

Global patterns of herbicide resistance evolution in *Amaranthus* spp.: an analysis comparing species, cropping regions and herbicides

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Abstract: Herbicide resistance in weeds is an evolutionary process. Although there is a great global diversity of weeds, independent origins of herbicide resistance evolution have been shown to converge into similar molecular and physiological resistance mechanisms in geographically distant weed populations. *Amaranthus* species have shown an extraordinary ability to evolve herbicide resistance and invade new environments at a global scale, which represents an opportunity for identifying adaptive evolutionary patterns. The most frequent cases of herbicide-resistant *Amaranthus* species have been identified in North America, where *A. hybridus*, *A. palmeri*, *A. tuberculatus* and *A. retroflexus* comprise more than 90% of them. Meanwhile, *A. retroflexus*, *A. hybridus* and *A. palmeri* have been the most reported species in South America. Around 70% of the cases of herbicide-resistant *Amaranthus*

species have been identified in global soybean and corn crops. The higher fecundity and adaptability of plants to a broad range of environments would make populations more likely to persist and be selected for herbicide resistance. Co-evolution of multiple herbicide resistance mechanisms at the plant and/or population level is evident in weed species. For *Amaranthus* spp., resistance cases highlight evolutionary responses to herbicide use with clear patterns of selection for multiple herbicide resistance in particular regions and spread to new areas within and between global cropping systems. Seed-mediated gene flow is an important component to the spread of herbicide resistant *Amaranthus* spp. populations. Reduction of the intensity of herbicide selection by combining diverse and integrated weed control practices should be a common goal in weed management programs.

Keywords: *Amaranthus hybridus*; *A. palmeri*; *A. retroflexus*; *A. tuberculatus*; multiple herbicide resistance

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1. Introduction

Herbicide resistance in weeds is an evolutionary process, where the selection pressure is the driving force favouring individuals with reduced herbicide sensitivity in plant populations (Délye et al., 2013). Although there is a great global diversity of weeds, some species have evolved herbicide resistance more often than others, even among species of the same genus (Heap, 2014). Biological, genetic and molecular traits linked to the selecting herbicide dose and environment conditions influence the rate of herbicide resistance evolution (Powles, Yu, 2010).

In some regions, particular weeds are often pointed out as prone to evolve herbicide resistance where gene flow ensures a rapid spread of resistance at the agricultural landscape. Similarly, independent origins of herbicide resistance evolution have been shown to converge into similar resistance mechanisms in geographically distant weed populations (Baucom, 2016; Gaines et al., 2019). In this context, several questions emerge: (1) Why does a species evolve herbicide resistance more rapidly than others within the same genus? (2) Why does resistance seem less likely to evolve for some particular herbicides? and (3) Why are similar herbicide resistance mechanisms involved in non-related spatially disconnected weed populations spread globally across cropped fields from different latitudes?

A weed species that evolves a particular herbicide-resistant biotype and the region where the selection occurs, define a novel case of herbicide resistance with both a negative crop productivity impact and academic curiosity. Several *Amaranthus* species have evolved resistance to many herbicides in croplands of four continents, which represents an opportunity for identifying similar and different adaptive evolutionary patterns (Heap, 2014). The aim of this review is to analyse the patterns of herbicide resistance evolution in *Amaranthus* weed species with emphasis on the ecology, biochemical resistance mechanisms and environments where *Amaranthus* species occur, persist or invade, highlighting the current challenges for herbicide resistance management in cropping systems.

2. *Amaranthus* species as current and potential herbicide-resistant weeds

The genus *Amaranthus* L. (Caryophyllales: Amaranthaceae) includes species native to the Americas, as well as worldwide distribution of around 70 species that range from pre-Columbian crops to currently important weeds. *Amaranthus* species include both monoecious and dioecious flowering type (Bayón et al., 2022). The first group is represented by species endemic to every continent and dioecious species are natives to North America. Among them, *Amaranthus palmeri* and *A. tuberculatus* are dioecious plants and *A. albus*, *A. blitoides*, *A. blitum*, *A. crispus*, *A. deflexus*, *A. hybridus*, *A. muricatus*, *A. powellii*, *A. retroflexus*, *A. spinosus*, *A. standleyanus* and *A. viridis* are monoecious weed species found in extensive crops. From that, *A. crispus*, *A. deflexus*, *A. hybridus*, *A. muricatus*, *A. spinosus*, *A. standleyanus* and *A. viridis* are native to South America; however, only *A. hybridus* and *A. viridis* have evolved herbicide resistance in this region (Table 1). The most frequent cases of herbicide-resistant *Amaranthus* species have been identified in North America, where *A. hybridus*, *A. palmeri*, *A. tuberculatus* and *A. retroflexus* group more than 90% of them (Table 1).

2.1 Why does a species evolve herbicide resistance more rapidly than others within the same genus?

The evolution of herbicide resistance is conditioned by the intensity of herbicide selection and both the standing

and *de novo* genetic variation within weed populations (Jasieniuk et al., 1996; Neve et al., 2014). The intensity of selection for a particular herbicide (or herbicides with the same site of action) depends on the herbicide dose and frequency of herbicide use (which accounts for rotation with herbicides of different site of action and use of non-chemical methods). The range of genetic variation associated with a particular weed species is broadly determined by the rate of spontaneous new mutations at the herbicide target gene that may arise in individuals within the local population and/or brought by gene flow processes such as gene (seed and/or pollen) introgression, genetic recombination and genetic drift (Gressel, 2009; Powles, Yu, 2010). The degree of genetic variability brought by the mentioned genetic processes is highly influenced by the size of local populations and reproductive biology (fecundity level and self vs outcrossing) of weed species. An analysis of factors leading to herbicide resistance evolution deserves attention for *Amaranthus* species.

