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RESEARCH ARTICLE

Hydrogen Peroxide Affects Plant Growth Promoting Effects of *Azospirillum*

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Abstract

This study aimed to investigate if pretreatment seed with H_2O_2 affects promoting effect of *Azospirillum* on plant. The experiment was a factorial combination of two main factors, *Azospirillum* and hydrogen peroxide (0, 25, 50, and 80%), carried out under field conditions. Soaking seed in various concentrations of H_2O_2 solution resulted in enhanced stomata density and increased length and histological components of leaf. These changes in histological components were found to be positive since plants of H_2O_2-soaked seeds (H_2O_2 pretreatment) had higher fresh and dry weights, seed number per spike, and higher grain yield. *Azospiriilum* enhanced number of grain spikes^{-1} and total grain yield by 3% and 7%, respectively. Soaking seeds in 25% H_2O_2 solution enhanced the promoting effect of *Azospirillum* on increased vessels area and diameter. Soaking seeds in 50% H_2O_2 solution resulted in enhanced stimulating effect of *Azospirillum* on enhanced areas of vascular bundle and upper epidermis. The promoting effect of *Azospirillum* on the area of lower epidermis was largest when seed was pretreated with 80% H_2O_2. All three concentrations of H_2O_2 used had the same effect on *Azospirillum*–inoculated plant in terms of leaf area and chlorophyll *a*. Soaking seed in 80% H_2O_2 solution and then inoculated with *Azospirillum* resulted in the highest grain yields.

Key words : *Azospirillum,* hydrogen peroxide, leaf anatomy, wheat

Introduction

Seed priming is a pre-sowing strategy to influence seed germination and seedling development by modulating pre-germination metabolic activity prior to emergence of the radicle and generally enhances germination rate and plant performance (Bradford 1986; Taylor and Harman 1990). Hydrogen peroxide is often used as a disinfectant at wound sites (Halliwel and Gutteridge 1999). The role of hydrogen peroxide in plant biochemistry and physiology, and various functions of H_2O_2 in plants have been described in many review papers (Apel and Hirt 2004; Hung et al. 2005; Kuzniak and Urbanek, 2000; Neill et al. 2002a, 2002b). It is well known that hydrogen peroxide plays a vital role in the response to both abiotic and biotic stresses in plants (Slesak et al. 2007). In contrast to animal cells, plants seem to be much more resistant to high concentrations of H_2O_2. Experiments with plant material have demonstrated that plant tissues can tolerate high concentrations of H_2O_2 in the range 10^2-2×10^5 μM. Moreover, plants pre-treated with H_2O_2 were more resistant to excess light and chilling stresses (Karpinska et al. 2000; Karpinski et al. 1999; Prasad et al. 1994; Yu et al. 2003). It has been suggested that H_2O_2 could be an early electron donor to PSII (Olson and Blankenship 2004). Hydrogen peroxide regulates other hormonal responses in plants, therefore it can play an imperative role as a 'master hormone' (Slesak et al. 2007). Genetic systems controlling H_2O_2 signalling in plants have been suggested (Mateo et al. 2004, 2006).

In this study we used saline-adapted *Azospirillum* strains isolated from a saline area of southern Iran which were previously described to enhance wheat growth and yield under non-stressed and stressed conditions (Haji Nia et al. 2012; Zarea et al. 2012, 2013). The *Azospirillum* genus has been

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studied as plant growth-promoting rhizobacteria (PGPR; Glick et al. 1999). *Azospirillum* spp. are widespread colonizer bacteria that have been isolated from the root surface or the rhizosphere of a wide variety of plant species (Umali-García et al. 1980). In general, inoculation with PGPB can enhance germination, seedling emergence, and modify growth and yield of various cereal and non-cereal crops (Zahir et al. 2004). Regarding *Azospirillum*, the most researched associative bacterium (Bashan and Holguin 1997), stress conditions appear to emphasize its growth-promoting effects on plants (Barassi et al. 2000).

It is frequently assumed that *Azospirillum* enhances crop tolerance to stress. However, the positive response of various crops to plant growth-promoting bacteria under non-stressed and stressed conditions have been well established but there is no research ≠ comparing the leaf anatomical performance of *Azospirillum*-inoculated plants under field conditions and in response to seed pretreament with H_2O_2.

Most studies carried out under controlled conditions and in sterilized soils demonstrated a pronounced beneficial effect of *Azospirillum* inoculation on enhanced growth plants. It seems that under greenhouse conditions, introduced *Azospirillum* receives less environmental stress and the bacterial competition between the introduced bacteria and indigenous bacteria is lower than under field conditions. 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 20 years of field experiment data 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, *Azospirillum* conclusions, proper agronomical practices, and using indigenous efficient species may be also lead to more success of *Azospirillum* performances under field conditions.

The objectives of this study were to investigate to what extent the effects of *Azosperillum* under field conditions are affected by (i) soaking seeds with hydrogen peroxide on leaf anatomic features and grain yield of bread wheat and (ii) pre-soaking seed with hydrogen peroxide.

