

3 B.20 - GLYPHOSATE RESISTANCE OF TWO ITALIAN *LOLIUM* POPULATIONS

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Abstract: Greenhouse and laboratory experiments were conducted to investigate two *Lolium* spp. populations from a northern Italian vineyard (biotype "R-332") and a southern Italian olive grove (biotype "R-336") that were not controlled by the registered rates of glyphosate. The fraction of resistant (R) plants of each population was determined through screenings using two doses of glyphosate and three doses of ACCase inhibitors: cycloxydim and fluazifop. Shikimate accumulation at two doses was measured spectrophotometrically at 24, 48, 72, 96 and 216 hours after treatment. A quick germination dose-response test in petri dishes using glyphosate solutions at different concentrations was set up to select the R individuals within the population for further investigations. The R seedlings were discriminated on the basis of shoot and root development. The screening results confirmed resistance outlining the following profile for the northern R-332 biotype: 84% (Standard Error 1.4) and 56% (3.2) of survivors and 104% (6.6) and 43% (3.7) of fresh weight at lower (360 g a.e. ha⁻¹) and higher doses (1440 g a.e. ha⁻¹) respectively, referred to the untreated check. Southern R-336 biotype: 98% (0.25) and 88% (3.1) of survivors and 110% (2.9) and 60% (3.5) of fresh weight. Both ACCase inhibitors controlled the glyphosate resistant biotypes. The shikimate accumulation clearly discriminated the R populations from the susceptible (S-204) at the lower dose while at the maximum dose applied the northern population had an accumulation comparable to the S pop but R plants fully recovered in 21 days. The southern population did not accumulate shikimate as the S population neither at the higher level. The quick test outlined a concentration above 100 µM to discriminate S from R populations.

Keywords: shikimate, quick test, herbicide, selection, sustainability, evolution

INTRODUCTION

In Italy glyphosate is often used 2-4 times a year in perennial plantations to burn-down cover crops. Glyphosate kills plants by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs). EPSPs is a key enzyme in the shikimate biosynthetic pathway which is necessary for the production of aromatic amino acids, auxin, phytoalexins, folic acid, lignin, plastoquinones and many other secondary products (HERRMAN and WEAVER, 1999). For many years after its introduction glyphosate was considered as unaffected by resistance but after two decades the first resistant biotype was reported: in 1996 a *Lolium rigidum* population from Victoria, Australia, was confirmed to be resistant. According to what is reported in www.weedscience.org, *Lolium* spp. is the number one of the herbicide-resistant worst weed (HEAP, 2009). *Lolium* spp. is characterised by intra-species and intra-genus cross-breeding producing viable seeds. Potentially, it can spread resistance through its pollen for long distances (BUSI, 2008) frustrating those farmers that manage their fields according to the principles of the Good Agricultural Practices and the Integrated Pest Management.

In Italy *Lolium* is spread from north to south: usually the populations from north can be assigned to the species *multiflorum* while the one from south to *rigidum*. Intermediate forms are quite spread all over the country because of the high hybridisation typical for this genus. The aim of this study was to investigate two populations of *Lolium* spp. surviving the doses which normally control susceptible populations in northern vineyards and in southern olive groves. Greenhouse pot experiment, shikimate accumulation and dose response tests in petri dish were conducted. Greenhouse experiments gave an estimation of the fraction of individuals in the populations which survived the doses applied by farmers in the field. Shikimate accumulation during the first days after treatment gave an indication of the glyphosate injury and discriminated between susceptible and resistant plants. The dose response in Petri dishes was set up to identify the glyphosate concentration(s) which could discriminate between resistant and susceptible individuals within a population.

MATERIAL AND METHODS

Lolium spp. seeds were collected from plants surviving a glyphosate treatment. Two biotypes were investigated: one selected in vineyards from north Italy and the other in a southern olive orchard. The first biotype, referred to as R-332 can be ascribed mainly to *L. multiflorum* while the second, referred to as R-336 to *L. rigidum*. Seeds collected during May and June were cleaned, conserved in dry and dark conditions until late September, then they were vernalised for 4 days at 4 °C and finally placed in a germination cabinet with a photoperiod day/night of 12 hours and 25/15 °C respectively.

