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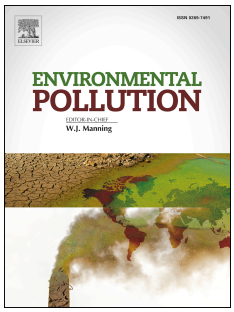

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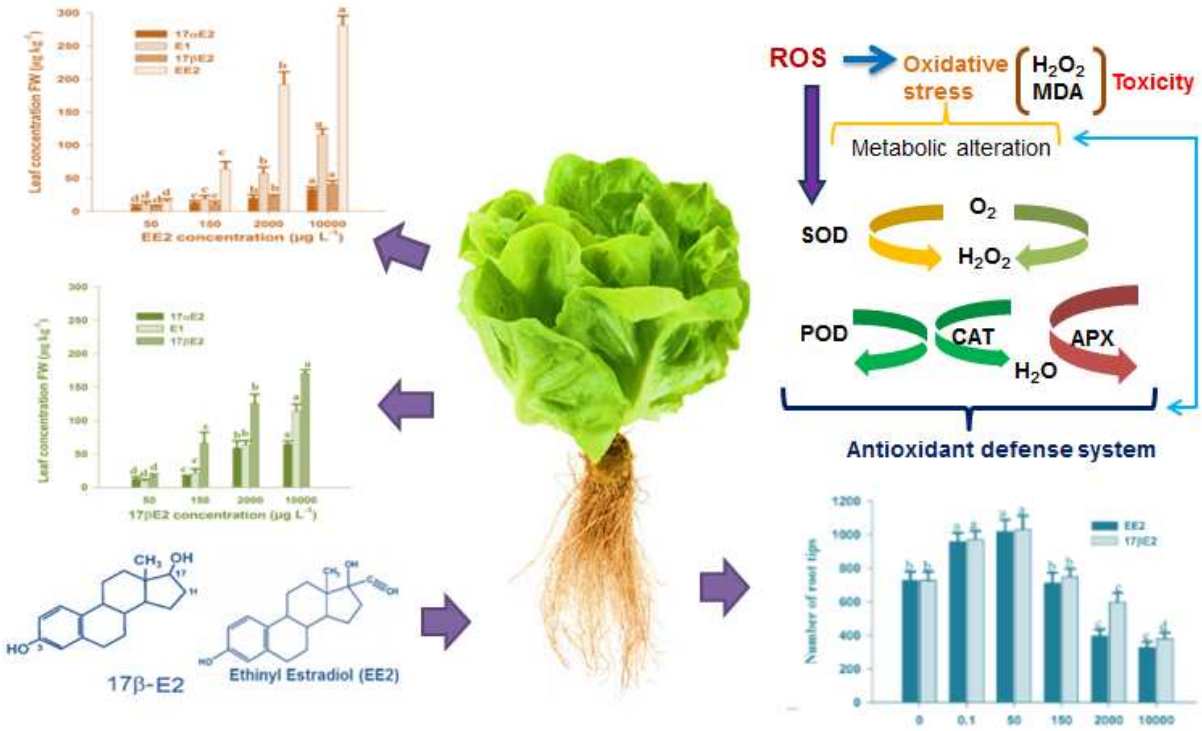

Uptake and transformation of steroid estrogens as emerging contaminants influence plant development

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Abstract

Steroid estrogens are emerging contaminants of concern due to their devastating effects on reproduction and development in animals and humans at very low concentrations. The increasing steroid estrogen in the environment all over the world contrasts very few studies for potential impacts on plant development as a result of estrogen uptake. This study evaluated the uptake, transformation and effects of estradiol (17β-E2) and ethinyl estradiol (EE2) (0.1-1000 µg/L) on lettuce. Uptake increased in leaves and roots in a dose-dependent manner, and roots were the major organ in which most of the estrogen was deposited. The transformation of estrogens to major metabolite and their further reverse biotransformation in lettuce tissue was identified. At low concentrations (0.1 and 50 µg/L) estrogens resulted in enhanced photosynthetic pigments, root growth and shoot biomass. Application of higher concentrations of estrogens (10 mg L^{-1}) significantly reduced total root growth and development. This was accompanied by increased levels of hydrogen peroxide (H_2O_2), and malondialdehyde (MDA), and activities of antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX). Taken together, these findings suggest that at low concentrations estrogens may biostimulate growth and primary metabolism of lettuce, while at elevated levels they have adverse effects.

