

Mitigating Cadmium Stress in Milk Thistle through Plant Growth-Promoting Rhizobacteria and Radiated Achenes: A Biochemical and Physiological Perspective

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Abstract

Soil contamination with cadmium (Cd) as a significant environmental challenge, has become a persistent and highly toxic heavy metal due to increased industrial and agricultural activities. Cd exposure negatively impacts plant growth, photosynthetic efficiency, nutrient uptake, and oxidative balance, which reduce crop productivity and harmful effects to human health. This study aimed to evaluate the effect of two eco-friendly approaches, plant growth-promoting rhizobacteria (PGPR) and gamma-radiated achenes (0-10 GY) on growth indices, biochemical, and yield attributes of milk thistle (*Silybum marianum*) under 200 μM Cd stress. Results revealed that combination of PGPR inoculation and 4 GY radiation improved shoot length by 31%, photosynthetic rate by 7.08%, and silymarin content by 28%, while reducing oxidative stress markers H_2O_2 by 33%, MDA by 7.89% under Cd stress. Enhanced activity of antioxidant enzymes such as, superoxide dismutase, catalase, by 97% and 50%, respectively, and accumulation of phenolics and flavonoids by 100% and 33% were also observed. Mineral nutrients, osmolytes, photosynthetic pigments, gas exchange parameters, silymarin content, and oil content revealed significant improvement with the application of PGPR and achene radiation treatments. Overall, treatment effectiveness ranked as radiation doses (GY): $4 > 2 > 0 > 6 > 8 > 10$, with 4 GY + PGPR showing the greatest improvement in Cd tolerance and silymarin accumulation. However, growth parameters, biochemical, and yield attributes were significantly increased with the application of PGPR under non-stressed plants. These findings highlight the potential of integrating PGPR and gamma radiation to improve milk thistle resilience and productivity in Cd-contaminated environments.

1. Introduction

Abiotic stresses such as extreme temperatures, elevated CO_2 , heavy metals (HMs), salinity, drought, waterlogging or pathogen infection could have a devastating effect on plant physiological functions and production. Among these stressors, HMs are a major abiotic constraint that affects agricultural productivity and human health [1]. Notably, cadmium (Cd), lead (Pb), aluminum (Al), copper (Cu), chromium (Cr), manganese (Mn), mercury (Hg) and zinc (Zn) are among the main hazardous HMs [2], due to their inability to undergo biodegradation. These metals exhibit high environmental mobility [3] and cause severe consequences on plant growth and development, plant metabolism and agriculture productivity. They also enhance the production of reactive oxygen species (ROS), which impairs key cellular components in plants [4, 5]. Among HMs, Cd is particularly more dangerous consequences on plant growth and yield and causes chlorosis and necrosis which limit crop growth and yield because it competes with other essential nutrients and prevents their uptake by plants [4].

Microbes-assisted soil remediation involves the detoxification, removal, and sequestration of HMs by an eco-friendly approach [6]. Agricultural scientists have found that plant growth-promoting rhizobacteria (PGPR) mitigates HMs toxicity and enhances plant growth in HMs polluted soils [7, 8]. It is interesting to note that microorganisms have resistance tactics due to their ability to create a variety of metabolites [9]. For instance, seed priming with *Azotobacter chroococcum* and *Piriformospora indica* in milk thistle plants under salt stress significantly enhanced relative water content, membrane stability index,

osmoprotectant accumulation, antioxidant capacity, synthesis of flavonoids, phenylpropanoids, silydianin, taxidoline and flavolignans, fatty acid composition and key enzymes such as rubisco and phenyl ammonium lyase contents [10].

Gamma irradiation, a safe and sustainable approach for mitigating abiotic stresses, is typically derived from radioactive sources such as cobalt-60 [11]. It has been successfully used to enhance prolong shelf life, maintain postharvest quality under storage stress, and improve tolerance to drought, salinity, and other stresses, as well as to enhance secondary metabolite production in crops such as wheat, rice, fenugreek, and milk thistle [11, 12]. For instance, low-dose gamma irradiation of seeds has been shown to improve the antioxidant defense system and bioactive compound accumulation, regulate reactive oxygen species (ROS) homeostasis, and promote osmoregulation, thereby protecting chloroplast ultrastructure, upregulating abscisic acid (ABA) biosynthesis genes, and suppressing ABA catabolic genes in sunflower and fenugreek under HMs stress conditions [13, 14]. Recent studies also demonstrate that irradiated milk thistle (*Silybum marianum*) achenes exhibit elevated phenolic content [15]. Similarly, Gamma radiation application in *in vitro* callus of milk thistle improved important medicinally secondary metabolites and critical flavonoids, such as rutin, naringin, rosmarinic acid, apigenin, kaempferol quercitrin, apigenin-7-glucoside, quercetin, naringenin and kaempferol-3-(2"-p-comaroyl) glucoside [12]. Nevertheless, the effectiveness of radiation-primed achenes in improving growth, silymarin contents, yield potential, and other physiological and biochemical performance is still unexplored under *in vivo* conditions.

Milk thistle [*Silybum marianum* (L.) Gaertn. family *Asteraceae*] is cultivated as a herbaceous annual or biennial dicotyledon plant [16]. This species is widely cultivated worldwide for its high medicinal value, particularly in the pharmaceutical sector [17]. Its achenes are rich in bioactive compound, such as apigenin, chrysoeriol, dihydrokaempferol, eriodictiol, flavonolignans, kaempferol, isosilybin, naringin, quercetin, silybin, silychristin, silydianin and taxifolin [18]. These compounds are employed in the treatment of numerous disorders such as fatty liver disease, viral hepatitis, and mushroom poisoning [19]. However, limited research has examined the effects of Cd stress on morphology, growth, physicochemical mechanisms, and yield of milk thistle, and no studies have investigated the effect of PGPR in this species. Moreover, the combined impact of radiation-treated achenes and PGPR application under Cd stress has not been reported. Therefore, this study aims to evaluate the effects of different radiation treatments and PGPR applications on growth performance, yield, oil content, silymarin content, photosynthetic attributes, antioxidant activity, and osmotic regulation in milk thistle plants exposed to Cd stress.

2. Material and methods

The potential of milk thistle to withstand Cd (200 μ M) stress was assessed by using PGPR and different radiation achene pre-treatment in a pot study conducted at the wire-house (control conditions) of Botanical Garden of Government College Women University Faisalabad (located at 31.4180°N, 73.0790°E, altitude; 184 meters), Punjab, Pakistan. The soil was analyzed for its physicochemical

properties before sowing. The soil electric conductivity was 1.2 dS/m, and pH was 6.8 suitable for milk thistle growth. The NPK ratio was 300:12:110 mg/kg (total nitrogen: available phosphorus: exchangeable potassium). Achenes of milk thistle were radiated at NIBGE (National Institute for Biotechnology and Genetic Engineering) in the section of their mutation breeding program, radiation measurement is a critical component of their mutation breeding program. Gamma-radiated achenes of milk thistle (0 GY, 2 GY, 6 GY, 8 GY, and 10 GY) were sterilized in 20% sodium hypochlorite for 10 minutes, afterward washed with deionized water and planted in pots filled with 10 Kg soils. After 30 days of germination, Cd stress was applied and *Bradyrhizobium* stain japonicum-S377 was applied in soil media.

