

Article

Nitro-Oleic Acid in Seeds and Differently Developed Seedlings of *Brassica napus* L.

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Abstract: Similar to animals, it has recently been proven that nitro-fatty acids such as nitro-linolenic acid and nitro-oleic acid (NO₂-OA) have relevant physiological roles as signalling molecules also in plants. Although NO₂-OA is of great therapeutic importance, its presence in plants as a free fatty acid has not been observed so far. Since *Brassica napus* (oilseed rape) is a crop with high oleic acid content, the abundance of NO₂-OA in its tissues can be assumed. Therefore, we quantified NO₂-OA in *B. napus* seeds and differently developed seedlings. In all samples, NO₂-OA was detectable at nanomolar concentrations. The seeds showed the highest NO₂-OA content, which decreased during germination. In contrast, nitric oxide (•NO) levels increased in the early stages of germination and seedling growth. Exogenous NO₂-OA treatment (100 μM, 24 h) of *Brassica* seeds resulted in significantly increased •NO level and induced germination capacity compared to untreated seeds. The results of in vitro approaches (4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) fluorescence, •NO-sensitive electrode) supported the •NO liberating capacity of NO₂-OA. We observed for the first time that *Brassica* seeds and seedlings contain free NO₂-OA which may be involved in germination as an •NO donor as suggested both by the results of exogenous NO₂-OA treatment of seeds and in vitro approaches. Due to their high NO₂-OA content, *Brassica* sprouts can be considered as a good source of dietary NO₂-OA intake.

Keywords: *Brassica napus*; germination; nitro-oleic acid; nitric oxide; seedlings; seeds

1. Introduction

Nitro-fatty acids (NO₂-FA) as endogenous signal molecules in animals and humans have gained great attention, since these nitrated lipid derivatives exert relevant bioactivity in association with anti-thrombotic, cytoprotective, and anti-inflammatory processes (recently reviewed in Ref. [1]). The addition reaction of nitric oxide (•NO) and •NO-derived higher oxides of nitrogen (peroxynitrite, nitrogen dioxide) with conjugated double bond-containing, unsaturated fatty acids results in the formation of NO₂-FA; although, the in vivo mechanism is still unknown [2]. During the first proposed mechanism, radical hydrogen abstraction from a bis-allylic carbon takes place resulting in the formation of an alkyl radical which is followed by the formation of a peroxy radical via double bond rearrangement and molecular oxygen insertion. The insertion of nitrogen dioxide (•NO₂) and the consequent formation of a non-electrophilic nitroalkane-alkene product is also possible [3]. The second mechanism includes

the formation of a carbon-centred radical as the result of the direct addition of $\bullet\text{NO}_2$. This can be followed by further oxidation steps yielding the electrophilic nitro-alkene [4].

Although the occurrence and bioactivity of nitrated fatty acids is well-characterized in animals, these interesting molecules have recently been the focus of attention in plants. The presence of endogenous NO_2 -FAs such as nitro-conjugated linoleic acid (NO_2 -cLA) and nitro-oleic acid-cysteine (NO_2 -OA-Cys) adducts have been detected in extra-virgin olive oil (EVOO) and fresh olives [5]. By incubating EVOO with nitrating agents, Fazzari et al. [4] was able to detect nitro-linolenic acid (NO_2 -Ln) and nitro-linoleic acid (NO_2 -LA). Later, in *Arabidopsis thaliana* at different developmental stages [6], in pea roots and leaves as well as in rice leaves, NO_2 -Ln proved to be the major endogenous NO_2 -FA [7]. Recently, Di Palma et al. [8,9] quantified NO_2 -OA in tomato cell suspensions treated with exogenous NO_2 -OA supporting the internalization of this nitrated derivative in tomato cells.

Nitro-fatty acids show electrophilic reactivity towards cellular nucleophilic targets such as reduced glutathione or protein cysteine (Cys) and histidine (His) residues. This results in the reversible formation of protein- NO_2 -FA adducts, which serve as a reservoir and also control the size of the free NO_2 -FA pool. The fast adduction of NO_2 -OA to thiol containing proteins and to glutathione has been revealed in mice plasma [10], while recently NO_2 -OA-glutathione adducts have been identified in tomato cell suspension [8].

At the same time, the electrophilic reactivity causes post-translational modifications of Cys and His containing proteins affecting their distribution and/or function [11]. These nitroalkylation reactions are partly responsible for the biological role of NO_2 -FA [12]. Additionally, NO_2 -FA have been reported to release $\bullet\text{NO}$ in aqueous environments thus acting as endogenous $\bullet\text{NO}$ donors and transducing the $\bullet\text{NO}$ signal [13–16]. The signalling role of NO_2 -FA (especially NO_2 -Ln) was further supported by the fact that NO_2 -Ln content was modified during plant development and by abiotic stress [6]. Moreover, transcriptomic analysis revealed several genes, the expression of which was positively or negatively modulated by NO_2 -Ln, indicating the signalling role of NO_2 -Ln in plant cells [6]. Recently, the signalling role of NO_2 -OA has also been revealed since it induces the production of reactive oxygen species possibly through the reduction of glutathione pool or via the activation of NADPH oxidase and triggers plant defence responses in tomato cells [8,9].

Due to its role in animal signal transduction [17–19] NO_2 -OA is the most intensively studied NO_2 -FA with potent anti-inflammatory effects [20]. Studies in animal models pointed out the protective role of NO_2 -OA in cardiovascular, renal, and metabolic diseases [10,21,22], therefore its therapeutic application is promising. Identifying plant-derived dietary sources of NO_2 -FAs, specially the well-studied NO_2 -OA and its beneficial effects, would constitute a suitable way to access to the valuable properties described for these relevant molecules.

Brassica sprouts are rich sources of nutrients, phytochemicals, vitamins, minerals, enzymes, amino acids, and fatty acids, therefore they have high nutritional value [23]. The most abundant fatty acids in *Brassica* sprouts are oleic, linoleic, linolenic, erucic, palmitic, and stearic acids which account for 89–94% of the total fatty acid content [24]. *Brassica napus* (oilseed rape) seedlings are especially rich in oleic acid (61% of the total fatty acids, [24]), but the endogenous presence of NO_2 -OA in this species as well as in other plants has not been observed so far. Therefore, our aim was to examine the presence of NO_2 -OA in differentially developed *Brassica* plants. Furthermore, we aimed to examine the putative biological functions of NO_2 -OA in *Brassica* seeds and seedlings.

