Scientific Research Publishing

American Journal of Plant Sciences, 2019, 10, 2366-2381
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Variation in 5-Enolpyruvylshikimate-3-Phosphate Synthase (*EPSPS*) Coding Sequences and Glyphosate Response among *Cyperus rotundus* L. Populations

William T. Molin*, Charles T. Bryson

Crop Production Systems Research Unit, USDA-ARS, 141 Experiment Station Rd, Stoneville, MS, USA
Email: *william.molin@ usda.gov

How to cite this paper: Molin, W.T. and Bryson, C.T. (2019) Variation in 5-Enolpyruvylshikimate-3-Phosphate Synthase (*EPSPS*) Coding Sequences and Glyphosate Response among *Cyperus rotundus* L. Populations. *American Journal of Plant Sciences*, **10**, 2366-2381.
https://doi.org/10.4236/ajps.2019.1012164

Received: November 3, 2019
Accepted: December 28, 2019
Published: December 31, 2019

Copyright © 2019 by author(s) and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).
http://creativecommons.org/licenses/by/4.0/

Open Access

Abstract

The gene sequence encoding 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), the enzymatic target site of the herbicide glyphosate, was determined for several purple nutsedge (*Cyperus rotundus* L.) accessions from geographically distant locations and these were aligned to generate a consensus sequence. The *EPSPS* sequences each had single nucleotide polymorphisms (SNPs) only a few of which were predicted to cause an amino acid change in the EPSP synthase. None had the proline to serine substitution or other substitutions responsible for glyphosate resistance reported in other species. A dendrogram generated from the cluster analysis of the *EPSPS* gene sequences indicated similarities between accessions from Tanzania, Indonesia, California-2, Greece, Brazil, Argentina and Iran much like cluster analysis previously reported based on RAPD scores and morphological traits possibly indicating a common genetic background or origin. Considering the differences in *EPSPS* sequences, the response of these purple nutsedge accessions to 0.84 kg·ae·ha^{-1} of glyphosate was assessed to determine whether differential tolerance was present. At 7 days after the first application control ranged from 9% for the accession from Greece to 73% for the accession from Tanzania. Control of these accessions increased to 45% and 93% respectively by 14 days after the second application. The I_{50}'s for glyphosate inhibition of growth for four accessions from geographically distant countries (Mississippi, Brazil, Indonesia and Tanzania) were 0.21, 0.10, 0.25 and 0.06 kg·ha^{-1}, respectively, which represented a 4-fold difference. The difference in sensitivity to glyphosate may be a result of a non-target site mechanism such as differences in se-

questration, translocation or cuticle thickness rather than alterations in *EPSPS*.

Keywords

Purple Nutsedge, Glyphosate Tolerance, Genetic Diversity, 5-Enolpyruvylshikimate-3-Phosphate

1. Introduction

Purple nutsedge (*Cyperus rotundus* L.) is a perennial weed that can reduce yields in many crops by depleting soil moisture and nutrients as well as by releasing allelopathic compounds that inhibit growth of neighboring plants [1]. It has been found in 92 countries and is a problematic weed in 52 crops [1] [2] [3]. Although purple nutsedge might be expected to be genetically and morphologically diverse considering its worldwide distribution, it reproduces primarily by asexual means from tubers and rhizomes and rarely produces viable seed [4] [5]. Hence, purple nutsedge populations may be largely clonal exhibiting limited gene flow and diversity [1] [5] [6] [7]. Dormant tubers buried deep in the soil profile may escape tillage and herbicide applications, thus preserving the genetic background until favorable conditions for growth return [8]. Tubers may survive for 42 months in the soil and each can give rise to several rounds of shoot, root and rhizome formation [5] [8].

Variations among purple nutsedge populations and clonal relationships have been derived from cluster analyses of morphological traits, chemical analysis of sesquiterpenes and random amplified polymorphic DNA (RAPD) patterns with decamer primers and microsatellite DNA markers [6] [7] [9] [10] [11]. Traditional morphological taxonomy recognized eight varieties and four subspecies based on morphology [12]. Morphological variation among 34 purple nutsedge accessions from 20 countries and 14 states in the USA was reported based on plant habit, tuber and shoot morphology and floral characteristics [13]. The variations among accessions were greater for those accessions from geographically distant locations compared to those within the continental USA [13]. Although morphological variation may be an indicator of genetic diversity for taxonomic diversification, plasticity among floral characteristics in *C. esculentus* varied with growth under different environmental conditions which may skew interpretations of relatedness based on morphological data [14]. Separation of purple nutsedge accessions based on essential sesquiterpene oils rather than morphologies was also used successfully to identify four major chemotypes from Pacific Rim countries [9]. This approach was different in that it examined differences based on expression of gene products.

Cluster analysis of RAPD data indicated that a global population of purple nutsedge consisted largely of two clades consisting of 11 and 33 accessions [10].

Except for accessions identified as California-2 and Arizona, all USA accessions were clustered with accessions from Taiwan China, Western Samoa, New Zealand, Malaysia, Japan, El Salvador, Columbia, Australia, Thailand and West Indies [10]. A second cluster included accessions from Sudan, Greece, Iran, California-2, Arizona, Brazil, Argentina, Mauritius, Philippines, Indonesia and Tanzania [10]. The accessions from Sudan, Greece, Iran, Mauritius, and Tanzania were distributed along a similar longitudinal axis possibly indicating a common origin [10]. Similarly, no intraspecific variation was observed among purple nutsedge populations collected from the USA and the Caribbean region although limited variation was observed among two Brazilian and one Indian accession by RAPD analysis [6]. A high level of genetic variation among 66 Brazilian accessions was observed possibly indicating the presence of different clones [7]. Cluster analysis based on RAPD data from simple sequence repeat markers microsatellite DNA was also applied to a subset of 12 purple nutsedge accessions and one yellow nutsedge from the Wills collection [13]. Four accessions were from the USA and 8 were from outside of the USA. Yellow nutsedge was clearly separated from the purple nutsedge accessions and the purple nutsedge was divided into 3 clusters [11]. In this study, Brazil was grouped with Indonesia, Sudan was with Greece, and Taiwan China, Thailand Australia and El Salvador were grouped with the USA accessions. The use of microsatellite markers represented a new approach for separation of nutsedge accessions which may offer an additional estimation of genetic relationships to those reported with binary data from RAPDs. Taken together, although these studies confirm genetic diversity among purple nutsedge populations, a common origin could not be established.

