Integrative roles of nitric oxide and hydrogen sulfide in melatonin-induced tolerance of pepper (*Capsicum annuum* L.) plants to iron deficiency and salt stress alone or in combination

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There seems to be no report in the literature on the effect of melatonin (MT) in relieving the detrimental effects of combined application of salt stress (SS) and iron deficiency (ID). Furthermore, the effect of MT on the accumulation/synthesis of endogenous nitric oxide (NO) and hydrogen sulphide (H_2S) and how far these molecules are involved in MT-improved tolerance to the combined application of ID and SS in pepper (*Capsicum annuum* L) were tested. Hence, two individual trials were set up. The treatments in the first experiment comprised: Control, ID (0.1 mM $FeSO_4$), SS (100 mM NaCl), and ID + SS. The detrimental effects of combined stresses were more prominent than those by either of the single stress, with respect to growth, oxidative stress and antioxidant defence attributes. Single stress or both in combination improved the endogenous H_2S and NO, and foliar-applied MT (100 µM) led to a further increase in NO and H_2S levels. In the second experiment, 0.1 mM scavenger of NO, cPTIO and that of H_2S, hypotuarine (HT) were applied along with MT to get further evidence that whether NO and H_2S involved in MT-induced tolerance to ID and SS. Melatonin combined with cPTIO and HT under a single or combined stress showed that NO effect was reversed by the NO scavenger, cPTIO alone, but the H_2S effect was inhibited by both scavengers. These findings suggested that tolerance to ID and SS induced by MT may be involved in downstream signal crosstalk between NO and H_2S.

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Abbreviations - CAT, catalase, cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt, EL, electrical leakage, Fv/Fm, maximum quantum efficiency of photosystem II, H_2S, hydrogen sulfide, HT, hypotuarine, H_2O_2, hydrogen peroxide, MDA, malondialdehyde, MT, melatonin, NO, nitric oxide, POD, peroxidase, SOD, superoxide dismutase.

Introduction

Both salinity stress and iron deficiency are crucial factors that limit crop growth and yield globally (Shahbaz and Ashraf 2013, Shrivastava and Kumar 2015, Briat et al. 2015). Although there are many reports in the literature on plant responses to either iron (Fe) deficiency (Marschner et al. 1986, Morales et al. 1990, Jin et al. 2011a, Chen et al. 2015a) or salinity stress (Oueslati et al. 2010, Syvertsen and Gracia-Sanchez 2014, Rebey et al. 2017), limited research has been carried out to examine plant responses to the combined application of salt stress and Fe deficiency (Rabhi et al. 2007). Most of those results cannot fully explain the effects of two or more stress combinations on crops (Atkinson et al. 2013, Zandalinas et al. 2018), due to the fact that crops are often subjected to more than one abiotic stress factor simultaneously (Mittler and Blumwald 2010, Suzuki et al. 2014, Zandalinas et al. 2018).

Being an essential element, iron (Fe) has a pivotal role in photosynthesis, DNA synthesis, respiration and hormone synthesis (Nagajyoti et al. 2010, Leroux et al. 2016). Iron deficiency is one of the major nutrient disorders deleteriously affecting growth of numerous plants because of the restriction of absorption and availability of this element by the plants rather than being present at low levels in soils (Ravet et al. 2009, Jin et al, 2009, Briat et al. 2015). Dissolved Fe in soil solution decreases at high soil pH and crops grown in such soils generally show chlorosis, a specific sign of Fe deficiency (Kakei et al. 2012, Pestana et al. 2012).

In dicotyledonous plants including pepper, more iron uptake in Fe-deficient soils can occur if the root zone (rhizosphere) is acidified by proton release by the plants. Lower rhizospheric pH enhances Fe solubility and makes Fe more available (Santi and Schmidt, 2009). Another strategy is to convert ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron by the synthesis of ferric-chelate reductase, thus promoting uptake of Fe^{2+} into root cells (Ding et al. 2009, Brumbarova et al. 2015).

Saline stress leads to micronutrient disorders because of low solubility of those nutrients (Page et al. 1990, Rabhi et al. 2007). Excess levels of sodium in saline soils limits uptake and utilization of iron and other inorganic minerals by inducing hyperosmotic stress in the plant as well as oxidative stress (Zhu 2001, Abbas et al. 2015). Therefore, salinity stress is another major soil problem adversely affecting growth of most plants (Meloni et al. 2003, Pandolfi et al. 2012, Ruiz-Lozano et al. 2012, Rebey et al. 2017). Salinity directly induces both osmotic and ionic stresses, which restrict the uptake of nutrients and water (Ashraf 2004, Khan et al. 2012, Ahmed et al. 2015). In addition, high Na^+

accumulated in the cytosol of plant cells exposed to saline regimes leads to electrolytic leaching due to cell membrane damage. This detrimentally affects metabolic activities in the cytosol including protein metabolism, CO_2 assimilation, N assimilation and lipid metabolism (Woo et al. 2004, Chen et al. 2007, Allakhverdiev et al. 2008, Ahmad 2010, Pandolfi et al. 2012, Ahmad et al. 2014). Salinity stress has also been linked to accumulation of reactive oxygen species (ROS) (Mittler 2002, Liu et al. 2018). Although ROS are obviously required for metabolic activities in the cell as secondary messengers (Choudhury et al. 2017), overproduction of can damage cellular membranes through oxidation of key organic molecules such as proteins, nucleic acids and lipids (Bose et al. 2014, Sharma et al. 2012, Shi et al. 2015a). So, there needs to be a balance between ROS generation and antioxidants including catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX), as well as a range of non-enzymatic antioxidants scavenging ROS generation for plants to survive under stressful cues (Khan et al. 2012, Baxter et al. 2014, Martinez et al. 2018). Melatonin (MT) has recently been reported as a strong antioxidant, particularly in vertebrate animals (Arnao et al. 2014). It was discovered in plants in the late 1990s (Zhang et al. 2015). Investigations into melatonin in plants only started in 2009 (Arnao et al. 2014, Martinez et al. 2018).

Melatonin is now known to be one of the common molecules synthesized naturally in plants (Iriti et al. 2006, Martinez et al. 2018). Over the past few years, the intensity of research on uncovering the role of melatonin in plant functions has increased greatly (Martinez et al. 2018). For instance, Huang et al. (2017) have revealed that pre-treatment with MT mitigated the deleterious effect of cadmium stress in kiwifruit by enhancing antioxidant defence system and induce defence response in peach fruit during cold storage (Cao et al. 2016). The involvement of melatonin in fruit development processes including ripening and improving fruit quality is also widely reported (Hattori et al. 1995, Tan et al. 2012).

Several reports in the literature indicate the involvement of MT in the stress-linked signaling mechanisms between ROS and melatonin (e.g. Martinez et al. 2018), and MT is also known as an antioxidant for ROS scavenging (Tan et al. 2000, Allegra et al. 2003). Despite its effective function in plant growth, melatonin is also believed to improve plant tolerance against harsh environmental cues such as water stress in cucumber (Zhang et al. 2013), freezing stress (Shi et al. 2015b), salinity stress in cucumber (Zhang et al. 2014), salt, drought, and cold stress in Bermuda grass (Shi et al. 2015a), heat shock in *Arabidopsis thaliana* (Shi et al. 2015c), and iron deficiency in *A. thaliana* (Zhou et al. 2016).

Nitric oxide (NO) is one of the crucial signalling molecules controlling perturbance in biochemical and physiological responses in plants under both stress and non-stress regimes. Research linked with the roles of NO in plants is constantly increasing (Romero-Puertas and Sandalio 2016). In the higher plants, NO is produced over oxidative and reductive biosynthetic processes developed under stress conditions (Gupta et al. 2011, Mur et al. 2013). Because of reaction of NO with ROS, it improves redox homeostasis, tolerance to oxidative stress and enhancement of the antioxidant

capacity of plants (Anjum et al. 2012, Correa-Aragunde et al. 2015). The generation of NO in plant cells probably comes from non-enzymatic or enzymatic substances where the basic supply originates from the activity of nitrate reductase (NR) (Modolo et al. 2005, Planchet et al. 2005).

Hydrogen sulfide (H_2S) has recently been recognized as a chief metabolic controller in plants (García-Mata and Lamattina 2010, Hancock et al. 2011, Filippou et al. 2012, Christou et al. 2013, Hancock and Whiteman 2014). There are at least five enzymes including sulfite reductase capable of generating H_2S (Calderwood and Kopriva 2014). Cysteine desulfhydrase seems to play a fundamental role in H_2S production and homeostasis (Riemenschneider et al. 2005). It has also been shown that H_2S can mitigate harmful effects of abiotic stresses including salinity, heavy metals, drought, osmotic stress, hypoxia and heat by disrupting the activities of antioxidant enzymes (Jin et al. 2011, Dawood et al. 2012, Cheng et al. 2013, Li et al. 2013, Hancock and Whiteman 2014, Duan et al. 2015).

Although MT is reported to be an important component of signal transduction occurring in plants under iron deficiency (Zhou et al. 2016) or salinity stress (Zhang et al. 2014), there seems to be little information available in the literature relating to the effect of MT on both stresses applied together. Furthermore, as explained above, some investigations reveal that NO and H_2S play significant roles in hormonal, developmental and ecological responses in plants. However, the effect of MT on the synthesis/accumulation of endogenous hydrogen sulfide (H_2S) and nitric oxide (NO) and how far these metabolites play a role in regulating growth and some key metabolic processes needs to be clarified. We have investigated this in pepper (*Capsicum annuum* L. cv Semerkand) plants under the combined effect of iron deficiency and saline stress.

