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AUGUSTO KALSING

ECOLOGICAL AND GENETIC FACTORS DETERMINING THE DISPERSION OF HERBICIDE RESISTANCE IN *Conyza* spp. ACROSS SOYBEAN CROPPING REGIONS AND SEASONS IN BRAZIL AND PARAGUAY

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Botucatu, 06 de janeiro de 2023

To my children,

Luisa and Lucas,

I dedicate

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ABSTRACT

Soybean has been grown in about 45 million hectares in Brazil and Paraguay in 2022. This crop is cultivated across regions that differ by environment and management. Herbicides are one of the main methods to protect soybean yields against weeds. However, over-reliance of herbicides imposed high selection pressure on weeds. *Conyza* spp. have been exhibited multiple-resistance to herbicides in these countries. Frequency and dispersion of resistance are variable among soybean cropping regions. Thus, the general objective of the study was to access the frequency and dispersion of herbicide-resistant *Conyza* spp. across regions and seasons in Brazil and Paraguay. Seeds from mature plants that have escaped the control by diverse herbicide programs were sampled from more than 400 preharvest soybean fields in 2019, 2020 and 2021. In the first assay, five DNA barcodes and 32 morphological traits were used to identify the *Conyza* species associated to soybeans and their dispersion across the regions. After, 2,998 single-nucleotide polymorphisms were found by genotyping by sequencing to investigate the genetic diversity and structure among accessions of *Conyza* spp. Finally, in the third assay, the frequency and dispersion of *Conyza* accessions resistant to five synthetic auxins were evaluated using pre-characterized discriminating doses. The combination of *its* and *rps16-trnQ* gene regions and reproductive traits supported the taxonomic resolution of *C. bonariensis* and *C. sumatrensis* in the sampled fields. Strong genetic structure has separated *C. bonariensis* from *C. sumatrensis* and clustered individuals into three genetic groups associated to the cropping regions. However, individuals of *C. bonariensis* were structured within *C. sumatrensis* clusters, which indicate interspecific gene flow of resistance alleles between *Conyza* species. Resistance to 2,4-D was widespread in Brazil, mainly in in the Southern of the country, while resistance to dicamba and triclopyr was significantly less frequent and dispersed. Resistance to auxin herbicides in *Conyza* spp. is a growing problem in Brazil and must be managed based on strict herbicide rotation and integrated weed management.

Keywords: *Conyza bonariensis*; *Conyza sumatrensis*; DNA barcoding; DNA sequencing; hybridization.

RESUMO

A soja foi cultivada em cerca de 45 milhões de hectares no Brasil e Paraguai em 2022. O seu cultivo ocorre em regiões que se diferenciam entre si em ambiente e manejo. Os herbicidas são um meio para evitar perdas de produtividade por plantas daninhas. Mas, o uso excessivo de herbicidas impõe pressão de seleção sobre estas espécies. *Conyza* spp. evoluíram para resistência múltipla a herbicidas no Brasil e Paraguai. A frequência e dispersão da resistência é variável entre as regiões de cultivo de soja. Assim, o objetivo geral do estudo foi o de avaliar a frequência e a dispersão de *Conyza* spp. resistente a herbicidas em regiões e safras no Brasil e no Paraguai. Sementes de plantas que escaparam do controle por programas de herbicidas foram amostradas em mais de 400 lavouras de soja em pré-colheita em 2019, 2020 e 2021. No primeiro ensaio, cinco códigos de barra de DNA e 32 caracteres morfológicos foram usados para identificar espécies de *Conyza* em soja e sua dispersão nas regiões de produção. No segundo ensaio, 2.998 polimorfismos de nucleotídeo único foram obtidos por sequenciamento para investigar a diversidade e estrutura genética entre acessos de *Conyza* spp. No terceiro ensaio, a frequência e dispersão de acessos de *Conyza* resistentes foram avaliados frente à cinco auxinas sintéticas por meio de doses discriminantes caracterizadas. A combinação das regiões gênicas *its* e *rps16-trnQ* e caracteres morfológicos reprodutivos suportou à resolução taxonômica de *C. bonariensis* e *C. sumatrensis* em áreas de soja. Uma forte estruturação genética separou *C. bonariensis* de *C. sumatrensis* e agrupou os indivíduos em três grupos genéticos que foram associados às regiões de cultivo de soja. Todavia, indivíduos de *C. bonariensis* foram agrupados junto à indivíduos de *C. sumatrensis*, o que indica fluxo gênico interespecífico de alelos de resistência entre as espécies. A resistência ao 2,4-D encontra-se disseminada no Brasil, principalmente na região Sul do país, enquanto a resistência ao dicamba e ao triclopir foi menos frequente e dispersa. A resistência às auxinas sintéticas em *Conyza* spp. é um problema crescente no Brasil e deve ser gerido com rotação de herbicidas e manejo integrado de plantas daninhas.

Palavras-chave: *Conyza bonariensis*; *Conyza sumatrensis*; código de barra de DNA; sequenciamento de DNA; hibridização.

SUMMARY

GENERAL INTRODUCTION

Conyza bonariensis (L.) Cronquist and *C. sumatrensis* (Retzius) E. H. Walker are two of the most frequent, dispersed, and problematic weeds in the soybean cropping systems in South America (BAJWA et al., 2016; HEAP, 2022). These weeds contain varieties, morphological similarities and are often misidentified as *C. canadensis* (L.) Cronquist, and has been collectively reported as *Conyza* spp. or *Conyza* complex (MENDES et al., 2021; PRUSKI; SANCHO, 2006). In Brazil, the estimated soybean yield losses of one plant m^2 of *C. bonariensis* established at 81, 38 and 0 days prior to crop sowing was of 36, 12 and 1%, respectively (TREZZI et al., 2015). Both species are highly prolific, and plants can produce more than 200 thousand seeds that germinate in a wide range of soil and weather conditions mainly from late fall to spring (VIDAL et al., 2007). *Conyza* weeds are also well adapted to the tropical climate regions and conservation cropping systems, such as no- or reduced-tillage systems largely used to cultivate soybean in South America.

Accessions of *Conyza* spp. resistant to herbicides have emerged as one of the major threats of soybeans in the early 2000s in South America since they have evolved resistance against several key herbicides. *C. bonariensis* resistant to glyphosate has been recorded from Argentina, Brazil, and Colombia, while *C. sumatrensis* resistant to the same herbicide has been reported in Brazil and Paraguay (HEAP, 2022). In recent years, these accessions have been managed in approximately half of Brazilian soybean area, where the weed control costs have risen by 42% due to the alternative herbicides (ADEGAS et al., 2017; LUCIO et al., 2019). Other important herbicides against which accessions of *C. sumatrensis* have evolved resistance are chlorimuron-ethyl, diuron, paraquat, saflufenacil and 2,4-D in Brazil, as well as chlorimuron-ethyl and paraquat in Paraguay (HEAP, 2022). Herbicide-resistant *C. sumatrensis* has been characterized for six sites of action herbicides and cases of multiple resistance evolution to three and even five herbicides have been detected (ALBRECHT et al., 2020b; PINHO et al., 2019).

In recent years, soybeans have been cultivated in approximately 41 million hectares in Brazil (USDA, 2022) across five macroregions (MRSs) that differ by both climate and latitude (KASTER; FARIAS, 2012). In addition to their climatic characteristics, these regions differ by the technological level of production, which influences the weed flora and tactics for its management (LUCIO et al., 2019). Soybean

has been also cultivated in Paraguay on approximately 3,5 million hectares, where climatic variables and cropping practices also differ among cropping regions (USDA, 2022). We hypothesized that closely related species of *Conyza* spp. co-occur across the landscape, forming unique population structures and experiencing some gene flow and local adaptions. In addition, we hypothesized that severity and scale of herbicide-resistance *Conyza* spp. depend on the herbicide, its usage patterns and production region (MRS).

Because of *Conyza* spp. evolved resistance to six mode-of-action herbicides and possess high dispersal potential, it is important to evaluate the mechanisms related to the spread of resistance. Thus, the present research aimed to characterize accessions of *Conyza* spp. from soybean fields in Brazil and Paraguay to assess the factors and risks of resistance dispersal. The thesis was divided in three chapters to cover aspects of spread of herbicide resistance. The first chapter was entitled “Taxonomic resolution of *Conyza* spp. through morphological and molecular markers and their dispersion across soybean cropping regions and seasons in Brazil” written according to Weed Science’s guidelines; the second was entitled “Population genomics of *Conyza* species in soybean macroregions in Brazil suggests spread of herbicide resistance though both intraspecific and interspecific gene flow", written according to Molecular Ecology ’s guidelines; and, the third chapter entitled “Frequency and dispersion of auxin synthetic resistance in *Conyza* spp. across soybean cropping regions and seasons in Brazil and Paraguay” written according to Weed Technology’s guidelines.

CHAPTER 1

TAXONOMIC RESOLUTION OF *Conyza* spp. THROUGH MORPHOLOGICAL AND MOLECULAR MARKERS AND THEIR DISPERSION ACROSS SOYBEAN CROPPING REGIONS AND SEASONS IN BRAZIL[1]

ABSTRACT

The *Conyza* genus includes near 150 species, comprising closely related weedy species. Proper *Conyzas* identification is needed to develop effective strategies for its management. However, the overlap of traits, species varieties and *Conyza* hybrids have difficult this task. Herein, we used five DNA barcodes and 32 morphological traits to classify *Conyza* species and survey their dispersion in soybean fields (*Glycine max* L.) in Brazil in 2019, 2020 and 2021. *Conyza* accessions included two species, hairy fleabane (*Conyza bonariensis* L.) and sumatran fleabane (*Conyza sumatrensis* Retz.), and each species comprised two varieties. The *it*s and *rps16-trnQ* gene regions showed ability to distinguish between the two *Conyza* species but *matK*, *rbcL*, and *trnF-trnF* gene regions were not polymorphic. Out of 32 morphological traits, phyllary color, involucre shape, capitulescence and inflorescence type were the most polymorphic and reliable markers. Combining *its* and *rps16-trnQ* regions and the four morphological markers discriminated 81 of 89 individuals (91%) of both *Conyza* spp. (except eight individuals *C. bonariensis* var. *bonariensis*). *C. sumatrensis* was detected in 353 of 374 (94%) soybean fields across regions and season, while *C. bonariensis* was sparsely dispersed mainly in the southern of the country (MRS 1). These results support the discrimination between *C. bonariensis*, *C. sumatrensis*, and other closed related weed species. The benefits and obstacles of validating DNA barcodes for *Conyza* species are discussed.

Keywords: DNA barcoding, *its*, morphology, phylogeny, *rps16-trnQ*.

[1] Chapter written according to the *Weed Science* Journal (1550-2759).

1.1 INTRODUCTION

Conyza Less. (Asteraceae: Astereae) is a genus that comprises about 150 species worldwide (CWG, 2022), including troublesome weedy species with accessions resistant up to six site of action herbicides (Heap, 2022). Broadly, *Conyza* weeds are closely related species because of shared traits, environments, and niches, and often overlaps in cropping regions across the globe (Thébaud and Abbott, 1995). For example, hairy fleabane [*Conyza bonariensis* (L.) Cronq.) and horseweed [*Conyza canadensis* (L.) Cronq.] co-occur in several field crops in at least 36 countries (Bajwa et al., 2016). In addition, species varieties of *C. bonariensis*, *C. canadensis* and Sumatran fleabane [*Conyza sumatrensis* (Retz.) E. Walk.] are often stated in floristic analysis of the genus (Sancho, 2014). For instance, *C. sumatrensis* var. *leiotheca* is a glabrous variety restricted to the Americas, while *C. sumatrensis* var. *sumatrensis* is hirsute and globally dispersed (Pruski and Sancho, 2006). The presence of species and varieties may create complex infestations of *Conyza* weeds containing individuals with differential responses to the weed control practices, mainly chemical control.

As with any pest, *Conyza* spp. properly identification is critical for early development of effective strategies; however, these weeds have often been identified to the genus level in South America (Mendes et al., 2021). In fact, *Conyza* weeds at seedling and rosette growth stages are hardly distinguished due to the paucity of morphological traits useful for identifying them in field conditions. Although several dichotomous keys are available for the identification of *Conyza* weeds, they require flowering plants and standards morphotypes for comparison (Wang et al., 2018). Even though, the taxonomy is not resolute due to overlapping of traits among species, existence of varieties within species and interspecific hybrids (Thébaud and Abbott, 1995). Due to the obstacles for morphological classification and the relevance as weeds for several crops, *Conyza* spp. are prime candidates for the use of molecular tools to support their taxonomic resolution.

By the 1980s, many molecular tools based on frequency data from markers were developed to support the identification and characterization of plant materials (Glover and Sharma, 2016). These tools include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites, and single nucleotide polymorphisms (SNP). However, despite these markers have had valuable contributions to resolve phylogenetic issues, they could be problematic and even

misleading for taxonomy (Arif et al., 2010). In this context, novel molecular techniques based on gene sequencing such as DNA barcoding and even whole-genome sequencing have emerged (Hebert et al., 2003). The introduction of gene sequencing approaches featuring error lower database was instrumental for easy assignment of unknown plant samples into appropriate species.

The DNA barcoding is a method that uses universally amplified, short, and polymorphic DNA markers (DNA barcodes) for genetically identifying taxonomically organisms at the species level (Hebert et al., 2003). The DNA barcodes mostly reported for plants include the ribosomal gene *its*, the plastid genes *matK* and *rbcL*, and the plastid intergenic spacer *psbA-trnH* (Li et al., 2015; Yang et al., 2020). For example, five of eight *Conyza* spp. in Australia were genetically identified by the combination of *its* and *rbcL* gene regions as an adequate two-loci DNA barcode (Alpen et al., 2014). In other case, the chloroplast genome sequencing of *C. bonariensis* revealed the plastid intergenic region *rps16-trnQ* as a barcode region to separate three *Conyza* spp. (Wang et al., 2018). Using entire chloroplast genome as a unique 'super DNA barcode' for species of the Asteraceae family has also been reported (Chen et al., 2018; Gichira et al., 2019; Wang et al., 2018).

Although DNA barcodes resolved taxonomic issues for several closely related species, the species delimitation using only molecular data is still a major challenge in some genera (Guo et al., 2016; Starr et al., 2009). In addition, plastid gene regions are mostly inherited uniparentally and cannot reliably distinguish interspecific hybrids from the parent species (Daniell et al., 2016; Park et al., 2021). Thus, morphological markers have been studied in association with molecular markers as a suitable method of species classification (Han et al., 2021; Li et al., 2019; Yang et al., 2022). Combining a variety of morphological markers across the weed life cycle with both plastid and nuclear gene regions may generate an accurate platform to distinguish *Conyza* weeds. This strategy would allow to identify *Conyza* spp. at field level and at any time across the season, mainly at early season for the development of effective strategies against the local *Conyza* weeds.

In recent years, soybeans have been cultivated in approximately 41 million hectares in Brazil (USDA, 2022) across five macroregions (MRSs) that differ by the climate and management (Kaster and Farias, 2012). Field surveys have reported *Conyza* spp. infestations in almost half of the Brazilian's soybean area, with higher frequencies in the southern region (Lucio et al., 2019; Silva et al., 2021). Thirteen

species of the genus *Conyza* are found in croplands, urban, and natural lands in Brazil, among which four are reported as weeds in field crops (Flora e Funga do Brasil, 2022). These weedy species include 'fleabane' [*Conyza blakei* (Cabr.) Cabr.], *C. bonariensis*, *C. canadensis*, and *C. sumatrensis* (Piasecki et al., 2019; Santos et al., 2014; Vidal et al., 2007). There is no available information about the distribution of each *Conyza* weed across Brazil, as well as the frequency of species overlaps in soybean fields across MRSs is unclear.

In this study, we have identified *Conyza* spp. by combining two approaches and surveyed their spatiotemporal dispersion in soybeans, using the Brazilian example as a case study with worldwide significance. We hypothesized that (1) *Conyza* spp. can be suitable identified by associating morphological and molecular markers; (2) dispersion and species overlaps of the *Conyza* weeds depend on geography. Thus, the aim of the present study was to identify *Conyza* spp. through DNA barcodes and morphology and survey their geographical dispersion across soybean cropping regions and seasons in Brazil.

1.2 MATERIAL AND METHODS

1.2.1 Plant Material

A total of 394 soybean fields were surveyed throughout the five MRSs of Brazil in 2019, 2020 and 2021, as follows: 130 in MRS 1, 111 in MRS 2, 98 in MRS 3, 54 in MRS 4, and 1 in MRS 5 (Figure 1). The fields were selected based on the representativeness of cropping area and system as well as by the occurrence of mature plants that have escaped the control by herbicide programs. Each field was farmed by a different grower and was treated as an accession of *Conyza* spp., and sampling was not repeated in the same farm to explore the diversity of fields within MRSs. Two distinct sampling strategies were employed to address the objectives of the study (Supplementary Figure S1). In 20 out of 394 fields, 20 individuals were harvested separately (single-plant sample) to obtain plant material from distinct species for the morphology assay of *Conyza* spp. In 374 out of 394 fields, 10 weeds were harvested and combined into a sample (10-plants sample) to obtain plant material for the survey on geographical dispersion of *Conyza* spp. After sampling, samples were identified and stored as described in Burgos et al. (2013).

1.2.2 Growing Conditions and Species Identification

Whole-plant assays were carried out from May to August each year (late fall to winter in the Southern hemisphere), when *Conyza* spp. mainly germinate and stablish in the crop fields (Vidal et al., 2007). Seeds from single-plant samples were germinated in petri dishes in growth chambers at 22 ± 1°C and 10-h light, and then one seedling was transplanted to 1 dm^{-3} pots filled by soil potting mix. Equally, seeds from 10-plants samples were germinated in 1 x 1 cm cell trays at greenhouse and then four seedlings were transplanted in pots of 1.2 dm^{-3} size filled by same subtract. The potting mix was composed of 50% soil, 25% rice bark, 25% peat, and traces of NPK, with 162 kg m^{-3} density, 52% water retention, pH 5.5 and 0.6 ± 0.3 mS cm^{-1} electrical conductivity. Plants were grown in open greenhouses with natural air temperature (20 ± 13°C), relative humidity (65 ± 13%), and light (11 ± 1 h), and received 2 mm sprinkler irrigation four times a day (Supplementary Figure S2). The weed species were classified using the keys of Pruski and Sancho (2006) and Sancho (2014), in which the most useful traits were involucre shape, capitula type, and capitulescence type. Twelve voucher specimens comprising the species and varieties found in the study were deposited in the Irina Delanova Gemtchújnicov Herbarium/Botu of UNESP (BOTU 34833-34844).

1.2.3 Phylogenetic Analysis Based on Morphology

In this assay, 314 individuals that were established from 400 single-plant samples (20 fields x 20 samples per field) were evaluated for 32 morphological traits throughout five weed growth stages (Table 1). Qualitative traits were evaluated as described by Pruski and Sancho (2006) and Sancho (2014), and quantitative traits were measure with a vernier caliper (Insize, WorldTools, Joinville, BR). After that, data of qualitative traits was converted to numerical scores according to the Table 1 and data of all variables were normalized at the same scale to perform clustering analysis. Principal component analysis (PCA) was made to highlight the ‘morphological markers’ among the 32 distinct traits using the *adegenet* package in R environment (Jombart, 2008). The data was subject to build dendrograms to cluster *Conyza* spp. based on Euclidean distances method using the software PAUP (set as default; Wilgenbusch and Swofford, 2003).

1.2.4 DNA isolation, PCR Amplification, and Sequencing

Leaf tissue (100 mg) from the fourth leaf was freeze dried and ground into a powder to extract genomic DNA using DNeasy® Plant Mini Kit (Qiagen, Hilden, DE) following the manufacturer's protocol. DNA quantity and quality was assessed by spectrophotometer (NanoDrop®, Thermo Scientific, Waltham, US) and samples were considered suitable when the absorbance ratio 260/280 >1.8. DNA samples were resuspended in 100 ml water, and then dilutions were made up to 10 ng μl^{-1}, after which DNA samples were stored at -20°C until the step of the gene region amplification.

Primer sequences for *rbcL* and *trnF-trnL* were obtained from previous research on universal DNA barcodes for plant species, while primer sequences for *its*, *matK* and *rps16-trnQ* were designed (Table 2). In such cases, DNA coding sequences annotated in NCBI for *Conyza* spp. were aligned and homologue regions were used for primer design at Primer3Plus (Untergasser et al., 2012). Primers were designed based on three published sequences of *C. bonariensis*, *C. canadensis* and *C. sumatrensis* from the nucleotide database, according to the Supplementary Table 1. The polymerase chain reaction (PCR) optimized conditions were 95°C for 2 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, followed by an extension stage at 72°C for 5 min. PCR products were visualized for quality and size using a UV transilluminator (iBright® CL1500 Imaging System, Thermo Scientific, Waltham, US) after electrophoresis through a 1.5% agarose gel.

High quality amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, DE), and then sequenced in both directions by BPI (“Biotecnologia, Pesquisa e Inovação”, Botucatu, BR). The sequences were proofread using Chromas Lite v. 2.1.1 (Technelysium Pty Ltd, Brisbane, AU) and consensus sequences of both directions were built in BioEdit v.7.0.5. (Hall, 1999).

1.2.5 Phylogenetic Analysis Based on Barcoding Gene Regions

In this assay, the gene regions were examined by sequencing gene targets from 89 out of 314 individuals from taxonomy assay that represent the species and varieties found in the present study. Firstly, an exploratory assay was carried out employing three individuals of each *Conyza* species to assess the discrimination ability of each

gene region, and it was repeated twice. Consensus sequences were analyzed and aligned at the Guidance2 (Sela et al., 2015) and nucleotide diversity, Tajima's D and genetic distances were calculated in DnaSP v.6.3.3. After, the gene regions with this ability were amplified from all 89 individuals and external sequences from NCBI were added as outgroups for comparison (Supplementary Table 1). The sequences were aligned as described above and subject to construct dendrograms based on neighbor-joining (NJ) distance in PAUP (set as default; Wilgenbusch and Swofford, 2003). Node supports within the NJ trees were assessed by a 10,000 replicate bootstrap test. Given that gene regions with discrimination ability were sequenced for all individuals, a multi-locus combination of them was made and an additional NJ tree was constructed for the obtained concatenated sequence. In this case, outgroup sequences were not added since they only contained single locus.

1.2.6 Phylogenetic Analysis Combining Morphology and Molecular Approaches

In this analysis, morphology and molecular data of the 89 individuals described in the previous section were combined in a two-approach analysis to associate the discriminating ability of each method. Polymorphic sites of the barcoding gene sequences were converted to binary data (0 or 1) and then the molecular dataset was positioned in tandem with the matrix of morphological data. Combined dataset was subject to construct dendrograms to cluster *Conyza* spp. based on Euclidean distances distance as that previously described for the morphology assay of this study.

1.2.7 Dispersion of *Conyza* Weeds Across Soybean Fields in Brazil

Accessions of *Conyza* spp. (n = 374, 10-plants samples) from soybean fields across MRSs and seasons of Brazil were morphologically classified as described, using 16 replications by accession. Spatial maps were plotted to illustrate the spatio-temporal dispersion of *Conyza* spp. and their varieties on the respective geographical origins using the *ggplot2* package in R (Ginestet, 2011). A color-coded classification scheme was used to identify each specie and variety as well as maps were plotted by specie to visualize the regions in which species overlaps have occurred.

1.3 RESULTS AND DISCUSSION

1.3.1 Species Identification Through Morphology

Out of 314 *Conyza* individuals evaluated, 42 individuals were identified by taxonomy as *C. bonariensis* and 272 as *C. sumatrensis*, following the dichotomous keys of Pruski and Sancho (2006) Sancho (2014). *C. bonariensis* mainly varied from *C. sumatrensis* by reddish phyllaries (vs. green), disk-like involucre (vs. bell shaped), and corymbiform capitulescence (vs. thyrsoid-paniculate) (Figure 2). Two varieties were observed within *C. bonariensis* (var. *angustifolia*, n = 8; var. *bonariensis*, n = 34) as well as within *C. sumatrensis* (var. *leiotheca*, n = 28; var. *sumatrensis*, n = 244). C*. bonariensis* var. *angustifolia* basically differed from *C. bonariensis* var. *bonariensis* by thyrsoid-paniculate, pyramidal capitulescence (vs. corymbiform, frequently flat-topped). In turn, *C. sumatrensis* var. *leiotheca* basically differed from *C. sumatrensis* var. *sumatrensis* by leaves, stems, and involucres glabrous or subglabrous (vs. moderately to largely pilose).

We did not find *C. blakei* and *C. canadensis* individuals among the 314 samples from 20 fields across Brazil, although they were reported in field crops in the country (Vidal et al., 2007; Piasecki et al., 2019). C*. blakei* would differ from *C. bonariensis* and *C. sumatrensis* by pinnatisect lower leaves (vs linear or obovate) and narrow capitulescence (vs. moderately elongated) (Sancho, 2014). In turn, *C. canadensis* would differ from *C. bonariensis* and *C. sumatrensis* by subradiate (vs. disciform) capitula and long hair (vs subglabrous) leaf margins (Pruski and Sancho, 2016). Thus, given the distinctive morphological traits, both *C. blakei* and *C. canadensis* would probably be easily distinguished from *C. bonariensis* and *C. sumatrensis* regardless to the species variety. Despite *C. sumatrensis* var *leiotheca* is often misidentified as *C. canadensis* (Pruski and Sancho, 2016), the capitula type allowed to effectively identify the individuals evaluated in our study.

Principal component (PC) 1 of PCA was mainly correlated (≤ -0.70) with number of leaves and plant height in the rosette stage, and number of leaves when *Conyza* weeds were elongating the stem (Figure 3). Equally, PC2 was mostly correlated (≥ 0.70) with leaf angle in the rosette state, and phyllaries color, involucre shape, capitulescence and inflorescence types when individuals were flowering. The first two PCs of the PCA account for 37.5% of the total variance of the morphology data and indicate these eight

traits may act as markers to distinguish between *Conyza* spp. (Figure 3). Euclidean trees based on morphology separated *C. bonariensis* from *C. sumatrensis*, providing monophyletic clades by species, except for *C. bonariensis* var. *angustifolia* (Figure 4a). In fact, individuals of this species variety have clustered with *C. sumatrensis* clade because of many morphological overlaps such as number of leaves, leaf angle, and inflorescence type.

Overall, the traits related to reproductive structures were the most stable variables in our study and are very consistent with other studies (De Ulzurrun et al., 2018; Hao et al., 2009; Thébaud and Abbott, 1995). However, quantitative traits such as plant height, number of leaves, and leaf angle were quite variable, even though they showed high contribution to the total variance in the PCA (Figure 3). *Conyza* weeds have commonly featured extensive phenotypic plasticity for vegetative traits that are evidenced in sightings of variable forms in the same field (Thébaud and Abbott, 1995). Leaf color, cotyledon width, and number of tooths were not stable traits in our study but were recognized as key traits to distinguish *Conyzas* in Argentine (De Ulzurrun et al., 2018). Thus, we adopted a more conservative approach by selecting only phyllaries color, involucre shape, capitulescence and inflorescence type as reliable markers to be combined with molecular data.