2. Results and Discussion

2.1. Characterization of the Synthesized NO_2 -OA Standard

The synthesized compound was identified as (*E*)-9-nitrooctadec-9-enoic acid based on the identical ^1H and ^{13}C NMR data with those reported in the literature [25]. This was assured by mass spectrometric measurements where the measured molecule ion mass was shown to be m/z 326.5, calculated neutral molecule mass was 327.4589 Da, molecular formula $\text{C}_{18}\text{H}_{33}\text{NO}_4$ (Figure 1A,B).

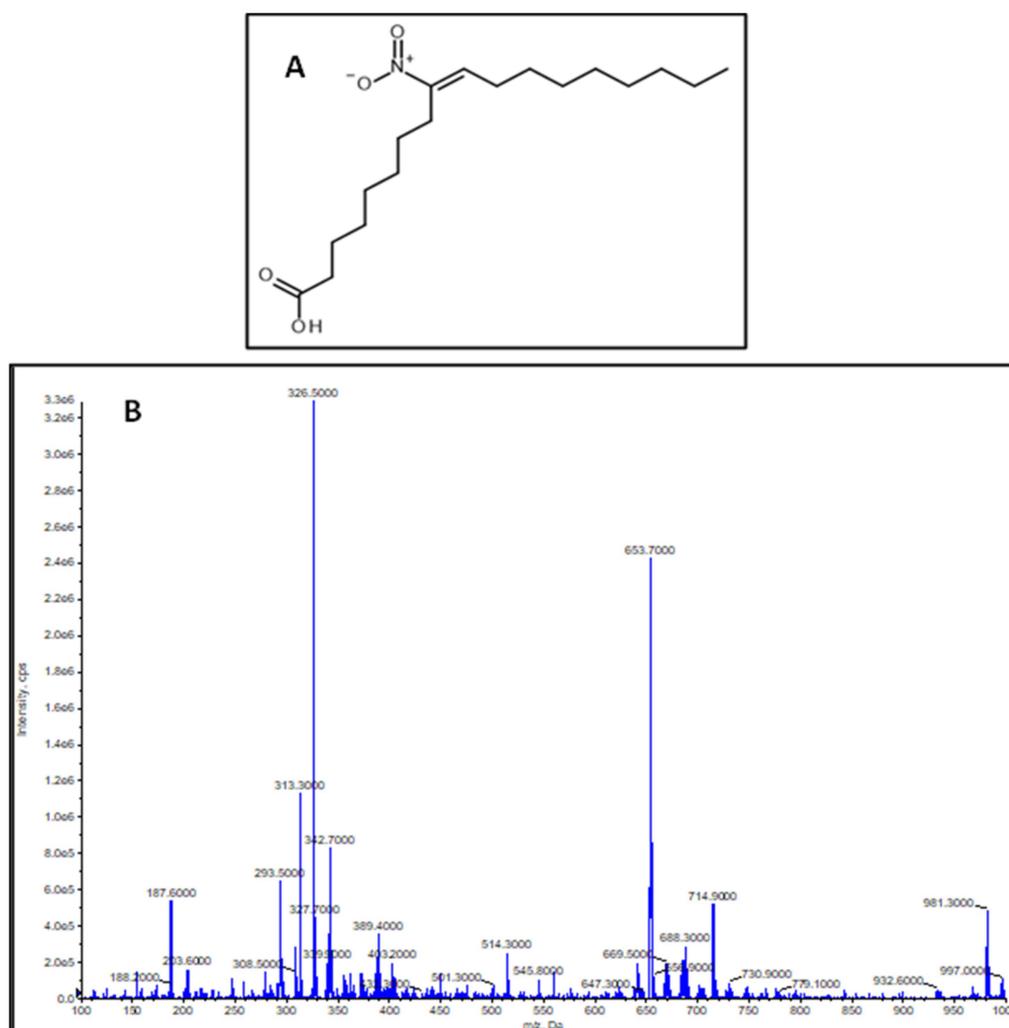


Figure 1. (A) Structure of (*E*)-9-nitrooctadec-9-enoic acid. (B) Q1 MS scan of nitro-oleic acid (NO₂-OA) standard. Scan range was set between *m/z* 100 and 1000 Da. Molecule ion peak [M – H][–] was *m/z* 326.5.

2.2. Calibration

From the synthesized standard a stock standard was prepared with methanol (HPLC grade) and working solutions were prepared (50–2500 ng/mL) by diluting the stock standard. Each solution was injected three times, the precision (Relative Standard Deviation, RSD, %) of the calibration measurements ranged between 1.2093–2.3633%. Calibration showed linear regression, the R² value was 0.9986, the limit of detection (LOD, S/N = 3.3) was 0.1184 nmol/mL, and the limit of quantitation (LOD, S/N = 10) was 0.3588 nmol/mL (Figure 2).

2.3. NO₂-OA Content of *Brassica napus* at the Seed and Seedling Stages

Seeds and seedlings of *Brassica napus* are remarkably rich in the unsaturated fatty acid, oleic acid, which implies the possibility of the presence of NO₂-OA in *Brassica* as well. Figure 3A represents *Brassica napus* seeds and seedlings at day 0, and 2nd, 4th, and 7th day after sowing. Therefore, we performed the analyses of NO₂-OA concentrations and the quantitative data are presented in Table 1, while the mean NO₂-OA concentrations with standard errors are shown in Figure 3. Ion chromatograms of the reference standard NO₂-OA and of the 7-day-old *Brassica napus* seedlings are presented in Figure 4. Further chromatograms are presented as Figure S1. In samples, one further unidentified isomer of NO₂-OA can be detected with higher retention time.

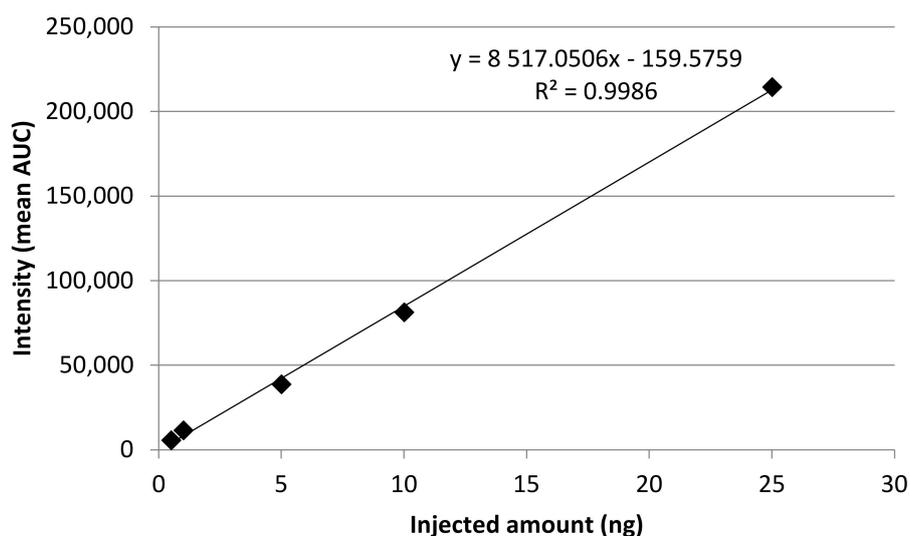


Figure 2. Calibration line of NO₂-OA standard. Axis X shows the range of injected NO₂-OA amount (ng) from working solutions, axis Y shows the mean area under curve (AUC) of the working solutions.

