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Study of the sensitivity of durum wheat varieties to herbicides inhibiting acetohydroxyacid synthase and acetyl-CoA carboxylase

Проучване на чувствителността на сортове твърда пшеница към хербициди инхибитори на ацетохидроксиацид синтаза и ацетил-КоА карбоксилаза

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ABSTRACT

A study on the sensitivity of four durum wheat varieties to herbicides inhibiting acetyl-CoA carboxylase (ACC) and acetohydroxyacid synthase (AHAS) was conducted in greenhouse pot experiments. Increased herbicide doses (double and quadruple) reduced photosynthetic activity in all tested varieties. The most sensitive variety was Helix, and the most resistant was Saya. The remaining varieties, Predel and Reyadur, occupy an intermediate position with respect to the analyzed physiological parameters. Regarding enzyme activity, although it performed well in photosynthesis, the most sensitive variety was Saya. Of the ACC inhibitor herbicides, Axial 050 EC had a more substantial adverse effect, and among the AHAS inhibitor herbicides, Pallas 75 WG + Dassoil 26-2N was the most effective. In addition, the genetic relationship among the studied genotypes was based on molecular markers.

Keywords: ACC, AHAS, DNA markers, herbicides, photosynthesis, *Triticum durum Desf.*

АБСТРАКТ

Проведено е проучване върху чувствителността на четири сорта твърда пшеница към хербициди, инхибиращи ацетил-CoA карбоксилазата (ACC) и ацетохидроксикиселинната синтаза (AHAS), в съдови опити в оранжерия. Повишените дози хербициди (двойни и четворни) намаляват фотосинтетичната активност във всички тествани сортове. Най-чувствителният сорт се оказа Хеликс, а най-устойчивият - Сая. Останалите сортове, Предел и Реадур, заемат междинно положение по отношение на анализираните физиологични параметри. По отношение на ензимната активност, въпреки че се представя добре във фотосинтезата, най-чувствителният сорт е Сая. От хербицидите инхибитори на ACC, Axial 050 EC има по-съществен неблагоприятен ефект, а сред хербицидите инхибитори на AHAS, Pallas 75 WG + Dassoil 26-2N е най-ефективен. Освен това, генетичната връзка между изследваните генотипове се основава на анализ с молекулярни маркери.

Ключови думи: АЦЦ, АХАС, ДНК маркери, хербициди,фотосинтеза, *Triticum durum Desf.*

INTRODUCTION

The progressive growth of the planet's population requires an increase in food production. By 2050, the UN predicts that the world population will reach approximately 10 billion people (UN, 2022), necessitating that scientists pay serious attention to meeting the growing global demand for food (Łaba et al., 2022). Wheat is among the primary cereals worldwide, providing nearly 55% of the world's carbohydrates and approximately 85% of the global population's daily protein (Alcamo et al., 2007; Zargar et al., 2017). This crop is directly related to food security and the global economy (FAO, 2021). Weeds are the main detrimental factor in wheat production. They have a significant negative impact on yield formation and quality, making harvesting equipment difficult and resulting in substantial losses (Jabran et al., 2017). The problem of weeds in winter cereal crops is a persistent issue of great importance. Its constant relevance is determined by the dynamic nature of weed species and communities in crops, as well as by their diverse biological characteristics and the emergence of herbicide resistance in recent years (Ghosh et al., 2020; Skalicky et al., 2020; Kubiak et al., 2022). Severe weed infestations can lead to a reduction in cereal yields, with wheat yields being reduced by up to 70% (Bekelle, 2004; Atanasova and Zarkov, 2005). The most widespread and frequently applied method of controlling weed infestations in crops is the use of herbicides. Herbicides are chemical compounds with biocidal action, and only their correct application, in accordance with the plant species, variety, and agrometeorological conditions, the optimally selected dose, the phenophase of the crop, the density, and species diversity of weeds, guarantees successful and safe work with them (Mesnage et al., 2021). However, the incorrect use of herbicides can cause stress in cultivated plants. In recent years, this problem has become increasingly relevant, as it negatively impacts crop yields and the quality of plant production. In this case, the term "phytotoxicity" is used instead of the term "stress"; however, both concepts reflect physiological states of plants that result from similar structural and functional disorders. Under herbicidal stress, plants typically produce large amounts of reactive oxygen species (ROS), leading to oxidative stress within the plant. The increase in ROS activates the plant's antioxidant system, enabling the removal of ROS (Jervekani et al., 2018; Li et al., 2022a). At the same time, plants can also respond to herbicidal stress by regulating hormonal activity and promoting or inhibiting the formation of key metabolites. Therefore, it is common to mitigate the toxicity of herbicides in crops by applying plant hormones (Li et al., 2022b). The tolerance of agricultural plants to various stress factors can be increased by two main approaches: plant breeding and technology. The first is associated with the purposeful creation and selection of tolerant varieties and hybrids, and the second includes various agrotechnical approaches. Genetic tolerance of crops to herbicides may have several advantages. It can increase the safety margins between weeds and crop sensitivity and also expand the applicability of a particular herbicide. The most appropriate molecular tool for primary screening of unstudied genotypes so far is ISSR marker system (Godwin et al., 1997; Pradeep Reddy et al., 2002). In the context of the study, ISSR analysis aims to assess the genetic distances between varieties and to relate it to the observed physiological and enzymatic responses to herbicide stress. Herbicide resistance is a vital quality of crops. The resistance allows plants to survive and reproduce even in increased doses of herbicides. Genetic tolerance of crops to herbicides may have several advantages. It can increase the safety margins between weeds and crop sensitivity, and also expand the applicability of a particular herbicide. This study aimed to investigate the sensitivity of different durum wheat genotypes to herbicide inhibitors of two key enzymes: acetyl-CoA carboxylase (ACC) and acetohydroxyacid synthase (AHAS).

