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Research Article

Effect of foliar application of *Azospirillum brasilense* and zinc sulfate on the grain-filling process of rainfed wheat

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ABSTRACT- Grain growth (by weight), grain-filling rate, and the duration of each grain-filling phase are crucial in determining the final grain weight. Two experiments were conducted to evaluate the effects of $ZnSO_4$ on grain filling in the wheat cultivar Sardari. In the first experiment, the grain-filling rate and grain growth were assessed following foliar application of $ZnSO_4$. The second experiment explored the effects of foliar *Azospirillum brasilense*, applied either alone or combined with $ZnSO_4$, on grain-filling rate, grain growth, and the duration of both the lag phase and the linear phase of grain filling. $ZnSO_4.7H_2O$ was applied at a rate of 3 kg ha^{-1} at the anthesis stage, and only the main stem spike was used for assessing the grain-filling process. Richards equation was employed to simulate grain filling. The results indicated that, compared to foliar *Azospirillum* alone, Zn application was more effective in enhancing grain growth (by weight) and 1000-grain weight. Foliar application of $ZnSO_4$ significantly increased the grain-filling rate, and $ZnSO_4$, whether applied alone or combined with *Azospirillum*, extended the lag phase duration. Additionally, the $ZnSO_4$ + *Azospirillum* treatment sustained higher relative water content in the flag leaves. While the time to reach maximum grain weight was shorter in untreated plants, $ZnSO_4$ combined with *Azospirillum* extended the lag phase and grain-filling rate. This combined treatment may benefit wheat production in semi-arid areas where soil drying during the grain-filling period often shortens this crucial phase.

INTRODUCTION

Wheat is the most important staple cereal crop worldwide, serving as the primary food source for over one-third of the global population (Saini et al., 2022). Wheat grain yield is attributed to two main components: (1) the number of kernels per head (spike), determined before anthesis, and (2) the 1000-grain weight, which depends on current photosynthesis and the re-translocation of photosynthetic assimilates stored before anthesis. These components collectively determine the final grain weight and yield of bread wheat (*Triticum aestivum* L.) (Baillot et al., 2018). Notably, the number of grains per unit area during the grain-filling period is considered a major factor influencing wheat yield variability (Reynolds et al., 2021). The final wheat grain weight is set during grain filling, a stage that spans from anthesis to physiological maturity (Baillot et al., 2018). Grain filling occurs through three distinct phases of seed growth: cell division, seed dry matter accumulation, and seed ripening (Shewry et al., 2012). To improve wheat performance, various agronomic approaches, such as mineral nutrition and the use of plant growth-promoting bacteria, have been widely adopted. Recent studies demonstrate that zinc sulfate ($ZnSO_4$) can increase wheat yield (Afshar et al., 2020; Shariatipour et al., 2020; Azeem et al., 2023; Zarea and Karimi, 2023) and improve Zn content in grains (Zarea and Karimi, 2023a). Several reports attribute the increase in wheat seed yield due to zinc application to a rise in 1000-seed weight (Zarea and Karimi, 2023b; Qamari et al., 2023; Shoormij et al., 2023). Even in soil conditions where zinc is adequate, $ZnSO_4$ application can still positively impact plant yield (Nanja Reddy et al., 2023). In many studies, $ZnSO_4$ fertilizer has been confirmed as an effective Zn source for wheat, with $ZnSO_4.7H_2O$ serving as a baseline Zn fertilizer (Joy et al., 2017). The beneficial impact of $ZnSO_4$ fertilization on wheat yield has been well-documented over recent decades; for example, $ZnSO_4$ use in wheat was estimated to increase Pakistan's annual grain supply by 19 kg per capita (Joy et al., 2017). While various Zn fertilizers exist, including inorganic sources like $ZnSO_4.7H_2O$ and chelated sources such as Zn-EDTA, studies suggest that Zn-EDTA may be a better option for Zn biofortification of wheat when applied in soil compared to $ZnSO_4$ (Zhao et al., 2019). Under laboratory conditions, zinc sulfate has been shown to maintain higher levels of Zn in soil solution than zinc oxide (Sharma et al., 1988). To further improve wheat performance, the role of growth-promoting bacteria, particularly in enhancing wheat

