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Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (Lactuca sativa) at environmentally relevant concentrations

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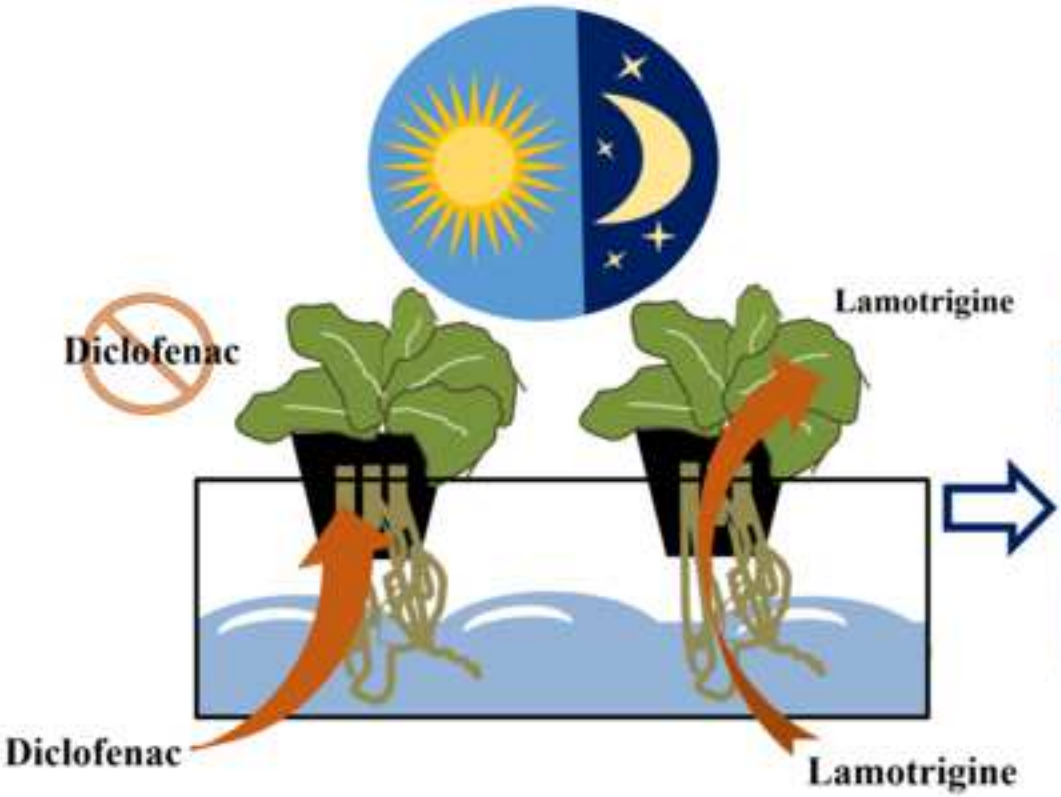
Graphical Abstract



Stress gene expression

	Day/Night
Control	rhythmically expressed
Lamotrigine	shifted pattern
Diclofenac	transient reduction

Abstract

Vegetable crops irrigated with treated wastewater can take up the environmentally persistent pharmaceuticals diclofenac and lamotrigine. This study aimed at quantifying the uptake and translocation of the two pharmaceuticals in lettuce (*Lactuca sativa*) as well as on the elucidation of the molecular and physiological changes triggered by them. Therefore, plants were cultivated in a phytochamber in hydroponic systems under controlled conditions and treated independently with diclofenac (20 µg L^{-1}) and lamotrigine (60 µg L^{-1}) for 48 h. A low translocation of lamotrigine but not of diclofenac or its metabolite 4′-hydroxydiclofenac to leaves was observed, which corresponded with the expression of stress related genes only in roots of diclofenac treated plants. We observed an oxidative burst in roots and leaves occurred around the same time point when lamotrigine was detected in leaves. This could be responsible for the significantly changed gene expression pattern in both tissues. Our results showed for the first time that pharmaceuticals like lamotrigine or diclofenac might act as signals or zeitgebers, affecting the circadian expression of stress related genes in lettuce possibly causing a repressed physiological status of the plant.

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Treated wastewater, Accumulation in plants, *Lactuca sativa*, Stress gene expression, Diurnal rhythm.

Highlights

- Translocation of lamotrigine, but not of diclofenac to lettuce leaves
- No direct triggering of oxidative stress but significant changes of gene expression
- Altered gene expression localized in root tissue where diclofenac was present
- Translocated lamotrigine to leaves triggered putative systemic response to roots
- Pharmaceuticals possibly act as zeitgebers affecting the expression of stress genes

Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (*Lactuca sativa*) at environmentally relevant concentrations

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Abstract

Vegetable crops irrigated with treated wastewater can take up the environmentally persistent pharmaceuticals diclofenac and lamotrigine. This study aimed at quantifying the uptake and translocation of the two pharmaceuticals in lettuce (*Lactuca sativa*) as well as on the elucidation of the molecular and physiological changes triggered by them. Therefore, plants were cultivated in a phytochamber in hydroponic systems under controlled conditions and treated independently with diclofenac (20 µg L^{-1}) and lamotrigine (60 µg L^{-1}) for 48 h. A low translocation of lamotrigine but not of diclofenac or its metabolite 4′-hydroxydiclofenac to leaves was observed, which corresponded with the expression of stress related genes only in roots of diclofenac treated plants. We observed an oxidative burst in roots and leaves occurred around the same time point when lamotrigine was detected in leaves. This could be responsible for the significantly changed gene expression pattern in both tissues. Our results showed for the first time that pharmaceuticals like lamotrigine or diclofenac might act as signals or zeitgebers, affecting the circadian expression of stress related genes in lettuce possibly causing a repressed physiological status of the plant.

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1. Introduction

Pharmaceuticals as contaminants in treated wastewater can become a serious problem for food safety when they are used for agricultural irrigation. These organic contaminants can be taken up by plants and trigger abiotic stress responses which can eventually affect plant growth and development. Plants have developed different strategies to adapt to abiotic stresses and environmental fluctuations by utilizing numerous molecular, biochemical, physiological and morphological changes to increase the probability of survival and competitive advantages (Pareek et al. 2009). These modulations in the plant might have fitness costs or effects on fruit quality attributes as has recently been shown in tomato plants (Christou et al. 2019).

Diclofenac ([2-(2,6-dichloroanilino) phenyl] acetic acid~~; DCF~~), is one of the most abundant pharmaceuticals in water derived from wastewater treatment plants and effluents (Pérez & Barceló 2008, Vieno & Sillanpää 2014). This compound can be taken up by plants and can induce oxidative stress. Kummerová et al. (2016) detected a significantly increased relative content of H_2O_2 in *Lemna minor* upon treatment with 10 µg/L diclofenac for 10 days. Moreover, other stress parameters like the ratio of oxidized/reduced thiols, and the peroxidation of lipids was significantly enhanced.

Apart from the oxidative stress induced by this compound, diclofenac can be rapidly metabolized in plants. This metabolization follows a pattern of three consecutive phases, first described as the “Green Liver” concept by Sandermann (1992). During phase I, compounds are activated by oxidation, reduction or hydroxylation for the conjugation to

reactive groups such as amino acids or sugars during phase II. Enzymes like glutathione *S*-transferases or glycosyltransferases catalyze these reactions. Conjugated phase II metabolites are sequestered in vacuoles or cell walls during phase III. In general the metabolization of foreign compounds will reduce their toxicity for the plant, although during phase I activation ROS may be produced, that need to be controlled by scavenging enzymes. Huber et al. (2012) observed phase I and phase II metabolization products of diclofenac in *Hordeum vulgare* (barley) and in the hairy root cell culture of *Armoracia rusticana* (horseradish). The activated hydroxylated metabolite 4′OH-diclofenac as well as the subsequently conjugated glucopyranoside were detected already after three hours of exposure.

Similar to diclofenac, the anti-epileptic drug lamotrigine ~~(LMG)~~ is highly persistent in the environment and could be detected in crops (Paz et al. 2016) even if the concentration found in plant tissue is low and the specific translocation mechanism still unknown. Therefore, Goldstein et al. (2018) hypothesized an adsorption of lamotrigine to the roots or a trapping in root vacuoles with only limited transport to the shoots. Information about lamotrigine-triggered stress responses in plants is lacking, but could provide useful hints for the translocation and perception of this pharmaceutical.

Genes involved in abiotic stress responses are often expressed in diurnal rhythms. Mutations in key circadian clock genes caused a greater sensitivity to salt, osmotic, and heat stress in *Arabidopsis thaliana*, which demonstrates the importance of the diurnal rhythms in the modulation of multiple stress responses (Kant et al. 2008). Many cold- and drought-responsive stress genes are rhythmically expressed in *A. thaliana* (Covington et al. 2008, Wilkins et al. 2010). Furthermore, Lai et al. (2012) demonstrated a circadian-regulation of reactive oxygen species (ROS) response. ROS act as secondary messengers involved in stress-response signaling but they are also cellular indicators of stress. High

levels of ROS cause oxidative damage such as membrane lipid peroxidation, protein oxidation, DNA and RNA damage and can lead to induced cell death. Consequently, scavenging of ROS in cells is essential and catalyzed by enzymes including peroxidases and catalases (Mittler 2002). The expression of a peroxidase (*NtPXC8.1*), a cytochrome P450 (*NtCYP71D21*) and different other genes involved in the metabolism of xenobiotic compounds and clock genes were significantly affected in *Nicotiana tabacum* hairy root culture under phenol treatment (Alderete et al. 2018). However, the putative influence of residual pharmaceuticals in wastewater on the expression of circadian controlled genes coding for stress enzymes in plants has not been investigated so far.

