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# Modulation of class III peroxidase pathways and phenylpropanoids in *Arundo donax* under salt and phosphorus stress

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**Abstract**

*Arundo donax* L. is an invasive species that has been recently employed for biomass production due to its well-known ability to colonize harsh environment. Based on previous observations, the present study investigated the potential role of phenylpropanoids and class III peroxidases to confer adaptation through biochemical and transcriptomic analysis in *A. donax* after $Na^+$ and P excess supply, both in single stress and in combination, and after growth at low P level.

The levels of hydrogen peroxide, flavonoids (i.e., quercetin, apigenin and kaempferol derivatives) and the activity of class III peroxidases, as well as the expression of several genes encoding for their enzymes involved in their biosynthesis, increased when $Na^+$ was supplied in combination with P. These results suggest that those biomolecules are involved in the response of *A. donax*, to the presence of +Na and P in the soil. Moreover, even though at the sampling time no significant accumulation of lignin has been determined, the trend of accumulation of such metabolite and most of all the increase of several transcripts involved in its synthesis was found. This work for the first time indicates the need for further investigation devoted to elucidating whether the strengthening of cell walls *via* lignin synthesis is one of the mechanisms used by *A. donax* to adapt to harsh environments.

**Keywords**

*Arundo donax*, stress tolerance, salt stress, phosphorous stress, peroxidase, transcriptome

## 1. Introduction

Soil salinity affects more than 1 billion of hectares worldwide and this global threat is gradually increasing. Soil salinization reduces soil quality and limits plant growth thus leading to the abandonment of agricultural soils. Salinity stress, as excess of sodium ($Na^+$), affects plant development and reproduction by reducing water uptake from soil, and induces to both ion toxicity and nutrient deficiency through the induction of osmotic and oxidative stress (Siddiqui et al., 2008; Almeida Machado and Serralheiro, 2017).

Even though many researches have been performed to elucidate salinity stress, many questions should be answered yet (Isayenkov and Maathuis, 2019). When exposed to salinity stress, plants employ multiple sensors and signalling mechanisms to face the stress, of which most are closely related to $Ca^{2+}$ sensing and signalling, Na displaces $Ca^{2+}$ from membranes, which also increases intracellular Na (Rahman et al., 2016). Root cytosolic $Ca^{2+}$ generally increases after few seconds from salt exposure and $Ca^{2+}$ signalling is triggered . Several response mechanisms are then coordinated by $Ca^{2+}$ signalling, such as osmoprotectant accumulation, increase of the antioxidant enzyme activities and others (Seifikalhor et al. 2019). Moreover, salinity stress significantly reduces phosphorus (P) uptake in plants because phosphate ions precipitate with calcium ($Ca^{2+}$) ions (Grattan and Grieve, 1998).

Therefore, high salinity and low levels of P are concomitant in calcareous and alkaline soils typical of Mediterranean area (Zribi et al., 2012). When a P excess occurs in soil, this element becomes toxic, thus causing eutrophication (Smith and Schindler, 2009) and reducing plant performances by interfering with the biosynthesis of starch (Cocozza et al., 2020), secondary metabolites (e.g. isoprenoids) (Cocozza et al., 2020), nitrate assimilation, and plant's nutrients absorption (Crouse, 2018). A direct result of these effects is the enhanced accumulation of reactive oxygen species (ROS) which can have a role both as signalling molecules (Foyer, 2018) and cause oxidative stress leading to programmed cell death (Van Breusegem and Dat, 2006). As direct consequence of ROS accumulation, plant metabolism is directed

toward the activation of a complex antioxidant machinery including enzymatic and non-enzymatic compounds. Among the non-enzymatic compounds, the biosynthesis of specific secondary metabolites, such as ascorbic acid and phenylpropanoids, in particular flavonoids which are rapidly activated in response to changes in redox state of the cell (Deng and Fu, 2017; Sharma et al., 2019).

The cultivation of marginal lands, characterized by edaphic and/or climatic limitations, using tolerant crops has been proposed to reverse non-sustainable management and land degradation (Schröder et al., 2018; Pulighe et al., 2019). Mediterranean marginal lands affected by salinity could be exploited by promising and valuable energy crop, such as *Arundo donax* L. (Pompeiano et al., 2017). The giant reed (*A. donax*) is one of the most promising among non-food and bioenergy crops that represent valid solutions for land recovery (Corno et al., 2014). It is a perennial and herbaceous plant, occurring over a wide range of climatic habitats and growing spontaneously and abundantly all over the Mediterranean region (Sánchez et al., 2016), due to its high performance. Despite being a C3 plant, *A. donax* shows very high photosynthetic rate (37 μmol $m^{-2}$ $s^{-1}$) and productivity like C4 species, with an average biomass yield ranging between 21 and 51 t $ha^{-1}$ under medium-low supply of nitrogen and water (Angelini et al., 2009). *A. donax* is known to be growing in all types of soil, from clay to sand, with presence of rocks or not, with soil pH ranging from 5 to 8.7, and in conditions of high salinity (Nackley and Kim, 2015; De Stefano et al., 2017).

Several investigations have been conducted to elucidate the mechanisms underlying the ability of *A. donax* to colonize harsh environments. The transcriptional response of *A. donax* to long-term salt stress was elucidated by Sicilia et al. (2020). Furthermore, Docimo et al. (2020) observed increased proline, abscisic acid (ABA) and leaf antioxidants and up-regulation of antioxidant genes in different genotypes of *A. donax* exposed to salinity stress. Negative effects on the physiological parameters and increase of

hydrogen peroxide and secondary compounds of phenylpropanoids family such as flavonoids, have been reported in *A. donax* under salinity stress (Cocozza et al 2019; 2020).

Flavonoids are mainly located in the vacuoles where they may scavenge high doses of $H_2O_2$ by forming a co-operative regenerating cycle with ascorbic acid and vacuolar peroxidase (Takahama, 2004).

Class III peroxidases are secretory enzymes which play role in a broad range of physiological and developmental processes (Lüthje and Martinez-Cortes, 2018). Peroxidases are responsible either for cell elongation or cell wall stiffening, affecting carbon allocation, auxin level and redox homeostasis, which implicates their key role as being in the regulation of growth and defense under stress condition (Kidwai et al., 2020) and salinity tolerance (Su et al., 2020). Class III peroxidases act through three different cycles, namely peroxidative cycle, oxidative and hydroxylic cycles. In their main peroxidative cycle, class III plant peroxidases act as antioxidant molecules involved in detoxification or generation of hydrogen peroxide ($H_2O_2$), catalysing the reduction of $H_2O_2$ by taking electrons from various donor molecules such as phenolic compounds, lignin precursors (i.e. caffeic acid), auxin, or secondary metabolites (Ferreres et al., 2011; Lüthje and Martinez-Cortes, 2018). Moreover, their role in the plant lignification has also been reported (Almagro et al., 2009; Marjamaa et al., 2009).

Ferreres et al. (2011), suggested that $H_2O_2$ may be scavenged by peroxidases using flavonols as substrates, and then flavonol radicals are recycled back by ascorbic acid to their reduced forms.