Material and Methods

Experimental location and field experiment

Experimental location was located in a semi-arid area in western Iran. Semi-arid areas of Iranaian cropland area are characterized by having constitutive stresses. In these areas, water and high-temperature occur at any life cycle of the plant from sowing to maturity, mainly in the terminal part of the growing season. Irregular and low precipitation are characteristics of these areas. Rainfall in these areas is a major concern limiting crop production. The field experiment was conducted at Ilam university research farm (37 °33" L, 33 °28" W, 1174 m above sea level) during the 2014-15 growing season. The soil was classified as clay-loamy. Recently the 30-yr-average annual precipitation was 600 mm. The experimental soil texture was loamy clay with pH 7, electrical conductivity 0.3 dS m^{-1}, 1.83% of organic matter, N 0.18%, available phosphorus 7 ppm, potassium 590 ppm, Fe 6.6 ppm, Mn 7.9 ppm, Cu 0.63 ppm, and available Zn 1.08 ppm. Rainfall during the growing season totaled 360 mm. The sowing date was November 9, 2015. Seed-bed preparation was made in the autumn when soil moisture was adequate for seed germination. Seed planting was made following the first rain in autumn. The amount of rain in these areas must be sufficient for adequate moisture for the seed to germinate. The experiment was a factorial combination of two main factors (*Azospirillum* and hydrogen peroxide) in complete randomized blocks, with three replications. Treatments included + *Azospirillum* (inoculated), -*Azospirillum* (non-inoculation), four concentrations of hydrogen peroxide (0, 25, 50, and 80%), and the combination of various levels of hydrogen peroxide with *Azospirillum*. Plot dimensions were 3.0 x 1.2 m consisting of six rows with a 20-cm-row space. Wheat was planted at 250 seeding rates m^{-2}. 40 kg P_2O_5 ha^{-1}, and 75 kg N ha^{-1} as fertilizer were applied with the seed at planting. Nitrogen and P_2O_5 fertilizers were side-banded at seedling at rates according to recommendations for dryland wheat production in these areas. Whole phosphorous was applied at seeding.

Hydrogen peroxide solution

Surface-sterilized seeds were first soaked in H_2O_2 for 8 h and then inoculated with the *Azospirillum.* Hydrogen peroxide concentrations of 0, 25, 50, and 80% were prepared with distilled water. Seeds of bread wheat c.v. *Kross sabalan* were pre-germinated after soaking for 8 h in either fresh water or in the required aqueous hydrogen peroxide solution of 0, 25, 50, and 80%. Seeds were surface-dried in the shade to facilitate clump-free sowing.

Azospirillum inoculation

Previously, hydrogen peroxide-soaked seeds and control seeds were inoculated by immersion in a total imbibition volume of autoclaved *Azospirillum* inoculants (control) or bacterial inoculants containing 10^7 bacterial cells $seed^{-1}$ for 1 h.

Leaf anatomical measurements

Leaf anatomy measurements were obtained when plants were at the tillering growth stage. At this growth stage, fresh and dried weights were also determined. For leaf anatomy studies, the top first leaves of inoculated and non-inoculated plants were randomly selected from each individual plot. The photo-synthetically active zone (> 60 mm above the leaf base, Hu et al. 2000) was chosen for leaf anatomical studies. Leaves were carefully freed from leaf sheaths and then cut from the stem at the base of the leaf blade. The blade was cut with a razor blade. Photosynthetically active segments were then prepared, cutting from the base of photosynthetically

active zone (> 60 mm above the leaf base). Leaf pieces were fixed for 48 h in 10% formalin solution. Tissue dehydration was carried out in an alcohol series. The leaf segments were then dehydrated in an alcohol series (30, 50, 70, 100%). samples were dehydrated once with 30% ethanol, once with 50% ethanol, once with 70% ethanol, once with 90% ethanol, and then twice with 100% ethanol. After this period, 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. Semithin 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 examinations were performed on five images randomly taken from each slide. Images were captured using an eye-piece digital camera, Dino-eye camera (AM423). All measurements were done using the software of the used camera (Dino-eye camera).

The estimation of stomata number and length methods described by Tear et al. (1971) as follows. The method to count stomata densities began with the application of a thick layer of clear nail polish to the upper epidermis of each leaf. The nail polish was allowed to dry. A section 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. Stomata density was calculated by counting the number of stomata on the leaf epidermis in a 10 × 40 microscopic field.

Dry matter weights were obtained from six plant samples of each plot at the tillering growth stage. Plant samples were oven-dried at 70°C for at least 72 h to a constant weight.

