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Characterization of Multiple-Herbicide-Resistant Echinochloa colona from Arkansas

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Characterization of Multiple-Herbicide-Resistant *Echinochloa colona* from Arkansas

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by

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Abstract

Echinochloa species are highly adaptive weeds that have the potential to impact crops in a variety of environments. This has positioned them as the most problematic weeds in a number of USA cropping systems with some species having the distinction of the 'worst herbicide-resistant weeds' in the world. Recent evidence has positioned *Echinochloa colona* (junglerice) as the most dominant in Arkansas and throughout the Mid-South, USA, especially in rice (*Oryza sativa L.*) and soybean (*Glycine max L.*) production fields. A history of extensive herbicide-use for management and a lack of integrated or diverse approaches to management have led to rampant herbicide resistance within production fields. The goal of this research is to assess herbicide-resistant *E. colona* from the field to the genomic level. Five objectives are the focus of this research: (1) characterize the current status of herbicide-resistant *Echinochloa* in Arkansas rice and assess the distribution of resistance patterns with time, (2) evaluate the underlying mechanisms driving multiple resistance in *E. colona* (3) assemble a *de novo* transcriptome of *E. colona* and assess the mechanisms of resistance to quinclorac, (4) use the transcriptome to characterize the response to propanil in multiple-resistant and susceptible *E. colona* and identify the basis for resistance to propanil, and (5) use the transcriptome analysis in response to multiple herbicides to identify the biological functions of susceptible and resistant *E. colona* following herbicide treatment. This research used a population that is highly resistant to propanil and quinclorac, and with elevated tolerance to cyhalofop and glufosinate. This *E. colona* accession has non-target site resistance via independent mechanisms involving cytochrome P450 enzymes and glycosyltransferase enzymes for propanil and quinclorac, respectively. Herbicide resistance co-evolved with abiotic stress tolerance potentially through the enhancement of the trehalose biosynthetic pathway. This research had generated the first assembled transcriptome of *E. colona*

and description of the transcriptomic responses to the common rice herbicides cyhalofop, propanil, and quinclorac, as well as the non-selective herbicide glufosinate. This research generated the first global transcriptome comparison across multiple herbicides, characterizing the patterns of gene expression following herbicide treatment with diverse herbicide modes of action.

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Dedication

My dissertation is dedicated to my parents, Sandra and Craig Rouse. They have been more than a support system and have never given up on me no matter how difficult I have been. They may not have always known what I am doing with my career but that has never mattered to them. Throughout my entire life they told me they didn't care if I was a farmer but I would be an educated farmer and go to college. I am not a farmer, but I have chosen to work for our farmers and make a difference through my profession as a weed scientist. Thank you.

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List of Published Papers

Rouse CE, Burgos NR, Norsworthy JK, Tseng TM, Starkey CE, Scott RC (2017) Echinochloa Resistance to Herbicides Continues to Increase in Arkansas Rice Fields. Weed Technol. https://doi.org/10.1017/wet.2017.82

Introduction

Arkansas is the leading producer of rice (*Oryza sativa L.*) and amongst the top producers of soybean in the USA. To maintain high yields with exceptional quality in the market, it is critical that the management of weedy species in crop production fields is of the highest priority. *Echinochloa* sp. are historically problematic in rice production and can persist within both lowland and upland agricultural systems. Barnyardgrass (*E. crus-galli*), in Arkansas, has been extensively investigated and targeted for management in both rice and soybean. Recently, the results of a statewide survey of rice fields revealed the predominance of junglerice (*E. colona*) as the most common *Echinochloa* species, followed by barnyardgrass and rough barnyardgrass (*E. muricata*). The re-classification of this species has not changed the management strategies, as their biology and response to control measures is the same. However, it has led to further investigation of the impact that complexes of these species have on production and more importantly evolutionary dynamics in these fields.

Herbicides are the most cost effective and widely used strategy for weed control in the state of Arkansas. Often paired with cultivation or crop rotations, herbicide-based programs are utilized in rice and soybean production with much success. These management programs have been instituted in rice and soybean rotations to manage *Echinochloa* species and continue to be the standard. In the 1950s, the first selective herbicide for *Echinochloa* control in rice, propanil, was released. To date, ten herbicides from five mode of action categories have been released, including an herbicide resistant crop technology- Clearfield® rice, which allowed for the use of the highly efficacious herbicide, imazethapyr. These compounds were released over the course of 50+ years, and were highly effective at their time of introduction. Due to their high efficacy and a lack of stewardship, these products soon became not just the capstone of a weed management

plan, but the only strategy used. The repeated and widespread use of these compounds led to the evolved resistance to the common rice herbicides: propanil, quinclorac, imazethapyr, and cyhalofop. To mitigate resistance evolution, extension and industry personnel recommend strategies including diversification of herbicide compounds and rotation to chemistries of different modes of action. Approaches such as these are effective at reducing the incidence of resistance but are still avenues for misuse or misapplication. Unfortunately, populations of *Echinochloa* throughout the state have been classified as multiple-resistant, or resistant to herbicides of two or more modes of action. The increasing presence of these populations is a concern for producers and researchers as the underlying cause of resistance has yet to be investigated and the threat of reduced efficacy to other herbicide products is of concern.

Mechanisms that enable herbicide resistance are broadly classified into two categories: target-site or non-target-site mechanisms. Target-site resistance is the modification of an herbicide site of action resulting in the reduced ability of the herbicide to interact with the target protein. This mechanism is specific to a single herbicide or group of herbicides from the same chemical family. Non-target-site mechanisms involve complex biological processes that result in either reduced herbicide activity or enhance physiological activity to allow for survival of the targeted species. This complex mechanism is not well understood and has resulted in broad resistance to herbicides from various modes of action and led to reduced efficacy to herbicides without a history of use on weed populations. *Echinochloa* populations, resistant to a single herbicide/ mode of action, in Arkansas have been identified with resistance due to both mechanisms. However, little investigation into the causal mechanisms within the multiple-resistant populations has occurred. A review of the literature reveals the necessity for investigating this type of resistance due to the complexity of the mechanism. Encouraging deeper

investigation into the biological and physiological processes that have an active role in survival. To achieve this goal, the use of next-generation-sequencing is now available to deeply probe and investigate the whole plant level response to herbicides and further detail the biological processes that are altered in resistant populations.

This research characterizes the evolution of herbicide resistance in Arkansas and provides a detailed analysis of the physiology of multiple-resistance using traditional whole plant and biochemical assays. This research has also produced the first assembled *de novo* transcriptome for multiple resistant *E. colona*. A detailed characterization of the biological networks employed by multiple-resistant *Echinochloa colona* have been described and presented. Herbicide resistance is complex and a holistic approach to understanding and interpreting the mechanisms utilized by weeds is critical for the future of weed management.

Review of Literature

Echinochloa spp.

The *Echinochloa* genus is a large group of species consisting of both beneficial and major weedy species. Species within the genus serve as a cereal grain in some countries, while in others they are major weed problems contributing to economic losses global food production [1]. Members of this genus were processed along with rice as long ago as 10,000 years, lending to their co-evolutionary adaptability and phenotypic similarities to rice [2,3]. Barnyardgrass [*Echinochloa crus- galli* (L.) Beauv] has been considered the most common and troublesome weed in Arkansas rice production [4]. Until recently, both researchers and crop consultants believed that barnyardgrass and junglerice [*Echinochloa colona* (L.) Link] were the most problematic members of the genus that impacted Arkansas rice producers [5]. Unfortunately, it is difficult to differentiate some species of the genus because of their morphologically integrating types, and much debate has occurred over their general taxonomy [6]. Following an extensive taxonomic investigation into the *Echinochloa* species from agricultural production areas throughout the southern USA, it was determined that at least five species were present and interfering in production systems throughout the south [7]. Junglerice was the most common species in agronomic crop fields, followed by rough barnyardgrass [*Echinochloa muricata* (Beauv.)], and then barnyardgrass. Unfortunately, due to the confusion in the literature, most research focuses on barnyardgrass and the other species have yet to be investigated at any considerable level.

Barnyardgrass (*Echinochloa crus-galli*)

Barnyardgrass is historically the most studied weed in rice production in the southern United States. Season-long interference of barnyardgrass can result in up to a 70% yield loss in

rice grain and as few as 52 plants m^{-2} can reduce yield by 50% [8]. Rice density, barnyardgrass density, duration of interference, nitrogen fertility, and growth habit of the rice cultivar all have an effect on how barnyardgrass will compete [9]. As a result of its widespread distribution and impacts on rice yield, a number of herbicide-based strategies have been employed to manage barnyardgrass. Propanil, and then quinclorac, were the two most effective herbicides used for barnyardgrass control in Arkansas rice production [9]. Due to continuous and widespread application, ecotypes resistant to both propanil [10] and quinclorac [11] had evolved throughout Arkansas. Alternative controls have since been instituted to manage resistant populations including acetyl-CoA carboxylase (ACCase) inhibiting herbicides and clomazone. The introduction of clomazone as a viable rice herbicide provided needed solutions for many small-seeded weeds. Clomazone provides excellent control (>90%) of barnyardgrass early in the season and sustained control (>85%) later in the season, without impacting yield [12]. A survey of crop consultants in Arkansas and Mississippi indicated that clomazone is the most recommended PRE-herbicide [5]. However, since its adoption and recurrent use in Arkansas, a population of barnyardgrass has been characterized as resistant to clomazone [13]. The introduction of imidazolinone- resistant (IR) rice technology also brought new herbicide options and programs for barnyardgrass control. As much as 90% control of barnyardgrass can be achieved utilizing sequential applications (2 weeks apart) of imazethapyr early in the rice season [14]. Imidazolinone (IMI)-resistant barnyardgrass populations have been identified in the Midsouth including in Arkansas, Louisiana, and Mississippi as well as other countries throughout the world [15,16]. Barnyardgrass has exhibited an ability to adapt to most herbicide-based management strategies.

Crop rotations are highly recommended to manage herbicide-resistant barnyardgrass populations. Rotations allow for the utilization of different herbicides with alternative modes of action that reduce the barnyardgrass infestation, delaying the evolution of resistance [9]. High yielding weed-suppressive rice lines have also been introduced with adequate control of barnyardgrass in recent years [17]. These varieties provide plenty of benefits; however, their adoption has been rather slow compared with new hybrid varieties because of the yield advantage with the latter. Barnyardgrass continues to be a major problem in mid-south rice production and new integrated strategies must be adopted to manage herbicide-resistant and problematic populations.

Junglerice (*Echinochloa colona*)

Junglerice is a major grass weed impacting rice producer's worldwide [6,18]. Little research has been conducted in the Mid-South characterizing it as a competing weed species in production systems; although Mississippi does classify it with barnyardgrass as the most common and troublesome weed in rice production [4]. Management of junglerice has been the same as with barnyardgrass, yielding similar results. Propanil-resistant populations of junglerice from rice production fields in Columbia were identified with varying levels of resistance; some populations had a resistance ratio 8.6 times greater than susceptible controls [19]. Glyphosate use in rice production is limited primarily to pre-plant burndown and to genetically modified crops used in rotation with rice. Junglerice populations resistant to glyphosate have been documented in California corn (*Zea mays L.*) fields with 6.6 times greater resistance than susceptible populations [20]. A moderately glyphosate-resistant population of junglerice was also documented in the Ord River Region of Australia where multiple crops, including cotton and rice, are produced [21].

Herbicides of Interest

Acetyl CoA Carboxylase Inhibitors

Acetyl COA carboxylase (ACCase) inhibiting herbicides are group 1 herbicides consisting of three families: aryloxyphenoxypropionate (FOPs), cyclohexanediones (DIMs), and phenylpyrazolins (DENs). Herbicides within this group inhibit the ACCase enzyme of grasses, preventing fatty acid synthesis, resulting in the limited production of phospholipids required for proper cell growth. Broadleaf species have an insensitive form of the enzyme providing tolerance to this group [22]. Cyhalofop and fenoxaprop are currently the only two ACCase herbicides registered for use in Arkansas rice production [23], however, the BASF corporation is preparing to release a new herbicide-resistant rice allowing for the application of quizalofop, another ACCase herbicide.

Due to the increasing presence of herbicide resistance in rice fields, particularly where Clearfield rice technology is used, growers are expected to increase the use of ACCase herbicides to control 'escaped' grass populations [24]. Fenoxaprop was marketed for use in rice production in 2002, followed closely with cyhalofop [9]. These two herbicides provide adequate control when applied in a program and as a tank mixture with other herbicide standards for rice weed control, with no impacts on rice yield [25]. A single application of cyhalofop (313 g ha $^{-1}$) 7 to 14 days post flood provides excellent barnyardgrass control (>90%) [26]. If the target 2-leaf growth stage of barnyardgrass is missed due to later planting or other factors, a second application (213 g ha^{-1}) within 14 days may be required for acceptable control. Observed barnyardgrass control in flooded rice culture with fenoxaprop is about 70% 10 DAT when applied alone [27].

Rice tolerance to cyhalofop is endowed by increased metabolism of the herbicide into an inactive form and a nonpolar metabolite [28]. Weed populations resistant to these herbicides usually tolerate application by target site mutations, or through herbicide detoxification by metabolism [29]. Devine [29] described target site mutations that result in the differential response of plant species to different families of ACCase inhibiting herbicides. Johnsongrass (*Sorghum halepense*) resistant to fluazifop was identified in northern Italy; resistance was due to an $Ile_{2041}Asp$ mutation [30]. This mutation also resulted in cross-resistance to other FOP herbicides. Quizalofop-resistant barnyardgrass populations were identified to have a less sensitive ACCase, but did not differentially express the enzyme, indicating a target site mutation [31]. Metabolic-based resistance to diclofop was identified in populations of *Lolium rigidum* [32]. RNAseq transcriptome analysis identified four contiguous sequences for two cytochrome P450s, one nitronate monooxygenase, and one glucosyltransferase that resulted in resistance to diclofop. As of now, resistance to ACCase herbicides in *Echinochloa* populations have not been documented in Arkansas. However, evolution modeling predicted that the co-application of ALS and ACCase herbicides in Clearfield rice will lead to resistance evolution within 14 years from the beginning of their use [24]. This has been observed previously in rice, whereby increases in ALS-resistant led to a concomitant increase in multiple resistant population to both ALS and ACCase, even with low ACCase herbicide inputs [33].

Glufosinate

Glufosinate is a phosphinic acid and member of the organophosphorus family. This is a group 10 herbicide which inhibits the glutamine synthetase (GS) enzyme, resulting in a lack of glutamine production and a buildup of toxic ammonia. Glufosinate is a nonselective foliar

applied herbicide registered for perennial fruit and nut crops, broadcast burndown applications, and as a postemergence application in genetically modified crops labeled as *LibertyLink*®.

Glufosinate is not registered for weed control during any stage of rice production, but it is labeled for use in LibertyLink® soybean; a crop grown in rotation and in close proximity to rice in Arkansas. Late season drift of glufosinate from neighboring fields is considered to be a major concern for rice producers because yield can be reduced by as much as 81% [34]. As in rice, barnyardgrass is documented as both a common and troublesome weed in Arkansas soybean production [4]. With barnyardgrass being associated with both rice and soybean, and the potential for uncontrolled overlapping populations between crops, it is important to investigate the possibility of glufosinate resistance in the *Echinochloa* populations infesting rice and soybean fields.

Transgenic crops are able to tolerate glufosinate application primarily though metabolism of glufosinate into less toxic compounds [35]. Investigation of glufosinate-resistant rice lines identified the resistance mechanisms to be metabolism and a low affinity of the GS enzyme for the herbicide molecule [36]. Glufosinate resistance in weed populations has only been documented in one species in the United States, *Lolium perenne ssp.multiflorum* (Italian ryegrass), and one species in Malaysia, *Eleusine indica* (goosegrass) [16]. Goosegrass populations found in Malaysia were also documented as multiple resistant to paraquat, another broad-spectrum herbicide [37]. Italian ryegrass populations resistant to glufosinate harbors a mutation in the GS enzyme resulting from a single site substitution of $Asp_{171}Asn$ [38]. The use of glufosinate over multiple cropping seasons may result in the build-up of herbicide-resistant populations.

Propanil

Propanil is a member of the amide family of herbicides. Members of this family are group 7 herbicides that inhibit electron transport of the photosystem II complex in the thylakoid membrane of chlorophyll cells and prevents the production of ATP, leading to the inability to produce needed compounds including proteins. Propanil does not have soil activity; therefore, it is registered for use in rice only as a postemergence treatment for control of grasses, broadleaves, and some sedges [23].

Following the introduction of propanil as the first viable and highly effective rice herbicide in 1959, propanil has been widely used throughout Arkansas [39,40]. Smith [39] first described the activity of propanil on barnyardgrass with greater than 80% control when applied at the appropriate rate and proper timing. Rice exhibited excellent tolerance to propanil, with minimal injury and no adverse effects on yield.

Rice is able to detoxify propanil through the aryl acylamidase enzyme that hydrolyzes the molecule into less harmful compounds- 3,4-dichloroaniline and propionic acid [41]. The concentration of the enzyme in the leaf tissues of rice is significantly greater than that in barnyardgrass, resulting in differential response to propanil. Propanil resistance was first identified in Arkansas in 1990; propanil at rates up to 11.2 kg ha^{-1} were unable to control barnyardgrass in a rice experiment [42]. The resistance mechanism was later identified as increased metabolism by the aryl acylamidase enzyme in barnyardgrass, the same mechanism by which rice detoxifies the herbicide [43]. Propanil resistance has since been documented in different species around the world including junglerice in Columbia and in *Cyperus difformis* L. in California [19,44]. An alternative metabolic pathway was identified in which junglerice was able to detoxify propanil through mono-oxygenase activity that reduced the molecule into similar

substrates as the aryl acylamidase enzyme [45]. Investigation into propanil-resistant *C. differmis* species of California showed that resistance is due to mechanisms (yet unknown) other than metabolism [44]. While there is information on the mechanisms to which plant species resist propanil, nothing currently describes the genetic mechanisms that induce these processes.

Quinclorac

Quinclorac is a synthetic auxin in the quinolone carboxylic acid family and a group 4 herbicide. This is a synthetic auxin that has been described and studied in multiple experiments and reviews [46,47]. Following root or shoot uptake by grass species, quinclorac induces the over production of ACC by the 1-aminocyclopropane-1-carboxylic (ACC) acid synthase enzyme; ACC is an intermediate compound for ethylene and cyanide production, thus, resulting in high levels of ethylene and cyanide which kills the plant [48]. The cyanide and subsequent ethylene production resulting from the application of quinclorac is unique to this herbicide within this mode of action. Quinclorac is registered for grass and broadleaf weed control in a number of grass crops including rice, wheat, turf, sorghum, and rangeland and has applications for fallow weed management and non-crop areas. It has both soil and foliar activity. Quinclorac controls barnyardgrass in rice, resulting in yields equal to that with propanil [42]. Quinclorac controls propanil-resistant barnyardgrass. Quinclorac is also a viable preemergence treatment for several broadleaf and other grass weed species. When applied in rice, at rates of 0.4 kg ha $^{-1}$ or higher, greater than 80% control of barnyardgrass, pitted morningglory (*Ipomoea lacunosa),* and hemp sesbania (*Sesbania herbacea)* is possible [49].

Rice tolerance to quinclorac is associated with an insensitive ACC synthase enzyme, preventing the over production of ACC and cyanide [46]. The differential response of rice varieties to quinclorac application may be the result of differential genetic controls in ethylene

pathway mediated by ACC synthase and other enzymes resulting in possible injury and yield reductions in some varieties [50]. Quinclorac-resistant barnyardgrass ecotypes were first identified in Arkansas in 1999 in Craighead County[51]. Barnyardgrass resistance to quinclorac is endowed by the insensitivity of the target site to the herbicide. Yasuor et al. [47] also investigated quinclorac resistance in the *Echinochloa phyllopogon* and identified two possible causes for resistance in the species. One mechanism was similar to previous studies and indicated an insensitive form of the enzyme. The other mechanism was identified as a P450 metabolism response in which the β- CAS activity reduces the concentration of cyanide in the plants, preventing any negative effects from the herbicide. Multiple mechanisms of resistance have been elucidated in grass weed species from different parts of the world. It is important to not only characterize the mechanism of resistance in the state of Arkansas but also understand the global implications of the nature of resistance evolution.

Herbicide Resistance

Herbicide resistance is a problem facing agriculture practitioners and researchers throughout the world. Repeated use of a single herbicide results in an unprecedented amount of selection pressure; shifting wild type and naturally susceptible weed populations to tolerant populations with evolved resistances to a given herbicide or mode of action [52]. According to the Weed Science Society of America, herbicide resistance is defined as *"the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type"*; meaning that a sensitive population has to undergo a selection process. Factors or mechanisms to which weed species resist herbicide application are classified as target-site or non-target-site [53].

Target Site Resistance

Target-site resistance involves an alteration or mutation in the genetic make-up of a herbicide target protein resulting in an altered conformation of the protein and reducing the affinity of the herbicide to its binding site [53,54]. This resistance mechanism has evolved within a number of monocot and dicot species, to almost all of the herbicide modes of action. A continuous high dose application of herbicide will exert selection pressure in favor of target site mutations endowing resistance [55]. Altered target sites have been identified in photosystem complexes, ACCase, ALS, α-tubulin, glutamine synthetase, PPO, and the 5-enolypyruvylshikimate-3-phosphate synthase (EPSPS) [54]. Some of these mutations have been discussed earlier in this proposal. The impact of target-site resistance on the competitive ability of weed species has been studied and reviewed in a number of publications [13,56–59]. Modification of an herbicide target (generally a key enzyme) usually impacts the functionality of that protein. By altering its function, the weed could incur a net loss in growth or competitive ability against sensitive biotypes when the herbicide is not being applied. Research into some biotypes of kochia (*Kochia scoparia*), indicated that the modified ALS target enzyme, endowing herbicide resistance, does not alter the competitive ability of the species and there is not a difference of biomass between resistant and susceptible biotypes [60].

Discovery of target-site mutations requires a multi-facetted approach that begins with resistance identification and ultimately the utilization of DNA-based techniques to characterize the modification of the target site. DNA-based approaches utilize single nucleotide polymorphisms (SNP's), which are most abundant in nature, to characterize substitutions in protein coding regions [61]. Following the nucleotide sequence analysis of the appropriate protein, researchers are able to understand what modification(s) in the genetic code result in

resistance evolution. As the frequency of target-site mutation increases under selection with multiple herbicide modes of action, research is needed to characterize these mutations.

Non-target Site Resistance

Non-target site resistance (NTSR) is the least understood and most unpredictable mechanism of resistance. Repeated low dose application of herbicides is believed to select for quantitative changes in the genetic composition of the plant, resulting in increased allelic frequency of non-target-site resistance-conferring mutations [55]. This mechanism may result in the ability to evolve cross and multiple resistances to all current and future herbicides [53,62]. NTSR is typically characterized as all other mechanisms that do not fall under the category of target-site mechanisms and involve mutations in multiple genes that, in concert, convey resistance to an herbicide. These mechanisms include altered compartmentation of the herbicide or its metabolites, increased metabolism of the herbicides, reduced herbicide uptake, or altered expression of the target protein. Gene amplification and altered protein expression are debated in the literature as also being target-site resistance mechanisms. Both of these will be considered NTSR mechanisms for the purposes of this research. It is the belief of the author that these mechanisms evolve through changes in non-target alleles, which result in the alteration of expression or function of the target protein and thus will be considered non-target-site mechanisms. Some of these mechanisms were described previously in this paper.