Control of purple nutsedge in row crops was a challenging task prior to the availability of glyphosate resistant crops. Commercialization of glyphosate-resistant soybeans in 1996 and then cotton and corn shortly thereafter permitted the use of more than one postemergence glyphosate applications to which purple nutsedge was susceptible [15] [16] [17]. Considering the genetic diversity reported in purple nutsedge, the question of whether purple nutsedge accessions might differ in response to glyphosate herbicide was of interest, and if so, might differential sensitivity to glyphosate be the result of changes at the target site of glyphosate action, the EPSP synthase.

This study aimed to determine whether there were *EPSPS* sequence variations among purple nutsedge accessions collected from diverse locations around the world, and if so, were the variations conserved between neighboring or regionally close countries. Genetic evolutionary distances indicated by *EPSPS* sequences may provide a better estimate of relationships among accessions than RAPD or morphological analyses [18]. Having the gene sequences in hand afforded a means to answer the question of whether nucleotide changes were present leading to mutations such as proline 106 to serine [Pro106Ser] substitution which confers resistance to the herbicide glyphosate [19]. Similarly, the innate tolerance among purple nutsedge populations from distant geographical locations to glyphosate could also be addressed.

2. Experimental Section

2.1. Plant Sources

The Wills purple nutsedge collection which had been maintained at the USDA-ARS research station in Stoneville, MS, USA and several additional accessions collected by the first author from across the southern United States, were used in this study (Table 1). A yellow nutsedge population from Stoneville, MS was included to serve as an outlier in *EPSPS* sequencing. Accessions collected by the first author were collected 10 years after the Wills collection and followed by the number 2 (Table 1). The Wills collection followed Tropicos [20] descriptions and all accessions were determined as "*C. rotundus* L." by T. G. Koyama (The New York Botanical Gardens, 2900 Southern Blvd., Bronx, NY 10458-5126 U.S.A.) and S. McDaniel (Institute of Botanical Exploration, Box EN, Mississippi State, MS 39762 U.S.A.). Herbarium vouchers for this collection are deposited at the Mississippi Museum of Natural Science, Jackson, MS, USA and are now available through the attached SERNEC network web site [21].

Purple nutsedge was grown in 2-gallon pots containing a 4:1 (w/w) mixture of soil (Dundee silty clay loam, fine-silty, mixed thermic Aeric Ochraqualf) and Jiffy Mix (Jiffy Products of America, Batavia, IL, USA). Pots were maintained in the greenhouse at 30˚C ± 2˚C and a photoperiod of 14 h. Pots were irrigated bi-weekly and fertilized monthly with 400 mL of 1% (w/v) General Purpose Water Soluble Fertilizer (N P K) (Scotts-Sierra Horticultural Products Corp., Marysville, OH, USA).

2.2. DNA and RNA Extractions

DNA was extracted using a modified protocol from the Qiagen DNeasy Plant Mini Kit (Qiagen, Germantown, MD USA) and was extracted from the most recently emerged leaf in a shoot whorl. Briefly, 100 mg of tissue was transferred to a 2 mL microcentrifuge tube containing DNA extraction buffer (prepared as described by [22]) and a ball bearing. Tubes were sandwiched between microcentrifuge tube racks and placed in a paint shaker. Tubes were shaken for three minutes, inverted, and shaken for another three minutes. The supernatant was separated from cellular debris and the ball bearing by centrifugation at maximum speed in a microcentrifuge tube for four minutes. The supernatant was transferred to a fresh tube and the Qiagen DNeasy plant mini kit was followed beginning at step 13.

To extract RNA, a modified version of the Qiagen RNeasy plant mini kit protocol was used. First, 100 mg of tissue was transferred to 1 mL of RNALater (Invitrogen) and incubated at 4˚C for three to four hours. The tissue was transferred to a 2 mL microcentrifuge tube containing 450 µL of prepared RLT buffer and a ball bearing and macerated as described previously. The supernatant was then transferred to a QiaShredder column (Qiagen) and the Qiagen RNeasy plant mini kit protocol was followed beginning with step 4.

The DNA and RNA were quantified using the Take 3 module of a plate reader

(Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) and the quality was assessed by absorbance at 260 and 280 nm reading and gel electrophoresis.

2.3. Sequencing EPSPS

RNA and DNA were isolated from accession from Mississippi 2 and used to establish a reference sequence as described above. The *EPSPS* cDNA was amplified with primers 5'GAGATCGTGCTGCAGCCCA3' and 5'GAAGGTCTTGCGGGT GCA3' which were designed for the conserved regions of the gene, using the Verso 1-step RT-PCR Reddy Mix (ThermoFisher Scientific). Reactions contained 10 - 20 ng of RNA, 10 μM primers, 1X Reddy Mix, 1 μL Verso enzyme mix, 2.5 μL RT Enhancer, and water to 50 μL. Cycle conditions were as follows: 50˚C for 15 min, 95˚C for 2 min, 35 cycles of 95˚C for 20 s, 59˚C for 30 s, and 72˚C for 3 min, 72˚C for 5 min, and a 4˚C hold. PCR products were gel purified using the Sigma GenElute™ Gel Extraction kit (SigmaAldrich, St. Louis, MO, USA).

The gel purified PCR products were ligated into the vector at a 1:1 ratio of vector to insert using a TA® Cloning kit with pCR™ 2.1 vector (Life Technologies) according to manufacturer's instructions. TOP10 electrocompetent cells were prepared [23] with a 10% glycerol wash solution. Two microliters of ligation reaction were added to the electrocompetent cells. The cells were transferred to an electroporation cuvette and were electroporated at 2.5 keV. One milliliter of SOC medium broth was added to the cells. The cells were transferred to a 15 mL tube and incubated at 37˚C and 300 rpm for one hour. Cells were then spread on LB plates (1% tryptone, 0.5% yeast extract, 0.5 NaCl, 1.0% agar) with 50 μg/mL ampicillin and incubated at 37˚C overnight. Colonies were streaked for isolation on LB plates with 50 μg/mL ampicillin and were screened by PCR for the presence of the insert. Positive colonies were stored as glycerol stocks by adding 800 μL of an overnight culture to 200 μL of glycerol and storing at −80˚C.

Plasmids were extracted from the positive clones using the Sigma Aldrich GenElute™ Plasmid Miniprep kit. Plasmids were submitted to the Genomics and Bioinformatics Research Unit, USDA-ARS, Stoneville, MS, USA for sequencing with M13R and M13F primers. The sequence data was analyzed using Geneious [24] and BLASTn confirmed that the cloned fragments were *EPSPS*.

As the entire *EPSPS* was not sequenced, the Genome Walker Kit (Clontech) was used to obtain sequences to the ends of the gene. The kit protocol was followed, using primers 5'CAGAGAGTGCGGCAAGGAG3' and 5'CTCCTGAGA AGCTAAACATAGCAG3' in the initial PCR and 5'CAGGCAAGTTGATAGTC CCAG3' and 5'CTAGCTGCATGTGCTGAAGTG3' in nested PCR. PCR products were gel extracted, cloned, and sequenced as described above.

