

# Progesterone and $\beta$ -Estradiol Stimulate Seed Germination in Chickpea by Causing Important Changes in Biochemical Parameters

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Effects of progesterone and  $\beta$ -estradiol on morphologic (germination velocity, root and shoot length) and biochemical (activities of  $\alpha$ -amylase, superoxide dismutase, peroxidase and catalase,  $H_2O_2$  content, lipid peroxidation) parameters during germination and post-germination stages of chickpea seeds were studied. The seeds germinated at various hormone concentrations ( $10^{-4}$ ,  $10^{-6}$ ,  $10^{-9}$ ,  $10^{-12}$ ,  $10^{-15}$  M) were harvested at the end of the 1st, 3rd, and 5th day. With comparison to the control, these hormones caused an increment in the number of germinating seeds at the end of days 1 and 3 by accelerating the seed germination. Root and shoot lengths were augmented by both hormones at all hormone concentrations tested. The highest elongation was recorded in  $10^{-6}$  M progesterone and  $10^{-9}$ – $10^{-12}$  M  $\beta$ -estradiol. Similarly, activities of  $\alpha$ -amylase and superoxide dismutase were increased by all concentrations of both hormones, and maximum increases were obtained with  $10^{-6}$  M progesterone and  $10^{-9}$ – $10^{-12}$  M  $\beta$ -estradiol. In the case of superoxide dismutase activity, not only the  $H_2O_2$  content but also the peroxidase and catalase activities increased. Lipid peroxidation decreased depending on an increase in the antioxidant enzyme activities. In the present study, it was demonstrated that progesterone and  $\beta$ -estradiol even at low concentrations increase the germination velocity and resistance to stress conditions by changing the activities of some biochemical pathways.

**Key words:** Chickpea, Seed Germination, Biochemical Activity

## Introduction

Steroids are lipophilic and low molecular weight compounds. These compounds, derived from isoprenoids, have important effects on differentiation, development, and homeostasis of higher eukaryotes (Kliwer *et al.*, 1998). They act in various roles like cell membrane constituents and bioregulators such as chemical messengers, vitamins, cytotoxins, and hormones (Barrington, 1979; Sandor and Mehdi, 1979). Steroid molecules have been identified as essential growth regulators in plants as well as in animals using many analytical tools (radioimmunoassay, thin-layer chromatography, mass spectrometry, ultra-performance liquid chromatography, high-performance liquid chromatography) (Geuns, 1978; Jones and Roddick, 1988; Simons and Grinwich, 1989; Janeczko and Skoczowski, 2005; Simersky *et al.*, 2009).

Mammalian sex hormones are a member of steroids and are naturally present in plants (San-

dor and Mehdi, 1979; Heftman *et al.*, 1965; Mordue and Stone, 1979; Geuns, 1978, 1982). They were first detected in plants by Dohrn *et al.* (1926) and Skarzynski (1933). Since then, many researches about the effects of these steroid hormones on growth and development of plants have been made. At the end of the studies, it was determined that steroid sex hormones applied exogenously (e.g. progesterone, estrone,  $\beta$ -estradiol, testosterone) stimulate growth and development (callus proliferation, cell division, root and shoot elongation, pollen germination flowering) (Kopcewicz, 1970; Geuns, 1978; Bhattacharya and Gupta, 1981; Shore *et al.*, 1992; Ylstra *et al.*, 1995; Hayat *et al.*, 2001; Janeczko and Skoczowski, 2005). However, there are a few reports on the effects of these hormones on seed germination. Seed germination is one of the most critical phases of plant life (Misra and Dwivedi, 2004) regulated by hormonal interactions and environmental factors (Paleg, 1960; Iglesias and Babiano, 1997; Atici *et al.*, 2005). Because there is not enough research

and knowledge on the effects of these hormones in seed germination, it is necessary to achieve advanced researches on biochemical parameters. The present studies about effects of mammalian sex hormones on seed germination demonstrated that these hormones positively affect some morphologic (root and shoot length, percentage of germination) and biochemical parameters (protein content, amylase and catalase activity) in germinating seeds. In previous studies, however, relatively high hormone concentrations (more than  $10^{-9}$  M) had been tested and only a few parameters had been studied (Martínez-Honduvilla *et al.*, 1976; Dogra and Thukral, 1991; Dogra and Kaur, 1994; Helmkamp and Bonner, 1952).

