Comparison of premix glyphosate and 2,4-D formulation and direct tank mixture for control of *Conyza canadensis* and *Epilobium ciliatum*

Candelario Palma-Bautista, Hugo E. Cruz-Hipolito, Ricardo Alcántara-de la Cruz,
José G. Vázquez-García, Marcos Yannicari, Rafael De Prado

PII: S0269-7491(21)00595-9

DOI: https://doi.org/10.1016/j.envpol.2021.117013

Reference: ENPO 117013

To appear in: *Environmental Pollution*

Received Date: 31 January 2021

Revised Date: 18 March 2021

Accepted Date: 21 March 2021

Please cite this article as: Palma-Bautista, C., Cruz-Hipolito, H.E., Alcántara-de la Cruz, R., Vázquez-García, J.G., Yannicari, M., De Prado, R., Comparison of premix glyphosate and 2,4-D formulation and direct tank mixture for control of *Conyza canadensis* and *Epilobium ciliatum*, *Environmental Pollution*, https://doi.org/10.1016/j.envpol.2021.117013.

Author Contribution Statement

HECH and RP: Conceptualization;

CPB, RAC, JGVG and MY: Investigation, Collection and analysis of the data, and validation;

CPB, HECH, RAC, JGVG, MY and RP: writing–original draft and –review and editing;

RP: Resources, supervision and funding acquisition.

Glyphosate + 2,4-D to control glyphosate resistant/tolerant broadleaf weeds

PREMIX		TANK MIX
✓	2,4-D suppressed glyphosate resistance/tolerance	✓
✓	herbicide retention	✗
✓	herbicide absorption and translocation	✗
Very good	weed control in the field	**Good**
✓	split applications prolonged weed control in the field	✓

Comparison of premix glyphosate and 2,4-D formulation and direct tank mixture for control of *Conyza canadensis* and *Epilobium ciliatum*

Candelario Palma-Bautista[1], Hugo E. Cruz-Hipolito[1], Ricardo Alcántara-de la Cruz[2]*, José G. Vázquez-García[1], Marcos Yannicari[3], Rafael De Prado[1]

[1]Department of Agricultural Chemistry, Edaphology and Microbiology, University of Cordoba, 14071 Cordoba, Spain

[2]Departamento de Química, Universidade Federal de São Carlos, São Carlos 13565-905, Brazil

[3]Chacra Experimental Integrada Barrow (MAIBA—INTA), National Scientific and Technical Research Council, Tres Arroyos 7500, Argentina

Corresponding author: ricardo.cruz@ufscar.br (RAC)

ABSTRACT

Premix or tank mix of glyphosate and 2,4-D are a good alternative to control glyphosate-resistant and -tolerant weeds; however, the combination of herbicides may increase the environmental impacts, since herbicide mixtures often have higher toxicity than single herbicide. In addition, antagonism between these herbicides has also been reported. We compared the efficacy of a premix glyphosate+2,4-D formulation with respect to the tank mix of both herbicides on glyphosate-resistant *Conyza canadensis* and -tolerant *Epilobium ciliatum* populations in laboratory and field experiments. 2,4-D suppressed the glyphosate-resistance/tolerance in both species, whose populations presented similar responses to their susceptible counterparts ($LD_{50} \geq 480+320$ g ha^{-1} glyphosate + 2,4-D, respectively). Premix treated plants of both species retained ~100-µL more herbicide, accumulated 20-25% and 28-38% more shikimate and ethylene, respectively, and greater ^{14}C-glyphosate absorption and translocation, depending on the species, compared to tank mix treated plants. Although doubling the field dose (720+480 g ha^{-1}) slightly improved (5-22%) the control of these weeds in the field, split applications of the premix formulation provided the best control (≤80%) for longer (120-d). No antagonism between glyphosate and 2,4-D was found. The addition of 2,4-D controlled both broadleaf species. For all parameters evaluated on the *C. canadensis* and *E. ciliatum* populations in the laboratory and in the field, the premix treatments showed better performance than the tank mix treatments. Premix formulations could reduce the environmental impact of herbicides used to control glyphosate resistant/tolerant weeds by decreasing the herbicide amount needed to achieve an acceptable weed control level.

Keywords: environmental impact; glyphosate resistance; herbicide tolerance; sequential applications; synthetic auxins

INTRODUCTION

Perennial crops in European Mediterranean areas include mainly olive, vineyard, citrus and stone and pip fruit trees. According to Eurostat data (Eurostat, 2020), around 6% of the European agricultural area was covered with perennial crops, which correspond to 11 million hectares in 2016. Spain (4,830,000 ha) and Italy (2,372,910 ha) have been the most important member countries of the EU-28 Mediterranean Region in terms of perennial crops (Eurostat, 2020).

Farmers invest keep crops free of pests, diseases and weeds to obtain high yields and high-quality products (Möhring et al. 2020). The most widely used weed control method is the application of herbicides at different times of the crop cycle (Kudsk and Mathiassen, 2020), with glyphosate being the main herbicide used in perennial crops for this purpose since its introduction in 1974 (Franz et al. 1997; Duke et al. 2018). Glyphosate is a foliar, systemic and broad-spectrum herbicide that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) causing the shikimate accumulation (Steinrücken and Amrhein, 1980). However, the continuous use of this herbicide, sometimes more than two applications a year in the same crop, has exerted a high selection pressure on the flora, causing the appearance of glyphosate-resistant and/or -tolerant weeds (Heap, 2020).

Acquired resistance to glyphosate is provided by target-site resistance (TSR) and non-target site resistance (NTSR) mechanisms (Sammons and Gaines, 2014; Gaines et al. 2019). NTSR mechanisms are caused, for example, by reduced absorption, impaired translocation, vacuolar sequestration and/or metabolism into non-toxic compounds (Ge et al. 2010; González-Torralva et al. 2010; Pan et al. 2019). The TSR mechanisms are caused by the increased expression of the target protein or structural changes in the herbicide-binding site (Gherekhloo et al. 2017; Gaines et al. 2019). On the other hand, glyphosate tolerant usually involves NTSR mechanisms (Rojano-Delgado et al. 2012). *Conyza canadensis* and *Epilobium ciliatum* are broadleaf weeds that have been confirmed as resistant and tolerant to glyphosate in different European countries, conferred by both TSR and/or NTSR mechanisms (Amaro-Blanco et al. 2018; Palma-Bautista et al. 2018; Tahmasebi et al. 2018).

Conyza canadensis is an annual Asteraceae weed found across temperate to tropical regions, that may have biannual habit, producing a large quantity wind-dispersed seeds, which has been found with resistance to glyphosate in Europe (Amaro-Blanco et al. 2018; Heap, 2020). *Epilobium ciliatum* is an Onagraceae perennial weed native to Central and North America that has been introduced to Europe, Australia and New Zealand, where it is spreading rapidly by producing large numbers of wind-dispersed seeds as *C. canadensis* (Mansanet-Salvador et al. 2014). *Epilobium ciliatum* was characterized as tolerant to glyphosate as it was observed that plants treated with six leaves were able to regrowth two weeks after treatment (Tahmasebi et al. 2018). Both broadleaf weeds present a major control Concern in Southern Spain and Northern Portugal (Fernández-Alonso et al. 2012), where thousands of hectares of orchard and almond are involved. Herbicide combination, premixed or tank mixed, has become a common practice to control glyphosate resistant/tolerant weeds (Beckie and Reboud, 2009; Amaro-Blanco et al. 2018).

One of the combinations most used by farmers to control glyphosate resistant/tolerant broadleaf weeds is glyphosate with 2,4-D (Franz et al. 1997; Wehtje and Gilliam, 2012). The last herbicide is a weak acid belonging to the synthetic auxins that is quite compatible with glyphosate. Mixing these herbicides generally produces a synergistic control of broadleaf weeds that is well documented (Gressel, 1993). However, punctual cases of antagonism have also been informed (Wehtje and Gilliam, 2012; Robinson et al. 2012; Li et al. 2020), reporting a reduction in glyphosate absorption and translocation as consequence of the incompatibility between these herbicides (Grossmann, 2010; Jugulam et al. 2011; Ganie and Jhala, 2017; Li et al. 2020). In addition, mixing multiple pesticides may increase the environmental impact (Choung et al. 2013; Sjollema et al. 2014), since the toxicity of mixtures is often greater than the toxicity of a single herbicide (Tang and Escher, 2014).

