

Metabolism and Photolysis of 2,4-Dinitroanisole in *Arabidopsis*

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Recipe for Murashige and Skoog (MS) basal medium (per liter of deionized water): 5.0 g sucrose, 4.43 g MS salts plus vitamins (Caisson labs, Smithfield, UT), 0.5 g 2-(N-morpholino)ethanesulfonic acid hydrate (MES). Adjust pH to 5.7 using 1 M KOH.

QTOF Parameters for untargeted metabolomics

To identify DNAN metabolites in whole plant extracts, we used a Waters (Milford, MA) Acquity ultraperformance liquid chromatography (UPLC) instrument with a quadrupole-time-of-flight mass spectrometer (Acquity QTof Premier). An Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 μ m) heated to 60 °C (0.6 mL/min) was used. Leucine enkephalin was infused (10 μ L/min) as the lock mass and samples were analyzed (in separate runs) in ESI⁻ and ESI⁺ modes. A mass to charge (m/z) range of 40–1000, a scan rate of 0.2 s/scan and a sampling cone voltage of 30 were used for ESI⁻ mode. A scan rate of 0.15 s/scan and a sampling cone voltage of 23 were used for ESI⁺ mode. Samples in the auto-sampler tray were maintained at 8 °C. The mobile phase was (A) 95% DI water with 5% acetonitrile and (B) 95% acetonitrile and 5% DI water, both containing 5 mM ammonium acetate for ESI⁻. For ESI⁺ mode, 0.05% formic acid was added to both mobile phases. The mobile phase gradient was: 0 min, 0% B; linear to 7 min, 100% B; 9 min, 100% B; 9.1 min 0% B; 13 min 0% B. The desolvation gas (400 °C) flow was 800 L/h and the capillary voltage was 2.5 kV.

Orbitrap parameters for tandem mass spectrometry and formula assignment

To elucidate accurate mass fragments of DNAN metabolites, we used a Thermo Scientific (Waltham, MA), Q Exactive quadrupole-Orbitrap mass spectrometer after separation with a Thermo Ultimate 3000 UHPLC⁺ Focused at the Center For Mass Spectrometry and Proteomics at the University of Minnesota. We used an Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 μ m) heated to 60 °C (0.4 mL/min). The mobile phase for positive mode was DI/ACN containing 10 mM ammonium acetate, and the gradient was as follows (%ACN): 0 min, 2%; linear to 3 min, 5%; linear to 11 min, 98%; 12 min, 98%, 13 min, 2%; 15 min 2%. For negative mode, the same gradient was used with 10 mM ammonium acetate in MeOH as the organic phase. The mass range was 65-975m/z and targeted MS/MS was conducted with a ramped normalized collision energy of 10, 35, 45.

Table S1. Chemicals and reagents utilized

IUPAC name	Abbreviation	CAS #	Purity	Source
2,4-dinitroanisole	DNAN	119-27-7	98%	Alfa Aesar
2,4-dinitrophenol	DNP	51-28-5	Analytical standard	Restek
2-methoxy-5-nitroaniline	2-ANAN	99-59-2	Analytical standard	SPEXOrganics
4-methoxy-3-nitroaniline	4-ANAN	577-72-0	97%	Accela ChemBio Co.
2-amino-4-nitrophenol	2-ANP	99-57-0	99%	Acros Organics
4-amino-2-nitrophenol	4-ANP	119-34-6	99%	Acros Organics
2-(N-acetyl)amino-4-nitroanisole	2-NAc-NAN	N/A	>99%	See ref. 30 of manuscript
Sucrose	N/A	57-50-1	ACS	Fisher Scientific
2-(N-morpholino)-ethanesulfonic acid	MES	4432-31-9	≥99%	Research Products International Corp.
Sodium Nitrite- ¹⁵ N	¹⁵ NO ₂	68378-96-1	98%+ atom	Cambridge Isotope Laboratories
2,3-diaminonaphthalene	DAN	771-97-1	97%	Alfa Aesar
Ammonium acetate	NH ₄ OAc	631-61-8	Optima LC/MS	Fisher Scientific