Materials and methods

Plant growth

This investigation was carried out under glasshouse conditions with Capia type red sweet pepper (*Capsicum annuum* L.) cv Semerkand. Before use, the seeds were surface sterilized in NaOCl solution (1% v/v). Three seeds were sown in each plastic pot (8.5 l) containing perlite and well-washed sand equally (v/v). Day and night temperatures during the experiment were maintained at 20–25 and 10ºC, respectively. The light period during the growing season was 11h per day.

For simulation of Fe-deficient conditions, 0.1 mM $FeSO_4$ was added (instead of 0.1 mM EDTA-Fe) to the Hoagland's nutrient solution. The treatments were control (neither salinity nor Fe deficiency), Fe deficiency (ID, 0.1 mM $FeSO_4$), salt stress (100 mM NaCl) and Fe deficiency plus salinity stress (ID + 100 mM NaCl) in Hoagland's nutrient solution. It has been reported that exogenous 100 µM melatonin applied to different crops such as Chinese licorice (Afreen et al. 2006), white lupin (Arnao and Hernández-Ruiz, 2013) and tomato (Yang et al. 2018, Martinez et al. 2018) improved tolerance to some abiotic stressors, so this level of MT was used in the present experiments. In our previous research for subjecting strawberry plants to iron deficiency, we used 0.1 mM $FeSO_4$ (Kaya et al. 2019). Furthermore, 100 mM NaCl was also chosen for pepper plants in previous studies

to create salinity stress (Özdemir et al. 2016, Yildiztekin et al. 2018), and so same concentrations were used in the present experiment for ID and salinity stress. Before starting stress treatments, half of the plants were sprayed every other day with 100 µM melatonin solution prepared in 0.01% tween-20 for 14 days. The other half of plants was sprayed with tween-20 (0.01%) solution prepared in distilled deionized H_2O (as a blank treatment). After two weeks of melatonin application, stress treatments were started and seedlings were grown for an additional 6 weeks.

Black plastic mulch was used to cover each pot in order to diminish both algal growth and evaporation. The composition of the nutrient solution used (containing macro- and micro-elements) was as depicted in Kaya and Ashraf (2015), and its pH was maintained at 5.5. Depending on plant size, 100-1000 ml of water was provided to each pot every two days during the trial.

Each treatment consisted of 3 replications and there were 5 pots in each replicate. The excess nutrient solution applied was allowed to flow through the pots so that salt stress could be maintained at the desired concentration in the root zone during the experiment. Excess water-induced anoxia (due to water-logging) was also prevented.

Three weeks after starting the stress treatments, three plants from each replicate (9 plants per treatment) were collected; fully expanded youngest leaves were used to record data of the below given parameters. The remaining two plants per replicate (6 plants per treatment) were further grown for three weeks and then picked to record total fruit weight per plant. The root and shoot samples were oven-dried at 75ºC for three days for dry mass determination.

Further trial

According to findings of the initial trial, elavated NO and H_2S levels in the leaves of plants subjected to ID, S or ID+S stresses, and exogenous application of MT caused additional raises in NO and H_2S contents. Therefore, an additional trial was carried out under the same conditions to further investigate the effect of MT on the synthesis/accumulation of endogenous hydrogen sulfide (H_2S) and nitric oxide (NO), and how far these metabolites play a role in regulating growth and some key metabolic processes in pepper plants under the combined effect of iron deficiency and saline stress. For this reason, the scavengers of NO and H_2S, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) and hypotuarine (HT), respectively were applied with MT treatments. Both scavengers (0.10 mM) were sprayed singly to the leaves of plants every other day for two weeks before starting the stress treatment as explained above. Correa-Aragunde et al. (2006) and Amooaghaie et al. (2017) used cPTIO and hypotuarine at 0.1 mM as scavenger of NO in tomato plants and scavenger of H_2S in *Sesamum indicum*, respectively. So this dose was chosen for both scavengers of NO and H_2S in the present experiment.

Chlorophyll content

Chlorophyll content was measured according to the method reported by Strain and Svec (1966). At the fruit set stage, a leaf sample (1.0 g each) was extracted with 5 ml of 90% acetone. The extract was filtered before storing in light-tight tubes. Chlorophyll extracts were read at 663.5 nm for chlorophyll a, 645 nm for chl *b* and expressed as mg g^{-1} FW. The formulae used to calculate chlorophyll *a* and *b* are:

$$\text{Chl } a = [11.64 \times (A663) - 2.16 \times (A645)] \times TV/ [FW]$$

$$\text{Chl } b = [20.97 \times (A645) - 3.94 \times (A663)] \times TV/ [FW]$$

A663: Optical density (OD) value at 663 nm, A645: OD value at 645 nm, TV: total volume of the extract (ml), FW = fresh leaf weight (g)

Chlorophyll fluorescence

For measurement of chlorophyll fluorescence, the leaves were exposed to dark for 30 min before taking readings. Fluorescence readings were taken using a chlorophyll fluorometer (Mini-PAM, Walz, Germany).

Measurement of Fe and Ferric reductase activity

Total Fe was determined following Lei et al. (2014). After washing the leaf samples with 1.5 mM $CaCl_2$ solution, they were digested in $HNO_3/HClO_4$ (4:1, v/v). Total Fe in the extracts was appraised using an ICP.

The method described by Gao and Shi (2007) was used to analyze active iron content in the leaves and ferric reductase activity in the roots.

Nitric oxide (NO) and hydrogen sulfide (H_2S) determination

Nitric oxide in the leaves was assayed following the modified protocol of Zhou et al. (2005) as previously reported by Ding et al. (1998) and Hu et al. (2003). Hydrogen sulfide in the leaf samples was quantified as described by Nashef et al. (1977) and subsequently modified by Christou et al. (2013).

Hydrogen peroxide (H_2O_2) and Leaf malondialdehyde (MDA)

Hydrogen peroxide in the leaf samples was appraised as described by Loreto and Velikova (2001).

The MDA concentration in the leaves was determined according to the method described by Weisany et al. (2012).

Electrolyte leakage (EL)

The method previously reported by Dionisio-Sese and Tobita (1998) was employed to appraise this parameter.

Antioxidant enzymes and soluble protein

Fresh leaf material (0.5 g each) was triturated in 50 mM of Na-P buffer consisting of soluble polyvinyl pyrolidine (1%). Homogenate solution was subjected for 15 min to centrifugation at 20,000 *g* under 4° C. The activity of CAT in the supernatant was appraised according to the method of Milosevic and Slusarenko (1996). The measurement of peroxidase (POD) activity followed the method of Chance and Maehly (1955) and that of SOD was assessed by determining the ability of SOD to block the nitroblue tetrazolium (NBT) photochemical inhibition (Beauchamp and Fridovich 1971). All antioxidant enzyme activities were expressed as Unit mg^{-1} protein. For quantifying total soluble proteins the Bradford (1976) protocol was employed.

Chemical analysis

For the determination of Na and K, powdered leaf samples were ashed at 550ºC in a muffle furnace. Five ml of hot HCl (2 M) were added to each sample and the final volume of the sample was brought up to 50 ml by adding distilled deionized H_2O (Chapman and Pratt 1982). An ICP was used to analyse all mineral elements in digested samples. Chloride was extracted with hot water and quantified using potassium chromate indicator titrated with $AgNO_3$ (Johnson and Ulrich 1959).

Statistical analysis

The data for all growth and biochemical attributes were subjected to a three-way analysis of variance using the statistical package (CoStat program version v 6.303). Significant differences among the mean values were appraised using the Duncan's test at 5% probability level. Data in all figures are presented as mean ± standard error.

Results

Preliminary experiment

Melatonin (MT) enhances key growth attributes under ID, SS alone or in combination

Iron deficiency decreased total, shoot and root dry mass by 32.05, 35.94 and 17.98%, respectively (Fig. 1A, B, C). When pepper plants were subjected to SS, total dry weight, and shoot and root dry weights reduced by 37.17, 39.97 and 27.09%, respectively compared with those of respective controls (Fig. 1A, B, C). The decreases in total, shoot and root dry mass by 59.61, 61.11 and 53.69%, respectively were most striking in the combined (S + ID) stress treatment. Foliar application of MT enhanced total, shoot and root biomass by 35.06, 45.82 and 37.99%, respectively in plants grown under Fe deficiency with respect to respective controls. Similarly, treatment of MT clearly alleviated the SS-induced detrimental effects. Total, shoot and root dry weights of pepper seedlings treated with MT were 31.04, 33.30 and 25.11% higher than those under SS alone, respectively. Enhancement of

total, shoot and root dry mass by 24.49, 25.19 and 23.93% low in the combination treatments (S + ID + MT). The control plants treated with MT did not show any change in total, shoot and root dry mass. These findings reveal that MT might promote the plant growth of pepper subjected to stress conditions.

Iron (Fe) deficiency reduced chlorophyll *a* and *b* contents and *F*v/*F*m by 42.75, 26.98 and 21.82%, respectively of pepper plants (Fig. 1D, E, F). Significant ($P \leq 0.05$) decreases in chlorophyll *a* and *b* content as well as *F*v/*F*m in pepper leaves were found in the plants grown under SS. As compared with control, the chlorophyll a and b content and *F*v/*F*m declined by 28.98, 26.50 and 21.97% under salt stress, respectively (Fig. 1D, E, F). Exogenously applied MT increased chlorophyll *a* and *b* contents and *F*v/*F*m in the leaves of plants subjected to ID and SS stress with respect to those in the control plants, but these improvements were more limited when both stresses applied in combination. Exogenous MT application to the control plants did not affect the chlorophyll contents and *F*v/*F*m.

Iron deficiency and saline stress applied individually or in combination significantly ($P \leq 0.05$) reduced fruit yield by 23.32, 25.29 and 33.65%, respectively. A maximal reduction was observed when both stresses were applied together (Fig. 1G) Exogenously applied MT partly enhanced fruit yield of plants subjected to either Fe deficiency or salinity stress, but had a very limited effect in the plants exposed to both stresses together where values were much lower than those in the control plants.

