

Insights into the role of cytokinin and gibberellic acid in improving waterlogging tolerance of mung bean

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Abstract

Mung bean (*Vigna radiata*) is one of the most important pulse crops, well-known for its protein rich seeds, which growth and productivity are severely undermined by waterlogging. In this study, we aim to evaluate how two promising phytohormones, namely cytokinin (CK) and gibberellic acid (GA₃), can improve waterlogging tolerance in mung bean by investigating key morphological, physiological, biochemical and yield-related attributes. Our results showed that foliar application of CK and GA₃ under 5-days of waterlogged conditions improved mung bean growth and biomass, which was associated to increased levels of photosynthetic rate and pigments. Waterlogged-induced accumulation of reactive oxygen species, and the consequent elevated levels of malondialdehyde, were considerably reduced by CK and GA₃ treatments. Mung bean plants sprayed with either CK or GA₃ suffered less oxidative stress due to the enhancement of total phenolics and flavonoids levels. Improvement in the contents of proline and total soluble sugars indicating a better osmotic adjustment following CK and GA₃ treatments in waterlogged-exposed plants. Most fundamentally, CK or GA₃-sprayed waterlogged-stressed mung bean plants demonstrated an increased tendency of the above-mentioned parameters after the 15-day recovery period as compared to water-sprayed waterlogged-exposed plants. Our results also revealed that CK and GA₃ treatments increased yield-associated features in waterlogged-stressed plant. Importantly, both phytohormones are efficient in improving mung bean resistance to waterlogging; however, CK was found to be more effective. Overall, our findings suggested that CK or GA₃ could be used for the management of waterlogging-induced damage in mung bean, and perhaps in other cash crops.

1. Introduction

Waterlogging has become a major environmental problem in global agriculture, arises from heavy rainfall, impromptu irrigation, inadequate drainage systems, unlevelled fields, and soil compaction (Kaur et al., 2020). Under waterlogged conditions, the mass of oxygen in the soil attenuates partially (hypoxic) or fully (anoxic) due to high microbial activity, and the resulting carbon dioxide accumulation in the root zone restricts root metabolism, aerobic respiration, ATP synthesis, and nutrient acquisition, resulting in a significant reduction in the growth, development and biomass of roots and shoots (Najeeb et al., 2015; Gill et al., 2018; Dossa et al., 2019). Moreover, waterlogging-induced excessive oxygen scarcity in water impairs root permeability and causes root injury, leading to a decrement of hydraulic conductivity and consequent stomatal closure, resulting in a significant reduction in net photosynthetic and transpiration rates (Else et al., 2001; Anee et al., 2019). While photosynthesis is disrupted in waterlogged plants, lethal levels of reactive oxygen species (ROS) such as superoxide (O₂^{•-}), singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂) and hydroxyl radical (•OH) are produced, which are harmful to cellular components and cause DNA and RNA damage, lipid peroxidation, protein oxidation, and enzyme inactivation (Yeung et al., 2018; Zhou et al., 2020).

Plants can acclimatise the deleterious consequence of waterlogging-mediated soil oxygen dearth through adapting various morphological, physiological and biochemical mechanisms. Increased adventitious root

development and faster stem elongation are some examples of morphological adaptations (Yamauchi et al., 2018), whereas reduced stomatal conductance and subsequent decrease in net photosynthetic rate are some short-term physiological adaptation process (Jacobsen et al., 2007; Bhusal et al., 2020). Additionally, to shield themselves from stress, waterlogged plants accumulate a number of osmoprotectants such as proline (Pro), soluble sugars, and sucrose (Tewari and Mishra, 2018). However, the most important adaptive mechanism is a well-balanced antioxidant defense system that involves both enzymatic (e.g., superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione S-transferase, GST; glutathione peroxidase, GPX) and non-enzymatic (e.g., total phenolics, total flavonoids, carotenoids) antioxidants that scavenge overaccumulation of ROS (Doupis et al., 2017; Fukao et al., 2019; Garcia et al., 2020).

Mung bean (*Vigna radiata*) is the third most important pulse crop after grass pea (*Lathyrus sativus*) and lentil (*Lens culinaris*) and considered as a major source of pulse protein because of its high protein content in the seeds (20.97–31.32%) (AESA, 2017; Yi-Shen et al., 2018; Rahman et al., 2019). Additionally, mung bean has been well-documented for its detoxification bioactivities along with its use as a treating agent for various health conditions, including stimulation of human mental function, allaying of heat stroke and regulation of gastrointestinal upset (Yi-Shen et al., 2018; Hou et al., 2019). More fundamentally, mung bean gaining its popularity in recent years and extensively cultivated throughout Bangladesh as a cash crop in the extensive rice-based cropping system, owing to its low fertilizer and pesticide requirements, which provides farmers with good economic benefits and nutritional security (Rahman et al., 2019). According to the estimation in the years 2016-17, cultivation of mung bean covered approximately 0.3188 million hectares (Mha) out of 0.9976 Mha of total pulse crop areas and yielded 0.21 million metric tonnes of mung bean (DAE. Krishi Dairy, 2018). However, recently the emerging waterlogging problem in major mung bean growing areas like Barishal, Patuakhali, Mymensingh, Sylhet, Chittagong hill tract, Natore, Khulna, Brahmanbaria and Pabna region make the land untenable for growing mung bean (<http://www.ffwc.gov.bd/index.php/map/inundation-map/bangladesh-today>). Therefore, the country must devise a strategy for effectively alleviating the effects of waterlogging stress in order to improve mung bean production in flash flood-prone areas. In this context, the search of a simple and cost-effective technology propelled us to use phytohormone such as cytokinin (CK) and gibberellic acid (GA₃) for overcoming the deleterious effects of waterlogging on the growth and yield-related attributes of mung bean.

In the present study, we first examined whether CK and GA₃ provides protection to mung bean from waterlogging stress, and to our expectations it really did. Next, we also examined the regulatory roles of CK and GA₃ in improving mung bean tolerance to waterlogging by investigating the morpho-physiological and biochemical mechanisms through assessments of the following key attributes: (i) plant growth performance and biomass production, (ii) gas-exchange features, (iii) photosynthetic pigment status, (iv) excessive waterlogging-mediated oxidative damage in terms of elevated ROS levels and lipid peroxidation, (v) different types of osmolyte accumulations, and (vi) improvement of non-enzymatic

antioxidant defense. Additionally, we also record the yield-associated features of waterlogged-stressed and waterlogged-devoid mung bean plants.