To grow rhizobium yeast mannitol (12.8 g) was added in 1000 mL of distilled water. After cooling the distilled water, a stain of rhizobium was poured into it and then the sample was placed on a shaker for two days. After maximum growth of rhizobium, treatment was applied through soil fertigation. The PGPR suspension solution was supplemented once a week for 3 times into the soil. To analyses physiochemical analysis and growth attributes one set of plants were harvested at BBCH principal growth stage 5, and one set was kept till maturity to collect achenes and determination of their oil and silymarin (Sil) contents.

2.1. Morphological measurements

Morphological traits recorded included number of leaves (NOL), shoot length (SL; g), shoot dry weight (SDW; g), achenes per capitulum (AC) and number of capitulum per plant (CP), root length (RL; cm), shoot fresh weight (SFW; g), root fresh weight (RFW; g), root dry weight (RDW; g), number of achenes per plant (AP), and capitulum diameter (CDi; cm²). For dry weight determination, shoots and roots were separated and oven-dried at 75°C for 72 h in a forced-air oven.

2.2. Photosynthetic and gas exchange measurements

Chlorophyll a (Chl a) and chlorophyll b (Chl b) pigments were quantified according to the method of Davis et al. [20], and carotenoid contents were measured following the prescribed method of Arnon [21]. For this process, fresh leaves (0.5 g) were chopped and added in 10 mL of 80% acetone. The absorbance was recorded at 645, 663, and 480 nm using spectrophotometer (Hitachi-U2001, Tokyo, Japan). Moreover, an infrared gas analyzer (IRGA), was used to record the following data: transpiration rate (E), photosynthetic rate (A), stomatal conductance (gs), and substomatal CO₂ concentration. These parameters were taken between 11 and 12 pm. For this purpose, the top third leaf of each plant was used.

2.3. Assessment of antioxidant enzyme activities

Fresh leaves (0.25 g) were homogenized with 5 mL of potassium phosphate buffer in a pre-chilled mortar and pestle. The activity of catalase (CAT) was determined using the methodology of Chance and Maehly [22]. CAT reaction mixture was prepared by adding, 0.1 mL of enzyme extract, 1 mL of hydrogen peroxide H₂O₂ (5.9 mM H₂O₂), and 1.8 mL of potassium phosphate buffer 50 mM (pH 7.8). The

absorbance was noted every 20 seconds for three minutes by using spectrophotometer at a wavelength of 240 nm and the results were expressed as μg protein per min.

Peroxidase (POD) activity was measured by following the protocol of Chance and Maehly [22]: 50 μL of enzyme extract was mixed with 750 μL of potassium phosphate buffer and 100 μL of guiacol. The absorbance of the reaction solution was then monitored for three minutes at 470 nm at every 20 seconds using spectrophotometer and the results were expressed as μg protein per min.

The activity of superoxide dismutase (SOD) was determined by adding the enzymatic extract (50 μL) with 400 μL distilled water, 250 μL potassium phosphate buffer, 50 μL riboflavin, 1000 μL L-methionine, 50 μL riboflavin, 75 μL triton-x, and 50 μL NBT (Giannopolitis and Ries, 1977). A UV-visible spectrophotometer was used to test the reaction mixture at 560 nm and the results were expressed as μg protein per min.

2.4. Determination of oxidative stress attributes

Malondialdehyde (MDA) was calculated using the method of Cakmak and Horst [23]. A 0.1 g fresh plant sample was ground in 1 mL of 1% trichloroacetic acid (TCA; 1% w/v). Accordingly, 1 mL of extract was mixed with 1 mL of 0.5% thiobarbituric acid and 20% TCA. After being heated to 95°C for 50 minutes, then the sample was cooled in an ice bath. The absorbance was measured at 532 and 600 nm.

Measurements of hydrogen peroxide (H_2O_2) were recorded according to Velikova et al. [24] methodology. A 0.1 g of fresh leave samples were extracted with 1 mL of 0.1% TCA. The mixture was centrifuged at 12,000 rpm for 15 minutes. Afterward, 1 mL of potassium iodide, 0.5 mL of phosphate buffer (pH 7.0), and 0.5 mL of the supernatant were mixed vigorously. The absorbance was measured at 390 nm.

2.5. Determination of ascorbic acid

Ascorbic acid (AsA) content was measured by using the method of Mukherjee and Choudhuri (1983). For extraction, 0.1 g of leaf and root sample was homogenized in 6% TCA. For 1 mL of the extract, 1 mL of Dinitrophenyl hydrazine was added, and then one drop of thiourea was added. Then sample was placed into water bath for 15 minutes; then cooled in an ice bath. By keeping the mixture on ice, 5 mL of H_2SO_4 (80%) was gently added; after that absorbance was measured at 530 nm.

2.6. Determination of osmoprotectants

The method of Bates et al. [25] was used to determine free proline (Pro) content. For this purpose, 0.25 g of fresh leaf tissue was homogenized in 10 mL of 3% sulfosalicylic acid and then filtered through Whatman No. 2 filter paper. Subsequently, 2.0 mL of the filtrate was mixed with 2 mL of 6 M orthophosphoric acid and 2.0 mL of ninhydrin reagent. The reaction mixture was incubated at 100°C for 60 minutes and then rapidly cooled in an ice bath. After that, 2.0 mL of toluene was added, and the mixture was vortexed. The absorbance of the upper organic phase was recorded at 520 nm using a spectrophotometer.

Glycine betaine (GB) content was quantified according the method of Grieve et al. [26]. A 0.5 g of fresh plant material was homogenized in 5 mL of distilled water and filtered. Then, 0.5 mL of the filtrate was mixed with 0.5 mL of 2 N HCl. Subsequently, 0.25 mL of the mixture was poured in a test tube, followed by the addition of 0.1 mL potassium tri-iodide solution. The reaction mixture was combined with 10 mL of chilled 1,2 dichloromethane and 1.0 mL of ice-cooled distilled water. The optical density was recorded at 365 nm using a spectrophotometer.

Total free amino acids (FAA) was determined according to the method of Hamilton et al. (1943). Briefly, 1 g of fresh plant tissue was extracted using a phosphate buffer (pH 7.0). Thereafter, 1 mL of the extract was mixed with 1 mL of 2% ninhydrin solution and 1 mL of 10% pyridine solution. The reaction mixture was heated for 30 minutes and then diluted up to a final volume of 25 mL with distilled water. The absorbance was measured at 535 nm using a spectrophotometer.

The content of soluble sugar (SS) was examined following the method of Yoshida [27]. A 0.1 g of fresh plant material was ground in 2 mL of potassium phosphate buffer (50 mM, pH 7.8). The extract was centrifuged at 14,000 rpm for 10 minutes. Then, phosphate buffer solution, 1.25 mL H₂SO₄ (96%), phenol (25 µL; 5%), and extract (500 µL) were added to test tubes and reading absorbance was taken at 490 nm using a spectrophotometer.

2.7. Determination of secondary metabolites

Total phenolic content was measured according to the method of Julkunen-Tiitto (1985). For this purpose, 0.1 g of frozen leaf samples were homogenized in 4 mL methanol. After that, 800 µL of 700 mM Na₂CO₃, 100 µL of Folin-Ciocalteu reagent, and 100 µL of the supernatant were added to each test tube. The absorbance was measured at 765 nm using a spectrophotometer.

