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Resveratrol exerts beneficial effects on the growth and metabolism of Lactuca sativa L

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(Article begins on next page)

1	RESVERATROL EXERTS BENEFICIAL EFFECTS ON THE GROWTH AND				
2	METABOLISM OF Lactuca sativa				
3					
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- 17 ABSTRACT

20 INTRODUCTION

21

22 Resveratrol (3,5,4'-trihydroxystilbene) is a phenolic micronutrient naturally 23 found in a few plant species, including grapes, berries, peanuts, and pines (Harikumar 24 and Aggarwal, 2008; Shishodia and Aggarwal, 2006). Over the last 50 years, the 25 resveratrol research has increased due to its promising human health benefits such as the 26 antioxidant, anticarcinogenic, antibacterial, anti-inflammatory, cardioand 27 neuroprotective properties (Vestergaard and Ingmer et al., 2019; Salehi et al., 2018; Shi 28 et al., 2014; Belchi-Navarro et al., 2012). The mechanism underlying these beneficial effects was its ability to activate sirtuin-like protein deacetylases, redox-sensing 29 30 enzymes involved in modulating metabolism regulation, stress responses, ageing 31 processes, and longevity (Gertz et al., 2012; Halls and Yu, 2008).

32 In plants, resveratrol plays a crucial role in plant response to biotic and abiotic 33 stress (Liu et al., 2019), such UV radiation and pathogens attacks (Vestergaard and 34 Ingmer, 2019; Elshaer et al., 2018), boron toxicity (Sarafi et al., 2017), ozone (Grimmig 35 et al., 2002) and saline stress (Kostopoulou et al., 2014). In particular, under stress 36 conditions, plants trigger a complex biochemical system to increase the resveratrol 37 synthesis and accumulation to confer protection (Vestergaard and Ingmer, 2019; Elshaer 38 et al., 2018; Ahuja et al., 2012; Dednarek and Osbourn, 2009; Hammerschimidt, 1999). 39 Some authors suggested that this protection was due to its ability to scavenge diverse 40 reactive oxygen species (ROS), thereby increasing the cellular defense system by 41 oxidative stress (Truong et al., 2018; Chang et al., 2009). King et al. (2006) 42 demonstrated that resveratrol reduced the cell membranes damage maintaining their 43 stability and limiting ROS stress in transgenic plants. Moreover, in tomato plants, the 44 resveratrol accumulation caused an increase in ascorbic acid, glutathione, and 45 antioxidant enzymes, which limited ROS damages (D'Intronio et al., 2009).

46 The beneficial resveratrol effects, as a potential natural crop protector, were also achieved by its exogenous application (Sarafi et al., 2017; Pociecha et al., 2014). In 47 48 particular, Pociecha et al. (2014) observed that the resveratrol applied on wheat leaves, 49 infected by powdery mildew, increased the phenolics metabolism and photosynthetic efficiency, reducing the damage during pathogenesis. Furthermore, the resveratrol 50 51 application to peanut plants, before UV-C treatment, mitigated the damage symptoms of rusty spots and leaf wilt (Tang et al., 2010) and also delayed the decay process during 52 53 apple fruit storage (Urena et al., 2003).

For all these reasons, researchers are focused on transgenic plants production in which the resveratrol synthase gene was overexpressed (Delaunois et al., 2009). The *sts* overexpression in tobacco, rice, apple and grape increased resveratrol content conferring higher resistence to abiotic and biotic stress (Chu et al., 2017; Dai et al., 2015; Zheng et al., 2015). For example, in transgenic rice seedlings, the resveratrol content was significantly increased (5–8 fold) under UV-C exposure compared to those grown under normal conditions (Zheng et al., 2015).

61 Alongside these benefits, resveratrol seems to inhibit weed growth. Recently, 62 Mantovanelli et al. (2020) studied the effect of exogenous resveratrol application on 63 seed germination, seedling growth, and mitochondrial energy metabolism in the 64 crop/weed system, maize/ *Ipomea grandifolia*. They demonstrated that resveratrol 65 stimulated maize seedlings growth, inhibiting, at the same concentration, the weed *I*. 66 *grandifolia*, confirming its potential as crop protector.

67 Despite the stimulatory activity excercted by resveratrol has been largely demonstrated,68 limited knowledge are available on its effects on plant metabolism.

In this respect, the present study aimed to evaluate the effect of resveratrol on
lettuce growth and development through a physiological and metabolomic approach to
deeply insight into the mechanisms underlying its action.

72

73 MATERIALS AND METHODS

74 Dose-response curves

75 Lactuca sativa L. (var. Parris Island COS) seeds were sterilized with 2.0% 76 sodium hypochlorite solution for 10 min and washed in distilled water. Then, 15 77 sterilized seeds were sown in square Petri dishes (100 x 100 mm) containing a double 78 layer of filter paper, moistened with 6 ml of sterile deionized water (control) and 79 acqueous solution of resveratrol (6.25, 12.5, 25, 50, 100, 200 and 400 µM) and 80 transferred into a ventilated climatic chamber with 16/8 h (light/dark) photoperiod, 25±1°C temperature, 120 µmol m⁻² s⁻¹ light intensity provided by a cold white 81 82 fluorescent lamp (Polylux XL FT8, 55 W 8440) and 55% relative humidity for 6 days.

