sources and bacteria inoculants.

AS=ammonium sulfate, AN=ammonium nitrate, SCU= sulfur coated urea. AMF= arbuscular mycorrhiza fungi, Azc= *Azotobacter chroococcum*.

**Number of Grain per Spike:** Significant difference in number of grain per spike between N sources was observed in this study (Table 2). Ammonium nitrate and ammonium sulfate fertilizers produced more grain per spike than urea and SCU fertilizers. Azotobacter inoculation was significantly affected on

grain per spike. The interaction effect of biofertilizers and N sources was significant at 5% probability level (Table 2). Mean comparisons showed that applying of Azotobacter and N sources not only did not increase the grain per spike but also in some cases decreased it (Fig 1).

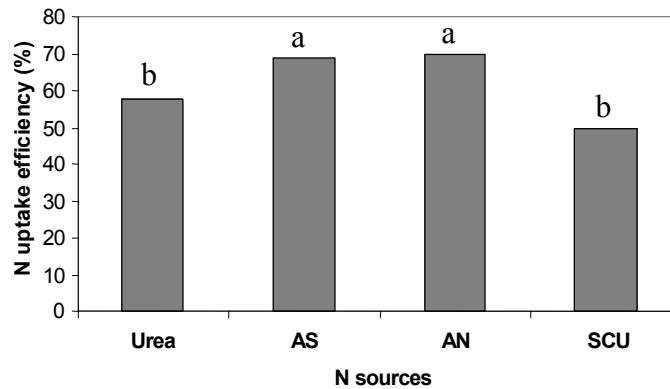


Fig. 2: Relative N uptake efficiency for wheat fertilized with various N sources. AS=ammonium sulfate, AN=ammonium nitrate and SCU= sulfur coated urea. Same letters are not significantly different at  $P \leq 0.05$ .

Table 4: Simple correlation coefficients between traits.

	1	2	3	4	5	6
GY	1.00					
HI	0.15	1.00				
GP	-0.85	0.05	1.00			
MC	0.35	-0.10	0.65	1.00		
DTF	0.05	0.07	0.25	0.06	1.00	
DTM	-0.67	0.15	0.39	0.16	0.08	1.00

1: Grain Yield (GY);  
 2: Harvest index (HI);  
 3: Grain protein (GP);  
 4: Micorrhizal colonization (MC);  
 5: Days to flowering (DTF);  
 6: Days to maturity (DTM).

various N sources and bacteria inoculants.  
 AS=ammonium sulfate, AN=ammonium nitrate, SCU= sulfur coated urea.  
 AMF= arbuscular mycorrhiza fungi, Azc= *Azotobacter chroococcum*.

**Kernel Weight:** Main effects of treatments had significant effect on kernel weight in and and 1 and 5 % probability levels (Table 2). The highest value of kernel weight was obtained with urea and ammonium nitrate fertilizers and the lowest value was belonged to SCU fertilizer. Maximum kernel weight was found in Azotobacter and Azotobacter+Micorrhiza and minimum in control and Micorrhiza treatments. (Table 3).

**Grain Yield:** The data (Table 2) indicated that there is significant difference in bacteria inoculation and N sources treatments. The greatest grain yield obtained in ammonium nitrate and Azotobacter + Micorrhiza treatments (Table 3).

**Straw Yield and Harvest Index:** There was only significant difference between N sources treatments in these two traits (Table 2). Ammonium nitrate and urea

fertilizer treatments were more successful than other treatments to transport of assimilate from sources to plant sinks (Table 3).

**Grain Protein Percent:** Both N sources and bacteria inoculation had significant effect of grain protein percent (Table 2). Grain protein percent increased up to 19 % in ammonium nitrate than urea and SCU fertilizers. Azotobacter + Micorrhiza treatment increased grain protein by 13 % than control.

**Relative Efficiency of Nitrogen Sources:** AN and AS showed greater N uptake efficiency (Fig. 2). In general, SCU and urea showed the lowest efficiency of N uptake. Greater N uptake efficiencies reported by Osborne *et al.*[32], who showed N recoveries varying from 29 to 45% for AN and from 16 to 27% for urea. For instance, Westerman *et al.*[33] observed relatively lower efficiency of N uptake with AS (37-48%), UAN and (45-36%) and urea (31-38%).

The results of correlation coefficients between traits showed that grain yield had a positive and significant correlation with days to maturity and mycorrhizal colonization at 1 and 5% probability levels, respectively and a negative signification with grain protein at 1% probability level. Also mycorrhizal colonization had positive and significant correlation with grain protein and days to maturity (Table 4).

## DISCUSSION

The improved performance with bio-inoculants for grain yield, yield components and grain protein percent was probably due to the absorption of more nutrients by

wheat plants because Azotobacter + AMF treatment provided access to more soil volume as extra matrical hyphae of AM fungi enlarge the effective surface outside of the roots [34, 35]. Rhizobial inoculation increased sink size by increasing either panicle number or spikelet number per panicle. The increase in grain yield was due to an increase in total biomass production rather than harvest index. Yanni *et al.* [36] also reported higher grain yield following inoculation with E12 in a field experiment in Egypt.

The importance of additive effects of bio-inoculants was reported by earlier workers for component traits like plant height [37], spike length [38], grain weight [38, 39], flag leaf area [40] and grains per spike [41].

