African Journal of Agricultural Research Vol. 6(17), pp. 4091-4098, 5 September, 2011
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.336
ISSN 1991-637X ©2011 Academic Journals

Full Length Research Paper

Gibberellin A_3 pretreatment increased antioxidative capacity of cucumber radicles and hypocotyls under suboptimal temperature

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Accepted 21 June, 2011

The effects of GA_3 on the growth and antioxidant capacity of cucumber hypocotyls and radicles under sub-optimal temperature were investigated. The elongation of hypocotyls and radicles was significantly promoted by GA_3 treatment under sub-optimal temperature, and the effects greatly depended on GA_3 concentrations. In the previous reported investigation, there is not much information on the role of GA_3 in modulating reactive oxygen species (ROS) under stress conditions, the present study indicated that GA_3 treatment could decrease excess accumulation of ROS and alleviate lipid peroxidation which was induced by sub-optimal temperature in cucumber hypocotyls and radicles, the alleviating effects were highly correlated with the increasing activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) as well as antioxidative activity indicated as α,α-diphenyl-β-picryllhydrazyl (DPPH) scavenging activity, hydroxyl radical (·HO) scavenging activity and ferrous ion chelating activity. Furthermore, the increasing antioxidative activity was positively related with amylase activity in cucumber cotyledons, suggesting that the distribution of carbohydrate from cotyledons to hypocotyls and radicles might be responsible for higher antioxidant activity induced by GA_3.

Key words: *Cucumis sativus* L., gibberellin, suboptimal temperature, hypocotyls, radicles, antioxidative activity.

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one important vegetable cultivated in solar-greenhouses through the winter and early spring in the north of China. In the process of cultivation, due to the protective effect of greenhouse, extreme low temperature seldom appears, however, suboptimal temperature (below 20/12℃, day/night) may be a limited factor for cucumber production because cucumber is the warm season crop (Liang et al., 2009). When cucumber is sown directly in the field, seed germination and seedling emergence is often slow and non-uniform due to suboptimal soil temperatures. The problems caused by delaying emergence will be aggravated because the probability of soil crust formation becomes greater and the chances of germinating seeds and seedlings to be infected by damping-off causing pathogens such as *Pysthium* increase (Hendrix et al., 1973). Therefore, early and uniform germination, adequate seedling emergence and establishment are critical stage for the commercial growth of cucumber. A common biochemical change occurring when plants are subjected to stress conditions is the accumulation of ROS, which unbalances the cellular redox in favor of oxidized forms, thereby creating oxidative stress that can damage DNA, inactivate enzymes and cause lipid peroxidation (Moldovan et al., 2004). The concentrations of ROS are controlled by antioxidant defense systems, comprised of both enzymatic and non-enzymatic components.

Many studies have shown a correlation between

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resistance to environmental stress and the efficiency of the antioxidant systems (Golan-Goldhirsh et al., 2004; Shi et al., 2007; Khan and Panda, 2008). There are also a great number of indications that chilling causes oxidative stress and the possible involvement of antioxidant systems in the prevention of damage by chilling in crops such as *Digitatia eriantha* (Garbero et al., 2010), cucumber (Kang et al., 2002a) and alfalfa (Wang et al., 2009). Many studies indicate that the regulation of ROS greatly depends on phytohormones including SA and ABA (Bari et al., 2009; Syeed et al., 2010; Shi et al., 2006), GA_3 is an endogenous hormone, whose function mainly includes the stimulation of seed germination and stem elongation (Hartweck, 2008), modulation of flowering (Mutasa-Göttgens et al., 2009; Sharp et al., 2010), breaking seed dormancy and delaying senescence (Hartweck, 2008; Li et al., 2010), however, there is shortage of investigation on the role of GA_3 in mediating the ROS scavenging capacity under suboptimal temperature. Therefore, the objectives of this study are to investigate if GA_3 is involved in the regulation of antioxidant capacity in the process of modulating cucumber hypocotyl and radicle growth under suboptimal temperature.