Adverse effects (pollutants or toxic to non-target organisms) of herbicide mixtures with a similar chemical class or mode of action can be predictable, however, the pesticide combination of different chemical class they are unpredictable and can be reductives, neutrals, additives or synergistics (Magnusson et al. 2010; Tang and Escher, 2014). In order to avoid these problems, some pesticide manufacturers have developed formulations that combine optimal bioactivity with smaller amounts of active ingredients than those of four individual formulations, but that at the same time have less environmental impact (Spaunhorst and Johnson, 2017; Busi and Beckie, 2020). Additionally, divided herbicide applications at reduced rates can also effectively control weeds (Lockhart and Howatt, 2004; Svobodová et al. 2018).

Although glyphosate and 2,4-D are widely used herbicides for weed control in perennial crops, the differences between the application of trade formulations (premix) or tank mix of these herbicides is unknown. Therefore, the aims of this study were: (a) to evaluate the efficacy of premix compared to tank mix treatments of glyphosate + 2,4-D on glyphosate-resistant (R) and -susceptible (S) *C. canadensis* populations from Spain, and *E. ciliatum* collected from a field never treated (NT) with glyphosate and from a field treated (T) with glyphosate in the last four years from Portugal in greenhouse; (b) to compare shikimic acid accumulation (in response to glyphosate), ethylene accumulation (in response to 2,4-D), foliar retention and ^{14}C-glyphosate absorption and translocation patterns in both species and populations; and (c) compare the efficacy of both premix and tank mix in two different fields at different application times and rates to determine the most environmentally friendly application form.

MATERIAL AND METHODS

Chemical

The trade formulation Kyleo® (240 g ae L^{-1} glyphosate as isopropylamine salt + 160 g ai L^{-1} 2,4-D acid (alkylamidopropyl and dimethylamine salts)) was used for the premix treatments, and Clinic® (360 g ae L^{-1} of glyphosate as salt isopropylamine) and U-46 D Complet® (600 g ai L^{-1} of 2,4-D as dimethylamine salt) were used for the tank mix treatments. The three trade formulations were purchased form Nufarm, España S.A. (Barcelona, Spain). ^{14}C-glyphosate (glycine-2-^{14}C), with a radiochemical purity of 95% and specific activity 273.8 MBq mmol^{-1}, was obtained from the Institute of Isotopes Co., Ltd. (Budapest, Hungary).

Plant material

Four populations of *C. canadensis* and *E. ciliatum,* two of each species, were used in this study (**Table 1**). One population of *C. canadensis* resistant (R) to glyphosate was collected in olive orchards (~15 years using 1080 g ae ha^{-1}) of the southern Spain (Sevilla), and one susceptible (S) population was collected in a new olive organic plantation (Amaro-Blanco et al. 2018). For *E. ciliatum*, a population (referred to as T) was collected in three almond plantations in Alentejo, northern Portugal, which had a history of glyphosate application of 4 years (720 g ae ha^{-1}); and the second population (referred to as NT), that was never exposed to glyphosate, was collected in the experimental field of the University of Córdoba (Spain). Mature seeds of the different population were randomly collected in 50 m^{2} from ~25 plants in different fields of southern Spain and northern Portugal in the summer of 2017.

Seeds were cleaned and conditioned for germination in 663 cm^{3} trays filled with peat and were covered with transparent film until emergence. Trays were placed in a growth chamber at 28/18 ºC (day/night), 16-h photoperiod, 850 µmol m^{-2} s^{-1} irradiance, and 80% relative humidity. The seedlings were transplanted individually into pots containing sand/peat in a 1:2 (*v/v*) ratio and placed in a greenhouse at 28/18 ºC (day/night) with a 16 h photoperiod. The *C. canadensis* and *E. ciliatum* plants used in the different laboratory experiments had 6-8 true leaves (BBCH 16-18).

Dose response bioassays

Premix or tank mix glyphosate + 2,4-D treatments on the R and S *C. canadensis* and T and NT *E. ciliatum* plants were performed in a laboratory chamber sprayer (SBS-6010 De Vries Manufacturing, Hollandale, MN, USA), equipped with an 8002 flat fan nozzle (TeeJet® Spraying System Spain, S.L., Madrid, Spain) at a pressure of 250 kPa and calibrated to deliver 200 L ha^{-1} at a height of 50 cm. The glyphosate + 2,4-D doses tested were: 0, X/64, X/32, X/16, X/8, X/4, X/2 and X, based on the minimum field dose (X= 3 L ha^{-1}, equivalent to 720 g ae of glyphosate + 480 g ai of 2,4-D) of Kyleo® trade premix formulation or its equivalents in g ha^{-1} of Clinic® and U-46 D Complet® for tank mix treatments. Zero (0) were the untreated plants used as control. Plant mortality and dry weight reduction (plant tissue dried at 60 °C for 72 h) were evaluated 21 days after treatment (DAT). Plant mortality (LD_{50}) and dry weight reduction (GR_{50}) at 50% were estimated by subjecting the percentage data of these parameters with respect to the control to nonlinear regression analysis (three-parameter function). Ten plants of each populations were treated per herbicide dose and the experiments were repeated twice.

Accumulation of shikimic acid and ethylene

For these experiments, sets of plants of the R and S *C. canadensis* and T and NT *E. ciliatum* populations were sprayed separately with the premix or the tank mix treatment of glyphosate + 2,4-D (240 + 160 g/ha, respectively) using the same media as those used for the dose-response assays.

Glyphosate-induced accumulation of shikimic acid: 50-mg samples of treated and non-treated plant tissue were taken at 96 h after treatment (HAT), frozen in liquid N_2, and stored at -40 °C until analysis. Accumulation of shikimic acid was determined according to González-Torralva et al. (2010). Results were expressed in mg g^{-1} fresh weight.

2,4-D-induced ethylene accumulation: 400-mg samples of leaf tissue were harvested at 24 HAT and placed in a 10-mL syringes with 1 mL of distilled water and sealed. Syringes were incubated at 27°C in the dark for 4 hours. The ethylene (C_2H_4) in 1 mL of the gas at the top of the syringe was analyzed by gas chromatography (Palma-Bautista et al. 2020). The C_2H_4 content was expressed as nL g^{-1} fresh weight h^{-1}.

Both experiments had a random design with five premix or tank-mix treated and non-treated samples per population/species with three technical replicates.

Glyphosate + 2,4-D foliar retention in premix and tank mix

The plants T and NT of *E. ciliatum* and R and S of *C. canadensis* were also treated with 240 + 160 g ha^{-1} (glyphosate + 2,4-D) in both premix and tank mix treatments. Before application, 100 mg L^{-1} Na-fluorescein, used as a colorimetric labeling reagent, was added to the herbicide solutions. Once the plants were treated, it waited for the herbicide solution on the foliage to dry. Then, plants were cut off at ground level and immersed in 50-mL of 5 mM NaOH and the samples were vigorously shaken for 30 s. Fluorescence of the rinse solutions was measured in a spectrofluorometer at 490/510 nm. Plants were dry-oven at 60 °C for 72 h and weighted. Foliar retention was expressed in µL of herbicide solution g^{-1} dry weight. Ten replications were used for each treatment and species and the experiments were repeated twice.

