CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE (CZU)
Faculty of Agrobiology, Food and Natural Resources

Molecular basis of herbicide resistance in newly identified cases in the Czech Republic

Doctoral dissertation thesis

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Praha, 2022

DECLARATION

I hereby declare that the present dissertation work "**Molecular basis of herbicide resistance in newly identified cases in the Czech Republic"** is my own research work and I have properly acknowledged all the sources of materials used in this dissertation.

Place and date: In Prague; 20.07.2022 **Signature:** Madhab Kumar Sen

DEDICATIONS

In loving memory of

my dear grandfather (Late Bijoy Kumar Sen)

&

maternal grandparents

(Late Jatindra Mohan Achcharya & Late Pratibha Achcharya).

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I extend my humbleness to Prof. Petr Sklenička (the honourable Rector) and Prof. Iva Langrová (previous Dean) for giving me the opportunity to complete my dissertation and for availing all the requirements. I would like to express my deepest sense of gratitude to my supervisor, Prof. Josef Soukup (the Dean), for accepting me as his doctoral student and giving me the opportunity to pursue this doctoral program, for his constant support, guidance as well as freedom throughout my research. Without his kindness and constant encouragement, it would have been impossible to complete this research work.

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ABSTRACT

Herbicide-resistant arable weeds are continuing to increase rapidly worldwide and hence posing a major threat to the global food security. Currently, resistance has been reported against all major herbicidal modes of actions. The dissertation thesis starts with a brief introduction and a short literature review on the subject of the motivation for this research. Following the literature review, the objectives and the hypotheses of the Ph.D. doctoral thesis are discussed. The primary goal of this dissertation research was to explore the molecular mechanisms responsible for the herbicide resistance in the most important grass weeds in the Czech Republic. Thereafter, 4 published studies (1 bioinformatic study, 1 technical study and 2 research studies) has been discussed. The first study aimed to identify the best optimal codons for *acetolactate synthase* gene (an important herbicide-target) in weedy species. In the second study, we discovered the resistance mechanism to pyroxsulam (an herbicidal molecule) in *Bromus sterilis* collected from the winter wheat field of the Czech Republic. The third study aimed to find a solution to the missing fact that there are no suitable reference gene in any brome species. This study solved the problem of normalisation of the real-time polymerase chain reaction for herbicide -resistance studies in *B. sterilis*. Finally, in the fourth study, we report the first case of *Apera spica-venti*, which had developed resistance to three different herbicide modes of action. The results of this doctoral thesis will assist increasing our understanding in using omics-technologies to solve the mechanisms underlying herbicide resistance development (in weeds occurring in the Czech republic) and propose its prospects for planning superior weed management strategies.

TABLE OF CONTENTS

1. INTRODUCTION

One of the major plant protection problems encountered by the farmers worldwide are the weeds. Weeds are considered as undesirable plants, which are known to compete with the crops for water, light and nutrients and result in decrease in the yields and productivity of the major crops. Moreover, weeds also interfere by providing hosts and vectors for plant pathogens, providing food or shelter for animal pests, causing irritation to some body parts or digestive tracts of people or animals etc. (Zimdahl, 2018; Navas, 1991; Harlan, 1982). According to the Weed Science Society of America, weeds are defined as "plants growing where it is not desired". Although, drop in the crop yield was the main reason for efforts to reduce weed populations in arable crops, but effects on the crop quality for the horticultural crops are equally important. Baker (1965) hypothesized that the "ideal weedy plant species" (both annuals and perennials) generally have certain traits. Some of these weedy characteristics are:

- ✓ early germination and ability to germinate in various environment
- ✓ easy cross-pollination
- ✓ rapid growth from seedling to reproductive maturity
- ✓ prolonged period of seed production
- ✓ self-compatibility and
- ✓ seed production even in adverse conditions.

Among the various commonly used weed management strategies, pesticides are widely used to manage weeds and increase the crop yields (Sharma *et al.*, 2019). Herbicides represent almost 60% (by volume) of the pesticides used worldwide (Sharma *et al.*, 2019). Compared to the developed countries like Australia, USA and some parts of Europe, in the developing countries, due to the small size of the farms, weeds are removed manually by ploughing, hoeing, hand-pulling and mulching. However, with the increase of urbanization and wage costs in agriculture, manual weeding is getting replaced by herbicide use (Gianessi, 2013). In the developed countries, herbicides are used widely to manage weeds. However, over-dependence on herbicides with analogous modes of action has resulted in the evolution of herbicide-resistant weeds. Hence, this doctoral work thesis will aim on herbicide and their mechanisms of action and will also provide examples of how omics-technologies can be used to decrypt the existing as well as novel mechanisms of herbicide target sites, which will be critical to decipher the ways by which the new herbicides may exert their action.

2. LITERATURE REVIEW

2.1 Herbicides and their modes of action

Although the occurrence of a single weed plant is not important, its incessant growth can reduce the yield of the crop it grows in. Thus, the expansion of the weed population can be remarkably rapid (Moss, 2002). A tremendous revolutionary measure in agriculture is documented in 1940, with the introduction of synthetic herbicides as a primary tool for weed management (Kraehmer *et al.*, 2014). Herbicides have been used extensively and successfully to manage weeds and magnify the crop yield quality and quantity. 2,4-Dichlorophenoxyacetic acid (2,4-D) and 4-Chloro-2-methylphenoxyacetic acid (MCPA) were the first herbicides to be commercialized. Phenoxyacetic acids, 2,4-D and various analogues, were widely used during World War II and were further developed after the war (Reade & Cobb, 2002). Subsequently, within the mid-1950s, these auxinic herbicides were followed by two new of action herbicides: PS II inhibiting herbicides (ureas) and the herbicides inhibiting the mitosis phase of the cell division. Most of the presently used herbicide modes of actions were introduced and developed between 1955 and 1975. Since the 1980s, only three new herbicide modes of actions were introduced: acetolactate synthase (ALS) inhibitors, glutamine synthase inhibitors and the 4-Hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (Reade & Cobb, 2002). Currently, there are only 26 known herbicide sites of action available to farmers worldwide (www.weedscience.org). Based on their modes of action, herbicides can be classified as: lipid biosynthesis inhibitors, amino acid biosynthesis inhibitors, inhibitors of plant growth regulators, photosynthesis inhibitors, nitrogen-metabolism inhibitors, pigment inhibitors, cell-membrane disruptors etc (Heap, 2014; Gaines *et al.*, 2020). However, compared to the thousands of reactions occurring within the plants, this number is extremely low. Notwithstanding the massive success in significantly reduced losses, continuous use of similar herbicides has developed herbicide resistance in many weeds (Košnarová *et al.*, 2021; Sen *et al.*, 2021a; Gaines *et al.*, 2020). The modes of action discussed in this thesis, are amino acid biosynthesis inhibitors, lipid biosynthesis inhibitors and photosynthesis inhibitors.

2.1.1 Acetolactate synthase (ALS) inhibitors: These types of herbicides are known as ALS inhibitors. ALS {also known as acetohydroxyacid synthase (AHAS)} catalyzes the first step in the synthesis of the branched-chain amino acids (valine, leucine, and isoleucine) (Powles & Yu, 2010; Chipman *et al.*, 1998). This group of herbicides inhibit the action of the ALS enzyme. ALS

inhibitors are a part of the largest group of herbicides, which comprise of chemical families such as imidazolinones, pyrimidinyl benzoates, sulfonylureas, triazolinones, triazolopyrimidines etc (Sherwani *et al*., 2015; Reade & Cobb, 2002). Some examples of the active ingredients of this type of herbicides along with their chemical structures are shown in figure 1.

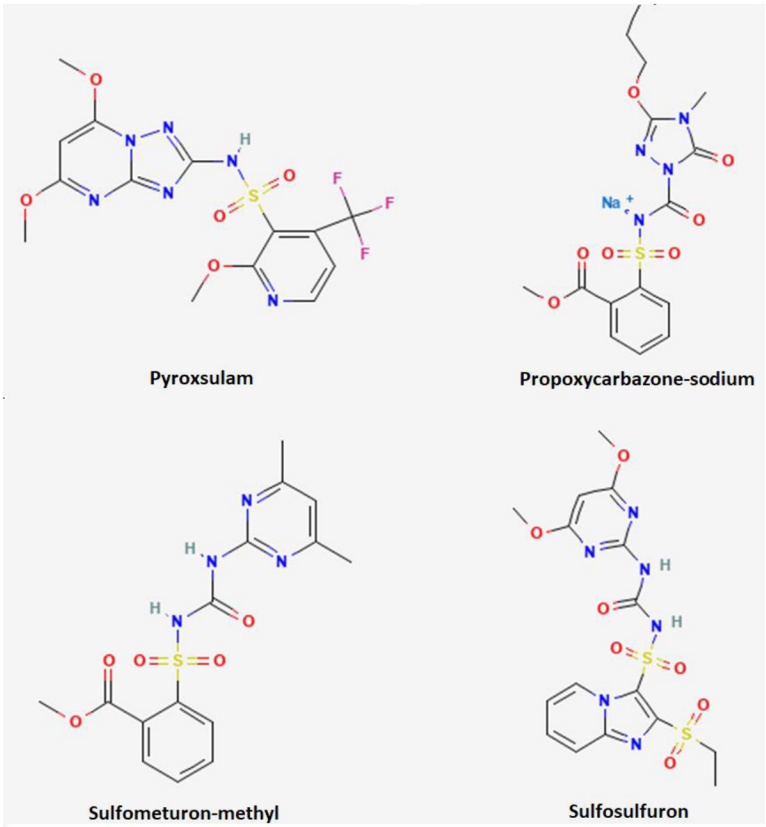

Figure 1: ***Chemical structures of some acetolactate synthase (ALS)- inhibiting herbicides.*** Picture source: PubChem (https://pubchem.ncbi.nlm.nih.gov/).

2.1.2 Acetyl-CoA carboxylase (ACCase) inhibitors: Malonyl-CoA is an intermediate substrate that plays an important role in the regulation of fatty acid metabolism. The process of carboxylation of acetyl-CoA to malonyl-CoA is catalyzed by a biotin-dependent enzyme, known as acetyl-CoA carboxylase (ACCase), which is a multi-subunit enzyme in most prokaryotes and the chloroplasts of most plants and algae. In the inactive state of this enzyme, another enzyme called, carnitine acyltransferase, catalyzes the transfer of the fatty acyl group from acyl CoA to carnitine, which further proceeds to the beta-oxidation of fatty acids in the mitochondria. ACCase is known to catalyze the first committed step of fatty acid synthesis and hence is very essential for the survival

of plants (Tong, 2005; Sasaki & Nagano, 2004). Because of its importance in plant metabolism, ACCase is one of the most frequently targeted enzymes of herbicides used against weedy grasses (Kaundun, 2014; Kukorelli, 2013). Three chemical families of graminicides (aryloxafenoxypropionates – fops, cyclohexandiones – dims, and phenylpyrazoline -dens) are known, which inhibit the ACCase. Some examples of the active ingredients of this type of herbicides along with their chemical structures are shown in figure 2.

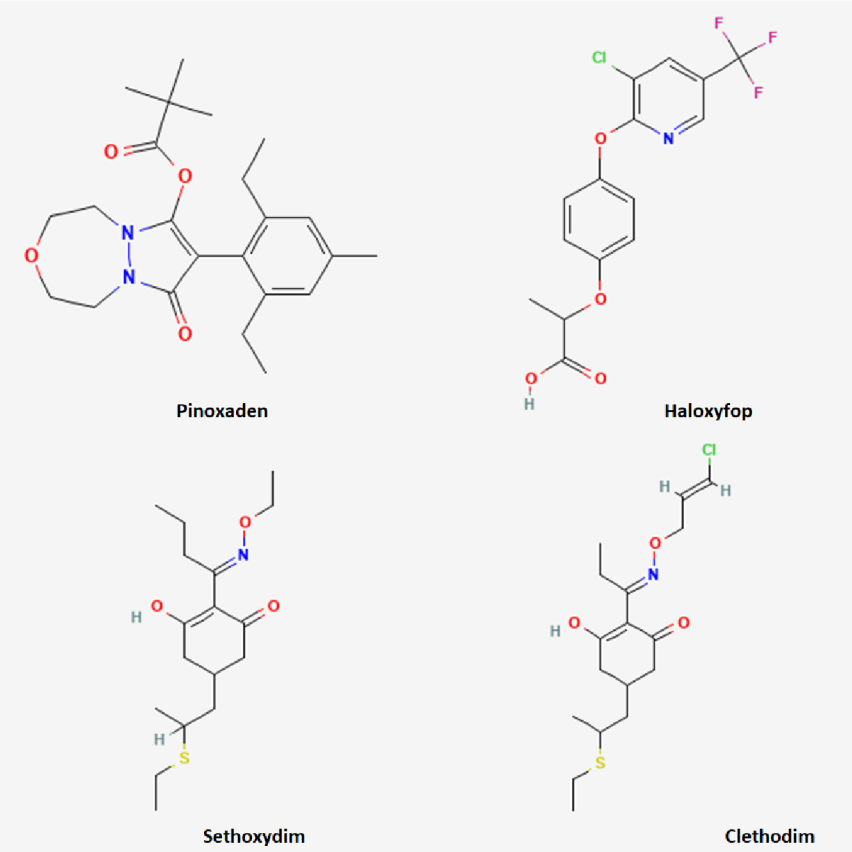

Figure 2: ***Chemical structures of some acetyl-CoA carboxylase (ACCase) inhibiting herbicides.*** Picture source: PubChem (https://pubchem.ncbi.nlm.nih.gov/).

2.1.3 PSII inhibitors: These mode of action herbicides is known to inhibit the photosynthetic pathway, specifically the Photosystem II (PSII). These classes of herbicides usually bind to the QB-binding site of the D1 protein complex present within the chloroplast thylakoid membrane. Following the binding of the herbicide molecule to the target protein, the electron transport system (ETS) from QA to QB gets disrupted. As a result of this, CO_2 fixation, ATP generation, and

nicotinamide adenine dinucleotide hydrogen phosphate ($NADPH_2$) production gets impeded. Ultimately, the biochemical pathways required for the plant's growth and development get hindered, resulting in the organism's death (Fedtke 2012; Fuerst, 1991; Draber, 1991). These herbicides comprise of chemical families such as phenyl-carbamates, pyridazinones, triazines, triazinones, uracils, ureas etc. Some examples of the active ingredients of this type of herbicides along with their chemical structures are shown in figure 3.

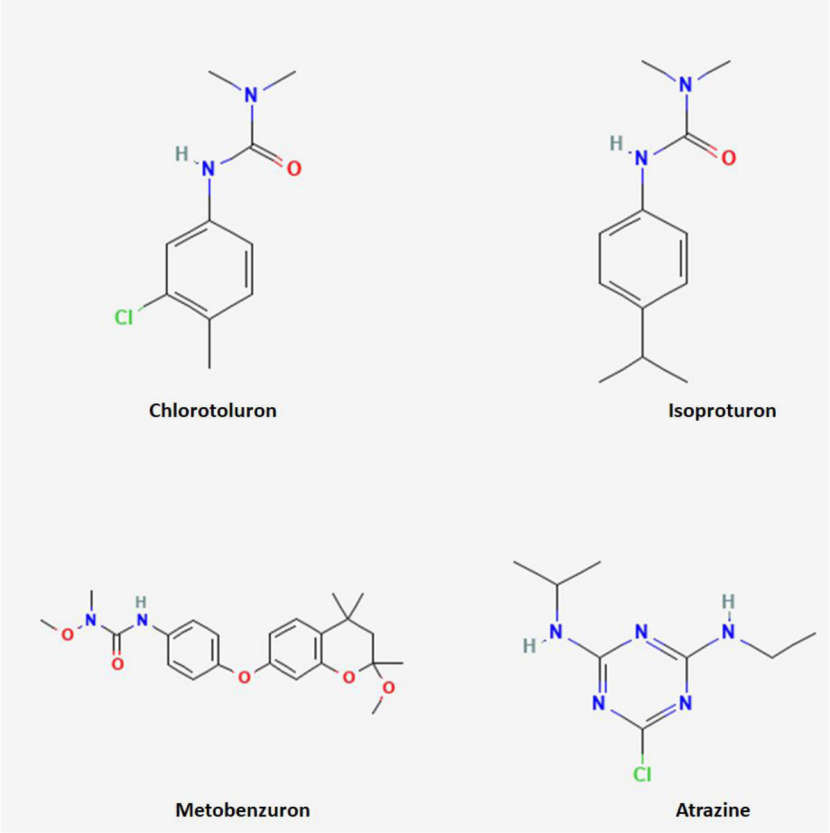

Figure 3: ***Chemical structures of some photosynthesis inhibiting herbicides.*** Picture source: PubChem (https://pubchem.ncbi.nlm.nih.gov/).

2.2 The status of herbicide resistance across the globe

According to the International Herbicide-Resistant Weed Database (http://www.weedscience.org), till 10th June 2022, 512 unique cases of herbicide-resistance have been reported in 96 crops from 71 countries, which had evolved resistance to 165 distinct different herbicides. Resistance has been reported in 153 dicots and 113 monocots, against 21 of 31 known herbicide modes of action (MOA). Figure 4 represents the alarming increase in the number of herbicide resistant weeds,

globally, in the last 30 years. Evolution of herbicide-resistance has been reported in many weeds, such as, *Lolium rigidum* (resistant against 14 MOAs), *Echinochloa crus-galli var. crus-galli* (resistant against 11 MOAs), *Poa annua* (resistant against 10 MOAs), *Amaranthus palmeri* (resistant against 8 MOAs), *Avena fatua* (resistant against 8 MOAs), *Eleusine indica* (resistant against 8 MOAs), *Alopecurus myosuroides* (resistant against 7 MOAs), *Amaranthus retroflexus* (resistant against 5 MOAs) etc. (data source: http://www.weedscience.org). Even though resistance has been reported against various herbicide mode of actions, however, this doctoral dissertation thesis will deal with three groups of herbicides (ALS inhibitors, ACCase inhibitors and PS II inhibitors) and two weedy species (*Apera spica-venti* and *Bromus sterilis*). Figure 5 represents a schematic diagram showing the increasing number of herbicide resistant species against Acetolactate synthase-inhibitors, Acetyl CoA Carboxylase-inhibitors and Photosystem II inhibitors, globally.

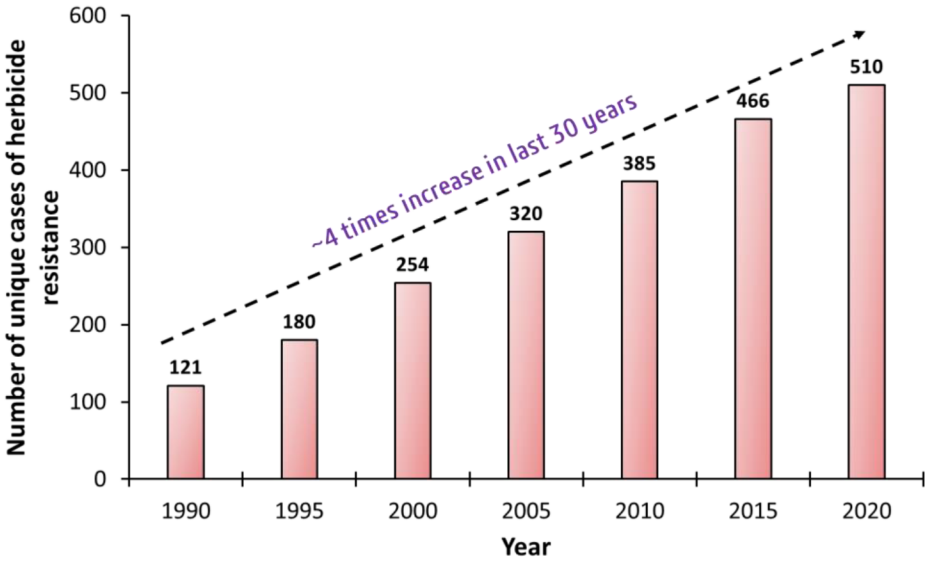

Figure 4: ***Schematic diagram showing chronological increase in the number of herbicide resistant weeds, globally.*** Picture source: International Herbicide-Resistant Weed Database (http://www.weedscience.org/home.aspx).

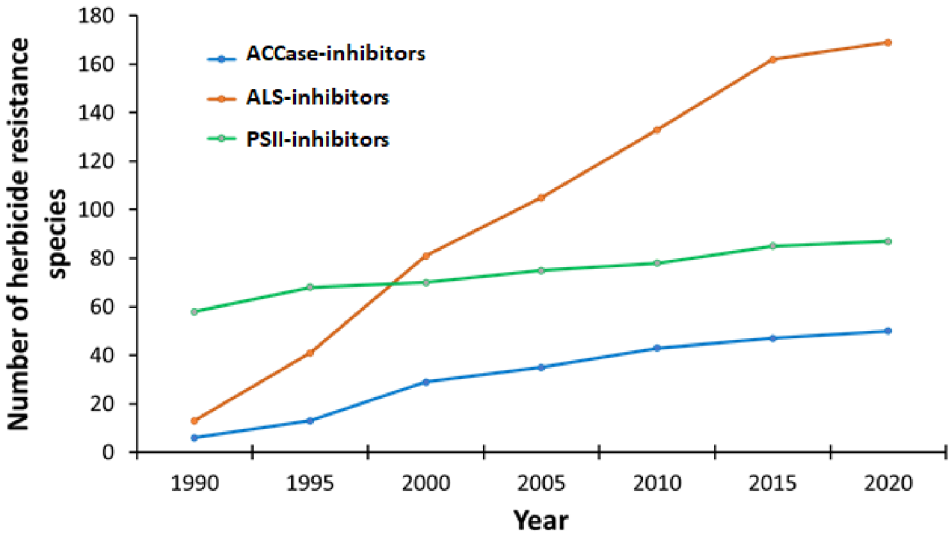

Figure 5: Schematic diagram showing the increasing number of herbicide resistant species against Acetolactate synthase-inhibitors, Acetyl CoA Carboxylase-inhibitors and Photosystem II inhibitors, globally. Picture source: International Herbicide-Resistant Weed Database (http://www.weedscience.org/home.aspx).

