Journal of Plant Interactions

ISSN: 1742-9145 (Print) 1742-9153 (Online) Journal homepage: https://www.tandfonline.com/loi/tjpi20

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To cite this article: Razieh Khalilzadeh, Raouf Seyed Sharifi & Jalal Jalilian (2016) Antioxidant status and physiological responses of wheat (*Triticum aestivum* L.) to cycocel application and bio fertilizers under water limitation condition, Journal of Plant Interactions, 11:1, 130-137, DOI: 10.1080/17429145.2016.1221150

To link to this article: https://doi.org/10.1080/17429145.2016.1221150

Published online: 23 Aug 2016.

JOURNAL OF PLANT INTERACTIONS, 2016
VOL. 11, NO. 1, 130–137
http://dx.doi.org/10.1080/17429145.2016.1221150

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RESEARCH ARTICLE

OPEN ACCESS

Antioxidant status and physiological responses of wheat (*Triticum aestivum* L.) to cycocel application and bio fertilizers under water limitation condition

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ABSTRACT

In order to study antioxidant status and physiological responses of wheat to cycocel (CCC) and bio fertilizers application under water limitation condition, a factorial experiment was conducted based on randomized complete block design with three replications in 2015. Treatments included water limitation in three levels [normal irrigation (I_1) as control; moderate water limitation (I_2) or irrigation withholding at 50% of heading stage; severe water limitation (I_3) or irrigation withholding at 50% of booting stage]; four bio fertilizer levels [(no bio fertilizer (F_0), seed inoculation by *Azotobacter chrocoocum strain* 5 (F_1), *Pseudomonas putida strain* 186 (F_2), *Azotobacter* + *Pseudomonas* (F_3))] and four CCC levels [(without CCC as control (C_0), application of 400 (C_1), 800 (C_2) and 1200 (C_3) mg/l)]. The results showed that water limitation decreased the chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, carotenoid, stomata conductance, leaf area index (LAI) and relative water content of wheat, but activity of catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO) enzymes and proline content were increased. Similar results were observed in CAT, POD and PPO activities due to bio fertilizers and CCC application. Besides the water limitation effects, CCC-treated plants displayed a significant decrease in stomata conductance and LAI. Generally, it was concluded that the application of bio fertilizers and CCC can be a proper tool for increasing wheat yield under water limitation.

ARTICLE HISTORY
Received 9 March 2016
Accepted 2 August 2016

KEYWORDS
Catalase; growth regulator; stomatal resistance; water deficit

1. Introduction

Drought stress is one of the most important abiotic factors that can limit plant growth and yield. The response of plants to water limitation has been evaluated based on genetic, biochemical and morpho-physiological traits. Among them, relative water content (RWC), antioxidant enzymes activity, chlorophyll and proline content, stomata conductance have been used as indicators of plant stress (Maccaferri et al. 2011).

Exposure of plants to stress is known to induce the formation of reactive oxygen species (ROS), which are involved not only in damage mechanisms but also in cell growth processes (Bernstein et al. 2010). ROS such as superoxide, hydrogen peroxide and hydroxyl radical are highly reactive and can seriously disrupt normal metabolism through oxidative damage of lipids, proteins and nucleic acids (Ashraf 2009). Also water deficit can damage pigments and plastids, reduce chlorophyll-*a*, chlorophyll-*b* and other carotenoids, hydrolyze proteins and prevalent photochemical reactions in most plants (Reddy et al. 2004). Recent investigations have shown that chlorophyll and its derivatives act as antioxidants to prevent oxidative DNA damage and lipid peroxidation both by chelating reactive ions and by scavenging free radicals (Hsu et al. 2013).

To minimize the damages due to oxidative stress, plants have evolved a complex enzymatic and non-enzymatic antioxidant systems, such as low-molecular mass antioxidants (glutathione and carotenoids) and ROS-scavenging enzymes [superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)]. The enzymatic components may directly scavenge ROS or may act by producing a non-enzymatic antioxidant (Apel & Hirt 2004). An increase in the activity of anti-oxidative enzymes under salinity and water limitation could be indicative of an increased production of ROS and a build-up of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants (Meloni et al. 2003).

Morpho-physiological changes occur rapidly after the onset of water limitation in order to maintain high tissue water potential. One of the swiftest responses is a reduction of transpiration through reduced leaf area following stomata closure. This response is often associated with an accumulation of abscisic acid (ABA) or enhancement of sensitivity to this hormone in the leaf cells, leading to the induction of related signaling genes (Harb et al. 2010). Rodriguez et al. (2005) reported that chlorophyll content, leaf area and stomatal conductance declined under drought stress.

