Pak. J. Bot., 46(6): 2151-2156, 2014.

ENDOGENOUS ANTIOXIDANTS AND PHYTOHORMONAL REGULATION INDUCED BY SPERMIDINE IMPROVE CUCUMBER PLANT GROWTH

RAMALINGAM RADHAKRISHNAN AND IN- JUNG LEE*

School of Applied Biosciences, Kyungpook National University, Sangyuk-Dong, Buk-gu, Daegu 702-701, South Korea,
**Corresponding author e-mail: ijlee@knu.ac.kr; Tel.: +82 53 950 5708 (Office); fax: +82 53 958 6880*

Abstract

Involvement of exogenously applied low dose of spermidine (Spd) on the regulation of endogenous chlorophylls, reactive oxygen species (ROS), antioxidants, gibberellins (GAs), jasmonic acid (JA) and salicylic acid (SA) was investigated in cucumber plants. The plants were exposed to low concentrations of 0.06 and 0.3 µM Spd. The length of shoots, leaf area, fresh and dry weight of leaves was greater in Spd treatments than their controls. A remarkable increase of chlorophyll and protein content was noticed in plants treated with 0.3 µM Spd. However, superoxide content and lipid peroxidation were moderately declined and the activities of peroxidase, polyphenol oxidase and acid phosphatase were elevated in plants treated with 0.3 µM Spd. The plants treated with 0.06 µM Spd also showed the greater level of antioxidants over the untreated controls. A higher accumulation of non-13-hydroxylated and 13-hydroxylated GAs such as GA_{24}, GA_9, GA_4, GA_{20}, GA_8 and GA_7 was observed in Spd treated plants than their controls. Consequently, the concentration of JA and SA declined significantly in 0.3 µM Spd treated plants. These results suggest that exogenous application of 0.3 µM Spd increases plant growth through the enhancement of photosynthetic pigments, protein, enzyme activities and GAs, and reduction of ROS, JA and SA content and it could be useful to enhancement of crop plants cultivation.

Key words: Antioxidants; Chlorophylls; Gibberellins (GAs); Jasmonic acid (JA); Salicylic acid (SA); Spermidine (Spd).

Introduction

Polyamines (PAs) are secondary messengers and can bind strongly with nucleic acids, proteins and phospholipids (Bouchereau *et al*., 1999) to implicate cellular metabolism. PAs play an important role in many physiological functions, such as organogenesis, embryogenesis, leaf senescence, floral and fruit development and ripening of fruits (Kusano *et al.*, 2008; Alcazar *et al.*, 2010). In addition, PAs can act indirectly as reactive oxygen species (ROS) scavengers and affect the activity of antioxidant enzymes and stress related hormones (Verma & Mishra, 2005; Zhang *et al.*, 2009a; Radhakrishnan & Lee, 2013a, 2013b). Spermidine (Spd) is one of the polyamines, it effectively scavenges ROS and to elicit several physiological and biochemical functions. Kubis (2008) suggested that exogenous application of Spd altered the activities of scavenging enzymes and reactive oxygen species in water stressed cucumber plants. Recently, Gill and Tuteja (2010) observed the cross talk between ABA and PAs signaling pathways and Yamaguchi (2008) suggested that Put and ABA reciprocally promote each other's biosynthesis under stress to improve plant adaptive potential. Similarly, numerous proteins produced in plants under stress are induced by ABA (Jin *et al.*, 2000) and SA (Hoyos & Zhang, 2000) signals.

Moreover, very few reports have documented that Spd (0.1 to 0.5 mM) could improve the cucumber plant growth (Duan *et al.*, 2008; Kubis, 2008; Zhang *et al.*, 2009a) and it suggested that more research is needed in exogenous application of Spd, due to expensive price of Spd, processing time and level of dosage. To date, very little is known about the role of PAs in the modulation of phytohormones biosynthesis and no study has explored very low concentrations of Spd and its interaction with crop plants. In the present study, we used very low quantity of Spd (0.06 to 0.3 µM) to improve the cucumber plant growth. The objective of this study is to confirm the plant growth promoting ability of low concentration of Spd on cucumber by regulating endogenous gibberellins, jasmonic acid, salicylic acid and enzyme activities.

