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Research article

Short-term soil-waterlogging contributes to cotton cross tolerance to chronic elevated temperature by regulating ROS metabolism in the subtending leaf

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ABSTRACT

Chronic elevated temperature and soil-waterlogging events often occur concomitantly in the Yangtze River Valley; however, a clear understanding of the effects of aforementioned co-occurring stresses on antioxidant defense in cotton has not been attained. To address this, two temperature conditions during the whole flowering and boll development periods, and three soil-waterlogging levels (0, 3, 6 d) starting on the day of anthesis were established. In the current study, no siginificant difference was observed on plant performance for 3 d soil-waterlogging, whereas 6 d soil-waterlogging event and elevated temperature in isolation negatively affected plant performance (i.e. leaf area declined by 33.3% and 14.7% in AW_6 (soil waterlogging for 6 d under ambient temperature regime) and EC (soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C) relative to AC (soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime)) and induced ROS (reactive oxygen species) production and scavenging mechanisms in the subtending leaf of cotton. SOD (superoxide dismutase), CAT (catalase), and POX (peroxidase) activities were increased, and ASA (ascorbic acid) concentration was enhanced due to higher H_2O_2 (hydrogen peroxide) and O_2^- accumulations. Whereas, APX (ascorbate peroxidase), DHAR (dehydroascorbate reductase) and GR (glutathione reductase) activities were inhibited under elevated temperature regime or waterlogging event, especially in the treatment of EW_6 (soil waterlogging for 6 d under elevated temperature for 2–3 °C), which resulted in increasing H_2O_2 concentration and higher O_2^- generation rate. However, plants acclimated to a short-term waterlogging stress (3 d) performed a cross tolerance to chronic elevated temperature regime (leaf number increased by 11.4%, whereas the abscission rate decreased by 4.6% in EW_3 (soil waterlogging for 3 d under elevated temperature for 2–3 °C) compared with EC (soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C)). Moreover, plants undergone a brief soil-waterlogging (3 d) induced higher GR activity and increased ASA concentration, along with enhanced SOD, CAT, POX activities, limiting H_2O_2 and O_2^- accumulation and reducing oxidative damage to membrane lipids as evidenced by reduced MDA (malondialdehyde) concentration when cotton was subsequently exposed to chronic elevated temperature regime.

1. Introduction

With global climate change, soil-waterlogging events are happening more and more frequently (Bailey-Serres et al., 2012). Meanwhile, chronic elevated temperature often occurred from May to October in the Yangtze River Valley, China. Futhermore, in the coming decades, global average air temperature is expected to increase by 1.8–4.0 °C (Stocker et al., 2013). Inevitably, co-occurring elevated temperature and soil-waterlogging events will significantly affect cotton production. Specifically, at the early anthesis stage, cotton is often affected by short-term waterlogging after a heavy rainfall (Kuai et al., 2015), and is subsequently exposed to continuous elevated temperature conditions for the whole anthesis and boll development periods (Dai et al., 2015; Hu et al., 2017). Recent publications have addressed the impact of these

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Abbreviations: MDT_{day}, mean daytime air temperature; MDT_{night}, mean nighttime air temperature; MDT, mean daily air temperature; SRWC, soil relative water content; SLW, specific leaf weight; OA, osmotic adjustment; MDA, malondialdehyde; ROS, reactive oxygen species; O_2^-, superoxide radicals; H_2O_2, hydrogen peroxide; ASA, ascorbic acid; CAT, catalase; SOD, superoxide dismutase; POX, peroxidase; APX, ascorbate peroxidase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; DPA, days post anthesis; LRW, leaf relative water; FW, fresh weight

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two stresses on fiber development, nitrogen and carbon metabolism in the subtending leaf of cotton (a dominant source leaf for the developing boll which it subtends) (Chen et al., 2017a, 2017b; Wang et al., 2017, 2018). By comparison, the effects of aforementioned co-occurring stresses on reactive oxygen scavenging processes is relatively unexplored.

Plants' cross-tolerance has been addressed a lot by previous publications (Shaaltiel et al., 1988; Malan et al., 1990), which reported that plants tolerant to man-made stress (such as herbicides and atmospheric contaminants) were tolerant to other, seemingly unrelated, environmental stresses (drought, high light or salinity). It has been considered that ROS (reactive oxygen species) metabolism plays essential roles in the cross acclimation of plants to various abiotic stresses (Suzuki et al., 2011; Bartoli et al., 2013). In rapeseed (*Brassica napus*) seedlings, Se-pretreated plants suffered from drought indicated higher activities of GR (glutathione reductase), DHAR (dehydroascorbate reductase), CAT (catalase) and APX (ascorbate peroxidase), and increased ASA (ascorbic acid) content, relative to the drought-stressed plants without Se, suggesting that application of Se promoted plants' tolerance to drought-induced oxidative damage (Hasanuzzaman and Fujita, 2011). Also, elevated level of proline and higher activities of CAT, POX (peroxidase), and SOD (superoxide dismutase) induced by salicylic acid treatment were reported to contribute to the amelioration of Al stress in mung bean seedlings (Ali, 2017). Therefore, a positive correlation between plants’ cross-tolerance and antioxidant defense capacity has been suggested.

In our previous study (Wang et al., 2017), it was observed that exposure to a brief soil-waterlogging (3 d) promoted photosynthetic response to chronic elevated temperature stress in cotton boll development and enhanced sucrose accumulation, indicating cross-tolerance in the subtending leaf for carbohydrate metabolism in cotton. Photosynthesis is a well-established source of ROS in plants, and recent study has shown that antioxidant network plays an important role in maintaining high rates of photosynthesis (Foyer and Shigeoka, 2011). By comparison, the roles of ROS scavenging systems played in the cross-tolerance to soil-waterlogging and elevated temperature in cotton leaves currently stay unknown. Therefore, we hypothesized that enhanced thermotolerance in photosynthesis by moderate waterlogging would be associated with alterations in antioxidant defense systems in the subtending leaf. Thus, the objectives of our study were (1) to quantify plant performance responses to soil-waterlogging and elevated temperature stresses in cotton; (2) to characterize changes in antioxidative defense systems of the subtending leaf under aforementioned combined stresses.

2. Materials and methods

2.1. Plant material and study site

In this experiment, pools (4 × 4 × 1.5 m per pool) with a transparent waterproof top were used from 2013 to 2015 cotton growing seasons at Pailou Experimental Station (118°50′ E, 32°02′ N), Nanjing, China. The soil type was yellow-brown loam soil (Dystrudept). The silt, sand and clay percentages in the soil were 35.2%, 57.0% and 7.8%, respectively. The soil chemical properties were presented in our previous publication (Chen et al., 2017a). Cotton seeds (*Gossypium hirsutum* cv. Siza 3, 180 d growth period and commonly planted) were planted in a nursery bed on 1 April in three years, and then healthy and uniform seedlings with three true leaves were transplanted for 5 rows in each pool (plot). The inter-row plant spacing was 75 cm, and intra-row was 25 cm. Thus, 16 plants were included in a row and 80 plants per plot. Concrete walls were used to completely prevent soil water movement between plots.

