Effects of mammalian sex hormones on antioxidant enzyme activities, H_2O_2 content and lipid peroxidation in germinating bean seeds

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ABSTRACT: In this study, effects of mammalian sex hormones (progesterone, β-estradiol and androsterone) on activities of superoxide dismutase, peroxidase and catalase, H_2O_2 content and lipid peroxidation level in germinating bean seeds were investigated. The bean seeds were germinated at various hormone concentrations between 10^{-4} and 10^{-15}M. Activities of superoxide dismutase, peroxidase and catalase significantly increased at all the concentrations tested compared to controls. The maximum enzyme activities were recorded at the 10^{-9} M concentration for all of three hormones. On the other hand, depending on increase in the activities of antioxidant enzymes, lipid peroxidation level and H_2O_2 content significantly decreased at hormone treated seeds.

Key words: Mammalian sex hormones, Germination, Antioxidant enzymes, Stress parameters

Çimlenen Fasulye Tohumlarının Antioksidan Enzim Aktiviteleri, H_2O_2 İçeriği ve Lipid Peroksidasyonu Üzerine Memeli Cinsiyet Hormonlarının Etkileri

ÖZET : Bu çalışmada, çimlenen fasulye tohumlarında süperoksit dismutaz, peroksidaz ve katalaz enzim aktiviteleri ile hidrojen peroksit (H_2O_2) içeriği ve lipid peroksidasyon seviyesi üzerine memeli cinsiyet hormonları olan progesteron, , β-östradiol ve androsteronun etkileri araştırıldı. Fasulye tohumları 10^{-4} ile 10^{-15} M konsantrasyon aralığında değişen hormon çözeltilerinde çimlendirildi. Uygulanan tüm konsantrasyonlarda süperoksit dismutaz, peroksidaz ve katalaz enzim aktiviteleri kendi kontrollerine oranla önemli derecede arttı. Maksimum enzim aktiviteleri her üç hormon içinde 10^{-9} M konsantrasyonda kaydedildi. Diğer yandan, hormon uygulanan tohumlarda antioksidan enzim aktivitelerindeki artışa bağlı olarak lipid peroksidayon seviyesi ve hidrojen peroksit içeriği önemli ölçüde azaldı.

Anahtar Kelimeler: Memeli cinsiyet hormonları, Çimlenme, Antioksidan enzimler, Stres parametreleri

INTRODUCTION

Environmental factors affect the plant growth and development together with own internal structure of plant positively or negatively (Kocacaliskan, 2004). Increasingly deteriorating environmental conditions have caused negative effects such as germination difficulty, inhibition of growth and development, and yield loss. Besides, negative environmental conditions significantly increase production of reactive oxygen species (ROS) in plants. The ROS including superoxide radicals (O_2^-), hydroxyl radicals (•OH), and hydrogen peroxide are produced by many metabolic pathways (e.g. electron transport systems in the cell membrane) at very high rates even under optimal conditions (Mittler *et al.* 2004; Verma and Mishra, 2005). They cause very important impairments such as enzyme inactivation and membrane damage by reacting lipids, proteins and nucleic acids [Foyer *et al.*, 1994; Blokhina *et al.*, 2003). Plants have an antioxidant defense system including enzymatic and non enzymatic mechanisms to protect themselves by scavenging the ROS. The enzymatic ROS scavenging mechanism is consist of superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) (Apel and Hirt, 2004).

Since this substance is toxic for cells, it is metabolized to H_2O by POX and CAT (Singh *et al.*, 2006). Thus, possible damages which the ROS caused are prevented by these enzymes.