2.3 Molecular mechanisms of herbicide resistance

The efficacy of herbicides mainly depends upon the quantity of the herbicide moving into a plant cell. Additionally, how long the herbicidal molecule's active form remains accessible to interact with its target site, is also a very crucial factor. A full knowledge of the mechanism of resistance to a herbicide involves understanding the mechanism of action of the herbicide. The mechanisms of herbicide resistance can be either target site-based and/or non-target site-based (Figure 6).

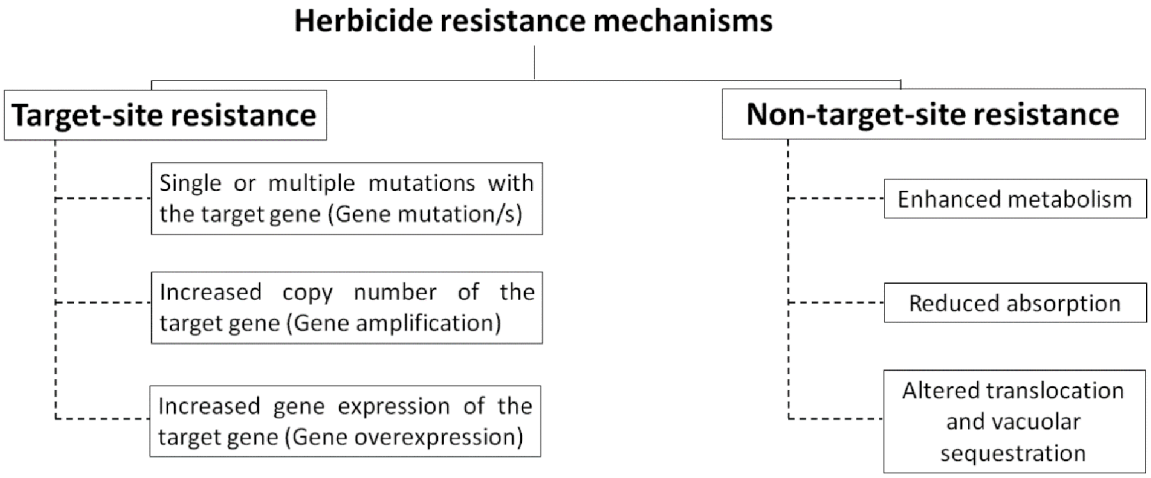

Figure 6: ***Brief outline showing the various mechanisms of resistance.***

2.3.1 Target-site resistance mechanism

Target-site resistance (TSR) mechanisms mainly include target protein mutations, target gene amplification and overexpression. Mutations within the target protein result in a change to the protein that binds the herbicide molecule, resulting in a lack of inhibition of the biochemical pathway (Sen *et al.*, 2021a; Rey-Caballero *et al.*, 2017). Even though most of the mutations associated with the herbicide resistance occur around the herbicide binding site of the target enzyme, however some mutations are also known to occur elsewhere within the protein structure. In addition to the single or polynucleotide polymorphisms, target-site mutations might also involve whole-codon deletions. Whole-codon deletions reduce herbicide's affinity towards the target-site enzyme (Gaines *et al.*, 2020). The first discovered nucleotide polymorphism related to the herbicide resistance was associated with D1 protein encoded by the *psbA* gene. Following this, several known mutation points were reported for herbicide target enzymes like ACCase, ALS etc. To date, codon deletions resulting in herbicide resistance has been reported only in the case of protoporphyrinogen oxidase (PPO; EC 1.3.3.4) in *Amaranthus sp.* (Patzoldt *et al.*, 2006). In addition to single and multiple nucleotide mutations, TSR also involves increased expression of the target-site gene. The overexpression of the target-site gene can be either due to changes in the regulatory regions and/or increased genomic copy numbers of the particular target gene. Gene duplication will result in an extra copy of the coding segment of DNA, which cause increased gene expression at the mRNA levels. Moreover, mutations might also accumulate in these new copies, resulting in the gain in function or loss of function over time. Evidence for elevated expression of the target gene has been reported in several species like *Bromus sterilis* L. (Sen *et al.*, 2021a), *Lolium rigidum* (Baerson *et al.*, 2002), *Conyza canadensis* (Gaines *et al.*, 2020), *Conyza bonariensis* (Gaines *et al.*, 2020), *Hordeum leporinum* (Yu *et al.*, 2007) etc.

2.3.2 Non-target-site resistance mechanism

The most common non-target site resistance mechanisms (NTSR) mechanisms are enhanced metabolism, reduced absorption, increased detoxification and reduced translocation and vacuolar sequestration (Sen *et al.*, 2021a). NTSR does not allow sufficient herbicide to reach the target site. NTSR also frequently leads to cross-resistance to herbicides of different modes of action, thereby making resistance management severely complicated (Yuan *et al.*, 2007). Cross-resistance patterns are highly variable and unpredictable. In general, herbicide detoxification can be separated into

three different phases: Phase I involves addition of a functional group to the herbicide. This is mediated by cytochrome P450 monooxygenases. Phase II involves addition of water-soluble metabolites. This is mediated by GSH S-transferases (GSTs) and glucosyltransferases (GTs)] and finally the phase III involving compartmentalization of the herbicide metabolites). Phase III is mediated by ABC-transporters and other transporters (Ghanizadeh & Harrington, 2017; Gaines *et al.*, 2020). Rapid detoxification of the herbicide molecules has been reported in many weeds like *Lolium rigidum* (Christopher *et al.*, 1991) and *Alopecurus myosuroides* Huds (Hall *et al.*, 1995).

2.4 Methods for detecting herbicide resistance

Unwavering tests for resistance are indispensable pre-requisites for the sensible operation of effective integrated weed control strategies. Ideally, the diagnostic tests must be quick, precise, cheap and readily available. Alongside these, the tests must give a consistent hint of the likely impact of resistance on herbicide activity in the field. Some of the many methods for detecting herbicide resistance are shown in the figure 7. Weed resistance diagnostics based on greenhouse bioassays include dose-response and cross-resistance studies. These usually allow us to get information on whole plant responses to the herbicides. However, the greenhouse bioassays in pots are time and space consuming, besides being quite expensive. To overcome these shortcomings, occasionally weed scientists prefer petri dish bioassays (using filter paper or agar). However, the accuracy and ability to distinguish between resistance mechanisms still remains a question for these bioassays.

Besides greenhouse bioassays biochemical, molecular and analytical assays had also proven their abilities in detecting herbicide resistance. Biochemical assays include herbicide-target enzyme assays such as ALS assay, ACCase assays etc. Even though we can expect a good correlation between the relative resistance at the enzyme and whole plant level, however, these assays afford to provide information mostly about TSR. But NTSR and multiple resistance to herbicides mostly remains undetected. Nevertheless, recently various cytochrome and GST-based had already grabbed the eyes of the weed scientists. Molecular techniques rely mostly on PCR-based pyrosequencing and qRT-PCR experiments to detect herbicide resistance mechanisms. Additionally, with the reduction of prices of next-generation sequencing technologies, interests in transcriptomics or RNA-seq have increased. Besides cost-reduction, with better availability of the reference genomes and transcriptomes of model crops, the number of studies based on novel RNA-

seq approach has been intensified. RNA-seq approach offers better insights to develop new knowledge about NTSR, as compared to the other diagnostic techniques. Analytical studies based on radioactive ^{14}C-radiolabelled compounds, HPLC and GC-MS allows analyzing the metabolic degradation of the herbicides and hence at present, these techniques are therefore the only choice to conduct metabolite-resistance studies. More details of the methods for detecting herbicide resistance can be found in Beffa *et al.*, 2012.

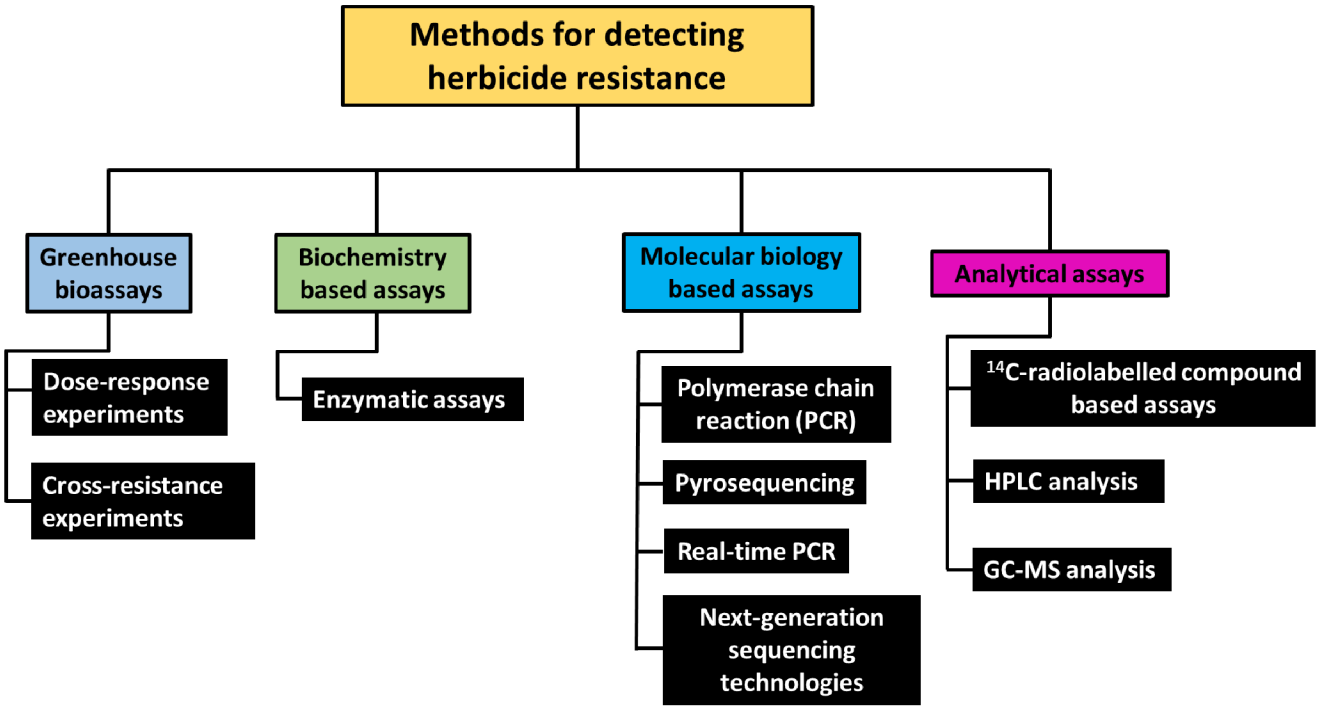

Figure 7: ***Brief outline showing the various methods for detecting herbicide resistance.***

2.5 Weeds studied in this Ph.D. work

2.5.1 *Apera spica-venti* (L.) P. Beauv.

Loose silky bent grass {*Apera spica-venti* (L.) P. Beauv.} (figure 8) is a winter annual grass, found in the Northern, Central and East European countries, mainly, in Germany, Poland and the Czech Republic (Auškalnienė *et al.*, 2020; Soukup *et al.*, 2006). Currently, the loose silky bent grass weed is the most prevalent grass weed in the Czech Republic. An year old loose silky bent grass is expected to form tufts of around 30-120 cm high. Their stems are usually erect, light green with a yellowish tinge. This weed mainly blooms during June to autumn and are known to be spread predominantly by wind and water. Their propagation is by seeds (only), which are usually

produced in a huge number. The seeds of this species emerge mainly in autumn, particularly under warm and rainy weather conditions. Hence, it can be predicted that the weediness of *A. spica-venti* varies usually from one year to the next. Silky bent grass often occurs in winter cereals, especially in winter wheat and winter barley. However, it might also cause damage to the yield and productivity of winter rape, forage crops, and early sown spring cereals (Soukup *et al.*, 2006).

Several herbicides, including ureas, dinitroanilines, thiocarbamates, sulfonylureas and triazolopyrimidines are mainly used to control these weedy species. Although, these herbicides were used successfully against this species, however, various cases of growing resistance in these species were reported (Košnarová *et al.*, 2021; Hamouzová *et al.*, 2011; Krysiak *et al.*, 2011).

Figure 8: ***Apera spica-venti.*** Picture source: https://www.flickr.com.

2.5.2 *Bromus sterilis* (L.)

Bromus sterilis L. (barren brome), is an annual or biennial grass weed, which is known to be found in northern Africa, western and middle Asia and northern, central and eastern Europe (figure 9). In the Czech Republic, these problematic weeds are known to occur in the lowland and hilly areas. *B. sterilis* usually blooms from May to July. This grass usually ranges from 20 to 90cm in height and its leaf blades are approximately 6–25 cm long containing short, soft hair. The leaves are usually rough and hairy with green to purplish in colour. Their spikelet is approximately 6 cm long (maximum). Although, these weeds mostly infest field margins, but it can also invade within the fields, causing severe infestations, resulting into high yield losses in cereal crops (Valičková *et al.*,

2017; Jursík *et al*., 2016; Moray *et al*., 2003; Allen & Meyer, 2002). These weeds are known to cause up to 30–60% yield losses in the productivity of winter wheat.

In the Europe, ALS-inhibiting herbicides such as pyroxsulam, propoxycarbazone, mesosulfuron, and sulfosulfuron are mainly used, to control this species. The evolution of resistance against these herbicides in these species has jeopardized their management in Europe. Resistance against glyphosate has also been reported in UK, in the recent years. Additionally, populations of *B. sterilis* resistant to ACCase- and ALS-inhibiting herbicides have also been reported from Germany and France (Sen *et al*., 2021a; Davies *et al*., 2020).

Figure 9: ***Bromus sterilis.*** Picture source: http://www.jvsystem.net.

3. OBJECTIVES & HYPOTHESES

3.1 DISSERTATION OBJECTIVES

- The main objective of this thesis is to investigate the molecular mechanisms which are responsible for the herbicide resistance in the most important grass weeds in the Czech Republic.
- To use the molecular techniques to identify any inhibitor binding site that binds compounds with physicochemical properties compatible with good uptake and translocation.
- To come-up with new molecular tools to detect the reasons behind the herbicide resistance and also to come-up with more efficient and cost-effective weed management strategies.

3.2 DISSERTATION HYPOTHESES

- Genetic mutations leading to structural change is responsible for the resistance to particular herbicide with a particular mode of action. Many of the mutations had been detected, but many more are yet to be detected.
- Higher expression of herbicide target genes leads to increased concentration of target enzyme and inadequate effect of herbicides.
- The metabolic resistance to a particular herbicide is due to the level of expression of specific genes encoding a particular protein, associated with that herbicide.

4. LIST OF STUDIES

4.1 Study 1

- **Title:** Identification of the optimal codons for acetolactate synthase from weeds: an in-silico study.
- **Brief description:** Although various studies of codon usage bias have been reported in a broad spectrum of organisms, no studies to date have examined codon usage bias for herbicide target genes. In this study, we analysed codon usage patterns for the *acetolactate synthase (ALS)* gene in eight monocot weeds and one model monocot. Additionally, the optimal codons, along with over- and under-represented codons, were identified. Gene design using optimal codons rather than overall abundant codons produce improved protein expression results.

4.2 Study 2

- **Title:** Enhanced metabolism and target gene overexpression confer resistance against acetolactate synthase-inhibiting herbicides in *Bromus sterilis*.
- **Brief description:** Frequent usage of pyroxsulam, an ALS inhibiting herbicide has resulted in development of herbicide-resistance in *Bromus sterilis*. The present study is the first detailed study on elucidating the mechanism of ALS inhibiting herbicide resistance in *Bromus sterilis* biotypes from the Czech Republic.

4.3 Study 3

- **Title:** Identification of the most suitable reference gene for gene expression studies with development and abiotic stress response in *Bromus sterilis*.
- **Brief description:** qRT-PCR and the next-generation sequencing technologies can illuminate more and decrease the knowledge gap in the field of the herbicide resistance. Although qRT-PCR can calculate accurate fold changes, however, its accuracy depends on the expression of reference genes. To our knowledge, there are no reports on the suitable reference gene in any brome species so far. Hence, the present study is the first detailed study on the identification of the most suitable reference gene/s in *Bromus sterilis*.

4.4 Study 4

- **Title:** *Apera spica-venti* in the Czech Republic develops resistance to three herbicide modes of action.
- **Brief description:** *Apera spica-venti* is widespread in many Central and East European countries, like Germany, Poland and the Czech Republic. It can be controlled with numerous herbicides like ALS inhibitors, ACCase inhibitors and PSII inhibitors. However, with the overuse of these herbicide modes of action, multiple resistances to ALS and ACCase inhibitors were detected. The present study is the first study reporting resistance to three different herbicide modes of action.

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Identification of the optimal codons for acetolactate synthase from weeds: an *in-silico* study

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Abstract: Although various studies of codon usage bias have been reported in a broad spectrum of organisms, no studies to date have examined codon usage bias for herbicide target genes. In this study, we analysed codon usage patterns for the acetolactate synthase (ALS) gene in eight monocot weeds and one model monocot. The base composition at the third codon position follows C3 > G3 > T3 > A3. The values of the effective number of codons (ENC or Nc) indicate low bias, and ENC or Nc *vs.* GC3 plot suggests that this low bias is due to mutational pressure. Low codon adaptation index and codon bias index values further supported the phenomenon of low bias. Additionally, the optimal codons, along with over- and under-represented codons, were identified. Gene design using optimal codons rather than overall abundant codons produce improved protein expression results. Our results can be used for further studies, including eliciting the mechanisms of herbicide resistance (occurring due to elevation of gene expression levels) and the development of new compounds, their efficiency and risk assessment for herbicide resistance evolution.

Keywords: herbicide-resistant weeds; heterologous gene expression; primer designing; recombinant *ALS* protein

Weeds compete with the major crop, thus reducing their yield and productivity. Economically, they can be regarded as a more damaging agent than other crop pests (in several situations). Globally, herbicide resistance has been documented in a wide range of weed species. Acetolactate synthase (ALS) catalyses the first step in the synthesis of the branched-chain amino acids (Duggleby et al. 2008, Hamouzová et al. 2014) and is the target for a large number of herbicides. Continuous use of the same herbicide with the same mode of action has allowed for the selection of weed populations resistant to the overused herbicide or mechanism of herbicide action. The widespread evolution of multiple-herbicide resistance in weedy species makes their control more difficult. The most common mechanisms of evolving resistance to herbicides by plants include metabolic changes, mutations in the DNA of the target gene and overexpression of the target protein (Jugulam and Shyam 2019, Murphy and Tranel 2019). Unfortunately, for nearly 20 years, no new mode of action has been introduced into the market. Furthermore, with the release of glyphosate-resistant crops, the efforts for herbicide discovery reduced significantly (Powles and Yu 2010).

Due to their favourable effects on the efficiency and accuracy of the translation, certain codons are preferred over the others, leading to differential codon usage patterns (codon-usage bias) (Je et al. 2019). Optimal codons contribute to the accuracy as well as the speed of the translation elongation (Wright 1990). Thus, it is very useful to know the rules which govern the synonymous codon selection of the target gene. This knowledge can be extremely useful to design a heterologous gene, having the most efficient expressional efficiency. Apart from playing important roles in various physiological processes,

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codon bias and codon optimisation, finds huge applications in industrial biotechnology, whose major goal is to produce recombinant proteins. Even though heterologous gene expression studies are among the most well-appreciated studies related to specific protein interactions, efficiency in the expression of heterologous genes remains the most challenging part. Codon usage patterns in non-model plants, especially weeds, are not well understood, mainly due to limitations in data availability. The development of new compounds with herbicidal properties along with an assessment of their efficiencies and risk of resistance may require designing synthetic genes based on their codon usage patterns. Synthetic genes find important applications in heterologous gene expression experiments. Heterologous gene expression studies can be very useful in basic biological research areas, including protein interactions studies and the development of new herbicidal compounds (Quax et al. 2015, Zhou et al. 2016).

In this study, we analysed codon usage patterns for ALS in eight monocot weeds (*Alopecurus myosuroides* Huds., *Apera spica-venti* L., *Beckmannia syzigachne* L., *Bromus tectorum* L., *Echinochloa crus-galli* (L.) P. Beauv., *Echinochloa oryzicola* (Vasinger) Ohwi, *Poa annua* L. and *Lolium rigidum* Gaud.) and one model monocot (*Zea mays* L.). Relative synonymous codon usage (RSCU), codon adaptation index (CAI), codon bias index (CBI), the effective number of codons (ENC or Nc), positional GC contents and CG dinucleotide suppression values, were analysed for ALS-coding sequences. Optimal codons, along with over- and under-represented codons, were identified. Our results would help in additional investigations with *acetolactate synthase* gene, including eliciting the mechanisms of herbicide resistance (occurring due to elevation of gene expression levels) and development of new herbicidal compounds with synthetic genes based on their codon usage pattern.

MATERIAL AND METHODS

Retrieval of sequences. Full-length *ALS* coding sequences were retrieved from the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/). The list of organisms and their accession numbers are in Table 1.

Base composition parameters. Total GC content, the GC content at the different positions of a codon (GC1, GC2 and GC3) were calculated using MEGAX (Kumar et al. 2018). Because of the degenerate property of the codons, the 3rd position of a codon (also called as wobble position) has less discriminatory for an amino acid than the other two bases (Elhaik and Tatarinova 2012). Moreover, CG dinucleotides are the potential target sites for methylation (Elhaik et al. 2014). The XCG/XCC ratio based on RSCU values was used to calculate CG dinucleotide suppression values (Mazumdar et al. 2017).

Analysis of codon usage for acetolactate synthase in weeds. MEGAX and CodonW (http://codonw.sourceforge.net), with in-house PERL script and standard genetic codon table, were used for computing the RSCU values. RSCU value of 1 indicates no codon usage bias, while values above and below 1 indicate codons are utilised more and less frequently (respectively) than expected (Mondal et al. 2016). Based on the RSCU values, optimal codons and their frequencies (F_{op}) were calculated. CAI and CBI values were determined using CodonW. CAI values range from 0 (random codon usage) to 1 (extreme codon bias) (Mondal et al. 2016). CBI values measure the extent to which a gene uses the set of its optimal codons. CBI values range between 0 (random codon usage) and 1 (extreme codon bias) (Bennetzen and Hall 1982).

