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Original paper

A new approach for phytotoxicity testing using Allium cepa **bulbs**

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Abstract This study examined the phytotoxic effects of two herbicides, glyphosate (Glifotim) and 2,4-D (DMA 6) on onion bulbs (*Allium cepa*). Our aim was the identification of an adequate parameter to estimate phytotoxic effects on *Allium cepa* bulbs directly exposed to herbicides solutions. The experiment was conducted during 2019. The short-term phytotoxic consequences on *A. cepa* were determined after a 4-day exposure to varying concentrations of the herbicides. A gravimetric method was used for biomass (fresh, dried, organic, and mineral) determination. Eleven physiological parameters were calculated. The most sensitive parameter for all analyzed sets was relative growth rate. This parameter could represent a completion and optimization of phytotoxicity assays.

Keywords Phytotoxic effects, relative growth rate, *Allium* test.

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Introduction

The increase in the number of xenobiotic compounds influences the environment, many of the existing chemicals representing toxicological threats to the biosphere. For ethical and economic reasons, but also due to the new laws on animal protection, the number of animal experiments should be reduced as much as possible when testing xenobiotics. The mode of action of the different substances, their long-term implications for human health, are priority scientific objectives (ȘUȚAN et al, 2014). Dramatic expansion of xenobiotic compounds through anthropic activities compromised the environment through the introduction of millions of chemical substances with toxic potential for biological systems (KRISTEN, 1997). In the last decades, numerous comparative studies were realized to evaluate toxicological effects of different herbicides classes and their risks on the environment using different plant species (KIELAK et al, 2011). JURADO et al, (2011), affirmed that when the herbicides are applied in agricultural lands, these can take different destinations due to the fact that these are degraded by microorganisms or can be transported in water far away from the de application place. Thus, organisms can be exposed to a large number of herbicides, but also to their metabolites.

For the purpose of toxicity testing of certain chemicals, higher plants represent systems suitable for a wide range of toxicological tests applicable in estimating risks to the environment and, in some cases, to vertebrates. The roots of young seedlings represent suitable testing methods, not only for the screening of pollutants, but also for the study of the mechanisms of toxic effects. Different tests, based on pollen germination and / or the test based on the growth of pollen tubes, also contributed to the understanding of the toxic action mechanisms of environmental pollutants. Other materials collected from higher plants such as segments of shoots, leaves and green seedlings, are specialized for the detection of the toxicity of chemicals that affect photosynthesis. Although these tests are not candidates for vertebrate / human toxicity testing, they are not completely insignificant in terms of testing chemicals that are toxic to the environment and indirectly to humans (DRAGOEVA et al, 2015).

Roots of cereal seedlings grown in various solutions represent a simple system for estimating the toxicity of metals in soil and water, by determining the root weight. Interactive effects of aluminum, cadmium, manganese, nickel and zinc on root growth of *Triticum aestivum* were analyzed as a model for plant response to metal stress and for the identification of additive, antagonistic, synergistic or multiplier interactions (TAYLOR, 1989). The inhibitory effect of herbicides for the control of *Poaceae* was also tracked by measuring root regeneration after root removal for *Avena* and of seedlings for *Glycine* (FEDTKE, 1987).

Fast-growing plants, such as *Sinapis alba* or *Lactuca*, have been used to test the toxic effects of benzonitrile esters, anesthetics and barbiturates (KORDAN, 1988). In addition to the inhibition of root growth, the ability to produce anthocyanins in roots and the seedling sprouting of maize showed a decrease with increasing lipid solubility due to the effects of barbiturates (KORDAN & RENGEL, 1998).

Occasionally the coleoptiles of cereal seedlings, shoots or shoot segments, leaves from monocotyledonous and dicotyledonous seedlings were used for toxicity screening (JUNG et al, 1986; CUTLER & JARVIS, 1985). In an early approach of phytotoxic effects examination of surfactants, the entire growth of seedlings grown in vitro was estimated by determining the weight of fresh plants after a long incubation time (150-270 days) with different concentrations of the test substance (ERNST et al, 1971). By this test, it was demonstrated that non-ionic surfactants reduce seedlings growth and viability at lower concentrations than ionic surfactants.