Materials and Methods

Plant growth condition and Spd treatment: Seeds of cucumber cv. chung-pung-chung-jang were immersed in distilled water for 30 min and surface sterilized with 50% ethanol for 30 sec, and thoroughly rinsed with distilled water. The seeds were placed to substrate composed of peat moss (13-18%), perlite (7-11%), coco-peat (63-68%) and zeolite (6-8%), NH^{4-} ~90 mg Kg^{-1}; NO_{3-} ~205 mg Kg^{-1}; P_2O_5 ~350 mg Kg^{-1} and K_2O ~100 mg Kg^{-1} and kept in the dark at 25°C for 3 days. Plants were grown in a growth chamber equipped with lamps of an irradiance approximately 200 mmol m^{-2} s^{-1}, kept at 28 ±3°C (day) /18 ± 3°C (night) under 10L/14D photoperiod. Relative aerial humidity fluctuated between 60% and 75%. After 7 days, plants were used as experimental materials to the treatments of PAs. The experiment (20 replication) was arranged as a randomized complete block design with Spd treatments. Spd was purchased from Sigma Aldrich, USA. To perform Spd treatments, 0.06 and 0.3 µM Spd were applied at three times with four days interval to the cucumber plants. Control plants received same amount of distilled water. After 10 days, aerial parts of plants were harvested to determine the chlorophylls, protein, enzymes activities and plant growth regulators. All the experiments were performed in triplicate.

Antioxidants and phytohormones analyses: The content of chlorophyll a, b and total chlorophyll were determined spectrophotometrically at 663 and 645 nm by following the method of Arnon (1949) and proteins content by the method of Bradford (1976). Electrical

conductivity was measured as the leakage percentage of electrolytes, as described by Gong and Chen (1998). The detection of O_2^- was based on its ability to reduce nitro blue tetrazolium (NBT) by the method of Doke (1983). The lipid peroxidation was determined by the method of Ohkawa *et al*., (1979). Reduced glutathione content was measured according to the method of Ellman (1959). Total polyphenol was determined by Folin-Ciocalteau colorimetric method of Kumazawa *et al*., (2004). Catalase activity was assayed by the method of Aebi (1984), Peroxidase and polyphenoloxidase activity was measured by Kar & Mishra (1976). Activity of acid and alkaline phosphatase was studied according to the modified method of Ikawa *et al*., (1964). The lyophilized samples were used for the extraction and quantification of endogenous gibberellins by the method of Lee *et al*., (1998). The endogenous JA was extracted according to the protocol of Mc-Cloud and Baldwin (1997). SA was extracted and quantified by Enyedi *et al*., (1992) and Seskar *et al*., (1998) method.

Statistical analysis: All of the presented data are mean values of a representative experiment (n = 3) and shown as the mean ± SE. The data were analyzed using SPSS software version 11.5 (SPSS Inc., Cary, NC, USA) and analysis of variance (ANOVA) was used to compare the statistical difference based on Duncan's multiple range test (DMRT), at significance level of $p<0.05$.

Results and Discussion

Exogenous application of Spd improved the shoot length, leaf area, leaf fresh and dry weight in cucumber plants than their controls (Fig. 1). The low concentration of Spd (0.06 µM and 0.3 µM) treated plants exhibited greater level of shoot length, leaf area, leaf fresh weight when compared with their untreated plants. The Spd (0.06 and 0.3 µM) treatments accelerated the accumulation of photosynthetic pigments such as total chlorophyll, chlorophyll a and chlorophyll b content (Table 1). PAs strongly affect photosynthetic apparatus and enhance photochemical quenching of absorbed light energy (Kotzabasis *et al.*, 1999), which lead to improve the photosynthetic capacity of cucumber plants (Zhang *et al.*, 2009b). However, an increment of protein content was observed in 0.3 µM Spd subjected plants. Zhang *et al*., (2009a) also reported that significant elevation of protein content in cucumber plants treated with high concentration of Spd under normal and salt stress condition. The result suggests that Spd treatment could either induce the protein synthesis or prevent the protein degradation.

