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## First cases of herbicide resistance of *Tripleurospermum inodorum* in the Czech Republic

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**Abstract:** *Tripleurospermum inodorum* (L.) Sch. Bip. is one of the most economically important and yield-reducing weeds in cereals in Europe. Random and systematic monitoring of this weed might provide an early warning for the farmers and slow down the pace of the evolution of herbicide resistance. This study aimed to identify resistant populations of *T. inodorum* in the Czech Republic and elucidate their possible resistance mechanism/s. Monitoring and screening of *T. inodorum* for herbicide resistance against acetolactate synthase (ALS) inhibitors and synthetic auxins was carried out. Greenhouse experiments and molecular-genetics studies were conducted to characterise the resistance. While all the tested populations were found to be susceptible to synthetic auxins, two populations (MATIN-2 and MATIN-4) were found to be resistant against tribenuron (ALS inhibitor). However, their cross-resistance to florasulam was not confirmed. The resistance mechanism detected was the target-site substitution of Pro197 at the ALS gene. The two populations carried different point mutations: Pro197Ser (MATIN-2) and Pro197Gln (MATIN-4). This is the first study in the Czech Republic to identify the survival mechanism in *T. inodorum* for resistance to ALS-inhibiting herbicides. Our research results will provide a basis for resistance management in *T. inodorum* in the Czech Republic and other countries.

**Keywords:** ALS-inhibitors resistance; Pro197 mutations; scentless mayweed; target-site resistance; weed control

Scentless mayweed (*Tripleurospermum inodorum* (L.) Sch. Bip.) is a competitive weed in the disturbed and agricultural areas of Europe, including the Czech Republic (Štrobach and Mikulka 2019). This weed is difficult to manage, particularly in cereals such as winter wheat and winter rape (Nadtochii 2009, Reiss et al. 2018). Since this weed germinates at almost the same time to that of the crop and produces high biomass, it significantly affects the yield of these crops (Adamczewski et al. 2014). The prevalence of this weed is mostly due to minimum or no-tillage systems. In general, scentless mayweed is well controlled by the most widely used herbicides in cereals, especially inhibitors of enzyme acetolactate synthase (ALS) such as active ingredients tribenuron, pyrox-sulam or florasulam (Majcen et al. 2013, Jursík et

al. 2018). Some herbicides, such as carfentrazone, pendimethalin, and growth regulator-inhibiting herbicides, were found to be less effective against this weed. Pre-emergence herbicides containing metazachlor, dimetachlor and pethoxamide usually give the most effective scentless mayweed control in winter rape. In case of control failure or the absence of preemergent treatment, herbicides containing clopyralid, picloram and halauxifen (synthetic auxins) can be applied after emergence during autumn or spring (Jursík et al. 2018).

Acetolactate synthase is a vital enzyme involved in the synthesis of the branched-chain amino acids valine, leucine and isoleucine (Duggleby and Pang 2000). Since the first cases of resistance against ALS-inhibiting herbicides, there has been a continuous rise

in this number (especially among the grass weeds). Herbicides that inhibit acetolactate synthase have the most reported cases of resistance worldwide, especially in grass weeds. They belong to the most prevalent herbicide group, and their application is increasing every year (Powles et al. 1997). Hence the emergence of new cases of resistance to this mode of action is expected in many weed species, including *T. inodorum*. The first case of the herbicide-resistant population in scentless mayweed to ALS inhibitors was reported in the United Kingdom (Moss et al. 2011). Since then, resistance in this weedy species has been described in seven other European countries (such as Denmark, the UK, Germany, Poland etc.). These populations have been discovered in the fields of wheat and spring barley and are resistant to the active ingredients florasulam, tribenuron and iodosulfuron (Heap 2022).

In the Czech Republic, resistant populations of *T. inodorum* against the above-mentioned active ingredients are yet to be found. The reason for this might be due to a lack of monitoring of resistance. Discovery of the mechanism of resistance might help the farmers in the Czech Republic/central Europe to implement the best management practices, before they become a serious threat. The main objectives of this study were to determine the occurrence of the scentless mayweed resistance in our country to ALS inhibitors (florasulam and tribenuron) and synthetic auxins (2,4-D and dicamba) and to identify the possible mechanisms of resistance.