2. Materials And Methods

2.1. Plant growth conditions and treatments imposition

Mung bean (*Vigna radiata*) genotype, VC6173-B, was used in the present study owing to their properties of short duration (80 days to maturity), greater grain weight (44g/1000 grain) and high yield potential (1.5 tons ha⁻¹). Healthy seeds of VC6173-B were germinated following the procedures of Rahman et al. (2019). Afterward, six germinated seeds were sown in each plastic pot (height × diameter = 20 cm × 16 cm) having 8 kg of soil per pot. The soil used was prepared by intermixing of cow dung, sand and soil at the ratio of 1:1:3 (in a weight basis). Additionally, the soil of the pots was also fertilized with urea, triple super phosphate (TSP) and muriate of potash (MP) at the rate of 0.176, 0.400 and 0.347 g corresponding to 40-60-40 Kg N, P₂O₅ and K₂O per hectare (Islam et al., 2008). It is worth noting that one-third of urea and the whole amount of TSP, MP and gypsum were mixed with the soil as basal dose during final pot preparation. The remaining amount of urea was applied at 10 and 25 days after emergence. In order to protect the seedlings from insect-induced damage, Ripcord 10 EC (BASE Bangladesh Limited) at 1 mL L⁻¹ water was sprayed at vegetative stage of plant growth. The average maximum and minimum temperature of the experimental area were lies in the range of 32.6 to 22.8°C, with 85.6% of relative humidity. Notably, the number of seedlings was thinned to two in each pot after eighth day of germination and continues to grow in normal condition up to the imposition of treatments.

Prior to waterlogging exposure for the next five days, fifteen-day-old mung bean seedlings at vegetative V1 stage (when first trifoliolate were fully developed) were grouped into four sets as follows:

1. water-sprayed waterlogged-stress-free plants (Control)
2. water-sprayed waterlogged-stressed plants (WL)
3. cytokinin (CK)-sprayed (50 mg L⁻¹) waterlogged-stressed plants (CK + WL)
4. gibberellic acid (GA₃)-sprayed (50 mg L⁻¹) waterlogged-stressed plants (GA₃ + WL)

It is worth noting that foliar spray with either CK or GA₃ or water (20 mL to each pot) was done for two times in a day (9.00 AM to 10.00 AM, and 3.00 PM to 4.00 PM). Tween-20 surfactant (0.2%, v/v) was used to ensure maximum adherence of CK, GA₃ and water to the leaves. Notably, the level of water for creating waterlogging stress was maintained at 2.5 cm above the soil surface. Following the stress period, one set of seedlings (Set I) were immediately harvested, and another set (Set II) was harvested 15-days after recovery, to record the morphological, physiological and biochemical parameters. Importantly, after stress exposure, one set of seedlings (Set III) was allowed to grow until harvest with normal irrigation to determine yield-related attributes, including pod length, total number of pods per plant, thousand seed weight, and seed yield per plant. Each set of experiments were repeated four times under

the same experimental conditions. Importantly, for determining various morphological, physiological and biochemical parameters, the first trifoliolate leaves from the bottom of the plants were collected.

2.2. Determination of growth-related parameters

The growth performance of mung bean plants was assessed by measuring the shoot height, and fresh weight (FW) of both shoots and roots. Additionally, stem girth of the mung bean plants was determined using a slide caliper.

2.3. Measurement of gas exchange features

Gas exchange features, including photosynthesis rate (Pn), stomatal conductance to water (H_2O) (g_s) and transpiration rate (E) of mung bean plants were measured using a LI-6400XT portable photosynthesis system (LI-COR Biosciences, Lincoln, Nebraska, USA) from 11.00 AM to 2.00 PM under a full sun-light condition. The parameters Pn , g_s and E were used for assessing the instantaneous water-use efficiency (WUE_{ins} ; ratio Pn/E) and intrinsic water-use efficiency (WUE_{int} ; ratio Pn/g_s).

2.4. Quantification of photosynthetic pigments, and total phenolic and flavonoid contents in mung bean leaves

The freshly harvested mung bean leaves were used to determine the contents of chlorophylls (Chls) [Chl a , Chl b and Chl ($a + b$)] by using the visible spectrophotometer (Model: T60 UV, PG Instruments Limited, Leicestershire, UK) according to the protocols described by Lichtenthaler and Wellburn (1983). The contents of total phenolic and total flavonoid were quantified following the methods of Ainsworth and Gillespie (2007), and Zhishen et al. (1999), respectively.

2.5. Quantification of H_2O_2 , MDA, proline and total soluble sugars

Freshly harvested leaf tissues were used for the quantification of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) (a lipid peroxidation product), proline (Pro) and total soluble sugars levels following the comprehensive protocols described by Yu et al. (2003), Heath and Packer (1968), Bates et al. (1973) and Somogyi (1952), respectively.

2.6. Statistical analysis

The obtained data were subjected to a one-way analysis of variance (ANOVA) using Statistix software (version 10). Different alphabetical letters were used to denote the significant variations among different treatments at the $P < 0.05$ level following a least significant difference (LSD) test. All the numerical data in figures and tables are presented as means \pm standard errors (SEs) of four independent replications.

3. Results

3.1. CK and GA₃ supplementation improved morphological features of mung bean plants under waterlogging stress and recovery period

In comparison with 'Control' plants, 'WL' plants displayed significant decrease in shoot height, shoot FW, root FW and stem girth width by 33.46, 37.04, 41.50 and 37.07%, respectively; however, following 15-days of recovery, these parameters were reduced by 35.31, 44.05, 35.88 and 32.18%, respectively (Table 1). On the other hand, 'CK+WL' and 'GA₃+WL' plants showed noteworthy improvement in shoot height by 35.46 and 25.54%, shoot FW by 37.93 and 26.87%, root FW by 36.05 and 25.58%, and stem girth width by 36.06 and 30.61%, respectively, when compared with 'WL' plants (Table 1). Following the recovery period, a substantial enhancement in shoot height (34.70 and 23.90%), shoot FW (37.93 and 26.87%), root FW (36.05 and 25.58%) and stem girth width (36.06 and 30.61%, respectively) was observed in the 'CK+WL' and 'GA₃+WL' plants, in comparison with 'WL' plants (Table 1). It was also observed that 'CK+WL' and 'GA₃+WL' plants exhibited notable decrease in shoot height by 9.86 and 16.46%, shoot FW by 13.17 and 20.13%, root FW by 20.41 and 26.53% and stem girth width by 14.40 and 17.83%, respectively, relative to that of 'Control' plants (Table 1). Similarly, in contrast to 'Control' plants, significant reductions in shoot height (by 12.86 and 19.85%), shoot FW (24.21 and 29.94%), root FW (20.58% for GA₃+WL) and stem girth width (8.99 and 15.86%, respectively) were observed following recovery period in the 'CK+WL' and 'GA₃+WL' plants (Table 1).