83 After the treatments, roots and aerial parts were collected, and their fresh weight 84 (FW) was evaluated separately, and root length was measured. Plant material was then 85 oven-dried for one week at 60° C in order to determine the dry weight (DW). The 86 average of aerial part FW in response to each resveratrol concentration allowed us to **Commentato** [HC1]: X Ana: Please include the date of germination analysis and the following sentence: Then, the germinated seeds were counted using any extrusion of the radicle as a criterion,

and the total germination index (GT %) as described by Chiapusio et al. (1997) was calculated. The seedling were then treated for 6 days and roots and aerial parts werre collected.....Need to be correctly described!! determine the ED₅₀ (dose causing 50% stimulation of the total response), which was
then used for all the physiological and metabolic experiments.

89

90 *Leaf osmotic potential* [$\Psi(\pi)$]

91 After 6 days of treatment, leaf $\Psi\pi$ was measured on four treated (100 µM) and 92 non-treated (0 µM) leaves according to Araniti et al. (2016). Treated and untreated 93 leaves were collected and frozen at -20°C. After 24 h, leaves were squeezed into a 94 syringe (the first drop was thrown away to avoid broken cells fluid contamination), the 95 extract was collected, and the $\Psi\pi$ was measured with a cryoscopic osmometer 96 (Osmomat 030, Gonatec). The $\Psi\pi$ leaf was expressed in mega pascal (MPA).

97

98 In situ semi-quantitative determination of H_2O_2 and O_2^-

99 Hydrogen peroxide was determined based on Araniti et al. (2016) with some 100 modifications. After 100 μ M resveratrol treatment for 6 days, four fully expanded 101 treated (100 μ M) and non-treated (0 μ M) leaves were cut, vacuum infiltrated for 5 min 102 in 3,3'-diaminobenzidine (DAB) (1 mg ml⁻¹) solution (pH 3.8), and incubated for 8 h in 103 the same solution in the dark. After the incubation period, leaves were illuminated for 1 104 h and rinsed twice in pure ethanol to remove the pigments. Bleached leaves were stored 105 in 80% glycerol.

For O_2^- determination, four fully expanded treated (100 μ M) and untreated (0 106 107 μ M) leaves were vacuum infiltrated for 5 min with a 0.65 mg ml⁻¹ solution of sodium 108 azide (NaN₃) in potassium phosphate buffer (pH 7.8) containing 0.1% of nitroblue 109 tetrazolium (NBT) (Halliwell and Gutteridge, 1985) and incubated in darkness for 20 110 min in the same solution. After the incubation, leaves were illuminated until the 111 appearance of stains. For both H2O2 and O2, stained areas were determined by image 112 analysis with the software Image ProPlus v.6.0 (Media Cybernetics Inc., Bethesda, MD, 113 USA).

114

115 Chlorophyll a fluorescence parameters

116 The chlorophyll *a* fluorescence in treated (100 μ M) and untreated (0 μ M) 117 lettuce seedlings was monitored at the end of the treatment (6d), using the Maxi-118 Imaging-PAM Chlorophyll Fluorescence System fluorometer (Walz, Effeltrich, 119 Germany), as previously described by Araniti et al. (2017c). The maximum efficiency 120 of photosystem II (PSII) in dark-adapted state (F_v/F_m), the effective PSII photochemical 121 quantum yield (ϕ_{II}), the quantum yield of regulated (ϕ_{NPQ}) and no-regulated no-122 photochemical energy loss in PSII (ϕ_{NO}), the no-photochemical quenching coefficient 123 (q_N); the fraction of open PSII reaction centers based on a lake model (q_L) were 124 evaluated. The photosynthetic response was monitored for 5 min, and fifteen 125 measurements were obtained for each parameter at each measuring time.

126

127 Stomatal density and size

128 Immediately, after leaf detaching, both stomatal density (number of stomata per unit 129 leaf area) and size (length between the junctions of the guard cells at each end of the stomata and width between the distal side of the guard cells) were evaluated on 130 131 untreated and treated plants using an epifluorescence microscope system (Olympus 132 bx53) used in bright field and expressed as a percentage compared to the control (Malone et al., 1993; Xu and Zhou, 2008). It should be specified that stomatal length 133 134 might indicate the maximum potential opening of the stomatal pore, but not the aperture 135 that actually occurs.

136

137 Samples extraction, derivatization, and analytical conditions

138 To evaluate the impact of resveratrol on plant metabolism, untreated $(0 \ \mu M)$ and 139 treated $(100 \ \mu M)$ leaves were collected after 6 d, and the metabolome was extracted and 140 derivatized as previously described by Lisec et al. (2006).

