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Studies on toxic effects of nitrogenous compound, putrescine and spermine on cucumber plant growth

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Abstract

The present study was conducted to determine the effects of putrescine (Put) and spermine (Spm) on cucumber plant growth. The low concentration of Put and Spm (0.02, 0.1 and 0.5 mM) was applied to cucumber seedlings. Put and Spm treatments reduced the length and fresh weight of shoots, leaf area, fresh and dry weight of leaves compared to their controls. The enhancement of electrical conductivity and lipid peroxidation was shown in cucumber plants exposed with Put and Spm. However, plants treated with Put and Spm showed low concentration of polyphenol contents with declined level of catalase and peroxidase activity than their controls. These results suggest that application of low concentrations of Put and Spm exhibit detrimental effects on plant growth through the enhanced level of lipid peroxidation and reduction of antioxidants activities.

Key words

Antioxidants, Cucumber, Putrescine, Spermine, Toxicity

Introduction

Polyamines (PAs) are nitrogenous compounds, which are present in prokaryotic and eukaryotic organisms. PAs often exist not only as free form but also bound with biomolecules, such as phenolic acids, proteins and cell wall. They are important modulators of biological processes such as plant growth and development (Amin *et al.*, 2011), and also involve in structure and functions of nucleic acids, modulation of enzyme activities, regulation of physical and chemical properties of membranes and plant responses to environmental stress (Bouchereau *et al.*, 1999). PAs have been detected in significant quantities in soils (Ishii *et al.*, 2008; Yao *et al.*, 2010).

Putrescine (Put), Spermidine (Spd) and Spermine (Spm) are most common polyamines present in plants. Biosynthesis of Put is initiated from decarboxylation of arginine or ornithine by arginine decarboxylase or ornithine decarboxylase. It is subsequently converted to triamine Spd and Spm (Yamaguchi *et al.*, 2006). Although, the functions of PAs on plants are investigated in various levels, the precise role of PAs in living organisms remain elusive (Hussain *et al.*, 2011). However, exogenous application of Put probably participates in regulation of photoadaption in plants (Ioannidis and Kotzabasis, 2007). Stress tolerance and susceptibility of plants depend on the exogenous treatment of PAs concentration. Accumulation of reactive oxygen species (ROS) such as O_2^-, H_2O_2, and OH^- in tissues causes membrane damage by lipid peroxidation (Hussain *et al.*, 2011). Exogenous treatment of PAs enhance the growth of wheat plants under control and salt stress conditions (Iqbal and Ashraf, 2005). Moreover, PAs can act as ROS scavengers and affect the activity of antioxidant enzymes (Verma and Mishra, 2005), stress related hormones (Radhakrishnan and Lee, 2013a; b), and enhance proline and betaine accumulation in plants to survive under stress conditions. Under stress condition, the effect of Spm modulates the activities of certain ion channels, cytoplasmic Ca_2^+ concentration and stomata closure (Wang *et al.*, 1993) to induce stress tolerance. However, PAs act as endogenous anti-senescence agents to delay fruit ripening of tomatoes (Law *et al.*, 1991), apples (Wang *et al.*, 1993), and used

to increase flower intensity in citrus orchards. Due to this physiological importance of PAs, exogenous application of PAs is widely suggested for agronomical purposes (Yao *et al.*, 2010).

Cucumber is a common vegetable crop and good source of vitamins A, C, minerals like molybdenum, potassium, manganese, magnesium, silica, folate and dietary fiber. Duan *et al.* (2008) reported high concentration of Put, Spm and Spd mitigates stress effects in cucumber under salt, water and chilling stress condition. The growth promoting effects of exogenous application of PAs are still not confirmed in many plants. For example, Put at low concentration inhibited root growth, whereas high concentration increased root length in *Arabidopsis* (Mirza and Bagni, 1991), and no distinct improvement was observed in elongation of shoots and roots of Spm applied sorghum plants when compared to their control (Chai *et al.*, 2010). However, very limited studies have been conducted to find the role of different concentrations of PAs for crop improvement. Therefore, the present study was conducted to evaluate the role of different concentrations of Put and Spm treatments in ROS production and antioxidants on cucumber plants.

