

## Article

# Stimulation of Early Post-Emergence Growth of *Alopecurus myosuroides* and *Apera spica-venti* Following Spray Application of ACCase Inhibitors

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**Abstract:** Resistance of blackgrass (*Alopecurus myosuroides* Huds.) and silky bentgrass (*Apera spica-venti* (L.) P. Beauv.) to ACCase inhibitors is a serious issue in winter cereals throughout Europe, especially as hormesis induced by herbicides has been observed in some populations. According to the literature, growth-stimulating herbicide rates are on the rise. The aim of this study was to assess the responses of *A. myosuroides* and *A. spica-venti*, which are potentially resistant to ACCase inhibitors, to fenoxaprop-P-ethyl and pinoxaden applied at rates up to 8 times greater than their registered rates. The reaction of *A. myosuroides* to fenoxaprop-P-ethyl and pinoxaden resulted in an increase in biomass gain in six and four populations, respectively. In one population of *A. myosuroides*, this increase was statistically significant (46.4% and 55.3%). All three potentially resistant *A. spica-venti* populations tested were at least partially stimulated by fenoxaprop-P-ethyl, while pinoxaden only stimulated the APSII population (significant increase of 43.8%). Predictions of the possible impact of herbicides on the reproductive potential of the tested populations allow genotypes to be identified whose reproduction may be stimulated by the herbicides. The results of this study indicate that the tested populations can induce mechanisms that reduce the negative impact of the applied herbicides, with some populations demonstrating the effect of stimulating the accumulation of biomass in the treated plants.

**Keywords:** blackgrass; silky bentgrass; graminicides; biomass; fitness; hormesis



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## 1. Introduction

Weeds are the most important biotic factor affecting crop production in the world. Therefore, the prevention of crop losses caused by undesirable vegetation in winter cereal crops, especially wheat, is of great importance for the sustainable development of food production in the world. Weed pressure affecting crop growth and yield is a relevant problem in modern agriculture [1,2]. Herbicides that target specific metabolic pathways, e.g., acetolactate synthase (ALS), acetyl-coenzyme A carboxylase (ACCase) and D1 protein in photosystem II [3], play a crucial role in all the latest weed control programs. Consequently, their frequent use combined with a lack of or insufficient rotation and the use of herbicides with different modes of action lead to resistance developing in weed populations.

Blackgrass (*Alopecurus myosuroides* Huds.) and silky bentgrass (*Apera spica-venti* L.) are weeds commonly found in winter crops. Even sensitive biotypes of both of these weeds pose a significant competitive threat to crops, especially for winter wheat [4,5]. *A. spica-venti* is an annual overwintering grass that is considered one of the most important and widespread weeds in winter cereals in Central and Northern Europe [6]. One plant can produce up to 16,000 seeds; seed shed has been reported in the field approximately two weeks before grain harvest [4]. Competition due to *A. spica-venti* presence in the field can reduce yield by up to 30% [7]. Due to the high degree of morphological and genetic variability [8], *A. spica-venti* adapts to the realities of current agriculture, leading to a quick selection

of biotypes resistant to ALS, ACCase and photosystem II inhibitors [9–11]. The logistic regression model described in 2013 by Masa et al. [12] for the whole of Europe showed that crop rotation dominated by winter cereals and a conventional tillage system significantly increase the likelihood of *A. spica-venti* resistance selection. In recent years, due to the increased share of winter plants in the structure of agriculture crops, *A. myosuroides* has become one of the most noxious herbicide-resistant weeds in Europe [5,13]. Its populations can be very diversified in terms of growth rate. With seed production of 935–3337 seeds plant<sup>-1</sup> [4] germinating predominantly in autumn (only about 20% of seeds germinate in spring), this weed species poses a serious risk to winter crops [14]. Both species, but *A. myosuroides* in particular, are very competitive in relation to the winter crops. They significantly reduce crop yield because they very successfully compete with crops for habitat resources. Additionally, unfavorable weather conditions, such as high temperatures or drought, weaken crops and make them less likely to compete effectively with weeds [15,16]. For this reason, the damage threshold for these species is less than 15 pieces per square meter [4]. *A. myosuroides* populations that are herbicide-resistant and well adapted to local agricultural conditions have been found in numerous European countries [5,17,18].

Inspection of the available data in the literature reveals common agreement that mutation-based resistance to pesticides generates an adaptation cost in the absence of selection pressure [19]. However, an increase in the fitness of herbicide-resistant populations has also frequently been observed [20]. Finally, resistance acquisition can lead to the development of resistant and highly productive weed biotypes, where attempts at chemical control unintentionally promote the growth and development of such weed biotypes. Such a phenomenon is known as hormesis, and its historical definition is attributed to Paracelsus (1493–1541) who stated that “The poison is in the dose” [21]. This definition describes the observed increase in the biomass of the sprayed weed from below 10% [22] to as high as 150% [23] of the control, especially when the herbicide is underdosed [21]. In some cases, the mechanism of herbicide hormesis appears to be related to the target site of the herbicide, whereas in other examples, hormesis may be due to overcompensation, a mechanism of moderation of stress triggered by the herbicides or a response to disturbed plant homeostasis [21].

