Ministry of Environment
of Denmark
Environmental
Protection Agency

Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies

RELIUM

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Preface

In the European Union, the Directive 2009/128/EC on the sustainable use of pesticides makes it mandatory to implement the principles of Integrated Pest Management (IPM). To face this challenge, member states of the European Union has co-funded an initiative to coordinate Integrated Pest Management (C-IPM) through an ERA-NET funding programme under the Seventh Framework Programme on research and development.
The present report is the result of a project with participating institutes from and co-funded by parties from Italy, Greece, and Denmark.
The project aimed at monitoring, mapping, developing innovative detection tools and characterizing (patterns, levels and resistance mechanisms) selected resistant populations as well as devising resistance management strategies for *Lolium* in various agronomic situations.
In Denmark, herbicide resistant *Lolium* is not as widespread as in Italy or Greece. However, several populations have been found to be resistant to both ALS and ACCase inhibitors. In contrast to Italy and Greece, only one mutation in the ALS gene was identified in the Danish populations, and it was present in only one population, indicating that the main resistance mechanism in Denmark is non-target site. Furthermore, no glyphosate resistance was found in Denmark.
The lower incidence of herbicide resistance in Denmark compared to Italy and Greece is probably attributed to a lesser use of winter annual crops and a higher use of crop rotation in Denmark.
Based on the results of this collaborative project, country specific guidelines to manage herbicide resistance *Lolium* have been developed, published, and distributed to grower associations.

See also:
Scarabel L., Panozzo, S., Loddo, D., Mathiassen S.K., Kristensen M., Kudsk P., Gitsopoulos T., Travlos I., Tani E., Chachalis D. & Sattin M. (2020). Diversified resistance mechanisms in multi-resistant *Lolium* spp. in three European countries.
Frontiers in Plant Science, 11, 608845.
https://doi.org/10.3389/fpls.2020.608845

Gerakari M, Cheimona N, Tani E, Travlos I, Chachalis D, Loddo D, Mathiassen SK, Gitsopoulos TK, Scarabel L, Panozzo S, Kristensen M, Kudsk P and Sattin M. (2022). Biochemical and Rapid Molecular Analyses to Identify Glyphosate Resistance in *Lolium* spp.
Agronomy, 12, 40.
https://doi.org/10.3390/agronomy12010040

Contents

C-IPM
Coordinated Integrated Pest Management in Europe
EU Grant agreement no.: 618110

Final report

Title of project

Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies

Period covered

From 06 June 2017 to 06 October 2020
(including four months postponement
of the end of the project)

Project information

Project acronym:	RELIUM	**Project ID:**	41
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Short description on the elaboration of the proposal (earlier projects this project is based on, involvement of end users/farmers/other stakeholders in the design of the project)
The project aimed at monitoring, mapping, developing innovative detection tools and characterizing (patterns, levels and resistance mechanisms) selected resistant populations as

[1] University, Public research centre, Private research centre, Company, Other

[2] PC = Project coordinator, WPL = Work package leader, WPCL = Work package co-leader, P = Participant

well as devising resistance management strategies for *Lolium* in various agronomic situations.
Relevant stakeholders, farmers, farmers' organisations, farmers' advisors, and national herbicide resistance action groups were involved in collecting seed samples, recording history of field management, devising resistance management strategies, and discussing and disseminating the outcomes of the project.

Outputs - results of the activities undertaken in the project

Final project summary suitable for web publication
194 populations of *Lolium* suspected to be resistant to ALS, and/or to ACCase inhibitors or to glyphosate were collected in cereal or perennial crops. Whole plant bioassays confirmed that 21% of these populations were resistant only to an ALS inhibitor, 9% were resistant to at least one ACCase inhibitor and 38% were multi-resistant to both modes of action. Three *Lolium* populations out of 9 populations collected in olive grove or orchards were resistant to glyphosate.
The interactive web-based application, iMAR (interactive MApping of Resistance), previously developed by CNR for mapping herbicide resistant populations was developed and adapted for Danish and Greek partners. The URLs to the working web-platforms are: relium-gr.agriserv.org for Greece and relium-dk.agriserv.org for Denmark.
The dose-response experiments with ALS- and ACCase inhibitors confirmed multi-resistance in populations from all three countries. High levels of resistance to Atlantis was found in all countries. The resistance level to ACCase was lower in the Danish populations compared to the Italian and Greek populations. The molecular analyses confirmed the presence of mutated alleles at several different amino acid positions in the ALS- and ACCase genes in populations from Greece and Italy indicating target-site resistance to be the main resistance mechanism. In contrast only one mutation in the ALS gene was identified in the Danish populations and it was present in only one population indicating that the main resistance mechanism in Denmark is non-target site. A wide range of responses to glyphosate was recorded in the 6 populations tested with resistance indexes (RIs) ranging from 1 to 6.
Validation of LAMP tests showed a potential for the identification of mutations in the ACCase gene. On the contrary, protocols for detecting ALS mutations gave less reliable results.
The expression patterns of four HMR genes and the reference gene IDE showed significant differences between plants from the same population implying that the *Lolium* populations in general are heterogenous for the gene expression of one or more HMR genes. There is therefore a potential for evolution of metabolic based resistance in most populations.
Sequencing of the EPSPS gene of six populations of L. rigidum revealed no point mutations in the Pro106 position thus indicating non-target site resistance to be the most likely mechanism. The impact of four different ABC transporter genes on resistance was studied. ABC transporter gene type 3 to be the best candidate for a marker gene discriminating resistant and susceptible populations at early stages which was confirmed by studies of a resistant population from conservation agriculture in Italy.

Main results, conclusions and fulfilment of objectives by Work Package

WP1 Monitoring and mapping of resistant *Lolium* spp. populations from three European countries

WPL: Laura Scarabel – CNR
Responsible partners: CNR, AU, DEMETER

Overall summary of main results and conclusions WP1

During the 3 years of the project, 185 *Lolium* populations suspected to be resistant to ALS and /or to ACCase inhibitors have been collected in cereal (mainly wheat) fields. In addition, 9 suspected resistant *Lolium* populations have been collected in perennial crops (olive grove or orchards) where of glyphosate had been intensively used. The seed sampling in the 3 countries was done following farmers' complaints and most of the collected samples were accompanied with the historical field data provided by farmers. From this information it appears that the most common herbicides used in the 3 countries to control *Lolium* plants in wheat fields belong to two modes of action, ALS and ACCase inhibitors. In perennial

crops the main herbicide used in Greece and Italy is glyphosate. Due to the repeated use of these herbicide families, the selection pressure is high.
Overall, whole plant bioassays conducted on the collected samples confirmed that 21% of these populations were resistant only to the ALS inhibitor, iodosulfuron + mesosulfuron, 9% were resistant to at least one of the ACCase inhibitors tested (clodinafop, pinoxaden or cycloxydim) and 38% of the populations were multi-resistant to the above mentioned modes of action. Three *Lolium* populations out of 9 populations collected in olive grove or orchards were found to be resistant to glyphosate. No multi-resistance including glyphosate was observed in the tested populations.
The confirmation in the 3 countries of a high number of *Lolium* populations being resistant to both modes of action, ALS and ACCase inhibitors, is worrying and indicates that the chemical control based only on post-emergence herbicides is not sustainable.
The interactive web-based application, iMAR, previously developed by CNR for mapping herbicide resistant populations was developed and adapted for Danish and Greek partners. The URLs to the working web-platforms are: relium-gr.agriserv.org for Greece and relium-dk.agriserv.org for Denmark.
The interactive web platform, based on free software tools, includes two sections:

- Data management where input and editing of the sample data is done.
- Dynamic mapping, generating customized maps based on a few selection criteria.

Currently, all the *Lolium* populations tested during this project were entered in the respective databases of the three countries participating in the project. The system can be extended to include larger datasets (i.e. other weed species, herbicides, types of resistance).
A relevant issue of the map system is the low cost. The system has been developed entirely with the use of open-source technology, and this makes it fully adaptable to most hardware and software configurations. It has been optimized for Firefox and Chrome browsers and to run on a Microsoft Windows operating system (Windows 7 or later).
The freely available and regularly updated system allows to show in real time maps of diffusion of *Lolium* resistant populations at national level. This allows all stakeholders to be timely informed on the presence and spread of resistant weed populations. Potentially this will increase the awareness of the resistance problem and will facilitate the adoption of appropriate management tools to delay the development and spread of resistant weeds.

Detailed results are reported in deliverables 1.1 and 1.2 attached to this report.

Report on the results obtained (A) and changes to the original plan/ WP objectives (B) by tasks and partners:

WP1- Task 1: Monitoring of resistant *Lolium* spp. populations from the 3 countries

Partner: AU

A. Results obtained: In 2017, one hundred and twenty six (126) putative resistant *Lolium* populations have been collected in Danish fields, mainly from winter wheat and spring barley, where resistant was suspected: 94 samples of L. multiflorum and 32 samples of L. perenne. Most of these samples were accompanied with information on location (address and GPS coordinates), crop, cultivation system (no till, reduced tillage, ploughing) and herbicide use from 2014 to 2017. In addition to the 126 samples from 2017, the database includes at present 26 samples of L. multiflorum and 15 samples of L. perenne collected in a monitoring project running from 2013-2016 (A status on herbicide resistance in Denmark, 2013-2016).
The collected samples represented localities over the whole country though more samples were from Eastern part of Jutland where *Lolium* is most common.
Screening assays conducted at whole plant level revealed that 66% of the L. multiflorum populations were resistant or partly resistant to Atlantis OD (0.5L/ha), with 38% of the populations being classified as resistant, 28% as partly resistant and 34% as susceptible. Resistance to Topik (0.25 L/ha) was found for 42% of the populations with 30% partly resistant, and 12% fully resistant. Only 28% of the populations were susceptible to both herbicides, while 35% of the populations were resistant or partly resistant to both herbicides.
In L. perenne, 26% of the populations were resistant (23%) or partly resistant (3%) to Atlantis OD. Resistance to Topik was less frequent with 3% of the populations classified as resistant and 16% as partly resistant.

In total 74 % of the populations were susceptible to Atlantis OD, 81% were susceptible to Topik, and 18% of the populations were resistant to both herbicides.
Overall, the results of the screening showed resistance to one or both herbicides in 72% of the populations of L. multiflorum and 26% of the L. perenne populations. In general, the frequency of resistance was higher than found in the previous monitoring project. This is not surprising as the samples in the screening were collected in fields with suspected resistance. Further, the seed samples were collected in herbicide sprayed crops in which resistant individuals have survived and susceptible individuals have been controlled.

B. **Comments on deviations from original plan:** none

Partner: CNR

A. **Results obtained**: From 2017 to 2019, seed samples of 29 suspected resistant L. rigidum and L. multiflorum populations were collected from Italian fields, mainly cultivated with durum wheat crop, after famers' complaints for poor *Lolium* control. Field records of herbicide use and cropping systems involved were gathered from farmers.
The samples collected during this project, were added to those previously collected and confirmed resistant that are present in the Italian database (www.resistenzaerbicidi.it). The collected samples came from different regions of the country mainly from central and southern of Italy but there are also *Lolium* samples from the Piedmont region in the North. Results from the greenhouse bioassays showed that almost all the collected samples from wheat fields (25 out of 28) were resistant to at least one of the two most common herbicide modes of action used to control grass weeds in cereal crop, i.e. ALS and ACCase inhibitors.
From the *Lolium* populations collected in 2017, one was highly resistant only to Atlantis with 98 % of survival, one was resistant only to ACCase inhibitors with 50% of survival to clodinafop and 3 populations showed a multiple resistance to both Atlantis and clodinafop. The only population collected in olive grove was resistant to glyphosate with 68% of survival. Finally two populations were susceptible to all the herbicides tested.
In 2018, six populations were sampled and four of them resulted to be cross resistant to the 3 ACCase inhibitors, clodinafop, cycloxydim and pinoxaden with survival values varying according to the herbicide and the population (from 38 to 100%). One population was multiple resistant to Atlantis and clodinafop and another one was susceptible to all the tested herbicides.
Among the fifteen samples collected in 2019, six were resistant to the ACCase inhibitors clodinafop with survival values ranging between 74 and 94 % and cross-resistant to pinoxaden and cycloxydim in most of these populations with overall lower survival values ranging between 22 and 63% and between 36-90% for pinoxaden and cycloxydim, respectively. Eight populations were multiple resistant to ACCase inhibitors and Atlantis and one was highly resistant only to Atlantis (about 90 % of survival).

B. **Comments on deviations from original plan: none**

PARTNER: DEMETER

A. **Results obtained:** Thirty-nine (39) L. rigidum populations were collected from different Greek fields in summer 2017 after farmers' complaints for not effective control of ryegrass. For each population, historical field data was also collected. The crop systems involved were mainly wheat and orchard.
The results of the screening assays revealed low efficacy of Atantis OD (0.75L/ha) on L. rigidum populations that came from arable crop fields. More specifically, there were 25 resistant (<50% efficacy) populations, 1 partial resistant (50-80% efficacy) population and 13 susceptible (>80% efficacy) populations.
The efficacy of the ACCase inhibitor pinoxaden on the *Lolium* populations was more pronounced compared to the ALS inhibitor Atlantis OD. There were 14 resistant, 5 partial resistant and 20 susceptible populations.
All the 19 pinoxaden-resistant populations were also resistant to mesosulfuron + iodosulfuron (Atlantis OD) indicating the presence of multiple resistant populations (i.e. resistance to two mode of action herbicides).
The treatment with glyphosate at 720 g a.e./ha showed that 2 populations from orchards were resistant.

Overall, in the tested populations resistance to ALS inhibitors was more evident compared to the ACCase inhibitors. The latter can be still an option for the control of L. rigidum however, this will probably not last, based on the 19 pinoxaden resistant populations that came from arable (not orchards) fields. The 19 multi-resistant populations to ALS and ACCase indicate the need for different mode of action or alternative to chemical weed control method. Two glyphosate resistance cases were evident in orchard crops in Greece, due to the repeated use of this herbicide. However, these populations were susceptible to both ALS and ACCase inhibitors.

B. **Comments on deviations from original plan: none**

WP1- Task 2:

Partner: CNR

A. **Results obtained:** Thirty-nine (39) L. rigidum populations were collected from different Greek fields in summer 2017 after farmers' complaints for not effective control of ryegrass. For each population, historical field data was also collected. The crop systems involved were mainly wheat and orchard.
The results of the screening assays revealed low efficacy of Atantis OD (0.75L/ha) on L. rigidum populations that came from arable crop fields. More specifically, there were 25 resistant (<50% efficacy) populations, 1 partial resistant (50-80% efficacy) population and 13 susceptible (>80% efficacy) populations.
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All the 19 pinoxaden-resistant populations were also resistant to mesosulfuron + iodosulfuron (Atlantis OD) indicating the presence of multiple resistant populations (i.e. resistance to two mode of action herbicides).
The treatment with glyphosate at 720 g a.e./ha showed that 2 populations from orchards were resistant.
Overall, in the tested populations resistance to ALS inhibitors was more evident compared to the ACCase inhibitors. The latter can be still an option for the control of L. rigidum however, this will probably not last, based on the 19 pinoxaden resistant populations that came from arable (not orchards) fields. The 19 multi-resistant populations to ALS and ACCase indicate the need for different mode of action or alternative to chemical weed control method. Two glyphosate resistance cases were evident in orchard crops in Greece, due to the repeated use of this herbicide. However, these populations were susceptible to both ALS and ACCase inhibitors.

B. **Comments on deviations from original plan: none**

PARTNER: AU

A. **Results obtained:** The iMAR application has been developed for Denmark and it is currently functioning at the following URL: relium-dk.agriserv.org.
Overall, 167 entries for the Danish database (126 L. multiflorum and L. perenne samples collected in 2017 and 41 samples collected before the project (2013-2016)) were inserted. The data inputs include all information about the collected *Lolium* populations: weed name, year, sample code, sub code (sub-category for a sample), municipality, locality, crop system, sample origin, coordinates, sampling date and comments. The data management system is flexible and allows to add new active ingredients, weed species or cropping systems. The database can be exported and edited in an Excel spreadsheet format.
In addition, for each population the result of resistance assessment was reported. A population is ascribed as resistant when more than 20% of the treated plants survived the recommended herbicide field dose.
Each *Lolium* population has a unique identifier which is geo-localized through the geographic database that contains the geographic information (physical boundaries) of the Danish municipalities.
The section of the dynamic mapping is accessible to end users and it allows to create customized maps of herbicide resistance distribution in Denmark. Through a multiple query including four drop-down menus, the user is guided to generate the desired map by selecting at least one criterion among cropping system, region, weed species or type of resistance.

B. **Comments on deviations from original plan: none**

Partner: Demeter

A. **Results obtained:** The iMAR application has been developed for Greece and it is currently functioning at the following URL: relium-gr.agriserv.org
The application is available and operative also in Greek version.
All the *Lolium* samples collected during the project were inserted in the database with all information: weed name, year, sample code, sub code (sub-category for a sample), municipality, locality, crop system, sample origin, coordinates, sampling date and comments. The data management system is flexible and allows to add new active ingredients, weed species or cropping systems. The database can be exported and edited in an Excel spreadsheet format.
In addition, for each population the result of resistance assessment was reported. A population is ascribed as resistant when more than 20% of the treated plants survived the recommended herbicide field dose.
Each *Lolium* population has a unique identifier which is geo-localized through the geographic database that contains the geographic information (physical boundaries) of the Danish municipalities. The geographic and resistance databases are related through the id- municipality.
The section of the dynamic mapping is accessible to end users and it allows to create customized maps of herbicide resistance distribution in Greece. Through a multiple query including four drop-down menus, the user is guided to generate the desired map by selecting at least one criterion among cropping system, region, weed species or type of resistance.

B. **Comments on deviations from original plan:** none

WP2 - Characterization of the resistance mechanisms involved

WPL: Solvejg K. Mathiassen – AU
Responsible partners: DEMETER, CNR, AU, AUA

Overall summary of main results and conclusions WP2

The dose-response experiments with ALS- and ACCase inhibitors confirmed multi-resistance in populations from all three countries. High levels of resistance to Atlantis was found in all countries. The resistance level to ACCase was lower in the Danish populations compared to the Italian and Greek populations. The molecular analyses confirmed the presence of mutated alleles at several different amino acid positions in the ALS- and ACCase genes in populations from Greece and Italy indicating target-site resistance to be the main resistance mechanism. In contrast only one mutation in the ALS gene was identified in the Danish populations and it was present in only one population indicating that the main resistance mechanism in Denmark is non-target site. A wide range of responses to glyphosate was recorded in the 6 populations tested with resistance indexes (RIs) ranging from 1 to 6. For the Greek populations, one was susceptible to glyphosate, two were moderately resistant, and two were highly resistant. The Italian population was moderately resistant to glyphosate. In conclusion, the resistance pattern varied between the countries.
The validation of the LAMP tests showed a potential for the identification of mutations in the ACCase gene. On the contrary, the design of LAMP protocols for detecting ALS mutations was more challenging and the validation tests gave less reliable results.
In conclusion, the LAMP protocols appear most suitable for monitoring the presence of ACCase mutant *Lolium* spp. plants in the field and this early detection allows farmers to adopt management strategies at earlier stages.
The expression patterns of four HMR genes, glycosyl-transferase (GT), nitronate monooxygenase (NMO), cytochrome P450 (CYP72A-1) and cytochrome P450 (CYP72A-2) and the reference gene IDE showed significant differences between plants from the same population implying that the *Lolium* populations in general are heterogenous for the gene

expression of one or more HMR genes. Consequently, there is a potential for evolution of metabolic based herbicide resistance in most populations. The results of the present study do not support the development of a diagnostic tool for NTSR based on consistent constituent overexpression of specific HMR genes.
Mutations in the EPSPS gene are known to confer target-site resistance to glyphosate. Sequencing of six populations of L. rigidum revealed no point mutations in the Pro106 position known to confer resistance thus indicating non-target site resistance to be the most likely mechanism. The impact of four different ABC transporter genes on resistance was studied indicating ABC transporter gene type 3 to be the best candidate for a marker gene discriminating resistant and susceptible populations at early stages which was confirmed by studies of a resistant population from conservation agriculture in Italy.

Report on the results obtained (A) and changes to the original plan/ WP objectives (B) by tasks and partners:

WP2- Task 1: Determination of pattern and level of resistance to ALS, ACCase and glyphosate
Partner: CNR, AU, DEMETER

A. **Results obtained**: Dose response experiments were conducted to examine the level of resistance of *Lolium* populations from Greece, Italy and Denmark to ALS- and ACCase inhibitors. Multi-resistance to an ALS inhibitor (Atlantis WG containing i.e. mesosulfuron-methyl + iodosulfuron-methyl-sodium) and two ACCase inhibitors (clodinafop and pinoxaden) was confirmed in all the populations tested, however a different resistance pattern was observed within the 3 countries. The Danish populations were highly resistant to mesosulfuron + iodosulfuron (RI > 37), moderately resistant to clodinafop (2 < RI < 7), whereas they showed no or a low level of resistance to pinoxaden (2 < RI < 5). The Greek populations were highly resistant to mesosulfuron + iodosulfuron (9 < RI < 7 1) and to clodinafop (17 < RI < 32), while resistance indexes to pinoxaden varied from a low level (RI= 3) to a high level (RI > 39) between populations. Similarly, the Italian populations were highly resistant to mesosulfuron + iodosulfuron (20 < RI< 68) and to clodinafop (8 < RI < 27), with a lower level of resistance to pinoxaden (6 < RI < 20), except for one population showing no resistance to pinoxaden. Multi-resistance to ALS and ACCase inhibitors was confirmed in all the populations tested, however a different resistance pattern was observed in the different countries. The results revealed that herbicide resistance to ACCase inhibitors was more pronounced in Greece and Italy compared to Denmark.
   Dose response experiments with glyphosate were conducted with six populations (five from Greece and one from Italy). A wide range of responses was recorded with resistance indexes (RIs) ranging from 1 to 6. Four of these populations had survival rates higher than 70% at the recommended dose (1N) and in two of the populations more than 60% of the plants survived the 8N dose. However, the growth of the surviving plants was strongly reduced and biomass was reduced by more than 75%. For the Greek populations one was susceptible to glyphosate, two were moderately resistant, and two were highly resistant. The Italian population was moderately resistant to glyphosate.

   Detailed report is available in deliverable D2.1

B. **Comments on deviations from original plan**: none

WP2- Task 2: TSR in *Lolium* spp. population resistant to glyphosate, ALS and ACCase inhibitors
Partner: CNR, AU, DEMETER

Results obtained: Twelve *Lolium* spp. populations, multi-resistant to ACCase (acetyl-CoA carboxylase) and ALS (acetolactate synthase) inhibitors from Italy, Denmark and Greece, were selected within WP1 and task 2.1. Genomic DNA (gDNA) of 5 to 10 selected ALS resistant plants from each population were analysed for mutations in the ALS and ACCase genes. If no ACCase mutations were found in the ALS-selected plants, five additional plants selected with an ACCase inhibitor were also analysed.
The most frequently identified mutations endowing resistance were considered for setting up the LAMP (Loop Mediated Isothermal Amplification) tests. This molecular diagnostic tool has been developed to detect the point mutation in position

1781 of the ACCase gene in Beckmannia syzigachne (Pan et al. 2014). In this study, *Lolium* spp. specific LAMP protocols were set-up for three ALS (197, 376 and 574) and four ACCase (1781, 2041, 2078 and 2096) point mutations endowing herbicide resistance.
The validation of the LAMP tests showed a potential for the identification of mutations in the ACCase gene. On the contrary, the design of LAMP protocols for the individuation of ALS mutations was more challenging and the validation tests gave less reliable results.
In summary, the LAMP protocols appear most suitable for monitoring the presence of ACCase mutant *Lolium* spp. plants in the field and this early detection allows farmers to adopt management strategies at earlier stages.
Detailed report is available in deliverable 2.2.

C. **Comments on deviations from original plan:** Deliverable delayed due to COVID19

WP2- Task 3: Study of NTSR in *Lolium* spp. populations resistant to ALS and ACCase inhibitors
Partner: AU, CNR, DEMETER

Results obtained: Non- target site resistance (NTSR) is considered to be polygenic inherited involving various genes encoding metabolic enzymes. Previous studies have identified over-expression of herbicide metabolism related (HMR) genes in resistant individuals of *Lolium* species. We examined the expression pattern of HMR genes in nine populations of L. multiflorum from Greece, Italy and Denmark and two L. perenne populations from Denmark. The expression patterns of the four HMR genes, glycosyl-transferase (GT), nitronate monooxygenase (NMO), cytochrome P450 (CYP72A-1) and cytochrome P450 (CYP72A-2) and the reference gene IDE showed significant differences between plants from the same population implying that the *Lolium* populations in general are heterogenous for the gene expression of one or more HMR genes. Consequently, there is a potential for evolution of metabolic based herbicide resistance in most populations. The results of the present study do not support the development of a diagnostic tool for NTSR based on consistent constituent overexpression of specific HMR genes.

Detailed report is available in deliverable D2.3.1

Comments on deviations from original plan: It was planned that AU should have conducted comparative transcriptomics of resistant populations collected in Denmark, Greece and Italy. The main purpose of the transcriptome analyses was to identify novel genes across different countries playing role in evolution of herbicide resistance and use them as diagnostic tool for non-target herbicide resistance. Unfortunately, the post doc who had experience in transcriptome analysis left AU for another position and we did no longer have the expertise in-house to conduct comparative transcriptomics.
Previously we had identified four herbicide metabolism related (HMR) genes in L. multiflorum (Mahmoud et al., 2016. Frontiers in Plant Science, 7, 1160). Instead of mining genes once again using RNAseq and check expression pattern of the genes in resistant populations collected from different countries, we analysed the expression patterns of these genes in the different populations. In case of consistent constituent over-expression of any these genes in examined resistant population, this would be ideal to use as diagnostic tool for NTSR. The name of these genes was glycosyl-transferase (GT), nitronate monooxygenase (NMO), cytochrome P450 (CYP72A-1) and cytochrome P450 (CYP72A-2). Rab GTPase (RGTP) and isocitrate dehydrogenase (IDE) was used as internal reference genes.

WP2- Task 4: Detection of resistant mechanisms in glyphosate-resistant *Lolium* spp.
Partner: AUA, Benaki Phytopathological Institute (BPI), CNR

A. **Results obtained:** Rigid ryegrass (L. rigidum Gaud.) is considered to be one of the major grass weed species in the Mediterranean region. Herbicide resistance in *Lolium* species has been reported in several habitats, such as agricultural fields, orchards, vineyards, and road sides. Rigid ryegrass is considered to be one of the most economically important weeds in several countries (Travlos et al. 2018) including Greece. Mutations in the EPSPS gene are known to confer target-site resistance to glyphosate. Sequencing of six populations of L. rigidum revealed no point mutations in the Pro106 position known to confer resistance thus indicating non-target site

resistance to be the most likely mechanism. The impact of four different ABC transporter genes on resistance was studied indicating ABC transporter gene type 3 to be the best candidate for a marker gene discriminating resistant and susceptible populations at early stages which was confirmed by studies of a resistant population from conservation agriculture in Italy. Determination of shikimic acid's concentration in the plants' tissue is a reliable and easy way to investigate the level of resistance to glyphosate. Seven biotypes, four resistant and three susceptible, were studied and their shikimate accumulation was estimated 4 days after the plants were treated with the recommended dose of glyphosate (N = 720 g. a.i. ha-1). The results indicated only two significant differences between populations, with population 12 being the most susceptible and population 16 being the most resistant populations.

Detailed report is available in deliverable 2.3.1

B. **Comments on deviations from original plan:** None

WP3 Resistance management guidelines and dissemination
WPL: Thomas Gitsopoulos, HAO-DEMETER
Responsible partners: DEMETER, CNR, AU

Overall summary of main results and conclusions WP3
Pan-European, Country- and cropping system-specific guidelines for herbicide resistant *Lolium* spp. management in winter cereals and perennial crops and guidelines for management of herbicide resistant *Lolium* used as cover crop in conservation agriculture have been developed. The guidelines aim at combining cultural tools with mechanical and chemical control in the context of Integrated Weed Management (IWM).
Pan-European guidelines have been listed depending on the status of herbicide resistance in the fields (confirmed or not identified yet) for both ALS/ACCase inhibitors and glyphosate (EPSP inhibitor) in winter cereals and perennial crops (orchards and vineyards), respectively. In case of confirmed herbicide resistance (re-active situation), the guidelines aim at managing resistance, preventing further spread and depleting the soil weed seed bank. In fields where no herbicide resistance has been identified (pro-active situation), guidelines aim at preventing or to delaying its development.
Country- and cropping –system guidelines for managing *Lolium* spp. herbicide resistance have been written separately for each country in each local language based on local policies, agronomic conditions, cropping systems, available sites of action of herbicides and resistance mechanisms involved.
Guidelines for management of herbicide resistant *Lolium* used as cover crop in Conservative Agriculture have been developed due to recent case of a glyphosate *Lolium* spp. selected from *Lolium* cover crop in Italy. Similarly, as indicated above, these guidelines have been separated in pro- and re-active strategies.
Open days and final workshop were cancelled due to Covid-19 pandemic.

Detailed results are reported in deliverables 3.1.1, 3.1.2 and 3.2.1 attached to this report.

Report on the results obtained (A) and changes to the original plan/ WP objectives (B) by tasks and partners:

WP3- Task 1: Development of guidelines for managing herbicide resistance in *Lolium* spp. and optimizing its control in winter cereals and perennial crops
Partner: HAO-Demeter, CNR, AU

A. **Results obtained:** Pan-European guidelines for the management of herbicide resistance *Lolium* spp. in winter cereals and perennial crops and Country- and cropping systems specific guidelines have been developed. Pattern and level of resistance of *Lolium* spp. populations from the 3 countries studied in WP1 and WP2 have been associated with specific management factors and general principles of managing herbicide resistance. Historical field data on crop and resistance management, crop rotation and tillage practices, herbicide use and cropping systems in each country have been used to develop the Pan-European guidelines for managing resistance to both ALS/ ACCase inhibitors and glyphosate (ESPS inhibitor) in winter cereals and perennial crops

(orchards and vineyards), respectively. The unifying strategy of the pro- and re-active guidelines written in English has been the reduction of viable weed seeds into the soil seed bank and maintaining low weed seed banks to minimize population proliferation, evolution of herbicide resistance to additional sites of action and spread. Country- and cropping system-specific guidelines have been written in Greek, Italian and Danish based on local policies, country specific crops and crop rotations, registered site of actions, level of *Lolium* spp. resistance and cropping systems in each country. These guidelines refer to management of *Lolium* spp. resistance both in winter cereals (ALS/ACCase inhibitors) and orchards/vineyards (glyphosate) in Greece and Italy, and, to management of *Lolium* spp. resistance in winter cereals (ALS/ACCase inhibitors) in Denmark.

B. Comments on deviations from original plan: delay of Deliverable due to Covid-19 pandemic

WP3- Task 2: Development of guidelines for managing herbicide resistance of *Lolium* spp. used as a cover crop in conservation agriculture
Partner: CNR

A. **Results obtained:** Guidelines for management of herbicide resistant *Lolium* used as a cover crop in Conservation Agriculture have been developed based on the status of glyphosate resistance of *Lolium* spp. populations from results derived from WP2. Guidelines have been listed in two tables; one regarding cases of confirmed resistance, with curative strategies aiming at alternative tactics in the frame of IWM to prevent further spread and deplete the soil seed bank, and the other with proactive strategies aiming at delaying or avoiding the development of glyphosate resistance. In most fields where no glyphosate resistance has been identified, proactive strategies should be adopted to prevent or to delay glyphosate resistance evolution.

B. Comments on deviations from original plan: delay of Deliverable due to Covid-19 pandemic.

WP3- Task 3: Communication and dissemination
Partner: HAO-Demeter, CNR, AU

C. Results obtained: Communication and dissemination mainly through websites and scientific papers.

D. **Comments on deviations from original plan:** final workshop and field days were cancelled due to Covid-19 pandemic.

Status of milestones and deliverables

Milestone No.	Milestone name	Planned delivery month[1)]	Actual delivery month[1)]	Reasons for changes/delay and explanation of consequences
M1.1.	List of resistant *Lolium* populations and field information for each country	M15	M15	
M1.2.1	Technical report on required adaptations and available web facilities in DK and GR	M4	M4	
M1.2.2	First version of web-based application adapted to Danish and Greek situations	M12	M12	
M1.2.3	Final version of the web-based	M18	M18	

	application in Denmark and Greece			
M2.1.1	Harmonized protocols for dose-response experiments defined and agreed	M6	M6	
M2.2.1	Mutations endowing TSR to ALS and ACCase inhibitors in selected populations identified	M18	M18	
M2.2.2	LAMP protocol for detection of ALS-resistant mutant plants defined	M20	M31	Technical problems in the lab, and maternity leave of a researcher
M2.2.3	LAMP protocol for detection of ACCaseresistant mutant plants defined	M25	M32	Technical problems in the lab, and maternity leave of a researcher
M2.2.4	LAMP protocol for detection of glyphosateresistant mutant plants defined	M30	-	No mutations causing TSR to glyphosate were found
M2.3.1	RNA extracted from selected samples for RNAseq	M12	M18	Growing plants for extraction took longer time than expected
M2.3.2	Genes and microRNA endowing metabolic resistance identified	M24	-	This milestone was deleted due to a change for this task as explained under Task 2.3
M2.3.3	Genes endowing NTSR validated through quantitative real time PCR	M30	-	As above
M2.3.4	Chromatographic technique to compare transcript based diagnostic tool developed	M34	-	As above
M2.4.1	Mutations endowing TSR to glyphosate identified	M18	-	No mutations present
M2..4.2	Protocols for shikimate analysis finalized	M20	M20	
M2.4.3	Information on expression levels of key genes for NTSR acquired	M30	M30	
M2.4.4	Characterization of *Lolium* population from conservation agriculture	M30	M36	Delayed due to shortage of seeds

	determined			
M3.1.1 and M3.1.2	Field data and resistance mechanisms analysed and integrated into strategies for *Lolium* management in winter cereals and perennial crops, respectively.	M33	M39	Due to Covid-19 pandemic
M3.2.1	Field data on crop management and resistance mechanisms integrated into strategies for *Lolium* management in conservation agriculture.	M33	M39	Due to Covid-19 pandemic
M3.3.1	National stakeholders identified.	M6	M6	
M3.3.2	Open-day meetings announced	M32	Not reached	Due to Covid-19 pandemic

Deliverables

Deliverable No.	Deliverable name	Planned delivery month[1)]	Actual delivery month[1)]	Reasons for changes/delay and explanation of consequences
D1.1	Updated database of herbicide resistant *Lolium* spp. for each country	M33	M33	
D1.2	Maps of herbicide resistant *Lolium* biotypes generated by the web-based application in the three countries	M33	M33	
D2.1	Report of *Lolium* resistance indexes to glyphosate, ALS and ACCase.	M18	M24	Time to reproduce the Italian populations for the dose-response experiments
D2.2	Molecular tool to detect ALS-, ACCase and glyphosate TSR plants	M32	M39	Technical problems in the lab, maternity leave of a researcher
D2.3.1	Molecular tool to detect ALS- and ACCase-NTSR plants	M34	M38	This deliverable was changed as explained under task 2.3
D2.4.1	Report on resistance	M35	M38	Technical problems in the

	mechanisms endowing glyphosate resistance			lab
D3.1.1	Pan-European guidelines for the management herbicide resistant *Lolium* written in English and published (web).	M35	M40	Delays in results of WP2. Covid-19 pandemic
D3.1.2	Country- and cropping system-specific guidelines for the management herbicide resistant *Lolium* spp. written in local languages and published (web).	M35	M41	Delays in results of WP2. Covid-19 pandemic, therefore the deliverable was finalised a month later than the official end of the project
D3.2.1	Guidelines for management of herbicide resistant *Lolium* used as a cover crop in conservative agriculture published (web).	M35	M41	Covid-19 pandemic, therefore the deliverable was finalised a month later than the official end of the project
D3.3.1	Open-day meetings organised and presentations made available on the web	M36	Not delivered	Due to restrictions consequent to Covid-19 pandemic

[1] Measured in months from the project start date (month 1)

Outputs of the consortium

Publications

List of published scientific papers in peer-reviewed journals. Please indicate accessibility of the publication (Open Access, Thomson Reuters Web of Science, SCOPUS etc.)

- Scarabel L., Panozzo S., Loddo D., Mathiassen S.K., Kristensen M., Kudsk P., Gitsopoulos T., Travlos I., Tani E., Chachalis D, Sattin M., 2020. Diversified resistance mechanisms in multi-resistant *Lolium* spp. in three European Countries. Frontiers in Plant Science 11, 608845. Doi: 10.3389/fpls.2020.608845 Open access).
- Gerakari M., Tani E., Chachalis D., Travlos I., Loddo D., Mathiassen S.K., Gitsopoulos T., Scarabel L., Panozzo S., Kristensen M., Kudsk P., Sattin M. (in preparation). Rapid identification of glyphosate resistant biotypes of *Lolium* rigidum through biochemical and molecular analyses.
- Gitsopoulos T., Travlos I., Tani E., Chachalis D., Scarabel L., Panozzo S., Loddo D., Mathiassen S.K , Kristensen M., Kudsk P., Sattin M. (in preparation). Guidelines for managing resistance of *Lolium* spp. in winter cereals in Greece, Italy and Denmark based on research and farmers' practice in each country.
-

Total number of items at this level: 1 published and 2 in preparation

List of <u>non-peer-reviewed scientific publications, proceedings and books</u>:

-
-
-

Total number of items at this level:

List of non-scientific publications:

-
-
-

Total number of items at this level:

List of press releases, interviews and TV appearances:

-
-

Total number of items at this level:

Events with stakeholders (if applicable): cancelled due to COVID19

Event	Aim/location/date	Approximate number of attendees
All planned events were cancelled due to COVID-19 pandemic		

Training sessions conducted (if applicable)

Description of training course	Approximate number of attendees

Methods, techniques, tools etc.

Description of methods, techniques, tools etc. developed in the frame of the project:

	Description
New methods, techniques, tools e.g. a method to monitor or attract a specific pest species	To confirm the resistance status of the suspected resistant *Lolium* populations collected during the project, a common bioassay at whole plant level was followed. To break dormancy, seeds are vernalized at 4°C for 3 days and then moved to a germination chamber and kept for 5 days at the following conditions: temperature (day/night) 25/15 °C, 12 hour photoperiod. Germinated seedling are then transplanted into pots (20/25 plants/pot) filled with a standard potting mix. The substrate is maintained at or near field capacity. When the seedlings are at the 3-4 true leaf stage, they are treated with an ALS inhibitor, an ACCase inhibitor and glyphosate at the maximum recommended field rates. Herbicide efficacy was assessed four weeks after treatment based on the number of surviving plants and the visual estimation of their biomass. Set up the LAMP (Loop Mediated Isothermal Amplification) tests for detecting resistance endowing mutation in the ACCase and ALS genes.
Patent applications, other IP e.g. patent for extraction process of lure/attractant	
Prototypes, pilots e.g. a trap prototype	Interactive web platform developed for Danish and Greek partners to create customized maps of herbicide resistance distribution at national level.
Marketable product/service e.g. a trap, lure, pheromone etc.	

Explanation of the use of resources

Funding

(All requested amounts should be expressed as thousands of euros. E.g.: 1.357.900 euro should be written as € 1357.9 in the answer box.)

Effective funding sources

Partner no.	EU funds (ERA-NET)		Other external public funds		External private funds		Own funds		**Total funds €**
	€	%	€	%	€	%	€	%	
P1 CNR	49.85	99					0.6	1	50.35
P2 DEMETER	25.0	50					25.0	50	50.0
P3 AU	184.3	85					18.3	15	202.6
TOTAL	259.2						43-9		303.1

Some of funds for travelling/meetings were not used due to cancellation of the final meeting of the project as well as the field days due to Covid-19 pandemic.

Effective costs

Partner no.	Personnel	Travelling/meetings	Consumables/Equipment	Subcontracts	Other costs	**Total effective Costs**
P1 CNR	33.77	0.42	7.33	-	3.39 (Coordination) +4.98(overheads)	49.8
P2- HAO-DEMETER	-	5.383	12.0	6.0	-	23.383
P3	109.4	4.0	17.9	11.9	57.7	200.9
TOTAL	143.17	9.803	37.23	17.9	66.07	274.173

Human resources

Total number of people in partner teams

Indicate the number of employees of the following positions that were permanent staff members / that were hired especially for the project *(only include people that were paid by ERA- NET funds):*

Partner no.	Researchers with PhD more than 3 years / experienced scientists		Researchers PhD post- docs / young scientists		PhD students		Master students		Support or technical staff		Other	
	Permanent	Hired	Permanent	Hired	Permanent	Hired	Permanent	Hired	Permanent	Hired	Permanent	Hired
P1	3	1							1			

P2- HAO-Demeter	3+3*								2			
P3	3								3			
P4												
P5												
P6												
P7												
P8												
TOTAL	9+3*	1							6			

*The 3 additional researchers are not staff of HAO-DEMETER but they belong to the permanent staff of Benaki Phyto-pathological Institute -Greece (1) and to Agricultural University of Athens-

How many people completed any of the following qualifications through their work on the ERA-NET funded project and/or using funding from the ERA-NET project?
Number of PhDs: Number of MSc, MEng:

Outcomes - effects of the project on the team and the institutions

Knowledge

Short description of the effects the project had (regarding skills, understanding of the concerned research fields, stakeholder expectations, end users's needs and consortium
partner's expertise). Did the research quality increase? *(Please compare to the period before the project started)*

The project was a unique opportunity for gaining a better understanding of the evolutionary process leading to herbicide resistance and develop transnational management strategies that were adapted to national conditions.
This was a European project where each participating country focused on specific tasks and complemented each other to devise novel weed management strategies. Partners continuously exchanged information and plant material. Using an interdisciplinary approach, all important issues and aspects relevant to herbicide resistance management in *Lolium* in the three participating countries were considered.
The interactive web-based applications developed during the project for Greek and Danish partners are freely available and regularly updated to show in real time maps of the spread of *Lolium* resistant populations at national levels.
The improved the knowledge on herbicide resistance in each country partner to better define specific resistance management strategies aiming at delaying the evolution of resistant populations.
The overall research quality significantly increased.

Network and cooperation

Short description of the effects the project had on networking and cooperation (cooperation of consortium partners, formation of new R&D partnerships, improved public-private cooperation, increased transnationality or transdisciplinarity, access to complementary expertise) (Please compare to the period before the project started)

RELIUM was a great opportunity for the partners to increase networking and cooperation which allowed to produce innovative tools (i.e. iMAR application, LAMP test for resistance and scientific papers)

Economy and strategy

Additional funding received through the achievement of this ERA-NET project (during or after
the completion of the project). Please indicate the source(s) and amount(s) of funding received
for carrying out (a) new project(s).

(All requested amounts should be expressed as thousands of euros. E.g.: 1.357.900 euro should be written as € 1357.9 in the answer box.)

Project acronym and approach[1)]	Participating partners (partner no.)	EU Framework Programmes / Horizon 2020 €	Other EU funds €	National funds €	Other public funds €	Private funds €	Own funds €	TOTAL €

[1)] R&D, Implementation, Commercialisation

Impacts - effect of the project on users and society at large

General Questions

How do you judge the information transfer of your results among the user communities? To what extent did your results reach the desired circles?

The dissemination of the developed iMAR application in Greece and Denmark will be faciltated as it is freely available on the web. To reach a broader audience and the end users, the application in Greece is also available in the local language.
The COVID-!) pandemic significantly impacted on the information transfer to the end users. However, most of information produced by the project is will shortly be available online.

Impacts on the research community

Do you know of any projects that were launched based on the results of your project?
Yes/No
If yes, please name them:

Please indicate the number of students/staff who have worked on the ERA-NET funded project (only if staff members were hired especially for the project, no permanent staff members), that chose the following (first) career destinations after finishing their involvement with the project.

Partner no.	Employment: private sector research	Employment: private sector non-research	Employment: public sector research	Employment: public sector non-research	Further study	Seeking employment	Don't know
P1						1	
P2							
P3							

Short description of the impacts the project had on the research environment (increased mobility of researchers, increased research activities, improved information exchange)

The web-based application for mapping herbicide resistant cases may be of interest to other researchers who would like to adopt similar web-based platform in their countries.
RELIUM significantly improved the information exchange between the partners and ultimately the quality of research.

Impact on industry/ service sector

Short description of the impacts resulting from the project (requests received from end users/companies concerning the use of your results, further development or commercialisation of results by industry)
Herbicide resistance has a significant impact on agrochemical industry. Some of the results produced by RELIUM are being used to produce a fact-sheet on resistance in *Lolium* spp. on collaboration with the European Herbicide resistance Action Committee (HRAC)

Anticipated impact on farmers and society at large

Short description of the anticipated impacts resulting from the project (anticipated implementation of your solution by farmers, improvement of the situation of farmers, impact on society at large)
RELIUM provided important information to improve herbicide resistance management of one of the key weeds in Europe. The information produced will be available online to the public. The freely available web application to create customize maps of the spread of weed resistant populations at national level will improve the awareness of farmers and all the stakeholders on herbicide resistance spread in their country. This will allow to improve weed control strategies that are not only based on herbicides having the same mode of action.

1. Updated database of herbicide resistant *Lolium* spp. for each country

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D1.1**
Deliverable title:	**Updated database of herbicide resistant *Lolium* spp. for each country**
WP number:	**WP1**
Lead beneficiary:	**Laura Scarabel**
Main author(s):	**Thomas Gitsopoulos, Solvejg Mathiassen, Laura Scarabel**
Delivery date:	**06.03.2020**
Actual delivery date:	**26.03.2020**

Executive Summary

During the 3 years of the project, 185 *Lolium* populations suspected to be resistant to ALS and /or to ACCase inhibitors have been collected in cereal (mainly wheat) fields. In addition, 9 suspected resistant *Lolium* populations have been collected in perennial crops (olive grove or orchards) where there has been a wide used of glyphosate. The seed sampling in the 3 countries has been performed following farmers' complaints and most of the collected samples were accompanied with the historical field data provided by farmers. From this information it appears that the most common herbicides used in the 3 countries to control *Lolium* plants in wheat fields belong to two modes of action, ALS and ACCase inhibitors. In perennial crops the main herbicide used in Greece and Italy is glyphosate. Due to the repeated use of these herbicide families, the selection pressure is high.

Overall, whole plant bioassays conducted on the collected samples confirmed that 21% of these populations were resistant only to the ALS inhibitor, iodosulfuron + mesosulfuron, 9% were resistant to at least one of the ACCase inhibitors tested (clodinafop, pinoxaden or cycloxydim) and 38% of the populations were multiresistant to the above mentioned modes of action. Three *Lolium* populations out of 9 populations collected in olive grove or orchards were found to be resistant to glyphosate. No multiresistance including glyphosate was observed in the tested populations.

The confirmation in the 3 countries of a high number of *Lolium* populations being resistant to both modes of action, ALS and ACCase inhibitors, is worrying and indicates that the chemical control based only on post-emergence herbicides is not sustainable. A greater effort has to be made to better diversify the *Lolium* management by integrating different chemical and non-chemical tools.

The lists of the *Lolium* cases confirmed resistant during the project have been entered into the database of each country enabling the production of resistance risk maps described in Deliverable 1.2.

Introduction

Lolium species are widespread and are considered as noxious weeds in Denmark, Greece and Italy. The evolution of resistant populations is becoming an issue and a major threat to the sustainability of cultivating winter cereals and perennial crops. Due to more stringent regulation of pesticide use in Europe, few herbicide modes of action are available and their intensive use to control grass weeds in winter cereals imposes a high selective pressure on weed populations.

The sampling of suspected resistant *Lolium* populations based on farmers' complaints and the subsequent identification of resistance through whole plant bioassays will provide the opportunity to create or update any existing national database of the resistant *Lolium* cases in the three countries and to visualize these resistant cases on the map system developed for the Greek and Danish partners in task 1.2. In addition, the historical field data collected from the farmers allows the evaluation of the main factors that lead to resistance and is a good background for preparing specific *Lolium* management strategies (activity of WP3).

