Sektion 5: Resistenzanalyse

Session 5: Resistance analysis

Resistance patterns of a bur chervil (*Anthriscus caucalis* M. Bieb.) population with a point mutation within the acetolactate synthase gene in comparison with sensitive bur chervil and further weedy Apiaceae species

Resistenzmuster einer Hundskerbel- (Anthriscus caucalis M. Bieb.) Population mit Punktmutation innerhalb des Acetolactat-Synthase-Gens im Vergleich mit sensitivem Hundskerbel und weiteren Schaddoldenblütlern

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Abstract

Weeds of the umbelliferous family (Apiaceae) are gaining importance in several crops and regions and are often difficult to control chemically. For example, the occurrence of bur chervil (*Anthriscus caucalis* M. Bieb.) is not only increasing as a ruderal plant, but is also establishing as an arable weed in winter crops. There, it is often not sufficiently controlled by standard measures and beyond that frequently confused with other weedy umbellifers. In addition, the first *A. caucalis* populations with resistance to inhibitors of the acetolactate synthase (ALS) have been described. For this reason, we sequenced the ALS gene of a sensitive and a resistant *A. caucalis* population and found a mutation at position Pro197. This population was compared with a sensitive one, as well as other umbellifers such as hedge parsley (*Torilis arvensis* (Huds.) Link), wild carrot (*Daucus carota* L.) and hemlock (*Conium maculatum* L.) in a greenhouse trial with 14 herbicides. There were clear differences between the treatments of sensitive and resistant *A. caucalis* and the other umbellifers. The sensitive *A. caucalis* population was effectively controlled by the ALS inhibitors thifensulfuron, tribenuron and metsulfuron, but not by florasulam. Of these herbicides, only metsulfuron had a partial efficacy on the resistant population with 71.8% freshwater reduction. Notably, the efficacy profiles of ALS inhibitors differed significantly between species, while several synthetic auxins and chlortoluron effectively controlled most populations tested. Finally, the correct identification of the umbellifer species, as well as the resistance status, is necessary to carry out targeted chemical control.

Keywords: Acetolactate synthase, Apiaceae, bur chervil, herbicide resistance

Zusammenfassung

Unkräuter der Familie der Doldenblütler (Apiaceae) gewinnen in einigen Kulturen und Regionen immer mehr an Bedeutung und sind chemisch z.T. nur schwer zu bekämpfen. Beispielsweise nimmt der Hundskerbel (*Anthriscus caucalis* M. Bieb.) nicht nur als Ruderalpflanze zu, sondern etabliert sich auch als Ackerunkraut in Winterungen. Er wird dort durch Standardmaßnahmen häufig nicht ausreichend erfasst und darüber hinaus gelegentlich mit anderen Doldenblütlern verwechselt. Zudem wurden erste Hundskerbelpopulationen mit Resistenz gegen Hemmer der Acetolactatsynthase (ALS) beschrieben. Aus diesem Grund haben wir das ALS-Gen einer sensitiven und einer resistenten Hundskerbelpopulation

sequenziert und eine Mutation an der Position Pro197 festgestellt. Diese Population wurde im Vergleich mit einer sensitiven sowie mit Klettenkerbel (*Torilis arvensis* (Huds.) Link), wilder Möhre (*Daucus carota* L.) und geflecktem Schierling (*Conium maculatum* L.) untersucht. Dabei zeigten sich deutliche Unterschiede zwischen den Behandlungen von sensitivem und resistentem Hundskerbel und den anderen Doldenblütlern. Die sensitive Hundskerbelpopulation wurde effektiv durch die ALS-Hemmer Thifensulfuron, Tribenuron und Metsulfuron bekämpft, jedoch nicht durch Florasulam. Von diesen Herbiziden hatte lediglich Metsulfuron mit 71,8 % Frischmassereduktion eine Teilwirksamkeit auf die resistente Population. Vor allem die Wirksamkeitsprofile der ALS-Hemmer unterschieden sich maßgeblich zwischen den Arten, während mehrere synthetische Auxine und Chlortoluron die meisten getesteten Populationen effektiv kontrollierten. Schlussendlich ist die korrekte Identifikation der Doldenblütlerarten sowie des Resistenzstatus notwendig, um eine gezielte chemische Kontrolle durchführen zu können.