Hence, in the present study we aimed to search the effect of progesterone and  $\beta$ -estradiol on morphologic (germination velocity, root and shoot length) and biochemical (activities of  $\alpha$ -amylase, peroxidase and catalase) parameters in chickpea seeds. To the best of our knowledge, there has not been any report on the effect of mammalian sex hormones on germination in chickpea seeds, one of the most important legume crops of the world. Besides, the effects of these hormones on  $H_2O_2$  content, lipid peroxidation and superoxide dismutase activity in germinating chickpea seeds were studied for the first time.

## Material and Methods

### *Plant culture and steroid treatment*

Chickpea seeds (*Cicer arietinum* cv. Aziziye-94) were immersed in 1% sodium hypochlorite solution for 20 min at room temperature (Aisien *et al.*, 1983) and rinsed four times into sterile distilled water. All further manipulations were carried out under sterile conditions. The steroids progesterone and  $\beta$ -estradiol 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 for about 6 h (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 included a small volume of methanol; they were identified as control group. All seeds were germinated in the dark at 25 °C. The seeds germinated were counted at the 1st, 3rd, and 5th day in order to determine the percentage of germination.

Root and shoot lengths were measured on the 1st, 3rd and 5th day. Steroids used in this work were obtained from Sigma-Aldrich Co., St. Louis, MO, USA.

### *$\alpha$ -Amylase activity*

For determining the  $\alpha$ -amylase activity, endosperms of seeds were cut out at the end of the 1st, 3rd, and 5th days. Crude extracts of these seeds were prepared according to Juliano and Varner (1969). The  $\alpha$ -amylase activity was spectrophotometrically measured according to de Moraes and Takaki (1998). One unit (U) of enzyme was considered as the quantity that caused an alteration of 0.1 in absorbance.

### *Antioxidant enzyme activity (POX, CAT, SOD)*

For determining the peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) enzyme activities, seeds with germinating plant were harvested at the end of the 1st, 3rd, and 5th day. Seeds (500 mg) were homogenized in 5 ml 10 mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at  $12,000 \times g$  for 30 min at 4 °C, and the supernatant obtained was used as an enzyme extract.

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

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

The SOD activity was estimated according to the method of Agarwal and Pandey (2004). One unit (U) of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

### *Lipid peroxidation*

For measuring the lipid peroxidation in seeds, the thiobarbituric acid (TBA) test, which deter-

mines malondialdehyde (MDA) as an endproduct of lipid peroxidation, was used. Lipid peroxidation was assessed as described by Velikova *et al.* (2000) measuring the MDA content. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

#### Determination of $\text{H}_2\text{O}_2$ content

Hydrogen peroxide levels were measured by monitoring the absorbance at 410 nm of the titanium peroxide complex according to He *et al.* (2005). Absorbance values were calibrated to a standard curve generated with known concentrations of  $\text{H}_2\text{O}_2$ .

#### Statistical analysis

Each experiment was repeated at least three times with two replicates. Statistical analysis was performed using one way analysis of variance (ANOVA).  $P \leq 0.05$  was considered as significant.

#### Results and Discussion

As seen from the results summarized in Table I, at all concentrations, progesterone and  $\beta$ -estradiol augmented the germination speed of chickpea seeds compared to the control. The maximum seed germination was recorded as 78 and 100% for  $10^{-6} \text{ M}$  progesterone-treated seeds and 76 and 100% for  $10^{-9} \text{ M}$   $\beta$ -estradiol-treated seeds at the

Table I. Effects of progesterone and  $\beta$ -estradiol on morphologic and biochemical parameters of germinating chickpea seeds.