Today, many studies have been done in order to increase the resistance of plants and productivity against to negative environmental conditions. A lot of chemical substances and hormones have been used for this purpose. Mammalian sex hormones are also one of these substances. Effects of MSH on seed germination, plant growth and development have been studied since they were identified for the first time in plants (Dohrn *et al.*, 1926). The previous studies elucidated that MSH have a stimulating effect which resulted in increases in root and shoot lengths, protein content and activities of some enzyme (α-amylase, CAT and POX) in stages of the seed germination and plant growth (Martínez-Honduvilla *et al.*, 1976; Dogra and Thukral, 1991; Dogra and Kaur, 1994; Helmkamp and Bonner, 1952; Kopcewicz, 1970; Janeczko and Skoczowski, 2005); however, their action mechanism remains relatively sparse. They may directly affect the physiological processes. But, there are also other possibilities such as their effects on membranous properties or their conversion to other physiologically active compounds. Especially, the studies in relation to effects of MSH on seed germination are very scarce. In those studies, only a few parameters (germination rate, root and shoot lengths, protein content, activities of α-amylase, POX and CAT) have been studied in *Pinus pinea* and *Triticum aestivum* seeds (Martínez-Honduvilla *et al.*, 1976; Dogra and Thukral, 1991; Dogra and Kaur, 1994; Helmkamp and Bonner, 1952). Whereas, effects of these hormones on the activities of antioxidant enzymes and other stress

parameters (e.g. lipid peroxidation and H_2O_2 content) are still unclear.

We think that the stimulating effect of MSH may be related with increase in activities of antioxidant enzymes which enhance the plant resistance to environmental conditions. Therefore, in the present paper, we aimed to determine effects of MSH on activities of antioxidant enzymes (SOD, POX and CAT) in germinating bean seeds.

MATERIALS AND METHODS
Plant culture and steroid treatment

Bean seeds *(Phaseolus vulgaris* L.) were immersed in 1% sodium hypochlorite solution for 20 min at the room temperature (Aisien and Stark, 1983) and rinsed into sterile distilled water. The hormones (progesterone, β-estradiol and androsterone) were dissolved first in a small volume of methanol (Kato-Noguchi and Macias, 2005) and then diluted in water in order to obtain the following concentrations: 10^{-4}, 10^{-6}, 10^{-9}, 10^{-12} and 10^{-15}M. Seeds were soaked in prepared solutions about 6 hours (Marero *et al.,* 1988) and then moved in Petri dishes on filter paper moistened with 10 ml distilled water (10 seeds per dish). Seeds were soaked in distilled water, which include a small volume of methanol. They were identified as control group. All seeds were germinated in the dark at 25 °C. Steroids used in this work were obtained from the Sigma-Aldrich Co., St. Louis, MO, USA.

Determination of activities of antioxidant enzymes

For determining of peroxidase, catalase and superoxide dismutase enzyme activities, seeds were harvested at the end of 1nd, 3rd and 5th days of germination. Seeds (500 mg) were homogenized in 5 ml 10 mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidon. The homogenate was centrifuged at 12,000 x *g* for 30 minutes at 4 °C, and the supernatant obtained was used as an enzyme extract.

The POX activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM H_2O_2 (Janda *et al.,* 2003). One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01/min.

The CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) containing 20 mM H_2O_2. One unit of CAT activity was defined as the amount of enzyme that used 1 μmol H_2O_2/min (Gong *et al.,* 2001).

The SOD activity was estimated according to the method of Agarwall and Pandey (2004). The 3.5 cm^3 reaction mixture contained 0.05 M sodium carbonate pH 10.2, 13 mM methionine, 0.1 M EDTA, 63 μM nitroblue tetrazolium chloride (NBT), 13 μM riboflavin and 0.4 cm^3 enzyme extract. Reaction was started by placing tubes below two 15 W fluorescent lamps for 10 min. Reaction was stopped by keeping the tubes in dark for 10 min. Absorbance was recorded at 560 nm. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50 % inhibition of reduction of NBT and the specific enzyme activity was expressed as units per mg protein.

Determination of H_2O_2 content

Hydrogen peroxide levels were measured by monitoring the A_{410} of the titanium-peroxide complex according to He *et al.* (2005). One mililiter of cold acetone extracted supernatant was added to 0.1 mL 20% titanium reagent (20% w/v $TiCl_4$ in 12.1 mol HCl) and 0.2 mL 17 mol ammonia solution. The solution was centrifuged at 3000*g* at 4°C for 10 min and the supernatant was discarded. The pellet was dissolved in 3 mL 1 mol sulfuric acid. The absorbance of the solution was measured at 410 nm. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2-.