Stichwörter: Acetolactatsynthase, Apiaceae, Herbizidresistenz, Hundskerbel

Introduction

Bur chervil (*Anthriscus caucalis* M. Bieb.) is a native winterannual weed species which has been increasing for about one decade on ruderal and arable land with high proportions of winter crops such as wheat or oilseed rape (METZING et al., 2018). This Apiaceae species survives (mild) winters and its bur-covered fruits allow effective zoochorous dispersal. The fruits ripen from June to July (BRANDES, 2007) and thus typically start enriching the seedbank already before harvest. The weed is often not controlled with standard weed management strategies, which is particularly problematic as it is frequently not recognised when it first appears in the field. However, correct identification of umbelliferious weeds faciliates the choice of effective weed control measurements e.g., herbicides. Despite morphological similarities, their seedlings differ in their cotyledon and leaf shapes, venation and trichome density. Additionally, the different species comprise various seed shapes, and also the distribution of weedy umbellifers across Germany varies considerably from species to species (Fig. 1).

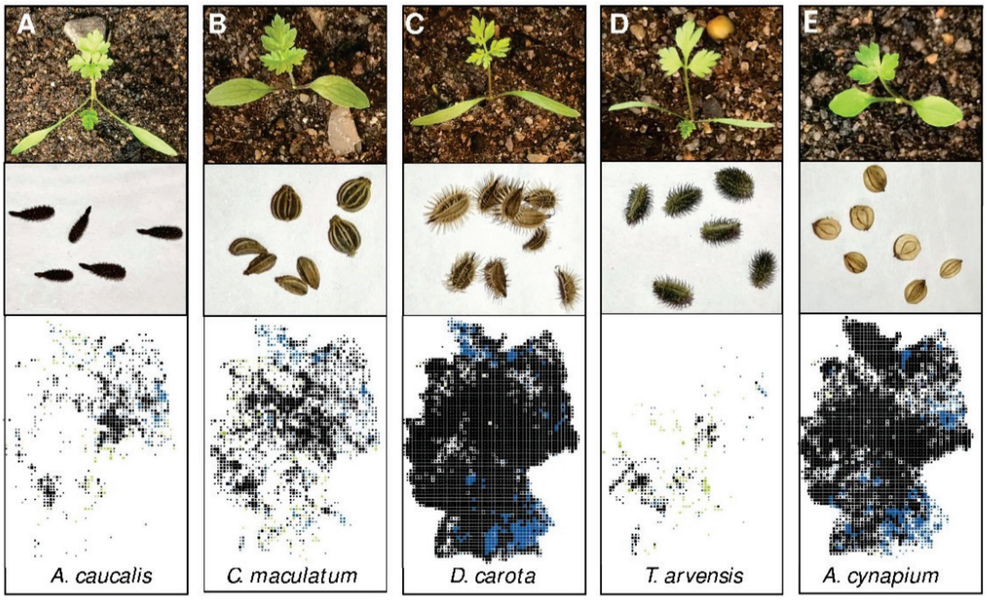

Figure 1 Seedlings (top), fruits (middle) and distribution across Germany (bottom) of *Anthriscus caucalis* (A), *Conium maculatum* (B), *Daucus carota* (C), *Torilis arvensis* (D) and *Aethusa cynapium* (E). Distribution before 1950 (■, ▲), between 1950 and 1980 (■, ▲), and after 1980 (■, ▲) is indicated by squares for native occurrence and by triangle in case of unsteady occurrence (modified with kind permission of BfN, 2021). The symbol size reflects the number of quadrants in which individuals were found.

Abbildung 1 *Keimlinge (oben), Früchte (mittig) und deutschlandweite Verbreitung (unten) von* Anthriscus caucalis *(A),* Conium maculatum *(B),* Daucus carota *(C),* Torilis arvensis *(D) und* Aethusa cynapium *(E). Verbreitung vor 1950 (■, ▲),* *zwischen 1950 and 1980 (■, ▲), und nach 1980 (■, ▲) wird bei einheimischem Vorkommen durch Quadrate und im Fall von unbeständigem Vorkommen durch Dreiecke angezeigt (modifiziert mit freundlicher Genehmigung des BfN, 2021). Die Größe der Symbole gibt die Anzahl der Quadranten an, in denen Individuen gefunden wurden.*

However, in addition to general gaps in effectiveness, in some cases, resistance to inhibitors of the acetolactate synthase (ALS) also complicates control of *A. caucalis*. Therefore, in the present study, the Pro197 position within the ALS gene of a resistant *A. caucalis* population has been investigated. Then, the efficacy of 14 herbicides with different modes of action was examined on the resistant and sensitive *A. caucalis* populations in the greenhouse. In addition, the efficacy of these herbicides was compared with other umbellifers relevant as weeds.

