

Unlocking the Green Solution: Progesterone and Brassinosteroids Synergistically Enhance Progesterone Phytoremediation and Antioxidant Capacity of *Solanum nigrum* L.

Ana Pinto

Daniela Correia da Silva

Ana Cardoso

Fátima Fernandes

Cristiano Soares

Patrícia Valentão

Fernanda Fidalgo

Jorge Teixeira (✉ agteixe@fc.up.pt)

GreenUPorto - Centro de Investigação em Produção Agroalimentar Sustentável - Inov4Agro

<https://orcid.org/0000-0002-2631-2112>

Research Article

Keywords: Animal sex hormones, Environmental clean-up, Gene expression, Oxidative stress, Black nightshade

Posted Date: October 27th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3460474/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Aims: Progesterone (PROG) has been detected in various concentrations in the environment, with adverse effects on humans and wildlife. So, tactics to reduce the accumulation of this steroid in the environment must be studied. This work aimed the evaluation of the phytoremediation potential of PROG by *Solanum nigrum* L. plants and the understanding of how 2,4-epibrassinolide (24-EBL) would enhance this species' remediation capacities.

Methods: Three treatments were assembled: 1) control; 2) plants irrigated with 0.8 μM PROG; and 3) plants treated with 0.8 μM PROG but pre-treated with a foliar application of 1 μM 24-EBL (PROG/24 EBL). PROG levels, and several antioxidant-related biochemical and molecular endpoints in roots and shoots were evaluated after 20 days of treatment.

Results: No PROG was detected in the nutrient solution and plant tissues at the end of the treatment, indicating that plants were able to remove PROG from the irrigating solution. Lipid peroxidation significantly decreased in response to PROG in shoots and roots and the pretreatment with 24-EBL was even more effective in both organs. Also, a decrease in H_2O_2 levels was observed in both treatments in both organs. Plus, the exogenous application of both steroid hormones increased the plants' antioxidant system both at the biochemical and gene expression levels.

Conclusions: *S. nigrum* can swiftly phytoremediate PROG *per se* with no negative effects to its growth and the use of 24-EBL synergistically decreases oxidative damage by increasing the AOX system and enhances the PROG removal ability of the plants.

1. Introduction

Steroid hormones such as oestrogens, progesterone and testosterone are, by definition, crucial substances for the proper function of organisms, mediating a wide variety of vital physiological functions, like sexual differentiation, maintenance of cellular homeostasis and regulation of events associated with growth (Janeczko, 2021). Besides their natural excretion by animals, but mainly due to the widespread use of birth-control pills and other analogous drugs used for the treatment of hormonal disorders, steroid sex hormones have been detected in wastewater effluents and surface water. Consequently, they end up accumulated in the biosphere, being already reported progesterone concentrations ranging from 0.59 to 2.33 $\mu\text{g}.\text{mL}^{-1}$ in several influents of water-treatment plants in South-Eastern Finland (Sirén and El Fellah, 2016) and 10 to 25 $\mu\text{g}.\text{kg}^{-1}$ of agricultural soils in Malaysia (Ho et al., 2014), as examples.

The presence of natural and synthetic steroids in the environment has initiated great concerns among the scientific community and the general public, since they are endocrine disrupting chemicals (EDCs) (Rocha and Rocha, 2022). EDCs' harmful effects undergo through the mimic or simulation of natural hormones, changing the standard pattern of organisms' biosynthesis and metabolism, as well as hormone receptor levels. Previous studies indicate that human health can be affected by EDCs, increasing the chance of

incidence for breast cancer and endometriosis, testicular and prostate cancers and reduced male fertility (Solomon and Schettler, 2000; Nohynek et al., 2013; Rocha and Rocha, 2022). In addition to the potential effects on human health, several studies suggest that synthetic androgens and progestins can disrupt normal endocrine function in a wide range of aquatic organisms, causing adverse effects on fish reproduction, even at low ng.L^{-1} levels (Zeilinger et al., 2009; Söffker and Tyler, 2012; Rocha and Rocha, 2022).

To limit the biological impact of these steroid hormones, it is essential that residual traces of these products are eliminated from the biosphere. Phytoremediation is the use of plants and their associated microbes to degrade, stabilize and/or remove contaminants (metals and organic compounds) from the environment. This method offers several advantages over other remediation strategies: low costs, because of the absence of energy-consuming equipment and maintenance; none or limited negative impact on the environment, due to the *in situ* nature of the process; and large public acceptance as an attractive green technology (Pilon-Smits, 2005).

Solanum nigrum L., or black nightshade as it is commonly known, has good key features to be used as a phytoremediation tool. Several studies conducted with this species have demonstrated its ability to accumulate and/or degrade both organic and non-organic pollutants (Kucerova et al., 2000; Marques et al., 2008; Teixeira et al., 2011; de Sousa et al., 2013; Soares et al., 2016; Sousa et al., 2020b).

Plants are commonly exposed to several biotic and abiotic stress factors that lead to the overproduction of reactive oxygen species (ROS), such as hydroxyl radical ($\text{OH}^\cdot$), anion superoxide ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), among others. In excess, ROS can trigger cellular damage through oxidation of membrane lipids, affecting their permeability and structural integrity, and also through direct interaction with various macromolecules (proteins, DNA, etc.). In plants, the toxic effects of ROS can be counteracted by non-enzymatic antioxidants and by enzymatic antioxidants. A common adaptive response of plants to oxidative stress is the increase of antioxidant compounds levels, as well as the increase in the activity and/or expression of one or more antioxidant enzymes (Soares et al., 2019).

ROS detoxification pathways entail on high GSH:GSSG ratios and the participation of related enzymes such as GR, GST and glutathione peroxidase (GPX, EC 1.11.1.9) that play an indispensable role in detoxification of ROS (Nogueirol et al., 2016). In plants, GSH is considered a major intracellular redox buffer. Increased activities of the enzymes responsible for GSH biosynthesis and regeneration, as well as maintaining a high GSH:GSSG ratio, enhance the resistance and adaptation of plants to various environmental stresses (Labrou et al., 2015; Rabêlo et al., 2017; Soares et al., 2019).

Acting together with the antioxidant system, several phytohormones have been implicated in modulating plant responses to oxidative stress, such as salicylic acid (SA) (Hayat et al., 2010) and brassinosteroids (BRs) (Bari and Jones, 2009; Fariduddin et al., 2014; Sousa et al., 2020b). BRs are steroidal plant growth regulators distributed ubiquitously throughout the plant kingdom well known to play a critical role in plant growth, development and seed germination. It has also been demonstrated that the application of BRs

has a specific antioxidant effect against environmental stresses. In fact, exogenous application of 24-epibrassinolide (24-EBL), a class of BRs, detoxified the stress generated by NaCl in *Brassica juncea* (Ali et al., 2008), and by Ni (Soares et al., 2016) and Zn in *S. nigrum* (Sousa et al., 2020b), by increasing their antioxidant responses.

To date, very few reports were published regarding progesterone actions in plants (Janeczko, 2021). PROG increased seed germination and plant defence against abiotic and biotic stress by stimulating the activity of antioxidant enzymes (Genisel et al., 2013). Addition of PROG mitigated cold stress in maize seedlings by activating the cytochrome pathway and especially the alternative respiratory pathway of the mitochondrial respiratory pathway (Erdal and Genisel, 2016). Still, most of the present investigation on progesterone in plants is limited to the physiological level, and a more molecular level investigation is in need to elucidate progesterone signalling pathways (Li et al., 2022).

Bearing in mind the important role of the antioxidant system in plant tolerance against several kinds of stresses and the protective effects of BRs, the main goal of this study was to evaluate the effect of PROG in *S. nigrum*'s physiological responses and its ability to phytoremediate this hormone, as well as to determine if the pre-treatment with 24-EBL would enhance this species' remediation capacity for PROG. For that, growth parameters, photosynthetic pigments, several enzymatic and non-enzymatic antioxidant mechanisms and oxidative stress markers were analysed both at the biochemical and at the molecular level, plus the determination of PROG levels in the nutrient solutions and in plant tissues.

