SUPPLEMENTAL MATERIAL

Chitosan/glyphosate formulation as a potential, environmental friendly herbicide with prolonged activity

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Materials and methods

Incubation of CH/GL formulations in soil and phytotoxicity (plant growth) test.

The polypropylene pots of diameter 90 mm and of capacity 300 mL were filled with the control soil or the soil loaded with CH/GL formulations. The obtained above solutions (separately in the ratio 1:1 and 2:1) were added into the pots at concentrations of 300, 600, and 1000 mg per kg of dry weight of soil. Next, the soil with loaded dissolved CH/GL formulations was thoroughly mixed and left in the pots for 3 months. Each concentration was in three replicates (3 pots per each concentration for both ratios and both plants). Soil used to incubate the formulations and for phytotoxicity test met the OECD 208 Guideline Terrestrial Plants Growth Test criteria and were characterized with the following parameters: granulometric composition of soil was 77% sand, 16% dust and loam, organic carbon content of approx. 1.6%, and pH (KCl) equal to 6.6.

All pots were regularly watered every day with the same amount of water (20 ml) in order to maintain uniform soil moisture conditions during the whole experiment.

Phytotoxicity assessment was performed at the initial time (0 months) and after 3 months of incubation of CH/GL formulations in soil. For comparison, phytotoxicity of neat high, medium and low molecular weight CH/GL, at the initial time was also evaluated.

The plant growth test of neat chitosans and glyphosate was conducted by introducing to the soil amounts corresponding to the 2:1 ratio formulations of CH/GL. It means that concentrations of formulations 300, 600, and 1000 mg/kg of soil corresponded to 100, 200, and 333mg/kg of soil of pure glyphosate and 200, 400, and 667 mg/kg of soil of each chitosan.

The plant growth test of all samples was performed in laboratory conditions following the OECD 208 Guideline using oat (*Avena sativa*) recommended by the standard as a monocotyledonous plant, and radish (*Raphanus sativus L. subvar. radicula Pers*.), a dicotyledonous plant.

Twenty seeds of each of the selected plant species were sown in the soil. The seeds originated from the same source. The plants were grown for 14 days under controlled conditions: constant humidity content at the level required for the plants (70% field water capacity), temperature (22 ± 2°C), light intensity (7000 lux) in the system of 16 h/day and 8 h/night.

The evaluation of phytotoxicity of the studied samples at applied concentrations was made by comparing the percentage inhibition rate of germination, fresh mass (yield), root length, shoot height and leaf pigment content of treated plants with untreated, control plants. The length of plants is defined as the length of the tip of the longest leaf from the base of culms, while root length is measured from the tip of the longest root to the root-shoot junction.

The growth inhibition (% GI) ratio of shoots, roots and fresh matter (yield) was calculated according to equation (1):

$$\text{GI \%} = \frac{Cp - Tp}{Cp}\; x\; 100\% \qquad (1)$$

where C_p is the height/length of shoot/roots (cm) in control plants, T_p is the height/length of shoot/roots (cm) in tested plants. In case of fresh matter, C_p is the weight of yield (g) in control plants and T_p is the weight of yield (g) in tested plants.

Pigment assay

Photosynthetic pigments content was determined according to method reported by Oren et al.[1] Briefly, 200 mg of fresh leaves was thoroughly homogenized in 20 ml of 80% acetone by means of a cooled mortar and centrifuged afterward. The content of chlorophyll a, chlorophyll b and carotenoids was calculated based on the absorbance at wavelength 470, 647 and 664 nm. The content of photosynthetic pigments were expressed in mg/g of dry weight.

Results

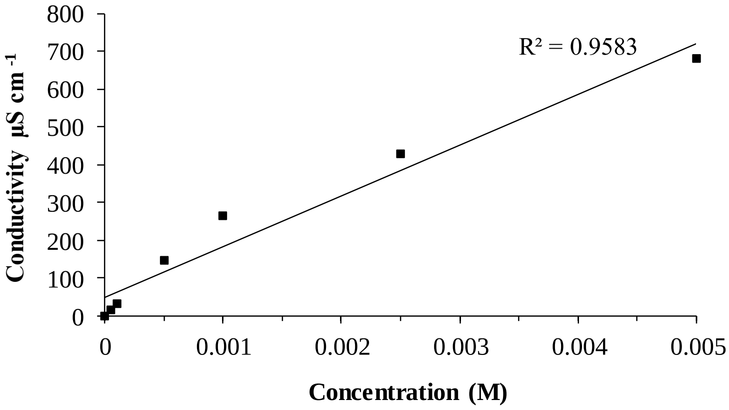


Figure 1S. Conductivity-concentration curve of glyphosate water solution

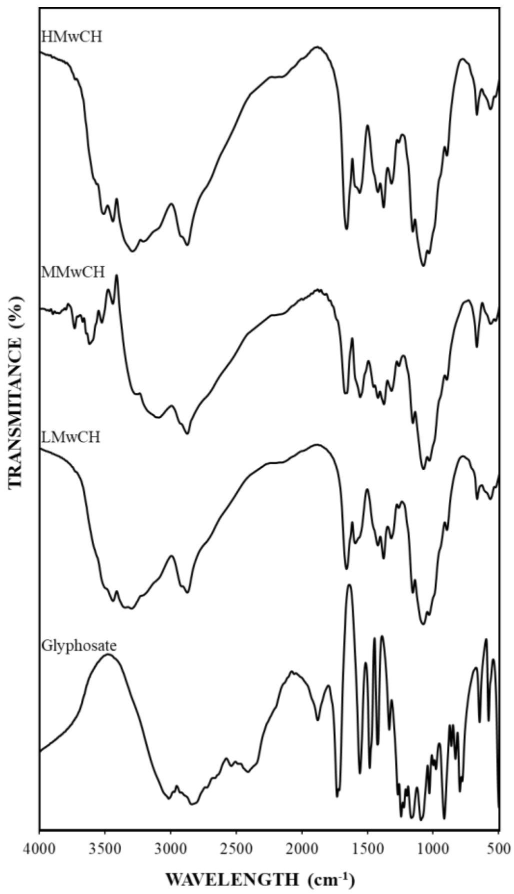


Figure 2S. IR spectra of pure low (LMwCH), medium (MMwCH) and high (HMwCH) molecular weight chitosan and glyphosate.

Figure S2 represents IR spectra of neat chitosans and glyphosate.

IR bands assignments (cm^{-1}) for glyphosate are already described in literature and in a region between 2000 and 900 cm^{-1} are as follows: C=O of free COOH (1732), C=O of H bonded COOH (1720), C-O asymmetric vibration (1568), NH_2 deformations (1557, 1483), CO and OH of free COOH (1434), CO and OH of H-bonded COOH (1424), CH_2 deformations (1336), P-O of PO_3H (1268), CH_2 groups (1243), P-OH (1223), CO, OH groups (1203), P-OH (1170), $P-O^-$ (1090), CCNC skeletal vibrations (1081), P-OH (1000), CCNC (916).[2-4]

IR bands assignments (cm^{-1}) for chitosan are commonly known and are show the following characteristic bands : 3500-3300 cm^{-1} (NH- and OH- stretching vibrations), 2921 and 2877 cm^{-1} can be attributed to C-H symmetric and asymmetric stretching, respectively. 1645 cm^{-1} (C=O stretching of amide I) and 1325 cm^{-1} (C-N stretching of amide III), respectively. The CH_2 bending and CH_3 symmetrical deformations were confirmed by the presence of bands at around 1423 and 1375 cm^{-1}, respectively. The absorption band at 1153 cm^{-1} can be attributed to asymmetric stretching of the C-O-C bridge. The bands at 1066 and 1028 cm^{-1} correspond to C-O stretching. Obtained IR spectra are in accordance with other reports.[5,6]

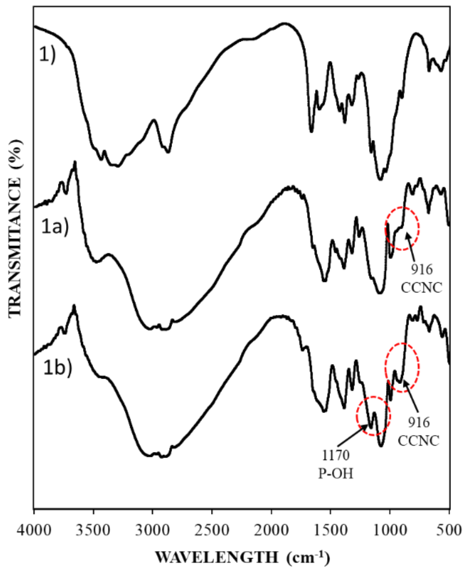
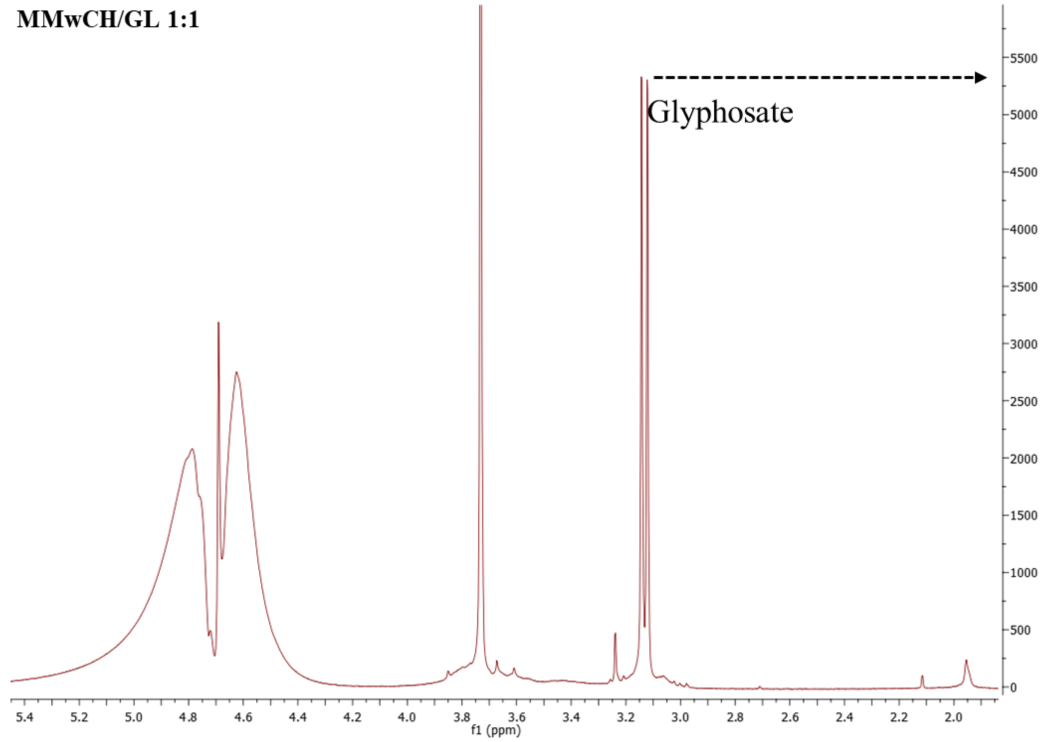


Figure 3S. FTIR spectra of 1) pure low molecular weight chitosan (LMwCH), 1a) LMwCH/GL 2:1 and 1b) LMwCH/GL 1:1.



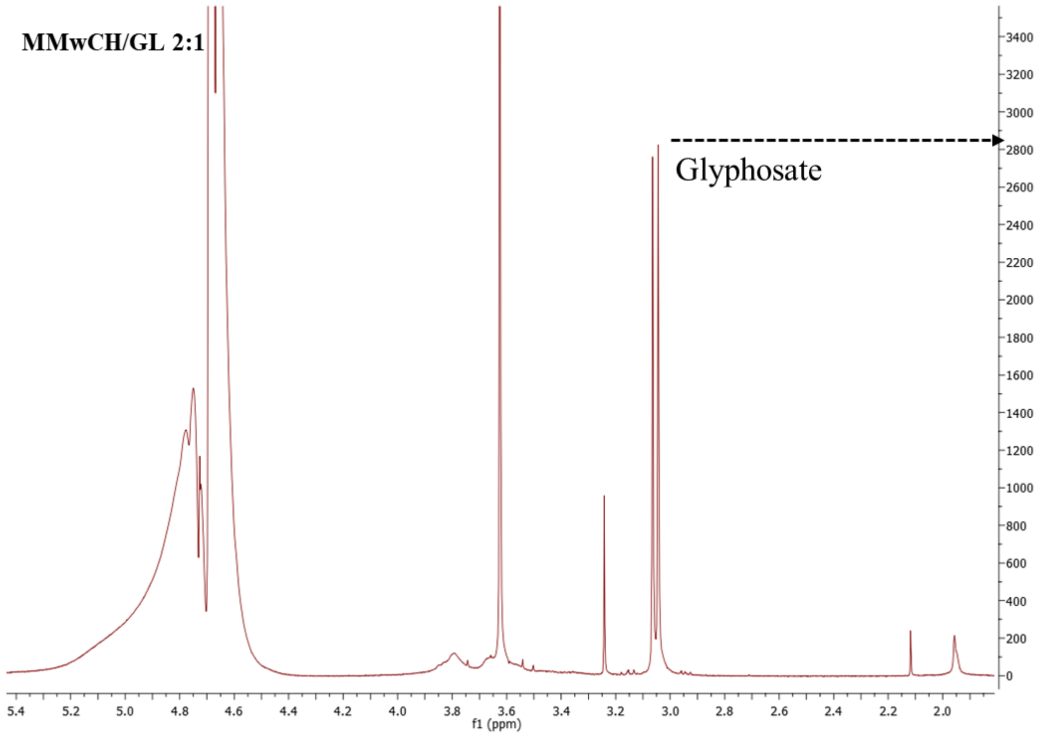


Figure 4S. [1]HNMR spectrum (films dissolved in D_2O) of MMwCH/GL in ratios 1:1 and 2:1.