Determination of lipid peroxidation (TBARS content)

The final product of lipid peroxidation, malondialdehyde (MDA) is poisonous to plant cells. The MDA amount could be expressed as degree of lipid peroxidation. For measuring of the lipid peroxidation in seeds, the thiobarbituric acid (TBA) test, which determines malondialdehyde (MDA) was used. Lipid peroxidation was assessment as described by Velikova *et al.* (2000) measuring MDA content. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Statistical analysis

Each experiment was repeated at three times and two parallels. Analysis of variance was conducted using one-way *ANOVA* test using SPSS 13.0 for *Microsoft Windows* and means were compared by Duncan test at the 0.05 level of confidence. Error bars mean±SD.

RESULTS
Effects of mammalian sex hormones on the SOD activity

As seen from Fig. 1, activities of SOD were statistically high in the hormone-treated seeds as compared with their respective controls. The maximum activities were recorded as 32.12, 42.4 and 59.88 $U.g^{-1}$ for 10^{-9} M of progesterone, 24.75, 41.12 and 55.62 $U.g^{-1}$ for 10^{-9} M of β-estradiol and 30.68, 42.9 and 58.84 $U.g^{-1}$ for 10^{-9} M of androsterone at the

end of the 1st, 3rd and 5th day, respectively. On the other hand, SOD activities in control seeds were

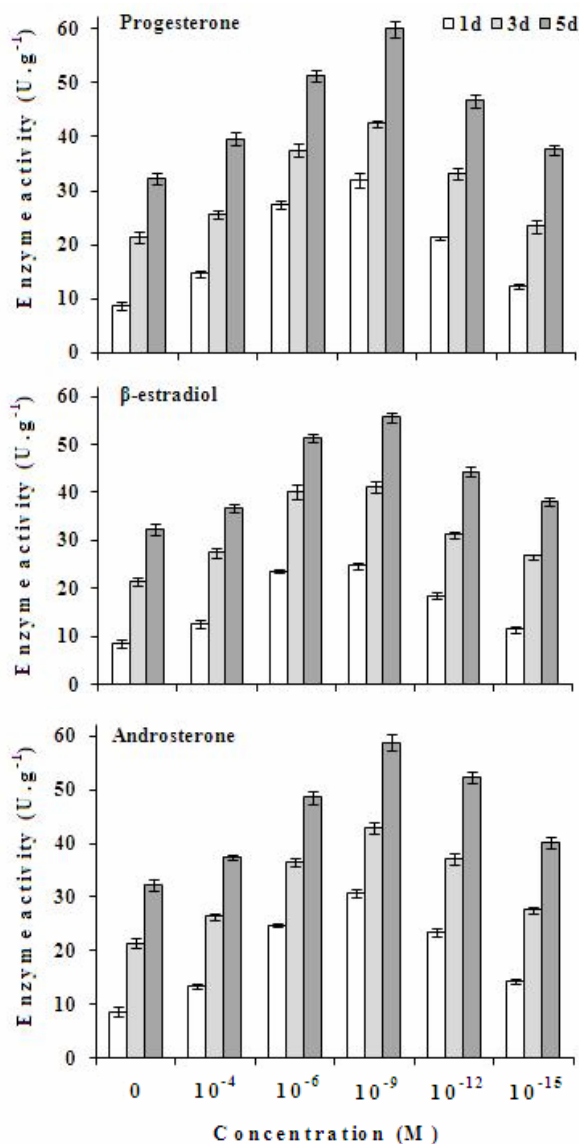8.68, 21.45 and 32.24 $U.g^{-1}$ at the end of the 1st, 3rd and 5th day, respectively.

Figure 1. Activity of superoxide dismutase in bean seeds treated with progesterone, β-estradiol and androsterone at 1st, 3rd and 5th day of germination.

