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# RESPONSE OF WHEAT CULTIVAR (SABER BEG) TO INOCULATION WITH NITROGEN FIXING *AZOSPIRILLUM* SPECIES (LOCAL ISOLATES)

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## Abstract

Investigating the impact of the inoculant *Azospirillum* spp. isolate on wheat plant (*Triticum aestivum* cv. Saber Beg) root hair deformation, growth parameters and detecting host-specificity phenomenon. The nitrogenase activity ($C_2H_2$ reduction) of *Azospirillum* Spp. isolate was measured using gas chromatography method, and the impact of these isolate on the wheat cultivar (Saber Beg) were studied using hydroponic culture experiments. Emphasis was attempted on the impact of inoculation on the root hair deformation, root length, root fresh weight, and location of bacteria in infected root, also the response of the green part (fresh, dry weight and total nitrogen). Results showed that all isolate of *Azospirillum* spp. used could fix nitrogen and deform wheat root hair cv. Saber Beg forming forks or unequal branching phenomenon. Also, almost all isolates produce a significant increase in the root mass of *Triticum aestivum* cv Saber Beg. The same patterns of affect were found in the root length. Considering the green part of inoculated plant, positive response in early growth stage was observed, which was enhancing the shoot giving rise to a significant increase in fresh and dry weight and height of plant compared with the non-inoculated control. Microscopic examination of cross section of infected roods showed that the bacterial cells was localized in the cortex layer.

***Keywords*** **:** *Azospirillum*, Saber Beg, nitrogen fixation, wheat cultivar.

## Introduction

Inoculation of crop plants with $N_2$–Fixing bacteria of the genus *Azospirillum* was proposed in the mid-1970s as a new approach in providing Fixed nitrogen, thereby decreasing fertilization requirements or increasing yield, or Both (Döbereiner and Day, 1976; Okon *et al*., 1977). The establishing of inoculated *Azospirillum* spp. in the rhizosphere and in root of field grown wheat and sorghum was studied, it was found that strains SP7 and Cd established poorly in wheat root, *Azospirillum lipoferum* SP S82 and natural *Azospirillum* infection became concentrated in the upper parts of the root system (Baldani *et al.,* 1986). Mixed cultures of dual inoculation of wheat seedling may improve infection (Yegorenkova *et al.,* 2016). Also, nitrogen fixing bacteria were isolated from the roots and rhizosphere of several agricultural important crops which found in associative symbiosis form (Lindberg and Granhall, 1984; Jagnow, 1990; Skvortsov and Ignatov, 1998; Al-Maadhidi, 1989). A significant enhancement in the mineral ions uptake by roots of *Zea mays* and *sorghum* as well as increasing in the plant growth after inoculation with *Azospirillum spp*. was found (Okon *et al.,* 1983; Bashan *et al.,* 1990). Although, increment in dry weight and total nitrogen were also observed (Cohen *et al.,* 1980; Kapulnik *et al.*, 1981; Tilak and Annapurna 1993; Baldani and Baldani 2005; Veresoglou and menexes 2010). Root hairs play an important role in the uptake of minerals and other nutritional requirements of plant growth, so, any increase in the surface area of the root hairs will enhance the absorption of nutrient and then the plant growth. *Azospirillum* exhibits a strain –specific effect on root hair deformation in wheat similar to the strain specific effect of *Rhizobia* on root hair of legumes (Patriquin *et al*., 1983; Baldani and Baldani, 2005; Bashan *et al.,* 1990; Jagnow, 1990). Several *Azospirillum* strain induced more deformational root hairs of wheat plant, *Azospirillum* sp., strain SP 245 caused the most deformation in wheat root hairs compared with strain SP7 isolated from the rhizosphere of a forage grass (Jain and Partriguin 1984), also, (lareen *et al.,* 2016) discussing the effects of microbe–microbe interaction on the shape of microbial communities at the root surface and modification to benefit agriculture and food production. A best result for lateral and tertiary root formation was found when sweet corn verity inoculated with *Azospirillum* spp, in present of nitrogen (fertilizer 0.06g/plant) (Faruq *et al.,* 2015). The deformations including tuning forks (branched root hair are of equal length) and unequally branched root hair form when seeding of wheat inoculated with *Azospirellum* (Jain and Patriquin, 1984). These changes have been proposed to improve mineral and water uptake by the inoculated plant (kapulnik *et al.,* 1985; Sarig *et al.,* 1988; Bashan and Levanony, 1989). Meanwhile, the effects of *Azospirellum* strains on root mass of wheat grown to maturity in pots was not homologues (Jain and Partiquein, 1984), such effects are thought to be depending on the production of hormones by *Azospirellum* or the extracellular polysaccharides, polysaccharide–lipid complex (Venkateswarlu and Rao, 1983; Skvortsov and Ignatov, 1998). However, growth of inoculated wheat and soybean plant (shoot root dry weight) was significantly improved (Bashan *et al.,* 1990). In this study the effects of 10 isolates of *Azsopirillum* spp. on root hair branding, root mass, and growth of green parts of wheat plant cultivar (Saber Beg) were studied.

