Journal of Plant Physiology and Breeding
2017, 7(2): 67-79 ISSN: 2008-5168

Histological Responses of Two Wheat Species to *Azospirillum* Inoculation under Dryland Farming

Taiebeh Jafarian[1], Mohammad Javad Zarea[1*] and Adel Siosemardeh[2]

Received: November 28, 2016 Accepted: May 21, 2017
[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture, Ilam University, Ilam, Iran
[2]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran
*Corresponding author; mj.zarea@ilam.ac.ir

Abstract
In this experiment the effect of inoculation with *Azospirillum* on the flag leaf and spike rachis anatomical features and also on grain yield and grain weight was investigated for the first time in bread and durum wheats during 2015-2016 growing season under semi-arid condition. The crop yield increased due to the inoculation with *Azospirillum* with a maximum yield increase of about 8.0 per cent for bread wheat. *Azospirillum* led to an increase in grain weight per spike. Yield increase due to *Azospirillum* inoculation was higher for bread wheat than durum wheat. The two wheat species exhibited differences in flag leaf and spike axis anatomic features. Although *Azospirillum* inoculation decreased the number of flag leaf stomata by 11.5% irrespective of the wheat type, stomatal length of the inoculated plants increased by about 3.6 and 11.5% for bread wheat and durum wheat, respectively. *Azospirillum* inoculation significantly enhanced the area and length of xylem and phloem vessels by about 12.5 and 12.39% and 14.42 and 33.33%, respectively. Area of the bundle sheath and mesophyll layers of inoculated plants was increased by 20 and 31.5%, respectively. *Azospirillum* inoculation enhanced the area of upper and lower epidermis of the bread wheat much more than the durum wheat. Anatomic changes due to *Azospirillum* was not limited to the flag leaf but also extended to the spike rachis and, therefore the inoculated plants had higher area of vascular bundle, xylem vessel and phloem vessel. Overall, the effect of *Azospirillum* on anatomical features and grain yield of wheat was slightly species specific and it was more pronounced in the bread wheat as compared to the durum wheat.

Keywords: Anatomical features; Bread wheat; Durum wheat; Grain yield

Introduction

Semi-arid areas of Iran's cropland are characterized by the occurrence of water stress and high-temperature during the life cycle of the plant from sowing to maturity, especially in the terminal part of the growing season. These areas are characterized by irregular and low precipitation, which is regarded as the major crop production constraint. Wheat is the most important cereal cultivated in Iran. In most parts, the wheat crop is affected by several environmental factors, from which drought and salinity stress are the most prevalent.

Azospirillum spp. are widespread colonizing bacteria that have been isolated from the root surface or the rhizosphere of a wide variety of plant species (Umali-García *et al.* 1980). The *Azospirillum* genus has been reported as the plant growth-promoting rhizobacteria (PGPB) (Glick *et al.* 1999). Inoculation with PGPB can enhance germination, seedling emergence and modifies growth and yield of various cereal and non-cereal crops (Zahir *et al.* 2004). Several researchers have reported that *Azospirillum* enhances wheat growth and yield under non-stress and stress conditions (Haji Nia *et al.* 2012; Zarea *et al.* 2012, 2013).

Most studies carried out under controlled conditions or in sterilized soils, demonstrated a pronounced beneficial effect of *Azospirillum* inoculation on enhancing the plant growth. However, in some studies, introduced microbe strains did not remain alive because of competition with indigenous species (van Elsas and Heijnen 1990; Asad *et al*. 1991). The unpredictability and the inconsistency of the results have been mentioned as the main factors preventing the introduction of *Azospirillum* into the agricultural market. Evaluating the data from 20 years of field experimentation, demonstrated that 60-70% of all field experiments were successful with significant yield increases ranging from 5 to 30% (Okon and Itzigsohn 1995). The development of better bacterial carriers, efficient bacterial culture, proper agronomical practices and using indigenous efficient species may also lead to further success of *Azospirillum* performance under field condition.

Although the positive response of various crops to plant growth-promoting bacteria under non-stressed and stressed conditions have been well established but, based on our knowledge, there is no research about the leaf and spike anatomical characters of wheat plants inoculated with *Azospirillum* under field conditions. Furthermore, durum wheat is frequently assumed to be more tolerant to stress than bread wheat (Marti and Slafer 2014). However, there is no published research paper that compares the performance of durum wheat and bread wheat under *Azospirillum* inoculation. Therefore, the objectives of this study were to investigate the effects of *Azosperillum* on histological features of flag leaf and spike and on grain yield of wheat in the field condition and also to compare the effect of *Azospirillum* on performance of these two species in terms of anatomical characters.