In this work to gain more information about the mechanisms used by *A. donax* to face harsh environments, the modulation of antioxidant scavengers, class III peroxidases and phenylpropanoids, and their encoding genes were determined in *A. donax* after $Na^+$ and P excess supply, both in single stress and in combination, and after growth at low P level. The long-term experimental treatment (43 days) allowed to elucidate how class III peroxidases and phenylpropanoids, as well as their potential substrates act into the mechanisms conferring plasticity to environmental stress to *A. donax.*

## 2. Materials and Methods

### *2.1. Plant material and growth conditions*

*Arundo donax* (L.) plants were obtained by rhizomes collected in Sesto Fiorentino (Italy). Rhizomes were hydrated in tap water for one day and then planted in 6 L pots in quartz sand. The experiment was carried out in a conditioned climatic chamber, where temperature ranged between 24°C and 26°C, relative air humidity between 40% and 60%, photosynthetic photon flux density (PPFD) was 700 μmol $m^{-2}$ $s^{-1}$ for 14 h per day. Plants were watered twice per week with half strength Hoagland solution for two months before the beginning of the experiment. The experiment was performed for 43 days by applying four different nutrient conditions: Hoagland solution (control); Hoagland solution deprived of phosphorus (-P); Hoagland solution complemented with 8.0 mM $KH_2PO_4$ (+P), very high phosphorus levels, as commonly observed in water (Neal et al., 2003); Hoagland solution complemented with 200 mM NaCl (+Na), by considering that 100 mM NaCl in Hoagland solution causes electrical conductivity of 13 dS $m^{-1}$ (Carrasco-Ríos et al. 2013); and Hoagland solution complemented with both 200 mM NaCl and 8.0 mM $KH_2PO_4$ (+NaP). All different nutrient conditions were supplied twice a week by causing an "acute" stress to plants, as found in Cocozza et al. (2020). Four replicates (plants) per treatments were sampled for the following analyses. Fully expanded young leaves of *A. donax* plants were sampled after 43 days of treatment (stress point, St), and after a recovery period, after stopping the treatments, lasting 14 days (Re). In the companion study, plant phenotyping (biometrics and gas-exchange) of *A. donax* in response to Na and P high supply dropped at 43 days and it was restored after 14 days of suspension of stress conditions (Cocozza et al. 2020).

### *2.2 Determination of hydrogen peroxide and ascorbic acid and dehydroascorbate*

Endogenous $H_2O_2$ content was determined according to the method of Velikova et al. (2000), with slight modifications. Briefly, leaves (0.25 g) were ground to a powder by mortar and pestle in liquid nitrogen and then, homogenized with 3 mL of 5 % (w/v) trichloroacetic acid (TCA) at 4 °C. The homogenate was centrifuged at 12,000g for 15 min. To 0.5 mL aliquot of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 0.75 ml of 1 M KI were added, and the absorbance was measured at 390 nm (EasySpec UV-Vis spectrophotometer, SAFAS). The relative absorbance (sample absorbance minus the absorbance of the same supernatant aliquot without KI) was used to determine the $H_2O_2$ content against a $H_2O_2$ standard curve. Data were expressed as µmol $g^{-1}$ DW.

Ascorbic acid (AS) and dehydroascorbate (DHA) were quantified following the method of Law et al., (1983). Briefly, leaves (0.5 g) were crushed with liquid nitrogen and then homogenized in 400 µL of 10% (w/v) trichloroacetic acid (TCA). After vortexing, the homogenate was kept in ice for 5 min and 10 µL of 5M NaOH solution was added. Then, samples were vortexed and centrifuged for 2 min at room temperature (RT). Two hundred microliters of 150 mM-$NaH_2PO_4$ buffer (pH 7.4) and 200 µL of distilled water were added to 200µL of homogenated sample. To another homogenate sample aliquot (200µL), 200 µL of 150 mM-$NaH_2PO_4$ buffer and 100µL of 10 mM-dithiothreitol were added. The solution was vortex-mixed and incubated at RT for 15 min, then 100 µL of 0.5 (w/v) N-ethylmaleimide was added. Both samples were vortex-mixed, incubated for 2 min at RT, and then 400 µL of 10% (w/v) TCA, 400 µL of 44% (w/v) $H_3PO_4$, 400 µL of bypiridyl in 70% (v/v) ethanol and 200 µL of 3% (w/v) $FeCl_3$ were added. Samples were vortex-mixing and incubated at 37°C for 60 min. The absorbance was measured at 525 nm and a standard curve with AS and DHA was used for the calibration. AS and DHA content was expressed as µg $g^{-1}$.

*2.3 Class III peroxidase assay*

Stored leaves samples at -80°C were grounded to a powder by mortar and pestle in liquid nitrogen. Then, 100 mg of leaf samples were solubilised in 500 µl of 50 mM Tris-HCl pH 7.5 and 1% (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS) for enzyme assay for 2h and then centrifuged at 14 g for 30 min at 4°C. The protein concentration was determined by using Bradford assay (Bradford, 1976) using bovine serum albumin to generate a standard curve.

The activity of class III peroxidases of *A. donax* (AdPrx) was determined using three different substrates, guaiacol, syringaldazine and coniferyl alcohol, as described in Cesarino et al. (2012), and adding to the assay solution a volume of leaf homogenate corresponding to 10µg of protein.

The activity of AdPrx using guaiacol was determined measuring the increase of absorbance at 470 nm for 2 min relative to guaiacol oxidation to tetraguaiacol ($\varepsilon$ = 26.6 m $M^{-1}$ $cm^{-1}$). The assay (1 mL) contained the leaf homogenate, 8.26 mM guaiacol and 0.03% $H_2O_2$ (v/v) in 50 mM Na-acetate buffer (pH 5.0) and the guaiacol activity was expressed as µmol tetraguaiacol $min^{-1}$ $mg^{-1}$ total protein.

The AdPrx activity using syringaldazine as substrate, was measured by following the increase of absorbance at 530 nm ($\varepsilon$ = 27.1 $mM^{-1}cm^{-1}$) for 2 min in 1 mL reaction mixture containing the leaf homogenate, 50 mM of syringaldazine and 0.03% $H_2O_2$ in 20 mM Tris HCl buffer (pH 7.5). Syr activity was expressed as µmol SYR oxidized $min^{-1}$ $mg^{-1}$ total protein.

The AdPrx activity when coniferyl alcohol was used as substrate was determined by following the decrease of absorbance at 262 nm ($\varepsilon$= 9.6 $mM^{-1}cm^{-1}$) for 2 min in 1 mL reaction mixture containing the leaf homogenate, 50 mM coniferyl alcohol and 0.03% $H_2O_2$ in 50 mM Na-acetate buffer (pH 5.0). Ca activity was expressed as µmol coniferyl alcohol oxidized $min^{-1}$ $mg^{-1}$ total protein.

*2.4 Quantification of flavonoids*

Individual phenylpropanoids were analysed following the protocol previously reported in Cocozza et al., 2020. In brief, 300 mg of leaf tissue was grounded with liquid nitrogen, placed in a centrifuge tube and

extracted three times with 4 mL of 75% EtOH/$H_2O$ adjusted to pH 2.5 with formic acid. The supernatant was partitioned with *n*-hexane to remove chlorophyll and carotenoids, reduced to dryness, and finally rinsed with 2 mL of $CH_3OH/H_2O$ (8:2). The extracts were injected (5 μL) into a Perkin Elmer liquid chromatograph equipped with a quaternary 200Q/410 pump and a LC 200 diode array detector (DAD) (all from Perkin Elmer, Bradford, CT, USA). Phenylpropanoids were separated using a 250 x 4.6 mm Zorbax SB-C18 column (5 μm) operating at 40 °C with a flow rate of 1 mL $min^{-1}$. The mobile phases were (A) $H_2O/CH_3CN$ (9:1, added with 0.1% of HCOOH) and (B) $CH_3CN/ H_2O$ (9:1, added with $CH_3CN$ 0.1% of HCOOH). Phenylpropanoids were separated using a linear gradient elution from A to B over a 45 min run and chromatograms recorded at 330 nm and 350 nm. Individual phenylpropanoids were identified using retention times and comparison of UV spectral characteristics of those of authentic standards (caffeic acid, apigenin-7-*O*-glucoside, quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside, all from Extrasynthese, Lyon-Nord, Genay, France). The phenylpropanoids consisted of three apigenin derivatives, one quercetin derivative and three kaempferol derivatives, all quantified with the external standard method and reported as mg $g^{-1}$ DW.



