MAMMALIAN SEX HORMONES AFFECT REGENERATION CAPACITY AND ENZYMES ACTIVITY OF *TRITICALE* L. *IN VITRO* CULTURE.

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ABSTRACT

This study is the first report that determines the effects of 17β-estradiol, estrone, progesterone and androsterone among the mammalian sex hormones on *in vitro* regeneration of *Triticale* mature embryos. A range of parameters which were 0 (control), 10^{-4}, 10^{-8} and 10^{-12} m mol L^{-1} doses of 4 different mammalian sex hormones were investigated. It was clear that mature embryo interacted with the mammalian sex hormones. In mammalian sex hormones hormone applications, estron group gave the best result in terms of explant percentage forming shoots, followed by of progesterone group. Moreover, effects of mammalian sex hormones on proline and activities of enzymes *in vitro* regenere plantlets were investigated. Proline and activities of enzyme significantly increased at all the concentrations tested compared to control group. The maximum regeneration and enzyme activities were observed at the 10^{-12} m mol L^{-1} concentrations for all of four hormones *in vitro* culture.

Key words: Triticale, mammalian sex hormones, enzyme activity, *in vitro* culture.

INTRODUCTION

Mammalian sex hormones (MSH) play vital and necessary role in mammalian reproduction. They are also synthesiz by plants, in 128 species representing more than 50 families. The hormones such as estrone, progesterone, testosterone, androsterone, β-estradiol and 17β-estradiol are steroidal compounds naturally occurring in plants, but their content depends on species, cultivar, plant tissue and stage of development (Milanesi and Boland 2004; Janeczko and 2008, Erdal and others 2012; Erdal 2012). Plants synthesized numerous steroids and sterols. Steroids also play a role as a fundamental hormone in plants as well as in animals (Bishop and Koncz 2002). Especially while pregnenolone and progesterone were found in more than 80% of the investigated plant species, testosterone and estrogens (estrone and 17β- estradiol) were found in 70% and 50% of them respectively (Speranza 2010). In plants, the steroid sex hormones play an important role in regulation of biosynthesis process and generative reproduction and they are also physiological characteristics of plants are affected by extension and division in cell level, hormonal balance, enzyme activity, protein metabolism, control of mineral and nucleic acids, increase in photosynthetic activity, the amino acid composition of protein, the cell membrane and the fatty acid compositions (Khripach and others 2000, Janeczko and Skoczows 2005, Janeczko and others 2008, Speranza 2010, Khripach and others 2000, Janeczko and others 2012 and Erdal 2010). The compounds exhibiting estrogenic activity and defined as the phytoestrogen has more than 300 plants (Adams 1995). Several studies have been described for presence of the evaluation of numerous quantitative and mechanism of action since they were first emerged in plants. Recently the studies have focused on their receptors and specific binding sites in order to elucidate the effect mechanism of MSH however, the extent of such changes in various plant species has not been yet understood. In addition, exogenous application of MSH by seed soaking or foliar spray has been also studied on plant growth and development in various plant species. The studies demonstrated that exogenous application of MSH substantially induced production of plant growth and development, and stimulated oxidative enzyme activities under in vivo conditions. Janeczko and others (2002) stated that *in vitro* callus culture of immature wheat embryos is strongly stimulated by androsterone but regeneration is similar to control estrone and progesterone inhibits the first leaf and a callus growth of immature embryos in *in vitro* culture. Progesterone and 17β estradiol applied in *in vitro* culture of mature embryos of winter wheat stimulate the generative development of plants by increasing the percentage of heading plants and accelerating the heading. *Triticale* is an artificial species that originated 130 years ago from a cross between wheat and rye, with the first cultivars useful for breeders available in the 1960s (Mergeum et al 2009). It exhibits high yield potential, grain quality, resistant to pathogens favorable amino acid composition and adaptation to adverse conditions. The expansion of triticale cultivation has increased the need for improved classical breeding techniques, one such technique is *in vitro* culture plant regeneration can provide with MSH in triticale. Since last decade genetic studies, molecular