Table S2. LC-QQQ parameters

LC-QQQ, Agilent 1260 LC and Agilent 6460 QQQ, Agilent Poroshell 120 EC-C18 (3x50 mm, 2.7 µm) with 3x5 mm guard column and 0.5 mL/min flow rate (35 °C), 10 µL injection						
Analyte	ESI mode	LOQ ^a (ppb)	Mass transitions (m/z)	Mobile Phases (A/B)	Gradient Time %B	
DNAN	-	5	183>109 183>123	DI/MeOH 10mM ammonium acetate in both	0	10
[¹⁵ N ₂]DNAN	-	5	185>110 185>123		5	95
[¹³ C ₆]DNAN	-	5	189>114 189>129		7	95
DNP	-	0.5	183>109 183>123		7.1	10
					12	10
2-ANAN	+	0.5	169.1>123 169.1>108	DI/ACN 0.1% formic acid in both		
4-ANAN	+	0.5	169.1>122.0 169.1>94.1			
2-amino-4-nitrophenol	+	1	155.1>109 155.1>92		0	5
					3	5
4-amino-2-nitrophenol	+	1	155.1>109 155.1>80.1		10	15
					11	98
2-(N-acetyl)amino-4-nitroaniso	+	10	211.07>169.1 211.07>123		13	98
					13.1	5
					17	5
M330	+	N/A	331>169.1 331>123	DI/MeOH 10mM ammonium acetate in both		
[¹⁵ N ₂]M330	+	N/A	333>171 333>124			
M568	+	N/A	569.2>321 569.2>304 569.2>289		0	2
					12	2
M524	-	N/A	523.2>303 523.2>289 523.2>273		13	98
					16	98
					20	2

^aLimit of quantification defined here as lowest concentration used in a linear calibration curve

Table S3. XCMS Online parameters for untargeted metabolomics analysis

Feature detection	Method	matchedFilter
	FWHM	6
	step	0.1
	max # chrom. Peaks	50
	mzdiff	0.01
	S/N ratio cutoff	10
Retention time correction	method	obiwarp
	profStep	1
Alignment	minfrac	0.5
	bw	5
	mzwid	0.015
	minsamp	1
	max	300
Statistics	Statistical Test	unpaired parametric t-test
	p-value threshold (highly significant features)	0.01
	fold change threshold	1.5
	p-value threshold (significant features)	0.5
Miscellaneous	Correct mass calibration gaps	Yes
	Bypass file sanity check	No

Table S4. Summary of [¹⁵N₂]DNAN metabolites identified in *Arabidopsis* plant tissue after 48 hours of exposure.