2.1.1 *De novo* genetic variation

The main evolutionary forces determining genetic variation are mutation, selection, genetic drift and gene flow. The mutation rate leading to new resistance alleles has been pointed out as the most difficult estimation in evolutionary models (Diggle, Neve, 2001; Friesen, Hall, 2004). A susceptible-to-resistance mutation rate of 5×10^{-9} was assumed in modelling of herbicide-resistant *A.*

Table 1 - Origin, reproductive system and herbicide resistance cases reported in *Amaranthus* species from different regions of the world (Heap, 2022)

<i>Amaranthus</i> species	Origin	Reproductive system	Herbicide resistance cases				
			Africa	Asia	Europe	North America	South America
<i>albus</i>	N	M	0	0	1	0	0
<i>blitoides</i>	N	M	0	2	1	1	0
<i>blitum</i>	M	M	0	1	2	1	0
<i>crispus</i>	S	M	0	0	0	0	0
<i>deflexus</i>	S	M	0	0	0	0	0
<i>hybridus</i>	N,C,S	M	1	1	5	18	8
<i>muricatus</i>	S	M	0	0	0	0	0
<i>palmeri</i>	N	D	0	1	1	67	3
<i>powellii</i>	N	M	0	0	3	10	0
<i>retroflexus</i>	N	M	0	3	14	29	3
<i>spinosus</i>	N,C,S	M	0	0	0	2	0
<i>standleyanus</i>	S	M	0	0	0	0	0
<i>tuberculatus</i>	N	D	0	1	0	61	0
<i>viridis</i>	S	M	0	0	0	0	2
Total			1	9	27	189	16

N: North America; C: Central America; S: South America; M: monoecious; D: dioecious.

palmeri (Neve et al., 2011). Most recently, an empirical study evaluating spontaneous mutants with resistance to acetolactate synthase (ALS)-inhibiting herbicides in *A. hypochondriacus* concluded that the *de novo* mutation rate was lower than 1.4×10^{-8} (Casale et al., 2019). Considering a mutation rate of 1×10^{-8} , a herbicide-resistant *Amaranthus* spp. plant by *de novo* mutation would be expected in 10 or 100 thousand hectares if the density were 1 or 0.1 plant m^{-2} , respectively. Hence, the greatest risks of herbicide resistance have been associated with weeds comprising large populations (Jasieniuk et al., 1996; Neve et al., 2011).

2.1.2 Gene flow

The magnitude and quality of gene flow determine the evolutionary pathway that unfolds (Ellstrand, 2014). Most mutations conferring herbicide resistance can spread among populations through pollen produced by herbicide-resistant plants or their seeds. Logically, pollen-mediated gene flow processes will be most common in *A. palmeri* and *A. tuberculatus* as obligate-outcrossing dioecious species. The dispersion rate of resistance alleles would depend on the distance between herbicide-resistant and -susceptible plant receptors as well as the wind speed and direction, pollen viability and pollen competition of neighbour susceptible plants (Dafni, Firmagi, 2000; Liu et al., 2012; Beckie et al., 2019). For instance, *A. tuberculatus* pollen can remain viable for up to 5 days after release and it can reach plants as far as 800 m away (Liu et al., 2012). However, Sarangi et al. (2017) determined that half of the effective pollen mediating gene flow occurred within around 3 m from the pollen source. Meanwhile, pollen of glyphosate-resistant *A. palmeri* has been shown to disperse up to hundreds of meters, but approximately half of the progeny of susceptible plants located at 5 m distance from the pollen source of glyphosate-resistant plants were resistant to this herbicide (Sosnoskie et al., 2012).

The impact of pollen-mediated dispersal of resistance genes would be mainly associated with the development of patches of resistant plants at the farm level and this would likely increase the initial frequency of resistant plants within populations (Gressel, 2009; Beckie et al., 2019). Pollen spread contributes to a higher frequency of herbicide-resistant plants compared to the expected initial frequency of herbicide-resistant individuals (10^{-8} to 10^{-9}) in herbicide unselected populations, and it plays an important role in resistance evolution by increasing the incidence of herbicide-resistant weeds across a region (Sarangi et al., 2017; Beckie et al., 2019; Shimono et al., 2020). However, the impact of the pollen-mediated gene flow process on herbicide-sensitivity of receptor susceptible populations is modulated by several factors such as particular gene and alleles, species ploidy, genetic inheritance of resistance, fitness costs, species ecological traits (seed fecundity, seed-bank ecology and outcrossing rate) and agricultural

management practices (Maxwell, Mortimer, 1994; Jasieniuk et al., 1996).

Comparatively, seed-mediated gene flow involves the dispersal of individuals selected in a particular ecological environment. In the case of self-pollinated *Amaranthus* species (monoecious *Amaranthus* species are largely self-pollinated [Franssen et al., 2001]), seed dispersal would represent the main avenue of gene flow to new agroecosystems, and the primary way for herbicide-resistance spread. Herbicide resistance spread following this process would require not only the dispersal of one seed but also the ability to germinate, survive, and reproduce in the new environment.

In the last years, a triple amino acid substitution in the EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase) has been identified in two glyphosate-resistant *A. hybridus* populations from the central region of Argentina (Córdoba province) (García et al., 2019; Perotti et al., 2019). The same triple EPSPS mutation was detected in three *A. hybridus* accessions collected in farms located 500-800 km south-east of these populations in 2016 (Figure 1). In the three farms, glyphosate-resistant plants were found in soybean crops that were preceded by wheat crops. Coincidentally, the harvest operation of the winter crop was performed by hired machinery from the central region of Argentina where glyphosate-resistant *A. hybridus* was broadly dispersed (García et al., 2019; Perotti et al., 2019). In the Northern hemisphere, *A. palmeri* seeds were moved by harvesting equipment from the southern to northern states of USA (Beckie et al., 2019). At a global scale, analyses using reduced representation sequencing and genotyping have implied the likely dispersal of *A. palmeri* seeds from the USA to Brazil, Argentina and Uruguay through commercial trade of crop seed and agricultural machinery between North and South America (Gaines et al., 2021).