Capsule: EDC estrogens (17β-E2 and EE2) stresses influence lettuce growth with a dose-dependent negative effect

Keywords: Estrogens; Plant uptake; Bioavailability; Antioxidant system; Biotransformation

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Abbreviations: E1, estrone; E2, estradiol; 17β-E2, 17β-estradiol; 17α-E2, 17α-estradiol; E3, estriol ; EE2, ethinyl estradiol ; CAFOs, Concentrated animal feeding operations; WWTPs, waste water treatment plants; MSH, mammalian sex hormones; CAT, catalase; POX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; GPX, guaiacol peroxidase; APX, ascorbate peroxidase; MDA, mono dehydro ascorbate; MSTFA, N-Methyltrimethylsilyltrifluoroacetamide; TRL, total root length; RV, root volume; RD, average root diameter; RSA, root surface area; RTs, number of root tips; SPE, solid phase extraction.

Introduction

A major challenge for the agricultural sector today is to produce more and safe food for a growing global population. Meat and dairy products are parts of the livestock industry and the use of synthetic steroid hormones as growth promoters (Bartelt-Hunt et al., 2012), increasing the muscle mass (Biswas et al., 2013) are the mostly adopted practices in the developed countries.

The world human population of about 7 billion is estimated to discharge 30,000 kg/yr. of natural estrogens (E1, E2, and E3) and an additional 700 kg/yr. of synthetic estrogens (EE2) from contraceptive pill practice (Adeel et al., 2017). However, the possible input of estrogens to the environment from livestock is much greater, where it is calculated in the U.S and European Union alone, the annual estrogen excretion by livestock, at 83000 kg/yr., is more than double that produced by the world human population. Indeed, possible relations have been made between animal feeding operations and the detection of estrogens in the aquatic environment (Shrestha et al., 2012). Naturally produced hormones excreted from animal and human waste pose serious effect to the environment, since applying animal manure or sludge bio-solids onto agricultural land as alternative fertilizers to organic products is a widely adopted practice in modern agriculture (Xuan et al., 2008).

Studies have documented the occurrence in reclaimed water of many classes of organic pollutants, including steroid estrogens. In addition to wastewater or effluent from WWTP, treated sewage sludge is also widely used all over the world in agriculture and for the latter, land application is the most adopted practice of disposal (Calderón-Preciado et al., 2012; Zhou et al., 2012; Calderón-Preciado et al., 2013; Gabet-Giraud et al., 2014). Previous studies indicate that steroid estrogens can be taken up, accumulated in, or metabolized in beans, aquatic macrophytes, and algae (Lai et al., 2002; Imai et al., 2007; Shi et al., 2010; Card et al., 2012). For example, some steroid estrogens derived from animal excrement and reclaimed water were taken up in terrestrial plants including leafy vegetables and fruits (Karnjanapiboonwong et al., 2011; Zheng et al., 2014). Thus, land application of reclaimed water and animal manure can result in these

emerging pollutants entering terrestrial food chains. The bioavailable concentrations of estrogens in soil also affect their ability to be taken up by plants. This concentration is difficult to measure, so it tends to be estimated (Dodgen, 2014). Recently, our study found 69 ng L^{-1} and 74 ng g^{-1} 17β-E2 in groundwater and soil respectively (Song et al., 2018).

Steroid hormones are essential factors responsible for the regulation of normal development in both the plant and animal kingdom. These compounds participate in many physiological processes such as development and reproductive processes as well as protein, sugar, and mineral management. Plants and animals produce hundreds of types of steroid estrogenic compounds (Janeczko et al., 2012; Sherafatmandjour et al., 2013). Steroid estrogens E1, E2 and E3 lie on interconnecting metabolic pathways. In aerobic conditions reverse transformation of E2 to E1 occurs under microbes and latter can be degraded to E3. Similarly, synthetic EE2 can be converted to E1 by *Sphingobacterium sp.* (Adeel et al., 2017). Treatment of plants with steroid estrogens affects root and shoot growth (Hewitt and Hillman, 1980;Guan and Roddick, 1988b), pollination in flowers (Ylstra et al., 1995) and seed germination (Janeczko and Skoczowski, 2011). Interestingly, at the biochemical level, mammalian sex hormones (MSH) significantly improve the inorganic contents of barley, maize, chickpea and beans seeds (Dumlupinar et al., 2011;Erdal and Dumlupinar, 2011a;Erdal et al., 2012), and chlorophyll, carotenoid, sugar, and protein in lentil seed,duckweed, soybean and fennel (Czerpak and Szamrej, 2003b;Dumlupinar et al., 2011;Chaoui and El Ferjani, 2013;Sherafatmandjour et al., 2013).