Materials and Methods

Seeds of cucumber cv. chung-pung-chung-jang were surface sterilized with 50% ethanol for 30 sec and washed 3 times with distilled water. The seeds were placed in 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 dark at 25 °C for 3 days. The seedlings 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 to 75%. In the study, 7 day old cucumber plants were used as experimental materials to evaluate the role of PAs on cucumber growth. Put and Spm were purchased from Sigma Aldrich, USA. To perform PAs treatment, 3.0 ml of 0.02, 0.1 and 0.5 mM of Put and Spm was applied three times with four days interval to seedlings containing pots. After 10 days, aerial parts of plants were harvested to determine the plant growth and analysis of ROS and antioxidants. For biochemical analysis, the plants were stored at -80 °C until use.

The growth parameters like length and fresh weight of shoots, leaf area, fresh and dry weight of fully expanded leaves were measured in cucumber plants treated with or without Put and Spm. Total leaf area was measured with Laser Leaf Area meter (CI-203 model, CID Inc., USA) and dry weight was measured after drying the leaves at 70 ° C for 3 days.

Electrical conductivity (EC) was calculated from the leakage percentage of electrolytes as described by Gong and Chen (1998). Fresh leaves were cut into pieces (1.0 cm) and placed in test tubes containing deionized water. The tubes were incubated in a water bath at 30 °C for 2 hr and the initial electrical conductivity of the medium (EC_1) was measured. The samples were boiled at 100 °C for 15 min to release all electrolytes, cooled and then the final electrical conductivity (EC_2) was measured. The leakage percentage of electrolytes was calculated by the following formula :

$$\frac{Ec_1}{EC_2} \times 100\%$$

The extent of lipid peroxidation was determined by the method of Ohkawa *et al.,* (1979). For this assay, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.81% thiobarbituric acid aqueous solution were added in succession in a reaction tube. To this reaction mixture, 0.2 ml of tissue homogenate was added. The mixture was then heated in boiling water for 60 min. After cooling to room temperature, 5 ml of butanol: pyridine (15:1v/v) solution was added. The upper organic layer was separated and the intensity of the resulting pink colour was read at 532 nm. Tetramethoxy-propane was used as an external standard. The level of lipid peroxides was expressed as mg of malondialdehyde (MDA) g^{-1} f.wt.

Table 1 : Effect of Put and Spm on plant growth in cucumber

Treatments (mM)	Shoot length (cm)	Shoot fresh weight (g)	Leaf area (cm^2)	Leaf fresh weight (g)	Leaf dry weight (mg)
Control	19.99±0.29^{a}	3.74±0.12^{a}	49.48±2.9^{a}	0.77±0.01^{a}	67.8±3.44^{a}
Put 0.02	17.28±0.39^{c}	2.38±0.18^{bc}	41.16±4.0^{ab}	0.61±0.01^{b}	49.4±1.36^{b}
Put 0.1	17.40±0.60^{bc}	2.23±0.17^{c}	38.22±3.4^{bc}	0.52±0.01^{c}	43.0±2.61^{bc}
Put 0.5	19.01±0.31^{ab}	2.72±0.14^{b}	44.22±3.9^{ab}	0.61±0.22^{b}	46.8±1.74^{bc}
Spm 0.02	19.05±0.38^{ab}	2.63±0.13^{bc}	42.94±1.4^{ab}	0.58±0.01^{bc}	43.2±2.52^{bc}
Spm 0.1	18.50±0.31^{b}	2.36±0.13^{bc}	40.06±1.3^{b}	0.57±0.02^{bc}	42.2±2.71^{bc}
Spm 0.5	19.50±0.25^{ab}	2.37±0.06^{bc}	39.84±1.4^{bc}	0.55±0.01^{bc}	39.6±2.06^{c}

Values are mean of four replicates ± SE. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test