Two replication of 20 plants per pot were treated with the minimum and maximum doses of glyphosate reported on the label othe the Italian Roundup 450 formulation (360 and 1440 g a.e. ha⁻¹) at a water volume of 154 L ha⁻¹. One replication of 30 plants per dose was treated with two ACCase inhibitors at three doses (fluazifop, 47 - 94 and 156 g a.i. ha⁻¹ and cycloxydim, 75 - 150 and 250 g a.i. ha⁻¹), spray volume used was of 300 L ha⁻¹.

Shikimate accumulation analysis were done on 3 plants/pots, 3 replications, Plants were treated at 5-7 leave stage with a dose of 360 and 1080 g ae ha⁻¹ of glyphosate in a volume of 200 L of water. Fifty mg of plant tissue/replication was extracted at 1, 2, 3, 4 and 9 days after treatment. Shikimate was determined spectrophotometrically following the procedure of CROMARTIE and POLGE (2000).

The dose-response was done in Petri dishes (Ø 9 cm) with two replicates of 35 seeds. Each Petri dish contained two filter papers with 4 mL of glyphosate solution added. The 9 doses ranged between 10 and 1000 µM plus an untreated check. The experiment was repeated twice. Seedlings with developed roots were considered viable.

RESULTS AND DISCUSSION

The greenhouse pot experiment confirmed the resistance of *Lolium* populations R-332 and R-336 to glyphosate (Table 1). The minimum dose completely controlled the susceptible population, but the resistant populations were not killed. Biotype R-332 had a mortality close to 50 % while biotype R-336 showed a higher level of resistance with 88 % of survivors.

The ACCase inhibitor cycloxydim completely controlled all the biotypes at all the applied doses. Fluazifop controlled all biotypes at the minimum recommended field dose reported on commercial label (Fusilade 1.25 L ha⁻¹ corresponding to 156 g a.i. ha⁻¹), except for the population 332 at the minimum dose applied (0.375 L ha⁻¹). This biotype showed a lower mortality (69.9%, data not shown) which could reveal a lower susceptibility of the population, which needs to be taken into account when considering a rotation with herbicides of this mode of action. Further studies on the mechanisms of resistance are needed to evaluate the resistance differences between the two populations.

The shikimate levels outlined that the highest accumulation is observed 72 hours after treatment (HAT) in the susceptible population but at 96 HAT in the resistant ones (Figure 1). Population R-332 had a behaviour similar to the susceptible when treated at the higher dose (1080 g

a.e. ha⁻¹), but 67% of the treated plants survived at 21 DAT. Biotype R-336 had a full recovery 9 DAT even at the higher dose.

The Petri dish dose response outlined that the best way to discriminate susceptible from resistant population was a concentration above 100 μM. In order to develop a method that selects resistant from susceptible plants within a population and to have results related to realistic field doses, it might be better to use higher concentrations. Preliminary results indicate that the concentration has to be at least 250 μM. Further studies are needed to confirm this hypothesis.

Table 1. Survival and Fresh weight of *Lolium* biotypes referred to the untreated check (NT). Treatments at minimum and maximum dose. Standard errors in brackets.

biotype	Survival		Fresh weight	
	360 g a.e. ha ⁻¹	1440 g a.e. ha ⁻¹	360 g a.e. ha ⁻¹	1440 g a.e. ha ⁻¹
R-332	84.4 (1.42)	56.3 (3.24)	104.5 (6.56)	42.8 (3.68)
R-336	98.2 (0.25)	88 (3.13)	110.5 (2.88)	60 (3.45)

