Supplementary Information

Mg-Al LDH nanosheets as a nanotechnological tool in agriculture: An exploratory toxicity evaluation study

Rafael Salinas-Jiménez[a,b,†] , Gloria Vera[a,b,†], Mariola Tobar[b,c,†], Jeimy Moscote[b,c], Giovana Acha[b], Ariel Herrera-Vásquez [c,d], Diego Rojas-Rivera[e,f], Elena A. Vidal,[b,c,f] Andrea Miyasaka Almeida[,b,g,]*, Manuel Ahumada[a,f,]*

[a]Centro de Nanotecnología Aplicada, Facultad de Ciencias, Ingeniería y Tecnología, Universidad Mayor, Santiago 8580745, Chile

[b]Centro de Genómica y Bioinformática, Facultad de Ciencias, Ingeniería y Tecnología, Universidad Mayor, Santiago 8580745, Chile

[c]ANID-Millennium Science Initiative Program-Millennium Institute for Integrative Biology (iBio), Santiago 8331150, Chile

[d]Centro de Biotecnología Vegetal, Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago 8370146, Chile

[e]Laboratorio de Muerte Celular y Biomedicina, Vicerrectoria de Investigación, Universidad Mayor, Santiago 8580745, Chile

[f]Escuela de Biotecnología, Facultad de Ciencias, Ingeniería y Tecnología, Universidad Mayor, Santiago 8580745, Chile

[g]Escuela de Agronomía, Facultad de Ciencias, Ingeniería y Tecnología, Universidad Mayor, Santiago 8580745, Chile

† Denotes equal contributions.

Corresponding author´s emails: andrea.miyasaka@umayor.cl; manuel.ahumada@umayor.cl
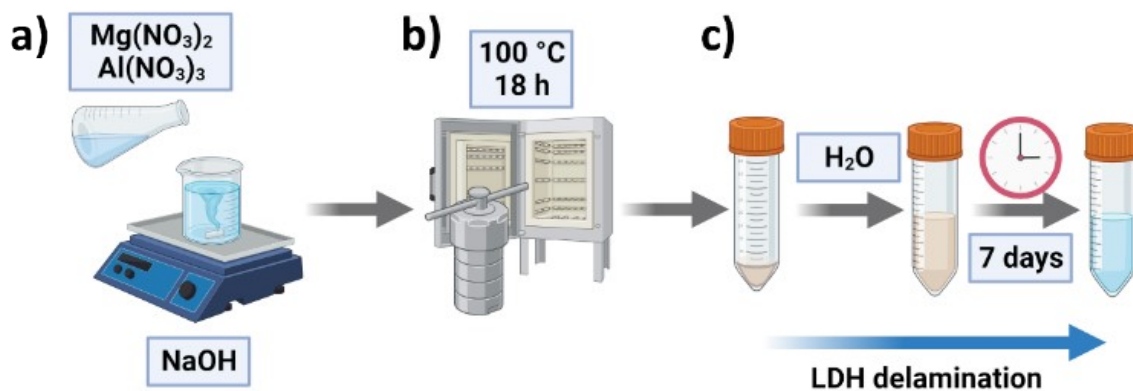
Figure S1. Schematic representation of the followed protocol used in this work. a) LDH nanosheets were prepared by co-precipitation method followed by b) a hydrothermal treatment. Posteriorly, c) LDH purification was performed using several centrifuge and water resuspension cycles.
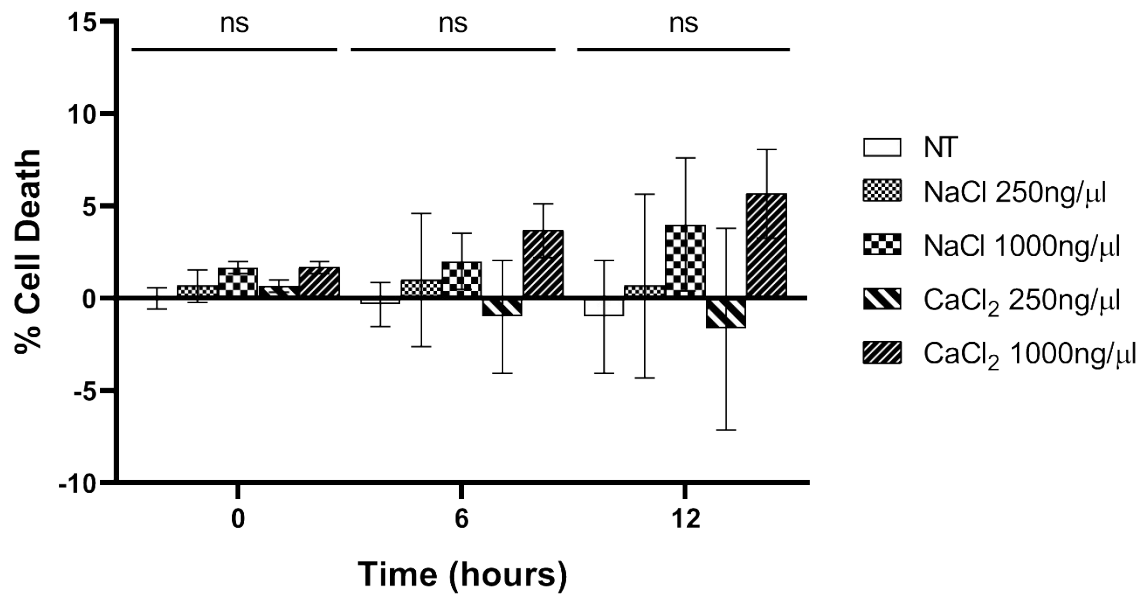
Figure S2. Effect of ionic solutions of NaCl and CaCl$_2$ in *A. thaliana* protoplast in the absence (NT) and presence of NaCl and CaCl$_2$ at concentrations of 250 ng/μL and 1000 ng/μL. Statistical significance was calculated using two-way ANOVA with Dunnett´s multiple comparisons test, being non-significant within the times evaluated. The values shown are means with SEM of three independent sets of experiments.