MATERIALS AND METHODS

Seed germination assay

Seeds of cucumber (*C. sativus* L.) were soaked for 8 h at 28℃ in 100, 200, 400 mg L^{-1} GA_3 or distilled water. After the pre-sowing treatment, all seeds were rinsed with distilled water and germinated on petri dishes containing moisture Whatman 3 mm filter paper and then incubated in a growth chamber at 18/12℃ (suboptimal temperature), and a 13/11 h light/dark photoperiod of 140 µmol m^{-2} s^{-1}. The germination assay was carried out with three replicates in each replicate dish containing 20 seeds. The germination rate (GR), germination velocity index (GVI) and vigor index (VI) were assessed to evaluate the impact of GA_3 on cucumber seed germination under suboptimal temperature. GR was calculated from the proportion of seeds with radicles to the total seeds number. GVI and VI were modified from the method of Raizada and Raghubanshi (2010). GVI = ∑Gt/Dt, here, Gt are the number of germinants on days 1, 2, 3, etc., following the start of the germination test. If more seeds germinate in the fewest number of days, the value of GVI is higher. VI = GVI × S, here S is average length of seedlings at the age of 7 days, the VI shows seedling growth potential. The radical length (RL), hypocotyl length (HL), radical weight (RW), hypocotyl weight (HW) and seedling weight (SW) were measure in the 7th day of germination test.

H_2O_2 concentration assay

The H_2O_2 level was measured according to the method of Patterson et al. (1983). 1 g tissue was homogenized in 3 ml ice-cold acetone. The 1 ml supernatant was mixed with 0.1 ml of 5% (w/v) titanylsulphate and 0.2 ml conc. NH4OH solution, the peroxide-titanium complex was precipitated and this sediment was dissolved in 3 ml 1 M H_2SO_4. The absorbance was read at 415 nm against water. The H_2O_2 content was calculated from a standard curve prepared in a similar way.

Determination of thiobarbituric acid-reactive substances (TBARS) concentration

TBARS were measured according to the method of Shalata et al. (1998). 0.3 g fresh hypocotyl or radicle was homogenized in 3 ml 1.0% (w/v) trichloroacetic acid (TCA) at 4℃. The homogenate was centrifuged at 12,000 g for 20 min and 1 ml of the supernatant was mixed with 3 ml 20% TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated at 95℃ for 30 min and the reaction was stopped by quickly placing in an ice-bath. The cooled mixture was centrifuged at 10,000 g for 10 min, and the absorbance of the supernatant at 532 and 600 nm was read. After subtracting the no-specific absorbance at 600 nm, the TBARS concentration was determined by its extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of antioxidant enzyme activities

For enzyme activity determination, 0.3 g fresh hypocotyl or radicle was ground in 3 ml ice-cold 25 mM PBS buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% PVP. The homogenates were centrifuged at 4℃ for 20 min at 12,000 g and the supernatants were used for determination of enzymatic activities. All spectrophotometric analyses were done by a SHIMADZU UV-2450PC spectrophotometer. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart et al. (1980). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of H_2O_2 according to the method of Patra et al. (1978). GPX activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel et al., 1969). APX activity was measured by the decrease in absorbance at 290 nm as ascorbate was oxidized (Nakano et al., 1981).

Determination of antioxidative activity

The determination of antioxidative activity was done by mixing 0.3 g cucumber hypocotyl or radicle tissue with 3 ml serine borate buffer (100 mM Tris-HCl,10 mM borate, 5 mM serine and 1 mM diethylenetriaminepentacetic acid, pH 7.0). The slurry was centrifuged at 5,000 g for 10 min at 4℃ and the supernatant assayed for the in vitro antioxidant activity. Radical scavenging power (RSP) of extracts were determined as reported by Manda et al. (2010). The 100 µl extract was added to 2.9 ml 0.1 mM DPPH solution and kept for 30 min at room temperature under the darkness; the resulting color was measured at 520 nm against blanks. The radical scavenging activity was calculated by the following formula:

$$RSP = [1-(A_{s30}/B_{s30})] \times 100\%$$

Where A_{s30} is absorbance of sample and B_{s30} is absorbance of blank.