Of all the genotoxicity tests used over time, only the *Allium* test, the *Tradescantia* micronucleus test, and the *Arabidopsis* mutagenicity test were evaluated alongside the carcinogenicity tests applied to vertebrates. However, with the exception of the *Arabidopsis* test, the ability of these tests to predict the carcinogenic capacity was relatively low (ENNEVER et al, 1988).

The *Allium* test was introduced by Levan (LEVAN, 1938) to study the effects of colchicine on root growth and was then used as a standard method in the study of chromosomal aberrations (GRANT, 1982). Currently, the *Allium* test combines two objectives: mutagenicity and toxicity (TEDESCO & LAUGHINGHOUSE, 2012). Toxicity is measured by observing root growth inhibition and mutagenicity is correlated with the rate of chromosome breakdown. The sensitivity of the *Allium* test is at the same level as, for example, testing systems that use algae or human lymphocytes. Many tests on various organisms have given similar results, comparable to the results of the *Allium* test, which makes this test a reliable test as a screening test (FISKESJO, 1985).

Any harmful effect has direct or indirect repercussions on root growth inhibitions (FISKESJO, 1995). In order to assess the toxicity of some compounds, standard macroscopic parameters (tumors formation, root or root tip bending and the root length) are proposed. Also, there are other indices which can be used in preliminary assays, required for the establishment of the investigated substances concentrations, like: green leaves growth restriction, turgescence and color change. Root length can be measured in two ways: normally, after the extraction from the test tube, full root length is determined using a ruler and this method gives a value for a root and allows study continuance. A more accurate method is

represented by cutting and measuring all the roots from a bulb and this leads to the end of the experiment.

The herbicides can induce modifications on organisms that are not necessary the target, modifying ecosystem surviving and equilibrium, for both cases, aquatic and terrestrial. In this study we tested the effects of some known herbicides on Allium bulbs. Our aim was the identification of an adequate parameter to estimate phytotoxic effects on *Allium cepa* bulbs directly exposed to herbicides solutions.

Materials and Methods

The study was conducted in April 2019. In our experiment 50 *Allium cepa* bulbs were used, with an average weight of 1.6 grams. These were divided in 5 sets. The bulbs were maintained in tap water for 7 days, to form roots. In the seventh day the treatments were applied, as follows: the first set was treated with distilled water, the second was treated with an herbicide solution of DMA6, 4 mg L^{-1}, the third set with DMA6, 2 mg L^{-1}, the fourth set was treated with Glifotim herbicide 250 mg L^{-1} and the fifth set with Glifotim 500 mg L^{-1}.

The DMA herbicide contains 2,4D dymethylamine salt in a concentration of 660 g/L. It is a systemic herbicide used in agriculture for annual and perennial dicotyledonous weed control in cereals crops (OZKUL et al, 2016).

Glifotim formulation contains glyphosate (glyphosate-isopropylamine salt) in a concentration of 360 g/L. Glifotim is a total, systemic, non-selective, herbicide used in agriculture to combat annual or perennial weeds, monocotyledonous or dicotyledonous (CAREUSAGLU et al, 2011).

For the fresh biomass determination (FB), the bulbs were weighted using an analytical balance (Kern Model) in the first day of the experiment, and then reweighted in the third, the sixth and the eleventh days.

After de completion of plant exposure to treatment, the probes were dried in an oven, Sauter Model, at 100°C, for 2 hours, to obtain plants dry biomass (DB) and water quantity (WQ). Next, the samples were introduced into a calcinator, Nabertherm model, at 500°C, for 2 hours in order to obtain plants mineral content (MC). Organic biomass (OB) was calculated as a difference, ash content being subtracted from dry biomass.

