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Effect of 17β-estradiol on seedling and callus growth of German chamomile (*Matricaria chamomilla* L.)

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Abstract

To study the effect of 17β-estradiol on seedling growth, antioxidant enzyme activity and also, on callus induction from leaf explants of German Chamomile (*Matricaria chamomilla* L.), an experiment was conducted as a completely randomized design with three replications using MS medium containing different concentrations of 17β-estradiol (0, 0.01, 0.1, 1 and 10 mg/l) alone or in combination with 3 mg/l Benzylaminopurine (BAP) + 0 or 1.5 mg/l 1-naphthalene acetic acid (NAA). The results showed that 17β-estradiol at 0.01 and 0.1 mg/l increased root and shoot length and weight, respectively and at high concentration (10 mg/l) increased peroxidase, polyphenol oxidase and catalase activity of German chamomile seedlings. Also, the callus induction was observed after one to two weeks in all media, but the growth varied depending on the presence or absence of plant growth regulators and different concentrations of 17β-estradiol. Maximum callus weight was obtained in 0.01 mg/l of 17β-estradiol with about three-fold increase in comparison with the control (MS without17β-estradiol). This indicates that the 17-beta-estradiol at lower concentrations (0.01 mg/l) can significantly improve callus growth in the presence of plant growth regulators such as NAA and BAP. The results of this study indicate that using steroidal hormone 17β-estradiol can be used to optimize German chamomile cell growth under *in vitro* conditions.

Keywords: Antioxidant enzymes, Callus induction, *In vitro* culture, Steroid hormone

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Introduction

17β-estradiol, a steroid hormone with low molecular weight has key roles in controlling growth, development, reproduction and control of protein and mineral metabolism in mammals. In recent years, this hormone has been recognized in many plant species and extracted from tissues and organs such as roots, leaves and flowers (Janeczko and Skoczowski 2005; Genisel *et al.* 2013). Recent studies on the effects of this hormone have shown that its exogenic use in plants, stimulate cell division, root and shoot growth, embryo development, pollen tube growth, flowering, proliferation and callus growth and increase the activity of antioxidant enzymes (Erdal 2012a; Erdal and Dumlupinar 2011b; Genisel *et al.* 2013). It has also been reported that 17β-estradiol is effective in callus induction and proliferating in *Polygonatum verticillatum* L. (Janeczko and Szybka 2001). Furthermore, the application of β-estradiol leads to a significant increase in plant growth, protein and soluble sugars contents and antioxidant enzymes activity such as superoxide dismutase, peroxidase and catalase in *Cicer*

arietinum L. (Erdal and Dumlupinar 2011a) and wheat seedlings under salt stress (Erdal 2012b). It has also been reported that this hormone can improve sugar, proline, protein, chlorophyll, glutathione and dry matter contents in wheat seedlings under salt stress conditions as compared with the control plants. Furthermore, lipid peroxidation and production of hydrogen peroxide and superoxide content induced by salt stress are decreased in wheat by using this hormone (Erdal 2012b). The effects of the 17β-estradiol hormone on plant life have not been fully studied. Recent research shows the existence and effects of this hormone on plant growth under *in vitro* conditions (Janeczko 2012).

It was shown that *in vitro* callus induction and shoot regeneration in plants are affected by the application of proper plant growth regulators (Fazeli Behgo and Alizadeh Ajirlo 2015). Due to the lack of adequate research on the effect of this hormone on medicinal herbs, this study aimed to investigate the effect of mammalian sex hormones on callus and seedling growth and antioxidant enzymes activity in German chamomile (*Matricaria chamomilla* L.) under *in vitro* culture. German chamomile from the Asteraceae family is widely used in the pharmacopeia of many countries in the world due to its essential oils and secondary metabolites with pharmaceutical importance (Petronilho *et al.* 2012). In a previous study (Koohi *et al.* 2014), we investigated the callus induction from leaf explants of German chamomile on MS medium supplemented with different concentrations of plant growth regulators, and it was concluded that 1.5 mg/l NAA and 3 mg/l BAP is the most proper medium for the callus induction. So, the main aim of the present study was to investigate the effect of different concentrations of 17β-estradiol on callus growth, antioxidant enzymes activity and plantlet growth of German chamomile under the *in vitro* culture conditions in the presence or absence of these phytohormones.

