

Genetic Variability of Acetolactate Synthase (ALS) Sequence in *Centaurea Cyanus* Plants Resistant and Susceptible to Tribenuron-methyl

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Short Report

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Abstract

Centaurea cyanus, belonging to the Asteraceae family, is an arable weed species being encountered mainly in the fields with cereals, sugar beet, and corn. *C. cyanus* high genetic variability has recently been reported, however, little is known about sequence variability in the context of herbicide resistance. *C. cyanus* resistance was found mainly against acetolactate inhibitors (ALS) inhibitors, but no ALS sequence information concerning herbicide resistance mechanism has been published yet. Therefore, the aim of this study was to determine ALS sequences for biotypes susceptible and resistant to tribenuron-methyl in order to identify possible mutations conferring the resistance. DNA isolation from susceptible and resistant plants was followed by PCR amplification and sequencing of ALS sequence. As a result different lengths of DNA products were obtained. Moreover, both nucleotide and amino acid sequence analysis revealed high sequence variability within one plant as well as between plants from the same biotype. In a few resistant plants, six changes in amino acid sequence were identified in comparison to susceptible ones. However, these preliminary studies require further investigation toward confirming the significance of these mutations in herbicide resistance development.

This study provides the first attempt in the research on *C. cyanus* target-site resistance mechanism.

Introduction

Centaurea cyanus L is an annual weed species belonging to the Asteraceae family. It is commonly found in the fields with cereals, sugar beet, and corn. In certain countries, it is increasingly rare, while in the others, such as in Central and Eastern Europe, more frequently encountered. Currently, 22 active ingredients of herbicides are recommended for controlling *C. cyanus* in Europe [1]. Herbicides that are recommended for the control of this weed belong mainly to acetolactate synthase (ALS) and photosystem II inhibitors, and synthetic auxins groups. However, frequent use of herbicides with the same mechanism of action leads to the emergence of weed resistance. First reports concerning *C. cyanus* resistance to chlorsulfuron (ALS inhibitor) are dated to 2008 [2] and its cross-resistance to chlorosulfuron and tribenuron-methyl was detected in 2010 [3]. So far, the resistant *C. cyanus* biotypes have been identified only in Poland [4].

The mechanism of the herbicide resistance emergence in *C. cyanus* plants has been unknown yet. There are two types of resistance mechanisms: target-site resistance (TSR) and non-target-site resistance (NTSR). TSR is associated with the target enzyme of the herbicide active ingredient, which includes changes in the enzyme amino acid sequence (contributing to the changes in the protein conformation within the herbicide action site) as well as an increased expression of the target enzyme gene [5]. NTSR mechanism is associated with an increased herbicide metabolism or sequestration, or decreased rates of herbicide uptake, translocation, penetration, or activation [5]. Early studies on *C. cyanus* resistance towards ALS inhibitors suggested both TSR and NTRS mechanisms [6].

Thus far, there is a lack of information concerning the nucleotide sequences of herbicide target enzyme genes of susceptible and resistant to the herbicide *C. cyanus* plants in gene repository databases. Only one *ALS* sequence (accession number: MK941142) was deposited in GenBank, however, no resistance status of the source plant was provided. Therefore, the aim of this study was to analyse and compare the *ALS* sequences of *C. cyanus* plants that belong to susceptible and resistant to tribenuron-methyl biotypes.