Whereas intraspecific gene flow tends to homogenize the genetic variation within populations at the spatial level, interspecific gene flow has also an important role for the evolution of weediness and invasiveness and can provide a substrate for adaptive evolution (Ellstrand, 2014). The interspecific gene flow exchange leading to natural hybridizations between herbicide susceptible and -resistant *Amaranthus* individuals has been associated with herbicide resistance evolution (Nandula et al., 2014; Tranel, 2021). Although multiple resistance mechanisms to different herbicides have been identified in different *Amaranthus* weeds, evidence of natural interspecific hybridizations within the same genus has been recently communicated. Nie et al. (2019) found typical allele variants of the *PPX2* gene, coding for protoporphyrinogen oxidase (PPO) of *A. albus* and *A. palmeri* widespread in 66% of *A. tuberculatus* populations collected in the Midwest of the United States.

In field studies using a donor receptor design, the hybridization and effective glyphosate-resistance gene flow were evident between *A. palmeri* as pollen donor and *A. spinosus* (<0.4%), *A. tuberculatus* (<0.2%) or *A. hybridus*

(<0.01%) as receptors (Gaines et al., 2012). Field-level interspecific hybridization between *A. spinosus* and natural infestations of glyphosate-resistant *A. palmeri* was found when the EPSPS amplicon from *A. palmeri* was detected in glyphosate-resistant *A. spinosus*. The latter is regarded as a weed of minor importance compared to *A. palmeri* or *A. tuberculatus*, but the hybridization event would lead to the selection of glyphosate resistance under glyphosate treatment (Nandula et al., 2014). Also, hybridization between *A. hybridus* and *A. tuberculatus* was evidenced reaching a mean frequency of 0.4 to 2.3%, depending on the proximity between parental plants (Trucco et al., 2005). Interestingly, the genetic exchange between these species seems to be unidirectional: *A. hybridus* alleles transfer to *A. tuberculatus*, but the reciprocal exchange was noticeably distorted (Trucco et al., 2009). However, despite the low frequencies of hybridization and the minimal fertility of the progeny, these studies clearly demonstrate that hybridization could play an important role in adaptive evolution of herbicide resistance in *Amaranthus* species.

Anthropogenic and/or natural seed-mediated gene flow facilitate the spread and colonization of new environments of herbicide-resistant *Amaranthus* species (Shimono et al., 2020). Pollen-mediated gene flow (i.e. genetic crossing) between *Amaranthus* plants will not only lead to *de novo* evolution of herbicide resistance in new areas but also facilitate the stacking of multiple resistance mechanisms within individuals and/or populations (Tranel, 2021). Pollen-mediated gene flow can contribute to interspecific hybridization among populations of *Amaranthus* species at regional level and thus facilitate the spread of herbicide resistance alleles at the field, farm and landscape scale (Nandula et al., 2014). In addition, the efficient distal herbicide-resistance gene flow, across regions, would mainly be linked to seed dispersal both in dioecious and monoecious *Amaranthus* species, resulting in the original source of herbicide resistance in new agroecosystems (Gaines et al., 2021).

2.1.3 Adaptive fitness

The level of population genetic variation is predicted to correlate with adaptive evolution in response to changing environments. In this regard, species with high survival and fecundity rates (i.e. high fitness) are expected to lead this evolutionary path and dominate at the vegetation community level. Seed fecundity of six *Amaranthus* species was evaluated in experiments carried out in the Midwestern region of USA. *Amaranthus hybridus*, *A. palmeri*, *A. retroflexus*, and *A. tuberculatus* individuals produced 250,000 seeds, while a half and a fifth of this amount was recorded in *A. spinosus* and *A. albus*, respectively (Sellers et al., 2003). Consistently, these most fecund species have shown higher cases of resistance evolution than *A. spinosus* or *A. albus* in North America (Table 1). The growth and development of *A. deflexus*, *A. hybridus*, *A. retroflexus*, *A.*

spinosus and *A. viridis* was compared, with *A. retroflexus* and *A. hybridus* showing the highest aerial biomass production (Carvalho, 2005). Considering the *Amaranthus* genus, *A. retroflexus* and *A. hybridus*, together with *A. palmeri*, registered the most reported cases of herbicide resistance in South America (Table 1). Nevertheless, Carvalho et al. (2005) highlighted that *A. viridis* would show the highest ecological adaptability to South-eastern Brazil. After that, cases of *A. viridis* resistant to ALS, photosystem II (PSII) and glyphosate herbicides were detected in the South of Brazil (Francischini et al., 2014; Cruz et al., 2020). Interestingly, this species is a tropical plant distributed in both hemispheres, but it also occurs as weed in fields of temperate regions (Sánchez-del Pino et al., 2013). It seems evident that the most adapted *Amaranthus* species to a particular habitat or cropped region are more likely to evolve herbicide resistance more rapidly.

2.1.4 Agricultural environment and the selection process

The genetic variation associated herbicide resistance *de novo* mutations, intra or interspecific gene flow will likely have no adaptive function in environments under no herbicide selection (Vila-Aiub, 2019). Interestingly, around 70% of the cases of herbicide-resistant *Amaranthus* species worldwide have been recorded in soybean and corn crops (Figure 2). Agricultural areas suitable for soybean and corn represent environments in which natural (temperature, irradiance, etc.) and human (weed chemical control) factors make them of potential spread and evolution of herbicide resistance in *Amaranthus* spp.

Although *Amaranthus* spp. have been shown to evolve herbicide resistance since 1970s, the first case of glyphosate-resistant in the *Amaranthus* genus can be considered a milestone with serious ramifications for weed management, especially in light of the widespread planting of glyphosate-resistant crops (Culpepper et al., 2006). Back then, glyphosate-resistant corn, cotton, and soybeans led to farmers to adopt a single weed control strategy based on the repeated use of glyphosate which resulted in environments with a high selection pressure for glyphosate resistance evolution in *Amaranthus* spp., even under corn, cotton, and soybeans crop rotation (Price et al., 2011; Streck, 2014).