Materials and Methods

The chamomile seeds provided from Pakanbazr Co. (http://www.pakanbazr.com/), were washed with distilled water, sterilized for 10 minutes in 2.5% sodium hypochlorite and then were immersed for 30 seconds in 70% ethanol. Then, the seeds were rinsed three times with sterilized distilled water and were cultured in vessels containing MS medium (Murashige and Skoog 1962) in a growth chamber at 25±2 °C and the photoperiod of 16/8 h light and darkness, respectively. After germination, 10 days old seedlings were transferred to an MS medium containing different concentrations (0, 0.01, 0.1, 1 and 10 mg/l) of 17β-estradiol (Sigma-Aldrich, E8875). Due to the water insolubility of 17β-estradiol in ethanol, it was first dissolved in one drop of 96% ethanol then the required distilled water was added to reach the proper concentration. It should be noticed that for negligible adverse effects of ethanol, it was added to the control treatment.

After 40 days, shoot and root length and weight and the activity of antioxidant enzymes were measured. To measure the activity of antioxidant enzymes including catalase (CAT), polyphenol oxidase (PPO) and peroxidase (POX), leaf tissues from 40-day-old seedlings were

ground in liquid nitrogen and homogenized in 50 mM Tris-HCl buffer. The enzymes were extracted according to Sudhakar *et al.* (2001). After centrifugation at 12000 rpm for 20 min at 4 °C, the supernatant was taken to measure the activity of peroxidase and polyphenol oxidase based on Kar and Mishra (1976) and catalase activity according to Chance and Maehly (1955), by recoding absorbance at 425, 420 and 240 nm, respectively.

In addition, the effect of the 17β-estradiol hormone on callus induction and growth from leaf explants of chamomile was studied on MS medium containing different concentrations (0, 0.01, 0.1, 1, 10 mg/l) of this hormone alone or in combination with 3 mg/l BAP + 0 or 1.5 mg/l NAA according to Koohi *et al.* (2014). Five weeks after culture, callus induction rate and callus fresh weight (per explant) was recorded. Analysis of variance and comparison of means was done with the SAS software (Version 9.1). The data were reported as the mean ± standard error (SE) based on three replications.

Results

Seedling growth

The *in vitro* growth of German chamomile seedlings and the calluses induced from its leaf explants under different concentration of 17β-estradiol are shown in Figure 1. The effect of 17β-estradiol on all of the seedling characteristics was significant ($p \leq 0.05$). The 17β-estradiol hormone was effective in increasing the shoot and root length of German chamomile seedlings. The highest shoot length and weight of chamomile seedlings were observed at 0.1 mg/l of 17β-estradiol. However, the highest concentration significantly differed from the control regarding these characteristics (Figure 2). The root length and weight of German chamomile seedlings were also significantly increased at 0.01 and 0.1 mg/l of 17β-estradiol as compared to the control, but at higher concentrations (1 and 10 mg/l) its difference with the control was not significant.

On the other hand, the activity of peroxidase, polyphenol oxidase and catalase enzymes increased as the concentration of 17β-estradiol hormone was increased, so the maximum activity was observed at 10 mg/l concentration of this hormone. The lowest concentration of 17β-estradiol (0.01 mg/l) was not significantly different from the control concerning all antioxidant enzymes activities (Figure 3).

Callus induction

Callus induction from leaf explants of the German chamomile in all media containing various concentrations of 17β-estradiol alone, or in combination with NAA (0 and 1.5 mg/l) and BAP (3 mg/l) was observed after two weeks. However, callus growth varied depending on the concentration of the steroid hormone and the presence or absence of phytohormones (Figure 4). On the MS medium containing 3 mg/l BAP plus 0 or 1.5 mg/l NAA, all of the leaf explants induced callus. The highest callus induction on MS medium containing 17β-estradiol without the phytohormones was only 37%, however, it was significantly greater than the control. On the MS medium containing both NAA and BAP, the addition of 17β-estradiol increased the callus weight. The maximum callus weight was obtained