Several strategies have been developed in order to decrease the toxic effects caused by severe water limitation on plant growth. Among them the use of bio fertilizers such as plant growth promoting rhizobacteria (PGPR) plays a very important role in yield improvement. Some PGPR strains produce cytokinin and antioxidants, which result in ABA accumulation and degradation of ROS (Timmusk & Wagner 1999). Also it is reported that inoculated seeds with PGPR showed high antioxidant enzyme activities against water and salt stress (Wang et al. 2012). Heidari and Golpayegani (2012) reported that PGPR application increased the proline,

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chlorophyll and water content of basil (*Ociumum basilicum* L.) under water stress conditions.

Proline has a function of osmotic adjustment in plants, but it also protects enzymes and membranes against oxidative stress (Agarwal & Pandey 2004). A higher level of proline was observed in PGPR-treated plants subjected to drought stress (Heidari & Golpayegani 2012). El-Zieny et al. (2001) indicated that the application of bio fertilizers such as *Azotobacter* improves leaf area and plant growth.

It is well known that cycocel (CCC) treatment could induce changes in the physiological traits of wheat plants under stress condition (Meera & Poonam 2010) and may increase wheat yield and quality. Hoque and Haque (2002) reported that CCC prevents ent-kaurene synthesis in the GA_3 biosynthetic cycle leading to GA_3 deficiency and the subsequent reduced vegetative growth potential. Nejadsahebi et al. (2010) reported that the application of CCC in water limitation, increased RWC and stomata resistance. Wang and Xiao (2009) imply that treatment of plants with CCC may increase the number of chloroplasts, elevate the concentration of chlorophyll and carotenoids, accelerate the process of photophosphorylation, and stimulate the photosynthetic rate. CCC has the ability to delay senescence of leaf, arresting chlorophyll degradation and promoting the synthesis of soluble proteins and enzymes, resulting in more assimilation surface area (Attia 2004).

A better understanding of wheat antioxidant status and physiological responses may help programs whose objective is to improve the grain yield under water limitation. Therefore, the aim of this study was to evaluate the effects of bio fertilizers and CCC on the physiological responses (i.e. antioxidant enzyme activity, chlorophyll, proline, chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, carotenoid and stomata conductance) of wheat under water limitation.

2. Materials and methods

2.1. Materials used in experiment

A factorial experiment was conducted based on randomized complete block design with three replications in 2015. The area is located at 38°15′N latitude and 48°15′E longitude with an elevation of 1350 m above mean sea level. Climatically, the area is situated in the semi-arid temperature zone with cold winter and moderate summer in north-western Iran. Treatments included water limitation in three levels [normal irrigation (I_1) as control; moderate water limitation (I_2) – irrigation withholding at 50% of heading stage; severe water limitation (I_3) –irrigation withholding at 50% of booting stage], bio fertilizer at four levels [without inoculation (F_0) as control, inoculation with *Azotobacter chrocoocum* strain 5 (F_1), *Pseudomonas putida* strain 186 (F_2) and *Azotobacter* + *Pseudomonas* (F_3)] and foliar application of cycocle at four levels [without cycocle (C_0) as control, application of 400 (C_1), 800 (C_2) and 1200 (C_3) mg/l]. The soil was silty clay, with pH about 8.2 and EC about 2.68 dS/m. There were 5 rows with 2 m long in each plot. Plots and blocks were separated by 1 m unplanted distances. Fertilizer basic dose of N.P.K. at the rate of 90–70–60 kg/ha was applied in the form of urea, triple super phosphate, and nitrate potassium. All of phosphorus and potassium were applied at the time of seedbed preparation. Nitrogen fertilizer was applied as ½ at sowing, ½ at 6–8 leaves. The wheat cultivar 'Atila 4' was used in the experiment with a plant density of 400 seeds/m^2. Seeds were sown on 10 April 2015. For inoculation, seeds were coated with gum Arabic as an adhesive and rolled into the suspension of bacteria until uniformly coated (Seyed Sharifi & Khavazi 2011). The strains and cell densities of microorganisms used as PGPR in this experiment were 1×10^8 colony forming units. Foliar application with CCC was done in two stages of period growth (4–6 leaf stage and before the booting stage). At the mid of the booting stage, the flag leaves of plants were separated for measuring the following determinations (Zayed et al. 2014).

