



## Article

# Effects of Metribuzin Herbicide on Some Morpho-Physiological Characteristics of Two *Echinacea* Species

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**Abstract:** *Echinacea* is a perennial plant that is used for its phytotherapeutic potential. *Echinacea* crops are often affected by invasive weeds. One of the most effective strategies in weed control is the use of chemicals such as herbicides. However, herbicides also affect the physiological and morphological processes of *Echinacea*. For this reason, the aim of this study was to determine the effects of different dosages (0, 250, 500, 750, 1000, and 1250 g ha<sup>-1</sup>) of the postemergent herbicide metribuzin on some morphological and physiological characteristics of *Echinacea purpurea* and *Echinacea angustifolia* collected from different locations in Iran (*E. purpurea* from the Shiraz and Isfahan regions and *E. angustifolia* from the Ardestan and Kazerun regions). Application of metribuzin decreased leaf dry weight for both *Echinacea* species at high doses (750 and 1250 g ha<sup>-1</sup>). At high metribuzin dose (1250 g ha<sup>-1</sup>), *E. purpurea* Shiraz leaves showed an increase in MDA (malondialdehyde) up to 9.14, while in other species the MDA content was lower. Minimum and maximum fluorescence increased at both the registered dosage (500 g ha<sup>-1</sup>) and at high doses (750–1250 g ha<sup>-1</sup>) of metribuzin treatments in both species. The Fv/Fm (maximum quantum yield) value was reduced in herbicide treated species, compared to the control, starting at the 250 g ha<sup>-1</sup> dose, and was lowest at 750 g ha<sup>-1</sup> dose. The results of this study indicate that metribuzin has adverse effects on the physiology and morphology of *Echinacea* species at dosages above 500 g ha<sup>-1</sup>.

**Keywords:** *Echinacea* spp.; dose response; metribuzin herbicide; post emergence; weed



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## 1. Introduction

*Echinacea* is a perennial plant of the Asteraceae family found naturally in parts of North America [1]. Three species of *Echinacea*, *E. pallida* (Nutt) Nutt, *E. purpurea* (L.) Moench, and *E. angustifolia* (DC) Hell, are used for their medicinal aspects [2]. All three were traditionally used by native Americans in medicinal remedies for cold viruses, pain, coughing, influenza, and snake bites [3]. Additionally, *Echinacea* has been found to have antibacterial, antiviral, anti-inflammatory, and antioxidant properties [4]. With the growing demand for *Echinacea*, the cultivation of these species has increased worldwide for several years [5]. Unfortunately, relatively little is known on the effects of plantation management on the physiological aspects and yield of *Echinacea* [6]. One of the primary aspects of management that is least addressed is weed control, particularly in newly established crops [7–12]. Because of the effectiveness and ease of application of herbicides for weed control, farmers have been repeatedly using herbicides to control adventitious weeds in different cropping systems [5,13]. Use of herbicides for weed control in medicinal plants has also been reported [14]. Pre-planting and post-planting application of oxadiazon- (dichlorobenzenes group) and oxyfluorfen- (ether group) based herbicides on *Origanum syriacum* L. indicates both molecules to be effective in controlling weeds in this species [14]. Forcella et al. (2012)

evaluated the tolerance of pot marigold (*Calendula officinalis* L.) to postemergence herbicides, phenmedipham- (carbamate acid group) and desmedipham- (carbamate acid group) based herbicides, and reported tolerance of this species to both compounds [15]. *Foeniculum vulgare* Mill was identified to be tolerant to pendimethalin- (dinitroaniline group) and trifluralin- (aniline group) based herbicides, and the treatment provided substantial weed control without *Foeniculum vulgare* Mill injury. Moreover, dinitroaniline group-based herbicide (pendimethalin) was found to be more effective than aniline group-based herbicide (trifluralin) [16].

Cultivars with herbicide tolerance provide an effective way to manage weeds and at the same time protect the yield and quality of Echinacea. The effects of herbicides on crops may be assessed through agronomic traits (leaf area, plant growth, biomass, and yield, etc.) and physiological variations (chlorophyll content, photosynthesis, and lipid peroxidation) [17,18]. Chlorophyll fluorescence analysis may be a useful tool to detect adverse effects of herbicides belonging to the chemical group of photosynthetic inhibitors. These herbicides cause damage to photosystem II (PSII) complex, blocked PSII electron transport, enhanced  $F_0$ , decreased  $F_m$  and  $F_v/F_m$ , and reduced photosynthetic efficiency [17,19]. Moreover, herbicide application resulted in peroxidation and destruction of lipids in plant leaves [20]. Malondialdehyde (MDA) is the most important and is an indicator of lipid peroxidation. MDA causes adverse effects in plants, thus, free MDA has been determined in various sources as an oxidative stress marker [21]. It is a product of lipid peroxidation, and its levels are shown to rise when a plant is exposed to herbicide stress [20].

Among the herbicides that could be potentially labeled in Echinacea production is metribuzin, a postemergent herbicide, having the ability to effectively control a broad spectrum of dicot weeds [22]. In particular, metribuzin is a herbicide that blocks photosynthetic processes by inhibiting the electron transport of PSII [23]. This inhibiting action causes an alteration of the physiology and morphology of the leaf structures that turn yellow and fall (abscission) [24]. The PSII inhibition generated by metribuzin is not species specific, and many other plants can be affected by its toxic action [24]. Moreover, the production of secondary metabolites is also affected by the action of metribuzin. Indeed, metribuzin acts on the metabolism of mevalonate by reducing the synthesis of secondary metabolites [25]. Furthermore, metribuzin reduces the synthesis of jasmonates making plants more vulnerable to biotic and abiotic stress [25]. *Echinacea* also is sensitive to metribuzin [26] and in some cases metribuzin application has led to noticeable seed yield reduction in *Echinacea* [27]. However, some plants have developed a certain tolerance to metribuzin, thanks to the increase in detoxification processes. Indeed, Xu and colleagues identified in some metribuzin-resistant species of *Triticum aestivum* L. an increase in the expression of genes associated with metabolic detoxification, such as those of the P450 pathway and those of the xenobiotic transmembrane transporter activity [28].

