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MAMMALIAN SEX HORMONES AFFECT REGENERATION CAPACITY AND ENZYMES ACTIVITY OF *TRITICALE* (X TRITICOSECALE WITTMACK) *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

Plants encounter many physiological and biochemical factors during their life cycle. This process cycling hormones plays one of the predominant roles for survival and plant development events. Five groups recognized of natural plant hormones have been known in regulation of plant development of the auxins, gibberellin, absisic acid, ethylene, cytokinins until recently. Nowadays another signal molecule has been recognized such as jasmonic acid (JA), salicylic acid (SA) including polypeptide system that plays important roles in transducing the activation of plant defense systems against pathogen attacks (Taiz and Zeiger 2008). Steroids are one of these hormones. A large group of steroids has properties of hormones activity. Phytosteroids are produced in many plant species (Sarin 2005; Milanesi and Boland 2004). Previously, both flora and fauna have specifically reported on a wide of chemically diverse of steroids. Although, there are small variations of chemical structure and large variations in physiological properties of steroids, plants and animals were more closely than previously thought the results of new studies. (Pauli *et al.,* 2010). Mammalian steroids hormones also known as mammalian sex hormones estrone, progesterone, testosterone, androsterone, β-estradiol and 17β-estradiol species synthesized by many plant species. MSH is well known for its important effect on mammalian reproduction and metabolism (Milanesi *et al*., 2001). The ongoing research has allowed new approaches and revised of available information the role of steroids on plant and animals.

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 *et al.* (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 (Mergoum *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, 2006). 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 (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.

MATERIALS AND METHODS

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 determined 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 (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 Nakano 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).

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). aValues are mean ± Std Dev

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

In the present study, calli from mature embryos of triticale cultured on MS medium supplemented with different concentration of 17β-estradiol, estrone, progesterone and androsterone were applied in tissue culture. . The results of this study displayed that calli increased regeneration capacity and that the antioxidants enzyme activities (Proline, APX, CAT and Peroksidase) studied increased; and these increases were seen to have changed depending on the type and dosage of the hormone applied. This increase was observed changeable depending on the applied MSH type and doses. Exogenously trearment MSH has positive or negative effects in enhancing the plant growth and development, morphology, polen tube development, generative stage and resistance of stress factors parameters of in vitro regeneration used in tissue culture and plant spceies, treatment hormone, doses effects of these were reported by many researchers (Ylstra *et al.,* 1995; Janeczko *et al.,* 2002; Janeczko and Filek 2002; Janeczko *et al.,* 2003; Hakk *et al*., 2005; Janeczko and Skoczowski 2005; Brown 2006; Lino *et al*., 2007; Erdal and Dumlupinar 2010; Janeczko and Skoczowski 2011; Hacıbektaşoğlu 2011; Pekşen 2011; Janeczko *et al*., 2012; Otaran 2012; Shemesh and Shore 2012, Uysal 2014; Bowlin 2014; Adeel *et al*., 2017).

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±0.21) and progesterone (45.23±0.64). When concentration was getting increased, the promotion effect of mammalian sex hormones was slightly decreased. Similarly, Uysal (2014) investigated the effect of mammalian sex hormones on the in vitro regeneration of birdsfoot trefoil (*Lotus corniculatus* L.) She observed that high concentration treatment the inhibition effect of mammalian sex hormones was increased when concentration was increasing. Lino *et al*. (2007), In their study investigating the effect of progesterone on vegetative development of the Arabidopsis shoots, that the vegetative growth increased in low concentrations of progesterone, that both in dark and light conditions the growth was under pressure by high concentration. Shore *et al*. (1992), investigated the effects of estrone and β-estradiol on the vegetative development of alfalfa. They reported that applying these two estrogens to the plant in very low concentrations increased the dry weight of both the roots and shoots, whereas the high concentrations decreased that.

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} mol L^{-1} 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 study is important in terms of interaction MSH and calli obtained mature embryo of triticale. This situation points out mammalian sex hormones binding regions and binding degrees to triticale tissues are the matters requiring the further studies. Besides, mammalian sex hormone applications can be used in triticale in vitro culture studies.

