Cereal Research Communications (2024) 52:1201–1213
https://doi.org/10.1007/s42976-023-00456-5

ORIGINAL PAPER

Enhanced growth and stress tolerance in Barley (*Hordeum vulgare*) through biopriming with *Aspergillus niger* CSR3: a promising approach for sustainable agriculture in saline environments

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Received: 30 May 2023 / Accepted: 10 October 2023 / Published online: 6 November 2023

Abstract

Barley (*Hordeum vulgare*) is the fourth largest cereal crop in the world, with considerable nutritional value. Recently more studies on the toleration of barley to salt stress have been published, indicating an increased concern for food safety. Salt stress is an increasing threat to agricultural productivity; thus, an attempt was made to explore the growth-promoting capacities of an endophytic fungal strain *Aspergillus niger* CSR3 in *H. vulgare*. In the current study, we investigated various physiological and biochemical characteristics of two *H. vulgare* varieties, namely OM-80 and OM-82, under 300 mM NaCl and 100% seawater treatments with and without the inoculation of CSR3. Our results showed that biopriming of *H. vulgare* seeds with CSR3 enhanced germination ratio both in control and salt treated conditions. Under salt stress, the growth of *H. vulgare* plants was significantly reduced; however, CSR3 alleviated the salt stress and significantly increased root/shoot length and weight compared to their respective counterparts both under control and stress conditions. The fungal strain showed an ameliorated response to salt stress by improving the photosynthetic machinery. Results demonstrate that accumulation of reduced glutathione (GSH), catalase (CAT), and flavonoids decreased in inoculated plants as compared to non-inoculated under saline conditions indicating the potential of CSR3 in maintaining cellular homeostasis against salinity stress. Moreover, our finding also revealed that starch accumulation decreased with a gradual increase of salt treatment; however, CSR3 inoculation enhanced starch and decreased sugar level, indicating its potential to convert excess sugar to starch. In conclusion, CSR3 can improve plant performance significantly and can greatly improve sustainable agricultural production in saline marginal lands.

Keywords Barley · Endophytes · Salinity · Sustainable agriculture · Oxidative stress · Biopriming

Introduction

With dramatic expansion in human population, the proportion of various abiotic stresses including salinity is also rising, adversely affecting crops germination and productivity (Khan et al. 2022). Salinity is the most serious abiotic stress that prevails more than 30% arable and 7% dry lands. In order to meet the increasing demands for food supplements, it is urgent issue to minimize the adverse effects of salinity stress on plants (Khan et al. 2021a). Salinity stress induces an imbalance of ions and water deficiency inside plant cells, leading to reduced uptake and transport of other essential ions for plant processes and functions (Arif et al. 2020). Moreover, high salinity alters the ultrastructure of cellular components, damages the membranous structure, disrupts the photosynthetic machinery, decreases enzymatic

Communicated by Katalin Posta.

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activity, increases the production of the reactive oxygen species, which adversely affects seed germination, growth, and development and ultimately declines productivity of the crop plants (Hasanuzzaman et al. 2014). So far, significant efforts have been made to address the issue of high salinity by applying various methods like applying chemicals and improving drainage, developing salt-tolerant cultivars by traditional breeding and genetic enhancement (Huang 2018; Khan et al. 2021b). However, such approaches are not always feasible and might negatively impact the ecosystem. Therefore, finding and developing eco-friendly and cost-effective strategies to manage high salinity are vital for agriculture systems (Mishra et al. 2023). The application of salt-tolerant endophytic fungal strains has emerged a promising approach to improve plant growth and adaptation to hostile environments (Jhuma et al. 2021; Lubna et al. 2018b). Lesser work has been done to explore endophytic fungi's potential in alleviating salt stress in crops.

Endophytic fungi colonize the intercellular spaces of healthy tissues of the host plants without causing disease symptoms (Khan et al. 2011). A few endophytic fungal strains have been investigated for their plant growth promoting traits (Ismail et al. 2021). Extensive colonization of the fungal endophytes may profit host plants by secreting antibacterial antifungal compounds that inhibit pathogenic organisms' growth (Yan et al. 2018). Endophytic fungi produce several secondary metabolites, hormones and enzymes that can greatly influence the host plant growth by the enhancement of nutrients absorption, which ultimately confer resistance against various stress conditions and reflects rise in yield (Asaf et al. 2023a; Waqas et al. 2012). Endophytes usually form mutualistic relationship with the host plant, in which the host plants provide nutrients and shelter for the fungi while the endophytic fungi promote plant growth and health (Bamisile et al. 2021).

Barley (*Hordeum vulgare*) is the fourth most important cereal crop just after maize (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) in terms of total production (Yang et al. 2022). Barley is cultivated widely throughout the world because of its high productivity and adaptability to stressful conditions such as salinity, drought, and extreme temperatures (Schulte et al. 2009). Interest in barley cultivation has increased due to its wide usage as a feed and fodder for animals as well as its significant consumption in the production of alcoholic drinks and human diet such as bread, biscuits, and snack-type products (Jaeger et al. 2021; Newton et al. 2011). Despite giving nutritional value to our diet, barley also offers a wealth of potential health benefits in several parts of the world (Badea and Wijekoon 2021). Barley is considered a semi-halophyte and extensive research has been conducted for the generation of new high yielding varieties with enhanced salt stress resistance through traditional breeding and genetic improvement however, understanding the adaptation of these cultivars to salt stress is not yet studied comprehensively (Elsawy et al. 2018; Salih et al. 2022).

Previously many strains of *Aspergillus* fungi have been extensively studied for their plant growth promoting potentials (Araújo et al. 2020; Gujar et al. 2013; Hung and Rutgers 2016). Therefore, the current study was designed to determine the co-inoculation effects of an important endophytic fungal strain (*A. niger* CSR3) on barley growth attributes, including stomatal conductance, chlorophyll contents, different antioxidants, length and weight of shoot and root. Previous studies demonstrated that some *Aspergillus* species including *A. niger* are safe for plant and human health. *A. niger* has been widely used in biotechnology to produce extracellular (food) enzymes and citric acid (Attia et al. 2022; Schuster et al. 2002). Our previous research demonstrated that *A. niger* CSR3 has phosphate solubilizing and siderophore producing capability as well as the ability to produce the well-characterized plant growth regulators IAA and GA (Lubna et al. 2018a). In the current study, we hypothesized that the CSR3 endophytic fungal strain could be a suitable alleviator of salt stress on crops such as barley and might influence gene expression, modulate the antioxidative system and production of phytohormones by developing an eco-friendly phytoremediation strategy to for efficiently coping salt stress and promote barley plant growth. Therefore, we investigated the in vitro and in vivo plant growth promoting abilities of the CSR3 strain on the growth of the two barley varieties in a greenhouse experiment under control and high salinity conditions.