Description of work
Denmark sample collection and screening

Material and Methods
In 2017, Institute of Agroecology, Aarhus University (AGRO) offered farmers a free of charge resistance test on *Lolium* multiflorum and *Lolium* perenne from fields where resistance was suspected. Farmers were asked to send seed samples to AGRO with information on location (address and GPS coordinates), crop, cultivation system (no till, reduced tillage, ploughing) and herbicide use from 2014 to 2017. The offer was launched via the agricultural advisory system.
AGRO received in total 94 samples of L. multiflorum and 32 samples of L. perenne. Most of the samples were accompanied with the requested information. The samples were collected in herbicide sprayed fields, consequently the composition of resistant and susceptible individuals in the samples was influenced by the selection pressure of the herbicide applied in the fields in the year of sampling.
In addition to the 126 samples from 2017, the database includes at present 26 samples of L. multiflorum and 15 samples of L. perenne collected in a monitoring project running from 2013-2016 (A status on herbicide resistance in Denmark, 2013-2016). These samples were collected in untreated plots in GEP registration trials conducted all over Denmark and therefore present the actual composition of resistant and susceptible individuals in the year of sampling.

Historical field data
The available historical field data for the samples collected in the RELIUM project are shown in Table 1.

TABLE 1. Historical field data for the samples collected to the RELIUM project

Weed species	**Sampling year**	**Number of samples**	**Sampling crop**	**Tillage system**	**Information on herbicide use**
L. multiflorum	2017	94	winter wheat (69) winter barley (2) spring barley (12) no info (10)	no till (11) reduced till (10) conventional (63) no info (10)	82 samples
L. perenne	2017	32	winter wheat (21) winter rye (1) winter oilseed rape (2) no info (8)	no till (4) conventional (18) no info (10)	23 samples

Screening

A screening for resistance to ALS- and ACCase inhibiting herbicides was conducted for all samples collected in the RELIUM project. The seeds were cleaned and sown in trays with 124 holes in a potting mixture. The trays were watered and placed in a glasshouse at a temperature of 14°/10°(day/night). Supplemental light to a day length of 14 hours. Each population was sown in three trays. In addition to the collected samples trays with susceptible and resistant populations of L. multiflorum and L. perenne were included in the screening.
The trays were sprayed when the plants had 2-3 leaves. For each population one tray was sprayed with 0.25 L/ha Topik (100 g/L clodinafop + 25 g/L cloquintocet-mexyl) + 0,5 L/ha Renol, one tray with 0.5 L/ha Atlantis OD (10 g/L mesosulfuron + 2 g/L iodosulfuron + 30 g/L mefenpyr-diethyl) and one tray remained untreated. Application was conducted in a

cabinet pot sprayed equipped with two ISO-02 flat fan nozzles at a pressure of 3 bars and a velocity of 5.2 km/h delivering a volume of 172.8 L/ha.
The plants were harvested 4 weeks after herbicide application. A visual assessment of the effect was made before harvest. For each population the biomass of treated trays was given a score compared to the untreated using a scale from 0 to 10 (0 = no reduction of biomass, 10= total control). Values between 0 and 10 show the relative reduction of biomass. The number of dead and alive plants in each tray was counted. The plants were cut at the soil surface and fresh and dry weights were recorded. The effect on number of plants (= % dead plants) and the effect on biomass (% reduction in fresh and dry weight per plant compared to untreated) was calculated. The effects were compared with effects on susceptible and resistant reference populations.

Results and discussion
The collected samples represented localities over the whole country though more samples were from Eastern part of Jutland where *Lolium* is most common.

TABLE 2. Criteria for classification of populations

	Resistant	Partly resistant	Susceptible
Reduction in biomass (FW)	<50%	50% - 80%	>80%
Dead plants	<50%	50% - 80%	>80%
Visual assessment	0-4	5-8	>8

The applied herbicides represent the two main modes of action used for control of grass weeds in Denmark. Resistance to both groups of herbicides has been identified in previous screenings. Topik belongs to the group of ACCase inhibitors and Atlantis OD to the group of ALS inhibitors. Previous monitoring in Denmark showed, resistance to ALS inhibitors to be more common than resistance to ACCase inhibitors and indicated that the prevalent resistance mechanism was enhanced metabolism.

Lolium multiflorum

The results showed that 66% of the populations were resistant or partly resistant to Atlantis OD, with 38% of the populations being classified as resistant, 28% as partly resistant and 34% as susceptible (Figure 2). Resistance to Topik was found for 42% of the populations with 30% partly resistant, and 12% fully resistant. Only 28% of the populations were susceptible to both herbicides, while 35% of the populations were resistant or partly resistant to both herbicides (Figure 3).

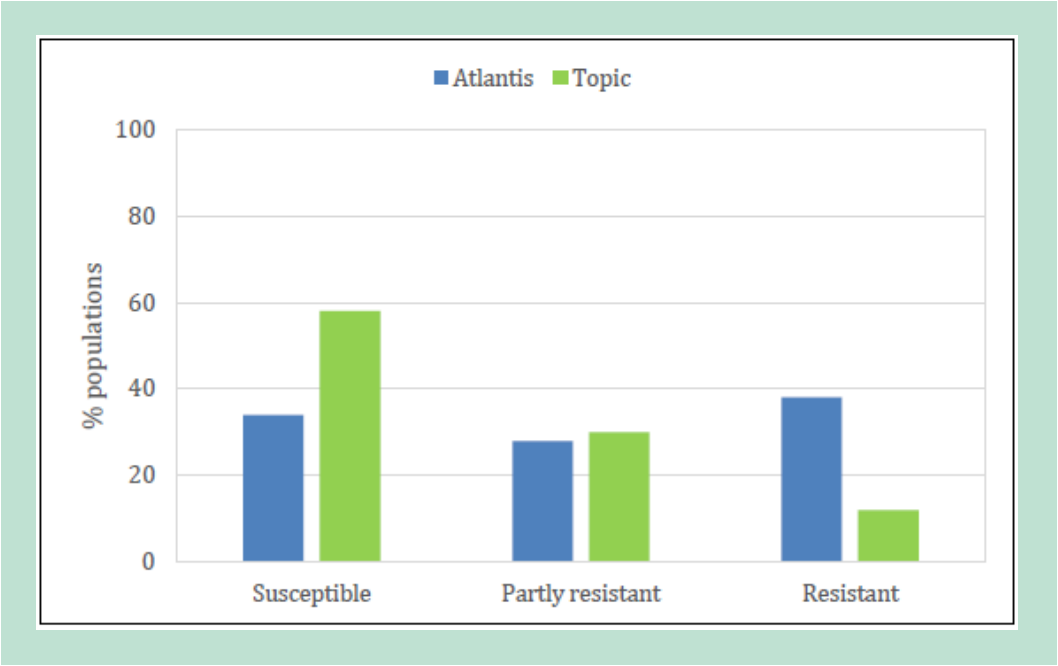


FIGURE 2. Percent populations of L. multiflorum classified as susceptible, partly resistant and resistant to Atlantis OD (blue plots) and Topik (green plots).

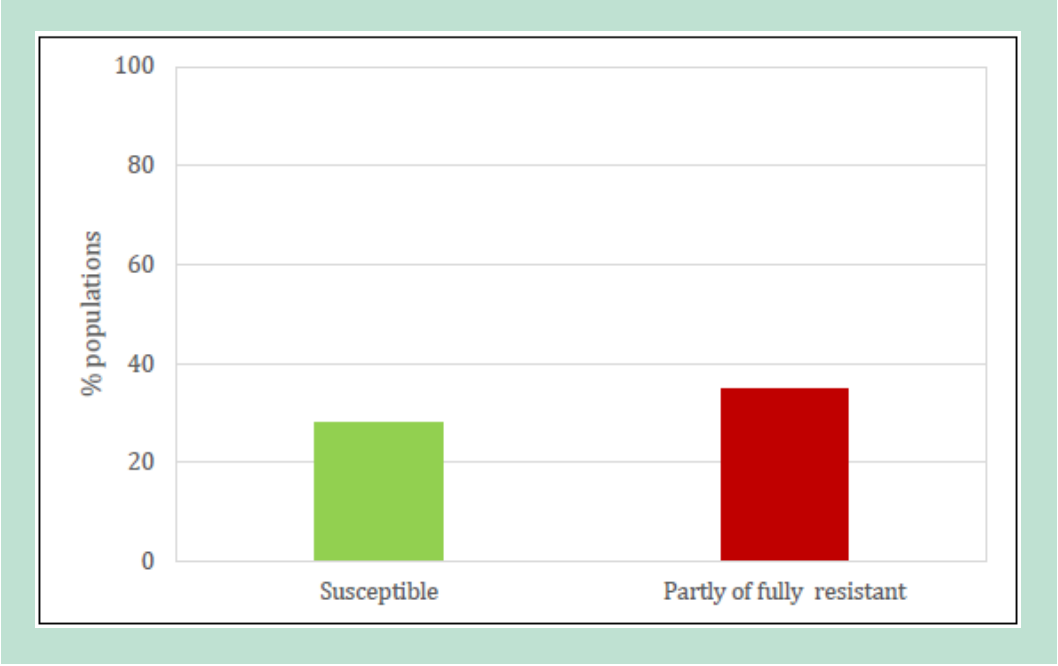


FIGURE 3. Percent populations of L. multiflorum being susceptible (green bar) and fully or partly resistant (red bar) to both Atlantis OD and Topic

Lolium perenne

In L. perenne 26% of the populations were resistant (23%) or partly resistant (3%) to Atlantis OD (Figure 4). Resistance to Topik was less frequent with 3% of the populations classified as resistant and 16% as partly resistant.

In total 74 % of the populations were susceptible to Atlantis OD, 81% were susceptible to Topik, and 18% of the populations were resistant to both herbicides (Figure 4 and 5).

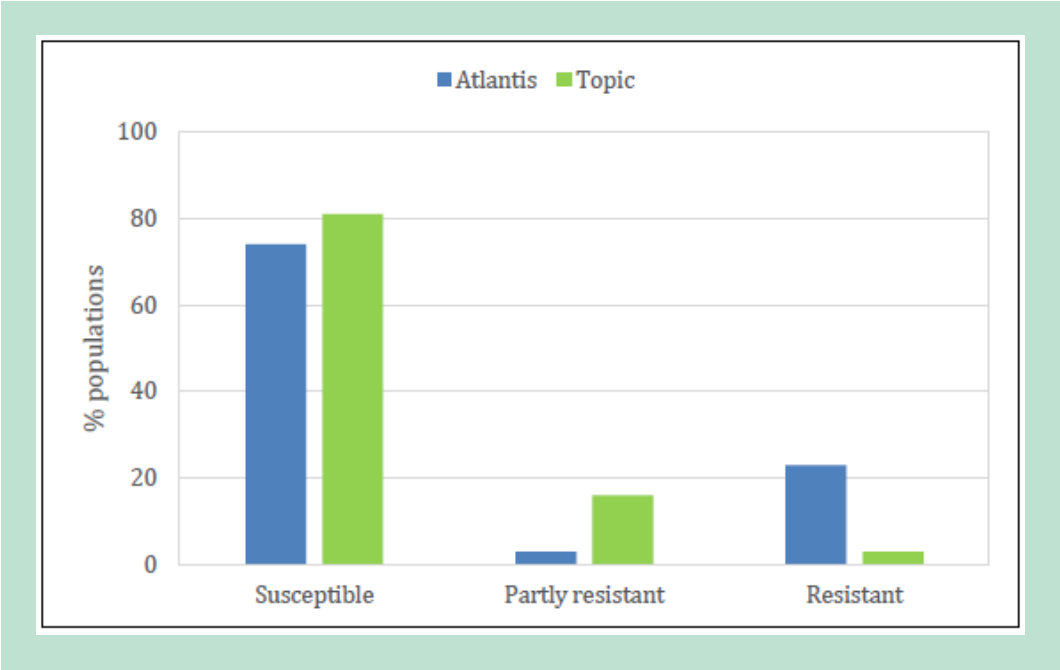


FIGURE 4. Percent populations of L. multiflorum being susceptible (green bar) and fully or partly resistant (red bar) to both Atlantis OD and Topic.

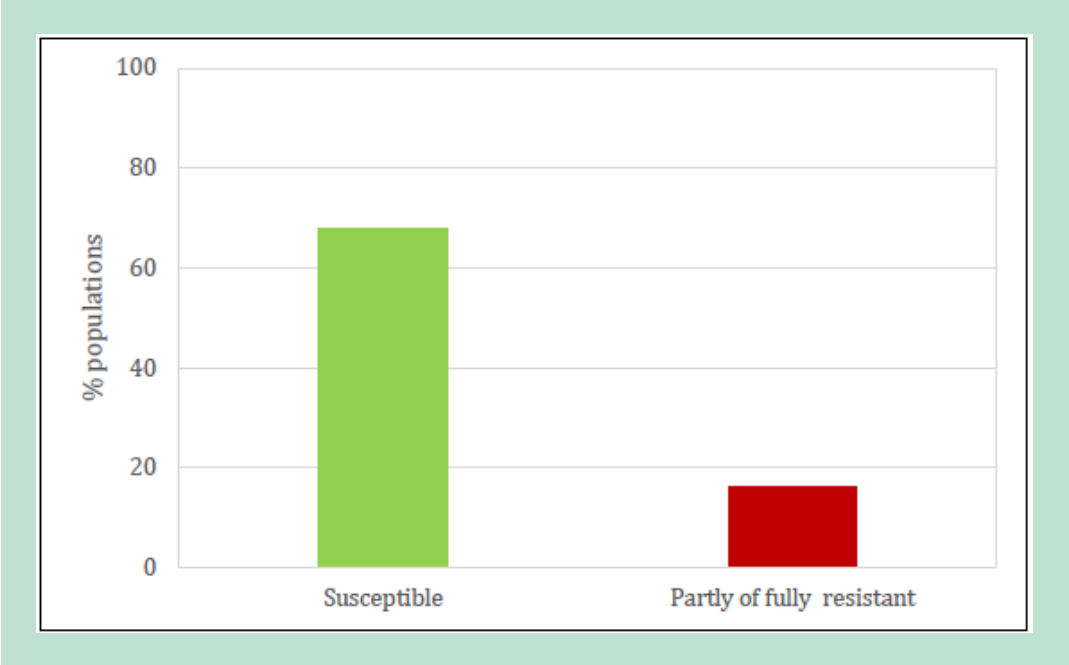


FIGURE 5. Percent populations of L. perenne susceptible (green bar) and fully or partly resistant (red bar) to both Atlantis OD and Topic.

Conclusion

The results of the screening showed resistance to one or both herbicides in 72% of the populations of *L. multiflorum* and 26% of the *L. perenne* populations. In general, the frequency of resistance was higher than found in the previous monitoring project. This is not surprising as the samples in the screening were collected in fields with suspected resistance.

Further, the seed samples were collected in herbicide sprayed crops in which resistant individuals have survived and susceptible individuals have been controlled.

Greek sample collection and screening

Material and Methods

Thirty-nine (39) L. rigidum populations (GR) were collected from 39 different Greek fields in summer 2017 after farmers' complaints for not effective control of ryegrass. The crops where ryegrass populations were collected from, the crops grown in these fields the previous three years and the herbicides applied to control ryegrass are listed in Table 1.

Three herbicides, an ALS inhibitor (mesosulfuron + iodosulfuron), an ACCase inhibitor (pinoxaden) and an EPSP inhibitor (glyphosate) were used to evaluate their efficacy (maximum recommended field rate used) on the L. rigidum populations under glasshouse conditions (Table 2). Boxes of 4lt (0.27mx0.18mx0.08m) were filled with a mixture of loam soil (40%), perlite (10%), turf (30%) and sand (20%). The soil used was loam soil with 50% sand, 14% silt, 36% clay, pH=7.61 and O.M = 2.42%. Seeds of L. rigidum were seeded in four rows in each box in order to get 20 ryegrass seedlings (4 rows with 5 plants in each row). There were two replicates for each treatment and for each population there was an untreated control. The population GR11 was included as a sensitive reference population to three herbicides used. When ryegrass plants reached the 3rd to 5th leaf stage, herbicides were applied with a boom sprayer equipped with 6 Teejet twin-jet nozzles at 400 lt/ha water volume. Forty (40) days after herbicide application (DAT) visual assessment of herbicide efficacy was performed for each population. A 0-100% scale was used to evaluate the efficacy where 0%= no herbicide effect, 100%= dead plants, compared with the untreated control of each population. The population were classified as Resistant, Partial resistance and Susceptible according to visual estimation criteria. Resistant population was considered one when a herbicide caused <50% efficacy, Partly Resistant when the efficacy ranged between 50-80% and Susceptible when the efficacy was >80%.

The trial was replicated in time. Arcsine square root transformation of the percentages of herbicide efficacy was used before analysis of variance (ANOVA). ANOVA was performed separately for each herbicide. Since there was no significant interaction effect between trials and populations, the data were pooled over the two trials. Least significant differences (5%) were used to separate difference between treatments. The results were used to create maps for herbicide L. rigidum resistance in Greece.

TABLE 1. *L. rigidum* populations (GR), the crops by the time of ryegrass seed collection, the previous crops grown in these fields and the herbicides applied the last three years against L. rigidum

Greek (GR) populations of *Lolium* rigidum*	**Sampling crop**	**Crops grown the previous 3 years****	**Herbicides used the previous three years to control ryegrass**
GR1	lupine	wheat, wheat, sunflower	Accase & ALS inhibitors
GR3	wheat	wheat, sunflower, barley	Accase & ALS inhibitors
GR4	oil-seed rape	wheat, oil-seed rape, wheat	Accase & ALS inhibitors
GR5	oil-seed rape	common vetch, wheat, chickpea	Accase & ALS inhibitors
GR6	wheat	wheat	Accase & ALS inhibitors
GR7	wheat	wheat	Accase & ALS inhibitors
GR9	oregano	wheat	Accase & ALS inhibitors

GR10	wheat	wheat	Accase & ALS inhibitors
GR11 (sensitive)	olive groves	Olive grove	No herbicides used
GR12	wheat	wheat, barley, wheat	Accase & ALS inhibitors
GR13	wheat	wheat	Accase & ALS inhibitors
GR14	wheat	oat, sunflower, wheat	Accase & ALS inhibitors
GR15	sunflower	wheat	Accase & ALS inhibitors
GR16	wheat	wheat, cotton	Accase & ALS inhibitors
GR16	wheat	wheat	Accase & ALS inhibitors
GR17	wheat	wheat	Accase & ALS inhibitors
GR18	lupine	wheat, lupine, wheat	Accase & ALS inhibitors
GR19	wheat	sunflower, wheat, sunflower	Accase & ALS inhibitors
GR20	wheat	sunflower, wheat, sunflower	Accase & ALS inhibitors
GR21	wheat	wheat	Accase & ALS inhibitors
GR22	wheat	wheat	Accase & ALS inhibitors
GR23	wheat	wheat	Accase & ALS inhibitors
GR24	wheat	pea, wheat, pea	Accase & ALS inhibitors
GR25	wheat	oil-seed rape, wheat, cotton	Accase & ALS inhibitors
GR26	No cultivation	wheat	Accase & ALS inhibitors
GR27	wheat	cotton, wheat, oil-seed rape	Accase & ALS inhibitors
GR28	oilseed rape	wheat, cotton, wheat	Accase & ALS inhibitors
GR29	wheat	sunflower, wheat, sunflower	Accase & ALS inhibitors
GR30	wheat	sunflower, wheat, sunflower	Accase & ALS inhibitors
GR31	wheat	wheat	Accase & ALS inhibitors
GR32	barley	cotton, barley, cotton	Accase & ALS inhibitors
GR33	barley	cotton, barley, cotton	Accase & ALS inhibitors
GR34	olive grove	olive groove	glyphosate
GR35	olive grove	olive groove	glyphosate
GR36	olive grove	olive groove	glyphosate
GR37	grape	grape	glyphosate

GR38	olive grove	olive groove	glyphosate
GR39	grape	grape	glyphosate
GR40	olive grove	olive groove	glyphosate
GR42	apricot	apricot	glyphosate

*GR2, GR8 and GR41 populations are not included in this research

** When one crop is listed, this indicated there was monoculture the previous 3 years

TABLE 2: herbicides and rates applied

Herbicide (Commercial name)	**formulation**	**Screen rates**
10 g/L mesosulfuron + 2 g/L iodosulfuron (Atlantis OD)	OD	15+3 g a.i./ha
pinoxaden 6% (Axial 60 EC)	EC	45 g a.i./ha
glyphosate 36% (Round UP Gold 36 SL)	SL	720 g a.e./ha

Results

Mesosulfuron +iodosulfuron: The results revealed low efficacy of the ALS inhibitor on *L. rigidum* populations that came from arable crop fields (Table 3). More specifically, there were 25 Resistant (<50% efficacy) populations, 1 Partial Resistant (50-80% efficacy) population and 13 Susceptible (>80% efficacy) populations. The susceptible populations were those from orchards where no ALS herbicide, but glyphosate, was used.
Pinoxaden: the efficacy of this herbicide was more pronounced compared to the ALS inhibitor. There were 14 Resistant, 5 Partial Resistant and 20 Susceptible populations (Table 3).
Mesosulfuron +iodosulfuron & pinoxaden: 19 populations showed resistance to both ALS and ACCase inhibitors, indicating multiple resistance. These populations were all the pinoxaden- resistant populations.
Glyphosate: only 2 populations were resistant both from orchards. The GR38 showed no symptom compared to the untreated control (Table 3).

From the results above it is concluded that in the tested populations resistance was more evident to ALS inhibitors compared to the ACCase inhibitors. The latter can be still an option for the control of *L. rigidum* however, this will probably not last, based on the 19 pinoxaden resistant populations that came from arable (not orchards) fields. The 19 multiresistant populations to ALS and ACCase indicate the need for different mode of action or alternative to chemical weed control method. Two glyphosate resistance cases were evident in orchard crops in Greece, due to the repeated use of this herbicide. However, these populations were susceptible to both ALS and ACCase inhibitors.

TABLE 3: herbicide effect* on *L. rigidum* population

Populations of *Lolium rigidum*	**Mesosulfuron + iodosulfuron**	**Pinoxaden**	**Glyphosate**
GR1	0** f***	57 e	100 a
GR3	0 f	100 a	100 a

GR4	2 de	95 abc	100 a
GR5	5 ef	10 fg	100 a
GR6	0 f	15 fg	100 a
GR7	0 f	8 gh	100 a
GR9	20 d	10 f	100 a
GR10	7 ef	100 a	100 a
GR11 (sensitive)	97 a	100 a	100 a
GR12	85 b	97 abc	100 a
GR13	13 e	18 fg	100 a
GR14	7 ef	72 de	100 a
GR15	12 e	50 e	100 a
GR16	0 f	30 f	100 a
GR17	3 ef	92 bc	100 a
GR18	3 ef	100 a	100 a
GR19	0 f	3 gh	100 a
GR20	3 e	9 g	100 a
GR21	7 ef	9 g	100 a
GR22	0 f	20 f	100 a
GR23	0 f	95 ab	100 a
GR24	0 f	0 h	100 a
GR25	50 c	85 cd	100 a
GR26	37 cd	53 e	100 a
GR27	8 e	3 gh	100 a
GR28	10 e	70 de	100 a
GR29	0 f	0 h	100 a
GR30	0 f	5 g	100 a
GR31	82 b	100 a	100 a
GR32	97 a	100 a	100 a

GR33	97 a	100 a	100 a
GR34	88 ab	100 a	40 b
GR35	95 ab	98 ab	100 a
GR36	93 ab	100 a	100 a
GR37	95 ab	100 a	100 a
GR38	95 ab	100 a	0 c
GR39	95 ab	100 a	100 a
GR40	95 ab	100 a	100 a
GR42	93 ab	100 a	100 a

*0%= no symptom, 100%=dead plant
** re-transformed data are presented in table (arcsice square root transformed data were used for the analysis)
***Same letter in the each column indicates no significant difference between means

Italian sample collection and screening

Material and Methods

From 2017 to 2019, seed samples of 29 suspected resistant *L. rigidum* and *L. multiflorum* populations were collected from Italian fields, mainly cultivated with durum wheat crop, after famers' complaints for poor *Lolium* control. Field records of herbicide use and cropping systems involved were gathered from farmers and reported in Table 1.
The samples collected during this project, were added to those previously collected and confirmed resistant that are present in the Italian database.

TABLE 1 Historical field data for the samples collected to the RELIUM project

Collection Year	Code	Municipality	Previous crops	Herbicides used the previous years
	204-L	Legnaro	Susceptible check	
2017	608	Quattordio	wheat, oilseed rape, wheat	ACCase and ALS inhibitors
2017	609	Alessandria	wheat, oilseed rape, wheat	ACCase and ALS inhibitors
2017	610	Bagnacavallo	wheat, sugarbeet, wheat	ACCase and ALS inhibitors
2017	617	Vicari	barley, wheat, bean	ACCase and ALS inhibitors
2017	619	Jelsi	wheat	ACCase and ALS inhibitors
2017	620	Caragna Piemonte	wheat	ACCase and ALS inhibitors
2017	621	Castellazzo Bormida	wheat, maize, wheat	ACCase and ALS inhibitors

2017	623	Francavilla Fontana	olive grove	Glyphosate
2018	624	Ravenna	wheat, sugarbeet, wheat	ACCase and ALS inhibitors
2018	625	Ravenna	wheat, maize, tomato	ACCase and ALS inhibitors
2018	626	Ravenna	wheat, flax, wheat	ACCase and ALS inhibitors
2018	627	Ravenna	wheat, alfaalfa, alfaalfa	ACCase and ALS inhibitors
2018	628	Russi	wheat, pea, wheat	ACCase and ALS inhibitors
2018	648	Pozzolo Formigaro	wheat, lupin, wheat	ALS inhibitor
2019	650/1	Troia	Wheat	ALS inhibitor
2019	650/2	Troia	Wheat	ALS inhibitor
2019	651	Cerignola	wheat (current crop)	not available
2019	652	Cerignola	wheat (current crop)	not available
2019	653	Castelnuovo della Daunia	wheat (current crop)	not available
2019	669	Matera	durum wheat	ACCase and ALS inhibitors
2019	670	Imola	durum wheat	ACCase and ALS inhibitors
2019	671	Faenza	wheat, sunflower	ACCase and ALS inhibitors
2019	672	Pienza	barley, wheat	ACCase inhibitors
2019	673	Pienza	wheat, bean	ACCase inhibitor and glyphosate
2019	674	Tolentino	wheat, bean, barley	ACCase inhibitor
2019	675	Macerata	wheat, oilseed rape	ACCase inhibitors
2019	676	Tolentino	wheat, bean	ACCase inhibitor and glyphosate
2019	677	Loreto	wheat, bean	ACCase and ALS inhibitors
2019	678	Alessandria	maize, wheat, sunflower, wheat	ACCase and ALS inhibitors

Seeds of each population were cleaned and preserved in paper bags at room temperature in a low humidity environment. A *Lolium* population (204L) that had never been treated with herbicides was used as susceptible reference in all the screening experiments. To break dormancy, the *Lolium* seeds were vernalized at 4°C on wet filter paper in dark condition for 3 days. Then germinated seedlings of similar growth stage were transplanted in plastic trays (325 x 265 x 95 mm) filled with a standard potting mix (silty loam soil (60%), perlite (15%), sand (15%) and peat (10%) and placed in the greenhouse. The experimental layout was a completely randomized design with two replicates of twenty five *Lolium* spp. seedlings for each tray. At the 3-5 leaf stage, the plants were treated with an ALS inhibitor (mesosulfuron + iodosulfuron), two ACCase inhibitors (clodinafop and pinoxaden) and an EPSP inhibitor (glyphosate) (see Table 2). Herbicides were applied using a precision bench delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet®, 11002). Four weeks after treatment, survival and visual estimate of biomass (VEB) in relation to the untreated check were recorded. VEB was determined giving a

score of 10 to the untreated check and 0 to replicates where all plants were clearly dead. Survival records have been expressed as percentage of n° of treated plants. Standard error was calculated per each mean value.

TABLE 2 Herbicides and rates used in the screening experiment

Herbicide (commercial name)	**Herbicide (active ingredient)**	**Italian recommended field dose**
Topik 240 EC	clodinafop 24%	60 g a.i./ha
Atlantis WG	mesosulfuron (3%) + iodosulfuron (0.6%)	15+3 g a.i./ha
Axial Pronto	pinoxaden 6%	45 g a.i./ha
Stratos ultra	cycloxydim 10%	200 g a.i./ha
MON 79351	glyphosate 48%	720 g a.e./ha

Results

The collected samples came from different regions of the country mainly from central and southern of Italy but there are also *Lolium* samples from the Piedmont region in the North.
Results from the greenhouse bioassays showed that almost all the collected samples from wheat fields (25 out of 28) were resistant to at least one of the two most common herbicide modes of action used to control grass weeds in cereal crop, i.e. ALS and ACCase inhibitors (Table 3).
From the *Lolium* populations collected in 2017, one was highly resistant only to Atlantis with 98 % of survival, one was resistant only to ACCase inhibitors with 50% of survival to clodinafop and 3 populations showed a multiple resistance to both Atlantis and clodinafop. The only population collected in olive grove was resistant to glyphosate with 68% of survival. Finally two populations were susceptible to all the herbicides tested.
In 2018, six populations were sampled and four of them resulted to be cross resistant to the 3 ACCase inhibitors, clodinafop, cycloxydim and pinoxaden with survival values varying according to the herbicide and the population (from 38 to 100%). One population was multiple resistant to Atlantis and clodinafop and another one was susceptible to all the tested herbicides.
Among the fifteen samples collected in 2019, six were resistant to the ACCase inhibitors clodinafop with survival values ranging between 74 and 94 % and cross-resistant to pinoxaden and cycloxydim in most of these populations with overall lower survival values ranging between 22 and 63% and between 36-90% for pinoxaden and cycloxydim, respectively. Eight populations were multiple resistant to ACCase inhibitors and Atlantis and one was highly resistant only to Atlantis (about 90 % of survival).

TABLE 3 Percentage of survival of the *Lolium* populations after treatments with the ALS inhibitor (iodosulfuron+ mesosulfuron), the ACCase inhibitors (clodinafop, pinoxaden and cycloxydim) and the EPSP inhibitor (glyphosate). The standard errors were reported in bracket.

		% survival (ES)				
Collectio n Year	**Code**	**Mesosulfuron + iodosulfuron**	**Clodinafop**	**Pinoxaden**	**Cycloxydim**	**Glyphosate**
2017	608	20 (8)	92 (4)	62 (10)	92 (4)	0
2017	609	94 (2)	98 (2)	54 (6)	0	0

2017	610	8 (8)	0	0	0	0
2017	617	0	0	0	0	0
2017	619	0	50 (0)	12 (12)	42 (8.3)	0
2017	620	82 (10)	84 (4)	60 (4)	10 (10)	0
2017	621	98 (2)	16 (4)	8 (4.3)	0	0
2017	623	2 (2.2)	0	0	0	68 (7.6)
2018	624	0	96 (4.2)	48 (0)	100 (0)	0
2018	625	0	2 (2)	0	0	0
2018	626	0	100 (0)	60 (0)	94 (6)	0
2018	627	0	100 (0)	44 (16)	98 (2)	0
2018	628	0	78 (2.5)	38 (18)	72 (7.6)	0
2018	648	52 (0)	46 (6)	18 (2)	0	0
2019	650/1	18 (2.6)	82 (3.1)	60 (20)	0	0
2019	650/2	30 (3.6)	94 (6)	59 (9.2)	0	0
2019	651	88 (4.3)	82 (2.4)	70 (2)	46 (2)	0
2019	652	40 (8)	70 (2)	52 (4)	40 (8)	0
2019	653	0	74 (6)	31 (14.6)	70 (5.5)	0
2019	669	0	76 (0.5)	22 (14)	36 (0)	0
2019	670	94 (2)	6 (2)	4 (4)	0	0
2019	671	39 (9.4)	92 (0)	94 (2)	27 (4.6)	0
2019	672	2 (2.1)	81 (2.9)	52 (0)	90 (2)	0
2019	673	40 (6.7)	77 (16.7)	60 (0)	96 (3.6)	0
2019	674	0	92 (3.9)	63 (4.2)	88 (4.2)	0
2019	675	37 (8.9)	94 (2)	53 (11.2)	96 (0.1)	0
2019	676	2 (2)	88 (4)	50 (2)	88 (3.8)	0
2019	677	34 (10.1)	22 (2)	2 (2)	10 (6)	0
2019	678	86 (14.3)	30 (16.7)	17 (3.3)	3 (3)	0

Discussion and conclusions

In the three countries, there are some differences among the herbicides used to control *Lolium* plants in wheat fields but they all belong to two modes of action, ALS and ACCase inhibitors. Therefore the selective pressure imposed by these herbicides is very high. A third mode of action herbicide that is widely used in perennial crop in Greece and Italy is glyphosate. This herbicide is also sometimes used in wheat as pre-emergence herbicide and substitute of soil tillage. Overall, the whole plant bioassays conducted on the suspected samples have confirmed that 66% of these populations are resistant to one or both modes of action herbicides. 21% are resistant to only the ALS inhibitor, iodosulfuron + mesosulfuron, 9% are resistant to at least one of the ACCase inhibitor tested (clodinafop for Denmark and Italy or pinoxaden for Greece) and 38% of the populations are multi-resistant to both modes of action herbicides (see Table below).
In the three countries, there are some differences among the herbicides used to control *Lolium* plants in wheat fields but they all belong to two modes of action, ALS and ACCase inhibitors. Therefore the selective pressure imposed by these herbicides is very high. A third mode of action herbicide that is widely used in perennial crop in Greece and Italy is glyphosate. This herbicide is also sometimes used in wheat as pre-emergence herbicide and substitute of soil tillage. Overall, the whole plant bioassays conducted on the suspected samples have confirmed that 66% of these populations are resistant to one or both modes of action herbicides. 21% are resistant to only the ALS inhibitor, iodosulfuron + mesosulfuron, 9% are resistant to at least one of the ACCase inhibitor tested (clodinafop for Denmark and Italy or pinoxaden for Greece) and 38% of the populations are multi-resistant to both modes of action herbicides (see Table below).

				[a]Resistant populations			
Country	**Main crop system**	***Lolium* species**	**Collected Samples**	**ALS**	**[b]ACCase**	**ALS + ACCase**	**EPSP**
Denmark	Winter wheat and spring barley	*L. perenne*	32	2	0	6	0
	Winter wheat	*L. multiflorum*	94	29	6	33	0
Greece	Wheat, monoculture or rotation with oilseed rape, sunflower or cotton	*L. rigidum*	31	7	0	19	0
	Perennial crop: olive grove, grape, apricot	*L. rigidum*	8	0	0	0	2
Italy	Wheat, monoculture or rotation with oilseed rape, bean barley or maize	*L. rigidum L. multiflorum*	28	2	11	12	0
	Olive grove	*L. rigidum L. multiflorum*	1	0	0	0	1
Overall			194	40	17	70	3

[a]Resistant populations when amost 20 % of the treated plants survived at the recommended herbicide field rate (i.e. herbicide efficacy less than 20%)
[b]ACCase inhibitor: clodinafop for Denmark and Italy; pinoxaden for Greece.

The high confirmed cases in the 3 countries of *Lolium* populations resistant to both modes of action, ALS and ACCase inhibitors, is worrying and indicates that the chemical control based only on post-emergence herbicides is not sustainable. A greater effort have to be made to better diversify the *Lolium* management by integrating different chemical and non-chemical tools.
The lists of the *Lolium* cases confirmed resistant during the project have been enter in the database of each country and these are linked to the map visualization system presented in DeliverableTask 1.2

2. Mapping of resistant *Lolium* spp. populations from the 3 countries.

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D1.2**
Deliverable title:	**Mapping of resistant *Lolium* spp. populations from the 3 countries.**
WP number:	**WP1**
Lead beneficiary:	**Laura Scarabel**
Main author(s):	**Thomas Gitsopoulos, Solvejg K. Mathiassen, Laura Scarabel, Michele Colauzzi**
Delivery date:	**31.03.2020**
Actual delivery date:	**22.04.2020**

Executive Summary

Description.
Herbicide resistant *Lolium* spp. is a major threat to the sustainability of annual and perennial crops in the EU. In the 3 countries involved in the project, different *Lolium* species have been reported to be resistant to ACCase inhibitors, ALS inhibitors or glyphosate: *L. multiflorum* in Denmark and Italy, *L. rigidum* in Greece and Italy and *L. perenne* in Denmark. The monitoring, mapping and collection of information related to agronomic practices are important in order to evaluate the spread of resistance in each country and to adopt specific resistance management strategies aiming at delaying the evolution of resistant populations. The development of a technological information and communication system is of great importance in communicating the information to farmers and improve their awareness of the problem. Interactive web-based applications, adapted from the Italian application, iMAR, have been developed for Denmark and Greece. The applications are freely available and regularly updated to show in real time maps of the spread of *Lolium* resistant populations at national levels. The use of this computer tool, continuously updated and promptly disseminated, can support farmers, stakeholders and decision makers of the Regional Phytosanitory Services in herbicide resistance issues.

Introduction

Cases of herbicide resistance is increasing worldwide involving many weed species and various cropping systems. The high reliance and repeated use of limited herbicide modes of action, very effective to control weeds, are prone to evolve resistance. To maintain the effectiveness of herbicides, the adoption of specific strategies aiming at diversifying the management practices by integrating them with non-chemical practices is crucial. It is important to develop technological information and communication approaches to present the information on the spread of resistance to farmers and all stakeholders in order to promote their awareness of the problem, The mapping of the resistant weed populations, continuously updated and promptly disseminated is one way to reach this goal.

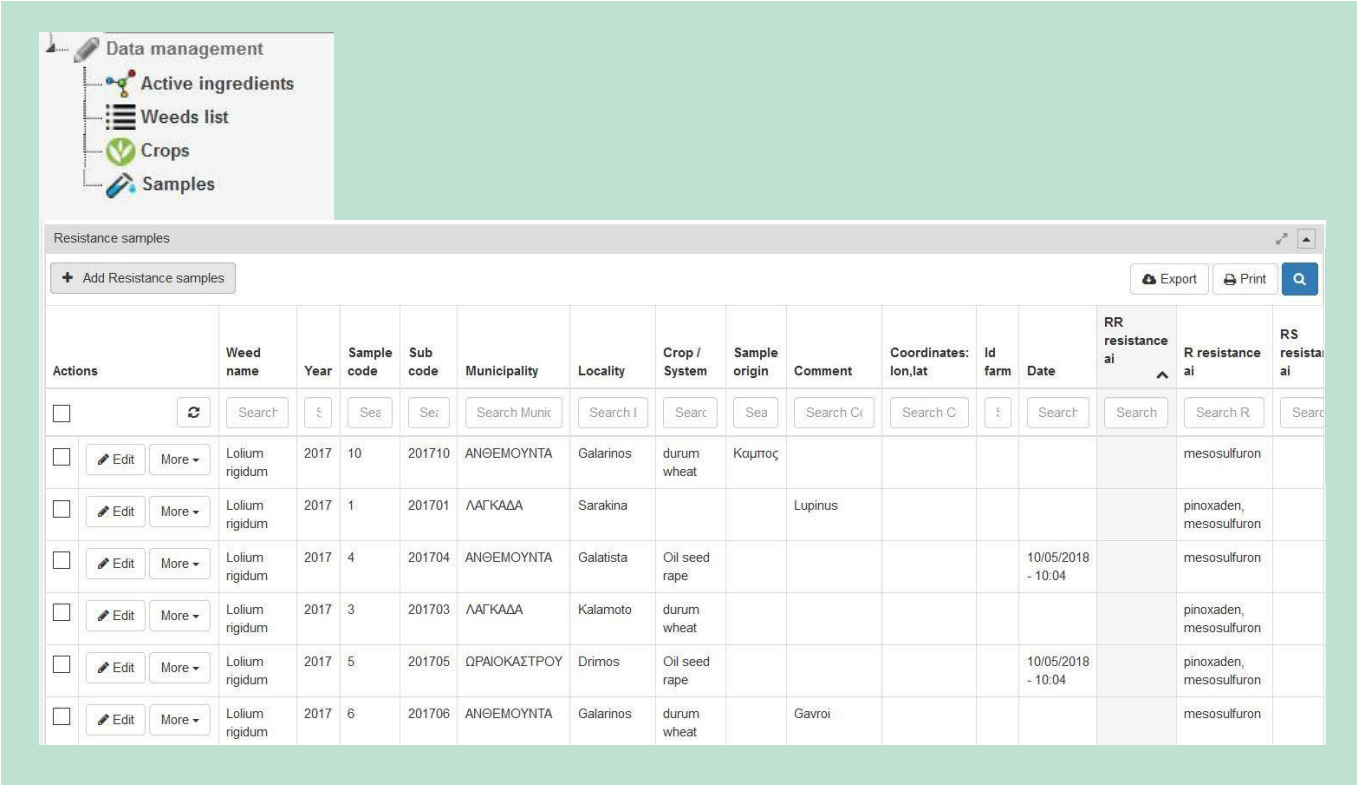
Description of work

The interactive web based application, iMAR, developed by the Italian partner for mapping herbicide resistant weed (Panozzo et al. 2015), has been made available to the project partners and has been adapted to Greek and Danish conditions.
The iMAR application is currently available on the GIRE web site ww.resistenzaerbicidi.it
All the data of *Lolium* populations collected in Italy and confirmed to be herbicide resistant during this project were entered to update the existing Italian database.

Interactive web based applications developed for Danish and Greek partners
The interactive web platform (iMAR) was created by the web developer for Danish and Greek partners. The system is based on free software tools and the infrastructure includes two sections:
Data management where input and editing of the sample data is done.
Dynamic mapping, generating customized maps based on a few selection criteria.

The URLs to the working web-platforms are : relium-gr.agriserv.org and relium-dk.agriserv.org

Data management
All the *Lolium* cases confirmed to be resistant through bioassay trials conducted in Task 1.1 were entered in the respective databases of the web-based applications. Overall 167 entries for the Danish database (126 *L. multiflorum* and *L. perenne* samples collected in 2017 and 41 samples collected before the project (2013-2016)) and 35 entries for the Greek database were inserted. The data inputs include all information about the collected *Lolium* populations: weed name, year, sample code, sub code (sub-category for a sample), municipality, locality, crop system, sample origin, coordinates, date and comments (as an example, Fig. 1).The data management system is flexible and allows to add new active ingredients, weed species or cropping systems.



Actions	Weed name	Year	Sample code	Sub code	Municipality	Locality	Crop / System	Sample origin	Comment	Coordinates: lon,lat	Id farm	Date	RR resistance ai	R resistance ai	RS resistance ai
Edit More	Lolium rigidum	2017	10	201710	ΑΝΘΕΜΟΥΝΤΑ	Galarinos	durum wheat	Κομπος						mesosulfuron	
Edit More	Lolium rigidum	2017	1	201701	ΛΑΓΚΑΔΑ	Sarakina			Lupinus					pinoxaden, mesosulfuron	
Edit More	Lolium rigidum	2017	4	201704	ΑΝΘΕΜΟΥΝΤΑ	Galatista	Oil seed rape					10/05/2018 - 10:04		mesosulfuron	
Edit More	Lolium rigidum	2017	3	201703	ΛΑΓΚΑΔΑ	Kalamoto	durum wheat							pinoxaden, mesosulfuron	
Edit More	Lolium rigidum	2017	5	201705	ΩΡΑΙΟΚΑΣΤΡΟΥ	Drimos	Oil seed rape					10/05/2018 - 10:04		pinoxaden, mesosulfuron	
Edit More	Lolium rigidum	2017	6	201706	ΑΝΘΕΜΟΥΝΤΑ	Galarinos	durum wheat		Gavroi					mesosulfuron	

FIGURE 1. Data input

In addition, for each population the result of resistance assessment was reported. A population is ascribed as resistant when more than 20% of the treated plants survived the recommended herbicide field dose.

Each *Lolium* population has a unique identifier which is geo-localized through the geographic database that contains the geographic information (physical boundaries) of Greek prefectures or Danish municipalities. The geographic and resistance databases are related through the id- municipality.
For the Danish map, the current geographic boundaries, based on municipalities, will be substituted with smaller geographic areas, based on postal codes.

The database can be exported and edited in an Excel spreadsheet format.

Map generation and visualization
The section of the dynamic mapping allows to create customized maps of herbicide resistance distribution in Denmark and Greece. This part is accessible to end users. Through a multiple query including four drop-down menus, the user is guided to generate the desired map by selecting at least one criterion among cropping system, region, weed species or type of resistance (Figure 2). The date is operative if associated with one of these criteria.
For each criteria there is the option “all” that allows to create maps that include all types of resistance, all weed species, all regions or all cropping systems.

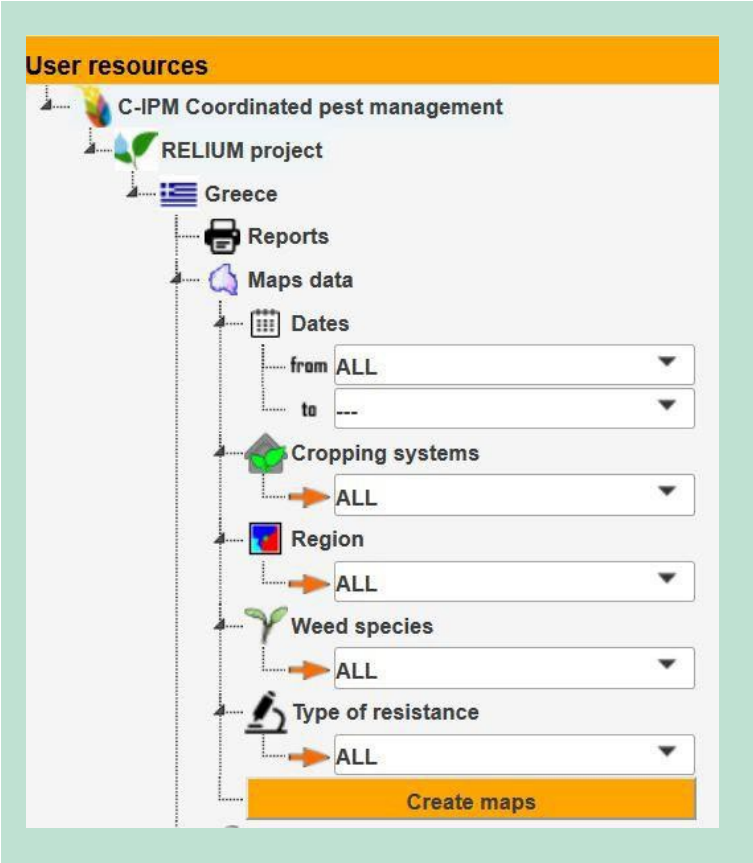


FIGURE 2. The drop-down menu available to create the maps

The distribution maps of the resistant *Lolium* biotypes appear by changing the color of the municipality (for Denmark) or prefecture (for Greece) where at least one population has been confirmed to be resistant. Different types of resistance appear with different colours (i.e yellow= resistance to ALS inhibitors; red=resistance to ACCase inhibitors; orange= multiple resistance to ALS and ACCase inhibitors; green=resistance to glyphosate) (Figure 3).
For each municipality or prefecture, a popup window appears indicating the number of resistant cases that have been detected for each type of resistance.