A number of genetic factors may contribute to herbicide resistance evolution. Epigenetic mutations have the potential to alter the expression of a key gene in a biochemical pathway that results in increased tolerance to an herbicide. Genes that confer tolerance to abiotic stresses (i.e. chilling, drought, flooding, heat) could also endow increased tolerance to an herbicide by interacting or actively metabolizing the compound during normal physiological processes not

related to herbicide tolerance. Environmental stress may result in DNA-methylation within the genome, modifying gene expression, endowing resistance to some herbicides [63]. These modifications to the plant genome in relation to herbicide resistance evolution have gone primarily unstudied. The potential for these plant responses and tolerance build up may be heritable and lead to resistance to herbicides that are not even in development at this point.

The competitive ability of weed species with NTSR has also been evaluated. Populations of Palmer amaranth (*Amaranthus palmeri)* from Georgia with 76-fold EPSPS gene amplification had similar biomass distribution between vegetative and reproductive organs as susceptible plants [64]. This study indicates that the plants were able to fully develop and reproduce even with the increase in enzyme production.

Delye [53] suggests a 3-step approach to identifying NTSR in a plant population: (1) collect weed genotypes and characterize their resistance profile, (2) evaluate the phenotype and the genotypic variation, (3) validate and characterize the NTSR alleles. Ultimately, the goal for proper identification of NTSR is to identify which genes are controlling the expression of other genes that eventually result in increased metabolism of an herbicide, increased expression of an herbicide target protein, reduced herbicide uptake or transport, tagging and sequestration of herbicides into vacuoles, and other plant adaptation traits. Proteins associated with NTSR can be identified using a number of methods including direct identification using proteins in mass spectrometry [65] or using RNA transcripts for quantification and analysis of expression [32]. Identification and verification of NTSR is critical for managing herbicide-resistant weed populations. The buildup of these ecotypes with the potential to resist a wide range of herbicides may become even bigger problems for agriculture producers in the future.

Next Generation Sequencing

Next generation sequencing (NGS) is an overarching term describing various strategies that rely on methods to sequence and analyze DNA templates; subsequently assembling a genome from a series of fragmented pieces [66]. The need arose for a next generation technology that would allow researchers to move beyond traditional Sanger sequencing methods, increase the speed of DNA sequencing, and also reduce its cost. There are multiple commercial technologies that are considered to be NGS technologies and all analyze a given sequence differently. Each of the technologies relies on the same series of methods to produce results: preparation of DNA template, sequencing and imaging, and analysis of the genomic data [66].

A number of different methods are used and considered NGS approaches. For NGS to be applicable in a research environment, sequencing and imaging of DNA and RNA transcripts must occur. The library or complimentary DNA (cDNA) library serves as a template or reference genome for which comparisons are to be made for characterization of the transcripts of interest. A common procedure for both the cDNA construction and transcript analysis is the use of 454 pyrosequencing. 454 pyrosequencing machines have been commercialized and are available for use at a cost. Pyrosequencing requires many different steps but can be summarized as follows: immobilization of amplified DNA on PicoTiterPlate, dNTP's are pulsed over the plate and incorporated into the building strand, their incorporation releases pyrophosphate into a solution covering the surface of the plate converting it into ATP; that ATP excites luciferase and it reacts with luciferin releasing a flash of light [66,67]. This flash of light is then analyzed based on its intensity and its cluster in the array. An alternative approach, which is employed by the Illumina sequencer, is reverse terminator sequencing. The process for the reverse terminator sequencing is summarized as follows: the sequencing primer which is complimentary to the known DNA

fragment is fixed to plate, a solution of fluorescently tagged dNTP's with DNA polymerase is passed over the plate, as the dNTP's are incorporated into the strand they release a colored flash of light which is analyzed for its sequence. Both of these methods serve as the basis for general transcript sequencing. Following the genome wide sequencing and construction of the library, this library is compared, computationally, against other sequenced genomes and specific sequences are tagged with a given function based on the compared genome.

RNA-sequencing (RNAseq) is a method that was developed to provide information about the transcriptome of an organism and transcripts of interest. The transcriptome can hold information that allows for interpretation of the functional elements of a genome, gives information about the constituents of a cell, provide an understanding of development, or even provide information on how an organism will respond to different factors [68]. In general, RNAseq requires similar steps as library construction and identification: RNA is converted into cDNA and each molecule is then sequenced in a high throughput method to obtain short sequences [68]. Following the sequencing, the fragments are aligned and the compared with a reference cDNA library for identification. The previously mentioned methods can be used to sequence the RNA transcripts.

NGS allows plant scientists, especially weed scientist which lack model species for comparion, to explore areas of the plant genome that were previously unavailable. The low cost and ease of application of NGS allows for research into non-model organisms, such as weed species, that do not have a sequenced genome. NGS technology is a viable means to study the non-model species because it does not require the complete genomic sequence for comparison and is able to reduce costs by only analyzing transcribed regions of an organism [69]. Weed scientists are only now starting to utilize this technology to investigate genomic assembly

[70,71], herbicide resistance evolution [32], and herbicide target-site gene identification [72], as well as developing genomic resource database for comparative analysis and further exploration of weediness traits [73]. The transcriptome of both herbicide-susceptible and -resistant barnyardgrass ecotypes was assembled utilizing 454 pyrosequencing [70]. This assembly allowed for successful identification of target-site and non-target-site gene groups associated with herbicide resistance. Investigation into non-target-site resistance mechanisms of diclofop-resistant *Lolium rigidum* using RNAseq successfully identified four metabolism transcripts associated with herbicide detoxification [32]. These are only a couple of examples of how NGS has advanced the understanding of herbicide resistance at a previously unknown or understood genetic level. The use of NGS will undoubtedly provide more information on the mechanisms of resistance among current resistant weed populations. Transcriptome level investigation could provide information on any future resistance risks and improve our understanding of weed evolution.

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Echinochloa Resistance to Herbicides Continues to Increase in Arkansas Rice Fields

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Abstract

Herbicide-resistant *Echinochloa spp*. pose a significant threat to USA rice production. Two surveys were conducted to characterize *Echinochloa* resistance to common rice herbicides and provide important demographic information on the populations in Arkansas: one was the *Echinochloa Herbicide Resistance Confirmation Survey* conducted annually since 2006; the other was the *Echinochloa Herbicide Resistance Demographics Survey* conducted since 2010. The *Resistance Confirmation Survey* showed that resistance to propanil (50%) was most prevalent, followed by quinclorac (23%), imazethapyr (13%), and cyhalofop (3%). Multiple resistance increased with time, with 27% of accessions being multiple-resistant, mostly to propanil + quinclorac (12%). The parallel *Resistance Demographics Survey* tested resistance by species. Of the 264 accessions collected, 73% were junglerice, 14% were rough barnyardgrass, and 11% were barnyardgrass. Overall, this survey also showed resistance to propanil (53%) and quinclorac (28%) being most prevalent, with low frequencies of resistance to cyhalofop (12%) and imazethapyr (6%). Resistance to herbicides was less frequent with barnyardgrass (54%) and rough barnyardgrass (28%) than with junglerice (73%). Multiple resistance was most frequent with junglerice (33%) and least with rough barnyardgrass (8%). Across both surveys, the resistance cases were clustered in the northeast and Grand Prairie regions of the state. Herbicide resistance among *Echinochloa* populations in rice fields is continuing to increase in frequency and complexity. This is a consequence of sequential selection with different major herbicide sites of action, starting with propanil followed by quinclorac and others.

Introduction

Globally, rice is a major agricultural commodity produced in lowland and upland cropping systems across a wide range of environments. Rice production in the United States (USA) is localized in two regions – California, in the West, and in the Midsouth. The Midsouth consists of four states including Arkansas, Mississippi, Louisiana, and Missouri. Collectively these states produce 6.6 million metric tons of rice equating to 65% of USA rice produced, and contributing $1.9 billion USD to the world market (Workman 2017, USDA ERS 2016). Arkansas consistently ranks 1st in overall production, and accounts for half the USA area and production. Arkansas producers can take advantage of several strategies to maximize production including the adoption of ideal varieties, optimal location-specific fertilizer recommendations, and flooding as a primary means to reduce weed infestation. While rice variety selection and cultural management are critical to improve production, weed management is often considered the leading factor that limits productivity.

Weed species in rice are diverse, consisting of grasses, broadleaf weeds, and sedges that can survive in aerobic or anaerobic conditions or both. Among these, the *Echinochloa* genus is the most widespread and damaging to rice yield (Danquah et al. 2002). *Echinochloa* and rice are morphologically and biologically similar. They tolerate flooded culture and co-exist under similar environments. Members of this genus have been classified consistently as primary weed problems in USA rice fields. In California, early watergrass (*E. phyllopogon*), late watergrass (*E. oryzoides*), and barnyardgrass (*E. crus-galli*) are the primary species; while in the Midsouth, barnyardgrass and junglerice (*E. colona*) are more prevalent (Fischer et al. 2000; Van Wychen 2015). Historically, barnyardgrass has been identified and ranked as the predominant weed species in Arkansas rice production fields. Season-long interference of barnyardgrass can result

in up to a 70% loss in grain yield with a 50% yield reduction from a density of 52 plants m^{-2} (Smith 1988). A recent study sought to assess the *Echinochloa* spp. present in Arkansas rice fields, identifying junglerice as the dominant species (Tahir et al. 2014). While this reclassification has not changed the recommendations for management, it does require updating the literature and the description of the impact of this species on rice production in the Midsouth.

In USA rice production, herbicides have been used since the 1950s to selectively manage *Echinochloa* and other major species including weedy rice (*Oryza sativa* L.), sprangletops (*Leptochloa* spp.), hemp sesbania (*Sesbania herbacea* [P. Mill] McVaugh), and northern jointvetch (*Aeschynomene virginica* [L.] B.S.P) (Talbert and Burgos 2007). Propanil is a photosystem II inhibitor (WSSA Group 7) introduced in 1959 with excellent control of barnyardgrass and the added benefit of hemp sesbania control (Scott 2017). In 1992, quinclorac, an auxinic herbicide (WSSA Group 4), was introduced specifically to mitigate propanil-resistant barnyardgrass with the added benefit of controlling other grasses. While these two herbicides have been the standard for rice weed control, clomazone (WSSA Group 13), cyhalofop (WSSA Group 1), and fenoxaprop (WSSA Group 1) also have been introduced for management of grasses in rice. Clearfield® technology was introduced in the early 2000s as the first non-genetically-modified, HR rice with resistance to acetolactate synthase (ALS) inhibitors (WSSA Group 2), specifically imidazolinones- imazethapyr, imazamox, and imazapic (not used in the USA). The Clearfield® rice technology improved the management of weedy rice throughout the Midsouth and also provided an additional mode of action for *Echinochloa* management. Despite crop rotation (primarily rice-soybean (*Glycine max* L. Merr) in Arkansas) and the diversity of herbicides used to manage grass weeds across both crops (Hardke 2016), resistance to herbicides has evolved.

A survey of Arkansas and Mississippi rice crop consultants conducted in 2012 (Norsworthy et al. 2013) listed barnyardgrass as the most problematic (63% of respondents), with 58% and 52% of respondents, respectively, listing propanil- and quinclorac-resistant barnyardgrass as the common problem. It should be noted that *Echinochloa* species have been collectively called barnyardgrass; thus, the term includes junglerice. Barnyardgrass with resistance to propanil, quinclorac, clomazone, and imazethapyr have been reported and documented in Arkansas rice fields beginning in the early 1990s (Heap 2017). More recently, barnyardgrass populations with multiple resistance to propanil and quinclorac, as well as a junglerice population with three-way resistance to propanil, quinclorac, and imazethapyr have been reported (Heap 2017). Worldwide, barnyardgrass and junglerice have been documented with resistance to six modes of action in 34 countries throughout a variety cropping systems (Heap 2017). Its widespread distribution and ability to evolve resistance to the diverse herbicides used for management, is a great concern to both producers and researchers.

Surveys were conducted to 1) confirm the occurrence of herbicide resistance in *Echinochloa*, 2) assess the distribution and track the evolution of resistance patterns with time, and 3) improve demographic knowledge on the *Echinochloa* populations.

Materials and Methods

The surveys conducted from 2006 to 2016 with the goal of identifying and reporting herbicide resistance will be referred to as the "*Echinochloa Herbicide Resistance Confirmation Survey*". The surveys conducted from 2010 to 2016 with the goal of characterizing the herbicide resistance profiles of common *Echinochloa* species in Arkansas, will be referred to as the "*Echinochloa Herbicide Resistance Demographics Survey*". Bioassays conducted for both surveys followed similar methodologies unless otherwise described in the following sections.

Echinochloa collection and field sampling

Rice field surveys and *Echinochloa* sampling were conducted according to Burgos (2015) as weeds began maturing during the crop season until harvest. Sampling occurred in fields reported to crop consultants or University Extension personnel as having populations that survived at least one herbicide application. For the *Resistance Confirmation Survey*, seeds were bulk-sampled per field, without discriminating among species. Samples were sent to the University of Arkansas by consultants and Extension personnel. For the *Resistance Demographics Survey*, samples were bulked by site in the field and plant morphotype. University of Arkansas Faculty led the collection of most samples for this survey. Sample size ranged from panicles of a few plants (all that existed in a small patch) to about 200 g of seed (representing a large patch of one plant type); independent samples were collected within the same field and from separate fields. Samples were placed in paper bags and allowed to dry at room temperature. When possible, field history, including crop and herbicide programs were obtained. The identity of species evaluated in the *Resistance Demographics Survey* was determined using taxonomic features, specifically the panicle structure and inflorescence features. Henceforth, each bulked sample from a field, or separate bulk samples from multiple sites in a field, will be referred to as accessions.

Herbicide resistance profiling

Major rice herbicides were used in the bioassays at field use rates, with recommended adjuvants (Tables 1 and 2). Herbicide resistance bioassays were conducted in the greenhouses at the University of Arkansas Altheimer Laboratory in Fayetteville. The greenhouses were set at 14-h daylength with supplemental lighting and maintained at a temperature of 30 to 35° C. The bioassays occurred from January to March for initial reporting. For the demographic studies, a

second run of the bioassays was conducted later in the year. Seeds were sown into pots containing a commercial potting mix with 75%-85% peat (Sungro Horticulture, Seba Beach, Canada). Each experiment contained a non-treated control for each accession and a susceptible standard. For the *Resistance Confirmation Survey* of postemergence (POST) herbicides, seedlings were thinned to 5 plants per pot within 1 wk of emergence, with each pot serving as one experimental unit and replicated twice. The response to a preemergence herbicide, clomazone, was evaluated by applying the herbicide to the surface of field soil (Captina silt loam- fine-silty, siliceous, active, mesic typic fragiudults) in which approximately 50 seeds were planted per replication. For the *Resistance Demographics Survey* involving POST herbicides, plants were thinned to 20 per pot, with each pot serving as one experimental unit, replicated three times, and the experiment was conducted twice. All herbicide applications were made in an air-propelled, motorized spray chamber calibrated to deliver 187 L ha^{-1}. Plants were sprayed when seedlings had 1 to 2 visible leaf collars. Following herbicide application, treated plants were left to dry before returning them to the greenhouse, and irrigated as necessary. Clomazone-treated pots were lightly misted following herbicide application to activate the herbicide and allowing it to percolate to the seed zone.

Data collection and analysis

Treatment effects were evaluated 21 d after herbicide application. For the *Resistance Confirmation Survey,* injury/control (0%=no injury to 100%= complete plant death) was evaluated visually. Data were averaged across runs. Accessions showing less than 70% control were classified as resistant; thus, generating a matrix of resistance confirmation across various herbicides. A description of the herbicide resistance profile is presented. For the *Resistance Demographics Survey,* the surviving plants were counted and the level of visible injury on

surviving plants was recorded (0%=no injury to 100%= plant death). Survivors (%) and injury data were averaged across replications and runs for analysis. Similar analysis was performed as described in the *Resistance Confirmation Survey*; further, cluster analysis was also performed to statistically delineate the accessions into different resistance groups, by herbicide, based on the injury (%) of survivors and frequency of surviving plants for the accession (%).

Results and Discussion

***Echinochloa* herbicide resistance confirmation survey**

A total of 450 accessions from 27 counties were tested. The rice herbicides evaluated were clomazone, cyhalofop, imazethapyr, penoxsulam, propanil, and quinclorac. Resistance to propanil was confirmed in 50% of the accessions tested and quinclorac resistance was confirmed in 23% of the accessions from 2006 to 2016 (Table 2). Resistance to clomazone or cyhalofop was rare, at 2% and 3%, respectively. Resistance to ALS inhibitors imazethapyr and penoxsulam occurred in 14 and 20% of the accessions, respectively. While both herbicides belong to Group 2, they are from different chemical families; 13% of the accessions were cross-resistant to these herbicides. From 2013 to 2016, cross resistance to ALS inhibitors increased to 18% or more of the accessions. Multiple resistance was identified each year, totaling 27% of the accessions: 37% were resistant to a single herbicide and 28% were resistant to herbicides belonging to two or more modes of action (Figure 1). Resistance to propanil or quinclorac occurred at a higher frequency (57 or 12% of accessions) than resistance to other herbicides due to their long history of use in Arkansas (Figure 2). None of the accessions were resistant to only cyhalofop; rather, resistance to cyhalofop occurred along with resistance to other herbicides, indicating an excessive selection pressure by cyhalofop after failure of other herbicides to control the *Echinochloa*. ALS-inhibitor-resistant accessions were also resistant to propanil 5% of the time

and to a lesser extent, resistant to both propanil and quinclorac (2%). Only about one-third (35%) of accessions tested were susceptible to all herbicides evaluated.

Sampling fields was nonrandom as the accessions were submitted by growers, extension personnel, or independent consultants who observed *Echinochloa* infestations in the field after herbicide applications. However, important information can be gleaned from the distribution and characterization of these accessions (Figure 3). Sixty-five of the 450 accessions submitted did not have county information; thus, could not be shown on the maps. Herbicide resistance occurs throughout the major rice-producing areas of eastern Arkansas. The highest number of accessions submitted were from Arkansas (45), Cross (23), Greene (49), Jefferson (20), Lawrence (42), Poinsett (22), and Prairie (44) counties (data not shown). Greene and Lawrence counties, located at the northeast corner of the state, had the highest number of confirmed resistance cases. Approximately 50% of the accessions in these two counties were multiple-resistant. Another area of high frequency for resistance is in the central part of the state, along the I-40 corridor, in what is collectively referred to as the Grand Prairie region. Monroe County, which had only 14 accessions submitted for testing, consistently had a higher number of accessions with cyhalofop-, propanil-, quinclorac-, and multiple resistance. To better develop an integrated and community-driven herbicide-resistance management approach, it is necessary to identify the locations with high frequencies of resistance to improve the strategies used in these areas while reinforcing effective management strategies in low resistance areas to prevent the spread of resistance.

Echinochloa herbicide resistance demographics survey

For the *Resistance Demographics Survey,* 258 accessions from 28 counties were collected (Table 3). Testing for resistance to cyhalofop, imazethapyr, propanil, and quinclorac

were prioritized in this survey because of their widespread use in rice production. Resistance to propanil and quinclorac was similar to the data from the *Herbicide Resistance Survey,* with propanil and quinclorac resistance confirmed in 53% and 28% of accessions, respectively. A higher proportion of cyhalofop-resistant accessions (12%) and a lower proportion of imazethapyr-resistant accessions (6%) were detected in this survey relative to the data from the *Resistance Confirmation Survey*. Multiple resistance was confirmed in 28% of the accessions, almost identical to that of the *Resistance Confirmation Survey*. Resistance to propanil and quinclorac was the dominant multiple-resistance profile, observed in 16% of the accessions (Figure 4a). This was followed by multiple resistance to propanil, quinclorac, and cyhalofop, which was confirmed in 5% of the accessions. Only 36% of accessions were deemed susceptible to the herbicides tested, similar to the *Resistance Confirmation Survey*.

Three primary species characterized in the *Resistance Demographics Survey* were junglerice (N=187), barnyardgrass (N=28), and rough barnyardgrass (N=36) (Figure 4). A fourth grouping is also included in the analysis (n=7) that could not be identified unequivocally and is signified as ECH. The presence of multiple *Echinochloa* species in Arkansas was reported previously, but the resistance profiling had not been done by species (Bryson and Reddy 2009; Burgos et al. 2015 Tahir et al. 2014). The survey could not determine, without bias, whether rough barnyardgrass was more common than barnyardgrass because of the relatively small sample size of these species. A more extensive survey is needed to answer this question. The resistance profile of junglerice aligned with the whole collection, showing high resistance frequency to propanil only (32%), followed by resistance to quinclorac only (6%), and multiple resistance to both herbicides being prevalent (18%) (Figure 4b). Considering that junglerice comprised 73% of the total collection, it should dictate the overall resistance pattern. Resistance

to only propanil is higher in barnyardgrass (18%) and rough barnyardgrass (8%) than to the other herbicides evaluated (Figures 4c & 4d). This is expected since propanil was the primary selector for resistance. Resistance to imazethapyr was not observed among the rough barnyardgrass accessions. Approximately 40% of the junglerice accessions were resistant only to a single herbicide, while 33% were resistant to two or more herbicides (Figure 5). Barnyardgrass accessions had similar frequencies of single resistance (29%) and multiple resistance (25%). The frequency of three-way resistance in barnyardgrass (18%) was higher than that in other species. Only three accessions of rough barnyardgrass (8%) were confirmed as multiple-resistant, which was substantially lower than for the other species. Both barnyardgrass and rough barnyardgrass had a higher frequency of susceptible individuals than junglerice.

The occurrence of resistance was concentrated in the northeast and Grand Prairie regions of the state (Figure 6). Greene (56%) and Lawrence (55%) counties had higher proportions of accessions with resistance to the four herbicides tested. Prairie County in the Grand Prairie region, had more accessions with resistance to these herbicides, with propanil- (75%) and quinclorac- (35%) resistant individuals being predominate. Multiple-resistant populations were distributed across the rice-producing regions of the state with the top four counties being Greene, Lawrence, Jackson, and Prairie (Figure 7a). In the southern region of the state, multiple resistance was detected in Ashley and Chicot counties. Junglerice was distributed evenly throughout the rice-producing regions of Arkansas, with the occurrence of multiple resistance following a similar distribution as the whole collection (Figure 7b). Higher frequencies of multiple resistance in junglerice were observed in the northeast and Grand Prairie. Barnyardgrass and rough barnyardgrass appeared to be mostly present in the northeast corner of the state; except for a few barnyardgrass observed in Ashley County. Again, the highest proportion of

accessions with multiple resistance in both species was in Greene and Lawrence counties. The data represent a relatively small nonrandom sampling of *Echinochloa* spp. populations in the state of Arkansas; thus, data should be interpreted within these limits.