In this exploratory research, we aimed to elucidate the influence of environmentally relevant concentrations of diclofenac and lamotrigine on the physiology and biochemistry of edible plants under controlled conditions. Hydroponic systems therefore offer several advantages like the usage of nutrient solution, which can be modified easily and homogeneously to test toxic effects of elements and different contaminants under controlled and known conditions. However, the results may vary in magnitude compared to plants grown in soil experiments (Nguyen et al. 2016). Lettuce, the species used in our experiment is frequently grown in hydroponic systems in commercial production, as growth and yield are independent of soil type and quality of the cultivated area (Maucieri et al. 2019). Therefore, the usage of hydroponic systems for lettuce experiments represent a realistic growing scenario for the food producing industry.~~In this context~~ A multidisciplinary approach was used, (1) ~~we quantified~~ to quantify the concentrations of the two pharmaceuticals and key metabolites in lettuce roots and leaves to investigate their uptake and translocation. These results were related (2) to the analysis of the oxidative stress level in the plant and, (3) to the investigation of the expression of genes involved in abiotic stress response and the metabolization of xenobiotics such as

peroxidase (*PER50*), catalase (*CAT1*), and glutathione *S*-transferases (*GST-F6*, *GST-F8*, *GST-U5*).

2. Materials and methods

2.1. Experimental design

Lettuce (*Lactuca sativa* var. capitata cv. 'Tizian', Syngenta, Bad Salzuflen, Germany) was grown for 21 days after germination in hydroponic systems in a phytochamber with 16/8 h light/dark cycle at 20/15°C, and an average humidity of 50%. Each pot contained one plant and was filled with clean perlite to avoid possible adsorptions of the pharmaceuticals to the substrate. Modified 0.5 × Johnson`s solution pH 5.4 containing 20 µM $FeSO_4 \times 7\ H_2O$ was used as nutrient media. The experiment was performed in triplicates. For the treatments the nutrient media was renewed and either lamotrigine (60 µg L^{-1}), diclofenac (20 µg L^{-1}) or pure ethanol (control) was added to it. Plant leaves and roots were harvested separately at time points 0, 6, 12, 24, 30, 36 and 48 hours post treatment, snap frozen in liquid nitrogen and stored at – 80°C until processing. Frozen material was ground in liquid nitrogen with mortar and pestle into a fine powder for either RNA, enzyme or H_2O_2 extraction. For the analytical procedure, the plant cultivation and treatments were repeated and samples of time points 0, 6, 12, 24 and 48 hours were lyophilized for further processing.

2.2. Extraction and analysis of diclofenac & lamotrigine and metabolites

Extraction of pharmaceuticals from lettuce root and leaf samples was carried out using the Original QuEChERS extraction kit (Bekolut, Hauptstuhl, Germany) followed by LC/QTOF-MS analysis according to (Nicola Montemurro et al. in prep.). Briefly, 1 g of homogenized freeze-dried lettuce leaves was placed in 50-mL Falcon tube and 9 mL of HPLC water were added. Then, the tubes were vortexed for 2 minutes at 2500 rpm using

a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US). After 1 hour from the complete hydration, 50 µL of internal standard (IS) mix were added to achieve the final concentration 10 ng mL^{-1}, vortexed (2500 rpm, 2.5 min) and rested for another 30 minutes. Then 10 mL of acetonitrile and 50 µL of concentrated formic acid were added and the tubes were vortexed again. After that, the Original QuEChERS extraction kit was added directly into the tubes and instantly hand shaken for 30 seconds. All tubes were vortexed another time and centrifuged (4000 rpm, 10 min, 4 º C). The supernatant was transferred into a glass tube and left overnight at -20°C, to promote the precipitation of co-extractives like waxes and sugars contained in lettuce leaves. After 12 h, 6 ml of the organic phase were transferred into PSA tube (150mg PSA, 150mg C18, 900mg $MgSO_4$), vortexed for 2 min, and centrifuged at 4000 rpm for 5 min, 4°C. One mL of the supernatant was transferred to a 2-mL vial and evaporated until total dryness under a nitrogen stream and then reconstituted with 1 mL of water/MeOH (90:10) solution before it was injected for LC-MS/MS analysis. For the roots, a similar modified QuEChERS procedure was used which consists of a single extraction step according to the following protocol (Manasfi et al., In preparation). Briefly, 1 g of homogenized freeze-dried root tissue was transferred in a 50-mL falcon tube and hydrated with 8 mL of EDTA-McIlvaine buffer (pH=4), vortexed, and rested for 30 minutes. After adding 50 µL of IS mix, the tubes were vortexed (2500 rpm, 2.5 min) and rested for another 30 minutes. Then, 10 mL of acetonitrile were added to the samples and they were vortexed for 2 minutes at 2500 rpm. Finally, the Original QuEChERS extraction kit was transferred into the falcon tubes, hand shaken and vortexed another time and finally, the tubes were centrifuged (4000 rpm, 10 min, 4 º C) as for lettuce. No freezing or cleanup step took place in this case. Just 1 mL of the supernatant was transferred to a 2-mL vial, evaporated to dryness under a nitrogen stream, reconstituted with 1 mL of water/MeOH (90:10)

171 solution and injected for LC/QTOF-MS/MS analysis. Details about chemicals, EDTA-
172 McIIvaine buffer preparation, LC/QTOF-MS/MS conditions are reported in (see
173 Supplementary Methods (SM)). An one-way ANOVA with corresponding post-hoc
174 Lincon testing was performed to determine significant differences between time points
175 within the diclofenac or lamotrigine treated samples ($n = 3$). Significant differences were
176 indicated with different letters (p–value ≤ 0.05)
177 Liquid media samples were collected for each exposure time point, mixed 1:2 with
178 200 mM 5-sulfosalicylic acid and centrifuged at 16,100 x g for 10 min at 4°C for protein
179 precipitation. Afterwards supernatants were injected for LC-MS/MS analysis. Further
180 details are described in SM.

181 **2.3. Quantitative-PCR analysis of gene expression**

182 Target genes involved in oxidative stress reactions and the detoxification of xenobiotics
183 were selected based on the comparison with functional genes from *A. thaliana* using 'The
184 Arabidopsis Information Resource' (www.arabidopsis.org, Berardini et al. 2015).
185 Complete sequences of those genes were acquired from the *Lactuca sativa* whole genome
186 sequencing project at NCBI (www.ncbi.nlm.nih.gov/bioproject/PRJNA68025). All
187 primer pairs for qPCR (Table S4) were designed by Primer3Plus software (Untergasser
188 et al. 2007) and validated (Applied Biosystems Real-time PCR handbook guidelines,
189 Thermo Fisher Scientific). Afterwards primer/gene-specificities were checked by PCR on
190 cDNAs. The housekeeping gene, coding for the glyceraldehyde-3-dehydrogenase
191 (*GAPDH*), was used as an endogenous control for the qPCR analyses.
192 The RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract
193 RNA from 100 mg pulverized lettuce leaves and roots. After quantification of RNA by
194 NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), cDNA
195 was synthesized from 2 µg of RNA with the High Capacity cDNA Reverse Transcription

196 Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). The following
197 qPCR of the three biological replicates was performed as described previously
198 (Chowdhury et al. 2019) in three technical replicates. Specific PCR products were
199 confirmed by melting curve analysis and gel electrophoresis before the relative
200 quantification by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). ΔCt values were
201 calculated relative to the endogenous control and subsequently the data of each time point
202 was normalized to the initial time point 0. The standard error of the mean was calculated
203 from the average of the triplicates.
204 To compare which genes were differentially expressed in the diclofenac and lamotrigine
205 treatments compared to control, one-way ANOVA with post-hoc Tukey's HSD tests were
206 performed based on ΔCt data.

207 **2.4. Quantification of H_2O_2**

208 H_2O_2 production in roots and leaves was measured according to Shin et al. (2005) using
209 the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes,
210 Invitrogen, Carlsbad, CA). Ground frozen plant tissue was mixed with 20 mM potassium-
211 phosphate buffer pH 6.5 and centrifuged. Supernatants were incubated with 100 µM
212 Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.2 U ml^{-1} horseradish
213 peroxidase at room temperature for 30 min in the dark before quantifying with a
214 fluorescence/absorbance microplate reader (TECAN Spark®, Tecan Group Ltd.,
215 Switzerland) at excitation/emission at 530/590 nm against a H_2O_2 standard curve (0 –
216 10 µM).

