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Article

Galium spurium AND *G. aparine* RESISTANCE TO ALS-INHIBITING HERBICIDES IN NORTHERN GREECE

Resistência de Galium spurium e G. aparine a Herbicidas Inibidores da ALS no Norte da Grécia

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ABSTRACT - Knowledge of the level of resistance of weed populations and the herbicides to which they survive is important for recommending suitable advice to farmers and allowing the selection of appropriate management strategies. Whole-plant dose response experiments were carried out to assess the resistance status of eight putative resistant *Galium spurium* L. populations and one *G. aparine* L. population, originating from northern Greece. High levels of resistance of both species to the ALS-inhibiting herbicides chlorsulfuron and tribenuron were found, while their susceptible populations were controlled. Three *G. spurium* (GS) populations showed additional cross-resistance to [florasulam + 2,4-D], whereas the remaining five resistant GS populations were controlled with [tribenuron + mecoprop-p], [florasulam + 2,4-D], and [florasulam + aminopyralid]. Also, [florasulam + fluroxypyr] was very effective against two resistant GS populations tested. DNA sequence alignment of the three GS populations (GS 1, GS 6, and GS 8) with cross-resistance to chlorsulfuron, tribenuron, and florasulam revealed a point mutation at Trp-574 (tryptophan-574), causing amino acid substitution by Leu (leucine). The *G. aparine* (GA) population showed cross-resistance to chlorsulfuron and tribenuron, but it was controlled with [tribenuron + mecoprop-p], [florasulam + aminopyralid], [florasulam + 2,4-D], and [florasulam + fluroxypyr]. The confirmed cross-resistance of both GS and GA species to chlorsulfuron and tribenuron in northern Greece is the first report of *Galium* spp. resistance to ALS-inhibiting herbicides in Europe. Finally, all populations (8 GS and 1 GA) that showed resistance to chlorsulfuron and tribenuron were controlled with the mixtures [tribenuron + mecoprop-p] and [florasulam + fluroxypyr].

Keywords: ALS gene sequencing, cross-resistance, target-site mutation.

*RESUMO - O conhecimento do nível de resistência das populações de plantas daninhas e dos herbicidas aos quais eles sobrevivem é importante para recomendar conselhos adequados aos agricultores e permitir a seleção de estratégias de gestão apropriadas. Experimentos de resposta de plantas inteiras foram realizados para estudar os níveis de resistência de oito populações supostamente resistentes de **Galium spurium** L. e uma população de **G. aparine** L., originários do norte da Grécia. Altos níveis de resistência de ambas as espécies aos herbicidas inibidores de ALS clorsulfuron e tribenuron foram encontrados, enquanto suas populações suscetíveis foram controladas. Três populações de **G. spurium** (GS) mostraram resistência cruzada adicional a [florasulam + 2,4-D], enquanto as cinco populações restantes de GS resistentes foram totalmente controladas com [tribenuron + mecoprop-p], [florasulam + 2,4-D] e [florasulam + aminopyralid]. Além disso, o tratamento com [florasulam + fluroxypyr] foi muito eficaz contra*

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duas populações GS resistentes testadas. A sequência de DNA das três populações GS com resistência cruzada a clorsulfuron, tribenuron e florasulam revelou uma mutação pontual na posição Trp-574 (triptofano-574), causando a substituição de aminoácidos por Leu (leucina). A suposta população de ***G. aparine*** *(GA) apresentou resistência cruzada a clorsulfuron e a tribenuron, mas foi controlada com [tribenuron + mecoprop-p], [florasulam + aminopyralid], [florasulam + 2,4-D] e [florasulam + fluroxypyr]. A resistência cruzada confirmada das espécies GS e GA ao clorsulfuron e tribenuron no norte da Grécia é o primeiro relato de resistência de* ***Galium*** *spp. a herbicidas inibidores da ALS na Europa. Finalmente, todas as populações (8 GS and 1 GA) que apresentaram resistência foram controladas com as misturas [tribenuron + mecoprop-p] e [florasulam + fluroxypyr].*

Palavras-chave: sequenciamento do gene ALS, resistência cruzada, mutação no sítio-alvo.

INTRODUCTION

Galium spp., such as *G. spurium* and *G. aparine*, are annual broadleaf weeds with reproduction by seed (Malik et al., 1988). Both species are native to Eurasia, whereas *G. aparine* is also native to North America (Malik et al., 1988; Defelice, 2002). *Galium aparine* occurs in temperate zones and in high altitudes in the tropics (Holm et al., 1978), whereas *G. spurium* is widely distributed in Europe (Hanf, 1983). *G. aparine* is found in arable fields and native habitats, waste ground, fence rows, barnyards, and pastures (Moore, 1975). This weed can grow in various soil types, but it is mostly found in nutrient-rich, deep, loamy and clayey soils, containing humus, and moist habitats (Holm et al., 1978). On the other hand, *G. spurium* prefers dry and sunny habitats and does not tolerate shade (Malik et al., 1988).

