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**Research Article**

# Changes in anatomy, pigment contents, and nutrients of barley cultivars biofertilized by *Azotobacter chroococcum* and *Pseudomonas fluorescens*

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## Abstract

**Biofertilizers can enhance crop nutrient availability and grain yield. A barley greenhouse experiment was laid out to measure the effect of biofertilizers on flag leaf anatomical structure, pigment contents, grain yield, and nutrient content. Treatments included barley cultivars (Zehak and Nimrooz), and fertilizer treatments consisted of control (no fertilizer applied) (C), seed inoculation by *Azotobacter chroococcum* (Azt) and *Pseudomonas fluorescens* (Psd) alone, *Azotobacter* with *Pseudomonas* (Azt+Psd), *Azotobacter* and *Pseudomonas* with 100 kg ha$^{-1}$ urea (Azt+Psd+100U), application of 100 (100U), and 200 kg ha$^{-1}$ urea (200U). The flag leaf midrib, metaxylem, and protoxylem areas were the largest in the 200U and Azt+Psd+100U in Zehak, whereas in Nimrooz the leaf areas tended to be lower. Increasing flag leaf area was related to increasing protoxylem and metaxylem area of the midrib. The stomatal area reached maximum values in Zehak, increasing by 47 and 40% in 200U and 100U treatments, respectively. Azt+Psd+100U affected total chlorophyll, carotenoid content, and relative water content, positively. Azt+Psd+100U treatment was able to reach similar grain yields to 200U. The highest grain yield was obtained in 200U (108% increase) and Azt+Psd+100U (102% increase) in Zehak. Azt had a greater effect on grain N content, while Psd had a stronger influence on P content. A significant relationship was observed between grain yield and midrib area, total chlorophyll, relative water content, and grain N and P content. The co-application of Azt+Psd and a reduced quantity of mineral N fertilizer (100 U) could be a suitable strategy to improve the sustainability of barley production.**

**Keywords: Midrib area, Nitrogen content, Protoxylem, Relative water content, Stomata area**

## Introduction

Nitrogen (N) and phosphorus (P) are two major nutrients required by crops to achieve optimum yields and quality. To ensure high crop yields, farmers apply synthetic chemical fertilizers, which are costly, have a high carbon (C) footprint, and may have negative environmental impacts. Due to the associated environmental concerns, application of chemical fertilizers, especially urea (46% N), is under discussion (Chiquito-Contreras *et al.*, 2017; Galindo *et al.*, 2020). Use of biofertilizers with a reduced application rate of synthesized fertilizers may be a suitable alternative to achieve more sustainable agriculture. Biofertilizers contain beneficial microorganisms such as *Azotobacter*, *Pseudomonas*, and *Azospirillum, which*, when added to seed, plant surfaces, or soil, increase plant nutrient availability and growth (Jayant, 2012; Nosheen *et al.*, 2016; Zeffa *et al.*, 2018).

Barley (*Hordeum vulgare* L.) is the fourth main grain cereal produced in the world. Barley monoculture produced only by means of chemical fertilizers has been shown to gradually decrease soil fertility and quality (Mercado-Blanco *et al.*, 2016). Integrated nutrient management (INM) is a suitable strategy in sustainable barley production (Salama and Badry, 2021). In INM system, synthesized chemical fertilizers combined with biofertilizers is applied to improve crop production and sustainability (Kamali and Mehraban, 2020).

*Azotobacter* spp. are aerobic bacteria that can fix an average of 20 kg N ha$^{-1}$ year$^{-1}$ and play a main role in the N cycle (Jnawali *et al.*, 2015). Application of high levels of mineral N to the soil can decrease nitrogen-fixation by *Azotobacter* because of limitation of

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nitrogenase activity. *Azotobacter* can colonize the rhizosphere of many plants and improve nutrient availability and biomass production (Nosheen *et al.*, 2016). *Pseudomonas* species are aerobic bacteria known as phosphate solubilizing microorganisms, which are able to solubilize P and increase P availability of the soil for plants (Chiquito-Contreras *et al.*, 2017; Qessaoui *et al.*, 2019).

*Pseudomonas fluorescens* has been used as a biofertilizer due to its excellent rhizosphere colonization ability and catabolic versatility (Hernandez-Montiel *et al.*, 2020; Mayak *et al.*, 2004). Febri *et al.* (2014) reported that *Pseudomonas* significantly increased rice biomass and seedling growth, and suggested that this biofertilizer could be investigated as a suitable rhizobacterial inoculant in rice fields. Also, Frohlich *et al.* (2012) declared that barley yield and straw weight were improved by seed inoculation of barley with *Pseudomonas* under nutrient deficient conditions. Rhizobacteria, such as *Azotobacter* and *Pseudomonas*, can promote nutrient uptake in the plant and enhance the availability of N, P, K and some micronutrients (Hernandez-Montiel *et al.*, 2020; Nosheen *et al.*, 2016). Application of biofertilizers with a reduced dosage of chemical fertilizers, has been shown to lower crop production costs and mitigate the harmful environmental effects of chemical fertilizers (Chiquito-Contreras *et al.*, 2017; Kamali and Mehraban, 2020).

Investigating the improvement of barley grain yield by N- and P-fixing bacteria is critical for sustainable agriculture due to environmental benefits and chemical fertilizer costs. On the other hand, little information has been published about the effect of biofertilizers on leaf expansion and anatomy of barley cultivars, and its relationship with barley yield. We hypothesized that application of biofertilizers combined with urea influenced the leaf anatomy, pigment content, RWC, and nutrient uptake, which are traits linked to barley grain yield. Thus, the main aim of the current study was to consider the biofertilizer application of (*Azotobacter chroococcum* and *Pseudomonas fluorescens*) alone, or in combination with 50% of the recommended dose of urea, and 100% of the crop's urea requirement alone, on the anatomy and biochemical characteristics of the flag leaf, grain yield attributes, and nutrient uptake of barley cultivars.

## Materials and methods

**Site description:** A pot experiment was conducted to consider the performance of *Azotobacter chroococcum* and *Pseudomonas fluorescens* with chemical N fertilizer as urea (46%) on barley cultivars at the greenhouse of the Agroecology Department of Shiraz University, Fars Province, Iran. The soil used in the trial was a loamy, carbonatic, hyperthermic, typic Torriorthent that had the following properties: 37.2% sand, 43.1% silt, and 19.7% clay; 0.86% organic C content; electrical conductivity of 1.08 (dS $m^{-1}$), pH of 7.42; 0.071% total N; 14 mg $kg^{-1}$ available P; and 212 mg $kg^{-1}$ available K content.

**Treatments and plant material:** Experimental treatments consisted of two barley cultivars (Zehak and Nimrooz) and fertilizer treatments including a control (no fertilizer applied) (C), seed inoculation by *Azotobacter chroococcum* alone (Azt), seed inoculation by *Pseudomonas fluorescens* (Psd) alone, seed inoculation with a combination of *Azotobacter* and *Pseudomonas fluorescens* (Azt+Psd), seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% barley N requirement (100 kg $ha^{-1}$ urea N) (Azt+Psd+100U), application of 50% barley mineral N requirement (100 kg $ha^{-1}$ urea N source alone) (100U), and application of 100% barley mineral N requirement (200 kg $ha^{-1}$ urea N source alone) (200U). The pot experiment was arranged as a factorial study using a completely randomized design (RCD) with four replicates.

The *Azotobacter chroococcum* and *Pseudomonas fluorescens* bacterial strains were prepared at the Soil and Water Research Institute, Tehran, Iran. First, seeds were sterilized by sodium hypochlorite solution (1%) for 10 minutes and then washed several times by deionized water. The washed seeds were then transferred into a polyethylene bag, and 20 ml of sugar adhesive solution (20%) was added, and shaken for 30 seconds to completely immerse the seeds. Twenty grams of inoculum was then added to the sticky seeds and shaken for 45 seconds. Finally, the treated seeds were transferred onto aluminum foil and put in the shade to dry (Somasegaran and Hoben, 2012).

Zehak and Nimrooz barley cultivars have different morphologies, growing season lengths, and yield attributes and were expected to react differently to the fertilizer treatments. Zehak is a six-rowed barley cultivar with a medium plant height of 50-80 cm, and an average grain yield of 3846 kg/ha. The growing season length for Zehak is 130-138 days, which is adapted to the warm and arid regions (Ghazvini *et al.*, 2014). Nimrooz is a two-rowed barley cultivar that matures early with approximately 3431 kg $ha^{-1}$ grain yield. It tolerates water stress, which makes it appropriate for arid and semi-arid regions of Iran (Koohkan *et al.*, 2012). The seeds of Zehak and Nimrooz were prepared at the Agriculture and Natural Resources Research Center of Darab, Fars Province, Iran. Ten uniform seeds of each cultivar were sown in 5 kg pots filled with the loamy, calcareous soil. The seedlings were thinned to four seedlings at the three-leaf stage. The temperature in the greenhouse was 25±5°C, with 60±10% relative humidity, and light intensity was in the range of 600-1100 µmol. $m^{-2}$ $s^{-1}$. To avoid volatilization of urea, it was used in a split application, i.e., half at the tillering stage (Zadoks Growth Stage; ZGS21), and half at stem elongation (ZGS31) (Zadoks *et al.*, 1974).