Table 1. Concentrations of nitro-oleic acid (NO₂-OA) in *B. napus* seeds and differently developed seedlings. N/A (not available) indicates data under the detection limit. Samples were injected three times, the precision (RSD, %) of the sample measurements ranged between 0.1348–3.9822 RSD%. The precision between the parallel samples ranged between 9.4828–26.6930 RSD%.

Sample	NO ₂ -OA Concentration (nmol/g Fresh Weight)	Mean NO ₂ -OA Concentration (nmol/g Fresh Weight)	RSD (%)
Seed 1	1.4582		
Seed 2	1.8293	1.6987	12.2753
Seed 3	1.8086		
Seedling 2nd day 1	0.4378		
Seedling 2nd day 2	0.3475	0.3701	16.1281
Seedling 2nd day 3	0.3250		
Seedling 4th day 1		N/A	
Seedling 4th day 2			
Seedling 4th day 3			
Seedling 7th day 1	0.5569		
Seedling 7th day 2	0.8663	0.6622	26.6930
Seedling 7th day 3	0.5635		
Shoot 7th day 1	0.3124		
Shoot 7th day 2	0.3943	0.3470	12.2250
Shoot 7th day 3	0.3343		
Root 7th day 1	0.4681		
Root 7th day 2	0.3876	0.4260	9.4828
Root 7th day 3	0.4221		

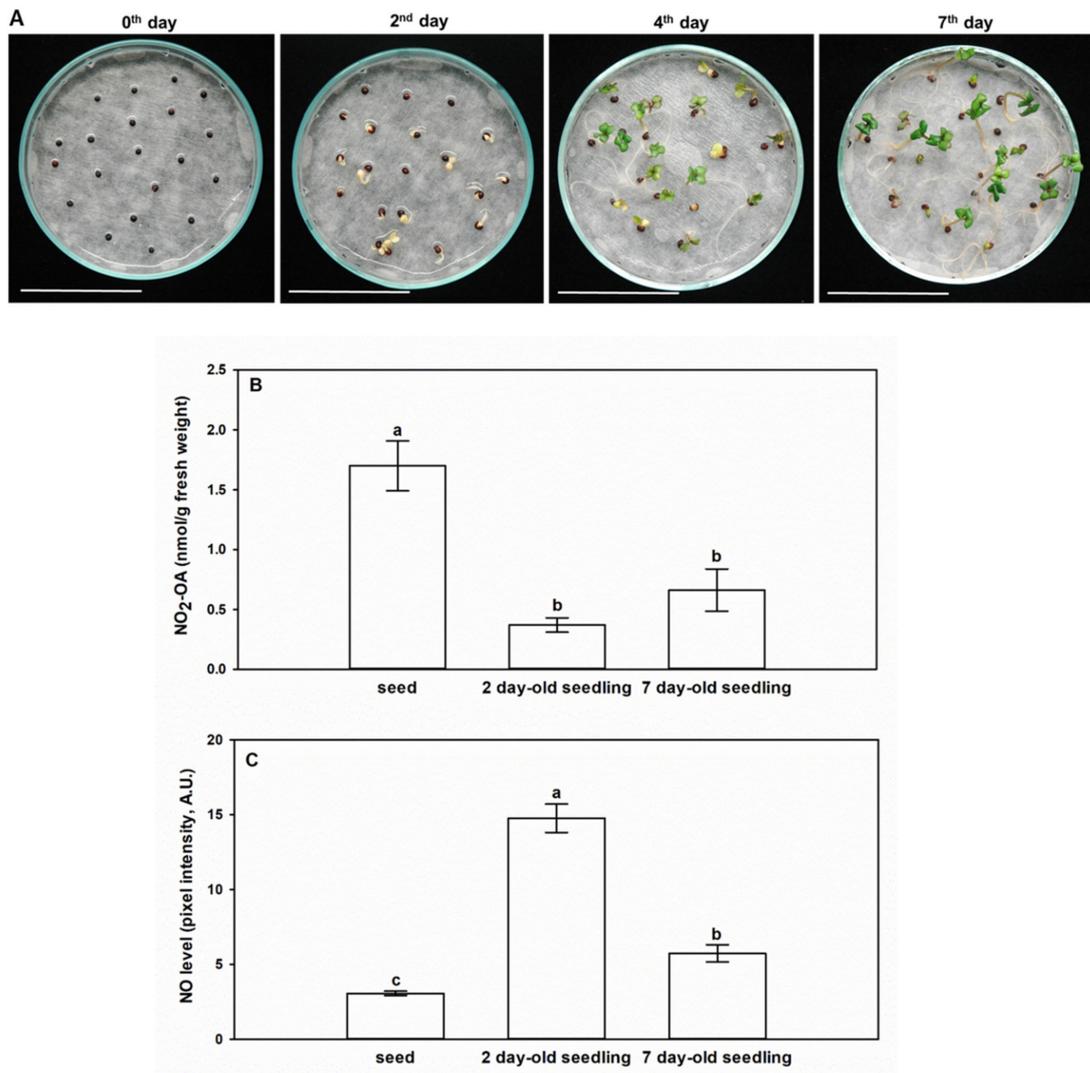


Figure 3. Levels of NO₂-OA and •NO change in opposite ways in the early stage of germination (day 0) and during seedling growth (2nd, 4th and 7th days). (A) Representative photographs taken from *Brassica napus* seedlings on day 0, and 2nd, 4th, and 7th day after sowing. Bars = 4.5 cm. (B) Mean concentrations of NO₂-OA (nmol/g fresh weight, with standard errors) in seeds and differently developed seedlings of *Brassica napus*. (C) •NO levels (pixel intensity, arbitrary unit) in *Brassica napus* seeds, 2 day-old and 7 day-old seedlings. Different letters indicate significant differences according to the Holm–Sidak test ($P \leq 0.001$).