1.3.2 Characteristics of Barcoding Gene Regions

In the exploratory assay, high amplification success rates were observed for the *matK*, *rbcL*, and *trnL-trnF* gene regions, while for *its* and *rps16-trnQ* those rates ranged from 55 to 67% (Table 2). All five DNA barcodes had a high sequencing success rate that ranged from 90 to 100% of PCR products, which shows that overall gene regions were more easily sequenced than amplified. There were not found sequence deletions, stop codons and unusual site substitutions in the five cases, confirming that the sequences consisted of the DNA barcodes and not pseudogenes. Nucleotide diversity by loci was calculated as 2.4 and 4.9% for *its* and *rps16-trnQ*, respectively, while no segregating sites were found in the other three barcoding gene regions (Table 3). Also, intraspecific, and interspecific distances were only found in the same two gene regions, in which the mean interspecific distance was greater than the intraspecific one at each gene.

Beyond simple DNA sequence variability, crucial characteristics for barcoding loci include primer universality and easy amplification and sequencing (Fazekas et al., 2008; Kress and Erickson, 2007). In this study, *its* and *rps16-trnQ* were the more variable gene regions and also the most difficult to amplify, even with adjustments in PCR protocols and design of new primer sequences. Even so, these DNA barcodes were chosen to identify the 89 individuals of the study since the other gene regions showed no ability to separate *Conyza* spp. found in Brazil. In other study, the *its* gene region had the highest genetic variability among *Conyza* spp. (0.3 to 15%), while the *rbcL* showed interspecific distances ranging from 0 to 0.8% (Alpen et al., 2014). Therefore, the data from our and other studies have indicated that some universal DNA plant barcodes such as *rbcL* are likely not capable to resolute the taxonomic obstacles of *Conyza* spp.

1.3.3 Species Identification Through Barcoding Gene Regions

Phylogenetic analysis based on *its*, *rps16-trnQ*, and *its* + *rps16-trnQ* gene regions were used to distinguish 89 individuals comprising the species and varieties identified with morphology data (Figure 5). The individuals of *C. bonariensis* (var. *angustifolia*, n = 8; var. *bonariensis*, n = 30) and *C. sumatrensis* (var. *leiotheca*, n = 28; var. *sumatrensis*, n = 23) are detailed in Supplementary Table S2. The *its* gene region exhibited the highest separation of *C. bonariensis* from *C. sumatrensis*, supporting the monophyly for all individuals of *C. bonariensis* var. *bonariensis* (Figure 5a). The *rps16-trnQ* gene region did not supported monophyletic clusters for each species hence this proposed DNA barcode was unable to discriminate between the species (Figure 5b). The concatenation of the two gene regions failed to improve on the ability of *its* individually, and even smaller bootstrap support was observed in the clade of *C. bonariensis* (Figure 5c).

Neighbor-joining analysis of the *its* gene region supported a clearly monophyly for five of the eight *Conyza* spp. in Australia, including individuals of *C. bonariensis* and *C. sumatrensis* (Alpen et al., 2014). A similar analysis based on the *rps16-trnQ* gene region separated 13 individuals of *C. bonariensis*, *C. canadensis* and *C. sumatrensis* from Australia into monophyletic clades (Wang et al., 2018). In our research, these two gene regions were not capable to distinguish the species since not all individuals formed a single cluster in the tree, as expected for a universal DNA plant barcode.

Curiously, only the individuals featuring the morphotype of *C. bonariensis* var. *angustifolia* were spread across the clades even in the analysis based on *its* or *its* + *rps16-trnQ* gene regions. We have hypothesized at least three different reasons for these findings in the present study: misidentification of species, incomplete lineage sorting, and interspecific hybridization.

The incorrect *C. bonariensis* var. *angustifolia* identification is unlikely since individuals taxonomically differed as the plant matures, comprising a well-defined plant morphotype, identified according to Sancho (2014). In addition, we submitted three of these individuals as voucher specimens at the Herbarium where the plant species identification was confirmed, and vouchers annotated appropriately. Incomplete lineage sorting is more likely because *Conyza* comprise paraphyletic species that have recently speciated from a common ancestor (Alpen et al., 2014; Marochio et al., 2017). Thus, DNA barcodes may not delimit species if the mutation rate at the target gene regions are insufficient to allow novel diversity to emerge (Simeone et al., 2013; Van Velzen et al., 2012). Interspecific hybridization is equally likely since there are strong evidence that *Conyza* hybrids can be generated from individuals that can interact freely in field crops (Zelaya et al., 2007).

1.3.4 Species Identification Through Molecular and Morphological Approaches

The *its* and *its*+*rps16-trnQ* gene regions were able to differentiate *C. bonariensis* var. *bonariensis* from *C. sumatrensis* while failing to separate *C. bonariensis* var. *angustifolia* from *C. sumatrensis* (Figure 5). To efficiently differentiate this unclassified variety of the species *C. bonariensis* in our study, we assessed 32 traits and chosen four of them as morphological markers (Figure 4). The combined, two-approach analysis considering the polymorphic sites of each gene region and the four morphological markers did not differ *C. bonariensis* var. *angustifolia* (Figure 6). In fact, this variety of *C. bonariensis* remained spread among cluster and thus unclassified, which did not confirm our hypothesis about the combination of morphology and molecular data. Even though, this joint analysis was capable to discriminate 81 of 89 samples (91%) and demonstrate the potential of the combination between DNA barcodes and morphology to identify weeds.

There was not found studies to date that have combined DNA barcodes and morphological traits to classify *Conyza* spp., despite of the global relevance of these

widespread weedy species in several cropping systems. In other closely related weeds, such as *Citrullus* spp., *Echinochloa* spp., and *Gallium* spp., this approach supported taxonomic resolution (Deroo et al., 2019; Shaik et al., 2016; Tabacchi et al., 2006). Thus, this study revealed a potential tool to differentiate species of *Conyza* in Brazil and worldwide because molecular and morphological markers will apply for any species of the genus. For example, we believe that the identification of other weeds like *C. canadensis* may be easier, since it appears to be less related to the genus than other weeds (Thébaud and Abbott, 1995). This study could also be a valuable tool not only to identify *Conyza* spp. in field crops but also for ecological and conservation purpose due to the size (c. 150) of this genus (CWG, 2022).

1.3.5 Dispersion of *Conyza* Weeds Across Brazil

C. sumatrensis was the prevailing *Conyza* species found in the field survey across five MRSs and three seasons in Brazil, with high frequency and dispersion regardless to the region and season (Figure 6). This weed was detected in 166 of 177 fields in 2019, 99 of 104 fields in 2020, and 88 of 93 fields in 2021, among which *C. sumatrensis* var. *sumatrensis* was the most common species variety. *C. bonariensis* was the other *Conyza* weed found throughout the three seasons in the country but was only sparsely dispersed in MRS 1 and in a few numbers of sites in another regions. In this case, the weed occurred in14 of 177 fields in 2019, 6 of 104 fields in 2020, and 6 of 93 fields in 2021, in which the species variety mostly noted was *C. bonariensis* var. *bonariensis*. The overlaps between these species were relatively rare and were only detected in 3 of 177 fields in 2019, 1 of 104 fields in 2020, and 1 of 93 fields in 2021.

While the dispersion of *C. bonariensis* in Brazil was dependent on the geography, the dispersion of *C. sumatrensis* did not, which partially confirm our hypothesis about the overlaps between these species. Accessions of *C. sumatrensis* were detected over the seasons under high frequency and dispersion across Brazil and therefore there were not found large differences among the five soybean MRSs (Figure 6). If the environment has had slight or even no influence over the proportion of the *Conyza* weeds in Brazil, we can assume that multiple resistance to herbicides is the main factor to explain these findings. In fact, cases of accessions of *C. sumatrensis* featuring resistance to herbicides from different sites of action have been often documented in Brazil (Albrecht et al., 2020; Mendes et al., 2021; Pinho et al., 2019). While accessions

of glyphosate-resistant *C. bonariensis* are being well controlled by alternative herbicides in soybean fields, multiple-resistant *C. sumatrensis* are probably surviving to a variety of herbicide programs.

As earlier shown in the morphology study, we also did not identify individuals of *C. blakei* and *C. canadensis* over the 374 soybean fields across different cropping regions and seasons. Among them, C*. canadensis* was reported in at least 16 studies from Brazil in *Web of Science* database, including cases of characterization of accessions resistant to glyphosate. Despite the possibility of occurrence at very low frequencies and eventual weed species shifts over time, it is likely that misidentification occurred in most of these researches from Brazil. In fact, a variety of *C. sumatrensis* generally has glabrous involucres, is restricted to the Americas, and is often misidentified as *C. canadensis* (Pruski and Sancho, 2006). It is necessary to improve the scientific rigor applied to species identification in studies with *Conyza* spp. to avoid producing further inconsistent information, as this might affect management actions.

1.4 CONCLUSIONS

In conclusion, the *Conyza* spp. identification is a fundamental step in developing effective strategies against these weeds, and species misidentification may result in lower-than-expected efficacy of control. Combining *its* and *rps16-trnQ* regions and the four morphological markers discriminated 81 of 89 individuals (91%) of both *Conyza* spp. (except eight individuals *C. bonariensis* var. *bonariensis*). *C. sumatrensis* was detected in 353 of 374 (94%) soybean fields across regions and season, while *C. bonariensis* was sparsely dispersed mainly in the southern of the country (MRS 1). These results support the discrimination between *C. bonariensis*, *C. sumatrensis*, and other closed related weed species in soybeans in Brazil and other cropping systems worldwide. Our study presented here brings relevant information to support the species identification and its management towards minimizing the dispersion of herbicide resistance in *Conyza* weeds.

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1.6 APPENDICES

Table 1 List of morphological features and their scores.

No.	Growth stage	Feature	Feature scores
1	Seedling	Cotyledon length	mm
2	Seedling	Cotyledon width	mm
3	Rosette	Rosette diameter	cm
4	Rosette	Rosette height	cm
5	Rosette	Number of leaves	n
6	Rosette	Leaf insertion angle	0, prostrate; 1, moderately erect or erect
7	Rosette	Number of tooths	n
8	Stem elongation	Growth habit	0, climbing; 1, erect or ascending
9	Stem elongation	Plant diameter	cm
10	Stem elongation	Plant height	cm
11	Stem elongation	Number of leaves	n
12	Stem elongation	Leaf insertion angle	0, prostrate; 1, moderately erect or erect
13	Stem elongation	Number of tooths	n
14	Flowering	Inflorescence type	0, anthelate; 1 cylindrical or pyramidal
15	Flowering	Secondary inflorescence type	0, corimbiform; 1, paniculiform
16	Flowering	Inflorescence length	cm
17	Flowering	Inflorescence width	cm
18	Flowering	Branches by inflorescence	n
19	Flowering	Involucre type	0, cup-shaped; 1, disk-like
20	Flowering	Phyllaries color	0, white green or green; 1, reddish green
21	Main stem capitulum	Capitulum type	0, disciform; 1, liguliform
22	Main stem capitulum	Capitulum length	cm
23	Main stem capitulum	Capitulum width	cm
24	Main stem capitulum	Cypselas by capitulum	n
25	Senescence	Bottom leaves shape	0, linear; 1, linear-obovate or obovate
26	Senescence	Bottom leaves margin	0, entire; 1, lobate or serrate
27	Senescence	Top leaves shape	0, linear; 1, linear-obovate or obovate
28	Senescence	Top leaves margin	0, entire; 1, lobate or serrate
29	Senescence	Number of secondary stems	n
30	Senescence	Leaf vestiture	0, glabrous or subglabrous; 1 hirsute-pilose
31	Senescence	Stem vestiture	0, glabrous or subglabrous; 1 hirsute-pilose
32	Senescence	Life form	0, annual, 1, biannual or perennial

Table 2 Primer sequences and characteristics of the barcoding gene regions

Gene region	Primer (direction)	Sequence (direction 5' - 3')	TM (°C)[f]	AS (bp)	Amp Ef (%)[f]	Seq Ef (%)[f]
its[a]	*its_br(F)*[d]	TTGTCGAAGCCTGCAAAG	60	585	56	90
	its_br(R)[d]	AACGCGTTGGGTCAATAA				
matK[b]	*matK_br(F)*[d]	TACAGTACTTTTGTGTTTACG	55	771	94	100
	matK_br(R)[d]	CAGTCCATCTGGAAATCTTGG				
rbcL[b]	*rbcLF(F)*[e]	ATGTCACCACAAACAGAGACTAAAGC	50	646	94	100
	rbcLajf634(R)[e]	GAAACGGTCTCTCCAACGCAT				
rps16-trnQ[c]	*rps16-trnQ_br(F)*[d]	TCGATATAGAAATCGAAAGGAT	50	513	67	100
	rps16-trnQ_br(R)[d]	CCCTAGAACCGTATAGGAAG				
trnF-trnL[c]	*Ucp-e(F)*[e]	GGTTCAAGTCCCTCTATCCC	55	447	100	100
	Ucp-f(R)[e]	ATTTGAACTGGTGACACGAG				

[a] Internal transcribed spacer region of the nuclear ribosomal cistron (18S-5.8S-26S).
[b] Plastid gene regions (*matK* - Maturase K; *rbcl* - Ribulose 1,5-biphosphate carboxylase).
[c] Intergenic spacer regions (between *rps16 and trnQ*; and, between *trnF* and *trnL* gene regions).
[d] Primer sequences re-designed in the present study based on external DNA sequences of *Conyza* spp.
[e] Primer sequences obtained from Kress and Erickson (2007), Fazekas et al. (2008) and Taberlet et al. (1991).
[f] Melting temperature (TM), amplicon size (AS), amplification efficiency (Amp Ef) and sequencing efficiency (Seq Ef).

Table 3 Diversity and variation of the barcoding gene regions.

Parameter	*its*	*matK*	*rbcL*	*trnL-rps16*	*trnL-trnF*
Segregating Sites	32	0	0	56	0
Nucleotide Diversity (Pi)	0.0239	0	0	0.0489	0
Tajima´s test (D)	-1.1363	-[a]	-[a]	-0.5903	-[a]
Intraspecific distance	0.0450	0	0	0.0500	0
Interspecific distance	0.0597	0	0	0.0647	0
Interspecific/Intraspecific distance	1.3266	0	0	1.2940	0

[a] Not calculated due the absence of diversity.

List of figures

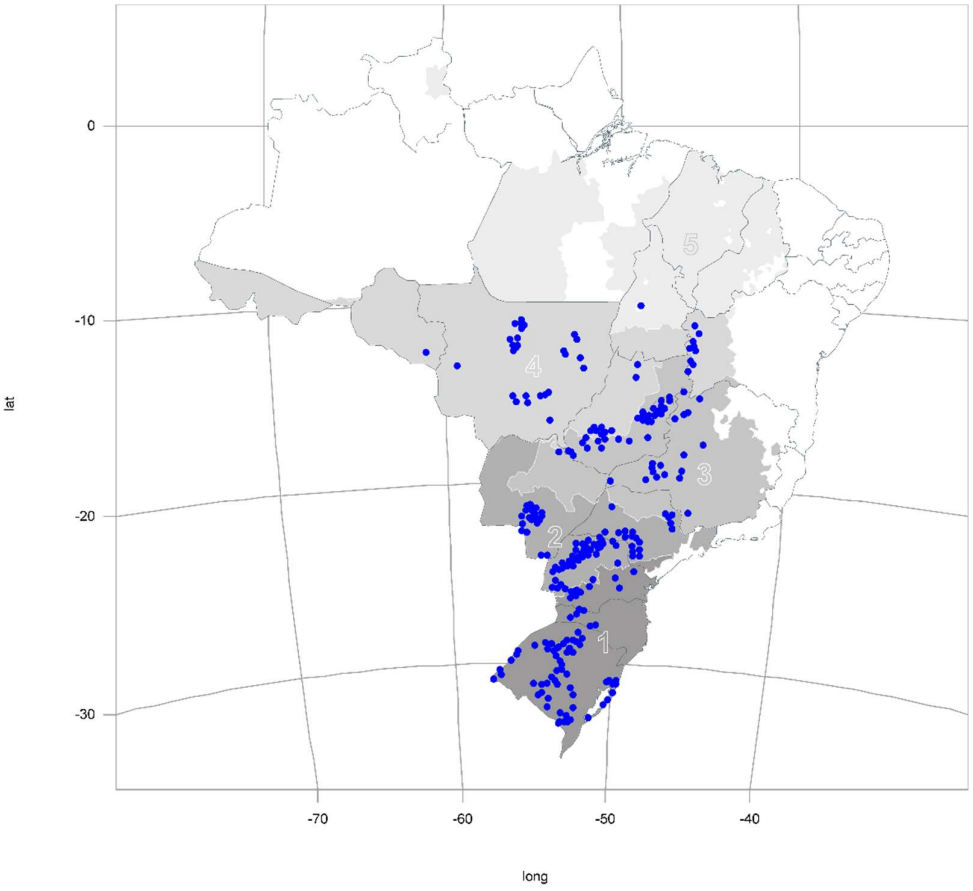
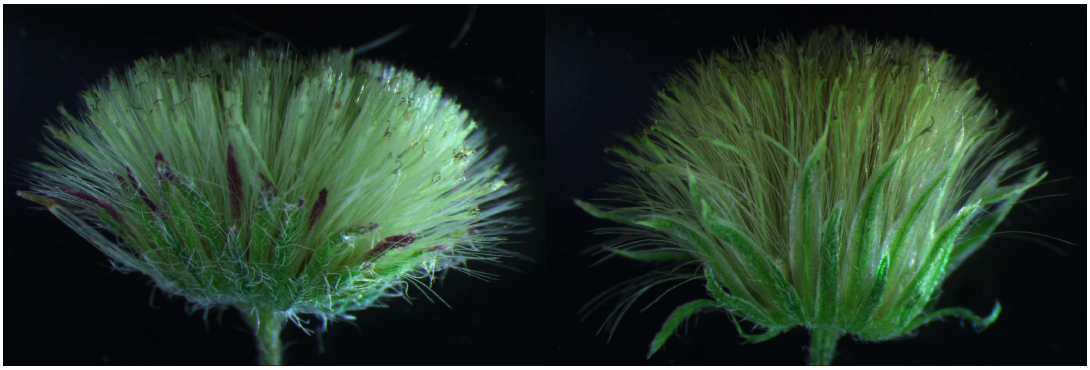


Fig 1.

(a) Phyllaries color



(b) Involucre shape

(b) capitulescence type

Fig. 2

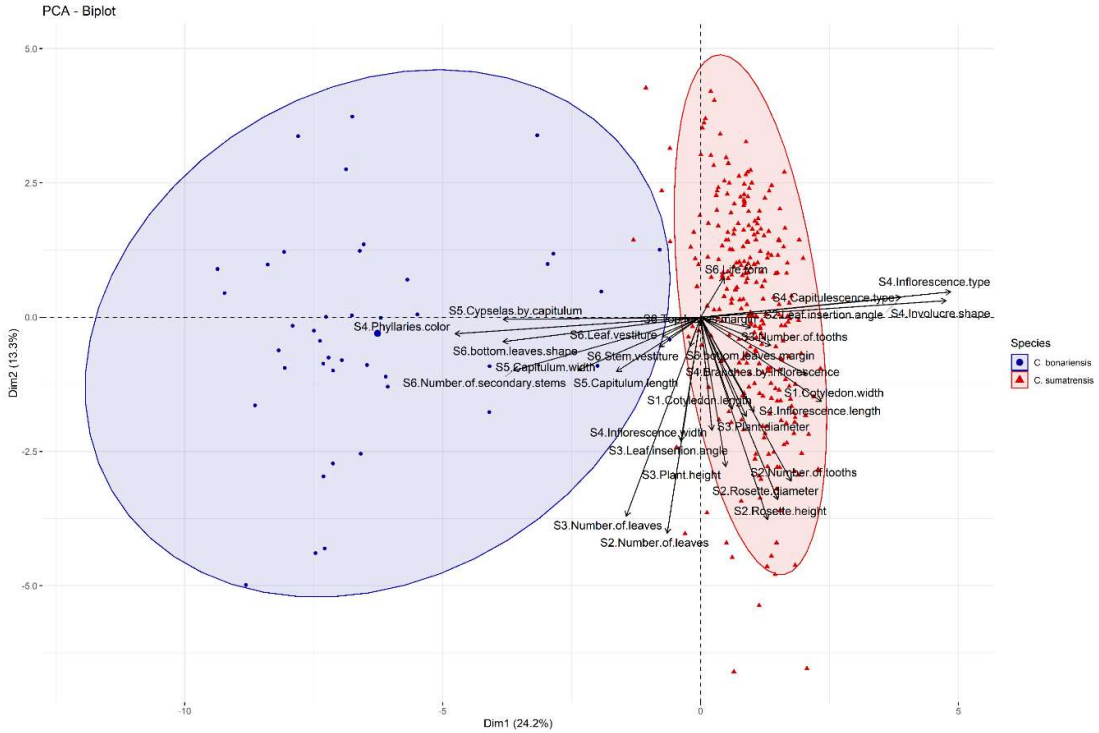

PCA - Biplot
Dim2 (13.3%)
Dim1 (24.2%)
5.0
2.5
0.0
-2.5
-5.0
-10
-5
0
5
S6.Life.form
S4.Inflorescence.type
S4.Capitulescence.type
S2.Leaf.insertion.angle
S4.Involucre.shape
S5.Cypselas.by.capitulum
S4.Phyllaries.color
S6.Leaf.vestiture
S3.Number.of.tooths
S6.bottom.leaves.shape
S6.Stem.vestiture
S5.bottom.leaves.margin
S5.Capitulum.width
S4.Branches.by.Inflorescence
S6.Number.of.secondary.stems
S5.Capitulum.length
S1.Cotyledon.length
S1.Cotyledon.width
S4.Inflorescence.length
S3.Plant.diameter
S4.Inflorescence.width
S3.Leaf.insertion.angle
S3.Plant.height
S2.Number.of.tooths
S2.Rosette.diameter
S2.Rosette.height
S3.Number.of.leaves
S2.Number.of.leaves
Species
C. bonariensis
C. sumatrensis

Fig. 3

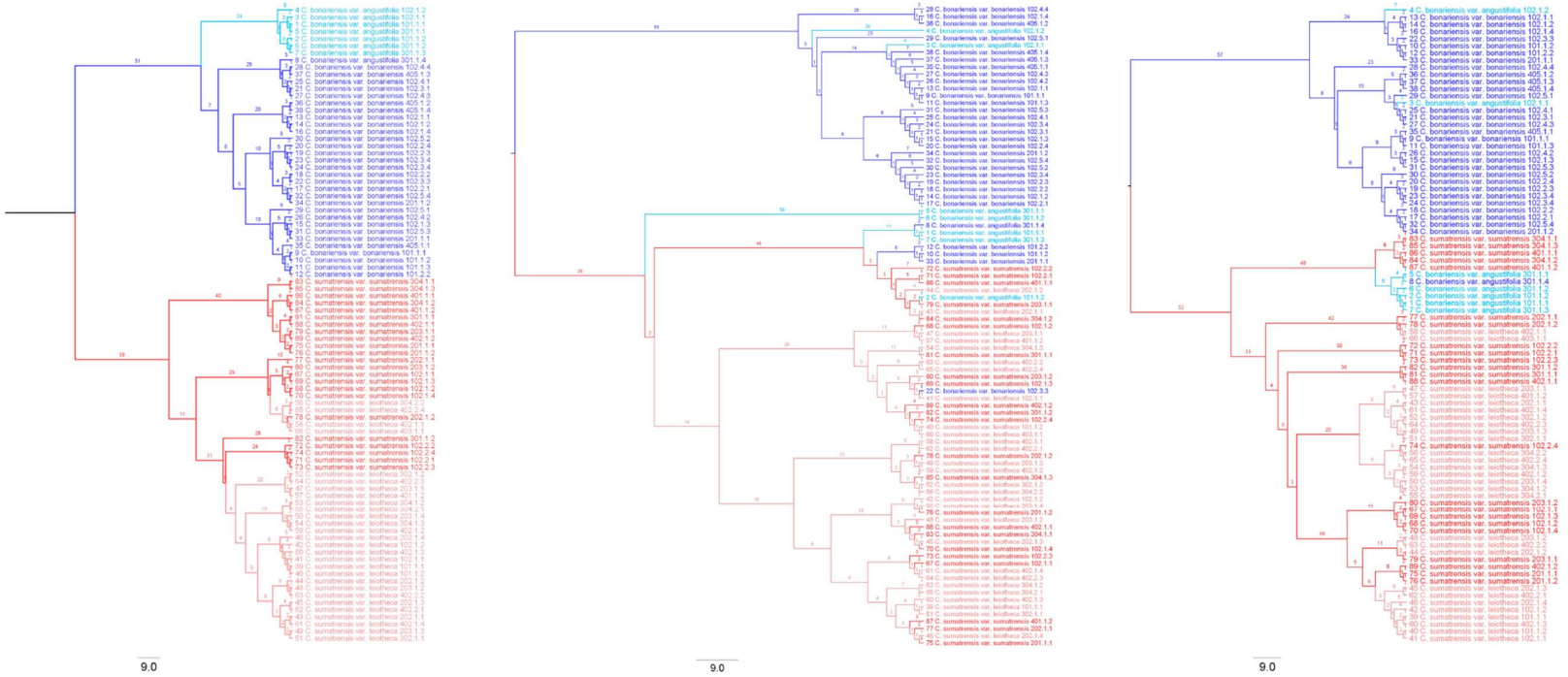


Fig. 4

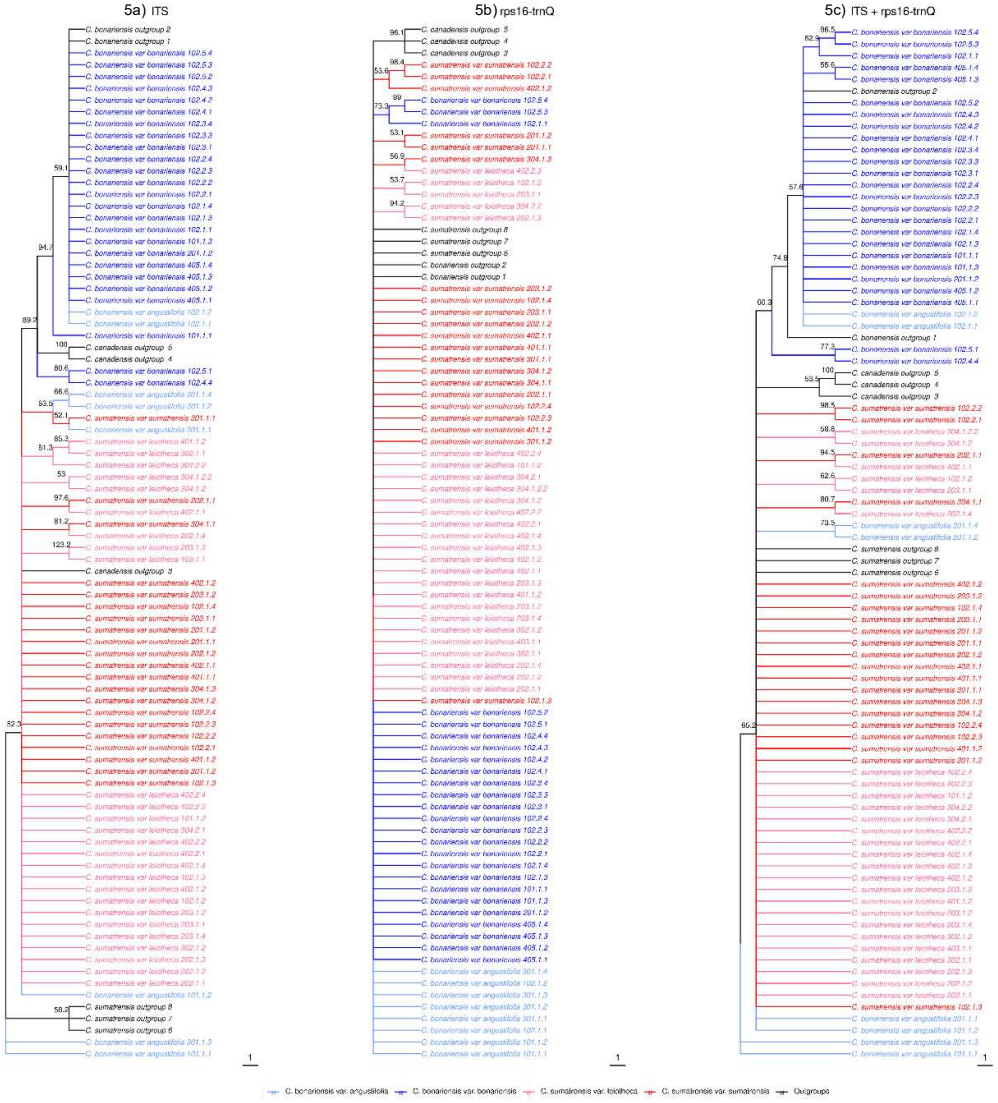

5a) ITS
5b) rps16-trnQ
5c) ITS + rps16-trnQ
C. bonariensis var. angustifolia
C. bonariensis var. bonariensis
C. sumatrensis var. leiotheca
C. sumatrensis var. sumatrensis
Outgroups

