Supplementary Information (SI) for Environmental Science: Nano.

A multi-technique approach for nanoherbicide tracking: uptake and translocation pathways of metribuzin nanocarrier in weed plants

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Abstract

Understanding the strategies of delivering active ingredients with nanoparticles to plant species is crucial to implementing a safe-by-design approach for pesticides. Here, we used metribuzin (MTZ) as a study model to understand polymeric nanocarriers' plant uptake and distribution pathways. We investigated the weed-control efficacy, uptake, internalization, and distribution of the (nano)herbicide MTZ in *Amaranthus viridis* (C4 species) and *Bidens pilosa* (C3 species), after soil and foliar application. Radiolabeled herbicide and fluorescent probes were used as complementary tools to track both MTZ and nanoparticles in plants. The weed-control results indicated significant dose reductions with MTZ nanoencapsulation (from ½ to 10-fold doses). Root uptake was an efficient pathway for *A. viridis* and *B. pilosa* entry of nanoMTZ was preferentially by stomata and was internalized in leaf mesophyll cells. No differences in herbicide uptake were observed in the soil, and nanoMTZ distribution was 1.3-1.5 lower than MTZ, with nanoparticle concentration in vascular cells. After foliar application, nanoMTZ was absorbed 2.5 times more than MTZ in *A. viridis* and was similar in *B. pilosa*, following the stomata entrance in the leaves. For *B. pilosa*, the internalization in the leaf mesophyll was more evident than in *A. viridis*; however, for *A. viridis*, the foliar pathway was essential to improve herbicide delivery by the nanocarrier. Our findings highlight the role of target weed species and application mode in plants' nanoherbicide efficacy, uptake, and distribution. It is an innovative and multi-technique perspective that contributes to a safe-by-design approach while emphasizing the significance of nanoherbicides in sustainable agriculture.

Keywords: Weed control; nanotechnology; radiometric techniques; fluorescent marker; nanoformulation design.

Table S1. Nanoparticles characterization by nano tracking analysis (NTA) and dynamic light scattering (DSL). Hydrodynamic size, polydispersity index (PDI), and surface charge (zeta potential) of MTZ nanoparticles.

Technique	Nanoformulation characteristics*		
	Hydrodynamic size (nm)	PDI**	Zeta potential (mV)
DLS	304.7 ± 2.3	0.08 ± 0.01	-37.6 ± 0.3
NTA	333.2 ± 13.5	---	---

*Measurements before preparation of work application solutions.
**Dimensionless values.

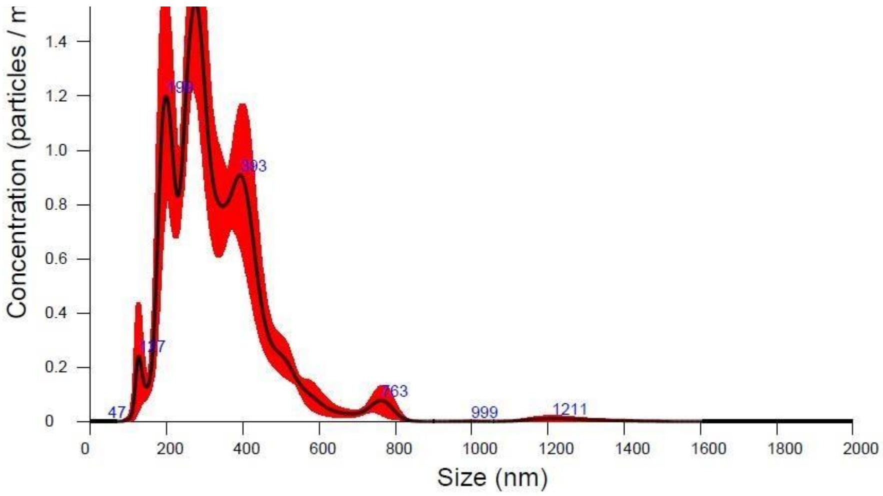


Figure S1. Average concentration in function of nanoparticles size by Nano Tracking Analysis (NTA). Error range (red) indicate the standard error of the mean (± 1).

Table S2. Physical-chemical properties of soils. Sandy loam soil was used in studies of weed control, morphology analysis, and radiolabeled metribuzin uptake and distribution. The clay soil was used in nanoformulation uptake and distribution (fluorescent probe) studies. Sandy loam soil was collected from Escola Superior de Agricultura "Luiz de Queiroz", of Universidade de São Paulo, Piracicaba-SP. Clay soil was collected from Fazenda Escola, of Universidade Estadual de Londrina, Paraná-SP. All soils were from an agricultural area without previously metribuzin application.

Parameters[a]	Soil[b]	
	Sandy loam	Clay
Total sandy ($g.kg^{-1}$)	710	68.9
Silt($g.kg^{-1}$)	64	153.5
Clay ($g.kg^{-1}$)	226	778
pH ($CaCl_2$)	4.6	4.83
O.M. ($g\ dm^{-3}$)	9	28.21
P ($mg\ dm^{-3}$)	36	7.63
K ($mmolc\ dm^{-3}$)	1.3	6.5
Ca ($mmolc\ dm^{-3}$)	15	39.6
Mg ($mmolc\ dm^{-3}$)	12	18
H+Al ($mmolc\ dm^{-3}$)	12	46.1
SB ($mmolc\ dm^{-3}$)	28.3	6.41
CEC ($mmolc\ dm^{-3}$)	40.3	110.19
V (%)	70	58.19

[a]Sand loam soil was analyzed at the Laboratory of Mineral Fertilizers of the Superior School of Agriculture "Luiz de Queiroz", University of São Paulo, Piracicaba, São Paulo, Brazil. The clay soil was analyzed at the Institute of Technology and Laboratory, Londrina, Paraná, Brazil.
[b]Soil classification according to the Brazilian Soil Classification System (Embrapa, 2018). Dystrophic Red-Yellow Argisol (ARGISSOLO VERMELHO-AMARELO Distrófico – PVAd) (Sandy loam), Red Latosol (LATOSSOLO VERMELHO) (Clay).

Plant morphology characteristics

The leaf surface of *A. viridis* is smooth, with few trichomes and epicuticular wax crystals (Fig. S2 A). The stomata on the adaxial surface in leaves are in a depression about the level of the leaf surface (Fig. S2 C). The stomata density of *A. viridis* is 102.6 stomata units per mm^2 (n=3). The trichomes on the adaxial surface of this species are multicellular and 138.1 units per mm^2 (Fig. S2 E and G).