^{14}C-glyphosate absorption, translocation and visualization in plants

The premix and tank mix treatments of glyphosate + 2,4-D, which were radiolabeled only with ^{14}C-glyphosate, were compared with isolated ^{14}C-glyphosate application. The three radiolabeled solutions were prepared at a specific activity of 0.834 kBq µL^{-1}(González-Torralva et al. 2010). This concentration corresponded to 240 + 160 g ai ha^{-1} of glyphosate + 2,4-D, respectively for the premix and tank mix treatment and for the isolated application 240 g ai ha^{-1} of commercial glyphosate and an application volume of 200 L ha^{-1}. R and S *Conyza canadensis* and NT and T *E. ciliatum* plants received one-µL drop (0.834 kBq) on the adaxial surface of the second leaf of the plants using a micropipette (LabMate + HTL). Unabsorbed ^{14}C-glyphosate was removed by washing three times the treated leaf with 1 mL of water–acetone (1:1, v/v) each time at 48 HAT. Plants were then sectioned into treated leaf, rest of the plant, and roots, and subsequently placed in cellulose cones. The resulting rinsate of each wash was mixed with 3 mL of scintillation liquid and analyzed by liquid scintillation spectrometry (LSS) for 10 min per sample. Plant tissues were dried at 60 ◦C for 72 h and combusted in a Packard Tri Carb 307 biological sample oxidizer during 3 min. $^{14}CO_2$ from combustion were trapped into 18 mL Carbo-Sorb E and Permafluor (1:1, v/v; Perkin-Elmer, Packard Bioscience BV) and measured by LSS (10 min sample^{-1}). The percentages of ^{14}C-glyphosate recovered, absorbed, and translocated were calculated using the radioactive values in disintegration per minute (dpm). The equipment efficiency correction factor was calculated to be between 92-95%. The experiment was arranged in a completely randomized design with 4 repetitions per herbicide application form (premix or tank mix) for each population.

^{14}C-glyphosate movement was visualized in *C. canadensis* and *E. ciliatum* plants that were treated and removed from pots at the same time, as described in the absorption and translocation assays. Whole plants were gently rinsed, pressed on paper filter and dried at room temperature for one week. Then, plants were pressed for 4 h under a phosphor store film and radioactivity distribution was scanned using a phosphor imager Cyclone (Perkin-Elmer, Packard BioScience BV, MA, USA). The experiment was carried out with three plants per biotype.

Field trials

Two field trials were carried out on olive and almond farms. Trial 1 was an olive grove located in southern Spain (37°46'49.7''N, 5°00'46.2''W) with infestation of *C. canadensis* characterized as resistant to glyphosate (Amaro-Blanco et al. 2018). Trial 2 was established on an almond farm in northern Portugal (38° 03'51.7''N, 7° 48'28.4''W) and the control target weed was *E. ciliatum*.

The experiments were carried out for two consecutive seasons (2018 and 2019). Six different premix and tank mix treatments of glyphosate + 2,4-D were evaluated in single and split applications (**Table 2**). Single herbicide applications, premix or tank mix, and the first split application were made in early March when the *C. canadensis* plants were in the rosette stage (BBCH 16-18) and those of *E. ciliatum* had 3 to 6 true leaves. The second split application was made after 60 days. Herbicide treatments were made with a Pulvexper spray backpack, at a pressure of 200 KPa, equipped with four-flat fan nozzles 11002, calibrated to deliver 250 L ha^{-1}. In each case, the six treatments plus one untreated control were arranged in a randomized complete block design with four replications, where each experimental unit was a 4 x 5 m plot that included a row of trees. Visual control evaluations, rating from 0 to 100%, were performed at 15, 30, 60, 90 and 120 days after treatment (DAT), where 0% corresponded to a null control and 100% to a total control. An additional evaluation at 90 DAT was performed for the split treatments considering that the second application was made 30 DAT of the first.

Statistical analyses

All tests of significance were reported using ANOVA followed by the Tukey´s test (p≤0.05). In addition, Student's t test was performed to compare in pairs between populations of a species that received the same treatment. Statistical analyses were performed using Statistix 9 (Analytical Software, USA) and plotted using Sigma Plot 11.0 (Systat Software Inc., USA).

RESULTS

Dose response assays

The R and T populations of *C. canadensis* and *E. ciliatum* showed a similar response to glyphosate + 2,4-D that their counterparts S and NT, respectively. The premix treatment was at least 3-fold more effective in reducing the dry weight by 50%

in all populations than tank mix. The GR_{50} of these populations, regardless of glyphosate resistance/tolerance status, ranged from 30+20 to 45+30 g ha^{-1} of glyphosate + 2,4-D in premix, and from 90+60 to 156+104 g ha^{-1} in the tank mix treatments. Based on plant mortality, these differences were not so pronounced and the LD_{50} values ranged from 368+253 to 390+260 g ha^{-1} of glyphosate + 2,4-D for premix treatments, and from 405+270 to 480+320 g ha^{-1} for the tank mix applications (**Table 3**).

Foliar retention

Glyphosate + 2,4-D foliar retention differed between treatments (premix versus tank mix) and between weed species, but not among populations within each species. Overall, plants of *C. canadensis* and *E. ciliatum* treated with the premix formulation retained ~100 µL more of herbicide solution g^{-1} dry weight than plants treated with the tank mix treatment. The mean foliar retention of *C. canadensis* and *E. ciliatum* was 747 and 553 µL herbicide solution g^{-1} dry weight, respectively, i.e., NT and T *E. ciliatum* plants retained ~200 µL more of herbicide solution g^{-1} dry weight compared to R and S *C. canadensis* plants (**Figure 1**).

Accumulation of shikimic acid and ethylene

Glyphosate-induced accumulation of shikimic acid differed between premix and tank mix treatments as well as between species and between populations. Shikimate accumulation was similar in the T and NT *E. ciliatum* populations within each glyphosate + 2,4-D treatment, but both populations accumulated ~20% more shikimic acid with the premix treatment. In *C. canadensis*, this accumulation differed between populations. The highest level of shikimate (11.9 mg g^{-1} fresh weight) was quantified in S plants treated with the premix, accumulation that was ~25% greater than that of tank mix treated plants. The R plants accumulated little shikimate (~1.35 mg g^{-1} fresh weight) both in premix or tank mix treatments (**Figure 2A**).

2,4-D-induced ethylene accumulation differed between premix and tank mix of glyphosate + 2,4-D in both species, but there were no differences between populations within each species. The trade formulation used in the premix treatment induced a 38% and 28% higher synthesis of ethylene in the S and R *C. canadensis* and NT and T *E. ciliatum* populations, respectively, compared to the tank-mix treatment (**Figure 2B**).

^{14}C-glyphosate absorption, translocation and visualization in plants species

The lowest absorption level (64%) was observed both NT and T *E. ciliatum* plants treated only with ^{14}C-glyphosate. Plants treated with glyphosate + 2,4-D, in premix or tank mix, of this species absorbed ~9% more ^{14}C-herbicide, showing no differences between treatments. In the case of *C. canadensis,* the R plants absorbed between 67 to 82% of ^{14}C-glyphosate, and the S plants absorbed between 82 to 95%. R plants absorbed 13-18% less ^{14}C-herbicide in relation to the S plants, depending on the treatment. In this species, the highest ^{14}C-glyphosate absorption rates were observed in the premix treated R and S plants (**Figure 3**).

Most of the absorbed ^{14}C-glyphosate was retained in the treated leaf in T and NT *E. ciliatum* and R *C. canadensis* plants. These populations translocated only 26% or less, ^{14}C-glyphosate to the shoots and roots. Plants treated only with ^{14}C-glyphosate moved only between 9 and 12% of the ^{14}C-herbicide off the treated leaf, while those that received the glyphosate + 2,4-D premix and tank mix treatments translocated at least 8% more ^{14}C-glyphosate to the rest of the plant and roots. The S *C. canadensis* population showed the highest translocation rates (45-55%), and up to 28-33 and 18-23% of 14C-glyphosate were found in the rest of the plant and roots, respectively. In global terms, the best translocation ^{14}C-glyphosate rates were recorded in the premix treated plants, followed by tank mix treated ones (**Figure 4A**). This translocation patters were corroborated qualitatively in the autoradiographs (**Figure 4B**).

Field trials

As the control of *C. canadensis* and *E. ciliatum* with glyphosate + 2,4-D was similar in 2018 and 2019, the crop cycle data were pooled for each species. For each pair of treatments (same dose or application time), the premix treatments controlled both species better (up to 19%) than the tank mix in all cases. In single applications, the best control was observed at 30 and 60 DAT varying from 66 to 99%, but from 90 DAT, such control decreased considerably. This is explained by the fact that neither herbicide is residual enough to provide control for more than 60 days. Such decrease in weed control was higher in *C. canadensis,* which was only 17% controlled at 120 DAT with 720 + 480 g ha^{-1} (field dose). Doubling the field dose (1440 + 960 g ha^{-1}) improved the weed control between 5 and 22% in relation to the single application of field dose, depending on the evaluation period. However, the split application of the field dose (360 + 240 g ha^{-1} each time) provided the best control of *C. canadensis* and *E. ciliatum* during the entire period evaluated in both premix (76 to 98%) and tank mix (66 to 88%) treatments (**Table 4**).