2.2. Photosynthetic pigment content assay

Chlorophyll content was measured in 0.2 g fresh leaf tissue, and gradually adds about 10 ml of acetone 80%. Then it was centrifuged for 10 min at 400 rpm and the absorbance at 645, 663, and 470 nm was recorded by a spectrophotometer. Chlorophyll and carotenoids were obtained based on the following equations (Arnon 1949):

$$\text{Chlorophyll-}a = (19.3 \times \text{A663} - 0.86 \times \text{A645})\text{V}/100\ \text{W}$$

$$\text{Chlorophyll-}b = (19.3 \times \text{A645} - 3.6 \times \text{A663})\text{V}/100\ \text{W}$$

$$\text{Total Chlorophyll} = \text{Chlorophyll-}a + \text{Chlorophyll-}b$$

$$\text{Carotenoid} = (1000\ \text{A470} - 1.82\ \text{C}_a - 85.02\ \text{C}_b)$$

2.3. Protein assay and antioxidant enzymes activity

The evaluation of protein was carried out by Bradford's (1976) method; 0.2 g of the plant tissue was squashed with 0.6 ml of extraction buffer and was centrifuged at 11,500 rpm for 20 min at 4°C. The supernatant was transferred to the new tubes and centrifuged for 20 min at 4000 rpm. To measure the protein amount, 10 µl of the obtained extract was added to 5 µl of Bradford solution and 290 µl of extraction buffer and the absorbance rate was read at 595 nm. To measure the enzyme activity, 0.2 g of fresh tissue was used for the enzyme activity. Also, in order to extract the protein, 0.2 g of fresh tissue was crushed using liquid nitrogen and then 1 ml of Tris-HCl buffer (0.05 M, pH = 7.5) was added. The obtained mixture was centrifuged for 20 min (13,000 rpm and 4°C), then the supernatant was used for enzyme activity measurements (Sudhakar et al. 2001).

2.3.1. Catalase assay

CAT activity was assayed according to Karo and Mishra (1976). Sixty microliter of the protein extract was added to Tris buffer (50 mM, pH = 7) and 0.3 ml H_2O_2 5 mM in the ice bath, then the absorbance curve was read at 240 nm. The enzyme activity was obtained for (OD/mg protein/min) from fresh tissue.

2.3.2. Peroxidase assay

POD activity was measured as described by Karo and Mishra (1976). Fifty microliters of protein extracts was added to 2.5 ml of extraction buffer, containing 100 µM Tris buffer 100 mM and H_2O_2 5 mM and 10 mM pirogalol in the ice bath and absorbance was read at 425 nm.

2.3.3. Polyphenol oxidase assay

Polyphenol oxidase enzyme activity was measured by Karo and Mishra's (1976) method, as follows: 100 µl of the protein extract was dissolved in 1.5 ml Tris 0.2 M and 0.3 ml pirogallol 0.02 M and the resulting composition was placed in the bain-marie bath at 25°C for 5 min and then the absorbance at 420 nm was recorded.

2.4. Stomatal conductance, relative water content and LAI assay

The fully developed flag leaf of the main tillers was randomly selected from five plants of each plot for the determination of stomata conductance with a leaf Porometer (Model SC-J Eijkelkamp, Netherlands). At this stage, RWC was estimated according to the method of Tambussi et al. (2005). Leaf area index (LAI) was determined at the flowering stage on the fully developed flag leaf by dividing the leaf area over ground area. Proline content was determined based on the method of Bates et al. (1973). At plant maturity, grain yield in each plot was harvested, three central rows each 1 m long.

A factorial experiment was conducted based on randomized complete block design with three replications. Analysis of variance and mean comparisons were performed using SAS computer software packages. The main effects and interactions were tested using the least significant difference (LSD) test at the 0.05 probability level.

3. Results and discussion

Analysis of variance showed a significant interaction effect between irrigation and bio fertilizer on the CAT, POD and PPO enzymes (Table 1). Interaction of water limitation and CCC significantly affected chlorophyll-*b*, stomata conductance and LAI (Table 1). Chlorophyll-*b* and total chlorophyll were affected by the interaction of bio fertilizer and CCC (Table 1). There were significant interactions between irrigation, bio fertilizer and CCC on proline and grain yield (Table 1).

Our results showed that the highest chlorophyll-*a* and total chlorophyll content (6.50 and 8.73 mg/g FW, respectively) was obtained in application of CCC and bio fertilizer as C_3F_3. Whereas the lowest values (4.02 and 4.92 mg/g FW, respectively) were observed in control treatment (C_0F_0) (Table 2). There was an increase of about 168% in the content of chlorophyll-*b* in I_3C_3 application in comparison with I_3C_0 (Table 3). Results showed that the application of bio fertilizer as F_3 increased carotenoid content by about 12% in comparison with control. Water limitation decreased the carotenoid content, and the lowest (0.22 mg/g FW) was observed under severe water limitation (Table 1). Liu et al. (2011) reported a decrease in carotenoid content due to drought stress.