## MATERIAL AND METHODS

**Plant material.** Twenty-eight samples of scentless mayweed (in the text as MATIN-No) were collected from winter wheat, spring barley and oilseed rape fields in different regions of the Czech Republic during 2019 and 2020. Fields where ALS inhibitors and synthetic auxins had been applied in the past and where more than 100 plants survived the ap-

plication were selected for sampling. Approximately one hundred random plants were taken from these survivors, and their seeds were used to form a mixed sample for the experiments. The sensitive control population was collected from the experimental field of the Czech University of Life Sciences Prague from a place where herbicides had never been applied in the past.

**Effect of registered and double rates and selection of resistant populations.** Pot experiments were conducted with four replicates per treatment in a randomised block design. *T. inodorum* seeds were sown in plastic pots (250 mL) containing chernozem soil with a clay content of 46%, soil pH<sub>KCl</sub> 7.5, sorption capacity of soil: 209 mmol<sub>+</sub>, 87 ppm P, 203 ppm K, 197 ppm Mg, and 8 073 ppm Ca. Emerged seedlings were thinned to ten plants/pot. The experiment was conducted under greenhouse conditions, under controlled temperature and light regime (16 h light at 20 °C and 8 h dark at 16 °C). The seedlings were fertilised (NPK 7-7-6) and watered when necessary. Four different herbicidal ingredients (two ALS inhibitors and two synthetic auxins) were selected: tribenuron (Nuance 750 g a.i. (active ingredient), Cheminova A/S), florasulam (Fragma, 50 g a.i., Cheminova A/S), 2,4-D (Esteron, 600 g a.i., Corteva Agriscience) and dicamba (Banvel 480 S, 480 g a.i., Syngenta Crop Protection AG). All herbicide treatments were applied at the 3–4 leaf stage with field recommended and double doses (Table 1). Herbicides were applied in a laboratory spray chamber equipped with a Lurmark 01F80 nozzle. The nozzle was calibrated to the spray volume of 250 L/ha at a spraying pressure of 250 kPa. Plants treated with water served as an untreated control. The visual plant injury scores were performed 30 days after application. Visible symptoms on surviving plants were expressed as a percentage where zero corresponded to no damage and 100% to dead plants.

**Dose-response assay.** A dose-response experiment was established for the estimation of effective dose (ED50) and resistance factors (RF) in two populations

Table 1. Doses of used active ingredients in whole-plant bioassay

Active ingredient (a.i.)	Site of action	1N (L a.i./ha, g a.i./ha)	2N (L a.i./ha, g a.i./ha)
Florasulam	Inhibition of acetolactate synthase	5	10
Tribenuron		11.25	22.5
2,4-D	Auxin mimics	420	840
Dicamba		288	576

1N – recommended field dose in the Czech Republic; 2N – double dose

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(MATIN-2 and MATIN-4) selected from a previous experiment in which resistance was observed. This experiment was conducted as described in the previous section. Nine increasing doses of florasulam and tribenuron were sprayed at the 3–4 leaf stage (Table 2). The whole experiment was conducted twice. Synthetic auxins were not used in the dose-response assay because there were no cases of resistance for 1N (recommended field dose) and 2N (double dose). Visual evaluation of treatment effects was conducted 28 days after application. Visible symptoms on surviving plants were expressed as percentage values (100% no survival, 0% plants without any visible damage) of the untreated control. Above-ground biomass from all the treatments was harvested to determine the production of shoot dry weight. Dry weight was recorded after drying the plants at 65 °C in an oven for 48 h when the plants were completely dried and moisture-free.

**Partial ALS gene sequencing.** The R populations (MATIN-2 and MATIN-4) were subjected to PCR analysis to detect target-site substitution of Pro197 and Trp-574 at the *ALS* gene. Ten leaves from each population were collected from the individual plants that survived the treatments and were sent to IDENTXX GmbH company (Stuttgart, Germany) for single nucleotide polymorphism analysis by pyrosequencing. The pyrosequencing reactions were carried out according to the manufacturer's instructions using PyroMark Q24 (Qiagen, Hilden, Germany) with sequence-specific primers and the PyroMark Q24 Gold Q24 Reagents (Qiagen, Hilden, Germany) according to Löbmann et al. (2021). Consequently, the genotypes were analysed using the supplied SNP Software (Qiagen, Hilden, Germany). Details of reactions and primers are under trade secret (IDENTXX GmbH Company). PyroMark Q24-software (v. 2.0.7) was used for further analysis.

