agronomy

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Susceptibility of Selected Crops to Simulated Imazethapyr Carryover: A Morpho-Anatomical Analysis

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Abstract: A bioassay study was conducted to determine the differences in the susceptibility of selected crops to simulated imazethapyr residues based on morphological and anatomical parameters. Sugar beet, white mustard, and rapeseed oil were found to be the most sensitive based on ED_{50} values for the root length and the root fresh weight. Corn and sunflower were less sensitive, and wheat was the least sensitive. The measured anatomical parameters confirmed the different sensitivities of the tested plants, as evidenced by a shortening of the meristem and elongation zones, a reduction in the distance between the root tip and the absorption zone, and the distance between the root tip and the point where the primordium of the lateral root appears. An imazethapyr residue level equivalent to ED_{20} (for root length) did not cause serious morphological changes in the less sensitive plants, nor did it cause significant changes in the length of the root cap, the beginning of the root absorption zone (root hair region), the beginning of lateral root formation (i.e., the permanent region), and the number of root primordia per root length. Therefore, ED_{20} could be proposed as an acceptable residue level (ARL) or herbicide residue level at which these plants can be safely sown.

Keywords: acceptable residue level; dose–response curve; imazethapyr; phytotoxicity; root anatomy

Citation: Jovanović-Radovanov, K.; Rančić, D. Susceptibility of Selected Crops to Simulated Imazethapyr Carryover: A Morpho-Anatomical Analysis. *Agronomy* **2023**, *13*, 1857. https://doi.org/10.3390/agronomy13071857

Academic Editor: Tomasz Głąb

Received: 15 June 2023
Revised: 30 June 2023
Accepted: 6 July 2023
Published: 13 July 2023

1. Introduction

Imazethapyr [*(RS)*-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-il)nicotinic acid)] is a systemic imidazolinone herbicide that belongs to the B/2 group (HRAC/WSSA) and is used for the selective control of a broad spectrum of broadleaf and grassy weeds in leguminous and imidazolinone-tolerant crops [1–7]. It has both soil and foliar activities and inhibits the synthesis of the branched-chain amino acids (valine, leucine, and isoleucine), by inhibiting the enzyme acetolactate synthase (acetohydroxyacid synthase AHAS; E.C.4.1.3.18) [8].

The fate and behavior of imazethapyr in soil depend on numerous factors. According to previous studies, imazethapyr hardly moves downward in the soil, even under conditions that favor leaching [9–11], with residues remaining in a layer of 10–20 cm in most soils [12,13] and being evenly distributed in the upper 15 cm [14,15]. Imazethapyr is strongly adsorbed to soil colloids at a low pH [16], and adsorption is positively correlated with organic matter content, clay content, and cation exchange capacity (CEC) [17–28]. Desorption hysteresis is more pronounced in higher pH soils [26,29,30]. The degradation of imazethapyr depends on climatic and meteorological conditions, so the herbicide is more persistent in dry and cold soils [13,29,31–34]. The amount of rainfall in the summer, after application, has a significant effect on faster degradation [18,35,36]. The degradation of imazethapyr is negatively correlated with soil adsorption capacity [37], making it less available to microorganisms under conditions that favor sorption [26]. Bacterial strains of *Bacillus* sp., *Alcaligenaceae* sp., *Achromobacter* sp., and *Pseudomonas* sp. are capable of degrading up to 80–90% of the applied imazethapyr [38–40]. Degradation of imazethapyr by evaporation is not of concern (less than 2%); however, photo-degradation is slightly

higher (about 8%), especially when imazethapyr is applied to the soil surface and to coarse-textured, moist soils with low clay and organic carbon content. Degradationis higher with natural irradiation than with artificial UV light [29,41–43]. The influence of the application method and subsequent tillage system on the persistence of imazethapyr is neither consistent nor significant [41,42,44–47]. The half-life of imazethapyr varies from 8 to 120 days and depends primarily on soil moisture, but also always increases with the increasing organic matter content. Different half-lives have been determined in different soil types and different years in field studies using bioassays and first-order kinetics [12,36,42,48–50]. In laboratory studies, the half-life varies from 30 days to 10.6 months, depending on the incubation conditions [18,31,51,52].

There are numerous and various reports of carryover and phytotoxicity of imazethapyr to various crops in rotation. Several researchers documented moderate to severe plant injuries or even a risk of yield loss [53–61]. On the other hand, there are reports of phytotoxicity from imazethapyr residues that occurred early in the growing season (at early growth stages) but had no effect on the yield [46,48,59,62–65]. Some researchers have identified extremely sensitive crops, while others have identified less sensitive crops [49,56,66–71]. In addition, there are numerous studies with varying and/or inconsistent results [16,30,50,72–80]. There is still no information on what residue level of imazethapyr is acceptable for different crops to be safely sown.

The objectives of this study were (1) to determine the sensitivity of different crops based on morphological parameters; (2) to investigate the effects of low concentrations of imazethapyr on some root anatomy parameters and compare them with the control and the recommended application dose; (3) and to determine whether the ED_{20} value (determined for the most sensitive morphological parameter and based on the measured anatomical parameters) can be used and recommended as an acceptable residue level (ARL) with respect to the safe sowing of crops in rotation after imazethapyr application.

2. Materials and Methods

2.1. Plant Material, Soil, and Herbicide

The following plant species/varieties were used for all studiesas test plants: maize (*Zea mays* L. var. NS 444), sunflower (*Helianthus annuus* L. var. Bačvanin), sugar beet (*Beta vulgaris* L. *ssp. saccarifera* var. Lara), wheat (*Triticum aestivum* L. var. Venera), rapeseed oil (*Brassica napus* L. *ssp. oleifera* var. Slavica), and white mustard (*Sinapis alba* L. var. NS bela). Seeds of the selected varieties were obtained from the Institute of Field and Vegetable Crops in Novi Sad.

The soil was taken from a field in Sremska Mitrovica (45°00′06.6″ N; 19°37′50.3″ E, and altitude: 96 ± 5 m), where no herbicides had previously been applied. The soil was a sandy loam with the following properties: pH 7.47, 51.96% sand, 38.08% silt, 9.96% clay, and 2.69% organic matter. The soil was air dried, then passed through a 3-mm sieve and divided into 600-g portions (one portion represents one replication for each concentration used).

The commercial formulation of imazethapyr (Pivot 100-E, 40 g-L; BASF) was used to prepare a standard herbicide solution in aqueous solutions. Application rates of imazethapyr were established at 0, 1.875, 3.75, 7.5, 15, 30, 60, 120, and 240 µg a.i./kg soil.

2.2. Bioassay under Controlled Conditions: Differences in Sensitivity of Selected Crops to Imazethapyr Based on Morphological Parameters

The bioassay procedure used has been described in detail previously [36]. Each sample of 600 g of soil was treated with 6 mL of an appropriately diluted herbicide solution for each test plant and herbicide dose. A thin-layer chromatography sprinkler connected to a compressor delivered the herbicide solution at a constant pressure of 120 kPa. After the herbicide application, the treated soil sample was mixed manually and then placed in a rotating mixer, where it was mixed for an additional 7 min (60 rpm). The soil was

then divided into three 200-g portions and transferred to plastic pots. The seeds were planted at the appropriate depth for each of the selected crops so that there were three plants per pot for corn and sunflower, five plants per pot for sugar beet and wheat, and seven plants per pot for rapeseed oil and white mustard. Soil moisture was brought to field capacity (24% vol.) [81], and then the pots were irrigated daily up to 70% of the field capacity. Plants were grown for 14 days in a growth chamber at 25 °C/18 °C (day/night) with a 16-h photoperiod of 300 μE/m^2s.The plants in untreated soil served as the controls. Treatments were arranged in a completely randomized block design with four replications. After 14 days, the plants were removed from the pots intact, and the soil was removed from the root system by careful washing. Top root length (RL) (mean root length in wheat), root fresh weight (RFW) and shoot fresh weight (SFW) were measured, and these data were converted to percent inhibition compared to the control treatment. Each bioassay was repeated twice, and two sets of data were pooled and subjected to nonlinear regression analysis to calculate ED_{50}, ED_{20} and ED_{10}. The four-parameter log-logistic model was used (Equation (1)):

$$Y = C + \frac{D - C}{1 + \exp\{b \cdot [\log(X) - \log(E)]\}}, \quad (1)$$

where Y is the test plant response (i.e., inhibition of the measured parameter) as a function of the herbicide of dose X; C is the lower limit of plant response (lower asymptote); D is the upper limit of plant response (upper asymptote); b is the proportional slope of the curve around ED_{50} (the inflection point); and E is the herbicide dose required to achieve half the plant response between the upper and lower limits, i.e., ED_{50}. All statistical analyses and graphs were performed in the R Software Program using the dose–response curve (drc) statistic package [82]. The ED_{50}, ED_{20}, and ED_{10} values were calculated for each measured parameter and all herbicide–crop combinations. All calculated ED values were used to rank crops in terms of sensitivity to imazethapyr, while ED_{10} and ED_{20} were used to examine the effects of imazethapyr on root anatomy.