Galium spp. are among the most common and important annual broadleaf weeds in winter cereals (mainly wheat) grown throughout the world and in Greece (Malik et al., 1988). The three *Galium* spp., which commonly occur in Greece, are *G. spurium* L., *G. aparine* L., and *G. tricornutum* Dandy. *G. spurium* is the predominant *Galium* species, infesting winter cereals in Greece (Giannopolitis, 1982), along with corn poppy (*Papaver rhoeas* L.) and wild mustard (*Sinapis arvensis* L.). *Galium* spp. share similar morphological characteristics, which makes them difficult to distinguish, particularly at the vegetative stage (Malik et al., 1988). *Galium* spp. are very competitive with wheat causing high yield losses, especially as N inputs increases (Baylis and Watkinson, 1991). The *Galium* plants compete effectively with and cause substantial losses (30-60%) in cereal crops, as they grow over the top of cereal canopies, causing lodging, delaying harvest, resulting in serious interference with mechanical harvesting operations, and reducing marketable yield by 30-60% (Froud-Williams, 1985).

Several acetolactate synthase (ALS) inhibitors (mainly chlorsulfuron, tribenuron, and florasulam) have been widely used to control *Galium* spp. in cereals for the past 20 years. Moreover, formulated mixtures of ALS-inhibiting herbicides with auxin-type herbicides (e.g., [tribenuron + mecoprop-p], [florasulam + 2,4-D], [florasulam + aminopyralid], [florasulam + fluroxypyr], [florasulam + dicamba]) are currently used to manage populations of serious common weed species (corn poppy, wild mustard) infesting winter cereals (mostly wheat) grown in Greece that display reduced levels of control with the use of ALS-inhibiting herbicides (Kaloumenos et al., 2009, 2011; Ntoanidou et al., 2017).

ALS (EC 2.2.1.6) is a key enzyme in the biosynthesis of the essentials branched-chain amino acids valine, leucine, and isoleucine (Duggleby and Pang, 2000). This enzyme is the target site of herbicides belonging to five distinct chemical families: sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl-benzoates (thio- and oxy-benzoates), and sulfonyl-aminocarbonyl-triazolinones (Yu et al., 2010). Sensitive plants show various injury symptoms by the ALS-inhibiting herbicides (reduction of plant growth, shortening of internodes, purplish foliage, and shortening of lateral roots), resulting in plant death, caused by deficiency in branched-chain amino acids or by production and build-up of toxic compounds like α-amino-butyrate and α-ketobutyrate (Van Eerd et al., 2004).

ALS-inhibiting herbicides are used in all major agronomic crops and have been widely adopted due to their low dose rate and high efficacy against a broad spectrum of weeds, relatively low

mammalian toxicity, mild toxicological profile, and excellent crop selectivity (Whitcomb, 1999). However, the widespread use of ALS-inhibiting herbicides led to rapid selection of many resistant weed populations. ALS-resistant weeds represent the fastest-growing group of herbicide-resistant weeds worldwide, with 159 monocot and dicot related weeds (Heap, 2018). Herbicides with this specific site of action can select resistant weed biotypes when the initial weed population is treated with lesser than ten applications (Beckie and Tardif, 2012). The herbicide resistance follows two groups: the target-site resistance mechanisms and the non-target site resistance mechanisms. The target-site mechanisms of resistance are due to mutations encounter in the gene that encodes a target enzyme (Yu and Powles, 2014a), while the non-target site mechanisms occur by reduced absorption and translocation or by enhanced metabolism of the herbicide through cytochrome P450 monoxygenases (Yu and Powles, 2014b). Non-target site mechanisms can entail resistance to ALS-inhibitors along with other herbicides with diverse modes of action. Field-selected resistance in most weed species is conferred by a modified ALS coding gene, resulting from a single nucleotide change or point mutation, which renders the enzyme insensitive to the herbicide (Tranel and Wright, 2002). So far, 26 resistance-endowing amino acid substitutions have been identified at eight sites across the ALS enzyme (Yu and Powles, 2014b). The most frequently identified amino acid substitutions are at Pro-197 and Trp-574-Leu (Yu and Powles, 2014a).

Published research on *Galium* spp. resistance is limited. In Canada, high levels of resistance to ALS inhibitors triasulfuron, thifensulfuron plus tribenuron, and sulfometuron, whereas moderate levels of resistance to imazethapyr with simultaneous resistance to the auxin herbicide quinclorac have been reported in a biotype of *G. spurium* (Hall et al., 1998; Van Eerd, 2004; Van Eerd et al., 2005; Beckie and Tardif, 2012). A *G. aparine* population resistant to tribenuron-methyl has been reported in winter wheat fields of China (Sun et al., 2011). Reports of *Galium* spp. resistance to other ALS-inhibitors and the auxin herbicide fluroxypyr also exist in Canada (Heap, 2018). There are no confirmed cases of *Galium* spp. resistance in Europe. However, numerous growers of winter cereals in central and northwestern Greece expressed severe concerns of unsatisfactory control of *G. spurium* and *G. aparine* populations with the use of chlorsulfuron and other ALS-inhibiting herbicides. Knowledge of the level of resistance of weed populations and the herbicides to which they are resistant is important for offering adequate advice to farmers and allowing the selection of suitable management strategies.

The aim of this research was to examine whether: 1) the poor control of *G. spurium* and *G. aparine* in northern Greece can account for development of cross-resistance to ALS-inhibiting herbicides, 2) to clarify the genetic basis of resistance by sequencing the *als* gene, and 3) to determine the effectiveness of mixtures of ALS-inhibitors with auxin-type herbicides as alternatives for control of the studied populations, bearing different herbicide resistance traits due to various histories of exposure to ALS-inhibiting herbicides.