Anthocyanin content was determined according to the protocol described by Strack and Wray [28]. Briefly, 0.1 g of fresh plant tissue was ground in 2.5 mL of acidified methanol and heated at 50°C for 1 hour. Following centrifugation, the absorbance of the supernatant was measured at 535 nm.

Flavonoid content was determined following the method of Zhishen et al. [29]. 0.1 g of fresh plant tissue was homogenized in 80% acetone. Subsequently, 1 mL of extracted solution and 4 mL of distilled water were mixed in test tubes. After that, 0.6 mL of 5% NaNO₂, 0.5 mL of 10% AlCl₃, and 2 mL of 1 M NaOH were added sequentially. The reaction mixture was diluted with 2.4 mL of distilled water and absorbance was recorded at 510 nm.

Silymarin content was determined using Soxhlet extraction following the procedure described by Saleh et al. (2017). For extraction, 10 g of achene samples were placed in a thimble holder within a Soxhlet apparatus. Approximately 200 mL of 80% methanol (prepared in distilled water) was used as the extraction solvent, and the extraction process was continued for 6 h. The absorbance of the extracted solution was subsequently measured at 530 nm according to the method described by Rahman et al. (2004).

2.8. Determination of nutritional elements

According to Wolf [30] method, 0.1 g of dried plant shoot material was added with 5 mL of HNO_3 , and the mixture was heated at 250°C until fumes began to form. This process was repeated until the digestion material became transparent. After that, the sample was filtered and diluted with 50 mL of distilled water. After that, sodium (Na), calcium (Ca), potassium (K), chloride (Cl), and magnesium (Mg) contents were measured by using a flame photometer.

2.9. Statistical Analysis

The efficacy of PGPR potential and radiation dose was evaluated for the growth, yield and physiochemical attributes of milk thistle under Cd stress by using five plants of each treatment as replicates. For each treatment, three independent replicates were applied. The experimental design was CRD factorial (3-way). Analysis of variance (ANOVA) at a 5% probability level through the software "Statistix" (version 8.1, Tallahassee, FL, USA) was performed. The least significant differences (LSD) test was utilized to compare relative means. Heatmaps and principal component analysis were generated by using R programming language. Distinct letters in tables and figures indicate significant differences ($p \leq 0.05$), whereas identical letters represent no significance.

3. Results

3.1. Morphological and yield parameters

Results obtained for NOL and SL manifested highly significant ($p < 0.001$) differences in radiation and PGPR under Cd stress and the interaction among them was also highly significant ($p < 0.001$). Statistical results for SDW displayed highly significant ($p < 0.001$) differences in radiation and PGPR under Cd stress and the interaction among them was non-significant ($p > 0.05$), however, statistical data for number of AC and CP demonstrated non-significant ($p > 0.05$) differences in radiation and PGPR under Cd stress and the interaction among them was highly significant ($p < 0.001$). While data regarding RL, SFW, RFW, RDW, NOF, number of achenes per plant AP, CDi showed non-significant ($p > 0.05$) difference in PGPR and radiation under Cd stress and the main interaction among them was also non-significant (Table 1). Under control conditions, PGPR supplementation was very beneficial for improving these parameters in all (0 GY, 2 GY, 4 GY, 6 GY, 8 GY, and 10 GY) radiated plants but 4 GY radiated plants showed remarkable results, and the percent increase was 31.1475%, 81.8%, 53.8%, 41.66%, 200%, 40%, 84.21%, 84%, 54.54%, 37.7% and 33.33% for SL, RL, SFW, RFW, SDW, RDW, NOL, NOF, AC, AP and CDi, respectively under Cd stress (Tables 1 & 2). Data further revealed that the order of improvement in 4 GY > 2 GY > 0 GY > 6 GY > 8 GY > 10 GY respectively, under control and Cd stressed condition respectively. Overall, Cd stress decreased all these morphological parameters and caused severe yield penalty.

3.2. Photosynthetic attributes and Gas exchange (IRGA) attributes

Statistical analysis for Chl a, Chl b, Caro, TChl, E, Pn, iWUE, gs, and Ci revealed non-significant ($p > 0.05$) differences in radiation and PGPR under Cd and the interaction among them was also non-significant (Figs. 1 & 2). Photosynthetic parameters increase with the increase in radiation levels and the maximum increase was observed at 4 GY after higher doses these attributes significantly decreased. However, PGPR treatments particularly at 4 GY significantly increased Chl a, Chl b, TChl, Caro, E, Pn, iWUE, gs and Ci and the percent increase was 85%, 36.66%, 42%, 30%, 91%, 7.08%, 53%, 77% and 9.37% for respectively under Cd stress condition. Under control conditions, the order of improvement of the effectiveness of increase was: 4 GY > 2 GY > 0 GY > 6 GY > 8 GY > 10 GY. However, Cd stress deteriorates all the photosynthetic and gas exchange attributes irrespective of PGPR application and radiation achene pre-treatments.

3.3. Oxidative stress and antioxidants

Statistical analysis for H_2O_2 , MDA, SOD, POD, CAT and AsA revealed non-significant ($p > 0.05$) differences in radiation and PGPR. Antioxidants increase with the increase in Cd stress, PGPR, and achene pre-treatment especially at 4 GY, and the present increase was 97%, 35%, 50%, and 100% for SOD, POD, CAT, and AsA respectively (Fig. 3). Meanwhile, H_2O_2 and MDA decreased at 4 GY, and more reduction was observed with the application of PGPR. The percent reduction in H_2O_2 and MDA was 33% and 7.89%, respectively, with the application of PGPR at 4 GY achene pre-treatment. Overall, Cd stress increased antioxidants and ROS irrespective of achene pre-treatments and PGPR applications.

3.4. Osmoprotectants

Statistical analysis for GB revealed highly significant ($p < 0.001$) differences in radiation and PGPR under Cd stress and the interaction among them was also significant. Statistical analysis for Pro, TFAA and SS revealed non-significant ($p > 0.05$) differences in radiation and PGPR under cadmium, and the interaction among them was also non-significant. Osmoprotectants increased under Cd stress; however, PGPR treatments, particularly at 4 GY, significantly increased 97%, 22%, 23%, and 36% for GB, Pro, TFAA, and SS contents, respectively, with PGPR application under Cd stress (Figs. 4–5).

3.5. Secondary metabolites

The contents of phenolics, flavonoids, and anthocyanin were not significantly affected ($p > 0.05$) by radiation or PGPR treatments under Cd stress and the interaction among them was also non-significant (Fig. 4). Nevertheless, Cd stress promoted the accumulation of these secondary metabolites; However, under Cd stress and PGPR treatments, particularly at 4 GY, significantly increased phenolic, flavonoid, and anthocyanin contents by 100%, 33%, 110%, and 100%, respectively, compared with the control.

3.6. Nutritional relations

Statistical analysis for K, Ca, Na and Cd revealed non-significant ($p > 0.05$) effects in response to radiation or PGPR treatments under Cd stress treatment, and the interaction effect was also non-

significant. K, Ca, and Na nutrient uptake were decreased, while Cd content increased under Cd stress. However, PGPR treatments, particularly at 4 GY led to significant enhancements in K, Ca, Na, and Cd concentrations—by 28%, 17%, 28%, and 14%, respectively—relative to the stressed plants (Fig. 5).