For *B. pilosa*, the leaf surface shows a layer of wax that appeared rough, distributed in parallel, as if they were fibers (Fig. S2 B). The stomata are located above the level of the leaf surface (Fig. S2 D) and the calculated stomata density was 38.2 units per mm^2. The trichomes on the adaxial surface have more than one type of morphology (Fig. 2S F and H). Most are pointed and number 485.4 units per mm^2 (Fig. S2 F).

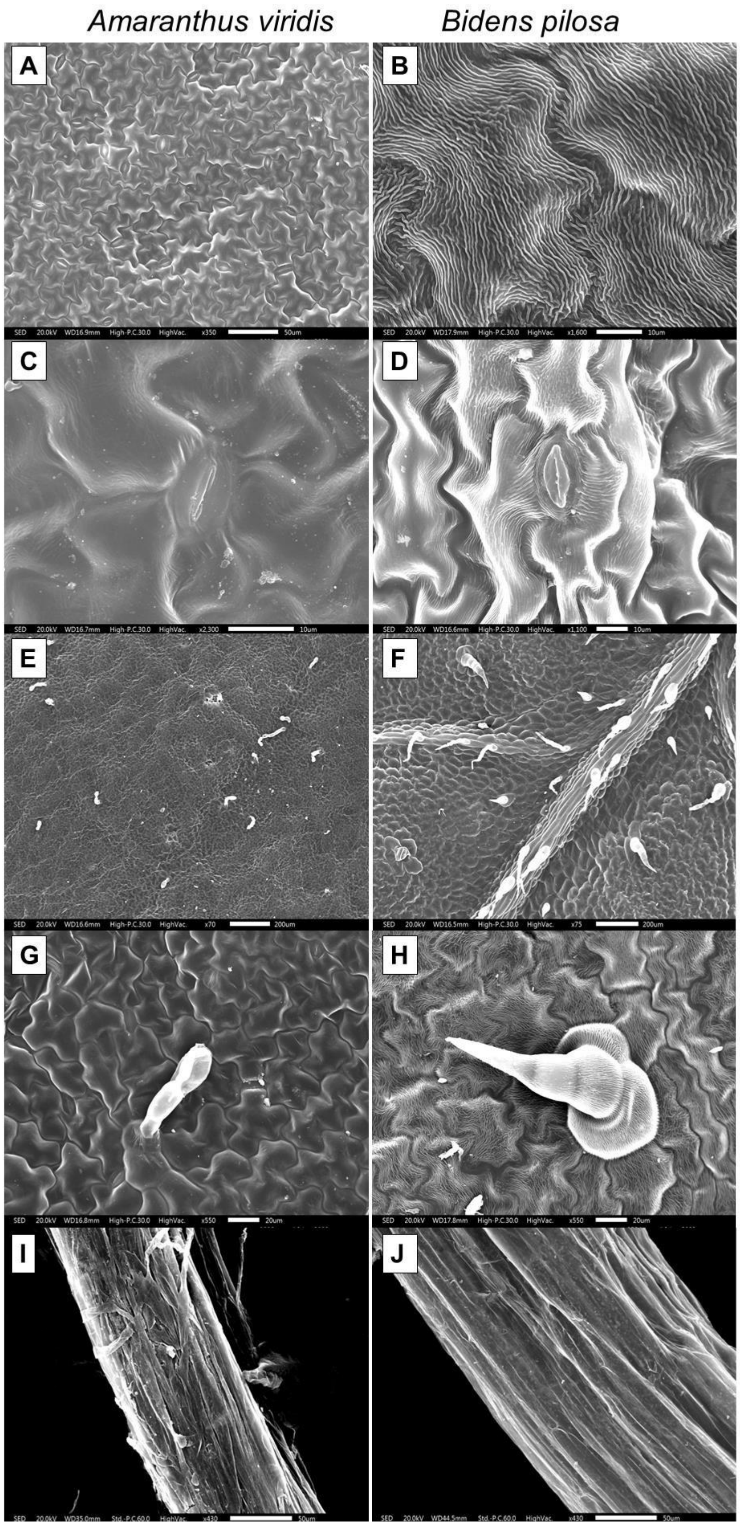


Figure S2. Representative SEM images for each plant species (*Amaranthus viridis* and *Bidens pilosa* – column B) related to the morphological characterization. A and B - the leaf surface. C and D - the stomata morphology. E and F - the trichomes distribution. G and H - trichomes morphology. I and J - Root surface.