**Statistical analysis.** R software version 3.6.3 (R Development Core Team) was used to analyse the data from the dose-response assay (Baty et al. 2015). Dose-response curves were obtained by nonlinear regression using a log-logistic equation:

$$f(x) = c + ((d - c)/(1 + \exp(b(\log(x) - \log(e)))))) \quad (1)$$

where:  $d$  – upper limit;  $c$  – lower limit;  $b$  – slope of the curve;  $e$  – inflection point of the curve. The four-parameter log-logistic function where the lower limit equals 0 was found to be the "best-fit" distribution for our data. The quality of each set of dose-response models was compared with an analysis of variance by a lack-of-fit  $F$ -test. The regression parameters and standard errors for each scentless mayweed population were obtained using the package *drc* in the statistical program R. The herbicide doses that caused the median effective dose (ED50) were computed for each herbicide and population. The resistance factor (RF) was calculated as a ratio of the ED50 of a test population to the ED50 of a susceptible standard.

## RESULTS AND DISCUSSIONS

**Effect of registered and double rates and selection of resistant populations.** Synthetic auxins with registered and double doses showed very high efficacy in all the populations in our experiment. The average efficacy values for 2,4-D and dicamba were 98% and 95% (Table 3) at the recommended doses, respectively, in 29 tested populations. In contrast to our findings, Kay reported in 1994 that scentless mayweed has a high innate tolerance to synthetic auxins, including active ingredients 2,4-D and MCPA. However, the resistance of scentless mayweed to this herbicide group has not been described in any recent studies, including ours; all tested samples in our study were sensitive.

In the case of florasulam, an ALS inhibitor, no resistance has been identified for any tested populations. The average efficacy value with the recommended dose ranged from 94–96% in all tested samples. The majority (~82%) of the populations tested were also susceptible to tribenuron, and the failure of herbicide efficacy in field conditions in these populations might occur for reasons other than herbicide resistance. However, a significant difference in the average efficacy value of tribenuron between the two populations (MATIN-2, MATIN-4)

Table 2. Increasing doses of herbicides used in a dose-response assay

Herbicide	0.00316N	0.01N	0.0316N	0.1N	0.316N	1N	3.16N	10N	31.6N
Florasulam (g a.i./ha)	0.058	0.050	0.158	0.500	1.580	5	15.800	50	158
Tribenuron (g a.i./ha)	0.036	0.126	0.355	1.125	3.555	11.250	35.550	112.500	355.500

1N – recommended field dose in the Czech Republic; xN – multiple of the recommended dose; a.i. – active ingredient

Table 3. Average efficacy of the recommended and double doses for the four active ingredients tested

Active ingredient (a.i.)	Average efficacy recommended dose	Average efficacy double dose
	(%)	
Florasulam	95	95
Tribenuron	86	88
2,4-D	98	100
Dicamba	95	98

and sensitive control was detected. Both MATIN-2 and MATIN-4 survived both a single and double dose of tribenuron. The mean survival rate of MATIN-2 and MATIN-4 was 60% and 55%, respectively. In line with our findings, Tiede et al. (2014) did not reveal the cross-resistance of tribenuron and florasulam on *T. inodorum*. However, they detected a reduced efficacy of tribenuron (only 13%) in two populations of closely related species, *Matricaria chamomilla* (L.) and *Matricaria recutita* (L.). Their results, as ours, could not confirm cross-resistance between florasulam and tribenuron because greenhouse trials confirmed full florasulam efficacy.