**Flag leaf dimensions and anatomy structure:** For tissue sampling at the end of the flowering stage (ZGS

Cultivar



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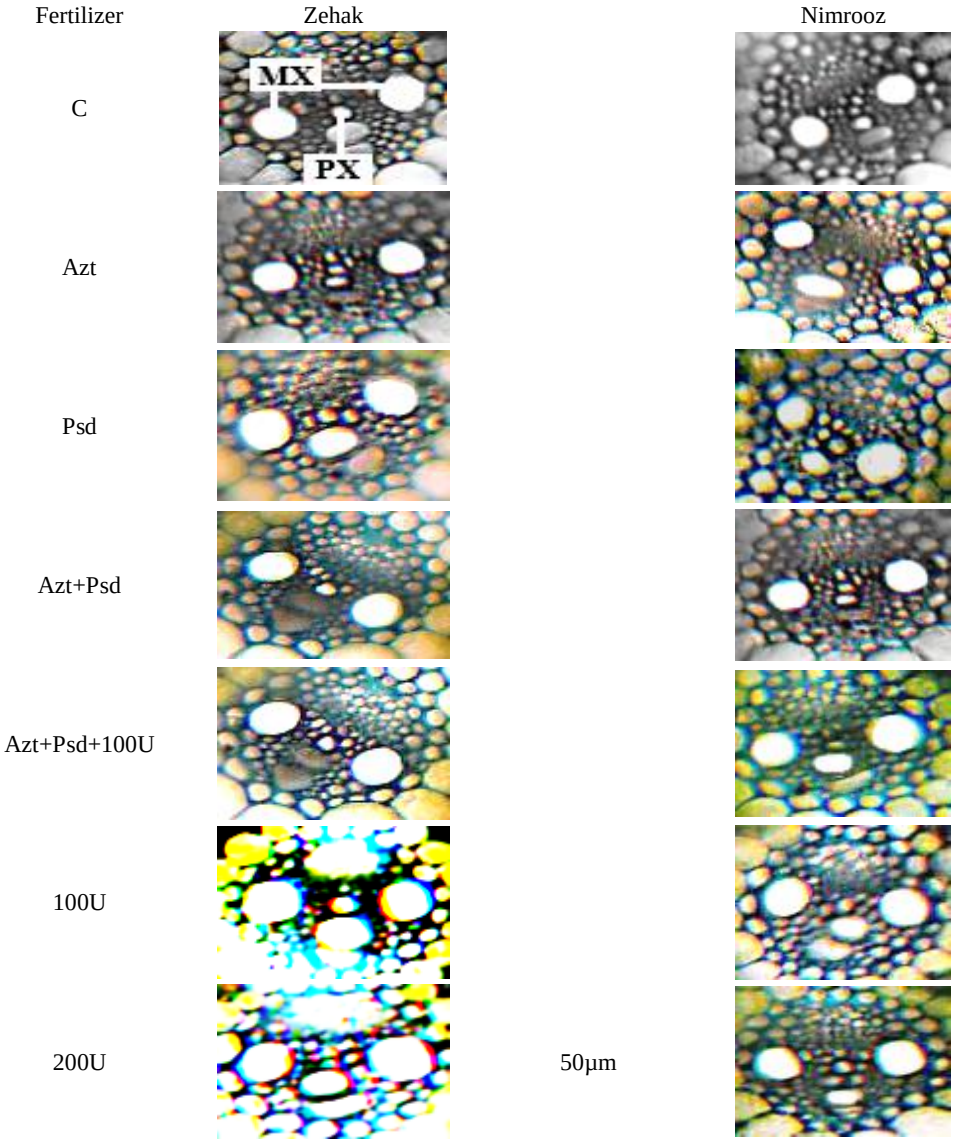


**Figure 1. Flag leaf cross-sectional micrographs of xylem, including metaxylem and protoxylem vessels of the midrib at the end of the flowering stage (ZGS 69), of two barley cultivars under different fertilizer treatments. Leaf sections were taken from 5-7 mm above the leaf base. The scale bar represents 50 µm. MX: Metaxylem, PX: Protoxylem. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas*; Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N source); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone); 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

69), the flag leaf was detached because leaf elongation has stopped at this stage (Hu *et al.*, 2000a). First, the area of the flag leaf was determined by a leaf area meter (Kaiser, RS1, Germany). Then, to investigate the flag leaf anatomy, sections were taken from 5-7 mm above the leaf base (Hu *et al.*, 2000b). Segments immediately were transferred to phosphate buffered saline (PBS) supplemented with 3% formaldehyde and incubated overnight. Samples were washed in PBS and dehydrated in a graded series of ethanol (Hu *et al.*, 2005). Then, segments were cut with a razor and stained with 0.5% toluidine blue (TB) for one minute (Hachez *et al.*, 2006). A Ceti bright-field microscope was used to observe leaf dimensions. Images were taken with a camera (X60 HS, Canon, Inc., Ota, Tokyo, Japan) to consider the midrib area (Figure 1) and stomatal dimensions of the flag leaf (Figure 2).

**Stomatal measurement:** Flag leaf stomatal area was evaluated by epidermal impression at the end of the flowering stage (ZGS 69) from 5-10 mm above the leaf base. Fingernail varnish was applied to the adaxial (upper) leaf surface and permitted to dry. Clear tape was used to peel the hardened varnish from the leaf, and then used to make a microscope slide for viewing (Wu and Zhao, 2017).

**Chlorophyll and carotenoid content**

Cultivar



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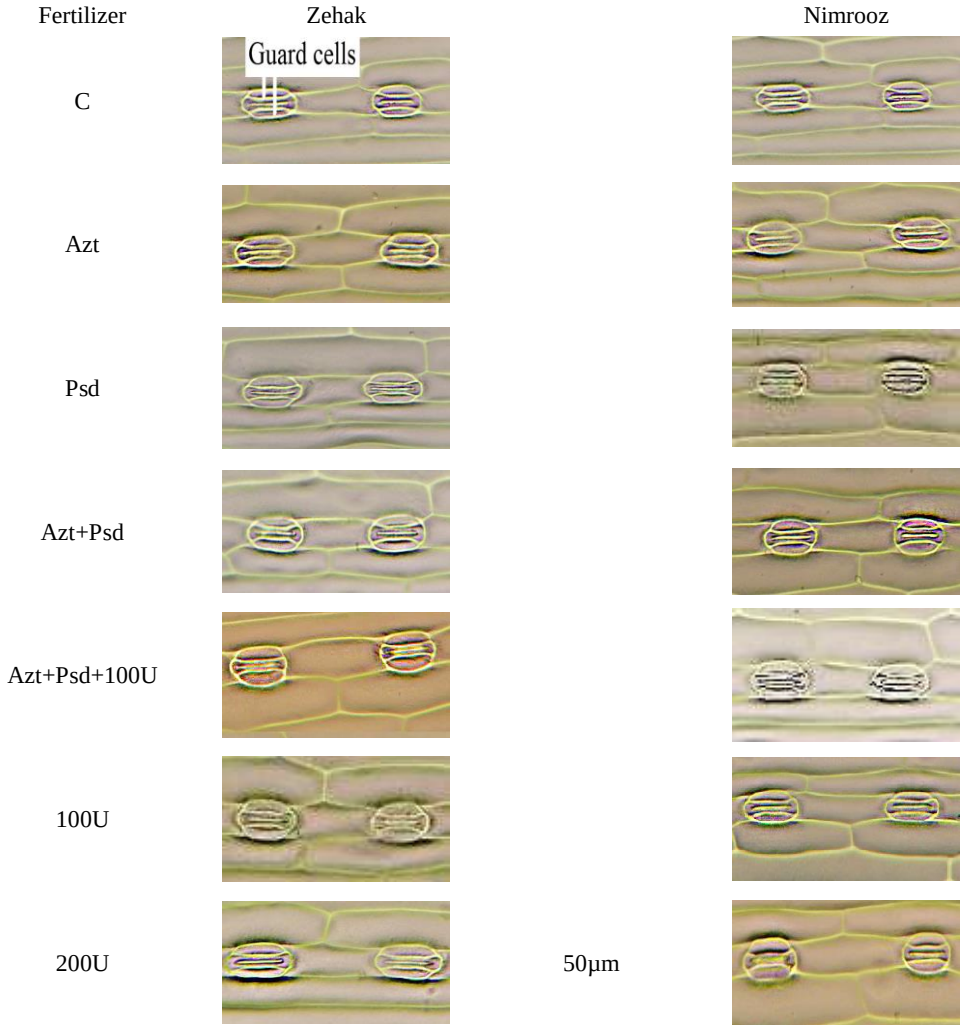