Table S1. Dunnett´s multiple comparisons test. Within each time point, a comparison in different concentrations of NaCl and $CaCl_2$. The "Summary" column indicates the conclusion about the significance of the data, with "ns" having no significance and "*" as a p-value < 0.05.

Within each row, compare columns (simple effects within rows)					
Number of families	3				
Number of comparisons per family	4				
Alpha	0,05				
Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 hrs					
NT vs. NaCl 250ng/μl	-0,6667	-5,009 to 3,676	No	ns	0,7434
NT vs. NaCl 1000ng/μl	-1,667	-6,009 to 2,676	No	ns	0,271
NT vs. $CaCl_2$ 250ng/μl	-0,6667	-6,411 to 5,078	No	ns	0,8566
NT vs. $CaCl_2$ 1000ng/μl	-1,667	-6,009 to 2,676	No	ns	0,271
6 hrs					
NT vs. NaCl 250ng/μl	-1,333	-18,29 to 15,62	No	ns	0,9485
NT vs. NaCl 1000ng/μl	-2,333	-11,02 to 6,351	No	ns	0,443
NT vs. $CaCl_2$ 250ng/μl	0,6667	-11,42 to 12,75	No	ns	0,9832
NT vs. $CaCl_2$ 1000ng/μl	-4	-13,95 to 5,949	No	ns	0,2524
12 hrs					
NT vs. NaCl 250ng/μl	-1,667	-20,22 to 16,88	No	ns	0,925
NT vs. NaCl 1000ng/μl	-5	-27,87 to 17,87	No	ns	0,5571
NT vs. $CaCl_2$ 250ng/μl	0,6667	-14,99 to 16,32	No	ns	0,9932
NT vs. $CaCl_2$ 1000ng/μl	-6,667	-18,16 to 4,822	No	ns	0,1368

Table S2. Dunnett´s multiple comparisons test. Within each concentration, a comparison of the different time points effect. The "Summary" column indicates the conclusion about the significance of the data, with "ns" having no significance and "*" as a p-value < 0.05.