Leaf relative water content, leaf area and photosynthetic pigments contents

Leaf relative water content was measured when plants were at the tillering growth stage. The following equation was used to estimate the water content of leaf.

$$RWC\,(\%) = [(FM - DM)/(TM - DM)] \times 100$$

Where, FM is fresh weight, DM is dry weight, and TM is weight of turgid leaf samples. A leaf sample was made up of nine leaves. Leaf area (cm^2) was measured by scanning photocopies of 12 leaves, senescent or damaged leaves were avoided, randomly sampled from each plot and analyzing the images with Scion Image software (Scion Corporation, Frederick, Maryland). Photosynthetic pigments (Chlorophyll a and b and carotenoid) concentrations were measured on fresh fully expanded leaves as described by Lichtenthaler and Wellburn (1983). Fresh tissue (0.25 g) was extracted with 90% acetone, and read using a UV/visible spectrophotometer at 663, 646 and 450 nm wavelengths. Chlorophyll *a*, chlorophyll b, and carotenoid concentrations were calculated using the formulae:

$$Chlorophyll\ a\ (\mu g\ ml^{-1}(= 12.21(A_{663}) - 2.81(A_{646})$$

$$Chlorophyll\ b\ (\mu g\ ml^{-1}) = 20.13(A_{646}) - 5.03(A_{663})$$

$$Carotenoids = [(1000A_{470}{}^{-1}.8\ Chl.\ a-85.02\ Chl.\ b) \div 198]$$

Catalase and ascorbate peroxidase

Catalase and ascorbate activities were measured as according to the methods described by Velikova et al. (2000) and Nakano and Asada (1981), respectively. The initial rate of the disappearance of hydrogen peroxide was measured. To prepared the catalase assay reaction mixture, 10 mM potassium buffer (pH 7.0), with an appropriate aliquot (100 μl) of enzyme extract, and 33 mM hydrogen peroxide were used. Hydrogen peroxide was assayed from the decrease in absorbance in optical density at 240 nm, and the activity was calculated using the extinction coefficient of 40 mM cm^{-1} of hydrogen peroxide. Ascorbate peroxidase was assayed from the decrease in absorbance at 290 nm (an absorbance coefficient of 2.8 mM^{-1} cm^{-1}) as ascorbate was oxidized.

Total grain yield

A 1 m^{-2} of central each plot was chosen as total grain yield. Plants were separated into spikes and stems. Grain yield and seed number per spike were determined at grain maturity. From each treatment a bundle sample was cut from an area of 1 m^{-2}. All samples were cut from the middle rows to avoid border effects. Before threshing the bundles, six spikes were randomly selected from each bundle and each spike was threshed separately by hand. The seed number of each spike was then measured and recorded. Finally, the average grain yield were measured and expressed as kg h^{-1}.

Statistical analysis

Differences among treatments were analyzed for main effects (hydrogen peroxide and *Azospirillum*) and their interaction by a two-way ANOVA using the SAS software package. Treatment effects were considered significant at $P < 0.05$. L.S.D.s ($P < 0.05$) were used to compare means within and among treatments. Correlation coefficients were calculated between each pairs of various parameters.

Results

Statistical findings

Tables 1 and 2 show the summary of the two-way ANOVA of the effect of *Azospirillum* and hydrogen peroxide and their nitration effect on leaf histological component and photosynthetic pigment contents, activity rate of catalase and ascorbate enzymes and grain yield. *Azospirillum* and hydrogen peroxide as main effects and their interactions were significant in the final ANOVAs on most studied traits (Tables 1 and 2). ANOVAs showed that main effect of *Azospirillum* was significant on all studied leaf histological components at

Table 1. A two-way ANOVA of the effects of microbial-inoculation and hydrogen peroxide and their interaction effects on leaf area, stomata density, stomata length, and leaf histological component.

Sources of variation	df	LAI	Stomata		Xylem		Phloem		Vascular bundle sheath	Mesophyll	Upper epidermis	Lower epidermis
			density	length	Area	Length	Area	Length				
Azospirillum (AZ)	1	ns	ns	ns	*	*	**	*	*	**	ns	*
H_2O_2	3	**	**	**	*	**	**	**	**	*	**	**
$H_2O_2 \times AZ$	3	**	**	**	*	*	**	**	*	**	*	**
CV	-	5	5	3	12	5	6	4	8	6	3	4

^{ns}, not significant; * and **: significant at 5% and 1%, respectively.
CV, Coefficient of variance

Table 2. A two-way ANOVA of the effects of microbial-inoculation and H2O2 and their interaction effects on photosynthetic pigments (chlorophyll *a* and *b* and carotenoid content), fresh and dry weight of seedlings at tillering growth stage, seed number per spike, and grain yield.

Sources of variation	df	Pigments contents			Antioxidant activity		Weight		Seed per spike	Grain yield
		Chl *a*	Chl *b*	Carotenoid	catalase	Ascorbate	Fresh	Dry		
Azospirillum (AZ)	1	ns	ns	**	ns	ns	**	ns	**	*
H_2O_2	3	**	ns	*	*	**	*	**	**	**
$H_2O_2 \times AZ$	3	*	**	*	**	**	**	ns	**	*
CV	-	8	7	4	26	26	5	4	3	8

