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Lactofen tolerance in commercial chickpea (*Cicer arietinum* l.) genotypes: the role of herbicide metabolism

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Abstract: Background: Chickpeas (*Cicer arietinum*) are an important type of pulse whose production is hindered by poor weed control. **Objective:** To assess lactofen selectivity levels and to uncover tolerance mechanisms in commercial genotypes. **Methods:** Four genotypes and weed species were grown under controlled-environment conditions and subjected to lactofen dose-response assays (11.2–2,880 g a.i. ha^{-1}). Visual evaluations performed until 35 days after application, when biomass was quantified and selectivity indexes obtained using regression analysis. Following ^{14}C-lactofen application, absorption and translocation were assessed until 168h after treatment (HAT), when chickpea plants were washed to remove the unabsorbed herbicide or subjected to autoradiography and tissue combustion. Lastly, ^{14}C-lactofen was applied and metabolites extracted at 96 HAT, and their retention factors (Rf) determined by thin layer chromatography and radioscanner reading. **Results:** *Tridax procumbens* and *Amaranthus spinosus* were highly susceptible to lactofen. Chickpea genotypes tolerated lactofen at the highest labelled used rate in soybeans (180 g a.i. ha^{-1}). Lactofen absorption was high (88.1%–94.6%) and translocation was limited (< 2.5%) across genotypes - a similar trend observed in susceptible species, and hence are not implicated in lactofen tolerance. One main metabolite was found in chickpea genotypes, accounting for 12.5%–19.4% of applied radioactivity; the chickpea genotype with the highest tolerance level (BRS Cicero) was the only one in which two metabolites were quantified. Altogether, these results suggest that lactofen tolerance in chickpeas could be conferred by enhanced herbicide metabolism. **Conclusions:** Lactofen could become an option for selective, post-emergence weed control in chickpeas.

Keywords: Protox; *Bidens*; *Amaranthus*; Absorption; Translocation; Metabolite

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1. Introduction

Chickpeas (*Cicer arietinum* L., also known as garbanzo beans in the U.S.) are the third most cultivated legume (Fabaceae) worldwide and an important type of pulse, providing between 17–22% protein content whose quality is ranked above other legume crops due to its higher digestibility (Jukanti et al., 2012). In 2018, chickpeas were grown on over 17 mi ha worldwide for a total production of 14.6 mi t, with average yields of 850 kg ha^{-1} (Food and Agriculture Organization of the United Nations, 2020).

Chickpea yields can be severely lowered in the field due to weed interference, which, accordingly, has been considered as the most important factor limiting its production (Solh, Pala, 1990). According to Oerke (2006), productivity levels of legume crops worldwide could decrease by up to 38% should no crop protection and weed management measure be employed.

In Brazil, yield losses of up to 70% have been reported in chickpeas regardless of nitrogen fertilization levels (Amaral et al., 2018), providing further evidence that weed interference can greatly impact the economic feasibility of chickpea cultivation. This annual legume crop is widely regarded as being highly susceptible to weed interference-related yield losses, which can be explained by the slow initial growth of its shoots as well as its open canopy architecture and reduced height of mature plants; these, combined, might explain chickpea's low competitiveness against weeds (Knights, 1991). Given the low competitiveness nature of this pulse – and the subsequent impaired cultural weed control that follows, greater importance is placed on other weed control methods as a means to prevent significant weed losses from taking place in chickpea fields.

Amongst many weed control methods, managing weeds chemically with herbicides is currently the most widely used one in large agricultural areas since it generally constitutes the quickest, cheapest and most effective weed control tool available to growers (Oliveira Jr., 2011a). However, crop selectivity levels must be considered when selecting herbicides for spraying onto chickpeas. Selectivity is defined as the differential response between plants with commercial interest (i.e. the crop) and unwanted plant species (i.e. weeds) to a chemical treatment. Therefore, the greater the difference, the safer the herbicide treatment to the crop (Oliveira Jr., Inoue, 2011).

Achieving satisfactory control levels of broadleaf (dicot) weeds in Brazilian chickpea fields is often difficult, owing to the limited number of registered postemergence herbicide molecules since only graminicides (ACCase-inhibiting herbicides; HRAC group 1) are currently available for usage (Ministério da Agricultura Pecuária e Abastecimento, 2023). However, reports in the literature indicate that chickpeas can tolerate diphenyl ethers when sprayed following emergence, despite low levels of injury (Malik et al., 2001; Boydston et al., 2017; Nath et al., 2018).

Diphenyl ethers are a chemical group of herbicides that achieve weed control by inhibiting protoporphyrinogen oxidase (E.C. no. 1.3.3.4), an enzyme involved in chlorophyll biosynthesis in plants (Oliveira Jr., 2011b). In Brazil, lactofen (IUPAC name ethyl O-[5-(2-chloro-α,α,α-trifluoro-p-tolyloxy)-2-nitrobenzoyl]-DL-lactate) is a diphenyl ether herbicide which is registered for postemergence weed control in soybeans (*Glycine max* (L.) Merr.) when sprayed at rates between 120–180 g a.i. ha^{-1}, achieving satisfactory control of dicot weeds without significant soil residual activity (Rodrigues, Almeida, 2018).