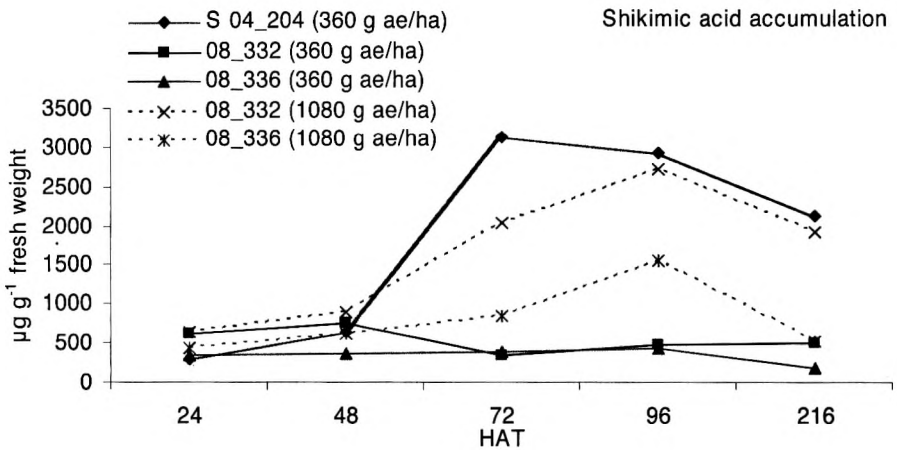


Figure 1. Shikimate accumulation measured as a function of time after treatment (HAT). Values are reported in μg of shikimic acid per mg of fresh tissue.

CONCLUSIONS

These preliminary investigations proved the capability of these two *Lolium* biotypes to survive glyphosate doses that completely controlled them in the past. This is the results of a continuous glyphosate use for weed control in this cropping system. The issue can be solved by diversifying weed control with different agronomical techniques like mowing, spray sequences and tank mixes of glyphosate with other herbicides like flazasulfuron and cycloxydim. Good Agricultural Practices and Integrated Pest Management need to be applied.

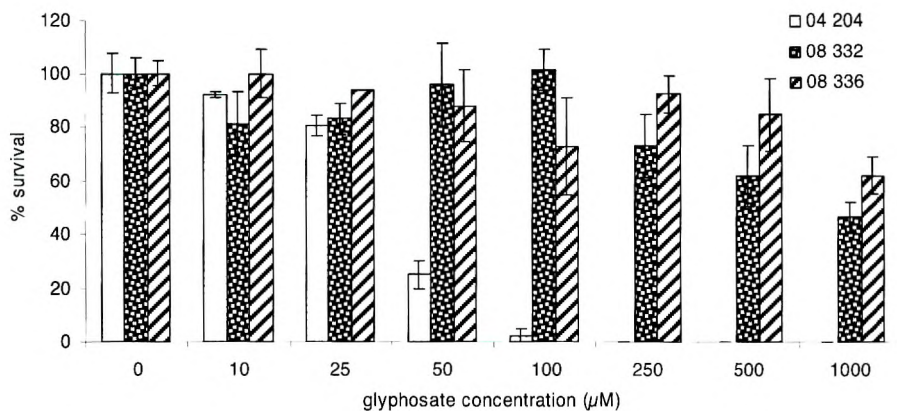


Figure 2. Percentage of survival referred to untreated control at different glyphosate concentrations.

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Resumen: Resistencia a glifosato en dos poblaciones de *lolium* spp recolectadas en Italia. Ensayos de de invernadero y laboratorion han sido realizados sobre dos biotipos de *Lolium* spp encontrados en Italia con el fin de conocer el nivel de resistencia a glifosato. El primero (biotipo R-332) fue recolectado en el Norte de Italia en un viñedo, mientras que el segundo (R-336) fue recolectado en el Sur de Italia en un campo de olivar, en ambos casos las plantas sobrevivieron a la aplicación de dosis usuales de glifosato en campo. Los ensayos realizados sobre plantas enteras (% sobrevivientes y % reducción de peso fresco con respecto control no tratado) mostraron que ambos biotipos tienen un alto nivel de resistencia, siendo mayor para el biotipo R-336 que para R-332. La acumulación de shikimato en plantas tratadas con 360 g a.e. ha⁻¹ de glifosato fue mayor para el biotipo sensible (S-204) que para los dos biotipos resistentes (R-332 y R-336), confirmando que el orden de resistencia es R-336 > R-332. La aplicación de cicloxidim y fluzafop sobre plantas enteras realizada sobre los tres biotipos (S-204, R-336, R-332) mostró una alta eficacia a diferentes dosis de campo. Este resultado nos induce a pensar que no existe múltiple resistencia a los herbicidas inhibidores de la EPSPS y a los inhibidores de la ACCasa.

Palabras claves: acumulación shikimato, test rapido, EPSPS, ACCasa, selección