2. Materials and methods

2.1 Growth conditions and biometric analysis

Seeds of *S. nigrum* were collected from plants grown at Foz do Douro, Porto, Portugal (41°09'17,80"N and 8°39'47,91"O). Seed disinfection and stratification was performed as previously described (Moreira et al., 2018). Twenty-days-old seedlings were selected and transplanted to plastic pots with a mixture of expanded vermiculite and perlite (2:1) as root substrate. These were grown in a growth chamber at 24°C, with Hoagland solution (HS) being supplied when needed, under a 16h/8h day/night photoperiod with a photosynthetically active radiation of 120 mmol.m².s⁻¹ until seedlings reached 1.5 cm tall, at which point they were divided in the following groups: without any phytohormone supplied and watered with HS (control); exposure to 0.8 µM progesterone (PROG) (water-soluble, HBC complex, Sigma-Aldrich, Germany, dissolved in the HS), to simulate a soil contaminated with this hormone based on data previously reported (Rocha and Rocha, 2022); the third situation consisted in plants also exposed to 0.8 µM PROG in HS but pre-treated with a single foliar application of 1 µM 24-epibrassinolide (PROG/24-EBL; dissolved in a small amount of ethanol and the final volume completed with deionized water) prior to PROG exposure; the choice for using 1 µM 24-epibrassinolide was based on previous reports where it was shown that a pre-treatment with 1 µM 24-epibrassinolide increased *S. nigrum* tolerance to Ni (Soares et al., 2016) and Zn (Sousa et al., 2020b). Whenever the HS solutions were running low, their remaining was discarded and replaced by a new one.

Additionally, a “no plants” situation was used to determine if PROG removal could be due to non-plant-related factors, like microbiological degradation or adsorption to the vermiculite:perlite matrix. For this situation, samples for PROG quantifications in the HS solution were made before their replacement by new ones, along with the sampling from the two treatments (see below).

At the end of the treatment period (20 days after exposure), shoots and roots of at least six plants from each condition were used for the determination of some growth parameters: length (cm), fresh weight (g) and dry weight (g). Also, both shoots and roots ($n \geq 3$) were ground to a fine powder in liquid nitrogen and subsequently stored at -80°C for later biochemical and molecular studies and PROG quantifications. Additionally, HS from “no plants”, PROG and PROG/24-EBL treatments was collected throughout 6 days (days 10 to 15; before the solution was discarded and 20 minutes after its replacement), and at the end of the treatment (day 20), for PROG determination by HPLC-DAD.

2.2. Progesterone determination

2.2.1. Standards and reagents

Progesterone (water-soluble, HBC complex) was purchased from Sigma-Aldrich (Steinheim, Germany). Potassium hydroxide was acquired from Pronalab (Lisboa, Portugal), hexane was from Merck (Darmstadt, Germany) and ethanol and anhydrous sodium sulphate from Panreac (Castellar del Vallès, Spain). HPLC-grade methanol and acetonitrile were obtained from Merck. Water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2.2. Hydrolysis and HPLC-DAD analysis

To increase the solubility of PROG in water, a complex form of the hormone with methyl- β -cyclodextrin was acquired. From 2 mL samples of Hoagland's solution supplemented with PROG, a purification step was performed with an organic solvent: the solutions were transferred to a separation funnel to extract the lipophilic components with 4 mL of n-hexane for 3 times. The organic phase was dehydrated with anhydrous sodium sulphate and evaporated to dryness under reduced pressure, at 30°C . The residue was redissolved in acetonitrile:methanol (70/30, v/v) and filtered through a $0.45\ \mu\text{m}$ membrane (Millipore). Tissues from each treatment (ca. 5 g) were extracted with 100 mL of 80% MeOH for 2 h, under stirring (300 rpm). The extract was centrifuged (10 min, 4000 xg) and the material was re-extracted with 100 mL of 80% MeOH (15 min). The supernatants were evaporated to dryness under reduced pressure, at 30°C . The residue was dissolved in acetonitrile:methanol (70/30, v/v) and filtered through a $0.45\ \mu\text{m}$ membrane (Millipore).

Twenty microliters of each extract were analysed at room temperature in a HPLC unit (Gilson), using a reversed phase Hypersil ODS ($20 \times 0.4\ \text{cm i.d.} \times 5\ \mu\text{m}$) column. The mobile phase was

methanol:acetonitrile (30/70, v/v) at a flow rate of 0.8 mL.min⁻¹. Detection was achieved with a Gilson diode array detector (DAD). Spectral data from all peaks were accumulated in the 190-400 nm range. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. PROG was identified by comparison of retention time and UV absorption spectrum with one obtained for an authentic standard. Quantification was achieved by the absorbance recorded in the chromatograms registered at 240 nm. The calibration curve ($y=1152372.5574x$) ($r^2=0.9971$) was obtained with standard solutions (seven different concentrations), prepared according to the conditions described above.

2.3. Chlorophylls and carotenoids content

Photosynthetic pigments were extracted and quantified using the formulas of Lichtenthaler (1987).

2.4. Determination of H₂O₂, lipid peroxidation and proline

H₂O₂ was extracted of a proportion of 0.3 g of frozen tissue (shoots or roots) to 1 mL 0.1% (w/v) trichloroacetic acid (TCA) and the amount of H₂O₂ was calculated using $e=0.28 \mu\text{M}^{-1}.\text{cm}^{-1}$ (Alexieva et al., 2001). Results were expressed as pmol.g⁻¹ fresh weight (f.w.).

Determination of thiobarbituric acid reacting substances (TBARS), as an indicator of membrane lipid peroxidation, was measured in terms of malondialdehyde (MDA) content as described by Heath and Packer (1968). The molar extinction coefficient of $155 \text{ mM}^{-1}.\text{cm}^{-1}$ was used and results were expressed as nmol MDA.g⁻¹ f.w.

Proline concentration in both shoots and roots was determined by the ninhydrin-based colorimetric assay (Bates et al., 1973). Results were expressed as $\mu\text{g proline.g}^{-1}$ f.w.

2.5. Glutathione (GSH) determination

GSH extraction was accomplished by homogenizing 0.5 g of plant tissue with 2.5 mL of 3 % (w/v) sulfosalicylic acid. After 10 min of incubation at 4°C, the homogenate was centrifuged at 10,000 xg for 10 min at 4°C. GSH levels were determined quantifying the amount of DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] directly reduced by GSH at 412 nm. For that, 750 μL of a working solution [1 mM EDTA in 100 mM phosphate buffer and 400 μL of DTNB (1.5 mg.mL^{-1})] were added to 50 μL of extract and H₂O in a final volume of 1 mL. For the blank, 3% (w/v) sulfosalicylic acid was used instead of extract. For the standard curve known GSH concentrations were used instead of extract. GSH levels were expressed as nmole GSH.g⁻¹ f.w..

2.6. Ascorbate peroxidase (APX), Glutathione S-transferase (GST), Gamma-glutamylcysteine synthetase (γ -ECS) and Glutathione reductase (GR) activity determinations

APX extraction from roots and shoots was performed according to Fidalgo et al. (2011). GST was extracted and quantified as described previously by Sousa et al. (2021).

γ -ECS extraction and activity was performed based on the protocol described by Sengupta et al. (2012). Frozen aliquots (0.5 g) were homogenized with 1 mL of 100 mM Tris-HCl (pH 8.0) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged for 10 min at 13,000 $\times g$ (4°C). The reaction for γ -ECS activity was started by the addition of the enzyme extract (20 μ L) to give a 400 μ L-assay mix containing 100 mM Hepes (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 1 mM cysteine, 5 mM ATP, 5 mM phosphoenolpyruvate, 5 mM DTT and 10 U.mL⁻¹ pyruvate kinase. The reaction was incubated at 37°C for 45 min, and was stopped by adding 200 μ L of 50% (w/v) TCA. After centrifugation, the supernatant was used for the determination of the phosphate content using the phosphomolybdate method (De Groeve et al., 2010). The results were expressed in nmol.min⁻¹.mg⁻¹ protein. GR activity was determined following the oxidation of NADPH at 340 nm, using the extinction coefficient 6.22 mM⁻¹.cm⁻¹ (Yannarelli et al., 2007). The results were expressed in nmol.min⁻¹.mg⁻¹ protein.

