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Genetic Variations among Fleabane (*Conyza bonariensis* (L.) Cronquist) Populations in Jordan and Their Susceptibility Levels to Contact Herbicides

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Abstract: A field demonstration and pot experiments were implemented to assess the effect of paraquat, oxadiazon, and oxyfluorfen herbicides in controlling selected populations of fleabane *Conyza bonariensis* (L.), grown in the central valley of Jordan. Conyza mature seeds were collected from six investigated sites (five from Jordan valley named P1, P2, P3, P4, P5, and one from the University of Jordan Campus named P6). Only populations proved to be *C. bonariensis* via ITS assessment were involved in the glasshouse experiments at the University of Jordan in 2017 and 2019. Results showed that recommended or two-fold higher rates (2.5 and 5 kg ha^{-1}) of paraquat failed to affect weed plants in a date palm orchard located at Tal-al-Ramel in the Central Jordan Valley. Paraquat, oxyfluorfen, and oxadiazon (2.5, 3.3, and 5 kg ha^{-1}, respectively), failed to control plants of the same weed population grown in pot experiments. Treated plants at Tal-al-Ramel grew similarly to untreated control, mostly due to different genetic backgrounds. The other *C. bonariensis* populations (University Research Station, al-Twal, and University Campus) were effectively controlled with all herbicides. The application of recommended or 10-fold higher rates of herbicides failed to control or slightly injured the resistant population. Seed DNA analysis of the ITS region showed genetic differences among the investigated populations. It indicated that four populations are *C. bonariensis* (P1, P3, P4, and P6). At the same time, two are *C. canadensis* (a closely related species) collected from the University Research Station (P2) and al-Twal sites (P5), and also that the population of *C. bonariensis* in the date palm orchard was genetically distinct from the other *C. bonariensis* populations. It is concluded that *C. bonariensis* population in the Tal-al-Ramel site developed resistance to paraquat, oxadiazon, and oxyfluorfen herbicides. Thus, novel alternative practices in controlling the resistant weed population are necessary to prevent its possible spread to other regions in the country and obstruct the development of new herbicide-resistance weed populations.

Keywords: genetic variations; paraquat; oxadiazon; oxyfluorfen; herbicide resistance; ITS

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1. Introduction

Chemical weed control using herbicides as a cost-effective, not exhaustive, easy-to-implement, and rapid solution for weed problems is the farmer's preference. However, heavy reliance on herbicides created unforeseen problems for agriculture and farmers worldwide [1], including selection pressure leading to the development of herbicide-resistant populations of weeds. Herbicide resistance is the inherited ability of a plant or a biotype to survive and reproduce after exposure to a herbicide dose normally lethal to the

wild type [2,3]. In many cases, restrictions on production, development, and legislation of new herbicides to break the resistance barrier lead to failure in weed eradication practices. Therefore, a decreased response of a weed population to the applied herbicides is observed. Notably, repeated use of one or more herbicides with a similar mechanism of action, with usually multiple applications in one season, will lead to the selection of resistant individuals until they are completely dominant in the local environment. At the same time, the frequency of herbicide-sensitive individuals is gradually decreasing. However, this development usually takes a long time for the population to shift and depends on various factors, i.e., weed species more prone to the selection of herbicide-resistant biotypes, the herbicide/herbicides used, the timing/frequency of herbicide applications, concentration, and quantity used, etc. [4].

Conyza plants are weeds in more than 40 crops in 70 countries [5]. Herbicide resistance within *Conyza* populations is a common phenomenon [6] since these are weeds of good fitness and particularly successful in spreading due to a large number of wind-dispersed seeds (up to 200,000 per plant), which can be effectively spread to a distance of 100 km [7]. They are annuals able to germinate over a wide period from late autumn through to spring. Although several species belong to the *Conyza* genus, only five, i.e., *Conyza aegyptiaca* (L.) Aiiton, *Conyza albida* Sprigel, *Conyza bonariensis* (L.) Conquist, *Conyza canadensis* (L.) Conquist, and *Conyza stricta* Willd occur in Jordan while *C. bonariensis* is the most prevalent. Many farmers have reported *Conyza bonariensis* to resist different herbicides, including glyphosate [8–19] and paraquat [20,21]. The glyphosate rates required to control resistant populations were 7–10 times higher than those for susceptible populations [8]. Several reported cases in which biotypes showed resistance to herbicides have been documented in different parts of the world [9,10,22]. The mechanism of *C. bonariensis* resistance to glyphosate was due to the low translocation and high basal insensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) transcript levels [9]. These reasons suggest that similar agricultural practices (repeated application of glyphosate, no crop and herbicide rotation, no-tillage) have effectively contributed to developing herbicide-resistant species from the resistance-prone *Conyza* genus. The resistance mechanism has also been mentioned due to the differential translocation of glyphosate in the weed biotypes [10]. In paraquat-resistant *Conyza* weeds, high constitutive levels of chloroplast enzymes protect the plant from paraquat damage by detoxifying the applied herbicide. In the same context, herbicide toxification or sequestration can only come after paraquat temporarily inhibits chloroplast activity, and thus internal chloroplast protection reactions have temporal primacy over sequestration [23]. The high genetic constitutiveness of the enzyme levels in the resistant biotype allows rapid protection of resistant plants until paraquat sequestration, measured at 4 h [24].

