

Phytohormonal Regulation of Antioxidant Systems in Petals of Drought Stressed Pot Marigold (*Calendula officinalis* L.)

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ABSTRACT

Drought is an important abiotic stress limiting plant performance. Generation of reactive oxygen species (ROS) is enhanced under stresses. Two greenhouse experiments were conducted to evaluate the effect of phytohormones on the changes of antioxidant enzymes and carotenoids in petals of pot marigold (*Calendula officinalis* L.) under drought stress. Results showed that the activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) increased 47 and 73%, respectively, in petals under water deficit conditions compared with the control plants. Spraying with gibberellic acid (GA₃) and benzyl amino purine (BAP) alleviated drought effects, but application of abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and brassinolid (BR) induced the activity of these enzymes. In the case of peroxidase (POD, EC 1.11.1.7), GA₃ enhanced the enzyme activity unlike the other enzymes and the rest of the phytohormones had no significant effect on POD activity under either stressed or non-stressed condition. Concentration of carotenoids was affected by drought and hormone treatments. Concentration of carotenoids increased under water deficit but, GA₃, BAP and JA had inhibitory effects on lycopene and carotene synthesis, while the rest of the hormones increased them. Spraying with GA₃ increased luteoxanthin concentration in petals by 35 and 20% in comparison with the non-stressed and stressed environments, respectively. The decrease in POD activity under stress suggests that other mechanisms might be involved for ROS scavenging in petals of pot marigold.

Keywords: Antioxidant, Carotenoid, Drought, Petal, Phytohormone.

INTRODUCTION

Biotic and abiotic stresses adversely affect growth, metabolism and plant yields (Yildiz Aktas *et al.*, 2007), and prevent them from expressing their full genetic potentials (Zhu, 2002). Reactive oxygen species (ROS) are produced in normal and stressed cells (Alscher *et al.*, 2002). Under stressful conditions, ROS is accumulated biochemically in cells, however, it is kept under tight control by antioxidant system. ROS is linked to phytohormones like abscisic acid (ABA) and

other ions and organic molecules. If cellular quantities of ROS are kept at relatively low levels, they would act as a part of stress signaling pathway, but if increased to a certain level, they could phytotoxically damage the cell compartments (Cruz de Carvalho, 2008). The ROS is produced from partial reduction of molecular oxygen (Unyayar and Ozlem, 2005). In arid and semi arid regions, plants undergo oxidative stress that is a secondary drought stress. Plants protect themselves by increasing scavenging capacity of ROS via antioxidant enzymes and molecules (Yildiz Aktas *et al.*, 2007).

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Exogenous ABA can result in increased production of ROS and subsequently induction of the antioxidant system. It has been reported that exogenous application of ABA significantly increases the activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APOX, EC 1.11.1.11), peroxidase (POD, EC 1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2) in the leaves. Under this condition, increases in quantities of ascorbate, reduced glutathione, tocopherole and carotenoids as non-enzymatic antioxidants were also reported (Unyayar and Ozlem, 2005; Yildiz Aktas *et al.*, 2007).

Down regulation of photosystem II (PSII) activity occurs under drought stress due to imbalance between light capture and its utilization, resulting in an imbalance between electrons generation and utilization (Peltzer *et al.*, 2002). Electrons leaked from these chains can react with O₂ during normal and abnormal metabolism to produce ROS (O₂⁻, H₂O₂, HO[•] and ¹O₂) (Pan *et al.*, 2006). Mehler reaction or water – water cycle occurs in chloroplast and produces super oxide as side effect that is dismutated by SOD and APOX (Cruz de Carvalho, 2008). Water deficit forces the root system to send stress signals up to the leaves, and phytohormone ABA is the major signal in this event (Zhang and Davies, 1987; Jiang and Hartung, 2008).

Carotenoids are accessory pigments in light harvesting complexes (LHC) and effective protectants against photo-oxidative damage by quenching triplet state of chlorophyll and scavenging singlet oxygen (Sun *et al.*, 1996; Baranski *et al.*, 2005). They are the substrates for the biosynthesis of some phytohormones (Kim and Dellapenna, 2006). Lutein is a critical structural component of the LHC, whereas Zeaxanthin plays a role in non-photochemical quenching and is derived from β-carotene (Tian *et al.*, 2003).