^{ns,} not significant; * and **: significant at 5% and 1%, respectively.
CV, Coefficient of variance

the 0.005 level or smaller, except for leaf area and stomata characteristics. Main effect of hydrogen peroxide was significant on all studied leaf anatomic features (Table 1). *Azospirillum* ´ hydrogen peroxide interaction for all studied histological components was also significant (Table 1). In contrast to *Azospirillum,* the main effect of hydrogen peroxide was significant on leaf area (Table 1). Table 2 summarized two-way ANOVAs of the main effect of *Azospirillum* and hydrogen peroxide and their nitration effect on photosynthetic pigments (Chlorophylls *a* and *b* and carotenoids), activity of catalase and ascorbate, plant dry and fresh matter and grain yield. Among photosynthetic pigments, only the carotenoid pigment was significantly ($P < 0.05$) affected by the main effect of *Azospirillum* (Table 2). The main effect of *Azospirillum* was not significant on activity of catalase and ascorbate (Table 2). Seed number spike^{-1} and grain yield significantly affected by the main effect of *Azospirillum* at the 0.005 levels or smaller (Table 2). When the data was analyzed, two-way interaction of hydrogen peroxide with *Azospirillum* was significant (at probability levels of 0.05 or smaller) in the case of photosynthetic pigments, catalase and ascorbate peroxidase activities, seedling fresh weight, seed number per spike, and grain yield (Table 2).

Obtained grain yield

The cropping season had a low yield (1100 kg ha^{-1} on average). This was likely because of low rainfall occurring during growth season, especially at the tillering growth stage, stem elongation, and grain-filling stage.

Azospirillum performances

Differences in the area of upper epidermis layer between *Azospirillum*-inoculated plants and non-inoculated plant were not observed. Length and density of guard cells were the same among inoculated and non-inoculated plants (control plants). Significant differences were observed in diameter and length of xylem. The greater diameter and area of xylem vessel were found in plants inoculated with *Azospirillum* (Table 3). Inoculating *Azospirillum* resulted in enhanced area and diameter of xylem vessel by 10 and 15%, respectively, as compared to control plants (Table 1). *Azospirillum* increased area and diameter of phloem tissue by 12.3 and 4.8%, respectively (Table 3). Plants of *Azospirillum*-inoculated seeds had a significantly ($P < 0.05$) higher area of mesophyll tissue (18.5%) than those of non-inoculated control seeds (Table 3). *Azospirillum* significantly ($P < 0.05$) enhanced areas of vascular bundle by 8.4% compared to non-inoculated plants (Table 3). Among photosynthetic pigments only carotenoid content was significantly ($P < 0.05$) affected by *Azospriilum* inoculation (Table 3). The rate of increase in carotenoid content of *Azospirillum*-inoculated plant leaf was 9.2% in compared to that of non-inoculated plant leaf (Table 3). Fresh weight of *Azospirillum*-inoculated plants was higher than that of non-inoculated plants (Table 3). *Azospiriilum* enhanced the number of grain spikes^{-1} and total grain yield by 3% and 7%, respectively, compared to the non-inoculated treatment.

H_2O_2 performances

Soaking seeds in various concentrations of H_2O_2 solution resulted in enhanced leaf histological components (stomatal density and length, xylem diameter and area, phloem area and diameter, mesophyll tissue area, and vascular bundle area) (Table 4). These changes in histological components were positive since plants of H_2O_2-soaked seeds had higher

Table 3. Mean stomatal density, stomatal length, xylem area and length, phloem area and length, vascular bundle sheath area, mesophyll area, upper and lower epidermis areas, carotenoid content, fresh and dry matter, seed number per spike and grain yield of inoculated and non-inoculated wheat with *Azospirillum* spp.

	Stomata		Xylem		Phloem		Vascular bundle sheath area (μm^2)	Mesophy ll area (μm^2)	Upper epider-mis (μm^2)	Lower epider-mis (μm^2)	Caroteno id	Fresh weight (g $plant^{-1}$)	Seed $spik^{-1}$	Grain yield (kg ha^{-1}
	density	Length (μm)	Area (μm^2)	density	Area (μm^2)	density								
Control	8.5 ± 0.5^a	24 ± 1.6^a	84^b	10^b	13^b	3.9^b	59^b	389^b	352^a	328^b	6.5^b	6.9^b	33^b	1832^b
Azospirillum	7.9 ± 0.5^a	25 ± 1.2^a	97^a	11^a	14.6^a	4.09^a	64^a	461^a	354^a	345^a	7.1^a	7.5^a	34^a	1961^a

Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

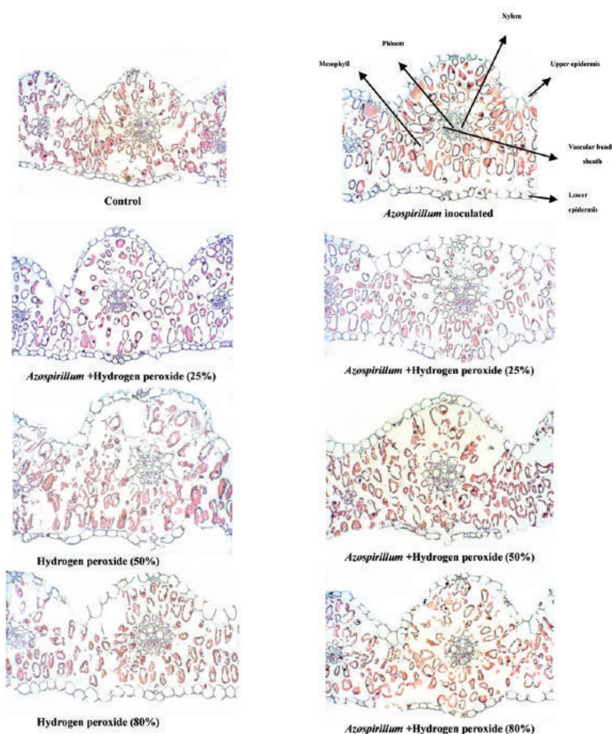

Fig. 1. Comparative leaf anatomy in blade cross-sections (cutting from the base of photo-synthetically active zone; > 60 mm above the leaf base) of wheat plant leaves of pre-soaked seeds in different peroxide hydrogen concentrations (0, 25, 50, and 80%) and inoculation with *Azospirillum* under dry land farming.

fresh and dry weights, seed number $spike^{-1}$, and grain yield than those of non-H_2O_2 primed seed. Soaking seed in 80% H_2O_2 solution was more effective in enhanced density (number) of stomatal, diameter and area of xylem, area and diameter of phloem and area of mesophyll tissue than other used H_2O_2 concentration (25 and 50%) (Table 5). Soaking seed in 50% H_2O_2 solution increased area of leaf, stomatal length, vascular bundle area, and upper and lower epidermis areas compared to other concentrations of H_2O_2 (Table 5). Soaking seeds in 80% H_2O_2 was more effective in increased photosynthetic pigments (chlorophyll *a* and carotenoid but not chlorophyll *b*) (Table 5). Activates of catalase and ascorbate peroxidase were more enhanced by 80% H_2O_2. Pre-soaking seed in 80% H_2O_2 increased seed number $spike^{-1}$ and grain yield more than other concentrations of H_2O_2 solution used (Table 5). All three concentrations of H_2O_2 had the same effect on increased fresh weight and on average enhanced plant fresh weight by about 10% compared to control plants. H_2O_2, especially at the concentration of 50%, increased total dry weight of plants compared to the control treatment (control). Soaking seed in 80% concentration of H_2O_2 had a more promoting effect on enhanced seed number $spike^{-1}$ and grain yield than other concentrations of H_2O_2. Soaking seed in 80% concentration of H_2O_2 resulted in enhanced number grain $spike^{-1}$ and total grain yield by 11% and 56%, respectively (Table 5).

H_2O_2 affecting *Azospirillum* performances

Fig. 1 shows comparative leaf anatomy in blade cross-sections of *Azospirillum*-inoculated and non-inoculated plant leaves of pre-soaked and non-pre-soaked seeds in different peroxide hydrogen concentrations (0, 25, 50, and 80%). The trend chaining pattern in leaf histological components of the plants in response to H_2O_2 soaking and inoculation with *Azospirillum* are shown in Figs. 2-7. H_2O_2 changed the performance effect of *Azospirillum* on length and density of stomata, diameter and area of xylem, areas of mesophyll, vascular bundle, and upper and lower epidermis tissues. The highest length and density of stomata were recorded in the leaf of plants of 50% H_2O_2-soked seeds and inoculated with *Azospirillum* (Fig. 2). Presoaking seeds in 25% H_2O_2 solution increased the promoting effect of *Azospirillum* on enhanced area and length of xylem (Fig. 3) and length of phloem (Fig. 4a). Pre-treating seeds with 50 and 80% H_2O_2 had the same effect on enhanced phloem area in plants inoculated with *Azospirillum* (Fig. 4b). Soaking seeds with H_2O_2 (25, 50, and 80%) solution negatively affected the promoting effect of *Azosperillum* on enhanced area of mesophyll tissue area (Fig 5a). Soaking seeds in 50% H_2O_2 solution increased induced effect of *Azospirillum* on enhanced vascular bundle area (Fig. 5b) and upper epidermis area (Fig. 6a). The most promoting effect of *Azospirillum* on enhanced area of lower epidermis layer was gained in plants of 80% H_2O_2- soaked seeds (Fig. 6b).

All three concentration levels of H_2O_2 had the same effect on promoted efficiency of *Azospirillum* on enhanced relative

Table 4. Mean leaf area, stomatal density, stomatal length, xylem area and length, phloem area and length, vascular bundle sheath area, mesophyll area, and upper and lower epidermis areas of soaking wheat seed into different concentrations of hydrogen peroxide.