DISCUSSION

In previous studies, the R population of *C. canadensis* presented GR_{50} and LD_{50} values of 791 and 2600 g ae ha^{-1}, respectively (Amaro-Blanco et al. 2018), and populations of *E. ciliatum* populations, defined as tolerant to glyphosate, of 270-310 (GR_{50}) and 904-989 (LD_{50}), g ae ha^{-1} (Tahmasebi et al. 2018). Observing only the GR_{50} and LD_{50} values of glyphosate of both *C. canadensis* and *E. ciliatum* populations compared in this study, they were much lower than those described above. Such a decrease in the GR_{50} and LD_{50} values of glyphosate can be attributed to the phytotoxic effect caused by 2,4-D. However, it cannot be stated whether the effect was exclusive of the 2,4-D, or there it was an additive/synergistic when mixed with glyphosate, because depending on the weed species, antagonistic or synergistic interactions between these two herbicides have been documented (Wehtje and Gilliam, 2012; Li et al, 2020; Merritt et al. 2020). That the populations R and T have shown a response similar to their counterparts S and NT indicates that the glyphosate tolerance/resistance was suppressed by 2,4-D, since these populations do not have a history of resistance to synthetic auxins. This reinforces that the combination of herbicides with different modes of action improves herbicide resistance management (Alcántara-de la Cruz et al. 2019; Han et al. 2020).

Regardless of the weed species, the premix of glyphosate + 2,4-D provoked greater weight reduction and plant mortality rates compared to the tank mix treatment. That contrast could be linked to differential foliar retention between treatments, since premix treated plants retained in average 17% more herbicide solution. Cuticle is the first barrier for post-emergence herbicides, determining the amount of principle active available to be absorbed by the plant (Michitte et al. 2007). Adjuvants present in each formulation could have played a key role in the foliar retention of the herbicide solution, since these substances, either in an herbicidal formulation or added to the spray tank, are intended to improve the performance of the active ingredient (Pacanoski, 2015). Possibly the adjuvants present in the premix formulation tested in this study contributed to improve the foliar retention of the active ingredients and the control of *C. canadensis* and *E. ciliatum* than those present in the single active ingredient formulations. However, the technical specifications of the products do not specify which adjuvants are present in each formulation (Nufarm, 2016, 2017a,b)

In addition to increasing foliar retention, it is possible that the adjuvants of the premix formulation also help to overcome the barrier imposed by the cuticle by increasing the deposition and the wetting behavior of the pesticide spray liquid on the leaf tissues, increasing, in this way, the permeability of the active ingredient through the plant surfaces (Räsch et al. 2018), which could have contributed to improve the absorption of the active ingredients, as corroborated by the biochemical markers assayed. It was expected that S *C. canadensis* plants had a high accumulation of shikimic acid induced by glyphosate than R plants, but such accumulation was higher (20-25%) in premix treated plants, including T and NT *E. ciliatum* plants. Regarding to ethylene accumulation induced by 2,4-D, there it was also a greater accumulation (28-38%) in both *E. ciliatum* and *C. canadensis* premix treated plants, regardless of glyphosate resistance/tolerance status. Adjuvants also improve the mixing, handling, spraying, efficacy and safety of pesticide formulations (Ramsey, 2005; Pacanoski, 2015). In this context, adjuvants of the premix formulation fulfilled these objectives better than those of the tank mix, since they amplified the biochemical responses.

Absorption and translocation of ^{14}C-glyphosate differed between treatments and some cases among populations within species. The addition/combination of 2,4-D with glyphosate did not affect, or even improve, the absorption and translocation of the second herbicide in both premix and tank mix treatments in the *C. canadensis* and *E. ciliatum* populations. These results seem to diverge from those observed in *Kochia scoparia*, where dicamba reduced glyphosate translocation (Ou et al. 2018). These divergences could be due to the relatively early time (48 HAT) in which these parameters were evaluated in *C. canadensis* and *E. ciliatum*, since in *K. scoparia*, the reduced translocation depended on the evaluation time. At short intervals, glyphosate absorption was enhanced by dicamba, but after 72 HAT, there was an antagonistic effect (Ou et al. 2018), i.e., the mixture of glyphosate with synthetic auxins enhance the absorption and translocation of both herbicides immediately after treatment, as corroborated in our experiments. However, synthetic auxins trigger metabolic and physiological reactions rapidly reducing transpiration and carbon assimilation and inducing and abnormal growth (Grossmann, 2010), which ends up affecting the translocation of glyphosate after a certain period of time. Thus, it cannot rule out that some 2,4-D-induced antagonistic effect can be found in glyphosate translocation in *C. canadensis* and *E. ciliatum* at intervals greater than that evaluated in this study.

Regard to differences between species and between populations, *E. ciliatum* populations showed lower ^{14}C-glyphosate absorption compared to the *C. canadensis* populations, which could explain the natural tolerance of the first species (Tahmasebi et al. 2018). This difference could reflect the intra-specific variation in glyphosate-sensitivity associated to the selection pressure exerted on weeds of agro-ecosystems versus uncultivated lands (Pazuch et al. 2017; Bracamonte et al. 2018). On the other hand, 2,4-D improved glyphosate uptake in *E. ciliatum* but not translocation. Restricted translocation observed in plants T and NT of *E. ciliatum* and R of C*. canadensis* could be due to the sequestration of herbicide in the vacuole as the main candidate to lead glyphosate tolerance/resistance in these populations, as this mechanism restricts the herbicide mobility by isolating it near the area where it was deposited (Ge et al. 2010). Mechanisms that define the glyphosate tolerance or resistance of a weed species are not easily determined, and in some cases, it has been analyzed by inter-specific and intra-specific contrasts (Ribeiro et al. 2015). In reference to *C. canadensis*, absorption and translocation were lower in the R population, regardless of the treatment, showing that these NTSR mechanisms conferred it resistance to glyphosate.

Mixtures using different herbicide sites of actions are a common practice to management of herbicide resistance (Busi and Beckie, 2020; Vencill et al. 2012). However, the effectiveness will be limited if the mixed herbicides do not have similar efficacy and residual soil activity but different propensities to select for resistance in the target species (Beckie and Harker, 2017; Gandini et al. 2020). In this sense, the combination of glyphosate and 2,4 D meet with these criteria; however, the effectiveness of this mixture to control *C. canadensis* and *E. ciliatum* depends on several factors such as the dose, form and time of application. Herbicide formulations are designed to ensure that the active ingredient (s) is retained, absorbed and translocated in a concentration enough to be lethal to weeds (Nandula and Vencill, 2015), so it can claim that commercial glyphosate + 2,4-D premix formulation meets this goal better than tank mix of separate products. 2,4-D causes metabolic and growth disorders after 24 HAT (Grossmann, 2010), while plants treated with glyphosate show symptoms only after 4-7 DAT (Singh et al. 2020). As *C. canadensis* and *E. ciliatum* did not have a history of resistance to synthetic auxins, when doubling the dose, the effects of 2,4-D were greater, which diminished the effects of glyphosate, explaining the low increase in the control of these weeds. This coincides, partially, with the reports of antagonism between these herbicides observed by others researchers (Ou et al. 2018; Li et al. 2020; Merritt et al. 2020). Such antagonism by increasing the dose can be avoided by making split applications both premix or tank mix of the field dose, improving and maintaining the control level for a longer time, which must be equal to or greater than 80% (Vanhala et al. 2004). The control escapes of the first application were reinforced with the second application. Throughout the literature, various positive experiences can be found to control glyphosate-resistant weeds, mainly in dicots, by applying the mixture of glyphosate and 2,4-D, achieving controls of up to 100% (Chahal et al. 2015; Kruger et al. 2010; Merritt et al. 2020; Vargas et al. 2007). Results of this field study confirmed that the combination of glyphosate + 2,4-D is efficient in controlling glyphosate resistant/tolerant broadleaf weeds. Additionally, the use of premix formulations or split applications may contribute to reducing environmental impact, since they employ doses lower than those recommended for single active ingredient formulations (Spaunhorst and Johnson, 2017; Svobodová et al. 2018; Busi and Beckie, 2020), while obtaining an acceptable level of weed control.