Water limitation caused the reduction in total chlorophyll, while the application of bio fertilizers increased this trait values. Chlorophyll loss was shown to be accompanied by the damage of the mesophyll chloroplasts, which led to a lower photosynthetic rate (Wang & Xiao 2009).

Several studies reported that chlorophyll content is higher in plants treated with bio fertilizer (Belimov et al. 2009). Also the reduction in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation (Oraki et al. 2012). El-Ghinbihi and Hassan (2007) found that drought stress caused reduction in photosynthetic pigments [(chlorophyll-*a*, chlorophyll-b, total chlorophyll (*a* + *b*) and carotenoids)] of pepper plants. Relative chlorophyll content has a positive relation with photosynthetic rate. Probably, the positive effect of CCC on enzyme activity results in an increase in the chlorophyll content (Memari et al. 2011). It is defined that under severe water limitation, foliar application of CCC as C_3 reduces stomatal conductance by 26% (Table 7) and also partial stomatal closure can lead to a decrease in transpiration and, possibly, an increase in chlorophyll-*b* content (Table 3). Also high chlorophyll content under the application of CCC due to the small leaf area could be considered as an avoidance mechanism which minimizes water losses (Rodriguez et al. 2005).

Also, it has been suggested that lower stomatal conductance and LAI in the application of CCC as C_3 led to higher chlorophyll-*a*, chlorophyll-*b* and total chlorophyll contents (Table 1). The increased level of total chlorophyll concentration in leaves of all the CCC-treated plants might be due to the influence of growth retardant on delaying leaf senescence (Guerfel et al. 2009), chlorophyll synthesis by high Rubisco activity (Osman 2014), promoting the synthesis of soluble proteins and enzymes (Wafsy & El-Din 1995). CCC may also promote cell growth by causing a decrease in the osmotic potential of cells (Attia 2004). Moreover, the increase in chlorophyll in the presence of bio fertilizers could be due to the effective symbiosis and positive effects of bio fertilizers on growth and development of plants (Namvar et al. 2013).

Means comparison showed the maximum proline (9.66 mg/g FW) was obtained in severe water limitation, application bio fertilizers as F_3 and CCC as C_3 (Table 4). Therefore, the lowest proline (5.25 mg/g FW) was observed in normal irrigation and application of bio fertilizers as F_0 and CCC as C_0 (Table 4). When plants were subjected to water limitation conditions, the proline content of leaves increased. Furthermore, under water limitation, bio fertilizer inoculation enhanced the proline and water contents of plants under stress conditions. Many studies have shown that the presence of high proline levels in tolerant plants contribute to the occurrence of osmotic adjustment. However, in most cases, osmotic adjustment was not the main consequence of proline accumulation, which was involved in other mechanisms such as protection against oxidative damage (De Campos et al. 2011). In the same line, Chookhampaeng et al. (2008) stated that the accumulation of nitrogen-containing compatible solutes including proline is known to function in osmotic adjustment, protection of cellular macromolecules from damage by salts, storage of nitrogen and scavenging of free radicals. In the present study, combined application of bio fertilizer and CCC as F_3C_3 showed a significant increase in the content of proline about 60% in comparison with F_0C_0 under severe water limitation, which could be taken as an indicator of enhanced plant tolerance to water stress and consistent with previous reports (Maiti et al. 2002).

In this study, the activity of CAT, POD and PPO enzymes was increased with the increase in water limitation, the application of bio fertilizers and CCC in comparison with control (Table 1). The highest of CAT (optical density at 240 nm) and POD (optical density at 425 nm) activity (52.56 and

Table 1. Means comparison and variance analysis effects of bio fertilizers × cycocle on activity of CAT, POD and PPO enzymes, chlorophyll, carotenoid, proline, stomata conductance and LAI of wheat under water limitation.