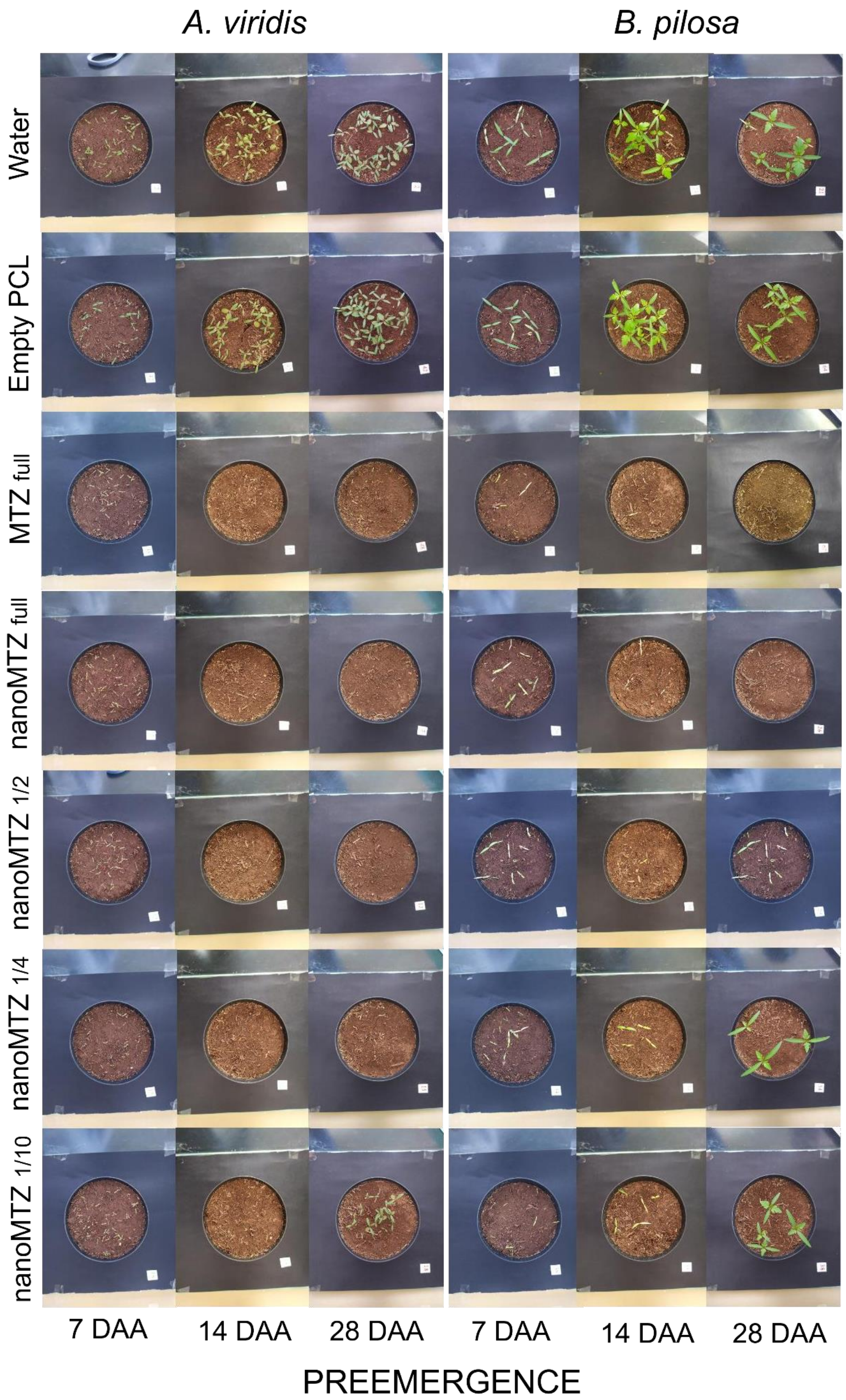


Figure S3. Symptoms evolution after conventional (MTZ) and metribuzin nanoformulation (nanoMTZ) preemergence application, at 7, 14, and 28 days after application (DAA) in the soil. Plants were harvested at 14 DAA and new sowing was performed to observe the residual effects of herbicide in the soil.

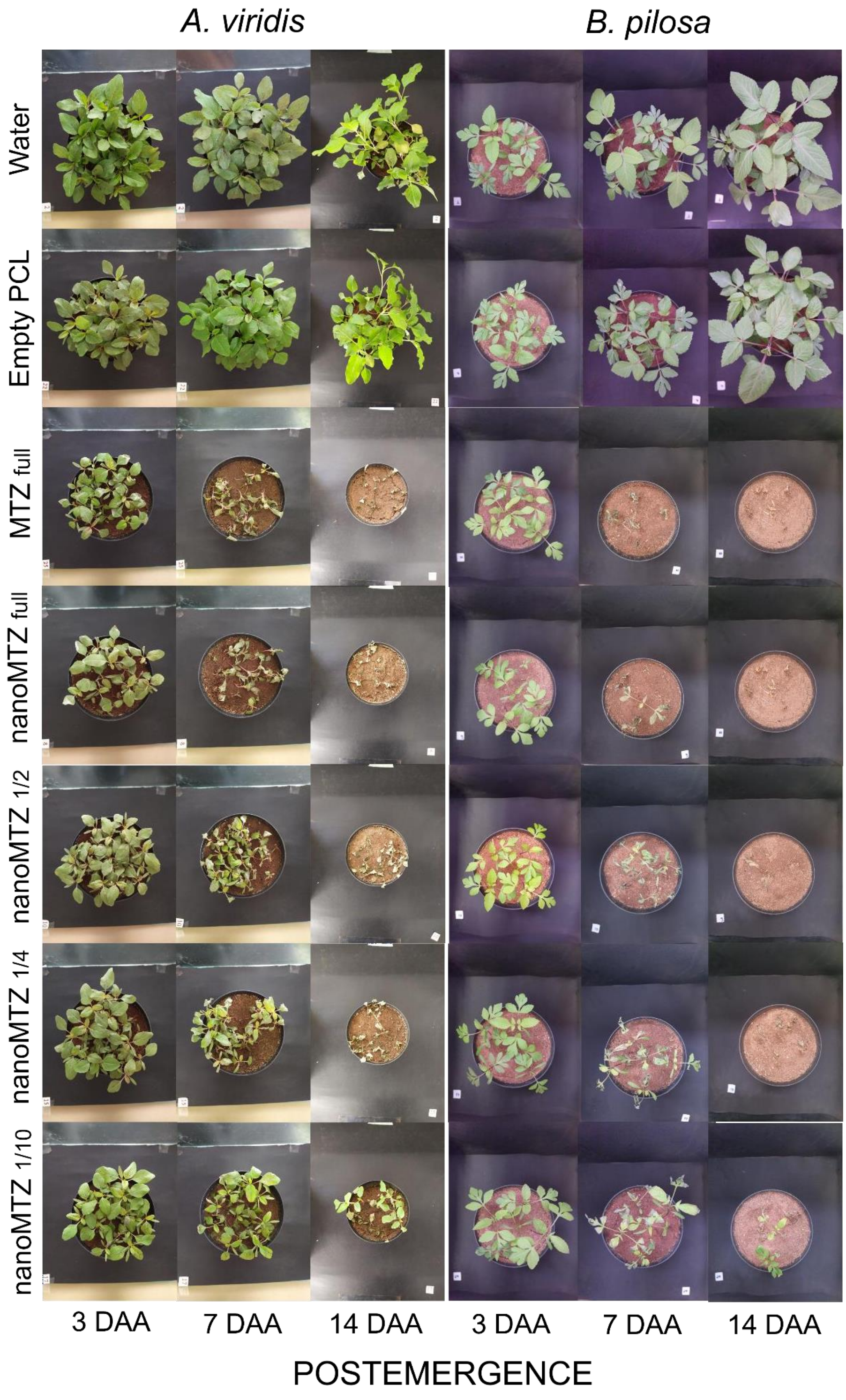


Figure S4. Symptoms evolution after conventional (MTZ) and metribuzin nanoformulation (nanoMTZ) postemergence application, at 3, 7, and 14 days after application (DAA) in the soil.

Table S3. Weed control percentage of *Amaranthus viridis* and *Bidens Pilosa* in pre-emergence (at 14 and 28 days after application - DAA) and in post-emergence at 14 DAA. Lowercase letters differ between treatments by Tukey's test (p<0.05 with adjustment to Sidak's test), for each plant species and time point evaluation. Values represent the mean and standard error (n=4).