Fig. 5

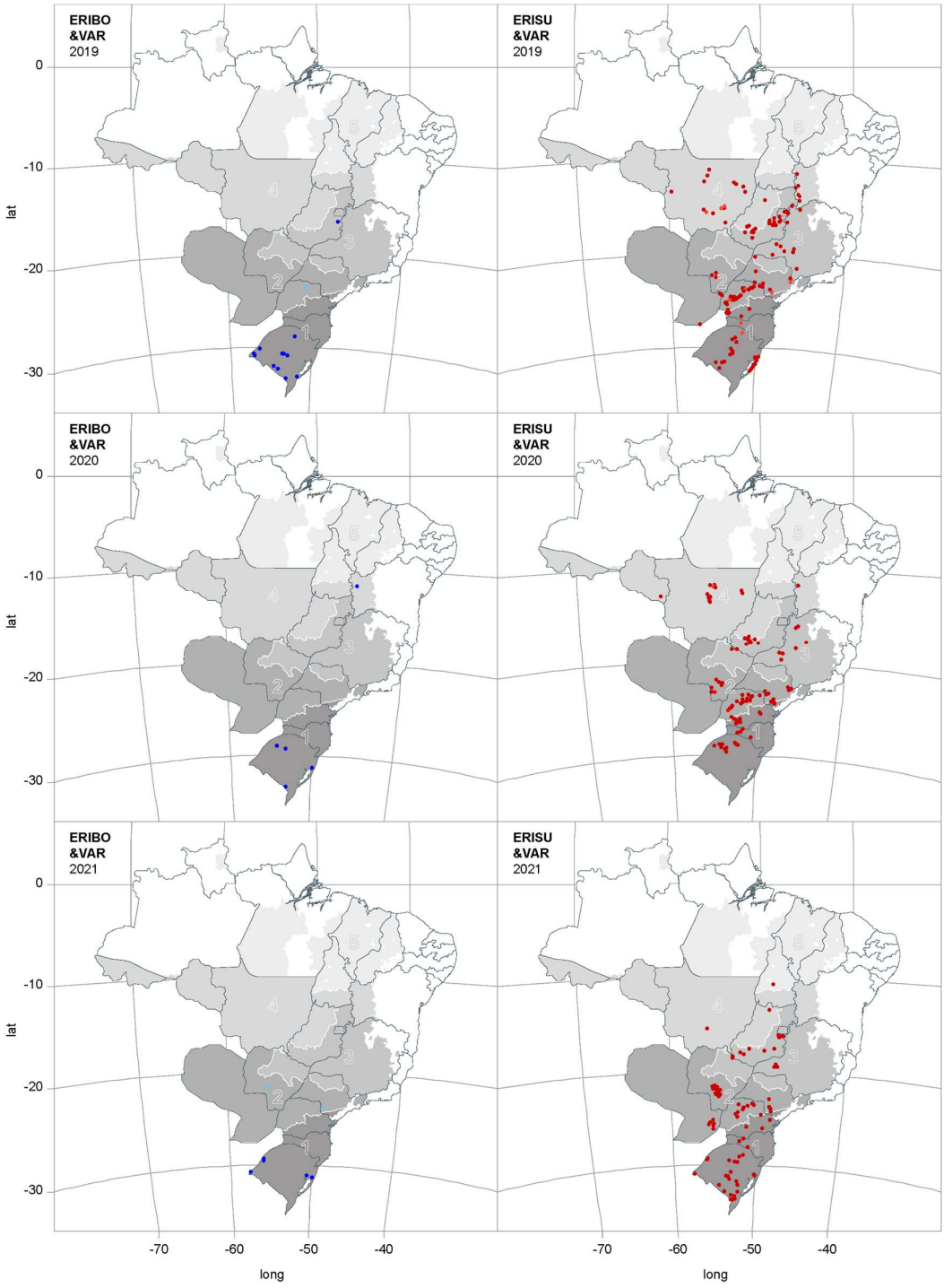

ERIBO
&VAR
2019
ERISU
&VAR
2019
ERIBO
&VAR
2020
ERISU
&VAR
2020
ERIBO
&VAR
2021
ERISU
&VAR
2021
lat
long
0
-10
-20
-30
-70
-60
-50
-40

Fig. 6

Supplementary Table 1 Sequence numbers of the *Conyza* spp. sequences used to design primers and construct dendrograms in the study.

Species	Outgroup	*its*	*matK*	*rps16-trnQ*
C. bonariensis	1	AF118513.1	JX518235.1	MH559523.1
	2	KP175228.1	MF694842.1	MH559529.1
	3	KX420707.1	MK125134.1	MH559530.1
C. canadensis	4	AF046987.1	HQ593246.1	MH559524.1
	5	AY875695.1	KJ204460.1	MH559527.1
	6	KP175229.1	MF350068.1	MH559528.1
C. sumatrensis	7	AY875698.1	KX671989.1	MH559521.1
	8	JN315923.1	-[a]	MH559531.1
	9	MH050152.1	-	MH559532.1

[a] No additional sequences were found in the database.

Supplementary Table 2 List of 89 individuals of *Conyza* spp. utilized in the morphology and molecular studies with geographic origins.

#	Individual	Specie and variety	Geographic origins
1	ERIBOvAn 101.1.1	*Conyza bonariensis* var. *angustifolia*	-30.67833, -52.76556
2	ERIBOvAn 101.1.2	*Conyza bonariensis* var. *angustifolia*	-30.67833, -52.76556
3	ERIBOvAn 102.1.1	*Conyza bonariensis* var. *angustifolia*	-28.81913, -53.38753
4	ERIBOvAn 102.1.2	*Conyza bonariensis* var. *angustifolia*	-28.81913, -53.38753
5	ERIBOvAn 301.1.1	*Conyza bonariensis* var. *angustifolia*	-17.77417, -51.03250
6	ERIBOvAn 301.1.2	*Conyza bonariensis* var. *angustifolia*	-17.77417, -51.03250
7	ERIBOvAn 301.1.3	*Conyza bonariensis* var. *angustifolia*	-17.77417, -51.03250
8	ERIBOvAn 301.1.4	*Conyza bonariensis* var. *angustifolia*	-16.52083, -48.28722
9	ERIBOvBo 101.1.1	*Conyza bonariensis* var. *bonariensis*	-30.67833, -52.76556
10	ERIBOvBo 101.1.2	*Conyza bonariensis* var. *bonariensis*	-30.67833, -52.76556
11	ERIBOvBo 101.1.3	*Conyza bonariensis* var. *bonariensis*	-30.67833, -52.76556
12	ERIBOvBo 101.2.2	*Conyza bonariensis* var. *bonariensis*	-32.56611, -53.37583
13	ERIBOvBo 102.1.1	*Conyza bonariensis* var. *bonariensis*	-28.81913, -53.38753
14	ERIBOvBo 102.1.2	*Conyza bonariensis* var. *bonariensis*	-28.81913, -53.38753
15	ERIBOvBo 102.1.3	*Conyza bonariensis* var. *bonariensis*	-28.81913, -53.38753
16	ERIBOvBo 102.1.4	*Conyza bonariensis* var. *bonariensis*	-28.81913, -53.38753
17	ERIBOvBo 102.2.1	*Conyza bonariensis* var. *bonariensis*	-28.23528, -52.38056
18	ERIBOvBo 102.2.2	*Conyza bonariensis* var. *bonariensis*	-28.23528, -52.38056
19	ERIBOvBo 102.2.3	*Conyza bonariensis* var. *bonariensis*	-28.23528, -52.38056
20	ERIBOvBo 102.2.4	*Conyza bonariensis* var. *bonariensis*	-28.23528, -52.38056
21	ERIBOvBo 102.3.1	*Conyza bonariensis* var. *bonariensis*	-28.51361, -53.49583
22	ERIBOvBo 102.3.3	*Conyza bonariensis* var. *bonariensis*	-28.51361, -53.49583
23	ERIBOvBo 102.3.4	*Conyza bonariensis* var. *bonariensis*	-28.51361, -53.49583
24	ERIBOvBo 102.3.4	*Conyza bonariensis* var. *bonariensis*	-28.51361, -53.49583
25	ERIBOvBo 102.4.1	*Conyza bonariensis* var. *bonariensis*	-28.61897, -52.46786
26	ERIBOvBo 102.4.2	*Conyza bonariensis* var. *bonariensis*	-28.61897, -52.46786
27	ERIBOvBo 102.4.3	*Conyza bonariensis* var. *bonariensis*	-28.61897, -52.46786
28	ERIBOvBo 102.4.4	*Conyza bonariensis* var. *bonariensis*	-28.61897, -52.46786
29	ERIBOvBo 102.5.1	*Conyza bonariensis* var. *bonariensis*	-27.72333, -52.29472
30	ERIBOvBo 102.5.2	*Conyza bonariensis* var. *bonariensis*	-27.72333, -52.29472
31	ERIBOvBo 102.5.3	*Conyza bonariensis* var. *bonariensis*	-27.72333, -52.29472
32	ERIBOvBo 102.5.4	*Conyza bonariensis* var. *bonariensis*	-27.72333, -52.29472
33	ERIBOvBo 201.1.1	*Conyza bonariensis* var. *bonariensis*	-24.46111, -53.31306
34	ERIBOvBo 201.1.2	*Conyza bonariensis* var. *bonariensis*	-24.46111, -53.31306
35	ERIBOvBo 405.1.1	*Conyza bonariensis* var. *bonariensis*	-12.09944, -45.79833
36	ERIBOvBo 405.1.2	*Conyza bonariensis* var. *bonariensis*	-12.09944, -45.79833
37	ERIBOvBo 405.1.3	*Conyza bonariensis* var. *bonariensis*	-12.09944, -45.79833
38	ERIBOvBo 405.1.4	*Conyza bonariensis* var. *bonariensis*	-12.09944, -45.79833
39	ERISUvLe 101.1.1	*Conyza sumatrensis* var. *leiotheca*	-28.72425, -52.87345
40	ERISUvLe 101.1.2	*Conyza sumatrensis* var. *leiotheca*	-28.72425, -52.87345
41	ERISUvLe 102.1.1	*Conyza sumatrensis* var. *leiotheca*	-28.18361, -52.34194
42	ERISUvLe 102.1.2	*Conyza sumatrensis* var. *leiotheca*	-28.18361, -52.34194
43	ERISUvLe 202.1.1	*Conyza sumatrensis* var. *leiotheca*	-23.22806, -51.79556
44	ERISUvLe 202.1.2	*Conyza sumatrensis* var. *leiotheca*	-23.22806, -51.79556

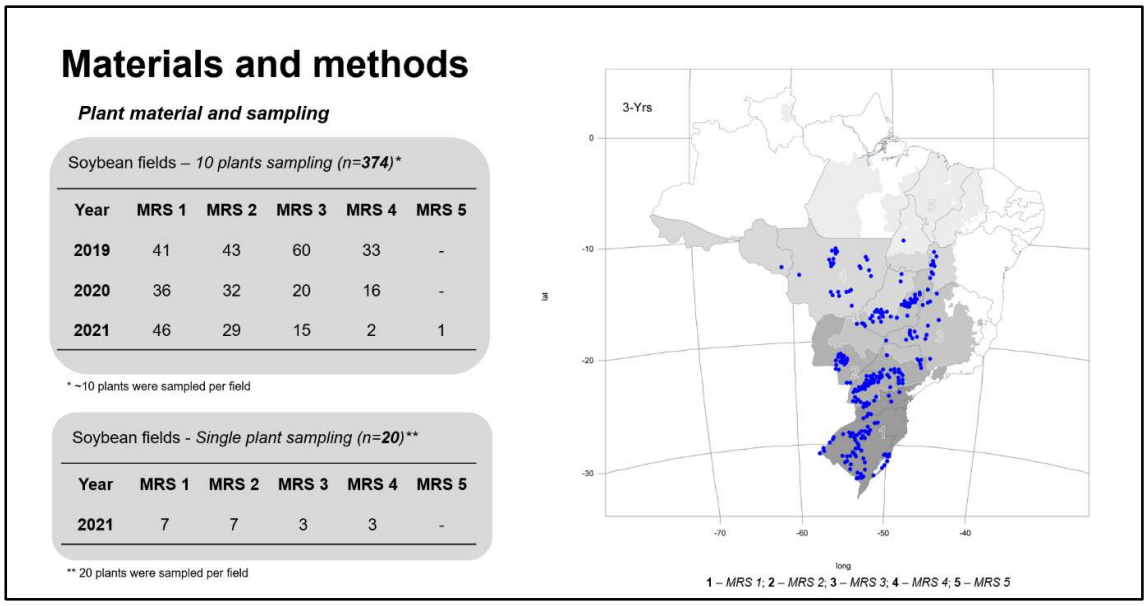
Supplementary Table 2 Continuation.

#	Individual	Specie and variety	Geographic origins
45	ERISUvLe 202.1.3	*Conyza sumatrensis* var. *leiotheca*	-23.22806, -51.79556
46	ERISUvLe 202.1.4	*Conyza sumatrensis* var. *leiotheca*	-23.22806, -51.79556
47	ERISUvLe 203.1.1	*Conyza sumatrensis* var. *leiotheca*	-23.30167, -48.78111
48	ERISUvLe 203.1.2	*Conyza sumatrensis* var. *leiotheca*	-23.30167, -48.78111
49	ERISUvLe 203.1.3	*Conyza sumatrensis* var. *leiotheca*	-22.50722, -46.94222
50	ERISUvLe 203.1.4	*Conyza sumatrensis* var. *leiotheca*	-22.50722, -46.94222
51	ERISUvLe 302.1.1	*Conyza sumatrensis* var. *leiotheca*	-17.78722, -51.00500
52	ERISUvLe 302.1.2	*Conyza sumatrensis* var. *leiotheca*	-17.78722, -51.00500
53	ERISUvLe 304.1.2	*Conyza sumatrensis* var. *leiotheca*	-15.72111, -47.60833
54	ERISUvLe 304.1.3	*Conyza sumatrensis* var. *leiotheca*	-15.72111, -47.60833
55	ERISUvLe 304.2.1	*Conyza sumatrensis* var. *leiotheca*	-15.99861, -47.60417
56	ERISUvLe 304.2.2	*Conyza sumatrensis* var. *leiotheca*	-15.99861, -47.60417
57	ERISUvLe 401.1.2	*Conyza sumatrensis* var. *leiotheca*	-15.56778, -54.44194
58	ERISUvLe 402.1.1	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
59	ERISUvLe 402.1.2	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
60	ERISUvLe 402.1.3	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
61	ERISUvLe 402.1.4	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
62	ERISUvLe 402.2.1	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
63	ERISUvLe 402.2.2	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
64	ERISUvLe 402.2.3	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
65	ERISUvLe 402.2.4	*Conyza sumatrensis* var. *leiotheca*	-11.75194, -55.60917
66	ERISUvLe 403.1.1	*Conyza sumatrensis* var. *leiotheca*	-13.24750, -53.08722
67	ERISUvSu 101.1.1	*Conyza sumatrensis* var. *sumatrensis*	-28.81913, -53.38753
68	ERISUvSu 101.1.2	*Conyza sumatrensis* var. *sumatrensis*	-28.81913, -53.38753
69	ERISUvSu 102.1.2	*Conyza sumatrensis* var. *sumatrensis*	-30.67833, -52.76556
70	ERISUvSu 102.1.2	*Conyza sumatrensis* var. *sumatrensis*	-30.67833, -52.76556
71	ERISUvSu 102.2.1	*Conyza sumatrensis* var. *sumatrensis*	-28.81913, -53.38753
72	ERISUvSu 102.2.2	*Conyza sumatrensis* var. *sumatrensis*	-28.81913, -53.38753
73	ERISUvSu 102.2.3	*Conyza sumatrensis* var. *sumatrensis*	-28.38787, -53.92017
74	ERISUvSu 102.2.4	*Conyza sumatrensis* var. *sumatrensis*	-28.38787, -53.92017
75	ERISUvSu 201.1.1	*Conyza sumatrensis* var. *sumatrensis*	-24.33694, -54.87639
76	ERISUvSu 201.1.2	*Conyza sumatrensis* var. *sumatrensis*	-24.29306, -52.58167
77	ERISUvSu 202.1.1	*Conyza sumatrensis* var. *sumatrensis*	-23.39333, -51.47028
78	ERISUvSu 202.1.2	*Conyza sumatrensis* var. *sumatrensis*	-23.39333, -51.47028
79	ERISUvSu 203.1.1	*Conyza sumatrensis* var. *sumatrensis*	-22.84556, -50.00750
80	ERISUvSu 203.1.2	*Conyza sumatrensis* var. *sumatrensis*	-22.84556, -50.00750
81	ERISUvSu 301.1.1	*Conyza sumatrensis* var. *sumatrensis*	-16.52083, -48.28722
82	ERISUvSu 301.1.2	*Conyza sumatrensis* var. *sumatrensis*	-16.52083, -48.28722
83	ERISUvSu 304.1.1	*Conyza sumatrensis* var. *sumatrensis*	-17.29645, -49.01055
84	ERISUvSu 304.1.2	*Conyza sumatrensis* var. *sumatrensis*	-17.29645, -49.01055
85	ERISUvSu 304.1.3	*Conyza sumatrensis* var. *sumatrensis*	-17.29645, -49.01055
86	ERISUvSu 401.1.1	*Conyza sumatrensis* var. *sumatrensis*	-15.56778, -54.44194
87	ERISUvSu 401.1.2	*Conyza sumatrensis* var. *sumatrensis*	-15.56778, -54.44194
88	ERISUvSu 402.1.1	*Conyza sumatrensis* var. *sumatrensis*	-13.07611, -56.10000
89	ERISUvSu 402.1.2	*Conyza sumatrensis* var. *sumatrensis*	-13.07611, -56.10000

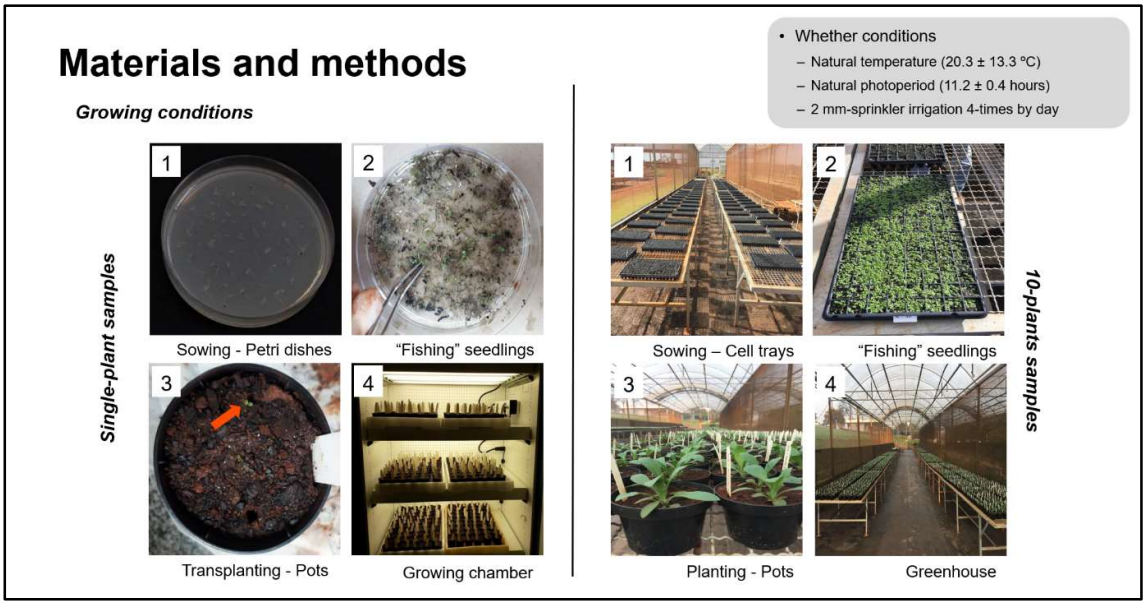
List of supplementary figures

Supplementary Figure S1 Sampling strategies employed to address the objectives of the study.

Supplementary Figure S2 Plant growth conditions and plant material to address the objectives of the study.



Supplementary Fig. S1



Supplementary Fig. S2

CHAPTER 2

POPULATION GENOMICS OF *Conyza* SPECIES IN SOYBEAN MACROREGIONS IN BRAZIL SUGGESTS SPREAD OF HERBICIDE RESISTANCE THOUGH BOTH INTRASPECIFIC AND INTERSPECIFIC GENE FLOW[1]

ABSTRACT

Herbicide-resistance *Conyza* spp. are a growing threat to many crops in Brazil and worldwide. These widespread weeds are closely related species and often co-occur across landscapes. The processes governing *Conyza* spp. coexistence and population structuring are unknown. To characterize the origins of resistance and elucidate the mechanisms for its spreading, we accessed the genetic diversity and structure of glyphosate-resistance *Conyza* spp. in Brazil. Twenty populations were sampled from soybean fields across four macroregions (MRS). Using the genotyping by sequencing method, 2,998 single-nucleotide polymorphisms (SNPs) were obtained across the genome of *C. bonariensis* and the closely related *C. sumatrensis*. The SNP data was used to examine genetic diversity, structure, and gene flow among MRSs. Higher genetic diversity (π), heterozygosity (H_O/H_E), and lower inbreeding coefficient (F_{IS}) were detected in *Conyza* populations in the MRS 1 (southern) than in the three other MRSs. There was found strong genetic structure clustering individuals into three genetic groups (F_{ST} = 0.22; *p*-value = 0.000) associated to the MRS 1, MRS 2, and MRS 3 plus MRS 4. Thus, the resistance to glyphosate in *Conyza* spp. was originated from independent selections. Our dataset supports the occurrence of intraspecific gene flow across the landscape in Brazil, and cluster analysis revealed individuals of *C. bonariensis* that did not group within-species. These findings suggest that allelic introgressions within and among species have likely impacted the evolution and spread of resistance to glyphosate in *Conyza* spp. in Brazil. We discuss the implications of these findings to avoid the evolution of new resistance cases, particularly in the context of the recent released stacked traits herbicide-resistant in soybeans.

Keywords: closely related species, genotyping by sequencing, interspecific hybridization, population structure, single-nucleotide polymorphism.

[1] Chapter written according to the *Molecular Ecology* Journal *(1365-294X)*.

2.1 INTRODUCTION

Habitats and landscapes comprising extensively cropland foist new challenges upon ecologists and weed scientists every time a new technology emerges and is incorporated into the set of weed control tactics. The usage of such technologies – including no-tillage, novel herbicides, and herbicide-resistant crops – modify the cropland setting the stage for new weed problems (Chauhan et al., 2012). In fact, changes in ecological dynamics (e.g., shift of weed flora) and evolutionary adaptations (e.g., herbicide resistance) are mostly associated to farming practices (Radosevich et al., 2007). These changes have been frequently detected in cropping systems in South America where intensive and large-scale farming impose a fierce selection pressure on pests (Zucchi et al., 2019). Although innovations have led to steadily increasing yields for such crops like soybeans, weed control failures are still more often than would be desirable (Heap, 2022; Heap and Duke, 2018).

Whether or not a weed population is capable to adapt in response to herbicides depends on whether that population contains the necessary genetic variation (Delye et al., 2013; Karn and Jasieniuk, 2017). Population size, ploidy level, epigenetic regulation, gene flow, fitness costs, and selection play a role in the evolved adaptive traits (Leimu et al., 2006; Markus et al., 2018; Smith et al., 2020). The use of herbicides decreases genetic diversity of susceptible weed populations because of recurrent population bottlenecks and strong positive selection (Neve et al., 2009). Each new strong selection will favor alleles that confer resistance to herbicides, if present in the population, reducing genetic variation as the frequency of resistance individuals increases. Even though, high genetic diversity may occur regardless the resistance frequency because weed populations are also influenced by other environmental factors (Karn and Jasieniuk, 2017).

Nowadays, soybean has been cultivated in approximately 41 million hectares in Brazil (USDA, 2022) across five macroregions (MRSs) that differ by the latitude and climatic conditions (Kaster and Farias, 2012). These production regions differ by cropping systems and technological level of soybean production, which influences the weed flora and prevailing agricultural practices (Lucio et al., 2019). Despite this, glyphosate-resistant soybean varieties are widely adopted across Brazilian MRSs and glyphosate has been used in about 98% of the soybean fields last three years (Spark, 2022). Cases of resistance to glyphosate emerged exponentially across MRSs since

2005 and to date is second herbicide mode of action in the number of resistant weedy species (Heap, 2022). Meanwhile, glyphosate is still the most used herbicide in soybeans despite ubiquitous resistance in weed populations, especially in the *Conyza* genus (Lucio et al., 2019; Silva et al., 2021).

Conyza (Asteraceae: Astereae) is a New World genus which consists of as many as 150 dicot species (CWG, 2022), among which there are some weedy species among the most widespread throughout the world. *C. bonariensis* and the closely related *C. sumatrensis* are the main *Conyza* weeds in soybeans in Brazil, and most populations are glyphosate-resistant (Lucio et al., 2019; Mendes et al., 2021). These *Conyza* weeds co-occur in similar environments because of shared traits and niches, and commonly overlap, forming unique population structures across distinct regions of the globe. In fact, *C. bonariensis* and *C. sumatrensis* have recently speciated from a common ancestor and therefore there has been insufficient time for the complete lineage sorting (Alpen et al., 2014). Although populations of these species have evolved to herbicide resistance concomitantly, the processes governing species coexistence and population structuring of *Conyza* spp. are unknown.

In plants, mating system is by far the most important life history trait influencing genomic structure since determine the level of gene flow with other populations and even species (Duminil et al., 2009). *Conyza* spp. are primarily self-compatible species through both intra- and inter-capitulum geitonogamy but outcrossing within species frequently occur (Henry et al., 2008; Zelaya et al., 2007). For instance, self- and cross-pollination yielded 59 and 48 seeds per capitulum in C*. sumatrensis*, respectively, due a versatile mating system that allow intraspecific gene flow (Hao et al., 2009). In addition, given that several weedy species of this genus comprise similar level of ploidy and number of chromosomes, some outcrossing rates can likely happen even among different species. When capitula of the diploids *C. canadensis* and *C. ramosissima* have interacted freely, 3% of the ova were fertilized by the other species, generating interspecific hybrids (Zelaya et al., 2007). However, little is known about interspecific gene flow among *C. bonariensis* and *C. sumatrensis* that are hexaploid species with 54 chromosomes and interact freely in cropping systems worldwide.