Primers 5'CAGGATCTACGTCTCCG3' and 5'CACATCAAAGTAATCCGG GAAG3' were designed based on the sequence obtained using the Genome Walker kit and captured most of the *EPSPS*. The Thermo Scientific Verso 1-step

RT-PCR Master mix kit was used as described above except with the new primers and for the cycle conditions the annealing temperature was reduced to 52˚C and the extension time reduced to 2 minutes. PCR products were gel extracted, cloned, and sequenced as described above. Three clones were sequenced per accession. Geneious® [24] was used to align sequences from forty-five accessions and to identify sequence differences and SNPs. Geneious® [24] was also used to generate a phenogram to estimate genetic distances among purple nutsedge accessions based the *EPSPS* sequences using the unweighted pair group method with arithmetic means.

2.4. Purple Nutsedge Response to Glyphosate Dosage

To evaluate the relative sensitivity of the accessions to glyphosate, plantlets at the two-leaf stage were transplanted from the large pots into 9 x 9 x 9 cm pots containing the same potting mix as previously described. Plants were grown to the five-leaf stage with the sixth leaf emerging and then glyphosate (Makaze (356 $g{\cdot}l^{-1}$ of glyphosate acid) Loveland Products, Inc., Loveland, CO) was applied at 0.84 $kg{\cdot}ae{\cdot}ha^{-1}$ with a pneumatic track sprayer (Generation III Research Sprayer, DeVries Manufacturing, Hollandale, MN, USA) equipped with a Teejet 8002 flat fan spray tip delivering 187 $L{\cdot}ha^{-1}$ water at 179 kPa. At 7 days after treatment (DAT), a visual estimate of percent control (0 = no effect on growth to 100 = complete kill) and a second application of glyphosate was made. Percentage control ratings were estimated 14 days after the second application. There were six replications per treatment and the experiment was conducted twice.

In the second experiment, glyphosate was applied at rates of 0, 0.01, 0.03, 0.06, 0.11, 0.23, 0.45, 0.91, 1.81, and 3.63 $kg{\cdot}ha^{-1}$ with a pneumatic track sprayer as previously described to five-leaf plants having an emerging 6th leaf to plants from Mississippi, Tanzania, Brazil and Indonesia. Induce surfactant was added to all treatments at a rate of 0.25%. These purple nutsedge were selected based on sensitivity to glyphosate in the first experiment at 7 DAT and having global distribution. At 21 DAT, above ground biomass was harvested, separated from dead tissue, dried and weighed in an oven at 60˚C for 3 days. Percentage control was calculated based on dry weight. There were three replications per treatment and the experiment was conducted twice.

Data from the whole-plant dose-response experiments were subjected to an analysis of variance using SAS 9.3 [25] to determine whether there were significant differences between repetitions. The results showed no significant differences so the data were pooled and fitted to the four-parameter nonlinear logistic-regression model presented below that was calculated using SigmaPlot 12.0 (SigmaPlot Software Inc., Chicago, IL, USA) to determine the effective dose of glyphosate to reduce growth by 50% based on fresh weight (I_{50}):

$y = C + (D - C) \Big/ 1 + \exp\left[b\left(\log\left(x - \log\left(I_{50}\right)\right)\right)\right]$, where the response is related to dose x, D is the mean response of the control, C is the mean response at very high doses, and b is the slope of the curve around the I_{50}.

3. Results

3.1. EPSPS Sequence Determination

The entire *EPSPS* gene for purple nutsedge was initially sequenced from an accession from Mississippi (GenBank Accession KM052381) (**Supplementary Figure S1**). The coding domain was 1530 bp in length and the corresponding protein was predicted to be 510 amino acids in length. BLASTn analysis revealed that the purple nutsedge *EPSPS* had 78% identity to rice (*Oryza sativa*) and goosegrass (*Eleusine indica*).

EPSPS sequences for 44 purple nutsedge accessions from 14 states, Puerto Rico, and eighteen countries were obtained using primers based on the entire *EPSPS* sequence. The *EPSPS* gene from yellow nutsedge was also sequenced (**Table 1**). Alignment of the *EPSPS* sequences for these accessions revealed some sequence variation (**Figure 1**) and these differences are presented in **Table 2**. Most notable was that both the accession from Tanzania and the yellow nutsedge accession each had an additional codon. These additional codons were located within the first 150 base pairs of sequence, the region which contains the chloroplast transit peptide sequence. In Tanzania the codon encoded an additional valine and in yellow nutsedge an arginine in the amino acid sequence. In yellow nutsedge this extra codon was only present in one of the three clones sequenced indicating that there are at least two alleles of *EPSPS* in the yellow nutsedge accession: one with the extra codon and one without (GenBank accessions KM052382, KM052383, KM052384 and KM052385). Of the purple nutsedge accessions, Tanzania exhibited the greatest number of

Table 1. Areas of origin for the accessions used in this study. California-2 was designated California* in reference 10.

Alabama	Georgia 2	North Carolina 1
Argentina	Georgia 3	North Carolina 2
Arizona	Greece	Philippines
Arkansas	Hawaii 1	Puerto Rico
Australia	Hawaii 2	South Carolina 1
Brazil	Indonesia	South Carolina 2
California 1	Iran	Sudan
California 2	Japan	Taiwan
Colombia 1	Louisiana	Tanzania
Colombia 2	Mauritius	Tennessee
El Salvador	Mississippi 1	Texas
Florida 1	Mississippi 2	Thailand
Florida 2	New Mexico 1	Western Samoa
Georgia/Alabama	New Mexico 2	West Indies
Georgia 1	New Zealand	Yellow Nutsedge (MS)