141 One µl of the derivatized extract was injected into a GC-MS apparatus (Thermo 142 Scientific) equipped with a MEGA S.r.l. 5MS capillary column (30 m×0.25 143 mm×0.25µm) equipped with 10 m of pre-column. Injector and source were settled at 250 °C and 260 °C temperatures, respectively. Samples were injected in splitless mode 144 145 with helium as a carrier gas with a flow of 1 ml/min. They were then analyzed using the 146 programmed temperature proposed by Landi et al. (2020): isothermal 5 min at 70 °C 147 followed by a 5 °C/ min ramp to 350 °C and a final 5 min heating at 330 °C. Mass spectra were recorded in electronic impact (EI) mode at 70 eV, scanning at 40-600 m/z 148 149 range and scanning time 0.2 s. The mass spectrometric solvent delay was settled as 7 150 min. n-Alkane standards (C10-C40 all even) and blank solvents were injected at scheduled intervals for instrumental performance, tentative identification, and 151 monitoring shifts in retention indices. 152

153

154 Analyses of GC-MS Metabolomics Data

Raw GC-MS data were analyzed using the software MS-DIAL ver. 4.48 coupled
with a home built EI spectra libraries based on GOLM database, MassBank; Mass Bank
of North America, etc. (Tsugawa et al., 2015; Tanaka et al., 2010; Kopka et al., 2005).

158 MS-DIAL analysis was settled as previously reported by Landi et al. (2020), and metabolite annotation was carried out comparing the retention index and the spectra 159 similarity of the samples with those of the libraries, following the Metabolomics 160 161 Standards Initiative (MSI) levels of the International Metabolomics Society: reported 162 annotations were considered at level 2 (putative annotation based on spectral library 163 similarity) or level 3 (putatively characterized compound class based on spectral 164 similarity to known compounds of a chemical class) as suggested by Summer et al. 165 (2007).

166

168 Experimental design and statistical analysis

169 All the experiments were carried out in a completely randomized design with N 170 = 3 for dose-response curves, N = 5 for leaf osmotic potential, and N = 6 for chlorophyll 171 *a* fluorescence parameters, leaf stomatal density, width and length, and metabolomic 172 analysis.

Dose-response curves and physiological data were expressed as mean \pm standard errors (SE) and were analyzed using analysis of variance (ANOVA) with Tukey's test as post-hoc ($P \le 0.05$). The ED₅₀ parameter was calculated, tightening the dose-response curve's raw data through a non-linear regression log-logistic equation model. The equation was chosen from those that had the highest determination coefficient (r²) (*best fit*) (Software GraphPad Prism).

179 Metabolomic experiments were carried out using a completely randomized design 180 with six replications for each treatment (N = 6). Metabolomic data were analysed using 181 the software Metaboanalyst 5.0 (Chong and Xia, 2020). Metabolomics data were 182 normalized using the internal standard (Ribitol 0.02 mg mL⁻¹) based normalization functions in the MS-DIAL software. The internal standard normalized dataset was 183 transformed through "Log2 normalization," and Pareto scaled. The data were then 184 classified through unsupervised multivariate Principal Component Analysis (PCA). The 185 186 output comprised score plots to visualize the contrast between different samples and 187 loading plots to explain the cluster separation. Metabolite variations were presented as a 188 heatmap reporting only the *t*-test analysis's significant features (see below).

Partial Least-Squares discriminant analysis (PLS-DA) was used to highlight differences
between the two treatments (0 and 100μM).

191 Data were then analysed through the univariate *t*-test ($P \le 0.05$) to highlight 192 statistical differences among single metabolites and treatment. A False Discovery Rate 193 (FDR) was applied to the nominal *P*-values to control for false-positive findings.

Finally, to identify the metabolites coverage and the main altered pathways under resveratrol treatment, data were analysed using the Metaboanalyst enrichment analysis and pathway analysis tools.

- 197
- 198

199 RESULTS

200

201 Germination and seedlings growth bioassays

202 Resveratrol did not affect lettuce seed germination at all the concentrations 203 applied. Conversely, resveratrol treatment caused a strong stimulatory effect on *L.* 204 *sativa* growth, especially on the aerial part, where, at all concentrations (6.25-400 μ M), 205 it significantly increased fresh biomass compared to the control (Fig. 1F). The raw data 206 obtained from the aerial part fresh biomass allowed us to estimate the ED₅₀ equal to 100 207 μ M (Fig. 1C). This concentration was used in all the subsequent experiments. The 208 highest resveratrol doses (100-400 μ M) also significantly increased the dry biomass of

the aerial part (Fig. 1F)

210 Resveratrol treatment also increased the root fresh biomass at 25 and 200 µM

211 concentrations, while both root length (Fig. 1B) and dry weight (Fig. 1E) were not

affected by resveratrol.

Commentato [Fa2]: X Ana: We can't probably use the ED50 parameter, here there is a stimulatory effect. So, you can find the correct parameter, using GraphPAD prism software and explain it on mat and met





214Figure 1. Dose-response curve of initial growth of *L. sativa* seedlings exposed to increasing doses of215resveratrol: A) germination; B) root length; C) root fresh biomass; D) aerial part fresh biomass, E) root216dry biomass, and F) aerial part dry biomass. ED₅₀: dose causing 50% stimulus of stem fresh biomass with217respect to the control. Significant differences between means were identified by ANOVA with Tukey's218test ($P \le 0.05$). * ($P \le 0.05$), ** ($P \le 0.01$), *** ($P \le 0.001$). N = 3.

223 In situ semi-quantitative determination of O_2^- and H_2O_2

As shown in Figure 2, leaves of control (Fig. 2A), and resveratrol (Fig. 2B) treated seedlings showed the same color and intensity, indicating that this potential

226 elicitor did not alter the production of H₂O₂ in *L. sativa* leaves.