XRD analysis of glyphosate and neat low, medium and high chitosan

XRD patterns of glyphosate and neat chitosans are presented on Fig. S5.

The glyphosate crystalize in monoclinic space group *$P2_1/c$* with unit cell parameters: a=8.682 Å, b=7.973 Å, c=9.875 Å, β=105.74º.[7] The typical XRD pattern for crystalline glyphosate is given in Fig. 1a. According to the crystal structure data the molecules in unit cell are hydrogenated bonded to their neighbours forming a hydrogenated bonded lattice via O-H···O and N-H···O bonds between the phosphono, amino and carboxyl groups.

The XRD pattern of neat low, medium and high Mw chitosan illustrates two characteristic broad diffraction peaks at (2θ) = 10 (weak peak)° and 20°(strong peak) that are typical fingerprints of crystalline chitosan. The peaks around 10° and 20° are related to crystal I and crystal II in chitosan structure.[8,9] The XRD pattern indicated that the low, medium and high molecular weight chitosan displays an amorphous form, but in this series there is an increase of degree of crystallinity.

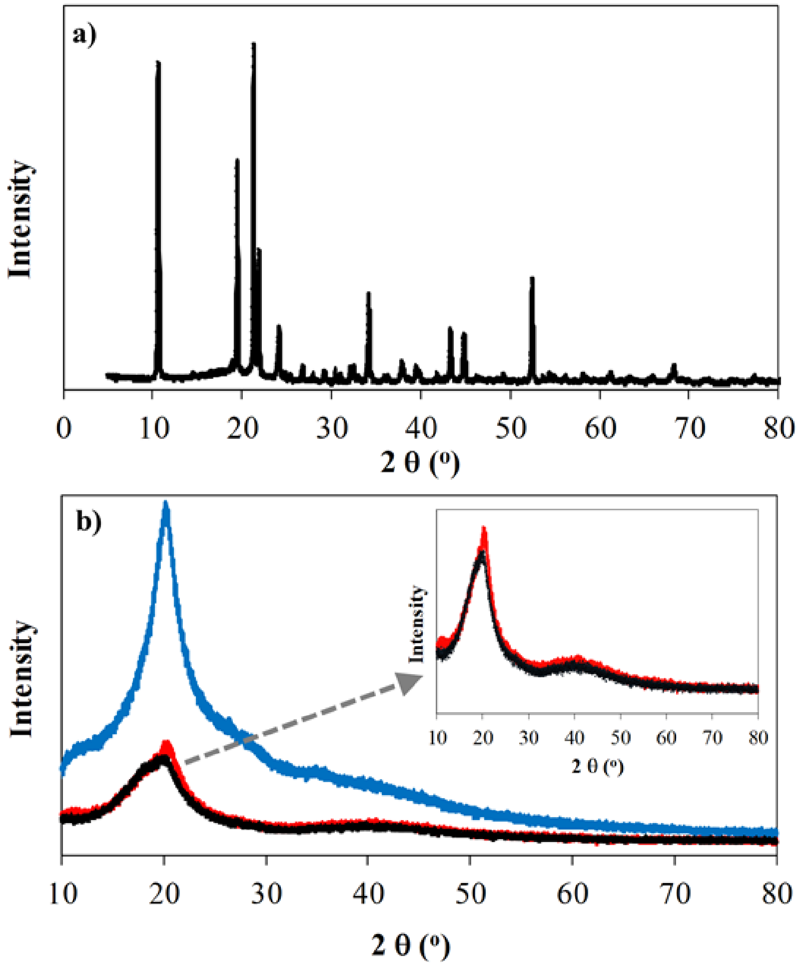


Figure 5S. XRD patterns of a) glyphosate and b) neat chitosans. Colours blue, red and black on figure b represent low, medium and high molecular weight chitosan respectively.

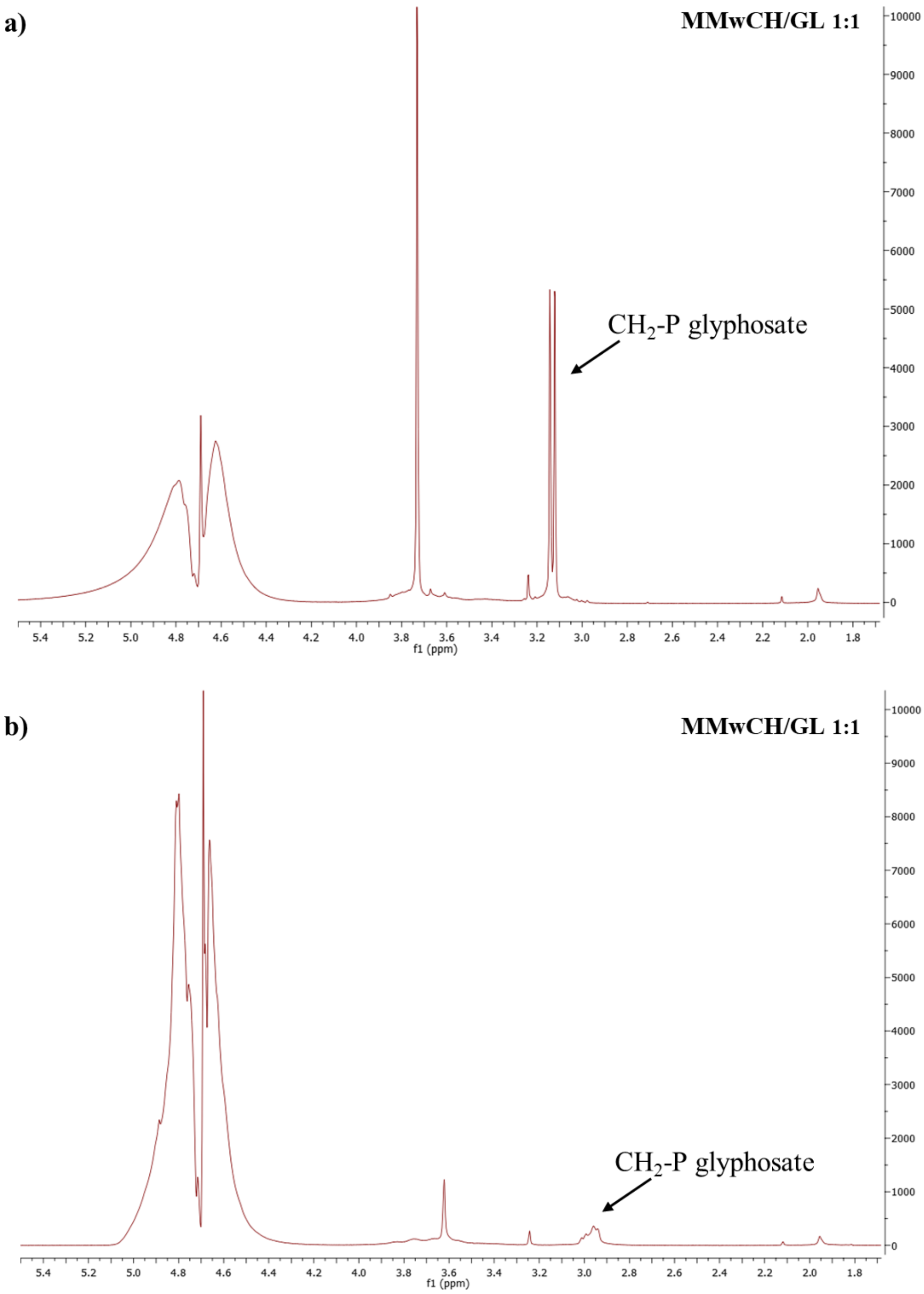


Figure 6S. [1]HNMR spectrum (films dissolved in D_2O) of MMwCH/GL 1:1 before (a) and after (b) 30 days of release experiment into water.

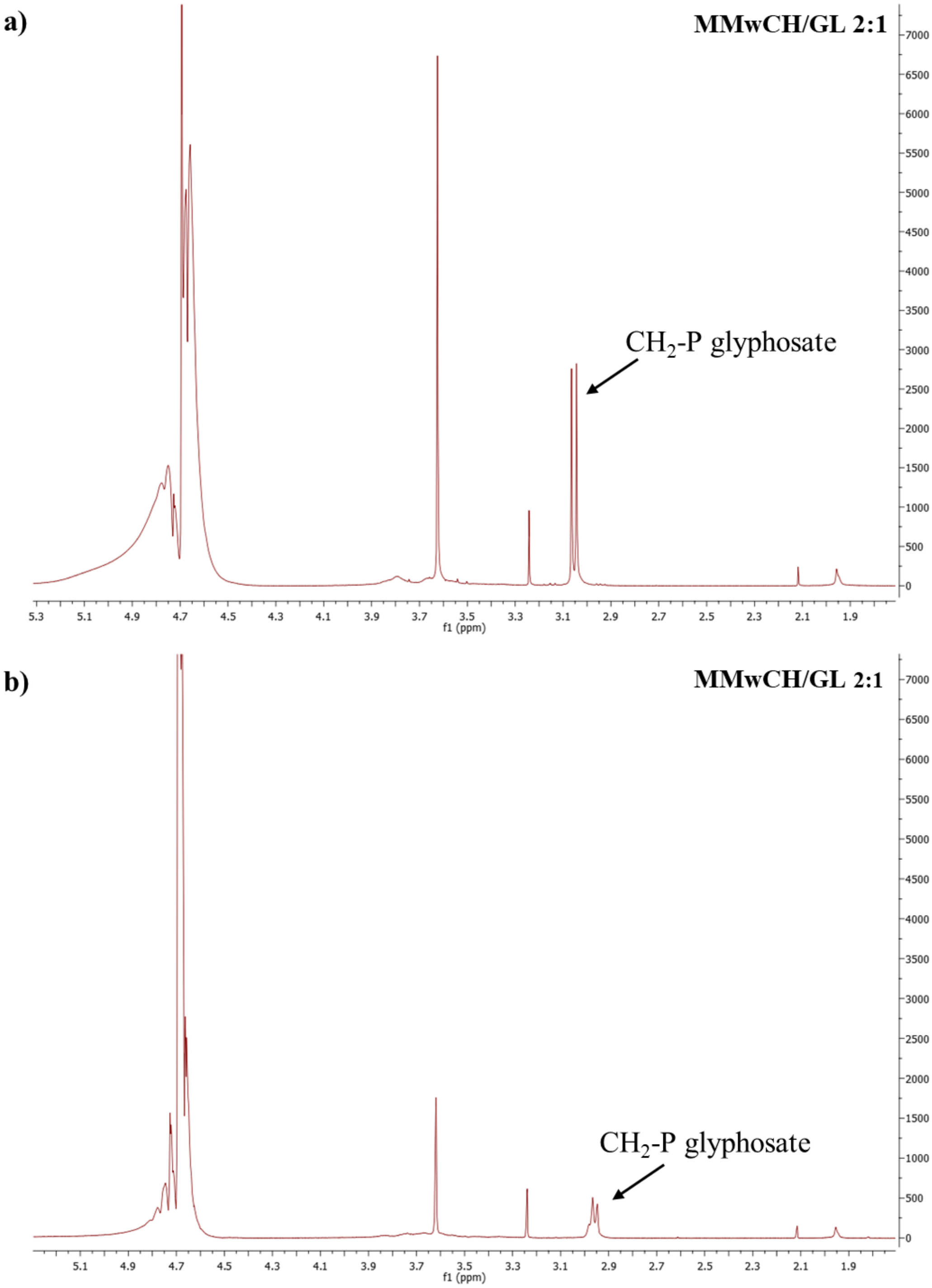


Figure 7S. ^{1}HNMR spectrum (films dissolved in D_2O) of MMwCH/GL 2:1 before (a) and after (b) 30 days of release experiment into water.