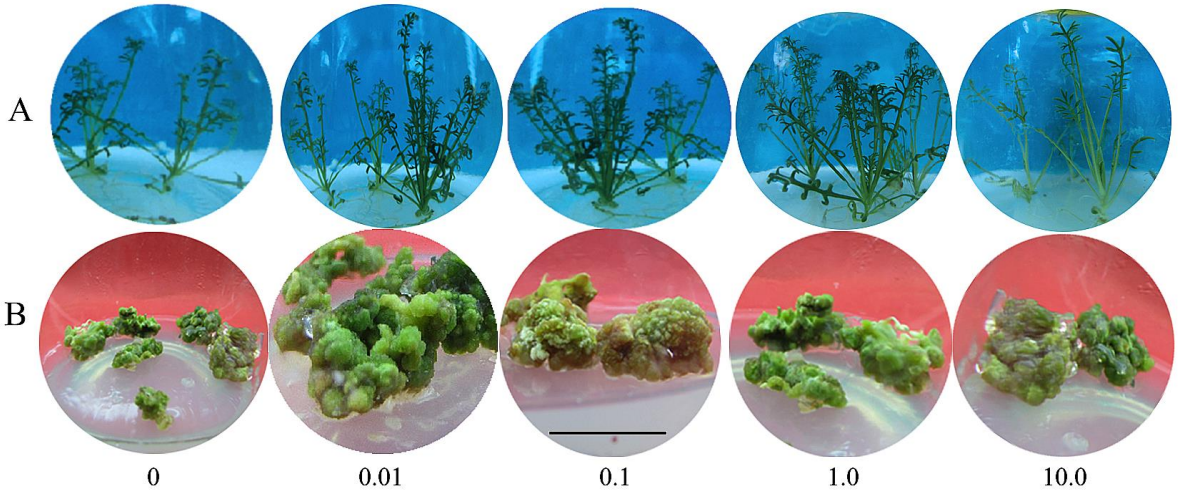

Figure 1. *In vitro* growth of German chamomile seedlings (A) and induced calluses from its leaf explants on MS medium containing different concentrations of 17β-estradiol (0, 0.01, 0.1, 1 and 10 mg/l) in combination with a 1.5 mg/l NAA and 3 mg/l BAP (B). Scale bar 50 mm.