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growth and yield, has gained global interest (Zarea, 2017). *Azospirillum* is a well-known plant growth-promoting bacterium, and its growth-enhancing effects on many agricultural plants have been widely reported (Raffi and Charyulu, 2021). Specifically, in wheat, *Azospirillum* reportedly increased grain yield by raising grain weight (Zaheer et al., 2019; Iqbal et al., 2024). Some studies also report that *Azospirillum* inoculation boosts seed yield through increased seed count (Alhammad et al., 2023; Zaheer et al., 2022). Yield increases due to *Azospirillum* inoculation have been attributed to several factors, including growth hormone production (Alhammad et al., 2023), a higher leaf area index under drought stress (Alhammad et al., 2023), and enhanced leaf relative water content (Zaheer et al., 2019). A recent study (Iqbal et al., 2024) attributed the improved performance of *Azospirillum brasilense*-inoculated wheat to enhanced photosynthetic rate, leaf relative water content, and stomatal conductance. The application of $ZnSO_4$ as a chemical fertilizer or zinc source has been suggested to increase biomass accumulation in grains during the grain-filling phase (Liu et al., 2020). Additionally, *Azospirillum* shows promising beneficial effects on wheat performance (Iqbal et al., 2024). However, no studies have investigated the effectiveness of combining *Azospirillum* with $ZnSO_4$ as a foliar spray treatment during the grain-filling process in wheat. This study, therefore, aimed to examine how the foliar inoculation of *Azospirillum brasilense* Sp7, applied alone or in combination with $ZnSO_4$, influences the grain-filling process in the rain-fed winter wheat cultivar Sardari. Given that both $ZnSO_4$ and *Azospirillum* can be absorbed by the leaves, we hypothesized that their foliar application at anthesis could improve wheat grain weight by enhancing the grain-filling process.

MATERIALS AND METHODS

The experiment was conducted under rain-fed (dryland) conditions during the 2021-2022 (Experiment 1) and 2022-2023 (Experiment 2) wheat growing seasons. It took place on a wheat farmer's field in the Dehgolan region (35° 16′ 40″ N, 47° 25′ 3″ E) of Kurdistan province, Iran. Soil properties across the experimental site included total N (0.16%), P_2O_5 (12.1 ppm), K_2O (132 ppm), and Zn (0.76 mg kg^{-1}) with sulfur content at 17.56 mg kg^{-1}. Average monthly precipitation and minimum and maximum monthly temperatures during the experiment are shown in Tables 1 and 2. The wheat cultivar used in this study was 'Sardari,' which is susceptible to yellow leaf rust but tolerant to cold temperatures and drought stress. Under rain-fed conditions, this cultivar has a potential grain yield of over 1900 kg ha^{-1} with an average thousand-grain weight of 44 g. With one supplemental irrigation, yield potential can reach up to 3221 kg ha^{-1}.

In Experiment 1, two treatments were applied: foliar $ZnSO_4$ and a control treatment. Experiment 2 included three treatments: (1) control (no inoculation or $ZnSO_4$ foliar spray), (2) inoculation with *Azospirillum brasilense* strain Sp7 LMG 13127 (Tarrand, Krieg, and Dobereiner, 1979 AL) through foliar spraying, and (3) foliar application of $ZnSO_4.7H_2O$ (0.3%, w/v) combined with *Azospirillum* bacteria. The choice of treatments was based on previous reports on the positive effects of foliar $ZnSO_4$ (Shoormij et al., 2023; Zarea and Karimi, 2023b) and *Azospirillum* inoculation (Iqbal et al., 2024; Alhammad et al., 2023; Kazi et al., 2016) on wheat performance. The preceding crop was potatoes, which had received 400 kg S ha^{-1}, ensuring sufficient sulfur content in the soil as verified by soil analysis. For both experiments, foliar treatments were applied at the beginning of anthesis (Zadoks GS 61). A 0.3% (w/v) zinc solution, prepared from zinc sulfate, was used based on prior studies showing its efficacy in increasing wheat yield (Zarea and Karimi, 2023b). The experiments followed a randomized complete block design with three replications. The experimental site was chosen to ensure uniform plant growth, verified through random sampling of plant fresh weight at the stem elongation stage. Fresh weights of plant samples from different site areas were collected and weighed to confirm minimal growth variation across the plots. Each experimental plot measured 6 m by 1 m, with 1 m spacing between plots and 0.5 m between blocks. Planting was done mechanically, and weed control was managed with common herbicides used by local farmers. Plant density was approximately 500 plants m^{-2}.