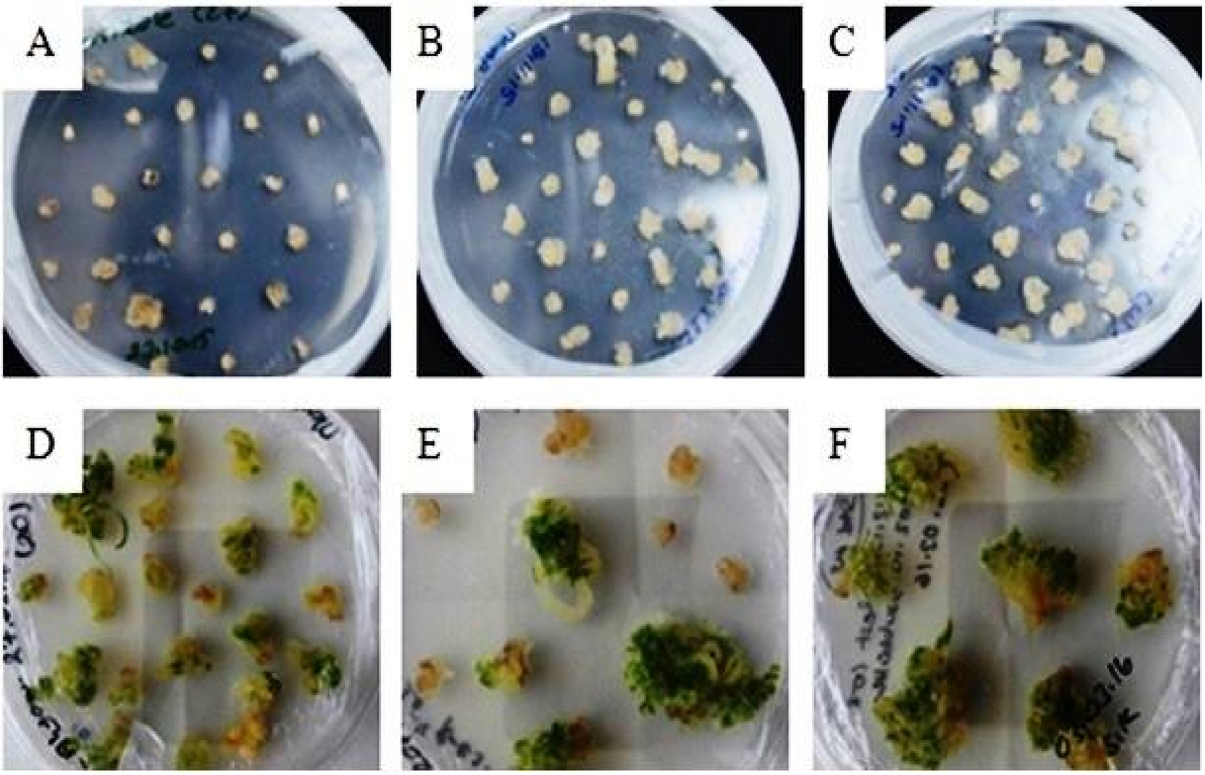
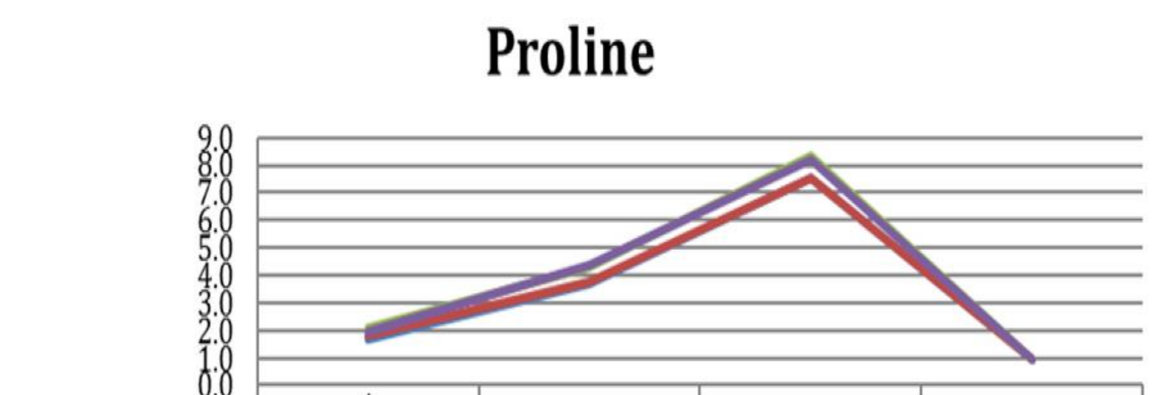
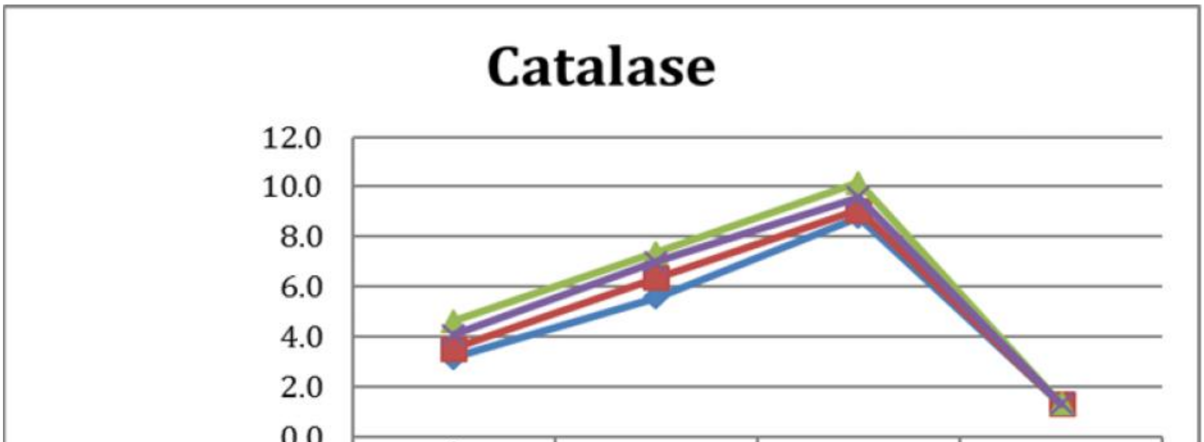