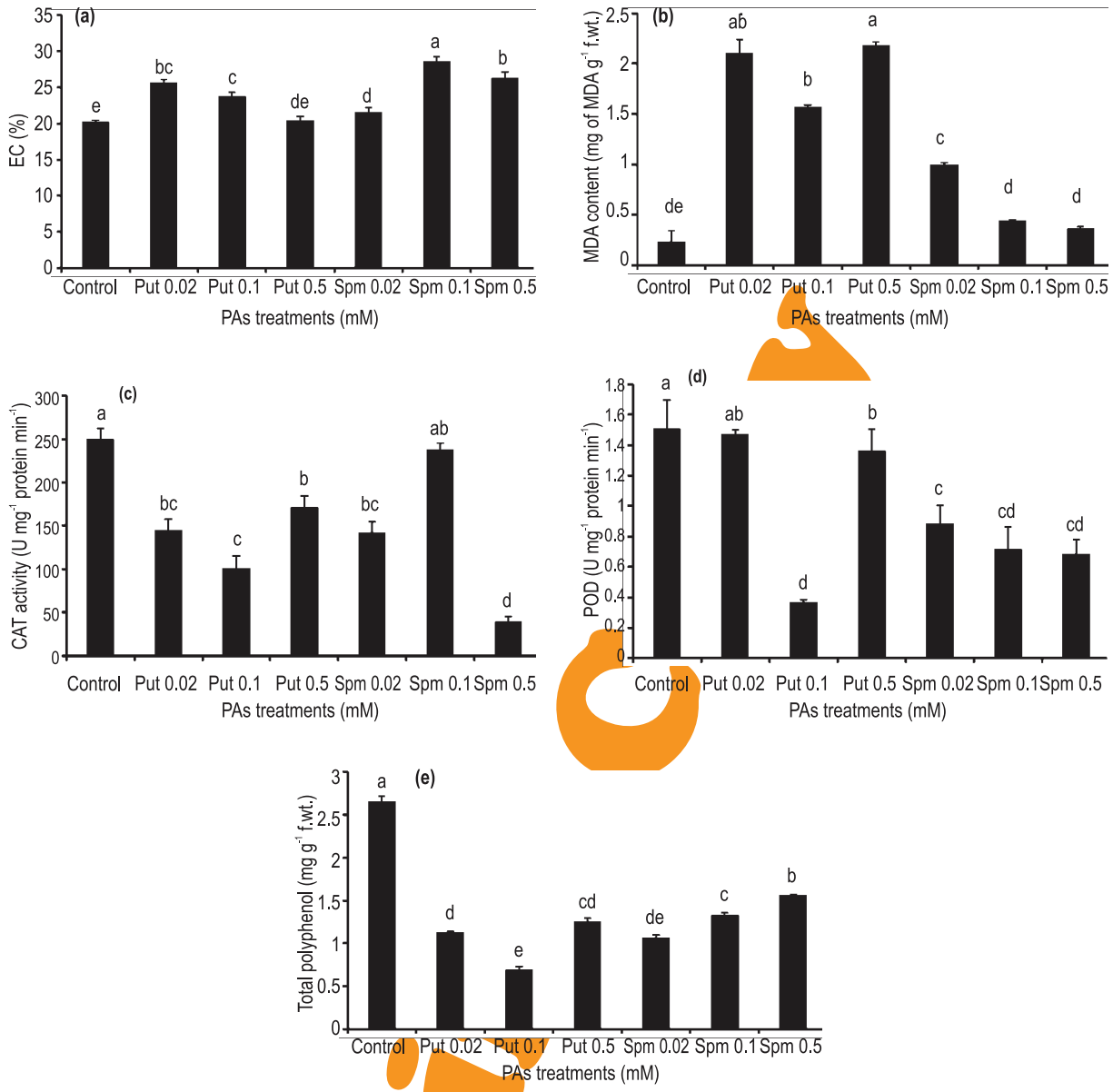

Fig. 1 : Effect of Put and Spm on **(a)** EC, **(b)** MDA content, **(c)** CAT activity, **(d)** POD activity and **(e)** total polyphenol contents in cucumber plants. Bars represent mean of four replicates ±SE. Means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test

For determining catalase (CAT) activity, the leaf samples were homogenized in 50 mM Tris-HCl buffer (pH 7.0) containing 3mM $MgCl_2$, 1mM EDTA and 1.0% PVP and then centrifuged at 10000 rpm for 15 min at 4 °C; the obtained supernatant was used for biochemical analysis. All parameters were expressed as activity per mg protein. The protein concentration in each fraction was determined by method of Bradford (1976) using bovine serum albumin as standard. Catalase activity was assayed by method of Aebi (1984), the crude enzyme extract was added with 0.5 ml of 0.2 M H_2O_2 in 10 mM phosphate buffer (pH 7.0). CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm and one unit of CAT was defined as µg of H_2O_2 released mg protein^{-1} min^{-1}.