To the authors’ knowledge, hormesis in *A. spica-venti* due to herbicides has not yet been described. Publications about hormesis observed in *A. myosuroides* deal with fenoxaprop-P-ethyl, clodinafop and cycloxdim [24], but not pinoxaden. As can be seen in studies reported in the literature, hormetic doses of herbicides increase, especially in herbicide-resistant populations [24,25]. Therefore this study tested stimulation not only at sublethal doses, but also at field doses and above. Moreover, research of this type has not been conducted on herbicide-resistant populations originating from Poland.

The aim of the study was to evaluate the response of *A. myosuroides* and *A. spica-venti*, which are potentially resistant to ACCase inhibitors, to different doses of fenoxaprop-P-ethyl and pinoxaden.

## 2. Materials and Methods

This trial tested populations of *A. myosuroides* (AMI–AMVII) and *A. spica-venti* (APSI–APSIIV), which are potentially resistant to ACCase inhibitors. The AMVIII and APSIV populations constituted the controls that are susceptible to herbicides. Seed samples representative of local populations were collected in 2017 from winter wheat fields in central (Masovia region) and northern Poland (Pomerania and Warmia-Masuria regions). According to information provided by the farmers, ACCase inhibitors have been used in these fields for at least three seasons and reduced efficacy has been observed. Since both fenoxaprop-P-ethyl and pinoxaden predominantly act against weeds that have already emerged, this study focused on the impact on the growing plants’ vegetative vigor.

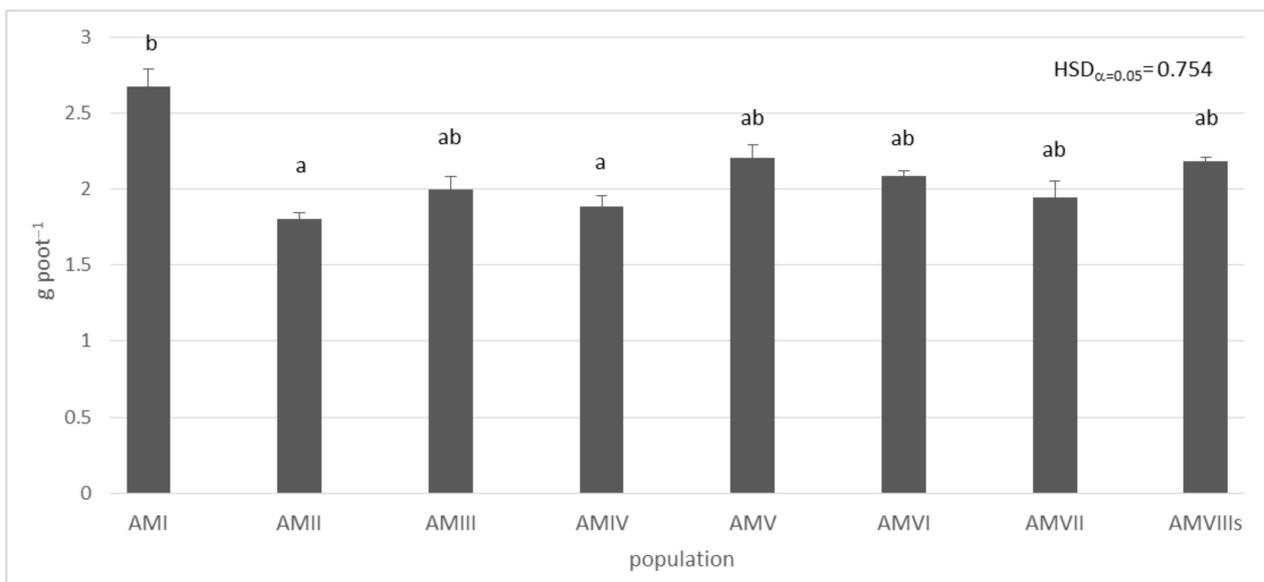
The collected seeds were stored at a temperature of about  $-4$  °C for seven days to break seed dormancy. The experiment was performed in ventilated glasshouses with a 14/10 h photoperiod and temperature of 16–25 °C. The seeds were sown in pot trays

