

# 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

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

45

## 46 **Abbreviations**

47 •NO<sub>2</sub>: nitrogen dioxide

48 •NO: nitric oxide

49 FA: fatty acid

50 GSH: reduced glutathione

51 H<sub>2</sub>O<sub>2</sub>: hydrogen peroxyde

52 NO<sub>2</sub>-FA: nitro fatty acids

53 NO<sub>2</sub>-Ln: nitro-linolenic acid

54 NO<sub>2</sub>-OA: nitro-oleic acid

55 OA: oleic acid

56 ROS: reactive oxygen species

57

58

## 59 **ABSTRACT**

60 Nitrated fatty acids (NO<sub>2</sub>-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 NO<sub>2</sub>-FAs in  
 64 plant systems constitutes an interesting and emerging area. The presence  
 65 of NO<sub>2</sub>-FA has been reported in olives, peas, rice and in Arabidopsis. To  
 66 gain a better understanding of the role of NO<sub>2</sub>-FA on plant physiology, we  
 67 analyzed the effects of exogenous application of nitro-oleic acid (NO<sub>2</sub>-OA)  
 68 to tomato cell cultures. We found that NO<sub>2</sub>-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. NO<sub>2</sub>-OA induced ROS production,  
 73 expression of plant defense genes and led to cell death. The mechanism  
 74 of action of NO<sub>2</sub>-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 NO<sub>2</sub>-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 (NO<sub>2</sub>-FAs) are formed by the addition  
89 reaction of nitric oxide (•NO)- and nitrite (NO<sub>2</sub><sup>-</sup>)-derived nitrogen dioxide  
90 (•NO<sub>2</sub>) 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, NO<sub>2</sub>-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 NO<sub>2</sub>-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, NO<sub>2</sub>-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 (NO<sub>2</sub>-Ln). The levels of  
110 these NO<sub>2</sub>-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 NO<sub>2</sub>-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, NO<sub>2</sub>-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 Ca<sup>2+</sup> 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 NO<sub>2</sub>-OA, with a particular focus on the induction of plant  
130 defense responses.

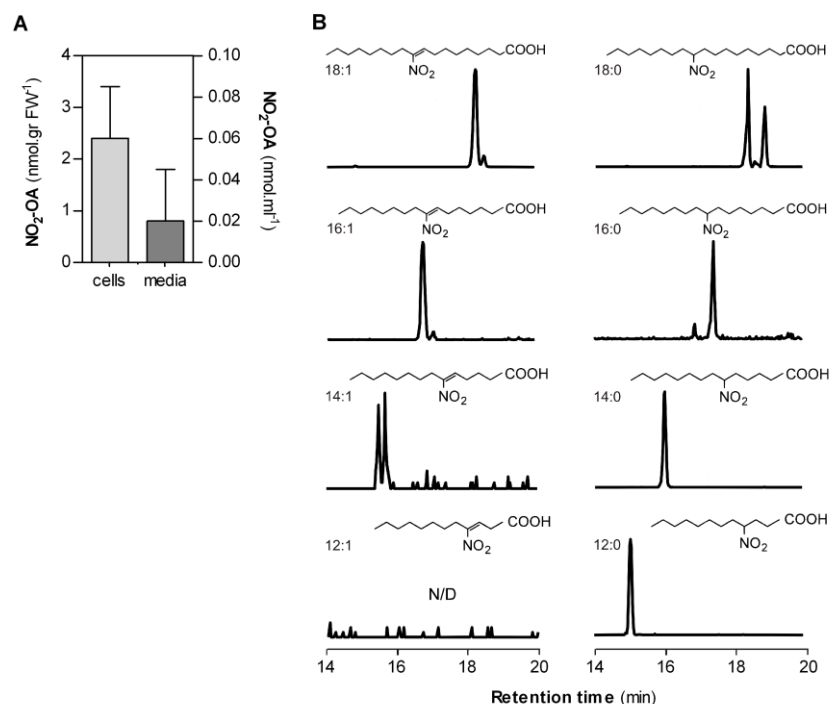
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## 133 **RESULTS**