metabolite	confidence level	RT (min)	proposed ion	proposed ion formula	accurate mass	deviation mDa (ppm)	fragments (nominal m/z)
M330	2	3.4	[M+H] ⁺	C ₁₃ H ₁₉ ¹⁵ N ₂ O ₈	333.1070	0.67 (2.0)	171
M568	3	4.4	[M+H] ⁺	C ₂₄ H ₂₉ ¹⁵ N ₂ O ₁₄	571.1545	0.90 (1.6)	323
							306
							291
							275
M524	3	3.9	[M-H] ⁻	C ₂₃ H ₂₇ ¹⁵ N ₂ O ₁₂	525.1513	1.4 (2.6)	321
							305
							291
							154
M699	3	3.0	[M+H] ⁺	C ₂₈ H ₃₈ ¹⁵ N ₂ ¹⁴ N ₃ O ₁₄ S	702.2057	1.4 (2.0)	395, plus same as Table 1
			[M-H] ⁻	C ₂₈ H ₃₆ ¹⁵ N ₂ ¹⁴ N ₃ O ₁₄ S	700.1936	2.1 (3.0)	
M669	3	3.1	[M+H] ⁺	C ₂₇ H ₃₆ ¹⁵ N ₂ ¹⁴ N ₃ O ₁₃ S	672.1951	1.5 (2.2)	365, plus same as Table 1
			[M-H] ⁻	C ₂₇ H ₃₄ ¹⁵ N ₂ ¹⁴ N ₃ O ₁₃ S	670.1825	1.6 (2.4)	
M513	4	3.4	[M+H] ⁺	C ₂₁ H ₂₈ ¹⁵ N ₂ ¹⁴ N ₁ O ₁₀ S	516.1423	0.76 (1.5)	N/A
			[M-H] ⁻	C ₂₁ H ₂₆ ¹⁵ N ₂ ¹⁴ N ₁ O ₁₀ S	514.1286	1.2 (2.3)	
M483a	4	3.4	[M+H] ⁺	C ₂₀ H ₂₆ ¹⁵ N ₂ ¹⁴ N ₁ O ₉ S	486.1321	0.40 (0.82)	N/A
			[M-H] ⁻	C ₂₀ H ₂₄ ¹⁵ N ₂ ¹⁴ N ₁ O ₉ S	484.1183	1.5 (3.0)	
M483b	4	3.5	[M+H] ⁺	C ₂₀ H ₂₆ ¹⁵ N ₂ ¹⁴ N ₁ O ₉ S	486.1323	0.20 (0.40)	N/A
			[M-H] ⁻	C ₂₀ H ₂₄ ¹⁵ N ₂ ¹⁴ N ₁ O ₉ S	484.1185	1.7 (3.4)	
M375	4	2.4	[M-H] ⁻	C ₁₄ H ₁₉ ¹⁵ N ₂ O ₁₀	377.0973	0.19 (0.51)	N/A
M808	5	2.4	N/A	N/A	809.1894	N/A	N/A

Table S5. Summary of [¹³C₆]DNAN metabolites identified in *Arabidopsis* plant tissue after 48 hours of exposure.

metabolite	confidence level	RT (min)	proposed ion	proposed ion formula	accurate mass	deviation mDa (ppm)	fragments (nominal m/z)
M330	2	3.4	[M+H] ⁺	¹³ C ₆ C ₇ H ₁₉ N ₂ O ₈	337.1332	1.1 (3.3)	175
M568	3	4.4	[M+H] ⁺	¹³ C ₆ C ₁₈ H ₂₉ N ₂ O ₁₄	575.1804	1.1 (1.8)	327
							310
							295
							280
M524	3	3.9	[M-H] ⁻	¹³ C ₆ C ₁₇ H ₂₇ N ₂ O ₁₂	529.1774	1.4 (2.7)	325
							309
							295
							158
M699	3	3.0	[M+H] ⁺	¹³ C ₆ C ₂₂ H ₃₈ N ₅ O ₁₄ S	706.2317	1.5 (2.1)	399, plus same as Table 1
			[M-H] ⁻	¹³ C ₆ C ₂₂ H ₃₆ N ₅ O ₁₄ S	704.219	1.5 (2.1)	
M669	3	3.1	[M+H] ⁺	¹³ C ₆ C ₂₁ H ₃₆ N ₅ O ₁₃ S	676.2214	1.2 (1.8)	369, plus same as Table 1
			[M-H] ⁻	¹³ C ₆ C ₂₁ H ₃₄ N ₅ O ₁₃ S	674.2080	1.0 (1.5)	
M513	4	3.4	[M+H] ⁺	¹³ C ₆ C ₁₅ H ₂₈ N ₃ O ₁₀ S	520.1684	0.72 (1.4)	N/A
			[M-H] ⁻	¹³ C ₆ C ₁₅ H ₂₆ N ₃ O ₁₀ S	518.1539	0.43 (0.83)	
M483a	4	3.4	[M+H] ⁺	¹³ C ₆ C ₁₄ H ₂₆ N ₃ O ₉ S	490.1578	0.76 (1.5)	N/A
			[M-H] ⁻	¹³ C ₆ C ₁₄ H ₂₄ N ₃ O ₉ S	488.1446	1.7 (3.5)	
M483b	4	3.5	[M+H] ⁺	¹³ C ₆ C ₁₄ H ₂₆ N ₃ O ₉ S	490.1583	0.23 (0.52)	N/A
			[M-H] ⁻	¹³ C ₆ C ₁₄ H ₂₄ N ₃ O ₉ S	488.1446	1.7 (3.5)	
M375	N/A	N/A	N/A	N/A	nd	N/A	N/A
M808	5	2.4	N/A	N/A	813.2102	N/A	N/A