CONCLUSIONS

2,4-D suppressed resistance and tolerance to glyphosate in the R and T populations of *C. canadensis* and *E. ciliatum* populations, respectively, leading them to present responses similar to their susceptible and natural tolerant counterparts. The combination of 2,4-D and glyphosate in premix has a better performance in foliar retention, absorption and translocation of herbicides than tank mixes of separate formulations, which was reflected in the levels of accumulated shikimic acid and ethylene. The best treatment to control of these glyphosate-resistant and -tolerant weeds was the split application of glyphosate + 2,4-D, preferably using the premix formulated products; therefore, premix formulations could reduce the environmental impact of herbicides by reducing the herbicide doses used to control glyphosate resistant/tolerant weeds.

Funding: This work was funded partially by Nufarm Europe and the Asociacióń de Agroquímicos y Medio Ambiente.

Notes: The authors have declared that no competing interests exist and the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Acknowledgments: RAC thanks to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2018/15910-6) for support.

REFERENCES

Alcántara-de la Cruz R, Domínguez-Martínez PA, Silveira HM, et al. 2019. Management of glyphosate-resistant weeds in Mexican citrus groves: Chemical alternatives and economic viability. Plants *8*, 325.

Amaro-Blanco I, Fernández-Moreno PT, Osuna-Ruiz MD, Bastida F, De Prado R. 2018. Mechanisms of glyphosate resistance and response to alternative herbicide-based management in populations of the three *Conyza* species introduced in Southern Spain. Pest Manag. Sci. *74*, 1925–1937.

Beckie HJ, Harker KN. 2017. Our top 10 herbicide-resistant weed management practices. Pest Manag. Sci. *73*, 1045–1052.

Beckie HJ, Reboud X. 2009. Selecting for weed resistance: Herbicide rotation and mixture. *Weed Technol. 23*, 363–370.

Bracamonte E, Silveira HM, Alcántara-de la Cruz R, et al. 2018. From tolerance to resistance: Mechanisms governing the differential response to glyphosate in *Chloris barbata*. Pest Manag. Sci. *74*, 1118–1124.

Busi R, Beckie HJ. 2020. Are herbicide mixtures unaffected by resistance? A case study with *Lolium rigidum*. *Weed Res.* (In Press) doi: 10.1111/wre.12453.

Chahal P S, Aulakh JS, Rosenbaum K, Jhala AJ. 2015. Growth stage affects dose response of selected glyphosate-resistant weeds to premix of 2,4-d choline and glyphosate (Enlist Duo^{TM} Herbicide*). J. Agric. Sci. *7*, 1-10.

Choung CB, Hyne RB, Stevens MM, Hosea GC. 2013. The ecological effects of a herbicide–insecticide mixture on an experimental freshwater ecosystem. Environ. Poll. *172*, 264–274.

Duke SO, Powles SB, Sammons RD. 2018. Glyphosate-how it became a once in a hundred year herbicide and its future. Outlooks Pest Manag*.* *29*, 247–251.

Eurostat 2020. *Agriculture, Forestry and Fishery Statistics*, 2020 edition; Cook E, Ed.; Luxemburgo.

Fernández-Alonso JL. 2012. *Epilobium ciliatum* Rafin. (*Onagraceae*), a new adventive species potentially invasive in the Iberian Peninsula. Acta Bot. Malacit. *37*, 179–184.

Franz JE, Sikorski JA, Mao MK. 1997. *Glyphosate: A unique global herbicide*; ACS Monogphap 189: Washington, DC, 653 p.

Gaines TA, Patterson EL, Neve P. 2019. Molecular mechanisms of adaptive evolution revealed by global selection for glyphosate resistance. New Phytol. *223*, 1770–1775.

Gandini EMM, Costa ESP, Santos JB, et al. 2020. Compatibility of pesticides and/or fertilizers in tank mixtures. J. Clean. Prod. 122152.

Ganie ZA, Jhala AJ. 2017. Interaction of 2,4-D or dicamba with glufosinate for control of glyphosate-resistant giant ragweed (*Ambrosia trifida* L.) in glufosinate-resistant maize (*Zea mays* L.). Front. Plant Sci. *8*, 1207.

Ge X, d'Avignon DA, Ackerman JJH, Sammons RD. 2010. Rapid vacuolar sequestration: The horseweed glyphosate resistance mechanism. Pest Manag. Sci. *66*, 345–348.

Gherekhloo J, Fernández-Moreno PT, Alcántara-de la Cruz R, et al. 2017. Pro-106-Ser mutation and EPSPS overexpression acting together simultaneously in glyphosate-resistant goosegrass (*Eleusine indica*). Sci. Rep. *7*, 6702.

González-Torralva F, Cruz-Hipolito H, Bastida F, et al. 2010. Differential susceptibility to glyphosate among the *Conyza* weed species in Spain. J. Agric. Food Chem. *58*, 4361–4366.

Gressel J. 1993. Synergizing pesticides to reduce use rates. In *Pest Control with Enhanced Environmental Safety*; Duke SO, Menn JJ, Plimmer JR, Eds.; ACS Symposium Series: Washington, DC. *524*, 48–61.

Grossmann K. 2010. Auxin herbicides: Current status of mechanism and mode of action. Pest Manag. Sci. *66*, 113–120.

Han H, Picoli Jr GJ, Guo H, Yu Q, Powles SB. 2020. Mechanistic basis for synergism of 2,4-D amine and metribuzin in *Avena sterilis*. J. Pestic. Sci. *45*, 216–222.

Heap I. The International Survey of Herbicide Resistant Weeds www.weedscience.org (accessed Jan 15, 2021).

Jugulam M, Hall JC, Johnson WG, Kelley KB, Riechers DE. 2011. Evolution of resistance to auxinic herbicides: Historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed Sci. *59*, 445–457.

Kruger GR, Davis VM, Weller SC, Johnson WG. 2010. Control of horseweed (*Conyza canadensis*) with growth regulator herbicides. Weed Technol. *24*, 425–429.

Kudsk P, Mathiassen SK. 2020. Pesticide regulation in the European Union and the glyphosate controversy. *Weed Sci. 68*, 214–222.

Li J, Han H, Bai L, Yu Q. 2020. 2,4-D antagonizes glyphosate in glyphosate-resistant barnyard grass *Echinochloa colona*. J. Pestic. Sci. *45*, 109–113.

Lockhart SJ, Howatt KA, 2004. Split applications of herbicides at reduced rates can effectively control wild oat (*Avena fatua*) in wheat. Weed Technol. *18*, 369–374.Mansanet-Salvador CJMS, Ferrer-Gallego PP, Ferrando I, Laguna E. 2014. Primera cita de *Epilobium ciliatum* Raf. (*Onagraceae*) en la comunidad Valenciana. Flora Montiberica. *57*, 17–23.

Magnusson M, Heimann K, Quayle P, Negri AP. 2010. Additive toxicity of herbicide mixtures and comparative sensitivity of tropical benthic microalgae. Mar. Pollut. Bull. *60*, 1978–1987.Merritt LH, Ferguson JC, Brown-Johnson AE, et al. 2020. Reduced herbicide antagonism of grass weed control through spray application technique. Agronomy *10*, 1131.

Michitte P, De Prado R, Espinoza N, Ruiz-Santaella JP, Gauvrit C. 2007. Mechanisms of resistance to glyphosate in a ryegrass (*Lolium multiflorum*) biotype from Chile. Weed Sci. *55*, 435–440.

Möhring N, Dalhaus T, Enjolras G, Finger R. 2020. Crop insurance and pesticide use in

European agriculture. Agric. Syst. *184*, 102902.

Nandula VK, Vencill WK. 2015. Herbicide Absorption and translocation in plants using radioisotopes. Weed Sci. *63*, 140–151.

