Differences in metabolism of three *Conyza* species to herbicides glyphosate and triclopyr revealed by LC-MS/MS

Christos Anagnostopoulos¹, Panagiota Stasinopoulou², Panagiotis Kanatas³, and Ilias Travlos²*

¹Benaki Phytopathological Institute, 8 St. Delta str., GR-145 61 Kifissia (Athens), Greece.
²Agricultural University of Athens, 75, Iera Odos St., 11855 Athens, Greece. *Corresponding author (travlos@aua.gr).
³Agricultural Cooperative of Mesolonghi-Nafpaktia, 30200 Mesolonghi, Greece.

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**ABSTRACT**

*Conyza* spp. are among the most common weeds found in perennial crops, and resistance to several herbicides has already occurred worldwide. In the present study, pot and laboratory experiments were conducted to evaluate differences in glyphosate and triclopyr susceptibility between different biotypes of *Conyza* spp. Plant material was obtained from seeds collected in perennial crops in Greece. Seeds of four *Conyza* spp. biotypes were sown in separate pots and treated with glyphosate at the rosette stage, while seeds of six different *Conyza* spp. biotypes were sown and treated with triclopyr. Twenty-four hours after the herbicide treatment, the aboveground part of the weeds was manually collected and plant samples were prepared to determine the analytes with LC-MS/MS. In two *C. bonariensis* biotypes, the glyphosate concentration was 50% to 52% lower than for other biotypes; therefore, they could be considered as potentially resistant to glyphosate because the resistant biotype had a stronger and faster metabolism than the sensitive biotype, transforming glyphosate into non-toxic metabolites. The higher glyphosate concentration was detected in *C. canadensis* biotypes, which proved to be potentially susceptible to glyphosate. Three *C. bonariensis* biotypes and one *C. albida* biotype were less susceptible to triclopyr because analytes were detected in 63% to 82% higher concentrations than the two *C. canadensis* biotypes that were susceptible to triclopyr. These results reveal significant differences between the three species, confirm the problem of herbicide resistance, and suggest that glyphosate and triclopyr efficacy in *Conyza* spp. is merely due to herbicide metabolism to other compounds.

**Key words:** *Conyza* spp., glyphosate, herbicide resistance, LC-MS/MS, triclopyr.

**INTRODUCTION**

Different species of the genus *Conyza* are found in Europe that were introduced as neophytes from both North and South America. These invasive species are now considered to be among the most common plant species in the recipient territory. *Conyza* plants occur as weeds in more than 40 crops in 70 countries worldwide (Holm et al., 1997). *Conyza bonariensis*, *C. canadensis*, and *C. albida* (or *C. sumatrensis*) are the three main species of *Conyza* commonly found in perennial crops. *Conyza bonariensis* and *C. canadensis* are the most common and abundant of the three species, while *C. albida* was more recently introduced (Travlos and Chachalis, 2010). *Conyza* weed plants are considered highly adaptable, extremely competitive, and easily dispersed; they are particularly successful in extending herbicide resistance. They are often characterized by several ecological properties such as prolific seed formation, wide range of pollinating insects or self-fertilization, ability to outcross, short period from flowering to production of viable seed, non-specific habitat requirements, ease and distance of seed dispersal, long germination period, and low dormancy and viability in the soil for
several years (Hao et al., 2009). There are currently many reports from farmers that Conyza spp. have become increasingly difficult to control with several herbicides, especially in no-tillage or minimum tillage systems (Urbano et al., 2007; Travlos et al., 2009; Travlos and Chachalis, 2013).

Glyphosate (N-(phosphonomethyl)glycine) is a non-selective herbicide with little or no activity in the soil that acts as a potent inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) by blocking the synthesis of major compounds in the shikimic acid pathway, thereby instigating ultrastructural atrophy and arresting protein synthesis (Nandula et al., 2007). Glyphosate has been used extensively on both agricultural and non-agricultural land for more than 35 yr (Powles, 2008). No cases of resistant weeds were reported during the first years of use and the evolution of glyphosate resistance was considered unlikely (Bradshaw et al., 1997). To date, more than 30 weed species have reportedly developed resistance to glyphosate worldwide (Heap, 2019). Although its use in glyphosate-resistant crops has resulted in the evolution of weed resistance (Owen and Zelaya, 2005), cultivation of glyphosate-resistant crops cannot be considered as the only cause (Dill, 2005). Repeated use of glyphosate in no-tillage systems can greatly increase the risk of resistance, even in the absence of glyphosate-resistant crops (Neve et al., 2003; Travlos and Chachalis, 2010).