Given that the literature is still lacking concerning tolerance of commercial chickpea genotypes to broadleaf herbicides, studies focused on crop selectivity mechanisms to the same molecule can be seen as key to offering alternative for chemical weed control. Due to the phylogenetic proximity between soybeans and chickpeas, we have hypothesized that the latter might display tolerance to lactofen such that it might become an option for broadleaf weed control in this important pulse.

The present work aimed at assessing lactofen selectivity levels in commercial chickpea genotypes as well as gaining key knowledge concerning lactofen absorption, translocation, and metabolism in these cultivars. Priority was then given to commercial cultivars as these are readily available for growers.

2. Material and Methods

Plant material and plant growth conditions. Four commercial chickpea genotypes (BRS Aleppo, BRS Cícero, BRS Kalifa, and BRS Toro) were employed at the present study, as well as the weed species coat buttons (*Tridax procumbens* L., EPPO Code TRQPR), spiny amaranth (*Amaranthus spinosus* L., AMASP), hairy beggarticks (*Bidens pilosa* L., BIDPI), and carb white (*Alternanthera tenella* Colla, ALRTE). Weed seeds had been previously collected from mature plants growing in an experimental area within the University of Sao Paulo campus, in Piracicaba/SP, Brazil.

Dose-response assays aimed to study lactofen selectivity levels were conducted under controlled-environment conditions. For the absorption, translocation and metabolism assays, plants were cultivated in a growth chamber with a mean temperature of 25 °C, relative humidity of 65% and under fluorescent and artificial incandescent lights with a photoperiod of 12 h.

Weed seeds and chickpea genotypes were placed to germinate in plastic trays filled with coconut fiber substrate. Fourteen days after sowing, weed and chickpea seedlings were selected for transplanting at the rate of one plant per pot (experimental unit), each containing 2.8 dm^3 of loamy soil (Table 1). Weeds were then grown until they reached the 6-8 fully expanded leaf stage (~10 cm in height) whereas chickpea genotypes were grown until displaying 3 basal branches (~15 cm in height); such represents the growth stage at which the weeds and the crop were treated with at different rates.

Lactofen selectivity index: dose-response curves. Dose-response trials were performed simultaneously for each chickpea genotype (BRS Aleppo, BRS Cícero, BRS Kalifa, and BRS Toro) and weed species (*T. procumbens*, *A. spinosus*, *B. pilosa*, and *A. tenella*). The lactofen herbicide (Drible, 240 g a.i. L^{-1}, Sumitomo Chemical, Maracanaú/CE, Brazil) was applied at the following rates: 0, 11.25, 22.5, 45, 90, 180 (1X), 360, 720, 1,440, and 2,880 g a.i. ha^{-1}. Dose-response studies were completely randomized and were replicated four times for each treatment and plant material used. Herbicide application was performed using a CO_2-pressurized backpack sprayer equipped with a spray boom and four Teejet 110.02 flat-fan nozzles, spaced 0.5 m apart. The sprayer operated with a pressure of 196 kPa, providing an equivalent volume of 200 L ha^{-1}.

Injury and control levels were visually graded at 3, 7, 14, 21, 28, and 35 days after treatment (DAT) using a scale from 0 to 100%, at which 0 indicates lack of injury and/or control, and 100% means plant death (Velini et al., 1995). Dry mass quantification was carried out at 35 DAT by cutting plants at the soil surface level and storing harvested material in paper bags, with subsequent drying in an oven with forced air circulation at 65°C for 72 h until a constant weight was reached. Weighing was then performed on a semi-analytical balance.

The selectivity index of each chickpea genotypes was derived from dose-response trial results. Data were subject

Table 1 - Chemical and physical properties of the soil employed in this work

pH[1]	H + Al	Ca	Mg	K	CEC[2]	BS[3]	P	SOM	Sand	Silt	Clay	Texture
	---------- $mmol_c\ dm^{-3}$ ----------						$mg\ dm^{-3}$	------- % --------				Loam
5.4	10.9	26.0	7.0	1.3	62.3	34.3		1.8	74.0	5.8	20.1	

[1] pH measured in $CaCl_2$; [2] cation exchange capacity; [3] base saturation

to non-linear logistic regression analysis using the *drc* package in R (Ritz et al., 2015) according to equation 1:

$$y = \frac{a}{\left[1 + \left(\frac{x}{b}\right)^{c}\right]} \quad (1)$$

at which y is injury or control, x is lactofen rate (g a.i. ha^{-1}), a represents the maximum value, b is the dose that provides 10, 50, or 90% response (ED_{10}, ED_{50}, or ED_{90}, respectively), and c is the slope of the curve around b.

Based on the non-linear logistic regression parameters, the ED_{10}, ED_{50} and ED_{90} values were estimated and the selectivity index (SI) for each chickpea genotype was determined according to equation 2:

$$\frac{ED_{10(crop)}}{ED_{90(weed)}} \quad (2)$$

at which ED10 is the herbicide dose that provides 10% injury in the chickpea genotypes whereas ED90 is the herbicide dose that provides 90% weed control in the experiment (Ritz, Streibig, 2005). The larger the SI value, the greater the levels of lactofen selectivity (Tind et al., 2009).