For all enzymes, the protein content of the extracts was measured according to Bradford (1976) using BSA as standard.

2.7. Statistical analysis

All assays and measurements were performed at least in triplicate ($n \geq 3$). Variance analysis was made using the Fisher test and the means were statistically analysed using a two-sided *t*-Student test. Statistical significance was assumed at $p < 0.05$.

2.8. RNA extraction and semi-quantitative RT-PCR

Total RNA extraction was performed using the Trizol reagent (Invitrogen, USA), according to the supplier's instructions. The RT-PCR reactions consisted in two steps: a reverse transcription (RT) reaction (using the primer R9 – 5'-TTT TTT TTT TTT TCG AAC TCG AGC TCA GGA GCA GTG AGA CGA GTG ACC-3') (Fidalgo et al., 2011), followed by cDNA-specific PCR reactions. The RT reactions were performed using NZY M-MuLV Reverse Transcriptase (NZYTech®, Portugal), according to the recommended protocol.

The specific PCR reactions were performed in a total volume of 20 μ L with Kapa Taq ReadyMix (Kapa Biosystems, UK) and assembled with 1 μ L of a 1:10 dilution of the RT reaction as template. The primers' sets and programs used for the amplification of APX and tubulin (Tub) were those described by Fidalgo

et al. (2011). Those used for the amplification of GST and -ECS cDNA's were previously published (Teixeira et al., 2015; Martins and Teixeira, 2021). Because there is only available one (incomplete) cDNA nucleotide sequence for *S. nigrum*'s GR, the primers' sets used for the detection GR transcripts were designed based on the alignment of several GR-encoding sequences from other *Solanum* species in *PrimerIdent* (Pessoa et al., 2010), resulting in the following sequences (forward/reverse): 5'-TGG TGA ACT TGA GGA TGC-3'/ 5'-CAA CTA GTG CCC TCA TTT CG -3'. The GR-related PCR reactions were set on a S-96 thermocycler (Quanta Biotech, UK) with the following program: 94°C - 2', following by 28 cycles of 94°C - 30", 50°C - 30" and 72°C - 1'. The final extension was performed at 72°C for 5' and the reaction mixtures were stored at 4°C until analysed by agarose gel electrophoresis. All RT-PCR were performed at least in triplicates.

PCR reactions were loaded in 0.8% (w/v) agarose gels and DNA electrophoresis was performed according to standard molecular biology procedures. Subsequent electrophoresis, using the same PCR products, were performed with adjusted volumes taking into consideration those needed to see uniform bands for the Tubulin amplicon in all wells/situations, so that semi-quantitative results for the studied genes could be attained. All gel images were acquired with the ChemiDoc™ MP System (Bio-Rad Laboratories, USA).

3. Results

3.1. Growth parameters

No significant differences were observed in the biometry of *S. nigrum* plants from both treatments. Additionally, PROG- and PROG/24-EBL-treated plants did not show any signs of toxicity (data not shown).

3.2. Progesterone quantification

The HPLC-DAD analysis of the hydrolysed commercial standard showed progesterone eluting at 2.8 min at 240 nm (λ_{max}), as previously reported (Almeida and Nogueira, 2006). By a 6-days period (day 10 to 15) evaluation of the PROG removal efficiency by the plants it was possible to observe that PROG levels in the nutrient solution were reduced to non-detectable levels after one day of exposure, independently of the plants being pre-treated or not with 24-EBL (Fig. 1). Moreover, at the end of the experiment, no progesterone was detected in all analysed samples (plant tissues (data not shown), and nutrient solutions). Considering the "No plants" situation, PROG levels remained always high throughout the experimental period, with a slight, but not statistically significant, increase throughout time.

The pre-treatment with 24-EBR resulted in a significant reduction in PROG levels in the solution, when compared to the PROG treatment, by 1.33- and 2-fold, on days 11 and 14, respectively.

3.3. Biochemical determinations

3.3.1. Total chlorophyll and carotenoid content

Chlorophyll levels (Chl a and Chl b) and carotenoids in the shoots of plants exposed to PROG and to PROG/24-EBL were similar to those of control plants (Table 1).

3.3.2. H₂O₂ quantification

The spectrophotometric detection of shoots' H₂O₂ levels revealed a significant decrease of 26% and 52% for the PROG and PROG/24-EBL treatments, respectively, whilst a significant decrease of 35% and 54% was detected for the roots of those treatments (Table 1).

Table 1. Effect of PROG (0.8 µM) and PROG (0.8 µM)/24-EBL (1 µM) on chlorophyll (a+b) and carotenoids contents (mg.g⁻¹ f.w.), H₂O₂ (pmoles.g⁻¹ f.w.), MDA levels (nmol.g⁻¹ f.w.), proline (µg.g⁻¹ f.w.) and GSH contents (nmol.g⁻¹ f.w.) in shoots and roots of *S. nigrum* L. plants after 20 days of treatment. Data presented are mean ± SE (n≥3). ^aSignificant statistical difference from control at p<0.05. ^bSignificant statistical difference from PROG at p<0.05.

Parameters	Control	PROG	PROG/24-EBL
Shoots			
Total chlorophyll	1.35 ± 0.13	1.31 ± 0.04	1.33 ± 0.07
Carotenoids	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.02
H ₂ O ₂	4.54 ± 0.10	3.36 ± 0.35 ^a	2.20 ± 0.13 ^{a,b}
MDA	22.20 ± 0.46	14.76 ± 0.52 ^a	7.41 ± 0.60 ^{a,b}
Proline	0.06 ± 0.0025	0.069 ± 0.0023	0.077 ± 0.0026 ^{a,b}
GSH	48.38 ± 0.81	44.31 ± 0.59 ^a	37.82 ± 0.29 ^{a,b}
Roots			
H ₂ O ₂	1.53 ± 0.06	0.99 ± 0.06 ^a	0.70 ± 0.04 ^{a,b}
MDA	11.52 ± 0.49	5.74 ± 0.58 ^a	4.19 ± 0.11 ^{a,b}
Proline	0.035 ± 0.0025	0.041 ± 0.002	0.058 ± 0.001 ^{a,b}
GSH	23.78 ± 0.48	19.6 ± 0.97 ^a	14.46 ± 0.73 ^{a,b}

3.3.3. MDA and free proline content

The lipid peroxidation suffered a significant decrease in shoots and roots of both treatments (Table 1), being this reduction in shoots of 33.5% and 66.5% in the PROG and PROG/24-EBL treatments, respectively; while in roots it decreased 50% and 64% in the PROG and PROG/24-EBL-treated plants, respectively. As shown in Table 1, proline levels significantly increased only with the PROG/24-EBL treatment: shoots by 1.3-fold; roots by 1.7-fold.

3.3.4. Glutathione levels

GSH levels decreased with both treatments, both in shoots and roots (Table 1). It significantly decreased 9% and 18% in shoots and roots, respectively, in plants exposed to 0.8 μM of PROG and 22% and 40% in shoots and roots in plants treated with the two hormones.

3.3.5. Antioxidant enzyme activities and transcript accumulation patterns

γ -ECS, APX, GR, and GST activities were determined spectrophotometrically for shoots and roots of *S. nigrum* plants (Table 2). PROG exposure did not alter γ -ECS activity, both in shoots and in roots. However, in the PROG/24-EBL treatment, the activity of this enzyme increased 2.4-fold in shoots and decrease 1.21-fold in roots.