Hydroxyl radical (·OH) scavenging activity was measured according to Manda et al. (2010). The reaction mixture (2.1 ml) contained 1 ml 1.5 mM $FeSO_4$, 0.7 ml 6 mM H_2O_2, 0.3 ml 20 mM sodium salicylate and 100 µl of the cucumber tissue extracts. This mixture was incubated at 37℃ for 1 h and then the absorbance was measured at 562 nm. The percentage scavenging effect was calculated as:

$$\text{Scavenging effect} = [1-(A_1-A_2)/A_0] \times 100\%$$

Where A_0 was the absorbance of the control (without extract), A_1 the absorbance in the presence of the extract and A_2 the absorbance without sodium salicylate. Fe^{2+}-chelating activity was

Table 1. Effect of GA_3 on the germination rate (GR), germination velocity index (GVI) and vigor index (VI) of cucumber under suboptimal temperature (day/night temperature 18/12℃).

GA_3 cocentrations ($mg \cdot L^{-1}$)	GR (%)	GVI	VI
0	90.91±4.55[b]	32.08±2.78[b]	164.25±8.19[d]
100	92.75±5.02[b]	32.97±3.62[b]	194.85±6.37[c]
200	97.10±2.51[a]	43.73±3.94[a]	321.85±6.04[a]
400	92.75±7.14[b]	43.80±4.19[a]	302.66±7.81[b]

The result was measured in 7th day after experiment beginning. Data are means ± SD of three replicates in one experiment. Value in each column by different letters (a to d) are significantly different at $P<0.05$.

measured according to Manda et al. (2010). The reaction mixture (2.0 ml) contained 100 µl of cucumber tissue extracts, 100 µl 0.6 mM $FeCl_2$ and 1.7 ml deionised water. The mixture was shaken vigorously and left at room temperature for 5 min; 100 µl of ferrozine (5 mM in methanol) were then added, mixed and left for another 5 min, then the absorbance was read at 562 nm against a blank. Disodium ethylenediamineteracetic acid (EDTA-Na_2) was used as the control. The chelating activity of the extract for Fe^{2+} was calculated as:

Chelating effect = $[1-(A_1-A_2)/A_0] \times 100\%$

Where A_0 was the absorbance of the control (without extract), A_1, the absobance in the presence of the extract and A_2 the absorbance without ferrozine.

Total amylase activity assay

For total amylase activity assay, 1 g cotyledons were homogenized with 5 ml 20 mM sodium acetate buffer (pH 5.0) containing 1 mM $CaCl_2$ (Miyagi et al., 1990). The mixture was aged for 1 h at room temperature, centrifuged at 10,000 g and the supernatant was used for the amylase activity assay. To 0.5 ml of soluble starch (2%) in 50 mM sodium acetate buffer (pH 5.0) containing 1 mM $CaCl_2$, 0.5 ml of enzyme extract was added and incubated at 35℃ for 20 min. Reducing sugars formed were measured (Nelson, 1944).

Statistical analysis

Data were analyzed by variance using the SPSS 11.0 statistical software package, differences were assessed by the Ducan's test and they were significant with *p* values <0.05.

RESULTS

Cucumber germination and seedling growth

To investigate the physiological effects of GA_3 on germination under suboptimal temperature, increasing concentrations of GA_3 were applied for cucumber seeds pretreatment, As shown in Table 1, all GA_3 treatments significantly increased the germination rate (GR), germination velocity index (GVI) and vigor index (VI) of cucumber under suboptimal temperature, however, its effects were closely related with concentrations, among all treatments, 200 mg L^{-1} GA_3 treatment showed the strongest effect, and increased GR, GVI and VI by 6.81, 36.32 and 95.95% compared to the control ($P<0.05$), respectively. The growth of cucumber seedling was shown in Table 2, GA_3 treatment significantly increased elongation of cucumber radicle and hypocotyl as well as the fresh weight under suboptimal temperature, in the 7th day of the treatment, the radicle length was the largest in the 200 mg L^{-1} GA_3 treatment, and was increased by 43.75% compared to the control, while the hypocotyl length was the largest in the 400 mg L^{-1} GA_3 treatment, and was increased by 80.88% ($P<0.05$). As for the fresh weight, the 200 mg L^{-1} GA_3 treatment exhibited the most positive effects and increased radical fresh weight, hypocotyl fresh weight and total fresh weight by 105.07, 36.69 and 51.49% compared to the control ($P<0.05$), respectively.