Hydrogen peroxide	Leaf area	Stomata		Xylem		Phloem		Vascular bundle sheath	Mesophyll	Upper epidermis	Lower epidermis
		density	Length	Area	density	Area	density				
0%	5.8±0.19^{c}	8.5±0.34^{a}	21±0.54^{b}	77±3.4^{b}	9±0.25^{b}	9.8±1.4^{b}	3.4±0.24^{c}	57±4.3^{b}	438±49^{a}	314±7.9^{c}	301±8.07^{c}
25%	6.4±0.17^{b}	7.5±0.22^{b}	23±0.42^{a}	97±9.6^{a}	11±0.33^{a}	14±0.11^{a}	4.2±0.12^{a}	65±2.47^{a}	404±7.7^{b}	353±5.8^{b}	335±3.9^{b}
50%	7.1±0.23^{a}	7.5±0.48^{b}	23±0.42^{a}	90±6.2^{ab}	10.7±0.36^{a}	15±0.32^{a}	4±0.06^{b}	69±1.7^{a}	416±8.5^{ab}	406±8.5^{a}	374±10^{a}
80%	6.7±0.17^{ab}	8.5±0.22^{a}	22±0.25^{b}	97±5.3^{a}	11.3±0.25^{a}	15±0.27^{a}	4.3±0.11^{a}	57±0.76^{b}	441±14^{a}	341±4.08^{b}	336±16^{b}

Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

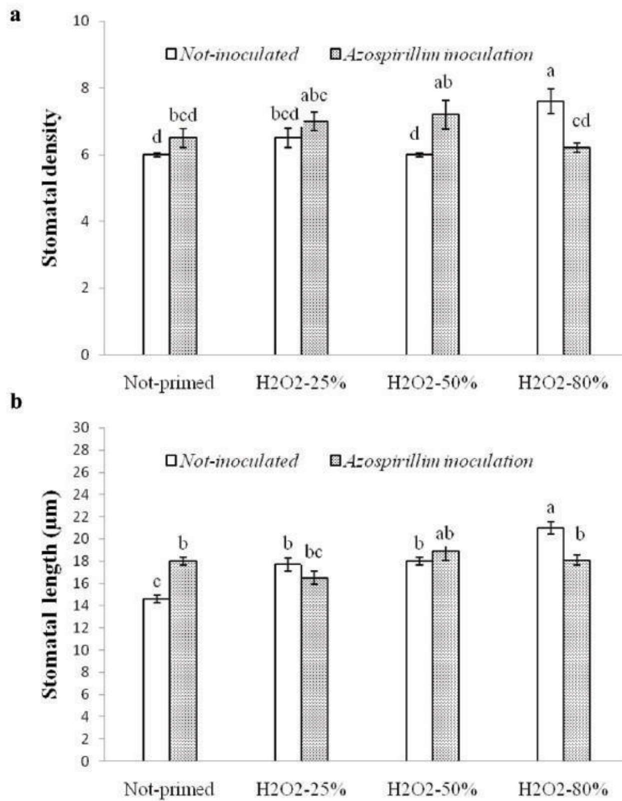

Fig. 2. Effect of soaking seed in 0, 25, 50, and 80% hydrogen peroxide solutions and *Azospirillum* inoculation on wheat leaf stomatal density (a) and stomatal length (b). Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

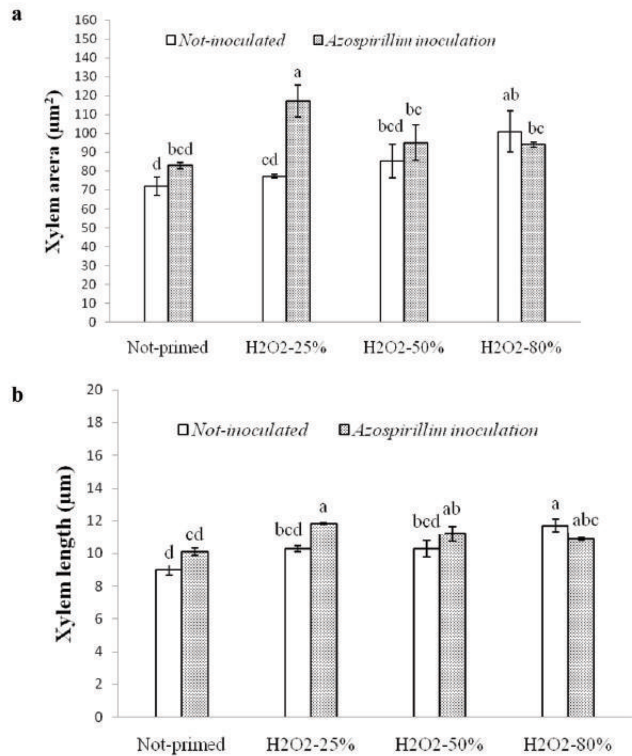

Fig. 3. Effect of soaking seed in 0, 25, 50, and 80% hydrogen peroxide and *Azospirillum* inoculation on xylem area (a) and xylem length (b) of wheat flag leaf. Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

water content of leaf, leaf area, and chlorophyll pigment of *a* (Table 6). Soaking seeds in 25 or 50% concentration of H_2O_2 and then inoculating with *Azospirillum* gained the highest content of chlorophyll *b* (Table 6). Soaking seeds in H_2O_2 did not lead to any significant advantages of *Azospirillum* in terms of carotenoid content, plant fresh weight, and activates of catalase and ascorbate (Table 6). The highest total grain yield of 2090 kg ha^{-1} was obtained from *Azospirillum* inoculation + 80% of H_2O_2 treatment (Table 6) .