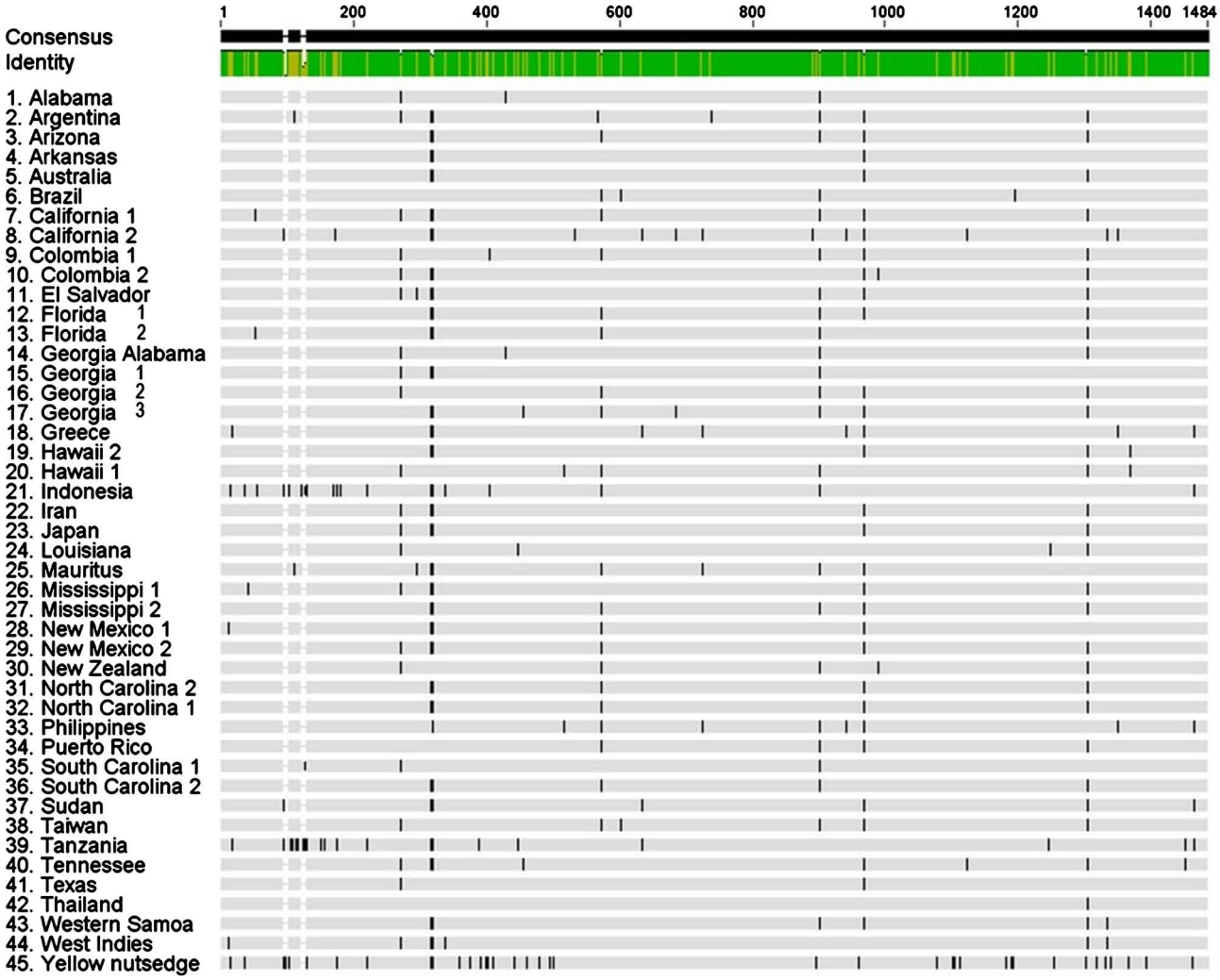

Figure 1. The alignment of partial *EPSPS* for 44 purple nutsedge accessions and yellow nutsedge. At the top of the alignment, the black line represents the consensus identity and the two gaps are due to the additional codons in the Tanzania and yellow nutsedge accessions. The green line indicates homology among accessions whereas brown gaps represent areas of nonhomologous sequence. The light gray lines represent the sequence and the darker bands represent sequence differences.

sequence differences, followed by Indonesia (Table 2).

Alignment of EPSPS sequences by accession provided information on the presence of single nucleotide polymorphisms (SNPs, Table 2). The accessions from the Philippines, California-2 and Indonesia exhibited the most SNPs. Seven of the SNPs were very common. C/T266 occurred in 15 of the accessions, T/C311 in 22, T/C314 in 23, C/T566 in 21, A/G893 in 24, A/T960 in 24, and G/C1295 in 28 accessions. Of these SNPs only two occurred together. In every sequence where T/C311 occurred, T/C314 was also present, except for the accession from the Philippines where only position T/C314 was variable. None of these common SNPs changed the amino acid sequence except for A/T960, in which case a threonine was replaced with a serine residue. Although these SNPs were not present in some accessions, that does not mean they are not there.

Table 2. Single nucleotide polymorphisms (SNPs) by nucleotide position in the *EPSPS* from purple and yellow nutsedge accessions and the corresponding amino acid change if present. The first nucleotide is the reference allele nucleotide in the consensus sequence followed by a slash (/) and the replaced nucleotide which is then followed by the numerical nucleotide position in the sequence at which it occurred (X/X###).

Accession	Polymorphism	Amino Acid Change
Alabama	C/T266, T/C422, A/G893	V/A147
Argentina	C/T109, A/G893, A/T960, G/C1295	A/V36, T/S320
Arizona	T/C311, T/C314, C/T566, A/G893, A/T960, G/C1295	T/S320
Arkansas	T/C311, T/C314, A/T960	T/S320
Australia	A/T960, G/C1295	T/S320
Brazil	C/T566, C/T596, A/G893	
California 1	T/C53, C/T266, T/C311, T/C314, C/T566, A/G893, A/T960, G/C1295	T/S320
California 2	A/G95, A/G96, C/T626, A/G679, T/C717, G/A932, A/G1112, T/C1322, G/A1340	N/D32, K/R226, E/G371, I/T441
Colombia 1	C/T266, T/C397, C/T566, A/G893, A/T960, G/C1295	V/A132, T/S320
Colombia 2	T/C311, T/C314, A/T960, A/G982, G/C1295	T/S320, E/G327
El Salvador	C/T266, T/C289, T/C311, T/C314, A/G893, A/T960, G/C1295	V/A96, T/S320
Florida 1	T/C311, T/C314, C/T566, A/G893, A/T960, G/C1295	T/S320
Florida 2	T/C53, T/C311, T/C314, C/T566, A/G893, G/C1295	
Georgia/Alabama	C/T266, T/C422, A/G893, G/C1295	
Georgia 1	C/T266, T/C311, T/C314, A/G893	
Georgia 2	C/T266, C/T566, A/G893, A/T960, G/C1295	T/S320
Georgia 3	T/C311, T/C314, T/C448, C/T566, A/G679, A/G893, G/C1295	L/P149, K/R226
Greece	C/A17, C/T717, G/A932, G/A1340, G/A1454	
Hawaii 1	C/T266, T/C509, C/T566, A/G893, G/C1295, A/G1360	D/G453
Hawaii 2	G/C1295, A/G1360	D/G453
Indonesia	A/G95, T/C122, T/C215, T/C311, T/C314, C/T332, T/C397, C/T566, A/G893, G/A1454	V/A132
Iran	C/T266, T/C311, T/C314, A/T960, G/C1295	T/S320
Japan	C/T266, T/C311, T/C314, G/C1295	
Louisiana	T/C422, G/C1295	V/A147
Mauritius	C/T109, T/C289, T/C311, T/C314, C/T566, C/T717, A/G893, A/T960	A/V36, V/A96, T/S320
Mississippi 1	C/T266, T/C311, T/C314, A/T960	T/S320
Mississippi 2	T/C311, T/C314, C/T566, A/G893, A/T960, G/C1295	T/S320
New Mexico 1	T/C12, T/C311, T/C314, C/T566, A/T960	S/P4, T/S320
New Mexico 2	C/T266, T/C311, T/C314, C/T566, A/T960, G/C1295	T/S320
New Zealand	C/T266, C/T566, A/G893, A/G982, G/C1295	T/S320, E/G327
North Carolina 1	T/C311, T/C314, C/T566, A/T960, G/C1295	T/S320
North Carolina 2	T/C311, T/C314, C/T566, A/T960, G/C1295	T/S320
Philippines	T/C314, T/C509, C/T566, C/T717, A/G893, G/A932, A/T960, G/A1340, G/A1454	T/S320
Puerto Rico	C/T566, A/G893, A/T960, G/C1295	T/S320

Continued

South Carolina 1	T/C122, C/T266, A/G893	
South Carolina 2	T/C311, T/C314, C/T566, A/G893, G/C1295	
Sudan	A/G95, A/G96, C/T626, A/T960, G/C1295, G/A1454	N/D32, T/S320
Taiwan China	C/T566, C/T596, A/G893, A/T960, G/C1295	T/S320
Tanzania	A95G, G103C, G107C, G110A, G113C, G115C, GCT codon inserted at 121, G125T, G146C, T152C, G170C, T311C, T314C, A381G, C626T, G1238A, G1454A C/A17, T/C215, T/C422, T/C1442	G34A, S38T, V between 40 and 41, K127E V/A147
Tennessee	C/T266, T/C311, T/C314, T/C448, A/T960, A/G1112, G/C1295, T/C1442	L/P149, T/S320, E/G371
Texas	A/T960	T/S320
Thailand	G/C1295	
Western Samoa	T/C311, T/C314, A/G893, A/T960, G/C1295, T/C1322	T/S320, I/T441
West Indies	T/C12, C/T266, T/C311, T/C314, C/T332, G/C1295, T/C1322	S/P4, I/T441
Yellow Nutsedge	C16A, GGA codon inserted at 96, C100G, T124C, G125T, G170C, T215C, T311C, T314C, C353G, G368T, G386A, G392C, T397C, T404C, C436A, G455A, T473C, G488T, A494G, A890G, C953T, C1070T, G1094A, A1097T, T1106A, A1175G, C1181T, C1184T, T1247C, C1294A, T1310C, T1322C, T1331A, T1358A, T1385C, G1454A	T5N, R between 30 and 31, A33G, V41A, V132A, T431K

Additional sequencing of individuals from each location may be necessary to assess the full extent to which SNPs are distributed within populations.

Cluster analysis of the *EPSPS* sequences was performed in Geneious [24] (**Figure 2**). Accessions from Brazil, Mississippi 1, Iran, Louisiana, Greece, Argentina, California-2, Indonesia and Tanzania clustered together. The US purple nutsedge accessions were less diverse compared to accessions from around the world (**Figure 2**). Most notable in the analysis was that California-2, Mississippi-1 and Louisiana clustered with accessions from Brazil, Tanzania, Greece, Iran and Sudan.

3.2. Response of Purple Nutsedge to Glyphosate

The genetic diversity in the EPSPS gene prompted the question of whether there may be differential glyphosate tolerance between accessions. Purple nutsedge accessions were initially screened for tolerance to glyphosate at 0.84 $kg \cdot ha^{-1}$. There were no glyphosate by experiment interactions for *C. rotundus* response to glyphosate so data were combined over experiments. The *C. rotundus* accessions differed in response to the first glyphosate application (**Table 3**). Percentage control ranged from 9% for the accession from Greece to 73% for the accession from Tanzania. The accession from Greece and the yellow nutsedge accessions had similar responses, although by 14 days after the second application the growth inhibition of the accession from Greece increased whereas that of yellow nutsedge was little changed. Yellow nutsedge is more tolerant of glyphosate so this result was expected. The rates of control increased following the second application of glyphosate where control increased from 42% for the low for Indonesia and to 95% for Tanzania. These results indicated there were considerable

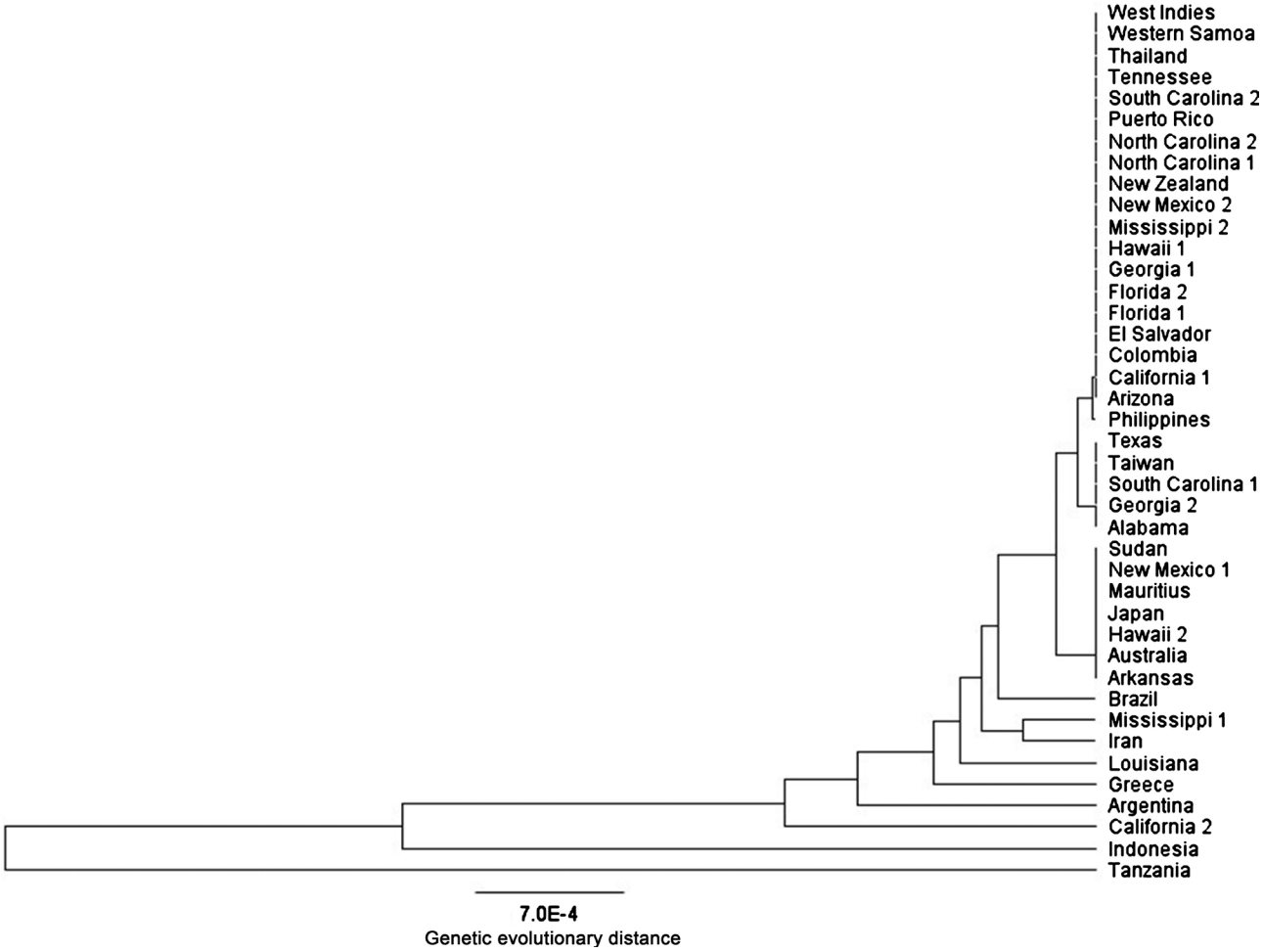

Figure 2. Cluster analysis of *EPSPS* gene sequences.

innate differences in sensitivity to glyphosate among *C. rotundus* accessions.

3.3. Glyphosate Dose Response

There was no glyphosate dose by experiment interactions, so data were combined across experiments. Dose response curves were generated by nonlinear log-logistic regression. I_{50} values for the Mississippi, Brazil, Indonesia and Tanzania accessions, based on percentage control, were 0.21, 0.10, 0.25 and 0.06 kg·ha^{-1} glyphosate, respectively. The accession from Tanzania was more sensitive to glyphosate compared to Indonesia as was observed in the fixed concentration experiment by a factor of 4 (**Figure 3**). This indicates that the Mississippi, Indonesia and Brazil accessions were less sensitive to glyphosate compared.

4. Discussion

The phylogenetic tree based on *EPSPS* sequences indicated homogeneity among many accessions which may be indicative of a common origin. The countries of Sudan, Tanzania, Iran, and Greece align along a common longitude and accessions from these regions were grouped together herein and by cluster analysis of

Table 3. Purple nutsedge response to glyphosate at 7 days after the first glyphosate application and 14 days after the second application. USA accessions followed by a 1 were collected by Wills [13] and those by a 2 were collected by the author. Plants from a yellow nutsedge population, indicated by an *, were included to demonstrate the relative glyphosate tolerance compared to purple nutsedge. Values followed by the same letter are not significantly different at $P \leq 0.05$; ns = not significant.

Accession	Control (%)	
	7 DAT	14DAT
Tanzania	73.2a	95.0ns
Iran	67.8ab	91.3ns
Texas	65.8ab	78.8ns
Puerto Rico	59.6ab	76.7ns
Georgia 1	58.5ab	85.4ns
Arizona	58.1ab	94.6ns
Hawaii 2	51.0abc	74.7ns
Alabama	50.8abc	77.3ns
North Carolina 2	49.2abc	70.4ns
Tennessee	47.8abc	73.2ns
Florida 1	45.8abc	66.3ns
Columbia	42.5bc	65.8ns
California 1	41.7bc	68.3ns
New Mexico 1	40.4bc	59.2ns
Mississippi 1	39.6bc	70.0ns
Taiwan China	38.5bc	87.6ns
New Mexico 2	38.2bc	70.5ns
Argentina	38.0bc	72.9ns
South Carolina 2	36.7bc	61.7ns
California 2	35.4bc	65.8ns
Sudan	33.3bc	61.7ns
North Carolina 1	32.1bc	57.5ns
Mississippi 2	32.1bc	57.1ns
El Salvador	30.8bc	81.7ns
Western Samoa	29.6bc	85.8ns
Hawaii 1	24.6c	54.6ns
Louisiana	26.3c	54.6ns
South Carolina 1	24.6c	61.7ns
Japan	23.9c	60.4ns
Arkansas	23.8cd	58.8ns
Indonesia	22.5cd	60.0ns
West Indies	22.5cd	63.3ns
Mauritius	22.5cd	55.0ns
Georgia 2	21.7cd	42.9ns
Florida 2	20.5cd	49.1ns
Brazil	16.3cd	43.3ns
Australia	14.5cd	41.3ns
New Zealand	12.9cd	63.1ns
Thailand	11.7d	52.5ns
Greece	9.0d	45.4ns
Yellow nutsedge	7.1*	9.6*

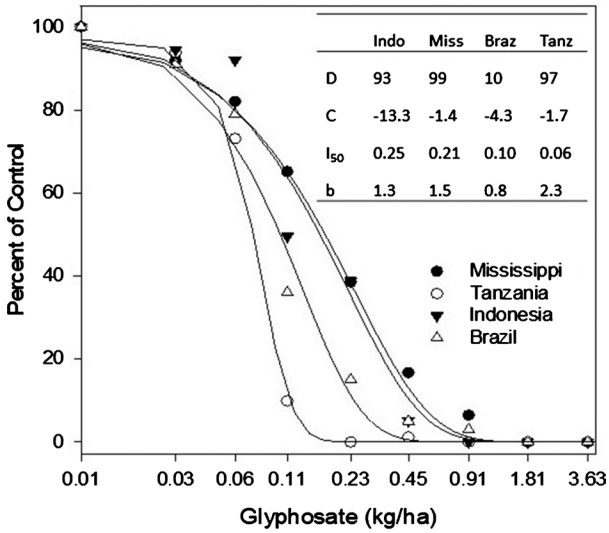

Figure 3. Dose response curves for the effect of glyphosate on the growth of purple nutsedge plants treated at the six-leaf stage of development. I_{50} (dose required to reduce plant growth by 50%) values for the Mississippi 1, Brazil, Indonesia and Tanzania accessions, based on percentage control, were 0.21, 0.10, 0.25 and 0.06 kg·ha^{-1} glyphosate, respectively. The results were fitted to the four-parameter nonlinear logistic-regression model (I_{50}): $y = C + (D - C)/1 + \exp\left[b\left(\log\left(x - \log\left(I_{50}\right)\right)\right)\right]$, where the response is related to dose x, D is the mean response of the control, C is the mean response at very high doses, and b is the slope of the curve around the I_{50}. Values are indicated in the inset table.

RAPD and morphological traits [10] [11] [13]. Many of the New World accessions clustered together as well as those from Mauritius to Hawaii. The presence of sequence differences in the *EPSPS* indicated that some measure of sexual reproduction had occurred rather than reproduction and spread based solely on tuber proliferation. These results may support previously reported separation of some accessions into sub species [12].

In studies where little or no diversity was observed, the number of populations sampled may have been too small to perceive diversity or there may have been too few primers capable of delineating accessions prior to initiating the study [7]. In order to separate the accessions by RAPD analysis 240 primers had to be screened first to find 14 primers that would provide novel RAPD products [10]. Except for accessions from California-2, Mississippi-1 and Louisiana, the variation and alignment of among US accessions were similar as estimated by *EPSPS* sequence, RAPD and morphological analysis despite accessions spanning a distance of 4000 km. Little variation among US accessions is also consistent with a recent introduction into the US from a common source.

Differential tolerance of purple nutsedge accessions to glyphosate was confirmed but was unrelated to mutations known to confer glyphosate resistance. At amino acid position 106, where a proline to serine mutation is known to cause glyphosate resistance in some species [18], a proline was present. Other known mutations known to confer resistance to glyphosate were also not present [26] [27]. Multiple SNP differences in the *EPSPS* gene sequences were found in ac-

cessions from across the US and around the world, seven of which were common among accessions, but did not confer any amino acid change. The causes of the differential response to glyphosate among this collection of accessions is unknown.

5. Conclusion

Purple nutsedge spread and proliferation were largely due to spread of clones. The genetic evolutionary distance approximation based on *EPSPS* gene sequences indicated that most populations were highly similar but there had been mutation and genetic drift among the purple nutsedge originating along the longitudinal axis from Greece south to Tanzania. For this to occur, there had to have been conditions supporting greater incidence of mutation and selection in that region of the world. These results are consistent with the findings determined by RAPD and morphological trait analysis [6] [7] [9] [10] [11]. Mutations conferring resistance to glyphosate had not occurred.

Acknowledgements

The author would like to thank Fanny Liu and Dr. Brian Scheffler of the Genomics and Bioinformatics Research Unit, USDA-ARS for assistance with Sanger sequencing, and Chris Suddoth and Collin Rounsavall for assistance with experiments on glyphosate tolerance in purple nutsedge.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Holm, L.G., Plucknett, D.L., Pancho, J.V. and Herberger, J.P. (1977) The World's Worst Weeds: Distribution, and Biology. University Press of Hawaii, Honolulu.

[2] Holm, L., Pancho, J.V., Herberger, J.P. and Plucknett, D.L. (1979) A Geographical Atlas of World Weeds. John Wiley and Sons, New York.

[3] Bendixen, L.E. and Nandihalli, U.B. (1987) Worldwide Distribution of Purple and Yellow Nutsedge (*Cyperus rotundus* and *C. esculentus*). *Weed Technology* **1**, 61-65. https://doi.org/10.1017/S0890037X00029158

[4] Thullen, R.J. and Keeley, P.E. (1979) Seed Production and Germination in *Cyperus esculentus* and *C. rotundus*. *Weed Science*, **27**, 502-505. https://doi.org/10.1017/S0043174500044489

[5] Horowitz, M. (1972) Growth, Tuber Formation and Spread of *Cyperus rotundus* L. from Single Tubers. *Weed Research*, **12**, 348-363. https://doi.org/10.1111/j.1365-3180.1972.tb01229.x

[6] Okoli, C.A.N., Shilling, D.G., Smith, R.L. and Bewick, T.A. (1997) Genetic Diversity in Purple Nutsedge (*Cyperus rotundus* L.) and Yellow Nutsedge (*Cyperus esculentus* L.). *Biological Control*, **8**, 111-118. https://doi.org/10.1006/bcon.1996.0490

[7] Pereira, W., Tessmann, D.J. and Charudattan, R. (2000) Analysis of Genetic Varia-

tion in *Cyperus rotundus* Accessions Using Molecular Markers. *Third International Weed Science Conference*, Foz do Iguassu, 47.

[8] Neeser, C., Aguero, R. and Swanton, C.J. (1997) Survival and Dormancy of Purple Nutsedge (C*yperus rotundus*) Tubers. *Weed Science*, **45**, 784-790. https://doi.org/10.1017/S0043174500088974

[9] Komai, K., Tang, C.-S. and Nishimoto, R.K. (1991) Chemotypes of *Cyperus rotundus* in Pacific Rim and Basin: Distribution and Inhibitory Activities of Essential Oils. *Journal of Chemical Ecology*, **17**, 1-8. https://doi.org/10.1007/BF00994417

[10] Molin, W.T., Kronfol, R.R., Ray, J.D., Scheffler, B.E. and Bryson, C.T. (2019) Genetic Diversity among Geographically Separated *Cyperus rotundus* Accessions Based on RAPD Markers and Morphological Characteristics. *American Journal of Plants Sciences*, **10**, 2034-2046. https://doi.org/10.4236/ajps.2019.1011143

[11] Arias, R.S., Molin, W.T., Ray, J.D., Peels, M.D. and Scheffler, B.E. (2011) Isolation and Characterization of the First Microsatellite Markers for *Cyperus rotundus*. *Weed Research*, **51**, 451-460. https://doi.org/10.1111/j.1365-3180.2011.00861.x

[12] Kükenthal, G. (1935-1936) Cyperaceae-Scirpoideae-Cypereae. In: Engler, A., Ed., *Das Pflanzenreich* 4(20) [*Heft* 101], Engelmann, Berlin, 1-671.

[13] Wills, G.D. (1998) Comparison of Purple Nutsedge (*Cyperus rotundus*) from around the World. *Weed Technology*, **12**, 491-503. https://doi.org/10.1017/S0890037X00044201

[14] Schippers, P., Ter Borg, S. and Bos, J.J. (1995) A Revision of the Infraspecific Taxonomy of *Cyperus esculentus* (Yellow Nutsedge) with an Experimentally Evaluated Character Set. *Systematic Botany*, **20**, 461-481. https://doi.org/10.2307/2419804

[15] Wang, C.-Y. (2002) Effects of Glyphosate on Tuber Sprouting and Growth of Purple Nutsedge (*Cyperus rotundus*). *Weed Technology*, **16**, 477-481. https://doi.org/10.1614/0890-037X(2002)016[0477:EOGOTS]2.0.CO;2

[16] Webster, T.M., Grey, T.L., Davis, J.W. and Culpepper, A.S. (2008) Glyphosate Hinders Purple Nutsedge (*Cyperus rotundus*) and Yellow Nutsedge (*Cyperus esculentus*) Tuber Production. *Weed Science*, **56**, 735-742. https://doi.org/10.1614/WS-07-188.1

[17] Bryson, C.T., Reddy, K.N. and Molin, W.T. (2003) Purple Nutsedge (*Cyperus rotundus*) Population Dynamics in Narrow Row Transgenic Cotton (*Gossypium hirsutum*) and Soybean (*Glycine max*) Rotation. *Weed Technology*, **17**, 805-810. https://doi.org/10.1614/WT02-177

[18] Nei, M. (1987) Molecular Evolutionary Genetics. Columbia University Press, New York. https://doi.org/10.7312/nei-92038

[19] Baerson, S.R., Rodriguez, D.J., Tran, M., Feng, Y., Biest, N.A. and Dill, G.M. (2002) Glyphosate-Resistant Goosegrass. Identification of a Mutation in the Target Enzyme 5-Enolpyruvylshikimate-3-phosphate Synthase. *Plant Physiol*ogy, **129**, 1265-1275. https://doi.org/10.1104/pp.001560

[20] Tropicos (2019) Tropicos.org. Missouri Botanical Garden, Saint Louis. http://www.tropicos.org/Name/22200058

[21] SERNEC Data Portal. http://sernecportal.org/portal/index.php

[22] Paterson, A.H., Brubaker, C.L. and Wendel, J.F. (1993) A Rapid Method for Extraction of Cotton Genomic DNA Suitable for RFLP or PCR Analysis. *Plant Molecular Biology Reports*, **11**, 122-127. https://doi.org/10.1007/BF02670470

[23] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor,

1.76-1.78.

[24] Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., *et al.* (2011) Geneious, v5.4. http://www.geneious.com

[25] (2012) Data Analysis for This Paper Was Generated Using SAS Software. SAS Institute Inc., SAS Campus Drive, Cary.

[26] García, M.J., Palma-Bautista, C., Rojano-Delgado, A.M., Bracamonte, E., Portugal, J., Alcántara-de la Cruz, R. and De Prado, R. (2019) The Triple Amino Acid Substitution TAP-IVS in the EPSPS Gene Confers High Glyphosate Resistance to the Superweed *Amaranthus hybridus*. *International Journal of Molecular Science*, **20**, 2396. https://doi.org/10.3390/ijms20102396

[27] Eschenburg, S., Healy, M., Priestman, M., Lushington, G. and Schönbrunn, E. (2002) How the Mutation glycine96 to Alanine Confers Glyphosate Insensitivity to 5-Enolpyruvyl Shikimate-3-Phosphate Synthase from *Escherichia coli*. *Planta*, **216**, 129-135. https://doi.org/10.1007/s00425-002-0908-0

Supplementary

```
1         10         20         30         40         50
|         |          |          |          |          |
ACGTCTCCGGCTCAACCCTCTCCAGATCTCGCGCCCAGGCCGTCCCCATC
GCTTCTTCGCTGCGATTTTTCAAATCGGGGTCGCTGAGGTGCAGAAATGC
GGGCTCGGCGTCGAGTAGGGGTGTGGTTGTCAGGGCTTCTGTCGCGGCTG
CTCCCGAGAAACCGTCCACGGTGCCGGAGATTGTGCTCCAGCCCATCAAG
GAGATTTCTGGGACTATCAACTTGCCTGGATCTAAATCCCTCTCCAATCG
CACGCTTCTCCTTGCCGCACTCTCTGAGGGAACAACTGTTGTGGATAACC
TGTTAAGCAGTGATGACATACACTACATGCTCGAGGCTTTGAAAACGCTT
GGCCTTTCTGTAGAGGTGGACAAAGAGGCTAAAAGGGCAGTGGTTGTGGG
GTCTGGCGGCCAGTTCCCAGCTGGTAAAGAATCAACGGAGGTGGAACTCT
TCTTGGGTAATGCTGGCACTGCTATGCGCCCTTTGACGGCTGCATTGACT
GTCGCTGGTGGAAATGGAAGTTATGTGCTTGATGGAGTCCCACGCATGAG
GGAAAGACCGATAGGCGATTTGGTTGTGGGCTTAAAACAACTTGGCGCTG
ATGTGGATTGTTTTTTGGGTACCAACTGCCCACCAGTTCGTGTTAGAGGC
ATTGGCGGCCTTCCTGGTGGAAAGGTTAAGCTGTCGGGTTCTATTAGTAG
TCAGTACTTGAGTGCATTGCTCATGGCTGGCCCACTTGCCCTAGGAGATA
TCGAAATAGAAATCATCGATAAACTAATATCTGTCCCTTACGTGGAAATG
ACCATAAAATTAATGGAACGCTTCGGTGTCAGTGTTGAGCATACTGACAG
CTGGGATCGCTTCTTCATTAAAGGTGGTCAGAAGTACAAATCACCTGGAA
AGGCTTTCGTAGAAGGTGATGCATCCAGTGCGAGTTATTTCTTGGCTGGT
GCCGCTATAACTGGCGGTACAGTCACTGTAGAAGGCTGTGGTACAGCCAG
CTTACAGGGAGATGTGAAATTTGCTGAAGTACTTGAGAAAATGGGAGCAA
AGGTTACATGGACTGAGAACAGTGTAACTGTCACTGGTCCACCGCGAGGT
TCTTCTTTCAGAGAGAAACACTTGAAAGCTGTTGATGTGAACATGAACAA
GATGCCAGATGTTGCCATGACCTTAGCAGTCGTCGCACTTTTTGCTGATG
GCCCTACTGCTATCAGAGATGTGGCTTCTTGGAGAGTGAAGGAGACTGAG
AGAATGGTTGCCATTTGCACTGAACTGAGAAAGTTGGGTGCAACGGTAGA
AGAAGGGCCTGACTATTGCATTATCACTCCTCCTGAGAAGCTAAACATAG
CAGCAATTGACACCTATGATGACCATAGAATGGCTATGACATTTTCTCTA
GCTGCATGTGCTGAAGTGCCGGTAACTATAAATGACCCTGGTTGCACACG
CAAGACCTTCCCGGATTACTTTGA
```

Figure S1. Consensus nucleotide identity sequence of purple nutsedge *EPSPS*.