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Impact of Biofertilizers Application on Improving Barley (*Hordeum vulgare*) and the Presence of Dehydrin Genes

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ABSTRACT

Two field experiments were conducted during two successive seasons, 2006-2007 and 2007-2008, to investigate the effectiveness of biofertilizer treatment i.e., plant growth-promoting rhizobacteria species (*Azospirillum brasilense*, *Azotobacter chrococcum*, and *Klebsiella pneumoniae*), chemical fertilizer and their combination on four barley (*Hordeum vulgare*) cultivars. These cultivars showed a characteristic response under different fertilizer treatments. In general, 'Sahrawy' and 'KSU 101' produced the highest grain yield. 'KSU 101' gave the highest harvest index (43.3%) in the second season. Also, 'KSU 102' and 'KSU 101' had more grains per spike. The application of nitrogen alone (at 200 kg N ha^{-1}) and biofertilizer treatment (a mixture of three growth-promoting nitrogen-fixing rhizobacteria) significantly increased grain yield and grains per spike of all four barley cultivars. SSR markers for the amplification of dehydrin genes revealed the presence of two dehydrin genes (*HVDHN7* and *HVDHN9*). 'Sahrawy' had homologous dehydrin genes and produced higher grain yield.

Keywords: barley cultivars, *HVD* genes, plant growth-promoting rhizobacteria, SSR markers

INTRODUCTION

Barley ranks second after wheat among the most important crops in Saudi Arabia (Motawei and Abdalla 2003). In order to increase crop production, the system is relying exclusively on the use of chemical fertilizers. The use of chemical fertilizers and other pesticides has caused tremendous harm to the environment by pollution and water and soil contamination (Al-Redhaiman *et al.* 2005). There is thus a need to search for alternative methods of increasing plant production in an eco-friendly manner, with adequate management of natural, renewable resources and to reduce chemical inputs (Al-Otayk *et al.* 2008). Without doubt, all chemical fertilizers support plant growth and development, resulting in higher production, and are thus beneficial if used judiciously, such as bio-fertilizers that are considered to be totally safe and now used in most countries because of their environmentally-friendly fertilizer property. Bio-fertilizers are 100% natural and organic fertilizers that enrich the nutrient quality of soil and are organisms that help to provide and keep in the soil all the nutrients and micro-organisms required for the benefits of plants (http://www.biofertilizer.com/biofertilizer.html). Bacteria, fungi and blue-green algae (*Cynobacteria*) are the main sources of bio-fertilizers. Symbiosis of these organisms with plants is the most striking relationship, in which the partners derive benefits from each other, without any damage/hazards. The disease-causing organisms (pathogenic bacteria, fungi, etc.) are totally different to the non-pathogenic strains used in bio-fertilizers.

Secretion of vitamins and amino acids, auxins, and fixing atmospheric nitrogen by *Azotobacter* and *Azospirillum* are among the direct mechanisms of increasing root development and plant growth (Radwan 2002; Khavazi *et al*. 2005; Akbari *et al*. 2007). Secretion of siderophores and hydrogen cyanides and antibiotics that control some plant diseases are additional effects of improving the growth rate and yields of crops such as wheat and barley (Khavazi *et al*. 2005). Recent studies detected the synergistic effects of plant growth-promoting rhizobacteria (PGPR) (such as *Azospirillum* and *Azotobacter*) and *Rhizobium* on nodulation and nitrogen fixation of legumes (Tilak *et al*. 2006). Also, Canbolat *et al*. (2006) showed that PGPR strains as bio-fertilizer stimulated barley growth and could be used as an alternative to chemical fertilizer. Kaci *et al*. (2005) reported that these microorganisms deliver a number of benefits, including plant nutrition and tolerance to adverse soil conditions.

Moreover, barley production is limited by the availability of water resources. Plants have developed different strategies to face water deficit and over the past few years much attention has been focused on the identification of genes induced in response to environment stress (Zhu 2002). Inheritance studies, QTL analysis, in several crop plants have revealed apparent co-segregation of dehydrin genes (*HVA*) with phenotypes associated with dehydrative stress, such as drought (Sivamani *et al.* 2000). Among genes induced by drought and low-temperature stress, dehydrin proteins are produced in response to drought, low temperature, and salinity. Dehydrins are intracellular stabilizers, acting upon targets in both the nucleus and cytoplasm (Svensson *et al.* 2002; Koag *et al.* 2003). Dehydrin loci are multigenic and present in clusters on different chromosomes in barley (Tommasini *et al*. 2008).