*Echinacea* species need to be evaluated for tolerance to metribuzin to ensure crop safety under different field conditions and to improve weed control. Furthermore, there is little information on the effects of metribuzin in postemergence phases in *Echinacea*. Therefore, the aim of the present study was (i) to evaluate the morphological and physiological responses of *Echinacea* species to metribuzin treatment and (ii) to establish the maximum dose of metribuzin tolerated by *E. purpurea* and *E. angustifolia*.

## 2. Materials and Methods

### 2.1. Experimental Setup and Plant Material

A pot experiment was conducted from May to October 2018 (i.e., 170 days) to investigate the effects of metribuzin on four *Echinacea* cultivars collected from different geographical regions in Iran, namely Isfahan, Shiraz, Ardestan, and Kazerun. The experiment was conducted as a two-factor factorial based on completely randomized design with three replications, in the research greenhouse located in the Faculty of Agriculture, Isfahan University of Technology, in 2018. The experimental treatments were five

metribuzin doses (0, 250, 500, 750, and 1250 g ha<sup>-1</sup>) and four *Echinacea* cultivars including *Echinacea purpurea* Shiraz, *Echinacea purpurea* Isfahan, *Echinacea angustifolia* Ardestan, and *Echinacea angustifolia* Kazerun. The recommended rate for metribuzin is 750 g ha<sup>-1</sup> based on Sencor 70 WP (Bayer, Tehran, Iran) as its commercial formulation in Iran. Metribuzin is a herbicide belonging to the triazines group. *Echinacea* seeds were treated with 5% NaOCl solution for 2 min, followed by washing with distilled water three times to remove excess sodium hypochlorite solution. Seeds were then pre-germinated in mixed media (80% cocopeat and 20% perlite) and seedlings were transferred to pots when they reached the 2–3 leaf stage. Pots were filled with 8.5 kg of air-dried soil and the plants were grown under 16 h:8 h (light:dark) photoperiod. Each replication consisted of three pots with three plants per pot. During the growing season, pots were irrigated every day and metribuzin was applied to all nine plants individually transferred to a pot. Since the growth area of the plants was about 50 cm<sup>2</sup>, intermediate dilutions (in irrigation water) were performed starting from Sencor 70 (metribuzin 70%) in order to reach doses of 0, 250, 500, 750, and 1250 g ha<sup>-1</sup>. Plants were subjected to a single metribuzin treatment with an electric backpack sprayer at the 4–6 leaf stage of *Echinacea* plant. For the application of metribuzin a diffuser (twin flat-fan nozzle) was used which emits the herbicide at a pressure of 250 kPa, in a calibrated nebulization chamber to deliver 250 L water ha<sup>-1</sup>.

## 2.2. Leaf Area and Growth Parameters

Initially, all *E. purpurea* and *E. angustifolia* leaves (aerial parts of the plant, about 10 leaves per plant) were collected in spring 2018 and were used to measure the effect of metribuzin treatment on the leaf area. The leaf area was measured by a Leaf Area Meter (WINAREA-UT-11, Isfahan University, Isfahan, Iran). Subsequently, the aerial parts of the plant (about 20 g of *E. purpurea* and 5 g of *E. angustifolia*) were oven-dried (Incubator-Model of Arvin Tajhiz Spadan, Isfahan, Iran) completely at 70 °C for 48 h and the dry weight was measured.

Other growth traits such as, stem and root length, root and shoot fresh weight, and root volume, were also assessed. In particular, a precision ruler was used to measure the height of the root and the weight of all the roots of the plants tested. To estimate the root volume the following equation was applied to calculate the volume of the cone:

$$Volume = \frac{\pi \times r^2 \times h}{3}$$

where  $r$  is the radius of the root and  $h$  is the height of the root.

The dry weight of both the leaves and the root is about 80% less than their fresh weight.

## 2.3. Malondialdehyde (MDA) Content

For estimating the amount of MDA production, leaf material was collected 48 h after herbicide application and frozen in liquid N<sub>2</sub> and preserved in –80 °C. The thiobarbituric acid (TBA) test was used [29] with slight modification. Briefly, leaf sample (200 mg) was homogenized in 2 mL 0.1% (*w:v*) TCA (trichloroacetic acid) solution. The homogenate was centrifuged at 10,000× *g* for 20 min at 4 °C (Eppendorf, Teheran, Iran) and 250 µL of the supernatant was added to 1 mL 0.5% (*w:v*) TBA in 20% TCA. The mixture was incubated in a water bath at 95 °C for 30 min, and the reaction stopped by cooling the tubes in an ice bath. Samples were then centrifuged at 10,000× *g* for 15 min, and the absorbance of supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted and the MDA (red pigment) dose was calculated from the extinction coefficient (155 mM<sup>-1</sup> cm<sup>-1</sup>).

## 2.4. Chlorophyll Fluorescence

Measurements of F<sub>0</sub> (minimum fluorescence, arbitrary units), F<sub>m</sub> (maximum fluorescence, arbitrary units), maximum quantum yield (F<sub>v</sub>/F<sub>m</sub>, ratio that measures the efficiency of Photosystem II) were randomly taken from the top-most developed leaves of the

seedlings using a chlorophyll fluorometer (Opti-Sciences, model OS-30p, Boston, MA, USA). The fluorescence signal was measured at a standard position on the dark adapted leaf, approximately in the middle of the adaxial surface. Plants were covered using a dark plastic sheet for 20 min before the data were recorded. Data for chlorophyll fluorescence were recorded 10 days after application of metribuzin.

### 2.5. Statistical Analysis

Data analysis was performed after normality test by SPSS 19.0 software and analysis of variance was carried out to determine differences among the treatments using the GLM (general linear model) method in SAS 9.1 software. If the analysis of variance indicated statistically significant differences, the mean comparisons were completed using least significant difference (LSD) test at 0.05 level of probability. All data were normalized as a percentage of the control mean for each parameter. This processing allows to evaluate the percentage increase and decrease with respect to the control group. Moreover, for leaf area, leaf dry weight, malondialdehyde, root dry weight, root volume, root length, and photosynthesis parameters a two-way ANOVA was performed using the GraphPad Software, San Diego, CA, USA followed by Dunnett's multiple comparisons test for the comparison of individual means.