H_2O_2 concentration and lipid peroxidation

Figure 1A shows the changes of H_2O_2 concentration in radicle and hypocotyl of cucumber seedlings, pretreatment with GA_3 showed obvious effects on H_2O_2 accumulation under suboptimal temperature, H_2O_2 concentrations of radicle were decreased by 22.62 and 24.40% in the 100 and 200 mg L^{-1} treatments compared to the control ($P<0.05$), respectively, while 400 mg L^{-1} treatment did not significantly affect the H_2O_2 accumulation in cucumber radicle. Compared with the radicle, GA_3 pretreatment had much more effects on the H_2O_2 accumulation of hypocotyl, the H_2O_2 levels in 100, 200 and 400 mg L^{-1} GA_3 treatment were34.05, 81.83 and 61.09% lower than that of the control ($P<0.05$), respectively. As an indicator of lipid peroxidation, TBARS concentration was measured. TBARS accumulation greatly depended on GA_3 concentration, 100 mg L^{-1} GA_3 treatment did not significantly affect the TBARS level of cucumber radicle, and 200 and 400 mg L^{-1} GA_3 treatment decreased TBARS level of cucumber radicle by 23.01 and 29.86% compared to the control ($P<0.05$), respectively. Like the change of H_2O_2, all GA_3 treatments significantly decreased TBARS accumulation of cucumber hypocotyl, 100, 200 and 400 mg L^{-1} GA_3 treatment led to 20.83, 29.26 and 28.76% decrease in

Table 2. Effects of GA_3 on the radicle length (RL), hypocotyl length (HL), radical fresh weight (RW), hypocotyl fresh weight (HW), seedling fresh weight (SW) of cucumber under suboptimal temperature (day/night temperature 18/12℃).

GA_3 concentration ($mg \cdot L^{-1}$)	RL(cm)	HL(cm)	RW(mg/seedling)	HW(mg/seedling)	SW(mg/seedling)
0	5.12±0.24^{c}	0.68±0.02^{c}	27.6±2.97^{c}	14.28±1.43^{b}	90.5±4.36^{c}
100	5.91±0.17^{b}	1.05±0.08^{b}	46.7±3.30^{b}	16.67±2.97^{ab}	119.0±4.59^{b}
200	7.36±0.10^{a}	1.17±0.07^{a}	56.6±4.37^{a}	19.52±3.29^{a}	137.1±7.55^{a}
400	6.91±0.19^{a}	1.23±0.07^{a}	44.3±6.54^{b}	17.62±2.97^{ab}	131.4±6.54^{ab}

The result was measured in 7th day after experiment beginning. Data are means ± SD of three replicates in one experiment. Value in each column by different letters (a to c) are significantly different at $P<0.05$.