genetic and plant transformation studied have been reported intensive research on triticale (Lelley 1992). Few studies have been carried out to investigate the effects of different concentration MSH on accumulation of enzyme activity *in vitro* culture. *In vitro* culture is one of latest tools necessary for successful conducting plant basic research and widely used in commercial biotechnology. In vitro culture have become worth studying as a useful alternative because of unequal quality of products caused by environmental conditions. It is well known that plant regenerated *in vitro* undergo physiological and biochemical changes of the plants (Meja Orzechowska et al 2013). The objectives of this research were to study the influence of selected steroids on the *in vitro* regeneration of *Triticale* L. In addition, it was to determine differences between enzyme activity and the effect of the mammalian sex hormone on differentiation of regenere plantlets.

MATERIAL AND METHOD

Explants Source: In our study, mature seeds were surface sterilized with %70 ethanol for 5 min, washed several times with sterile distilled water, treated for 20 min, 33% with commercial bleach, and rinsed with several changes of sterile distilled water for overnight at 4°C The mature embryos placed scutellum up were cultivated in Petri dishes containing full MS medium 30 days at 26±1 and in 16 hour light / 8 hour dark photoperiod at 1500 lux illumination intensity.

Tissue Culture Media: Culture media used in all stages of experiment was MS medium (Murashige and Skoog 1962) with 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, 0,5 mg L^{-1} nicotinic acid, 0,5 mg L^{-1} pyridoxine HCl, 0.1 mg L^{-1} of thiamine HCl vitamins, 1.95 g L^{-1} of MES, 50 mg L^{-1} of ascorbic acid, 20 g L^{-1} of sucrose, solidified with 7 g L^{-1} of agar and the pH adjusted to 5.8 prior to autoclaving. In order to sterilize the vitamins and hormones, 0,22 µm of porous cellulose nitrate filters were used.

Culture of Mature Embryo and Observations: The triticale mature embryos were cultured in MS medium containing with 12 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) for 30 days in dark. Later calli were transferred to MS medium containing 17β-estradiol, estrone, progesterone and androsterone with four different doses (0, 10^{-4} m mol L^{-1} and 10^{-8} m mol L^{-1} and 10^{-12} m mol L^{-1}) for 50 days and subcultured in 30 days. All calli were kept under fluorescent light with 62 µmol m^{-2} s^{-1} and 16 h/8 h light/dark cycle in 26±1°C. Total culture duration was 80 days.

Proline estimation: Samples for the assay of proline content was obtained from green colour embryogenic calli at the end of culture 80th days. Proline content was measured with the method of Bates et al (1973). 100 mg of plant material was homogenized in 5 ml of 3% aqeuos sulfosalcylic acid and centrifuged at 4°C for 15 min at 4800 g. 2 ml of extract was mixed with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid in test tubes. Samples were kept for 1h at 100°C. The reaction was terminated in an ice bath. 4 ml of toluene was used for reaction mixture extraction. The absorbance of colour reaction product was measured at 520 nm using toluene for a blank. The proline concentration was determinated from a calibration curve.

Antioxidant enzyme assay: Samples for the assay of super oxidase (SOD), ascorbate peroksidaze (APX), catalase (CAT), peroxidase (POX) contents were collected from green colour embryogenic calli at the end of culture 80th days. The calli (500 mg) was homogenized in 5 ml 10 mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12000 g for 30 minutes at 4 °C, and the supernatant obtained was used as an enzyme extract.

Superoxide dismutase activitiy was assayed by monitoring the inbihition of photochemical reduction of NBT at 560 nm as described by Agarwall and Pandey et al 2004, in a reaction mixture containing 13 mM methionine, 75mM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50mM phosphate buffer (pH 7.8), 2 µM riboflavin, 0.02 cm^3 of enzyme extract.