Triclopyr ([3,5,6-trichloro-2-pyridinyl]oxy]acetic acid) is a pyridine-based herbicide used to control woody plants and annual and perennial broadleaf weeds. It is absorbed through the leaves or roots and is translocated primarily through the symplastic pathway and accumulates in the growing points of the plant (Radosevich and Bayer, 1979). Its mechanism of action is not fully understood, but it appears to involve cell wall plasticity and nucleic acid metabolism (Ahrens, 1994). Triclopyr can damage plants due to root uptake but does not work effectively in that way (Newton et al., 1990). Triclopyr acts as a synthetic auxin by giving the plant an auxin overdose of 1000 times its natural levels, which disrupts the hormonal balance and interferes with growth. No research of the reasons for the species selectivity of triclopyr has been undertaken. One of the studies using cell suspension of soybean cultures showed that triclopyr acid was easily absorbed and metabolized. The major metabolic products were the aspartate and glutamate conjugates of triclopyr, which were not released from the cells (Lewer and Owen, 1987).

There are several plausible herbicide resistance mechanisms for weeds, including herbicide absorption (De Prado et al., 2005) and reduced herbicide movement from the site of absorption to the target site due to vacuolar herbicide sequestration (Ge et al., 2010; Yu et al., 2010). Rapid metabolic detoxification of the herbicide (De Prado et al., 2005; Busi et al., 2011), herbicide target site alteration (Powles and Yu, 2010), and gene amplification (Gaines et al., 2010) are other mechanisms.

The present study was conducted to evaluate several Conyza spp. biotypes in terms of their susceptibility to herbicides glyphosate and triclopyr. The specific objectives of this work were (a) to evaluate the different responses of three Conyza species to the herbicides glyphosate and triclopyr, and (b) to study the herbicide metabolism in R and S biotypes of Conyza spp. as a potential source of glyphosate and triclopyr resistance of this weed. The evaluation was performed in pot experiments and the analysis was performed by complex analytical techniques.

**MATERIALS AND METHODS**

**Pot experiments**

Conyza spp. seeds were collected from perennial crops in Greece. Seeds of four different biotypes of Conyza spp. (Conyza bonariensis [L.] Cronquist, C. canadensis [L.] Cronquist, C. albida Willd. ex Spreng., and C. sumatrensis (Retz.) E. Walker) were sown in separate pots to study weed susceptibility to glyphosate (samples 3, 4, 9, 10), while seeds of six different biotypes of Conyza spp. were sown in separate pots to study triclopyr (samples 2, 3, 4, 7, 9, 10) (Table 1). Seeds were placed on the substrate surface and watered. When seedlings were at the rosette stage (7 to 9 cm diameter and 10 to 15 leaves), the abovementioned Conyza spp. biotypes were treated with the recommended rates of glyphosate and triclopyr (0.72 kg ai ha⁻¹ and 0.96 kg ai ha⁻¹, respectively), using a custom built, compressed-air, low-pressure flat-fan nozzle experimental sprayer. Twenty-four hours after the herbicide treatment, the aboveground part of weeds was manually collected. The pot experiment was carried out and repeated in the field at the Agricultural University of Athens in the summer of 2016. The experiment had a completely randomized design with a split-plot arrangement, and biotypes were the main plots and herbicide treatments were the subplots. There was also an untreated control in which no herbicide was applied. Each pot was an independent sample.
Sample preparation to determine glyphosate
A simple and easy procedure was used to extract glyphosate from the plant samples. The protocol is based on the QuPPE2 method (quick polar pesticides method) developed by the European Reference Laboratory of Single Residue Methods (EURL-SRM) and described as follows. In a 50 mL Teflon centrifuge tube, 2 ± 0.02 g weed tissues was weighed and 10 mL water was added. The mixture was shaken by hand for 1 min to create slurry. Then, 10 mL MeOH 1%HCOOH was added and the mixture was shaken by hand for 1 min. The tube was capped and centrifuged at 4000 rpm for 5 min. An aliquot of the extract was filtered through a 0.45 μm syringe filter (cellulose) into a 2 mL autosampler vial (amber plastic), capped with a polytetrafluoroethylene (PTFE) cap, and placed in the freezer until injection.

Determination of glyphosate by LC-MS/MS
Glyphosate was determined using a combination of liquid chromatography (LC) and mass spectrometry (MS). An LC system was equipped with two pumps (ProStar 210; Varian, Palo Alto, California, USA) and an automatic sampler (ProStar 420) that was coupled with an electrospray ionization (ESI) source working in the positive mode and a triple quadrupole mass spectrometer (1200L, Varian) in the multiple reaction monitoring (MRM) mode. The transitions 170 > 60 and 170 > 88 were used for glyphosate. Separation was performed with a Hypercarb (P/N 35005-102130) column (100 mm × 2.1 mm × 5 μm), and the column temperature was 40 °C. The elution solvents were solvent A as H2O, 1%HCOOH and solvent B as MeOH 1%HCOOH. A gradient program was used that initially consisted of 100% solvent A held for 4 min (flow rate: 0.2 mL min⁻¹), and linearly ramped over 4 min to 90% solvent B. This composition was held for an additional 7 min before returning to the initial condition (flow rate: 0.4 mL min⁻¹). The column was re-equilibrated for 5 min at the initial mobile phase composition. Total runtime was 20 min and the injection volume was 10 μL. Before each injection, the autosampler was washed with a methanol:water (50:50) solution to prevent carry-over.