Within each column, compare rows (simple effects within columns)					
Number of families	6				
Number of comparisons per family	4				
Alpha	0,05				
Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 ng/µl					
0 hrs vs. 6 hrs	-0,05824	-2,527 to 2,411	No	ns	0,9415
0 hrs vs. 12 hrs	-0,1966	-5,171 to 4,778	No	ns	0,8182
0 hrs vs. 18 hrs	-0,2448	-6,595 to 6,105	No	ns	0,8263
0 hrs vs. 24 hrs	-0,2456	-6,572 to 6,081	No	ns	0,8240
25 ng/µl					
0 hrs vs. 6 hrs	-0,1827	-0,7525 to 0,3872	No	ns	0,3522
0 hrs vs. 12 hrs	-0,2476	-0,7107 to 0,2155	No	ns	0,1578
0 hrs vs. 18 hrs	-0,3175	-0,9127 to 0,2777	No	ns	0,1584
0 hrs vs. 24 hrs	-0,3973	-1,032 to 0,2369	No	ns	0,1194
250 ng/µl					
0 hrs vs. 6 hrs	-0,1059	-0,1839 to -0,02795	Yes	*	0,0277
0 hrs vs. 12 hrs	-0,05913	-0,1633 to 0,04504	No	ns	0,1422
0 hrs vs. 18 hrs	-0,03455	-0,3249 to 0,2558	No	ns	0,8481
0 hrs vs. 24 hrs	-0,06854	-0,2928 to 0,1557	No	ns	0,3758
500 ng/µl					
0 hrs vs. 6 hrs	-0,1663	-0,4723 to 0,1397	No	ns	0,1534
0 hrs vs. 12 hrs	-0,2386	-0,5825 to 0,1053	No	ns	0,0992
0 hrs vs. 18 hrs	-0,2621	-0,7317 to 0,2076	No	ns	0,1465
0 hrs vs. 24 hrs	-0,3032	-0,7390 to 0,1325	No	ns	0,0987
750 ng/µl					
0 hrs vs. 6 hrs	-0,2206	-0,5251 to 0,08397	No	ns	0,0917
0 hrs vs. 12 hrs	-0,2609	-0,5528 to 0,03095	No	ns	0,0619
0 hrs vs. 18 hrs	-0,2546	-0,5486 to 0,03945	No	ns	0,0657
0 hrs vs. 24 hrs	-0,2522	-0,5724 to 0,06809	No	ns	0,0785
1000 ng/µl					
0 hrs vs. 6 hrs	-0,3028	-0,5600 to -0,04568	Yes	*	0,0365
0 hrs vs. 12 hrs	-0,3307	-0,5301 to -0,1313	Yes	*	0,0187
0 hrs vs. 18 hrs	-0,3107	-0,5085 to -0,1130	Yes	*	0,0208
0 hrs vs. 24 hrs	-0,2923	-0,4538 to -0,1308	Yes	*	0,0158

Table S3. Two-way ANOVA of concentrations and time exposure of Mg-Al LDH treatments on protoplast cell death. "Summary" column indicates the conclusion about significance of data, with "ns" a no significant, "*" as p-value < 0.05, "**" as p-value <0.01 and "***" as p-value < 0.0001.

| Table Analyzed	Transform of Data (log(Y))				
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	No				
Alpha	0,05				
Source of Variation	% of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Interaction	3,550	0,0293	*	Yes	
Time	13,13	<0,0001	****	Yes	0,3208
Concentration	56,46	0,0088	**	Yes	
Subject	22,56	<0,0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0,1972	20	0,009862	F (20, 44) = 1,983	P=0,0293
Time	0,7293	4	0,1823	F (1,283, 14,11) = 36,67	P<0,0001
Concentration	3,137	5	0,6274	F (5, 11) = 5,506	P=0,0088
Subject	1,254	11	0,1140	F (11, 44) = 22,92	P<0,0001
Residual	0,2188	44	0,004972		
Data summary					
Number of columns (Concentration)	6				
Number of rows (Time)	5				
Number of subjects (Subject)	17				