5 cm in diameter filled with potting mixture (Kronnen, Poland). After germination, the number of plants was thinned to five per pot, with a pot considered a replicate, and with three replicates per herbicide dose and an untreated control. Both herbicides were applied at the 2–3-leaf stage at five doses (0.5 N, 1 N, 2 N, 4 N, 8 N), where N is the highest application rate authorized in the European Union. For fenoxaprop-P ethyl, the N rate was 82.8 g active substance (a.s.) ha<sup>-1</sup> [26], and for pinoxaden, it was 45 g a.s. ha<sup>-1</sup> [27], with distilled water as the control. Plants were sprayed with a laboratory chamber sprayer (FHU KAMA, Poland) fitted with a single flat-fan hydraulic Teejet XR 11,002 VP nozzle (200 L ha<sup>-1</sup>, pressure of 200 kPa). Twenty-one days after treatment, plant shoots were harvested and weighed. Since the available resources [26,27] indicated that the lowest vegetative vigor endpoints were observed for the biomass, effects on shoot length were not assessed. ER50, ER25 and ER10, i.e., the doses resulting in a 50, 25 and 10% reduction, respectively, in the biomass of the herbicide-treated plant, were calculated using the log-logistic regression model of the “drc” package in R version 4.0.1 [28]. Additionally, the surrogate reproductive endpoint, ERrepro10 (i.e., the dose resulting in a 10% reduction in the reproductive potential of the herbicide-treated plant), was calculated using the extrapolation factor (EF) determined by the EFSA’s Panel on Plant Protection Products and their Residues (PPR) as covering 95% of the 37 different species studied in 54 independent studies [29]. The no observed effect rate (NOER) value was not calculated since ER10 is deemed to be a more precise and reliable estimator of negligible effects [29–31]. Statistical analyses were performed with one-way ANOVA, and a post hoc Tukey test was used for mean comparison at  $\alpha < 0.05$  (SPSS.1 IBM SPSS Statistics 26, Armonk, NY, USA).

### 3. Results

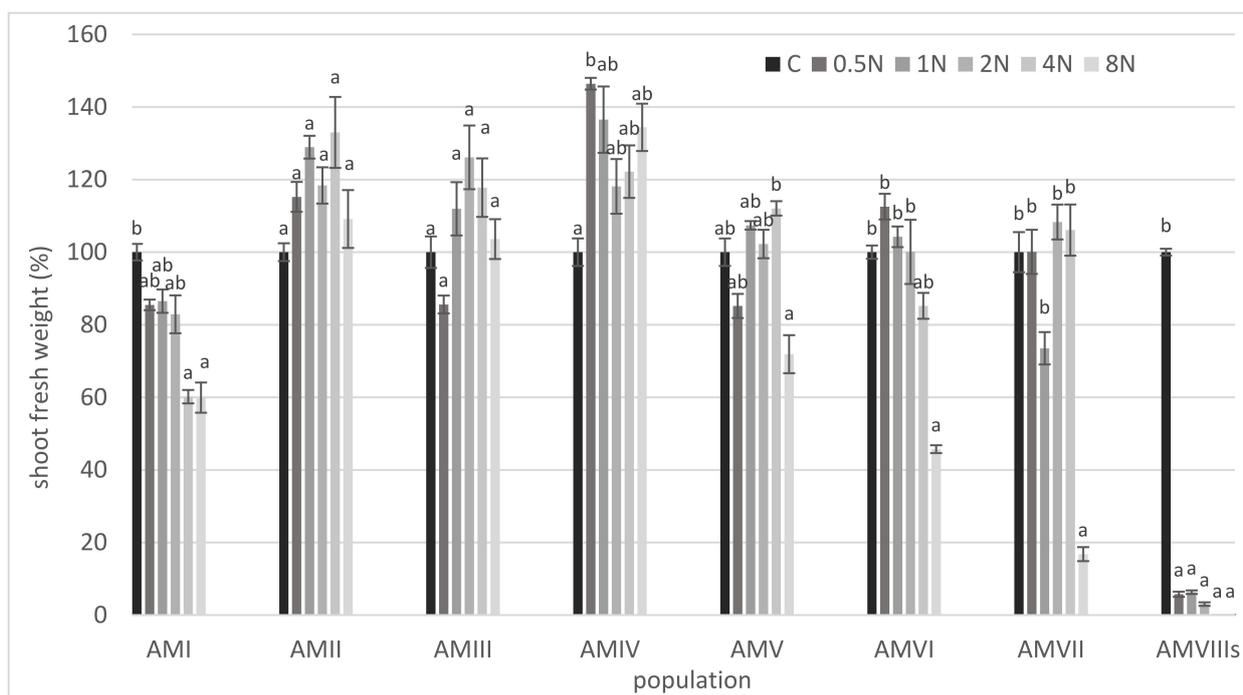
Tested populations of *A. myosuroides* differed in the accumulation of shoot fresh biomass (Figure 1), with the highest biomass of unsprayed plants found for the AMI plants (2.68 g per replicate), while the AMII and AMIV populations had the lowest shoot fresh biomass of 1.80 and 1.89 g per replicate, respectively.

The unsprayed *A. spica-venti* did not show any differences in biomass, which was about 1.4 g per replicate (data not shown).