	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoid (mg/g FW)	Proline (mg/g FW)	CAT (OD µg protein/min)	POD (OD µg protein/min)	PPO (OD µg protein/min)	Stomata conductance (mmol/m^2/s)	LAI	Relative water content (%)	Grain yield (kg/ha)
Water limitation												
I_1 = normal irrigation	5.66a	1.85a	7.52a	0.30a	7.44c	30.97b	125.48b	39.95c	46.96a	4.79a	84.21a	2897.16a
I_2 = moderate water limitation	5.10b	1.16b	6.26b	0.27b	7.95b	31.78b	127.61b	49.12b	42.55b	4.31b	79.35b	2288.11b
I_3 = severe water limitation	4.37c	0.97c	5.34c	0.22c	8.753a	41.40a	160.53a	75.27a	37.96c	3.86c	75.00c	1886.25c
LSD ($p < 0.05$)	0.44	0.14	0.56	0.013	0.43	3.52	10.75	5.43	1.38	0.14	2.66	197.53
Bio fertilizers												
F_0 = no inoculation as control	5.79b	1.25b	6.05b	0.25b	7.76bc	27.31c	130.07b	43.41b	42.39a	4.32a	78.30b	2146.9b
F_1 = *Azotobacter*	5.21a	1.32b	6.54a	0.27ab	8.25ab	36.18b	135.74ab	49.13b	42.51a	4.31a	80.75a	2167.9b
F_2 = *Pseudomonas*	4.91b	1.31b	6.22b	0.26ab	7.61c	32.57b	138.48ab	59.48a	42.52a	4.32a	79.18ab	2411.4b
F_3 = *Azotobacter* + *Pseudomonas*	5.26a	1.41a	6.68a	0.28a	8.58a	42.80a	147.21a	67.11a	42.54a	4.32a	79.85ab	27.2.4
LSD ($p < 0.05$)	0.28	0.07	0.30	0.021	0.53	3.81	13.67	8.44	2.37	0.24	3.54	283.01
Cycocle (g lit^{-1})												
C_0 = without cycocle as control	4.09c	0.98c	5.07d	0.26a	7.19c	32.10b	130.07b	51.84a	45.99a	4.68a	74.73c	2068.6c
C_1 = 400	4.32c	1.07c	5.39c	0.26a	7.91b	31.98b	135.74ab	54.21a	44.13a	4.49a	76.61c	2176.5bc
C_2 = 800	5.57b	1.49b	7.06b	0.27a	8.44a	35.59ab	138.48ab	55.89a	40.63b	4.13b	81.25b	2402.3b
C_3 = 1200	6.20a	1.77a	7.97a	0.27a	8.65a	39.19a	147.21a	57.18a	39.21b	3.98b	85.5a	2781.3a
LSD ($p < 0.05$)	0.39	0.19	0.53	0.021	0.49	4.44	14.31	9.45	1.99	0.20	2.96	273.28
I × F	ns	ns	ns	ns	**	*	*	**	ns	ns	**	ns
I × C	ns	*	ns	ns	**	ns	ns	ns	**	**	ns	ns
F × C	**	ns	**	ns	**	ns	ns	ns	ns	ns	ns	*
I × F × C	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	*
C.V.	11.98	11.42	11.98	13.15	2.92	13.07	18.02	15.22	2.53	2.37	6.77	13.08

The same letters in each column show non-significant difference at $p \leq 0.05$ by LSD test.
(ns) and (*, **) show no significant and significant differences at 0.05, 0.01 probability level, respectively.
C.V.: coefficient of variation; CAT: catalase; POD: peroxidase; PPO: polyphenol oxidase; LAI: leaf area index; RWC: relative water content.

Table 2. Means comparison the effects of bio fertilizers × CCC on chlorophyll a and total chlorophyll of wheat.

Treatment	Chlorophyll *a* (mg/g FW)				Total chlorophyll (mg/g FW)			
Bio fertilizers	C_0	C_1	C_2	C_3	C_0	C_1	C_2	C_3
F_0	4.02 ± 1.10	4.38 ± 0.98	5.42 ± 1.92	5.35 ± 1.66	4.92 ± 1.70	5.44 ± 1.46	6.76 ± 2.30	7.03 ± 2.10
F_1	4.17 ± 0.83	4.55 ± 0.97	5.22 ± 1.00	6.91 ± 1.60	5.10 ± 1.31	5.59 ± 1.39	6.74 ± 1.58	8.35 ± 1.57
F_2	3.91 ± 0.96	4.01 ± 0.92	5.68 ± 1.05	6.05 ± 0.91	4.97 ± 1.47	5.08 ± 1.46	7.13 ± 1.37	7.79 ± 1.40
F_3	4.24 ± 1.10	4.33 ± 0.90	5.96 ± 1.38	6.50 ± 1.00	5.30 ± 1.56	5.44 ± 1.34	7.62 ± 1.92	8.73 ± 2.19
$LSD_{0.05}$	0.75				1.05			

Notes: F_0, F_1, F_2 and F_3 indicative without inoculation, seed inoculation with *Azotobacter*, *Pseudomonas* and inoculation with *Azotobacter* + *Pseudomonas*, respectively. C_0, C_1, C_2 and C_3 indicative no application, application of 400, 600 and 1000 mg/l CCC, respectively.

Table 3. Means comparison the effects of water limitation × CCC on chlorophyll b of wheat.