Catalase 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 1µmol H_2O_2/min (Gong et al 2001).

The POX activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1mM 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.

APX activitiy was measured according to Nakaro and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0,5 mM ascorbic acid, 0,1 mM hydrogen peroxide and 0,1 mL of enzyme extract in a total volume of 1 mL. The concentration of oxizied ascorbate was calculated by decrease in absorbance at 290 nm.

Statistical Analysis: The experiment was conducted in factorial design using Completely Randomized Design (CRD) with 4 replications of 20 explants per Petri dish. Each petri dish is considered as one experimental unit. Twenty explants were placed in each petri dish. Analysis of variance and the Waller-Duncan K-ratio t-test were used to determine significant differences. Statistical analysis was carry out using SPSS (IBM-SPSS statistic for windows version 20.0).

RESULTS

Potential for callus induction and embryogenic calli: Different mammalian sex hormones and their doses affected percentage (%) of embryogenic calli, antioxidant enzyme activity and proline content was measured green colour embryogenic calli in media containing different mammalian sex hormones and their doses at the end of culture duration (80 days) (Fig. 1). Induction of callus was initiated after 12-15 days culture. 12 mg L^{-1} in media containing 2,4-D was 93% the response of callus formation of mature embryos the end of 30 days. Formation of embryogenic callus on callus from mature embryos after 80 days of callus differentiation induction were recorded and there were significant differences between different mammalian sex hormone and interactions hormonexdoses ($p<0,05$). Duncan multiple range test results of the means belonging to embryogenic calli percentage of different mammalian sex hormone types and doses embryogenic calli are summarized in the table below (Table 1).

Potential for regeneration: As shown in Table 1, Fig 1, the degree of regenerate shoots observed in the callus induction, depended on the different concentration of different MSH in the culture medium. The highest regeneration of 56% occurred in 10^{-12} mg/L estrone concentrations and was recorded at the end of third months. Control groups also showed regeneration of shoots in thirds months but there was notable change to the discolouration or browning of all remaining shoots over the 3 months culture period. On the other hand in the experimental group treated with different concentration MSH, plant regeneration ranged from 21,90% to 56,19% depending on the hormone concentration. Rate of plant regeneration was higher on the medium containing estrone and progesterone than the medium containing 17β-estradiol and androsterone. The highest plant regeneration was observed on MS medium supplemented with estrone and progesterone whereas the lowest plant regeneration occurred on the medium containing 10^{-4} mg/L androsterone. In addition increase in hormone concentration decreased the rate of plant regeneration and 10^{-12} mg/L concentration of estrone was found to be best for plant regeneration.

Proline Estimation: Proline in the selected plantlets was higher than the control group regeneration when grown on different levels of MSH supplemented media. Proline contents were significantly ($p<0,05$) affected with an increase in different concentration of MSH. Similarly a gradual increase in the proline content of green colour embryogenic calli was noticed with an various in the concentration of MSH from 10^{-4} to 10^{-12} mg/L, maximum being 10^{-12} mg/L estrone level (Fig 2). The differences between hormone types, doses and hormonexdose interactions in terms of proline level were very highly significant ($p<0,05$).

Antioxidant Enzyme Activity: The level of activities of antioxidant enzymes such as catalase, ascorbate peroxidase, superoxide dismutase and peroxidase was investigated green colour embryogenic calli *in vitro* culture at the end of three months. All tested enzyme activities were enhanced different of concentration during *in vitro* culture. The maximum catalase activity was observed 10^{-12} mg/L estrone level *in vitro* embryogenic calli. APX activity was significantly the highest in low dose calli as compared to control group. POX activity was also increased considerably under 10^{-12} mg/L concentration of MSH application both estrone and progesterone. The activity of SOD increased progressively and significant at 10^{-12} mg/L estrone level, however, the activity significantly decreased in plantlets of 10^{-4} mg/L estrone level compare to 10^{-12} mg/L estrone level (Fig 3.a,b,c,d).

DISCUSSION

Exogenous application of MSH was reported to have effects on a wide range physiological process including plant growth and development. This study clearly indicated that mammalian sex hormones affected the *in vitro* parameters of *Triticale* mature embryo culture. In our study, callus culture and their transfer to regeneration medium including MSH were carried out within eighty days to ensure that embryogenic calli at the each plate.

Otaran (2012) worked out an efficient protocol for MSH from mature wheat embryo by means of *in vitro* culture. In the study investigated the effect of mammalian sex hormones (progesterone, testesterone, estrogen and 17β-estradiol) and doses (10^{-4}, 10^{-5} and 10^{-6} m mol L^{-1}) by using mature wheat embryo culture. They reported that mature wheat embryos responded differently depending on the type of the mammalian sex hormone, their concentration and genotype. In our experiment, similar results were obtained when steroid hormone *in vitro* culture medium was used. This difference could be due to plant species, explant types or other factors such as concentration MSH and culture medium. Similar results were also reported by various researchers in many studies dealing with different perspectives of plant physiology and development under normal conditions. (Janeczko and others 2002; Janeczko and Filek 2002, Hakk et al. 2005; Lino et al. 2007; Pekşen 2011; Hacıbektaşoğlu 2011; Erdal and Dumlupinar 2011a, Erdal and Dumlupinar 2011b; Dumlupinar and others 2011, Shemesh and Shore 2012, Erdal 2012a, Erdal 2012b). Janeczko and others (2008) who investigated in plant body binding regions of the mammalian sex hormones reported that there were specific binding regions for progesterone and 17β-estradiol in *Triticum aestivum* L.

cells. The researchers emphasized that this binding occurs in membrane and cytosols. They identified more special bindings of 17β-estradiol in membranes than cytosols. In our experiment, control dose gave the lowest result in terms of percentage of embryogenic calli (PEC) parameter, and followed by 10^{-4} m mol L^{-1} dose of androsterone and estrone. The best hormone group in terms of PEC was estrone and 10^{-12} m mol L^{-1} dose was ranked the first and the difference between control significant ($P > 0.05$). When general means were paid attention, the lowest dose of estrone and progesterone gave the best results (10^{-12} m mol L^{-1}) and followed by the doses of 10^{-8} m mol L^{-1}. Regeneration development was influenced by MSH, although no significant the high level effects of different hormones was observed. The value was statistically significant the medium and low level of MSH treatment. The highest number of developed 10^{-12} m mol L^{-1} level was observed on the estrone (56.19) and progesterone (54,76). Addition of 10^{-8} m mol L^{-1} level to the media resulted development of estrone (48.10) and progesterone (45.23). When concentration was getting increased, the promotion effect of mammalian sex hormones was slightly decreased. Janeczko and others (2003), Hakk and others (2005), Lino and others (2007), Guan and Roddick (1988), reported that there were some changes based on genotype and type and concentration of hormones used in studies related to mammalian sex hormones. In addition, similar effects of mammalian sex hormones were observed in plant development when lower doses were applied. The fact that the induction in promotion effect was closely related with hormone concentrations in the relatively increasing was summarized in Table 1. In adverse with our findings, the study conducted by Shore and others (1992) about the effect of estrone and 17β-estradiol on vegetative development of alfalfa indicated that the amount of estrogen in plant content was not increased in the vegetative growth-enhancing concentrations (0,005 µg/l-0,5µg/l) and internal estrogen concentrations of plants were increased in estrogen concentrations (50 µg/l-500 µg/l) by inhibiting vegetative development and nodule weight. Previous researchers displayed that exogenously treated MSH increased proline, protein and activities of catalase, peroxsidase, acid phosphatase in wheat seedlings under normal conditions. They were determined that the best concentrations were 10^{-8} and 10^{-6} mol L^{-1}. Therefore from a wide range of concentrations of MSH, 10^{-4},10^{-8} and 10^{-12} were studied in our study. MSH application considerably increased the promotion effects of mature embryo culture, and significantly enhanced enzyme activities as compared to control groups. Proline has been widely considered to be maintaining membrane integrity and in stabilization of macromolecules or molecular assemblies. In addition, proline is important component of the defense system of plants. It occurs widely in higher plants and accumulates in larger amounts in stressed plants. The increase in proline content under *in vitro* culture could be attributed to growth of leaf area expansion. The maximum proline content was recorded at 10^{-12} m mol L^{-1} estrone and progesterone. Based on our findings, increases in proline content in MSH-treated seedlings may be related to promotion of growth parameters and protective role of MSH as direct or indirect but when concentration increased, the inhibitory effect of mammalian sex hormones was slightly increased.