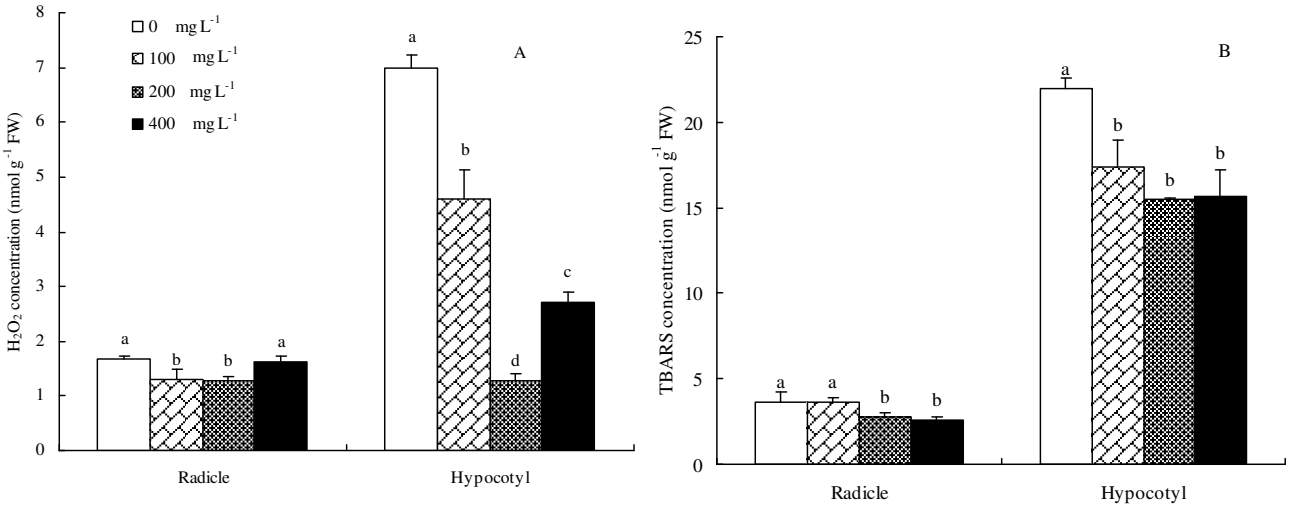

Figure 1. Effects of GA_3 on the concentrations of H_2O_2 (A) and TBARS (B) in cucumber hypocotyls and radicles under suboptimal temperature (day/night temperature 18/12℃). Data are means ± SD of three replicates in one experiment. Mean values by different letters (a to d) are significantly different at $P<0.05$.

TBARS level of cucumber hypocotyl ($P<0.05$) (Figure 1B).

Antioxidant enzyme activities

Pretreatment with GA_3 showed similar effects on activities of SOD and GPX in cucumber radicle and hypocotyl under suboptimal temperature, and their activities were the highest in the 200 mg L^{-1} GA_3 treatment, in which SOD activities were 4.10 and 4.41 times of the control in radicle and hypocotyl ($P<0.05$), respectively (Figure 2A); and GPX activities were 1.84 and 1.40 times of the control in radicle and hypocotyl ($P<0.05$), respectively (Figure 2C). The effects of GA_3 treatment on CAT and APX activities in cucumber radicle were not significant, while their activities in cucumber hypocotyls were greatly induced by GA_3, especially the 200 mg L^{-1} treatment, in which CAT and APX activities were 1.41 and 1.40 times of the control ($P<0.05$), respectively (Figure 2C and D). Antioxidative activity in general, the DPPH radical scavenging activity, hydroxyl radical (·HO) scavenging activity and ferrous ion chelating activity were lower in the radicals than in the hypocotyls (Figure 3). And the three indexes exhibited the same trends: with the treatment of GA_3 concentration, the activity increased except 400 $mg \cdot L^{-1}$ treatment, in which antioxidative activity was lower than that in 200 $mg \cdot L^{-1}$, but was still higher than that in the control (Figure 3).

The most significant increases in antioxidant activity were all observed at 200 $mg \cdot L^{-1}$, especially in hypocotyls, where DPPH radical scavenging activity, hydroxyl radical (HO·) scavenging activity and ferrous ion chelating activity increased by 95.73, 43.54 and 205.76% compared to the control ($P<0.05$), respectively (Figure 3A, B and C).

Amylase

Compared with the control, GA_3 treatment increased activity of amylase in the cotyledons under suboptimal