MATERIAL AND METHODS

Plant material and experimental design

A pot experiment was conducted in a steel-glass greenhouse with four varieties of durum wheat: Predel (standard), Saya, Reyadur, and Helix. To determine the sensitivity of the varieties, herbicides with different active substances acting on the same enzyme were used,

namely Pallas 75 WG (75 g/kg Pyroxulam + 75 g/kg Cloquintocet-mexil (antidote)) + Dassoil 26-2N (99.6% w / w alkylphenolalkoxylate) and Flyer (50 g/l florasulam) on AHAS, and Axial 050 EC (50 g/l pinoxaden) and Sword 240 EC (240 g/l clodinafop-propargyl) on ACC. The herbicides were applied at a double dose recommended by the manufacturer (this situation occurs more often in field conditions when the treated areas overlap), which will provide information about the sensitivity of the varieties, and also at a quadruple dose, which will provide information about the tolerance of the varieties to the tested herbicides. Each variant was set up in 4 repetitions with 10 plants per pot. Each pot has a volume of 10 liters and contains drainage and soil. The soil is an alluvial meadow with a crumbly, granular structure, containing 24.41 mg/kg of mobile nitrogen, 34.35 mg/100 g of mobile phosphorus, and 86.10 mg/100 g of mobile potassium. Pre-sowing fertilization was carried out with N:P:K - 15:15:15 at a rate of 0.95 g/pot. The variants of the experiment for each variety are presented in Table 1. The treatment was carried out with a sprayer, with a consumption of the working solution of 3.8 mL per variant.

Analyses

Physiological analyses

For rapid screening of the herbicidal effect on wheat plants, physiological parameters such as leaf gas exchange were measured with a portable infrared gas analyzer LCpro+ (ADC BioScientific Ltd., England) and chlorophyll content with a portable chlorophyll meter CCM300 (ADC BioScientific Ltd., England).

Enzyme analyses

To determine the resistance of plants, the activities of the target enzymes of the specific herbicides were analyzed. The activity of the enzyme Acetyl-CoA carboxylase (ACC) (EC 6.4.1.2) was measured spectrophotometrically with a UV/Vis INNO-S Plate reader (LTEK Co., Ltd., Republic of Korea) by the increase in inorganic phosphorus. At the end of the reaction period, the dye reagent formed a colored compound with the released phosphate, which was measured at a wavelength of 635 nm using the method described by Howard and Ridley (1990).

Table 1. Variants and an experimental design

Variants	Common name		
	Chemical name	Time of application In the BBCH scale	Rate of application of herbicides
1. Untreated control	-	-	-
2. Pallas 75 WG + Dassoil 26-2N	75 g/kgPiroxulam + 99.6% w/w alkylphenolalkoxylate	BBCH 29-31	0.50 kg/ha + 1.00 L/ha (double dose)
3. Pallas 75 WG + Dassoil26-2N	75 g/kgPiroxulam + 99.6% w/w alkylphenolalkoxylate	BBCH 29-31	1.0 kg/ha + 2.00 L/ha (quadruple dose)
4. Flyer	50 g/L florasulam	BBCH 29-31	0.30 L/ha (double dose)
5. Flyer	50 g/L florasulam	BBCH 29-31	0.60 L/ha (quadruple dose)
6. Axial 050 EC	50 g/L pinoxaden	BBCH 29-31	1.80 L/ha (double dose)
7. Axial 050 EC	50 g/L pinoxaden	BBCH 29-31	3.60 L/ha (quadruple dose)
8. Sword 240 EC	240 g/L clodinafop-propargyl	BBCH 29-31	0.50 L/ha (double dose)
9. Sword 240 EC	240 g/L clodinafop-propargyl	BBCH 29-31	1.00 L/ha (quadruple dose)

