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Effects of 17β-Estradiol on Growth, Primary Metabolism, Phenylpropanoid-Flavonoid Pathways and Pathogen Resistance in *Arabidopsis thaliana*

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Abstract

Mammalian sex hormones are spread in the environment from natural and anthropogenic sources. In the present study, the effect of estradiol on *Arabidopsis thaliana* growth primary metabolism, phenylpropanoid and flavonoid pathways and pathogen resistance were investigated. Treatments of *Arabidopsis* plants with 10 and 100 nM 17β-estradiol resulted in enhanced root growth and shoot biomass. In addition, treated plants had an increased rate of photosynthesis with a concomitant increase in carbohydrate and protein accumulation. Plants exposed to higher concentrations of 17β-estradiol (10 μM) had significantly lower root growth, biomass, photosynthesis rate, primary metabolite and phenylpropanoid and flavonoid contents indicating a toxic effect of estradiol. Treatments with increasing estradiol concentrations (10 nM, 100 nM and 10 μM) resulted in the downregulation of phenylpropanoid-flavonoid pathway genes (*PAL1*, *PAL4*, *CHI* and *ANS*) and subsequent decreased accumulation of phenolics, flavonoids and anthocyanins. Estradiol-treated plants were inoculated with *Pseudomonas syringae* pv. Tomato DC3000 and basal resistance was determined. Estradiol treatments rendered plants susceptible to the pathogen, thus compromising the plant defense mechanisms. These results indicate that at low concentrations, estradiol functions as a biostimulant of growth, yield and primary metabolism of *Arabidopsis*. However, estradiol functions as a potential transcriptional regulator of the phenylpropanoid pathway genes in *Arabidopsis*, having a negative effect on the phenylpropanoid and flavonoid biosynthetic pathways.

Keywords

Plant Ecology, Plant Physiology, Plant Host-Pathogen Interactions

1. Introduction

In vertebrates, estrogen and androgen steroidal hormones have important functions in development and reproduction. Estrogens and estrogen-like compounds (xenoestrogens) from livestock manure, animal waste, and human waste (especially pharmaceutical waste), are being disposed of and excreted at high rates into the agricultural soil and ground water all over the world [1]-[6]. Thus, estrogen concentrations in the soil can range from 0.5 ng/L to 70 ng/L depending on the soil type and pollutant source [7].

A number of studies have shown that application of mammalian sex hormones affect the growth and development of plants, from cell division to flowering, sex expression, embryo growth and modulation of stress responses, concluding that mammalian sex hormones act as potential plant growth regulators [8]-[11]. In a study on the effect of mammalian steroidal hormones on sunflower (*Helianthus annuus*) seedlings, the application of 17β-estradiol (ES) promoted shoot growth [12]. Application of 1 μM estrogen to winter wheat seedlings promoted leaf and root growth [13]. ES induced flowering in several plant species, such as *Cichorium intybus* [14] and *Arabidopsis thaliana* [15]. Ylstra and coworkers [16] found that treatment of tobacco plants with animal hormones increased pollen germination. It was observed that irrigation of *Medicago sativa* plants with sewage water, which contained 0.3 $\mu g \cdot L^{-1}$ estrogen, resulted in increased vegetative growth [17]. Although significant progress has been made in establishing the biological and ecological consequences of animal exposure to environmental estrogens (EEs) [6] [18], there is still a big gap of knowledge regarding the effects of EEs on plants, especially crop plants as sources of food for an exponentially growing human population.

More is known about the effect of estrogens on plant growth and development, but very little is known about the effect of estradiol application on secondary metabolism, which is plant specific and very important for plant life. Secondary metabolites affect both plant growth and development, as well as defense responses [19]. Phenylpropanoids (PPs) such as phenolics and lignins and flavonoids, including tannins, belong to the largest and most diverse group of secondary metabolites. It has been reported that during plant-microbe interaction, the process of lignification is upregulated [20]. Similarly, many PP and flavonoid compounds are shown to have antimicrobial properties and are known to accumulate during pathogen and herbivore attacks [21]. Plant secondary metabolism, specifically accumulation of PPs and flavonoids, is negatively affected by xenobiont pesticides [22]. Relatively less is known about the effects of exogenous estrogens on plant secondary metabolism and stress responses.

In the present study, we describe the effects of ES application on the growth, yield, photosynthesis, primary metabolism, phenylpropanoid and flavonoid pathways and pathogen resistance of *Arabidopsis* in a study design simulating plant exposure to EEs.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Arabidopsis thaliana ecotype Columbia wild type (WT) seeds (Lehle Seeds, TX, USA)

were used for this study. The study design simulated plant exposure to EEs in that *Arabidopsis* seeds were germinated on agar medium containing ES to simulate soil exposure to EEs and, during vegetative development, plants were sprayed with ES solutions to simulate irrigation conditions. Seeds were surface sterilized according to Kagale *et al.* [23] and grown in Murashige and Skoog nutrient medium [24] solidified with 1% agar and containing increasing ES concentrations (10 nM, 100 nM, 10 μM). *Arabidopsis* plants germinated on MS plates with 0.01% ethanol were used as control. At day 14 post germination, the seedlings were transferred from MS plates into pots in a Percival growth chamber under long-day conditions at 22˚C, 50% humidity, and 200 μmol/m^2/s. Foliar spray treatments with above-mentioned ES concentrations were applied to plants two more times once a week until maturity. Root length and lateral root numbers were recorded using Image J software (rsweb.nih.gov/ij) at 14 d post germination. Tissues were collected at day 21 for biochemical assays and q PCR. Fresh and dry shoot biomass (mg) and number of siliques were recorded at 35 days post germination. All experiments and analyses were conducted in triplicate and repeated thrice unless otherwise mentioned.

2.2. Chlorophyll Content and Photosynthesis Rate

Total leaf chlorophyll content was determined in 21-day old plants by the method of Lichtenthaler and Buschmann [25] and expressed as $\mu g \cdot g^{-1}$ fresh weight (F. W.). Photosynthesis rate was estimated on 21-day-old *Arabidopsis* plants by using the LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) and calculated based on leaf biomass according to the manufacturer's instructions.