Materials And Methods

Biotypes of *C. cyanus* that were found to be susceptible (2 biotypes) and resistant (3 biotypes) to ALS inhibitor - tribenuron-methyl, obtained from different locations in Poland, were taken to the analysis. Their susceptibility to herbicide treatment was assessed by the determination of ED50 (effective dose of active ingredient (a. i.) causing a 50% of reduction in plant biomass). For this purpose, the seeds were sown in the pots placed under controlled conditions in the greenhouse. Plants at the 12-13 growth stage (according to BBCH scale) were treated with Lumer 50 WG (a.i. tribenuron-methyl 500 g kg⁻¹, ADAMA Agriculture B.V., Schaffhausen, Switzerland) at doses: for resistant populations: 0N, 0,5N, 1N, 2N, 4N, 8N, 16N; for susceptible populations: 0N, 1/16N, 1/8N, 1/4N, 1/2N, 1N, 2N, 4N; where N – the maximal recommended dose of the herbicide (30 g ha⁻¹, i.e. 15 g ha⁻¹ of active substance). Leaves from four plants from each biotype treated with a 1N dose of the herbicide were harvested for molecular analyses.

Leaves were ground in a mortar using liquid nitrogen. Genomic DNA was isolated using NucleoSpin Plant II, Mini kit for DNA from plants (Mecherey-Nagel, Düren, Germany). To amplify *C. cyanus ALS* sequence, a pair of degenerated primers was designed based on the alignment of the *ALS* coding sequences of plant species belonging to the Asteraceae family, deposited in GenBank. The alignment was done using BioEdit Sequence Alignment Editor (version 7.5.5) [7]. PCR was carried out in 50 µl reaction mixture containing 1X Q5 Reaction Buffer (NEB, Ipswich, MA, USA), 200 µM dNTPs, 0.5 µM forward primer (5' CGTKCTBGTRGAAGCCYTSGA 3'), 0.5 µM reverse primer (5' TCAATATTKYGTTCTKCCATCDCC 3'), 200 ng of genomic DNA, and 1 U of Q5 High-Fidelity DNA Polymerase (NEB). PCR was done in a Mastercycler nexus (Eppendorf, Hamburg, Germany) with an initial denaturation at 98°C for 30 s, followed by 35 cycles of amplification: 10 s at 98°C, 30 s at a 63°C, and 1 min at 72°C, with a final step of 2 min at 72°C. The reaction products were separated with 1% gel electrophoresis, purified from the gel with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), ligated to pJET1.2 plasmid using CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA), and cloned into DH10B *Escherichia coli* competent cells. The colony PCR method was used for positive colonies verification. The plasmids containing the inserted *ALS* gene sequence were isolated from *E. coli* cells using NucleoSpin Plasmid (Mecherey-Nagel). The presence of the insert in plasmids was confirmed by the digestion with *Bgl*III. Three plasmids were sequenced per one plant. DNA inserts were sequenced by Genomed (Warsaw, Poland). Sequencing data were analysed using the BioEdit Sequence Alignment Editor 7.5.5 [7] and visualised by GeneDoc (version 2.7.000) [8].

Results And Discussion

Herbicide resistance of weeds poses a threat to agricultural production, therefore, a particular emphasis on the causes of the emergence of this phenomenon is taken. It is essential in order to establish effective weed management procedures. One of the approaches aimed at explaining the herbicide resistance mechanisms is the analysis of the sequences of the herbicide target enzymes. TSR mechanism was scarcely examined in *C. cyanus*, in which resistance to ALS inhibitors has been detected in Poland [2,3]. Here, we have undertaken to analyse and compare plants belonging to two susceptible to ALS inhibitor (tribenuron-methyl) and three resistant biotypes of *C. cyanus*. Their susceptibility to the herbicide was assessed by the determination of ED50. The measurement of an effective dose of the tribenuron-methyl causing 50% loss in plant biomass showed that the low doses of the a.i. such as 4.73 g h⁻¹ and 6.08 g h⁻¹ were necessary for S1 and S2 biotypes biomass reduction, respectively. Whereas, the application of the 16 N dose of the a. i. on all plants of resistant biotypes was insufficient for ED50 determination. The dose of 16 N did not cause visible signs of the herbicide treatment.