2.3. Differences in Crop Sensitivity to Imazethapyr Based on Anatomical Parameters

Separate tests were performed with the same crop species. Three herbicide solutions were prepared in aqueous solution, and the concentrations of imazethapyr in the growing media were established at 0 (untreated control), ED_{10} (corresponding to the available residue level causing 10% inhibition of root length—calculated for each given species), ED_{20} (corresponding to the available residue level causing 20% inhibition of root length—calculated for each given species), and 240 μg a.i./kg (the highest dose in the previous bioassay corresponding to the recommended application dose (RAD)). To simulate the bioavailable residues at the desired level (i.e., ED_{10} and ED_{20}), these experiments were performed with sand instead of soil (to avoid the sorption of imazethapyr). For each treatment, 600 g of sand was placed in a glass Petri dish (d = 15 cm) and seeded with 15 seeds. Treatments were applied by watering the pots with a pre-calculated amount of each herbicide solution. The pots were placed in the growth chamber under the same conditions as previously described and left for 14 days to allow the plants to grow. The pots were watered daily. Treatments were arranged in a completely randomized block design with four replications. After 14 days, the plants were removed from the pots intact, and sand was removed from the root system by thorough washing. A root tip no longer than 2 cm was cut from each plant and stored in a 50% ethanol solution until the microscopic preparations were made (both temporary and permanent). For permanent microscopic preparations, 20 roots per plant species and treatment (5 plants/roots per plant species and treatment, with 4 replications) were used, and for temporary microscopic preparations, 40 roots per plant species and treatment (10 plants/roots per plant species and treatment, with 4 replications) were used. Permanent microscopic preparations were made using the standard paraffin method [83]. Paraffin blocks were cut out with a LEICA SM 2000 R sliding microtome, and the longitudinal sections (approximately 7 μm thick) were stained with the histological dyes toluidine blue, safranin, and alcian blue. All microscopic preparations

were used to analyze the root tip anatomy of all tested crops and to measure the relevant parameters: root cap length (RCL), distance from the root tip to the beginning of the root hair region or simply the beginning of the absorption zone (AZ), distance from the root tip to the beginning of the permanent region or simply the beginning of lateral root formation (LRF), and the number of lateral root primordia ($LRPN_o$) per 0.5 mm root length (this short length was used because of the strong inhibition of root growth in treatments with 240 µg imazethapyr/kg soil). The preparations were examined under a light microscope LEICA DMLS, photographed with a digital camera LEICA DC 300, and measured with the software LEICA IM 1000. The results were processed with the statistical package SPSS, and the average values for each parameter and the standard error were calculated. The Tuckey test was used to determine the significance of the differences between the treatments studied for each parameter analyzed for all crops.

3. Results and Discussion

3.1. Differences in Crop Sensitivity to Imazethapyr Based on Morphological Parameters

The overall response, expressed by morphological parameters, varied according to the plant species and imazethapyr dosage. In general, wheat was the least sensitive, while sugar beet was the most sensitive to imazethapyr (Figure 1). Root growth parameters (length and fresh weight) were more sensitive than the shoot growth parameter (fresh weight) based on ED_{50}, ED_{20}, and ED_{10} (Table 1). The observed difference between the parameters has been previously demonstrated [19,84–86].

Table 1. Regression parameters (Equation (1)) and imazethapyr doses (µg a.i./kg soil) that caused 10%, 20%, and 50% inhibition [ED_{10} (±SE); ED_{20} (±SE) and ED_{50} (±SE)] of all measured morphological parameters for all crops tested.

Plant Species	Parameter Measured	Regression Parameters (±SE)			ED_{50}	ED_{20}	ED_{10}
		B	*D*	*C*			
Wheat (n = 60)	RL	−0.6 (0.7)	122.9 (254.2)	0.8 (10.1)	109.8 (12.5)	13.7 (5.8)	3.1 (7.8)
	RFW	−0.4 (0.1)	139.8 (69.7)	1.7 (8.1)	90.9 (8.5)	6.9 (1.4)	0.7 (1.4)
	SFW	1.8 (1.3)	13.9 (3.8)	29.1 (24.6)	3131 (79)	413 (26)	260 (18.9)
Corn (n = 48)	RL	−0.8 (0.2)	88.7 (9.1)	0.4 (4)	16.0 (5.4)	2.9 (0.9)	1.1 (0.5)
	RFW	−0.9 (0.2)	96.1 (6.6)	0.8 (4)	11.9 (2.7)	2.5 (0.7)	1.0 (0.4)
	SFW	−1.3 (0.5)	30.3 (5.7)	−5.4 (2.8)	24.5 (10.9)	86.3 (3.5)	46.9 (2.4)
Sunflower (n = 48)	RL	−1.1 (0.2)	87.3 (5.9)	−2.5 (3.8)	14.3 (3.1)	3.9 (0.9)	1.8 (0.6)
	RFW	−1.0 (0.2)	84.8 (7.3)	−0.8 (4.1)	17.8 (4.7)	4.2 (1.2)	1.8 (0.7)
	SFW	−1.7 (0.9)	39.0 (9.7)	−1.7 (2.4)	61.7 (25.2)	176 (9)	72.7 (8.1)
Rapeseed oil (n = 84)	RL	−1.0 (0.1)	86.6 (2.7)	−0.1 (3.0)	3.4 (0.4)	0.9 (0.2)	0.4 (0.1)
	RFW	−1.4 (0.2)	91.6 (1.9)	−0.4 (3.0)	3.2 (0.3)	1.2 (0.2)	0.6 (0.1)
	SFW	−1.5 (0.2)	61.8 (2.4)	−2.4 (2.7)	7.7 (1.0)	3.0 (0.5)	1.7 (0.4)
White mustard (n = 84)	RL	−0.6 (0.1)	91.5 (4.9)	0.0002 (2.9)	2.2 (0.5) [1]	0.2 (0.1)	0.1 (0.04) [1]
	RFW	−1.0 (0.2)	91.7 (2.4)	−0.03 (2.9)	1.6 (0.2)	0.4 (0.1)	0.2 (0,1)
	SFW	−0.7 (0.1)	63.9 (5.8)	0.04 (2.9)	7.6 (2.6)	1.0 (0.4)	0.3 (0.2)
Sugar beet (n = 60)	RL	−0.6 (0.2)	83.8 (6.1)	−0.1 (3.2)	2.1 (0.6)	0.2 (0.1)	0.05 (0.05)
	RFW	−1.1 (0.5)	88.6 (2.4)	−0.02 (3.2)	0.7 (0.4)	0.2 (0.2)	0.1 (0.1)
	SFW	−0.7 (0.2)	77.0 (4.3)	−0.1 (3.2)	1.2 (0.3)	0.2 (0.1)	0.05 (0.06)

n—number of plants per treatment; RL—root length; RFW—root fresh weight; SFW—shoot fresh weight; *B*—the slope of the line; *D*—upper limit; *C*—lower limit. [1] These data have already been mentioned in [36], but only as a justification for the method used and without any further comment that could be related to this work.

Wheat was the least sensitive crop, with a root growth inhibition of 11.1–65.96% and 22.53–73.99% for root length and root fresh weight, respectively (with no significant inhibition at concentrations below 30 ppbw). Inhibition of shoot fresh weight was observed at imazethapyr concentrations >30 ppbw but in a very narrow range (0.8–26.15%). These results are consistent with those of Moyer and Esau [58] and with the fact that no apparent

injuries occurred in wheat sown one year after imazethapyr application [66,68,69]. Wheat could be classified as moderately sensitive to imazethapyr.

