

Tolerance of common oregano (*Origanum vulgare*) to seven widely used post- and pre-emergence herbicides

ÁDÁM KOCZOR¹, SZILVIA VAJDA¹, PÉTER RYBALTOVSZKI², GÁBOR MARKÓ^{1,3*}

¹*Department of Plant Pathology*

Institute of Plant Protection, Hungarian University of Agriculture and Life Sciences

Ménesi út 44, Budapest, 1117-H Hungary

**(e-mail : marko.gabor3@gmail.com)*

²Genetics and Biotechnology Research Group, Hungarian University of Agriculture and Life Sciences
Páter Károly utca 1, Gödöllő, 2100-H Hungary.

³Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd
University, Pázmány P. stny 1/C, Budapest, 1118-H Hungary.

ABSTRACT

Herbicides are often used to support weed control in herbicide-sensitive medicinal herb productions; however, the herbicide tolerance of these plants is less known. To reduce the chemical hazard risk, a study was conducted in 2018 at University Research Centre, Budapest, Hungary, to explore the tolerance of the cultivated oregano to seven specific and widely used herbicides. In addition, the validity and usability of the applied bioassay procedures were compared for future methodological purposes. In laboratory and greenhouse studies, we applied three different bioassay tests, in which oregano leaves or plants were treated by different herbicides (*i.e.*, glyphosate, MCPA, bromoxynil, oxyfluorfen, phenmedipham-desmedipham, propyzamide, pendimethalin). The tissue damage represented the level of sensitivity analyzed by dose-response curve model fitting. The post-emergence herbicides such as MCPA, bromoxynil and glyphosate imposed a much higher technological risk for the oregano production than the others, such as pendimethalin, phenmedipham-desmedipham and oxyfluorfen, primarily used in pre-emergence technology, while the propyzamide was harmless. Nevertheless, all bioassays were considered to be valid, showing high repeatability, providing consistent tolerance ranks. The pre-emergence weed control could offer more, less harmful herbicide alternatives for the oregano farmers than the post-emergence technology. The applied bioassays could support the labour-intensive toxicological studies where the rapidity, accuracy and genetic control is especially crucial.

Key Words : Bromoxynil, dose-response, glyphosate, medicinal plants, post-emergence herbicides

INTRODUCTION

Cultivation of aromatic and medicinal plants are especially crucial to supply the food industry and pharmacology with standard, high-quality crop. The genus *Origanum* is one of the most distributed aromatic perennial plant groups used for medicine and spice. The oregano species occupied several natural habitats throughout the Mediterranean area, in the Irano-Turanian, in most parts of the Euro-Siberian region and Northern Africa (Marrelli *et al.*, 2018). The increasing economic interest of the oregano plant, for instance, *Origanum vulgare* (L.) (*Lamiaceae*) is driven by the abundance of its unique secondary metabolites (primarily essential oils), bearing antimicrobial and antioxidant properties (de Torre *et al.*, 2020; Simirgiotis *et al.*, 2020). As a result of its beneficial pharmacological properties, the oregano received closer attention recently as a potential alternative to medicines replacing antibiotics or other synthetic medicines in animal husbandry (e.g., Micciche *et al.*, 2019).

The oregano production successfully integrated into the agricultural system as a crop due to the intensive breeding efforts. Domesticated cultivars helped fulfil the high-yield demands and quality standard requirements in essential oil production while keeping protected the wild populations in natural habitats. Oregano farming mainly characterized by the following technological framework (e.g. Marzi, 1996). An oregano cultivation's lifespan is approximately 3-4 years or more if the applied crop management is efficient to limit the intensive growth and maintain essential oil production. However, the harvesting procedure is similar; the shoots cut very close to the ground, while the harvest time depends on the production purpose. The harvest is due twice a year for dry herbs, each starting between bud-formation and flowering stages, while for essential oil extraction, the plants are cut only once a year in full bloom. An aromatic plant enjoys a relatively high chemical defence due to its high yield of essential oils, so these plants are less exposed to plant pathogens and pests. Thus, oregano cultivation demands only a mild pest control level (e.g. Makri, 2002); however, weed control causes difficulties in plant protection.

Weeds often responsible for a significant part of crop loss, so effective weed control is crucial. In oregano or related herbs, induced weed emergence and maintenance sourced mainly for two reasons. There are specific features of crop management (i.e., wide-row spacing, low mechanical disturbances, long-term production at the same site) and the high limitation in herbicide usage. There are some controversial opinions about the balance between applying mechanical and chemical weed control management in herbicide-sensitive herbs. Some studies highly recommended the mechanical dominated (i.e., 3 to 5 times) weed control instead of

herbicides (Hartley, 1993; Marzi, 1996; Skoufogianni *et al.*, 2019). Contrary, other studies suggested less strict recommendations regarding applying chemical weed control, if it is reasonable in terms of economically mostly due to the limited availability of human labour or other financial demands (Karkanis *et al.*, 2018). Non-chemical weed control is much desirable on medicinal herbs for the pharmaceutical and food industry due to their top-quality standards. Therefore, both the herbicide tolerance of medicinal herbs and production security are still the central issues in horticulture research (Jervevani *et al.*, 2020; Karkanis *et al.*, 2018; Mitchell *et al.*, 1995). Performing a chemical weed control could also be challenging, mainly due to the falling number of available and licensed herbicides on the market in the last decade, especially in the European Union (Kudsk and Mathiassen, 2020). Moreover, both the toxicological and empirical knowledge about oregano's herbicide tolerance were highly underrepresented in the literature.

The available experimental studies in medicinal herbs showed highly diverse and herbicide-specific responses. Both the oxadiazon; used in pre-planting technology) and the oxyfluorfen; used in pre- and post-planting technology) showed successful weed control and yield increase in Syrian marjoram (*Origanum syriacum* L.) in Jordan (Karkanis *et al.*, 2018; Qasem and Foy, 2006). In another study, the prometryn and linuron were also efficient; the trifluralin and pendimethalin presented only moderate results, while the lenacil and pyridate showed less satisfactory results against the most broadleaf weeds (Zumelzú *et al.*, 1997). In both oregano and Dalmatian sage (*Salvia officinalis* L.), the pendimethalin treatment slightly reduced the plant vigour, causing a detectable yield loss (Mitchell *et al.*, 1995). Moreover, both pendimethalin and oxyfluorfen are the most frequently used and investigated herbicides in medicinal herb cultures (Karkanis *et al.*, 2018). As a related cultivated herb, peppermint (*Mentha piperita* L.) frequently treated by herbicides, such as bromoxynil and terbacil in Oregon (USA) (Mallory-Smith, 1998), without decreasing the quality or the quantity of the essential oils (Vaverkova *et al.*, 1997). In Dalmatian sage, as another medicinal herb culture, phenmedipham and desmedipham combined treatment was suitable for weed control, while the oxyfluorfen treatment caused visual symptoms showing necrosis and cupping leaves (Jervevani *et al.*, 2020). These herbicides were successfully applied in medicinal plants and could also be promising candidate herbicides in oregano, but the oregano tolerance still has some knowledge gaps.

Therefore, due to the lack of information, a series of detailed toxicological bioassays would require technical support of the cultivated oregano's chemical weed control. In the present

experimental study, we provided the herbicide tolerance of the cultivated oregano using the dose-response analysis approach. This approach is the most relevant and widely used statistical procedure in pharmacology and biology for analyzing nonlinear concentration- and dose-responses (Ritz *et al.*, 2019). The model-fitting function of the dose-response curve (DRC) describes the response of a group of a living organism (e.g., individual, tissue, cells) along a concentration gradient objectively mostly against potential toxic materials within a given period (Gharde *et al.*, 2017; Lutz *et al.*, 2020). The present bioassay series mainly focused on both the most commonly used and the most perspective herbicide with high expectations for potential application in the field. So, the herbicide selection based on the following chemical and technological aspects: 1) spectrum of the target weed species, 2) the action mode of the active ingredients, 3) being harmless for the oregano crop (i.e., without causing phytotoxic effects after unwanted contamination), and 4) previous experiences in the literature to compare the results. We summarised the essential characteristics of tested herbicides in Table 1.

Therefore, the specific aim of this study was twofold. First, we compared the oregano herbicide tolerance using the effective concentration (EC; *i.e.*, a higher value represents a higher tolerance) quantified in three different assay procedures. Second, we also evaluated the performed bioassays for methodological purposes. So, we calculated the repeatability of bioassay measurements to detect the consistency. We also tested whether the single-leaf (damage in leaf tissue) or the whole-plant (change of aboveground green biomass) bioassays could better reflect the herbicides' tolerance response against the herbicides. In general, finding a valid substitute for the whole-plant bioassays would be relevant for those labour-intensive toxicological studies if the control for the genetic and phenotypic variances among individuals is especially required.

MATERIALS AND METHODS

Plant Material and Growing Conditions

In this study, we examined pot-grown oregano (*Origanum vulgare* 'Supreme') plants provided by a local commercial producer (Zöldpont Kft., Albertirsa, Hungary). All plants were in the same phenology with standard-sized biomass (around six months old, 20 cm high) grown individually in a standard plastic pot (diameter: 13 cm, standard growing media) and labelled individually. The plants grown under standard conditions (natural light environment, temperature: 17±3°C; watering: 0.25 L/pot, twice a week; nutrient supply: 8 mL/L Blüh-Fix NPK 4:3:3, Terrasan Hungária Kft., Hungary, once a month) in the institute greenhouse (Hungarian University of Agriculture and Life Sciences, Budapest, Hungary). In all tests, only

the healthy, pest-free plants and leaves participated. We performed the herbicide treatments in a separate room, and we established the treated plants in an isolated part of the same greenhouse growing under the same conditions.

We performed dose-response bioassay series using three different valid procedures to quantify the tolerance level against different herbicides in oregano, designing our experimental setup to fulfil the dose-response analysis's statistical requirements. We followed the experimental design of a similar study in all bioassays with a few modifications due to species-specific differences in the applied model organisms (Massa and Gerhards, 2011)

Single-Leaf Bioassays (SLB)

We designed our single-leaf bioassays (SLB) to reveal the herbicide-specific tolerance over an extended concentration range. The applied setup allowed us to test the dose-responses on a relatively large sample size and create a statistical control for the genotypic variation (i.e., within- and between-individual variances) along with the concentration groups in each herbicide test due to the hierarchical nature of the experimental design. We used the following experimental design in hierarchical order: ‘herbicide’ (7 brands) × ‘plant’ (3 individuals) × ‘concentration’ (7 groups) × ‘repetition’ (3 Petri-dishes) × ‘leaves’ (2 leaves by a plant individual). We treated whole leaves to test the level of tissue damage, similar to the other studies using excised plant materials (Gonzalez-Torralva *et al.*, 2017; Ma *et al.*, 2015). Note that only in MCPA, more detailed data was available to study the dose-effect on the concentration range (13 groups using six individuals) measured for other scientific purposes (not mentioned here).

Before each herbicide test, we selected three oregano plants for harvesting their leaves. A plant presented only one test to avoid the unwanted variance in the stress responses due to leaf removal. Before establishing the leaves into a Petri-dish, we submerged them into the concentration-specific herbicide solution and dried up the wet leaf surface (around 10 min). We placed wet filter paper at the bottom of each Petri-dish and sealed them, maintaining the optimal moisture environment for the leaves. Leaves were marked individually, allowing us to identify them during the repeated measurements. We used preliminary experiments to determine the concentration gradients in each herbicide.

We measured the tissue damage (necrosis) by visual evaluation expressed in percentage in each leaf on 1, 2, 3, and 5 days after the treatments (DAT) with herbicides. Before the statistical analyses, we calculated the means of the within-individual repetitions within the same concentration group in each herbicide.

Whole-Plant Bioassays (WPB)

In the series of whole-plant bioassays (WPB), we examined the response of the whole-plant, measuring the change of the green aboveground biomass after the herbicide treatment according to previous studies (Massa and Gerhards, 2011). We used the following experimental design in hierarchical order: ‘herbicides’ (7 brands) $\times$ ‘concentration’ (7 groups) $\times$ ‘plant’ (3 individuals). We treated oregano plants by hand sprayer, applying homogeneous and full cover and similar substrate volume. In order to avoid chemical cross-contamination, new nozzles in each concentration group have been used. We determined the herbicide-specific concentration gradients based on the results provided by the SLB.

As the leaf and shoot necrosis describe well the sensitiveness, the green aboveground biomass change can be a good proxy. We used digital image analysis software (ImageJ 1.50i) to calculate the green coverage (applied settings; hue: 53-100, saturation: 0-255 brightness: 0-170) and expressed in percentage. We took digital images 1, 2, 3, 5, and 17 DAT.

Parallel Single-Leaf Bioassays (PSLB)

To compare methodological differences in dose-responses, we performed parallel single-leaf bioassays (PSLB) along with the WPB. After the herbicide treatment applied in the WPB (within 30 min), we collected two leaves from each treated plant, labelled and placed them individually into Petri-dishes. So, the dose-responses were gained from identical plants for the WPB and the PSLB methods. In the PSLB, we used the experimental and measuring protocol described earlier in the SLB, and the collected data on the tissue damage was processed as detailed in the WPB.

Statistical Analysis

The statistical procedure contained three main steps due to the reasons listed in the introduction.

First, we tested the potential effect of the concentration on the response variables running generalized mixed effect model (GLMM) in each herbicide, separately. This preselection approach was required to determine the tests with detectable tissue damage in further statistical analyses to avoid the irrelevant model fits. As the response variable had non-normal distribution in the most test, before performing the GLMMs, we transformed the response variable (such as level of the leaf or shoot damage) into a binomial variable based on the median of the given test (0: low response < median, 1: median < high response). In the model, we entered the binarised response variable as the dependent variable, the herbicide concentration and the time presented as covariates, while the plant ID represented the random factor. In the analyses of WPB, we also entered the initial aboveground coverage as a covariate for taking into account the effect of the individual variance in biomass. In these statistical analyses, the models included data from all temporal moments (SLB: 1, 2, 3, and 5 DAT; WPB and PSLB:

1, 2, 3, 5, and 17 DAT). Based on the results, we excluded from the dose-response analyses those herbicide tests, which response variable showing zero variance along the addressed concentration gradient or the concentration unaffected ($p > 0.1$).

Second, we applied specific DRC model-fitting on the response variables (Gharde *et al.*, 2017; Lutz *et al.*, 2020; Ritz *et al.*, 2019). We selected the best function during the DRC model fitting procedure (e.g., log-logistic and Weibull functions with three- and four-parameters, abbreviated as LL.3, LL.4, W2.3, W2.4, respectively) based on the Akaike Information Criteria indexes complemented by visual examinations. We statistically tested the function parameters of the selected DRC models. We calculated the EC estimates based on these model parameters at three representative values, such as 10, 50, and 90%. An EC10, EC50, and EC90 values corresponded to the concentration at which 10%, 50%, and 90% of efficacy were observed, respectively. The EC values allowed us to compare each herbicide treatment's relative tolerance at the exact temporal moment within the same bioassay. We interpreted the results as the higher EC value represents higher relative tolerance and vice versa (Focke *et al.*, 2017). We analyzed the response data measured on the same temporal moments to validate the level of tolerance (SLB: 2 DAT; WPB and PSLB: 2 and 17 DAT). Based on the EC estimates and their standard errors, pairwise comparisons allowed us to compare the herbicides' relative toxicity within the same bioassay (e.g., $EC10^{SLB}_{\text{glyphosate}}$ and $EC10^{SLB}_{\text{bromoxynil}}$) and also to compare the same herbicides provided by different bioassays (e.g., $EC10^{SLB}_{\text{glyphosate}}$ and $EC10^{WPB}_{\text{glyphosate}}$).

A repeatability value reflects the level of consistency of a given measurement series assessing within-observer reliability or the random noise loaded by the measuring procedure if measurements are available more than once for the same object (Hargreaves *et al.*, 2019; Nelson *et al.*, 2019). Therefore, we calculated the repeatability of the tested variables (such as tissue damage, area of green cover) using repeated measurements of the same object in each bioassay separately. The repeatability value was often calculated as a proportion of the between-individual variance relative to the total variance (Nakagawa and Schielzeth, 2010). For extracting the model variances, we used the linear mixed effect model (LMM) in which the focal variable was the response variable, and the sample identifier of the repeated observations represented the random factor. To derivate the 95% confidence interval (CI95) range around the repeatability estimates, we used a parametric bootstrap method applying 1000 iterations in which the 2.5 and 97.5 percentiles represented the lower and upper values of the CI95 range.

All statistical analyses performed in the R environment (version 3.6.2, 12-12-2019; R Development Core Team, 2019), using the 'drc' (Ritz *et al.*, 2019) and 'lme4' packages (Bates

et al., 2015). To test the fitted DRC concentration effect, we used the ‘*noEffect*’ function within the ‘*drc*’ package. This statistical approach provides a significance test for the model comparison between the fitted dose-response model and a linear regression model with a zero slope (i.e., equivalent to no dose effect) (Ritz *et al.*, 2019).

RESULTS AND DISCUSSION

Repeatability

Regarding the methodology, several toxicological and crop tolerance studies apply only a single procedure; however, different simultaneous procedures should provide more sophisticated results. To avoid such limitations, we followed a multi-test framework using novel and traditional bioassay procedures to check the measured responses in terms of consistency and validity. In general, studies using different experimental procedures could also generate variances among bioassays, which are often neglected to be taken into account. Our results showed that all bioassay provided highly repeatable and consistent measurements to support the same rank in herbicide tolerance, despite the bioassays slightly varied in sensitivity and rapidity. Specifically, the visual estimation method to evaluate the level of tissue damage in oregano leaves was highly repeatable ($R=0.987$, $N=25$, CI_{95} : 0.975/0.993). All bioassays showed very high repeatabilities as well. In the SLB, we could estimate the within-plant repeatability at two DAT with herbicides ($R=0.868$, $N=171$, CI_{95} : 0.908/0.937). In the WPB, we calculated the within-individual repeatability based on the repeated measurements at two and 17 DAT ($R=0.843$, $N=312$, CI_{95} : 0.819/0.863). Similarly to the WPB, we also computed the within-individual repeatability in the PSLB ($R=0.925$, $N=280$, CI_{95} : 0.908/0.937).

Therefore, we could conclude that both the herbicide treatment technique and the different herbicide exposition (i.e., spraying or submerging) could generate such systematic differences between bioassays. In line with our results, a submerged tissue was exposed more massively by the active ingredients covering both leaf surfaces than vertical spraying (Gao *et al.*, 2019). Moreover, during spray application in an open-field practice, the evaporation depended on the active ingredients' volatility properties reducing the dose exposition due to the chemical loss (Carlsen *et al.*, 2006; Jerome, 1989). For similar reasons, the test environment's ventilation properties could also generate substantial differences in the efficiency of a volatile active agent and additives, overestimating its performance in a poorly ventilated environment (e.g., Petri-dish, greenhouse) compared to well-ventilated conditions (e.g., open-field) (Jerome, 1989). On the other hand, the differential herbicide tolerance could also be the subject to the herbicide-plant interactions due to their specific differences in action modes and the phenology-dependent herbicide tolerance (Adami *et al.*, 2017). Consistently with our results, a whole-

plant could better tolerate the herbicide exposition than a single leaf because of the relatively lower dosage exposition and the higher ability to neutralize specific herbicide agents. Consequently, an adult oregano was less sensitive to herbicides designed to inhibit germination and seedling emergence than post-emergence herbicides to control weeds. These examples drew the attention that testing plants in different phenological statuses, although oregano produced slight differences on an absolute scale in herbicide tolerance, the relative tolerance order was consistent and unchanged.

Herbicide Tolerance

The present dose-response bioassay study provides an insight into the tolerance in cultivated oregano against seven widely used herbicides, detecting high production risk among post-emergence herbicides but the relatively low risk in herbicides against emerging weeds. Generally, our results reinforced previous studies related to the herbicides against the emerging weeds (such as oxyfluorfen, propyzamide, and pendimethalin) caused no or only negligible tissue lesions and necrosis. These mild symptoms were only provoked by a very high concentration, which was much higher than the maximum limit permitted to apply in practice. Although previous studies showed broadly similar crop tolerances to herbicide treatments for emerging weeds, the results slightly varied due to the different species or site-specific characteristics.

The tested herbicides revealed a considerable difference in their dose responses based on the applied dose-response bioassays' results due to the variance in the action modes and oregano tolerance. First, to separate the valid dose-response from the harmless ones, we included only the tests with detectable (or marginally insignificant) concentration effects in the further DRC analyses (see *Table 2*). In this preselection process, we found a significant concentration sensitivity in MCPA and glyphosate in all bioassays. Bromoxynil and oxyfluorfen showed a strong concentration-dependent effect in both the SLB and the WPB but not in the PSLB. The phenmedipham-desmedipham caused a strong dose-response effect in SLB only, similarly as pendimethalin. In all bioassays, the propyzamide treatments were ineffective for the oregano. We considered all herbicide tests to analyze further by DRC analyses that their p-values were lower than 0.1. Second, we could fit the DRC successfully at the herbicide tests that passed our preselection criteria. The applied herbicide-specific concentration ranges covered a substantial part of dose responses (i.e., including both the maximum and the minimum), serving an adequate fit of the statistical model. Due to the considerable diversity in herbicides' dose-response patterns, we paid particular attention to find the adequate curve type and check the regression parameters for each test. Finally, all model

adequately fitted the response variables for all the curves tested (lack-of-fit tests: $p > 0.06$), except for an MCPA test in the WPB (only at 17 DAT) due to a single outlier value (after exclusion: $p > 0.17$).

We found strong and herbicide-specific concentration-dependent responses in all bioassays ($p < 0.01$) (see Fig. 1, 2, 3, Table S1). The herbicide tolerance showed considerable differences in the temporal scale, both between- and within-herbicides. For instance, bromoxynil showed only nearly significant effects in both the SLB ($p = 0.054$) and the WPB ($p = 0.073$), measuring at the two DAT, but not at 17 DAT (WPB and SLB: $p < 0.001$).

We emphasized the specific differences with previous studies, focussing primarily on oregano cultivation or other medicinal plants if an underlying study was unavailable. Similarly, the pre-emergence application of oxyfluorfen was interpreted as a useful tool over a wide range of weeds and persisted for an extended period in crops *Lamiaceae*, such as in oregano (Vouzounis *et al.*, 2003) and Syrian marjoram (Qasem and Foy, 2006). Contrary to our results, other studies showed that oxyfluorfen could cause necrosis and cupping leaves by membrane peroxidation within the permitted dose range (Jervevani *et al.*, 2020). The pendimethalin also showed a satisfying pre-emergence control over gramineous with other broadleaf species, including the oregano-related species *Lamium amplexicaule*, without reducing plant number or yield (Zumelzú *et al.*, 1997). Both the pendimethalin and the propyzamide were considered a suitable herbicide applied in pre-planting treatment due to the oregano's high tolerance; however, their control efficiency was only moderately and mildly satisfying, respectively (Hartley, 1993). Despite the weak weed control, propyzamide is often used in small-scale agriculture, mainly producing perennial crops with less developed weed control technology (Hartley, 1993). Although these herbicides did not generate necrotic or other direct symptoms in our tests, we highlighted that these pre-emergence applications could decrease plant vigour and yield loss depending on the degree of herbicide tolerance (Mitchell *et al.*, 1995). In concordance with our results, other studies found these herbicides helpful, providing an efficient weed control coupled with high herbicide tolerance not only in oregano but also in other medicinal herbs.

Comparisons: Bioassay and Herbicide

Based on the EC estimates' statistical comparisons (i.e., EC10, EC50 and EC90), we compared the degree of oregano tolerance of the tested herbicides (see Fig. 4, 5, 6). The tolerance ranks against the herbicides were consistent along with the bioassays independently on the damage evaluation time. In all bioassays, the MCPA, the bromoxynil, and the glyphosate showed the most robust concentration effects. These herbicides are considered the most

harmful herbicides than the other ones, such as phenmedipham-desmedipham, oxyfluorfen, and pendimethalin, showing reduced or minor damage.

Bioassays provided more complex differences among herbicides based on the leaf tests (especially SLB) than using pot plants in terms of sensitivity.

Moreover, we compared the relevant EC evaluates based on different bioassays provided by identical herbicide tests. A complete methodical comparison was available only in MCPA, bromoxynil, and glyphosate representing detectable dose-responses in all bioassays (for contrasts, see *Fig. 4, 5, 6*). Overall, we found consistent concentration-responses between bioassays suggested by the undetectable significant differences between EC estimates. Here, we detailed all the exceptions that appeared. In MCPA, we found the following significant differences at two DAT comparing SLB and PSLB (EC50: Estimate difference ($\pm$ SD): -1.85 ($\pm$ 0.22), CI₉₅: 2.293/1.409, $p < 0.001$), while in the comparison of WPB and PSLB measured at 17 DAT, all EC estimates were also significant (EC10: Estimate difference ($\pm$ SD): -0.99 ($\pm$ 0.39), CI₉₅: -1.766/-0.231, $p < 0.001$; EC50: Estimate difference ($\pm$ SD): -1.61 ($\pm$ 0.27), CI₉₅: -2.158/-1.068, $p < 0.001$; EC90: Estimate difference ($\pm$ SD): -2.13 ($\pm$ 0.49), CI₉₅: -3.093/-1.068, $p < 0.001$). Glyphosate showed a detectable difference only in the comparison of WPB and PSLB measured at 17 DAT (EC10: Estimate difference ($\pm$ SD): -4.56 ($\pm$ 1.62), CI₉₅: -7.761/-1.372, $p < 0.001$).

In our study, most post-emergence herbicides caused a severe tissue lesion and necrosis even at low concentrations, although their efficiency was slightly varied. Expectedly, glyphosate caused tissue necrosis due to its well-known toxic properties, despite its extensive worldwide usage, including herbal cultivation. Moreover, some studies considered glyphosate an appropriate chemical tool to control weeds over winter weeds before plantation (Shiwakoti *et al.*, 2016) or crop growth (Sotiropoulou and Karamanos, 2010) but also avoiding the post-emergence or post-planted applications. Therefore, production risks might be kept low by glyphosate applications considering both the appropriate timing and the crop phenology.

Considering the practical aspects, we suggested a differential approach in weed control depending on the plantation age. Generally, a plantation can be more sensitive to any herbicide, including the sprouting inhibitors in the first year than an older one. So, these herbicides could be perspective against annual weeds using them in pre-emergence technology. In oregano production, the post-emergence herbicides should require high awareness, specific limitations or total avoidance due to the low herbicide tolerance. Despite these herbicides' hazards, specific control managements might reduce the unwanted crop loss using spatially and temporally selective applications. For instance, a perspective solution can be the non-spraying

technologies such as using the wiper-bar applicator (Harrington and Ghanizadeh, 2017) and spraying the plantation within specific temporal windows such as post-harvest or dormant stages. Due to some herbicides' diverse toxicological performance (e.g., MCPA, bromoxynil) and less clear species-specific sensitivity or tolerance features for closely related *Lamiaceae* species includes several aromatic herbs and weeds, wide-scale comprehensive toxicological surveys would be required to fill the knowledge gaps.

CONCLUSIONS

This study evaluated the herbicide tolerance of seven potential herbicides unknown or less studied in oregano to reveal the potential safety risk for production. The tested post-emergence herbicides could impose a much higher toxicological risk for the oregano than the pre-emergence herbicides. Thus, pre-emergence herbicides suggested safer chemical weed control in oregano farming after considering the general facts and potential risks in chemical hazards. Despite the relatively higher tolerance of the oregano against some specific herbicides, mechanical and agrotechnical weed control should be dominated in oregano and other herbicide-sensitive medicinal herb plants. Moreover, emphasizing the methodological aspects of the study, the applied leaf-bioassays provided exceptionally rapid, sensitive, and highly repeatable dose-dependent responses, so we highly recommend their use for labour-intensive toxicological studies where accuracy and genetic control is crucial.

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Table 1. Short description of the herbicides used in the experiments, including weed management and relevancies.

Substance (Chemical group†) Brand	Active ingredient [^a g/L, ^b g/kg] (Water amount ranges [L])	Authorized dose ranges [^c L/ha, ^d kg/ha] (in [%])	Weed control technology	Target weed group	Relevance, reference
Glyphosate (Glycines ^A) Total®	360 ^a (50 / 300)	0.5 / 6 ^c (0.2 / 10)	post-emergence	non-selective	Used worldwide; reference herbicide for comparisons
MCPA (Phenoxy carboxylates ^B) Mecomorn 750 SL®	750 ^a (200 / 400)	0.4 / 1.4 ^c (0.133 / 0.5)	post-emergence	broadleaf	Ineffective against genus <i>Lamium</i> ; Short withdrawal period for human consumption; permitted in aromatic and medicinal herbs in EU
Bromoxynil (Nitriles ^C) Emblem®	200 ^b (200 / 400)	1 / 1.5 ^d (0.25 / 0.75)	post-emergence	annual broadleaf	No information in oregano; commonly used in peppermint (<i>Mentha piperita</i> L.) in (USA) (Mallory-Smith, 1998)
Phenmedipham, Desmedipham, (Phenylcarbamates ^D) Beetup Compact SC®	80 + 80 ^a (80 / 200)	1.5 ^c (0.6 / 1.875)	post-emergence	annual broadleaf	Phenmedipham is recommended in oregano (Italy, EU) (Marzi, 1996), and other herbicide-sensitive crops (Gouda, 2019)
Oxyfluorfen (Diphenyl ethers ^E) Goal Duplo®	480 ^a (200 / 300)	0.4 / 0.5 ^c (0.13 / 0.25)	pre-emergence, post-emergence	annual broadleaf	Previous studies in oregano and other related herbs (Qasem and Foy, 2006; Vouzounis <i>et al.</i> , 2003)
Propyzamide (Benzonitrile amides ^F) Kerb 50 WP®	500 ^b (227.2 / 568.25)	1.125 / 2,275 ^d (0.198 / 1.001)	pre-emergence, early post-emergence	annual and perennial grass, annual broadleaf	Previous studies in oregano and other related herbs (Hartley, 1993)
Pendimethalin (Dinitroanilines ^F) Stomp 400 SC®	400 ^a (200 / 300)	3 / 3.5 ^c (1.167 / 2)	pre-emergence, post-emergence	annual grass, certain broadleaf	Previous studies in oregano (Zumelzú <i>et al.</i> , 1997); permitted in aromatic and medicinal herbs in EU

†Capital letters labelled the Mode of Action Classification provided by HRAC and WSSA : (Heap, 2020); ^A Inhibition of Enolpyruvyl Shikimate Phosphate Synthase (EPSP); ^B Auxin mimics, ^C Inhibition of photosynthesis (Histidine 215); ^D Inhibition of photosynthesis (PSII – Serine 264); ^E Inhibition of Protoporphyrinogen Oxidase (PPO); ^F Inhibition of microtubule assembly.

Table 2. Effects of the applied concentrations on the response variables using generalized mixed effect model (GLMM-b) in each herbicide treatment by bioassays.

Bioassay and Herbicide	Estimate (SE)	z-value	p-value	DRC analyses ^a
Single-leaf bioassay (SLB)				
MCPA	0.313 (0.062)	5.01	<0.001	Yes
Glyphosate	0.203 (0.056)	3.66	<0.001	Yes
Bromoxynil	177.204 (7.392)	23.97	<0.001	Yes
Oxyfluorfen	0.147 (0.047)	3.11	0.002	Yes
Phenmedipham-desmedipham	0.115 (0.028)	4.14	<0.001	Yes
Pendimethalin	226.3 (2.203)	102.74	<0.001	Yes
Propyzamide	NA			No
Whole-plant bioassay (WPB)				
MCPA	1.794 (0.586)	3.06	0.002	Yes
Glyphosate	3.387 (0.592)	5.72	<0.001	Yes
Bromoxynil	3.574 (0.002)	1846	<0.001	Yes
Oxyfluorfen	0.059 (0.018)	3.34	<0.001	Yes
Phenmedipham-desmedipham	0.737 (0.673)	1.1	0.273	No
Pendimethalin	0.062 (0.217)	0.29	0.775	No
Propyzamide	0.001 (0.401)	0	0.999	No
Parallel single-leaf bioassay (PSLB)				
MCPA	3.506 (1.443)	2.43	0.015	Yes
Glyphosate	0.114 (0.03)	3.74	<0.001	Yes
Bromoxynil	0.73 (0.41)	1.78	0.075	Yes
Oxyfluorfen	-0.002 (0.007)	-0.29	0.776	No
Phenmedipham-desmedipham	0.036 (0.023)	1.55	0.121	No
Pendimethalin	-0.044 (0.029)	-1.51	0.131	No
Propyzamide	-0.053 (0.05)	-1.05	0.292	No

^a This column contains the decisions for performing further statistical analyses by DRC.

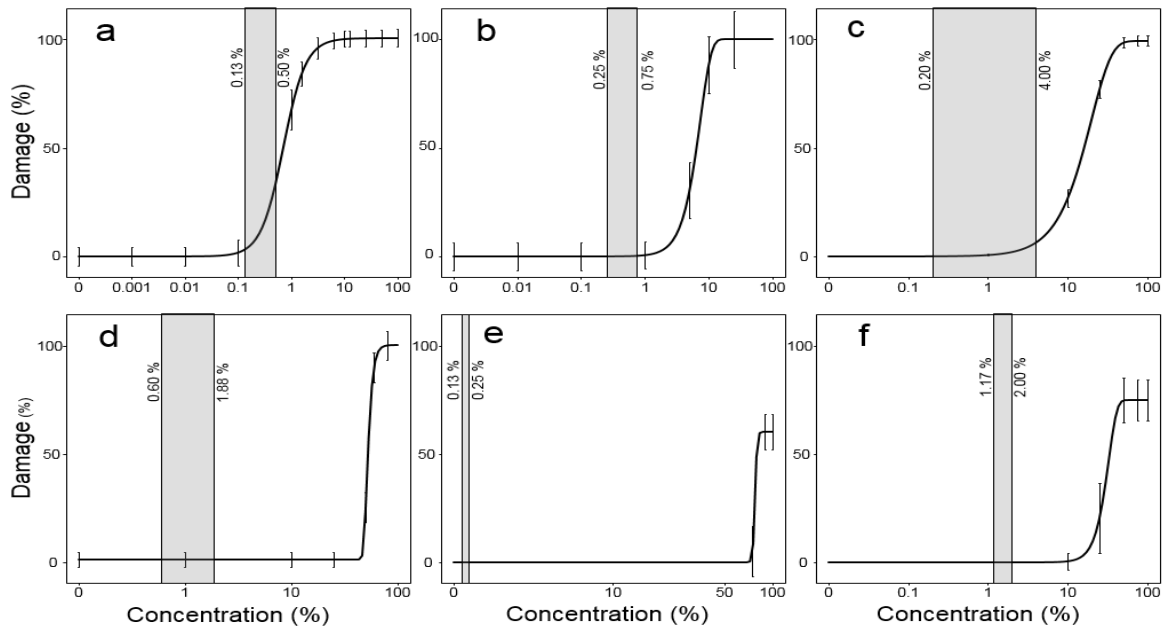


Fig. 1. Herbicide tests in the single leaf bioassay (SLB). The tissue damage of oregano leaves was evaluated at two days after treatment (DAT) with herbicides. The dose-response regression curves (with standard errors) calculated by DRC analyses in each tested herbicides, separately. The shaded area represented the authorized ranges of herbicide rates for field use. The herbicide-specific dose-responses are labelled by different letters: a) MCPA, b) bromoxynil, c) glyphosate, d) phenmedipham-desmedipham, e) oxyfluorfen, and f) pendimethalin. The values of the model parameter were recorded in supporting information Table S1.

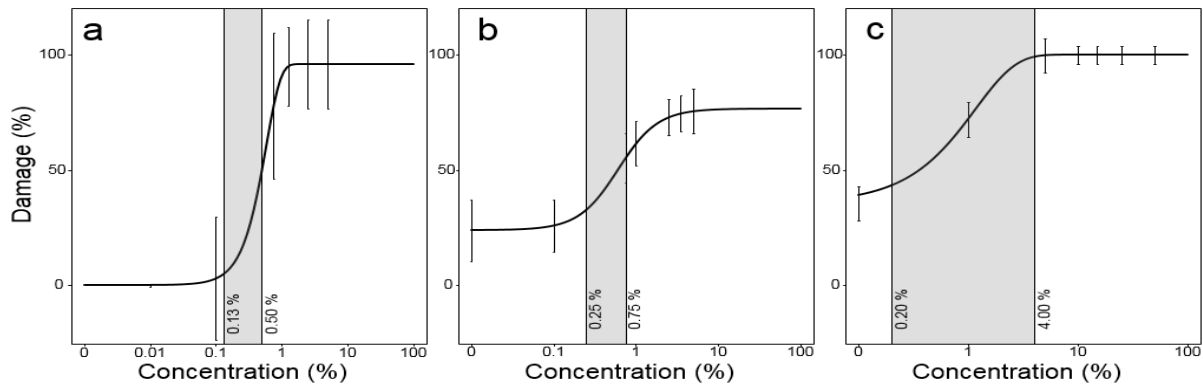


Fig. 2. Herbicide tests in the whole-plant bioassay (WPB). The reduction of the aboveground green biomass was evaluated at 17 days after treatment (DAT) with herbicides. The dose-response regression curves (with standard errors) calculated by DRC analyses in each tested herbicides, separately. The shaded area represented the authorized ranges of herbicide rates for field use. The herbicide-specific dose-responses are labelled by different letters: a) MCPA, b) bromoxynil, and c) glyphosate. The values of the model parameter were recorded in supporting information Table S1.

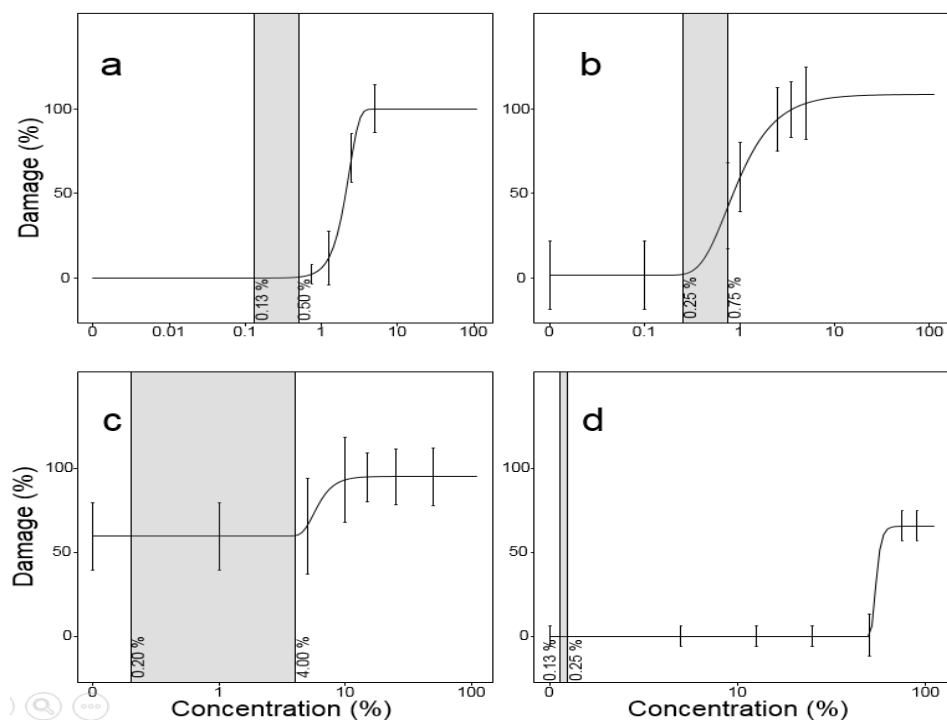


Fig. 3. Herbicide tests in the parallel single leaf bioassay (PSLB). The tissue damage of oregano leaves was evaluated at 17 days after treatment (DAT) with herbicides. The dose-response regression curves (with standard errors) calculated by DRC analyses in each tested herbicides, separately. The shaded area represented the authorized ranges of herbicide rates for field use. The herbicide-specific dose-responses are labelled by different letters: a) MCPA, b) bromoxynil, c) glyphosate, and d) oxyfluorfen. The values of the model parameter were recorded in supporting information Table S1.

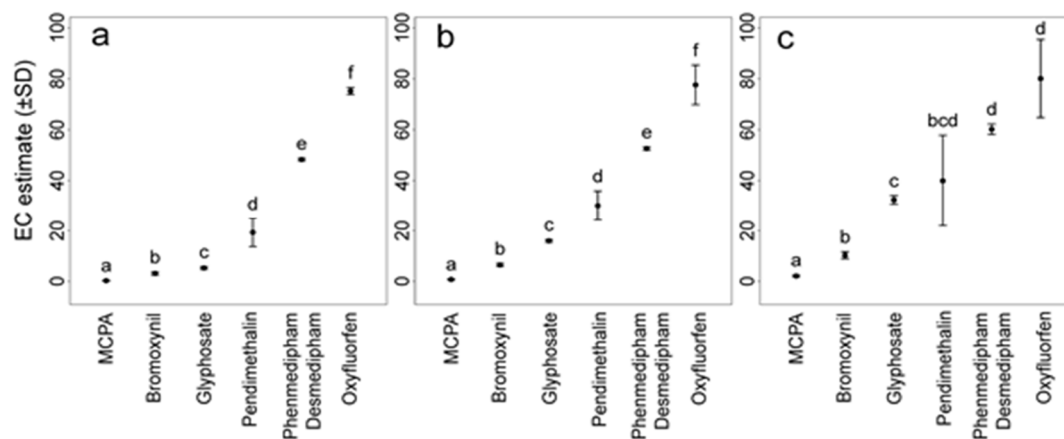


Fig. 4. The herbicide-specific EC estimates revealed from the single leaf test bioassay (SLB) at two days after treatment (DAT). The points with error bars represent the effective concentration (EC) estimates with standard deviation, which were calculated from the fitted statistical models: a) EC10, b) EC50, and c) EC90. The letters above the points indicate homogenous subgroups.

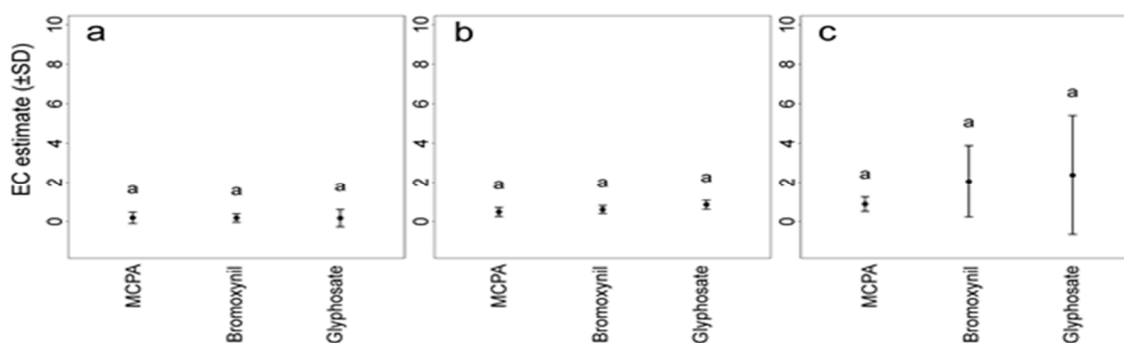


Fig. 5. The herbicide-specific EC estimates revealed from the whole-plant bioassay (WPB) at 17 days after treatment (DAT). The points with error bars represent the effective concentration (EC) estimates with standard deviation, which were calculated from the fitted statistical models: a) EC10, b) EC50, and c) EC90.