The activity of acetohydroxyacid synthase (AHAS) (EC 2.2.1.6) was determined from the reaction product acetoin by the method of Ray (1984). The increase in absorbance at 525 nm was directly proportional to the enzyme activity. The enzyme activity was expressed as a percentage of the untreated control for each variety. Physiological and enzyme analyses were performed on the fifth and twentieth days after herbicide treatment to monitor early and late effects.

Genetic analyses

To assess the genetic relationship between the studied durum wheat cultivars, an ISSR (Inter Simple Sequence Repeat) marker system was applied. Genomic DNA was extracted from young leaf tissue collected from plants of the untreated control variants using the Jena Bioscience DNA Preparation Kit according to the manufacturer's instructions (Jena BioSciences).

PCR amplification was conducted using a set of ISSR primers containing dinucleotide repeat motifs anchored at the 3′ or 5′ end. Reactions were carried out under optimized conditions. PCR was performed at temperatures appropriate for the primers used, and the resulting products were separated by electrophoresis on 2% agarose gels. (Table 2).

Table 2. ISSR primers used for genetic analysis

Primer	DNA sequence	Annealing temperature (Ta)
ISSR 1	$(AG)_8C+TG$	56°C
ISSR 2	$(AG)_8C$	52°C
ISSR 3	$(GA)_8T$	52°C
ISSR 4	$(GA)_9C$	55°C
ISSR 5	$(GA)_9T$	54°C
ISSR 6	$(GA)_9A$	54°C

The PCR reaction mixture included: 1 µl gDNA, 1.5 µl primer, 8 µl Horse-Power Taq DNA Polymerase MasterMix (CanvaxBiotech), 9.5 µl dH_2O. Reaction total volume: 20 µl. A standard PCR amplification program consists of an initial denaturation (94 - 98 °C), followed by 35 cycles of denaturation (94 - 98 °C), annealing (50 - 65 °C), and extension (72 - 78 °C), ending with a final extension and 4 °C hold. This process exponentially amplifies specific DNA sequences using thermal cycling and a thermostable polymerase.

Statistical analysis

The experiment was carried out twice, with each variant having four repetitions. The results are presented as average values of four measurements. The data were statistically processed using the free online statistics app. "Statisty" with a One-Way ANOVA. For each variety, a one-way analysis of variance was performed separately for a specific indicator to account for the influence of the herbicides used. The four varieties were compared separately for one indicator of a specific herbicide, in order to account for the sensitivity of each variety to the herbicide. For this purpose, the Dunn-Bonferroni post-hoc test was also used to compare the groups in pairs and to determine which of them differs significantly.

Genetic closure was performed using the unweighted pair group method with arithmetic means (UPGMA) to generate a dendrogram illustrating genetic relationships among the studied cultivars.

RESULTS AND DISCUSSION

Physiological analyses

As a sensitive and non-destructive method for determining the condition of plants, the analysis of leaf gas exchange provides information about the influence of the herbicides used. The results presented in Table 3 demonstrate the influence of the double and quadruple doses of herbicides on leaf gas exchange, as well as the instantaneous water uptake efficiency (WUEinst), calculated based on the rate of photosynthesis and transpiration for the time of measurement. This indicator provides information about the plasticity and adaptability of plants to environmental conditions.

Table 3. Results of leaf gas exchange and water use efficiency in durum wheat varieties treated with different herbicides (double and quadruple dose) (the analyses were performed twice to monitor the early and late effects of the herbicide impact (5 and 20 DAT)

