Mediterranean Agronomic Institute of Chania

Department of Sustainable Agriculture

Towards a Molecular Characterization of Glyphosate-resistance in

FCKW

Acknowledgements

I express my greatest gratitude to ALLAH for helping me to finish this work.

Foremost, I would like to express my infinite thanks to my supervisor Dr. Ioannis Livieratos

Abstract

Table of contents

List of Figures

F(Figure 1. 6.) Tj -0.013 0.053133 Tw 559 TD 0 TD /F9 12 Tf [(Conyza c)16(anadensis)] TJ 0.12 Tc -1. TD [

List of Tables

CHAPTER 1

Literature review

1.1. Glyphosate – a once-in-a-century herbicide

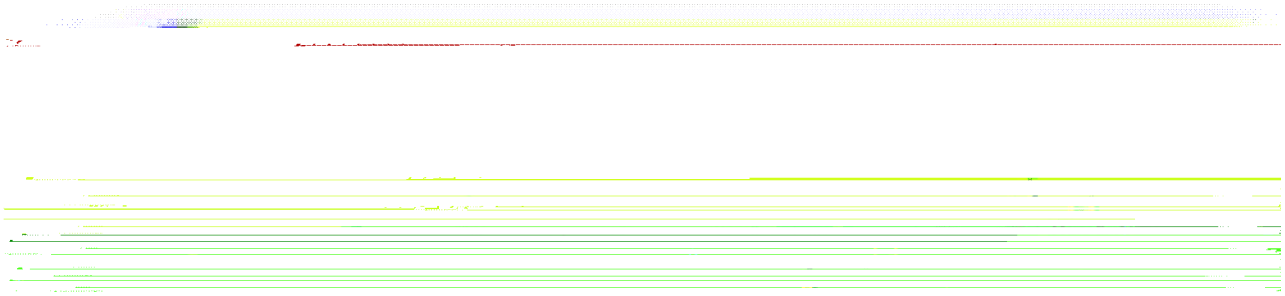
glyphosate. Glyphosate inhibits EPSPS (Steinrucken & Amrhein, 1980), resulting in the accumulation of shikimate, the dephosphorylated substrate of the enzyme (Amrhein et al., 1980).

1.2.2. Translocation of glyphosate

Glyphosate is comparatively weakly absorbed through leaves, but the amount of

Following sucrose movement, glyphosate is translocated in the phloem from the source leaves to sink tissues (Gougler & Geiger, 1984; McAllister & Haderlie, 1985). The phloem mobility of glyphosate is due to its unique combination of three acidic and one basic

Table 1. 2

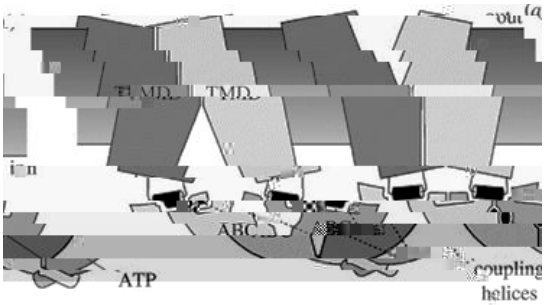


Many plant detoxifying proteins might be involved in non-target-site herbicide resistance. However, to date, participation in non-target herbicide resistance has been well

Figure 1. 3.

1.4.3.2. ABC-Transporter Mechanism

ABC transporters are active transporters, which require energy in the form of adenosine triphosphate (ATP) to translocate substrates across cell mem


TMD
ABC
ATP
coupling
helices

Herbicide metabolites have long been identiýed in plant vacuoles; finite research has linked ABC transporters with aon-target herbicide resistance in weeds. Nevertheless, ABC

Species: *Conyza canadensis* (L.) Cronquist – Canadian horseweed

Common names include Horseweed, Canadian Horseweed, Canadian Fleabane, Coltstail, Marestail and Butterweed.