**Figure 2. Stomatal micrograph dimensions of flag leaf in two barley cultivars under different fertilizer treatments at the end of the flowering stage (ZGS 69). Leaf sections were taken from 5-10 mm above the leaf base. The scale bar represents 5 µm. C: Control (no inoculation); Azt: Seed inoculation by Azotobacter chroococcum alone; Psd: Seed inoculation by Pseudomonas fluorescens alone; Azt+Psd: Seed inoculation with a combination of Azotobacter and Pseudomonas; Azt+Psd+100U: Seed inoculation with a combination of Azotobacter and Pseudomonas with 50% crop N requirement (100 kg ha$^{-1}$ urea N source); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone); 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

**determination:** The chlorophyll content of the flag leaf was evaluated at the end of the flowering stage (ZGS 69). Chlorophyll was extracted using 10 ml of 80% acetone that was gradually added to 200 mg of leaf tissue, and ground with a mortar and pestle. After creating a homogenized solution, which was described by Arnon (1949), the absorbance was determined by a double-beam UV-VIS spectrophotometer (UV-1900, Shimadzu, Japan) at λ = 645, 663, and 470 nm. Then, the chlorophyll *a*, *b* and total (Arnon, 1949) and carotenoid (Lichtenthaler and Buschmann, 2001) were calculated for pigment assay.

**Leaf relative water content (RWC) measurement:** The leaf RWC was measured by the method of Machado and Paulsen (2001) at the end of the flowering stage (ZGS 69). Eight leaf discs (8 mm in diameter) were taken from fully expanded flag leaves in each pot, and were weighed for determination of fresh weight (FW).

Then, the leaf discs were put in deionized water for 6 h, and dried on filter paper and weighed for turgid weight (TW). The samples were oven dried for a dry weight measurement (DW). Finally, the RWC was determined as:

RWC= [(FW-DW)/ (TW-DW)] ×100

**Measurement of yield attributes and yield:** At the end of the ripening stage (ZGS 99), the plants in each pot were harvested manually, and plant height was measured in the laboratory. The samples were then oven dried at 72°C for 48 h, and grain and biological yield, and yield components consisted of 100-grain weight and grain number spike$^{-1}$ determined.

**Grain nutrient analysis:** After harvesting, the macro and micro-nutrients of barley grain were



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measured by the following methods. First, the dry grain of each pot was powdered by an electric mill, then ashed at 550˚C and digested with 2 M HCl. Total N content of the grain was measured by the Kjeldhal method (Nadeem *et al.,* 2006). Also, total P concentration was measured colorimetrically and total K by flame photometer (Corning 510, UK). Finally, the total concentration of micro-nutrients including Mn, Cu, Zn, and Fe in the acid extract was measured by atomic absorption spectroscopy (PG 990, PG Instruments Ltd. UK) (Boostani *et al.,* 2021).

**Statistical analysis:** Analysis of variance (ANOVA) was done on all experimental data and the means were compared by the least significant differences (LSD) test at $P \leq 0.05$ using SAS software 2012 (version 9.4).

## Results and discussion

**Grain nutrient content:** The type of cultivar and fertilizer treatments significantly affected the grain N, P and K contents ($P \leq 0.05$) (Table 1). The highest grain N content was observed in 200U, Azt+Psd+100U, and 100U treatments of Zehak (1.51-1.78%) which was significantly higher than in the Nimrooz cultivar (1.09-1.31%). Application of Azt alone or combined with Psd, enhanced grain N content more than Psd alone. All of the fertilizer treatments had significant effects on grain P content enchantment in comparison to control (Table 1). In both barley cultivars, the 200U, Azt+Psd+100U, 100U and Azt+Psd significantly increased the grain P content by 92-109% in Zehak and 83-106% in Nimrooz in comparison to control (Table 1). In Zehak and Nimrooz, application of each bacteria alone resulted in lower grain P content related to the co-application.

All of the fertilizer treatments in Zehak, enhanced grain K content significantly, while, it had less of an effect on grain K content improvement in Nimrooz. Also, in Zehak, Azt+Psd+100U treatment resulted in the highest grain K content (48% increase), with significant difference with 200U treatment (Table 1). In both of the cultivars, there were no significant differences in grain K content between Azt and Psd single strain treatments. Overall, the grain N and K contents of Zehak were more than Nimrooz (Table 1).

The fertilizer treatments significantly affected grain micronutrient contents such as Fe, Cu, Zn, and Mn ($P \leq 0.05$) (Table 2). The highest grain Fe contents ($P \leq 0.05$) were found in Azt+APsd+100U (137% increase) and Azt+Psd (130%) treatments of Zehak. In both cultivars, the urea chemical fertilizer treatments (100 and 200 U) had less of an effect on Fe content enhancement. In both cultivars, the highest grain Cu content ($P \leq 0.05$) was observed in the Azt+Psd+100U treatment. Similar to Cu, application of Azt or Psd alone, had less effect on Zn content compared to co-application of bacteria. In both cultivars, the Mn grain content was enhanced by all fertilizer treatments significantly (Table 2). The Mn content in all the fertilizer treatments of Zehak was higher than in Nimrooz, and achieved maximum values in the Azt+Psd+100U (61.2 mg $kg^{-1}$ DW) and 200U (60.3 mg $kg^{-1}$ DW) treatments.

Previous studies have described that seed inoculation by N-fixing bacteria resulted in a significant effect on grain micro- and macronutrient contents. The application of Azt and Psd biofertilizers has been shown to enhance N and or P availability, and the root dimensions, consequently promoting water and nutrient uptake from the rhizosphere (Mercado-Blanco *et al.,* 2016; Sultana *et al.,* 2016). Bageshwar *et al.* (2017) showed that seed inoculation by Azt alone in wheat, created more biomass and grain N content compared to the control. Sultana *et al*. (2016) reported that the application of *Pseudomonas putida* and *Azotobacter chroococcum* in sorghum promoted the uptake of Fe, Zn, and Cu, while the increase in Mn content was not significant. They suggested that the increase in nutrient uptake might be attributed to co-inoculation of these bacteria in promoting division and elongation of cell roots. The Psd strains are able to enhance P solubility by secretion of organic acids and phosphatase enzymes, which likely also enhances micronutrient availability. Shaharoona *et al.* (2008) found that in wheat, Psd could promote the N, P and K uptake in comparison to control. Jayant (2012) showed that the maximum content of N, P, K, Cu, and Zn in the leaf was obtained in combination with *Azotobacter chroococcum* + *Pseudomonas striata* + *Trichoderma viride*. Also, *Azotobacter chroococcum* and *Pseudomonas striata* were the second-best combination in achieving optimal growth of apple seedlings. Similar to our results, they found that Azt fixed additional N via roots from the atmosphere, and that *Pseudomonas striata* solubilized extra P, and that both of the bacteria enhanced K content. In the current study, Azt had a more positive effect on grain N content, while Psd more positively influenced grain P content. Biological N fixation by bacteria can enhance the synthesis of growth promoters and availability of micro- and macronutrients while decreasing the amount of ethylene, which is known as a growth inhibitor (Galindo *et al.,* 2020, Yadav and Sarkar, 2019). Likewise, P-fixing bacteria by modifying the root zone acidity can change the soil dissolved P to organic phosphorus acids and improve P availability of the soil (Delshadi *et al.,* 2017). These bacteria also can promote auxin and cytokinin production in the plant, which enhances nutrient uptake by roots. Generally, we found that barley grain yield was increased when Azt and Psd were applied together compared to the sole application. This was in relation to the better performance of the barley under co-application, demonstrating the presence of a synergistic effect between two bacteria in the rhizosphere.