3.2. CK and GA₃ supplementation improved gas exchange features of mung bean plants under waterlogging stress and recovery period

Mung bean plants subjected to waterlogging stress and followed by stress recovery period showed a decrease in P_n (by 44.12 and 41.97%), g_s (90.28 and 53.25%) and E (73.92 and 35.56%, respectively) when compared with 'Control' plants (Fig. 1A-C). Nonetheless, the levels of WUE_{int} and WUE_{ins} in 'WL' plants increased by 514.96 and 117.90%, respectively relative to 'Control' plants, whereas the level of WUE_{int} and WUE_{ins} in 'Control' and 'WL' plants was comparable after recovery period (Fig. 1D,E). On the other hand, 'CK+WL' and 'GA₃+WL' plants exhibited decreased levels of g_s (by 40.03 and 27.36%) and E (32.87 and 22.55%), and increased levels of P_n (by 36.80 and 26.65%), WUE_{int} (119.44 and 63.82%) and WUE_{ins} (100.06 and 61.36%, respectively) when compared with that of 'WL' plants (Fig. 1A-E). Similarly, following the recovery period, decreased levels of g_s (by 35.60 and 27.61%) and E (34.14 and 21.37%), and increased levels of P_n (36.80 and 26.65%), WUE_{int} (114.55 and 72.68%) and WUE_{ins} (107.42 and 59.09%, respectively) were recorded in 'CK+WL' and 'GA₃+WL' plants, as compared with that of 'WL' plants (Fig. 1A-E). In relation to the 'Control', a sharp reduction in the levels of P_n (by 23.56 and 29.23%), g_s (94.17 and 92.94%) and E (82.49 and 79.80%) was noticed in 'CK+WL' and 'GA₃+WL' plants, respectively. Similarly, the levels of P_n , g_s and E were noticeably reduced by 20.85, 69.89 and 57.56% in 'CK+WL', and by 27.28, 66.15 and 49.33% in 'GA₃+WL' plants followed by the recovery period over the corresponding

values of 'Control' plants (Fig. 1A-C). Additionally, in relation to the 'Control', the level of WUE_{int} and WUE_{ins} was substantially enhanced by 1249.50 and 335.94% in 'CK+WL' and by 907.44 and 251.60% in 'GA₃+WL' plants followed by exposure to waterlogging stress. Whereas after recovery period, augmented level of WUE_{int} (by 165.23 and 113.47%) and WUE_{ins} (87.07 and 43.47%) was observed in 'CK+WL' and 'GA₃+WL' plants, respectively (Fig. 1D,E).

3.3. CK and GA₃ supplementation improved photosynthetic pigments status of mung bean plants under waterlogging stress and recovery period

In relation to 'Control' plants, a sharp decline in the contents of Chl *a* (by 50.10 and 43.29%), Chl *b* (65.44 and 62.99%) and Chl (*a+b*) (54.35 and 48.96%) were observed in the leaves of 'WL' plants following stress and recovery periods, respectively (Fig. 2A-C). In contrast, CK and GA₃ supplementation protected the photosynthetic pigments from waterlogged-induced deleterious effects by enhancing the contents of Chl *a* (by 74.01 and 60.52%), Chl *b* (142.38 and 124.40%) and Chl (*a+b*) (88.37 and 73.94%, respectively) in the leaves of 'CK+WL' and 'GA₃+WL' plants, respectively (Fig. 2A-C). Furthermore, following the recovery period, the leaves of 'CK+WL' and 'GA₃+WL' plants also displayed a significant rise in the content of Chl *a* (by 59.75 and 49.54%), Chl *b* (134.31 and 120.36%) and Chl (*a+b*) (75.32 and 64.33%, respectively) in comparison with 'WL' plants (Fig. 2A-C). On the other hand, CK and GA₃ supplementation resulted in a decrement of Chl *a* (by 13.16 and 19.90%), Chl *b* (16.23 and 22.44%) and Chl (*a+b*) (14.01 and 20.60%) in the leaves of 'CK+WL' and 'GA₃+WL' plants, respectively, when compared with 'Control' plants (Fig. 2A-C). Likewise, following the recovery period, a noteworthy decrement in the contents of Chl *a* (by 9.40 and 15.19 %), Chl *b* (13.28 and 18.44%) and Chl (*a+b*) (10.51 and 16.13%) were observed in the leaves of 'CK+WL' and 'GA₃+WL' plants, respectively, in comparison with 'Control' plants (Fig. 2A-C).

3.4. CK and GA₃ supplementation suppressed the levels of oxidative stress markers of mung bean plants under waterlogging stress and recovery period

Imposition of waterlogging stress and following recovery period substantially increased the contents of H₂O₂ (by 113.40 and 62.08%) and MDA (178.67 and 100.18%, respectively) in the leaves of 'WL' plants, relative to that of 'Control' plants (Fig. 3A,B). Fascinatingly, CK and GA₃ treatment resulted in reductions of H₂O₂ (by 57.31 and 45.40%) and MDA (29.01 and 19.04%, respectively) contents in the leaves of 'CK+WL' and 'GA₃+WL' plants, in comparison with 'WL' plants (Fig. 3A,B). Similarly, compared with the 'WL' plants, substantial decreases in the contents of H₂O₂ (by 36.61 and 26.44 %) and MDA (33.79 and

20.70%, respectively) were noticed in the leaves of 'CK+WL' and 'GA₃+WL' plants followed by the recovery period (Fig. 3A,B). On the other hand, 'CK+WL' and 'GA₃+WL' plants exhibited noteworthy improvements in the levels of H₂O₂ (by 38.52 and 59.69%) and MDA (71.34 and 102.46%, respectively), when contrasted with that of 'Control' plants (Fig. 3A,B). After the recovery period, the contents of H₂O₂ (by 2.75 and 19.22%) and MDA (32.53 and 58.74%, respectively) in the leaves of 'CK+WL' and 'GA₃+WL' plants were substantially higher compared with the respective value of 'Control' plants (Fig. 3A,B).