### 134 **NO<sub>2</sub>-OA is Internalized and Metabolized in Tomato Cells.**

135 NO<sub>2</sub>-FAs are hydrophobic fatty acids with poor solubility in aqueous  
136 solutions. Thus, we first sought to analyze binding and internalization of  
137 NO<sub>2</sub>-OA by tomato cell suspensions. Figure 1A shows that NO<sub>2</sub>-OA  
138 effectively bound to tomato cells, reducing the remaining levels in media.  
139 Moreover, analysis of metabolic products of NO<sub>2</sub>-OA in treated cells  
140 revealed that NO<sub>2</sub>-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 NO<sub>2</sub>-OA is effectively internalized into the cell and therefore



**Figure 1.** Detection and quantification of exogenous NO<sub>2</sub>-OA added to tomato cell suspensions and metabolic products. Tomato cell suspensions were incubated with 10 μM NO<sub>2</sub>-OA for 1 h and then NO<sub>2</sub>-OA and its metabolic products were analyzed by HPLC-MS/MS. A, Quantification of NO<sub>2</sub>-OA in tomato cells or in the suspension media. Graph shows media with standard errors (n=3). B, Representative chromatographic profiles of NO<sub>2</sub>-OA (left panel) and NO<sub>2</sub>-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 NO<sub>2</sub>-FA physiological responses associated with its exogenous application.

### NO<sub>2</sub>-OA Induces ROS but not •NO Production in Tomato Cells

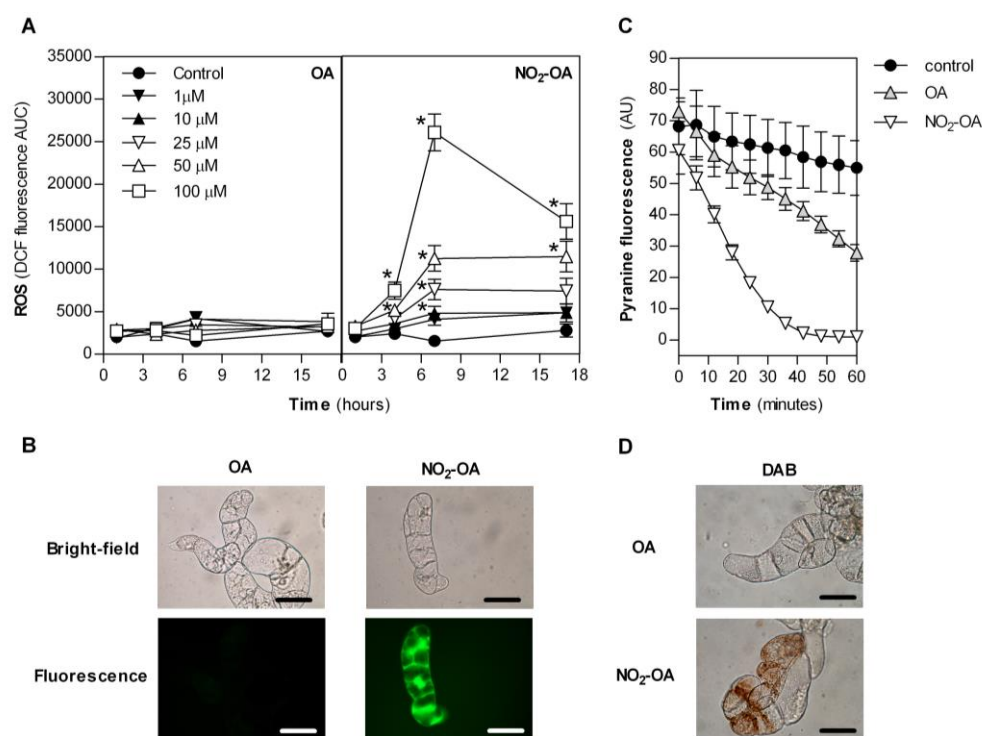
Bioinformatics analysis of RNAseq data in Arabidopsis cell suspensions revealed that a large number of NO<sub>2</sub>-Ln-induced genes were related oxidative stress response, mainly depicted by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and reactive oxygen species (Mata-Perez et al., 2015). In this regard, we tested if NO<sub>2</sub>-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 NO<sub>2</sub>-OA. Figure 2A shows an increase in the fluorescence signal of NO<sub>2</sub>-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  $\mu$ M NO<sub>2</sub>-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  $\mu$ M of NO<sub>2</sub>-OA for 6 h showed  
182 a significant increase in the fluorescent signal (Figure 2B).