Pan *et al.* (2006) reported that the activity of SOD and POD were up regulated by salt and drought stress, while CAT activity was decreased. Application of ascorbate and salicylic acid on drought stressed Okra (*Hibiscus esculentus* L.) plants mitigated the

stress effects and increased carotenoid biosynthesis (Baghizadeh *et al.*, 2009). Somasundaram *et al.* (2009) found an increase in activities of SOD, APOX and POD in root, stem, and leaf of *Sesamum indicum* (L.) under water deficit conditions and with simultaneous application of pachlobutrazol and ABA. Seed soaking and foliar spraying of salt stressed maize plants with brassinolid and salicylic acid significantly increased the activity of antioxidant enzymes and endogenous levels of auxine, gibberellin and zeatin (El-Khallal *et al.*, 2009). Chaparzadeh *et al.* (2004) reported decreases in SOD and POD activities under salinity in roots and leaves of pot marigold (*Calendula officinalis* L.).

Pot marigold belongs to the Asteraceae and is an important medicinal and ornamental plant (Kishimoto *et al.*, 2005). The extracts obtained from pot marigold possess a wide range of pharmacological effects such as wound-healing, anti-inflammatory, antibacterial, immunostimulative and antitumoral effects (Pintea *et al.*, 2003). There is a great deal of information found in the literature on drought stress, but the reports on the effects of water deficit and the role of hormonal regulation in this plant are rare. To the best of our knowledge, there is not any evidence on the effect of drought induced activity of antioxidant mechanisms in pot marigold, especially in petals which are the major parts of the plants for utilization.

Therefore, in this study, activity of enzymatic and content of non-enzymatic antioxidants in petals of pot marigold have been discussed for the first time under water limitation, and the effect of major phytohormones on the activity of antioxidants has been described.

MATERIALS AND METHODS

Plant Materials and Treatments

Pot marigold (*C. officinalis*) was grown in a greenhouse at 25/15°C day/night temperature and 14/10 hours day/night photoperiod. Pots were divided into two groups-four replicates

each- both well watered until flower unfolding stage (when the first flowers were seen). At this stage, a group of pots was exposed to drought stress until the termination of flowering (about 14 days) while the second group was kept as control. After two weeks, soil water content was measured by MPkit (MPM160 Moisture Probe Meter, ICT international, Australia) to ensure the occurrence of the drought stress. Well watered pots had 92% water content, while in stressed pots water content decreased to 51%. At this stage plant samples were taken for analysis and then pots were watered to recover the stress condition. Phytohormones used in this study contained GA₃ (50 μM), BAP (40 μM), ABA (100 μM), BR (20 μM), JA (10 μM) and SA (10 μM) in addition to the control (Davies, 2007) that was sprayed three times in the early morning, 1, 6, and 11 days after the flower unfolding stage. Control plants were sprayed with pure water. Sampling for enzyme assay and carotenoid measurement was done at the full flowering stage (day 15 of the first flower unfolding). The greenhouse experiment was repeated in two growing seasons (2008-2009) with four replications of the treatments in each factorial experiment.

Antioxidant Enzyme Assay

The petal samples were frozen in liquid nitrogen and stored at -30°C. One g of the frozen petals was homogenized in mortar with 5 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol and 2% polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000g for 25 min and the supernatant was used for SOD, CAT, and POD assay.

SOD Assay

The activity of SOD (EC 1.15.1.1) was determined according to Beyer and Fridovich (1987). In small glass tubes, 20 μL of enzyme supernatant were added to 50

mM potassium phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 μM nitro blue tetrazolium (NBT), and 0.025% triton-X-100. Reaction was started under fluorescent light for 10 minutes by adding 10 μL of riboflavin solution. Absorbance of the solution was measured at 560 nm for both blank and control. SOD activity was expressed as Unit mg⁻¹ DW.