217 **2.5. Protein extraction and enzyme activity analysis**

218 Soluble protein was extracted according to Schröder et al. (2005), and protein content
219 was quantified (Bradford 1976) before assaying enzyme activities in a 96-well
220 spectrophotometer (Spectra MAX 190, Molecular Devices, Germany). GST activity was

221 determined at 400 nm (ε = 17.2 mM^{-1} cm^{-1}) using the model substrate 1-chloro-2,4-
222 dinitrobenzene (CDNB) and reduced glutathione (GSH) as a co-substrate (Habig et al.
223 1974). Peroxidase (POX, EC 1.11.1.7) activity was evaluated by the oxidation of guajacol
224 to tetraguajacol in the presence of H_2O_2 at an extinction of 420 nm (ε = 26.6 mM^{-1} cm^{-1},
225 Diekmann et al. 2004).

226 **2.6. Statistics**

227 Statistical analyses were performed with the software R version 3.6.1. If not indicated
228 differently, a two-way analysis of variance (ANOVA) with Bonferroni post-test was
229 applied to determine significant differences between control plants and treated groups
230 (n = 3). Significance levels were determined as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for
231 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001.

232

233 **3. Results and Discussion**

234 **3.1. Uptake and translocation of pharmaceuticals in lettuce**

235 The highest concentration of diclofenac was detected in root tissue 6 h after treatment
236 (6.02 µg g^{-1} DW) and a significant reduction of this concentration occurred during the
237 experiment. Simultaneously the analysis of diclofenac treated root samples revealed the
238 formation of the metabolite 4'-hydroxydiclofenac at the same time point and onwards
239 (Figure 1). Corroborating our results, hydroxylated metabolites had been already detected
240 after 3 h of exposure in a hairy root cell culture of *Armoracia rusticana* (horseradish)
241 (Huber et al. 2012). We observed a rapid metabolization of diclofenac and a higher
242 concentration of the phase I metabolite than the initial compound after 24 h, similar to
243 results published for *Typha latifolia* by Bartha et al. (2014).

244 However, we were not able to detect diclofenac and the phase I metabolite
245 4'-hydroxydiclofenac in leaves of the treated lettuce plants at any time point. Similar to

246 our observation, in *Typha latifolia* exposed to high concentrations of diclofenac (1
247 mg L^{-1}) under hydroponic conditions, barely small amounts of the pharmaceutical (4% of
248 amount in roots after 24 h) were quantified in shoots (Bartha et al. 2014). Additionally, it
249 has been reported that only when plants were treated with diclofenac for a prolonged
250 period, this compound was translocated to tomato fruits (Christou et al. 2017) or to the
251 leaves of *Scirpus validus* (Zhang et al. 2012) in higher rates.
252 Unlike diclofenac, the concentration of lamotrigine in lettuce roots increased during the
253 first 6 h but stayed constant at a similar concentration (2.14 ± 0.22 µg L^{-1}) afterwards
254 until the end of the experiment (Figure 2). Moreover, a translocation of lamotrigine to the
255 leaves in low but increasing concentrations was detected. It has been proposed that
256 lamotrigine may be restricted from passing through plant cell walls or membranes
257 because of its ionic character and therefore might rather accumulate in roots than in shoots
258 (Chuang et al. 2019). At the initial pH of the liquid media at pH 5.4, ~ 50% of lamotrigine
259 (pKa 5.7) is charged to form a cation. Charged lamotrigine putatively remains in the
260 apoplastic space and is adsorbed to the root surface, whereas uncharged lamotrigine might
261 be transported by passive diffusion into root cells (pH 7 – 7.4) or to the leaves. After
262 entering root vacuoles (pH 4 – 5.5) the molecule is again charged and cannot pass the
263 tonoplast (Nason et al. 2018). Consequently, the highest accumulation of lamotrigine was
264 detected in roots and only low concentrations were translocated to leaves (Figure 2).
265 In general, our findings highlight a putative passive transport of lamotrigine to leaves,
266 occuring in low concentrations and at slow rates. This reduced mobility might be caused
267 by the cationic charge of the molecule depending on the pH. The high hydrophobicity of
268 diclofenac is hypothesized to be the main reason for the lacking translocation of this
269 compound to aboveground tissues. As already reported in previous studies, the octanol-
270 water partitioning coefficient (log *Kow*) plays a crucial role to predict the uptake of

xenobiotics by plants (Briggs et al. 1982). Therefore, highly hydrophobic substances (like diclofenac; log *Kow* = 4.51) have a large potential for bioconcentration in roots but a low possibility for translocation to shoots and leaves. Moreover, when diclofenac had entered plant tissue, the molecule underwent rapid metabolization, as was observed by a decrease of the parent compound and a simultaneous increase of the phase I metabolite (Figure 1). Such a decrease was not verified for lamotrigine in the present study, but there is also no information about possible metabolism in plants available in literature.

None of the pharmaceuticals was present in control plants growing in liquid media only. Moreover, for the tested concentrations and exposure time of lamotrigine or diclofenac neither visual signs of toxicity nor changes in growth were observed in lettuce (Figure S1). However, exposure was only for 48 h and at low concentrations (diclofenac: 20 µg L^{-1}; lamotrigine: 60 µg L^{-1}).

Concentrations of lamotrigine were also analyzed in liquid media of treated plants and plant-free control groups. During the 48h-experiment, we detected a relatively stable concentration of lamotrigine in the plant-free control groups (Figure S2), showing there was negligible loss of the pharmaceutical by sorption to perlite or non-plant related photo- or biodegradation. In the presence of plants, the initial concentration of 58.32 ± 6.74 µg L^{-1} of lamotrigine in nutrient media was reduced to 45.48 ± 2.96 µg L^{-1} within 48 h (Figure S3).

3.2. H_2O_2 production

H_2O_2 is an important signaling molecule in plant cells that can cause damage to various cell structures in high concentrations. On the one hand, H_2O_2 in high concentrations mediates oxidative stress, which causes damage to cellular components such as proteins, DNA or lipids (Moller et al. 2007). On the other hand, H_2O_2 also acts as a secondary messenger for further downstream signaling, leading to plant responses and to diverse

functions of growth and development (Choudhury et al. 2013). After specific perception, H_2O_2 as one of the primary reactive oxygen species (ROS) in plants, is formed as an initial reaction in almost all plant compartments during different enzyme reactions e.g. by plasma membrane bound NADPH oxidases. The apoplastic ROS accumulation can activate ion channels leading to an influx of calcium (Ca^{2+}) into the cytoplasm, which can then vice versa enhance the induction of the apoplastic ROS production during abiotic stress conditions (Lamers et al. 2020). Consequently, these common ROS-calcium signaling pathways enable cell-to-cell communication and thereby long-distance transmission besides the signaling on the single-cell level (Steinhorst and Kudla 2013, Mittler, 2017). The information presented in the Ca^{2+} signatures can be decoded by diverse Ca^{2+} sensors (e.g. calcium dependent protein kinases (CDPKs), Calmodulins (CaM) or Calmodulin-like proteins (CMLs)) into phosphorylation events, changes in protein–protein interactions or regulation of gene expression by binding at Ca2+/calmodulin-binding transcription factors (Hashimoto and Kudla 2011). The concentration of H_2O_2 in lamotrigine-treated roots and leaves was significantly elevated (p–values ≤ 0.001) after 12 h compared to control plants (Figure 3). For the other time points no difference was detected, indicating that lamotrigine is not triggering cellular ROS production but rather a transient oxidative burst, as has been shown for *Salvia officinalis* leaves after they were exposed to ozone for 5 hours (Marchica et al. 2019). Interestingly, this transient oxidative burst was detected in roots and leaves at the same time point, when we were also able detect lamotrigine for the first time in the lettuce leaves. We postulate that this oxidative burst appeared due to systemic signaling activities from leaves to roots triggered by the presence of lamotrigine or its metabolites in the leaves. Whether lamotrigine or its degradation products have a direct influence on a leaf

specific cell structure remains to be elucidated. Since there are no plant related metabolites of lamotrigine published to date, we were not able to test this hypothesis.

In contrast, upon diclofenac treatment we observed a trend of a reduced H_2O_2 concentration in roots but not in leaves during the experiment (Figure 3), indicating that the pattern was only detected in the tissue where we were able to quantify the compound.