Parameter	Time (day)	Hormone concentration [M]					Range of standard deviation
		Control	Progesterone		$\beta$ -Estradiol		
		0	$10^{-9}$	$10^{-15}$	$10^{-9}$	$10^{-15}$	
Seed germination (%)	1st	62	75	63	76	66	2.80–2.88
	3rd	85	96	88	100	90	0.25–2.89
	5th	100	100	100	100	100	0–0
Root length [cm]	1st	0.41	0.53	0.435	0.63	0.625	0.01–0.04
	3rd	3.36	4.25	3.28	3.88	3.55	0.04–0.07
	5th	5.70	6.41	6.02	6.88	5.88	0.01–0.06
Shoot length [cm]	3rd	0.74	0.94	0.77	1.17	1.02	0.01–0.02
	5th	2.52	2.84	2.56	3.19	2.72	0.01–0.04
$\alpha$ -Amylase activity [U g <sup>-1</sup> ]	1st	157	180	166	237	227	1.52–3.51
	3rd	166	236	203	265	261	1.52–4.04
	5th	200	263	235	269	258	1.15–4.16
SOD activity [U g <sup>-1</sup> ]	1st	13.6	41	21	29.3	16.4	0.57–1.54
	3rd	29.4	48.2	24	53.8	34	1.12–1.52
	5th	38.1	57.4	41.4	60.5	46.6	0.78–1.96
H <sub>2</sub> O <sub>2</sub> content [ $\mu$ mol g <sup>-1</sup> FW]	1st	94	122	98	119	99	0.57–2.08
	3rd	177	277	211	256	209	1.73–4.61
	5th	116	142	123	233	149	0.57–4.35
POX activity [U g <sup>-1</sup> ]	1st	325	328	325	331	325	0.57–1.15
	3rd	3356	4800	4130	4430	4360	21–134
	5th	10900	15580	12300	17580	14410	80–196
CAT activity [U g <sup>-1</sup> ]	1st	145	228	168	236	150	1.01–4.35
	3rd	1023	1170	1065	1213	1078	2.08–11.52
	5th	1233	1300	1240	1365	1265	2.64–6.51
MDA content [nmol ml <sup>-1</sup> FW]	1st	0.645	0.465	0.59	0.48	0.59	0.014–0.021
	3rd	0.97	0.8	0.93	0.62	0.88	0.015–0.020
	5th	2.48	2.32	2.39	2.14	2.38	0.011–0.025

All values are means of at least three determinations with 2 replicates.

end of the 1st and 3rd day, respectively; 62 and 85% germination in control seeds was observed in the same time periods. All groups (control and steroid-treated seeds) showed 100% germination at the end of the 5th day. These results were in agreement with those reported in previous studies, but the obtained optimal concentrations were lower than in the prior studies. Some researchers informed that pea embryos were stimulated by estrone (Helmkamp and Bonner, 1952; Kögl and Haagen-Smit, 1936). They claimed that best optimal concentration range was  $10^{-6}$ – $10^{-8}$  M. Besides, Martínez-Honduvilla *et al.* (1976) reported that the mammalian steroids (estrone,  $\beta$ -estradiol and testosterone) increased the germination degree and growth rate compared to the control.

Similarly, at all tested concentrations, although, both progesterone and  $\beta$ -estradiol meaningfully augmented the root and shoot lengths of chickpea seeds compared to the control,  $10^{-6}$  M progesterone and  $10^{-12}$  M  $\beta$ -estradiol supplied the best ameliorative effects on these parameters. These results were similar to previous studies, which showed that estrone ( $1 \text{ mg l}^{-1}$ ) stimulated the growth of an isolated pea embryo *in vitro* and accelerated the meristem activity in the roots of *Melandrium dioecium*, *Rumex acetosa*, and *Anthoxanthum aristatum*. In germinating wheat seeds, root and shoot lengths increased at low concentrations ( $10^{-8}$  and  $10^{-6}$  M) more than at higher concentrations ( $10^{-4}$  M) of the steroids (Dogra and Kaur, 1994). These results suggested that progesterone and  $\beta$ -estradiol might stimulate the root and shoot growth depending on the increment of germination velocity. It can be also said that these steroids (especially  $\beta$ -estradiol) may be used to accelerate the seed germination and seedling growth.

Seeds gather the needed storage materials in cotyledons or endosperm (Bewley and Black, 1994). Embryos provide energy that is needed for germination from the degradation of storage forms such as carbohydrates. The beginning of germination activates the production of hydrolyzed enzymes, especially  $\alpha$ -amylase. This enzyme plays a major role in degradation of reserve carbohydrates to soluble sugars, which are used for providing the needed energy in germination (Perata *et al.*, 1997; Vartapetian and Jackson, 1997). Hence,  $\alpha$ -amylase is critical in seed germination. The  $\alpha$ -amylase activity was augmented by all concentrations of both progesterone and  $\beta$ -estradiol. The highest activity values were determined in the

$10^{-6}$  M progesterone-treated seeds as 203, 259 and  $272 \text{ U g}^{-1}$  and in the  $10^{-12}$  M  $\beta$ -estradiol-treated seeds as 270, 275 and  $281 \text{ U g}^{-1}$ , respectively, at the end of the 1st, 3rd and 5th day. This finding is in accordance with the results of preceding investigators, who showed that these steroids stimulated the  $\alpha$ -amylase activity in germinating seeds. But, the optimal hormone concentrations obtained from the present study were lower than the optimal values  $10^{-6}$ – $10^{-8}$  M by these investigators (Dogra and Kaur, 1994; Dogra and Thukral, 1991). With respect to these results, an increase in germination velocity may attribute to an increment in  $\alpha$ -amylase activity.