183 In order to further validate ROS production in NO<sub>2</sub>-OA-treated cells,  
184 we used two alternative methodologies. First, H<sub>2</sub>O<sub>2</sub> 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  $\mu$ M  
187 NO<sub>2</sub>-OA-treated cells. To further confirm this increase in ROS, a second  
188 method based on 3,3'-diaminobenzidine (DAB) staining to detect H<sub>2</sub>O<sub>2</sub>  
189 was used (Daudi and O'Brien, 2012). Again, NO<sub>2</sub>-OA treated cells showed  
190 positive staining with DAB when compared to OA-treated tomato cells  
191 (Figure 2D). Altogether these results show that NO<sub>2</sub>-OA but not OA  
192 triggers a dose- and time-dependent production of ROS in tomato cell  
193 suspensions.

194 Previous reports suggest that NO<sub>2</sub>-FA could act as a •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 NO<sub>2</sub>-  
198 OA and •NO production analyzed using the fluorescent probe DAF-FM-  
199 DA. NO<sub>2</sub>-OA was unable to induce •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 NO<sub>2</sub>-OA does not act as a •NO donor and/or induce •NO production.

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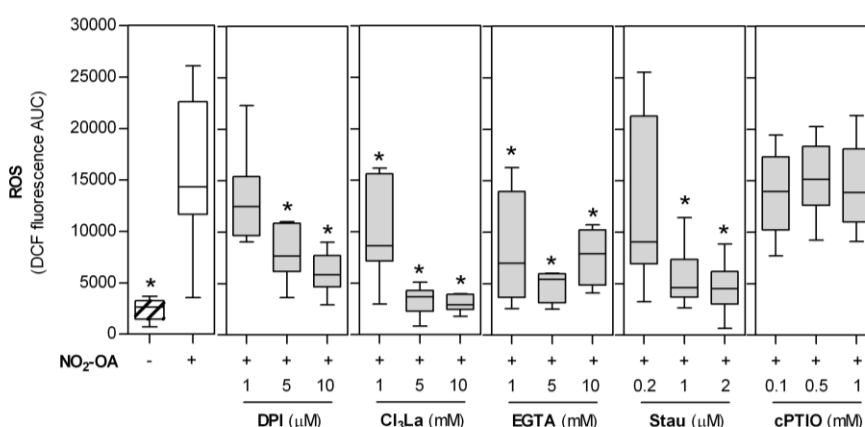
**Figure 2.** Reactive oxygen species (ROS) production in tomato cell suspensions treated with NO<sub>2</sub>-OA. A, Tomato cell suspensions were treated with OA or NO<sub>2</sub>-OA, or non-treated as a control. At 0, 3, 6 or 16 h of treatment 4  $\mu$ M H<sub>2</sub>DCF-DA was added and the fluorescence was measured. The fluorescence was determined as the area under the curve (accumulated fluorescence within one hour). Data represents media and error standard of 4 independent experiments. \* indicated significant difference ( $p < 0.05$ ) from control for each time (One way ANOVA post-hoc Holm-Sidak). B, ROS production on tomato cells suspensions treated for 6 h with 100  $\mu$ M OA or NO<sub>2</sub>-OA and then incubated with 4  $\mu$ M H<sub>2</sub>DCF-DA for 1 h. A representative light and epifluorescence microscope picture of experiments is shown. C, Oxidative burst. Cell suspensions were treated for 6 h with 100  $\mu$ M OA or NO<sub>2</sub>-OA and then the quenching of pyranine fluorescence was recorded as a measure of the oxidative burst. Data represent media and error standard of 2 independent experiments. D, H<sub>2</sub>O<sub>2</sub> detection by DAB stain on tomato cells treated with 100  $\mu$ M OA or NO<sub>2</sub>-OA for 6h. Bars= 5  $\mu$ m in panels B and D.