To determine *ALS* nucleotide and amino acid sequences of *C. cyanus*, a pair of primers was designed based on the *ALS* sequences from Asteraceae plant species deposited in GenBank. *ALS* nucleotide sequence amplification resulted in the generation of 1699 to 1708 bp fragments that encompass amino acids from 109 to 663 of *ALS* protein sequence (according to *Arabidopsis thaliana* amino acid numbering in *ALS* sequence, accession number: P17597). An involvement of P197 mutation in TSR against sulfonylurea herbicides in *C. cyanus* was previously indicated, however, no more detailed results were presented [9]. The amplified sequences were sufficient to screen for the presence of mutations that were found to be involved in resistance development to *ALS* inhibitors in other weed species (A122, P197, A205, D376, R377, W574, S653, and G654)[4].

To the analyses, two susceptible and three resistant to tribenuron-methyl biotypes were taken. Four plants from each biotype and 3 plasmids from each plant were analysed. Nucleotide sequence analysis revealed high variability between the obtained sequences. Different lengths of the analysed fragments were found as well, which was the effect of the presence of three-nucleotide indels in the sequence fragments located between *ALS* functional regions as shown in Figure 1. Moreover, changes of the nucleotides at multiple positions within the analysed fragments were observed. Overall, out of the obtained 60 nucleotide sequences from all plasmids, there were 27 different sequence variants including 3 sequences that were found in plasmids obtained from both susceptible and resistant plants. Of these 27 different sequence variants, 8 were unique for susceptible plants, whereas, 16 – for the resistant ones. Totally, at 146 positions within the nucleotide sequences, synonymous changes were found, whereas, at 71 positions, nucleotide changes resulted in the changes to other amino acids. The majority of the differences in amino acid sequences were present in both susceptible and resistant biotypes, which indicates that their significance in herbicide resistance emergence may not be vital. However, 8 mutations (L179I, S314T, N404R, I468V, T475M, V525I, A605D, and L621) that were located in the functional regions of *ALS* were found only in the resistant plants (Figures 2-4). Six of these amino acid changes were present in 1 or 2 plasmids (out of 3) from certain plants, while mutations N404R and V525I were found in 1 out of 3

plasmids in 4 resistant plants, which implies their heterozygosity. Additionally, these two changes were identified in the same plasmids simultaneously. No previously reported amino acid mutations in the ALS sequence associated with the herbicide resistance in other weed species [4,10] were found. Also, the presence of P197 mutation, which was suggested to be present in one resistant biotype in Poland [9], was not confirmed. P197 mutation is one of the most commonly identified amino acid substitutions, and together with A205 is considered to confer sulfonylurea-specific resistance [10]. N404R and V525I mutations constitute novel changes within ALS amino acid sequence in biotypes resistant to ALS inhibitors, therefore, more detailed studies involving numerous samples should be carried out. The analysed nucleotide sequences were deposited in GenBank under accession numbers MZ561651-MZ561687.

In the case of 7 plants out of 20, all 3 sequences (obtained from 3 plasmids from the same plant) were the same, but within the same biotype, the sequences derived from different plants significantly differed. In some cases, the sequencing resulted in the identification of 3 divergent sequences from one plant. Such a high number of polymorphisms in ALS nucleotide sequence was also observed in other Asteraceae family species, namely in *Ambrosia artemisiifolia* L.[11,12], as well as, in other plant families and species such as *Alopecurus aequalis* [13] or *Zea mays* [14]. The reason for such differences in the obtained sequences may be copy number variation (CNV). Multiple gene copies may increase the effective dosage of a gene, which may influence the phenotype [15]. This mechanism was described in the context of the evolution of *Amaranthus palmeri* resistance to glyphosate. It was revealed that *A. palmeri* resistance to glyphosate was driven by the elevated 5-enolpyruvylshikimate-3-phosphate synthase gene copy number, followed by increased EPSPS transcript and protein levels along with enhanced glyphosate dose survival rate [16]. In the case of *C. cyanus* high variability between *ALS* sequences was observed in both biotypes, susceptible and resistant to tribenuron-methyl, therefore, the possibility of copy number variation involvement in resistance emergence cannot be excluded, but also cannot be confirmed. This phenomenon can be explained by the natural variability of *ALS*. High genetic variability of *C. cyanus* plants was confirmed in the analysis of ten microsatellite markers where high polymorphism was detected [17]. Another study concerning the analysis of leaf isozyme markers also highlighted the high genetic diversity of *C. cyanus* populations [18]. It should be noted, that despite low levels of genetic differentiation between populations, fine-scale spatial genetic structure was observed within populations [17].