Conyza species can hybridize with each other and include diploids and polyploids [25,26]. Admixed individuals have been characterized in many invasive populations. Nevertheless, admixture may occur in the invaded range, or admixed individuals can be introduced to a specific region [27]. Estimated heterozygosity indicated a relatively low population differentiation between horseweed (*C. canadensis*) and hairy fleabane (*C. bonariensis*). Therefore, a substantial genetic exchange between the two *Conyza* species is proposed as an estimate of gene flow and was found to be high for many alleles. That provides additional evidence of the occurrence of outcrossing between populations or dispersion of samples of one for other sites. Previous studies reported that outcrossing is a common process in *Conyza* spp., effectively contributing to novel variability and enhancing herbicide resistance [28]. The internal transcribed spacer (ITS) is an effective DNA barcoding tool widely used to identify plant species, especially wild and weed populations [29]. The ITS region is a highly sensitive domain of approximately 100 copies per single genome positioned between the 18S and 28S rRNA genes. This conservative region clearly defines the possible inter- and intra-specific variation; furthermore, it offers several advantages over other traditional molecular tools, which make it suitable for identifying the genetic basis of speciation and understanding the evolutionary history of closely related wild species. [30]. Based on

any differences in weed populations' responses to the herbicides, molecular assay on the DNA level may be conducted further to investigate the genetic basis for any resistance to herbicides. Therefore, the objectives of this study were to examine the effects of paraquat with two other foliage-applied contact herbicides (oxyfluorfen and oxadiazon) widely used by farmers on populations of *Conyza* spp. collected from different regions in the country. Further investigation on the DNA molecular level was conducted to investigate the different evolutionary paths these populations may go through.

2. Materials and Methods

2.1. Conyza Bonariensis Populations

Mature seeds of six *Conyza* spp. populations (Table 1) were collected from wildly growing plants in four sites. Five weed populations were from Jordan valley: one (P1) from a date palm orchard at Tal-al-Ramel, two (P2 and P3) from the University of Jordan Research Station in Central Jordan Valley, and two (P4 and P5) from al-Twal, about 40 km north of the first site. Seeds of the sixth weed population, P6, were gathered from the University of Jordan Campus at al-Jubeiha, Amman.

Table 1. The collected populations of *Conyza* spp. and their geographical distribution.

Site of Collection	*Conyza* spp.	Symbol	Field Description	Geographical Coordinates
Tal-al-Ramel	*Conyza bonariensis*	P1	Date palm orchard	32.059821° N, 35.598875° E
University of Jordan Research Station	*Conyza canadensis*	P2	Cultivated fields	32.145968° N, 35.696554° E
	Conyza bonariensis	P3	Cultivated fields	
Al-Twal	*Conyza bonariensis*	P4	Uncultivated fields	32.147574° N, 35.692870° E
	Conyza canadensis	P5	Uncultivated fields	
The University of Jordan Campus	*Conyza bonariensis*	P6	Gardens	32.0161° N, 35.8695° E

2.2. DNA Analysis of Conyza spp. Populations

2.2.1. Extraction Quantification and Qualification of Total Genomic DNA

DNA analysis was carried out on seeds of all six populations (P1–P6) of *Conyza* spp. Each six-seed lot was split into three replicates and prepared for total genomic DNA extraction using ZR Plant/Seed DNA MiniPrep™ (Zymo, Irvine, CA, USA). The extraction protocol was followed literally as the supplier instructed. Quantification and qualification of the extracted DNA were accomplished accordingly with the aid of Nanodrop, where reads ranged between 1.7–2 and were adjusted to a final concentration of 50 ng μL^{-1}.

2.2.2. PCR Conditions

The PCR amplification was accomplished in a total volume of 25 µL, containing 1.5 µL DNA, 5 µL Taq PCR PreMix (Intron, Seongnam, Republic of Korea), 1 µL of each primer (10 pmol), then distilled water was added to reach a total volume of 25 µL. A pair of primers were used to amplify the ITS region, ITS1 as forward (F: 5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 as a reverse (R: 5′-TCCTCCGCTTATTGATATGC-3′) supplied by Integrated DNA Technologies Company-IDT, Kanata, ON, Canada.

The thermal cycling conditions were as follows: denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 1 min, and 72 °C for 1 min with final incubation at 72 °C for 7 min. using a thermal cycler (Gene Amp, PCR system 9700; Applied Biosystem, Waltham, MA, USA). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ultraviolet light (302 nm) after red stain staining (Intron, Seongnam, Republic of Korea).

2.2.3. Preparation of Agarose Gel

According to Sambrook and Russell [31], the agarose gel has been made in 1.5% condensation by melting 1.5 g of agarose in 100 mL of previously prepared TBE buffer. Subsequently, the electrophoresed gel was stained in a pool of a staining solution

(3 µL red safe nucleic acid and 500 mL of distilled water), ultimately tested with a 336 nm UV light, and photographed.

2.2.4. Sequencing Protocol

The procedure followed for Gel Extraction DNA was as stated by Vogelstein and Gillespie [32]. Sanger sequencing method was applied to sequence the gel extracted DNA in the National Instrumentation Center for Environmental Management (NICEM) (http://nicem.snu.ac.kr/main/?en_skin=index.html, accessed on 15 June 2020), biotechnology lab using 3730XL Genetic Analyzer (Applied Biosystems, USA). Homology investigation was conducted using Basic Local Alignment Search Tool (BLAST) software at the National Center Biotechnology Information (NCBI), (www.ncbi.nlm.nih.gov, accessed on 15 June 2020) and BioEdit and MEGA6 software [33].

2.3. Field Trial at Tal-al-Ramel

The experiment started on 18 April 2017, in a date palm orchard at Tal-al-Ramel, in the central Jordan valley. The area is 255 m below sea level and is characterized by its tropical climate. Date palm trees were 15 years old. *Conyza bonariensis* plants at full vegetative or pre-flowering growth stages were spread almost over the entire field and growing in the pure stand at an average density of 30 plants m^{-2}, except for some spots where *Cynodon dactylon* and *Prosopis farcta* were found. A weed-infested piece of land was divided into 33 plots of 4 m^2 each. Herbicides paraquat (Gramaxon, Sandoz, UK); oxadiazon (Ronstar, Bayer, Germany), and oxyfluorfen (Goal; Rohm and Haas, Mozzata, Italy) available in local markets were used (Table 2).

Table 2. Herbicides used for controlling *Conyza bonariensis* populations.