3.5. CK and GA₃ supplementation improved the levels of Proline (Pro), total soluble sugars, total phenolics and flavonoids of mung bean plants under waterlogging stress and recovery period

Exposure of mung bean plants to waterlogging stress and followed by a recovery period exhibited a rise in the contents of Pro (63.01 and 42.17%), total soluble sugars (33.20 and 20.29%), total phenolics (57.65 and 23.16%) and total flavonoids (30.98 and 15.89%, respectively) in the leaves of 'WL' plants, when compared with that of 'Control' plants (Fig. 3C-F). In comparison with 'WL' plants, 'CK+WL' and 'GA₃+WL' plants displayed enhanced levels of Pro (by 33.75 and 21.01%), total soluble sugars (32.24 and 21.79%), total phenolics (36.48 and 24.53%) and total flavonoids (37.34 and 28.19%, respectively) (Fig. 3C-F). Similarly, in relation to the 'WL' plants, the levels of Pro, total soluble sugars, total phenolics and flavonoids were noticeably enhanced in 'CK+WL' leaves by 34.61, 35.02, 34.59 and 35.04%, respectively, and in 'GA₃+WL' leaves by 23.45, 27.21, 23.45 and 25.08%, respectively, followed by a recovery period (Fig. 3C-F). Significant improvement in the contents of Pro (by 118.02 and 97.25%), total soluble sugars (76.15 and 62.23%), total phenolics (115.16 and 96.32%) and total flavonoids (79.90 and 67.91%, respectively) were recorded in the leaves of 'CK+WL' and 'GA₃+WL' plants, when contrasted with the corresponding 'Control' values (Fig. 3C-F). Likewise, in contrasted with the corresponding values obtained from the 'Control', the contents of Pro, total soluble sugars, total phenolics and total flavonoids increased by 91.38, 62.41, 65.76 and 56.51%, respectively, in 'CK+WL' plants, and by 75.50, 53.02, 52.04 and 44.96%, respectively, in 'GA₃+WL' plants, followed by the stress recovery period (Fig. 3C-F).

3.6. CK and GA₃ supplementation improved yield-related features of mung bean plants

Compared to the 'Control', 'WL' plants displayed notable decrements in pod length (by 13.18%), total number of pods per plant (36.36%), thousand seed weight (13.48%) and seed yield per plant (42.23%) (Table 2). Intriguingly, significant improvement in pod length (by 9.29 and 7.77%), total number of pods per plant (23.81 and 19.05%), and seed yield per plant (25.52 and 20.76%, respectively) were observed in 'CK+WL' and 'GA₃+WL' plants when compared with that of 'WL' plants (Table 2). On the other hand,

'CK+WL' and 'GA₃+WL' plants displayed noticeable reduction in total number of pods per plant (21.21 and 24.24%) and seed yield per plant (27.49 and 30.24%, respectively), in relation to the 'Control' plants (Table 2). Nonetheless, no distinct difference was observed in pod length between 'Control' and 'CK+WL' plants, while 6.43% reduction in pod length was recorded in 'GA₃+WL' plants versus 'Control' plants (Table 2).

4. Discussion

Waterlogging in agricultural field is generally triggered by climate change-induced intensive and/or extensive rainfall over a period of time, which has detrimental consequences on a number of crop plants, including mung bean (Donat et al., 2016; Amin et al., 2015). The current study investigated the effective mitigation measure of waterlogged-induced damage to mung bean plants by exploring the potential role of CK and GA₃.

In the present study, waterlogging-induced deleterious effects was evident as a reduction of shoot height, shoot and root FW, and stem girth width; however, intriguingly the foliar application of CK and GA₃ alleviated those waterlogged-mediated detrimental effects (Table 1). More fundamentally, the positive effects of CK and GA₃ also remain persistent after 15-days of recovery (Table 1). Our results also clearly indicated that CK was more effective than GA₃ in ameliorating waterlogged-induced growth retardation (Table 1). It is well reported that both CK and GA₃ played a pivotal role in protecting plants from a variety of stresses (Ahanger et al., 2018; Wang et al., 2019; Rady et al., 2021; Saleem et al., 2020); nonetheless, their underlying mechanisms in alleviation of waterlogged-induced damages are yet to be investigated. Thus, we used various physiological and biochemical assay to investigate on how CK and GA₃ could improve the growth response of mung bean plants in response to waterlogging stress.

The waterlogged-induced growth inhibition and biomass reduction in mung bean plants might be a consequence of impeded photosynthesis (Table 1; Fig. 1A). It is envisaged that at the beginning of waterlogging stress, plant roots rapidly transmit a xylem-borne signals to the leaves in the form of hormones, most notably abscisic acid (ABA) to slow down the process of transpiration through stomatal closure, and thus attenuating carbon dioxide availability in leaves (Jackson et al., 2003; Najeeb et al., 2015). Coupled with decreased transpiration rate because of enhanced stomatal closure, the waterlogged-mediated anaerobic respiration triggered a drop in root hydraulic conductivity and an upsurge in root cell death, resulting in inadequate ATP for active delivery of water and nutrients to the shoots, thereby diminishing net photosynthetic rate (Steffens et al., 2005; Kaur et al., 2019). Henceforth, the supply of photoassimilates from leaves to different plant parts is also reduced, which ultimately leading to poor plant growth and biomass production (Kogawara et al., 2006). Apart from these, waterlogged-induced demolition of photosynthetic pigments, including Chl *a* and *b*, also responsible for reduced net photosynthetic rate (Ren et al., 2016; Zhang et al., 2020), as also observed in the present study (Fig. 1A and 3A-C). On the other hand, mung bean plants treated with CK and GA₃ significantly improved the net photosynthetic, as well as the contents of Chl *a* and Chl *b* under waterlogged conditions, implying that CK and GA₃ played a pivotal role in improving photosynthetic process during waterlogging