2.3. Primary Metabolite Estimation

Total proteins and total carbohydrates in leaf tissues from 21-day old ES-treated and untreated *Arabidopsis* plants were estimated. Total soluble protein content was estimated according to the Bradford method [26] and protein concentration was expressed as $mg \cdot g^{-1}$ F. W. For carbohydrate analysis, 0.1 g leaf tissue was ground in 80% acetone and extracted with 80% ethanol. The extracts were mixed with 5 ml anthrone reagent, heated at 100˚C (10 min) and allowed to cool on ice. Absorbances were read at 630 nm and total carbohydrate content was expressed as $mg \cdot g^{-1}$ F. W. based on a glucose standard curve [27].

2.4. Phenylpropanoid-Flavonoid Estimation

Estimation of total phenolics was performed according to Velioglu *et al.* [28] with some modifications. Leaf tissues (0.2 g) were extracted in 2 ml acidic methanol (80% methanol with 1% HCl) at room temperature for 2 h. Extracts were centrifuged at 1000 × g for 15 min. Supernatants (100 μl) were mixed with Folin-Ciocalteu reagent (0.75 ml) and incubated at 22˚C for 5 min. Sodium bicarbonate (0.1 M, 0.75 ml) was added to the reaction mixture, which was allowed to stand at 22˚C for 90 min. Absorbances were measured at 725 nm and total phenolics content was estimated using a *p*-coumaric acid standard curve and expressed as $\mu g \cdot g^{-1}$ fresh weight.

Flavonoid estimation was performed according to Chang *et al.* [29]. Flavonoid extraction from 0.5 g leaf tissues was carried out overnight in 95% ethanol. Flavone and flavonol contents were estimated by the $AlCl_3$ colorimetric method against a quercetin standard at 415 nm and flavanones were measured by the 2, 4-diphenylhydrazine colorimetric method against a naringenin standard at 495 nm. Flavonoid content was calculated as sum of naringenin and quercetin equivalents and expressed as $\mu g \cdot g^{-1}$ fresh weight.

Total anthocyanin estimation was performed according to Laxmi *et al.* [30]. Anthocyanins were extracted from 0.2 g leaves with 3 ml 1% acidic methanol overnight. Phase separation was performed next day by adding 3 ml chloroform and 2 ml water. Absorbance of the aqueous phase was measured at 530 nm and 657 nm. Anthocyanin content was calculated using the formula $\lambda_{530} - \lambda_{657}$ and expressed per g^{-1} fresh weight.

2.5. Pathogen Inoculation

Leaves of six 21-day old plants for each ES treatments and controls were infiltrated with a suspension (OD_{600} = 0.0002) of the virulent *Pseudomonas syringae* pv. tomato DC 3000 (*Pst*) strain in 10 mM $MgCl_2$. To determine bacterial growth, inoculated leaves were harvested at 3-days-post inoculation and 1cm leaf discs were homogenized in 10 mM $MgCl_2$. Diluted leaf extracts were plated on King's B medium supplemented with kanamycin (100 mg/ml) and incubated at 28˚C for 2 days before counting the colony-forming units (cfu) as previously described [31].

2.6. RNA Isolation and qPCR

RNA was isolated from leaves of 21-day old ES-treated and untreated plants using the Plant RNA isolation reagent (Life Technologies, CA, USA) following the manufacturer's protocol. RT-PCR was performed using the RETRO script reverse transcription kit (Thermo Fisher, USA). Primers were designed using the Primer-3 software. The following primer sequences were used for the PCR analysis: *PHENYLALANINE AMMONIA LYASE1,* PAL*1*F: 5'CGGTGTCGCACTTCAGAAGGAA3', PAL*1*R: 5'GGATACCGGAAAATCCTTGGAGGAG3'; *PHENYLALANINE AMMONIA LYASE4,* PAL*4*F: 5'CCGAGGAACGGACAGTTATGGAG3', PAL*4*R: 5'GGGCCAAATATTCCGGCATTCAAG3'; *CHALCONE ISOMERASE*, CHIF: 5'CGGCCTCCTCCAATCCATTATTCC3', CHIR: 5'GCTCCTCCGTAGTTTTTCCCTTCCA3'; *ANTHOCYANIDIN SYNTHASE,* ANSF: 5'TGGGTCACTGCAAAATGTGT3', ANSR: 5'TCACAAAACACAGCCCAAGA3'; *PATHOGENESIS RELATED1,* PR1F: 5'GGTA GCGGTGACTTGTCTGG3', PR1R: 5'ACTTTGGCACATCCGAGTCT3'; *ELONGATION FACTOR1a,* EF1*a*F: 5'TTCACCCTTGGTGTCAAGCAGATG3', EF1*a*R: 5'TCAGGGTTGTATCCGACCTTCTTCA3'. The real-time PCR experiments were carried out using the iQ SYBR® Green supermix and the BioRAD CFX 96 RT-PCR detection system (BioRad, USA) according to the manufacturers' instructions. Reaction parameters were set as follows: initial denaturation at 95˚C for 5 min followed by 35 cycles of 30 s at 95˚C, 30 s at 55˚C and 30 s at 72˚C. The relative RNA levels in each sample were cali-

brated and normalized against *EF*1*α* expression, which was used as an internal control.

2.7. Statistical Analysis

Data representing the means ± SD of three independent replicates were subjected to a one-way analysis of variance (ANOVA) and the mean differences were compared using Tukey test. Comparisons with $P < 0.05$ were considered significantly different.

3. Results

3.1. Estradiol Application Enhanced Growth and Development in *Arabidopsis*

Significantly increased root length was observed in 14-day old seedlings exposed to the low ES concentrations of 10 nM (7.43 ± 0.41 cm) and 100 nM (7.51 ± 0.4 cm) as compared to control plants (4.14 ± 0.29 cm) (Figure 1(A), Figure 1(C)). However, treatments with higher concentration of ES (10 μM) resulted in a significant decrease in root length (2.4 ± 0.22 cm) (Figure 1(A), Figure 1(C)). A similar trend was observed with respect to lateral root growth in that seedlings treated with 10 and 100 nM ES displayed a significantly higher number of lateral roots (6.8 ± 0.91 − 7.1 ± 0.56) as compared to the control treatments (4.2 ± 0.91) and plants treated with 10 μM ES, which displayed the lowest lateral root number (3.2 ± 0.63) (Figure 1(B), Figure 1(C)).