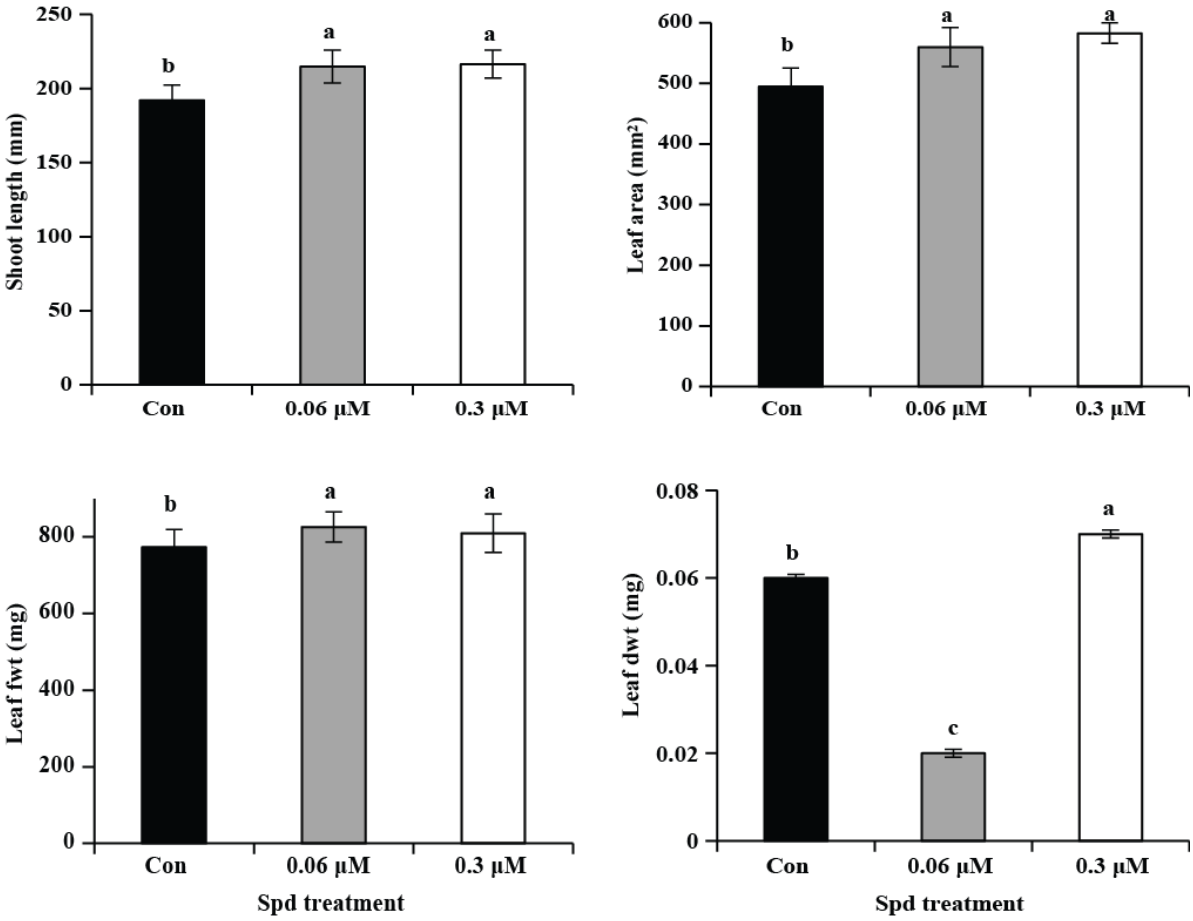

Fig. 1. Influence of Spd on shoot length, leaf area, leaf fresh and dry weight in cucumber plants. Means with standard error followed by the same letter are not significantly different ($p<0.05$) as determined by Duncan's multiple-range test.

Table 1. Effect of Spd on biochemical content in cucumber plants.