Steroidal estrogens found in sewage water inhibit vegetative growth of alfalfa plants (Shore et al., 1992). At a concentration of 1 µM, steroid estrogen reduced root growth and also caused morphological abnormalities including epinasty in tomato plants (Guan and Roddick, 1988b). Hence it is important to evaluate their disruptive potential in various ecological environments (Chaoui and El Ferjani, 2013).

To date, few studies have described the effects of these hormones as stresses to plants or their uptake from irrigation water containing environmental-level emerging pollutants. Of particular interest is their effect on the plant's antioxidant system, one of the chief phyto mechanisms for dealing with environmental stress. MSH including estrogens enhanced antioxidant enzymes, such as catalase (CAT) and peroxidase (POX) during germination of chickpea, maize and wheat seeds and enhanced plant growth and development by affecting

biochemical parameters including components of the antioxidative system (Erdal and Dumlupinar, 2011b). However, to our knowledge, the effects of steroid estrogens (E2, EE2) on leafy vegetables such as lettuce have not been reported. Our work has addressed this specific problem by analyzing the response of lettuce under stress of steroid estrogen (17β-E2 and EE2). Lettuce (*Lactuca sativa L.)* was chosen for the study because this crop is one of the most widely cultivated salad crops world-wide (Trujillo-Reyes et al., 2014). The study was carried out to investigate the effect of steroids i.e. estradiol and ethinyl estradiol on lettuce plant growth, photosynthetic pigments, and the role of antioxidant activities in protecting the plants against estrogen toxicity. Furthermore, we have investigated the uptake and transformation product concentrations in the root and shoot tissues of lettuce.

Materials and methods

2.1 Chemicals

E1 (≥99.5%), 17α-E2 (≥99%), 17β-E2 (≥98.4%), E3 (≥98.8%), and EE2 (≥98.2%) were purchased from Sigma-Aldrich (USA). Methanol, ethyl acetate, n-hexane, acetonitrile and acetone, purchased from Merck (Germany). N-Methy-N-(trimethylsilyl) trifluoroacetamide (MSTFA, ≥98.5%), used as the derivatization reagent, was obtained from Sigma-Aldrich (USA); pyridin (≥99.5%) was purchased from Kermel (China). SPE cartridges containing Oasis HLB cartridges (150 mg, 6 cc) were supplied by Waters (USA); for cleanup, CARB cartridges (500 mg, 6 ml) were purchased from WG Labs (China). The stock solutions of individual estrogens were prepared by dissolving each compound in methanol at a concentration of 1000 mg L^{-1} and stored at -20 °C.

2.2 Plant materials, growth conditions and treatments

Lettuce seeds (*Lactuca sativa* cv., cream lettuce, Yu He vegetable breeding center, China) were obtained from Shenyang Agriculture University and germinated in trays containing sandy soil in control conditions. After 14 days of sowing, uniform seedlings measuring 4 cm in height with two leaves were briefly rinsed in milliQ water and transferred to sterile amber 2000 mL glass jars (Supporting Information Fig. S1.2-3). Each jar was watered with ½-strength Hoagland's nutrient solution (pH 5.5- 6.3, Supporting Information Table S1-1). Experiments were performed in the controlled environmental conditions: 16 h light/8 h dark cycle, with constant 50% relative air humidity, 21-25 °C temperature; illumination was provided by

fluorescent tubes. After one-week acclimation, steroid hormones, 17β-E2 or EE2 (Sigma-Aldrich, USA, dissolved in methanol) were added at a final concentration of 0, 0.1, 50, 150, 2000 and 10,000 µg L^{-1} to the nutrient medium in the glass jars. Five treatments, four replications and a blank control were included, each bottle containing two plants. 17β-E2, EE2 solutions were prepared by dissolving them in methanol. The nutrient solutions were renewed once per week to avoid nutrient depletion and restrict bacterial growth. Plants were grown for a total of 21 days, a total growth time that corresponds to growth periods used commercially. At given time intervals, plants were destructively sampled. The growth of lettuce plants was investigated by evaluating the fresh weight (FW), number of leaves, leaf area and root length then leaves was stored at -80 °C for further analysis.