For the four herbicides, the accessions separated into five distinct clusters (Table 4). Within each herbicide, the clusters were tabulated from the lowest to highest mean injury. With respect to propanil, the majority of accessions (55%) fell into clusters 1 to 3 where the average injury of survivors ranged from 4 to 51%. Cluster 1 included accessions with 21% survivors, but with negligible injury from the field use rate of propanil. Cluster 2 had the highest frequency of survivors (83%) which also had barely perceptible injury. This cluster was highly resistant to the field use rate of propanil. Twenty-six percent of accessions belonged to Cluster 3 characterized by having few survivors (4%) that incurred substantial (50%) injury. Low frequency of resistant plants in a population usually indicates an early phase of selection (Salas et al. 2016). This indicates continuing evolution of resistance to propanil because it is still being used in combination with other herbicide modes of action. Propanil resistance was reported first in 1994 among populations evaluated between 1991 and 1992 in Poinsett County, Arkansas (Baltazar and Smith 1994). Following this initial discovery, the first statewide survey revealed 16 counties with at least one propanil-resistant population (Carey et al. 1995). In 20 years since this initial description, the evolution of resistance to propanil has occurred in 28 counties within Arkansas.

Treatment with a field use rate of quinclorac placed the largest group of accessions (67/178) in cluster 5 (Table 4). This was the susceptible group, with few survivors (3%) and high injury (97%). Accessions in cluster 4 (22/178) were still susceptible as indicated by having high frequency of live plants (97%) 21 DAT, but with high injury (96%). Cluster 1 contained the most resistant accessions, with 93% of plants remaining, but with 22% injury. Resistance to

quinclorac in Arkansas was first characterized from a single population collected in 1999 from Craighead county (Lovelace et al. 2007; Malik et al. 2010). Accessions from 22 counties were confirmed resistant to quinclorac, this being the second most common resistance problem in Arkansas. The high frequency of accessions with multiple resistance to propanil and quinclorac is a concern, but not unexpected. These surveys indicated that 127 accessions were resistant to both herbicides and, while propanil resistance was high, there were a greater number of accessions resistant only to propanil than there were accessions resistant only to quinclorac. The historic use of these herbicides has undoubtedly resulted in the evolution of multiple-resistant populations (Talbert and Burgos 2007). Based on the current literature, the mechanisms of resistance to each herbicide in barnyardgrass or junglerice appear to be independent, with propanil being metabolism-based and with quinclorac being yet undetermined (Carey et al. 1997; Lovelace et al. 2007). However, new technologies have arisen since the early characterization of these populations allowing for better investigation into the molecular basis of resistance. Hence, more research is needed to elucidate the causal mechanisms.

The activity of cyhalofop, overall, was lower than for most other herbicides because cyhalofop is comparatively weaker, or inconsistent, on *Echinochloa* than propanil or quinclorac. One issue often noted by university extension personnel, and documented by Jha et al. (2010), is the poor activity of cyhalofop under drought-like environmental conditions. Pre-flood applications generally result in poor control (<50%). Cluster 5 was the largest group (119/190) composed of the most susceptible accessions (9% survivors, 97% injury). Both surveys detected low resistance frequency to cyhalofop, but the occurrence of several survivors from 10% of accessions (clusters 1 and 2) is a concern. Cyhalofop-resistant *Echinochloa spp.* had not been

reported previously in the state of Arkansas. The data indicate that it is an increasing problem in the rice-producing regions, having been confirmed in 13 counties.

Echinochloa species responded similarly to imazethapyr as they did to cyhalofop wherein the majority of accessions fell into the 5th cluster, which showed less survivors but were highly injured. Imazethapyr-resistant accessions with a high number of survivors, in cluster one, were less frequent, indicating that this herbicide is still effective in most fields. *Echinochloa* populations with cross resistance to ALS herbicides in Arkansas was first reported in 2012 from Greene and Prairie counties (Riar et al. 2012). Accessions evaluated in both surveys exhibited single- or cross resistance to ALS-inhibiting herbicides; 114 accessions across 20 counties were confirmed with ALS resistance in Arkansas. The evolution of resistance to imazethapyr coincided with the adoption and utilization of Clearfield® technology in rice. Peak Clearfield® rice production occurred in 2011 with approximately 70% of production hectarage in Clearfield® production, which declined by 5% each subsequent year (Hardke 2016). Prior to 2011, less than 10% of Arkansas *Echinochloa* submitted for testing were classified as resistant to one or both ALS herbicides. From 2013 to 2016, over 20% were identified with resistance to one of these two herbicides. In the *Resistance Demographics Survey*, most of the imazethapyr-resistant accessions were among those collected in 2011 and 2012. It is possible that in these years, selection of fields to accession was biased toward those with a history of ALS herbicide use in anticipation of resistance evolution in these fields.

Multiple-resistance evolution may occur via simple accumulation of independent target-site or non-target-site resistance mechanisms as exemplified by the occurrence of *Echinochloa* spp. with multiple NTSR mechanisms to propanil and quinclocrac (Malik et al. 2010). During field sampling in 2001 and 2002, Malik et al. (2010) reported that 76% of farmers in the counties

where fields were sampled had been using propanil for more than 20 yr and quinclorac for around 5 yr. In that sampling period, two *Echinochloa* samples were confirmed multiple-resistant to propanil and quinclorac. Resistance to propanil was documented in the early 1990s (Carey et al. 1995) and is due to enhanced detoxification by aryl acylamidase (Carey et al. 1997). Many populations were already resistant to propanil when farmers started using quinclorac. Resistance to quinclorac is due to enhanced activity of another enzyme, *β*-cyanoalanine synthase (*β*-CAS) as observed by Burgos et al. in *E. colona* (unpublished data) and by Yasour et al. (2011) in *E. phyllopogon*. However, induction of (β-CAS) accounted only for low level of resistance to quinclorac; extreme high resistance is facilitated by other CytP450 enzymes (Yasour et al. 2011), or other mechanisms yet unknown. Since resistance to quinclorac did not evolve until after about eight years of use (Talbert and Burgos 2007), resistance to quinclorac is independent from resistance to propanil. However, multiple resistance may also occur if the resistance mechanism to the first selector is mediated by NTSR genes that endow broad resistance to abiotic stressors, including herbicides. If this were the case for resistance mechanism to propanil, then quinclorac would not have been effective on the propanil-resistant populations from the beginning. Similarly, multiple resistance involving TSR + NTSR mechanisms can occur via successive or simultaneous selection. Another means of acquiring stacked resistance traits is via gene flow. This occurs quickly and is a major avenue for spread of resistance.

A predictive model was developed to estimate the potential time frame for resistance evolution to occur among Arkansas *Echinochloa* populations, given the increased adoption of ALS herbicides (Group 2) with Clearfield® rice and the use of ACCase herbicides (Group 1), including cyhalofop, for management (Bagavathiannan et al. 2014). The assumption was that resistance to each group would be by a different mechanism. With the parameters used, the

model predicted multiple resistance to ACCase (Group 1)- and ALS (Group 2) herbicides by yr 16 of adoption. Given that Clearfield® rice was commercialized in 2002 and has since been widely adopted, the surveys showed that multiple resistance to ACCase- and ALS herbicides occurred several years earlier than the model predicted. While multiple resistance to ALS- and ACCase inhibitors was identified in this research, it often occurred with other resistance traits and represented <1% of the total accessions evaluated. Coevolution of resistance to ALS- and ACCase herbicides in barnyardgrass was documented by Panozzo et al. (2013) in rice production, where multiple resistance to both herbicides was noted in low frequencies. The populations resistant to the ACCase herbicides showed low-level resistance, indicating a non-target-site, polygenic mechanism, which was not included in the model by Bagavathiannan et al. (2014). Given the criterion of the surveys at 70% injury as an indicator of resistance, it is possible that some accessions with low-level resistance to ACCase herbicide were excluded from the analysis. This evolutionary process has also been characterized in Australian populations of rigid ryegrass (*Lolium rigidum Gaudin*) where resistance to as many as three modes of action were endowed by similar degrading metabolic enzymes (Preston et al. 1996; Owen et al. 2007). A multiple-resistant population of prostrate pigweed (*Amaranthus blitoides S. Wats.)* was characterized as having a mutation in two herbicide target sites , one in the ALS enzyme and one in the *psbA* gene for the photosystem I complex, endowing resistance to ALS inhibiting herbicides and atrazine, respectively (Sibony and Rubin 2003). The selection of these mutations in two target sites could occur simultaneously if both herbicides are used sequentially in a cropping season or in tank mixes. More research needs to be done to understand the process of coevolution of resistance traits as it poses a much larger threat to crop production than independent evolution of single resistance traits.

Herbicide resistance frequency and distribution provides insight into management of problematic weed species and on the evolution of resistance within a species. This research presents the trend in resistance evolution to multiple herbicides and characterization of multiple resistance in Arkansas *Echinochloa* populations. The Weed Science Society of America has outlined best management practices (BMPs) that focus on reducing the evolution of resistance and recommend effective strategies for improving sustainable weed control (Norsworthy et al. 2012). Among these recommendations is the use of alternate effective modes of action to extend the efficacy of herbicides and reduce or delay the evolution of resistance. Given that Arkansas rice producers have at least five modes of action to integrate in weed management programs, the potential for herbicide resistance evolution should be minimized. However, *Echinochloa spp*. in Arkansas have evolved resistance to all major herbicides and modes of action currently used in rice production. The distribution of resistance is widespread and appears to be concentrated heavily in the northeast and Grand Prairie regions of the state, which have been the leading rice production areas. Given the presence of single-, multiple-, and cross resistance, growers can still manage problematic species by using a combination of herbicides and increasing rotation to other crops such as soybean. While this research provides information on the status of resistance, it provides no information on the genetic or physiological mechanisms that endow resistance. Further research is required to improve our understanding of the underlying mechanisms, which allow *Echinochloa* spp. to adapt to diverse abiotic stressors such as herbicide application.

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Tables and Figures

Table 1. Herbicide common name, trade name, application rate, timing, and adjuvant (if necessary) used in the *Echinochloa Herbicide Resistance Confirmation Survey* from 2006 to 2016 and the *Echinochloa Herbicide Resistance Demographic Survey* from 2010 to 2016.

Common Name	Trade Name	Application Timing[a]	Application Rate	Adjuvant[b]	Survey[c]
			g ha^{-1}	v/v	
clomazone	Command 3ME®	PRE	336	-	Confirmation
cyhalofop	Clincher®	POST	314	1% COC	Both
imazethapyr	Newpath®	POST	110	0.25% NIS	Both
penoxsulam	Grasp SC®	POST	49	0.25% NIS	Confirmation
propanil	Riceshot®	POST	4500	-	Both
quinclorac	FacetL®	POST	560	1% COC	Both

[a] Application timings: PRE= following planting; POST= 2 to 3 leaf *Echinochloa*
[b] Adjuvant: NIS= nonionic surfactant, Induce ®; COC= crop oil concentrate, Agridex ®
[c] Indicated the survey in which the herbicide was included for screening: Both= included in both surveys; Confirmation= included in the *Echinochloa Herbicide Resistance Confirmation Survey*

Table 2. Herbicide resistance profile of Arkansas *Echinochloa spp.* accessions from in the *Echinochloa Herbicide Resistance Confirmation Survey* from 2006 to 2016 treated with common rice herbicides.

Sampling year	No. Acc.	Proportion of resistant accessions						Cross –resistant, ALS	Multiple-resistant	Susceptible
		Clomazone	Cyhalofop	Imazethapyr	Penoxsulam	Propanil	Quinclorac			
		---------- % ----------								
2006	20	-	-	-	-	50	30	-	20	40
2007	18	6	-	-	-	44	28	-	22	44
2008	23	4	-	4	4	52	17	4	22	48
2009	18	-	-	6	6	11	17	6	11	83
2010	106	-	-	6	8	42	27	2	18	48
2011	22	-	-	-	-	73	23	-	23	27
2012	11	-	-	-	-	100	36	-	36	-
2013	26	-	-	35	35	38	23	27	31	27
2014	40	3	13	25	25	68	35	20	48	23
2015	100	4	6	27	27	72	7	26	33	19
2016	66	-	2	18	18	18	30	18	30	35
Total[a]	***450***	***2***	***3***	***14***	***20***	***50***	***23***	***13***	***27***	***35***

[a] Total percentage of accessions with resistance to the respective herbicide, based on the total number of accessions from 2006 to 2016.

Table 3. Herbicide resistance to common rice herbicides of Arkansas *Echinochloa* spp. accessions profiled in the *Echinochloa Herbicide Resistance Demographics Survey* from 2010 to 2016.

Sampling year	Acc.	Proportion of resistant accessions				Multiple-resistant	Susceptible
		Cyhalofop	Imazethapyr	Propanil	Quinclorac		
	N	%					
2010	83	14	2	81	29	33	19
2011	18	0	22	50	11	17	28
2012	24	8	13	58	54	46	17
2013	69	14	7	26	26	20	58
2014	56	11	4	43	20	21	52
2015-2016	8	0	0	75	63	63	0
Total[b]	***258***	***12 (30)***	***6 (16)***	***53 (138)***	***28 (73)***	***28 (72)***	***36 (94)***

[b] Total percentage of the accessions with resistance to the respective herbicide from the total number of collections from 2006 to 2016; numbers in the parenthesis indicated the number of accessions with resistance to the respective herbicides

Table 4. Cluster analysis summary for the four common rice herbicides evaluated in the *Echinochloa Herbicide Resistance Demographics Survey* 2010 to 2016.

Herbicide	Cluster	Injury	Survivors	No. Accessions	Accessions by species		
					E. colona	*E. crus-galli*	*E. muricata*
		%					
cyhalofop	1	51	96	10	6	3	1
	2	59	14	9	7	2	0
	3	76	67	26	22	0	4
	4	83	37	26	22	1	2
	5	97	9	119	86	13	17
imazethapyr	1	29	22	5	5	0	0
	2	71	52	21	11	5	5
	3	92	79	18	13	2	3
	4	92	26	28	19	6	3
	5	99.5	1	48	34	3	11
propanil	1	4	21	20	18	2	0
	2	7	83	26	20	4	1
	3	51	4	43	32	5	6
	4	57	70	44	35	4	4
	5	96	5	30	21	3	6
quinclorac	1	22	93	36	33	2	1
	2	62	65	26	23	3	0
	3	81	29	27	20	4	3
	4	96	97	22	12	3	7
	5	98	1	67	51	6	8

Figure 1. Frequency (%) of *Echinochloa* accessions showing different resistance profile categories, collected from Arkansas rice fields, and tested in the *Echinochloa Herbicide Resistance Confirmation Survey* 2006 to 2016.

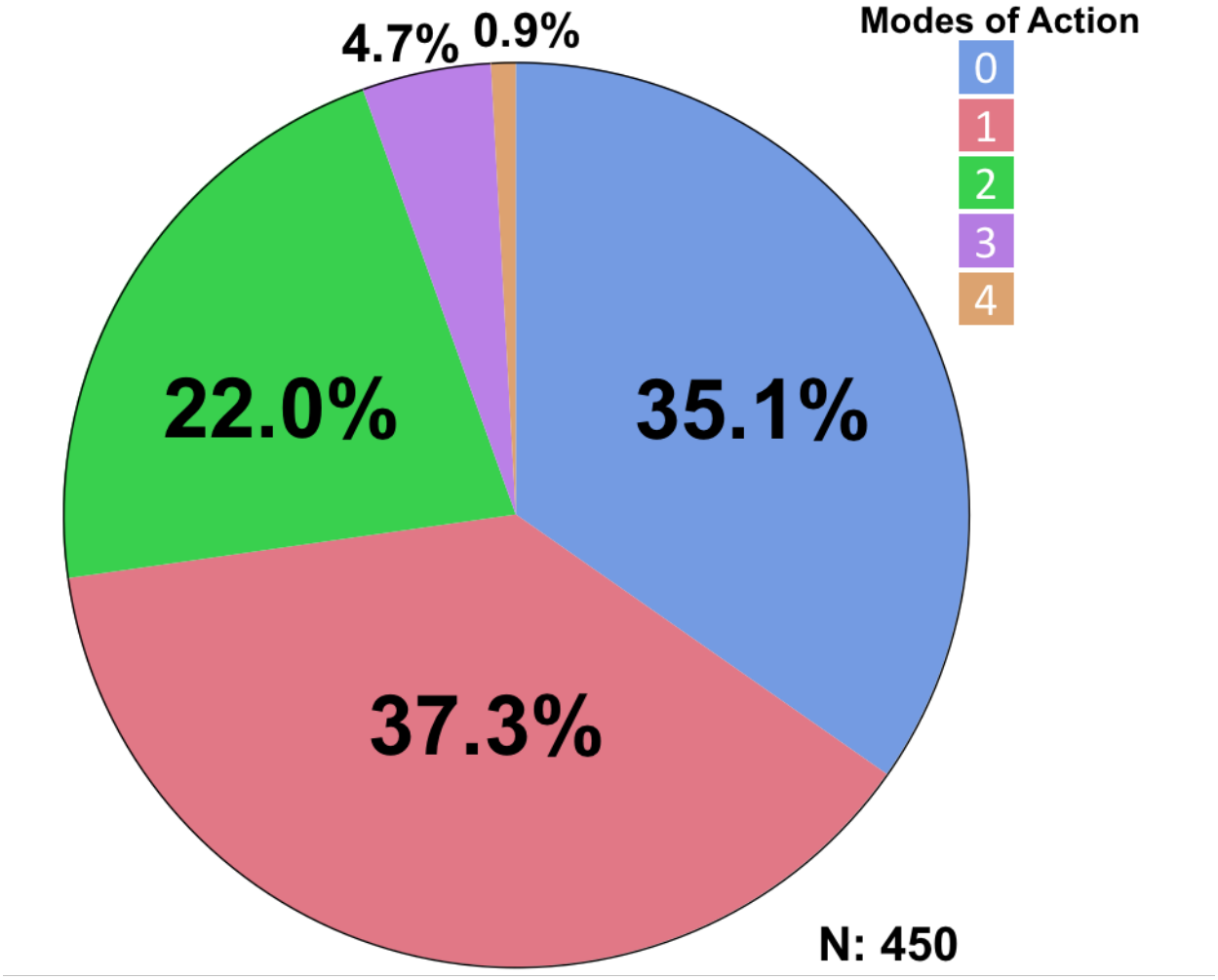


Figure 2. Number of *Echinochloa* accessions with resistance to common rice herbicides used in Arkansas, collected from Arkansas rice fields, and tested in the *Echinochloa Herbicide Resistance Confirmation Survey* from 2006 to 2016. Each oval represents one herbicide. Overlapping ovals indicate that the accessions within a given group are multiple-resistant to the respective herbicides. The oval for ALS herbicides contains the number of accessions with cross resistance to both imazethapyr and penoxsulam.

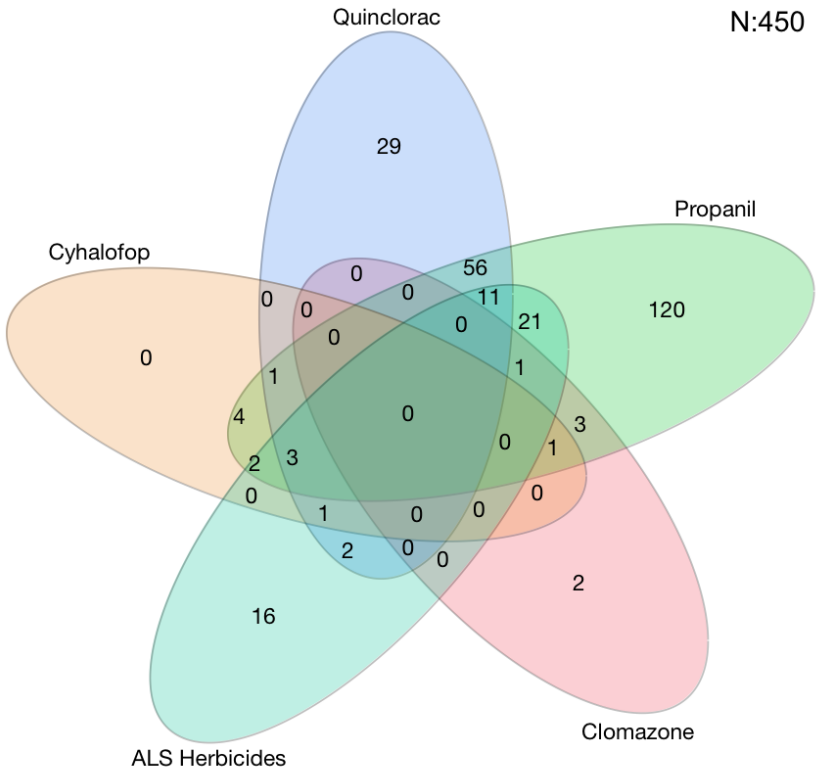


Figure 3. Arkansas maps showing the distribution of the accessions of *Echinochloa* spp. resistant to five common rice herbicides from the *Echinochloa Herbicide Resistance Confirmation Survey* 2006 to 2016.

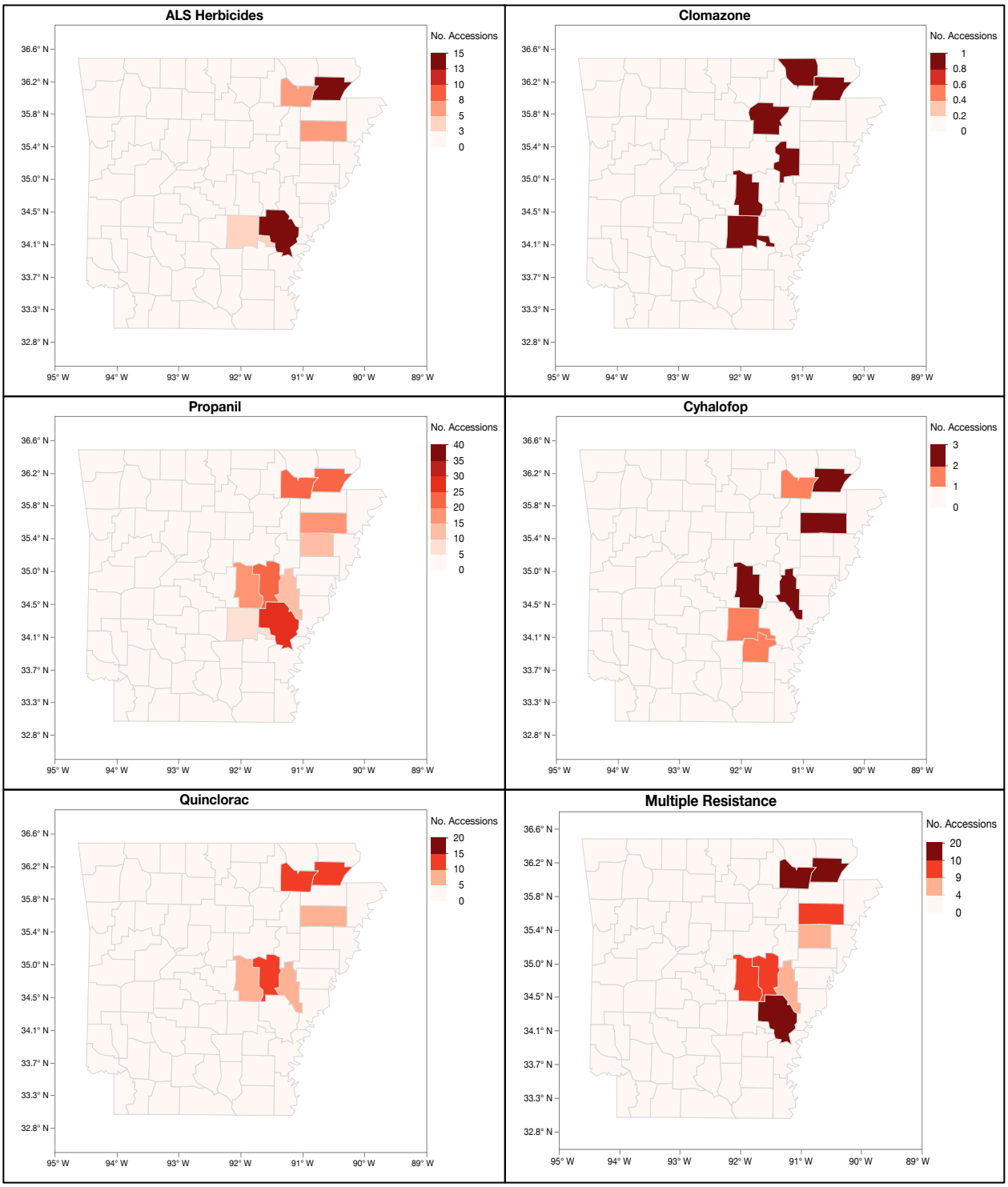


Figure 4. Number of *Echinochloa* spp. accessions with resistance to the 4 most common rice herbicides used in Arkansas tested in the *Echinochloa Herbicide Resistance Demographics Survey* from 2010 to 2016. (A) All *Echinochloa spp.* accessions; (B) junglerice (*E. colona)*; (C) barnyardgrass (*E. crus*-galli); (D) rough barnyardgrass (*E. muricata*).