1.5.2. Characteristics of *Conyza canadensis*

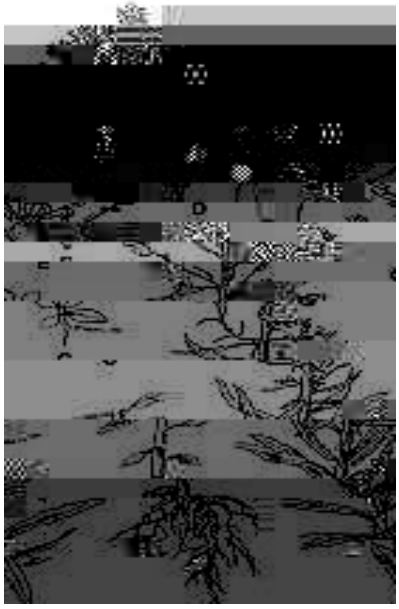


Figure 1. 6.*Conyza canadensis*

1.6. Scope of investigation

CHAPTER 2

Materials and Methods

2.1. Plant origin and glyphosate application

2.1.1. *Conyza canadensis* seed sources

Conyza canadensis seeds originating from biotypes with tested and confirmed reduced

sprayer was calibrated to deliver 50.74ml/m^2 on the working pressure of 2 bars with 70cm

3. Total RNA extraction
4. Measuring the concentration of the total RNA and purity of product
5. RT- Reverse Transcriptase - cDNA
6. PCR – amplification of the DNA
7. Electrophoresis
8. Agarose Gel Extraction
9. DNA Cloning - pDNA
10. DNA Sequencing (conserved region of the EPSPS gene containing the Proline-106 codon, known as the resistance-endowing mutation site; M10 and M11 gene sequence data)

2.2.1. Design of oligonucleotidesDNA tene pDNAgo] TJhJD ucTJ0l(

EPSPS (TIB – Molbiol Berlin)
EPSPS Conyza – F: 5’- ATGGCAGTTCACATCAACAACT -3’ 22- mer

TaKaRa LA Taq ™ (5 units/ml)	0.5 µl
2 X GC Buffer I or ll *	25 µl

DyNAzyme EXT DNA Polymerase	1 µl
Sterilized distilled water	up to 50 µl

The general reaction mixture was prepared by adding, in the following order, the following reagents: first water was added according to the volumes of the other reagents, to a total of up to 50 µl; 5 µl specific buffer, 1 µl dNTP mixture, cDNA (RT product) in the range of 1-5 µl depending on the quality of the previous results or the success of previous PCR reaction volumes, 1 µl F and R oligonucleotides, and finally 1 µl enzyme. The final PCR products were stored at -20°C. The PCR program was as follows:

No. of cycles – 34
Predenaturation: 95°C – 2 min
35 cycles: 94°C – 1min
X°C – X min*
72°C –X min**
Delay: 72°C – 10 min
Soak: 4°C – end

***** Annealing Temperature was different from one gene to the other; it was 50°C for EPSPS and M10R1 & M10R2, while for M11R1 & M11R2 it was 55°C.

** Extension time was 1 min for M10R1 & M11R1, 1.5 min for M10R2 & EPSPS and 2.5 min for M11R2.

2.2.7. Electrophoresis - Analysis of PCR products

Protocol description:

For making a 1% agarose gel, 1g agarose was mixed in a flask with 98 mL distilled water and 2mL of 50 x TAE Buffer [40 mM Tris-acetate, 1 mM ethylenediaminetetra acetic

Buffer and 3 Weiss units of T4 DNA ligase (Promega). The mixture was incubated at 4^{o}C overnight.

2.2.9.2. Bacterial transformation protocol

Materials:

- 1% (w/v) NaCl
- 1% (w/v) bacto-tryptone
- Luria-Bertani (LB) medium

2.2.9.3. Plasmid DNA purification

A single recombinant colony was grown overnight at 37°C in 3.5-4 ml LB medium supplemented with 100 εg/ml ampicillin. For each test biotype, four colonies were produced (four replications). The isolation of plasmid DNA was done using the QIAprep Spin miniprep Kit (QIAGEN) according to the manufacturer's instructions without any alteration.