Weed species	Treatment	Weed control (%)					
		Preemergence				Postemergen	
		14 DAA		28 DAA		14 DAA	
Amaranthus viridis	Empty PCL nanoparticles	35.5 ± 11.7	b	15.5 ± 6.5	c	15.6 ± 2.5	b
	MTZ (480 g a.i. ha^{-1})	91.6 ± 2.1	a	88.9 ± 2.1	ab	80.8 ± 3.3	a
	nanoMTZ (480 g a.i. ha^{-1})	89.2 ± 2.8	a	96.4 ± 2.2	ab	65.1 ± 9.9	a
	nanoMTZ (240 g a.i. ha^{-1})	83.5 ± 6.5	a	93.8 ± 1.7	ab	71.8 ± 4.6	a
	nanoMTZ (120 g a.i. ha^{-1})	81.1 ± 5.8	a	94.3 ± 1.4	ab	67.3 ± 6.2	a
	nanoMTZ (48 g a.i. ha^{-1})	81.4 ± 3.9	a	84.3 ± 4.4	b	64.5 ± 8.2	a
Bidens pilosa	Empty PCL nanoparticles	34.7 ± 12.0	b	60.1 ± 15.9	bc	16.8 ± 7.3	c
	MTZ (480 g a.i. ha^{-1})	88.2 ± 5.4	a	96.5 ± 1.6	a	85.1 ± 1.6	ab
	nanoMTZ (480 g a.i. ha^{-1})	93.0 ± 1.6	a	91.3 ± 3.8	abc	89.1 ± 1.0	a
	nanoMTZ (240 g a.i. ha^{-1})	93.7 ± 4.2	a	93.3 ± 0.9	ab	89.0 ± 1.1	a
	nanoMTZ (120 g a.i. ha^{-1})	82.5 ± 6.5	a	64.8 ± 11.1	c	90.7 ± 0.7	a
	nanoMTZ (48 g a.i. ha^{-1})	77.7 ± 7.6	a	73.9 ± 8.9	bc	81.8 ± 2.3	b

Table S4. Absorption over time of conventional metribuzin (MTZ) and nanometribuzin (nanoaMTZ), with soil application, in *Amaranthus viridis* and *Bidens pilosa* weed plants. Lowercase letters represent the differences between formulations in function of each time after application and uppercase letters represent the differences between time in function of each formulation (p<0.05, with adjustment to Sidak's test), ns represents non-significative interaction and also in each factor alone (p>0.05). Values represent the mean and standard error (n=3).

Treatments	Hours after application	Non-absorbed		Absorbed	
		Amaranthus viridis			
nanoMTZ	24 h	93.30 ± 4.10*	ns	3.33 ± 0.86	ns
	48 h	89.97 ± 2.87	ns	6.09 ± 2.51	ns
	72 h	91.59 ± 11.31	ns	6.46 ± 2.71	ns
	96 h	87.72 ± 4.45	ns	8.50 ± 3.31	ns
	120 h	83.26 ± 6.65	ns	9.56 ± 5.14	ns
MTZ	24 h	97.58 ± 7.52	ns	2.47 ± 1.32	ns
	48 h	82.47 ± 2.93	ns	3.20 ± 1.86	ns
	72 h	77.63 ± 4.50	ns	5.44 ± 1.86	ns
	96 h	85.82 ± 8.01	ns	6.13 ± 3.01	ns
	120 h	81.72 ± 4.19	ns	8.03 ± 0.27	ns
		Bidens pilosa			
nanoMTZ	24 h	85.50 ± 8.15*	ns	1.72 ± 0.52	Ba
	48 h	90.30 ± 4.99	ns	2.18 ± 0.56	Ba
	72 h	93.56 ± 10.87	ns	4.09 ± 0.42	Aa
	96 h	88.16 ± 7.00	ns	4.80 ± 0.29	Aa
	120 h	80.41 ± 6.76	ns	5.43 ± 0.50	Aa
MTZ	24 h	94.3 ± 3.45	ns	0.94 ± 0.31	Bb
	48 h	93.08 ± 3.09	ns	1.60 ± 0.16	Bb
	72 h	97.50 ± 4.69	ns	2.31 ± 0.29	Ba
	96 h	96.17 ± 6.71	ns	1.95 ± 0.39	Ba
	120 h	87.54 ± 4.03	ns	3.94 ± 0.38	Aa

Table S5. Distribution over time of conventional metribuzin (MTZ) and nanometribuzin (nanoaMTZ), with soil application, in *Amaranthus viridis* and *Bidens pilosa* weed plants. Lowercase letters represent the differences between formulations at each time after application (p<0.05, with adjustment to Sidak's test). Uppercase letters represent the differences between the herbicide accumulation at the time after each formulation application (p<0.05, with adjustment to Sidak's test). Values represent the mean and standard error (n=3).

Treatments	Hours after application	Leaves		Stem		Roots	
			Amaranthus viridis				
nanoMTZ	24 h	54.53 ± 3.45*	b	20.13 ± 2.24	a	25.34 ± 1.37	a
	48 h	50.26 ± 5.20	b	20.06 ± 3.92	a	29.68 ± 1.66	a
	72 h	53.15 ± 1.40	b	14.52 ± 2.54	a	32.32 ± 1.16	a
	96 h	57.62 ± 3.36	b	11.69 ± 0.85	a	30.68 ± 2.98	a
	120 h	53.29 ± 2.00	b	11.97 ± 1.52	a	34.74 ± 0.58	a
MTZ	24 h	79.74 ± 5.58	a	9.54 ± 3.43	b	10.72 ± 2.38	b
	48 h	80.21 ± 1.84	a	9.64 ± 1.37	b	10.15 ± 0.74	b
	72 h	78.61 ± 4.12	a	9.63 ± 3.28	a	11.77 ± 0.94	b
	96 h	82.79 ± 2.68	a	6.20 ± 0.34	a	11.01 ± 2.34	b
	120 h	83.07 ± 1.56	a	6.26 ± 1.12	a	10.66 ± 0.82	b
			Bidens pilosa				
nanoMTZ	24 h	22.68 ± 6.08	b	13.94 ± 0.93	A	65.16 ± 7.97	a
	48 h	30.46 ± 7.57	b	12.23 ± 1.21	A	57.31 ± 7.26	a
	72 h	18.20 ± 8.48	b	7.32 ± 1.35	A	53.23 ± 18.80	a
	96 h	29.64 ± 6.14	b	6.88 ± 0.67	A	63.48 ± 5.58	a
	120 h	39.73 ± 8.25	b	7.02 ± 0.46	A	53.26 ± 8.33	a
MTZ	24 h	66.27 ± 5.66	a	11.05 ± 2.41	A	22.68 ± 3.85	b
	48 h	70.15 ± 1.61	a	7.37 ± 1.01	AB	22.48 ± 2.34	b
	72 h	71.05 ± 1.40	a	8.09 ± 1.32	AB	20.87 ± 2.09	b
	96 h	78.20 ± 1.24	a	5.68 ± 1.35	B	16.12 ± 2.13	b
	120 h	71.19 ± 5.25	a	6.07 ± 0.81	AB	22.74 ± 4.49	b

Table S6. Absorption over time of conventional metribuzin (MTZ) and nanometribuzin (nanoaMTZ), with foliar application, in *Amaranthus viridis* and *Bidens pilosa* weed plants. Lowercase letters represent the differences between formulations in function of each time after application and uppercase letters represent the differences between time in function of each formulation (p<0.05, with adjustment to Sidak's test). Values represent the mean and standard error (n=3).