2.2. Experimental treatments and microclimate data

Three soil-waterlogging events (0, 3 and 6 d) and two temperature conditions (ambient and elevated) were established in this study. A split plot, randomized complete block design with two replications was arranged, where temperature condition was the whole plot factor and soil-waterlogging treatment was the subplot factor. No treatments were imposed until 50% of the flowers had bloomed in the first position of fruiting branches at 6–7 mainstem nodes. Soil-waterlogging was imposed for 3 or 6 d, while temperature treatment was maintained until boll maturity. A temperature control system (OTC, South-east Co. Ltd, Ningbo, China) was involved in this study to ensure higher of 2–3 °C in elevated temperature zone relative to ambient temperature zone. The operating principle of the temperature control system was described in detail in our previous publication (Chen et al., 2017b). The MDT_{day}/MDT_{night} in the ambient temperature zone was 34.0/28.3 °C, 29.9/25.2 °C and 31.2/26.3 °C, and 36.4/30.8 °C, 32.7/28.0 °C and 33.9/28.8 °C in the elevated temperature zone in the three years, separately. More specific temperature data were provided in our previous publication (Chen et al., 2017b).

Experimental treatments were: ambient temperature regime and well-watered control (AC), where the soil relative water content kept at 70–80% of water capacity; ambient temperature regime with two soil-waterlogging events (AW_3, AW_6), where the soil relative water content maintained 100% for 3 or 6 d, and then soil relative water content was decreased to 70–80% by opening valves; chronic elevated temperature regime and well-watered control (EC); chronic elevated temperature regime with the two aforementioned soil-waterlogging events (EW_3, EW_6). There were 160 plants totally with two plots per treatment in this experiment.

2.3. Sampling

White flowers and its subtending leaves at the first position in the fruiting branches at 6–7 mainstem nodes were tagged. At 9:00–10:00 a.m., about 6–8 tagged leaves were sampled from plants in the center three rows per plot, every 7 d from 10 to 38 days post anthesis (DPA) in 2013 and 2015, and from 10 to 45 DPA in 2014. The leaves were immediately carried to the lab on ice. In the lab, samples were cleaned and cutted into two halves without the main vein. Then one half was directly frozen by liquid nitrogen and then put in a freezer (− 80 °C) for assessments of enzyme activities, malondialdehyde (MDA), total chlorophyll, ascorbic acid (ASA), hydrogen peroxide (H_2O_2), and $O_2{}^-$ production; the rest of samples were used to determine osmotic adjustment (OA), leaf area and dry weight to calculate specific leaf weight (SLW).

2.4. Chlorophyll

Chlorophyll contents (n = 6 per treatment) were determined by the method of Dillenburg et al. (1995) with some modifications. 0.2 g fresh leaf tissues were included in a dark test tube with 10 mL of pure ethanol and kept in a 4 °C freezer for 24 h. Then the supernatant was detected at 663 nm and 645 nm by a spectrophotometer (UV-2450, Shimadzu, Japan), with pure ethanol as blank. Chlorophyll a, chlorophyll b, and total chlorophyll contents were calculated according to Arnon (1949).

2.5. Osmotic adjustment

Osmotic potential was measured according to Hummel et al. (2010). Two leaf discs were quickly sampled from fresh excised leaves using a 1 cm^2 borer (n = 6 for each treatment), and then put into microtubes within liquid nitrogen. Centrifugation 10,000 × *g* for 5 min to extract cell sap. 10 μL of the sap were assessed by a vapor pressure osmometer (Wescor Vapor 5520, ELITechGroup Inc., Logan, UT, USA). Osmotic potential (MPa) was deduced from osmolarity by the Van't Hoff equation at 20 °C.

The leaf relative water (LRW) content was measured according to Barrs and Weatherley (1962), and was provided in supplementary. Osmotic potential at full turgor (Ψs^{100}) was calculated by multiplying LRW content and osmotic potential. Osmotic adjustment (OA) was deduced as the difference in Ψs^{100} between the control ($\Psi {s_c}^{100}$) and the stressed ($\Psi {s_s}^{100}$) as described by Zahoor et al. (2017): $OA = \Psi {s_c}^{100} - \Psi {s_s}^{100}$.

2.6. Malondialdehyde (MDA), ascorbic acid (ASC), hydrogen peroxide (H_2O_2) contents and O_2^- generation rate

MDA content was assayed according to Zhao et al. (2013). 0.2 g leaf tissue (n = 6 for each treatment) was ground with 5 mL of 10% (w/v) trichloroacetic acid (TCA). Then centrifuged at 12,000 × *g* for 10 min 2 mL supernatant was included in 4 mL of 0.6% (w/v) thiobarbituric acid, waiting for 15 min at 100 °C. Then the mixture was placed at room temperature to stop the reaction. A450, A532 and A600 were observed. MDA content was calculated and expressed in μmol g^{-1} FW.

Ascorbic acid (ASC) was determined (Hodges and Forney, 2003). 0.3 g leaf tissue (n = 6 for each treatment) was homogenized in 5 mL of 5% (w/v) *m*-phosphoric acid. Then centrifugation (15 min, 22,000 × *g*). 300 μL supernatant fluid was added in a 700 μL mixture (containing 100 mM KH_2PO_4, 3.6 mM EDTA, and 2 mM dithiothreitol). After 10 min, included 100 μL of 0.5% (w/v) *N*-ethylmaieimide. Color was showed up with 400 μL of 65 mM α, α′-dipyridyl in 70% ethanol, 400 μL of 10% (w/v) TCA, 200 μL of 110 mM $FeCl_3$, and 400 μL of 44% *o*-phosphoric acid. After 1 h at 40 °C, A525 was measured.

H_2O_2 was extracted according to Okuda et al. (1991) (n = 6 for each treatment). The mixture contained 80 μL MBTH, 400 μL of 12.5 mM DMAB, 20 μL peroxidase, and 1 mL extraction solution. The peroxidase was included to initiate reaction at 25 °C. Alterations of A590 was monitored.

The generation rate of O_2^- was assayed (Yang et al., 2008). 0.2 g leaf tissue (n = 6 for each treatment) was homogenized in 1.8 mL of 50 mM potassium phosphate. Then centrifugation (10 min, 5,000 × *g*). Mixtures (containing 1 mL of 1 mM hydroxylamine hydrochloride, 0.5 ml extraction solution, and 0.5 mL potassium phosphate) were lay for 20 min at 25 °C. After that, 1 mL of 7 Mm α-naphthylamine and 1 mL of 17 mM sulfanilamide were included. After 20 min, A525 was recorded. A NO_2^- standard curve was necessary for the O_2^- generation rate calculation.

