Role of reactive oxygen species and isoflavonoids in soybean resistance to the attack of the southern green stink bug

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Abstract

22 Southern green stink bugs (*Nezara viridula* L.) are one of the major pests in many soybean
23 producing areas. They cause a decrease in yield and affect seed quality by reducing viability and
24 vigor. Alterations have been reported in the oxidative response and in the secondary metabolites
25 in different plant species due to insect damage. However, there is little information available on
26 soybean-stink bug interactions. In this study we compare the response of undamaged and
27 damaged seeds by *Nezara viridula* in two soybean cultivars, IAC-100 (resistant) and Davis
28 (susceptible), grown under greenhouse conditions. Pod hardness, H_2O_2 generation, enzyme
29 activities in guaiacol peroxidase (GPOX), catalase (CAT) and superoxide dismutase (SOD) as
30 well as lipoxygenase expression and isoflavonoid production were quantified. Our results
31 showed a greater resistance of IAC-100 to pod penetration, a decrease in peroxide content after
32 stink bug attack, and higher GPOX, CAT and SOD activities in seeds due to the genotype and to
33 the genotype-interaction with the herbivory treatment. Induction of *LOX* expression in both
34 cultivars and higher production of isoflavonoids in IAC-100 were also detected. It was then
35 concluded that the herbivory stink bug induces pathways related to oxidative stress and to the
36 secondary metabolites in developing seeds of soybean and that differences between cultivars
37 hold promise for a plant breeding program.

38

39 **Keywords**: Plant–insect interaction − *Glycine max* - *Nezara viridula* − Oxidative stress
40 − Herbivory − Lipoxygenase

41

Introduction

43 The stink bug complex is one of the most detrimental pests that affects soybean (*Glycine max* L.)
44 yields in many countries. In South America, the main species that impair soybean yields are
45 *Nezara viridula*, *Piezodorus guildinii* and *Euschistus heros*. They adversely affect seed vigor and
46 may even kill the embryo and stop germination. Brazil and Argentina are two of the most
47 important producers of this legume and damage caused by the mentioned species can result in
48 significant economic losses (Schaefer & Panizzi, 2000). Soybean crops destined for seed
49 production are less tolerant to stink bug damage than soybean crops used for animal feed (Jensen
50 & Newsom, 1972). While stink bugs can be controlled by spraying pesticides, there are
51 environmental and economic issues concerning the frequent use of these products. Biological
52 control can be mentioned as an alternative method to fight against this complex of insects. For
53 example, the well-studied egg-parasitoid *Trissolcus basalis* (Wollaston) has been used
54 successfully by farmers in Brazil (Corrêa-Ferreira & Moscardi, 1996). However, soybean host
55 plant resistance is an interesting strategy for managing plant breeding programs (Boerma &
56 Walker, 2005), and it is necessary to first understand the mechanisms related to stink bug
57 resistance in soybean breeding lines.
58 The soybean cultivar IAC-100 has been defined as resistant against stink bug attack (Campos *et*
59 *al.*, 2010; McPherson, Buss & Roberts, 2007; Piubelli *et al.*, 2003a; de Souza *et al.*, 2014;
60 Michereff *et al.*, 2011) and is used in breeding programs as a source of resistance (McPherson *et*
61 *al.*, 2007). This variety was developed by the Agronomic Institute of Campinas in Sao Paulo
62 (Brazil) by crossing breeding lines IAC 78-2318 and IAC-12 (Priolli *et al.*, 2002). Studies on this
63 genotype showed that stink bug attack caused less seed damage and less seed weight loss than
64 other genotypes (Campos *et al.* 2010). Previous studies found lower adult fresh weight of *Nezara*
65 *viridula* when fed on 'IAC-100' and less lipids in females than in those fed on the other
66 genotypes (Piubelli *et al.,* 2003b). However, the mechanisms through which soybean reacts to
67 stink bug attack are still unknown.
68 Plants respond to herbivore injury through several direct and indirect morphological,
69 biochemical and molecular mechanisms that help them avoid herbivore attack or affect its
70 performance (Fürstenberg-Hägg, Zagrobelny & Bak, 2013). Direct defense mechanisms affect
71 insect performance and feeding behavior, while indirect defense mechanisms can attract the
72 natural enemies of the herbivores and thus reduce plant loss (War *et al.*, 2012). Insect herbivory
73 induces early responses such us the oxidative burst (production of reactive oxygen species -
74 ROS), the expression of defense-related genes, and late responses such as callose deposition and
75 accumulation of proteinase inhibitors (Savatin *et al.*, 2014). Infestation of *Vicia faba* by *N.*
76 *viridula* stimulates significantly the production of H_2O_2 (Ederli *et al.*, 2017). Moreover, in poplar
77 the concentrations of H_2O_2 and malondialdehyde and the activities of ROS-scavenging enzymes,
78 such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), were enhanced by
79 herbivore wounding, suggesting that they are associated with insect resistance. The above-
80 mentioned enzymes are related to plant signaling, synthesis of defense compounds, and to
81 oxidative stress tolerance (Bi & Felton, 1995; Zhang, Hua & Zhang, 2008; Bednarski *et al.*,
82 2013; Zebelo & Maffei, 2014).
83 After insect attack the activation of defense genes that stimulate the production of the
84 antiherbivory compounds is induced due to internal signals such as calcium ion flow, a
85 phosphorylation cascade and responses mediated by hormones: jasmonic acid (JA) and ethylene
86 (ET) (Waternack, 2007; Howe & Jander, 2008; Browse, 2009). JA and its methyl ester, methyl
87 jasmonate (MJ), participate in the activation of plant defense mechanisms as signaling
88 compounds in processes related to production of various secondary metabolites (i.e. terpenoids,