MT reverses mineral nutrition under ID, SS alone or in combination

Iron deficiency and salinity stress led to significant decreases in both absorption and transportation of Fe in pepper plants. Iron deficiency and salinity stress reduced leaf total Fe and active Fe content of plants compared with that by the control treatment (Fig. 2A, B). Reduction in total Fe content was 2-fold higher than that in leaf active Fe in plants subjected to Fe deficiency or SS stress. In contrast, Fe deficiency significantly increased the ferric reductase activity (Fig. 2C), while salinity stress did not change ferric reductase activity in the roots of pepper plants compared with that in the control treatment. Exogenously applied MT increased total Fe, active Fe content, and ferric reductase activity under Fe deficiency and also in all other treatments. In the control treatment, these parameters were not significantly affected by MT application.

Salinity stress increased leaf sodium (Na^+) and chloride (Cl^-) concentrations, but reduced those of potassium (K^+). Iron deficiency did not change the levels of leaf Na^+ and Cl^-, but it increased those of K^+ (Fig. 2D, E, F). Exogenously supplied MT reduced both leaf Na^+ and Cl^- and improved K^+ content in plants exposed to saline stress, but it did not notably alter those element concentrations in the leaves of plants exposed to Fe deficient conditions. However, MT did not change concentrations of these elements in the control plants.

Salinity stress and SS + ID increased leaf Na^+: K^+ ratio with respect to that in the control plants, but ID stress did not change the Na^+: K^+ ratio. The leaf Na^+: K^+ ratios decreased considerably in the plants sprayed with melatonin under the SS and SS + ID (Fig. 2G).

MT further enhances nitric oxide (NO) and hydrogen sulfide (H_2S) under ID, SS, or ID + SS

Both NO and H_2S contents increased in the leaves of pepper plants subjected to ID and SS applied singly or in combination (Fig. 3A, B). Salinity stress, ID and both stresses in combination increased the levels of NO by 2.5-, 3.5- and 4.0-fold compared with that in the non-stressed plants, respectively. Similarly, leaf H_2S levels in pepper plants subjected to salinity stress, ID and both stresses in combination were 1.6-, 1.3- and 2.1-fold higher than those in the control plants, respectively. Exogenously applied MT further increased both leaf NO and H_2S levels in the plants, but it did not increase H_2S levels in the control plants. These results indicate that MT-generated NO and H_2S could be involved in the mitigation of ID and SS. However, for further evidence of the role of these two signalling molecules in overcoming of both ID and S or ID + S by MT, scavengers of NO and H_2S, cPTIO and HT, respectively were applied in the second experiment.

MT mitigates oxidative stress under ID, SS, or ID + SS

To study the effect of MT treatment on oxidative stress parameters in pepper plants stressed with ID or SS, MT was sprayed to the leaves of plants for 14 day before starting the SS treatment. Iron deficiency and salinity stress alone or especially in combination led to significant increases in H_2O_2, MDA, and EL. Exogenously-applied MT mitigated the oxidative damage caused by the stresses of Fe deficiency and salinity by decreasing H_2O_2, MDA and EL (Fig. 3C, D, E), but it did not change the contents of H_2O_2 and MDA as well as the extent of EL in the control plants.

MT improves antioxidant defence systems under ID, SS or ID + SS

The chief defensive enzymes in an antioxidant defense system such as SOD, POD and CAT can efficiently scavenge the active oxygen species. So, the role of MT was also tested on antioxidant defense system under ID, SS alone or in combination. Iron deficiency decreased the activities of catalase (CAT) by 32.67% and peroxidase (POD) by 28.25%, while it increased the activities of leaf superoxide dismutase (SOD) by 38.18% compared with those in the control treatment (Figure 3F, G, H). Salinity stress reduced SOD and CAT activities by 22.22 and 47.24%, respectively, but increased that of POD by 173% with respect to those in the control plants. Exogenously applied MT promoted the activities of leaf CAT and POD, while it suppressed that of SOD in plants exposed to Fe deficiency. MT increased leaf SOD and CAT activities, but decreased that of POD in plants subjected to salinity stress or salinity stress + Fe deficiency. These enzyme activities were not affected by MT application in the control plants.

Second experiment

The involvement of MT-generated NO and H_2S in enhancing key growth parameters under ID, SS, or ID + SS

To investigate the involvement of endogenous NO and H_2S induced by MT in improving key growth parameters, dry mass, chlorophyll content and *F*v/*F*m of pepper plants were determined.

As presented in experiment 1, stress and MT treatments showed similar effects on dry mass and chlorophyll content and *F*v/*F*m of pepper plants (Fig. 4A, B, C, D, E, F). Total, shoot and root dry mass, chlorophyll *a* and *b* contents and *F*v/*F*m of pepper plants decreased significantly ($P \leq 0.005$) by ID, SS alone or in combination stress relative to those in the control plants. Foliar application of MT was effective in enhancing these parameters under ID and SS alone rather than the combined application of these stresses relative to those in stressed plants receiving no MT treatment. However, these attributes did not change by MT treatment in the control plants.

The affirmative effects of MT on the key growth parameters were almost completely reversed by the application of the scavenger of NO, cPTIO along with MT. These findings revealed that the endogenous NO is involved in the stimulatory effect induced by MT on key growth parameters of stressed plants. However, application of the scavenger of H_2S, hypotuarine (HT), partly reversed the alleviation effects of MT treatments on these parameters. As presented in the later section, HT did not completely reverse the endogenous NO levels but in contrast it completely reversed H_2S levels in the leaves of plants treated with MT under stress conditions. So, the reasonable levels of NO maintained due to such treatments may still support the alleviation effect of MT. These results clearly showed that endogenous NO and H_2S jointly contribute to the positive effect of MT on key growth parameters.

The involvement of NO and H_2S induced by MT in enhancing mineral nutrition under ID, SS or ID + SS

Leaf total Fe, leaf active Fe and root ferric reductase activity were determined in MT supplied plants exposed to ID, SS or ID + SS in order to understand whether or not the endogenous NO and H_2S generated by MT played a role in elevating Fe acquisition. Iron deficiency and SS significantly ($P \leq 0.005$) reduced total and active Fe in the leaves, but ID increased ferric reductase activity in the roots of pepper plants as observed in the previous experiment (Fig. 5A, B, C). The MT supply significantly increased total and active Fe in the leaves, and ferric reductase activity in the root of pepper plants exposed to all stress treatments relative to these stress treatments without MT. However, MT treatments did not alter these parameters in the control plants. Applications of cPTIO along with MT treatment completely reversed the positive effects of MT on these parameters, but HT along with MT treatment partly reversed the mitigation effects of MT on these parameters under ID.

Furthermore, as presented in the first experiment, all stress and MT treatments showed similar effects on leaf Na^+, Cl^- and K^+ contents of pepper plants in the second experiment. So, to investigate the involvement of MT-mediated generation of NO and H_2S in enhancing mineral nutrition under all

stress conditions, the scavengers of NO and H_2S, cPTIO and HT, respectively were applied along with MT. As shown in Fig. 5D, E, F, applications of cPTIO along with MT treatment completely reversed the positive effects of MT on leaf Na^+, Cl^- and K^+ contents under all stresses, but HT along with MT treatment partly reversed the mitigation effects of MT on these parameters under all stresses. These results show that endogenous NO and H_2S work jointly to make MT fully effective in improving tissue K^+ status and reducing Na^+ and Cl^-.

The involvement of NO and H_2S induced by MT in stress tolerance

In this trial, cPTIO and HT, scavengers of NO and H_2S (0.10 mM), respectively were supplied to pepper plants exposed to ID, SS or ID + SS along with MT treatment so as to understand whether or not MT-induced generation of NO and H_2S as signal molecules were involved in alleviation of these stresses in pepper seedlings. Iron deficiency and SS enhanced leaf NO and H_2S in pepper plants (Fig. 6A, B). These enhancements in NO and H_2S were slightly higher in plants subjected to both stresses applied jointly than those in ID or SS alone. Exogenously applied MT further increased both leaf NO and H_2S levels in the plants subjected to all stresses, but it did not increase H_2S levels in the control plants. These results indicate that NO and H_2S may be involved in the mitigation of all stresses by MT. The MT treatments combined with cPTIO and HT under all stresses showed that the effect of NO was specifically reversed by the NO scavenger, cPTIO, alone while the H_2S effect was inhibited by both scavengers.

The involvement of NO and H_2S induced by MT in reversing oxidative stress under ID, SS, or ID + SS

In order to understand the role of NO and H_2S in tolerance to ID, SS or ID + SS-induced oxidative stress in pepper plants, the oxidative stress parameters such as hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) as well as electrolyte leakage (EL) were determined.

All stresses, especially ID + SS caused significant increases in H_2O_2 and MDA contents as well as EL in the leaves of pepper plants relative to those in the control plants. However, MT treatments reduced H_2O_2, MDA and EL in the leaves of pepper seedlings subjected to all stresses relative to those in the stressed plants not treated with MT, but these parameters were not changed by the MT treatment in the control plants. While MT applied along with cPTIO completely reversed the reductions in these oxidative stress attributes to the levels of those in plants subjected to any of the stresses. On the other hand, MT applied along with HT partly reversed the decrease in these parameters by MT relative to those in the stressed plants (Fig. 6C, D, E).