GC3 *vs.* the effective number of codons (ENC or Nc) plot. Mutational pressure and natural selection are the two well-known core factors responsible for codon biasness. Plot between GC3 and expected the effective number of codons can be a good measure to determine that among the mentioned core factors, which factor is the driving force (Sharp et al. 1993). ENc values

Table 1. List of organisms used for analysis along with their GenBank accession numbers

Organism	Accession number (CDS)
Zea mays ALS1	NCVQ01000006.1
Z. mays ALS2	NM_001148702.2
Apera spica-venti	JN646110.1
Bromus tectorum	MK492423.1
Alopecurus myosuroides	AJ437300.2
Beckmannia syzigachne	MG891930.1
Lolium rigidum	MK492446.1
Echinochloa crus-galli ALS1	KY071206.1
E. crus-galli ALS2	KY071207.1
E. crus-galli ALS3	KY071208.1
E. oryzicola ALS1	KY071209.1
E. oryzicola ALS2	KY071210.1
Poa annua ALSa	KT346395.1
P. annua ALSb	KT346396.1

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Table 2. Nucleotide composition for acetolactate synthase of the weeds of interest

Organism		T-3	C-3	A-3	G-3	GC1	GC2	GC3	GC	Nc	Codon adaptation index	Codon Bias index
		(%)										
Apera spica-venti		23.2	35.4	10.8	30.7	58.3	63.2	45.7	66	55.9	0.3	0.1
Bromus tectorum		20.5	37.2	11.3	31	59.1	63.6	45.4	68.2	54.6	0.3	0.1
Alopecurus myosuroides		23.6	34.3	12.8	29.3	57.4	62.7	45.9	63.7	57.3	0.2	0.1
Beckmannia syzigachne		23.3	35.8	11.3	29.7	57.9	62.3	45.8	65.5	56.3	0.3	0.1
Lolium rigidum		18.7	42	8.6	30.8	60.6	63	46	72.8	51.4	0.3	0.1
Echinochloa crus-galli	*ALS1*	18.8	40.6	9.3	31.3	60.6	63.3	46.7	71.9	52.1	0.3	0.1
	ALS2	18.5	40.4	9.3	31.8	60.5	63.2	46.1	72.2	51.9	0.3	0.1
	ALS3	19.3	39.8	8.7	32.2	60.7	63.6	46.5	72	52	0.3	0.2
Zea mays	*ALS1*	21.1	37.1	10	31.8	59.3	63.5	45.4	68.9	54.3	0.2	0.1
	ALS2	22.8	36.5	11.6	29.1	57.9	62.8	45.4	65.6	56.2	0.2	0.1
Echinochloa oryzicola	*ALS1*	18.8	40.4	9.3	31.4	60.4	63.1	46.2	71.9	52.2	0.3	0.1
	ALS2	18.5	40.4	9.2	32	60.6	63.2	46.3	72.4	51.7	0.3	0.1
Poa annua	*ALSa*	22.4	36.9	8.9	31.8	59	62.8	45.5	68.7	54.3	0.3	0.1
	ALSb	19.7	40.1	9.0	31.2	60.0	62.6	46.1	71.3	52.5	0.3	0.1

Nc – number of codons

were calculated from GC3s under the null hypothesis (i.e., no selection) according to the given equation by Wright (1990). The values of ENc (or Nc) might vary from extreme (20) to least bias (61) (Mondal et al. 2016).

RESULTS AND DISCUSSIONS

Base compositional parameters and correlation analysis. For these species, the base composition at the third codon position follows C3 > G3 > T3 > A3 (Table 2). Analysis of XCG/XCC ratio showed values of 0.4 (*A. spica-venti* L., *Z. mays* L. *ALS2* and *P. annua* L. *ALSb*), 0.5 (*B. tectorum* L., *A. myosuroides* Huds., *B. syzigachne* L., *E. crus-galli* (L.) P. Beauv., *E. oryzicola* (Vasinger) Ohwi, *P. annua* L. *ALSa* and *Z. mays* L. *ALS1*) and 0.6 (*L. rigidum* Gaud.). Except for *A. spica-venti*, *Z. mays* L. *ALS2* and *P. annua* L. *ALSb*; our results indicate moderate CG dinucleotide suppression. The values of Nc varied from 51.4 to 57.3. This indicates weak bias. Moreover, the Enc (or Nc) *vs.* GC3 plot suggests that the low bias might be due to mutational pressure and not a natural selection (Figure 1). The CAI values ranged from 0.2–0.3 (i.e., random codon usage). Low CBI values further supported the fact of random codon usage (Table 2).

Analysis of relative synonymous codon usage and determination of optimal codons. To analyse the codon usage patterns in the selected weed species, RSCU values were calculated. Based on the cluster analysis, primarily two clusters were formed: one with maize ALS and the other with the rest species. Within the cluster containing weedy species ALS, two further sub-clusters were formed. ALS from *B. tectorum* L. and *L. rigidum* Gaud. showed similar patterns with the ALS from the *Echinochloa* sp. (Figure 2). In all the cases, the number of codons having an RSCU value less than 1 is found to be greater than the codons having an RSCU value higher than optimum. Nine codons (GAA, GGA, AAA, UUA, CUA, CAA, AGA, CGA and GUA) were under-represented in all cases, whereas CAG, CGC and UCC were over-represented in all cases. Interestingly, all the nine

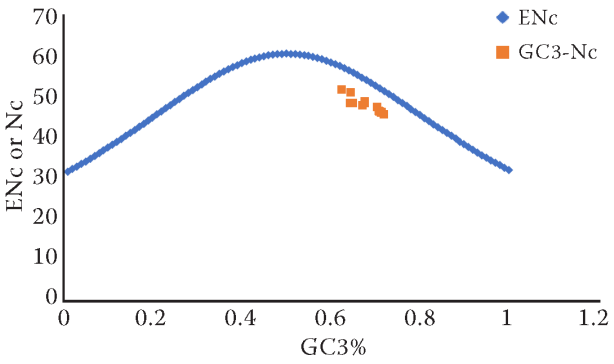

Figure 1. GC3 vs. number of codons (ENC or Nc) plot. The acetolactate synthase (ALS) genes which are positioned on or close to the curve line are considered to be under mutational pressure

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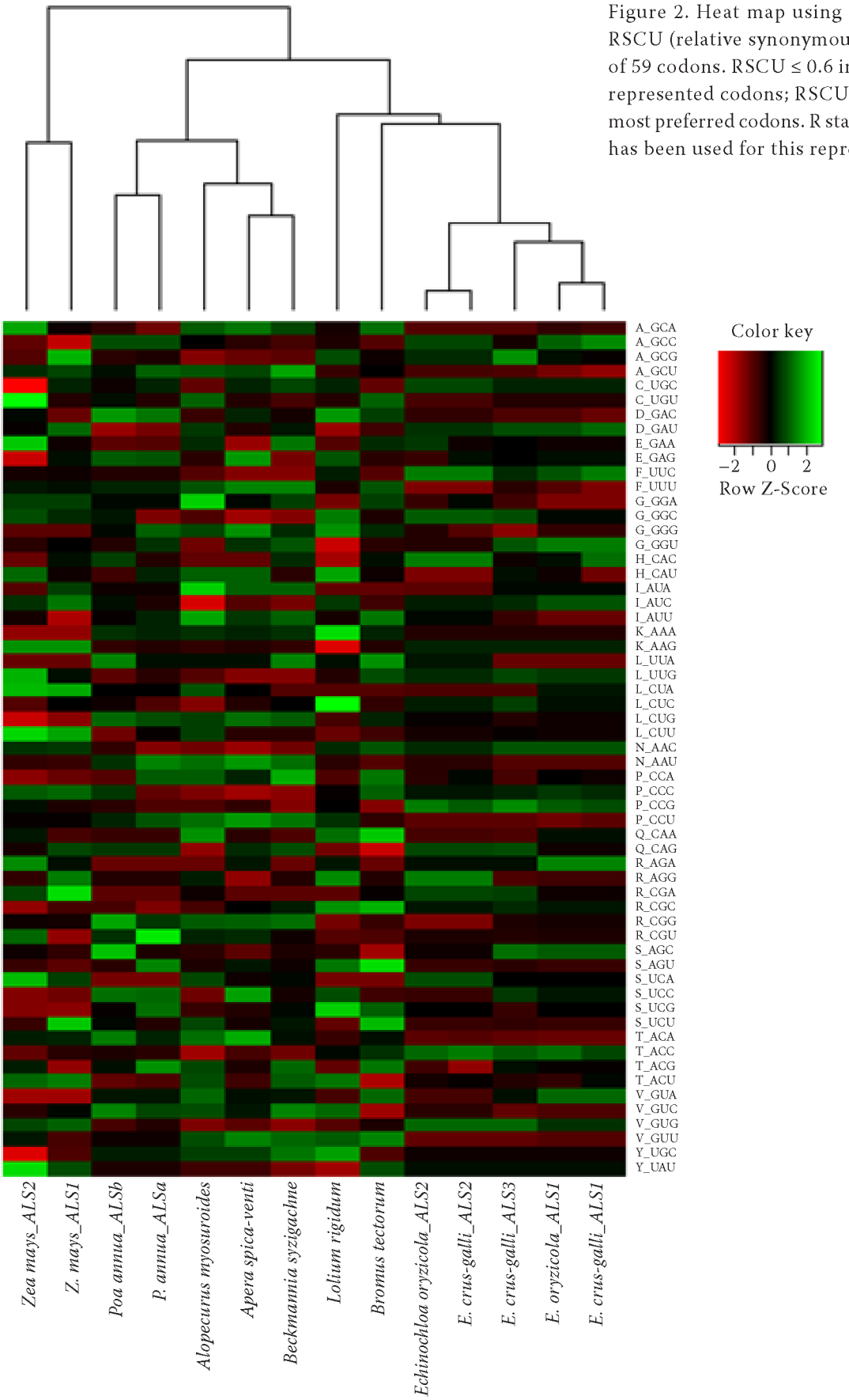

Figure 2. Heat map using raw Z-score of RSCU (relative synonymous codon usage) of 59 codons. RSCU ≤ 0.6 indicates under-represented codons; RSCU ≥ 1.6 indicates most preferred codons. R statistical software has been used for this representation

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Table 3. Optimal codons and their frequencies

Organism	A	F_{op}	C	F_{op}	D	F_{op}	E	F_{op}	F	F_{op}	G	F_{op}
Apera spica-venti	GCC	0.41	TGC	0.80	GAC	0.53	GAG	0.91	TTT	0.57	GGC	0.28
Bromus tectorum	GCC	0.39	TGC	0.67	GAC	0.55	GAG	0.82	TTT	0.52	GGC	0.35
Alopecurus myosuroides	GCC	0.43	TGC	0.67	GAT	0.52	GAG	0.82	TTT	0.52	GGC	0.33
Beckmannia syzigachne	GCC	0.40	TGC	0.83	GAC/GAT	0.50	GAG	0.79	TTT	0.57	GGC	0.30
Lolium rigidum	GCC	0.42	TGC	0.80	GAC	0.60	GAG	0.88	TTC	0.59	GGC	0.43
Echinochloa crus-galli ALS1	GCC	0.49	TGC	0.80	GAT	0.55	GAG	0.85	TTC	0.70	GGC	0.37
E. crus-galli ALS2	GCC	0.46	TGC	0.83	GAT	0.52	GAG	0.85	TTC	0.70	GGC	0.42
E. crus-galli ALS3	GCC	0.42	TGC	0.80	GAT	0.53	GAG	0.84	TTC	0.61	GGC	0.41
Zea mays ALS1	GCC	0.34	TGC	0.80	GAT	0.55	GAG	0.85	TTC	0.55	GGC	0.39
Z. mays ALS2	GCC	0.39	TGC/TGT	0.50	GAC	0.52	GAG	0.75	TTC	0.54	GGC	0.41
Echinochloa oryzicola ALS1	GCC	0.48	TGC	0.80	GAT	0.53	GAG	0.85	TTC	0.65	GGC	0.37
E. oryzicola ALS2	GCC	0.46	TGC	0.83	GAT	0.52	GAG	0.82	TTC	0.70	GGC	0.42
Poa annua ALSa	GCC	0.46	TGC	0.80	GAC	0.58	GAG	0.88	TTC	0.52	GGC/GGG/GGT	0.30
P. annua ALSb	GCC	0.47	TGC	0.75	GAC	0.60	GAG	0.89	TTC	0.52	GGC	0.38
Organism	H	F_{op}	I	F_{op}	K	F_{op}	L	F_{op}	N	F_{op}	P	F_{op}
Apera spica-venti	CAC	0.69	ATC	0.52	AAG	0.80	CTG	0.40	AAC/AAT	0.50	CCA	0.33
Bromus tectorum	CAC	0.77	ATC	0.54	AAG	0.80	CTG	0.35	AAC	0.82	CCC	0.38
Alopecurus myosuroides	CAC	0.69	ATC/ATT	0.44	AAG	0.79	CTG	0.37	AAC	0.56	CCA	0.35
Beckmannia syzigachne	CAC	0.79	ATC	0.50	AAG	0.79	CTG	0.39	AAC	0.56	CCA	0.39
Lolium rigidum	CAC	0.64	ATC	0.61	AAG	0.71	CTC	0.44	AAC	0.76	CCC	0.31
Echinochloa crus-galli ALS1	CAC	0.83	ATC	0.63	AAG	0.83	CTC/CTG	0.31	AAC	0.82	CCC	0.35
E. crus-galli ALS2	CAC	0.85	ATC	0.59	AAG	0.83	CTC/CTG	0.31	AAC	0.76	CCC	0.33
E. crus-galli ALS3	CAC	0.75	ATC	0.60	AAG	0.83	CTC	0.33	AAC	0.82	CCC	0.34
Zea mays ALS1	CAC	0.77	ATC	0.65	AAG	0.88	CTC	0.30	AAC	0.79	CCC	0.39
Z. mays ALS2	CAC	0.69	ATC	0.61	AAG	0.88	CTC	0.25	AAC	0.78	CCC	0.38
Echinochloa oryzicola ALS1	CAC	0.77	ATC	0.63	AAG	0.83	CTC/CTG	0.31	AAC	0.82	CCC	0.36
E. oryzicola ALS2	CAC	0.85	ATC	0.59	AAG	0.83	CTC/CTG	0.31	AAC	0.76	CCC	0.33
Poa annua ALSa	CAC	0.73	ATC	0.56	AAG	0.80	CTG	0.38	AAC	0.53	CCA	0.35
P. annua ALSb	CAC	0.80	ATC	0.58	AAG	0.79	CTG	0.40	AAC	0.65	CCC	0.35
Organism	Q	F_{op}	R	F_{op}	S	F_{op}	T	F_{op}	V	F_{op}	Y	F_{op}
Apera spica-venti	CAG	0.90	CGC	0.54	TCC	0.44	ACC	0.33	GTC	0.42	TAC	0.72
Bromus tectorum	CAG	0.79	CGC	0.64	TCC	0.35	ACC	0.42	GTC	0.36	TAC	0.59
Alopecurus myosuroides	CAG	0.82	CGC	0.50	TCC	0.33	ACC/ACT	0.27	GTC	0.44	TAC	0.72
Beckmannia syzigachne	CAG	0.92	CGC	0.56	TCC	0.37	ACC	0.31	GTC	0.46	TAC	0.78
Lolium rigidum	CAG	0.83	CGC	0.62	TCC	0.41	ACC	0.39	GTC	0.45	TAC	0.82
Echinochloa crus-galli ALS1	CAG	0.88	CGC	0.55	TCC	0.39	ACC	0.43	GTC/GTG	0.39	TAC	0.65
E. crus-galli ALS2	CAG	0.92	CGC	0.55	TCC	0.35	ACC	0.47	GTG	0.42	TAC	0.65
E. crus-galli ALS3	CAG	0.92	CGC	0.56	TCC	0.40	ACC	0.44	GTG	0.42	TAC	0.65
Zea mays ALS1	CAG	0.92	CGC	0.50	TCC	0.33	ACC	0.35	GTC/GTG	0.42	TAC	0.59
Z. mays ALS2	CAG	0.88	CGC	0.46	TCC	0.32	ACC	0.31	GTC/GTG	0.40	TAT	0.56
Echinochloa oryzicola ALS1	CAG	0.88	CGC	0.55	TCC	0.39	ACC	0.46	GTC/GTG	0.39	TAC	0.65
E. oryzicola ALS2	CAG	0.92	CGC	0.55	TCC	0.35	ACC	0.46	GTG	0.42	TAC	0.65
Poa annua ALSa	CAG	0.91	CGC	0.47	TCC	0.42	ACC	0.35	GTC	0.44	TAC	0.69
P. annua ALSb	CAG	0.91	CGC	0.50	TCC	0.43	ACC	0.36	GTC	0.46	TAC	0.69

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under-represented are A-ending codons, whereas the three over-represented codons are G/C-ending. Furthermore, based on the RSCU values, the F_{op} values were calculated (Table 3). Overall, the set of optimal codons for *ALS* gene in weeds is as follow: Ala (GCC), Cys (TGC/TGT), Asp (GAC/GAT), Glu (GAG), Phe (TTT/TTC), Gly (GGC/GGT/GGG), His (CAC), Ile (ATC/ATT), Lys (AAG), Leu (CTG/CTC), Asn (AAC/AAT), Pro (CCA/CCC), Gln (CAG), Arg (CGC), Ser (TCC), Thr (ACC/ACT), Val (GTC/GTG), Tyr (TAC/TAT).

Despite of its ubiquitous nature, the mechanism of codon bias is not fully understood. Studies showed that synonymous codon usage may alter the expression of the gene of interest, and this effect can reach up to 1 000-fold or even more (Stoletzki and Eyre-Walker 2007, Quax et al. 2015). Although several field-based studies on weeds and their herbicide-resistant properties were conducted but work related to their molecular properties are still at their infancies. Heterologous gene expression studies can be very useful to study specific protein interactions. Studies involving codon optimisation will allow researchers to develop synthetic heterologous genes involved in herbicide resistance with the most efficient expressional efficiencies. These heterologous expression studies with optimised codons will have the potential to prove their efforts in the development of new herbicidal compounds. Hence, the present study was conducted to gain insight into the codon usage pattern of the acetolactate synthase gene in weedy species. The results obtained from the current study will enhance our understanding of the major factors and the pattern of codon usage in the ALS gene of weeds. Additionally, these results will help further investigations with the ALS gene and the development of new herbicidal compounds, which may require synthetic gene design based on codon usage patterns.

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Enhanced metabolism and target gene overexpression confer resistance against acetolactate synthase-inhibiting herbicides in *Bromus sterilis*

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Abstract

BACKGROUND: Intensive application of acetolactate synthase (ALS)-inhibiting herbicides has resulted in herbicide-resistance in many weeds, including *Bromus sterilis*. The present study was conducted to identify the mechanisms conferring resistance to ALS-inhibiting herbicides in a *Bromus sterilis* biotype.

RESULTS: Dose–response studies revealed the resistant biotype to be 288 times less sensitive to pyroxsulam than the susceptible biotype. Furthermore, experiment with a single-dose, proved this biotype was also cross-resistant to propoxycarbazone, iodosulfuron plus mesosulfuron and sulfosulfuron. Prior treatment with malathion, a known inhibitor of cytochrome P450s, reduced the level of resistance to pyroxsulam. No mutations were detected from the partial *ALS* gene sequencing. Flow cytometry and chromosome counting rejected ploidy level variation between the susceptible and resistant biotypes. Relative copy number variation ruled out gene amplification. Quantitative real-time polymerase chain reaction (PCR) detected a significant difference in *ALS* gene expression between the susceptible and resistant biotypes.

CONCLUSIONS: Target gene overexpression and enhanced metabolism by cytochrome P450s are likely mechanisms of resistance to pyroxsulam in *Bromus sterilis*. The current findings highlight the need to monitor additional brome populations for herbicide resistance in Europe and endorse the need for alternate herbicides in integrated weed management to delay the possible evolution of herbicide resistance in these species.

Supporting information may be found in the online version of this article.

Keywords: *Bromus sterilis*; herbicide resistance; chromosome counting; copy number variation; *ALS* gene overexpression; CytP450s

1 INTRODUCTION

Bromus sterilis L. (barren brome), is an annual or biennial grass native to northern Africa, western and middle Asia and northern, central and eastern Europe.[1] In the Czech Republic, these troublesome weeds are known to occur in the lowland areas. Since the first reports in France (http://www.weedscience.org/Pages/Case.aspx?ResistID=9931), data pinpoint towards an astonishing rise in these noxious weeds triggering 30–60% yield losses in the productivity of winter wheat.[2,3] Apart from yield losses, previous studies also indicated that these weeds could cause lodging and interfere with the harvesting processes.[4] The evolution of herbicide resistance in *Bromus* species has jeopardized their management in Europe where pyroxsulam, propoxycarbazone, mesosulfuron, and sulfosulfuron are mainly used, to control this species.[3,5]

Since their discovery and commercialization, acetolactate synthase (ALS)-inhibiting herbicides have been widely used for weed management due to their broad-spectrum weed control and low toxicity to mammals.[6,7] The ALS enzyme catalyses the first step in the synthesis of the branched-chain amino acids valine, leucine, and isoleucine.[8–11] Currently, herbicides that target ALS

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are the most widely used herbicides in the world, resulting in maximum large number of weed species resistant to these herbicides.[6] Herbicide resistance may evolve as a result of target-site resistance (TSR) or non-target site resistance (NTSR). TSR involves mutations within the protein-coding regions, which alter the conformations of the target protein and ultimately lead to a decreased affinity for the herbicide.[6,8] The most common mutations linked with TSR to ALS-inhibiting herbicides in grass weeds are as follows: Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653 and Gly-654[8,12,13] (the numbers are based on *Arabidopsis thaliana* ALS). TSR may also occur due to overexpression of the target gene, but these cases are much less frequent than the former.[14] NTSR works by reducing the amount of herbicide which reaches the target site.[14] The most common NTSR mechanisms are increased detoxification, enhanced metabolism, or reduced herbicide uptake and translocation.[15–18] Among the NTSRs found in weed species, enhanced metabolism of the herbicides by the enzymes like CytP450[15,19,20] is the most widely discussed mechanism. CytP450-mediated NTSR had been reported in many species like *Lolium rigidum*,[21] *Alopecurus myosuroides*,[22] etc. In addition to the CytP450s, glutathione-*S*-transferase (GSTs), glycosyltransferases and ABC transporters are also known to be involved with NTSRs.[23] Resistant populations of *Bromus sterilis* from the United Kingdom showed elevated amounts of an orthologue of the glutathione transferase phi (F) class 1 protein.[24]

Recently, farmers in the Czech Republic have also reported of resistance to ALS-inhibiting herbicides in *Bromus sterilis* biotypes. The recommended doses of pyroxsulam have failed to control this weed species in winter wheat fields of the Czech Republic. The involved resistance mechanisms must be discovered in these species before it becomes a global threat. The present study is the first detailed study on elucidating the mechanism of ALS-inhibiting herbicide resistance in *Bromus sterilis* biotypes from the Czech Republic/central Europe. Single gene mutation, *ALS* gene overexpression, and enhanced metabolism via detoxification enzymes (i.e. CytP450s) were suspected as a putative source of herbicide resistance in *Bromus sterilis* biotypes. Potential cross-resistance to other relevant herbicides (commonly used ALS-inhibitors in many parts of Europe) were also examined. Current findings not only fill the existing knowledge gap in the field but also provide a basis for further research using comparative proteomic or epigenetic approaches to investigate how target gene overexpression is orchestrated at the molecular level.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The susceptible (S) biotype was collected from the campus of the Czech University of Life Sciences Prague (50.1317956 N, 14.3692800E) and the resistant (R) biotype was collected from a winter wheat field in the Ústecký (Louny) region of the Czech Republic (50.2612525 N, 13.4818572E), where the farmer had reported resistance to pyroxsulam. The collected R and S biotype seeds were kept in the dark at room temperature until further use. Seed samples in bulk were collected from at least 100 plants, from the same field (as described earlier), to ensure variability in the field collection. Ten seeds were directly sown in pots of approximately 343 cm^3, filled with chernozem soil [high fertility property and moisture storage capacity, clay content 46% (loamy soil), soil pH (potassium chloride) 7.5, sorption capacity of soil: 209 mmol (+), 87 mg kg^{-1} phosphorus, 203 mg kg^{-1} potassium, 197 mg kg^{-1} magnesium, 8073 mg kg^{-1} calcium].The pot experiments were conducted in an open-air vegetation hall (with roof-top, to avoid the rain). The seedlings were regularly watered, and fertilizers were applied as required.