89 phenylpropanoids, and alkaloids) (Wang *et al.*, 2015; Misra *et al.*, 2014). In the case of soybean,
90 isoflavones are the main chemical defense compounds against insects because they affect the
91 performance and survival of the herbivores (Piubelli *et al.*, 2003a). It is well known that MJ and
92 JA induce the accumulation of proteinase inhibitors as direct defense mechanisms against
93 herbivorous insects (Farmer & Ryan 1990; Farmer, Johnson & Ryan, 1992). Studies on soybean
94 analyzed the effects of jasmonic acid induction and showed that the soybean looper
95 (*Chrysodeixis includens* Walker) selects plant leaves that have not been induced by JA
96 (Accamando *et al.* (2012). In addition, soybean crops grown under conditions of high
97 concentrations of ambient CO_2 were unable to express genes related to the JA synthesis, which
98 made them highly susceptible to insect attack (Zavala *et al.*, 2008). The synthesis of jasmonates
99 and many other oxilipins is initiated by lipoxygenases (LOXs), which catalyze dioxygenation of
100 polyunsaturated fatty acids (reviewed by Blee, 2002, Feussner & Wasternack, 2002, Howe &
101 Schilmiller, 2002, Wasternack, 2007). LOX activity was higher in soybean plants that were less
102 vulnerable to the attack of *A. gemmatalis* when compared to controls (Fortunato *et al.* 2007).
103 Since three lipoxygenases, Lox1, Lox2 and Lox3 were detected in soybean seeds (Axelrod,
104 Cheesbrough & Laakso, 1981), it is possible that seed lipoxygenases regulate defenses against
105 stink bug attack. As a first step to develop a resistant cultivar, it is necessary to identify the
106 sources of resistance for any breeding program (de Morais & Pinheiro, 2012). Therefore, it is
107 important to determine the different characteristics of the diverse soybean cultivars as regards
108 plant defense against insect attack.
109 The aim of this study is to identify and quantify physical and biochemical soybean defense
110 mechanisms against the southern green stink bug, *Nezara viridula* attack. Our hypothesis is that
111 herbivory by stink bugs lead to biochemical changes and that there are differences between
112 cultivars at both biochemical and physical level. In this study we have compared soybean seed
113 response to stink bug damage of two cultivars, IAC-100 (resistant) and Davis (susceptible; Orr,
114 Boethel & Jones, 1985; Kester, Smith & Gilman 1984). Our results show that there are
115 differences between genotypes as regards the oxidative stress response and isoflavonoid
116 production in response to stink bugs attack. A better understanding of the resistance pathways
117 can help find ways to improve varieties.
118
Materials & Methods
120 *Insect and plant material*
121 In order to determine soybean resistance to stink bug damage (*Nezara viridula* L.) in developing
122 seeds, two soybean (*Glycine max* L.) cultivars, IAC-100 and Davis, were grown under
123 greenhouse conditions and stink bug adults were placed and allowed to feed on pods. Plants were
124 grown with 16:8 L:D light regime at 26±3°C, in pots with 3:1:1:1 soil:peat:sand:perlite mixture.
125 The colony of stink bugs consisted of field-collected individuals from the city of Chacabuco
126 (34°38′00″S 60°28′00″O) in the province of Buenos Aires (Argentina). The insects were reared
127 at our laboratory (School of Agronomy, University of Buenos Aires) on a diet based on hydrated
128 soybean seeds (cv. Williams) and peeled sunflower seeds, and were then provided water with
129 ascorbic acid (0.5 w/v %) (Giacometti *et. al.*, 2016).
130
131 *Experimental design*
132 Four experiments were carried out under the same greenhouse conditions in a completely
133 randomized design. In the first experiment, seventeen replications of each cultivar were used,
134 pod hardness was determined by collecting pods at the R6 soybean development stage (Fehr *et*

135   *al.*, 1971) and by using a texturometer. In order to determine oxidative stress response of
136   developing seeds to stink bug damage in the second experiment, a single adult stink bug was
137   bagged in on one soybean pod of plants (n=5) at the R6 development stage (Fehr *et al.*, 1971).
138   Pods of control plants were covered with tulle bags and collected 72 h after stink bugs started
139   feeding. Soon after, they were fast frozen in liquid nitrogen and stored at −80°C until analysis. In
140   addition, attacked and unattacked pods (one per plant) were collected for H_2O_2 detection in
141   developing seeds. Five replications of each cultivar were used for stink bug infestation with an
142   equal number of control plants. In the third experiment undamaged and damaged seeds by stink
143   bugs were collected to determine isoflavonoid accumulation after 72-hr herbivory. Finally, a
144   fourth experiment was performed to determine the LOX expression level in damaged and
145   undamaged seeds, and pods were collected 24-hr after herbivory and were fast frozen in liquid
146   nitrogen.

147

148   *Hardness determination on pods*
149   Hardness, the peak force required to penetrate a soybean pod, was measured with an INSTRON
150   4442 tensile tester calibrated to penetrate 12 mm at a rate of 2.0 mm sec^{-1} with a needle. The
151   tests were performed on both soybean cultivars at the R6 phenological stage and the needle was
152   inserted in pods at the second seed position. Results were expressed as gram-force per square
153   centimeter (gf cm-^{2}) and represented the maximum force required to penetrate a pod with the tip
154   needle in order to simulate the situation in which the sting bugs insert the stylus (de Santana
155   Souza *et al.*, 2013).