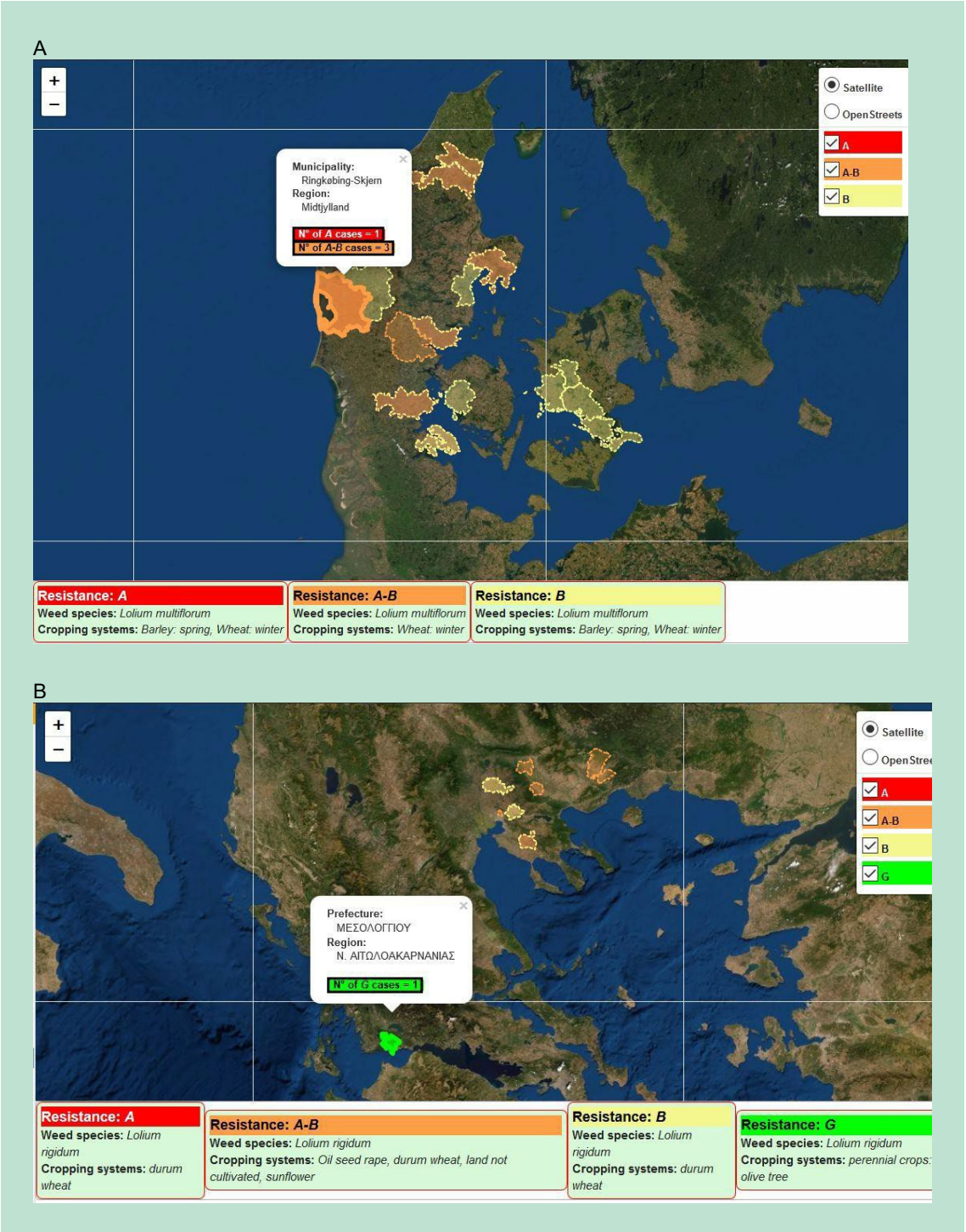


FIGURE 3. Examples of *Lolium* resistance maps created by using the Danish (A) and Greek (B) web- based application.

Below the map a number of boxes equal to the types of resistance (single or multiple) reported in that municipality appears summarizing the weed species involved and the cropping system affected.
In the white box in the upper right part of the generated map it is possible to select or deselect all the types of resistance visualized on the map.

Discussion and conclusions

The interactive web-based application, iMAR, for mapping herbicide resistant populations was developed and adapted to the Greek and Danish conditions.

Currently, the input data include the resistant *Lolium* populations tested during this project but the system can be extended to include larger datasets (i.e other weed species, herbicides, types of resistance…etc).

A relevant issue of the map system is the low cost. The system has been developed entirely with the use of open source technology, and this makes it fully adaptable to most hardware and software configurations. It has been optimized for Firefox and Chrome browsers and to run on a Microsoft Windows operating system (Windows 7 or later).

The freely available and regularly updated system allows to show in real time maps of diffusion of *Lolium* resistant populations at national level. This allows all stakeholders to be timely informed on the presence and spread of resistant weed populations. Potentially this will increase the awareness of the resistance problem and will facilitate the adoption of appropriate management tools to delay the development and spread of resistant weeds.

References

Panozzo S., Colauzzi M., Scarabel L., Collavo A., Rosan V., Sattin M. (2015) iMAR: An Interactive Web-Based Application for Mapping Herbicide Resistant Weeds. PoS ONE 10(8): e0135328. Doi:10. 137/journal.pone.0135328.

3. Report of *Lolium* resistance indexes to glyphosate, ALS and ACCase inhibitors

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D2.1**
Deliverable title:	**Report of *Lolium* resistance indexes to glyphosate, ALS and ACCase inhibitors**
WP number:	**WP2**
Lead beneficiary:	**Solvejg K. Mathiassen**
Main author(s):	**Thomas Gitsopoulos, Ilias Travlos, Solvejg K. Mathiassen, Donato Loddo, Laura Scarabel**
Delivery date:	**06.10.2019**
Actual delivery date:	**24.04.2020**

Executive Summary

Dose response experiments were conducted to examine the level of resistance of *Lolium* populations from Greece, Italy and Denmark to glyphosate and ALS- and ACCase inhibitors. Six populations (five from Greece and one from Italy) were tested for susceptibility to glyphosate and a wide range of responses was recorded with resistance indexes (RIs) ranging from 1 to 6. For the Greek populations one was susceptible to glyphosate, two were moderately resistant, and two were highly resistant. The Italian population was moderately resistant to glyphosate. Multi-resistance to an ALS inhibitor (Atlantis WG containing i.e. mesosulfuron-methyl + iodosulfuron-methyl- sodium) and two ACCase inhibitors (clodinafop and pinoxaden) was confirmed in all the populations tested, however a different resistance pattern was observed within the 3 countries. The Danish populations were highly resistant to mesosulfuron + iodosulfuron (RIs > 37), moderately resistant to clodinafop (2 < RI < 7), whereas they showed no or a low level of resistance to pinoxaden (2 < RI < 5). The Greek populations were highly resistant to mesosulfuron + iodosulfuron (9 < RI < 7 1) and to clodinafop (17 < RI < 32), while resistance indexes to pinoxaden varied from a low level (RI= 3) to a high level (RI > 39) between populations. Similarly, the Italian populations were highly resistant to mesosulfuron + iodosulfuron (20 < RI< 68) and to clodinafop (8 < RI < 27), with a lower level of resistance to pinoxaden (6 < RI < 20), except for one population showing no resistance to pinoxaden.

Introduction

Reliance on glyphosate as a principal tool for weed control in perennial crops for the last 30 years has resulted in the evolution of several resistant weed species and changes in the weed flora composition. In winter cereals ALS- and ACCase inhibitors have been widely used as the main weed control method against grass weeds for many years. The continuous use of the same modes of action has led to the development of resistance to both of these herbicide groups.

Seeds of *Lolium* populations collected from fields grown with perennial and arable crops with incidence of low level of weed control in Denmark, Greece and Italy were used to determine the pattern and level of resistance to glyphosate and to, ALS- and ACCase inhibitors, respectively in dose response experiments. Effective doses for obtaining 50% reduction in number of surviving plants (LD50) and in biomass (GR50) were calculated together with the resistance indexes (RIs) based on LD50 data.

Description of work

Glyphosate

Five biotypes of *Lolium* from Greece (G33, G34, G36, G37, G40) and one from Italy (IT581) were included in the dose response experiment (Table 1). To break seed dormancy, seeds were placed in petri dishes on wet filter paper and vernalized in a fridge at 4 °C under dark conditions for 3 days. Then, the seeds were placed in a germination chamber and kept for 5 days at 25/15 (day/night) °C with a 12 hours photoperiod. Germinated seedlings at similar growth stages were transplanted into pots (15 x 15 x 20 cm) filled with a standard potting mix (50% silty loam soil, 25% perlite and 25% peat). The pots were placed outdoors and the moisture was maintained at field capacity. Each pot contained 10 seedlings. Eight glyphostate doses were applied: 1/16 N, 1/8 N, 1/4 N, 1/2 N, 1 N, 2 N, 4 N and 8 N (Table 2) with 4 replicate pots per treatment plus untreated. The glyphosate doses were applied at BBCH 14-21 (from 4 true leaves up to 1 tiller, usually 15-20 days after seedling transplanting) in a spray volume of 300 L/ha.

TABLE 1: List of *Lolium* populations used in the glyphosate dose response experiment.

Population	Country
G33	Greece
G34	Greece
G36	Greece
G37	Greece
G40	Greece
IT581	Italy

TABLE 2: Glyphosate doses applied in dose response experiment

Treatment	1/16 N	1/8 N	¼ N	½ N	1 N*	2 N	4 N	8 N
Glyphosate dose (g ae/ha)	45	90	180	360	720	1440	2880	5760

*dose that corresponds to the field dose

Dose response data were recorded 4 weeks after the treatment (4 WAT). Plant survival and fresh weight of biomass were recorded for each pot. Plant survival (%) was calculated as the number of survived plants after treatment x 100/ number of alive plants before treatment. Fresh biomass was measured after collecting and weighting all plant material, including dead plants. Fresh biomass was expressed as percentage of the untreated control. Effective dose values based on plant survival (LD50, LD60, LD80, LD90) and biomass (GR50, GR60, GR80, GR90) were calculated. Resistance indexes were calculated for each population as the ratio of the LD50 of the test population/ LD50 of the susceptible reference. The G33 population served as susceptible reference for the calculation of resistance indexes.

ALS and ACCase inhibitors

In order to determine the pattern and level of resistance to ALS- and ACCase inhibitors twelve *Lolium* populations (four from each country, Table 3) were chosen based on the initial screening results (Task 1.1) for the outdoor dose response experiment.

TABLE 3: List of *Lolium* populations used in the dose-response experiment

Country	**DENMARK**	**GREECE**	**ITALY**
Code	DK100LM[a]	G33[a]	IT204L[a]
	DK06LP	G9	IT533
	DK29LM	G20	IT595
	DK47LM	G24	IT609
	DK90LM	G30	IT620

[a] Susceptible reference populations

Vernalization was performed as described for glyphosate. Eight herbicide doses were applied: 1/16 N (only for the susceptible populations), 1/8 N, ¼ N, ½ N, 1 N, 2 N, 4 N and 8 N (only for the resistant populations). There were three replicates per treatment, each one with 9 seedlings. The dose response experiment was performed twice, with one experiment in the spring 2018 and one in the autumn 2018. All populations were treated with two ACCase inhibitors, pinoxaden (Axial pronto, 60 g a.i./L, field dose 0.75 L/ha) and clodinafop (Topik 240, 240 g a.i./L, field dose 0.25 L/ha) and one ALS inhibitor, (Atlantis WG, 30 g a.i./kg mesosulfuron-methyl + 6 g a.i./kg iodosulfuron methyl-sodium (in the following refered to as mesosulfuron + iodosulfuron), field dose= 500 g/ha). The doses are shown in Table 4.

TABLE 4: Mesosulfuron + iodosulfuron, pinoxaden and clodinafop doses used in the dose response experiments

	1/16 N	1/8 N	¼ N	½ N	1 N*	2 N	4 N	8 N
Mesosulfuron + iodosulfuron (g a.i./ha)	0.94 + 0.19	1.88 + 0.38	3.75 + 0.75	7.5 + 1.5	15 + 3	30 + 6	60 + 12	120 + 24

Pinoxaden (g a.i./ha)	2.81	5.63	11.25	22.50	45	90	180	360
Clodinafop (g a.i./ha)	3.75	7.5	15	30	60	120	240	480

*dose that corresponds to the field dose.

The herbicides were applied at BBCH 21-22 (1-2 tillers) in the spring experiment and at BBCH 13- 14 (3-4 true leaves) in the autumn experiment. All treatments were applied in a spray volume of 300 L/ha.

The herbicide effect was evaluated 4 WAT. Plant survival and fresh biomass were recorded for each pot (replicate). Plant survival (%) was estimated as the number of surviving plants after the treatment x100/number of alive plants before the treatment. Fresh biomass was measured collecting and weighting all plant material including dead plants. Fresh biomass was then expressed as % of the untreated control. The dose response data were analysed using a log-logistic equation in the macro BIOASSAY®, developed by Onofri and running in Windows Excel® environment. LD50 (based on plant survival data), GR50 (based on fresh weight data) and relative standard errors were estimated. Resistance indexes were calculated as the ratio between the LD50 (or GR50) of each resistant population and the LD50 of the susceptible reference specific for each country. The results reported here are RIs calculated on LD50 data.

Results

Glyphosate

The responses of the different populations to the applied glyphosate doses are shown in Figures 1 to 12.

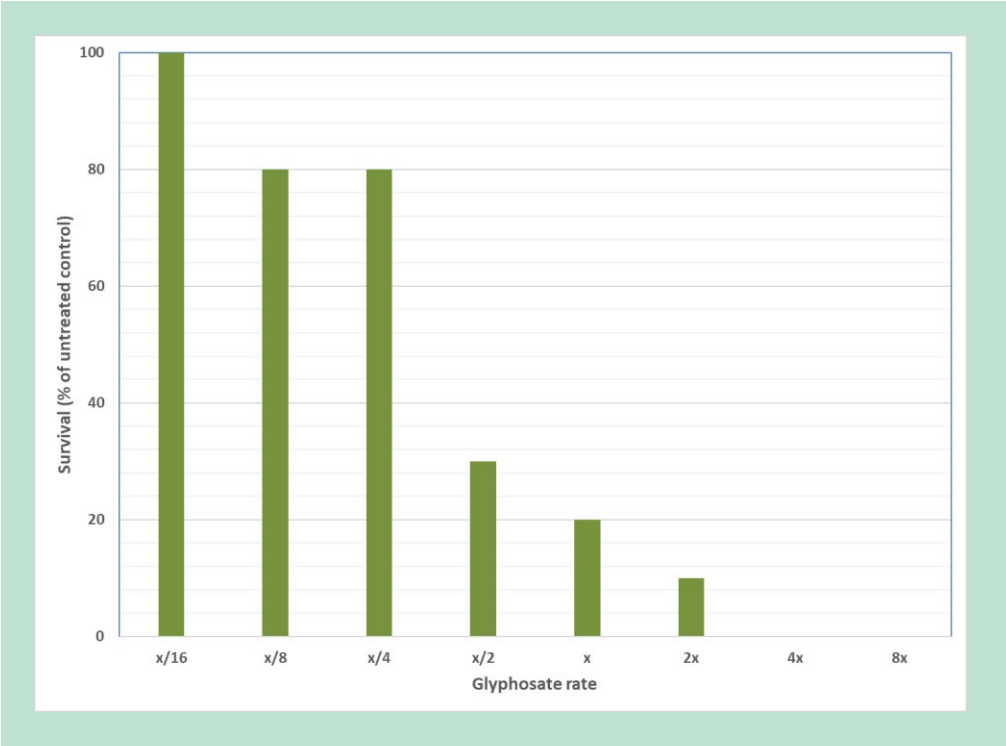


FIGURE 1. Effect of glyphosate on survival of population G33 at 4 WAT

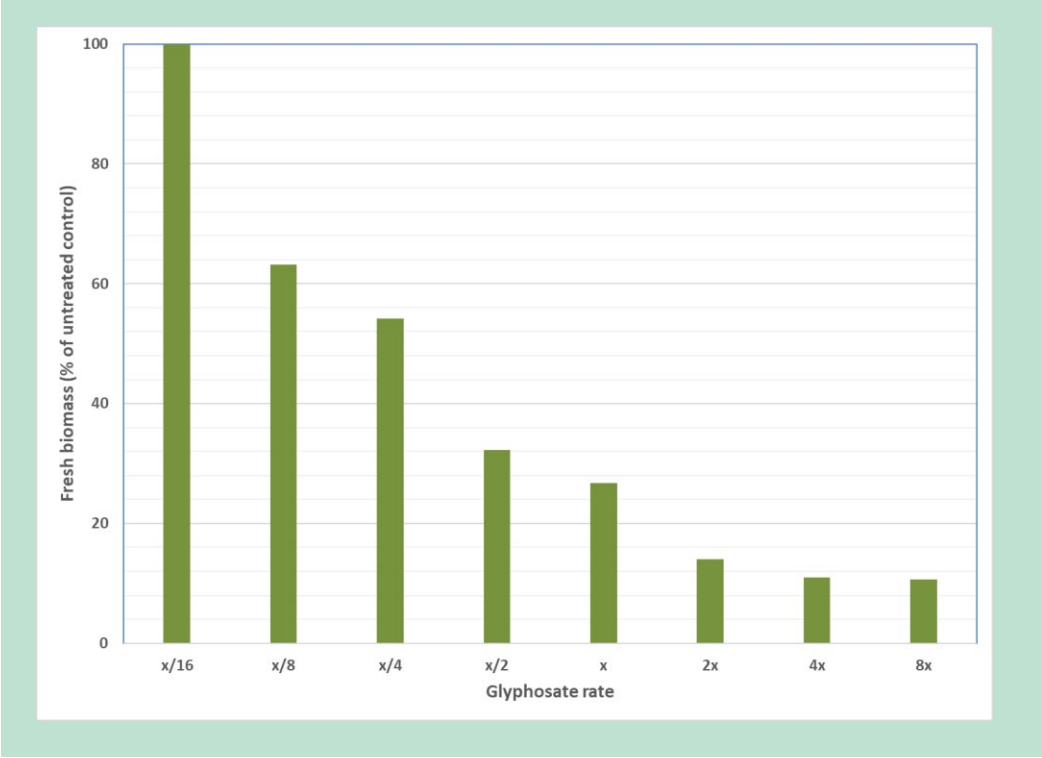


FIGURE 2. Effect of glyphosate on biomass of population G33 at 4 WAT.

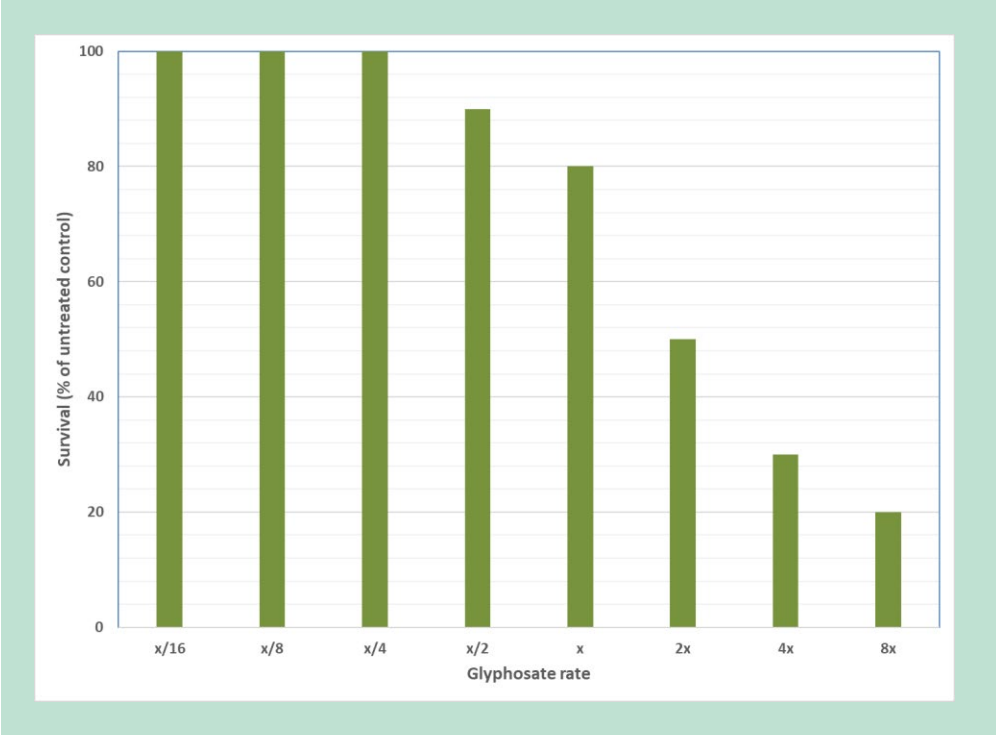


FIGURE 3. Effect of glyphosate on survival of population G34 at 4 WAT

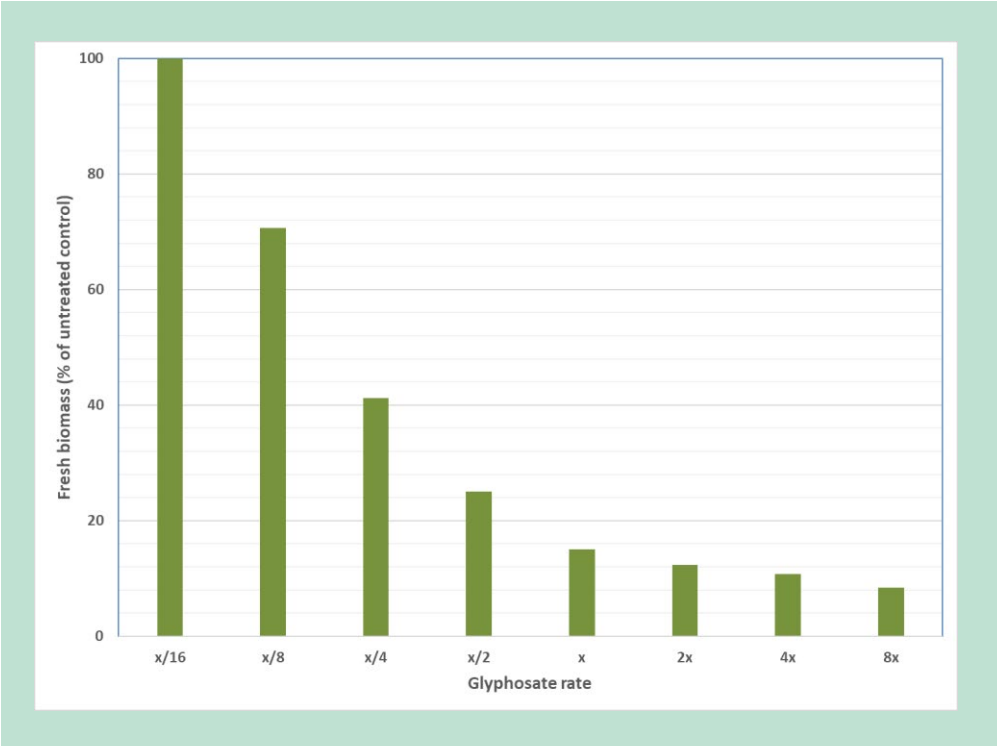


FIGURE 4. Effect of glyphosate on biomass of population G34 at 4 WAT.

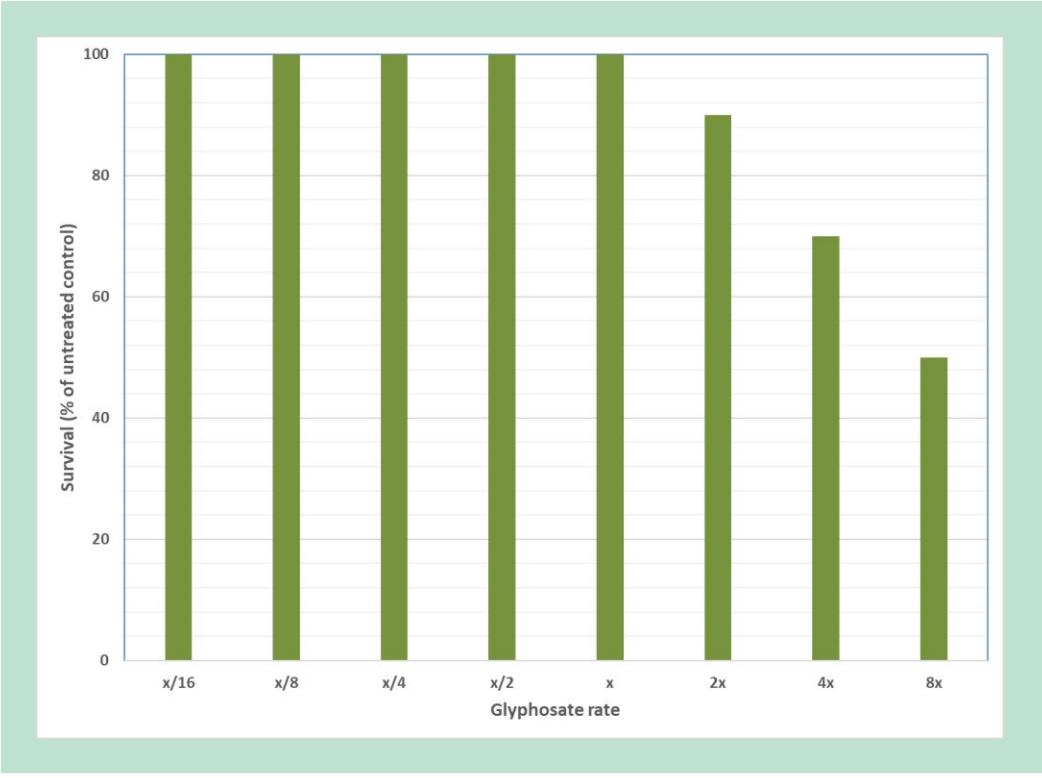


FIGURE 5. Effect of glyphosate on survival of population G36 at 4 WAT

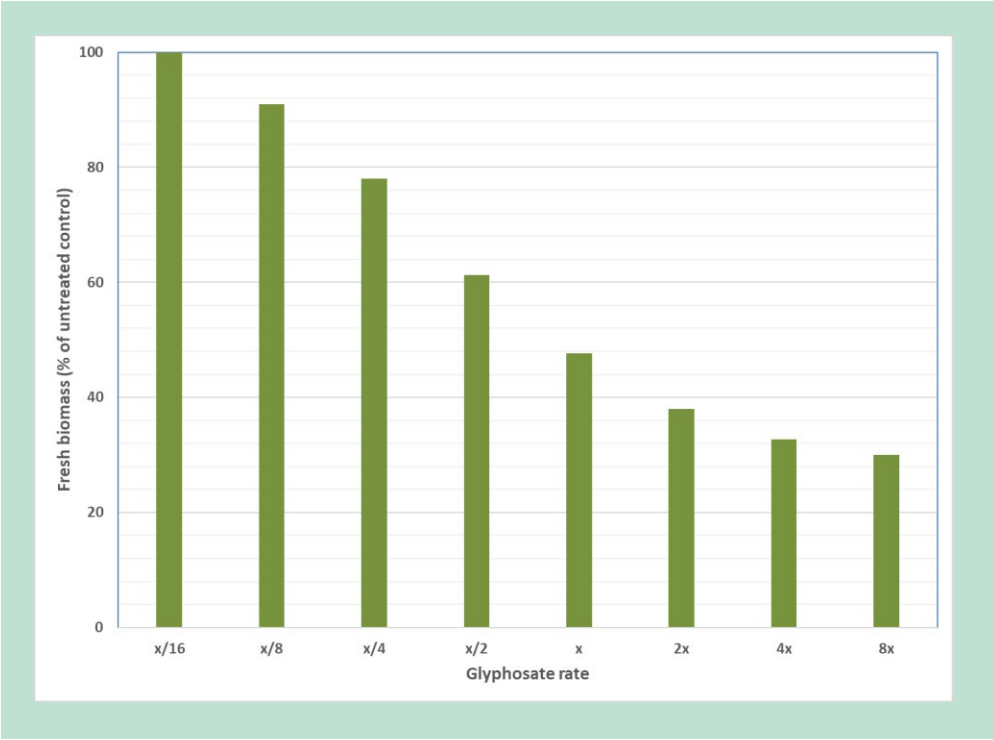


FIGURE 6. Effect of glyphosate on biomass of population G36 at 4 WAT

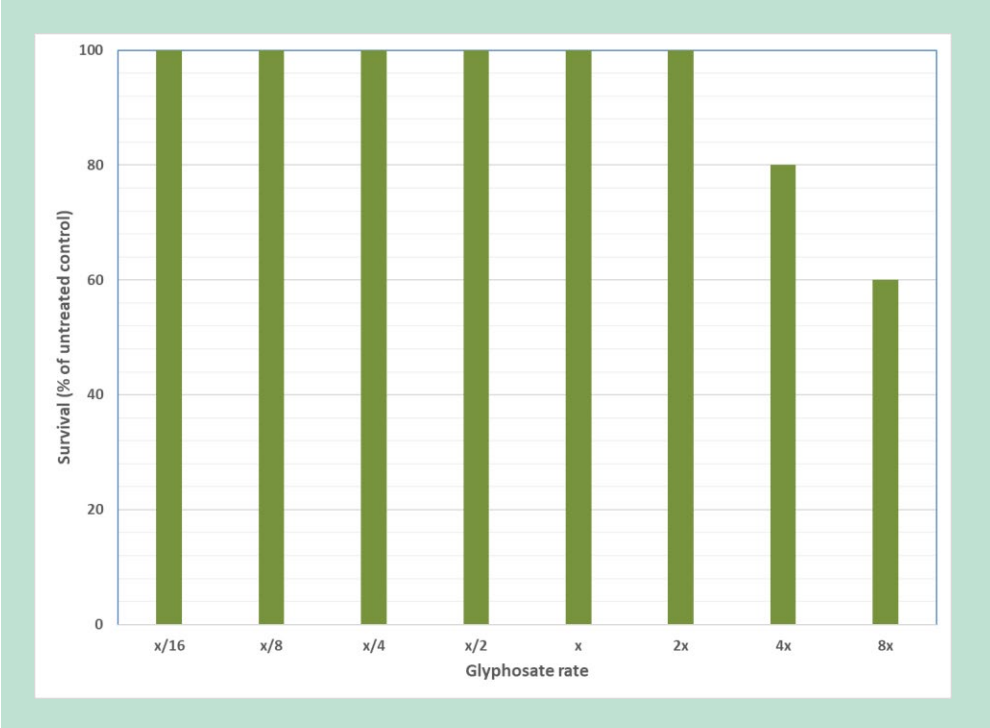


FIGURE 7. Effect of glyphosate on survival of population G37 at 4 WAT

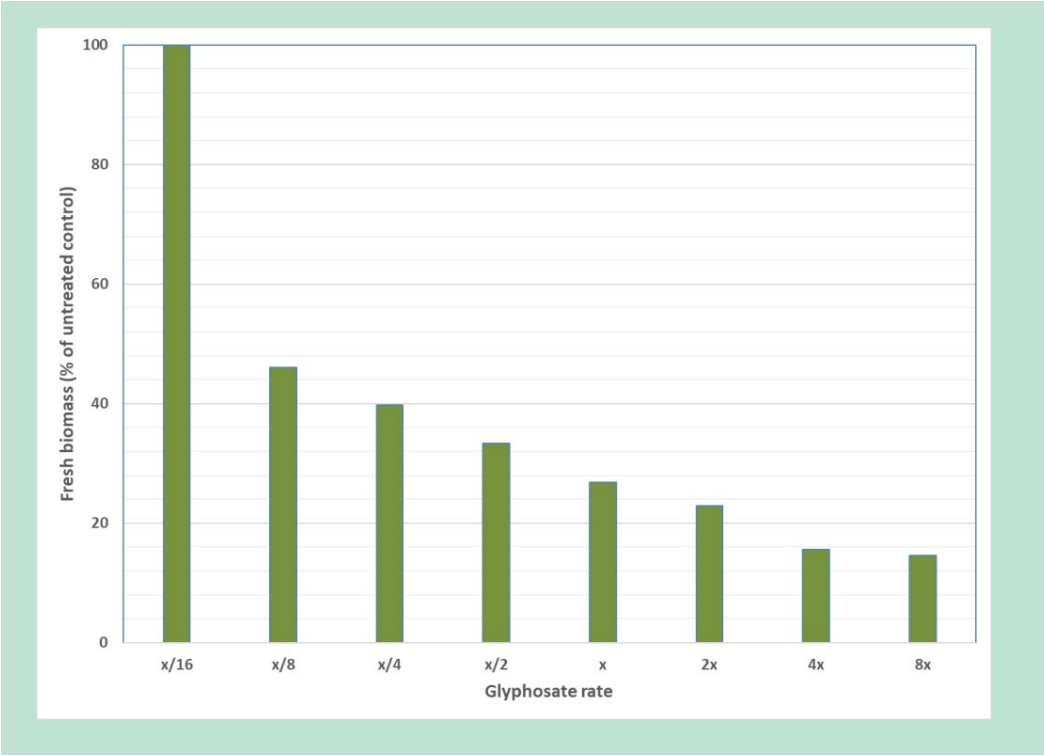


FIGURE 8. Effect of glyphosate on biomass of population G37 at 4 WAT.

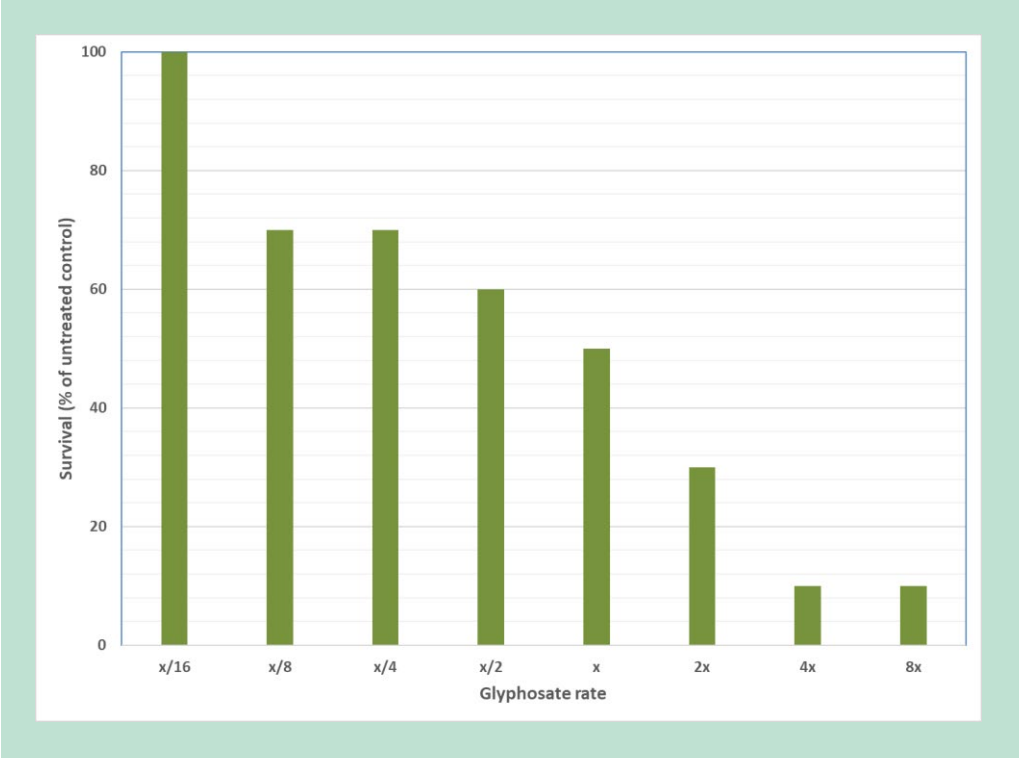


FIGURE 9. Effect of glyphosate on survival of population G40 at 4 WAT

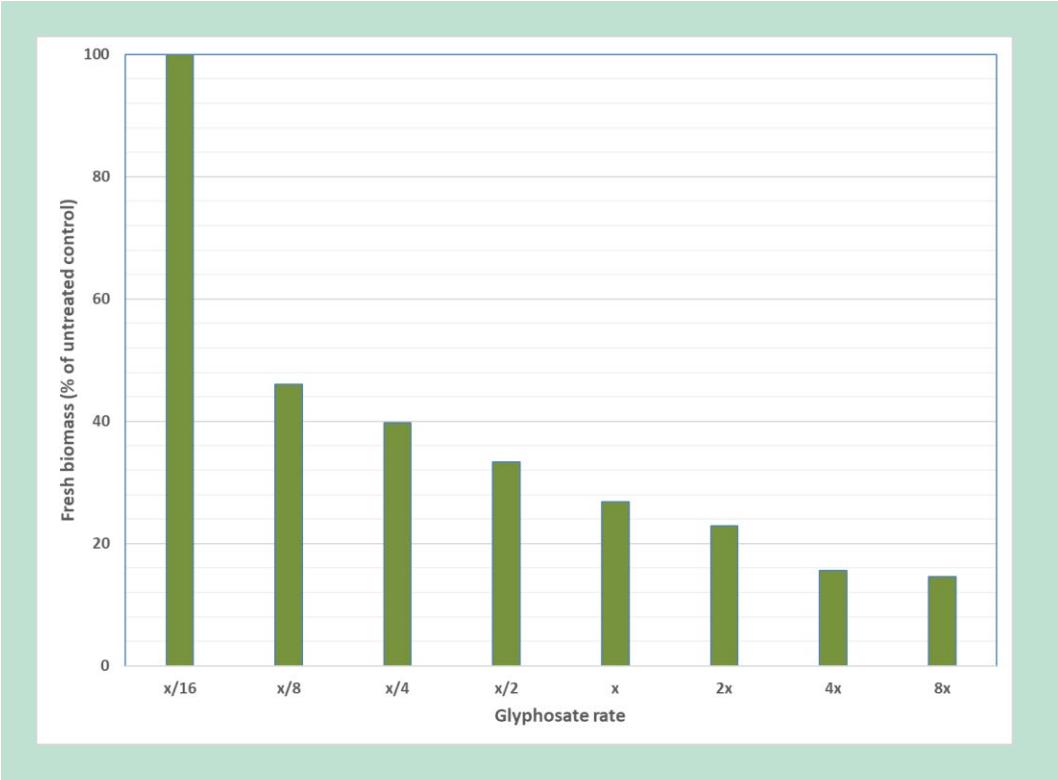


FIGURE 10. Effect of glyphosate on biomass of population G40 at 4 WAT.

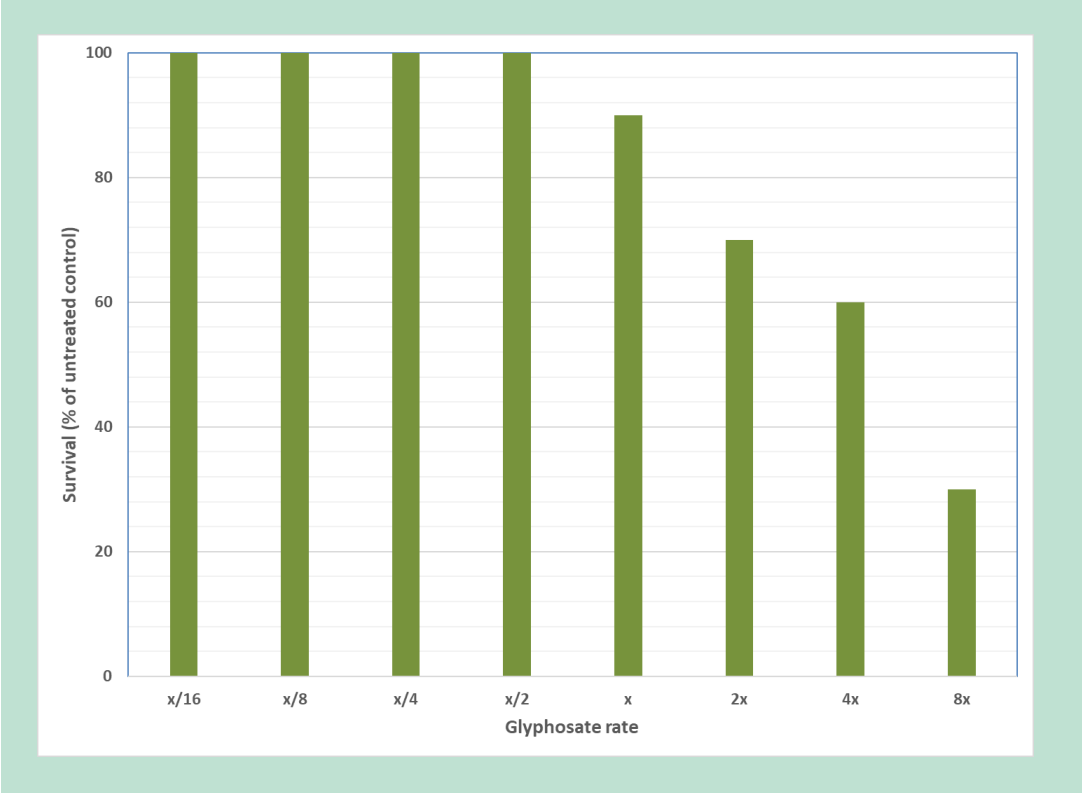


FIGURE 11. Effect of glyphosate on survival of population IT581 at 4 WAT

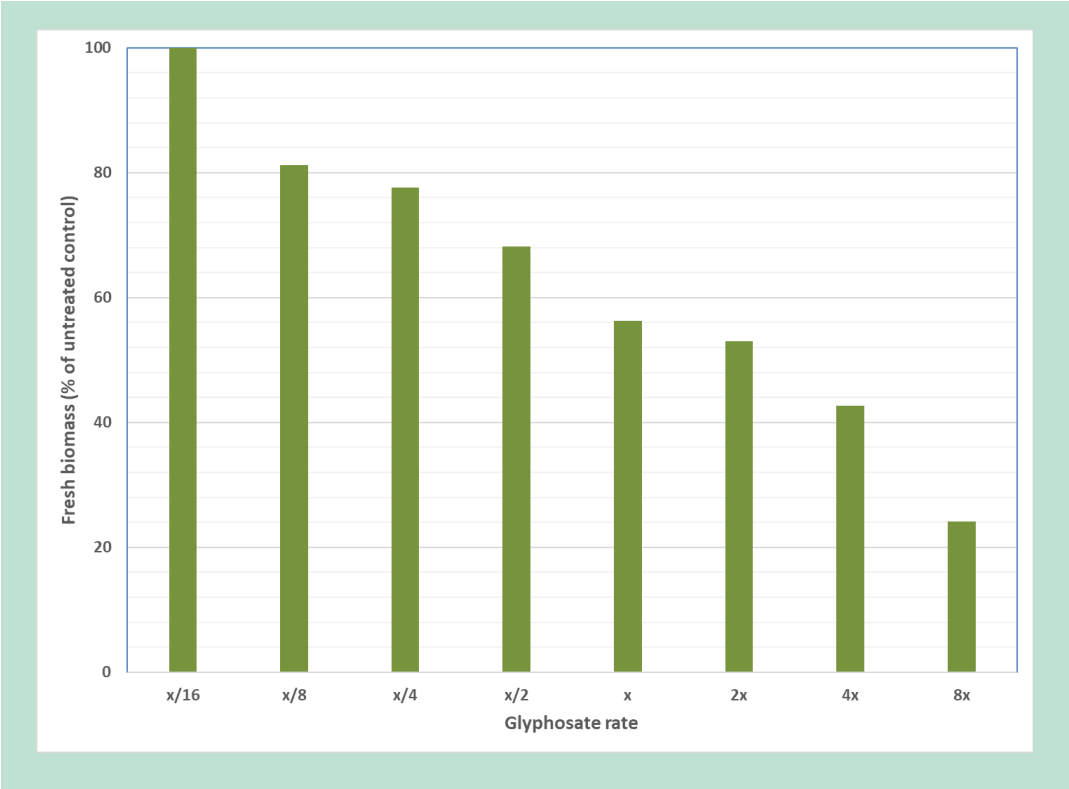


FIGURE 12. Effect of glyphosate on biomass of population IT581 at 4 WAT

Four out of six populations showed survival equal to or higher than 80% at the 1 N dose (G34, G36, G37, IT581, Fig. 1, 3, 5, 7, 11). In some cases (G36, G37 and IT581) the survival at the 8 N dose was equal to or higher than 30% (Fig. 5, 7, 11). LD50, LD60, LD80 and LD90 values revealed differences among populations with the G33 population being the most susceptible, whereas the populations G36, G37 and IT581 were the most resistant (Table 5). The RIs based on LD50 values with G33 as the susceptible reference were 5.3, 22.2, 23.7, 2.5 and 10.51 for the G34, G36, G37, G40 and IT581 populations, respectively.

TABLE 5. LD50, LD60, LD80 and LD90 values (g ae/ha) for the tested populations. RIs in parentheses

	Populations					
	G33	**G34**	**G36**	**G37**	**G40**	**581**
LD50	270 (1)	1440 (5.3)	6000 (22.2)	6440 (23.7)	680 (2.5)	2850 (10.6)
LD60	360	2160	6320	7030	810	4820
LD80	880	4810	7510	8120	2280	6680
LD90	1580	5920	8080	8420	3640	7350

Growth reduction (GR) at the recommended dose (1 N) ranged from 44 to 85% compared to the untreated control (Fig. 2, 4, 6, 8 and 10). Even at the 8 N dose biomass of population G36 and IT581 was only reduced by 70 and 76%, respectively (Fig. 6 and 12). The GR values revealed that populations G33, G34 and G40 were the most susceptible ones, whereas the populations G36, G37 and 581 were the most resistant populations (Table 6). The RIs based on GR50 values with population G33 population as the susceptible one were 1, 3.9, 2.9, 1 and 5.9 for the GR34, GR36, GR37, GR40 and IT581 populations, respectively.

TABLE 6. GR50, GR60, GR80 and GR90 values (g ae/ha) for the tested populations. RIs in parentheses

	Populations					
	G33	**G34**	**G36**	**G37**	**G40**	**581**
GR50	126 (1)	120 (0.95)	490 (3.9)	360 (2.9)	95 (0.75)	740 (5.9)
GR60	174	164	1045	680	180	1890
GR80	720	345	6210	2780	680	5870
GR90	1240	1380	6450	6140	1420	6320

In summary, the results showed a wide range of responses to glyphosate for the populations tested with population G34 and G40 being moderately resistant, whereas the G36, G37 and the Italian 581 were highly resistant.

ALS and ACCase inhibitors

The three susceptible references, one for each country, were entirely controlled with half the recommended field dose of mesosulfuron + iodosulfuron (i.e 7.5 + 1.5 g a.i./ha). The LD50 values of Atlantis WG for the susceptible populations were 80.6 g (=2.4 g mesosulfuron + 0.5 g iodosulfuron) for the Danish one, 56 g/ha for the Greek (=1.7 g mesosulfuron + 0.3 g iodosulfuron) and 58.9 g/ha for the Italian population (=1.8 g mesosulfuron + 0.4 g iodosulfuron) (Table 7). The four Danish populations were confirmed as being highly resistant to mesosulfuron + iodosulfuron, and even at the highest herbicide doses tested (i.e. 120 + 24g a.i./ha of mesosulfuron + iodosulfuron), plant survival was above the 50%. The RIs were >50 for three populations (DK06LP, DK47LM and DK90LM) and >37 for population DK29LM. Two Greek populations (G24 and G30) out of four were also highly resistant to mesosulfuron + iodosulfuron with 54 < RI <71. The other two were less resistant but still had RIs of 10 and 7 for population G20 and G9, respectively. The four Italian populations were highly resistant to mesosulfuron + iodosulfuron with a RI of 20 for population IT533 and RIs > 68 for the other three populations. In general the RI's were higher for all populations in the autumn experiment compared to the spring experiment, however the ranking was consistent.

The response of the susceptible populations to the clodinafop showed that the plants were not completely controlled at the recommended field dose (i.e. 60 g a.i./ha) in the first experiment, while they were killed in the second experiment. This difference is probably due to the different development stages of the plants at the time of treatment; in the first experiment, plants were at a more advanced development stage than in the second experiment (1-2 tillers versus 3-4 leaves) but both stages were within the recommended range for clodinafop application (BBCH 13-30). The four Danish populations were moderately resistant to clodinafop with RIs ranging between 2 and 7 (Table 8). The Greek populations were highly resistant to clodinafop with RIs >17 for the four populations, while the RIs for the Italian populations were from 8 for population IT595 to>11 for the other three populations. In the second experiment where the LD50 of the susceptible populations were lower (between 15 and 19 g a.i./ha), the RIs were on average higher with RIs between 3 and 7 for the Danish populations, >32 for the Greek populations and 8< RI<27 for the Italian populations.

Overall, the responses of the susceptible populations to pinoxaden showed a good control at the ½ N dose (i.e. 22.5 g a.i./ha). The LD50 values of the three susceptible populations ranged between 9 and 13 g a.i./ha in both experiments (Table 9). Three Danish populations (DK06LP, DK29LM and DK47LM) were susceptible to pinoxaden (1 < RI <3). The fourth population DK90LM had a RI of 4 to 5 and was classified as resistant to pinoxaden. Three Greek populations (G24, G30 and G9) were highly resistant to pinoxaden, with 6 < RI < 13 in the first experiment and 19 < RI < 39 in the second experiment. The fourth population (G20) had an intermediate resistance level (3 < RI < 11). Finally, three (IT533, IT609 and IT620) of the four Italian populations were moderately resistant to pinoxaden, with RIs ranging between 6 and 7 in the first experiment and between 6 and 20 in the second one. The fourth Italian population (IT595) was susceptible with a RI around 2.