## Materials and Methods

### Microorganisms and growth conditions

*Azospirillum* spp. (isolated and characterized previously by Al-Maadhidi, 1989) were subculture in nitrogen free medium (NFB) containing one gm/L of ammonium chloride at 30°C for 24hrs, which used as inoculum. *Triticum*

*aestivum* seeds were surface sterilized by 95% ethanol for 3 minutes then washed four times with sterile distilled water and sucked for four hours. The sucked seeds were germinated in dark incubator on agar – water plates for two days. Seedlings (2-3) plants per bottle were transferred to a 1000 ml hydroponic culture bottle containing 1000 ml of half strength Hoagland solution (0.025gm/L $NH_4$ Cl). The bottles were maintained in green house at 30°C with 14 hrs. of light duration. Bacterial inoculum (10ml) was added to the bottles after 6 days and 16 days of transplantation.

**Nitrogenase activity ($C_2H_2$ reduction) of the isolates**

Nitrogenase activity of the isolates was measured using a method described by (Hardy *et al.,* 1968). A Shimadzu gas chromatography, model GC- 7AG with a hydrogen flame ionization detector, equipped with a 3.2 mm x 2.1 m column packed with 80 – 100 mesh Porapak-T and maintained at 150 °C to analyze the $C_2H_4$ and $C_2H_2$ gases. Nitrogenase activity of the *Azospirillum* isolates was characterized by sub culturing the isolates on nitrogen free medium (NFB) for 18 hrs. at 30°c with shaking (150 rpm). A 10% from the culture gas face was replaced by $C_2H_2$ and incubated for one hour. At the end of incubation period, one ml gas phase withdrawn and analysis for $C_2H_4$ produced, which representing the nitrogenase activity of the isolates. Nitrogen fixing activity of each isolate was calculated according to the number of cells counted per ml of culture.

**Effect of isolates on root hair branching**

Wheat cultivar seeds (*Tritium aestivum* Saber Beg) were surface sterilized in ethyl alcohol (95%) for three minutes, then germinated on water – agar plates. Culture suspension of the isolates or its filtrate were added to the 48 hrs. old sterile seedlings in plates. Branched root hairs were counted after 48 hrs. of treatment at the zone of the root hair elongation under light microscope.

**Ability of *Azospirillum* isolates for infecting root system**

Hydroponic cultivar of inoculated seedling of wheat cultivar was grown for three weeks at green house. Root segmented of inoculated plants were fixed in formaldehyde–acetic acid (FAA) 5:5 V/V for 24 hours. After gradual dehydration with ethanol, root segments were transferred to 100% xylol, then infiltrated with paraffin at 60°C. Paraffin blocks containing root segments were prepared and then solidified in a deepfreeze. Root sections (15µm) were Fixed on slid by hot plate (40 °C), then stained with safranin and fast green. The section was examined using light microscope to detect the localization of bacterial cells inside the root tissue.

**Measurement of growth parameter**

Plant growth represented by shoot (Height, fresh weight, dry weight and total nitrogen), roots length and fresh weight were measured at the end of the experiment. shoot dry weight were obtained by drying the green parts of the plant at 80°C for 48hrs in a forced air oven. Percentage of total nitrogen in dry material was measured using micro kjeldahl.

## Results and Discussion

In order to study the impact of inoculation by *Azospirillum* spp. on wheat cultivar (Saber Beg). Nitrogen fixation ($C_2 H_2$ reduction) activity for isolates was measured using gas chromatography technique. Results optioned showed that the isolates 1 and 9 are the most active once in reducing acetylene to ethylene (table 1), the isolates 3, 5, 12 and 14 showed moderate activity, while, the isolates 6,7 and 10 showed the lowest activity (table 1). The impacts of inoculation had been conducted in hydroponic culture. In general, all isolates or it filtrates shows positive impact on cultivated seedling and progressive growth stages, but not at the same extent. Almost all isolates (*Azospirillum* spp.) affect the root hairs and modified it's to tuning forks form (branched root hairs are of equal length) and unequally branched root hairs (Fig. 1). This phenomenon had observed previously (Patriquin *et al.,* 1983; Lareen *et al.*, 2016). The filtrates of *Azospirillum* culture also cause root hair branching. Jain and Patriquin, 1984 make a comparison studies between two *Azospirillum* strains on four wheat cultivars and they found the induction in seedling root hair branching was not at the same order, it depends on specificity between *Azospirillum* strain and wheat cultivar (Baldani and Baldani, 2005). Root mass of wheat cultivars affected as well.