To sum up, our study revealed high variability in the obtained *ALS* nucleotide as well as amino acid sequences within and between the analysed plants. Multiple changes were observed both in susceptible and resistant to tribenuron-methyl biotypes, therefore this phenomenon can be treated as the natural variability in this species. Few mutations were found only in resistant plants, among which N404R and V525I were observed in 4 plants but no mutations previously associated with conferring resistance to *ALS* inhibitors were observed. Currently, the connection between the found mutations and their contribution to the herbicide resistance emergence is hard to prove and thus it requires further analyses to confirm the presence of these mutations in a greater number of biotypes and plants. Owing to the fact that there has been scarce information about the *ALS* sequence of susceptible and resistant *C. cyanus*

biotypes, these studies provide the first comparative analysis between susceptible and resistant *C. cyanus* plants, which may be useful for future studies concerning herbicide resistance of this plant. Moreover, our work also confirms high genetic variability in this species.

Declarations

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Conflicts of interest /Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and material

The fragments of *Centaurea cyanus* acetolactate synthase gene sequences are available in the GenBank database under accession numbers MZ561651-MZ561687.

Code availability

Not applicable.

Authors' contributions

AOS and BW designed the experiment and methodology of the analysis. TP provided samples of weed populations. BW performed the study and analysed the results. BW and AOS wrote the manuscript. BW analysed sequence data. BW, AOS, and TP revised the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors have consented to this publication.