Treatment	Chlorophyll *b* (mg/ g FW)			
Water limitation	C_0	C_1	C_2	C_3
I_1	1.57 ± 0.27	1.64 ± 0.30	1.98 ± 0.42	2.22 ± 0.47
I_2	0.82 ± 0.31	0.84 ± 0.33	1.33 ± 0.43	1.63 ± 0.44
I_3	0.54 ± 0.41	0.73 ± 0.30	1.16 ± 0.45	1.45 ± 0.44
$LSD_{0.05}$	0.12			

Notes: I_1, I_2 and I_3 indicative normal irrigation, irrigation withholding in heading and booting stages. C_0, C_1, C_2 and C_3 indicative no application, application of 400, 600 and 1000 mg/l CCC, respectively.

175.11 OD µg protein/min) was observed in severe water limitation and bio fertilizers application as F_3 (Table 5). The lowest of these values (23.93 and 98.3 OD µg protein/min) was obtained at normal irrigation, application of bio fertilizer as F_0 (Table 5). Our results also showed that the highest activity of PPO (optical density at 420 nm) (92.08 OD µg protein/min) was obtained in the severe water limitation and bio fertilizer as F_3 or (I_3F_3) (Table 6). Based on our results, bio fertilizers had approximately 1.3- to 1.78-fold higher activities under water limitation stress in comparison with control plants.

An increase in the activity of POD and PPO enzymes under water limitation could be indicative of an increased production of ROS and a build-up of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants (Meloni et al. 2003). An increase in POD activity was also observed by different authors during drought and salt stress (Wang et al. 2012). Some PGPR strains improve plant enzyme activity, such as CAT or SOD, which alleviates the oxidative damage induced by drought (Wang et al. 2012). It has been found that plants infected with PGPR strains showed high antioxidant enzymes activity which contributed to enhance plant protection against drought and salt stress (Wang et al. 2012).

Based on our results, it is obvious that the reduced level of oxidative stress enzymes suggests a low level of stress convened to the PGPR-applied plants. Similar results were also observed by Rakshapal et al. (2013). These researchers showed that *Pseudomonas* application to *O. basilicum* L. increased the nutrient uptake and reduced the antagonistic effects of abiotic stress. Several bio fertilizers can also improve plant tolerance to salinity, drought, flooding, and heavy metal toxicity and enable plants to survive under unfavorable environmental conditions (Ma et al. 2011). Wang et al. (2012) found that application of PGPR strains improve plant enzyme activity, which alleviates the oxidative damage induced by drought and salinity. Belimov et al. (2009) have reported beneficial effects of PGPR for improving plant growth under normal as well as stressful environment. Noorieh et al. (2013) have also reported that PGPRs species like *Pseudomonas* sp. increased the growth and biomass of canola plants by regulating the oxidative stress enzymes and essential nutrient under water limitation.

The stomatal conductance was significantly decreased with severe water limitation. Besides the water limitation effects, CCC-treated plants displayed a significant decrease in LAI and stomatal conductance. Moderate (I_2) and severe water limitation (I_3) in comparisons with normal irrigation decreased stomatal conductance as much as 10.7% and 39.72%, respectively (Table 7) when plant was treated with 1200 mg/l CCC. Similar results have been reported by Rodriguez et al. (2005).

The highest LAI (4.97) was obtained in normal irrigation and no-application of CCC. The lowest value (3.23) was observed in severe water limitation and during the application of CCC as C_3 (Table 7). The reduction in LAI under water limitation could be considered as an avoidance mechanism which minimizes water losses (Rodriguez et al. 2005). Leaf area reduction due to retardant application has been shown to be due to inhibition of gibberellin synthesis, increment of ABA content and cell elongation prevention within the leaf (Gopi et al. 2005). It can be assumed that the increase in proline content in the leaves of CCC-treated plants (Table 4) can be explained in terms of retarded growth (smaller plants with more concentrated nitrogen content) and delayed senescence with longer retention of nitrogen in the leaves before translocation into generative organs.

Considerable decrease in RWC was observed, depending on the increase in water limitation levels (Table 1). Means comparison for the effects of bio fertilizer and irrigation on RWC are given in Table 6. In general, the highest RWC (87.50%) was observed in plants with the application of bio fertilizer as F_3 under normal irrigation and the lowest (73.25%) was observed in severe water limitation and application of bio fertilizer as F_0. Plant water status is intimately related to several physiological variables, such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration. It seems that the inhibitory and deleterious effects of water stress can be decreased by seed inoculation with plant regulation. Indeed, bio fertilizer inoculation promote plant growth by improving root growth and production of plant growth stimulating compounds (Zahir et al. 2004).