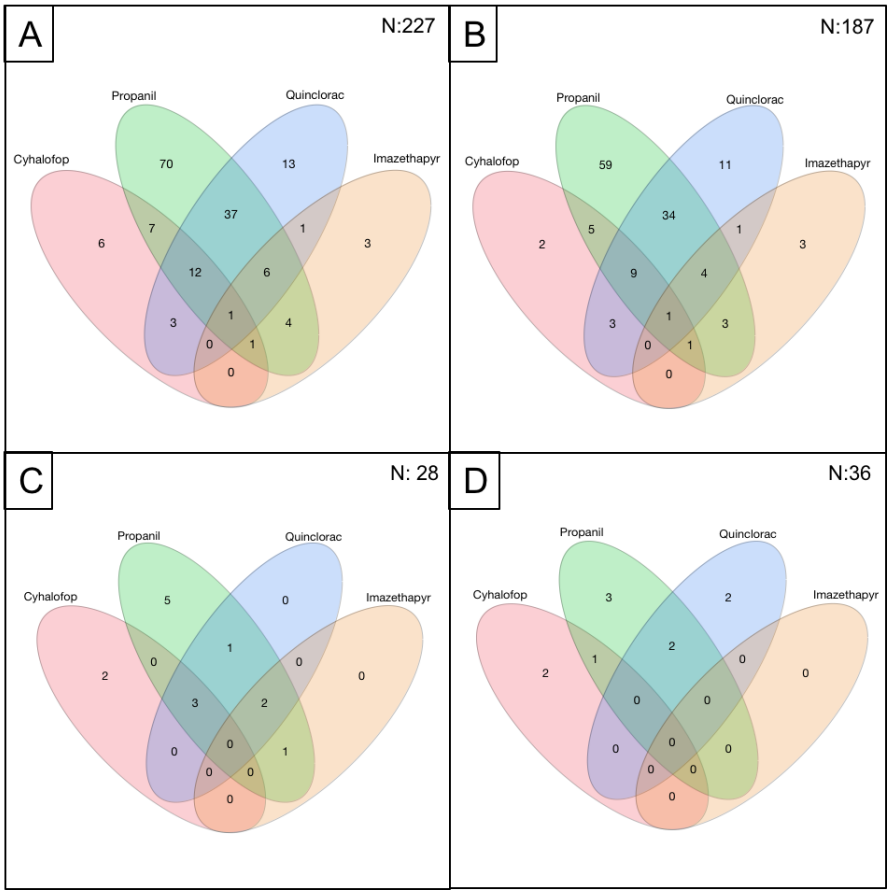


Figure 5. Frequency (%) of *Echinochloa* accessions in each resistance profile category, from Arkansas rice fields, tested in the *Echinochloa Herbicide Resistance Demographics Survey* from 2010 to 2016. ECH= Unknown *Echinochloa spp.;* ECO= junglerice (*E. colona*); ECR= barnyardgrass (*E. crus-galli*); EMU= rough barnyardgrass (*E. muricata)*.

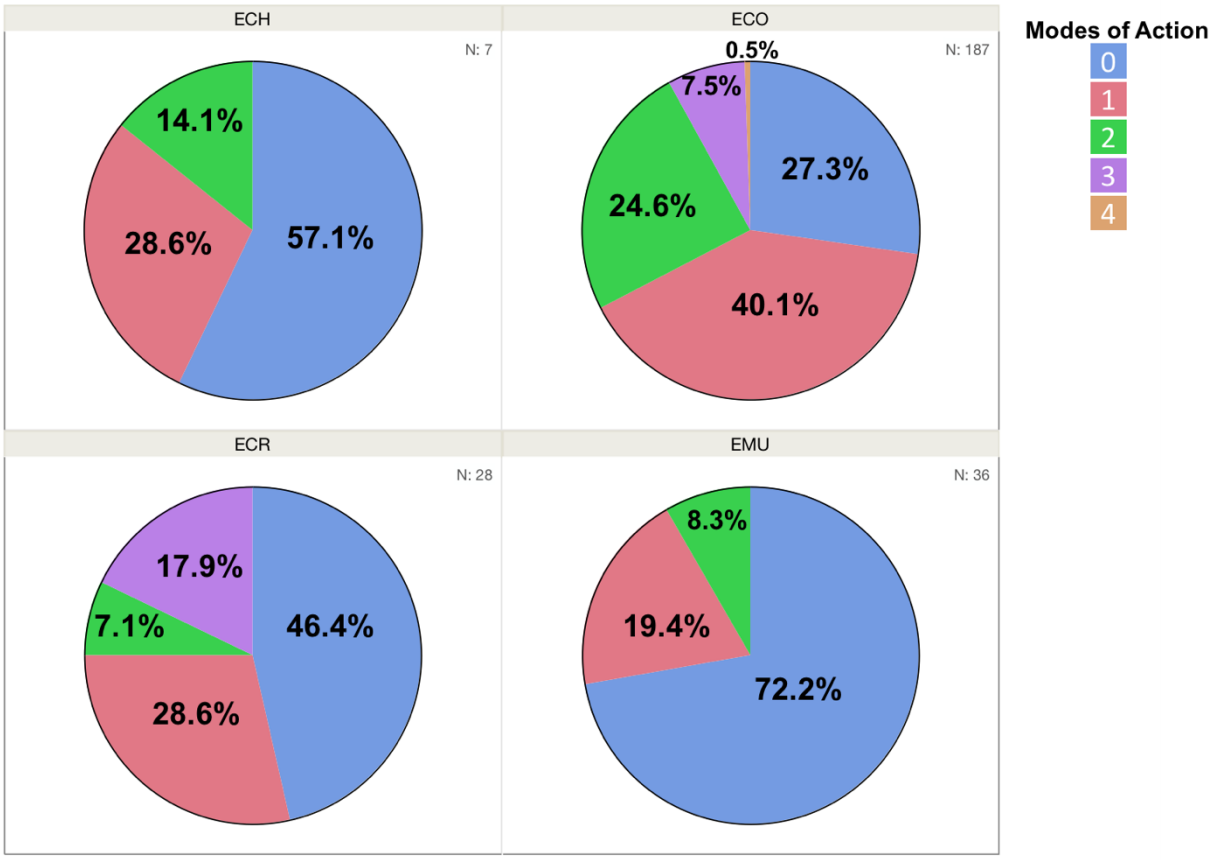


Figure 6. Arkansas maps the distribution of the accessions of *Echinochloa* spp. resistant to the four common rice herbicides tested in the *Echinochloa Herbicide Resistance Demographics Survey* from 2010 to 2016.

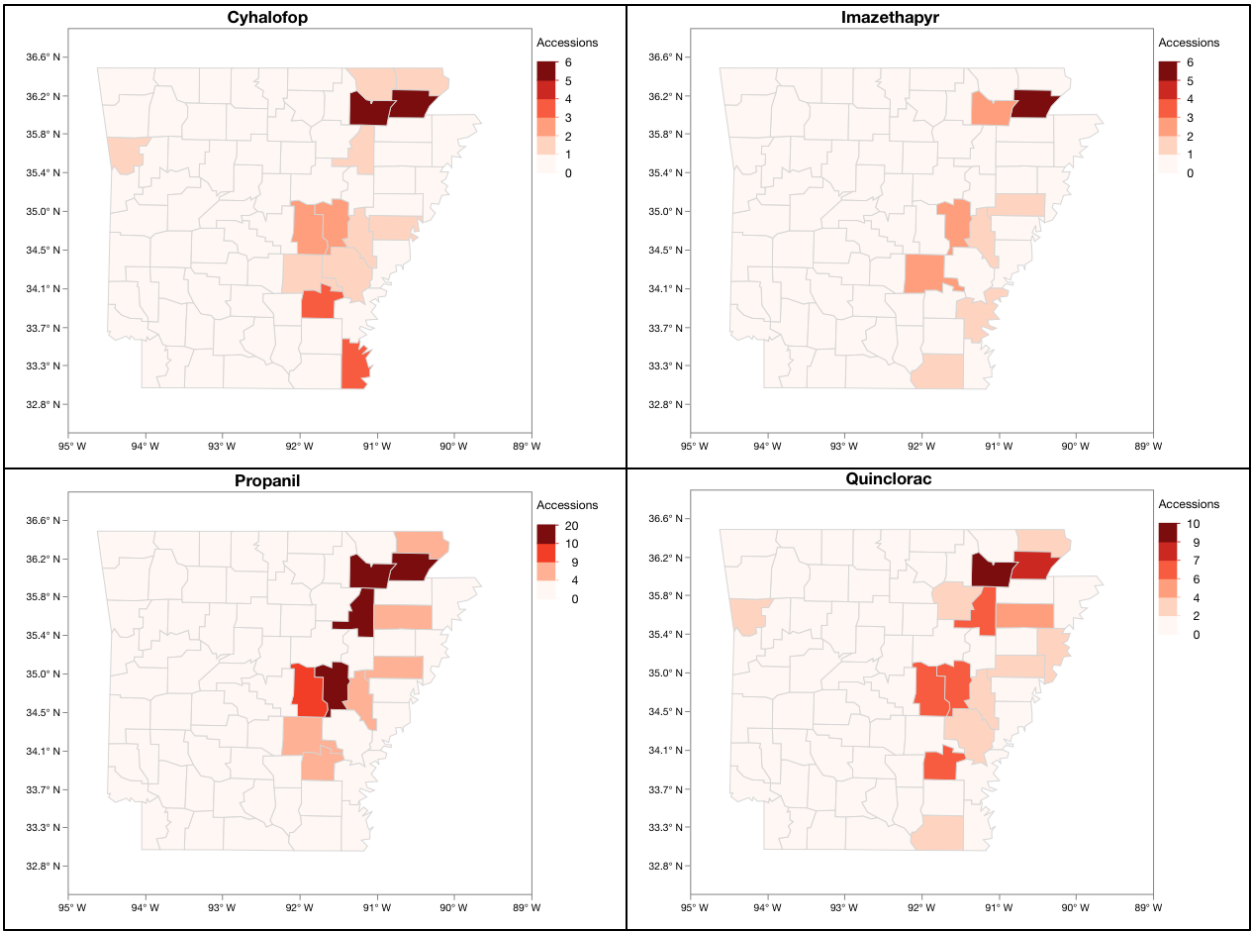
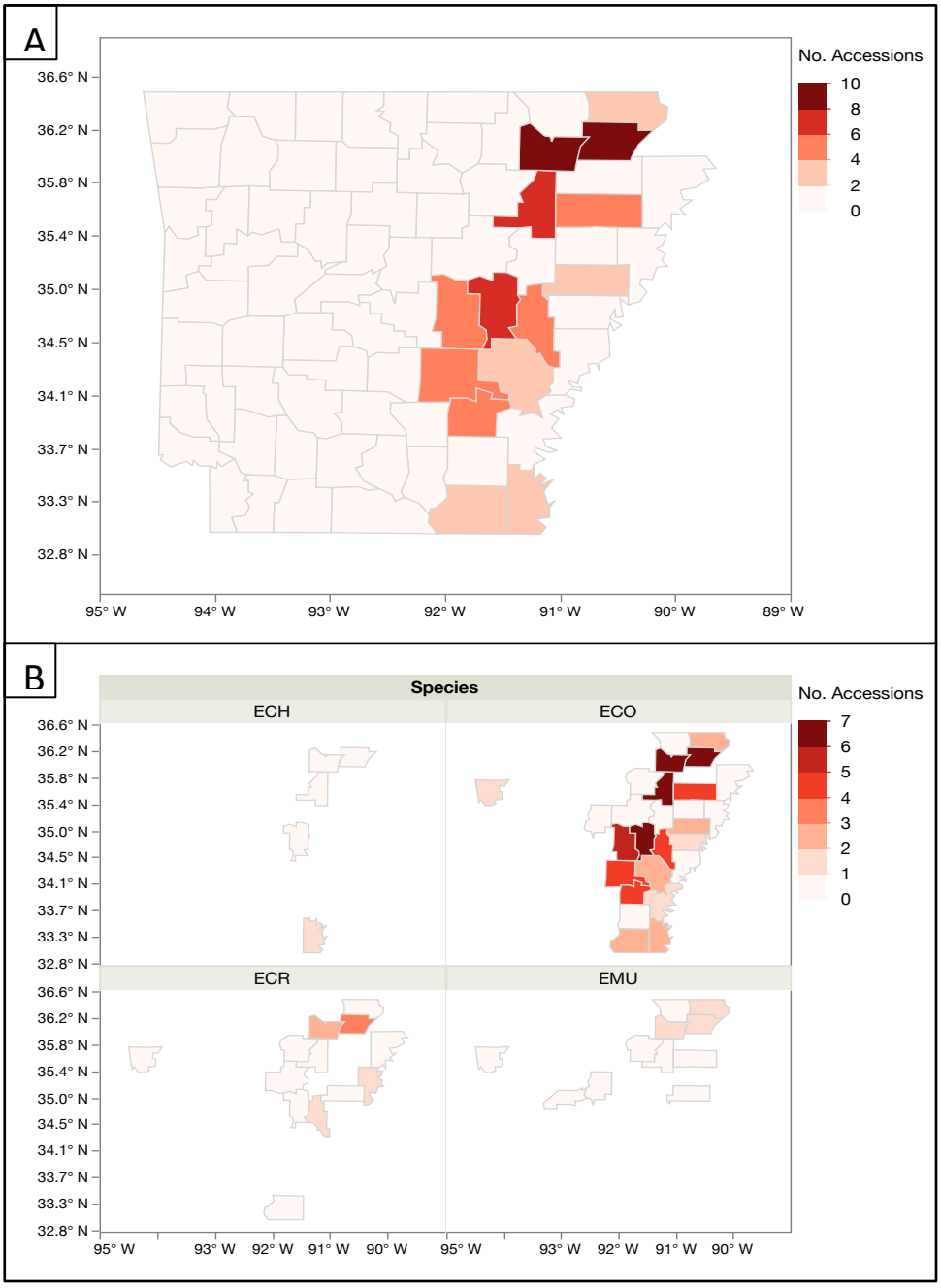


Figure 7. Arkansas maps showing the occurrence of multiple-resistance from the accessions of *Echinochloa* spp. evaluated in the *Echinochloa Herbicide Resistance Demographics Survey* from 2010 to 2016. (A) Distribution of multiple-resistant accessions of *Echinochloa* spp. (B) Distribution of the multiple resistance of the accessionss by species: ECH= species not identified; ECO= junglerice (*E. colona*); ECR= barnyardgrass (*E. crus-galli*); EMU= rough banyardgrass (*E. muricata*)



Co-evolution of independent resistance mechanisms to propanil and quinclorac in multiple-resistant *Echinochloa colona*

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Abstract

Herbicide-resistant *Echinochloa spp.* are amongst the most problematic weeds in the global agricultural landscape. A history of herbicide use and repeated selection pressure in the absence of diverse management, have resulted in over 20% of sampled populations identified as multiple-resistant in Arkansas, USA. We investigated a multiple-herbicide-resistant *E. colona* (ECO-R) collected from a rice field to assess the level and potential mechanisms of resistance in this population. ECO-R was highly resistant to propanil (>37800 g ha^{-1}) and quinclorac (>17920 g ha^{-1}), but tolerant to cyhalofop (R/S=1.9) and glufosinate (R/S=1.2) when applied separately. The addition of glufosinate (590 g ha^{-1}) to cyhalofop (314 g ha^{-1}), propanil (4500 g ha^{-1}), or quinclorac (560 g ha^{-1}) killed ECO-R. However, cyhalofop applied with propanil (48%) or quinclorac (15%) was antagonistic; treating ECO-R with quinclorac followed by cyhalofop increases control (45%). The application of malathion or carbaryl, known detoxifying enzyme inhibitors, one hour prior to propanil application synergized the herbicide and increased control of ECO-R (>75%). The inhibitors were not effective for any of the other herbicides. Using radiolabeled herbicides, neither the absorption nor translocation of ^{14}C-cyhalofop or propanil was different between ECO-R or ECO-S. The absorption of ^{14}C-quinclorac was similar between ECO-R and ECO-S. However, redistribution of the herbicide in tissues above the treated leaf of ECO-R increased (>20%) and herbicide remaining in the treated leaf decreased (<60%) relative to ECO-S. The abundance of metabolites was higher (especially for two unknown breakdown products, about 10%) in the treated leaves of ECO-R relative to ECO-S beginning 48-hours after treatment. The activity of the β-cyanoalanine synthase enzyme, capable of detoxifying hydrogen cyanide, was not different between ECO-R or ECO-S following quinclorac treatment. Propanil and quinclorac resistance appear to be caused by two independent metabolic enzymes. The

reduction in sensitivity to cyhalofop and glufosinate are unique and may be a secondary effect of the high herbicide resistance to propanil and quinclorac.

Introduction

Rice (*Oryza sativa L.*) is a global agricultural commodity, feeding over 50% of the world's population, and produced across all six arable continents [1]. Global trade is concentrated into five exporter countries representing 85% of the net trade including Thailand, Vietnam, India, Pakistan, and the United States [2]. The USA accounts for 10% of the global export market which arises mostly from the mid-south region consisting of Arkansas, Mississippi, Missouri, and Louisiana [3]. Maximizing yield within these regions is critical and eliminating weeds is of the utmost importance as they are the greatest yield-limiting biotic factor [4]. Weedy species are diverse across the rice production areas because of the differences in environment and management systems employed within various countries. However, *Echinochloa spp.* are consistently ranked as the most common weeds impacting rice production, and domestically in the USA, they are the most common and troublesome weeds in several major cropping systems [5,6]. This global threat has a unique biology that originates from its early co-domestication with rice and its adaptive evolutionary traits which allow for aggressive competition and phenotypic plasticity leading to crop mimics, making management difficult [7,8].

The most dominant of these species in rice and rice-based production systems are *E colona* (junglerice) and *E. crus-galli* (barnyardgrass), which share similar morphological traits making identification difficult but allowing for similar management [9,10]. In Arkansas, USA, herbicides and cultural management via flooding and crop rotation to soybeans are the primary methods of weed management in rice production. The focus of herbicide-based strategies has been centered on *Echinochloa* management since the early 1950s when propanil, a photosystem, II herbicide (WSSA Group 7), was released [11,12]. Following propanil in the early 1990's,

quinclorac, an auxinic herbicide (WSSA Group 4), and several graminicide or acetyl CoA-carboxylase (ACCase) inhibitor herbicides (WSSA Group 1) were released. Finally, in the early-2000s acetolactate synthase (ALS) inhibitor (WSSA Group 2) herbicides were available for use in rice with the advent of the Clearfield ® rice technology providing the first non-transgenic, herbicide-resistant rice. While each new herbicide provided excellent control of *Echinochloa spp.,* the over-use, ease of application, and lack of diversity in herbicide products resulted in rampant herbicide resistance in Arkansas beginning in the 1990s [13,14]. This is not a unique problem to Arkansas nor the USA, as herbicide-resistant *Echinochloa* were first identified in 1986 and have expanded to 14 countries [15]. This genus has been deemed to contain several of the worst herbicide-resistant weeds in the world, primarily attributable to the high degree of genetic diversity and adaptive abilities [16]. Recent evidence suggests that single herbicide resistance is of concern, but more importantly, multiple-herbicide resistant populations with resistance to two or more herbicide modes of action is increasing in prominence [10]. To investigate the cause of multiple resistance, comprehensive physiological and genomic assessment of these populations is of the utmost importance.

Genetics and plant physiology play a significant role in herbicide resistance evolution among weedy species. Two terms are often used to categorize the underlying mechanisms of resistance- target site (TSR) and non-target-site (NTSR). TSR, resulting from high dose selection leading to modifications in herbicide target proteins, is the most prevalent and results in only single and/or cross-resistance to herbicides from the same mode of action category [17,18]. NTSR is a more complex and polygenic response to herbicide activity and action, involving several processes which limit the presence or concentration of the active herbicide at its target [19]. NTSR is often observed due to the elevation in enzymes associated with one or more of the

xenobiotic detoxification phases [20]. Much less is known about NTSR but the threat to the evolution of multiple resistance is much greater due to the general substrate nature of these enzymes. Multiple resistance may be endowed by a single mechanism or by multiple independent mechanisms providing the resistant phenotype for different herbicides [21]. The threat of a single mechanism resulting in multiple resistance is of great concern as it may pose a risk for weed control options including herbicides, limiting their potential utility. More importantly multiple herbicide resistance has the potential to have an impact on the biology of weedy species through either increasing or decreasing fitness [22]. In some cases, the impact on fitness may be overcome through compensatory evolution that allows it to be resilient to abiotic stressors, an even greater concern for management [23]. TSR and NTSR has manifested in *Echinochloa spp.* in a number of ways to a variety of herbicide modes of actions, however, little research probing the multiple-resistant populations has been conducted. Characterization of this evolved phenomena may be key in ascertaining the evolutionary processes that have a role in both compensatory evolution but also herbicide resistance.

The continuous selection pressure on recurrent generations of *E. colona* in rice fields in Arkansas has led to widespread resistance within this species. The goal of this research is to provide an understanding of the physiological NTSR mechanisms employed by multiple herbicide-resistant *E. colona* and evaluate if there is potential in shared resistance mechanisms amongst the herbicides of interest. Based on the preliminary results used to assess the level of resistance in this population and the potential mechanism, a series of biochemical and physiological assays were conducted to target the mechanisms related to quinclorac resistance. The results of these experiments will assist in characterizing this population and attempt to

identify potential mitigating control options for multiple herbicide- resistant *E. colona* which have evolved under similar selection pressures.