The involvement of NO and H_2S in melatonin-mediated antioxidant defence system under ID, SS or ID + SS

For a further evidence on the involvement of endogenous NO and H_2S generated by MT on antioxidant defence system, we determined the activities of some key enzymes involved in the antioxidant defence system. Application of cPTIO totally reversed ($P < 0.05$) MT-induced alteration in the activities of SOD, CAT and POD, but HT could not completely reverse the promoting effects by MT on the antioxidant enzymes activities (Fig. 6F, G, H). Such an effect of HT may have been due to the inability of HT to completely scavenge the endogenous NO content, even if it blocks the accumulation of H_2S. However, both scavengers did not change the activities of the antioxidant enzymes in the unstressed-plants. These results obviously show that MT can regulate antioxidant enzymes, causing a reduction in H_2O_2 accumulation in ID and salinity-stressed pepper plants.

Discussion

The primary objective of the present research was to examine whether or not exogenously applied melatonin (MT) as a foliar spray could alleviate the detrimental effects of Fe deficiency and salinity stress individually or in combination on pepper plants. Although there are many reports in the literature on plant responses to either iron (Fe) deficiency, e.g. in cucumber (Donnini et al. 2010), maize (Hopff et al. 2013), and rice (Chen et al. 2015a) or salinity stress (Tao et al. 2015, Coskun et al. 2016, Martinez et al. 2018), limited research has been conducted to examine plant responses to the combined application of saline stress and Fe deficiency (Rabhi et al. 2007).

Exogenously applied MT has been tested as a key biomolecule effectively associated with signal transduction involved in alleviation of iron deficiency (Zhou et al. 2016) and salinity stress (Zhang et al. 2014, Shi et al. 2015a) individually. However, there seem to be no reports in the literature relating to the effect of MT on both stresses applied together. In this study, foliar application of MT partially improved tolerance of iron deficiency and salinity stress, but it was not so effective when the two stresses were applied together (Fig. 1A, B, C). Previous findings reported by Martinez et al. (2016, 2018) in tomato and Li et al. (2016) in cucumber, both indicated that MT improved biomass production in plants subjected to saline stress, thus showing that MT has a possible role under saline conditions. However, the addition of a scavenger of NO, cPTIO along with MT reversed mitigation effects of MT on plant growth, but application of HT along with MT did not completely reverse the mitigation effect prompted by MT on the plant growth attributes (Fig. 4) These findings suggest that the endogenous NO and H_2S are both also needed for the stimulatory effect induced by MT on the plant growth of ID and S–stressed plants.

It has been reported that the specific symptoms of Fe deficiency appear on young leaves as chlorosis, the leaves of Fe-deficient pepper plants lose their bright green colour and turn to light green (Landsberg 1986). Similar symptoms appeared on leaves of pepper plants subjected to Fe-deficient alone and also with Fe deficiency plus salinity stress visually (Fig. 7). It is likely that these symptoms are linked to leaf chlorophyll and iron concentrations as explained by Chen et al. (2015b). Analogous findings have also been reported in a broad range of crops such as peach (Molassiotis et al. 2005), pear

(Morales et al. 2000), chinese cabbage (Ding et al. 2008), strawberry (Pestana et al. 2012), and peanut (Zhang et al. 2012). Exogenous MT enhanced chlorophyll content in the leaves of pepper exposed to Fe-deficiency, but chlorophyll values were still lower than those in control plants (Fig. 1D, E). The findings of our experiment evidently show that melatonin has a crucial role in modulating the antioxidant defense mechanism under Fe deficiency and salinity stress. Exogenous MT effectively alleviated chlorosis induced by Fe deficiency improving Fe absorption and translocation, stimulating Fe activation and relieving the oxidative stress induced by Fe deficient situations in the pepper leaves. This probably caused elevated Fe nutrition and chlorophyll synthesis, and hence enhanced plant growth under Fe-deficient conditions. Furthermore, our findings showed a marked suppression in chlorophyll a and b in pepper plants exposed to salt stress. Similar results were recorded by Taffouo et al. (2010) on *Vigna subterranean* L. and Turan et al. (2007) on *Phaseolus vulgaris* L. The reduction in chlorophyll contents of leaves subjected to salinity has been linked to oxidative stress (Smirnoff, 1996) and might be due to both reduction of chlorophyll synthesis and degradation of chlorophyll by high activity of chlorophyllase enzyme (Santos, 2004). On account of either restraint of blend or fast degradation of chlorophyll, it has been demonstrated that there may be a photoprotection system that declines light absorbance by diminishing chlorophyll content (Elsheery and Cao 2008). Reduction in the chlorophyll contents also showed that light reactions and electron transport in PS II were reduced by salinity (Li et al. 2017). However, melatonin application improved chlorophyll content in the present experiment. Earlier reports have indicated that exogenously applied melatonin improved Chl *a* and Chl *b* levels under abiotic stresses (Wang et al. 2016, Li et al. 2017, Martinez et al. 2018). The decrease in chlorophyll content might have been due to decreased active Fe content under both salinity stress and Fe deficiency (Figs 1D, E and 2B). There is an effective association between active Fe in leaf tissues, chlorophyll biosynthesis and electron transport (Celik and Katkat 2007, Zhang et al. 2011). Melatonin application to the plants exposed to both salinity and Fe deficiency improved active Fe and chlorophyll content in the present experiment. This could have been due to exogenous MT lowering the degradation of chlorophyll and enhancing active Fe levels in the leaves. This indicates that exogenous MT can stimulate more Fe translocation into plant cells and also enhance the activation of Fe within leaves.

The *F*v/*F*m values measured in the dark-adapted leaves are a well-known parameter relating to the quantum efficiency of photosystem II (Maxwell and Johnson 2000, Zhou et al. 2010, Wang et al. 2013a). Under normal conditions, the ratio of *F*v/*F*m is in the range of 0.76 to 0.85, but the ratio is lowered under stress conditions (Liang et al. 2010). It has been reported that reduced PSII activity leads to imbalance between the production and utilization of electrons in plants subjected to saline conditions (Zhou et al. 2016). Exogenously applied melatonin increased the maximum photochemical efficiency of PSII (*F*v/*F*m), which was reduced in the pepper plants subjected to both stressors (Fig. 1F). These findings are analogous to those reported by Han et al. (2017) in cold-stressed rice, by Wang et al. (2013b) in drought-stressed apple and by Zhang et al. (2013) in water-stressed cucumber.

It is known that nitric oxide (NO) and hydrogen sulfide (H_2S) play crucial roles as vital signal molecules in the tolerance mechanisms of plants subjected to Fe-deficient regimes (Graziano et al. 2002, Graziano and Lamattina 2007, Chen et al. 2010, 2015b). It has also been shown that NO and H_2S increase the active Fe within plants, remedying both leaf chlorosis and oxidative stress caused by Fe deficiency (Ramirez et al. 2010, Chen et al. 2015b). Moreover, under saline conditions, increased NO (Li et al. 2012, Chen et al. 2017, Zhao et al. 2018) and H_2S (Mostofa et al. 2015, Jovelina da Silva et al. 2017) levels have been reported in different plants. In the present experiment, MT treatment led to further increases in the NO and H_2S levels at single and combined stress as well as control treatments except for H_2S in control treatment (Fig. 3A, B). The increases in NO levels in control treatments could not effectively stimulate the ferric reductase activity compared with the increases of NO and H_2S levels under stress conditions (Figs 2C and 3A, B). This suggests that ID and SS induced by MT might be attained by elevations of endogenous NO and H_2S. However, excess generation of NO and H_2S may lead to harmful effects in plants (Corpas et al. 2011, Jovelina da Silva et al. 2017). So, a precise balance of cellular NO and H_2S levels is required for tolerance of plants to different stresses. The generation of NO and H_2S stimulated by MT did not exceed critical levels causing detrimental physiological results (Liu et al. 2015, Zhou et al. 2016, 2018).

In the present study, we set up an additional experiment to understand the underlying mechanisms and to provide evidence that NO and H_2S accumulations were associated with mitigation of ID and S by melatonin. Therefore, the scavengers of NO and H_2S, cPTIO and hypotuarine (HT), respectively were used with the MT treatments. The affirmative influence of applied MT was totally reversed by the application of cPTIO (scavenger of NO) and partly by HT (scavenger of H_2S) (Fig. 4A, B, C). This indicates that both NO and H_2S might be downstream signal molecules in MT-induced ID and SS. The cPTIO can straightforwardly enter biomembranes and so it is the most commonly used for NO distinct scavengers. cPTIO can react with NO, so various physiological progressions prompted by NO were reversed by adding cPTIO (Wang et al. 2012, Li et al. 2013). Moreover, treatment considerably enhanced the tolerance of a broad range of plant species to NaCl, water deficit, and Cd stress, while mitigation effects of NO were inverted by addition of cPTIO (Xiong et al. 2009).

One deleterious effect of salinity in plants is oxidative stress, which is related to the production of a large amount of ROS (Mittler 2002). The present results indicate that salinity stress increased the H_2O_2 content of pepper plants (Fig. 6C), thereby inducing a high level of ROS (Liu et al. 2018). Over-generation of ROS can inhibit electron transport by causing degradation of proteins and influencing the repair process of PSII (Allakhverdiev et al. 2008). This could cause increased membrane lipid peroxidation and membrane permeability (Mittler 2002, Sharma et al. 2012). In the present experiment, salinity stress and Fe deficiency caused a high accumulation of H_2O_2 as well as increased MDA and electrolyte leakage (Fig. 3C, D, E), which indicates that accumulation of ROS could be responsible for salinity stress and Fe deficiency-induced membrane injury. It is well reported that melatonin is an antioxidant and has a crucial role in mitigating oxidative stress by removing most

of the ROS accumulated within plants (Li et al. 2016). In the present experiment, it has been found that salt stress or iron deficiency induced a large amount of ROS causing membrane injuries, which were mitigated by exogenously applied melatonin. It has been shown that melatonin does not remove hydrogen peroxide (Bonnefont-Rousselot et al. 2011, Li et al. 2016), but it induces oxidative defence systems including enzymatic and non-enzymatic antioxidants (Li et al. 2017). However, using scavengers of NO and H2S, cPTIO and HT, respectively reversed the reduced ROS by melatonin in total and partly, respectively (Fig. 6C, D, E). This indicates that accumulation of endogenous NO and H_2S induced by MT can react with ROS and it suggests that endogenous NO and H_2S play a critical role in the oxidative defence in the plant (Wang et al. 2010, Gupta et al. 2012, Qiao et al. 2014, Corpas 2017). Taking into account the powerful oxidative element of several stress situations, it has been reported that both NO and H_2S have the ability to stimulate antioxidant systems which alleviate the deleterious effect of ROS accumulation (Sang et al. 2008, Manai et al. 2014). Nitric oxide can react directly with ROS and lipid radicals to counteract and enhanced the activities of antioxidant enzymes (Corpas 2017). This has also been shown in our experiment, where endogenous NO and H_2S were positively correlated ($P \leq 0.005$) with H_2O_2 content ($r = 0.435$ and 0.449, respectively) (Figs 8A and 9A). This suggests that when H_2O_2 content increases in the leaves of plants under stress, stressed plants also produce NO and H_2S to reverse the oxidative stress.