Sample preparation to determine triclopyr
Sample preparation was performed according to the description by Anagnostopoulos et al. (2012). In a 50 mL Teflon centrifuge tube, an aliquot of 10 ± 0.1 g weed tissues was weighed and 10 mL cold water (< 4 °C to compensate heat increase caused by adding salts) was added. The mixture was shaken by hand for 1 min to create slurry. Then, 10 mL acetonitrile was added and the mixture was shaken again by hand for 1 min. A mixture of the following salts was added: 4 g ± 0.2 g anhydrous magnesium sulphate, 1 g ± 0.05 g sodium chloride, 1 g ± 0.05 g trisodium citrate dihydrate, and 0.5 g ± 0.03 g disodium hydrogen citrate sesquihydrate. The tubes were immediately shaken by hand for 1 min and centrifuged for 5 min at 4000 rpm. An aliquot of the extract was filtered through a 0.45 μm syringe filter (PTFE) into a 2 mL autosampler vial and placed in the freezer until injection.

Determination of triclopyr with LC-MS/MS
Triclopyr was determined with a Varian LC system equipped with two pumps (ProStar 210) and an automatic sampler (ProStar 420) that was coupled with an electrospray ionization (ESI) source working in the negative mode and a triple quadrupole mass spectrometer (1200L, Varian) in the multiple reaction monitoring (MRM) mode. The transitions 256 > 220, 256 > 198, and 254 > 196 were used for triclopyr. Analytes were separated with an Atlantis C₁₈ column (150 mm × 2.1 mm × 3.5 μm), and the column temperature was 25 ± 4 °C. The elution solvents were solvent A as acetonitrile:H2O (10:90), 1 mM HCOONH₄, and 0.5% HCOOH and elution solvent B was acetonitrile:H₂O (90:10), 1 mM HCOONH₄, 0.5% HCOOH. A gradient program was used that initially consisted of 80% solvent A and 20% solvent B and ramped linearly over the course of 8 min to 95% solvent B. This composition was held for an additional 7 min before returning

| Code | Biotypes    | Treatment               |
|------|-------------|-------------------------|
| Sample 2 | Conyza bonariensis | Triclopyr               |
| Sample 3 | C. bonariensis             | Glyphosate, Triclopyr |
| Sample 4 | C. bonariensis             | Glyphosate, Triclopyr |
| Sample 7 | C. albida                 | Triclopyr               |
| Sample 9 | C. canadensis             | Glyphosate, Triclopyr |
| Sample 10 | C. canadensis           | Glyphosate, Triclopyr |

Table 1. Biotypes sprayed with maximum recommended rates of glyphosate and triclopyr.
to the initial condition. The column was re-equilibrated for 10 min at the initial mobile phase composition. The flow rate was stable at 0.2 mL min\(^{-1}\) and total runtime was 25 min. The injection volume was 5 μL and the autosampler was purged with a mixture of methanol:water (50:50 v/v) before sample injection to prevent carry-over.

**Identification and quantification procedure**

For both methods, quality control samples were performed to ensure validity of the results. Analytes were identified according to EU guidelines (European Commission, 2017) using retention times (RT) and ion ratio. The presence of an analyte in the sample was identified by the presence of a chromatographic peak at the time close to the RT of the reference standard (± 0.1 min) and the ratio of the two ion transitions should not deviate by more than 30% compared with the reference standard.

The analyte concentration (mg kg\(^{-1}\)) in the sample was performed using single point calibration or bracketing. When using single point calibration, the surface area of the chromatographic peak of the sample is compared with the surface of the chromatographic peak of the reference standard when the surfaces of the sample peaks and standard solution do not differ by more than 50%. When using bracketing, a comparison is made using two reference standards. The analyte concentration in the sample should be between the concentrations of the standards.