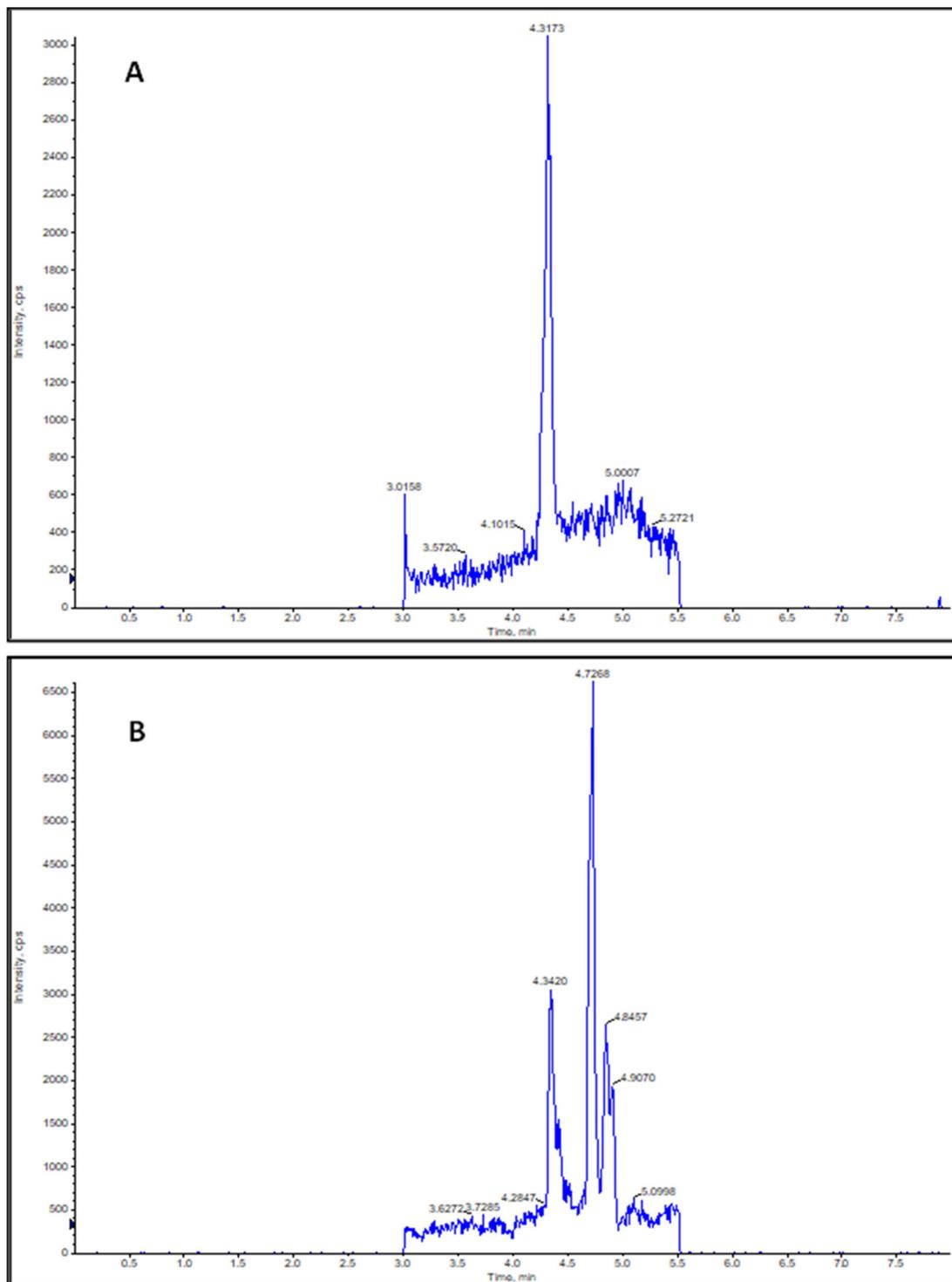


Figure 4. (A) Ion Chromatogram of m/z 326.5 of NO_2 -OA working solution (100 ng/mL). Peak retention time (RT) = 4.3173 min. (B) Ion Chromatogram of m/z 326.5 of a sample derived from 7- day- old *Brassica napus* seedlings. Peak retention time (RT) = 4.3420 min.

Brassica napus seeds contained a notably high amount of NO_2 -OA compared to seedlings (Table 1, Figure 3B). During the early phase of seedling growth (2nd day), the high NO_2 -OA content of *Brassica* seeds decreased by 78%, and it continued to decline in the following two days (4th day). At the 7th day of seedling growth, the seedlings showed an increased NO_2 -OA content compared to the

2- and 4-day-old seedlings, but it was only approximately 40% of the NO₂-OA content of the seed. Separated analysis of the shoot and root material indicated that both organs of 7-day-old *Brassica napus* seedlings contained NO₂-OA in similar quantities (Table 1). In *Arabidopsis* tissues, NO₂-OA could not be detected, but NO₂-Ln content was 2.9-fold higher in the seeds than in the 14-day-old seedlings ([6], Table 2). The relatively high NO₂-Ln and NO₂-OA contents of *Arabidopsis* and *Brassica* seeds (Table 1, Table 2) indicate the involvement of NO₂-FA in seed germination possibly as endogenous •NO donors [16]. To examine this hypothesis, we detected •NO levels in *Brassica* seeds and seedlings (2nd, 7th days) (Figure 3C). Compared to seeds, on the 2nd day the •NO content increased by almost 5-fold. The opposite changes in NO₂-OA and •NO levels suggest that following the induction of germination NO₂-OA as endogenous donor in the seed may release NO resulting in the decrease in its own free level and concomitant •NO accumulation. Furthermore, it is important to note, that the measured NO₂-OA concentrations in *Brassica* tissues are in the nanomolar range (Table 1, Figure 3B), that is an order of magnitude higher than the picomolar NO₂-Ln concentrations of *Arabidopsis* seedlings, cell suspension, pea roots, leaves and rice leaves ([6,7], Table 2). In case of NO₂-OA supplemented tomato cell suspension, the endogenous NO₂-OA content was similar to that of the untreated *Brassica napus* seeds and seedlings ([8,9], Table 2). From the above comparisons, it can be concluded that the oleic acid content of *Brassica napus* seeds and seedlings is susceptible to physiological nitration making the plant a rich source of NO₂-OA.

Table 2. Concentration values of free NO₂-FA determined in different plant species and experimental systems (modified from Ref. [7]). Abbreviations: ACSC = *Arabidopsis* cell suspension culture.