Material and methods

The seed material of an ALS-sensitive (ANRCA S) and an ALS-resistant (ANRCA R) *A. caucalis* population was collected in different fields in Germany in 2017. The DNA of the *A. caucalis* populations was extracted using the CTAB method as described by BRANDFASS and KARLOVSKY (2008) and subsequently sequenced by Sanger sequencing . A pairwise sequence alignment was conducted using the Emboss Needle method (MADEIRA et al., 2019). In 2020 and 2021, seeds of *Aethusa cynapium* L. (fool's parsley), *Conium maculatum* L. (hemlock), *Daucus carota* L. (wild carrot) and *Torilis arvensis* (Huds.) Link (hedge parsley) were collected in different fields across Germany. The seeds were sown in seed trays containing a 3:3:1 mixture of compost, potting soil (Hawita, Vechta, Germany) and sand with 0.5 g NPK (Mg) 16-9-12 (2) pelleted fertilizer L^{-1} and kept at 5°C for five to eight days, depending on the expected speed of germination. Each four seedlings per 9 cm pot containing the same soil mixture were transplanted under greenhouse conditions at the cotyledon stage, except for *A. cynapium* which did not germinate due to dormancy. For each experiment, three pots per population were prepared.
In a first approach, each population was treated at BBCH 11 with dose rates of different herbicides at dose rates relevant for arable crops (see Tab. 1). For the application (three bar), a laboratory sprayer with an ES 90-04 nozzle (Lechler, Metzingen, Germany) was used. The fresh weight of the treated plants and twelve untreated plants per population was assessed four weeks after application. The experiment was repeated with additional dose rates of clomazone, isoxaben and metazachlor (see Tab. 1). Data of both experiments were pooled. Subsequently, the data were visualized with the 'ggplot' package in R (version 4.0.0, (R CORE TEAM, 2021).

In a second approach, the *A. caucalis* populations ANRCA S and ANRCA R were pregerminated and transplanted as described. Subsequently, the plants were treated with the ALS-inhibitors metsulfuron, tribenuron, thifensulfuron and florasulam with the dose rates displayed in Table 1 and four additional dilutions of a 1:4 dilution series. The experiment was repeated. The plants were harvested as described above and the data were log-transformed prior to analysis with the three-parameter log-logistic model (see Form. 1) and the 'confint()' function of the 'drc' package in R (version 4.0.0, RITZ et al., 2015; R CORE TEAM, 2021). Resistance factors were calculated based on the quotient of effective dose rates leading to 50% fresh weight reduction.

Formula 1 Log-logistic function for dose-response analysis with 0 as lower asymptote.

***Formel 1** Log-logistische Funktion für Dosis-Wirkungsanalysen mit 0 als untere Asymptote.*

$$f(x) = 0 + \frac{d - 0}{1 + \exp(b(\lg(x) -)}$$

Table 1 Herbicides with corresponding active ingredients, mode of action and applied rate

***Tabelle 1** Herbizide mit entsprechenden Wirkstoffen, Wirkungsmechanismus und Anwendungsrate*

Herbicide	Active ingredient (a.i.)	Mode of action (HRAC class)	g a.i. ha^{-1}
Gropper	Metsulfuron	ALS (2)	5.8
Pointer	Tribenuron	ALS (2)	28.9
Harmony	Thifensulfuron	ALS (2)	7.2
Primus	Florasulam	ALS (2)	6.3
Kerb FLO	Propyzamide	Microtubule assembly (3)	750.0
GF2573	Halauxifen	Auxin mimics (4)	75.0
Starane	Fluroxypyr	Auxin mimics (4)	180.0
Lontrel 600	Clopyralid	Auxin mimics (4)	120.0
CTU 700	Chlorotoluron	PS II (5)	2100.0
Fuego Top	Metazachlor+quinmerac	Auxin mimics (4)+VLCFA (15)	1000.0
Butisan	Metazachlor	VLCFA (15)	750.0
Cadou	Flufenacet	VLCFA (15)	254.0
Clomazone	Clomazone	DOXP (13)	118.8
Flexidor	Isoxaben	Cellulose synthesis (29)	500.0