3.3. Gene expression analysis

Our earlier work showed that the two genes *GST-F6* and *GST-U5* were induced in roots in *Brassica* upon Paracetamol treatment (Bartha 2012). However, the influence of residual pharmaceuticals in water on the circadian rhythm/control of stress signaling genes in plants has not been investigated so far. We determined the expression of these two genes as well as of an additional GST (*GST- F8*) and two other genes involved in the detoxification of ROS (*PER50 and CAT1*) in lettuce after the exposure to diclofenac or lamotrigine over a time period of 48 h. The expression of all tested genes in the control plants, without exposure to any pharmaceuticals, followed a diurnal pattern over the duration of the experiment (Figure 4; A and B). In lettuce roots, all five tested genes showed maximal expression in the last hour before subjective dusk (T12 and T36), whereas in the leaves the peaks of the expression were observed at different time points for different genes. We detected the highest expression of the genes coding for the two GSTs belonging to the plant specific phi class (*GST-F6* and *GST-F8*) during the first 8 h after subjective dawn (T6 and T30), the one coding for the peroxidase (*PER50*) in the last hour before subjective dusk (T12 and T36) (Figure 4; B and D). The diurnal cycle of gene expression in shoots and roots of plants are not usually in-sync. This had been demonstrated in a previous study comparing the circadian clock in roots and shoots in *Arabidopsis*. The rhythmic behavior of the gene expression markedly differed between the tissues. Furthermore, a photosynthesis-related signal from the shoots was identified,

345 affecting the setting of the clock in the roots (James et al. 2008). However, the rhythmic
346 diurnal expression of these genes in lettuce has not been described so far, which makes
347 this an interesting observation.
348 As an exception to the obvious diurnal expression pattern, the gene coding for the tau-
349 class GST (*GST-U5*) was expressed at constant levels in lettuce leaves in control plants.
350 A constitutive expression of the gene *GST-U5* in leaves had been reported previously,
351 suggesting its housekeeping functions (Wagner et al. 2002) although it was also found to
352 be induced by auxin in roots by another study (van der Kop et al. 1996). Interestingly, the
353 expression of *GST-U5* was significantly increased over all analyzed time points in
354 lamotrigine treated lettuce leaves compared to control plants, indicating a lamotrigine-
355 triggered effect on *GST-U5* (Figure 4; D).
356 All other tested genes (*PER50*, *CAT*, *GST-F6* and *GST-F8*) measured in lamotrigine
357 treated plant roots, which were previously shown to be induced by H_2O_2 (Chen et al.
358 1996, Guan et al. 2000, Wagner et al. 2002) had a similar expression pattern, differing
359 from the control plants (Figure 4; C). In general, we observed a phase shift in the diurnal
360 expression of the genes. There was a trend for an earlier increased expression after 6 h
361 and an enhanced expression over time for *PER50*, *CAT1* and *GST-F6* in roots. The
362 expression high and low peaks in the circadian rhythm were shifted for most of the genes
363 and their expression at T24, T36 and T48 was significantly different to that in the control
364 plants in roots and leaves (Figure 4 A-D; Table S5). Shortly before this significant change
365 in gene expression, we detected a significant increase of the H_2O_2 concentration in both
366 tissues at T12 in lamotrigine treated plants, highlighting the role of H_2O_2 in intracellular
367 communication and its connection to subsequent downstream signaling like changes in
368 gene expression (Choudhury et al. 2017).

369 It has been proven that amongst several other signals, ROS, metabolism and nutrients can
370 act as zeitgebers (external or internal signals acting as time cues) which can affect the
371 functioning of circadian clock of the plants. They can affect a shift in the phase, period
372 or the amplitude of the circadian clock (Lai et al. 2012). The circadian clock has been
373 shown to influence several biological processes in plants, within a complex network of
374 pathways which has been studied in detail for Arabidopsis (Harmer et al. 2000, Lai et al.
375 2012). However, since such information is lacking for lettuce, we may only postulate that
376 lamotrigine or its metabolites could either directly or indirectly act as a stimulus
377 (zeitgeber) or cause a disruption of the circadian clock in lettuce plants.
378 A significant transient reduction of the expression of all genes was observed at T6 in roots
379 of diclofenac treated plants (Figure 5). Moreover, the expression of *CAT1*, *PER50*, *GST-*
380 *F6* and *GST-F8* was also significantly reduced at T12. With decreasing concentrations of
381 diclofenac we detected a reduced influence on stress gene expression compared to control
382 plants in lettuce roots. In leaves, where we were not able to detect diclofenac or its
383 metabolite 4'-hydroxydiclofenac, the influence on the expression of stress genes was
384 generally low (Figure 6). Nevertheless, a reduced expression of stress genes might lead
385 to a decreased defense status against biotic and abiotic stressors and therefore to a higher
386 susceptibility of the plant when the compound was present.
387 **3.4. Stress enzyme activity**
388 Since reactive oxygen species in high concentrations produced during the activation of
389 xenobiotics can cause oxidative stress to the plant, it is crucial to strictly regulate
390 intracellular H_2O_2 concentrations because of its additional role in cell signaling.
391 Peroxidases (POX) are important enzymes involved in the antioxidant network and
392 catalyze the conversion of H_2O_2 to water (Mittler 2002). We observed a significantly

393 reduced POX activity in roots exposed to lamotrigine during the whole experiment
394 (Figure 7).
395 In *Typha latifolia*, POX activity was inhibited during the first 14 days of the exposure and
396 began to increase only after 21 days of exposure to carbamazepine (Dordio et al. 2011).
397 This change was detected also in leaves, since carbamazepine is taken up by the plants'
398 roots and translocated to the aerial parts of the plants. However, since the translocation
399 of lamotrigine to lettuce leaves is relatively low; hence we measured no change of POX
400 activity in the leaves compared to control plants. Plant peroxidases were reported to
401 oxidize diclofenac to activate the molecule for further conjugation (Huber et al. 2016).
402 When *Typha latifolia* was incubated with 1 mg L^{-1} of diclofenac, enzyme activities were
403 significantly increased after 24 h (Bartha et al. 2014). In the present case, exposing plants
404 to a much lower concentration (20 µg L^{-1}) for up to 48 h, we were not able to detect
405 differences in POX activities in roots or leaves (Figure 7).
406 The activity of enzymes involved in the conjugation of activated xenobiotics to
407 glutathione during detoxification processes was comparable between diclofenac
408 (20 µg L^{-1}) treated and control plants in lettuce, as also shown for a concentration of 10
409 µg L^{-1} in *Lemna minor* (Kummerová et al. 2016). Only higher diclofenac concentrations
410 (100 µg L^{-1}) caused significantly increased *Lemna* GST activities. Moreover, no change
411 of GST activities was caused by the exposure to lamotrigine, as this compound might not
412 be a substrate for these enzymes.
413 The present observations showed that the alterations of the antioxidant enzyme POX
414 might be explained as a reaction to the uptake of lamotrigine by lettuce roots and the low
415 translocation to the leaves. In contrast, the concentration of diclofenac in the tissue
416 seemed too low to induce a change of enzyme activities.
417

4. Conclusions

Our results indicate that low concentrations of diclofenac and lamotrigine do not trigger measurable inductions of stress enzyme activities in lettuce, but a significant change in the expression of several stress related genes. The alterations of gene expression in case of diclofenac were predominantly pronounced in the roots where the pharmaceutical was localized whereas lamotrigine triggered a putative systemic response after the pharmaceutical was translocated to the leaves. We show for the first time that pharmaceuticals like lamotrigine and diclofenac can possibly act as signals or zeitgebers, which affect the circadian expression of the selected genes in lettuce plants.

Irrigation of vegetable crops using treated wastewater is a common growing practice in modern agriculture. The constant presence of various pharmaceuticals in the wastewater and their uptake by crops may influence the expression of plant stress genes in different ways. Especially circadian dysfunction of the stress gene expression could lead to chronic reactions and cause a repressed physiological status resulting in a reduced resistance to biotic stresses, an inferior tolerance to other abiotic stresses or in general to reduced growth and yields.

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446

447 **Conflict of Interest Statement**

448 The authors declare that this research was conducted in the absence of any commercial
449 or financial relationships that could be construed as a potential conflict of interest.
450

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456

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*Novelty Statement (maximum limit:100 words)

Statement of Novelty

This study showed for the first time that environmentally relevant concentrations of pharmaceuticals can significantly influence the expression of genes involved in the metabolization of xenobiotics in lettuce, even though concentrations were probably too low to induce measurable oxidative stress reactions. Moreover, these compounds possibly act as zeitgebers affecting the circadian expression of these genes. We also detected that the pharmaceuticals triggered different signal transductions. In the case of diclofenac alterations in gene expression were predominantly pronounced in the roots where the compound was localized, while lamotrigine caused a putative systemic response after its translocation to the leaves.

Figures

Elucidating stress responses in lettuce exposed to the pharmaceuticals diclofenac and lamotrigine using a multidisciplinary approach

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Contents

S-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in controls and diclofenac treated plants. Error bars indicate 95% confidence interval. Significant differences between treated groups and control plants are indicated according to Tukey`s HSD pairwise testing as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001. Grey bars: subjective night.

Figure 6: : Line diagram showing changes in relative gene expression (log fold change) in lettuce leaves over the measured time period as compared to time 0, of three glutathione *S*-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in controls and diclofenac treated plants. Error bars indicate 95% confidence interval. Significant differences between treated groups and control plants are indicated according to Tukey`s HSD pairwise testing as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001. Grey bars: subjective night.

Figure 7: Stress enzyme activities of (A+B) guajacol-peroxidase and (C+D) glutathione S-transferase in different plant tissues ((A+C) roots and (B+D) leaves) in control and treated plants. Data are mean activities ± standard error (n = 3). Significant differences between treated groups and control plants are indicated according to ANOVA as "*" for 0.01 ≤ p–value ≤ 0.05 and "**" for 0.001 ≤ p–value ≤ 0.01~~, and "***" for p–value ≤ 0.001~~.