Plant Species	Organ or Xperimental System	Type of NO ₂ -FA Detected	Concentration of NO ₂ - FA Detected (pmol/g Fresh Weight)	Refs.
<i>Arabidopsis thaliana</i>	seed	NO ₂ -Ln	11.18	[6]
	14-day-old seedling	NO ₂ -Ln	3.84	
	30-day-old leaves	NO ₂ -Ln	0.36	
	45 day-old leaves	NO ₂ -Ln	0.54	
	9-day-old ACSC	NO ₂ -Ln	0.28	
<i>Pisum sativum</i>	root	NO ₂ -Ln	0.072	[7]
	leaf	NO ₂ -Ln	0.084	
	mitochondria	NO ₂ -Ln	0.282	
<i>Oryza sativa</i>	peroxisomes	NO ₂ -Ln	0.282	[7]
	leaf	NO ₂ -Ln	0.748	
<i>Solanum lycopersicum</i>	cell suspension treated with NO ₂ -OA (0.5, 5, 10, 12.5, 25, 50 μM, 1 h or 6 h)	NO ₂ -OA	~2500	[8] [9]

2.4. Exogenous NO₂-OA Treatment of *Brassica* Seeds Positively Influences •NO Levels and Germination Capacity

The 24-hour-long treatment with 100 μM NO₂-OA caused 10-fold increment in the endogenous •NO level of seeds (Figure 5A,B), while NO₂-OA at 50 μM or 500 μM concentrations did not cause significant changes in •NO levels compared to controls (50 or 500 μM DMSO, respectively). The significant effect of 100 μM NO₂-OA on •NO level prevailed also on the 2nd day after sowing, since the •NO level of NO₂-OA-supplemented seedlings was 4.7 times that of the control (100 μM DMSO, Figure 5A,B). The NO₂-OA-induced DAF-FM fluorescence was quenched by the •NO scavenger cPTIO (Figure 5B) indicating that the alterations in DAF fluorescence correspond to •NO level changes. Oleic acid (OA) treatments applied as controls did not influence •NO levels either in seeds or in 2-day-old seedlings (Figure 5A).

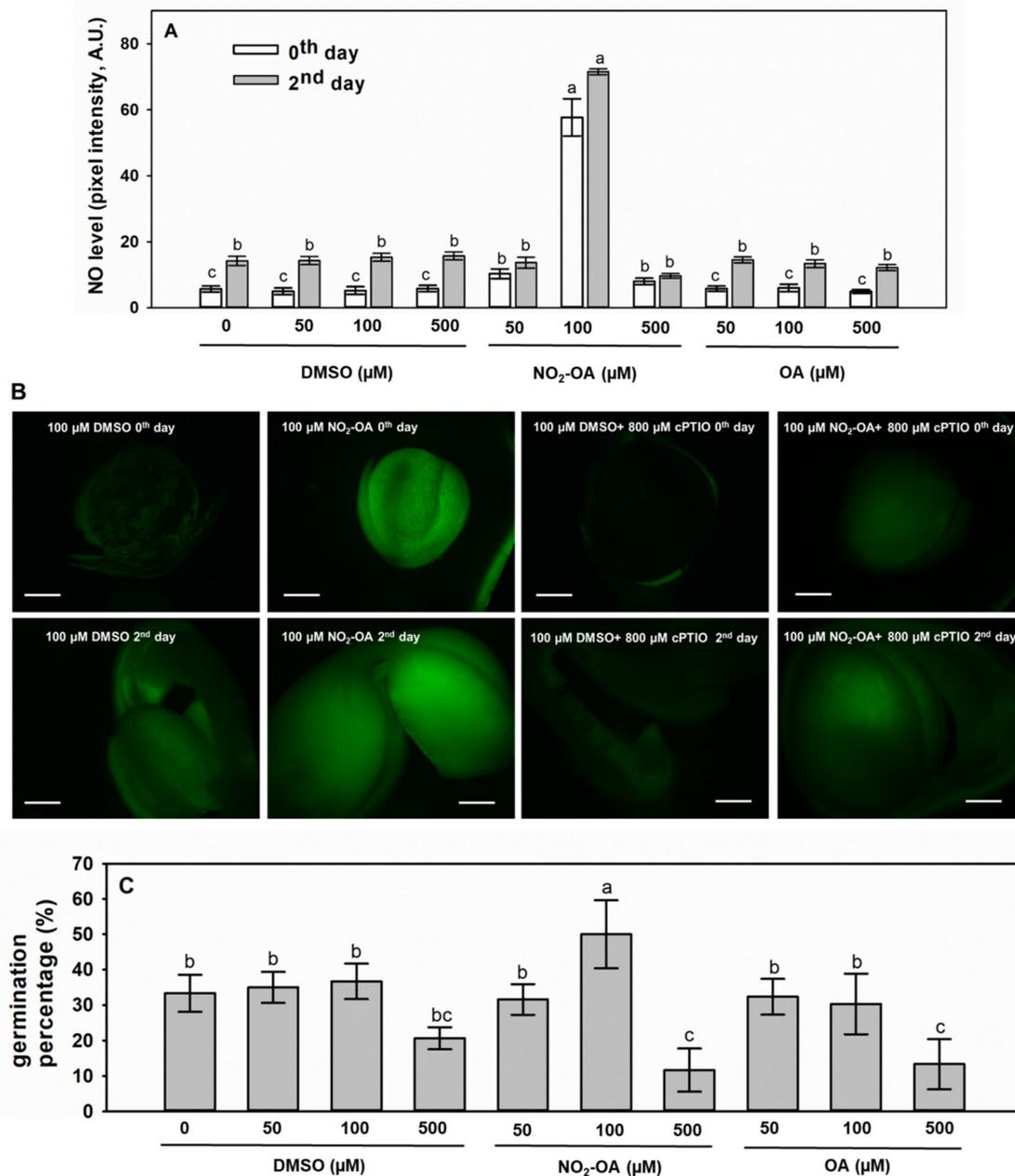


Figure 5. Exogenous NO₂-OA increases endogenous •NO levels and triggers germination of *Brassica napus* seeds. (A) Nitric oxide level in seeds (day 0 of germination) and seedlings (2nd day after sowing) of *Brassica napus* treated for 24 h with 50, 100 or 500 μM NO₂-OA or with equal concentrations of OA. As controls, seeds were treated with 0, 50, 100 or 500 μM DMSO. Different letters indicate significant differences according to Holm–Sidak test ($P \leq 0.001$). (B) Representative fluorescent microscopic images of DAF FM-stained *Brassica* seeds and seedlings treated with 100 μM NO₂-OA or 100 μM DMSO for 24 h in the presence or absence of 800 μM cPTIO. Bars = 800 μm. (C) Germination percentage (determined at the 2nd day after sowing) of *Brassica* seeds treated with 50, 100, or 500 μM NO₂-OA or with equal concentrations of OA. As controls, seeds were treated with 0, 50, 100, or 500 μM DMSO. Different letters indicate significant differences according to the Holm–Sidak test ($P \leq 0.001$).