2.3 Root morphometry

Root scanning was carried out using an Epson Perfection V700 Photo, Dual Lens system (Regent Instruments Company, Canada) equipped with a water tray, into which the roots were placed, and a positioning system. The following root parameters were measured: total root length (TRL), root volume (RV), average root diameter (RD), root surface area (RSA) and number of root tips (RTs) with a root image analysis system using image analysis software WinRHIZO (version Pro 2007d, Regents Instruments, Quebec, Canada). The average root diameter was expressed as the total root width divided by the length of roots.

2.4 Photosynthetic pigments

The chlorophyll content was determined according to the method of Knudson et al. (1977). Fresh lettuce leaves (0.5 g) were extracted in 10 mL of 96 % ethanol for 24 h in the dark. The amounts of chlorophyll a, b and carotenoids were determined spectrophotometrically (U- 2910, Double Beam UV/VISspectro, 2JI-0013, Tokyo, Japan), by reading the absorbance at 665, 649 and 470 nm. Chlorophyll content was expressed as mg g FW^{-1}. The amount of photosynthetic pigments was calculated by using the following formulae:

$$C_a = (13.95A_{665} - 6.88\,A_{649})\,V/1000M$$

$$C_b = (24.96A_{649} - 7.32A_{665})\,V/1000M$$

$$C_{Total} = C_a + C_b$$

$$C_{x+c} = (4.08A_{470} - 11.56A_{649} + 3.29A_{665})\,V/1000M$$

where C_a is chlorophyll a, C_b is chlorophyll b, C_{Total} total chlorophyll, Cx+c total carotenoids, V volume of extraction (ethanol), and M mass of fresh leaf.

2.5 Determination of antioxidative and oxidative enzyme activity

All the biochemical analyses were carried out using fresh leaf samples. Activities of enzymatic antioxidants were assessed using commercial kits in accordance with the manufacturer's instructions. Kits for analysis of superoxide dismutase (SOD) (A001-1), peroxidase (POD) (A084-3), catalase (CAT) (A007-1), malondialdehyde (MDA) (A003-1), ascorbate peroxidase (APX) (50/48), protein (A045-3-2) and H_2O_2 (A064-1) were obtained from the Nanjing Jiancheng Bioengineering Institute, China (www.njjcbio.com). The absorbance readings of SOD, POD, CAT, APX, MDA and protein were detected at 550, 420, 405, 290, 532, and 562 nm respectively (U- 2910 Hitachi, Tokyo, Japan). The SOD, POD and CAT activities were expressed as unit mg^{-1}protein.

2.6 Sample preparation for estrogen testing

2.6.1 Preparation of Plant samples

After harvesting, all plants were rinsed under a stream of deionized water for 5 min, left to drain, and then blotted dry. The lettuce plants were separated into roots and leaves and stored at -80°C until used for extraction. The extraction and clean-up procedure were modified from (Karnjanapiboonwong et al., 2011; Zheng et al., 2014). Briefly, control plant samples (2.5 g) were weighed into centrifuge tubes spiked with 500 µg L^{-1} of each hormone standard. After 24 h, 5 mL of 1:1 (v/v) acetonitrile: water was added to samples for extraction. Plant samples were sonicated for 30 min, shaken for 30 min, and then centrifuged (Huanan Herexei instrument & Equipment Co., Ltd) at 10,000 RPM for 15 min. The supernatant was filtered through a GF/F filter (0.22 µm) and transferred to amber glass bottles. The solid phase of the samples was further extracted three more times by adding 5 mL of extraction solvent followed by sonicating, shaking, and centrifuging. The aqueous layer was filtered into the same amber glass bottle. The mixed supernatant was evaporated to 1 mL under a gentle stream of nitrogen, and diluted with 10 mL of ultrapure water. The solid phase extraction (SPE) procedures were modified as previously described (Zhang et al., 2015). The analytes were further cleaned-up by Oasis HLB cartridges (see Supporting Information). The extracts were then evaporated under a gentle nitrogen flow until 2 ml was left. For chlorophyll removal, samples were extracted through CARB cartridges

(Weifang Pufen Instrument Co., LTD). CARB cartridges were conditioned with 10 ml n-hexane:acetone (1:1) and eluted by very low vacuum. For estrogen recoveries in plant tissue see Supporting Information.

2.6.2 Derivatization

The eluted fractions from SPE were evaporated with nitrogen until near to dryness then the residues were transfer to a 1.5 mL reaction vial and further dried under a gentle stream of nitrogen. Derivatisation was performed by addition of 50 µL of pyridin and 100 µL MSTFA. The vial was capped and vortexed for 30 s and heated in an oven for 20 min at 40°C. The derivatives were cooled to room temperature and subjected to GC–MS analysis.