## 3. Results

From the general analysis of the collected results, it is highlighted that the herbicide had a significant effect in all *Echinacea* cultivars. In particular, the analysis of variance revealed a significant effect of metribuzin dose on all measured traits. Additionally, the effect of *Echinacea* cultivars and *Echinacea* cultivars  $\times$  herbicide was significant for all traits (Table 1).

**Table 1.** Analysis of variance for morphophysiological traits of four *Echinacea* cultivars (G) evaluated at five doses of metribuzin (H).

SOV	df	MS								
		LDW	RDW	RL	RV	LA	MDA	F <sub>0</sub>	F <sub>m</sub>	Fv/F <sub>m</sub>
G	3	40.35 **	140.83 **	856.51 **	1637 **	15,445,432 **	7.49 **	1131.2 **	3972 **	0.0246 **
H	4	2.78 **	15.01 **	49.79 **	236 **	1,082,277 **	30.43 **	9525.2 **	5140 **	0.0917 **
G $\times$ H	12	0.36 **	4.53 **	26.41 **	215 **	272,880.2 **	0.68 **	349.4 **	780 **	0.0134 **
Error	40	0.033	0.102	3	14.35	1638.48	0.151	37.51	42.57	0.00071
CV	-	8.65	5.90	6.42	15.20	3.20	6.45	5.54	2.68	4.91

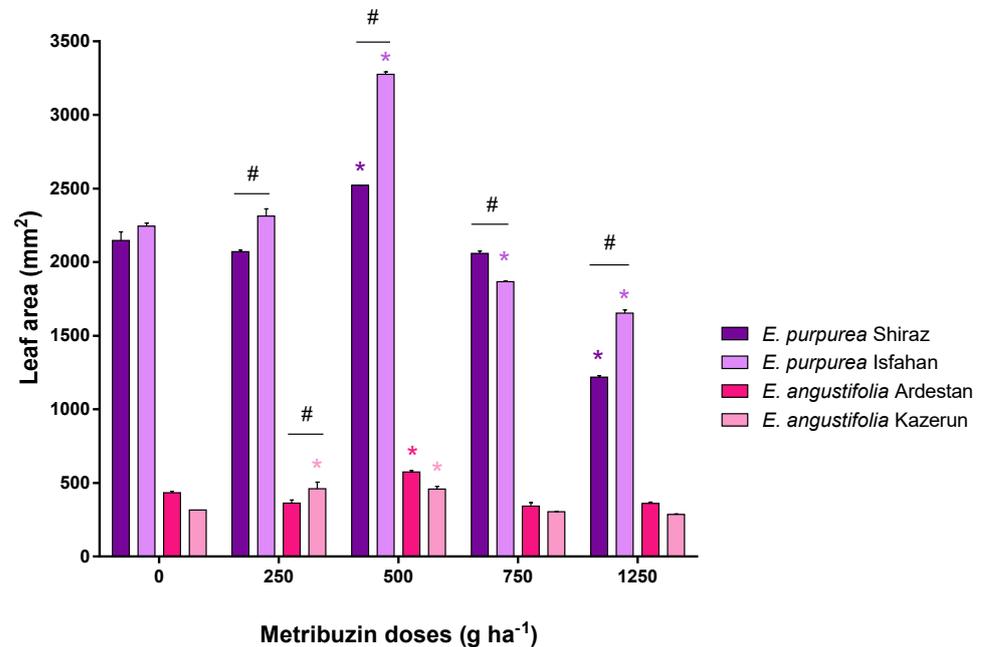
\*\*  $p \leq 0.01$ ; MS—mean squares; SOV—source of variation; df—degrees of freedom; LDW—leaf dry weight; RDW—root dry weight; RL—root length; RV—root volume; LA—leaf area; MDA—malondialdehyde; F<sub>0</sub>—minimum fluorescence; F<sub>m</sub>—maximum fluorescence; Fv/F<sub>m</sub>—maximum quantum yield; CV—coefficient of variation.

### 3.1. Leaf Parameters: Leaf Area and Leaf Dry Weight

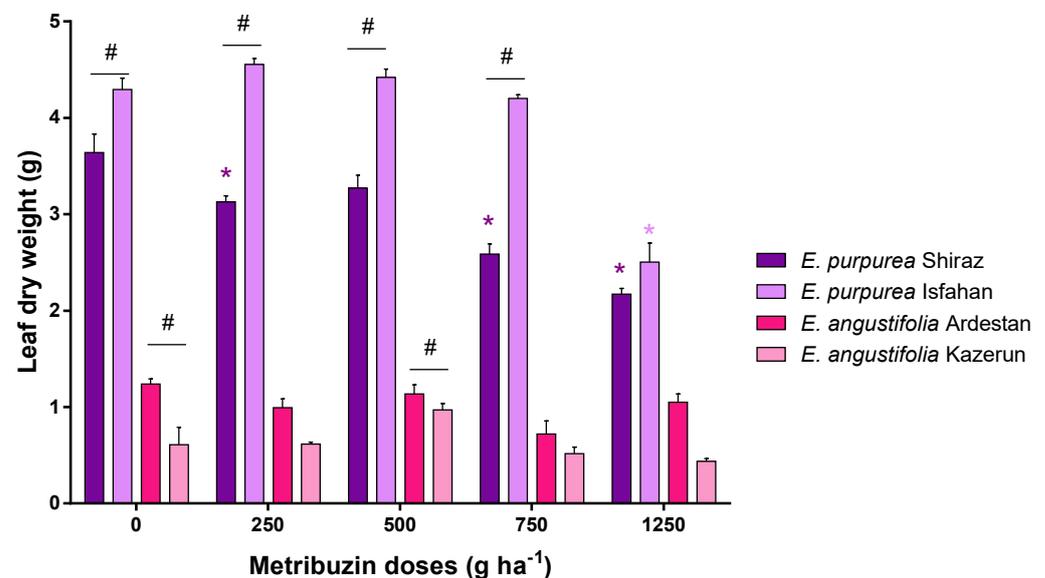
Analyzing in detail the effects of metribuzin on the studied *Echinacea* cultivars, statistically significant effects were highlighted at high dosages. Regarding leaf area (Figure 1), both *E. angustifolia* and *E. purpurea* showed an increase after treatment with 500 g ha<sup>-1</sup> of metribuzin compared to control. Moreover, statistical analysis revealed significant differences between the cultivars of *E. purpurea* and *E. angustifolia*. At 250, 500, 750, and 1250 g ha<sup>-1</sup> metribuzin doses, *E. purpurea* Shiraz showed significant differences in the leaf area compared to Isfahan. Above the 500 g ha<sup>-1</sup> metribuzin dose, the significant differences between the two *E. purpurea* cultivars were maintained and the leaf area decreased. A similar trend was observed in *E. angustifolia* Ardestan and Kazerun at 250 g ha<sup>-1</sup>. Finally, at high dosages of metribuzin, no significant differences in the leaf area were observed with respect to the control in both *E. angustifolia* cultivars.