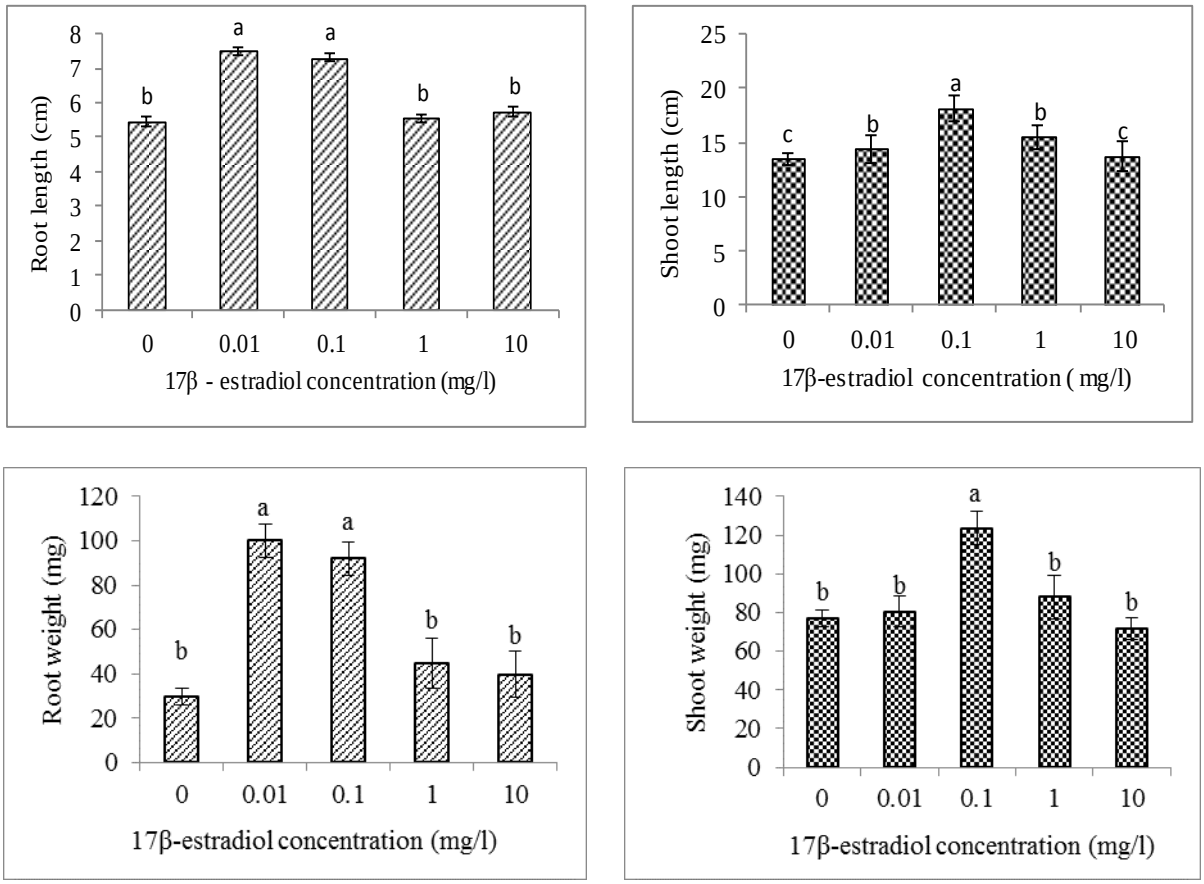

Figure 2. Effects of 17β-estradiol treatment on root and shoot length and weight of German chamomile seedlings. The error bars represent standard errors; Means with different letters are significantly different at $p \leq 0.05$.

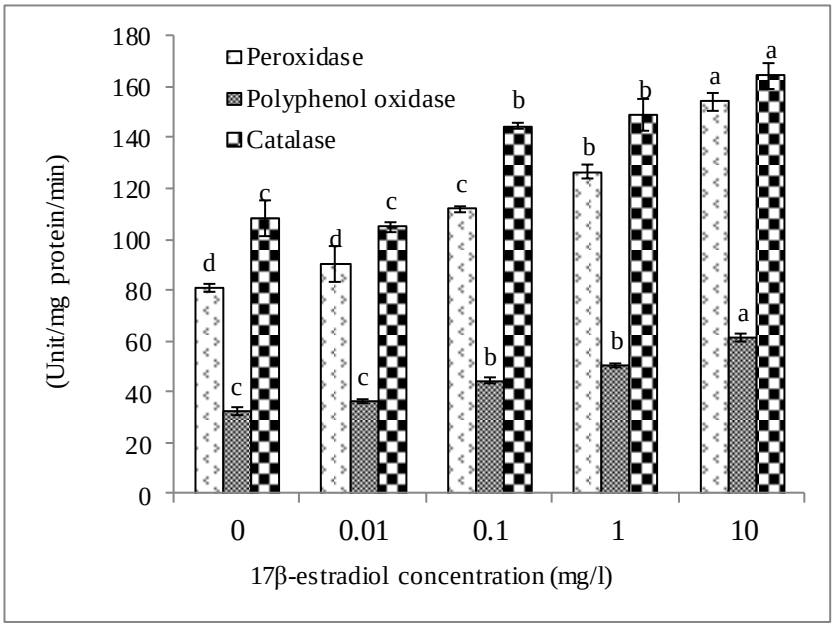

Figure 3. Effects of 17β-estradiol treatment on peroxidase, polyphenol oxidase and catalase enzymes activity of the German chamomile seedlings. The error bars represent standard errors; Means with different letters are significantly different ($p \leq 0.05$) in each enzyme.