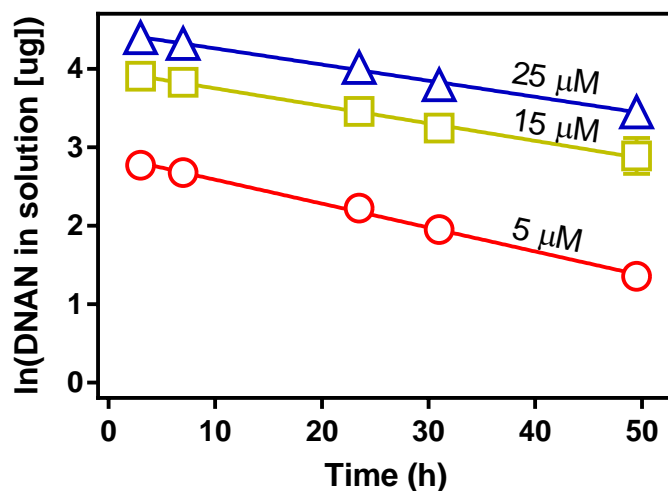


Figure S1. DNAN uptake kinetics at varying initial concentrations ($n=3$). Error bars of one standard deviation from the mean of triplicate flasks are not shown because they are smaller than the symbols. Experiments were conducted with 20 seedlings in 25 mL of MS media shaking at 120 rpm in 50 mL Erlenmeyer flasks after 14 days of pre-growth. DNAN was added in MeOH and an equivalent amount of MeOH was added to all conditions. Samples (0.5 mL) were withdrawn, analyzed by HPLC, and were not corrected for evaporation. Lines are simple linear regression ($R^2 = 0.997$ for all three), indicating pseudo-first-order uptake kinetics. The slopes of the linear regression (k_{obs}) were significantly different between the three initial concentrations ($p < 0.0001$); $k_{obs,5\mu M} = 0.031 \pm 0.001 \text{ h}^{-1}$, $k_{obs,15\mu M} = 0.022 \pm 0.001 \text{ h}^{-1}$, $k_{obs,25\mu M} = 0.021 \pm 0.001 \text{ h}^{-1}$. The difference in observed rates is most likely due to DNAN toxicity. Plants showed minor chlorosis at 15 μM initial DNAN and some chlorosis and plant death at 25 μM initial DNAN, so 5 μM was selected for further experiments.

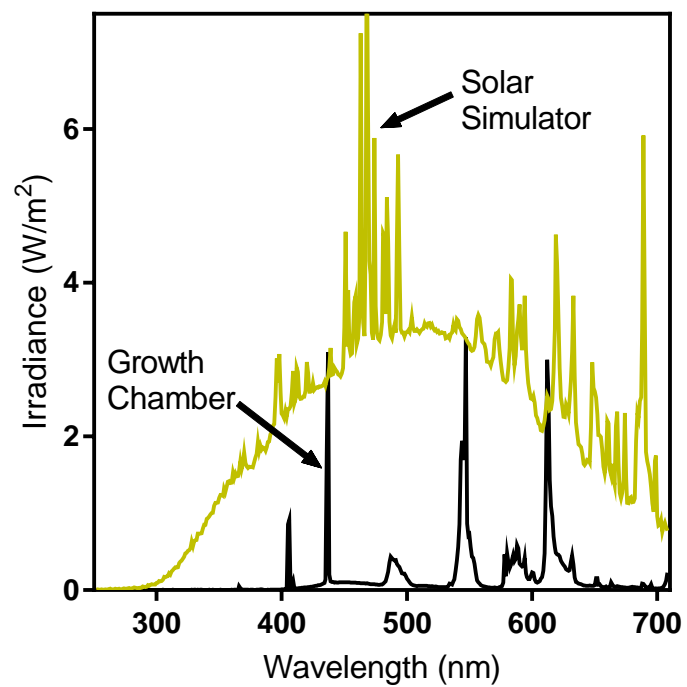


Figure S2. Irradiance of Suntest CPS+ at 750 W/m² and growth chamber lights as measured by a spectral radiometer (International Light Technologies, Peabody, MA, USA).