Results

Resistance level to various herbicides

Herbicide response was assessed using dose response analysis to obtain LD_{50} values, or lethal dose that causes 50% injury, for both the resistant (ECO-R) and susceptible populations (ECO-S). The response to cyhalofop was fitted with a 4-parameter Gompertz model producing an R/S value of 1.9 (Fig 1a). The response to glufosinate best fit a 4-parameter logistic model resulting in an R/S value of 1.2 (Fig 1b). The response of ECO-S to propanil was higher than anticipated; however, data was best fit by a 4-parameter logistic model (Fig1c). The R/S value for ECO-R was 2, but both the ECO-S and ECO-R accession were not controlled by the 1x field application rate. The LD_{50} value for ECO-S was approximately 4.2X the field dose rate of propanil or 18900 g ha^{-1}, whereas the LD_{50} for ECO-R was approximately 8.4X the field dose or 37800 g ha^{-1}, a significantly higher application rate. The response to quinclorac was different from those of the other herbicides and the data were fitted with a 3-parameter logistic model. The LD_{50} for ECO-S was 0.33X of the field dose or 185 g ha^{-1} quinclorac. ECO-S was controlled 100% by approximately 280 g ai ha^{-1} quinclorac; however, ECO-R was not controlled by the highest dose evaluated. The highest dose, 32X or 17920 g ha^{-1}, caused less than 20% injury. Therefore, an LD_{50} value for ECO-R could not be attained and the R/S value could not be calculated.

Efficacy of herbicide mixtures

The field dose rate of propanil (29%) and quinclorac (1%) were ineffective and cyhalofop (62%) provided only moderate control (Fig 2a). In these experiments, glufosinate was

able to provide near complete control of ECO-R across all runs of the experiment. Cyhalofop applied with propanil increased the control of ECO-R by 5% relative to cyhalofop applied alone and by over 30% relative to propanil applied alone (Table 1). Cyhalofop + quinclorac reduced the control of cyhalofop to less than 20% and only marginally increased quinclorac activity (18%), again an antagonistic relationship. The propanil and quinclorac tank mixture had an additive effect on ECO-R (42%). Quinclorac (138%) and cyhalofop + quinclorac (114%) application resulted in more biomass than the nontreated control (Fig 2b). Regardless of the tank-mixture companion, glufosinate application resulted in 100% control of ECO-R. The sequential applications of cyhalofop fb quinclorac and quinclorac fb cyhalofop were also tested for antagonistic interactions using Colby's method. The observed control was 45% and 0% for quinclorac fb cyhalofop and cyhalofop fb quinclorac, respectively. Quinclorac applied before cyhalofop had an additive effect but applying cyhalofop first was antagonistic.

Detoxification enzyme inhibitor assessment

The use of known detoxifying enzyme inhibitors- malathion and carbaryl resulted in increased visible injury from the propanil application (Figure 3a). No other herbicide application was synergized in this way. The application of carbaryl prior to propanil application increased visual injury to 93%, approximately 55% more than propanil applied alone. This was comparable to the effect of malathion (78%) but better than that of piperonyl butoxide (PBO) (~60%). Without herbicide, PBO stunted the plants 7%. All three enzyme inhibitors reduced biomass when applied prior to propanil by over 90% (Fig 3b). The presence of these compounds greatly reduced the plants ability to tolerate and/or recover from the herbicide treatment. With quinclorac, only malathion (83%) and PBO (45%) reduced biomass relative to quinclorac alone. However, quinclorac alone induced growth and resulted in 13% more biomass than the

nontreated control plants. This has not been observed previously in any *Echinochloa* population response to quinclorac. Propanil application was synergized by all three enzyme inhibitors tested resulting in almost complete control of ECO-R. This provides an indication that propanil resistance is due to enzymatic detoxification. The three remaining herbicides either possess a different resistance mechanism or these inhibitors were not effective on their respective enzymes.

^{14}C-herbicide absorption and translocation

Cyhalofop. Cyhalofop absorption was maximized 72-hours after treatment, with 68% in ECO-R and 78% in ECO-S (Table 2). The absorption was not significantly different at any time except for 72-hours after treatment, whereby ECO-S had a greater quantity of ^{14}C-cyhalofop inside the plant. The majority of the absorbed ^{14}C-cyhalofop remained in the treated leaf (>90%) (Appendix Table 1). Soon after treatment 6- and 12-hours after application, both ECO-R and ECO-S had moved some of the herbicide into leaves above the treated leaf (2% to 4%). By 24 hours, the herbicide was more concentrated below the treated leaf in the plant shoot than above the treated leaf; this level of portioning remained the same 72-hours after treatment. Very little ^{14}C-cyhalofop (<1%) was translocated into the roots. Absorption and translocation of ^{14}C-cyhalofop was not different between ECO-S and ECO-R.

Propanil. Propanil absorption was also maximized 72-hours after treatment, with ECO-R (42%) having a numerically higher concentration of ^{14}C-propanil than ECO-S (32%) (Table 2). Significant differences in absorption were only observed 48-hours after treatment with ECO-R (34%) being greater than ECO-S (25%). As with cyhalofop, the majority of propanil was retained in the treated leaf. From 12- to 72-hours after treatment, the proportion of herbicide in

the tissues below the treated leaf was less than 0.5% of the absorbed herbicide. The absorption or translocation of ^{14}C-propanil did not differ between ECO-S and ECO-R.

Quinclorac. Quinclorac absorption at 24-, 48-, 72-, and 96-hours after application was not significantly different between ECO-R and ECO-S (Table 3). However, by 120-hours after treatment ECO-R had absorbed more quinclorac (72%) than ECO-S (64%). Beginning 72-hours after treatment, ECO-R had a lower concentration (<60%) of ^{14}C-quinclorac in the treated leaf but a higher concentration (>24%) in the tissues above the treated leaf than ECO-S (table 4). Also, 48- and 120-hours after treatment, there was a greater concentration of the ^{14}C-quinclorac in the roots of ECO-R than in ECO-S. The pattern of movement favors translocation out of the treated leaf and into the rest of the plant sections. This was validated by phosphorimaging (Fig 4).

^{14}C-herbicide quinclorac metabolism

Profiling of the quinclorac metabolites following treatment revealed three unique metabolites in both ECO-R and ECO-S**.** The metabolites were not identified, but the relative quantities were analyzed. At all four timings, the majority (>70%) of the ^{14}C-quinclorac remained as the parent molecule (Fig 5). The amount of parent quinclorac molecule did not differ significantly between ECO-R and ECO-S, but was numerically less in ECO-R than ECO-S beginning 48-hours after treatment. The parent molecule remained relatively the same in ECO-S, approximately 75% to 80%. Of the three metabolites, metabolite 2, was consistently present in quantity in ECO-R 24-, 48-, and 72-hours after treatment; by 72-hours after treatment it equated to about 9% of the absorbed parent molecule. Metabolite 1, was significantly greater in ECO-R at 96-hours after treatment (10%) than ECO-S.

β-cyanoalanine synthase enzyme activity

The β-CAS enzyme activity did not differ between quinclorac-treated ECO-R (0.319373 M Na_2S) and ECO-S (0.319206 M Na_2S). For ECO-R, there were no differences between the nontreated (0.319376 M Na_2S) and quinclorac-treated (0.319373 M Na_2S) plants. The same was observed for ECO-S, with the quinclorac-treated (0.319120 M Na_2S) β-CAS enzyme activity being lower than the nontreated (0.319206 M Na_2S). Slightly elevated activity was observed in the ECO-R plants without herbicide and following treatment, indicating higher biological activity by ECO-R.

Foliar/ root absorbed cyanide toxicity

Results for the potassium cyanide (KCN) topical absorption assays did not yield quantifiable differences in injury or plant growth and thus were not analyzed but are presented pictorially for reference (Appendix Fig 1). At the highest concentration, 100 parts per million (PPM), notable differences in the formation of adventitious roots were observed. ECO-R had a higher concentration of fibrous and lateral roots than ECO-S, but shoot mass was similar. In the first run of the assay, ECO-R produced a higher number of relatively larger shoots than ECO-S, but this was not observed in the second run of the experiment. The suppression of growth by elevated cyanide concentrations would be expected as cyanide disrupts cell membrane integrity.

Discussion

The multiple-resistant *Echinochloa colona* population under evaluation, ECO-R, is a very unique population from Arkansas. The high levels of resistance to propanil and quinclorac are a concern to rice producers in the state, but, the potential low-tolerance to both cyhalofop and glufosinate may be of greater concern. High levels of propanil [14,24] and quinclorac [25,26] resistance have been reported previously in Arkansas, but not to the level at which we observed

in this experiment. Previous research identified that 'moderate' resistance to propanil was observed at approximately 20 kg ha^{-1}, which was comparable to what was observed in ECO-S, but significantly less than the 37.8 kg ha^{-1} LD_{50} observed by ECO-R [14]. Cyhalofop nor glufosinate resistance has been reported in Arkansas populations. Given the dose response analysis, with the low R/S values and the control of the populations at a field dose application, these populations cannot be considered as resistant to either herbicide. However, the separation in the response between ECO-R and ECO-S does warrant further characterization as this may be an indication of early evolutionary responses to the herbicides. Propanil and quinclorac multiple-resistant populations are among the most common in the state of Arkansas [10]. Given the results in this experiment it is possible that multiple resistance is of a greater concern than previously anticipated and optimizing control of *Echinochloa spp.* must be prioritized to reduce the impact they have.

The use of tank-mixtures to improve weed control is an effective recommendation to reduce the evolution of herbicide resistance and was thus evaluated in this research [13]. Glufosinate is still an effective herbicide control option for ECO-R in burndown or Liberty Link® soybean systems, and the application of it with cyhalofop, propanil, or quinclorac provides good control, significantly reducing *E. colona* biomass. Unfortunately, the use of cyhalofop with propanil or quinclorac is much less effective and antagonistic to the herbicide efficacy. Propanil and cyhalofop are known to antagonize each other, potentially due to reduced translocation of the herbicide [27]. This interaction with graminicide compounds is common and is not unexpected for auxin herbicide compounds[28]. But given that this population is already multiple-resistant it may be best to recommend not applying these compounds in fields with high levels of resistance. This may aid in the selection for NTSR mechanisms caused by ineffective

herbicide controls [19]. More importantly the interactions between quinclorac and cyhalofop were investigated further, and a unique response pattern whereby the application of quinclorac followed by cyhalofop is a more effective combination, while still not adequate. This has not been reported previously and may have a role in explaining the underlying resistance mechanism employed against quinclorac. Further research needs to be conducted to characterize the role this type of application may have.

Enzyme inhibitors have been used to study metabolic based resistance previously in order to ascertain the potential mechanisms utilized by herbicide-resistant *Echinochloa* [14,29,30]. Cyhalofop and glufosinate were unaffected by any of the evaluated inhibitors in ECO-R. Both propanil and quinclorac resistance mechanisms have been inhibited through the use of different known enzymatic inhibitors in previous research. For ECO-R, both malathion and carbaryl were effective at synergizing the activities of propanil, leading to the conclusion that a metabolic based mechanism is a component of resistance. Previous research found that aryl acylamidase, the enzyme involved in propanil detoxification in rice and *Echinochloa,* is inhibited by malathion and carbaryl [12,31]. The response by ECO-R may be a result of the inhibition of this enzyme, but further research is required to verify this. Quinclorac injury was not increased from any of the applications even though the biomass was reduced in ECO-R. The known HCN detoxifying enzyme $\boldsymbol{\beta}$-CAS, which is an identified quinclorac-resistance mechanism, is inhibited by malathion [30]. The lack of synergism with quinclorac and the results of the $\boldsymbol{\beta}$-CAS enzyme assay lead USA to the conclusion that this enzyme does not have a role in resistance for ECO-R. While these inhibitors interact with a range of xenobiotic detoxification enzymes, they do not account for all possible mechanisms and thus metabolic resistance cannot be ruled out for quinclorac. It is also important to note that in several of these experiments the application of

quinclorac alone encouraged the growth and/or vigor of ECO-R following treatment. This has not been described previously but may implicate a non-target-site resistance mechanism that is not metabolic but a component of a large abiotic stress pathway or physiological pathway [19]. Results from the ^{14}C-herbicide experiments provided more information on the activities of quinclorac in the plant but to a lesser extent describe the actions of propanil or cyhalofop. Cyhalofop was distributed more within the plant, away from the treated leaf, than was propanil. Absorption and translocation of ^{14}C-cyhalofop was similar to previous research in Arkansas populations for susceptible and propanil-resistant populations [27]. For propanil, the observed absorption by ECO-R and ECO-S was greater than in previous experiments but the translocation patter was similar, with little being moved outside of the treated leaf [14]. This is expected given that propanil is a PSII herbicide, with low translocation, which usually moves via mass flow and not active carbohydrate loading in the phloem. The response for quinclorac has not been described previously in the literature. Not only did quinclorac move out from the treated leaf, it accumulated in the new growth above the treated leaf, indicating active movement. This can occur as either the parent quinclorac compound or as a polar metabolite. The ^{14}C-quinclorac metabolism experiment identified two potential unknown metabolites of quinclorac in the ECO-R population, present at higher concentrations than in ECO-S. Given the high distribution of the herbicide throughout the plant by 120-hours, this may have a role in the resistance mechanism. Quinclorac has been shown to be highly mobile in the plant from root applications, but not the extent observed from the foliar applications in this experiment [32,33]. Further research needs to be conducted to examine the identity of the metabolite and determine if its polar nature has a role in the redistribution of the herbicide following treatment. The conjugation of quinclorac to a polar metabolite may be the quinclorac resistance mechanism in ECO-R.

Conclusions

This research provides the first in-depth investigation into the physiological basis for resistance in a multiple-resistant *E. colona* from Arkansas. While propanil and quinclorac resistant populations are present in a number of Arkansas fields, ECO-R has an abnormally high resistance level compared to previous research. The responses to cyhalofop and glufosinate were less than expected from the initial field screen conducted by our lab, however, they do provide an indication of potential co-evolutionary adaptation from the two primary herbicides. Using the litany of experiments in this research both propanil and quinclorac resistance appear to be caused by non-target-site resistance mechanisms potentially involving two independent xenobiotic detoxifying enzymes. Further physiological and biochemical assays should be conducted to asses which enzymes have a role in resistance, but a novel genomics approach may be more beneficial.

Materials and Methods

Plant Materials

Beginning in 2010, the University of Arkansas Weed Physiology research group began a statewide herbicide resistance and species demographic survey aimed at assessing the distribution and status of herbicide resistance in Arkansas [10]. This research resulted in the characterization of approximately 200 populations of *Echinochloa spp.* collected from rice or historical rice production areas of the state which had survived the standard weed management employed by the contributing rice farmers. Details on the screening procedure and herbicide resistance profiling can be found in Rouse et al. [10]. From this collection, two populations of *E. colona* were selected for further characterization in this research: ECO-R and ECO-S. ECO-R was collected in 2010 from a rice field in Lincoln County, AR and was flagged as putatively resistant to three rice herbicides based on the herbicide screen- cyhalofop, propanil, and

quinclorac. The herbicide screen also assessed glufosinate, a common soybean herbicide, as these populations may have been exposed to this herbicide in their history; ECO-R was also flagged as potentially glufosinate-resistant based on the moderate control and high level of survivors in the screen (data not shown). ECO-S was collected in 2011 from a field in Prairie county, AR and based on the field history and results of the herbicide resistance screen was a susceptible counterpart to ECO-R. Following screening, single plants from both ECO-R and ECO-S were grown in isolation to produce a single generation of self-pollinated offspring. Due to the low outcrossing exhibited by *E. colona*, it has been determined that a single generation is enough to produce near homozygous individuals for further research. All research following this initial selection was conducted using this 'pure-line' generated seed.

Dose response analysis

ECO-R and ECO-S were grown under greenhouse controlled environmental conditions with 14-hour days set to a constant temperature of 30 to 35°C. Regardless of the run, approximately 10 to 20 seeds of either ECO-R or ECO-S were planted into individual square pots containing commercial potting soil (Sungro Horticulture) measuring 7.6 cm wide and 10.2 cm in height. Approximately one week after planting, each pot was thinned to a single plant per pot, with either six or nine replicates depending on the run of the experiment, the final run contained 20 individual plant replications. Plants were maintained in the greenhouse and sub-irrigated until each plant had reached the 2 to 3-leaf stage for treatment. All replications were treated simultaneously in an air pressurized, stationary spray chamber, calibrated to deliver 187 L ha^{-1}. Herbicide application rates were determined by the standard use rates for the four herbicides of interest: cyhalofop- 314 g ha^{-1}, propanil- 4500 g ha^{-1}, quinclorac- 560 g ha^{-1}, and glufosinate- 590 g ha^{-1}. These rates served as the 1x dose evaluated in all of the experiments. In

the initial run of the experiment, all herbicides were evaluated at eight gradual doses from 0x to 16x for cyhalofop, glufosinate, and quinclorac, and 0x to 32x for propanil, to define an initial curve for the response of ECO-R and ECO-S. Following this initial assay, follow-up runs evaluated a greater number of doses within these respective bounds, with the exception of quinclorac which was extended to 32x the field dose. Following herbicide treatment, plants were returned to the greenhouse and maintained for three weeks. Three weeks after treatment the experiment was terminated and an assessment of visual injury (0%= no symptoms to 100%= complete plant death) and fresh biomass was collected. Fresh biomass was converted to a percentage of the nontreated control to best evaluate the herbicide response. This experiment was established as a completely randomized design with each pot serving as a single replication of the experimental unit with five runs of the experiment being conducted. Data across all runs of the experiment were combined for analysis as the mean responses were similar across the runs and to best evaluate all of the herbicide doses in the experiment. The results for ECO-R and ECO-S, for each of the herbicides of interest, were fit using a non-linear logistic model. From these models and LD_{50}, or lethal dose resulting in 50% injury to plant, was inversely calculated from the model. Using the LD_{50} value from both ECO-R and ECO-S, an R/S ratio was calculated to determine the difference in the herbicide response between the two populations.

Tank mixture assessment

Approximately 25 seeds were germinated in square pots (10.2 cm wide by 10.2 cm tall) containing commercial potting soil 10.2 cm wide by 10.2 cm in height. At approximately one week after planting, each pot was thinned to a density of five plants per pot; each pot served as one replication or one experimental unit, with four replications total. In the same manner as described previously plants were treated at the 2 to 3-leaf stage. Treatments included the field

application rate of all four herbicides of interest and a tank mixture of each herbicide with one of the other herbicides at the same field application rates, for 10 treatments in total for the first run of the experiment. Four pots maintained in a similar manner were left untreated and served as controls for comparison. Two more runs were conducted with the same treatments, however, in these runs the application of cyhalofop and quinclorac were expanded into three treatments: cyhalofop + quinclorac, cyhalofop followed by (fb) quinclorac, and quinclorac fb cyhalofop; each application was split by 60 mins. Previous research and experience has identified antagonism between tank mixture of graminicides and auxin compounds [28]. To best assess this interaction, the treatments were separated to insure chemical interactions did not increase or decrease the herbicide activity. The experiment was terminated three weeks after application and visual injury and fresh biomass were assessed, with fresh biomass converted to a percentage of a non-treated control. The experiment was established as a completely randomized design with three runs and four replications. The runs were analyzed together due to similarities in the observed responses. An analysis of variance (ANOVA) was conducted for both visual injury and biomass with significant means separated using Fisher's Protected LSD ($\alpha \leq 0.05$). A follow-up analysis using a modified Colby's method for assessing tank mixture interactions were calculated to determine if the interaction between the chemicals was antagonistic, additive, or synergistic [34,35].

Detoxification enzyme inhibitor assessment

An assessment of detoxification enzyme inhibition was conducted for the two populations of interest and all four herbicides under evaluation. Seed for ECO-R and ECO-S were germinated and grown in a similar manner to the tank mixture assays previously described. At the 2 to 3-leaf stage the plants were treated according to the respective treatments listed in

Table 4. The known detoxification inhibitors were applied in a manner similar to the herbicides 60 minutes prior to the herbicide application. After 60 mins, when the plants had dried, the herbicide treatments were made for all four herbicides at the field application rate, a no inhibitor and no herbicide set of plants were left untreated for comparison. Three weeks after application, the experiment was terminated and a visual assessment of injury was made and fresh biomass was collected and weighed. The experiment was a completely randomized design, with four replications consisting of a single experimental unit of one pot containing five plants. The analysis was conducted by herbicide, with the single fixed factor of inhibitor. An ANOVA was conducted for both visual injury and biomass as a percent of the nontreated control, significant means were separated using Fisher's Protected LSD ($\alpha \leq 0.05$).

^{14}C-herbicide absorption and translocation

All experiments, regardless of herbicide, were conducted in the same way except when specified according to modified procedures from Vencill et al. [36] . Radiolabeled ^{14}C-herbicide was used to measure the absorption and translocation of cyhalofop, propanil, and quinclorac in ECO-R and ECO-S. Individual plants were grown, maintained, and treated in manner similar to the previous experiments. At the three-leaf stage the plants were over-sprayed using a field application rate of the three herbicides of interest. After the plants had dried, they were moved into laboratory for treatment. A spotting solution containing 0.24 kBq μL^{-1} was formulated with a subsample of the 'cold' herbicide solution applied to the plants. Five droplets were applied within a 2.54 cm area on the adaxial surface of the second fully expanded leaf. A total 1.7 kBq of radiolabeled herbicide was applied. For cyhalofop and propanil, plants were harvested at 6-, 12-, 24-, 48-, and 72-hours after treatment. For quinclorac, plants were harvested at 24-, 48-, 72-, 96-, and 120-hours after treatment. At each time point, the treated leaf was removed from the plant

and rinsed in a vial containing deionized water (cyhalofop and propanil) or 70% acetonitrile (quinclorac). The leaf tissues were shaken gently to wash the leaf surface to remove the unabsorbed ^{14}C-herbicide; the tissue was removed from the vile to dry. A subsample of the leaf wash was drawn from the vile and counted using a liquid scintillation counter for a quantification of the absorbed herbicide. The remaining plant was removed from the pot and the roots were washed thoroughly to remove the soil. The plant was sectioned into three parts- above treated leaf, below treated leaf, and roots. Samples were then air-dried prior to oxidizing for a quantification of the ^{14}C-herbicide within each of the plant parts. The quantity in each respective tissue section was converted to a percentage of the absorbed concentration in the plant for analysis. Data were analyzed by tissue and harvest timing. A t-test was performed to determine if the mean absorption or concentration for the plant tissue was different between ECO-S and ECO-R.

^{14}C-herbicide quinclorac metabolism

Plants of ECO-R and ECO-S were grown and maintained in a similar manner as the previously described absorption and translocation experiments. However, the plants were not over sprayed with a cold herbicide solution prior to treatment. When the plant had reached the 3-leaf stage they were spotted with approximately 14.3 kBq of ^{14}C-quinclorac. At 24-, 48-, 72-, 96-, and 120-hours after treatment, the treated leaf was removed, washed and placed into a extraction tube. The tissue was homogenized with 3 mL 70% acetonitrile for extraction of the ^{14}C-quinclorac. The extraction solution was then dried under vacuum using a rotavaporator until dry and re-suspended in a methanol: acetonitrile (40:60) buffer. A 150 µL sample was then analyzed using a reverse-phase HPLC. Data were analyzed by harvest timing and quinclorac

parent molecule or metabolite. A t-test was performed to determine if the mean of the respective metabolite was significantly different between ECO-R and ECO-S.