Comparing a herbicide use along a sequence of crops and seasons and the pattern of herbicide rotation of different sites of action can be a useful exercise to understand the ongoing selection pressure for a particular specialist resistance mechanism. However, the strategy of herbicide rotation among cropping seasons might not be good enough to minimize the rate of herbicide resistance evolution as weed populations are subjected to single herbicides at a time, potentially allowing the resistant individuals to set seeds (Norsworthy et al., 2012). Sequential applications of herbicides of different sites of action within the same cropping season could further reduce the risk of resistance compared to the above strategy (Willemse et al., 2021).

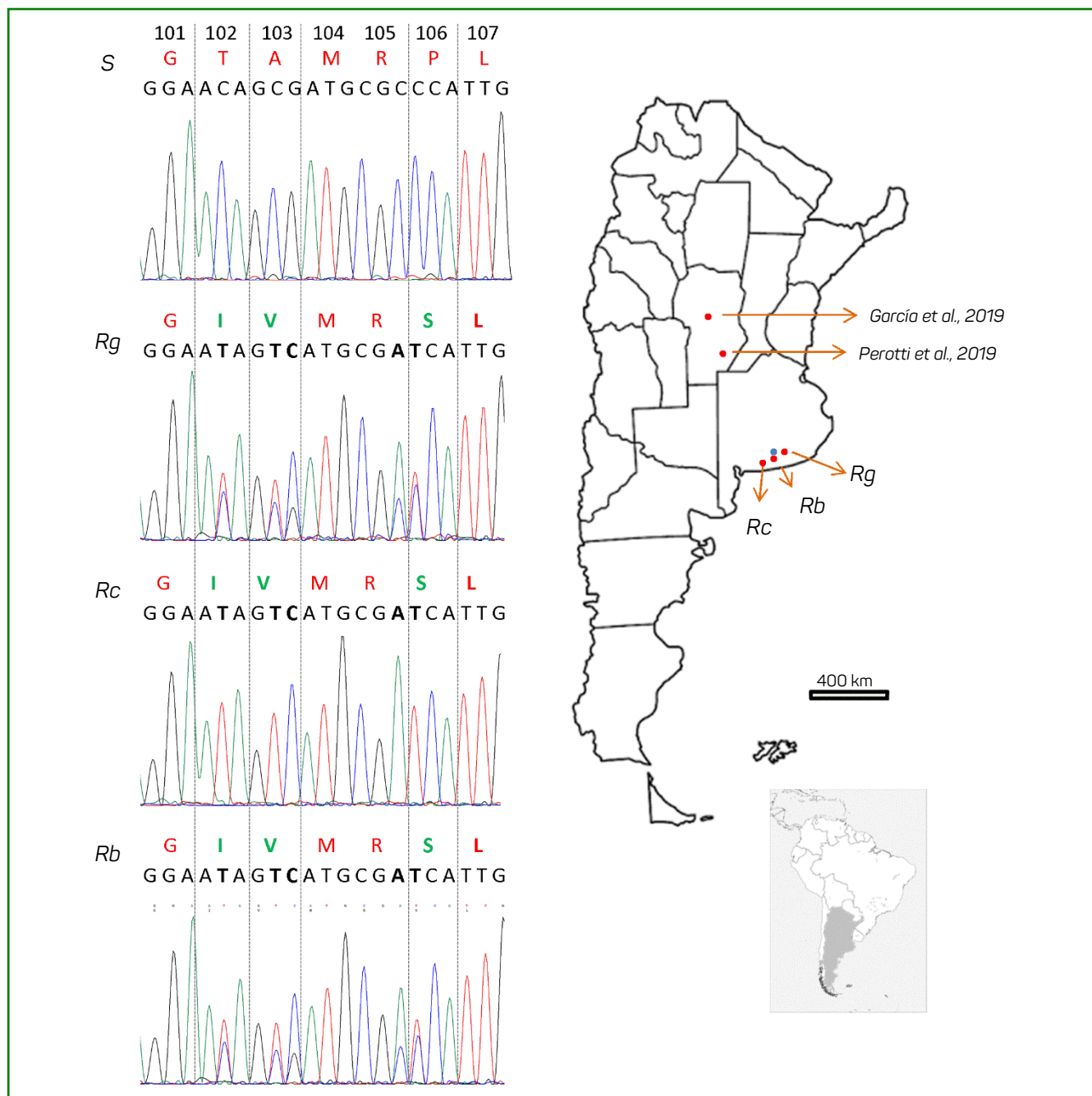


Figure 1 - Sequences and chromatograms of the partial *EPSPS* gene of *Amaranthus hybridus* plants from a susceptible population (blue point on the map) and putative field collected glyphosate-resistant populations (Rg, Rc and Rb). The conceptual translation of the amino acid sequence is shown. The resistance-conferring codon is shown in green letters. Numbers refer to amino acid positions of full-length *EPSPS*. The origin of plant material studied is pointed out on the map and the location of the glyphosate-resistant *A. hybridus* populations studied by Garcia et al. (2019) and Perotti et al. (2019) are indicated

However, both herbicide use approaches are still likely to select for single specialist resistance type mechanisms over time especially if the interval between herbicide treatments is long enough enabling the emergence of different plant cohorts in that period (Norsworthy et al., 2012; Comont et al., 2020). The co-existence of single resistance mechanisms at the plant and/or population level each endowing resistance to a particular site of action herbicide is

named multiple resistance. Still, sequential use of herbicide with different site of action can potentially select for cross-resistance where herbicide metabolism by P450s or GST is possible. Herbicide treatments should be designed based on the most common resistance mechanism associated with each site of action herbicide in order to alternate herbicides with the least probability of resistance evolution. Mixtures of active ingredients, each with different site of