Next, instantaneous relative growth rate R_1 (between April 12 and April 15), R_2 (between April 15 and April 18), R_3 (between April 18 and April 22) and R_4 (between April 12 and April 22) were determined. Instantaneous relative growth rate (relative growth speed, g g^{-1} day^{-1}) can be calculated using next formula (POMMERENING and MUSZTA, 2015):

$$RGR = \frac{\ln(W2)-\ln(W1)}{t2-t1} \quad (1)$$

where W1 and W2 are dry weights at times t1 and t2.

The next physiological parameters were also determined: final growth rate, increase in fresh biomass, fresh biomass/dry biomass ratio, % root biomass/ dry biomass, % minerals/ fresh biomass, % organic biomass from fresh biomass, % water from fresh biomass, organic content/mineral content ratio, tissues minerals deposition (TDM=CM/DB*1000 - in g/kg dry biomass) and tissues density (TD=DB/FB*1000: in g/kg fresh biomass) (IANOVICI, 2016). Succulence was defined as the ratio between water quantity (WQ) and organic biomass (OB) (IANOVICI et al, 2012).

Analysis of variance (Kruskal-Wallis test) was realized using PAST software (HAMMER et al, 2001). P values below 0.05 were considered significant.

Results and Discussions

Descriptive statistical data of the calculated physiological parameters are presented in Table 1 and 2. Shapiro-Wilk testing indicated that the data for gravimetric parameters and for those calculated do not have a normal distribution. Kruskall-Wallis test for medians can be considered a reserve method for ANOVA and is a nonparametric approach for comparing the probes from two or many independent groups.

Instantaneous growth rate for the plants from 2, 3, 4 and 5 sets are affected by the used herbicide concentration. After substances administration, mean values of growth rate (R3) present a significant decrease (Table 1).

Regarding final growth rates (for the entire experimental period) analysis, we noticed that there are not significant differences between the five samples sets.

Regarding the increase in fresh biomass, significant differences were observed between sets 3 and 4 (p=0.04854). Samples from the third set treated with DMA6 (2 mg/L) have the highest biomass increase at the end of the experiment (62.8925%).

For fresh biomass/dry biomass ratio, the highest value was obtained for the fifth experimental set and the lowest for the second set, without any significant difference.

The highest percentage of root from dry biomass was calculated for the first set (1.7422%). There are no significant differences for this parameter.

Regarding organic biomass % and organic content/ mineral content we observed that the highest mean values were obtained for the second set treated with DMA6 (4 mg L^{-1}). These plants presented the lowest water percentage from fresh biomass, but also the lowest value of tissues minerals deposition. Nevertheless, the differences between the experimental values are not significant.

Table 1. Comparative results for calculated growth rates for the five sets of probes

		R_1	R_2	R_3	Kruskal–Wallis test	
					H	p
set 1 Control	Mean	0.039655	0.048697	0.031394	5.128	0.07701
	Std. error	0.007944	0.004942	0.005197		
	Stand. dev	0.025121	0.015629	0.016435		
	Coeff. Var	63.34882	32.09361	52.35129		
set 2	Mean	0.057424	0.047996	0.027908	17.04	**0.0001992**
	Std. error	0.005211	0.002228	0.002594		
	Stand. dev	0.016478	0.007045	0.008202		
	Coeff. var	28.69508	14.67864	29.38824		
set 3	Mean	0.062502	0.053968	0.037895	6.582	**0.03721**
	Std. error	0.008787	0.00467	0.002319		
	Stand. dev	0.024852	0.014011	0.006956		
	Coeff. var	39.76213	25.96145	18.3559		
set 4	Mean	0.056548	0.049955	0.030133	9.529	**0.008525**
	Std. error	0.011633	0.005494	0.003126		
	Stand. dev	0.026012	0.016483	0.009377		
	Coeff. var	46.00003	32.99623	31.12029		
set 5	Mean	0.060785	0.053695	0.025934	16.64	**0.0008374**
	Std. error	0.006695	0.005327	0.00288		
	Stand. dev	0.018935	0.016845	0.009106		
	Coeff. var	31.15149	31.37167	35.11417		

Tabel 2. Comparative results for other physiological parameters calculated for the five sets of probes