MATERIALS AND METHODS

Seed source

Three roadside surveys (namely, members of the research team traveling through specified study areas) were carried out during early summer of 2014, 2015, and 2016, respectively, in wheat monoculture fields of northern Greece (counties of Pieria, Serres, Kozani, and Florina), where failure of *Galium* spp. control with chlorsulfuron and other ALS-inhibiting herbicides had been reported. These surveys aimed at locating and marking fields with poor *G. spurium* and *G. aparine* control. Before wheat harvest, mature seeds of *Galium* spp. were collected from representative fields that had been marked during the roadside surveys. The specific sites located for this study were: Kitros (Pieria) 40.3724 N, 22.5779 E, Kolindros (Pieria) 40.4702 N, 22.4846 E, Melinikitsi (Serres) 41.1470 N, 234429 E, Trigoniko (Kozani) 40.1108 N, 21.9323 E, Frourio (Kozani) 40.063 N, 21.8089 E, and Tripotamos (Florina) 40.8242 N, 21.5009 E. Seeds were collected manually from individual plants of each field and pooled together (characterized as population). Moreover, mature seeds were collected from *Galium* populations grown in non-cultivated areas with no history of exposure to herbicide applications. Those *G. spurium* and *G. aparine* were considered as susceptible populations. The seed material was collected in big

plastic bags and transported to the laboratory. They were then air-dried, placed in paper bags, and saved at 3-5 °C (refrigerator) until initiation of the experiments.

Whole-plant response to herbicides [*G. spurium* (GS) experiments]

Eight putative resistant (R) and one susceptible GS populations were studied in two whole-plant response experiments. The first experiment was carried out at the farm of Aristotle University of Thessaloniki from winter 2014 to spring 2015, where six putative GS resistant populations and one susceptible (S) GS population were studied. The second experiment was conducted in 2015-2016 growing period at the farm of Technological Educational Institute of West Macedonia, Florina, where two additional putative GS resistant populations were studied. Experiments at Thessaloniki and Florina were established in 0.9 L plastic pots filled with a clay loam soil (31.6% clay, 48.0% silt, 20.4% sand, 1.3% organic matter, 7.8 pH) and a peat:sand mixture (1:1 v/v), respectively. Each pot was seeded with approximately 25 *Galium* seeds (at 1 cm depth), which were carefully covered with soil. When GS seedlings passed the cotyledon stage, they were carefully thinned to six plants per pot.

The six putative resistant GS populations were treated with the recommended (X), twice (2X), four times (4X), and eight times (8X) of the recommended field rate of the herbicides: chlorsulfuron (Glean 75 WG, DuPont Hellas) (15, 30, 60, 120 g a.i. ha^{-1}), tribenuron methyl (Granstar 50 SG, DuPont Hellas) (15, 30, 60, 120 g a.i. ha^{-1}), [tribenuron + mecoprop-p] (Granstar Combi SG, DuPont Hellas) ([10.9 + 800], [21.8 + 1600], [43.6 + 3200], [87.2 + 6400] g a.i. ha^{-1}), [florasulam + 2,4-D] (Mustang 306 SE, Dow Elanco Hellas) ([5 + 240], [10 + 480], [20 + 960], [40 + 1920] g a.i. ha^{-1}), and [florasulam + aminopyralid] (Lancelot 450 WG, Dow Elanco Hellas) ([4.95 + 9.9], [9.9 + 19.8], [19.8 + 39.6], [39.6 + 79.2] g a.i. ha^{-1}). The susceptible GS population was treated with one eighth (1/8X) of, a quarter (1/4X) of, half (1/2X) of, and the recommended rate (X) of the above herbicides, i.e., chlorsulfuron (1.87, 3.75, 7.5, 15 g a.i. ha^{-1}), tribenuron (1.87, 3.75, 7.5, 15 g a.i. ha^{-1}), [tribenuron + mecoprop-p] ([1.4 + 100], [2.7 + 200], [5.4 + 400], [10.9 + 800] g a.i. ha^{-1}), [florasulam + 2,4-D] ([0.62 + 30], [1.25 + 60], [2.5 + 120], [5 + 240] g a.i. ha^{-1}), [florasulam + aminopyralid] ([0.62 + 12.3], [12.3 + 24.7], [24.7 + 49.5], [4.95 + 9.9] g a.i. ha^{-1}). For obtaining additional information on the resistance status of the GS populations, the two putative resistant GS populations evaluated at the farm of Technological Educational Institute of West Macedonia, Florina, in addition to the herbicide treatments described above for the six putative resistant GS populations, were also exposed to the pre-package mixture of [florasulam + fluroxypyr] applied at X, 2X, 4X, and 8X rates ([0.45 + 45], [0.90 + 90], [1.8 + 180], [3.6 + 360] g a.i. ha^{-1}).

Herbicide applications were performed when GS plants were at the 2-3 whorl (a circular pattern of leaves occurring in *Galium* spp.) growth stage. Untreated plants of each population served as control. Herbicides were applied with a portable 2.4 m wide boom field plot propane-pressurized sprayer, carrying six 8002 flat-fan nozzles. The sprayer was calibrated to deliver a water volume of 300 L ha^{-1} at a pressure 280 kPa. Pots were moved outdoors in a net protected area and watered regularly. A randomization of pots was conducted weekly to ensure uniform growth conditions for all plants. Weed control was assessed by determining the aboveground fresh weight of plants, four weeks after treatment (WAT). Fresh weight is recommended in the assessments of herbicide efficacy, irrespective of herbicide systemic activity (translocation), according to international standards (EPPO, 2012). Two runs of each experiment were conducted, established in a completely randomized design with four replicates, respectively.