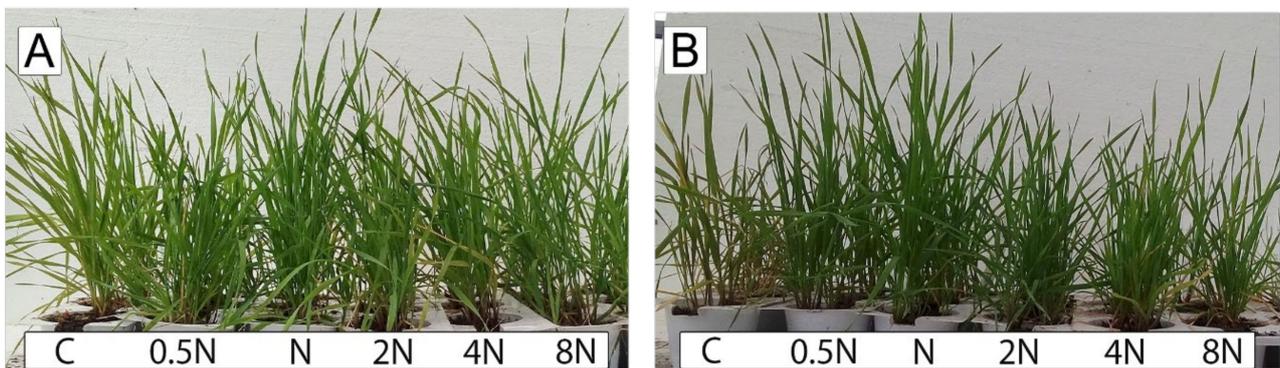


**Figure 1.** Shoot fresh weight of *A. myosuroides* plants without herbicide treatment. Means labeled with the same letter do not differ significantly. The statistical analysis of the significance of differences was performed using the Tukey test for each population separately. Data are means  $\pm$  SE,  $n = 3$ . Means marked by the same letter do not differ significantly.

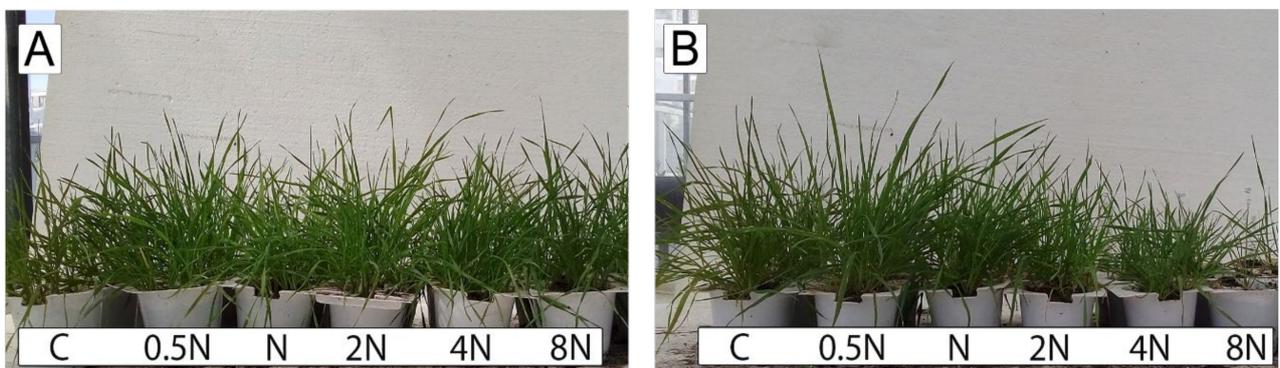
All potentially resistant *A. myosuroides* populations were shown to be resistant to fenoxaprop-P-ethyl in this study (Figure 2). In the case of *A. myosuroides*, a reduction in the biomass of plants treated with fenoxaprop-P-ethyl at all doses compared with the control was only observed for the susceptible AMVIII and AMI populations. The biomass of the remaining populations was unaffected or even increased, especially for doses of 0.5 N–4 N (Figure 2). The greatest stimulation that was statistically significantly different from the control was recorded for the AMIV population (118–146% of the control) (Figures 2 and 3A), where the ER10 value  $> 662.4 \text{ g a.s. ha}^{-1}$  indicated no significant effects at rates 8-fold the registered rate. Plants of the AMII population treated with fenoxaprop-P-ethyl also had 9–33% greater biomass than the control plants; however, these differences were not statistically significant (Figure 2). Moreover, ER10 of  $8.59 \text{ g a.s. ha}^{-1}$  indicated that the biomass gain was similar to that in the control plants. In the case of the AMV–AMVII populations, the biomass of the treated plants increased unevenly, and it reached values of 104 and 113% of the control in AMVI 2 N and AMVI 0.5 N, respectively, with ER10 values exceeding the registered herbicide rate by 4 to 7 times. As shown in Figures 4A and 5, the reaction of population APSIII of *A. spica-venti* was similar to that of the AMII population of *A. myosuroides* (Figure 2). In the APSII population, an increase in the biomass of the sprayed plants was noted in the range of 106 to 133.5% of the control (Figure 5), and ER10 was  $> 662.4 \text{ g a.s. ha}^{-1}$ ; thus, it could be considered that there was a stimulating effect. In the other two populations, the effect was less pronounced (105–113%) and was only noted with the 0.5 N dose (Figure 5). However, the assessment of ERx values showed that these populations had a relatively low susceptibility to the toxicant.