Seed sowing was conducted in October 2021 and 2022, following the local planting schedule. Zinc sulfate ($ZnSO_4 \cdot 7H_2O$, molecular weight: 287.54 g/mol, Merck) was used as the source of zinc, as previously reported by Afshar et al. (2020), Shariatipour et al. (2020), and Azeem et al. (2023). In this study, a 0.3% (w/v) zinc solution was prepared using $ZnSO_4 \cdot 7H_2O$, which contained approximately 22% zinc and 11% sulfur. Tween-20 (0.02% v/v) was added as a surfactant. For the control treatment, distilled water was used with 0.02% Tween-20. Foliar spraying was carried out using a hand sprayer during anthesis initiation in the evening when winds had ceased. The spraying volume was 1000 liters per hectare (100 mL/m^2), delivering 3 kg of zinc per hectare. This amount ensured thorough wetting of the entire wheat leaf surface. Inoculation with *Azospirillum brasilense* (strain Sp7) was also performed via foliar spraying at anthesis initiation. A fresh bacterial culture was grown overnight on semi-solid nitrogen-free medium (NFb) at 120 rpm. The bacterial culture was diluted to a concentration of 1×10^7 colony-forming units (CFU) per mL using phosphate buffer. A 10% bacterial solution was prepared by adding 10 mL of this culture to 90 mL of distilled water, with 0.02% Tween-20 added as a surfactant. For the control treatment, an autoclaved bacterial culture (dead bacteria) was used at the same concentration, along with 0.02% Tween-20. For the combination treatment of zinc and bacteria, the zinc solution and bacterial culture were mixed just before spraying to prevent any negative effects of high zinc concentrations on the bacteria. Thus, the foliar solution contained 10% bacterial culture (1×10^7 CFU) and 0.02% Tween-20. To study grain filling, only the main stem's spike was sampled. At the late stem elongation stage, the main stem was identified and tagged for subsequent sampling. Plants with delayed spike emergence were excluded, as were spikes from the border rows. In Experiment 1, the first spike sample was taken 5 days after anthesis (the zero point) and subsequent samples were collected at 5-day intervals until 45 days after anthesis. Each sample consisted of 5 spikes. In Experiment 2, the zero point was also set at 5 days post-anthesis, and the first spike sampling was used to determine seed weight. Sampling was then carried out at 4-day intervals until the grain weight stabilized. Initially, 5 spikes were sampled from each replication, but this was reduced to 3 spikes per replication for subsequent samples. To separate the three phases of seed filling, i.e., the lag phase, linear growth, and ripening, specific time points were used. The lag phase was considered to begin when the grain weight reached 5% of the final weight, and ripening was deemed complete when the grain weight reached 95% of its final weight. At harvest, the 1000-seed weight was also measured.

The equations in Table 3 were used for computing grain-filling processes and various grain growth parameters. The grain-filling process was modeled using the Richards growth equation (Richards, 1959), where A represents the final grain weight (mg). The parameters B, k, and N are coefficients that require computation through regression analysis, as they are not fixed values and must be determined for each dataset. At harvest, the number of seeds per spike and the 1000-seed weight were determined from five main spikes randomly sampled from the center of each plot. To assess the effect of the two treatments in Experiment 1 on grain growth (by weight) and growth rate, Student's t-test was applied. For Experiment 2, statistical analysis was conducted using the SAS software package, version 9. Treatment comparisons were made using the least significant difference (LSD) test at a 5% probability level.

Table 1. Average monthly rainfall and average monthly maximum and minimum temperatures during the wheat growing season in 2022-23.

Month	Temperature (°C)		Rainfall (mm)
	Minimum	Maximum	
November	-0.8	12.2	50.2
December	-2.9	10.8	27.4
January	-6.5	6.2	33.6
February	-11	4	26.6
March	-2.1	10.1	72
April	0.5	17.6	13.4
May	3.3	19.3	40
June	7.3	28.9	0.4
July	9.9	33.5	0

Table 2. Average monthly rainfall and average monthly maximum and minimum temperatures during wheat growing season in 2022-23

Month	Temperature (°C)		Rainfall (mm)
	Minimum	Maximum	
November	-0.8	15.4	18.2
December	-2.3	7.3	34.8
January	-8.5	0.8	51.8
February	-13.2	1.4	33.8
March	-1.5	11.9	92
April	1.2	14.9	70.6
May	3.1	19.9	87.2
June	7.3	26.4	19
July	26.5	45.8	0

Table 3. Representation of equations used in the current study to compute the grain weight process, grain filling, some parameters of grain growth

Equation	Equation description
$W = A/(1 + Be^{-kt})^{1/N}$	Grain-filling process (by weight)
$G = AKBe^{-kt}/(1 + Be^{-kt})^{(N+1)/N}$	Grain-filling rate
$Tmax = \frac{(\ln B - \ln N)}{k}$	Time to reach maximum grain-filling rate
$Wmax = A(N+1)^{-1/N}$	Grain weight at maximum grain-filling rate
$GRmax = \frac{Ak}{(1+N)^{\frac{(N+1)}{N}}}$	Maximum grain-filling rate
$GRmean = \frac{Ak}{2(N+2)}$	Mean grain-filling rate
$T1 = \frac{-\ln[(N^2+3N+N*\sqrt{N^2+6N+5})\ /2B]}{K}$	Linear growth stage time
$T2 = \frac{-\ln[(N^2+3N-N*\sqrt{N^2+6N+5})\ /2B]}{K}$	End of the linear growth

RESULTS AND DISCUSSION

Experiment 1

Rainfall during grain filling was lower in Experiment 1 than in Experiment 2. As illustrated in Fig 1, maximum grain growth (by weight) was reached 25 days after anthesis in both treatments; however, plants treated with foliar $ZnSO_4$ showed higher grain weight at this time. The grain-filling rate is shown in Fig 2 (the equations of the curves used in Fig. 2 is presented in Table 4), where it is evident that the maximum rate occurred earlier in control plants than in those treated with $ZnSO_4$. The maximum grain-filling rate was also higher in $ZnSO_4$-treated plants compared to the control (Fig. 2).