The germination percentages were altered in accordance with •NO levels, since 100 μM NO₂-OA treatment doubled the percentage of germinated seeds compared to control (100 μM DMSO) (Figure 5C). DMSO alone at 50 μM or 100 μM concentrations had no effect on seed germination, although in the presence of 500 μM DMSO germination percentage of *Brassica* seeds slightly decreased compared to control (0 μM

DMSO). Moreover, OA at 50 or 100 μM concentration exerted no effect on germination of *B. napus* seeds (Figure 5C). The effect of DMSO could dominate in case of 500 μM $\text{NO}_2\text{-OA}$ or 500 μM OA treatments, since in these seeds the germination ability was weaker than in control and $\bullet\text{NO}$ levels remained at control level (Figure 5A). These data clearly indicate that exogenous $\text{NO}_2\text{-OA}$ can result in the long-term increase of endogenous $\bullet\text{NO}$ level in *Brassica napus* seeds and seedlings, i.e., it may act as a $\bullet\text{NO}$ donor. The fact that nitro-fatty acids may act as $\bullet\text{NO}$ donors in plants has been previously raised [16]. It was revealed that $\text{NO}_2\text{-Ln}$ has the ability to release in vivo, in leaves and roots of old *Arabidopsis* plants [26], in *Arabidopsis* seedlings [16], and in *Arabidopsis* cell-suspension cultures [27]. Contrary to $\text{NO}_2\text{-Ln}$ and to $\text{NO}_2\text{-OA}$ in our study, exogenous application of $\text{NO}_2\text{-OA}$ (0.5, 5, 10, 12.5, 25, or 50 μM , 1 h or 6 h) did not increase $\bullet\text{NO}$ level in tomato cell suspension as was recently reported by Di Palma et al. [8,9]. Contradiction of the results can be explained by that here higher concentration of $\text{NO}_2\text{-OA}$ (100 μM) were applied for longer time period (24 h) than in the studies of Di Palma et al. [8,9].

2.5. $\text{NO}_2\text{-OA}$ Releases $\bullet\text{NO}$ in Vitro

Additionally, the $\bullet\text{NO}$ donor nature of $\text{NO}_2\text{-Ln}$ was proved by using several different in vitro approaches (DAF fluorescence, oxyhaemoglobin method, ozone chemiluminescence, [27]). In this work, we performed in vitro tests to support the hypothesis regarding the $\bullet\text{NO}$ donor role of $\text{NO}_2\text{-OA}$ (Figure 6). Spectrofluorometric measurement of DAF-FM-associated fluorescence revealed that the sample containing 10 μM $\text{NO}_2\text{-OA}$ liberated a relatively small amount of $\bullet\text{NO}$ during the 80 min period, while the same dosage of OA did not induce $\bullet\text{NO}$ level increase compared to the blind sample containing only buffer and the fluorophore (Figure 6A). Moreover, $\text{NO}_2\text{-OA}$ liberated $\bullet\text{NO}$ in a concentration-dependent manner and the fluorescence increase could be quenched by cPTIO. Elevating doses of OA did not increase $\bullet\text{NO}$ levels (Figure 6B).

Using the more sensitive $\bullet\text{NO}$ electrode, we could quantify $\bullet\text{NO}$ liberation in $\text{NO}_2\text{-OA}$ solution (pH 5.8) within 5 min reaching its maximum (~ 30 nM $\bullet\text{NO}$) after 20 min incubation. The produced $\bullet\text{NO}$ concentrations (20–30 nM) quantified by ISO-NOP electrode in this study are similar to those measured by $\bullet\text{NO}$ autoanalyzer in the case of 100 μM $\text{NO}_2\text{-Ln}$ or 80 μM $\text{NO}_2\text{-LA}$ [27]. The same concentration of OA showed no relevant $\bullet\text{NO}$ releasing capacity in the solution (Figure 6C). These in vitro data support the $\bullet\text{NO}$ donor character of $\text{NO}_2\text{-OA}$ in solutions and the degree of $\bullet\text{NO}$ liberation is similar to other $\text{NO}_2\text{-FAs}$. However, the same dosage of S-nitrosoglutathione (GSNO) or sodium nitroprusside (SNP) produced approx. 10-fold higher $\bullet\text{NO}$ concentration in solutions following 20 min incubation in light (data not shown) indicating that $\text{NO}_2\text{-FAs}$ (including $\text{NO}_2\text{-OA}$) $\bullet\text{NO}$ donor capacity is much lower compared to “classical $\bullet\text{NO}$ donors”.

In our experimental system, $\text{NO}_2\text{-OA}$ (100 μM , 24 h) treatment of *Brassica* seeds also promoted germination, presumably through the induction of high $\bullet\text{NO}$ levels. In vitro tests revealed that the concentration of liberated $\bullet\text{NO}$ is relatively low (Figure 6), but in seeds $\text{NO}_2\text{-OA}$ treatment caused intense $\bullet\text{NO}$ formation (Figure 5). Therefore, we assume that beyond direct $\bullet\text{NO}$ emission, secondary signal processes may also be activated in the presence of $\text{NO}_2\text{-OA}$ leading to the activation of $\bullet\text{NO}$ metabolic routes in seeds and seedlings. It is well known, that $\bullet\text{NO}$ attenuates seed dormancy and promotes germination and we are beginning to recognize also the molecular mechanisms of $\bullet\text{NO}$ action [28].

