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## Letter to the Editor

# UV-B radiation-mediated stress ethylene emission is regulated by ACC deaminase producing bacteria in rice (*Oryza sativa* L.)

**Running title:** Bacteria-mediated UV-B stress alleviation in rice

Dear Editor,

Industrial emissions have played a major role in damaging the ozone layer and led to the penetration of ultraviolet-B (UV-B) (290 – 320 nm) radiation (Jordan et al., 2002). The recovery rate of ozone layer in the mid latitudes and tropics have been insignificant since 1979 (Chipperfield et al., 2017), even after the adoption of Montreal protocol (Protocol M, 1987). Hence, the recovery process might take decades and UV-B radiation will continue to penetrate the atmosphere (McKenzie et al., 2011; Bais et al., 2019). The characteristic short length and high energy radiation can directly affect major molecules such as DNA, proteins, and lipids which are important to living beings (Hideg et al., 2013). Rice is one of the major crops across the globe which is generally cultivated at lower latitudes and potentially can get exposed to a higher flux of UV radiation due to greater solar angles and longer duration of sunlight (Kataria and Guruprasad 2012).

UV-B radiation exposure shows incurs decrease in plant height, total leaf area and biomass (Newsham and Robinson, 2009). The initial responses are elevated ethylene emission and ROS accumulation (An et al. 2006; Czégény et al., 2016), which can eventually result in accelerated damage to DNA and cell membrane (Wang et al., 2010; Surjadinata et al., 2021). Additionally, prolonged exposure to UV-B radiation can also lead to destabilization of the cell membrane through enhanced lipid peroxidation (Berli et al., 2010; Li et al., 2014). On the other hand, plants show a myriad of responses to cope up with UV-B radiation mediated stress. For example, plants tend to enhance the deposition of polyphenols such as flavonoids in the epidermal tissues which acts as a “sunscreen” to prevent penetration of UV rays as well as a potential antioxidant (Surjadinata et al., 2021). However, regulating ROS and ethylene emission can also be attained by inoculation of ACC deaminase (ACCd) producing plant growth promoting bacteria (PGPR) for enhancing stress tolerance; and its effectiveness have been extensively studied in a plethora of abiotic and biotic stresses (Glick 2014). Additionally, designing effective synthetic communities (SynComs) for alleviation of stresses (Trivedi et al., 2020) is regarded as a sustainable and eco-friendly approach. *Brevibacterium linens* RS16 is an ACCd producing plant growth promoting bacteria (PGPB) which has the ability to alleviate salt and heat stress on plants by ROS detoxification and regulation of ethylene and volatile organic compound (VOC) emissions (Siddikee et al., 2010; Chatterjee et al., 2020). The bacterium can also be regarded as a multifunctional PGPB which possesses ACC deaminase activity (4.13 μmol α-KB $mg^{-1}$ protein $h^{-1}$), nitrogen fixing, IAA production, ammonia production and thiosulfate oxidation abilities. (Siddikee et al. 2010). Hence, this study was conducted to evaluate the efficiency of *B. linens* RS16 in regulating UV-B stress mediated ethylene emission, ROS accumulation and cell damage for enhancing stress tolerance of rice plants.

Rice (*Oryza sativa* L.) seeds were soaked in water and kept for germination in a dark chamber at 30 °C for 72 hrs. The germinated seedlings were transplanted in a seedling tray and transferred to the greenhouse with 32°C/28°C day/night temperature and natural illumination. *B. linens* RS16 was grown in nutrient broth until $OD_{600}$ reached ~ 0.8 and subsequently the inoculum was prepared by