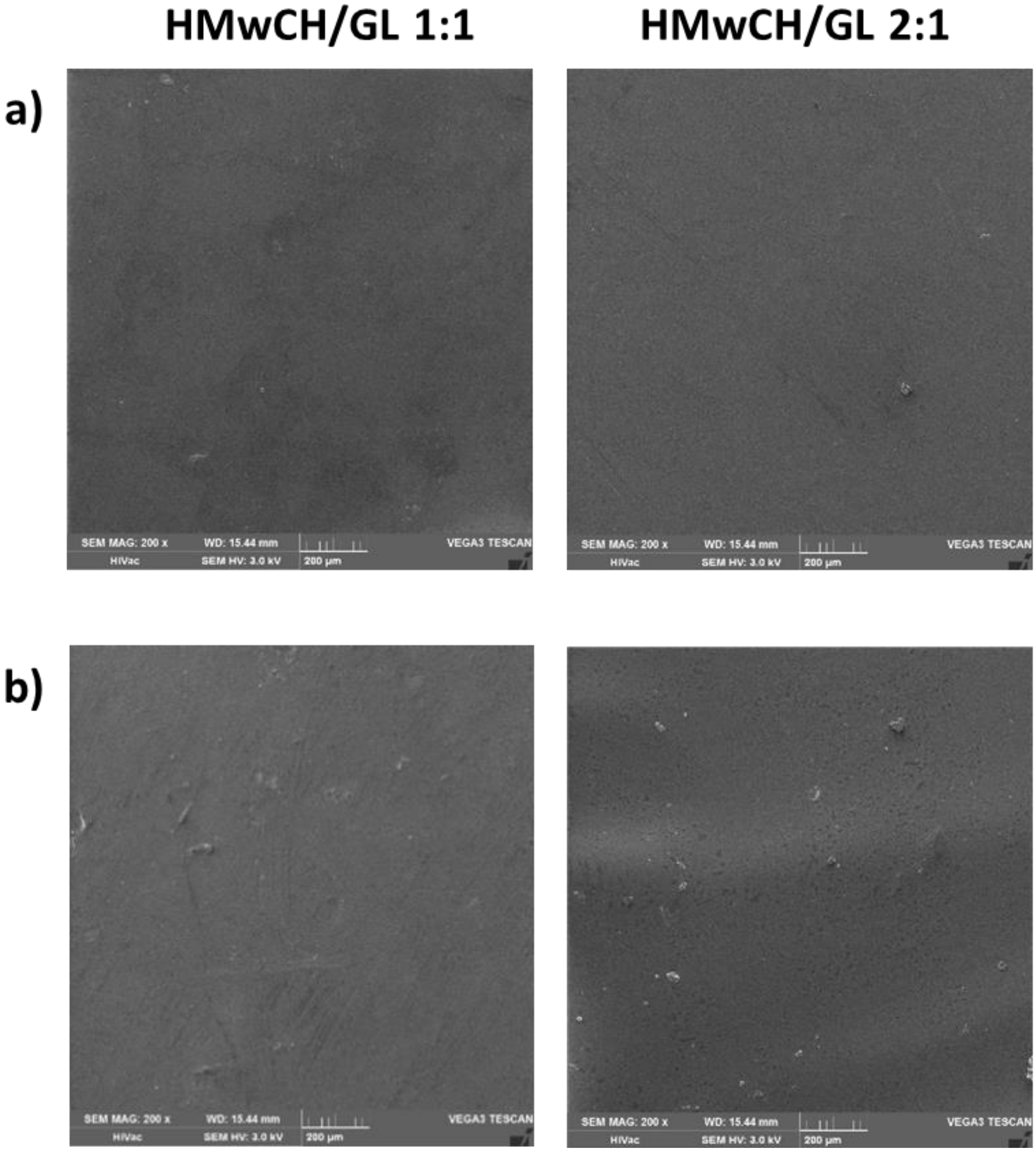


Figure 8S. SEM pictures of HMwCH/GL formulation before (a) and after (b) release experiment (30 days)

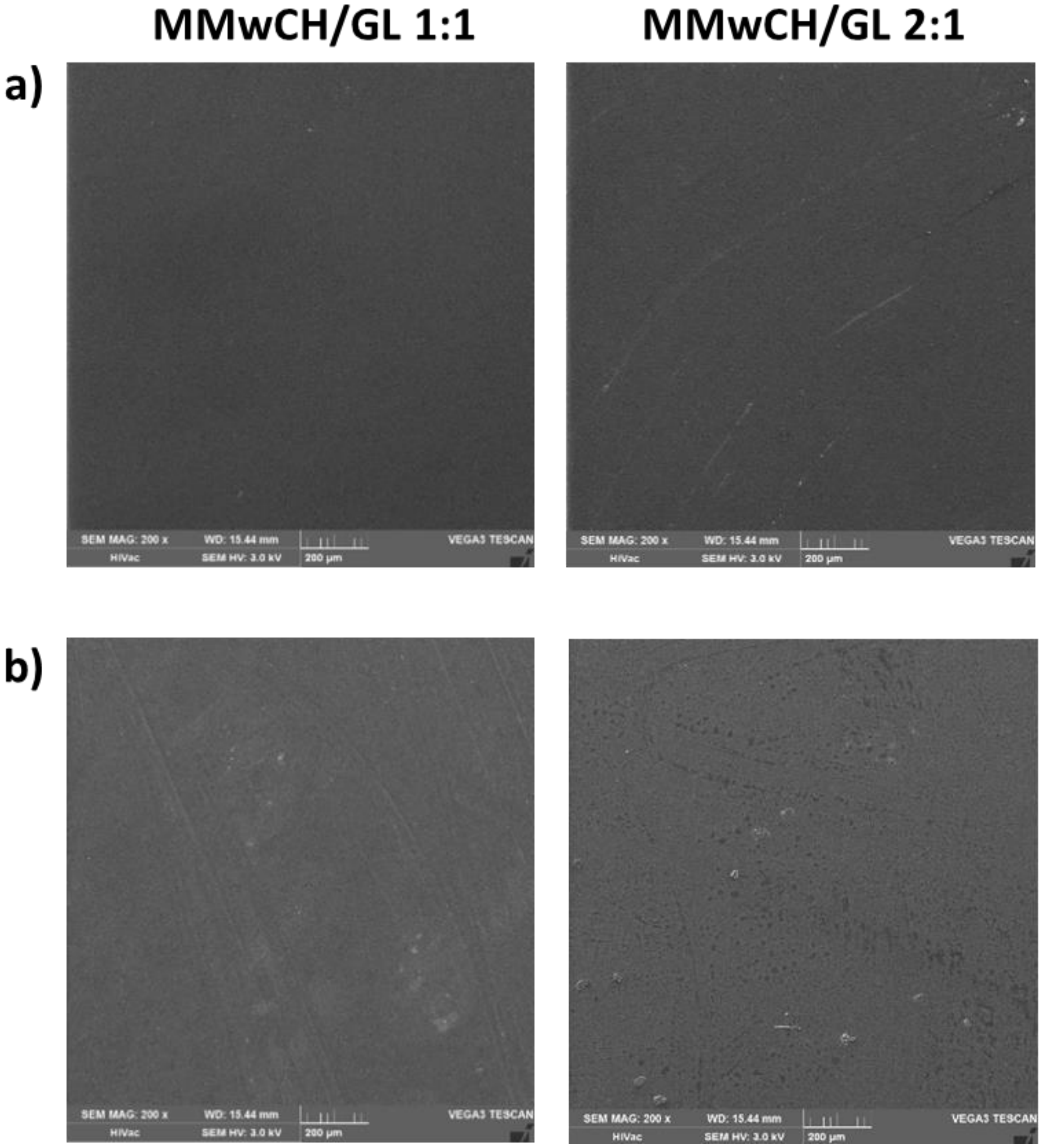


Figure 9S. SEM pictures of MMwCH/GL formulation before (a) and after (b) release experiment (30 days)

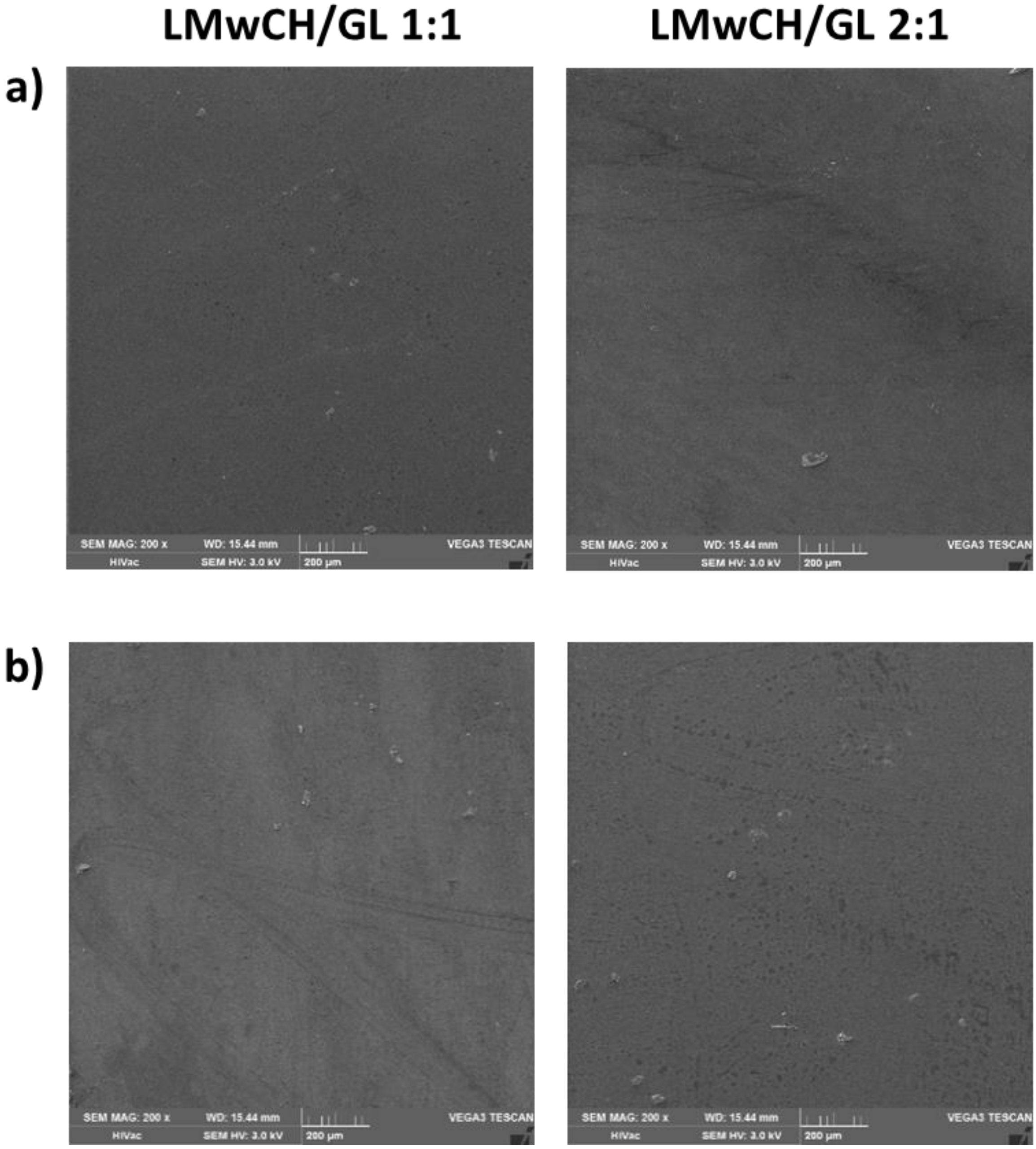


Figure 10S. SEM pictures of LMwCH/GL formulation before (a) and after (b) release experiment (30 days)

Table 1S: Effect of formulation of high, medium and low molecular weight chitosan with glyphosate on the **shoot height** of oat and radish sprouts (mean ± SD) determined after 14 days of growth in the soil containing different amounts of formulations in soil. These values were determined by equation (1) and represent the percentage of growth inhibition for a tested parameter compared to the control. Negative values mean that examined parameter was higher (promoted growth) compared to the control plants.

Concentration (mg/kg of soil dry weight)	***Avena sativa***					
	LMwCH/GL		**MMwCH/GL**		**HMwCH/GL**	
	1:1	**2:1**	**1:1**	**2:1**	**1:1**	**2:1**
	0 months					
300	0.9±0.1	0.3±0.1	1.9±0.9	-0.9±0.3	0.6±1.1	-4.0±0.2
600	-3.3±0.2	-4.2±0.3	-1.2±0.7	-4.8±0.3	-3.1±0.3	-5.8±0.1
1000	8.3±0.8	6.7±0.7	7.6±0.8	4.5±0.8	6.1±0.6	-3.4±0.9
	After 3 months					
300	1.1±0.5	0.8±0.4	0.3±0.2	-1.1±0.7	-1.4±0.1	-4.8±0.5
600	-0.3±0.4	-0.6±0.3	-2.2±0.6	-2.3±0.8	-2.9±0.7	-5.7±0.2
1000	-0.5±0.3	-0.9±0.2	-3.2±0.2	-3.4±0.8	-4.2±0.8	-6.8±0.4
	Raphanus sativus					
	LMwCH/GL		**MMwCH/GL**		**HMwCH/GL**	
	1:1	**2:1**	**1:1**	**2:1**	**1:1**	**2:1**
	0 months					
300	4.7±0.3	3.8±0.8	2.5±0.4	-0.9±0.5	1.3±0.2	-1.6±0.5
600	2.8±0.2	7.0±0.4	1.9±0.2	-1.9±0.5	0.3±0.4	-5.1±0.8
1000	15.8±0.5	7.9±0.1	10.1±0.5	1.3±0.3	1.3±0.2	-6.3±0.3
	After 3 months					
300	1.0±0.7	2.3±0.6	-0.7±0.2	-1.3±0.2	-3.0±0.5	-3.3±0.4
600	3.0±0.7	3.3±0.7	-1.3±0.1	-2.6±0.4	-3.9±0.3	-4.9±0.4
1000	3.3±0.7	6.3±0.5	-2.0±0.4	-3.6±0.4	-4.9±0.5	-5.6±0.2

Table 2S: Effect of formulation of high, medium and low molecular weight chitosan with glyphosate on the **yield (fresh mass)** of oat and radish sprouts (mean ± SD) determined after 14 days of growth in the soil containing different amounts of formulations in soil. These values were determined by equation (1) and represent the percentage of growth inhibition for a tested parameter compared to the control. Negative values mean that examined parameter was higher (promoted growth) compared to the control plants

Concentration (mg/kg of soil dry weight)	***Avena sativa***					
	LMwCH/GL		**MMwCH/GL**		**HMwCH/GL**	
	1:1	**2:1**	**1:1**	**2:1**	**1:1**	**2:1**
	0 months					
300	0.5±0.2	-1.5±0.3	0.4±0.2	-2.0±0.2	0.5±0.2	-9.7±0.4
600	-6.7±0.4	-9.4±0.0	-7.3±0.2	-9.2±0.1	-7.6±0.1	-8.7±0.1
1000	23.3±0.2	9.5±0.2	21.6±0.0	4.7±0.1	21.2±0.2	-6.5±0.2
	After 3 months					
300	0.2±0.1	-0.4±0.3	-0.4±0.1	0.5±0.1	-0.3±0.2	-0.2±0.2
600	1.6±0.2	2.5±0.2	-0.5±0.2	-0.8±0.2	-0.9±0.2	-2.4±0.1
1000	4.4±0.2	-2.3±0.0	-1.4±0.1	-2.2±0.1	-0.8±0.1	-4.9±0.1
	Raphanus sativus					
	LMwCH/GL		**MMwCH/GL**		**HMwCH/GL**	
	1:1	**2:1**	**1:1**	**2:1**	**1:1**	**2:1**
	0 months					
300	4.6±0.3	-2.6±0.3	3.0±0.2	-6.4±0.2	0.3±0.3	-7.3±0.4
600	4.4±0.1	-6.3±0.3	2.2±0.2	-4.6±0.3	-1.1±0.2	-3.6±0.2
1000	55.4±0.2	42.0±0.2	54.0±0.3	20.5±0.1	53.4±0.2	-0.7±0.7
	After 3 months					
300	3.3±0.6	2.1±0.0	-0.4±0.1	-0.7±0.2	-3.1±0.2	-2.7±0.3
600	8.3±0.1	4.5±0.4	0.3±0.3	-1.6±0.1	-5.0±0.5	-5.7±0.0
1000	15.4±0.1	6.5±0.1	4.2±0.2	-2.9±0.7	-3.9±0.5	-1.0±0.2

Effect of formulations and neat substances on germination, plant pigments content and visual evaluation of plants.