3.7. Chord diagram and heatmap

The chord diagram revealed significant variability in the effects of GY on the measured parameters, underscoring treatment-specific interactions. Notably, treatments like CC0 and CR8 demonstrated pronounced contributions, as evidenced by their wider arcs, indicating a potentially higher impact on all the studied parameters (Fig. 6). The heatmap correlation matrix revealed that Cd4GY, CdR4GY, CR4GY, CC4GY, and CR2GY showed a strong positive relationship with all the studied parameters except for ROS and Cd stress indicators (Fig. 7). While CC0GY, CC2GY and CR0GY showed a strong negative relationship with CAT, phenol, flavonoid, POD, SOD, Pro, FAA, TSS, GB, AsA, and ROS, and positive and no relationship with the rest of the parameters, while the opposite trend was observed for Cd10GY, CdR10GY, CC10GY, CR8GY, CC8GY, CR10GY, Cd8GY, CdR8GY, Cd6GY and CdR6GY.

4. Discussion

Gamma radiation has been considered as a rapid and effective method for inducing variations in plants' physiological and metabolic processes [31]. The achene pericarp of milk thistle contains silymarin, which is used to treat hepatitis patients and has earned a lavish reputation due to its economic profits and its potential uses in the pharma and cosmetics industry. Limited research has explored strategies to enhance silymarin accumulation and achene productivity through the combined use of plant growth-promoting rhizobacteria (PGPR) and gamma irradiation treatments. In order to ascertain the milk thistle potential for Cd stress tolerance as well as the effects of medium supplementation PGPR on growth, yield, physiological characteristics, and silymarin concentration, the achenes were treated with different gamma radiation doses (0, 2, 4, 6, 8, and 10 GY). The main hypothesis proposed that gamma irradiation and PGPR inoculation would synergistically enhance silymarin biosynthesis and improve plant tolerance to Cd-induced stress.

Cadmium stress severely impaired plant growth and yield-related parameters such as RL, SL, SFW, SDW, CDi, AP, AC, CP, and NOL. However, gamma irradiation at 4 GY effectively ameliorated the toxic effects of Cd stress in combination with PGPR (Table 1–2). The remarkable improvement in growth performance, yield attributes, and Cd tolerance at 4 GY might be due to the transduction of intertwined signaling pathways. Notably, all the irradiated achenes employed in this experiment consistently outperformed in enhancing all the growth and yield attributes as compared to non-treated achenes plants, regardless of Cd exposure. These findings align with Atteh and Adeyeye [32], who demonstrated that low-dose gamma radiation effectively improved the germination potential and growth attributes of pea (*Pisum sativum* L.) and mung bean (*Vigna radiate* L.) seedlings.

Moreover, *Bradyrhizobium* inoculation plants significantly improved all the growth and yield attributes irrespective of radiation doses as compared to uninoculated control plants under Cd stress and non-

stressful conditions. The enhancement growth might be due to improved root development and the synthesis of phytohormones like gibberellins and auxins that improve plant resilience in Cd-contaminated soils [33, 34]. This study further confirmed that PGPR inoculation not only led to higher growth and yield attributes but also elevated photosynthetic pigment content (Chl a, Chl b, TChl, and carotenoid contents) and gas exchange parameters (A, E, gs, Ci, and iWUE) under Cd stress (Fig. 1–2). Similar results were reported by Bhardwaj et al. [35] in *Brassica juncea* L. where PGPR improved photosynthetic attributes. Moreover, achene-irradiated plants showed higher gas exchange attributes at all levels, but remarkable results were ascertained at 4 GY treatment and these findings are aligned with the study of Salloom et al. [36]. However, Cd stress decreased photosynthetic efficiency that might be due to slow growth rate, lower number of leaves, and accumulation of ROS (MDA and H₂O₂) as shown in Fig. 3. These results are in agreement with the findings of Luo et al. [37] who reported that Cd stress-induced ROS production that leads to growth inhibition, chlorosis, and potentially plant death. To counteract these ROS, plants utilized a complex network of non-enzymatic (AsA, phenolics, flavonoids, silymarin, and anthocyanins) and enzymatic antioxidant systems (CAT, POD, and SOD) to manage ROS production and protect plants from oxidative stress (Fig. 3). Enhanced production of osmoprotectants (GB, Pro, SS, and TFAA) was reported as a defensive strategy in milk thistle plants to counteract the adverse effect of abiotic stress [38, 39].

PGPR application further enhanced antioxidant activity, secondary metabolite synthesis, and osmoregulation in the present study which thereby resulted in lower ROS-induced oxidative damage. These findings align with the study of AbuQamar et al. [40], who reported that PGPR employed a wide range of mechanisms to alleviate the toxic effects of abiotic stress by siderophores production, secondary metabolites, ion homeostasis, phytohormones synthesis and enzymatic activation. In the present study, higher ionic homeostasis and lower Cd accumulation were confirmed with the application of PGPR. Certain secondary metabolites produced by PGPR directly participate in HMs bioaccumulation, bioabsorption, altering meta(loid), sequestration, mobilization, and chelation thus inhibiting the bioavailability of these HMs in plants [41]. Moreover, achene gamma radiation treatments especially at 4 GY with PGPR outperformed in terms of secondary metabolite production, osmoregulation, nutrient uptake and ROS scavenging (Figs. 4–5). Khalifa et al. [12] also revealed that the application of γ -radiation as an elicitor improved medicinally important secondary metabolites and flavonoids such as rutin, naringin, rosmarinic acid, apigenin, kaempferol quercitrin, apigenin-7-glucoside, quercetin, naringenin and kaempferol-3-(2"-p-comaroyl) glucoside in milk thistle callus. Similarly, [42] reported that gamma radiation-induced increases in chemical composition of antioxidants, secondary metabolites, polyphenols, and flavonoids.

5. Conclusion

This study demonstrates the detrimental effect of Cd stress on plant growth and yield attributes by overproduction of reactive oxygen species, nutrient imbalance, reduced intrinsic water use efficiency, degradation of photosynthetic pigments, impaired gas exchange parameters. These disruptions hinder physiological and metabolic equilibrium, ultimately diminishing plant productivity. However, the

application of PGPR effectively counteracted Cd-induced damage by scavenging reactive oxygen species (hydrogen peroxide and malondialdehyde), enhancing secondary metabolite synthesis, improving osmoregulation, restoring ionic homeostasis, and increasing antioxidant activity. Moreover, achene irradiation treatment 4 GY followed by 2 GY also enhanced these attributes, especially with the application of PGPR, and demonstrated a remarkable ability to mitigate the adverse effect of Cd stress. Therefore, the integration of PGPR with radiated achene provides a promising strategy to enhance medicinally important constituents of milk thistle such as flavonoids, phenolics, silymarin and oil contents, boost plant resilience against heavy metals and optimize agriculture productivity in contaminated environments. So, PGPR and radiation treatment at 4 GY is recommended to enhance achene production and photoactive compounds under abiotic stresses. However, further research can be conducted by upscaling the findings to field conditions to validate the efficiency of PGPR and radiation treatments under real-world agriculture settings. Moreover, genetic exploration of Cd stress-responsive genes may also provide clearer proof of stress tolerance under application of PGPR and irradiation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Author Contribution

AK and NZ were responsible for supervising this research jointly. AK, NZ, and SZ, provided constructive guidelines on the development of research study. AB conducted the study and AA and Shabana E helped in laboratory work. MBH, FR, SZ, SE, and KTK, helped in data analysis, reviewed and editing the manuscript. All authors contributed to the improvement of this manuscript through proofreading.