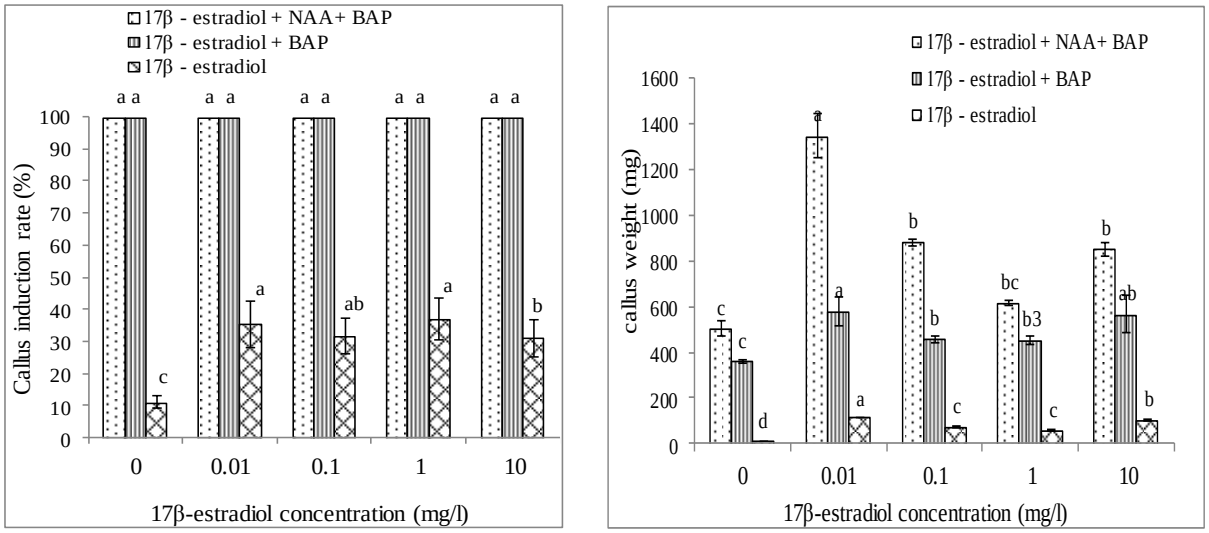

Figure 4. Callus fresh weight and induction rate (%) and German chamomile leaf explants in MS medium supplemented with NAA and BAP, MS medium with BAP only and MS medium without NAA and BAP under treatment with different concentrations of 17β-estradiol. In each trait, means with different letters show significant differences by the LSD test at the 5% probability level.

at 0.01 mg/l of 17β-estradiol with about three-fold increase. This indicates that the 17-beta-estradiol at lower concentrations (0.01 mg/l) can significantly improve callus growth in the presence of phytohormones such as NAA and BAP. Furthermore, on the MS medium supplemented with only BAP (3 mg/l without NAA) and various concentrations of 17β-estradiol, the callus weight was significantly increased. So that, 17β-estradiol at 0.01 mg/l significantly increased callus weight in the auxin-free medium (about 50%) as compared with the medium consisted of BAP only. This represents an effective role of 17β-estradiol in improving the callus growth at the auxin-free medium. On the other hand, although leaf explants on MS medium without NAA or BAP, can produce callus as maximum as 37% as compared with the control (MS medium free of any hormones), the callus growth was much more reduced in comparison with the MS medium containing NAA and BAP. Maximum callus weight (114.6 mg) was obtained on the MS medium containing 0.01 mg/l, which was significantly greater than other concentrations of 17β-estradiol (Figure 4).

Discussion

The results of this study about the effect of 17β-estradiol on seedling growth of the German chamomile showed that at the low concentration of 17β-estradiol (0.01 and 0.1 mg/l), the root length and weight increased, and at 0.1 mg/l, the shoot length and weight were increased. It has been shown that external use of this steroid hormone significantly increases plant growth, protein, soluble sugars and antioxidant enzymes (Erdal and Dumlupinar 2011a). Treatment of corn seedlings with this hormone under salinity stress has significantly alleviated the adverse effects of salinity (Erdal 2012c). In addition, the application of 17β-estradiol on wheat seedlings under salinity stress improved dry weight, glucose, proline, protein and chlorophyll contents (Erdal and Dumlupinar 2011b). Furthermore, spraying alfalfa (*Medicago sativa* L.) with nutrient solution containing 0.005 and 0.05 mg/l of 17β-estradiol and estrone increased root and shoot weight, but at 50 and 500 mg/l, the growth of this plant was inhibited (Shore *et al.* 1992). Some studies show that foliar application of hormones such as gibberellic acid, epibrassinolide and acetylsalicylic acid on plants under stress conditions can improve plant physiological and agro-morphological characteristics (Fathi *et al.* 2019; Pourasadollahi *et al.* 2019).