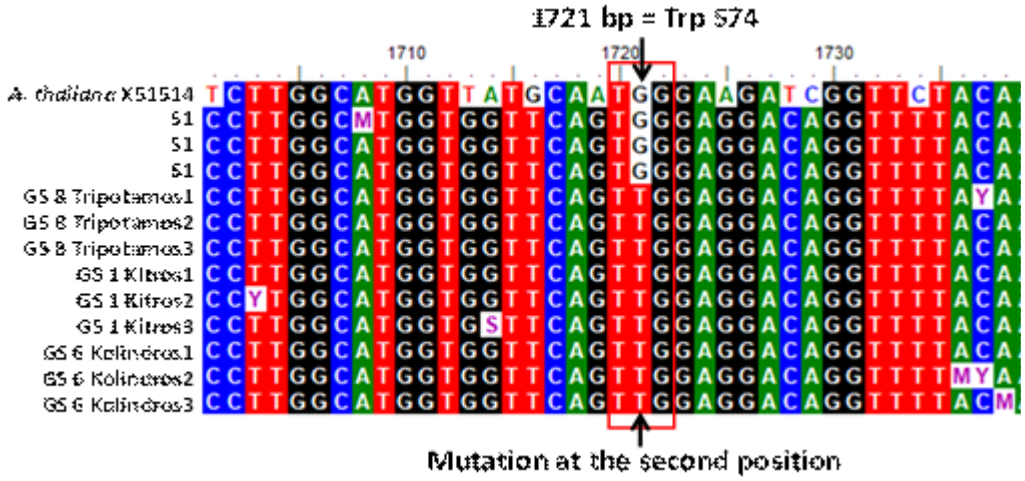
Whole-plant response to herbicides [*G. aparine* (GA) experiments]

The putative resistant GA Frourio (R) population, which was evaluated in the whole-plant response pot experiment conducted at the farm of Technological Educational Institute of West Macedonia, Florina during 2016-2017, was treated with the same field rates (X, 2X, 4X, and 8X) of all the above herbicides used for the evaluation of the two putative resistant GS populations in Florina. On the other hand, the susceptible GA Frourio (S) population received 1/8X, 1/4X, 1/2X, and X field rate of the same herbicides used for the putative resistant GA population. An untreated control was included for each population.

Two runs of each experiment were carried out in a completely randomized design with three replicates, whereas the herbicide application, the pot randomization, and the weed control assessments were performed in the same way as described above.

Amplification and sequencing of the ALS gene fragment

For the amplification of the *als* gene, plant material was collected from six plants per pot, from four pots per each abovementioned resistant or susceptible population. All resistant plants and six susceptible plants in each pot (replicated four times) were treated with the label rate of tribenuron (15 g a.i. ha^{-1}) at the 2-3 whorl growth stage, whereas six susceptible plants in each pot (replicated four times) were left untreated. This treatment was applied for eliminating individual susceptible plants from the resistant populations and ensuring susceptibility of the susceptible population. Leaf parts of surviving plants from the three resistant GS populations were collected and immediately stored at -28 °C. They were then used for extraction of the DNA. Additionally, leaf parts of single plants from the susceptible population (i.e., plants not exposed to tribenuron) were harvested. Leaf samples from three different plants were collected from each resistant and susceptible GS population for sequencing. A quantity of leaf tissue 100 mg (three plants for each population) was used for extraction of genomic DNA with the NucleoSpin® Plant II kit (MACHEREY NAGEL GmbH & Co. KG, Düren, Germany) following manufacturers' protocol. The *als* gene fragment (434 bp) (the fragment that includes the Trp-574 codon) was amplified from the genomic DNA of the tested populations (R and S) with two primers: 52 - GGTTGGGAGCAATGGGGTT-32 and the reverse primer 52 - ACTTGGAATCATCGGCAGCA-32 . The two primers (F and R), produced by INVITROGEN, were designed based on the JN038048.1 *G. spurium* isolate CHO_sequence of acetolactate synthase (ALS). These primers were designed to investigate the region 1504-1938bp of the *als* gene that includes the Trp-574 codon. The polymerase chain reaction (PCR) included 10 µm of each forward and reverse primer, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 2 µL of the supplied 10^x thermophilic buffer, 1 U Kapa Taq DNA polymerase in 20 mL mixture 1, and mL of genomic DNA diluted at 20 ng/µL. Amplification was performed in a Veriti 96 wells model thermocycler using the following cycles: DNA denaturation for 3 min at 95 °C, denaturation for 30 s at 95 °C (35 cycles), annealing at 59 °C for 20 s and elongation at 72 °C for 30 s, with a final elongation at 72 °C for 1 min. The derived products were separated in agarose gel (1.5%) and then purified according to the NucleoSpin® Extract II kit protocol. The sequencing of the purified PCR product was performed in the Immunology and Histocompability Department, School of Medicine, University of Thessaly. The forward primer was used for each PCR product. The sequences were aligned with the CLUSTAL W software (multiple alignment of nucleic acid and protein sequences) using the MEGA5 and/or Bioedit. The sequence of each sample was performed in both strands (F and R), but the F strand is presented in this study (Figure 1).