Additionally, two enzymes of the ascorbate–glutathione cycle were studied: APX and GR. In shoots, the activity of both enzymes was significantly increased by both treatments, with PROG/24-EBL-treated plants presenting the highest values. Shoot APX activity increased almost 2-fold in the PROG treatment and 2.25-fold with both hormones; shoot GR activity increased 1.3- and 2.4-fold in the respective treatments. Root APX activity of the PROG treatment significantly increased 2.8-fold, and a higher increase (3.8-fold) was detected in the PROG/24-EBL-treated plants. In contrast, root GR activity did not present variations in the PROG-treated plants and showed significant 1.9-fold decrease in plants treated with the two hormones, when compared to the control, and 1.1-fold decrease when compared to the PROG-treated plants.

When compared to the control, shoot GST activity significantly decreased 2.4-fold in PROG-treated plants, but increased 2.6-fold with the PROG/24-EBL treatment. PROG/24-EBL shoot GST activity significantly increased 6.1-fold when compared to the PROG treatment. In roots, both treatments lead to significant decreases of GST activity: 1.8- and $\approx$ 2-fold, PROG and PROG/24-EBL, respectively.

Table 2. Effect of PROG (0.8 μM) and PROG (0.8 μM)/24-EBL (1 μM) on APX ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ prot), GR, γ -ECS and GST activities ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ prot) in shoots and roots of *S. nigrum* plants after 20 days of

treatment. Data presented are mean $\pm$ SE ($n \geq 3$). ^aSignificant statistical difference from control at $p < 0.05$.
^bSignificant statistical difference from PROG at $p < 0.05$.

Parameters	Control	PROG	PROG/24-EBL
Shoots			
APX	9.38 $\pm$ 2.05	18.40 $\pm$ 0.16 ^a	21.10 $\pm$ 1.22 ^{a,b}
GR	9.97 $\pm$ 0.90	12.67 $\pm$ 0.76 ^a	24.16 $\pm$ 0.56 ^{a,b}
γ -ECS	1.22 $\pm$ 0.03	1.18 $\pm$ 0.05	2.93 $\pm$ 0.01 ^{a,b}
GST	2.83 $\pm$ 0.56	1.20 $\pm$ 0.46 ^a	7.35 $\pm$ 0.42 ^{a,b}
Roots			
APX	3.72 $\pm$ 0.37	10.50 $\pm$ 0.58 ^a	13.96 $\pm$ 2.10 ^a
GR	10.46 $\pm$ 0.70	9.63 $\pm$ 0.69	5.38 $\pm$ 0.63 ^{a,b}
γ -ECS	16.10 $\pm$ 0.15	15.55 $\pm$ 0.17	13.29 $\pm$ 0.25 ^{a,b}
GST	25.96 $\pm$ 1.93	14.63 $\pm$ 0.94 ^a	13.12 $\pm$ 5.13 ^a

The expression of four antioxidant enzymes encoding genes was studied in shoots and roots of control, PROG and PROG/24-EBL-treated plants by semi-quantitative RT-PCR (Fig. 2). The analysis of the GR mRNA accumulation patterns revealed no changes among the three groups of plants and for both organs. Only the plants treated with both hormones increased APX's mRNA accumulation in roots and no variation was observed for the other situations.

The levels of γ -ECS mRNA did not change in response to PROG in both shoots and roots, but when the plants were pre-treated with 24-EBL an increase was observed in shoots and a decrease was observed in roots. In shoots, GST-related transcripts accumulation was negatively affected by PROG but an increase was observed in with the 24-EBL pre-treatment. Concerning roots, a decrease in GST-encoding mRNA accumulation was observed in plants exposed to both treatments, with PROG/24-EBL presenting the lowest accumulation (Fig. 2).

Discussion

Plant growth is the result of integrated and regulated physiological processes. To assess the effects of PROG on *S. nigrum* plants, 0.8 μ M PROG was added to the nutrient solution while another similar set was made, but with a foliar pre-treatment with 24-EBL to determine if this steroid hormone would enhance this species' tolerance or remediation capacity towards PROG, similarly to when this species was exposed to

high levels of Ni (Soares et al., 2016) or Zn (Sousa et al., 2020b). Exposure of *S. nigrum* to 0.8 μM PROG did not trigger a reduction on biomass and did not increase any of the physiological stress-related parameters tested (lipid peroxidation and H_2O_2). Hence, it can be assumed that *S. nigrum* plants' growth was not compromised by the PROG concentration used, which corresponds to the average concentration found in PROG-contaminated sites (Rocha and Rocha, 2022).

The HPLC-DAD analysis showed that no PROG was detected in the nutrient solutions one day after the exposure to the plants, but remained always high in the "no plants" situation. This shows that: the substrate used had no effect on the PROG levels in the nutrient medium; no PROG degradation occurred throughout time by external factors to the plants; the plants were the only responsible factor for the removal of PROG from the solution; and that the PROG removal rate was very high (Fig. 1). Moreover, because the PROG levels on the days when the nutrient solution was added to the tray were consistently higher on the non pre-treated plants with 24-EBL situation, one can conclude that the 24-EBL pre-treatment boosted this removal capacity. Plus, since no PROG was detected on the nutrient solutions and plant tissues of both treatments at the end of the treatment period, it further supports the belief that the plants examined were able to uptake and metabolise all added PROG, independently of being pre-treated with 24-EBL or not. Supporting this thought, PROG conjugates were found as a product of the conversion of exogenously applied PROG (Asthana & Asthana 2015). Considering this information, the obtained results imply that *S. nigrum* plants were capable to absorb and metabolise this hormone and that the remediation of PROG-contaminated sites using *S. nigrum* is, therefore, possible.

Photosynthesis is a major physiological process. Nowadays, photosynthetic pigment contents are good criteria for assessing the effect of organic pollutants on the photosynthetic apparatus. As already mentioned, no significant changes in the photosynthetic pigments' levels were observed with the described treatments. Regarding the action of 24-EBL, these findings are in agreement with those reported by earlier researches that showed that a pre-treatment with brassinosteroids did not change photosynthetic pigment contents (Chl a, Chl b and carotenoids) (Ahammed et al., 2013), implying that BRs are not capable to improve photosynthetic apparatus under non-stressful conditions.

Contrarily to other ROS, H_2O_2 can easily cross biological membranes causing phytotoxic reactions such as lipid peroxidation and protein degradation far from the site of its formation. For this reason, MDA content, an indicator of membrane lipid peroxidation, is a good index to measure the toxic effects of pollutants (Soares et al., 2019). PROG exposure did not increase H_2O_2 levels. In fact, PROG alone significantly decreased both MDA and H_2O_2 levels in both organs studied. To date, there are only a few reports concerned with the effects of PROG on plant oxidative stress. A study with several mammalian sex hormones, including PROG, showed that a co-treatment with these hormones significantly ameliorated the negative effects of plants exposed to salt stress and that this effect was associated with a decrease in H_2O_2 and MDA levels (Erdal, 2012). In accordance to the present results, a previous report showed that the lower levels of ROS and lipid peroxidation in plants treated with PROG were due to an increase in the activity of ROS-scavenging enzymes such as SOD and CAT (Erdal and Dumlupinar, 2011).

Such results reveal more positive effects of PROG on the plant antioxidant system, rather than oxidative damages. Accordingly, the biochemical data obtained in this study for total chlorophyll and carotenoids, together with a decrease in MDA and H₂O₂ levels, strengthen the notion that *S. nigrum* plants did not suffer oxidative stress when exposed to PROG.