Germination of seeds

The germination study revealed that chitosan/glyphosate formulations regardless the type of chitosan, ratio of polymer/herbicide and time of phytotoxcity assessment, the inhibition of seeds germination did not exceed 10% as compared to control plants (Tab. S3) which is considered as the non toxic in germination process. The same data were found for neat chitosans and glyphosate (not shown).

Table 3S: Average values (mean of three replicates) referring to germination of oat and radish seeds germinated in the soil treated with chitosan/glyphosate formulations. The percentage of germination refers to the number of emerged plants compared to the control. Values of LSD_s and LSD_c represent the least significant difference for samples and concentrations respectively. Given values e.g. 20/100 represent 20 seeds germinated, which corresponds to 100% as compared to control germinated seeds.

Concentration (mg/kg of soil dry weight)	***Avena sativa***					
	LMwCH/GL		**MMwCH/GL**		**HMwCH/GL**	
	1:1	**2:1**	**1:1**	**2:1**	**1:1**	**2:1**
	0 months					
300	20/100	19/95	20/100	20/100	20/100	20/100
600	19/95	19/95	19/95	19/95	19/95	19/95
1000	19/95	19/95	19/95	19/95	19/95	19/95
	After 3 months					
300	20/100	20/100	20/100	20/100	20/100	20/100
600	19/95	19/95	19/95	19/95	19/95	20/100
1000	19/95	19/95	19/95	19/95	19/95	20/100
	LSD_s =1 LSD_c =1					
	Raphanus sativus					
	LMwCH/GL		**MMwCH/GL**		**HMwCH/GL**	
	1:1	**2:1**	**1:1**	**2:1**	**1:1**	**2:1**
	0 months					
300	19/95	19/95	19/95	19/95	19/95	19/95
600	19/95	19/95	19/95	19/95	19/95	19/95
1000	18/90	18/90	19/95	18/90	18/90	18/90
	After 3 months					
300	19/95	19/95	19/95	19/95	19/95	19/95
600	19/95	19/95	19/95	19/95	19/95	19/95
1000	19/95	19/95	19/95	19/95	19/95	19/95
	LSD_s =1 LSD_c =1					

Effect of neat substances and formulations of CH/GL on plant pigment content in leaves. Changes of chlorophyll and carotenoids content in plants leaves treated with chitosans/glyphosate formulations as well as neat substances are presented on Figs. S19-S24. Chlorotic symptoms like yellowing or whitening of normally green plant tissue appearing on plant leaves indicates decrease in chlorophyll resulting in disease (chlorosis) or nutrient deficiency. The ratio of chlorophylls to carotenoids is a crucial factor responsible for the proper functioning of the photosynthesis system. Carotenoid pigments are critical for plant survival; therefore their composition and amount strongly depends on the physiological and pathological conditions they live in.[10,11] At the initial time, the content of both the plant pigments in leaves, regardless of the concentration in the soil and the kind of chitosan, was comparable to that in control plants. For comparison, as expected, total chlorophyll in both plants decreased with the increasing concentration of glyphosate in soil wherein, the strongest effect was noticed for dicotyldenous radish than monocytlodenous oat. For comparison, the level of carotenoids in leaves increased with increasing concentration of this herbicide in soil. This response mechanism of plants growing in stressful conditions was already described in literature by Jarvis and Lopez-Juez.[12] They reported that one of the most important environmental factors affecting the amount and metabolism of carotenoids is oxidative stress which occurs when the absorbed energy exceeds the capacity of the plant's photosynthetic apparatus. The reason for the level of carotenoids of tested seedlings increasing in the presence of increasing concentrations of glyphosate may be the fact that carotenoids are antioxidants and, consequently, they induce the appearance of complex protective systems within the leaf tissue which minimizes the effect of oxidative stress.

In this respect, the effect of formulations CH/GL was herbicide content dependent. Briefly, among the tested samples, the least harmful effect on chlorophyll content for both plants was found for formulations of CH/GL in the ratio 2:1; however it is worth noting that the formulation with high molecular weight was found to have the least effect on chlorophyll levels.

No significant changes were noticed in the carotenoid content of leaves of radish treated with all the formulations. For comparison, the carotenoid level in oat seedlings was dependent on the type of chitosan used and the negative effect increased with its reducing molecular weight.

After 3 months of incubation of the formulations in the soil, the same tendency was observed in the pigment changes of both plants. The HMwCH/GL protected both plants from the harmful effect of glyphosate because the most negative effect on chlorophyll level was noticed for the formulation with LMwCH/GL. After 3 months of incubation of formulation in soil there was a significant change in the carotenoid content in radish leaves. Meanwhile, a slightly less harmful effect of LMwCH/GL on carotenoids in oat leaves was noticed compared to the test after the initial time of experiment.

Formulations in the ratio 1:1 had much worse influence on the total chlorophyll content in both plants at each term of determination. This fact demonstrates that chlorophyll content is dependent both on the molecular weight of chitosan and the ratio of the CH/GL (formulations of CH/GL 2:1 were much less harmful when compared to ratio 1:1).

Visual evaluation of plants

Regardless the time of phytotxicity evaluation (0 or 3 months), any symptoms of chlorosis and necrosis of oat seedlings treated with formulations CH/GL were identified (Figs. S11-S18). For comparison, after 3 months of incubation of formulations of LMwCH/GL and MMwCH/GL (in the ratio 2:1) in soil, a slight yellowing of the surface of radish leaves, considered as a symptom of chlorosis, was observed, indicating that this plant was more sensitive to the formulations used.

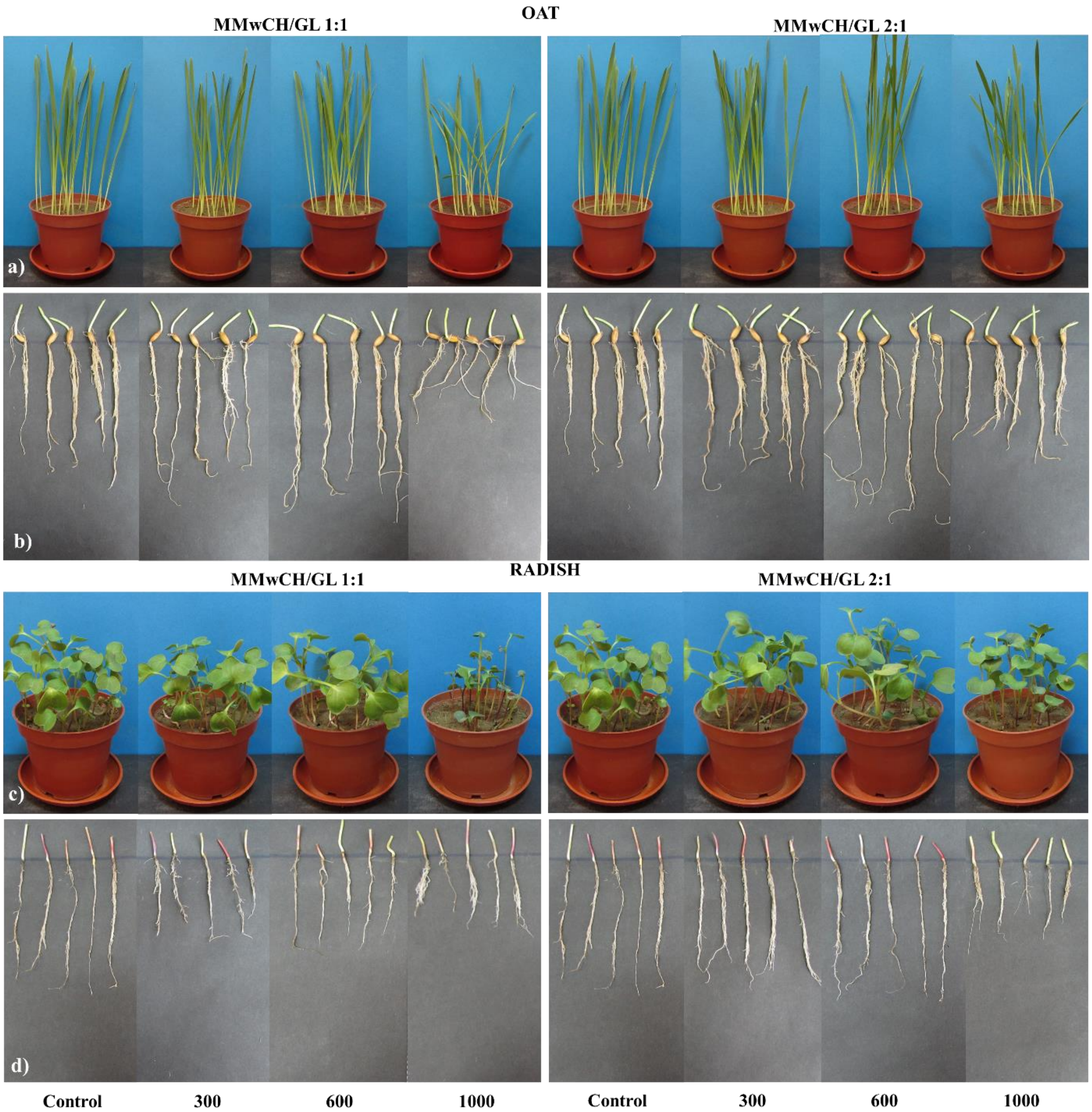


Figure 11S. Photographs of selected oat seedlings (a), oat roots (b), radish sprouts (c) and radish roots (d) at the 14th day growing in the presence of various MMwCH/GL concentrations (expressed in mg/kg of soil dry weight).

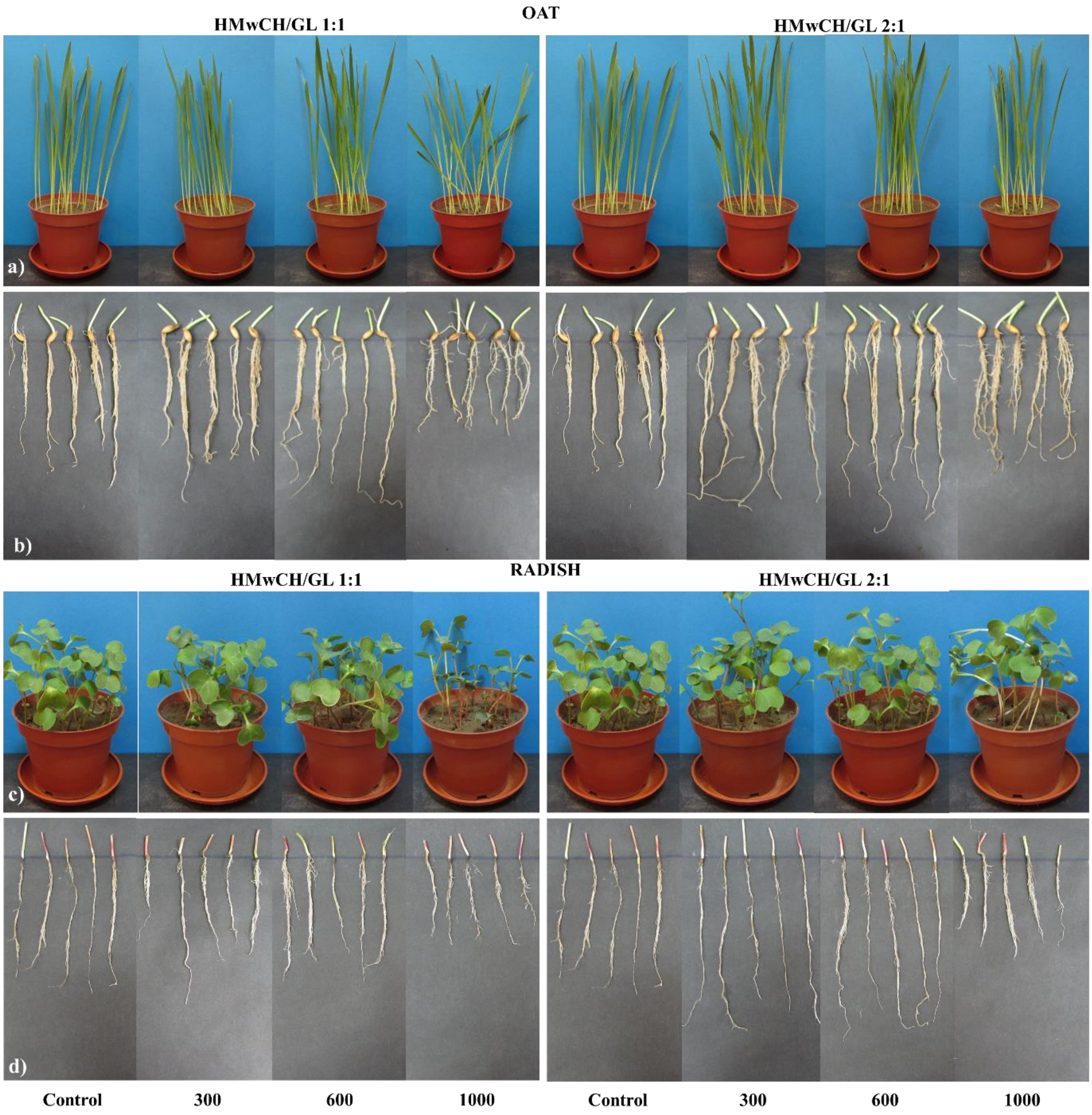


Figure 12S. Photographs of selected oat seedlings (a), oat roots (b), radish sprouts (c) and radish roots (d) at the 14th day growing in the presence of various HMwCH/GL concentrations (expressed in mg/kg of soil dry weight).