washing the bacterial cells in 0.03 M $MgSO_4$ thrice and resuspension in the same by maintaining $OD_{600}$ ~ 0.8. Five-day old seedlings (30 rice seedlings per replicate) were transplanted into a 60 mL pot containing 50 g nursery soil (Doobaena, Nongkyung Co., Jincheon-gun, Republic of Korea) and 10 mL of bacterial inoculum was inoculated in the root zone and kept in greenhouse conditions for 2 days. The control plants were treated with 0.03 M $MgSO_4$. The pots containing 7-day old seedlings (2 days after inoculation) were transferred to growth chambers (DS 54 GLP, DASOL Scientific Co., Ltd., Korea) additionally equipped with UV-B lamps (306 nm, G20T10E, Sankyo Ultraviolet, Co., Ltd., Kanagawa, Japan) of two different intensities, viz., 0.5- and 1-W $m^{-2}$ in two different growth chambers, and the control plants were kept in a growth chamber without any UV lamps. The intensity of UV-B light was measured by a spectroradiometer (JAZ-EL 200, Ocean Optics, Dunedin, FL, USA). The plants were kept at 32°C/28°C and 16h-8h day/night conditions with ~70% relative humidity. The UV-B stress was applied by turning on the lamps for 12 hrs daily in addition to the illumination.

Plants were harvested at 2, 3, and 4 days after UV-B stress treatment for measuring the hydrogen peroxide concentration, MDA concentration, electrolyte leakage and polyphenol concentration. Hydrogen peroxide concentration was measured by taking 0.5 g of leaf samples and grinding with liquid nitrogen. The ground leaf samples were homogenized with 1 mL cold acetone and centrifuging at 6000 x *g* for 10 mins at 4 °C. The supernatant (1 mL) was carefully taken in a test tube and 250 µL of cold distilled water, 0.1 mL 5% (w/v) titanium sulfate and 0.5 mL 1 N ammonium hydroxide were added. The resultant mixture was centrifuged at 10,000 x *g* for 5 mins at 4 °C and the supernatant was discarded. The pellet was dissolved with 2 N $H_2SO_4$ and the final volume was adjusted to 2 mL with cold distilled water. The absorbance was recorded at 415 nm with UV-Vis spectrophotometer and the hydrogen peroxide concentration was determined by using a standard curve plotted with known concentrations of $H_2O_2$ (Theocharis et al., 2012). MDA concentration was determined by using ground leaf samples (0.5 g) homogenized with ice-cold phosphate buffer and centrifuged at 6000 x *g* for 10 mins at room temperature. The supernatant (1 mL) was taken into a test tube and 4 mL of reagent (0.5% (w/v) thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA)) was added into it. The reaction mixture was incubated at 95 °C for 30 mins and the reaction was terminated by transferring the tubes into a cold-water bath. Absorbance was recorded by using a UV-Vis spectrophotometer at 532 nm and 600 nm. The resulting MDA concentration was calculated by subtracting $A_{532}$ from $A_{600}$ and multiplied by the extinction coefficient 155 $mm^{-1}$ $cm^{-1}$ (Samaddar et al., 2019). To determine the electrolyte leakage, 1 g of leaf samples were taken and cut into small segments. The cut samples were placed in a conical tube containing 10 mL sterile deionized water and incubated for 24 h at 25 °C. The electrical conductivity of the sample was measured and followed by incubation of the same samples at 100 °C for 30 mins to determine the total electrical conductivity. The relative electrical conductivity was determined in terms of percentage (Subramanian et al., 2016). Polyphenol concentration was determined by taking 0.5 g fresh leaf samples and by homogenization with 80% ethanol, followed by agitation at 70 °C for 20 mins. The mixture was centrifuged at 9000 x *g* for 10 mins and 2 mL of supernatant was carefully taken into a test tube. The supernatant was mixed with 10 mL distilled water and 500 µL Folin-Ciocalteau reagent and incubated in dark for 30 mins at room temperature. Absorbance was recorded at 750 nm with UV-Vis spectrophotometer and the concentration was determined by plotting a known concentration of phenol (Berli et al., 2009).

Ethylene emission was measured by transferring the plants into a customized 1-L GC bottle and closing the lid for 2 hrs, thereby collecting 1 mL of air from the headspace and injecting it into a gas chromatograph (dsCHROM 6200, Donam Instruments Inc., Republic of Korea) equipped with Poropak-Q column. The study was carried out in a randomized block design and three independent trials were conducted for data validity. The data obtained were subjected to one-way analysis of variance (ANOVA) and significant differences between the means within the treatments were determined by Tukey's test at $P < 0.05$ using SAS package, Version 9.4.