Table S4. Dunnett´s multiple comparisons test. Within each time point, a comparison in different concentrations. The "Summary" column indicates the conclusion about the significance of the data, with "ns" having no significance and "*" as a p-value < 0.05.

| Within each row, compare columns (simple effects within rows)					
Number of families	5				
Number of comparisons per family	5				
Alpha	0,05				
Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
0 hrs					
0 ng/µl vs. 25 ng/µl	0,2131	-6,791 to 7,217	No	ns	>0,9999
0 ng/µl vs. 250 ng/µl	-0,03705	-6,933 to 6,858	No	ns	>0,9999
0 ng/µl vs. 500 ng/µl	-0,1652	-7,429 to 7,099	No	ns	>0,9999
0 ng/µl vs. 750 ng/µl	-0,2787	-8,415 to 7,858	No	ns	>0,9999
0 ng/µl vs. 1000 ng/µl	-0,2511	-8,018 to 7,516	No	ns	>0,9999
6 hrs					
0 ng/µl vs. 25 ng/µl	0,08863	-5,980 to 6,157	No	ns	>0,9999
0 ng/µl vs. 250 ng/µl	-0,08473	-4,424 to 4,255	No	ns	0,9932
0 ng/µl vs. 500 ng/µl	-0,2732	-3,708 to 3,162	No	ns	0,8071
0 ng/µl vs. 750 ng/µl	-0,4410	-5,357 to 4,475	No	ns	>0,9999
0 ng/µl vs. 1000 ng/µl	-0,4957	-4,954 to 3,963	No	ns	0,5374
12 hrs					
0 ng/µl vs. 25 ng/µl	0,1621	-2,598 to 2,923	No	ns	0,7874
0 ng/µl vs. 250 ng/µl	0,1004	-1,632 to 1,833	No	ns	0,9437
0 ng/µl vs. 500 ng/µl	-0,2072	-1,532 to 1,117	No	ns	0,7377
0 ng/µl vs. 750 ng/µl	-0,3430	-2,754 to 2,068	No	ns	0,4624
0 ng/µl vs. 1000 ng/µl	-0,3852	-2,414 to 1,644	No	ns	0,4062
18 hrs					
0 ng/µl vs. 25 ng/µl	0,1404	-0,7398 to 1,021	No	ns	0,6722
0 ng/µl vs. 250 ng/µl	0,1732	-0,6156 to 0,9619	No	ns	0,5742
0 ng/µl vs. 500 ng/µl	-0,1824	-0,9255 to 0,5606	No	ns	0,6770
0 ng/µl vs. 750 ng/µl	-0,2884	-1,371 to 0,7936	No	ns	0,3159
0 ng/µl vs. 1000 ng/µl	-0,3171	-1,169 to 0,5352	No	ns	0,2642
24 hrs					
0 ng/µl vs. 25 ng/µl	0,06138	-0,9456 to 1,068	No	ns	0,9484
0 ng/µl vs. 250 ng/µl	0,1400	-0,6544 to 0,9345	No	ns	0,6988
0 ng/µl vs. 500 ng/µl	-0,2228	-0,9770 to 0,5315	No	ns	0,5630
0 ng/µl vs. 750 ng/µl	-0,2852	-1,220 to 0,6494	No	ns	0,3158
0 ng/µl vs. 1000 ng/µl	-0,2978	-1,136 to 0,5400	No	ns	0,2932

Table S5. Dunnett´s multiple comparisons test of different Mg-Al LDH concentrations. The "Summary" column indicates the conclusion about the significance of the data, with "ns" having no significance and "*" as a p-value < 0.05.