2.2 Dose–response experiments

Pyroxsulam was applied to the biotypes (using a laboratory spray chamber equipped with a Lurmark 015F80 nozzle with spray volume of 250 L ha^{-1} and pressure 120 kPa, as described by Hamouzová *et al.*,[12] at the two- to three-leaf stage at rates of 0.05925, 0.1875, 0.5925, 1.875, 5.925, 18.75, 59.25, 187.5 and 592.5 g a.i. (active ingredient) ha^{-1}, with four-pot replicates for each rate. Malathion (Malathion, PESTANAL®, analytical standard, Sigma-Aldrich, Merck Group, St Louis, MO, USA) was sprayed at 1000 g ha^{-1} of active ingredient, as described by Hamouzová *et al.*[12]

Biotypes were treated at the two- to three-leaf stage with sulfometuron-methyl at rates of 0.1659, 0.525, 1.659, 5.25, 16.59, 52.5, 165.9, 525 and 1659 g a.i. ha^{-1} and propoxycarbazone at rates of 0.13272, 0.42, 1.3272, 4.2, 13.272, 42, 132.72, 420 and 1327.2 g a.i. ha^{-1}, with four pot replicates for each rate. Herbicide efficacy was evaluated on the 28th day after treatment through biomass reduction in treated pots compared with untreated control.

2.3 Cross-resistance studies to other herbicides

Four different herbicides were used for cross-resistance studies, based on their frequency of use in the fields. The chosen herbicides were: sulfosulfuron, (applied at 19.5 g a.i. ha^{-1}) and a mixture of iodosulfuron-methyl-sodium, mesosulfuron-methyl and thiencarbazone-methyl (9 g + 45 g + 22.5 g; mixture applied at 25.47 g a.i. ha^{-1}). These experiments were conducted with four pot replicates for each herbicide. Herbicide efficacy was evaluated 28 days after treatment as biomass reduction in treated pots compared with untreated control.

2.4 ALS gene partial sequencing, determination of relative copy number and expression studies

Shock frozen leaf tissues (±80 mg per sample) from R and S plants were collected for genomic DNA (gDNA) extraction using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. RNA from the fresh leaf tissues (±80 mg per sample) was extracted using RNeasy Mini Kit (QIAGEN). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe the RNA templates. The gene-specific degenerate primers were designed based on the publicly available sequences of the *ALS* gene from *Apera spica-venti* (JN646110.1), *Alopecurus myosuroides* (AJ437300), *Oryza sativa* (AY885674, AY885675, AB049822, AB049823), *Bromus tectorum* (MK492423.1) and *Beckmannia syzigachne* (MG891930.1). The primers were designed using Primer-BLAST and Primer3 software. Polymerase chain reaction (PCR) was performed using a C1000 thermocycler (Bio-Rad, Hercules, CA, USA), using 50 ng of total gDNA per reaction. The thermocycler was programmed at an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 59 to 60 °C (based on the annealing temperature of the primer pair) and 30 s at 72 °C along with a final extension step for 10 min at 72 °C. The PCR amplified products were separated in the 1.5% agarose gel and subsequently purified using GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Finally, the gel-purified product was sent for custom DNA Sequencing (Eurofins Genomics Germany GmbH,

Ebersberg, Germany). The 3′ UTR region of *Bromus sterilis* ALS was obtained using Gene Specific Primers (3′ RACE outer primer and 3′ RACE inner primer) and First Choice ® RLM-RACE Kit (Ambion, Austin TX, USA). Relative copy number and expression was conducted with StepOne™ Real-Time PCR System (Applied Biosystems) using genomic DNA (~10 ng), and complementary DNA (cDNA, ~10 ng) respectively as templates and gene-specific primers (GSP) (ALS_ Forward primer and ALS_ Reverse primer). For selecting the suitable reference gene, cycle threshold (Ct) values before (water-treated) and after herbicide treatment were compared with the four commonly used software: BestKeeper, NormFinder, geNorm and RefFinder.[25,26] Ubiquitin was chosen as an internal standard as it was the most stable reference gene based on the number of times a gene has secured top or second position (Supporting Information Fig. S1 and Table S1). Partial *ubiquitin* gene sequence (168 bp) was submitted to the GenBank with an accession number MT193724 and primers (Ubiquitin_Forward primer and Ubiquitin_Reverse primer) were designed for real-time PCR-based gene expression normalization. The relative copy number and expression level of the *ALS* gene in S and R biotype, after herbicide treatment, were calculated using the $2^{-\Delta\Delta Ct}$ method.[27,28] The real-time PCR experiments were conducted with nine biological replicates from each biotype. DNA and RNA integrity was confirmed by running the samples on 0.8% and 1.2% agarose gel, respectively. The list of primers is provided in Table S2.

2.5 Estimation of ploidy level and chromosome counting

Leaf samples from each R and S biotypes were analysed by flow cytometry, using a Partec PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a high-pressure mercury arc lamp, as described by Zahumenická *et al.*[29] Chromosome counting was performed using α-bromonaphthalene pretreatment.[30]

2.6 Statistical analysis

Data analysis and dose–response curves were plotted by non-linear regression model using R-Studio program (https://www.r-project.org/) as described by Hamouzová *et al.*[12] GR_{50} (50% growth inhibition) values were calculated for each biotype and compared. The resistance factor (RF) ratios were calculated as the ratio of GR_{50} of the R biotype and the GR_{50} of the S biotype. For the cross-resistance studies, the selected herbicides were applied at their recommended field doses, and the results were verified by one-way analysis of variance (ANOVA) in R-Studio program and were compared based on Tukey's *post hoc* analysis (5% significance level).[31] The results for relative *ALS* copy number and relative *ALS* expression were compared using two-sample *t*-test in Excel with XLSTAT (version 2020.3) (https://www.xlstat.com/en/).

3 RESULTS AND DISCUSSION

3.1 Whole plant dose–response to pyroxsulam (with and without malathion) and sensitivity to sulfometuron-methyl and propoxycarbazone

From the dose–response experiments the GR_{50} of the R biotype (25.89 g a.i. ha^{-1}) was higher than the recommended field dose for pyroxsulam, while the S biotype GR_{50} value was 0.09 g a.i. ha^{-1}.The resulting resistance index was 287.67 (Table 1). These results indicate high resistance to pyroxsulam in the R biotype. When malathion (at the rate of 1000 g a.i. ha^{-1}) was applied prior to pyroxsulam, the resistance index decreased by almost 1.6-fold compared to the plants treated with pyroxsulam alone (Fig. 1(A, B)). The GR_{50} values and resistance index values with and without malathion are shown in Table 1. Applying malathion alone at the same rate resulted in no effect (data not shown). The reduction in resistance with malathion indicated the involvement of Cytochrome P450s in resistance to pyroxsulam. The R biotype was also resistant to sulfometuron-methyl with a resistance index of 88.10 (Fig. 1(C)). The GR_{50} values for R and S biotypes were 31.46 and 0.36, respectively, which is an indication that the resistant plants may also possess TSR. The GR_{50} of the R biotype to propoxycarbazone (181.12 g a.i. ha^{-1}) was higher than the recommended field dose whereas the GR_{50} of the S biotypes was only 0.32 g a.i. ha^{-1}, giving a resistance index of 574.980, confirming a high level of resistance to propoxycarbazone (Fig. 1(D)). Therefore, the pyroxsulam resistant biotype is cross-resistant to propoxycarbazone and sulfometuron-methyl.

3.2 Cross-resistance to other herbicides

Iodosulfuron, mesosulfuron and sulfosulfuron belong to sulfonylurea group of herbicides, whereas pyroxsulam and propoxycarbazone belong to triazolopyrimidines and sulfonylaminocarbonyltriazolinone group of herbicides, respectively. Based on the dry weight of surviving plants, we found that the pyroxsulam and propoxycarbazone R biotype was also resistant to iodosulfuron plus mesosulfuron and sulfosulfuron (Table 2). Similar results were also reported by Nakka *et al.*; wherein the resistance to chlorsulfuron in a R biotype of *Amaranthus palmeri* S. Watson was contributed by enhanced metabolism and also conferred cross-resistance to other chemical classes of ALS inhibitors.[32] Beckie and Tardif have suggested that enhanced metabolism can be responsible for cross-resistance.[33]

3.3 ALS gene mutations

TSR was tested for by partial amplification of the *ALS* gene using different primer pairs covering mutation points such as Ala-122, Pro-197 and Ala-205 (first primer pair), Asp-376 and Arg-377 (second primer pair), Trp-574, Ser-653 and Gly-654 (third primer pair). The resulting ALS gene sequence (1779 bp partial) from a combination of all three primer pairs based 3' RACE PCR was submitted to the GenBank (an accession number MT113952). Comparison among the R and S biotypes, based on the partially amplified ALS gene showed no mutations at the positions Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653 and Gly-654 (Fig. 2). Despite the variation in gene length, the mature ALS protein consists of five conserved domains in higher plants.[34] Single point mutations in each domain have the potential to confer resistance in resistant weeds.[34,35] Although no mutation was found here, ALS mutations conferring resistance to ALS inhibitors have been identified in weed species, like *Bromus tectorum* L.,[35] *Scirpus juncoides*,[36] *Hordeum leporinum*,[37] *L. rigidum*[38] and *Alopecurus myosuroides*.[39]

3.4 Investigation on ploidy level variation along with chromosome counting and ALS gene copy number variation analysis

Yu *et al.* had suggested that polyploid weed species show lower levels of TSR, owing to their multiple homoeologous copies of the target gene.[40] To investigate the ploidy level variation, we used flow-cytometry and cell counting. The flow cytometry results revealed no difference in DNA content between the biotypes (Fig. S2). Therefore, no ploidy level variation was present. Furthermore, chromosome counting (picture not shown) was done to confirm the flow cytometry results. Our result showed that both R and S biotypes are diploid, with 14 chromosomes in each cell.

Table 1. Result from the non-linear regression analysis of pyroxsulam dose–response experiment for resistant (R) and susceptible (S) biotype

Biotype	b (SE)	d (SE)	GR_{50} (SE)	RF
R_ pyroxsulam	1.67 (0.38)	1.20 (0.03)	25.89 (2.71)	287.67
S_ pyroxsulam	0.39 (0.05)	1.54 (0.09)	0.09 (0.04)	
R_pyroxsulam + malathion	3.64 (1.11)	1.01 (0.03)	8.98 (1.36)	179.60
S_pyroxsulam + malathion	1.77 (0.52)	1.46 (0.06)	0.05 (0.01)	
R_ sulfometuron-methyl	1.50 (0.29)	1.25 (0.30)	31.46 (3.77)	88.10
R_ sulfometuron-methyl	0.97 (0.14)	1.46 (0.05)	0.36 (0.05)	

The parameters used are as follow: 'd' is the upper limit, 'b' is the slope around the GR_{50}, 'SE' represents the standard error, 'GR_{50}' is the rate of herbicide (g a.e. ha^{-1}) required to reduce shoot dry weight by 50%, 'resistance factor (RF)' is calculated with resistant/susceptible based on GR_{50} ratios.

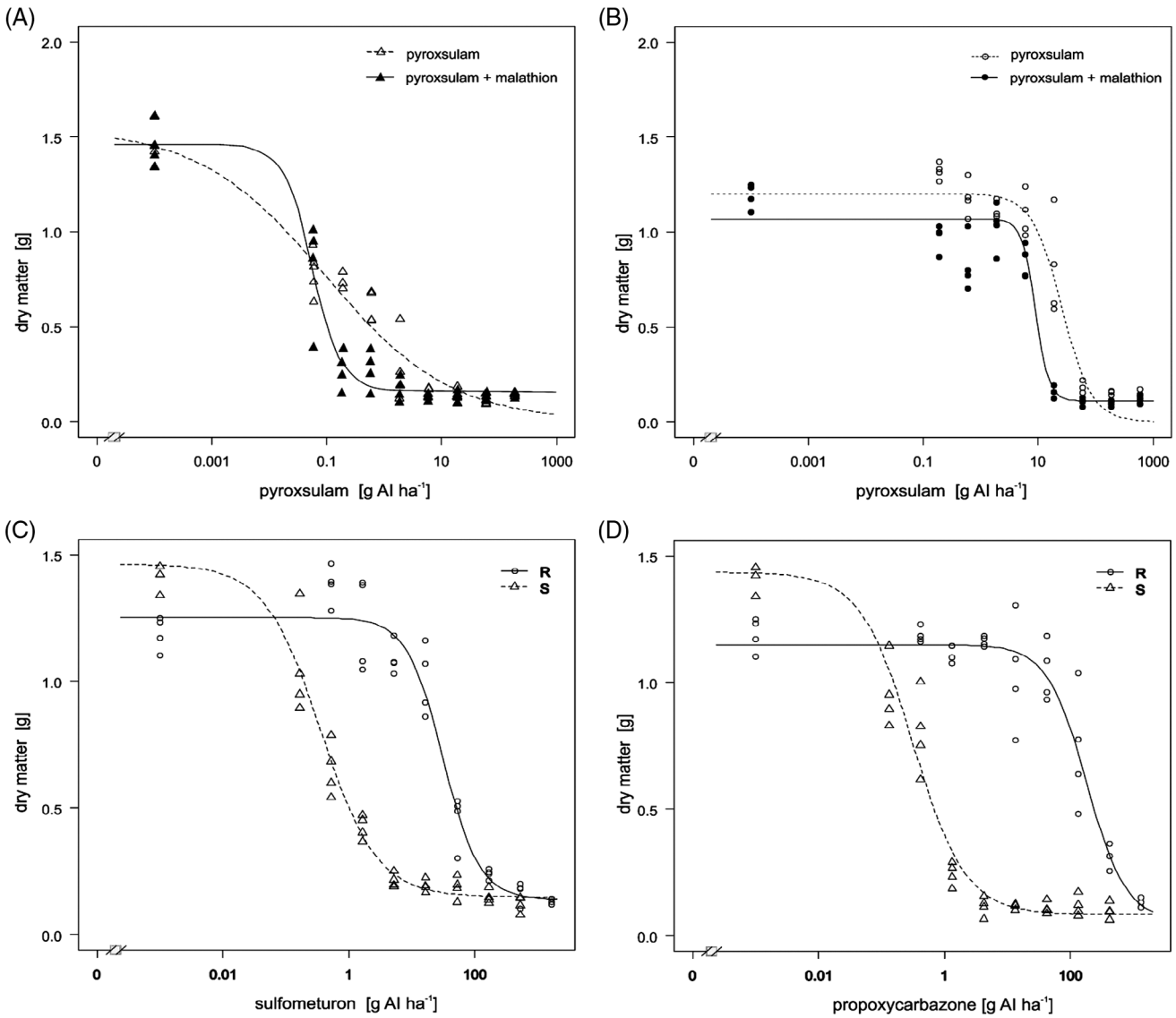

Figure 1. Fitted logarithmic dose–response curves for *Bromus sterilis* (A) susceptible (S) biotype (with and without malathion), (B) the resistant (R) biotype (with and without malathion), (C) with sulfometuron-methyl and (D) with propoxycarbazone.

Reports have suggested the link between copy number variation (CNV) of herbicide target genes and the evolution of herbicide resistance.[41] Target gene CNV has been observed in glyphosate resistance, further emphasizing the importance to determine copy numbers of genes which code for the herbicide target enzyme.[42] In the present study, amplification of the

Table 2. Results for cross-resistance studies

Biotype	Dose (g a.e. ha^{-1})	Average biomass weight (standard deviation)	Significance level (at 5% significance level)
R_ Untreated	0	1.19 (0.06)	e
S_ Untreated	0	1.46 (0.16)	e
R_iodosulfuron+mesosulfuron	25.47	0.67 (0.04)	c
S_iodosulfuron+mesosulfuron	25.47	0.08 (0.01)	a
R_sulfosulfuron	19.5	0.98 (0.07)	d
S_sulfosulfuron	19.5	0.13 (0.02)	a

The dry biomasses from resistant (R) biotype and the susceptible (S) biotype of *Bromus sterilis* were compared using Tukey's test for *post hoc* analysis (5% significance level).

Figure 2. Partial ALS gene sequencing results. The ALS sequences obtained from the 30 individuals of each biotype were compared. The numbers are based on *Arabidopsis thaliana* ALS.

genomic DNA by quantitative real-time PCR showed that the relative *ALS* gene copy did not differ between R and S biotypes (Fig. 3(A)), confirming that gene amplification is not responsible for pyroxsulam resistance.

3.5 *ALS* gene expression

A significant difference in *ALS* gene expression was detected between the biotypes. The *ALS* gene in the R biotype showed almost 2× expression, compared to the S biotype (Fig. 3(B)). Hence, our results indicate that ALS gene overexpression (but not due to CNV) might contribute to resistance to pyroxsulam. A plausible explanation for this phenomenon of ALS gene overexpression (without gene amplification), might be due to involvement of transcriptional regulation and/or epigenetic regulation of gene expression. However, such possibilities demand further experimental validation. Despite its genetically homogeneous nature, cells within multicellular organisms are functionally different from each other due to the differential expression patterns of the genes.[43] Epigenetic changes include changes due to DNA and histone modifications, non-coding RNAs (like microRNA, small interfering RNA), methylation patterns and others, which result in alleles with a similar sequence of DNA but with differential expression patterns.[43,44] Earlier studies on model plant species have already demonstrated that plants could cope with abiotic stresses by reorganizing gene expression patterns through epigenetic changes.[45,46] Markus *et al.*, suggested that epigenetic changes might help weeds to rapidly mitigate herbicidal stresses by various mechanisms as gene duplication, differential target gene expression or transposable element changes.[47] Further research is necessary to confirm the role of higher ALS gene expression in resistance.

4 CONCLUSIONS

Our results indicate that the biotype from the Czech Republic is resistant to pyroxsulam and cross-resistant to propoxycarbazone, iodosulfuron plus mesosulfuron and sulfosulfuron. We did not detect a mutation in the ALS gene in *Bromus sterilis*. Flow cytometry and relative copy number determination result excluded ALS gene amplification as a putative pyroxsulam resistance

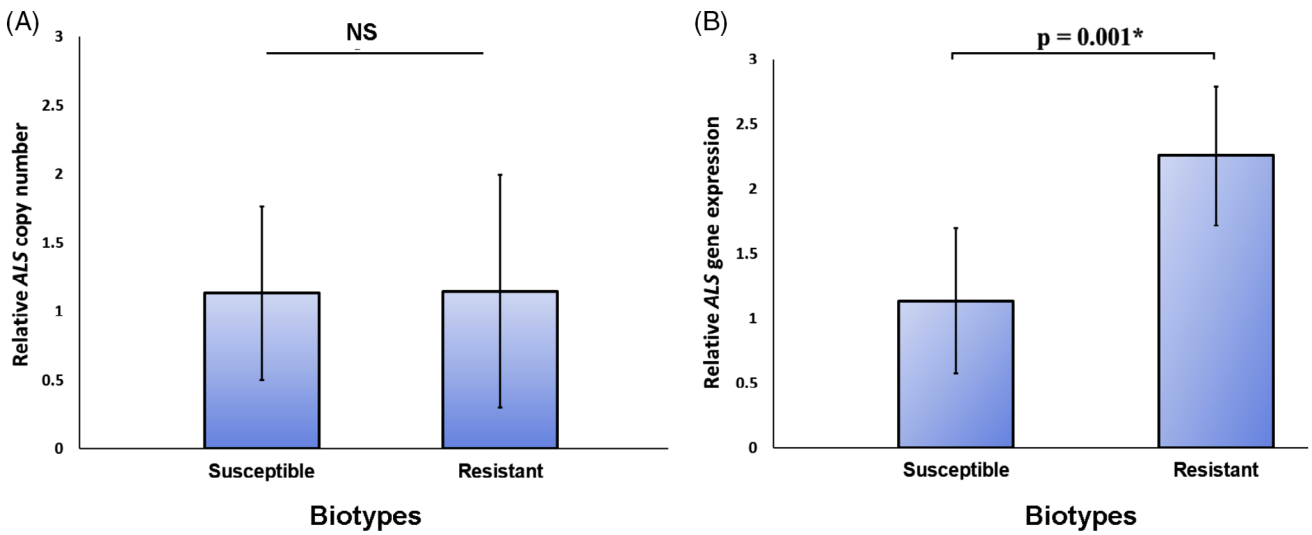

Figure 3. Target gene copy number and expression analysis. (A) Relative *ALS* gene copy number. (B) Relative *ALS* gene expression level. The results obtained by quantitative real time PCR were compared by two sample *t*-test at 5% significance level. * and NS represents significant at 5% significance level and not significant, respectively.

mechanism in this biotype. Current findings indicated that the resistance is likely associated with overexpression of the ALS gene (almost two-fold overexpression) and enhanced metabolism (by CytP450s). Future RNA-sequencing based transcriptome analysis to identify the differential expression of different cytochrome P450s or studies elucidating epigenetic mechanisms underlying the observed overexpression of ALS gene can shed light on the precise mechanism of herbicide resistance in *Bromus sterilis*. As only ALS inhibiting herbicides are registered products for selective control of *Bromus sterilis* in cereals, the results of the study confirming strong ALS resistance are an early warning for farmers and a challenge for the systematic use of integrated weed management strategies.