CAT Assay

The activity of CAT (EC 1.11.1.6) was assayed according to Chance and Maehly (1955). A 1.5 mL reaction mixture containing 30 μL water, 50 μL 1M Tris-HCl buffer (pH 8.0), 5 mM EDTA and 900 μL 10 mM H₂O₂ was added to 20 μL of enzyme supernatant. The decrease in the absorbance at 240 nm was recorded for 60 seconds. CAT activity was expressed as absorbance in mg protein per min.

POD Assay

The activity of POD (EC 1.11.1.7) was determined spectrophotometrically at 470 nm according to Yamane *et al.* (1999) in a 3 mL reaction mixture containing 1.5 mL 0.1 M potassium phosphate buffer (pH 7.0), 600 μL 10 mM guaiacol, 800 μL 4 mM H₂O₂ and 100 μL crude enzyme. POD activity was expressed as μmols of guaiacol oxidized to tetraguaiacol by a unit of enzyme per min.

Lipid Peroxidation Assay

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content of petals as described by Stewart and Bewley (1980) in a colorimetric method. Petal samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA) and centrifuged. Then, 0.5 ml of supernatant was mixed with 2 ml of 20% TCA containing 0.5% thiobarbituric acid. The mixture was incubated at 95°C for 30 minutes. The



samples were centrifuged at 10,000g for 10 minutes. The absorbance of the supernatant was read at 532 and 600 nm. The amount of MDA was calculated from the extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Carotenoid Extraction and Measurement

The carotenoids measured in this study were selected from a list prepared by Kishimoto *et al.* (2005) based on their concentrations in petals of orange-flowered pot marigold. They included flavoxanthin, luteoxanthin, total lycopene, total carotene and lutein which have the highest percentage of total carotenoids. These compounds were purified and fractionated by high performance liquid chromatography (HPLC) as described by Kishimoto *et al.* (2005).

Statistical Analysis

The data were obtained from two consecutive factorial experiments based on completely randomized design (CRD). Each separate greenhouse experiment consisted of four replicates. Combined analysis procedure was performed after normality and homogeneity of variances tests. Means

were compared with Tukey's honestly test at 5% statistical probability level.

RESULTS

SOD Activity

Interaction of drought and hormones on the SOD activity was significant. In the petals of pot marigold, SOD activity increased under drought stress in comparison with control at any hormone application level. GA_3 and BAP increased the enzyme activity less than other hormones and control under drought and GA_3 treated petals had lower SOD activity than BAP treated ones in both stressed and non-stressed pots. Other hormones increased the enzyme activity and the ABA application had the highest activity (Figure 1).

CAT Activity

There was a significant difference in the interaction of drought and hormones on the CAT activity. The activity of CAT in petals of pot marigold was similar to SOD under drought condition and the enzyme activity increased under water limitation and

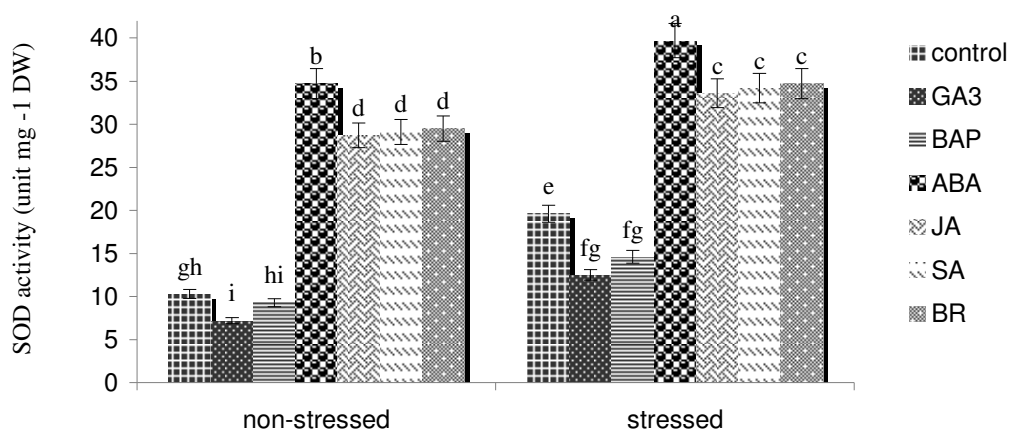


Figure1. Changes in SOD (Superoxide dismutase) activity under drought condition as affected by phytohormones ($P < 0.05$).

hormone application comparing to the non-stress environment. However, ABA and JA had the same effect (Figure 2).