The plants can remove a large amount of accumulated ROS if the activity of enzymes such as, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) is enhanced (Liu et al. 2018). Fe deficiency is believed to elevate SOD activity and reduce those of CAT and POD in peanut plants (Zhang et al. 2012). These results are analogous to ours, which showed that SOD was increased, and CAT and POD decreased by Fe deficiency (Fig. 3F, G, H). It has also been reported that melatonin increased CAT activity in salt-stressed *Malus hupehensis* and watermelon plants (Li et al. 2012, 2017), a similar finding to our results where MT enhanced the activities of both SOD and CAT in the salt-stressed plants. These findings clearly indicate that melatonin can enhance the antioxidant enzymes' activities under both stresses in the pepper plants protecting cell membranes against the detrimental effects of oxidative stress induced by salinity and Fe deficiency. However, treatments of scavengers of NO and H2S, cPTIO and HT, respectively caused a complete and partial alteration in the antioxidant enzyme activities induced by MT (Fig. 6F, G, H). These results show that when both levels of endogenous NO and H_2S were co-blocked, it caused increases in oxidative stress attributes and altered levels of antioxidants led to reduced stress tolerance. This suggests that the endogenous NO and H_2S variations in the ROS detoxification system and improving antioxidant defense system might be directly related to the effects of H_2S and NO on the stress tolerance. Enhanced antioxidant defense system induced by NO treatment has been stated by some of researchers in broad range of species under various stress conditions (Fan et al. 2013, Groß et al. 2013, Fu et al. 2016, Ahmad et al. 2018). In the present experiment, endogenous NO and H_2S were negatively correlated ($P \leq 0.001$) with CAT enzyme activity (r = -0.531, -0.559, respectively) (Figs 8C and 9C) and not significantly correlated

with SOD and POD enzyme activities (Figs 8B, D and 9B, D). This reveals that when CAT activity decreases in plants, they synthesize both NO and H_2S to improve antioxidant systems including improved activity of CAT.

Furthermore, H_2S is well known as a regulator of antioxidant enzymes and it reduces the deleterious effect of stress-induced oxidative stress (Liu et al. 2016). As an example, in an earlier study, an elevation in POD activity was reported in the roots and leaves of barley plants exposed to exogenous application of H_2S (Chen et al. 2012).

A further detrimental effect of salinity stress is to induce Na^+ and Cl^- accumulation causing mineral nutrient disorders and an imbalance in cellular ion homeostasis (Munns and Tester 2008). Salinity also reduces K^+ uptake, damages cell membranes and as a consequence causes cell death. So, a high K^+/Na^+ ratio can improve tolerance of plants to salinity (Shabala and Cuin 2008, Munns and Tester 2008). In our study, salinity stress led to increased Na^+ and decreased K^+ in the plants (Figure 5D, F). Such a salinity-induced regulation in ion homeostasis has been reported in a variety of plants such as *Aloe vera* (Jiang et al. 2014), poplar (Jiang et al. 2011, 2012) and tomato (Zhang and Blumwald 2001) under saline stress. Exogenous MT stimulated these plants to sustain markedly higher K^+ and lower leaf Na^+ and Cl^- compared to untreated plants. Parallel findings have been also reported by Liu et al. (2015) in tomato and Jiang et al. (2016) in maize. A low ratio of Na^+: K^+ in the cytosol is crucial in order to sustain enzymatic activities (James et al. 2006). The ratios of Na^+: K^+ were considerably decreased in the leaves of plants sprayed with melatonin in our study (Fig. 2G), this is in agreement with Li et al. (2012, 2015), Jiang et al. (2016).

It can be concluded that MT can effectively enhance tolerance of pepper plants to Fe deficiency and salinity stress by increasing active Fe, K, endogenous NO and H_2S as well as activities of antioxidant enzymes and reducing the levels of H_2O_2 and MDA, while abolished by addition of cPTIO and HT combined along with MT. These findings suggested that tolerance to ID and SS induced by MT may be involved in downstream signal crosstalk between NO and H_2S. However, melatonin was not so effective when Fe deficiency and salinity stress were applied together. Further studies are needed to improve tolerance of plants subjected to both stresses together perhaps by using melatonin combined with other signal molecules or hormones. The detailed molecular mechanism of crosstalk among MT, NO, and H_2S involved in both stresses needs to be investigated in future study.

Author contributions

C.K. conducted the experimentation and carried out data analysis. C.K. also wrote up the initial draft of manuscript. M.A., D.H., M.N.A. and P.A. helped in designing the study and critically edited the whole manuscript. All authors read and approved the final manuscript.

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Figure legends

Fig. 1. Total plant (A), shoot (B), and root (C) dry matter (DM), chlorophyll a (D), chlorophyll b (E) on fresh weight (FW) basis, chlorophyll fluorescence parameters [*F*v/*F*m (F)] and fruit yield (G) of pepper plants grown under iron deficiency and salinity stress applied individually or in combination, and supplied with 100 µM melatonin (+MT) or no melatonin (-MT) as foliar spray. Mean ± S.E; Mean pairs followed by different letters are significantly different ($P \leq 0.05$) by the Duncan's multiple range test. C: Control treatment – Hoagland's nutrient solution containing 0.1 mM EDTA-Fe with no addition of NaCl; ID: Iron deficiency treatment – Hoagland's nutrient solution containing 0.1 mM $FeSO_4$; SS: 100 mM NaCl.

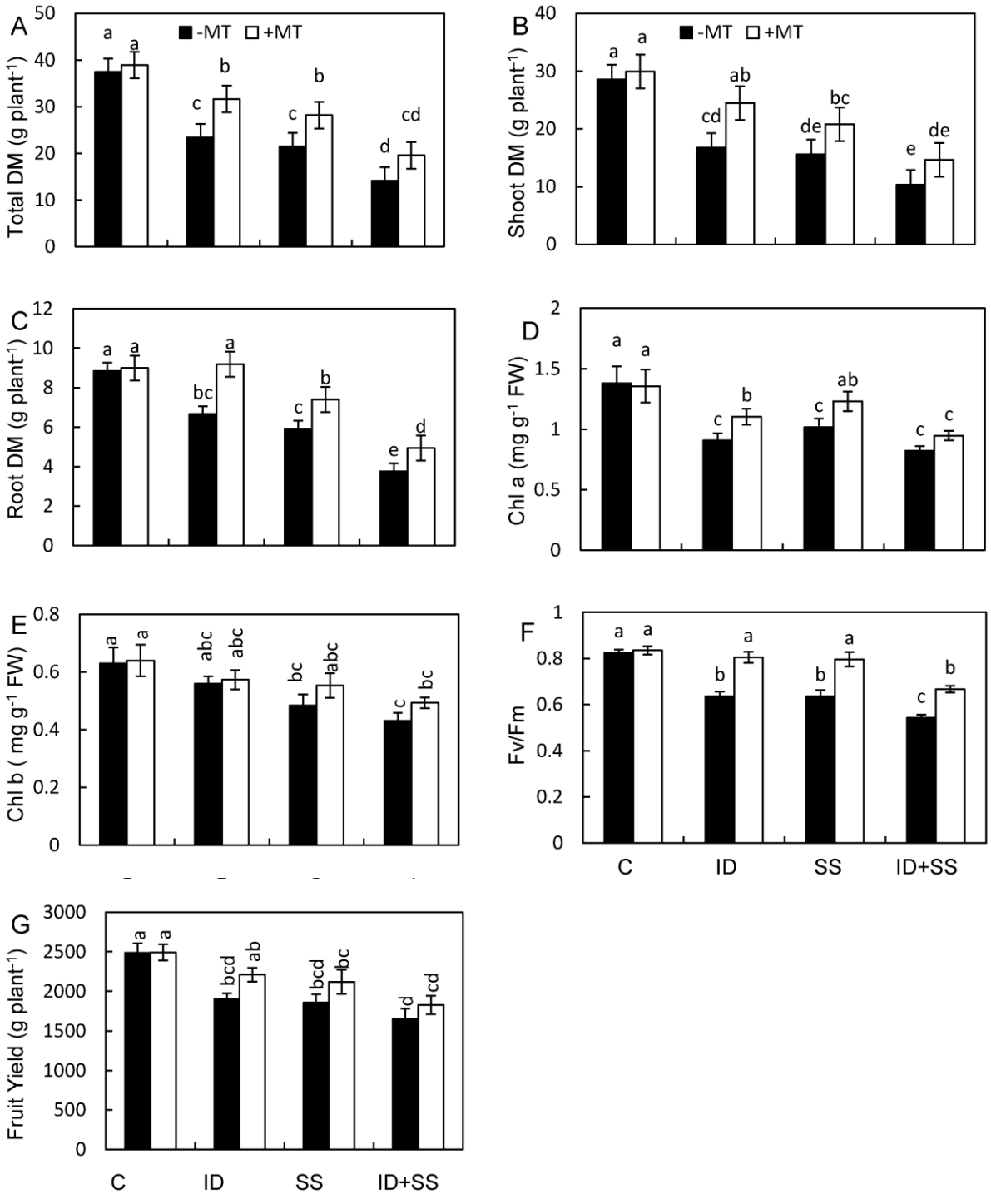

Fig. 2. Total Fe (A) and active Fe (B) contents on dry weight (DW) basis in leaves, ferric reductase activity in the roots (C), leaf Na^+ (D), leaf Cl^- (E), leaf K^+ (F) on dry weight (DW) basis and leaf Na^+/K^+ (G) ratio of pepper plants grown under iron deficiency and salt stress applied individually or in combination and supplied with 100 µM melatonin (+MT) or no melatonin (-MT) as foliar spray. Mean ± S.E; Mean pairs followed by different letters are significantly different ($P \leq 0.05$) by the Duncan's multiple range test. C: Control treatment – Hoagland's nutrient solution containing 0.1 mM EDTA-Fe with no addition of NaCl; ID: Iron deficiency treatment – Hoagland's nutrient solution containing 0.1 mM $FeSO_4$; SS: 100 mM NaCl.