stress (Fig. 1A,C and 3A-C). Improvements of photosynthesis as well as photosynthetic pigments were also observed after recovery period in response to CK and GA₃ supplementation (Fig. 1A,C and 3A-C). Importantly, our results clearly showed that CK supplementation plausibly played more decisive role than GA₃ supplementation in repairing the growth and photosynthetic performance of mung bean under waterlogging stress. Similar to our findings, protection of photosynthetic pigments and consequent improvement in plant growth performance were also reported in drought-stressed pomegranate (*Punica granatum*) and salt-stressed tomato (*Solanum lycopersicum*) plant in response to CK application, while GA₃ provided positive results in salt-stressed tomato, drought-stressed faba bean (*Vicia faba*) and copper-exposed jute (*Corchorus capsularis*) plants (Bompadre et al., 2015; Ahanger et al., 2018; Siddiqui et al., 2020; Saleem et al., 2020; Rady et al., 2021).

We also observed that, in comparison with 'WL' plants, CK or GA₃-treated plants demonstrated remarkably improved WUE (Fig. 2D,E), indicating that CK and GA₃ might enabled mung bean plants to produce more biomass under the conditions of limited water uptake. Surprisingly, after recovery period, we have seen similar positive effects of CK and GA₃ in boosting WUE (Fig. 2D,E). Importantly, our findings revealed that the spraying of mung bean plants with CK was more efficient than GA₃ in preventing waterlogged-induced deterioration of water use efficiency (Fig. 2D,E). It is well-known that improving WUE in abiotic-stressed plants without deteriorating growth and yield is scrutinized as a paramount goal of current plant breeding programs (Yang et al., 2019). Our findings support this phenomenon and corroborate with the previous findings where external application of CK to cold-stressed coffee (*Coffea arabica*) and GA₃ to drought-stressed faba bean seedlings improved WUE (Acidri et al., 2020; Rady et al., 2021).

Water stagnation because of waterlogging conditions causes poor gas exchange between soils and plant roots, resulting in hypoxia or anoxia in plant tissues (Matin and Jalali, 2017). The scarcity of oxygen induces leakage of electrons from mitochondria, which together with impaired photosynthesis, triggers a burst of excessive ROS production, causing oxidative damage to waterlogged-stressed seedlings (Xu et al., 2013), and was clearly manifested in our study with increasing H₂O₂ and MDA in 'WL' plants (Fig. 3A,B). Intriguingly, external application of either CK or GA₃ to waterlogged-stressed mung bean plants resulted in a dramatic reduction in ROS accumulation (Fig. 3A,B), implying that CK and GA₃ plays an important role in reducing oxidative burden induced by ROS and providing protection to plasma membrane integrity. In line with our study, previous reports also demonstrated that the contents of H₂O₂ and MDA were noticeably diminished in salt-stressed tomato and okra (*Abelmoschus esculentus*) by CK application, and in drought-stressed wheat (*Triticum aestivum*) and boron-stressed tomato by GA₃ application (Ahanger et al., 2018; Wang et al., 2019; Moumita et al., 2019; Javed et al., 2021).

Our results also demonstrated that, compared with 'WL', the leaves of 'CK + WL' and 'GA₃ + WL' plants accumulated higher levels of non-enzymatic antioxidants, namely total phenolics and total flavonoids (Fig. 3E,F). More fundamentally, following the recovery period, there was a similar pattern of improvement in total phenolics and total flavonoids levels in response to CK or GA₃ application (Fig. 3A,B,E,F). Phenolics and flavonoids are two well-known secondary metabolites that play a pivotal role in

scavenging free radicals and preventing lipid peroxidation, thus maintaining membrane fluidity and shielding cell membrane damage from oxidative damage under different abiotic stresses, including waterlogging (Ahmad et al., 2010; Elkelish et al., 2020; Patel et al., 2020). These results implied that either CK or GA₃-induced alleviation of ROS burden was most likely due to the heightened level of non-enzymatic antioxidants (Fig. 3A,E,F). It is worth noting that the application of CK to mung bean plants displayed greater role in alleviating the contents of H₂O₂ and MDA by enhancing the levels of total phenolics and flavonoids when compared with the plants treated with GA₃ (Fig. 3A,B,E,F). Nonetheless, in consistent with our findings, Ahanger et al. (2018) reported that CK application to salt-stressed tomato plants increased the level of total phenolics and flavonoids, while Rady et al. (2021) revealed that GA₃ supplementation to drought-stressed faba bean plants increased phenolics accumulation.

To overcome waterlogged-induced osmotic stress, plants also accumulate a number of compatible solutes, including Pro and total soluble sugars, which play vital roles in maintaining water balance, retrieving photosynthetic functions, stabilizing cellular components, ROS scavenging, improving cellular signalling and secondary metabolite biosynthesis (Sami et al., 2016; Barickman et al., 2019; Chávez-Arias et al., 2019; Xiao et al., 2020). Our data on compatible solutes revealed that the levels of Pro and total soluble sugar were dramatically enhanced in 'WL' plants (Fig. 3C,D). Importantly, exogenous application of CK and GA₃ further enhanced the contents of Pro and total soluble sugars in the leaves of 'CK + WL' and 'GA₃ + WL' plants, and this trend was found surprisingly similar following the recovery period (Fig. 3C,D). The results of our study clearly indicated that CK-treated waterlogged-stressed mung bean plants were able to accumulate more Pro and soluble sugars than GA₃-treated plant (Fig. 3C,D). Our findings coincided with the results of Sarafraz-ardakani et al. (2014), who also observed that CK supplementation increased the amount of Pro and soluble sugars in drought-stressed wheat plants.