Calculated physiological parameters		Set 1 Distilled water	Set 2 DMA6, 4 mg/L	Set 3 DMA6, 2 mg/L	Set 4 Glifotim 250 mg/L	Set 5 Glifotim, 500 mg/L	Kruskal–Wallis test	
							H	p
Final growth rate	Mean	0.0412	0.0422	0.0480	0.0363	0.0457	3.132	0.5359
	Std. error	0.0036	0.0027	0.0043	0.0037	0.0057		
	Stand. dev	0.0108	0.0083	0.0131	0.0098	0.0182		
	Coeff. var	26.3199	19.7588	27.3457	27.0268	39.9588		
Increase in fresh biomass (%)	Mean	48.8006	49.4922	62.8925	41.5796	49.0963	4.078	0.3955
	Std. error	5.6774	5.2572	7.1816	5.4332	6.9837		
	Stand. dev	17.9536	16.6248	21.5449	15.3676	19.7531		
	Coeff. var	36.7897	33.5908	34.2568	36.9595	40.2334		
Fresh biomass/ Dry biomass	Mean	2.8739	2.0918	2.7102	2.754145	2.9702	7.289	0.1214
	Std. error	0.5884	0.0820	0.1946	0.4141	0.3129		
	Stand. dev	1.7652	0.2593	0.6153	1.309584	0.9897		
	Coeff. var	61.4239	12.3983	22.7060	47.5495	33.3230		
Root biomass percentage from dry biomass (%)	Mean	1.7422	1.2623	1.6113	1.3063	1.6827	1.143	0.8875
	Std. error	0.4429	0.2039	0.3122	0.1827	0.3598		
	Stand. dev	1.0849	0.5768	0.8830	0.5168	1.0796		
	Coeff. var	62.2757	45.6980	54.8002	39.5688	64.1622		
Minerals from fresh biomass %)	Mean	0.9037	0.9132	0.8747	1.0376	0.8672	1.024	0.9061
	Std. error	0.1057	0.0994	0.0654	0.1313	0.0792		
	Stand. dev	0.3342	0.3143	0.2070	0.4152	0.2505		
	Coeff. var	36.9895	34.4216	23.6761	40.0209	28.8952		
Organic biomass percentage from fresh biomass (%)	Mean	44.2684	47.6388	37.6643	39.5732	37.6594	8.211	0.0841
	Std. error	5.1577	2.1540	2.5899	2.2331	3.0023		
	Stand. dev	14.5882	6.8116	8.1901	6.3164	9.0069		
	Coeff. var	32.9541	14.2985	21.7450	15.9613	23.9168		
Water percentage from fresh biomass (%)	Mean	60.8715	53.0605	60.2436	59.3768	61.4667	9.935	0.1393
	Std. error	4.9048	1.559188	2.5865	2.3065	3.0667		
	Stand. dev	10.9675	4.6775	7.7596	6.5237	9.2002		
	Coeff. var	18.0174	8.8155	12.8803	10.9870	14.9678		
Organic content/Mineral content	Mean	46.6057	57.8059	44.3986	42.1584	44.6551	3.03	0.5528
	Std. error	6.47568	6.3264	3.2782	4.7831	3.3015		
	Stand. dev	19.4270	20.0060	10.3665	13.5287	9.9045		
	Coeff. var	41.6838	34.6090	23.3488	32.0903	22.1800		
Succulence	Mean	1.756634	1.172007	1.649704	1.564675	1.780778	6.788	0.1475
	Std. error	0.331365	0.069205	0.193696	0.164949	0.24314		
	Stand. dev	0.740954	0.207614	0.581088	0.466547	0.729419		
	Coeff. var	42.18031	17.7144	35.22379	29.81747	40.96069		
TD (g/kg)	Mean	422.9343	485.5203	385.3908	422.3809	366.6116	7.921	0.09452
	Std. error	54.523	21.31954	26.14158	52.61856	33.20931		
	Stand. dev	163.569	67.41829	82.66692	166.3945	105.0171		
	Coeff. var	38.6748	13.88578	21.45015	39.39442	28.64531		
TDM (g/kg)	Mean	24.2771	19.2493	23.2758	26.9074	24.6041	3.641	0.4568
	Std. error	3.1340	2.3898	1.9616	3.7860	2.2885		
	Stand. dev	9.4022	7.5573	6.2032	11.9725	7.2371		
	Coeff. var	38.7289	39.2603	26.6507	44.4951	29.4141		