Cultivar	Variants	Double dose						Quadruple dose					
		5 DAT			20 DAT			5 DAT			20 DAT		
		A	E	W	A	E	W	A	E	W	A	E	W
Predel	1. Control	28.79^{a}	2.08^{a}	13.84^{a}	29.00^{a}	1.87^{a}	15.51^{b}	26.86^{a}	1.88^{a}	14.3^{a}	28.08^{a}	1.87^{a}	15.0^{a}
	2. Pallas 75 WG + Dassoil 26-2N	25.00^{b}	2.07^{a}	12.07^{a}	25.05^{b}	1.69^{a}	14.82^{b}	23.00^{a}	1.71^{a}	13.4^{a}	24.05^{a}	1.69^{a}	14.2^{a}
	3. Flyer	26.56^{a}	2.05^{a}	12.96^{a}	26.52^{a}	1.59^{a}	16.67^{b}	22.56^{b}	1.74^{a}	12.9^{a}	23.73^{a}	1.59^{a}	15.0^{a}
	4. Axial 050 EC	22.35^{c}	1.58^{b}	14.15^{a}	24.14^{b}	1.13^{b}	21.36^{a}	17.35^{c}	1.08^{b}	16.0^{a}	20.86^{b}	1.13^{b}	18.5^{a}
	5. Sword 240 EC	22.37^{c}	1.72^{b}	13.01^{a}	25.11^{b}	1.13^{b}	22.22^{a}	18.37^{c}	1.12^{b}	16.4^{a}	21.71^{b}	1.13^{b}	19.2^{a}
Saya	1. Control	29.85^{a}	2.09^{a}	14.24^{a}	28.92^{a}	1.34^{b}	21.58^{a}	26.45^{a}	2.41^{a}	11.0^{b}	26.79^{a}	1.34^{b}	19.9^{a}
	2. Pallas 75 WG + Dassoil 26-2N	26.76^{a}	2.01^{a}	13.31^{a}	25.42^{b}	1.41^{b}	18.03^{a}	23.76^{a}	2.41^{a}	9.9^{b}	23.21^{b}	1.41^{a}	16.5^{a}
	3. Flyer	26.71^{a}	1.98^{a}	13.49^{a}	24.81^{b}	1.25^{b}	19.85^{a}	25.62^{a}	2.08^{a}	12.3^{a}	24.81^{a}	1.25^{b}	19.8^{a}
	4. Axial 050 EC	24.34^{b}	1.88^{a}	11.88^{a}	24.42^{b}	1.23^{b}	19.85^{a}	22.28^{b}	1.38^{b}	16.1^{a}	23.17^{b}	1.23^{b}	18.9^{a}
	5. Sword 240 EC	25.00^{b}	1.67^{b}	14.97^{a}	25.53^{b}	1.85^{a}	13.8^{b}	24.09^{a}	1.87^{b}	12.9^{a}	24.27^{a}	1.85^{a}	13.1^{b}
Reyadur	1. Control	28.77^{a}	1.90^{a}	15.14^{a}	27.79^{a}	1.20^{a}	23.16^{a}	25.77^{a}	1.50^{a}	17.2^{a}	25.46^{a}	1.20^{a}	21.2^{a}
	2. Pallas 75 WG + Dassoil 26-2N	26.58^{a}	1.75^{a}	15.19^{a}	26.29^{a}	1.19^{a}	22.09^{a}	20.58^{b}	1.38^{a}	14.9^{a}	20.88^{b}	1.19^{a}	17.5^{a}
	3. Flyer	24.20^{b}	1.63^{a}	14.85^{a}	25.54^{a}	1.11^{a}	23.01^{a}	23.20^{a}	1.33^{a}	17.4^{a}	22.44^{a}	1.11^{a}	20.2^{a}
	4. Axial 050 EC	22.30^{b}	1.08^{b}	17.87^{a}	23.36^{b}	1.01^{b}	23.13^{a}	12.30^{c}	0.81^{b}	15.2^{a}	19.31^{c}	1.01^{b}	19.1^{a}
	5. Sword 240 EC	23.70^{b}	1.19^{b}	17.39^{a}	24.43^{b}	1.15^{a}	21.24^{a}	14.70^{c}	0.93^{b}	15.8^{a}	20.25^{b}	1.15^{a}	17.7^{a}
Helix	1. Control	27.97^{a}	1.72^{a}	16.26^{a}	29.29^{a}	1.13^{a}	25.92^{a}	26.75^{a}	1.42^{a}	18.8^{a}	26.18^{a}	1.13^{a}	23.2^{a}
	2. Pallas 75 WG + Dassoil 26-2N	21.94^{c}	1.61^{a}	13.63^{b}	23.37^{b}	1.12^{a}	20.87^{b}	17.94^{c}	1.41^{a}	12.7^{b}	20.14^{b}	1.12^{a}	18.0^{a}
	3. Flyer	24.59^{b}	1.35^{b}	18.21^{a}	23.57^{b}	1.12^{a}	21.04^{b}	20.59^{b}	1.35^{a}	15.2^{a}	20.23^{b}	1.12^{a}	18.1^{a}
	4. Axial 050 EC	21.31^{c}	1.58^{a}	13.49^{b}	22.97^{b}	1.11^{a}	20.69^{b}	17.31^{c}	1.38^{a}	12.5^{b}	18.35^{c}	1.11^{a}	16.5^{b}
	5. Sword 240 EC	21.09^{c}	1.53^{a}	13.78^{b}	27.36^{a}	1.13^{a}	24.21^{a}	17.09^{c}	1.33^{a}	12.8^{b}	20.36^{b}	1.13^{a}	18.1^{a}

A – photosynthesis (mmol $CO_2/m^2/s$), E (mmol $H_2O/m^2/s$), W (WUEinst) – instantaneous water use efficiency (*WUEinst = A/E*). The different letters (a, b, c) after the numbers for the same variety for a certain indicator indicate statistical significance at $P<0.001$.