Nufarm. 2017a. Clinic® (TF) [glyphosate (N-phosphonomethyl glycine) 36% w/v (360 g/L) as isopropylamine salt] (Technical sheet) https://cdn.nufarm.com/wp-content/uploads/sites/32/2018/09/24050429/FTCLINICTFV1130317.pdf (accessed Jan 15, 2021).

Nufarm. 2017b. Kyleo® [2,4-D acid (alkylamidopropyl and dimethylamine salts) 16% + glyphosate (isopropylamine salt) 24%] (Technical sheet) https://cdn.nufarm.com/wpcontent/uploads/sites/32/2018/09/24050407/FichaTcnica-KYLEOvs2ok.pdf (accessed Jan 15, 2021).

Nufarm. 2016. U-46 D Complet® [2,4-D as dimethylamine salt 60% w/v (600 g/l)] (Technical sheet) https://cdn.nufarm.com/wp-content/uploads/sites/32/2018/09/24050150/FichaTcnicaU-46DCOMPLET.pdf (accessed Jan 15, 2021).

Ou J, Thompson CR, Stahlman PW, et al. 2018. Reduced translocation of glyphosate and dicamba in combination contributes to poor control of kochia scoparia: Evidence of herbicide antagonism. Sci. Rep. *8*, 5330.

Pacanoski Z. Herbicides and adjuvants. In *Herbicides, Physiology of Action, and Safety*; Price A., Ed.; IntechOpen, 2015.

Palma-Bautista C, Belluccini P, Gentiletti V, et al. 2020. Multiple resistance to glyphosate and 2,4-D in *Carduus acanthoides* L. from Argentina and alternative control solutions. Agronomy. *10*, 1735.

Palma-Bautista C, Tahmasebi BK, Fernández-Moreno PT, et al. 2018. First case of *Conyza canadensis* from Hungary with multiple resistance to glyphosate and flazasulfuron. Agronomy. *8*, 157.

Pan L, Yu Q, Han H, et al. Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona*. Plant Physiol. 2019, *181*, 1519–1534.

Pazuch D, Trezzi MM, Guimarães ACD, et al. 2017. Evolution of natural resistance to glyphosate in morning glory populations. Planta Daninha. *35*, 1–9.

Ramsey RJL, Stephenson GR, Hall JC. 2005. A review of the effects of humidity, humectants, and surfactant composition on the absorption and efficacy of highly water-soluble herbicides. Pestic. Biochem. Physiol. *82*, 162–175.

Räsch A, Hunsche M, Mail M, et al. 2018. Agricultural adjuvants may impair leaf transpiration and photosynthetic activity. Plant Physiol. Biochem. *132*, 229–237.Ribeiro DN, Nandula VK, Dayan FE, et al. 2015. Possible glyphosate tolerance mechanism in pitted morningglory (*Ipomoea lacunosa* L.). J. Agric. Food Chem. *63*, 1689–1697.

Robinson AP, Simpson DM, Johnson WG. 2012. Summer annual weed control with 2,4-D and glyphosate. Weed Technol. *26*, 657–660.

Rojano-Delgado AM, Cruz-Hipolito H, De Prado R, Luque de Castro MD, Franco AR. 2012. Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *uilis* plants. Phytochemistry. *73*, 34–41.

Sammons RD, Gaines TA. 2014. Glyphosate resistance: State of knowledge. Pest Manag. Sci. *70*, 1367–1377.

Singh S, Kumar V, Datta, S.; et al. 2020. Glyphosate uptake, translocation, resistance emergence in crops, analytical monitoring, toxicity and degradation: A review. Environ. Chem. Lett. *18*, 663–702.

Sjollema SB, Martínezgarcía G, van der Geest HG, et al. 2014. Hazard and risk of herbicides for marine microalgae. Environ. Poll. *187*, 106–111.

Spaunhorst D, Johnson W. 2017. Variable tolerance among palmer amaranth (*Amaranthus palmeri*) biotypes to glyphosate, 2,4-D amine, and premix formulation of glyphosate plus 2,4-D choline (Enlist Duo®) herbicide. Weed Sci. *65*, 787-797.

Steinrücken HC, Amrhein N. 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. *94*, 1207–1212.

Svobodová, Z, Skoková HO, Holec J, et al. 2018. Split application of glyphosate in herbicide-tolerant maize provides efficient weed control and favors beneficial epigeic arthropods. Agric. Ecosyst. Environ. *251*, 171–179. Tahmasebi BK, Alcántara-de la Cruz R, Alcántara E, et al. 2018. Multiple resistance evolution in bipyridylium-resistant *Epilobium ciliatum* after recurrent selection. Front. Plant Sci. *9*, 695.

Tang JYM, Escher BI. 2014. Realistic environmental mixtures of micropollutants in surface, drinking, and recycled water: herbicides dominate the mixture toxicity toward algae. Environ. Toxicol. Chem. *33*, 1427-36.Vanhala P, Kurstjens D, Ascard J, et al. 2004. Guidelines for physical weed control research: flame weeding, weed harrowing and intra-row cultivation. Proc. 6th EWRS Work. Phys. Cult. Weed Control. No. March, 194–225.

Vargas L, Bianchi MA, Rizzardi MA, Agostinetto D, Dal Magro T. 2007. *Conyza bonariensis* resistant to glyphosate in southern Brazil. Planta Daninha. *25*, 573–578.

Vencill WK, Nichols RL, Webster TM, et al. 2012. Herbicide resistance: Toward an understanding of resistance development and the impact of herbicide-resistant crops. Weed Sci. *60*, 2–30.

Wehtje G, Gilliam CH. 2012. Cost-effectiveness of glyphosate, 2,4-D, and triclopyr, alone and in select mixtures for poison ivy control. Weed Technol. *26*, 469–473.

Table 1. Location of the *C. canadensis* and *E. ciliatum* species and their glyphosate-sensitivity state studied

Species	**Country**	**Crop**	**Application years /dose**[a]	**Coordinates**	**Status**
C. canadensis (S)	Spain	New plantation	Organic system	37°47'N, 4°20'W	Susceptible
C. canadensis (R)	Spain	Olive orchard	15 years /1080	37°46'N, 5°00'W	Resistant[b]
E. ciliatum (NT)	Spain	No crops	Non herbicide	38°03'N, 7°57'W	Natural tolerant[c]
E. ciliatum (T)	Portugal	Almond	4 year /720	38°03'N, 7°48'W	Tolerant

[a] g ae ha^{-1}; [b] Resistance to glyphosate (Amaro-Blanco et al. 2018); [c] Natural tolerance to glyphosate (Tahmasebi et al. 2018).

Table 2. Doses of glyphosate + 2,4-D applied in premix and in tank mix used in the field trials during 2018 and 2019

No	Treatment[a]	Application time[b]	Rate (g ha^{-1})	Liters of PC
1	Untreated	-	-	-
2	Premix	Single	720 + 480	3
3	Tank mix	Single	720 + 480	2 + 0.8
4	Premix	Single	1440 + 960	6
5	Tank mix	Single	1440 + 960	4 + 1.6
6	Premix	Split application	720 + 480	3
7	Tank mix	Split application	720 + 480	2 + 0.8

[a] Premix, trade formulation Kyleo® (240 g ae L^{-1} glyphosate + 160 g ai L^{-1} 2,4-D); Tank mix, Clinic® (360 g ae L^{-1} glyphosate) + U-46 D Complet® (600 g ai L^{-1} of 2,4-D). PC, commercial product. [b] Split applications consisted in two sequential treatments, applying the half of the final rate each time.