Biochemical content	Control	0.06 µM Spd	0.3 µM Spd
Total Chlorophyll (mg^{-1} g FW)	1.30 ± 0.08^{b}	1.75 ± 0.08^{ab}	1.88 ± 0.10^{a}
Chlorophyll a (mg^{-1} g FW)	0.94 ± 0.07^{b}	1.30 ± 0.01^{a}	1.31 ± 0.05^{a}
Chlorohyll b (mg^{-1} g FW)	0.39 ± 0.06^{b}	0.45 ± 0.03^{a}	0.46 ± 0.09^{a}
Protein (mg^{-1} g FW)	5.56 ± 0.17^{b}	6.20 ± 0.07^{a}	6.82 ± 0.16^{a}
Electrical conductivity (%)	20.24 ± 0.24^{b}	23.56 ± 0.44^{a}	20.37 ± 0.50^{b}
Superoxide (Abs^{-1} 0.1 g DW)	0.55 ± 0.007^{a}	0.44 ± 0.01^{b}	0.5 ± 0.008^{ab}
Lipid peroxidation (mg of MDA^{-1} g FW)	0.23 ± 0.003^{a}	0.19 ± 0.01^{b}	0.17 ± 0.01^{b}
Reduced glutathione (mg^{-1} g FW)	0.29 ± 0.06^{b}	0.68 ± 0.006^{a}	0.28 ± 0.004^{b}
Total polyphenol (mg^{-1} g FW)	1.66 ± 0.05^{b}	1.75 ± 0.08^{a}	1.80 ± 0.002^{a}
Catalase ($Unit^{-1}$ mg $protein^{-1}$ min)	119.8 ± 12.8^{a}	123.0 ± 15.8^{a}	121.6 ± 4.9^{a}
Peroxidase ($Unit^{-1}$ mg $protein^{-1}$ min)	1.30 ± 0.18^{b}	0.95 ± 0.18^{c}	1.91 ± 0.04^{a}
Polyphenol oxidase ($Unit^{-1}$ mg $protein^{-1}$ min)	1.64 ± 0.32^{b}	1.39 ± 0.26^{c}	4.27 ± 0.12^{a}
Acid phosphatase (µmol^{-1} mg $protein^{-1}$ min)	0.19 ± 0.007^{b}	0.18 ± 0.005^{b}	0.431 ± 0.008^{a}
Alkaline phosphatase (µmol^{-1} mg $protein^{-1}$ min)	3.26 ± 0.14^{c}	3.6 ± 0.15^{b}	4.09 ± 0.17^{a}

Means with standard error followed by the same letter are not significantly different ($p<0.05$) as determined by Duncan's multiple-range test

Electrical conductivity (EC) and superoxide radical (O_2^-) analysis are useful to evaluate the stress condition. In the present study, the EC of leaves was higher in 0.06 µM Spd treatments than their controls, but 0.3 µM Spd maintained the same level of EC in treated plants (Table 1). Interestingly, superoxide anion and malondialdehyde (MDA) were lower in all concentrations of Spd treated plants, when compared to controls. This result is contrary with Duan *et al*., (2008) report, who observed the exogenous Spd (0.1 mM) increased the O_2^-, and declined the EC in cucumber plants. PAs can able to form a ternary complex with Fe^{2+} to auto-oxidation, and protect the membrane from attack (Velikova *et al.*, 2000), which indicates that application of Spd inhibits the damage of plasma membrane by reducing the level of ROS (Xu *et al.*, 2011).

The concentration of reduced glutathione in plants was accelerated by the effect of 0.06 µM Spd. However, the total polyphenol content was not changed by the exposure of Spd (0.06 and 0.3 µM). The activity of catalase was slightly enhanced in Spd treatments. The effect of 0.3 µM Spd in plants showed an elevation in the activities of peroxidase, polyphenol oxidase, acid phosphatase and alkaline phosphatase than their controls (Table 1). PAs may act as antioxidant, a free radical scavenger and a membrance stabilizer (Larher *et al.*, 2003). Moreover, the plants exposed to stress condition, increased the acid and alkaline phosphatase activity for maintaining the certain level of inorganic phosphate in the cell (Hamayun *et al*., 2010a; Olmos & Hellin, 1997). Our data allow us to hypothesize that application of low dose of Spd (0.06 and 0.3 µM) differentially alters the pattern of activities of antioxidants and adjust the stress level to improve the plant growth.