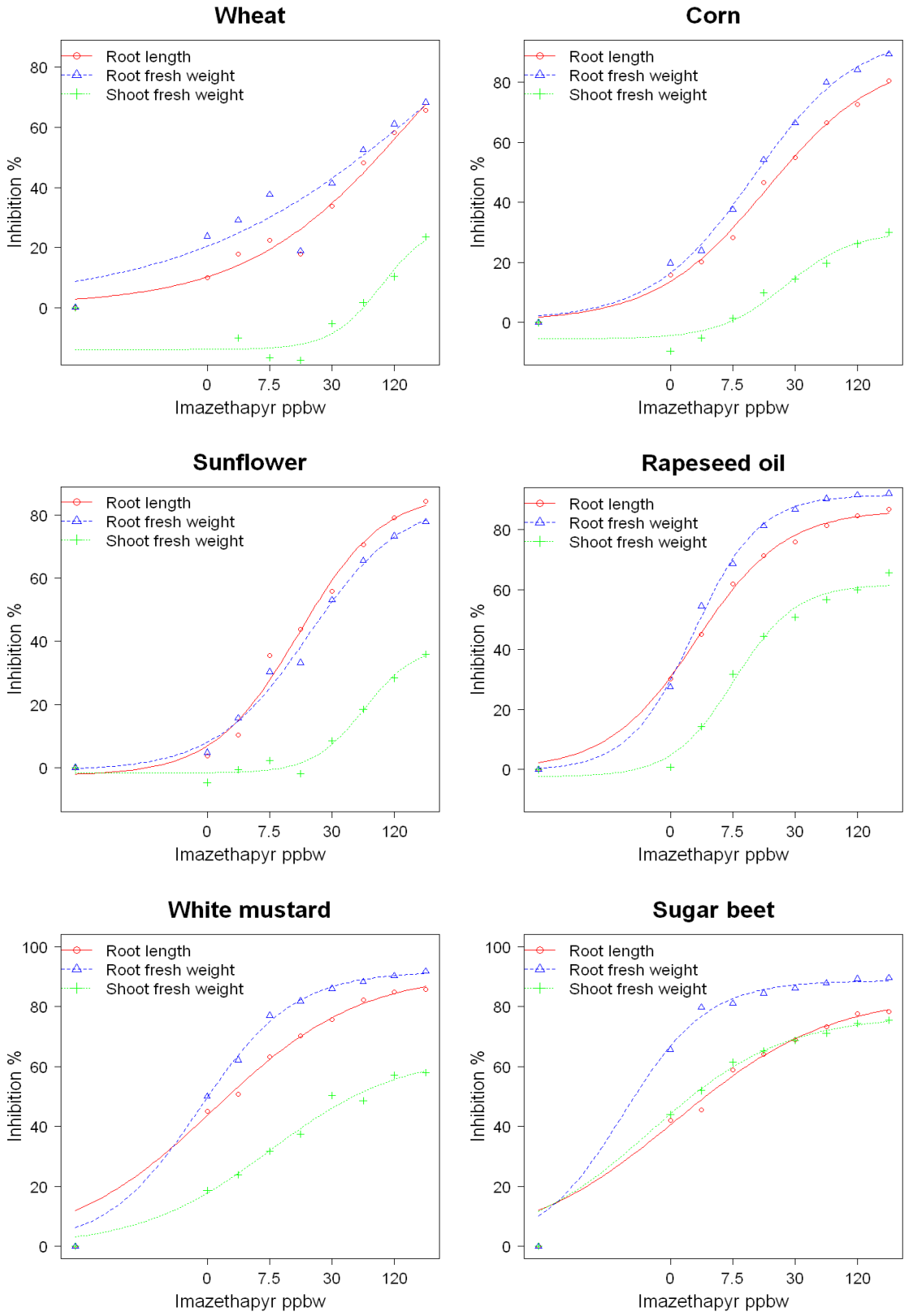

Figure 1. Dose–response curves for crop sensitivity to imazethapyr. The regression lines are plotted using Equation (1), and the parameter values are recorded in Table 1.ppbw—parts per billion by weight (was used as equivalent for μg/kg due to software requirements).

The root growth inhibition of corn ranged from 17.21–80.61% as well as 23.32–89.78% for root length and root fresh weight, respectively. Shoot fresh weight inhibition ranged from 1.5–30.48% (Table 1 and Figure 1), with hormesis manifested at two initial concentrations (i.e., 1.875 and 3.75 ppbw).

The inhibition of sunflower root growth ranged from 7.74–84.39% as well as 5.97–78.21% for root length and root fresh weight, respectively. Shoot fresh weight inhibition was observed at imazethapyr concentrations greater than 30 ppbw ranging from 9.19–36.92% (Table 1 and Figure 1), with hormesis occurring at initial concentrations.

Corn and sunflower were found to be more sensitive than wheat and could be classified as sensitive to imazethapyr. A similar classification was found by Moyer and Esau [58], Onofri [49], and Brighenti et al. [71]. The ED_{50}, ED_{20} and ED_{10} values obtained for these two crops were very similar for root growth parameters, while the differences were more pronounced for shoot fresh weight (Table 1). Significantly, a stimulatory effect of low imazethapyr concentrations on shoot growth (i.e., shoot fresh weight) was observed in both maize and sunflower, as has been observed previously for the same species–herbicide combinations as well as for several other herbicides [49]. Corn has often been used as a test plant (especially with ALS-inhibiting herbicides). There are numerous and varied data describing the sensitivity of corn to imazethapyr [16,19,57,85,87]. These results agree well with the data obtained in this study, with some differences due to soil type (in terms of organic matter content) and hybrids used.

Rapeseed oil, white mustard, and sugar beet were the most sensitive to imazethapyr and, therefore, can be classified as very sensitive crops. There were some differences between rapeseed oil on the one hand and white mustard and sugar beet on the other, with respect to ED_{50}, ED_{20}, and ED_{10} for root and shoot growth parameters; however, overall, their sensitivities were in the same range (Table 1 and Figure 1). These results are in agreement with the findings of Onofri [49], who had reported that rapeseed oil is the most sensitive and, thus, the most suitable test plant for this type of research. Similarly, Szmigielska and Schoenau [88], in developing a reliable bioassay for the detection of imazethapyr residues, found that the detection limit for canola root length was 0.3 µg a.i./kg soil. This is quite close to the ED_{10} value for rapeseed oil root length (0.39 µg a.i./kg soil) determined in this study, considering the differences between the two varieties, and especially considering that the ED_{10} value was considered as NOEL (no observable effect level), i.e., the only safe herbicide residue value for future sowings [49]. The high sensitivity of rapeseed oil was also confirmed by measuring the dry matter of roots and shoots [86]. The results obtained are somewhat different due to the parameter measured, the varieties used, and probably the soil, the properties of which are unknown. The sensitivity of white mustard to imazethapyr was studied earlier, but only on the basis of the fresh weight of shoots [42]. The ED_{50} value determined there differed somewhat from the ED_{50} value determined here because the soil types differed significantly in terms of organic matter content. Nevertheless, white mustard proved to be a good test plant for studying the degradation of imazethapyr in the bioassay [36]. Sugar beet was the most sensitive to imazethapyr for all measured parameters. This is in agreement with the results of some authors [49,58] and with the results on the injury of sugar beet sown one and/or two years after imazethapyr application [60–62,73].

It is well-known that different plant varieties respond differently to herbicides in terms of selectivity or phytotoxicity [57,85]. However, it is not clear whether this phenomenon is due to micro-morphological characteristics, as seen in some weeds [89], or subtle differences in physiology, gene expression, and other factors, making this topic an interesting opportunity for further research.

3.2. Differences in Crop Sensitivity to Imazethapyr Based on Anatomical Parameters

In general, the results indicate that the changes in root anatomy are consistent with the observed degrees of sensitivity of the tested crops to imazethapyr. The changes in root anatomy observed in this study are a direct result of the mode of action of imazethapyr. In addition to the primary inhibition of branched-chain amino acid synthesis (valine, leucine, and isoleucine), mitosis is known to arrest in interphase in the root meristem of roots that have absorbed an ALS inhibitory herbicide [84]. Thus, there are fewer meristematic cells capable of cell division and subsequent differentiation. Another consequence is the shortening of the meristematic and elongation zones, reducing the distance between the root tip and the zone of absorption and the distance between the root tip and the point where the primordium of the lateral root appears. There were no statistically significant differences between the control and the treatments defined as ED_{10} and ED_{20} for most of the measured parameters and the tested crops, while there were always significant differences between the control and the 240 µg a.i./kg treatment. Sensitive crops showed a higher degree of variation in response to different concentrations of imazethapyr, as well as more frequent differences between the applied treatments (Table 2 and Figures 2–6).