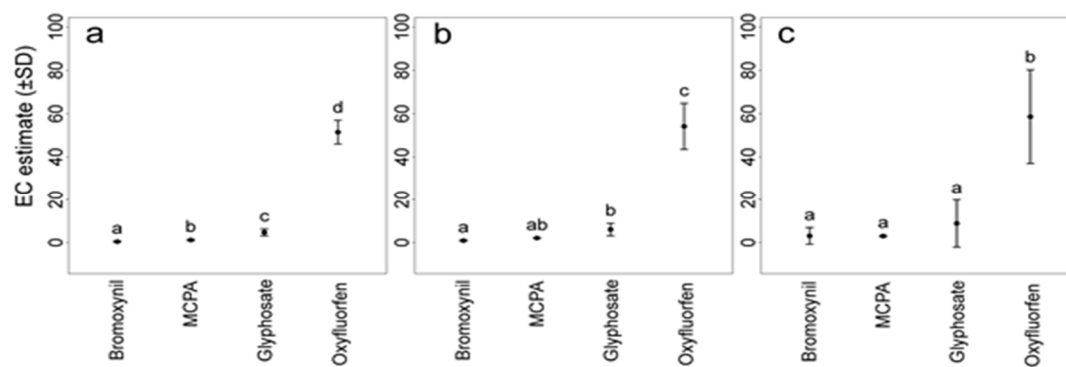


Fig. 6. The herbicide-specific EC estimates revealed from the parallel single leaf bioassay (PSLB) at 17 days after treatment (DAT). The points with error bars represent the effective concentration (EC) estimates with standard deviation, which were calculated from the fitted statistical models: a) EC10, b) EC50, and c) EC90. The letters above the points indicate homogenous subgroups.

Table S1 (Supplementary). Regression parameters and herbicide treatments provided by different bioassays based on different measuring days for estimating the damage in oregano.

Bioassay and Herbicide (DAT ^a , fitted model ^b)		Parameter	Estimate (SE)	t value	P value
Single-leaf bioassay (SLB)					
MCPA (2, LL.4)	Slope	-2.05 (0.64)	-3.2	0.003	
	Minimum	-0.08 (2.19)	-0.04	0.97	
	Upper Limit	100.71 (2)	50.27	<0.001	
	EC50	0.71 (0.13)	5.33	<0.001	
Bromoxynil (2, W2.4)	Slope	2.57 (0.55)	4.64	<0.001	
	Minimum	-0.11 (3.13)	-0.04	0.972	
	Upper Limit	99.99 (6.18)	16.19	<0.001	
	EC50	7.43 (0.69)	10.75	<0.001	
Glyphosate (2, W2.3)	Slope	1.7 (0.11)	14.9	<0.001	
	Upper Limit	99.53 (1.21)	82.6	<0.001	
	EC50	19.79 (0.66)	30.18	<0.001	
Pendimethalin (2, W2.3)	Slope	4.24 (4.14)	1.02	0.317	
	Upper Limit	74.96 (4.56)	16.45	<0.001	
	EC50	32.78 (8.72)	3.76	0.001	
Phenmedipham-desmedipham (2, W1.4)	Slope	-14 (2.51)	-5.59	<0.001	
	Minimum	1.15 (1.6)	0.72	0.483	
	Upper Limit	100.19 (3.36)	29.79	<0.001	
	EC50	51.24 (0.43)	119.35	<0.001	
Oxyfluorfen (2, LL.3)	Slope	-69.13 (200.06)	-0.35	0.734	
	Upper Limit	60.22 (3.94)	15.28	<0.001	
	EC50	77.62 (7.76)	10	<0.001	
Whole-plant bioassay (WPB)					
Bromoxynil (2, W2.3)	Slope	25.93 (84.04)	0.31	0.761	
	Upper Limit	8.55 (3.67)	2.33	0.032	
	EC50	0.96 (0.19)	5.09	<0.001	
	MCPA (2, LL.4)	Slope	-15.34 (52.17)	-0.29	0.772
Minimum		2.26 (3.69)	0.61	0.549	
Upper Limit		38.25 (8.25)	4.64	<0.001	
EC50		2.26 (0.81)	2.81	0.012	
Glyphosate (2, W2.3)	Slope	1.06 (0.35)	3.06	0.007	
	Upper Limit	51.38 (8.42)	6.1	<0.001	
	EC50	13.92 (5.42)	2.57	0.019	
	MCPA (17, W2.3)	Slope	1.99 (2.36)	0.85	0.409
Upper Limit		96.14 (9.17)	10.49	<0.001	
EC50		0.58 (0.19)	3.09	0.006	
Bromoxynil (17, LL.4)		Slope	-1.8 (1.47)	-1.23	0.238
	Minimum	23.82 (6.27)	3.8	0.002	
	Upper Limit	76.79 (6.98)	11	<0.001	
	EC50	0.61 (0.2)	3	0.009	
Glyphosate (17, W2.4)	Slope	1.17 (1.7)	0.69	0.499	
	Minimum	35.51 (3.58)	9.92	<0.001	
	Upper Limit	99.97 (1.79)	55.82	<0.001	
	EC50	1.17 (0.32)	3.65	0.002	
Parallel single-leaf bioassay (PSLB)					
MCPA (2, W2.3)	Slope	7.37 (20.08)	0.37	0.718	
	Upper Limit	100 (7.66)	13.05	<0.001	
	EC50	2.69 (0.54)	4.98	<0.001	
	Bromoxynil (2, LL.3)	Slope	-4.57 (6.34)	-0.72	0.481
Upper Limit		2670.26 (29600.65)	0.09	0.929	
EC50		14.49 (19.36)	0.75	0.464	
Glyphosate (2, W2.3)		Slope	3.87 (3.97)	0.98	0.342
	Upper Limit	439.97 (8618.58)	0.05	0.96	
	EC50	103.94 (565.54)	0.18	0.856	
	Bromoxynil (17, W1.4)	Slope	-1.56 (1.35)	-1.15	0.264
Minimum		1.7 (9.52)	0.18	0.86	

	Upper Limit	108.7 (21.78)	4.99	<0.001
	EC50	0.73 (0.16)	4.58	<0.001
MCPA	Slope	3.31 (1.05)	3.15	0.005
(17, W2.3)	Upper Limit	100.16 (6.81)	14.72	<0.001
	EC50	2.34 (0.16)	15.03	<0.001
Glyphosate	Slope	-4.91 (10.5)	-0.47	0.646
(17, W1.4)	Minimum	59.76 (9.48)	6.3	<0.001
	Upper Limit	94.96 (8.09)	11.74	<0.001
	EC50	5.61 (2.07)	2.71	0.015
Oxyfluorfen	Slope	-23.73 (54.89)	-0.43	0.671
(17, W1.4)	Minimum	0.52 (2.98)	0.17	0.863
	Upper Limit	65.76 (4.23)	15.53	<0.001
	EC50	53.31 (8.82)	6.05	<0.001

aDay after treatment, bDose-response models fitted on data: LL.3: three-parameter log-logistic, LL.4: four-parameter log-logistic, W2.3: three-parameter Weibull, W1.4 and W2.4: four-parameter Weibull.