Most of the non-13-hydroxylated gibberellins GA_{24}, GA_9 and GA_4, and 13-hydroxylated gibberellins such as GA_{20}, GA_1 and GA_8 were higher in their concentration due to the effect of Spd (Fig. 2). GA_1 and GA_4 are considered as bioactive GAs in plants (Kang *et al.*, 2012; Kanno *et al.*, 2010), and GA_4 is reported as a major bioactive GA in *Arabidopsis thaliana* and some cucurbitaceae members (Yamaguchi, 2008). The current study would confirm the positive changes of GA biosynthetic pathway under Spd treatment. The dose of 0.3 µM Spd induced more accumulation of GA_{24}, GA_9 and GA_4. Although the physiological roles of the two pathways are not well understood, some reports suggested that non-13-hydroxylated GA pathway is predominant in plants (Yamaguchi, 2008). Our results showed that 0.3 µM Spd effectively promoted the non-13-hydroxylated gibberellins metabolism in cucumber plants.

When the distribution of 13-hydroxylated gibberellins was compared between Spd treated and untreated plants, we found higher concentration of GA_{20} and GA_8 in the treatment of 0.3 µM Spd. During plant developmental process, GA_1 and GA_{20} are increased in the vegetative phase, which indicate that these GAs are more important for vegetative development, whereas the GAs from non-13-hydroxylation pathway are involved in the regulation of reproductive development (Meijon *et al.*, 2011). Interestingly, low concentration of Spd (0.06 µM) caused a significant increase of GA_{12}, the precursor of both 13-hydroxylated and non-13-hydroxylated gibberellins. In addition, the analysis of GA_7 in cucumber plants showed that exogenous application of Spd positively influenced the GA metabolism by elevated level of GA_7 in the treatment of 0.3 µM Spd. These results suggest that exogenous treatment of Spd could interact with GA biosynthetic pathway and stimulate both bioactive and non-biotic GAs, which might be a reason for the enhancement of plant growth.