156

157   *Oxidative stress response*
158   Extracts for determination of catalase (CAT), superoxide dismutase (SOD) and Guaiacol
159   peroxidase (GPOX) activities were prepared from 0.3 g of soybean seeds homogenized under
160   ice-cold conditions in 3mL of extraction buffer, containing 50mM phosphate buffer (pH 7.4),
161   1mMEDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4°C. The homogenates were centrifuged
162   at 10 000 × g for 20 minutes and the supernatant fraction was used for the assays. Total protein
163   content was determined by following the Bradford method for protein quantification (1976).
164   CAT activity was determined according to a modified protocol of Chance, Sies & Boveris
165   (1979). The reaction was conducted in a quartz cuvette by measuring the decrease in absorption
166   at 240 nm in a reaction medium containing 150 μL of enzyme extract, 50mM potassium
167   phosphate buffer (pH 7.2) and 2mM $H_2O_2$for 2 minutes. CAT activity was determined using the
168   molar absorptivity of H_2O_2 at 240 nm and expressed as μmoles mg protein^{-1}min^{-1}.
169   SOD activity was assayed by the inhibition of the photochemical reduction of NBT (Becana *et*
170   *al.*, 1986). The reaction mixture consisted of 50, 100 and 200 μL of enzyme extract and 3.5mL
171    $O_2{}^{\bullet-}$ generating solution which contained 14.3mM methionine, 82.5 μM NBT, and 2.2μM
172   riboflavin. Extracts were brought to a final volume of 0.3mL with 50mM K-phosphate (pH 7.8)
173   and 0.1mM Na_2EDTA.
174   Sample tubes were shaken and placed in front of fluorescent lamps during 10 minutes. The
175   reduction in NBT was followed by reading absorbance at 560 nm. Blanks and controls were run
176   in the same way but without illumination and enzyme, respectively. One unit of SOD was
177   defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under the
178   assay conditions.
179   GPOX activity was determined according to the protocol devised by Shannon *et al.* (1966) which
180   consists in measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol

181 at 30°C in a reaction extract, 50 mM buffer K-phosphate 50 mM, pH 7, 0.1 mM EDTA, 10 mM
182 guaiacol and 10 mM H_2O_2. One unit of POD forms 1 μmol of guaiacol oxidised per min under
183 assay conditions.

184
185 *Detection of H_2O_2*
186 To visually analyze H_2O_2 generation, stink bug damaged seeds and their respective controls were
187 excised and immersed in a 1% solution of 3,3-Diaminobenzidine (DAB) for 24 h under light at
188 25 °C (Zilli *et al*., 2008). DAB generates a reddish-brown compound that could be detected at
189 the site of H_2O_2 formation in the presence of endogenous peroxidase activity. After staining,
190 formation of brown precipitates was documented by photography.

191
192 *Lipoxygenase expression*
193 The expression level of Lipoxygenase 1 (*LOX1*) and Lipoxygenase 2 (*LOX2*) genes in soybean
194 seeds was determined. Briefly, total RNA was isolated using the RNeasy Plant Mini kit
195 (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. RNA
196 concentration was determined with a fluorometer QubitTM (Invitrogen), and quality and quantity
197 were assessed spectrophotometrically before the cDNA was synthesized using the Thermo
198 Scientific RevertAid Reverse Transcriptase in a Bio-Rad My CyclerTM Termal Cycler.
199 An ABI 7500 Fast Real-Time PCR system was used to perform the qRT-PCR (Applied
200 Biosystems, Foster City, CA, USA) with the SYBR Green Real-time Master Mix. The *LOX1* and
201 *LOX2* primer sequences in this study are detailed in Table 1. The housekeeping soybean
202 elongation factor (*ELF1b*) was used as a reference gene for relative quantification and the target
203 expression relative to the housekeeping was used for ANOVA.

204
205 *Isoflavonoid determination*
206 Isoflavonoid accumulation was determined after 72 h of herbivory treatment in seeds at R6
207 according to Fehr et al (1971), the lipid phase of seeds was removed with cyclohexane (Zavala *et*
208 *al*., 2015). 0.2 g of pulverized seeds were placed in a 50 ml tube and mixed with 10 mL of
209 cyclohexane and incubated for 6 hours. Then, tubes were centrifuged at 1500 rpm for 10
210 minutes. Supernatant was discarded and the pellet was used for the extraction step. Isoflavonoids
211 were extracted with methanol plus rutin that was used as an extraction internal control (0.1 g/ 5
212 ml MeOH). 10 mL of methanol were added to 50 mL tubes and incubated for 6 hours at room
213 temperature. Then tubes were centrifuged at 1500 rpm for 10 minutes. Supernatant was saved.
214 The solvent was then evaporated at 40 °C and samples were re-dissolved with 500 µL of MeOH.
215 Samples were purified with a C18 Silica Cartridge (Agilent Technologies, Palo Alto, CA, USA)
216 using different mixtures of MeOH–H2O. Aliquots of 5 µL were subjected to high-performance
217 liquid chromatography (HPLC; Agilent 1100 A series) using a reverse-phase octadesyl column
218 (Eclipse XDB-C18 4.6 × 150 mm, 5 µm). Isoflavonoids were eluted using a mobile phase
219 gradient of 15–60% acetonitrile in 0.1% acetic acid for 60 min at a flow rate of 1 mL min−1.
220 Compounds were measured with the detector set at λ 270 nm. Retention times and quantitative
221 data for daidzin, daidzein, genistin and genistein were obtained by comparison to known
222 standards (all from Sigma-Aldrich, St. Louis, MO, USA). External standard curves were
223 performed for isoflavonoid quantification.

224
225 *Statistical analysis*

226 The experiments were set up as a completely randomized design with factorial treatments. One
227 factor was the genotype (IAC-100 and Davis) and the second was insect damage (control and
228 treated). Data were analyzed with the Infostat v. 2011 statistical package (Di Rienzo *et al*.,
229 2011). Hardness analysis of pods, and SOD, CAT and GPOX activities as well as seed
230 isoflavonoid contents in each experiment were analyzed using analysis of variance (ANOVA).
231 Data on isoflavonoid content were transformed as needed to meet the assumptions of ANOVA
232 by square root transformation. We applied the Tukey pairwise multiple comparisons procedure
233 for studying GENOTYPE and TREATMENT effects.
234 Hierarchical clustering of oxidative stress enzymes was performed with the same data used for
235 ANOVA using Euclidean distances in Infostat.
236