Likewise, the levels of H₂O₂ and lipid peroxidation on plants exposed to PROG, but pre-treated with 24-EBL (Table 2), presented a decrease for the same biochemical parameters that was even more pronounced than with PROG alone, suggesting a synergistic effect of PROG and BR on *S. nigrum* plants' ROS removal capacity. It is known that BRs are capable to decrease lipid peroxidation even without any adverse situations (Sharma et al., 2015). This decrease in lipid peroxidation could be regarded as evidence for BR-induced efficient ROS scavenging mechanisms, like proline, which possesses the ability to scavenge ROS (Hayat et al., 2012), and which levels increased in both organs of the plants treated with the two hormones (see below). So, the presence of major reductions in H₂O₂ and lipid peroxidation levels observed in this study, more pronounced in plants from the PROG/24-EBL treatment, must be a result of an efficient ROS scavenging response synergistically induced by both hormones.

Proline is a proteinogenic amino acid that under stressful conditions acts like an osmoprotector and as a potent antioxidant, participating in repair processes of membranes and in ROS scavenging. Elevation in proline content has been reported under several types of stresses (Gill and Tuteja, 2010; de Sousa et al., 2013; Sharma et al., 2015; Sousa et al., 2020a; Martins et al., 2021). In the present study, the increased levels of proline in both organs of PROG/24-EBL-treated plants suggest that it may be protecting *S. nigrum* plants by acting as a scavenger of H₂O₂, thus contributing more to the lower MDA levels detected. Nevertheless, the two treatments tested may also induce other antioxidant defence mechanisms in order to decrease the levels of H₂O₂ and lipid peroxidation, like AsA, thiols or peroxidases (Moreira et al., 2018).

In this study, the activity of several antioxidant enzymes and their relative transcript accumulation were studied. In PROG and PROG/24-EBL-treated plants, the reduction in H₂O₂ levels can also be related to the enhanced activity of the H₂O₂-scavenging enzyme APX. In fact, the increase observed in APX activity suggests that the ROS scavenging function of this enzyme is improved by both treatments for both organs. In accordance to these results, it was previously demonstrated that a pre-treatment with BR is capable to increase APX activity in *Solanum lycopersicum* L. plants when exposed to polychlorinated biphenyls (Ahammed et al., 2013) and in *S. nigrum* plants exposed to Zn (Sousa et al., 2020b). Despite the increased APX activity, there was no correlation with the corresponding mRNA levels in most situations, suggesting that post-transcriptional and post-translational regulations were involved in promoting this enzymes' activity and stability. In fact, a similar pattern of regulation was suggested for APX in *S. nigrum* leaves of Cd- or Ni-treated plants (Fidalgo et al., 2011; Soares et al., 2016). Nevertheless, further research should be pursued by native PAGE to identify potential APX isoenzymes responsible for the increased activities recorded.

The significant decrease in lipid peroxidation observed in this study could be also due to an effective removal of lipid peroxides by GST, and/or a result of the protective actions of proline, APX and

GSH. Interestingly, both treatments lead to reductions of the GSH levels in both organs analysed. PROG-treated plants had a decrease in GST activity in both organs analysed, which suggests that GSH may be protecting *S. nigrum* cells by acting as an antioxidant, removing ROS, and not so much involved in detoxification processes (conjugation) of PROG. Supporting this hypothesis comes the fact that two pathways have been described for the processing of PROG by plant cells, and that all final transformation products are posteriorly glycosylated and not glutathiolated (Asthana and Asthana, 2015). Shoot GST was upregulated in response to BR in tomato (Ahammed et al., 2012) and in the present study the PROG/24-EBL treatment greatly increased shoot GST activity and its transcript levels. Such increased activity presumably contributed for the observed reduction in GSH levels, thus suggesting a potential enhancement in the shoot cellular detoxification processes through conjugation of GSH with potentially toxic products, like lipid peroxides, being activated by BR (Ahammed et al., 2012). Moreover, the correlation between mRNA levels and GST activities in shoots and roots from both treatments denotes that this enzyme was regulated by both hormones at the transcriptional level.

GSH is maintained in a predominantly reduced state by the enzyme GR (Soares et al., 2019). The increase recorded for GR activity in shoots of both treatments was of great importance to provide GSH to function as an antioxidant, although no differences were detected regarding its mRNA accumulation, thus suggesting that this enzyme was regulated post-transcriptional and/or post-translationally, as already proposed for the GR in the bundle sheath cells of maize (Pastori et al., 2000).

The obtained results with the PROG/24-EBL treatment reveal that the increased shoot γ -ECS activity (which is the critical enzyme in the GSH biosynthesis process) was a response to the depletion in the GSH levels. Moreover, there was a strong correlation between the activity of γ -ECS and its encoding mRNA accumulation in all organs and situations analysed, which strongly suggests that γ -ECS was mainly regulated at its transcription by both hormones. γ -ECS primary regulation at its transcription has also been proposed for *S. nigrum* exposed to the end-products of the photo-Fenton process of metalaxyl (Teixeira et al., 2015). Nevertheless, in this study, there was an organ-dependent differential response to the PROG/24-EBL treatment, as the biosynthesis, reduction and usage of GSH increased in shoots and decreased in roots. This can be explained by the fact that BR exogenously applied to shoots barely suffer translocation to other parts of the plant (Taiz and Zeiger, 2010), thus exerting their beneficial effects mainly at the site of application, which in this case corresponds to the shoots, being the untreated parts of the plant (roots) depleted in the GSH-related biosynthesis necessary resources.

This study unequivocally establishes that *S. nigrum* plants are exceptional for the remediation of PROG-contaminated sites without experiencing (oxidative) stress. Furthermore, it shows that the exogenous application of both steroid hormones (PROG and BR) increased the plants' antioxidant response system, with γ -ECS and GST being directly controlled at the transcription level, while APX and GR were regulated post-transcriptionally. Additionally, it demonstrates that although these two steroidal hormones have distinct modes of action, both leading to a better performance against ROS, when supplied simultaneously they have a synergic effect on such performance and also on the remediation of progesterone.

An important part of hormone action is its perception in plant cells. Previous studies prove the presence of PROG-binding sites at the cell membrane and in the cytosolic fraction (Yang et al., 2005; Janeczko et al., 2008; Janeczko, 2021). The presence of similar binding sites for these two steroid hormones (PROG and BR) highlights the convergence of similar transduction pathways for the regulation of the enzymes studied, as well as other cellular processes. As such, more studies are still needed to better understand their signalling pathways.

Declarations

Funding

This research was partially supported through national funds by Foundation for Science and Technology (FCT), within the scope of UIDB/05748/2020 and UIDP/05748/2020 (GreenUPorto).

Declaration of Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

CRediT authorship contribution statement

Ana Pinto: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Daniela Silva:** Investigation, Formal analysis, Writing - original draft. **Ana Cardoso:** Investigation. **Cristiano Soares:** Investigation. **Patrícia Valentão:** Investigation, Formal analysis, Writing - original draft. **Fátima Fernandes:** Investigation. **Fernanda Fidalgo:** Funding acquisition, Resources, Writing - review & editing, Supervision. **Jorge Teixeira:** Funding acquisition, Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

This research was partially supported through national funds by Foundation for Science and Technology (FCT), within the scope of UIDB/05748/2020 and UIDP/05748/2020 (GreenUPorto). GreenUPorto and FCUP are highly recognized for equipment support.

Acknowledgments

This research was partially supported through national funds by Foundation for Science and Technology (FCT), within the scope of UIDB/05748/2020 and UIDP/05748/2020 (GreenUPorto). GreenUPorto and FCUP are highly recognized for equipment support.