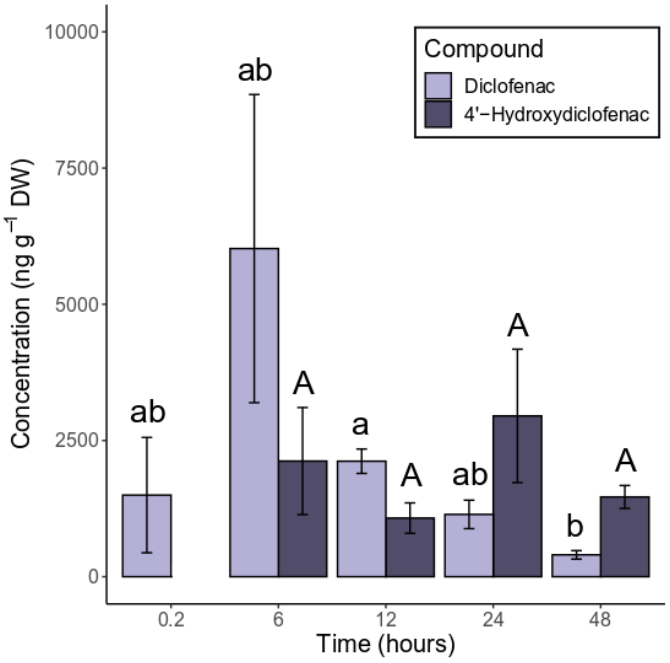


Figure 1: Concentration of diclofenac and its metabolite 4'-hydroxydiclofenac (ng g^{-1}) in lettuce roots of diclofenac treated groups. Data are mean concentrations (dry weight, DW) ± standard error (n = 3). Different letters indicate statistical significance among different time points after exposure to diclofenac (one-way ANOVA, p–value ≤ 0.05).

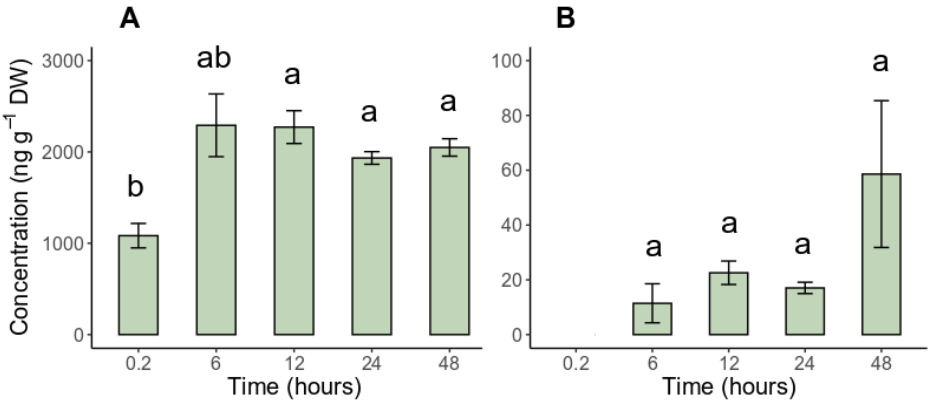


Figure 2: Concentration of lamotrigine (ng g^{-1}) in lettuce tissue ((A) roots, (B) leaves) of lamotrigine treated groups. Data are mean concentrations (dry weight, DW) ± standard error (n = 3). Different letters indicate statistical significance among different time points after exposure to lamotrigine (one-way ANOVA, p–value ≤ 0.05).

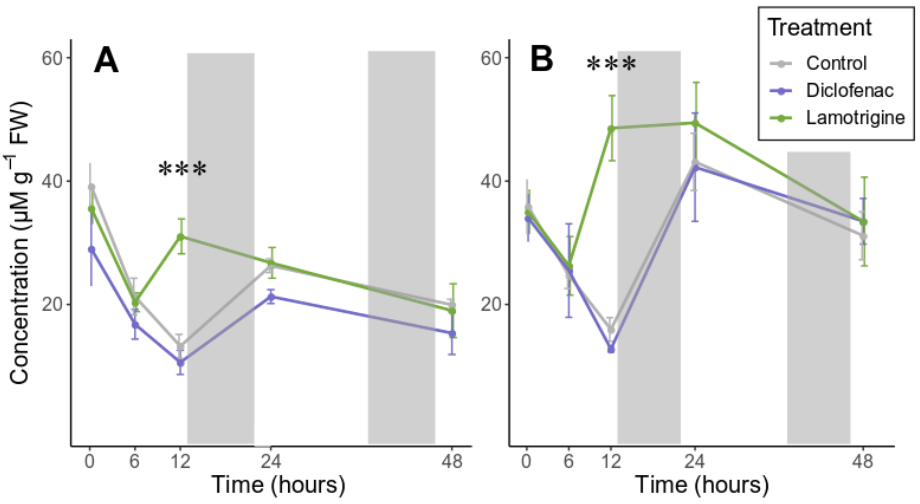


Figure 3: Line diagram showing change in the concentration of hydrogen peroxide (µM g^{-1}) in lettuce tissue ((A) roots, (B) leaves) in control plants and diclofenac or lamotrigine treated groups as measured over a time period of 48 hours. Data are mean H_2O_2 concentrations (g^{-1}fresh weight, FW) ± standard error (n = 3). Significant differences between samples of treated groups and control plants are indicated according to ANOVA as "***" for p–value ≤ 0.001. Grey bars: subjective night.

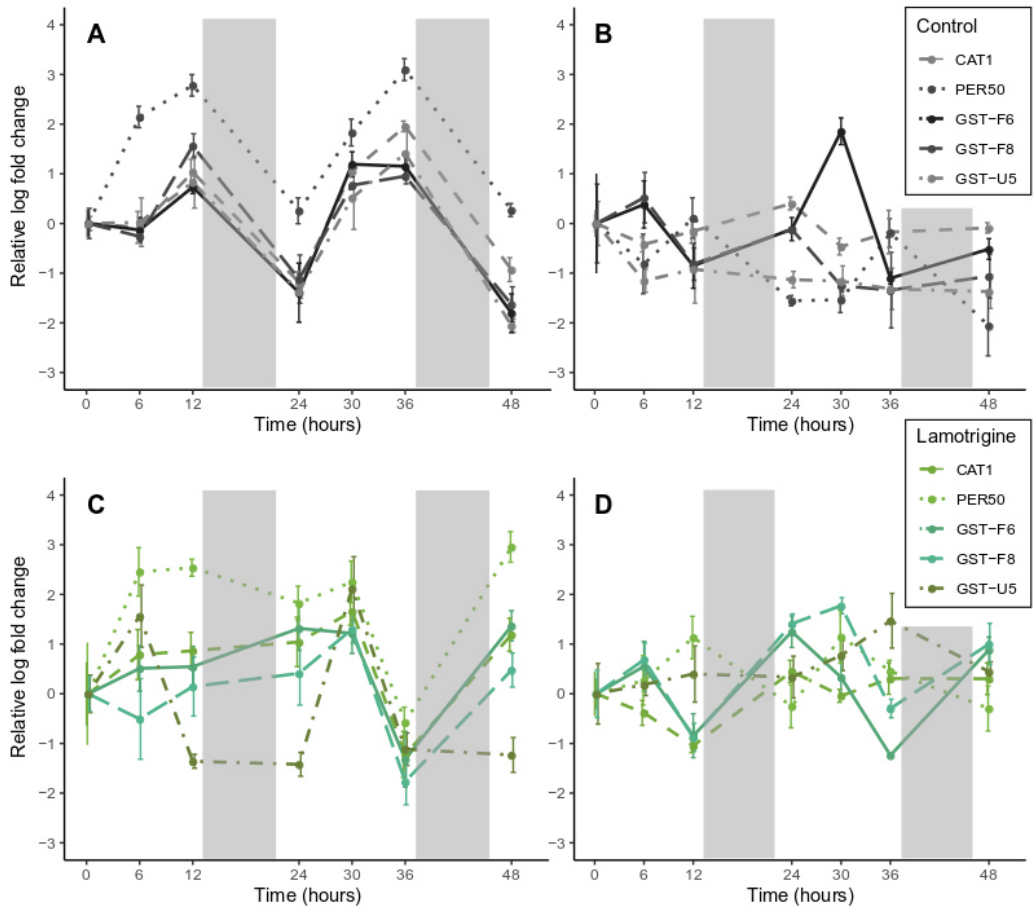


Figure 4: Line diagram showing changes in relative gene expression (log fold change) over the measured time period as compared to time 0, of three glutathione S-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in (A+B) control and (C+D) lamotrigine treated lettuce in different plant tissues ((A+C) roots and (B+D) leaves). Error bars indicate 95% confidence interval. Significant differences were observed in the expression pattern of all genes when compared to control plants at different time points revealed by Tukey`s HSD pairwise testing (Supplementary Table S 1). Grey bars: subjective night.