Summing up, multi-resistance to ALS and ACCase was confirmed in all the populations tested, however a different resistance pattern was observed in the different countries. The Danish *Lolium* populations were highly resistant to

mesosulfuron + iodosulfuron, resistant to clodinafop, but only one population was resistant to pinoxaden. The four Greek populations were highly resistant to mesosulfuron + iodosulfuron and to clodinafop and moderately resistant to pinoxaden. Similarly, the Italian populations were highly resistant to mesosulfuron + iodosulfuron and to clodinafop and moderately resistant to pinoxaden, except for one population which showed no resistance to pinoxaden. Consequently, the results revealed that herbicide resistance to ACCase was more pronounced in Greece and Italy compared to Denmark.

TABLE 7. Atlantis WG (30 g/kg mesosulfuron + 6 g/kg iodosulfuron) doses that caused 50% or 90% reduction of the percentage of surviving plants relative to untreated controls (LD50, LD90 and standard errors) and resistance indexes. Field dose of Atlantis WG is 500 g/ha (= 15 g mesosulfuron
+ 3 g iodosulfuron)

First experiment (spring)

DK	Atlantis				
	ED50	E.S.	ED90	E.S.	**R.I. ED50**
06_LP	>4000		>4000		**>50**
29_LM	>3000		>3000		**>37**
47_LM	>4000		>4000		**>50**
90_LM	>4000		>4000		**>50**
100_LM	80.6	2.5	161.2	10.6	

GR	ED50	E.S.	ED90	E.S.	**R.I. ED50**
GR20	568.3	113.7	>900		**10**
GR24	>4000		>4000		**>71**
GR30	>1500		>4000		**>54**
GR9	412.7	87.0	>2000		**7**
GR33	56.0	7.4	146.6	44.1	

IT	ED50	E.S.	ED90	E.S.	**R.I. ED50**
IT533	1159.5	124.8	>2500		**20**
IT595	>4000		>4000		**>68**
IT609	>4000		>4000		**>68**
IT620	>4000		>4000		**>68**
IT204	58.9	4.7	131.6	24.0	

Second experiment (autumn)

DK	Atlantis				
	ED50	E.S.	ED90	E.S.	**R.I. ED50**
06_LP	>4000		>4000		**>109**
29_LM	2509.0	234.6	>3000		**68**
47_LM	>4000		>4000		**>109**
90_LM	>4000		>4000		**>109**
100_LM	36.8	0.9	85.8	5.1	

GR	ED50	E.S.	ED90	E.S.	**R.I. ED50**
GR20	630.6	61.4	2867.1	625.5	**12**
GR24	>4000		>4000		**>76**
GR30	>3000		>4000		**>57**
GR9	443.2	120.1	>2000		**8**
GR33	52.3	2.7	128.5	14.8	

IT	ED50	E.S.	ED90	E.S.	**R.I. ED50**
IT533	2015.2	157.9	>4000		**36**
IT595	>4000		>4000		**>71**
IT609	2747.4	193.9	>4000		**49**
IT620	>4000		>4000		**>71**
IT204	56.4	0.5	104.7	1.9	

TABLE 8. Clodinafop doses that caused 50% or 90% reduction of the percentage of surviving plants relative to untreated controls (LD50, LD90 and standard errors) and resistance indexes. Field dose of clodinafop is 60 g a.i./ha

First experiment (spring)

DK	clodinafop				
	ED50	E.S.	ED90	E.S.	R.I. ED50
06_LP	237.8	32.1	>480		4
29_LM	129.3	15.4	>480		2
47_LM	146.6	10.2	>480		3
90_LM	371.7	13.9	>480		7
100_LM	55.1	2.8	110.4	12.7	

GR	ED50	E.S.	ED90	E.S.	R.I. ED50
GR20	>480		>480		>17
GR24	>480		>480		>17
GR30	>480		>480		>17
GR9	>480		>480		>17
GR33	28.6	3.6	116.6	32.3	

IT	ED50	E.S.	ED90	E.S.	R.I. ED50
IT533	>480		>480		>11
IT595	337.2	61.1	>480		8
IT609	>480		>480		>11
IT620	>480		>480		>11
IT204	41.8	4.6	139.8	34.0	

Second experiment (autumn)

DK	clodinafo				
	ED50	E.S.	ED90	E.S.	R.I. ED50
06_LP	122.7	17.7	>480		7
29_LM	124.1	16.0	>480		7
47_LM	63.1	10.2	229.3	81.7	3
90_LM	136.6	6.4	>480		7
100_LM	18.8	0.9	36.2	3.7	

GR	ED50	E.S.	ED90	E.S.	R.I. ED50
GR20	>480		>480		>32
GR24	>480		>480		>32
GR30	>480		>480		>32
GR9	>480		>480		>32
GR33	14.8	0.5	21.9	3.9	

IT	ED50	E.S.	ED90	E.S.	R.I. ED50
IT533	>480		>480		>27
IT595	134.7	27.9	>480		8
IT609	>480		>480		>27
IT620	>480		>480		>27
IT204	17.5	2.5	49.9	15.5	

TABLE 9 Pinoxaden doses that caused 50% or 90% reduction of the percentage of surviving plants relative to untreated controls (LD50, LD90 and standard errors) and resistance indexes. Field dose is 45 g a.i./ha

First experiment (spring)

DK	pinoxaden				
	ED50	E.S.	ED90	E.S.	R.I. ED50
06_LP	15.4	0.2	23.0	0.6	1
29_LM	27.7	1.5	40.8	4.9	2
47_LM	14.5	0.0	21.2	0.1	1
90_LM	46.4	5.3	138.5	34.6	4
100_LM	11.8	0.0	13.8	0.1	

GR	ED50	E.S.	ED90	E.S.	R.I. ED50
GR20	27.3	1.0	62.6	5.0	3
GR24	94.3	15.4	>360		11
GR30	109.2	22.8	>180		13
GR9	52.5	8.3	84.9	35.1	6
GR33	8.7	0.0	12.8	0.0	

IT	ED50	E.S.	ED90	E.S.	R.I. ED50
IT533	85.1	28.2	>180		7
IT595	20.5	0.3	30.2	1.2	2
IT609	84.2	12.4	>180		7
IT620	76.8	4.7	241.1	32.4	6
IT204	12.1	0.2	16.4	1.5	

Second experiment (autumn)

DK	pinoxaden				
	ED50	E.S.	ED90	E.S.	R.I. ED50
06_LP	13.7	0.2	19.3	0.8	1
29_LM	32.9	0.9	45.9	1.5	3
47_LM	19.7	2.7	24.0	1.6	2
90_LM	60.1	4.8	161.2	27.9	5
100_LM	12.7	0.1	20.4	0.6	

GR	ED50	E.S.	ED90	E.S.	R.I. ED50
GR20	105.8	16.3	>360		11
GR24	300.5	23.2	>360		32
GR30	>360		>360		>39
GR9	177.3	10.4	>360		19
GR33	9.3	0.6	27.0	4.0	

IT	ED50	E.S.	ED90	E.S.	R.I. ED50
IT533	211.3	26.2	>360		20
IT595	18.4	1.7	76.8	16.3	2
IT609	68.5	10.9	260.1	91.6	6
IT620	130.0	15.7	>360		12
IT204	10.6	0.0	17.0	0.2	

4. Reliable molecular tool to detect ALS- and ACCase-resistant mutant plants

C-IPM	**Coordinated Integrated Pest Management in Europe** **Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D2.2**
Deliverable title:	**Reliable molecular tool to detect ALS- and ACCase-resistant mutant plants**
WP number:	**WP2**
Lead beneficiary:	**Silvia Panozzo/ Solvejg K. Mathiassen**
Main author(s):	**Silvia Panozzo, Silvia Farinati, Laura Scarabel**
Delivery date:	**30/08/2020**
Actual delivery date:	**30/09/2020**

Executive Summary

Description.

Twelve *Lolium* spp. populations multi-resistant to ACCase (acetyl-CoA carboxylase) and ALS (acetolactate synthase) inhibitors from Italy, Denmark and Greece were selected within WP1 and task 2.1. Genomic DNA (gDNA) of 5 to 10 selected ALS resistant plants from each population were analysed for mutations in the *ALS* and *ACCase* genes. If no ACCase mutations were found in the ALS-selected plants, five additional plants selected with an ACCase inhibitor were also analysed.
The most frequently identified mutations endowing resistance were considered for setting up the LAMP (Loop Mediated Isothermal Amplification) tests. This molecular diagnostic tool has been developed to detect the point mutation in position 1781 of the *ACCase* gene in *Beckmannia syzigachne* (Pan et al. 2014). In this study, *Lolium* spp. specific LAMP protocols were set-up for three ALS (197, 376 and 574) and four ACCase (1781, 2041, 2078 and 2096) point mutations endowing herbicide resistance.
The validation of the LAMP tests showed a potential for the identification of mutations in the *ACCase* gene. On the contrary, the design of LAMP protocols for the individuation of *ALS* mutations was more challenging and the validation tests gave less reliable results.
In summary, the LAMP protocols appear most suitable for monitoring the presence of ACCase mutant *Lolium* spp. plants in the field and this early detection allows farmers to adopt management strategies at earlier stages.

Introduction

In cereal crops, ALS and ACCase inhibitors are the two groups of herbicides most widely used to control *Lolium* spp. Populations with multiple resistance to ALS and ACCase inhibitors have been reported from many countries in the world and this presents a major practical problem, because it significantly reduces the herbicides available for the control of this weed. Several resistance mechanisms have been reported for *Lolium* spp. Among them gene mutations reducing or inhibiting herbicide binding by conferring amino-acid changes in a target enzyme (Target Site Resistance, TSR) and detoxification of the herbicide (Enhanced Metabolic Resistance, EMR) are the main mechanisms in grass weeds such as *Lolium* spp. (Tan et al. 2007;Yu et al. 2009). TSR has been extensively studied in the last fifteen years and many of the known mutations responsible for herbicide resistance in the *ALS* and *ACCase* genes were found in *Lolium* spp. populations.
The development of analytical tools for a rapid TSR diagnosis have gone on over years. Many techniques are now available for the detection of target-site mutations causing herbicide resistance
(Baker et al. 2007) but their reliability differ. Loop-mediated isothermal amplification (LAMP) is a novel method that rapidly amplifies target nucleic acids under isothermal conditions (i.e. a single incubation temperature) (Notomi et al. 2000). In contrast to the PCR techniques, the LAMP method is more specific because it uses 4 to 6 different primers that specifically recognize 6 to 8 discrete regions of the target gene (template strand) (Figure 1). The sample mixture containing template DNA, primers, DNA polymerase with high strand displacement activity and dNTPs is incubated at a constant temperature (60 to 66 °C) for amplification and detection of nucleic acids in a single step. Usually, around 50-fold more amplicon is produced in LAMP than in PCR techniques (Kumar et al. 2017). Results can be visualized on the conventional gel electrophoresis or using several alternative methods such as turbidity, colour development or fluorescence. The endpoint detection or real-time monitoring of the LAMP reaction can also be done by various approaches like for example optical methods, electrophoresis, immuno-detection (lateral flow assay) or electrochemical processes.

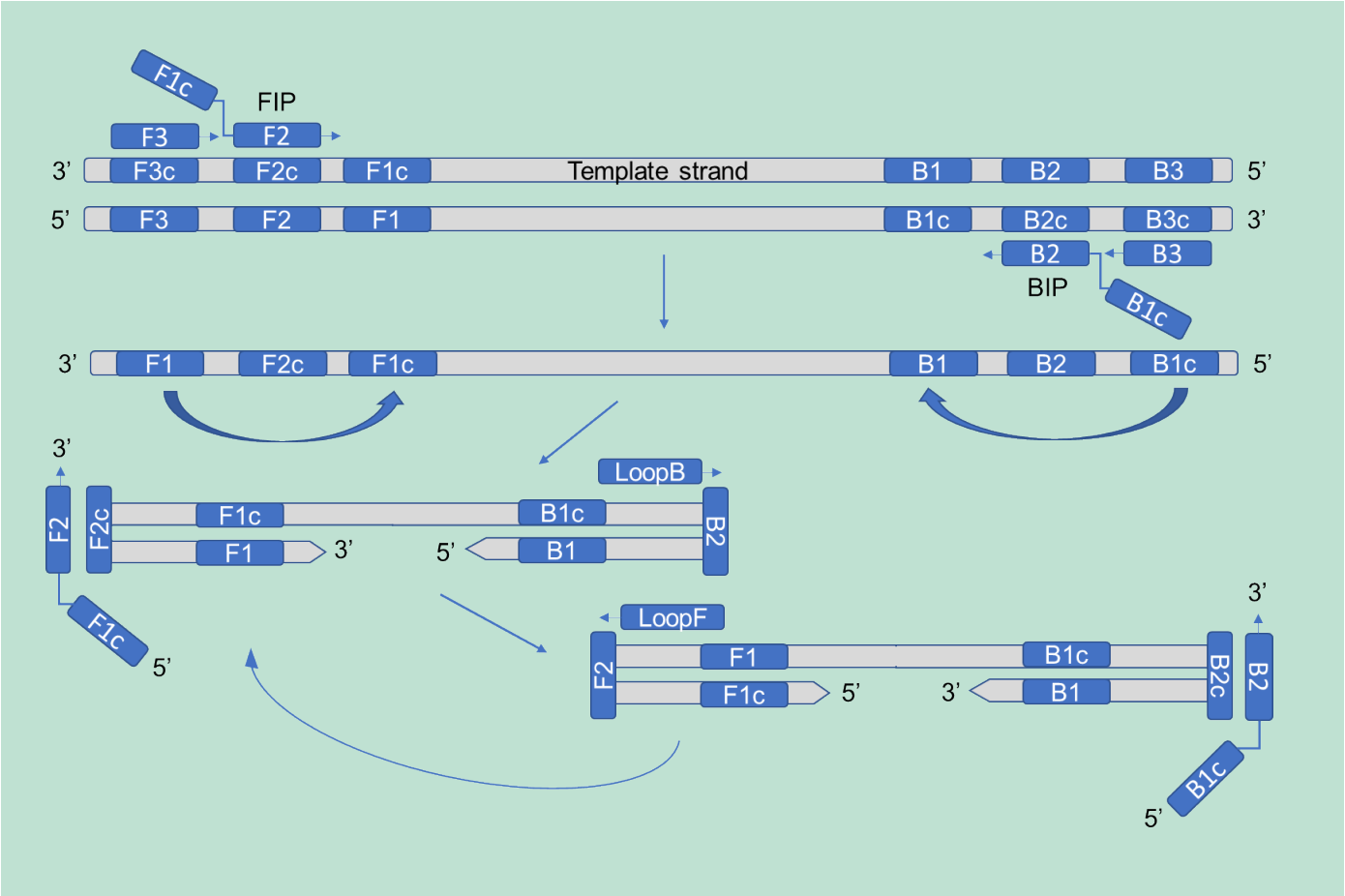


FIGURE 1. Diagram of operation of LAMP method and primers involved. Four specie-specific primers are involved in the template strand amplification: the two external primers, F3 and B3, and the two internal primers, FIP and BIP (which are made-up linking two complementary regions of the template strand), that during the amplification step will create a loop which is exponentially amplified. Other two primers, LoopB and LoopF, may be designed for the stabilization of the amplification.

Description of work

Methods

Genotyping of ALS and ACCase genes in multi-resistant populations

Twelve populations showing multi-resistance to ALS and ACCase inhibitors in the dose-response experiments in task 2.1 were analysed for the identification of resistance mechanisms. Populations originated from the three countries participating in the RELIUM project, four from Italy (IT533, IT595, IT609 and IT620), four from Denmark (DK6, DK29, DK47 and DK90) and four from Greece (GR9, GR20, GR24 and GR30). Ten plants from each population, surviving an herbicide treatment with Atlantis WG (mesosulfuron-methyl 30 g L-1 + iodosulfuron-methyl 6 g L-1), were sampled and gDNA was extracted using the CTAB method (Aras and Duran 2003). ALS gene sequence, 1700 bp, was amplified using the primer couple LOL_ALS_F and ALS_LOL_R and sequenced using the primers LOL_ALS_F and ALS_LOL_FS (Table 1). On the same samples, a 1600 bp amplicon of the carboxyl-transferase (CT) domain of ACCase gene was amplified using the primer couple acclr6 and acclr9 and sequenced using the primers LOL_FOR and LOL_FOR_SEQ (Table 1). All PCR analyses were performed using GoTaq DNA Polymerase kit (Promega, USA) in a 25 µL mixture including 5 µL of 5x Green GoTaq Flexi Buffer, dNTPs mix (0.2 mM each), $MgCl_2$ (3 mM), forward and reverse primers (0.4 µM each), 0.125 µL GoTaq DNA Polymerase, and 25 ng of gDNA. The thermocycler program was as follows: 95 °C for 2 min; 45 cycles of 95 °C for 30 s, 57

°C (for *ACCase* gene) or 60 °C (for *ALS* gene) for 30 s, 72 °C for 2 min; 72 °C for 5 min. PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co., Germany) following the manufacturer's instructions. Once purified, PCR products obtained from each plant were sequenced by BMR Genomics (Padova, Italy).

LAMP set-up: primer sets design, testing and reaction setting

Specie-specific LAMP primer sets for *Lolium* spp., including external primers F3 and B3, internal primers FIP and BIP, and loop primers LoopF and LoopB (Fig. 1), were designed using Primer explorer V5 software (http://primerexplorer.jp/lampv5e/index.html), a specific tool for the design of LAMP primers. The primer sets were designed to specifically distinguish the seven most frequent point mutations detected in *ALS* (197, 376 and 574) and *ACCase* (1781, 2041, 2078 and 2096) genes during the genotyping experiment.

TABLE 1. List of primers used for the *ALS* and *ACCase* gene fragments amplification and sequencing.

Primer name	Sequence 5'-3'	Target
acclr9	ATGGTAGCCTGGATCTTGGACATG	Forward primer, *ACCase* CT domain amplification (Zhang and Powles 2006)
acclr6	GGAAGTGTCATGCAATTCAGCAA	Forward reverse, *ACCase* CT domain amplification (Zhang and Powles 2006)
LOL_FOR	CTGTCTGAAGAAGACTATGGCCG	Sequencing *ACCase* gene
LOL_FOR_SEQ	GAGGTGGCTCAGCTATGTTCCTG	Sequencing *ACCase* gene
LOL_ALS_F	CCGCAAGGGCGCCGACATCCTCGT	Forward primer, *ALS* amplification
ALS_LOL_R	CGAAATCCTGCCATCACCTTCCAT	Reverse primer, *ALS* amplification
ALS_LOL_FS	TCCATCACCAAGCACAACTACCTC	Sequencing *ALS* gene

To select the primers that could successfully differentiate the mutant allele (MUT, responsible for the resistant phenotype) from wild type one (WT, responsible for the susceptible phenotype), at least two sets of specific primers were designed for each target mutation, according to Primer explorer V5 software manual. Several strategies were used and specific primers were designed for a specific MUT allele: in the primers FIP and BIP the mutation was included in 5'end of F1c or B1c (FIP5' and BIP5' primers, respectively) or in 3'end of F2 or B2 (FIP3' and BIP3' primers, respectively) (Fig. 1); in these cases the mutant allele was generally amplified while the wild type amplification was hindered. The same strategies were used for the corresponding WT allele. The WT/MUT primer sets were designed for each tested point mutation using as input for Primers explorer 5 software the sequences produced during the genotyping experiment. The primer set list designed with the different strategies and subsequently tested is reported in Table 2.

TABLE 2. List of specific primer sets designed for distinguishing between wild type and mutant alleles for each point mutation in both considered genes. (*) The suffixes 5' and 3' indicate the position of WT or MUT nucleotide in FIP and BIP sequence primers, respectively.

Gene	Substitution point	Primer set	Specific primer (*)
	197	#1	FIP5'

ALS		#2	BIP5’
	376	#1	FIP5’
		#2	FIP3’
	574	#1	FIP3’
		#2	BIP3’
		#3	BIP5’
		#4	FIP5’
ACCase	1781	#1	BIP5’
		#2	FIP5’
		#3	FIP3’
	2041	#1	BIP5’
		#2	FIP5’
	2078	#1	BIP5’
		#2	FIP5’
	2096	#1	BIP5’
		#2	FIP5’

The testing phase of each primer set listed in Table 2 was performed using the gDNA samples used for primer design by software. Each primer set was investigated on at least one known wild type sample and one/two mutant samples (one heterozygous and one homozygous for the specific point mutation when available).

Real-time LAMP assays were carried out on a Genie II instrument (OptiGene, Horsham, United Kingdom) in 25 µL reaction mixtures containing 15 µL of isothermal master mix at 1x concentration (OptiGene), 200 nM each external primer, 2 µM each internal primer, and 1 µM each loop primer. The isothermal master mix contained a fluorescent double-stranded DNA binding dye to permit the real-time detection of the results. To evaluate the sensitivity of the LAMP assays, serial dilutions of

template gDNA were tested, and assays were optimized also in terms of reaction time and temperature. The optimum conditions were obtained with 25 ng of template gDNA added per reaction and keeping the reaction at 60 °C for 60 min. After amplification, the nature of the amplification products was confirmed by subjecting the reactions to a slow annealing step (0.05 °C per s) from 95 °C to 75 °C with fluorescence monitoring.

Validation of LAMP method

Seven populations (576, 594, 602, 648, 651, 670 and 678), collected in different agricultural fields in Italy and already characterized for resistance patterns and levels (GIRE 2020), were considered for the validation of LAMP methods designed for the detection of ALS and ACCase point mutations responsible for resistance in these two groups of herbicides.

Seeds of each population were pre-germinated, transplanted in trays in the greenhouse and treated with specific herbicides to confirm resistance (Panozzo et al. 2015). From each population gDNA was extracted from five to ten plants which survived the ACCase or ALS treatment. LAMP primer sets for detecting ACCase

mutations 1781, 2041 and 2078 were tested on gDNA extracted from plants surviving an Axial Pronto (pinoxaden 60 g L^{-1}) treatment at 0.75 L ha^{-1}, whereas LAMP primer sets for detecting ALS mutations 197, 376 and 574 were tested on gDNA extracted from plants that survived an Atlantis WG (mesosulfuron 30 g L^{-1} + iodosulfuron 10 g L^{-1}) treatment at 1.2 L ha^{-1}. Subsequently, the two genes (*ACCase* and *ALS*, respectively) were amplified from each single plant as described in section 2.1.1 and sequenced to compare the results obtained with the LAMP analyses.

Results

Mutant ALS and ACCase alleles

Primers acclr9/acclr6 amplified a 1600 bp amplicon encompassing all codons of the *ACCase* gene already known to confer resistance. Similarly, primers LOL_ALS_F/ALS_LOL_R amplified a 1719 bp amplicon encompassing all codons of the *ALS* gene conferring resistance. Both amplicons were sequenced for a total of 83 plants (Table 3).

TABLE 3. *ALS* and *ACCase* allelic variants identified in resistant *Lolium* spp. plants from Danish, Greek and Italian populations compared to the susceptible plant. For each codon, the amino acid substitution identified is indicated and the number of plants encompassing the mutation is reported in bracket. Dashes indicate amino acids identical to those in susceptible plant from IT-204[a].

ACCase allelic variants							ALS allelic variants					n° mutant p/n° analyzed p
Pop	**1781**	**2027**	**2041**	**2078**	**2088**	**2096**	**122**	**197**	**205**	**376**	**574**	
DK6	-	-	-	-	-	-	-	-	-	-	Leu (5)	5/5
DK29	-	-	-	-	-	-	-	-	-	-	-	0/5
DK47	-	-	-	-	-	-	-	-	-	-	Leu (4)	4/5
DK90	-	-	-	-	-	-	-	-	-	-	-	0/5
GR9	Leu (3)	-	Val (1)	-	-	-	-	Ser (3) Thr (2)	-	Asn (1)	-	5/5
GR20	-	Cys (1)	Asn (3)	Gly (1)	Arg (1)	-	-	Ser (5)	-	-	-	5/5
GR24	Leu (1)	Cys (1)	Asn (1)	Gly (1)	-	-	-	Ala (1) Gln / Leu (1)	-	-	-	5/7
GR30	Leu (3)	-	-	-	-	-	-	Ser (1)	-	-	-	4/8
IT533	Leu (2)	-	-	Gly (1)	-	Ala (1)	-	-	-	Glu (1)	-	5/10
IT595	Leu (5)	-	-	-	-	-	-	-	-	Glu (4)	Leu (4)	9/10
IT609	-	-	Asn (1)	-	-	-	-	Gln (1) Ser (1)	-	-	-	3/10
IT620	-	-	-	-	-	Ala (3)	Gly (2)	Leu (1)	Val (1)	-	-	5/8
[a]IT204	Ile	Trp	Ile	Asp	Cys	Gly	Ala	Pro	Ala	Asp	Trp	

The sequencing of the ACCase amplicons revealed that all Greek and Italian populations included plants having a mutated *ACCase* allele (Table 3). Overall, in the Greek populations, six different types of *ACCase* mutant alleles were detected: Leu_{1781}, Cys_{2027}, Asn_{2041}, Val_{2041}, Gly_{2078} and Arg_{2088}. In addition, in population GR20, different mutant *ACCase* alleles were found in the same plant. Overall, four different types of *ACCase* mutant alleles were found in plants from Italy. Populations IT595, IT609 and IT620 showed only one *ACCase* mutant allele, Leu_{1781}, Asn_{2041} and Ala_{2096}, respectively. In population IT533 three different mutant alleles were identified (Leu_{1781}, Gly_{2078} and Ala_{2096}). In contrast, no mutant *ACCase* alleles were identified in any of the 20 plants analysed from the Danish populations (Table 3).
Five types of mutant *ALS* alleles (Ala_{197}, Gln_{197}, Leu_{197}, Ser_{197}, Thr_{197}) were identified in the plants from the Greek populations. In population GR20 and GR30 only one type of *ALS* mutant allele was detected (Ser_{197}), while populations GR9 included three types in two different point (Ser_{197}, Thr_{197} and Asn_{376}) and population GR24 had three types of mutant *ALS* alleles at position 197 (Ala_{197}, Gln_{197} and Leu_{197}) . Plants of these latter populations showed different mutant *ALS* alleles in the same plant. In three Italian populations out of four, more than one mutant *ALS* allele was detected especially Glu_{376} and Leu_{574} in population IT595, Gln_{197} and Ser_{197} in population IT609, and Gly_{122}, Leu_{197} and Val_{205} in population IT620. The fourth population IT533 showed only the Glu_{376} allelic variant. Conversely, the plants of the Danish populations showed only one type of mutant *ALS* allele (Leu_{574}) and only in two populations (DK6 and DK47). No mutant *ALS* alleles were detected in the other two populations DK29 and DK90 (Table 3).

Identification of mutated allele through LAMP method

To confirm the effectiveness of each primer set, they were firstly tested on samples deriving from the genotyping process and for each point mutation the best primer set able to distinguish the two allelic variants (WT and MUT) was identified. Regarding the *ALS* gene, the set #1 FIP5' for position 197 and 376 and the set #3 BIP5' for position 574 were the most successful sets for distinguishing between the different allelic variants; for the *ACCase* gene, the #1 BIP5' was the best set for all tested nucleotide substitutions. The working success of a specific primer set was translated with a delay time, Δt (at least of 5 minutes), of target amplification (i.e. evaluated on the inflection point, I50, of the amplification curve), observed for example when the sample WT is questioned with MUT primer set in comparison to the relative WT primer set (and *vice versa*) (Figure 2).

Once the best working sets had been identified for each point mutation for both genes, they were tested on gDNA extracted from plants which showed a resistant phenotype to ACCase or ALS inhibitors, respectively (described in section 2.1.3).

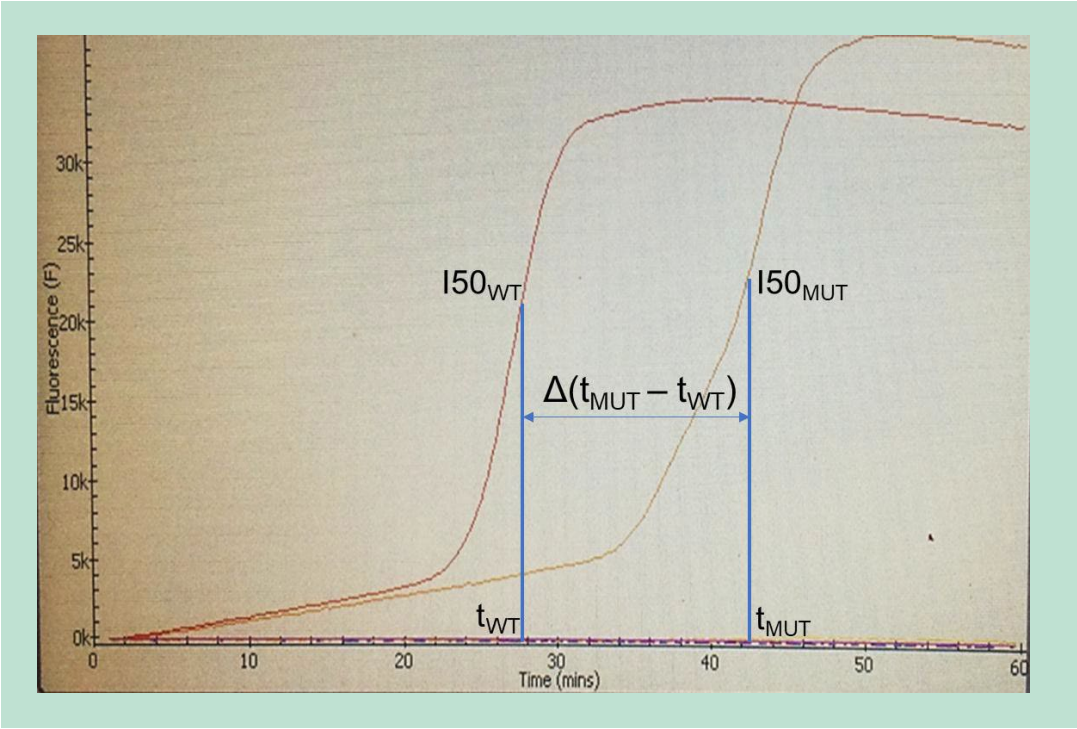


FIGURE 2. Example of graphic output obtained after specific LAMP reaction. Amplification plots represent the reaction trend of fluorescence emitted during the time (run of 60 min) for a sample with a WT genotype tested with WT set primers (in red) and with MUT set primers (in yellow). When a Δ (t_{MUT} - t_{WT}) of at least 5 minutes was observed, the sample was ascribed as WT (or MUT, depending on which curve started first), as described in the text.

Validation of the LAMP method

For the first step in the validation process, three populations were examined: populations 670 and 678 showing resistance only to the ALS inhibitor Atlantis, whereas population 651 was classified as multi-resistant to both the ACCase inhibitor Axial and the ALS inhibitor Atlantis. Five ALS-resistant plants from each population and in addition ten ACCase-resistant plants from population 651 were sampled to extract gDNA. Each plant was investigated with both LAMP primer sets, WT and MUT, for all point mutations available, without knowing the *ALS* and *ACCase* gene sequences of the different samples. In this part of the development, the attention was focused on LAMP primer sets for detecting 1781, 2041 and 2078 *ACCase* mutations and 197, 376 and 574 *ALS* mutations. In each reaction run, a positive control and a negative control, (a WT and/or MUT known genotype), identified previously, were added for each primer set. Subsequently, from each tested plant, the *ALS* and *ACCase* genes were amplified and sequenced in order to reveal the presence of mutated alleles. The genotyping results were then compared with those obtained from the LAMP analyses (Table 4 and 5).

The results obtained from genotyping of each plant are reported in Table 4 (*ALS* gene) and Table 5 (*ACCase* gene). The results obtained from LAMP reactions were added in the same tables with a color code. The cases for which it was not possible to distinguish the WT from MUT allele are highlighted in YELLOW (i.e. results were not clear or ambiguous), in WHITE are reported the cases for which no amplification curves were obtained neither from WT nor from MUT primers sets, in GREEN when the LAMP reaction suggested the presence of only WT alleles and in RED when the LAMP reaction suggested the presence of a MUT allele (in homo or heterozygous status). There is consistency between the results of genotyping and those of the LAMP when there is a dash in the green box or a codon in a red box. All other cases indicate a contrast between the results of genotyping and those of LAMP or impossibility of a diagnosis with the LAMP method (yellow boxes).

TABLES 4-5. *ALS* and *ACCase* allelic variants identified in resistant *Lolium* spp. plants of populations 651, 670 and 678 in heterozygous or homozygous conditions; in bold the nucleotide which gives the mutated codon. Dashes indicate amino acids identical to those in susceptible plants, so wild type allelic variants. See text for colour codes.

4)	ALS		
	197	376	574
651-1	-	-	-
651-2	C(C/**A**)G	-	-
651-3	C**A**G	-	-
651-4	-	-	-
651-5	C**A**G	-	-
670-1	-	-	-
670-2	-	-	-
670-3	-	-	-
670-4	-	-	-
670-5	-	-	-
678-1	-	-	-
678-2	C(C/**G**)G	-	-
678-3	-	-	-
678-4	-	-	-
678-5	C**A**G	-	-

5)	ACCase		
	1781	2041	2078
651-1	(A/**T**)TA	-	-
651-2	(A/**T**)TA	A(T/**A**)T	-
651-3	-	A(T/**A**)T	G(**G**/A)T
651-4	Not Detected		
651-5	**T**TA	-	-
651-6	(A/**T**)TA	(A/**G**)TT	-
651-7	(A/**T**)TA	-	-
651-8	-	A(T/**A**)T	G(**G**/A)T
651-9	(A/**T**)TA	-	-
651-10	-	A**A**T	-

Overall, the results were not very consistent between the genotyping and the LAMP analyses when considering the *ALS* gene (Table 4). In most plants, the LAMP results were often not clear enough to distinguish if a plant had a specific mutation or not (yellow boxes). In point mutation 197, the results were consistent in only one sample out of 15 (678-5) and in four cases, the results were in contrast (point mutation present in the sequence of the gene but WT allele for LAMP results, e.g. plant 651-3, or *vice versa*, e.g. plant 678-4). None of the analyzed plants carried the point mutations 376 and 574, and the LAMP results were in accordance with the genotyping results in 9 plants out of 15. Further, the results for mutation 376 were contrasting for plant 678-4, while for all other cases the results of LAMP analyses were not clear, or the reaction did not start (plants 651-2 and 3 in position 376).

Regarding point mutations in the *ACCase* gene (Table 5), generally the results were more consistent between the genotyping and the LAMP analyses. In total, 9 plants were genotyped (plant 651-4 sequence was not readable). In position 1781 results were consistent in 8 plants out of 9, only in plant 651-1 results of LAMP were not clear. In position 2041 results were consistent in 7 plants out of 9, in 2 plants results of LAMP were not clear and in position 2078 results were consistent in 6 plants out of 9, whereas in 3 plants LAMP results were confusing.

Due to the results obtained in the first part of validation, the second part was focused only on plants resistant to ACCase inhibitors. Four populations were examined - 576, 594, 602 and 648 - all of them showed multi-resistance to the ALS inhibitor Atlantis and to the ACCase inhibitor Axial. Five ACCase-resistant plants from each population were sampled to extract gDNA. Each plant was investigated with both LAMP primer sets, WT and MUT, for the three most frequent ACCase point mutations (1781, 2041 and 2078). As in the first part of validation, *ACCase* genes were amplified and sequenced and the genotyping results were compared with those obtained from the LAMP analyses (Table 6). The colour codes for the LAMP results are the same as used in Tables 4 and 5. As observed in Table 5, generally the results were quite consistent between the genotyping and the LAMP analyses. In total, 20 plants were genotyped: in position 1781 the results were consistent in 11 plants out of 20, whereas in three plants the results were in contrast and in two plants the results of LAMP were not clear. Unfortunately, in four plants the reaction did not start. In position 2041 the results were consistent in 14 plants out of 20, in five plants the results of LAMP were not clear and in one plant results were in contrast. In position 2078 results were consistent in 14 plants out of 20, whereas in two plants LAMP and genotyping results were in contrast, in three cases results of LAMP were confusing and in one case reaction did not start (Table 6). It should be noted that in two plants of population 602, different mutations (in position 2027 and 2088) compared to those detected with LAMP method were found.

TABLES 6. *ACCase* allelic variants identified in resistant *Lolium* spp. plants of populations 576, 594, 602 and 648 in heterozygous or homozygous conditions; in bold the nucleotide which gives the mutated codon. Dashes indicate amino acids identical to those in susceptible plants, so wild type allelic variants.

6)	ACCase		
	1781	2041	2078
576-1	**T**TA	-	-
576-2	(A/**T**)TA	-	-
576-3	(A/**C**)TA	A(T/**A**)T	-
576-4	-	-	-
576-5	(A/T)TA	-	-
594-2	**T**TA	-	-
594-3	**T**TA	-	-
594-4	**T**TA	-	-
594-5	(**T**/A)TA	-	-
594-6	(**T**/A)TA	-	-
602-1*	-	-	-
602-2	-	-	-
602-3	-	-	-
602-4	-	-	-
602-5**	-	-	-
648-2	-	-	-
648-3	-	-	-
648-4	-	-	-
648-5	-	-	-
648-6	-	-	G(**G**/A)T

but 2088

Overall, the LAMP and the genotyping results were consistent in 65% of the cases while in 10% of the cases, they were not consistent. As the percentage of plants in which the reaction has not started was never very high (3- 14%), the cases in which it was not possible to give an answer with the LAMP method reached up to 25% for one of the mutations examined (2041).

TABLES 7. Overall comparison between LAMP and genotyping results for the *ACCase* gene. Number of plants and percentage
where results were consistent (green), in contrast (red), where LAMP gave results not clear or did not give results were reported.

LAMP and genotyping results		***ACCase* position**		
		1781	**2041**	**2078**
Consistent 	n. plants	19	21	20
	%	**66**	**72**	**69**
In contrast 	n. plants	3	1	2
	%	**10**	**3**	**7**
LAMP results were not clear	n. plants	3	7	6
	%	**10**	**25**	**21**
LAMP reaction did not start	n. plants	4	-	1
	%	**14**	**-**	**3**
TOT	n. plant	29	29	29

Discussion and conclusions

Overall, seven different types of *ACCase* variant alleles were detected in the *Lolium* spp. populations from Greece and Italy: Leu_{1781}, Cys_{2027}, Asn_{2041}, Val_{2041}, Gly_{2078}, Arg_{2088} and Ala_{2096}. Depending on the population considered, one to four different *ACCase* alleles were observed in the same population. In contrast, no ACCase variant alleles were detected in the resistant plants of the four Danish populations indicating that this type of TSR is not present. The analysis of the *ALS* gene indicated that only one *ALS* variant allele (Leu_{574}) endowing resistance to iodosulfuron- methyl + mesosulfuron-methyl was present in two Danish populations while in the other two no *ALS* variants were detected. Most likely in these two populations (DK29 and DK90) the mechanism of resistance is non-target site (NTS). Conversely, in the Greek and Italian populations different *ALS* variant alleles were found: $Gly_{122,}$ 5 different allelic variants in position 197 (Ala_{197}, Gln_{197}, Leu_{197}, Ser_{197} and Thr_{197}), Val_{205}, Glu_{376} and Leu_{574}. Notably, the Italian populations showed amino acid substitutions at four different codons of the *ALS* gene.
According to our results and the literature, the most frequent mutations in the *ALS* gene are those in position 197, 376 and 574, whereas for the *ACCase* gene the mutations were in position 1781, 2041, 2078 and 2096. For all these positions a series of LAMP protocols were designed and subsequently tested.
Overall, the LAMP method was successful for the detection of TSR in *Lolium* species. Results of LAMP method on the ACCase point mutations were more consistent with results of the genotyping compared to those obtained on ALS point mutations. Consequently, the LAMP method is more feasible for detection of TSR to ACCase inhibitors than to TSR to ALS inhibitors. This result could be explained imputing a strategic role assumed by the nucleotide context (*ALS* gene has sequences rich in GC which often makes primers design difficult or leads to poor quality primers) around the investigated region. The quality of results on *ALS* gene could probably be improved to decrease at least the not clear results (in yellow in the table 4) by designing more specific primers.
For these reasons, in the validation step, we decided to focus the study of LAMP on TSR in the *ACCase* gene. Percentage of correspondence between LAMP and genotyping results were high (Table 7) and in all point mutations analysed more than 65% of plants were correctly assigned. Therefore, the analyses of a certain number of plants in an unknown population would provide a good indication of the presence of TSR mechanism and which are the most frequent mutations.
Anyway, some limits of LAMP method need to be considered:

- results are very specific for an allelic variant: if the specific primer (FIP or BIP) is designed for a specific allelic variant only that allelic variant will be detected. Plants including a different allelic variant in the same point will be recognised as not mutated: e.g. position 2041 of *ACCase* gene has two mutant allelic variant knows, AAT and GTT, but primer BIP5' were designed to be specific only to detect the triplet AAT. In fact, the result of the unique plant found carrying the triplet GTT was not correct with the LAMP method (plant 651-6, Table 5) causing an increase of false negatives which in reality are possibility resistant samples. Therefore, results of the LAMP method are feasible only to detect point mutation where a unique allelic variant was reported. The same is true for the position 197 of the *ALS* gene where 5 different allelic variants were detected in this study, but many other are reported in the literature. False negatives are underestimated also because the LAMP method was not available for all point mutations known in *ACCase* (or *ALS*) gene, e.g. in two plants of population 602 (Table 6) two different point mutations were found in position 2027 and 2088;
- using a real time detection method as GeneII, it is indispensable to have both set primers WT and MUT, in order to compare the amplification profiles of both and have a complete analysis to detect WT and MUT plants. This detection method is very sensitive and a different method for gDNA extraction (i.e. faster method with "Tissue Laser" and/or different "Two-Step" extraction buffer) is not usable because a good quality gDNA is required. In some cases, most likely due to the DNA quality, the LAMP reactions did not start neither with WT nor with MUT primer sets;
- the primers specificity is a variable to be considered: the primer sets WT and MUT are not always enough specific to give a clear distinction between the I50 of the two curves in the amplification plot (Figure 2). This is the reason why we put a threshold of at least 5 min for the Δt of the curves to assign a sample to WT or MUT phenotype. When this requisite is not applied, two almost overlapping curves were obtained and the results could not be assigned (yellow boxes in the Tables 4, 5 and 6);
- due to the lack of the method for all mutations known, a certain percentage of false negative (plants resulted WT for the LAMP method but carrying a point mutation not frequent) will always be possible. On the other hand, we detected the presence of some false positive (plants responded as mutated in the LAMP method which did not carry the mutation), also if in low percentage (3-10%), and also this have to be considered in a possible analyses of an unknown population.

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5. Expression pattern of individuated genes involved in ALS- and ACCase non-target site resistance in three countries.

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse Euro-pean countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D2.3.1**
Deliverable title:	**Expression pattern of individuated genes involved in ALS- and ACCase non-target site resistance in three countries.**
WP number:	**WP2**
Lead beneficiary:	**Solvejg K. Mathiassen**
Main author(s):	**Michael Kristensen & Solvejg K. Mathiassen**
Delivery date:	**06.04.2020**
Actual delivery date:	**24.07.2020**

Executive Summary

Non- target site resistance (NTSR) is considered to be polygenic inherited involving various genes encoding metabolic enzymes. Previous studies have identified over expression of herbicide metabolism related (HMR) genes in resistant individuals of *Lolium* species. We examined the expression pattern of HMR genes in nine populations of *Lolium multiflorum* from Greece, Italy and Denmark and two *L. perenne* populations from Denmark. The expression patterns of the four HMR genes, glycosyl-transferase (GT), nitronate monooxygenase (NMO), cytochrome P450 (CYP72A-1) and cytochrome P450 (CYP72A-2) and the reference gene IDE showed significant differences between plants from the same population implying that the *Lolium* populations in general are heterogenous for the gene expression of one or more HMR genes. Consequently, there is a potential for evolution of metabolic based herbicide resistance in most populations. The results of the present study do not support the development of a diagnostic tool for NTSR based on consistent constituent overexpression of specific HMR genes.

Introduction

Non-target site herbicide resistance can confer cross-resistance to herbicides of different modes of actions. NTSR is considered to be polygenic inherited involving various genes encoding metabolic enzymes such as cytochrome P450 monooxygenase (P450s), glucosyl transferases (GTs), glutathione S-transferases (GSTs), esterases and ABC transporters (Yuan et al., 2007; Duhoux and Délye 2013; Duhoux et al., 2017). Four consistently over-expressed genes were identified in resistant individuals of *Lolium rigidium*, a close relative of *Lolium multiflorum*. These include two- cytochrome P450 (P450), one nitronate monooxygenase (NMO) and one glycosyl-transferase (GT) (Gaines et al., 2014). In *L.* multiflorum higher expressions of these four herbicide metabolism related (HMR) genes were revealed in individuals of certain resistant populations from Denmark (Mahmood et al. 2016).

Description of work

Plant material

Eleven populations of *Lolium ssp*. were chosen for this study including three populations (one susceptible and two resistant) of *Lolium multiflorum* from each of the countries Greece, Italy and Denmark and two populations of *Lolium perenne* from Denmark (one susceptible and one resistant). The resistant populations were identified in previously conducted dose response experiments (see D.2.1). All populations were sown in trays placed in a glasshouse. At BBCH 21 twenty plants from each population were cloned and transplanted in pots. The remaining plants in the trays were sprayed with 1L/ha Atlantis OD (2 g/L iodosulfuron + 10 g/L mesosulfuron + 30 g/L mefenpyr-diethyle). The number of dead and surviving plants were counted three weeks later.

A week after transplanting one plant from each clone was sprayed with 1 L/ha Atlantis OD while the other one remained untreated.Plant responses to herbicide treatment was visually assessed four weeks after application. The individual sprayed plants (and their unsprayed twin) were rated as susceptible or resistant. The unsprayed plants were cut at the soil surface and frozen immediately after harvest.

Gene expression

Total RNA was extracted from 50 mg of leaf material of individual plants of eleven selected populations using RNeasy Plant Mini Kit (Qiagen, Stanford, California, USA). The quality and concentration of RNA samples were determined as Mahmood et al. (2016).

The qPCR reactions were performed with the GoTaq 1-step RT-qPCR System (Promega, Madison, Wisconsin, USA) using an Applied Bioscience ViiATM7 real-time PCR system with 384 wells (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Reactions were done in triplicate and a negative control consisting of reaction without template was also included for each primer. Briefly, 20 µL volume of reaction included 10 µL GoTaq qPCR MasterMix 2X, 4 µL RNA template, 4 µL 0.5 pmol µL primers (1:1 mix of forward and reverse primers), 0.4 µL GoScript RT Mix 1-Step RT-qPCR 50X, 1.6 µL nuclease-free distilled water. Reaction conditions included 50°C for 5 min followed by 10 min incubation at 95°C, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Two internal control genes, Rab GTPase (RGTP) and isocitrate dehydrogenase (IDE) and four herbicide metabolism genes nitronate monooxygenase (NMO), glutathione S-transferase

(GST), cytochrome P450s CYP72A1 and CYP72A2 were chosen. Primer sequences are the same described by Gaines et al. 2014 and available in Mahmood et al. 2016.
Threshold-cycle (C_t) values were calculated for each reaction. Gene-specific PCR efficiency was used to calculate the expression of target genes relative to the expression of internal reference genes. Equivalent slopes for target and internal control genes were observed in amplification plots. The ΔC_t value was calculated as follows: ΔC_t (target genes) = C_t (target gene) - C_t (reference gene), where C_t is the cycle number at which PCR product exceeded a set threshold. Relative transcript level (RTL) was calculated through = $1\times 2^{-\Delta Ct}$ (Pfaffl 2006).

Results and discussion

Plant material
The populations included in the study were collected in farmer fields and therefore consist of a mix of susceptible and resistant seeds. Results on surviving plants in the sprayed trays showed that 98 to 100% of the plants from the susceptible reference populations were controlled by the herbicide treatment verifying a high proportion of seeds from susceptible individuals in these populations. Conversely, 80 to 100% of the plants in the resistant populations survived the herbicide treatment these field populations to include a mix of ALS resistant and susceptible seeds. The cloning and subsequent herbicide treatment of one of the twins allowed selection of resistant individuals for the gene expression study.