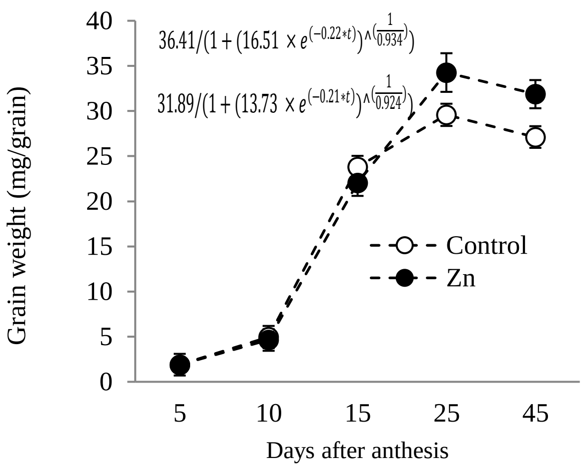

Fig. 1. Changes in grain growth by weight (mg per grain) of wheat grains under foliar spraying with or without 0.3% (w/v) ZnSO4 foliar spraying. The rate of grain growth (changes in grain weight) was fitted with the Richards equation.

Table 4. The equations of the curves in fig 2

Equation	Equation description
$36.41 \times 0.22 \times 16.51e^{-0.22t} / (1 + 16.51e^{-0.22t})^{\frac{0.22+1}{0.22}}$	Zn
$31.89 \times 0.21 \times 13.73e^{-0.21t} / (1 + 13.73e^{-0.21t})^{\frac{0.21+1}{0.21}}$	Control

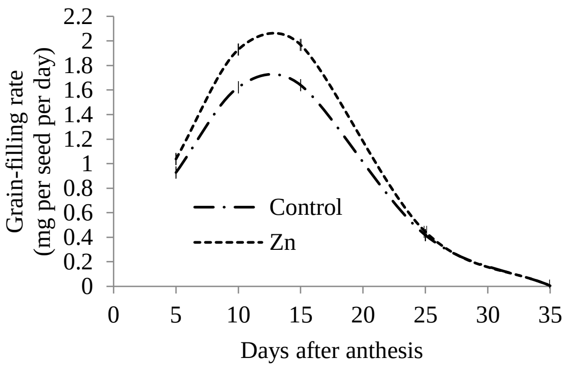

Fig. 2. Wheat grain-filling rate under foliar application of $ZnSO_4$ (0.3%) compared to control plants. Zn foliar application 0.3% (w/v) $ZnSO_4$.

Table 5 presents the effects of foliar Zn application on the timing of maximum grain-filling rate (Tmax), grain weight at the maximum rate (W_{max}), maximum grain-filling rate (GRmax), and the duration of both the lag phase (LP) and linear growth phase (LGP). Only W_{max} was significantly influenced by foliar-applied Zn. various environmental factors can significantly impact both the grain-filling period and grain-filling rate; drought, for example, can accelerate the translocation of soluble carbohydrates from source organs (stem and leaves) to sink organs (grains). Rainfall during grain filling was recorded at only 0.4 mm.

Table 5. Effect of foliar application of $ZnSO_4$ on time (days) to reach maximum grain-filling rate (Tmax), grain weight at maximum grain-filling rate (Wmax), maximum grain-filling rate (GRmax), and duration of to lag phase (LP) and linear growth phase (LP)

Treatment	Tmax (mg)	Wmax	GRmax	LP	LGP
Control	12.5	18.1	1.17	6.56	12.03
$ZnSO_4$	12.6	20.22	1.41	6.94	11.39
Sig.	ns	*	ns	ns	ns

Sig, significance; ns: no significant; *Significant at $p < 0.05$

Experiment 2

The data on the water content of the flag leaf at the beginning of the seed milking stage were collected at three time points, i.e., morning, noon, and evening, in response to the applied treatments and were subjected to analysis of variance. The results showed a significant effect of the treatments on the relative water content (RWC) of the flag leaf (Table 6). Specifically, plants inoculated with *Azospirillum* and foliar-sprayed with zinc exhibited the highest RWC at 6 am, 12 pm, and 6 pm (Fig. 3). The relationship between zinc and increased wheat yield under rain-fed conditions has been documented in prior research (Zarea and Karimi, 2023b). Foliar zinc application has been found to improve wheat's water status and increase tolerance to drought stress (Sattar et al., 2022). Previous studies indicate that zinc ($ZnSO_4$) enhances leaf water content by promoting the accumulation of compatible osmolytes (Zarea and Karimi, 2023a; Sattar et al., 2022). Consequently, the higher flag leaf water content observed at different times of day may be associated with zinc's role in osmolyte accumulation within the plant. Additionally, *Azospirillum* has been shown to support osmotic adjustment under drought stress by increasing proline levels, which aids in maintaining cell water balance (Zarea et al., 2012).