227

228

232



Figure 2. Semi-quantitative determination of H₂O₂ in *L. sativa* leaves treated with resveratrol 100 μM,
 showing the localization of the hydrogen peroxide on leaf surface after DAB staining: A) control leaf, and
 B) treated leaf. Image magnification 4X, scale bar 200 μm.

By contrast, resveratrol markedly reduced the superoxide production in *L. sativa* leaves. Indeed, treated leaves showed less and weaker color regions (Fig 3B) than the control (Fig 3A), supporting a reduction in the O_2^- production under resveratrol treatment.



Figure 3. In situ O₂⁻ localization in resveratrol 100 μM treated and untreated *L. sativa* leaves, showing the
 localization of the superoxide on leaf surface after NBT staining: A) control leaf, B) treated leaf (Image

- 240 magnification 4X, scale bar 200 µm).
- 241

²⁴² Leaf stomatal density, size and width and leaf osmotic potential ($\Psi \pi$)

- 243 In both untreated and treated plants, stomata were open with turgid guard cells
- 244 (Fig 4A and B). Resveratrol treatment did not alter stomatal density and length (Fig 4C
- 245 and E), but increased stomatal width (Fig 4D).
- 246 Concerning leaf $\Psi\pi$, the data pointed out that resveratrol (100 μ M) did not alter leaf
- 247 osmotic potential of *L. sativa* compared to the control (Fig 5).
- 248
- 249







251

252Figure 4. Micrograph of stomatal density of untreated (A) and treated (B) leaves of L. sativa.Stomatal253density (C), width (D) and length (E) in treated and untreated leaves with 100 μ M resveratrol Asterisks254indicate significant differences between mean values (N = 6) of treated and control plants after t-test (P ≤2550.05): * (P ≤ 0.05), ** (P ≤ 0.01), *** (P ≤ 0.001). Magnification 20X, scale bar 20 μ m.

- 256
- 257



Figure 5. Effects of resveratrol 100 μ M on the leaf osmotic potential [$\Psi(\pi)$] of *L. sativa*. Significant differences between means were identified by *t*-test ($P \le 0.05$). N = 5.

261

262 Chlorophyll a fluorescence parameters

Among all the parameters, only Fv/Fm, the maximum quantum efficiency of dark-adapted PSII, was significantly increased by resveratrol treatment (Fig 6A), which did not alter the apparent electron transport rate (ETR), the effective PSII photochemical quantum yield (ϕ_{II}), the quantum yield of regulated (ϕ_{NPQ}) and the nonregulated energy emission in the form of fluorescence (ϕ_{NO}) (Fig 6B-E).



271

Figure 6. The maximum quantum efficiency of dark-adapted PSII (Fv/Fm) (A), the apparent electron transport rate (ETR) (B), the effective PSII photochemical quantum yield (C) (ϕ_{II}), the quantum yield of regulated emission of energy in the form of heat (ϕ_{NPQ}) (D), and the non-regulated emission of energy in the form of fluorescence (ϕ_{NO}) (E) in the untreated and treated lettuce seedlings with resveratrol (100 µM). Significant differences between means were identified by *t*-test with $P \le 0.05$: * ($P \le 0.05$), ** ($P \le$ 0.01), *** ($P \le 0.001$). AU = Arbitrary Units. N = 6.

279 Untargeted-Metabolomic analysis

280 The GC-MS-driven untargeted metabolomic analysis of resveratrol-treated 281 seedlings allowed us to annotate and quantify 116 metabolites and extract 1005 282 unknown EI-MS shared features (Supplementary Table SXXX file excel).

Both annotated and unknown metabolites (Supplementary Table SXXX file excel), processed through MS-DIAL, were reported as supplementary data displaying their retention times, quantmass, signal/ noise ratio (S/N), RI similarity, total similarity, total spectrum similarity, and relative abundances.

287 A KEGG-based enrichment analysis (a method to identify classes of metabolites that 288 are over-represented in a large set of metabolites and might have an association with 289 treated seedlings phenotype) of the metabolic pathway revealed enrichment of galactose 290 metabolism, amino sugar and nucleotide sugar metabolism, ascorbate and aldarate 291 metabolism, among others (Figure 6a and Supplementary table SXXX). Most of these 292 annotated metabolites belonged to the primary metabolism (amino acids, sugars, 293 organic acids etc.) and in minor part to plant specialized metabolites (e.g., 2,3-294 Dihydroxybenzoate, quinic acid etc.).

The pathway analysis, which combines enrichment and topology analysis, pointed out that 28 pathways were significantly changed between the two treatments (Figure 6b and Supplementary table SXXX). Still, only 8 were characterized by an impact higher than 0.2 (starch and sucrose metabolism, alanine aspartate and glutamate metabolism, glycine serine and threonine metabolism, arginine biosynthesis, galactose metabolism, beta-alanine metabolism, glyoxylate and dicarboxylate metabolism, pantothenate and CoA biosynthesis) (Figure 6b and Supplementary table SXXXX).

The *t*-test analysis pointed out that 68 out of 116 metabolites were differentially produced between treatments. These metabolites mainly belonged to chemical classes of the amino acids (aspartic acid, glutamic acid, alanine, serine, among others), organic acids (tartaric acid, succinic acid, glyceric acid, among others), sugars and sugar alcohols (fructose, cellobiose, arabinose, galactinol, xylitol, among others), polyamines (putrescine and ornithine), etc., (Table 1).