The results showed that almost all the isolates produced a significant increase in the root fresh weigh of Saber Beg wheat cultivar (table 2). However, high significant pattern of effect was found in the Length of the roots belonging to the same cultivar. Also, isolate No.1 was the most effective once in root Length and fresh weight, while isolate No.5 induced high elongation in roots but to less extent in fresh weight (table 2). This effect on length and fresh weight due to the promoting growth substances excreted by *Azospirillum* spp. used in this study or enhancement of mineral uptake (Veresoglou *et al.,* 2010; Bashan *et al.,* 1990). The same positive results induction was found in Malaysian sweet corn variety (J58) seedling inoculated with *Azospirillum* spp., it induced longer roots compared to the control, and the highest biomass was obtained from *A. brasilense* CCM with nitrogen treatment (Faruq *et al.,* 2015). The shoots part of the plant was also affected as a result of inoculation with *Azospirillum* isolates. Both shoots biomass and plant height were affected positively. All isolates showed significant increase in fresh weight of wheat plant, the most effective isolates are No. 1, 6, 12 and 14, it causes significant increase under 1% of statistical level (Table 3). The increase in biomass was observed at the beginning of the growth stages and continued up to the tillering stage (Fig. 2, 3). The significant increase in shoot and root dry weight of inoculated seedling cultivated in nitrogen free medium due to inoculation with *Azospirillum* spp. (Christiansen-Weniger and Van Veen, 1991; Tilak and Annapurna 1993; Veresoglou and Menexes, 2010). Measurement of total nitrogen showed the isolates No. 6, 7, 12 and 14 had most impact leading to 16–55% increment in accumulated nitrogen of wheat cultivar (Table 3). Also, most isolates which showing positively impacts on roots and shoots have the same effects on total nitrogen, except isolate No.1 which showed significant increase in all parameter studied except total nitrogen. Also, isolate No.1, 6 and 10 showed significant increase in shots height, fresh weight and dry weight of wheat cultivar.

The effect of *Azospirillum* isolates on tillering and yield of wheat cultivar (Saber Beg) is show in Fig. 4. Most of the isolate used as inoculum for Saber Beg cultivar caused early tillering (isolates No.1, 7, 10 and 14), while isolate No.6 and 12 had no effect comparing with untreated control. However, isolate No.1 is the most effective one, it caused early tillering and appearance of spike by 10 days earlier rather than the

untreated control (Fig 3). This tiller escaping and head emergence possibly reflect an improvement in the hormonal balance of the inoculated plant rather than the contribution coming from fixed nitrogen (Tilak and Annapurna, 1993; Skvortsov and Ignatov, 1998).

The infectivity of isolate and location of infected bacteria inside the root tissue was studied microscopically. Cross and longitudinal section of root segments showed that, all isolates have the ability to infect the roots, and the location of infected bacteria were found in the cortex zone (Fig. 5). The bacterial cells occupied the lumen of cortex cells, then reproduced and fixing nitrogen. The efficiency of isolates for fixing nitrogen depending on: number of bacterial cells per plant cell and the frequency of infected plant cells (Döbereiner and day, 1976; Okon, 1982; Baldani and Baldani 2005).

As a conclusion, the infectivity of *Azospirillum* isolates used against wheat cultivar (Saber Beg) was not similar and properly sometime not reflecting the nitrogenase activity of such isolates in vitro, it depend on the plant genome (host specificity). Isolate No.1 is the most effective once in fixing nitrogen in vitro, and showing high impact on all character properties of plant in vivo, which consider more specific for Saber Beg cultivar.

**Conclusion significance and impact of the study**

There is an indication for strain-host specificity between wheat cultivar (Saber Beg) and *Azospirillum* isolate no.1 due to the high impact of this isolate on plant yield, early spike emergence and high frequency of infected root hair in inoculated plant.