Materials and methods

Salt tolerance evaluation in *A. niger* CSR3

To investigate the resistance of *A. niger* to salt, we prepared a potato dextrose agar (PDA) medium containing a concentration of 300 NaCl and approximately 600 mM of seawater (100% seawater).. Small plugs of agar from the edge of stock culture with immature mycelium were finely chopped and placed on the surface of the solid medium. The plates were incubated at 30 °C for 7 days and were examined daily for hyphal growth. Simultaneously, culture without NaCl was carried out as a control. The diameter of the radial growth was measured (in millimeters) across the center of the infected section. The initial diameter was subtracted from the portion's starting diameter. On the 7th day, the average of the perpendicular diameter measurements of each plate was noted. To determine the salt tolerance index (TI) of each isolate as indicator of the endophytic strain's tolerance to salt, growth of CSR3 exposed to salt was divided by the CSR3 growth measurement in the control plate (Errasquın and Vazquez 2003).

Seed biopriming and germination in salt stress and CSR3 inoculation

Barley seeds of two different varieties (OM-80 and OM-82) were obtained from the Seed Gene Bank Istiama at the University of Nizwa Oman. Seeds were thoroughly surface sterilized with 70% ethanol and 2.5% sodium hypochlorite and then washed four times with autoclaved distilled water. In conical falcon tubes, 10 seeds were soaked each with 10 mL distilled water, 300 mM NaCl, 100% seawater, and 10 days old CSR3 culture filtrate supernatant and kept at 28 °C in the dark for 12 h. After priming, the germinated seeds were carefully transferred to Petri dishes with respective treatment labeling. The Petri dishes were added with 5 mL of distilled water and placed in a germination chamber at 25 °C in the dark. Germination percentage was calculated every 24 h for up to 5 days using the formula GP = seeds germinated/total seeds × 100. After 5 days of seed germination, the shoot and root lengths were measured with a ruler.

Experimental design and assessment of growth attributes

Seedlings at the bifoliate leaves stage were transferred to plastic pots filled with autoclaved organic soil containing sphagnum peat (40%), light peat (60%), organic matter (91.4%), 38.5% moisture contents, 410.0µS/cm electrical conductivity, 340 kg/m^3 density and 4.5–5.5 pH. After 3 days of transplanting, plantlets of both varieties were exposed to 6 different growth conditions: (a) control plants (distilled water), (b) plants inoculated with CSR3, (c) plants with 300 mM NaCl, (d) plants with 300 mM NaCl + CSR3, (e) plants with seawater and (f) plants with seawater + CSR3. These plant groups were irrigated with 20–30 mL of distilled water, 300 mM NaCl, 100% seawater stress and inoculated with freshly diluted fungal culture (10^{-6} spores/mL) at appropriate intervals for 20 days. The pots were kept in a plant growth chamber under 16 h of light/day and 60–70% relative humidity at a temperature of 26 °C. The experiment was performed with 5 replicates per treatment. After 1 month of planting, growth attributes (root/shoot length) and biomass (root/shoot weight) were measured. The harvested plants were immediately frozen in liquid nitrogen and then preserved at − 80 °C until further analysis.

Assessment of chlorophyll a, b, and carotenoids

Freshly grinded plant samples (200 mg) from each treatment group were added with 1 mL of 80% acetone. After being mixed using a vortex, the homogenate was centrifuged at 4 °C for 15 min. 100 µL supernatant from each sample was transferred in triplet into a 96-well microplate and measured using a spectrophotometer at an absorbance of 663 nm, 645 nm, and 470 nm to determine the concentration of chlorophyll-a, chlorophyll-b, and carotenoids respectively. Photosynthetic pigment concentrations were calculated using the extension coefficients and equation given in Barnes's method (Barnes et al. 1992) with slight modifications.

Assessment of reduced glutathione

Tissue reduced glutathione (GSH) contents were assessed according to the method outlined by Ellman (1959) with slight modifications. In brief, 500 mg of powdered ingredients were added with 1 mL of 10% trichloroacetic acid (TCA). The mixture was centrifuged for 15 min at a speed of 10,000 rpm. The collected supernatants (350 µL) were mixed with 150 µL of the Ellman's reagent, and the final volume was made up to 1.5 mL by adding 1 mL of 150 mM phosphate buffer. The optical density of the samples was measured at 420 nm spectrophotometrically. The resulting contents were estimated by comparing the absorbance values with a standard curve obtained from known GSH.

Assessment of catalase activity

Freshly preserved leaves (200 mg) were crushed into fine powder under liquid nitrogen and mixed with 1 mL extraction buffer containing 50 mM Tris HCl (pH 7.0), 10% glycerol, 3 mM $MgCl_2$, 1 mM EDTA and 1% pvp). After centrifugation at 10,000 rpm at 4 °C for 15 min the collected supernatant was mixed with equal volume (240 µL) of 0.1 mM phosphate buffer (pH 7.0). The sample extract was quickly mixed with 120 µL, 0.2 M H_2O_2 and the optical density measurement was carried out at 240 nm.

Assessment of total flavonoid contents

A method adopted by Park et al (2008) was used to extract the total flavonoid contents. Approximately 0.5 g of leaf sample was ground finely in liquid nitrogen and homogenized in 1 mL 80% methanol. After 24 h of incubation at room temperature, the extract was centrifuged for 15 min at 10,000 rpm and 25 °C. The resultant 0.5 mL supernatant (having flavonoid contents) was collected and mixed well with an equal volume of 2% aluminum chloride diluted in 95% ethanol. After 20 min of incubation at room temperature, the OD was recorded at 390 nm.

Assessment of total protein contents

The analytical spectroscopic method of Bradford (1976) was used to assess the total protein contents. Fresh leaves were grounded in liquid nitrogen, added with 1 mL extraction buffer formulated by Arulsekar and Parfitt (1986), and gently mixed. The homogenate samples were centrifuged at

4000 rpm for 10 min at 2 °C. Supernatants were collected in fresh tubes and added with 150 µL of Bradford reagent, followed by a five-minute incubation. The solutions were pipetted into a 96-wells microplate in triplet, and absorbance was measured at 595 nm using a spectrophotometer.