2.6.3 GC-MS analysis

The GC-MS system (Thermo Electron Corporation, USA) consisted of a gas chromatograph (TRACE GC Ultra), a quadrupole mass spectrometer (PolarisQ), an auto sampler (AI/AS 3000), and a TR5-MS quartz capillary column (30m×0.25 mm, 0.25 µm). High purity helium gas (99.999%) was used as carrier gas at a constant flow rate of 1.0 mL min^{-1}. Samples (1 µL) were injected into the GC splitlessly for 0.75 min. The GC oven temperature was programmed as follows: starting from 50 °C and equilibrated for 2 min, then ramped to 260 °C at 12 °C min^{-1} and equilibrated for 8 min, then further ramped to 280°C at 3 °C min^{-1} and maintained at this temperature for 5 min. For MS detection, the electron impact (EI) ionization was adopted, and electron impact energy was 70 eV. The inlet and MS transfer line temperatures were maintained at 280 °C, and the ion source temperature was 250 °C. The solvent delay time was 15.0 min. The MS was operated in total ion chromatogram (TIC) mode for qualitative analysis from m/z 50 to 600 and selected ion monitoring (SIM) mode for quantitative analysis. The TIC chromatograms of derivatized estrogens and internal standards by full scan and selected ion monitoring are shown in the Supporting Information.

2.7 Statistical Analyses

Data were analyzed statistically using one way analysis of variance (ANOVA) and Fisher's least significant test (LSD) using Statistix 8.1 software (Analytical Software, Tallahassee, FL, USA) and different letters show significant differences amongst treatments at $P < 0.05$. All data represented are means ± standard deviations (SD) of four replicates for each treatment.

3. Results

3.1 Hormone uptake and biotransformation in plant

The uptake of two estrogens by lettuce plants was investigated in hydroponic culture, to test for toxicity, transformation and distribution among the plant parts (Fig. 1). Uptake and accumulation of both of the two estrogens, 17β-E2 and EE2 arise in roots and leaves in a dose-dependent manner (see Figs. 1 C-D). No estrogen was detected in control plants. The uptake of 17β-E2 in lettuce root was slightly higher than EE2 while biotransformation of both hormones was detected in roots. At low treatment concentrations 17β-E2 was transformed into E1 and at higher concentration treatments (2000 and 10000 µg L^{-1}) into E1 and 17 α E2 (Fig. 1 C), although concentrations of E1 recovered from17β-E2 were higher than those of EE2 treatments. Interestingly, estrogen EE2 was transformed into E1, 17β-E2 and 17α-E2 in roots. At a low treatment concentration (0.1 µg L^{-1}) EE2 was transformed into E1 with a concentration of 6.45 µg kg^{-1}.

In leaves EE2 concentration was higher than 17β-E2 (Fig. 1 B). However, transformation of EE2 was low as compare to 17β-E2 treatments for leaves. The uptake of both estrogens at 0.1 µg L^{-1} treatments was not detected in leaves.

3.2 Negative dose-effect of steroid estrogens on growth and biomass

Both hormone treatments exerted a dose-dependent negative effect on both roots and leaves although there was no significant difference in effect between the two hormones tested (see supporting information Fig. S1-2).

3.2.1 Leaf number, area and fresh weight

Treatments of 50-10000 µg L^{-1} of EE2 or 17β-E2 significantly inhibited the number of leaves formed and both the leaf area and leaf fresh weight in 21 day old plants compared with the controls ($P < 0.005$). At 10000 µg L^{-1} this resulted in a 53-77% decrease in leaf number a 60-66% decrease in leaf area and 80-85% decrease in leaf fresh weight with both hormones (Figs. 2 A, B). The effect was less severe at lower concentrations but the 50 µg L^{-1} treatments still exerted a significant negative effect with an approximately 33% decrease in leaf number , a 28-34% decrease in leaf area and a 23% decrease in fresh weight with both hormones compared

with the controls (Figs. 2 A-C). However, the 0.1 µg L^{-1} treatments did not have any significant effect on leaf number ($P < 0.005$).