**Dose-response assays.** The dose-response analysis demonstrated that the ED<sub>50</sub> values in MATIN-2 and MATIN-4 were higher than for the susceptible

Table 4. Calculated ED<sub>50</sub> (effective dose, g a.i./ha) and RF (resistance factor) values from tribenuron dose-response assay for MATIN-2, MATIN-4 and the sensitive control

Populations	ED <sub>50</sub>	SE	CI	RF
MATIN-2	87.25	15.47	40.95–115.44	101.64
MATIN-4	20.36	6.28	11.80–32.54	23.73
MATIN-S	0.86	0.11	0.64–1.078	–

SE – standard error; CI – confidence interval (95%)

population (MATIN-S). The ED<sub>50</sub> value for MATIN-2 was calculated as 87.25 g a.i./ha, which is ~102 times higher than that of MATIN-S (ED<sub>50</sub> is 0.86), whereas the ED<sub>50</sub> value for MATIN-4 was calculated as 20.36 g a.i./ha, which is ~24 times higher than that of MATIN-S (Table 4 and Figure1). We noted plants that survived a 10-fold dose with almost no damage in both of these populations. In a study conducted by De Mol et al. (2015), herbicide sensitivity with florasulam and tribenuron was tested in dose-response experiments for *T. inodorum* collected from three different European countries (Denmark, Germany and Poland). According to their studies, for tribenuron, RF values ranged from 0.24 to 2.71 (De Mol et al. 2015). In another study conducted by Pannacci et al. (2010), the ED<sub>50</sub> values for tribenuron applied

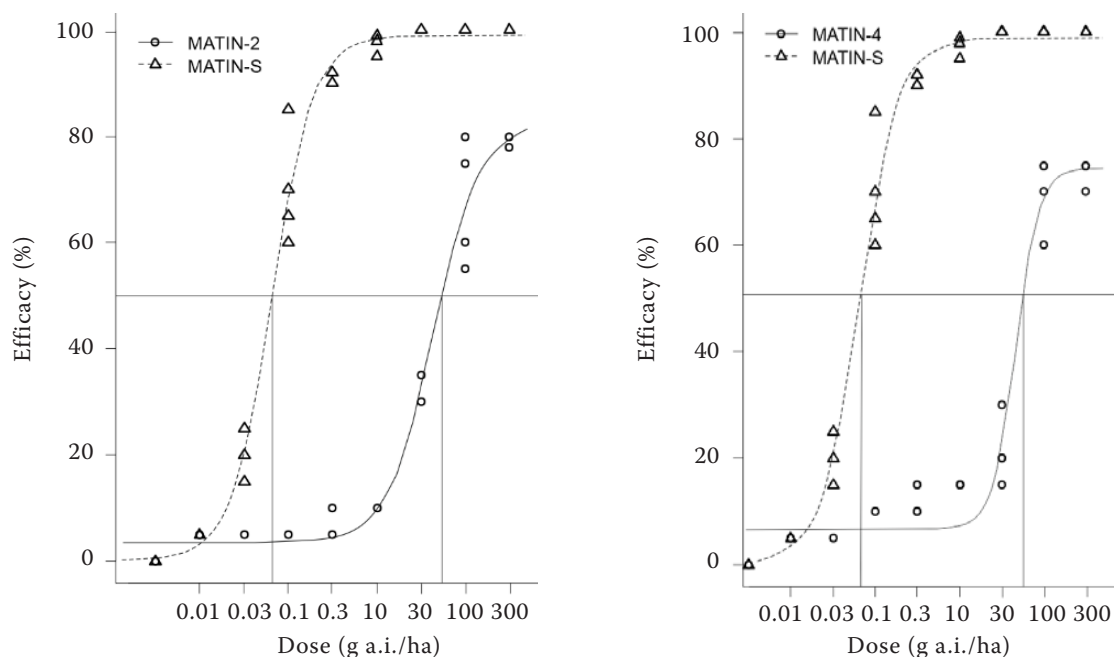


Figure 1. Dose-response curves for survival (efficacy %) of sensitive (MATIN-S) and test populations (MATIN-2, MATIN-4) of *Triplerospermum inodorum* treated with a range of dose rates covering from 0.00316 – 31.6x recommended field rate of tribenuron. a.i. – active ingredient