The growth-promoting activities (GPA) of bacterial inoculants on crop plants may be manifested in several ways. For example, their production of iron-sequestering siderophores and antimicrobial compounds may hinder colonization of hosts by phytopathogens [42], thereby suppressing the diseases they cause [12]. Other mechanisms of GPA include the induction of host systemic disease resistance [43], N<sub>2</sub> fixation [44], solubilization of precipitated mineral nutrients [45] and/or production of plant growth regulators [46, 4] that induce additional root hairs and/or lateral root formation [46], thereby enhancing the plant's ability to take up nutrients and water from soil and increase yield.

Most of the researchers suggested that VAM symbiosis increased the photosynthesis and increase the rates of photosynthetic storage and export at the same time [47]. It has been proved that concentration of chlorophyll in VAM plants was higher than their control plants. Therefore it can produce larger grains and enhance economical yield. DeJong and Phillips [48] reported higher leaf apparent photosynthesis and increased leaf N content in Alaska pea (*Pisum sativum* L.) following rhizobial inoculation. In that study, both leaf N content and photosynthetic rate increased linearly with symbiotically fixed N<sub>2</sub>. A close relationship between photosynthetic rate and leaf N content was reported for both greenhouse and field-grown rice plants [49, 50].

In a greenhouse experiment, *Azotobacter chroococcum* increased wheat grain yields by 12.6 to 14.0% at N fertilizer rates of 60 to 120 kg ha<sup>-1</sup> [51]. In a field experiment in Iran, yield improvements of more than 20% have been obtained for wheat as a result of Azotobacter and Azospirillum inoculation. Narula *et al.* [52] observed a net saving of 25-30 kg nitrogen by using Azotobacter inoculants for wheat.

Manske *et al.* [53] and Behl *et al.* [54,55] have also observed synergistic effects between AMF and Azc. The productiveness of rhizosphere for AMF may be attributed to favorable influence exerted by root exudates [56], which contain amino acids, carbohydrates, organic acids and growth promoting substances and also phytohormones produced by Azotobacter. It is a well known fact that wheat roots secrete carbonaceous exudates, which could help in proliferation of AMF and Azotobacter [53]. Azotobacter excretes phytohormones, which improves growth of plant roots and AMF may solubilize P from surrounding areas and makes it available to the roots. Dual inoculation of efficient strains of *Azotobacter chroococcum* and *Glomus fasciculatum* in responsive wheat genotypes adapted to low input stress conditions could be profitably used to maximize wheat production. However, intense AMF infected roots even at moderate nutrient deficiency are important during early plant growth when roots are too small to provide a high demand for minerals for shoot growth.

Brennan [57] reported that phosphate utilization efficiency in grain yield production was more enhanced (average 13%) than N utilization efficiency (5%). Furthermore, N uptake was not qualitatively enough improved by Azotobacter inoculation. This supports the hypothesis that Azotobacter acts through the production of phytohormones, which stimulate root growth and VAM infection, rather than as an associative dinitrogen fixer. Biswas *et al.* [10] found that the growth promotion in rice plants by rhizobial inoculation was associated with indole-3- acetic acid accumulated in the external root environment of rice plants when grown gnotobiotically with rhizobia [10]. Biswas *et al.* [10,58] suggest that certain strains of rhizobia can promote wheat growth and yield through mechanisms that improve single-leaf net photosynthetic rate rather than biological N<sub>2</sub> fixation.

Narula *et al.* [29] detected a significant interaction between the inoculants and N fertilizer rate on N uptake and yield of wheat. The effect of Azotobacter and Azospirillum on grain yield and N uptake was most pronounced without fertilizer application (+57% and +94%, respectively), but the effect gradually declined with increasing amounts of N application up to 120 kg N ha<sup>-1</sup> where no differences could be observed. Highest biological yields for Azotobacter and Azospirillum inoculated wheat and barley were recorded at moderate fertilizer doses in greenhouse and field trials in Northern Sinai [8].

Muazu [59] by applying N fertilizer sources reported a linear response to nitrogen application in grain yield and straw yield. Also, response of grain yield to ammonium nitrate and urea was the same and was the lowest at SCU fertilizer.

Ammonium fertilizers may reduce take-all severity because of a decrease in rhizosphere pH that promotes more vigorous root growth, allowing roots to escape severe disease damage [57]. However, where take-all was at high levels, ammonium forms of N were ineffective in reducing take-all severity [20]. Also, soil acidity should be carefully monitored, especially when annually applying N sources such as AS that can significantly reduce soil pH. Selection of N source should be carefully planned to avoid detrimental effects on soil acidity and, consequently, fertilizer efficiency. On low-buffer capacity, acid sandy soils, use of AS as the sole source of N throughout the growing season should be limited.

With respect to this matter that ammonium nitrate did not need to special enzyme, such as Ureas enzyme, providing nutritional conditions during floret initiation stage and less competition in this stage led to more grain per spike, resulting to more grain yield.

Result from the present study indicated that grain yield and yield components of wheat have been affected significantly by the inoculation with Azotobacter and Mycorrhiza, because these biofertilizers can fix the atmospheric nitrogen and increase phosphorus availability in soil and enhance absorb elements by plant. Seed inoculation at sowing date with Azotobacter and Mycorrhiza increased grain yield about 7.13%. Also, AN and AS resulted in increased yields, N uptake and fertilizer efficiency compared with urea and SCU.

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