Apart from the physiological and biochemical assay, we also focused on the yield-related attributes of mung bean plants to confirm the beneficiary role of the CK and GA₃ application. Waterlogged-stressed mung bean plants showed a decrease in yield-associated features such as pod length, total number of pods per plant, thousand seed weight, and seed yield per plant (Table 2), which might be correlated with poor plant growth because of impaired photosynthesis and enhanced accumulation of ROS (Fig. 2A-C and 3A). In contrast, foliar application of either CK or GA₃ to waterlogged-exposed mung bean plants resulted in a dramatic increase in pod length, total number of pods per plant, thousand seed weight, and seed yield per plant (Table 2). These results suggested that CK or GA₃ supplementation improved photosynthetic efficiency of mung bean to sustain higher levels of total soluble sugars (Fig. 3F), as well as heightened the levels of Pro and non-enzymatic antioxidants to resist the phytotoxic effects of waterlogging through the reductions of ROS-mediated oxidative stress (Fig. 3A, C-E), resulting in improvement of yield performance (Table 2). Importantly, CK-treated waterlogged-stressed mung bean plants provided greater yield-associated features compared with GA₃-treated plants. Yield improvement in wheat plants under heat and drought stress was also reported upon supplementation of CK and GA₃, respectively (Gupta et al., 2012; Yang et al., 2016).

5. Conclusion

In summary, our results concluded that treating plants with either CK or GA₃ positively regulates defense responses in waterlogged-stressed mung bean plants through regulating several physiological and biochemical mechanisms. In comparison with water-sprayed stressed plants, CK and GA₃-sprayed plants demonstrated improvement in plant height, stem girth width and plant biomass, which might be interlinked with delayed of photosynthetic pigment destruction, improvement of photosynthetic rate and WUE. Under waterlogged conditions, application of CK and GA₃ successfully decreased the levels of ROS accumulation and consequent MDA level; implying that CK and GA₃ played a decisive role in controlling ROS-induced cellular damage. These observations were closely interlinked to heightened levels of non-enzymatic antioxidants, including total phenolics and total flavonoids. Elevated levels of proline and total soluble sugars in CK and GA₃-sprayed plants also contributed to the maintenance of better water status and osmotic adjustment under waterlogging. Intriguingly, the positive regulatory roles of CK and GA₃ in the improvement of above-mentioned parameters were also persist after 15-days of recovery period. All of these physiological and biochemical mechanisms in CK and GA₃-sprayed plants contribute to improve yield-associated features, including pod length, total number of pods per plant, thousand seed weight, and seed yield per plant, in relation of water-sprayed plants. Importantly, plants treated with CK performed better compared with GA₃-treated plants. Taken together, our findings suggests that either CK or GA₃ could be a potential chemical for mitigating waterlogged-induced detrimental effects on crop performance to ensure sustainable agriculture in flash flood-prone areas. However, together with field trials, comprehensive studies are recommended to decipher the underlying molecular mechanisms that are regulated by either CK or GA₃ under waterlogged conditions.

Declarations

Author Contributions

MRI: conceived, designed and supervised the experiment, wrote and edited the manuscript, and gave final approval; **MMR:** analyzed and interpreted the data, wrote and revised the manuscript; **MA and EJ:** performed the experiment, prepared the materials, collected and analyzed the data; **SSK:** helped in statistical analysis, interpretation of the data and results, and writing the manuscript; **MH:** monitoring the experiment and reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1. The effects of exogenous cytokinin and gibberellic acid on shoot height, shoot dry weight, root dry weight and stem girth width of mung bean plants after 5-days of stress and

15-days of recovery periods

Treatments	Shoot height (cm)		Shoot FW (g)		Root FW (g)		Stem girth width (mm)	
	After stress	After recovery	After stress	After recovery	After stress	After recovery	After stress	After recovery
Control	16.96 ± 0.65 ^a	33.21 ± 1.58 ^a	2.34 ± 0.09 ^a	9.68 ± 0.44 ^a	0.37 ± 0.02 ^a	1.90 ± 0.07 ^a	4.82 ± 0.31 ^a	7.04 ± 0.04 ^a
WL	11.29 ± 0.42 ^c	21.49 ± 0.90 ^d	1.47 ± 0.09 ^c	5.42 ± 0.17 ^c	0.22 ± 0.01 ^c	1.22 ± 0.10 ^c	3.03 ± 0.14 ^c	4.77 ± 0.27 ^c
CK+WL	15.29 ± 0.46 ^b	28.94 ± 0.58 ^b	2.03 ± 0.06 ^b	7.34 ± 0.26 ^b	0.29 ± 0.01 ^b	1.65 ± 0.06 ^{ab}	4.12 ± 0.08 ^b	6.41 ± 0.20 ^b
GA₃+WL	14.17 ± 0.33 ^b	26.62 ± 0.48 ^c	1.87 ± 0.03 ^b	6.78 ± 0.30 ^b	0.27 ± 0.01 ^b	1.51 ± 0.12 ^{bc}	3.96 ± 0.12 ^b	5.92 ± 0.18 ^b

Values are means ± standard errors ($n = 4$). Different alphabetical letters as superscripted within the same column indicate significant differences among various treatments according to a least significant difference test (LSD) at $P < 0.05$. WL, water-sprayed waterlogged-stressed plants; CK+WL, cytokinin (CK)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; GA₃+WL, gibberellic acid (GA)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; FW, fresh weight.

Table 2. The effects of exogenous cytokinin and gibberellic acid on yield-contributing parameters of stressed and stress-free mung bean plants

Treatments	Pod length (cm)	Total number of pods per plant	Thousand seed weight (g)	Seed yield (g per plant)
Control	8.46 ± 0.19 ^a	33.00 ± 1.29 ^a	51.10 ± 0.86 ^a	16.47 ± 0.59 ^a
WL	7.35 ± 0.19 ^c	21.00 ± 0.41 ^c	44.21 ± 1.07 ^b	9.51 ± 0.30 ^c
CK+WL	8.03 ± 0.10 ^{ab}	26.00 ± 1.47 ^b	47.12 ± 2.31 ^{ab}	11.94 ± 0.64 ^b
GA₃+WL	7.92 ± 0.09 ^b	25.00 ± 0.41 ^b	46.23 ± 1.88 ^{ab}	11.49 ± 0.33 ^b

Values are means ± standard errors ($n = 4$). Different alphabetical letters as superscripted within the same column indicate significant differences among various treatments according to a least significant difference test (LSD) ($P < 0.05$). WL, water-sprayed waterlogged-stressed plants; CK+WL, cytokinin (CK)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; GA₃+WL, gibberellic acid (GA)-sprayed (50 mg L⁻¹) waterlogged-stressed plants.