**Flag leaf anatomy:** At the end of the flowering stage (ZGS 69), the cross-section areas of the midrib were sampled from 5-7 mm above the flag leaf base (Figure 1). Each midrib usually had two metaxylem and one protoxylem. The metaxylem area was influenced by



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**Table 1. Interaction effect of cultivar and fertilizer on grain macronutrient contents of two barley cultivars**

| Cultivar | Fertilizer | Grain N content (%) | Grain P content (%) | Grain K content (%) |
|---|---|---|---|---|
| Zehak | C | 0.54±0.03$^{g}$ | 0.102±0.006$^{e}$ | 1.36±0.05$^{d}$ |
| | Azt | 1.23±0.06$^{de}$ | 0.151±0.004$^{d}$ | 1.71±0.03$^{c}$ |
| | Psd | 1.01±0.02$^{f}$ | 0.182±0.008$^{bc}$ | 1.72±0.07$^{c}$ |
| | Azt+Psd | 1.41±0.03$^{cd}$ | 0.196±0.005$^{a}$ | 1.86±0.05$^{b}$ |
| | Azt+Psd+100U | 1.68±0.04$^{ab}$ | 0.213±0.006$^{a}$ | 2.01±0.02$^{a}$ |
| | 100U | 1.51±0.03$^{bc}$ | 0.202±0.002$^{a}$ | 1.71±0.06$^{c}$ |
| | 200U | 1.78±0.05$^{a}$ | 0.207±0.002$^{a}$ | 1.86±0.04$^{b}$ |
| Nimrooz | C | 0.41±0.03$^{h}$ | 0.112±0.001$^{e}$ | 1.03±0.03$^{h}$ |
| | Azt | 0.98±0.03$^{f}$ | 0.159±0.003$^{cd}$ | 1.24±0.03$^{fg}$ |
| | Psd | 0.73±0.02$^{g}$ | 0.192±0.004$^{b}$ | 1.20±0.01$^{g}$ |
| | Azt+Psd | 1.05±0.04$^{ef}$ | 0.206±0.006$^{a}$ | 1.31±0.02$^{efg}$ |
| | Azt+Psd+100U | 1.23±0.02$^{de}$ | 0.231±0.005$^{a}$ | 1.36±0.03$^{de}$ |
| | 100U | 1.09±0.01$^{ef}$ | 0.218±0.004$^{a}$ | 1.29±0.02$^{efg}$ |
| | 200U | 1.31±0.02$^{d}$ | 0.223±0.002$^{a}$ | 1.32±0.04$^{ef}$ |

**Means in each column followed by the same letters are not significantly different at the 5% probability level using the LSD test. The data included ±SE. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas;* Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone); 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

**Table 2. Interaction effect of cultivar and fertilizer on seed micronutrient contents of two barley cultivars**

| Cultivar | Fertilizer | Grain Fe content (mg kg$^{-1}$ DW) | Grain Cu content (mg kg$^{-1}$ DW) | Grain Zn content (mg kg$^{-1}$ DW) | Grain Mn content (mg kg$^{-1}$ DW) |
|---|---|---|---|---|---|
| Zehak | C | 40.1±2.9$^{i}$ | 9.6±0.11$^{i}$ | 30.2±1.3$^{h}$ | 30.1±0.98$^{h}$ |
| | Azt | 65.1±3.6$^{efg}$ | 14.3±0.15$^{g}$ | 35.6±1.5$^{g}$ | 49.8±1.1$^{de}$ |
| | Psd | 71.2±2.7$^{de}$ | 15.2±0.16$^{fg}$ | 40.1±1.9$^{f}$ | 52.3±2.1$^{cd}$ |
| | Azt+Psd | 92.3±3.1$^{a}$ | 18.6±0.12$^{c}$ | 55.3±2.1$^{a}$ | 55.6±2.2$^{bc}$ |
| | Azt+Psd+100U | 95.3±4.9$^{a}$ | 22.9±0.015$^{b}$ | 56.7±2.6$^{a}$ | 61.2±1.7$^{a}$ |
| | 100U | 85.4±3.5$^{b}$ | 17.2±0.16$^{d}$ | 50.2±2.4$^{bc}$ | 50.2±1.4$^{de}$ |
| | 200U | 83.1±3.1$^{b}$ | 19.3±0.18$^{c}$ | 50.9±3.1$^{b}$ | 60.3±1.5$^{ab}$ |
| Nimrooz | C | 47.2±2.4$^{h}$ | 11.5±0.09$^{h}$ | 39.5±2.7$^{g}$ | 20.3±1.9$^{i}$ |
| | Azt | 68.2±1.7$^{def}$ | 15.8±0.16$^{f}$ | 40.1±1.1$^{f}$ | 33.5±0.8$^{h}$ |
| | Psd | 70.1±2.8$^{def}$ | 16.7±0.13$^{e}$ | 44.6±1.3$^{e}$ | 40.3±1.4$^{g}$ |
| | Azt+Psd | 72.3±4.1$^{d}$ | 18.6±0.14$^{c}$ | 46.5±1.5$^{cd}$ | 45.9$^{e}$±0.9$^{f}$ |
| | Azt+Psd+100U | 76.3±3.5$^{c}$ | 24.3±0.13$^{a}$ | 49.2±1.8$^{bcd}$ | 49.6±0.7$^{de}$ |
| | 100U | 62.3±0.5$^{g}$ | 19.1±0.15$^{c}$ | 45.1±1.3$^{e}$ | 42.2±0.8$^{f}$ |
| | 200U | 63.9±0.6$^{f}$ | 19.7±0.12$^{c}$ | 46.1±0.9$^{de}$ | 43.1±0.6$^{f}$ |

**Means in each column followed by the same letters are not significantly different at the 5% probability level using the LSD test. The data included ±SE. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas;* Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N), 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

fertilizer treatments, so that the maximum areas were obtained in the 200U and Azt+Psd+100U treatments of Zehak, with no significant difference ($P \leq 0.05$) between them (Table 3 and Figure 1). The metaxylem area in Zehak was larger than in Nimrooz in all the fertilizer treatments. Similar to the metaxylem results, the highest protoxylem area was observed in 200U (34% increase) and Azt+Psd+100U (32% increase) of Zehak, which significantly ($P \leq 0.05$) differed from Nimrooz (Table 3 and Figure 1). In Zehak and Nimrooz, no significant difference was observed between Azt and Psd application alone in terms of protoxylem area. All the fertilizer treatments enhanced the flag leaf midrib area in comparison to the control, so that in 200U and Azt+Psd+100U increased 15-16% in Zehak and 20-22% in the Nimrooz cultivar (Table 3 and Figure 1). Application of Azt increased the midrib area more than Psd, while their combined application significantly increased the midrib area compared to their separate applications. Fertilizer treatments significantly affected flag leaf area, and the highest flag leaf area was obtained with 200U of Zehak (33% increase) and Nimrooz (18% increase). In both barley cultivars, application of Azt+Psd and/or 100U increased the flag leaf area, in comparison to application of bacteria, alone. Additionally, in each fertilizer treatment, the flag leaf area of Zehak was more than Nimrooz ($P \leq 0.05$).

Biofertilizers such as bacteria can release nutrients from a source, slowly compared to chemical fertilizers (Yadav and Sarkar, 2019). Also, the high cost of



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**Table 3. Metaxylem, protoxylem, midrib and total areas of flag leaf of two barley cultivars under different fertilizer treatments at the end of flowering stage (ZGS 69).**

| Barley cultivar | Fertilizer | Metaxylem area ($\mu m^2$) | Protoxylem area ($\mu m^2$) | Midrib area ($\mu m^2$) | Flag leaf area ($cm^2$) |
|---|---|---|---|---|---|
| Zehak | C | 3980±33$^{g}$ | 996±23$^{de}$ | 46491±126$^{f}$ | 6.03±0.26$^{k}$ |
| | Azt | 4541±56$^{d}$ | 1044±11$^{d}$ | 50236±143$^{c}$ | 6.51±0.11$^{h}$ |
| | Psd | 4347±75$^{e}$ | 1003±15$^{de}$ | 48278±101$^{d}$ | 6.54±0.16$^{h}$ |
| | Azt+Psd | 4736±66$^{b}$ | 1141±31$^{c}$ | 52721±66$^{b}$ | 7.15±0.15$^{ef}$ |
| | Azt+Psd+100U | 4955±96$^{a}$ | 1318±22$^{a}$ | 53844±91$^{a}$ | 7.89±0.11$^{b}$ |
| | 100U | 4703±86$^{bc}$ | 1226±10$^{b}$ | 52891±112$^{b}$ | 7.41±0.08$^{c}$ |
| | 200U | 4976±76$^{a}$ | 1341±24$^{a}$ | 53946±143$^{a}$ | 8.05±0.04$^{a}$ |
| Nimrooz | C | 3791±33$^{i}$ | 861±7$^{h}$ | 40102±145$^{j}$ | 6.15±0.09$^{j}$ |
| | Azt | 4021±29$^{fg}$ | 936±9$^{fg}$ | 42627±201$^{h}$ | 6.31±0.14$^{i}$ |
| | Psd | 3812±65$^{h}$ | 899±16$^{gh}$ | 41450±236$^{i}$ | 6.23±0.07$^{ij}$ |
| | Azt+Psd | 4113±45$^{f}$ | 984±20$^{ef}$ | 44765±213$^{g}$ | 6.89±0.03$^{g}$ |
| | Azt+Psd+100U | 4653±63$^{c}$ | 997±15$^{de}$ | 48493±146$^{d}$ | 7.08±0.05$^{f}$ |
| | 100U | 4404±13$^{e}$ | 911±17$^{fg}$ | 47641±211$^{e}$ | 7.21±0.09$^{de}$ |
| | 200U | 4647±49$^{c}$ | 1001±31$^{de}$ | 48944±91$^{d}$ | 7.24±0.10$^{d}$ |