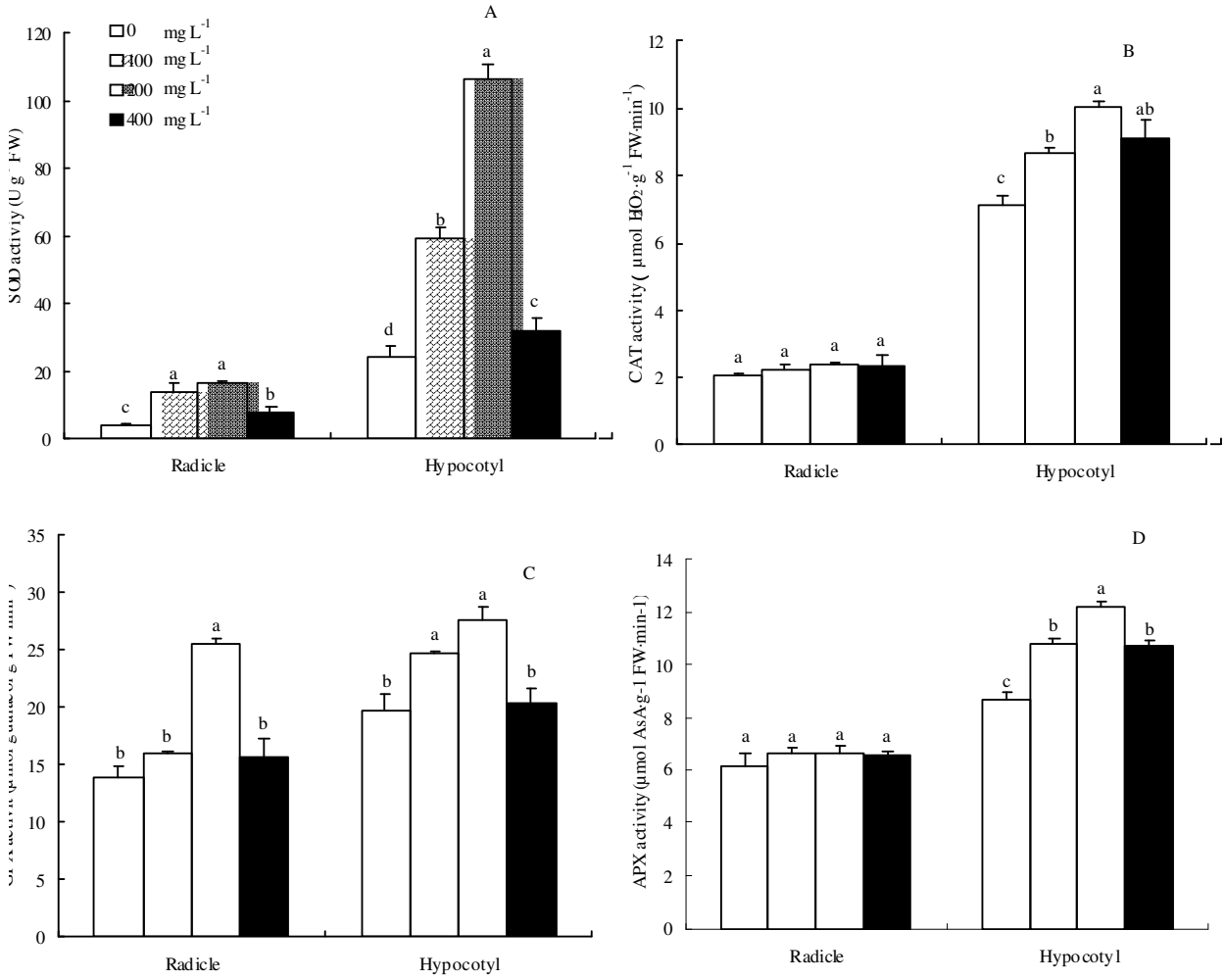

Figure 2. Effects of GA_3 on the activities of SOD (A), CAT (B), GPX (C) and APX (D) in cucumber hypocotyls and radicles under suboptimal temperature (day/night temperature 18/12℃). Data are means ± SD of three replicates in one experiment. Mean values by different letters (a-d) are significantly different at P<0.05.

temperature (Figure 4), and the increasing level depended on the GA_3 concentrations. There were no significant difference between the control and 100 mg L^{-1} GA_3 treatment, and amylase activities in 200 and 400 mg L^{-1} GA_3 treatments were 100 and 46% higher than that in the control (P<0.05), respectively.

DISCUSSION

The results obtained in the present study indicated that GA_3 application to a different degree could promote the growth and physio-biochemical processes in cucumber seedlings under suboptimal temperature. The similar role of GA_3 has been obtained in salt-treated chickpea (Kaur et al., 1998). Under suboptimal temperature, plants are overloaded with ROS, which inhibits several plant processes and imposes damage to the plants in different ways. It has been reported that plant growth regulators such as gibberellins (GA_3) can play a vital role in the tolerance to abiotic stress including heavy metals, salt stress by improving activities of antioxidant enzymes and by preventing lipid peroxidation (maggio et al., 2010; Siddiqui et al., 2010). In the present study, application of GA_3 significantly decreased accumulation of H_2O_2 in cucumber hypocotyls and radicles under suboptimal temperature, and lower accumulation of H_2O_2 may be mainly responsible for lower lipid peroxidation which was confirmed by lower accumulation of TBARS. It is well known that the induced increase of antioxidant enzyme activities including SOD, CAT, GPX and APX can be considered as an important mechanism in the cellular defense strategy against oxidative stress, in the present study, application of GA_3 increased the activities of all these enzymes, this might be one important mechanism of GA_3 decreasing accumulation of ROS and lower lipid