The effects of high dosages of metribuzin on the leaf area also affected the dry weight of *Echinacea* leaves. Particularly, application of metribuzin at high doses (750–1250 g ha<sup>-1</sup>) resulted in drastic reduction in leaf dry weight of all *Echinacea* species (Figure 2). Furthermore,

all the experimental groups of *E. purpurea* Isfahan show a higher and significant dry weight of the leaf compared to *E. purpurea* Shiraz. *E. angustifolia* Kazerun also shows statistically higher foliar dry weight values than Ardestan, but only at 250 g ha<sup>-1</sup> metribuzin.



**Figure 1.** Effect of different metribuzin doses (0, 250, 500, 750, 1250 (g ha<sup>-1</sup>)) on leaf area of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

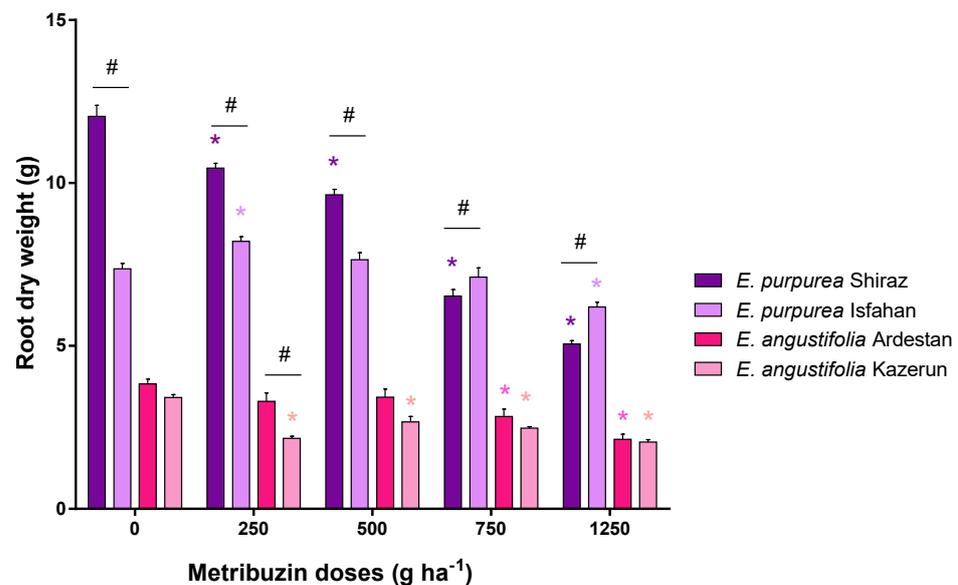


**Figure 2.** Effect of different metribuzin doses on leaf dry weight of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

These results suggest that application of high doses (750–1250 g ha<sup>-1</sup>) of metribuzin adversely affects the biomass accumulation in *Echinacea* species.

### 3.2. Root Parameters: Dry Weight, Length, and Volume

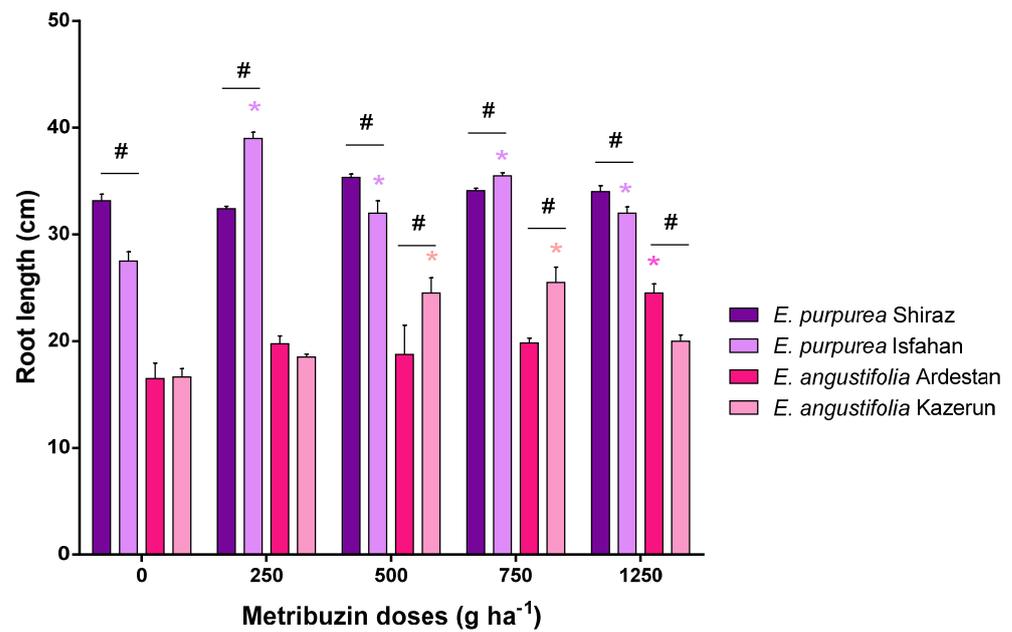
The effect of metribuzin was also evaluated at the root level of the studied *Echinacea* species. In particular, the highest root dry weight was represented by *E. purpurea* Isfahan compared to the control group (0 g ha<sup>-1</sup> metribuzin) (Figure 3). At 1250 g ha<sup>-1</sup> metribuzin, all species showed the lowest root dry weight except *E. purpurea* Isfahan. In *E. purpurea* Shiraz, *E. angustifolia* Kazerun and Ardestan, root dry weight significantly decreased with increasing herbicide dose, while in *E. purpurea* Isfahan, root dry weight increased up to 250 g ha<sup>-1</sup> dose after which it decreased with increasing metribuzin dose. Root dry weight was significantly different between the two *E. purpurea* cultivars in all experimental groups. Finally, the response to herbicide dose was statistically different between the two *E. angustifolia* plants Kazerun and Ardestan only at 250 g ha<sup>-1</sup>.



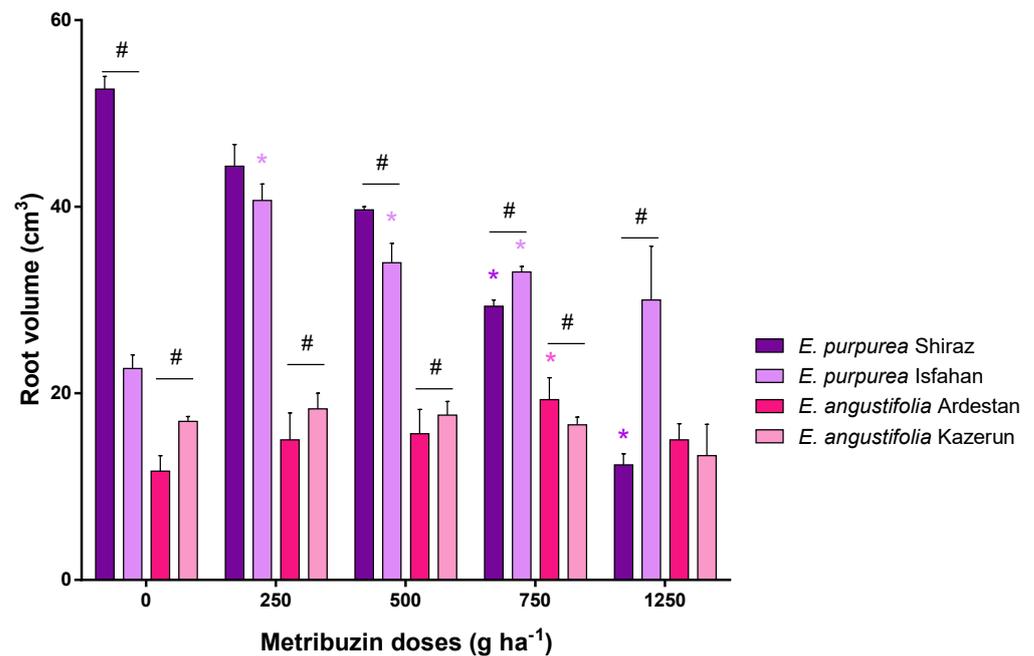
**Figure 3.** Effect of different metribuzin doses on root dry weight of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

Regarding the root length (Figure 4), in *E. purpurea* Shiraz no significant differences were found compared to the control group (0 g ha<sup>-1</sup> metribuzin). On the contrary, all doses of metribuzin significantly increased root length with the highest value being 250 g ha<sup>-1</sup> in *E. purpurea* Isfahan. On the other hand, *E. angustifolia* Ardestan showed a significant increase in root length only at 1250 g ha<sup>-1</sup> and Kazerun at 500 and 750 g ha<sup>-1</sup>, compared to the control. Regarding the differences between *Echinacea* cultivars, Shiraz and Isfahan differed significantly between all experimental groups. On the other hand, Kazerun showed a significant increase in root length compared to Ardestan at 500, 750, and 1250 g ha<sup>-1</sup>.

Regarding the root volume (Figure 5), metribuzin induces a reduction in root volume in *E. purpurea* Shiraz starting from 750 to 1250 g ha<sup>-1</sup> compared to the control group. On the contrary, *E. purpurea* Isfahan appears to tolerate metribuzin treatment better. Indeed, a significant increase in root volume is observed at all dosages compared to the untreated control plants. Additionally, metribuzin 250 g ha<sup>-1</sup> enhanced the root volume of *E. purpurea* Isfahan and resulted in twice the root volume (around 40 mm<sup>2</sup>) as compared to the control group. Application of metribuzin had a similar effect on *E. angustifolia* Ardestan. Regarding the differences between cultivars of *Echinacea*, Shiraz and Isfahan differ significantly in all metribuzin-treated groups. Same significant differences were observed between the Ardestan and Kazerun cultivars.



**Figure 4.** Effect of different metribuzin doses on root length of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

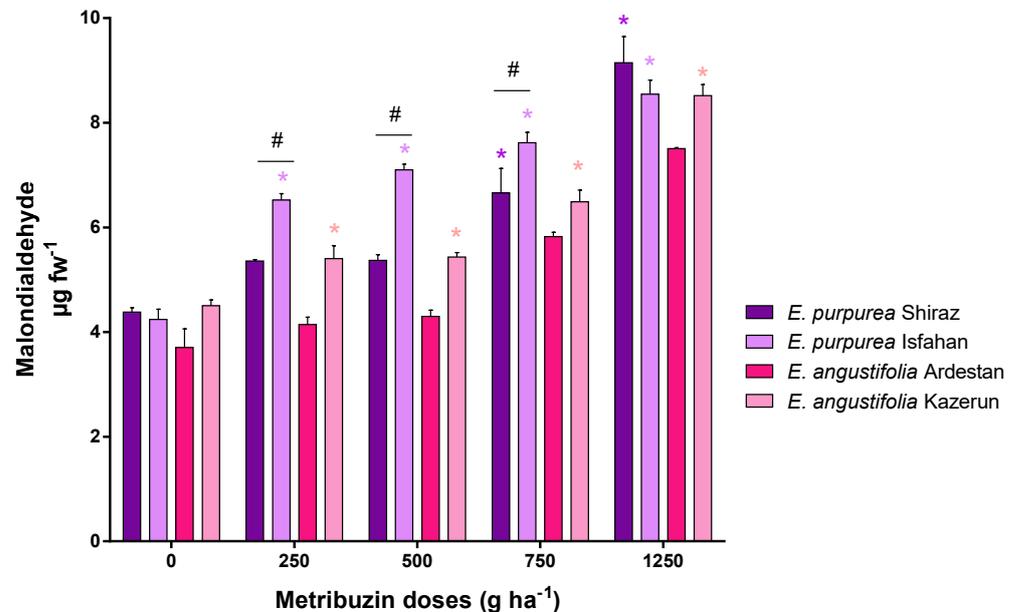


**Figure 5.** Effect of different metribuzin doses on root volume of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

### 3.3. Oxidative Stress: Malondialdehyde (MDA)

High dosage of metribuzin increased the oxidative stress which led to the increase in malondialdehyde (MDA) as shown in Figure 6. MDA accumulation in leaves increased in response to increasing metribuzin doses and was observed in all *Echinacea* cultivars. The increase in MDA content seemed species dependent. Indeed, *E. purpurea* Isfahan and

*E. angustifolia* Kazerun showed a significant increase, compared to control, starting as early as metribuzin 250 g ha<sup>-1</sup>. On the contrary, *E. purpurea* Shiraz and *E. angustifolia* Ardestan showed a significant increase in MDA compared to control only starting at metribuzin 750 g ha<sup>-1</sup>. Furthermore, *E. purpurea* Isfahan showed significantly higher levels of MDA than *E. purpurea* Shiraz at metribuzin 250 and 500 g ha<sup>-1</sup>. Finally, the amount of MDA did not show significant differences between *E. angustifolia* Kazerun and Ardestan at all doses of metribuzin.



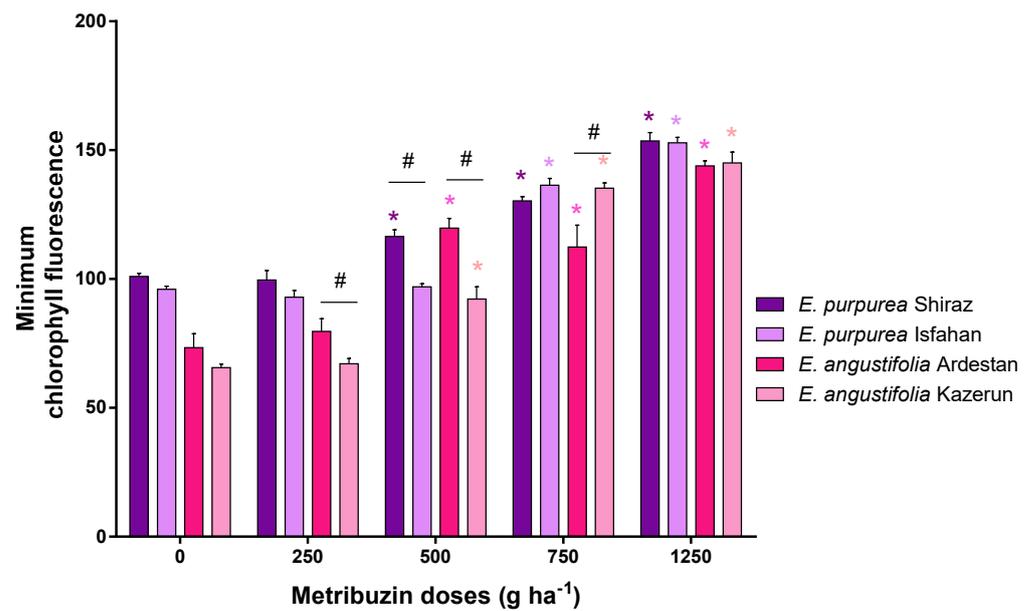
**Figure 6.** Effect of different metribuzin doses on malondialdehyde content of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

### 3.4. Chlorophyll Fluorescence and Maximum Quantum Yield

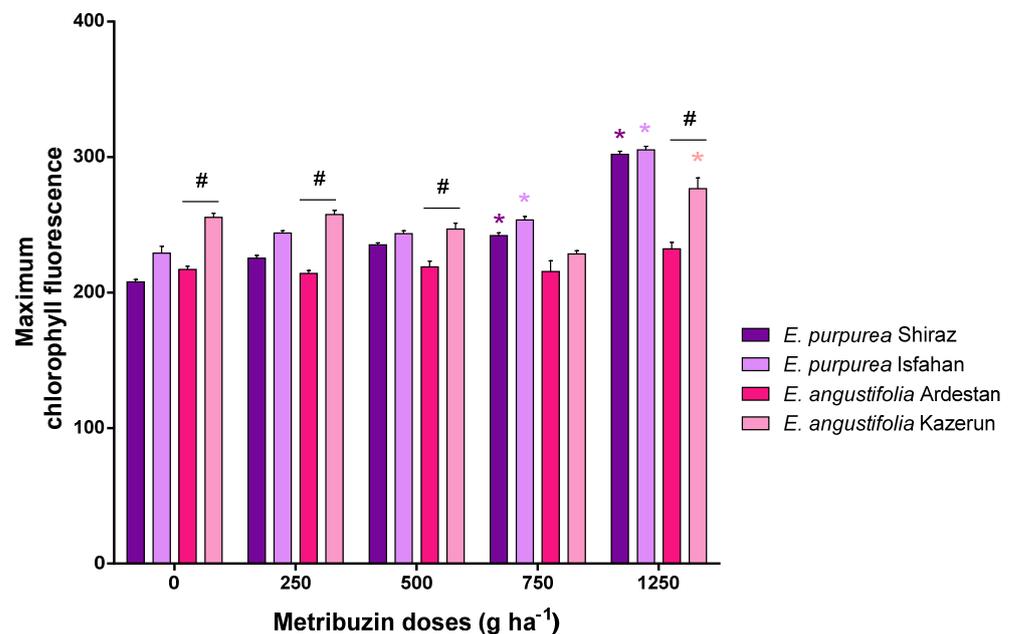
Minimum chlorophyll fluorescence (Figure 7) in all *Echinacea* species increased with increasing metribuzin dose. For all species, minimum fluorescence at 250 g ha<sup>-1</sup> dose was similar to the untreated control. Regarding the differences between cultivars, Shiraz and Isfahan differed at metribuzin 500 and 750 g ha<sup>-1</sup>. Furthermore, the minimum chlorophyll fluorescence of *E. angustifolia* Ardestan and Kazerun differed at metribuzin 750 and 1250 g ha<sup>-1</sup>.