Absorption and translocation of [^{14}C]-lactofen in chickpea genotypes. Pots containing one plant per commercial chickpea genotype were cultivated in triplicates following the aforementioned growth conditions and parameters. At the 5-to-7 fully expanded leaf stage, non-radiolabeled lactofen herbicide was applied at 180 g a.i. ha^{-1} using a CO_2-pressurized backpack sprayer equipped with a spray boom and four Teejet 110.02 nozzles, spaced 0.5 m apart and calibrated to deliver 200 L ha^{-1} at 196 kPa. The third leaf, counted from the apex to the base of each plant was protected with a plastic bag to avoid direct contact with the spray jet. Afterwards, the ^{14}C-lactofen was mixed into the solution with the commercial herbicide and applied five drops (one drop on each of the 5 leaflets closest to the stem) of 1.0 µL of the radiomarked solution on the previously protected leaf using a microapplicator (Hamilton PB6000 Dispenser, Hamilton Co., USA), equaling ~101,342.00 DPM plant-1.

Treated plants were taken back to the growth chamber and assessed at either 12, 24, 48, 96, or 168 hours after treatment (HAT). At each evaluation timing, chickpea plants were removed from the pots and split into three sections, *viz*. treated leaf, other leaves, and roots. The amount of unabsorbed herbicide was determined by washing the leaf that received the radiolabeled herbicide with 5 mL $plant^{-1}$ of a solution containing 1:1 of methanol and deionized water. Two 500 µL aliquots of the leaf wash solution were obtained upon washing. Afterwards, 10 mL of scintillating solution were added to the aliquots, followed by liquid scintillation spectrometry analysis (Tri-Carb 2910 TR contain, LSA Perkin-Elmer, Waltham, MA, USA). Results for each aliquot were normalized in relation to the total solution volume.

Results for each aliquot were normalized in relation to the total solution volume. The translocation of radiolabeled herbicides was observed qualitatively by autoradiography and quantitatively by combustion of plant tissues. To this end, plants were dried in a forced ventilation oven for 48 h at 50 °C, after which these were developed on Super Resolution plates (Sr type) for 24 h and analyzed by autoradiography on the Cyclone® Plus radio scanner. Each plant was separated into treated leaves, other leaves, roots and cotyledons for plant tissue combustion, and a biological oxidizer was used (OX500, RJ Harvey Instrument Corporation, Tappan, NY, USA); results of roots and cotyledons were summed at the end of the analysis. Radioactivity was then quantified by liquid scintillation spectrometry. Absorption by the treated leaf was determined as the percentage of radioactivity present inside the plant. Herbicide translocation was determined as the percentage of radioactivity in each sectioned part of the plant. For the mass balance of the radiolabeled herbicide, the sum of the radioactivity inside the plant with the radioactivity in the washing solution of the treated leaf was calculated. The proportion of absorbed herbicide was determined using the following equation:

$$\%H_{abs} = \left[\frac{ot}{(ot + wl)}\right] \times 100 \quad (3)$$

at which $\%H_{abs}$ = herbicide absorbed by plants; *ot* = amount of ^{14}C detected in oxidized tissues; and *wl* = amount of ^{14}C detected in the treated leaf wash.

For lactofen translocation the following equation was used:

$$\%H_{abs} = 100 - \left[\frac{al}{(al + ol)} \times 100\right] \quad (4)$$

where $\%H_{tr}$ is the proportion of herbicide translocated, *al* = the amount of ^{14}C measured in the treated leaf, and *ol* = the amount of ^{14}C detected in other untreated tissues of the plant.

Absorption and translocation over time were analyzed using the *drc* package in the R software, according to Kniss et al. (2011) following the criteria of Spiess and Neumeyer (2010). The model that best represented the data was the rectangular hyperbolic regression, according to the equation below:

$$Absorption\ or\ translocation = \frac{(b \times t)}{\{[1 + (b \times t)]/A_{max}\}} \quad (5)$$

where absorption or translocation is expressed as a percentage of the dose applied, Amax = maximum percentage of absorption or translocation, b = relative slope

of the curve when t approaches zero or the rate at which the herbicide is absorbed or translocated after application and t = time after application. This model was chosen due to the lower Akaike information criterion corrected (AICc) value compared to the asymptotic regression model, with an evidence index of 7,015, i.e. 7,015 times more likely for the rectangular hyperbolic regression model to be more suitable than the asymptotic regression model.

Metabolism of ^{14}C-lactofen in chickpea genotypes. Three replicates of each chickpea genotype were cultivated in growth chambers as previously mentioned. At the 5-to-7 fully expanded leaf stage, each chickpea plant received the application of 16 drops of 1.0 µL each of a solution containing ^{14}C-lactofen. Droplets were distributed on the third and fourth leaves when counted from the top of the plant, representing one drop to each of the 8 most basal leaflets within each leaf for an estimated total of 257,072 dpm plant^{-1}. Herbicide application was carried out as previously described concerning the absorption and translocation studies, with the application of the non-radiolabeled herbicide in advance with protection of the leaves that would receive ^{14}C-lactofen.