Effects of mammalian sex hormones on the POX activity

Changes in POX activity are shown Fig. 2. At all the concentrations tested, POX activities were very high compared to control seeds. The maximum POX activities were determined as 288, 2945 and 11245 $U.g^{-1}$ for 10^{-9} M of progesterone, 321, 2989 and 10899 $U.g^{-1}$ for 10^{-9} M of β-estradiol and 336, 3065 and 11125 $U.g^{-1}$ for 10^{-9} M of androsterone at the end of the 1st, 3rd and 5th day, respectively. POX activities in control seeds were only 226, 1842 and 7424 $U.g^{-1}$ at the end of the 1st, 3rd and 5th day, respectively.
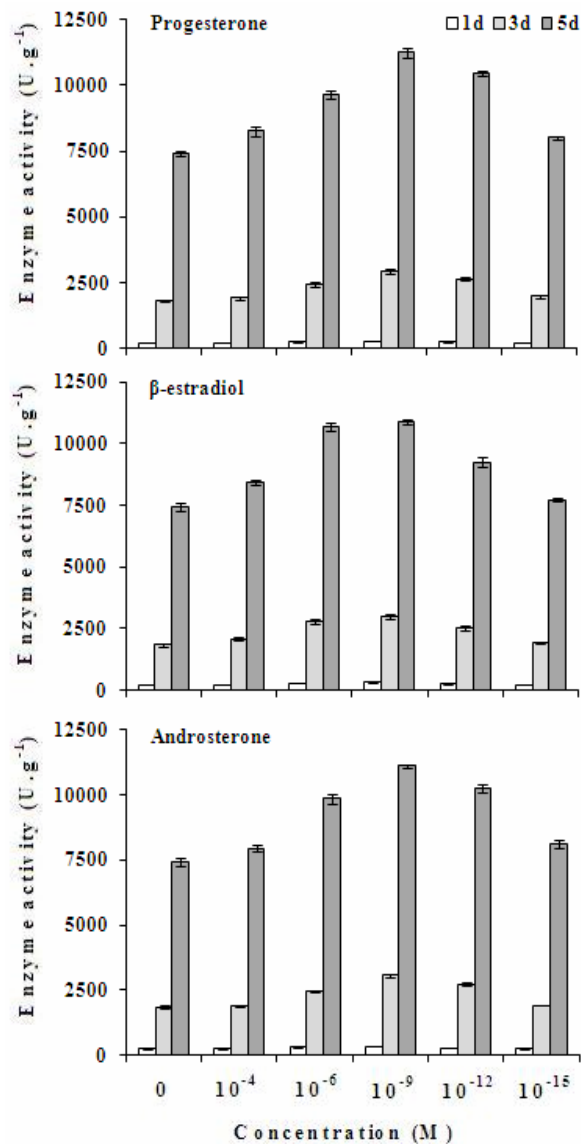
Figure 2. Effects of progesterone, β-estradiol and androsterone on peroxidase activity in bean seeds at 1nd, 3rd and 5th day of germination.