Common Name	Trade Name and a.i Percentage	Chemical Name	Rate of Application (kg ha^{-1})	Mode of Action
Paraquat	Gramaxon 20% (*v*/*v*)	1,1′-dimethyl-(4,4′-bipyridiniom) dichloride	2.5	Contact
Oxadiazon	Ronstar 25%(*v*/*v*)	2-tert-butyl-4-(2,4-dichloro-5-isopropyloxyphenyl)-1,3,4-oxadiazolin-5-one	5	Contact
Oxyfluorfen	Goal 24%(*v*/*v*)	[2-chloro-N-[[4-methoxy-6-methyl-I,3,5-triazine-2-yl)-amino]carbboennyzl]esulphonamide	3.3	Contact

The herbicides were applied at recommended, double, and 10-fold rates in 3 repetitions: paraquat 2.5, 5.0, or 25 kg ha^{-1}; oxadiazon 5.0, 10, or 50 kg ha^{-1}; oxyfluorfen 3.3, 6.6, or 33 kg ha^{-1}. The remaining plots were included as untreated control. The herbicides were applied as an aqueous spray on the foliage parts of the weed and at a constant pressure in the morning without air movement, using a Knapsack sprayer with a single nozzle at a volume rate of 1083.3 l ha^{-1}. Visual estimation, on a scale of 0 to 10, of the effects of herbicides on weed injuries was carried out two weeks after their application. In this scale, zero denotes the plants were completely controlled. Scores 1–2 mean plants were almost killed/desiccated with no possible recovery; 3–4 plants were severely harmed with no possible resuming normal growth; 5–6 plants were harmed by necrotic spots scattered on their vegetative parts but had moderate possible growth recovery; 7–8 plants showed some scattered necrotic spots on foliage parts but with high possible growth recovery, resumed normal growth and seeding; 9–10 plants were slightly or not affected and set seeds [4]. Three persons conducted the evaluation, and the average of the three scores was considered for each plot. Representative photos of severely affected or completely controlled *C. bonariensis* plants were included for documentation (Figure 1).

Figure 1. Field demonstration showing; (**A**) *C. bonariensis* untreated with paraquat, (**B**) treated with paraquat at 2.5 kg ha^{-1}, and (**C**) treated with paraquat at 5 kg ha^{-1}.

2.4. Herbicide Treatment Pots Experiments

The four populations (P1, P3, P4, P6) that proved to be *Conyza bonariensis* (L.) through molecular analysis were subjected to further investigation with herbicides paraquat, oxadiazon, and oxyfluorfen in a glasshouse.

PVC pots of ten-centimeter diameter were filled with soil/peat mixture (1:1 v/v) to grow seeds of each of the four *C. bonariensis* populations on 15 January 2019. Five plants were growing in each pot in an unconditioned glasshouse at an average day temperature of 24 °C and irrigated as needed.

At full vegetative growth (about 25 cm tall), the pots were divided into two groups, each of which included 80 pots.

Herbicides were applied using shoulder-held sprayers. Each contained 570 mL solution calculated to cover an area of 2 m^2. Five pots of each population (a total of twenty pots for each population) were placed and treated with a single herbicide.

2.4.1. Experiment 1

Three groups, each of twenty pots, were treated with paraquat, oxyfluorfen, or oxadiazon, and the fourth group (twenty pots) was kept untreated and considered a control. The herbicides were applied at recommended doses (2.5, 3.3, and 5.0 kg ha^{-1} for the three herbicides, respectively). The effect of herbicides on the growth of weed plants was visually estimated five days after application using a zero to ten scale (the same as in experiment 2.3). Two people carried out the evaluation, and the average of the two estimated scores was considered for each herbicide on each weed population. The same herbicides were re-applied on the same plants two weeks after the first application using the same rates as in the first spray. The effect of herbicides was visually estimated five days after application using a zero to ten evaluation scale. In this experiment, each herbicide was applied twice

and exactly as carried out in the field. However, since herbicides tested are all of the contact actions, complete coverage treatment was performed on each weed population.

2.4.2. Experiment 2

The second group of 80 pots of each population of *C. bonariensis* raised in the glasshouse was divided, as before, into 4 groups for each weed population, each of 5 pots. Three groups of each population were treated with ten-fold the recommended rate, equal to 25, 33, and 50 kg ha^{-1} of paraquat, oxyfluorfen, and oxadiazon, respectively. While the fourth group (20 pots) was kept untreated and considered a control.

As in experiment 1, visual estimation of weed injuries was performed five days after treatment using the same scale and methods to evaluate the effect of herbicides on weed growth. On the same plants, another application of the same herbicides was made two weeks after the first application using the same rates. The effect was visually estimated after five days of application, and a similar evaluation was performed.

2.5. Statistic Analysis

Treatments in both glasshouse experiments were laid out in a complete randomized block design with five replicates. Recorded data of the studied parameters to estimate the effects of herbicides used on weed populations were subjected to analysis of variance (ANOVA) using SAS® software version 9.1 [34]. Treatments were compared at $\geq$0.05 significance level using the least significant difference test (LSD).

Based on the results of ITS sequences, the maximum likelihood method was adopted to generate the evolutionary tree of investigated weed populations [35].

$$P(r\ \tau) = \exp(r\ \tau\ \mathrm{Q})$$

where $P_{ij}(r\ \tau)$ is the probability that nucleotide i will be replaced by nucleotide j after τ units of time at relative rate r.

3. Results

3.1. Molecular Analysis of C. Bonariensis Seed Samples

ITS Sequencing

The amplified ITS region was about 550 bp in molecular size (Figure 2) in all three replicates of the six collected seed lots of *Conyza* spp. The amplified sequences were registered in the DDBJ (DNA Data Bank of Japan) under the two accession numbers of (LC574985) and (LC574986).