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Figures

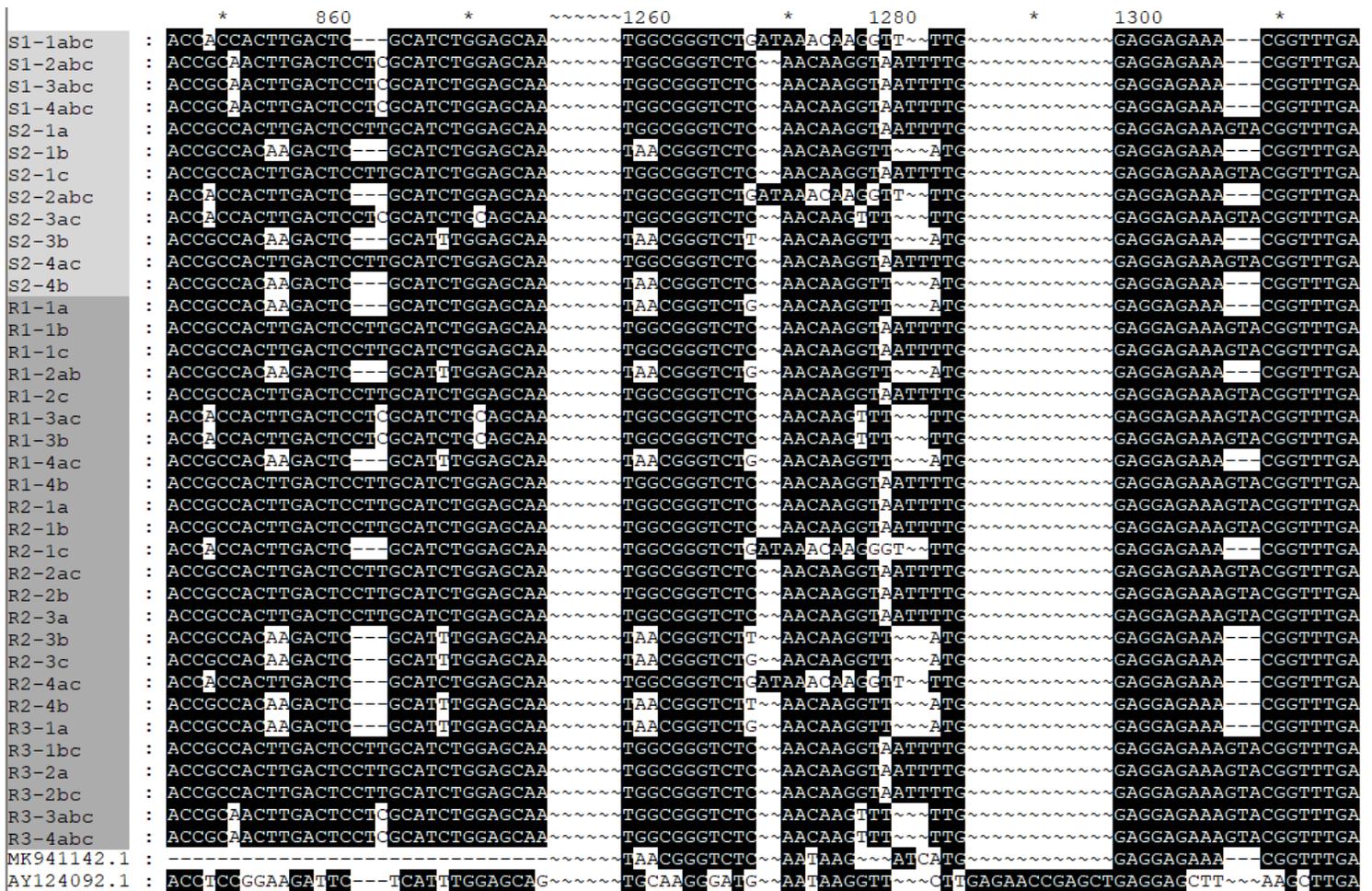


Figure 1

The alignment of the chosen fragments of *Centaurea cyanus* ALS nucleotide sequence. The sequences were derived from susceptible (S) and resistant (R) to Lumer 50 WG plants. The numbers in sequence names represent the plant number; a, b, c – clone name; MK941142 – *C. cyanus* ALS sequence GenBank

accession number; AY124092.1 – Arabidopsis thaliana ALS amino acid sequence GenBank accession number. The nucleotides numbering refers to A. thaliana ALS sequence.



Figure 2

The alignment of *Centaurea cyanus* ALS amino acid sequences encompassing pyrimidine (PYR) binding domain of pyruvate oxidase (POX) and related proteins region. The sequences were derived from susceptible (S) and resistant (R) to Lumer 50 WG plants. The numbers in sequence names represent the plant number; a, b, c – clone name; MK941142 – *C. cyanus* ALS sequence GenBank accession number (the nucleotide sequence was translated to amino acid sequence); P17597 – *Arabidopsis thaliana* ALS amino acid sequence GenBank accession number. The red frames and red arrows indicate amino acid mutations found in *C. cyanus* R plants. The blue arrows indicate positions of mutations known for conferring resistance to ALS-inhibiting herbicides in positions A122, P197, and A205, respectively. The amino acid numbering refers to *A. thaliana* ALS sequence.



Figure 3

The alignment of *Centaurea cyanus* ALS amino acid sequences encompassing thiamine pyrophosphate enzyme, central domain. The sequences were derived from susceptible (S) and resistant (R) to Lumer 50 WG plants. The numbers in sequence names represent the plant number; a, b, c – clone name; MK941142 – *C. cyanus* ALS sequence GenBank accession number (the nucleotide sequence was translated to amino acid sequence); P17597 – *Arabidopsis thaliana* ALS amino acid sequence GenBank accession number. The red frames and red arrows indicate amino acid mutations found in *C. cyanus* R plants. The blue arrows indicate positions of mutations known for conferring resistance to ALS-inhibiting herbicides in positions D376 and R377, respectively. The amino acid numbering refers to *A. thaliana* ALS sequence.