The 10 and 100 nM ES treatments significantly increased the fresh and dry weight biomass of *Arabidopsis* plants (Table 1). The 10 nM ES treatments increased the plant F.W. by 26% and dry weight by 42%. Plants treated with 100 nM ES increased their F.W. by 17% and dry weight by 33%. In addition, treatments with low concentrations of estradiol stimulated the reproductive development of *Arabidopsis*, reflected in increased silique yield (Table 1). The 10 and 100 nM ES-treated plants generated a significantly higher number of siliques, 87.68 ± 13.53 and 81.79 ± 13.52, respectively, than control plants with no ES treatment, which yielded 71.11 ± 12.71 siliques. When com-

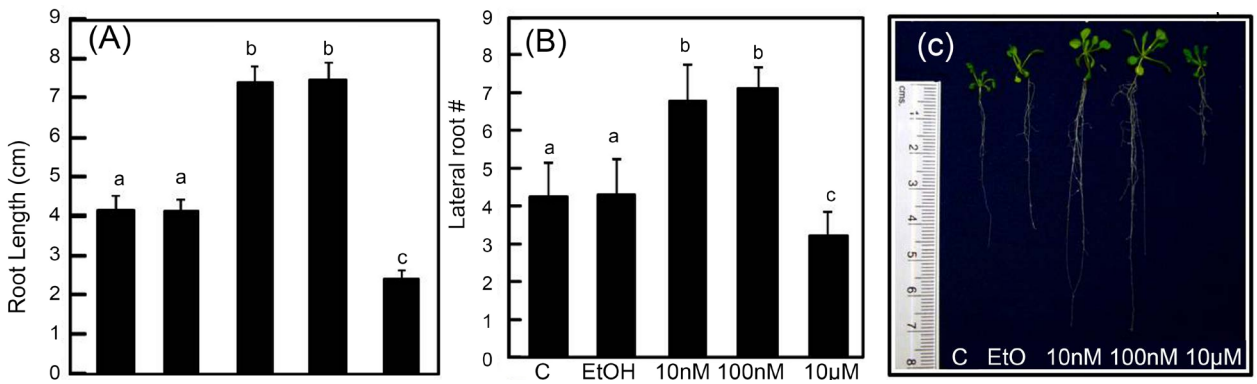

Figure 1. Effect of 17*β*-estradiol applications on *Arabidopsis* root growth. (A) Root length of 14-day old control and ES-treated *arabidopsis* seedlings. (B) Number of lateral roots in 14-day old, control and ES-treated *arabidopsis* seedlings. (C) Representative image of the roots in 14-day old, control and ES-treated *arabidopsis* seedlings. Results are means ± SD (n = 9). Different alphabets represent statistically significant differences ($P < 0.05$, ANOVA). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied ES treatments.

Table 1. Effect of 17β-estradiol applications on biomass accumulation and silique generation in *Arabidopsis* plants.

Treatment	Fresh weight (g)	Dry weight (g)	Silique #
Control	$2.89 \pm 0.71^{*}$	0.33 ± 0.12^{a}	$71.11 \pm 12.71^{\Psi}$
EtOH	$2.76 \pm 0.81^{*}$	0.31 ± 0.18^{a}	$68.20 \pm 15.04^{\Psi}$
10 nM	$3.65 \pm 0.96^{**}$	0.47 ± 0.16^{b}	$87.68 \pm 13.53^{\Delta}$
100 nM	$3.40 \pm 0.89^{**}$	0.44 ± 0.20^{b}	$81.79 \pm 13.52^{\Delta}$
10 μM	$1.98 \pm 0.63^{***}$	0.20 ± 0.14^{c}	$38.77 \pm 12.97^{\delta}$

Different superscript symbols represent statistically significant differences ($P < 0.05$, ANOVA). The results are means ± SD (n = 30).

pared to the controls and the other ES treatments, the application of 10 μM ES resulted in the lowest biomass accumulation and silique number (Table 1).

3.2. Estradiol Application Enhanced Photosynthesis Rate in *Arabidopsis*

Chlorophyll content was not significantly affected by 10 and 100 nM ES applications (Figure 2(B)). However, a significantly higher photosynthesis rate was observed in these plants when compared to the controls or the 10 μM ES-treated plants (Figure 2(A)). The increased rate of photosynthesis correlated with enhanced shoot biomass of 10 and 100 nM ES-treated plants (Table 1).

The negative trend in growth and development of 10 μM ES-treated plants as compared to control and 10 and 100 nM ES-treated plants was also reflected in the total chlorophyll content and photosynthesis rate. The 10 μM ES-treated plants had a 38% decrease in chlorophyll content and 40% decrease in photosynthesis rate compared to control plants (Figure 2(A), Figure 2(B)).

3.3. Estradiol Application Enhanced Primary Metabolism in *Arabidopsis*

The increased biomass was accompanied by an enhanced rate of photosynthesis, which resulted in significantly higher accumulation of both carbohydrates and proteins in the 10 and 100 nM ES-treated plants when compared to the control and 10 μM ES-treated plants (Figure 3). Plants treated with 10 and 100 nM ES accumulated significantly higher levels of both total carbohydrates (6.87 ± 0.5 mg/g and 6.86 ± 0.29 mg/g F. W., respectively), representing a 43% increase, and proteins. The 10 nM ES-treated plants had a 30% increase (7.51 ± 1.41 mg/g) and the100 nM ES-treated plants a 17% increase (6.88 ± 0.7 mg/g F. W.) in total protein content when compared to control plants (Figure 3). High concentration ES (10 μM ES) applications resulted in a significant decrease in total carbohydrates (3.03 ± 0.4 mg/g F. W.) and proteins (3.6 ± 1.1 mg/g F. W.) (Figure 3(A), Figure 3(B)).