**Means in each column followed by the same letters are not significantly different at the 5% probability level using the LSD test. The data included ±SE. Leaf sections were taken from 5-7 mm above the leaf base. MX: Metaxylem, PX: protoxylem. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas;* Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg $ha^{-1}$ urea N); 100U: Application of 50% crop mineral N requirement (100 kg $ha^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg $ha^{-1}$ urea N source alone).**

chemical synthesized fertilizers for crop producers has encouraged some researchers to consider biofertilizers as a suitable strategy to replace or combine them with chemical fertilizers (Vaxevanidou *et al.,* 2015). Many researchers have previously shown that tissue architecture variations are related to stress level, plant type, and variety. Some abiotic stresses, such as water stress or nutrient deficiency, have also been shown to significantly decrease leaf photosynthesis and pigment contents, which subsequently negatively affects plant performance (Bijanzadeh *et al.,* 2022; Sitko *et al.,* 2019). However, little knowledge has been published on the effect of plant nutrients on flag leaf dimensions and midrib area. In peanut (*Arachis hypogaea* L.), water deficit declined leaf cross section area by 89% (Sankar *et al.,* 2013). The variation in cross-sectional area of the third leaf of barley was observed under salt stressed plants, especially at 0-25 mm from the leaf base. Different vein types play different roles in the leaf, and midribs are mostly for water transport (Bijanzadeh and Kazemeini, 2014).

**Stomata size:** The stomatal area of flag leaves from 5-10 mm above the leaf base of the two barley cultivars under different fertilizer treatments at the end of the flowering stage (ZGS 69) is shown in Figures 2 and 3. The chemical fertilizer application significantly enhanced the stomatal area compared to the other treatments, so that in Zehak, it increased by 47 and 40% in 200U and 100U treatments. In the fertilizer treatments, stomatal area in Zehak was higher than in Nimrooz. In Nimrooz, there were no significant differences ($P \leq 0.05$) among the Azt+Psd+100U, 100U, and 200U treatments in terms of stomatal area. Fu *et al.* (2013) declared that regardless of growth stage, severe water stress declines stomatal size of eggplant (*Solanum melongena* L.). In strawberry (*Fragaria* × *ananassa* Duch.), a smaller stomatal size was observed in plants grown under water stress (Klamkowski and Treder, 2006). Likewise, under water deficit, stomata are smaller than under well-watered conditions due to decreased water transpiration (Sarker and Hara, 2011). This decline in stomatal aperture may therefore negatively affect biomass production. In a similar study on corn, Sitko *et al.* (2019) showed that macronutrient deprivation (Ca, K, Mg, and P) had an adverse effect on stomata properties, pigment content, photosynthetic characteristics, and water transpiration. Bijanzadeh *et al.* (2022) declared that under water stress, application of 1.0 mM silicon at the seedling stage, increased the stomatal area of corn by 24% in comparison to the control. In our study, differences in flag leaf stomatal size depended on cultivar type and fertilizer treatments so that, under nutrient deficiency, stomatal size declined, sharply due to lower flag leaf.

**Chlorophyll and carotenoid contents:** The mean data of the interaction effect of barley cultivar × fertilizer on chlorophyll *a*, *b* and carotenoid is shown in Table 4. In both of the barley cultivars, the maximum chlorophyll *a* content was observed in Azt+Psd+100U and 200U, while in Nimrooz these treatments did not differ significantly ($P \leq 0.05$). In both cultivars, the co-application of biofertilizers with 100U, significantly increased the chlorophyll *a* content compared to the application of Azt and or Psd, alone. Also, in Zehak, chlorophyll *b* content in Azt+Psd+100U, 100U, and 200U treatments reached maximum values, increasing by 177-261% compared to the control. The combination of Azt with Psd increased total chlorophyll (Figure 4) and carotenoid contents (Table 4) in comparison to the control. There were significant differences in total chlorophyll content between the single strain treatments compared to the control. Azt was more effective in



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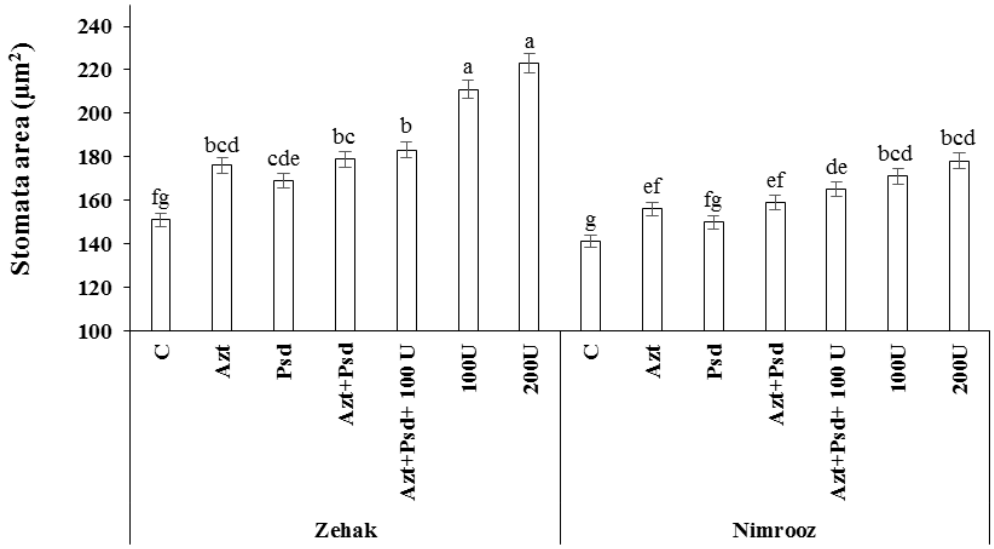


**Figure 3. Stomatal area of flag leaf in two barley cultivars under different fertilizer treatments at the end of the flowering stage (ZGS 69). Means followed by the same letters are not significantly different at the 5% probability level using the LSD test. Vertical bars represent ±SE. Leaf sections were taken from 5-10 mm above the leaf base. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas*; Azt+Psd+100U: seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N source); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

**Table 4. Interaction effect of cultivar and fertilizer on chlorophyll *a*, *b* and carotenoid content of flag leaf of two barley cultivars under different fertilizer treatments at the end of flowering stage (ZGS 69).**

| Cultivar | Fertilizer | Chlorophyll a (mg g$^{-1}$ FW) | Chlorophyll b (mg g$^{-1}$ FW) | Carotenoid (mg g$^{-1}$ FW) |
|---|---|---|---|---|
| Zehak | C | 0.192±0.05$^{g}$ | 0.136±0.06$^{ef}$ | 0.256±0.02$^{c}$ |
| | Azt | 0.320±0.04$^{de}$ | 0.202±0.04$^{de}$ | 0.315±0.01$^{bc}$ |
| | Psd | 0.2360.06$^{ef}$ | 0.1580.02$^{ef}$ | 0.323±0.02$^{b}$ |
| | Azt+Psd | 0.332±0.06$^{cde}$ | 0.229±0.03$^{cd}$ | 0.342±0.03$^{b}$ |
| | Azt+Psd+100U | 0.568±0.03$^{b}$ | 0.377±0.02$^{a}$ | 0.469±0.02$^{a}$ |
| | 100U | 0.432±0.02$^{c}$ | 0.386±0.03$^{a}$ | 0.478±0.05$^{a}$ |
| | 200U | 0.733±0.05$^{a}$ | 0.429±0.05$^{a}$ | 0.536±0.01$^{a}$ |
| Nimrooz | C | 0.1620.03$^{g}$ | 0.101±0.01$^{f}$ | 0.251±0.02$^{c}$ |
| | Azt | 0.297±0.04$^{ef}$ | 0.181±0.02$^{de}$ | 0.298±0.02$^{bc}$ |
| | Psd | 0.201±0.02$^{f}$ | 0.134±0.01$^{ef}$ | 0.286±0.01$^{bc}$ |
| | Azt+Psd | 0.301±0.02$^{ef}$ | 0.192±0.02$^{de}$ | 0.301±0.03$^{bc}$ |
| | Azt+Psd+100U | 0.534±0.04$^{b}$ | 0.227±0.01$^{cd}$ | 0.486±0.02$^{a}$ |
| | 100U | 0.407±0.07$^{cd}$ | 0.295±0.03$^{bc}$ | 0.477±0.03$^{a}$ |
| | 200U | 0.586±0.09$^{b}$ | 0.306±0.02$^{b}$ | 0.511±0.02$^{a}$ |

**Means in each column followed by the same letters are not significantly different at the 5% probability level using the LSD test. The data included ±SE. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas;* Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone); 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

increasing chlorophyll content, especially in Zehak (59%) compared to Nimrooz (21%) (Figure 4). In both cultivars, the highest carotenoid content was observed in Azt+Psd+100U (83-93% increase), 100U (86-90% increase), and 200U (103-109% increase) in comparison to the control (Figure 4).