β-cyanoalanine synthase enzyme activity assay

A biochemical assessment of the activity of the β-cyanoalanine synthase (β-CAS) enzyme was made using a colorimetric assay similar to Grossman and Kwiatkowski [37] and Yasuor et al. [30]. Trays containing commercial potting medium, two per accession, were used to germinate seed of ECO-R and ECO-S, trays were thinned to prevent overcrowding of the plants. When plants reached the 2-leaf stage they were treated with 560 g ha^{-1} of quinclorac. At 24-hours after application, five plants per replication, three replications total, were harvested and immediately frozen in liquid nitrogen. Plants were homogenized using a mortar and pestle and 100 mM Trip buffer (pH 8.5). Homogenized tissues were kept on ice and centrifuged for 10 min at 6708 g and 4C. The supernatant was transferred to a fresh centrifuge tube. Fresh substrate solution was prepared by mixing 50 mM NaCN and 10 mM Tris-HCl (pH 8.5). Both the substrate solution and supernatant was equilibrated at 30C for 10 mins. The reaction was started in a sealed test tube where 0.5 mL of the enzyme extract to 4 mL of substrate solution for 60 mins at 30°C. After this incubation period, the color was developed by adding a 1 mL aliquot of the reaction mixture (30 mM $FeCl_3$ in 1.2 N HCL+40 mM N,N-dimethyl-phenylenediamine sulfate salt in 7.2 N HCl) to the substrate and enzyme solution. The sample was vortexed and left in the dark at room temperature for 1 to 2 hours to allow color to develop. The enzyme activity based on the conversion of cysteine to methylene blue and the release of hydrogen sulfide. The absorbance of each sample was measured at 650 nm using a Pharma Spec UV-100 (Shimadzu Columbia, MD). The absorbance reading was converted to 'M Na_2S' based on a standard curve. A t-test was performed to determine if the mean concentration was significantly

different between ECO-R and ECO-S and if there was a difference between the treated and nontreated samples.

Foliar/ root absorbed cyanide toxicity assay

An agar based topical absorption assay was conducted similar to the RISQ assay developed by Kaundun et al. [38] and described by Burgos [39]. A solution containing 0.5% wt/v agar was prepared. For the initial run of the experiment, the agar solutions were mixed with a potassium cyanide (KCN) solution to a concentration of 0, 4, and 100 PPM; in the second run, an increasing dose of KCN was used including 0, 10, 25, 50, and 100 PPM KCN. ECO-R and ECO-S were grown in a similar manned as previously described in the $\boldsymbol{\beta}$-CAS enzyme assays. When the plants were at the one true leaf stage and the first collar was visible, four plants were gently removed from the soil, rinsed in deionized water, and placed onto the petri plate containing 75 mL of agar solution. The roots were pushed into the medium and the leaf tissue was lightly pressed onto the surface of the medium. Three plates per treatment were used as individual experimental units or replications. The plates were placed into the greenhouse for one week. At the end of the week the plants were assessed for injury based on the health of the shoot tissue and estimated root growth compared with the 0 PPM.

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Tables and Figures

Table 1. Visible injury (%) to ECO-R following the application of the tank mixture treatments of the four herbicides of interested 3-weeks after application.

Herbicide[1,2]	Cyhalofop	Glufosinate	Propanil	Quinclorac	fb Cyhalofop	fb Quinclorac
Cyhalofop	-	70 (88)	48 (72)	15 (61)		0 (58)
Glufosinate	70 (88)	-	68 (77)	75 (72)		
Propanil	48 (72)	68 (77)	-	28 (25)		
Quinclorac	15 (61)	75 (72)	28 (25)	-	45 (58)	

[1] Colors signify if the interaction was antagonistic (red) or additive (yellow) according to Colby's method of assessing tank mixture interactions ($p \leq 0.05$)
[2] Numbers indicate the observed value and numbers in parenthesis are the expected values used for Colby's method

Table 2. Cyhalofop and propanil absorption as a percentage of the total applied ^{14}C-radiolabeled herbicide compounds at the five timings.

	Cyhalofop[2]		Propanil	
Timing[1]	**ECO-R**	**ECO-S**	**ECO-R**	**ECO-S**
Hours	----------%----------			
6	35	57	14	13
12	43	41	*15*	*13*
24	53	55	20	20
48	60	52	*34*	*25*
72	*68*	*78*	42	32

[1] Timing is presented as hours after treatment
[2] Means in italics for ECO-R and ECO-S within the respective herbicide are different according to a t-test ($p \leq 0.05$)

Table 3. Quinclorac absorption as a percentage of the total applied ^{14}C-radiolabeled herbicide at the five timings.

	Quinclorac[2]	
Timing[1]	**ECO-R**	**ECO-S**
Hours	----------%----------	
24	45	52
48	61	75
72	59	67
96	58	62
120	*73*	*64*

[1] Timing is presented as hours after treatment
[2]Means in italics for ECO-R and ECO-S are different according to a t-test ($p \leq 0.05$)

Table 4. Concentration (%) of the absorbed ^{14}C-quinclorac in the respective plant tissues for the five harvest timings

		Quinclorac[3]	
Timing	**Section[2]**	**ECO-R**	**ECO-S**
Hours		----------%----------	
24	Trt	79	81
	AL	3	7
	BL	11	6
	RT	1	1
48	Trt	63	90
	AL	23	3
	BL	9	5
	RT	*2*	*1*
72	Trt	*58*	*89*
	AL	*25*	*3*
	BL	11	6
	RT	2	0
96	Trt	*57*	*85*
	AL	*24*	*6*
	BL	13	5
	RT	2	2
120	Trt	*43*	*83*
	AL	*41*	*7*
	BL	12	6
	RT	*2*	*1*

[1] Timing is presented as hours after treatment

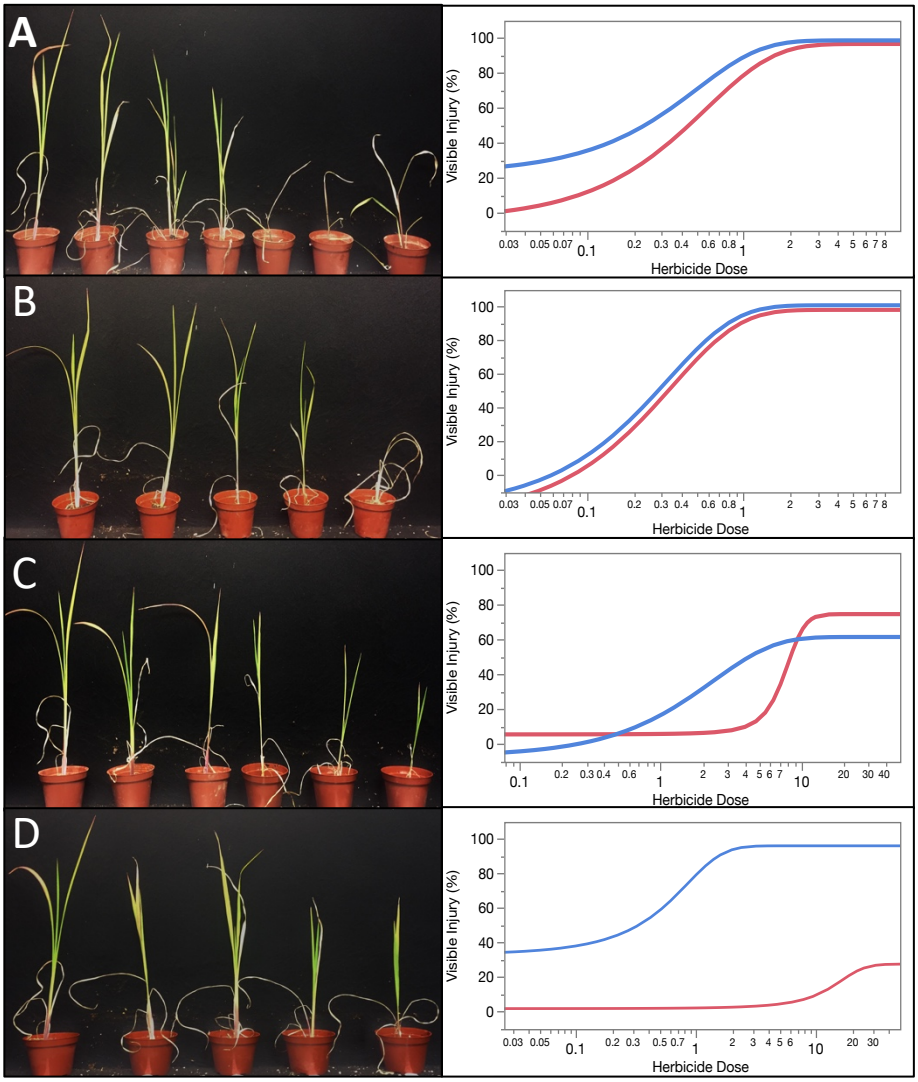
[2] Abbreviations for the plant sections: Trt=Treated leaf, AL= Tissues above the treated leaf; BL= Tissues below the treated leaf; RT= roots

[3] Means in italics for ECO-R and ECO-S are different according to a t-test ($p \leq 0.05$)

Table 5. Enzyme inhibitors used to assess the potential involvement of enzymatic detoxification for herbicide resistance.

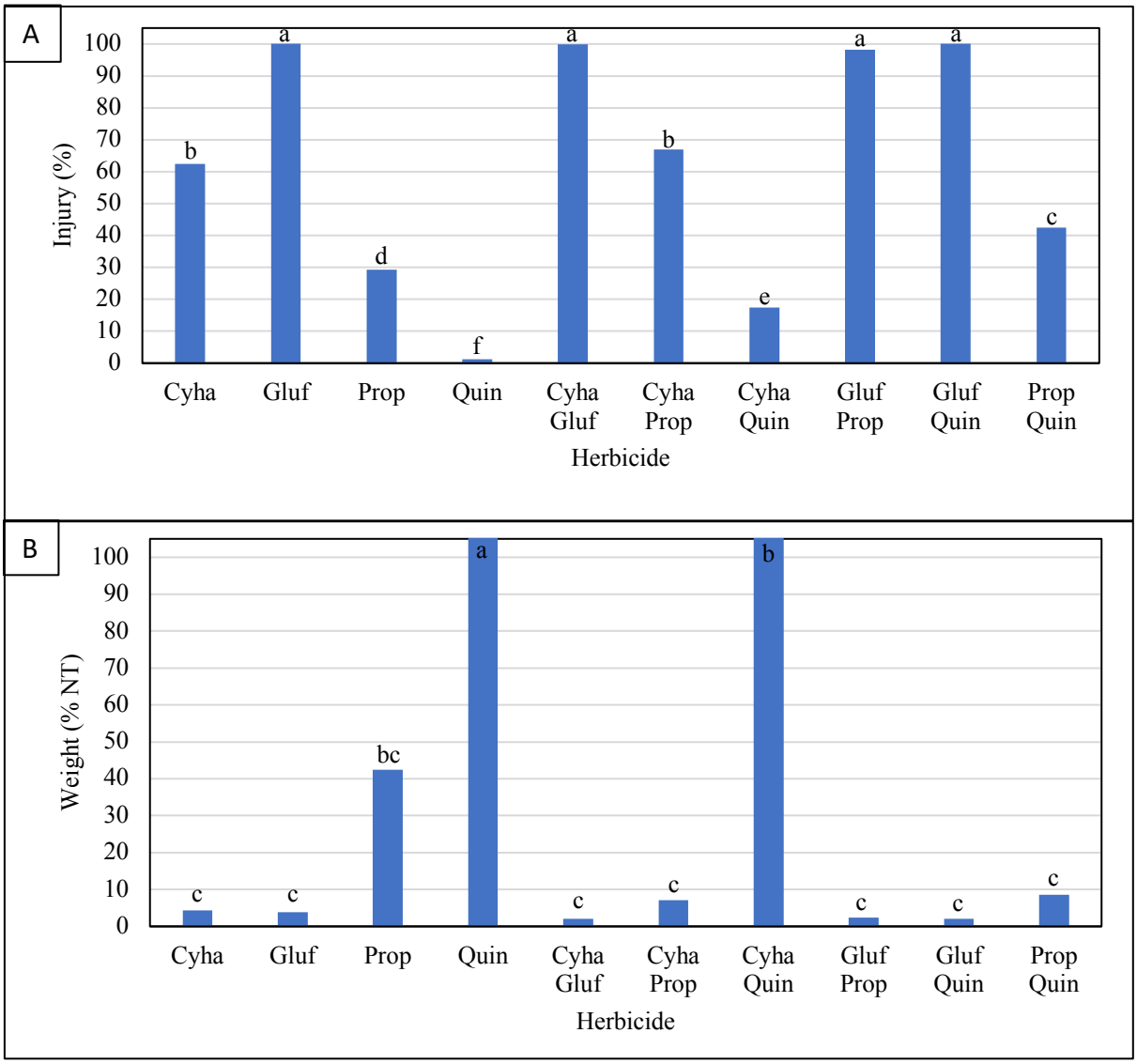
Inhibitor	**Rate**	**Trade name**
	kg ai ha^{-1}	
No inhibitor	-	
Carbaryl	1.1	Sevin®
Malathion	0.99	Hi-Yield®
Piperonyl butoxide (PBO)	1.2	exponent®

Figure 1. Nonlinear regression analysis of the herbicide dose (x-axis) on a log scale and the visible injury (y- axis) for ECO-R (red) and ECO-S (ECO-S), including the visual depiction of ECO-R to cyhalofop (A), glufosinate (B), propanil (C), and quinclorac (D) 3-weeks after application.



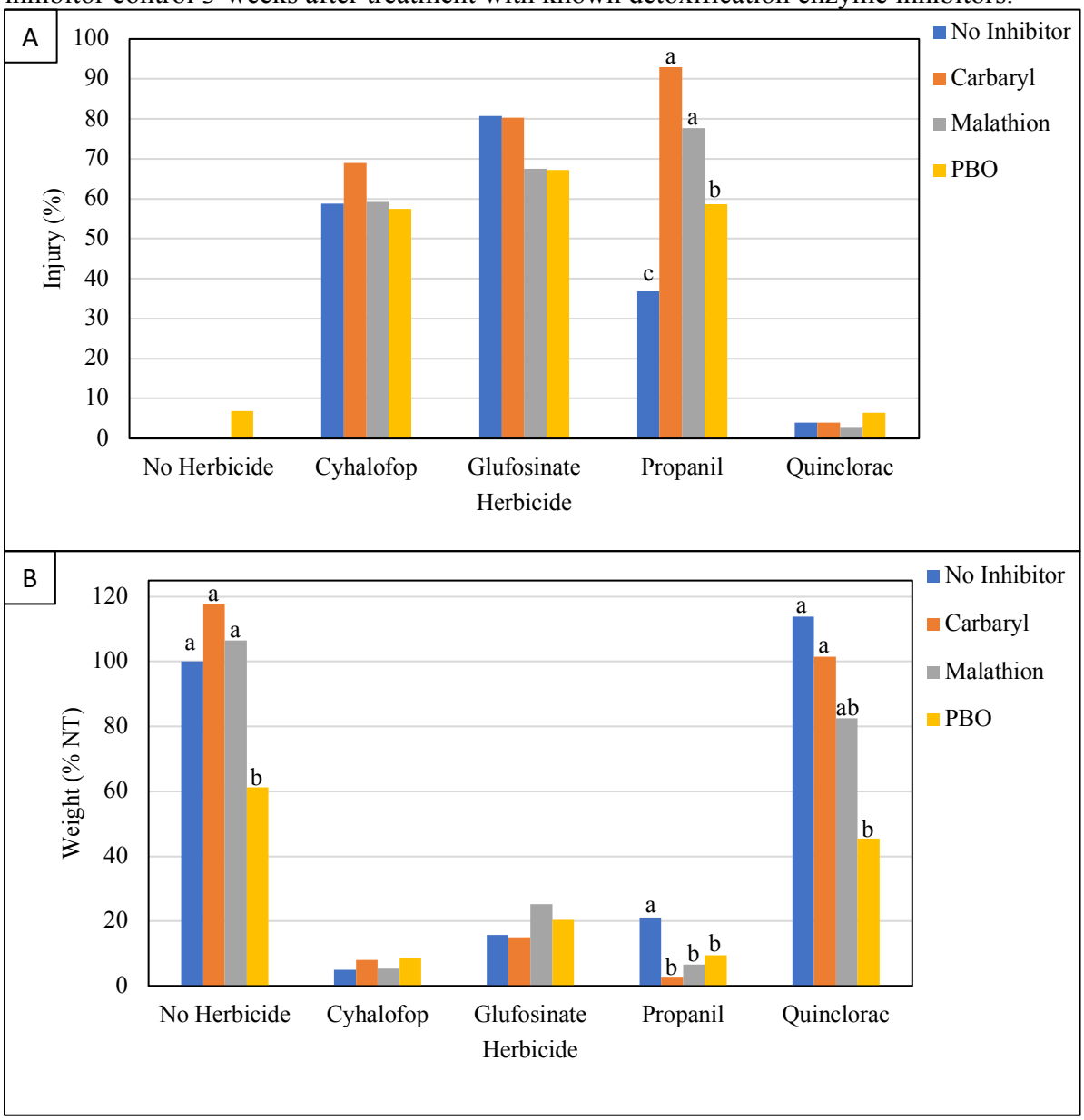
[1] Pictures presented include the control plant on the far left with the increasing doses to the right for each respective herbicide.

Figure 2. Visible injury (A) and fresh biomass (B) as a percentage of the no-herbicide control 3-weeks after treatment for the tank mixture evaluation conducted on ECO-R.



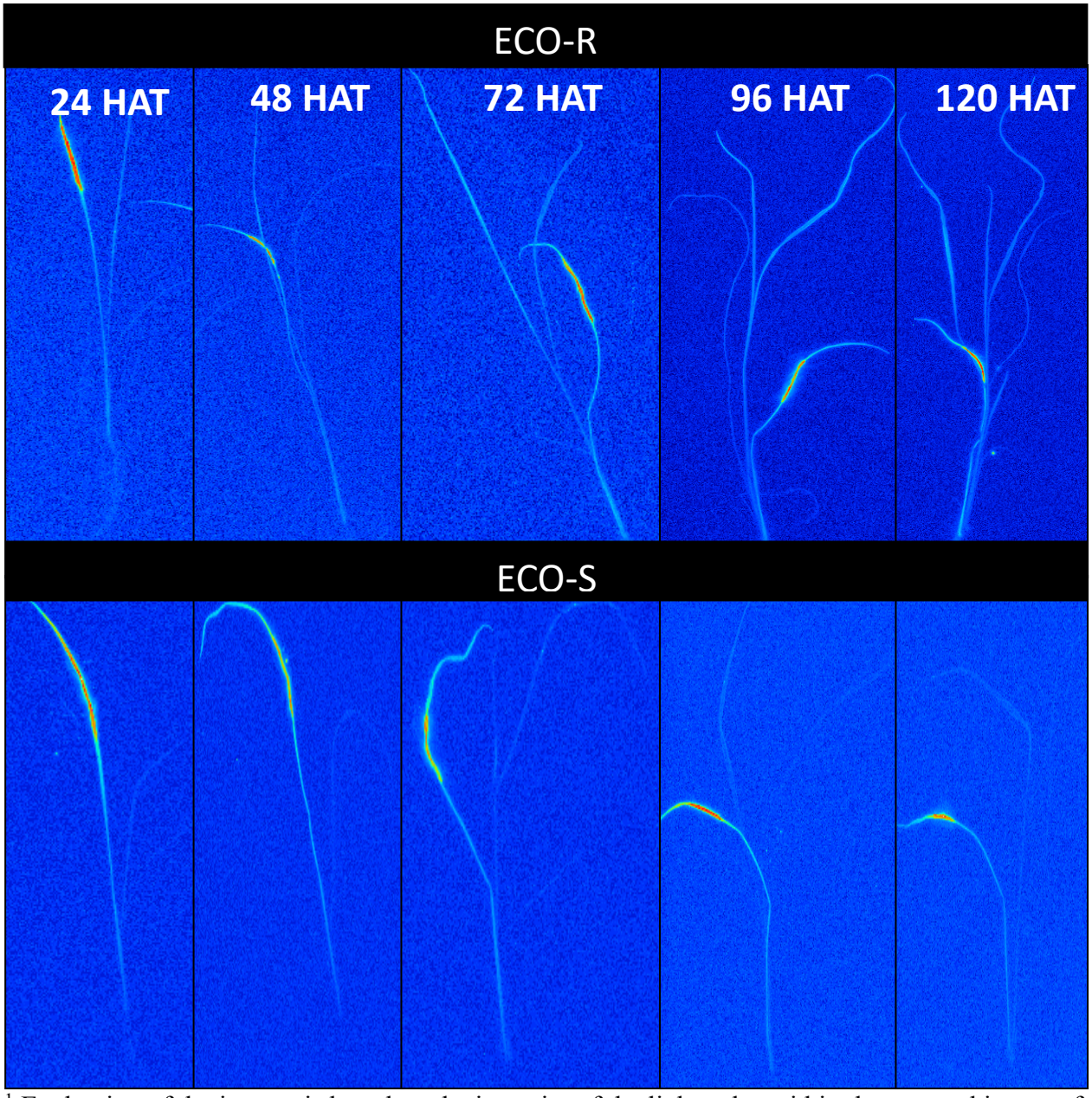
[1] Bars with the same letters are not significantly different according to Fisher's Protected LSD (α=0.05)

Figure 3. Visible injury (A) and fresh biomass (B) as a percentage of the no-herbicide and no-inhibitor control 3-weeks after treatment with known detoxification enzyme inhibitors.