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Availability of data and materials

All data generated or analyzed during this study are included in the published article and its Supplementary Information.

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Tables

Table 1 to 2 are not available with this version.

Figures

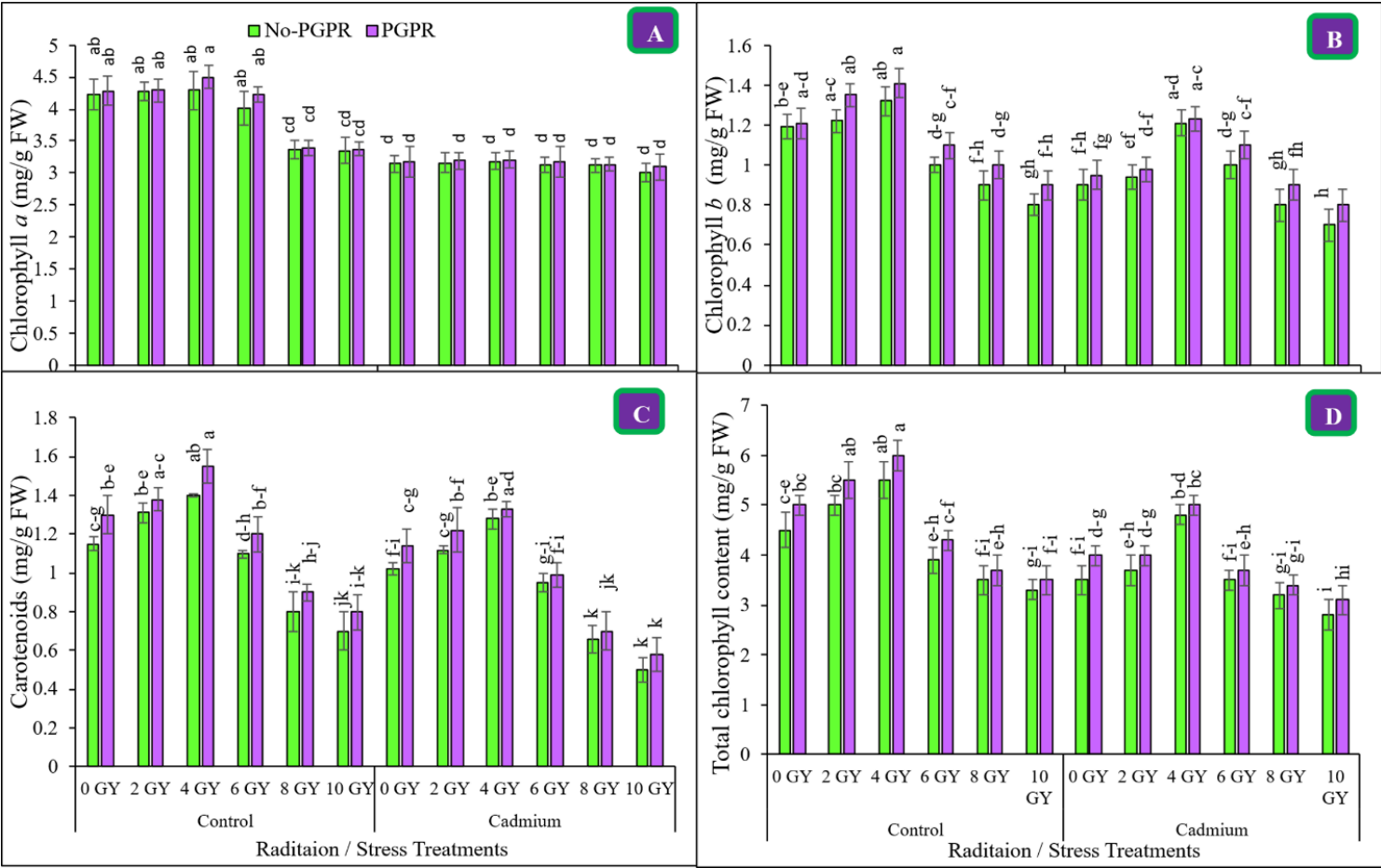


Figure 1

Effect of PGPR application on chlorophyll a (A), chlorophyll b (B), carotenoids (C) and total chlorophyll (D) of radiated achenes of milk thistle under cadmium stress.