*2.5 Quantification of total lignin content*

Lignin content was determined using a protocol based on the extraction of lignothioglycolic acid with minor modifications (Trupiano et al., 2012). Analysis of lignin content in leaves of *Arundo* was performed to obtain preliminary data in salt experiment, given that literature is missing and leaves, as well as stems, define the characterization of the *Arundo* saccharification efficiency in bioenergy crop species. Briefly, 0.5 g of lyophilized leaf tissue was pulverized and homogenized in 10 mL of extraction buffer (50 mM Tris–HCl, 0.01% Triton X-100, 1 M NaCl, pH 8.3). The suspension was stirred, centrifuged at 10000 g for 10 min, washed twice with 4 mL of extraction buffer, 4 mL of 80% (v/v)

acetone, 4 mL of acetone and then dried in a concentrator. Each pellet was then treated with 0.2 ml of thioglycolic acid and 1 mL of 2 M HCl, for 4 h, at 100 °C, centrifuged at 15000 g for 10 min and washed three times with distilled water. Lignothioglycolic acid from each pellet was then extracted with 1.5 mL of 0.5 M NaOH, under stirring for 16 h, at 25 °C. The supernatant was acidified with 0.2 mL of 37% (v/v) HCl. Lignothioglycolic acid was precipitated at 4°C, for 4 h, recovered by centrifugation at 15000 g for 15 min, and dissolved in 1 mL of 0.5 M NaOH. Lignin content was obtained by absorbance measurement at 280 nm, using a specific absorbance coefficient of 6.0 $g^{-1}$ L cm (Doster and Bostock, 1988).

*2.6 RNA extraction, sequencing and differential expression analysis*

RNA extraction was performed using TRIzol® Reagent (Ambion). A total of 15 RNA samples, 3 biological replicates for each of the 5 treatments collected at 43 days of treatment /stress point, were sent to HuGeF sequencing service (http://www.hugef-torino.org, Human Genetics Foundation, Turin, Italy), for paired-end libraries (2 x 75bp) construction and sequencing with Illumina NextSeq 500. Raw data have been deposited in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) with SRA accession SRP145569. After low-quality filtering and adapters removal, *de novo* transcriptome assembly was performed through Trinity software (version 2.0.6). A total of 184,849 transcripts were assembled, reduced to 120,553 after redundancy removal. Analysis of differential expression of transcripts was performed with edgeR (version 3.16.5, R version 3.3.2), using a false discovery rate cut off 0.05 (5% FDR). The differential expression is reported as fold change (FC), i.e. the ratio between the expression value in the treatment and the expression value in the control of a transcript. A detailed description of the protocol can be found in Cocozza et al. (2019).

*2.7 Statistical analyses*

Descriptive statistics (means, standard errors) of all the measured parameters were calculated. To account for variability in the samples, a pooled standard deviation was computed from the four replicated plants from each experimental condition. Normality of the population distribution was tested using the Shapiro–Wilk test. The homogeneity of variances was performed with the Levene's test. One-way ANOVA with post-hoc Tukey HSD Test was applied to test the effect of Na+ and P supply in *A. donax* plants, and two-way ANOVA was performed to define the effects of treatments in the experimental points, stress point and recovery. An LSD post-hoc test was applied to assess significantly different means among treatments ($P < 0.05$ level). Statistical analyses were performed using OriginPro 8 program (OriginLab Corporation, Northampton, UK). For transcriptomic analysis, the false discovery rate (FDR) represents an adjusted p-value, obtained applying a multiple testing correction (method of Benjamini and Hockberg, 1995). Indeed, when a large number of genes/transcripts are tested, a certain number of false positives is expected. In these cases, the FDR is used to lower the number of false positives. The FDR calculation is done by edgeR analysis according to Benjamini and Hockberg (1995). An FDR under 0.05 is usually used and means that we accept that 5 out of 100 measures (differential expression) can be false positive. The lower the FDR, the lower is the probability that the differential expression observed is a false positive.



## 3. Results

### *3.1 Hydrogen peroxide, ascorbic acid and dehydroascorbate content in* A. donax *under stress conditions*

The $H_2O_2$ concentration was increased of 2-fold by +NaP respect to the levels measured in control plant. Under recovery, the $H_2O_2$ concentration decreased in plants deprived of P respect to the levels measured in the other stress conditions and in control plants (Fig. 1).

Ascorbate (AS) and dehydroascorbate (DHA) concentrations did not changes among the treatments (-P, +P, +Na, +NaP) after 43 days (stress point, St) or after 14 days from the end of treatments (Recovery, Re) (Table S1).

*3.2 Content of phenylpropanoids and related biosynthetic pathway in* A. donax *under stress conditions*

At the end of stress, the apigenin derivatives increased in plants treated with +NaP, and decreased in –P and +P treatments compared to control plants (Fig. 2A). After recovery, the apigenin derivatives significantly increased in +NaP and –P plants, whereas +P plants showed lower content of these metabolites compared to control ones (Fig. 2A). The quercetin derivatives increased in Na+ and +NaP plants at the end of stress treatment and after recovery. No significant change was induced by the other treatments (Fig. 2B). The content of kaempferol derivatives was significantly increased only in +NaP plants , both at stress and recovery, while in –P plants an increment in these metabolites was observed only at recovery (Fig. 2C). Transcriptomic analysis showed the upregulation of the transcripts of the gene coding for the chalcone synthase (isoforms TR30713|c0_g1_i2 and TR30713|c0_g1_i3, FC=12.1 and FC=8.2, respectively), the first enzyme of the flavonoid biosynthetic pathway in +NaP plants.

No increased or decreased expression of the genes related to flavonoid biosynthesis was found in –P, +P or +Na treatment with respect to C plants (Fig. S1; Table 1).

In plants treated with +NaP the transcripts of genes involved in caffeic acid synthesis and its derivatives, i.e. the trans-cinnamate 4-monooxygenase (TR12947|c1_g1_i1 FC=3.8, TR22013|c1_g1_i1 FC=2.9, and TR22013|c1_g1_i3 FC=4.9) and shikimate O-hydroxycinnamoyl transferase (TR5007|c0_g1_i1 FC=2.8, TR3575|c0_g1_i1 FC=3.9), were up-regulated. No increased or decreased expression of these genes was observed in –P, +P or +Na treatment. Transcripts coding for Chalcone-flavanone isomerase (TR14275|c1_g1_i4 FC=3.9, TR15117|c1_g1_i2 FC=9.2 and TR15117|c1_g1_i3 FC=7.9), involved in the synthesis of apigenin, were also up-regulated in +NaP treatments. Transcripts coding for the

anthocyanidin 3-O-glucosyltransferase 2, involved in apigenin derivatives, were up-regulated in +NaP (five transcripts with FC between 4.2 and 12.6), +Na (TR136|c4_g3_i3 FC=2.9) and –P (TR12985|c2_g1_i1 FC=6.8) treatments. The transcriptomic data agreed with the levels of apigenin measured in +NaP plants, whereas there was no correlation between the induction of the anthocyanidin 3-O-glucosyltransferase 2 transcript (Table 1) and the decrease of apigenin derivatives measured in –P plants (Fig. 2B).