Fig 1. A. Induction of callus from triticale mature embryos after 1 weeks of culture. B. Callus formation of mature embryos explants after 2 weeks of culture. C. Callus formation of mature embryos after 1 months D. E. F. Formation of embriyogenic callus from callus on MSH after 3 months of culture.



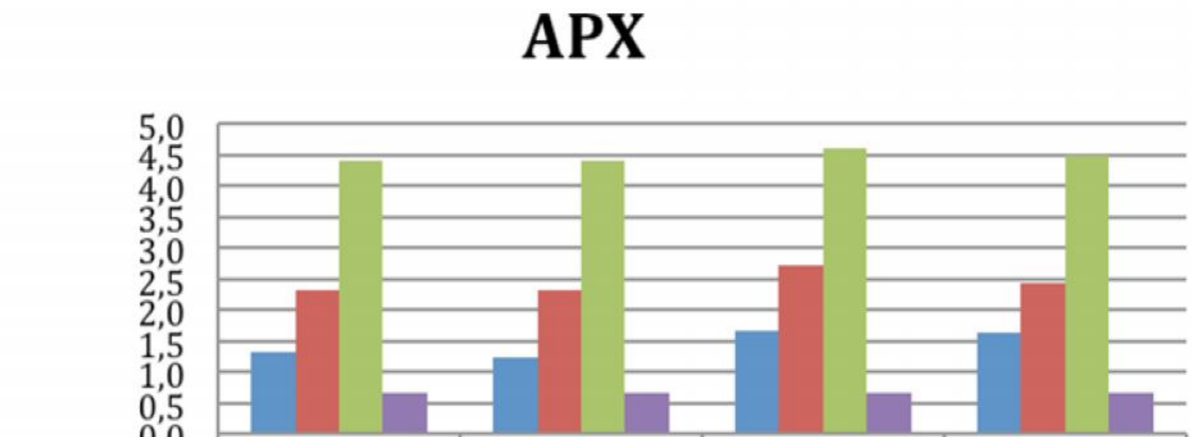
	10^{-4}mM	$10^{-}8$mM	$10^{-}12$mM	0 mM
Androsterone	1.7	3.7	7.5	1.0
17β- Estradiol	1.8	3.7	7.5	1.0
Estrone	2.1	4.3	8.4	1.0
Progesterone	1.9	4.4	8.2	1.0

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



	10^{-4}mM	10^{-8}mM	10^{-12}mM	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^{-8}mM	2,3	2,3	2,7	2,4
10^{-12}mM	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

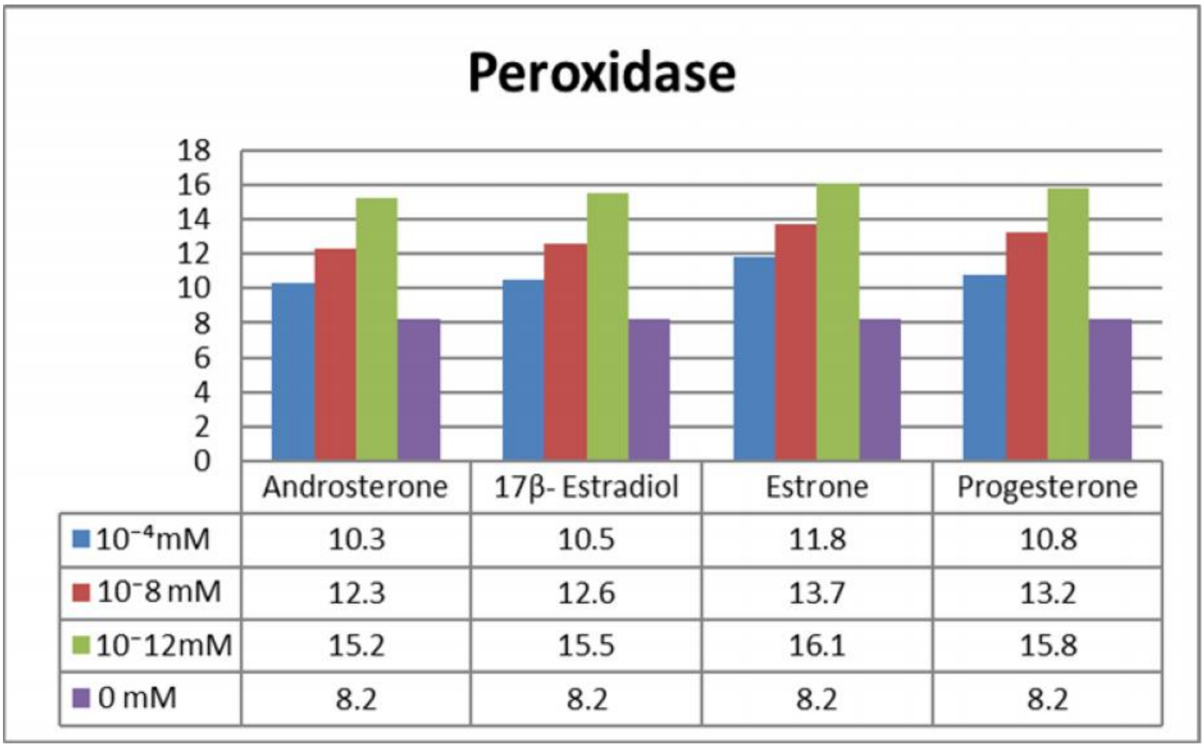


Fig 3c. Antioxidant enzyme POX activities (nmol/g FW) of Triticale regenere plantlets under different MSH concentrations.

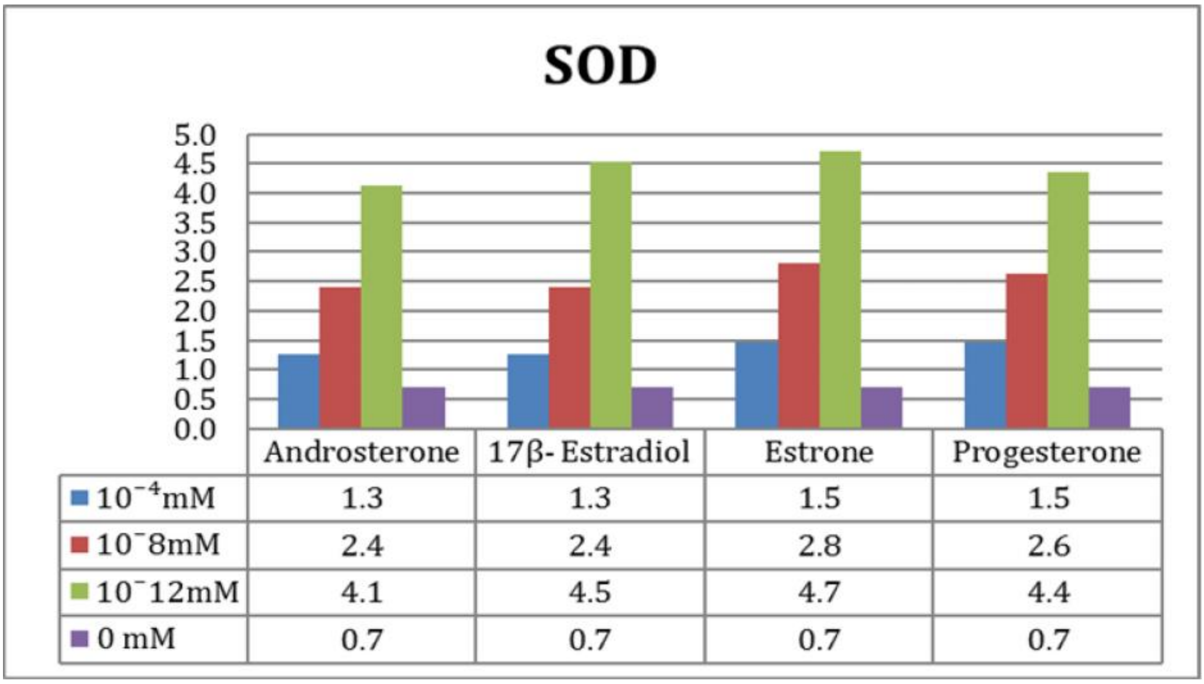


Fig 3d. Antioxidant enzyme SOD activities (nmol/g FW) of Triticale regenere plantlets under different MSH concentrations

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