2.2.9.4. Restriction endonuclease digestion of plasmid DNA

The restriction digestion of plasmid DNA was carried out in a reaction mixture containing: 5 εl of pDNA, 1.5 εl of the appropriate 10x restriction enzyme buffer, 0.6εl restriction enzya39(n.)] TJ-0.24 c 9-5.51909 TD /91 12 Tf EcoRl

2.3. Additional study- Nicotiana benthamiana M10 & M11 gene sequencing

N. benthamiana is considered a model organism for performing plaat research. The glyphosate-treated plaats were selected, harvested, and M10 aad M11 genes were amplified

2.4.2 RT-PCR

First-strand cDNA was synthesized using 2 ε

The thermal cycler was programmed as follows:

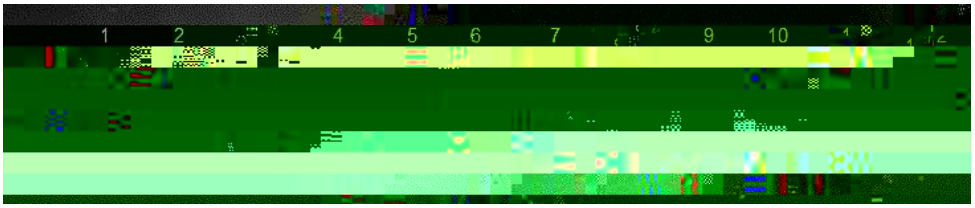
CHAPTER 3

Figure 3. 2. Electrophoresis analysis in 1% agarose gel of the *Eco*RI digestions of the EPSPS cloned products: Lane 1- molecular weight marker; Lanes 2 & 3 - EPSPS cloned product.

Comparisons of the amino acid sequences of the specific fragment were made between three Cretan *C. canadensis* biotypes, namely the glyphosate-susceptible biotype OL, the

spectrophotometry (Table 3.1) and agarose gel electrophoresis (Figure 3-4), and used as a template for the first-strand cDNA s,nthesis.

Table 3. 1



The thermal cycler was programmed in 40 cycles (Figure 3-6) as mentioned

the same between treated and untreated plants. The differences between biotypes regarding the EPSPS gene were statistically insignificant consistently implying that the EPSPS gene is not involved in the glyphosate resistance mechanism in horseweed.

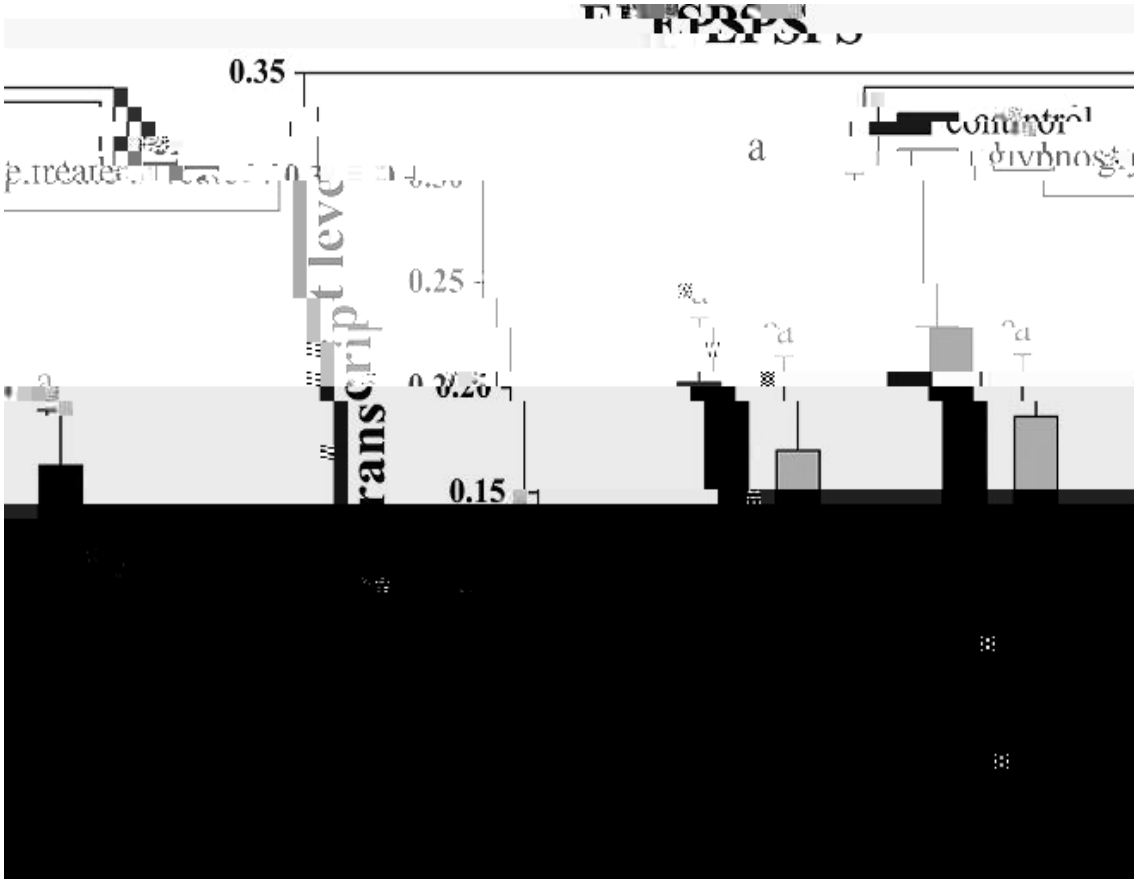


Figure 3. 7

another ABC transporter gene (M11) may also play an essential role in the glyphosate resistance mechanism of *C. canadensis*.

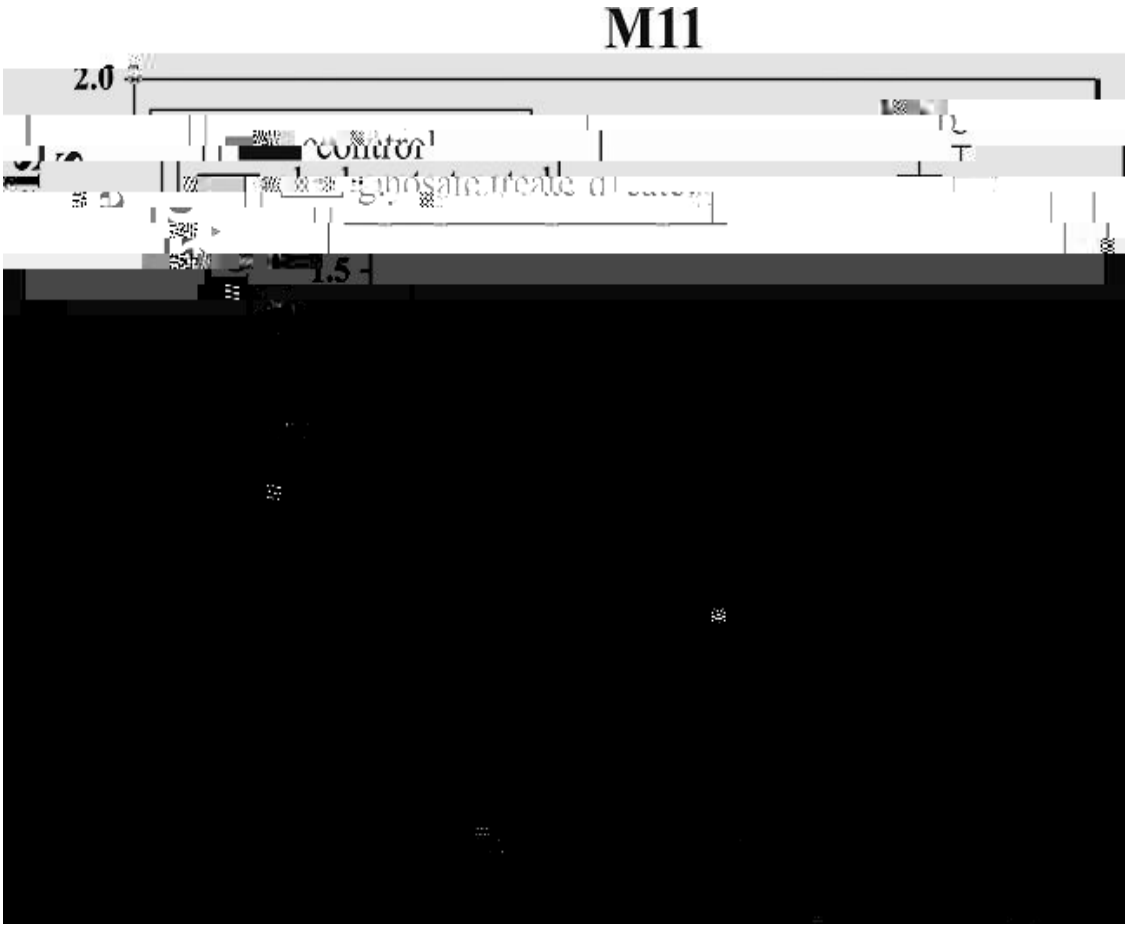


Figure 3. 9. M11 gene relative transcript levels in young leaves of treated and untreated (OL, B, L-19) biotypes of *C. canadensis*. Transcript levels in the different samples were normalized to those of the constitutive gene,

3.4. M11 partial gene amplification and sequence (C. Canadensis)

3.4.1. M11R1

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Crete, M11R1       1 ---------------------------------------------ATGC      4
                                                                    ||||
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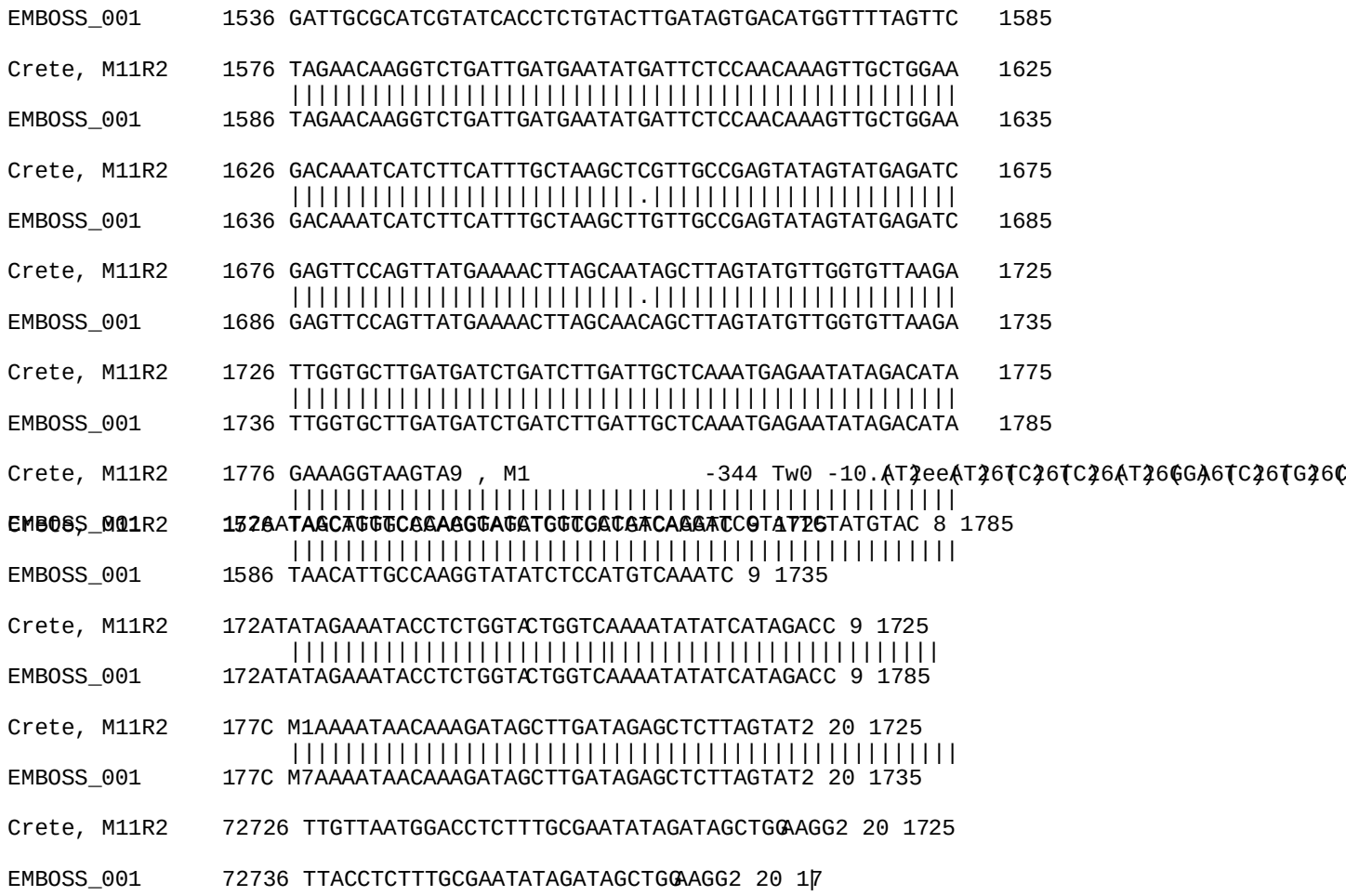
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EMBOSS_001 1586 TAGAACAAGGTCTGATTGATGAATATGATTCTCCAACAAAGTTGCTGGAA 1635
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EMBOSS_001 1636 GACAAATCATCTTCATTTGCTAAGCTTGTTGCCGAGTATAGTATGAGATC 1685
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Crete, M11R2 1726 TTGGTGCTTGATGATCTGATCTTGATTGCTCAAATGAGAATATAGACATA 1775
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Crete, M11R2 72726 TTGTTAATGGACCTCTTTGCGAATATAGATAGCTGGAAGG2 20 1725
EMBOSS_001 72736 TTACCTCTTTGCGAATATAGATAGCTGGAAGG2 20 17

3.5. M10 and M11 partial genes amplification and sequence (Nicotiana benthamiana)

3.5.1 M10-R3 gene

In order to include an additional negative control in future reverse genetics approaches, the homologous genes from *N. benthamiana* plants were amplified using oligonucleotide primers (Material and Methods, section 2.3.1.) deriv nucleotide sequences between different plant species.

we got only 393 bp from 563 bp of this gene and the obtained sequence of this biotype is presented in Appendix.

oligonucleotides (Material and Methods, section 2.3.1.) were designed for the

3.5.2. M11R1 gene

CHAPTER 4

Conclusions

The results of EPSPS protein sequence alignment between OL, B and L-19 (as sequenced in MSc Thesis: Glyphosate Resistance of *Conyza* spp. Plants in Crete, Nol Nevena, MAICh, 2010) *C. canadensis*

and M11 from *C. canadensis* and *N. benthamiana*, the last to be used as an external negative control. Nucleotide sequence alignments have proved that the correct products were amplified and cloned in qEM-T plasmids allowing future experimentation.

References

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Salazar, L.C. and Appleby, A. P. (1982).

(2006).

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Published data M11R2 Protein translation

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