Table 6. Analysis of variance for the effect of foliar *Azospirillum*, foliar *Azospirillum* + foliar $ZnSO_4$ on leaf relative water content at three different hour of the day and 1000-seed weight of wheat

S.O.V.	*d.f.*	Leaf relative water content			Seed weight
		6 pm	12	6 am	
Block	2	80.2^{ns}	39.9*	14.4^{ns}	25.9*
Treatment	2	158*	74.8*	55.8*	33.38*
Error	4	22.9	5.85	3.43	3.92
C.V.		6.1	4.1	3.43	5.34

Sig, significance; ns, no significant; *, significant at p<0.05

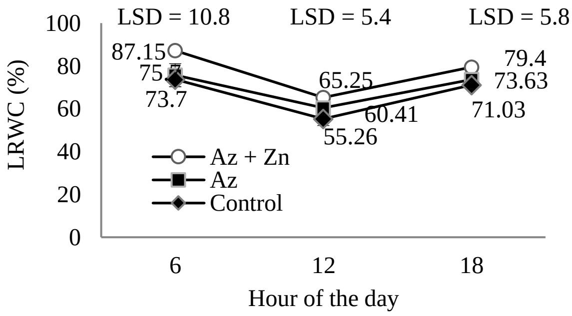

Fig. 3. Effect of foliar *Azospirillum* inoculation singly or in combination with application of $ZnSO_4$ via foliar spray on flag leaf relative water content (LRWC) at early dough phase. Az, *Azospirillum*; Az + Zn, *Azospirillum* + foliar Zn.

At harvest, the 1000-grain weight was measured. The analysis of variance results for the effect of treatments on the final grain weight are presented in Table 6, showing a significant impact of the treatments on 1000-grain weight. A mean comparison test (LSD) indicated that the combined application of *Azospirillum* and zinc resulted in a significantly higher 1000-grain weight compared to both the control and *Azospirillum* inoculation alone (Fig. 4). The positive influence of zinc (Shoormij et al., 2023; Zarea and Karimi, 2023b) and *Azospirillum* (Zaheer et al., 2019; Iqbal et al., 2024) on grain growth has been highlighted in recent studies. However, the underlying mechanisms contributing to increased wheat grain yield in these studies have not been thoroughly investigated. In this experiment, the improved water status observed in plants treated with Zn + *Azospirillum* may have facilitated enhanced translocation of sucrose from source organs (leaves) to the sink (grains) and sustained the photosynthesis process, potentially contributing to increased grain weight.

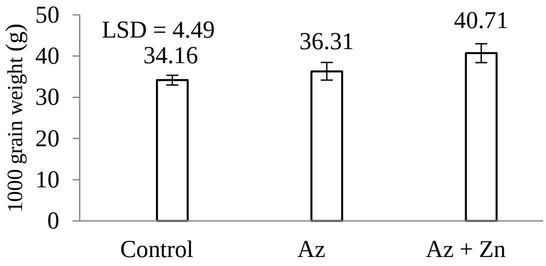

Fig. 4. Effect of foliar *Azospirillum* inoculation, foliar *Azospirillum* + foliar $ZnSO_4$ on 1000-grain weight. Az, *Azospirillum*; Az + Zn, *Azospirillum* + foliar Zn.