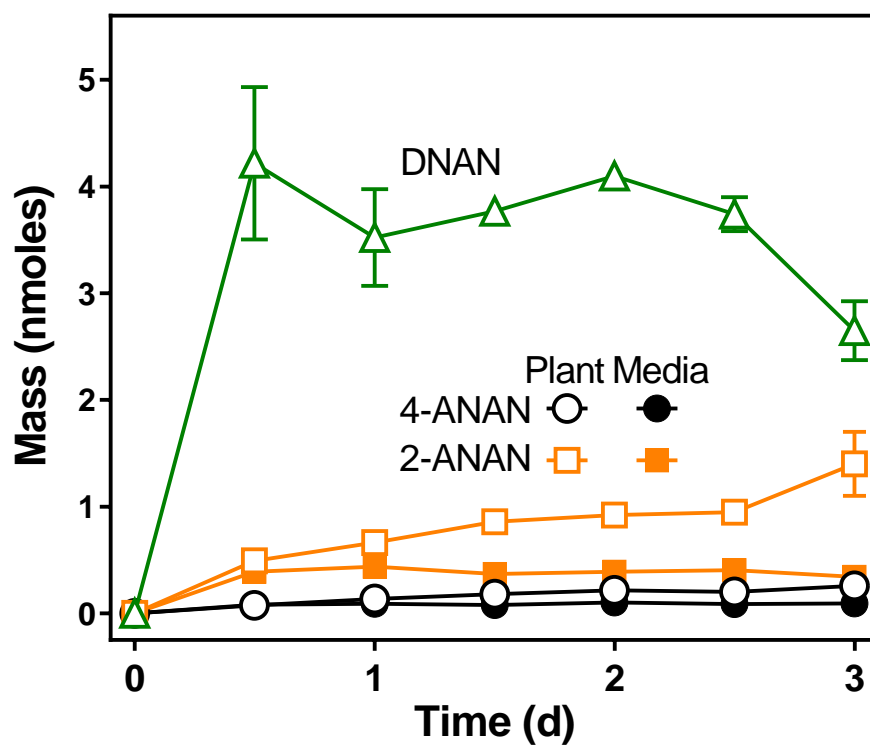


Figure S3. Mass of DNAN and phase I metabolites in plant tissues and media. DNAN data are the same as Figure 1 from manuscript, zoomed in on the y-axis for detail. Error bars are \pm standard error of the mean ($n=3$) and are not displayed when smaller than the symbol.