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AUTHOR CONTRIBUTIONS

M.K.S., K.H., A.R. and J.S. planned the experiments. M.K.S., K.H., A.R., J.M., R.B., P.K. and P.H. conducted the experiments. M.K.S., K.H., R.B and A.R. performed the bioinformatics and statistical analysis. M.K.S., K.H., A.R., J.M., R.B. and J.S. analysed the results and wrote the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

COMPLIANCE WITH ETHICS REQUIREMENTS

This article does not contain any studies with human or animal subjects.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Identification of the most suitable reference gene for gene expression studies with development and abiotic stress response in *Bromus sterilis*

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Bromus sterilis **is an annual weedy grass, causing high yield losses in winter cereals. Frequent use of herbicides had led to the evolution of herbicide resistance in this species. Mechanisms underlying herbicide resistance in *B. sterilis* must be uncovered because this problem is becoming a global threat. qRT-PCR and the next-generation sequencing technologies can elucidate the resistance mechanisms. Although qRT-PCR can calculate precise fold changes, its preciseness depends on the expression of reference genes. Regardless of stable expression in any given condition, no gene can act as a universal reference gene. Hence, it is necessary to identify the suitable reference gene for each species. To our knowledge, there are no reports on the suitable reference gene in any brome species so far. Thus, in this paper, the stability of eight genes was evaluated using qRT-PCR experiments followed by expression stability ranking via five most commonly used software for reference gene selection. Our findings suggest using a combination of *18S rRNA* and *ACCase* to normalise the qRT-PCR data in *B. sterilis*. Besides, reference genes are also recommended for different experimental conditions. The present study outcomes will facilitate future molecular work in *B. sterilis* and other related grass species.**

One of the major plant protection problems encountered by farmers across the globe is regarding weeds. Herbicides have been widely used to manage weeds and magnify the main crop's yield quality and quantity. Despite their success in managing weeds, constant use of similar herbicides has evolved resistance in many weedy species. Owing to its rapid population dynamics and lack of efficient herbicides, barren brome (*Bromus sterilis* L.) has grown into a troublesome weed in winter cereals in many south and north American countries, middle and west Europe[1–3]. Besides the most frequent acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) resistance in Europe (http://www.weedscience.org/Home.aspx), United Kingdom also reported *B. sterilis* resistance against glyphosate in 2019[4]. These results indicate the prerequisite for monitoring more barren brome populations. Gene expression studies have contributed immensely in elucidating the target gene amplification and expression and the over-expression of detoxifying enzyme genes related to herbicide resistance and other abiotic stresses[5,6]. Moreover, with the development of next-generation sequencing technologies, there is a need to validate the expression of a greater number of genes involved in abiotic stresses[7,8]. qRT-PCR is widely used for such comparative gene expression studies. However, the reliability of the qRT-PCR depends on the selection of a stable reference gene.

Compared to the traditional polymerase chain reaction (PCR), quantitative real-time polymerase chain reaction (qRT-PCR) has many advantages like high specificity, rapidity and sensitivity, making it an essential part of comparative expression studies[9,10]. Previously, the relative quantification of gene expression was done either by Northern blot or by reverse transcription-polymerase chain reaction (RT-PCR). The most important limitation

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of these methods is their inability to detect extremely low expression, resulting in replacing the pre-existing methods with microarrays and qRT-PCR[9,11]. Even though these modern techniques are highly sensitive and can calculate precise fold changes, their preciseness is highly dependent on the expression of a reference gene. Ideally, a reference gene refers to constitutive genes required to maintain the basic cellular functions of an organism. These genes are known to have stable gene expression in all cells under both normal and pathophysiological conditions[12–14]. However, the steps of qRT-PCR are reclined to variations; therefore, to overcome these variations, target gene transcription levels must be normalised to reference genes transcription levels. Any error in selecting a suitable reference gene may lead to misleading results. Hence, selecting a reliable reference gene is necessary for molecular biology-oriented studies[9,14–17]. The most commonly used references genes for normalisation of plant gene expression studies are *ubiquitin* (*UBQ*), *β-tubulin* (*β-TUB*), *ribosomal RNA genes* (*18S rRNA* and *25S rRNA*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *eukaryotic elongation factor* (*eEF*), *eukaryotic translation initiation factor 1 (eIF1), actin* (*ACT*), *acetyl-CoA carboxylase* (*ACCase*) etc[9,18]. Although these genes are known to have a stable expression in any given condition, several studies documented variability in their expression level between species of plants or different stress conditions or developmental stages[19–21]. As no gene can act as a universal reference, it is necessary to systematically select and identify the suitable reference gene for each species[22,23].

There are no reports of a suitable reference gene in *B. sterilis* or any other brome species. Our study aims to identify a suitable reference gene for gene expression studies in *B. sterilis* (or barren brome). Increasing the number of treatments might lead to more variations in results, which decreases the chance of identifying a suitable reference gene[9]. In this study, we had selected eight common candidate reference genes (*UBQ*, *ACT*, *GAPDH*, *18S rRNA*, *25S rRNA*, *ACCase*, *β-TUB* and *eEF*) identified in *B. sterilis* and evaluated the stability of their gene expression in three developmental stages (two-leaves, three-leaves and four-leaves), two different plant organs (shoots and leaves) and one abiotic stress (drought stress). Among the various severe issues with detrimental effects, climate change has remained a top priority. Global warming has resulted in an increase of air temperature and evapotranspiration, leading to agricultural droughts, affecting both crops and weeds.

Low soil moisture increase the competition for water and nutrients between weeds and the crop, thus making weed management complicated. Some (usually C4) weed species gain profit from this situation. Uptake and translocation of herbicides within the plant is reduced, thereby affecting the efficacy of the applied herbicides. Hence, interest for studies under drought is recently rising[24], which might require expression studies with several genes of interest. Therefore, drought stress has been included in the present study, and our recommended reference genes will be helpful in future drought-related studies.

The most suitable candidate was selected based on the ranking provided by different widely used statistical software for reference gene analysis (comparative ΔCt, BestKeeper, NormFinder, geNorm and RefFinder). Additionally, the most suitable reference gene was used to validate a herbicide-stress experiment. Thus, our study provides a basis for identifying the suitable reference gene for future gene expression studies in *B. sterilis* and will aid in impending studies on the molecular basis underlying the herbicide resistance in barren brome.

Results

Primer efficiency and candidate genes expression. 1.2% agarose gel electrophoresis was used to check the integrity of the RNA. In addition, the quantity and quality of RNA were evaluated by a nanodrop spectrophotometer (Thermo Scientific™, US). The A260/A280 values ranged from 1.90 to 2.05. These samples were further used to synthesise cDNA, which was used for the qRT-PCR experiments. In all the qRT-PCR amplification, a single peak was obtained (supplementary Fig. 1). The selected primers for this study showed a single band in the 1.5% agarose gel (supplementary Fig. 2) and had efficiency values ranged between 92.32 and 106.79%, which falls under the acceptable range. The correlation coefficient values ranged from 0.980 to 0.999 (Table 1). The expression profile of the 8 candidate genes under different experimental conditions is shown in the Fig. 1. *18s rRNA* showed the lowest cycle threshold value (Ct), indicating high expression of the gene, whereas *ACT* showed the highest Ct value indicating low expression.

Gene expression stability analysis. *Developmental stages-related experiments.* *18S rRNA* was identified as the stable reference gene by comparative ΔCt and RefFinder. BestKeeper software identified *ACCase* as the most stable reference gene (Table 2). NormFinder analysis revealed *18S rRNA* and *eEF* as the most stable genes, whereas geNorm analysis ranked *18S rRNA* and *ACCase* as the best reference gene for developmental stages-related experiments in *B. sterilis* (Table 2, Fig. 2). Except, comparative ΔCt, all the used software identified *GAPDH* as the least stable gene. According to the comparative ΔCt analysis, *eEF* is the least stable gene.

Plant organs related studies. In gene expression studies with the plant organs, *18S rRNA* has been ranked as the most stable gene by comparative ΔCt, BestKeeper and RefFinder (Table 2). NormFinder analysis identified *ACCase* and eEF as the most stable genes (Table 2). Based on the geNorm analysis, *18S rRNA* and *ACCase* might be the best reference gene for plant organs-related studies in *B. sterilis* (Fig. 2). *β-TUB* was identified as the least stable gene by comparative ΔCt, BestKeeper and RefFinder, whereas NormFinder and geNorm analysis identified GAPDH as the least stable gene.

Under drought stress. For studies under drought stress, comparative ΔCt and RefFinder identified *18S rRNA* as the most suitable reference gene (Table 2). BestKeeper software identified *ACCase* as the most stable reference gene (Table 2). NormFinder analysis revealed *GAPDH* and *18S rRNA* as the most stable genes, whereas geNorm analysis ranked *18S rRNA* and *β-TUB* as the best reference gene (Table 2, Fig. 2). *eEF* was identified as the least

Gene	Sequence	Annealing temperature (°C)	Amplicon length (bp)	Primer efficiency (%)	R^2 value
*Ubiquitin*_forward primer	GCACAAGCACAA GAAGGTGA	60	120	99.46	0.997
*Ubiquitin*_reverse primer	AGTGGTTTGCCA TGAAGGTC				
*Actin*_forward primer	ATGCGATTCTTCGTT TGGAC		172	102.34	0.980
*Actin*_reverse primer	GATGTCTCCAGC TCCTGCTC				
*GAPDH*_forward primer	AGCGACATCAAG CTCAAGAA	58	241	92.44	0.994
*GAPDH*_reverse primer	CGTTGACACCAA CCACAAAC				
*18S rRNA*_forward primer	AAACGGCTACCA CATCCAAG		154	92.42	0.999
*18S rRNA*_reverse primer	CCTCCAATGGATCCT CGTTA				
*25S rRNA*_forward primer	CCCAGTGCTCTG AATGTCAA		211	92.32	0.999
*25S rRNA*_reverse primer	GTCTTCTTTCCC CGCTGATT				
*ACCase*_forward primer	GCTGCTATTGCCAGT GCTTA	57	171	95.77	0.989
*ACCase*_reverse primer	AAGCTTGTTCAG GGCAGAAA				
*β-Tubulin*_forward primer	AGTACCGTGCCC TCACAGTC		150	106.79	0.996
*β-Tubulin*_reverse primer	TCTGCTCGTCAACCT CCTTT				
*eukaryotic elongation factor*_forward primer	CCTGCACTGTCATTG ATGCT		185	94.51	0.988
*eukaryotic elongation factor*_reverse primer	CTGCCTGACACC AAGAGTGA				

Table 1. Primer information of the eight candidate reference genes.

stable gene by ΔCt, BestKeeper and RefFinder, whereas NormFinder and geNorm analysis identified *UBQ* as the least stable gene for studies under drought stress on *B. sterilis*.

Combined conditions. When all the conditions were taken together, *18S rRNA* was identified as the most stable reference gene, irrespective of the method (Table 2, Fig. 2). NormFinder analysis results for combined conditions revealed that *UBQ and ACCase* could be considered the best reference gene, and the geNorm algorithm ranked *18S rRNA* and *ACCase*, as the best reference gene (Table 2, Fig. 2). *β-TUB* was identified as the least stable gene by comparative ΔCt, BestKeeper and RefFinder, whereas NormFinder and geNorm analysis identified *GAPDH* as the least stable gene.

Pairwise variation analysis. The pairwise variation (Vn/Vn + 1) was calculated based on the geNorm algorithm. The optimal number of the reference genes required for the normalisation were determined from the pairwise variation results, based on the average expression stability (M) values (cutoff: M < 1.5). The optimal number of the reference genes required for the normalisation for experiments related to the developmental stages and plant organs are 1 and 2, respectively. However, to avoid any biases in the normalization, we recommend using 2 reference genes for developmental stages. Hence, we recommend using *18S rRNA* and *ACCase*, as housekeeping genes for developmental stages and plant organ-related studies in *B. sterilis*. Under drought stress, 4 candidate genes (*18S rRNA*, *β-TUB*, *25S rRNA* and *ACCase*) were considered suitable for normalisation. When all the conditions were considered together, the pairwise variation result suggested that 2 reference genes will be required for the normalisation (Fig. 3). Therefore, *18S rRNA* and *ACCase* were identified as the most suitable gene when all the conditions were considered together.

Relative expression of the *acetolactate synthase* (*ALS*) gene under herbicide stress. Based on the analysis of the commonly used software for reference gene analysis, *18S rRNA* and a combination of *18S rRNA* and *ACCase* were identified as the most suitable candidate genes for gene expression studies in *Bromus sterilis,* whereas *β-TUB* as the most unstable gene. To validate the reliability of the candidate genes, relative expression of the *acetolactate synthase* under herbicide stress was evaluated using the best and the least stable candidate genes. When normalised with *18S rRNA* and a combination of 18S rRNA and ACCase, *B. sterilis* biotype showed twofold *ALS* gene overexpression after herbicide treatment compared to the control, whereas with *β-TUB*, the result is almost eight times (Fig. 4).

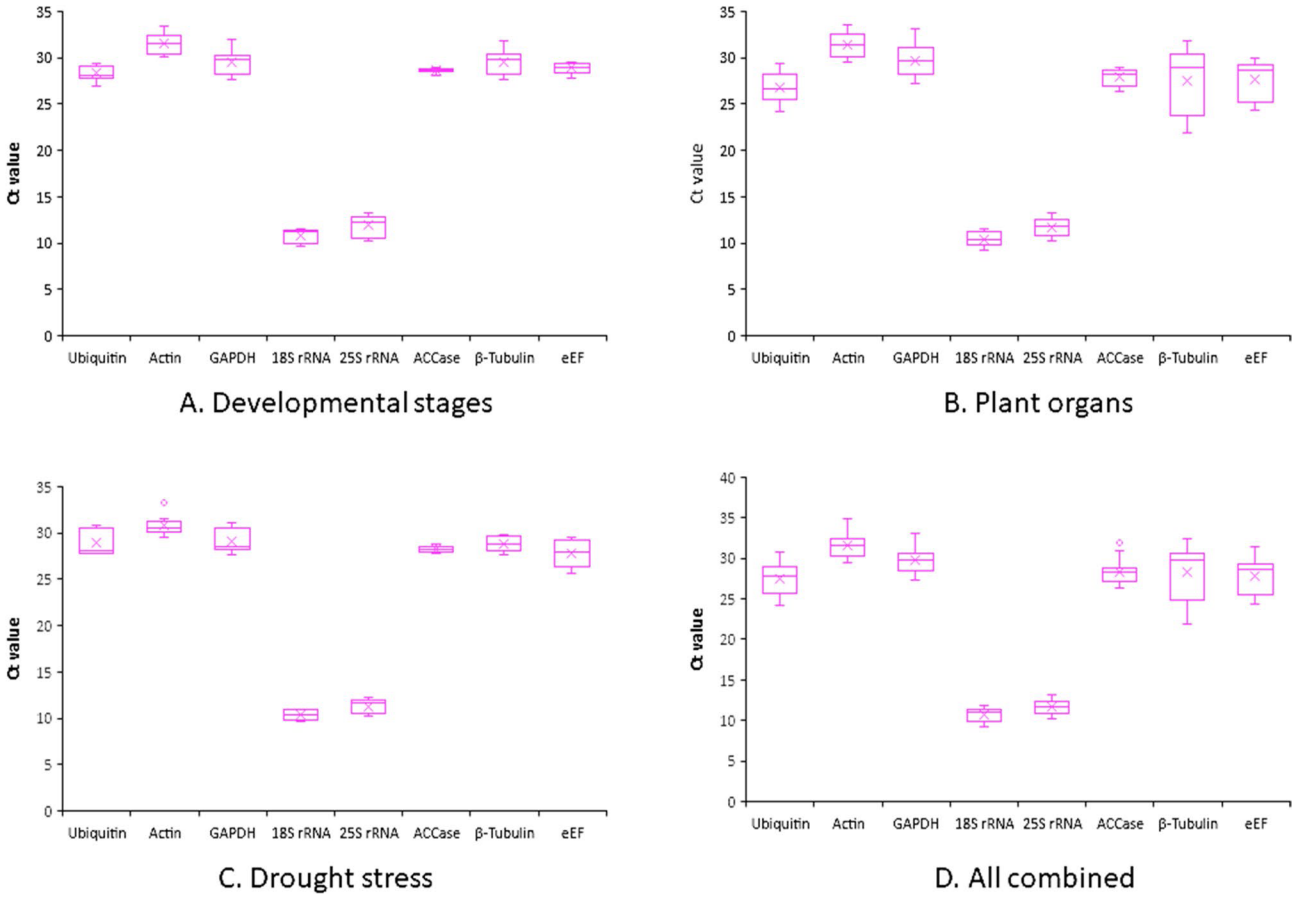


Figure 1. Expression levels of the eight candidate genes. Ct values obtained from three developmental stages (2nd, 3rd and 4th leaves), two different plant organs (shoots and leaves) and abiotic stress (drought stress) were compared and plotted.

Discussion

Recent reports from the United Kingdom and the Czech Republic on *B. sterilis*, developing resistance against commonly used herbicides, indicate that if they remained uncontrolled, these species might become a concern worldwide[25,26]. Herbicide resistance mechanisms can be target-site based (TSR) and/or non-target site-based (NTSR). Target-site based mechanisms involve nucleotide polymorphisms[27], gene amplification[6] and gene over-expression[28], whereas increased detoxification by enhanced metabolism[29,30] and/or reduced herbicide uptake and translocation[29] fall under non-target site-based herbicide resistance. Irrespective of the mechanism/s of resistance, qRT-PCR and the next-generation sequencing technologies have been used recently as a common technique to investigate the resistance mechanism in different weed species[5]. qRT-PCR experiments require an appropriate reference gene to normalise the target transcript levels. Any misapprehension in selecting a stable reference gene might lead to ambiguous results. Hence, the selection of a reliable reference gene is obligatory. Even though suitable candidate genes under different experimental condition were identified in many weedy species, like *Alopecurus sp.*[20], *Eleusine* sp.[8], *Avena* sp.[31,32,33], *Descurainia* sp.[34] etc., but to date, there are no reports on the systematic selection of stable reference genes under any conditions for barren brome or any other related brome species.