The double dose of the tested herbicides showed a rapid effect on photosynthetic rate across different varieties of durum wheat. When comparing the action of herbicides in each variety, Axial 050 EC showed the strongest inhibitory effect. This herbicide reduced photosynthesis in the Predel variety by 22.37% (5 days after treatment) and by 16.76% (20 days after treatment) compared to the control. In the Saya variety, Axial 050 EC reduced photosynthesis by 18.46% and 15.56%, respectively, in both measurements. In the Reyadur variety, the same herbicide reduced photosynthesis by 22.49% and 15.59%, respectively, in both measurements. In the Helix variety, the decrease by Axial 050 EC was 23.82% and 21.53%, respectively, in the two consecutive measurements. The results show that among all varieties, Helix is the most sensitive, with the most significant inhibition of photosynthesis already in the early stages of exposure to the herbicide Axial 050 EC, which also affects the instantaneous efficiency of water absorption. In all varieties, a significant recovery of photosynthetic activity over time was observed, as well as WUEinst. However, only the Helix variety showed a retention of the negative effect on photosynthesis, albeit at the expense of improved WUEinst. The rapid recovery of photosynthetic activity and the improved WUEinst values are indicators of the plasticity of the studied durum wheat genotypes. In both measurements, the results for stomatal conductance did not show statistically significant differences between the individual variants (data not shown).

When using a higher dose (quadruple) of the tested herbicides, a more pronounced difference in the reaction of the different varieties was observed (Table 3). The testing with such a dose aims to analyze the possibilities for a variety with higher adaptability and resistance. The herbicide Axial 050 EC also appears to be the most effective across all varieties. The Helix variety again appears as sensitive, in which a more substantial decrease in photosynthesis was observed as a result of more than one herbicide, namely Sword 240 EC, Axial 050 EC, and Pallas 75 WG + Dassoil 26-2N, respectively by 36%, 35%, 33% compared to the control, and the negative impact was maintained during the second measurement. In the Predel variety, Axial 050 EC also inhibits photosynthesis by 35% compared to the control. The strongest inhibition was observed in the Reyadur variety, in which Axial 050 EC and Sword 240 EC reduced photosynthesis by 52% and 43%, respectively, compared to the control. The Saya variety showed the greatest resistance to Axial 050 EC, with photosynthesis decreasing by only 16% in the first measurement and 14% in the second, accompanied by high WUEinst values. In this case, the WUEinst values in both measurements were less variable than in the variants treated with a double dose of herbicides, in which a significant increase in WUEinst was observed in the later stages.

Another important indicator characterising the physiological state of plants is the content of plastid pigments. Since the synthesis of plastid pigments depends largely on the synthesis of fatty acids (Fudji et al., 2025) and some amino acids (Deveikite et al., 2025), the use of herbicides that suppress key enzymes in the synthesis of these metabolites would also affect the content of plastid pigments. Table 4 presents the effects of double and quadruple doses of the studied herbicides on the content of plastid pigments.

As shown in Table 4, the double dose of the various herbicides reduces chlorophyll content to some extent. A more significant decrease was observed under the influence of the herbicide Axial050 EC, which inhibits ACC and, respectively, fatty acid synthesis, as well as Pallas75 WG + Dassoil 26-2N, which inhibits AHAS and the synthesis of branched-chain amino acids. Under the influence of the quadruple dose, a decrease in chlorophyll content by 25% was observed in the Predel variety as a result of Pallas 75 WG + Dassoil 26-2N and Axial 050 EC, and this effect was maintained with Axial 050 EC in the second measurement. Pigments also decreased by 25% in the Helix variety treated with Axial 050 EC. In the Reyadur variety, under the influence of Axial 050 EC, the pigment content was 32% lower than the control, and under the influence of Pallas 75 WG + Dassoil 26-2N, it was reduced by 21%. In the Saya variety, a decrease in chlorophyll content was observed only in the variant treated with Axial 050 EC, to 21% compared to the control, further defining it as less sensitive than the other varieties.