POD Activity

Under water deficit condition, POD activity in petals decreased. GA₃ increased POD activity but BAP and the rest of the hormones had no significant effect on its activity (Figure 3).

POD/CAT Ratio

Increasing the ratio of POD/CAT enzymes

indicates the oxidative stress in plants. Significant interaction was seen between drought and hormones on POD/CAT ratio. In both environments the greatest ratio of POD/CAT was related to GA₃ application (Table 1).

Lipid Peroxidation

Degree of lipid peroxidation in terms of MDA concentration increased under drought stress and reached 25.6 nmol g⁻¹ in stressed sample, which was three fold greater than the non-stressed control. The lowest value for MDA content was recorded in GA₃ spraying under non-stress environment (Table 1).

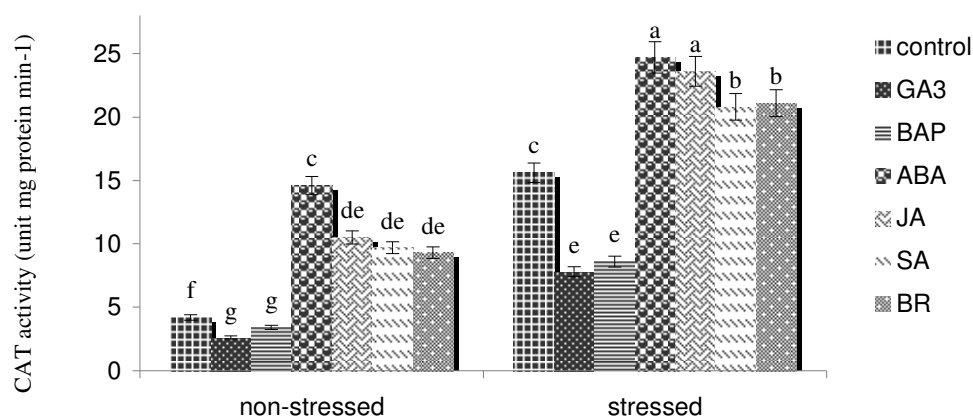


Figure2. Changes in CAT (Catalase) activity under drought condition as affected by phytohormones (P<0.05).

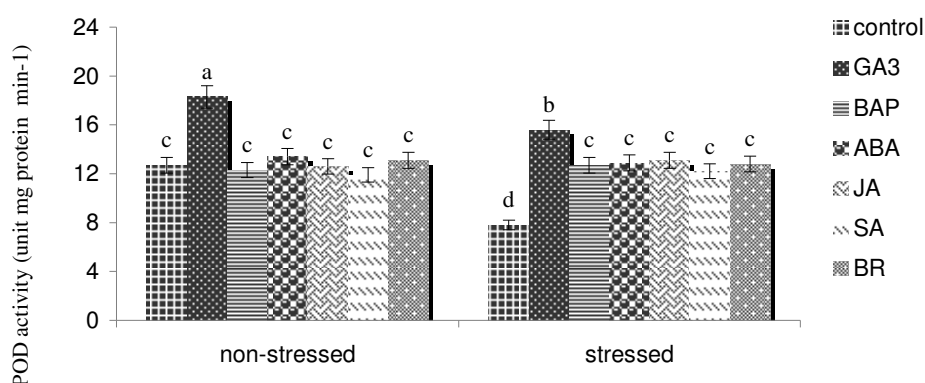


Figure3. Changes in POD (Peroxidase) activity under drought condition as affected by phytohormones (P<0.05).