Herein, it has been hypothesized that gene flow within and among populations of closely related species may spread herbicide resistance and govern population structuring across an agricultural landscape. Thus, we characterized the genetic variation of glyphosate resistant populations of *Conyza* spp. across Brazil with

genotyping by sequencing (GBS) to address the following questions: (i) did occur intraspecific gene flow of herbicide resistance alleles across the landscape? (ii) is there interspecific gene flow and spread of resistance among polyploid *Conyza* spp.? We present the first study that compares the genomic variation of mixed weedy species as the closely related *Conyza* species co-occur in the soybean in Brazil and other regions of the globe. Our data allow to infer about tactics to avoid spread of resistance to the novel technologies, such as resistance to synthetic auxins in the recently released 2,4-D and dicamba-resistant soybeans.

2.2 MATERIAL AND METHODS

2.2.1 Plant material

A total of 314 individuals of *Conyza* spp. were sampled from 20 soybean fields across four MRSs in Brazil in 2021 growth season: 7 in MRS 1, 7 in MRS 2, 3 in MRS 3, and 3 in MRS 4 (Figure 1; Table S1). The fields were selected based on the representativeness of cropping area and system as well as by the occurrence of mature plants that have escaped the control by glyphosate. Seeds from mature inflorescences were sampled and placed in a paper bag (single-plant sample), identified with their geographic origins, and stored at -2°C until the plant material sowing.

2.2.2 Growing conditions and species identification

A plant by sample was stablished in pots of 1 dm^{-3} size filled by potting soil mix in greenhouse at 25 ± 3°C temperature, 65 ± 13% humidity, and daily irrigation until the end of the reproductive growth stage (Figure S1). The *Conyza* species were taxonomically classified by the dichotomous key of Pruski and Sancho (2006), in which the main morphological traits were involucre, capitula and inflorescence types. Twelve voucher specimens representing the species and varieties found in the study were deposited in the Irina Delanova Gemtchújnicov Herbarium/Botu of UNESP (BOTU 34833-34844).

2.2.3 Whole plant assay to screen for glyphosate resistance

Conyza individuals (n=314) and putative resistant and susceptible accessions (n=4) were screened for glyphosate at 1,200 g ha^{-1} when the plants achieved the 4- to 6-leaf stage according to Mendes et al. (2021). The level of plant mortality (0-100%) was visually rated 42 days after glyphosate treatment and data was subjected to hierarchical cluster analysis to distinguish herbicide-resistant individuals. An individual was screened as glyphosate- resistant when was grouped in cluster 1 and its level of mortality was meaningly lower than the susceptible reference accessions of the assay.

2.2.4 DNA isolation and genomic library preparation

Leaf tissue (100 mg) of the fourth leaf was sampled and then freeze dried and ground into a powder to extract genomic DNA using DNeasy® Plant Mini Kit (Qiagen) following the manufacturer's protocol. DNA quality and quantity were assessed by electrophoresis on agarose gels (1% w v^{-1}) stained with SYBR Safe DNA Gel Stain, and by visual comparison with phage lambda DNA (Invitrogen). DNA samples were resuspended in 100 ml water, and then dilutions were made up to 30 ng μl^{-1}, after which DNA samples were stored at -20°C until the sequence-based genotyping step.

The GBS libraries were prepared using *PstI* and *MseI* restriction enzymes according to the protocol of Poland et al. (2012) and then digested DNA sequences were polymerase chain reaction (PCR) amplified. Sequencing was performed in an Illumina NextSeq2000 with single-reads 101 nt in length and libraries were quantified using the KAPA Library Quantification kit (KAPA Biosystems). Illumina generated reads with the samples of *Conyza* species investigated in present study will be submitted at the NCBI Sequence Read Archive (SRA) (Figure S2).

2.2.5 Genotyping and loci filtering

Samples were demultiplexed with *process_radtag* module from STACKS v. 1.42 (Catchen et al., 2013). Only cases with at least 150,000 sequencing reads were retained for bioinformatic processes. Raw reads were trimmed to 70 bp and shorter reads were discarded with CUTADAPT (Martin, 2011). After, reads were aligned to the reference genome of *C. canadensis* (NCBI Accession GCF 010389155.1; see Laforest

et al. 2020) using BWA-MEN set to default parameters (Li, 2013). Aligned reads were processed with STACKS in which a minimum stack depth of three (*M*) and a maximum of four mismatches (*m*) were allowed to generate the RADloci (Ilut et al., 2014). The *population* module filtered the data to limit missing loci to maximum 50% within fields and 50% among fields, as well as minor allele frequency of at least 5% (Roesti et al., 2012). In few cases, more stringent filtering tactics were used to reduce the impact of missing data. Outputs were saved in genepop, vcf, and structure (.str) formats for downstream analyses.

2.2.6 Population genomics analysis

Genomic diversity of *Conyza* weeds, estimated for each MRS of Brazil, included observed heterozygosity (H_O), expected heterozygosity (H_E), nucleotide diversity (π), and the inbreeding coefficients (F_{IS}). Likewise, we tested the standardized index of association, rBarD, (Agapow and Burt, 2001), and the genetic differentiation through the pairwise Fixation Index F_{ST} (Weir and Cockerham, 1984). H_O, H_E, π, F_{IS}, and F_{ST} values were determined using the R library *adegenet* (Jombart, 2008), while for rBarD the R library *poppr* was used with 1,000 permutations (Kamvar et al., 2014). A Mantel test with 9,999 permutations was made between the matrix of F_{ST} described above and a matrix of geographic distances using the library *ecodist* of R (Goslee and Urban, 2007). We also performed a hierarchical analysis of molecular variance (AMOVA) in the software GenAlEx to examine the distribution of genetic variation among MRSs (Peakall and Smouse, 2012).

The genetic structure of *Conyza* weeds was examined in the software STRUCTURE v.2.3.4 according to Pritchard et al. (2000) using a model where admixture and correlated allele frequency were allowed. Five rounds of 10^5 burn-in interactions and 10^6 Markov chain Monte Carlo steps were used. We simulated the number of clusters ranging from 1 to 15 (K = 1 to K = 15) and then most likely value of *K* was estimated based on the ad hoc statistic Delta *K* (Evanno et al., 2005). Lastly, the genetic structure was also examined though principal component analysis (PCA) using *adegenet* library of R to compare the clustering within and among species and populations.

2.2.7 Phylogenetic analysis

A phylogenetic analysis was made to cluster the genotypes based on their similarity and identify potential interspecific hybridization when individuals of one species group in the cluster of another species. Thus, a distance-based dendrogram was created using the neighbor-joining method based on the Jaccard similarity index in the DARwin program (Perrier and Jacquemoud-Collet, 2006). Clade supports within the neighbor-joining trees were assessed by a 10,000 replicates bootstrap test, and dendrograms were build using the *GTR* model in MEGA v. 7 (Kumar et al., 2016).

2.3 RESULTS

2.3.1 Screening for glyphosate resistance

Out of 314 individuals from 20 fields, 42 individuals were taxonomy classified as *C. bonariensis* (sampled in 8 of 20 fields) and 272 were identified as *C. sumatrensis* (sampled in all the 20 fields) (Table S1). Among them, 269 *Conyza* individuals (86%) were screened as glyphosate-resistant in the single-dose assay, comprising 30 individuals of *C. bonariensis* and 233 of *C. sumatrensis* (Figure 2). Resistant individuals were then genotyped without distinction of the weedy species since the study aimed to assess the genomic variation as the *Conyza* populations co-occur in soybean fields.

2.3.2 SNP discovery and data processing

A total of 430,431,768 raw reads were generated in the GBS after samples multiplexing and checking, and sequencing was successful, considering the high number of reads obtained in the study. After demultiplexing, quality control, and filtering strategies to solve bias corrections due to missing data, 2,998 high quality nonduplicated SNPs were kept for the population genomics analysis. Out of 269 *Conyza* individuals, 67 were excluded for downstream bioinformatic processes due to low genome coverage (<150,000 reads), excess of missing data, and deviation of CG content.

2.3.3 Genomic diversity

The r_d index was significant ($p < 0.001$) in all situations and revealed disequilibrium between genetic markers in Brazilian MRSs, indicating sexual reproduction is predominant in *Conyza* weeds (Table 1). Great variation in *Conyzas* nucleotide diversity was observed throughout the landscape, which were higher in MRS 1 ($\pi = 0.122$), compared to the other three MRSs ($\pi \leq 0.061$) (Table 2). Observed and expected heterozygosity revealed the same pattern verified of the nucleotide diversity, with larger values related to higher proportion of *C. bonariensis* individuals in the MRS. The inbreeding coefficient also differed substantially among MRSs and ranged from 0.372 to 0.778, showing the populations are not in equilibrium due to both inbreed and outbreed (Table 2). Therefore, our plant material has exhibited high genomic diversity and a hybrid mating system, consistent with recent population expansion due to gene flow within and among MRSs.

2.3.4 Genomic differentiation and genetic structure

For subsequent analyses of population, we did not discriminate SNPs into datasets of putatively neutral or influenced by selection to not favor either of the closely related species that were assessed concomitantly. A significant genetic structure in *Conyza* weeds was found in AMOVA ($F_{ST} = 0.220$, $p < 0.001$) where 78% of the variance was distributed within MRSs rather and 22% among MRSs (Table 3). These (mixed) populations had a considerable degree of differentiation with F_{ST} ranging from 0.084 to 0.274 ($p < 0.001$), mainly comparing the southern versus northern MRSs (Table 4). In fact, the lower pairwise F_{ST} estimate was found between the MRS 3 and 4 ($F_{ST} = 0.084$) that comprise the populations of *Conyza* spp. sampled in the Cerrado Region in the northern Brazil. Mantel test revealed a weak but significant correlation ($r = 0.49$; $p = 0.048$) and supports that genetic variation (F_{ST}) among MRSs was also related with geographical distances (Figure 3).

The Delta *K* of the Evanno's method showed the highest peak at $K = 8$ (8 clusters), followed by lower peaks from $K = 6$, 4 and 2, in which many cases purebred individuals were apparent across MRSs (Figure 4). *Conyza* spp. and populations were strongly structured within MRSs according to the admixture analysis, except *C. sumatrensis* samples at MRSs 3 and 4 that belongs to a same ancestral group. Migration of few

individuals among MRSs were noted driven mainly from the MRSs 1 and 2. In addition, *C. bonariensis* individuals differed from the *C. sumatrensis* ones across all cluster but were not always genetically purebred or shared the same genomic background (Figure 4). The PCA analysis (PC1 = 34.5%; PC2 = 3.8%) showed two clusters for *C. sumatrensis* weeds, separating individuals from southern (MRS 1 and 2) and northern (MRS 3 and 4) (Figure 5). A third cluster composed by individuals of *C. bonariensis* was revealed in the analysis, although six individuals were grouped within the clusters of the closely related *C. sumatrensis*.

2.3.5 Phylogenetic analysis

Phylogenetics based on genetic similarity divided *Conyza* weeds from 20 populations and two species into four major clusters corresponding to the MRSs of Brazil with bootstrap support of at least 70% (Figure 6). While individuals from MRS 1 were divided into two clades mostly according to the weedy species, those from MRS 2 had a specific clade and those from MRSs 3 and 4 shared a clade. In addition, this analysis allowed to identify the six individuals of *C. bonariensis* that were not grouped within-species: 101.1.8, 101.1.6, 201.3.3, 201.3.5, 301.1.9, and 301.1.12 (Figure 6). The first four individuals were taxonomically classified as *C. bonariensis* var. *bonariensis*, while the last two individuals were classified as *C. bonariensis* var. *angustifolia* (Table S1). Lastly, phylogenetics also identified seven individuals of *C. sumatrensis* that have migrated among MRSs, as follows: 201.1.11, 301.1.8, 301.1.13, 303.1.2, 303.1.5, 304.1.9, and 405.1.5.

2.4 DISCUSSION

2.4.1 Intraspecific gene flow

Did occur intraspecific gene flow of herbicide resistance alleles (among populations) across the landscape in Brazil? Our findings support that closely related *Conyza* species have formed unique structures of mixed but often well-differentiated populations experiencing high admixture and local adaptation. Overall, we found high genomic diversity associated to both inbreeding and crossbreeding behaviors, which enable the occurrence of moderate intraspecific gene flow (Tables 1 and 2). Almost a

quarter of the genetic variation occurred among the MSRs in Brazil, and genetic and spatial variation correlated, indicating gene flow among macroregions (Figure 3; Tables 3 and 4). Lastly, the genetic structuring did not differ between MRSs 3 and 4 and there were migrants among MRSs, which also reinforce the occurrence of regional gene flow (Figures 4, 5 and 6). Thus, our dataset supports the evidence of intraspecific gene flow by pollen and seeds across the landscape in Brazil.

In *C. sumatrensis*, the analysis microsatellite polymorphism showed moderate gene flow (Nm = 0.5535) of six biotypes from MRS 2, suggesting out-crossing among plants from this region (Marochio et al., 2017). In other study, with the same species and analysis, moderate allele transfer (Nm = 0.3441) was suggested among 50 biotypes sampled from MRSs 1, 2, and 3 of Brazil (Ruiz et al., 2022). In C. bonariensis, multiple recombinant alleles were detected within 35 populations in California, United States, indicating gene flow of herbicide resistant genes (Okada et al., 2015). These findings from different countries are consistent with those found in our study and confirm that *C. bonariensis* and *C. sumatrensis* display a versatile mating system allowing gene flow. Interesting, this feature appears to be specific for these *Conyza* weeds since other ones like C. *canadensis* are largely selfers with limited outcrossing (Okada et al., 2013; Ye et al., 2016).

Nowadays, populations of *Conyza* spp. resistant to glyphosate are widespread across most of soybean cropping regions in Brazil, as reported in resistance surveys in Lucio et al. (2019) and Mendes et al. (2021). It was proposed resistance evolved once in the south, where it was first reported, and then spread to north; but resistant to herbicides may also evolve concomitantly in multiple locations. Although *Conyza* weeds show low to moderate degree of gene flow among the four MRSs of Brazil, our findings suggest multiple independent origins of resistance across the landscape. In fact, structure analysis releveled at least three genetic groups of *Conyza* weeds in Brazil rejecting the possibility of a single founder effect that evolved and spread resistance (Figure 5). Resistance to glyphosate in *C. bonariensis* and *C. canadensis* in California, United States, was related to multiple independent origins according to multilocus data (Okada et al., 2013; 2015).

After resistance was evolved and detected, the pace with which resistance has evolved over a landscape with extensive cropland was mainly driven by the degree and ways of gene flow to the surrounding regions. As discussed, our data have showed moderate gene flow among populations of *Conyza* spp. across MRSs and assisted the

spread of resistance through pollen, seeds, or vegetative propagation. In *C. bonariensis*, the analysis of shared multilocus genes indicated higher influence of seeds than pollen to spread resistance to glyphosate across a large landscape (Okada et al., 2015). In fact, *Conyza* weeds display a robust seed production with an estimated 200,000 seeds per plant and long-ranged seed dispersal are wind assisted (Huang et al., 2015; Ye et al., 2016). Likewise, seeds of *Conyza* spp. may have been transported with agricultural machinery among distinct soybean cropping regions, as evidenced for *Digitaria insularis* (Netto et al., 2021).

2.4.2. Interspecific gene flow (hybridization)

Is there interspecific gene flow (hybridization) and spread of resistance among polyploid *Conyza* spp.? In our study, observed and expected heterozygosity within populations varied from 0.028 to 0.084, with larger values related to higher proportion of *C. bonariensis* individuals in the MRS (Table 2). The inbreeding coefficient were positive and significant in all MRSs, ranging from 0.372 to 0.778, which evidence both selfing and outcrossing mating systems, mainly in MRS 1 (Table 2). These findings have suggested strong potential for intraspecific gene flow between *Conyza* spp. and introgression of herbicide resistant alleles even in the absence of selection pressure. In fact, *C. bonariensis* individuals did not cluster within-species in three analysis (Figure 4, 5 and 6), which is unusual considering the number of loci that were sequenced in the present study. We have hypothesized at least three different reasons for these findings in the present study: misidentification of species, incomplete lineage sorting, and interspecific hybridization.

The incorrect *C. bonariensis* identification is unlikely since individuals differed as the plant matures, comprising a well-defined plant morphotype, identified according to the key of Pruski and Sancho (2006). Also, we submitted six of these individuals as voucher specimens at the Herbarium where the plant species identification was confirmed and then vouchers were annotated properly. Incomplete lineage sorting is also unlikely even though *C. bonariensis* is paraphyletic to *C. sumatrensis* because of the recent speciation from a common ancestor (Alpen et al., 2014). In fact, the number of loci studied would be enough to detect genomic differences among individuals once we observed high genomic diversity within populations in all regions (Table 2). Thus, the *C. bonariensis* individuals not grouped within-species are possibly interspecific

Conyza hybrids generated from individuals that interact freely in soybean fields across Brazil.

C. bonariensis and *C. sumatrensis* are, according to the samples obtained in our study, genetically compatible, capable of transferring alleles, and producing interspecific hybrids that are vigorous and fertile. These weeds exhibit similar phenology, inflorescence traits and pollen/ovule ratio (Sancho, 2014), and shared pollinators (Hao et al., 2009), that assist pollen transfer between the two species. In addition, *C. bonariensis* and *C. sumatrensis* feature a common hexaploidy structure of 2n = 54 that allow for successful chromosome pairing during cell meiosis (Thébaud and Abbott, 1995). Both weeds represent sibling species as ascertained by different DNA barcode gene regions in which they commonly have clustered within a monophyletic clade (Alpen et al., 2014). This phylogenic analysis estimated a recent speciation event between *C. bonariensis* and *C. sumatrensis* and provides further support to our thesis of genetic compatibility between them.

In *Conyza* genus, evidence of interspecific hybridization is at present restricted to Europe and United States, mainly for diploid species such as *C. canadensis* (Thébaud and Abbott, 1995; Zelaya et al., 2007). To our knowledge, there was a unique report of hybrids between *C. bonariensis* and *C. sumatrensis*, namely *C. daveauiana* in France, with unknown fertility (McClintock and Marshall, 1988). In cropped areas, *C. bonariensis* and *C. canadensis* have crossed and generated hybrid progenies, with likely reduced fitness due to the difference in ploidy level (Okada et al., 2013; 2015). If mutation for herbicide resistance occurs frequently in both *Conyza* weeds, the interspecific gene flow may not be a major factor adding to the evolution and spread of resistance. Otherwise, introgression from the other species might be a source of alleles and have had an impact on the evolution of resistance in one or both *Conyza* species (Okada et al., 2015).

2.4.3. The implications for weed management

The three well-defined genetic backgrounds of mixed populations of *Conyza* spp. in MRS 1, MRS 2 and MRS 3 plus 4 of Brazil should be considered for the adoption of new practices and technologies. For example, new technologies should ideally be tested in these three distinct scenarios of *Conyzas* and their technical positionings and stewardship should be customized by macroregion. Weed shifts and resistance

evolution in *Conyza* spp. are expected to occur first in MRS 1 and 2 while adaptations to the cropping practices are expected to happen slowly in MRS 3 and 4. As resistance originated from multiple selections and then spread by gene flow in *Conyza* weeds, new technologies should be based on reduction of selection pressure by herbicides. Thus, recently released stacked trait 2,4-D- or dicamba-resistant soybeans should diversify weed management practices to keep resistance of *Conyza* spp. and other weedy species under control.

In conclusion, *C. bonariensis* and the closely related *C. sumatrensis* have formed unique structures of mixed but often well-differentiated populations experiencing high admixture and local adaptation. Three genetic backgrounds of mixed populations were noted in MRS 1, MRS 2 and MRS 3 plus 4 of Brazil but occurrence of gene flow and even migrants were detected among the four MRSs. The origin of the resistance to glyphosate was related to multiple independent selections that influenced the different genetic backgrounds found in the current mixed populations of *Conyza* spp. After resistance was evolved and detected, evidence suggest the spread of resistance within MRSs through of gene flow among populations and species (interspecific hybridization). *C. bonariensis* and *C. sumatrensis* sampled in this study were genetically compatible, capable of transferring alleles, and producing interspecific hybrids that are vigorous and fertile.

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2.6 APPENDICES

Table 1 Index of association of mixed populations of the closely related species *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) from four soybean macroregions (MRS) in Brazil estimated from RADseq data (202 individuals and 2,998 SNPs included).

Macroregion	n	r_d	*p*-value
MRS 1	7 (26/54)	0.308	0.001
MRS 2	7 (02/80)	0.009	0.001
MRS 3	3 (02/18)	0.027	0.001
MRS 4	3 (00/20)	0.040	0.001

Abbreviations: n, number of populations (number of ERIBO individuals / number of ERISU individuals); r_d, index of association.

Table 2 Genetic diversity statistics of mixed populations of the closely related species *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) from four soybean macroregions (MRS) in Brazil estimated from RADseq data (202 individuals and 2,998 SNPs included).

Macroregion	n	H_O	H_E	F_{IS}	π
MRS 1	7 (26/54)	0.080	0.084	0.372	0.122 ± 0.003
MRS 2	7 (02/80)	0.036	0.040	0.567	0.061 ± 0.002
MRS 3	3 (02/18)	0.030	0.033	0.699	0.049 ± 0.002
MRS 4	3 (00/20)	0.028	0.029	0.778	0.042 ± 0.002

Abbreviations: n, number of populations (number of ERIBO individuals / number of ERISU individuals); H_0, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficients; and π, nucleotide diversity (mean ± SE).

Table 3 Hierarchical analysis of molecular variance (AMOVA) of mixed populations of the closely related species *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) from four soybean macroregions (MRS) in Brazil estimated from RADseq data (202 individuals and 2,998 SNPs included).

Source of variance	df	SS	VC	PV	ϕ	*p*-value
Among MRSs	3	4,530	39	22	0.220	0.0001
Among fields within MRSs	201	27,826	138	78		
Total	204	32,356	177			

Note: ϕ refers the F_{ST} population differentiation statistics to test hypotheses about population differentiation.

Abbreviations: df, degrees of freedom; SS, sum of squares; VC, variance components; PV, percentage of variation.

Table 4 Pairwise fixation index for mixed populations of the closely related species *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) from four soybean macroregions (MRS) in Brazil estimated from RADseq data (202 individuals and 2,998 SNPs included).

Macroregion	MRS 1	MRS 2	MRS 3	MRS 4
MRS 1	---	0.229	0.230	0.238
MRS 2	<0.001	---	0.252	0.274
MRS 3	<0.001	<0.001	---	0.084
MRS 4	<0.001	<0.001	<0.001	---

Note: Upper values, fixation index (F_{ST}); bottom values, *p*-values.

List of figures

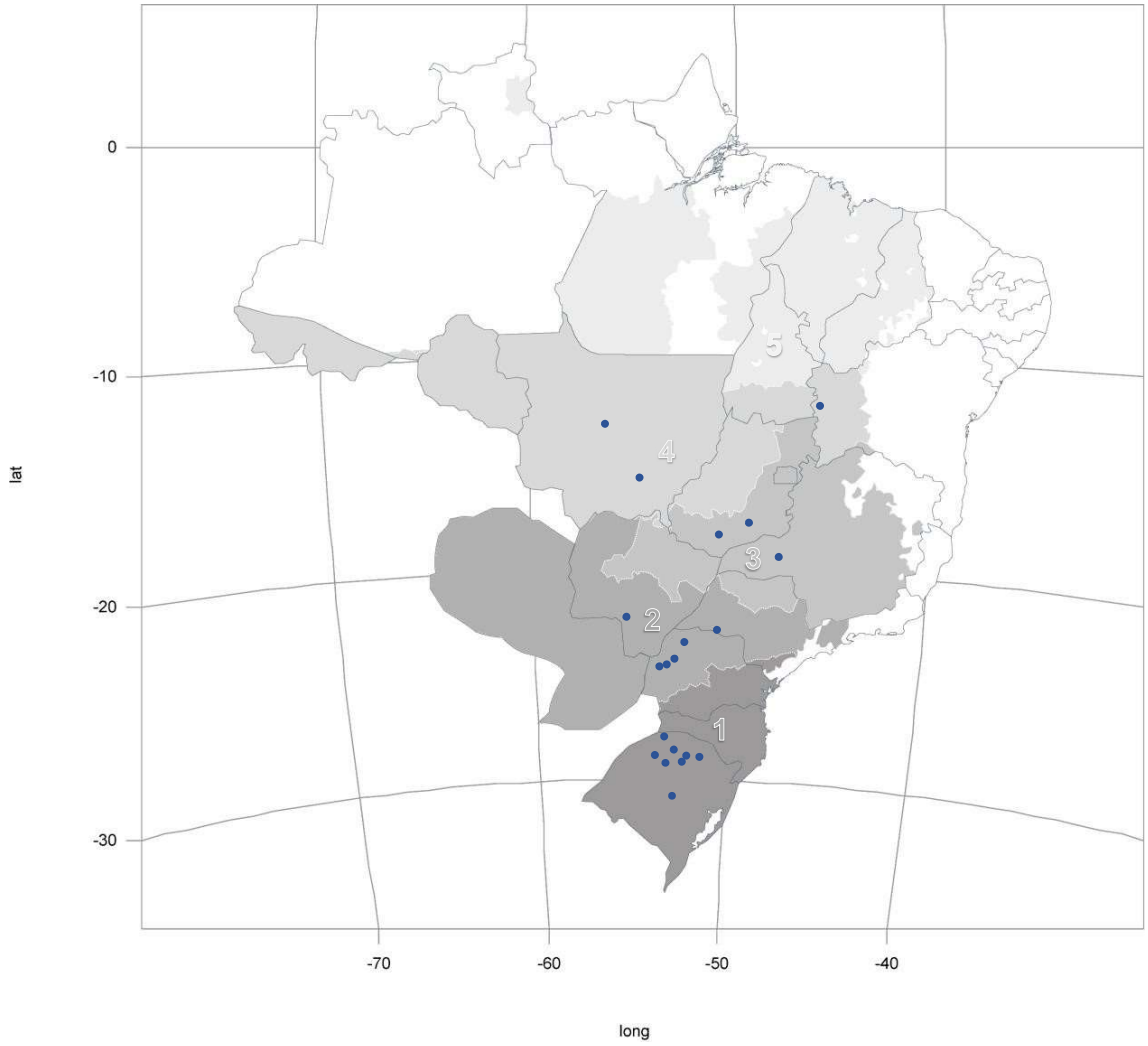

Fig 1.