Assessment of total sugar and starch

Zavřel et al. (2018) was slightly modified to extract and measure total sugar content in plant samples. Approximately 0.5 g of frozen leaf samples were crushed in liquid nitrogen and poured into Eppendorf tubes. 1 mL of 80% ethanol was added and incubated at room temperature. After 24 h of incubation, the extracts were centrifuged at 3000 rpm for 10 min. The absorbance of the collected supernatant was read at 490 nm and 630 nm for sugar and starch, respectively.

Statistical analysis

The mean values of the three-replicate data of all the conducted assays were combined and subjected to Duncan's multiple range test (DMRT) for statistical analysis. The mean values of different treatments were compared by adopting a completely randomized design. Principle component analysis (PCA) was performed to generate the correlation matrix between various traits of the selected varieties under control, salt stress (300 mM NaCl and 100% seawater, CSR3 inoculated and non-inoculated conditions. The Pearson's correlation coefficient was used to determine variance significance in various morpho-physiological, biochemical, and antioxidative traits under salt-stressed inoculated and non-inoculated conditions.

Results

Salt tolerance and bioaccumulation of CSR3

The salt tolerance index of CSR3 was determined by adding 300 mM NaCl and 100% seawater to PDA medium. The results revealed that CSR3 can grow both on 300 mM NaCl and seawater supplemented media. However, its growth was reduced significantly in as compared to the control. A minimum inhibitory concentration of CSR3 was found at seawater stress (Fig. 1).

Seed biopriming and effect of CSR3 inoculation on *H. vulgare* seed germination and seedling length

CSR3 culture filtrate was used for seed biopriming to observe its effect on barley seed germination and seedling growth under control and salt stress conditions. Results revealed that barley seed germination of both the verities (OM-80 and OM-82) was significantly increased when treated with the cultural filtrate of CSR3 as compared NaCl and seawater treated ones. The results showed that after 24 h, CSR3 inculcation enhanced germination of the NaCl and seawater treated seeds of OM-80 variety up to 50% and 80%, respectively as compared to their non-inoculated counterparts. A similar trend was also observed after 48 h. More interestingly, after 72 h, almost 100% of seeds inoculated with CSR3 of the OM-80 variety were germinated in control as well as in sea water treated plants. Seeds of the OM-82 variety of barley inoculated with CSR3 showed a significant ameliorative impact in comparison to non-inoculated seeds in both control (distilled water) and seawater stress conditions. However, unexpectedly, seeds inoculated with CSR3 and treated with seawater stress germinated 100%

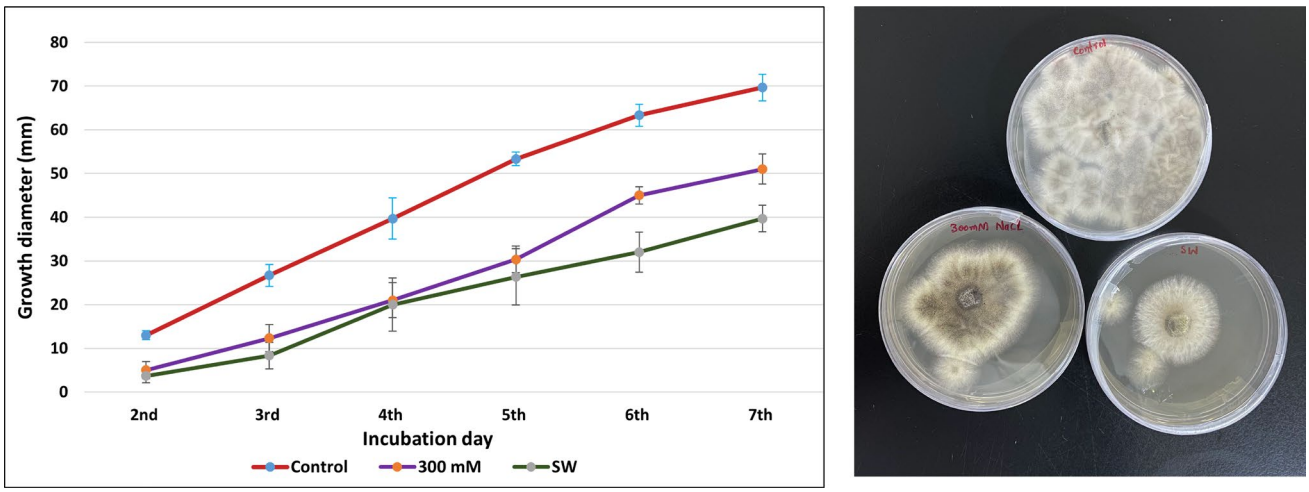

Fig. 1 CSR3 growth on PDA medium under varying conditions of salt stress

after 96 h, showing significant salt stress alleviation ability of the CSR3 fungal strain (Table 1). We have verified the endophytic characteristic and the presence of the CSR3 fungal strain within the inoculated plants through a process that involves reisolation, followed by PCR amplification and sequencing of the ITS gene.

Effect of endophytic co-inoculation on *H. vulgare* growth promoting attributes under salt stress

Inoculation of CSR3 endophytic fungal strain on barley plants disclosed its significant growth promoting attributes on the barley plants compared to the non-inoculated plants. As shown in Fig. 2A, B, the endophytic co-inoculation significantly enhanced the shoot length of OM-80 variety by 9.7%, 9.2%, and 5.3% and the root length by 29.6%, 40%, and 110% when compared with the shoot and root lengths of their respective non-inoculated counterparts. The fresh weights of shoots and roots also increased significantly by 19.7%, 45.3%, and 17.07% in the CSR3 inoculated plants, compared to the fresh weights of their non-inoculated counterparts both under control and stress conditions (Fig. 2C, D). Similarly, the inoculation of CSR3 substantially mitigated the adverse effects of salt stress in the OM-82 variety by displaying significantly higher growth attributes in inoculated plants compared to their non-inoculated counterparts. Our results indicated that salt stress considerably reduced fresh shoot and root weights of both the varieties, but the CSR3 inoculation mitigated it to some extent.