This 17β-estradiol hormone had a significant effect on the activity of antioxidant enzymes. The activity of peroxidase, polyphenol oxidase and catalase enzymes increased in the German chamomile seedlings. The highest activity of these enzymes was obtained at the highest concentration of 17β-estradiol (10 mg/l). Researches by Erdal (2012a, b) and Erdal and Dumlupinar (2011a) have also shown that the use of 17β-estradiol increases the activity of antioxidant enzymes. These enzymes provide tolerance and adaptation to environmental conditions mainly by purifying reactive oxygen species and reducing the harmful effects of stress on plants. Erdal and Dumlupinar (2011b) reported that this hormone increased

concentrations of potassium, calcium, magnesium, manganese, aluminum, zinc, iron, copper, phosphorus and sulfur. It can be stated that this steroidal hormone, along with the increase of organic matter and minerals and the increase of antioxidant enzyme activity, improves the conditions for plant growth (Dumlupinar *et al.* 2011). The application of other steroidal hormones such as androsterone has also significantly increased the growth and activity of antioxidant enzymes in *Cicer arietinum* L. (Erdal and Dumlupinar 2011a).

As was shown, the highest callus weight of the German chamomile was obtained at the low concentration (0.01 mg/l) of 17β-estradiol. This suggests that 17β-estradiol can significantly increase the weight of callus in combination with NAA and BAP more than the amount reported previously in this plant in the presence of NAA and BAP (Koohi *et al.* 2014). In addition, increasing the callus weight in the auxin-free medium indicated the effective role of this hormone in improving callus growth conditions in the absence of auxin. Induction and growth of callus from leaf explants in the NAA-free medium seem to have been influenced by this steroidal hormone. In addition, callus induction of leaf explants in the MS medium without phytohormones showed that in the absence of BAP and NAA, it can induce callus formation in the leaf explants although, its growth was weak. Still, this hormone significantly increased callus growth if callus induction was made in the presence of BAP and NAA phytohormones. The results of the present study confirm the effective role of 17β-estradiol in the growth of calli obtained from German chamomile, which was in agreement with the results of studies by Janeczko *et al.* (2002) that showed increased callus growth under the influence of 17β-estradiol and androstenedione. It has been shown that androstenedione at a rate of 1 μM induces callus growth from the scutellum of the immature wheat (*Triticum aestivum* L.) embryos. It has been also reported that 17β-estradiol at the concentration of 2–12 mg/l, increases the growth of *Daucus carrota* L. in the tissue culture medium by 100% (Janeczko and Skoczowski 2005). Janeczko and Szybka (2001) also showed that this hormone induces callus in *Polygonatum verticillatum* L.

Although the presence of steroid hormones in plants has been established (Geuns 1978; Simons and Grinwich 1989; Janeczko and Skoczowski 2005; Iino *et al.* 2007; Simersky *et al.* 2009; Janeczko 2012), little is known about the major role and effects of these hormones in plants. Similar to mammalian sex hormones, plant steroidal hormones play a key role in the flexibility of plants under varying environmental conditions. At the low concentrations, they affect plants by controlling cell division (Iino *et al.* 2007). It was reported that when brassinosteroids bind to a receptor on the cell wall, they trigger a multi-level cascade of reactions that regulates the activity of several transcription factors especially CESTA (CES) (Khan *et al.* 2014). In animals, steroidal hormones are involved in regulating the transcription of specific genes through the plasma membrane and attaching to cytoplasmic or nuclear receptors. Milanesi *et al.* (2001) used the calli of *Solanum glaucophyllum* to study the expression of estrogen-binding proteins. They first used the

radioligand binding assay to investigate the specific binding site of 17β-estradiol and then used western blotting and ligand blotting methods to confirm the possible association of these loci with known isoforms. The calli of *Solanum glaucophyllum* not only contain 17β-estradiol and estrone hormones but also have abundant binding sites. These findings indicate the existence of estrogen receptor molecules in the plant. In addition, the presence of 17β-estradiol internal receptors was demonstrated in the pistil of *Gladiolus primulinus* (Janik and Adler 1984).