On the other hand, it has been known well that in plants reactive oxygen species (ROS) are permanently produced in cells even under optimal conditions (Atıcı and Nalbantoglu, 2003; Kang *et al.*, 2003). The tolerance of plants to oxidative stress conditions may be attributed to the increase in the activity of one or more antioxidant enzymes preventing cell and tissue damage. To determine the effects of progesterone and  $\beta$ -estradiol on the antioxidant enzymes in germinating chickpea seeds, the activities of three antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), were studied. SOD transforms superoxide to  $\text{H}_2\text{O}_2$  by acting as the first line of defence against ROS. Since  $\text{H}_2\text{O}_2$  is a substance toxic to living cells, POX and CAT metabolize  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  (Willekens *et al.*, 1997). In the present study, it was determined that the SOD, POX, and CAT activities were significantly increased by all concentrations of progesterone and  $\beta$ -estradiol compared to the control. The maximum SOD activities were 47, 53,  $62 \text{ U g}^{-1}$  for  $10^{-6}$  M progesterone and 29, 54,  $61 \text{ U g}^{-1}$  for  $10^{-9}$  M  $\beta$ -estradiol at the end of the 1st, 3rd, and 5th day, respectively. The highest CAT activities were recorded as 230, 1200, and  $1336 \text{ U g}^{-1}$  for  $10^{-6}$  M progesterone and 236, 1213, and  $1365 \text{ U g}^{-1}$  for  $10^{-9}$  M  $\beta$ -estradiol at the end of the 1st, 3rd, and 5th day, respectively. The maximum POX activities were determined as 328, 4800, and  $15580 \text{ U g}^{-1}$  for  $10^{-6}$  M progesterone and 333, 4640, and  $17230 \text{ U g}^{-1}$  for  $10^{-12}$  M  $\beta$ -estradiol at the end of the 1st, 3rd, and 5th day, respectively. On the other hand, the  $\text{H}_2\text{O}_2$  content was also augmented by these steroids. The maximum values for the  $\text{H}_2\text{O}_2$  level were achieved with  $10^{-6}$  M progesterone and  $10^{-9}$  M  $\beta$ -estradiol that resulted in the maximum SOD activities. The increment of

the  $H_2O_2$  level may be attributed to the increment of SOD activity.

Oxidative damage, caused by ROS, interrupts the lipid metabolism (Hernandez and Almansa, 2002; Misra and Gupta, 2006). The final product of lipid peroxidation, malondialdehyde (MDA), is poisonous to plant cells. The MDA amount could be expressed as degree of lipid peroxidation. In the present study, it was found that the MDA contents of chickpea seeds at the 1st, 3rd, and 5th day of germination were significantly decreased by progesterone and  $\beta$ -estradiol. MDA contents were lowest at the concentrations  $10^{-6}$  M progesterone and  $10^{-9}$  M  $\beta$ -estradiol, where antioxidant enzyme activities were the highest. The decrease of MDA content may be attributed to the increasing activities of antioxidant enzymes (SOD, CAT and POX) detoxifying ROS. Considering these results, it is possible to say that progesterone and  $\beta$ -estradiol may prevent possible damages, caused by ROS, by increasing the SOD, POX and CAT activities.

It was determined that at all tested concentrations of both progesterone and  $\beta$ -estradiol accelerated the seed germination and post-germination growth in chickpea by causing important biochemical changes. These results are basically in agreement with previous studies. However, the optimal concentrations of steroids obtained in the present study were lower than those obtained in

previous studies. This finding is very important due to the fact that, when the steroids are applied at lower concentrations, their possible negative side effects may decrease. Therefore, steroids may be used at low concentrations in agriculture. Because of positive effects on the germination velocity of the steroids, they may be used to accelerate the seed germination in some plant species which have germination difficulty. On the other hand, the steroids may be used to supply resistance to plants under various stress conditions since they have a stimulating effect on the antioxidant enzyme activity. The optimal concentrations of steroids were determined as  $10^{-6}$ – $10^{-8}$  M in previous studies, while the optimal concentrations in the present study were  $10^{-6}$  M progesterone and  $10^{-9}$  and  $10^{-12}$  M  $\beta$ -estradiol. From the results mentioned above, we believe that the usage of these steroids in agriculture can be very advantageous owing to roles at both the acceleration of germination and reduction of oxidative damage by increasing enzyme activity. However, further studies are needed to prove the usability of steroids in agriculture.

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