Table 4. Results for the content of photosynthetic pigments (chlorophyll mg/m^2) in durum wheat varieties treated with different herbicides (double and quadruple dose). The analyses were performed twice to monitor early and late effects of the herbicide impact (5 and 20 DAT)

Cultivar	Variant	Double dose		Quadruple dose	
		5 DAT	20 DAT	5 DAT	20 DAT
Predel	1. Control	495^a	497^a	495^a	497^a
	2. Pallas 75 WG + Dassoil 26-2N	430^b	465^a	370^c	453^b
	3. Flyer	471^a	483^a	464^b	466^b
	4. Axial 050 EC	423^b	436^b	370^c	396^c
	5. Sword 240 EC	468^a	456^b	455^b	451^b
Saya	1. Control	468^a	463^a	468^a	463^a
	2. Pallas 75 WG + Dassoil 26-2N	433^b	434^b	427^b	414^b
	3. Flyer	444^a	454^a	433^b	425^b
	4. Axial 050 EC	418^b	427^b	372^c	377^c
	5. Sword 240 EC	451^a	445^a	436^b	425^b
Reyadur	1. Control	469^a	473^a	469^a	473^a
	2. Pallas 75 WG + Dassoil 26-2N	402^b	408^b	358^c	406^b
	3. Flyer	464^a	459^a	471^a	436^b
	4. Axial 050 EC	415^b	421^b	315^c	361^c
	5. Sword 240 EC	451^a	447^b	431^b	425^b
Helix	1. Control	498^a	486^a	498^a	486^a
	2. Pallas 75 WG + Dassoil 26-2N	482^a	473^a	470^a	450^b
	3. Flyer	480^a	477^a	475^a	447^b
	4. Axial 050 EC	422^c	431^c	373^c	399^c
	5. Sword 240 EC	464^b	457^b	452^b	433^b

DAT – days after treatment. The different letters (a, b, c) after the numbers for the same variety for a certain indicator indicate statistical significance at $P<0.01$.

Enzyme assays

In addition to non-destructive assays that determine the response of varieties to increased doses of herbicides, the activity of the target enzymes of the used herbicides was also analyzed to better understand these reactions. The active substances in the herbicides Axial 050 EC (pinoxaden) and Sword 240 EC (clodinafop-propargyl) target and inhibit the enzyme acetyl-CoA carboxylase, which catalyzes the carboxylation reaction in fatty acid synthesis (Tong, 2005). The active substances in the herbicides Pallas 75 WG (75 g/kg Piroxulam) and Flyer (florasulam) inhibit the activity of the enzyme acetohydroxiacid synthase, which is responsible for the synthesis of branched-chain amino acids, such as valine, leucine, and isoleucine (Jeffers et al., 1996). The herbicides tested exhibit dose-dependent inhibition of enzyme activity (Table 5). Regarding the ACC enzyme, both herbicides (Axial 050 EC and Sword 240 EC) applied in double and quadruple doses had an inhibitory effect on all tested varieties, with the inhibition being more pronounced with Axial 050 EC in the first measurement (early effects).

The Saya variety was the most sensitive, with 48% (5 days after treatment) and 35% (20 days after treatment) inhibition by Axial 050 EC at the double dose and 86% and 59%, respectively, at the quadruple dose. The next most sensitive variety to Axial 050 EC was the Predel variety, with 61% and 36% inhibition at the first and second measurements at the quadruple dose. The remaining varieties – Helix and Reyadur – exhibit 15% and 11% inhibition at the second measurement, respectively, indicating higher resistance to this herbicide. The herbicide Sword 240 EC has a weaker inhibitory effect compared to Axial 050 EC. The highest sensitivity to it was observed in the variety Predel, with 22% inhibition in the second measurement. In the other varieties – Helix, Reyadur, and Saya - the ACC activity 20 days after treatment with the quadruple dose was inhibited by 9%, 4%, and 3%, respectively.

Among the targeted enzymes, AHAS appeared less sensitive to elevated herbicide levels. In terms of AHAS, Predel stands out as a sensitive variety, with 15% inhibition by Flyer and 14% by Pallas 75 WG+Dassoil 26-2N in the second quadruple-dose measurement. In Helix and Reyadur, the inhibition by Flyer is 12% and 11%, respectively, and by Pallas 75 WG+Dassoil 26-2N, with 9% and 8% in the second measurement. Sayashows the greatest resistance to both herbicides, with 8% inhibition by Pallas 75 WG + Dassoil 26-2N and 9% by Flyer in the second measurement at the quadruple dose. The increased activity of the enzymes in the second measurement compared to the first, even at the quadruple dose, indicates the compensatory capabilities of the genotypes and their ability to neutralize herbicides or compensate through increased de novo synthesis of enzymes. Establishing resistance in the studied varieties is a prerequisite for identifying mutations in the genes encoding these enzymes, leading to amino acid replacements in the enzymes (Wang et al., 2019; Gaines et al., 2020).