2.7. Enzyme extraction and analysis

Superoxide dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC 1.11.3.6), Peroxidase (POX, EC 1.11.1.7), Ascorbate peroxidase (APX, EC 1.11.1.11), Glutathione reductase (GR, EC 1.11.1.11) and Dehydroascorbate reductase (DHAR, EC 1.8.5.4) enzymes were extracted (Djanaguiraman et al., 2009). 0.3 g frozen leaf tissue (n = 6 for each treatment) was ground with 5 mL of 0.1 M Tris-HCl buffer. Then centrifugation at 20,000 × *g* for 20 min. Keep the supernatant for enzymes activity determinations. (a) SOD activity was estimated by recording the restraint of photochemical decrease in nitro blue tetrazolium (Lei et al., 2006). (b) CAT activity was analyzed according to Djanaguiraman et al. (2009) and expressed as μM H_2O_2 g^{-1} FW min^{-1}. (c) POX activity was determined by the degradation rate of H_2O_2. Guaiacol was used as hydrogen donor, monitoring the alteration rate at A436 (Lin and Kao, 1999). (d) APX activity was deduced as the reduction difference of A290 with 3 mL of reaction mixture (0.1 mM sodium ascorbate, 0.1 mM EDTA, 2.5 mM H_2O_2, 50 mM sodium phosphate buffer, and 200 μL of extraction solution) for 1 min (Nakano and Asada, 1981). (e) GR activity was estimated as the oxidation of NADPH by recording A340 with 3 mL mixture (2 mM Na_2EDTA, 0.5 mM GSSG, 0.15 mM NADPH, 50 mM potassium phosphate buffer (pH 7.8), and 200 μL of enzyme extraction solution) for 3 min (Lei et al., 2006). (f) DHAR activity was assayed as decrease difference in DHA by recording A265 (Nakano and Asada, 1981). The mixture included 0.85 mL of 100 mM potassium phosphate, 0.05 mL of 4 mM DHA, 0.05 mL of 50 mM reduced glutathione, and 0.05 mL of enzyme extraction solution.

2.8. Biomass accumulation and partitioning

When tagged bolls had opened (reached maturity), five plants per plot were harvested. Leaf number was counted, and leaf area was assessed by a LI-3100 leaf area meter (LiCor, Linoln, NE, USA). After drying at 80 °C, reproductive organs weight (including floral buds, flowers, and bolls) was recorded, and then boll load per unit leaf area was calculated by reproductive organs weight and leaf area. 10 plants per plot were used to measure abscission rate, which was deduced as the number of shed reproductive organs divided by the total number of shed and harvested reproductive organs (Ertek and Kanber, 2003).

2.9. Data analysis

The effects of treatment (AC, AW_3, AW_6, EC, EW_3, and EW_6) on all parameters of interest across three years were analyzed using a one-way ANOVA and JMP Pro 13 software. Post-hoc analysis for differences between means was performed by Fisher's least significant difference (LSD) test at 0.05 alpha level.

3. Results

3.1. Plant performance

As shown in Table 1, with the exception of leaf area in 2015, 3 d waterlogging at the onset of flowering under ambient temperatures did not influence the leaf number, leaf area, boll load, or abscission rate relative to AC across all three years. However, AW_6 and EC significantly decreased leaf number, leaf area, and boll load relative to AC in all three years. When averaged across all sampling dates in the three years, decline of 30.0%, 33.3%, and 15.4% were observed in leaf number, leaf area, and boll load in AW_6 relative to AC, and 16.3%, 14.7%, and 7.9% decline in chronic elevated temperature (EC) compared with AC, respectively. In contrast, the abscission rate was increased by 7.5% and 7.2% in AW_6 and EC relative to AC, across three years. Besides, 3 d waterlogging and chronic elevated temperature combined (EW_3) did not produce statistically comparable leaf number and leaf area to the EC treatment in 2013. However, a 6.2% increase in boll load and 3.9% decline in abscission rate were observed in EW_3 compared with EC in 2013. In 2014 and 2015, leaf number, leaf area, and boll load were significantly increased in EW_3 relative to EC (not significant for boll load in 2015), while the abscission rate was reduced by 4.6% in EW_3 relative to EC. In contrast, EW_6 produced the lowest values for leaf number, leaf area, and boll load and the highest abscission rate of any treatment in all three years.

3.2. Chlorophyll content and osmotic adjustment in the subtending leaf

As shown in Table 2, total chlorophyll content was decreased, while Chl a/b was increased from 10 days post anthesis (DPA) to 38 DPA in AC in all three years. No difference was found for total chlorophyll content for 3 d waterlogging alone (AW_3) and elevated temperature (EC) compared with AC in most situation. However, 6 d waterlogging alone (AW_6) significantly reduced total chlorophyll content and pronouncedly increased Chl a/b relative to AC in the three years (with the exceptions of Chl a/b at 10 DPA and 38 DPA in 2015). Besides, a reduction in total chlorophyll content and an increase in Chl a/b is also observed in the treatment of EW_6 in all three years for all DPA. Specific leaf weight (SLW) was significantly enhanced in 6 d waterlogging treatment (AW_6), especially in EW_6, relative to AC during 2013–2015

Table 1
Effect of short-term soil waterlogging coupled with elevated temperature during the flowering and boll-forming stages on leaf num., leaf area, boll load, and abscission rate in cotton during 2013–2015.

Treatments	2013				2014				2015			
	Leaf num. no. plant^{-1}	Leaf area cm^2 plant^{-1}	Boll load g m^{-2}	Abscission rate %	Leaf num. no. plant^{-1}	Leaf area cm^2 plant^{-1}	Boll load g m^{-2}	Abscission rate %	Leaf num. no. plant^{-1}	Leaf area cm^2 plant^{-1}	Boll load g m^{-2}	Abscission rate %
AC	70.3a	7125.0a	187.1a	64.1c	65.2a	6751.1a	175.2a	63.5b	60.3a	6259.3a	180.3a	64.3b
AW$_3$	68.6a	6987.3a	180.6 ab	64.3c	64.1a	6622.3a	170.0a	64.2b	57.1a	5922.3b	176.1a	65.2b
AW$_6$	51.2c	5003.9c	155.3d	68.9a	45.8c	4425.0d	150.6c	68.6a	40.6c	3995.0d	153.2c	68.7a
EC	60.5b	6231.0b	167.2c	68.7a	52.3b	5584.6c	163.2b	67.9a	51.0b	5366.7c	169.2 ab	69.2a
EW$_3$	61.7b	6335.7b	177.6b	66.1b	62.4a	6389.2b	171.2a	64.8b	58.3a	5998.6b	175.3a	66.0b
EW$_6$	40.1d	3952.1d	150.3d	69.2a	39.0d	3808.0e	140.3d	68.9a	40.2c	3881.0d	143.0d	69.7a

Boll load is expressed as dry weight of reproductive organs per unit leaf area.
Values not sharing a common letter within the same column in a given year are significantly different ($P < 0.05$).
The data for abscission rate are the means for 20 replications, and other parameters are 10 replications.
AC represents soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime.
AW$_3$ and AW$_6$ represent soil waterlogging for 3 d and 6 d under ambient temperature regime, respectively.
EC represents soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C.
EW$_3$ and EW$_6$ represent soil waterlogging for 3 d and 6 d under elevated temperature for 2–3 °C.