Material and Methods

Experimental location and field experiment

The field experiment was conducted at the research station of Ilam University, Ilam, Iran (latitude of 33°38´ north and longitude of 46°26´ east, and 1174 m above sea level) during the 2014-15 growing season on a clay-loamy soil. The 30-year average annual precipitation in this area was 600 mm (Ilam Meteorological Office, Personal Communication). The experimental soil was characterized with a pH of 7, electrical conductivity of 0.3 dS m^{-1}, 1.83% organic matter, 0.18% N, 7 ppm available phosphorus, 590 ppm potassium, 6.6 ppm Fe, 7.9 ppm Mn, 0.63 ppm Cu and 1.08 ppm available Zn. The rainfall during the growing season was 360 mm. Seedbed preparation was made in the autumn when soil moisture was adequate for seed germination. The sowing date was November 9, 2015, following the first rain. The experiment was conducted as factorial based on randomized complete block design with three replications. Four treatment combinations of two factors, *Azospirillum* (with or without inoculation) and wheat species (bread wheat and durum wheat) were used in this experiment. Plot dimensions were 3.0 x 1.2 m consisting of six rows with 20 cm between rows. The seeding rate was 250 m^{-2}. At the planting time, nitrogen and phosphorous fertilizers were applied as 40 kg P2O5 ha^{-1} and 75 kg N ha^{-1}, respectively.

Azospirillum inoculation

The *Azospirillum* species used in this experiment were saline-adapted *Azospirillum* strains isolated from a saline area of southern Iran which were previously described to enhance wheat growth and yield under non-stress and stress conditions (Haji Nia *et al.* 2012; Zarea *et al.* 2012, 2013). Seeds of bread wheat, cv. Cross-Sabalan, and durum wheat, cv. Saji, were inoculated by immersion in a total imbibition volume of autoclaved *Azospirillum* inoculants (control) or bacterial inoculants containing 10^7 bacterial cells per seed for 1 h.

Leaf anatomical measurements

Leaf anatomy characters were measured at the tillering growth stage. For this purpose, the flag leaves of inoculated and non-inoculated plants were randomly selected from each individual plot. Leaves were carefully freed from leaf sheaths and then cut with a razor blade from the stem at the base of the leaf blade. Photosynthetically active segments (Hu *et al*. 2000) were then prepared. Leaf pieces were fixed for 48 h in 10% formalin solution. A series of alcohol solutions were used to dehydrate the tissues. Thereafter, samples were twice embedded in pure xylol. Samples were then submerged in melted paraffin inside the blocking cassettes. Paraffin blocks were then fixed in the microtome clump and were transversely sectioned while the blade was adjusted at 5 μm. Semi-thin sections of leaves (5 μm thick) were obtained in a rotary microtome (4055 Slee, Germany), stained with hematoxylin and eosin, and examined with a photomicroscope (at 10 × 10 magnification). Anatomical features were measured on five images randomly taken from each slide. Images were captured using an eye-piece digital camera, Dino-eye camera (AM423) and the data was recorded by the software installed in the camera.

Stomata number and length were determined according to Tear *et al.* (1971). In order to count the stomata density, a thick layer of clear nail polish was applied on the upper epidermis of each leaf. Then, the nail polish was allowed to dry. After that, a piece of clear tape was firmly stuck to the section of nail polish and then carefully peeled away from the leaf, leaving a leaf impression. The impression was then placed on a slide and viewed under 10 × 40 magnification with a light microscope. Stomatal density was calculated by counting the number of stomata on the leaf epidermis in a 10 × 40 microscopic field.

Grain yield and seed number per spike were obtained from the innermost rows of each plot at maturity. Samples were oven-dried at 70 °C for at least 72 h to obtain a constant weight.

Anatomic features of spike axis and grain weight

The central axis of the spike, rachis, between spikelets 4 and 8 was selected to study histological and grain weight changes affected by *Azospirillum* and wheat species. There is a gradient of size and maturity along the ear, with the largest and most advanced spikelets situated on the mid-part of the ear. Under unfavorable growing conditions, the lowermost spikelets and those at the top may be poorly developed and devoid of fertile florets. The spike axis was carefully freed from the spikelets and then samples were prepared by cutting with a razor blade. The method utilized for measuring the anatomical characters was similar to the procedure used for the flag leaf.

Statistical analyses

Analysis of variance of the factors (*Azospirillum* and wheat species) and their interaction was carried out using the SAS software (SAS Institute 2008). Means were compared by the least significant difference method at the 5% probability level.

Results

Stomata number and length

Based on the analysis of variance in Table 1, the effects of *Azospirillum* and wheat species were significant for both length and number of leaf stomata. However, *Azospirillum* × wheat species interaction was only significant for length of stomata but not for stomata number. Stomata number was higher in bread wheat as compared to durum wheat (Table 2). In contrast, length of stoma was higher for durum wheat than that of bread wheat (Table 2). *Azospirillum* inoculation decreased the number of stomata by 11.5% irrespective of the wheat species (Table 3). On the other hand, *Azospirillum* increased the length of stomata by 3.6 and 11.5% for bread wheat and durum wheat, respectively. *Azospirillum* enhanced the stomatal length of bread wheat much more than that of durum wheat (Figure 1).

Xylem and phloem vessels

The effect of *Azospirillum* was significant for the area and length of xylem and phloem vessels. However, the effect of wheat species and the interaction of *Azospirillum* × wheat species were not significant (Table 1). The length and area of xylem and phloem were higher in the inoculated plants as compared to the non-inoculated control plants (Table 3). *Azospirillum* inoculation significantly increased the area and length of xylem and phloem vessels by about 12.5 and 12.39% and 14.42 and 33.33%, respectively (Table 3).