| Number of families	1							
Number of comparisons per family	5							
Alpha	0,05							
Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value	A-?		
0 ng/µl vs. 25 ng/µl	-2,321	-25,59 to 20,95	No	ns	0,9983	B	25 ng/µl	
0 ng/µl vs. 250 ng/µl	-7,509	-30,78 to 15,76	No	ns	0,8128	C	250 ng/µl	
0 ng/µl vs. 500 ng/µl	-16,21	-39,48 to 7,065	No	ns	0,2186	D	500 ng/µl	
0 ng/µl vs. 750 ng/µl	-25,62	-48,90 to -2,350	Yes	*	0,0298	E	750 ng/µl	
0 ng/µl vs. 1000 ng/µl	-30,45	-53,73 to -7,179	Yes	*	0,0103	F	1000 ng/µl	
Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	n1	n2	q	DF
0 ng/µl vs. 25 ng/µl	7,153	9,474	-2,321	8,022	3	3	0,2894	12
0 ng/µl vs. 250 ng/µl	7,153	14,66	-7,509	8,022	3	3	0,9360	12
0 ng/µl vs. 500 ng/µl	7,153	23,36	-16,21	8,022	3	3	2,021	12
0 ng/µl vs. 750 ng/µl	7,153	32,78	-25,62	8,022	3	3	3,194	12
0 ng/µl vs. 1000 ng/µl	7,153	37,61	-30,45	8,022	3	3	3,796	12
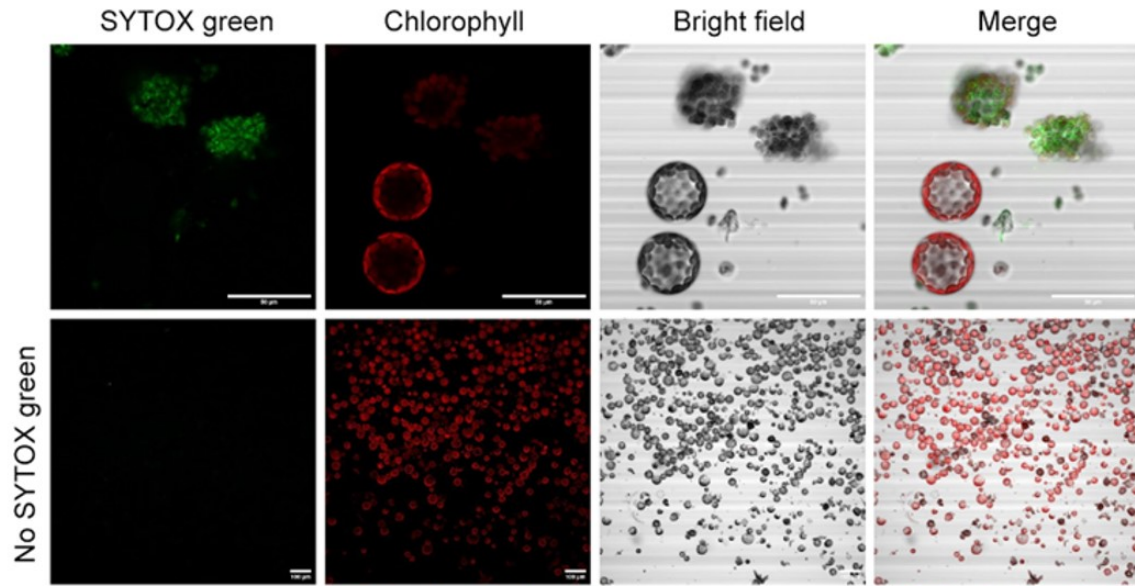