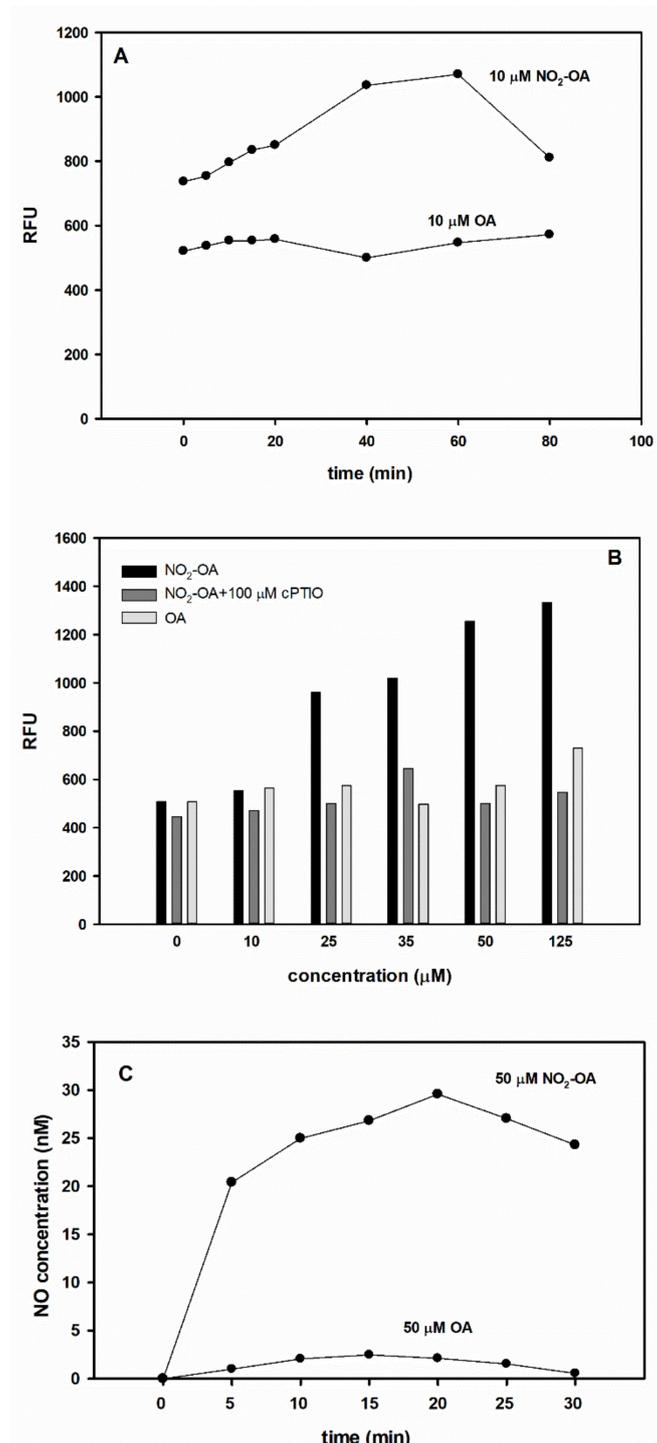


Figure 6. NO₂-OA liberates •NO in solutions (A) Relative fluorescence units (RFU) of DAF-FM fluorescence in solutions in the presence of 10 μM NO₂-OA or 10 μM OA measured at different time points. (B) Relative fluorescence units (RFU) of DAF-FM fluorescence in solutions containing different concentrations of NO₂-OA (with or without 100 μM cPTIO) or OA. Data were recorded after 20 min incubation. (C) •NO concentration (nM) measured by ISO-NOP (2 mm) electrode in solutions containing 50 μM NO₂-OA or OA measured at different time points.

3. Materials and Methods

3.1. Plant Material and Growing Conditions

Experiments were carried out on *Brassica napus* L. (cv. GK Gabriella) seedlings. The seeds were obtained from the Cereal Research Non-profit Ltd., Szeged, Hungary. *Brassica* seeds were surface sterilised in 70% (v/v) ethanol and 5% (v/v) sodium hypochlorite, then placed on moistened filter paper in Petri dishes (9 cm diameter, 30 seeds/Petri dish). Germination took place under controlled conditions (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density, 12 h/12 h light/dark cycle, relative humidity 55–60% and temperature 25 ± 2 °C). For the NO₂-OA analysis, the samples were taken at the 2nd, 4th and 7th day after sowing. Additionally, seeds after imbibition, at the early stage of germination (day 0) were also sampled for NO₂-OA quantification. Plant material (5 g) was collected, frozen in liquid nitrogen and stored at -80 °C until the analyses.

3.2. Synthesis and Structure Determination of 9-Nitro-Oleic Acid Standard

Using the slightly modified method of Woodcock et al. [29], 9-nitro-oleic acid was synthesized. Bromononanoic acid was used as starting material for allylization to gain 9-bromononanoic acid allyl ester. This compound was nitrated using silver nitrite. In the next step, 10-hydroxy-9-nitro-octadecanoic acid allyl ester was synthesized by the addition of nonyl aldehyde and 1,8-diazabicyclo[5.4.0]undec-7-ene. This compound was acylated with acetic anhydride in the presence of *p*-toluenesulfonic acid to gain 10-acetoxy-9-nitro-octadecanoic acid allyl ester. Deacylation in the presence of Na₂CO₃ led to the synthesis of 9-nitro-oleic acid allyl ester. The final product 9-nitro-oleic acid was gained by a catalytic hydrolysis using palladium tetrakis(triphenylphosphine). Purification was carried out by flash chromatography on silica gel (Merck, 40–63 μm) using a gradient of 0.5% acetic acid. Fractions with similar compositions were combined and the combined fraction containing a spot with remarkable absorption at 254 nm and with an R_f approximately 0.5 (silica gel plate, eluent CHCl₃–MeOH 95:5) was subjected to NMR identification and purity check.

¹H (500.1 MHz) and ¹³C (125.6 MHz) NMR spectra were recorded in CDCl₃ on a Bruker Avance DRX-500 spectrometer. The peaks of the residual solvent were taken as reference points. The compound was identified by comparison of its chemical shifts with literature data [25,30]. Mass spectrometric identification was performed on an API 2000 triple quadrupole tandem mass spectrometer (MDS Sciex, Toronto, ON, Canada) equipped with electrospray ion source. Mass spectrometric measurement was carried out by direct infusion in Q1 MS scan type in negative mode. Flow rate was set to 40 $\mu\text{L}/\text{min}$, scan range was set from *m/z* 100 to 1000, ion source temperature was set to 100 °C. The nebulizer gas was set to 18 psi. Measured molecule ion mass shown to be *m/z* 326.5, calculated neutral molecule mass was 327.4589 Da.