The irrigation, bio fertilizers and CCC application significantly affected the grain yield (Table 1). The highest yield (3822.2 kg/ha) was obtained in normal irrigation (I_1), the application of bio fertilizer as F_3 and CCC as C_3 (Table 4). The lowest yield (1409.7 kg/ha) was determined in severe water limitation (I_3), without the application of bio fertilizer and CCC. Under severe water limitation, the application of bio fertilizer and CCC as F_3C_3 had 66% more grain yield in comparison with F_0C_0 (Table 4). Based on these results, the stimulatory effect of bio fertilizer has been attributed to several mechanisms that increase plant yield, including

Table 4. Means comparison the effects of bio fertilizers × CCC on leaf proline and soluble carbohydrate of wheat under water limitation.

Treatment		Proline (mg/g FW)				Grain yield (kg/ha)			
Water limitation	Bio Fertilizers	C_0	C_1	C_2	C_3	C_0	C_1	C_2	C_3
I_1	F_0	5.25 ± 1.05	8.40 ± 1.68	8.43 ± 1.69	8.99 ± 1.80	2423.7 ± 242.3	2636.4 ± 401.6	2799.3 ± 467.1	2942.4 ± 211.4
	F_1	7.61 ± 1.52	6.20 ± 1.24	8.45 ± 1.69	8.94 ± 1.78	2079.2 ± 326.8	2485.4 ± 98.1	2776.4 ± 402.1	3167.4 ± 202.1
	F_2	5.33 ± 1.07	5.70 ± 1.14	7.30 ± 1.46	8.23 ± 1.65	2564.6 ± 460.6	3286.1 ± 16.83	2760.4 ± 504.2	3234.7 ± 403.4
	F_3	7.42 ± 1.48	8.04 ± 1.61	6.52 ± 1.30	8.38 ± 1.68	2736.1 ± 502.7	2957.6 ± 177.5	3682.6 ± 266.1	3822.2 ± 209.3
I_2	F_0	5.82 ± 1.16	8.08 ± 1.62	8.12 ± 1.62	8.17 ± 1.63	1813.2 ± 186.1	1833.4 ± 217.0	2345.3 ± 411.4	2477/1 ± 113.7
	F_1	8.21 ± 1.64	8.32 ± 1.66	8.31 ± 1.66	8.35 ± 1.67	1574.3 ± 132.0	2071/8 ± 428.1	1756.9 ± 183.7	2519 ± 405.0
	F_2	5.60 ± 1.12	6.38 ± 1.28	8.12l ± 1.62	9.40 ± 1.88	2137.5 ± 122.1	2419.2 ± 657.2	2070.8 ± 164.6	2715.3 ± 520.4
	F_3	7.52 ± 1.50	8.86 ± 1.77	8.63 ± 1.73	9.43 ± 1.89	2107.3 ± 126.3	2313.2 ± 100.4	3028.5 ± 472.5	3427.2 ± 47.6
I_3	F_0	6.40 ± 1.28	8.44 ± 1.68	8.49 ± 1.70	8.53 ± 1.70	1409.7 ± 178.8	1468.5 ± 63.8	1567.4 ± 4.2	2298.6 ± 444.8
	F_1	8.58 ± 1.71	8.62 ± 1.72	8.67 ± 1.73	8.71 ± 1.74	1842.4 ± 376.8	1518.8 ± 322.9	1929.9 ± 260.1	2041.7 ± 218.1
	F_2	8.76 ± 1.75	8.81b-f ± 1.76	8.85 ± 1.77	8.90 ± 1.78	1659 ± 199.2	1770.1 ± 241.0	1940.3 ± 138.5	2379.2 ± 447.2
	F_3	9.44 ± 1.89	9.51 ± 1.90	9.6 ± 1.92	9.66 ± 1.93	1789.6 ± 27.2	2043.8 ± 173.0	2170.1 ± 138.8	2351.0 ± 197.6
$LSD_{0.05}$				0.095				125.03	

Notes: I_1, I_2 and I_3 indicative normal irrigation, irrigation withholding in heading and booting stages. F_0, F_1, F_2 and F_3 indicative without inoculation, inoculation with *Azotobacter*, *Pseudomonas* and inoculation with *Azotobacter* + *Pseudomonas*, respectively. C_0, C_1, C_2 and C_3 indicative no application, application of 400, 600 and 1000 mg/l CCC, respectively.

Table 5. Means comparison the effects of water limitation × bio fertilizers on activity of CAT and POD enzymes of wheat.