Gene expression
The expression patterns of four herbicide metabolism genes, NMO, GST, CYP72A1, CYP72A2 as well as the reference gene IDE were tested in eleven populations of *Lolium* sp. (Table 1). For all five genes we observed significant difference between plants from the same population e.g. plant 7-3 was significant different ($p<0.0010$) from both plant 7-5 and 7-6 in NMO expression (Table S1) and plant 16-5 was significant different ($p<0.0010$) from plant 16-3, but not plant 16-4. These differences were observed for within all populations except population 15, implying that the *Lolium* populations in general are heterogenous for the gene expression of one or more herbicide metabolism genes (Table 1).
In the gene expression analysis the NMO gene showed significantly ($p<0.0010$) increased expression in most comparisons for 7-3, 14-1 and 16-5, whereas 18-1 showed very low relative expression (Table 1, Table S1). The GST gene showed significantly ($p<0.0010$) increased expression in most comparisons for 16-5 and 18-5, whereas three plants (3-6, 17-8, 18-1) from three different populations showed very low relative expression (Table 1, Table S2). The CYP72A1 gene was expressed at a relative low level as indicated by its generally low RTL values (Table 1). Plants 16-4, 16-5 and 18-5 showed significantly ($p<0.0010$) increased expression in most comparisons. Plant 14-5 showed very low relative expression (Table 1, Table S3). In the present study the CYP72A2 gene is expressed at the highest level of the genes investigated. Plant 16-5 exhibited the highest expression of CYP72A2 and plants 13-4, 14-1, 16-4, 19-11 all had relative high expression (table 1). Plants 16-5 and 18-5 showed significantly ($p<0.0010$) increased expression in most comparisons (Table 1, Table S4). Mostly the alternative reference gene IDE was not showing significant difference in expression, conforming its status as a potential reference gene. Only one outstanding population appears, 16-5, with significantly ($p<0.010$) elevated RTL in comparison to most other plants (Table 1, Table S5).
In conclusion, metabolic herbicide resistance is very heterogenous distributed among plants and populations, which highlights the potential for evolution of metabolic based herbicide resistance in most populations. In the present material plants 16-5 and 18-5 with high expression of NMO, GST, CYP72A1 and CYP72A2 represent the metabolic herbicide resistance type from Italy and Denmark, respectively.

Table 1. Gene expression of herbicide metabolism genes in *Lolium* ssp. from Greece (plants 3, 13, 14), Italy (plants 15, 16) and Denmark (plants 7, 9, 12, 17, 18, 19).*

		NMO	GST	CYP72A1	CYP72A2	IDE
Population	Plant	RTL (ΔC_t ± STD)	RTL (ΔC_t ± STD)	RTL (ΔC_t ± STD)	RTL (ΔC_t ± STD)	RTL (ΔC_t ± STD)

GR39 (S)	3-6	0.63 (0.66±0.54)	0.04 (4.79±0.53)	nd	2.14 (-3.29±0.64)	0.57(0.80±0.01)
	3-13	0.25 (2.01±1.35)	0.66 (0.60±0.99)*	nd	1.82 (-2.58±0.98)	0.29 (1.80±1.09)
DK100LM (S)	7-3	1.55 (-0.63±0.51)**	1.15 (-0.21±0.46)	0.25 (2.00±0.42)*	3.85 (-5.83±0.49)**	0.49 (1.03±0.41)
	7-5	0.31 (1.67±1.54)	0.38 (1.41±1.47)	0.04 (4.56±1.47)	0.77 (1.15±1.48)	0.44 (1.18±1.49)
	7-6	0.25 (2.00±0.16)	0.58 (0.80±0.17)	0.15 (2.77±0.17)	0.93 (0.33±0.19)	0.40 (1.33±0.16)
DK22LM (S)	9-3	0.65 (0.63±1.17)	0.50 (0.99±1.25)	0.23 (2.09±1.15)*	1.93 (-2.85±1.18)	1.09 (-0.12±1.16)**
	9-5	0.57 (0.81±0.51	1.09 (-0.12±0.72)	0.08 (3.56±0.78)	2.38 (-3.75±0.69)	0.26 (1.93±0.23)
	9-6	0.31 (1.69±0.06	0.15 (2.75±0.05)	0.03 (5.30±0.13)	1.12 (-0.48±0.06)	0.30 (1.75±0.05)
DK22LP (S)	12-8	0.46 (1.12±0.14)	0.14 (2.85±0.11)	0.04 (4.76±0.45)	0.72 (1.44±0.17)	0.45 (1.14±0.09)
	12-9	0.95 (0.08±0.13)	0.33 (1.58±0.42)	0.09 (3.42±0.42)	3.21 (-5.04±0.23)*	0.42 (1.24±0.08)
	12-11	0.28 (1.86±0.11)	0.11 (3.17±0.13)	0.04 (4.72±0.41)	1.27 (-1.02±0.21)	nd
GR24 (R)	13-4	1.11 (-0.15±0.13)	0.20 (2.32±0.05)	0.03 (5.28±0.15)	5.30 (-7.22±0.08)*	0.54 (0.89±0.06)
	13-5	0.43 (1.23±0.44)	0.23 (2.14±0.12)	nd	1.17 (-0.67±0.21)	0.68 (0.56±0.09)
GR30 (R)	14-1	1.41(-0.49±0.01)	0.55 (0.85±0.33)*	0.16 (2.69±0.33)	6.77 (-8.27±0.23)	0.60 (0.73±0.05)
	14-5	0.80 (0.32±0.08)	0.05 (4.28±0.16)	0.01 (6.24±0.24)**	1.70 (-2.29±0.10)	0.52 (0.93±0.09)
	14-7	1.51 (-0.59±0.05)	0.15 (2.77±0.09)	0.23 (2.15±0.45)	3.91 (-5.90±0.12)	0.37 (1.43±0.03)
IT533 (R)	15-5	0.23 (2.15±0.12)	0.05 (4.21±0.06)	0.09 (3.42±0.03)	1.64 (-2.15±0.05)	0.45 (1.14±0.04)
	15-7	0.28 (1.82±0.01)	0.06 (4.00±0.06)	0.03 (5.22±0.04)	1.77 (-2.46±0.17)	0.47 (1.08±0.06)
	15-11	0.31 (1.69±0.50)	0.48 (1.05±0.14)	0.05 (4.29±0.21)	3.10 (-4.89±0.50)	0.52 (0.94±0.49)
IT609 (R)	16-3	0.19 (2.42±0.06)	0.25 (2.02±0.03)	0.06 (4.07±0.03)**	1.73 (-2.36±0.12)	0.48 (1.07±0.01)
	16-4	0.46 (1.13±0.05)	1.53 (-0.61±0.01)	0.39 (1.37±0.15)	6.87 (-8.34±0.20)	0.34 (1.55±0.05)
	16-5	1.71 (-0.77±0.04)*	5.46 (-2.45±0.05)*	0.46 (1.13±0.21)	24.99 (-13.93±0.15)*	1.96 (-0.97±0.17)**
DK29LM (R)	17-8	0.18 (2.50±0.03)	0.01 (6.69±0.18)**	0.04 (4.73±0.07)	1.38 (-1.38±0.05)	0.18 (2.51±0.03)
	17-15	0.43 (1.23±0.06)	0.58 (0.78±0.04)	0.26 (1.96±0.00)*	2.44 (-3.87±0.09)	0.69 (0.53±0.09)

	17-17	0.55 (0.86±0.95)	0.88 (0.18±0.70)	0.09 (3.40±0.95)	4.03 (-6.03±0.83)	1.34 (-0.42±0.77)**
DK90LM (R)	18-1	0.09 (3.51±0.19)	0.03 (5.28±3.28)	0.09 (3.53±0.18)	1.77 (-2.47±0.03)	0.48 (1.07±0.04)
	18-3	0.16 (2.66±0.08)	0.14 (2.80±1.00)	0.04 (4.82±0.26)	4.17 (-6.18±0.11)	0.48 (1.04±0.01)
	18-5	0.57 (0.80±0.02)*	4.04 (-2.02±0.25)**	0.73 (0.45±0.30)**	12.50 (-10.93±0.07)*	0.18 (2.48±0.03)
DK13LP (R)	19-1	0.22 (2.18±0.06)	0.85 (0.23±0.25)	0.11 (3.16±0.37)	0.86 (0.68±0.12)	0.30 (1.76±0.12)
	19-3	0.46 (1.12±0.18)	0.82 (0.29±0.14)	0.17 (2.59±0.20)	2.17 (-3.35±0.03)	0.59 (0.76±0.03)
	19-11	0.53 (0.91±0.12)	1.20 (-0.26±0.10)	0.20 (2.35±0.29)	5.93 (-7.71±0.11)*	1.01 (-0.02±0.03)

*Transcript levels were assessed in 10 weeks old plants. Relative transcript levels (RTL) were based on reference to internal reference gene Rab GTPase (RGTP). NMO, GST, CYP72A1 and CYP72A2 are possible herbicide metabolism genes and IDE was another reference control gene.

The ΔCt ± STD used for statistical analysis are shown in parenthesis. The significance levels for each gene were calculated for all pairwise comparisons by an initial analysis of variance by a single factor Anova followed by Tukey HSD (Honestly Significant Difference) test. They are presented as supplementary tables Table S1-Table S5.

The nd indicates samples with Ct values below “no template” control.

One asterisk (*) Indicates where one plant is significant different ($p<0.0010$) to a plant from the same population. Two asterisks (**) indicates where one plant is significant different ($p<0.0010$) to two plants from the same population.

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Supplementary data Table S1-5. The significance levels for each gene were calculated for all pairwise comparisons by an initial analysis of variance by a single factor Anova followed by Tukey HSD (Honestly Significant Difference) test [http://www.real-statistics.com/one-way-analysis-of- variance-anova/unplanned-comparisons/tukey-hsd/].

TABLE S1. Pairwise comparison of NMO expression level Tukey p-values are indicated for all comparisons. The orange colour indicates significant different plants p < 0.0010.

NMO	P3-6	P3-13	P7-3	P7-5	P7-6	P9-3	P9-5	P9-6	P12-8	P12-9	P12-11	P13-4	P13-5	P14-1	P14-5	P14-7	P15-5	P15-7	P15-11	P16-3	P16-4	P16-5	P17-8	P17-15	P17-17	P18-1	P18-3	P18-5	P19-1	P19-3
P3-6																														
P3-13	0.2765																													
P7-3	0.3442	1.13E-05																												
P7-5	0.8246	1.0000	0.0003																											
P7-6	0.2844	1.0000	1.2E-05	1.0000																										
P9-3	1.0000	0.2294	0.4036	0.7703	0.2364																									
P9-5	1.0000	0.5102	0.1644	0.9607	0.5207	1.0000																								
P9-6	0.7881	1.0000	0.0002	1.0000	1.0000	0.7294	0.9458																							
P12-8	1.0000	0.9433	0.0221	1.0000	0.9471	1.0000	1.0000	0.9999																						
P12-9	0.9998	0.0056	0.9967	0.0670	0.0059	1.0000	0.9939	0.0565	0.7629																					
P12-11	0.5142	1.0000	4.58E-05	1.0000	1.0000	0.4485	0.7683	1.0000	0.9943	0.0180																				
P13-4	0.9774	0.0009	1.0000	0.0136	0.0009	0.9876	0.8737	0.0111	0.3780	1.0000	0.0031																			
P13-5	0.9999	0.9875	0.0099	1.0000	0.9887	0.9998	1.0000	1.0000	1.0000	0.5811	0.9995	0.2299																		
P14-1	0.5721	0.0000	1.0000	0.0008	4.2E-05	0.6392	0.3249	0.001	0.0570	0.9999	0.0002	1.000	0.0271																	
P14-5	1.0000	0.0348	0.8903	0.2666	0.0363	1.0000	1.0000	0.2344	0.9807	1.0000	0.0942	1.000	0.9233	0.9782																
P14-7	0.3983	1.56E-05	1.0000	0.0003	1.65E-05	0.4615	0.1983	0.0003	0.0284	0.9985	6.29E-05	1.000	0.0129	1.0000	0.9222															
P15-5	0.1307	1.0000	3E-06	1.0000	1.0000	0.1043	0.2865	1.0000	0.7935	0.0018	1.0000	0.0002	0.9179	1.08E-05	0.0124	4.17E-06														
P15-7	0.5709	1.0000	6.14E-05	1.0000	1.0000	0.5037	0.8147	1.0000	0.9970	0.0228	1.0000	0.0040	0.9998	0.0002	0.1145	8.42E-05	1.0000													
P15-11	0.7881	1.0000	0.000199	1.0000	1.0000	0.7295	0.9458	1.0000	0.9999	0.0565	1.0000	0.0111	1.0000	0.0007	0.2345	0.0003	1.0000	1.0000												
P16-3	0.0217	1.0000	2.27E-07	0.9920	1.0000	0.0164	0.0610	0.9951	0.3399	0.0002	0.9999	2.11E-05	0.5170	8.28E-07	0.0014	3.16E-07	1.0000	0.9998	0.9951											
P16-4	1.0000	0.9501	0.0205	1.0000	0.9536	1.0000	1.0000	0.9999	1.0000	0.7462	0.9954	0.3613	1.0000	0.0531	0.9773	0.0263	0.8087	0.9976	0.9999	0.3561										
P16-5	0.1682	2.91E-06	1.0000	6.82E-05	3.08E-06	0.2062	0.0689	5.371E-05	0.0074	0.9628	1.2E-05	0.9996	0.0031	1.0000	0.6881	1.0000	7.62E-07	1.62E-05	5.37E-05	5.68E-08	0.0068									
P17-8	0.0118	1.0000	1.04E-07	0.9706	1.0000	0.0088	0.0350	0.9798	0.2312	7.982E-05	0.9992	9.89E-06	0.3797	3.81E-07	0.0007	1.45E-07	1.0000	0.9983	0.9798	1.0000	0.2439	2.6E-08								
P17-15	0.9999	0.9882	0.0096	1.0000	0.9893	0.9997	1.0000	1.0000	1.0000	0.5752	0.9996	0.2261	1.0000	0.0265	0.9206	0.0125	0.9207	0.9998	1.0000	0.5228	1.0000	0.0030	0.3850							
P17-17	1.0000	0.5894	0.1274	0.9789	0.6000	1.0000	1.0000	0.9693	1.0000	0.9865	0.8317	0.8183	1.0000	0.2638	1.0000	0.1554	0.3507	0.8707	0.9694	0.0813	1.0000	0.0513	0.0477	1.0000						
P18-1	1.62E-06	0.1181	7.3E-12	0.0115	0.1140	1.13E-06	6.6E-06	0.0140	0.0001	5.819E-09	0.0450	6.7E-10	0.0003	2.59E-11	5.92E-08	1.01E-11	0.2544	0.0361	0.0140	0.6953	0.0001	1.93E-12	0.8203	0.0003	1E-05					
P18-3	0.0033	0.9990	2.25E-08	0.8385	0.9989	0.0024	0.0108	0.8700	0.0940	1.84E-05	0.9805	2.2E-06	0.1757	8.26E-08	0.0002	3.14E-08	1.0000	0.9691	0.8699	1.0000	0.1004	5.63E-09	1.0000	0.1789	0.0152	0.9645				
P18-5	1.0000	0.4924	0.1738	0.9553	0.5029	1.0000	1.0000	0.9390	1.0000	0.9950	0.7525	0.8847	1.0000	0.3399	1.0000	0.2092	0.2730	0.8005	0.9390	0.0570	1.0000	0.0735	0.0326	1.0000	1.0000	6E-06	0.0100			
P19-1	0.1069	1.0000	2.18E-06	1.0000	1.0000	0.0846	0.2432	1.0000	0.7420	0.0013	1.0000	0.0002	0.8858	7.83E-06	0.0095	3.03E-06	1.0000	1.0000	1.0000	1.0000	0.7588	5.52E-07	1.0000	0.8892	0.3014	0.2989	1.0000	0.2311		
P19-3	1.0000	0.9442	0.0219	1.0000	0.9480	1.0000	1.0000	0.9999	1.0000	0.7608	0.9944	0.3758	1.0000	0.0565	0.9802	0.0281	0.7954	0.9971	0.9999	0.3419	1.0000	0.0073	0.2328	1.0000	1.0000	0.0001	0.0948	1.0000	0.7442	
P19-11	1.0000	0.6805	0.0931	0.9910	0.6906	1.0000	1.0000	0.9860	1.0000	0.9702	0.8922	0.7411	1.0000	0.2028	0.9998	0.1150	0.4340	0.9216	0.9860	0.1121	1.0000	0.0359	0.0674	1.0000	1.0000	1.63E-05	0.0224	1.0000	0.3789	1.0000

TABLE S2. Pairwise comparison of GST expression level Tukey p-values are indicated for all comparisons. The orange colour indicates significant different plants p < 0.0010.

GST	P3-6	P3-13	P7-3	P7-5	P7-6	P9-3	P9-5	P9-6	P12-8	P12-9	P12-11	P13-4	P13-5	P14-1	P14-5	P14-7	P15-5	P15-7	P15-11	P16-3	P16-4	P16-5	P17-8	P17-15	P17-17	P18-1	P18-3	P18-5	P19-1	P19-3
P3-6																														
P3-13	4.07E-06																													
P7-3	2.55E-08	1.0000																												
P7-5	0.0005	1.0000	0.7398																											
P7-6	1.32E-05	1.0000	0.9989	1.0000																										
P9-3	4.24E-05	1.0000	0.9861	1.0000	1.0000																									
P9-5	4.33E-08	1.0000	1.0000	0.8215	0.9998	0.9949																								
P9-6	0.2846	0.1972	0.0052	0.9461	0.3603	0.5733	0.0080																							
P12-8	0.3764	0.1395	0.0031	0.8951	0.2710	0.4632	0.0049	1.0000																						
P12-9	0.0013	0.9993	0.5488	1.0000	1.0000	1.0000	0.6465	0.9890	0.9709																					
P12-11	0.7354	0.0365	0.0005	0.5826	0.0841	0.1762	0.0008	1.0000	1.0000	0.7695																				
P13-4	0.0557	0.6303	0.0432	0.9998	0.8289	0.9498	0.0625	1.0000	1.0000	1.0000	0.9999																			
P13-5	0.0242	0.8215	0.0945	1.0000	0.9462	0.9916	0.1316	1.0000	1.0000	1.0000	0.9983	1.0000																		
P14-1	1.89E-05	1.0000	0.9974	1.0000	1.0000	1.0000	0.9993	0.4217	0.3239	1.0000	0.1066	0.8755	0.9669																	
P14-5	1.0000	8.74E-05	6.04E-07	0.0080	0.0003	0.0008	1.02E-06	0.8217	0.8953	0.0187	0.9943	0.3520	0.1974	0.0004																
P14-7	0.3052	0.1821	0.0046	0.9359	0.3377	0.5468	0.0071	1.0000	1.0000	0.9859	1.0000	1.0000	1.0000	0.3972	0.8412															
P15-5	1.0000	0.0001	9.61E-07	0.0117	0.0004	0.0012	1.62E-06	0.8809	0.9368	0.0267	0.9980	0.4299	0.2532	0.0006	1.0000	0.8963														
P15-7	1.0000	0.0005	3.61E-06	0.0328	0.0014	0.0039	6.08E-06	0.9766	0.9916	0.0693	1.0000	0.6739	0.4631	0.0019	1.0000	0.9813	1.0000													
P15-11	6.26E-05	1.0000	0.97340	1.0000	1.0000	1.0000	0.9888	0.6486	0.5376	1.0000	0.2207	0.9711	0.9963	1.0000	0.0012	0.6223	0.0018	0.0056												
P16-3	0.0136	0.9095	0.14972	1.0000	0.9813	0.9983	0.2024	1.0000	1.0000	1.0000	0.9916	1.0000	1.0000	0.9900	0.1279	1.0000	0.1688	0.3378	0.9994											
P16-4	1.99E-09	0.9825	1.00000	0.2973	0.9135	0.7583	1.0000	0.0006	0.0003	0.1693	4.84E-05	0.0060	0.0151	0.8754	4.74E-08	0.0005	7.57E-08	2.88E-07	0.6890	0.0268										
P16-5	5.22E-14	0.0031	0.13945	2.96E-05	0.0011	0.0004	0.1005	6.916E-09	3.791E-09	1.057E-05	5.12E-10	1.05E-07	3.31E-07	0.000781	5.66E-13	5.99E-05	8.69E-13	3.09E-12	0.0002	6.96E-07	0.4881									
P17-8	0.4177	2.8E-11	2.31E-13	4.25E-09	9.14E-11	0.0000	3.65E-13	1.85E-05	3.306E-05	1.23E-08	0.0002	1.28E-06	4.09E-07	1.31E-10	0.0731	2.13E-05	0.0531	0.0199	4.51E-10	1.94E-07	4.31E-14	2.51E-14								
P17-15	1.2E-05	1.0000	0.9992	1.0000	1.0000	1.0000	0.9998	0.3441	0.2574	1.0000	0.0786	0.8146	0.9392	1.0000	0.0002	0.3223	0.0004	0.0013	1.0000	0.9780	0.9225	0.0012	8.26E-11							
P17-17	2.9E-07	1.0000	1.0000	0.9798	1.0000	1.0000	1.0000	0.0352	0.0225	0.9183	0.0045	0.2033	0.3606	1.0000	6.69E-08	0.0317	1.06E-05	3.86E-05	0.9995	0.4890	1.0000	0.0267	2.13E-12	1.000						
P18-1	1.0000	1.84E-07	1.13E-09	2.72E-05	6.1E-07	2.02E-06	1.91E-09	0.0419	0.0639	7.528E-05	0.2189	0.0050	0.0019	8.8E-07	0.9990	0.0464	0.9968	0.9659	3.02E-08	0.0010	9.02E-11	2.63E-14	0.9147	5.51E-07	1.28E-08					
P18-3	0.3285	0.1665	0.0040	0.9235	0.3139	0.5179	0.0063	1.0000	1.0000	0.9817	1.0000	1.0000	1.0000	0.3713	0.8612	1.0000	0.9116	0.9858	0.5935	1.0000	0.0004	5.12E-09	2.47E-05	0.2990	0.0282	0.0518				
P18-5	3.8E-13	0.0280	0.5205	0.0004	0.0110	0.0041	0.4255	1.057E-07	5.792E-08	0.0001	7.75E-09	1.58E-06	4.93E-06	0.0082	7.49E-12	9.16E-08	1.18E-11	4.36E-11	0.0029	1.02E-05	0.9148	1.0000	2.51E-14	0.0119	0.1655	4.31E-14	7.83E-08			
P19-1	4.08E-07	1.0000	1.0000	0.9886	1.0000	1.0000	1.0000	0.0449	0.0290	0.9447	0.0059	0.2438	0.4172	1.0000	9.33E-08	0.0408	1.47E-05	5.34E-05	1.0000	0.5515	0.9999	0.0206	2.94E-12	1.0000	1.0000	1.8E-08	0.0362	0.1354		
P19-3	5.64E-07	1.0000	1.0000	0.9938	1.0000	1.0000	1.0000	0.0565	0.0369	0.9638	0.0078	0.2873	0.4746	1.0000	1.28E-05	0.0511	2.02E-05	7.29E-05	1.0000	0.6118	0.9998	0.0160	4.03E-12	1.0000	1.0000	2.49E-08	0.0458	0.1109	1.0000	
P19-11	1.76E-08	0.9999	1.0000	0.6763	0.9974	0.9750	1.0000	0.0038	0.0023	0.4815	0.0004	0.0331	0.0743	0.9942	4.19E-07	0.0034	6.67E-07	2.52E-06	0.9557	0.1200	1.0000	0.1729	1.71E-13	0.9979	1.0000	7.82E-10	0.0029	0.5884	1.0000	1.0000

TABLE S3. Pairwise comparison of CYP72A1 expression level Turkey p-values are indicated for all comparisons. The orange colour indicates significant different plants $p < 0.0010$.

CYP72A1	P3-6	P3-13	P7-3	P7-5	P7-6	P9-3	P9-5	P9-6	P12-8	P12-9	P12-11	P13-4	P13-5	P14-1	P14-5	P14-7	P15-5	P15-7	P15-11	P16-3	P16-4	P16-5	P17-8	P17-15	P17-17	P18-1	P18-3	P18-5	P19-1	P19-3
P3-6																														
P3-13																														
P7-3																														
P7-5			0.0000																											
P7-6			0.9736	0.0100																										
P9-3			1.0000	0.0000	0.9945																									
P9-5			0.0547	0.7364	0.9670	0.0988																								
P9-6			0.0000	0.9826	0.0000	0.0000	0.0139																							
P12-8			0.0000	1.0000	0.0020	0.0000	0.3891	0.9998																						
P12-9			0.1294	0.4969	0.9970	0.2153	1.0000	0.0048	0.2039																					
P12-11			0.0000	1.0000	0.0029	0.0000	0.4628	0.9993	1.0000	0.2553																				
P13-4			0.0000	0.9870	0.0000	0.0000	0.0159	1.0000	0.9999	0.0055	0.9996																			
P13-5																														
P14-1			0.9936	0.0052	1.0000	0.9992	0.9107	0.0000	0.0010	0.9851	0.0014	0.0000																		
P14-5			0.0000	0.0232	0.0000	0.0000	0.0000	0.8378	0.0905	0.0000	0.0684	0.8139		0.0000																
P14-7			1.0000	0.0000	0.9982	1.0000	0.1349	0.0000	0.0000	0.2791	0.0000	0.0000		0.9998	0.0000															
P15-5			0.1320	0.4909	0.9972	0.2191	1.0000	0.0047	0.2002	1.0000	0.2510	0.0054		0.9859	0.0000	0.2837														
P15-7			0.0000	0.9962	0.0000	0.0000	0.0257	1.0000	1.0000	0.0092	1.0000	1.0000		0.0000	0.7131	0.0000	0.0090													
P15-11			0.0002	1.0000	0.0713	0.0003	0.9859	0.7152	1.0000	0.9135	1.0000	0.7442		0.0406	0.0027	0.0005	0.9104	0.8394												
P16-3			0.0010	1.0000	0.2506	0.0022	0.9999	0.3408	0.9931	0.9970	0.9972	0.3681		0.1607	0.0004	0.0034	0.9968	0.4768	1.0000											
P16-4			0.9979	0.0000	0.1425	0.9871	0.0004	0.0000	0.0000	0.0012	0.0000	0.0000		0.2251	2.43E-14	0.9711	0.0012	7.23E-11	0.0000	3.3E-06										
P16-5			0.9039	0.0000	0.0291	0.7916	0.0000	0.0000	0.0000	0.0001	7.99E-10	4.5E-12		0.0521	1.57E-14	0.7095	0.0001	8.03E-12	4.38E-08	3.38E-07	1.0000									
P17-8			0.0000	1.0000	0.0025	0.0000	0.4320	0.9996	1.0000	0.2333	1.0000	0.9998		0.0012	0.0768	9.41E-06	0.2292	1.0000	1.0000	0.9959	6.51E-09	6.77E-10								
P17-15			1.0000	8.75E-06	0.9557	1.0000	0.0422	8.266E-09	1.363E-06	0.1029	2.05E-06	0.0000		0.9871	0.0000	1.0000	0.1051	0.0000	0.0001	0.0007	0.9992	0.936	1.74E-06							
P17-17			0.1443	0.4641	0.9980	0.2371	1.0000	0.0041	0.1844	1.0000	0.2323	0.0047		0.9892	9.43E-07	0.3049	1.0000	0.0080	0.8957	0.9956	0.0014	0.000	0.2117	0.1153						
P18-1			0.0674	0.6839	0.9791	0.1195	1.0000	0.0110	0.3402	1.0000	0.4097	0.0125		0.9364	0.0000	0.1614	1.0000	0.0205	0.9768	0.9998	0.0005	5.32E-05	0.3804	0.0523	1.0000					
P18-3			1.13E-06	1.0000	0.0012	2.65E-06	0.3005	1.0000	1.0000	0.1476	1.0000	1.0000		0.0006	0.1291	0.0000	0.1447	1.0000	0.9999	0.9810	2.96E-09	3.11E-10	1.0000	0.0000	0.1324	0.2585				
P18-5			0.0538	0.0000	0.0001	0.0285	0.0000	0.0000	0.0000	0.0000	0.0000	2.61E-14		0.0002	1.41E-14	0.0195	2.56E-07	3.52E-14	0.0000	5.74E-10	0.8503	0.9941	1.43E-12	0.0693	0.0000	9.25E-08	6.9E-13			
P19-1			0.4647	0.1440	1.0000	0.6282	1.0000	0.0005	0.0403	1.0000	0.0543	0.0006		1.0000	9.57E-08	0.7179	1.0000	0.0011	0.5169	0.8673	0.0099	0.0014	0.0481	0.4003	1.0000	1.0000	0.0266	2.9E-06		
P19-3			0.9993	0.0023	1.0000	1.0000	0.7932	0.0000	0.0004	0.9414	0.0006	0.0000		1.0000	4.72E-10	1.0000	0.9438	6.42E-06	0.0202	0.0898	0.3582	0.0982	0.0005	0.9982	0.9533	0.8367	0.0003	0.0005	0.9996	
P19-11			1.0000	0.0003	1.0000	1.0000	0.3805	0.0000	0.0000	0.6180	0.0001	0.0000		1.0000	5.19E-11	1.0000	0.6241	6.73E-07	0.0030	0.0169	0.7719	0.3549	6.16E-05	1.0000	0.6512	0.4320	2.85E-05	0.0039	0.9546	1.0000

TABLE S4. Pairwise comparison of CYP72A2 expression level Turkey p-values are indicated for all comparisons. The orange colour indicates significant different plants p < 0.0010

CYP72A2	P3-6	P3-13	P7-3	P7-5	P7-6	P9-3	P9-5	P9-6	P12-8	P12-9	P12-11	P13-4	P13-5	P14-1	P14-5	P14-7	P15-5	P15-7	P15-11	P16-3	P16-4	P16-5	P17-8	P17-15	P17-17	P18-1	P18-3	P18-5	P19-1	P19-3
P3-6																														
P3-13	1.0000																													
P7-3	0.9316	0.5866																												
P7-5	0.0770	0.3057	0.0001																											
P7-6	0.3642	0.7846	0.0007	1.0000																										
P9-3	1.0000	1.0000	0.7499	0.1896	0.6268																									
P9-5	1.0000	1.0000	0.9937	0.0255	0.1642	1.0000																								
P9-6	0.8358	0.9928	0.0074	0.9999	1.0000	0.9670	0.5752																							
P12-8	0.0386	0.1819	0.0000	1.0000	1.0000	0.1048	0.0118	0.9982																						
P12-9	0.9996	0.9517	1.0000	0.0006	0.0070	0.9879	1.0000	0.0569	0.0003																					
P12-11	0.9809	0.9999	0.0311	0.9895	1.0000	0.9991	0.8712	1.0000	0.9521	0.1812																				
P13-4	0.2160	0.0482	1.0000	0.0000	0.0000	0.0899	0.4474	0.0001	0.0000	0.9888	0.0006																			
P13-5	0.9119	0.9983	0.0127	0.9992	1.0000	0.9886	0.6967	1.0000	0.9924	0.0891	1.0000	0.0002																		
P14-1	0.0200	0.0028	0.9554	0.0000	0.0000	0.0062	0.0620	0.0000	0.0000	0.5982	0.0000	1.0000	0.0000																	
P14-5	1.0000	1.0000	0.4084	0.4680	0.9086	1.0000	1.0000	0.9993	0.3046	0.8614	1.0000	0.0234	0.9999	0.0012																
P14-7	0.9117	0.5434	1.0000	0.0000	0.0006	0.7107	0.9901	0.0061	0.0000	1.0000	0.0262	1.0000	0.0106	0.9675	0.3696															
P15-5	1.0000	1.0000	0.3276	0.5595	0.9483	1.0000	0.9999	0.9998	0.3826	0.7928	1.0000	0.0159	1.0000	0.0008	1.0000	0.2932														
P15-7	1.0000	1.0000	0.5122	0.3679	0.8422	1.0000	1.0000	0.9969	0.2267	0.9222	1.0000	0.0361	0.9994	0.0020	1.0000	0.4699	1.0000													
P15-11	0.9999	0.9761	1.0000	0.0010	0.0106	0.9954	1.0000	0.0802	0.0004	1.0000	0.2386	0.9744	0.1228	0.5047	0.9153	1.0000	0.8622	0.9578												
P16-3	1.0000	1.0000	0.4501	0.4257	0.8839	1.0000	1.0000	0.9986	0.2708	0.8890	1.0000	0.0280	0.9998	0.0015	1.0000	0.4096	1.0000	1.0000	0.9352											
P16-4	0.0169	0.0023	0.9413	0.0000	0.0000	0.0051	0.0532	0.0000	0.0000	0.5573	1.71E-05	1.0000	0.0000	1.0000	0.0010	0.9561	0.0006	0.0017	0.4647	0.0012										
P16-5	2.34E-10	0.0000	0.0000	2.54E-14	0.0000	0.0000	0.0000	4.9E-14	2.498E-14	8.95E-08	1.6E-13	0.000124	7.06E-14	0.0032	8.54E-12	1.62E-06	5.26E-12	1.51E-11	5.39E-08	1.08E-11	0.0038									
P17-8	0.9984	1.0000	0.0746	0.935276	0.9997	1.0000	0.9693	1.0000	0.8292	0.3420	1.0000	0.0019	1.0000	6.97E-05	1.0000	0.0637	1.000	1.0000	0.4256	1.0000	5.65E-05	4.63E-13								
P17-15	1.0000	1.0000	0.9974	0.019	0.1281	1.0000	1.0000	0.4983	0.0084	1.0000	0.8169	0.5228	0.6216	0.0820	0.9999	0.9956	0.9997	1.0000	1.0000	1.0000	0.0708	0.0000	0.9465							
P17-17	0.8643	0.4627	1.0000	0.000	0.0004	0.6317	0.9791	0.0042	0.0000	1.0000	0.0187	1.0000	0.0074	0.9835	0.3014	1.0000	0.234337	0.3930	1.0000	0.3374	0.9765	0.0000	0.0469	0.9896						
P18-1	1.0000	1.0000	0.5161	0.364457	0.8394	1.0000	1.0000	0.9968	0.2242	0.9240	1.0000	0.0367	0.9994	0.0020	1.0000	0.4737	1.000	1.0000	0.9589	1.0000	0.0017	1.54E-11	1.0000	1.0000	0.3965					
P18-3	0.7944	0.3747	1.0000	0.000	0.0002	0.5370	0.9563	0.0027	0.0000	1.0000	0.0125	1.0000	0.0048	0.9935	0.2328	1.0000	0.177	0.3119	1.0000	0.2635	0.9901	4.17E-06	0.0324	0.9757	1.0000	0.3151				
P18-5	0.0000	0.0000	0.0149	0.000	0.0000	0.0000	0.0000	4.41E-10	7.918E-13	0.0016	2.73E-09	0.3143	8.56E-10	0.8970	2.06E-07	0.0178	1.25E-07	3.7E-07	0.0010	2.62E-07	0.9181	0.7391	9.45E-09	3.97E-05	0.0250	3.78E-07	0.0366			
P19-1	0.2038	0.5807	0.0002	1.000	1.0000	0.4144	0.0794	1.0000	1.0000	0.0026	0.9998	0.0000	1.0000	0.0000	0.7557	0.0002	0.830695	0.6547	0.0040	0.7155	5.78E-08	2.64E-14	0.9948	0.0600	0.0001	0.6508	7.74E-05	9.41E-12		
P19-3	1.0000	1.0000	0.9464	0.067	0.3321	1.0000	1.0000	0.8071	0.0333	0.9998	0.9738	0.2406	0.8917	0.0234	1.0000	0.9293	1	1.0000	1.0000	1.0000	0.0198	2.87E-10	0.9974	1.0000	0.8877	1.0000	0.824036	7.16E-06	0.1820	
P19-11	0.0799	0.0140	0.9988	0.000	0.0000	0.0284	0.2049	0.0000	0.0000	0.8956	0.0001	1.0000	0.0000	1.0000	0.0063	0.9993	0.004153	0.0102	0.8352	0.0077	1.0000	0.0006	0.0004	0.2555	0.9998	0.0103	1.0000	0.6007	4.96E-07	0.0913

TABLE S5. Pairwise comparison of IDE expression level Tukey p-values are indicated for all comparisons. The orange colour indicates significant different plants p < 0.0010.

IDE	P3-6	P3-13	P7-3	P7-5	P7-6	P9-3	P9-5	P9-6	P12-8	P12-9	P12-11	P13-4	P13-5	P14-1	P14-5	P14-7	P15-5	P15-7	P15-11	P16-3	P16-4	P16-5	P17-8	P17-15	P17-17	P18-1	P18-3	P18-5	P19-1	P19-3
P3-6																														
P3-13	0.5688																													
P7-3	1.0000	0.9399																												
P7-5	1.0000	0.9957	1.0000																											
P7-6	0.9997	1.0000	1.0000	1.0000																										
P9-3	0.7273	0.0005	0.2799	0.1117	0.0370																									
P9-5	0.3280	1.0000	0.7809	0.9558	0.9975	0.0001																								
P9-6	0.6689	1.0000	0.9708	0.9988	1.0000	0.0008	1.0000																							
P12-8	1.0000	0.9898	1.0000	1.0000	1.0000	0.1460	0.9249	0.9966																						
P12-9	1.0000	0.9991	1.0000	1.0000	1.0000	0.0727	0.9833	0.9998	1.0000																					
P12-11																														
P13-4	1.0000	0.7439	1.0000	1.0000	1.0000	0.5503	0.4937	0.8273	1.0000	1.0000																				
P13-5	1.0000	0.1708	1.0000	0.9959	0.9418	0.9837	0.0719	0.2308	0.9985	0.9855		1.0000																		
P14-1	1.0000	0.4225	1.0000	1.0000	0.9974	0.8490	0.2187	0.5203	1.0000	0.9998		1.0000	1.0000																	
P14-5	1.0000	0.8168	1.0000	1.0000	1.0000	0.4642	0.5806	0.8858	1.0000	1.0000		1.0000	1.0000	1.0000																
P14-7	0.9941	1.0000	1.0000	1.0000	1.0000	0.0151	0.9999	1.0000	1.0000	1.0000		0.9994	0.8150	0.9758	0.9999															
P15-5	1.0000	0.9886	1.0000	1.0000	1.0000	0.1513	0.9197	0.9961	1.0000	1.0000		1.0000	0.9987	1.0000	1.0000	1.0000														
P15-7	1.0000	0.9703	1.0000	1.0000	1.0000	0.2121	0.8561	0.9876	1.0000	1.0000		1.0000	0.9998	1.0000	1.0000	1.0000	1.0000													
P15-11	1.0000	0.8331	1.0000	1.0000	1.0000	0.4434	0.6023	0.8981	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	0.9999	1.0000	1.0000												
P16-3	1.0000	0.9657	1.0000	1.0000	1.0000	0.2241	0.8431	0.9852	1.0000	1.0000		1.0000	0.9998	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000											
P16-4	0.9557	1.0000	0.9998	1.0000	1.0000	0.0055	1.0000	1.0000	1.0000	1.0000		0.9904	0.6059	0.8896	0.9962	1.0000	1.0000	1.0000	0.9971	1.0000										
P16-5	0.0021	0.0000	0.0002	4.18E-05	0.0000	0.8473	0.0000	8.01E-08	6.356E-05	2.208E-05		0.0009	0.0182	0.0041	0.0006	2.69E-06	6.73E-05	0.0001	0.0005	0.0001	0.0000									
P17-8	0.0036	0.9729	0.0285	0.0894	0.2337	0.0000	0.9981	0.9435	0.0668	0.1353		0.0081	0.0003	0.0018	0.0118	0.4093	0.0641	0.0422	0.0129	0.0392	0.6340	1.77E-11								
P17-15	1.0000	0.1389	0.9999	0.9917	0.9133	0.9913	0.0566	0.1906	0.9966	0.9746		1.0000	1.0000	1.0000	1.0000	0.7613	0.9970	0.9993	1.0000	0.9995	0.5406	0.0238	0.0002							
P17-17	0.1832	0.0000	0.0335	0.0095	0.0024	1.0000	0.0000	0.0000	0.0135	0.0055		0.1018	0.5916	0.2811	0.0748	0.0009	0.0141	0.0225	0.0692	0.0243	0.0003	0.9995	0.0000	0.6563						
P18-1	1.0000	0.9637	1.0000	1.0000	1.0000	0.2289	0.8377	0.9841	1.0000	1.0000		1.0000	0.9998	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0001	0.0381	0.9996	0.0250					
P18-3	1.0000	0.9472	1.0000	1.0000	1.0000	0.2654	0.7971	0.9750	1.0000	1.0000		1.0000	0.9999	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9999	0.0002	0.0309	0.9998	0.0310	1.0000				
P18-5	0.0047	0.9836	0.0361	0.1097	0.2754	0.0000	0.9991	0.9626	0.0827	0.1636		0.0104	0.0005	0.0023	0.0152	0.4654	0.0795	0.0529	0.0166	0.0493	0.6917	0.0000	1.0000	0.0003	9.75E-09	0.0479	0.0390			
P19-1	0.6513	1.0000	0.9664	0.9985	1.0000	0.0007	1.0000	1.0000	0.9958	0.9998		0.8135	0.2191	0.5024	0.8748	1.0000	0.9952	0.9853	0.8879	0.9826	1.0000	7.26E-08	0.9499	0.1804	0.0000	0.9814	0.9712	0.9674		
P19-3	1.0000	0.4905	1.0000	1.0000	0.9990	0.7954	0.2669	0.5912	1.0000	0.9999		1.0000	1.0000	1.0000	1.0000	0.9870	1.0000	1.0000	1.0000	1.0000	0.9260	0.0030	0.0025	1.0000	0.2311	1.0000	1.0000	0.0033	0.5731	
P19-11	0.8856	0.0013	0.4587	0.2142	0.0803	1.0000	0.0004	0.0020	0.2693	0.1472		0.7499	0.9983	0.9535	0.6686	0.0352	0.2776	0.3674	0.6475	0.3842	0.0137	0.6714	0.0000	0.9993	1.0000	0.3909	0.4399	8.85E-07	0.0019	0.9263

6. Report on resistance mechanisms endowing glyphosate resistance

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse Euro-pean countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D2.4.1**
Deliverable title:	**Report on resistance mechanisms endowing glyphosate resistance**
WP number:	**WP2**
Lead beneficiary:	**Solvejg K. Mathiassen**
Main author(s):	**Demosthenis Chachalis, Eleni Tani & Solvejg K. Mathiassen**
Delivery date:	**06.08.2020**
Actual delivery date:	**20.09.2020**

Executive Summary

Rigid ryegrass (*Lolium rigidum Gaud*.), belonging to the *Poaceae* family, is considered to be one of the major grass weed species in the Mediterranean region. Herbicide resistance in *Lolium* species has been reported in several habitats, such as agricultural fields, orchards, vineyards, and road sides.Rigid ryegrass is considered to be one of the most economically important weeds in several countries (Travlos et al. 2018) including Greece. Mutations in the EPSPS gene are known to confer target-site resistance to glyphosate. Sequencing of six populations of *L. rigidum* revealed no point mutations in the Pro106 position known to confer resistance thus indicating non-target site resistance to be the most likely mechanism. The impact of four different ABC transporter genes on resistance was studied indicating *ABC transporter gene type 3* to be the best candidate for a marker gene discriminating resistant and susceptible populations at early stages which was confirmed by studies of a resistant population from conservation agriculture in Italy. Determination of shikimic acid's concentration in the plants' tissue is a reliable and easy way to investigate the level of resistance to glyphosate. Seven biotypes, four resistant and three susceptible, were studied and their shikimate accumulation was estimated 4 days after the plants were treated with the recommended dose of glyphosate (N = 720 g. a.i. ha- 1). The results indicated only two significant differences between populations, with population 12 being the most susceptible and population 16 being the most resistant populations.

Introduction

Rigid ryegrass or annual ryegrass (*L. rigidum* Gaud.) is a winter annual grass weed which causes severe problems mainly in winter cereal crops and, to a lesser extent, in other winter and early spring crops. Rigid ryegrass is nowadays present in almost every country in the world and it is also reported in high frequency in Greece. The effective control of this weed is a matter of great importance for the cereal crop farmers because it has developed several mechanisms of resistance towards the most commonly used herbicides worldwide. The herbicide glyphosate kills the plants by blocking a reaction on the shikimic acid pathway. The known glyphosate resistance mechanisms include target-site mutation and target-site gene duplication. Mutations in the *EPSPS* gene are known to develop target- site resistance to glyphosate. The region of the gene that might potentially carry the mutation is Proline 106. Non-target site resistance (NTSR) mechanisms include reduced translocation as a consequence of active vacuole sequestration, limited cellular uptake, a rapid necrosis response and prevention or reduction in the amount of glyphosate entering the cell in resistant plants. Different metabolic genes have been identified to be involved in herbicide resistance - among these the *ABC transporter genes* (Cechin et al., 2020). Due to the competition of the EPSPS enzyme with glyphosate, the concentration of shikimic acid in the herbicide-treated parts of the plant is affected in case of resistance. Measuring of the shikimic acid's concentration is one way of monitoring plant resistance (Tani et al. 2020). In the present study, seven *L .rigidum* biotypes, both resistant and susceptible to glyphosate, were studied in order to determine the shikimate concentration and estimate their level of resistance to the recommended dose (N = 720 g. a.i. ha^{-1}) of glyphosate.

Description of work

This study includes several ways to to determine glyphosate resistance. One part investigated the target-site resistance mechanism to glyphosate in different *L. rigidum* populations while another part compared the level of different transporter genes known to be conferred with NTSR in resistant and susceptible plants. A third part investigated the shikimate level in different glyphosate resistant populations of *L. rigidum* and finally a *Lolium* population from conservation agriculture was characterized. The first part of this deliverable will present a short summary of the studies Full reports of the two studies are enclosed as supplemental information in the end of this deliverable.

Target-site resistance mechanisms in different populations of *L. rigidum*

The aim of this study was to sequence several population of *L. rigidum* to identify point mutations in resistant populations. Mutations in the EPSPS gene are known to be responsible for development of target-site resistance to glyphosate.The region of the gene that might potentially carry the mutation is Proline at position 106.

Six populations were included in the experiment (5 resistant, 1 susceptible) – five of these populations were from Greece.

The results of the PCR sequencing showed that the majority of populations carried the proline amino acid encoded by the CCA codon at 106, thus no mutation was observed in the resistant populations. However, it was interesting to notice that in the Italian population and one sample of population 93, proline was encoded by the CCG codon instead of CCA codon. Previously this has been interpreted as a silent point mutation that occurs predominantly in sensitive populations and confers a different level of resistance (Jasieniuk et al. 2008). However, in the present work this silent mutation appears to be consistent with resistant populations which is interesting for further investigations.

In conclusion, the Greek populations did not carry the mutation at Pro 106 knowing to confer resistance to glyphosate. Further experiments need to be conducted with the Italian population in order to certify that this population carry a silent mutation.

Expression of key genes involved in NTSR in *L. rigidum*

The relative expression of four *ABC transporter* genes in a susceptible and a resistant population of *L. rigidum* was studied in order to investigate their impact on conferring resistance. The plants were treated with 720 g a.e. ha^{-1} glyphosate and leaf samples were harvested 3, 6 and 12 h after treatment. The genes of interest were selected from the NCBI database.

The most profound difference between the two populations was observed for the relative expression of the *ABC transporter gene type 3* at 12 h after treatment. This gene seems to be a good candidate for being a marker gene discriminating glyphosate resistant and susceptible populations at very early stages.

Characterization of a *Lolium rigidum* population from conservation agriculture

The relative expression of the ABC transporter genes were studied in a putative resistant *Lolium rigidum* population from Italy in order to identify marker genes that could discriminate resistant from susceptible populations at early growth stages. The plants were treated as described above.