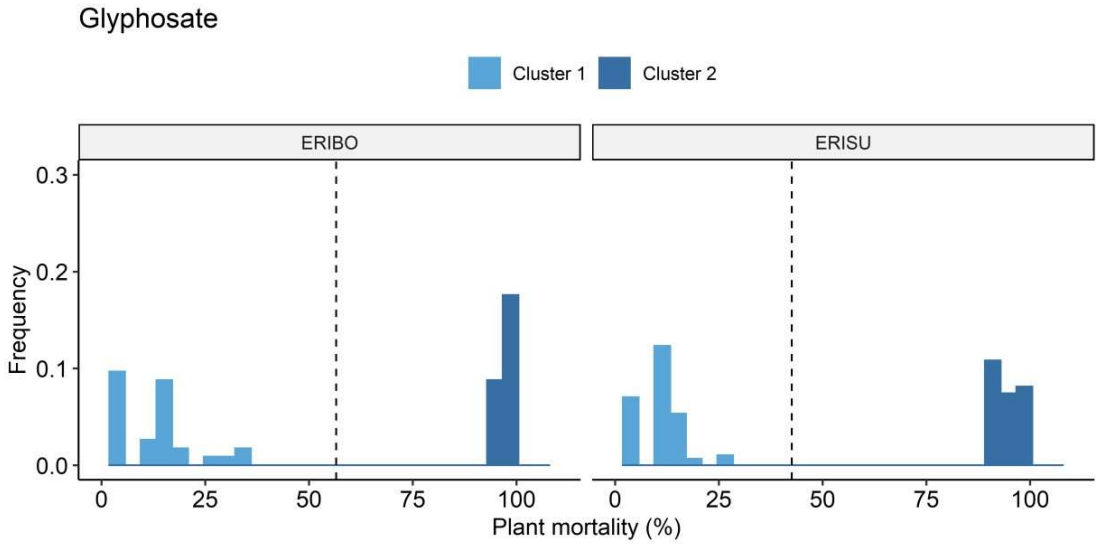
Glyphosate
Cluster 1
Cluster 2
ERIBO
ERISU
Frequency
0.3
0.2
0.1
0.0
0
25
50
75
100
Plant mortality (%)

Fig 2.

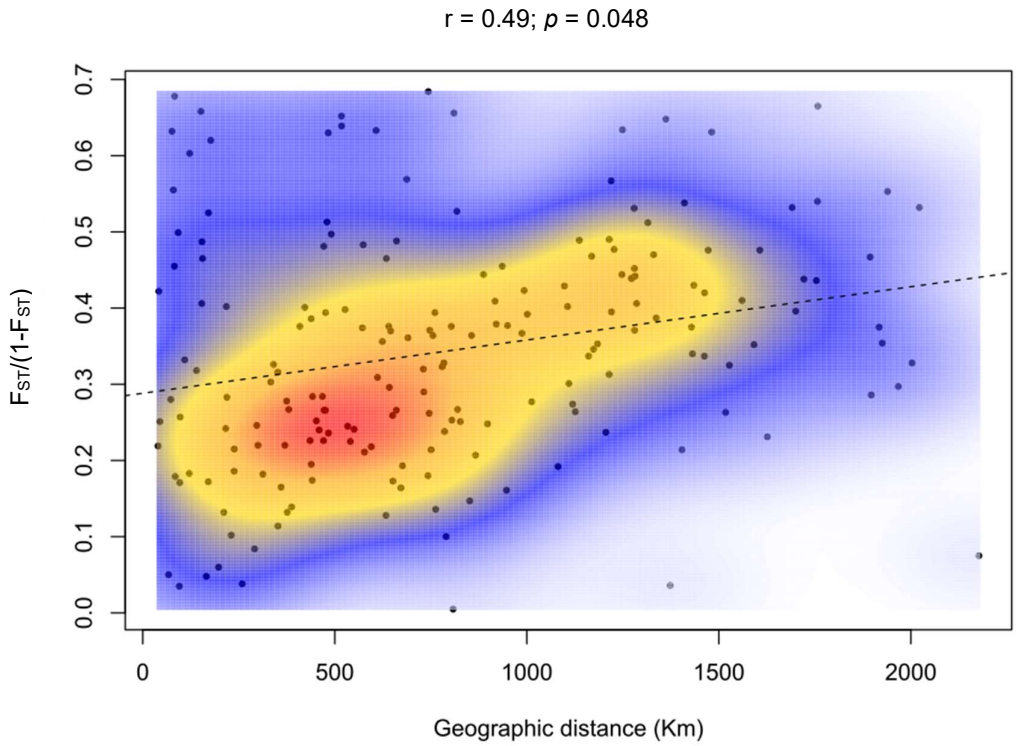
r = 0.49; p = 0.048
FST/(1-FST)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
500
1000
1500
2000
Geographic distance (Km)

Fig 3.

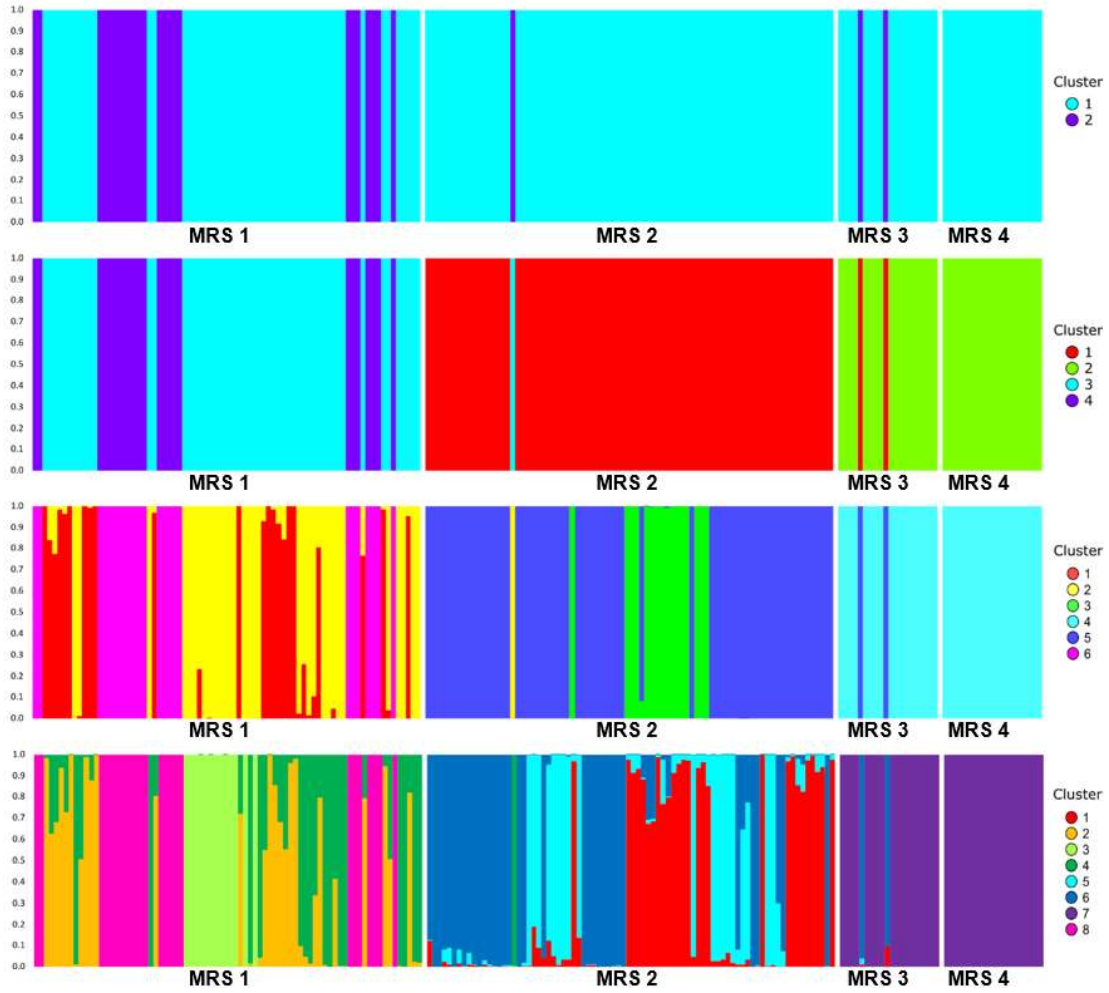

Fig 4.

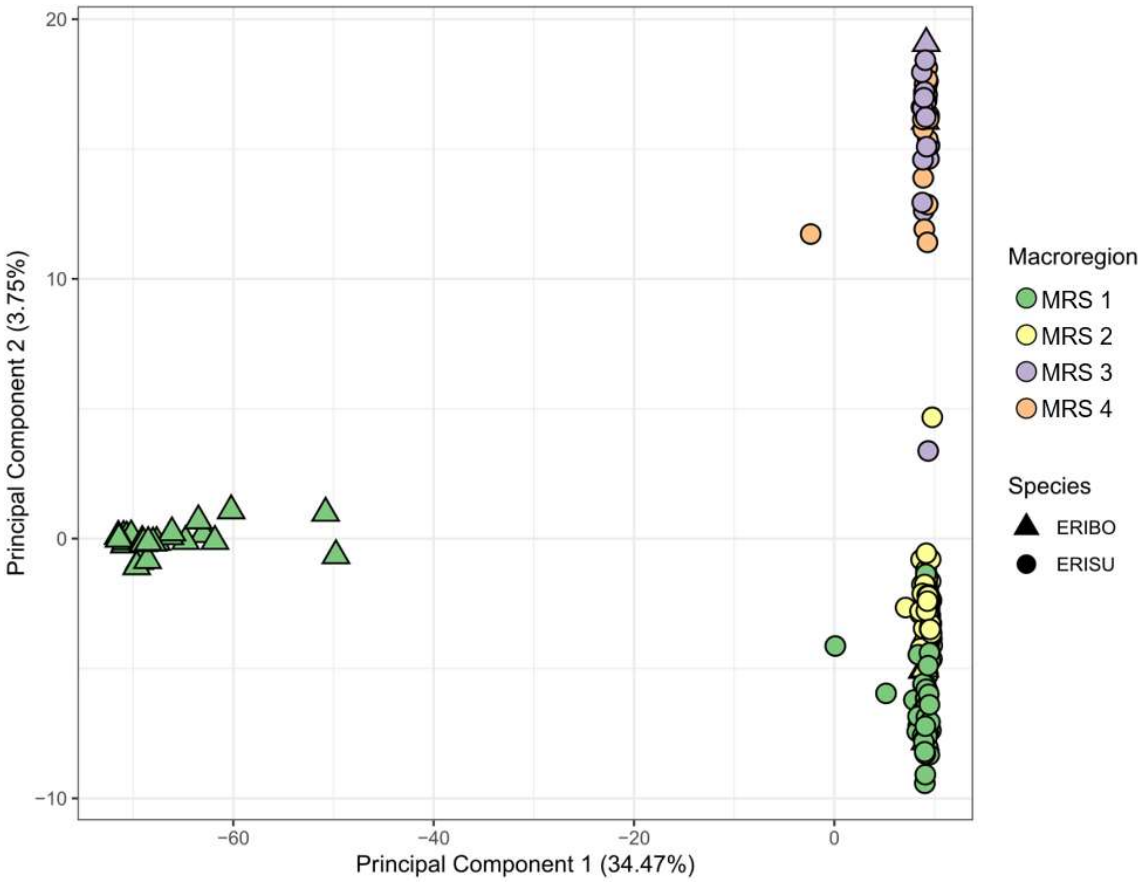
Principal Component 2 (3.75%)
20
10
0
−10
−60
−40
−20
0
Principal Component 1 (34.47%)
Macroregion
MRS 1
MRS 2
MRS 3
MRS 4
Species
ERIBO
ERISU

Fig. 5

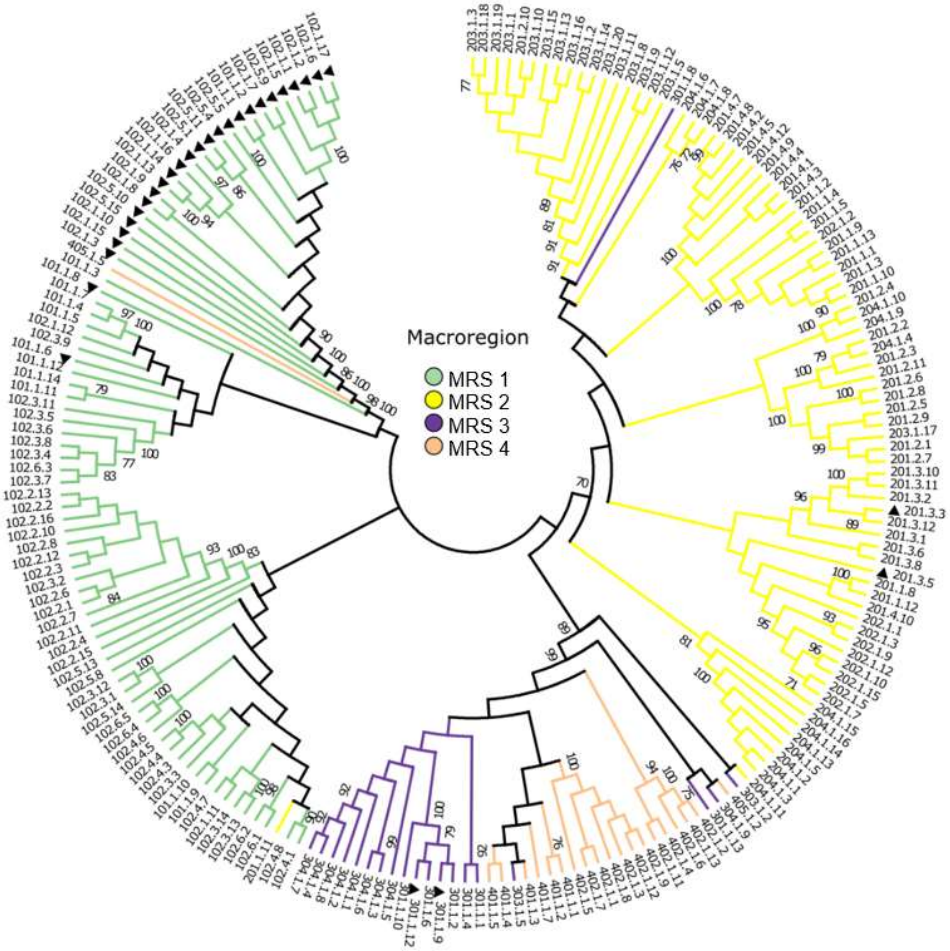

Fig. 6

Supplementary Table 1 (Table S1) Sampling sites of *Conyza* spp. with geographic origins.

Site	Species	Geographic origins
1	*Conyza bonariensis* and *Conyza sumatrensis*	-30.67833, -52.76556
2	*Conyza bonariensis* and *Conyza sumatrensis*	-28.81913, -53.38753
3	*Conyza sumatrensis*	-28.38787, -53.92017
4	*Conyza sumatrensis*	-28.36862, -51.64128
5	*Conyza bonariensis* and *Conyza sumatrensis*	-28.61897, -52.46786
6	*Conyza sumatrensis*	-28.13389, -52.56694
7	*Conyza sumatrensis*	-24.40668, -53.51424
8	*Conyza sumatrensis*	-18.96111, -47.85806
9	*Conyza sumatrensis*	-17.29645, -49.01055
10	*Conyza bonariensis* and *Conyza sumatrensis*	-16.52083, -48.28722
11	*Conyza sumatrensis*	-15.56778, -54.44194
12	*Conyza sumatrensis*	-13.07611, -56.10000
13	*Conyza sumatrensis*	-12.09945, -45.79835
14	*Conyza sumatrensis*	-23.39333, -51.47028
15	*Conyza sumatrensis*	-24.33694, -54.87639
16	*Conyza sumatrensis*	-24.29306, -52.58167
17	*Conyza bonariensis* and *Conyza sumatrensis*	-24.46111, -53.31306
18	*Conyza sumatrensis*	-22.84556, -50.00750
19	*Conyza sumatrensis*	-21.47611, -55.39139
20	*Conyza sumatrensis*	-28.13139, -53.03167

List of supplementary figures

Supplementary Figure 1 (Figure S1) Plant growth conditions and plant material to address the objectives of the study.

Supplementary Figure 2 (Figure S2) Genotyping by sequencing protocol adopted in the study.

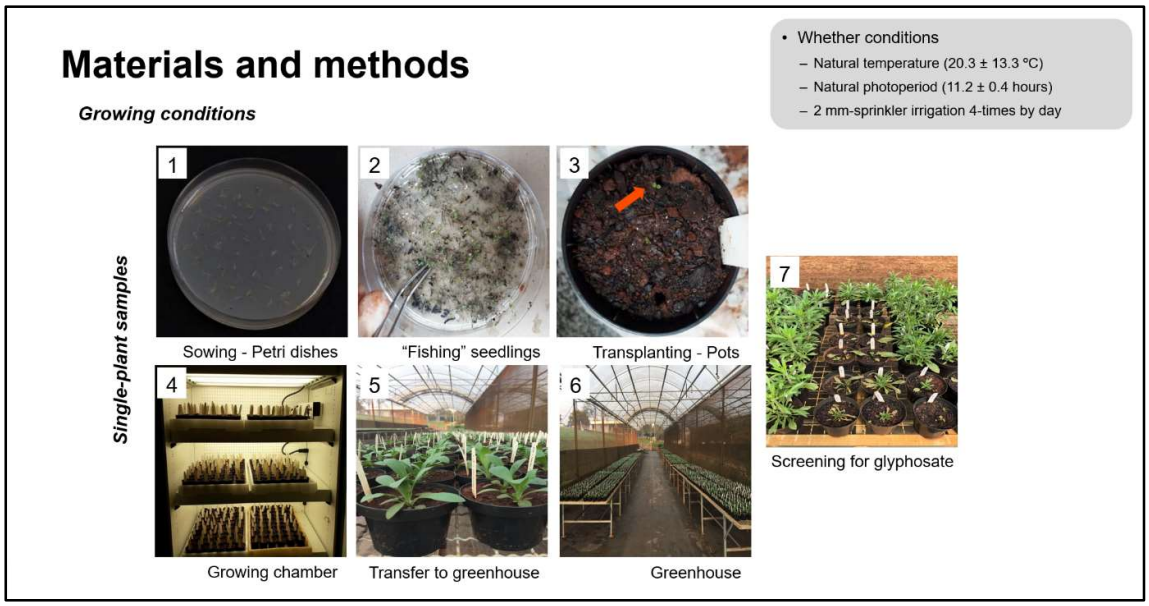
Materials and methods
Growing conditions
• Whether conditions
– Natural temperature (20.3 ± 13.3 °C)
– Natural photoperiod (11.2 ± 0.4 hours)
– 2 mm-sprinkler irrigation 4-times by day
Single-plant samples
1
Sowing - Petri dishes
2
"Fishing" seedlings
3
Transplanting - Pots
4
Growing chamber
5
Transfer to greenhouse
6
Greenhouse
7
Screening for glyphosate

Figure S1.

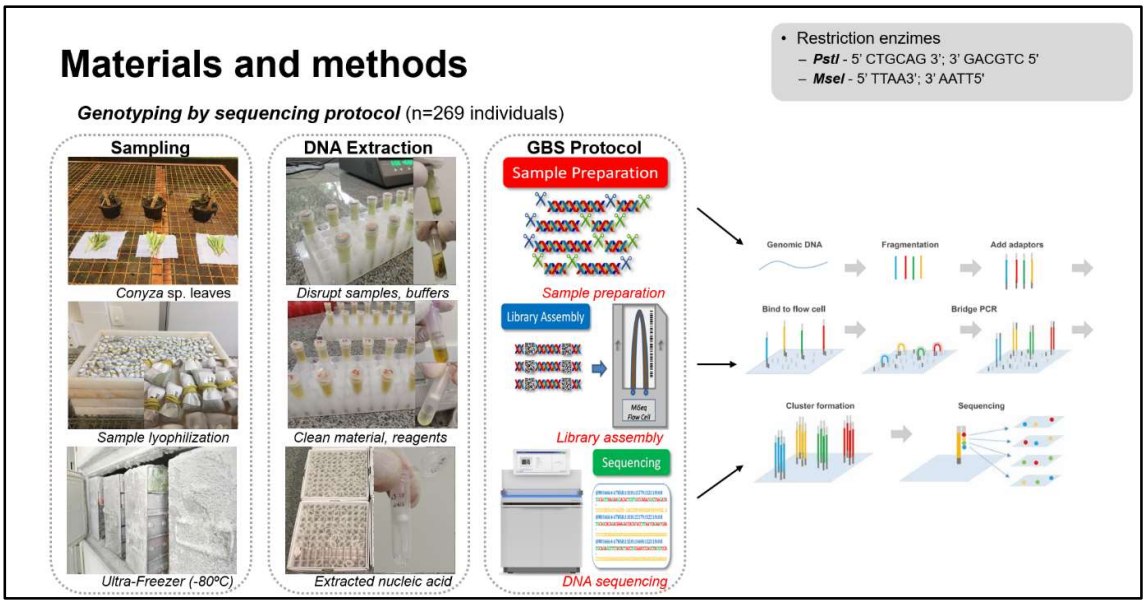
Materials and methods
• Restriction enzimes
– PstI - 5' CTGCAG 3'; 3' GACGTC 5'
– MseI - 5' TTAA3'; 3' AATT5'
Genotyping by sequencing protocol (n=269 individuals)
Sampling
Conyza sp. leaves
Sample lyophilization
Ultra-Freezer (-80ºC)
DNA Extraction
Disrupt samples, buffers
Clean material, reagents
Extracted nucleic acid
GBS Protocol
Sample Preparation
Sample preparation
Library Assembly
Library assembly
Sequencing
DNA sequencing
Genomic DNA
Fragmentation
Add adaptors
Bind to flow cell
Bridge PCR
Cluster formation
Sequencing

Figure S2.

CHAPTER 3

FREQUENCY AND DISPERSION OF AUXIN SYNTHETIC RESISTANCE IN *Conyza* spp. ACROSS SOYBEAN CROPPING REGIONS AND SEASONS IN BRAZIL AND PARAGUAY [1]

ABSTRACT

2,4-D resistant *Conyza sumatrensis* due to rapid necrosis is a recent phenomenon in Brazil. The extension of these 2,4-D-resistant cases and existence of cross-resistance are unknown. Thus, we surveyed 382 soybean fields across regions (MRS1-5) and seasons (2019-2021) to assess the frequency and dispersion of *Conyza* spp. resistant to five synthetic auxins in Brazil and Paraguay. Whole plant assays were carried out with plants at the 4- to 6-leaf stage (7-17 cm) including accessions of *C. sumatrensis* (356 fields) and the closely related *C. bonariensis* (26 fields). The herbicides consisted of 2,4-D, dicamba, halauxifen-methyl, florpyrauxifen-benzyl, and triclopyr. The dataset was clustered by *k*-means to proper discriminate herbicide-resistant individuals. Resistant individuals were grouped into Cluster 2 for rapid necrosis and Cluster 1 for plant mortality. In the dose-response assay, the double of the label dose was chosen as the discriminant dose once it was the lowest rate that resulted in 80% mortality of susceptible reference accessions. In the single dose assays, 302 out of 382 accessions were susceptible to the five herbicides. Single resistant to 2,4-D (63), dicamba (4), and triclopyr (1) totalled 68 of 80 accessions. Three cross-resistance patterns to 2,4-D, dicamba, and/or triclopyr were detected in 12 cases, including an accession with triple-resistance. Further studies are needed to confirm these data. Halauxifen and florpyrauxifen not caused rapid necrosis and resulted at least 72% mortality. Resistance was more frequent in *C. sumatrensis* (77 of 356) than in *C. bonariensis* (3 of 26). High frequency and dispersion of resistance occurred in MRS 1 and 2 (southern Brazil), compared to the other soybean macroregions. No resistance cases were found in Paraguay. Resistance to auxin herbicides in *Conyza* spp. is a growing problem in Brazil and must be managed based on strict herbicide rotation and integrated weed management.

Keywords: 2,4-D, *Conyza sumatrensis*, dicamba, rapid necrosis, triclopyr.

[1] Chapter written according to the *Weed Technology* Journal (1550-2740).

3.1 INTRODUCTION

Synthetic auxins (HRAC Group 4) are a class of herbicides that mimic the plant hormone auxin indole-3-acetic acid and comprise key tools for weed control in grain cereals, fallow, and pastures (Todd et al., 2020). These herbicides have been utilized for more than 75 years since the introduction of 2,4-D in 1945 to the present, with the commercial release of florpyrauxifen-benzyl in 2018. Globally, synthetic auxin (366×10^6 ha) use ranks third only behind acetolactate synthase inhibitors (508×10^6 ha) and glyphosate (477×10^6 ha) in the crop area treated (Busi et al., 2018). Among them, 2,4-D has been the most utilized in soybeans [*Glycine max* (L.) Merr] in South America to control glyphosate-resistant and -tolerant weeds early prior to crop planting. A survey with 3,723 Brazilian farmers in 2021 indicated that 47% of the burndown herbicide programs for soybeans comprised single or mixed sprays containing 2,4-D (Spark, 2022).

Conyza bonariensis (L.) Cronquist and *C. sumatrensis* (Retzius) E. H. Walker are two of the most frequent and problematic weedy species in soybeans in South America (Bajwa et al., 2016; Santos et al., 2014). In fact, *Conyza* species were the most abundant weeds in a survey of 2,481 soybean fields in Brazil and glyphosate-resistant cases were found in almost half of the area (Lucio et al., 2019). These weeds are also known to exhibit resistance up to six site-of-action herbicides across the region, including cases of *C. sumatrensis* resistant to 2,4-D (Heap, 2022; Queiroz et al., 2020). 2,4-D-resistant *Conyza* was recently reported and is characterized by a rapid necrosis phenotype approximately 2-h after exposure to this synthetic auxin (Leal et al., 2022; Queiroz et al., 2020). In addition, high level of resistance to 2,4-D (resistance factor of 19-fold) and the absence of cross-resistance patterns to six auxin herbicides were documented (Queiroz et al., 2020).

The resistance to 2,4-D in *Conyza* weeds has prompted farmers to adopt alternative synthetic auxins prior to soybean planting, mainly dicamba and triclopyr, in early burndown applications (Cantu et al., 2021). However, reports of *Conyza* accessions showing the same symptoms of 2,4-D resistance after sprays of those auxin herbicides have occurred in Paraná and Mato Grosso do Sul states, Brazil. In addition, new technologies based on synthetic auxins, such as halauxifen-methyl compound and 2,4-D- and dicamba-resistant soybeans, were recently released in South America. Because auxinic compounds are key tools with which efficiently

manage *Conyza* weeds in soybeans, there is a need to further confirm the cross-resistance to other synthetic auxins. In addition, surveying resistance to herbicides is critical for detecting weed resistance early as well as understanding the severity and scale of the resistance problem (Beckie et al., 2020; Squires et al., 2021).

In recent years, soybeans have been cultivated in approximately 40 million hectares in Brazil (USDA, 2022) across five macroregions (MRSs) that differ by climate and latitude (Kaster and Farias, 2012). In addition to their climatic characteristics, these regions differ by the technological level of production, which influences the weed flora and strategies for its management (Lucio et al., 2019). Soybean is also cultivated in Paraguay on approximately 3.5 million hectares, where climatic variables and cropping practices differ among cropping regions (USDA, 2022). To date, no research has assessed the severity and scale of 2,4-D-resistant *Conyza* spp. across these countries, as well as the occurrence of cross-resistance to other synthetic auxins. Thus, this study surveyed the frequency and dispersion of accessions of *Conyza* spp. resistant to auxin herbicides across soybean cropping regions and seasons in Brazil and Paraguay.

3.2 MATERIAL AND METHODS

3.2.1. Plant Material

A total of 374 soybean fields were surveyed throughout the five MRSs in Brazil in 2019, 2020 and 2021, as follows: 123 in MRS 1, 104 in MRS 2, 95 in MRS 3, 51 in MRS 4, and 1 in MRS 5 (Figure 1; Supplementary Figure S1). In addition, eight fields were surveyed across five provinces over the same seasons in eastern Paraguay, where climate-soil conditions and cropping practices resemble Brazilian's MRS 2. The fields were selected based on the representativeness of cropping area and system as well as by the occurrence of mature plants that have escaped the control by synthetic auxins. Each field was farmed by a different grower and was treated as an accession of *Conyza* spp. and sampling was not repeated over seasons to explore the variety of scenarios within each region. Seeds from 10 mature plants were sampled and then combined into a single composite sample, placed in individual paper bag, and identified with their geographic origins (Beckie et al., 2000).