One-way analysis of variance revealed a significant difference between the variants in ACC ($P<0.001$). The Bonferroni post hoc test revealed that the pairwise comparisons between the groups Predel-Saya, Predel-Reyadur, Predel-Helix, Saya-Reyadur, and Saya-Helix had p-values less than 0.05; therefore, these groups were significantly different in pairs.

The negative effect of herbicides that inhibit AHAS on photosynthesis is not due to a direct influence on the process, but rather to an indirect effect by reducing the synthesis of branched amino acids (valine, leucine, isoleucine). This reduces protein synthesis, including components of the photosynthetic apparatus, chlorophyll, and ETB transporters (Todorova et al., 2022; Dong et al., 2024). In the present study, a similar negative effect of high herbicide doses inhibiting AHAS was observed (Table 3, 4). Despite the resistance of durum wheat genotypes to AHAS inhibitors declared by many scientists (Jimenez et al., 2016; Zobiole et al., 2018), the application of high doses (2 and 4 times the recommended dose) reduces photosynthetic efficiency and lowers chlorophyll content.

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Table 5. Activity of the enzymes ACC and AHAS, expressed as % of the control, in durum wheat varieties treated with different herbicides (double and quadruple dose). The analyses were performed twice to monitor early and late effects of the herbicide exposure (5 and 20 DAT)

Cultivar	Variant (specific herbicide)	ACC				Variant (specific herbicide)	AHAS			
		Double dose		Quadruple dose			Double dose		Quadruple dose	
		5 DAT	20 DAT	5 DAT	20 DAT		5 DAT	20 DAT	5 DAT	20 DAT
Predel	Control	100^{a}	100^{a}	100^{a}	100^{a}	Control	100^{a}	100^{a}	100^{a}	100^{a}
	Axial 050 EC	65^{c}	73^{c}	59^{c}	64^{c}	Pallas 75 WG + Dassoil 26-2N	81^{b}	94^{b}	73^{c}	86^{b}
	Sword 240 EC	74^{b}	85^{b}	66^{c}	78^{b}	Flyer	79^{b}	90^{b}	72^{c}	85^{b}
Saya	Control	100^{a}	100^{a}	100^{a}	100^{a}	Control	100^{a}	100^{a}	100^{a}	100^{a}
	Axial 050 EC	52^{c}	65^{d}	14^{c}	50^{b}	Pallas 75 WG + Dassoil 26-2N	92^{b}	98^{a}	88^{b}	92^{a}
	Sword 240 EC	90^{b}	99^{a}	88^{b}	97^{a}	Flyer	89^{b}	98^{a}	86^{b}	91^{a}
Reyadur	Control	100^{a}	100^{a}	100^{a}	100^{a}	Control	100^{a}	100^{a}	100^{a}	100^{a}
	Axial 050 EC	82^{b}	92^{b}	76^{b}	89^{b}	Pallas 75 WG + Dassoil 26-2N	91^{b}	95^{a}	88^{b}	92^{a}
	Sword 240 EC	88^{b}	97^{a}	85^{b}	96^{a}	Flyer	83c	90^{b}	77^{c}	89^{b}
Helix	Control	100^{a}	100^{a}	100^{a}	100^{a}	Control	100^{a}	100^{a}	100^{a}	100^{a}
	Axial 050 EC	75^{c}	91^{b}	70^{b}	85^{b}	Pallas 75 WG + Dassoil 26-2N	94^{b}	96^{a}	86^{b}	91^{a}
	Sword 240 EC	86^{b}	97^{a}	82^{b}	91^{a}	Flyer	83^{c}	8^{b}	74^{c}	88^{b}

DAT – days after treatment. The different letters (a, b, c) after the numbers for the same variety for a certain enzyme indicate statistical significance at $P<0.001$.

Genetic analysis

ISSR (Inter-Simple Sequence Repeats) analysis was performed to determine polymorphism using short repetitive regions of the genome. This method was preferred due to its high informativeness, cost-effectiveness, and ease of application in selection studies.

The set of ISSR primers (Table 2) used for the purposes of this study revealed significant levels of polymorphism between the durum wheat varieties studied. Only clear, reproducible amplification fragments (Figure 1) were evaluated. Based on this, a binary matrix of data 1 (present) and 0 (absent) was created. Data conversion was necessary to prepare a dendrogram that reveals the levels of genetic distance between genotypes.