Figures

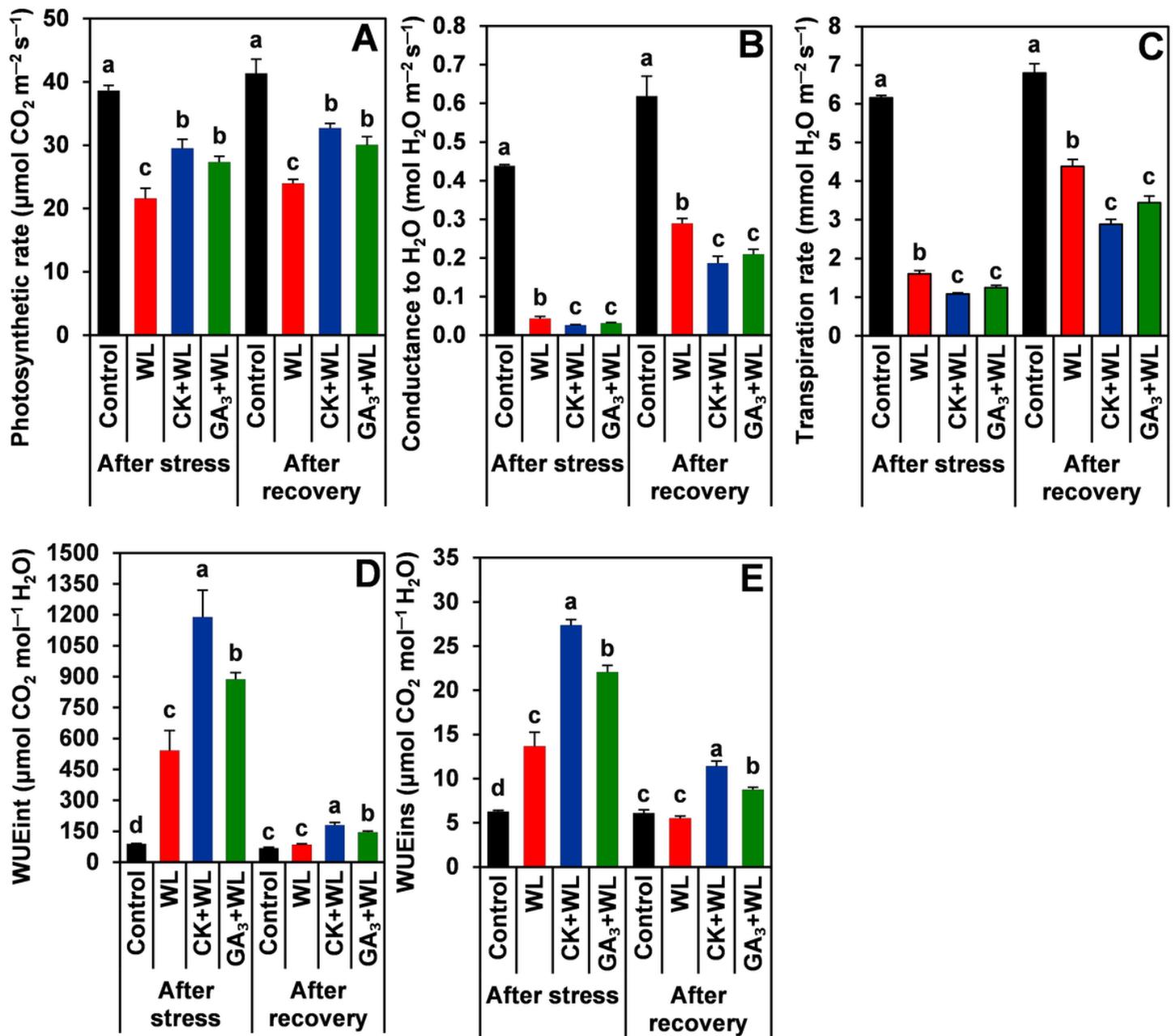


Figure 1

Effects of exogenous cytokinin and gibberellic acid on (A) photosynthetic rate (P_n), (B) stomatal conductance to H_2O (g_s), (C) transpiration rate (E) (D) intrinsic water-use efficiency (WUE_{int}) and (E) instantaneous water-use efficiency (WUE_{ins}) in the leaves of mung bean plants after 5-days of stress and 15-days of recovery period. Data represent means of four independent replicates ($n = 4$). Vertical bars indicate standard errors. Different letters represent significant differences at $P < 0.05$ (least significant difference test). WL, water-sprayed waterlogged-stressed plants; CK+WL, cytokinin (CK)-sprayed (50 mg L^{-1}) waterlogged-stressed plants; GA₃+WL, gibberellic acid (GA)-sprayed (50 mg L^{-1}) waterlogged-stressed plants.