These investigations along with the genetic mapping of *B. linens* RS16 had assisted in addressing our objectives. The genetic mapping of the bacterium (NCBI accession no. NZ_CP030797.1) has revealed its potentiality in alleviating UV-B radiation mediated stress as it contains three specific genes, viz., phytoene desaturase (protein ID: WP_139908826.1), phytoene synthase (protein ID: WP_139908827.1) and ectoine synthase (protein ID: WP_135810511.1). These genes are important for protecting the DNA from the damage induced by ionizing radiation (Klassen 2010; Schröter et al., 2017).

Ethylene emission increased significantly for rice plants exposed to UV-B stress after 3 and 4 days of exposure compared to the control plants (Fig. 1). The duration of exposure also resulted in a significant increase in ethylene emission. The two different levels of UV-B stress treatment had shown similar trends in the induction of ethylene emission, but the plants exposed to 1-W $m^{-2}$ UV-B treatment had shown the highest emission rates. The plants exposed to 1-W $m^{-2}$ UV-B treatment had shown a 26% higher ethylene emission level compared to the plants exposed to 0.5-W $m^{-2}$ UV-B treatment after 4 days of stress exposure. These observations are in line with previous study where elevated ethylene emission levels were observed under UV-B exposure (An et al. 2006). Moreover, the inoculation of *B. linens* RS16 had shown a 12.2% and 16.4% decrease in ethylene emission at 3 and 4 days, respectively, after 0.5-W $m^{-2}$ of UV-B treatment compared to non-inoculated plants. On the other hand, the bacterial inoculation resulted in a 19.4% and 19.2% decrease in ethylene emission at 3 and 4 days after 1-W $m^{-2}$ of UV-B treatment. The inoculation of *B. linens* RS16 was able to reduce the ethylene emission rates, as it has the ability to produce ACCd enzyme which can scavenge the precursor of ethylene, ACC, and use it as a nitrogen source for its growth and development (Siddikee et al., 2010; Glick 2014).

**Figure 1.** Ethylene emission from rice plants inoculated with *B. linens* RS16 exposed to UV-B radiation at 2, 3 and 4 days of stress treatment. Different letters indicate significant differences $P < 0.05$ among the treatments at each day.

The elevated ethylene emission levels due to UV-B exposure can also lead to oxidative stress by higher accumulation of ROS *in planta* by the up-regulation of membrane bound RBOHD protein (Yao et al. 2017). In this study, UV-B induced significantly higher ROS accumulation measured in terms of hydrogen peroxide ($H_2O_2$) concentration in rice plants irrespective of bacterial inoculation throughout the stress exposure period (Fig. 2a). Furthermore, the *B. linens* RS16 inoculation had significantly reduced $H_2O_2$ concentration compared to the non-inoculated plants (Fig 2a and 2b). At 4 days of UV-B stress exposure, the bacterial inoculation had resulted in a 30% and 25% reduction in $H_2O_2$ concentration for 0.5-W $m^{-2}$ and 1-W $m^{-2}$ UV-B exposure, respectively, compared to the non-inoculated plants (Fig. 2a). *B. linens* RS16 has been accounted for enhancing the antioxidant enzyme activities such as catalase, superoxide dismutase and ascorbate peroxidase under stress conditions, which might be a potential mechanism for scavenging ROS and decreasing its detrimental effect on plant physiological processes (Chatterjee et al., 2018).

**Figure 2.** Hydrogen peroxide (a) and malondialdehyde (b) concentrations of rice plants inoculated with *B. linens* RS16 exposed to UV-B radiation at 2, 3 and 4 days of stress treatment. Different letters indicate significant differences $P < 0.05$ among the treatments at each day.