Effects of inoculation with CSR3 on photosynthetic pigments of *H. vulgare* under salinity stress

The current investigation has clearly revealed that the inoculation of CSR3 fungal strain has resulted in a significant increase in photosynthetic pigments as compared to non-inoculated plants. Our results revealed that the contents of the photosynthetic pigments of plants of both the OM-80 and OM-82 *H. vulgare* varieties decreased significantly with gradual exposure to increasing levels of salinity stress. It was observed that plants treated with 300 mM NaCl and inoculated with CSR3 had the highest chlorophyll-a and b levels, respectively. Surprisingly, the highest concentrations of carotenoids were found in non-inoculated plants treated with distilled water (control plants), followed by CSR3 inoculated plants treated with 300 mM NaCl salt. The results demonstrated that seawater stress caused a highly significant reduction in the level of the three photosynthetic pigments in both OM-80 and OM-82 varieties of *H. vulgare* (Fig. 3A–C).

Effect of the endophytic CSR3 on reduced glutathione under salinity stress

Assessment of the GSH contents in *H. vulgare* plants further illustrates the oxidative stress mitigated by the CSR3 fungal strain. Our findings showed that salt treated non-inoculated plants have higher levels of GSH contents as compared to inoculated plants, determining the potential of CSR3 fungus to ameliorate salinity stress (Fig. 3D). Similar to other studies, our results indicated that accumulation of GSH contents was inversely proportional to the salt concentration. The fungal inoculation showed a significant decrease in GSH accumulation compared to their respective control plants (Khan and Panda 2008; Meng et al. 2016).

Effect of the endophytic CSR3 on catalase activity under salinity stress

In both varieties, an increased level (13.6 and 11.2%) of catalase (CAT) was observed in inoculated plants during normal conditions. Similarly, subject to both NaCl and SW stress, the CAT activity of the plants increased significantly. However, a significant decrease was also observed

Table 1 Effect of CSR3 inoculation on barley seeds germination percentage

Variety	Treatment	24 h	48 h	72 h	96 h	120 h
OM-80	Control	50 ± 0.7	70 ± 0.6	80 ± 0.1	100 ± 0	100 ± 0
	CSR3	66.66 ± 0.1	73.33 ± 0.7	100 ± 0	100 ± 0	100 ± 0
	300 mM	33.33 ± 1.4	50 ± 0.7	66.66 ± 0.7	66.66 ± 0.1	66.66 ± 0.1
	CSR3-300 mM	50 ± 0.7	83.33 ± 0.6	83.33 ± 0.7	83.33 ± 0.1	83.33 ± 0.2
	SW	33.33 ± 0	66.66 ± 0.1	66.66 ± 0.1	73.33 ± 0.2	83.33 ± 0.1
	CSR3-SW	60 ± 0.7	80 ± 1.4	100 ± 0	100 ± 0	100 ± 0
OM-82	Control	25 ± 0.1	37.5 ± 0.7	50 ± 0.1	80 ± 0.2	100 ± 0
	CSR3	57.14 ± 1.4	57.14 ± 1.4	57.14 ± 1.4	85.14 ± 1.4	100 ± 1.4
	300 mM	14.28 ± 0.7	28.57 ± 0.1	42.85 ± 0.2	42.85 ± 0.7	42.85 ± 0.7
	CSR3-300 mM	16.66 ± 0.7	32.66 ± 0.6	48.66 ± 0.7	68.66 ± 0.7	68.66 ± 0.7
	SW	33.33 ± 0.7	50 ± 0.1	66.66 ± 0.1	66.66 ± 0.2	66.66 ± 0.7
	CSR3-SW	62.5 ± 0.7	87.5 ± 0.7	87.5 ± 0.5	100 ± 0	100 ± 0