A significant up-regulation (43 fold compared to control) of the *ABC transporter gene type 3* was recorded 12 hours after glyphosate treatment. The results support the hypothesis that this gene is a good candidate for a marker gene discriminating glyphosate resistant and susceptible populations

Shikimate level in different populations of *L. rigidum*

The herbicide glyphosate kills the plants by blocking a reaction on the shikimic acid pathway. Due to the competition of the EPSPS enzyme with glyphosate, the concentration of shikimic acid in the herbicide-treated parts of the plants is affected. Determination of shikimic acid concentration in plant tissue is a reliable and easy way to investigate the level of resistance to glyphosate. In this study two different protocols were compared – one was based on the method described by Shaner et al. (2005) and the other one on a similar study on Conyza ssp. (Tani et al. 2015).

Seven populations were included in the study (4 resistant and 4 susceptible) The plants were sown in pots and were sprayed with glyphosate 12 weeks after sowing. Plant tissue was collected 4 days after treatment.

The results following the use of protocol 1 were not consistent. Further investigation and standardization of this protocol is required in order to obtain a better profile and repeatability of the results. Results of the second protocol presented significant differences in shikimate concentration for two populations. The susceptible population 12 accumulated the highest level of shikimic acid and the resistant population 16 the lowest level. All other populations showed no difference in shikimic acid accumulation. Standardization of the test (t.e. collected plant tissue, growth stage of plants) is suggested to obtain a reliable separation of resistant and susceptible populations based on sole biochemical test.

Conclusion

The results of the target-site resistance study confirm that the known mutations in position 106 of the EPSPS gene conferring resistance to glyphosate were not present in the resistant populations. Consequently, resistance may be due to other mutations, or more likely, to non-target site resistance mechanisms. Studies on the expression pattern of four genes suggested involved in glyphosate NTSR showed the *ABC transporter gene type 3* to be the best candidate for a marker gene. This gene was upregulated in resistant plants at 12 hours after treatment and the response was confirmed in a resistant population of L. rigidum from Italy. One way to assess the level of resistance is by measuring the level of shikimic acid in glyphosate treated plant tissue. In resistant plants a slight increase in shikimic acid appeared within 24 h after treatment and the level remained stable while in susceptible plants an accentuated increase occurs within 72 h after glyphosate treatment and stabilizes at a high level. The two protocols tested in our study both required further standardization to obtain a reliable separation of resistant and susceptible populations based sole on biochemical test.

Appendix 1. INVESTIGATIONS ON MUTATIONS ENDOWING TARGET-SITE RESISTANCE TO GLYPHOSATE

The purpose of the present study was to investigate the target-site resistance mechanism to glyphosate in different populations of the annual ryegrass, *Lollium rigidum*. More specifically, to study mutations on the EPSPS gene which is responsible for developing glyphosate resistance.. The aim was to sequence various populations to identify point mutations in resistant populations so that the development of resistance to glyphosate could be investigated and understood better. The region of the gene that might potentially carry the mutation is proline 106. The procedure followed and the results obtained are presented in detail below.

Materials and Methods

At first it was expected to have results from three individuals per biotype but not all samples worked effectively. So the results from those biotypes, which gave us the best sequencing results are mentioned below. Table 1 lists the biotypes used in this experiment as well as the number of individuals sequenced per biotype.

Table 1 Biotypes used, the indication that they are likely to be resistant or susceptible, and the number of plants used for sequencing per biotype.

POPULATIN	POSIBLE RESISTANCE/SUSCEPTABILITY TO GLYPHOSATE	NUMBER OF INDIVIDUALS SEQUENCED/ BIOTYPE
Italian	RESISTANT	2
93	RESISTANT	3
82	RESISTANT	3
70	RESISTANT	1
27	SUSCEPTIBLE	2
21	RESISTANT	4

RNA Isolation

Prior to RNA isolation, tips, Eppendorf tubes, and other tools (spatulas, tongs, mortar, pestle, etc.) were sterilized at high temperature using a sterilizer. The procedure was performed at a fume hood to avoid inhalation of the chemical reagents used.

The RNA isolation procedure for each sample is as follows:

1) Grind plant tissue weighing about 0.1 g in a mortar with continuous addition of liquid nitrogen so as the tissue does not thaw. Placement of grinded plant tissue (powder) using a spatula in a 1.5 ml microtube, where 1 ml of NucleoZOL was then prepared. The microtube was shaken with a mild VORTEX.

2) Add 400μl of water (H2O) to each microtube and shake gently by hand for 15 seconds.

3) The samples are kept at room temperature for 30 minutes.

4) Place samples in a cooled centrifuge set at 12000G at 4 ° C for 15 minutes.

5) Remove the supernatant, approximately 1ml, in a new labeled microtube and add 5μl of 4- bromoanisole. Then gently mix by hand for 15 seconds.

6) The samples are allowed to stand again at room temperature for 15 minutes.

7) Place the samples in a cooled centrifuge set at 12000G at 4 ° C for 10 minutes.

8) Take 750μl of supernatant into a new labeled microtube and add 750μl of isopropanol.

9) The samples are then allowed to stand again at room temperature for 10 minutes and then re- centrifuged at 12000 g at 4 ° C for 10 minutes.

10) Wash the pellet formed with 500 μl of 75% ethanol followed by centrifugation at 6.000G at 4 ° C for 2 minutes.

11) Repeat step 10.

12) The ethanol is removed and the samples are allowed to air dry in the fume hood to evaporate the entire amount of ethanol for about 5-7 minutes.

13) Finally, make up to a volume of 300 μl of water in the tubes of the samples and heat in a water bath for 10 'at 55 ° C.

The samples were then stored at -80 ° C until re-used in the next step.

1.1 Identification and assay of RNA

The qualitative and quantitative determination of the RNA

a) Using Nanodrop 1000 spectrophotometer and ND-1000 v software. 3.3.1. for each sample. The concentration and purity of the eluted RNA were determined using the Nanodrop 1000 spectrophotometer and the N.D.-1000 v software 3.3.1. .

Using the spectrophotometer, the optical density of the sample was measured at 230, 260 and 280 nm. The purity of each sample was estimated from the ratio of A260 / A280 which indicates contamination with proteins, and from the ratio A260 / A230 which indicates contamination with high concentration of salts or phenolic substances.

Acceptable values for A260 / A280 range from 1.8 to 2.0, while acceptable values for A260 / A230 are around 1.0. These two values indicate that the sample is of good purity.

Concerning the process of using the spectrophotometer, the following is a summary:

Since the software N.D. table task pane -1000 v 3.3.1. Nucleic acids, RNA (RNA-40) was selected. Measurement of each sample was performed by placing the 1.0 μL RNA sample spectrophotometer on the podium and pressing MEASUREMENT on the task pane. Each time before the next sample was placed, the "pedestal" was cleaned so as not to interfere with the results.

b) By visualizing the results through the agarose gel electrophoresis technique. Preparation of the gel

A) In a conical flask are added:

- 100 ml of 1X TBE Buffer (Tris / Borate / EDTA (Ethylenediaminetetraacetic acid)).
- 0.8 g of agarose

The conical flask is heated in a microwave oven so that the solution becomes completely clear.

B) Then add to the conical 4-6 μl of MIDORigreen Advance pigment, which is a safe alternative to traditional ethidium bromide. It is a non-carcinogenic and less mutagenic pigment for the detection of dsDNA, ssDNA and RNA in a very high sensitivity agarose gel. MIDORIGreen Advance can be used with ultraviolet (UV) radiation.

C) The gel is placed on the special form of the electrophoresis apparatus to obtain the desired shape and to form the desired "wells" on which the specimens will be placed, and is allowed to cool.

D) Samples are placed on agarose gel together with a specific blue dye (3μl sample + 2μl dye).

E) Allow the gel to run on the electrophoresis device for at least 30'-40 '.

Finally, the results are visualized in a dark room with the help of a UV lamp and the GELCAPTURE program on the computer screen, in order to confirm if there is any RNA product at all.

1.2 Reverse transcription reaction to generate cDNA

cDNA is a single stranded DNA sequence, complementary to plant mRNA.

• The first step before cDNA preparation begins is to calculate the volume of RNA sample used to reach a final concentration of 0.5 μg RNA for each cDNA preparation reaction.

• Reaction materials for cDNA fabrication are contained in TAKARA BIO INC's “PrimeScriptTM RT Reagent KIT with gDNA Eraser”.

• All materials (RNA samples, KIT reaction materials) are always on ice during preparation.

The RNA volume required for each sample was calculated to have a final RNA concentration of 0.5μg. In the final reaction solution, followed by removal of the genomic DNA that may exist in the RNA samples according to the following reaction (step 1):

• 5X gDNA Eraser Buffer: 2μL

• gDNA Eraser: 1μL

• Total RNA: ~ of the initial RNA concentration (Nanodrop), so that the final sample concentration is 0.5 μg.

• RNase Free dH2O: XμL.

• TOTAL SOLUTION: 10μL.

* The first two components of the reaction are MasterMix 1, the amount of which is calculated for all cDNA samples to be constructed and then shared by 3μL. of MM1 in each microtubule. The RNA is added last in sequence, followed by good pipetting to mix the materials well.

They are then transported (always in grated ice) to the thermocycler where they remain at 42 ° C for 3 minutes. After this time, the tubes are re-placed in the ice to continue the process. Genomic DNA removal is complete.

After successful removal of genomic DNA, we proceed with the reverse transcription-RT reaction, where the following materials were used (step 2):

• Reaction solution from Step 1: 10μL.

• 5X PrimeScript Buffer 2 (for Real Time): 4μL.

- PrimeScript RT Enzyme Mix 1: 1μL.
- RT Primer Mix: 1μL.

RNase Free dH2O: 4μL.

- TOTAL SOLUTION: 20μL.

* The latter components constitute MasterMix 2, their total is calculated for all cDNA samples to be manufactured, and then divides by an amount of MM2 equal to 10μL / sample. Stir well with the pipette. The microtubules are then transferred back to the thermocycler where they remain at 37 °
C. for 15 minutes. After this time has elapsed, the process in the thermocycler ends with the microwave for another 5 seconds at 85 ° C. With this reaction the cDNAs were constructed.

The microtubes with the specimens remain in the freezer at -20oC until re-used.

1.3 Primers selection:

After extensive research in the existing published literature, it was decided to choose primers that had been used in the recent past for the purpose of tracking glyphosate-targeted resistance to Lollium rigidum, and more specifically to the proline 106 gene EPSPS. The primers where they were used were derived from the published work of Kaundun et. al., 2011 and are as follows:

Epsps forward primer 5' TCTTCTTGGGGAACGCTGGA 3' Epsps

reverse primer5' TAACCTTGCCACCAGGTAGCCCTC 3'

1.5 Preparation and Execution of Polymerase Chain Reaction (PCR)

The preparation of the samples as well as the process of performing the reaction cycles were based on the published work of Kaundun, et.al., 2011.

Sample preparation:

In 0.2ml microtubules were placed:

3μl sample cDNA, 50ng

22μl Master Mix containing the following:

- 2mM MgCl2
- 0.2mM dNTPs
- 0.2mM from each primer
- 1X Buffer with Mg
- 0.625 units polymerase enzyme
- H2O, so that the final volume per sample is 25 μl.

The samples were then placed in the thermocycler and the program applied to them was set as follows:

1. A cycle at 95 °C for 5 minutes
2. 40 cycles with the following stages:

- 95°C for 30 "
- 60°C for 30 "
- 72°C for 2 '

3. Last cycle at 72 ° C for 10'

After completion of the procedure, the samples are frozen at -20 ° C.

1.6 Check the finished product

Before sending the samples under study for sequencing, it should be checked whether or not the procedure set out above has been successfully completed. For this purpose, the PCR product is screened and visualized by agarose gel electrophoresis

The procedure is exactly the same as described above (paragraph 1.2) with the only difference being the placement in the first well of the series, a DNA Ladder. The DNA Ladder is a set of template molecules and is used to determine the size of molecules that "run" on an electrophoresis gel, based on the principle that the molecular weight is inversely proportional to the rate of migration through an agarose gel and therefore the molecules with higher molecular weight are those that appear in the above bands, whereas smaller molecular weight molecules "run" faster, since they can penetrate more easily from the pores of the gel, thus visualizing them as lower zones of the agarose gel.

Results

Listed below is that fragment of the amino acid sequence of the EPSPS gene, which contains the proline P106,

FLGNAGTAMR**P**LTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGANVDCFLGTDCPPVRI NGIGGLPGGKV

According to our samples the results obtained for each biotype were as follows:

- **Italian biotype, sample 1**

```
L.rigidum          ------------------------------ FLGNAGTAMRPLTAAVVAAGGNATYVLDGV  30
Italiansample1     KYKFXGTLKXRNEGGLPWKQVXXPCQQVAFFLGNAGTAMRPLTAAVVAAGGNATYVLDGV  60
                                                  ******************************
```

- **Italian biotype, sample 2**

```
L.rigidum          ------------------------------ FLGNAGTAMRPLTAAVVAAGGNATYVLDG   29
Italiansample2     SFHHISGIXXDGGGTXRQVASKVMPRKAXTFFLGNAGTAMRPLTAAVVAAGGNATYVLDG   60
                                                  ******************************
```

- **Biotype 93 sample 1**

```
L.rigidum       --FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGANVDCFLGT  58
93sample1       SFFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPIGK-LSV-NN--------VRT  50
                  ************************************. :    ::        : *
```

- **Biotype 93 sample 2**

```
L.rigidum       -------------------------FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRER  36
93sample2       VALPCHQVAFPCHQVALPXHQVAFFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMREX  60
                                         **********************************
```

- **Biotype 93 sample 3**

```
L.rigidum       ---------------------------------------FLGNAGTAMRPLTAAVVAAGG  21
93sample3       AIYPPPXAPLSNPPHEAALXHQVAXPCHQVALPCHQVAFFLGNAGTAMRPLTAXVVAAGG  60
```

```
                                                              ************** ******
```

- **Biotype 82 sample 1**

```
L.rigidum       ---------------------------------FLGNAGTAMRPLTAAVVAAGGNATYVLD  28
82sample1       PLFSGXRGXLPPQERAFPCQQVALLPXHXXAFFLGNAGTAMRPLTAXVVAAGGNATYVLD  60
                                                 ************** *************
```

- **Biotype 82 sample 2**

```
                 ------------------------------------------------------------
L.rigidum     FLGNAGTAMR
```

```
L.rigidum       TAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGANVDCFLGTDCPPVRINGIGGLP  72
82sample2       TAAVVAAGGNATYVLDGVPRMRERPIGALXVGXXVPX-----LXGSRRQLFHPNX ------ 110
                ************************** * **          : *:    .: *
```

- **Biotype 82 sample 3**

```
L.rigidum       MRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGANVDCFLGTDCPPVRINGI  68
82sample3       MRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGANVEGSPGCQXSTVXX ---- 117
                ****************************************:    * :   *
```

- **Biotype 70 sample 1**

```
L.rigidum       --------------------------FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMR  34
70sample1       RTLSCQQVALLPCHQVALIPGHQAAFFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMR  60
                                          **********************************
```

- **Biotype 27 sample 1**

```
L.rigidum       -------------FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQ  47
27sample1       EKXMEXAKEXVKLFLGNXXTAMRPXTXAXXXAXXNATYVLDGXXRMRERPIXDLXVGLKQ  60
                      ****  ***** * *   *  ********  ******* ** *****
```

- **Biotype 27 sample 2**

```
L.rigidum       ----------------FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVG  44
27sample2       GTRXMERXPXXXSKALLGXQXXTAMRPXXXAVXAXGGNXXYVXDXXXRMXERPXGDLXVG  60
                         :  :  *****   ** * ***  ** *   ** *** *** **
```

- **Biotype 21 sample 1**

```
L.rigidum       ---------------------------FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRM  33
21sample1       QVALPCHQVALLPXHQVAXLXGHPXAFFLGNAGTAMRPLTAAVVAAGGNATYVKDGGPRX  60
                                           ************************** ** **
```

- **Biotype 21 sample 2**

```
L.rigidum       MRPLTAAVVAAGGNATYVLDGVPRMRERPIGDLVVGLKQLGANVDCFLGTDCPPVRINGI  68
21sample2       MRPVAAAVVVEGGNPTYVKDGGPRXRKGPIXXG--------GN----------------V  96
```

```
***::****. *** *** ** ** *: **          .*              :
```

- **Biotype 21 sample 3**

```
L.rigidum       ------------------------FLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRER  36
21sample3       ALKXHRVAFLPWHQVALPGHPVAFFLGNAGTAMRPLTAAVVAAGGNATYVLDGVPRMRER  60
                                        ************************************
```

Discussion and Conclusions

From the above results it is clear that the biotypes used in the present experiment, in the majority, carry the proline amino acid encoded by the CCA codon, thus, no mutation was observed in the resistant biotypes, as reported in similar studies. The only exception is the Italian biotype, which will be discussed below.

However the Italian biotype, as well as one sample from biotype 93, were the only ones that did contain the proline amino acid, nevertheless it was not encoded by the CCA codon, but from the CCG codon (Figure 1). This is interpreted as a point silent mutation that occurs predominantly in sensitive biotypes and confers a different level of resistance (Jasieniuk et.al., 2008). In the present work, this silent mutation appears to be consistent with resistant biotypes and that fact seems interesting for further investigation.

Comparison of Italian & 93 biotype' sequences at the corresponding amino acid sequence (CCA) that is translated to the

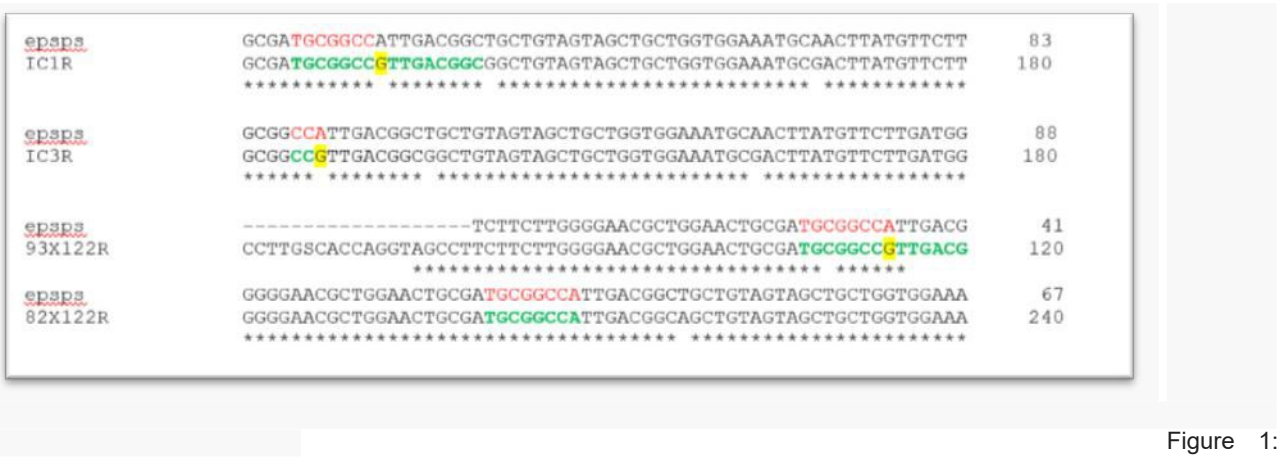

Figure 1: Comparison of Italian & 93 biotype' sequences at the corresponding amino acid sequence (CCA) that is translated to the target amino acid proline 106.

In general, there is a correlation between positions 106 and 109. Susceptible plants with CCG at site 106 have GCG at site 109. One other hand, all R alleles with G in the third position of codon 106 (i.e., GCG or TCG) have GCG at position 109 and only one base substitution at site 106 would have been required to result in resistance. (Jasieniuk et.al., 2008). As shown in Table 2:

- In the resistant Italian biotype, the silent mutation of proline at position 106 from CCA to CCG is also accompanied by a silent mutation of alanine at position 109 from GCT to GCG

- In resistant biotype 93, it was observed that in two of the three samples proline had no change in CCA codon while alanine showed a point mutation from GCT to GCA, whereas sample two (s2) of biotype 93 although silent proline from CCA to CCG, alanine remains unchanged.

- In the samples of resistant biotypes 82, 70 and 21 no mutations in proline 106 were observed, however alanine is coded in all samples with the GCA codon over GCT.

- In sensitive biotype 27, sequencing could not give us a clear result.

Table 2 Proline (Pro 106) and Alanine (Ala 109) amino acid codons found in the populations (Resistant= R, susceptible= S)

Population	Pro 106 CCA	Ala 109 GCT
Italian s1 (R)	**CCG**	**GCG**
Italian s2 (R)	**CCG**	**GCG**
93 s1(R)	CCA	GCA
93 s2 (R)	**CCG**	GCT
93 s3 (R)	CCA	GCA
82 s1 (R)	CCA	GCA
82 s2 (R)	CCA	GCA
82 s3 (R)	CCA	GCA
70 s1 (R)	CCA	GCA
27 s1 (S)	CCA	_
27 s2 (S)	CCA	_
21 s1 (R)	CCA	GCA
21 s2 (R)	CCA	GCA
21 s3 (R)	CCA	GCG
21 s4 (R)	CCA	GCG

From the above results it can be assumed that resistance is not due to a point mutation at proline 106 in the examined biotypes. Moreover the nucleotide substitutions at the nucleotide triplet that is translated to ala 109, are not giving straightforward results.

On the other hand, the Italian biotype, as long as one sample from the biotype 93, carry a silent mutation at proline 106 (CCG codon). As it was mentioned before, we wanted to send more individuals per biotype to be sequenced but unfortunately some of the samples did not work so well as we expected to, so in order to get more accurate results, it is suggested to sequence more individuals from each biotype, particularly from biotype 70, from which only one sample worked well.

Finally, from the present study it is concluded that the Greek resistant biotypes do not carry the mutation at Pro 106. Further experiments should be conducted for the Italian biotype in order to certify that this biotype carries a silent mutation which could be useful in order to identify this biotype in future studies by sequencing. Of course it would be very useful to sequence the same region from the susceptible Italian biotypes as well in order to compare the sequences.

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Appendix 2. EXPRESSION LEVEL OF SOME KEY GENES INVOLVED IN GLYPHOSATE NTSR IN *L. RIGIDUM*

Aim of the study

The expression of four different *ABC transporter* genes following exposure to glyphosate was studied in two *Lolium rigidum* populations from Greece GR21 (resistant) and GR27 (susceptible)) in order to investigate their impact on conferring non-target site resistance.

Materials and Methods

Glyphosate resistant (GR21) and susceptible (GR27) plants were treated with 720 g a.e./ha glyphosate. Leaf samples were harvested 3, 6 and 12 hours after treatment. These time points were chosen based on a previous study reporting the highest expression ratio of *ABC transporters* at 3 and 6 hours after glyphosate application (González-Torralva et al. 2017). Triplicate biological samples, each of these consisting of 5 leaves from 5 independent plants, were harvested at each time-point and used for RNA extraction. Total RNA was isolated using RNA Nucleosol protocol

https://www.mn- net.com/Portals/8/attachments/Redakteure_Bio/Protocols/RNA%20and%20mRNA/UM_TotalRNA_NucleoZOL.pdf).

For first-strand cDNA synthesis we used 0.5 µg RNA, and PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara).

Real-time quantitative-PCR was performed using SyberSelect Mix (Invitrogen) on a Step-One-Plus Real Time PCR system (Applied Biosystems). The reaction was carried out with 2µL of a 4-fold dilution of cDNA, 10µl SYBR™ Select Master Mix, 7,6µl µM Nuclease-Free Water, 0,2µl from each primer, 0.4µM in a 20 µL reaction volume. Cycling conditions were as follows: hold temperature at
95 °C for 2min, followed by 40 cycles: denaturation at 95 °C for 15 s; 60°C for 1min (annealing/extension). Primers were designed using the NBCI/Primer-Blast tool.

Primers selection:

After extensive research in the existing published literature (Byrne et.al., 2010, Salas et.al. 2015; Tani et al. 2015), and in NCBI database, it was decided to choose the genes listed below.

The primers used were as follows: (reference gene CCR-Salas et al. 2015)

LpCCR-F: GATGTCGAACCAGAAGCTCCA (21)

LpCCR-R: GCAGCTAGGGTTTCCTTGTCC (21)

Genes of interest:

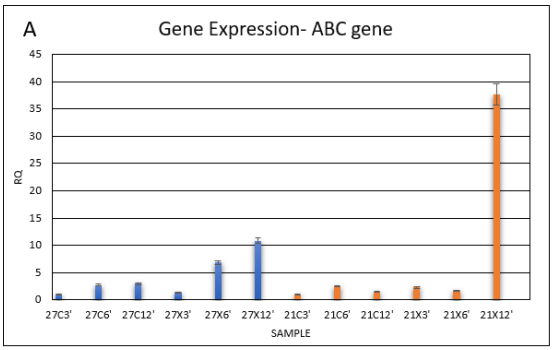
Lpmultidrugprotein-F: GGTCATGGACTGCGACAGAG (20)

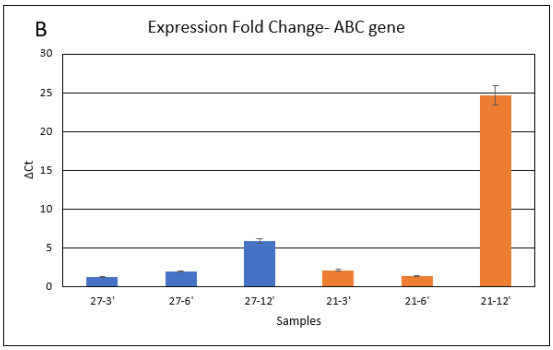
Lpmultidrugprotein-R: CACGTCAGATGACCGGTTTG (20)

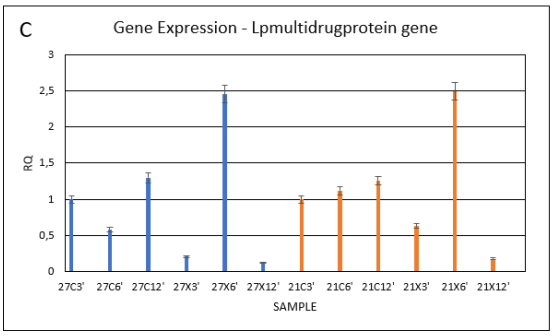
A. LpABC1-F: AGAGCTGCAAAGGCTGGTAG (20)
LpABC1-R: TCTAAGCGGAAGCAAAGCCA (20)

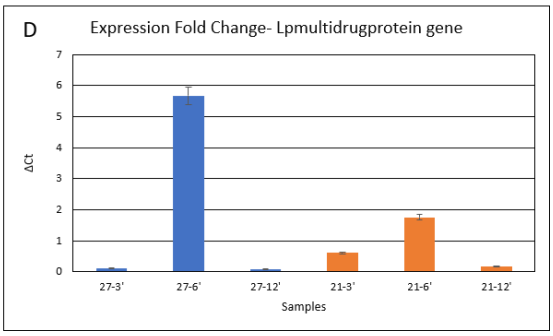
B. LpironinhibitedABC-F: TAAACTCCCACCACCAGTGC (20)
LpironinhibitedABC-R: TCACCGGTCATGAGCTTCAG (20)

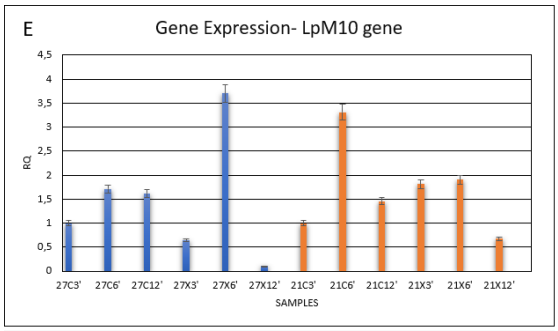
LpM10-F: TATGTTGTGGCTGACACGCT (20) LpM10-R: ATCGGCGTTGTGCAAGAAAT (20)

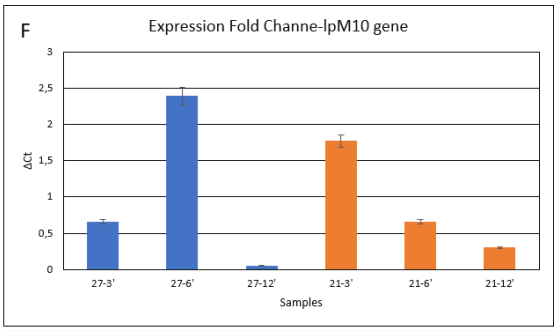


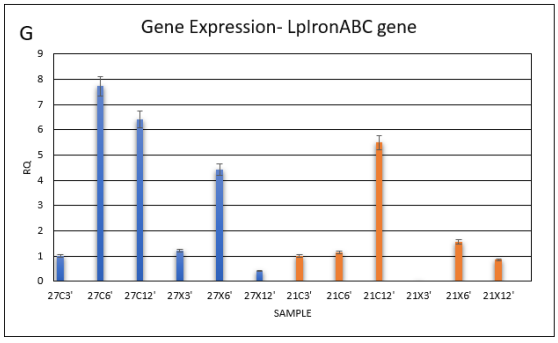












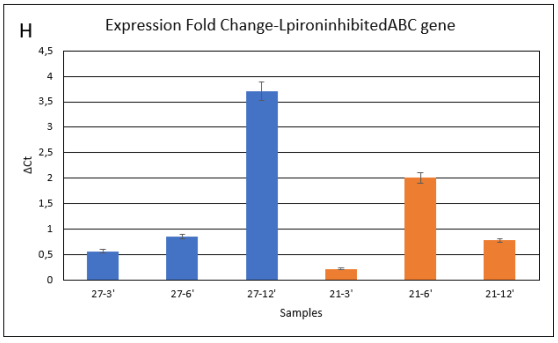


Figure 1. ABC transporter gene expression for the resistant and the susceptible biotype. Quantitative-PCR analysis of ABC transporter gene type 3 (a,b), Lpmultidrug gene (c,d),LpM10 gene (e,f) and iron inhibited ABC gene (g,i),in glyphosate-treated leaves harvested at the indicated time-points (3,6,12 hours after glyphosate treatment) The different graphs show the different ways of estimating the relative expression ratio. The graphs on the left are originated based on Step-One Plus Software. The graphs on the right originated by conducting ΔΔCT method (Livak and Schmittgen 2001). (The abbreviations are as follows: graphs on the left 27C3-27 biotype Control 3hours-27X3- 27biotype 3 hours after treated, etc-, graphs on the right- 27'-3: biotype 27 3 hours after treatment, e.t.c.).

Discussion

The most profound difference between the two populations was observed for the relative expression of *ABC transporter* gene type 3 (graphs A and B). At 12 hours after glyphosate treatment the ABC transporter gene type 3 was increased by a factor 24 in the resistant population and by a factor 6 in the susceptible population. This gene is a good candidate for being a marker gene discriminating susceptible and glyphosate resistant populations at early stages.

The *LpM10* and *iron inhibited ABC transporter* genes were up-regulated at earlier time points in the resistant population compared to the sensitive one (at 3 and 6 hours after glyphosate spraying respectively).

Regarding *Multidrug resistance* gene, no clear conclusion could be made.

Future work

Relative expression ratio of the Italian resistant biotype (581), after glyphosate spraying at the same time points is under way in order to confirm the aforementioned results.

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Appendix 3. EXPRESSION OF ABS TRANSPORTER GENE TYPE 3 IN AN ITALIAN POPULATION OF L. RIGIDUM

Aim of the study

The expression of the *ABC-like transporter* genes *ABC III, Lpmultidrugprotein, LpM10, and LproninhibitedABC* in an Italian population of *Lolium rigidum* (IT581) putative resistant to glyphosate was studied in order to identify marker genes that could discriminate susceptible populations from glyphosate resistant populations at early stages after spraying.

Materials and Methods

Plants of the Italian population IT581 were sprayed with 720g a.e./ha glyphosate and leaf samples were harvested 3, 6, and 12 hours after treatment. Three different biological samples, each of them consisting of a bulk of leaves from 5 different plants were harvested at each time-point and used for RNA extraction. Total RNA was isolated using RNA Nucleosol protocol (https://www.mn- net.com/Portals/8/attachments/Redakteure_Bio/Protocols/RNA%20and%20mRNA/UM_TotalRNA_ NucleoZOL.pdf).

For first-strand cDNA synthesis 0.5 µg RNA, and PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara) was used.

Real-time quantitative-PCR was performed using Sybr-select Mix (Invitrogen) on a Step-One-Plus Real Time PCR system (Applied Biosystems). The reaction was carried out with 2µL of a 4-fold dilution of cDNA, 10µl SYBR™ Select Master Mix, 7,6µl µM Nuclease-Free Water, 0,2µl from each primer, (0.4µM) in a 20 µL reaction volume. Cycling conditions were as follows: hold temperature at 95 °C for 2min, followed by 40 cycles: denaturation at 95 °C for 15 s; 60°C for 1min (annealing/extension). Primers were designed using the NBCI/Primer-Blast tool. Three individual biological replicates were assayed per q-PCR run, in three technical replicates. The relative gene expression was determined with the comparative Ct method (Livak and Schmittgen, 2001), calculating the mean threshold cycle (Ct) values of the target and endogenous control genes for three individual biological replicates and three technical replicate runs.

Primers selection:

The primers used were taken from:-*ABC transporter* gene *type III*, *Lpmultidrug protein*, *LpM10*, *Lp iron inhibited ABC* (Byrne et al. 2010); reference gene *CCR* (Salas et al. 2015) and were as follows:

LpCCR-F: GATGTCGAACCAGAAGCTCCA (21)

LpCCR-R: GCAGCTAGGGTTTCCTTGTCC (21)

Gene of interest:

LpABCIII-F: AGAGCTGCAAAGGCTGGTAG (20)

LpABCIII-R: TCTAAGCGGAAGCAAAGCCA (20)

Lpmultidrugprotein-F: GGTCATGGACTGCGACAGAG (20)

Lpmultidrugprotein-R: CACGTCAGATGACCGGTTTG (20)

LpM10-F: TATGTTGTGGCTGACACGCT (20)

LpM10-R: ATCGGCGTTGTGCAAGAAAT (20)

LpironinhibitedABC-F: TAAACTCCCACCACCAGTGC (20)

LpironinhibitedABC-R: TCACCGGTCATGAGCTTCAG (20)

Results

ABC transporter gene type III

Three hours after glyphosate treatment a small induction of *ABC transporter gene type III* was recorded. At six hours after treatment the relative expression was lower compared to 3 hours after spraying. A strong upregulation of gene expression was observed at twelve hours after glyphosate treatment. At 12 hours after glyphosate spraying the *ABC transporter type III* was increased by a factor 43 compared to the control (Figure 1).

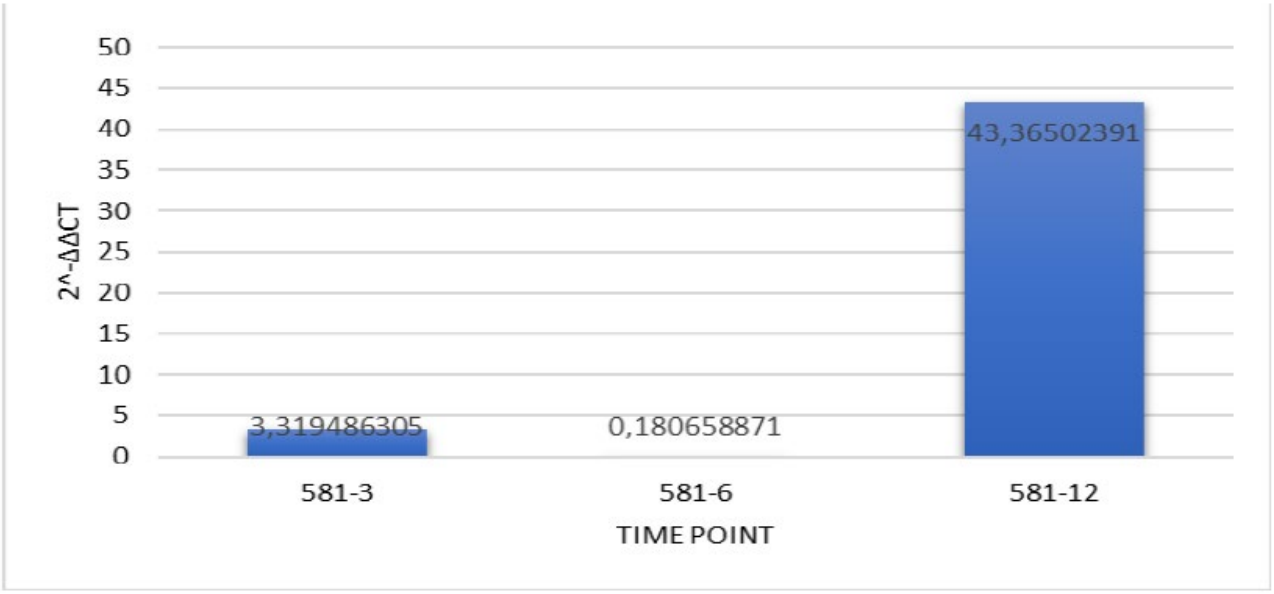

Figure 1: Gene expression fold change of the *ABC transporter* gene type III for the Italian biotype, 581, at 3, 6 and 12 hours after glyphosate spraying.

Lpmultidrugprotein, LpM10 & LpironinhibitedABC genes

Three hours after glyphosate treatment, an overexpression of the *Lpmultidrugprotein* was recorded. This expression decreased dramatically at six and twelve hours after glyphosate treatment. Specifically, there was a 17 fold change of *Lpmultidrugprotein* gene expression compared to the control three hours after spraying with glyphosate. Conversely, at 6 and 12 hours after glyphosate spraying the relative expression dropped (Figure 2). Similar results were observed for both *LpM10* and *LpironinhibitedABC* genes. Three hours after glyphosate spraying *LpM10* and *LpironinhibitedABC* genes increased by a factor 15.7 and 8 respectively, compared to the control. At six and twelve hours after spaying gene expression decreased dramatically (Figures 3 and 4).

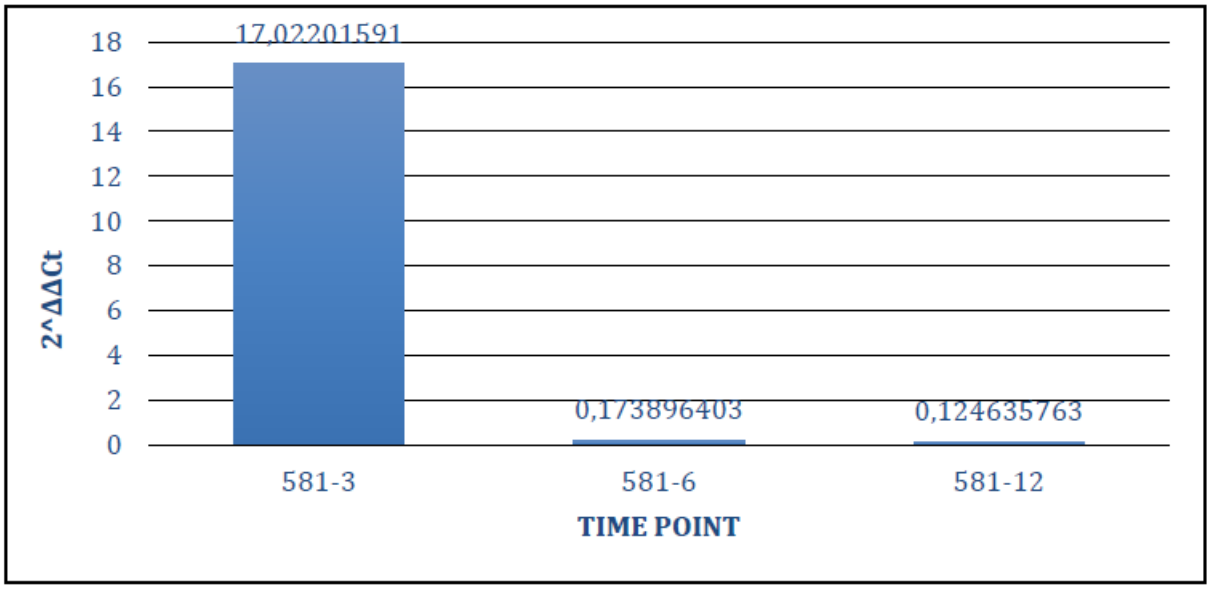

Figure 2: Gene expression fold change of the *Lpmultidrugprotein* gene for the Italian biotype, 581, at 3, 6 and 12 hours after glyphosate spraying.

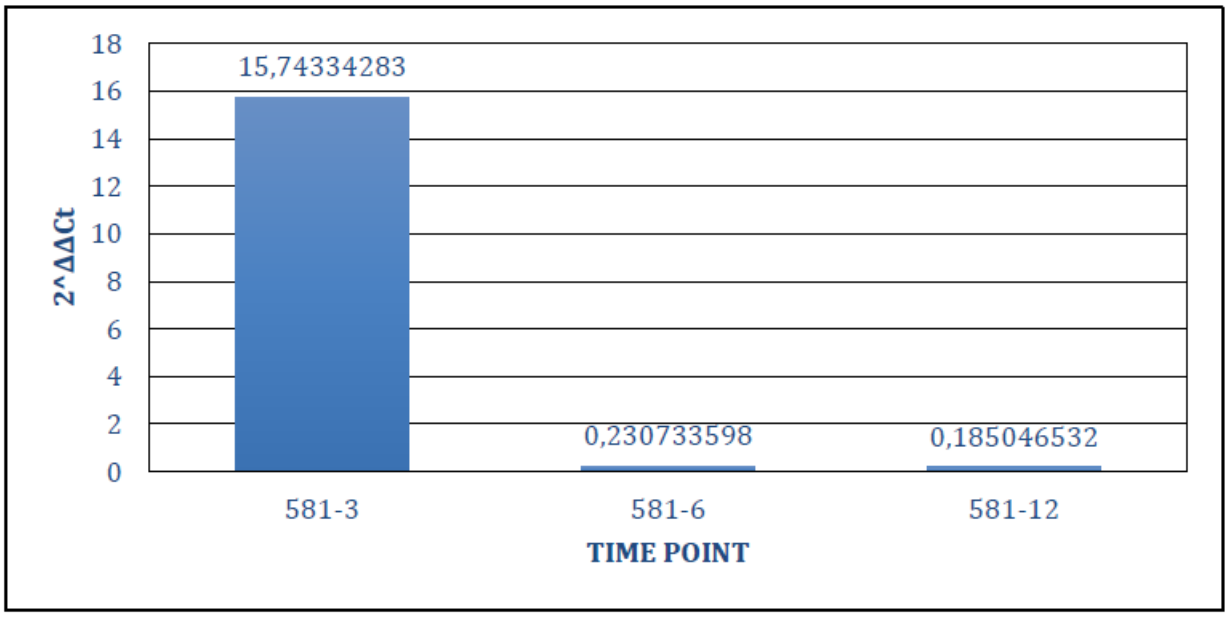

Figure 3: Gene expression fold change of the *LpM10* gene for the Italian biotype, 581, at 3, 6 and 12 hours after glyphosate spraying.

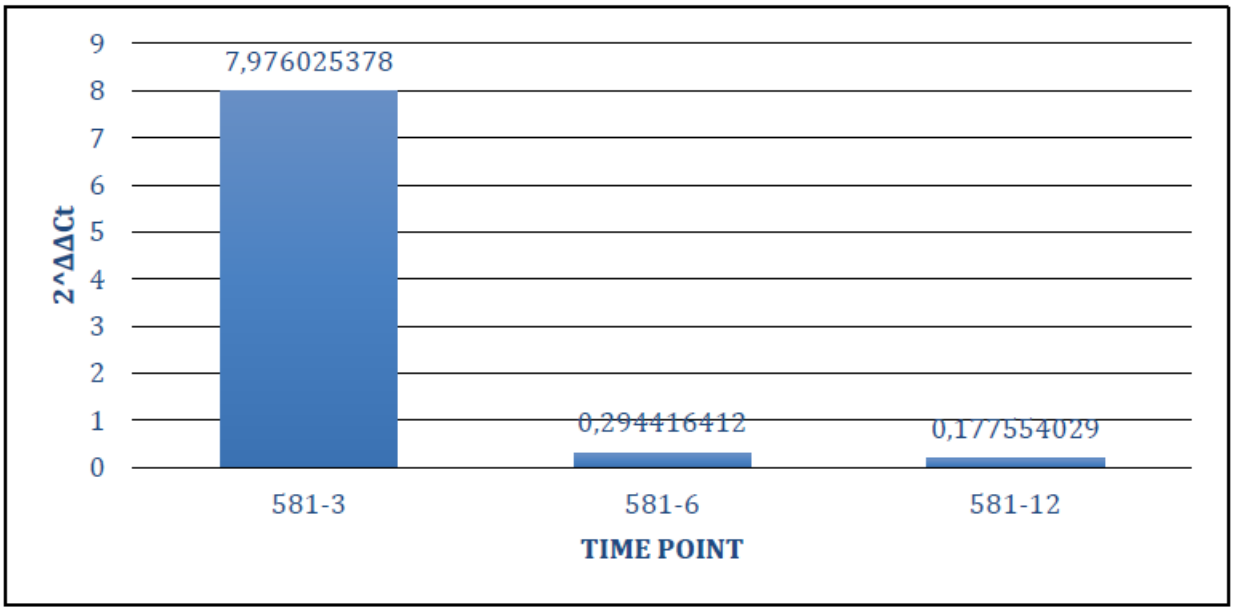

Figure 4: Gene expression fold change of the *LpironinhibitedABC* gene for the Italian biotype, 581, at 3, 6 and 12 hours after glyphosate spraying.

Discussion

A significant up-regulation of the *ABC transporter gene type 3* was recorded 12 hours after glyphosate treatment (43-fold change compared to the control). This is in accordance with our previous results on the relative expression ratio of this gene in the Greek resistant population (population GR21 with a 25- fold change compared to control 12 hours after treatment). These results support the hypothesis that this gene is a good candidate for being a marker gene, discriminating susceptible populations from glyphosate resistant populations at very early stages after spraying. Results for the *ABC-like* transporter genes *Lpmultidrugprotein, LpM10 and LpironinhibitedABC* showed an upregulation (by a factor 17, 15.7 and 8, respectively) compared to the controls at three hours after glyphosate treatment. The up-regulating of these genes was temporary and six and twelve hours after glyphosate treatment, the expression of these genes was similar in glyphosate treated and control plants.

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Appendix 4. REPORT ON SHIKIMIC ACID EXPERIMENT

Introduction

The herbicide glyphosate kills the plants by blocking a reaction on the shikimic acid pathway. Due to the competition of the EPSPS enzyme with glyphosate, the concentration of shikimic acid in the herbicide-treated parts of the plant is affected. Determination of the concentration of shikimic acid in glyphosate treated plant tissue is a reliable and easy way to investigate the level of resistance to glyphosate (Tani et al. 2020). In the present study, seven *L .rigidum* biotypes, both resistant and susceptible to glyphosate, were studied in order to determine the shikimate concentration and estimate their level of resistance to the recommended dose (N = 720 g. a.i. ha^{-1}) of glyphosate.

Materials and Methods

Two different protocols were used to determine the shikimic acid concentration in the plant tissue of seven populations of *L. rigidum*, in order to evaluate the level of resistance to glyphosate. These populations have previously been characterized as resistant in dose- response experiments, as summarized in Table 1. The plants were sown in pots and 12 weeks after sowing (WAS) they were treated with the recommended dose of glyphosate. Plant tissue was collected 4 days after treatment (DAT) with glyphosate.