Discussion

In this study, wheat was grown under dry land farming conditions. Rainfall during the wheat-growing season was irregular and inadequate. During some growth stages, wheat plants experienced drought conditions. Precipitation was low especially at the growth stage of stem elongation and grain filling. In this study, pre-sowing treatment of H_2O_2 eventually enhanced grain yield of wheat through modulation of leaf histological components, enhanced photosynthetic pigments, and induced activity of catalase and ascorbate peroxidase. *Azospirillum* inoculation also improved grain yield of wheat, and its promoting effect on enhanced grain yield was more pronounced when accompanied with H_2O_2 pretreatment. *Azospiriilum* also affected some features of leaf anatomic components. Many works have reported that exogenous application of H_2O_2 can improve seed germination in many plant species (Fontaine et al. 1994; Zeinalabedini et al. 2009). An acceleration of the germination process of pea

Table 4. Mean leaf chlorophyll pigments and antioxidant activity of catalase and ascorbate, fresh and dry matter, seed number per spike and grain yield of soaking wheat seed into different concentrations of hydrogen peroxide.

Hydrogen peroxide	Chlorophyll *a* (μg ml^{-1})	Chlorophyll *b* (μg ml^{-1})	Carotenoid	Antioxidant activity		Fresh weight (g plant^{-1})	Dry weight (g plant^{-1})	Seed number spike^{-1}	Grain yield (kg ha^{-1})
				density	Length				
0%	16±0.7^{c}	6.2±0.17^{b}	6.46±0.27^{b}	0.005±0.001^{c}	0.06±0.007^{b}	6.7±0.22^{b}	2.3±0.03^{c}	31±1.9^{b}	1390±134^{c}
25%	17±0.5^{bc}	6.7±0.17^{ab}	71±0.08^{a}	0.008±0.0005^{bc}	0.08±0.008^{ab}	7.2±0.39^{a}	2.5±0.06^{b}	34.6±0.41^{a}	1946±32^{b}
50%	18±1^{ab}	6.6±0.34^{ab}	6.7±0.25^{ab}	0.0086±0.0008^{ab}	0.08±0.003^{ab}	7.5±0.24^{a}	2.8±0.07^{a}	34.9±0.44^{a}	2070±87^{ab}
80%	19±0.9^{a}	6.9±0.36^{a}	7±0.05^{a}	0.011±0.002^{a}	0.1±0.02^{a}	7.5±0.2^{a}	2.7±0.05^{ab}	34.5±0.29^{a}	2181±51^{a}

Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

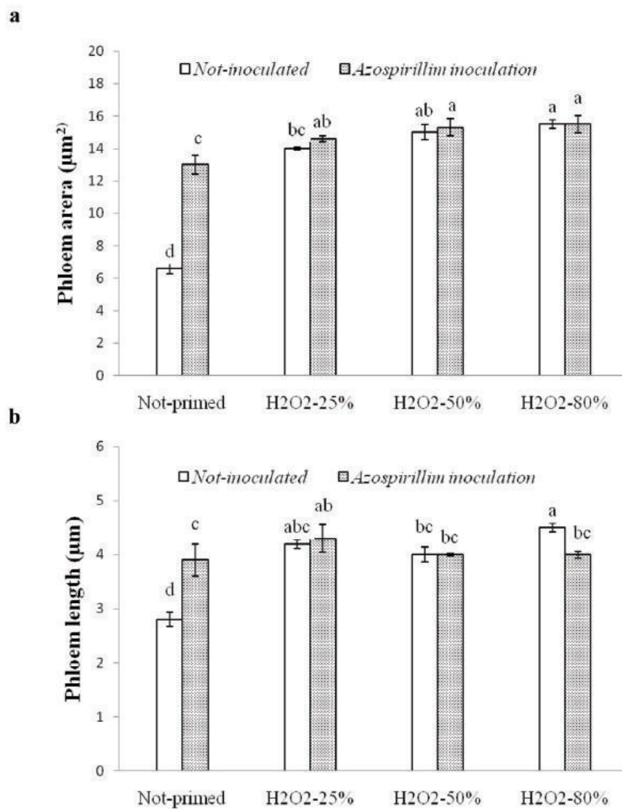

Fig. 4. Effect of soaking seed in 0, 25, 50, 80% and hydrogen peroxide and *Azospirillum* inoculation on phloem area (a) and length of phloem (b) in flag leaf of wheat. Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