## NADPH Oxidase is Involved in NO<sub>2</sub>-OA-induced ROS Production

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 NO<sub>2</sub>-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 NO<sub>2</sub>-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  $\text{Ca}^{2+}$ , protein kinases and  
244 •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  $\text{NO}_2$ -OA-induced ROS  
247 production. Both, the calcium channel blocker  $\text{Cl}_3\text{La}$  and extracellular  
248 calcium chelator EGTA reduced ROS production triggered by  $\text{NO}_2$ -OA  
249 (Figure 3). Thus, we conclude that ROS production in response to  $\text{NO}_2$ -OA  
250 is triggered by  $\text{Ca}^{2+}$  entry from the extracellular compartment.  
251 Furthermore, the protein kinase inhibitor staurosporine decreased  $\text{NO}_2$ -  
252 OA-induced ROS production (Figure 3) highlighting the requirement of  
253 phosphorylation events for the  $\text{NO}_2$ -OA-dependent activation of NADPH  
254 oxidase. Finally, incubation of cells with the •NO scavenger cPTIO did not  
255 affect  $\text{NO}_2$ -OA-induced ROS production (Figure 3). In aggregate, our  
256 results suggest that •NO is not involved in signaling responses leading to  
257 increased ROS formation elicited by  $\text{NO}_2$ -OA in tomato cell suspensions.



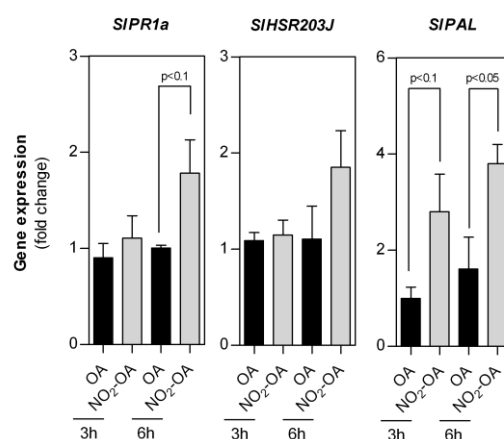
**Figure 3.**  $\text{NO}_2$ -OA induced ROS production requires NADPH oxidase,  $\text{Ca}^{2+}$  and phosphorylation.

Tomato cell suspensions were incubated with 100  $\mu\text{M}$   $\text{NO}_2$ -OA for 6 hours (+) and as control, non-treated cells were incubated the same time (-). To 5 hours  $\text{NO}_2$ -OA treated cells, different concentrations of NADPH oxidase inhibitor (DPI), calcium channel blocker ( $\text{Cl}_3\text{La}$ ), extracellular calcium chelator (EGTA), protein kinase inhibitor staurosporine (Stau) or •NO scavenger (cPTIO) were added for another hour. Then, cells were incubated with 4  $\mu\text{M}$   $\text{H}_2\text{DCF-DA}$  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 de median of 6 experiments. \* indicated significant difference from  $\text{NO}_2$ -OA treated cells (One way ANOVA, post-hoc Holm-Sidak test,  $p < 0.05$ ).