3.4. Estradiol Application Resulted in Decreased Accumulation of Phenylpropanoids and Flavonoids

To determine the effect of ES on secondary metabolite production, total leaf phenolics,

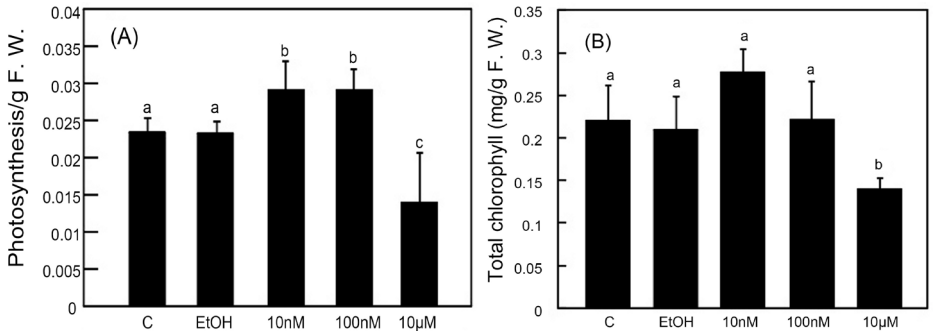

Figure 2. Effect of 17β-estradiol applications on photosynthesis rate and chlorophyll content of *Arabidopsis*. (A) Photosynthesis rate and (B) total chlorophyll content of 21-day old, control and ES-treated *arabidopsis* leaves. Results are means ± SD (n = 9). Different alphabets represent statistically significant differences ($P < 0.05$, ANOVA). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied ES treatments.

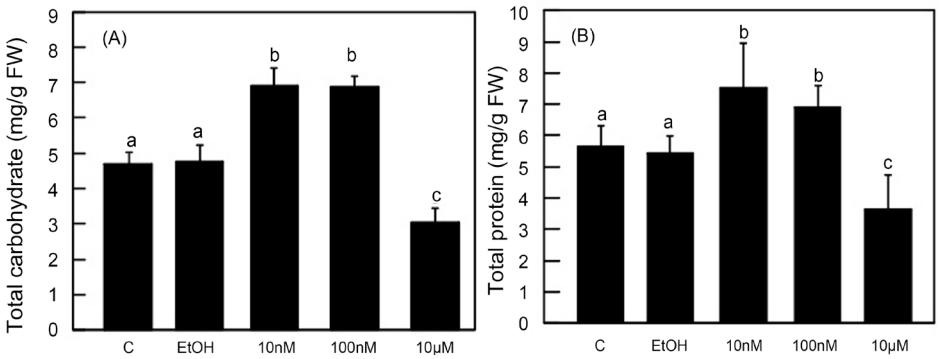

Figure 3. Effect of 17β-estradiol applications on carbohydrate and protein contents in *Arabidopsis*. (A) Total carbohydrate content and (B) total protein content of 21-day old, control and ES-treated *Arabidopsis* plants. Results are means ± SD (n = 9). Different alphabets represent statistically significant differences ($P < 0.05$, ANOVA). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied ES treatments.

flavonoids and anthocyanins in 21-day old *Arabidopsis* leaves were estimated (**Figure 4**). A significant decline in the phenylpropanoid (PP) and flavonoid synthesis was observed in response to increasing ES treatments. Total PP and flavonoid contents were found to be the lowest in 10 μM ES-treated plants in comparison with the 10 and 100 nM ES-treated and control *Arabidopsis* plants. Although the decrease in total phenolics contents was relatively small (4% - 5%), the decrease in the level of the downstream products of the flavonoid pathway was significantly higher: total flavonoids decreased by 7% - 15% and total anthocyanins by 37.5% - 67.5%.

3.5. Decreased Expression of the Phenylpropanoid Pathway Genes by Estradiol Application Was Transcription Ally Controlled

The decrease in PP and flavonoid accumulation as a result of ES treatments suggests that ES could downregulate transcription of key genes in the PP and flavonoid biosynthesis pathways in *Arabidopsis*. This was found to be the case, as the expression of

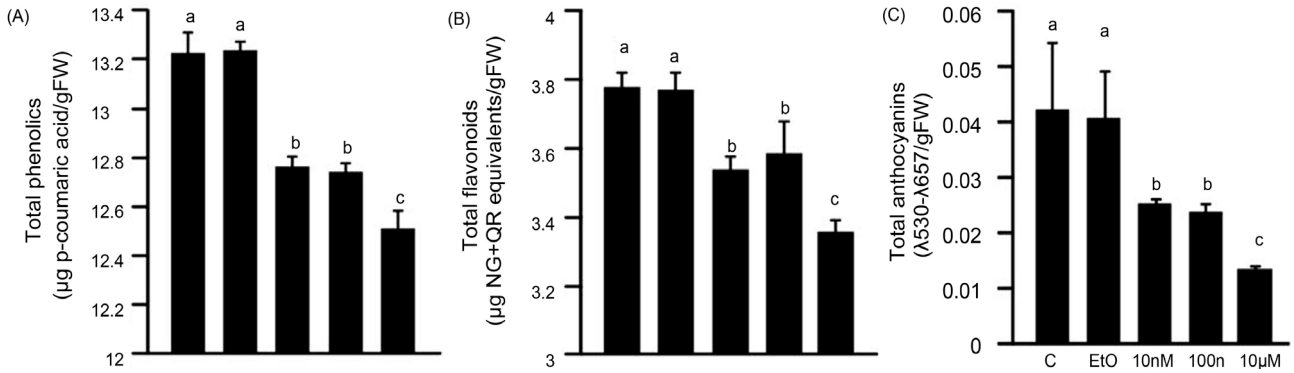

Figure 4. Estradiol negatively impacts phenylpropanoid and flavonoid production in *arabidopsis*. (A) Total phenolics content; (B) Total flavonoid content; and (C) total anthocyanin content in 21-day old, control and ES-treated *arabidopsis* leaves. Different alphabets represent statistically significant differences (means ± SD; $P < 0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol treatments. Flavonoid content is presented as sum of naringenin (NG) and quercetin (QR) equivalents.