(Table 2). When averaged across all sampling dates in the three years, increase of 16.2% and 29.3% were observed in SLW in AW$_6$ and EW$_6$ relative to AC. Elevated temperature also pronouncedly increased SLW relative to ambient temperature in nearly all sample dates (not significant at 10 DPA in 2013). EW$_3$ markedly decreased SLW relative to elevated temperature alone (EC) in most situations (not significant at 10 DAP in 2013 and 2014; Table 2). When averaged across all sampling dates in the three years, a 14.9% decline was found in SLW in EW$_3$ relative to EC.

Similar to SLW, osmotic adjustment (OA) was increased significantly by waterlogging treatment, especially when combined with elevated temperature (Table 2). OA ranged from 0.23 to 0.42, 0.20–0.40, and 0.23–0.64 MPa in AW$_6$, and 0.52–0.55, 0.54–0.65, and 0.58–0.76 MPa in EW$_6$ from 10 to 38 DPA in 2013, 2014, and 2015, respectively. Interestingly, EW$_3$ significantly decreased OA relative to EC at 24 and 38 DPA in all three years. When averaging these two sample dates, decline of 28.8%, 42.7%, and 24.7% were observed on OA in EW$_3$ relative to EC in 2013, 2014, and 2015, separately.

3.3. Lipid peroxidation and enzymatic antioxidant activities in the subtending leaf

As an indicator of lipid peroxidation, MDA concentration was markedly enhanced in plants either under soil-waterlogging or under chronic elevated temperature for 2–3 °C relative to AC in three years (Fig. 1A). When averaged across sampling dates in a given year, increase of 20.0%, 47.3%, and 39.1% in AW$_3$, 61.7%, 87.8% and 101.0% in AW$_6$, and 60.4%, 105.0%, and 88.2% in EC were found for MDA concentration compared with AC in 2013, 2014, and 2015, respectively. EW$_6$ increased MDA concentration to a greater extent than any

Table 2
Effect of short-term soil waterlogging coupled with elevated temperature during the flowering and boll-forming stages on total chlorophyll content (Chl a + b), chlorophyll a to chlorophyll b ratio (Chl a/b), specific leaf weight (SLW), and osmotic adjustment (OA) in the subtending leaf of cotton at 10 DPA, 24 DPA, and 38 DPA during 2013–2015.

Year	Treatments	10 DPA				24 DPA				38 DPA			
		Chla + b mg g^{-1} FW	Chla/b	SLW μg cm^{-2}	OA Mpa	Chla + b mg g^{-1} FW	Chla/b	SLW μg cm^{-2}	OA Mpa	Chla + b mg g^{-1} FW	Chla/b	SLW μg cm^{-2}	OA Mpa
2013	AC	1.66a	3.64c	46.0b	control	1.70a	4.33a	48.9cd	control	1.48a	4.12bc	50.0d	control
	AW$_3$	1.65a	3.79b	47.9b	0.14c	1.53b	3.75b	45.6d	0.13e	1.33b	4.43b	51.2d	0.12d
	AW$_6$	1.51b	3.94a	53.2a	0.35b	1.29c	4.16a	56.0b	0.42b	1.14c	4.49 ab	58.8c	0.23c
	EC	1.63a	3.18d	49.0b	0.16c	1.50b	3.81b	60.1a	0.30c	1.39 ab	3.94c	64.8b	0.43b
	EW$_3$	1.49b	3.29d	48.1b	0.17c	1.40b	4.10a	50.0c	0.25d	1.25b	3.96c	53.3d	0.27c
	EW$_6$	1.31b	4.36a	54.0a	0.52a	1.16c	4.66a	63.2a	0.54a	1.05c	5.16a	67.0a	0.55a
2014	AC	1.33a	4.49b	43.2b	control	1.27 ab	4.77b	45.1d	control	1.18 ab	5.60b	49.3c	control
	AW$_3$	1.29a	4.28c	44.5b	0.15d	1.23b	4.49b	44.2d	0.16d	1.12b	6.03b	50.0c	0.15d
	AW$_6$	1.21b	5.49a	50.2a	0.40b	1.10c	5.66a	52.2b	0.21c	1.04c	6.80a	58.0b	0.20c
	EC	1.39a	4.36c	53.7a	0.18d	1.33a	4.59b	54.9b	0.43b	1.25a	5.03c	60.3 ab	0.53a
	EW$_3$	1.28a	4.78b	49.8a	0.27c	1.22b	4.71b	48.0c	0.27c	1.12b	5.51b	51.0c	0.28b
	EW$_6$	1.14b	5.08a	55.0a	0.61a	1.07c	6.16a	58.6a	0.65a	0.99c	7.06a	63.2a	0.54a
2015	AC	1.78a	3.57a	40.6b	control	1.63a	3.41b	46.2c	control	1.48a	3.50a	48.9c	control
	AW$_3$	1.67b	3.29a	43.0b	0.15d	1.58a	3.40b	48.0c	0.13d	1.37b	3.47a	47.1c	0.13d
	AW$_6$	1.54c	3.62a	50.1a	0.64b	1.36b	3.46b	56.7b	0.33c	1.15c	3.29a	60.0b	0.23c
	EC	1.80a	3.68a	49.0a	0.19d	1.70a	3.27c	55.0b	0.42b	1.55a	3.51a	63.1 ab	0.55a
	EW$_3$	1.68b	3.40a	42.3b	0.36c	1.52a	3.18c	49.0c	0.37c	1.39b	3.36a	50.0c	0.36b
	EW$_6$	1.40c	3.45a	53.2a	0.76a	1.29b	3.81a	60.3a	0.66a	1.04c	3.43a	67.1a	0.58a

The data are the means for six replications.
Values not sharing a common letter within the same column in a given year are significantly different ($P < 0.05$).
AC, AW$_3$, AW$_6$, EC, EW$_3$, EW$_6$ were explained in Table 1.