## Induction of Plant Defense Gene Expression and Cell Death by NO<sub>2</sub>-OA

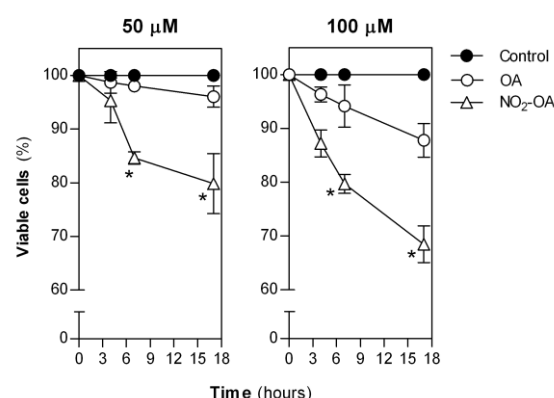
In tomato cells, we reported a rapid ROS production associated with the induction of gene expression and cell death upon treatments with the fungal elicitor xylanase (Laxalt et al., 2007; Gonorazky et al., 2014). Figure 4 shows the expression pattern of salicylic acid (SA)-dependent gene *SLPR1a*, a gene marker for hypersensitive response *SIHSR203J* and a jasmonic acid (JA)-dependent gene *SIPAL* at 3 h or 6 h upon treatment with NO<sub>2</sub>-OA or OA. No significant differences were found for any of the genes analyzed 3 h post treatment with NO<sub>2</sub>-OA. However, an increase in gene expression was observed for *SIPAL* and *SIPR1a* at 6 h.

The ROS burst and the increased expression of the above-analyzed genes suggest that NO<sub>2</sub>-OA could induce cell death. To evaluate the role of NO<sub>2</sub>-OA in this pathway, we determined cell death in tomato cells upon treatment with 50 or 100 μM NO<sub>2</sub>-OA or OA for 4, 7 and 17 h (Figure 5). Cells treated with NO<sub>2</sub>-OA at both tested concentrations lead to an increased rapid cell death rate compared to the corresponding OA treatment.



**Figure 4.** NO<sub>2</sub>-OA induces plant defence gene expression.

Tomato cells suspensions were treated with 100 μM OA or NO<sub>2</sub>-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. *SIAC1* (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).



**Figure 5.** NO<sub>2</sub>-OA induces cell death.

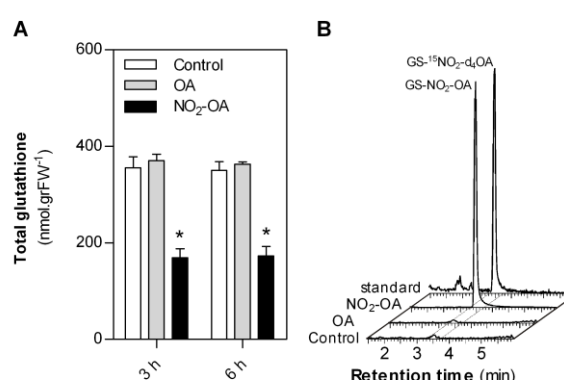
Tomato cells suspensions were incubated with OA or NO<sub>2</sub>-OA (at 50 μM or 100 μM) or without treatment as a control for 3, 6, and 17 h. Then, cells were stained with Evan Blue, incubated at room temperature for 5 minutes and observed under optical microscope. Live (none stained) and dead (blue stained) cells were manually counted on 10 random optical fields (40x). Data are expressed as percentage of viable cells respect to non-treated cells (black circles, control) at each time point. Data represents percentage average and standard error of 4 (0 and 3h) and 3 (6 and 17h) independent experiments. \* indicated significant difference from control (z-test, p<0.05)

## NO<sub>2</sub>-OA Reduces Total GSH Content and Forms GS-NO<sub>2</sub>-OA and Protein-NO<sub>2</sub>-OA Adducts

NO<sub>2</sub>-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 NO<sub>2</sub>-OA or OA for 3 h or 6 h to evaluate the extent of these reactions. Figure 6A shows that NO<sub>2</sub>-OA treatment led to a ~50 % decrease in total GSH. As this decrease was most likely associated with the formation of glutathione-NO<sub>2</sub>-OA adduct (GS-NO<sub>2</sub>-OA), we sought to detect their formation in tomato cells suspensions. In this regard, HPLC-MS/MS analysis demonstrated the presence GS-NO<sub>2</sub>-OA adducts in NO<sub>2</sub>-OA treated cells (Figure 6B).