Bundle sheath and mesophyll tissue area

The effect of *Azospirillum* on bundle sheath area was significant, however, the effects of wheat species and *Azospirillum* × wheat species interaction were not significant (Table 1). The area of bundle sheath of the inoculated plants increased up to 20% as compared with the non-inoculated control plants (Table 3).

Area of the mesophyll tissue was significantly affected by *Azospirillum* and wheat species and their interaction (Table 1). Area of the mesophyll tissue for durum wheat was higher than that of bread wheat (Table 2). Area of the mesophyll tissue was increased 31.5% following the *Azospirillum* inoculation (Table 3). *Azospirillum*-inoculated bread wheat had the highest area of the mesophyll tissue in the flag leaf (Figure 2).

Upper and lower epidermis area

The effects of *Azospirillum* and *Azospirillum* × wheat species interaction on lower epidermis area were significant. However, none of these sources of variation were significant for the upper epidermis area (Table 1). Furthermore, the upper epidermis area, but not lower epidermis area, was significantly affected by the type of wheat species. (Table 1). Areas of the upper and lower epidermis layers were significantly higher for bread wheat than those of durum wheat (Table 2). *Azospirillum* inoculation enhanced the area of the lower epidermis layer in both wheat species, however, it was more pronounced in bread wheat

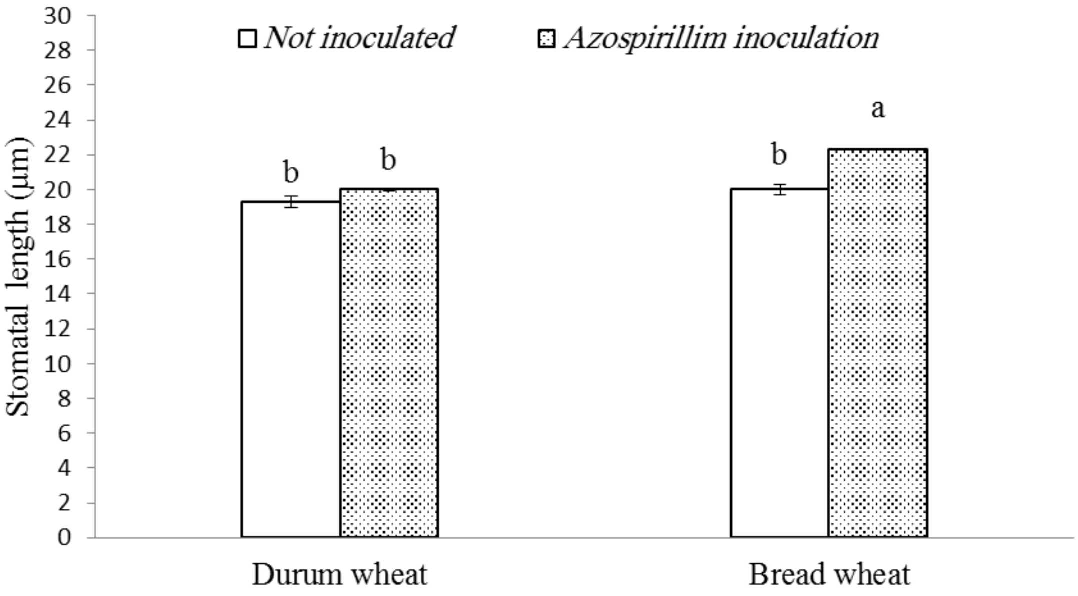

Figure 1. Effect of *Azospirillum* inoculation on stomatal length of the flag leaf in bread and durum wheats. Means followed by the same letter do not differ at $p \leq 0.05$ based on the least significant difference method.