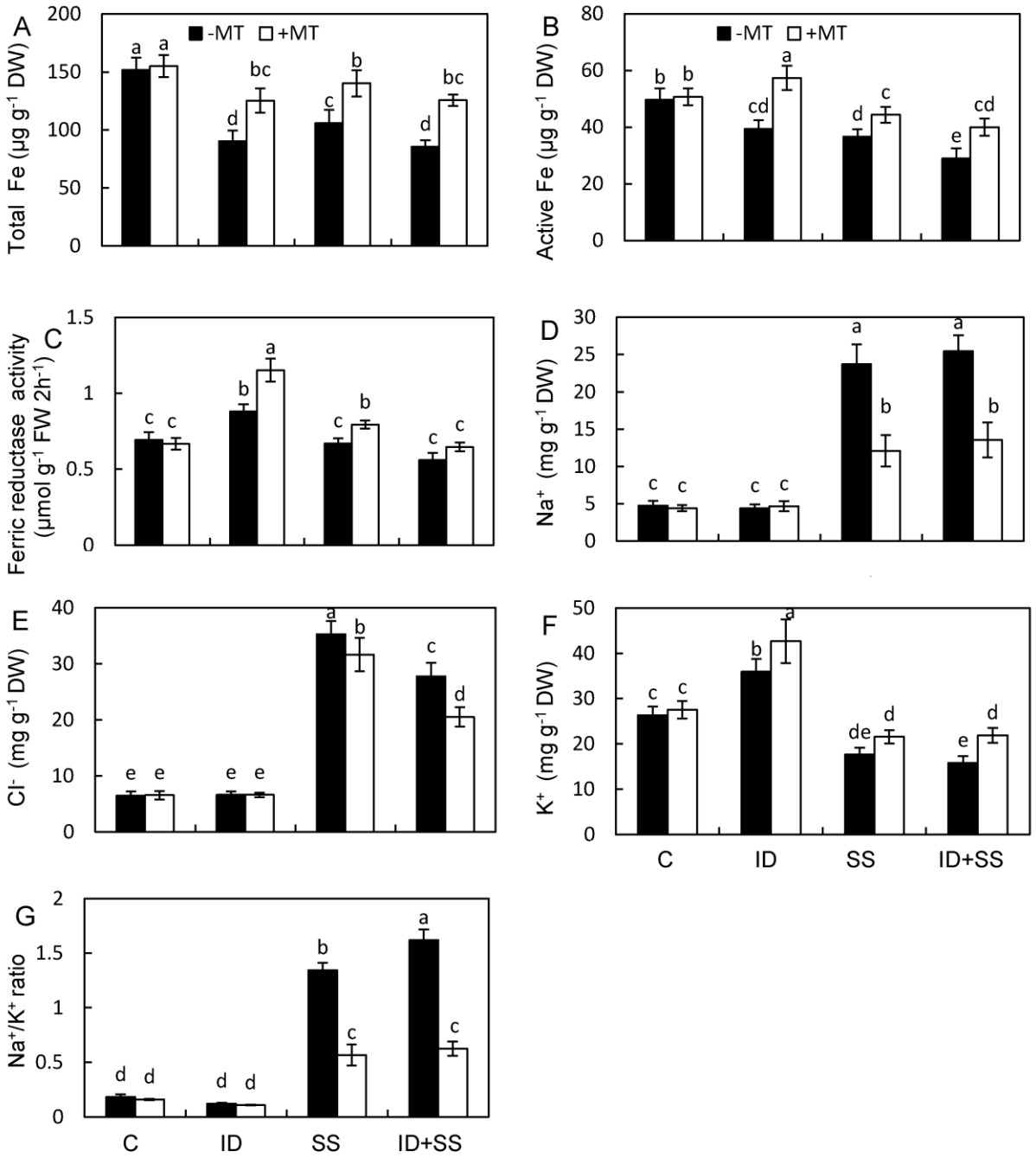

Fig. 3. Nitric oxide [NO (A)], hydrogen sulfide [H_2S (B)], hydrogen peroxide [H_2O_2 (C)], malondialdehyde [(MDA (D)] on fresh weight (FW) basis, electrolyte leakage [EL (E)], activities of superoxide dismutase [SOD (F)], catalase [CAT (G)] and peroxidase [POD (H)] in the leaves of pepper plants grown under iron deficiency and salt stress applied individually or in combination, and supplied with 100 µM melatonin (+MT) or no melatonin (-MT) as foliar spray. Mean ± SE; Mean pairs followed by different letters are significantly different ($P \leq 0.05$) by Duncan's multiple range test. C: Control treatment – Hoagland's nutrient solution containing 0.1 mM EDTA-Fe with no addition of NaCl; ID: Iron deficiency treatment – Hoagland's nutrient solution containing 0.1 mM $FeSO_4$; SS: 100 mM NaCl.

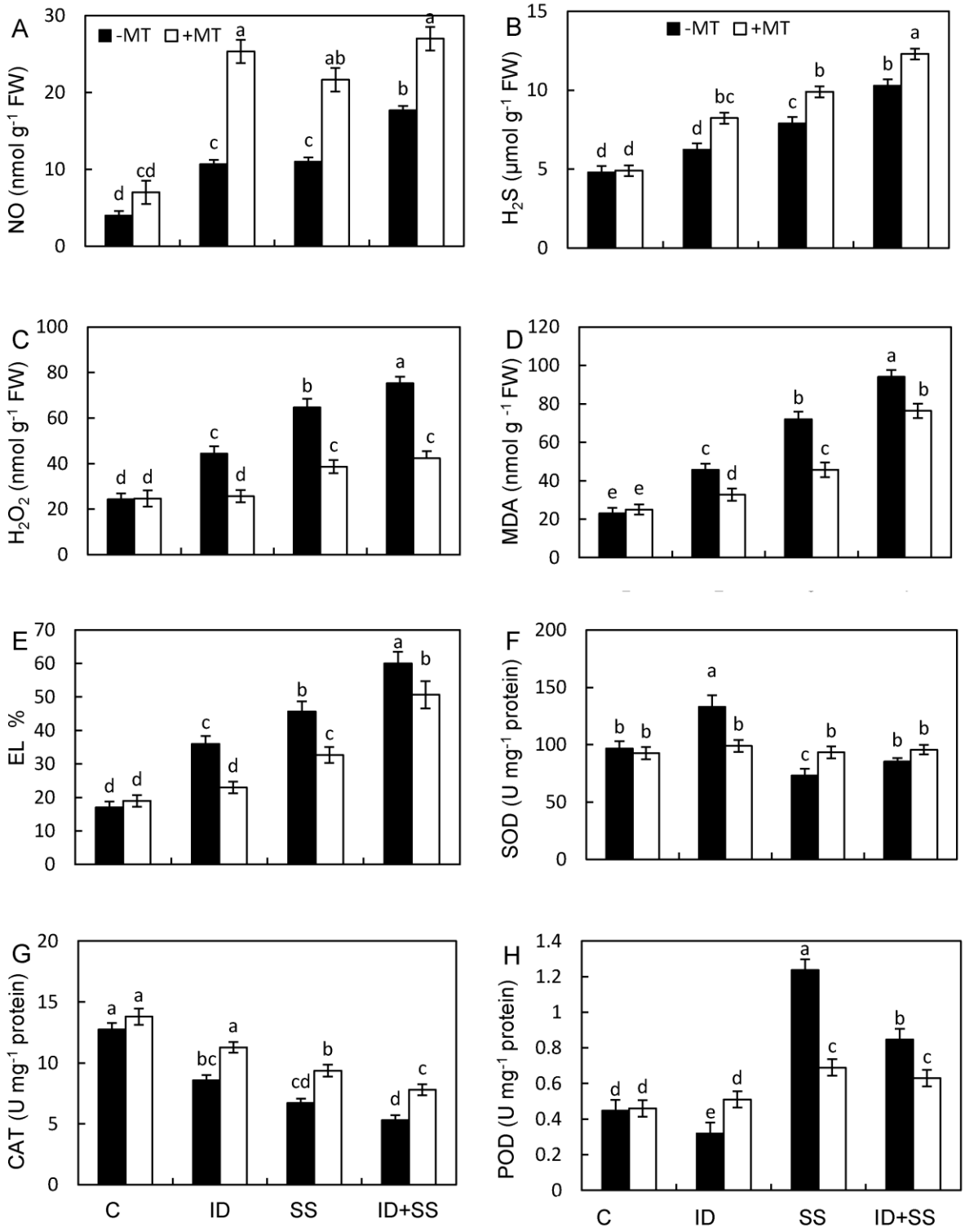

Fig. 4. Total (A), shoot (B), and root (C) dry matter (DM), chlorophyll a (D), chlorophyll b (E) on fresh weight (FW) basis, and chlorophyll fluorescence parameters [*Fv*/*Fm* (F)] of pepper plants grown under ID, SS or S+ID with 100 µM melatonin (MT) combined with 0.1 mM cPTIO and hypotuarine (HT) scavenger of NO and H_2S, respectively. (Mean ± S.E). Mean values carrying different letters within each parameter differ significantly ($P \leq 0.05$) based on the Duncan's multiple range test. C: Control treatment – Hoagland's nutrient solution containing 0.1 mM EDTA-Fe with no addition of NaCl; ID: Iron deficiency treatment – Hoagland's nutrient solution containing 0.1 mM $FeSO_4$; SS: 100 mM NaCl; NS: not sprayed.