**RESULTS AND DISCUSSION**

The sample residue analysis identified significant differences in the glyphosate concentration. The presence of glyphosate in the sample was recognized by the presence of the chromatographic peak over time near the RT of the reference standard (± 0.1 min) (Figure 1). The highest concentration of glyphosate was detected in samples 10 and 9, while both compounds were determined at lower concentrations in samples 3 and 4 (Table 2). In accordance with González-Torralva et al. (2012),

![Figure 1. Chromatograms of sample 9 (Conyza canadensis) for the glyphosate transitions (170 > 60, 170 > 88).](image-url)
the resistant biotype has a stronger and faster metabolism than the sensitive biotype, transforming glyphosate into the aminomethylphosphonic acid (AMPA), glyoxylate, and sarcosine metabolites, thus reducing the amount of intracellular glyphosate that reaches the target enzyme EPSPS. The non-target-site-based mechanisms have been reported most frequently in glyphosate-resistant *C. canadensis* and *C. bonariensis* weed biotypes (Powles and Preston, 2006). Based on the results, samples 3 and 4 of *C. bonariensis* were potentially resistant to the herbicide glyphosate, whereas samples 9 and 10 (*C. canadensis*) were potentially susceptible to glyphosate; our findings completely concur with previous studies (Travlos et al., 2009; Travlos and Chachalis, 2010; 2013). These results indicated that *C. bonariensis* biotypes have a stronger metabolism than *C. canadensis* biotypes, transforming glyphosate into non-toxic metabolites. Based on some reports (Dinelli et al., 2006; Shrestha et al., 2007; VanGessel et al., 2009), *C. canadensis* is more susceptible at the rosette stage than at later growth stages.

Glyphosate degradation and transformation have been examined in previous studies as potential glyphosate resistance mechanisms (Lorraine-Colwill et al., 2002; Feng et al., 2004; Dinelli et al., 2006). Glyphosate is enzymatically metabolized to other nontoxic compounds such as glyoxylate, sarcosine, and formaldehyde (Rueppl et al., 1977), and a mildly toxic compound, AMPA. Our results confirm the involvement of glyphosate metabolism in the resistant biotype. Glyphosate-resistant *Digitaria insularis* biotypes have shown their ability to degrade glyphosate into AMPA, glyoxylate, and sarcosine. Furthermore, other authors working with glyphosate-tolerant plants (e.g., *Ipomoea purpurea*, *Convolvulus arvensis*, and *Neonotonia wightii*) have demonstrated the ability of these plants to metabolize glyphosate into AMPA, sarcosine, and glycine (Sprankle et al., 1978; Sandberg et al., 1980; Cruz-Hipolito et al., 2011).

Our results also revealed significantly different concentrations of triclopyr. The presence of triclopyr in the sample was recognized by the presence of the chromatographic peak over time near the RT of the reference standard (± 0.1 min) (Figure 2). The highest concentration of triclopyr was detected in samples 10 and 9, while both analytes were determined at lower concentrations in samples 2, 3, 4, and 7 (Table 3). Non-target-site resistance involves mechanisms that minimize the amount of active herbicide reaching the target site (Yu and Powles, 2014). Specifically, non-target-site resistance mechanisms to the synthetic auxin herbicides, such as triclopyr, have frequently been associated with their metabolism by enzymes into harmless products. The major route for deactivating the endogenous auxin, indole-3-acetic acid (IAA), is by conjugation. The enzymes responsible for conjugating IAA can be considered as candidates for deactivating synthetic auxin herbicides and a potential source for resistance (Busi et al., 2018). Despite the frequent and repeated use of auxin herbicides, there are few reports of auxin herbicide-resistant weed biotypes that have been selected for use under these conditions that appear to be ideal for the evolution of resistance (Mithila et al., 2011). Based on the results, samples 2, 3, and 4 (*C. bonariensis*) and sample 7 (*C. albida*) were less susceptible to the herbicide triclopyr, whereas samples 9 and 10 (*C. canadensis*) were susceptible to triclopyr.

| Code   | Biotypes        | Findings  | mg kg⁻¹ |
|--------|-----------------|-----------|---------|
| Sample 2 | *Conyza bonariensis* | Triclopyr | 14.00   |
| Sample 3 | *C. bonariensis* | Triclopyr | 29.70   |
| Sample 4 | *C. bonariensis* | Triclopyr | 16.30   |
| Sample 7 | *C. albida*    | Triclopyr | 22.00   |
| Sample 9 | *C. canadensis* | Triclopyr | 75.90   |
| Sample 10 | *C. canadensis* | Triclopyr | 44.00   |

Table 3. Concentration of glyphosate residues found in samples 2, 3, 4, 9, and 10.
CONCLUSIONS

To date, available information is limited in the published literature on the role of the metabolism of glyphosate associated with glyphosate resistance in *Conyza* spp. This study is probably among the first to examine the response of three different species of *Conyza* to the herbicides glyphosate and triclopyr with complex analytical techniques (LC-MS/MS). As such, this study provides some potentially useful information for the quick and accurate biotype screening of the three *Conyza* species. *Conyza* spp. seem to have developed resistant biotypes in glyphosate and this resistance means that there are now fewer herbicidal control agents. Furthermore, *Conyza* spp. biotypes have been recorded as resistant to synthetic auxin herbicides. To determine the range of the resistant biotypes and better understand the biology and ecology of resistant and susceptible biotypes is essential for developing effective long-term integrated management strategies.

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