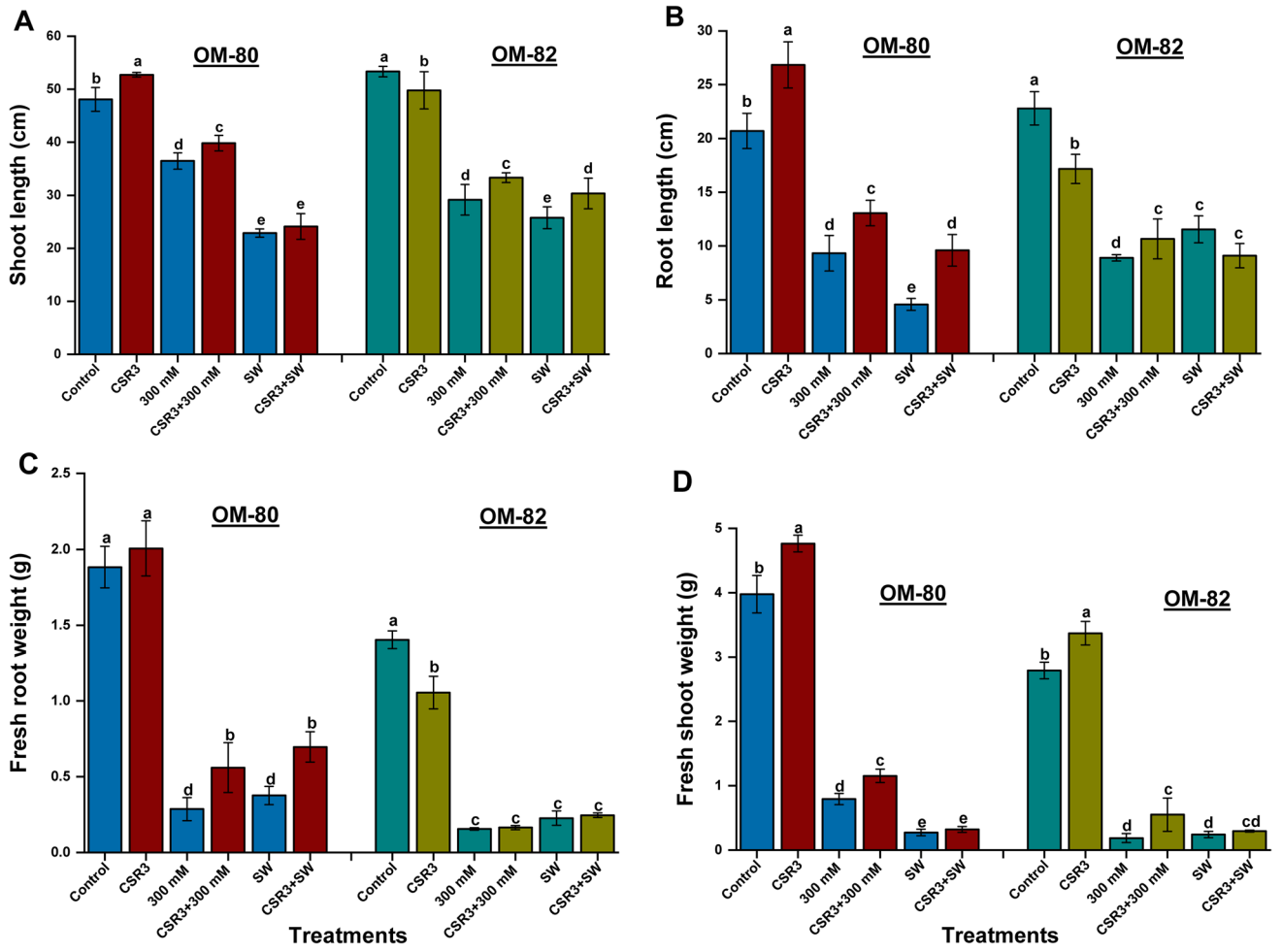

Fig. 2 Effect of inoculation of the halotolerant CSR3 fungal strain on growth of the two barley varieties under normal and salt stress (300 mM NaCl and 100% seawater) conditions **A** shoot length, **B** root length, **C** fresh root weight and **D** fresh shoot weight

after CSR3 inoculation in both OM-80 and OM-82 plants (Fig. 4A).

Effect of CSR3 on the total flavonoid contents

In both CSR3 inoculated and non-inoculated plants, the flavonoid content was examined with and without NaCl and SW stress. The results revealed that flavonoid contents in normal conditions were higher in non-inoculated plants than inoculated counterparts. Our finding revealed that flavonoids were enhanced in salt-treated plants. However, with the inoculation of CSR3 the flavonoid contents of plants of both OM-80 and OM-82 varieties were reduced to almost equal level that of the control plants. Compared to OM-82 plants, higher accumulation level was found in OM-80 plants showing higher adaptability of OM-80 plants to salt stress compared to OM-82 plants (Fig. 4B).

Effect of the endophyticCSR3 on total protein contents under salinity stress

Our results showed that total protein contents were inversely proportional to the salt concentration. However, inoculation of the CSR3 fungal strain significantly increased protein contents compared to their respective non-inoculated counterparts. The most remarkable result was observed in inoculated plants of OM-80 variety treated with seawater, whose protein contents accumulation increased by 31.6% compared to respective control plants. Our results also revealed that salt stress did not affect the accumulation of protein contents in OM-80 variety and found almost similar levels in inoculated and non-inoculated plants. Contrastingly in OM-82 plants, a reduction in total protein contents was recorded under salt conditions; however, the fungal inoculation significantly enhanced the level in inoculated plants (Fig. 4C).

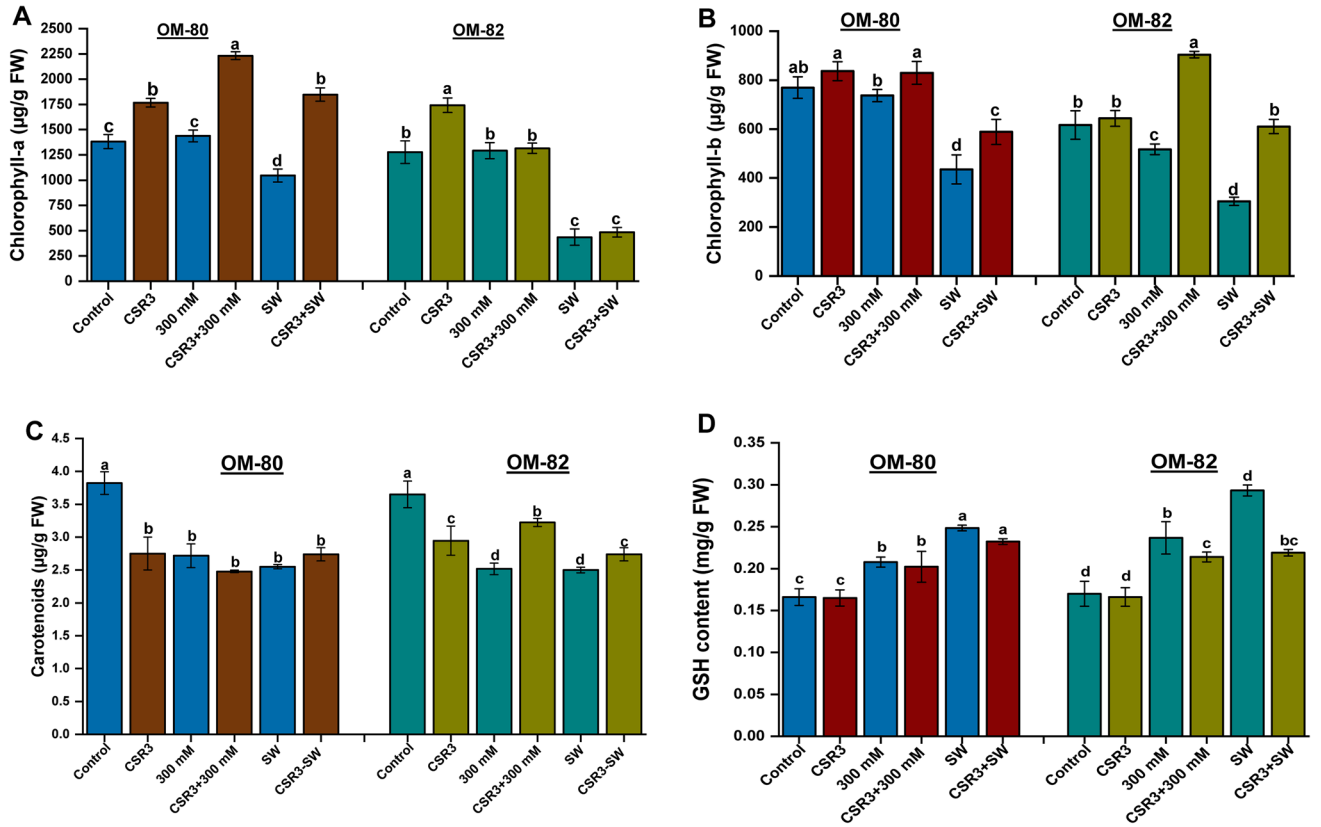

Fig. 3 Effect of inoculation of the halotolerant CSR3 fungal strain on photosynthetic pigments and GSH **A** chlorophyll-a, **B** chlorophyll-b, **C** carotenoids and **D** GSH contents in barley plants under normal and salt stress (300 mM NaCl and 100% seawater) conditions