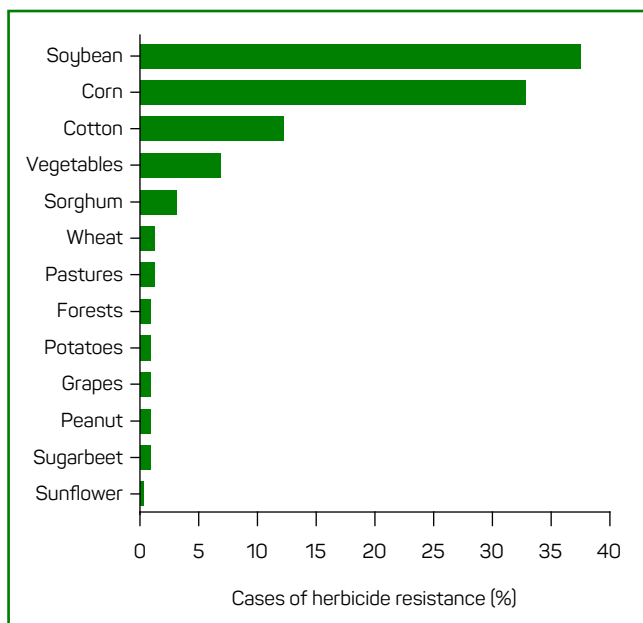


Figure 2 - Comparison of percentage of field crops where herbicide-resistant *Amaranthus* species were reported ($n = 236$) (Heap, 2022)

action and potentially metabolized by distinct biochemical mechanisms, would invariably lead to multiple selection pressure at one time, limiting the chance of resistant plants to arise (Neve et al., 2014).

Why does a species evolve herbicide resistance more rapidly than others within the same genus? Some *Amaranthus* species have evolved herbicide resistance more rapidly than others. Although our analysis attempted to separate the single effect of the main factors driving herbicide resistance, it seems clear that in a environment under herbicide selection, the most prolific and genetically diverse *Amaranthus* species (*A. palmeri*, *A. tuberculatus*) will exhibit higher chances of successful local adaptation and herbicide resistance evolution (Table 1).

3. Evolution of herbicide resistance in *Amaranthus* spp.

About 50 years ago, the first cases of herbicide-resistant *Amaranthus* spp. were reported when resistance to atrazine (i.e. PSII inhibitor) was evidenced in biotypes from North America (Thompson et al., 1974; Radosevich et al., 1977; Weaver et al., 1982). Almost simultaneously, several cases of resistance to PSII-inhibiting herbicides were identified in Europe with target-site resistance (TSR) mechanisms revealed in both continents (Hirschberg, McIntosh, 1983; McNally et al., 1987). More than half of the reports of resistance to PSII-inhibitors date from before 1995, where monoecious *Amaranthus* species prevail (Heap, 2022). Fitness costs associated with PSII-TSR and the maternal inheritance seemed to explain the uncommon atrazine-resistance in the dioecious species until then (Vila-Aiub et al., 2009; Tranel, 2021). Enhanced atrazine metabolism via

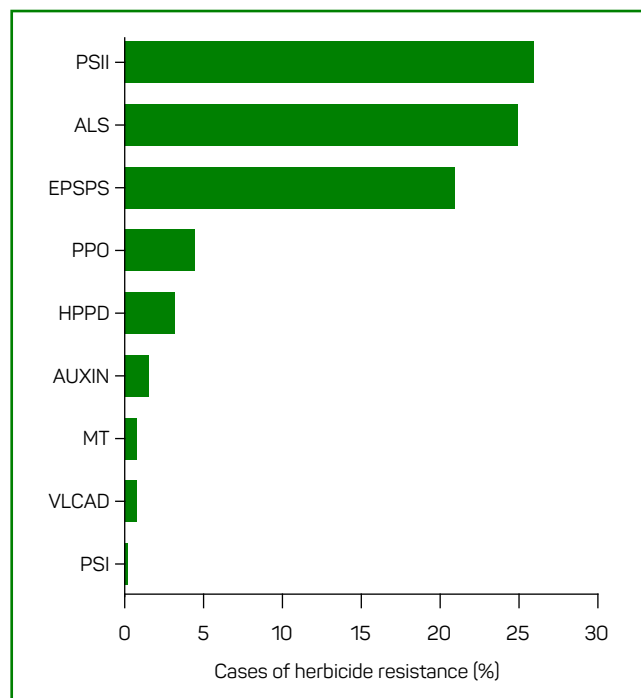


Figure 3 - Cases (%) of herbicide-resistant *Amaranthus* species according to the site of action. Data based on Heap (2022) (236 total cases reported)

glutathione S-transferase (GST) conjugation would be the mechanism most involved in *A. tuberculatus* and *A. palmeri* (Vennapusa et al., 2018; Tranel, 2021). Currently, at least 25% of the cases of herbicide-resistant *Amaranthus* spp. show resistance to PSII-inhibitors (Figure 3) (Heap 2022).

Herbicides that inhibit the biosynthesis of branch chain amino acid (i.e. ALS-inhibiting herbicides), have been commercialized since 1980s and *Amaranthus* species have rapidly evolved resistance to these herbicides. Point mutations causing amino acid substitutions (Ala122, Pro197, Ala205, Asp376, Trp574 and Ser653) in the ALS gene have been reported as the most common mechanism of resistance (Schmenk et al. 1997; Sibony et al., 2001; McNaughton et al., 2005; Whaley et al., 2006; Larrán et al., 2018; Palmieri et al., 2022). However, non-target site resistance (NTSR) mechanisms as rapid herbicide metabolism via cytochrome P450 monooxygenase (P450) and GST have also been reported in dioecious *Amaranthus* species (Guo et al., 2015; Nakka et al., 2017; Figueiredo et al., 2018; Küpper et al., 2018; Oliveira et al., 2018; Shyam et al., 2021). Evidence of enhanced ALS-inhibiting herbicide metabolism in monoecious *Amaranthus* species is limited and NTSR could be masked by TSR (Cao et al., 2021).