Phytotoxicity assays require special attention in order to model and optimize the necessities which appear due to the influence of anthropic factors. Phytotoxicity assays represent efficient and cheap alternatives to classical toxicity tests. Research direction from the last years implies the development of some testing systems as part of a test battery in order to obtain a partial or total replacement of the experiments on vertebrates.

Allium test has multiple domains of applicability and through this known substances can be tested (e.g. for the determination of pH range, soluble and insoluble substances in water), but also unknown substances, generally found in tap water, natural wasters or household (FIRBAS & AMON, 2013).

Allium test received much attention after its adaptation in soil and water pollution screening programs, for pollutants like chlorophenoxyacetic acids and chlorophenols (FISKESJO et al, 1981), aluminum (BERGGREN & FISKESJO, 1987), heavy metal salts (LIU et al, 1995), other industrial chemical waste (FISKESJO, 1985) and pesticides (FRANEKIC et al, 1994).

Chemical products like glyphosate and 2,4 D can kill aboveground leaves, but the underground bulbs remain active and will generate new plants. In this study we have analyzed some physiological and gravimetric parameters to evaluate direct exposure effects caused by herbicides solutions on *Allium cepa* roots. The concentrations were chosen based on literature data. In a study, the results showed that clear negative cytogenetic effects for a 4.02 mg L^{-1} 2,4-D applied 48 h on vegetal tissues could lead to unwanted variations that could affect genetic purity of *Allium cepa* (ÖZKUL et al, 2016). Other researches showed that every glyphosate dose leads to severe toxic effects on *A. cepa* cells and the most toxic effect was obtained for 500 mg L^{-1} dose. These effects induce physiological, anatomical, biochemical, cytological and genetical changes on *A. cepa* (ÇAVUŞOĞLU et al, 2011). A significant increase of the glyphosate amount translocated from the root was observed when herbicide total absorption increased. For *Zea* roots, a linear relation between glyphosate concentration and absorption was noticed, in 2-30 mg L^{-1} range (WAGNER et al, 2003).

Our results indicated an adequate parameter for the phytotoxic effects estimation on *A. cepa*: instantaneous relative growth rate. RGR is used on a large scale for the quantification of plant growth speed (HOFFMANN and POORTER, 2002). RGR represents the increase of plant size when compared with the same plant, in a given time interval. We calculated RGR using fresh biomass values of the same plant, in three distinct moments, without a destructive approach.

RGR was used to express the effect of fertilizers, weed control, shadow, soil humidity carbon dioxide, sulphur dioxide and ozone on growth.

Relative growth rate is also used to compare the differences caused by genotype and seedlings size. This technique can be seen as a valuable method when comparing seedlings with different sizes. Relative growth rates examination is a major indicator of productivity plant strategies in a stressing and disturbed environment (KARADAVUT et al, 2010), like in our experiment due to herbicide use. Herbicide concentrations significantly affected the growth rate of *Allium cepa* bulbs. We consider that this parameter can be successfully included in phytotoxicity assays of different xenobiotics using *Allium* test.

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Conclusions

The aim of the present study was to evaluate toxicity induced by glyphosate and 2,4-D in *A. cepa.* Two different doses of both Glifotim (250 and 500 mg L^{-1}) and DMA 6 (2 and 4 mg L^{-1}) were applied. Physiological parameters could be relatively quickly and cheaply evaluated by *Allium* test. Relative growth rate gives very important data about plant growth and is the most important index of productivity. This physiological parameter could represent a completion and optimization of phytotoxicity assays.

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