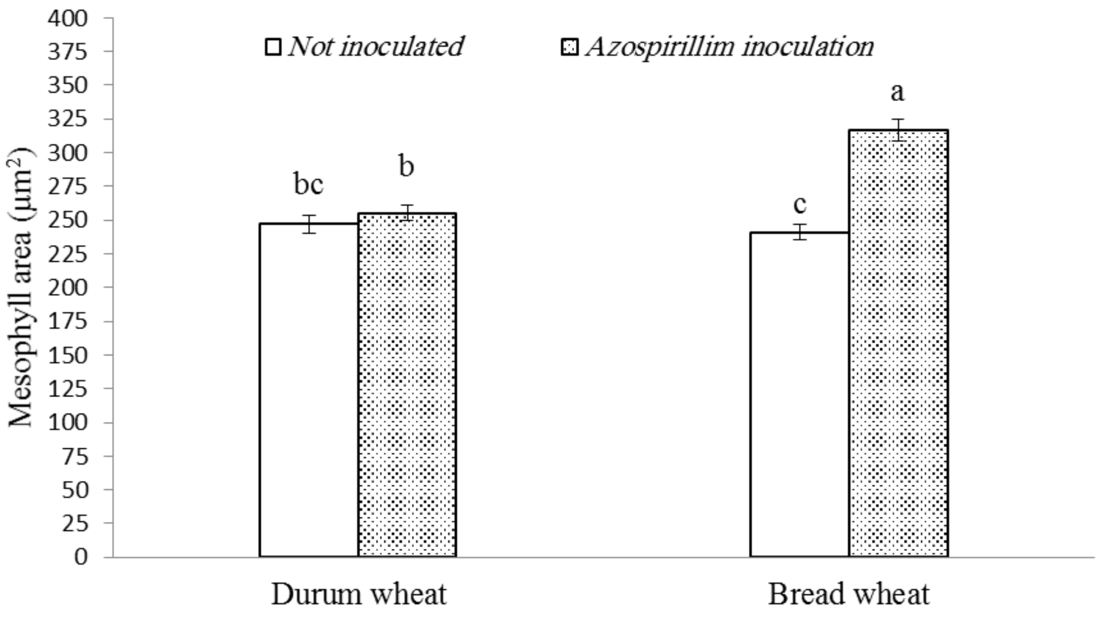

Figure 2. Effect of *Azospirillum* inoculation on mesophyll area of the flag leaf in bread and durum wheats. Means followed by the same letter do not differ at $p \leq 0.05$ based on the least significant difference method.

Table 1. Analysis of variance for the effects of *Azospirillum* inoculation and wheat species (bread and durum wheat) on stomatal density, stomatal length and flag leaf histological features

Sources of variation+	df	Stomata Density	Stomata Length	Xylem Area	Xylem Length	Phloem Area	Phloem Length	Vascular bundle sheath	Mesophyll area	Upper epidermis area	Lower epidermis area
Wheat Species (WT)	1	27^{**}	6.75^{**}	40^{ns}	1.5^{ns}	1.69^{ns}	0.02^{ns}	18.7^{ns}	2324^{**}	7550^{**}	1073^{**}
Azospirillum (Az)	1	3^{*}	6.75^{**}	2640^{**}	5.64^{**}	42.2^{**}	1.01^{**}	432^{*}	5250^{**}	494^{ns}	2067^{**}
WT×Az	1	0.01^{ns}	2.08*	533^{ns}	1.18^{ns}	0.19^{ns}	0.02^{ns}	0.01^{ns}	3434^{**}	2.1^{ns}	697^{**}
Error	6	0.25	0.19	184	0.33	0.92	0.01	56.9	34	297	26.3
CV (%)		5	2	5	3	6	3	11	2	11	3

Ns: Not significant; * and **: Significant at 5% and 1% probability levels, respectively.
+Blocks are not included in the table; CV: Coefficient of variation

Table 2. Stomatal density, stomatal length and leaf histological features of the bread and durum wheat species under study

Inoculation	Stomata Density	Stomata Length	Xylem Area	Xylem Length	Phloem Area	Phloem Length	Vascular bundle sheath area	Mesophyll area	Upper epidermis area	Lower epidermis area
Control	10±0.69a	19±0.2b	234±4b	16±0.2b	12±0.5b	4.02±0.07b	59±2b	244±4b	138±14a	120±2b
Azospirillum	9.5±0.67b	21±0.5a	263±8a	18±0.4a	16±0.4a	4.6±0.04a	71±4a	286±14a	151±14a	146±8a

Means followed by the same letter in each column do not differ at $p \leq 0.05$ based on the least significant difference method

Table 3. Stomata density, stomata length and histological features of flag leaf in bread and durum wheat species as affected by *Azospirillum* inoculation

Species	Stomata Density	Stomata Length	Xylem Area	Xylem Length	Phloem Area	Phloem Length	Vascular bundle sheath area	Mesophyll area	Upper epidermis area	Lower epidermis area
Bread wheat	11.5±0.3a	19±0.2b	247±4a	17.2±0.3a	14.2±0.1a	4.2±0.2a	66±5a	251±4b	120±4b	123±3b
Durum wheat	8.5±0.2b	21±0.5a	250±12a	18±0.5a	15±0.9a	4.3±0.1a	64±3a	279±17a	170±12a	142±9a

Means followed by the same letter in each column do not differ at $p \leq 0.05$ based on the least significant difference method

(Figure 3). *Azospirillum* increased the area of the lower epidermis by about 33.6 and 9.32% for bread wheat and durum wheat, respectively. Figure 4 represents anatomic features of two wheat species as affected by the *Azospirillum* inoculation.

Grain weight and anatomic features of the spike axis

The effect of *Azospirillum* and wheat species on the xylem area and phloem area of the spike rachis (between spikelets 4 and 8) were significant, however, the effect of *Azospirillum* × wheat species interaction on these characters was not significant (Table 4). Figure 5 represents the rachis anatomic features of two wheat species in response to *Azospirillum* inoculation. Area of the xylem and phloem was higher for bread wheat than that of durum wheat (Table 5). Irrespective of wheat type *Azospirillum* enhanced area of both xylem and phloem of spike rachis by about 24.8 and 48.2%, respectively (Table 5).