A transcript coding for a flavonoid 3–hydroxylase, one of the enzymes involved in the synthesis of quercetine, as well as one coding for a flavonol sulfotransferase, involved in quercetine derivatives synthesis, were up-regulated in +NaP treatment (TR18107|c0_g2_i1 FC=11.2 and TR24551|c3_g2_i3 FC=7.7, respectively) (Fig. S1). The same flavonol sulfotransferase transcript was induced in +Na treatment (FC=4.3) (Table 1). On the other hand, one other transcript coding for flavonol sulfotransferase and two isoforms of a transcript coding for an anthocyanidin 5,3-O-glucosyltransferase, also involved in the biosynthesis of quercetine derivatives, were down-regulated in +NaP (TR24226|c1_g1_i1 FC=0.1, TR5705|c4_g3_i2 FC=0.25, TR5705|c4_g3_i3 FC=0.07) (Table 1). No up- or down-regulation was observed in –P or +P with respect to C plants (Table 1).

In +NaP treatment, the transcripts coding for enzymes involved in the synthesis of kaempferol and its derivatives, i.e. flavonol synthase flavanone 3-hydroxylase-like and anthocyanidin 5,3-O-glucosyltransferase, were either up-regulated (TR296|c0_g3_i1 FC=34.5) or down-regulated (five transcripts with FC between 0.1 and 0.5), respectively. No up- or down- regulation of these transcripts was measured in the other treatments (Table 1).

*3.3 Peroxidase activities and related transcript content in* A. donax *under stress conditions*

The activity of *Arundo* peroxidases when coniferyl alcohol (Fig. 3A) or siryngaldazine (Fig. 3B) were used as substrates increased in the plants exposed to high concentrations of Na+ and +P, both in single stress and in combination. The lower increment was observed in the plants deprived of P (-P plants), whereas the same plants presented high levels of Prx at recovery when siryngaldazine was provided. The activity of peroxidases in presence of guaiacol increased quite 2-fold in +NaP plants respect to the control at the end of treatment, and more moderately as consequence of all the other treatments (Fig. 3C). At recovery, increased activity was also observed in all treatments compared to control plants, with the highest values recorded in -P plants.

Transcriptomic analysis showed that in +NaP treatment, four peroxidase encoding genes were up-regulated, and 8 were down-regulated (Table 2). Among these, four transcripts were of particular interest: peroxidase 17, known to be involved in lignin deposition (Cosio et al., 2016), was up-regulated (isoforms TR19645|c0_g1_i5 FC=2.4 and TR19645|c0_g1_i6 FC=2.7), while peroxidase P7-like (TR482|c0_g1_i4) and peroxidase 66 (TR5652|c2_g2_i1), involved in the deposition of syringal monomers (GO:1901430, positive regulation of syringal lignin biosynthetic process), were down-regulated (FC=0.14 and FC=0.12, respectively) (Table 1). A transcript coding for a peroxidase 5-like was down-regulated in –P and in +P (TR23604|c5_g1_i4 FC=0.08, in both) treated plants. In +Na treated plants, no differential expression among peroxidase genes was found (Table 2).

*3.4 Lignin content and related biosynthetic pathway in* A. donax *under stress conditions*

Total lignin content in leaves did not change significantly among plant treatments, even though a tendence through lignin accumulation was evident when *A. donax* in all the treatment respect to the control (Table S2). Data obtained from transcriptomic analysis showed that the expression of several genes encoding the enzymes involved in the lignin biosynthetic pathway, part of the phenylpropanoid

biosynthetic pathway, were induced by the +NaP treatment, while slightly impaired by the other treatments (Fig. S2; Table S3) respect to control. In detail, in +NaP plants, the transcripts of phenylalanine ammonia-lyase, the first enzyme of the lignin biosynthetic pathway, was down-regulated (TR3072|c2_g2_i4 FC=0.19) (Fig. S2, Table S4). Among 6 transcripts coding for 4-coumarate-CoA ligase, two were down-regulated (TR19035|c0_g1_i1 FC=0.24 and TR3027|c2_g2_i1 FC=0.25) and four were up-regulated (TR22604|c3_g2_i1 FC=4.2, TR5718|c1_g1_i5 FC=3.1, TR7497|c3_g2_i3 FC=6.7 and TR7497|c3_g2_i7 FC=4.2). Three transcripts for cinnamoyl-CoA reductase, the first key enzyme for monolignol biosynthesis, were up-regulated (TR6509|c1_g1_i1 FC=4.3, TR6509|c1_g1_i6 FC=2.7 and TR9597|c1_g4_i1 FC=2.3) and 2 transcripts codifying for caffeoyl-O-methyltransferase 1 show opposite regulation (TR18821|c1_g3_i8 FC=0.07 and TR30139|c0_g1_i3 FC=7.1); 2 transcripts codifying for caffeoylshikimate esterase were down-regulated (TR1036|c1_g1_i10 FC=0.11 and TR11203|c3_g1_i3 FC=0.35) and one was up-regulated (TR1036|c1_g1_i12 FC=2.41). Finally, 4 transcripts for laccase genes, involved in the polymerization of monolignol subunit, were up-regulated (TR18961|c2_g1_i1 FC=2.9, TR18961|c2_g1_i4 FC=2.2, TR6554|c0_g1_i2 FC=41.3 and TR7969|c1_g1_i3 FC=37.8) and 2 were down-regulated (TR6546|c1_g1_i2 FC=0.15 and TR6546|c1_g1_i3 FC=0.08) (Fig. S2, Table S4). In +Na treated plants, transcript coding for cinnamoyl-CoA reductase 1-like (TR6509|c1_g1_i7 FC=3) and for cinnamyl alcohol dehydrogenase (TR24767|c0_g1_i4 FC=7.6), involved in the last step of monolignol biosynthesis, were up-regulated, while a laccase gene (TR6546|c1_g1_i3 FC=0.09) was down-regulated (Fig. S2, Table S4). In -P treated plants, a cinnamoyl-CoA reductase, a caffeoylshikimate esterase were down-regulated (TR9597|c1_g4_i1 FC=0.3, TR11203|c3_g1_i6 FC=0.44, TR23604|c5_g1_i4 FC=0.08, respectively). One transcript codifying for a peroxidase (TR23604|c5_g1_i4 FC=0.08) which was down-regulated both in -P and +P plants (Table S4).