Results

238 *Damage and hardness assessment of pods*
239 Oxidative damage caused by stink bugs was observed by visual analyses of H_2O_2 generation.
240 Davis seeds affected by *Nezara viridula* exhibited highly enhanced brownish staining compared
241 to the IAC-100 genotype (Figure 1). Textural analysis of pods showed that IAC-100 has greater
242 resistance to penetration than Davis ($p < 0.05$; Figure 2). This can be a physical barrier to the
243 insect's stylet penetration.
244

245 *Stress metabolism*
246 According to our results, the cluster analysis of oxidative stress enzyme activities (CAT, SOD,
247 GPOX) that was carried out using the statistical package Infostat v. 2011 (Di Rienzo *et al*., 2011)
248 showed two main clusters that grouped the genotypes with the treatments. Figure 3 A shows one
249 cluster containing IAC-100 treated with stink bugs, and the second cluster containing IAC-100
250 control, Davis treated and Davis control. When exposed to insect treatment, the cluster
251 distribution suggests a differential induction of enzyme activities between the resistant and
252 susceptible genotypes. In addition, there is no Genotype x Treatment interaction in CAT activity
253 and the differences are due to Genotype and Treatment ($p < 0.05$; Figure 3 B). Although the CAT
254 enzyme is induced in both genotypes, its activity is always higher in IAC-100 than in Davis.
255 SOD activity was significantly greater in IAC-100 ($p < 0.05$) when affected by stink bugs (Figure
256 3 C). When comparing cultivars, GPOX showed a higher activity in IAC-100 both with and
257 without damage caused by the insect ($p < 0.05$, Figure 3 D).
258

259 *Lipoxygenase expression quantification*
260 Expression of *LOX1* and *LOX2* was induced by herbivory in both soybean cultivars when
261 compared to their respective controls (Fig 4). It suggests that soybean plants respond to this type
262 of stress by increasing *LOX* expression after 24 h following treatment ($p < 0.05$).
263

264 *Isoflavonoids*
265 Concentrations of isoflavonoids in soybean seeds showed differences between cultivars and
266 types of compounds (Figure 5). After herbivory, the concentration of daidzin and genistin
267 increased in both genotypes and constitutive concentrations were similar and very low ($p < 0.05$;
268 Figure 5 A and C). Higher levels of daidzein were found in the IAC-100 genotype, regardless of
269 the treatment, with a strong uprising trend under herbivory ($p < 0.05$; Figure 5 B). Except for one
270 repeat of the cultivar Davis genistein was not detected in any treatment (supplementary
271 information).

272

Discussion

274 Previous experiments with the IAC-100 soybean genotype reported low incidence of stink bugs
275 (McPherson *et al.*, 2007). This cultivar has PI 229358 (Clement & Quisenberry, 1998) and PI
276 274454 (Souza *et al.*, 2015) in its genealogy (Carrao-Panizzi & Kitamura 1995), which exhibits a
277 high degree of resistance to the stink bug complex. Some studies have shown antixenosis in IAC-
278 100 against *N. viridula, P. guildinii* and *E. heros* (Campos *et al.*, 2010; Silva *et al.*, 2014 and de
279 Souza *et al.*, 2014), and biological effects through isoflavonoids on *N. viridula* (Piubelli *et al.*,
280 2003a). Our study suggests that cultivar IAC-100 maybe more tolerant to the southern green
281 stinkbug due to greater hardness in pods, which serves as the first line of defense. In addition, a
282 higher antioxidative enzyme activity was involved in plant defense, and it showed high levels of
283 isoflavonoids in response to insect attack which, according to some authors (Lane *et al*., 1985;
284 Murakami *et al.*, 2014; Simmonds & Stevenson, 2001) affect the performance and survival of the
285 herbivores.
286 Antixenosis in resistant plants can be related to morphological factors such as thickened plant
287 epidermal layers, waxy deposits on leaves, stems, or fruits, nutritional deficiency, and chemical
288 compounds (Smith, 2005). The thickness of various plant tissues influences the degree of
289 resistance in some crop cultivars. Stink bug (*N. viridula*) damage elicits activation of MAPK
290 signal in soybean seeds and induced salicylic acid that induced genes related with cell wall
291 restructuration and increased lignin content, which can increase resistance to new insect attack
292 by hardening cell walls (Giacometti *et al.*, 2018). In this study, pods of IAC-100 cultivar showed
293 greater resistance to penetration when compared to those of Davis, suggesting that hardening
294 may be a physical barrier to insect feeding (Figure 2).
295 Under stress conditions in plants, such as insect feeding, many signaling pathways are activated
296 by different types of ROS causing "oxidative burst" (Maffei, Mithöfer & Boland, 2007). After
297 insect attack, ROS are accumulated acting as the first barrier against the attack of pathogens and
298 herbivores. However, to avoid self-toxicity by ROS, plant cells have ROS scavenging systems
299 that help to remove the excess concentration and maintain a relatively low and constant one (War
300 *et al.*, 2012). Tolerance in plants is generally related to the control capacity of the cellular redox
301 state resulting in lower oxidative damage (Slesak *et al.* 2007). ROS accumulation was detected
302 after the feeding period of the chewing caterpillar *S. littoralis* or the piercing-sucking action of
303 the spider mite *Tetranychus* urticae (Maffei *et al.,* 2006; Afify *et al.,* 2011). Infestation of lima
304 beans by *S. littoralis* revealed, trough diaminobenzidine (DAB) staining, accumulation of H_2O_2
305 around the wounded area (Maffei *et al.,* 2006). In our study we found that IAC-100 had less
306 accumulation of H_2O_2 after insect feeding when compared to the susceptible genotype, which
307 suggests that it has a higher capacity to control the redox state. A previous study demonstrated
308 that ROS detoxification enzymes were only induced in H_2O_2-accumulating resistant lines (Moloi
309 & van der Westhuizen 2008). POD is one of such group of antioxidative enzymes, which
310 scavenges the ROS besides having other defensive roles because they are also an important
311 component of the immediate response of plants to insect damage (War *et al.*, 2012). Catalases
312 remove the H_2O_2 and reduce H_2O_2 to $2H_2O$. And SOD constitutes a frontline in the defense
313 against ROS as they catalyze the dismutation of O_2^-. (superoxide radical) to H_2O_2 (Caverzan,
314 Casassola & Brammer, 2016). In wheat, an increase in *SOD* transcript in response to differential
315 heat shock treatment was related to an enhanced tolerance to environmental stresses (Kumar *et*
316 *al.*, 2013). The present study demonstrates that the resistant cultivar had more control of the