Similarly, maximum chlorophyll fluorescence (Figure 8) was significantly increased with increasing metribuzin dose in all *Echinacea purpurea* cultivars. Maximum chlorophyll fluorescence response was statistically relevant between *E. purpurea* Shiraz and Isfahan in the plants treated with metribuzin 500, 750, and 1250 g ha<sup>-1</sup>. Furthermore, *E. angustifolia* Kazerun showed maximum chlorophyll fluorescence values higher than *E. angustifolia* Ardestan at 1250 g ha<sup>-1</sup>. No significant differences were observed in the other experimental groups.

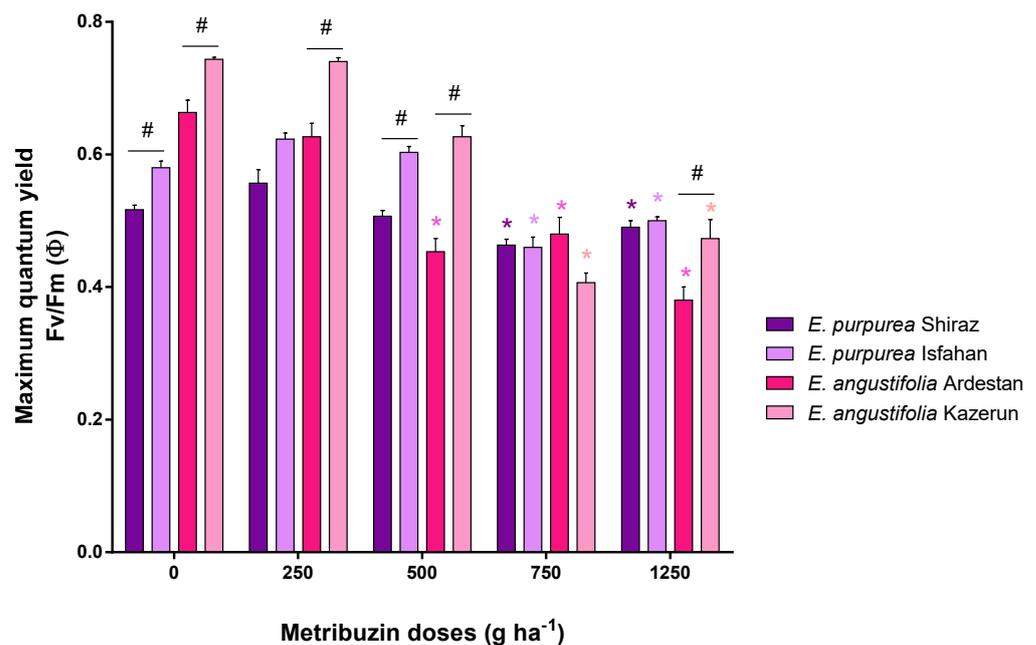
As regards photosynthetic efficiency (Figure 9), metribuzin significantly reduced the values of Fv/Fm in the species of *E. purpurea* studied at dosages of 750 and 1250 g ha<sup>-1</sup> compared to the control group. The effects of metribuzin are most noticeable in *Echinacea angustifolia*. Indeed, both the samples collected in Ardestan and those collected in Kazerun showed a significant reduction starting from 500 g ha<sup>-1</sup> compared to the control. Furthermore, significant differences were found between Shiraz and Isfahan in plants receiving metribuzin 750 and 1250 g ha<sup>-1</sup>. Likewise, significant differences were found between Ardestan and Kazerun at 500 and 750 g ha<sup>-1</sup>.



**Figure 7.** Effect of different metribuzin doses on minimum chlorophyll fluorescence of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.



**Figure 8.** Effect of different metribuzin doses on maximum chlorophyll fluorescence of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.



**Figure 9.** Effect of different metribuzin doses on maximum quantum yield of *Echinacea purpurea* and *Echinacea angustifolia* species. Data are shown as the mean  $\pm$  SEM, and ordinary two-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical significance; \*  $p < 0.005$  vs. control (0 g ha<sup>-1</sup> metribuzin). Significant differences between *Echinacea* cultivars are indicated with #.

### 3.5. Correlation between Traits

The correlation coefficients between measured traits were calculated and are shown in Table 2. Negative correlations were observed between Fv/Fm and MDA (−0.47), and between Fv/Fm and F<sub>0</sub> (−0.88); whereas, significant positive correlations were observed between F<sub>0</sub> and MDA (0.766), and between Fm and MDA (0.746). Furthermore, significant positive correlations were found between leaf dry weight and root dry weight (0.854), root length (0.796), root volume (0.740), and leaf area (0.937), in the presence of herbicide (Table 2).

**Table 2.** Correlation coefficients among measured traits in four *Echinacea* cultivars treated by metribuzin.

	RDW	RL	RV	MDA	LA	Fv/Fm	F <sub>0</sub>	Fm
RDW	-							
RL	0.760 **	-						
RV	0.888 **	0.702 **	-					
MDA	−0.08 <sup>ns</sup>	0.407 **	−0.05 <sup>ns</sup>	-				
LA	0.868 **	0.810 **	0.775 **	0.119 <sup>ns</sup>	-			
Fv/Fm	−0.03 <sup>ns</sup>	−0.299 *	−0.04 <sup>ns</sup>	−0.47 **	−0.03 <sup>ns</sup>	-		
F <sub>0</sub>	−0.01 <sup>ns</sup>	0.377 **	−0.03 <sup>ns</sup>	0.766 **	0.05 <sup>ns</sup>	−0.88 **	-	
Fm	−0.14 <sup>ns</sup>	0.21 <sup>ns</sup>	−0.17 <sup>ns</sup>	0.746 **	0.02 <sup>ns</sup>	−0.00 <sup>ns</sup>	0.460 **	-

\*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; <sup>ns</sup> not significant; RDW—root dry weight; RL—root length; RV—root volume; MDA—malondialdehyde; LA—leaf area; Fv/Fm—maximum quantum yield; F<sub>0</sub>—minimum fluorescence; Fm—maximum fluorescence.