## 4. Discussion

*Arundo donax* is known as a moderately sensitive species in response to high NaCl concentrations by maintaining relative growth (Nackley and Kim, 2015) and preserving the photosynthetic efficiency of photosystem II (Pompeiano et al. 2017); therefore as a stress resistant plant, it is prone to be used to restoring salt-affected marginal areas. However, the combination of +Na and P impaired *A. donax* photosynthesis (Cocozza et al., 2019, 2020), and such imbalance might be the reason of the observed increment in ROS (i.e. $H_2O_2$) levels (Fig. 1). In particular, $H_2O_2$, diffusing across biological membranes, can be harmful for plant membranes and organelles (Groβ et al., 2013), however it can also play a role as secondary messenger (Foyer and Noctor, 2003). Therefore, a fine-tuning regulation of its levels is of relevance for cell adaptation to stress condition. Accumulated evidence indicated that a increase in ROS levels is generally overcomed by plants using enzymatic and non-enzymatic compounds (Gupta et al., 2018). In this work, no increase of ascorbic acid has been found in *A. donax* treated with +Na and P in any of the imposed experimental condition (Fig S1), therefore we hypothesized the presence of other scavenging mechanisms used by *A. donax* to minimize deleterious effects of intracellular generation of $H_2O_2$. Among them, one mechanism could be the involvement of secondary compounds belonging to the phenylpropanoid pathway. Indeed, in a previous investigation, high levels of caffeic acid derivatives were reported in plants treated with +Na and P (Cocozza et al. 2019), suggesting their potential role in regulating and supporting the acclimatation of *A. donax* to salinity stress (Isah, 2019). The over-expression of stress response genes has been observed in *Stevia rebaudiana* (Azzam et al. 2021) under salinity stress and considered a common adaptive response of plants such as *A. donax* capable to face harsh environments. Despite those genes are part of multigenic families and RNA-seq cannot identify what gene is exactly involved, the observed change might be correlated with the accumulation of leaf phenylpropanoids (quercetin, apigenin, kaempferol derivatives, Fig. S3) in +Na and P treated plants. However, differential expression of several genes encoding the enzymes involved in the synthesis of phenylpropanoid (Fig. S3 and Table 1) has been observed above all in the plants exposed to a

combination of +Na and P and two genes, cinnamoyl-CoA reductase cinnamyl alcohol dehydrogenase also after +Na excess. Notably, P limitation frequently leads to upregulated expression of genes and accumulation of secondary metabolites synthesized in the phenylpropanoid pathway (Alexova et al., 2017; Muller et al., 2015). However, since P deprivation (-P) affects only slightly photosynthetic efficiency in *Arundo donax* (Cocozza et al., 2019), our findings may suggest that excess or deprivation of P do not stimulate differential changes in the biosynthesis of phenylpropanoids. Indeed, phosphorous deficiency does not always elicit an increase in leaf phenylpropanoid content (Stewart et al., 2001; Lillo et al., 2007) or that the lack of response could be due to a shift between the expression time and sampling time (Morcuende et al., 2006).

Quercetin derivatives, which possess a higher antioxidant activity than kaempferol derivatives (Nakabayashi et al., 2014), might play a direct ROS scavenging activity in *A. donax* exposed only to excess of salinity (Na+) as a specific increment of these compounds under Na+ treatment was found in *A. donax*, in accordance with the suggested role of this molecule against photo-oxidative damages and its distribution in mesophyll cells (Agati et al., 2011). Furthermore, besides the well-studied role of flavonoids as effective antioxidants, they are also considered natural regulators of auxin gradients (by inhibiting polar auxin transport), local auxin concentrations (inhibiting peroxidase-mediated IAA oxidation), and thus could be also potentially involved in the modulation of "stress-induced morphogenic responses" and in the regulation of cell expansion (Agati et al., 2012).

On the other hand, phenylpropanoids might be used to scavenge oxidative stress by the activity of class III peroxidases. Plant peroxidases have been used as biochemical markers of stress due to their important role in lignification, catabolism of auxin and ROS scavenging. In 2011, Ferreres et al. proposed that the vacuolar class III peroxidases were involved in the homeostasis of $H_2O_2$ cellular concentrations, using secondary organic compounds as substrates. Peroxidase levels and activity are not always correlated, as enzyme activity can be stimulated by posttranslational modification (Ryšlavá et al., 2013). However, the

increased expression of several peroxidase encoding genes in the plants exposed to +Na and P might reinforce our hypothesis of a possible involvement of class III peroxidases in *A. donax* adaptation to the salinity stress. Moreover increased activities of POX have been observed in our samples ancd it is in accordance to the induced POX activity reported by Azzam et al. (2021) in white clover under salinity stress. In the cell, class III peroxidases have rather wide range of substrate spectrum and POX activity is often determined using an artificial electron donor (Jovanoniv et al. 2018). Therefore, in this work three different substrates were used to measure the differential POX activity in *A donax*. Taking into account that protein extract is a mixture of isoenzymes, this estimation does not give information about what specific POX isoform was involved (Jovanovic et al., 2018), which would be out of the goal of our investigation, but only information that POX could use the phenylpropanoid compound similar to the specific used substrate.

The increased activity of class III peroxidases (AdPrx) when guaiacol (Fig. 3) was used as substrate in *A. donax* under combined exposition to +Na and P (+NaP), might suggest the involvement of these enzymes in scavenging $H_2O_2$ utilizing glycosylated quercetin derivatives, which structure is similar to that of guaiacol, as substrates (Ferreres et al., 2011). Indeed, previous *in vitro* and *in vivo* studies demonstrated that these forms also act as substrates of class III plant peroxidases in addition to the aglycone (Rácz et al., 2008). However, in *A. donax* experienced with P starvation or excess, the increase of AdPrx activity was also determined without accumulation of apigenin, quercetin and kaemferol derivatives and $H_2O_2$ when guaiacol and syringaldazine were used as substrates. Nevertheless, in those treatments, high levels of caffeic acid derivatives, which structure reminds to that of guaiacol, were found (Cocozza et al., 2019). Indeed, caffeic acid derivatives (e.g. chlorogenic acid) have been found to act as optimal substrate for POX (Takahama et al., 1999).

*A. donax* has been proposed among the non-food crop for energy and biofuel production. Nevertheless the presence of lignin is still a major bottleneck for the utilization of plant biomass as a source for biofuels

and bio-based materials (Vanholme et al., 2010). Lignin adversely impacts biomass enzymatic hydrolysis resulting in an increased dose of cellulases, which accounts for almost half of bioethanol cost (Luterbacher et al., 2014) and reviewed by Yao et al. (2022). Despite lignification is a phenomenon mostly occurring in stems and roots, increase of the peroxidase activity and leaf lignification have been reported in white clover under drought stress (Lee et al., 2007). Moreover correlation between POX activity and lignification in needles of Norway spruce has been reported (Polle et al., 1994). Therefore, we also explored the possibility that the observed increase of the flavonoid levels and POX activities might drive with leaf lignification. Both flavonoids and lignin are important in counteracting environmental stresses due to their ability to neutralize ROS by donating electrons to hydrogen atoms (Rice-Evans et al., 1996; Kieffer et al., 2008).

Although in *A. donax* under all the treatments no significant change in lignin was observed at the sampling time, data showed a trend of lignin accumulation suggesting that enhanced leaf lignin accumulation might be occurred under the imposed stress. Alternatively, as recently reported for maize plants, *A. donax* under stress might be undergoing only subtle alterations in lignin composition without significant changes in its content (Oliveira et al., 2020).



## 5. Conclusions

Our data shed a light on the mechanism used by *A. donax* to adapt to harsh environments. The observed differential changes in the expression of several genes involved in the synthesis of phenylpropanoids and peroxidases, even if they do not indicate the changes of any specific gene suggest the involvement of the phenylpropanoids pathway in the response of *A. donax* to salinity stress. ROS (i.e., $H_2O_2$ produced by an excess of +Na in single stress and in combination with P, might be scavenged by phenylpropanoids, such as quercetin, apigenin and kaempferol both directly acting as antioxidant compounds or through the activity of class III peroxidases. Moreover, a possible accumulation of leaf lignin and consequently the

strengthening of the mechanical properties of cell wall when the plants are exposed +Na and/or P stress should be further explored. Mechanism that, on one hand, might help *A. donax* to face stressful conditions, on the other hand, might limit its potential use in energy and biofuel production. Therefore, our data underline for the first time, the need of performing further investigation to the possible role of leaf lignification in the response mechanisms of A. *donax* to adaptation to harsh environment whether its use in biomass production will be further exploited.