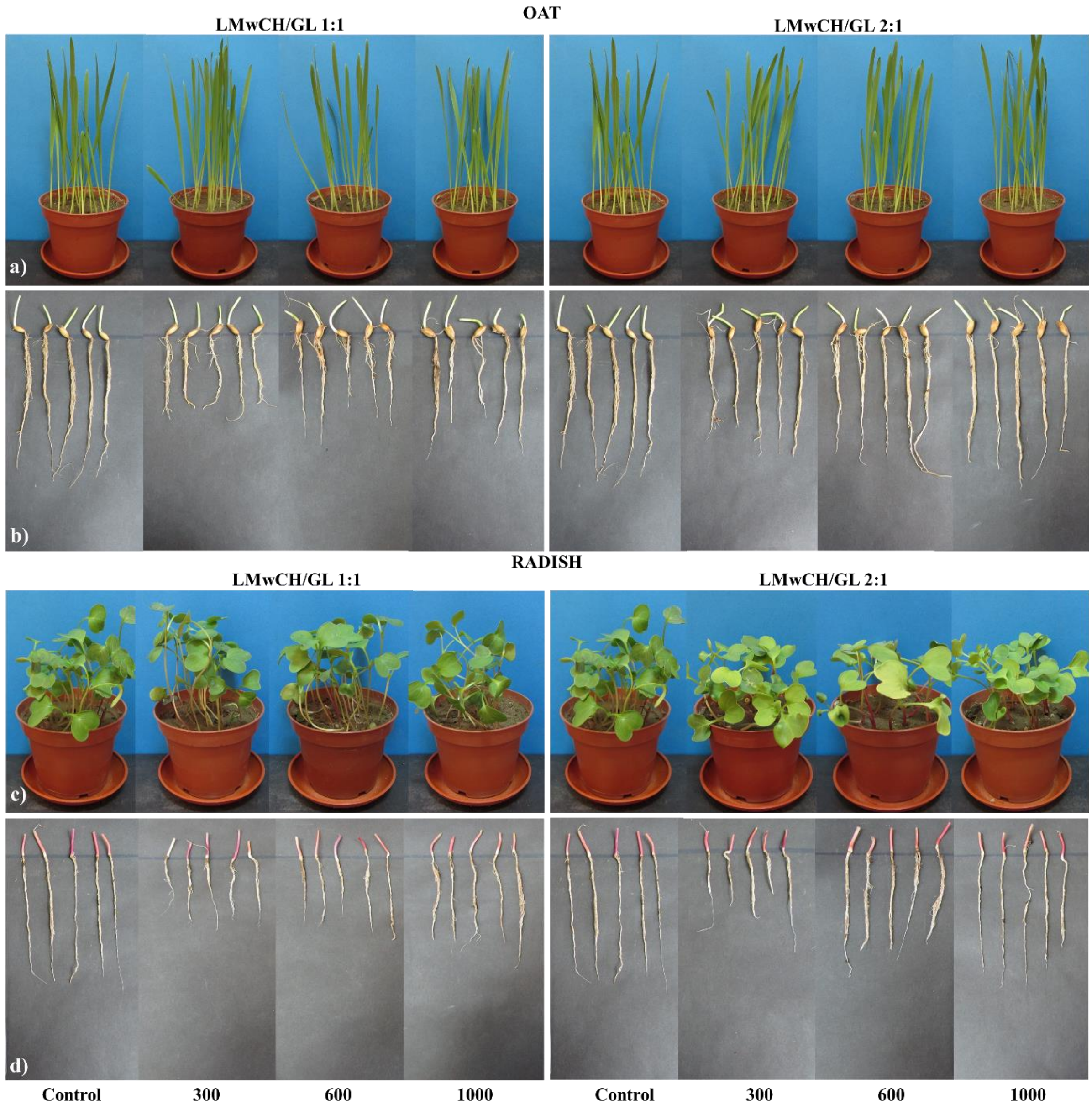


Figure 13S. Photographs of selected oat seedlings (a), oat roots (b), radish sprouts (c) and radish roots (d) at the 14th day growing in the presence of various LMwCH/GL concentrations (expressed in mg/kg of soil dry weight), after their 3 months incubation in the soil.

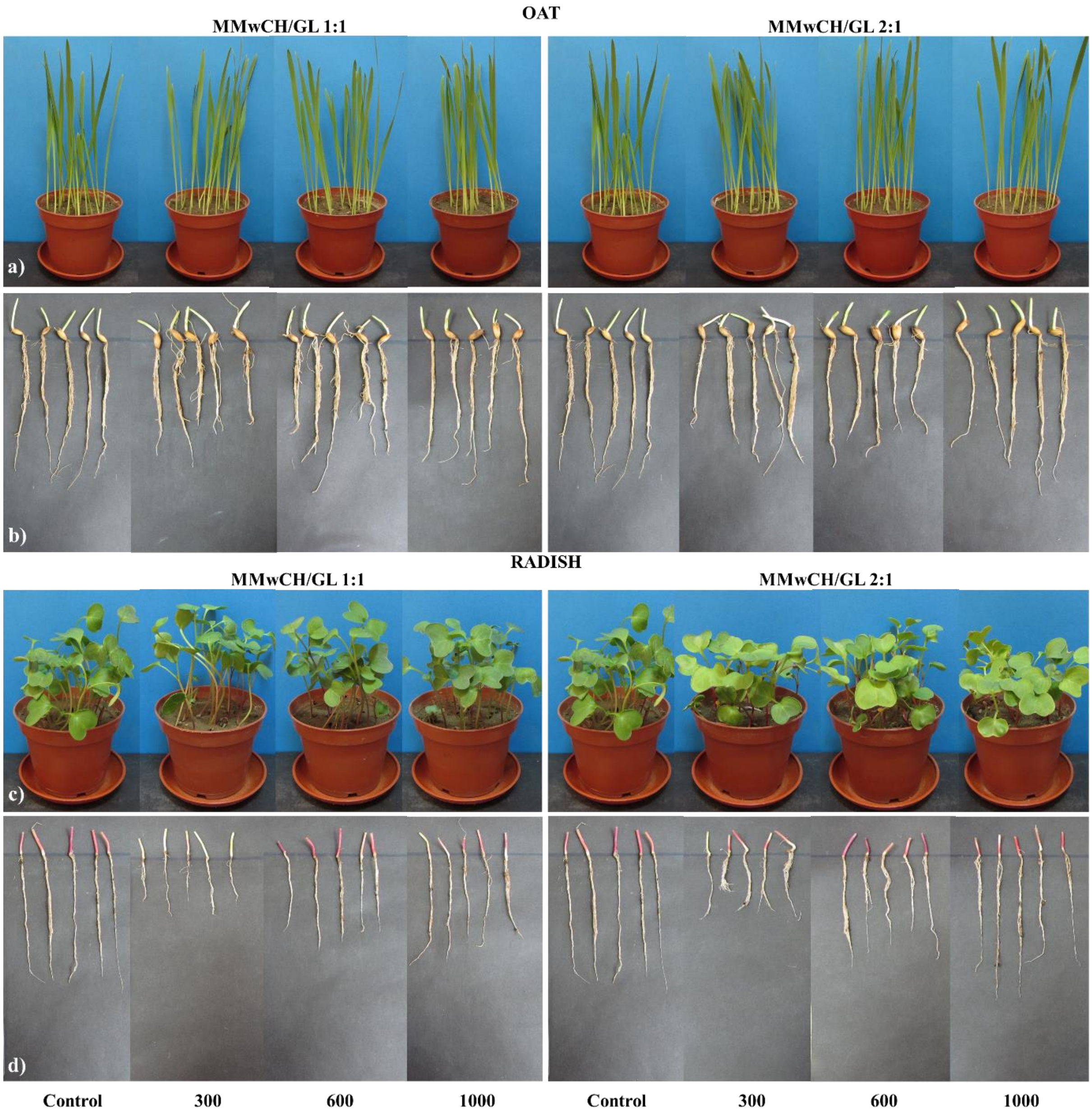


Figure 14S. Photographs of selected oat seedlings (a), oat roots (b), radish sprouts (c) and radish roots (d) at the 14th day growing in the presence of various MMwCH/GL concentrations (expressed in mg/kg of soil dry weight), after their 3 months incubation in the soil.

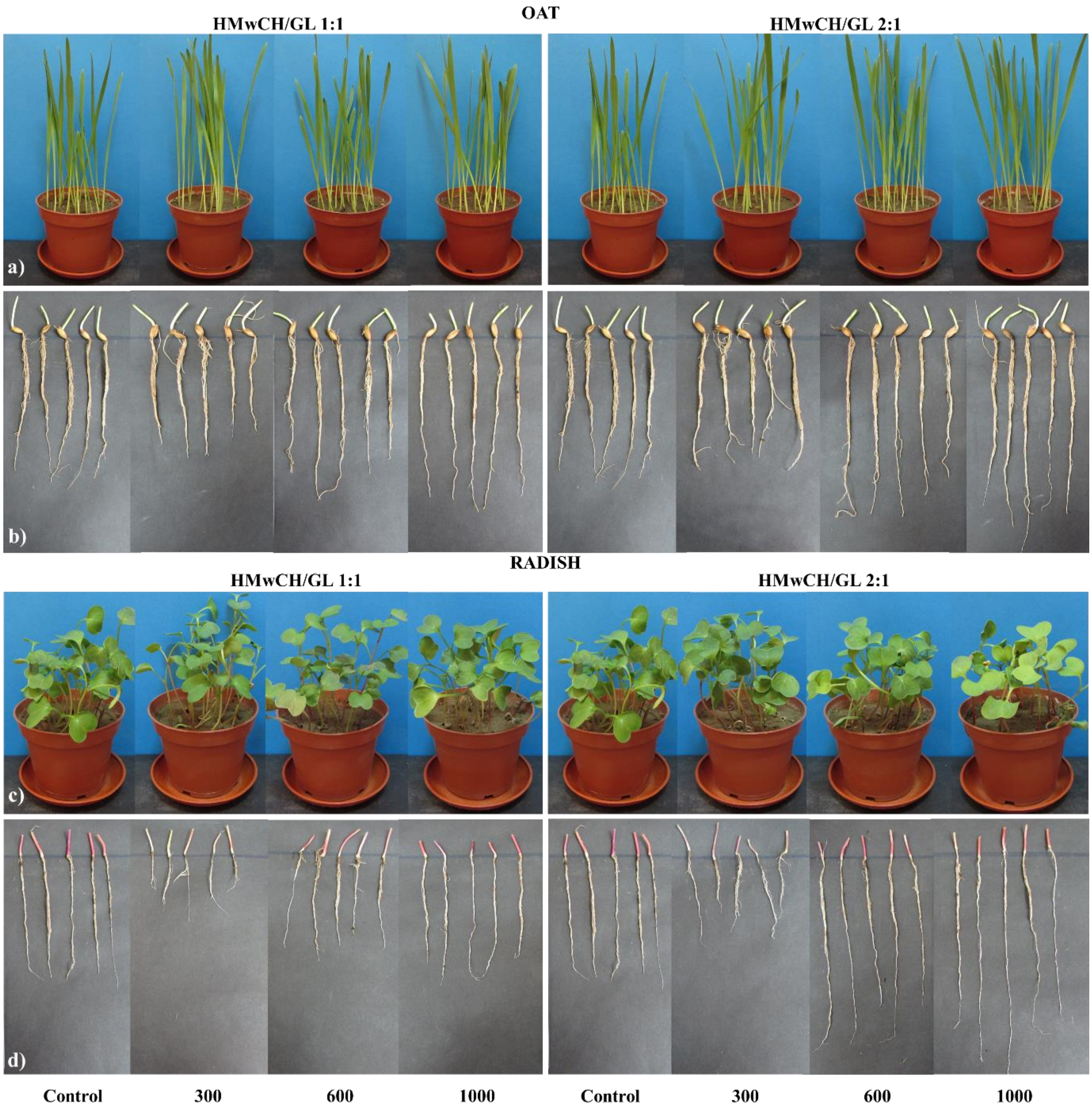


Figure 15S. Photographs of selected oat seedlings (a), oat roots (b), radish sprouts (c) and radish roots (d) at the 14th day growing in the presence of various HMwCH/GL concentrations (expressed in mg/kg of soil dry weight), after their 3 months incubation in the soil.