Effects of mammalian sex hormones on the CAT activity

The data presented in Fig. 3 clearly shows that even if CAT activities were increased by all the concentrations tested, they reached to maximum levels at 10^{-9} M concentrations for all the hormones. The maximum CAT activities were 142, 579 and 938 U.g^{-1} for progesterone, 155, 584 and 922 U.g^{-1} for β-estradiol and 144, 574 and 942 U.g^{-1} for androsterone at the end of the 1st, 3rd and 5th day, respectively. Whereas, CAT activities in control seeds were recorded as 75, 468 and 803 U.g^{-1} at the end of the 1st, 3rd and 5th day, respectively.
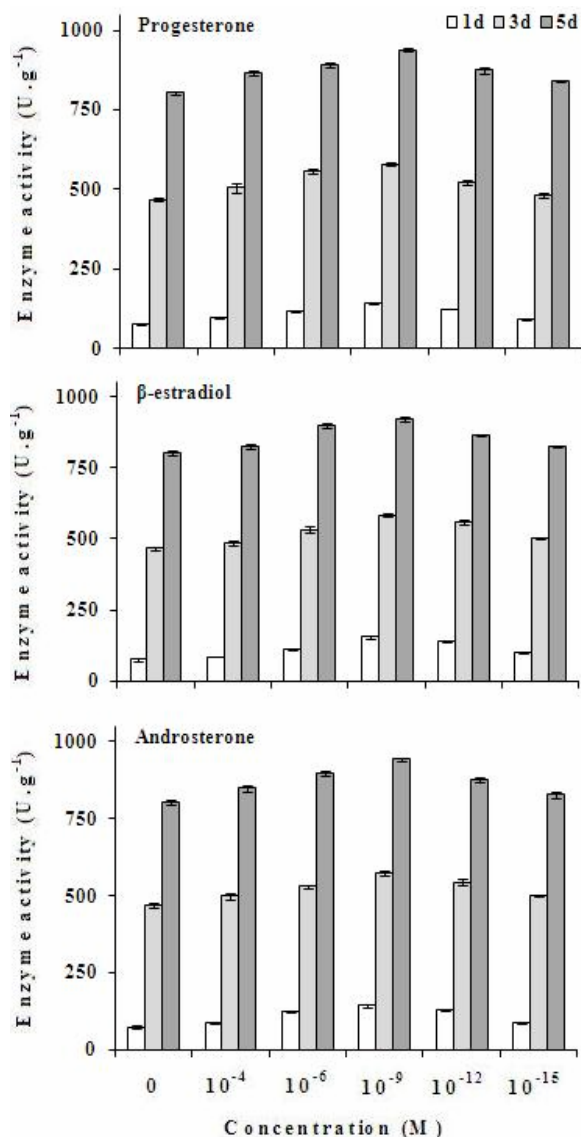
Figure 3. Effects of progesterone, β-estradiol and androsterone on catalase activity in bean seeds at 1nd, 3rd and 5th day of germination

Effects of mammalian sex hormones on H_2O_2 content

H_2O_2 content was significantly low in hormone-treated seeds with comparison to control seeds (Table 1). The lowest H_2O_2 contents were measured as 69.47, 114.76 and 132.3 $\mu mol.g^{-1}$ for 10^{-9} M of progesterone, 71.84, 119.04 and 132.18 $\mu mol.g^{-1}$ for 10^{-9} M of β-estradiol and 70.22, 118.41 and 128.74 $\mu mol.g^{-1}$ for 10^{-9} M of androsterone at the end of 1st, 3rd and 5th day, respectively. Whereas, H_2O_2 content was the highest (84.16, 132.26 and 149.62 $\mu mol.g^{-1}$) in control seeds at the end of 1st, 3rd and 5th day, respectively.

Effects of mammalian sex hormones on lipid peroxidation

Mean values of MDA content are presented in Table 1. MDA content was significantly decreased by all the concentrations tested of these hormones at the end of 1st, 3rd and 5th day of germination (Table 2). While MDA content of control seeds was 0.397, 0.746 and 1.974 $nmol.ml^{-1}$ at the end of 1st, 3rd and 5th day of germination, respectively, those of the hormone-treated seeds were 0.224, 0.612 and 1.792 $nmol.ml^{-1}$ for 10^{-9}M progesterone, 0.257, 0.622 and 1.826 $nmol.ml^{-1}$ for 10^{-9}M β-estradiol, and 0.236, 0.644 and 1.816 for 10^{-9}M androsterone.

Table 1. Effects of mammalian sex hormones on H_2O_2 content and lipid peroxidation at 1nd, 3rd and 5th day of germination in bean seeds.