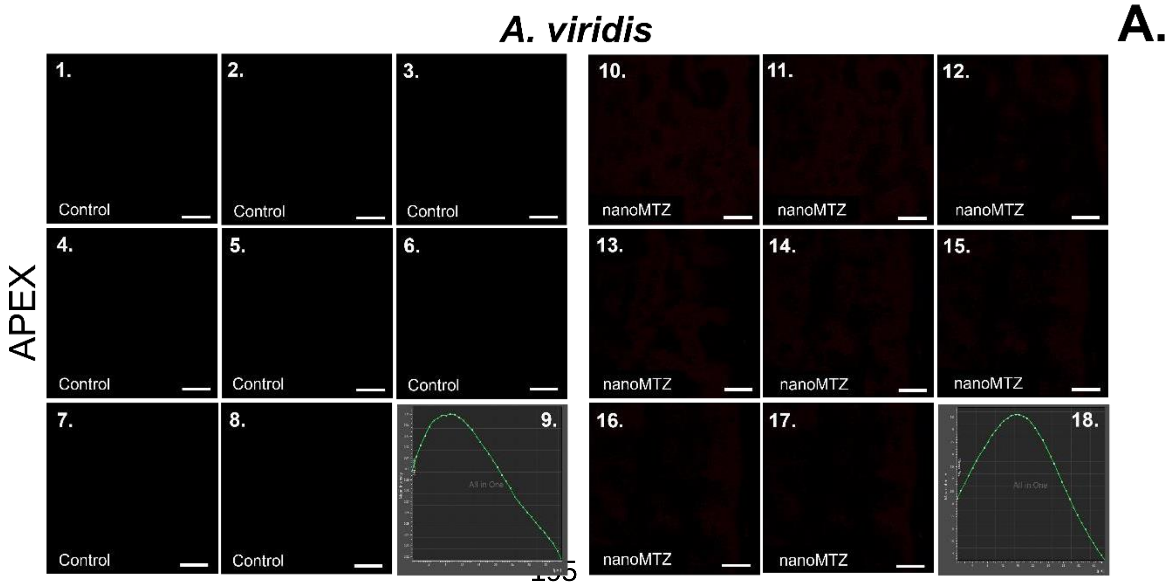
Treatments	Hours after application	Non-absorbed		Absorbed	
		Amaranthus viridis			
nanoMTZ	4 h	28.10 ± 2.94	Ab	65.22 ± 3.71	Ca
	8 h	13.33 ± 1.67	Bb	84.14 ± 2.08	Ba
	24 h	7.62 ± 0.88	Cb	90.97 ± 1.18	ABa
	48 h	6.75 ± 0.58	Cb	91.08 ± 0.78	ABa
	72 h	1.72 ± 0.22	Db	97.85 ± 0.29	Aa
MTZ	4 h	64.48 ± 1.25	Aa	11.19 ± 1.57	Bb
	8 h	42.78 ± 4.68	ABCa	22.39 ± 6.17	Bb
	24 h	53.22 ± 2.83	ABa	18.54 ± 3.69	Bb
	48 h	41.20 ± 3.24	BCa	27.56 ± 2.01	ABb
	72 h	29.83 ± 1.85	Ca	37.37 ± 2.97	Ab
		Bidens pilosa			
nanoMTZ	4 h	27.23 ± 5.47	Aa	65.88 ± 7.13	Ca
	8 h	14.84 ± 2.49	Ba	82.00 ± 3.36	BCa
	24 h	7.33 ± 1.94	BCa	90.88 ± 2.66	ABa
	48 h	5.05 ± 0.68	Ca	93.85 ± 0.97	Aa
	72 h	2.35 ± 0.51	Ca	97.17 ± 0.66	Aa
MTZ	4 h	15.57 ± 3.20	Ab	67.23 ± 6.13	Ca
	8 h	11.91 ± 0.43	ABa	75.21 ± 2.08	BCa
	24 h	8.64 ± 2.05	ABa	78.37 ± 6.81	BCb
	48 h	6.82 ± 0.46	Ba	83.84 ± 2.06	ABb
	72 h	4.87 ± 0.65	Ba	88.79 ± 2.29	Ab

Table S7. Distribution of conventional metribuzin (MTZ) and nanometribuzin (nanoaMTZ), with foliar application, in *Amaranthus viridis* and *Bidens pilosa* weed plants. Lowercase letters represent the differences between formulations at each time after application (p<0.05, with adjustment to Sidak's test). Uppercase letters represent the differences between the herbicide accumulation at the time after each formulation application (*p*<0.05, with adjustment to Sidak's test). Values represent the mean and standard error (*n*=3).