3.2.2. Whole-Plant Assay and Species Identification

Whole-plant assays were carried out from May to August each year (late fall to winter in the Southern Hemisphere), when *Conyza* weeds mainly germinate and are stablished under field conditions (Vidal et al., 2007). The plants were grown in open greenhouses with natural humidity (65.1 ± 13.2%), photoperiod (11.2 ± 0.4 h), and air temperature (20.3 ± 3.3 °C), with 2 mm irrigation four times a day. Seeds were germinated in 1 x 1-cm cell trays, and two seedlings were then transplanted into pots of 1-dm^{-3} capacity filled by commercial potting mix (50% soil, 25% rice bark, and 25% peat) (Supplementary Figure S2). The species were taxonomically classified by the dichotomous key of Pruski and Sancho (2006), in which the main morphological traits were involucre, capitulum and capitulescence types. In all cases, twenty individuals per accession were grown until full flowering and their reproductive structures were studied using a stereomicroscope (Olympus SZ61, Olympus, Tokyo, JP).

3.2.3. Dose-Response Assay for Discriminating Doses

Six accessions of *C. sumatrensis* whose herbicide-resistance patterns had been characterized in previous studies were chosen to define the discriminating doses of the auxin herbicides in 2019 (Supplementary Table S1). The assay was designed as a two-factor factorial completely randomized with six replications, in which the Factor A was the accessions at five levels and Factor B was the doses at 10 levels (Table 1). The herbicides were applied to plants at the 4- to 6-leaf stage and 7 to 17 cm tall under indoor conditions at a 25°C temperature, 80% air relative humidity and 665 lux light intensity. The applications were made in a chamber sprayer (SBS-060 De Vries Mfr., Hollandale, USA) equipped with 100.015E nozzles delivering 100 L ha^{-1} at 225 kPa and at a height of 50 cm. The level of mortality (%) and plant dry weight were evaluated 56 days after treatment (DAT) (Supplementary Figure S2). The plant dry weight was measured for the aboveground plant tissue after drying at 60 °C for 7-d.

3.2.4. Single-Dose Assays for Screening Herbicide Resistance

Accessions of *Conyza* spp. (n=382) were screened for 2,4-D, dicamba, florpyrauxifen-benzyl, halauxifen-methyl and triclopyr from 2019 to 2021 (an assay by

season), except for triclopyr in the first season (2019). These assays were carried out in a randomized complete block design with four replications, in which each accession was treated with the discriminant dose of the different auxin herbicides. An optimized number of replications was used to enable the workflow with thousands of potted plants, and replications were blocked since assays required more than one greenhouse. The application procedures were the same as those previously described, except for the use of backpack sprayer (Nevoa Comercial, Campinas, BR), and the applications always occurred at a temperature ≤ 25 °C and humidity ≥ 65%. The symptom of rapid necrosis (%) was assessed visually at 8 and 24 hours after treatment (HAT), and the level of mortality (%) was then evaluated at 56 DAT in the final of the study (Supplementary Figure S2). In both cases, the six reference accessions from the dose-response assay and untreated checks by each accession were included as standards to compare the effect of the chemistries.

3.2.5. Data analysis

Data from the dose-response assay were first subjected to analysis of variance, and when the effect was significant ($P \leq 0.05$), the four-parameter log-logistic model was adjusted according to Equation 1:

$$fx = c + (d - c / 1 + \exp(b(\log x - \log e))) \quad (1)$$

where fx = treatment output, x = herbicide dosage, c = lower asymptote, d = upper asymptote, b = relative slope around e, and e = relative dose to reach 50% of weed mortality (LD_{50}) or plant dry weight reduction (GR_{50}). The resistance factors (resistant/susceptible) were computed as resistant-to-susceptible LD_{50} and GR_{50} ratios using the *drc* library in the R environment (Anunciato et al., 2022). The discriminant herbicide dose was chosen as the minimum dose that provides the largest vertical difference between dose-response curves of resistant and susceptible accessions and that results in at least 80% mortality of the susceptible references (Beckie et al., 2000). In cases in which resistance accessions were not detected in the dose-response assays, it was chosen as the minimum dose that provided at least 80% mortality of all susceptible references.

Data from single-dose assays were first grouped by species, herbicide, and season, and the relative frequencies were then calculated (number of individuals in each group/total number of individuals). The datasets were subjected to hierarchical cluster analysis as a tool with which detect herbicide-resistant weeds, and the number of clusters was estimated by the elbow criterion (Madhulatha, 2012). This resulted in two (k = 2) or three (k = 3) clusters according to the interaction among the classes, and then k-means clustering was performed using the R library *Nbclust* (Charrad et al., 2014). An individual was considered resistant when it was in Cluster 2 for injury at 24 HAT, and in Cluster 1 for mortality at 56 DAT, and its survival was meaningly higher than that of the susceptible references. Accessions were classified as resistant when at least one individual was screened as resistant. Finally, spatial maps were plotted to show the dispersion of accessions and their mortality level on the respective geographical origins using the *ggplot2* package of R (Ginestet, 2011).

3.3 RESULTS AND DISCUSSION

3.3.1. Discriminant Herbicide Dose

Accessions did not significantly differ in response to the doses of dicamba, florpyrauxifen-benzyl, halauxifen-methyl and triclopyr; however, for 2,4-D, two cases of resistance were observed (Supplementary Figures S3 and S4). In fact, accessions Ref4 and Ref5 showed resistance factors up to 3.2-fold and 4.5-fold based on LD_{50} and GR_{50}, respectively, compared with the other reference accessions (Figure 2 and 3). Only doubling the dose on the label resulted in 80% mortality of the susceptible cases by 2,4-D; thus, this dose was used as the discriminant dose in the single-dose assays (Table 2). By replacing the independent variable by the label dose of the other synthetic auxins assessed, we found cases with mortality levels lower than the criterion of 80% (Supplementary Figures S3 and S4). Thus, double the label dose was also chosen for dicamba, florpyrauxifen-benzyl, halauxifen-methyl and triclopyr due to the higher consistency of mortality (>80%) among accessions. The discriminant doses were 2,010, 960, 10, 10 and 1,440 g ha^{-1} for 2,4-D, dicamba, florpyrauxifen-benzyl, halauxifen-methyl and triclopyr, respectively.

Overall, single-dose assays used to screen for weed resistance have usually used label doses as discriminant doses since they equal each other in many cases (Beckie

et al., 2020; Geddes et al., 2022; Mendes et al., 2021). In fact, herbicides that inhibit amino acid synthesis exhibit a strong effect on susceptible weeds while being frequently ineffective on resistant accessions when applied at the label doses. However, stand-alone auxin herbicides do not always provide high levels of mortality in *Conyza* weeds, mainly in situations in which weeds exceed the rosette growth stage (Osipe et al., 2017). For example, the efficacy of stand-alone 2,4-D at 670 g ae ha^{-1} (label dose) was 99, 85 and 30% for *Conyza* spp. at < 6 cm, 6-15 cm, and > 15 cm tall, respectively (Takano et al., 2013). Thus, our results from the dose-response assay are consistent with those from previous studies and demonstrated that discriminant doses of auxin herbicides need to be higher than the label doses for *Conyza* spp.

3.3.2. Screening for Auxin Herbicide Resistance

Single-dose assays associated with cluster analysis were able to reliably screen almost 400 *Conyza* accessions and revealed different patterns of resistance to 2,4-D, dicamba and triclopyr (Figure 4; Table 3). Among the 382 accessions, 302 were found to be susceptible to the five synthetic auxin herbicides studied, which represents 79% of the cases screened throughout various regions and seasons. Of the 80 resistant accessions, 68 contained individuals resistant only to 2,4-D, dicamba or triclopyr, and 12 included individuals resistant to at least two of these herbicides (Figure 4; Table 3). Overall, higher resistance frequencies were detected in Brazilian's MRS 2 (49 of 104 accessions) and MRS 1 (19 of 123 accessions) compared with the other four soybean cropping regions. Likewise, a greater proportion of resistant cases was verified in 2020 (53 of 104 accessions), than in 2019 (20 of 179 accessions) and 2021 (7 of 99 accessions) (Figure 4; Table 3).

Assuming that *Conyza* spp. resistant to 2,4-D and other synthetic are a recent phenomenon in soybean fields in Brazil, we expected greater frequency and dispersion of cases of resistance over time. Although a higher number of cases of resistance to these herbicides were noted in 2020 compared to 2019, fewer number cases were detected in 2021, in comparison to 2020. In 2021, there was different weather condition of cold and cloudy soon after the spray of the treatment, that have persisted until the fifth day after application (data not shown). We believe that these weather condition has pronounced the effect of the treatments because lower proportion of resistance cases were found compared to the previous years. It may be related to the mechanism

of resistance of *Conyza* ssp. to synthetic auxins and suggest that environmental conditions can influence the level of resistance.

Rapid necrosis at 8 and 24 HAT were variable across accessions and even individuals within accessions after 2,4-D treatment, with a greater data range in *C. sumatrensis* than in *C. bonariensis* (Figure 5a and 5b). Overall, this injury was up to 6-fold and up to10-fold higher in Cluster 2 than in Cluster 1 for *C. bonariensis* and *C. sumatrensis*, respectively, indicating the phenotype of resistance to 2,4-D. However, Cluster 2 individuals for injury were not always grouped in Cluster 1 for mortality, since some of the injured plants did not recover and then died over time (data not shown). Mortality at 56 DAT was also variable among and within accessions mainly for *C. sumatrensis*, with an average of 87% and ranging from 5 to 100%, over all species and seasons (Figure 5c). Single and cross-resistance to 2,4-D totalled 7 of 179 accessions in 2019, 51 of 104 in 2020, and 7 of 99 in 2021, mostly dispersed in MRS 1 and MRS 2 of Brazil (Figure 4, Table 3).

In our study, 2,4-D resistant *C. sumatrensis* was found in MRS 2 of Brazil since the first cropping season, and the frequency and dispersion increased in most MRSs in 2020, compared with 2019 (Figure 6, Table 3). Glyphosate-resistant *C. canadensis* found in Delaware, US, in 2001 also rapidly spread in a few years, covering an area greater than 44,000 hectares of annual crops (Shields et al., 2006). *Conyza* weeds display robust seed production with an estimated 200,000 seeds per plant, and both grain pollen and seed dispersal are wind-assisted (Huang et al., 2015; Weaver, 2001). Thus, in addition to seed dispersal from resistant individuals to surrounding fields, gene flow occurs by pollen dispersion, and *Conyza* hybrids can naturally be generated (Zelaya et al., 2007). Interspecific hybridization can explain the accessions of *C. bonariensis* resistant to 2,4-D found in our study, but the possibility of independent selection cannot be ruled out (Table 3).

Overall, rapid necrosis after exposure to dicamba did not occur or was very low (<5%) at 8 and 24 HAT, except in *C. sumatrensis* in 2019 and 2020, when Cluster 2 ranged from 11 up to 55% (Figure 5d and 5e). Similarly, *Conyza* plants mostly exhibited zero or low levels of rapid necrosis after triclopyr treatment but some accessions of *C. sumatrensis* showed up to 40% injury in 2020 (Figure 5n and 5o). Dicamba and triclopyr were generally effective against both weed species, with average mortalities of 92 and 94%, respectively, and at least 74% mortality was observed even in Cluster 1 (Figure 5f and 5p). However, accessions of *C. sumatrensis* showed atypical survival

to dicamba in 2019 and 2020 and to triclopyr in 2020, with mortality ranging from 55 to 69% and from 49 to 67%, respectively. Thus, resistance to dicamba was detected in 4 of 179 accessions in 2019 and 9 of 104 in 2020, and resistance to triclopyr was found in 5 of 104 accessions in 2020 (Figure 4, Table 3).

Herein, we reported that accessions of *C. sumatrensis* from Brazil displayed rapid necrosis (Supplementary Figures S5 and S6) and poor mortality (Figures 7 and 8) after the application of double the label dose of dicamba and triclopyr. The rapid necrosis symptoms were similar to those described by Queiroz et al. (2020) and Leal et al. (2022), but we often noted slightly levels of epinasty regardless of the herbicide (Supplementary Figure S7). Thus, the present study is the first report of resistance to both dicamba and triclopyr in *C. sumatrensis*, although additional studies are required to properly confirm these findings (Heap, 2005). Resistance to 2,4-D in *C. sumatrensis* was not related to 2,4-D detoxification but may be related to changes in auxin transporters, receptors, and stress-related proteins (Queiroz et al., 2022). The consistent patterns of resistance to 2,4-D, dicamba and triclopyr found in our study suggest that resistance to these herbicides may be conferred by a common mechanism of resistance.

No symptoms other than epinasty were observed at 8 and 24 HAT in the *Conyza* spp. after the application of florpyrauxifen-benzyl and halauxifen-methyl (Figure 5g, 5h, 5j and 5k). Overall, both auxin herbicides were effective and reached 93% mortality at 56 DAT, with at least 72% mortality in Cluster 1; thus, no resistant cases were detected (Figure 5i, 5l; Table 2). Florpyrauxifen-benzyl and halauxifen-methyl are arylpicolinates synthetic auxins and differ from 2,4-D and dicamba in cellular uptake and primary receptor (Walsh et al., 2006). This may explain the absence of single and cross-resistance to these herbicides in our study and support the hypothesis of the rapid necrosis mechanism described by Queiroz et al. (2022). Thus, arylpicolinates are options to control synthetic auxin-resistant *C. sumatrensis* because they resulted in high levels of weed mortality without generating rapid necrosis (Figures 9 and 10).

Of the 80 herbicide resistant accessions, 12 had individuals with resistance to 2,4-D and dicamba (8 accessions), 2,4-D and triclopyr (3 accessions) or 2,4-D, dicamba and triclopyr (1 accession) (Figure 4). Although cross-resistance was restricted to *C. sumatrensis* and 2,4-D was involved in all detected cases, the incidence of cross-resistance and its pattern were accession-dependent in our study. In another study, rapid necrosis symptoms did not occur for six auxin herbicides in a unique evaluated

2,4-D-resistant accession of *C. sumatrensis* collected in 2016, indicating the absence of cross-resistance (Queiroz et al., 2020). To date, cross-resistance was found in 26 of the 83 reports of resistance to synthetic auxins, including almost 20 weed species worldwide but not species of the *Conyza* genus (Heap, 2022). This study is thus the first report of cross-resistance to auxin herbicides in *Conyza* spp. and the second report of resistance to triclopyr after field burweed (*Soliva sessilis* Ruiz & Pav.).

3.3.3. Implications for Weed Management in Soybeans

Accessions of *Conyza* spp. resistant to 2,4-D, dicamba and triclopyr limit the herbicide options for preplant burndown programs, as well as for stacked trait 2,4-D or dicamba-resistant varieties in soybeans. In fact, *Conyza* weeds are becoming an increasing threat in most soybean cropping regions in Brazil, as evidenced in this study and recent surveys (Lucio et al., 2019; Mendes et al., 2021). Although we did not find accessions resistant to synthetic auxins from Paraguay in this study, other research has reported cases of multiple-resistant *C. sumatrensis* (Albrecht et al., 2020a). Farmers and their consultants must consider proactive chemical and nonchemical weed control methods to help maintain the efficacy of the remaining herbicide options for *Conyza* control. The use of alternative such as halauxifen-methyl associated nonchemical weed control methods can enable an effective and sustainable way to control resistant *Conyza* spp. Key nonchemical weed control tools include off-season cover crops, early preplant burndown, double knockdown sprays, pre-emergent herbicides, no-tillage, use of certified seeds, competitive varieties, machine cleaning, and crop rotations (Kalsing et al., 2020; Lamego et al., 2013; Marochi et al., 2018; Zobiole et al., 2018).

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3.5 APPENDICES

Table 1 Auxin herbicides evaluated.

Comon name	Trade name[1]	Concetration	Formulation	Label rate[2]
2,4-D	DMA® 806 BR	670 g ae L^{-1}	SL	1,005
Dicamba	Atectra®	480 g ai L^{-1}	SL	480
Florpyrauxifen-benzyl	Loyant®	25 g ai L^{-1}	EC	5
Halauxifen-methyl	Elevore®	68 g ai L^{-1}	SC	5
Triclopyr	Triclon®	480 g ae L^{-1}	EC	720

[1]Manufacter: DMA® 806 BR, Loyant® and Elevore® (Corteva Agriscience™, Indianapolis, IN, US), Atectra® (Basf™, Ludwigshafen, RP, GE) and Triclon® (UPL™ OpenAg (Mumbai, MA, IN).

[2]Baseline (1x) for the dose-response study with 0, 1/16, 1/8, 1/4, 1/2, 1, 2, 4, 8 and 16x (n=10). Added adjuvants according to the herbicide label or manufacturer recommendation.

Table 2 Auxin herbicides, label doses, and characterization of reference accessions.

Auxin herbicide[1]	Discriminating dose[2] g ai or ae ha^{-1}	Reference accessions					
		Ref1	Ref2	Ref3	Ref4	Ref5	Ref6
2,4-D	2,010	S	S	S	**R**	**R**	S
Dicamba	960	S	S	S	S	S	S
Florpyrauxifen-benzyl	10	S	S	S	S	S	S
Halauxifen-methyl	10	S	S	S	S	S	S
Triclopyr	1,440	S	S	S	S	S	S

Ref, reference accession; *R*, herbicide-resistant; *S*, herbicide-susceptible.

Table 3 Incidence and frequency of herbicide resistance to five synthetic auxin herbicides of 382 accessions of *Conyza bonariensis* and *C. sumatrensis* sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay (PY).

Auxin herbicides	Season	Soybean production macroregions MRS 1[1]	MRS 2	MRS 3	MRS 4	MRS	PY
Conyza		*(n=23)*	*(n=1)*	*(n=1)*	*(n=1)*	*(n=0)*	*(n=0)*
	2019	0 (0%)	0 (0%)	0 (0%)	-	-	-
2,4-D	2020	**2**	0 (0%)	0 (0%)	**1**	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	-	-	-
	2019	0 (0%)	0 (0%)	0 (0%)	-	-	-
Dicamba	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	-	-	-
	2019	0 (0%)	0 (0%)	0 (0%)	-	-	-
Florpyrauxifen-benzyl	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	-	-	-
	2019	0 (0%)	0 (0%)	0 (0%)	-	-	-
Halauxifen-methyl	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	-	-	-
	2019	-[4]	-	-	-	-	-
Triclopyr	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	-	-	-
	2019	0 (0%)	0 (0%)	0 (0%)	-	-	-
Two or more[2]	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	-	-	-
Conyza		*(n=100*	*(n=103)*	*(n=94)*	*(n=50)*	*(n=1)*	*(n=8)*
	2019	0 (0%)	**15**	0 (0%)	**1 (3%)**	-	0
2,4-D	2020	**13**	**14**	**5**	**5**	-	-
	2021	0 (0%)	**7**	0 (0%)	0 (0%)	0	0
	2019	0 (0%)	**3 (7%)**	0 (0%)	0 (0%)	-	0
Dicamba	2020	0 (0%)	**1 (3%)**	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0
	2019	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	0
Florpyrauxifen-benzyl	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0
	2019	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	0
Halauxifen-methyl	2020	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0
	2019	-	-	-	-	-	0
Triclopyr	2020	0 (0%)	**1 (3%)**	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0
	2019	0 (0%)	**1 (2%)**	0 (0%)	0 (0%)	-	0
Two or more	2020	**4**	**7**	0 (0%)	0 (0%)	-	-
	2021	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0

[1]Soybean cropping macroregions according to Kaster and Farias (2012).
[2]Cases of cross-resistance to two or more auxin herbicides assessed in the study.
[3]Frequency data calculated by each dataset of species, herbicide, season, and region.
[4]No collected samples.

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correspond to the cluster limits. $\bar{x}1$, $\bar{x}2$ and $\bar{x}3$ refer to the average values of Clusters 1, 2 and 3, respectively.

Figure 6 Plant mortality (%) due to 2,4-D on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refer to MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represent the plant mortality at 56 days after treatment.

Figure 7 Plant mortality (%) due to dicamba on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refer to MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represent the plant mortality at 56 days after treatment.

Figure 8 Plant mortality (%) due to florpyrauxifen-benzyl on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refer to MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represent the plant mortality at 56 days after treatment.

Figure 9 Plant mortality (%) due to halauxifen-methyl on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refer to MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represent the plant mortality at 56 days after treatment.

Figure 10 Plant mortality (%) due to triclopyr on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refer to MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represent the plant mortality at 56 days after treatment.

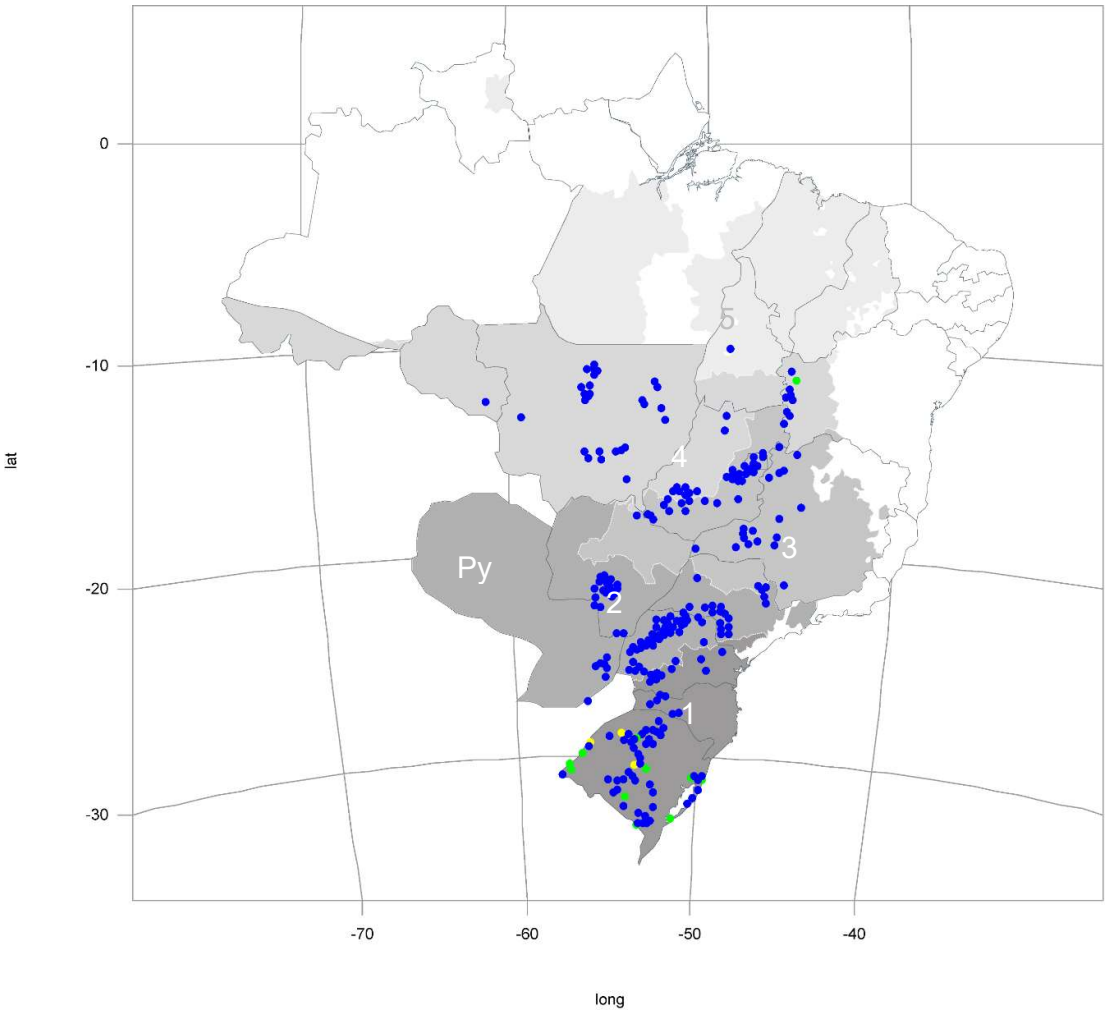


Fig. 1

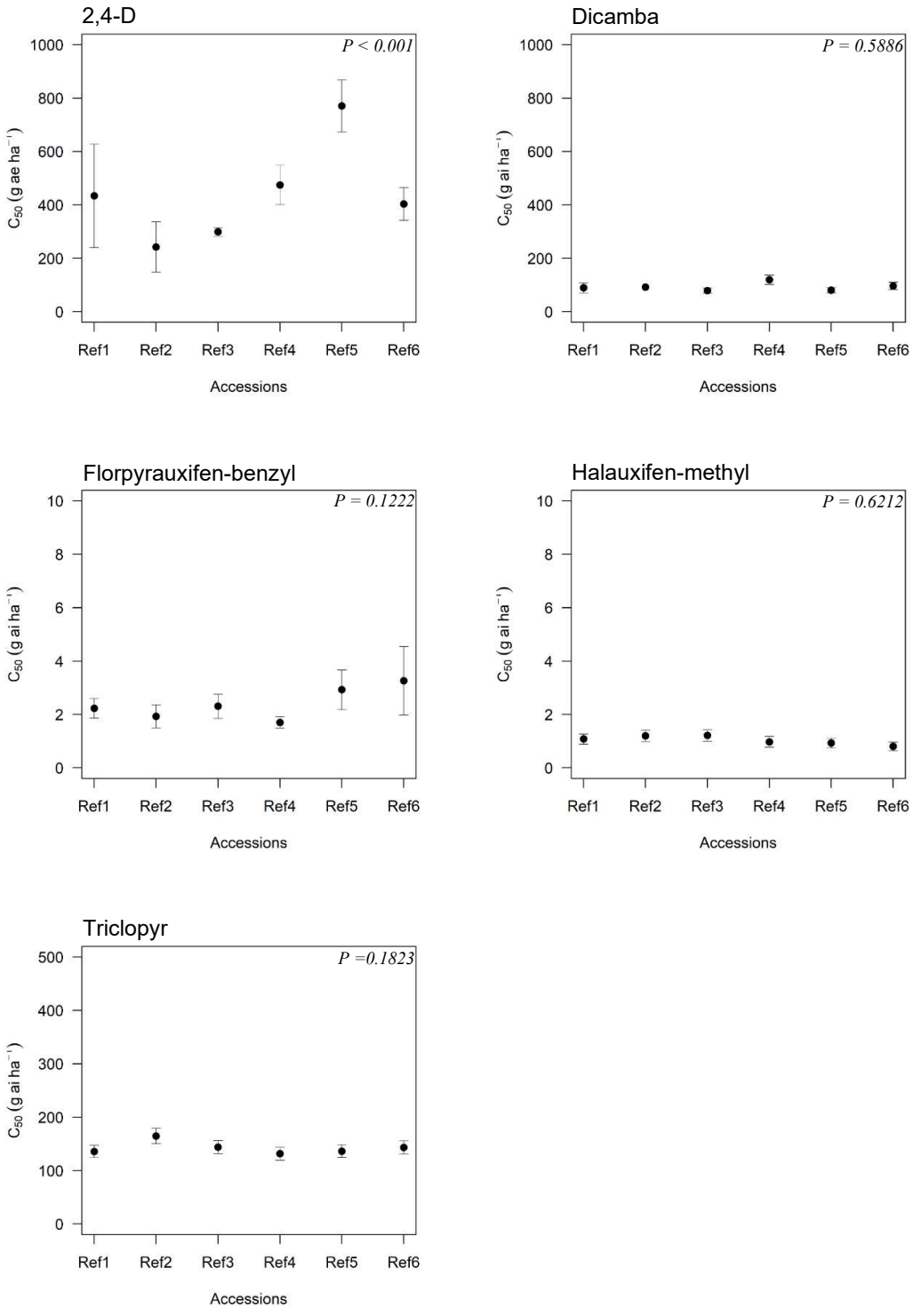

2,4-D
P < 0.001
C50 (g ae ha−1)
Dicamba
P = 0.5886
C50 (g ai ha−1)
Florpyrauxifen-benzyl
P = 0.1222
C50 (g ai ha−1)
Halauxifen-methyl
P = 0.6212
C50 (g ai ha−1)
Triclopyr
P =0.1823
C50 (g ai ha−1)
Ref1
Ref2
Ref3
Ref4
Ref5
Ref6
Accessions