Table 2. Imazethapyr effects on root anatomy (n = 15).

Plant Species	Imazethapyr Treatments	RCL (µm) Mean ± (SD) (SE)	AZ (mm) Mean ± (SD) (SE)	LRF (mm) Mean ± (SD) (SE)	$LRPN_o$/ 0.5 mm Root Lenght Mean ± (SD) (SE)
Corn	Control	49.80 (3.30) ^{a}	2.58 (0.46) ^{a}	10.53 (1.16) ^{a}	0.0 (0) ^{a}
	ED_{10}	36.85 (4.30) ^{b}	2.60 (0.47) ^{a}	9.89 (0.71) ^{a}	0.0 (0) ^{a}
	ED_{20}	34.69 (3.23) ^{b}	2.15 (0.11) ^{a}	9.82 (0.24) ^{a}	0.0 (0) ^{a}
	RAD	21.97 (3.77) ^{c}	0.27 (0.03) ^{b}	4.99 (0.42) ^{b}	1.45 (0.76) ^{b}
$HSD_{0.05/0.01}$		(2.5994) 7.72/10.11	(0.2377) 0.71/0.92	(0.5115) 1.52/1.99	(0.2684) 0.51/0.41
Sunflower	Control	26.21 (1.41) ^{a}	2.23 (0.13) ^{a}	12.40 (0.93) ^{a}	0.55 (0.3) ^{a}
	ED_{10}	24.08 (4.28) ^{a}	2.42 (0.28) ^{a}	11.90 (1.43) ^{a}	0.32 (0.10) ^{a}
	ED_{20}	21.99 (2.62) ^{a}	2.06 (0.29) ^{a}	5.98 (1.27) ^{b}	0.45 (0.13) ^{a}
	RAD	9.81 (1.44) ^{b}	1.46 (0.33) ^{b}	2.58 (0.40) ^{c}	5.45 (0.40) ^{b}
$HSD_{0.05/0.01}$		(1.9134) 5.68/7.44	(0.1906) 0.57/0.74	(0.7645) 2.27/2.97	(0.1868) 0.56/0.73
Wheat	Control	35.71 (7.82) ^{a}	2.32 (0.73) ^{a}	10.54 (2.15) ^{a}	0.0 (0) ^{a}
	ED_{10}	41.36 (2.60) ^{a}	1.93 (0.23) ^{a}	7.74 (3.36) ^{b}	0.0 (0) ^{a}
	ED_{20}	40.53 (5.27) ^{a}	1.82 (0.37) ^{a}	5.60 (0.33) ^{bc}	0.0 (0) ^{a}
	RAD	15.58 (1.95) ^{b}	0.39 (0.04) ^{b}	4.94 (1.06) ^{c}	0.45 (0.21) ^{b}
$HSD_{0.05/0.01}$		(3.5275) 10.48/13.72	(0.2992) 0.89/1.16	(0.8642) 2.57/3.36	(0.0736) 0.22/0.29
Sugar beet	Control	17.26 (4.30) ^{a}	1.51 (0.22) ^{a}	4.33 (0.46) ^{a}	1.75 (0.56) ^{a}
	ED_{10}	22.10 (4.21) ^{ab}	1.13 (0.10) ^{b}	3.08 (0.18) ^{b}	2.45 (0.44) ^{ab}
	ED_{20}	25.73 (5.51) ^{ab}	0.70 (0.10) ^{c}	2.85 (0.46) ^{b}	4.00 (1.41) ^{bc}
	RAD	31.30 (5.58) ^{b}	0.62 (0.06) ^{c}	1.08 (0.11) ^{c}	4.50 (1) ^{c}
$HSD_{0.05/0.01}$		(3.4951) 10.38/13.59	(0.0967) 0.29/0.38	(0.2438) 0.72/0.95	(0.6621) 1.97/2.58
White mustard	Control	28.94 (1.88) ^{a}	1.91 (0.37) ^{a}	8.40 (0.84) ^{a}	0.45 (0.17) ^{a}
	ED_{10}	25.22 (3.07) ^{a}	0.61 (0.10) ^{b}	3.02 (0.22) ^{b}	2.75 (0.96) ^{b}
	ED_{20}	34.13 (3.99) ^{a}	0.47 (0.04) ^{b}	2.73 (0.29) ^{b}	3.55 (0.78) ^{bc}
	RAD	52.86 (15.50) ^{b}	0.33 (0.25) ^{b}	1.58 (0.29) ^{c}	5.00 (0.82) ^{c}

Table 2. *Cont.*

Plant Species	Imazethapyr Treatments	RCL (µm) Mean ± (SD) (SE)	AZ (mm) Mean ± (SD) (SE)	LRF (mm) Mean ± (SD) (SE)	$LRPN_o$/ 0.5 mm Root Lenght Mean ± (SD) (SE)
$HSD_{0.05/0.01}$		(5.8004) 17.23/22.56	(0.1621) 0.48/0.63	(0.3401) 1.01/1.32	(0.5264) 1.56/2.05
Rapeseed oil	Control	25.35 (5.44) ^{a}	1.43 (0.33) ^{a}	7.90 (0.35) ^{a}	3.10 (0.26) ^{a}
	ED_{10}	24.15 (1.80) ^{ab}	0.36 (0.05) ^{b}	2.63 (0.26) ^{b}	4.30 (0.42) ^{b}
	ED_{20}	22.89 (1.89) ^{abc}	0.27 (0.06) ^{b}	2.34 (0.10) ^{bc}	4.90 (0.27) ^{bc}
	RAD	16.84 (2.39) ^{c}	0.11 0.05) ^{b}	2.02 (0.16) ^{c}	5.40 (0.36) ^{c}
$HSD_{0.05/0.01}$		(2.2967) 6.82/8.93	(0.1222) 0.36/0.48	(0.1666) 0.50/0.65	(0.2363) 0.70/0.92

n—number of plants per plant species and treatment (see Section 2 for further explanations); SD—standard deviation; SE—standard error; ^{a,b,c}—values marked with different letters are statistically significantly different RCL—root cap length; AZ—distance from the root tip to the beginning of the absorption zone; LRF—distance from the root tip to the beginning of the lateral root formation; $LRPN_o$—number of lateral root primordia; ED_{10}—corresponds to the available residue level causing 10% inhibition of root length; ED_{20}—corresponds to the available residue level causing 20% inhibition of root length; RAD—recommended application dose, i.e., 240 µg a.i./kg soil.