Nitrogen-fixing bacteria, such as Azt, improve nutrient availability in plants, which increases pigment contents, especially chlorophyll (Nosheen *et al.*, 2016; Qessaoui *et al.*, 2019). Haneef *et al.* (2014) found that *in Plantago ovate*, the total leaf chlorophyll and carotenoid contents were enhanced by inoculation of seeds with Azt alone or combined with arbuscular mycorrhiza fungi (AMF). Rahimi *et al.* (2019) also found that the highest chlorophyll content in cephalaria (*Cephalaria syriaca* L.) was achieved with Azt with AMF treatment. Vatanpour *et al.* (2021) demonstrated that in the Gonbad wheat cultivar, the highest amount of



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chlorophyll *a* (6.31 mg/g FW) and total chlorophyll (7 mg $g^{-1}$ FW) was obtained by applying Azt, and in terms of chlorophyll *b*, the lowest amount (0.57 mg $g^{-1}$ FW) was observed from the use of Psd. Whereas, they found that Azt had a noticeable effect on the pigment contents of the Karim wheat cultivar. Similar to our findings, they concluded that the type of cultivar and bacteria influenced the pigment content and grain yield. In the current study, Zehak reacted more positively in terms of pigment increase to bacteria treatments than the Nimrooz cultivar. N-fixing bacteria is able to enhance the N uptake and its availability through the root system, which enhances photosynthetic pigments, directly (Sanchez *et al*., 2021; Zeffa *et al*., 2018). Overall, nitrogen is one key component of chlorophyll, so that a positive relationship was obtained between nitrogen uptake and chlorophyll content of leaves (Davaran-Hagh *et al.,* 2015; Niazi *et al.,* 2021). Bio-fertilizers have a main function in chlorophyll creation through increasing the activity of pyridoxal enzyme. In fact, this enzyme has a main role in the synthesis of α-aminolevulinic acid, which is an important compound in chlorophyll structure (Kahil *et al.,* 2017).

**Leaf relative water content (RWC):** The RWC was significantly affected by barley cultivar × fertilizer interaction (Figure 5). The highest RWC ($P \leq 0.05$) was obtained in the Azt+Psd+100U and 200U treatments, which increased by 48 and 47%, respectively. A similar trend was observed in the Nimrooz cultivar, so that the RWC in control was increased from 48.7% to 65.7% and 66.3% in Azt+Psd+100U (34% increase) and 200U (36% increase) treatments, respectively (Figure 5). Also, the RWC of Zehak in all the fertilizer treatments was higher than in the Nimrooz cultivar. Overall, Zehak and Nimrooz alone or in combination with Azt+Psd increased the RWC significantly.

The RWC is a suitable index to monitor the water status of the leaf and is directly related to cell turgor of the mesophyll. Also, cell division and elongation are mainly dependent on the cell turgidity, especially at the initial growth zone, and influence RWC and yield components positively (Sitko *et al.,* 2019). Kamali and Mehraban (2020) found that the Nitroxin application (mixture of Azt + *Azospirillum lipoferum*) with AMF on sorghum enhanced leaf RWC, especially under water stress. Kazeminasab *et al.* (2016) asserted that biofertilizers had an important function in enhancing RWC and assimilate production by increasing nutrient uptake, especially at the reproductive stage. In the present study, the single or co-application of Azt and Psd increased the RWC in barley, which directly related to grain yield improvement.

**Plant height, yield components, and yield:** Application of the biofertilizers alone or combined increased the plant height of both barley cultivars; however, Azt alone had a greater effect than Psd alone (Figure 6). In each fertilizer treatment, the plant height in the Nimrooz two-rowed barley cultivar was significantly greater than the Zehak six-rowed cultivar. In both cultivars, 200U, Azt+Psd+100U, and Azt+Psd had the greatest plant height compared to the other fertilizer treatments (Figure 6). Increment of plant height has a key function in remobilization of pre-anthesis of assimilate to grain at the grain filling stage (Sharma *et al.,* 2015). Hernandez-Montiel *et al.* (2020) observed that seed inoculation by a combination of rhizobacterial strains of *Pseudomonas putida* with 75% chemical fertilization dosage enhances the fresh weight and plant height of bell pepper (*Capsicum annuum* L.) by 25 and 19%, respectively, compared to 100% chemical fertilizer dosage. In a field study, Lally *et al.* (2017) reported that the Psd application enhanced the plant height and seed and oil yield of canola (*Brassica napus* L.). Sudhakar *et al.* (2008) stated that a single application of Psd enhanced the plant height of safflower (*Carthamus tinctorius* L.), which was equal to recommended synthesized N fertilization. In accordance with our results, Espidkar *et al.* (2017) demonstrated that Psd strains influence the plant height of barley positively.

The grain number $spike^{-1}$ is one of the most important yield components in grain yield determination. The highest ($P \leq 0.05$) grain number $spike^{-1}$ was obtained in the 200U and Azt+Psd+100U treatments in Zehak (Table 5). In contrast, Nimrooz had a lower grain number $spike^{-1}$ which increased from 17.1 in the control to 30.8 in 200U. In both barley cultivars, combined application of Azt with Psd significantly enhanced grain number $spike^{-1}$ in comparison to single-strain application. The changes in 100-grain weight to cultivar × fertilizer interactions were smaller than in grain number $spike^{-1}$, especially in Zehak (Table 5). The highest 100-grain weight was obtained in Azt+Psd, Azt+Psd+100U, and 200U treatments of the Nimrooz cultivar (34-39% increase compared to control). The interaction effect of cultivar × fertilizer significantly influenced biological yield (Table 5). In Zehak, combined application of Azt with Psd significantly increased the biological yield compared to single strain application, while in Nimrooz there was no significant difference ($P \leq 0.05$) between these treatments. The highest biological yield was observed in the 200U and Azt+Psd+100U treatments, resulting in increases of between 89-91% in Zehak and 28-31% in Nimrooz.

The cultivar type and fertilizer treatments had noticeable effects on grain yield (Figure 7). The application of Azt and Psd alone, significantly enhanced the grain yield of both barley cultivars compared to the control. In the control treatments, Zehak (six-rowed barley) created a higher grain yield in comparison to Nimrooz (two-rowed barley). In Zehak, the highest grain yields ($P < 0.05$) were observed in the 200U treatment (108% increase) and Azt+Psd+100U treatment (102% increase). Similarly, in Nimrooz, the highest grain yields were observed in 200U (142% increase) and Azt+Psd+100U (138% increase). In both barley cultivars, application of Azt or Psd alone causes lower grain yield in comparison to combined



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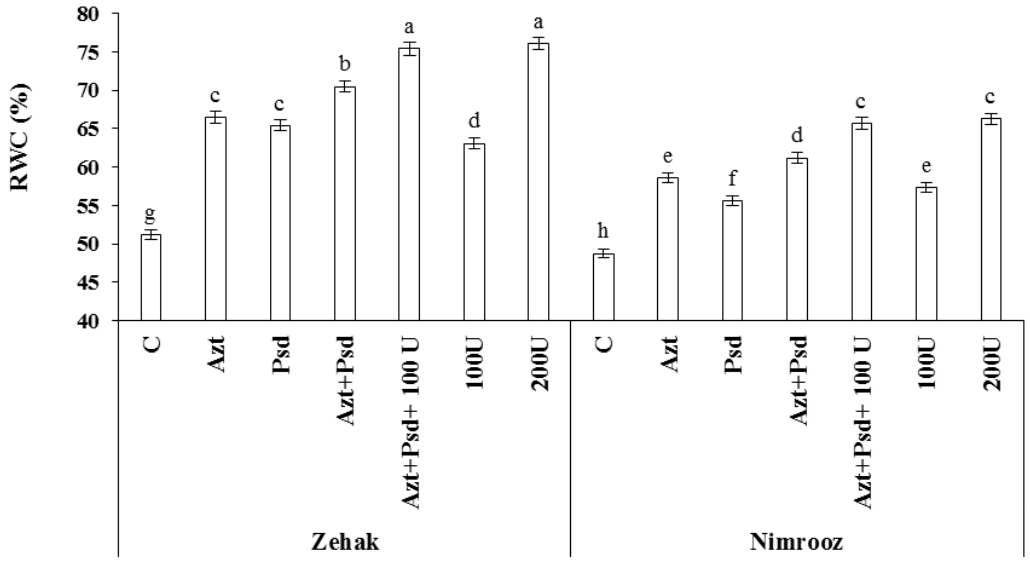