Table 1 Characterization of resistance in seven populations. R: Resistant S: Susceptible

BIOTYPE	**12**	**1**	**581**	**107**	**103**	**19**	**16**
CHARACTERIZATION OF RESISTANCE	S	R	R	R	S	S	R

PROTOCOL 1

A myriad of methods have been developed to quantify shikimate in leaf tissues. Most methods require 0.1 to 1 g of fresh tissue and includes a homogenization or grinding step. In our laboratory, we selected the Shaner et al. (2005) method because of its relative simplicity and good sensitivity. This method requires only single-leaf discs (i.e., 4 to 5 mg tissue) and does not involve grinding or homogenization of the sample. For optimum results, excise discs (4 mm in diameter) from young leaves, and place in separate wells of a microtiter plate each containing 100 µl of 10 mM ammonium phosphate monobasic (adjust to pH 4.4 with either 0.1 N HCl or NaOH). A nonionic surfactant, such as 0.1% (v/v) Tween 20, can be added to reduce the surface tension, which improves both the penetration of glyphosate and the extraction of shikimate. Full dose–response curves (1 to 500 mM glyphosate) can be done directly in the microtiter plates. Seal and wrap plates with plastic to minimize evaporation, and incubate under fluorescent lights (150 mM m^{-2} s^{-1}) at 25°C for up to 24 h. The format can be adapted if the objective is to determine the accumulation of shikimate over time. After incubation, place plates in a -20 °C freezer until the solution is frozen. Alternatively, shikimate accumulating in tissue samples from whole plants treated with glyphosate can be extracted in the same way. The samples can be kept frozen until further analysis. At that time, thaw samples at 60 °C for 30 min, add 25 ml of

1.25 N HCl into each well (final concentration of 0.25 N HCl per well), and incubate the plates at 60 ºC for 15 min. The tissues will become uniformly gray–green. Transfer 25-ml aliquots to new wells, and oxidize shikimate with 100 ml of a solution containing 0.25% (w/v) periodic acid and 0.25% (w/v) sodium m-periodate at 25 ºC for 90 min. Then, add 100 ml of 0.6 N sodium hydroxide in 0.22 M sodium sulfite to the wells to neutralize the extract and to quench the oxidative reaction. Determine shikimate spectrophotometrically at 380 nm within 30 min. using a microtiter plate reader. Background absorption values measured in wells containing discs not exposed to glyphosate are subtracted. Shikimate concentrations are calculated from a shikimate standard curve (Dayan et al 2015).

PROTOCOL 2

The protocol was used for the determination of shikimate accumulation in plant tissue and was based on a similar study in *Conyza sp*. (Tani et al. in 2015) with a few changes. Shikimic acid was isolated from the plant tissue using hydrochloride (HCl). The old and the very young leaves were excluded. Leaf samples were kept in a deep freezer (−80 °C) until further processed. Leaf sections (0.1 g) were grinded with 1 ml of HCl and left at room temperature for 24 hours. After the isolation of shikimic acid, the samples were centrifuged for 1 minute at 3.000 rpm and 75 µl of supernatant was transferred into a new tube. For the oxidation stage, deionized water and 500 µl of oxidation solution were added to each tube so as the total volume would reach 1 ml. The tubes were left at room temperature for 3 hours. Finally, for the chromophore stage, 300 µl of each tube was transferred into a new one and 700 µl of deionized water, 400 µl of sodium sulfate and 600 µl NaOH were added. The absorbance of each sample was determined throughout measurement with spectrophotometer at 380nm and the results are presented in Figure 1. A shikimate standard curve was developed by adding known amounts of shikimate to vials containing no leaves. Shikimate levels are presented as mM shikimic acid per ml of HCl solution.

Results and discussion:

Preliminary results from Protocol 1 were not consistent and are not presented in. Further investigation and standardization of this protocol is suggested in order to obtain a better profile and repeatability of the results.

Results of the second protocol indicated that shikimate concentration presented significant differences for two out of seven populations (Figure 1). Population 12 (S- population;) accumulated the highest concentration values of shikimic acid (= 924 Mm/ml), in contrast to population 16 (R- that had the the lowest level (425Mm/ml). The other five populations showed no difference (from 586 – 625 Mm/ml) in shikimic acid level accumulation. , Two of these five populations (103, 19) were previously characterized as susceptible, whereas three populations (1, 581, 107) were characterized as resistant.

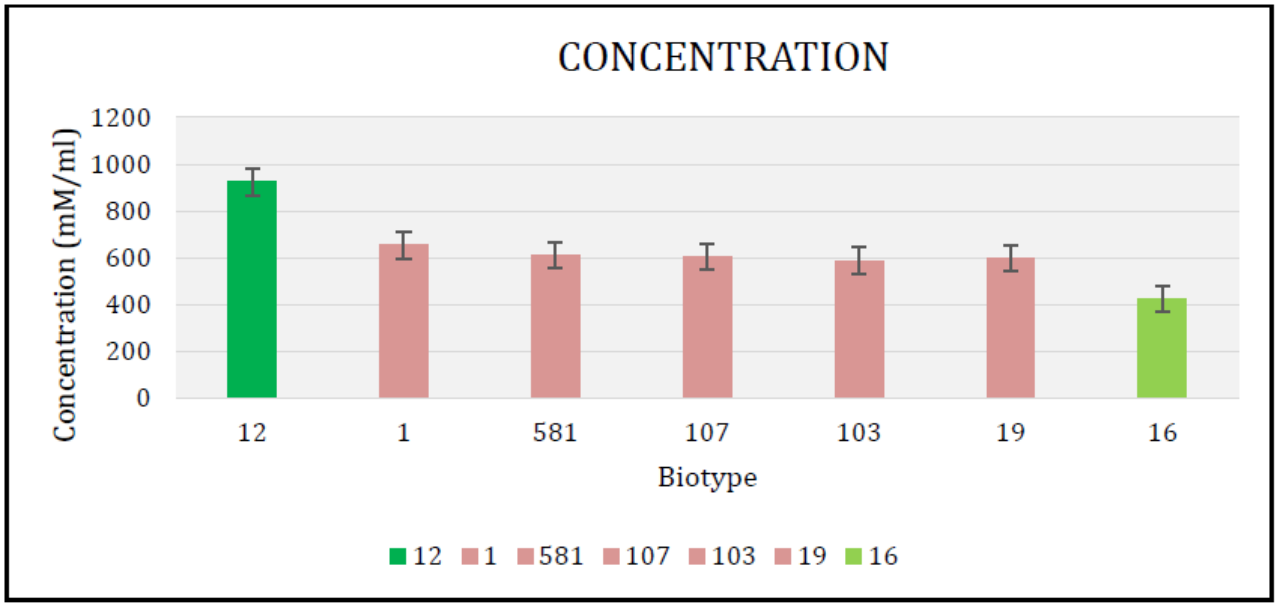

Figure 1. Level of shikimic acid concentration in the 7 studied biotypes.

Shikimic acid level determination could be a valuable biochemical test for further proving susceptibility or resistance to glyphosate in *Lolium spp.* populations. At the same time, shikimic acid test could produce some false positive or negative results. These contradictory results may be attributed to several reasons. Factors such as the collected part of plant tissue, as well as the growth stage that glyphosate was applied on plants are crucial and a better standardization of the method is required to ensure the repeatability of the results. In future experiments, lower doses of glyphosate should be included in order to get a better and more complete profile of the resistance. As such, standardization of shikimic acid test would be needed to ensure sound and reliable separation of R- and S- populations based on sole biochemical tests without the need to conduct pot experiments.

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Supplemental information

REAGENTS USED FOR THE METHOD

A) 1N HCl HYDROCHLORIC ACID:

- Put 40.98ml HCl in a volumetric flask and add up to 500ml of deionized water (459.02 ml H2O)

B) OXIDATION SOLUTION:

- For 100ml oxidation solution, weigh 0.5g periodic acid + 0.5g sodium periodate

C) 0.0056 N SODIUM SULFATE anydrus:

- 100 ml of deionized water and 0.71g Sodium Sulfate anydrus

D) 1M (NaOH):

- 4g of NaOH in 50ml of deionized water and dissolve with magnetic stirrer. After it is completely dissolved and the solution becomes clear, add deionized water up to 100ml total volume.

E) STANDARD CURVE 20Mm:

- Dissolve 0.0348g of 99% purity shikimic acid, in 20ml of deionized water.

7. Pan-European guidelines for the management herbicide resistant *Lolium* spp written in English and published

C-IPM	**Coordinated Integrated Pest Management in Europe** **Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
Starting date:	**06.06.2017**
Project duration:	**36 months plus 4 months postponement of the end of project**
Project end date:	**06.10.2020**
Deliverable number:	**D3.1.1**
Deliverable title:	**Pan-European guidelines for the management herbicide resistant *Lolium* spp written in English and published**
WP number:	**WP3**
Lead beneficiary:	**HAO-DEMETER**
Main author(s):	**Thomas Gitsopoulos, Solvejg K. Mathiassen, Donato Loddo**
Delivery date:	**M39**
Actual delivery date:	**30.11.2020**

Executive Summary

The deliverable presents pan-European guidelines in English for the management of herbicide resistant *Lolium* spp. The guidelines are based on the results of studies examining the patterns and levels of resistance, the mechanisms providing resistance against ALS, ACCase and EPSPs inhibitors, as well as on historical data of crop and resistance management in Italy, Denmark and Greece. Pan- European guidelines are presented depending on the status of herbicide resistance in the fields (confirmed or not identified) for both ALS/ACCase inhibitors in winter cereals and EPSP inhibitor (glyphosate) in orchards, respectively.
These guidelines will be published on the web and will be available to the public free of charge.

Introduction

Herbicide resistance is a threat to food supply. Winter cereals are very important for human nutrition and uncontrolled weeds can reduce production quantity and quality. Weed management practices largely rely on herbicides. Particularly, weed control in winter cereals is mainly based on herbicides and more specifically on post-emergence application of ALS or/and ACCase inhibitors.
This weed management in combination with the continuous monoculture of winter cereals in certain fields and the limited options for alternative non-chemical weed control methods have caused increased levels of ALS and ACCase resistance in *Lolium* spp. worldwide, threatening winter cereal production. Similarly, the continuous reliance on glyphosate for *Lolium* spp. control in orchards has contributed to the development of glyphosate resistance.
Although herbicide resistance has been worldwide reported, there is a lack in published guidelines concerning its management. The main objective of the Work Package 3 is to develop pan-European guidelines for management of *Lolium* spp. herbicide resistance. Experimental results in the patterns and level of resistance, characterization in resistance mechanisms and historical field data on crop management from Greece, Italy and Denmark were recorded and Pan-European guidelines for management of herbicide resistant *Lolium* spp. were designed

Description of work

Experimental results in the patterns and level of resistance and characterization of resistance mechanisms for ALS, ACCase and EPSPs resistance of *Lolium* spp.

Italy
Resistance to ALS and ACCase inhibitors

Four Italian *Lolium* populations (IT533, IT595, IT609, IT620) were tested to assess their resistance to ALS and ACCase inhibitors. Multi-resistance to ALS inhibitors (mesosulfuron-methyl + iodosulfuron-methyl-sodium) and ACCase inhibitors (clodinafop and pinoxaden) was confirmed in the populations. In particular, high resistance was confirmed to mesosulfuron + iodosulfuron (20 < RI< 68) and to clodinafop (8 < RI < 27), with a lower level of resistance to pinoxaden (6 < RI < 20), except for one population (IT595) showing no resistance to pinoxaden.
In all four Italian populations investigated, mutated ALS alleles were detected in some plants surviving application of ALS-inhibitors. These mutations involved different amino acidic positions in the different populations: 376 for population IT533; 376 and 574 for population IT595; 197 for population IT609; 122, 197 and 205 for population IT620. The sequencing of the ACCase gene revealed that in three populations out of four, plants surviving treatment with ALS-inhibitors had also mutant ACCase alleles. The 1781-Leu, 2078-Gly and 2096-Ala mutant ACCase alleles were detected in population IT533, the 1781-Leu and 1781-Asp mutations were detected in population IT595 and the 2096-Ala mutant ACCase allele in population IT620. Therefore, in these three populations there were multi-resistant plants having target-site resistance to ALS and ACCase. While population IT609 presents the 2041-Asn mutation in the ACCase gene only in plants surviving to clodinafop application but not in plants surviving ALS-inhibitors, indicating no multi-resistance at plant level. This population is multi-resistant at population level. In all the Italian populations investigated, several of the plants that survived treatment with ALS-inhibitors had no ALS or ACCase mutations, particularly in populations IT533 and IT609, indicating the presence of possible non-target site resistance (NTSR) mechanism. Analyses of the herbicide metabolism

genes glycosyl- transferase (GT), nitronate monooxygenase (NMO), cytochrome P450 (CYP72A1) and cytochrome P450 (CYP72A-2) confirmed an over-expression of NMO, GST, CYP72A1 and CYT72A2 in the IT609 population. The presence of multiple resistant (ALS and/ACCase) populations in Italian wheat fields was confirmed. Those populations present a diverse combination of target site resistance (TSR), involving a range of amino acidic substitutions at different positions, and NTS resistance mechanisms. This corroborates that several parallel processes of *in situ* evolution have led to the current spread of *Lolium* resistant populations in the different Italian regions.

Resistance to glyphosate

One population (IT581) was tested for glyphosate resistance. Population IT581 showed more than 80% survival and only 40% of biomass reduction at the field recommended rate of glyphosate (720 g ae ha^{-1}), with 30% of survival even at the highest rate (5760 g ae ha^{-1}). The partial EPSPS sequence of plants of the glyphosate resistant Italian biotype did not reveal any mutation at Proline106. However, a silent mutation at Proline106 (codon CCG) was detected in addition to nucleotide polymorphism (silent mutations) at Ala109.
The expression profiles of 4 ABC-transporter like genes at 3, 6 and 12 hours after glyphosate spraying showed that the gene with the highest upregulation was an ABC-type III transporter like gene (JZ166942.1) in the resistant biotypes compared to the susceptible ones (at 12 hours after spraying), thus this gene is a putative marker gene for the rapid identification of glyphosate resistant plants. Biochemical tests for proving glyphosate resistance was done by shikimate analysis. The Italian population showed lower level in shikimic acid as compared to the S-population. The results may indicate an early (some hours after herbicide application) sequestration of glyphosate possibly to vacuole leading to limited translocation within the resistant plants.

Denmark
Resistance to ALS/ ACCase

Four Danish populations were tested for resistance to ALS and ACCase inhibitors (DK06LP, DK47LM, DK90LM and DK29LM). Multiple resistance to an ALS inhibitor (mesosulfuron-methyl + iodosulfuron-methyl-sodium) and to an ACCase inhibitor (clodinafop) was confirmed in all the populations. The four Danish populations were confirmed as being highly resistant to ALS inhibitors (mesosulfuron
+ iodosulfuron), and even at the highest herbicide dose tested (i.e. 120 + 24g a.i./ha of mesosulfuron + iodosulfuron), plant survival was higher than 50%. The estimated RIs were >50 for three populations (DK06LP, DK47LM and DK90LM) and >37 for population DK29LM in the first experiment and >68 in a subsequent experiment.
The four Danish populations were moderately resistant to clodinafop with RIs ranging between from 2 to 7 in the two experiments. Three of the Danish populations (DK06LP, DK29LM and DK47LM) were susceptible to pinoxaden (1 < RI <3) while the fourth population (DK90LM) had a RI of 4 to 5 and was classified as resistant to pinoxaden.
In summary the Danish *Lolium* populations were highly resistant to mesosulfuron + iodosulfuron, moderately resistant to clodinafop, but only one population was resistant to pinoxaden. Overall, the results revealed a lower level of resistance to ACCase inhibitors in the Danish populations compared to those from Greece and Italy. In contrast, resistance to ALS inhibitors was at a high level in populations from Italy and Denmark compared to populations from Greece. The genomic DNA from plants resistant to iodosulfuron + mesosulfuron and clodinafop was analyzed for the presence of mutations known to cause target-site resistance. These analyses revealed that population DK06LP and DK47LM possessed the 574- Leu mutation known to cause ALS-resistance. No ALS mutated alleles were observed in populations DK29LM and DK90LM. Furthermore, none of the mutations causing resistance to ACCase inhibiting herbicides were found in the Danish populations. In conclusion, the results of the dose-response experiments and the molecular analyses indicate enhanced metabolism to be the main mechanism for resistance to ACCase inhibitors in Danish populations. This is in alignment with the relatively low level of resistance detected. Apparently, the enhanced metabolism is not affecting the activity of pinoxaden but seems to contribute to the ALS resistance observed in populations DK29LM and DK90LM having no mutated alleles.

Analyses of the herbicide metabolism genes glycosyl-transferase (GT), nitronate monooxygenase (NMO), cytochrome P450 (CYP72A1) and cytochrome P450 (CYP72A-2) confirmed an over-expression of GST, CYP72A1 and CYT72A2 in population DK90LM.
In summary, the results reflect that ALS inhibitors have been the most commonly used herbicides to control *Lolium* species in recent years and that pinoxaden has never been authorized in Denmark.

Resistance to glyphosate

The Danish populations (*L. multiflorum* and *L. perenne*) were not tested for resistance to glyphosate, as no cases of glyphosate resistance have been reported in Denmark.

Greece
Resistance to ALS and ACCase

Four Greek *L. rigidum* populations (GR-9, GR-20, G-24 and G-30) were tested to assess their resistance to ALS and ACCase inhibitors. Multi-resistance to ALS inhibitors (mesosulfuron-methyl + iodosulfuron-methyl-sodium) and ACCase inhibitors (clodinafop and pinoxaden) was confirmed in the four populations tested. In particular, resistance was confirmed to mesosulfuron + iodosulfuron (RI ranging from 8 to >70, to clodinafop (RI >17 for each population), with a lower level of resistance to pinoxaden (6 < RI < 20).
In all four Greek populations investigated, mutated ALS alleles were detected in some plants surviving application of ALS-inhibitors. More specifically, the 197-Ser mutant ALS alleles were detected in each population, whereas the 197-Thr and both 197-Ala and 197-Glu mutations were detected in population GR-9 and GR-24, respectively. The 376-Asn mutation was also detected in population GR-9. These mutations indicate high resistance to sulfonylureas (SU) herbicides.
Concerning ACCase resistance, six different types of ACCase mutant alleles were detected: 1781-Leu, 2027-Cys, 2041-Asn, 2041-Val, 2078-Gly and 2088-Arg. In population GR-30 only one type of mutant *ACCase* allele was detected (1781-Leu), instead in population GR-9 two types (1781-Leu and 2041-Val), in population GR-20 four types (2027-Cys, 2041-Asn, 2078-Gly, 2088-Arg) as well as in population GR-24 (1781-Leu, 2027-Cys, 2041-Asn, 2078-Gly). In addition, in population GR-20, different mutant ACCase alleles were found in the same plant. These mutations indicate high resistance to Aryloxyphenoxypropionates (APPs) and Cyclohexanediones (CHDs), however, less to Phenylpyrazolines herbicides.
Concerning NTSR, the analyses of the herbicide metabolism genes glycosyl-transferase (GT), monooxygenase (NMO), cytochrome P450 (CYP72A1) and cytochrome P450 (CYP72A-2) confirmed an over-expression of NMO and CYT72A2 in population GR-30. The results indicated both TSR and NTSR in the populations investigated.

Resistance to glyphosate

Regarding survival, three Greek populations showed survival equal to or higher than 80% at the recommended dose (720 g a.i. ha^{-1}). It has to be noted that in some cases the survival and growth of resistant plants at 8 fold the recommended dose were noticeably high. The partial *EPSPS* sequence of plants from 6 glyphosate- resistant Greek populations did not reveal any mutation at $Proline_{106}$. However, one Greek population carried a silent mutation at Proline106 (codon CCG). In addition, all biotypes showed nucleotide polymorphism (silent mutations) at Ala109. The expression profiles of 4 *ABC-transporter* like genes at 3, 6 and 12 hours after glyphosate spraying showed that the gene with the highest upregulation was an *ABC- type III transporter* like gene (JZ166942.1) in the resistant populations compared to the susceptible ones (at 12 hours after spraying), thus this is a putative marker gene for the rapid identification of glyphosate resistant plants. Biochemical tests for confirming glyphosate resistance were performed by shikimate analysis. The majority of R-populations (six Greek and one Italian populations) showed lower accumulation of shikimic acid as compared to the S-population. The results may indicate an early (some hours after herbicide application) sequestration of glyphosate possibly to vacuole leading to limited translocation within the resistant plants.

Historical field data on crop and resistance management in each country

Historical field data on crop management and resistance mechanism were collected from Italy, Denmark and Greece based on communication with of farmers concerning the crop and resistance management they had applied in their fields in previous years. More specifically, details about the herbicides applied over the previous 2-5 years in winter cereal and orchards fields in, Italy, Denmark and Greece, crop rotation and other cultural practices were collected. The individual management practices for each country are listed below:

Italy

The occurrence and diffusion of herbicide resistant *Lolium* spp. populations has increased during the past 20 years in many Italian region. This evolution began in the 1990s with the first reports of populations resistant to ACCase-inhibitors in the cereal monoculture areas of Southern and Central Italy. Since then the situation has progressively worsened due to the reliance on the solely post-emergence application of herbicides with the same MoA, and resistant populations have been identified also in Northern regions such as Emilia Romagna and Piedmont. The introduction of ALS- inhibitors temporarily improved control of *Lolium* spp. in cereals, however resistance soon evolved also against those herbicides because farmers still over-relied on post- emergence herbicides for weed control. As a consequence, populations with multiple resistance to ACCase/ALS inhibitors are common in the cereal production areas of Southern and Central Italy but they have also been reported in Northern regions. Similarly, in the same period glyphosate resistance started to spread in olive groves in Southern Italy where this herbicide was the only control tool applied. Further glyphosate-resistant populations of *Lolium* spp. have been identified in vineyards and hazelnut orchards in Piedmont region but their spread is still limited.

Farmer actions related to herbicide resistance management are primarily focused on chemical control, with three main cases:

1. Rotation or mixture (less often) of ALS and ACCase inhibitors in post-emergence applications. In case of already evolved herbicide resistance, farmers usually react switching to herbicides with a different site of action (SoA) (normally from ACCase to ALS inhibitors).
2. Pre-emergence or early post-emergence (autumn-winter applications). In case of *Lolium* populations with multiple ALS/ACCase resistance, farmers perform pre- emergence or early post applications of herbicides with different SoAs (eg triallate, chlortoluron) both in addition or as alternative to post-emergence applications.
3. Mixture of glyphosate with ALS-inhibitors in olive groves where glyphosate-resistant populations are present.

Crop Rotation

Farmers normally don't consider herbicide resistance when they plan crop rotation. Crop choice is usually driven by simple economic evaluations. In case of already evolved resistance, some farmers react by modifying rotations, such as reducing the frequency of cereal crops, but this is feasible only in the areas where spring crops can be cultivated. Oilseed rape could be an important alternative as autumn-sown crop, since some effective herbicides are available for *Lolium* control in this crop, but its cultivation has almost disappeared (less than 15,000 ha in Italy) due to economic reasons. In Southern regions where crop potential productivity is limited, farmers sometimes modify the management of field bean (*Vicia faba* var. *minor*), which is often in rotation with durum wheat, to reduce populations of resistant *Lolium*. Given that the economic value of field bean grain yield is low, farmers prefer to manage it as a cover crop for green manure incorporated before ripening, in order to avoid *Lolium* dissemination during the cropping season.

Tillage

Even if no-till agriculture is not very common in Italy, minimum tillage (eg chisel + harrowing) is commonly adopted for wheat seedbed preparation. In case of herbicide resistance evolution or dense *Lolium* populations, farmers tend to increase soil tillage intensity introducing ploughing, which is known to be very effective for *Lolium* control.

Cultural practices

In case of fields with high *Lolium* density, farmers sometimes try to anticipate seedbed preparation and postpone wheat sowing in order to eliminate *Lolium* seedlings before crop sowing. This is usually achieved by glyphosate pre-sowing application. There are some climatic constraints for this technique: in Southern regions autumn is sometimes rather dry

so weed germination is limited and false seedbed is not effective. In Northern regions, postponing cereal sowing can be risky because late sowing (from begin of November onwards) can cause relevant yield reduction.

Denmark

Currently, herbicide resistance is not restricting cropping practices but it is an emerging problem receiving a lot of attention of farm advisors, researchers and authorities. Farmers are less aware on resistance until they experience the problem in their own fields. The list of confirmed herbicide resistant species includes 8 weed species. Four of these are broadleaved weed species with resistance to ALS inhibitors (*Stellaria media, Capsella bursa-pastoris, Tripleurospernum inodorum* and *Papaver rhoeas*). The remaining four weed species are grasses with resistance to ALS- and ACCase inhibitors (*Alopecurus myosuroides, L .perenne, L. multiflorum, Apera spica- venti*). The first report dates back to 1991 and concerned *S. media*. Resistance in grass weeds was reported for *A. myosuroides* 10 years later and concerned ALS- as well as ACCase inhibitors.

A recent survey on the status on herbicide resistance in Denmark showed an average frequency of resistance of 8% among the collected seed samples. The survey included 8 weed species and a total of 330 samples from field plots not sprayed with herbicides in the year of sampling. The highest frequency of resistance was found in *A. myosuroides* with 30% of the samples showing resistance to ACCase and ALS inhibitors. For *L. perenne* 19% of the samples were resistant to ALS inhibitors, while the 15% of the *L. multiflorum* and *S. media* were resistant to ALS inhibitors. The high frequencies of resistance in *A. myosuroides* and *S. media* were not surprising as the first cases of resistance in these species were confirmed many years ago. In contrast, it was surprising to learn that 19% of the *L. perenne* samples were resistant because prior to the survey only one case of resistance had been reported in Europe. Results of a later survey including 126 samples of *Lolium* (94 *L. multiflorum* and 32 *L. perenne*) showed resistance to ALS- and ACCase inhibiting herbicides in 72% of the *L. multiflorum* populations and 32% of the *L. perenne* populations. The higher frequency of resistance found in this survey compared to the previous one was not surprising as the seed samples were collected in fields with suspected resistance and where herbicides had been applied in the year of sampling.

Cropping system

One of the reasons for herbicide resistance being less of a problem in Denmark compared to our neighboring countries is probably that the dominance of winter cereals in the crop rotation happened later in Denmark than in the UK and Germany. Spring cereals used to be the most widely cultivated crop until the 1980s and crop rotations were generally more diverse than today. Cereals is currently cultivated on more than 54% of the agricultural land in Denmark with winter cereals covering around 32% of the cultivated land. Other main crops are grass (28% of which 19% is in rotation), oilseed rape (7%), beets and potatoes (3.4%) and legumes (1%). The farmer's choice of crops is primarily governed by economic returns though the need for production of fodder for livestock is also important on mixed farms. Therefore crop rotations are not static but rather dynamic. Geographical differences exist for some crops. In the eastern part of the country the dominating crop rotation is cereal with winter oilseed rape every fourth or fifth year. Many farms also grow grass for seed production adding to the diversity. Sugar beets are mainly grown on the southern islands where they replace the winter oilseed rape. The main area for potatoes is in the western part of the country where also most of the dairy farm are located and grass and maize are therefore common crops in the rotation. A recent survey on crop rotations covering 20% of the agricultural area showed that winter wheat in monoculture was common on 10% of the agricultural area and winter wheat in rotation with winter oilseed rape on 14% of the area. More than 37% of the area had more than 75% winter crops.

On farms where herbicide resistance has been confirmed, farmers sometimes modify their rotations, e.g. reducing the frequency of winter cereal crops. However, the list of alternative crops is short. Oilseed rape is an important alternative to winter cereals, since effective herbicides are available for this crop, but it can only be cultivated every 4th year due to the risk of soil-borne pests and diseases. Potatoes, sugar beet and grass seed crops are only grown on contracts and the market for these crops has been saturated. Cultivation of broad bean has increased at the expense of peas. A potential expanding market is crops grown for bioenergy. The regulation on compulsory on cover crops limits crop choices.

Pesticide use

Farmers rely very much on herbicides for weed control. Pesticide regulation in Denmark is very strict. On top of the EU legislation on pesticides, national pesticide action plans have imposed further restrictions on pesticide use over the last 30 years. One consequence of this is that the number of available sites of action is lower in Denmark than in most other EU member states. For many years, the treatment frequently index (TFI) was used as an indicator for pesticide consumption encouraging farmers to reduce the dose. Pesticide taxes have been implemented as an incentive to make farmers reduce the use of pesticides. The latest pesticide tax is based on the new indicator, the pesticide load. Differences in pesticide load within a chemical group of pesticides with the same site of action tend to be lower than between chemical groups with different sites of action. Therefore, certain herbicide groups are more heavily taxed than others potentially leading to less diversity in the sites of action used by farmers and therefore a higher risk of herbicide resistance development.

Diversity in site of action is regarded as one of the most effective anti- resistance measures on the short term. ALS inhibitors are among the most resistant- prone herbicide sites of action and in Denmark ALS resistance in broadleaved weed species evolved due to the continuous use of ALS in cereals. However due to their benign environmental profile and low dose rates the pesticide tax is low and ALS inhibitors are therefore still widely used in Denmark as they combine a high effectiveness and a low cost. The recommendations to prevent and to overcome ALS resistance in broadleaved weed species have been to use ALS inhibitors in mixture with herbicides with other site of action.

The residual herbicide prosulfocarb applied early post- emergence has been a common part of a weed control strategy in winter cereals to control or at least suppress the first flushes of grass weed seedlings. Prosulfocarb is one of the herbicides most severely affected by the pesticide tax and although considered as a resistance breaker, the high cost might tempt farmers to reduce their use of prosulfocarb. Actually, the statistics show a reduction in the use of prosulfocarb from 2012 (before implementation of the new tax system) to 2017.

For post-emergence control of grass weeds only two sites of action are available – the resistance-prone ALS- and ACCase inhibitors. The resistance strategy has been to combine these herbicides with pre-emergence or early post-emergence herbicides – primarily prosulfocarb as a measure to reduce the first flushes of seedlings thereby creating a more uniform population of grass weeds for the later sprayings. For the later post-emergence applications the recommendation is to rotate between ALS- and ACCase inhibitors. In case of already evolved herbicide resistance, farmers usually switch to herbicides with a different site of action if available. This may imply choosing an alternative crop in which other sites of action can be used (i.e. propazymide in oilseed rape and mesotrione in maize).

Tillage

Main tillage systems include ploughing although reduced tillage/ no-till has increased during the last 10 years. Currently the area under reduced tillage covers around 15% of the agricultural land. In systems with reduced tillage ploughing is most often replaced by glyphosate. Stubble harrowing has decreased after new research has shown that survival of grass seeds is reduced by leaving the stubble undisturbed as long as possible Time for soil cultivation is short and further limited by the regulation on cover crops.

Cultural practices

In fields with grass weeds delayed sowing is recommended. Late sowing reduces seedling emergence, weed/crop competition and seed production in grasses. Emerged weed seedlings can be controlled before sowing. In wet autumns delayed sowing can be risky as seed bed preparation may be suboptimal and cause poor crop establishment and therefore yield reductions. Late sowing requires higher seed rates.

Characteristics for fields included in the* Lolium *survey

For fields in the *Lolium* survey the following characteristics can be derived from a questioner sent to the farmers:

Tillage: Conventional tillage including mouldboard ploughing was practiced in 63% of the fields. Seven percent used reduced tillage and 13% of the fields were not ploughed within the last 8 years. Seventeen percent of the farmers did not respond on this question.

Crops: Most samples were collected in winter cereals (73%), 10% were from spring cereals, 2% from winter oilseed rape and 14% did not respond on this question.

Crop rotation: The most common crop rotation for the fields included in the survey was winter cereals + winter oilseed rape covering 35% of the fields. The same percentage of fields was cultivated only with cereals; monoculture of winter wheat in 13% and winter cereals combined with spring cereals in 22% of the fields. Winter cereals combined with spring cereals and winter oilseed rape covered 10% of the fields. Only 5% of the fields had maize, grass or broad bean in the rotation. Information on crop rotation was not available for 16% of the farmers.

Pesticide use: Pesticide use for 4 years was available for 67 fields (53%) and showed that ALS inhibitors were used at least 3 out of 4 years in 74% of the fields whereas only 14% of the fields were sprayed with ACCase inhibitors 3 or more times within 4 years. In 68% of the fields ACCase inhibitors had not been used for 4 years. All fields were sprayed with prosulfocarb at least once within the last 4 years, while 38% of the fields were treated in 3 out of 4 years and 12% every year.

Greece

Resistance to ALS/ACCase

Herbicide resistance in Greece has become a big issue, especially in winter cereals crops. Since 1997, when resistant *L. rigidum* to diclofop-methyl was detected in winter cereals in north Greece, numerous cases of herbicide resistance have been reported, most of them referring resistant *L. rigium*, *Avena sterilis, Phalaris minor, Milium vernale* and *Apera spica-ventis* to ALS and ACCase inhibitors. Moreover, in winter cereals there have been reports about ALS resistant broad-leaves such *Papaver rhoeas* (resistance to 2,4-D as well), *Sinapis arvensis, Galium spurium, Galium rugosum, Bifora radians* and *Camelina microcarpa*. Concerning resistant *L. rigidum,* this weed species is mainly distributed in the north part of Greece and it has been reported that its resistance is attributed mainly to TSR mutations (Pro-197) and also to NTSR.

Winter cereals in Greece account for 25% of the cultivation area. Most of this area is monoculture rain-fed crop with little participation in crop rotation. The latter and the use of post-emergence herbicide as a solely weed control method have contributed to the development of herbicide resistance. The last years, some fields of winter cereals are rotated with oilseed rape or lupine, however, rotation plan is based mainly on subsidies

The results revealed that the populations were multiple resistant to ALS /ACCase inhibitors with TSR as the main resistance mechanism for both ALS/ACCase. NTSR seems to co-exist. These results are in agreement with previous reports. Greek farmers use post emergence herbicides as the main method to control *Lolium* spp and other weeds in winter cereals and they usually switch between ALS and ACCase inhibitors. The herbicides applied in previous years in these fields were the following: clodinafop-propargyl, pinoxaden, iodosulfuron-methyl, mesosulfuron- methyl, pyroxsulam, diclofop fenoxaprop and tralkoxydim. Due to increased cases of herbicide resistance the last 2 years some farmers switched to pre-emergence herbicides (prosulfocarb, diflufenican/chlotoluron mixture) in order to control *Lolium* spp. The high cost of some pre-emergence herbicides, a possible additional post-emergence application and possible risk of crop injuries caused by weather conditions (eg. extreme rainfall) have been reported as limiting factors for the use of pre- emergence herbicides.

Crop rotation

Rye (*Secale cereale*) in the place of winter wheat or barley is used in crop rotation to manage and reduce the infestation of *L. rigidum*. Rye is highly competitive against *L. rigidum* and keeps weed densities at very low levels the year of crop rotation fields. Oil-seed rape is also cultivated however at low extent due to economic reason, whereas there is still herbi-cide resistance problem in such fields. Lupine, beans or other pulses observed in crop rotation are cultivated mainly for subsidy reasons, are poor competitors against *L. rigidum*. In whole year crop rotation some farmers use cotton, sun-flower or oregano; however, the crop selection highly depends on economic reasons and not on herbicide management issues.

Tillage

No-till agriculture is not used in Greece apart from very few farmers who own
the specific machinery for direct drilling. Conventional tillage including moldboard ploughing is the most common practice for seedbed preparation by Greek farmers. Some farmers are advised to apply deep tillage to bury *Lolium* seeds in the fields.

Cultural practices

Techniques such as false-seedbed, increased crop density or use of competitive cultivars are not applied much in practice by Greek farmers, although many of them are aware of these practices.

Resistance to glyphosate

Most famers of perennial crops (olives, citrus, vines etc) control weeds both mechanically and chemically. Glyphosate is a broad-spectrum herbicide has a long history (more than 40 years) of use; many farmers usually apply glyphosate 1-3 times per year. The overreliance on this herbicide in combination with limited application of herbicides with a different mechanism of action, its high efficacy against many and also perennial weed species, application of suboptimal glyphosate rates, and the limited alternative integrated weed management approaches (such as crop rotation and use of mechanical weed control) have resulted in the development of glyphosate resistant *Conyza* spp. populations in perennial crops. In Greece, in 2016 *L. rigidum* was reported with resistance to glyphosate.

Characteristics for fields included in the* Lolium *survey

Thirty-nine (39) *L. rigidum* populations were collected from 39 different fields mainly after farmers' complains for ineffective control of ryegrass. The 30 out of 39 populations originated from arable crop fields, whereas the other 9 came from orchards (6 from olive groove fields, 2 from grape and 1 from apricot). The eight (8) orchards were located in the middle-south part of Greece, one (1) in north Greece, whereas more than 90% of the other fields were situated in north Greece as well.

Tillage: Conventional tillage including mouldboard ploughing was performed in all winter cereals and arable fields included in this study.

Crops: Most samples were collected from winter cereals (60%), mainly wheat, 7% from oil-seed rape, 16 % from olive groove, 5% from grape, 5% from lupine, 2% from apricot, 2% from oregano, 2% from sunflower and 2% from non-cultivated field.

Crop rotation: In 8 out of 30 fields of arable crops there has been no crop rotation the last 3 years and wheat was one of the crops in crop rotation the last 3 years in almost all these fields. In 4 out of 30 of these fields a summer crop (cotton) was included in the crop rotation.

Pesticide use: in all the orchards, except from where the sensitive population to ALS, ACCase and EPSP seeds came from and no herbicide has been used there so far, glyphosate was extensively used for years for weed control. In all other fields ALS and ACCase inhibitors were used the last three years.

In summary, multiple resistance in the three countries to ALS and ACCase was detected, endowed by amino acid substitutions. Concerning ALS resistance, it was more pronounced in Denmark compared to Greece and Italy. More particular, in Danish populations only one mutant allele was detected (574-Leu), whereas in Greek main mutations were detected mainly at 197-Pro (one more mutation at 376-Asp); in Italian populations, there were mutations at 122-Ala, 197-Pro, 205-Ala, 376-Asp and 574-Trp. Concerning ACCase resistance, no mutation was detected in Danish populations, while in Greek populations six different mutations were detected at 1781-Leu, 2027-Cys, 2041-Asn, 2041-Val, 2078-Gly and 2088-Arg, and in Italian populations five mutations were found at 1781-Leu, 1781-Asp, 2078-Gly, 2096-Ala and 2096-Ala. Concerning NTSR, the resistant populations from all countries were heterogenous for the gene expression of one or more herbicide metabolism genes. More specifically, NMO, GST, and CYP72A2 genes showed increased expression in plants in populations of the three countries, with the latter gene (CYP72A2) to express at the highest level.

CYP72A1 gene was expressed at a relative low level, except for plants in an Italian and a Danish population. In conclusion, metabolic herbicide resistance was very heterogeneous distributed among plants and populations, which highlighted the potential for evolution of metabolic based herbicide resistance in most populations. The results indicated existence of both TSR and NTSR in the populations of the three countries investigated. Concerning *Lolium* resistance to glyphosate, the results indicated that mutations were not responsible since no mutation was detected in population for Greece and Italy (no glyphosate resistance was evident in Denmark), with more resistance cases to reveal in Greece compared to Italy. The results indicate possible sequestration of glyphosate into the vacuole that confer the resistance. In Greece, glyphosate resistant *Lolium* cases were reported only in perennial crops and not in winter cereals, since reduce tillage methods with glyphosate application for termination of cover crops are not applied in Greece.

Pan-European guidelines for the management of the herbicide resistant *Lolium* spp

Herbicide resistance is currently a big issue, however, no guidelines for its management have been published in an EU level. Based on the research and the data collected from WP1 and WP2 of the RELIUM project, pan-European guidelines for herbicide resistant *Lolium* spp. are presented below.
These guidelines are listed depending on the status of herbicide resistance in the fields (confirmed or yet not identified) for both ALS/ACCase inhibitors in winter cereals and EPSP inhibitor (glyphosate) in orchards. In case of confirmed herbicide resistance (re-active situation), the aim is to manage the resistance, to prevent further spread and to deplete the soil seed bank. In fields where no herbicide resistance has been identified (pro-active situation), the aim is to prevent or to delay the development. However, the unifying strategy is reduction of viable weed seeds into the soil seed bank and maintaining low weed seed banks to minimize population proliferation, evolution of herbicide resistance to additional sites of action and spread.
Below Pan-European guidelines for *Lolium* spp. management are presented. In particular, Tables 1 & 2 present re-active strategies in cases of confirmed resistance to both ALS /ACCase in winter cereals and glyphosate in orchards; whereas, Tables 3 & 4 present pro-active strategies in cases of no resistant detected so far to ALS /ACCase and/or glyphosate.

TABLE 1: Pan-European guidelines for *Lolium* spp. management with confirmed resistance to ALS /ACCase inhibitors in winter cereals. Re-active strategies

Aim: to manage herbicide resistance and prevent further spread
Chemical methods
a. Use of registered pre-emergence or early- post herbicides with at least one site of action that controls *Lolium* spp. in place of ALS or ACCase inhibitors (eg. prosulfocarb, diflufenican / chlotolouron, diflufenican/flufenacet in winter cereals)*
b. In fields with confirmed ALS resistance but no resistance to ACCase inhibitors or vice versa, make use of any herbicide of the latter group that is known to control *Lolium* spp.
c. In cases with low resistance level to ACCase inhibitors, switch between Aryloxyphenoxypropionates (APPs) / Cyclohexanediones (CHDs) and Phenylpyrazolines herbicides
d. In delayed planting cases, the initial *Lolium* spp. flush can be controlled with non-selective non-residual herbicides. (Do not destroy the plants mechanically to avoid bringing *Lolium* seeds to the soil surface)
e. Site specific weed management to target weed patches with higher doses.
Cultural methods
f. Crop rotations that include crops with different life cycles, different requirements allowing herbicide rotation, as well as the application of other weed control methods. Particularly, crop rotation with broadleaf crops (eg. oil-seed rape) should be applied only in cases where herbicides of other chemical group have not been reported as a resistant case.
g. Increased frequency of spring sown crops in the crop rotation.
h. Use of false-seed bed technique combined with control of the initial flush of *Lolium* spp. with glyphosate.

i. Crop rotation with crops with allelopathic properties (eg. rye) that is highly competitive against *L. rigidum*.
j. Crop rotation with forages or silage cereals (eg. silage barley). Frequent mowing and early harvest for silage production prevent *Lolium* plant to produce seeds
k. Increase crop seeding rates to strengthen crop competition against *Lolium* spp.
l. Chose competitive crop cultivars to enhance crop competition against *Lolium* spp.

Mechanical methods

m. Mechanical mowing or hand-weeding of the *Lolium* populations found in patches before they set seeds
n. *Lolium* spp. control with shallow tillage (10 cm) during false seedbed technique
o. Apply deep tillage (>20 cm) to bury seeds of herbicide resistant *Lolium* spp. in winter cereals. To increase control efficacy, deep tillage should be applied only every 3-4 years.
p. Harvest weed seed can reduce the number of seeds in the chaff fraction emitted by the combine harvester. It requires that the species retain their seeds in sufficient quantities until harvest (limited seed shattering)

Prevention and additional practices

q. Avoid soil and plant material transportation and avoid movement of livestock from infested fields to clear fields
r. Keep field fences and bunds free from weeds
s. Remove seeds and plant parts from any machinery, especially harvesters to avoid spread of seeds of resistant *Lolium* spp.
t. Leave fields with history of confirmed *Lolium* spp. Resistance last for any crop practices to apply
u. Leave stubble undisturbed as long as possible after harvest to allow maximum seed predation and control emerged seedlings with glyphosate

**Herbicide selection is based on the registered products in each country*

TABLE 2. Pan-European guidelines for *Lolium* management with confirmed resistance to glyphosate in orchards. Reactive strategies

Aim: to manage herbicide resistance and prevent further spread
Chemical methods
a. Use alternative registered herbicides with at least one site of action that controls *Lolium* spp. in place of glyphosate
b. Site specific weed management with variable dose application on target weed patches with higher doses.
Cultural methods
c. Use and management of annual cover crops (eg. *Vicia sativa*) to suppress resistant *Lolium* and other weed species as well.
Mechanical methods
d. Mechanical mowing or hand-weeding of the *Lolium* populations found in patches before they set seeds
e. *Lolium* spp. control with shallow tillage (10 cm), avoiding ploughs and disk harrows in orchards located on steep slopes (caution in cases of presence of perennial species such as eg. johnsongrass).
Prevention and additional practices
f. Avoid soil and plant material transportation and avoid movement of livestock from infested fields to clean fields
g. Keep field fences and bunds free from weeds
h. Remove seeds and plant parts from any machinery to avoid spread of seeds of resistant *Lolium* spp.
i. Leave last for any crop practices to apply the fields with history of confirmed *Lolium* spp. resistance

TABLE 3. Pan-European guidelines for *Lolium* management with no herbicide resistance to ALS /ACCase inhibitors in winter cereals detected so far. Pro- active strategies

Aim: to prevent or delay herbicide resistance
Chemical methods
a. Use of herbicide mixtures with different sites of action
b. Follow herbicide use instructions and apply the recommended full rates at the correct timing as stated on the herbicide label
c. Switch periodically from post-emergence to pre-emergence herbicides
d. Do not use the same herbicide for more than 3 years in a row even if it is still effective. Switch to an alternative herbicide with different site of action
e. In fields with no resistance, use of glyphosate for the control of ALS and ACCase resistant *Lolium* spp. populations in fields with extreme *Lolium* density, under no-crop cultivation and before the set of *Lolium* seeds.
Cultural methods
f. Crop rotation that include crops with different life cycles, crops with different requirements allowing herbicide rotation.
g. In delayed planting cases, destroy the initial *Lolium* spp flush with glyhposate
h. Rotation with crops with allelopathic and competitive properties (eg. rye) that highly compete with *L. rigidum*
i. Application of integrated weed management and no reliance exclusively on chemical means for the control weeds
Mechanical methods
j. Deep tillage every 3-4 years to bury *Lolium* seeds and reduce its density every 3-4 years
Prevention and additional practices
k. Use of certified crop seeds
l. Keep field fences and bunds free from weeds
m. Removal of seeds and plant parts from any machinery to avoid spread of seeds of possibly resistant *Lolium* spp.
n. Use of increased crop densities or competitive cultivars to compete and suppress weeds

TABLE 4. Pan-European guidelines for *Lolium* management with no herbicide resistance to glyphosate in orchards detected so far. Pro-active strategies

Aim: to prevent or delay herbicide resistance
Chemical methods a. Replace glyphosate with other registered herbicide or herbicide mixtures with different site of action every 3 years. *Cultural methods* a. Application of integrated weed management and no reliance exclusively on chemical means in order to control weeds b. Use of cover crops (eg *Vicia sativa, Trifolium* spp., *Pisum sativum, Festuca* spp.) in orchards and vineyards to suppress weeds (only annual winter species between the rows in irrigated orchards and vineyards) *Mechanical methods* c. Mechanical weed control (shallow tillage or mowing) where possible in place of continuous glyphosate use in perennial crops before *Lolium* spp. set seeds *Prevention and additional practices* d. Keep field fences and bunds free from weeds e. Removal of seeds and plant parts from any machinery to avoid spread of seeds of possibly resistant *Lolium* spp.

Problems and obstacles associated with the adoption of these guidelines

Experiences on herbicide resistance management globally show that farmers deal with the problem after it occurs, not before. Proactive resistance management involves more diverse strategies to delay the evolution of herbicide resistant weeds but often these strategies are not economically attractive in the short term. Farmers perceive greater risks and little benefits of preventative tactic addressing herbicide resistant weeds. Those focusing on profitability are less likely to adopt diverse strategies particularly if they don't own the land. Tactics that enhance the diversity of weed management must not only be timely relative to crop and weed stage of development but farmer also need time and labour to adopt the diverse practices. This can be a limiting factor due to increasing farm sizes.

Economic issues and environmental constraints can hamper the adoption of crop rotation in Mediterranean areas. The lack of precipitation during summer months in those areas usually impedes the growth of spring-sown crops. There are limited alternatives for autumn sowing, mainly pulses such as broad bean, but those crops are not economically profitable. Besides, chemical weed control in pulses is usually based on ACCase-inhibitors so pulses do not allow the use of other herbicide sites of action in the cropping systems. Mediterranean areas are normally characterized by limited crop productivity, average wheat yields are usually around 3-4 t/ha. Thus low crop productivity, and consequently low economic margins, is often the limiting factor for the adoption of guidelines. Farmers can hardly afford the cost of herbicide mixtures or application of both pre-emergence and post-emergence herbicides. Environmental conditions, such as the presence of rocks in the soil or the excessive hill slope, can prevent the use of mechanical tools or soil tillage for *Lolium* control in vineyards or orchards.

8. Country- and cropping system-specific guidelines for the management herbicide resistant *Lolium* spp. written in local languages and published (web).

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
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Main author(s):	**Thomas Gitsopoulos, Donato Loddo, Solvejg Mathiassen**
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Executive Summary

The deliverable presents the country- and cropping system-specific guidelines in Greek, Italian and Danish for the management of herbicide resistant *Lolium* spp. A short description of the situation concerning *Lolium* spp. resistance and the management methods currently applied in Greece, Italy and Denmark is also presented in the introduction section in English and translation in each local language follows with the guidelines. Guidelines for Greece and Italy are presented in two tables in Greek and Italian, one table with *Guidelines for Lolium spp. management in winter cereals* and the other with *Guidelines for Lolium spp. management in orchards and vineyards*. For Denmark, only one guideline is presented in local language, that referring to *Guidelines for Lolium spp. management in winter cereals,* since Danish farmers are not facing a problem with resistance in orchards so far.