Effect of CSR3 on the total sugar and starch contents

The effect of CSR3 fungi was analyzed on the accumulation of the total sugar and starch contents of the selected barley varieties. Like flavonoid contents, CSR3 inoculation without salt stress showed an elevation in sugar contents compared to non-inoculated control plants. On the contrary, the sugar contents showed different results as sugar contents decreased when plants inculcated with fungi. In OM-80 variety the highest sugar accumulation was observed in 300 mM stress subjected plants. A significant increase in plant endogenous starch was observed after CSR3 inoculation in the varieties' stress-treated plants. The highest starch contents were observed in control and 300 mM stress treated plants (Fig. 4D, E).

PCA biplot analysis and correlation of traits

PCA biplot analysis was employed to assess the impact of salt stress and CSR3 inoculation on the seedling parameters of two barley varieties, including shoot length (SL), root length (RL), fresh weight (FW), dry weight (DW), chlorophyll content, carotenoids, flavonoids, catalase, reduced glutathione (GSH), and others. The results of the PCA demonstrated that for the OM-82 variety, the first two principal components (PCs) accounted for the highest variance (PC1: 71.6%; PC2: 12.2%), amounting to 83.8% of the total variation (Fig. 5A). The analysis further revealed that the first three principal components for the measured traits, with eigenvalues greater than 1, explained up to 94.32% of the variance among the various physiological and biochemical traits (Supplementary Table 1). The PCA results displayed distinct groupings of treatments, such as salt stress (300 mM and seawater) and CSR3 inoculation, in the PCA plot, indicating varying effects on barley growth parameters. These findings were consistent for the OM-80 variety as well, where the first two PCs with eigenvalues exceeding 1 accounted for 95.7% of the total variation. Notably, both varieties treated with CSR3 exhibited separation from the salt stress treatments in terms of growth parameters, resembling the control group. Furthermore, significant differences were observed between inoculated and non-inoculated stress plants regarding these growth parameters. Parameters like catalase, GSH, and flavonoids were distinctly separated from others and significantly influenced by the application of 300 mM and seawater. Moreover, the PCA analysis demonstrated that CSR3 treatments, both in control conditions and under salt stress, were well-represented in PC1 and PC2,

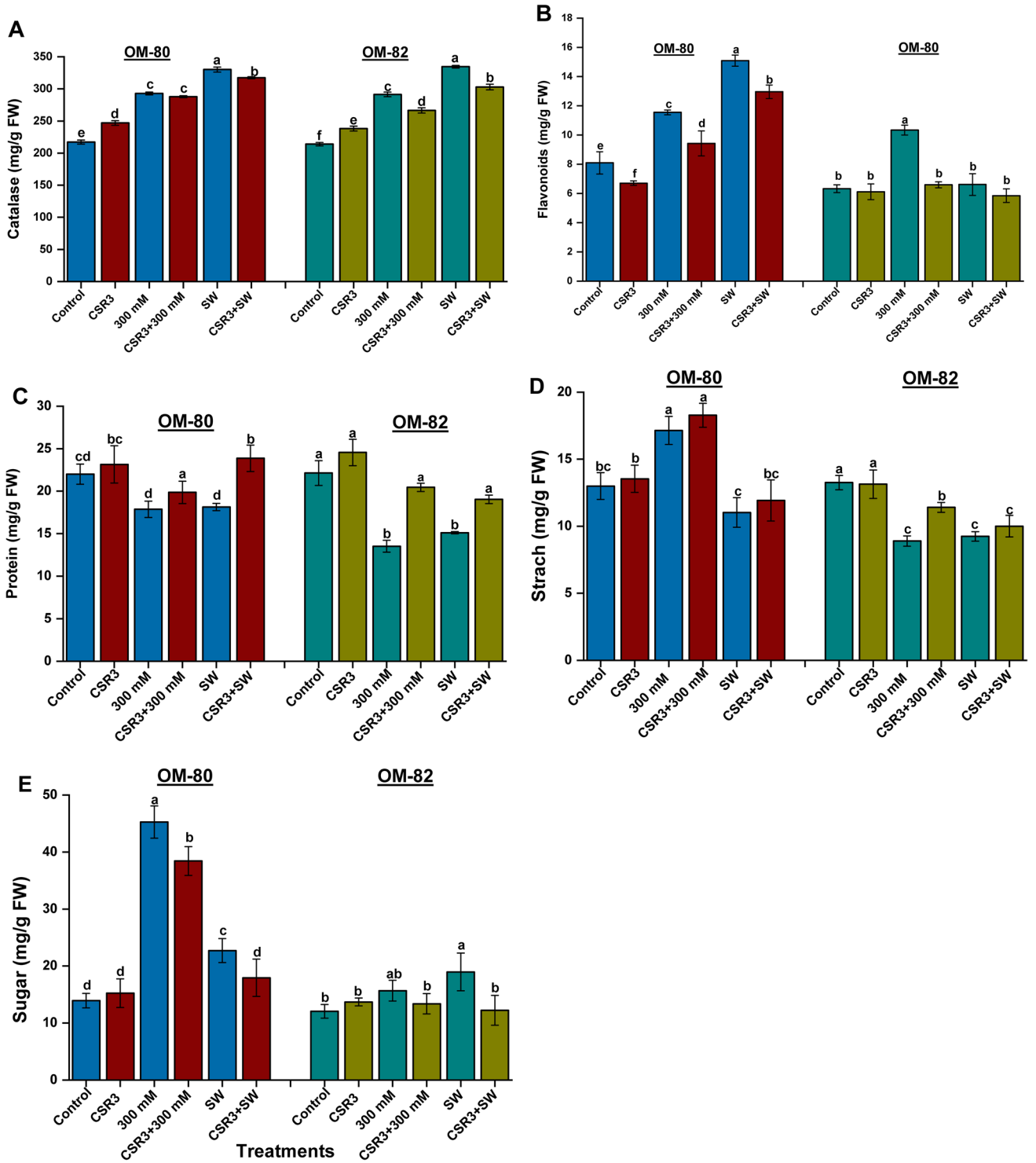

Fig. 4 Effect of inoculation of the halotolerant CSR3 fungal strain on different defensive enzymatic and non-enzymatic antioxidants **A** catalase (CAT), **B** flavonoids, **C** protein, **D** starch and **E** sugar

with a significant impact on SL, RL, FWR, FWS, chlorophyll, carotenoids, and proteins (Fig. 5C).