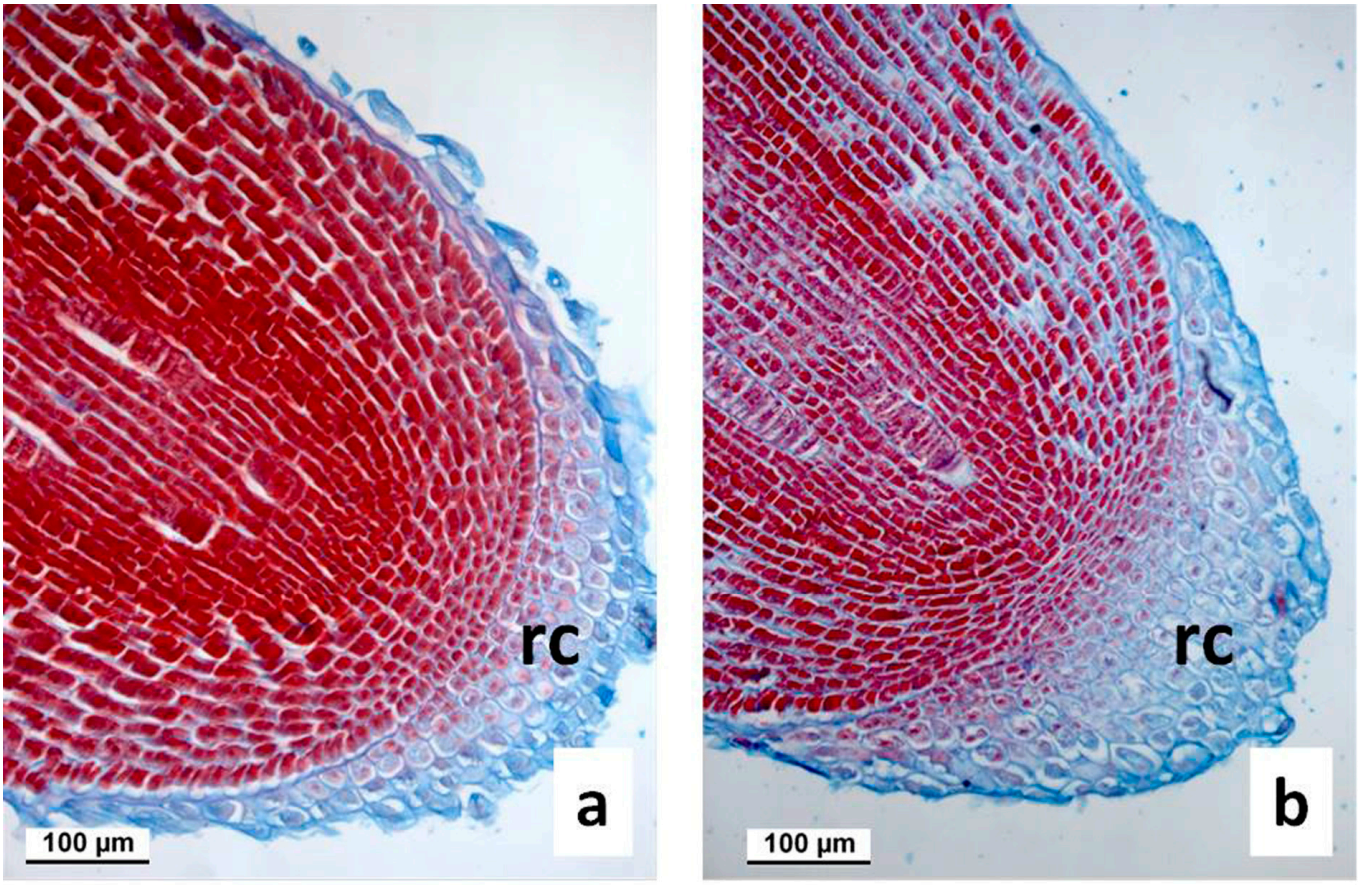

Figure 2. Effect of imazethapyr on root anatomy of maize(**a**) ED_{20} treatment; (**b**) control; rc—root cap (The Figure is representative of each treatment).

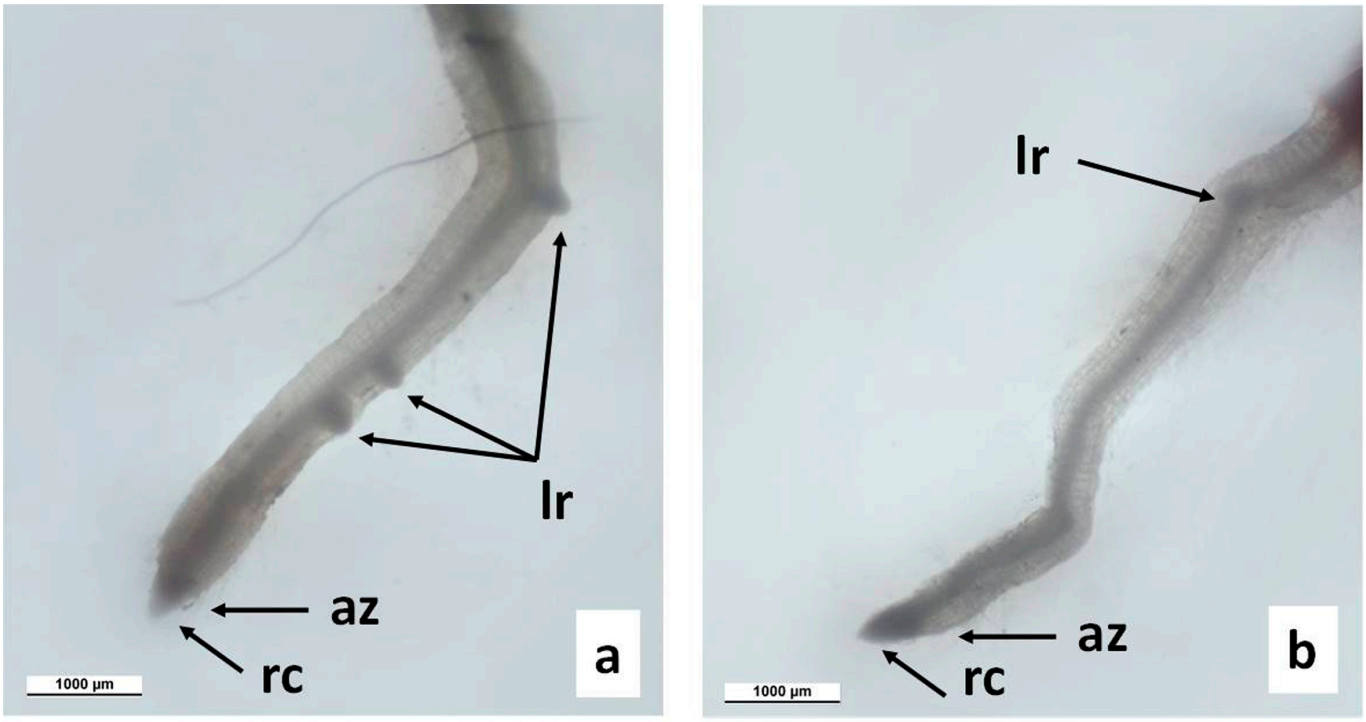

Figure 3. Effect of imazethapyr on root anatomy of rapeseed oil: (**a**) 240 µg a.i./kg soil; (**b**) EC_{20} treatment;rc—root cap; az—beginning of absorption zone; lr—lateral root primordia (The Figure is representative of each treatment).

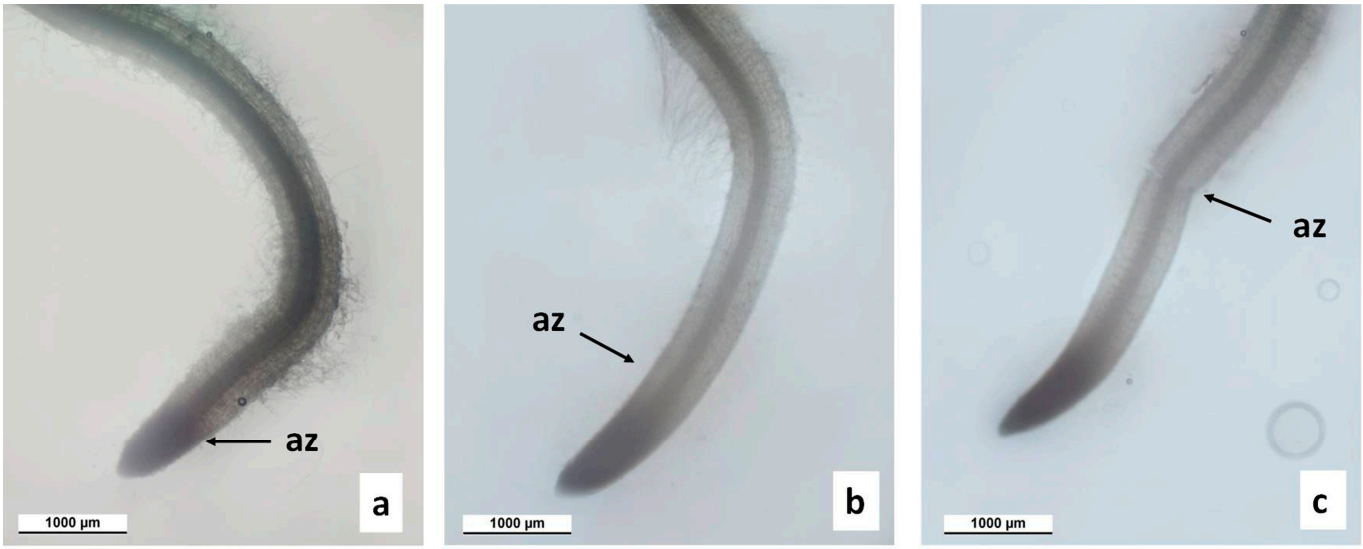

Figure 4. Effect of imazethapyr on root anatomy of wheat: (**a**) 240 µg a.i./kg soil; (**b**) ED_{20} treatment; (**c**) ED_{10} treatment; az—beginning of the absorption zone (The Figure is representative of each treatment).