Table 3. Parameters of the sigmoidal equation[a] used to estimate the dose of glyphosate + 2,4-D, applied in premix and tank mix, need to reduce the dry weight (GR_{50}) and plant mortality (LD_{50}) by 50% in glyphosate resistant (R) and susceptible (S) *Conyza canadensis* and tolerant (T) and non-tolerant (NT) *Epilobium ciliatum* plants

Treatment[b]	**Species/population**	**b**	**d**	**GR_{50}**	***P*-value**	**b**	**d**	**LD_{50}**	***P*-value**
Premix	*C. canadensis* (S)	1.1	99.8	40.0 + 26.6	0.0053	2.5	101.8	380.0 + 253.3	0.0001
	C. canadensis (R)	0.2	100.0	45.4 + 30.2	0.0001	1.0	99.9	368.3 + 245.5	0.0001
	E. ciliatum (NT)	1.3	100.0	30.8 + 20.5	0.0023	3.3	100.9	390.0 + 260.0	0.0001
	E. ciliatum (T)	1.3	100.1	32.5 + 21.6	0.0001	0.5	99.2	370.5 + 246.7	0.0001
Tank mix	*C. canadensis* (S)	2.2	104.3	140.0 + 93.3	0.0001	0.3	100.9	421.5 + 281.0	0.0001
	C. canadensis (R)	1.2	101.5	155.8 + 103.9	0.0008	1.2	100.5	405.9 + 270.6	0.0009
	E. ciliatum (NT)	3.6	100.3	90.0 + 60.0	0.0001	1.9	102.9	480.0 + 320.0	0.0002
	E. ciliatum (T)	2.2	101.4	105.8 + 70.5	0.0001	0.8	101.2	467.9 + 311.9	0.0001

[a] $y = d/\{1+\exp[b(\log x - \log e)]\}$, where b is the relative slope of the curve, d is the upper limit of y, e is the herbicide rate that reduces y by 50% and y is the dry weight (GR_{50}) or plant survival (LD_{50}) of a given population. [b]Premix, trade formulation Kyleo® (240 g ae L^{-1} glyphosate + 160 g ai L^{-1} 2,4-D); Tank mix, Clinic® (360 g ae L^{-1} glyphosate) and U-46 D Complet® (600 g ai L^{-1} of 2,4-D).

Table 4. Percentage of control of *C. canadensis* and *E. ciliatum* with glyphosate + 2,4-D in farms from Southern Spain and Northern Portugal, respectively, from 15 to 120 days after treatment (DAT) in 2018 and 2019.

Application time[a] (Rate g ha^{-1})	**Treatment[b]**	**DAT**				
		15	**30**	**60**	**90**	**120**
Conyza canadensis (Southern Spain)						
-	Control	-	-	-	-	-
Single	Premix	74±3.6b	90±4.2b	79±4.5b	37±4.1e	19±5.1e
(720 + 480)	Tank mix	55±2.1e	82±2.3c	66±3.2d	31±3.6e	17±2.6e
Single	Premix	79±4.2a	99±1.4a	90±2.6a	61±5.5c	40±4.8c
(1440 + 960)	Tank mix	69±2.8c	86±3.6b	78±4.1b	51±2.9d	31±2.1d
Split application	Premix	76±3.7b	91±2.3b	82±3.6b	98±2.0a	88±3.7a
(720 + 480)	Tank mix	66±1.8d	82±3.4c	71±3.4c	88±1.9b	75±2.5b
Epilobium ciliatum (Northern Portugal)						
-	Control	-	-	-	-	-
Single	Premix	82±3.6b	91±3.8b	82±2.9b	58±5.4ef	44±5.8e
(720 + 480)	Tank mix	72±2.1c	81±1.9c	73±3.7c	51±2.1f	36±2.7e
Single	Premix	87±4.9a	99±1.0a	93±2.0a	70±4.0c	59±5.2cd
(1440 + 960)	Tank mix	77±1.5b	91±3.2b	81±3.7b	60±2.9d	49±4.1d
Split application	Premix	85±3.5a	93±3.9b	83±5.2b	98±2.3a	89±3.8a
(720 + 480)	Tank mix	69±3.9c	84±2.7c	72±3.7c	86±2.5b	77±2.9b

Different letter per column shown differences by the Tukey test ($p < 0.05$). [a]Two applications of 360 + 240 g ha^{-1} were made each time for the split application. [b]Premix, trade formulation Kyleo® (240 g ae glyphosate L^{-1} + 160 g ai 2,4-D L^{-1}); Tank mix, Clinic® (360 g ae glyphosate L^{-1}) and U-46 D Complet® (600 g ai 2,4-D L^{-1}).

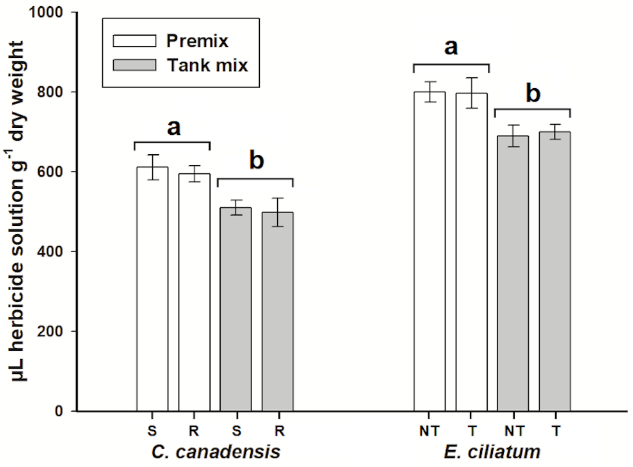
Figure 1. Foliar retention of glyphosate + 2,4-D (240 + 160 g ha^{-1}, respectively), applied in premix (Kyleo®) and tank mix (Clinic® + U-46 D Complet®), in glyphosate resistant (R) and susceptible (S) *Conyza canadensis* and tolerant (T) and non-tolerant (NT) *Epilobium ciliatum* plants. Same letter denotes no differences between treatments by the Tukey test (P> 0.05). Vertical bars ± standard error (n= 10).

Figure 2. Accumulation of shikimic acid (A) and ethylene (B) in glyphosate resistant (R) and susceptible (S) *Conyza canadensis* and tolerant (T) and non-tolerant (NT) *Epilobium ciliatum* plants, treated with glyphosate + 2,4-D (240 + 160 g ha^{-1}, respectively) in premix (Kyleo®) and tank mix (Clinic® + U-46 D Complet®). Same letter denotes no differences between treatments by the Tukey test (P> 0.05). *show differences between populations of a species within the same treatment by the Student's t test (P> 0.05). Vertical bars ± standard error (n= 5).

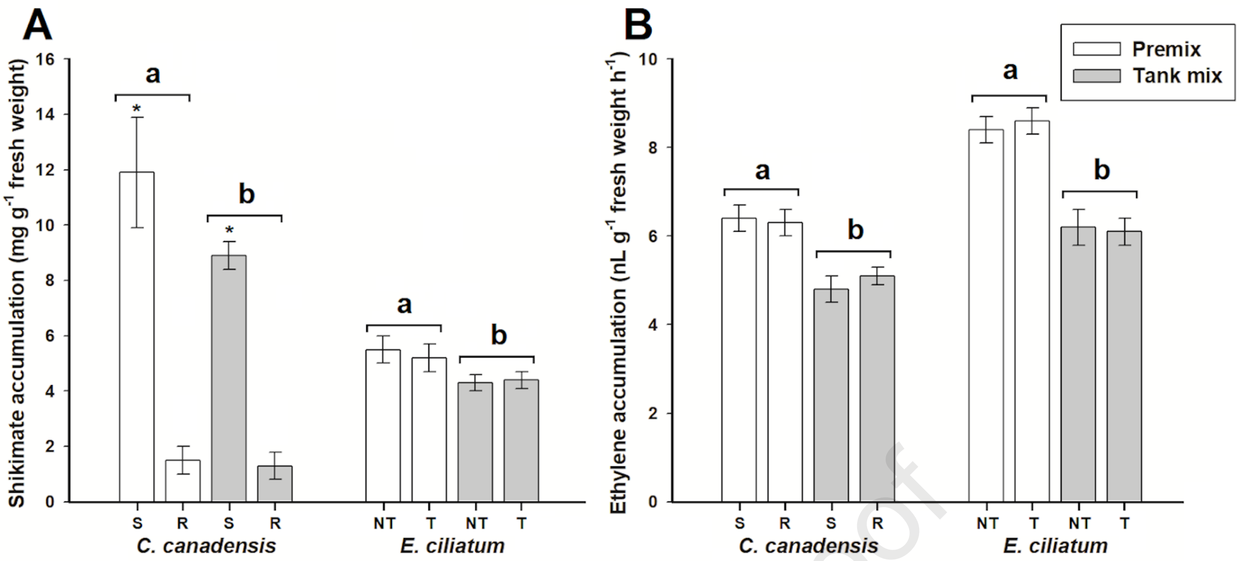
Figure 3. Absorption of ^{14}C-glyphosate in glyphosate resistant (R) and susceptible (S) *Conyza canadensis* and non-tolerant (NT) and tolerant (T) *Epilobium ciliatum* plants, treated with glyphosate and glyphosate + 2,4-D (240 + 160 g ha^{-1}, respectively) in premix (Kyleo®) and tank mix (Clinic® + U-46 D Complet®) at 48 hours after treatment. Same letter denotes no differences between treatments by the Tukey test (P> 0.05). * show differences between populations of a species within the same treatment by the Student's t test (P> 0.05).