In South America, *A. hybridus*, *A. viridis* and *A. palmeri* have evolved resistance to ALS-inhibiting herbicides (Tuesca, Nisensohn, 2001; Francischini et al., 2014; Heap, 2022) and TSR has been found in some populations of Argentina and Brazil (Küpper et al., 2017; Larran et al., 2017; 2018). Twenty five percent of the total herbicide

resistance cases of *Amaranthus* spp. worldwide, include resistance to this chemical herbicide class (Figure 3).

In 2005, an *A. palmeri* biotype from USA (Georgia) was detected as the world's first case of glyphosate resistance in the *Amaranthus* genus (Culpepper et al., 2006). *EPSPS* gene amplification (50 to more than 150 copies) was identified as the glyphosate-endowing resistance mechanism (Gaines et al., 2010). Thus, the level of *EPSPS* gene copies is proportional to *EPSPS* expression and the dose of glyphosate needed to control these plants (Gaines et al., 2010). An extrachromosomal circular DNA (eccDNA) that harbors the *EPSPS* gene was pointed out as the vehicle for the gene amplification (Molin et al., 2020). Jugulam (2021) posits that these eccDNA have not integrated into the host genome and the number of *EPSPS* copies may be dissipated in absence of glyphosate selection.

Molecular comparisons support that *EPSPS* gene amplification would evolve once and then spread by pollen and/or seed across the USA (Molin et al., 2018), but this resistance mechanism has been also identified in South American cropping systems where *A. palmeri* is not a native species but also evolving glyphosate resistance (Brazil and Uruguay) (Gaines et al., 2021). In contrast, *EPSPS* gene amplification has not been found in Argentinean glyphosate-resistant *A. palmeri* populations, which only exhibit TSR conferred by a point mutation (proline to serine at 106 codon of *EPSPS*) and reduced glyphosate absorption and translocation (Palma-Bautista et al., 2019; Kaundun et al., 2019).

Tandem *EPSPS* duplications, amino acid substitution due to proline to serine in 106 position of the target site enzyme, and reduced glyphosate translocation have been identified as resistance mechanisms in glyphosate-resistant *A. tuberculatus* populations from the Midwestern USA (Lorentz et al., 2013; Nandula et al., 2014; Dillon et al., 2017). Furthermore, multiple glyphosate-resistance mechanisms, such as *EPSPS* point mutations and *EPSPS* gene amplification, co-exist in *A. tuberculatus* populations (Wu et al., 2017).

Among monoecious *Amaranthus* species, glyphosate-resistance was reported in *A. spinosus*, *A. hybridus* and *A. viridis* populations from USA, Argentina and Brazil, respectively (Nandula et al., 2014; García et al., 2019; Perotti et al., 2019; Cruz et al., 2020). As mentioned before, the *EPSPS* amplicon from *A. palmeri* was found in an *A. spinosus* biotype as source of glyphosate resistance (Nandula et al., 2014). While a triple amino acid substitution in the *EPSPS* (T102I, A103V, and P106S) confers a high-level of glyphosate resistance in the *A. hybridus* populations (García et al., 2019; Perotti et al., 2019), results of enzyme activity at different glyphosate concentrations have suggested a TSR mechanism involved in the resistant *A. viridis* biotype (Cruz et al., 2020).

Glyphosate resistance represents 21% of the total cases of herbicide-resistant *Amaranthus* spp. and most reports of glyphosate resistance have been documented in soybean,

cotton and corn crops (Heap, 2022). Several populations have also evolved resistance to synthetic auxins, VLCFAs, PPO- and/or HPPD-inhibiting herbicides although these cases of resistance are the least common and represent less than 10% of total records.

Evolution of multiple herbicide resistance has been identified in *Amaranthus* spp, where enhanced herbicide metabolism is likely to play a significant role in endowing multiple resistance to several herbicides of dissimilar chemistry (Jugulam, Shyam, 2019; Shyam et al., 2020; Tranel, 2021; Heap, 2022).

3.1 Why does resistance seem less likely to evolve for some particular herbicides?

Multiple factors associated with herbicides such as chemical structure, target gene (i.e. site of action) and residual activity and the interplay with both genetic (ploidy, inheritance, dominance etc.) and ecological (fitness cost) factors, have a significant impact on the evolutionary process towards herbicide resistance evolution (Powles, Yu, 2010).

The likelihood of TSR evolution is different according to the site of action of the herbicide (Beckie, 2006). ACCase-, ALS- and PSII-inhibiting herbicides have been shown the most documented and rapid cases of resistance evolution in weeds. In addition to ALS- and PSII-inhibiting herbicides, glyphosate resistance in *Amaranthus* spp. has often evolved in contrast to other herbicide chemistries such as synthetic auxins and VLCFAs. Similarly to the latter group of herbicides, evolution of paraquat (PSI-inhibitor) and glufosinate (inhibitor of glutamine synthetase) resistance have been a relatively slow process or has not yet been confirmed in *Amaranthus* spp. (Figure 3) (Shyam et al., 2021; Nazish et al., 2022). Although glufosinate is intensively used in the Midwest and Southern regions of USA where glufosinate-resistant crops are grown (Takano et al., 2020), the rarity of glufosinate resistance in *Amaranthus* spp. could be related to the inherent difficulty for a plant to achieve resistance to this herbicide (Tranel, 2021). Paraquat exerts a relatively low selection pressure due to it is restricted use to control emerged *Amaranthus* plants prior to crop planting (Tranel, 2021; Liu et al., 2022). Contrary to 2,4-D, ALS-, PSII-, PPO- and HPPD-inhibiting herbicides, metabolism of glufosinate and paraquat has proved to be a biochemical limitation for most plants to evolve metabolic NTSR (Shyam et al., 2021).