PAL1, PAL4, CHI and *ANS* was downregulated in ES-treated plants (Figure 5). The expression of *PAL1*, *PAL4* and *ANS* was found to be nearly two-fold higher in the control plants than that observed in the ES-treated plants. The expression of *CHI* was found to be significantly lower in 10 µM ES-treated plants only. q PCR analysis of the RNA isolated from ES-treated and control plants demonstrated that the ES-induced downregulation of the PP and flavonoid pathway genes transcription is dose dependent for *PAL1*, *PAL4* and *ANS* (Figure 5).

3.6. Estradiol Application Resulted in Compromised Resistance against Bacterial Pathogen

The growth of a virulent bacterial pathogen, *Pseudomonas syringae* pv. Tomato DC 3000 (*Pst*), was studied to see the effects of ES-induced low PP and flavonoid levels on plant defense responses. At a low dose of inoculation (OD600 = 0.0002), the ES-treated plants were significantly more susceptible to the pathogen and displayed both enhanced disease symptoms and bacterial growth than control plants (Figure 6(A), Figure 6(B)). The compromised resistance to *Pst* correlated with the reduced expression level of *PATHOGENESIS RELATED*1 (*PR*1) in the ES-treated plants (Figure 7). In addition, the ES-treated plants accumulated *PAL1* and *PAL4* transcripts at a significantly lower level than that in the control plants in response to *Pst* infection and there were no significant differences in *PAL1* and *PAL4* transcript levels between the mock-treated and ES-treated pathogen inoculated plants. Untreated *Arabidopsis* plants showed up regulation of *PAL1* and *PAL4* expressions in response to pathogen inoculation (Figure 8).

4. Discussion

4.1. Low Estradiol Concentrations Have Positive Effects on *Arabidopsis* Growth and Development

The effects of mammalian sex hormones on plant growth and reproductive development have been studied before. It is evident from our results that application of low ES

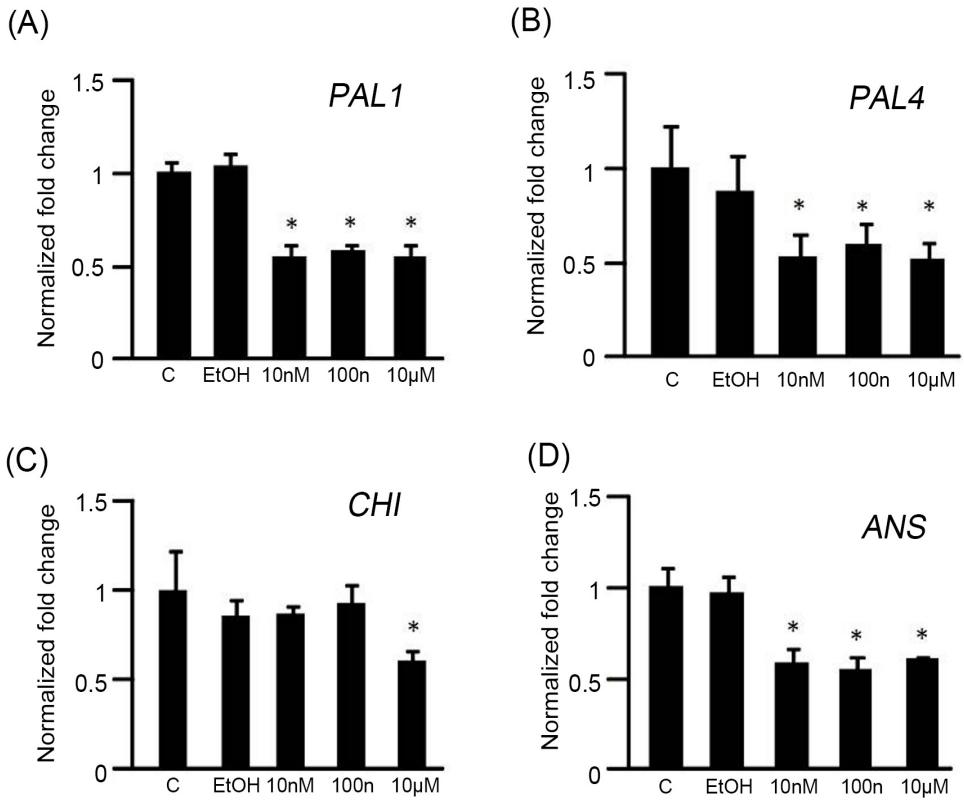

Figure 5. Reduced expression levels of the phenylpropanoid-flavonoid pathway genes (A) *PAL1*, (B) *PAL4*, (C) *CHI*, and (D) *ANS* by ES application is transcriptionally regulated. RNA was isolated from 21-day old, control and ES-treated *Arabidopsis* leaves. C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied estradiol treatments, *PAL* = phenylalanine ammonia lyase, *CHI* = chalcone isomerase, *ANS* = anthocyanidin synthase.

concentrations (10 and 100 nM) has a positive impact on the vegetative growth and fruit yield of *Arabidopsis* plants. The highest concentration of ES (10 μM) applied to plants was found to have a negative effect on the growth and physiology parameters studied, indicating a toxic effect. Similar results were obtained with *M. sativa* plants for which lower concentrations of ES had a positive impact on plant growth whereas higher concentrations of the hormone resulted in growth inhibition [17]. It has been reported that 10^{-6} M ES stimulated while 10^{-5} M ES inhibited winter wheat seedling growth [13]. The root growth results of this study are consistent with similar results obtained with chickpea seedlings in that exogenous application of 10^{-4} - 10^{-12} M ES as a foliar spray resulted in enhanced root and shoot length growth [32]. Application of selected estrogens on *Arabidopsis* plants grown on artificial medium [15], winter wheat [33], sage (*Salvia splendens*) [34] and chicory (*Cichorium intybus*) [14] was found to stimulate flowering. More recently, it was reported that *in vitro* culture of unpollinated *Arabidopsis* pistils on media supplemented with estrone and progesterone resulted in the development of autonomous endosperm [35].