Figure S3. Control microscopic analysis of *Arabidopsis* protoplasts. Arabidopsis protoplasts were extracted as indicated in methods, and cell death was evaluated using SYTOX Green stain. The first row shows an amplified image (Scale bar: 50 µm. Row 2 to 5: scale bar = 100 µm). The second row corresponds to a control sample without SYTOX green, which was needed as a background fluorescence sample. The corresponding channels are indicated at the top of each column.
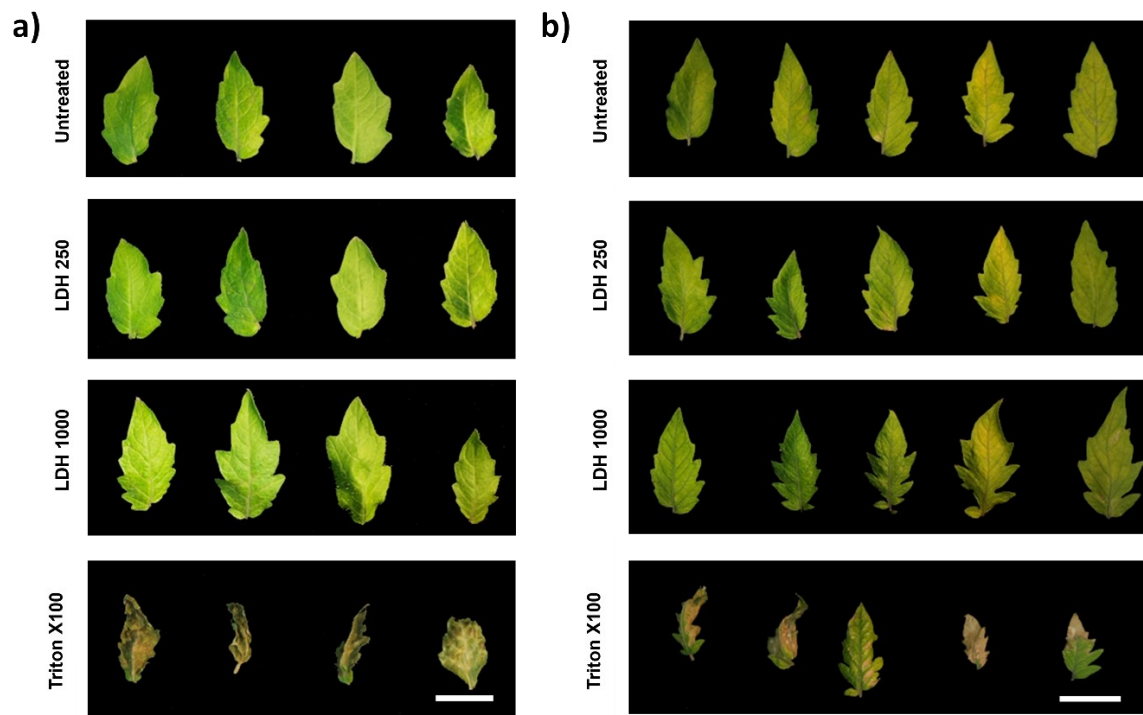
Figure S4. Nanoparticles' effect on tomato leaves. Fully expanded leaves from 6-week-old tomatoes A) ´Marmande´ and B) Rosade varieties. The leaves were infiltrated with water (mock), 250 ng/μL nanoparticles (LDH 250), 1000 ng/μL nanoparticles (LDH 1000), or Triton X-100. Plants were photo-documented after 48 h—scale bar: 1 cm.
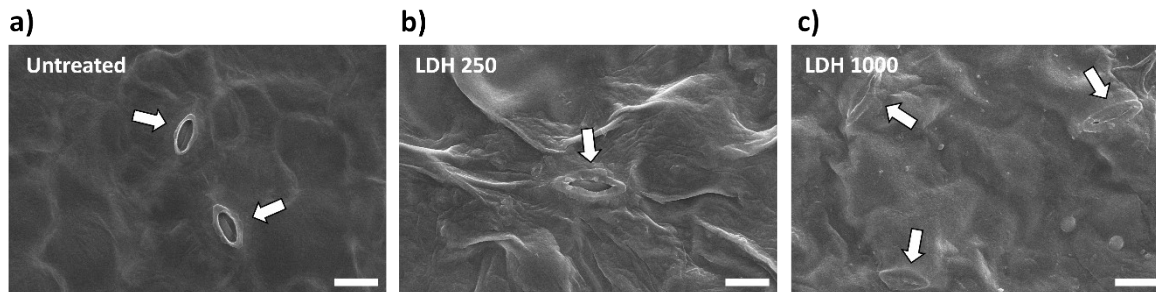
Figure S5. SEM images of LDH nanosheets on *A. thaliana* leaves. Leaves were incubated for 48 h with 250 ng/μL (LDH 250) and 1000 ng/μL (LDH 1000). Untreated protoplasts are shown as a negative control. The arrow within the images indicates the stomata location. Scale bars: 10 μm.