308 Except for eleven metabolites (putrescine, DL-beta-hydroxybutyric acid, L-309 rhamnose, succinic acid, glyceric acid, glycerol-3-galactoside, creatinine, uridine, 310 threonic acid, mannose and methylmalonic acid), all the statistically significant 311 metabolites were stimulated by resveratrol treatment (Table 1). 312 The unsupervised Principal Component Analysis (PCA) was carried out on 313 blank samples and all three samples group to demonstrate the system suitability. The 314 PCA Score Plot, built on the first (PC1) and the second component (PC2), revealed 315 clear discrimination of sample groups against blanks, highlighting model robustness 316 (Supplementary Figure S1a). The components separated control and treated groups with no outliers (Supplementary Figure S1), indicating that the metabolomic analysis was 317 318 reliable and could reflect the metabolic profile changes induced by the resveratrol 319 treatment.

320 Both unsupervised PCA runs on MS-DIAL suggested that metabolites 321 (Supplementary Figure S1b) and unknowns features (Supplementary Figure S1c) were 322 useful to a clear sample groups' discrimination. Further, both unsupervised PCA 323 analyses (Fig. 7a) and Supervised Partial Least Squares Discriminant Analysis (PLS-324 DA), carried out only on the annotated metabolites (Fig. 7b), demonstrated group 325 separation with the first 2 principal components (PCs), explaining 72.2% variance for PCA and 71.5% variance in PLS-DA score plots. The PLS-DA model's robustness was 326 327 validated by the permutation test, which highlighted a high R² and Q² for both latent 328 variables (Supplementary Figure S2).

PLS-DA derived variable importance of projection (VIP) scores (built on the
first 30 metabolites with a VIP score higher than 1.4) revealed melezitose, gallic acid,
glutamine, and aspartic acid, among others, like the ones with the highest VIP scores
between the two treatments (Fig. 7c).

Finally, the cluster analysis on the top of the heat map (reporting in a false scale
color the variation of significantly different metabolite concentrations for each sample
and replicate) further confirmed total discrimination between the two treatments, which
clustered separately (Figure 7d).



Glyoxylate and dicarboxylate metabolism

Pantothenate and CoA biosynthesis

339 Fig. 6: (a) Pathway enrichment analysis revealed different metabolic pathways enriched during resveratrol treatment (P value cut off ≤ 0.05). (b) Results from "Pathway 340 341 Analysis" carried on the concentrations of metabolite identified in resveratrol treated and untreated seedlings. Total Cmpd: the total number of compounds in the pathway; 342 Hits: the matched number from the uploaded data; Raw p is the original P value, -343 344 Log(P) value: the logarithm of the original P-value calculated from the enrichment analysis; Holm adjust: the Holm adjustment used to counteract the problem of multiple 345 comparisons, FDR: the false discovery rate applied to the nominal P-values to control 346 for false-positive findings; Impact: the pathway impact value calculated from the 347 combination of enrichment and topology analysis. 348

29

23

7

1.51E-05

0.015483

4.8217

1.8101

0.00037688 3.15E-05 0.22451

0.092898 0.017371 0.21166

349

351 Table 1: Metabolites differentially accumulated in the control and resveratrol-treated

352 samples. Data were analyzed through Student's t-test (P \leq 0.05). A False Discovery Rate

353 (FDR) was applied to the nominal P-values to control for false-positive findings.

354 Negative values of the t-stat indicate a significant increase of the specific metabolite in

355 resveratrol-treated seedlings. (N = 6).

Metabolites	t.stat	p.value	FDR	Class
Phosphate	-37.609	4.21E-12	4.76E-10	
Galactose	-30.953	2.91E-11	1.64E-09	Sugar
L-Arabinose	-29.163	5.24E-11	1.97E-09	Sugar
N-Acetyl-D-glucosamine	-27.628	8.95E-11	2.18E-09	
Putrescine	27.422	9.63E-11	2.18E-09	Polyamine
DL-beta-Hydroxybutyric acid	25.241	2.18E-10	4.11E-09	
Glucosaminic acid	-24.524	2.90E-10	4.68E-09	
Inositol	-16.876	1.12E-08	1.58E-07	Sugar alcohol
Sophorose	-16.15	1.72E-08	2.15E-07	Sugar
Lactitol	-15.483	2.58E-08	2.91E-07	Sugar alcohol
Uridine 5'-diphospho-N-acetylglucosamine	-15.304	2.88E-08	2.96E-07	
Xylose	-13.096	1.28E-07	1.20E-06	Sugar
Palatinitol	-12.85	1.53E-07	1.33E-06	Sugar alcohol
Melibiose	-12.685	1.73E-07	1.40E-06	Sugar
Gentiobiose	-12.547	1.92E-07	1.45E-06	Sugar
Maltitol	-12.149	2.60E-07	1.84E-06	Sugar alcohol
Lactobionic acid	-12.07	2.77E-07	1.84E-06	
Glucose 6-phosphate	-10.682	8.66E-07	5.43E-06	
L-Rhamnose	10.146	1.39E-06	7.55E-06	Sugar
Galactinol	-10.123	1.42E-06	7.55E-06	Sugar alcohol
L-Glutamic acid	-10.114	1.43E-06	7.55E-06	Amino acid
L-Ornithine	-10.033	1.54E-06	7.55E-06	Polyamine
Lactose	-10.017	1.57E-06	7.55E-06	Sugar
Succinic acid	9.9904	1.60E-06	7.55E-06	Organic acid
L-Alanine	-9.9057	1.73E-06	7.84E-06	Amino acid
Lactulose	-9.3589	2.91E-06	1.23E-05	Sugar
L-Iditol	-9.346	2.94E-06	1.23E-05	Sugar alcohol
4-Hydroxyphenylacetic acid	-9.1653	3.51E-06	1.42E-05	
Trehalose	-8.8309	4.91E-06	1.91E-05	Sugar
Glyceric acid	8.4732	7.09E-06	2.67E-05	Organic acid
Cellobiose	-7.9975	1.18E-05	4.30E-05	Sugar
2,3-Dihydroxybenzoate	-7.8281	1.42E-05	5.03E-05	
Glycerol-3-galactoside	7.3477	2.46E-05	8.42E-05	
L-Aspartic acid	-7.1904	2.96E-05	9.84E-05	Amino acid
Arabinose	-7.1229	3.21E-05	0.000103	Sugar
Lysine	-7.1036	3.28E-05	0.000103	Amino acid
DL-Allothreonine	-6.77	4.92E-05	0.00015	Amino acid
5-Keto-D-Gluconate	-6.518	6.74E-05	0.000197	