Given the detection of GS-NO<sub>2</sub>-OA adducts, we sought to evaluate the formation of protein-NO<sub>2</sub>-OA adducts in tomato cell suspensions. To this end, cells were incubated with NO<sub>2</sub>-OA conjugated to biotin for

different times and the formation of protein-NO<sub>2</sub>-OA-biotin adducts was assessed at different times using western blot. Supplemental Figure S3 shows several tagged proteins in treated cells, indicating that cellular proteins are targets of NO<sub>2</sub>-OA. This further supports a role for protein covalent modification induced by NO<sub>2</sub>-OA in the signaling activities identified for this post-translational modification.



**Figure 6.** NO<sub>2</sub>-OA modifies glutathione cellular pool and forms GS-NO<sub>2</sub>-OA adducts.

Tomato cell suspensions were treated with 100  $\mu$ M NO<sub>2</sub>-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-NO<sub>2</sub>-OA adducts by HPLC-MS/MS in tomato cell suspension treated with 100  $\mu$ M OA or NO<sub>2</sub>-OA or without treatment for 3 h. Representative chromatograph form one of four independent experiments is show. As internal standard GS-<sup>15</sup>NO<sub>2</sub>-d<sub>4</sub>OA was used.

## DISCUSSION

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, NO<sub>2</sub>-FA represent a new class of lipid molecules involved in plant signaling.

Sanchez-Calvo et al., (2013) proposed them to be novel mediators of •NO-dependent signaling pathways and metabolic processes in plant physiology. Later 9-NO<sub>2</sub>-cLA and 12-NO<sub>2</sub>-cLA isomers, were found for the first time in extra-virgin olive oil and NO<sub>2</sub>-OA was identified in whole olives adducted to cysteines (Cys-NO<sub>2</sub>-OA, Fazzari et al., 2014). In addition, NO<sub>2</sub>-Ln was detected in Pea, Rice and Arabidopsis. In the later, its levels changed during development and abiotic stress (Mata-Perez et al., 2015; Mata-Perez et al., 2017). Our attempts to detect free endogenous NO<sub>2</sub>-FAs in tomato cells suspension were unsuccessful. The source of plant fatty acids substrates to form nitroalkenes is an important aspect of these reactions that could involve membrane, mitochondrial and/or chloroplast phospholipids or triglycerides. In our experimental system, tomato cells are grown under dark conditions and have non-green plastids (Sello et al., 2017). Functional chloroplasts are very important for lipid signaling, particularly in defense responses to biotic stress (Serrano et al., 2016). In this sense, the fact that tomato cells have non-green plastids provides a plausible explanation for absence of NO<sub>2</sub>-FA in our measurements. We were unable to detected free NO<sub>2</sub>-FA in cells elicited with molecules derived from pathogens, such as xylanase, a condition that generates an oxidative and nitrosative stress (Laxalt et al., 2007) or during •NO donor treatments (data not show). However, when cells were pre-incubated with conjugated linoleic acid (cLA) and then treated with xylanase or •NO donors, cellular detection of NO<sub>2</sub>-cLA formation was observed (Supplemental Figure S4). This result indicates that tomato cells have the chemical environment required to endogenously nitrate fatty acids and generate electrophilic nitroalkenes. In humans cells, >99% of nitroalkenes are predicted to be covalently bound to thiols (Turell et al., 2017). The fact that we were unable to detect free NO<sub>2</sub>-FA could be due to the low levels of these nitro-lipids, their rapid metabolism, and/or the reversible chemical equilibrium established with thiols which favors adduct formation under cellular conditions. Given the uptake and metabolism of NO<sub>2</sub>-OA in tomato cell suspensions, we used it as a model system to study the effects of

401 nitrolipids on plant defense responses.