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to the scentless mayweed population from Denmark were found to be 0.13 g a.i./ha. Similarly, with the same herbicide-weed combination, Sønderskov et al. (2012) found ED50 values of 0.24 and 2.02 g a.i./ha in two pot experiments. As compared to these results, both the ED50 and RF values in our experiment are very high. This is an early indication for the farmers in the Czech Republic. Furthermore, high values of ED50 in our study may suggest that the resistance of these population types is due to target-site resistance. Our assumption is supported by the results of Ulber (2014), who in her study describes a population that survived a 16-fold dose of tribenuron than recommended and a very high value of RF and the study of Löbmann (2019), who confirmed this mechanism of herbicide resistance in molecular genetic studies. The differences between the ED50 values determined in other studies and the data shown here can be explained by differences in the herbicide resistance mechanism (e.g. non-target-site mechanisms or ALS gene over-expression) or experimental conditions and require further study. In addition to the NTSR mechanisms, ploidy level disparity between the R and S might also play a key role on herbicide resistance appearance in scentless mayweed populations. Polyploidisation offers several advantages to the new phenotypes and helps the novel phenotypes adapt easily to rapid environmental changes (Rutland et al. 2021, Tossi et al. 2022). One of these benefits might be increased resistance to herbicide stress in the R populations. Earlier, glyphosate resistance (due to mutations) had been detected in many weedy polyploid species like *Poa annua* L., *Echinochloa colona* (L.) Link, *Echinochloa crus-galli* (L.) P. B., etc. (Rutland et al. 2021). To date, there are no reports of the effect of ploidy level on the appearance of herbicide resistance in scentless mayweed. However, *T. inodorum* might occur in fields as diploid, tetraploid or mixed populations exhibiting varying temporal stabilities (Čertner et al. 2017). TSR mechanisms are more prevalent in polyploid weeds since the polyploids are expected to have a more flexible expression profile (Rutland et al. 2021). Nevertheless, these hypotheses will require further investigation.

**Partial ALS gene sequencing.** Herbicide-resistance mechanisms can be either target-site resistance (TSR) based or non-target-site resistance (NTSR) based. However, both TSR and NTSR can coexist within the same weedy species. Amino acid substitution in the ALS protein (domains A-E) is considered as the most common TSR mechanism (Park et al. 2004). In weed populations, either single or multiple amino

acid changes might result in resistance to ALS inhibitors (Tranel et al. 2021). Currently, substitutions at eight different sites (Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654) have been confirmed to confer resistance to ALS inhibitors (Sen et al. 2021, Tranel et al. 2021). Among these, Pro197 and Trp574 are the two most commonly reported positions. To elucidate the molecular mechanism of resistance, single nucleotide polymorphism (SNP) at Pro197 and Trp574 was analysed by pyrosequencing. No SNP at the Trp-574 position resulting in another amino acid substitution was detected in the R populations. However, comparison among the R and S population confirmed substitution at Pro197 in both MATIN-2 and MATIN-4. Pro197 can be substituted by many different types of amino acids in different species, such as Pro197Thr in *Bassia scoparia* (L.) A. J. Scott (Guttieri et al. 1995), Pro197His in *Papaver rhoeas* L. (Yu et al. 2003), Pro197Ser in *Bromus japonicus* Thunb. (Lan et al. 2022) etc. In our case, for MATIN-2, we found Pro197Ser, and for MATIN-4, we detected Pro197Gln, all plants tested were heterozygous. Identified SNP was founded in all surviving MATIN-2 and MATIN-4 plants that were partially sequenced, thus confirming target site resistance. Previously, substitution at the Pro197 position was also found in mayweed plants by Tiede et al. (2014). Although no Trp574 mutation was found here, ALS mutation at the 574<sup>th</sup> position has been identified in other weed species, like *Lithospermum arvense* (L.) I. M. Johnst. (Wang et al. 2019), *Sinapis arvensis* L. (Sin and Kadioglu 2021), *Apera spica-venti* (L.) P. Beauv. (Košnarová et al. 2021) etc. In addition to SNP, non-target resistance mechanisms might also play a role in conferring the resistance. However, this will require further investigation (Gaines et al. 2020).

In summary, this study confirms that the first resistance *T. inodorum* populations occur in the Czech Republic, and it is due to target-site substitution of Pro197 at the *ALS* gene. Our findings are an early warning for the farmers, and we strictly recommend using alternative herbicides in integrated *T. inodorum* management to impede the probable evolution of resistance to this herbicide group, e.g. synthetic auxins, which in our study confirmed high efficacy and no occurrence of resistance. Monitoring and detection of herbicide resistance in problematic weed species, including scentless mayweed, will help slow the development of herbicide resistance and support the implementation of strategies to rapidly manage the problem and reduce its economic impact.

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