3.2.2 Root fresh weights

Data for changes in root FW in response to the hormone treatments closely paralleled those for the leaf characters with approximate 85% reductions in FW in the 10000 µg L^{-1} treatments compared with the controls ($P < 0.005$). As with the data on leaves, root FW was unaffected by the 0.1 µg L^{-1} treatments (Fig. 2D)

3.3 Change of photo synthetic pigment in response to steroid estrogens

3.3.1 Total Chlorophyll, Chl a and Chl b

Treatments with 2000 and 10000 µg L^{-1} EE2 caused a significant decline of (55%, 40% and 71%) and (62%, 47% and 78%) in the levels of total chlorophyll, Chl a and Chl b respectively compared with the control. (Figs. 3 A and 4B). Treatments of either hormone up to 50 µg L^{-1} had little effect on Chl a, but both total chlorophyll and Chl b were significantly reduced in response to treatment with 50 µg L^{-1} EE2, but not 17β-E2. At 150 µg L^{-1} the effect was significantly greater on Chl b than Chl a. The two hormones had very similar effects on Chl a, however effects of EE2 on Chl b were significantly greater than 17β-E2 at all concentrations > 0.1 µg L^{-1} and also affected total chlorophyll more severely at the highest two concentrations tested.

3.3.2 Carotenoids

Treatments with the two hormones appeared to affect carotenoid content less that Chlorophyll content, and only at the highest concentration tested was there a significant reduction compared to the controls. There were no significant differences in the effect of EE2 or 17β-E2 on carotenoid content (Figs. 3, 4D).

3.4 Influence of steroid estrogens on root morphology

3.4.1 Total and primary root lengths

Total root length defines the all primary, secondary, tertiary roots and root length is the length of primary main root. The effect of the two hormone treatments on total root length (Figs. 4A and 4B) was very similar to that for root fresh weight (Fig. 2D) with a significantly negative effect only at hormone concentrations of $\geq$ 50 µg L^{-1}, and similar effects between the two

hormones. The effect on primary root length was more gradual than that on total root length with significant reductions at 150 and then again at 2000 µg L^{-1} (Figs. 4A and 4B).

3.4.2 Average root diameter and root tip number

The effect of the hormone treatments on these two root parameters was different to all the effects on leaves and other effects on roots in that there was a stimulatory effect of the lowest concentration tested (0.1 µg L^{-1}). Average diameter then fell back to control levels at 50 µg L^{-1}. In contrast, root tip number remained greater than the control also when plants were treated with 50 µg L^{-1} falling back to control levels at 150 µg L^{-1}. At the highest two concentrating tested, both root diameter and root tip number was reduced compared to the control. (Fig. 4C and 4D).

3.4.3 Root volume and surface area

Root volume and surface area were affected by the hormone treatment in a similar way to root number. There was a gradual reduction in both parameters with increasing hormone concentration and a severe reduction at the highest two concentrations tested (Fig. 4E and 4F). Again the effects of the two hormones were comparable at each concentration.

3.5 Estrogen upregulates antioxidant enzymes.

Activities of four antioxidant enzymes increased in response to the hormone treatments, in a dose-dependent manner.

3.5.1 SOD and POD activities

Both the SOD and POD activities increased significantly between 0.1 and 50 µg L^{-1} treatments of both hormones with approximately 2-fold increases in both enzyme activities. Thereafter was an approximately linear dose response to increasing hormone concentration. There was no significant difference in the response to the two hormones for either enzyme (Figs. 5A, 5B). At the highest concentration of hormone tested the induction of both enzymes was approximately 3.5 fold.

3.5.2 CAT and APX activities

Unlike SOD and POD, CAT activity increased significantly with a 0.1 µg L^{-1} treatment of both hormones with a significantly greater response to 17β-E2. However at higher treatment concentrations the response was reversed and was greater with EE2, although this difference was

only significant at 50 µg L^{-1}. At the highest concentration of hormone treatment activity was stimulated by approximately 7-fold compared with the controls. (Fig. 5C).

The pattern of APX activity differed from the other enzymes tested in that induction of activity increase significantly at >150 µg L^{-1} of both hormones. Again there was no significant difference in the induction of activity increase by the two hormones although, as seen with CAT activity, EE2 appeared to induce the enzyme a little more than 17β-E2. At the highest concentration tested the induction was 6-fold (Fig. 5 D)

3.6 Steroid estrogen treatment induced oxidative damage

Both lipid peroxidation and accumulation of ROS in the leaves of lettuce plants under steroid estrogen stress increased with the dose of hormone (Fig. 6 A and B). Both markers for oxidative stress increase significantly at treatments of 50 µg L^{-1} compared to the control. Both markers also increased up to the highest concentration of hormone tested and concentration was approximately a 3-fold stimulation. Interestingly there was a small decrease in the H_2O_2 concentration at 0.1 µgL^{-1} compared to the control. There was no significant difference in effect between the two hormones tested.