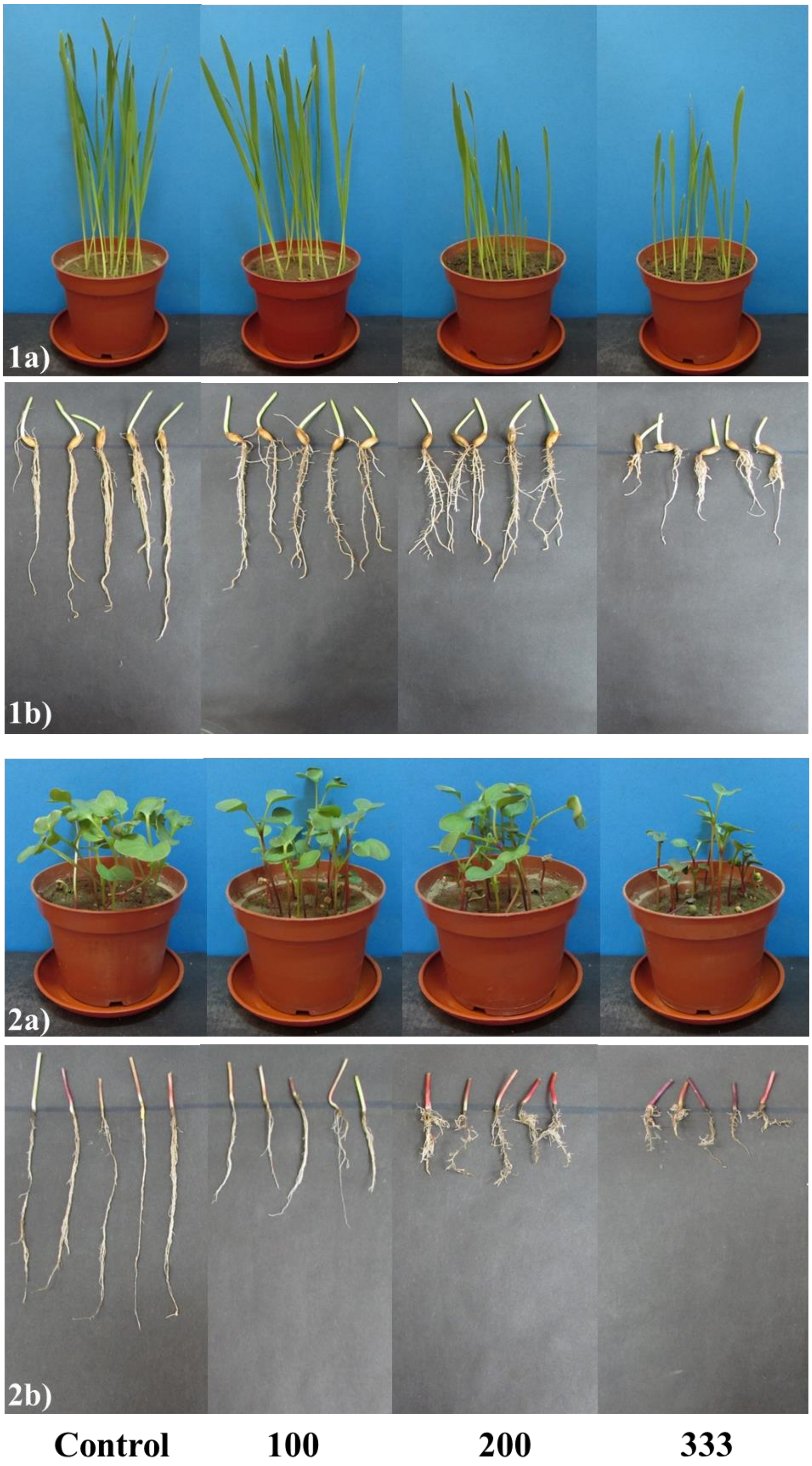


Figure 16S. Photographs of selected radish sprouts at the 14th day growing in the presence of glyphosate at various concentrations (expressed in mg/kg of soil dry weight).

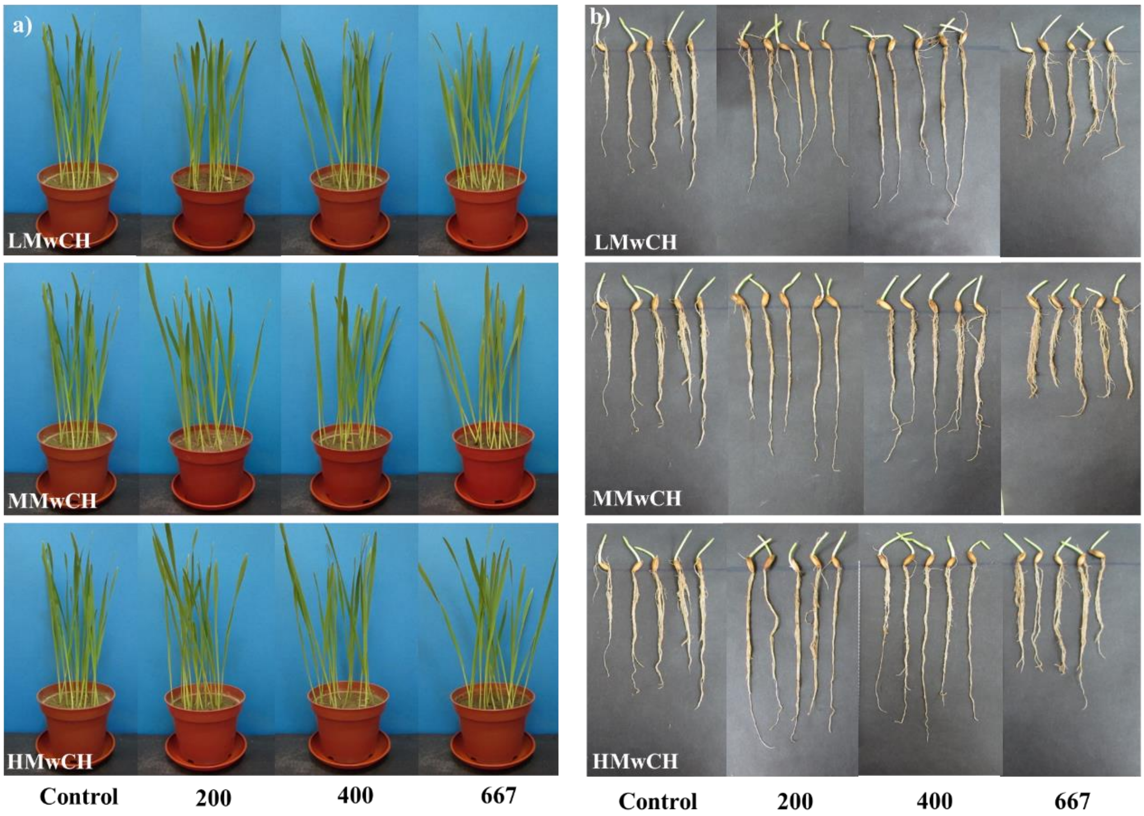


Figure 17S. Photographs of selected oat sprouts (a) and oat roots (b) at the 14th day growing in the presence of high (HMwCH), medium (MMwCH) and low (LMwCH) at various concentrations (expressed in mg/kg of soil dry weight).

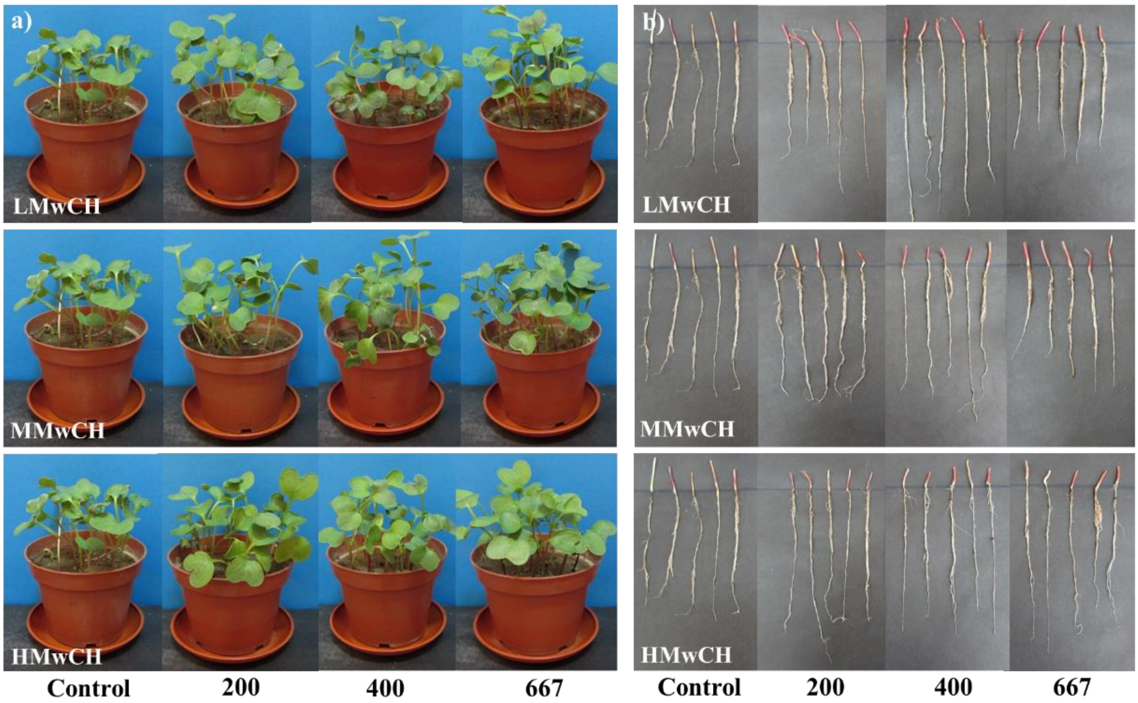


Figure 18S. Photographs of selected radish sprouts (a) and radish roots (b) at the 14th day growing in the presence of high (HMwCH), medium (MMwCH) and low (LMwCH) at various concentrations (expressed in mg/kg of soil dry weight).

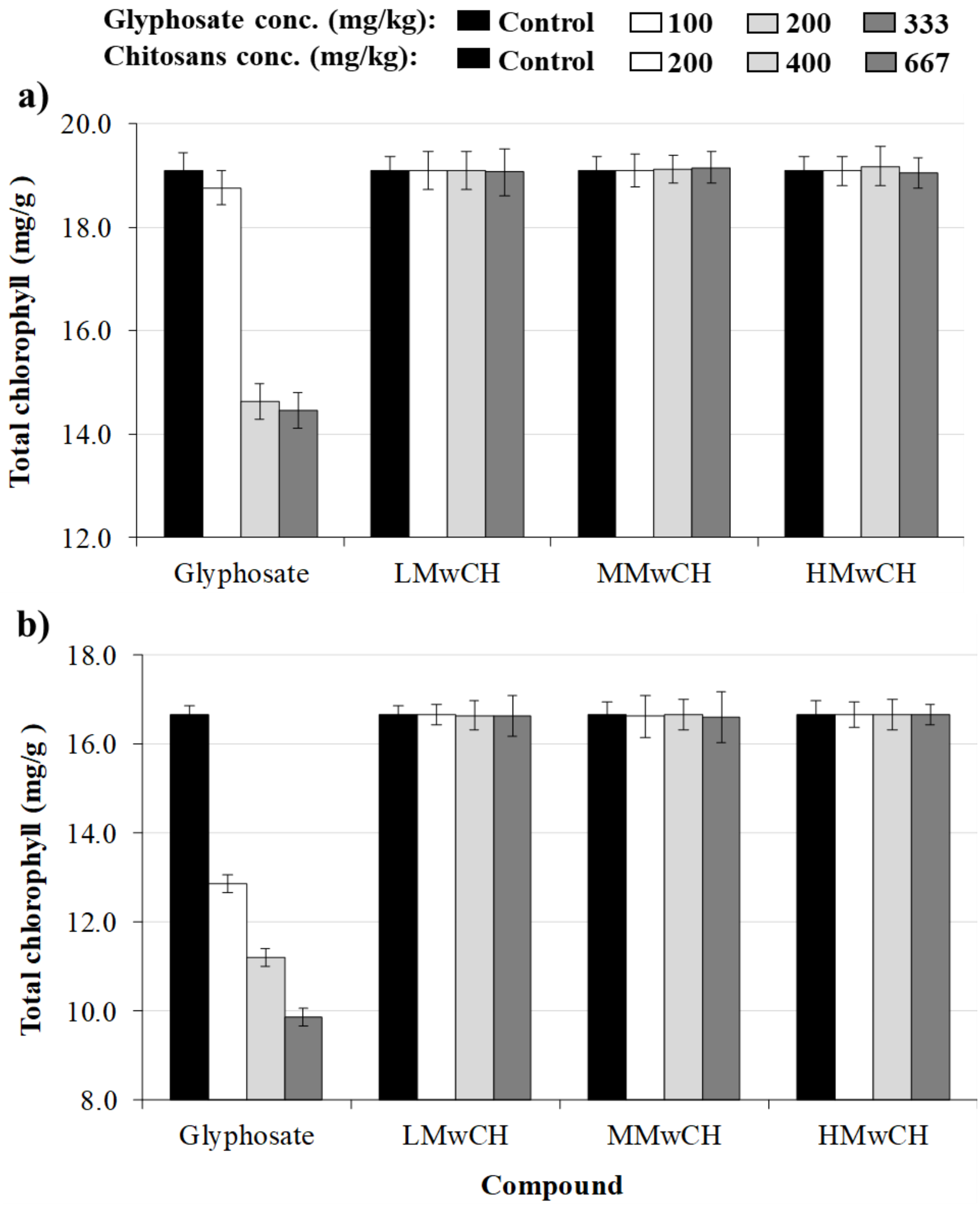


Figure 19S. Effect of the neat low (LMwCH), medium (MMwCH) and high molecular weight chitosan (HMwCH) as well as glyphosate on the content of total chlorophyll (expressed in mg/g of dry weight of plant) of oat seedlings (a) and radish leaves (b). Data are expressed as a mean ± SD (n = 3).