The present paper showed that lower dose of MSH caused noticeable increase in SOD, APX, CAT and POX; however, the highest doses reduced the CAT activity compare to lower doses. Similarly, MSH treatment increased considerably content of all antioxidant compound compared to control groups. Based on our findings, it can be said that amount of mammalian sex hormones applied at promotion level for the development may be accumulated within the body of the plant. This finding may explain the reason why the promotion effect of mammalian sex hormones various doses when applied at various doses of MSH on the explants of the culture taken to hormone-free medium in the third months. This situation points out the activity time of the mammalian sex hormones within plant, binding regions and binding degrees to plant tissues are the matters requiring the further studies. Besides, mammalian sex hormone applications can be used in *in vitro* culture studies. Further studies about the subject are needed. Again when high hormone doses applications are tried it may have positive effects on plant regeneration.

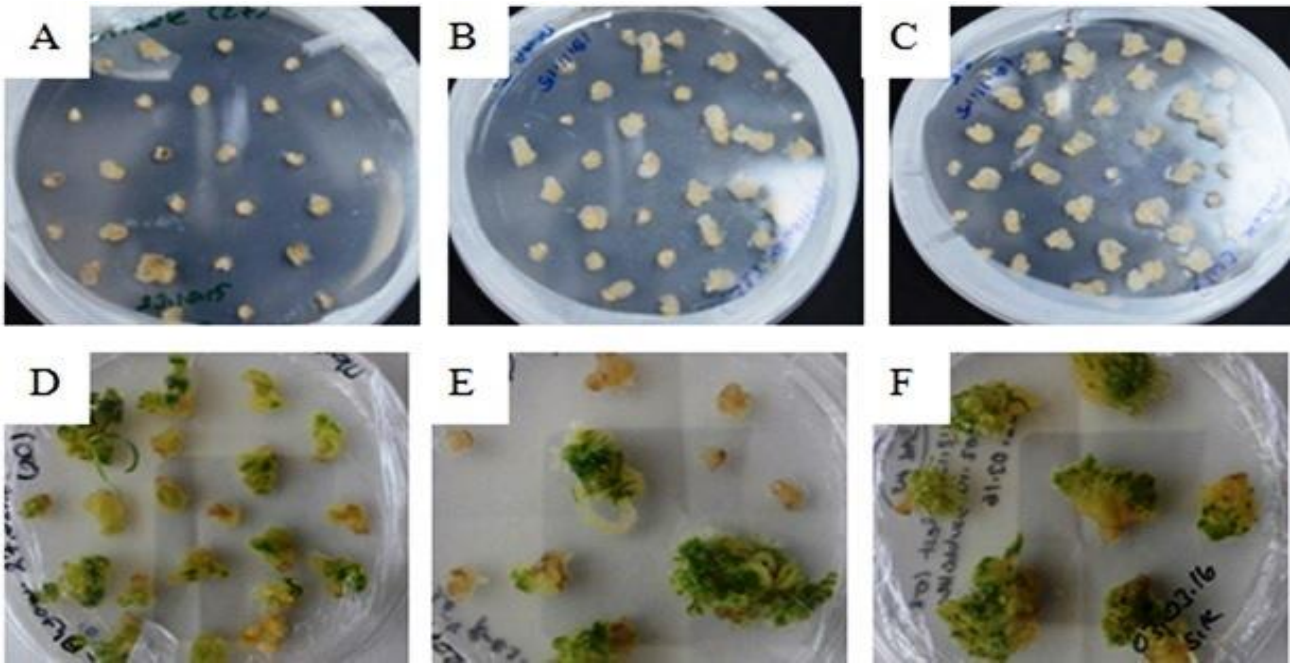


Fig 1. A. Induction of callus from triticale mature embryos after 1 weeks of culture. B. Callus formation of cotyledons explants after 2 weeks of culture. C. Callus formation of cotyledons explants after 1 months D. E. F. Formation of embriyogenic callus from callus on MSH after 3 months of culture.

Table 1. Effect of four mammalian sex hormones and their doses on percentage of regeneration capacity of callus

	Hormones[a]			
Doses	**17β-Estradiol**	**Estron**	**Progesteron**	**Androsteron**
0 m mol L−1	18,69±0,18Da	18,69±0,18Da	18,69±0,18Da	18,69±0,18Ca
10^{-4} m molL−1	25,24±2,97Ca	23.33±2,97Ca	25,97±0,93Ca	21,90±2,18Ca
10^{-8} m molL−1	36,19±0,82Bb	48,10±2,18Ba	45,24±6,44Ba	28,10±3,30Bc
10^{-12} m molL−1	45,24±3,60Ab	56,19±2,18Aa	54,76±0,82Aa	43,81±5,02Ab
Mean ± SD	31,34±10,82b	36,58±16,72a	36,17±15,37a	28,13±10,46c

*: The differences between the means shown with capital letters in the same column and the means shown with lower case on the same line are significant (P<0,05). a:Values are mean ± Std Dev.