^{14}C-lactofen extraction was performed following Silva et al. (2019) whose methodological procedures were originally adapted from Bell et al. (2011). At 96 HAT, leaves that had received the radiolabeled herbicide were washed with 16 ml of a 1:1 mixture of methanol and deionized water per plant, representing around 1 ml leaflet^{-1}. Right after washing, treated leaves from each plant were combined into a single 50 mL falcon tube and stored at -20 °C; importantly, only the treated leaf was selected for the extraction procedure due to the low (< 2.5%) translocation of lactofen molecules which had already been observed in previous steps of this research project. Afterwards, 10 ml of methanol were added to each falcon tube containing frozen leaves, along with five metal spheres to facilitate maceration. Tubes were then manually shaken for 2 minutes and centrifuged at 4,000 rpm for 5 minutes at 10 °C, with subsequent removal of the supernatant, which was placed in another tube. The process of adding methanol, agitating and centrifuging was performed three times consecutively.

Approximately 30 ml of solution were obtained at the end of the extraction phase, which were concentrated to 5 ml using a rotary evaporator (Rotavapor® R-215). Thin Layer Chromatography (TLC) was used to separate the metabolites, with the application of 100 µL of the extracted solution on silica gel plates as a stationary phase and a solvent system of benzene:acetone (2:1 v /v) as the mobile phase. The already applied TLC plates were placed in a glass vat containing 100 mL of mobile phase, and removed when the liquid reached 15 cm in height in relation to the applied region. Upon drying the plates, these were sensitized on Super Resolution plates for 24 h and submitted for analysis on a Cyclone® Plus radio scanner, where the metabolites were identified by comparing their retention factor (Rf) with that of an analytical standard. The Rf is calculated by dividing the distance traveled by each compound and the distance traveled by the solvent (Collins et al. 1993), as follows:

$$Rf = \frac{dc}{ds} \quad (6)$$

where *Rf* = Retention factor; *dc* = distance traveled by the compound, and *ds* = distance traveled by the solvent.

3. Results and Discussion

3.1 Selectivity index of commercial chickpea genotypes to lactofen

Lactofen rates considerably larger than the labelled use one were required to decrease dry mass accumulation of all commercial chickpea genotypes by 50% (Table 2 and Figure 1), indicating that chickpeas can tolerate this herbicide, corroborating reports by Lopes (2018) and Araujo (2017). Furthermore, a 10% reduction in dry mass was calculated to require 94.8 ± 21.5 (standard error), 41.7 ± 10.4, 39.3 ± 7.4, and 37.6 ± 8.9 g a.i. ha^{-1} for genotypes BRS Cicero, BRS Kalifa, BRS Toro, and BRS Aleppo, respectively, indicating that the former displayed the highest level of tolerance to lactofen among all the tested commercial genotypes (Figure 2).

The analysis of dry mass indicated that nearly all weed species employed at the present study were more susceptible to lactofen relative to chickpeas, as even very low rates (30 g a.i. ha^{-1}, representing ~0.16x of the field rate of this PPO inhibitor) were found to decrease dry mass by almost 90% relative to the untreated control plants (Table 3). The exception, however, was *B. pilosa*, which required a much

Table 2 - Model parameters and lactofen rates required for 50% (ED_{50}) or 10% (ED_{10}) crop injury, as observed for four commercial chickpea genotypes at 35 days after treatment in dose-response assays

Genotype	a	c	b (ED_{50}) [1]	ED_{10} [1]
BRS Cicero	82.9 ± 6.1	-1.4 ± 0.3	431.2 ± 66.9	94.8 ± 21.5
BRS Kalifa	96.8 ± 17.9	-0.8 ± 0.1	630.3 ± 350.4	41.7 ± 10.4
BRS Toro	109.5 ± 11.9	-0.9 ± 0.1	451.4 ± 144.3	39.3 ± 7.4
BRS Aleppo	100.7 ± 16.6	-0.8 ± 0.1	577.0 ± 289.5	37.6 ± 8.9

[1]herbicide dose needed to cause crop injury levels equal to 50% (ED_{50}) or 10% (ED_{10})

Figure 1 - Photographs taken at 35 days after lactofen spraying at increasing rates onto four commercial chickpea genotypes. "1 D" represents the labelled rate (180 g a.i. ha^{-1}) used for registered crops such as soybeans

higher rate (293.6 g a.i. ha^{-1}) for 90% dry mass reduction at 35 DAT; such lies beyond the labelled rate for this herbicide (180 g a.i. ha^{-1}). Accordingly, control levels for each weed species in response to a range of lactofen rates (Figure 3) varied significantly and further exacerbate *B. pilosa*'s lower susceptibility to lactofen relative to other three weed species studies (i.e. *A. spinosus*, *T. procumbens*, and *A. tenella*).

T. procumbens and *A. spinosus* displayed the highest levels of susceptibility to lactofen since a rate as low as 15 g a.i. ha^{-1} was required to cause a 90% decrease in dry mass accumulation (Figure 3). As a result, data regarding these weed species did not fit well into a 3-parameter log-logistic model, requiring Gompertz model instead (Ritz, Streibig, 2005) for estimation of their selectivity indexes (SI).