The current study aimed to: (1) investigate the effect of a mixture of three growth-promoting nitrogen-fixing rhizobacteria, namely *Azospirillum brasilense*, *Azotobacter chrococcum*, and *Klebsiella pneumoniae* on barley yield and its components, and (2) to investigate the presence of the dehydrin gene as a marker of drought tolerance in barley cultivars.

MATERIALS AND METHODS

Field trials

Field experiments were conducted at the Agricultural Research Station, College of Agriculture and Veterinary Medicine, Al-

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Table 1 Four barley cultivars studied for response to biofertilizer and chemical fertilizer applications.

Barley cultivars	Source
KSU. BL. 101	King Saud Univ., Saudi Arabia
KSU. BL. 102	King Saud Univ., Saudi Arabia
Gustoe	American
Sahrawy	ACR- Egypt

Qassim University, Saudi Arabia, during 2006/2007 and 2007/2008 winter seasons. Four barley cultivars (**Table 1**) were sown on the 1st and 15th of December 2006 and 2007, respectively, with a seeding rate of 140 kg h^{-1}. The plot size was 4 × 3 m^2 with a row-to-row spacing of 25 cm. The four fertilizer treatments used were: (1) 200 kg N h^{-1} applied as calcium nitrate (15% N); (2) 100 kg N h^{-1} applied as calcium nitrate (15% N) and PGPR species (*Azos. brasilense*, *Azot. chrococcum*, and *K. pneumoniae*) as biofertilizer at the rate of 2.5 kg h^{-1}; (3) PGPR species as biofertilizer at a rate of 2.5 kg h^{-1}; and (4) control (no nitrogen application). A split-plot in a randomized complete block design with three replicates was used. The four fertilizer treatments were considered as main plots, and the four barley cultivars were regarded as sub-plots. A computerized drip irrigation system was designed and built for this experimental field.

At harvesting time, 10 plants were randomly chosen to measure plant height, spike length, the number of spikelets per spike and the number of grains per spike. Also, harvest index and grain yield per square meter were recorded.

DNA extraction

Leaf samples from barley cultivars were used. The sample of leaves was first ground into a fine powder with liquid nitrogen. DNA was extracted in 10 ml of CTAB buffer consisting of 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA, and 1% CTAB. The homogenate was incubated for 2 hrs at 65°C with occasional mixing. Following incubation, 5 ml of chloroform: isoamylalcohol (24: 1) was added to the tubes, mixed, and centrifuged at 2600× *g* for 10 min. The aqueous phase was removed to a fresh tube and an equal volume of ice-cold isopropanol was added followed by centrifugation as above to precipitate the DNA. The pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The DNA concentration was assessed spectrophotometrically at 260 nm, and quality was assessed by the 260/280 ratio (Sambrook *et al.* 1989). The DNA was suspended to a final concentration of 10 ng/µL in 0.5X TE and stored at 4°C.

Simple sequence repeat (SSR) marker for dehydrin genes

SSR primers for amplification of dehydrin genes were *HVDHN9* (X152572, gene for dehydrin-9) and *HVDHN7* (X71362, gene for dehydrin-7). These primers were designed on the basis of published sequences (Becker and Heun 1995). Amplification was carried out in 25 µL reaction volumes, containing 1X *Taq* polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$) and 1 U of *Taq* polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each dNTPs (Pharmacia Biotech, Germany), 25 pmol primer, and 50 ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for 1 cycle of 30 s at 94°C, 40 cycles of {1 min at 94°C, 1 min at 55°C, and 1 min at 72°C} followed by 5 min at 72°C.

Statistical analysis

Standard analysis of variance was applied to all data, using PC SAS, version 6.12 (SAS Institute, Carry, NC). Comparisons of means were based on Duncan's Multiple Range test at ($P \leq 0.05$) for the main effects of each dependent variable.

RESULTS AND DISCUSSION

Grain yield and its components

The results presented in **Tables 2** and **3** indicate that barley cultivars showed different characteristic responses under different fertilizer treatments. In general, 'Sahrawy' and 'KSU 101' produced the highest grain yield (386.5 and 392.7 g/m^2) in the first and second seasons, respectively. 'KSU 101' gave the highest harvest index (43.3%) in the second season. 'Sahrawy' and 'Gustoe' were the tallest plants (83 and 81 cm) and (69.25 and 69.5 cm) in both seasons, respectively while the shortest plants were 'KSU 101' (67.4 cm) in the first season, and 'KSU 102' (59.12 cm) in the second season. 'KSU 102' and 'KSU 101' had more grains per spike (40.16 and 38.41, respectively) in the first season (**Table 3**). In the second season, 'Sahrawy', 'KSU 101', and 'KSU 102' had more grains per spike (45.1, 43.4, and 42.7, respectively). Also, the latter three cultivars had the highest spike length and number of spikelets per spike in the second season (**Table 3**).