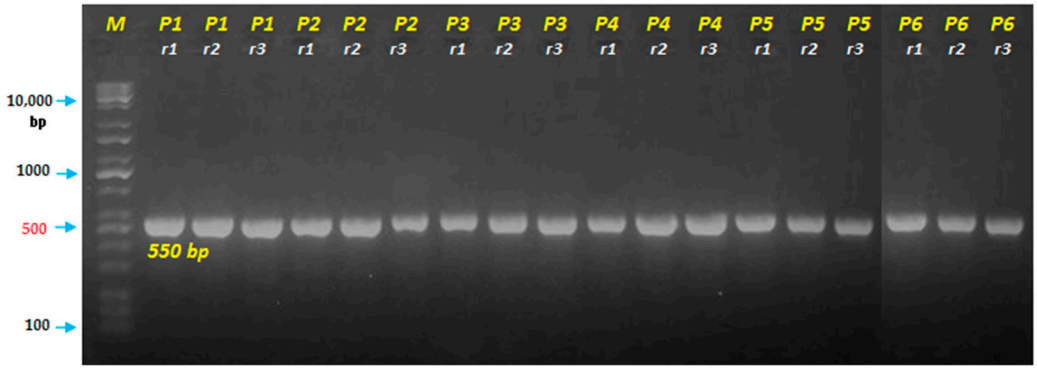

Figure 2. The amplified ITS region of the three replicates (r1 to r3) of each of the six seed lots (P1 to P6) of *Conyza* spp. run on 1.5% agarose gel.

From the viewpoint of nrDNA analysis, it was found that the first tested population P1 (date palm population, Tal-al-Ramel) in its three replicates (P1-r1, P1-r2, and P1-r3) has expressed a unique genetic background as it composed a single main cluster (Figures 3 and 4) according to the maximum composite likelihood model in characterizing ITS polymorphism. Although the ITS region includes a small area of the genome compared to the

total genome size, the genetic variation in such a highly conservative domain may play a crucial role in the exhibited phenotypic and physiologic plasticity, a common feature for the Conyza genus. Hence, this magnitude of genetic differences may partly reflect this genus's ability to cope with the toxic effect of different herbicides.

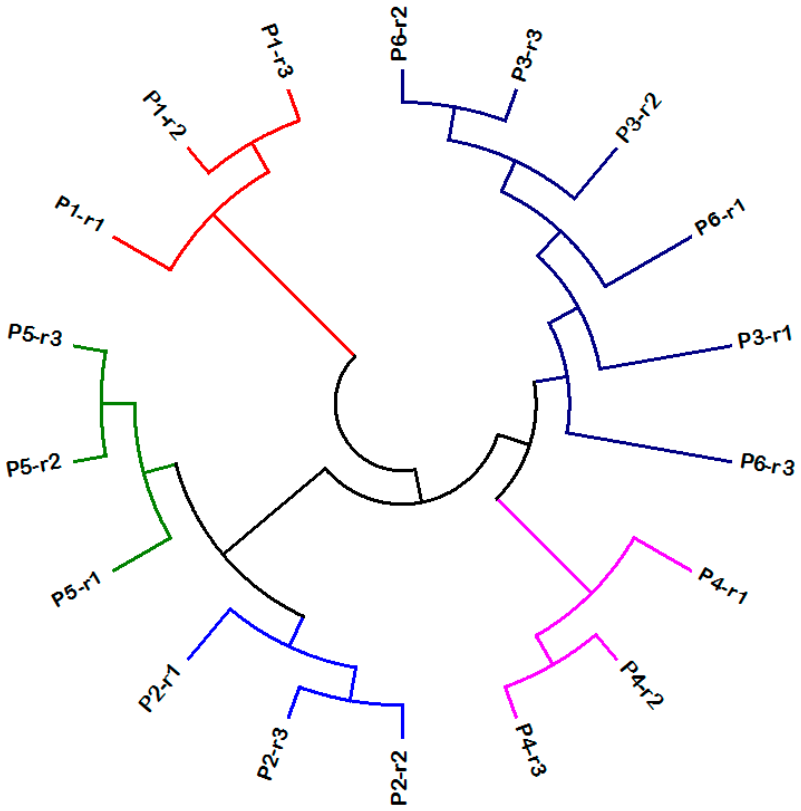

Figure 3. Cluster analysis using the maximum composite likelihood model based on ITS sequencing of the three replicates (r1 to r3) of each six seed lots (P1 to P6) of *Conyza* spp. P1 (*C. bonariensis*) = Tal-al-Ramel site, P2 (*C. canadensis*) and P3 (*C. bonariensis*) = University of Jordan Research Station site, P4 (*C. bonariensis*) and P5 (*C. canadensis*) = al-Twal site and P6 (*C. bonariensis*) = University of Jordan Campus site.

Yet, the comparative ITS DNA profile indicated that the three replicates (r1–r3) of the collected seeds of each P2 population (collected from the University of Jordan Research Station) and P5 (r1–r3) population (collected from al-Twal) showed a higher rate of common sequences in their ITS region. That made them eligible to combine one main cluster separated into two sub-clusters, one for the P2 and the other for the P5 population (Figures 3 and 4). The sequence of the ITS region indicates that both P2 and P5 seed lots were *C. canadensis*.

Regarding the P4 population (al-Twal site), although the entire tested replicates were also *C. bonariensis*, this population tended to separate in a single sub-cluster and highlight distinct ITS sequence (Figures 3 and 4). The updated ITS sequence may demonstrate a different evolutionary path of these populations, which may be affected by the practiced weed control methods, the type of herbicides used, and the local herbivores.

The ITS sequencing results indicated that the collected seeds lots from the fifth site (P5), which is raised from seeds collected from the University Research Station located in the Jordan Valley, and the sixth site (P6), raised from seeds collected from the University of Jordan Campus belong to *C. bonariensis*, hence, the same sub-cluster (Figures 3 and 4). According to the ITS sequence data, most of the different replicates of each seed lot still occupy the same sub-cluster, indicating the high genetic similarity of these samples.

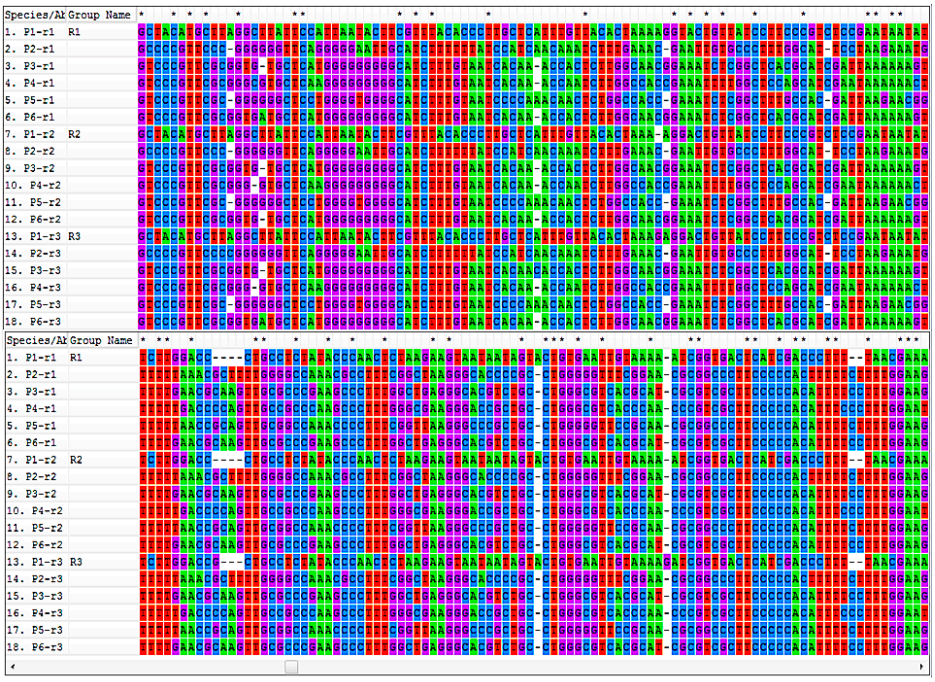

Figure 4. ITS sequencing of the three replicates (r1 to r3) of each six seed lots (P1 to P6) of *Conyza* spp. P1 (*C. bonariensis*) = Tal-al-Ramel site, P2 (*C. canadensis*) and P3 (*C. bonariensis*) = University of Jordan Research Station site, P4 (*C. bonariensis*) and P5 (*C. canadensis*) = al-Twal site and P6 (*C. bonariensis*) = University of Jordan Campus site. A, C, G and T are DNA nucleotides. * refers to identical nucelotides with same color.

3.2. Field Trial at Tal-al-Ramel

The effects of herbicides on *C. bonariensis* were visually estimated in the field two weeks after application (Table 3). The paraquat used at rates 2.5, 5.0, and 25 kg ha^{-1} caused no obvious phytotoxic effect on weeds. However, the level of injuries following oxyfluorfen in the highest dose and oxadiazon in all doses significantly differed from the control treatments. Additionally, oxadiazon and oxyfluorfen-treated plants showed a slight change in green color with some burning on leaf tips (Figure 5).

Table 3. Visual estimations of foliage-applied herbicides' effects on *Conyza bonariensis* of Tal-al-Ramel population (P1) control at 15 days after herbicides application (DAT).

Treatments	15 Days after Herbicides Application (DAT)
Untreated (control)	10.0 ^{a}
Paraquat (2.5 kg ha^{-1})	10.0 ± 0.4 ^{a}
Paraquat (5 kg ha^{-1})	10.0 ± 0.4 ^{a}
Paraquat (25 kg ha^{-1})	9.0 ± 0.6 ^{ab}
Oxadiazon (5.0 kg ha^{-1})	7.8 ± 0.5 ^{b}
Oxadiazon (10.0 kg ha^{-1})	7.4 ± 0.6 ^{b}
Oxadiazon (50 kg ha^{-1})	7.8 ± 0.6 ^{b}
Oxyfluorfen (3.3 kg ha^{-1})	9.8 ± 0.6 ^{a}
Oxyfluorfen (6.6 kg ha^{-1})	10.0 ± 0.5 ^{a}
Oxyfluorfen (33 kg ha^{-1})	7.2 ± 0.7 ^{b}
LSD ≤ p (0.05)	1.8

Mean values (±SD values) for three replicates treated with each herbicide applied at different rates and evaluated using a scale of 0–10, where 0 denotes that the weed was completely controlled, and 10 means that the weed was not affected. Mean values in the column followed by the same lower-case letter are not significantly different according to Fisher's LSD at p = 0.05.

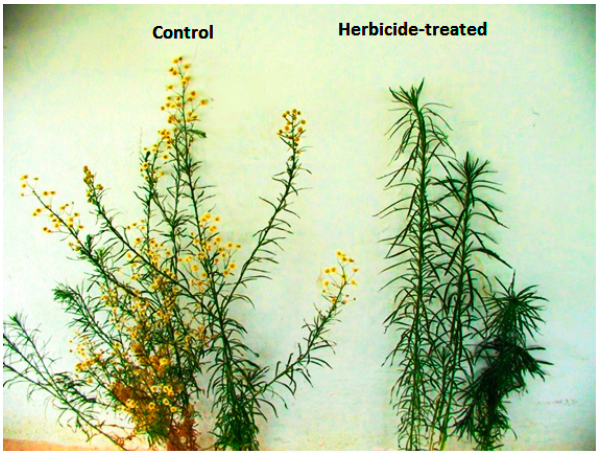

Figure 5. Control and recommended rate Paraquat-treated *Conyza bonariensis* plants from a population found in Jordan valley (Tal-al-Ramel population, P1).

3.3. Glasshouse Experiments

3.3.1. Experiment 1

The effect of paraquat, oxadiazon, and oxyfluorfen on *C. bonariensis* populations used at field rates at two spraying dates is shown in Table 4. Notably, herbicides used at recommended application rates revealed no significant effect on *Conyza bonariensis* plants grown from Tal-al-Ramel's date palm orchard population (Table 4 and Figure 5).

Table 4. The effect of field rate application of three herbicides sprayed at two dates at the full vegetative growth stage of four populations of *C. bonariensis* grown in a pot experiment.