SI is a commonly-used parameter for comparing herbicide tolerance levels in a given crop relative to susceptible weed species; the larger the value, the greater herbicide tolerance will be in the crop (Tind et al., 2009; Bartley, 1993). SI values above 1 were estimated for nearly all weed species employed at the present study, further suggesting lactofen could be sprayed selectively onto chickpeas. Given *B. pilosa*'s lower susceptibility to this herbicide (Figure 3), SI values relative to this Asteraceae weed species were many times lower than SI obtained for the remaining weeds (Table 4). When taking *A. spinosus* data into account, chickpea cultivars were found to display high SI values, ranging from 3.6 to 9.1 (BRS Cicero), which as previously mentioned displayed the highest levels of tolerance to lactofen amongst all cultivars tested. SI values

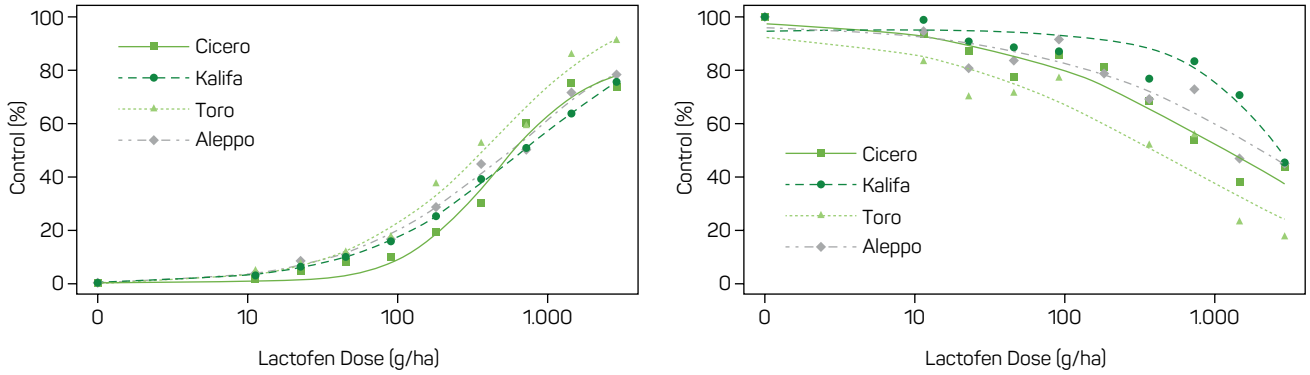

Figure 2 - Control (%) and dry mass reduction (%) levels observed for four commercial chickpea genotypes at 35 days after treatment with increasing rates of lactofen

Table 3 - Model parameters and lactofen rates required for 50% (ED_{50}) or 90% (ED_{90}) control of each of four broadleaf weed species, as observed 35 days after treatment in dose-response assays. Weed species were arranged from lowest to highest ED_{50}

Weed species	a	c	b (ED_{50}) [1]	ED_{90} [1]
Amaranthus spinosus	99.9 ± 0.6	-0.4 ± 0.0	4.1 ± 0.4	10.5 ± 5.7
Tridax procumbens	98.9 ± 1.2	-0.2 ± 0.0	5.6 ± 0.7	14.8 ± 17.7
Alternanthera tenella	100.5 ± 1.2	-1.7 ± 0.3	8.1 ± 0.9	29.3 ± 4.5
Bidens pilosa	105.2 ± 3.8	-0.8 ± 0.1	19.2 ± 3.0	293.6 ± 140.9

[1]herbicide dose that provides 50% (ED_{50}) or 90% (ED_{90}) control

relative to *T. procumbens* and *A. tenella* dose-response data were in between those already calculated for *B. pilosa* and *A. spinosus* (Table 4). Nonetheless, in spite of low SI values relative to *B. pilosa* (which could indicate the herbicide is less selective to the crop), one can still conclude that chickpeas can tolerate lactofen given the nature of the SI parameter (i.e. that comparisons be made using data from a susceptible weed species) and the resulting elevated SI values when other weed species were analyzed.

3.2 Selectivity mechanism of chickpea genotypes to lactofen

3.2.1 *Absorption*

Absorption is defined as the fraction of applied ^{14}C-lactofen which is absorbed by chickpea leaves (Oyan, 2019). Here, absorption of lactofen quickly started soon after application and followed a similar trend regardless of chickpea genotype (Figure 4), reaching maximum values close to 35 HAT. Total absorption ranged from 88.1% to 94.5% with no significant differences among genotypes (Table 5).

Total lactofen absorption values found at the present study (Table 5) greatly resemble those published by Shaw and Wesley (1993), who reported total absorption values of 81%, 83%, and 87% for common cocklebur (*Xanthium strumarium* L.), pitted morningglory (*Ipomoea lacunosa* L.), and prickly sida (*Sida spinosa* L.), respectively. Given similar total absorption levels between chickpea leaves (present study) and susceptible weeds (Shaw, Wesley, 1993) and also taking into account that those weeds were properly controlled, our results seem to altogether indicate that the mechanism of tolerance to lactofen in chickpeas is not related to differential (i.e. lower) herbicide uptake.

3.2.2 *Translocation*

Translocation can be defined as the sum of all the radioactivity that can be recovered by burning plant tissues, with the exception of the treated leaf (Oyan, 2019). Here, total ^{14}C-lactofen translocation values were found to be lower than 2.5% regardless of chickpea genotype (Table 5). Such, in turn, can be expected since PPO-inhibiting herbicides such as lactofen generally present little or no translocation capabilities due to their physico-chemical properties (Oliveira Jr., 2011b). Interestingly, total translocation levels obtained at this study resemble those published by Shaw and Wesley (1993) who reported lactofen translocation values ranging from 1.0% to 4.2% when studying the weed species *X. strumarium*, *I. lacunosa*, and *S. spinosus*.

Within the small amount of ^{14}C-lactofen that translocated out of the treated leaf, the majority of it was found to move to other leaves, with translocation values ranging from 1.3 to 1.4% depending on actual genotype (Table 5); roots were found to display low lactofen radioactivity (< 1% total translocation), with no differences across genotypes.

Low translocation of ^{14}C-lactofen within the plant was also observed qualitatively by autoradiography taken at 96 HAT (Figure 5). In fact, quantities found in non-treated

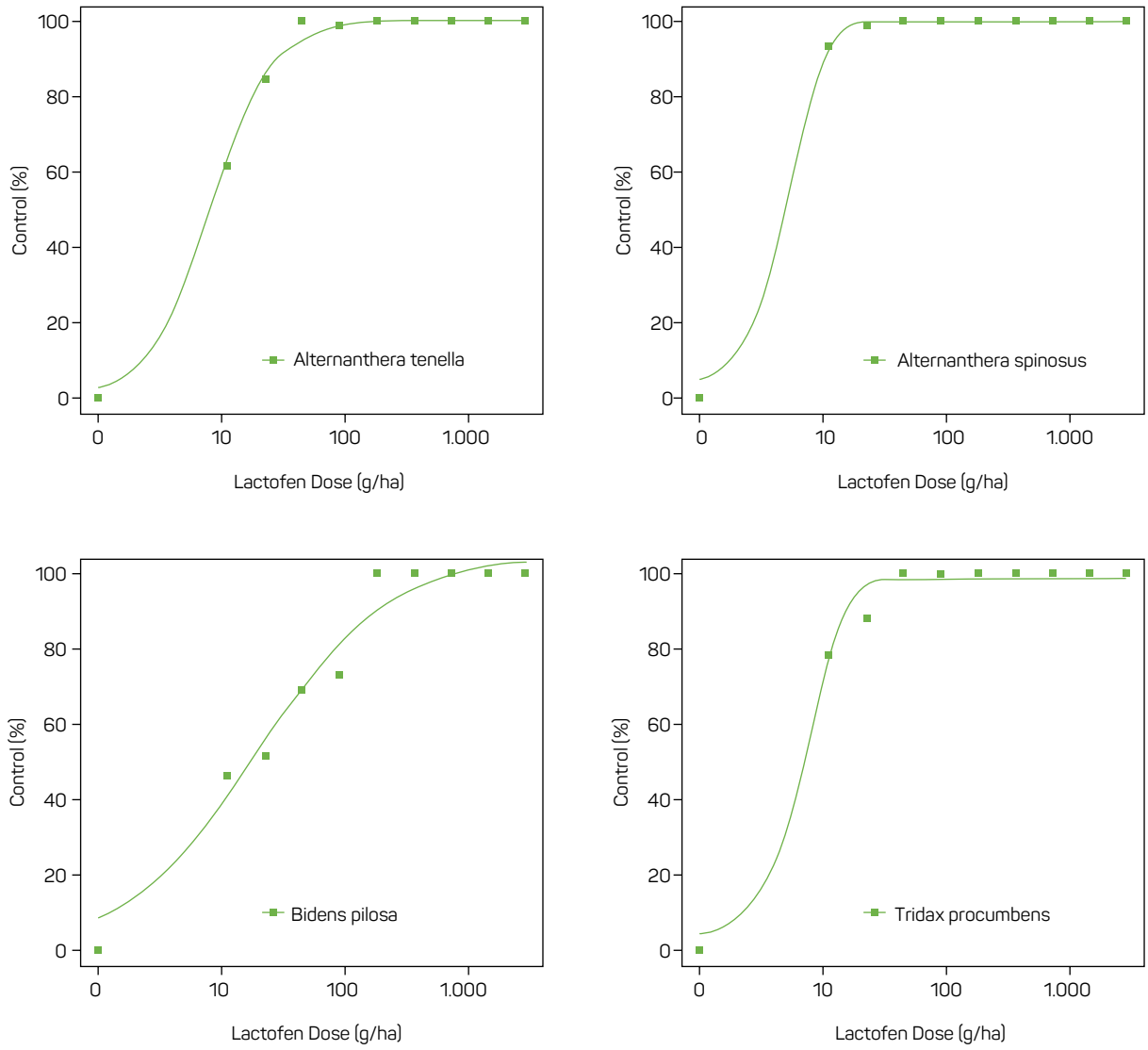

Figure 3 - Control (%) levels observed for each of four broadleaf (dicot) weed species at 35 days after treatment with increasing rates of lactofen