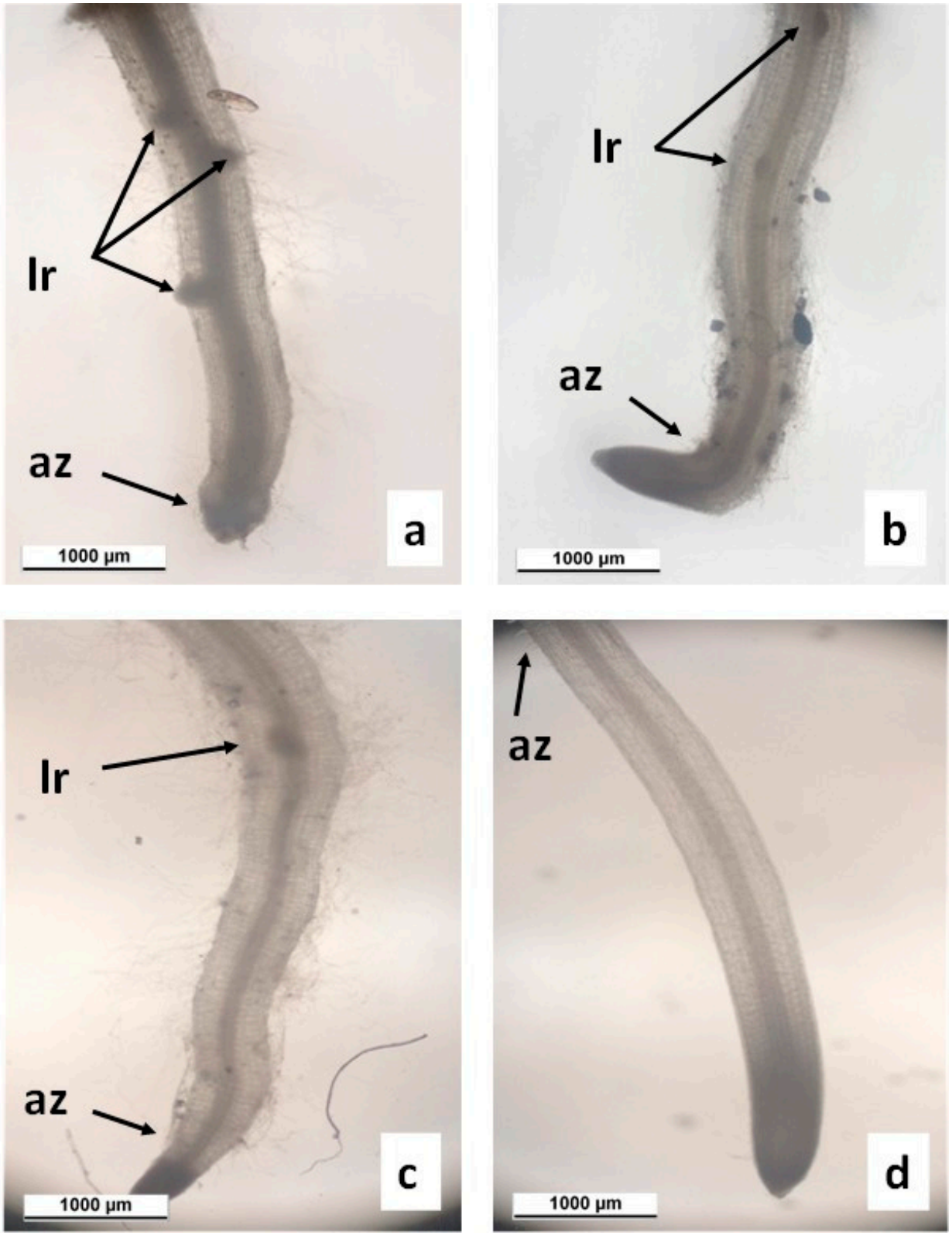

Figure 5. Effect of imazethapyr on root anatomy of white mustard: (**a**) 240 µg a.i./kg soil; (**b**) ED_{20} treatment; (**c**) ED_{10} treatment; (**d**) control; az—beginning of absorption zone; lr—lateral root primordia (The Figure is representative of each treatment).

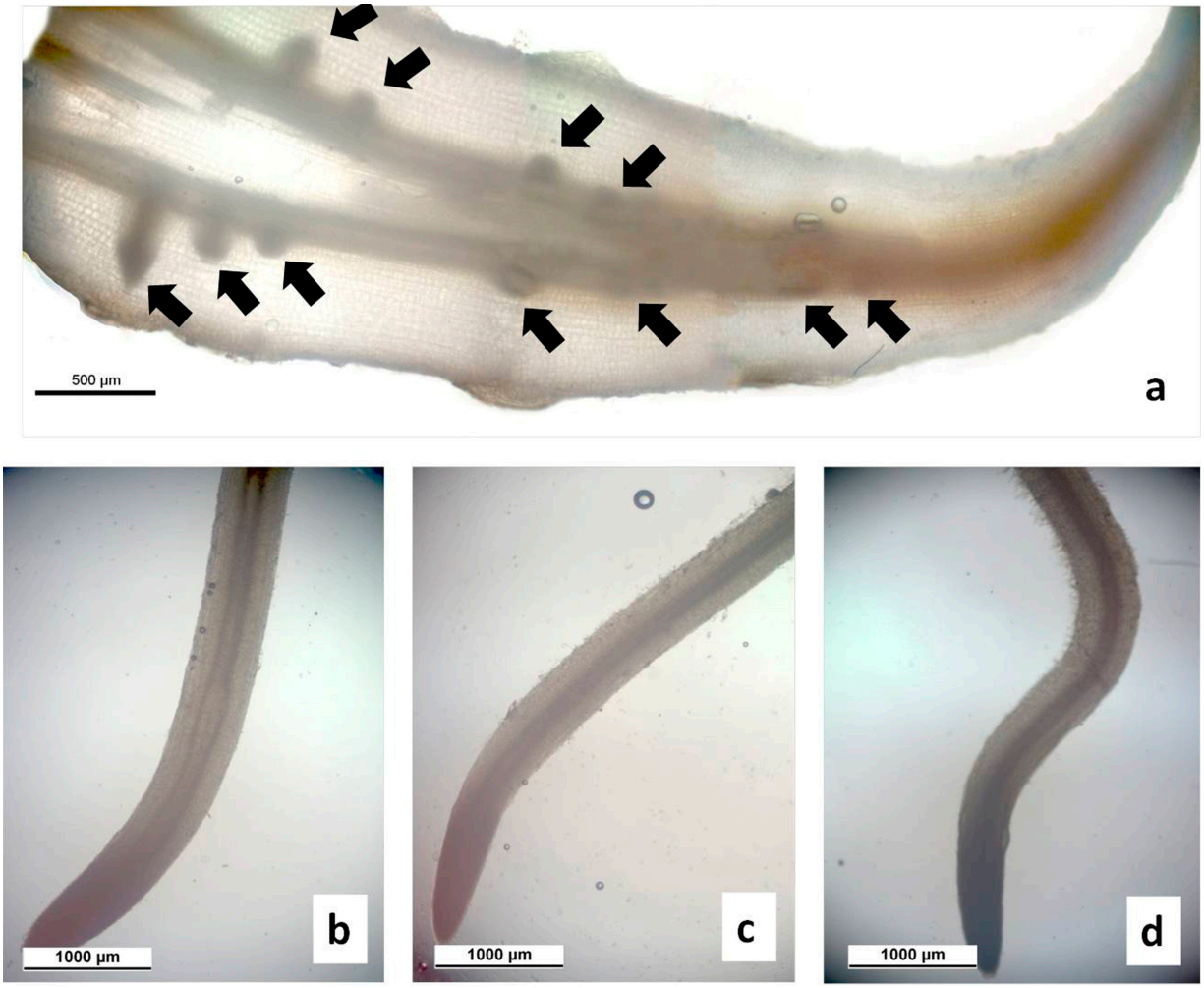

Figure 6. Effect of imazethapyr on root anatomy of sunflower: (**a**) 240 µg a.i./kg soil; (**b**) ED_{20} treatment; (**c**) ED_{10} treatment; (**d**) control; lateral root primordia are indicated by arrows(The Figure is representative of each treatment).