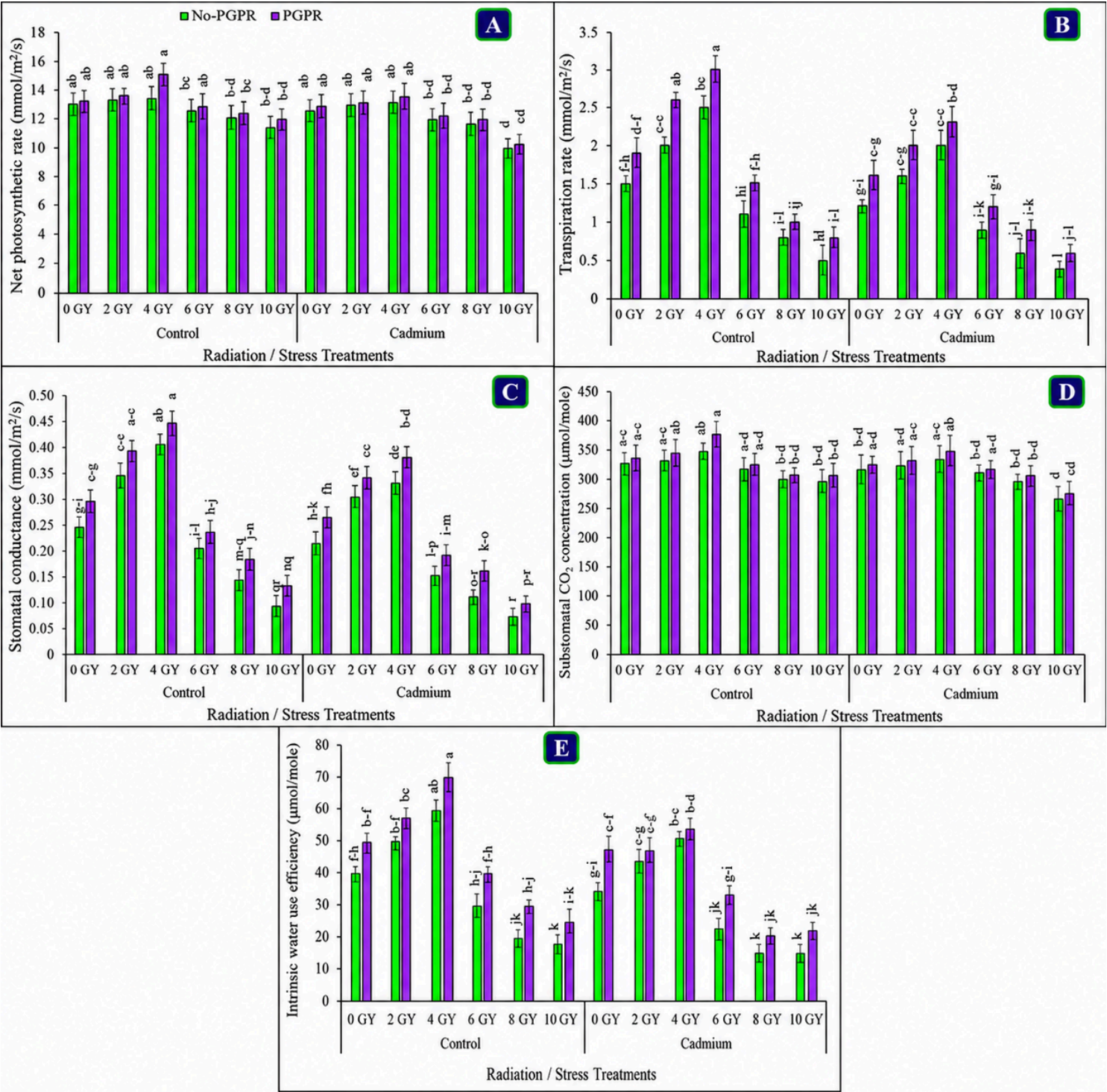


Figure 2

Effect of PGPR application on net photosynthetic rate (A), transpirational rate (B), stomatal conductance (C), substomatal conductance (D) and intrinsic water use efficiency (E) of radiated achenes of milk thistle under cadmium stress.

under cadmium stress.

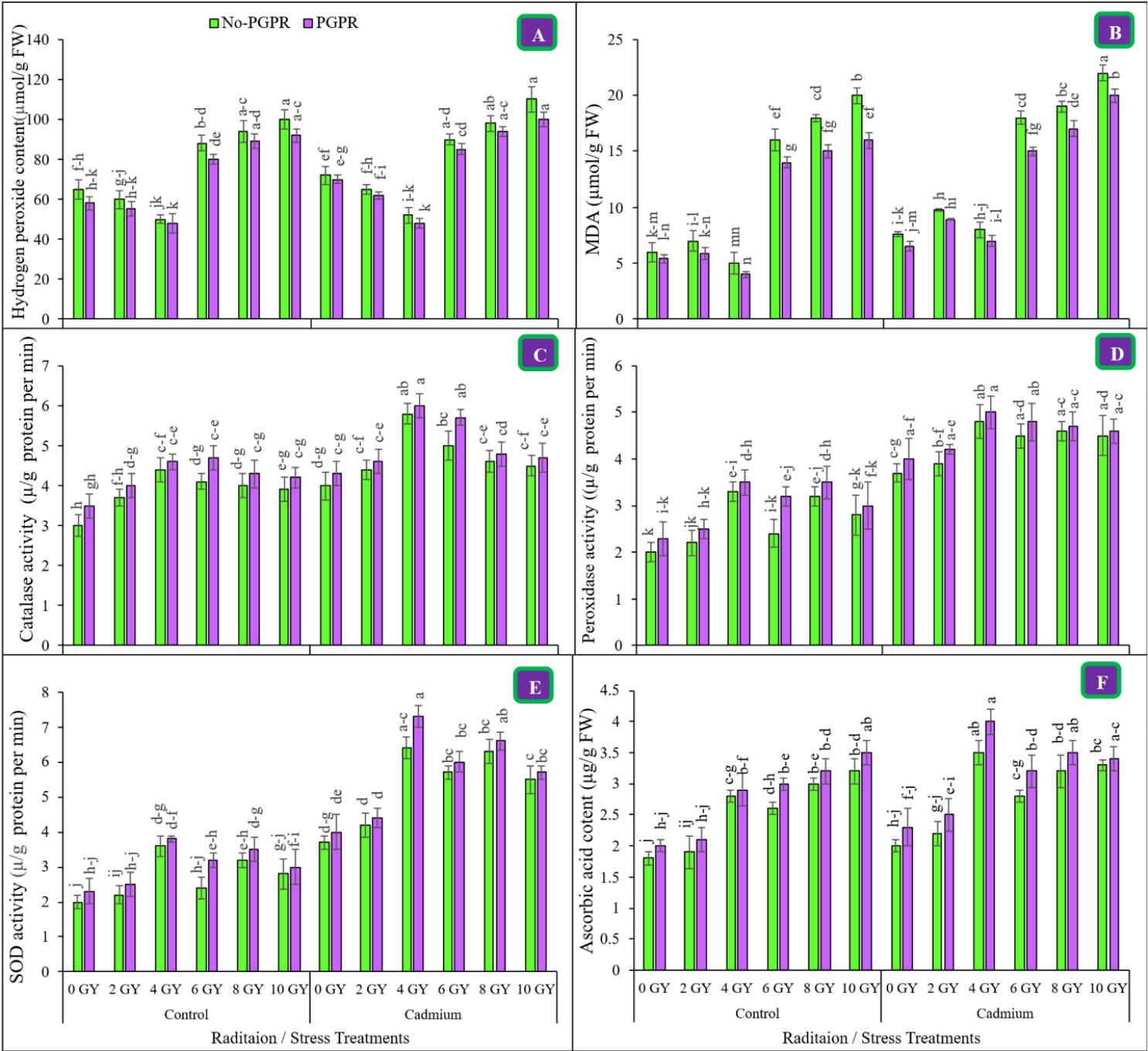


Figure 3

Effect of PGPR application on hydrogen peroxide (A), MDA (B), catalase (C), peroxidase (D), superoxide dismutase (SOD) (E) and ascorbic acid content (F) of radiated achenes of milk thistle under cadmium stress.