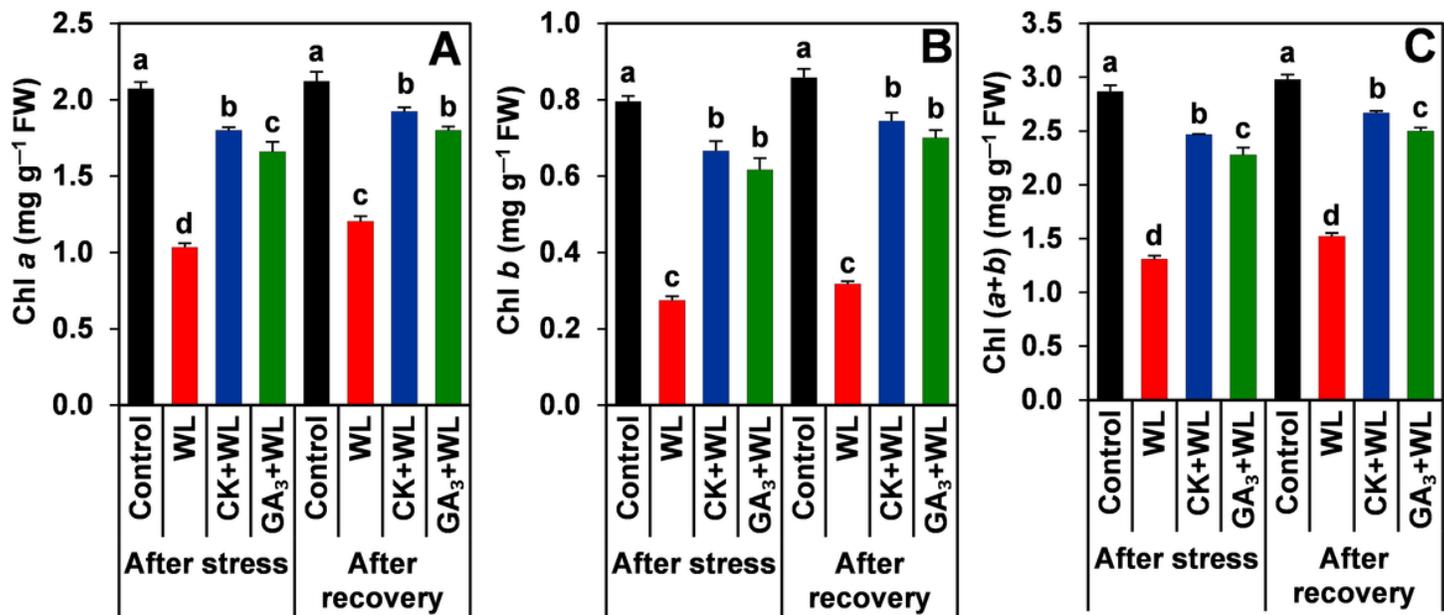


Figure 2

Effects of exogenous cytokinin and gibberellic acid on (A) Chlorophyll (Chl) a, (B) Chl b and (C) Chl (a+b) in the leaves of mung bean plants after 5-days of stress and 15-days of recovery period. Data represent means of four independent replicates (n = 4). Vertical bars indicate standard errors. Different letters represent significant differences at P < 0.05 (least significant difference test). WL, water-sprayed waterlogged-stressed plants; CK+WL, cytokinin (CK)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; GA₃+WL, gibberellic acid (GA)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; FW, fresh weight.

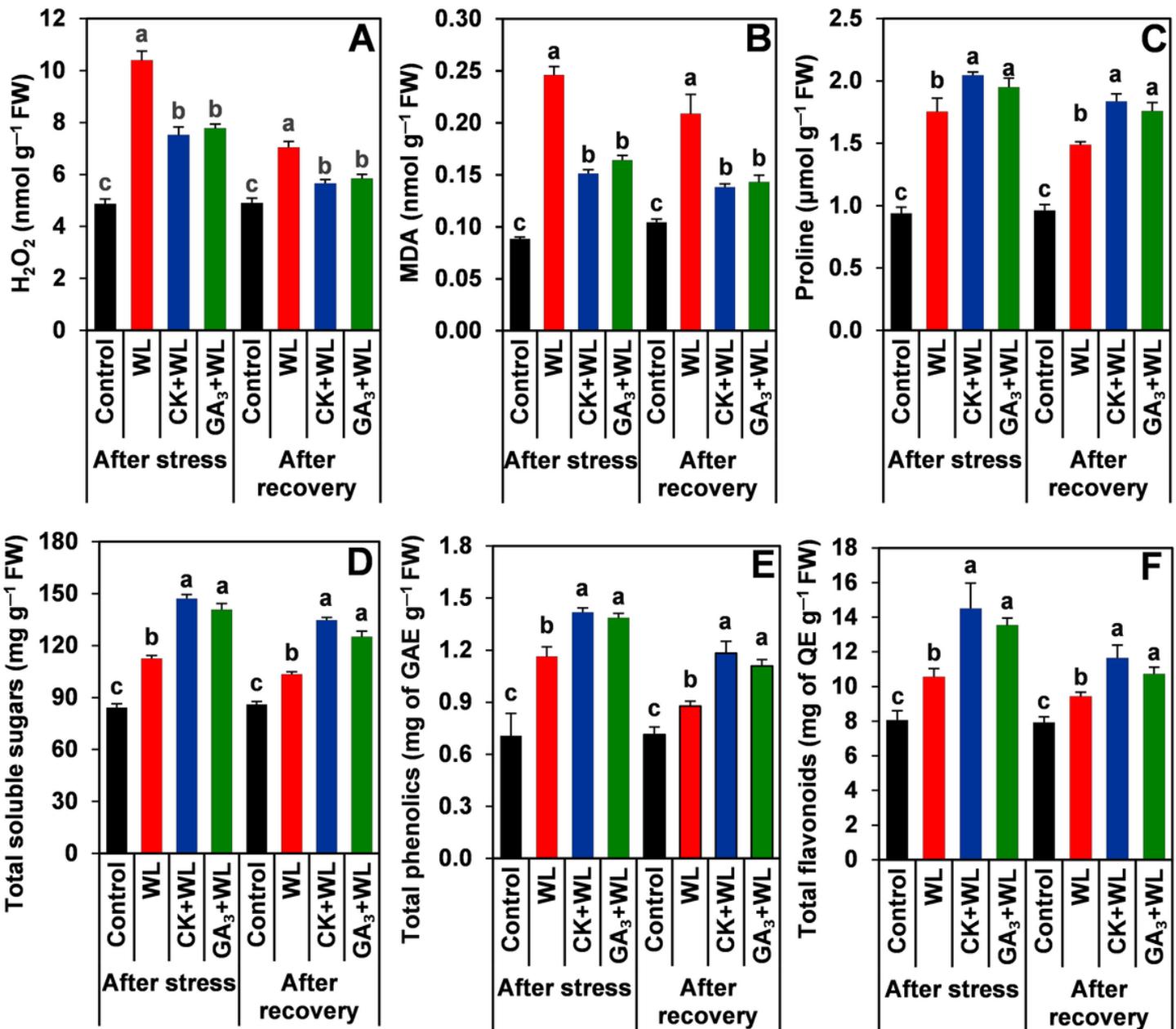


Figure 3

Effects of exogenous cytokinin and gibberellic acid on contents of (A) malondialdehyde (MDA), (B) hydrogen peroxide (H₂O₂), (C) proline, (D) total soluble sugars, (E) total phenolics and (F) total flavonoids in the leaves of mung bean plants after 5-days of stress and 15-days of recovery period. Data represent means of four independent replicates (n = 4). Vertical bars indicate standard errors. Different letters represent significant differences at P < 0.05 (least significant difference test). WL, water-sprayed waterlogged-stressed plants; CK+WL, cytokinin (CK)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; GA₃+WL, gibberellic acid (GA)-sprayed (50 mg L⁻¹) waterlogged-stressed plants; FW, fresh weight; GAE, gallic acid equivalent; QE, quercetin equivalent.