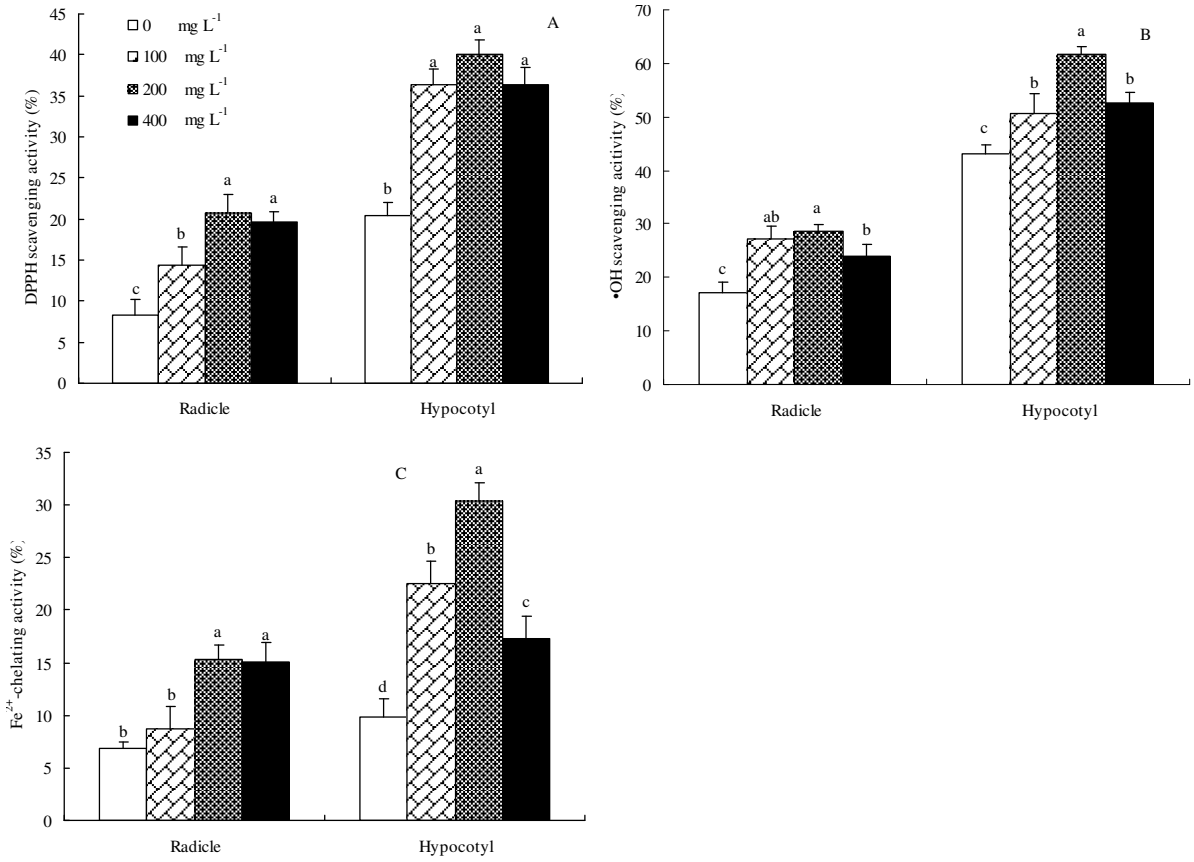

Figure 3. Effects of GA_3 on the activities of DPPH scavenging (A), ·OH scavenging (B) and Fe2+-chelating in cucumber hypocotyls and radicles under suboptimal temperature (day/night temperature 18/12℃). Data are means ± SD of three replicates in one experiment. Mean values by different letters (a to d) are significantly different at $P<0.05$.

peroxidation in cucumber hypocotyl and radicle. Compared to other growth regulators, there is not much information on the role of GA on abiotic stress responses, Heckman et al. (2002) have obtained that Kentucky bluegrass plants treated with a GA inhibitor were less heat tolerant than untreated plants, pointing out a role of GAs in thermotolerance.