In terms of the root cap length (RCL), there were no significant differences between the control, ED_{10}, and ED_{20} treatments in all crops except maize (control vs. ED_{10}: $p = 0.002$; control vs. ED_{20}: $p < 0.001$) (Table 2 and Figure 2). In all cases, there were statistically significant differences between the control and the treatment with the recommended application dose (RAD)($p = 0.014$, 0.007 and 0.008 for rapeseed oil, white mustard, and sugar beet, respectively, and $p < 0.001$ for maize, sunflower, and wheat). The differences between ED_{10} and RAD were statistically significant in all crops except sugar beet ($p = 0.034$ and 0.002 for rapeseed oil and white mustard, respectively, and $p < 0.001$ for maize, sunflower, and wheat), as well as in the case of ED_{20} and RAD ($p = 0.032$ and 0.002 for white mustard and maize, respectively; $p < 0.001$ for sunflower and wheat) (there were no significant differences in sugar beet and rapeseed oil). The root cap cells protect other tissues while the root continues to grow in the soil. They secrete a mucilaginous substance that helps the roots move through the soil. Root cap cells are in a continuous process of decay due to their constant friction with soil particles and are periodically replaced by new cells. It is known that ALS-inhibiting herbicides also inhibit cell division, which in turn can lead to less root cap formation. This was confirmed in all plants tested by treatment with RAD. A steady decrease in the root cap length was also observed in corn, sunflower, and rapeseed oil at higher imazethapyr concentrations. However, the most interesting result was observed in the most sensitive crops (i.e., sugar beet and white mustard), where the root cap length was longer in the herbicide treatments than in the control and were longest when the

recommended application dose was reached. It could be hypothesized that growth arrest was more pronounced in these plants due to reduced cell division (reductions in root length and fresh weight were most pronounced) so that root's cap cells were not exposed to friction with soil particles to the same extent as in less sensitive plants whose roots grew more vigorously. In the most sensitive plants, fewer cells decayed as a result of the reduced growth than had been produced by mitosis, so that these cells subsequently differentiated and formed root caps.

Regarding the distance from the root tip to the beginning of the absorption zone (AZ), there were no significant differences between the control and ED_{10} treatment and between the control and ED_{20} treatment in all moderately sensitive and sensitive crops.However, there were statistically significant differences in very sensitive crops (control vs. ED_{10}: $p = 0.009$ for sugar beet and $p < 0.001$ for white mustard and rapeseed oil; control vs. ED_{20}: $p < 0.001$ for the same three crops) (Table 2 and Figures 3–5). These results indicate that the concentrations of imazethapyr residue that are as high as their ED_{20} value do not significantly reduce meristematic cell activity in sensitive and moderately sensitive crops and, therefore, can be accepted as safe for seeding. In all cases, the differences between the control and the recommended application dose were statistically significant ($p = 0.008$ for sunflower; $p < 0.001$ for all other crops). The comparison between treatments ED_{10} and ED_{20} showed significant differences only in sugar beet ($p = 0.004$). The differences between ED_{10} and RAD were statistically significant in sugar beet and less sensitive crops ($p = 0.001$ for sugar beet, sunflower, and wheat and $p < 0.001$ for maize), while in the case of ED_{20} and RAD, the differences were significant only in the less sensitive crops ($p = 0.0037$, 0.002, and <<0.001 for sunflower, wheat, and maize, respectively). Based on these results, some injury in the form of root length reduction is expected only in very sensitive crops exposed to imazethapyr residues at ED_{20}. However, this may be overcome if root growth occurs below the herbicide layer in the soil or if there is leaching of herbicides or increased degradation due to environmental factors. Thus, this remains to be investigated because there are data on both severe injury (effects on plant height, fresh weight, number of leaves per plant, and yield measured 75 days after seeding or at maturity) and mild phytotoxicity (which declined within one month after seeding) in Indian (brown) mustard seeded in rotation after imazethapyr application. Phytotoxicity depends on soil type but more so on rainfall during the growing season (between the time of herbicide application and mustard seeding), which affects both the degradation and leaching of imazethapyr [90,91].

Regarding the distance from the root tip to the beginning of the lateral root formation (LRF), there were significant differences between the control and the ED_{10} treatment in all crops except maize and sunflower ($p = 0.031$ and 0.001 for wheat and sugar beet, respectively, and $p < 0.001$ for white mustard and rapeseed oil), while there was no difference between the control and the ED_{20} treatment only in maize ($p < 0.001$ for all other crops) (Table 2). The comparison between the ED_{10} and ED_{20} treatments showed significant differences only in sunflower ($p < 0.001$) (Table 2). The differences between ED_{10} and the recommended application dose were statistically significant in all crops ($p = 0.006$, 0.015 and 0.031 for white mustard, rapeseed oil, and wheat, respectively; $p < 0.001$ for maize, sunflower, and sugar beet), as well as in the case of ED_{20} and RAD ($p = 0.004$ and 0.024 for sunflower and white mustard, respectively; $p < 0.001$ for maize and sugar beet);there were no significant differences in wheat and rapeseed oil (Figures 3–5). These results agree well with the expected visible reduction in root length in very sensitive crops exposed to imazethapyr residues at ED_{20} but with the same possibility of overcoming them by root growth below the herbicide layer in the soil or by the leaching of the herbicide or an increased herbicide degradation.

There were significant differences in the number of lateral root primordia ($LRPN_o$) per 0.5 mm root length between the control and EC_{10} in white mustard ($p = 0.004$)and rapeseed oil($p = 0.001$), while there were statistically significant differences between the control and ED_{20} in all very sensitive crops ($p = 0.024$ for sugar beet; $p < 0.001$ for white mustard and rapeseed oil) (Table 2). The comparison between the ED_{10} and ED_{20} treatments showed

no significant differences, while there were significant differences between ED_{10} and the recommended application dose in all crops (p = 0.001, 0.003, 0,004 and 0.005 for maize, rapeseed oil, sugar beet and white mustard, respectively; $p < 0.001$ for sunflower and wheat). Between ED_{20} and RAD, the differences were significant only in the less sensitive crops (p = 0.001 for maize; $p < 0.001$ for sunflower and wheat) (Figures 3 and 5–7). This is further confirmation that less sensitive crops can tolerate imazethapyr residues up to ED_{20}.

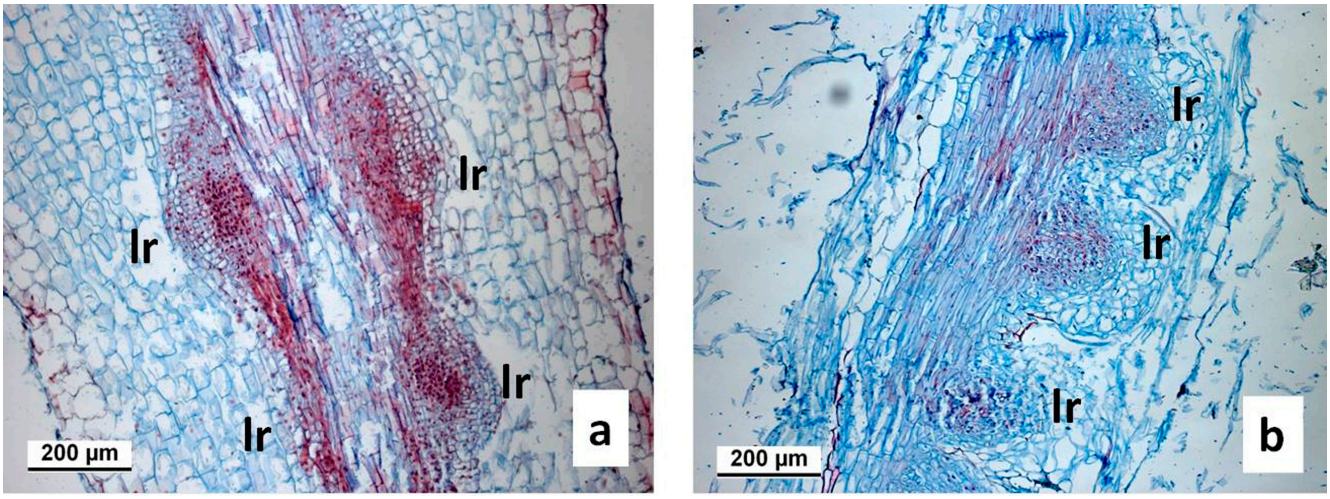

Figure 7. Effect of imazethapyr (240 µg a.i./kg soil) on root anatomy: (**a**) sunflower; (**b**) rapeseed oil; lr—lateral root primordia.