These guidelines will be published on the web and will be available to the public free of charge

Introduction

In Greece *L. rigidum* is the main species of *Lolium* genus mainly distributed in the central and northern part of the country. *L. rigidum* resistant populations were firstly found in 1996 in winter wheat. Since then, *L. rigidum* resistance to ALS and ACCase inhibitors has become an important issue in winter cereals. Winter cereals in Greece account for 25% of the cultivated area, the crop is rain-fed and mostly monoculture with little application of crop rotation. When crop rotation is adopted, it mainly consists of oilseed rape, forage crops (eg. *Trifolium* spp., alfalfa, fodder mixtures), cotton, or pulses. Among cereals rye (*Secale cereale*) is used to suppress *L. rigidum* and other weed species due to its competitive ability and to its allelopathic properties. However, crop choice is mostly based on economic returns, rather than solutions for weed management. Conservation Agriculture (CA) in general is limited in Greece, similarly the use of cover crops. Mouldboard ploughing is the main and conventional method for seedbed preparation for winter cereals, whereas the false seedbed technique for weed control is not adopted by the Greek farmers. In order to control grass weeds in cereals farmers used to apply ACCase or ALS-inhibitors for many years. In the last recent years many farmers have shifted to pre- or early post-emergence applications of herbicides with different mode of action of the above type of herbicides to control *L.rigidum* resistant populations. Regarding orchards, (they account for about the 30% of the cultivated area,), with olive groves being the main crop *L. rigidum* resistance to glufosinate ammonium and to glyphosate has been reported in orchards and vineyards due to the continuous use of these herbicides for weed control in place of mechanical method of weed management.
In Italy, *L. rigidum* and *L. multiflorum* are the main species of *Lolium* genus. Evolution of resistance was firstly reported in 1990s with *L. rigidum* ACCase-resistant populations found in cereal monoculture areas of southern and central Italy. Since then, many resistant populations of *L. rigidum* and *L. multiflorum* to both ALS and ACCase have been identified, even in northern regions of the country as well. Moreover, resistant *L. rigidum* to glyphosate has been detected in olive groves, hazelnuts, orchards and vineyards in southern and northern Italy, whereas *L. multiflorum* resistant to glyphosate have been detected in north-eastern Italy in fields under no-till management. *Lolium* spp. populations with multiple resistance to glyphosate, ACCase inhibitors and ALS inhibitors were found in wheat-based cropping systems in Tuscan due to continuous pre-sowing use of glyphosate at seedbed preparation under reduced tillage. Concerning cultural practices farmers apply crop rotation that includes maize and soybean in northern regions, sunflower and sorghum in central Italy; crop choice is mainly based on economic returns. Oilseed rape was used in crop rotation however it has disappeared due to economic reasons. In southern Italy farmers usually include field bean (*Vicia faba* var. *minor*) in rotation with durum wheat but this is not effective to reduce populations of resistant *Lolium* spp since the only herbicides available for grass weed control are ACCase-inhibitors (same MOA than in wheat). In Italy under Conservation Agriculture (CA) systems (mainly minimum tillage) *L. multiflorum* is frequently used as autumn-cover crop and it is terminated with glyphosate in mid-March before sowing spring crops. Glyphosate is used to terminate *Lolium* spp. and other weed species also in false bed technique in pre-sowing application. Deep ploughing with mouldboard is used to bury *Lolium* spp. seeds as weed control method in fields with herbicide resistance. Concerning herbicide use farmers mainly switch between ACCase and ALS inhibitors. In cases of resistant *Lolium* spp. populations farmers apply pre- or early post- emergence herbicides with different SoA (eg. chlorotoluron), either in addition to or as alternative to late post-emergence applications of ALS/ ACCase inhibitors.

In Denmark, *L. mutiflorum* and *L. perenne* are the main *Lolium* spp. weed species. Currently, herbicide resistance is not considered a problem compared to Greece and Italy, however, it is receiving a lot of attention the last years. Herbicide resistant *L. multiflorum* was firstly reported in 2010 in Denmark; since then, *L. multiflorum* populations with multiple resistance to ALS and ACCase have been found, whereas *Lolium* spp. with resistance to glyphosate has not be reported in Denmark yet. The lower level of herbicide resistance in Denmark is probably due to the delayed dominance of winter cereals in crop rotation. Today, crop rotations are not so diverse as in the past, however, wheat monoculture is less compared to other countries being common on 10% of the agricultural area in the country and on 14% in rotation with oilseed rape. Winter cereals currently cover around 32% of the agricultural area. Danish main crops consist of grasses, oilseed rape, beets, potatoes and legumes. Crop choice is based mainly on economic returns, however, on the need for production of fodder for livestock in mixed farming as well. Cultural practices include modification of crop rotation reducing the frequency of winter cereals. Reduced or no-till has been increased in Denmark during the last 10 year. More particularly, reduced tillage area currently accounts for 15% of the agricultural land. Delayed sowing is recommended to control grass and other weeds with glyphosate application or mechanically. ALS and ACCase inhibitors are generally used for grass *Lolium* spp. control, whereas a common part of weed control strategy is the use of prosulfocarb herbicide with SoA different to that of ALS and ACCase applied early post. Farmers' common practice is the use of early post emergence herbicides-primarily prosulfocarb in conjunction with ALS or ACCase inhibitors. In cases of *Lolium* spp. resistance farmers apply crop and herbicide rotation (propyzamide in oilseed rape and mesotrione in maize).
The management of *Lolium* spp. herbicide resistance imposes the development of country- and cropping system-specific guidelines separately for Greece, Italy and Denmark due to difference in status of resistance, climate, cropping systems, crops cultivated and methods for weed controlled more adopted in each country. These guidelines are presented below in each local language.

Οδηγίες για την Ελλάδα

Εισαγωγή

Στην Ελλάδα η ήρα, (*Lolium rigidum*) είναι το κύριο είδος του γένους *Lolium* που εμφανίζεται κυρίως στη βόρεια και κεντρική της χώρας. Ανθεκτικοί πληθυσμοί ήρας βρέθηκαν αρχικά το 1996 σε καλλιέργεια σιταριού. Έκτοτε, η ανθεκτικότητα ήρας σε ALS και ACCase αναστολείς έχει γίνει σημαντικό ζήτημα στα χειμερινά σιτηρά. Τα χειμερινά σιτηρά στην Ελλάδα αντιπροσωπεύουν το 25% της καλλιεργούμενης έκτασης, στο μεγαλύτερο μέρος τους αποτελούν μη-αρδευόμενη μονοκαλλιέργεια. Η αμειψιπορά δεν εφαρμόζεται σε μεγάλο βαθμό και όταν εφαρμόζεται κυρίως περιλαμβάνει καλλιέργειες ελαιοκράμβης, ζωοτροφές (τριφύλλια, μηδική, χορτοδοτικά μείγματα), βαμβάκι ή όσπρια. Μεταξύ των δημητριακών η σίκαλη (*Secale cereale*) χρησιμοποιείται για την αντιμετώπιση της ήρας και άλλων ζιζανίων λόγω της ανταγωνιστικής ικανότητάς της και των αλλοπαθητικών της ιδιοτήτων. Η επιλογή των καλλιεργειών στην αμειψισπορά βασίζεται κυρίως σε οικονομικές κριτήρια, παρά σε πλάνο αντιμετώπισης των ζιζανίων. Η διατήρηση εδαφών (Conservation Agriculture) γενικά είναι περιορισμένη στην Ελλάδα, όπως επίσης η χρήση φυτών εδαφοκάλυψης (cover crops). Το όργωμα με άροτρο είναι η κύρια και συμβατική μέθοδος για την προετοιμασία των αγρών για σπορά των χειμερινών σιτηρών, ενώ η ψευδοσπορά για τον έλεγχο των ζιζανίων δεν υιοθετείται από τους Έλληνες αγρότες. Για τον έλεγχο των ζιζανίων στα χειμερινά σιτηρά, οι παραγωγοί χρησιμοποιούν για χρόνια διάφορους ACCase ή ALS-αναστολείς. Τα τελευταία χρόνια πολλοί παραγωγοί εφαρμόζουν προφυτρωτικά ή πολύ νωρίς μεταφυτρωτικά ζιζανιοκτόνα, διαφορετικά των ALS/ACCase αναστολέων για να ελέγξουν την ανθεκτική στους παραπάνω αναστολείς ήρα. Όσον αφορά τους οπωρώνες, αυτοί αντιπροσωπεύουν περίπου το 30% της καλλιεργούμενης έκτασης, με τους ελαιώνες να είναι η κύρια καλλιέργεια. Ανθεκτικότητας ήρας στο glufosinate ammonium και στο glyphosate έχει αναφερθεί σε οπωρώνες και αμπελώνες λόγω της συνεχούς χρήσης αυτών των ζιζανιοκτόνων για τον έλεγχο ζιζανίων αντί για μηχανική καταπολέμηση.

Οδηγίες για την αντιμετώπιση της ήρας (*Lolium rigidum*) στα χειμερινά σιτηρά

Χημικές μέθοδοι

- Χρησιμοποιήστε ζιζανιοκτόνα που εφαρμόζονται προφυτρωτικά ή νωρίς μεταφυτρωτικά (πχ. Boxer, Carmina max, Herold Trio, Fosburi) και ελέγχουν την ήρα στη θέση των ALS ή ACCase αναστολέων σε χειμερινά σιτηρά όπου έχει επιβεβαιωθεί ανθεκτικότητα σε ALS ή/και σε ACCase αναστολείς. Επιλέξτε ζιζανιοκτόνο από τον επίσημο κατάλογο φυτοπροστατευτικών προϊόντων (***wwww.minag-ric.gr/syspest***) του Υπουργείου Αγροτικής Ανάπτυξης και Τροφίμων
- Χρησιμοποιήστε ζιζανιοκτόνα που εφαρμόζονται προφυτρωτικά ή νωρίς μεταφυτρωτικά (όπως αναφέρονται παραπάνω) που ελέγχουν την ήρα στη θέση των ALS ή ACCase αναστολέων ή σε συνδυασμό με τους ALS ή ACCase αναστολείς για καθυστέρηση ανάπτυξης ανθεκτικότητας.
- Εναλλάσσετε τα ζιζανιοκτόνα (ιδιαίτερα τους ALS και ACCase αναστολείς) και μην χρησιμοποιείτε την ίδια δραστική ουσία κάθε χρόνο. Σε περίπτωση επιβεβαιωμένης ανθεκτικότητας σε έναν ALS ή AC-Case αναστολέα σταματήστε να χρησιμοποιείτε αυτά τα ζιζανιοκτόνα και χρησιμοποιείστε κάποια διαφορετική δραστική ουσία (πχ. Boxer, Carmina max, Herold Trio, Fosburi)
- Χρησιμοποιήστε την μέγιστη επιτρεπόμενη δόση των ζιζανιοκτόνων όπως αναγράφεται στην ετικέτα του σκευάσματος. Μην χρησιμοποιείτε μειωμένες δόσεις ζιζανιοκτόνων.
- Εφαρμόστε ζιζανιοκτόνα στο συνιστώμενο στάδιο ανάπτυξης της ήρας, υπό κατάλληλες περιβαλλοντικές συνθήκες και κάνετε χρήση κατάλληλου εξοπλισμού ψεκασμού

Καλλιεργητικά μέτρα

- Εφαρμόστε αμειψισπορά με καλλιέργειες διαφορετικών κύκλων ζωής, με διαφορετικές απαιτήσεις που επιτρέπουν την εφαρμογή εναλλακτικών ζιζανιοκτόνων και την εφαρμογή μη χημικών μεθόδων αντιμετώπισης της ήρας. Εναλλακτικές καλλιέργειες μπορεί να είναι η ελαιοκράμβη, ανοιξιάτικες καλλιέργειες (πχ. βαμβάκι, ηλίανθος, καλαμπόκι), ετήσιες ή πολυετείς χορτοδοτικές καλλιέργειες (πχ. τριφύλια, μηδική, χορτοδοτικά μείγματα). Καλλιέργειες στις οποίες η ήρα έχει αναπτύξει ανθεκτικότητα σε

ζιζανιοκτόνα που εφαρμόζονται σε αυτές (διαφορετικά αυτών που εφαρμόζονται στα χειμερινά σιτηρά) δεν πρέπει να συμπεριλαμβάνονται στην αμειψισπορά. (π.χ. ελαιοκράμβη με ανθεκτικότητα ήρας στο ζιζανιοκτόνο cycloxydim)

- Σε όψιμη σπορά όπως πχ. σε εφαρμογή ψευδοσποράς, το πρώτο κύμα φυτρώματος ήρας μπορεί να ελεγχθεί με το glyphosate και να ακολουθήσει σπορά των χειμερινών σιτηρών. Μην καταστρέφετε τα φυτά μηχανικά τα ζιζάνια για να αποφύγετε τη μεταφορά σπόρων ήρας στην επιφάνεια του εδάφους.
- Χρησιμοποιήστε άλλα σιτηρά αντί του σιταριού με αυξημένη ανταγωνιστική ικανότητα ή με αλλοπαθητικές ιδιότητες (π.χ. σίκαλη, κριθάρι) σε αγρούς όπου δεν μπορούν να καλλιεργηθούν άλλες καλλιέργειες εκτός από χειμερινά σιτηρά.
- Χρησιμοποιήστε πολυετείς χορτοδοτικές καλλιέργειες (πχ. μηδική) ή ετήσιες χορτοδοτικές σιτηρών (π. βρώμη) που επιτρέπουν κοπή και πρώιμη συγκομιδή εμποδίζοντας την ήρα να σποροποιήσει. Μην χρησιμοποιείτε το είδος ήρα πολυανθής (*Lolium multiflorum)* ως χορτοδοτικό σε περιοχές με ανθεκτικότητα ήρας σε ζιζανιοκτόνα ALS ή ACCase αναστολείς.
- Αύξηση της πυκνότητας σποράς των χειμερινών σιτηρών στο μέγιστο επιτρεπτό (πχ. 20 kgr/στρ) για την ενίσχυση της ανταγωνιστικής ικανότητας των χειμερινών σιτηρών.
- Σε αγρούς χειμερινών σιτηρών με ανθεκτικότητα ήρας σε ALS ή /και σε ACCase αναστολείς που μπαίνουν σε αγρανάπαυση να γίνεται καταστροφή των φυτρωμένων φυτών ήρας με συνδυασμό glyphosate και μηχανικών μεθόδων, μη-επιτρέποντάς τα φυτά ήρας να φτάσουν στην σποροποίηση.

Μηχανικές μέθοδοι

- Εφαρμόστε βαθύ όργωμα (σε βάθος 30 cm) για να θάψετε τους σπόρους της ήρας σε βάθος που δεν μπορούν να βλαστήσουν. Η βαθιά άροση αυτή άροση να εφαρμόζεται κάθε 3-4 χρόνια και όχι κάθε χρόνο για να μην επαναφέρονται από μεγαλύτερα βάθη σπόροι του ζιζανίου κοντά στην επιφάνεια του εδάφους

Πρόληψη και πρόσθετες πρακτικές

- Αποφύγετε τη μεταφορά εδάφους και φυτικού υλικού όπως και τη μετακίνηση ζώων από αγρούς με ανθεκτικότητα σε αγρούς που δεν έχει αναφερθεί ανθεκτικότητα
- Διατηρήστε τα περιθώρια των αγρών καθαρά από φυτά ήρας. Καταστρέψτε (χειρονακτικά) επιζώντα φυτά ήρας στον αγρό μετά την εφαρμογή ζιζανιοκτόνων πριν αυτά τα φυτά σποροποιήσουν
- Καθαρίζετε τα αγροτικά μηχανήματα και ειδικότερα τις αλωνιστικές μηχανές με σκοπό την αποφυγή διασποράς σπόρων ήρας με ανθεκτικότητα στα ζιζανιοκτόνα.
- Αφήστε αγρούς με ιστορικό ανθεκτικότητας ήρας τελευταίους στη σειρά για καλλιέργεια
- Παρατείνετε στο μέγιστο δυνατό την κατεργασία εδάφους για την προετοιμασία σποροκλίνης για σπορά ώστε να καταστραφούν οι σπόροι της ήρας που έχουν πέσει στο έδαφος από φυσικές συνθήκες ή από προσβολές από έντομα ή μύκητες
- Χρησιμοποιήστε πιστοποιημένο σπόρο χειμερινών σιτηρών
- Διατηρείτε αρχεία αγρού με τα ζιζανιοκτόνα που χρησιμοποιείτε, τις ημερομηνίες εφαρμογής τους και καταγράψτε το επίπεδο αποτελεσματικότητάς τους.
- Εφαρμόζετε συχνές επισκοπήσεις στον αγρό για επιζώντα φυτά ήρας
- Επικοινωνήστε με τοπικούς γεωπόνους ή άλλες υπηρεσίες που μπορούν να βοηθήσουν στον ανίχνευση πιθανών περιπτώσεων ανθεκτικότητας

Οδηγίες για την αντιμετώπιση της ήρας (*Lolium rigidum*) σε οπωρώνες και στο αμπέλι

Χημικές μέθοδοι

- Χρησιμοποιήστε εναλλακτικά εγκεκριμένα ζιζανιοκτόνα που ελέγχουν την ήρα αντί του glyphosate σε οπωρώνες και αμπελώνες όπου έχει επιβεβαιωθεί ανθεκτικότητα στο glyphosate. Επιλέξτε ζιζανιοκτόνο για τον οπωρώνα ή τον αμπελώνα σας από τον επίσημο κατάλογο φυτοπροστατευτικών προϊόντων (***wwww.minagric.gr/syspest***) του Υπουργείου Αγροτικής Ανάπτυξης και Τροφίμων
- Χρησιμοποιήστε εναλλακτικά εγκεκριμένα ζιζανιοκτόνα που ελέγχουν την ήρα στη θέση του glyphosate κάθε 3-4 χρόνια για αποφυγή ή καθυστέρηση ανάπτυξης ανθεκτικότητας
- Εφαρμόζετε τις μεγαλύτερες συνιστώμενες δόσεις για τον έλεγχο της ήρας όπως αναγράφονται στην ετικέτα των σκευασμάτων. Μην εφαρμόζετε μειωμένες δόσεις ζιζανιοκτόνων.
- Εφαρμόστε τα ζιζανιοκτόνα στο κατάλληλο στάδιο ανάπτυξης της ήρας, κάτω από κατάλληλες περιβαλλοντικές συνθήκες και χρησιμοποιήστε κατάλληλο εξοπλισμό ψεκασμού.
- Όταν εφαρμόζετε το glyphosate, τότε προτιμήστε ο ψεκασμός να γίνεται σε χαμηλή θερμοκρασία (<12 ° C) για αποφυγή ή καθυστέρηση ανάπτυξης ανθεκτικότητας.

Καλλιεργητικά μέτρα

- Χρησιμοποιήστε καλλιέργειες φυτοκάλυψης (cover crops) (π.χ. *Vicia sativa, Trifolium spp., Pisum sativum, Festuca* spp.) σε οπωρώνες και αμπελώνες για τη διαχείριση της ήρας (μόνο ετήσια χειμερινά είδη με σπορά μεταξύ των γραμμών φύτευσης σε αρδευόμενους οπωρώνες και αμπελώνες)

Μηχανικές μέθοδοι

- Εφαρμόστε μηχανική καταπολέμηση της ήρας (επιφανειακή κατεργασία εδάφους σε βάθος έως 10 cm ή κοπή των ζιζανίων) πριν σποροποιήσει, αποφεύγοντας την κατεργασία εδάφους σε οπωρώνες που βρίσκονται σε απότομες πλαγιές

Πρόληψη και πρόσθετες πρακτικές

- Αποφύγετε τη μεταφορά εδάφους και φυτικού υλικού όπως και τη μετακίνηση ζώων από αγρούς με ανθεκτικότητα ήρας σε αγρούς που δεν έχει αναφερθεί ανθεκτικότητα
- Καθαρίζετε τα αγροτικά μηχανήματα και ειδικότερα τις αλωνιστικές μηχανές.
- Διατηρήστε τα περιθώρια των αγρών καθαρά από φυτά ήρας. Καταστρέψτε (χειρονακτικά) επιζώντα φυτά ήρας στον αγρό μετά την εφαρμογή ζιζανιοκτόνων πριν αυτά σποροποιήσουν
- Αφήστε αγρούς με ιστορικό ανθεκτικότητας της ήρας τελευταίους στη σειρά για καλλιέργεια
- Διατηρείτε αρχεία αγρού με τα ζιζανιοκτόνα που χρησιμοποιείτε, τις ημερομηνίες εφαρμογής τους και καταγράψτε το επίπεδο αποτελεσματικότητάς τους.
- Εφαρμόζετε συχνές επισκοπήσεις στον αγρό για επιζώντα φυτά ήρας
- Επικοινωνήστε με τοπικούς γεωπόνους ή άλλες υπηρεσίες που μπορούν να βοηθήσουν στον ανίχνευση πιθανών περιπτώσεων ανθεκτικότητας

Version in Italian

Introduzione

In Italia le principali specie del genere *Lolium* sono *L. rigidum* e *L. multiflorum*. La resistenza agli erbicidi è stata segnalata per la prima volta negli anni 90 con l'identificazione di popolazioni di *L. rigidum* resistenti agli inibitori ACCase nelle zone a monocoltura di cereali dell'Italia centrale e meridionale. Da allora, molte popolazioni di *L. rigidum* e *L. multiflorum* resistenti agli inibitori ACCase e ALS sono state confermate, anche nelle regioni settentrionali. Inoltre, popolazioni di *L. rigidum* resistenti al glifosate sono state identificate in oliveti noccioleti, frutteti e vigneti del sud e del nord Italia, mentre popolazioni di *L. multiflorum* resistenti al glifosate sono state ritrovate nel nord-est del paese in campi gestiti con la non lavorazione. Infine, popolazioni di *Lolium* con resistenza multipla a glifosate, ALS e ACCase sono state identificate in Toscana in aziende cerealicole, in cui l'evoluzione della resistenza era stata favorita dall'assenza di lavorazioni e dall'uso ripetuto del glifosate per la pulizia del letto di semina. Per quanto riguarda la gestione agronomica, gli agricoltori adottano rotazioni colturali che includono oltre al frumento mais e soia nelle regioni settentrionali, girasole e sorgo in quelle centrali; le scelte colturali sono dettate principalmente da ragioni economiche. Il colza era talvolta presente nella rotazione ma è praticamente scomparsa per la scarsa redditività. Nell'Italia meridionale il favino (*Vicia faba* var *minor*) viene normalmente inserito in rotazione con il grano duro ma questa soluzione non è efficacie per la gestione delle popolazioni resistenti di *Lolium* spp. perché i graminicidi utilizzabili nelle leguminose sono inibitori ACCase (quindi con lo stesso meccanismo di azione di quelli usati in frumento).

Nel caso dei sistemi di Agricoltura Conservativa (CA), *L. multiflorum* è spesso impiegato come cover crop autunnale, con terminazione mediante glifosate a metà Marzo prima della semina delle colture primaverili. Il glifosate viene inoltre utilizzato per eliminare *Lolium* spp. e le altre infestanti presenti anche nell'ambito di tecniche di falsa semina. L'aratura profonda (30-40 cm) viene utilizzata come strumento di controllo per interrare i semi di *Lolium* spp. negli appezzamenti interessati da casi di resistenza agli erbicidi.

Per quanto riguarda la gestione chimica della resistenza, gli agricoltori principalmente adottano la rotazione di inibitori ACCase e ALS. In caso di popolazioni già resistenti, gli agricoltori utilizzano applicazioni di pre-emergenza o post-precoce (applicazioni autunnali o invernali) di erbicidi con altri modi di azione (es. chlorotoluron), sia in aggiunta che in alternativa ai classici interventi di post-emergenza con gli inibitori ACCase o ALS.

Linee guida per L'Italia

Linee guida per la gestione di *Lolium* spp. nei cereali e per l'utilizzo di *L. multiflorum* come cover crop.

Metodi chimici

- Utilizzo di erbicidi di pre-emergenza o post-precoce efficaci contro *Lolium* spp. (http://www.fitosanitari.salute.gov.it/fitosanitariwsWeb_new/FitosanitariServlet) al posto degli inibitori ACCase o ALS nei campi di frumento dove è stata confermata la resistenza a questi erbicidi.
- Utilizzo di erbicidi di pre-emergenza o post-precoce. al posto o in aggiunta agli inibitori ACCase o ALS per evitare o ritardare l'evoluzione della resistenza
- Rotazione degli erbicidi (in particolare gli inibitori ALS e ACCase) e non usare lo stesso MOA ogni anno. In caso di resistenza confermata a un inibitore ALS o ACCase, interrompere l'uso di tale erbicida e passare agli altri erbicidi già menzionati.
- Utilizzare le dosi raccomandate nelle etichette degli erbicidi, non utilizzare dosi ridotte.
- Applicare gli erbicidi su infestanti al corretto stadio di sviluppo, nelle condizioni ambientali adeguate e utilizzando attrezzature appropriate.

Metodi colturali

- Rotazioni con colture con diverso ciclo colturale e diverse pratiche colturali per permettere la rotazione di erbicidi e di altri strumenti di controllo. Eventuali colture alternative sono il colza, le primaverili e le foraggere annuali o poliennali. Ricordarsi che colture in cui il controllo di *Lolium* spp. è basato su erbicidi con lo stesso MOA di quelli utilizzati nel grano non sono alternative efficaci per la gestione della resistenza agli erbicidi.
- Aumentare la frequenza di colture primaverili per interrompere il ciclo vitale del *Lolium* spp,
- Attraverso il ritardo delle semine, è possibile controllare il flusso iniziale di emergenza di *Lolium* spp. mediante l'utilizzo di glifosate, pirodiserbo o strumenti meccanici. Non adottare lavorazioni del suolo per evitare di riportare in superficie i semi interrati, stimolandone la germinazione.
- Adottare colture con una maggiore competitività o con caratteristiche allelopatiche (segale, orzo) al posto del frumento in zone in cui i cereali autunno-vernini sono le uniche colture praticabili
- Ricorrere a foraggere poliennali o cereali da insilare in modo che gli sfalci frequenti o la raccolta anticipata per l'insilamento impediscano la produzione di semi da parte di *Lolium* spp. Non utilizzare *L. multiflorum* come foraggera nelle zone con casi confermati di resistenza a ACCase, ALS o glifosate.
- Aumentare la dose di semina del grano (200 kg/ha) per aumentare la capacità competitiva della coltura nei confronti di *Lolium* spp.
- Ridurre l'uso di *L. multiflorum* come cover crop, anche nel caso di utilizzo in miscugli, soprattutto nel caso di non lavorazione del suolo.
- Utilizzare come cover crop cultivars di L. multiflorum con crescita più lenta e produzione di biomassa limitata.

Metodi meccanici

- Utilizzare l'aratura (30 cm) per interrare i semi di popolazioni resistenti di *Lolium* spp. per inibirne la germinazione e provocare la morte dei semi. Per evitare di riportare in superficie i semi interrati l'anno prima, l'aratura deve essere utilizzata ogni 3-4 anni.
- Nei sistemi di Agricoltura Conservativa sostituire il glifosate con metodi meccanici (trinciatura, roller crimper, erpicatura) per terminare le cover crops.

Pratiche preventive e ulteriori misure

- Evitare il trasporto di terreno e materiale vegetale o il movimento di bestiame dai campi infestati a quelli non infestati.

- Mantenere i bordi dei campi e le recinzioni liberi da piante di *Lolium* spp. Distruggere manualmente le macchie di *Lolium* spp. prima che disseminino.
- Rimuovere i semi o altri parti di piante dai macchinari, specialmente dalle mietitrebbie, per evitare la diffusione di semi di *Lolium* spp. resistenti. Nelle varie operazioni colturali lasciare per ultimi i campi con storia pregressa di resistenza agli erbicidi
- Mantenere indisturbate il più lungo possibile le stoppie dei cereali per aumentare la predazione dei semi e permettere l'emergenza di plantule e il loro successivo controllo con glifosate.
- Utilizzare sementi certificate non contaminate da semi di infestanti
- Tenere un registro dell'uso di erbicidi, date di applicazione e del livello di efficacia osservato. Monitorare i campi regolarmente per identificare eventuali piante di *Lolium* spp. sopravvissute ai diserbi.
- Contattare gli agronomi di zona o altri servizi per il supporto nell'identificazione di eventuali casi di resistenza.

Linee guida per la gestione di *Lolium* spp. in frutteti e vigneti

Metodi chimici

- Utilizzare altri erbicidi registrati per il controllo di *Lolium* spp. (http://www.fitosanitari.salute.gov.it/fitosanitariwsWeb_new/FitosanitariServlet) al posto del glifosate nei frutteti in cui la resistenza al glifosate è stata già confermata-
- Ruotare ogni 3 anni il glifosate con altri erbicidi registrati per evitare o rallentare l'evoluzione della resistenza da parte di *Lolium* spp.
- Utilizzare miscele di erbicidi (glifosate incluso) per evitare o ritardare l'evoluzione di resistenza..
- Utilizzare le dosi raccomandate nelle etichette degli erbicidi, non utilizzare dosi ridotte.
- Applicare gli erbicidi su infestanti al corretto stadio di sviluppo, nelle condizioni ambientali adeguate e utilizzando attrezzature appropriate.

Metodi colturali

- Utilizzare cover crops (es *Vicia sativa, Trifolium* spp*., Pisum sativum, Festuca* spp.) nell'interfila di frutteti e vigneti per contenere le infestanti
- Utilizzare la pacciamatura lungo il filare con teli plastici o materiali biodegredabili (corteccia, paglia) per inibire l'emergenza e la crescita di *Lolium* spp.

Metodi meccanici

- Utilizzare il controllo meccanico (lavorazioni superficiali fino 10 cm di profondità o sfalci) prima della disseminazione di *Lolium* spp., evitando l'aratura e erpicature pesanti in frutteti in collina
- Controllare con sfalcio meccanico o scerbatura manuale le eventuali macchie di *Lolium* spp. presenti prima che producano seme.

Pratiche preventive e ulteriori misure

- Evitare il trasporto di terreno e materiale vegetale o il movimento di bestiame dai campi infestati a quelli non infestati. Rimuovere i semi o altri parti di piante dai macchinari.
- Mantenere i bordi dei campi e le recinzioni liberi da piante di *Lolium* spp. Distruggere manualmente le macchie di *Lolium* spp. prima che disseminino.
- Nelle varie operazioni colturali lasciare per ultimi i campi con storia pregressa di resistenza agli erbicidi
- Tenere un registro dell'uso di erbicidi, date di applicazione e del livello di efficacia osservato. Monitorare i campi regolarmente per identificare eventuali piante di *Lolium* spp. sopravvissute ai diserbi.
- Contattare gli agronomi di zona o altri servizi per il supporto nell'identificazione di eventuali casi di resistenza.

Guidelines for Danmark

Italiensk rajgræs *(Lolium multiflorum)* og almindelig rajgræs (*L. perenne)* optræder som ukrudtsarter i Danmark. I øjeblikket er resistens hos disse arter ikke så udbredt som i Grækenland og Italien, men der er opmærksomhed på, at resistensproblemerne synes at øges. Det første tilfælde af resistens hos italiensk rajgræs blev fundet i 2010, og siden er der fundet en del populationer af denne art med resistens over for både ALS and ACCase hæmmere. Der er ikke fundet resistens over for glyphosat. Den lavere frekvens af resistens i Danmark end i de øvrige lande skyldes sandsynligvis, at andelen af vintersæd er lavere. Hovedparten af det dyrkede areal består af korn, græs, raps, sukkerroer, kartofler og bælgplanter. Vintersæd dækker ca. 32% af det dyrkede areal. Sædskifterne i Danmark er med tiden blevet mindre varierede, men arealet med vinterhvede i monokultur er kun ca. 5% af det dyrkede areal, mens 14% af arealet dyrkes med vinterhvede i sædskifte med vinterraps. Arealet med reduceret jordbearbejdning er øget i de seneste 10 år og omfatter i øjeblikket 15% af det dyrkede land.

Dækningsbidraget er den mest afgørende faktor for afgrødevalget, men på brug med husdyr har behovet for foder dog også stor betydning. De væsentligste dyrkningsmæssige tiltag til reduktion af problemer med rajgræs består i ændring af sædskiftet til en mindre andel af vintersæd. Sen såning kombineret med falsk såbed, hvor de fremspirede planter bekæmpes mekanisk eller med glyphosat, anbefales som en måde at reducere problemer med græsukrudt. ALS og ACCase hæmmere er de vigtigste herbicider til bekæmpelse af rajgræs. Ofte anvendes prosulfocarb før eller lige efter afgrødens fremspiring, og der følges op med ALS or ACCase hæmmere senere i vækstsæsonen. I tilfælde af resistens hos rajgræs anbefales ændringer af sædskiftet, som gør det muligt at anvende herbicider med andre virkemekanismer (f.eks. propyzamid i raps og mesotrion in majs).

Guidelines for håndtering af resistens hos rajgræs i Danmark er vist nedenfor.

Guidelines for Danmark

Guidelines for *Lolium* spp. management i vintersæd

Kemiske metoder

- Anvend herbicider med jordeffekt før eller lige efter fremspiring af afgrøden (https://middeldatabasen.dk/) i kombination med ALS eller ACCase hæmmere senere i vækstsæsonen for at forsinke resistensudvikling.
- Skift mellem ALS- og ACCase hæmmere og anvend ikke herbicider med samme virkemåde år efter år. Hvis der er konstateret resistens over for en bestemt gruppe herbicider, bør der skiftes til herbicider med en anden virkemekanisme.
- Brug doseringer, som er effektive over for de ukrudtsarter, som er tilstede.
- Anvend herbiciderne på det anbefalede vækststadie, under optimale klimaforhold og udbring dem med den rette sprøjteteknik.
- Ved reduceret jordbearbejdning bør glyphosat anvendes med omtanke for at forsinke resistensudvikling mod dette herbicid.

Dyrkningsmæssige metoder

- Anvend et sædskifte med afgrøder med forskellige livscyklus, som gør det muligt at skifte mellem herbicider og mekaniske bekæmpelsesmetoder. Alternative afgrøder kan være vinterraps, vårsæd og flerårige afgrøder (f.eks. græs til slæt, frøgræs).
- En øget andel af vårafgrøder i sædskiftet reducerer jordens frøbank af rajgræs.
- Ved sen såning kan de tidligt fremspirede rajgræsplanter bekæmpes med glyphosat. Undgå mekanisk bekæmpelse af planterne, da det kan bringe nye frø til spiring.
- Anvend afgrøder med god konkurrenceevne eller med allelopatiske egenskaber (f.eks. rug og byg) i marker, hvor det ikke er muligt at dyrke alternative afgrøder til korn.
- Dyrk flerårige foderafgrøder eller helsæd, som kan slås eller høstes tidligt, for at hindre, at rajgræsplanterne sætter frø. Dyrk ikke rajgræs som foder- eller efterafgrøde i områder, hvor der er fundet resistent rajgræs.
- Øg udsædsmængden for at styrke konkurrenceevnen over for rajgræs
- Braklæg marker med resistent rajgræs og bekæmp fremspirede planter mekanisk eller med glyphosat.

Mekaniske metoder

- Ved pløjning begraves frø af resistente planter i en dybde, hvorfra de ikke kan spire. For at undgå at bringe frøene op til overfladen bør dyb jordbearbejdning kun anvendes hvert 3.-4. år.
- Anvend mekaniske metoder i stedet for glyphosat til afslutning af efterafgrøder i CA systemer.

Forebyggelse og andre metoder

- Undgå at transportere jord og plantemateriale fra inficerede marker til marker uden resistens.
- Hold markkanter og hegn frie for rajgræs. Bekæmp pletter med rajgræs før de udvikler frø (håndlugning eller pletsprøjtning).
- Fjern frø og plantedele fra redskaber, specielt mejetærskere og halmpressere for at undgå spredning af frø af resistent rajgræs. Marker med resistent rajgræs bør jordbearbejdes og høstes sidst.
- Lad stubben være urørt så længe som muligt for at maksimere henfald som følge af predation og bekæmp fremspirede planter med glyphosat.
- Brug certificeret udsæd, som ikke er forurenet med ukrudtsfrø.
- Vær omhyggelig med sprøjtejournalen og tjek den opnåede effekt. Hold udkig i marken efter overlevende planter.
- Kontakt din planteavlskonsulent, hvis du har mistanke om resistens.

9. Guidelines for management of herbicide resistant *Lolium* used as a cover crop in conservation agriculture

C-IPM	**Coordinated Integrated Pest Management in Europe Grant agreement no.: 618110**
Full project title:	**Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies**
Project Acronym	**RELIUM**
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WP number:	**WP3**
Lead beneficiary:	**HAO-DEMETER**
Main author(s):	**Donato Loddo**
Delivery date:	
Actual delivery date:	

Executive Summary

The deliverable presents guidelines in English for the management of herbicide resistant *Lolium* spp. occurring in ryegrass cover crop in conservation agriculture. The guidelines are based on the results of studies examining the patterns and levels of resistance, the mechanisms providing resistance against EPSPs inhibitor, as well as on historical data of crop management in conservation agriculture.
These guidelines will be published on the web and will be available to the public free of charge.

Introduction

Cover crops are a pillar of Conservation Agriculture (CA) systems, since they increase soil fertility, reduce erosion, avoid nutrient leaching, and contribute to weed control in the intercropping periods. *Lolium multiflorum* Lam. (Italian ryegrass) is often used as autumn-sown cover crop in Italy for its early establishment and rapid growth, good nutrient uptake, and high competitiveness against weeds. Moreover, given that *L. multiflorum* is also a forage crop, several cultivars are easily available to farmers. Before the sowing of the subsequent spring crop, *L. multiflorum* cover crop should be terminated by glyphosate application or by mulching and soil incorporation (in the case of CA systems based on minimum tillage).Termination by glyphosate application can be a risky operation in the long term, considering that the *Lolium* genus is prone to evolve resistance to glyphosate. Many glyphosate resistant *Lolium* spp. populations have indeed been detected in Europe and worldwide. A single case of glyphosate resistant *Lolium* population evolved as consequence of cover crop termination has been reported in CA systems in Europe so far. This glyphosate resistant population (IT581) was detected in 2015 in North-Eastern Italy and was studied during the RELIUM project. However, given the increasing diffusion of CA and the repeated use of glyphosate in these cropping systems, further resistance cases will reasonably occur in the next years. Although glyphosate resistance in *Lolium* has been thoroughly investigated, there is a lack in information regarding its management in the case of CA. The specific objective of the Task 3.2 is to develop guidelines for management of glyphosate resistant *Lolium* occurring in cover crops in CA. Experimental results regarding level and mechanisms of resistance of the resistant population IT581, and historical field data on crop management provided useful information for the preparation of this specific guidelines.

Description of work

Historical field data

Population IT581 was detected in 2015 in North-Eastern Italy in fields managed according to no-till management for some years. Crop rotation included maize, silage barley (without herbicide application), and soybean and all crops were directly drilled. Diploid and tetraploid cultivars of *L. multiflorum* were repeatedly used over the years as autumn-winter cover crops and they were usually terminated by glyphosate application (1080-1440 g ae ha^{-1}) in mid-March before the sowing of spring crops. Starting from 2013-14, some plants of *L. multiflorum* were observed to survive to glyphosate application. This problem notably increased in spring 2015, when survivors to glyphosate were detected at high density on several hectares. Those survivors did not originate from the *L. multiflorum* sown as cover crop in autumn 2014. All plants belonging to the cover crop rows were indeed controlled by glyphosate and all survivors were localized in the inter-row, so they probably originated from seeds produced by the survivors observed in the previous years.

Experimental results in level of glyphosate resistance, and efficacy of alternative herbicides

Seeds were collected from plants of population IT581 surviving to field application of glyphosate. To evaluate the level of glyphosate resistance, whole plant experiments were conducted in a greenhouse. Response of population IT581 to different glyphosate doses (480, 1440 and 3600 g ae ha^{-1}) was assessed in comparison with a reference susceptible *L. multiflorum* population.

Sensitivity to other herbicides commonly adopted for *Lolium* control in cereals and legume crops was also investigated. In particular, ACCase inhibitors (cycloxydim, pinoxaden, and quizalofop) and ALS inhibitors (mesosulfuron-methyl + iodosulfuron-methyl-sodium) were tested.

While plants of the susceptible population were controlled by the lowest glyphosate dose (480 g ae ha^{-1}), high survival (>80% of treated plants) was observed for population IT581 at 480 and 1440 g ae ha^{-1}. Even at the highest glyphosate dose (3600 g ae ha^{-1}) 15-20% of the treated plants of population IT581 survived, confirming a high level of glyphosate resistance in this population. On the contrary, both populations were completely controlled by the other tested herbicides, proving that population IT581 was still susceptible to ALS and ACCase inhibitors.
To thoroughly evaluate the level of glyphosate resistance, population IT581 was included in a dose-response experiment with a susceptible reference population from Greece (G33). Both populations were treated with a range of glyphosate doses (0, 45, 90, 180, 360, 720, 1440, 2880, 5760 g ae ha^{-1}). Effective dose values based on plant survival (LD50) and biomass reduction (GR50) were calculated. Resistance indexes were estimated for IT581 as the ratio of the LD50 (GR50) of IT581 population/ LD50 (GR50) of the susceptible reference G33. Population IT581 showed more than 80% survival and only 40% of biomass reduction at the field recommended dose of glyphosate (720 g ae ha^{-1}), with 30% of survival even at the highest glyphosate dose (5760 g ae ha^{-1}). The RIs estimated for population IT581 were 10.5 and 5.9 on the base of LD50 or GR50, respectively. Given the high level of resistance, glyphosate is no longer effective for IT581 control under field conditions.

Characterization of the mechanisms for glyphosate resistance.
Population IT581 was investigated to identify the mechanism of glyphosate resistance. The partial EPSPS sequence of plants of IT581 did not reveal any mutation at Proline106. However, a silent mutation at Proline106 (codon CCG) was detected and also showed nucleotide polymorphism (silent mutations) at Ala109. Glyphosate resistance of IT581 populations is therefore not related to target site mechanisms.
The expression profiles of 4 ABC-transporter like genes at 3, 6 and 12 hours after glyphosate spraying showed that the gene with the highest upregulation was an ABC-type III transporter like gene (JZ166942.1) in the resistant IT581 plants compared to the plants of S-population (at 12 hours after spraying), thus this is a putative marker gene for rapid identification of glyphosate resistant plants. Biochemical tests for proving glyphosate resistance was done by shikimate analysis. The IT581 population showed lower level in shikimic acid as compared to the S-population. The results may indicate an early (some hours after herbicide application) sequestration of glyphosate possibly to vacuoles leading to limited translocation within the resistant plants.

Guidelines for the management of herbicide resistant Lolium spp
Two different guidelines have been prepared depending on the status of glyphosate resistance of *Lolium* spp. populations. In case of confirmed resistance, the aim of curative strategies is to find alternative tactics to ensure good weed control. This would prevent further spread and deplete the soil seed bank. In the vast majority of fields where no glyphosate resistance has been identified, proactive strategies should be adopted to prevent or to delay resistance evolution. This is mainly achievable by reducing the pressure of selection exerted by glyphosate use on local *Lolium* spp. populations and maintaining low weed densities.

Guidelines for curative strategies

Chemical methods

- No alternative herbicides are available for cover crop termination and herbicide application is not allowed during cover crop growth
- Chemical control of resistant *Lolium* should be performed in autumn sown crops (with ALS and ACCase inhibitors), adopting herbicide rotation and mixture to avoid evolution of resistance

Cultural methods

- Replace *Lolium* sp. with other cover crops
- Use competitive cover crops, such as white mustard, or species that tolerate mowing, such as clovers, to suppress growth of resistant *Lolium* in the autumn-winter period
- Increase frequency of autumn sown crops (winter cereals, winter oilseed rape) in the crop rotation to have more opportunities for selective chemical control of *Lolium*
- Adopt winter crops with allelopathic and competitive properties (eg. rye) that highly compete with *Lolium*
- Prefer silage to grain production for winter cereals to avoid *Lolium* dissemination

Mechanical methods

- If resistant *Lolium* plants are still present in small patches, perform hand-weeding before they set seeds
- If possible, adopt mechanical tools and shallow tillage (harrowing) to terminate cover crop instead of glyphosate application

Additional practices

- Avoid soil and plant material transportation and avoid movement of livestock from infested fields to clear fields
- Keep field fences and bunds free from weeds
- Remove seeds and plant parts from any machinery, especially harvesters to avoid spread of seeds of resistant *Lolium* spp.
- Leave last for any crop practices to apply the fields with history of confirmed *Lolium* spp. resistance

Guidelines for proactive strategies

Chemical methods

- No alternative herbicides are available for cover crop termination and herbicide application is not allowed during cover crop growth
- Follow herbicide use instructions and apply the recommended full rates. Low glyphosate doses can facilitate resistance evolution
- Anticipate application timing since glyphosate application in March usually provides better efficacy than in April, also due to the lower *Lolium* biomass.
- Monitor glyphosate efficacy

Cultural methods

- Reduce the use of *Lolium* sp. as cover crops, even in the case of mixtures with other species, especially in no-till conditions
- Select *Lolium* cultivars with slower growth and limited biomass production
- Use certified *Lolium* seeds to minimize the risk of introducing GR biotypes
- Use competitive cover crops, such as white mustard, or species that tolerate mowing, such as clovers, to suppress weeds in the autumn-winter period

Mechanical methods

- If possible, adopt mechanical tools and shallow tillage (harrowing) to terminate cover crop instead of glyphosate application
- In case of survivors following glyphosate treatment perform hand-weeding.

Additional practices

- Avoid soil and plant material transportation and avoid movement of livestock from infested fields to clear fields
- Keep field fences and bunds free from weeds
- Remove seeds and plant parts from any machinery, especially harvesters to avoid spread of seeds of *Lolium* spp.

Problems and obstacles associated with the adoption of these guidelines

Experiences on herbicide resistance management globally show that farmers deal with the problem after it occurs, not before. Proactive resistance management involves more diverse strategies to delay the evolution of herbicide resistant weeds but often these strategies are not economically attractive in the short term.

Lolium still represents a cheap and effective option as winter cover crops, farmers consequently are inclined to continue to use it. This issue should be addressed with a specific campaign of communication on the risk of using *Lolium* as cover crops under no-till conditions.

Regional programs for the support of Conservation Agriculture in Italy often establish higher financial supports for no-till than for minimum tillage. This discourage the adoption of tillage and promotes the use of glyphosate for cover crop termination, increasing the risk of resistance evolution.

Herbicide resistant *Lolium* spp. in climatically and agronomically diverse European countries: from developing quick and reliable detection tools to devising sustainable control strategies - RELIUM

In the European Union, the Directive 2009/128/EC on the sustainable use of pesticides makes it mandatory to implement the principles of Integrated Pest Management (IPM). To face this challenge, member states of the European Union has co-funded an initiative to coordinate Integrated Pest Management (C-IPM) through an ERA-NET funding programme under the Seventh Framework Programme on research and development.

The present report is the result of a project with participating institutes from and co-funded by parties from Italy Greece, and Denmark.

The project aimed at monitoring, mapping, developing innovative detection tools and characterizing (patterns, levels and resistance mechanisms) selected resistant populations as well as devising resistance management strategies for *Lolium* in various agronomic situations.

In Denmark, herbicide resistant *Lolium* is not as widespread as in Italy or Greece. However, several populations have been found to be resistant to both ALS and ACCase inhibitors. In contrast to Italy and Greece, only one mutation in the ALS gene was identified in the Danish populations, and it was present in only one population, indicating that the main resistance mechanism in Denmark is non-target site. Furthermore, no glyphosate resistance was found in Denmark.

The lower incidence of herbicide resistance in Denmark compared to Italy and Greece is probably attributed to a lesser use of winter annual crops and a higher use of crop rotation in Denmark.

Based on the results of this collaborative project, country specific guidelines to manage herbicide resistance *Lolium* have been developed, published, and distributed to grower associations.

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