Our study indicates that estradiol can stimulate photosynthesis, resulting in enhanced primary metabolism, growth and yield in *Arabidopsis*. Based on these results,

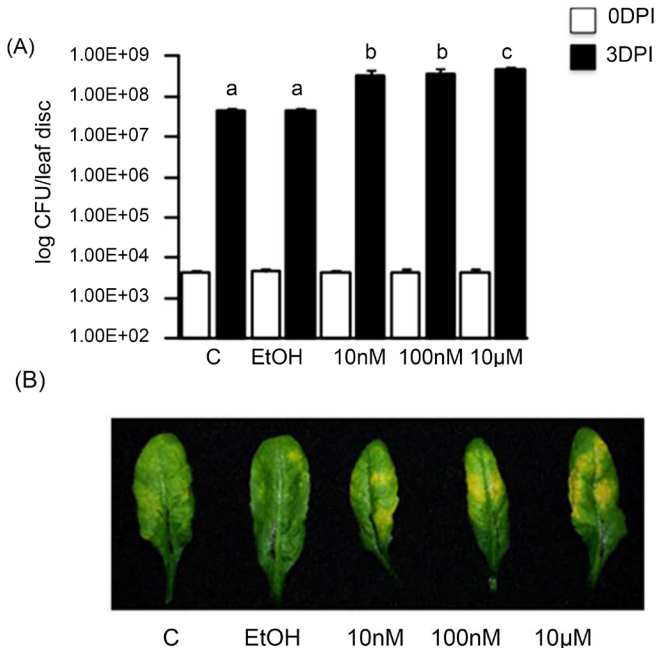

Figure 6. Estradiol application results in increased susceptibility to *P. syringae* pv. DC 3000 in *arabidopsis*. (A) Bacterial growth in 21-day old control and ES-treated *Arabidopsis* plants at 0 and 3 DPI. (B) Representative image of pathogen inoculated *Arabidopsis* leaves at 3 DPI showing pathogen induced chlorosis. Different alphabets represent statistically significant differences (means ± SD; $P < 0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied estradiol treatments.

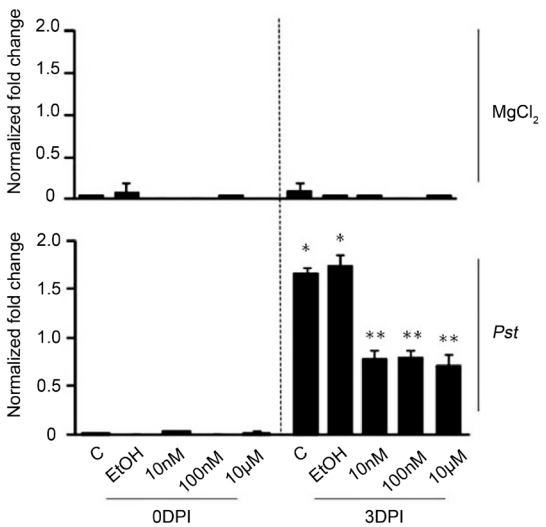

Figure 7. *PR*1 (pathogenesis-related protein 1) expression in 21-day old ES-treated and control *Arabidopsis* plants inoculated with *P. syringae* pv. DC 300 or $MgCl_2$ as mock. Asterisks represent statistically significant differences (means ± SD; $P < 0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied estradiol treatments.

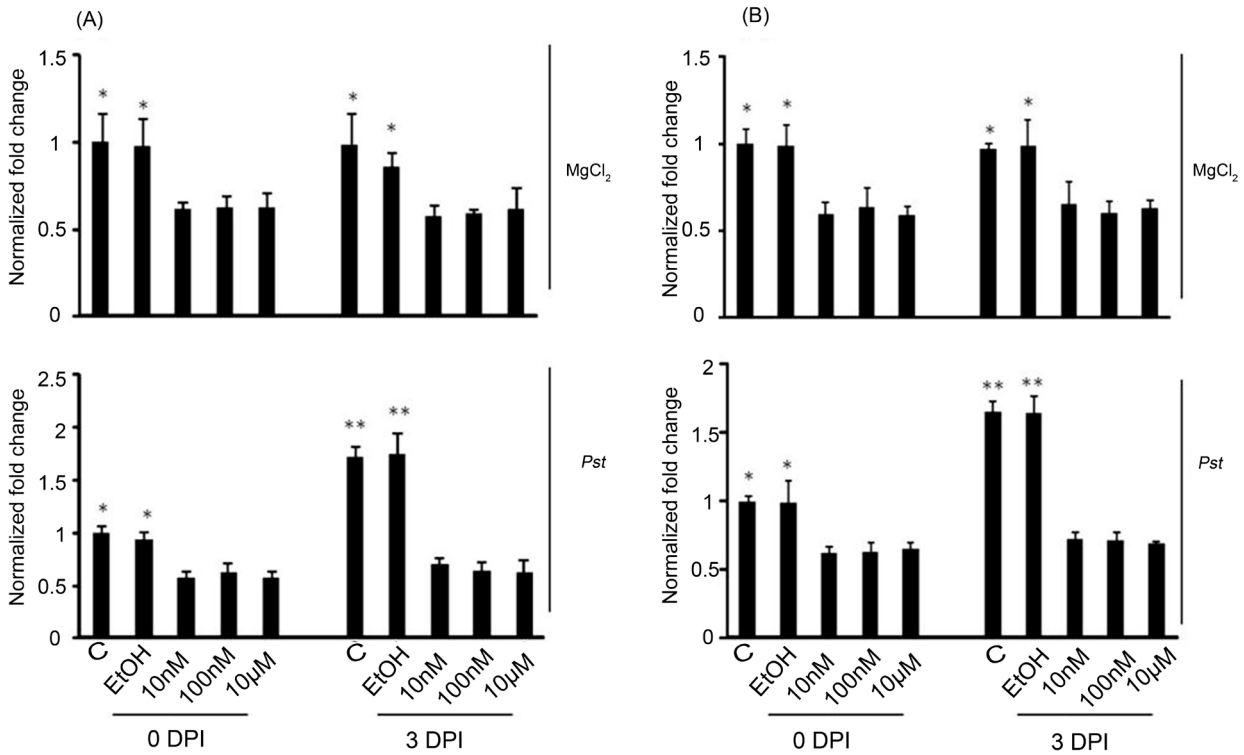

Figure 8. *PAL1* (A) and *PAL4* (B) expression in 21-day old ES-treated and control *Arabidopsis* plants inoculated with *P. syringae* pv. DC 300 or $MgCl_2$ mock. Results are cumulative of three independent experiments. Asterisks represent statistically significant differences (means ± SD; $P < 0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied estradiol treatments, *PAL* = phenylalanine ammonia lyase.