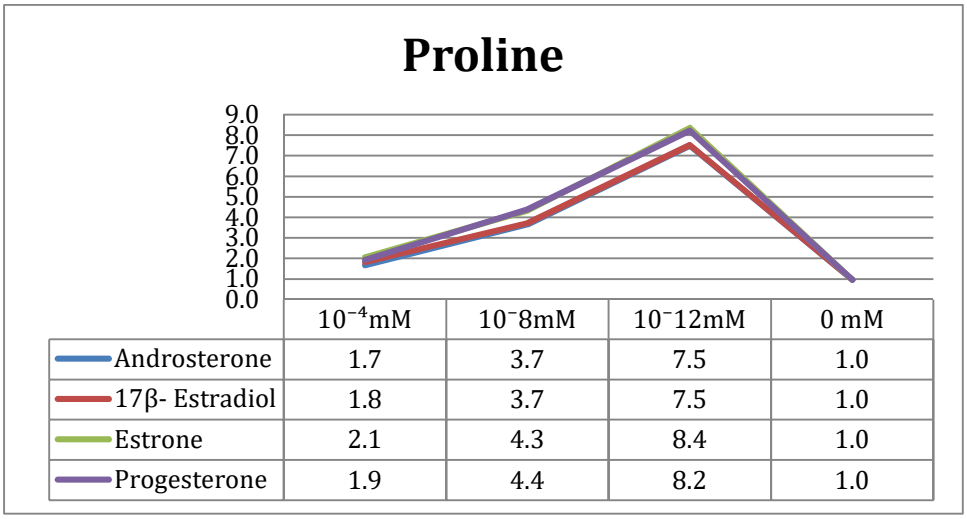
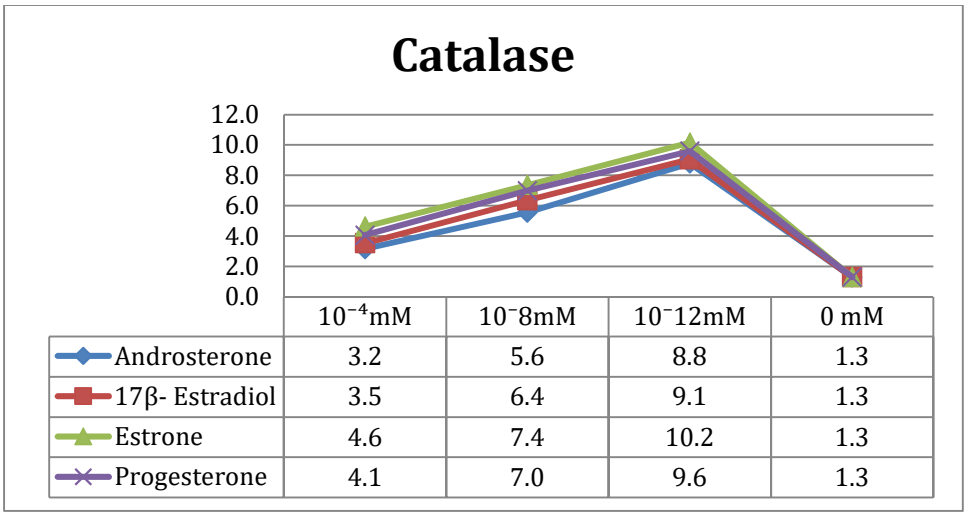
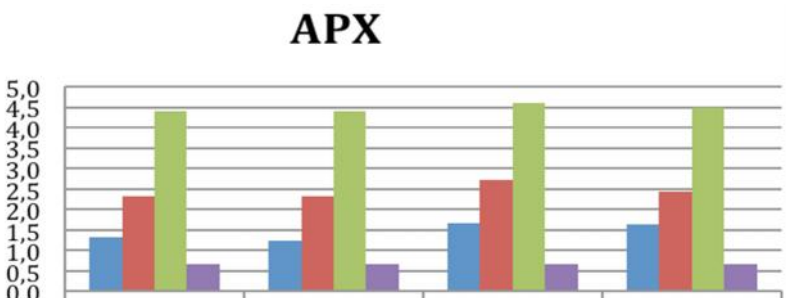


Fig 2. Proline contents of *Triticale* under different MSH concentrations. Proline content was measured regenere