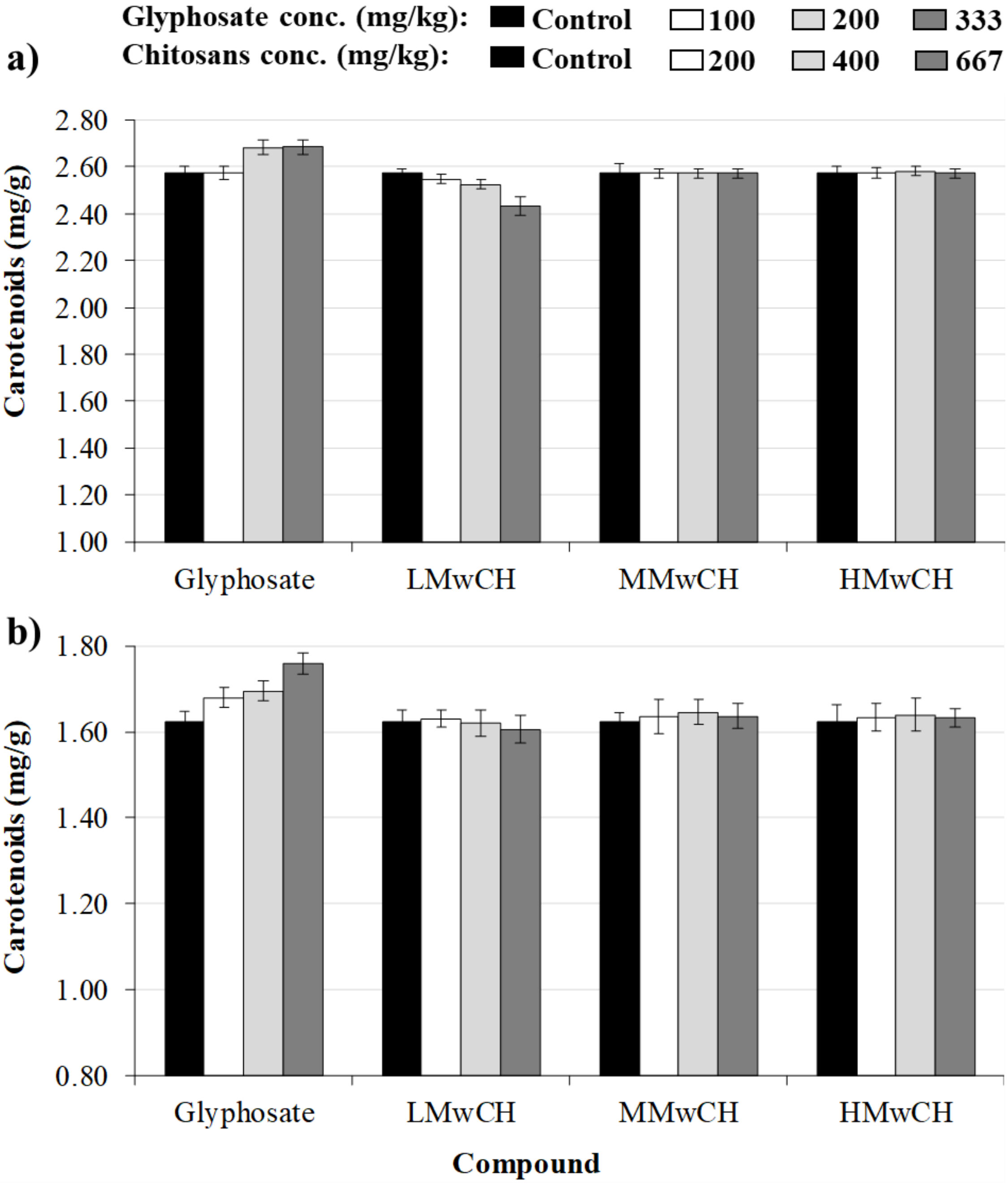


Figure 20S. Effect of the neat low (LMwCH), medium (MMwCH) and low molecular weight chitosan (LMwCH) as well as glyphosate on the content of carotenoids (expressed in mg/g of dry weight of plant) of oat seedlings (a) and radish leaves (b). Data are expressed as a mean ± SD (n = 3).

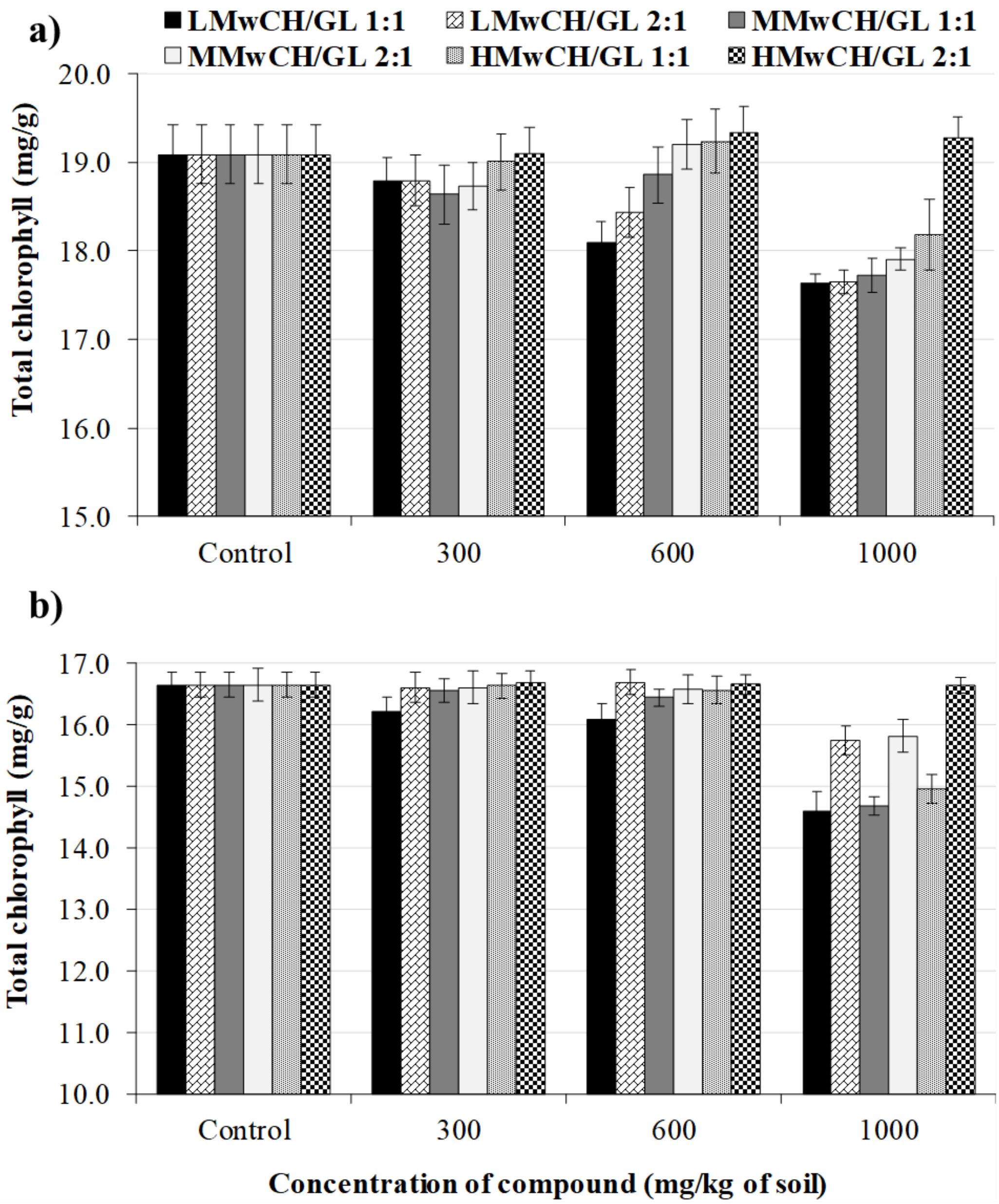


Figure 21S. Effect of the HMwCH/GL, MMwCH/GL and LMwCH/GL formulations on the content of total chlorophyll (expressed in mg/g of dry weight of plant) of oat seedlings (a) and radish leaves (b). Data are expressed as a mean ± SD (n = 3).

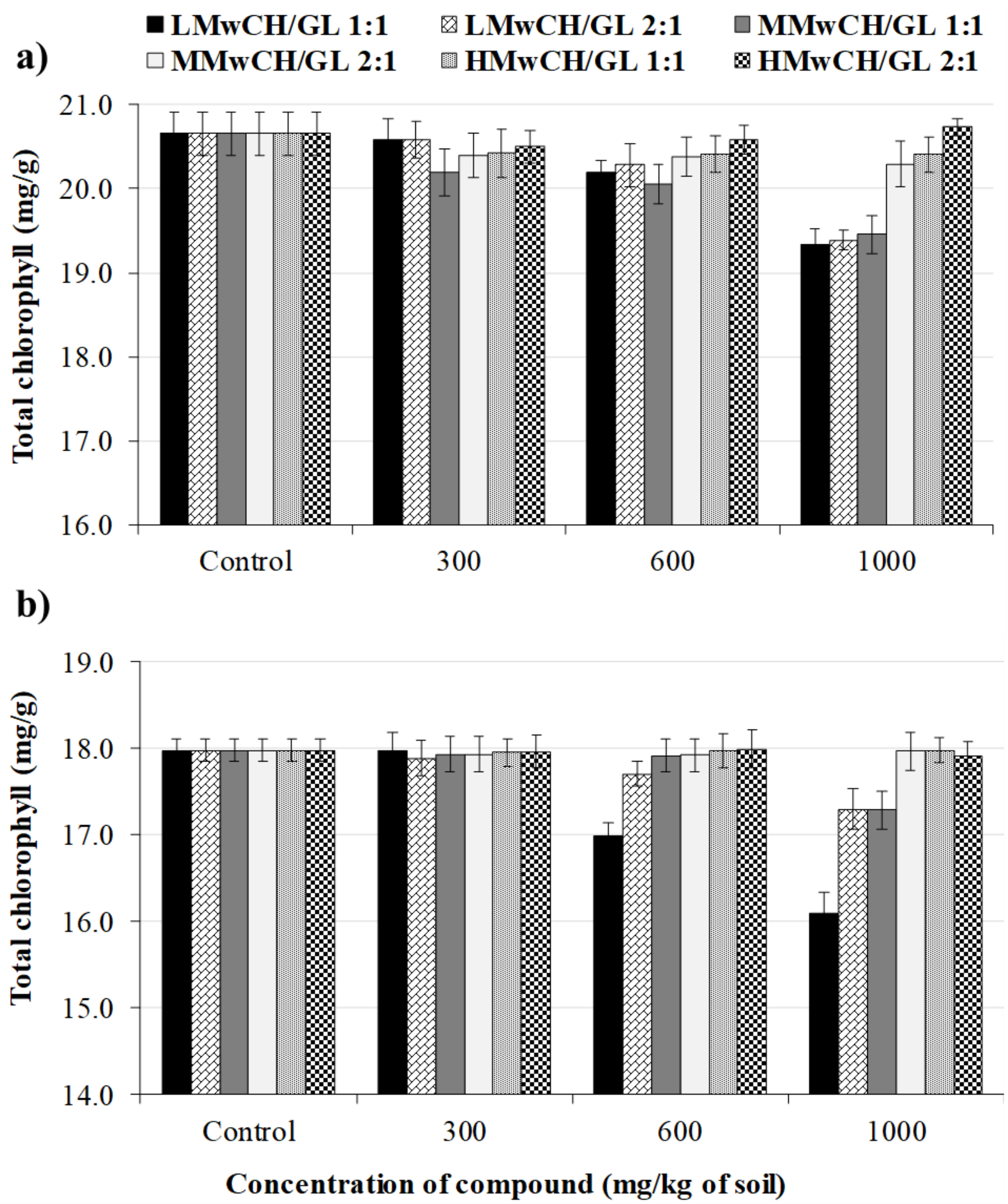


Figure 22S. Effect of the HMwCH/GL, MMwCH/GL and LMwCH/GL formulations on the content of total chlorophyll (expressed in mg/g of dry weight of plant) of oat seedlings (a) and radish leaves (b) after 3 months of their incubation in soil. Data are expressed as a mean ± SD (n = 3).