**Figure 5. Effect of barley cultivar and fertilizer on relative water content (RWC) of flag leaf. Means followed by the same letters are not significantly different at the 5% probability level using the LSD test. Vertical bars represent ±SE. Leaf sections were taken from 5-10 mm above the leaf base. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas*; Azt+Psd+100U: seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N source); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

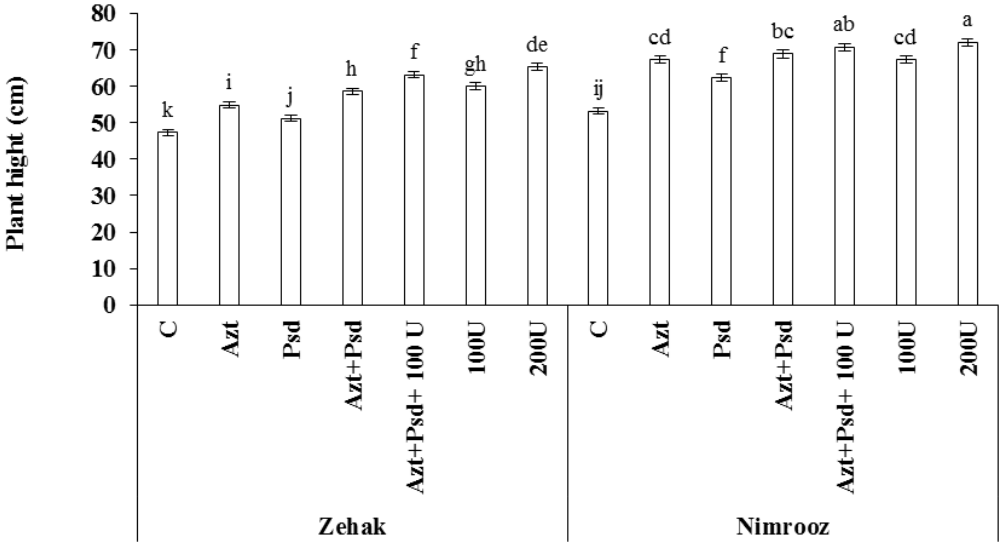


**Figure 6. Effect of barley cultivar and fertilizer on plant height. Means followed by the same letters are not significantly different at the 5% probability level using the LSD test. Vertical bars represent ±SE. Leaf sections were taken from 5-10 mm above the leaf base. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas*; Azt+Psd+100U: seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N source); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

application of them (Figure 7).

Enhancement in plant N availability and grain yield due to the application of Azt as N-fixing bacteria has been previously declared by some researchers (Jnawali *et al.*, 2015; Nosheen *et al.*, 2016; Sultana *et al.*, 2016). Also, the grain yield improvement may be correlated to the role of Azt or Psd in increasing nutrient uptake of Fe and P (Jnawali *et al.*, 2015). Improvements in grain yield may also be related to auxin secretion by plant growth stimulating microorganisms (Mayak *et al.*, 2004). Bageshwar *et al.* (2017) reported that using Azt caused a 60% increase in grain yield of wheat. They also found that the co-application of Azt with urea resulted in maximum grain yield by applying 85 kg



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**Table 5. Interaction effect of cultivar and fertilizer on yield components and biological yield of two barley cultivars.**

| Cultivar | Fertilizer | Grain no. spike$^{-1}$ | 100-grain weight (g) | Biological yield (g plant$^{-1}$) |
|---|---|---|---|---|
| Zehak | C | 22.3±0.5$^{i}$ | 3.21±0.09$^{fg}$ | 1.79±0.03$^{g}$ |
| | Azt | 30.2±0.6$^{e}$ | 3.62±0.11$^{e}$ | 2.60±0.02$^{ef}$ |
| | Psd | 34.8±0.4$^{d}$ | 3.11±0.08$^{g}$ | 2.53±0.02$^{f}$ |
| | Azt+Psd | 36.9±0.7$^{b}$ | 3.66±0.05$^{e}$ | 3.16±0.03$^{c}$ |
| | Azt+Psd+100U | 38.2±0.2$^{a}$ | 3.82±0.08$^{d}$ | 3.39±0.01$^{b}$ |
| | 100U | 36.2±0.3$^{c}$ | 3.33±0.07$^{f}$ | 2.76±0.02$^{d}$ |
| | 200U | 38.2±0.4$^{a}$ | 3.92±0.06$^{d}$ | 3.43±0.02$^{ab}$ |
| Nimrooz | C | 17.1±0.2$^{j}$ | 3.13±0.07$^{g}$ | 1.47±0.03$^{h}$ |
| | Azt | 25.3±0.3$^{g}$ | 4.11±0.05$^{bc}$ | 2.82±0.04$^{d}$ |
| | Psd | 24.3±0.2$^{h}$ | 4.15±0.04$^{bc}$ | 2.70±0.03$^{de}$ |
| | Azt+Psd | 26.9±0.4$^{f}$ | 4.22±0.02$^{abc}$ | 3.01±0.01$^{d}$ |
| | Azt+Psd+100U | 29.6±0.5$^{e}$ | 4.35±0.03$^{a}$ | 3.47±0.03$^{b}$ |
| | 100U | 25.2±0.6$^{g}$ | 4.09±0.05$^{c}$ | 2.78±0.02$^{d}$ |
| | 200U | 30.8±0.4$^{e}$ | 4.26±0.07$^{ab}$ | 3.54±0.04$^{a}$ |

**Means in each column followed by the same letters are not significantly different at the 5% probability level using the LSD test. The data included ±SE. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas;* Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

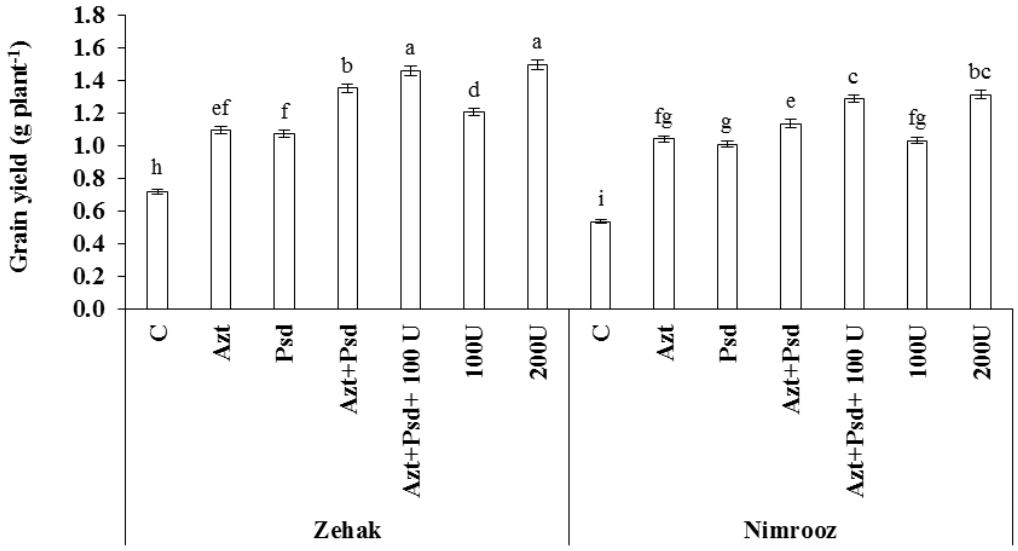


**Figure 7. Effect of barley cultivar and fertilizer on grain yield. Means in each column followed by the same letters are not significantly different at the 5% probability level using the LSD test. The data included ±SE. C: Control (no inoculation); Azt: Seed inoculation by *Azotobacter chroococcum* alone; Psd: Seed inoculation by *Pseudomonas fluorescens* alone; Azt+Psd: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas;* Azt+Psd+100U: Seed inoculation with a combination of *Azotobacter* and *Pseudomonas* with 50% crop N requirement (100 kg ha$^{-1}$ urea N); 100U: Application of 50% crop mineral N requirement (100 kg ha$^{-1}$ urea N source alone), 200U: Application of 100% crop mineral N requirement (200 kg ha$^{-1}$ urea N source alone).**

less urea compared to the recommended dose (257 kg U ha$^{-1}$). In another study on wheat seed inoculated with Azt helps in N, P, Fe and Zn uptake (Rajaee *et al.,* 2007). In addition, Azt and Psd can stimulate the synthesis of cytokinins and auxins, and these plant hormones, which originate from the root surface, are the primary substances controlling plant growth (Qessaoui *et al.,* 2019; Sultana *et al.,* 2016; Vatanpour *et al.,* 2021).