Furthermore, we performed a Pearson correlation analysis to investigate the extent of the relationship among the traits. The results showed significant correlations among the morphological, biochemical, and antioxidative traits under salt stress and control conditions in both CSR3 inoculated and non-inoculated plants (Fig. 5B, D). The

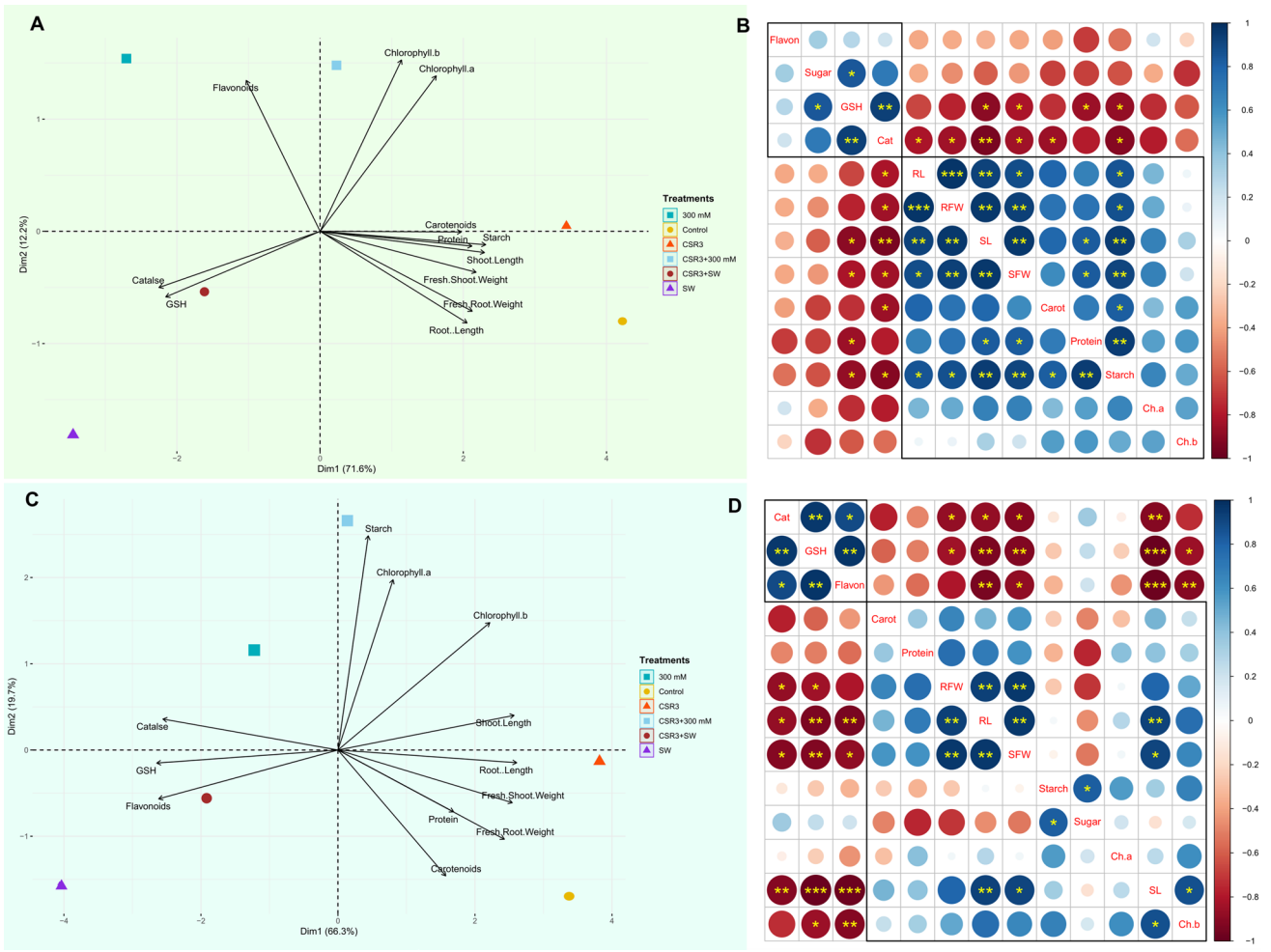

Fig. 5 **A** Principal component analysis (PCA) biplot of individual barley plant (OM-82 cultivar) based on the variance in morpho-physiological, biochemical, and antioxidative traits under control, salt stress (300 mM NaCl and seawater), CSR3 inoculated, and un-inoculated conditions. **B** Pearson's correlation matrix between plant growth attributes, antioxidant enzymes, and secondary metabolites in salt-stressed inoculated and non-inoculated plants. **C** Principal component analysis (PCA) biplot of individual barley plant (OM-80 cultivar) based on the variance in morpho-physiological, biochemical, and antioxidative traits under control, salt stress (300 mM NaCl and seawater), CSR3 inoculated, and un-inoculated conditions. **D** Correlations are displayed in blue (positive) and red (negative); color intensity and circle size are proportional to the correlation coefficient. RL: root length, SL: shoot length, FWS: shoot fresh weight, FWR: root fresh weight, protein, catalase, flavonoid, starch, sugar

results revealed that flavonoids, catalase, and GSH have significantly positive correlations with each other, but they are negatively correlated with all other morphological and metabolic traits in both varieties. Furthermore, the results also showed that the growth parameters, including root/shoot length and weight, have significantly positive correlations with each other. These findings provide insights into the complex interactions among different traits in these two barley varieties under different conditions and highlight the potential of using CSR3 inoculation to improve plant growth and tress tolerance.