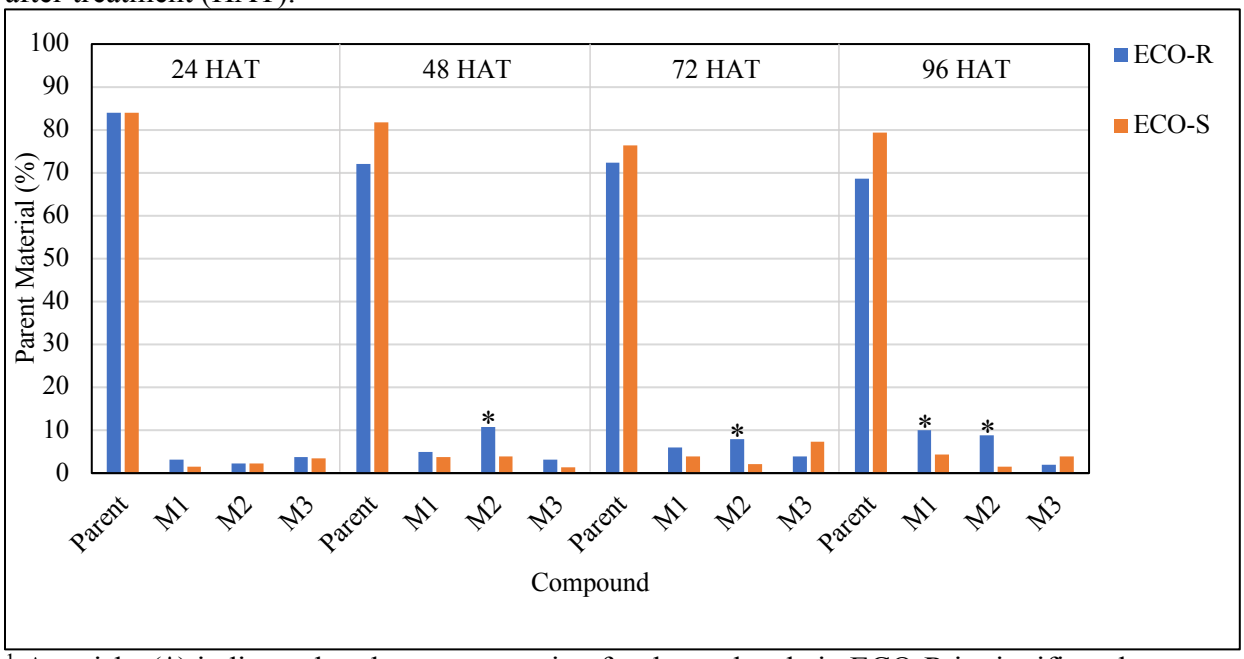
[1] Bars with the same letters are not significantly different according to Fisher's Protected LSD (α=0.05)

Figure 4. Phosphorimages depicting the quantity of ^{14}C-quinclorac and its distribution from the treated leaf throughout the plant at 24-, 48-, 72-, 96-, and 120-hours after treatment.



[1] Evaluation of the images is based on the intensity of the light color within the scanned image of the plants; areas of red contain the highest concentration of the ^{14}C-quinclorac.

Figure 5. Quantity of absorbed ^{14}C-quinclorac as the parent molecule and three unknown metabolites in the treated leaves of ECO-R and ECO-S harvested at 24-, 48-, 72-, and 96-hours after treatment (HAT).



[1] Asterisks (*) indicate that that concentration for the molecule in ECO-R is significantly different than in ECO-S according to a t-test ($p \leq 0.05$).

Appendix

Appendix Table 1. Quantity (%) of the absorbed ^{14}C-cyhalofop and propanil in the plant tissues at the five timings.

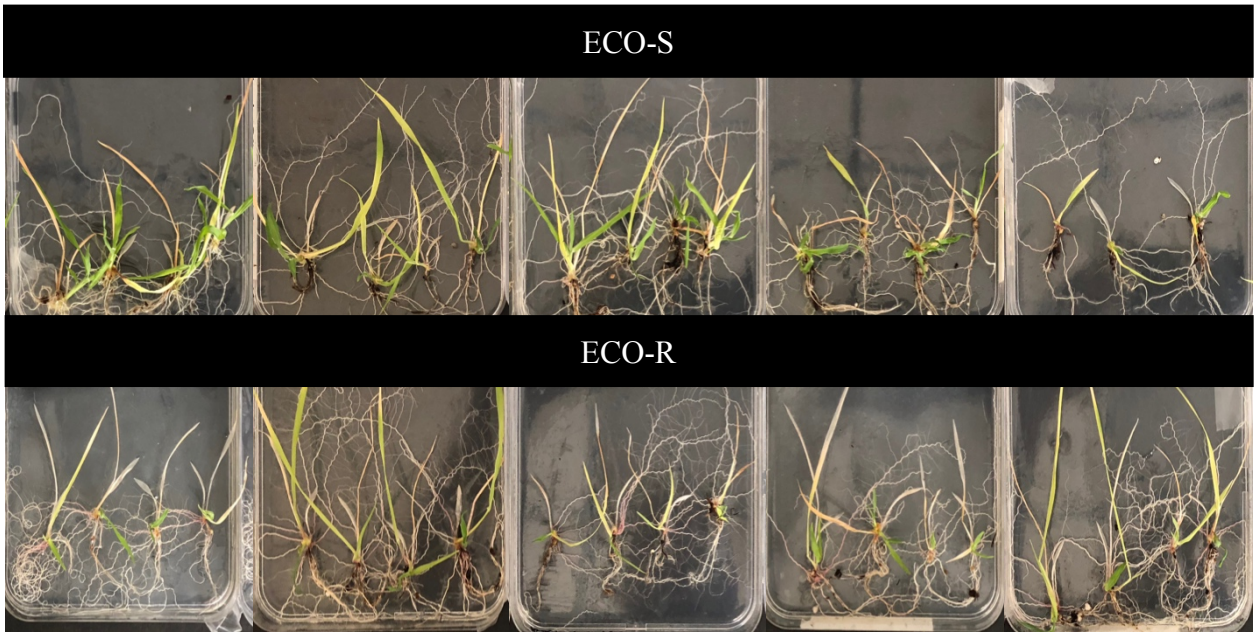
		Cyhalofop[3]		Propanil	
Timing[1]	**Section[2]**	**ECO-R**	**ECO-S**	**ECO-R**	**ECO-S**
Hours		----------%----------			
6	Trt	83	91	67	64
	AL	4	2	1	0
	BL	2	1	1	0
	RT	2	1	1	1
12	Trt	91	88	76	66
	AL	1	4	0	1
	BL	1	1	0	0
	RT	1	1	0	1
24	Trt	94	94	80	77
	AL	1	1	0	1
	BL	1	1	0	0
	RT	0	0	0	1
48	Trt	94	96	*90*	*84*
	AL	0	3	0	1
	BL	1	1	0	0
	RT	1	0	0	1
72	Trt	96	96	92	90
	AL	1	1	0	0
	BL	1	2	0	0
	RT	0	0	0	0

[1] Timing is presented as hours after treatment

[2] Abbreviations for the plant sections: Trt=Treated leaf, AL= Tissues above the treated leaf; BL= Tissues below the treated leaf to the soil line; RT= roots

[3] Italicized numbers for ECO-R and ECO-S within the respective herbicide indicate that the means were significantly different according to a t-test ($p \leq 0.05$).

Figure 1. Results of the topical foliar and root absorbed cyanide toxicity assay for ECO-S and ECO-R evaluated 1 week from transplanting at 0, 10, 25, 50, and 100 PPM KCN (left to right).



High resistance to quinclorac in multiple-resistant *Echinochloa colona* involves adaptive co-evolution of abiotic stress- and xenobiotic detoxification genes

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Abstract

Adaptation is a critical component of weed biology, allowing for the ability of weedy species to respond to adversity and evolve to persist within agricultural landscapes. A unique multiple herbicide-resistant population of *E. colona* (ECO-R) was collected from a rice field in Arkansas, USA, and previously profiled for its level and mechanisms of resistance. Results from these experiments implicated an unknown xenobiotic detoxification enzyme as the cause of resistance to quinclorac but further research into the specific gene was required. The following research presents the first *de novo* transcriptome from RNA-sequencing data and examination into the biological networks and gene expression patterns in a multiple-resistant and susceptible (ECO-S) *E. colona*. The *de novo* transcriptome identified 60,530 assembled genes from 109,539 transcripts. Constitutive gene expression, without herbicide treatment, was investigated between ECO-S and ECO-R implicating the induction of several plant growth and maintenance processes such as carbon metabolism and photosynthesis, as well as the trehalose biosynthetic processes which were enhanced by ECO-R. Following quinclorac treatment in ECO-S, 3,926 genes were induced and included several xenobiotic detoxification genes and the induction of the established quinclorac mediated ethylene pathway. ECO-R response to quinclorac was much different, with only 74 genes being induced following treatment. One gene of interest, a glycosyltransferase gene-UGT75D1, was upregulated near 9-fold following quinclorac treatment. The high levels of trehalose induction prior to herbicide treatment and lack of change following treatment, indicates that a ready source of UDP-glucose could serve as the conjugate required for modification via UGT75D1. This mechanism may be due to the presence of ALPL1, an antagonist of an epigenetic repressor protein, induced by stress. This research provides the first characterization of the potential association between an abiotic stress mediating process- trehalose biosynthesis,

and a xenobiotic detoxification gene-UGT75D1. The RNA-sequencing provides the first *de novo* transcriptome and subsequent global expression characterization of multiple-resistant *E. colona*.

Introduction

Echinochloa spp. include highly diverse weedy members that are distributed globally, posing a threat to upland and lowland agricultural systems [1,2]. The genus is composed of several species well adapted to both dryland and flooded agriculture. Some species within the genus are cultivated as millet crops in underdeveloped regions, providing a needed nutrition source; but the majority are weedy and invasive [3]. While there is significant diversity within the genus, several species including the dominate *E. colona* (junglerice) and *E. crus-galli* (barnyardgrass) are phenotypically similar [4]. A history of co-domestication and continued selection in rice (*Oryza sativa L.*) culture systems have resulted in crop mimics within these species [5,6]. In the USA, 13 *Echinochloa* species have been recognized in 48 of the contiguous United States [7]. Of these, the most impactful in agricultural areas, specifically in rice and rice-based rotation crop systems, include *E. colona, E. crus-galli, E. phyllopogon* (late watergrass), and *E. oryzoides* (early watergrass). These species impact every major agricultural commodity including alfalfa (*Medicago sativa L.*), cotton (*Gossypium hirsutum L.)*, nut, perennial fruit, rice, soybeans (*Glycine max L.*), and several vegetable crops [8]. A single *E. crus-galli* plant has the ability to reduce rice yield by up to 65 kg ha^{-1}; it is second only to weedy rice (*Oryza sativa L.*) in terms of impact to production [9,10]

Rice is considered a minor crop in the USA, however, the USA currently ranks third in export value contributing 10% of global exports [11]. In order to maximize production, weeds must be controlled as they are the most limiting biotic factor in rice production [1]. Propanil, a photosystem II inhibitor, was the first highly effective and selective *Echinochloa* herbicide in rice; this was followed by quinclorac, an auxin mimic, and several herbicide chemistries that disrupt fatty acid and amino acid synthesis [12]. Quinclorac has a unique mode of action in grass

species that makes it highly effective on *Echinochloa* (Fig 1). In dicots, quinclorac (like other auxin mimics, i.e., dicamba or 2,4-D) disrupts auxin regulation, causing elevated ethylene and abscisic acid (ABA) production, which results in uncontrolled cell elongation and growth, ultimately leading to plant death [13]. In monocots, quinclorac induces production of cyanide to toxic levels that results from excessive induction of ethylene in response to quinclorac [14]. Rice and other grass crops have a modification in the aminocyclopropane-1-carboxylic acid synthase (ACC synthase) enzyme that allows for selective induction of ACC synthase, providing insensitivity to the herbicide [15]. While the mechanisms have been described biochemically, transcriptome analysis may reveal details in the signal cascade that would improve or clarify our current understanding of how grass species respond, and adapt, to herbicidal auxin mimics.

Herbicide resistance in weedy species is an adaptive evolutionary trait selected for by repeated herbicide application. This is in contrast to herbicide tolerance in crop and weed species which results from underlying mechanisms that reduce herbicide response at the species level and develop independently in the absence of herbicide selectors [16]. Two terminologies are used to describe herbicide resistance: target-site resistance (TSR) and non-target-site resistance (NTSR). TSR pertains to a modification in amino acid sequence of an enzyme the herbicide inhibits, resulting in reduced binding efficiency of the herbicide. NTSR encompasses diverse mechanisms including a number of physiological, biochemical, and structural responses that work via cascading processes leading to detoxification, redistribution, or sequestration of an herbicide, reducing the concentration of the herbicide at the site of action [17,18]. These mechanisms are the least understood and most problematic as they may result in broad-spectrum resistance to other herbicides and enhanced abiotic stress tolerance. *Echinochloa* species have evolved herbicide resistance using both mechanisms: TSR to multiple amino acid synthesis

inhibitors [19], glyphosate [20] and photosystem II inhibitors [21]; and NTSR to amino acid synthesis inhibitors, clomazone, propanil [22], and quinclorac [23].

Historically, research into the mechanisms of herbicide resistance has been limited to monogenic or single trait response characterization in weedy species. This is due to both a limitation in resources to investigate global genetic response patterns and a lack of understanding of the potential role that these responses may have on herbicide resistance. Evolution occurs through adaptive responses that modify existing biological pathways and the underlying processes that contribute to these pathways, allowing for survival. These modifications not only change the pathway, which is being acted upon, but also the interconnection of biological networks. Herbicide resistance traits do not evolve independent of other genetic and physiological factors. Research using advanced genomics techniques in weed science is currently limited; however, the demand for understanding herbicide resistance at a higher level will increase the utility of this type of research.

In this work, we present the first assembled transcriptome of *E. colona* from a susceptible (ECO-S) and a multiple-resistant (ECO-R) population under herbicide stress. We provide an in-depth characterization of the *E. colona* gene expression profiles and use this information to describe and compare the response of these biotypes to quinclorac. We identified and mapped the constitutive biochemical pathways that are involved in herbicide resistance and plant response to abiotic stress. This resistance mechanism is dependent on the constitutive induction of trehalose biosynthesis in the absence of the herbicide and the induction, following herbicide treatment, of a specific glycosyltransferase gene to conjugate the quinclorac molecule with UDP-glucose. The biochemical response of the resistant phenotype is vastly different from that of the

susceptible one and demonstrates the divergence in evolution that occurs under immense herbicide selection pressure.

Results

De Novo transcriptome assembly and functional characterization of *E. colona*

The *de novo* transcriptome assembled for *E. colona* represents two-week-old leaf tissue, 24 hours after treatment with (ECO-R-T) and without quinclorac (ECO-R-N). The transcriptome was assembled from 545,000,000 raw read pairs, which generated over 109,000 transcripts (Table S1). Analysis of conserved plant ortholog sequences (BUSCO) revealed that approximately 75% of the transcriptome was resolved. Functional annotation revealed 60,530 genes retained, which were used to characterize the transcriptome. Homology to other organisms was as expected given the parameter of the annotation. However, sequence homology to *Oryza sativa* var. *japonica* (17.7%) is of value given the early co-domestication of these species, and their co-evolution throughout the history of rice production [5,6].

Constitutive difference in gene expression and gene networks between ECO-S-N and ECO-R-N

Gene network enrichment. Overall, transcription-, protein translation-, and protein synthesis-related terms were enriched in ECO-R-N and ECO-S-N (Figure 2). However, the gene ontology analysis yielded several biochemical pathway features that are enriched for ECO-R-N relative to ECO-S-N. A supercluster of terms identified as ‘trehalose metabolism in response to stress’ was enriched in ECO-R-N. Within this cluster were terms that include: response to herbicide and nitrate, nitrate assimilation, positive regulation of transcription factor catabolic process, and trehalose metabolism in response to stress. The nitrate responses were expected given the enriched nitrate transport and their connection to trehalose synthesis.

Plant growth and maintenance activity. Constitutive gene expression differed by 2,475 genes between ECO-R-N and ECO-S-N, with ECO-R-N having the greater gene expression (2,127); the majority of which were annotated (70%) (Table S2 and S3). Genes associated with growth functions such as carbon metabolism and photosynthesis were greatly enhanced in ECO-R-N. Photosynthesis-related genes such as ferredoxin-6 (4), ATP synthase subunits, (<4), NADH-cytochrome b5 reductase (5.4), and photosystem II core complex proteins psbY (4.8) were all elevated in ECO-R-N over ECO-S-N. Carbon assimilation genes were also induced: malate dehydrogenase (6.3), aspartate aminotransferase (5), phosphoenylpyruvate carboxylase (PEPC) kinase (4.7), pyruvate dehydrogenase subunits (>4.2), transketolase 1 (5.4) and the glycolysis component- triosephosphate isomerase (4.4). Both acetyl-CoA (3.4) and acetyl-CoA 2 (3.3) were induced. This indicates demand and utilization of products from carbon assimilation and photosynthesis in fatty acid metabolism. Nitrogen metabolism-related genes, specifically high affinity nitrate transporter-activating protein 2.1 (3.1 to 4.2), nitrate reductase (3.9 to 7.6), and glutamine synthetase (5.9) were induced. All these were indicative of higher level of biological activity in the resistant- than in the susceptible accession. Twelve DNA transcription factors, with ranging activities, were less abundant in ECO-R-N. Several MYB44 transcripts were induced (2.2 & 8.1). These have a role in abiotic stress response via ABA-inducible processes under drought stress [24]. The elevated activities of DNA ligase (4.2), DNA repair protein RAD16, and several DNA polymerase proteins, indicate higher-level activities of ECO-R-N.

Sugar metabolism and transport activity. Trehalose metabolism was a biological function supercluster that was significantly enriched containing multiple GO terms. Twenty-three transcripts, for eight genes in the trehalose pathway were enhanced in ECO-R-N compared to ECO-S-N. Five were α, α-trehalose-phosphate synthase UDP-forming enzymes (TPS) and three

were probable trehalose-phosphate phosphatases (TPP). These genes all feature in abiotic stress response and stress tolerance [23]. Their enhanced constitutive expression in ECO-R-N is unique given these plants were not grown under stress. Sugar transport protein 14 (3.3 & 7) and bidirectional sugar transporter SWEET2a (3.3) were also induced, serving as transporters for this elevation in trehalose sugar quantities.

Ethylene biosynthetic pathway activity. Induction of the ethylene biosynthetic pathway is a major component of plant response to quinclorac. The activity of ACC-synthase was repressed in ECO-R-N (-7.4). Two forms of ACC-oxidase homolog 11 were observed, one was repressed (-3.4) and the other enhanced (2.3). Several ethylene-responsive transcription factors (ERF) were constitutively expressed, indicating heightened transcriptional activity to effect ethylene-mediated responses. Six ERFs were repressed, all involved in transcriptional repression, while 11 ERFs related to transcriptional activation were enhanced. The majority of these ERFs, both repressors and activators, bind to the GCC-box pathogenesis-related promoter element. This promoter element is linked to stress tolerance and signal transduction in response to disease, cold, salt, and/ or water deprivation stress [25,26]. In ECO-R-N an elevated ethylene insensitive protein (EIN) 3 (2.7) gene was present; when in complex with ERF1 (2.5), both acts as component in ethylene signal transduction, bacterial defense, and hypoxia response, as well as sugar mediated signaling [27].

Xenobiotic detoxification genes present at the constitutive level. Genes within several xenobiotic detoxification gene families were differentially expressed in ECO-R-N relative to ECO-S-N (Table 1; Figure 3). Seven ABC transporters were identified, six of which were enhanced. Three cytochrome P450 enzymes were enhanced in ECO-R, including CYP90D2, CYP94C1, and CYP71A21. CYP90D2 is a component of brassinosteroid synthesis [28] ;

CYP94C1 is involved in the oxidation of the phytohormone jasmonyl-L-isoleucine and wound response [29]; and CYP71A21 is involved in secondary metabolite biosynthesis with no described functions. The cytochrome P450 enzymes are associated with transmembrane movement of compounds and phytohormones. Their constitutive upregulation indicates possible involvement in intrinsic stress tolerance in ECO-R.

Two glutathione-S-transferase (GST) enzymes were identified as well. One was GST1, which aids in glutathionylation of proteins, and the other was GST-T3, which conjugates glutathione to various hydrophobic electrophiles. GST-T3 has been implicated in detoxification of herbicides based on its sequence similarities to like proteins within the Uniprot database. Seven glucosyltransferase (GT) enzymes were induced in ECO-R. Three of these (UGT83A1, UGT73C2, and UGT90A1) are involved in the transfer of the glucosyl group from UDP-glucose to either the 3- or 7-hydroxy group on the quercetin molecule; a flavonol with auxin transport inhibitor and antioxidant activities. UGT73C1, has similar quercetin activity but also transfers glucose to cis- and trans-zeatin and can detoxify 2,4,6-trinitrotoluene (TNT) in plants by forming O- or C- glucosides [30]. UGT74D1, is unique in that it glycosylates indole-3-acetic acid (IAA), a natural auxin similar to quinclorac. Several aminotransferase, amylase, hydrolase, and peptidase enzymes were also expressed, indicating active modifications of biomolecules. These enzymes may also be involved in natural growth processes, but are not necessarily related to herbicide resistance.

Plant abiotic stress signaling activities. Several biological pathways, including some of the aforementioned gene families and genes of the auxin-, peptide-, and abscisic acid response pathways, are involved in plant signaling [31–33]. Enhanced auxin response factors such as ARFSAUR72, ARF13, IAA19, and IAA30 primarily serve as transcription factors that bind to

promoter sequences, modulating gene expression following auxin signaling. In the quinclorac response pathway the production of ABA results in stomatal closure, limiting photosynthetic activity and disrupting electron flow in the photosystem complexes, causing irreparable cellular damage [32,34]. Disruptions in ABA signaling could be a source for limiting the negative effects caused by herbicide application. This would result in less stomatal closure which may lead to a build-up of free energy which results in cell membrane disruption. Two ABA receptor proteins PYL8 (3.8) and PYL5 (2.1), had enhanced expression in ECO-R, indicating that the plant is producing the necessary components to receive ABA signals [35] . However, four forms of ABA 8'-hydroxylase 1 were also enhanced (4.5 to 6.4-fold change); these are oxidative enzymes involved in catabolizing ABA. This means that although some ABA receptors are produced, there was insufficient ABA to transport. Several calcium receptors, components of the ABA signaling process [36], were repressed in ECO-R: CML45, CML46, and CRLK1. CRLK1 is unique in that it is also required for cold tolerance, which is enhanced by increasing calcium concentrations. The majority of the calcium-signaling-related genes are involved in transport or calcium perception. CPK5 (2.2), is a receptor that regulates reactive oxygen species (ROS) by directing kinase activity to the NADPH-oxidase [37]. Given the elevated levels of ABA-catabolizing enzymes and a reduction in several calcium receptors, it is possible that ECO-R is less sensitive to stress-induced cellular destruction through avoidance mechanisms [36]. One protein of note, with several transcripts constitutively repressed (-12 to -7) and enhanced (3.5 to 6.4) in ECO-R-N, is protein ALP1-like (ALPL1). Not much is known about ALPL1 other than it is analogous to the ALP1 protein, which is a stress- responsive transcription factor that antagonizes Polycomb group (PcG) proteins [38,39]. PcG proteins rest on sections of target DNA repressing the transcription of the subsequent proteins. ALPL1 may possess significant

epigenetic functions that may assist in the herbicide resistance response via activation of DNA segments allowing for transcription of needed genes and enzymes.

Coordinated gene expression following quinclorac treatment in ECO-S

Gene network enrichment. ECO-S-T had enriched GO terms for 25 biological functions, 2 cellular components, and 16 molecular functions (Table 2). The frequency is presented and provides information on the frequency of the GO term in the underlying GOA database, the lower the value the more unique and specific the term is for its function[40]. The ethylene-activated signaling pathway and the abscisic acid-activated signaling pathway were enriched, both of which would be a direct response to the herbicide. Anaerobic respiration, detection of hypoxia, response to hypoxia, response to oxidative stress, and the oxidation-reduction enriched terms indicate severe abiotic stress. Molecular function terms related to stress response were enriched including oxidoreductase, heme binding, peroxidase, and ABA 8'-hydroxylase activities. The enrichment of heme binding and oxidoreductase activity implies that cytochrome P450 enzymes, which are primary agents of phase I degradation of xenobiotic compounds, were induced following treatment. Nitrate assimilation GO terms similar to those observed in ECO-R-N were also enriched in response to quinclorac.