The resulting fragments were separated by electrophoresis on a 2% agarose gel in buffer at 200 V. The visualization was made through a photo-documentation system under ultraviolet light, with an extra 4 µl Green Safe DNA gel stain.

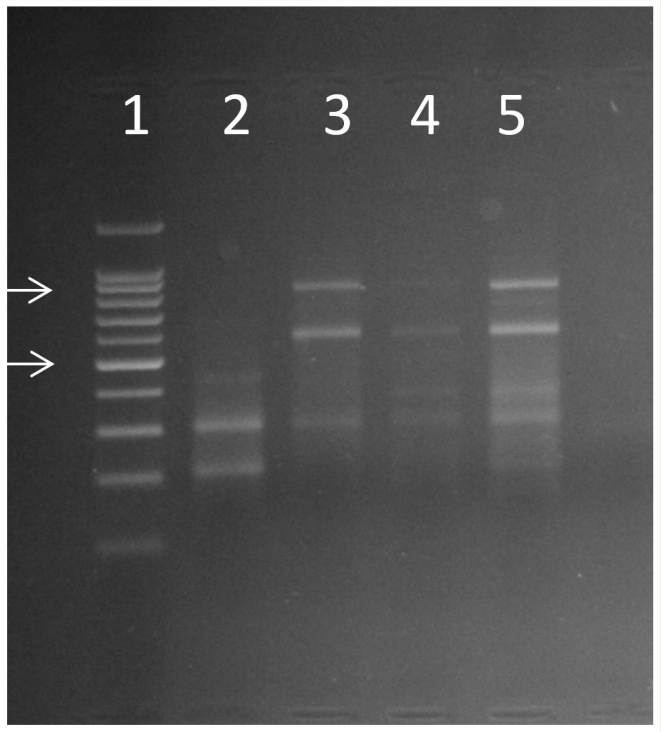


Figure 1. Polymorphisms among genotypes revealed by ISSR 6 primer amplification products. Start "1" - 1 500 bp. standard-sized DNA (marker), in which the bands of size 500 and 1000 bp are brighter, Start "2" - Sayacv, "3" - Predel cv, "4" - Reyadur cv, "5" - Helix cv

The Saya cultivar, which showed the highest tolerance to herbicide stress based on physiological parameters, chlorophyll content, and target enzyme activity, formed a separate subgroup genetically distant from Helix, the most herbicide-sensitive variety, inhibiting ACC and AHAS (Figure 2).

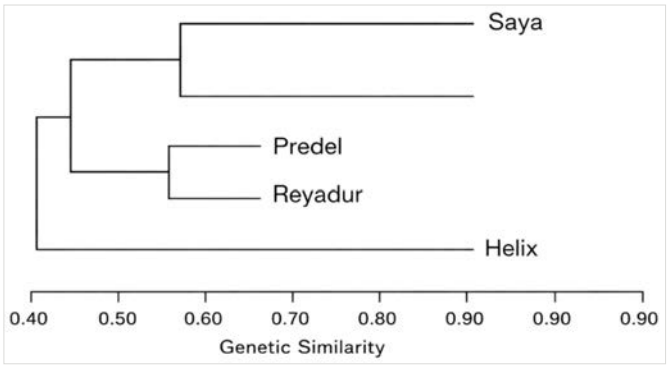


Figure 2. Dendrogram revealing the genetic distance between varieties based on ISSR profiling

This distribution of genotypes into clusters suggests that the increased tolerance observed in the Saya variety has a genetic basis and may be associated with variation in regions detectable by ISSR markers.

The Predel and Reyadur varieties form a separate cluster in the dendrogram. This corresponds to their intermediate phenotypic expression and physiological responses to herbicide treatment. The coincidence between molecular-genetic grouping and phenotypic responses supports the hypothesis that herbicide tolerance is a complex trait controlled by multiple genetic factors, not just changes in the target enzymes ACC and AHAS.

Although ISSR markers do not allow direct identification of specific genes responsible for herbicide resistance, they provide valuable information about genetic differences between varieties.

CONCLUSION

In general, despite the adverse effects of increased herbicide doses (double and quadruple), the studied durum wheat varieties were relatively resistant, as they compensated for or fully restored both photosynthetic activity and the activities of the enzymes ACC and AHAS over time. This is most evident in the Saya variety. The enzyme activity analysis manages an objective assessment of the response of cultivars and potential breeding lines in terms of their tolerance to stress factors, particularly herbicide resistance. This analysis, in combination with ISSR-based molecular characterization, represents an effective integrative approach for the preliminary screening of herbicide-tolerant genotypes and provides a basis for future breeding studies targeting specific genes.

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