Discussion

Salinity stress is one of the most serious environmental constraints which adversely affects normal morphological, phycological, and biochemical processes (Aizaz et al. 2023). Many conventional interventions like agrochemicals and modern molecular breeding techniques like genome editing technologies are used to develop salinity tolerant crop varieties. However, such approaches are not always feasible and might produce serious implications on

the ecosystem (Khan et al. 2021a). Sustainable agriculture is key to coping with the detrimental effects of high salinity. In this context, plant microbes play an important role in incorporating salt stress resistance and regulating the ecosystem's nutrient balance (Kumar and Verma 2018; Verma et al. 2022). Many endophytic microbes have been reported to enhance plants' tolerance to elevated salinity (Vaishnav et al. 2019). Microbes are known to restore ion homeostasis and lessen the negative consequences of ion toxicity and oxidative stress (Gupta et al. 2023). Evidence from the literature has shown that most of the endophytes have the characteristic to produce phytohormones like IAA and GA, which can boost up the host tolerance ability against various biotic and abiotic stress conditions resulting in higher plant development and growth (Asaf et al. 2023b). Our previous study revealed that *A. niger* CSR3 has phosphate solubilizing and siderophore producing capabilities as well as the ability to regulate plant endogenous hormones and secondary metabolites by producing IAA and GAs (Lubna et al. 2018a). The current study attempts to gain a holistic understanding of CSR3 endophytic fungal strain for its salt stress alleviation in two different barley varieties. The results obtained here showed that CSR3 can grow on media supplemented with 2 mM salt concentration. In the current study, we investigated that barley seeds biopriming with CSR3 facilitates germination by diminishing their susceptibility to salt stress. Our results are in agreement with previous studies that the endophytic fungus CSR3 showed a sufficient capability to alleviate salt stress via regulating plant endogenous hormones and antioxidant system (Lubna et al. 2018a, b, 2022). As compared to non-inoculated barley plants, CSR3 inoculated plants showed considerably better growth attributes such as root/shoot length and weight by alleviating the deleterious effects of salt stress. The growth promoting potential of CSR3 under salt stress reflects that this endophytic strain improves plant's ability to uptake Na+ from soil, increase water availability to plants and decrease salt ions absorption in the roots and their transportation to the shoots (Gupta et al. 2021). It has been hypothesized that endophytic microbes increase the photosynthetic content of plants, which in turn promotes photosynthetic activity (Shi et al. 2010). The results obtained and shown in Fig. 3A–C support this hypothesis, as CSR3 positively impacts photosynthetic pigments as a significant increase in chlorophyll and carotenoid contents was observed in inoculated than inoculated plants of both varieties. Enhanced chlorophyll content in inoculated plants could be due to alleviation of ion toxicity and oxidative stress from plants and improved chloroplast metabolism (Khan et al. 2020). Previous studies support the obtained results, that endophytic microbes increase photosynthetic translocation which helped to promote root growth and improved root system architecture to fight salinity (Gupta et al. 2023; Liu et al. 2022). Salt stress induced the overgeneration of oxygen radicals and their derivates called reactive oxygen species (ROS) (Lushchak 2014). Excessive cellular levels of ROS can cause direct injury to nucleic acids, proteins, lipids, and other organelles, leading to cell death (Auten and Davis 2009). A defensive enzymatic and non-enzymatic antioxidative systems are triggered to prevent the formation of these free radicals during stress and repair the damage caused by them (Abogadallah 2010). Increased antioxidant enzyme activities may be considered a protective acclimation response, resulting in lower ROS levels and higher tolerance to oxidative stress [46]. Our results showed that barley plants inoculated with CSR3 have lower CAT antioxidant enzyme activity, indicating less ROS generation, obviating the requirements to withstand salt stress. Furthermore, the ROS also scavenged by the synthesis of non-enzymatic antioxidants such as GSH, flavonoids, and soluble proteins allowing plants to withstand the toxic effects of salt stress (Ahmad et al. 2009; Sarker and Oba 2018). In the current study, barley plants treated with salt stress have significantly higher levels of GSH, flavonoids, and protein contents than CSR3 inoculated plants showing the amelioration ability of CSR3 to minimize the toxic impacts of salinity. These findings corroborate those of Asaf et al. (2023a) and Egamberdieva et al. (2021), who demonstrated that inoculation of endophytic fungus reduced level of the non-enzymatic antioxidants and eliminate Na^+ from the soil. Metabolites like starch and sugar were also significantly affected by the fungus alone and the combination of the fungus with salt treatments. Our results revealed a decrease in starch accumulation accompanied by an increase in sugar content in stress conditions confirming the adaptive response of the plants to water deficit conditions induced by salt stress. Similar results were also found in previous studies conducted by Ahmadi and Baker (2001), Canellas and Olivares (2014) and Dkhil and Denden (2010). However, CSR3 enhanced the level of starch and decreased sugar in both verities under salt stress indicating its ability to convert excess sugar to starch.

PCA was used to identify and evaluate barley plants' most important selection traits for salt tolerance. The multivariate analysis technique, PCA-biplot, was used to combine traits in two dimensions by minimizing overlapping variations and to determine main characters for selection (Arzu et al. 2018). The PCA revealed that PC1 had a positive and strong correlation with plants biomasses and photosynthetic pigments under control and CSR3 + 300 mM NaCl conditions, whereas in PC2, a negative and strong correlation with flavonoids, catalase, and GSH was measured both under 300 mM NaCl and SW treated conditions. These results reflect that the traits of PC1 can be considered for better performance

of plants under control and salinity conditions whereas, the traits disseminated in PC2 can be considered sensitive to salinity stress conditions.

Conclusion

The current study's finding revealed that the halotolerant endophytic *A. niger* CSR3 significantly increased germination percentage and growth of barley plants under control as well as salt stress conditions. The selected strain consistently showed the potential to maintain plants cellular homeostasis against salinity stress. The stress-adopted fungi resist salt stress in barley by improving various physiological and biochemical parameters such as modulation of enzymatic and enzymatic antioxidant systems and production of secondary metabolites. The conclusion of the current study provides a valuable eco-friendly and cost-effective approach to improve sustainable agricultural production in salt-affected areas. Research is still required to fully comprehend how CSR3 influences barley development and growth under salt stress conditions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42976-023-00456-5.

Author contributions IK, L, SSA, and SA performed experimental and analysis. RJ, SA, and SB performed antioxidant analysis and wrote the draft manuscript and statistical analysis. K-MK and AA-H, supervision and arranging resources.

Funding This work was supported by the Ministry of Higher Education, Research & Innovation and the Ministry of Agriculture, Fisheries & Water Resources under the project (MoHERI/SRPP/MoAFWR/1/2022/RI/01). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability Not applicable.

Declarations

Ethical approval Not applicable.

Informed consent Not applicable.

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