L-Norleucine	-6.5114	6.80E-05	0.000197	Amino acid
L-Isoleucine	-6.3214	8.67E-05	0.000239	Amino acid
dehydroascorbic acid	-6.2487	9.52E-05	0.000253	
Glutaric acid	-6.2388	9.65E-05	0.000253	Organic acid
Creatinine	6.1681	0.000106	0.000272	
Uridine	5.8723	0.000157	0.000394	
Tartrate	-5.821	0.000168	0.000413	Organic acid
Threonic acid	5.7757	0.000179	0.00043	Organic acid
L-Serine	-5.6616	0.000209	0.00049	Amino acid
1,6-Anhydro-beta-D-Glucose	-5.6503	0.000212	0.00049	
Methylamine	-5.5625	0.00024	0.000534	
Isomaltose	-5.5594	0.000241	0.000534	Sugar
Xylitol	-5.4559	0.000279	0.000605	Sugar alcohol
Xylonolactone	-5.1024	0.000462	0.000986	
meso-Erythritol	-4.3951	0.001345	0.002815	Sugar alcohol
Oxamic acid	-4.3671	0.001406	0.002888	Organic acid
L-Norvaline	-4.2893	0.001588	0.003204	Amino acid
L-Valine	-4.2538	0.001679	0.003329	Amino acid
Galactosamine	-4.2064	0.00181	0.003527	
Glycine	-3.866	0.003129	0.005994	Amino acid
Oxalic acid	-3.7346	0.003881	0.007309	Organic acid
Glycerol	-3.5777	0.005031	0.009319	Sugar alcohol
Mannose	3.5198	0.00554	0.010098	Sugar
Methylmalonic acid	3.474	0.005981	0.010728	Organic acid
Fructose	-3.0832	0.011578	0.020442	Sugar
Hexacosane	-2.9796	0.013818	0.024022	Alkane
N-acetylornithine	-2.9698	0.01405	0.024055	
DL-Pyroglutamic acid	-2.9538	0.014442	0.024357	
Cafferic acid	-2.9263	0.015137	0.025154	
3-Amino isobutyric acid	-2.7135	0.021801	0.035703	





Fig. 8: (a) Principal component analysis (PCA) and (b) Partial least square discriminant analysis (PLS-DA) showing score plots discriminating the control (C), and resveratrol-treated (T) groups by virtue of the first 2 PCs. (c) PLSDA derived analysis variable importance of projection (VIP) features for the groups, and (d) overlay heat map of the significantly affected metabolites (selected by *t*-test with $P \leq 0.05$). Each square represents the different stage's effect on every metabolite's relative abundance using a false-colour scale. Colours dark red and blue indicate relative metabolite abundances, increased and decreased, respectively (n = 6).