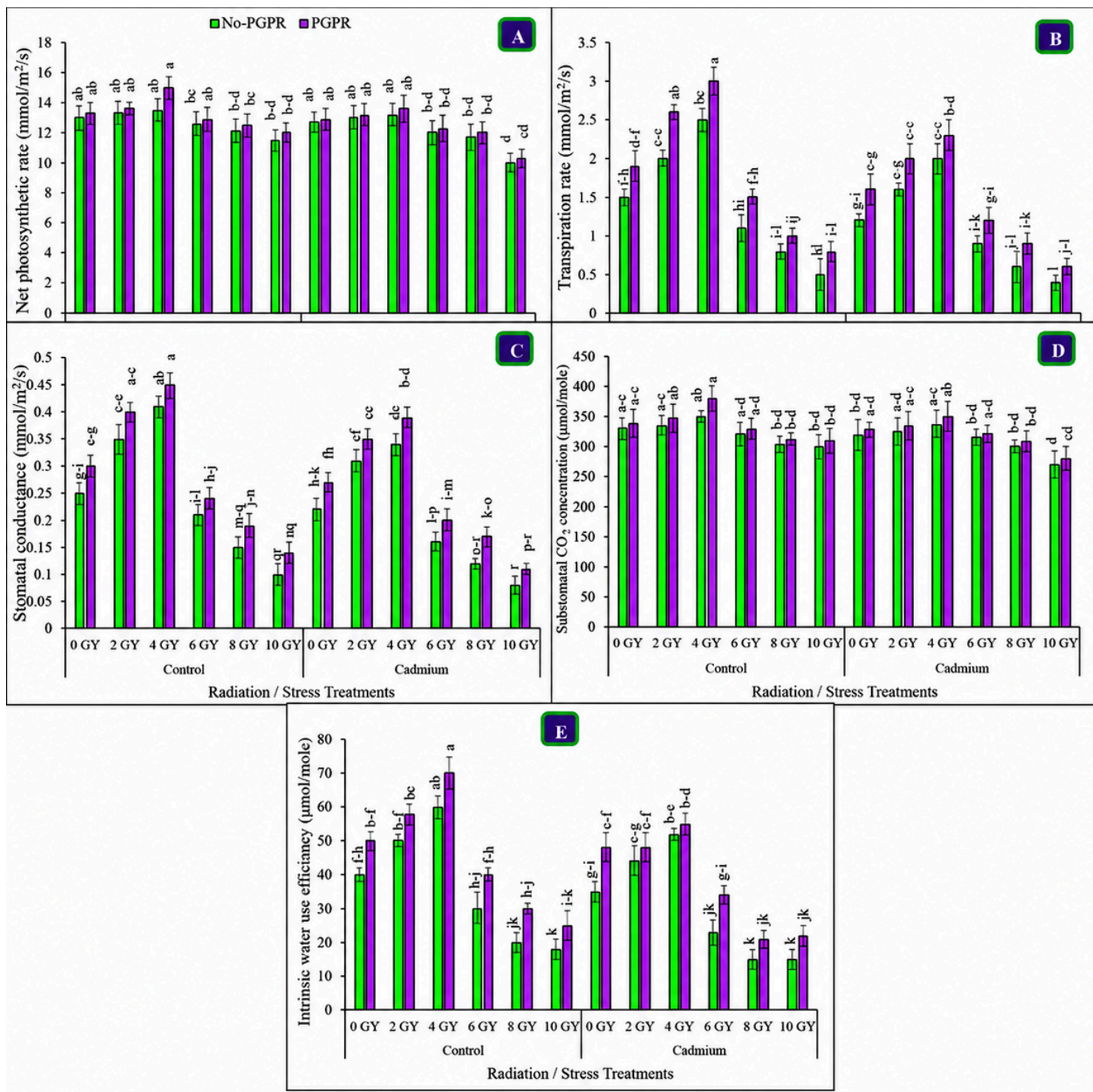


Figure 4

Effect of PGPR application on glycine betaine (A), proline (B), potassium (C), calcium (D), sodium (E) and cadmium content (F) of radiated achenes of milk thistle under cadmium stress.

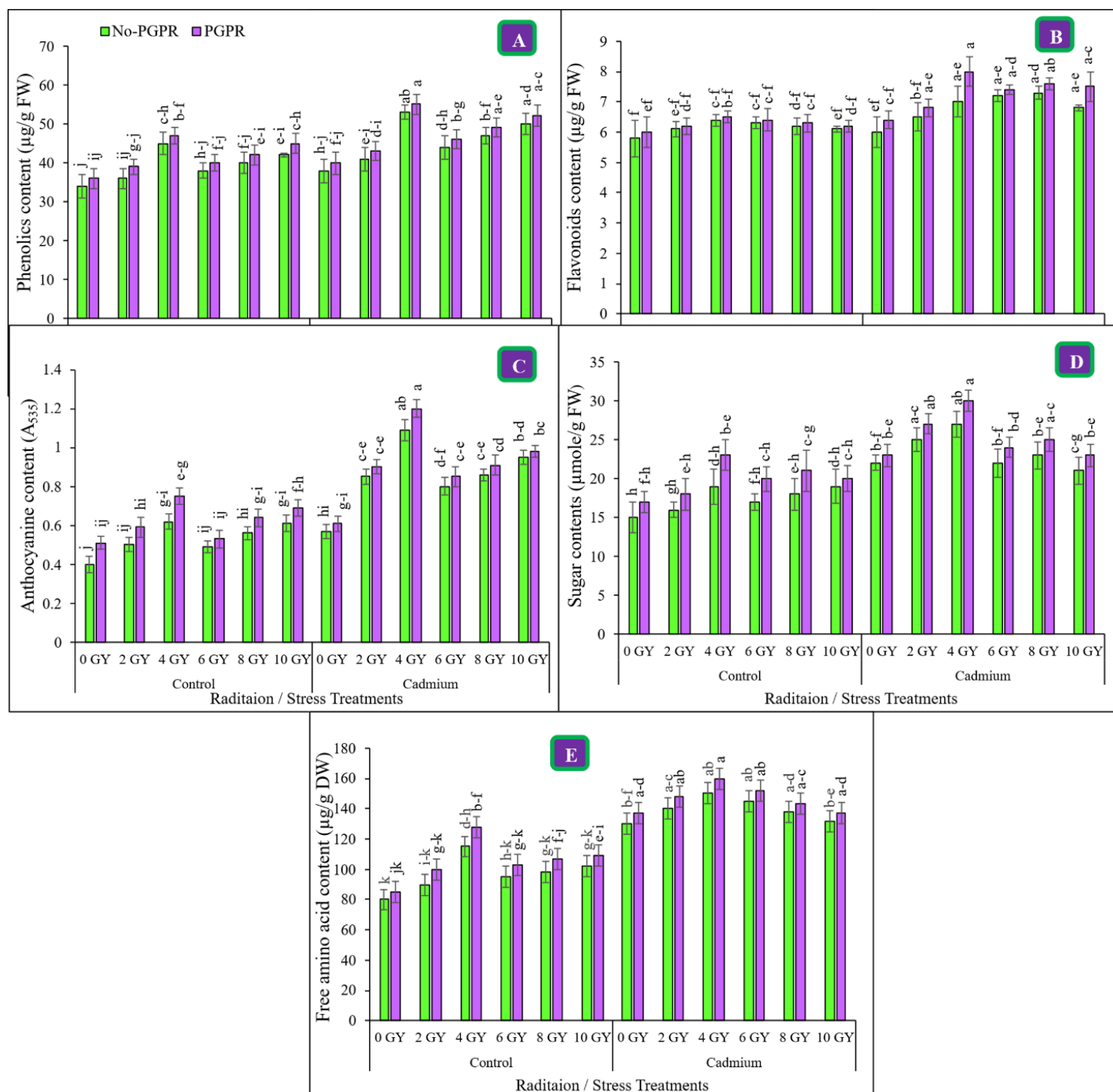


Figure 5

Effect of PGPR application on phenolics (A), flavonoids (B), anthocyanin (C), soluble sugars (D), and free amino acid contents (E) of radiated achenes of milk thistle under cadmium stress.