Fig. 2

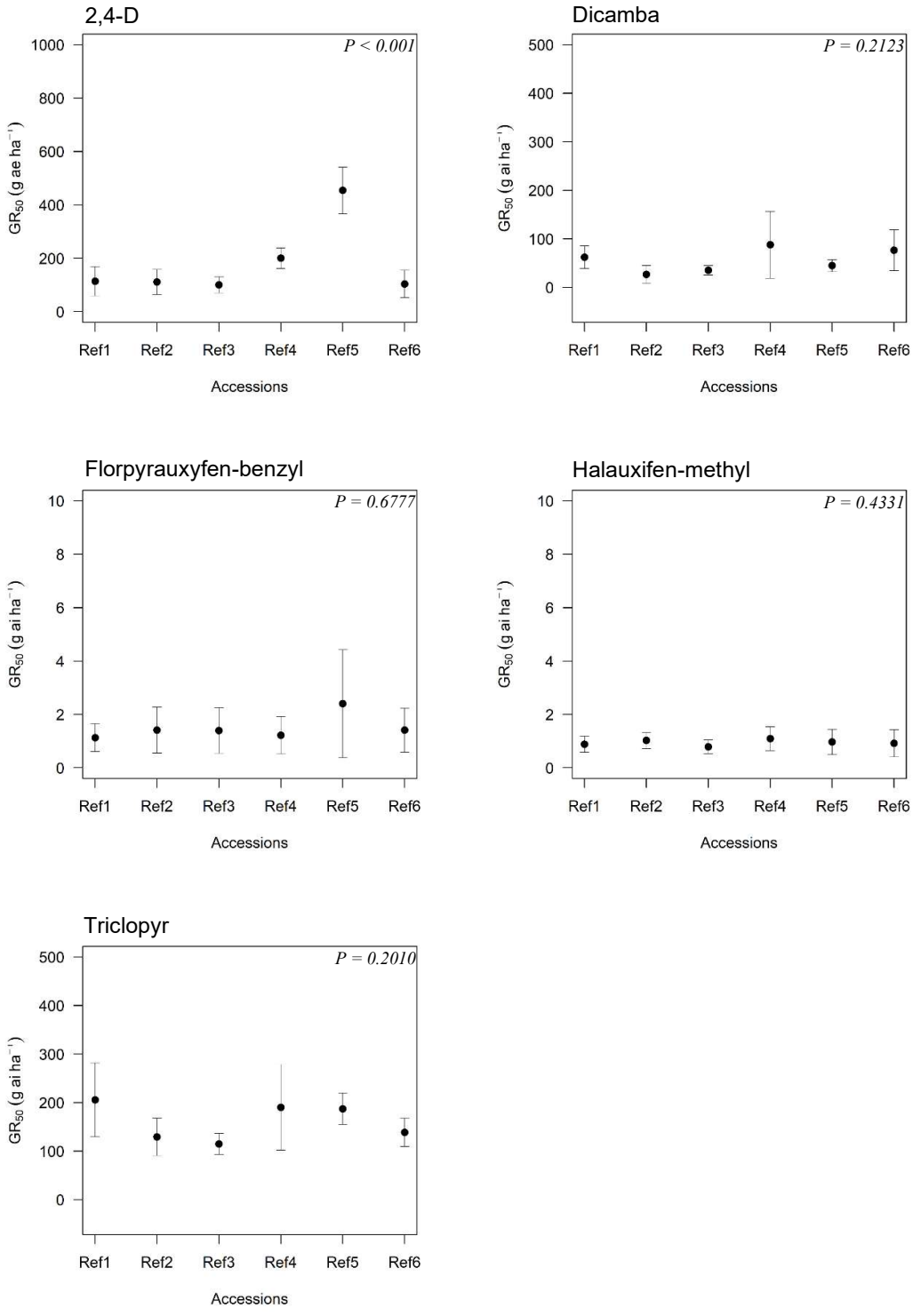

2,4-D
P < 0.001
GR50 (g ae ha−1)
Dicamba
P = 0.2123
GR50 (g ai ha−1)
Florpyrauxyfen-benzyl
P = 0.6777
GR50 (g ai ha−1)
Halauxifen-methyl
P = 0.4331
GR50 (g ai ha−1)
Triclopyr
P = 0.2010
GR50 (g ai ha−1)
Ref1
Ref2
Ref3
Ref4
Ref5
Ref6
Accessions

Fig. 3

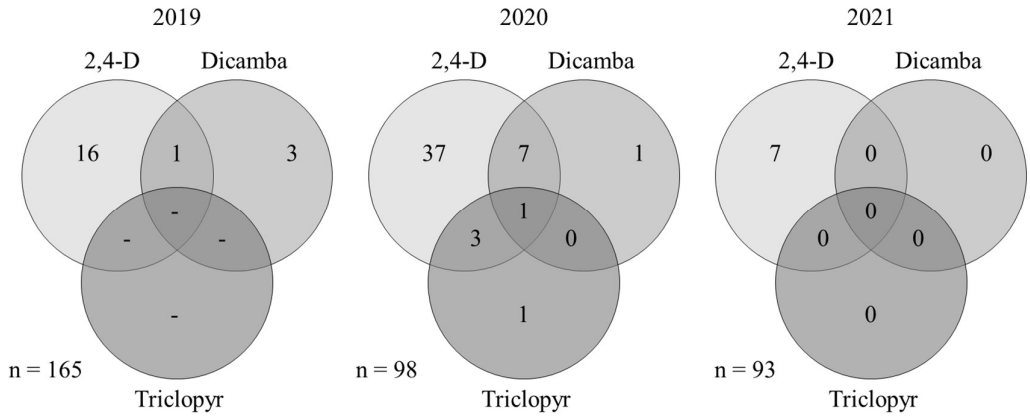
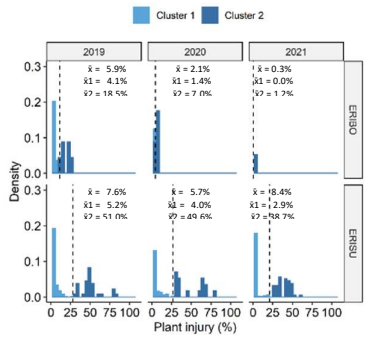
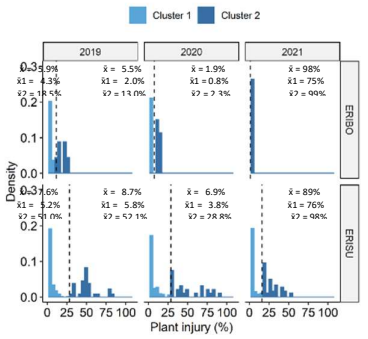

2019
2,4-D
Dicamba
16
1
3
-
-
-
-
n = 165
Triclopyr
2020
2,4-D
Dicamba
37
7
1
1
3
0
1
n = 98
Triclopyr
2021
2,4-D
Dicamba
7
0
0
0
0
0
0
n = 93
Triclopyr

Fig. 4

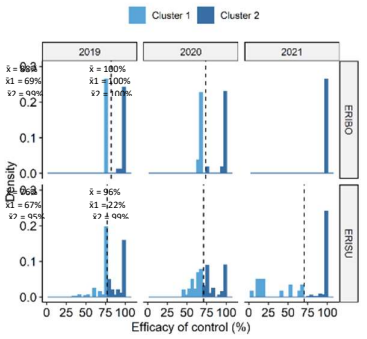
5a) 2,4-D, foliar necrosis at 8 HAT



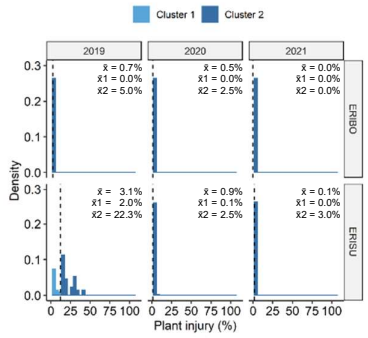
5b) 2,4-D, foliar necrosis at 24 HAT



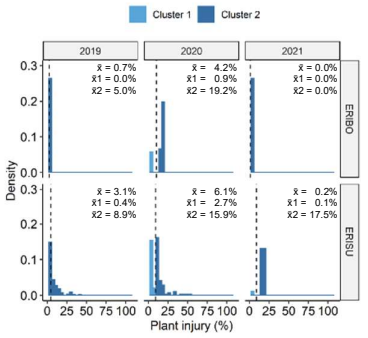
5c) 2,4-D, plant mortality at 56 DAT



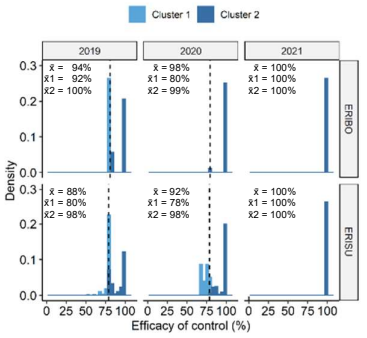
5d) Dicamba, foliar necrosis at 8 HAT



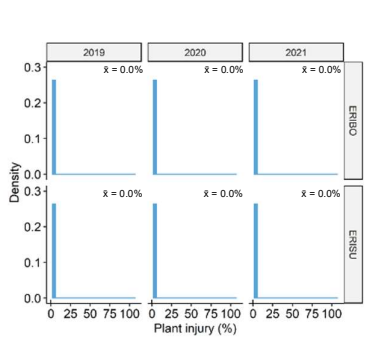
5e) Dicamba, foliar necrosis at 24 HAT



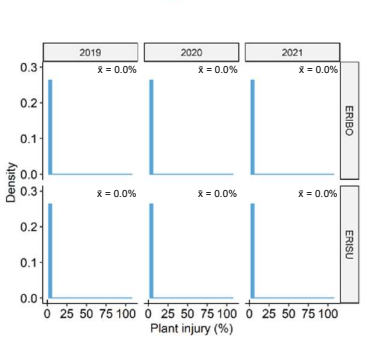
5f) Dicamba, plant mortality at 56 DAT



5g) Florpyrauxifen-benzyl, foliar necrosis at 8 HAT



5h) Florpyrauxifen-benzyl, foliar necrosis at 24 HAT



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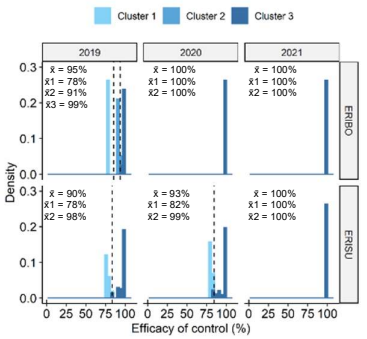
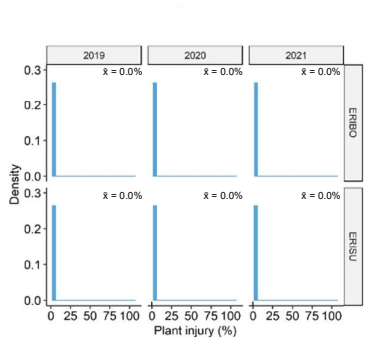


5j) Halauxifen-methyl, foliar necrosis at 8 HAT



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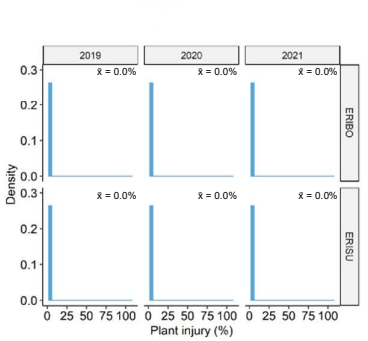
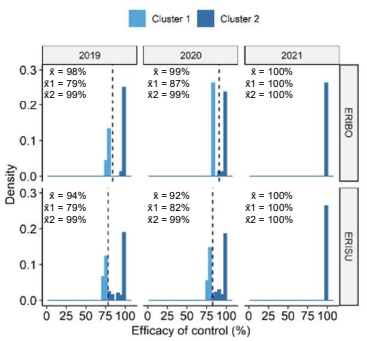
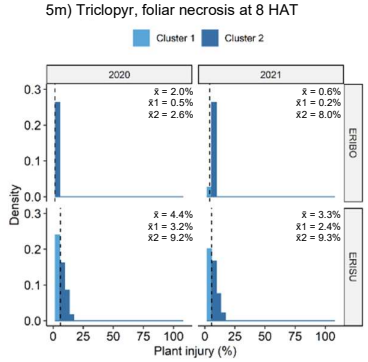


5l) Halauxifen-methyl, plant mortality at 56 DAT



5m) Triclopyr, foliar necrosis at 8 HAT



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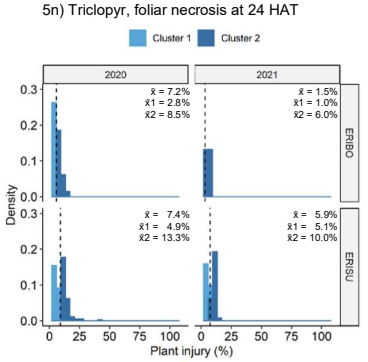
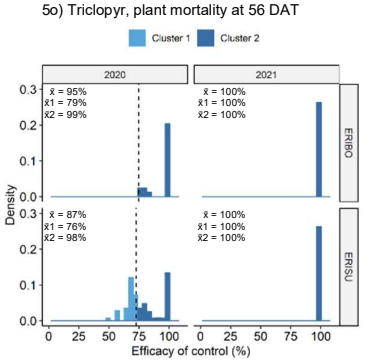


5o) Triclopyr, plant mortality at 56 DAT



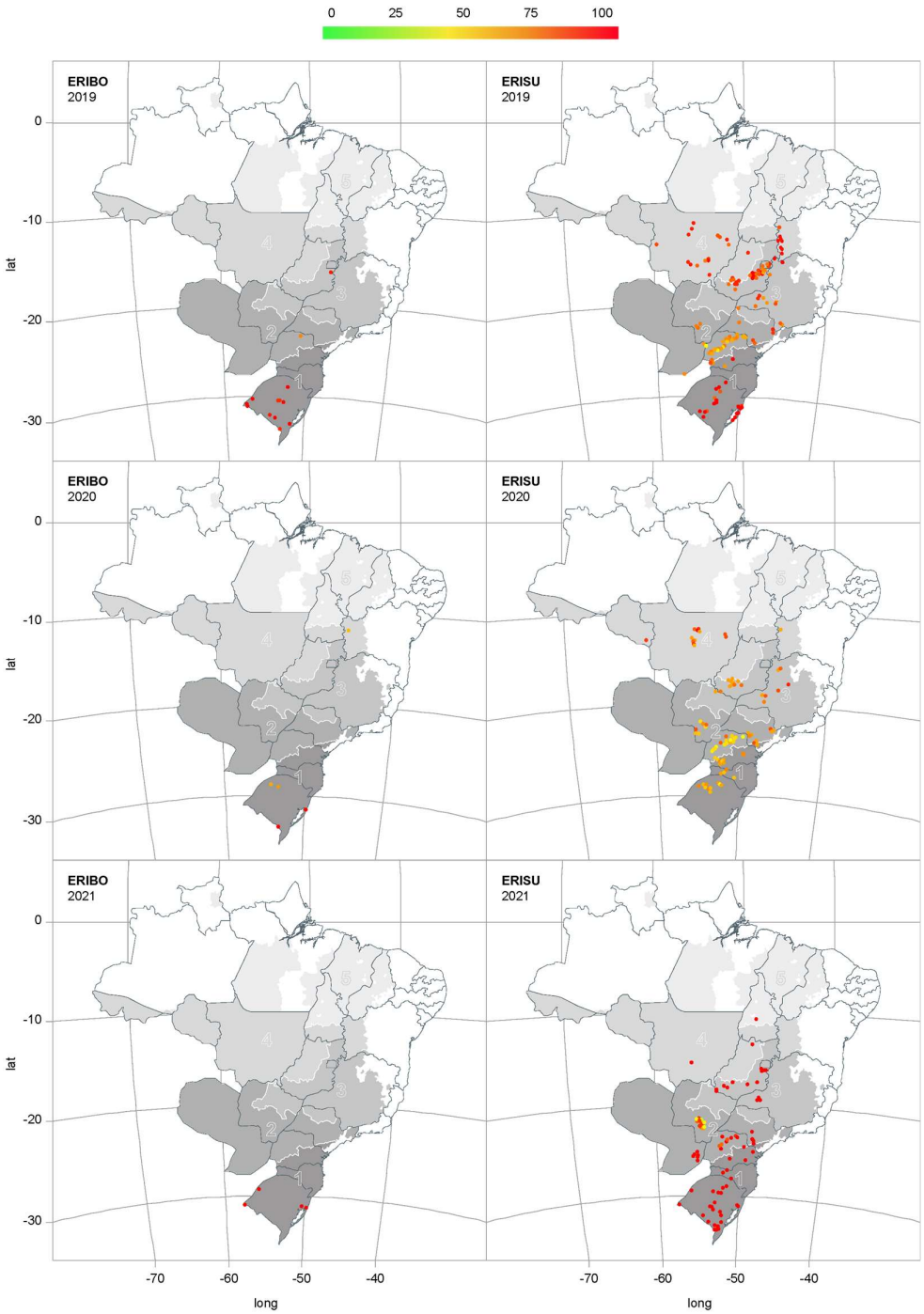

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Fig 6.

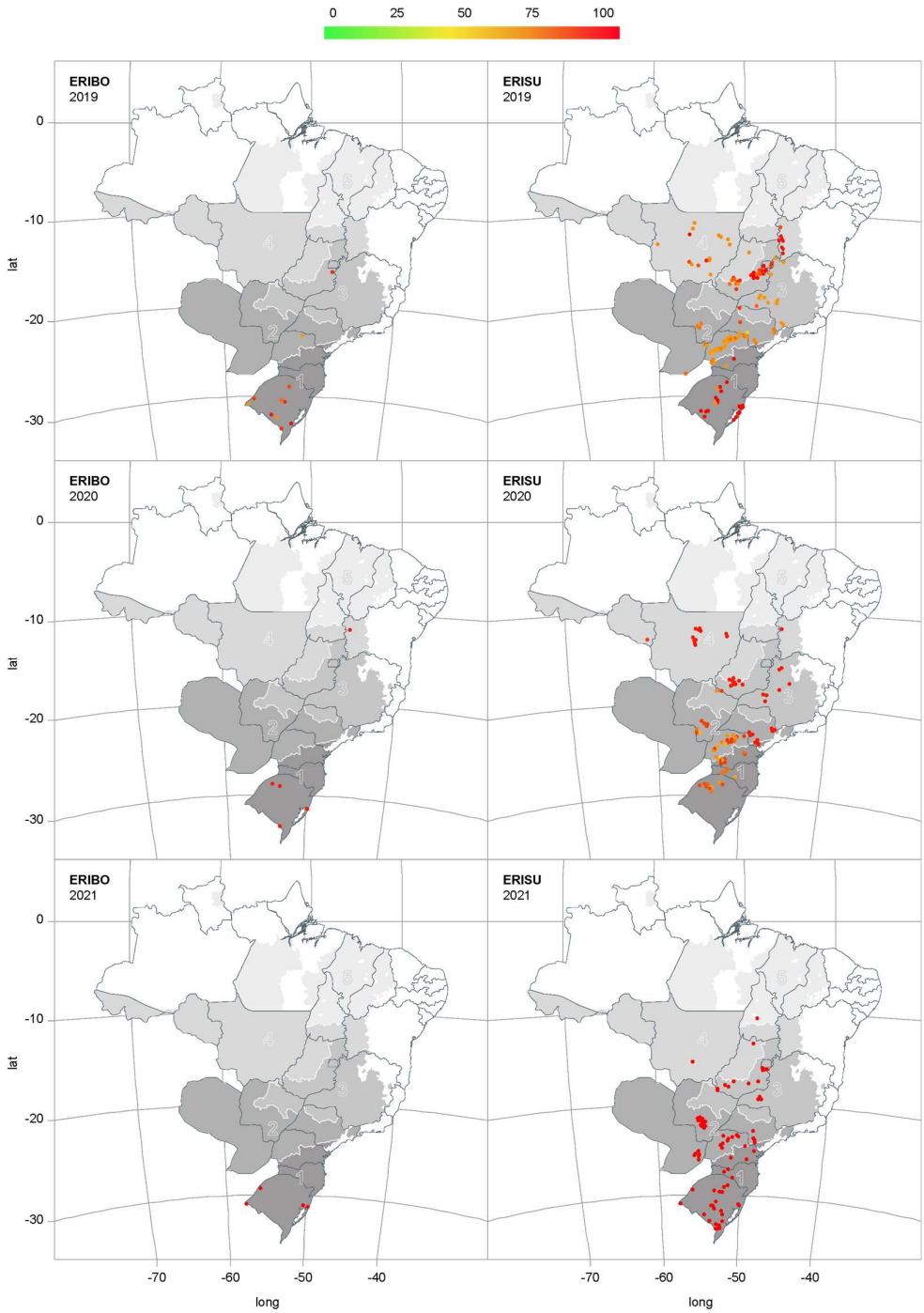
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Fig 7.

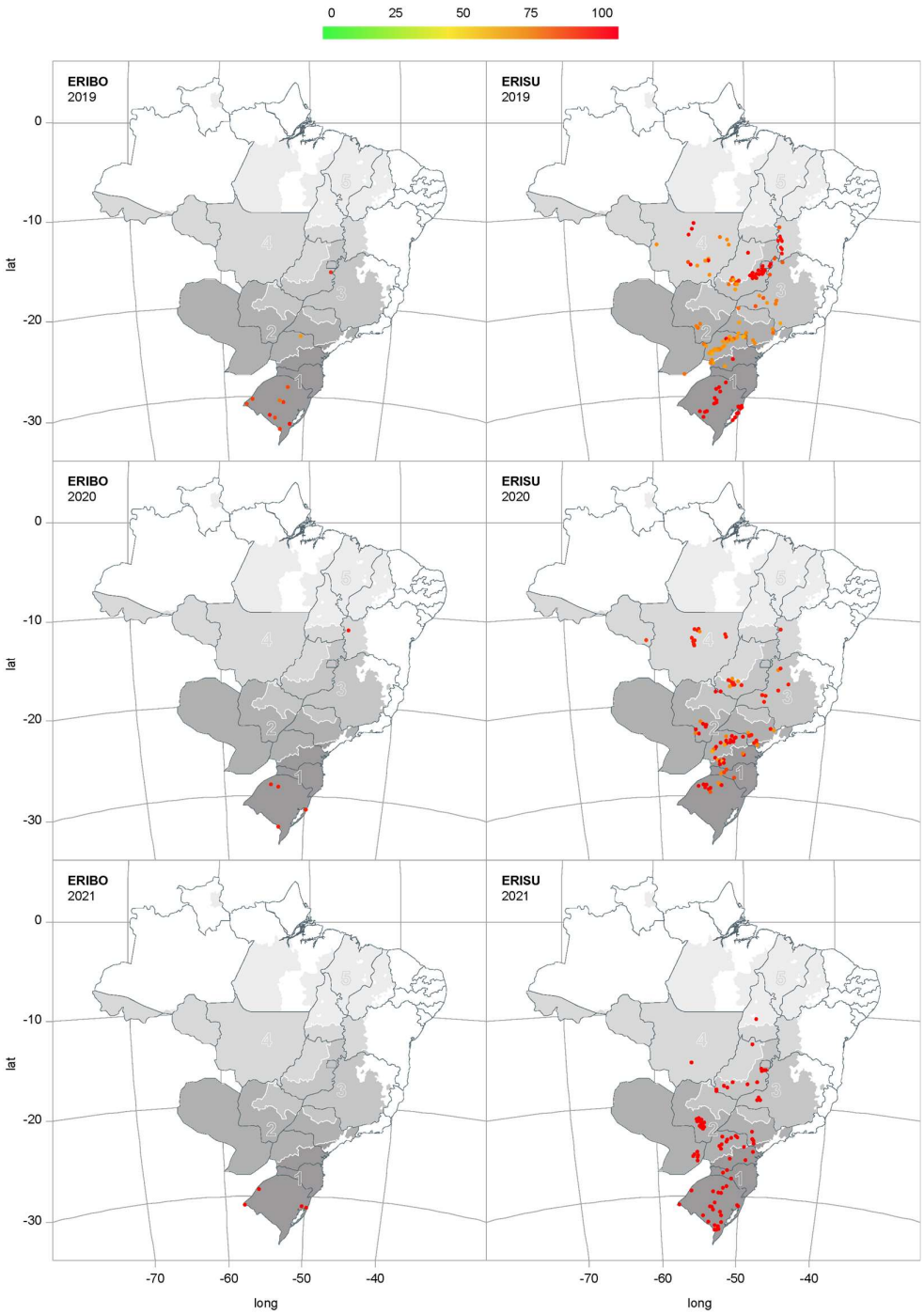
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Fig 8.

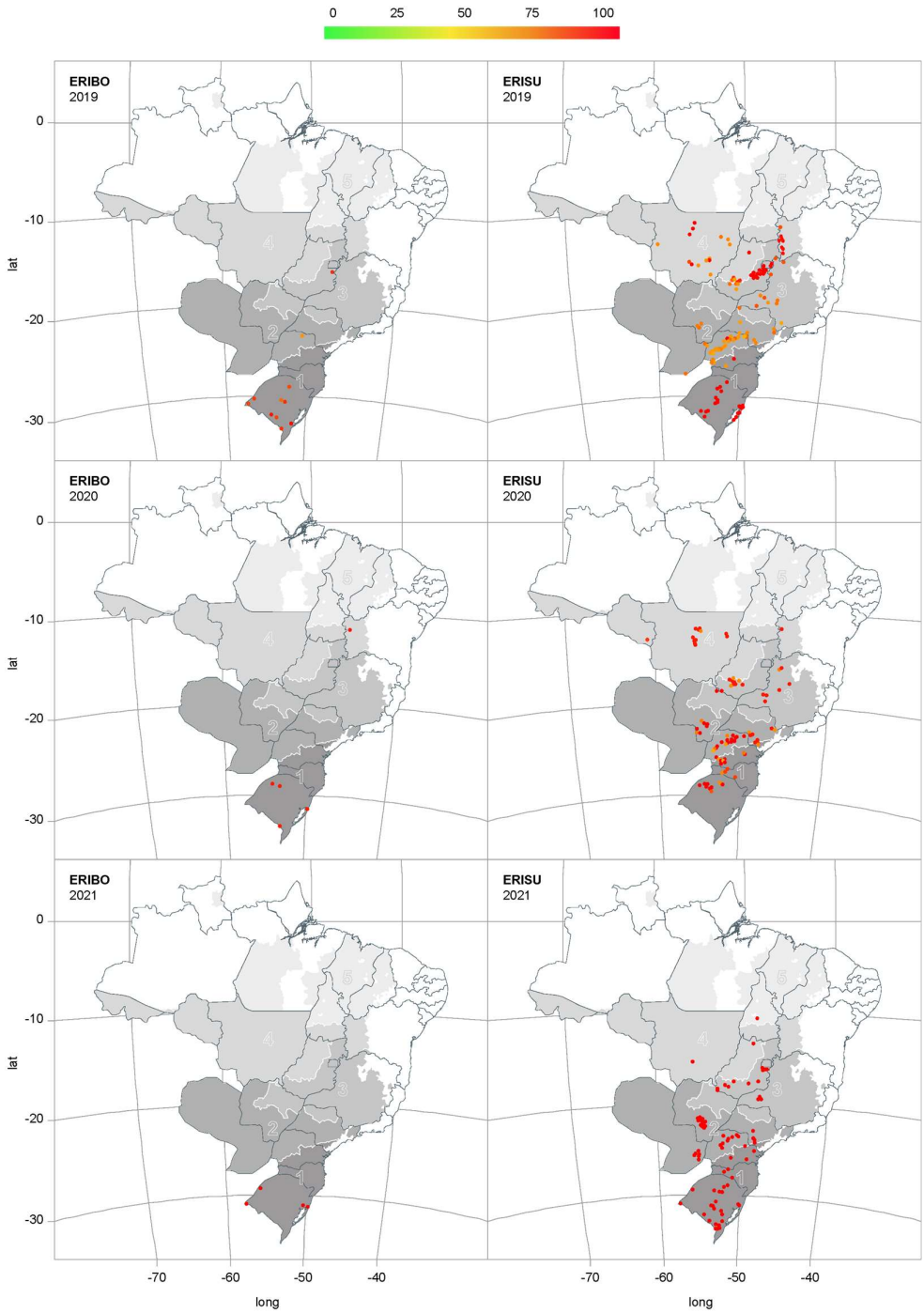
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Fig 9.