371 DISCUSSION

372 Resveratrol is a stilbenoid compound produced by plants involved in plant defense 373 responses to abiotic and biotic stress (Chang et al., 2011; Hasan and Bae, 2017). 374 Although most studies had mainly focused on the potential antimicrobial, antibacterial 375 (Mattio et al., 2020) antioxidant activity in response to abiotic and biotic stress (Hasan 376 and Bae, 2017), few investigations have been carried out on resveratrol effect on plant 377 growth, regardless its role in the induction of the protective mechanisms (Bruno and 378 Sparapano, 2006; Li et al., 2019). Here we used L. sativa, a sensitive crop species to 379 natural and synthetic compounds (Macías et al. 2000) to study resveratrol effects on 380 germination and early seedlings growth. According to Mantovanelli et al. (2020), 381 resveratrol did not significantly affect the lettuce seed germination. Similar results were 382 also reported in radish seeds where resveratrol did not have intensive germination-383 stimulating properties, unlike protectors to ethanol seed sterilization treatment (Balanov 384 et al., 2020). Conversely, it significantly stimulated fresh and dry biomass of the aerial 385 part of lettuce, already at low concentrations, leaving unchanged the length and biomass 386 of root system. This positive effect was already observed in maize seedlings, although 387 only at 440 µM resveratrol concentration (Mantovanelli et al. 2020). In particular, the 388 fresh weight biomass increased in a dose-dependent manner ranging from 6.25-400 µM. By contrast, the positive effect on dry weight was observed at 100-400 µM range. 389 390 According to Mantovanelli et al. (2020), both primary root length and root fresh weight 391 were not affected by resveratrol, although a significant increase in root dry weight 392 biomass was observed at 200 µM resveratrol, confirming the trend already reported in maize (Mantovanelli et al., 2020). However, these results appeared in contrast with 393 394 Fang et al. (2010), which observed an inhibitory effect of this phytoalexin on root 395 lettuce growth. The highest concentration adopted by these authors could justify this 396 contrasting response. Thereby, our results indicated that the aerial part could be considered a main target of resveratrol; thus, we focused on a better understanding of 397 398 the resveratrol activity using the ED₅₀ value. One hundred µM treatment confirmed the 399 beneficial effect of this elicitor on lettuce aerial part (stem and leaves) and its ability to stimulate plant growth when exogenously applied (Pociecha et al., Mantovanelli et al., 400 2021). Mantovanelli et al. (2020) hypothesized that resveratrol is structurally similar to 401 the synthetic oestrogen diethylstilbestrol, naturally produced by plants, which 402 403 stimulated plant growth, cell division and pollen germination (Janeczco et al., 2005). 404 They further suggested a behaviour similar to brassinosteroids, which induced plants 405 tolerance under stress conditions by increasing the antioxidant activity (Farriduddin et 406 al., 2013) and stimulated plant growth (Clouse and Sasse, 1998). Thus, we hypothesized 407 that the stimulatory effect of resveratrol on lettuce could be linked to its potent ROS 408 scavenger ability (Stojanovic et al., 2001). In plants, ROS, generated in several 409 organelles (Dietz et al., 2016; Huang et al., 2016), included hydroxyl radicals ('OH) and 410 superoxide anions (O⁻²), and molecular states, hydrogen peroxide (H₂O₂), and singlet oxygen (1O2) (Apel and Hirt, 2004; Mittler et al., 2004). While ROS are important for 411 412 plant growth, performing many physiological processes (Elstner, 1987; Choudhury et 413 al., 2017), their overproduction, under various biotic and abiotic conditions, causes lipid 414 peroxidation, DNA and protein damage, resulting in perturbation of the cellular redox state that can ultimately lead to oxidative stress and cell death (Gill and Tuteja, 2010; 415 Dumnod and Rivoal, 2019). Interestingly, 100 µM resveratrol reduced the O⁻² 416 production, leaving unchanged the H2O2 content. In particular, the superoxide anion, 417 418 produced in chloroplasts, mitochondria, endoplasmic reticulum, and peroxisomes under 419 their normal metabolism (Sharma et al., 2012), is an unstable molecule (Juan et al., 2021) rapidly converted to hydrogen peroxide, permeable to the membrane. Trans-420 membrane NADP-oxidases (NOXs) and the mithocondrial and chloroplastic electron 421 422 transport chain (ETC) are the most important enzymes and organelles producing O⁻² 423 and H₂O₂ (Fisher et al., 2006). However, it is not clear whether resveratrol acts directly 424 as anti-ROS, or indirectly by blocking ROS production by enzymes such as NADPH 425 oxidase (NOX) enzymes or by influencing the expression of cellular pro- and 426 antioxidants. The down-regulation of NOXs after resveratrol treatment to protect 427 mammalian cells from oxidative functional damages is strongly demonstrated (Block and Gorin, 2012). Mantovanelli et al. (2021) demonstrated that at concentrations above 428 429 440 µM, resveratrol inhibited the respiration coupled to ADP phosphorylation and 430 NADH-oxidase, succinate-oxidase and ATP synthase activities in mitochondria isolated from Z. mays roots, conferring a beneficial effect on plant growth. 431 432 The importance of the antioxidant network in maintaining high rates of photosynthesis

has been demonstrated in many studies (Foyer and Shigeoka, 2011) since ROS overproduction and accumulation can also inhibit photosynthesis, limiting plant growth and yield (Mittler and Blumwald, 2010). Thus, the maintenance of O^{-2} low concentration by resveratrol could induce a greater photosynthetic efficiency. For example, by preserving ROS homeostasis, melatonin also helps to maintain a better