Table 4 - Lactofen selectivity indexes (SI) estimated for four commercial chickpea genotypes in comparison to each of four weed species employed at the present study. Larger values indicate larger herbicide selectivity to the crop. Species were arranged from highest to lowest SI values

Comparative species	BRS Cicero	BRS Kalifa	BRS Toro	BRS Aleppo
Amaranthus spinosus	9.07	3.98	3.75	3.59
Tridax procumbens	6.39	2.8	2.64	2.53
Alternanthera tenella	3.24	1.42	1.34	1.28
Bidens pilosa	0.32	0.14	0.13	0.13

leaves as well as roots were so low that such could not be properly detected, being only visualized in the treated leaf instead. Overall, as low translocation is an inherent characteristic of this herbicide – which might also be observed in susceptible weed species (Shaw, Wesley, 1993), such cannot be considered as the mechanism of tolerance to lactofen in chickpeas.

3.2.3 *Metabolism*

Two main lactofen metabolites were found in plant extracts originating from commercial chickpea genotypes at 96 HAT, with a metabolite with Rf of 0.5 being observed in all the tested genotypes (Table 6). Interestingly, chickpea genotype BRS Cicero, which exhibited the highest level of

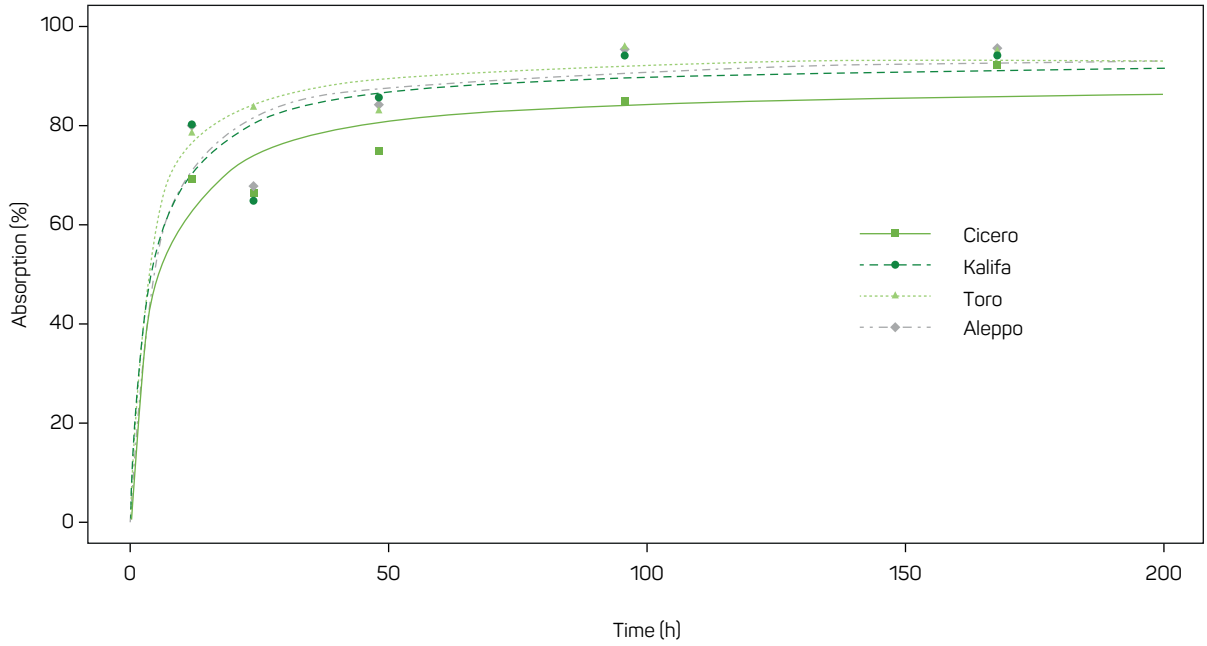

Figure 4 - ^{14}C-lactofen absorption (%) by each of four commercial chickpea genotypes, as observed throughout 196h after treatment with this PPO-inhibiting herbicide

Table 5 - Total ^{14}C-lactofen absorption (%) and translocation (%) values quantified for each of four commercial chickpea genotypes employed at this study

Genotype	Total absorption	Translocation		
		Leaves	Roots	Total
	---------- % ----------			
BRS Toro	94.5 ± 4.3 a[1]	1.3 ± NA a	0.6 ± NA a	1.8 ± NA a
BRS Aleppo	94.7 ± 4.5 a	1.4 ± 0.1 a	0.9 ± 0.2 a	2.2 ± 0.5 a
BRS Kalifa	93.0 ± 4.5 a	1.3 ± 0.3 a	0.8 ± NA a	2.1 ± 0.4 a
BRS Cicero	88.1 ± 4.6 a	1.3 ± 0.3 a	0.5 ± 0.3 a	1.8 ± 0.4 a

[1] Means followed by the same lower-case letter within columns are not statistically different according to Tukey´s HSD test (α = 0.05)

Figure 5 - Autoradiographies taken from each of four commercial chickpea genotypes at 96 h after treatment with ^{14}C-lactofen. Arrows indicate the treated leaf

tolerance to lactofen (Table 4), was also found to display a second metabolite with Rf equal to 0.4.