The data obtained from the seed-filling process (seed weight during the grain-filling period) in response to *Azospirillum* inoculation alone and in combination with zinc foliar application at anthesis were subjected to analysis of variance. The effect of the treatments was significant. Based on data from the first sampling, conducted 5 days after anthesis, treatment effects on grain weight were not significant; however, at subsequent stages, treatments significantly influenced the grain-filling process (Fig. 5). Both the lag phase and the linear growth phase were clearly visible in control plants, *Azospirillum*-inoculated plants, and *Azospirillum*-inoculated + Zn foliar-sprayed plants (Fig. 5). Seed growth in crops typically starts slowly, then enters a linear growth phase with a rapid growth rate, and finally progresses to maturity. Wu et al. (2018) identified three main stages of the wheat grain-filling period: a lag phase occurring shortly after spike appearance, a linear increase phase following grain setting (the longest phase), and a maturation phase. However, these grain-filling phases are often influenced by plant genotype and environmental conditions (Teng et al., 2023). In this study, foliar application of $ZnSO_4$ in combination with *Azospirillum* increased grain weight (Fig. 5). This may be due to zinc's role in enhancing plant function; for example, nano-ZnO has been shown to increase chlorophyll content in wheat (Sun et al., 2020) and improve antioxidant activity and proline content (Abbas et al., 2023). Sattar et al. (2022) also reported that foliar application of Zn improved wheat leaf water content. The increase in grain weight following zinc treatment is consistent with previous findings (Shoormij et al., 2023). *Azospirillum* inoculation alone also had a positive effect on grain weight compared to the control treatment (Fig. 5), supporting previous studies on *Azospirillum*'s ability to increase wheat grain weight (Zaheer et al., 2019; Iqbal et al., 2024). However, in this study, the effect of *Azospirillum* was less pronounced than that of foliar-applied Zn (Fig. 5). As root colonizers, *Azospirillum* may be less suited for activity in leaf tissues than in root tissues. Puente et al. (2021) used scanning electron microscopy to study the localization of *Azospirillum* inoculated on soybean leaves and observed that while bacterial cells were initially present on the leaf surface, they predominantly colonized the midrib and inner leaf epidermis. However, survival of the bacteria within leaf tissues remains uncertain, and it is unclear if the leaf environment supports optimal bacterial activity. Nevertheless, the results of this study suggest a moderate beneficial effect of foliar *Azospirillum* inoculation on grain weight.

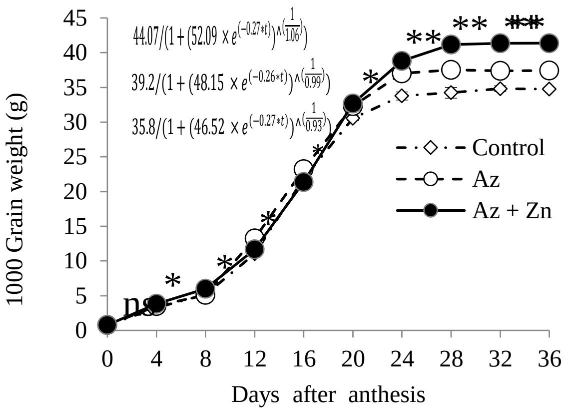

Fig. 5. Changes in grain weight (grams per thousand grains) of wheat grains under foliar spraying with *Azospirillum* alone or in combination with $ZnSO_4$ foliar spraying compared to control plants. The rate of grain filling (changes in grain weight) was fitted with the Richards equation. Az, *Azospirillum*; Az + Zn, *Azospirillum* + foliar Zn.

The data obtained from the grain-filling rate were also analyzed through analysis of variance. Fig 6 presents wheat grain-filling rate response to treatments. The equations of the curves in Fig 6 have been presented in Table 7. According to the LSD test, there was no significant difference in grain-filling rate between *Azospirillum*-inoculated and control plants up to 12 days after the first spike sampling (Fig. 6). However, at this stage, plants treated with *Azospirillum* + zinc solution exhibited a higher grain-filling rate than plants inoculated with *Azospirillum* alone and the control group. By 16 days post-anthesis, differences emerged between *Azospirillum*-inoculated plants and control plants, with *Azospirillum*-treated plants showing a higher grain-filling rate than the controls (Fig. 6). In later stages, although *Azospirillum*-inoculated plants maintained a higher grain-filling rate than the controls, this difference was not statistically significant. Notably, plants treated with both zinc and *Azospirillum* maintained a significant advantage in grain-filling rate throughout the entire grain-filling period, from 16 days after pollination to seed maturity (Fig. 6). This combined treatment demonstrated a pronounced effect, enhancing both the grain-filling period's duration (particularly during the linear growth phase) and the grain-filling rate itself, which occurred with a noticeable delay compared to control plants. Grain-filling is a critical determinant of cereal grain yield, influenced by both the rate and duration of the filling period (Teng et al., 2023). In this study, a correlation between grain-filling rate and final seed weight was observed, with plants treated with zinc and *Azospirillum* showing both higher seed weights and higher grain-filling rates. A similar relationship between grain weight and grain-filling rate in wheat has been previously reported (Wu, 2018).

Table 7. The equations of the curves in fig 6

Equation	Equation description
44.07×0.27×52.09e^(-0.27t)/([1+52.09e^(-0.27t))]^((1.06+ 1)/1.06)	Az+Zn
39.2×0.26×48.15e^(-0.26t)/([1+48.15e^(-0.26t))]^((0.99+ 1)/0.99)	Az
35.8×0.27×46.52e^(-0.27t)/([1+46.52e^(-0.27t))]^((0.93+ 1)/0.93)	Control