Treatments	Hours after application	Upper leaves		Treated leaves		Leaves above		Stem		Root	
				Amaranthus viridis							
nanoMTZ	24 h	0.29 ± 0.11	b	99.15 ± 0.03	a	0.50 ± 0.08	b	0.07 ± 0.00	Cb	0.09 ± 0.01	b
	48 h	0.24 ± 0.07	b	99.02 ± 0.11	a	0.58 ± 0.12	b	0.09 ± 0.01	BCb	0.07 ± 0.01	b
	72 h	0.42 ± 0.08	b	98.90 ± 0.08	a	0.46 ± 0.06	b	0.13 ± 0.03	ABb	0.07 ± 0.00	b
	96 h	0.43 ± 003	b	98.73 ± 0.02	a	0.50 ± 0.04	b	0.21 ± 0.02	Aa	0.13 ± 0.03	b
	120 h	0.32 ± 0.01	b	99.02 ± 0.02	a	0.44 ± 0.00	b	0.13 ± 0.01	ABb	0.09 ± 0.01	b
MTZ	24 h	0.77 ± 0.20	a	97.33 ± 0.52	b	1.06 ± 0.23	a	0.40 ± 0.07	Aa	0.43 ± 0.03	a
	48 h	1.23 ± 0.38	a	96.96 ± 0.27	b	1.15 ± 0.18	a	0.37 ± 0.07	Aa	0.28 ± 0.05	a
	72 h	0.96 ± 0.29	a	97.37 ± 0.57	b	0.90 ± 0.14	a	0.44 ± 0.11	Aa	0.33 ± 0.05	a
	96 h	0.92 ± 0.16	a	97.12 ± 0.29	b	1.37 ± 0.15	a	0.25 ± 0.03	Aa	0.33 ± 0.06	a
	120 h	1.16 ± 0.18	a	97.40 ± 0.26	b	0.93 ± 0.10	a	0.28 ± 0.03	Aa	0.24 ± 0.02	a
				Bidens pilosa							
nanoMTZ	24 h	0.49 ± 0.02	ns	99.21 ± 0.06	ns	0.12 ± 0.00	b	0.09 ± 0.03	Bb	0.08 ± 0.01	a
	48 h	0.79 ± 0.35	ns	98.98 ± 0.34	ns	0.09 ± 0.00	b	0.07 ± 0.00	Bb	0.07 ± 0.00	b
	72 h	0.91 ± 0.46	ns	98.84 ± 0.50	ns	0.09 ± 0.04	b	0.07 ± 0.01	Bb	0.09 ± 0.01	b
	96 h	1.55 ± 0.10	ns	97.78 ± 0.44	ns	0.15 ± 0.01	b	0.08 ± 0.00	Bb	0.11 ± 0.04	b
	120 h	0.54 ± 0.02	ns	98.72 ± 0.41	ns	0.11 ± 0.00	b	1.17 ± 0.15	Ab	0.10 ± 0.02	b
MTZ	24 h	0.49 ± 0.01	ns	97.98 ± 0.18	ns	1.21 ± 0.16	a	0.18 ± 0.00	Ba	0.13 ± 0.03	a
	48 h	0.32 ± 0.02	ns	98.28 ± 0.12	ns	1.12 ± 0.12	a	0.13 ± 0.01	Ba	0.15 ± 0.02	a
	72 h	0.47 ± 0.01	ns	97.42 ± 0.00	ns	1.75 ± 0.04	a	0.15 ± 0.00	Ba	0.21 ± 0.04	a
	96 h	0.52 ± 0.15	ns	98.19 ± 0.43	ns	0.93 ± 0.30	a	0.15 ± 0.01	Ba	0.21 ± 0.05	a
	120 h	0.41 ± 0.11	ns	93.52 ± 4.49	ns	0.94 ± 0.15	a	0.20 ± 0.03	Ba	0.18 ± 0.01	a

Table S8. Fluorescence intensity – IF in apex and base of leaf, at 120 h after soil application and 72 h after foliar application of nanometribuzin (nanoMTZ) and water (negative control). Values represent the mean and standard error (n=4).

Treatment	Weed plant	Application form	IF apex	IF base
Fluorescence intensity in the leaf				
Control (water)	*Amaranthus viridis*	Soil	1.07 ± 0.06	1.50 ± 0.49
		Foliar	1.06 ± 0.05	2.33 ± 1.38
nanoMTZ	*Bidens pilosa*	Soil	12.06 ± 2.09	10.62 ± 0.98
		Foliar	20.43 ± 2.91	26.43 ± 1.20


A. viridis
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APEX
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9. All in One
10. nanoMTZ
11. nanoMTZ
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14. nanoMTZ
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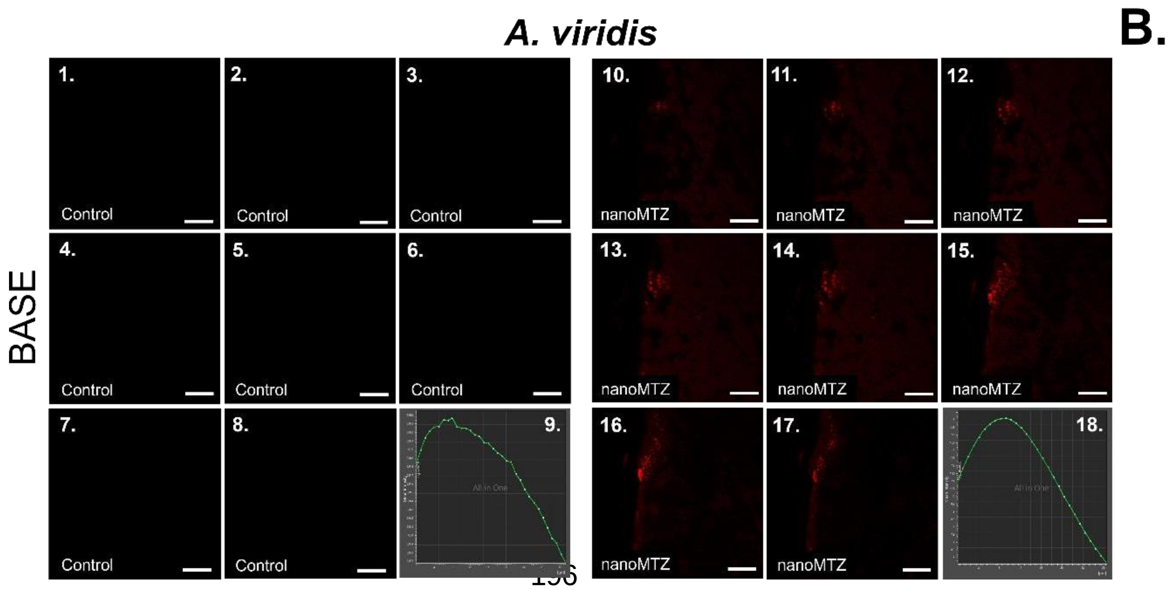
16. nanoMTZ
17. nanoMTZ
18. All in One


A. viridis
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BASE
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18. All in One