402 NO<sub>2</sub>-OA induced ROS production in tomato cell suspension. This  
403 observation is in line with enhanced expression of several genes  
404 associated to H<sub>2</sub>O<sub>2</sub> and ROS responses observed in Arabidopsis cell  
405 cultures (Mata-Perez et al. 2015). The inquiry of signaling downstream  
406 components of NO<sub>2</sub>-OA but upstream to ROS production, led us to find  
407 that calcium and phosphorylation events are required for ROS production.  
408 In plants, Ca<sup>2+</sup> 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 Ca<sup>2+</sup> and  
413 phosphorylation events. The presence of both signaling components in  
414 plant resembles the signaling pathway described in mammalian cells for  
415 NO<sub>2</sub>-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 NO<sub>2</sub>-OA triggered the expression of defense response genes and cell  
424 death. Thus, under this condition, NO<sub>2</sub>-OA could be considered as a  
425 signaling component in plant immune response.

426 One mechanism of action of NO<sub>2</sub>-FAs involves their reactivity as  
427 electrophiles through Michael addition reactions with cellular thiols. We  
428 show evidence that NO<sub>2</sub>-OA modify the GSH cellular pool forming adducts  
429 with this NO<sub>2</sub>-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

has a particularly high concentration in broccoli. Sulforaphane reduced the GSH pool in Arabidopsis and increased cell leakage and cell death probably associated with ROS burst (Andersson et al., 2015). We determined that in tomato cells, sulforaphane induced ROS production in a similar way as NO<sub>2</sub>-OA does (Figure S5). Interestingly, as we demonstrated for NO<sub>2</sub>-OA, sulforaphane can form adducts with cellular thiols thus generating post-translational modifications due to their electrophile nature (Groeger and Freeman, 2010). In summary, the post-translational modification of proteins and the GSH pool by Michael addition reactions of nitroalkene reveals a novel mechanism of action by which NO<sub>2</sub>-OA exert their activity in tomato cells. Future work will focus on the identification of protein targets adducted to NO<sub>2</sub>-FA. Altogether, we unravel the role of NO<sub>2</sub>-FA as a signal molecule in plant immune response.

## **MATERIALS AND METHODS**

### **Tomato Cell Suspensions Culture Conditions**

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

### **Chemicals and Reagents**

OA was purchased from Nu-Chek Prep (Elysian, MN). NO<sub>2</sub>-OA and biotinylated NO<sub>2</sub>-OA were synthesized and purified as previously described (Woodcock et al., 2013; Bonacci et al., 2011; respectively). GS-<sup>15</sup>NO<sub>2</sub>-d<sub>4</sub>-OA standard was generated by the reaction of 200 mM reduced glutathione with 100 μM <sup>15</sup>NO<sub>2</sub>-d<sub>4</sub>-OA in 50 mM phosphate buffer (pH 8 at 37°C) for 3 h. The lipid conjugates were loaded on a C18 SPE column pre-equilibrated with 10% methanol and then eluted with methanol. Solvents used for extractions and mass spectrometric analyses were of HPLC grade or higher from Burdick and Jackson (Muskegon, MI).

465

## 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  $^{15}\text{NO}_2\text{-d}_4\text{-OA}$  (100 nM). The organic phase was  
470 dried under  $\text{N}_2$  and reconstituted in methanol before MS analysis.

471

## 472 **Chromatography**

473 Nitro-FA and GS- $\text{NO}_2\text{-OA}$  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  $\mu\text{m}$  C18 Luna column; Phenomenex) at a 0.65 ml/min flow rate.  $\text{NO}_2\text{-FA}$   
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  $\text{NO}_2\text{-FA}$  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  $\text{NO}_2\text{-FA}$  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-NO<sub>2</sub>-OA and  
500 GS-<sup>15</sup>NO<sub>2</sub>-d<sub>4</sub>-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 NO<sub>2</sub>-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 H<sub>2</sub>DCF-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 H<sub>2</sub>DCF-  
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 H<sub>2</sub>DCF-DA.