**Table 1.** Changes in carotenoid composition and concentration (percent of total carotenoids), lipid peroxidation (amount of malondialdehyde, MDA) and ratio of POD/CAT (peroxidase to catalase) under drought stress as affected by phytohormones.

		Total lycopene (%)	Total carotene (%)	Lutein (%)	Flavoxanthin (%)	Luteoxanthin (%)	Lipid peroxidation (nmol MDA g ⁻¹ petal)	POD ^a CAT
Non-stressed	Control	5.3±0.15	2.7±0.06	2.5±0.03	26.2±1.84	12.3±0.48	8.1±1.24	3.02±0.13
	GA ₃	4.9±0.12	2.1±0.04	3.6±0.07	27.5±1.92	13.6±0.62	6.3±0.95	7.04±0.25
	BAP	4.8±0.12	2.0±0.04	3.2±0.04	27.6±1.92	12.9±0.6	6.5±0.96	3.62±0.14
	ABA	6.1±0.15	1.8±0.04	2.1±0.01	23.1±1.38	10.8±0.3	9.4±1.32	0.92±0.03
	JA	4.5±0.1	3.2±0.08	1.9±0.01	22.6±1.12	11.0±0.32	7.5±1.09	1.2±0.04
	SA	6.2±0.16	3.5±0.08	3.2±0.04	28.2±2.06	13.1±0.6	6.9±1.02	1.23±0.04
	BR	6.4±0.16	3.4±0.08	3.5±0.07	27.8±1.93	13.0±0.6	7.1±1.04	1.41±0.05
Stressed	Control	6.4±0.16	3.5±0.09	3.2±0.04	28.6±2.07	15.2±0.84	25.6±2.41	0.5±0.011
	GA ₃	5.1±0.15	3.0±0.08	4.5±0.13	30.3±2.38	18.9±1.08	16.2±2.02	2±0.10
	BAP	5.0±0.13	2.8±0.07	4.1±0.1	29.7±2.21	18.3±1.06	16.4±2.03	1.48±0.07
	ABA	7.1±0.18	3.1±0.08	2.5±0.02	25.4±1.56	13.5±0.61	15.1±1.85	0.52±0.01
	JA	5.3±0.15	4.2±0.11	2.8±0.03	26.1±1.8	12.7±0.51	18.5±2.11	0.56±0.012
	SA	7.5±0.19	4.0±0.11	3.9±0.09	29.6±2.19	16.7±0.95	15.6±1.92	0.59±0.019
	BR	7.8±0.21	3.9±0.11	3.5±0.06	30.0±2.37	16.3±0.95	15.3±1.9	0.61±0.022

^aPOD: Peroxidase, CAT: Catalase

Carotenoid Composition and Concentration

Interaction between drought stress and hormone application was significant on the carotenoids. Carotenoid concentration was higher in drought stressed petals with hormone spraying comparing to the non-stress condition. Phytohormones had different effects on carotenoid composition and concentration. Lycopene and carotene concentrations increased under BR, SA, and ABA spraying in comparison with control in both stressed and non-stressed conditions, while JA, GA₃, and BAP had inhibitory effects on lycopene biosynthesis. In the case of carotene, GA₃, BAP, and ABA inhibited the total carotene accumulation comparing to the control in both environments, whereas the rest of the hormones increased its concentration. JA and ABA decreased flavoxanthin and luteoxanthin concentrations compared with control in each environment while the rest of the hormones had stimulatory effects (Table 1).

DISCUSSION

Plants are the natural producers of ROS and SOD is the front line enzyme in ROS attack since it rapidly scavenges superoxide and dismutates it to oxygen and H₂O₂ (Cruz de Carvalho, 2008). There is a considerable number of reports in the literature on the fluctuations of SOD activity under stress conditions (Alscher *et al.*, 2002; Zhu, 2002; Yildiz Aktas *et al.*, 2007). In this experiment, generation of superoxide radical increased in pot marigold petals due to drought stress and consequently the activity of SOD was increased. Spraying with GA₃ caused the lowest SOD activity showing the potential of GA₃ for alleviation of drought stress effects. Somasundaram *et al.* (2009) reported that spraying with paclobutrazol increased the SOD activity in the stems of *Sesamum indicum* (L.). Paclobutrazol is a triazole compound inhibits gibberellin biosynthesis. Therefore, in the absence of GA₃, the ROS production increases and subsequently SOD activity will increase. It seems that BAP acts like GA₃ and ameliorates the drought effects in plants. There is a large body of literature on the effect of ABA action in abiotic stresses and