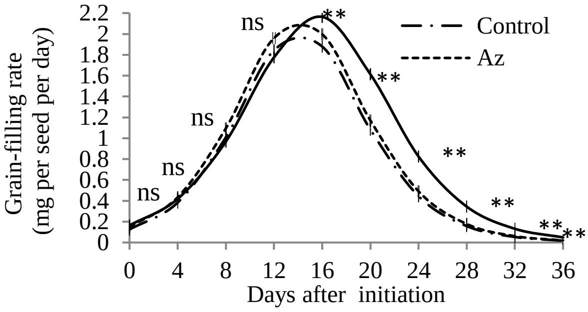

Fig. 6. Wheat grain-filling rate under foliar application of *Azospirillum* alone or in combination with foliar application with $ZnSO_4$ compared to control plants. Az, *Azospirillum*; Az + Zn, *Azospirillum* + foliar Zn

Table 8 shows Richard's equation parameters under foliar $ZnSO_4$ application plus *Azospirillum brasilense* and *A. brasilense* foliar application compared to the control treatment. Coefficients of determination (R^2) were above 0.9.

Table 8. Decisive factor (R^2), final weight (g/1000 seeds) of kernel (A), and coefficients of B and K computed by fitted curve under treatment of *Azospirillum* alone and foliar *Azospirillum* inoculation plus foliar application of $ZnSO_4$

Treatment	R^2	A	B	K
Az + Zn	0.94	42.8	53.3	0.20
Az	0.91	38.2	48.4	0.22
Control	0.90	35.2	47.2	0.22

Az, *Azospirillum*; Az + Zn, *Azospirillum* + foliar Zn; B and K are the coefficients determined by the regression.

The experiment's results further highlight the significant effects of *Azospirillum* inoculation and the combined *Azospirillum* + zinc foliar application on some grain growth characteristics (Table 9). Among the parameters evaluated, only the time to reach the maximum seed-filling rate (Tmax) was significantly affected by the treatments (Table 9). Specifically, Tmax was observed to be 17.6 days for the control, 17.7 days for *Azospirillum* alone, and extended to 19.64 days for the combined *Azospirillum* + Zn treatment (Table 10). Drought or soil drying during the seed-filling period in cereals is known to accelerate the grain-filling rate, as water stress typically shortens the grain-filling duration. However, there appears to be a positive association between zinc content in plants and improved water retention in wheat leaves (Sattar et al., 2021). Consequently, the delay in reaching Tmax in plants treated with both *Azospirillum* and zinc could likely be due to enhanced water retention, leading to improved physiological conditions that support a longer grain-filling period and a more gradual approach to peak filling speed. This may provide the plants with an extended duration for carbohydrate accumulation, potentially contributing to increased final grain weight.

Table 11 presents the analysis of variance for the effects of *Azospirillum* inoculation, combined *Azospirillum* + zinc foliar spraying, and control treatments on grain-filling parameters, the delayed (lag) phase, linear growth phase, and physiological maturity of the seeds. Fig 7 shows the grain-filling duration across different phases of this process. Plants treated with both *Azospirillum* and zinc demonstrated a notably extended lag phase compared to both the control and the plants treated solely with *Azospirillum* (Fig. 7). Differences were also observed in the linear growth phase across treatments, with a slight yet significant extension seen in plants receiving the combined *Azospirillum* + zinc treatment (Fig. 7). There are two primary carbon sources for grain filling: carbohydrates produced through ongoing photosynthesis, which are directly transferred to the seeds, and carbohydrates stored in vegetative tissues, which are later re-translocated (Pheloung and Siddique, 1991). Both sources are influenced by environmental conditions. For instance, reduced soil moisture and higher temperatures can shorten the grain-filling period. Soil water availability during grain filling has a complex role. On one hand, water stress during early grain development can limit endosperm cell formation and amyloplast development, reducing starch storage capacity and ultimately seed weight (Farooq et al., 2017). On the other hand, moderate soil drying post-anthesis can significantly enhance grain filling in cereal crops, such as rice and wheat, largely due to increased translocation of non-structural carbohydrates from vegetative tissues to grains, which accelerates the grain-filling rate. Therefore, the observed extension of the linear growth phase in plants treated with both zinc and bacteria could be attributed to the improved water status of the flag leaf, which likely helped maintain favorable conditions for continued grain filling

Table 9. Analysis of variance for the effect of foliar inoculation of *Azospirillum* alone and foliar *Azospirillum* inoculation plus foliar application of $ZnSO_4$ on some grain growth characteristics

		Sum of squares			
S.O.V.	*d.f.*	T_{max}	W_{max}	GR_{max}	GR_{mean}
Block	2	0.42*	0.55^{ns}	0.01^{ns}	0.026^{ns}
Treatment	2	3.9**	6.5^{ns}	0.005^{ns}	0.019^{ns}
Error	4	0.062	2.12	0.002	0.007^{ns}
C.V.		1.36	7.21	3.18	15.4

S.O.V., Source of variance; C.V., coefficient of variance; Ns, no significant; *, Significant at $p < 0.05$; **, Significant at $p < 0.01$.