Treatment	CAT (OD µg protein/min)				POD (OD µg protein/min)			
Water limitation	F_0	F_1	F_2	F_3	F_0	F_1	F_2	F_3
I_1	23.93 ± 4.51	31.84 ± 7.54	29.78 ± 5.74	38.34 ± 7.08	98.30 ± 21.46	131.97 ± 24.22	127.57 ± 14.40	144.07 ± 54.26
I_2	27.02 ± 8.01	35.44 ± 12.58	27.15 ± 9.05	37.52 ± 9.49	121.08 ± 25.41	143.06 ± 31.46	112.01 ± 23.10	134.29 ± 23.09
I_3	30.99 ± 6.02	41.26 ± 11.86	40.78 ± 9.31	52.56 ± 10.27	147.19 ± 40.46	156.68 ± 24.91	163.14 ± 65.88	175.11 ± 42.67
$LSD_{0.05}$	5.22				19.18			

Notes: I_1, I_2 and I_3 indicative normal irrigation, irrigation withholding in heading and booting stages. F_0, F_1, F_2 and F_3 indicative without inoculation, inoculation with *Azotobacter*, *Pseudomonas* and inoculation with *Azotobacter* + *Pseudomonas*, respectively.

Table 6. Means comparison the effects of water limitation × bio fertilizers on activity of PPO enzyme and RWC of wheat.

Treatment	PPO (OD µg protein/ min)				RWC (%)			
Water limitation	F_0	F_1	F_2	F_3	F_0	F_1	F_2	F_3
I_1	34.76 ± 6.01	36.64 ± 8.08	40.12 ± 8.22	42.76 ± 7.87	79.75 ± 5.97	83.00 ± 9.22	86.31 ± 7.80	87.5 ± 8.91
I_2	39.95 ± 9.68	42.17 ± 7.79	53.41 ± 10.13	66.48 ± 12.43	79.20 ± 6.91	81.95 ± 6.36	77.75 ± 8.29	78.5 ± 6.29
I_3	55.51 ± 14.54	68.57 ± 23.24	84.57 ± 14.91	92.08 ± 15.88	73.25 ± 10.46	77.3 ± 11.33	73.5 ± 4.12	75.9 ± 5.73
$LSD_{0.05}$	6.43				5.17			

Notes: I_1, I_2 and I_3 indicative normal irrigation, irrigation withholding in heading and booting stages. F_0, F_1, F_2 and F_3 indicative without inoculation, inoculation with *Azotobacter*, *Pseudomonas* and inoculation with *Azotobacter* + *Pseudomonas*, respectively.

Table 7. Means comparison the effects of water limitation × CCC on stomata conductance and LAI of wheat.

Treatment	Stomata conductance				LAI			
Water limitation	C_0	C_1	C_2	C_3	C_0	C_1	C_2	C_3
I_1	48.85 ± 8.34	47.94 ± 8.19	45.91 ± 7.86	45.14 ± 8.46	4.97 ± 0.84	4.88 ± 0.83	4.67 ± 0.80	4.63 ± 0.79
I_2	46.05 ± 7.86	41.74 ± 7.12	41.64 ± 7.11	40.77 ± 7.44	4.69 ± 0.80	4.24 ± 0.72	4.23 ± 0.72	4.07 ± 0.69
I_3	43.06 ± 7.34	42.70 ± 7.27	34.35 ± 5.86	31.72 ± 5.42	4.38 ± 0.74	4.34 ± 0.74	3.49 ± 0.59	3.23 ± 0.55
$LSD_{0.05}$	0.75				0.07			

Notes: I_1, I_2 and I_3 indicative normal irrigation, irrigation withholding in heading and booting stages. C_0, C_1, C_2 and C_3 indicative no application, application of 400, 600 and 1000 mg/l CCC, respectively.

enhancement in RWC, proline and photosynthetic pigments by plants. It has been suggested that improvement of the grain yield under CCC treatments might be associated with the enhanced activity of PPO, POD and CAT in the leaves thereby improving the performance of the plants under sub-optimal growth conditions.

4. Conclusion

The results showed that water limitation reduced grain yield, chlorophyll content, carotenoid, stomata conductance, LAI and RWC of the plants. But antioxidant enzymes activity and proline increased. Also, the application of bio fertilizer and CCC improved grain yield, chlorophyll content, antioxidant enzyme activity, proline, carotenoid, LAI and RWC under water limitation condition. Our results suggested that plants use defensive mechanisms, such as synthesis of antioxidant enzymes and proline to reduce effects of stress. We believe that the application of bio fertilizer and CCC might be recommended for profitable wheat production under water limitation condition.

Disclosure statement

No potential conflict of interest was reported by the authors.

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