plantlets content after 3 months.



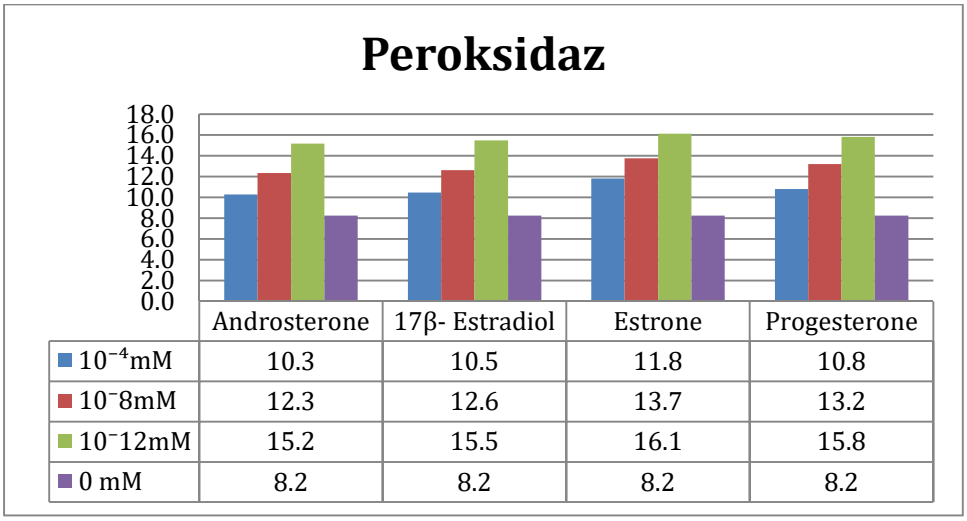
	10^{-4}mM	10^{-}8mM	10^{-}12mM	0 mM
Androsterone	3.2	5.6	8.8	1.3
17β- Estradiol	3.5	6.4	9.1	1.3
Estrone	4.6	7.4	10.2	1.3
Progesterone	4.1	7.0	9.6	1.3