The effect of *Azospirillum* and wheat species on vascular bundle area was significant, but their interaction was not significant (Table 4). Spike axis of bread wheat had higher area of

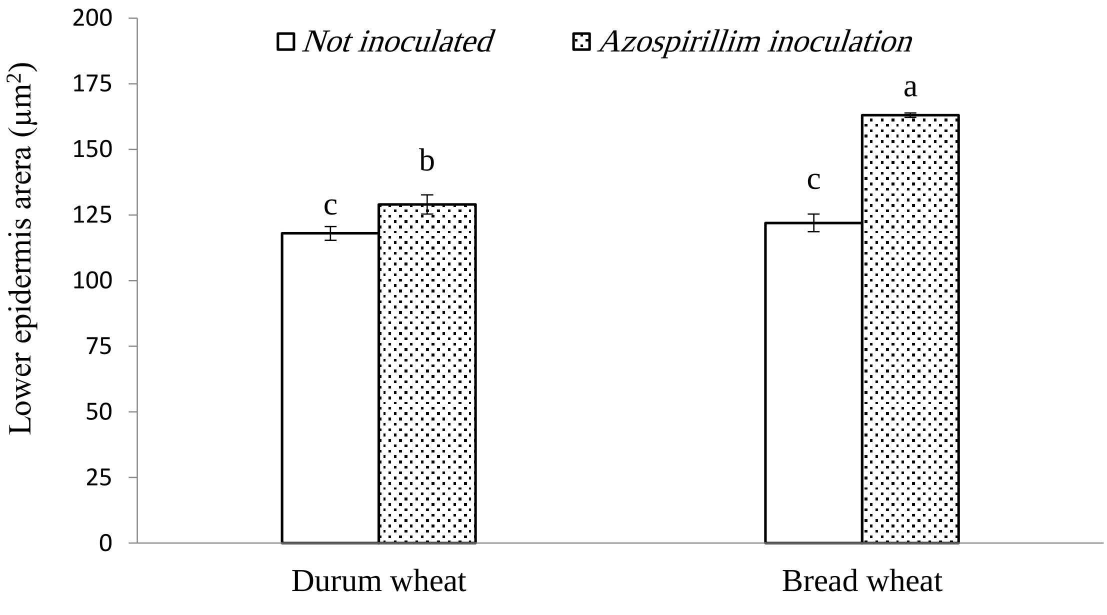

Figure 3. Effect of *Azospirillum* inoculation on lower epidermis area of the flag leaf in bread and durum wheats. Means followed by the same letter do not differ at p≤ 0.05 based on the least significant difference method.

Table 4. Analysis of the variance of the effects of microbial inoculation and wheat species (bread and durum wheat) on grain weight of spikelets 4 to 8, grain number per spike, grain yield, xylem area and phloem area of the spike rachis

Sources of variation	df	Rachis xylem vessel area	Rachis phloem vessel area	Grain weight of spikelet 4 to 8	Grain number per spike	Grain yield
Wheat species (WS)	1	103230**	220**	0.108**	97.4**	1302*
Azospirillum (Az)	1	27360**	568**	0.036*	154**	2054*
WS × Az	1	0.75ns	32.01ns	0.001ns	0.27ns	1800*
Error	6	553	7.38	0.004	4.2	191
CV (%)		5	7	17	6	9

Ns: Not significant; * and **: Significant at 5% and 1% probability levels, respectively. +Blocks are not included in the table; CV: Coefficient of variation

vascular bundle than that of durum wheat (Table 5). *Azospirillum* increased the vascular bundle area irrespective of the wheat species (Table 5). *Azospirillum* increased the vascular bundle area by 27.9%.

Grain yield, grain weight and kernel number per spike

Analysis of variance for the grain weight of seeds, between spikelets 4 and 8, and also kernel number per spike indicated the significant effect of *Azospirillum* and wheat species on these traits. However, interaction of the two factors was not significant for both characters (Table 4). Grain weight of bread wheat was higher than durum wheat (Table 5). Inoculated plants positively responded to *Azospirillum* and produced seeds with higher weight at the central spike axis (Table 5). The average grain weight of central spike axis increased by 27.9% following *Azopsirillum* inoculation, irrespective of the wheat species. Furthermore, durum wheat produced higher number of kernels per spike (18.4%) than bread wheat. *Azospirillum* led to the enhanced kernel number per spike by about 23.3% irrespective of the wheat species (Table 5).

The effect of *Azospirillum,* wheat species and their interaction were significant for the grain yield (Table 4). Treatment combination of wheat species with *Azospirillum* for grain yield is shown in Figure 4. The increase in grain yield due to inoculation by *Azospirillum* was more pronounced in bread wheat as compared with the *Azospirillum* inoculated durum wheat (Figure 6).

Discussion

In this study, wheat was grown under dryland farming conditions. Rainfall during the growing season of wheat was irregular and inadequate. During some growth stages, wheat plants experienced drought stress. The yield was low in the cropping season because of low rainfall, especially at the tillering, stem elongation and grain-filling periods.