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**Author contributions**

Conceptualization, C.C. and B.E.M; methodology, B.E.M., P. B., C. B., L. M., S. P., A. P., D. T., S. R.; data curation, C.C., B.E.M., C. B.; writing—original draft preparation, C.C. and B.E.M.; writing—review and editing, C.C., C. B., L. M., D. T., F. B., B.E.M., G.S.S.; project administration, C.C.; funding acquisition, C.C.. All authors have read and agreed to the published version of the manuscript.

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**Captions of figures**

**Figure 1.** $H_2O_2$ content of *A. donax* plants in control conditions (C), phosphorous-deprived (-P), phosphorous-enriched (+P), sodium chloride-enriched (+Na), and phosphorous and sodium-enriched (+NaP) soil measured after 43 days by applying different solutions (St) and following a recovery period of 14 days (Re). Data are means of 4 plants per treatment ± SE; different letters indicate statistical difference between treatments at $P < 0.05$; two-way ANOVA defines the significance of effects of treatments in time, the experimental points, at $P < 0.05$.

**Fig. 2** Apigenin (A), quercetin (B) and kaempferol (C) derivatives contents of *A. donax* plants in control conditions (C), phosphorous-deprived (-P), phosphorous-enriched (+P), sodium chloride-enriched (+Na), and phosphorous and sodium-enriched (+NaP) soil measured after 43 days (St) by applying different solutions and following a recovery period of 14 days (Re). Data are means of 4 plants per treatment ± SE; different letters indicate statistical difference between treatments at $P < 0.05$; two-way ANOVA defines the significance of effects of treatments in time, the experimental points, at $P < 0.05$.

**Figure 3.** Coniferyl alcohol peroxidase (B), syringaldazine peroxidase (C) and guaiacol peroxidase (D) activity of *A. donax* plants in control conditions (C), phosphorous-deprived (-P), phosphorous-enriched (+P), sodium chloride-enriched (+Na), and phosphorous and sodium-enriched (+NaP) soil measured after 43 days by applying different solutions (St) and following a recovery period of 14 days (Re). Data are means of 4 plants per treatment ± SE; different letters indicate statistical difference between treatments at $P < 0.05$; two-way ANOVA defines the significance of effects of treatments in time, the experimental points, at $P < 0.05$.

**Captions of tables**

**Table 1.** Metabolites, class III peroxidase and genes which levels differentially changed in *Arundo donax* plants with excess supply of sodium chloride (Na2+) or phosphorus (P+), without phosphorus supply (-P), and with an excess supply of both phosphorus and sodium chloride (+NaP) respect to the control, in stress (dark arrows) and recovery (dashed arrows) conditions. Increased levels (arrows towards) and decreased levels (arrows towards down).

**Captions of supplementary materials**

**Figure S1.** Expression of genes related to flavonoid biosynthesis in +NaP (excess of salt and phosphorus) plants; dark grey: up-regulated genes, light grey: down-regulated genes; EC:1.3.1.77 anthocyanidin reductase, EC:1.14.14.82 flavonoid 3 -hydroxylase, EC:1.14.14.91 trans-cinnamate 4-monooxygenase, EC:1.14.20.6 flavonol synthase flavanone 3-hydroxylase-like, EC:2.3.1.133 shikimate O-hydroxycinnamoyltransferase, EC:2.3.1.74 chalcone synthase, EC:5.5.1.6 chalcone-flavonone isomerase.

**Figure S2**. Expression of genes related to phenylpropanoid biosynthesis in A) +NaP (excess of salt and phosphorus) plants, B) +Na (excess of salt), C) +P (excess of phosphorus), D) –P (lack of phosphorus); dark grey: up-regulated genes, light grey: down-regulated genes. EC:1.1.1.195 cinnamyl-alcohol dehydrogenase, EC:1.11.1.7 peroxidase, EC:1.14.14.91 trans-cinnamate 4-monooxygenase, EC:1.2.1.44 cinnamoyl-CoA reductase, EC:2.3.1.133 shikimate O-hydroxycinnamoyltransferase, EC:3.1.1.-caffeoylshikimate esterase, EC:3.2.1.21 beta-glucosidase, EC:4.3.1.24 phenylalanine ammonia-lyase, EC:6.2.1.12 4-coumarate--CoA ligase.

**Figure S3.** Representative chromatogram of *Arundo donax* extracts with the main peaks identified on the basis of the comparison of their UV-spectral characteristics with those of authentic standards: caffeic

acid derivative (tR 12.04 min), apigenin derivatives (tR 14.13, 15,24 and 17,43 min), quercetin derivative (tR 20.98 min) and kaempferol derivatives (tR 22.36, 23.66 and 26.02 min).

**Table S1.** Ascorbic acid (AS), dehydroascorbate (DHA) and total ascorbate in *A. donax* plants in control conditions (C), phosphorous-deprived (-P), phosphorous-enriched (+P), sodium chloride-enriched (+Na), and phosphorous and sodium-enriched (+NaP) soil after 43 days by applying different solutions (St) and following a recovery period of 14 days (Re). Data are means of 4 plants per treatment ± SE; two-way ANOVA defines the significance of effects of treatments in time, the experimental points, at $P < 0.05$; one-way ANOVA defines the significance of effects of treatments in St and Re, at $P < 0.05$.

**Table S2**. Lignin content (mg/ml) in leaves of *A. donax* in control conditions (C), phosphorous-deprived (-P), phosphorous-enriched (+P), sodium chloride-enriched (+Na), and phosphorous and sodium-enriched (+NaP) soil measured after 43 days by applying different solutions (St). Data are means of 3 plants per treatment ± SE. ANOVA defines no effects of treatments in lignin content at $P < 0.05$.

**Table S3**. List of differentially expressed genes (DEGs) of flavonoids biosynthesis pathway in each treatment at false discovery rate (FDR) of 5%; FC=Fold Change relative to control.

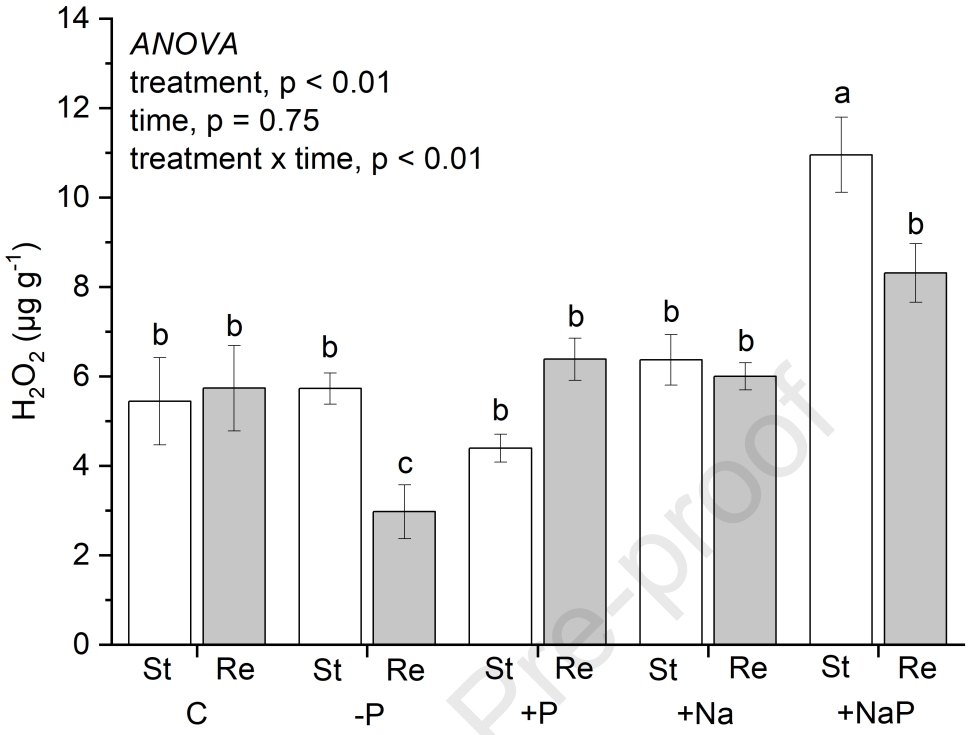
**Table S4**. List of differentially expressed genes (DEGs) in each treatment at false discovery rate (FDR) of 5%; FC=Fold Change relative to control.