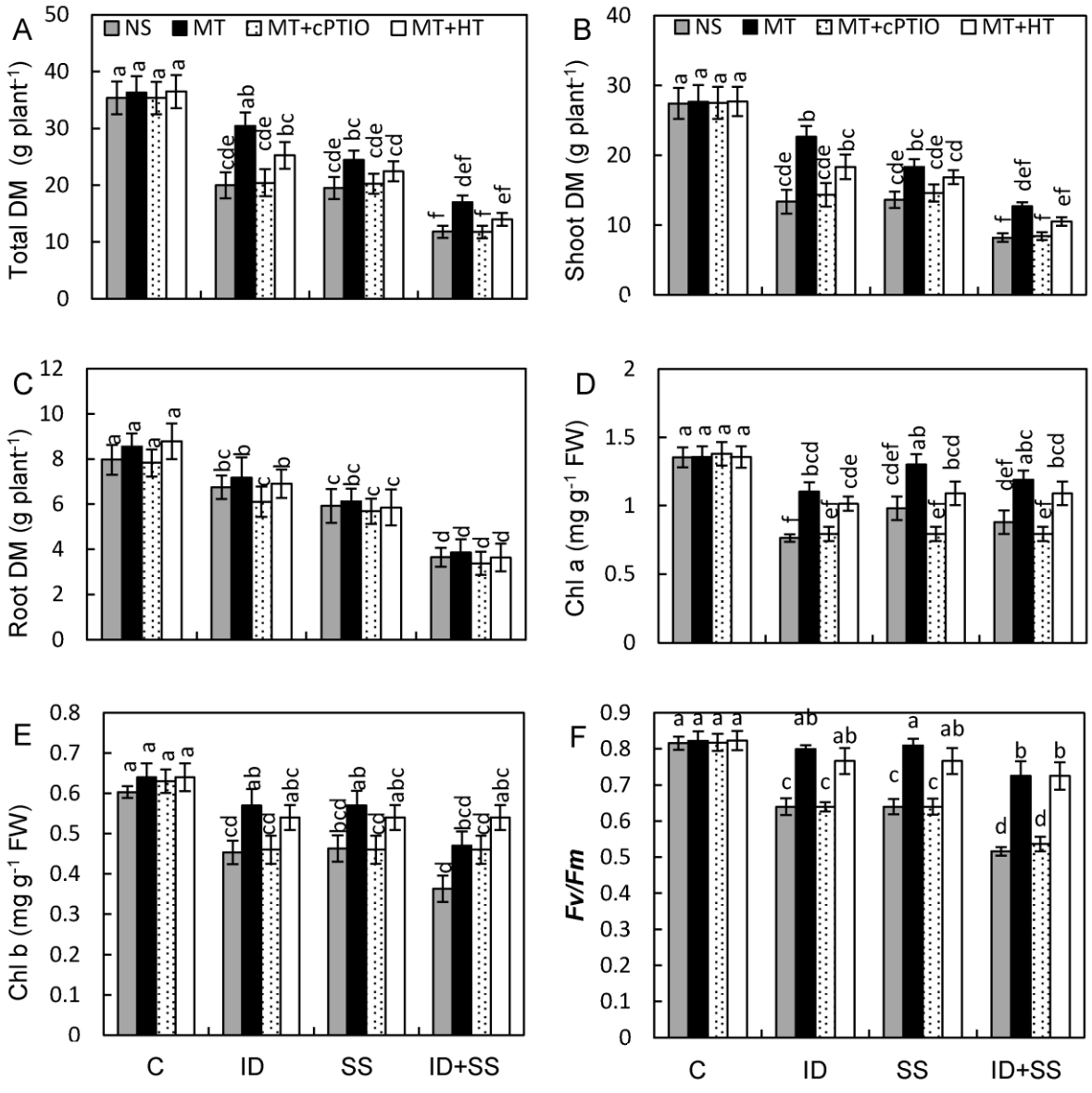

Fig. 5. Total Fe (A) and active Fe (B) contents on dry weight (DW) basis in leaves, ferric reductase activity in the roots (C), leaf Na^+ (D), leaf Cl^- (E) and leaf K^+ (F) on dry weight (DW) basis of pepper plants grown under ID, SS or SS+ID with 100 µM melatonin (MT) combined with 0.1 mM cPTIO and hypotuarine (HT) scavenger of NO and H_2S, respectively. (Mean ± S.E). Mean values carrying different letters within each parameter differ significantly ($P \leq 0.05$) based on the Duncan's multiple range test. C: Control treatment – Hoagland's nutrient solution containing 0.1 mM EDTA-Fe with no addition of NaCl; ID: Iron deficiency treatment – Hoagland's nutrient solution containing 0.1 mM $FeSO_4$; SS: 100 mM NaCl; NS: not sprayed.

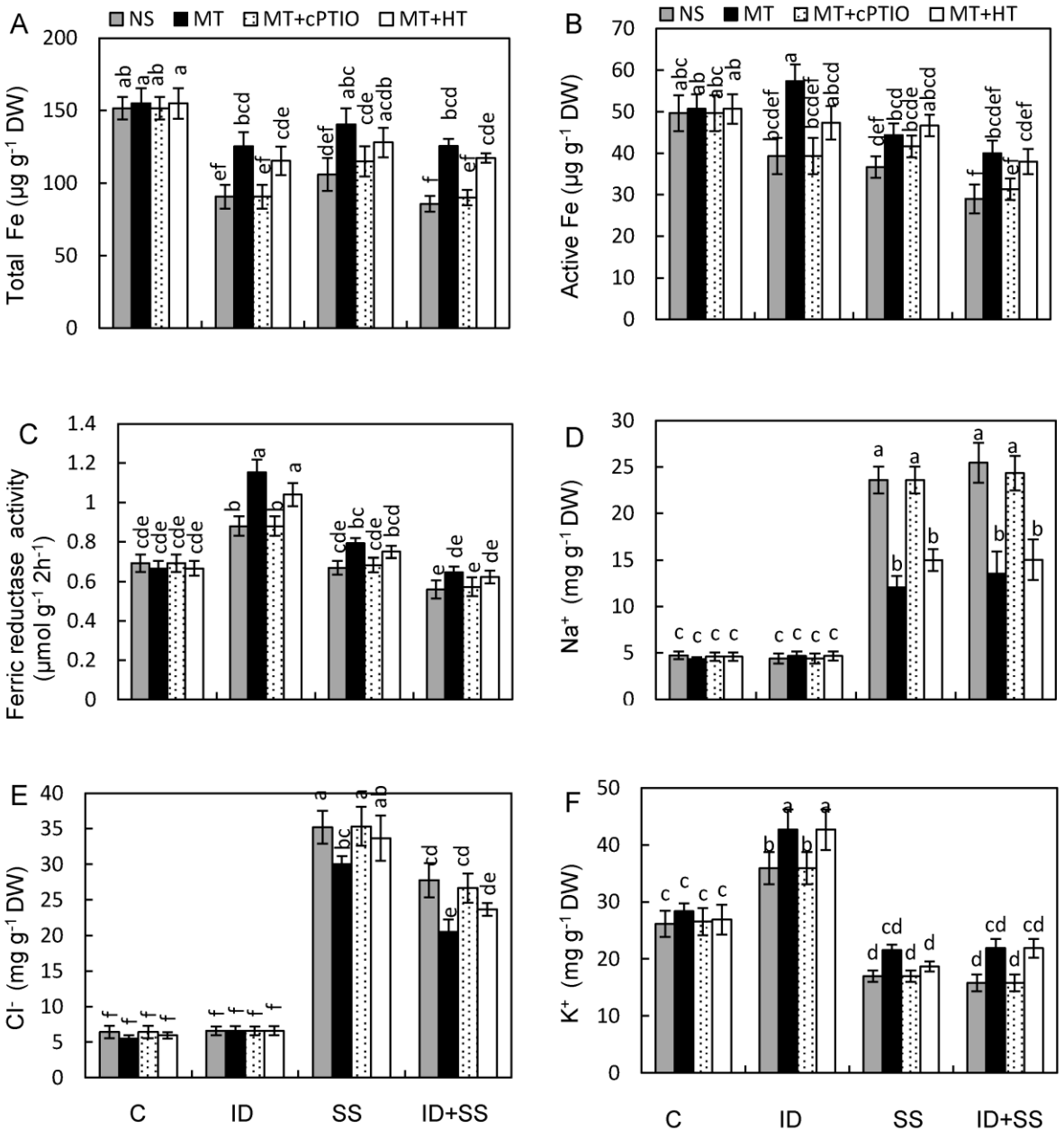

Fig. 6. Nitric oxide [NO (A)], hydrogen sulfide [H_2S (B)], hydrogen peroxide [H_2O_2 (C)], malondialdehyde [MDA (D)] on fresh weight (FW) basis, electrolyte leakage [EL (E)], activities of superoxide dismutase [SOD (F)], catalase [CAT (G)] and peroxidase [POD (H)] in the leaves of pepper plants grown under ID, SS or SS+ID with 100 µM melatonin (MT) combined with 0.1 mM cPTIO and hypotuarine (HT) scavenger of NO and H_2S, respectively. (Mean ± SE). Mean values carrying different letters within each parameter differ significantly ($P \leq 0.05$) based on the Duncan's multiple range test. C: Control treatment – Hoagland's nutrient solution containing 0.1 mM EDTA-Fe with no addition of NaCl; ID: Iron deficiency treatment – Hoagland's nutrient solution containing 0.1 mM $FeSO_4$; SS: 100 mM NaCl; NS: not sprayed.