## 4. Discussion

In this study, the morphological and physiological effects of an increasing dose treatment of metribuzin on cultures of *Echinacea purpurea* and *Echinacea angustifolia* were evaluated. Metribuzin is a herbicide that blocks photosystem II and leads to the degeneration of the leaf districts [23]. The toxic action of metribuzin on photosynthesis and leaves is not selectively targeted to weeds but can also affect crops of agronomic value such as *Echinacea*.

However, plants can develop resistance to metribuzin and need higher doses to block photosynthesis [30–34]. Here, we established the metribuzin doses most tolerated by the two *Echinacea* species. Regarding the foliar apparatus, the high doses (750 and 1250 g ha<sup>-1</sup>) reduced the growth (leaf area and dry weight) of all *Echinacea* cultivars. This reduction eventually resulted in biomass decrease, thus leading to a decline in biological weight of *Echinacea* species as observed previously [35]. Although metribuzin appears to be not particularly toxic at dosages of 250 and 500 g ha<sup>-1</sup>, in all the *Echinacea* plants studied there was an increase in oxidative stress in terms of increased levels of malondialdehyde. Indeed, accumulation of some metabolites is a strategy to combat oxidative stress within the plant cell [9–11,36–46]. Among these metabolites, MDA is the most important and is an indicator of lipid peroxidation [9,39]. Moreover, MDA has been identified in numerous sources as an oxidative stress marker [21,47,48]. However, the biological effects of MDA on plant functions are not yet known [39,49]. In this work, an increased amount of MDA was detected in metribuzin-treated leaves indicating higher lipid peroxidation and oxidative stress as reported previously [50,51]. Therefore, if on the one hand the dosages of metribuzin 250 and 500 g ha<sup>-1</sup> did not show particular foliar toxicity in *Echinacea* species, however, on the other hand, an increase in oxidative stress was observed. The increase in oxidative stress can be associated with the blockade of PSII, which degenerates and increases the release of free radicals [52,53]. For this reason, in order to define the physiological effects on photosynthesis, the minimum and maximum fluorescence of chlorophyll and the photosynthetic yield were evaluated. The data reported here show that treatment with metribuzin greater than 500 g ha<sup>-1</sup> induced an alteration in photosynthetic processes. In particular, the PSII of *E. angustifolia* Ardestan and Kazerun was more sensitive than that of *E. purpurea* Shiraz and Isfahan to treatment with metribuzin. With regard to photosynthetic efficiency, both *E. purpurea* and *E. angustifolia* reduced their photosynthetic yield starting from 250 g ha<sup>-1</sup>. The effectiveness of many herbicides is based on the alteration of photosynthetic functionality as reported by Wang and colleagues [17]. They reported that mesosulfuron-methyl and iodosulfuron-methyl sodium (herbicides that also act on the photosynthetic yield) were unsafe for wheat and decreased the fresh weight and yield compositions significantly. Reversible inactivation or destruction of PSII reaction center can cause an increase in F<sub>0</sub> [54]. Previous studies have shown that herbicides cause damage to the PSII complex, block photosynthetic electron transfer, and reduce Fv/Fm significantly [17]. For example, the use of selective herbicides for photosystems leads to an increase in F<sub>0</sub> and a decrease in Fm and Fv/Fm by blocking electron transport to PS II and inactivating the reaction center in wheat crops [17,19]. Similarly, our results indicate that F<sub>0</sub> and Fm increased while Fv/Fm decreased in leaves. Moreover, there is a positive correlation between both endpoints, i.e., inhibition of photosynthesis and biomass reduction. This suggests that measuring the quantum yield is a useful tool in predicting adverse herbicide effects on biomass growth. However, both dose–response curves differ from each other, and the quantum yield was more highly sensitive to metribuzin than biomass production.

As for the effects on the root system, metribuzin did not cause significant alterations in terms of length, volume, and dry weight of the roots. These data further confirm the action of metribuzin on the PSII system. Further measures should be carried out to assess the environmental impact of the herbicide. This aspect must be considered above all for the response of other plants and pollinating insects [55,56].

Differences in response to metribuzin treatment were noted in this manuscript between collection sites of the same *Echinacea* species. These differences may be due to the effect of metribuzin on secondary metabolism [57]. To date, there are no studies exploring the effect of metribuzin on the synthesis and degradation pathways of secondary metabolites of *Echinacea*. However, the application of metribuzin did not alter the phytochemical profile of *Silybum marianum* [58]. In addition, *Salvia officinalis* increases the content of essential oils after weed reduction due to treatment with herbicides (such as metribuzin) [59]. Although secondary metabolite levels have not been evaluated in this manuscript, we cannot exclude a modulatory action of metribuzin in the secondary metabolism of both species of *Echinacea*.

Further studies will be needed in the future to identify and quantify the main classes of secondary metabolites of the different cultivars of *Echinacea*.

## 5. Conclusions

The present study clearly indicated that photosynthesis and growth of *Echinacea* species are affected by metribuzin doses. Differences in sensitivity could be attributed to differences in uptake, translocation, metabolism, and/or dilution within the plant. Unfortunately, only a few of these factors have been thoroughly investigated. However, doses of metribuzin that did not significantly alter the physiology of the two *Echinacea* species ranged from 250 to 500 g ha<sup>-1</sup> for the parameters studied. Doses higher than 500 g ha<sup>-1</sup> of metribuzin can alter the foliar morphology, root volume, and PSII. Further studies will be needed to determine the molecular signaling involved in tolerance mechanisms to metribuzin treatment and to establish which weed species are sensitive to dosages below 500 g ha<sup>-1</sup>. It would be interesting to study the possibility of using metribuzin in combination with other herbicides.

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