This study used qRT-PCR to evaluate the expression stability of eight candidate reference genes in barren brome under different experimental conditions. The most stable reference genes for each experimental condition were identified exclusively. geNorm software identified the ideal pair of genes with the minor variation in their expression ratios for each experimental condition. For studies related to life stages, geNorm identified that combining two reference genes would be suitable for normalising the qRT-PCR based gene expression values. *18S rRNA and ACCase* was chosen as the best reference gene for the studies with life stages of *B. sterilis*. For studies related to plant organs and under drought stress, pairwise variation analysis recommended using two and four genes, respectively. *18S rRNA* and *ACCase* were chosen as the most suitable candidates for plant organs-related studies, whereas, for studies under drought stress, we recommend using *18S rRNA*, *β-TUB*, *25S rRNA* and *ACCase*. When all the conditions were considered together, *18S rRNA* and *ACCase* were identified as the most suitable gene. Validation under herbicide stress indicated that both *18S rRNA* and the combination of *18S rRNA* and *ACCase* could be suitable. *18S rRNA*, a component of the 40S ribosomal small subunit in eukaryotes, has been recognised to have a steady expression in grasses under different stresses in earlier studies[35,36]. 18S rRNA is a primary constituent of all eukaryotic cells. Hence, *18S rRNA* is known to have extremely high expression in most cell types, so it can be challenging to use it as an endogenous normaliser gene. Moreover, synchronized

Rank	Comparative ΔCt		BestKeeper		NormFinder		RefFinder	
	Genes	Average of SD	Genes	Std dev [+/− CP]	Gene name	Stability value	Genes	Geomean of ranking values
Life stages (two-leaves stage, three-leaves stage and four-leaves stage)								
1	*18S rRNA*	0.7	*ACCase*	0.19	*18S rRNA*	0.132	*18S rRNA*	1.73
2	*Actin*	0.79	*eEF*	0.47	*ACCase*	0.134	*25S rRNA*	2.71
3	*25S rRNA*	0.8	*18S rRNA*	0.65	*Actin*	0.154	*Actin*	2.99
4	*β-tubulin*	0.81	*Ubiquitin*	0.65	*eEF*	0.166	*β-tubulin*	3.25
5	*ACCase*	0.89	*Actin*	0.86	*β-tubulin*	0.215	*ACCase*	3.64
6	*GAPDH*	0.94	*25S rRNA*	0.88	*25S rRNA*	0.224	*Ubiquitin*	5.63
7	*Ubiquitin*	0.94	*β-tubulin*	0.97	*Ubiquitin*	0.248	*eEF*	5.66
8	*eEF*	1.18	*GAPDH*	0.99	*GAPDH*	0.275	*GAPDH*	6.4
Best combination of two genes					*18S rRNA* and *eEF*	0.102		
Plant organs (stem and leaf)								
1	*18S rRNA*	1.39	*18S rRNA*	0.76	*18S rRNA*	0.125	*18S rRNA*	1
2	*25S rRNA*	1.48	*ACCase*	0.83	*ACCase*	0.146	*25S rRNA*	1.86
3	*Actin*	1.67	*25S rRNA*	0.89	*β-tubulin*	0.204	*Actin*	3.46
4	*Ubiquitin*	1.72	*Actin*	1.1	*Ubiquitin*	0.206	*ACCase*	3.76
5	*ACCase*	1.79	*GAPDH*	1.38	*25S rRNA*	0.214	*Ubiquitin*	4.36
6	*GAPDH*	2.02	*Ubiquitin*	1.51	*Actin*	0.214	*GAPDH*	5.73
7	*eEF*	2.37	*eEF*	1.81	*eEF*	0.229	*eEF*	7
8	*β-tubulin*	2.89	*β-tubulin*	3.01	*GAPDH*	0.323	*β-tubulin*	8
Best combination of two genes					*ACCase* and *eEF*	0.113		
Drought stress (water-treated vs untreated)								
1	*18S rRNA*	0.93	*ACCase*	0.26	*eEF*	0.191	*18S rRNA*	1.19
2	*β-tubulin*	1	*18S rRNA*	0.54	*β-tubulin*	0.295	*β-tubulin*	2
3	*25S rRNA*	1.05	*25S rRNA*	0.73	*18S rRNA*	0.304	*25S rRNA*	3
4	*ACCase*	1.21	*β-tubulin*	0.75	*ACCase*	0.326	*ACCase*	3.25
5	*Actin*	1.24	*Actin*	0.77	*GAPDH*	0.349	*Actin*	5
6	*GAPDH*	1.27	*GAPDH*	1.02	*25S rRNA*	0.406	*GAPDH*	5.42
7	*Ubiquitin*	1.35	*Ubiquitin*	1.17	*Actin*	0.516	*Ubiquitin*	6.74
8	*eEF*	2.3	*eEF*	1.48	*Ubiquitin*	0.581	*eEF*	8
Best combination of two genes					*GAPDH* and *18S rRNA*	0.165		
All samples (plant life stages, plant organs and drought stress)								
1	*18S rRNA*	1.39	*18S rRNA*	0.72	*18S rRNA*	0.175	*18S rRNA*	1
2	*25S rRNA*	1.48	*ACCase*	0.73	*ACCase*	0.198	*25S rRNA*	1.86
3	*Actin*	1.69	*25S rRNA*	0.8	*B-Tubulin*	0.237	*Actin*	3.22
4	*ACCase*	1.75	*Actin*	1.06	*eEF*	0.249	*ACCase*	3.36
5	*Ubiquitin*	1.97	*GAPDH*	1.33	*25S rRNA*	0.257	*Ubiquitin*	5.48
6	*GAPDH*	1.97	*Ubiquitin*	1.68	*Actin*	0.303	*GAPDH*	5.48
7	*eEF*	2.39	*eEF*	1.77	*Ubiquitin*	0.323	*eEF*	7
8	*β-tubulin*	2.78	*β-tubulin*	2.75	*GAPDH*	0.369	*β-tubulin*	8
Best combination of two genes					*Ubiquitin* and *ACCase*	0.142		

Table 2. Expression stability of candidate genes analysed by ΔCt, BestKeeper, NormFinder and RefFinder. RefFinder compares the results evaluated by four different programs (comparative ΔCt method, geNorm, BestKeeper and NormFinder) and based on the geomean of ranking values, provides a comprehensive ranking. St. dev.: standard deviation; St. dev [+/− CP]: standard deviation of crossing point (CP) values, eEF: eukaryotic elongation factor, ACCase: Acetyl-CoA carboxylase.

use of multiple reference genes will also decrease the chance of biased normalisations. Finally, from our study results, *18S rRNA* and *ACCase* appeared to be the most suitable reference genes to normalise the qRT-PCR data in *B. sterilis*.

Rapid advances in molecular biology techniques in plant biotechnology have increased the demand for identification of reference genes, which will be more stable than the traditional reference genes. The reference genes identified and validated in our study will assist the studies related to the elucidation of abiotic stress and its regulatory mechanisms. Comparative RNA-seq transcriptome analysis between the control and experimental plants

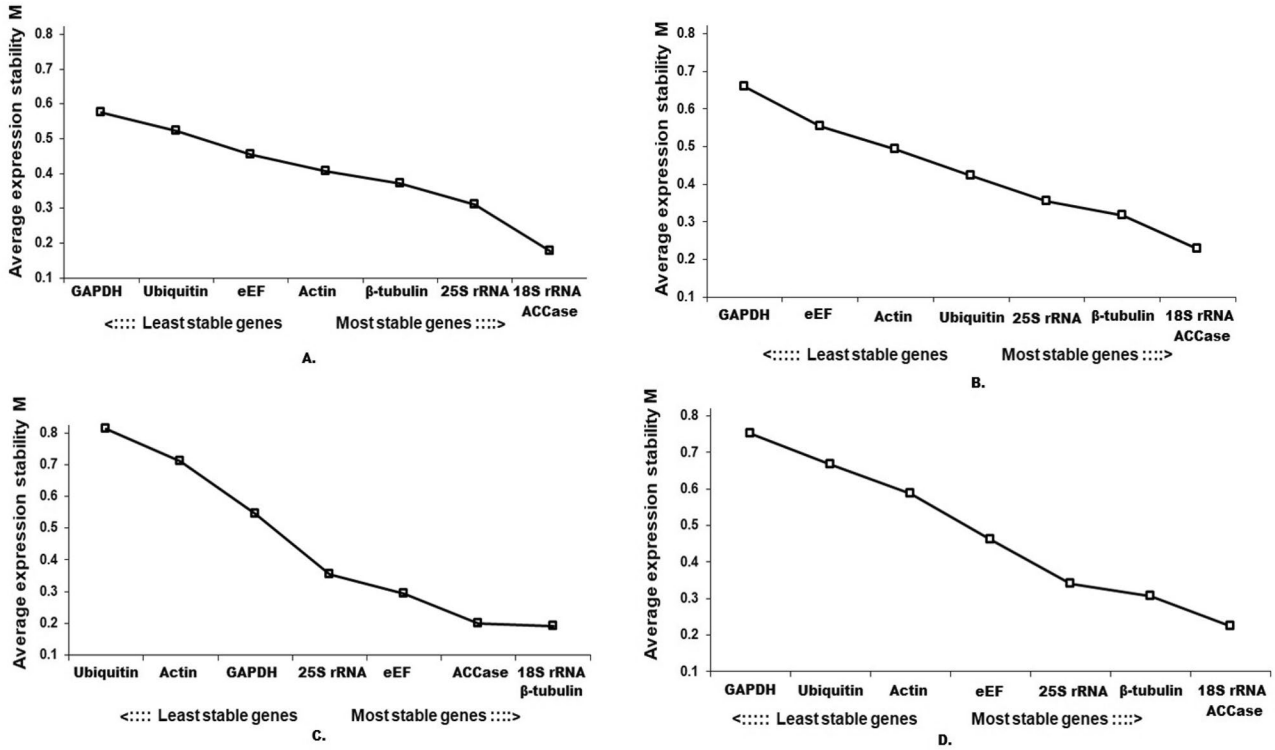
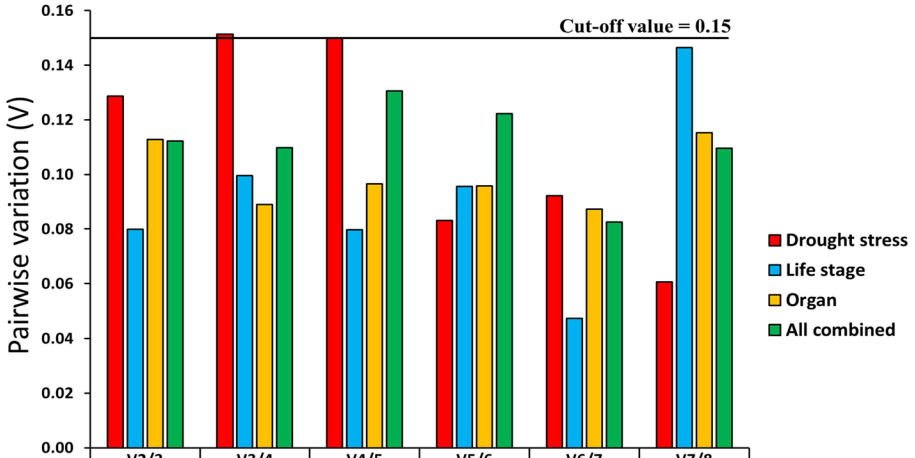


Figure 2. geNorm ranking of the candidate genes under different tested conditions. (**A**) Developmental stages, (**B**) plant organs, (**C**) Drought stress, and (**D**) All combined.



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
Drought stress	0.13	0.15	0.15	0.08	0.09	0.06
Life stage	0.08	0.10	0.08	0.10	0.05	0.15
Organ	0.11	0.09	0.10	0.10	0.09	0.12
All combined	0.11	0.11	0.13	0.12	0.08	0.11

Figure 3. Pairwise variation to determine the optimal number of reference genes. The recommended cutoff value under which there is no need for another gene is 0.15.

can be regarded as the most straightforward way to identify the genes involved in abiotic stresses like herbicide stress[20]. Recent studies on herbicide resistance mechanisms of *B. sterilis* suggest that both TSR and NTSR can be linked with the herbicide resistance in these species[25,26]. Nevertheless, detailed follow-up studies are essential to delineate further the regulatory mechanisms underlying the observed herbicide resistance mechanism[24]. However, among the herbicide resistance mechanisms, NTSR mechanisms are considered more complex to elucidate than the TSRs[20]. Comparative RNA-seq studies between the herbicide-resistant and susceptible plants will facilitate unravelling plausible resistance mechanisms in barren brome. Nevertheless, the RNA-seq data

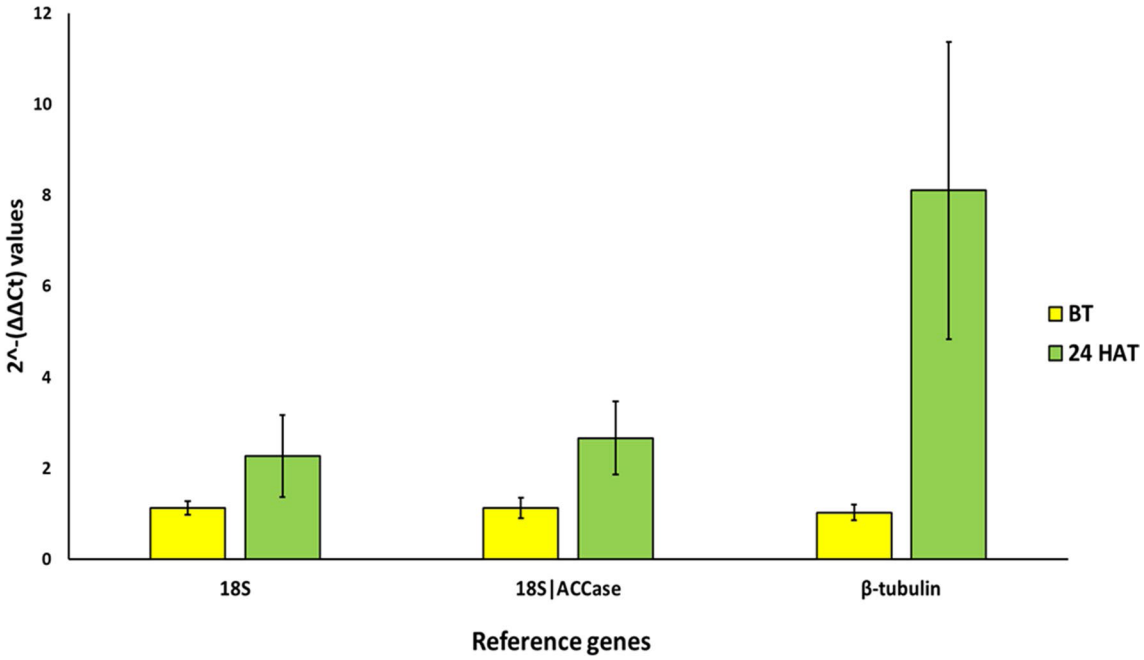


Figure 4. Relative expression of the acetolactate synthase gene under herbicide stress. Relative gene expression before herbicide treatment (BT) and 24 h after treatment (24 HAT) were compared, and normalization was done with *18S rRNA*, *18S rRNA|ACCase* and *β-tubulin*.

should be further cross-checked via qPCR, whose reliability depends on selecting the reference genes. This is the first study to evaluate and validate experiment-condition specific reference genes in brome species to the best of our knowledge. We had identified and validated internal reference gene suitable for normalising qRT-PCR experiments. Thus, our reference genes can be used during any RNA-seq based transcriptome or gene expression studies on *B. sterilis*. Our findings provide a basis for future molecular work on *B. sterilis* and can also be used during gene expression studies in other related species after preliminary validation.

Methods

Plant materials. A single population of *B. sterilis*, used for this study. *B. sterilis* was collected from winter wheat fields in the Ústecký region of the Czech Republic (50.2612525 N, 13.4818572 E). *Bromus sterilis* is an undesirable arable weed, so there are no specific country regulations for manipulation with it. No permissions were necessary to collect plant samples. 25 cm^2 pots (filled with chernozem soil, clay content 46% (loamy soil), soil pH (KCl) 7.5, sorption capacity of soil: 209 mmol (+), 87 mg kg^{-1} P, 203 mg kg^{-1} K, 197 mg kg^{-1} Mg, 8073 mg kg^{-1} Ca), were used to plant the seeds. The pots were kept in an open-air vegetation hall (with rooftop). Plant samples from three developmental stages (2-leaves stage, 3-leaves stage and 4-leaves stage), two different plant organs (shoots and leaves) and one abiotic stress (drought stress) were used for this study. For drought stress, watering was interrupted when the plants reach the three to four leaves stage, till symptoms of wilting were observed. Wilted leaves samples were collected and stored at − 80 °C (until further use). For herbicide stress, the plants were treated with pyroxsulam (a group of triazolopyrimidine sulfonamide ALS-inhibiting herbicide) at two to three leaf stage with recommended dose (1.875 g a.i. ha^{-1}). Herbicide was sprayed using a laboratory spray chamber equipped with a Lurmark 015F80 nozzle with a spray volume of 250 L ha^{-1} and pressure 120 kPa. The leaves samples were collected before treatment and 24 h after treatment and stored at − 80℃ for RNA extraction. All experiments conducted in this study, including the collection of plant material, are in compliance with relevant institutional, national, and international guidelines and legislation.

RNA extraction, complementary DNA (cDNA) synthesis and primer design. RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract RNA from the fresh tissues (± 80 mg per sample). TURBO DNA-free (Invitrogen, US) Kit was used to remove gDNA contamination. RNA integrity was verified by running the samples on 1.2% agarose gel electrophoresis. cDNA was synthesised by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) from the quality-checked gDNA-free RNA template (1 µg per reaction). Degenerate primers were designed for eight common candidate reference genes (*UBQ*, *ACT*, *GAPDH*, *18S rRNA*, *25S rRNA*, *ACCase*, *β-TUB* and *eEF*) based on their homologous sequences in other plants species (Table 1). The primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer3 software (https://bioinfo.ut.ee/primer3-0.4.0/). All the primers were tested by general PCR, performed using a C1000 thermocycler (Bio-Rad, Hercules, CA, USA), using cDNA template (10 ng per reaction). The thermocycler was programmed at an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 57 to 60 °C (based on the annealing temperature of the primer pairs), and 30 s at 72 °C along

with a final extension step for 10 min at 72 °C. The PCR amplified products were verified in the 1.5% agarose gel electrophoresis (data not shown).

qRT-PCR experiment and data analysis. PowerUp SYBR Green Master Mix (Applied Biosystems, USA) was used to conduct qRT-PCR assay in StepOne™ Real-Time PCR System (Applied Biosystems, USA). The reaction mixture contained 5 µL of SYBR Green Master Mix, 1 µL of primer mix and 4 µL of cDNA (2.5 ng μL^{-1}). For primer efficiency (E) and correlation coefficient (R^2) calculation, qRT-PCR assay was performed with diluted series of cDNA samples. $E = \{10^{(-1/slope)} - 1 * 100\%\}$ was used to calculate the values of E. The thermocycler was programmed at an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 57 to 60 °C (based on the annealing temperature of the primer pairs). To obtain the melting curves, stepwise heating was performed from 60 to 95 °C. All qRT-PCR experiments were conducted with 5 biological replicates. Quantification cycle threshold (Ct) values obtained from the StepOne Real-Time PCR System (Applied Biosystems, USA) was exported and used for further calculations. Gene expression stabilities of the eight candidate genes in the *B. sterilis* were examined by geNorm, NormFinder and BestKeeper, according to Chen et al.[8]. Besides, comparative ΔCt[37] and RefFinder[8] were also used. Before and after herbicide treatment, the relative ALS gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method[38,39]. NormFinder software estimates the intra- and intergroup variation. These variations are then combined into a stability value. The gene with minimal variation is ranked as the best by the software. geNorm program estimates an expression stability value (M) for each gene. Genes with the lowest M values have the most stable expression. BestKeeper ranks the candidate genes based on standard deviation values of cycle threshold (Ct) or crossing point values (CP) and coefficient of correlation (r) values. A gene with a standard deviation value below 1 and a coefficient of correlation value close to 1 is considered to have more stable gene expression than others. RefFinder integrates the available well-known programs for reference gene screening (geNorm, NormFinder, BestKeeper, and the comparative Delta-Ct method) and calculates the geometric mean of ranking values to give the overall ranking. The genes with a minimal geometric mean of ranking values are categorized as the best[37].

Ethical approval. No permissions were necessary to collect plant samples. All experiments conducted in this study, including the collection of plant material, are in compliance with relevant institutional, national, and international guidelines and legislation.

Ethics statement. This article does not contain any studies with human or animal subjects.

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Author contributions

M.K.S., K.H. and A.R. planned the experiments. P.K. had collected the samples. M.K.S. conducted the experiments. M.K.S., K.H. and A.R. performed the bioinformatics and statistical analysis. M.K.S., K.H., A.R. and J.S. analysed the results. M.K.S. and A.R. wrote the initial draft of the manuscript. M.K.S., K.H., A.R., P.K. and J.S. prepared the final manuscript. All authors have read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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ORIGINAL ARTICLE

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Apera spica-venti in the Czech Republic develops resistance to three herbicide modes of action

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Abstract

Investigation of molecular mechanisms of herbicide resistance to three modes of action was carried out in a population of *Apera spica-venti* with resistance to herbicides inhibiting acetolactate synthase (ALS), acetyl-CoA-carboxylase (ACCase) and inhibitors of photosystem-II (PSII). Greenhouse experiments were conducted to detect and characterize resistance pattern to pyroxsulam (ALS inhibitor), pinoxaden (ACCase) and chlorotoluron (PSII) using the recommended rate of each herbicide alone and in tank-mix, sequential application and dose–response tests. Metabolic detoxification and/or reduced herbicide uptake and translocation studies were conducted using dose–response tests with the herbicides in combination with malathion. After treatment, leaves from surviving plants were collected for pyrosequencing to identify target-site mutations in specific regions of *ALS*, *ACCase* and *psbA* genes. Among 32 analysed plants, target-site mutations in specific regions were detected for *ALS* and *ACCase* gene, but no *psbA* mutations were found. Dose–response assay showed high resistance factors for pyroxsulam (RF = 269.4), pinoxaden (RF = 66.3), but lower for chlorotoluron (RF = 14.3). Testing for metabolic detoxification by pre-treatment with malathion resulted in an increased susceptibility to pinoxaden in all doses, some increased susceptibility to chlorotoluron at the highest doses and no difference for pyroxsulam. Our results indicate that while target-site mutations were present for ALS and ACCase sites of action, metabolic detoxification does play a role for pinoxaden resistance. This research provided key insights into the resistance mechanisms in *Apera spica-venti* and will be important for developing control strategies for this weed in the Czech Republic.

KEYWORDS

chlorophyll fluorescence, dose–response test, herbicide resistance, loose silky bent grass, malathion

1 | INTRODUCTION

Loose silky bent grass (*Apera spica-venti* (L.) P. Beauv) is a winter outcrossing annual grass, widespread in the Central and East European countries, mainly, in Germany, Poland and the Czech Republic (Soukup et al., 2006). Although, it is also found in North European countries, such as Denmark, Sweden and Lithuania (e.g. Auškalnienė et al., 2020). It can be controlled with numerous herbicides, including ureas, dinitroanilines and thiocarbamates. The first case of resistance to ureas was found in Germany in 1996 (Niemann, 2000). Lately,

the sulfonylureas and triazolopyrimidines, two of the six chemical families collectively known as acetolactate synthase (ALS or AHAS) inhibitors, have become the most frequently used herbicides to control this grass in cereals. Shortly after the introduction of ALS inhibitors, the herbicide resistance in *Apera* spread rapidly (Nováková et al., 2006). After populations were confirmed to have high frequency of resistance to ALS inhibitors, many farmers began to use ACCase inhibitors and different herbicide combinations with often unpredictable results. Without an understanding of the resistance mechanism in a population, herbicide substitution may not improve control and instead increase the selection pressure on a resistant population (Petersen, 2018). As such, resistance to ACCase inhibitors was identified soon after their increased use (Adamczewski et al., 2016; Wolber, 2014). According to Heap (2021), resistance to ALS inhibitors in *A. spica-venti* is currently reported in ten European countries, including Czech Republic. Multiple resistance to ALS and PSII inhibitors has evolved in Austria and Denmark. Multiple resistance to ALS and ACCase inhibitors is present in Denmark, Poland and Germany. Resistance to three modes of action (MOA) namely ACCase, ALS and PSII inhibitors, was described in Germany.

None of these studies investigated the possible accumulation of resistance mechanisms in an individual *A. spica-venti* plant, as has been observed in other grasses, e.g. *Alopecurus aequalis* Sobol (Xia et al., 2015) and *Alopecurus myosuroides* Huds. (Marshall et al., 2013). Regarding resistance to ALS inhibitors, previous studies have shown varying, but very high levels of resistance to chlorsulfuron, sulfosulfuron and iodosulfuron, and cross-resistance to all three herbicides was confirmed in all populations tested (Hamouzová et al., 2011, 2014). The target-site resistance to sulfonylureas was attributed to four resistance-conferring ALS mutations: Pro-197-Ala, Pro-197-Thr, Trp-574-Leu and a novel Trp-574-Met substitution. Massa et al. (2011) detected the presence of additional Trp-574-Leu and Pro-197-Asn mutations in populations from Germany. Other German populations carried the Ile-1781-Leu and Ile-2041-Asn ACCase gene mutations (Wolber, 2014). The non-target-site mechanisms of herbicide resistance in *A. spica-venti* have not been described in detail until now, but there is suspicion of enhanced metabolism by the cytochrome P450s family and an involvement of glycosyltransferase and glutathion-S-transferase enzymes (Babineau et al., 2017). Our previous studies support this hypothesis – resistance to chlorsulfuron decreased when an organophosphate insecticide malathion was included, well known as inhibitor cytochrome P-450 (CYP450)-mediated metabolism of a sulfonylurea herbicide (Hamouzová et al., 2014).

The objectives of this study were (a) to evaluate the resistance profile in a new population of *A. spica-venti* for resistance to three modes of action of herbicides -qALS inhibitors (pyroxsulam), ACCase inhibitors (pinoxaden) and PSII inhibitors (chlorotoluron); (b) to clarify possible mechanisms of resistance to these three herbicide modes of action; (c) to assess the suitability and reliability of the method based on measurement of chlorophyll fluorescence for detection of herbicide resistance to the above-mentioned modes of action in *A. spica-venti*.

2 | MATERIALS AND METHODS

2.1 | Plant material

Seeds of an *A. spica-venti* population (from here after called LMC population) were collected in 2016 from a winter wheat field in South Bohemia, Czech Republic (49.0915833N, 14.7343022E) where the farmer had reported repeated control failures after the use of ALS, ACCase, and PSII inhibiting herbicides. In order to obtain a representative sample of the resistance population, seeds were collected from at least 100 plants in the field and combined to form a composite sample.

The standard susceptible reference population (S) was obtained from an integrated farm in South-eastern part of the Czech Republic (49.4105178N, 15.0198383E). Seeds from this population are used as a standard for most resistance studies in our laboratory.

2.2 | Effect of registered rates and selection of resistant individuals

This basic study was conducted to compare an effect of field rates of individual herbicides and their combinations on LMC population, and to select resistant individuals after different types of selection pressure for further molecular studies.

The experiment was conducted in a greenhouse where temperature and day length were controlled (16 hr light at 20°C and 8 hr dark at 16°C). *Apera spica-venti* seeds were sown in 8 × 8 cm plastic pots, containing chernozem soil (clay content 46% (loamy soil), soil pH (KCl) 7.5, sorption capacity of soil: 209 mmol (+), 87 ppm P, 203 ppm K, 197 ppm Mg, 8,073 ppm Ca). Emerged seedlings were thinned to ten plants per pot. Seedlings were regularly watered and fertilized. Herbicides pyroxsulam (Corello, 74 g a.i. (active ingredient)/kg, WG, ALS inhibitor, Dow AgroScience s.r.o., Prague, Czech Republic), pinoxaden (Axial Plus, 50 g a.i./l, OD, ACCase inhibitor, Syngenta Czech s.r.o., Prague, Czech Republic) and chlorotoluron (Lentipur 500 FW, 500 g a. i./l, SC, PSII inhibitor, FMC Agro Czech Republic s.r.o., Prague, Czech Republic) were used in the experiment. For the pyroxsulam treatment, the recommended adjuvant alkylphenol alkoxylate (Šaman 990 g a.i./L, Dow AgroScience s.r.o.) was added, at the dose of 990 g a.i. ha^{-1}. The herbicides pyroxsulam, pinoxaden and chlorotoluron were applied at 2–3 leaf stage (BBCH 12–13) with field recommended doses (Table 1). A laboratory spray chamber, equipped with a Lurmark 01F80 nozzle was calibrated to the spray volume of 250 L ha^{-1} at a spraying pressure of 250 kPa. Plants treated with water served as an untreated control. The experiment was not replicated in time.

Four replicates of the herbicide treatments were applied, as described in Table 1, for evaluation of resistance compared to the control and to select surviving plants for molecular studies. Three-way resistant individuals were selected for in two treatments, representing different approaches: tank-mix application and sequential application of all three herbicides. The sequential application of

TABLE 1 Herbicide dosage used for single dose treatment, tank-mix and sequence application together with specified numbers of *A. spica-venti* leaf samples collected from individual treatments for the partial *ALS*, *ACCase* and *psbA* gene sequencing

Treatment	Active ingredient	Recommended rate (g a.i. ha^{-1})	Samples tested (number of leaves)
1	Untreated control	–	0
2	Pyroxsulam	10	8
3	Pinoxaden	45	8
4	Chlorotoluron	1,000	8
5	Tank-mix (pyroxsulam + pinoxaden + chlorotoluron)	10 + 45 + 1,000	8
6	Sequence (pyroxsulam – pinoxaden – chlorotoluron)	10 – 45 – 1,000	0

herbicides on the population was carried out at 7-day intervals in the order: pyroxsulam–pinoxaden–chlorotoluron. Plant injury was visually estimated 28 days after herbicide treatment by comparing the biomass reduction based on dry weight and visual phytotoxicity symptoms of the treated plants *vs* the untreated control. Herbicide efficacy was visually estimated and expressed as percentage scores, where 0 corresponded to no damage and 100% to dead plants. Numbers of surviving plants were also recorded for individual pots.

2.3 | Dose–response assays

A dose–response experiment was conducted to estimate 50% growth reduction (GR50), resistance factors (RF), and an exclusion of metabolic resistance. For the latter purpose, malathion, an inhibitor of CYP450, was added to indicate cytochrome P450 (CYP450) involvement in metabolic resistance of herbicides (ALS inhibitors and other groups). Dose–response test was conducted in a greenhouse, between May and June 2018, in a completely randomised design with four replicates. The preparation of pots and assessments were identical as described in the previous section. The herbicide application was performed at 2–3 leaf stage (BBCH 12–13) of *A. spica-venti* plants with pyroxsulam (0; 0.0316; 0.1; 0.316; 1; 3.16; 10; 31.6; 100; 316 g a.i. ha^{-1}), pinoxaden (0; 0.1422; 0.45; 1.422; 4.5; 14.22; 45; 142.2; 450; 1,422 g a.i. ha^{-1}) and chlorotoluron (0; 3.16; 10; 31.6; 100; 316; 1,000; 3,160; 10,000; 31,600 g a.i. ha^{-1}), respectively. The herbicide treatments were performed one hour after treatment with malathion ([(dimethoxyphosphinothioyl)-thio] butanedioic acid diethyl ester, Supelco, USA). Malathion was applied at the rate of 1,000 g a.i. ha^{-1}, using a solution containing 0.005 g of malathion per ml in a carrier of isopropyl alcohol (78%), terpineol, dipentene and pine needle oil. The experiment was repeated twice to verify the data. Since no statistical differences were observed among replication, the most recent experiment is presented in this paper.

2.4 | Partial *ALS*, *ACCase* and *psbA* gene sequencing

Eight plants per treatment (32 in total) were selected among those that survived the treatments 1–4 according to Table 1 and were delivered to IDENTXX GmbH company (Stuttgart, Germany), which conducted the single nucleotide polymorphism analysis by pyrosequencing. Amino acid substitutions in Pro-197, Trp-574 of the *ALS* gene (the numbers are according to *Arabidopsis thaliana*), Ile-1781, Trp-2027, Ile-2041, Asp-2078 and Gly-2096 in the *ACCase* gene (the numbering according to *A. myosuroides*) and Val-219, Thr-220, Ala-251, Phe-255, Gly-256, Ser-264, Asn-266 and Leu-275 in the D1 protein of photosystem II (the numbers are according to *Amaranthus sp.*) were investigated. The pyrosequencing reactions were carried out according to the manufacturer's instructions using PyroMark Q24 (Qiagen, Hilden, Germany), specific sequence-primers and the PyroMark Q24 Gold Q24 Reagents (Qiagen, Hilden, Germany) in a similar manner as Löbmann et al. (2021). Details of reactions and primers are covered by trade secret (IDENTXX GmbH company). Subsequently, the genotypes were analysed using the supplied SNP Software (Qiagen, Hilden, Germany).

2.5 | Chlorophyll fluorescence measurement

The chlorophyll fluorescence of *A. spica-venti* plants was measured at days 1–7, 9 and 14 after the herbicide treatment. Untreated control plants and plants treated with recommended herbicide doses of chlorotoluron, pinoxaden and pyroxsulam were examined. All five plants in one pot were measured together representing one replicate. Six replicates per treatment were examined. Prior to measurement, the plants were dark-adapted for 20 min. The ground fluorescence (F_o) and the maximum fluorescence (F_m) were assessed with a pulse amplitude modulating imaging fluorometer (IMAGING-PAM MAXI, Heinz Walz GmbH, Effeltrich, Germany). In the case of open PSII centres of dark-adapted plants, the alternating fluorescence signal is referred to as minimal fluorescence F_0 and is excited by the measuring light. When a strong light pulse is applied, photosynthesis is saturated and all the reactions centres close. In this state, the alternating fluorescence signal appears to be strongly increased and achieves its maximal level, F_m. The chlorophyll fluorescence ratio F_v/F_m, indicating the maximum quantum yield of photosystem II, was measured according to the manufacturer's recommendation and calculated (Equation 1).

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \quad (1)$$

For F_v/F_m determination, a saturation pulse of 2000 μM s^{-1} m^{-2} of photosynthetic photon flux density was used at a wavelength of 450 nm.

2.6 | Statistical data analysis

R 2.8.0 software (R Development Core Team) was utilised to analyse the data from the dose–response assay, by non-linear regression (Baty et al., 2015). Dose–response curves were fitted to observed data. The three-parameter log-logistic function where the lower limit is equal to 0 was found to be the 'best-fit' distribution for our data.

$$f(x) = \frac{d}{1 + \exp(b)(\log(x) - e)}, \quad (2)$$

where d corresponds to the upper limit, b is the slope of the curve and e is the inflection point of the curve. The heterogeneity adjustment was performed through a Box-Cox transformation. The quality of each set of dose–response models was compared with an analysis of variance by a lack-of-fit F-test. The herbicide doses that caused 50% shoot growth inhibition (GR50) were computed for each herbicide and population. Resistance factor (RF) ratios were calculated by dividing GR50 of the LMC population by the GR50 of the susceptible (S) population. The comParm function (package drc) was used to compare parameter estimates of respective herbicide treatment with and without malathion ($p < 0.05$). For fluorescence measurements, the differences in the F_v/F_m values among treatments were analysed on a daily basis by ANOVA and Tukey's HSD test ($p < 0.05$).

3 | RESULTS

3.1 | Effect of registered rates and selection of resistant individuals

The tested herbicides did not have a significant effect on the growth of the LMC population, and the results confirmed a high level of resistance to all three active ingredients. The average efficacies after the single applications of pyroxsulam, pinoxaden and chlorotoluron in this population, reached 22.5%, 20% and 10% respectively. The S population was controlled by all tested herbicides in all experiments with efficacies ranging from 95% to 100% compared to the untreated control. Substantial differences were observed in sensitivity between individual plants and there were varying levels of resistance according to the production of above-ground biomass and phytotoxicity symptoms, especially after the application of pyroxsulam (data not shown). The highest rate of survival was found after the chlorotoluron application. No significant differences were found between the efficacy of tank-mix and the sequential application of these three active ingredients. On average, 30% of the plants survived the tank-mix application, resulting in 45% efficacy, and 28% of the plants survived the application of sequential application, which resulted in 50% efficacy. There were no statistically significant differences between single dose and tank-mix efficacy nor between single dose and sequential application.

3.2 | Dose–response assays

3.2.1 | Pyroxsulam

The whole-plant response experiments proved that the LMC population has evolved a high-level of resistance to pyroxsulam. The GR50 value reached 433.8 g a.i. ha^{-1} for this population while the dose 1.6 g a.i. ha^{-1} was needed to achieve a 50% growth reduction in the reference one. This resulted in RF of 269.4. The GR50 value showed minor reduction for both, R and S populations, when pyroxsulam was applied after the pre-treatment by 1,000 g ha^{-1} of malathion (Figure 1). The differences were, however, non-significant ($p > 0.05$). The values of GR50 for both populations, as well as the RFs are summarised in Table 2.

3.2.2 | Pinoxaden

The dose–response test also demonstrated that the LMC population had evolved resistance to pinoxaden. Our results showed relatively a high RF value (66.3) for this active ingredient when applied alone. After the malathion pre-treatment, the GR50 for pinoxaden decreased 18-fold to 11.36 g a.i ha^{-1} for LMC population and remained higher than the reference population (3.52 g a.i. ha^{-1}).

3.2.3 | Chlorotoluron

The dose–response analysis indicates that the GR50 values for the resistant plants were markedly higher than for the susceptible, although the resistance factor was lower (RF = 14.3) compared to pyroxsulam and pinoxaden. There was no difference between the GR50 values of LMC population treated with chlorotoluron alone and pre-treated with malathion (Table 2). The inhibiting effect of malathion pre-treatment occurred at the highest dose of 31,600 g a.i. ha^{-1} chlorotoluron ($p = 0.028$) (Figure 1).

3.3 | Partial *ALS*, *ACCase* and *psbA* gene sequencing

In total, pyrosequencing analysis discovered 14 plants with one of three mutations in 32 analysed plants. No individuals displayed more than one of the tested gene mutations. The highest occurrence of point mutations were associated with the *ALS* gene. Some plants are heterozygote with one wild allele and the other showing one of the expected mutations (e. g. CCC/ACC, CCC/TCC), while others are homozygous (e. g. ACC).

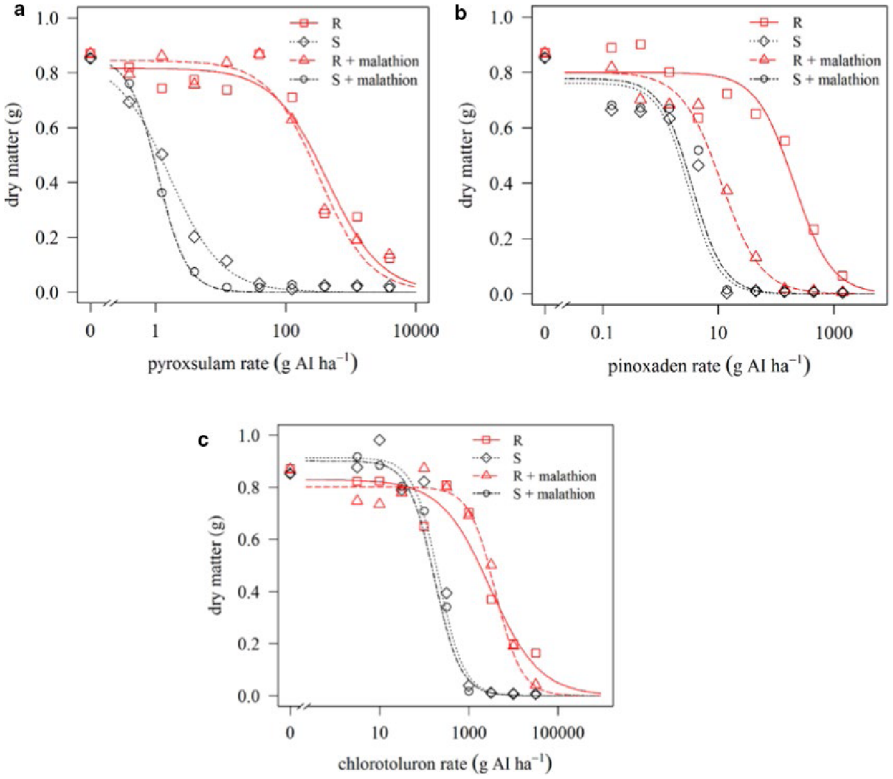

FIGURE 1 The total dry weight of the above-ground biomass after the application of pyroxsulam, pinoxaden and chlorotoluron with or without the addition of malathion at the dose 1,000 g/ha in the LMC (R) and the reference (S) population of *A. spica-venti*

TABLE 2 Calculated GR50 (g a.i. ha^{-1}) and RF values from dose–response assay with or without malathion pre-treatment, for the LMC and the reference populations.

Treatment	Recommended rate (g a.i. ha^{-1})	LMC population		Susceptible standard		RF
		GR50	CI	GR50	CI	
Pyroxsulam	10	433.86[a]	298.27–569.45	1.61[a]	1.03–2.18	269.4
Pyroxsulam + malathion	10 + 1,000	322.93[a]	229.87–415.99	1.08[a]	0.83–1.34	299.01
Pinoxaden	45	204.74[a]	119.29–258.22	3.09[a]	1.54–4.63	66.26
Pinoxaden +malathion	45 + 1,000	11.36[b]	9.69–17.21	3.52[a]	4.44–5.12	3.23
Chlorotoluron	1,000	2,999.57[a]	2,289.55–4,741.31	209.53[a]	231.37–276.02	14.32
Chlorotoluron + malathion	1,000 + 1,000	3,923.14[a]	3,096.57–5,318.77	170.878[a]	171.78–228.81	22.96

Note: The letters indicate homogeneous groups at 5% significance level.
Abbreviations: CI, confidence interval (95%); GR50, growth reduction; RF, resistance factor.

Both, heterozygotic mutations (C/A-CC and C/T-CC) and homozygotic mutations (ACC−−threonine) were detected for Pro-197 site and only heterozygotic mutations (T-G/T-G) were identified for Trp-574 site. More plants were associated with heterozygotic mutations at Pro-197 compared to homozygotes and among them, C/A-CC mutation type was more common than C/T-CC type. One case of a single heterozygotic mutation (A/T-TA) at Ile-1781 was found from the plants surviving the tank-mix doses. No single nucleotide mutations were detected to be responsible for the resistance to PS-II-inhibiting herbicides.

The distribution of mutations among herbicide treatments were relatively balanced and no apparent selection of relevant target-site mutations were observed for individual herbicides. The frequency of mutations after individual treatments is shown in Table 3.

3.4 | Chlorophyll fluorescence measurement

The untreated control plants of both populations, showed F_v/F_m, values ranging between 0.67 and 0.74, depending on the assessment day. Since these values were not significantly different, the data were pooled. In the reference population, F_v/F_m values of pyroxsulam- and pinoxaden-treated plants were different than the untreated control as early as 3 days after treatment (DAT), with the reduction in 19% and 21% respectively (Figure 2). From 4 DAT

TABLE 3 Amino-acid substitutions related to the genes of interest in 32 individual plants from the LMC population

Treatment	No. of analysed plants	Pro-197		Trp-574		Ile-1781	
		Codon	Number of plants	Codon	Number of plants	Codon	Number of plants
Tank-mix	8	C/ A-CC	2	—	—	A/T-TA	1
		ACC	1				
Pyroxsulam	8	C/A-CC	2	T-G/T-G	1	—	—
		ACC	1				
Pinoxaden	8	C/A-CC	2	T-G/T-G	1	—	—
		C/T-CC	1				
Chlorotoluron	8	ACC	1	T-G/T-G	1	—	—

Note: CCC-proline, ACC-threonine, TCC-serine, TGG-tryptophan, TTG-leucine, TTA-leucine, ATA-isoleucine.

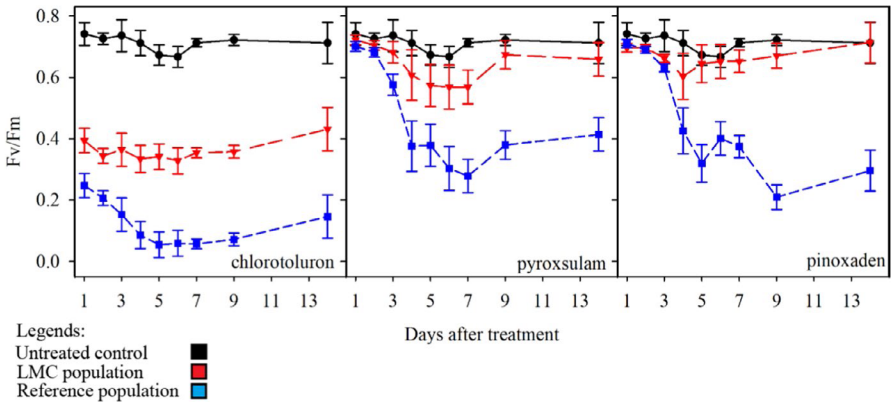

FIGURE 2 Trends in maximum quantum efficiency of PSII (F_v/F_m) in *A. spica-venti* untreated control plants from the LMC and reference populations (data pooled) and those treated with the recommended dose of chlorotoluron, pyroxsulam and pinoxaden, 1–14 days after treatment. The vertical bars represent standard errors

onwards, the F_v/F_m values for the reference population plants decreased by 40%–70%, compared to the untreated control, for both herbicide treatments. These differences were statistically significant and indicated a strong stress due to the herbicides in the reference population plants. On the other hand, the F_v/F_m value of the pyroxsulam-treated LMC population decreased only by 4% and 20% at 3 and 7 DAT, respectively, compared to the untreated control. The decrease in F_v/F_m values for the pinoxaden-treated LMC population was even lower than for pyroxsulam, throughout the assessment period, and exceeded a 10% decrease only at 4 DAT. There were no differences in F_v/F_m between treated and untreated control plants of the LMC population for both pyroxsulam and pinoxaden; however, a differentiation between resistant and susceptible treated plants was possible for both herbicides. For pinoxaden, F_v/F_m values differed by 5 DAT until the end of the experiment. In the case of pyroxsulam, there were differences at 4 DAT, but not at 5 DAT, and then from 6 DAT until the end of the experiment.

With regard to chlorotoluron, which inhibits the electron transport in PS II, a fast decrease in the F_v/F_m value was expected in both S and R plants. Indeed, only 1 DAT with chlorotoluron, the F_v/F_m value of the reference population plants decreased to 0.25, which corresponds to a decrease in 66% compared to the untreated control. In addition, the decrease of F_v/F_m value of S standard continued until 5 DAT followed by a period of stagnation, or non-significant recovery. The F_v/F_m value of the treated LMC population plants was only decreased to 0.40 at 1 DAT and was relatively constant in the following measurements. There were differences in F_v/F_m values between the herbicide-treated plants of the two populations for all measurements, starting at 1 DAT, where F_v/F_m values of the LMC population plants remained higher than for the reference population plants, indicating they were less affected by chlorotoluron.