Results and discussion

Bur chervil (*A. caucalis*) is an increasingly common weed in arable and ruderal habitats (BRANDES, 2007; METZING et al., 2018), which is adapted to oceanic climate, moderate temperature, pH, moisture, nitrogen levels and light and/or dry soil (BRANDES, 2007; ELLENBERG, 1991). As *A. caucalis* has been described to survive mild winters especially well (BRANDES, 2007; ELLENBERG, 1991), the change in climate over the past decades may be one possible reason for its more frequent occurrence in arable and ruderal areas. However, altered genetics are another possible reason. In arable fields, the loss of active ingredients such as chlortoluron, as well as reduced tillage could play a role in the spread of the species. Where *A. caucalis* occurs in arable fields, it is difficult to control with herbicides and especially prevalent in winter crops. In addition, ALS resistance has been identified in some populations in the past (ULBER and ZHANG, 2021). However, to date, no scientific literature is available on the mutation and potential cross-resistances. Therefore, in the present study, the sequence of the ALS gene in a sensitive and a resistant *A. caucalis* population was investigated. Additionally, the efficacy of different ALS-inhibitors and herbicides of several other modes of action in the control of the two populations were investigated in comparison with other umbellifers such as *C. maculatum*, *D. carota* and *T. arvensis*. Indeed, a substitution of proline to serine at position 197 of the ALS gene was confirmed and linked to the initially observed reduced effect of ALS inhibitors (Fig. 2).

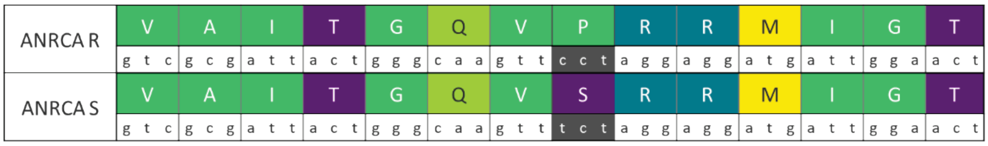

Figure 2 Amino acid (upper case, colored by physico-chemical characteristics) and nucleotide (lower case) sequence of the *ALS* gene of resistant (ANRCA R) and sensitive (ANRCA S) *Anthriscus caucalis* around the position Pro197 (dark shading of the nucleotide sequence).

***Abbildung 2** Aminosäure- (Großbuchstaben, nach physikalisch-chemikalischen Eigenschaften gefärbt) und Nukleotidsequenz (Kleinbuchstaben) des ALS-Gens von resistentem (ANRCA R) und sensitivem (ANRCA S)* Anthriscus caucalis *um die Position Pro197 (dunkle Schattierung der Nukleotidsequenz).*

This allele variation was first described in 1995 (GUTTIERI et al.) in a biotype of *Kochia scoparia* and responsible for resistance to various ALS inhibitors. *In vitro* activity assays and modeling of the inhibition of ALS by herbicides show that the replacement of the amino acid proline at position 197 by serine has a significant influence on the binding of various ALS inhibitors (LIU et al., 2019). In the herbicide trials conducted, the ALS inhibitors metsulfuron, tribenuron, thifensulfuron and florasulam showed a clearly reduced effect in the control of the *A. caucalis* population with the mutation. Yet, because of high variance differences between both populations were not significant in the dose-response assays despite the resistance factors of 2, 6, 7 and 28 for florasulam, tribenuron, metsulfuron and thifensulfuron, respectively (Fig. 4). However, metsulfuron achieved at least a partial effect with a fresh weight reduction to 28.2 ± 4.3% of the untreated control (Fig. 3 A). Under greenhouse conditions the sensitive population was well controlled with metsulfuron, tribenuron and thifensulfuron, but not with florasulam. The recommendations of the official extension services (LANDWIRTSCHAFTSKAMMER NRW, 2021; LANDWIRTSCHAFTSKAMMER SCHLESWIG-HOLSTEIN, 2021), however, attribute good efficacy to metsulfuron in particular (especially in combined products). Both biotypes of *A. caucalis* were similarly controlled with herbicides of alternative modes of action, indicating no other mechanism of resistance is present in the resistant biotype. The control with isoxaben (cellulose inhibition), flufenacet (inhibition of VLCFA biosynthesis), clomazone (DOXP inhibition) and propyzamide (inhibition of microtubule assembly) was insufficient (<90% efficacy) for both *A. caucalis* populations. However, the efficacy of propyzamide may have been further diminished by application under warm greenhouse conditions, as it generally benefits from temperatures >10°C (AUGUSTIN and HÜRSGEN, 2008). Good control of all populations of the different species tested was achieved with the photosystem II-specific inhibitor chlortoluron (not applicable on drained fields) and the auxin mimics such as fluroxypyr, which is in accordance with the efficacy rates of fluroxypyr described by NICKE and GLATTKOWSKI (2018).

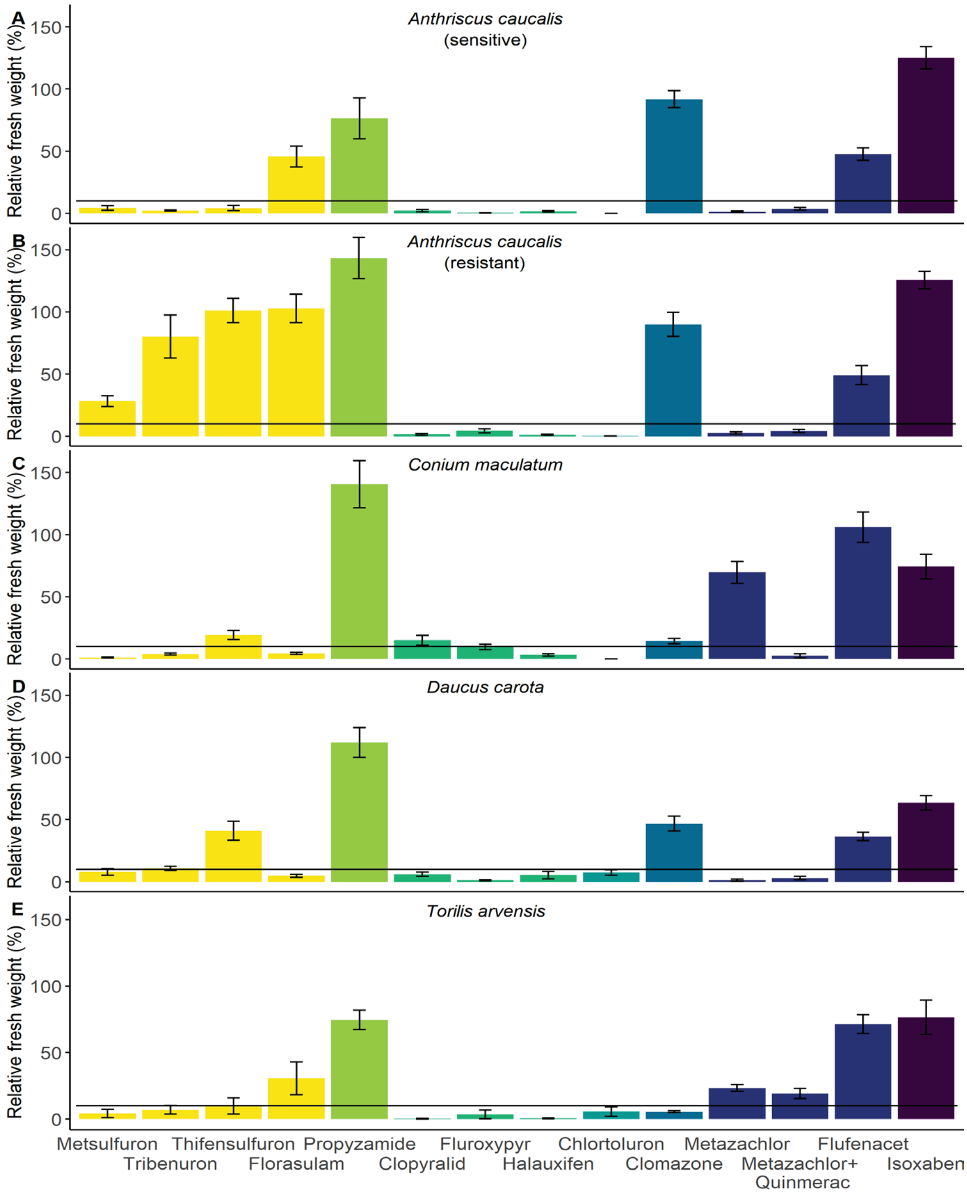

Figure 3 Relative fresh weight of sensitive (A) and resistant (B) *Anthriscus caucalis, Conium maculatum* (C*), Daucus carota* (D) and *Torilis arvensis* (E) four weeks after application with the herbicides of the HRAC groups 2 (▮), 3 (▮), 4 (▮), 5(▮),13 (▮), 15 and 15+4 (▮) and 29 (▮), n=24. Standard errors are indicated by error bars. Horizontal black lines indicate 90% fresh weight reduction.

Abbildung 3 *Relatives Frischgewicht von sensitivem (A) und resistentem (B)* Anthriscus caucalis, Conium maculatum *(C),* Daucus carota *(D) und* Torilis arvensis *(E) vier Wochen nach der Behandlung mit Herbiziden der HRAC Gruppen 2 (▮), 3 (▮), 4 (▮), 5(▮),13 (▮), 15 und 15+4 (▮) und 29 (▮), n=24. Fehlerbalken zeigen Standardfehler. Schwarze, horizontale Linien zeigen 90 % Frischmassereduktion.*

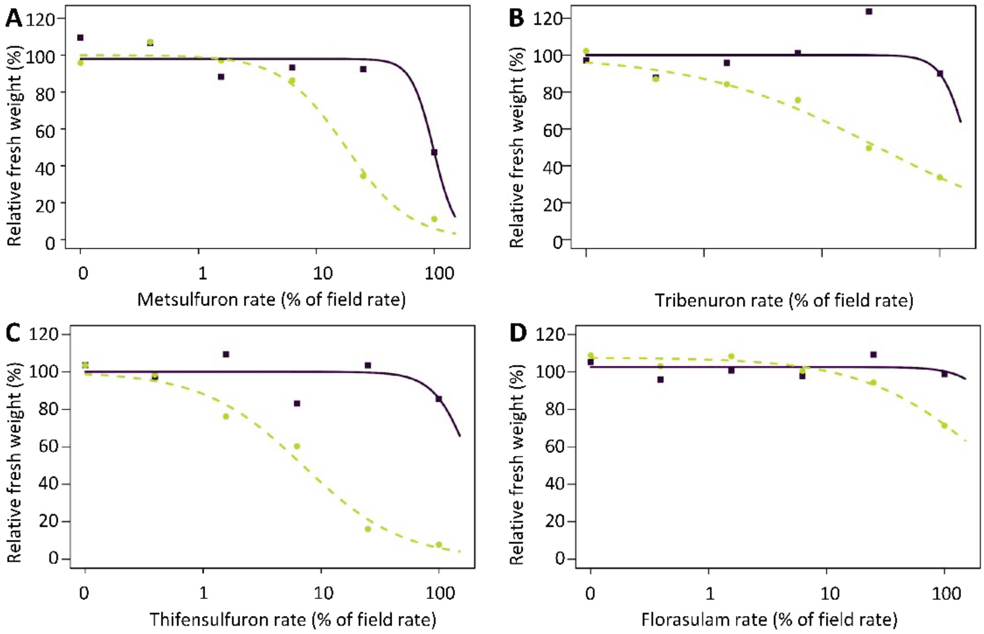

Figure 4 Response of sensitive (●, ANRCA S) and resistant (■, ANRCA R) *Anthriscus caucalis* to different dose rates of Metsulfuron (A), Tribenuron (B), Thifensulfuron (C) and Florasulam (D).

***Abbildung 4** Reaktion von sensitivem (●, ANRCA S) und resistentem (■, ANRCA S)* Anthriscus caucalis *auf verschiedene Dosierungen von Metsulfuron (A), Tribenuron (B), Thifensulfuron (C) und Florasulam (D).*

However, as synthetic auxins often have higher efficacies at warm temperatures (GROSSMAN, 1998; REED and MCCULLOUGH, 2012), those herbicides may perform less good under field conditions. In contrast to *A. caucalis*, *C. maculatum* was less good controlled with the synthetic auxin clopyralid, metazachlor (inhibition of VLCFAs) and thifensulfuron (inhibition of ALS), while the ALS inhibitor florasulam led to sufficient control with 95.5% efficacy on average. Additionally, clomazone had a partial effect on this species. *D. carota* showed a similar pattern like *C. maculatum*, however, it was better controlled with metazachlor and less good with chlortoluron. *T. arvensis* showed a similar pattern like the sensitive *A. caucalis* population, however, it was better controlled with clomazone and less good with metazachlor. Particularly as good or very good control rates are rarely observed under practical conditions, the correct identification and effective combination and timing of chemical measures is crucial for effective control of weedy Apiaceae species. However, as chemical approaches alone increase the pressure for selection of resistant populations, cultural control measures such as increased proportions of spring crops in the crop rotation could be a particularly effective tool for the control of winter annuals such as *A. caucalis*.

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