As for the very sensitive crops and the increase in the number of lateral root primordia, this could be discussed in light of the fact that imazethapyr induces significant morphological changes in root growth and the development associated with altered water use (i.e., these plants absorb less water due to reduced root growth and especially reduced root hair formation). Plants that take up less water are, to some extent, similar to plants exposed to drought. Two types of changes were observed in these plants. First, continuous amino acid synthesis was observed throughout the duration of the stress [92]. Such a response was observed not only in plant shoots but also in roots, suggesting that, overall, protein synthesis is either not disturbed or disturbed only to a minor extent [93]. The herbicides are considered an obvious stressor, but the fact is that some amino acid synthesis was increased in the presence of imazapyr [94], and the total protein content in roots exposed to imazethapyr did not correlate with herbicide concentration [95]. This allows for shoot development despite the inhibition of branched-chain amino acid synthesis and corresponding proteins, even when the plant roots are exposed to residues of an ALS-inhibiting herbicide. For this reason, the fresh weight of shoots is not as important in determining the sensitivity of different plants to the ALS-inhibiting group of herbicides (e.g., imazethapyr). On the other hand, Jupp and Newman [96] found a 3–8-fold increase in the number of lateral roots in *Lolium perenne* L. under increased drought stress compared to the control, as well as root tip decay. They also cite that it was previously found that soybean roots adapt to drought by increasing the number of lateral roots 2–3-fold. Considering that the root tip meristem inhibits lateral root formation at a certain distance from the root tip and that root tip removal stimulates lateral root formation, these authors hypothesized that root tip decay might be responsible for the development of new lateral roots and that moderate drought (in which root tips remain vital) might stop hormone synthesis and activity. This agrees very well with Shaner [84], who pointed out that imidazolinones have a secondary effect on the overall hormonal status of the plant (their site of action is the meristematic tissue, the very tissue where hormones are produced and active) so that plant death is a consequence of indirect disruption of hormonal balance and energy flow. This statement could be complemented by another finding from this study. Indeed, the early

differentiation of vascular tissue was observed in the primordium of lateral roots when treated with the recommended application dose (in all plants tested) (Figure 8), but in no other treatment, not even in lateral roots that had reached some initial growth.

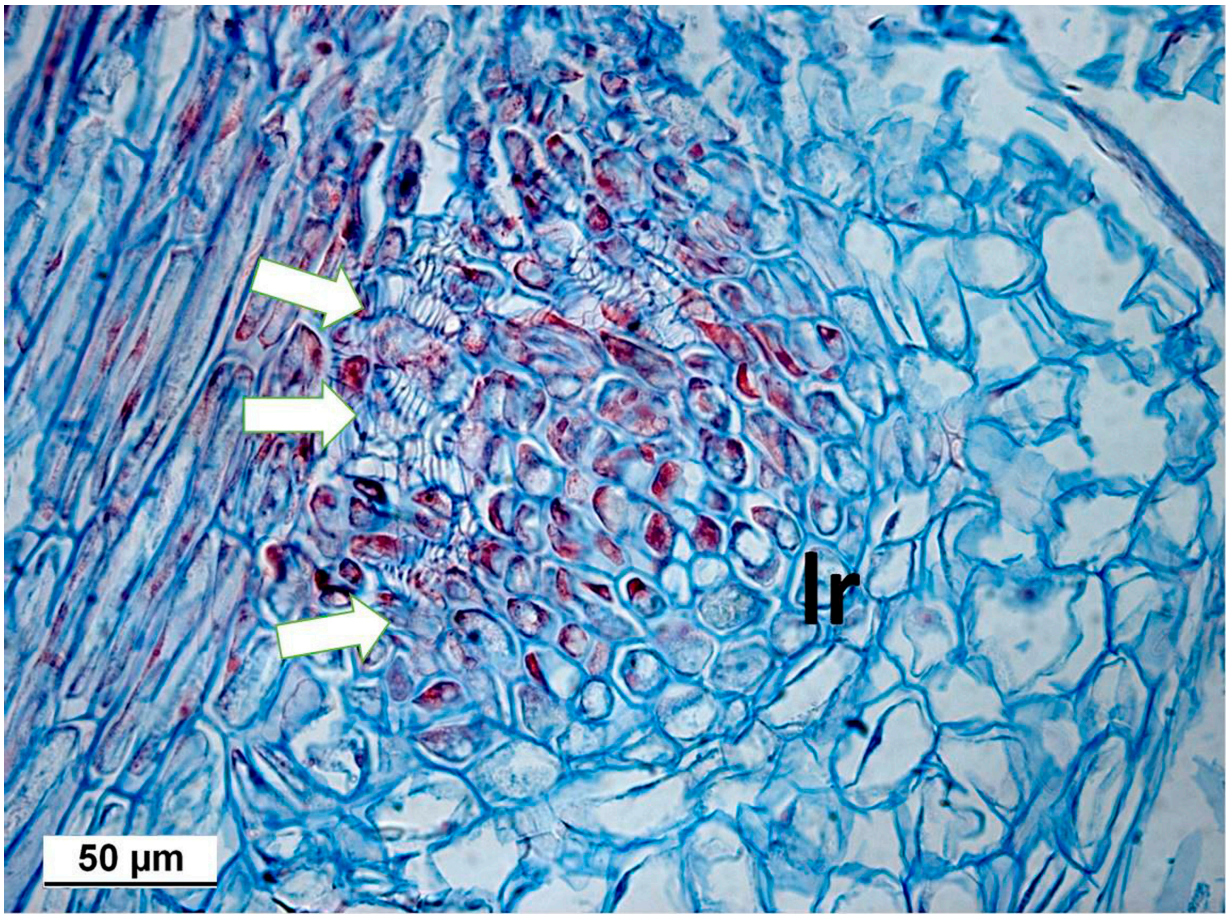

Figure 8. Early differentiation in lateral root primordia of rapeseed oil, induced by imazethapyr (lr—lateral root primordia; differentiation of vascular tissue is indicated by arrows).

In the scientific literature, the effects of imazethapyr on mitotic index reduction and genotoxic activity in non-target plants are well-documented, as evidenced by an increased frequency of chromosomal aberrations and micronucleus in the meristematic cells of roots exposed to imazethapyr concentrations as low as 0.001 mg a.i./L [97,98]. The authors observed a significant delay between prophase and metaphase, suggesting that the mitotic process was blocked at the end of prophase, leading to an accumulation of dividing cells at this stage. However, there are no findings on the early differentiation of vascular tissue, which requires further investigation and explanation.

4. Conclusions

Based on the measured morphological parameters, there are clear and obvious differences in sensitivity to imazethapyr among the selected crops, which can be divided into three categories: very sensitive (sugar beet > white mustard ≥ rapeseed oil), sensitive (maize ≥ sunflower), and moderately sensitive (wheat). The most sensitive and reliable parameters were root length and root fresh weight, which should be considered relevant in any bioassay with ALS-inhibiting herbicides. The main finding is that imazethapyr residue levels corresponding to the ED_{20} values for root length of corn, sunflower, and wheat could be proposed as acceptable residue levels for the safe seeding of these crops. These residue levels do not cause visible damage or changes in the root anatomy of these crops. Further

clarification is needed for rapeseed oil, white mustard, and sugar beet, particularly for those crops whose root systems cannot grow below the herbicide residue zone in the soil.

Author Contributions: Conceptualization, K.J.-R.; methodology, K.J.-R. and D.R.; software, K.J.-R. and D.R.; validation, K.J.-R. and D.R.; formal analysis, K.J.-R.; investigation, K.J.-R. and D.R.; resources, K.J.-R. and D.R.; data curation, K.J.-R.; writing—original draft preparation, K.J.-R.; writing—review and editing, K.J.-R. and D.R.; supervision, K.J.-R.; project administration, K.J.-R.; funding acquisition, K.J.-R. and D.R. All authors have read and agreed to the published version of the manuscript.

Funding: The research in this paper is part of the project of NIO in 2023:451-03-47/2023-01/200116 (to D.R), which is financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Radenko Radosevic for his help in microslide preparations and Tajma Hodžić for her assistance with the statistics.

Conflicts of Interest: The authors declare no conflict of interest.

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