317 cellular redox state given its high level of enzyme activity after herbivory in SOD, GPOX y CAT
318 (Figure 3).
319 Many studies have shown that LOXs are implicated in the defense systems of several plant
320 species against pathogens and insects (Koch *et al.*, 1992; Melan *et al.*, 1993; Felton *et al.*, 1994;
321 Christensen *et al.*, 2013). Induction of LOX activity after herbivory has previously been studied
322 in tomato in response to *Helicoverpa armigera* (Yan *et al.,* 2013), in Arabidopsis following
323 infestation by *Myzus persicae* and in lima bean in response to *Tetranychus urticae (*Moran &
324 Thompson, 2001; Arimura *et al.*, 2000*)*. In developing seeds of soybean, we found an induction
325 of *LOX1* and *LOX2* after herbivory in both genotypes under study suggesting that this pathway is
326 activated in response to the damage caused by *Nezara viridula*.
327 LOX activity regulates the production of the hormone JA, which modulates flavonoids and
328 isoflavonoids that protect plants against insect pests and affect the behavior, growth and
329 development of insects (Simmonds, 2003). In Arabidopsis, resistance against *Spodoptera*
330 *frugiperda* is enhanced overexpressing a transcription factor that controls flavonoid production
331 (Johnson & Dowd, 2004). In soybean leaves, flavonoids negatively affected the behavior of
332 *Aphis glycines* and *Trichoplusia ni* (Meng *et al.*, 2011; Neupane & Norris 1990). Moreover,
333 soybean seed damage caused by soybean pod borer (*Leguminivora glycinivorella*) was
334 negatively correlated with higher levels of isoflavonoids, and showed positive correlations
335 between isoflavonoid content in pods and undamaged seeds in treatments with stink bugs
336 (*Nezara viridula* and *Piezodorus guildinii*) (Zhao *et al.,* 2015; Zavala *et al.,* 2015). Our results
337 reveal that stink bug herbivory differentially promoted isoflavonoid production between
338 genotypes with variations according to the type of compound. It can thus be concluded that not
339 only total production of isoflavonoids but also the type of isoflavonoid produced can affect insect
340 behavior.
341

Conclusions
343 Taken the results together, it can be inferred that stink bug herbivory injury generates cell wall
344 changes, induces pathways related to oxidative stress and secondary metabolites in developing
345 seeds of soybean. As a result of the resistance characteristics to insects, such as control of
346 cellular redox state and production of secondary metabolites, IAC-100 is a promising cultivar for
347 a breeding program. However, further studies are still required to understand the functions of
348 genes and the regulatory factors involved in defense responses in order to develop molecular
349 markers for a breeding program.
350 The study of host plant resistance is an important alternative for producers in Argentina, but even
351 more in Brazil, not only because of the high insect pressure but also for the phytopathogenic
352 fungi that lead to the frequent use of chemical control.
353

Acknowledgements

355 GDM Seeds for the availability for the use of greenhouses and other infrastructure. Verónica
356 Feuring and Alina Crelier for technical assistance. Romina Giacometti and Natalia Ilina for the
357 help in molecular biology methods.

358

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Figure 1

H_2O_2 generation in sites damaged by stink bug.

Brown color shows a more affected condition. Images were acquired with a digital camera Nikon Coolpix L110.

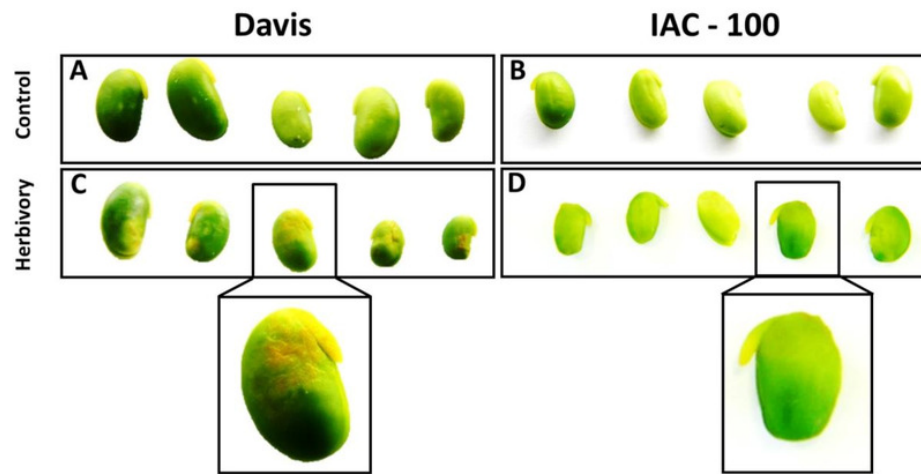



Figure 2

Mean hardness of soybean pods in R6

Means values (bars) and SEM (whiskers) are shown. Different letters are significantly different ($p<0.05$ - n=17). Data source in Supplemental Information.

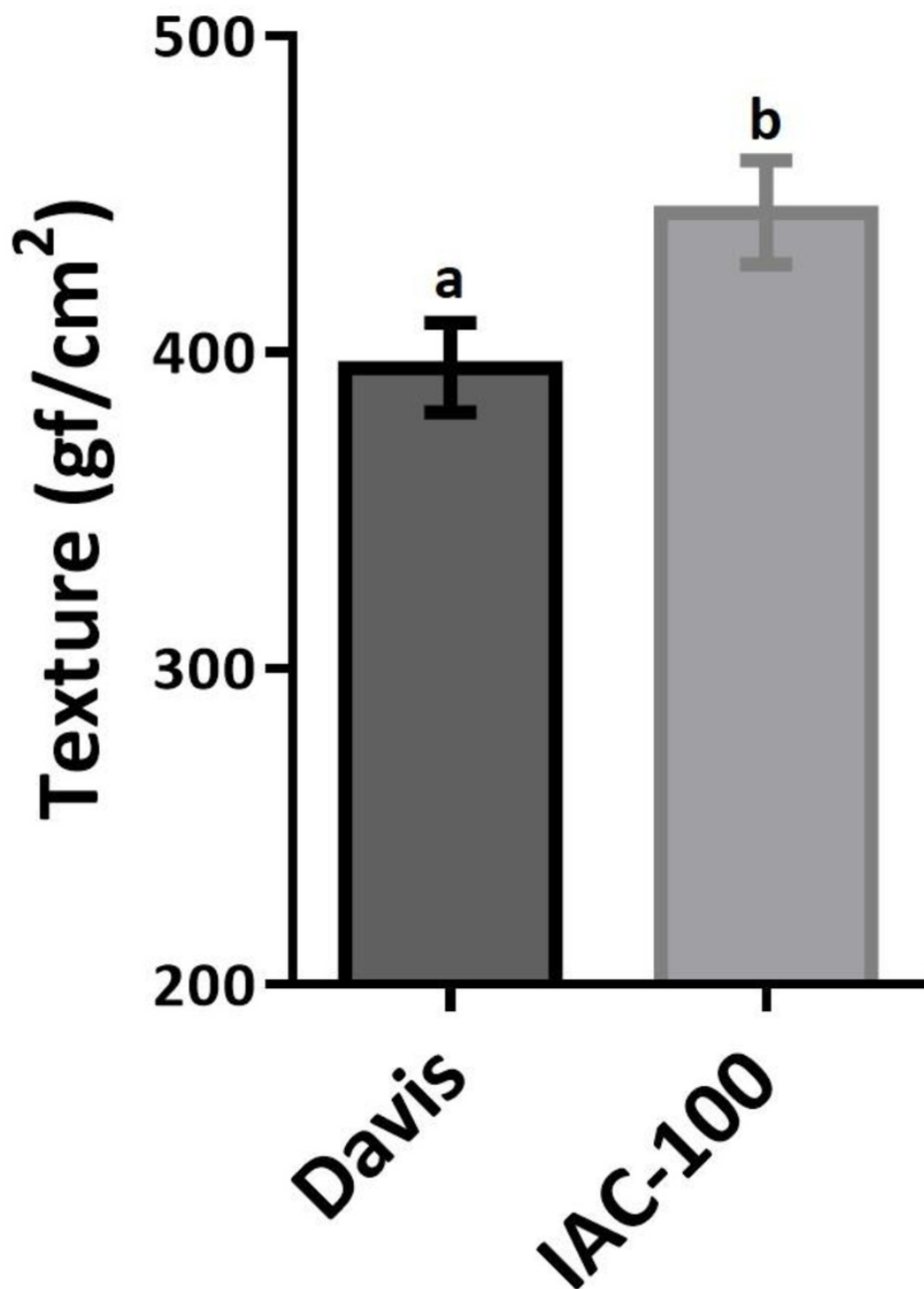



Figure 3

Oxidative stress analysis in seeds of different soybean genotypes damaged by *Nezara viridula* and their respective controls without herbivory

(A) Cluster analysis of oxidative stress enzymes. (**B**) Catalase activity. (**C**) Superoxide dismutase activity. (**D**) Guaiacol peroxidase activity. Means values (dots and squares) and SEM (whiskers) are shown. Different letters are significantly different ($p < 0.05$). Data source in Supplemental Information.

A *Average linkage*

Distance: Euclidean

IAC-100_herbivory

IAC-100_control

Davis_ herbivory

Davis_control

0.00   0.85   1.70   2.54   3.39

B

- ⊖ Davis
- ■ IAC-100

CAT (pmol.mg-1 protein)

300

200

100

0

ab

b

a

a

Control   Herbivory

C

- ⊖ Davis
- ■ IAC-100

SOD (U mg-1 protein)

2.0

1.5

1.0

0.5

0.0

a

a

b

a

Control   Herbivory

D

- ⊖ Davis
- ■ IAC-100

GPOX (µmoles/min*mg protein)

0.8

0.7

0.6

0.5

0.4

0.3

ab

a

b

a

Control   Herbivory

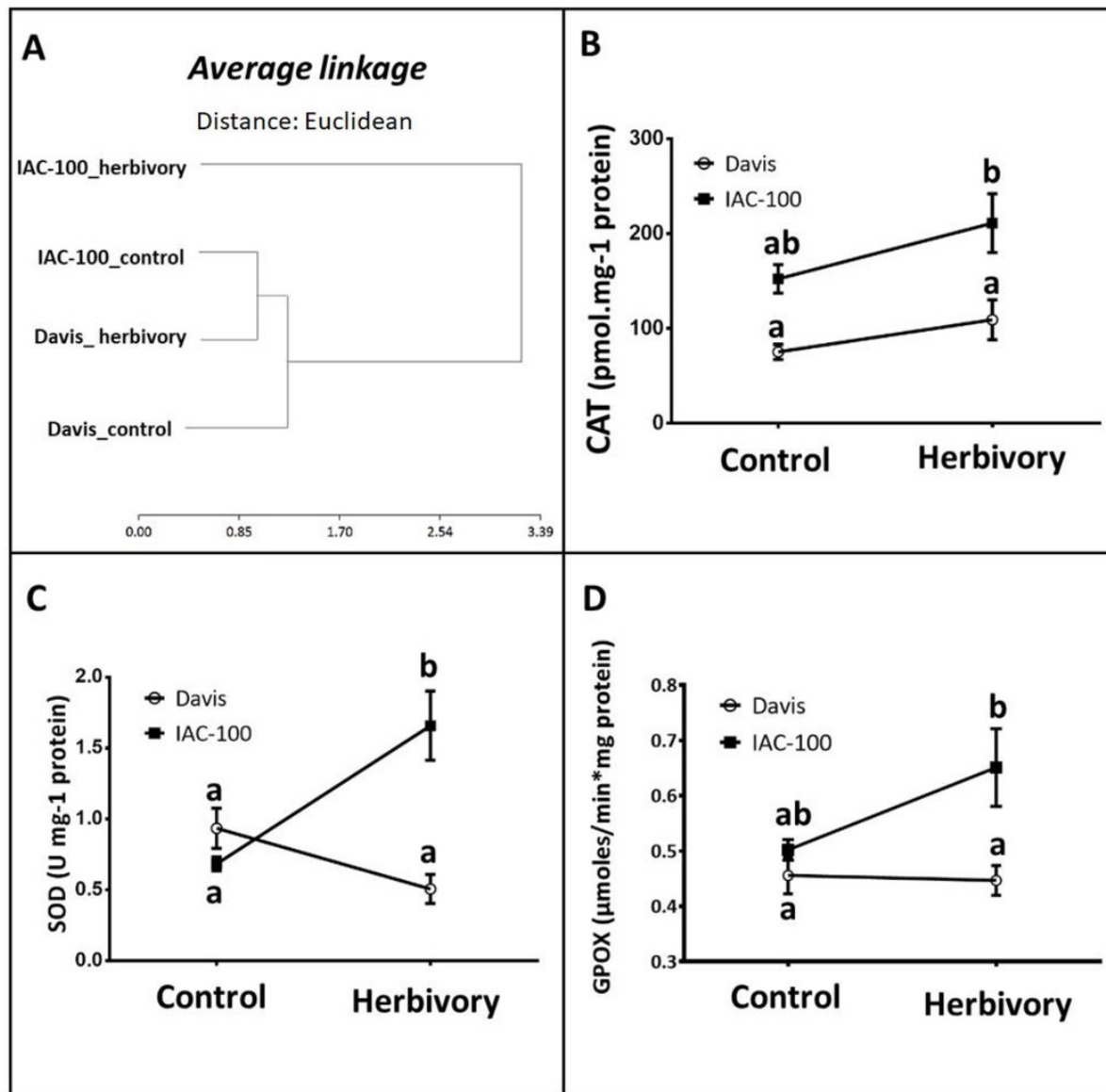



Figure 4

Gene expression by Real-Time PCR of LOX1 and LOX2 in soybean seeds, either undamaged and damaged by *Nezara viridula*

(A) LOX1. (B) LOX2. Gene expression is relative to the soybean elongation factor (**ELF1b**). Means values (bars) and SEM (whiskers) are shown. Different letters are significantly different ($p < 0.05$). Data source in Supplemental Information.

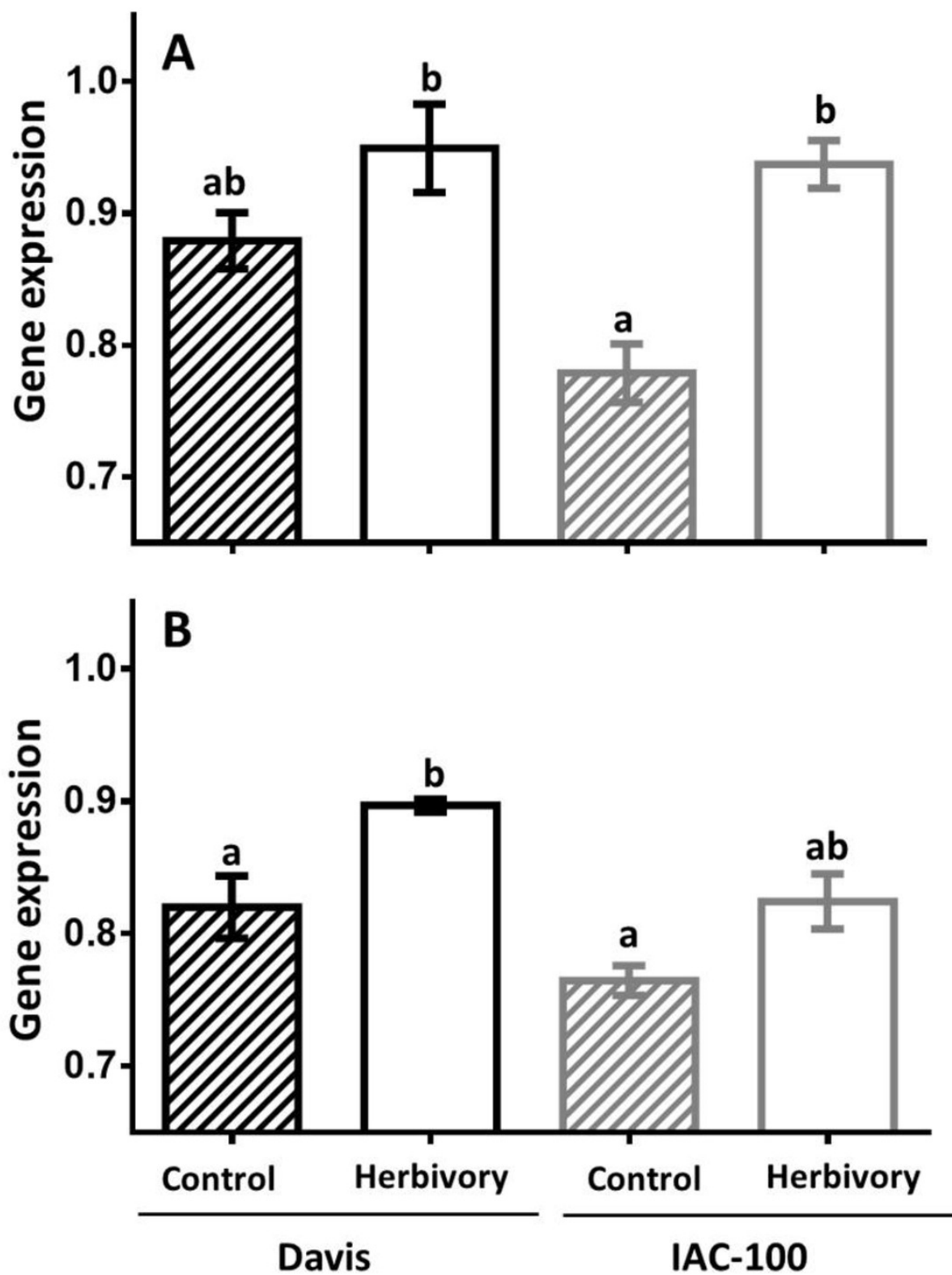
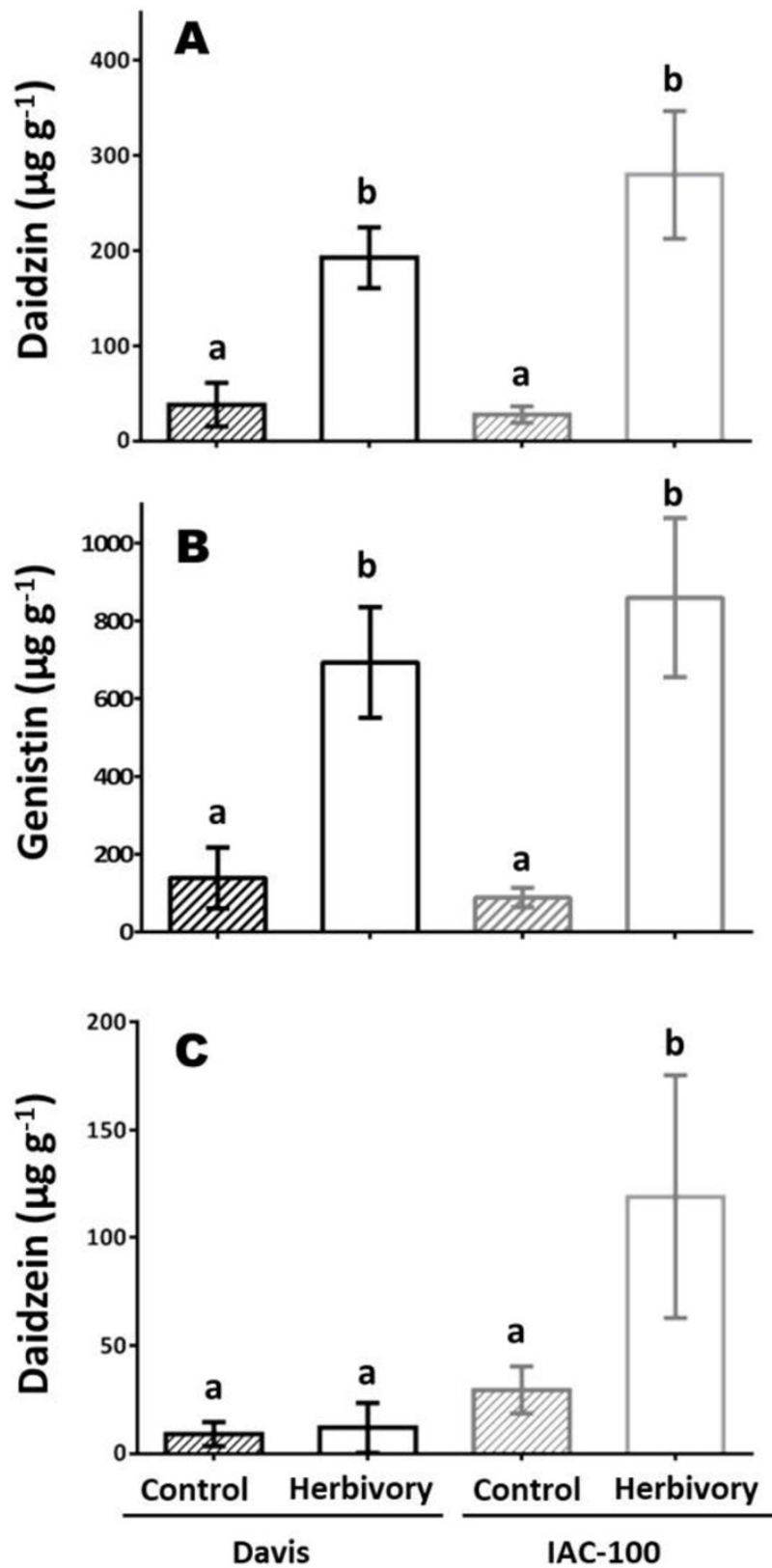



Figure 5

Isoflavonoid concentration in seeds of cultivars Davis and IAC-100 damaged by stink bugs and their respective controls without herbivory

(A) Daidzin concentration. **(B)** Genistin concentration. **(C)** Daidzein concentration. Means values (bars) and SEM (whiskers) are shown. Different letters are significantly different ($p<0.05$). Data source in Supplemental Information.

LOX1, LOX2 and ELF1b primer sequences

LOX1	Forward:	5' CTGGTGTAAATCCCTGCGTAA 3'	Chen *et al.* (2012)
	Reverse:	5' TACCAAGTGCCTCGTCCATT 3'	
LOX2	Forward:	5' AGATGGTTGCGGGTGTAAAT 3'	Chen *et al.* (2012)
	Reverse:	5' GGGCATCTGCTGTTATCTTAC 3'	
ELF1b	Forward:	5'- GTTGAAAAGCCAGGGGACA -3'	Jian *et al.* (2008)
	Reverse:	5'- TCTTACCCCTTGAGCGTGG -3'	