Treatments	Recommended Rates of Application (kg ha^{-1})	First Spray 15 October 2019 Score Out of 10	Second Spray 30 October 2019 Score Out of 10
Date palm population (Tal-al-Ramel) of *C. bonariensis* (P 1)			
Control	0.0	10.0 ± 0.6 ^{a}	10.0 ± 0.2 ^{a}
Paraquat	2.5	9.2 ± 0.3 ^{ab}	7.4 ± 0.5 ^{dc}
Oxadiazon	5.0	9.4 ± 0.5 ^{ab}	8.8 ± 0.2 ^{ab}
Oxyfluorfen	3.0	9.2 ± 0.3 ^{ab}	8.4 ± 0.3 ^{bc}
University of Jordan Research Station Population of *C. bonariensis* (P3)			
Control	0.0	10.0 ± 0.3 ^{a}	10.0 ± 0.3 ^{a}
Paraquat	2.5	0.0 ± 1.4 ^{e}	0.0 ± 1.2 ^{g}
Oxadiazon	5.0	7.0 ± 1.2 ^{c}	4.2 ± 1.0 ^{f}
Oxyfluorfen	3.0	6.8 ± 0.9 ^{c}	3.4 ± 1.3 ^{f}
Al-Twal Population of *C. bonariensis* (P4)			
Control	0.0	10.0 ± 0.8 ^{a}	10.0 ± 0.3 ^{a}
Paraquat	2.5	1.6 ± 1.3 ^{d}	0.4 ± 1.4 ^{g}
Oxadiazon	5.0	8.4 ± 0.4 ^{b}	8.4 ± 0.3 ^{bc}
Oxyfluorfen	3.0	0.6 ± 0.6 ^{de}	0.6 ± 1.2 ^{g}
The University of Jordan Campus Population of *C. bonariensis* (P6)			
Control	0.0	10.0 ± 0.5 ^{a}	10.0 ± 0.4 ^{a}
Paraquat	2.5	7.2 ± 0.3 ^{c}	6.2 ± 0.6 ^{de}
Oxadiazon	5.0	7.3 ± 0.6 ^{c}	5.6 ± 0.6 ^{e}
Oxyfluorfen	3.0	7.2 ± 0.3 ^{c}	3.8 ± 0.8 ^{f}
LSD (p = 0.05)	-	1.2	1.2

Mean values (±SD) for five replicate pots treated with each herbicide on a scale of 0–10, where 10 denotes the weed is not affected by herbicide, and 0 means that the weed was completely killed. The means within the column followed by the same letter are not significantly different using the least significant difference (LSD) at p = 0.05.

The three herbicides negatively affected the growth of other weed populations to various degrees. Paraquat was most effective on weed populations grown from Al-Twal and the University of Jordan Research Station. Paraquat reduced the growth of the Al-Twal population by 84% compared with the untreated control, while oxyfluorfen caused a 94% growth reduction in the same population. However, the lowest effect of paraquat was on the weed population obtained from the University of Jordan Campus. The effects of oxadiazon and oxyfluorfen were similar on weed populations of the University Research Station and the University Campus and reduced weed growth by 30–35%, respectively, compared with the untreated control. Oxadiazon appeared least effective on all populations of *C. bonariensis*.

Repeated application of the same herbicides using the same rates resulted in no phytotoxic effects on the weed population from the date palm orchard (Table 4). The al-Twal weed population was the most sensitive; the growth of this population was reduced by 96% and 94% with paraquat and oxyfluorfen herbicides, respectively.

Generally, all herbicides showed more phytotoxic effects on weed populations obtained from other sites, with the University of Jordan population being the least responsive. More weed growth reduction was obtained in all populations at the second spray time compared with the first application. Oxyflourfen appeared more effective than oxadiazon on all weed populations. At the two application dates, repeated application of all herbicides failed to effectively control the weed from the date palm orchard (Tal al-Ramel site) and, to a lesser extent, that of the University Campus population.

It was noticed that plants of the herbicide-resistant population were not branched or of a low number of branches, late flowering, and a low number of flowers that consequently produced lower seed numbers compared with herbicide-susceptible plants (Figure 5).

3.3.2. Experiment 2

Using ten-fold higher rates of the three herbicides than the recommended ones resulted in only partial control of weed populations (Table 5 and Figure 6). At first application, weed plants from the date palm population were the least responsive to herbicide treatments. Paraquat was most effective and caused a 36% growth reduction compared with the untreated control.

Table 5. The effect of high rate application of three herbicides sprayed at two dates at full vegetative growth stage of four populations of *C. bonariensis* grown in a pot experiment.

Treatments	High Rates of Application (kg ha^{-1})	First Spray 15 October 2019 Score Out of 10	Second Spray 30 October 2019 Score Out of 10
Date palm population of *C. bonariensis* (P1)			
Control	0	10.0 ±0.4 ^{a}	10.0 ±0.2 ^{a}
Paraquat	25	6.4 ± 0.8 ^{cd}	4.8 ± 1.3 ^{c}
Oxadiazon	50	8.8 ± 0.6 ^{ab}	7.4 ± 0.5 ^{b}
Oxyfluorfen	30	8.0 ± 0.7 ^{bc}	7.8 ± 0.6 ^{b}
University of Jordan Research Station Population of *C. bonariensis* (P3)			
Control	0	10.0 ± 0.3 ^{a}	10.0 ± 0.3 ^{a}
Paraquat	25	0.0 ± 1.3 ^{g}	0.0 ± 1.2 ^{e}
Oxadiazon	50	6.2 ± 0.8 ^{cd}	4.4 ± 0.9 ^{c}
Oxyfluorfen	30	6.0 ± 0.7 ^{d}	4.0 ± 0.8 ^{c}
Al-Twal Population of *C. bonariensis* (P4)			
Control	0	10.0 ± 0.3 ^{a}	10.0 ± 0.2 ^{a}
Paraquat	25	1.4 ± 0.5 ^{fg}	1.0 ± 1.3 ^{de}
Oxadiazon	50	7.2 ± 0.6 ^{bcd}	6.8 ± 0.7 ^{b}
Oxyfluorfen	30	2.0 ± 0.3 ^{ef}	1.6 ± 1.3 ^{d}
The University of Jordan Campus Population of *C. bonariensis* (P6)			
Control	0	10.0 ± 0.3 ^{a}	10.0 ± 0.1 ^{a}
Paraquat	25	3.6 ± 1.2 ^{e}	5.0 ± 1.0 ^{c}
Oxadiazon	50	6.6 ± 0.8 ^{cd}	5.2 ± 0.6 ^{c}
Oxyfluorfen	30	6.4 ± 0.7 ^{cd}	4.6 ± 0.7 ^{c}
LSD (p = 0.05)	-	1.9	1.4