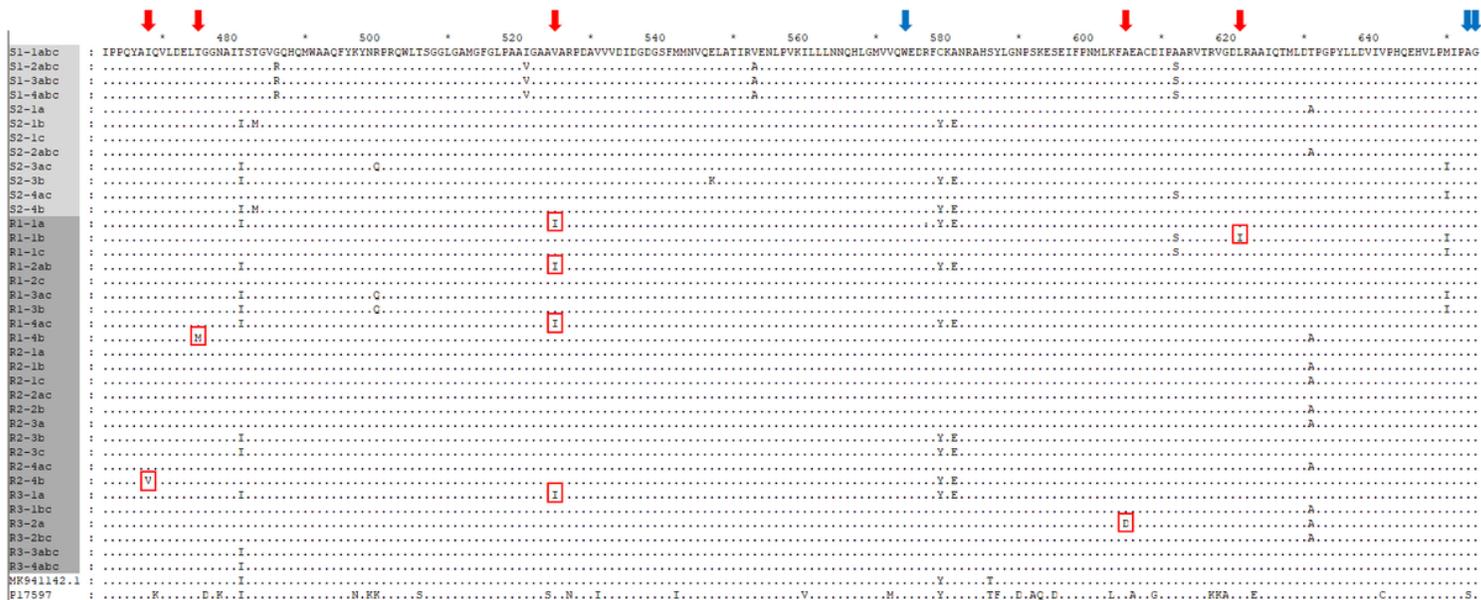


Figure 4

The alignment of *Centaurea cyanus* ALS amino acid sequences encompassing thiamine pyrophosphate binding domain. The sequences were derived from susceptible (S) and resistant (R) to Lumer 50 WG plants. The numbers in sequence names represent the plant number; a, b, c – clone name; MK941142 – *C. cyanus* ALS sequence GenBank accession number (the nucleotide sequence was translated to amino acid sequence); P17597 – *Arabidopsis thaliana* ALS amino acid sequence GenBank accession number. The red frames and red arrows indicate amino acid mutations found in *C. cyanus* R plants. The blue arrows indicate positions of mutations known for conferring resistance to ALS-inhibiting herbicides in positions W574, A653, and G654, respectively. The amino acid numbering refers to *A. thaliana* ALS sequence.