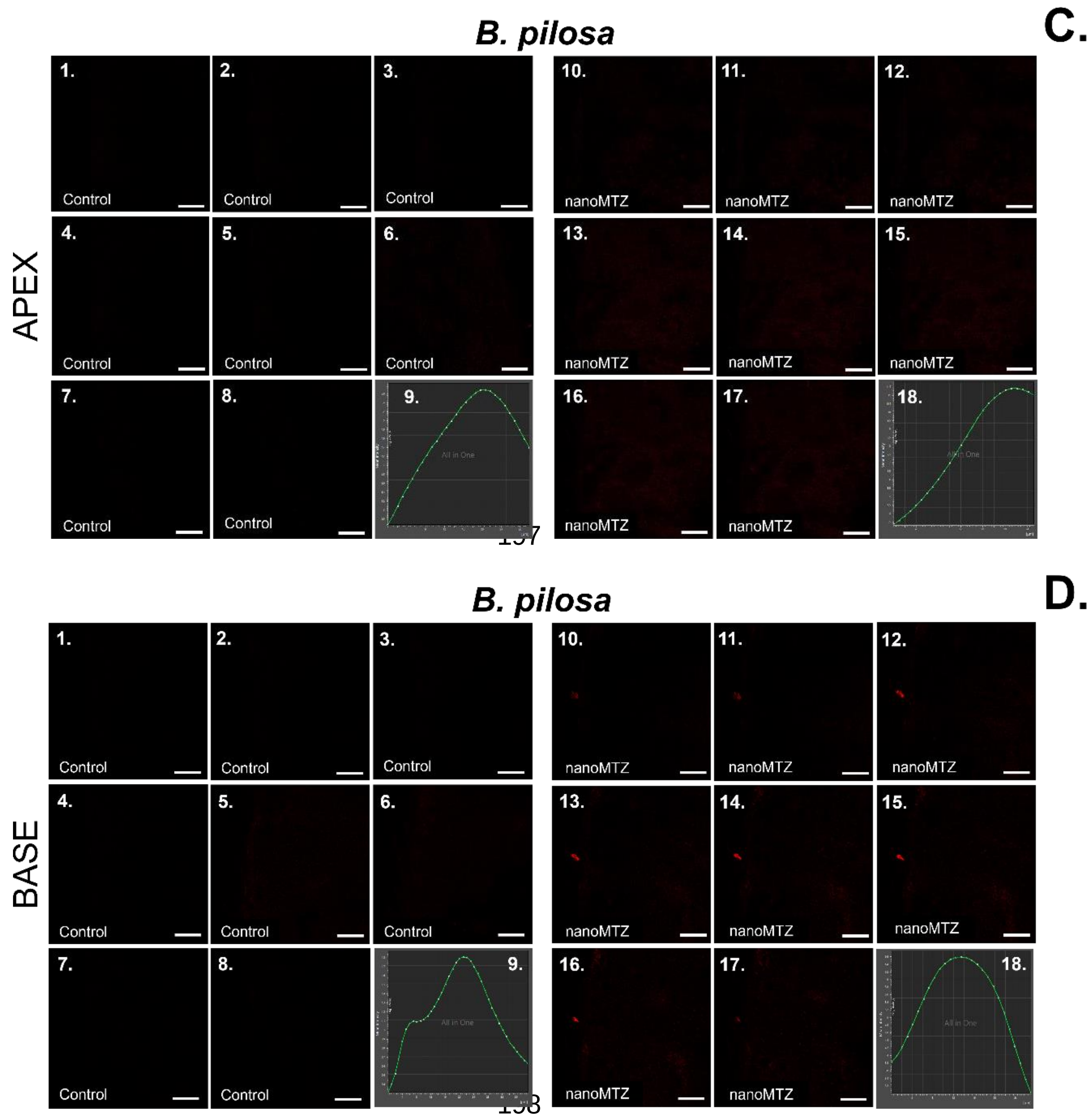
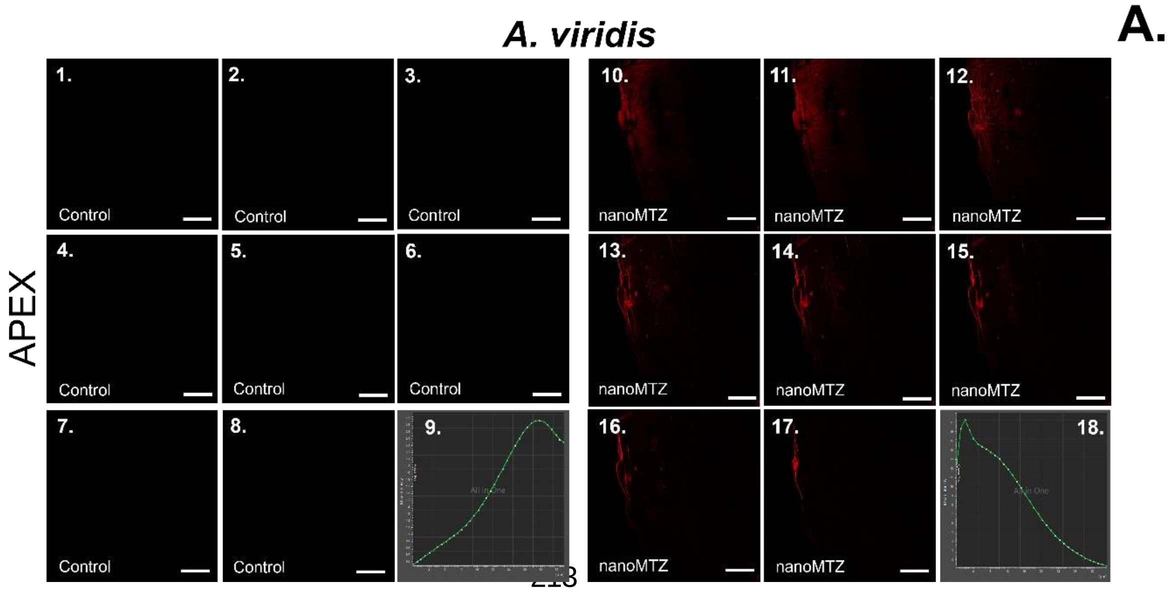


Figure S5. Fluorescence intensity in apex (A and C) and base (B and D) of *Amaranthus viridis* and *Bidens pilosa* leaf, respectively, 120 h after soil application of nanometribuzin (nanoMTZ) (*n*=4). **The images represent different depths of the Z axis, showing internalization throughout the thickness of the leaf.** Z analysis was used to determine the regions of interest. Representative images of water treatment as a control (1-8) and nanoMTZ (10-17). The graphs represent the internalization of the nanoparticles throughout the mesophyll for *A. viridis* (9) and *B. pilosa* (18). Bars = 100 µm.