**Table 1:** Nitrogenase activity ($C_2H_2$ reduction) of the *Azospirillum Spp.* isolated from roots of local Iraqi wheat cultivar

| Isolate No. | Nitrogenase activity (n mole/$10^6$ cells/h.) |
|---|---|
| 1 | 16.20 |
| 3 | 8.74 |
| 3" | 9.81 |
| 5 | 8.53 |
| 6 | 2.98 |
| 7 | 4.16 |
| 9 | 19.40 |
| 10 | 2.56 |
| 12 | 6.61 |
| 14 | 10.24 |

**Table 2 :** Effect of local isolates of *Azospirillum Spp*. on root length and fresh weight of wheat cultivar (Saber beg) in hydroponic culture.

| Isolate No. | X" Root length / cm | X" fresh weight/g. |
|---|---|---|
| 1 | 19.3 ± 1.15 ** | 1.67 ± 0.5498 * |
| 3 | 16.0 ±3.29 ** | 1.30 ± 0.1636 * |
| 3 | 13.3 ± 1.366 ** | 1.64 ±0.078 ** |
| 5 | 22.83 ± 0.408 ** | 1.362 ±0.2379 ** |
| 6 | 14.00 ± 1.414 ** | 1.347 ±0.5037* |
| 7 | 18.00 ± 1.414 ** | 0.61 ±0.2263 |
| 10 | 17.50 ± 2.7886** | 1.167 ±0.165* |
| 12 | 16.00 ±5.656 | 1.348 ±0.2949** |
| 14 | 17.60 ±o;8944** | 1.258 ±0.297** |
| Control | 8.66 ±0.577 | 0.663 ±0.4219 |

Roots fresh weight is mean of three replicates. * Significant at 5%.
Roots length is mean of three replicates. ** Significant at 1%.

**Tables 3 :** Effect of *Azospirillum Spp.* isolated from Iraqi soil on shoot height, fresh and dry weight, and total nitrogen of wheat cultivar (Saber beg) Cultivated in hydroponic culture.

| Isolates No. | X shoot Height/cm | X fresh wt/g | X dry wt/g | Total nitrogen (%) |
|---|---|---|---|---|
| 1 | 28.8 ± 4.3243** | 2.12 ±0.5053** | 0.494 | 2.030 |
| 3 | 22.16 ±2.4832 | 1.53 ± 0.2818* | 0.375 | 1.825 |
| 3 | 27.3 13.5024 | 2.03 ±0.1920 | 0.440 | 0.665 |
| 5 | 21.29 ± 6.183 | 1.53 ± 0.3656* | 0.360 | 1.750 |
| 6 | 28.20 ± 1.6431** | 2.165 ±0.3551** | 0.480 | 3.955 |
| 7 | 21.50 ±1.948 | 1.693 ±0.6886 | 0.313 | 2.975 |
| 10 | 29.875 ± 5.4625** | 2.196 ±0.4947 | 0.550 | 2.415 |
| 12 | 25.166 ± 3.2506* | 1.7516 ±0.3149** | 0.344 | 3.325 |
| 14 | 25.0 ± 3.8172** | 1.8440 ± 0.518** | 0.405 | 3.150 |
| Control | 20.160 ±4.3550 | 1.02 ±0.623 | 0.215 | 2.555 |

Shoots fresh weight is mean of three replicates. *Significant at 5%.
Shoot height is mean of three replicates. **Significant at *1%*.
Shoot dry weight is mean of three replicates.