Fig 3a. Antioxidant enzyme CAT activities (nmol/g FW) of Triticale regenere plantlets under different MSH concentrations



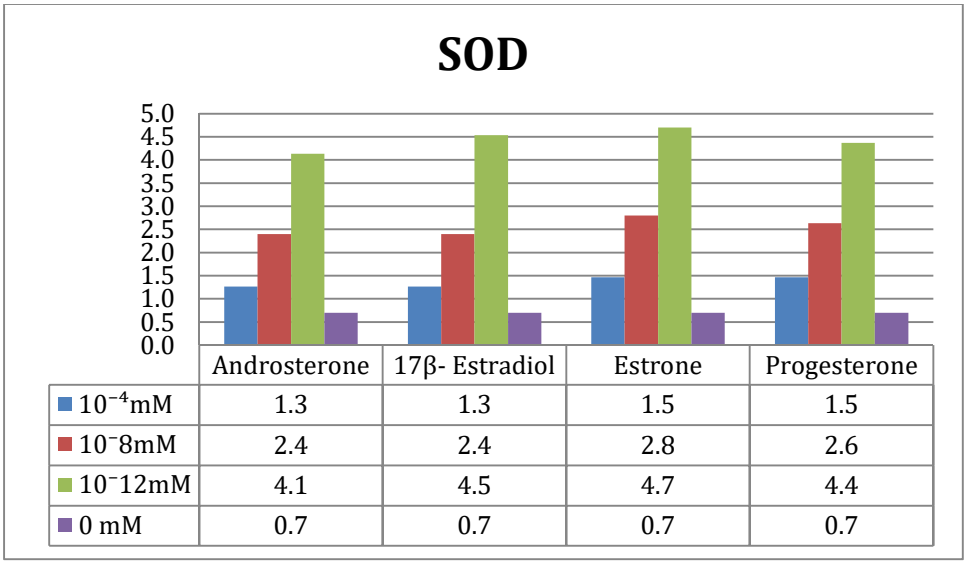
	Androsterone	17β- Estradiol	Estrone	Progesterone
10^{-4}mM	1,3	1,2	1,7	1,6
10^{-}8mM	2,3	2,3	2,7	2,4
10^{-}12mM	4,4	4,4	4,6	4,5
0 mM	0,7	0,7	0,7	0,7

Fig 3b. Antioxidant enzyme APX activities (nmol/g FW) of Triticale regenere plantlets under different MSH concentrations.



	Androsterone	17β- Estradiol	Estrone	Progesterone
10^{-4}mM	10.3	10.5	11.8	10.8
10^{-}8mM	12.3	12.6	13.7	13.2
10^{-}12mM	15.2	15.5	16.1	15.8
0 mM	8.2	8.2	8.2	8.2

Fig 3c. Antioxidant enzyme POX activities (nmol/g FW) of Triticale regenere plantlets under different MSH concentrations



	Androsterone	17β- Estradiol	Estrone	Progesterone
10^{-4}mM	1.3	1.3	1.5	1.5
10^{-}8mM	2.4	2.4	2.8	2.6
10^{-}12mM	4.1	4.5	4.7	4.4
0 mM	0.7	0.7	0.7	0.7

Fig 3d. Antioxidant enzyme SOD activities (nmol/g FW) of Triticale regenere plantlets under different MSH concentrations.

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