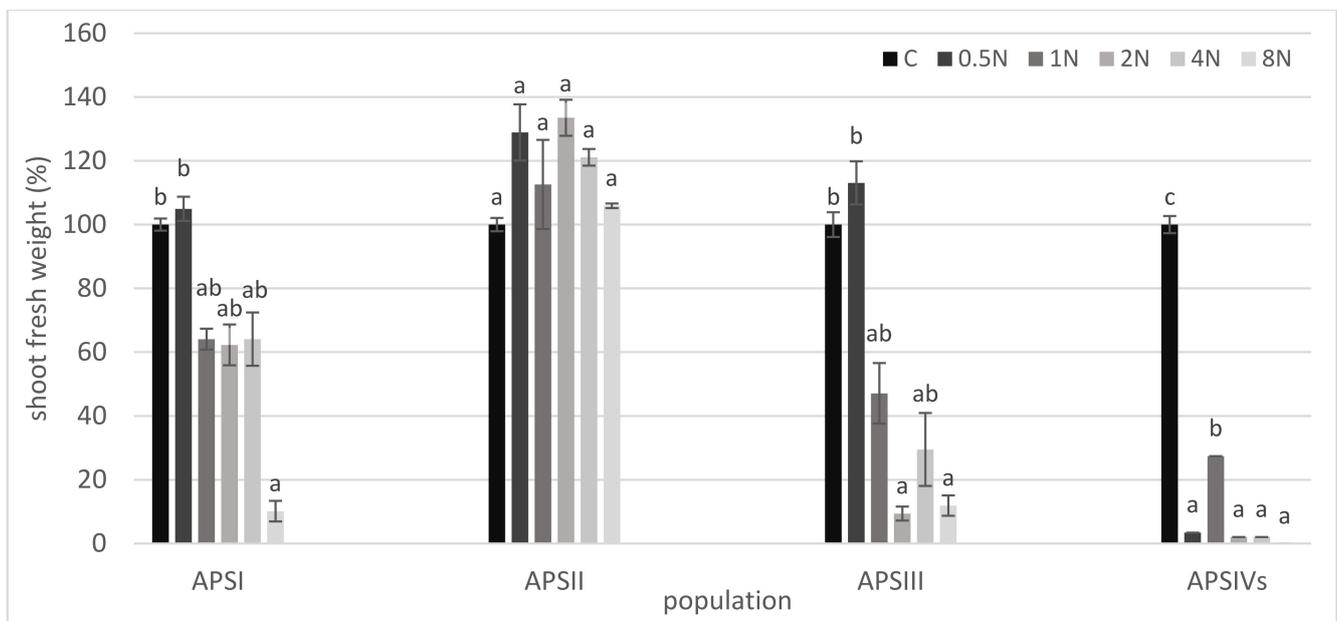
**Figure 2.** Shoot fresh weight of treated and untreated fenoxaprop-P-ethyl plants of *A. myosuroides*, expressed as a percentage of the control. The statistical analysis of the significance of differences was performed using the Tukey test for each population separately. Data are means  $\pm$  SE,  $n = 3$ . Means marked by the same letter do not differ significantly. C: untreated control.



**Figure 3.** *A. myosuroides* population AMIV seedlings sprayed with fenoxaprop-P-ethyl (A) and pinoxaden (B) at doses of 0.5 N, N, 2 N, 4 N and 8 N. C: untreated control.