3.2 Travelling the same pathway

The broad evolution of survival mechanisms for generalist- (NTSR) and specialist- (TSR and some NTSR) adaptation types can illustrate the evolutionary resilience of weeds to extreme selection pressures (Gaines et al., 2020). Currently, the last step on the road to herbicide resistance evolution in weeds is the resistance to either

multiple herbicides due to the stacking of specialist single mechanisms or a more generalist or cross resistance where a single mechanism endows resistance to several chemical class herbicides (Comont et al., 2020; Torra et al., 2021). A cluster analysis searching for associations among resistance mechanisms to different sites of action for the 58 cases of *Amaranthus spp.* with resistance to multiple herbicides compiled by Heap (2022) is shown in Figure 4. Mechanisms endowing resistance to EPSPS-, ALS- and PSII-inhibitors clustered together likely due to stacking of TSR mutations (Figure 4). Instead, resistance to VLCAFs and microtubules (MT) inhibiting herbicides, synthetic auxins and HPPD-inhibiting herbicides seem to be clustered where more generalist-type mechanisms would have been evolved. Resistance to PPO-inhibiting herbicides, which is often associated with both TSR and NTSR mechanisms, constitute a group closer to these clusters than PSII-, ALS- or EPSPS- inhibiting herbicides (Figure 4).

Cytochrome P450 and GST conform diverse superfamilies of enzymes that are able to metabolize many herbicides (Dixon et al., 2002; Nelson, Werck-Reichhart, 2011). It is broadly accepted that P450 and GST enzymatic complexes can confer broad-spectrum herbicide resistance. However the molecular basis of the NTSR conferred by herbicide enhanced metabolism is still unclear for most of the cases in weeds (but see Han et al., 2021), and the phenotypic distinction between a single metabolic mechanism involving a cross-resistance pattern and multiple distinct stacked herbicide metabolic mechanisms in a single biotype is challenging (Gaines et al., 2020). In this regard, distinct herbicide metabolism mechanisms have been involved in a resistant *A. tuberculatus* population to HPPD- and PSII-inhibitor herbicides (mesotrione and atrazine, respectively) (Ma et al., 2013) and evidence of cross-resistance has been reported in another population (Jacobs et al., 2020).

Interestingly, in a single *A. palmeri* population, metabolism of the same PPO-inhibiting herbicide was found to be mediated by both P450 and GST (Varanasi et al., 2018). In addition, resistance to herbicides from six sites of action has been recently reported in an *A. palmeri* population from USA (Kansas) which shows metabolic resistance mediated by P450 and GST enzymes that metabolize 2,4-D, PSII-, PPO- and HPPD inhibitor herbicides (Shyam et al., 2020).

These examples are cases of resistance that range from two metabolic mechanisms that explain resistance to two herbicides, one gene encoding metabolic resistance to distinct herbicides, to two metabolic mechanisms (P450 and GST) conferring resistance to one or more herbicides in a single population. In any case, this is robust evidence of the selection of metabolic mechanisms conferring resistance to key herbicides for the control of glyphosate and/or ALS resistant *Amaranthus spp.* populations.

At least, 75% of the cases of multiple herbicide resistance reported in *Amaranthus spp.* correspond to *A. palmeri* and *A. tuberculatus*. Most of these cases of multiple

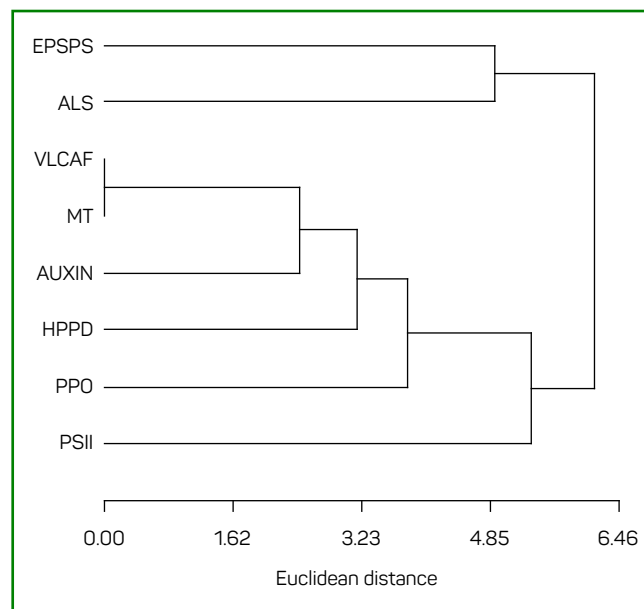


Figure 4 - Cluster analysis of variables of resistance to herbicides of different sites of action that occur in cases of *Amaranthus* species with resistance to multiple herbicides. Data compiled by Heap (2022) (n = 58)

resistance have occurred in regions of North America where resistance evolution to glyphosate in both doieicious *Amaranthus* species has been very frequent. The risks of the ever-growing cases of multiple resistance detected in North America seems to be mirrored in South America given the accelerated evolution rate in *Amaranthus spp.* populations reported recently (Dellafrera et al., 2018; García et al., 2019; Kaundun et al., 2019; Gaines et al., 2020; Heap 2022). Interestingly, in a recent survey carried out by Scursoni et al. (2022) across fifty *A. hybridus* accessions, 84% and 76% were susceptible to recommended field dose of 2,4-D and dicamba, respectively. Whereas more than 90% of the accessions showed high (>60%) survival to glyphosate and 43% and 72% exhibited survival greater than 60% to fomesafen and topramezone, respectively (Scursoni et al., 2022).

The pattern of herbicide use in many cropping systems seems to provide a proper environment for the selection of multiple-herbicide-resistant populations consisting of plants with generalist resistance mechanisms, including genotypes with stacked herbicide-resistance traits, a mix of plants with heterogenous herbicide-sensitivity or a combination of them. Anyway, resistance to more than one chemically unrelated herbicide emerges as a challenge for current and future resistance management in agroecosystems. By elucidating the biochemical and genetics of enhanced herbicide metabolism and patterns of herbicide-sensitivity, it may help identify alternative herbicides and thus decrease the intensity of selection pressure of the most used herbicides to control in *Amaranthus* populations (Shyam et al., 2021; Tranel, 2021).

3.3 Why are similar herbicide resistance mechanisms involved in non-related spatially disconnected weed populations spread globally across cropped fields from different latitudes?

As discussed above, gene flow tends to homogenize the genetic variation within populations and could be the cause of finding similar herbicide resistance mechanisms in geographically distant populations. However the evidence does not seem to associate all *Amaranthus* spp. populations with a same mechanism of resistance to a common origin (Gaines et al., 2021). Processes of convergent and parallel evolution of similar herbicide resistance mechanisms have been detected in non-related weed species and populations (Patterson et al., 2018; Kreiner et al., 2019).

Convergent phenotypic evolution can be explained by the generalized pattern use of herbicides that imposes a common selection pressure on weed populations of different regions as pointed out above (Powles, Yu, 2010). However, the specific repeatability of adaptive evolution is of particular interest when supported by genotypic convergences (Patterson et al., 2018).

Convergent genotypic evolution of *A. tuberculatus* populations was evidenced in North America, where distinct *EPSPS* amplification events were detected in populations from Canadian regions and US Midwest (Kreiner et al., 2019). This phenomenon is not exclusive to glyphosate selection, as parallel and convergent genotypic evolution of resistance to PPO inhibitors in *A. tuberculatus* and *A. palmeri* populations has also taken place within a single field (Lillie et al., 2019). Wide genetic diversity of these *Amaranthus* spp. would have facilitated the parallel and convergent adaptation. At the early stage of the evolutionary process, the pattern of herbicide use would select for genetic resistance mechanisms that endow an advantage but are not the ones necessarily conferring the most beneficial fitness effect. A second process of microevolution of the original mechanism endowing herbicide resistance overriding any possible trade-offs between fitness cost and benefit is possible and make a resistance mechanism more likely to be selected for (Uyenoyama 1986; Vila-Aiub et al., 2009; Vila-Aiub et al., 2014). The result of this trade-off would be similar in environments of different regions under a common herbicide use, leading to convergent herbicide resistance evolution.

4. Herbicide-resistant *Amaranthus* weeds in new environments

The spread of herbicide-resistant *Amaranthus* spp. to new environments is a great threat to sustainability of cropping systems. Both monoecious and dioecious *Amaranthus* species have been recorded as weeds in at least four continents, revealing their capacity to adaptation to different environments (Bayón, 2022). In this context, herbicide resistance genes are a trait that can shift the role of these species in the agroecosystem where *Amaranthus* species are currently classified as secondary weeds with low plant density (Torra et al., 2020).

Recently, three *A. palmeri* populations with cross-resistance to ALS-inhibiting herbicides have been reported in soybean crops in Italy (Milani et al., 2021), and low glyphosate sensitivity was demonstrated in *A. palmeri* accessions collected in Turkish citrus fields (Mennan et al., 2021). In agricultural farms located in North-eastern Spain, three *A. palmeri* populations were identified. This represents the introduction of a exotic weed into Spanish agroecosystems, which not only shows evolved resistance to ALS-inhibiting herbicides but also complete adaptation to this new environment. The origin of these *A. palmeri* populations would be likely related to dispersal and colonization events from America (Torra et al., 2020). An analysis of the potential global distribution of *A. palmeri* has revealed a real risk of expansion and invasion into Europe, North America and South America. Furthermore, large regions of Asia, Australia and Caribbean Islands and South Africa have been described as climatically suitable for *A. palmeri* (Kistner, Hatfield, 2018). In this last area, this species was recently found and reported invading ruderal and segetal plant communities (Sukhorukov et al., 2021).

The distribution and invasion of *A. palmeri* to new regions would be conditioned on the sufficient growing degree days accumulated to complete the cycle of the species. These new environments seem to be limited by some high altitude regions and the equatorial tropics where the cold and hot-wet stress are an ecological constraint for plant establishment. Nevertheless, global climate change associated with further increases in average temperature would help expand the potential of *A. palmeri* distribution northward into Canada and Europe (Kistner, Hatfield, 2018). Amplification of the *EPSPS* gene, together with extra genomic sequences as revealed by putative genes, tandem repeats and regulatory elements (“*EPSPS* cassette”) has been proposed not only as an adaptation conferring resistance to glyphosate with an adaptive value for *A. palmeri* to invade new environments (Jugulam, 2021).

The potential spread of *A. retroflexus* was studied by Liu et al. (2007). This monoecious species is currently distributed along USA, most Europe, Northern Africa, central and east of Asia and South-eastern Australia, but its maximum potential distribution highlights the risk of spread to most Asia, Southern Africa, north and south of Australia, large areas of North America and south central of South America (Liu et al., 2007). Several environmental variables such as temperature, rainfall, irradiance and altitude influence the actual and potential distribution of this species (Liu et al., 2007). Notwithstanding, climatic conditions are not the only barriers for the spread and naturalization of herbicide-resistant weeds as other factors can also favour this process at the farm scale (Bravo et al., 2018).

5. Conclusion

Amaranthus species have shown an extraordinary ability to evolve herbicide resistance and invade new environments

at a global scale. Resistance cases highlight the increasing and repetitive evolutionary response to global herbicide use with clear patterns for selection of multiple herbicide resistance in particular regions and spread to new areas within and between global cropping systems. In agricultural areas where resistance evolution of *Amaranthus* species has not been identified, there is a real threat and risk of both natural and human-driven seed-mediated gene flow from regions where resistance evolution is present together with pollen-mediated gene flow from other native or naturalized herbicide-resistant *Amaranthus* species. In this context, the management and prevention of herbicide resistance should consider agronomic strategies that minimize the intensity of herbicide selection

through the adoption of a correct use pattern of herbicides with different site of action aiming to minimize the evolution of both stacked multiple resistance mechanisms (TSR and NTSR) and single generalist (NTSR) resistance mechanisms endowing cross- and multiple resistance.

6. Author's contributions

All authors read and agreed to the manuscript. MY, TG, JS, RDP, and MVA: conceptualization of the manuscript. MY: data analysis. MY and MVA: data interpretation. MY and MVA: writing the original draft of the manuscript. MY, TG, JS, RDP, and MVA: writing, review and editing.

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