Mean values (± SD) for five replicate pots treated with each herbicide on a scale of 0–10, where 10 denotes no herbicide effects on the weed, and 0 means that the weed was completely killed. Mean separation was carried out using the least significant difference (LSD p = 0.05).

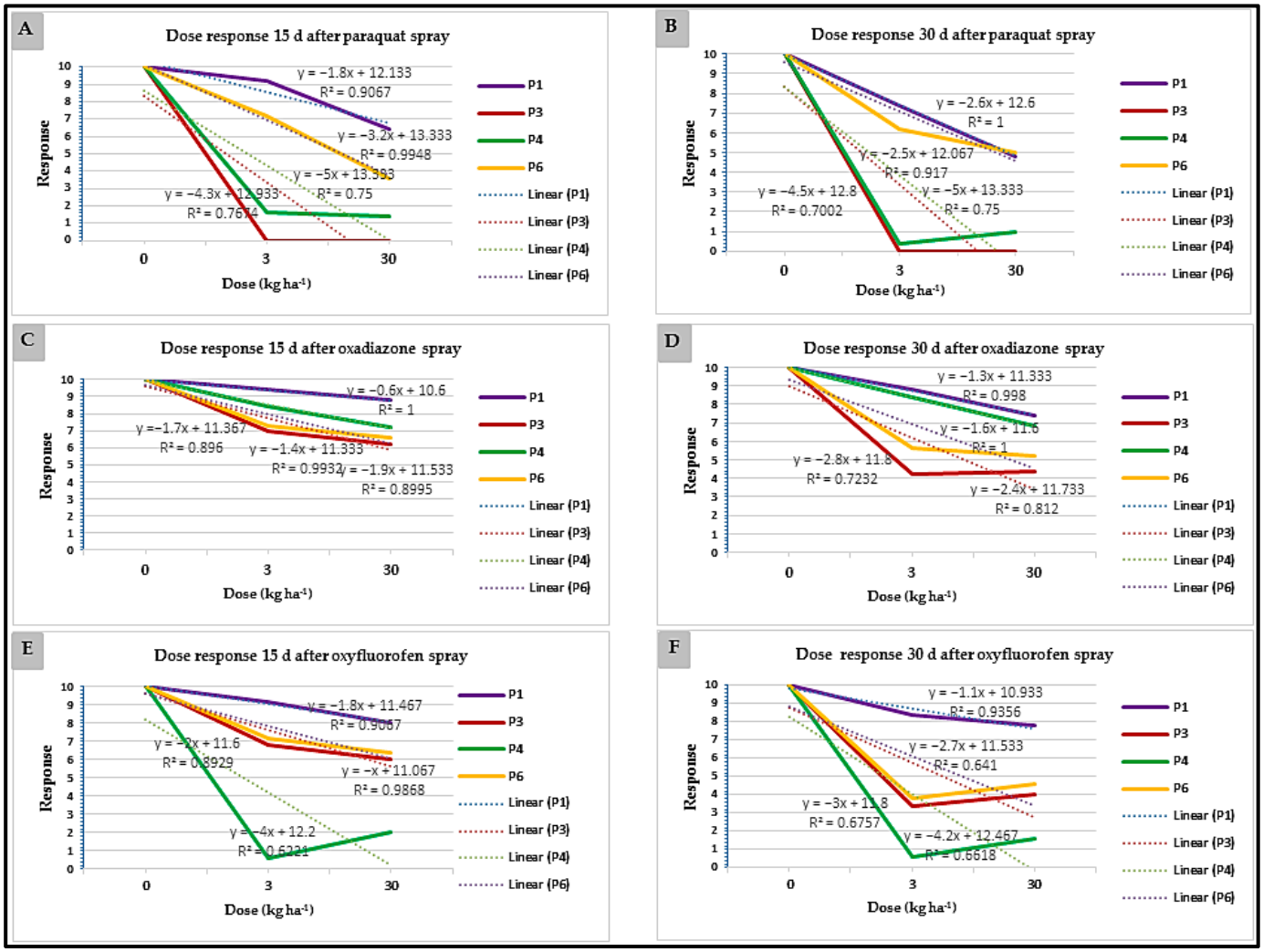

Figure 6. Dose–response curve of three herbicides applied on four populations of *C. bonariensis* (P1, P3, P4, and P6) sown in a pot experiment and sprayed at two dates at full vegetative growth stage (paraquat, (**A**,**B**); oxadiazon, (**C**,**D**); oxyfluorofen (**E**,**F**)).

Oxyfluorfen reduced weed growth by 20%, while the effect of oxadiazon was not more than 12%. The effect of the three herbicides on other weed populations varied. Paraquat was the most effective and almost eliminated weed population from the al-Twal site and, to a lesser extent, that of the University Research Station. This herbicide reduced the growth of the University Campus population of *C. bonariensis* by 64% compared with the control. The effect of oxyfluorfen on the al-Twal weed population was severe, resulting in an 80% growth reduction. In contrast, its effect on other populations was lower and similar to that of oxadiazon, showing little difference in its effect on the growth of weed populations from all sites. Still, paraquat remained more effective compared with the other two herbicides. It showed little difference in their effects on all populations except the al-Twal population, which was more affected by oxyfluorfen (Table 5 and Figure 6).