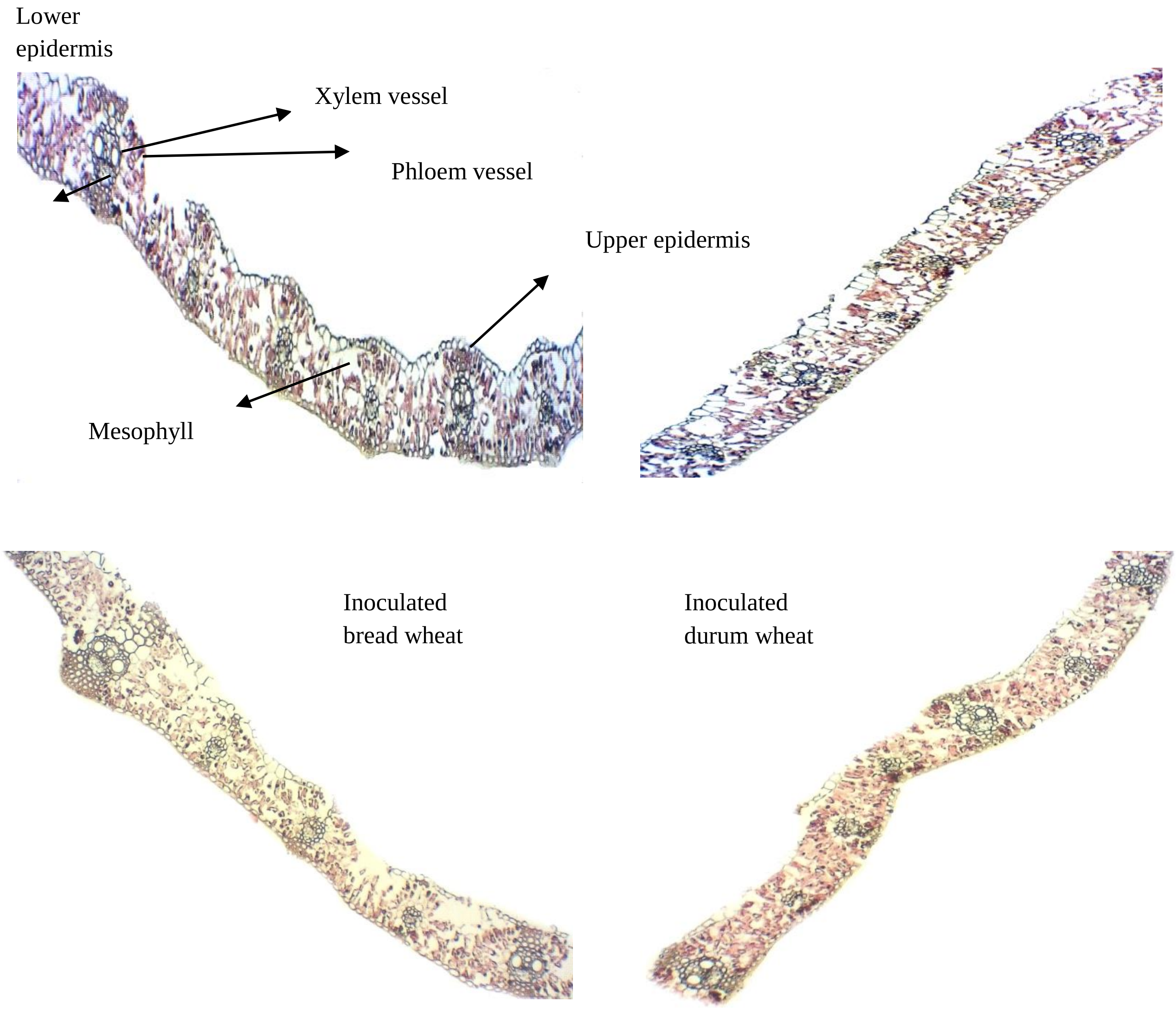

Figure 4. Comparative leaf anatomy for blade cross-sections (cutting from the base of photosynthetically active zone; >60 mm above the leaf) of flag leaf in *Azospirillum* inoculated and non-inoculated wheat species bread and durum wheat base) of wheat

Table 5. Effect of *Azospirillum* inoculation on the grain weight of spikelets 4 to 8, xylem area and phloem area of the spike rachis in bread and durum wheats

Treatment	Rachis xylem vessel area	Rachis phloem vessel area	Grain weight (spikelets 4 to 8)
Species			
Bread wheat	522±21a	39.8±4a	0.50±0.04a
Durum wheat	337±23b	31.2±3b	0.31±0.02b
Azospirillum inoculation			
Inoculated	477±41a	42.4±3a	0.45±0.05a
Not inoculated	382±43b	28.6±1b	0.34±0.03b

Means followed by the same letter for each column and each factor do not differ at p≤ 0.05 based on the least significant difference method