Alonso-Ramírez et al. (2009) has reported exogenous application of GA_3 was able to reverse the inhibitory effect of salt, oxidative and heat stress in the germination and seedling establishment of Arabidopsis, and this effect was attributed to the induced SA biosynthesis. To directly make clear the antioxidative activity induced by GA_3 in cucumber hypcotyls and radicles under suboptimal temperature, DPPH radical scaven-ging activity, hydroxyl Radical (·HO) scavenging activity and ferrous ion chelating ability were investigated in the present study. DPPH-radical scavenging activity is a measure of non-enzymatic antioxidant activity, it has been reported that the chilling-tolerant cucumber radicles had higher levels of DPPH scavenging activity (Kang et al., 2002b) and there is a correlation between DPPH scavenging activity in cucumber radicles and vigour difference in chilling sensitivity (Kang et al., 2002a), therefore, the increased DPPH-radical scavenging activity provided a further proof that GA_3 induced antioxidative activity in cucumber hypocotyls and radicles under suboptimal temperature. Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as iron and copper (Halliwell, 2006). Hydroxyl radicals actively react with lipid, polypeptides, proteins, and DNA, and the efficient scavenging of hydroxyl radicals is vital for maintaining normal metabolism of organisms. In the present study, GA_3 treatment could significantly increased hydroxyl radical scavenging activity and ferrous ions chelating activity in cucumber hypocotyls and radicles under suboptimal temperature, which could be partly response-ble for lower lipid peroxidation. In common, the germination stimulation by gibberellin is greatly attributed to increasing amylase expression and activity, in the present study, the similar results were obtained in cucumber cotyledon.

The amylase of cotyledon in the germination process plays an important role in the formation of glucose from starch, its decrease restricts thedistribution of carbohy-drates to the embryonic axis, which can result in reduced growth of hypocotyl and radicles. Kaur et al. (1998)

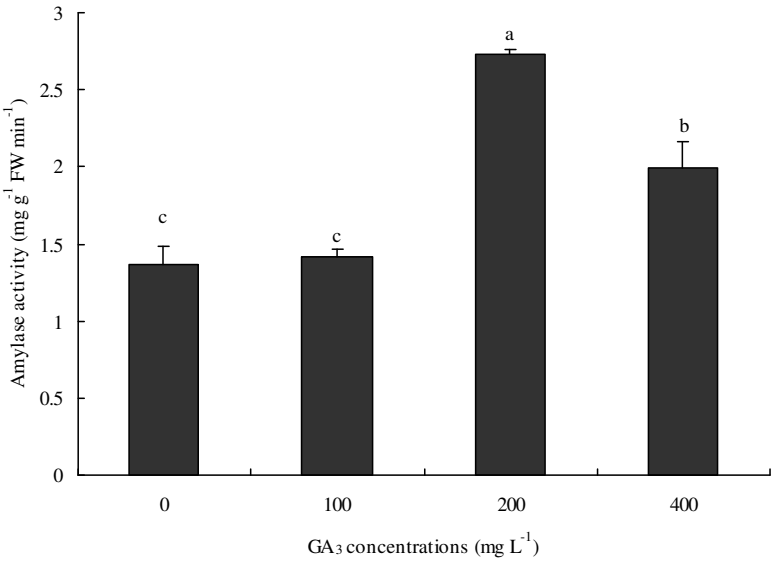

Figure 4. Effects of GA_3 on the activity of amylase in cucumber cotyledons under suboptimal temperature (day/night temperature 18/12℃). Data are means ± SD of three replicates in one experiment. Mean values by different letters (a to d) are significantly different at $P<0.05$.

obtained that GA_3 reversed the negative effects on salt stress in chickpea by enhancing amylase activity and mobilization of starch in cotyledons. Based on the observation, it could be concluded that the stimulated growth of cucumber hypocotyls and radicles by GA_3 under suboptimal temperature might also be partly due to the induced amylase activity. In dark germinated soybean, the close correlation between α-amylase and increased phenolic synthesis, antioxidant activity, and GPX activity were obtained (McCue et al., 2004), furthermore, McCue et al. (2004) attributed these phenomenon to carbohydrate mobilization, therefore, in the present study, antioxidative activity induced by GA_3 might be closely related with increasing amylase activity.

ACKNOWLEDGEMENT

This work was supported by the National Basic Research Program of China (2009CB119000).

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