we suggest that estradiol acts as a biostimulant for plant growth at lowers concentrations, similar to concentrations of EEs in contaminated environments [17]. It is suggested that mammalian sex hormones mediate their effects in plants at the level of gene transcription by means of specific receptors as in animal cells, as well as through non-genomic pathways. Yang *et al.* [36] characterized a putative Membrane Steroid Binding Protein (MSBP1) that binds progesterone with high affinity and functions in regulating growth in *Arabidopsis*. Estrogen binding proteins were reported in *Solanaceae* [37] and steroid binding proteins specific for progesterone and 17β-estradiol were reported in *Triticum aestivum* [38]. Other research implies that the stimulating effects of human steroid hormone application on plants are due to increase in antioxidant activities in plant cells, which in turn enhance the plant resistance to environmental stresses [32] [39]. Our results demonstrate that estradiol can influence growth and yield of *Arabidopsis* plants in a dose-dependent manner. The biostimulant effect of estradiol could be used in practice, especially in small-scale cultures of crop and horticultural plants.

4.2. Estradiol Has a Negative Effect on the Phenylpropanoid-Flavonoid Biosynthetic Pathways and Pathogen Resistance in *Arabidopsis*

The effect of xenoestrogens on plant secondary metabolism has not been studied be-

fore. The present study is the first report of the effects of exogenous ES on the phenylpropanoid and flavonoid biosynthetic pathways. It was observed that ES treatments of *Arabidopsis* plants resulted in the downregulation of PP and flavonoid pathway genes (*PAL1*, *PAL4* and *ANS*), which was accompanied by significantly lower accumulations of total phenolics, flavonoids and anthocyanins. Phenylalanine ammonia lyase (PAL*1*) is the first committed enzyme of the PP pathway, converting phenylalanine to cinnamic acid, reaction that also represents an important regulation point between primary and secondary metabolism. PAL is encoded in *Arabidopsis* by a four-member gene family, *PAL1-PAL4*. Chalcone isomerase (CHI) catalyzes cyclization of bicyclic chalcone into tricyclic (S)-flavanone, namely naringenin, which then becomes a source of diverse flavonoids such as flanones, flavonols, anthocyanins and condensed tannins. Anthocyanin synthase (ANS) catalyzes the transformation of leucoanthocyanidins into colored anthocyanidins. A disruption in the *PAL* transcription or PAL enzymatic activity induces reductions in PP and flavonoid products since the flow of precursors is disrupted. ES application to *Arabidopsis* reduced the flow of chemicals to the entire PP and flavonoid pathways by downregulating *PAL* and *ANS* and possibly other enzyme genes in these pathways.

PAL4 encodes for an active PAL enzyme that can potentially compensate for PAL*1* activity in *PAL1* plants [40]. Reduced expression of *PAL4* along with *PAL1* in ES-treated plants indicates a comprehensive repression of *PAL* gene expression by ES. A significant lower expression of *CHI* was observed only in 10 μM ES-treated plants. There was no significant difference in expression levels of *CHI* between untreated and 10 and 100 nM ES-treated plants. It is possible that ES acts differently on some PP and flavonoid enzyme genes as was the case with *CHI*.

It is also possible that ES interfered directly with the enzyme activities. It has been shown that inhibition of PP and flavonoid pathway enzymes by exogenous application of chemicals results in reduced accumulation of PP and flavonoid products in plants. Addition to tissue culture media of 2-aminoindan-2-phosphonic acid, a chemical xenobiont that inhibits PAL activity, led to significant decrease in the accumulation of total phenolics and flavonoids in *Ulmus americana* [41] and *Artemisia annua* [42]. Conversion of the PAL product trans-cinnamic acid to p-coumaric acid by cinnamate 4-hydroxylase (C4H), the second step in the PP pathway, was inhibited by the addition of piperonylic acid to *Nicotiana tabaccum* culture cells leading to decreased accumulation of PP pathway products [43]. In soybean, application of methylene dioxocinnamic acid, inhibitor of 4CL (PP enzyme responsible for the conversion of p-coumaric acid to coumaryl-CoA) even in the presence of exogenous caffeic acid led to decreased lignin production in roots [44]. Similarly, inhibition of PP pathway biosynthetic enzymes by herbicides such as glyphosate and alachlor led to decreased accumulation of phenolics and flavonoids [22]. Based on above-mentioned studies, it is evident that inhibition or reduction of early PP pathway biosynthetic enzyme activities has a drastic impact on the accumulation of the downstream flavonoid pathway products.

It has been reported that ES application has a protective effect on plants under oxida-

tive stress [45], which is similar to the effect of brassinosteriods (BRs), plant-specific steroid hormones, structurally similar to ES, on plant abiotic stress responses [23]. Exogenous application of BRs by themselves and in combination with other plant hormones resulted in increased accumulation of PP and flavonoids in tomato and sweet basil [46] [47]. In our study, however, it was observed that ES application resulted in a reduction of total phenolics, flavonoids and anthocyanins in *Arabidopsis*, implying that ES and BRs have different mechanisms of action on the PP and flavonoid pathways. ES may act as a transcriptional regulator of the PP and flavonoid pathway genes in similar mechanistic way as in mammals and/or possibly on the transcriptional regulators of the pathways. ES could also have an inhibitory effect on enzymatic activities of the pathway enzymes. Further studies are needed in order to establish the mechanism of action of ES in dowregulating the PP and flavonoid biosynthetic pathways.