Hormone Concentration [M]		H2O2 content [μmol.g⁻¹]			MDA content [nmol.ml⁻¹]		
		1 st day	3 rd day	5 th day	1 st day	3 rd day	5 th day
Progesterone	0	84,16[e]	132,26[d]	149,62[d]	0,397[e]	0,746[f]	1,974[e]
	10⁻⁴	80,29[d]	128,24[c]	144,04[c]	0,346[d]	0,726[e]	1,935[d]
	10⁻⁶	76,35[c]	119,42[b]	137,39[b]	0,309[c]	0,683[c]	1,861[b]
	10⁻⁹	69,47[a]	114,76[a]	132,31[a]	0,224[a]	0,612[a]	1,792[a]
	10⁻¹²	74,19[b]	120,84[b]	136,57[b]	0,278[b]	0,654[b]	1,844[b]
	10⁻¹⁵	80,66[d]	126,52[c]	142,64[c]	0,359[d]	0,701[d]	1,908[c]
β-estradiol	0	84,16[d]	132,26[d]	149,62[f]	0,397[d]	0,746[e]	1,974[d]
	10⁻⁴	79,43[c]	128,64[c]	142,14[d]	0,364[c]	0,694[d]	1,937[c]
	10⁻⁶	72,38[a]	120,56[a]	135,47[b]	0,278[a]	0,646[b]	1,845[a]
	10⁻⁹	71,84[a]	119,04[a]	132,18[a]	0,257[a]	0,622[a]	1,826[a]
	10⁻¹²	75,71[b]	124,88[b]	139,09[c]	0,319[b]	0,674[c]	1,892[b]
	10⁻¹⁵	78,19[c]	127,84[c]	145,84[e]	0,378[c]	0,703[d]	1,924[c]
Androsterone	0	84,16[f]	132,26[e]	149,62[e]	0,397[e]	0,746[d]	1,974[f]
	10⁻⁴	82,94[e]	127,36[d]	140,23[d]	0,339[d]	0,722[c]	1,943[e]
	10⁻⁶	76,68[c]	121,06[b]	136,29[c]	0,292[c]	0,671[b]	1,884[c]
	10⁻⁹	70,22[a]	118,41[a]	128,74[a]	0,236[a]	0,644[a]	1,816[a]
	10⁻¹²	74,56[b]	123,91[c]	132,39[b]	0,274[b]	0,678[b]	1,849[b]
	10⁻¹⁵	79,14[d]	129,93[d]	137,84[d]	0,345[d]	0,711[c]	1,917[d]

All values means of at least three determinations with 2 replicates. Means in the same column followed by the same letter are not significantly different at the ($p<0.05$) level.

DISCUSSION

The present paper focused on determining effects of mammalian sex hormones on the activities of antioxidant enzymes in germinating bean seeds. This work clarified that these hormones resulted in significant increments in activities of SOD, POX and CAT. Although all the concentrations tested stimulated enzyme activities, the maximum activities were achieved at 10^{-9}M concentration for all the hormones. These findings were consistent with those of previous study. But, the optimum concentrations obtained in the present study were lower than values obtained in the prior study. Dogra and Kaur (1994) reported that estrone, testosterone and 17 alpha-hydroxy progesterone stimulated the activities of CAT and POX in *Triticum aestivum* seeds. They informed that the maximum activities were obtained from the range of 10^{-6} and 10^{-8}M. Besides, the present paper showed that lipid peroxidation level and H_2O_2 content were significantly decreased by these hormones. The decrease in the lipid peroxidation level and H_2O_2 content might be attributed to increasing antioxidant enzyme activity. Because, lipid peroxidation level and H_2O_2 content were the lowest values at the concentrations which the maximum enzyme activities were achieved.

On the other hand, the some researchers informed that these hormones significantly stimulated seed germination. Martinez-Honduvilla *et al.* (1976) informed that estrone, β-estradiol and testosterone resulted in a higher growth rate and germination degree compared to their controls in *Pinus pinea* seeds. Dogra and Thukral (1991) and Dogra and Kaur (1994) reported that these hormones stimulated germination rate.

Furthermore, they determined the best stimulating effects were between 10^{-6} and 10^{-8}M, where the maximum CAT and POX activities were achieved. Similarly, the other researchers showed that the growth of pea embryo was stimulated by estrone (Bonner and Axtman 1937; Helmkamp and Bonner 1952; Kögl and Heagen-Smit 1936).

In conclusion, we may suggest usage of mammalian sex hormones in agriculture. Because, the present study demonstrated that these hormones might increase the plant resistance by stimulating activities of antioxidant enzymes. This assumption was also supported by decrease in lipid peroxidation level and H_2O_2 content. On the other hand, it was determined that they showed the maximum effects at very low concentrations such as 10^{-9}M. This finding is very important due to fact that their possible

negative effects for human and animal health may be prevented or reduced to minimal level. However, further studies are needed to prove the usability of these hormones in agricultural applications.

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