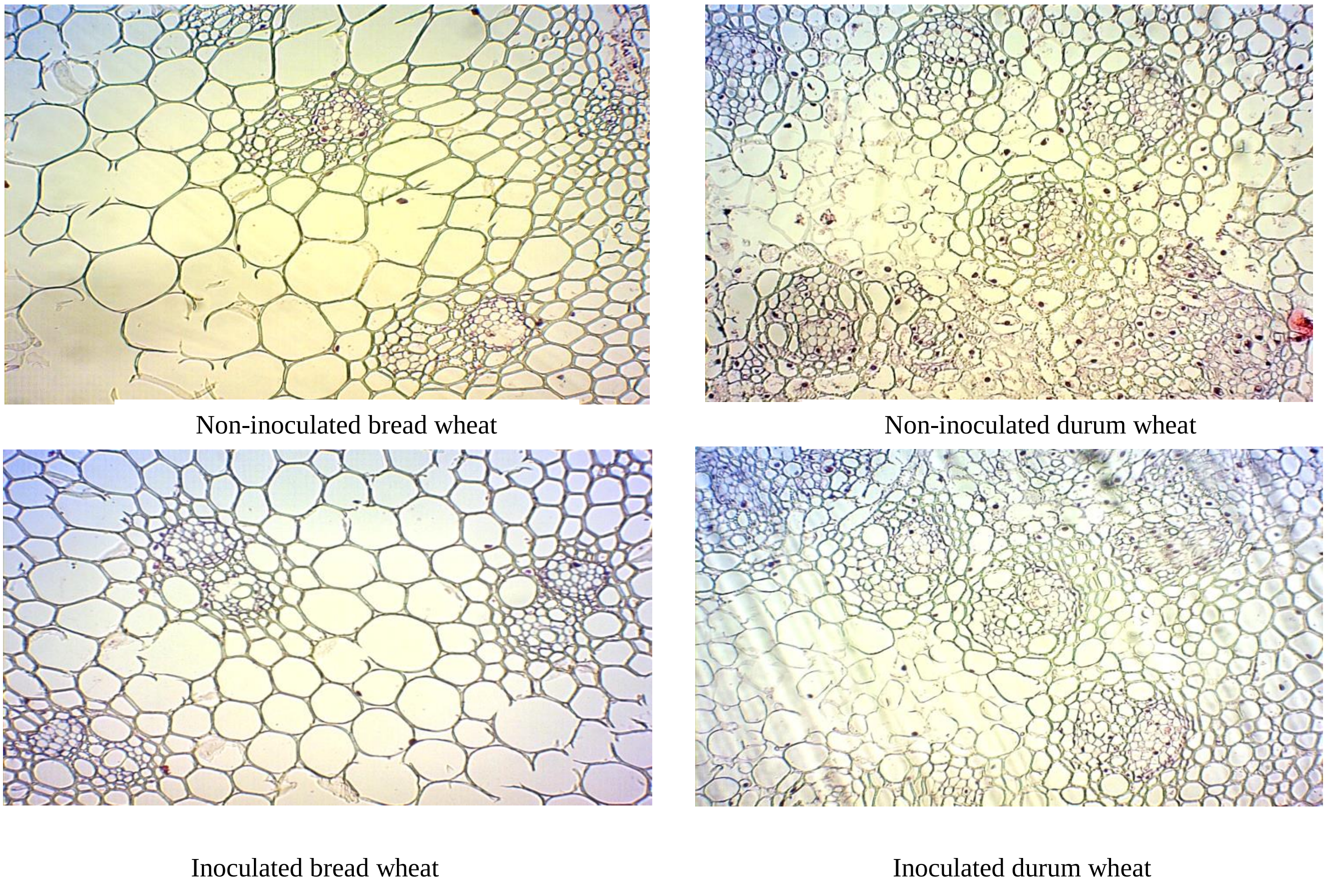

Figure 5. Comparative spike anatomy of rachis in non-inoculated and *Azospirillum* inoculated bread and durum wheat specie

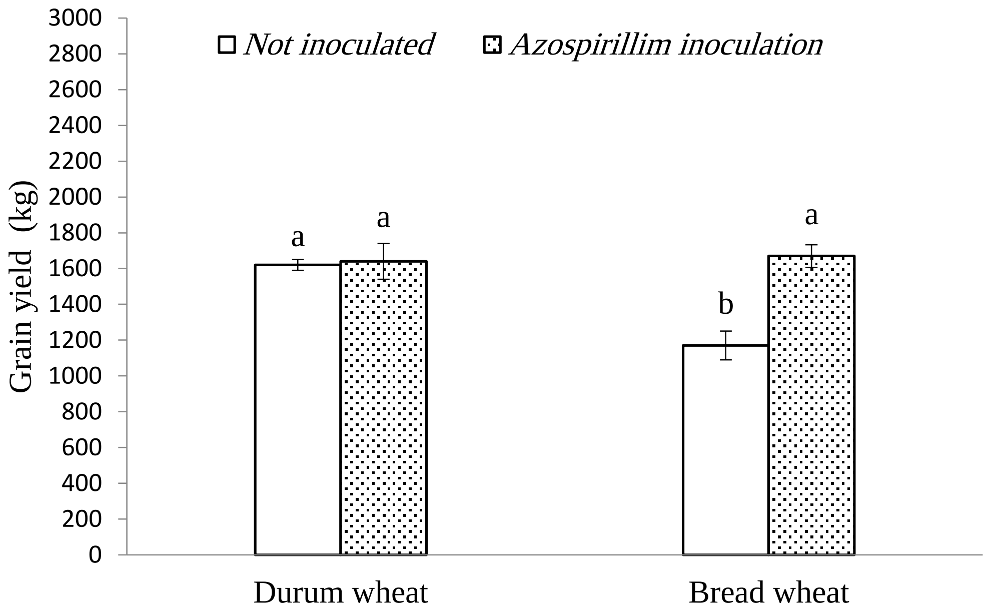

Figure 6. Effect of *Azospirillum* inoculation on the grain yield of bread and durum wheat species. Means followed by the same letter do not differ at $p \leq 0.05$ based on the least significant difference method.