Table 10. Influence of treatment with inoculation of *Azospirillum* bacteria, inoculation of *Azospirillum* bacteria + $ZnSO_4$ foliar spraying on Tmax, Wmax, GRmax and GRmean

Treatment	T_{max} (day)	W_{max} (g/1000-seed weight)	GR_{max} (g per 1000 grains per day)	GR_{mean} (mg/grain/d)
Az + Zn	19.64	20.29	1.41	0.61
Az	17.7	20.42	1.43	0.47
Control	17.64	19.91	1.34	0.61
LSD (0.5)	0.56	3.31	0.1	0.19

Table 11. Analysis of variance for the effect of foliar inoculation of *Azospirillum* alone and foliar *Azospirillum* inoculation plus foliar application of $ZnSO_4$ on the duration (day) of the three phase of grain-filling process based on Richards' equation

S.O.V.	*d.f.*	Sum of squares		
		Lag phase	Linear growth phase	Physiological maturity phase
Block	2	0.07ns	0.33*	0.65*
Treatment	2	0.93**	1.53**	4.83**
Error	4	0.016	0.052	0.075
C.V.		1.9	2.34	2.25

S.O.V., Source of variance; C.V., coefficient of variance;
Ns, no significant; *, Significant at $p < 0.05$; **, Significant at $p < 0.01$

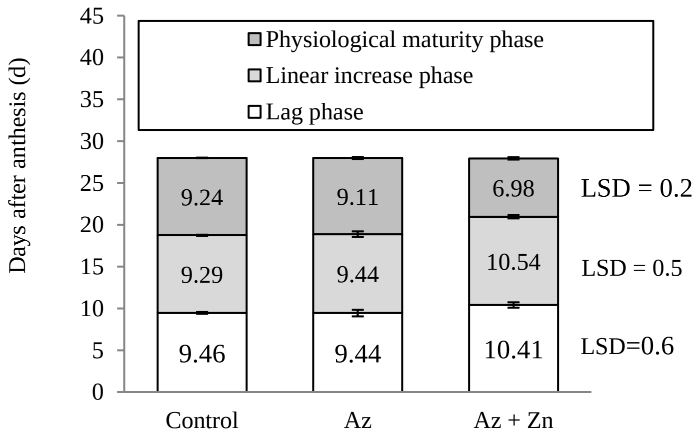

Fig. 7. Representation of wheat grain-filling phase duration (days) under the influence of inoculation with *Azospirillum*, and combined foliar application of $ZnSO_4$ + *Azospirillum* under rain-fed conditions. Az, *Azospirillum*; Az + Zn, *Azospirillum* + foliar Zn.

CONCLUSION

Grain weight is a key yield component determining total grain yield in cereals. While the genetic makeup of wheat influences the final grain weight, environmental factors also play a significant role. Nutrition through Zn-containing fertilizers or growth-promoting bacteria like *Azospirillum brasilense* has been shown to enhance wheat yield, primarily by increasing grain weight or grain count. The duration of the grain-filling period is critical for determining final grain weight, as shorter grain-filling durations, often due to soil moisture limitations, lead to accelerated grain filling and can limit seed development. This study aimed to evaluate the effectiveness of foliar-applied $ZnSO_4$ and *Azospirillum brasilense* SP7 on grain growth (by weight) and the grain-filling process under dryland conditions, specifically in winter wheat. Foliar treatments were administered at the onset of anthesis (Zadoks GS 61), with only the main stem spike used to monitor grain-filling dynamics. The grain-filling process was modeled using the Richards growth equation (Richards, 1959). Results from both experiments highlighted the benefits of $ZnSO_4$ application, which extended the lag phase and increased the grain-filling rate. Given that grain filling in wheat aligns with spring when rainfall is often limited and soil drying occurs, treatments that prolong grain-filling duration and enhance the filling rate have the potential to improve yield outcomes. This study found that the combined application of $ZnSO_4$ and *Azospirillum brasilense* significantly extended the seed-filling period (linear growth stage) compared to inoculation with *Azospirillum* alone, which is promising for maintaining yield performance in cereal crops facing drought stress toward the end of the season.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Conceptualization: Mohammad Javad Zarea; Methodology: Mohammad Javad Zarea; Software: Mohammad Javad Zarea; Validation: Mohammad Javad Zarea; Formal analysis: Mohammad Javad Zarea; Resources: Mohammad Javad Zarea; Data curation: Mohammad Javad Zarea; Writing—original draft preparation: Mohammad Javad Zarea; Writing—review and editing: Mohammad Javad Zarea; Visualization: Mohammad Javad Zarea; Supervision: Mohammad Javad Zarea; Project administration: Mohammad Javad Zarea; Funding acquisition: Mohammad Javad Zarea.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author on request.

ETHICAL STATEMENT

The conducted research is not related to either human or animals use. Author is aware of the content of the manuscript and consented to submit it to *Iran Agricultural Research* journal.

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