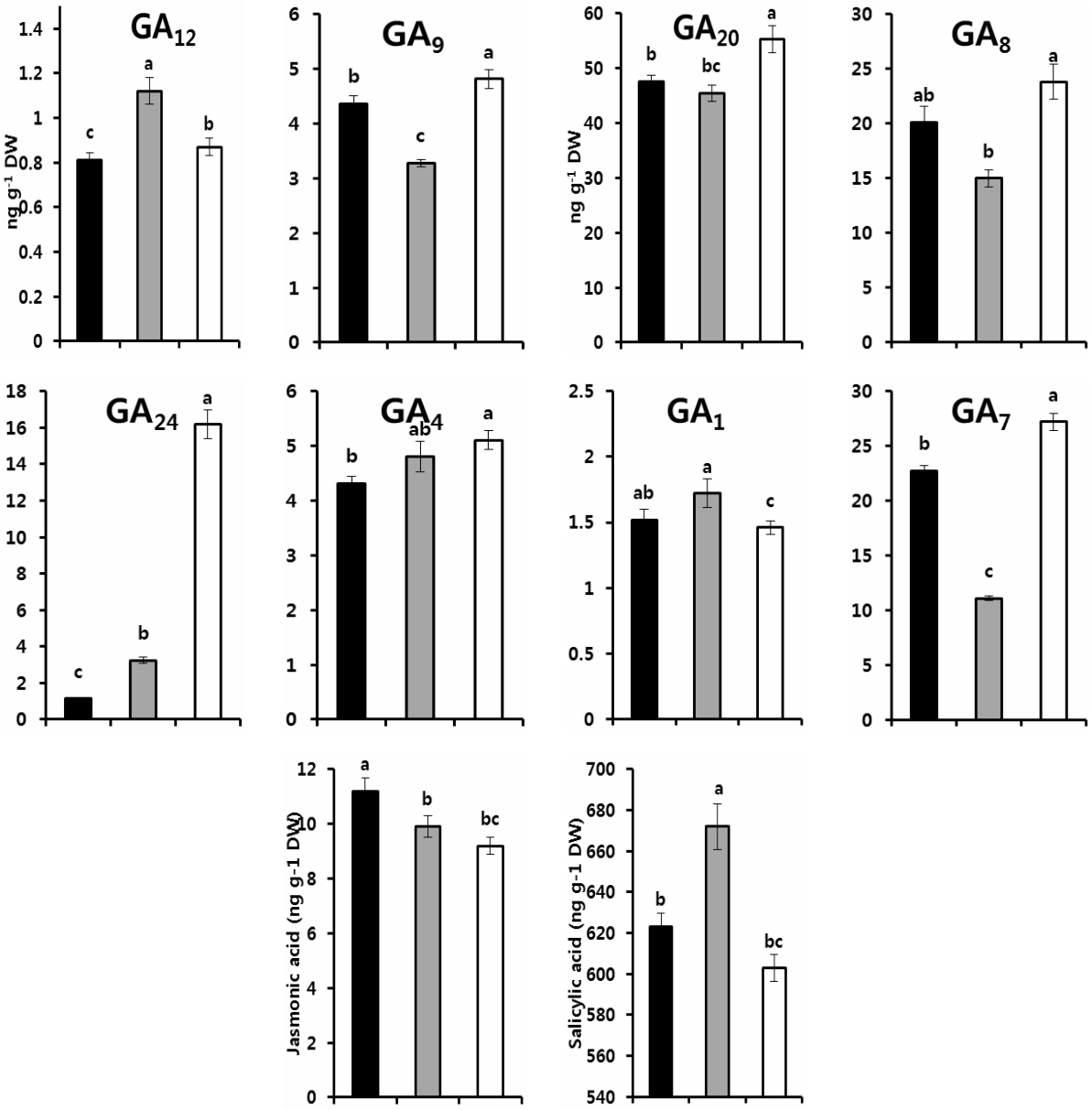

Fig. 2. Gibberellins (GA), jasmonic acid (JA) and salicylic acid (SA) levels in control (■), 0.06 µM (▒) and 0.3 µM (□) Spd treated cucumber plants (ng g $^{-1}$ dwt). Bars represent means plus standard error. Means followed by the same letter are not significantly different (p<0.05) as determined by Duncan's multiple-range test.

Generally, seeds have high rate of JA content and this content declined at seedling stage. Spd treatment reduced the JA content when compared to their controls (Fig. 2). Many researchers observed the over accumulation of JA in plants subjected with various biotic and abiotic stresses (Glazebrook, 2005; Hamayun *et al.,* 2010b; Kondo *et al.*, 2011). However, little is known about the physiological roles of SA during plant development. We found that the dual expression of SA accumulation, which depends on the exogenous concentration of Spd. The higher level of SA was observed in low dose of Spd (0.06 µM) treated plants than their controls (Fig. 2). The declined level of SA was found in plants subjected with 0.3 µM Spd. Recently, Santner *et al*., (2009) proposed that the understanding of hormonal interaction during plant growth will be a major challenge in the future. The results of SA analysis suggest that 0.3 µM Spd is more useful for cucumber plants improvement.

In conclusion, the results showed that exogenous treatment of Spd (0.06 and 0.3 µM) increased the shoot length, leaf area, leaf fresh and dry weight than control plants by differential interaction of antioxidants and hormonal signals. Among the various concentration of Spd, 0.3 µM Spd exposures significantly enhanced the protein content while it implicated to a decrease of ROS. Activities of peroxidase, polyphenol oxidase and acid phosphotase were elevated in plant treated with 0.3 µM Spd over the untreated controls. In addition, the

application of Spd would increase the growth promoting GAs to improve plant growth. However, JA and SA declined in plants exposed with Spd. Our findings suggest that low concentration of Spd (0.3 μM) would be useful to improve the crop plants.

Acknowledgments

This work was supported by Eco- Innovation Project, Korean Government's R & D program on Environmental Technology and Development.

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(Received for publication 30 June 2013)