UV-B radiation can accelerate destabilization of plants' membrane integrity through enhanced lipid peroxidation and electrolyte leakage due to ROS accumulation (Berli et al., 2010; Li et al., 2014; Ma et al. 2019). This study also reports the increase in lipid peroxidation measured in terms of MDA concentration (Fig. 2b). Furthermore, the decrease in $H_2O_2$ concentration with inoculation of *B. linens* RS16 significantly reduced the MDA concentration throughout the UV-B exposure period. It had

shown a reduction of 15% and 14% reduction in MDA content for 0.5-W $m^{-2}$ and 1-W $m^{-2}$ UV-B exposure respectively, compared to the non-inoculated plants (Fig. 2b). On the other hand, electrolyte leakage is a typical biomarker for monitoring damage to plant cell membrane due to enhanced hydrogen peroxide concentration (Ma et al. 2019). The electrolyte leakage enhanced significantly upon UV-B exposure irrespective of bacterial inoculation at 2, 3, and 4 days of stress treatment. The plants exposed to 1-W $m^{-2}$ UV-B radiation recorded electrolyte leakage compared to other treatments (Fig. 3b). However, bacterial inoculation had significantly decreased the electrolyte leakage under UV-B exposure compared to the non-inoculated plants. The inoculation of *B. linens* RS16 had decreased electrolyte leakage by 13.6%, 8.3%, and 13.4% for 1-W $m^{-2}$ UV-B exposure, whereas 15.6%, 15.4%, and 13% reduction were observed for plants exposed to 0.5 W $m^{-2}$ UV-B irradiation compared to non-inoculated plants at 2, 3 and 4 days of stress treatment, respectively. These results are in line with previous reports where the inoculation of ACCd producing bacteria results in lower MDA concentration and electrolyte leakage through enhancement of antioxidant defense mechanisms under environmental stress conditions (Chatterjee et al., 2018; Subramanian et al., 2016).

**Figure 3.** Polyphenol concentration (a) and electrolyte leakage from leaves (b) of rice plants inoculated with *B. linens* RS16 exposed to UV-B radiation at 2, 3 and 4 days of stress treatment. Different letters indicate significant differences $P < 0.05$ among the treatments at each day.

Polyphenols including flavonoids are regarded as a potential antioxidant and also restricts UV-B penetration through the epidermal tissues of plants (Surjadinata et al., 2021). In this study, polyphenol concentration enhanced significantly in the leaves of UV-B exposed rice plants compared to the control plants throughout the exposure period (Fig. 3a). The polyphenol concentration increased by 4.9%, 18.7%, and 28% for 0.5 W $m^{-2}$ UV-B exposure, whereas 13%, 35.8%, and 41.4% increase were observed for 1 W $m^{-2}$ UV-B exposure at 2, 3 and 4 days of stress treatment, respectively, compared to the control plants (Fig. 3a). Furthermore, the bacterial inoculation had significantly decreased polyphenol concentration under UV-B exposure during 2, 3, and 4 days of stress treatment compared to the non-inoculated plants. At 4 days of stress treatment, *B. linens* RS16 inoculation resulted in an 11.6% and 11.8% decrease in polyphenol concentration compared to the non-inoculated plants for 0.5 W $m^{-2}$ and 1 W $m^{-2}$ UV-B exposure, respectively. Our study corroborates to a previous report where PGPR-inoculated cucumber plants had shown lower polyphenol concentration under salinity and drought stress (Kang et al., 2014). The decrease in polyphenol deposition in bacteria inoculated plants can be attributed to enhanced tolerance against UV-B radiation.

This study has shown that ethylene emission from rice plants enhanced with the increase in the intensity of UV-B exposure. The rise in ethylene emission resulted in enhanced ROS accumulation, peroxidation of lipid residues that is present in the cell membrane, electrolyte leakage, and deposition of polyphenols. The inoculation of ACC deaminase producing bacteria was able to reduce the ethylene emission from plants exposed to UV-B radiation and subsequently assisted in decline in ROS accumulation, stabilization of cell membrane by decreasing lipid peroxidation and electrolyte leakage, and also reduced polyphenol deposition in the leaves. The collective study puts forward a new perspective in the characterization of UV-resistant ACC deaminase producing plant growth promoting bacteria for the amelioration of ionizing radiation in stress agriculture.

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**Figure 1.**

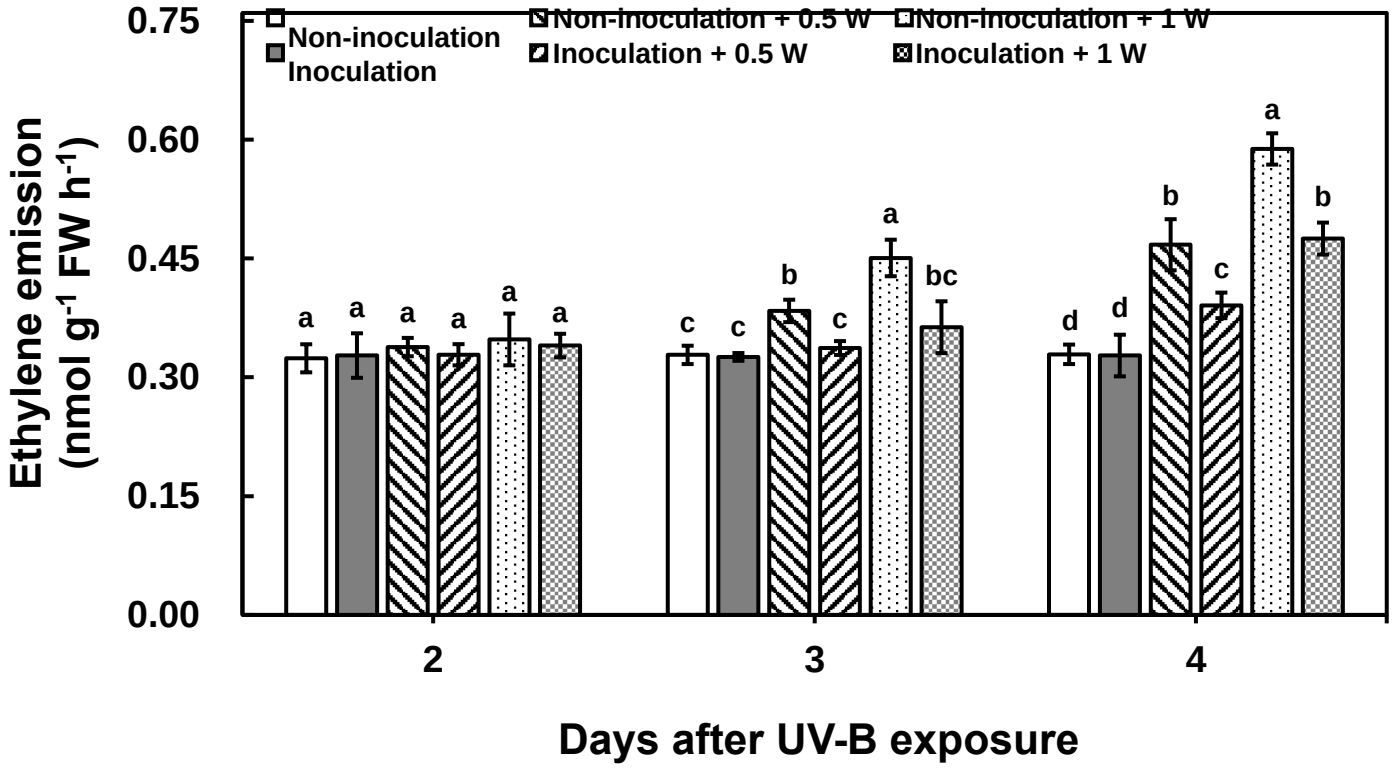


**Figure 2.**

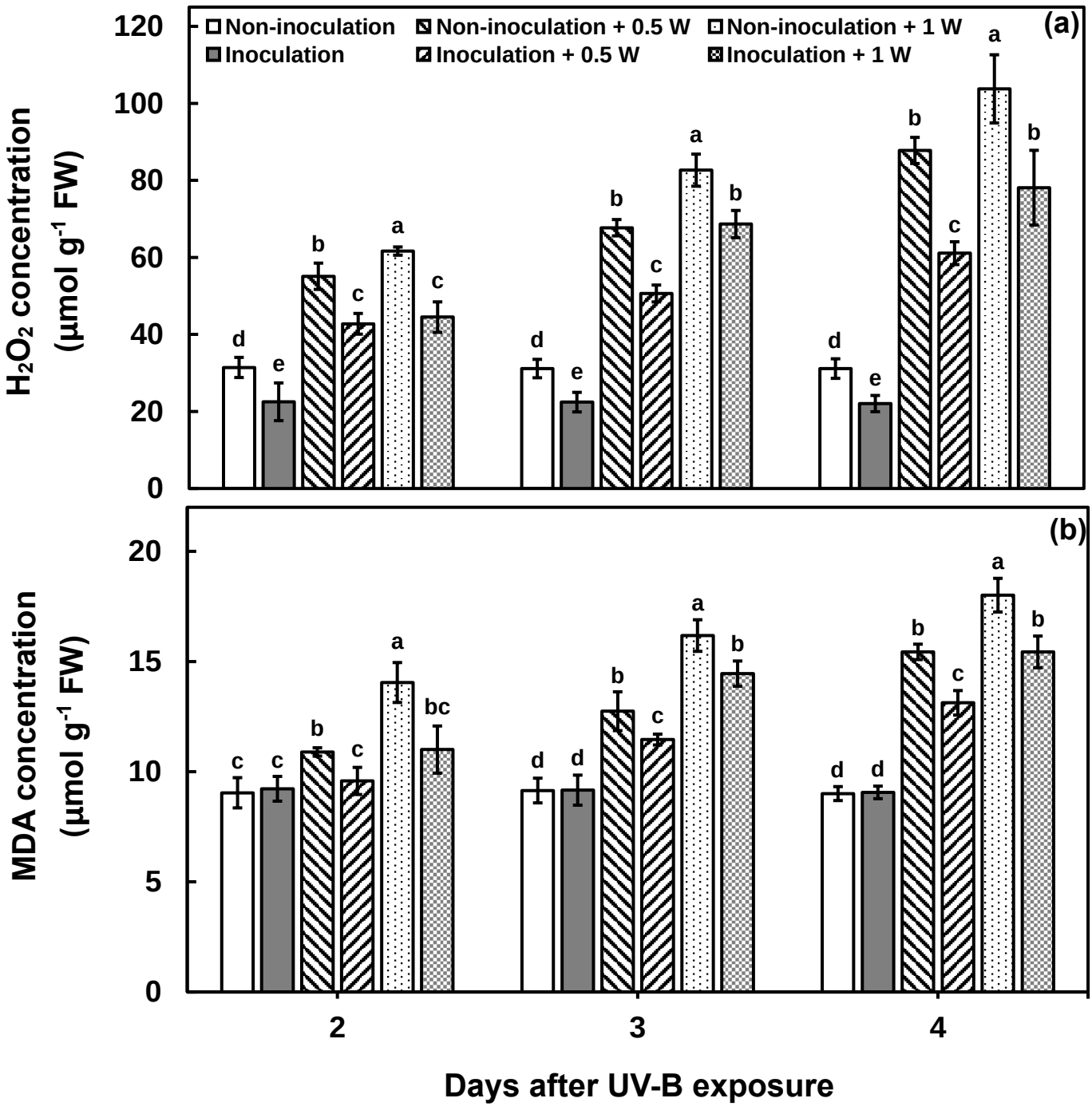
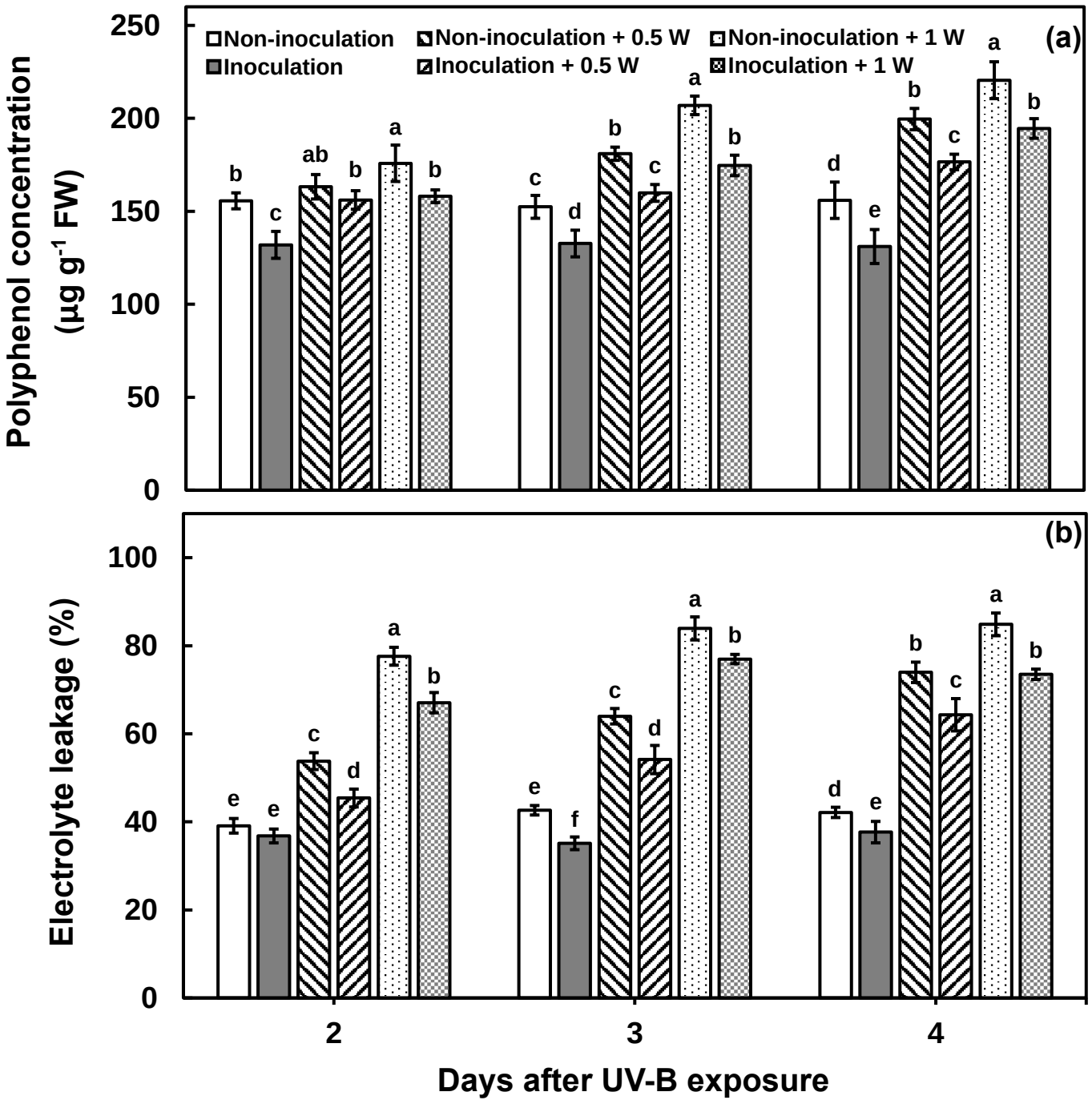

Non-inoculation
Non-inoculation + 0.5 W
Non-inoculation + 1 W
Inoculation
Inoculation + 0.5 W
Inoculation + 1 W
(a)
$H_2O_2$ concentration (µmol $g^{-1}$ FW)
(b)
MDA concentration (µmol $g^{-1}$ FW)
Days after UV-B exposure

**Figure 3.**


Non-inoculation
Non-inoculation + 0.5 W
Non-inoculation + 1 W
Inoculation
Inoculation + 0.5 W
Inoculation + 1 W
(a)
Polyphenol concentration (µg g-1 FW)
250
200
150
100
50
0
(b)
Electrolyte leakage (%)
100
80
60
40
20
0
2
3
4
Days after UV-B exposure