The analytical standard used for comparison purposes peaked at an Rf value equal to 0.0 which, in turn, correctly corresponds to its application timing, as well as an Rf value of 0.6 that corresponds to the lactofen herbicide, with 96.4% of the radiation found in the standard and ranging from 58.0% to 73.0% in the tested genotypes (Table 6).

Higgins et al. (1988) observed four metabolites at 96 h after lactofen application onto *Ipomoea lacunosa* and ivyleaf morningglory (*Ipomoea hederacea* L.), with Rf values equal to 0.03 (~15% of the total), 0.13, 0.26, and 0.43 (less than 3% each) which differs from results presented herein. Therefore, given that (i) all commercial chickpea genotypes displayed at least one metabolite (Rf equal to 0.5) in reasonable amounts (12.5% to

Table 6 - Metabolites (expressed in % of applied radioactivity) quantified at 96 h after treatment following extraction of ^{14}C-lactofen relative to its analytical standard and separated according to their retention factors (Rf), in four commercial chickpea genotypes

Genotype	Rf [1] = 0,0 (application)	Rf = 0,4 (metabolite 1)	Rf = 0,5 (metabolite 2)	Rf = 0,6 (lactofen)
BRS Aleppo	12.7 a[2]	0.0 b	15.2 a	72.1 ab
BRS Cicero	13.2 a	9.4 a	19.4 a	58.0 b
BRS Kalifa	14.6 a	0.0 b	12.5 a	73.0 ab
BRS Toro	14.0 a	0.0 b	13.4 a	72.6 ab
Analytical standard	*3.6* b	*0.0* b	*0.0 b*	*96.4* a

[1]Retention factors; [2]Means followed by the same lower-case letter within columns are not statistically different according to Tukey´s HSD test (α = 0.05)

19.4%) and that (ii) such differs from metabolites found in susceptible weed species (Higgins et al., 1998), lactofen tolerance in chickpeas could be at least partially conferred by enhanced metabolism of herbicide molecules. However, > 70% of the parent lactofen is still in the plant at 96 HAT – resembling lactofen-sensitive weeds quantified by Higgins et al. (1989). In fact, species where metabolism is clearly shown to confer tolerance – such as soybeans - can metabolize lactofen much more quickly, with 85-95% lactofen metabolized by 24 h (Frear et al. 1983). When lactofen´s quick herbicide action is also taken into account, it seems unlikely that enhanced lactofen metabolism is the sole contributor for this tolerance case. Since free-radical breakdown is not regarded as an important mechanism of protox tolerance or resistance, remaining possibilities are (i) herbicide sequestration; (ii) herbicide-insensitive protox enzyme; (iii) chloroplast and/or mitochondria protox overexpression; (iv) rapid scavenging of cytoplastic protoporphyrinogen and/or protoporphyrin; and (v) inactivation of the conversion of protoporphyrinogen into protoporphyrin in the cytoplasm.

4. Conclusions

Under the test conditions presented and discussed herein, *Tridax procumbens* and *Amaranthus spinosus* could be regarded as displaying the highest levels of susceptibility to lactofen amongst the tested weed species, as lactofen rates as low as 15 g a.i. ha^{-1} provided 90% weed control. Commercial chickpea genotypes, on the other hand, were tolerant to lactofen at the labelled rate (180 g a.i. ha^{-1}) that is used for crops at which this PPO-inhibitor is registered. Such is demonstrated by SI above 1 when the crop was compared to all weed species except *Bidens pilosa*, which surprisingly required lactofen rates above the labelled use one for proper control.

Lactofen absorption was high (88.1% to 94.6%) and its translocation was limited (< 2.5%) regardless of chickpea genotype. Such levels were similar to reports in the literature concerning lactofen susceptible species, indicating these are most likely not related to the tolerance mechanism in chickpeas. However, at least one main metabolite was found in all genotypes studied, accounting for 12.5%–19.4% of the applied radioactivity, strongly suggesting that lactofen tolerance in chickpeas could be at least partially conferred by enhanced metabolism of herbicide molecules.

We hypothesized that the second metabolite found in BRS Cicero – which was not observed in the remaining chickpea genotypes tested - is at least partially responsible for this genotype's greater tolerance to lactofen relative to the others. Such hypothesis, however, remains to be elucidated. Furthermore, results altogether point towards a potential safe use of lactofen as a selective, postemergence tool in chickpea fields. Ongoing research efforts are thus aimed at (i) determining chickpea yields in the field in response to a range of meaningful rates of lactofen and (ii) further clarifying lactofen degradation in chickpeas using metabolic inhibitors as well as genetics tools to pinpoint candidate genes that could be related to the tolerance trait.

Author's contributions

All authors read and agreed to the published version of the manuscript. MAD, RVF, VLT, and LSA: conceptualization of the manuscript and development of the methodology; data collection and curation; data analysis; data interpretation; funding acquisition and resources: not related to this project; RVF: supervision; project administration; MAD, RMP, BRC, and LRR: writing the original draft of the manuscript; writing, review and editing.

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