In a similar study on barley, Mercado-Blanco *et al.* (2016) reported a significant improvement in the grain number spike$^{-1}$ and 1000-grain weight up to 19 and 20%, respectively, by application of Psd. Some researchers reported a positive relationship between application of Azt and Psd bacteria and biological yield increment in plants (Chiquito-Contreras *et al.,* 2017, Espidkar *et al.,* 2017, Mercado-Blanco *et al.,* 2016). Vatanpour *et al.* (2021) reported that Psd and Azt application influenced the above-ground biomass of barley, which it attributed to improvement of soil properties in rhizosphere. Messele and Pant (2012) reported that in chickpea (*Cicer arietinum* L.) seed



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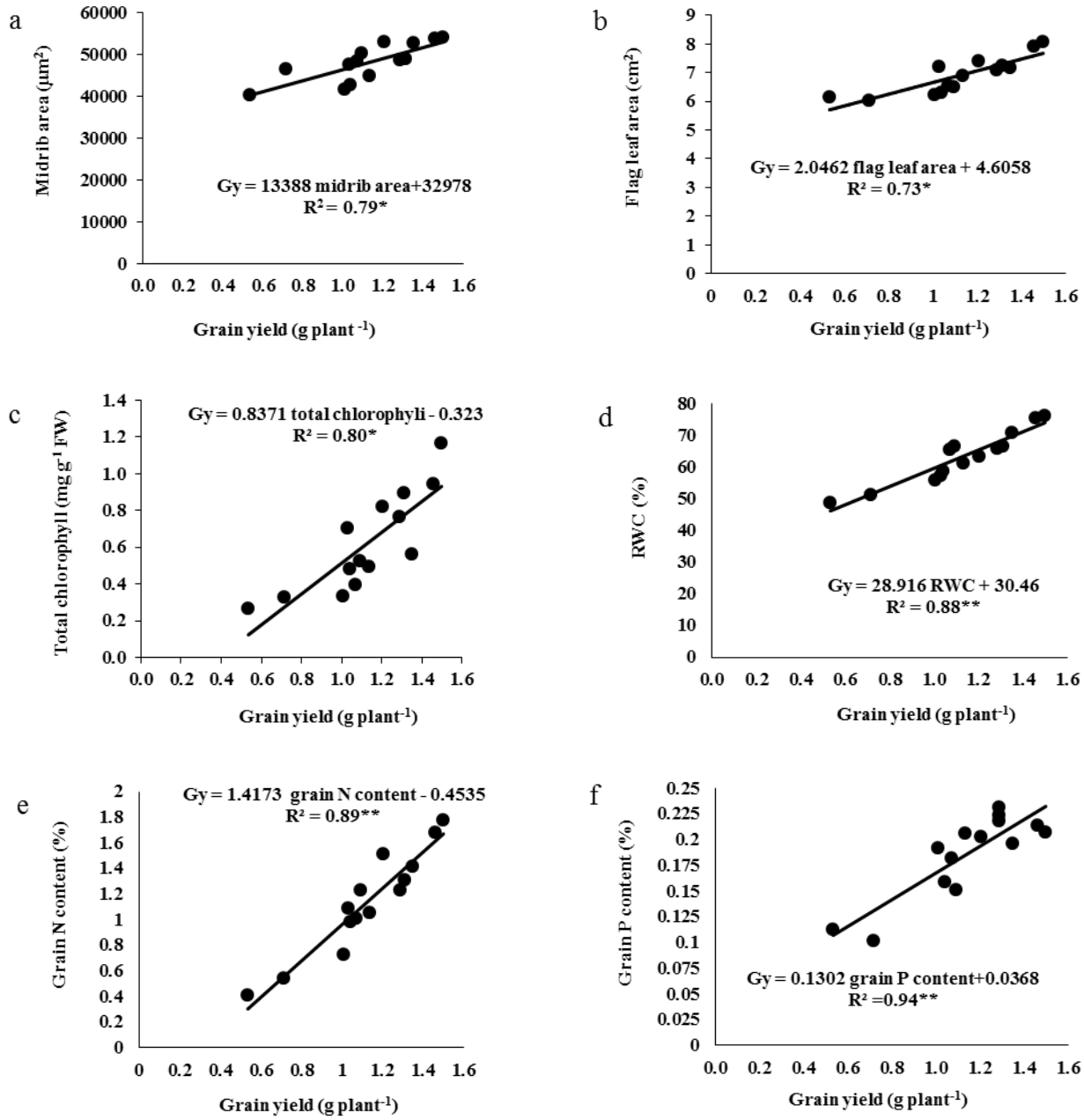


**Figure 8. Relationship between grain yield and midrib area (a), flag leaf area (b), total chlorophyll (c), RWC (d), grain N content (e), and grain P content (f) of barley.**

inoculation with Psd improved P content and biological yield. Indeed, Psd by mineralizing of the organic P and solubilizing of inorganic phosphates, can release of organic acids, which stimulate plant growth (Frohlich *et al.*, 2012). Qessaoui *et al.* (2019) in a study by evaluating 19 new rhizobacteria Psd isolates reported that some isolates increased tomato by growth promoting processes, such as phytohormones production, solubilization of insoluble P, ammonia production and colonization of root in the rhizosphere. In the current study, in both barley cultivars, single application of Azt and Psd could not meet the N requirement of barley, however in these treatments yield improves between 48 to 51% in Zehak and 87 to 92% in Nimrooz, in comparison to control. While the combination of Azt+Psd+100U showed maximum yield increment by 102 and 138% in Zehak and Nimrooz, respectively. There was a greater relative grain yield increase from the lower yielding Nimrooz cultivar as result of the combined application of Azt+Psd with half dose of mineral N fertilizer application (100U) than in Zehak cultivar. Thus, it is possible to reduce urea application by using Azt+Psd seed inoculation.

**Relationship between grain yield and other traits:** A linear relationship between grain yield and midrib area of flag leaf is shown in Figure 8a. By increasing the midrib area from 40101 to 53946 µm$^2$, grain yield improved sharply, so that it significantly correlated to



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midrib area ($R^2$=0.79$^*$). Similarly, flag leaf area significantly linearly correlated to grain yield ($R^2$=0.73$^*$) (Figure 8b). A highly significant ($R^2$=0.80$^{**}$) and positive relationship was observed between grain yield and total chlorophyll, by the following equation (Figure 8c):

Gy= 28.916 RWC +30.46

Likewise, a significant ($R^2$=0.88$^{**}$) and linear relationship was observed between the grain yield and RWC in the range of 48.7 to 76.1% (Figure 8d). The grain N content increment from 0.41 to 1.78 (%) resulted in a highly significant increase in the grain yield from 0.54 to 1.50 g plant$^{-1}$ ($R^2$=0.89$^{**}$) (Figure 8e). Similar to N content, a highly significant ($R^2$=0.94$^{**}$) and positive correlation was obtained between grain yield and grain P content (Figure 8f).

## Conclusions

Application of *Azotobacter* with *Pseudomonas* combined with urea affected the leaf anatomy, pigment content, relative water content, and nutrient uptake. This combination improved the metaxylem and protoxylem, were the main compartments of the midrib in water transport and nutrient uptake along the flag leaf. The flag leaf area, midrib area of the flag leaf, total chlorophyll, relative water content, and grain nitrogen and phosphorus content related to grain yield positively, which demonstrated the importance of these traits in yield improvement. In fact, biofertilizers combined with urea, by increasing the midrib dimensions, improve nutrient uptake and water, which causes grain yield enhancement, especially in Zehak. This combination can satisfy the N and P demand of barley, resulting in a significant positive effect on barley characteristics. Barley producers can practically apply the co-application of *Azotobacter* with *Pseudomonas* with 50% of the recommended dose of urea (100 kg ha$^{-1}$) to obtain optimal barley yields but at a potentially reduced economic and environmental cost. Further research will be needed to investigate the effect of the other N and P-fixing bacteria on plant anatomy and soil characteristics of barley.

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