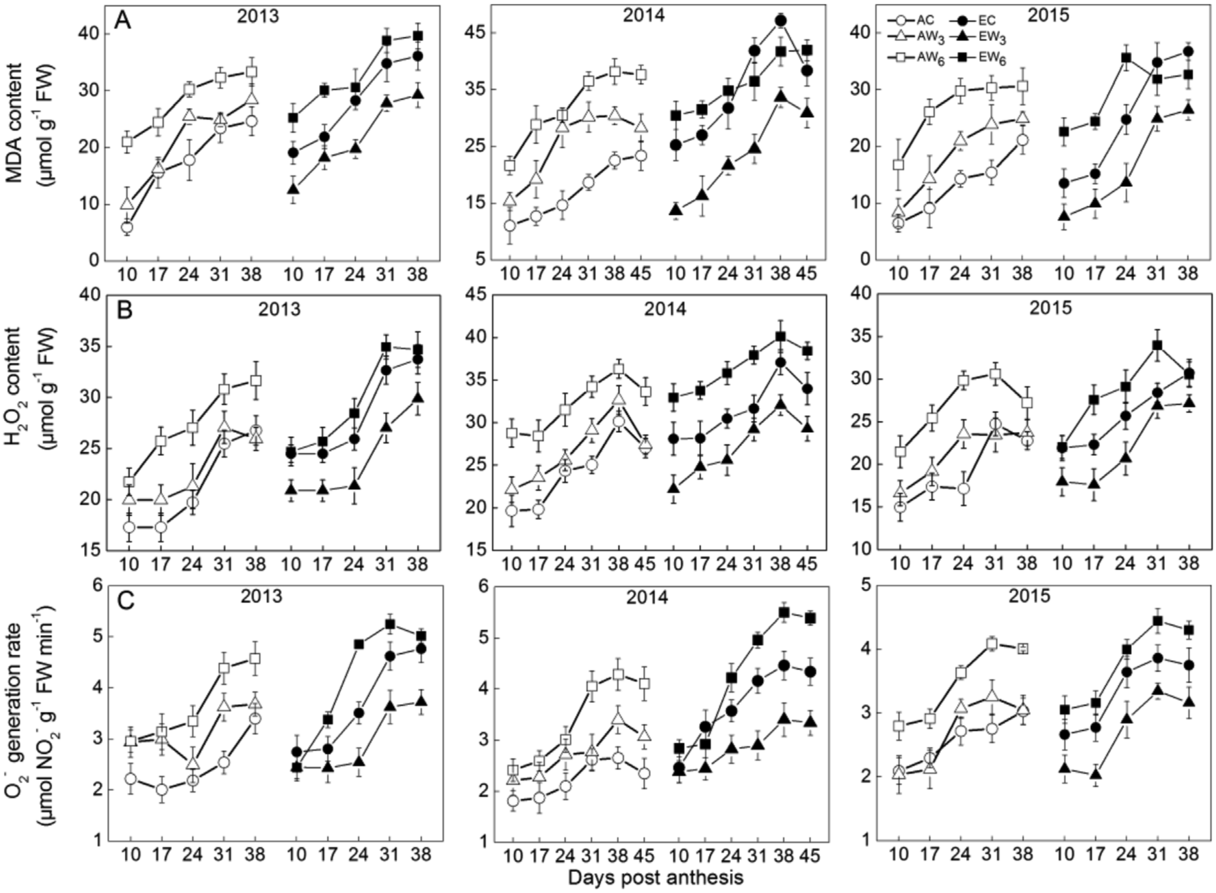

Fig. 1. Effect of short-term soil-waterlogging coupled with elevated temperature during the flowering and boll-forming stages on MDA content (A), H_2O_2 content (B), and O_2^- generation rate (C) in the subtending leaf of cotton during 2013–2015. The data are the means for six replications ± SD. AC represents soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime. AW_3 and AW_6 represent soil-waterlogging for 3 d and 6 d under ambient temperature regime, respectively. EC represents soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C. EW_3 and EW_6 represent soil-waterlogging for 3 d and 6 d under elevated temperature for 2–3 °C. FW represents fresh weight.

other treatment in the three years. Conversely, EW_3 significantly lower MDA concentration by 23.3%, 33.5%, and 34.0%, relative to EC in 2013, 2014, and 2015, separately. Similarly, H_2O_2 content and O_2^- generation rate were increased by 7.2%–9.8% and 4.9%–22.3% in AW_3, 28.6%–38.7% and 27.4%–52.7% in AW_6, and 29.8%–33.0% and 29.5%–66.1% in EC relative to AC from 2013 to 2015, respectively (Fig. 1B and C). Moreover, decline of 15.1%, 13.9%, and 14.6% in H_2O_2 content and 20.0%, 22.3%, and 18.8% in O_2^- generation rate were observed in EW_3 compared with EC in 2013, 2014, and 2015, separately.

The ASA content was increased with DPA (Fig. 2). Both soil-waterlogging and elevated temperature treatment significantly increased ASA content in the three years. Increase of 15.4%–20.4%, 24.4%–29.9%, 2.2%–14.0%, 22.3%–36.9%, and 32.5%–49.6% in AW_3, AW_6, EC, EW_3, and EW_6 relative to AC were observed for ASA content across DPA when averaged across the three years of the study.

In our study, antioxidant enzyme (SOD, CAT and POX) activities were evaluated (Fig. 3A–C). Both soil-waterlogging and elevated

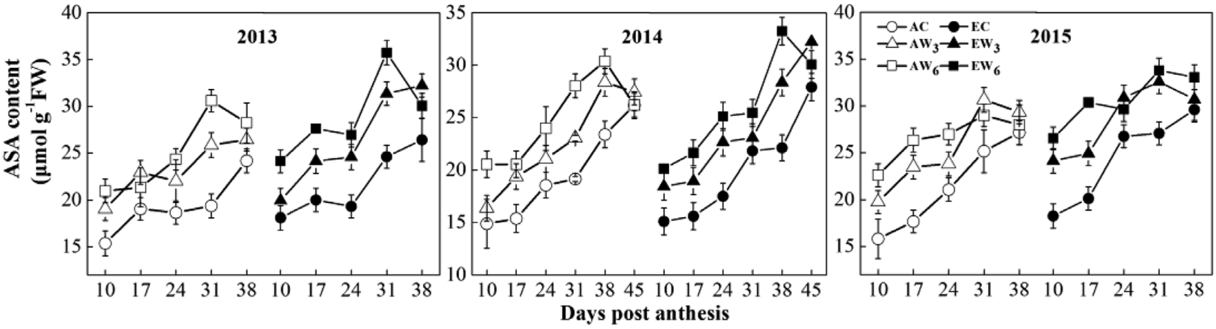

Fig. 2. Effect of short-term soil-waterlogging coupled with elevated temperature during the flowering and boll-forming stages on ASA content in the subtending leaf of cotton during 2013–2015. The data are the means for six replications ± SD. AC represents soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime. AW_3 and AW_6 represent soil-waterlogging for 3 d and 6 d under ambient temperature regime, respectively. EC represents soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C. EW_3 and EW_6 represent soil-waterlogging for 3 d and 6 d under elevated temperature for 2–3 °C. FW represents fresh weight.

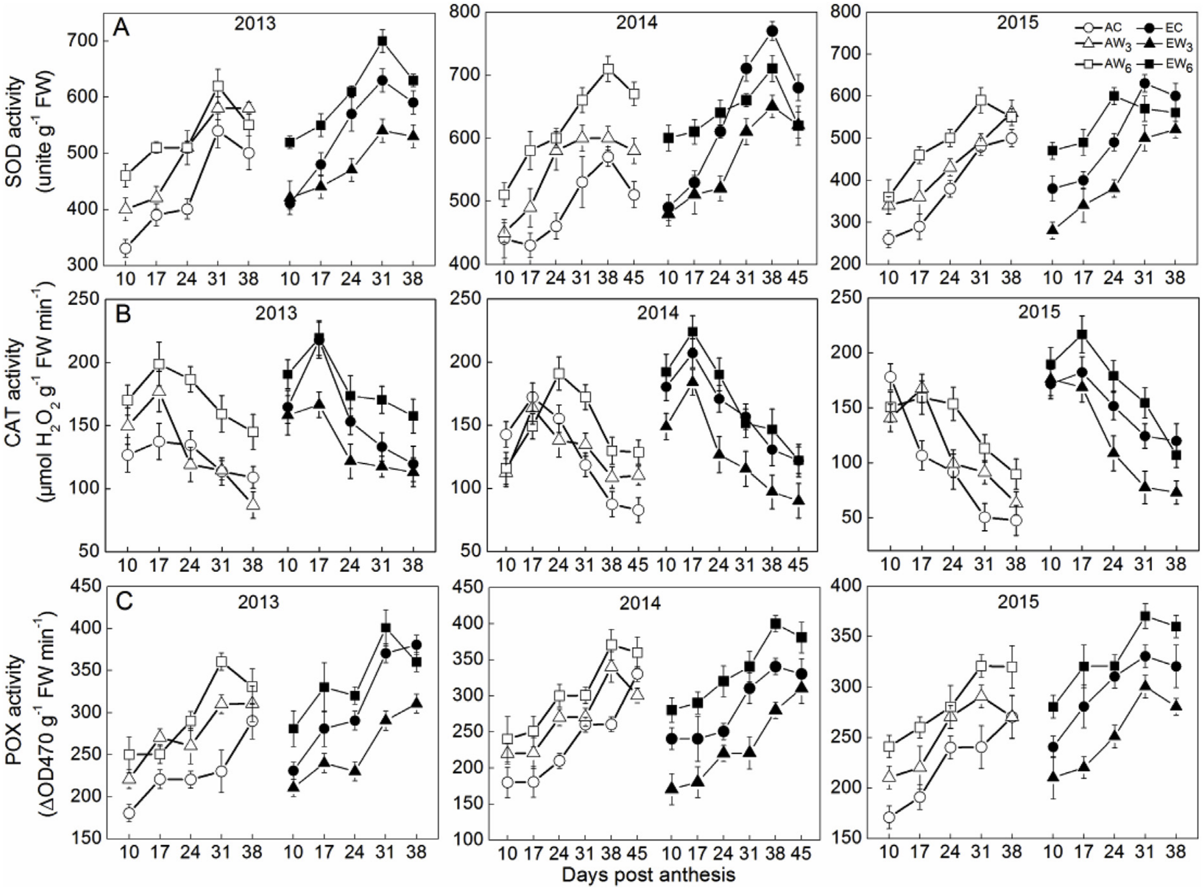

Fig. 3. Effect of short-term soil-waterlogging coupled with elevated temperature during the flowering and boll-forming stages on SOD activity (A), CAT activity (B), and POX activity (C) in the subtending leaf of cotton during 2013–2015. The data are the means for six replications ± SD. AC represents soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime. AW_3 and AW_6 represent soil-waterlogging for 3 d and 6 d under ambient temperature regime, respectively. EC represents soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C. EW_3 and EW_6 represent soil-waterlogging for 3 d and 6 d under elevated temperature for 2–3 °C. FW represents fresh weight.

temperature significantly increased SOD activity relative to AC. Increase of 15.3%, 12.2%, and 14.2% in AW_3, 22.6%, 26.9%, and 28.8% in AW_6, and 24.1%, 28.9%, and 31.0% in EC were observed on average SOD activity across DPA relative to AC in 2013, 2014, and 2015, respectively. EW_6 significantly enhanced SOD activity compared with all other treatments in 2013, whereas, SOD activity was lowered during later boll development in EW_6 relative to EC in 2014 and 2015 (from 31 DPA to boll opening). By comparison, SOD activity was lowered in EW_3 relative to EC at all DPAs in the three years. Average SOD activity across DPA was markedly reduced by 10.4%, 10.5%, and 19.2% in EW_3 relative to EC in 2013, 2014, and 2015, separately. Similar trends among treatments were found on CAT activity and POX activity. Increase of 4.2%–18.4% and 13.4%–20.2% in AW_3, 16.9%–40.4% and 27.8%–29.8% in AW_6, and 26.9%–58.0% and 20.5%–36.1% in EC were observed for average CAT activity and POX activity across DPA relative to AC from 2013 to 2015, respectively. Average CAT activity and POX activity across DPA in the subtending leaf were lowered by 14.1% and 17.5%, 21.2% and 19.3%, and 19.4% and 14.9% in EW_3 relative to EC in 2013, 2014, and 2015, separately.

In this study, APX activity and DHAR activity were increased with DPA (Fig. 4A and B). However, average APX activity and DHAR activity across DPA were significantly reduced by 7.2%–12.6% and 8.4%–12.7% in AW_3, 15.9%–20.0% and 21.5%–26.8% in AW_6, 6.6%–8.6% and 9.5%–13.0% in EC, 14.0%–15.9% and 20.7%–23.6% in EW_3, and 21.4%–25.2% and 31.1%–37.6% in EW_6 relative to AC from 2013 to 2015, respectively. Both soil-waterlogging and elevated temperature decreased GR activity across three years compared with AC, especially in EW_6 (Fig. 5). One exception was that EW_3 significantly increased GR activity by 12.3%, 26.4%, and 13.0% relative to EC in 2013, 2014, and 2015, separately.

4. Discussion

In the Yangtze River Valley Cotton Belt, soil-waterlogging often happens simultaneously with chronic elevated temperature at cotton reproductive growth stage, negatively influencing cotton production. Although the underlying mechanisms on carbon metabolism (Wang et al., 2017) and nitrogen metabolism (Wang et al., 2018) for short-term soil-waterlogging and chronic elevated temperature stresses have been addressed, by comparison, the co-effects of the aforementioned combined stresses on plant performance and ROS scavenge system were still left unaddressed.