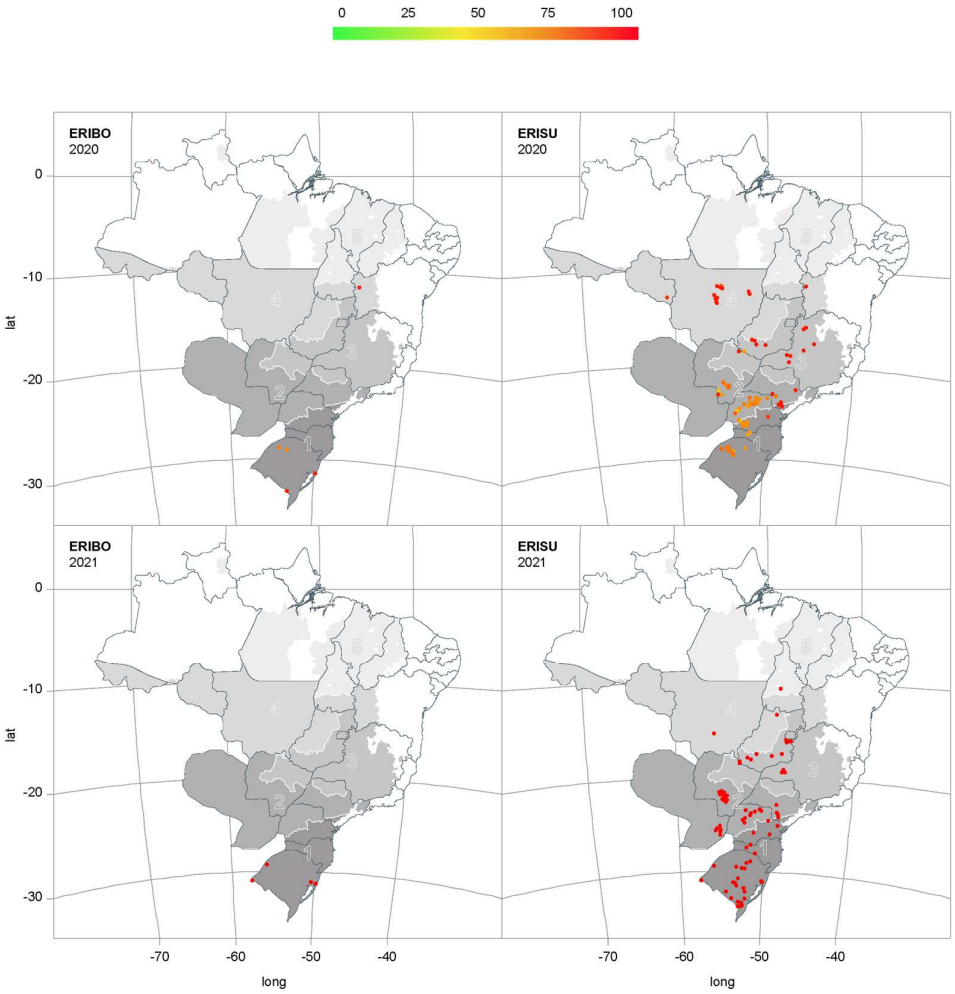
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Fig 10.

Supplementary Table 1 Sampling sites of reference accessions of *Conyza* spp. with geographic origins.

Accession	Geographic coordinates	Specie
Reference 1 (Ref1)	-25.31047, -53.49975	*Conyza sumatrensis*
Reference 2 (Ref2)	-24.12156, -52.28086	*Conyza sumatrensis*
Reference 3 (Ref3)	-28.30917, -52.31694	*Conyza sumatrensis*
Reference 4 (Ref4)	-23.10500, -50.36028	*Conyza sumatrensis*
Reference 5 (Ref5)	-24.09679, -51.54042	*Conyza sumatrensis*
Reference 6 (Ref6)	-14.53500, -49.09333	*Conyza sumatrensis*

List of supplementary figures

Supplementary Figure 1 Sampling strategy by macroregion employed to address the objectives of the study.

Supplementary Figure 2 Plant growth conditions and evaluation methods to address the objectives of the study.

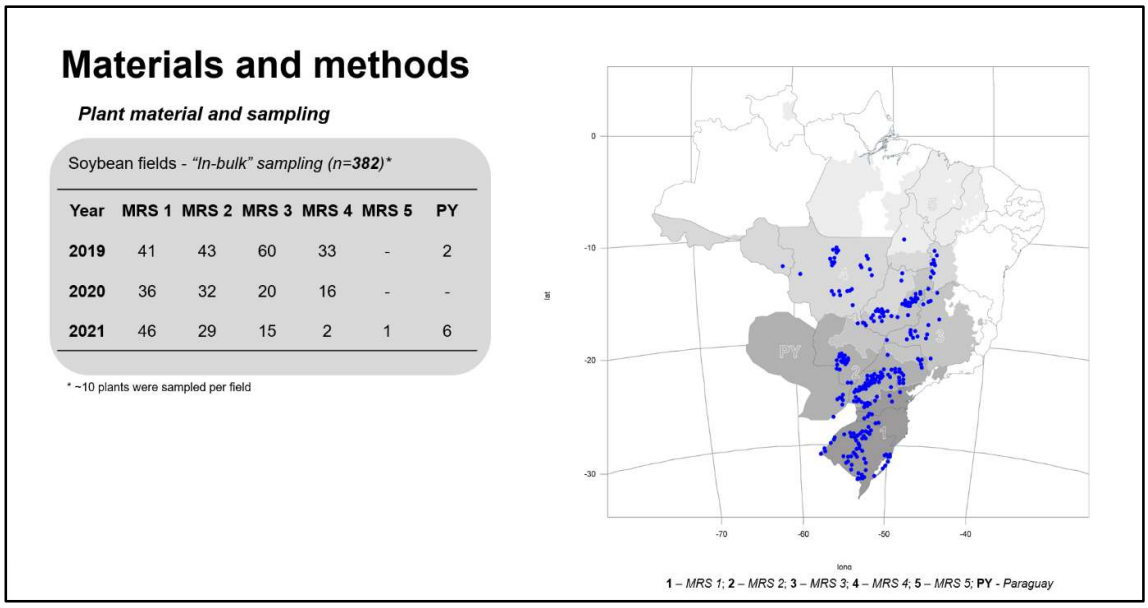
Supplementary Figure 3 Comparison of plant mortality (%) at 56 days after treatment of six reference (Ref) accessions of *Conyza sumatrensis* sampled from soybean cropping regions of Brazil in 2019 in response to ten doses of five synthetic auxins. Symbols denote mean of repetitions and vertical bars represent standard error of mean (n= 6).

Supplementary Figure 4 Comparison of plant dry weight (%) at 56 days after treatment of six reference (Ref) accessions of *Conyza sumatrensis* sampled from soybean cropping regions of Brazil in 2019 in response to ten doses of five synthetic auxins. Symbols denote mean of repetitions and vertical bars represent standard error of mean (n= 6).

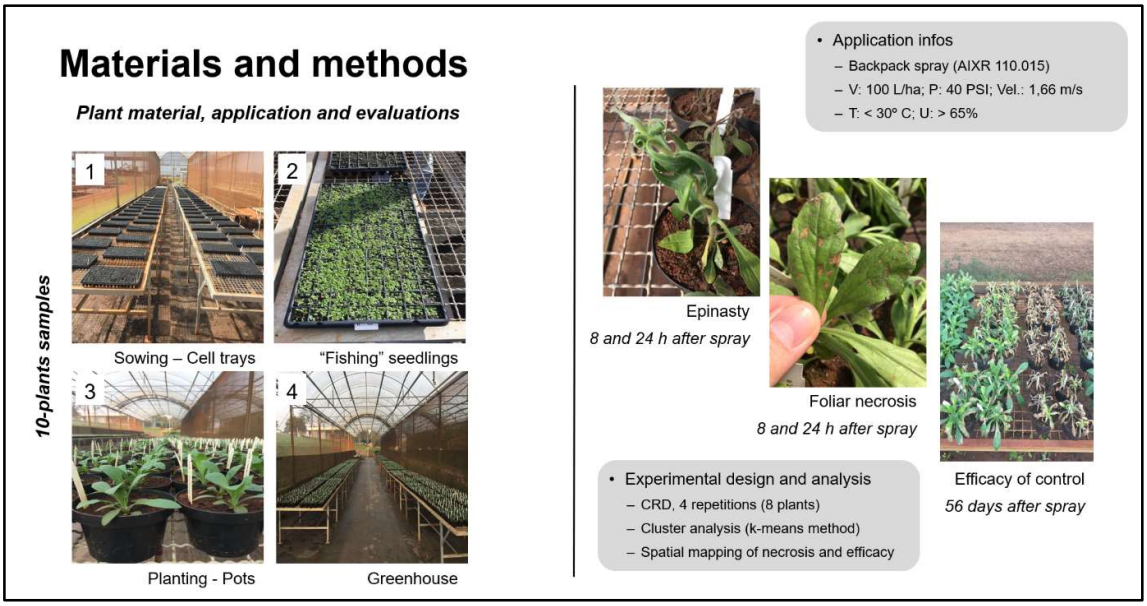
Supplementary Figure 5 Rapid necrosis (%) observed after dicamba spray on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refers to the MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represents the plant injury (foliar necrosis) at 24 hours after treatment.

Supplementary Figure 6 Rapid necrosis (%) observed after triclopyr spray on the respective geographical origins of 382 accessions of *Conyza bonariensis* (ERIBO) and *C. sumatrensis* (ERISU) sampled from five soybean cropping macroregions (MRSs) throughout three seasons in Brazil and Paraguay. 1, 2, 3, 4, and 5 refers to the MRS 1, MRS 2, MRS 3, MRS 4, and MRS 5, respectively. Legend colors represents the plant injury (foliar necrosis) at 24 hours after treatment.

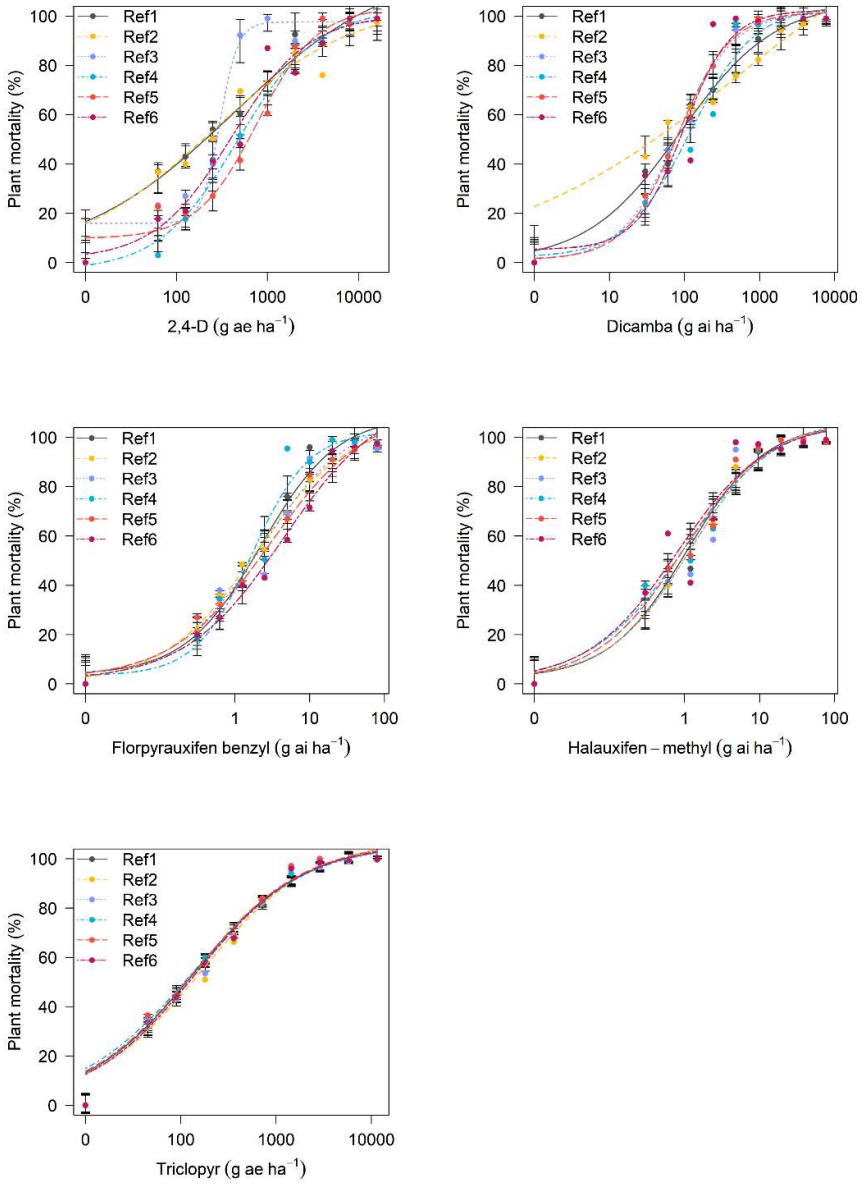
Supplementary Figure 7 Epinasty and necrosis symptoms at 8 and 24 hours after spray (A and B, respectively) and plant mortality at 56 days after spray (C) of accessions of *Conyza sumatrensis* resistant to 2,4 D, dicamba and triclopyr.



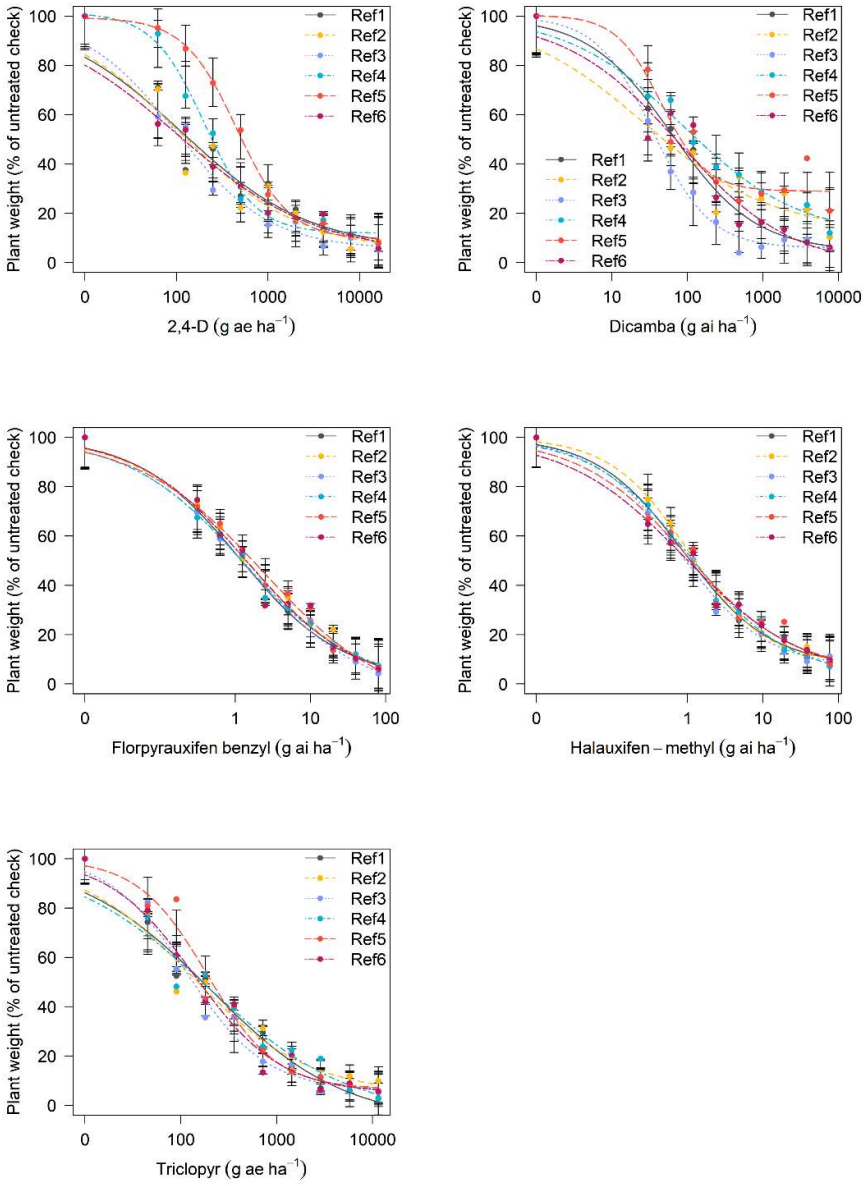
Supplementary Fig. 1



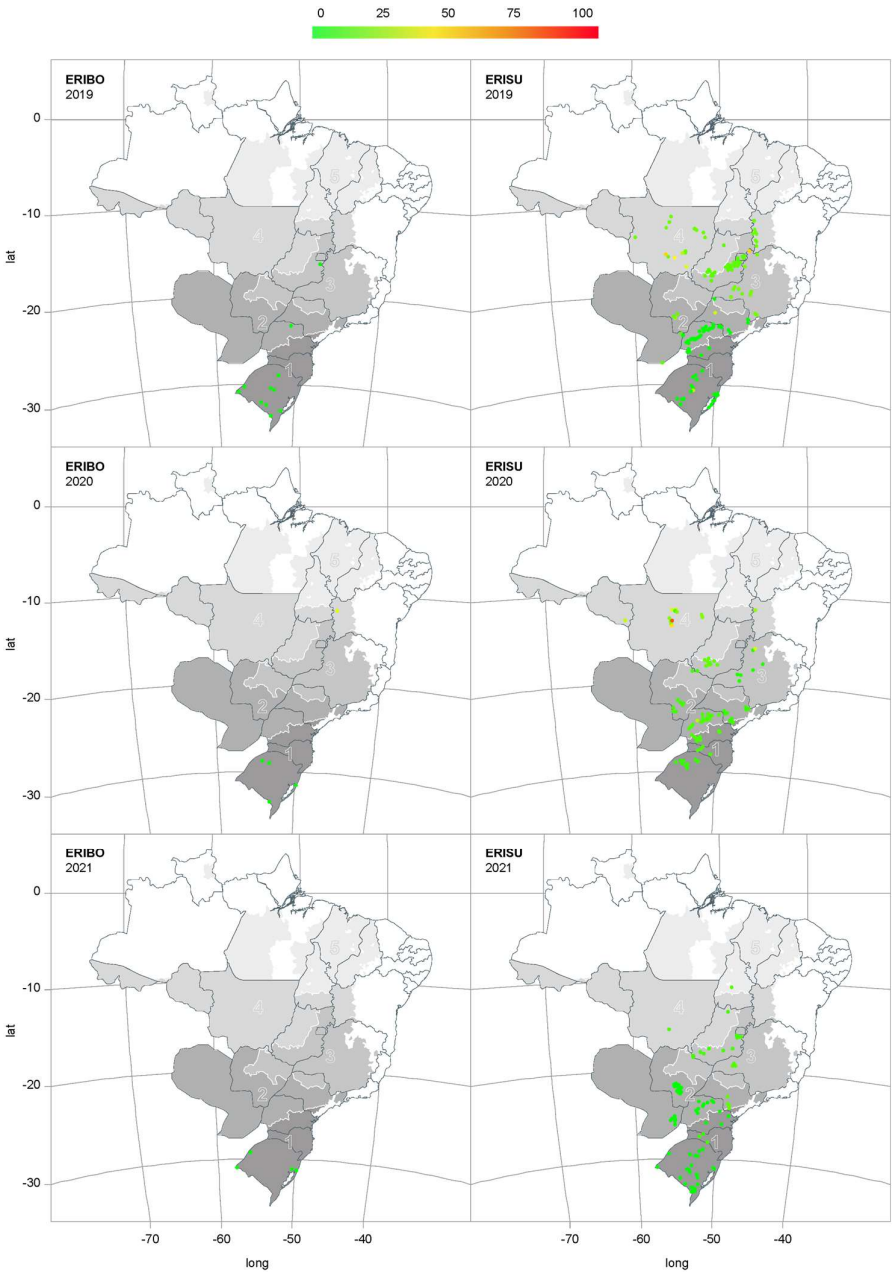
Supplementary Fig. 2



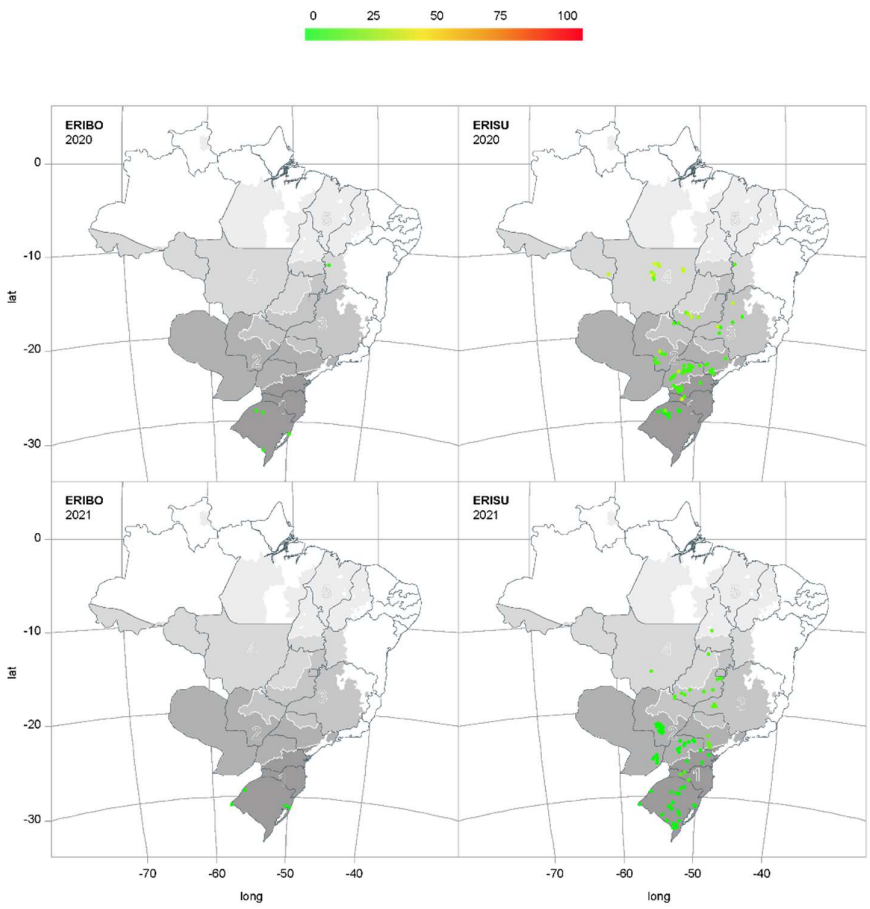
Supplementary Fig. 3



Supplementary Fig. 4

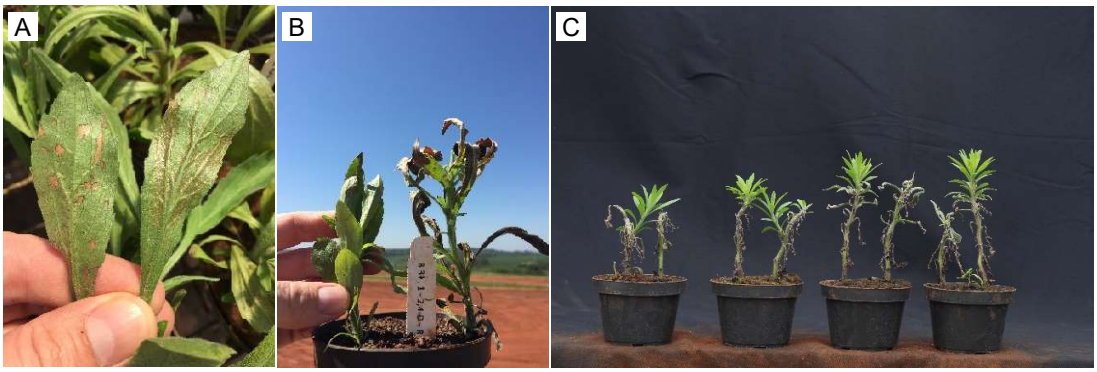


Supplementary Fig. 5



Supplementary Fig. 6

2,4-D



Dicamba

Triclopyr

Supplementary Fig. 7

FINAL CONSIDERATIONS

In this study, we report relevant aspects that support the taxonomic resolution of *Conyza* weeds associated to soybeans, as well as evidence that suggest gene flow within and among species. In addition, we report current scenario of frequency, dispersion, and patterns of *C. bonariensis* and *C. sumatrensis* resistant to five synthetic herbicides Brazil and Paraguay. *Conyza* weeds often pose one of the major threats to the soybean cropping systems due to their occurrence, competitiveness, and resistance against several herbicides. In recent years, the increasing number of official reports of multiple resistance in *C. sumatrensis* has risen many concerns regarding the possible impacts in Brazil and Paraguay. Thus, our study is instrumental to highlight the nature, severity, and scale of multiple-resistant *Conyza* spp. throughout three years in a soybean area of approximately 44 million ha^{-1}.

Based on the results obtained, the combination of *its* and *rps16-trnQ* gene regions and reproductive traits supported the taxonomic resolution of *C. bonariensis* and *C. sumatrensis* in the sampled soybean fields. Strong genetic structure separate *C. bonariensis* and *C. sumatrensis*; however, some individuals of *C. bonariensis* were structured within *C. sumatrensis* clusters. The incidence of interspecific *Conyza* hybrids explains the high geographical dispersion due to the gene flow to spread the resistance alleles between the two *Conyza* species. Resistance to 2,4-D was widespread in Southern Brazil, while resistance to dicamba and triclopyr are at initial evolution. Resistance to auxin herbicides in *Conyza* spp. is a growing problem in Brazil and must be managed based on strict herbicide rotation and integrated weed management, based on good agriculture practices.

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