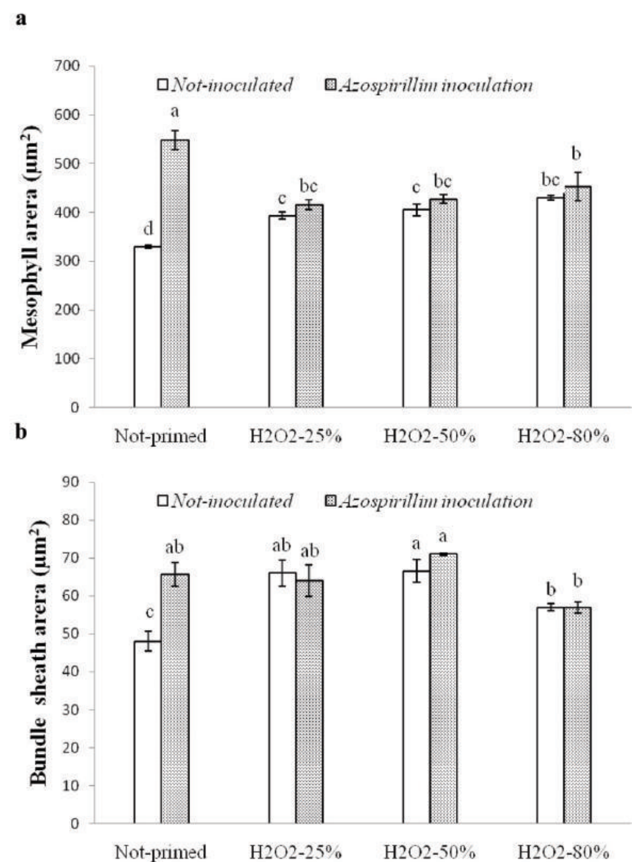

Fig. 5. Effect of soaking seed in 0, 25, 50, 80% and hydrogen peroxide and *Azospirillum* inoculation on mesophyll area (a) and bundle sheath area (b) of wheat flag leaf. Means followed by the same letter do not differ at $P < 0.05$ based on least significant difference comparisons.

seed due to invigoration of the seeds has been recently reported by Barba-Espin et al. (2011). H_2O_2 induces seed germination through impairing the abscisic acid transport from the cotyledon to the embryo, resulting in inducing a decrease in abscisic acid (Barba-Espin et al. 2011). H_2O_2-induced increase in pea seedling growth has been reported to correlate with the induction of proteins related to plant growth, cellular signaling, and cell cycle control (Barba-Espin et al. 2010). Recently, information on the role of H_2O_2/reactive oxygen species as signal molecules regulating growth and morphogenesis has emerged, suggesting that H_2O_2 is not only a stress signal molecule, but may also be an intrinsic signal in plant growth and development. H_2O_2 has also been shown to be involved in differentiation of the cellulose-rich cell wall (Potikha et al. 1999). Plants of H_2O_2-soaked seeds had higher leaf area, chlorophyll pigments contents, peroxidase and Catalase activity, xylem diameter and area, phloem area and diameter, mesophyll tissue area, vascular bundle area and upper and lower epidermis areas than those of non-primed seeds. Higher stomata density and smaller stomata size is a form of adaptation to drought because it enables plants to regulate water transport and transpiration more effectively (Dickison 2000; Fahn and Cutler 1992). A significant decreasing stomatal density can lead to a higher plant tolerance to drought stress (Miskin et al. 1972). Adequate available water increased 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). Higher density of stomata with wider length suggests that H_2O_2 improved water absorption and water content of plants. Results of this study showed better status of leaf water content in plants pre-treated with H_2O_2. In addition, plants of H_2O_2-soaked seeds had a higher area and length of xylem and phloem vessels. Xylem conductivity is determined by the structure and the size of the vessels (Schultz and Matthews 1993). It is assumed that xylem diameter and density in the petiole were reported to be positively related to hydraulic conductivity of leaves (Sack and Frole 2006). Xylem diameter possibly leads to better maintenance of leaf hydraulic conductance under drought stress. It was also reported earlier 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 are a key factor in determining the flow rate of photo assimilates from leaves to the sinks (Fitter and Hay 2002). It has been shown that the phloem sieve tube area depends on the sink with which they are related (Fitter and Hay 2002). *Azospirillum* inoculation and soaking seed in H_2O_2 increased the areas of upper and lower epidermis. Upper epidermis was thicker-walled than the lower epidermis. It is obvious that the upper surface receives more light and heat hence requires more effective protection against evaporation (Ehleringer and Mooney 1978). Therefore, it can be concluded that *Azospirillu*m and H_2O_2 through enhanced upper epidermis area help plant leaves to be more effective in protecting against evaporation. H_2O_2 increased mesophyll area and vascular bundle area of the leaf. Plants of *Azospirillum*-inoculated seed also had significantly higher mesophyll tissue areas and vascular bundle areas as compared to the non-inoculated control plants. Some of the anatomical characteristics representing adaptations to water deficit include: a greater stomata number per unit area, smaller dimensions, smaller epidermal cells, thicker cuticle, and a greater number of layers of smaller mesophyll cells (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 the vascular bundle area increased but was accompanied by higher mesophyll tissue areas.

Seed treatment with hydrogen peroxide had the potential to improve grain yield of wheat under dry land farming through modulation of leaf anatomic features, enhancing photosynthetic pigments, and increasing antioxidant activities. Relative water content, xylem and phloem area, mesophyll and bundle sheath area, and upper and lower epidermis areas of plant of H_2O_2-soaked seed were enhanced. The above-mentioned traits were also affected by *Azospirillum* inoculating. Also, soaking seed in hydrogen peroxide enhanced the promoting effect of *Azospirillum* on enhanced grain yield through more enhanced xylem and phloem area, mesophyll and bundle sheath area, and upper and lower epidermis areas. Seed per-treatment with hydrogen peroxide and then inoculation with *Azospirillum* could be suggested as a new strategy for cereal cropping under dry land farming.

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