There were differences in plant height among fertilizer treatments (**Table 2**). 200 kg N h^{-1} resulted in the tallest plants (81.75 and 72 cm) in both seasons, respectively. Plots that received no nitrogen had the shortest plants (67 and 63.5 cm in season 1 and 2, respectively).

Application of nitrogen alone (at 200 kg N h^{-1}) and biofertilizer treatment (mixture of three PGPR) significantly increased grain yield. This response was 71.4 and 70.4% in the first season and 82.9 and 30.9% in the second season, respectively as compared with the control. The incorporation of chemical fertilizer and biofertilizer increased grain yield by 41.8 and 19.5% in season 1 and 2, respectively. A possible explanation for these results may be due to enhanced root development in the early growth stages under biofertilizer treatment, which may be one of the factors responsible for the increases in yield (El-Hawary *et al*. 2002).

The number of spikelets per spike and grains per spike of the barley cultivars were affected by microbial treatment (a mixture of three PGPR) and application of N at a rate of 50 and 100% and their interactions (**Table 3**). Application of N alone (at 200 kg N h^{-1}) gave the highest number of

Table 2 The main effects of fertilizer treatments and barley cultivars on plant height, grain yield and harvest index during 2006/2007 and 2007/2008 seasons.

Treatments	Plant height (cm)		Grain Yield g/m^2		Harvest index (%)	
	2006/2007	2007/2008	2006/2007	2007/2008	2006/2007	2007/2008
Barley cultivars						
KSU. BL. 101	67.4 b	59.25 b	332.5 b	392.7 a	34.0 a	43.3 a
KSU. BL. 102	77.5 a	59.12 b	374.4 a	352.4 b	33.6 a	39.5 ab
Gustoe	83.0 a	69.25 a	386.5 a	359.1 ab	35.0 a	35.6 b
Sahrawy	81.0 a	69.50 a	386.0 a	342.1 b	32.0 a	35.3 b
Fertilizer treatments						
Control	67.0 b	63.5 b	253.5 c	271.2 c	33.3 a	39.7 a
Calcium nitrate	81.8 a	72.0 a	434.5 a	496.0 a	33.3 a	36.0 b
biofertilized + calcium nitrate	80.8 a	58.9 b	359.5 b	324.0 bc	33.3 a	40.1 a
Biofertilizer treatment	80.0 a	62.7 b	432.0 a	355.0 b	35.0 a	38.0 b

Data are expressed as mean
Means within the same column and followed by the same coefficient are not significant different from each other ($p \leq 0.05$).

Table 3 The main effects of fertilizer treatments and barley cultivars on number of grain per spike, spike length, number of spikelets per spike during 2006/2007 and 2007/2008 seasons.

Treatments	No. of Grains/Spike		Spike length (cm)		No. of Spikelets/Spike	
	2006/2007	2007/2008	2006/2007	2007/2008	2006/2007	2007/2008
Barley cultivars						
KSU. BL. 101	38.41 ab	43.4 a	6.0 a	8.0 a	14.50 a	16.73 ab
KSU. BL. 102	40.16 a	42.7 a	6.5 a	8.0 a	14.86 a	16.83 a
Gustoe	36.60 b	45.1 a	6.6 a	7.6 ab	13.38 a	16.7 ab
Sahrawy	37.17 b	37.9 b	6.5 a	7.0 b	13.43 a	14.3 b
Fertilizer treatments						
Control	33.65 c	33.1 c	6.25 a	6.5 c	12.37 b	13.25 c
Calcium nitrate	41.89 a	49.6 a	6.63 a	8.5 a	14.86 a	19.10 a
biofertilized + calcium nitrate	36.70 bc	41.7 b	6.25 a	7.5 b	14.4 a	15.0 bc
Biofertilizer treatment	40.16 ab	44.7 ab	6.50 a	8.1 ab	14.53 a	17.25 ab

Data are expressed as mean
Means within the same column and followed by the same coefficient are not significant different from each other ($p \leq 0.05$).

spikelets spike^{-1} (14.86 and 19.1) and number of grains spike^{-1} (41.89 and 49.6) in season 1 and 2, respectively. It was followed by biofertilizer treatment without a significant difference. These results might be due to that PGPR species stimulate barley growth and could be used as an alternative to chemical fertilizer (Canbolat *et al*. 2006). Also, the results of Ozturk *et al.* (2003) suggest that the application of *A. brasilense* Sp246 may be potentially used as a biofertilizer for spring wheat and barley cultivation in organic and low-N input agriculture.