3.3. LC-MS Quantification of NO₂-OA in Brassica Seeds and Seedlings

For the quantification of NO₂-OA, 5 g of fresh plant material (seeds, whole seedlings or separated root and shoot) was used and the analysis was conducted by LC-MS. Mass spectrometry measurement was performed using single ion monitoring (SIM) on an API 2000 triple quadrupole tandem mass spectrometer (MDS Sciex, Toronto, ON, Canada) equipped with electrospray ion source. The nebulizer and heater gas was nitrogen, generated from a Peak NM20Z nitrogen generator (Peak Scientific Instruments Ltd., Scotland, UK) coupled with an Atlas Copco SF 4FF compressor. The nebulizer gas was set to 30 psi, the heater gas was set to 80 psi. The ion source temperature was set to 300 °C. Measurement was in negative mode. The voltage volumes were adjusted to *m/z* 326.5, the collision energy was set to -5 V, focusing potential to -330 V, declustering potential to -61 V and entrance potential to -10 V. HPLC separation was performed with Shimadzu HPLC system (Kyoto, Kyoto Prefecture, Japan): DGU-20A3 degasser, CBM-20A controller, two LC-20AD pumps, SIL-20A HT autosampler, CTO-20AC column thermostat, SPD-20A UV-Vis detector, using Kinetex F5 (100 \times 4.6 mm, 2.6 μm , 100Å) (Phenomenex, Inc., Torrance, CA, USA). Elution was carried out with the gradient

system of H₂O–methanol (0 min: H₂O–methanol 2:8, 0.6 min: 2:8, 1.5 min: 0:1, 3 min: 0:1, 3.2 min: 2:8, 8.2 min: 2:8). The oven was set to 40 °C and the flow rate was 600 µL/min. The retention time of NO₂-OA was 4.32 min. Data acquisition was performed with Analyst software (ver. 1.6.3). Quantification of nitro-oleic acid was carried out by using the synthesized standard (see in Section 2.2).

Plant material was extracted with pure MeOH (HPLC grade) in a VWR Ultrasonic Cleaner USC 300D (capacity: 1 L, internal dimension: W × D × H 240 × 135 × 100 mm) ultrasonic bath for 10 min, with dual half-wave sound with sweep, frequency was set to 45 kHz, ultrasonic power to 80 W, temperature was set to 25 °C, and filtered through a syringe filter (PTFE, 0.45 µm pore size, Labex Ltd.), the first half ml of the filtrate was thrown into the waste. From each plant part, three parallel samples were prepared, and each sample was injected three times.

3.4. •NO Detection in *Brassica napus* Seeds and Seedlings

Nitric oxide levels were examined in seeds as well as in 2- and 7-day-old seedlings of *Brassica napus* using the 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) according to Kolbert et al. [31]. Plant samples were incubated in 10 µM dye solution for 30 min (darkness, 25 ± 2 °C) and washed twice with Tris-HCl (10 mM, pH 7.4). Microscopic analysis was accomplished under Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany) equipped with a high-resolution digital camera (AxioCamHR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (exc.: 450–490, em.: 515–565 nm). Pixel intensities were measured on digital photographs (at least 10 photographs per sample per experiment) using Axiovision Rel. 4.8 software (Carl Zeiss, Jena, Germany).

3.5. NO₂-OA Treatment of *Brassica napus* Seeds

The synthesized NO₂-OA or OA was dissolved in dimethyl sulfoxide (DMSO) in order to obtain a stock solution (10 mM). The NO₂-OA or OA stock was diluted with distilled water to the final concentrations (50, 100, 500 µM). Control solutions were prepared by measuring the volume of DMSO corresponding to the stock solutions (indicated as 0, 50, 100, or 500 DMSO). *Brassica napus* seeds (30 seeds per treatment) were incubated in NO₂-OA, in OA or in control solutions for 24 h on an orbital shaker and then were placed on moist filter paper in Petri dishes (20 seeds/Petri dish). Sets of seeds were treated with NO₂-OA or control solutions in the presence of 800 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO). Germination took place as indicated above. Nitric oxide levels were detected in seeds prior to germination (day 0), and in seedlings 2 days after sowing (2nd day). Germination percentages (%) were also calculated.

3.6. Spectrofluorometric Determination of •NO Levels

•NO liberation from NO₂-OA solutions were detected by fluorescence spectrophotometry (Ref. [27] with modifications). Reaction mixtures (2 mL final volume) containing 0 (blind) 10, 25, 35, 50, 125 µM NO₂-OA or the same concentrations of OA, plus 2 µM DAF-FM and Tris-HCl (pH 7.4) buffer were incubated at room temperature in the dark for several time periods (5–80 min) and the emitted fluorescence was recorded by a spectrofluorimeter (Hitachi F-4500, Hitachi Ltd., Tokyo, Japan). Excitation wavelength was set at 485 nm and emissions were measured at 515 nm. The NO₂-OA-induced fluorescence was quenched by the addition of 100 µM cPTIO.

3.7. Measurement of •NO Concentration by •NO-Specific Electrode

The •NO -sensitive electrode (ISO-NOP, 2 mm, World Precision Instruments Inc., Sarasota, FL, USA) was calibrated using a method based on S-nitroso-N-acetylpenicillamine (SNAP) decomposition to •NO in the presence of copper [32]. Two mL of NO₂-OA or OA (both at 50 µM concentration) solutions were prepared in a 5-mL glass bottle and were measured immediately after preparation. To ensure constant mixing of the solution a magnetic stirrer was applied during the measurement. •NO concentration (nM) was calculated from a standard curve.

3.8. Statistical Analysis

All results are shown as mean \pm SE. Data were statistically evaluated by the Holm–Sidak method (One-way ANOVA, $P \leq 0.001$) using SigmaPlot 12.

4. Conclusions

Following successful standard synthesis and method optimization, we have been the first to observe that *Brassica* seeds and seedlings contain free NO₂-OA. Exogenous treatment of *Brassica* seeds with NO₂-OA promoted germination and increased endogenous •NO level suggesting that NO₂-OA may be involved in germination as an •NO donor. The •NO liberating capacity of NO₂-OA was proved also by in vitro approaches (spectrofluorometric detection of DAF-FM fluorescence and •NO-sensitive electrode). Due to their relatively high NO₂-OA concentrations, *Brassica* sprouts can be considered as a good source of dietary NO₂-OA intake in addition to their nutrient, mineral and vitamin content. Therefore, future studies should quantify the NO₂-OA content of additional *Brassica* species and food plants.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2223-7747/9/3/406/s1>. Figure S1: Chromatograms of *B. napus* seeds or seedlings (A) Ion chromatogram of *m/z* 326.5 of a sample derived from *Brassica napus* seed. (B) Ion chromatogram of *m/z* 326.5 of a sample derived from 2-day-old *Brassica napus* seedling. (C) Ion chromatogram of *m/z* 326.5 of a sample derived from 4-day-old *Brassica napus* seedling. (D) Ion chromatogram of *m/z* 326.5 of a sample derived from 7-day-old *Brassica napus* shoot. (E) Ion chromatogram of *m/z* 326.5 of a sample derived from 7-day-old *Brassica napus* root.

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