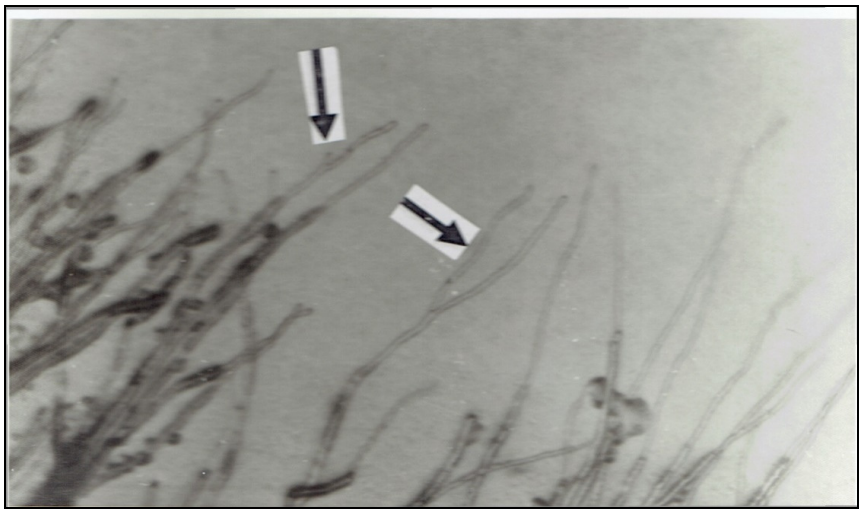

**Fig. 1 :** Modification of root hairs of wheat plants (Saber Beg) as a result of inoculation with *Azospirillum Spp.*

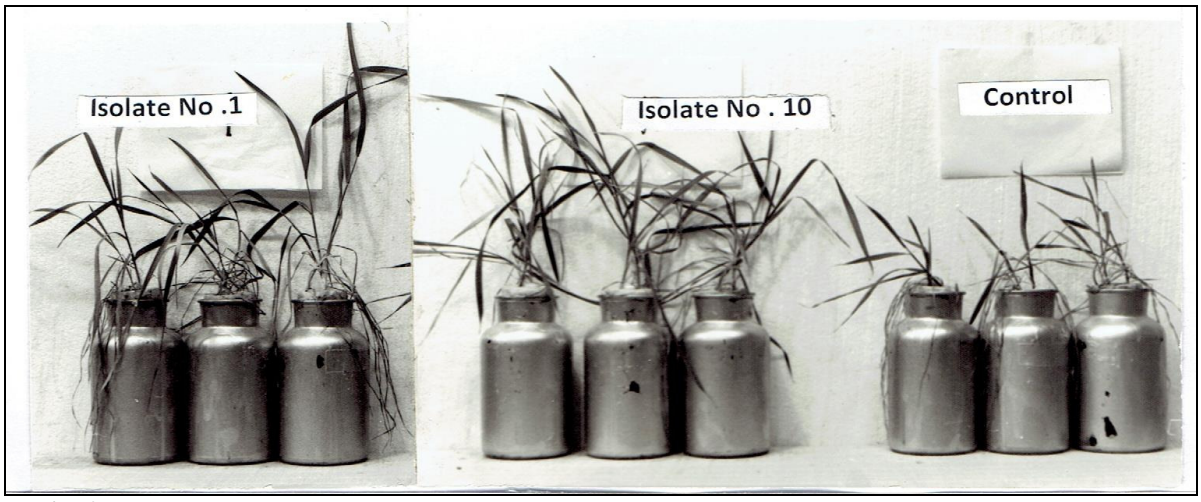


**Fig. 2 :** Growth response of wheat cultivar to inoculation with *Azospirillum* Spp. at early growth stage


**Fig. 3 :** Effect of inoculation with *Azospirillum* isolate No.1 on growth of wheat Cultivar (Saber beg) at tillering stage of growth

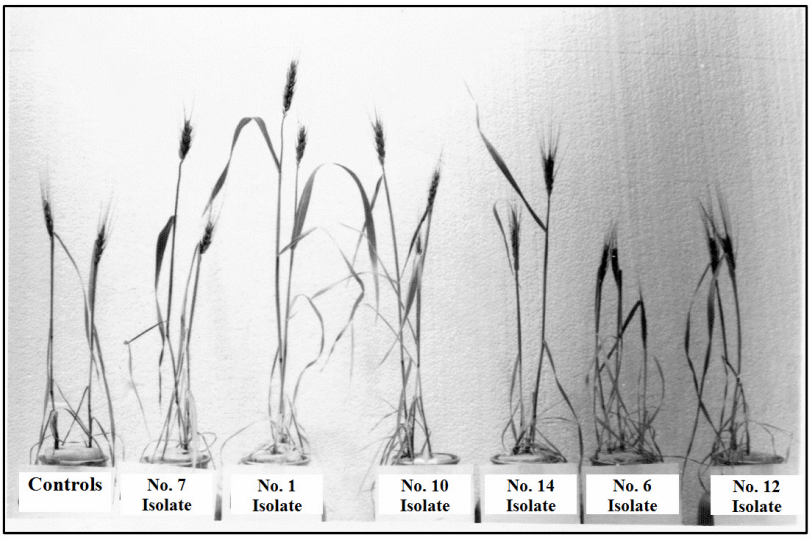


**Fig. 4 :** Response of wheat cultivar (Saber Beg) to inoculation with *Azospirillum Spp*. isolates at mature and heading stage

**Fig. 5 :** Location of *Azospirillm Spp*. cells inside the roots of wheat cultivar (Saber Beg)

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