Red boxed no mutation site (TGG = Trp) for *A. thaliana* X51514 and S population, and mutation TTG = Leu for R populations.

Figure 1 - Alignment of the sequenced genomic DNA originating from one susceptible (S) and three (GS 1, GS 6, GS 8) resistant (R) *G. spurium* populations.

Data analysis

Fresh weight data were expressed as percentages of control (based on fresh weight reduction % of untreated control), as a common approach in related studies of the literature (Ruiz-Santaella et al., 2006; Damalas et al., 2012) and were subjected to analysis of variance (ANOVA) separately for each experiment. First, data derived from the two dose-response experiments for the putative GS R populations carried out in Thessaloniki were analyzed over two runs using a 6 x 5 x 4 split-plot approach (6 populations x 5 herbicides x 4 herbicide rates), where populations were considered the main plots and herbicide by rate treatments the subplots. Second, data obtained from the GS S population were analyzed over two runs using a 5 x 4 factorial approach (5 herbicides x 4 herbicide rates). In addition, data obtained for the two putative GS R populations studied in Florina were analyzed over two runs using a 2 x 6 x 4 split-plot approach (2 populations x 6 herbicides x 4 herbicide rates), where populations were considered the main plots and herbicide by rate treatments the subplots. Third, regarding the GA experiments, an ANOVA was performed over two runs using a 6 x 4 factorial approach (6 herbicides x 4 herbicide rates) for putative R or S populations. Because no significant treatment by repeated runs interaction was found, the populations by herbicides by rates interaction means were averaged across two runs and compared with Fisher's protected LSD test (least significance difference) at $P<0.05$. Fourth, the pooled over two runs fresh weight data (% of untreated control) of each population were subjected to nonlinear regression analysis, where applicable, using the log-logistic equation 1 (Seefeldt et al., 1995).

$$y = C + \frac{D - C}{1 + \exp\{b[\log(x) - \log(GR_{50})]\}} \quad \text{(eq. 1)}$$

where C = the lower limit, D = the upper limit, b = the slope at the GR_{50}, and GR_{50} = the herbicide rate (g a.i. ha^{-1}) required for 50% reduction of fresh weight. The independent variable (x) was the herbicide rate and the dependent variable (y) was the fresh weight. The GR_{50} estimated for the first herbicide of each pre-packaged formulations.

RESULTS AND DISCUSSION

***G. spurium* experiments**

Chlorsulfuron and tribenuron applied at 4X rates reduced fresh weight of all six GS populations in the experiment of Thessaloniki by 25-73% and 12-47%, respectively (Table 1). However, all populations were totally controlled (100%) with [tribenuron + mecoprop-p], [florasulam + 2,4-D], and [florasulam + aminopyralid], except GS 1 Kitros and GS 6 Kolindros populations that were not controlled with [florasulam + 2,4-D] even with the 8X rate (Table 1). This was also confirmed by the estimated GR_{50} values for chlorsulfuron and tribenuron, which ranged from 26.9 to 195.5 and 56.9 to 330.3 g a.i. ha^{-1}, respectively. Among these populations, the GS 4 Trigoniko and GS 6 Kolindros showed the highest GR_{50} values for chlorsulfuron (195.5 and 192.9 g a.i. ha^{-1}) and tribenuron (203.4 and 330.3 g a.i. ha^{-1}). Regarding the GR_{50} values for the remaining herbicides, these were not estimated (except for florasulam + 2,4-D for GS 1 Kitros) as calculations were not applicable. On the contrary, the susceptible population GS Kitros was totally controlled with the X rates of all herbicides tested, showing high sensitivity even to X/2 rate in the case of mixtures with auxin-type herbicides (Table 2). This was the reason for the lower GR_{50} values (0.14 g a.i. ha^{-1} to 2.9 g a.i. ha^{-1}) as compared with the previously mentioned GR_{50} values for the resistant populations.

Both GS populations studied in Florina showed low and moderate levels of control with chlorsulfuron and tribenuron applied at 8X rates, with tribenuron being less effective than chlorsulfuron (Table 3). This difference was supported by the estimated GR_{50} values for chlorsulfuron, which were 31.1 and 73.4 g a.i. ha^{-1} for the two populations, whereas the respective values for tribenuron were 119.7 and 112.2 g a.i. ha^{-1}. However, the GS 7 Melinikitsi population was totally controlled (100%) with [tribenuron + mecoprop-p], [florasulam + 2,4-D], [florasulam + aminopyralid], and [florasulam + fluroxypyr], whereas this was not the case for GS 8 Tripotamos population that was not effectively controlled with any rate of [florasulam + 2,4-D] and for this reason the GR_{50} value for florasulam was 11.9 g a.i. ha^{-1} (Table 3). In addition, the X

Table 1 - Estimated GR_{50} values (g a.i. ha^{-1}), where applicable, of wheat herbicides used for control of *Galium spurium* (GS) populations (Thessaloniki experiments)