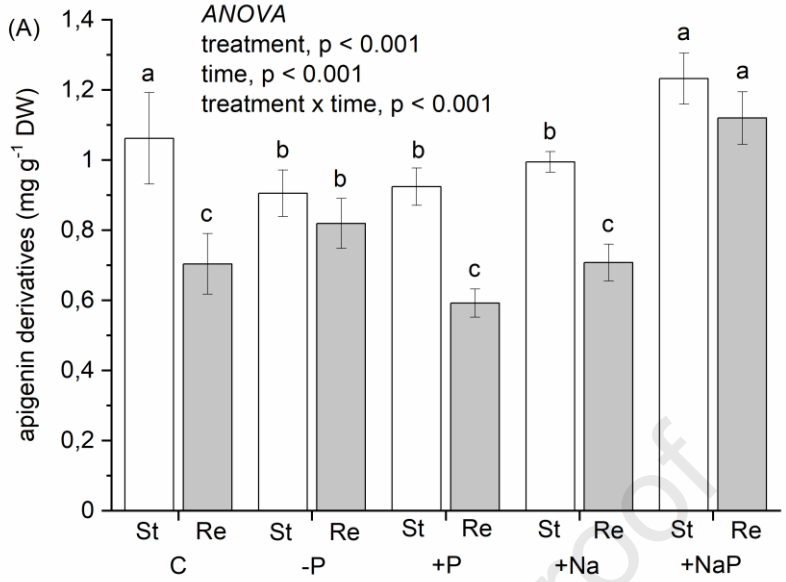
**Table 1**. Metabolites, class III peroxidase and genes which levels differentially changed in *Arundo donax* plants with excess supply of sodium chloride (Na2+) or phosphorus (P+), without phosphorus supply (-P), and with an excess supply of both phosphorus and sodium chloride (+NaP) respect to the control, in stress (dark arrows) and recovery (dashed arrows) conditions. Increased levels (arrows towards) and decreased levels (arrows towards down).

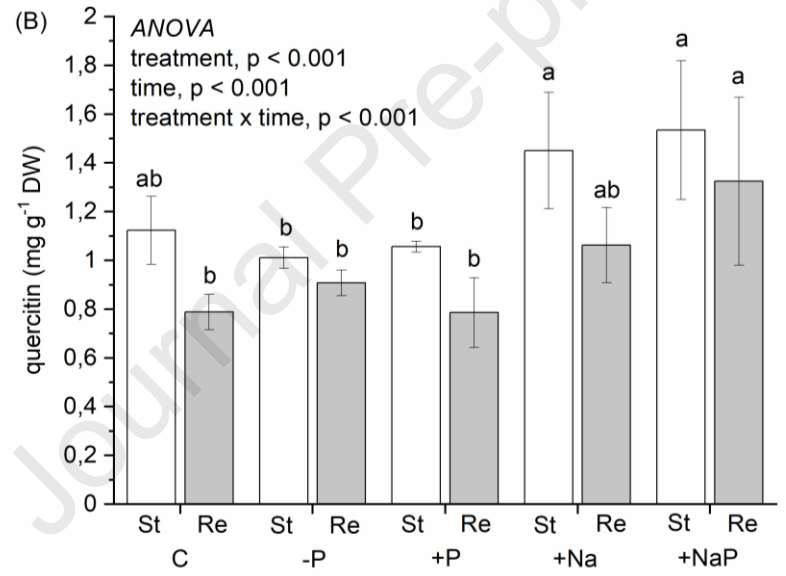
| | STRESS | | | | RECOVERY | | | |
|---|---|---|---|---|---|---|---|---|
| | **Na2+** | **+P** | **-P** | **+NaP** | **Na2+** | **+P** | **-P** | **+NaP** |
| Hydrogen peroxide | | | | ↑ | | | ⇣ | |
| Ascorbic acid | ↓ | ↓ | ↓ | ↓ | | | | |
| Dehydroascorbate | ↓ | ↓ | ↓ | ↓ | | | | |
| Isoforms of Chalcone synthase:<br>TR30713\|c0_g1_i2<br>TR30713\|c0_g1 | | | | ↑ | | | | |
| Apigenin derivatives | | ↓ | ↓ | ↑ | | ⇣ | ⇡ | ⇡ |
| Quercetin derivatives | ↑ | | | ↑ | ⇡ | | | ⇡ |
| Kaempferol derivatives | | | | ↑ | | | ⇡ | ⇡ |
| Peroxidase<br>Guaiacol substrate | ↑ | ↑ | ↑ | ↑ | ⇡ | ⇡ | ⇡ | ⇡ |
| Peroxidase<br>Syringaldazine substrate | ↑ | ↑ | ↑ | ↑ | ⇡ | ⇡ | ⇡ | ⇡ |
| Peroxidase<br>Coniferyl alcohol substrate | ↑ | ↑ | ↓ | ↑ | ⇡ | | ⇡ | |

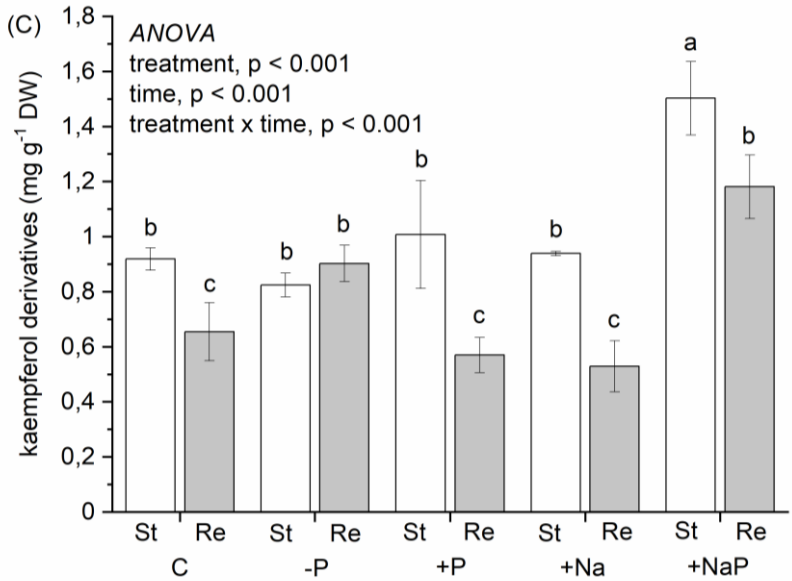
| | | |
|---|---|---|
| Peroxidase 17 gene<br>TR19645\|c0_g1_i5<br>TR19645\|c0_g1_i6 | ↑ | |
| Peroxidase P7-like<br>(TR482\|c0_g1_i4) | ↓ | |
| Peroxidase 66<br>TR5652\|c2_g2_i1 | ↓ | |
| Peroxidase<br>TR23604\|c5_g1_i4 FC=0.08 | ↓ ↓ | |
| Phenylalanine ammonia-lyase | ↓ | |
| 4-coumarate-CoA ligase<br>TR19035\|c0_g1_i1 FC=0.24<br>TR3027\|c2_g2_i1 FC=0.25 | ↓ | |
| 4-coumarate-CoA ligase<br>TR22604\|c3_g2_i1 FC=4.2,<br>TR5718\|c1_g1_i5 FC=3.1,<br>TR7497\|c3_g2_i3 FC=6.7<br>TR7497\|c3_g2_i7 FC=4.2 | ↑ | |
| cinnamoyl-CoA reductase<br>TR6509\|c1_g1_i1 FC=4.3<br>TR6509\|c1_g1_i6 FC=2.7<br>TR9597\|c1_g4_i1 FC=2.3 | ↑ | |
| cinnamoyl-CoA reductase<br>TR6509\|c1_g1_i7 FC=3 | ↑ | |
| cinnamoyl-CoA reductase<br>TR9597\|c1_g4_i1 FC=0.3 | ↓ | |