4 Discussion

4.1 Uptake and biotransformation of steroid estrogens in lettuce plants

Results clearly showed that both estrogens used to treat the plants were taken up in lettuce roots and transported to leaves. Moreover, their uptake increased with treatment concentration. These observations are consistent with previous data on both hormones in soil and hydroponic media (Karnjanapiboonwong et al., 2011;Card et al., 2012).

Biotransformation products of both estrogens were observed in both lettuce roots and leaves. Natural estrogen 17β-E2 was transformed, into its metabolite (E1), and a greater concentration of E1 was found in roots as compared in leaves. This is in agreement with previous studies that reported that natural and synthetic estrogen was bio transformed by poplar and maize plants in solution cultures (Card et al., 2013;Bircher et al., 2015). However, EE2 transformation to E1 was also detected, unlike in poplar root tissue. Biotransformation was observed in roots and leaves. However, these data do not explain which mechanism lettuce used to bio-transform the estrogens. It has been hypothesized that some plant organs may perform oxidation and reduction transformation (Card et al., 2013). This will need further investigation.

4.2 Estrogens concentrations have effects on plant biomass

To the best of our knowledge, this is the first time that an effect on lettuce growth, root morphology, ROI-production and the antioxidant defense system has been shown to occur as a consequence of uptake of the synthetic estrogen hormone, EE2 and natural estrogen 17β-E2. We show here that application of 17β-E2 and EE2 concentrations (0.1 and 50 µg L^{-1}) has a positive impact on the root growth. Similarly, studies reported that 17β-E2 had induced the growth at low concentration and detrimental effects at high concentration on *Medicago sativa* and *Arabidopsis thaliana* (Shore et al., 1992;Upadhyay and Maier, 2016b). The positive effect at low concentration may be caused by hormesis. Previous studies, proposed that low concentrations of toxic pollutants induce hermetic effects through activating defense mechanisms. However, further studies are needed to understand the mechanism of estrogen in plant physiology(Vargas-Hernandez et al., 2017).

Moreover, the present study indicates that EE2 is slightly more toxic to lettuce plants than 17β-E2 at elevated level.

4.3 Effects of estrogens on root morphology

Excessive estrogens can have negative effects on root architecture, which affects plants' capacity to absorb water and minerals (Adeel et al., 2017). We observed a significant effect of elevated level of estrogen on the root morphology of lettuce plants (Fig. 5.4). However interestingly, at the 0.1 µg L^{-1} treatment improve the root length, which is in agreement with results obtained with other plant species such as *A. thaliana* (Upadhyay and Maier, 2016b), and chickpea (Erdal and Dumlupinar, 2011b).

However, at doses higher than 50 µg L^{-1}, there was an inhibitory effect on root morphology. This is in agreement with a significant reduction in root length in response to estrogen exposure at 2704 µg L^{-1} in *Phaseolus aureus L.* and *A. thaliana* (Guan and Roddick, 1988a; Upadhyay and Maier, 2016b).

4.4 Effects on chlorophyll

Previous studies have shown that the photosynthetic performance of a plant under stressful conditions may reflect plants adaptability (Gururani et al., 2015) In general, the Chl a, Chl b, total chlorophyll and total carotene contents decreased with increasing estrogen levels. Chlorophyll b is more sensitive to 2 and 10 mg L^{-1} treatments. However, total carotene was only

affected by a high treatment with estrogens. These findings are in agreement with previous results that have shown a reduction of chlorophyll content in *A. thaliana* at 2704 µg L^{-1} and stimulation of carotenoids in *Wolffia arrhiza (Lemnaceae)* (at 10^{-6} M) in response to 17β-E2 exposure (Czerpak and Szamrej, 2003a;Upadhyay and Maier, 2016b). Similar findings of a decline in photosynthesis with synthetic estrogen (EE2) contamination were reported in green alga *Chlamydomonas reinhardtii* and *Dunaliella salina* at 1893 µg $L^{-1}$and 100 ng L^{-1} (Pocock and Falk, 2014;Belhaj et al., 2017).