4.1. Soil-waterlogging and chronic elevated temperature affected plant performance

Chronic elevated temperature condition and 6 d soil-waterlogging treatment in isolation negatively influenced leaf number, leaf area, and boll load, leading to higher boll abscission rates (increased by 7.5% and 7.2% in AW_6 and EC relative to AC). By comparison, leaf number, leaf area, and boll load were significantly increased, and abscission rate was pronouncedly decreased in EW_3 relative to EC in most situations. These results indicate that plants acclimated to 3 d soil-waterlogging improves cross tolerance to chronic elevated temperature stress, which was

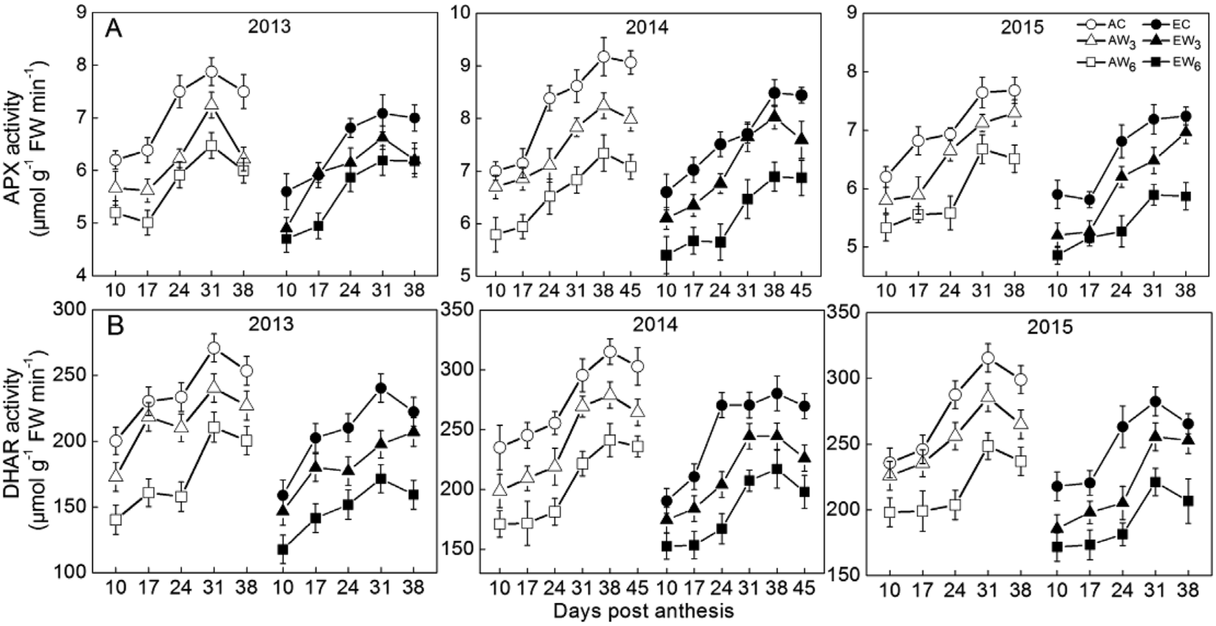

Fig. 4. Effect of short-term soil-waterlogging coupled with elevated temperature during the flowering and boll-forming stages on APX activity (A) and DHAR activity (B) in the subtending leaf of cotton during 2013–2015. The data are the means for six replications ± SD. AC represents soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime. AW_3 and AW_6 represent soil-waterlogging for 3 d and 6 d under ambient temperature regime, respectively. EC represents soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C. EW_3 and EW_6 represent soil-waterlogging for 3 d and 6 d under elevated temperature for 2–3 °C. FW represents fresh weight.

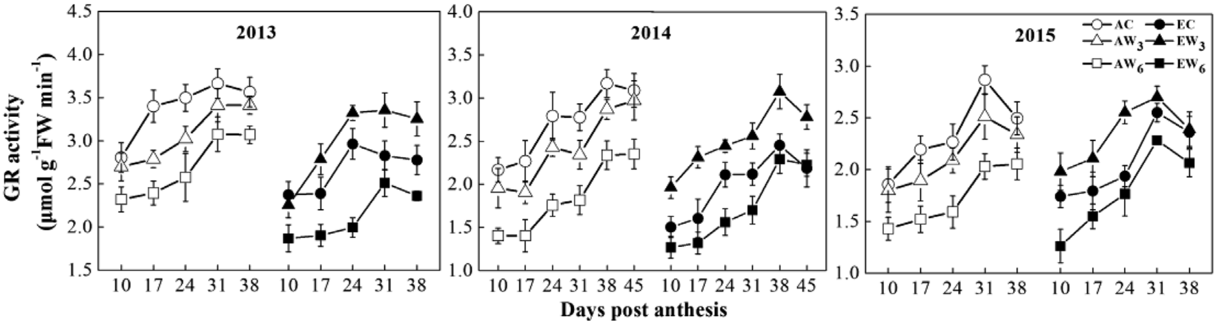

Fig. 5. Effect of short-term soil-waterlogging coupled with elevated temperature during the flowering and boll-forming stages on GR activity in the subtending leaf of cotton during 2013–2015. The data are the means for six replications ± SD. AC represents soil well-watered (SRWC(75 ± 5) %) under ambient temperature regime. AW_3 and AW_6 represent soil-waterlogging for 3 d and 6 d under ambient temperature regime, respectively. EC represents soil well-watered (SRWC(75 ± 5) %) under elevated temperature for 2–3 °C. EW_3 and EW_6 represent soil-waterlogging for 3 d and 6 d under elevated temperature for 2–3 °C. FW represents fresh weight.

consistent with our previous study addressing carbohydrate metabolism and cotton boll biomass (Wang et al., 2017). However, prior to the current study, the underlying stress physiological and biochemical alterations associated with the enhanced tolerance to elevated temperature by prior exposure to moderate waterlogging in cotton was still unaddressed.