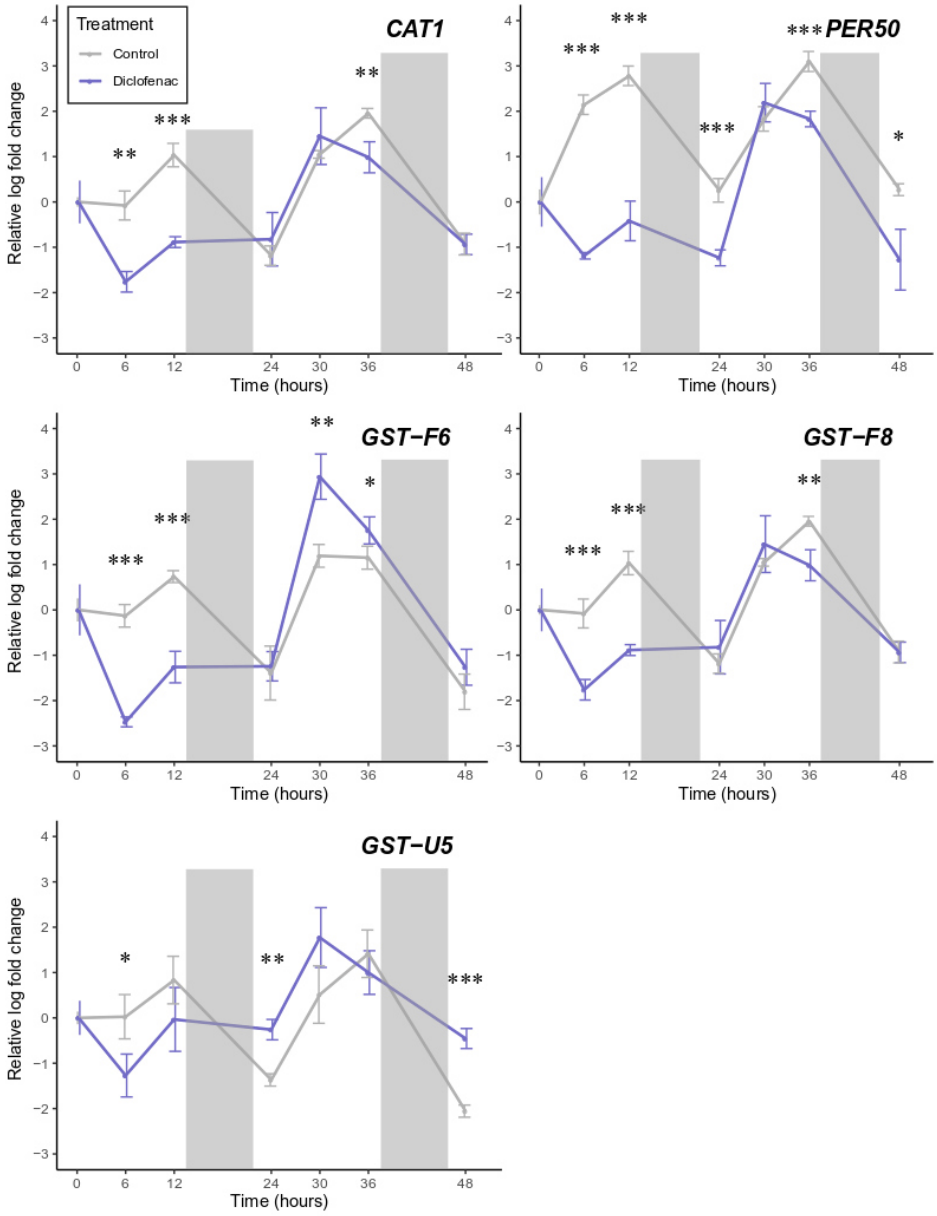


Figure 5: Line diagram showing changes in relative gene expression (log fold change) in lettuce roots over the measured time period as compared to time 0, of three glutathione *S*-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in controls and diclofenac treated plants. Error bars indicate 95% confidence interval. Significant differences between treated and control plants are indicated according to Tukey`s HSD pairwise testing as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001. Grey bars: subjective night.

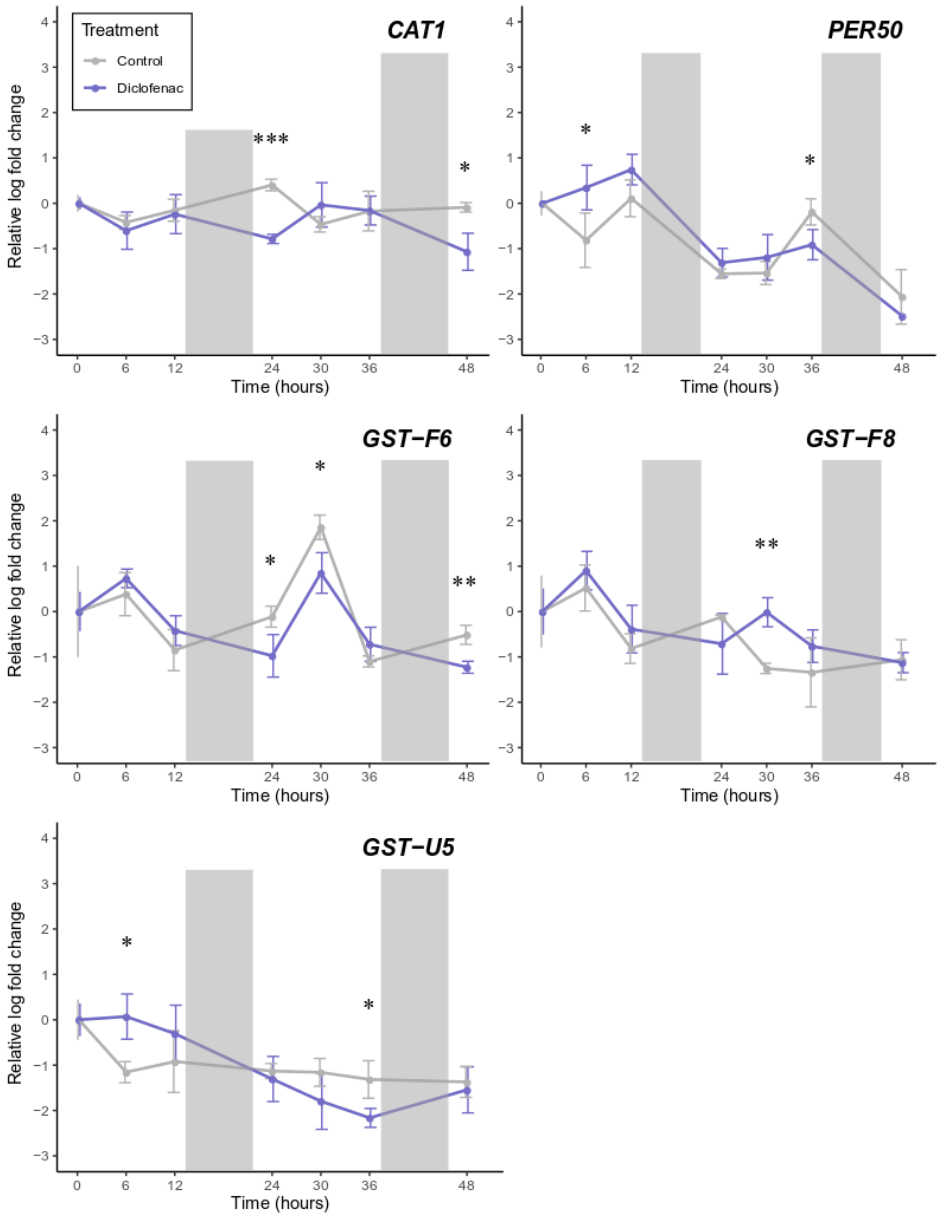
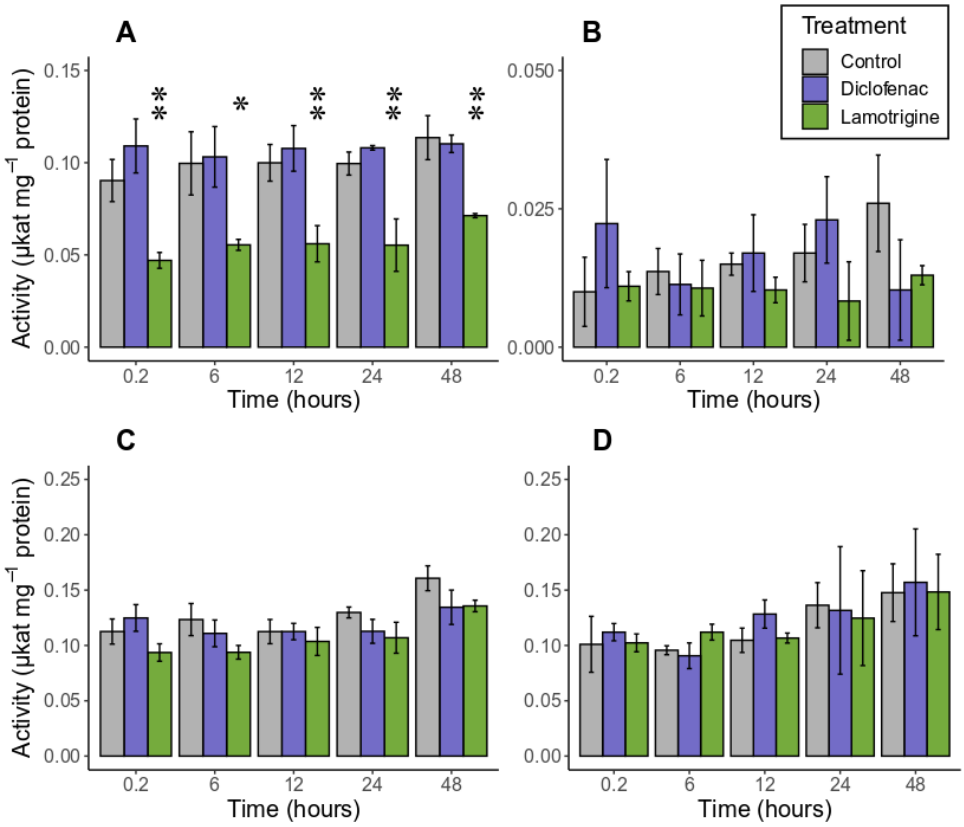


Figure 6: Line diagram showing changes in relative gene expression (log fold change) in lettuce leaves over the measured time period as compared to time 0, of three glutathione *S*-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in controls and diclofenac treated plants. Error bars indicate 95% confidence interval. Significant differences between treated and control plants are indicated according to Tukey`s HSD pairwise testing as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001. Grey bars: subjective night.