References

1. Ahammed, G.J., Ruan, Y.P., Zhou, J., Xia, X.J., Shi, K., Zhou, Y.H., Yu, J.Q., 2013. Brassinosteroid alleviates polychlorinated biphenyls-induced oxidative stress by enhancing antioxidant enzymes activity in tomato. *Chemosphere* 90, 2645-2653. doi:10.1016/j.chemosphere.2012.11.041
2. Ahammed, G.J., Yuan, H.L., Ogwen, J.O., Zhou, Y.H., Xia, X.J., Mao, W.H., Shi, K., Yu, J.Q., 2012. Brassinosteroid alleviates phenanthrene and pyrene phytotoxicity by increasing detoxification activity and photosynthesis in tomato. *Chemosphere* 86, 546-555. doi:10.1016/j.chemosphere.2011.10.038
3. Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell and Environment* 24, 1337-1344. doi:10.1046/j.1365-3040.2001.00778.x
4. Ali, B., Hayat, S., Fariduddin, Q., Ahmad, A., 2008. 24-epibrassinolide protects against the stress generated by salinity and nickel in *Brassica juncea*. *Chemosphere* 72, 1387-1392. doi:10.1016/j.chemosphere.2008.04.012
5. Almeida, C., Nogueira, J.M.F., 2006. Determination of steroid sex hormones in water and urine matrices by stir bar sorptive extraction and liquid chromatography with diode array detection. *Journal of Pharmaceutical and Biomedical Analysis* 41, 1303-1311. doi:10.1016/j.jpba.2006.02.037
6. Asthana, G.S., Asthana, A., 2015. Biotransformation of progesterone to 17 α -hydroxyprogesterone by using plant cell suspension culture of *Catharanthus roseus*. *International Journal of Pharmacy and Pharmaceutical Sciences* 7.
7. Bari, R., Jones, J., 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology* 69, 473-488. doi:10.1007/s11103-008-9435-0
8. Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid Determination of Free Proline for Water-Stress Studies. *Plant Soil* 39, 205-207. doi:10.1007/Bf00018060
9. Bradford, M.M., 1976. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Anal Biochem* 72, 248-254. doi:10.1006/abio.1976.9999
10. De Groeve, M.R.M., Tran, G.H., Van Hoorebeke, A., Stout, J., Desmet, T., Savvides, S.N., Soetaert, W., 2010. Development and application of a screening assay for glycoside phosphorylases. *Anal Biochem* 401, 162-167. doi:10.1016/J.Ab.2010.02.028
11. de Sousa, A., Teixeira, J., Regueiras, M.T., Azenha, M., Silva, F., Fidalgo, F., 2013. Metalaxyl-induced changes in the antioxidant metabolism of *Solanum nigrum* L. suspension cells. *Pesticide Biochemistry and Physiology* 107, 235-243. doi:10.1016/j.pestbp.2013.07.006
12. Erdal, S., 2012. Alleviation of salt stress in wheat seedlings by mammalian sex hormones. *Journal of the Science of Food and Agriculture* 92, 1411-1416. doi:10.1002/jsfa.4716
13. Erdal, S., Dumlupinar, R., 2011. Mammalian sex hormones stimulate antioxidant system and enhance growth of chickpea plants. *Acta Physiologiae Plantarum* 33, 1011-1017.

14. Erdal, S., Genisel, M., 2016. The property of progesterone to mitigate cold stress in maize is linked to a modulation of the mitochondrial respiratory pathway. *Theor Exp Plant Phys* 28, 385-393.
15. Fariduddin, Q., Yusuf, M., Ahmad, I., Ahmad, A., 2014. Brassinosteroids and their role in response of plants to abiotic stresses. *Biologia Plantarum* 58, 9-17. doi:10.1007/s10535-013-0374-5
16. Fidalgo, F., Freitas, R., Ferreira, R., Pessoa, A.M., Teixeira, J., 2011. *Solanum nigrum* L. antioxidant defence system isozymes are regulated transcriptionally and posttranslationally in Cd-induced stress. *Environ. Exp. Bot.* 72, 312-319. doi:10.1016/j.envexpbot.2011.04.007
17. Genisel, M., Turk, H., Erdal, S., 2013. Exogenous progesterone application protects chickpea seedlings against chilling-induced oxidative stress. *Acta Physiologiae Plantarum* 35, 241-251. doi:10.1007/s11738-012-1070-3
18. Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Bioch* 48, 909-930. doi:10.1016/j.plaphy.2010.08.016
19. Hayat, Q., Hayat, S., Irfan, M., Ahmad, A., 2010. Effect of exogenous salicylic acid under changing environment: A review. *Environmental and Experimental Botany* 68, 14-25. doi:10.1016/j.envexpbot.2009.08.005
20. Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., Ahmad, A., 2012. Role of proline under changing environments: a review. *Plant signaling & behavior* 7, 1456-1466. doi:10.4161/psb.21949
21. Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts .I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125, 189-&. doi:10.1016/0003-9861(68)90654-1
22. Ho, Y.B., Zakaria, M.P., Latif, P.A., Saari, N., 2014. Occurrence of veterinary antibiotics and progesterone in broiler manure and agricultural soil in Malaysia. *Science of The Total Environment* 488-489, 261-267. doi:https://doi.org/10.1016/j.scitotenv.2014.04.109
23. Janeczko, A., 2021. Estrogens and Androgens in Plants: The Last 20 Years of Studies. *Plants* 10, 2783. doi:https://doi.org/10.3390/plants10122783
24. Janeczko, A., Budziszewska, B., Skoczowski, A., Dybala, M., 2008. Specific binding sites for progesterone and 17 beta-estradiol in cells of *Triticum aestivum* L. *Acta Biochimica Polonica* 55, 707-711.
25. Kucerova, P., Mackova, M., Chroma, L., Burkhard, J., Triska, J., Demnerova, K., Macek, T., 2000. Metabolism of polychlorinated biphenyls by *Solanum nigrum* hairy root clone SNC-90 and analysis of transformation products. *Plant and Soil* 225, 109-115. doi:10.1023/a:1026551728196
26. Labrou, N.E., Papageorgiou, A.C., Pavli, O., Flemetakis, E., 2015. Plant GSTome: structure and functional role in xenome network and plant stress response. *Curr Opin Biotech* 32, 186-194. doi:10.1016/j.copbio.2014.12.024
27. Li, H., Chen, L., Chen, H., Xue, R., Wang, Y., Song, J., 2022. The Role of Plant Progesterone in Regulating Growth, Development, and Biotic/Abiotic Stress Responses. *International Journal of Molecular Sciences* 23, 10945. doi:https://doi.org/10.3390/ijms231810945