SSR marker for dehydrin gene

Dehydrins (DHNs) are one of the typical families of proteins that occur in plants as a consequence of dehydration and osmotic stress (Sivamani *et al*. 2000). The dehydrin genes were amplified from barley cultivars using *HVDHN7* and *HVDHN9* primers (**Fig. 1**). It should be noted that 'Sahrawy' had one band, while the other cultivars had two bands when *HVDHN7* primers were used (**Fig. 1**). Therefore, 'Sahrawy' was homologues for the dehydrin-7 gene. Provan *et al*. (1999) concluded that codominant SSR polymorphism can be detected without time-consuming and laborious processes usually associated with SSR development and characterisation. Moreover, polymorphism existed among barley cultivars at the dehydrin-9 gene locus (**Fig. 1**). *HVDHN7* and *HVDHN9* microsatellite markers were previously mapped to the chromosome 7(5H) region (Liu *et al*. 1996). Brini *et al*. (2007) concluded that the observed differential phosphorylation pattern of *HVDHN-5* in resistant and sensitive wheat varieties could be used as a basis for molecular screening of tolerance/sensitivity to drought and salt stresses in wheat germplasm. In addition, transgenic rice plants over-expressing the barley dehydrin gene *HVA*1 show enhanced tolerance to water and salt stress (Xu *et al*. 1996). The co-localization of QTLs controlling water-status and/or turgor with sequences corresponding to *DHN* genes on the same portion of chromosome 6H, was a great indication of the possible role of these genes in the variation of plant water-status under drought conditions (Teulat *et al*. 2002). DHNs are water-soluble lipid-associated proteins that accumulate in response to dehydration, low temperature, osmotic stress, or during seed maturation (Close *et al*. 1989). Several QTLs controlling tolerance traits, and particularly freezing tolerance, have already been identified close to *DHN* genes (Campbell and Close 1997). The first example was a QTL for winter-hardiness overlapping with a cluster of *DHN* genes, including *HVDHN1* on barley chromosome 5H associated with a cold-specific induction of a member of this DHN family (Pan *et al*. 1994; van Zee *et al*. 1995). Borovkova *et al*. (1998) detected a linkage between leaf rust resistance gene *Rph9* and the microsatellite marker dehydrin-9 (*HVDHN9*) at a distance of 10.2 cM in the Bowman x Hor 2596 cross. All of the *DHN* genes have been shown to be upregulated in barley under stress with no expression observed in well watered plants (Choi *et al*. 1999).

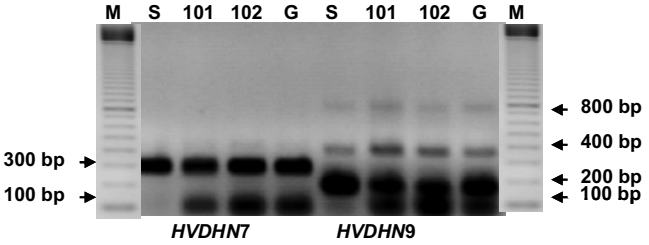

Fig. 1 Detection of dehydrin gene in wheat cultivars using SSR primers (*HVDHN*7 and *HVDHN*9). Cultivars of barley, S = 'Sahrawy', 101 = 'KSU.BL.101', 102 = 'KSU.BL.102', G = 'Gustoe'. M line is kbp DNA marker.

The genes *dhn2* and *dhn6* showed large increases in expression under polyethylene glycol stress in sorghum. The expression was greatest after 3 hrs of stress and declined after 27 hrs although the levels were still much higher than the control (Buchanan *et al*. 2005). In Suprunova *et al.* (2004) high correlations between the rates of expression level of these genes and the level of tolerance of tested barley varieties were detected. Evaluation of *Dhn1* gene expression could be used for preliminary prediction of sensitivity of barley genotypes to drought stress at a genetic level (Mikulková *et al.* 2007). Tommasini *et al*. (2008) concluded that the expression of 13 barley *Dhn* genes mirrored the global clustering of all transcripts, with specific combinations of *Dhn* genes providing an excellent indicator of each stress response.

In this study, 'Sahrawy' had homologous *DHN* genes and produced high grain yield. Therefore, SSR markers for amplifying the *DHN* gene could be valuable for barley breeding programs for selection of desirable *DHN* alleles under drought stress. In addition, the PGPR N-fixing rhizobacteria, namely *Azos. barasilense*, *Azot. chrococcum*, and *K. pneumoneaeon* may have the potential to be used as a biofertilizer for barley cultivation in organic agriculture.

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