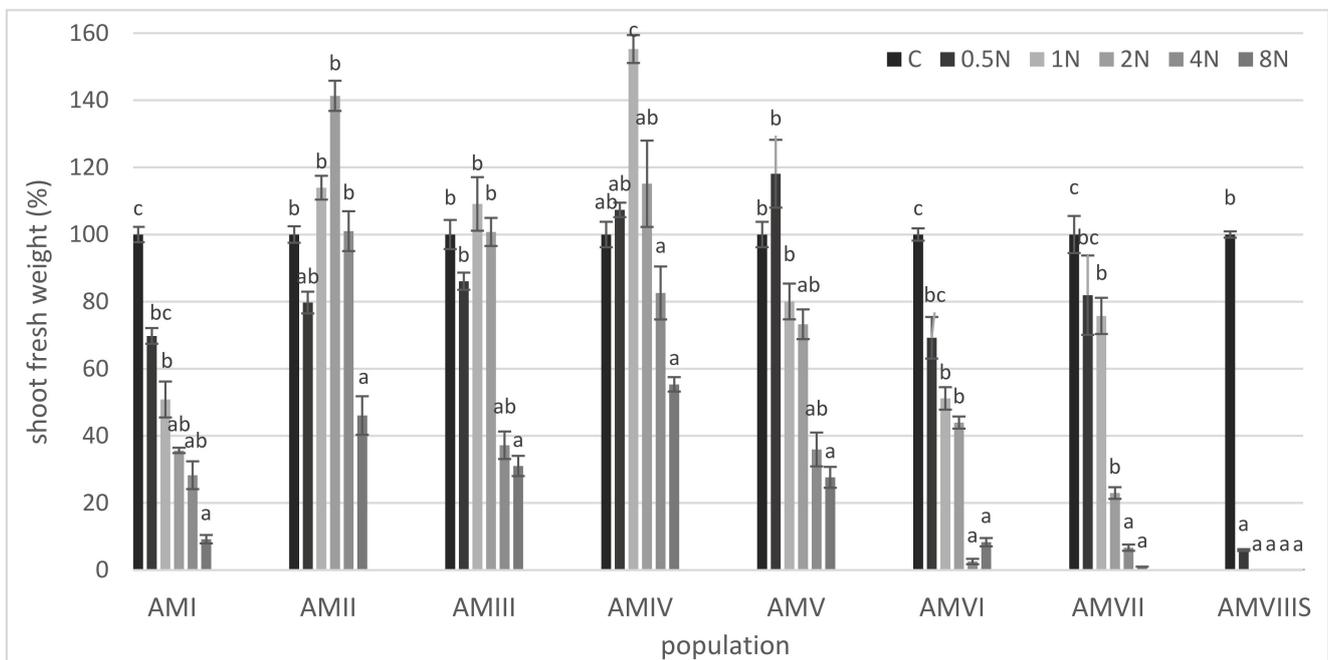


**Figure 4.** *A. spica-venti* population APSII seedlings sprayed with fenoxaprop-P-ethyl (A) and pinoxaden (B) at doses of 0.5 N, N, 2 N, 4 N and 8 N. C: untreated control.

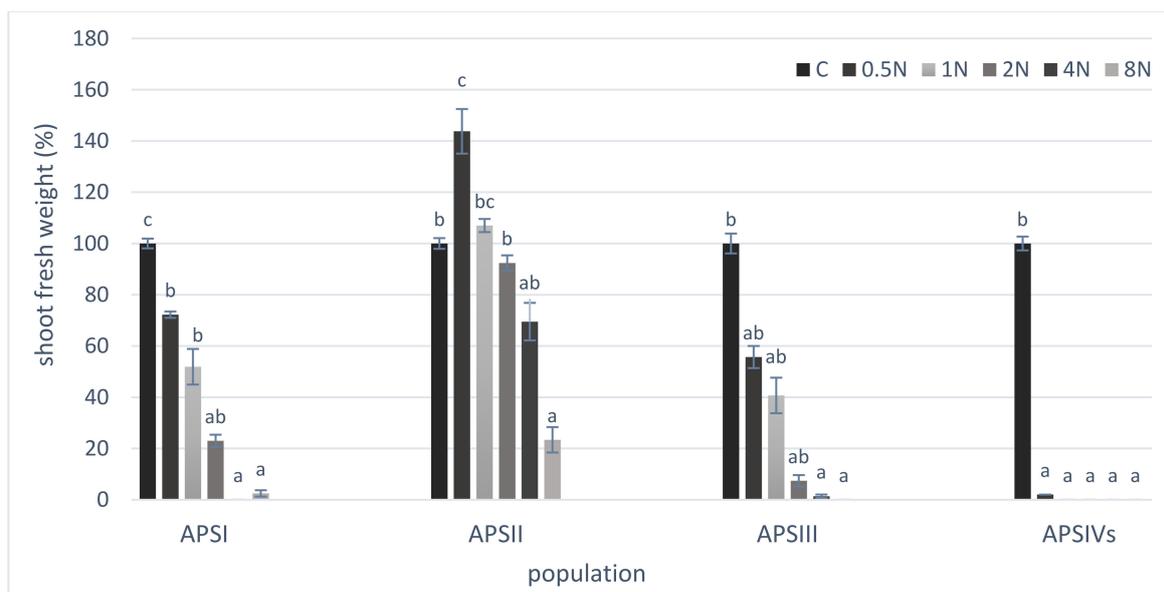


**Figure 5.** Shoot fresh weight of treated and untreated fenoxaprop-P-ethyl plants of *A. spica-venti*, expressed as a percentage of the control. The statistical analysis of the significance of differences was performed using the Tukey test for each population separately. Data are means  $\pm$  SE, n = 3. Means marked by the same letter do not differ significantly. C: untreated control.

Treatment of the tested *A. myosuroides* populations with pinoxaden resulted in a reduction in biomass accumulation in four populations (AMI, AMVI, AMVII, AMVIII). For the other four populations (AMII–AMV), the increase in observed biomass accumulation after herbicide treatment remained unaffected when compared with the untreated control (Figure 6). The largest statistically significant stimulation effect was observed for the AMIV population (Figures 6 and 3B) for the N dose, 45 g a.s. ha<sup>-1</sup>, with ER10 of 146.13 g a.s. ha<sup>-1</sup>. Similarly, an increase for the AMII population after the 2 N dose was also noted; however, the difference between this dose and the untreated control was not statistically significant (Figure 6), and the ER10 of 42.92 g a.s. ha<sup>-1</sup> essentially covered the standard application rate value. The biomass accumulation of the plants treated with the N dose was 57% greater than that in the untreated control (Figure 6). Among the examined populations of *A. spica-venti*, hormesis due to pinoxaden treatment can only be expected in the APSII population (Figure 4B). The biomass accumulation of APSII plants treated with the 0.5 N dose was significantly higher (by 43%) than that of the control (Figure 7), and the dose causing negligible effects was nearly double the application rate (ER10 = 88.23 g a.s. ha<sup>-1</sup>).



**Figure 6.** Shoot fresh weight of treated and untreated pinoxaden plants of *A. myosuroides*, expressed as a percentage of the control. The statistical analysis of the significance of differences was performed using the Tukey test for each population separately. Data are means  $\pm$  SE,  $n = 3$ . Means marked by the same letter do not differ significantly. C: untreated control.



**Figure 7.** Shoot fresh weight of treated and untreated pinoxaden plants of *A. spica-venti*, expressed as a percentage of the control. The statistical analysis of the significance of differences was performed using the Tukey test for each population separately. Data are means  $\pm$  SE, n = 3. Means marked by the same letter do not differ significantly. C: untreated control.

The *A. myosuroides* populations studied were highly resistant to the ACCase inhibitors used, especially to fenoxaprop-P-ethyl. In the case of six populations, the application of 8 times the authorized dose of the herbicide did not reduce plant biomass by half, and in one of the resistant populations, even the ER10 was above that rate. The level of tolerance to pinoxaden was lower, with ER50 for AMI and AMVI populations close to the label dose (36.82 and 45.30 g ha<sup>-1</sup>, respectively), and ER10 indicated negative effects at 5.88 and 10.26 g a.s. ha<sup>-1</sup>, respectively. The ER50 of the remaining populations ranged from less than 3 (AMV) to 8 times (AMII) the label dose of pinoxaden (Table 1).

**Table 1.** ERx (g a.s. ha<sup>-1</sup>) values for *A. myosuroides* (AMI–AMVII) and *A. spica-venti* (APSI–APSIII) populations treated with fenoxaprop-P-ethyl and pinoxaden.

Population	Fenoxaprop-P-ethyl			Pinoxaden		
	ER50	ER25	ER10	ER50	ER25	ER10
AMI	>662.40 (>8 N)	101.56	27.54	36.82	23.04	5.88
AMII	>662.40	52.24	8.59	351.03	55.25	42.92
AMIII	>662.40	65.69	59.71	203.74	188.41	174.23
AMIV	>662.40	>662.40	>662.40	312.78	182.08	146.13
AMV	>662.40	>662.40	642.61	129.34	75.62	44.88
AMVI	>662.40	396.71	246.34	50.20	29.40	10.26
AMVII	600.69	583.5	536.31	86.75	62.47	44.99
APSI	341.38	337.75	148.97	36.80	24.30	10.90
APSII	>662.40	>662.40	>662.40	291.22	160.29	88.23
APSIII	82.51	74.33	68.97	22.32	12.94	5.26

In the case of *A. spica-venti*, the highest ER50 values were recorded for the APSII population (about 3 N for pinoxaden and over an 8 N dose for fenoxaprop-P-ethyl), where ER10 values also indicated no negative impact of either herbicide, while the APSIII biotype was the most susceptible.

To assess the reproductive potential of resistant species, the ER10 estimated above was recalculated to the surrogate reproductive endpoint, ERrepro10, using an extrapolation factor (EF) of 3 [24].

In both species, the calculated surrogate ERrepro10 values indicated that the reproductive potential of the APSII, AMIV, AMV and AMVII populations may be unaffected by fenoxaprop-P-ethyl, while AMIII and AMIV may be unaffected by pinoxaden, applied at up to 2.75 times the label dose (Table 2).

**Table 2.** Calculated ERrepro10 (g a.s. ha<sup>-1</sup>) values for *A. myosuroides* (AMI–AMVII) and *A. spica-venti* (APSI–APSIII) populations treated with fenoxaprop-P-ethyl and pinoxaden.

Population	Fenoxaprop-P-ethyl		Pinoxaden	
	ER10 (Biomass)	ERrepro10	ER10 (Biomass)	ERrepro10
AMI	27.54	9.18	5.88	1.96
AMII	8.59	2.86	42.92	14.31
AMIII	59.71	19.90	174.23	58.08
AMIV	>662.40	220.80	146.13	48.71
AMV	642.61	214.20	44.88	14.96
AMVI	246.34	82.11	10.26	3.42
AMVII	536.31	178.77	44.99	15.00
APSI	148.97	49.66	10.90	3.63
APSI	>662.40	220.80	88.23	29.41
APSI	68.97	22.99	5.26	1.75

#### 4. Discussion

The results obtained in the present study indicated that the tested *A. myosuroides* and *A. spica-venti* populations differ in their sensitivity to the applied graminicides. Furthermore, the results showed that populations of *A. myosuroides* also differ in their biomass accumulation, while the fresh weight of all *A. spica-venti* samples without the application of herbicides was similar. The largest biomass of non-sprayed controls was produced by the AMI population, which showed sensitivity to both fenoxaprop-P-ethyl and pinoxaden. When analyzing the non-sprayed plants, a lower accumulation of biomass was found in the resistant AMII and AMIV populations. This confirms the results of other authors who associate the development of herbicide resistance with decreased plant fitness [20,32] and suggest that the acquisition of resistance can be associated with a fundamental, evolutionary “growth–defense” trade-off that contends that plant resource use is defined by two competing and mutually exclusive evolved functions: defense on one hand and growth and reproduction on the other. Sometimes, resistant biotypes which produce similar or even smaller amounts of biomass have different special characteristics such as earlier germination, flowering and seed maturation. These plant traits could be exploited by modifying the timing of herbicide application or other weed control treatments to reduce the pressure of resistant biotypes [20].

It is noteworthy that the lack of clearly pronounced positive effects or the presence of slightly negative effects, expressed in terms of ER10 value, do not indicate that there were no stimulating effects, since the ER10 values are driven by the effects observed at high concentrations (such an impact of chemicals or even non-herbicidal activity is well known from studies on the effects of insecticides, fungicides or foliar fertilizers; see, e.g., [33]). In this context, the surrogate ERrepro10 values are more informative. Here, no effects were expected for fenoxaprop-P-resistant APSII, AMIV, AMV or AMVII populations. It was noteworthy that even with the use of a more conservative EF of 5, which covered all of the ERveg10/ERrepro10 pairs measured and analyzed by the EFSA PPR, the surrogate ERrepro10 was still well above the application rate of N. The results for pinoxaden were less spectacular, but the negligible effects of N rate on reproduction were expected in the case of the AMIII and AMIV populations.

Beltz and Duke (2014) [21] stress that most common observations of hormesis have been in weeds exposed to subtoxic doses of herbicides (e.g., spray drift reaching adjacent fields, spray deposition lowered by interception by surrounding crops or other weeds); however, progress in resistance development may also result in hormetic responses to herbicides applied at the recommended rates. This is especially true in the case of populations with high resistance factors, where an expected or acceptable level of control is not achieved by application at the recommended rate. As such, recommended application doses become hormetic doses that are likely to influence a significant proportion of the herbicide-resistant weeds favorably, enhancing their propagation and spread [25]. Some of the *A. myosuroides* populations and one *A. spica-venti* population observed in this study showed hormesis for doses of fenoxaprop-P-ethyl and pinoxaden lower than, equal to or higher than the label dose. A similar stimulation has been described for ACCase target site-resistant *A. myosuroides* populations treated with fenoxaprop-P-ethyl and cycloxdim [24]. This suggests that in regions where the problem of weed resistance to herbicides has been observed for some time, plants of resistant populations have overcome the next stage of development. Populations very well adapted to the implemented chemical protection program are becoming dominant in crops. Hormetically enhanced resistant weeds might be of higher reproductive and competitive potential and thus also indirectly more resistant to a second weed control measure. These conclusions have unfortunately been confirmed by the results of studies monitoring the level of *A. myosuroides* and *A. spica-venti* resistance conducted in Germany in 2006–2018 and published by Petersen and Raffan [11]. On the basis of the results of greenhouse tests on 1137 populations of *A. myosuroides* and 737 populations of *A. spica-venti*, resistance to post-emergence herbicides was found to develop very quickly; this resistance can cause the complete loss of control of these weeds in a short time, especially in winter crops. It appears that this situation is being repeated in Poland as well and will require farmers to change their approach to weed control. Increasing the proportion of non-chemical methods will allow the weed population to be decreased without increasing the use of herbicides, and thus selection pressure can generally be reduced.

## 5. Conclusions

The increase in the number and abundance of herbicide-resistant populations of grass weeds, in particular *A. myosuroides* and *A. spica-venti*, is being observed in winter cereals throughout Europe, seriously affecting cereal production. In some populations, hormesis induced by herbicides has been noted, and this phenomenon should be considered one of the putative factors contributing to resistance evolution. The *A. myosuroides* populations studied were highly resistant to the ACCase inhibitors used, especially fenoxaprop-P-ethyl. The highest stimulation by fenoxaprop-P-ethyl that was statistically significantly different from the control was recorded for one population of this species. The level of tolerance of *A. myosuroides* populations to pinoxaden was lower than that to fenoxaprop-P-ethyl; however, significant stimulation by pinoxaden was noted for the same population as was the case with fenoxaprop-P-ethyl. Three *A. spica-venti* populations were shown to be resistant to fenoxaprop-P-ethyl, and an increase in the biomass of the treated plants was noted in all of them. In one population of this species, resistance to pinoxaden and stimulation in the treated plants were also recorded. The calculated surrogate ERrepro10 values indicate that the reproductive potential of three populations of *A. myosuroides* and one population of *A. spica-venti* may be unaffected by fenoxaprop-P-ethyl, while pinoxaden did not reduce this ability in two populations of *A. myosuroides*. As the results of the study show, ACCase inhibitors may not only be ineffective at controlling these weed species but can also aggravate the problem with resistant biotypes due to growth stimulation. Relying solely on chemical weed control is ineffective in the long term. Therefore, the use of non-chemical methods of weed control should be intensified in agricultural practice. Education and incentives for farmers are needed to direct them towards more sustainable weed management systems. Nevertheless, it should also be emphasized that the effects, or a lack thereof, on the biomass of resistant populations may not necessarily be reflected in their

reproductive capacity. While the goal of weed control practices is to eliminate the weeds competing with crop plants, the long-term approach is to reduce weed seeds in the seedbed. There is a need for further research, e.g., extended studies on the effect of herbicides on flowering, seed production and germination in resistant genotypes, inter-species relations under realistic laboratory conditions (microcosm studies) simulating the real field situation, and extended mesocosm and (semi)field studies, on possible stimulating effects on the reproductive potential of resistant weed populations.

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