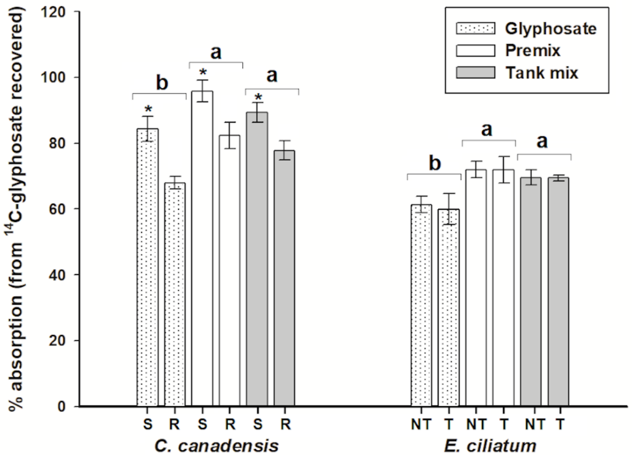
Figure 4. A) Translocation of ^{14}C-glyphosate (% from absorbed) in glyphosate resistant (R) and susceptible (S) *Conyza canadensis* and non-tolerant (NT) and tolerant (T) *Epilobium ciliatum* plants, treated with glyphosate and glyphosate + 2,4-D (240 + 160 g ha^{-1}, respectively) in premix (Kyleo®) and tank mix (Clinic® + U-46 D Complet®) at 48 hours after treatment. Single glyphosate or mix (^{14}C-Glyphosate + premix) and (^{14}C-glyphosate + (glyphosate+2,4-D)). B) Representative phosphor images revealing movement of ^{14}C-glyphosate in R and S *C. canadensis* and T and NT *E. ciliatum* plants. A darker red color indicates increased concentrations of ^{14}C-glyphosate. Same letter denotes no differences between treatments by the Tukey test (P > 0.05). * show differences between

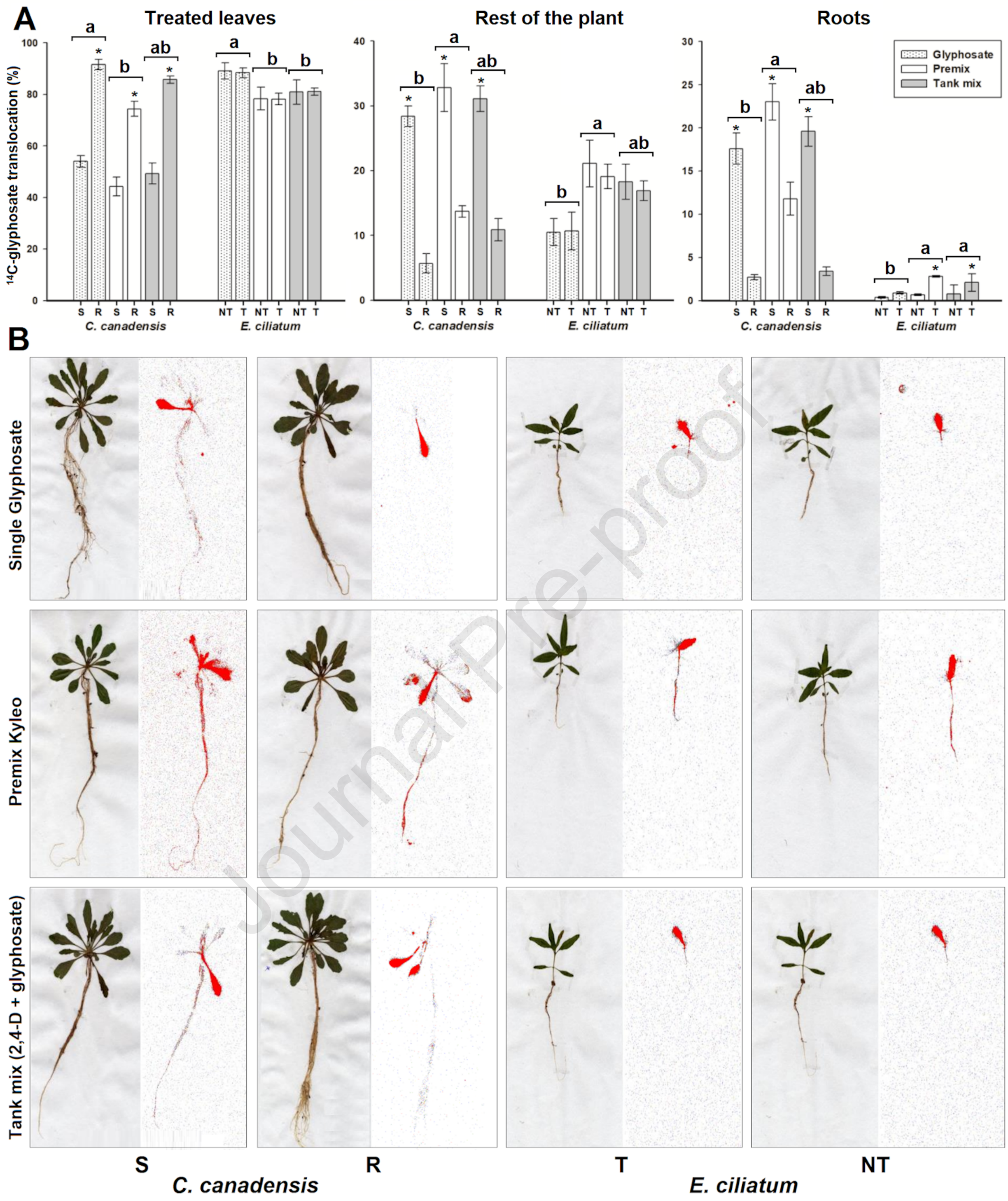
populations of a species within the same treatment by the Student's t test ($P > 0.05$).



1000
800
600
400
200
0
μL herbicide solution g-1 dry weight
Premix
Tank mix
a
b
a
b
S
R
S
R
NT
T
NT
T
C. canadensis
E. ciliatum


A
Shikimate accumulation (mg g-1 fresh weight)
0
2
4
6
8
10
12
14
16
a
b
a
b
*
*
S
R
S
R
NT
T
NT
T
C. canadensis
E. ciliatum
B
Ethylene accumulation (nL g-1 fresh weight h-1)
0
2
4
6
8
10
a
b
a
b
Premix
Tank mix
S
R
S
R
NT
T
NT
T
C. canadensis
E. ciliatum


% absorption (from ^{14}C-glyphosate recovered)
0
20
40
60
80
100
120
Glyphosate
Premix
Tank mix
b
a
a
*
*
*
b
a
a
S
R
S
R
S
R
C. canadensis
NT
T
NT
T
NT
T
E. ciliatum


A
Treated leaves
Rest of the plant
Roots
¹⁴C-glyphosate translocation (%)
Glyphosate
Premix
Tank mix
a
b
ab
*
S R S R S R
NT T NT T NT T
C. canadensis
E. ciliatum
0
20
40
60
80
100
10
30
5
15
25
B
Single Glyphosate
Premix Kyleo
Tank mix (2,4-D + glyphosate)
S
R
T
NT
C. canadensis
E. ciliatum

Highligths

- The combination of 2,4-D and glyphosate suppressed glyphosate resistance/tolerance.
- Premix formulations performed better than tank mixes from separate formulations.
- The divided application of glyphosate + 2,4-D controlled weeds for longer in the field.
- The use of premix formulations could help reduce environmental impact.
- Increasing herbicide doses reduces weed control and increases environmental impacts.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: