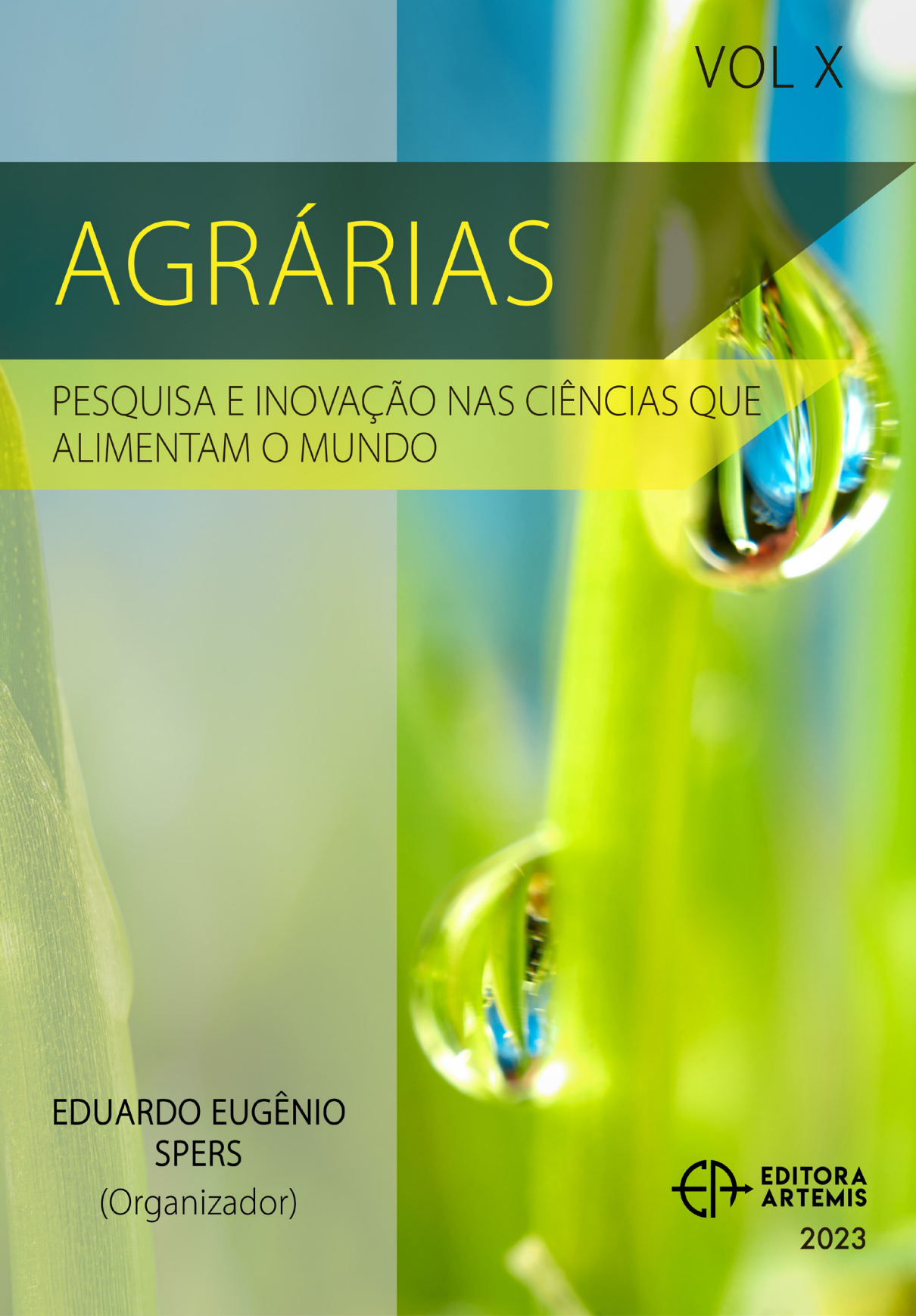
VOL X

AGRÁRIAS

PESQUISA E INOVAÇÃO NAS CIÊNCIAS QUE ALIMENTAM O MUNDO



EDUARDO EUGÊNIO SPERS
(Organizador)

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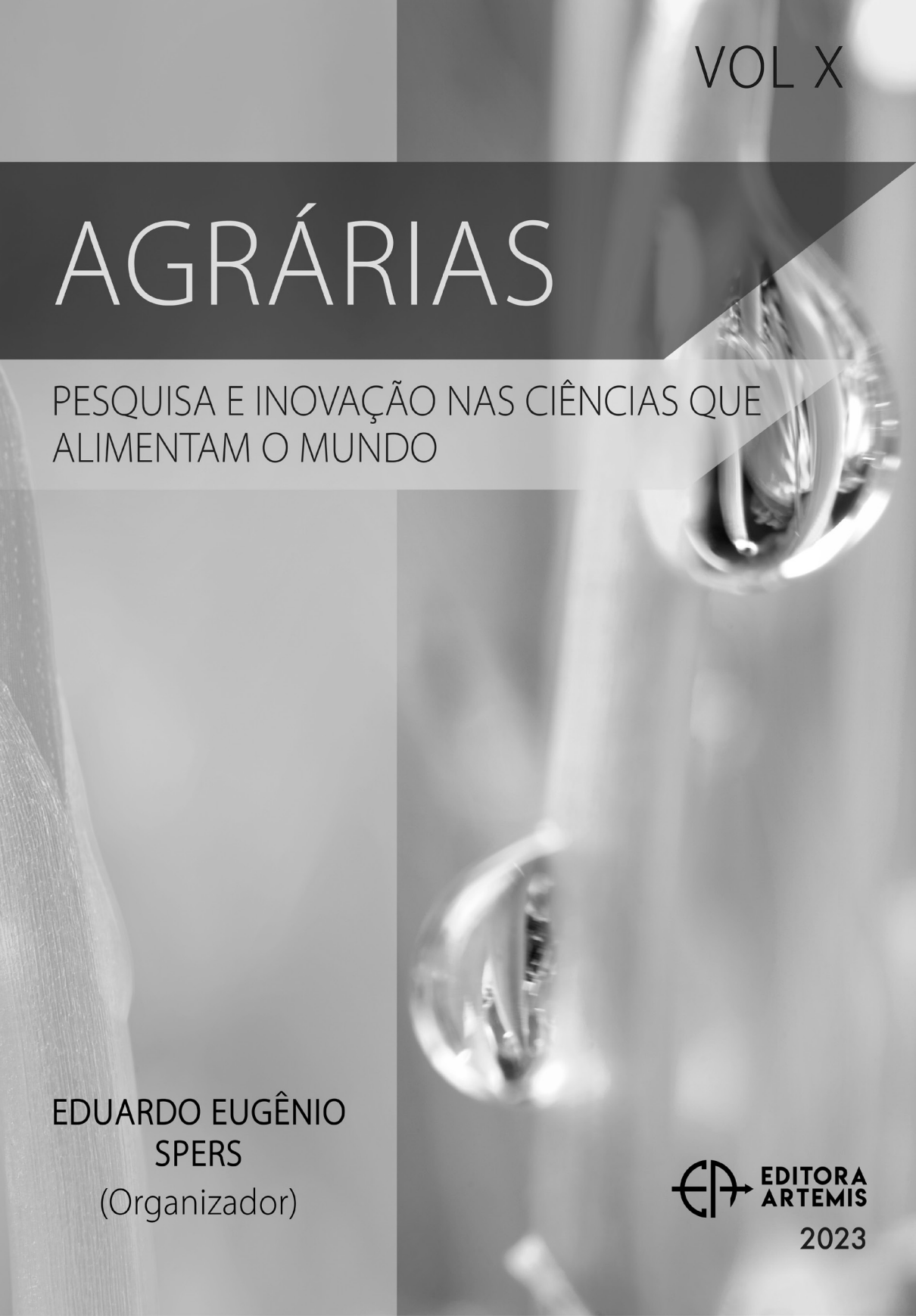

EDUARDO EUGÊNIO SPERS
(Organizador)

EDITORA ARTEMIS
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APRESENTAÇÃO

As Ciências Agrárias são um campo de estudo multidisciplinar por excelência, e um dos mais profícuos em termos de pesquisas e aprimoramento técnico. A demanda mundial por alimentos e a crescente degradação ambiental impulsionam a busca constante por soluções sustentáveis de produção e por medidas visando à preservação e recuperação dos recursos naturais.

A obra **Agrárias: Pesquisa e Inovação nas Ciências que Alimentam o Mundo** compila pesquisas atuais e extremamente relevantes, apresentadas em linguagem científica de fácil entendimento. Na coletânea, o leitor encontrará textos que tratam dos sistemas produtivos em seus diversos aspectos, além de estudos que exploram diferentes perspectivas ou abordagens sobre a planta, o meio ambiente, o animal, o homem e a sociedade no ambiente rural.

É uma obra que fornece dados, informações e resultados de pesquisas tanto para pesquisadores e atuantes nas diversas áreas das Ciências Agrárias, como para o leitor que tenha a curiosidade de entender e expandir seus conhecimentos.

Este Volume X traz 14 trabalhos de estudiosos de diversos países, divididos em dois eixos temáticos: *Produtividade e eficiência na produção vegetal* e *Sustentabilidade e reaproveitamento produtivo.*

Desejo a todos uma ótima leitura!

Eduardo Eugênio Spers

SUMÁRIO

PRODUTIVIDADE E EFICIÊNCIA NA PRODUÇÃO VEGETAL

CAPÍTULO 6

IDENTIFICACIÓN MOLECULAR DE MALEZAS TOLERANTES AL GLIFOSATO

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RESUMEN: El glifosato es un herbicida organofosforado utilizado mundialmente en la agricultura, el incremento en sus dosis de aplicación genera problemas ambientales y selecciona plantas más resistentes. Es necesario conocer el tipo de resistencia para generar estrategias de control de malezas. Así, el objetivo de este trabajo fue identificar molecularmente especies que resistan a este herbicida y analizar su mecanismo molecular de resistencia. En este estudiose colectaron 12 muestras de la zona oriente del estado de Morelos (México) con tolerancia a glifosato. Para la identificación se extrajo el ADN, se amplificó por PCR, se obtuvieron y analizaron las secuencias del gen *trn*. Para demostrar el mecanismo de resistencia se amplificó la

EPSPS, posteriormente se realizó un perfil de restricción con *AluI*, finalmente se generaron árboles filogenéticos de los biotipos que mostraron resistencia, resultando *Spermacoce* sp., *Helianthus* sp., *Ipomoea* sp., *Commelina* sp. *Echinochloa* sp. como géneros principales. Concluimos que son cinco especies que presentan resistencia, no obstante, no se encontró presencia de *EPSPS*, el crecimiento de los arvenses en cuestion podría deberse a una resistencia en sitio target o también por modificación fisiológica.
PALABRAS CLAVE: Herbicida. Glifosato oxidoreductasa. Plantas. Mecanismo de resistencia.

MOLECULAR IDENTIFICATION OF GLYPHOSATE-TOLERANT WEEDS

ABSTRACT: Glyphosate is an organophosphorus herbicide used worldwide in agriculture. In addition, the increase in application doses generates environmental problems and selects more resistant plants. It is necessary to know the type of resistance to develop weed control strategies. Thus, the objective of this work was to molecularly identify species that resist this herbicide, especially to analyze its molecular resistance mechanism. In this study, 12 samples, with apparent tolerance to glyphosate, were collected in different locations in the eastern part of the state of Morelos. At least 4 species were repeatedly found at the sampling sites. For the identification of each specimen the DNA was extracted, amplified by PCR, and visualized in agarose gel electrophoresis. In addition, the sequences of the trn gene were obtained and analyzed. To demonstrate the mechanism of resistance of the biotypes, an amplification of *EPSPS* was performed. However, it was not possible to amplify due to an incorrect performance of the techniques used or the oligonucleotides design. Subsequently, a restriction profile was performed with AluI, the in silico analysis agreed with these results. Finally, phylogenetic trees were generated to identify the biotypes that showed resistance; *Spermacoce* sp., *Helianthus* sp., *Ipomoea* sp., *Commelina* sp., and *Echinochloa* sp. were found as main genera. We conclude that there are five species that have resistance to glyphosate, tests are needed to study the *EPSPS* sequences to demonstrate the resistance mechanism of these biotypes. However, it could be due to resistance at the target site, where the objective of the process cannot be achieved at the site of action, since mutations occur in the DNA sequence, or also an expression of the gene that is inhibited by glyphosate.
KEYWORDS: Herbicide. Glyphosate oxidoreductase gene. Plants. Mechanism of resistance.

1 INTRODUCTION

Glyphosate is a non-selective herbicide commonly used by farmers to control weeds growth, as these are the most harmful biotic factors that may affect the yield in a crop (Chauhan, 2020). Glyphosate works by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) (Funke *et al.,* 2006), affecting the synthesis of chorismite, the precursor of aromatic amino acids (Dosselaere and Vanderleyden, 2001), leading

finally to plant death. Initially this herbicide was applied before the crops started to grow or after these were harvested, but with the creation of genetically modified glyphosate resistant crops glyphosate consumption increased, as this allowed the herbicide to be applied multiple times during crop growth (Benbrook, 2016).

At first glyphosate and glyphosate-based herbicides (GBH) were widely accepted as a harmless and very effective way to improve crop yields, however due to the increase on its use, concern about the effects of this compound on the environment and human health arose among scientists (Tarazona *et al.,* 2017). Further investigation has led to the conclusion that glyphosates and GBH affect non-target plants growth and health (Kanissery *et al.,* 2019; Martinez *et al.,* 2018) and its presence has been associated with various adverse health conditions including cancer (Meftaul *et al*., 2020), although that matter is still widely discussed (Tarazona *et al*., 2017). Although glyphosate has a relatively short half-life due to bacterial degradation (Kanissery *et al.,* 2019; Mercurio *et al.,* 2014), it has been discovered that it has a great capacity to distribute widely, and degradation may take longer under certain circumstances (Castrejón-Godínez *et al*., 2021; Kanissery *et al.,* 2019; Mercurio et al., 2014), like others organophosphates (Moreno-Medina *et al.,* 2014).

Besides the environmental and health problems that prolonged and extensive use of glyphosates imposes, a new economic challenge may begin. In 1996 Australian scientists reported the possibility that a species of weed present in the country, rigid ryegrass (*Lolium rigidum*), may have developed resistance to glyphosate, however in 1997 the possibility of weed resistance to glyphosate was still discussed and considered improbable, however in 1998 the presence of weed resistance in the plant mentioned before was demonstrated, encouraging more discussion on the matter (Bradshaw *et al.,* 1997; Powles *et al.,* 1998). By 2021 glyphosate resistance was found on at least 48 different species of weed within 30 countries, being Australia, USA, and Argentina the most affected with over 10 different weed-resistance species (Baek *et al.,* 2021).

Glyphosate resistance mechanisms in weeds are caused by different mutations across species which may change the active site of the targeted enzyme, making the herbicide useless. Another mechanism is the duplication of the *EPSPS* gene, that results in an overproduction of the enzyme that leads to an incomplete inhibition of the enzyme by using normal glyphosate doses. Other type of resistance known as non-target site mechanism consists in hampering glyphosate transportation across plant tissue by either sequestration or rapid plant tissue death. Some plants use both mechanisms, resulting in a more robust resistance mechanism (Duke, 2019).

In Mexico glyphosate and GBH products are widely used in the agricultural sector and weed resistance to glyphosate is reported in at least 4 species of weeds, *Lolium perenne, Bidens pilosa, Leptochloa virgata, and Aster squamatus* (Baek *et al.,* 2021; Domínguez-Valenzuela *et al.,* 2021). Although in Mexico around 13% of the workforce is dedicated to agriculture, this sector is one of the lowest contributors to national Gross Domestic Product (GDP) (*Higher Education in Mexico*, 2019). The presence of weed resistance in Mexico endangers this already vulnerable sector.

In this work the presence of weed in glyphosate treated land is studied, giving a wider insight on the presence of glyphosate-resistant weeds in Mexico.

2 MATERIALS AND METHODS

2.1 GENETIC MATERIAL EXTRACTION

Plant tissue was collected from weeds present in fields in Tetela del Volcán, Jonacatepec, Cuautla, Tepalcingo, and Ayala municipalities in Morelos state (Mexico), treated with glyphosate-based herbicides. A total of twelve samples were collected and kept at freezing temperatures until the extraction was made.

The sample was washed with sodium hypochlorite 0.5% and water. Then 200 mg were macerated with 1 mL of extraction buffer and transferred to a 2 mL centrifuge tube. The extraction buffer was prepared modifying the method reported by Doyle and Doyle *et al.* (1987). The sample was then vortexed until sample homogenization and heated at 65°C for 60 minutes, after which it was centrifuged at 8000 rpm for 1 minute. The upper phase was recovered and transferred to a new tube and isoamyl alcohol was added at a 24:1 ratio and it was mixed by inversion. The mix was then centrifuged at 8000 rpm for 15 minutes and the upper phase was transferred to a clean tube where 2 volumes of -20°C ethyl alcohol was added. Further mixing was done by inversion followed by a centrifugation at 8000 rpm for 2 minutes at 4°C and the supernatant was discarded. The DNA pellet was washed with 70% ethyl alcohol by inverting the tube several times and then centrifuged again at 8000 rpm for 2 minutes at 4°C. The ethanol was aspirated and let dry by leaving the tube open at room temperature for 15 minutes. Finally, the DNA was resuspended in TE solution and incubated for 1 hour at 65°C. The DNA was stored in a refrigerator at 4°C. Concentration and purity of the DNA extract were measured using NanoDrop® One spectrophotometer.

2.2 PCR ASSAYS

For species determination the *trnL* gene was amplified using the primers designed by Taberlet *et al.* (1991), while primers RR01 and RR04 reported by Tengel *et al.* (2001) were used for detection of possible glyphosate resistance amplifying *EPSPS* gene in the collected samples.

For the *trnL-F* gene amplification a mix of 5 µL of 5x Green GoTaq Buffer (Promega), 5 µL of $MgCl_2$ 50 mM, 1 µL of dNTPs, 1 µL of the forward primer 10 mM, 1 µL of the reverse primer 10 mM, 0.25 µL of Taq DNA polymerase (Thermo Scientific), and 31 µL of nuclease-free water was made. The PCR amplification was done with a first denaturing of the DNA at 94°C for 5 minutes, followed by 35 cycles of 1 minute at 94°C for denaturing, 48°C for 1 minute for the annealing step and an extending stage of 2 minutes at 72°C; at the end of these cycles there is a final annealing stage of 10 min at 72°C.

2.3 PHYLOGENETIC ANALYSIS

The *trnL-F* PCR products were purified using the AxyPrep MAG PCR Clean-Up Kit (Axygen) and the region was then sequenced by INSTITUTION. The sequences were inputted in the *Nucleotide Basic Local Alignment Search Tool (blastn)*. For every query sequence at least two different species were considered for the phylogenetic analysis, with BLAST similarity above 80%. The sequences were aligned using ClustalW for posterior phylogenetic analysis with MEGA X. For estimating distances 5 different models (Jukes-Cantor, Tajima-Nei, Kimura 2-Parameter, Tamura 3-Parameter & Tamura-Nei) were compared, choosing it by considering the Bayesian Information Criterion, Akaike Information Criterion and Maximum Likelihood value displayed by MEGA X. Finally, to construct the phylogenetic tree, the Maximum Likelihood Tree option was used. Bootstrap method was employed with 1000 replications using the Tamura 3-Parameter model with uniform rates among sites, as this had the best scores according to the model analysis.

3 RESULTS AND DISCUSSION

From the twelve samples, five were selected by morphology, avoiding the analysis of the same plant species. The DNA concentration upon extraction had values between 80 to 250 ng/µL and a A260/280 ratio value of ~1.8 supporting an absence of contaminants like proteins, however the A260/230 ratio value below 2.0 indicating

possible contamination. In spite of this, all further experiments were conducted successfully.

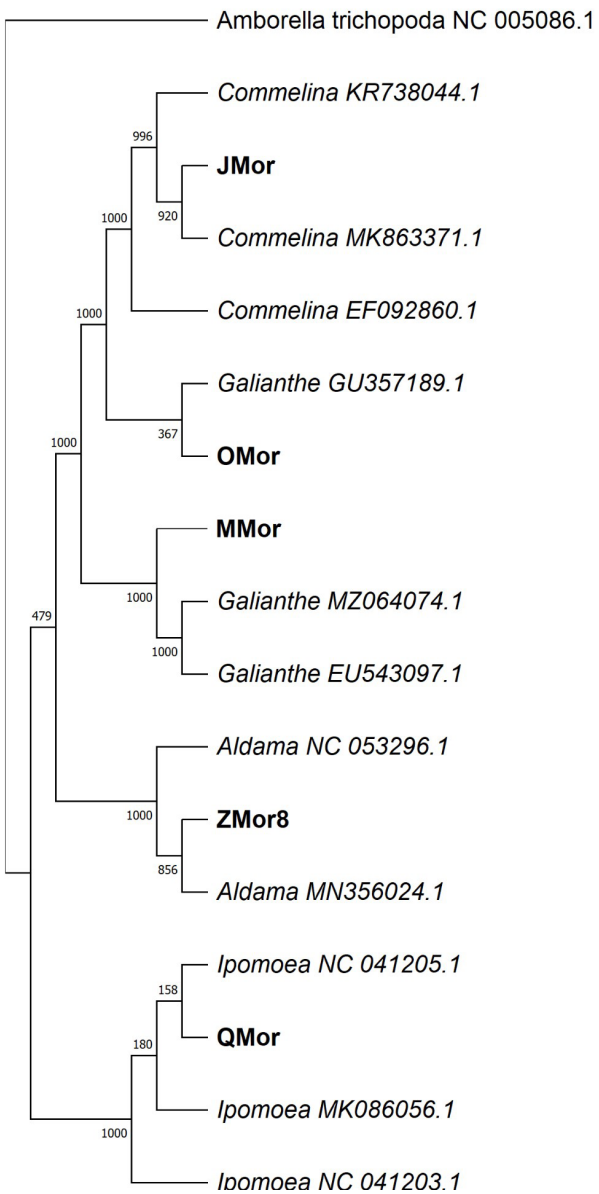
The PCR products of the *trnL* gene had a length of between 1000 and 1500 bp, which supports the information supplied by Taberlet *et al.* (1991) whose paper suggest that the expected product size could be around ~500-1050 bp depending on the species.

The *trnL* sequences firstly were registered in the GenBank of NCBI (applications code: 2761360, 2761358, 2761355, 2761284 and 2761077) and aligned with other registered using the BLAST software obtaining 10 results with an E-value of zero from which only three of these showed an identity percentage (IP) above 95% from which two belong to the *Commelina communis* species. As for MMor there are three results with an E-value of zero, which all have an IP below 86%. On the other hand, OMor sequence has more than a hundred results with an E-value of zero all with an IP above 94% that belong to various genus including *Xanthium spp., Ambrosia spp., Acmella spp.,* among others. QMor sequences equally had more than a hundred results with an E-value of zero, all of them with an IP above 96%, with most results showing a similarity to sequences of *Ipomoea spp.*

Finally, the ZMor BLAST also had more than a hundred results with an E-value of zero, all with a similarity above 98% that belong mainly to the genera *Aldama* and *Helianthus*.

As all the BLAST results were angiosperm, *Amborella trichopoda* was used as an outgroup for the phylogenetic analysis (Figure 1), as it is a representative sister lineage to all flowering plants (Albert *et al.,* 2013). The analysis of the *trnL* gene of the collected samples showed that these corresponded to different genus being JMor identified as *Commelina communis* with a bootstrap support of 92% and ZMor as *Aldama dentata* with a support of 85.6%. On the other hand, MMor and QMor could only be classified by genus as *Galianthe spp.* and *Ipomoea* spp. respectively with a bootstrap support of 100%. OMor could not be correctly grouped as the bootstrap support value for the nearest node was calculated below 50% and the next node included both *Commelina spp.* and *Galianthe spp*.

Figure 1. Phylogenetic analysis.



Glyphosate resistance has been reported in both the *Commelina* and *Ipmoea* genus in Brazil (Lucio *et al.,* 2019) and also specifically in *Commelina communis* in United States (Ulloa and Owen, 2009) supporting the possibility of glyphosate resistance presence in the analyzed samples. This is the first work describing potential glyphosate resistance of such species in Mexico and its presence on *Galianthe* genus and *Aldama dentata* species.

Commelina communis glyphosate resistance mechanisms have not been entirely determined; however, it has been reported that this genre does accumulate shikimate, showing that *EPSPS* inhibition does take place (Ulloa and Owen, 2009). Equally *Ipmoea* genus resistance mechanism has not been fully elucidated (Ribeiro *et al.,* 2015).

4 CONCLUSION

Intensive use of glyphosate-based herbicides has led to the development of glyphosate-resistant weed, which endangers the local economy. In this work the presence of such resistance in Mexico is suggested to be present in *Commelina, Ipmoea, Galianthe* and *Aldama* genus, which have not been reported in Mexico yet for the first two while for the other two there was no article suggesting such trait. Thus, this work is the first to report the potential presence of glyphosate resistance in these species in the country, suggesting that further tracking should be done to confirm the presence of glyphosate-resistant weeds and their mechanisms of resistance.

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SOBRE O ORGANIZADOR

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