Production of plant secondary metabolites in cell and tissue culture of medicinal plants is a major goal in the production of medicinally important compounds using biotechnological tools. The first step to do this is the establishment of well-growing cells and calli under *in vitro* conditions. The optimization of cell growth in plant tissue culture results in better growth of cells and improved production of metabolites in bioreactors for industrial-scale production of active compounds. The results of this study indicate that steroidal hormone 17β-estradiol can be used to optimize the German chamomile cell growth under *in vitro* conditions. However, more study and further research are needed in this and other plants to reach to a decisive conclusion.

Conclusion

The low concentration of 17β-estradiol increased 17-est increased root and shoot weight of German chamomile seedlings and at higher concentrations increased antioxidant enzymes activities. Also, the highest callus weight of leaf explants was obtained with NAA and BAP phytohormones by adding low concentration of 17β-estradiol (0.01 mg/l). In the absence of phytohormones such as BAP and NAA, this hormone can induce callus formation in the leaf explants of this plant, but the growth rate of callus was much lower than that used in combination with BAP and NAA in the culture medium. Our study indicates that using steroidal hormone 17β-estradiol will be beneficial to optimize the German chamomile cells' growth under *in vitro* conditions.

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Conflict of Interest

The authors declare that they have no conflict of interest with any people or organization concerning the subject of the manuscript.

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تأثیر ۱۷بتا-استرادیول بر رشد گیاهچه و کالوس در بابونه آلمانی (*Matricaria chamomilla* L.)

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چکیده

به منظور بررسی تأثیر ۱۷-β-استرادیول بر رشد گیاهچه، فعالیت آنزیم‌های آنتی‌اکسیدان و همچنین القای کالوس از ریزنمونه برگ گیاه بابونه آلمانی (*Matricaria chamomilla* L.)، آزمایشی به صورت طرح کاملاً تصادفی با سه تکرار با استفاده از محیط کشت MS حاوی غلظت‌های مختلف ۱۷-β-استرادیول (۰، ۰،۰۱، ۰،۱، ۱، و ۱۰ میلی‌گرم در لیتر) به تنهایی یا در ترکیب با ۳ میلی‌گرم در لیتر بنزیل‌آمینوپورین(BAP) به علاوه صفر یا ۱،۵ میلی‌گرم در لیتر ۱-نفتالین استیک اسید (NAA) انجام شد. نتایج نشان داد که ۱۷-β-استرادیول در ۰،۰۱ و ۰،۱ میلی‌گرم در لیتر، به ترتیب طول و وزن ریشه و اندام هوایی را افزایش داد و در غلظت زیاد (۱۰ میلی‌گرم در لیتر) باعث افزایش فعالیت پراکسیداز، پلی فنل اکسیداز و کاتالاز در گیاهچه‌های بابونه آلمانی شد. همچنین، القای کالوس پس از یک تا دو هفته در تمام محیط¬ها مشاهده شد، ولی رشد آن بسته به وجود یا عدم وجود تنظیم‌کننده‌های رشد گیاهی و غلظت‌های مختلف ۱۷-β-استرادیول متفاوت بود. حداکثر وزن کالوس در ۰،۰۱ میلی‌گرم در لیتر ۱۷-β-استرادیول با حدود سه برابر افزایش در مقایسه با شاهد (MS بدون ۱۷-β-استرادیول) به دست آمد. می توان گفت که ۱۷-β-استرادیول در غلظت‌های پایین‌تر (۰،۰۱ میلی‌گرم در لیتر) می‌تواند به طور قابل توجهی رشد کالوس را در حضور تنظیم‌کننده‌های رشد گیاه مانند NAA و BAP بهبود بخشد. نتایج این مطالعه نشان می‌دهد که می‌توان از هورمون استروئیدی ۱۷-β-استرادیول برای بهینه‌سازی رشد سلول‌های بابونه آلمان در شرایط درون شیشه‌ای استفاده کرد.

واژه‌های کلیدی: آنزیم‌های آنتی‌اکسیدان؛ القای کالوس؛ کشت درون‌شیشه‌ای؛ هورمون استروئیدی