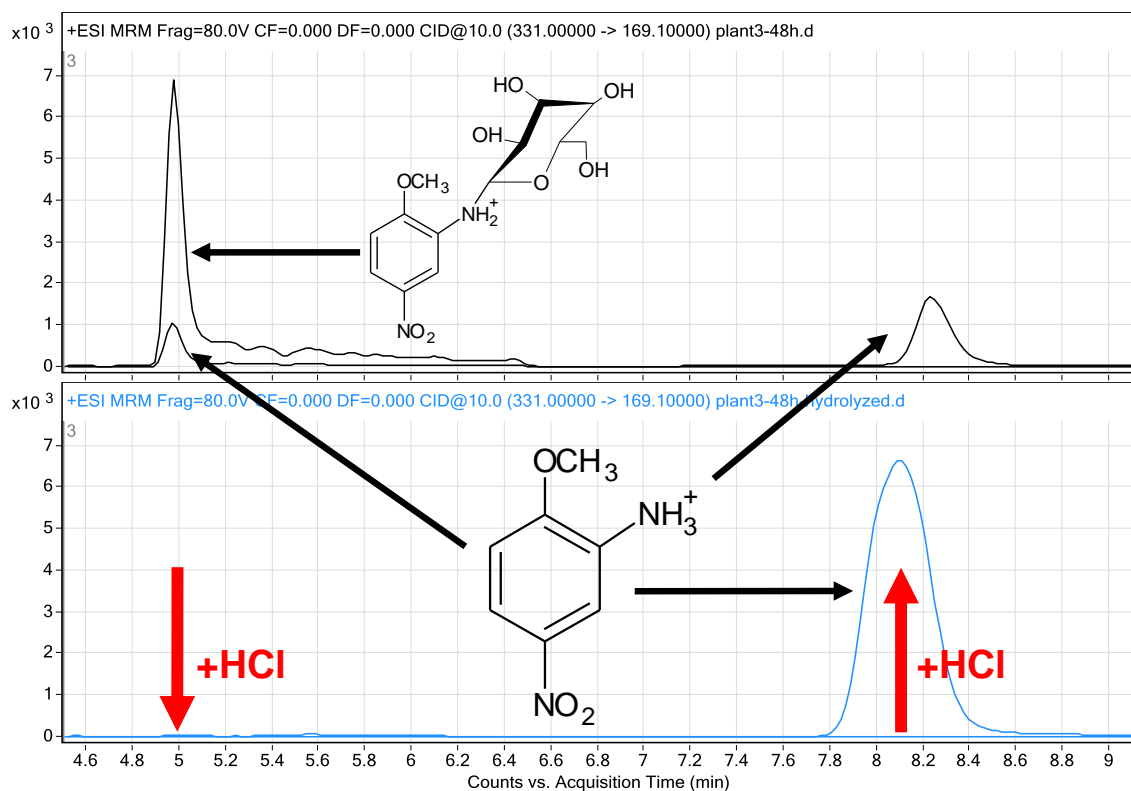


Figure S4. Hydrolysis of metabolite M330 to yield 2-ANAN. Shown are LC-MS/MS chromatograms of M330 in plant extract before (black lines, top panel) and after (blue lines, bottom panel) acid hydrolysis. M330 had a retention time of 5.0 minutes, while 2-ANAN had a retention time of about 8.1 minutes. The chromatograms shown are 331>169.1 for M330 and 169.1>108.0 for 2-ANAN. Some 2-ANAN is observed at 5 minutes due to in-source fragmentation of M330.

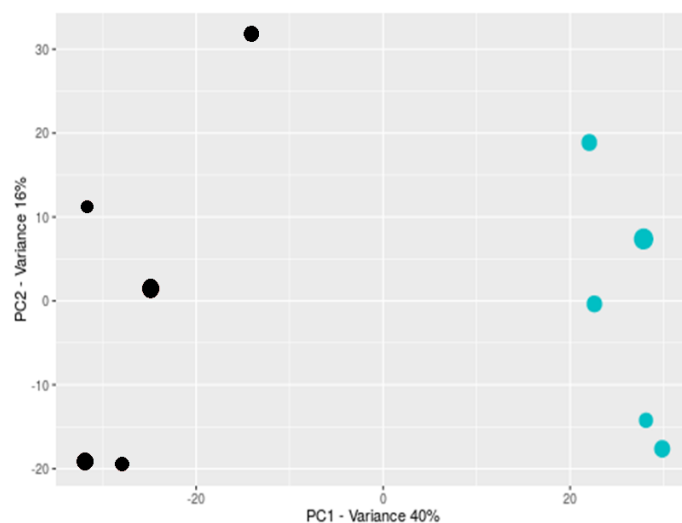


Figure S5. Principal components plot of photolyzed leaves (teal circles) compared to dark-control leaves (black circles) after 3 hours of simulated sunlight exposure, extraction, and analysis by LC-MS/QToF and XCMS online. The two treatments yielded two distinct groupings, but no photo-products of DNAN could be identified besides nitrite.