Peroxidase activity (POD) was measured by method of Kar and Mishra (1976). The leaf samples were homogenized with phosphate buffer pH 6.8 (0.1 M) and centrifuged at 2 °C for 15 min at 17000 rpm in a refrigerated centrifuge. The clear supernatant

was taken as the enzyme source. The assay mixture for POD activity comprised phosphate buffer (pH 6.8) 50 µm pyrogallol, 50 µm H_2O_2, and 0.1 ml enzyme extract. This was incubated for 5 min at 25 °C after which the reaction was stopped by adding 0.5 ml of 5 % (v/v) H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm. One unit of POD was defined as an increase of 0.1 units of absorbance.

Total polyphenol was determined by Folin-ciocalteau colorimetric method of Kumazawa *et al.*, (2004). One gram of tissue was extracted with 80% ethanol and 0.5 ml of extract was mixed with Folin-Ciocalteau reagent and 10% Na_2CO_3, the absorbance was measured at 760 nm after 1 hr incubation at room temperature. Total polyphenols contents were expressed as mg g^{-1} f. wt. (gallic acid equivalents).

All the experiments were conducted four times and expressed as mean ± SE. Analysis of variance was applied (ANOVA) using SPSS version 11.5 (SPSS Inc., Cary, NC, USA). The group means were compared by Duncan's multiple range test (DMRT). Values were considered statistically significant at $P < 0.05$.

Results and Discussion

The exogenous application of Put and Spm caused no obvious positive effect on cucumber plant growth. The length and fresh weight of shoots, fresh and dry weight of leaf and leaf area were inhibited at all the treatments of Put and Spm (Table 1). Our results revealed that Put application retarded shoot length and fresh weight of shoot and leaf as maximum level at 0.02 and 0.1 mM concentrations, while Spm also produced remarkable reduction in leaf area and dry weight at 0.5 mM treatment. These results were agreement with the previous report of Chai *et al.*, (2010), who found that Spm treatment reduced root length, plant fresh and dry weight in sorghum plants. Several studies reported that effect of PAs caused an increase of plant growth (Verma and Mishra, 2005; Terakado *et al.*, 2006; Xu *et al.*, 2011). In our previous study, we found that Spd treatment increased the growth and antioxidants system in cucumber plants than their control plants (data not shown). However, few studies suggested that exogenous application of PAs declined the growth of crop plants (Mirza and Bagni, 1991; Terakado *et al.*, 2006; Chai *et al.*, 2010).

Analysis of EC and lipid peroxidation can be useful to understand the range of stress condition in plants. Exogenous application of Put and Spm induced oxidative stress in plants through the enhancement of EC and accumulation of MDA (Fig. 1 a, b). In control plants, it was observed that very low quantity of MDA and EC and, the significant enhancement of EC was denoted at exogenous supplementation of Spm (0.1 and 0.02 mM), while elevated level of MDA concentration was found in plants exposed with 0.5 mM Put. Despite the fact that previous study showed exogenous application of Put and Spm slightly increased MDA accumulation in rice (Ndayiragije and Lutts, 2007) and sorghum (Chai *et al.*, 2010). The enhanced level of EC and MDA indicates the damaging effect of Put and Spm on cucumber plant growth. These results suggest that exogenous application of Put and Spm affects the balance of reactive oxygen species and antioxidants contents. This might be a reason for reduction of plant growth under Put and Spm treatments.

Regulation of ROS production, redox state of ascorbate and glutathione can act as signaling stimuli during plant growth (Vivancos *et al.*, 2010). The detrimental effects of Put and Spm on cucumber plants were measured by reduction of non-enzymatic and enzymatic antioxidants activities. As shown in Fig. 1 (c,d) plants under controlled condition exhibited significant increase in total polyphenol content and elevated activities of CAT and POD than PAs treatments. Application of 0.1 mM Put and 0.5 mM Spm significantly reduced the CAT activity compared with their controls, and there was no effect on the CAT activity in plants treated with 0.1 mM Spm. Similarly, POD activity was not affected by the treatment of 0.02 mM Put, whereas 0.1 mM Put drastically reduced the POD activity. However, maximum reduction of total polyphenol content was observed in plants treated with 0.1 mM Put as compared to their controls (Fig. 1e).

In conclusion, 0.02, 0.1 and 0.5 mM of Put and Spm reduced shoot length, fresh weight, leaf area, leaf fresh and dry weight, total polyphenol, CAT, POD activities, and enhanced electrical conductivity and lipid peroxidation in cucumber. Our findings suggest that low concentration of Put and Spm interrupts the regular mechanism of antioxidants and are harmful to plants and further molecular level intensive studies are needed to confirm the biological role of low dose of Put and Spm.

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