28. Lichtenthaler, H.K., 1987. Chlorophylls and Carotenoids - Pigments of Photosynthetic Biomembranes. *Methods in Enzymology* 148, 350-382.
29. Marques, A.P.G.C., Oliveira, R.S., Samardjieva, K.A., Pissarra, J., Rangel, A.O.S.S., Castro, P.M.L., 2008. EDDS and EDTA-enhanced zinc accumulation by *Solanum nigrum* inoculated with arbuscular mycorrhizal fungi grown in contaminated soil. *Chemosphere* 70, 1002-1014.
doi:<https://doi.org/10.1016/j.chemosphere.2007.08.045>
30. Martins, L., Teixeira, J., 2021. Gene- and organ-specific impact of paracetamol on *Solanum nigrum* L.'s γ -glutamylcysteine synthetase and glutathione S-transferase and consequent phytoremediation fitness. *Acta Physiol. Plant.* 43, 53. doi:10.1007/s11738-021-03224-2
31. Martins, M., Lopes, J., Sousa, B., Soares, C., Valente, I.M., Rodrigues, J.A., Fidalgo, F., Teixeira, J., 2021. Cr (VI)-induced oxidative damage impairs ammonia assimilation into organic forms in *Solanum lycopersicum* L. *Plant Stress* 2. doi:<https://doi.org/10.1016/j.stress.2021.100034>
32. Moreira, J.T., Moreira, T.M., Cunha, J.B., Azenha, M., Fidalgo, F., Teixeira, J., 2018. Differential effects of acetophenone on shoots' and roots' metabolism of *Solanum nigrum* L. plants and implications in its phytoremediation. *Plant Physiology and Biochemistry* 130, 391-398.
doi:10.1016/j.plaphy.2018.07.027
33. Nogueirol, R.C., Monteiro, F.A., Gratão, P.L., de Alcântara da Silva, B.K., Azevedo, R.A., 2016. Cadmium Application in Tomato: Nutritional Imbalance and Oxidative Stress. *Water, Air, & Soil Pollution* 227, 210. doi:10.1007/s11270-016-2895-y
34. Nohynek, G.J., Borgert, C.J., Dietrich, D., Rozman, K.K., 2013. Endocrine disruption: Fact or urban legend? *Toxicology Letters* 223, 295-305. doi:10.1016/j.toxlet.2013.10.022
35. Pastori, G.M., Mullineaux, P.M., Foyer, C.H., 2000. Post-Transcriptional Regulation Prevents Accumulation of Glutathione Reductase Protein and Activity in the Bundle Sheath Cells of Maize1. *Plant Physiol* 122, 667-676. doi:10.1104/pp.122.3.667
36. Pessoa, A.M., Pereira, S., Teixeira, J., 2010. PrimerIdent: A web based tool for conserved primer design. *Bioinformatics* 5, 52-54. doi:10.6026/97320630005052
37. Pilon-Smits, E., 2005. PHYTOREMEDIATION. *Annual Review of Plant Biology* 56, 15-39.
doi:<https://doi.org/10.1146/annurev.arplant.56.032604.144214>
38. Rabêlo, F.H.S., Azevedo, R.A., Monteiro, F.A., 2017. Proper supply of S increases GSH synthesis in the establishment and reduces tiller mortality during the regrowth of *Tanzania guinea* grass used for Cd phytoextraction. *Journal of Soils and Sediments* 17, 1427-1436. doi:10.1007/s11368-016-1429-y
39. Rocha, M.J., Rocha, E., 2022. Synthetic Progestins in Waste and Surface Waters: Concentrations, Impacts and Ecological Risk. *Toxics* 10, 163. doi:<https://doi.org/10.3390/toxics10040163>
40. Sengupta, D., Ramesh, G., Mudalkar, S., Kumar, K.R.R., Kirti, P.B., Reddy, A.R., 2012. Molecular Cloning and Characterization of γ -Glutamyl Cysteine Synthetase (V γ ECS) from Roots of *Vigna radiata* (L.) Wilczek Under Progressive Drought Stress and Recovery. *Plant Molecular Biology Reporter* 30, 894-903. doi:10.1007/s11105-011-0398-y

41. Sharma, I., Bhardwaj, R., Pati, P.K., 2015. Exogenous Application of 28-Homobrassinolide Modulates the Dynamics of Salt and Pesticides Induced Stress Responses in an Elite Rice Variety Pusa Basmati-1. *Journal of Plant Growth Regulation* 34, 509-518. doi:10.1007/s00344-015-9486-9
42. Sirén, H., El Fellah, S., 2016. Steroids contents in waters of wastewater purification plants: determination with partial-filling micellar electrokinetic capillary chromatography and UV detection. *International Journal of Environmental Analytical Chemistry* 96, 1003-1021. doi:https://doi.org/10.1080/03067319.2016.1232721
43. Soares, C., Carvalho, M.E.A., Azevedo, R.A., Fidalgo, F., 2019. Plants facing oxidative challenges-A little help from the antioxidant networks. *Environ Exp Bot* 161, 4-25. doi:10.1016/j.envexpbot.2018.12.009
44. Soares, C., de Sousa, A., Pinto, A., Azenha, M., Teixeira, J., Azevedo, R.A., Fidalgo, F., 2016. Effect of 24-epibrassinolide on ROS content, antioxidant system, lipid peroxidation and Ni uptake in *Solanum nigrum* L. under Ni stress. *Environ. Exp. Bot.* 122, 115-125. doi:http://dx.doi.org/10.1016/j.envexpbot.2015.09.010
45. Söffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish – a critical review on effects and possible consequences. *Critical Reviews in Toxicology* 42, 653-668. doi:https://doi.org/10.3109/10408444.2012.692114
46. Solomon, G.M., Schettler, T., 2000. Environment and health: 6. Endocrine disruption and potential human health implications. *Can. Med. Assoc. J.* 163, 1471-1476.
47. Sousa, B., Lopes, J., Leal, A., Martins, M., Soares, C., Azenha, M., Fidalgo, F., Teixeira, J., 2021. Specific glutathione-S-transferases ensure an efficient detoxification of diclofenac in *Solanum lycopersicum* L. plants. *Plant Physiology and Biochemistry* 168, 263-271. doi:10.1016/j.plaphy.2021.10.019
48. Sousa, B., Lopes, J., Leal, A., Martins, M., Soares, C., Valente, I.M., Rodrigues, J.A., Fidalgo, F., Teixeira, J., 2020a. Response of *Solanum lycopersicum* L. to diclofenac - Impacts on the plant's antioxidant mechanisms. *Environ Pollut* 258, 113762. doi:10.1016/j.envpol.2019.113762
49. Sousa, B., Soares, C., Oliveira, F., Martins, M., Branco-Neves, S., Barbosa, B., Ataíde, I., Teixeira, J., Azenha, M., Azevedo, R.A., Fidalgo, F., 2020b. Foliar application of 24-epibrassinolide improves *Solanum nigrum* L. tolerance to high levels of Zn without affecting its remediation potential. *Chemosphere* 244, 125579. doi:10.1016/j.chemosphere.2019.125579
50. Taiz, L., Zeiger, E., 2010. *Plant Physiology*, Fifth ed. Sinauer Associates, Sunderland.
51. Teixeira, J., de Sousa, A., Azenha, M., Moreira, J.T., Fidalgo, F., Silva, A.F., Faria, J.L., Silva, A.M.T., 2011. *Solanum nigrum* L. weed plants as a remediation tool for metalaxyl-polluted effluents and soils. *Chemosphere* 85, 744-750. doi:10.1016/j.chemosphere.2011.06.049
52. Teixeira, J., Ferraz, P., Gouveia, C., Azevedo, F., Neves, S., Fidalgo, F., Silva, A.M.T., 2015. Targeting key metabolic points for an enhanced phytoremediation of wastewaters pre-treated by the photo-Fenton process using *Solanum nigrum* L. *Ecotox Environ Safe* 120, 124-129. doi:10.1016/j.ecoenv.2015.05.035

53. Yang, X.H., Xu, Z.H., Xue, H.W., 2005. Arabidopsis Membrane Steroid Binding Protein 1 is involved in inhibition of cell elongation. *Plant Cell* 17, 116-131. doi:10.1105/tpc.104.028381
54. Yannarelli, G.G., Fernández-Alvarez, A.J., Santa-Cruz, D.M., Tomaro, M.L., 2007. Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. *Phytochemistry* 68, 505-512. doi:http://dx.doi.org/10.1016/j.phytochem.2006.11.016
55. Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Länge, R., 2009. Effects of synthetic gestagens on fish reproduction. *Environmental Toxicology and Chemistry* 28, 2663-2670. doi:https://doi.org/10.1897/08-485.1