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Supplementary Material

Elucidating stress responses in lettuce exposed to the pharmaceuticals diclofenac and lamotrigine using a multidisciplinary approach

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Contents

Supplementary Methods

1. Chemicals

Lamotrigine (3,5-Diamino-6-(2,3-dichlorphenyl)-1,2,4-triazin, pharmaceutical secondary standard) and Diclofenac sodium salt (2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid sodium salt) were purchased from Sigma-Aldrich (Germany). High purity reference standards (4'-hydroxydiclofenac, diclofenac, and lamotrigine) purchased from Sigma Aldrich (St. Luis, MO, U.S). Isotopically labelled compounds (IS) (diclofenac-$^{13}C_6$ and lamotrigine-$^{13}C_3$) were high purity (mostly 90%) and were obtained from Sigma-Aldrich (St. Luis, MO, USA) and Toronto Research Chemicals (Toronto, ON, Canada), respectively.

LC-MS grade acetonitrile (ACN) (≥99.9%), methanol (MeOH) (≥99.9%), HPLC water, and formic acid (98%) were purchased from Merck (Darmstadt, Germany). The Original (OR) QuEChERS extraction salts kit (4g $MgSO_4$ + 1g NaCl) was obtained from BEKOlut GmbH & Co. KG (Hauptstuhl, Germany). Working solutions mixture (2 µg mL^{-1}) and internal standard (IS) working solution (2 µg mL^{-1}), for analysis and calibration purposes, were prepared by diluting adequate volumes of the individual stock solutions (1000 mg L^{-1}) with MeOH. All the solutions were stored at -20 °C. For preparation of the EDTA-McIlvaine buffer (pH=4) for roots extraction, Di-Sodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) and ethylendiaminetetraacetic acid anhydrous (EDTA) (≥99%) were supplied by Sigma-Aldrich (St. Luis, MO, USA). The EDTA buffer was prepared by dissolving 1.5 g of disodium hydrogen phosphate dehydrate, 1.3 g of citric acid monohydrate, and 0.372 g EDTA in 100 mL HPLC water.

2. LC/QTOF-MS/MS analysis of lettuce samples

2.1. Method Performance

Qualitative and quantitative analysis were performed using SCIEX OS™ Software version 1.6 (Sciex, Redwood City, CA, U.S.). The two highest resolution ions were used for positive confirmation and identification through HR-QToF-MS analysis: the most abundant product ion for the quantification and the precursor ion for the confirmation (SANTE/11813) (Commission 2018). Calibration curves were constructed using linear weighted least-squares regression (1/x as weighting factor) by plotting the ratio of the analyte signal to that of its corresponding IS and presenting coefficients of determination (R^2) above 0.99. Linearity of the method was evaluated using calibration curves ranging between 0.5 and 2000 ng g^{-1} DW in lettuce tissues, with a minimum of eight calibration points. Sensitivity, Limits of Detection (LODs) and Limits of Quantification (LOQs) were estimated from the matrix-matched calibration curves using linear regression analysis and a signal-to-noise ratio of 3.3 and 10, respectively (Table S1).

Table S1: Linearity, LODs and LOQs of 4′-hydroxydiclofenac, diclofenac and lamotrigine extracted from lettuce roots and leaves.

	Linearity (ng g^{-1})	R^2	LOD (ng g^{-1})	LOQ (ng g^{-1})	Internal standard
LEAVES					
4'-Hydroxydiclofenac	2.5-2000	0.9935	0.09	0.26	Diclofenac-$^{13}C_6$
Diclofenac	5-2000	0.9917	0.05	0.17	Diclofenac-$^{13}C_6$
Lamotrigine	1-2000	0.9930	0.05	0.14	Lamotrigine-$^{13}C_3$
ROOTS					
4'-Hydroxydiclofenac	5-2000	0.9888	0.04	0.13	Diclofenac-$^{13}C_6$
Diclofenac	1-2000	0.9933	0.03	0.10	Diclofenac-$^{13}C_6$
Lamotrigine	5-2000	0.9957	0.02	0.05	Lamotrigine-$^{13}C_3$

2.2. Analysis of lettuce samples

Lettuce leaves and roots samples were analysed using a SCIEX X500R QTOF hybrid system (Sciex, Redwood City, CA, U.S.). Chromatographic separation was performed on a reverse phase Hibar® HR Purospher® STAR RP-C18 column (100 mm x 2.1 mm i.d., 2 µm particle size, Merck, Darmstadt, Germany), thermostated at 40 °C in the column oven. A 12 min fast elution was carried out using of ACN and water (5 mM ammonium acetate + 0.1% formic acid) as mobile phases, at a flow rate of 0.5 mL/min (Table S2).

Table S2: LC gradient for the elution of the target compounds extracted from lettuce roots and leaves.

Time (min)	Mobile phase composition/vol. %		Flow rate, (µL min^{-1})
	Water*	ACN	
0.0	95	5	0.5
0.1	95	5	0.5
6.0	60	40	0.5
10.0	2	98	0.5
10.9	2	98	0.5
11.1	95	5	0.5
12.0	95	5	0.5

*(5 mM ammonium acetate + 0.1% formic acid) for the positive electrospray ionization. ACN: acetonitrile.

The injection volume was 10 µL, and the auto-sampler temperature was maintained at 8 °C. High resolution data were acquired in positive electrospray ionization in MRM^{HR} acquisition using fragment scanning mode. Data acquisition method and source conditions are listed in Table S3. Exhaustive details of the methodology are reported elsewhere (Montemurro et al., in preparation; Manasfi et al., in preparation).

Table S3: Ion source parameters and MRM^{HR} acquisition parameters used for analysing lettuce sample.

Ion source Voltage:	5500V	**Source Temperature TEM:**	550°C
Atomizing gas GS1:	55 psi	**TOF-MS**	100 to 950 *m/z*, 0.12s acc. time
Auxiliary gas GS2:	55 psi	**Collision energy**	10 V
Air curtain gas:	30 psi	**Collision gas:**	7

Analyte	**4'-Hydroxydiclofenac**	**Diclofenac**	**Lamotrigine**
Chemical Formula	$C_{14}H_{11}Cl_2NO_3$	$C_{14}H_{11}Cl_2NO_2$	$C_9H_7Cl_2N_5$
Adduct/Charge	[M+H]+	[M+H]+	[M+H]+
Precursor Mass (m/z)	312.0188	296.0239	256.0151
Fragment Mass (m/z)	230.0277	214.0424	210.9719
Declustering potential (V)	65	55	145
Collision energy (V)	45	40	35
Retention Time (min)	7.54	8.49	4.02
IS Name	Diclofenac-$^{13}C_6$	Diclofenac-$^{13}C_6$	Lamotrigine-$^{13}C_3$

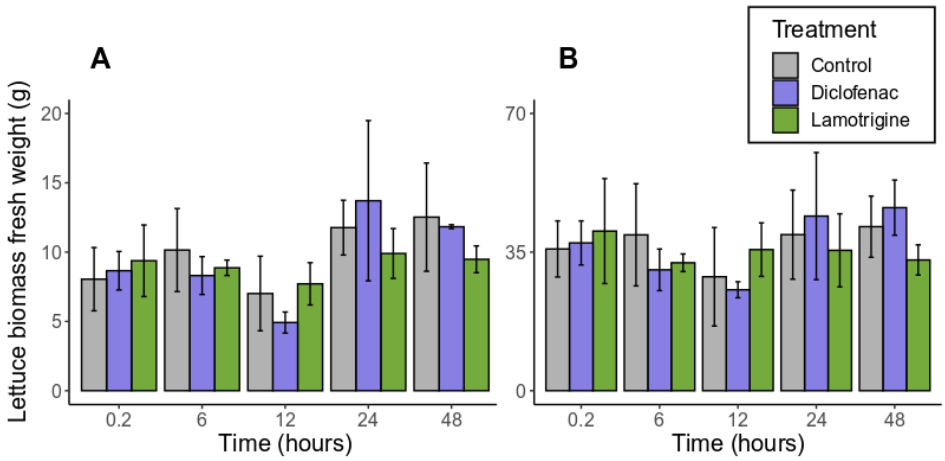
3. LC-MS/MS analysis of liquid media samples

3.1. Quality assurance procedures

The performance of the methods was checked daily, using method blanks (solvent controls), fortified samples spiked with internal standard using, new calibration curves weekly. The limits of detection (LODs) and quantification (LOQs) for each pharmaceutical were defined as LOD = 3.3(α /S) and LOQ= 10(α /S); here, α is the standard deviation slope and S is the average slope of the calibration curves. Precision and accuracy were evaluated following the criteria established by following the ICH (2005).