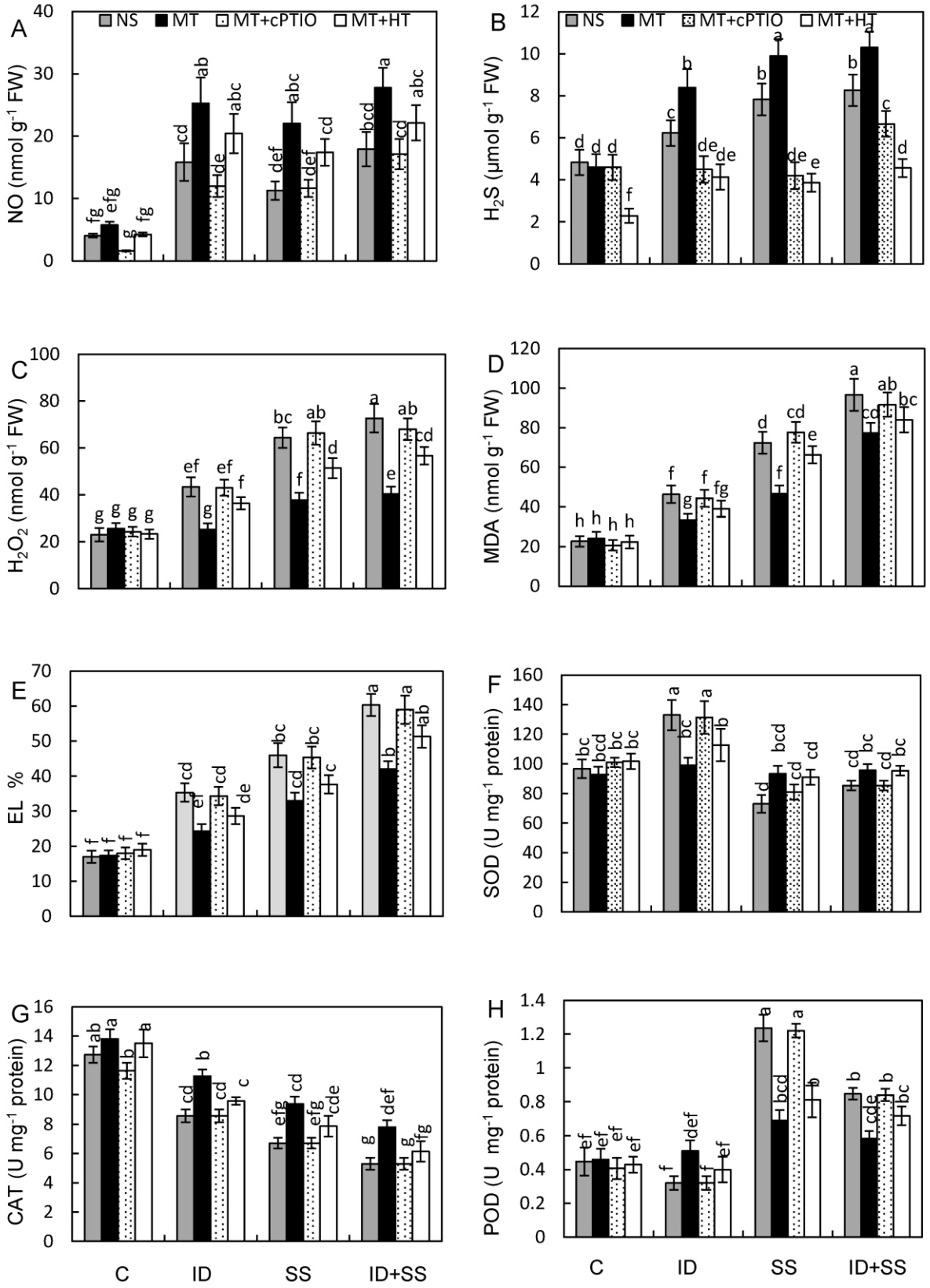

Fig. 7. Phenotypic effects of melatonin (MT), cPTIO and hypotuarine (HT) scavenger of NO and H_2S on pepper plants exposed to iron deficiency (ID) and salinity (S) stress applied individually or in combinations with respective controls. (A), Control treatment, (B) iron deficiency (ID), (C) ID+MT, (D) S stress, (E) S+MT, (F) ID +S, (G) S+ ID +MT, (H) ID+MT+cPTIO, (I) ID+MT+HT (Pictures were taken two weeks after starting the exogenous treatments).

Fig. 8. Correlation of nitric oxide (NO) on fresh weight (FW) basis with hydrogen peroxide [H_2O_2; (A)] superoxide dismutase [SOD; (B)], catalase [CAT; (C)] and peroxidase [POD; (D)] enzyme activities in the leaves of pepper plants grown under ID, SS or SS+ID with 100 µM melatonin (MT) combined with 0.1 mM cPTIO and hypotuarine (HT) scavenger of NO and H_2S, respectively. * and **: correlations are significant at $P \leq 0.05$ and $P \leq 0.001$, respectively.

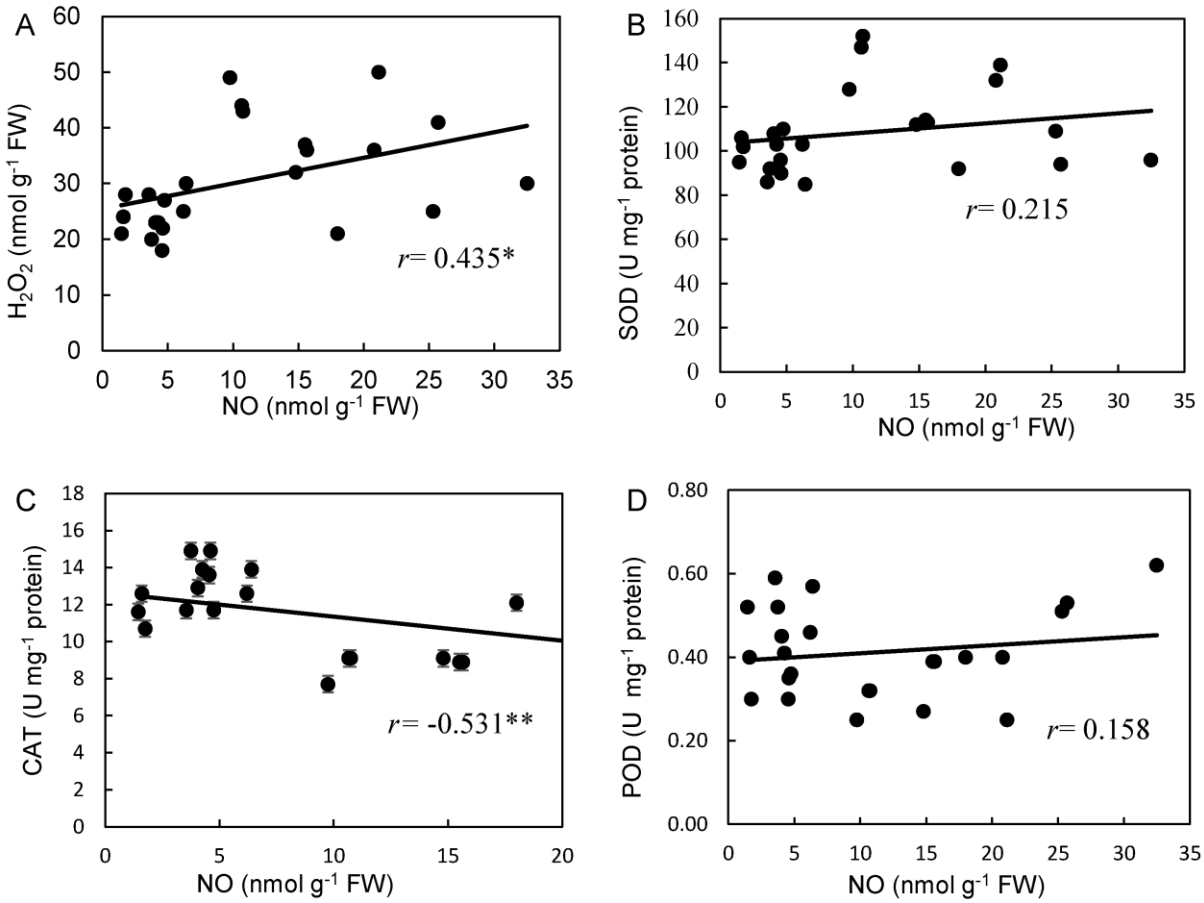

Fig. 9. Correlation of hydrogen sulfide (H_2S) on fresh weight (FW) basis with hydrogen peroxide [H_2O_2; (A)], superoxide dismutase [SOD; (B)], catalase [CAT; (C)], and peroxidase [POD; (D)] enzyme activities in the leaves of pepper plants grown under ID, SS or SS+ID with 100 µM melatonin (MT) combined with 0.1 mM cPTIO and hypotuarine (HT) scavenger of NO and H_2S, respectively. * and **: correlations are significant at $P \leq 0.05$ and $P \leq 0.001$, respectively.