Figures

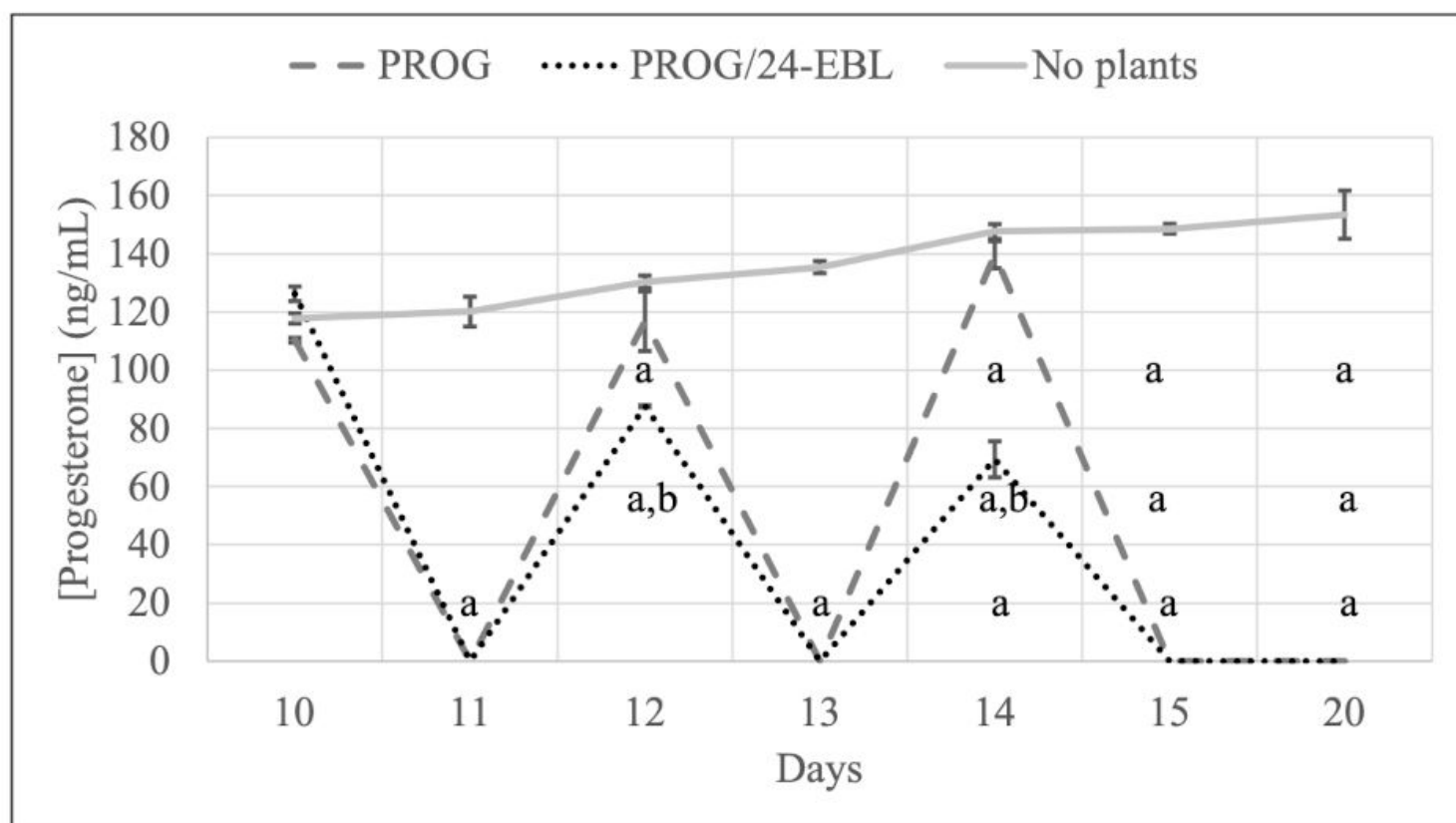


Figure 1

Progesterone levels quantified in the nutrient solutions added to the plants. In days 10, 12 and 14 aliquots were retrieved 20 minutes after the addition of the PROG-containing nutrient solution. In days 11, 13 and 15 aliquots were obtained before the addition of a fresh PROG-containing nutrient solution. PROG - plants exposed to 0.8 μ M PROG; PROG/24-EBL - plants exposed to 0.8 μ M PROG but pre-treated with a foliar application of 1 μ M 24-epibrassinolide; "No plants" – root support (vermiculite:perlite) watered with HS containing 0.8 μ M PROG. Data presented are mean $\pm$ SE ($n \geq 3$). ^aSignificant statistical difference from "No plants" at $p < 0.05$. ^bSignificant statistical difference from PROG at $p < 0.05$.

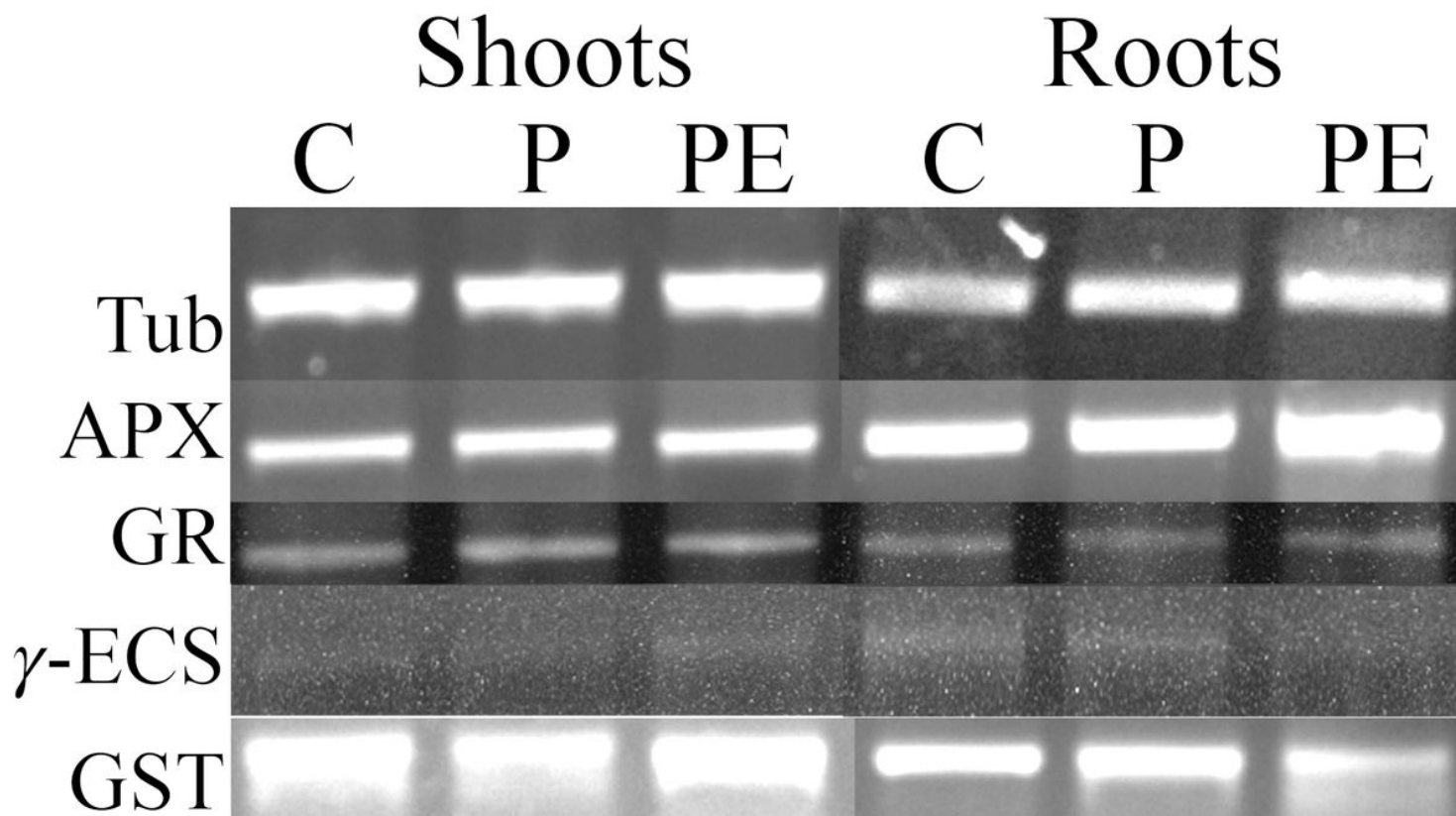


Figure 2

Typical transcript accumulation patterns for the APX-, GR-, γ -ECS- (GSH1) and GST-encoding genes in shoots and roots of *S. nigrum* plants, analysed by semi-quantitative RT-PCR followed by 0.8% (w/v) agarose gel electrophoresis. Tub - tubulin; C - control; P - 0.8 μ M progesterone; PE - 0.8 μ M progesterone + 1 μ M 24-epibrassinolide.