4.2. Soil-waterlogging and chronic elevated temperature influenced ROS-scavenging in the subtending leaf

Leaves play a primary role in plant carbon fixation and energy capture, and the subtending leaf produces 60%–87% of a cotton boll's dry-matter (Wullschleger and Oosterhuis, 1990; Liu et al., 2013), thus it is important to elucidate the mechanisms associated with enhanced tolerance to elevated temperature by moderate waterlogging in the subtending leaf in cotton. Soil-waterlogging significantly decreased total chlorophyll content and notably increased Chl a/b relative to well-watered plants, suggesting that waterlogging could accelerate chlorophyll degradation and Chl b was more sensitive to flooding stress, which was in accordance with previous work (Kuai et al., 2014). Other research reported that heat stress (38/20 °C) significantly decreased chlorophyll content in cotton leaves, since the chlorophylls were photo-oxidized in the presence of high temperature (Snider et al., 2009, 2010). Despite no significant effect on total chlorophyll content in this study, a decreasing Chla/b ratio was observed in the subtending leaf of plants exposed to 2–3 °C warmer conditions, reflecting alterations in concentrations and distributions of light-harvesting chlorophyll complexes (LHCII) relative to reaction-center complex (PSII and PSI). OA is defined as an accumulation of cell solute that induces alterations of cell osmotic potential, which may indicate cell stress level in some sense (Boyer et al., 2008). Specific leaf weight (SLW) and osmotic adjustment (OA) in the subtending leaf were significantly increased under soil-waterlogging and elevated temperature stresses, however, co-occurring of soil-waterlogging for 3 d and chronic elevated temperature considerably decreased SLW and OA in the subtending leaf relative to cotton exposed to elevated temperature alone, especially in 2014 and 2015. In our previous study, a prior exposure to 3 d soil-waterlogging stress significantly increased enzyme activities associated with carbon and nitrogen metabolism, and decreased the concentrations of micro-molecules such as nitrate and free amino acids under subsequent elevated temperature conditions (Wang et al., 2018), which may consequently result in lower OA in the subtending leaf for the treatment of EW_3 relative to EC in the current study. Nevertheless, higher OA for 6 d soil-waterlogging or elevated temperature suggested degradation of macromolecular substance, such as carbohydrate (Wang et al., 2017) and protein (Wang et al., 2018), and accumulation of micromolecules that decreases osmotic potential and may be termed plants' self-regulation under stress.

Leaf senescence was induced under soil-waterlogging and elevated temperature in isolation, indicated by reduced leaf area, decreased chlorophyll concentration, and enhanced MDA content relative to control conditions. As chloroplasts are the main ROS producing organelles, chlorophyll degradation is associated with the accumulation of ROS and cellular decline (Luo et al., 2016). A previous study has reported that 2 d waterlogging evidenced a reduction in superoxide radical concentrations in mung bean; however, further soil-waterlogging up to 8 d led to considerable increase in superoxide radical concentrations (Sairam et al., 2011). Similar results were found in our study. Only 18.2% increase was detected in O_2^- generation rate for 3 d waterlogging treatment relative to well-watered cotton, while 45.7% increase was observed for 6 d waterlogging when average across all sampling dates in the three years. The H_2O_2 is considered as relatively stable ROS (Sewelam et al., 2016). In our study, H_2O_2 concentration showed the same trend as O_2^- generation rate in the subtending leaf. Interestingly, H_2O_2 concentration and O_2^- generation rate were substantially lowered in the subtending leaf for the treatment of EW_3 compared with EC in three years, reducing oxidative damage to membrane lipids as evidenced by reduced MDA concentration (Fig. 1).

There are well-defined antioxidant defense systems to protect plants from ROS damage, for example enzymatic system. Superoxide dismutase (SOD), which catalyzes superoxide to molecular oxygen and hydrogen peroxide (H_2O_2), is the primary scavenger in detoxifying reactive oxygen species (ROS) (Scandalios, 1993). Ascorbate peroxidase (APX), converting H_2O_2 to water, is the most vital peroxidase (POX) in the detoxification of H_2O_2 (Foyer, 1996). In the Foyer-Halliwell-Asada pathway, APX, dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are together involved in reducing H_2O_2 to water (Halliwell, 1987; Foyer et al., 1994). Catalase (CAT) can also scavenge H_2O_2, it performs however a lower affinity for H_2O_2 relative to APX (Graham and Patterson, 1982). POX plays a role in lignin biosynthesis (Lee et al., 2007) but can also enhance plant defense by removing H_2O_2 under oxidative stress (Olmos and Hellin, 1996). In this study, the enhanced activities of SOD, CAT, and POX were recorded under both waterlogging and elevated temperature in isolation, as a response to accumulation of H_2O_2 and O_2^- in the subtending leaf; ROS, however, were not absolutely removed under environmental stresses, which lead to membrane lipid peroxidation (Fig. 1A) and chlorophyll degradation (Table. 2). Similar results were reported by Hu et al. (2016) in cotton plants under K deficiency stress. SOD, CAT, and POX activities were considerably decreased in the EW_3 treatment relative to elevated temperature alone, as a result of lower H_2O_2 concentration and O_2^- generation rate in the present study.

ASA-GSH cycle also plays an important role in antioxidant defense system against ROS-induced damage. Higher H_2O_2 and MDA concentrations and increased O_2^- generation rate (Fig. 1) indicated more oxidative damage under both soil-waterlogging and elevated temperature stresses, which was also associated with higher ASA concentration (Fig. 2), since ASA can facilitate ROS detoxification (Asada, 1999). Both soil-waterlogging and elevated temperature stresses significantly decreased APX and DHAR activities, and more negative effects were observed when these two stresses combined, which to some extent explained the higher O_2^- generation rate and H_2O_2 concentration. Enhanced GR activity in the treatment of EW_3 relative to EC was found in all three years, which functioned, along with increased SOD, CAT, POX activities, and higher ASA content, to lower ROS and MDA concentrations in the subtending leaf, leading to lesser oxidative damage. Thus, pre-exposure to 3 d waterlogging event improves cotton tolerance to chronic elevated temperature by limiting oxidative stress, thereby having a positive impact on plant performance relative to elevated temperature exposure alone.

5. Conclusions

In conclusion, 3 d soil-waterlogging did not produce statistically comparable plant performance parameters. Whereas, 6 d soil-waterlogging and elevated temperature in isolation can negatively affect plant performance and induce ROS production or ROS-scavenging mechanisms in the subtending leaf of cotton, especially in the treatment of 6 d soil-waterlogging and elevated temperature combined. By comparison, acclimation to 3 d soil-waterlogging stress improved cotton's performance on exposure to subsequently chronic elevated temperature (leaf number increased by 11.4%, whereas the abscission rate reduced by 4.6% in the treatment of 3 d soil-waterlogging and elevated temperature combined compared with the treatment of elevated temperature alone). Furthermore, exposure to 3 d soil-waterlogging induced higher GR activity and enhanced ASA content, together with increased SOD, CAT, POX activities, preventing H_2O_2 and O_2^- accumulation and membrane lipid peroxidation indicated by lower MDA content in the subtending leaf when plants were subsequently exposed to chronic elevated temperature condition.

Contribution

Conceived and designed the experiments: HW, ZZ. Performed the experiments: HW, YC. Analyzed the data: HW, WH. Contributed reagents/materials/analysis tools: HW, ZZ. Wrote the paper: HW, JLS. All the authors contributed to editing the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2019.03.038.

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