Results of herbicide applications at recommended and high rates and at two dates showed that the population grown from seeds collected from the date palm orchard (Tal-al-Ramel site) was least affected or irresponsive to the three herbicides followed by the University Campus. In contrast, the al-Twal site population was most susceptible to paraquat and oxyfluorfen and much reduced in growth at both application rates but showed a lower response to oxadiazon herbicide (Table 5 and Figure 6).

4. Discussion

Conyza spp. is among the world's worst weeds [5], well adapted to agricultural and marginal lands, roadsides, waste places, and uncultivated land. Certain species of this genera have been reported as resistant to herbicides [15–21]. In the last few years, fleabane (*Conyza bonariensis* (L.) Cronquist) population numbers dramatically increased in different locations in Jordan and became commonly observed in vegetable fields and fruit tree orchards. Many local farmers reported the ineffectiveness of paraquat, the common herbicide against fleabane. Although paraquat is recently banned, its stock is still available in local agricultural companies. Paraquat is a cheap, rapid desiccant and is thus favored by local farmers. Glyphosate was introduced as a suitable replacement since it takes over all vegetation and has a wider weed control spectrum. However, paraquat and glyphosate have been reported as resisted by certain species of *Conyza* [15,16,19,20].

A farmer field demonstration in the central Jordan valley confirmed resistance of at least one fleabane population to paraquat. A pot trial was conducted to investigate the possible resistance of different populations of *C. bonariensis* in Jordan to paraquat, oxyfluorfen, and oxadiazon. Although local farmers try other weed control methods, including tillage and grazing, both are often ineffective. That is because of the huge number of seeds produced by fleabane that can be transmitted through animal hairs or wool. In addition, the weed's ability to re-vegetate after animals partially graze shoot tips enhances the development of auxiliary buds and lateral shoots, enabling weed recovery, seed production, and re-introduction [36,37].

In the present study, three widely used herbicides were tested for possible control of *C. bonariensis* plants grown by seeds collected from weed populations in different geographical locations. Paraquat has been repeatedly mentioned and emphasized along with glyphosate as resisted by the same weed species [20,21,24]. Oxyfluorfen was suggested as a possible herbicide for managing the weed-resistant populations [14], while no reports on oxadiazon use on this weed. Our results were compatible with reports of other authors from different parts of the world [20,21] on fleabane resistance to paraquat. Results of the glasshouse experiment on four *C. bonariensis* populations showed that one of the populations was not readily controlled by paraquat, and the other two herbicides, at recommended and 10-fold rates. When the herbicides were re-applied, their effects on the same weed population were still poor. The current findings agreed with previous reports and confirmed the resistance of *C. bonariensis* to paraquat herbicide [20,21,24]. It is worth noting that oxadiazole and oxyfluorfen are widely used herbicides for weed control in vegetable fields (especially onions and garlic), orchids, and uncultivated areas across Jordan. This is a major factor in developing individuals and/or populations resistant to frequently applied pesticides. Conversely, results obtained on the oxyfluorfen effect on fleabane did not agree with previous results by [9], who reported managing resistant accessions of the weed species by the mixture of glufosinate + oxyfluorfen. The effect could be more due to glufosinate or the interaction between herbicides.

Applying 10-fold paraquat, oxadiazon, and oxyfluorfen herbicides did not improve herbicides' phytotoxicity to fleabane in the date palm weed population in Tal-al-Ramel (Table 5 and Figure 6). However, all treated plants continued growth at relatively lower levels than the untreated control. Other workers reported differences between populations of *C. bonariensis* in response to different herbicides [10,15].

A molecular analysis study has confirmed the genetic basis of herbicide resistance of at least one C. bonariensis population in Jordan (Figures 3 and 4). The resistant weed population, raised from seeds of the Tal-al-Ramel population, showed distinct genetic differences from other weed populations and the closely related populations of *C. canadensis*. In addition, the weed population of the al-Twal site was genetically different from others. It seems that it has developed its specific developmental characteristics to fit environmental conditions and agricultural practices in that site. Since paraquat-susceptible populations are not fully controlled with oxyfluorfen and oxadiazon herbicides, even though they were used at the 10-fold application rates, this raises great concerns about developing these

populations to new resistance mechanisms. Other workers reported on the genetic bases between populations of the same weed species' resistance to glyphosate [13]. At this stage, it may be necessary to diversify the herbicides used, especially those with a completely or partially different mode of action, to reduce the spreading of resistant populations. Accordingly, the efficacy of glyphosate and phenoxy herbicides can be improved in combination or integration with other weed control methods.

Despite the importance of single methods, integrating agronomic, herbicidal, and environmental factors will be more important in addressing the spreading of this harmful, herbicide-resistant weed in arable and perennial fields. Unfortunately, most Jordanian farmers do not have sufficient knowledge about *C. bonariensis* resistance to the commonly used herbicides. Nevertheless, this is the first report investigating the resistance of *Conyza* weeds to commonly applied herbicides. It is known that the three herbicides to which the studied weeds have shown resistance in the current study are a cornerstone in weed control programs in Jordan and have been in use for a long time. Therefore, priority should be given to diversifying agricultural management practices to prevent the development of herbicide-resistant populations of noxious weeds.

However, the herbicide rotation technique is emerging as a rapid alternative practice that prevents or limits the development of resistant *C. bonariensis* populations. It should also be indicated by warning labels on all chemical products explaining better the selection pressure resulting from these herbicides. To achieve a higher level of control, however, all chemicals used against C. bonariensis should be integrated with other weed control methods to prevent an escalation of herbicide resistance.

5. Conclusions

Results revealed that resistance to the three tested herbicides is well-developed in the Tal-al-Ramel population. The ITS genetic analysis efficiently distinguished between *C. bonarienses* and *C. canadensis* populations. The herbicide-resistant weed population from the Tal-al-Ramel site showed a distinct genetic background from other populations. In addition, the University Research Station's population showed tolerance to oxadiazon. Diverse management practices of *C. bonariensis* populations may counter the spread of resistant populations and reduce the weed effect on agriculture and the environment.

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