Treatment	Rate (g a.i. ha^{-1})	Fresh weight (% of untreated control)[1]					
		GS 1 Kitros	GS 2 Trigoniko	GS 3 Trigoniko	GS 4 Trigoniko	GS 5 Trigoniko	GS 6 Kolindros
Chlorsulfuron							
X	15	100	64	63	91	100	90
2X	30	88	44	59	73	93	84
4X	60	55	34	27	69	68	75
8X	120	49	27	20	59	55	60
	GR_{50}	98.6	26.9	30.7	195.5	127.1	192.9
	R^2	0.872	0.935	0.899	0.850	0.921	0.930
Tribenuron							
X	15	90	82	66	100	100	100
2X	30	86	64	60	97	93	100
4X	60	70	53	57	77	78	88
8X	120	60	27	36	69	42	81
	GR_{50}	180.6	56.9	61.8	203.4	104.8	330.3
	R^2	0.963	0.966	0.822	0.878	0.992	0.898
Tribenuron + mecoprop-p							
X	10.9 + 800	0	0	0	0	0	0
2X	21.8 + 1600	0	0	0	0	0	0
4X	43.6 + 3200	0	0	0	0	0	0
8X	87.2 + 6400	0	0	0	0	0	0
Florasulam + 2,4-D							
X	5 + 240	73	0	0	0	0	100
2X	10 + 480	59	0	0	0	0	100
4X	20 + 960	49	0	0	0	0	100
8X	40 + 1920	42	0	0	0	0	39
	GR_{50}*	21.1					
	R^2	0.938					
Florasulam + aminopyralid							
X	4.95 + 9.9	0	0	0	0	0	26
2X	9.9 + 19.8	0	0	0	0	0	0
4X	19.8 + 39.6	0	0	0	0	0	0
8X	39.6 + 79.2	0	0	0	0	0	0
$LSD_{0.05}$	3						

[1] Values are means of two identical experiments with 8 replications per combined treatment. * GR_{50}: Concentration of the first herbicide for 50% reduction of *Galium spurium* fresh weight.

and 2X of [florasulam + aminopyralid] indicated 80 and 91% control of GS 8 Tripotamos population, respectively, whereas the extra treatment of [florasulam + fluroxypyr] was highly effective against both populations. Because the lowest rate of the other herbicides did not reduce fresh weight by 50%, the GR_{50} values were not estimated as calculation was not applicable.

G. aparine experiments

The putative resistant GA Frourio (R) population studied in Florina showed low to moderate levels of control with up to 4X rate of chlorsulfuron and tribenuron, and this was confirmed by the estimated GR_{50} values, which were 60.3 and 40 g a.i. ha^{-1} for chlorsulfuron and tribenuron, respectively (Table 4). However, control of this weed population was excellent (100%) with the recommended rates of [tribenuron + mecoprop-p], [florasulam + 2,4-D], [florasulam + aminopyralid], and [florasulam + fluroxypyr], and this was the reason that GR_{50} values for these herbicides were

Table 2 - Estimated GR_{50} values (g a.i. ha^{-1}), where applicable, of wheat herbicides used for control of one *Galium spurium* (GS) population (Thessaloniki experiments)

Treatment	Rate (g a.i. ha^{-1})	Fresh weight (% of untreated control)[1]
		GS Kitros (S)
Chlorsulfuron		
X	15	0
X/2	7.5	22
X/4	3.75	30
X/8	1.87	73
GR_{50}		2.9
R^2		0.941
Tribenuron		
X	15	0
X/2	7.5	26
X/4	3.75	46
X/8	1.87	53
GR_{50}		2.5
R^2		0.867
Tribenuron + mecoprop-p		
X	10.9 + 800	0
X/2	5.45 + 400	0
X/4	2.72 + 200	1
X/8	1.36 + 100	18
Florasulam + 2,4-D		
X	5 + 240	0
X/2	2.5 + 120	3
X/4	1.25 + 60	8
X/8	0.62 + 30	14
Florasulam + aminopyralid		
X	4.95 + 9.9	0
X/2	2.47 + 4.95	4
X/4	1.24 + 2.47	17
X/8	0.62 + 1.24	35
$LSD_{0.05}$		1

[1] Values are means of two identical experiments with 8 replications per combined treatment.

Table 3 - Estimated GR_{50} values (g a.i. ha^{-1}), where applicable, of wheat herbicides used for control of *Galium spurium* (GS) populations (Florina experiments)

Treatment	Rate (g a.i. ha^{-1})	Fresh weight (% of untreated control)[1]	
		GS 7 Melinikitsi	GS 8 Tripotamos
Chlorsulfuron			
X	15	68	72
2X	30	51	67
4X	60	32	63
8X	120	21	34
GR_{50}		31.1	73.4
R^2		0.989	0.795
Tribenuron			
X	15	96	95
2X	30	85	73
4X	60	79	66
8X	120	48	49
GR_{50}		119.7	112.2
R^2		0.953	0.928
Tribenuron + mecoprop-p			
X	10.9 + 800	0	0
2X	21.8 + 1600	0	0
4X	43.6 + 3200	0	0
8X	87.2 + 6400	0	0
Florasulam + aminopyralid			
X	4.95 + 9.9	0	20
2X	9.9 + 19.8	0	9
4X	19.8 + 39.6	0	0
8X	39.6 + 79.2	0	0
Florasulam + 2,4-D			
X	5 + 240	0	57
2X	10 + 480	0	53
4X	20 + 960	0	49
8X	40 + 1920	0	31
GR_{50}*			11.9
R^2			0.869
Florasulam + fluroxypyr			
X	1.8 + 180	0	0
2X	3.6 + 360	0	0
4X	7.2 + 720	0	0
8X	14.4 + 1440	0	0
$LSD_{0.05}$		2	

[1] Values are means of two identical experiments with 8 replications per combined treatment. * GR50 : Concentration of the first herbicide for 50% reduction of *Galium spurium* fresh weight.

not estimated as calculation was not applicable. The susceptible GA Frourio population (S) was effectively controlled with the recommended rates of all herbicides (Table 5), and for this reason the estimated GR_{50} values for the herbicides tested were low and ranged from 0.14 to 2.4 g a.i. ha^{-1}.

Amplification and sequencing of the als gene fragment

The sequenced region (434 bp) of the *als* gene from the four GS individuals (three individuals from the susceptible and three from each of the resistant GS 1 Kitros, GS 6 Kolindros, and GS 8 Tripotamos populations), aligned with the *als* gene sequence obtained from GenBank (Accession

Table 4 - Estimated GR_{50} values (g a.i. ha^{-1}), where applicable, of wheat herbicides used for control of a *Galium aparine* (GA) population (Florina experiments)

Treatment	Rate (g a.i. ha^{-1})	Fresh weight (% of untreated control)[1]
		GA Frourio (R)
Chlorsulfuron		
X	15	100
2X	30	64
4X	60	53
8X	120	27
GR_{50}		60.3
R^2		0.917
Tribenuron		
X	15	88
2X	30	64
4X	60	28
8X	120	15
GR_{50}		40
R^2		0.990
Tribenuron + mecoprop-p		
X	10.9 + 800	0
2X	21.8 + 1600	0
4X	43.6 + 3200	0
8X	87.2 + 6400	0
Florasulam + aminopyralid		
X	4.95 + 9.9	0
2X	9.9 + 19.8	0
4X	19.8 + 39.6	0
8X	39.6 + 79.2	0
Florasulam + 2,4-D		
X	5 + 240	0
2X	10 + 480	0
4X	20 + 960	0
8X	40 + 1920	0
Florasulam + fluroxypyr		
X	1.8 + 180	0
2X	3.6 + 360	0
4X	7.2 + 720	0
8X	14.4 +1440	0
$LSD_{0.05}$		2

[1] Values are means of two identical experiments with 6 replications per combined treatment. * GR_{50}: Concentration of chlorsulfuron and tribenuron for 50% reduction of *Galium aparine* fresh weight.

Table 5 - Estimated GR_{50} values (g a.i. ha^{-1}), where applicable, of wheat herbicides used for control of a *Galium aparine* (GA) population (Florina experiments)

Treatment	Rate (g a.i. ha^{-1})	Fresh weight (% of untreated control)[1]
		GA Frourio (S)
Chlorsulfuron		
X	15	0
X/2	7.5	21
X/4	3.75	38
X/8	1.87	44
GR_{50}		1.8
R^2		0.850
Tribenuron		
X	15	0
X/2	7.5	19
X/4	3.75	26
X/8	1.87	62
GR_{50}		2.4
R^2		0.949
Tribenuron + mecoprop-p		
X	10.9 + 800	0
X/2	5.45 + 400	0
X/4	2.72 + 200	1
X/8	1.36 + 100	15
Florasulam + aminopyralid		
X	4.95 + 9.9	0
X/2	2.47 + 4.95	3
X/4	1.24 + 2.47	14
X/8	0.62 + 1.24	29
Florasulam + 2,4-D		
X	5 + 240	0
X/2	2.5 + 120	1
X/4	1.25 + 60	6
X/8	0.62 + 30	11
Florasulam + fluroxypyr		
X	1.8 + 180	0
X/2	0.9 + 90	0
X/4	0.45 + 45	0
X/8	0.22 + 22.5	2
$LSD_{0.05}$		1

[1] Values are means of two identical experiments with 6 replications per combined treatment. * GR_{50}: Concentration of chlorsulfuron and tribenuron for 50% reduction of *Galium aparine* fresh weight.

number: *Arabidopsis thaliana* X51514), showed 72-73% identity with the sequence of the *als* gene of *A. thaliana* X51514 (data not shown). *Arabidopsis thaliana* X51514 was used as the reference gene for the numbering of the amino acids, because is the most commonly used (standard) in the literature. Chromatograms of the DNA sequence revealed Trp (TGG) at position 574 in the three plants of the susceptible population as in the *A. thaliana*, while the respective DNA sequence of the three GS resistant populations (GS 1 Kitros, GS 6 Kolindros, and GS 8 Tripotamos) revealed a point mutation at the second base of the codon Trp-574 that resulted in the substitution of Trp-574 by Leu (TTG) in all plants (Figure 1). All plants belonging to three resistant populations had the point mutation resulting in substitution of Trp-574 by Leu in both alleles (homozygous plants), although certain other positions showed ambiguous base calls.

The low control levels of GS 1 Kitros, GS 6 Kolindros, and GS 8 Tripotamos populations, with up to 8X rates of the formulated mixture [florasulam + 2,4-D], confirms strong evidence for cross-resistance to both sulfonylurea (chlorsulfuron, tribenuron) and triazolopyrimidine (florasulam) herbicides. The amino acid substitution of Trp-574 by Leu in the analysed *als* sequences from these three GS resistant populations, along with the respective estimated high GR_{50} values, support the above confirmed cross-resistance to sulfonylurea and triazolopyrimidine herbicides at whole plant level. The same substitution of Trp-574 by Leu was also reported by Sun et al. (2011), Beckie et al. (2012), and Ntoanidou et al. (2017) for the *G. spurium, G. aparine,* and *Sinapis arvensis* cross-resistant species to ALS-inhibitors, respectively.

The high levels of cross-resistance of two *G. spurium* populations to chlorsulfuron, tribenuron, and florasulam are in agreement with results reported in a *G. spurium* population from Canada, which also showed high levels of cross-resistance to the ALS inhibitors triasulfuron, thifensulfuron/tribenuron, and sulfometuron (Hall et al., 1998; Van Eerd, 2004; Van Eerd et al., 2005; Beckie and Tardif, 2012). In addition, the *G. aparine* cross-resistance to chlorsulfuron, tribenuron, and florasulam agree partially with a resistant population to tribenuron in China (Sun et al., 2011). However, as there are no confirmed cases of *Galium* spp. resistance in Europe, this is the first report of *Galium* spp. resistance to ALS-inhibiting herbicides in Europe.

A number of auxin-type herbicides (e.g., 2,4-D, dicamba, fluroxypyr, aminopyralid, mecoprop-p) are registered as formulated mixtures with ALS-inhibiting herbicides aiming to broaden the spectrum of weed control and mitigate the evolution of herbicide resistance (Beckie and Tardif, 2012). Some of these mixtures of ALS-inhibiting herbicides with auxin-type herbicides were highly effective against the resistant *Galium* populations of the present study. Therefore, tank-mixing ALS-inhibiting herbicides with herbicides having different mode of action and more importantly with a less resistant-prone site of action could be an effective strategy for the control of the already resistant biotypes and thus delaying the evolution of herbicide resistance in key weed species of cereals. Unfortunately, certain auxin herbicides have been found to negatively affect the performance of ALS-inhibiting herbicides against important grass weed species (Damalas and Eleftherohorinos, 2001; Damalas et al., 2012) and this should be considered before different herbicides are mixed in the spray solution. Bromoxynil, a PS-II inhibitor that is highly effective on a wide spectrum of broadleaf weeds in cereals and rarely is involved in herbicide interactions in mixtures, could also be a valuable option for winter cereal growers either alone or as a mixture partner (Baghestani et al., 2008; Torra et al., 2010; Safdar et al., 2011; Kumar and Jha, 2015). Otherwise, non-chemical methods of weed control, such as systematic crop rotation, use of competitive cultivars, and on-time planting with correct seeding rates, should be implemented.

The excellent control of all and the four (2 GS and 2 GA) *Galium* populations with the respective formulated mixtures [tribenuron + mecoprop-p] and [florasulam + fluroxypyr] differs partially with results reported by Froud-Williams and Ferris-Kaan (1991) and Hill et al. (1996), who found a significant genetic variation within *G. aparine* biotypes in their response to these two auxin-type herbicides. It is worth noting that the application of MCPA, 2,4-D, and the formulated mixture [florasulam + 2,4-D] or [bromoxynil + 2,4-D] has been widely adopted in northern Greece for weed control in winter cereals to manage sulfonylurea-resistant *Papaver rhoeas* and *Sinapis arvensis* with cross-resistance to sulfonylureas due to Pro-197 mutation (Kaloumenos et al., 2009, 2011) and all chemistries of ALS-inhibiting herbicides due to a Trp-574-Leu mutation in the *als* gene, respectively (Ntoanidou et al., 2017).

The present study showed clearly that the poor control of 7 GS populations and 1 GA population reported by some cereal farmers in northern Greece is attributed to development of cross-resistance to ALS inhibitors. This could be the result of wheat monoculture as a main agronomic practice implemented in major wheat production areas, which were typically accompanied by the repeated use of those herbicides for at least 20 years. Indeed, chlorsulfuron was widely used due to long residual activity, enabling season-long control of weed flushes and due to excellent control of most broadleaf weeds and suppression of some important grasses in cereals. However, although the long residual activity of chlorsulfuron improved weed control, it exerted intense selection pressure on many weeds that have a prolonged germination period throughout the growing season and finally resulted in the development of weed resistance. This is confirmed by many populations of rigid ryegrass (*Lolium rigidum), Papaver rhoeas,* and *Sinapis arvensis* recorded

in wheat monocultures of northern and central Greece with cross-resistance to sulfonylurea herbicides and other ALS-inhibiting herbicides (Kotoula-Syka et al., 2000; Kaloumenos et al., 2011, 2012; Ntoanidou et al., 2017).

The high GR_{50} values for ALS-inhibiting herbicides used in this study provided strong evidence for high levels of resistance of both GS and GA species to the ALS-inhibiting herbicides chlorsulfuron and tribenuron, as well as additional cross-resistance to florasulam for three GS populations. Furthermore, DNA sequence of the last three cross-resistant GS populations revealed a point mutation at position Trp-574, causing amino acid substitution by Leu in the ALS enzyme. However, all *Galium* populations were controlled with the respective formulated mixtures of [tribenuron + mecoprop-p] or [florasulam + fluroxypyr]. In all cases, the resistance was closely associated with wheat monoculture and continuous use of the same most convenient herbicide (in terms of efficacy and costs) for years. These findings strongly suggest that actions should be taken for preventing further resistance evolution by implementing systematic crop rotation and herbicide rotation. Moreover, reliance on herbicides should be decreased and alternative tools of weed control, such as occasional deep ploughing, should be introduced. To achieve these goals, training of growers, crop consultants, and field practitioners in tackling herbicide resistant weed species and adopting integrated management practices for reducing herbicide selection pressure should be a top priority.

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