4 | DISCUSSION

Herbicide resistance in the LMC population evolved during a long period of farming under the selection pressure of different herbicides, especially the ALS inhibitors. Although PSII and ACCase inhibiting herbicides were previously efficacious for the LMC population after it had developed ALS resistance (Hamouzová et al., 2011), herbicide failures were observed in the last 8 years. The present study demonstrated that ALS, PSII and ACCase inhibitors currently have very low efficacy of 10%–22.5%, compared to the reference population, which is still fully controlled by these herbicides. Along with the active ingredients mentioned above, flumioxazin and flufenacet have been tested in a separate pot experiment. Their efficacy was high (>90%) and, thus, can be recommended to the farmer.

The survivors in this study showed varying levels of resistance, especially after the application of pyroxsulam. Tank-mixing of herbicides is recommended as the best strategy for delaying resistance by decreasing survival probabilities of all individuals (Evans et al.,

2016), but in this case, a high proportion of the population already contained multiple-resistant individuals. No differences were found in efficacy between the tank-mix and sequential application when all three herbicides, with different modes of action, were applied on the population. However, both tank-mix and sequential application resulted in fewer survivors compared to the application of single herbicides.

The background of the resistance in the tested LMC population seems to be different for each mode of action. In the case of the pyroxsulam, mutations known to confer resistance to ALS inhibitors were found at Pro-197 and Trp-574 in three out of eight plants analysed. On the other hand, malathion pre-treatment did not confirm any significant involvement of CYP450 mono-oxygenases in the metabolic detoxification of this herbicide, even though the synergistic effect of malathion with pyroxsulam had been reported in other studies (e.g. Feng et al., 2016). Therefore, decreased sensitivity of the target enzyme appeared to be the main mechanism of resistance present in the LMC population, which is supported by a high resistance factor (269.4). The resistance effect of the above-mentioned mutations had been reported in other grass weeds. Kaloumenos et al. (2013) showed that the Trp-574-Leu mutation in the *ALS* gene of *Echinochloa oryzicola* Vasing. conferred cross-resistance to several ALS inhibiting herbicide families, including triazolopyrimidines. Guo et al. (2015) confirmed that pyroxsulam resistance in a population of *A. aequalis* was very likely endowed by the Trp-574-Leu mutation. In the same species, the substitution Trp-574-Leu conferred broad-spectrum resistance to all ALS herbicides, while the substitution of Pro-197-Thr conferred high resistance to sulfonylureas and moderate resistance to triazolopyrimidines (Stankiewicz-Kosyl et al., 2017; Xia et al., 2015). In addition, although only heterozygous mutations were found for the Trp-574 site, their participation in herbicide resistance expression remains important. Therefore, both mentioned mutations seem to be responsible for resistance to pyroxsulam in the tested LMC population. As the percentage of surviving plants at the recommended rate of pyroxsulam is higher (90%) than the percentage of plants carrying relevant target-site mutations (37%), other resistance mechanisms are likely to be present in the tested LMC population. Specific P450 enzymes that are not inhibited by malathion, or other types of detoxification enzymes such as glutathione transferases (GSTs) may be involved in pyroxsulam resistance in *A. spica-venti*. Fang et al. (2019) described involvement of both P450 and GSTs in *Echinochloa crus-galli* resistance to another triazolopyrimidine herbicide, penoxsulam. Cummins et al. (2013) reported enhanced expression of glutathione s-transferases (GSTs) as an important mechanism of herbicide resistance to multiple herbicides in grasses.

The results discussed previously are consistent with the fluorescence measurement. The lack of difference between the F_v/F_m values and the untreated control throughout the measurement period, together with the relatively high standard errors of the measurements indicate that some plants were relatively unaffected by the pyroxsulam treatment. This supports the finding that target site resistant plants are also present in the LMC population.

For pinoxaden, both target-site and non-target-site mechanisms of resistance seem to be present in the LMC population. Ile-1781-Leu single heterozygotic mutation was confirmed by sequencing of the *ACCase* gene, although its frequency in the population was low (~3%). This mutation has been shown to confer resistance to pinoxaden in other grass species, such as *Digitaria ciliaris* (Retz.) Koeler and *Phalaris minor* L. (Basak et al., 2020). Similarly, the Ile-1781-Leu mutation in *Lolium rigidum* Gaud. plants endowed cross-resistance to pinoxaden, clodinafop, haloxyfop, sethoxydim and clethodim (Scarabel et al., 2011).

The effect of the heterozygotic mutation on resistance expression can be variable. In the dominant mode of resistance, the gene is expressed irrespective of its zygosity, whereas in the co-dominance mode, the level of resistance in the heterozygous plants is intermediate between the homozygous genotypes (Tal and Rubin, 2004). As no clear difference in sensitivity to pinoxaden was revealed between heterozygous and homozygous *Lolium* spp. plants with the Ile-1781-Leu mutation (Scarabel et al., 2011), a similar dominance mode could be suggested for *A. spica-venti*.

Irrespective to the zygosity effect, the proportion of plants in the population LMC carrying the Ile-1781-Leu mutation is small (~3%) and cannot be the only reason for the high-level of resistance observed in this population. A substantial decrease in GR50 value found in the malathion pre-treated plants indicates that metabolism plays an important role in their resistance to pinoxaden. For the S standard, malathion had no significant effect on herbicide efficacy. It has been found that malathion can reduce detoxification of herbicides in plants by inhibiting CYP450 and, thus, reverse the metabolism-based resistance (Beckie et al., 2012). They reported an increase in the efficacy of pinoxaden and flucarbazone after malathion pre-treatment in five Canadian *Avena fatua* L. populations with resistance to ACCase and ALS inhibitor. Involvement of CYP450 in the detoxification of pinoxaden was also confirmed by malathion pre-treatment in a non-target-site resistant population of *Brachypodium* sp. (Matzrafi et al., 2014).

Based on these results, the increased activity of P450 monooxygenases seems to be the predominant mechanism of resistance to pinoxaden in the tested LMC population. The fluorescence measurement also supports the evidence of an enhanced pinoxaden detoxification ability in this population. Non-significant decrease of F_v/F_m values is only traceable in data (Figure 2) during the first four days after treatment followed by a recovery to original values.

For chlorotoluron, the resistance mechanisms in the LMC population were not fully understood. The fact that no common mutations were found in the *psbA* gene combined with a relatively low resistance factor (14.3) indicates the non-target-site nature of the resistance. The resistance factors in other chlorotoluron non-target-site resistant grass weeds varied between 2.6 and 29.3 (Menéndez et al., 2006). No significant effect of malathion on GR50 of the chlorotoluron was confirmed in our assay. Nonetheless, the synergistic effect of malathion was significant at the highest dose of chlorotoluron. The reason for this could be partially explained by the relatively low synergism of malathion and chlorotoluron, which has been

reported e.g. by Preston et al. (1996), though other causes could also be possible. It is worth to mention that a high variability in the sensitivity of individual plants to chlorotoluron was traceable in the dose–response assay and the calculated resistance factor should be considered as a mean of the population. Most of the resistant individuals were not able to survive doses above 3,160 g a.i. ha^{-1}; however, some plants survived the highest applied dose (31,600 g a.i. ha^{-1}) and their resistance factor can be expected to be much higher. High variability in this population can be attributed to the biology of the species and conditions of the field; namely, while the species is self-incompatible and relies on wind pollination, it is geographically isolated from other populations. Chlorotoluron was the last effective herbicide used to control *Apera spica-venti* in this field, and perhaps at the beginning of selection pressure on this population. While the situation may have suggested target-site resistance, the effect of the malathion pre-treatment on the exhibited resistance in the population should preclude such a simplistic answer. Based on above-mentioned reasons, it seems that several pathways of herbicide detoxification or even other mechanisms may be involved in non-target-site resistance of the LMC population plants. Metabolic detoxification of chlorotoluron via P450 oxygenases was confirmed in resistant plants of *A. myosuroides* decades ago (Menéndez et al., 1994). More recently, Cummins et al. (2013) demonstrated an important role of GSTs in *A. myosuroides* and *Lolium multiflorum* populations exhibiting multiple resistance to many herbicides including chlorotoluron and suggested similar functions in other grass weeds.

The fluorescence measurements support the assumption of the non-target-site resistance to chlorotoluron. Any substantial changes in the affinity of chlorotoluron to the target D1 protein are unlikely since the F_v/F_m values of all measured plants were quickly decreased by the chlorotoluron treatment. The data of our study show that plants of LMC population were less affected by chlorotoluron than reference population plants 1 DAT, indicating a fast detoxification of the active ingredient. Similar response of the F_v/F_m parameter was observed for Zhang et al. (2016) in *Echinochloa colona* plants with non-target-site resistance to the PS II inhibitor after treatment with propanil. Surprisingly, the decrease of F_v/F_m in the LMC population was not followed by recovery. High standard error of F_v/F_m values observed at 14 DAT could, however, signal some recovery of individual plants at the end of the measurement period. The measurement of the maximum quantum efficiency of PS II proved to be a suitable method for fast detection of resistance. As the chlorotoluron inhibits the electron transport in PS II, a fast decrease of the F_v/F_m allowed reliable discrimination of resistant plants as early as 1 DAT. Similar results have been reported by Wang et al. (2018). Comparatively, the other herbicides tested in this study needed more time to identify resistant plants using the fluorescence parameter; five and six days were required for reliable discrimination between resistant and susceptible plants for pinoxaden and pyroxsulam respectively. A shorter discrimination period could be expected if individual plants had been assessed. Wang et al. (2018) found significantly higher F_v/F_m values in resistant biotype of *A. myosuroides* than in the susceptible biotype at 3 days after treatment with ACCase, ALS and PSII inhibiting herbicides. Likewise, Linn et al. (2019) successfully used the F_v/F_m parameter for the detection of herbicide resistant plants of *Stellaria media* L. Vill and *Papaver rhoeas* L. after ALS-inhibiting herbicide treatment. The discrimination between susceptible and resistant plants was possible 3 DAT (three days after the treatment). Averaging the F_v/F_m values of all plants present in the replicate may decrease statistical significance and prevent or delay achieving significant results if the ratio of resistant plants in the population is small. In this study, the F_v/F_m values were measured as a mean of five plants growing in each pot as the plants were inseparable in many cases. Although more time-consuming, measurement of individual plants as described, for example, by Wang et al. (2018) should be recommended in field conditions.

5 | CONCLUSIONS

This is the first case of *A. spica-venti* resistance to three MOA in the Czech Republic, confirmed by a combination of whole-plant dose–response assay, genetic analyses and fluorescence measurement. Changes in susceptibility of the target enzyme conferred by Trp-574-Leu and Pro-197-Thr mutations was determined to be the main mechanism of resistance to pyroxsulam. Mixed effect of target-site and non-target-site mechanisms is expected to be responsible for the resistance to pinoxaden. Because of low frequency of Ile-1781-Leu mutation in the population, the CYP450 detoxification of pinoxaden is probably the predominant cause of resistance. For chlorotoluron, non-target-site mechanisms seem to be the key reason of decreased susceptibility of R plants. Although, the inhibition of detoxification processes by the malathion pre-treatment was limited, and other detoxification mechanisms may be expected.

Measurement of maximum quantum efficiency of photosystem II (F_v/F_m) can be considered as a suitable method for fast identification of both, target site and non-target site herbicide resistance in *A. spica-venti*. Fast measurement of F_v/F_m parameter can be employed as a first-line method for preliminary identification of resistant plants before deeper analyses are conducted.

The population with herbicide resistance showed resistance to three groups of herbicides. While the target-site modes of action are usually specific for individual groups, the non-target-site mechanisms may be common for two or more herbicide groups. As shown in other studies, multiple resistance is typical for elevated levels of herbicide-detoxifying enzymes (Cummins et al., 2013). Apart from increased activity of P450 oxygenases, other detoxifying mechanisms are likely to be involved in multiple herbicide resistance of tested resistant population. Future research will focus on the role of GSTs in the expression of resistance to different herbicides in *A. spica-venti*.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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5. SUMMARY DISCUSSION

The modern input-intensive agriculture system aims to reduce economic losses in agricultural production. Weeds, if left uncontrolled, might cause huge agricultural economic loss (up to 50-60% or even 100%). Herbicides (represent almost 60% (by volume) of the pesticides used worldwide) have been used to manage weeds in agronomic crops. However, despite of their weed-killing efficiencies and cost-effectiveness, intensive and unplanned use of herbicides led to the evolution of herbicide-resistant weeds. Resistance has been reported against almost all known herbicide modes of action. Hence, herbicides with a novel mode of action are desperately needed to manage the evolution of resistance of weeds to existing herbicides.

In general, the mechanisms of herbicide resistance can be divided into TSR and NTSR mechanisms. NTSR mechanisms are more complex, when compared with the TSR. However, irrespective of the mechanisms, any mechanism that is present within the weed population can allow the weed to survive (Délye *et al.,* 2013). Why the knowledge of mechanism of herbicide resistance is so important? Good management of herbicide resistance generally depend on understanding the biology of the individual weed species and the herbicides that are still effective for control (Beckie, 2006; Délye *et al.,* 2013). Elucidation of the exact mechanisms of herbicide resistance will not only provide information about possible cross-resistance to other herbicides but also will provide alternative solutions about herbicide management. How omics-based technologies can be used to explicate the exact mechanism of herbicide resistance? High throughput molecular biology-based techniques and their integrations have drastically changed the way biologists carry out their research. Even though plant biology and genomics have already included these technologies, while some challenges remain for use in applied biology. Comparatively new subdiscipline, weed molecular biology is still learning how to integrate omics technologies into the discipline. Omics technologies such as transcriptomics, epitranscriptomics, proteomics, metabolomics and/or their integrations should be used and applied more often to address basic questions in weed biology (such as elucidating the mechanisms of resistance). Additionally, better utilization of these technologies may also help to answer some practical questions of improving weed management (Patterson *et al.,* 2019; Maroli *et al.,* 2018; Ravet *et al.,* 2018).

This doctoral thesis aims to use omics-technologies to unravel the molecular mechanisms underlying the herbicide resistance development (in weeds occurring in the Czech Republic) and propose its prospects for planning superior weed management strategies. In brief, this doctoral thesis contains 4 different scientific works (already published). The work 1 aims to solve the problem of primer designing for an important herbicide target gene (*acetolactate synthase*) (Sen *et al.*, 2021b). Codon usage is an important determinant factor of gene expression levels in eukaryotic and prokaryotic genomes, due to its effects on transcription (Liu *et al.,* 2021; Zhou *et al.,* 2016). Due to their favorable effects on the translation efficiency, particular codons are preferred by the organisms over the others. Hence, this lead to codon-usage bias. Codon usage biases are found in all eukaryotic and prokaryotic genomes. Optimal codons contribute to efficient and accurate translation. Hence, designing of genes of interest using optimal codons (rather than overall ample codons) might be a key strategy to boost protein expression for heterologous gene expression (Zhou *et al.,* 2016; Quax *et al.,* 2015). Heterologous protein expression studies find huge application while investigating cellular functions of proteins, genetic circuit engineering and in overexpressing proteins for various research. Even though there are several field-based studies published on weeds and their herbicide-resistant properties but studies related to their molecular properties are still at their initial stages. Development of new herbicidal compounds might require developing synthetic herbicidal-target heterologous genes. In this study, we had successfully identified the optimal codons for *ALS* gene in weeds, which will help the weed molecular biologists to design degenerate primers (independent of the availability of the genome sequences).

Work 2 aimed to elucidate the molecular mechanisms of resistance in a pyroxsulam-resistant *Bromus sterilis* biotype, collected from the winter wheat field within the Czech Republic (Sen *et al.*, 2021a). Frequent use of pyroxsulam has triggered the evolution of herbicide-resistance in many weeds, including *B. sterilis*. Dose–response and cross-resistance experiments implied that this biotype has developed resistance to pyroxsulam and cross-resistant to propoxycarbazone, iodosulfuron plus mesosulfuron and sulfosulfuron. Prior treatment with malathion resulted in the decrease of the resistance index compared to the plants treated with pyroxsulam alone. This confirmed the presence of metabolism-based resistance mechanism. Neither any differences in the ploidy level nor any mutations (for *ALS* gene) were detected between the resistant and susceptible biotypes. Almost two-fold overexpression of the *ALS* gene was detected in the resistant biotypes.

But our relative *ALS* gene copy number results confirmed that gene amplification is not responsible for the overexpression. In conclusion, these findings revealed that the pyroxsulam-resistance is expected to be associated with overexpression of the *ALS* gene and enhanced metabolism.

Work 3 was conducted based on the fact that, to date, there are no reports on appropriate reference gene in any brome species (Sen *et al.*, 2021c). To investigate the molecular mechanisms of herbicide-resistance, quantitative PCR (RT-qPCR) has been extensively used to assess the expression of candidate genes. Additionally, with the increasing number of transcriptome-based studies, the application of qRT-PCR has grown to validate the transcriptome data and to the study gene expression. Despite of being accurate and able to detect precise fold changes in gene-expression, the accuracy of this technique highly depends on the expression of reference gene/s. Any mistake in choosing a suitable reference gene might lead to deceptive results. Since there are no universal reference genes, it is necessary to methodically select the appropriate reference gene/s for each individual species. In this article, we had used five most commonly used software (ΔCt, BestKeeper, NormFinder, geNorm and RefFinder) for reference gene selection in *B. sterilis*. The candidate genes were *18S rRNA, 25S rRNA, Actin, eukaryotic elongation factor (eEF), ACCase, β-tubulin, Ubiquitin* and *GAPDH*. We had successfully identified and recommended suitable reference genes for *B. sterilis* gene expression studies under different experimental conditions (developmental stages, plant organs, drought stress, herbicide stress and all combined). Based on the ranking and validation studies, we recommend using a combination of *18S rRNA* and *ACCase* to normalise the qRT-PCR data in *B. sterilis*. The results from this study will expedite upcoming molecular works in *B. sterilis* and other related grass species.

In the work 4, we report the first case of *Apera spica-venti*, which had developed resistance to three herbicide modes of action (Košnarová *et al.*, 2021). Following the confirmation of the resistance, the investigation of the molecular mechanisms of herbicide resistance were carried separately for pyroxsulam (ALS-inhibiting herbicide), pinoxaden (ACCase-inhibiting herbicide) and chlorotoluron (PS II-inhibiting herbicide). Our results indicate that target-site mutations is the main mechanism for resistance against pyroxsulam and metabolic detoxification does play a role for pinoxaden resistance. However, we predict that neither *psbA* gene mutations nor enhanced metabolism is responsible for the resistance to chlorotoluron. Additionally, this study also showed that maximum quantum efficiency of photosystem II (Fv/Fm) can be considered as an important parameter for fast identification of both, TSR and NTSR in this weed species and must be tested

for its efficacy in other related species. This research provided important perceptions into the resistance mechanisms in *A. spica-venti*. Understanding the resistance mechanisms will be important for developing the existing control strategies for this weed in the Czech Republic as well as across the Europe.

6. CONCLUSIONS

Weeds are a major threat to food security. Herbicides were the most effective tool for the weed control ever developed. However, with the discovery of the first herbicide-resistant in 1957, the twist in the game began. This golden period of chemical weed control was quickly cut short and currently, the herbicide resistance has been reported in 267 weed species worldwide (www.weedscience.org). Moreover, no new herbicide modes of action had appeared in recent years. Discovery and development of new compounds with herbicidal properties along with an assessment of their efficiencies might necessitate designing of synthetic herbicidal target genes. In theory, designing of synthetic genes with optimal codons instead of random codons might produce impressive results. So, in our first study, we had focused on the identification of the optimal codons for an important herbicidal target gene (acetolactate synthase). Based on *in-silico* analysis, we recommended using the codons form our work for further studies, to avoid any delays while optimizing heterologous gene expression experiments in weedy species.

Rigorous application of ALS-inhibiting herbicides has sparked the evolution of herbicide-resistance in many weeds, including *Bromus sterilis*. In the Czech Republic, these troublesome weeds were well-controlled by ALS-inhibiting herbicides (mainly pyroxsulam and propoxycarbazone). But recently the farmers in the Czech Republic have reported that the recommended doses of pyroxsulam have failed to control this weed. So, following the collection of the seeds, based on the greenhouse dose-response experiments, we point out that this biotype is resistant to pyroxsulam and cross-resistant to propoxycarbazone, iodosulfuron plus mesosulfuron and sulfosulfuron. To understand the mechanism of resistance, we explored the possibilities such as ploidy level variation, *ALS*-gene mutations, relative copy number and expression. Finally, we discovered that enhanced metabolism and target gene overexpression are the main mechanism. In addition to these, we realised the fact that there were no suitable reference genes for qRT-PCR experiments in B. sterilis. Hence, in our third study, we had systematically identified the best reference genes in this species. We recommend using a combination of *18S rRNA* and *ACCase*. The obtained results of our studies will provide a basis for further research using comparative proteomic or epigenetic approaches to investigate how target gene overexpression is orchestrated at the molecular level.

Apera spica-venti is one the most economically important weed species in the Czech Republic. In our last study, we had investigated the molecular mechanisms of herbicide resistance to three modes of action (ALS-inhibiting, ACCase-inhibiting and PSII-inhibiting). Additionally, we had also tried to verify if measurement of chlorophyll fluorescence can be a reliable method for detection of herbicide resistance in this weed. Finally, we discovered that while genetic mutation is responsible for pyroxsulam-resistance, NTSR is involved in pinoxaden-resistance. However, the exact mechanism for chlorotoluron is still needed to be addressed. Moreover, we also recommend using measurement of chlorophyll fluorescence for detection of herbicide resistance.

In conclusion, in this Ph.D. thesis, we had successfully addressed all the hypotheses and fulfilled the objectives. The current findings highlight the need to monitor additional brome and common wind grass populations for herbicide resistance in Europe. Lack of new products with herbicidal properties and the imposed dose rate reduction of many active ingredients are likely to increase resistance cases in the near future. Moreover, herbicide resistance is as an evolutionary process driven by overuse of single site of action herbicides without additional weed control practices. Therefore, we recommend using alternative herbicides in integrated weed management to slow down the potential evolution of herbicide resistance in these species.

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