Our results showed the advantage of *Azospirillum* inoculation on increasing the grain yield, especially in bread wheat as compared to durum wheat. Previous studies indicated various mechanisms by which bacteria of the genus *Azospirillum* improves plant growth, dry weight, total nitrogen content and grain yield of cereals. (Bashan and Holguin 1997; Bashan *et al.* 2004). It has also been suggested that other factors such as plant growth promoting substances produced by *Azospirillum* can contribute to the yield increase (Tien *et al.* 1979). Furthermore, addition of *Azospirilum* can increase the uptake rate of nutrients by the crops such as wheat (Haji Nia *et al*. 2012; Zarea *et al.* 2012). Based on our study it can be suggested that *Azospirillum* may enhance grain yield through the improvement of the anatomical features of the wheat plant.

In this study we investigated the anatomic features of both flag leaf and spike axis to *Azospirillum* inoculation in bread and durum wheats. Flag leaf is one of the utmost important leaves in cereals providing assimilates to the seeds. Also, stomta have vital role in plants under drought stress (Chaves *et al.* 2009). One of the main strategies adapted by plants under water deficiency is to lower both density and length of stomata in order to decrease the water loss through transpiration (Belhadj *et al.* 2011) which in return leads to decrease in carbon dioxide diffusion and eventually decrease in the photosynthetic process (Galbiati *et al.* 2011). In our study *Azospirillum* increased the area of flag leaf by about 17%, irrespective of wheat species. Also, stomatal length in both inoculated plants of the bread and durum wheats was higher than that of non-inoculated plants which suggests the beneficial effect of *Azospirillum* on plants under dryland farming. Water deficiency leads to a decrease in leaf stomata number (van de Roovaart and Fuller 1935). Lower stomata density under dry condition is related to the decrease in leaf area (Blum *et al.* 1981). Adequate available water increases the density of the stomata, while under water deficit or drought stress, plants tend to have a lower stomatal density per area of leaf (van de Roovaart and Fuller 1935). In our study, flag leaf area and consequently stomata number per area of the flag leaf in the inoculated plants were higher than those of non-inoculated plants. Higher density and length of stomata in the inoculated plants with *Azospirillum* showed that this bacteria improved water absorption and water content of plants under study.

Under drought stress most anatomic features of the leaf are changed (Ghanem 2008) in order to assist the plant to better adapt itself to drought condition. These changes mainly have adverse effect on the net photosynthetic rate (Bertamini *et al.* 2007). In our study, *Azospirillum* inoculated plants had larger xylem and phloem area as compared with non-inoculated plants. Larger xylem diameter possibly leads to the better maintenance of leaf hydraulic conductance under drought stress. It was also reported that greater xylem area is associated with the ability to maintain functional conductance under stress, ensuring better water potential and stay-greenness (Oosterhuis and Wullschleger 1987). The diameter of phloem sieve tubes is a key factor in determining the flow rate of photo assimilates from leaves to the sinks (Fitter and Hay 2002).

Furthermore, the results showed the enhancement in the area of the bundle sheath, mesophyll tissue and lower epidermis layers in *Azospirillum*-inoculated plants as compared with non-inoculation plants. A greater stomata number per unit area, smaller stomata, smaller epidermal cells, thicker cuticle and a greater number of layers of smaller mesophyll cells are some of the anatomical characteristics representing adaptations to water deficit conditions (Merkulov *et al.* 1997). Since bundle sheath cells are not photosynthetically active, increasing their size in the cost of reducing mesophyll cell numbers may decrease the photosynthetic capacity of the leaf (McClendon 1992). However, the result of the present study showed that the increase in vascular bundle area was accompanied by the higher area of the mesophyll tissue.

Area of the xylem and phloem and vascular bundle area were higher for bread wheat than those of durum wheat. Irrespective of wheat species, *Azospirillum* enhanced the area of xylem, phloem and vascular bundle tissue of the spike rachis by about 24.8, 48.2% and 27.9%, respectively. The diameter of phloem sieve tubes determine the flow rate of photo-assimilates from leaves to the sinks (Fitter and Hay 2002). Enhanced area of the xylem and phloem and vascular bundle area was found to be a useful change since average grain weight of the central spike axis increased by 27.9% following *Azopsirillum* inoculation, irrespective of wheat species.

As indicated above, *Azospirillum* had various beneficial effects. However, results of this study elucidated another important role of *Azospirillum* for modifying the anatomic features of both flag leaf and spike rachis that can be important in the agronomic crop management under dryland farming.

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