Drought stress effects on *Calendula* antioxidant system

It is known that ABA is a stress hormone (Jiang and Hartung, 2008; Yildiz Aktas *et al.*, 2007; Cruz de Carvalho, 2008). In the present study, the application of ABA increased SOD activity. This is in agreement with other reports (Yildiz Aktas *et al.*, 2007), but it is considerable that SA, JA, and BR act like ABA and activate plant defense system against the ROS. It can be concluded that these hormones are responsible for more ROS generation. El-Khallal *et al.* (2009) found the same results with applying BR and SA under salinity in maize plants.

As SOD dismutates O_2^- to H_2O_2 , CAT is the enzyme involving in scavenging of this species of ROS in the peroxisomes. Similar to SOD, the CAT activity increased under water deficit in this study. It is believed that CAT activity is only enhanced under severe drought stress, and under moderate water deficit, H_2O_2 scavenging is preferably made by ascorbic acid through the ascorbate/glutathione cycle (Cruz de Carvalho, 2008). Increase in CAT activity in pot marigold petals probably indicates the severe stress conditions. On the other hand, it is proposed that CAT activity does not play a critical role in the wilting of florets (Yamane *et al.*, 1999). Therefore, an increase in the ratio of POD/CAT activity might promote oxidative stress in membranes of perianth. ABA and JA treated petals had the highest CAT activity. These results are in agreement with those of the others (Unyayar and Ozlem Cekic, 2005; Yildiz Aktas *et al.*, 2007).

Under drought stress, POD activity decreased in petals of pot marigold. Since CAT and POD act the same with different modes of action (Yamane *et al.*, 1999), this indicates that the importance of CAT in the pot marigold for scavenging ROS is greater than POD. Reduction in the ratio of POD/CAT confirms this indication. Many authors have shown that POD activity increased in the other parts of plants such as root, stem, and leaf (Chaparzadeh *et al.*, 2004; Somasundaram *et al.*, 2009). Among

phytohormones only GA_3 increased POD activity while the rest of the hormones had no significant effect on this enzyme. This result is in contrast with those of the others who demonstrated an increase in POD activity after SA, and BR applications (El-Khallal *et al.*, 2009). Treatment of root, stem, and leaf of *Sesamum indicum* (L.) with paclobutrazol (gibberellin inhibitor) and ABA increased POD activity (Somasundaram *et al.*, 2009). We concluded that GA_3 had a role in flower senescence because it increased the ratio of POD/CAT and enhanced the oxidative stress that lead to programmed cell death. Also, the POD activity at the sampling stage was probably low and increased afterwards. The work by Yamane *et al.* (1999) supports the second hypothesis.

Carotenoid biosynthesis occurs within the chloroplasts which is called isoprenoid pathway. It begins with mevalonic acid and in a series of reactions 40-carbon carotenoids (polyterpenes) are assembled. This pathway is linked to the biosynthesis of some phytohormones like gibberellins and ABA. In this pathway, firstly, lycopene and carotenes, then lutein, and finally, xanthins are synthesized (Rissler and Pogson 2001). Carotenoids as antioxidant molecules were accumulated in the petals of pot marigold in comparison with control plants. Munne-Bosch and Alegre (2000) reported that carotenoid increased under drought stress in *Rosmarinus officinalis*. Spraying of BR, SA, and ABA stimulated the lycopene and carotene synthesis in petals. Aberu and Munne-Bosch (2009) found that SA had an inhibitory effect on the lutein and β -carotene concentration in the seeds of *Arabidopsis thaliana*. In the present study, SA induced the biosynthesis of all studied carotenoids in the petals of pot marigold. GA_3 and BAP inhibited the biosynthesis of carotenoids at early steps (lycopene and carotene synthesis reactions) but final reactions (lutein, flavo- and luteoxanthin) were enhanced. JA had an inhibitory effect, except on lutein biosynthesis. BR was stimulant for biosynthesis of all carotenoids. ABA