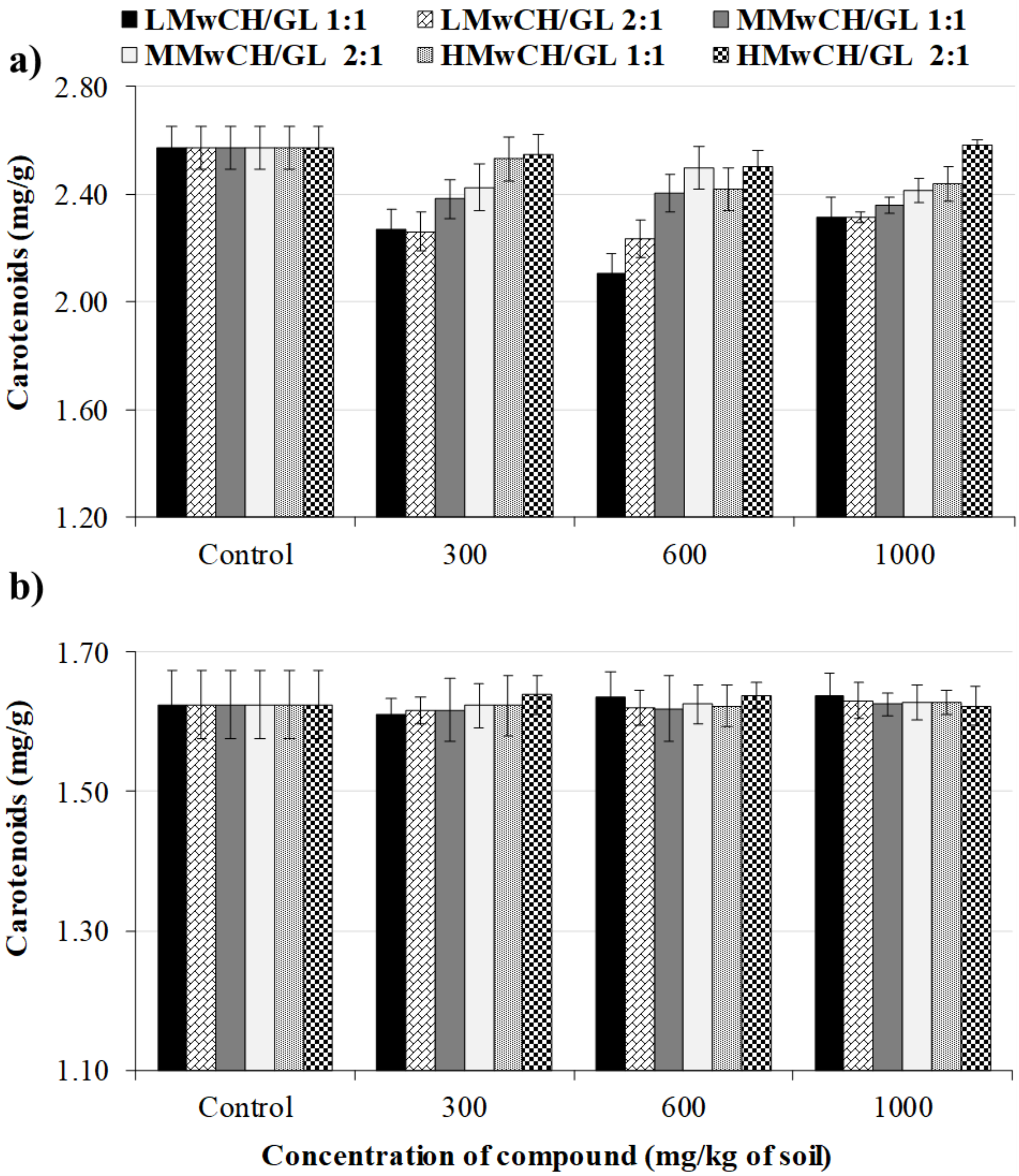


Figure 23S. Effect of the HMwCH/GL, MMwCH/GL and LMwCH/GL formulations on the content of carotenoids (expressed in mg/g of dry weight of plant) of oat seedlings (a) and radish leaves (b). Data are expressed as a mean ± SD (n = 3).

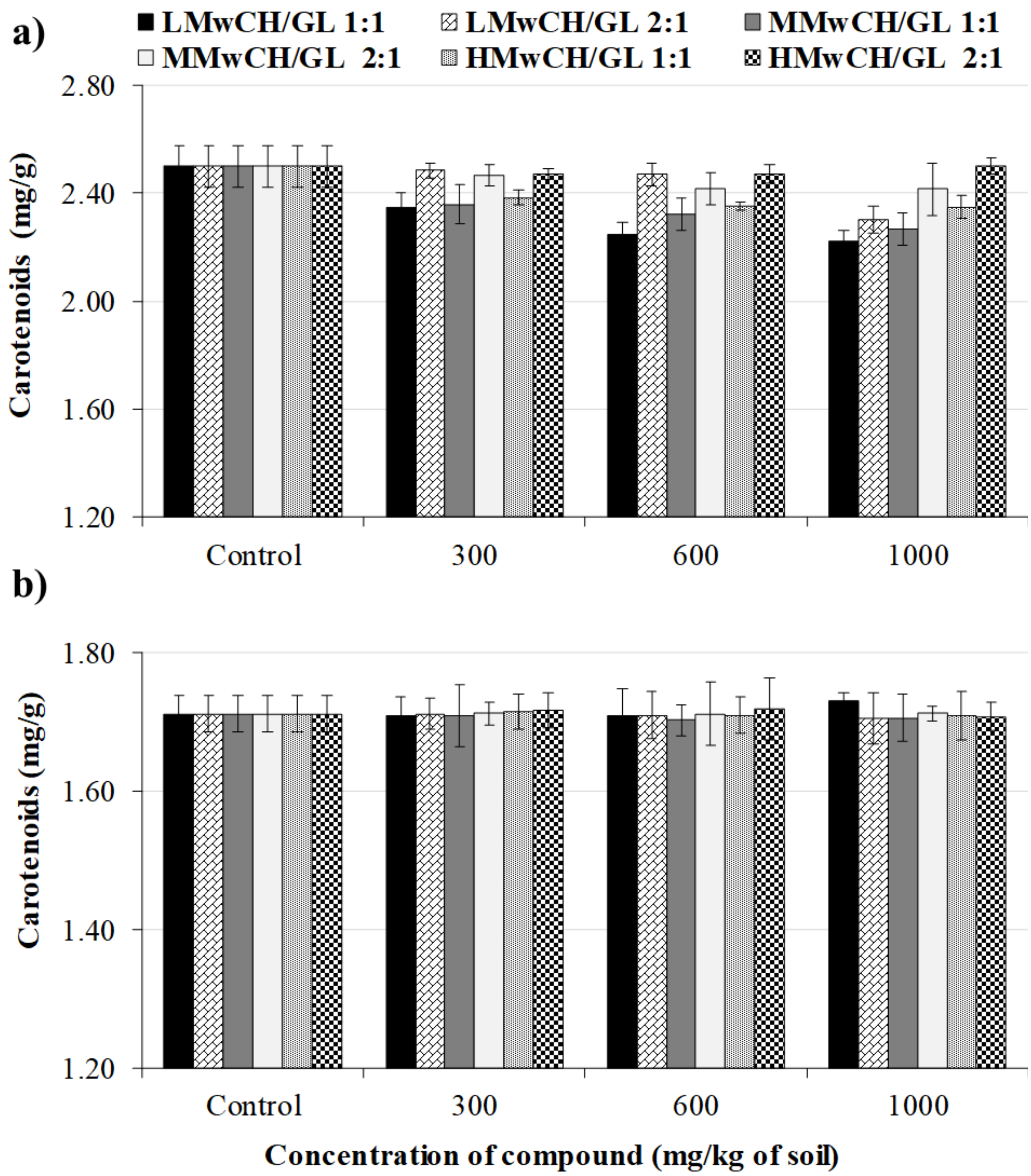
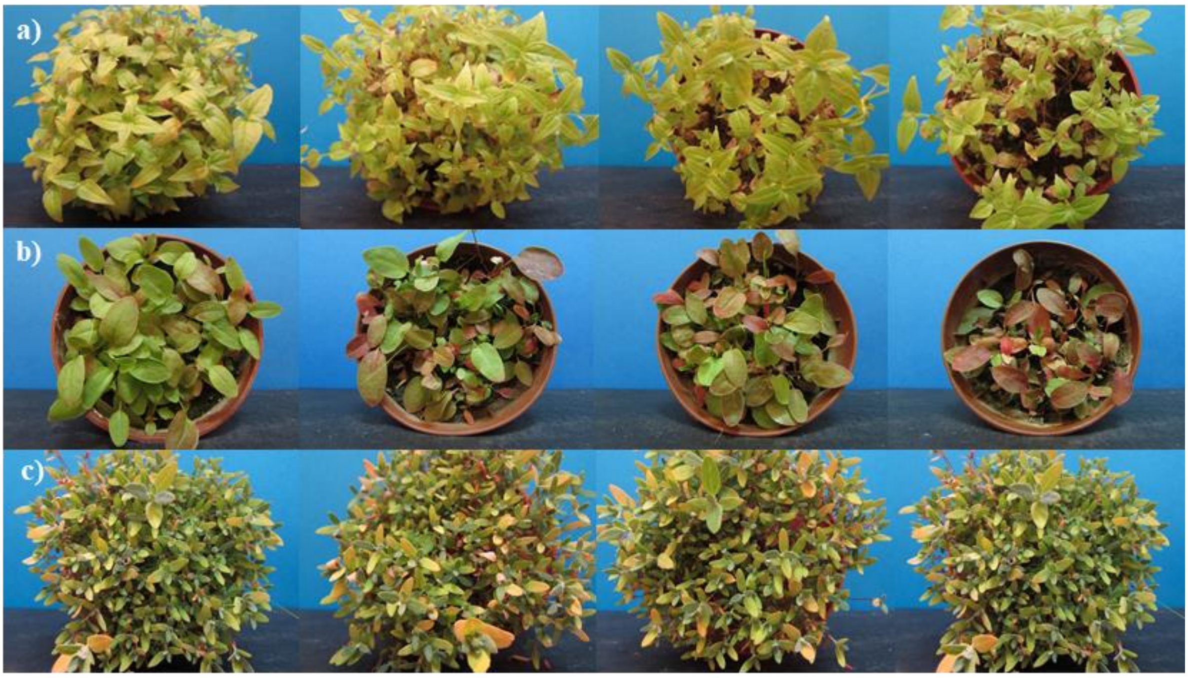


Figure 24S. Effect of the HMwCH/GL, MMwCH/GL and LMwCH/GL formulations on the content of carotenoids (expressed in mg/g of dry weight of plant) of oat seedlings (a) and radish leaves (b) after 3 months of their incubation in soil. Data are expressed as a mean ± SD (n = 3).

Table 4S: Photographs of selected weeds after their spraying with chitosan/glyphosate formulation in ratio 1:1. Phytointoxication was evaluated based on the EWRC rating scale: **1:** total plant death (100%); **2:** excellent (98.0 to 99.9%); **3:** very good (95.0 to 97.9%); **4:** good to acceptable (90 to 94.9%); **5:** moderate (82.0 to 89.9%); **6:** weak (70.0 to 81.9%); **7:** bad (55.0 to 69.9%); **8:** very bad (30 to 54.9%); and **9:** none (0.0 to 29.9%).

Formulation	***Gallant soldier*** **(G. parviflora Cav.)**			***Sorrel*** **(R. acetosa L.)**			**White goosefoot (Ch. album L.)**		
	2.5 L/ha	4.5 L/ha	6.0 L/ha	2.5 L/ha	4.5 L/ha	6.0 L/ha	2.5 L/ha	4.5 L/ha	6.0 L/ha
LMwCH/GL	9	9	8	9	9	8	9	9	9
MMwCH/GL	9	8	7	9	9	8	9	9	9
HMwCH/GL	8	7	6	9	9	8	9	9	9



Control 2.5 l/ha 4.5 l/ha 6.0 l/ha

Figure 25S. Digital photographs of a) gallant soldier *(G. parviflora Cav.)*, b) common sorrel (*R. acetosa L.)*, and c) white goosefoot *(Ch. album L.)* after foliar spraying with LMwCH/GL 1:1 formulation with various concentration, after the 28th day of growth.

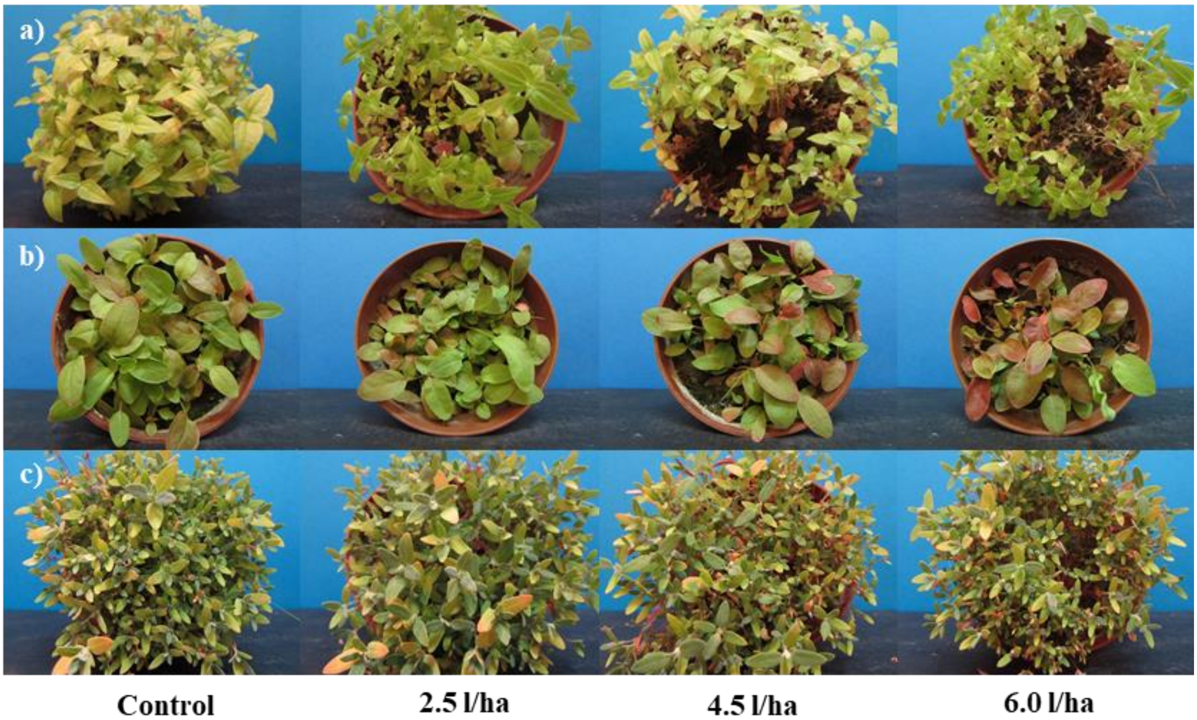


Figure 26S. Digital photographs of a) gallant soldier *(G. parviflora Cav.)*, b) common sorrel (*R. acetosa L.)*, and c) white goosefoot *(Ch. album L.)* after foliar spraying with MMwCH/GL 1:1 formulation with various concentration, after the 28th day of growth.

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