3.2. Analysis of liquid media samples

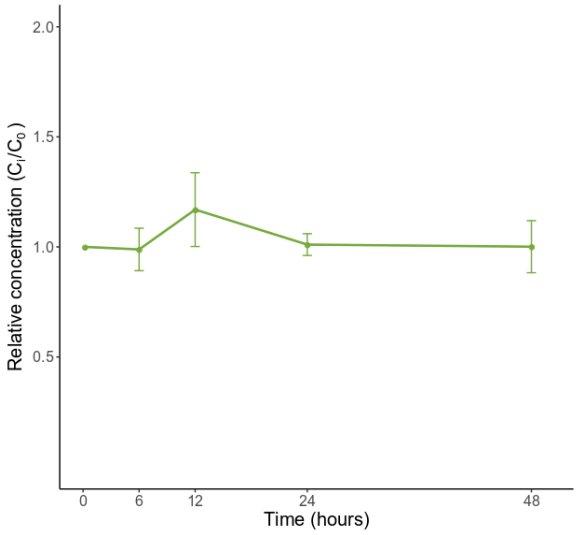
123 The protein precipitated liquid media samples were injected (10 µL) in triplicates by an
124 auto sampler (Dionex UltiMate 3000TRS, Gemering, Germany) into an UHPLC (Dionex
125 UltiMate 3000RS, Gemering, Germany) coupled to a triple quadrupole mass spectrometer
126 (HESI-MS/MS, TSQ Quantum Access Max, San Jose, USA) from Thermo Scientific.
127 An Accucore PFP column (100 mm x 2.1mm, 2.6 µm particle size, Thermo Scientific)
128 with an Accucore PFP pre-column (10 x 2.1mm, 2.6 µm particle size, Thermo Scientific)
129 at a flow rate of 0.450 mL min^{-1} was applied for chromatographic separation. For a linear
130 gradient elution, the mobile phases 0.1% formic acid in Mili-Q water (A) and 0.1% formic
131 acid in acetonitrile (B) were used to apply the following gradient program: 0–2 min 5%
132 Buffer B, 2-8 min 5-100% B, 8-9 min 100% B, 9-9.1 min 100-5% B, 9.1-10 min 5% B.
133 The mass spectrometer was operated in positive HESI mode with capillary voltage of
134 4000 V; nitrogen dumping gas temperature of 350 °C; sheath gas pressure 50 psi,
135 auxiliary gas pressure 5 psi, capillary temperature 380 °C, skimmer offset of 6, collision
136 energy of 28 eV and tube lenses of 97 V. Analysis of samples was in scheduled multiple-
137 reaction-monitoring (SMRM) mode following the precursor ion [M+H]+ 256.01 m/z and
138 the product ions 186.8 and 211.0 m/z. Afterwards, samples were quantified against a
139 calibration curve with five nominal concentrations from 7.5 to 120 µg L^{-1}, using
140 Lamotrigine-^{13}C as internal standard (20 µg L^{-1}).
141 Retention times and mass spectra were similar between standards and fortified matrices
142 (RSD<20%), thus proving that the chromatographic procedures were selective for the
143 quantification of all pesticides. The calibration curves proved to have good fits, with r^2
144 ranging from 0.0987. LOD and LOQ was 0.24 µg L^{-1} and 0.71 µg L^{-1}, respectively.
145



146

147 **Figure S1:** Lettuce biomass fresh weight (g) of (A) roots and (B) leaves of control plants
148 and diclofenac or lamotrigine treated groups over the time of the experiment of 48h. Data
149 are mean concentrations ± standard error (n = 3).

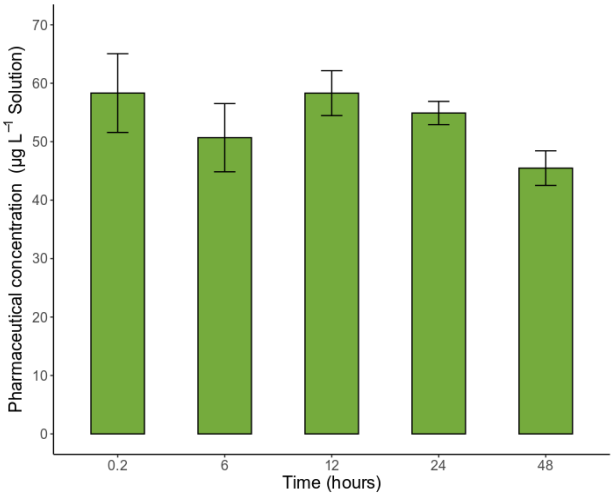
150



151

152 **Figure S2:** Relative concentration (Ci/C0) of lamotrigine in the plant-free control groups
153 over the time of the experiment of 48h. Ci is the measured concentration at a specific time
154 point, C0 the concentration at T0.

155



156

157 **Figure S3:** Concentration of lamotrigine (µg L^{-1}) in nutrient media over the time of the

158 experiment of 48h. Data are mean concentrations ± standard error (n = 3).

159

160 **Table S4:** List of plant genes selected for expression analysis with their corresponding

161 loci, functions in *A. thaliana* and primer sequences.

Name of gene (Locus tag in *Arabidopsis thaliana*)	**Documented functions in *Arabidopsis thaliana***	**Primer sequences (5‘ -3‘) All primers were designed in this study and have an annealing temperature of 55°C**
***CAT1* (AT1G20630)**	Catalase, induced by hydrogen peroxide, abscisic acid (ABA), drought, and salt stress.	5’ – GGTCCAAGGCGATGTCTTTG -3’ 5’ – ATGAACAGCTGGCGTTTTGT – 3’
***PER50* (AT4G37520)**	Peroxidase; Response to environmental stresses such as wounding, pathogen attack and oxidative stress.	5’ – CTGTCAACACATGGGCTTCC – 3’ 5’ – TCCCACTTCGACCCGTTTTA – 3’
***GST-F8* (AT2G47730)**	Glutathione S-transferase expressed in response to auxin, SA and hydrogen peroxide.	5’ – GCCCAAATACTTGCTCTCCG – 3’ 5’ – TTGGGATGACTACCGACGAG – 3’
***GST-U5* (AT2G29450)**	Tau Family, involved in glutathione metabolic processes, response to oxidative stress, toxin catabolic processes. Upregulated by Paracetamol Treatment in *A. thaliana*	5’- AGCATTGGACTTTTGTTTGGGA – 3’ 5’ - TGAAGCTATTGGGATTTTGGGG – 3’
***GST-F6* (AT1G02930)**	Phi class, involved in defense response to bacteria, glutathione metabolic processes, oxidative and water stress, toxin catabolic processes. Upregulated by Paracetamol Treatment in *A. thaliana*	5’ – TTGGGATGACTACCGACGAG – 3’ 5’ – RGCCCAAATACTTGCTCTCCG -3’
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase, used as internal standard /housekeeping gene	5’ – AGGTAGCGATCAACGGATTC – 3’ 5’ – AGGTGGGATGCTTGTTTGAC – 3’

162

Table S5: P values obtained from Tukey's HSD pairwise comparison of stress gene expression in lamotrigine treated lettuce tissue ((A) roots, (B) leaves) compared to control plants at different time points. Different significance graduation is indicated by different colors as "light green" for $0.01 \leq$ p–value ≤ 0.05, "light red" for $0.001 \leq$ p–value ≤ 0.01, and "dark red" for p–value ≤ 0.001.

A						**B**				
Time [h]	***CAT1***	***PER50***	***GST-F8***	***GST-U5***	***GST-F6***	***CAT1***	***PER50***	***GST-F8***	***GST-U5***	***GST-F6***
T6	0.0468	0.3169	0.5860	0.0191	0.0742	0.8021	0.0536	0.6069	0.0012	0.6546
T12	0.5066	0.1589	0.0123	0.0014	0.4384	0.0034	0.0271	0.8001	0.0426	0.9845
T24	0.0013	0.0020	0.0202	0.7355	0.0020	0.7560	0.0045	0.0001	0.0031	0.0026
T30	0.0149	0.1739	0.0209	0.0257	0.9241	0.0175	0.0009	0.0000	0.0009	0.0023
T36	0.0002	0.0001	0.0004	0.0013	0.0014	0.1540	0.0246	0.0594	0.0014	0.1030
T48	0.0005	0.0001	0.0011	0.0125	0.0002	0.0835	0.0100	0.0026	0.0008	0.0012

References

ICH Harmonised Tripartite Guideline. *International conference on harmonization, Geneva, Switzerland 2005,* 11.

Commission, European. 2018. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed (SANTE 11813/2017).