operated unlike GA₃ and produced more carotenoids from the first step reactions. Spraying with GA₃, kinetin and BR increased the total carotenoid concentration in rose plant (Kandil *et al.*, 2007) but the changes in carotenoid composition in response to hormones have not been reported.

From the results of this study, it can be concluded that drought stress enhances the ROS production and subsequently antioxidant enzymes and compounds. GA₃ and BAP have the potential to ameliorate water deficit effects, while POD activity decreases in the petals and subsequently lowers the POD/CAT ratio, which can be a mechanism for avoiding oxidative stress. GA₃, BAP, BR, and SA are more effective in carotenoid biosynthesis in pot marigold than JA and ABA. SOD and CAT might be the defensive enzymes against the ROS in the petals of pot marigold.

Abbreviations

ABA: Abscisic acid; APOX: Ascorbate peroxidase; BAP: Benzyl amino purine; BR: Brassinolid; CAT: Catalase; GA₃: Gibberellic acid; GR: Glutathione reductase; JA: Jasmonic acid; LHC: Light harvest complex; POD: Peroxidase; PSII: Photosystem II; ROS: Reactive oxygen species; SA: Salicylic acid, SOD: Superoxide dismutase.

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تنظیم هورمونی فعالیت سیستم‌های آنتی اکسیدانی گلبرگ همیشه بهار در تنش خشکی

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چکیده

تنش خشکی یکی از مهم‌ترین تنش‌های غیر زیستی است که عملکرد گیاهان را محدود می‌سازد. تولید گونه‌های فعال اکسیژن (ROS) در شرایط تنش افزایش می‌یابد. برای ارزیابی اثر فیتوهورمون‌ها بر تغییر فعالیت آنزیم‌های آنتی اکسیدانت و میزان کاروتنوئیدهای گلبرگ همیشه بهار، دو آزمایش گلخانه‌ای در شرایط خشکی انجام شد. نتایج نشان داد که فعالیت آنزیم‌های سوپراکسید دیسموتاز (SOD, EC 1.15.1.1) و کاتالاز (CAT, EC 1.11.1.6) به ترتیب ۴۷ و ۷۳ درصد در گلبرگ‌های تحت تنش نسبت به شاهد افزایش داشت. محلول‌پاشی اسید جیبرلیک (GA_3) و بنزیل آمینو پورین (BAP) اثر خشکی را تعدیل کرد، ولی اسید آبسزیک (ABA)، جاسمونیک (JA)، سالیسیلیک (SA) و براسینولید (BR) فعالیت آنزیم‌ها را افزایش دادند. اسید جیبرلیک فعالیت آنزیم پروکسیداز (POD, EC 1.11.1.7) را برخلاف سایر آنزیم‌ها افزایش داد در حالی که بقیه هورمون‌ها اثری بر این آنزیم نداشتند. میزان کاروتنوئیدها تحت تاثیر تیمارهای خشکی و هورمونی قرار نگرفت. در شرایط کمبود آب میزان کاروتنوئیدها افزایش نشان داد، ولی GA_3 ، BAP و JA اثر بازدارنده بر سنتز لیکوپن و کاروتن داشتند و سایر هورمون‌ها مقدار این کاروتنوئیدها را افزایش دادند. محلول‌پاشی GA_3 میزان لوتنوگراتین را در گلبرگ‌های همیشه بهار در شرایط شاهد و تنش به ترتیب ۳۵ و ۲۰ درصد افزایش داد. کاهش فعالیت POD در شرایط تنش به احتمال زیاد بیانگر نقش سایر مکانیسم‌ها در پاکسازی گونه‌های فعال اکسیژن در گلبرگ‌های همیشه بهار است.