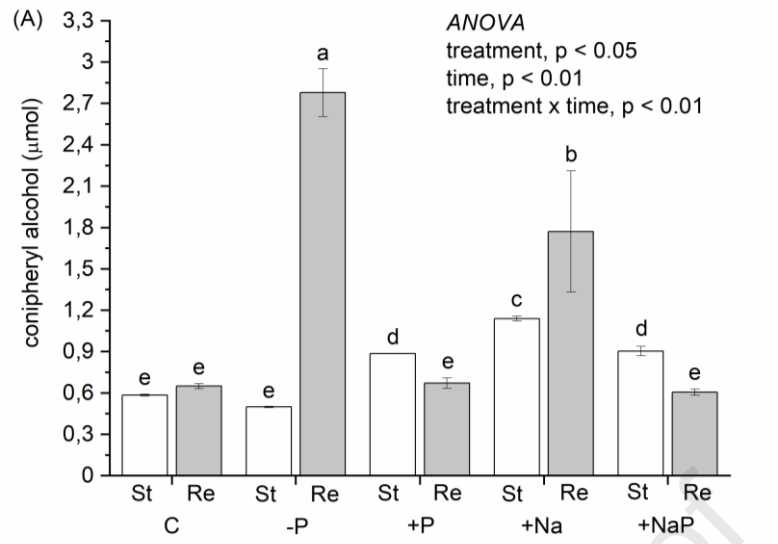
| | | | |
|---|---|---|---|
| cinnamyl alcohol dehydrogenase<br>TR24767\|c0_g1_i4 FC=7.6 | ↑ | | |
| caffeoyl-O-methyltransferase 1<br>TR30139\|c0_g1_i3 FC=7.1 | | ↑ | |
| caffeoyl-O-methyltransferase 1<br>TR18821\|c1_g3_i8 FC=0.07 | | ↓ | |
| caffeoylshikimate esterase<br>TR1036\|c1_g1_i12 FC=2.41 | | ↑ | |
| caffeoylshikimate esterase<br>TR1036\|c1_g1_i10 FC=0.11<br>TR11203\|c3_g1_i3 FC=0.35 | | ↓ | |
| caffeoylshikimate esterase<br>TR11203\|c3_g1_i6 FC=0.44 | ↓ | | |
| laccase genes<br>TR18961\|c2_g1_i1 FC=2.9<br>TR18961\|c2_g1_i4 FC=2.2<br>TR6554\|c0_g1_i2 FC=41.3<br>TR7969\|c1_g1_i3 FC=37.8 | | ↑ | |
| laccase genes<br>TR6546\|c1_g1_i2 FC=0.15,<br>TR6546\|c1_g1_i3 FC=0.08 | ↓ | ↓ | |
| laccase gene<br>TR6546\|c1_g1_i3 FC=0.09 | ↓ | | |

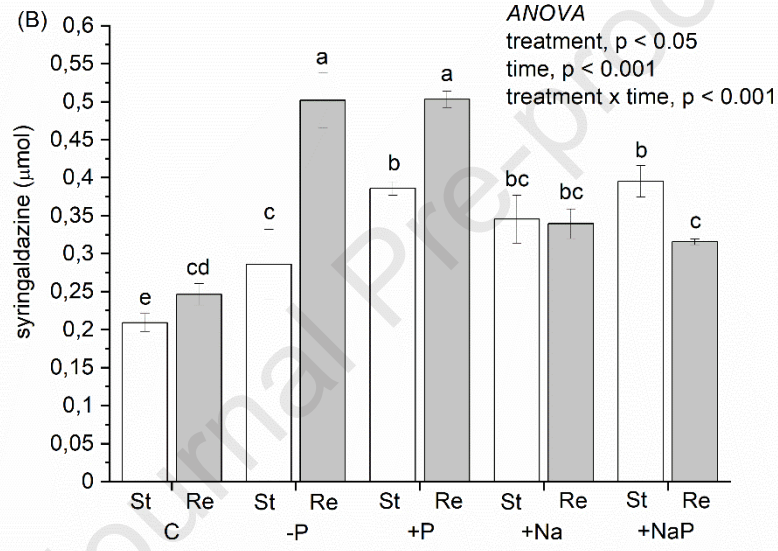


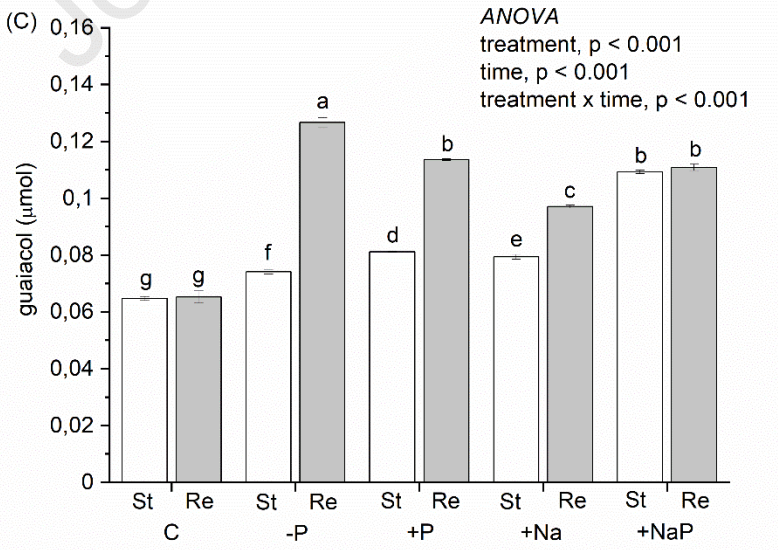




**Modulation of class III peroxidase pathways and phenylpropanoids in *Arundo donax* under salt and phosphorus stress**

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**Highlights**

- +Na and + NaP increased $H_2O_2$ levels, apigenin, quercitin and kaempferol
- Salt and phosphorus stress treatments activated Class III peroxidases
- P excess or starvation did not increased H2O2 and phenylpropanoid levels
- Class III peroxidases use phenylpropanoids to scavenge $H_2O_2$ in the peroxide cycle

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Conceptualization, C.C. and M.B.E.; methodology, M.B.E., B.P., B.C., M.L., P.S., P.A., T.D., R.S.; data curation, C.C., M.B.E., B.C.; writing—original draft preparation, C.C. and M.B.E.; writing—review and editing, C.C., B.C., M.L., T.D., B.F., M.B.E., G.S.S.; project administration, C.C.; funding acquisition, C.C.. All authors have read and agreed to the published version of the manuscript.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: