

1 **Nitro-Oleic Acid Induced Reactive Oxygen Species Formation and**
2 **Plant Defense Signaling in Tomato Cell Suspensions**

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25 *One-sentence summary:* Nitrated fatty acids act as signaling molecules in
26 tomato cells inducing ROS, reducing glutathione cellular pool, reacting
27 with protein thiols and free GSH and triggering plant defense responses.

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29 *List of author contribution:* AML and FJS conceived the project. AADP
30 performed most of the experiment and analyzed the data. LDF, JMD, SRS
31 and GG performed some of the experiment. CGM, AML and FJS designed
32 and supervised the experiments and analyzed the data. AADP and AML

33 wrote the article with contributions of all the authors.

34

35 *Funding information:* This work was supported by the UNMdP, Consejo
36 Nacional de Investigaciones Científicas y Técnicas, CONICET: PIP 219
37 and CONICET-NIH (to AML), Agencia Nacional de Promoción Científica y
38 Tecnológica, ANPCyT; PICT-Raices 2013-0800 (to AML), NIH GM125944
39 (to FJS) and AHA 17GRN33660955 (to FJS).

40

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43 *Key words:* nitro-oleic acid, tomato cell suspension, ROS, glutathione,
44 signalling, plant defense

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46 **Abbreviations**

47 •NO₂: nitrogen dioxide

48 •NO: nitric oxide

49 FA: fatty acid

50 GSH: reduced glutathione

51 H₂O₂: hydrogen peroxyde

52 NO₂-FA: nitro fatty acids

53 NO₂-Ln: nitro-linolenic acid

54 NO₂-OA: nitro-oleic acid

55 OA: oleic acid

56 ROS: reactive oxygen species

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59 **ABSTRACT**

60 Nitrated fatty acids (NO₂-FAs) are formed by the addition reaction of nitric
61 oxide- and nitrite-derived nitrogen dioxide with unsaturated fatty acids.
62 Nitrated fatty acids act as signaling molecules in mammals through the
63 formation of covalent adducts with cellular thiols. The study of NO₂-FAs in
64 plant systems constitutes an interesting and emerging area. The presence
65 of NO₂-FA has been reported in olives, peas, rice and in Arabidopsis. To
66 gain a better understanding of the role of NO₂-FA on plant physiology, we
67 analyzed the effects of exogenous application of nitro-oleic acid (NO₂-OA)
68 to tomato cell cultures. We found that NO₂-OA induced reactive oxygen
69 species (ROS) production in a dose-dependent manner via activation of
70 NADPH oxidases, which requires calcium entry from the extracellular
71 compartment and protein kinase activation, a mechanism that resembles
72 the plant defense responses. NO₂-OA induced ROS production,
73 expression of plant defense genes and led to cell death. The mechanism
74 of action of NO₂-OA involves a reduction in the glutathione cellular pool
75 and covalently addition reactions with protein thiols and reduced
76 glutathione. Altogether, these results indicate that NO₂-OA triggers
77 responses associated with plant defense, revealing its possible role as a
78 signal molecule in biotic stress.

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81 INTRODUCTION

82 Fatty acids (FA) not only provide structural integrity and energy for
83 various metabolic processes to the plant cell but can also function as
84 signal transduction mediators (Lim et al., 2017). As an example, oxylipins
85 are oxygenated FAs, many of which are electrophilic species involved in
86 plant defense against biotic and abiotic stresses (Lim et al., 2017; Farmer
87 and Mueller, 2013).

88 Electrophilic nitro-fatty acids (NO₂-FAs) are formed by the addition
89 reaction of nitric oxide (•NO)- and nitrite (NO₂⁻)-derived nitrogen dioxide
90 (•NO₂) to unsaturated fatty acids, in particular those containing conjugated
91 double bonds (Schopfer et al., 2011; Baker et al., 2009). Electrophiles
92 contain an electron-poor moiety, conferring attraction to electron-rich
93 nucleophiles that donate electrons to form reversible covalent bonds via
94 Michael additions (Chattaraj et al., 2006). In this regard, the electrophilic
95 reactivity of nitroalkenes facilitates reversible addition reaction with cellular
96 nucleophilic targets (e.g. protein Cys and His residues and reduced
97 glutathione, GSH, Baker et al., 2007; Batthyany et al., 2006). This
98 reactivity supports the post-translational modification of proteins, affecting
99 their distribution and/or function. In addition, NO₂-FA has been reported to
100 act as •NO donors under certain conditions (Schopfer et al., 2005;
101 Gorczynsk et al., 2007; Mata-Perez et al., 2016).

102 The study of NO₂-FAs in plant systems constitutes an interesting
103 and emerging area of investigation. The presence of nitroalkenes in plants
104 was first reported in extra-virgin olive oil and linked to the beneficial effects
105 of the Mediterranean diet on human health (Fazzari et al., 2014). In
106 addition, NO₂-FAs were later detected in Pea (*Pisum sativum*) and Rice
107 (*Oryza sativa*) (Mata-Perez et al., 2017). Likewise, in cell suspensions of
108 the model plant *Arabidopsis thaliana*, Mata-Perez et al., (2015) reported
109 the presence of the nitroalkene nitro-linolenic acid (NO₂-Ln). The levels of
110 these NO₂-FAs were modulated by both developmental stages and abiotic
111 stresses (NaCl, low temperatures, cadmium or wounding). Moreover,
112 treatments of *Arabidopsis* cell cultures with exogenous NO₂-Ln induced

113 differential gene expression related to oxidative stress responses as well
114 as up-regulation of several heat shock response genes (Mata-Perez et al.,
115 2015). In addition, in Arabidopsis roots and cell suspensions, NO₂-Ln
116 treatments induced •NO production (Mata-Perez et al., 2016).

117 Nitric oxide and reactive oxygen species (ROS) are signaling
118 molecules involved in abiotic and biotic stress responses in plants. In this
119 regard, tomato cell suspensions treated with pathogen-derived molecules,
120 called elicitors like xylanase or chitosan displayed increased ROS and
121 •NO production and induced plant-defense gene expression and cell death
122 (Laxalt et al., 2007; Raho et al., 2011). During plant defense, NADPH
123 oxidase activity of the Ca²⁺ and phosphorylation-dependent RBOHD (from
124 respiratory burst oxidase homolog D) is upregulated, leading to increases
125 in ROS production (Kadota et al., 2015). Thus, these physiological
126 conditions where •NO and ROS are produced, provide a favorable
127 chemical environment for the nitration of unsaturated fatty acids. Herein,
128 we analyzed the signaling effects of exogenous treatment of tomato cell
129 cultures with NO₂-OA, with a particular focus on the induction of plant
130 defense responses.

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133 **RESULTS**

134 **NO₂-OA is Internalized and Metabolized in Tomato Cells.**

135 NO₂-FAs are hydrophobic fatty acids with poor solubility in aqueous
136 solutions. Thus, we first sought to analyze binding and internalization of
137 NO₂-OA by tomato cell suspensions. Figure 1A shows that NO₂-OA
138 effectively bound to tomato cells, reducing the remaining levels in media.
139 Moreover, analysis of metabolic products of NO₂-OA in treated cells
140 revealed that NO₂-OA is internalized and metabolized. In this regard, β-
141 oxidation products and nitroalkene reduction products were detected
142 (Figure 1B). These metabolites are a consequence of enzymatic reactions
143 that take place in the cytoplasm and mitochondria of cells. These results
144 indicate that NO₂-OA is effectively internalized into the cell and therefore

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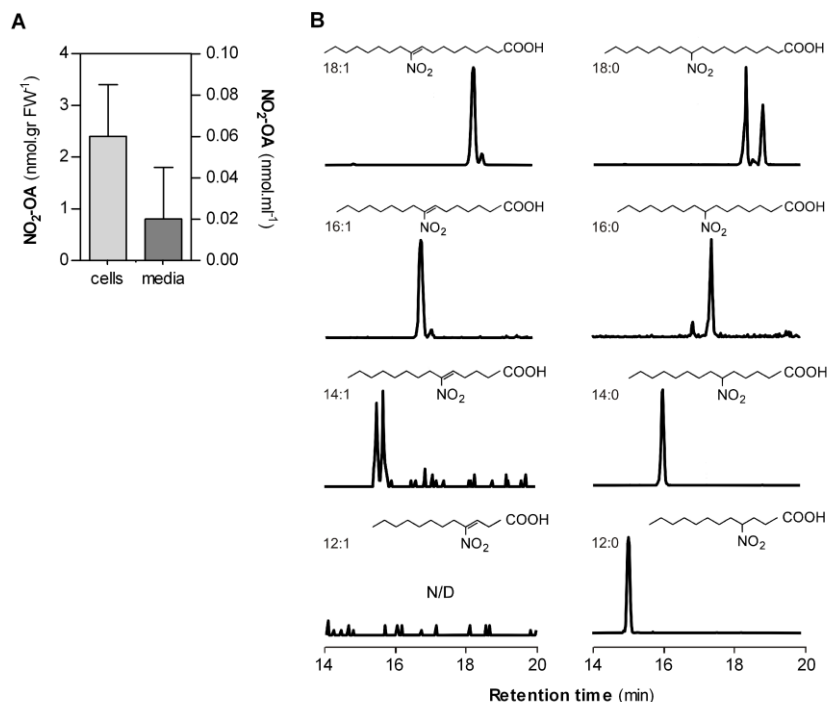


Figure 1. Detection and quantification of exogenous NO₂-OA added to tomato cell suspensions and metabolic products.

Tomato cell suspensions were incubated with 10 μM NO₂-OA for 1 h and then NO₂-OA and its metabolic products were analyzed by HPLC-MSMS. A, Quantification of NO₂-OA in tomato cells or in the suspension media. Graph shows media with standard errors (n=3). B, Representative chromatographic profiles of NO₂-OA (left panel) and NO₂-18:0 (right panel) and their β-oxidation products respectively found in tomato cells. N/D: not detected.

could be used as a model for the evaluation of NO₂-FA physiological responses associated with its exogenous application.

NO₂-OA Induces ROS but not •NO Production in Tomato Cells

Bioinformatics analysis of RNAseq data in Arabidopsis cell suspensions revealed that a large number of NO₂-Ln-induced genes were related to oxidative stress response, mainly depicted by hydrogen peroxide (H₂O₂) and reactive oxygen species (Mata-Perez et al., 2015). In this regard, we tested if NO₂-OA could induce ROS production in tomato cell suspensions. As a control, we compared the response to oleic acid (OA), the non-nitrated backbone of NO₂-OA. Figure 2A shows an increase in the fluorescence signal of NO₂-OA-treated cells in a dose-dependent manner. Time course analysis showed that extending incubation times led to an

177 increase in ROS production, with the exception of the 16 h treatment at
178 100 μM $\text{NO}_2\text{-OA}$, where a decrease in ROS production was observed
179 compared to 6 h. In the case of OA, none of the assayed conditions
180 displayed any change in ROS production (Figure 2A). Fluorescence
181 microscopy of tomato cells treated with 100 μM of $\text{NO}_2\text{-OA}$ for 6 h showed
182 a significant increase in the fluorescent signal (Figure 2B).

183 In order to further validate ROS production in $\text{NO}_2\text{-OA}$ -treated cells,
184 we used two alternative methodologies. First, H_2O_2 production was
185 analyzed using the pyranine quenching assay (Gonorazky et al., 2008).
186 Figure 2C shows a rapid quenching of pyranine fluorescence in 100 μM
187 $\text{NO}_2\text{-OA}$ -treated cells. To further confirm this increase in ROS, a second
188 method based on 3,3'-diaminobenzidine (DAB) staining to detect H_2O_2
189 was used (Daudi and O'Brien, 2012). Again, $\text{NO}_2\text{-OA}$ treated cells showed
190 positive staining with DAB when compared to OA-treated tomato cells
191 (Figure 2D). Altogether these results show that $\text{NO}_2\text{-OA}$ but not OA
192 triggers a dose- and time-dependent production of ROS in tomato cell
193 suspensions.

194 Previous reports suggest that $\text{NO}_2\text{-FA}$ could act as a $\bullet\text{NO}$ donor in
195 both, mammals and plants, a mechanism responsible for its physiological
196 responses in cells (review in Baker et al., 2009, Mata-Perez et al., 2016).
197 To test this hypothesis, tomato cells were treated for 1 and 6 h with $\text{NO}_2\text{-}$
198 OA and $\bullet\text{NO}$ production analyzed using the fluorescent probe DAF-FM-
199 DA. $\text{NO}_2\text{-OA}$ was unable to induce $\bullet\text{NO}$ production in tomato cell
200 suspensions at 1 h (data not shown) or 6 h of treatment (Supplemental
201 Figure S2). These results indicate that under our experimental conditions
202 $\text{NO}_2\text{-OA}$ does not act as a $\bullet\text{NO}$ donor and/or induce $\bullet\text{NO}$ production.

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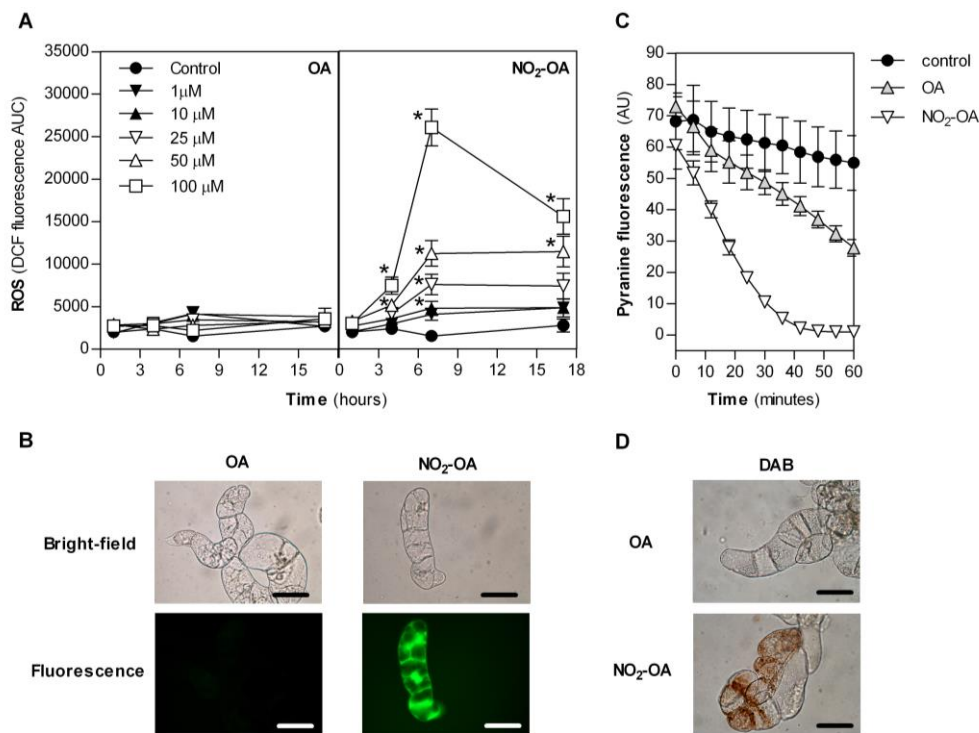
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NADPH Oxidase is Involved in NO₂-OA-induced ROS Production

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In plants, NADPH oxidase activation during plant defense is a key enzymatic source of ROS formation (Kadota et al., 2015). To specifically evaluate the role of NADPH oxidases as a source of ROS production triggered by NO₂-OA, tomato cell suspensions were treated with the inhibitor diphenyleneiodonium (DPI). DPI treatments have been successfully used previously in cell suspensions and entire plant systems (Piedras et al., 1998; Govrin and Levine 2000; Orozco-Cárdenas et al., 2001; De Jong et al., 2004). In this regard, Figure 3 shows that addition of DPI to NO₂-OA treated cells decreased ROS production in a dose-

241 dependent manner.

242 NADPH oxidase-dependent ROS production is finely tuned by
243 several signaling components, among them Ca^{2+} , protein kinases and
244 $\bullet\text{NO}$ -dependent posttranslational modifications (Kadota et al., 2015; Yun et
245 al., 2011). Thus, we used a pharmacological experimental approach to
246 assess the role of these signaling mechanisms on $\text{NO}_2\text{-OA}$ -induced ROS
247 production. Both, the calcium channel blocker Cl_3La and extracellular
248 calcium chelator EGTA reduced ROS production triggered by $\text{NO}_2\text{-OA}$
249 (Figure 3). Thus, we conclude that ROS production in response to $\text{NO}_2\text{-OA}$
250 is triggered by Ca^{2+} entry from the extracellular compartment.
251 Furthermore, the protein kinase inhibitor staurosporine decreased $\text{NO}_2\text{-OA}$ -
252 OA -induced ROS production (Figure 3) highlighting the requirement of
253 phosphorylation events for the $\text{NO}_2\text{-OA}$ -dependent activation of NADPH
254 oxidase. Finally, incubation of cells with the $\bullet\text{NO}$ scavenger cPTIO did not
255 affect $\text{NO}_2\text{-OA}$ -induced ROS production (Figure 3). In aggregate, our
256 results suggest that $\bullet\text{NO}$ is not involved in signaling responses leading to
257 increased ROS formation elicited by $\text{NO}_2\text{-OA}$ in tomato cell suspensions.

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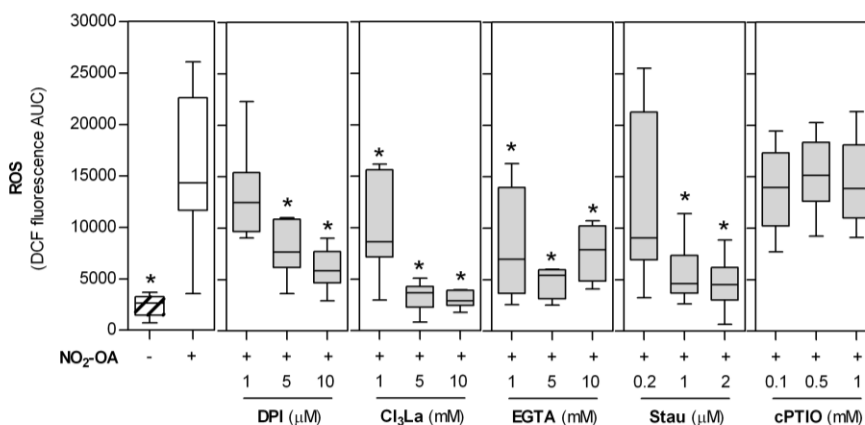


Figure 3. $\text{NO}_2\text{-OA}$ induced ROS production requires NADPH oxidase, Ca^{2+} and phosphorylation.

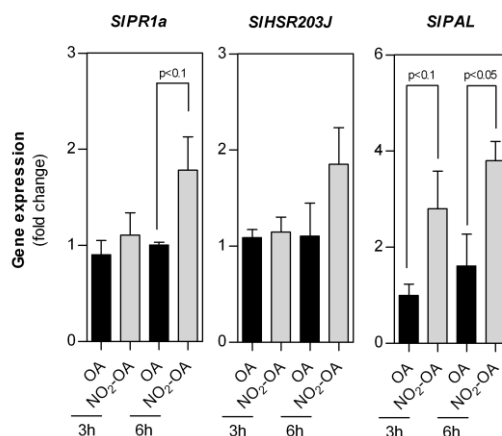
Tomato cell suspensions were incubated with 100 μM $\text{NO}_2\text{-OA}$ for 6 hours (+) and as control, non-treated cells were incubated the same time (-). To 5 hours $\text{NO}_2\text{-OA}$ treated cells, different concentrations of NADPH oxidase inhibitor (DPI), calcium channel blocker (Cl_3La), extracellular calcium chelator (EGTA), protein kinase inhibitor staurosporine (Stau) or $\bullet\text{NO}$ scavenger (cPTIO) were added for another hour. Then, cells were incubated with 4 μM $\text{H}_2\text{DCF-DA}$ and the accumulated fluorescence was determined. Data is presented by box-plot where the box is bound by the 25th to 75th percentile, whiskers span to minimum and maximum values, and the line in the middle is the median of 6 experiments. * indicated significant difference from $\text{NO}_2\text{-OA}$ treated cells (One way ANOVA, post-hoc Holm-Sidak test, $p < 0.05$).

273 Induction of Plant Defense Gene Expression and Cell Death by NO₂- 274 OA

275 In tomato cells, we reported a rapid ROS production associated
276 with the induction of gene expression and cell death upon treatments with
277 the fungal elicitor xylanase (Laxalt et al., 2007; Gonorazky et al., 2014).
278 Figure 4 shows the expression pattern of salicylic acid (SA)-dependent
279 gene *SLPR1a*, a gene marker for hypersensitive response *SIHSR203J*
280 and a jasmonic acid (JA)-dependent gene *SIPAL* at 3 h or 6 h upon
281 treatment with NO₂-OA or OA. No significant differences were found for
282 any of the genes analyzed 3 h post treatment with NO₂-OA. However, an
283 increase in gene expression was observed for *SIPAL* and *SIPR1a* at 6 h.

284 The ROS burst and the increased expression of the above-
285 analyzed genes suggest that NO₂-OA could induce cell death. To evaluate
286 the role of NO₂-OA in this pathway, we determined cell death in tomato
287 cells upon treatment with 50 or 100 μM NO₂-OA or OA for 4, 7 and 17 h
288 (Figure 5). Cells treated with NO₂-OA at both tested concentrations lead
289 to an increased rapid cell death rate compared to the corresponding OA
290 treatment.

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Figure 4. NO₂-OA induces plant defence gene expression. Tomato cells suspensions were treated with 100 μM OA or NO₂-OA. Cells were incubated for 3 or 6 h and total RNA was extracted. Transcripts levels of *SIPR1a*, *SIHSR203J* and *SIPAL* were analyzed by qPCR. *SIACT* (Actin) was used as a housekeeping gene. Data were analyzed by $\Delta\Delta C_t$ method and fold change was calculated. Error bars represent standard deviations of media from 4 independent experiments. P values for each comparison are indicated in figure (One way ANOVA, post-hoc Holm-Sidak test).

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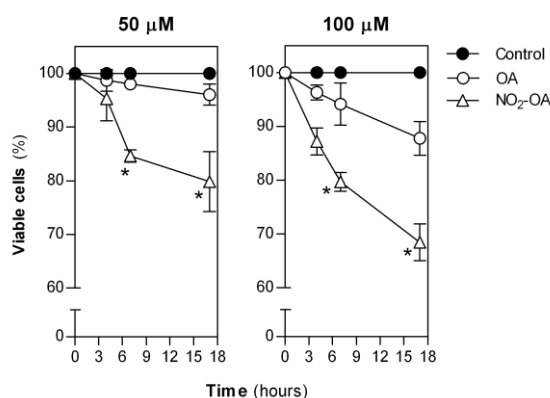
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Figure 5. NO₂-OA induces cell death.

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322 NO₂-OA Reduces Total GSH Content and Forms GS-NO₂-OA and 323 Protein-NO₂-OA Adducts

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NO₂-FAs are electrophiles that can form adducts with several cellular nucleophiles, in particular with GSH and protein thiols (Freeman et al., 2008). Thus, we quantified the GSH pool (reduced and oxidized) in cells treated with 100 μM NO₂-OA or OA for 3 h or 6 h to evaluate the extent of these reactions. Figure 6A shows that NO₂-OA treatment led to a ~50 % decrease in total GSH. As this decrease was most likely associated with the formation of glutathione-NO₂-OA adduct (GS-NO₂-OA), we sought to detect their formation in tomato cells suspensions. In this regard, HPLC-MSMS analysis demonstrated the presence GS-NO₂-OA adducts in NO₂-OA treated cells (Figure 6B).

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Given the detection of GS-NO₂-OA adducts, we sought to evaluate the formation of protein-NO₂-OA adducts in tomato cell suspensions. To this end, cells were incubated with NO₂-OA conjugated to biotin for

337 different times and the formation of protein-NO₂-OA-biotin adducts was
338 assessed at different times using western blot. Supplemental Figure S3
339 shows several tagged proteins in treated cells, indicating that cellular
340 proteins are targets of NO₂-OA. This further supports a role for protein
341 covalent modification induced by NO₂-OA in the signaling activities
342 identified for this post-translational modification.

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361 DISCUSSION

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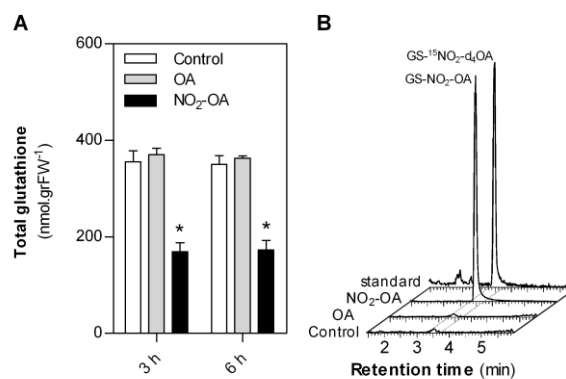


Figure 6. NO₂-OA modifies glutathione cellular pool and forms GS-NO₂-OA adducts.

Tomato cell suspensions were treated with 100 μM NO₂-OA or OA for 3 or 6 h. As control non treated cells were used. A, Total GSH pool was extracted and determinate by enzymatic GSH recycling method. Data represent media and standard error of 3 independent experiments. * indicated significant difference from control (One way ANOVA, post-hoc Holm-Sidak test, p <0.05). B, Detection of GS-NO₂-OA adducts by HPLC-MSMS in tomato cell suspension treated with 100 μM OA or NO₂-OA or without treatment for 3 h. Representative chromatograph form one of four independent experiments is show. As internal standard GS-¹⁵NO₂-d₄OA was used.

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361 DISCUSSION

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Lipids function as signaling mediators in various plant processes with an important role in signal transduction. Signaling lipids in plants include a wide range of molecules such as glycerolipids, sphingolipids, fatty acids, oxylipins and sterols that participates in the response to different stresses like temperature, drought, wounding, nutrition starvation and pathogens among others (Wang, 2004). In this regard, NO₂-FA represent a new class of lipid molecules involved in plant signaling.

369 Sanchez-Calvo et al., (2013) proposed them to be novel mediators of •NO-
370 dependent signaling pathways and metabolic processes in plant
371 physiology. Later 9-NO₂-cLA and 12-NO₂-cLA isomers, were found for the
372 first time in extra-virgin olive oil and NO₂-OA was identified in whole olives
373 adducted to cysteines (Cys-NO₂-OA, Fazzari et al., 2014). In addition,
374 NO₂-Ln was detected in Pea, Rice and Arabidopsis. In the later, its levels
375 changed during development and abiotic stress (Mata-Perez et al., 2015;
376 Mata-Perez et al., 2017). Our attempts to detect free endogenous NO₂-
377 FAs in tomato cells suspension were unsuccessful. The source of plant
378 fatty acids substrates to form nitroalkenes is an important aspect of these
379 reactions that could involve membrane, mitochondrial and/or chloroplast
380 phospholipids or triglycerides. In our experimental system, tomato cells are
381 grown under dark conditions and have non-green plastids (Sello et al.,
382 2017). Functional chloroplasts are very important for lipid signaling,
383 particularly in defense responses to biotic stress (Serrano et al., 2016). In
384 this sense, the fact that tomato cells have non-green plastids provides a
385 plausible explanation for absence of NO₂-FA in our measurements. We
386 were unable to detected free NO₂-FA in cells elicited with molecules
387 derived from pathogens, such as xylanase, a condition that generates an
388 oxidative and nitrosative stress (Laxalt et al., 2007) or during •NO donor
389 treatments (data not show). However, when cells were pre-incubated with
390 conjugated linoleic acid (cLA) and then treated with xylanase or •NO
391 donors, cellular detection of NO₂-cLA formation was observed
392 (Supplemental Figure S4). This result indicates that tomato cells have the
393 chemical environment required to endogenously nitrate fatty acids and
394 generate electrophilic nitroalkenes. In humans cells, >99% of nitroalkenes
395 are predicted to be covalently bound to thiols (Turell et al., 2017). The fact
396 that we were unable to detect free NO₂-FA could be due to the low levels
397 of these nitro-lipids, their rapid metabolism, and/or the reversible chemical
398 equilibrium established with thiols which favors adduct formation under
399 cellular conditions. Given the uptake and metabolism of NO₂-OA in tomato
400 cell suspensions, we used it as a model system to study the effects of

401 nitrolipids on plant defense responses.

402 NO₂-OA induced ROS production in tomato cell suspension. This
403 observation is in line with enhanced expression of several genes
404 associated to H₂O₂ and ROS responses observed in Arabidopsis cell
405 cultures (Mata-Perez et al. 2015). The inquiry of signaling downstream
406 components of NO₂-OA but upstream to ROS production, led us to find
407 that calcium and phosphorylation events are required for ROS production.
408 In plants, Ca²⁺ regulates ROS formation by NADPH oxidase, through
409 direct interaction with the Ct region of the protein, or by modulation of its
410 activity through the action of CDPks (Kadota et al., 2015; Sagi and Fluhr
411 2006). Our results show that ROS production is independent of •NO, and
412 occurs via activation of the NADPH oxidase, which requires Ca²⁺ and
413 phosphorylation events. The presence of both signaling components in
414 plant resembles the signaling pathway described in mammalian cells for
415 NO₂-FAs (Rudolph et al., 2010; Zhang et al., 2010).

416 ROS burst can lead to the up-regulation of several defense genes
417 and cell death in tomato cell suspensions (Gonorazky et al., 2014).
418 Particularly, we have previously demonstrated that upon xylanase
419 treatment, there is an induction of plant-defense gene expression and cell
420 death (Laxalt, et al., 2001; Laxalt et al., 2007). As mentioned above, in the
421 presence of cLA, xylanase treatments provided the chemical environment
422 required to generate electrophilic nitroalkenes. Exogenous addition of
423 NO₂-OA triggered the expression of defense response genes and cell
424 death. Thus, under this condition, NO₂-OA could be considered as a
425 signaling component in plant immune response.

426 One mechanism of action of NO₂-FAs involves their reactivity as
427 electrophiles through Michael addition reactions with cellular thiols. We
428 show evidence that NO₂-OA modify the GSH cellular pool forming adducts
429 with this NO₂-FA. A similar response to sulforaphane, an electrophilic
430 molecule was reported by Andersson et al., (2015) in Arabidopsis.
431 Sulforaphane is a naturally occurring isothiocyanate derived from
432 cruciferous vegetables that is present in widely consumed vegetables and

433 has a particularly high concentration in broccoli. Sulforaphane reduced the
434 GSH pool in Arabidopsis and increased cell leakage and cell death
435 probably associated with ROS burst (Andersson et al., 2015). We
436 determined that in tomato cells, sulforaphane induced ROS production in a
437 similar way as NO₂-OA does (Figure S5). Interestingly, as we demonstrated
438 for NO₂-OA, sulforaphane can form adducts with cellular thiols thus
439 generating post-translational modifications due to their electrophile nature
440 (Groeger and Freeman, 2010). In summary, the post-translational
441 modification of proteins and the GSH pool by Michael addition reactions of
442 nitroalkene reveals a novel mechanism of action by which NO₂-OA exert
443 their activity in tomato cells. Future work will focus on the identification of
444 protein targets adducted to NO₂-FA. Altogether, we unravel the role of
445 NO₂-FA as a signal molecule in plant immune response.

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448 **MATERIALS AND METHODS**

449 **Tomato Cell Suspensions Culture Conditions**

450 Tomato cell suspensions (*Solanum lycopersicum*, line Msk8) were
451 grown at 25°C in dark in MS medium (Duchefa Biochemie, Haarlem, The
452 Netherlands) as previously described (Laxalt et al., 2007). Cells of four-
453 day-old cultures were used for all experiments.

454

455 **Chemicals and Reagents**

456 OA was purchased from Nu-Chek Prep (Elysian, MN). NO₂-OA and
457 biotinylated NO₂-OA were synthesized and purified as previously
458 described (Woodcock et al., 2013; Bonacci et al., 2011; respectively). GS-
459 ¹⁵NO₂-d₄-OA standard was generated by the reaction of 200 mM reduced
460 glutathione with 100 μM ¹⁵NO₂-d₄-OA in 50 mM phosphate buffer (pH 8 at
461 37°C) for 3 h. The lipid conjugates were loaded on a C18 SPE column pre-
462 equilibrated with 10% methanol and then eluted with methanol. Solvents
463 used for extractions and mass spectrometric analyses were of HPLC
464 grade or higher from Burdick and Jackson (Muskegon, MI).

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466 **Lipid Extraction**

467 Lipid extraction from 100 mg of tomato cells were carried out using
468 hexane:isopropanol:1M formic acid (2:1:0.1, v/v/v). As internal standard
469 samples were spike with ¹⁵NO₂-d₄-OA (100 nM). The organic phase was
470 dried under N₂ and reconstituted in methanol before MS analysis.

471

472 **Chromatography**

473 Nitro-FA and GS-NO₂-OA were analyzed by HPLC-ESI-MS/MS
474 using gradient solvent systems consisting of water containing 0.1% acetic
475 acid (solvent A) and acetonitrile containing 0.1% acetic acid (solvent B),
476 and were resolved using a reverse phase HPLC column (100 × 2 mm x 5
477 μm C18 Luna column; Phenomenex) at a 0.65 ml/min flow rate. NO₂-FA
478 samples were applied to the column at 30% B (0.3min) and eluted with a
479 linear increase in solvent B (100% B in 14.7min) and GSH adducts were
480 applied to the column at 20% B (1.1 min) and eluted with a linear increase
481 in solvent B (20–100% solvent B in 5.9 min).

482

483 **Mass Spectrometry**

484 The NO₂-FA detection was performed using multiple reactions
485 monitoring (MRM) on an AB5000 triple quadrupole mass spectrometer
486 (Applied Biosystems, San Jose, CA) equipped with an electrospray
487 ionization source. MS analyses for NO₂-FA used electrospray ionization in
488 the negative ion mode with the collision gas set at 4 units, curtain gas 40,
489 ion source gas #1 55 and #260, ion spray voltage -4500 V, and
490 temperature 600 °C. The declustering potential was -100, entrance
491 potential -5, collision energy -35, and the collision exit potential -18.4.
492 MRM was used for sample analysis of nitrated fatty acids following the
493 charged loss of a nitro group (m/z 46) upon collision-induced dissociation.
494 An AB6500+ Q-trap triple quadrupole mass spectrometer (Applied
495 Biosystems, San Jose, CA) was used for GSH adducts detection in
496 positive ion mode using the following parameters: electrospray voltage 5.5

497 kV, declustering potential 60 eV, collision energy 30, gas1 45 and gas2 50
498 and de source temperature was set at 550°C. The following transitions
499 635.2/506.2 and 640.2/511.2 were used for detecting GS-NO₂-OA and
500 GS-¹⁵NO₂-d₄-OA respectively.

501

502 **Determination of ROS and •NO Production**

503 Tomato cells (90 µL per well in 96-well microtitre plate, DeltaLab)
504 were treated with 1, 10, 25, 50 or 100 µM of OA or NO₂-OA for 1, 4, 7 or
505 17 h. Plates were incubated at 25°C in darkness. ROS production was
506 detected by incubating cells with 4 µM H₂DCF-DA probe (Ubezio and
507 Civoli, 1994; Molecular Probe, Eugene, OR, USA) during the last hour of
508 each treatment. As an example, for 7 h treatment, at 6 h 4 µM of H₂DCF-
509 DA was added and ROS production was measured as follow. Cells were
510 immediately introduced in Fluoroskan Acsent microwell fluorometer
511 (Thermo Electron Company, Vantaa, Finland) and fluorescence (ex
512 485nm, em 525nm) was recorded every 2 minutes for 60 minutes. The
513 area under the curve (AUC, accumulated fluorescence) was calculated
514 according to equation showed in supplemental data and taken as an
515 accumulated florescence value (see supplemental Figure 1S). For •NO
516 determination 10 µM DAF-FM-DA was used as a probe (Kojima et al.,
517 1999, Molecular Probe, Eugene, OR, USA) and production was calculated
518 as indicated above for H₂DCF-DA.

519 For observation of ROS production, cells were treated with 100 µM
520 of OA or NO₂-OA for 6 h and then incubated with H₂DCF-DA for 1 h and
521 visualized under the epifluorescence microscopy with an excitation filter of
522 495 nm and a barrier filter of 515 nm according to Gonorazky et al.,
523 (2008).

524 Hydrogen peroxide determination was carried out by Pyranine
525 quenching assay according to Gonorazky et al., (2008, Pyranine Sigma-
526 Aldrich, St. Louis, MO, USA). Fluorescence quenching was recorded every
527 2 minutes for 60 minutes using Fluoroskan Acsent microwell fluorometer.

528 *In situ* hydrogen peroxide production was assayed by DAB staining.

529 Briefly, 100 μ l of treated cells were incubated with 50 μ l of 0.2% DAB
530 solution (Sigma-Aldrich) prepared according to Daudi and O'Brien, (2012).
531 Cells were incubated over night and observed under microscope.

532

533 **Inhibition Assays of ROS Production**

534 Tomato cell culture were treated in 96-well microtitre plate (90 μ L
535 per well) for 5 h with 100 μ M of NO₂-OA and then incubated with different
536 concentrations of NADPH oxidase inhibitor (DPI: 1, 5 or 10 μ M, Sigma),
537 calcium channel blocker (Cl₃La: 1, 5 or 10 mM, Sigma-Aldrich),
538 extracellular calcium chelator (EGTA: 1, 5 or 10 mM, Sigma-Aldrich),
539 protein kinase inhibitor (staurosporine: 0.2, 1 or 2 μ M, Sigma-Aldrich) or
540 •NO scavenger (cPTIO: 0.1, 0.5 or 1 mM, Invitrogene, Carlsbad, CA, USA)
541 for an additional hour in presence of 4 μ M H₂DCF-DA. Control cells (no
542 treatment, negative control) and NO₂-OA-only treated cells (positive
543 control) were incubated under the same conditions. Determination of ROS
544 production was performed as indicated above.

545

546 **qPCR Analysis of Gene Expression**

547 Three ml of tomato cells cultures were treated with 100 μ M OA, 100
548 μ M NO₂-OA or DMSO (Merk, Darmstadt, Germany) as a control for 3 or 6
549 h. Cells were washed with phosphate buffer (pH 7.5, 50 mM), frozen in
550 liquid nitrogen and total RNA was extracted using the Trizol method. cDNA
551 was synthesized according to manufactured instruction using M-MLV
552 enzyme (Invitrogene). Transcripts levels of *SIPR1a*, *SIHSR203J*, *SIPAL*,
553 and *SIACT* (Actin) genes were analyzed by qPCR (StepOne, Thermo).
554 Expression data are expressed as $\Delta\Delta C_t$ and *SIACT* was used a
555 housekeeping gene. Primers used are listed in supplemental Table S1.

556

557 **Cell Death Quantification**

558 Tomato cells were treated with 50 μ M or 100 μ M of OA or NO₂-OA
559 for 4, 7 or 17 h on 96-well microtitre plate (90 μ L per well). At each time,
560 50 μ l of 1% ^{w/v} Evans Blue solution (Fluka, Buchs, Switzerland) were

561 added to cells in wells, incubated at room temperature for 5 minutes and
562 observed under light microscope. Live (none stained) and dead (blue
563 stained) cells were manually counted on at least 10 random optical fields
564 (40x) for each treatment.

565

566 **GSH and GS-NO₂-OA Adduct Detection**

567 Three ml of tomato cell culture were treated with 100 μ M OA, 100
568 μ M NO₂-OA or DMSO as control for 3 or 6 h. Cells were collected, washed
569 and immediately frozen in liquid nitrogen. Total GSH was evaluated using
570 the enzymatic GSH recycling method (Griffith, 1980).

571 GS-NO₂-OA adducts were assessed by HPLC-MSMS. A mass of
572 0.4 mg of cell was spike with 30 fmol of GS-¹⁵NO₂-d₄-OA as internal
573 standard before extraction. GSH adducts was extracted using C18 SPE
574 columns. Columns were conditioned with 100% methanol, followed by 2
575 column volumes of 10% methanol. Samples were loaded into the SPE
576 column and washed with 2 column volumes of 10% methanol and the
577 column was dried under vacuum for 30 min. GSH adducts were eluted
578 with 3 ml methanol, solvent was evaporated, and samples were dissolved
579 in methanol for analysis by HPLC-electrospray ionization mass
580 spectrometry (ESI-MS/MS).

581

582 **Western Blot of Protein-NO₂-OA Adducts**

583 Tomato cell cultures (500 μ l) were treated with NO₂-OA-biotin at a
584 final concentration of 25 μ M for 4, 7, and 17 h. As a control, 500 μ l of cell
585 cultures were treated with DMSO. The cells were collected, subjected to
586 three cycles of freeze/thawed and ground under liquid nitrogen for
587 mechanical disruption. Proteins were extracted using phosphate buffer (50
588 mM pH 7.5) containing 20 mM NEM (Fluka). Total protein concentration
589 was determined by the bicinchoninic acid method (Smith et al., 1985,
590 bicinchoninic Sigma) and 100 μ g of proteins for each sample were
591 reduced by incubation with 10 mM BME (BioBasic, Ontario, Canada) for 5
592 minutes at 70°C (Schopfer et al., 2009). As a positive control, 100 μ g of

593 tomato proteins cell extract were treated with an excess of NO₂-OA-biotin
594 (125 μM final concentration) to induced nitroalkylation (room temperature
595 for 30 minutes in phosphate buffer). Samples were treated with BME and
596 heat as indicated above. All samples were mixed with protein loading
597 buffer without BME, separated in polyacrylamide gels, transferred to
598 nitrocellulose membrane and incubated with mouse anti-biotin primary
599 antibody overnight (Sigma-Aldrich). The membrane was incubated with a
600 secondary antibody coupled to phosphatase alkaline enzyme (Sigma-
601 Aldrich) for 3 h and developed over 5 minutes or 2 h (see supplemental
602 Figure S3).

603
604

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