Phenylpropanoids and flavonoids are not only required for normal plant growth and development but they also play an important role in protecting plants against various stresses, including pathogen attacks [48] [49]. It has been reported that *PAL1* expression is required for basal resistance against the virulent pathogen *Pseudomonas syringae* DC 3000 [40]. Overexpression of pepper *PAL1* gene in *Arabidopsis* resulted in increased resistance against *Pst* DC 3000 and *Hyalopernospora arabidopsidis* infections [50]. In addition, a positive correlation was found between *PAL1* expression and *PR*1 expression and SA accumulation in the infected pepper leaf tissue [50]. In another study, mutant *Arabidopsis* plants accumulating low levels of PP were found to be susceptible to *Botrytis cinerea* [51]. Plants are known to either induce or suppress the levels of certain flavonoids in response to pathogens [52]. Both PAL activity and PP accumulation were stimulated by *Pseudomonas fluorescens* infection in tomato plants [53]. In our study, ES application resulted in reduced *PAL1* expression levels and PP and flavonoid accumulation, rendering *Arabidopsis* plants susceptible to a virulent pathogen. ES-treated *Arabidopsis* plants showed a more severe phenotype in response to *Pst* inoculation and the bacterial growth 3 DPI was found to be significantly higher in ES-treated plants than in controls. The 10μM ES-treated plants showing lowest levels of total phenolics, flavonoids and anthocyanins were the most severely impacted by the pathogen. ES treatment of *Arabidopsis* plants compromised their resistance against the bacterial pathogen by downregulating the expression of PP and flavonoid biosynthesis pathway genes resulting in a reduction of PP and flavonoids.

Increased *PR*1 expression in response to pathogen infection is an important indicator of mounting plant defense responses [50]. Reduced expression of *PAL1* and *PAL4* in ES-treated pathogen inoculated *Arabidopsis* plants correlated with *PR*1 downregulation in those plants, as previously reported by Kim and Hwang [50]. In untreated *Arabidopsis* plants, *PAL1* and *PAL4* expressions were upregulated in response to pathogen inoculation. The significantly reduced expression levels of *PAL1* and *PAL4* in response to ES treatment remained unchanged in mock and pathogen inoculated plants, suggesting that repression of *PAL* gene expression by ES is independent of *Arabidopsis* defense response against *Pst*.

Figure 9 summarizes the effects of ES on *Arabidopsis*. ES altered the expression of genes related to plant stress responses (*PR*1) and secondary metabolism (*PAL1*, *PAL4*, *CHI*, *ANS*). The down regulation of PP and flavonoid biosynthesis genes resulted in the reduced accumulation of these pathways' products. A concurrent effect of low ES concentration applications was the increased plant growth as a result of increased allocation of carbon as evidenced by the higher accumulation of primary metabolic products like carbohydrates and proteins. As compared to controls, higher carbohydrate and protein contents in ES-treated plants further contributed to plant growth, which increased the photosynthesis rate. In the same instance, reduced activity of PP and flavonoid biosynthetic pathways, which are a significant carbon sinks, potentially contributed to the available carbon pool for the primary metabolism and growth. However low levels of PP and flavonoids resulted in compromised resistance to a bacterial pathogen in ES-treated plants. Therefore, ES may act as a metabolic switch that compromises secondary metabolism and biotic stress responses in favor of primary metabolism and growth in *Arabidopsis* (Figure 9).

5. Conclusions

In conclusion, our results demonstrate a unique effect of ES on plant PP metabolism. At low concentrations, ES acts as a biostimulant for plant growth and primary metabolism but negatively affects secondary metabolism and resistance to pathogen attacks.

The impact of mammalian sex hormones in the plant secondary metabolism and associated stress responses has not been studied in great detail. These are novel findings that contribute to our current knowledge of plant responses to xenoestrogens. Thus,

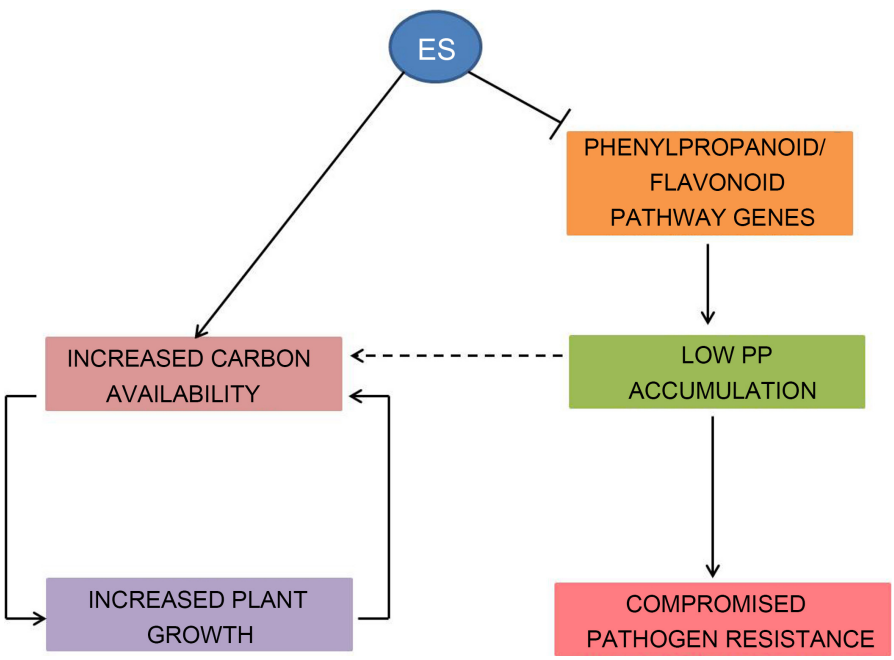

Figure 9. Summary of estradiol application effects on *Arabidopsis* growth, metabolism, PP-flavonoid pathway gene expression, and biotic stress responses.

these results will serve as a primer to study the effects of animal steroidal hormones and xenobionts on crop plants for the purpose of improving agricultural yield and productivity.

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