4.5 Relationship with detoxifying enzyme activity

A variety of environmental stresses cause an increase in H_2O_2 and MDA production leading to progressive oxidative injury and ultimately, cell death (Adeel et al., 2017). Accordingly in the present study, exogenous estrogens at elevated level triggered the production of H_2O_2 and MDA in lettuce plants. The increase in MDA might be due to membrane damage caused by ROS-induced oxidative damage. Similar results were found in *A. thaliana* when treated with 2704 µg L^{-1} 17β-E2 (Upadhyay and Maier, 2016a). However, in our study, there was a slight decrease in H_2O_2 levels at 0.1 µgL^{-1} of both estrogens in lettuce plants. These results are in agreement with previous studies that showed a reduction of MDA and H_2O_2 contents in chick pea plants (Erdal and Dumlupinar, 2011b), and in germinating bean seeds at 2.7×10^{-7} µg L^{-1} (Erdal, 2009). Moreover, Genisel *et al*., (2015) reported that 17β-E2 suppressed oxidative damage in wheat seedling at 2704 µg L^{-1}. The discrepancy with previous studies could result from differences in plant species. It is also possible that lettuce plants have different protective mechanism to combat the stress imposed by steroid estrogens.

4.6 Effect of steroid estrogens on the antioxidant defense system

Comparatively lower activities of SOD, POD, CAT and APX in lettuce plants were concomitant with the less H_2O_2 generation at 0.1 treatments. Similar results were obtained in different plant species, under estrogen low treatments (Erdal and Dumlupinar, 2011b;Chaoui and El Ferjani, 2014). Furthermore, at higher concentrations significantly enhanced these enzymes activities correlating with increased H_2O_2 concentration at these estrogens treatments. However, Genisel et al., (2015) reported that 17β-E2 improved the antioxidant enzyme activity in wheat seedlings at 2704 µg L^{-1}.

5. Conclusions

Uptake of steroid hormones increased in leaves and roots in a dose-dependent manner, and roots were the major organ in which most of the estrogen was deposited. At low concentrations estrogens may biostimulate growth and primary metabolism of lettuce, while at elevated levels they have adverse effects. This is some of the first research to demonstrate that the exposure of estrogens to lettuce is likely to cause impacts on plant development with unknown implications. Our findings suggest that overhead application of estrogens containing wastewater and animal manure could cause the negative physiological impact on plants. Further studies using soil culture media are required for better understanding of the uptake and biotransformation of estrogens.

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Figures captions

Figure 1. Concentrations of estrogens in lettuce leaf and root tissues following treatment with a range of concentrations of two estrogen hormones and after 21 days growth. Error bars represent the standard deviation (n= 4). Different letters above each column indicate statistically significant differences between a treatment at $P < 0.05$, according to Fisher's least significant test

Figure 2. Effects of estrogens on number of leaves (A), leaf area (B), root fresh weight (FW) (C) and leaf fresh weight (D) of 21-day-old lettuce plants treated with EE2 or 17β-E2. Error bars represent the standard deviation (n= 4). Different letters above each column indicate statistically significant differences between a treatment and the 0 control at $P < 0.05$, according to Fisher's least significant test.

Figure 3. Effects of a concentration range of estrogens (μg L^{-1}) on the levels of Chlorophylls and carotenoids (mg g^{-1} Fresh Weight) in leaves of 21 days old lettuce plants. Values are means ± SD; n = 4). Different letters above each column indicate statistically significant differences between a treatment and the 0 control at $P < 0.05$, according to Fisher's least significant test.

Figure 4. Effect of estrogens on root morphology. Total root length (A), root length (B), average diameter (C), number of root tips (D), root volume (E), and specific surface area (F), of 21- day-old lettuce plants. Error bars represent the standard deviation (n= 4). Different letters above each column indicate statistically significant differences between a treatment and the 0 control at $P < 0.05$, according to Fisher's least significant test.

Figure 5. Effects of estrogens on the activities of ROS detoxifying enzymes in the leaves of lettuce plants. (A) superoxide dismutase (SOD),(B) POD, (C) catalase (CAT) and (D) ascorbate peroxidase (APX). Error bars represent standard deviation (SD) of the mean (n = 4). Different letters (a–d) indicate significant differences among the treatments at $P < 0.05$, according to treatments.

Figure 6. Effects of estrogens on ROS in the leaves of lettuce plants with or without EE2 and 17β-E2 treatment. (A) malondialdehyde (MDA) and (B) Hydrogen peroxide (H_2O_2) . Bars represent standard deviation (SD) of the mean (n = 4). Different letters (a, b, c, d, e and f) indicate significant differences among the treatments at $P < 0.05$.

Highlights

- EDC estradiol (17β-E2) and ethinyl estradiol (EE2) stresses influence lettuce growth
- Estrogens biotransform to major metabolites and vice versa in lettuce tissue
- Both EDC treatments exerted a dose-dependent negative effect on both roots and leaves of lettuce