A. viridis
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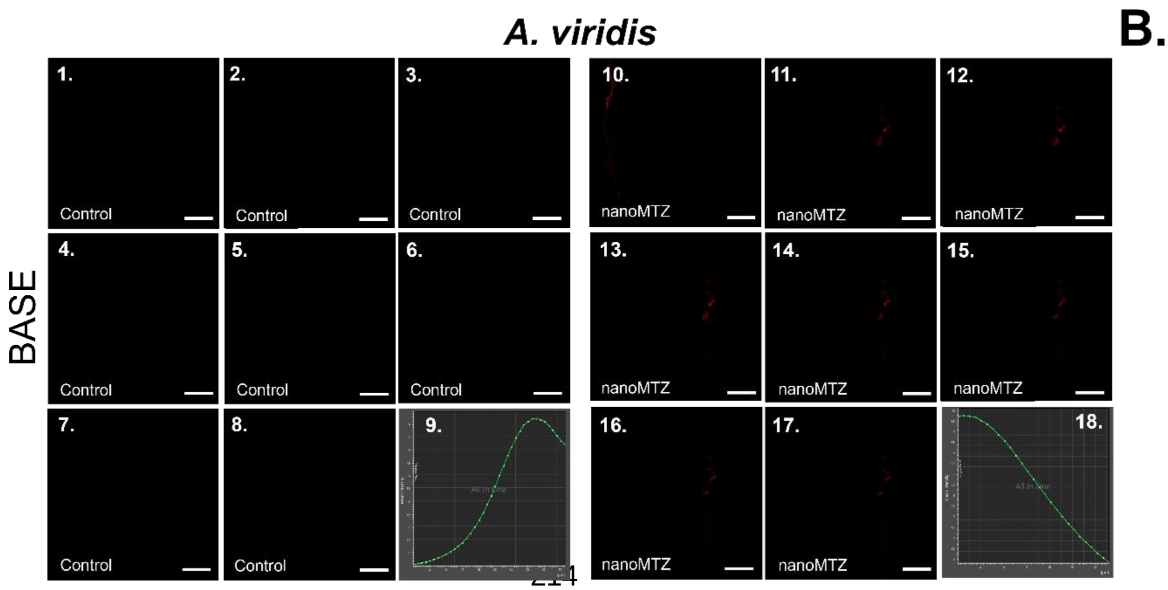
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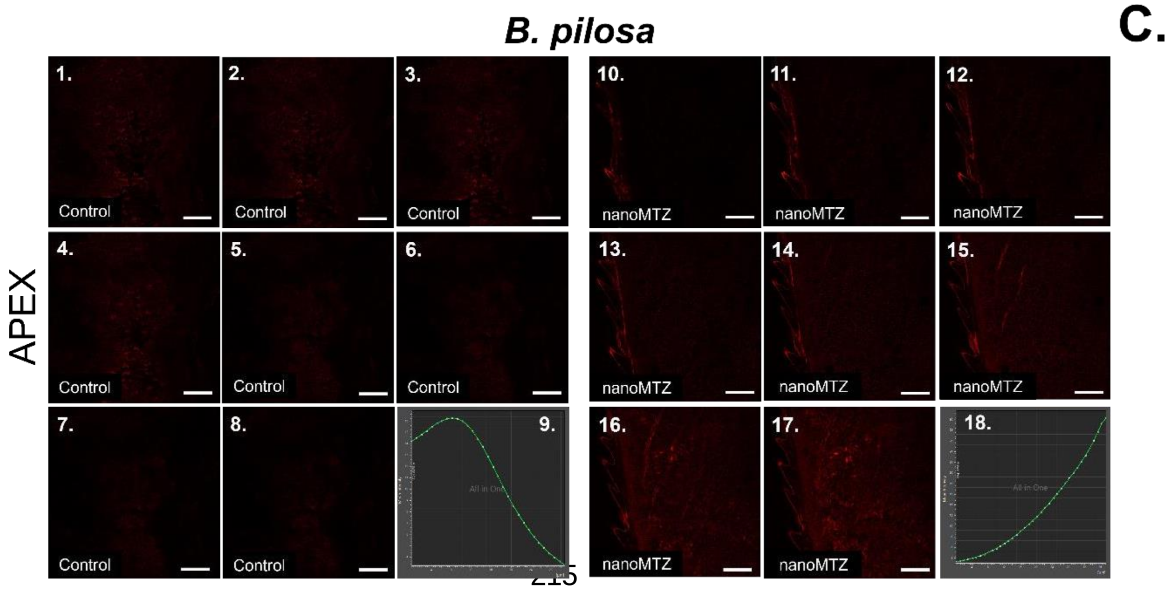
nanoMTZ



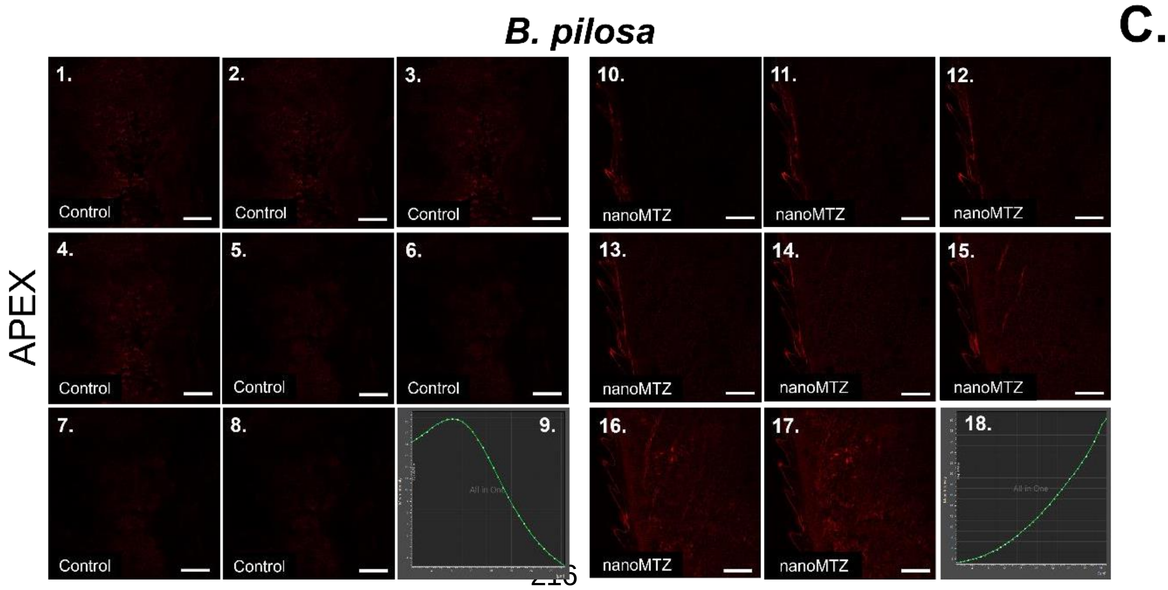


Figure S6. Fluorescence intensity in apex (A and C) and base (B and D) of *Amaranthus viridis* and *Bidens pilosa* leaf, respectively, 120 h after foliar application of nanometribuzin (nanoMTZ) (*n*=4). **The images represent different depths of the Z axis, showing internalization throughout the thickness of the leaf.** Z analysis was used to determine the regions of interest. Representative images of water treatment as a control (1-8) and nanoMTZ (10-17). The graphs represent the internalization of the nanoparticles throughout the mesophyll for *A. viridis* (9) and *B. pilosa* (18). Bars = 100 µm.