performance of the photosynthetic process under salinity stress (Yang et al., 2019). 438 439 Furthermore, Pociecha et al. (2014) demonstrated that resveratrol stimulated 440 photosynthetic efficiency during pathogenesis, influencing the energy flux parameter for 441 electron transport and improving the stability and efficiency of membranes. Among 442 Chlorophyll fluorescence (ChlF) parameters, the quantum efficiency of photostistem II 443 (PSII) in dark- and light-adapted conditions (Fv/Fm and Fv'/Fm') are usually good 444 indicators of photosynthetic activity, physiological function, as well as healthy and stress conditions, (Jia et al., 2019). In particular, Fv/Fm, which indicates the initial 445 maximal efficiency of photons captured by open PSII reaction centers (Butler, 2008), is 446 447 used as an indicator of health and plant growth (Feng et al., 2015) more than Fv'/Fm' 448 (Jia et al., 2019). For example, under a range of nitrogen (N) fertilizer, the Fv/Fm 449 increased along with N application (Liu et al., 2008). By contrast, a reduced value of 450 Fv/Fm was indicative of the probable physical damage at the level of the complex 451 antenna accompanied by a reduction in the PSII efficiency as observed under stress conditions such as drought (Maxwell and Johnson, 2000; Prieto et al., 2009). 452 453 Interestingly, the results indicated that all the chlorophyll fluorescence parameters 454 remained unchanged except for Fv/Fm whose ratio was higher in the resveratrol treated leaves, conferring a higher stability rate of the complex PSII/LHC and increasing lettuce 455 growth. The resveratrol action on photosyntesis may also be associated with the 456 457 stimulation of polyamines, which scavenge free radicals and activate some antioxidant 458 enzyme activities, subsequently reducing oxidative damage (Liu et al. 2015) and are 459 essential in the regulation of plant growth and development (Martin-Tanguy, 2001). In lettuce treated seedlings, a high level of L-ornithine, the precursor of polyamines, was 460 461 reported. Anyway, resveratrol's protective effect on PSII is negligible toto consider it as 462 a PSII enhancer.

On the other hand, resveratrol treatment did not affect stomatal density and size but 463 induced a higher stomatal width, suggesting an increased gas exchange in treated plants. 464 This phenomenon is commonly observed with natural compounds belonging to the 465 classes of phenols. For example, An et al. (2016) demonstrated that the accumulation of 466 467 flavonols in the guard cells, induced by an elicitor, is involved in ROS detoxification and the ABA-induced inhibition of stomatal closure. The stomatal width is an important 468 469 indicator of the stomatal aperture being related to a higher rate of CO2 exchange and photosynthetic efficiency. Therefore, the results suggest that resveratrol, more than 470

471 acting as a PSII protector and/or stimulating agent, could burst the metabolism, as also472 suggested by the metabolomic analysis.

473 Among the metabolic pathways, resveratrol significantly enriched the galactose 474 metabolism and the ascorbate and aldarate metabolism (the third most enriched 475 pathway). Both pathways are closely related to each other since -the galactose pathway 476 is involved in ascorbate biosynthesis (Smirnoff and Wheeler, 2000). Interestingly, 477 besides the high accumulation of galactose observed in resveratrol-treated plants showed an accumulation of dehydroascorbic acid (DHA). It should not be excluded 478 479 that the increase in DHA content could be due to the oxidation of AA during sample 480 handling and analysis, meaning that treated plants were particularly rich in ascorbate 481 content. In fact, it has been reported that AA is unstable in aqueous solutions under 482 aerobic conditions (extraction and derivatization conditions) being converted in DHA 483 (Levandoski et al., 1964; Dewhirst et al., 2018).

484 Among different pathways for ascorbate biosynthesis (Jain and Nessler, 2000; Agius et 485 al., 2003; Lorence et al., 2004), the galactose is one of the most important pathway 486 recently discovered. It is well known that high ascorbic acid content was positively 487 correlated with high galactose level induced by a higher activity of L-galactose-1-488 phosphate phosphatase (GPP) in rice (Zhang et al., 2015), or L-galactose 489 guanyltransferase, in Arabidopsis (Laing et al., 2007; Bulley et al., 2009) or Lgalactose DH, in tomato cultivars (Cervilla et al., 2007) or GPP and GME co-expression in 490 491 Nicotiana benthamiana (Laing et al., 2015).

492 The alterations in galactose and starch and sucrose metabolism were generally 493 underligned by a high accumulation of different classes of sugars including polyiols, 494 which play a pivotal role in providing carbon and energy for normal functioning of 495 cellular metabolism and in regulating growth and development of plants acting as signal 496 molecules. The osmoprotectant roles of sugars (glucose, fructose, threalose etc.) and 497 sugar alcohols (glycerol, inositol, maltitol etc), all stimulated by resveratrol treatment, have been widely accepted. They could regulate the osmotic adjustment and/or provide 498 499 membrane protection and ROS scavenging activity under stress (Kerepesi and Galiba 500 2000; Murakeozy et al. 2003Murakeozy et al. 2003; Ahmad and Sharma 2008; Livingston et al. 2009; Van den Ende and Valluru 2009; Koyro et al. 2012). Among 501 502 them, the trehalose should be mentioned in response to resveratrol treatment. . This 503 molecule plays an important role either in optimal or under stress conditions, acting as

504	an osmoprotectant or osmolyte protecting membranes, proteins and decreasing	
505	aggregation of denatured proteins (Ashraf and Harris 2004; Koyro et al. 2012).	
506	Furthermore, besides sugar accumulation, resveratrol-treated lettuce seedlings were	
507	characterized by an accumulation of several proteinogenic amino acids (glutamic acid,	
508	aspartic acid, alanine, among others), known to be involved either in osmoprotection or	
509	in protein biosynthesis and biomass production (Rai, 2002). The joint upregulation of	
510	the biochemical pathways involved in energetic and aminoacid metabolism could be the	
511	main reason for resveratrol-induced growth promotion.	
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604 Fig. S2: PLS-DA model's robustness was validation