519 For observation of ROS production, cells were treated with 100 µM  
520 of OA or NO<sub>2</sub>-OA for 6 h and then incubated with H<sub>2</sub>DCF-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.

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

### **Inhibition Assays of ROS Production**

Tomato cell culture were treated in 96-well microtitre plate (90  $\mu$ L per well) for 5 h with 100  $\mu$ M of NO<sub>2</sub>-OA and then incubated with different concentrations of NADPH oxidase inhibitor (DPI: 1, 5 or 10  $\mu$ M, Sigma), calcium channel blocker (Cl<sub>3</sub>La: 1, 5 or 10 mM, Sigma-Aldrich), extracellular calcium chelator (EGTA: 1, 5 or 10 mM, Sigma-Aldrich), protein kinase inhibitor (staurosporine: 0.2, 1 or 2  $\mu$ M, Sigma-Aldrich) or •NO scavenger (cPTIO: 0.1, 0.5 or 1 mM, Invitrogene, Carlsbad, CA, USA) for an additional hour in presence of 4  $\mu$ M H<sub>2</sub>DCF-DA. Control cells (no treatment, negative control) and NO<sub>2</sub>-OA-only treated cells (positive control) were incubated under the same conditions. Determination of ROS production was performed as indicated above.

### **qPCR Analysis of Gene Expression**

Three ml of tomato cells cultures were treated with 100  $\mu$ M OA, 100  $\mu$ M NO<sub>2</sub>-OA or DMSO (Merk, Darmstadt, Germany) as a control for 3 or 6 h. Cells were washed with phosphate buffer (pH 7.5, 50 mM), frozen in liquid nitrogen and total RNA was extracted using the Trizol method. cDNA was synthesized according to manufactured instruction using M-MLV enzyme (Invitrogene). Transcripts levels of *SIPR1a*, *SIHSR203J*, *SIPAL*, and *SI*ACT (Actin) genes were analyzed by qPCR (StepOne, Thermo). Expression data are expressed as  $\Delta\Delta C_t$  and *SI*ACT was used a housekeeping gene. Primers used are listed in supplemental Table S1.

### **Cell Death Quantification**

Tomato cells were treated with 50  $\mu$ M or 100  $\mu$ M of OA or NO<sub>2</sub>-OA for 4, 7 or 17 h on 96-well microtitre plate (90  $\mu$ L per well). At each time, 50  $\mu$ l of 1% <sup>w/v</sup> 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-NO<sub>2</sub>-OA Adduct Detection**

567 Three ml of tomato cell culture were treated with 100 μM OA, 100  
568 μM NO<sub>2</sub>-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-NO<sub>2</sub>-OA adducts were assessed by HPLC-MSMS. A mass of  
572 0.4 mg of cell was spike with 30 fmol of GS-<sup>15</sup>NO<sub>2</sub>-d<sub>4</sub>-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-NO<sub>2</sub>-OA Adducts**

583 Tomato cell cultures (500 μl) were treated with NO<sub>2</sub>-OA-biotin at a  
584 final concentration of 25 μM for 4, 7, and 17 h. As a control, 500 μl 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 μg 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 μg of

tomato proteins cell extract were treated with an excess of NO<sub>2</sub>-OA-biotin (125 μM final concentration) to induced nitroalkylation (room temperature for 30 minutes in phosphate buffer). Samples were treated with BME and heat as indicated above. All samples were mixed with protein loading buffer without BME, separated in polyacrylamide gels, transferred to nitrocellulose membrane and incubated with mouse anti-biotin primary antibody overnight (Sigma-Aldrich). The membrane was incubated with a secondary antibody coupled to phosphatase alkaline enzyme (Sigma-Aldrich) for 3 h and developed over 5 minutes or 2 h (see supplemental Figure S3).

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604

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