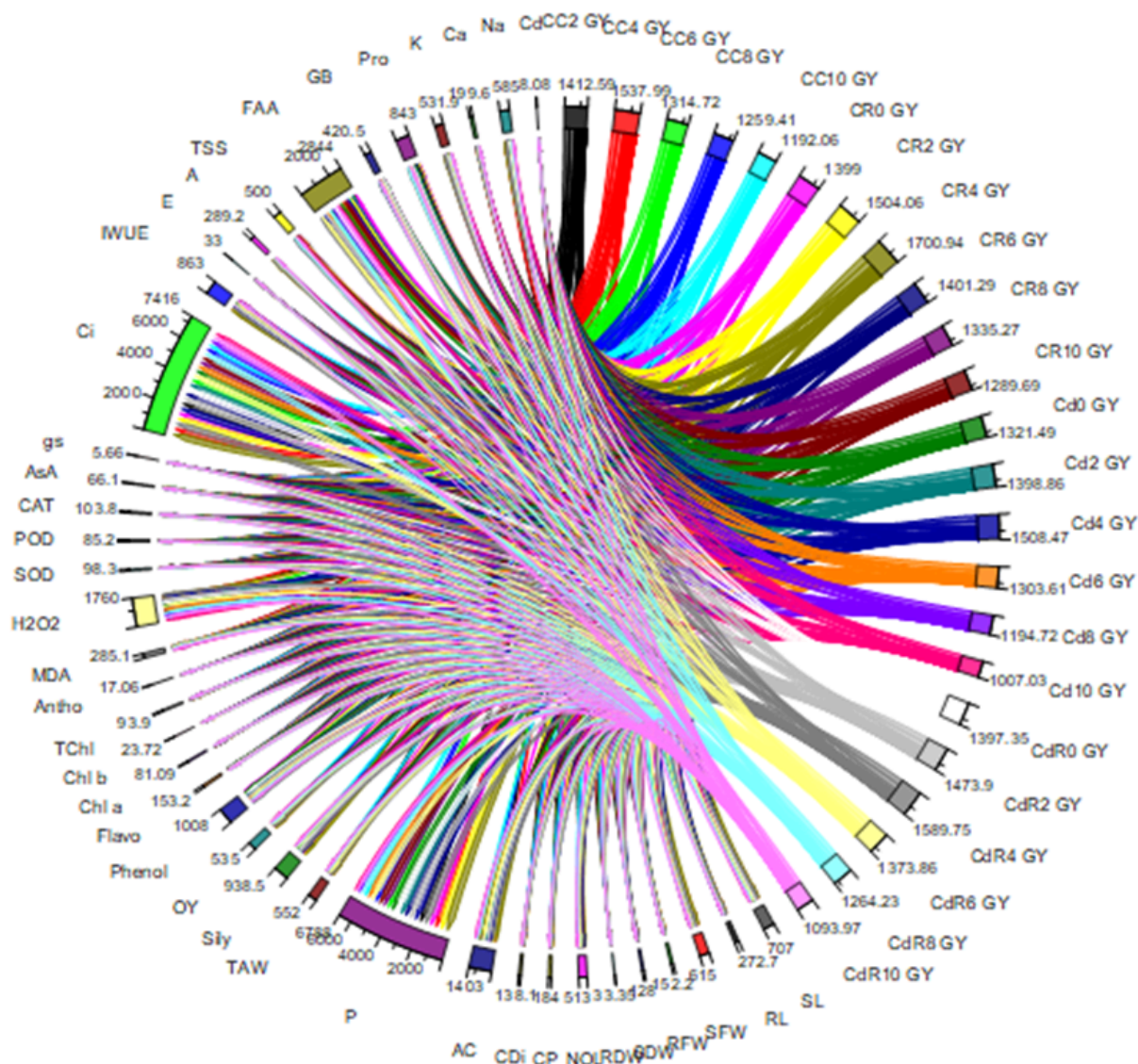


Figure 6

Chord diagram of the effect of PGPR application on all the growth, yield, and physiochemical parameters of leaves of radiated achenes of milk thistle under cadmium stress. CAT; catalase, Flavo, Flavonoids, Antho; anthocyanin, Phenol; phenolics, POD; peroxidase, SOD; superoxide dismutase, Pro; proline, FAA, total free amino acids, GB; glycine betaine, AsA; ascorbic acid, H₂O₂, hydrogen peroxide, MDA; malondialdehyde, Cd; cadmium content, IWUE; intrinsic water use efficiency, RL, root length, E; transpiration rate, gs; stomatal conductance, Na; sodium content, CP; number of capitulum per plant, Ca, calcium, K, potassium, SDW; shoot dry weight, TAW, thousand achene weight, NOL; the number of leaves, OY; oil content, TChl; total chlorophyll content; Chl b; chlorophyll b; sily; silymarin, CDi; capitulum diameter, SFW; shoot fresh weight; P; the number of achenes per plant; AC; the number of achenes per capitulum, RFW; root fresh weight; SL; shoot length, RDW; root dry weight, A; net photosynthetic rate, Ci; substomatal carbon dioxide concentration, Chl a; chlorophyll a, CC0GY; no-stress no PGPR 0GY, CC2GY; no-stress no PGPR 2GY, CC4GY; no-stress no PGPR 4GY; CC6GY; no-stress no PGPR 6GY, CC8GY; no-stress no PGPR 8GY, CC10GY; no-stress no PGPR 10GY, CR0GY; no-stress PGPR 0GY, CR0GY; no-stress

amino acids, GB; glycine betaine, AsA; ascorbic acid, H₂O₂, hydrogen peroxide, MDA; malondialdehyde, Cd; cadmium content, IWUE; intrinsic water use efficiency, RL, root length, E; transpiration rate, gs; stomatal conductance, Na; sodium content, CP; number of capitulum per plant, Ca, calcium, K, potassium, SDW; shoot dry weight, TAW, thousand achene weight, NOL; the number of leaves, OY; oil content, TChl; total chlorophyll content; Chl b; chlorophyll b; sily; silymarin, CDi; capitulum diameter, SFW; shoot fresh weight; P; the number of achenes per plant; AC; the number of achenes per capitulum, RFW; root fresh weight; SL; shoot length, RDW; root dry weight, A; net photosynthetic rate, Ci; substomatal carbon dioxide concentration, Chl a; chlorophyll a; CC0GY; no-stress no PGPR 0GY, CC2GY; no-stress no PGPR 2GY; CC4GY; no-stress no PGPR 4GY ; CC6GY; no-stress no PGPR 6GY, CC8GY; no-stress no PGPR 8GY, CC10GY; no-stress no PGPR 10GY, CR0GY; no-stress PGPR 0GY, CR0GY; no-stress PGPR 0GY, CR2GY; no-stress PGPR 2GY, CR4GY; no-stress PGPR 4GY, CR6GY; no-stress PGPR 6GY, CR8GY; no-stress PGPR 8GY, CR10GY; no-stress PGPR 10GY, Cd0GY; Cd stress no PGPR 0GY, Cd2GY; Cd stress no PGPR 2GY; Cd4GY; Cd stress no PGPR 4GY ; Cd6GY; Cd stress no PGPR 6GY, Cd8GY; Cd stress no PGPR 8GY, Cd10GY; Cd stress no PGPR 10GY, CdR0GY; Cd stress PGPR 0GY, CdR0GY; Cd stress PGPR 0GY, CdR2GY; Cd stress PGPR 2GY, CdR4GY; Cd stress PGPR 4GY, CdR6GY; Cd stress PGPR 6GY, CdR8GY; Cd stress PGPR 8GY, CdR10GY; Cd stress PGPR 10GY.

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