Quinclorac response pathway. Three transcripts within the ethylene response pathway were repressed following treatment: ACC synthase (-2.7), ACC oxidase (-2.8), and ACC oxidase homolog 3 (-2.2). Several transcripts were induced: two forms of ACC oxidase 1 (2.2 and 2.8) and four forms of ACC oxidase homolog 11 (1.6 to 6.4). The repression of ACC synthase, paired with the induction of multiple ACC oxidase transcripts, reflects the increase in ACC synthesis following quinclorac treatment. VP14 (9-cis-epoxycarotenoid dioxygenase [NECD]) is the first enzyme in ABA biosynthesis and is also a component of plant response to quinclorac [14].

Following treatment, VP14 was repressed (-6.4), indicating that by 24 hours sufficient ABA had been synthesized and feedback inhibition was occurring. Twelve ERF genes were repressed following treatment and 15 ERF genes were enhanced, similar to what was observed in ECO-R-N. The majority of the repressed genes were transcriptional repressors and the induced genes were transcriptional activators that interact with the GCC-pathogenesis promoter involved in stress signaling in plants. EIN2 (3.9) is a unique central factor in many signaling pathways including those related to plant development and defense as well as gene regulation and perception of environmental cues [41]. RAP2-2, another unique enzyme with enhanced expression (1.9), is a transcriptional activator for the promoter of phytoene synthase and desaturase enzymes in the carotenoid biosynthetic pathway [42]. This response has not been described previously; however, it is expected given the downstream effect of ABA synthesis resulting in stomatal closure. This has the potential to lead to an accumulation of light energy producing free radical or reactive oxygen species (ROS) following herbicide treatment.

Herbicide detoxification gene expression following quinclorac treatment. A total of 210 genes categorized as components of the detoxification process were identified in ECO-S-T (fig 3b). Fifty-two transcripts representing 33 ABC transporter genes were identified. The ABC transporters characterized in ECO-S-T perform various biological compound movement activities. ABCB5 (-6.5) has known auxin efflux transport activity and ABCC10 (-6.1), as well as several other repressed proteins, are glutathione S-conjugate pumps based on sequence homology. Seventy-four cytochrome P450 enzyme transcripts were differentially expressed; 47 were repressed. Sixteen annotated cytochrome P450 genes were induced (1.5 to 7.8). These have roles in secondary metabolite biosynthesis, brassinosteroid biosynthesis, and stress response. Eleven transcripts, representing six repressed genes and four induced genes, were GST enzymes.

The induced genes- GSTT3, GSTU8, and GST4, involve the conjugation of glutathione to hydrophobic electrophiles. The gene family of note, GT, comprised the second most observed transcripts (64) following treatment. UGT83A1, UGT74D1, UGT75C1, UGT73E1 were upregulated in both ECO-S-T and ECO-R-N, indicating their involvement in plant maintenance but also possibly in general plant stress response. The majority of these GT enzymes are involved in glycosylation to C- and O- side groups. Two genes of note, UGT74F2 and UGT74E2, have known interactions with auxin compounds, like quinclorac. UGT74F2 glycosylates benzoic acid and benzoic acid derivatives, similar to the herbicide dicamba, another plant growth regulator used in weed management [43]. UGT74E2 interacts with endogenously produced indole-3-butyric acid (IBA) altering auxin homeostasis which results in stress-induced morphology changes [44]. The variety and high quantity of xenobiotic detoxification transcripts observed following quinclorac treatment again indicate coping mechanisms against elevated stress, none of which were effective for quinclorac detoxification.

Stress responsive genes and signaling response. To best characterize the whole plant response to quinclorac, we need to study stress-specific genes. These stress-responsive genes may produce a wide variety of proteins that could potentially stabilize cellular structure and function or facilitate stress signaling. In total, 247 transcripts that could be categorized as abiotic or biotic stress proteins were differentially expressed; 99 were repressed and 142 were induced following treatment. Fifty-eight disease resistance genes with hypersensitive activity in response to bacterial avirulence proteins were enhanced. The hypersensitive response, which results from a buildup of hydrogen peroxide, could potentially limit the movement of herbicide in the plant [45]. Eight heat shock proteins were induced. A total of 64 peroxidase transcripts were induced (3.1 to 8). Peroxidases are protection agents against cellular damage by free radicals. Abscisic

stress-ripening protein 1 (ASR1) was also enhanced. This is associated with plant response to water deprivation, a process that leads to enhanced ABA production to mitigate water loss [46]. Five transcripts for ALPL1 were repressed by as much as -12 to -1.4-fold while three transcripts for were induced but only to as much as 3.5-fold.

Coordinated gene expression following quinclorac treatment in ECO-R

Gene network enrichment. None of the GO terms described in the ECO-S response to quinclorac were observed in ECO-R-T. In ECO-R-N, the majority of the enriched terms were involved with plant growth and maintenance processes, having no relationship to herbicide response. Fifty-three terms were significantly depleted in ECO-R-N relative to ECO-R-T. While most were irrelevant, several were related to stress responses including cold stress-, ABA-, and salicylic acid genes; plant-type hypersensitive response; and general plant defense response. No terms were enriched in ECO-R-T.

Plant growth and maintenance processes. The majority of genes coding for proteins in major metabolic pathways (photosynthesis, carbon metabolism, respiration, and fatty acid synthesis) were repressed in ECO-R-T; none were induced. Among the repressed transcripts were ATP synthase subunit (up to -12.3-fold), pyruvate dehydrogenase subunits (up to a -12-fold), cytochrome c oxidase proteins (-8.4-fold), and both acetyl-CoA and acetyl-CoA 2 proteins (up to -10.2-fold). In general, following quinclorac application, ECO-R appears to repress all non-essential processes.

Quinclorac-mediated response. Many genes in ECO-R were downregulated following treatment (5,311 transcripts), and only a minimal increase in gene expression (74 transcripts) was observed (Table S2). This pattern of expression implies that the constitutive upregulation of certain genes is a major mechanism contributing to quinclorac resistance in this plant. ACC

synthase was upregulated 6.3-fold in ECO-R-T. Given that there were no differences in the ACC oxidase transcripts without quinclorac, it appears that the plant is responding positively to quinclorac, but without the expected overload of ethylene. In ECO-R-T ALPL1 was present with a greater abundance in transcripts- 7.4-fold upregulation, implicating it in the *E. colona* response to quinclorac. However, given the significant increase in expression its potential value in ECO-R-T must be considered.

Xenobiotic detoxification gene expression. Over 100 detoxification-related transcripts were differentially expressed following quinclorac treatment; 84 were repressed and 17 were induced (Fig 3c). Only one of the ABC transporters, ABCD2 (-9.2) which were elevated in ECO-R-N was repressed following treatment. Four cytochrome P450 genes were upregulated following treatment: CYP709B1, four forms of CYP709B2, three forms of CYP72A15, and CYP89A2. CYP72A15 is the only gene, which was constitutively upregulated in ECO-R-N and upregulated further in ECO-R-T. This may indicate its necessity following herbicide application or that it is stabilizing an affected plant process. The three remaining CYP genes have stress response properties, potentially involved in phase I chemical degradation. UGT73D1 (5.3) with quercetin O- activity and UGT75D1 (7.3) with potential xenobiotic detoxification activity, based on homology, were upregulated in response to quinclorac. UGT75D1, with the greatest induction, is uniquely involved in glycosylation of indole-3-acetate, which is a growth hormone that is structurally analogous to quinclorac [47]. UGT73E1 was upregulated (2.6) following treatment in ECO-R-T. Transcripts for this gene were enhanced in ECO-R-N (6.3) and in two forms were present in ECO-S-T (-4.6 and 5.9). This gene may be involved in herbicide resistance given its elevated expression in ECO-R and repression in ECO-S following treatment. Given that multiple

transcripts for various forms of the gene are present, the polyploidy of *E. colona* may have a role in its action and the genome from which this gene is expressed may play a role in resistance.

Comparative network enrichment and gene expression following quinclorac treatment between ECO-S-T and ECO-R-T

Gene network enrichment. Comparison between ECO-S-T and ECO-R-T revealed five ontological terms that were enriched in ECO-R-T related to carbohydrate biosynthesis: galactose-1-phosphate guanalyltransferase activity, GDP-L-galactose phosphorylase activity, GDP-D-glucose phosphorylase activity, and the reductive pentose-phosphate cycle. The term L-amino acid efflux transmembrane transporter was also enriched in ECO-R-T. No other enriched or depleted terms for ECO-N-T or ECO-R-T were present in the comparison.

Quinclorac response pathway. Comparison across both of the treated samples provides an indication of the mechanisms that may be involved in herbicide resistance and/or general stress tolerance. In total, 595 transcripts were differentially expressed, 326 of which were repressed and 269 were upregulated (Appendix Table 2). 118 transcripts that were enhanced constitutively were repressed following treatment; 28 were that were repressed were enhanced following treatment in ECO-R (Fig 4). Of the 28 enhanced transcripts, one of note was the increased expression of ACC synthase (9), further suggesting that the quinclorac is reaching the target. There were six ERF transcripts induced for three genes: ERF4, EF8, and multiple forms of ERF11, all of which bind to the GCC-box pathogenesis promoter involved in stress response and signal transduction. ERF11 and ERF8 are transcriptional promoters while ERF4 is a repressor.

As previously noted, only one gene of significance within the ethylene pathway was induced at a higher level in ECO-R-T compared with ECO-R-N, ACC synthase (9), and again it was noted compared with ECO-S-T. The aforementioned VP14, involved in ABA synthesis and

induced in the quinclorac-mediated pathway, was not observed in this comparison but four forms of ABA 8'-hydroxylase were present at lower levels. Given this pattern, it did not appear as though there was a significant induction of the NECD required for ABA synthesis nor were the ABA concentrations high enough to warrant the hydroxylase enzyme. PYL5, an ABA receptor protein was also significantly repressed, -4.4-fold lower, in ECO-R-T compared to ECO-S-T. Reductions in ABA synthesis and reduced perception may increase abiotic stress tolerance and reduce the negative effects of herbicide application. The collective pattern of gene expression indicates that ECO-R-T is perceiving quinclorac at its expected target; however, there appears to be a significant reduction in auxin perception and signaling, which was reflected in reduced plant response to quinclorac.

Stress signaling. Several stress-related proteins were comparatively expressed including RVE2, part of cold-responsive gene expression and a response to auxin, NHL3 a bacterial resistance gene induced in response to wounding, and FAB1C a phosphorylating enzyme involved in stomatal closure. Several transcripts of note, which were comparatively repressed following treatment include RVE1, ILL4, ARF13, and ERF113. RVE1 regulates free auxin levels in a time-of-day manner and is a negative regulator of freezing tolerance, a counter to RVE2. ILL4 is a hydrolyzing enzyme of amino acid conjugates involving IAA, which may be of note considering the imbalanced perception of auxin caused by quinclorac. This is also evident in the repression of ARF13 transcripts whereby the auxin mediated pathways are not responding at the same level in ECO-R-T as they are in ECO-S-T. ERF113 is transcriptional activator involved in plant development and tolerance to abiotic stress specifically waterlogging; the expression of this gene in ECO-S-T and comparative repression in ECO-R-T further indicates a reduction in auxin signaling, which would be caused by a reduction in overall auxin perception. While little is

known about the function of ALPL1 it is evident based on the transcriptome profile, with transcripts ranging from 10.6 to 19.4-fold differences between ECO-S-T and ECO-R-T, that it possesses a major role in the ECO-R response to quinclorac. This high level of expression is the greatest among all transcripts in this different comparison condition.

Xenobiotic detoxification differences following quinclorac treatment. A greater number of xenobiotic detoxification genes were induced ECO-R-T than ECO-S-T, several of which had forms both repressed and induced (Table 3, Fig 3d). Aldolase, aminotransferase, amylase, hydrolase, peptidase genes are involved in the transfer of their respective conjugates or peptides to other proteins, which may or may not be directly involved in herbicide metabolism. While these may have a role in xenobiotic metabolism given the literature, more research needs to be conducted to adequately describe their roles in herbicide resistance. CYP89A1, CYP72A15, and CRYP71A9 were all repressed in ECO-R-T compared to ECO-S-T, consequently they are not involved in herbicide resistance. All of the cytochrome P450 genes with increased expression have been previously discussed and play a role in stress response except for CYP71A4. CYP71A4 was expressed to a greater extent in ECO-R-T (5.6) and has been described has having a role in maturation and metabolite production in older tissues[48]. This is interesting because these are young tissues and most maturation and secondary metabolite synthesis can be directed by ethylene under abiotic stress conditions. CYP71A1 was induced following treatment and is involved in the oxidation of flavoproteins during the fruit ripening process, this is important as this would indicate an ethylene induced response also characteristic of quinclorac activity [49]. CYP709B2 induction is also of interest given its induction by ABA and salt stress, both abiotic signals for plant response [50]. GSTU20, involved in toxic substance response and far-red light influence on development was present in ECO-R-T with a significantly higher

number of transcripts (6). The same GT enzymes enhanced in ECO-R-N and induced in ECO-R-T, compared with ECO-R-N, were present at higher levels in ECO-R-T compared with ECO-S-T. UGT75D1 is of great interest as a potential protein enabling resistance given the comparatively high expression in ECO-R-T (8.7) and its known activity on environmental toxins and xenobiotics.

Discussion

The role of constitutive gene induction in evaluating underlying differences in ECO-S and ECO-R

Transcriptome characterization of the physiological status of ECO-S and ECO-R, without herbicide, and its response to quinclorac, is key in understanding the signal cascade and whole-plant response of *E. colona* to this auxin-mimic herbicide. In ECO-R-N compared with ECO-S-N, DNA transcription and protein synthesis, as well as the ethylene activated signaling pathways were enriched. The enriched ethylene pathway involves several ethylene response transcription factor genes. They are linked to abiotic stress response as well as sequence-specific binding to a pathogenesis promoter sequence. A higher abundance of gene transcripts associated with plant processes associated with carbon uptake assimilation and energy production were observed in ECO-R-N. This high level of activities would support any number of functions necessary for resistance. More importantly, the elevated activities prior to herbicide action would allow the plant to tolerate adverse conditions following treatment. Among the constitutively enhanced genes in ECO-R-N were several associated with trehalose biosynthesis. This sugar produced by these enzymes, has been extensively studied for its role in abiotic stress tolerance but not in herbicide resistance. The increase in the number of transcripts prior to herbicide treatment may be an indication of the predisposition of this accession to tolerate negative herbicide actions

Immediate action following quinclorac treatment in ECO-S is a stark contrast to ECO-R ECO-S signal cascade. Ethylene- and ABA-activated signaling pathways were significantly enriched following quinclorac treatment indicating endogenous ethylene and ABA production. This was validated by several ABA mediated genes including ASR1, a water stress tolerance gene, which is stimulated by ABA concentrations [51]. Nitrate transporter activity was enriched, suggesting that the demand for proteins is elevated in response to quinclorac. This response is also linked to endogenous ethylene build-up, which has a stimulatory effect on nitrate uptake and assimilation within the plant [52]. It is evident that feedback inhibition of the ACC synthase has occurred by 24 hours resulting in depression of the ACC synthase due to the elevated ethylene concentrations [53]. To catabolize the built-up ACC from the initial stimulation by ACC synthase, two ACC oxidase genes were induced which would lead to the high ethylene and toxic cyanide concentrations. Concomitantly the NECD enzyme was also repressed to limit ABA production. Auxin and ABA catabolism responses were enriched to reduce the stimulatory effects of the exogenous auxin (quinclorac) response, and the endogenous ABA, respectively. ABA 8'-hydroxylase, was also induced to limit the concentration of ABA. ABA synthesis results in the closing of stomata, limiting water movement and gas exchange but also leads to the buildup of reactive oxygen species that cause tissue decay and senescence [54]. This was supported by several enriched processes related to anaerobic conditions and peroxidase activity, and further supported by the induction of RAP2-2. This gene is a transcriptional activator for the production of phytoene synthase and phytoene desaturase. Both enzymes are required for carotenoid biosynthesis, which would be necessary to mitigate the effects of excess energy build-up due to reduced electron flow resulting from stomatal closure. Collectively, these genes provide the underlying transcriptome response of *E. colona* following treatment. These are useful

in describing the herbicide action in the plant and may provide a basis for evaluating other herbicide typically found in rice production systems.

ECO-R Signal Cascade. The gene expression profile for ECO-R-T was somewhat unexpected given the high level of resistance in this population. Most genes were repressed following treatment and gene ontology terms were not enriched. The gene expression profile indicated that the plant is repressing most processes following treatment and energy is expended on only a small number of genes/ functions. The repressed pathways include photosynthetic, carbon assimilation, carbon metabolism, respiratory, and fatty acid synthesis pathways. Acetyl-CoA was induced following treatment indicating a buildup in fatty acid synthesis. The ACCase enzyme is the target for the cyhalofop herbicide, indicating a possible link between the response to the two herbicides, especially when applied together. Of the few genes which were induced by the treatment, those relating to the ethylene synthesis pathway, ACC synthase and ACC oxidase, were functioning. ACC synthase in particular was significantly induced following treatment indicating that the quinclorac reached its target. However, it does not appear given the comparison in responses between ECO-S-T and ECO-R-T that the downstream perception of the ethylene or ABA response is occurring. The general repression across most major gene families and in the functional transcripts indicates that the quinclorac-resistant plant averts lethal effects of the herbicide by limiting its biochemical output and entering a physiological ‘stasis’ state. This would mean not only a transient reduction in plant productivity but also a mitigation of the toxic production of cyanide and other harmful secondary effects.

Trehalose biosynthesis in ECO-R-N plays a significant role in the abiotic stress response by ECO-R

Several ontological terms and respective genes are presented in this research specifically related to trehalose metabolism in response to stress. Trehalose is a unique biological sugar which has been characterized as an important component in cellular metabolism and critical for proper plant growth and development [58,59]. Its role in rice abiotic stress tolerance has been investigated [60], but to date no research into herbicide activities have been described. This nonreducing sugar has several roles of interest to this research: its regulatory and signaling effect on sucrose, its role in membrane stability, and its ability to neutralize reactive oxygen species. The trehalose sugar and its precursor, intermediary, compound trehalose-6-P (Tre6P) both serve active roles in abiotic stress tolerance and may reveal a component in plant physiology that aids in herbicide resistance (Fig 6). The presence of the abundant trehalose biosynthetic genes which are highly express in ECO-R-N suggest a buildup of free trehalose and Tre6P in the plant. Tre6P is an intercellular signal for starch to sucrose conversion and is a direct measure of sucrose concentrations in the plant [58]. The build-up of Tre6P would occur from the presence of the TPP enzymes in ECO-R-N, which would partition the carbon/sugar production toward starch synthesis [59]. Given the elevated photosynthetic and carbon related processes of the plant prior to treatment, it should more than supplement its need for carbon precursors and energy. Elevated TPP also has a synergistic effect on the photosynthetic capacity of the plant by signaling a higher demand for carbon which is the rate limiting step under high light intensity [61,62]. Following herbicide application, when the plant is responding by repressing the photosynthesis and carbon assimilation processes, the decrease in Tre6P imparted by the lack of carbon, would induce a starch to sucrose conversion. This presence of sucrose would then be available for the several

critically induced processes that would need the carbon under the 'stasis' state exhibited by ECO-R-T. A second component of this trehalose build-up would be its role in the membrane stability following herbicide action including serving as a protectant against cyanide induced membrane decoupling, the production of ROS under high light intensity, or long-term water deprivation stress. The trehalose sugar is capable of forming hydrogen bonds with the hydrophobic head of the lipid bilayer, stabilizing it against oxidative and water deprivation stress, or potentially in the case of quinclorac against cyanide decoupling [63,64]. This will also stabilize membranes against destructive compounds such as free radicals and ROS. More importantly, trehalose has the ability scavenge both hydrogen peroxide and ROS, reducing the negative effects they may cause following herbicide action [65–67]. This would mitigate the destructive secondary or tertiary effects of the herbicide. This potential role for trehalose has not been described as a preventative measure against herbicide action nor has it been described in terms of herbicide response. This would require further investigation to validate the results, however, given the abundance of literature on the activities of trehalose under plant abiotic stress, there is potential for the role of this compound in co-evolutionary adaptation.

Proposed quinclorac detoxification mechanism

To investigate potential causal agents in resistance, the expression profile for ECO-R-T was surveyed for the known cyanide detoxification enzyme, $\boldsymbol{\beta}$-cyanoanaline synthase, previously implicated in quinclorac resistance [23]. This enzyme was not identified amongst the response transcripts. UGT75D1 was induced and acts on the IAA molecule with UDP-glucose to form 1-O-indole acetyl glucose ester, it also has been investigated for its role in xenobiotic detoxification [47]. Specifically, through interaction with the carboxylic acid side chains. This enzyme has not been described as a metabolic enzyme for quinclorac but GT enzymes have been

described as non-target-site resistance mechanisms specifically involved in phase II of xenobiotic detoxification [17]. Phase II GT activity requires the oxidation or hydrolysis of compounds to expose OH^- or NH_2 for conjugation. The quinclorac molecule contains an exposed OH^- side group for which UGT75D1 can interact, suggesting the phase I step would not be necessary. UGT75D1 will bind to IAA but preferentially binds to endogenous kaempferol and exogenous 2,4,5-trichlorophenol, another pesticide [55]. Both of these compounds contain similar phenolic ring structures, OH^- side groups, and exposed chloride groups. This reaction would require a ready source of free UDP-glucose for which the GT could conjugate to the quinclorac molecule. The trehalose biosynthetic pathway would provide this to the system. The limiting of the pathway by the post-application physiological cascade in ECO-R-N would lead to a build-up of the UDP-glucose, as TPP is repressed. Given the elevated expression in ECO-R-T following treatment (7.3) and the comparatively high expression to ECO-S-T (8.7), UGT75D1 is the most probable enzyme responsible for degradation of quinclorac. The quinclorac conjugation involving the interconnected trehalose biosynthesis with the potential endogenous (IAA and kaempferol) and exogenous (2,4,5 trichlorphenol) compounds with affinity for UGT75D1 are presented in figure 5.

The driving mechanism behind the elevated UGT75D1 and several other stress responsive proteins is also an important consideration. Given the response pattern of ALPL1, there is evidence to support its potential function in the ECO-R plant response. Further investigation into this protein revealed its structural similarity to the ANTAGONIST OF LIKE HETEROCHROMATIN PROETIN1 (ALP1), containing a unique harbinger transposase derived nuclease domain [38,56]. This domain allows for the targeting of specific regions of methylated DNA, including those being repressed by polycomb group proteins which repress transcriptional

activity. ALP1 has been identified in association with several critical growing regions of the cell including a cis-acting factor that modulate physiological activities and result in pleiotropic effects [39]. Given the notable response and comparative expression levels, it is possible that ALPL1 is stimulated by the quinclorac induced stress and antagonizing a polycomb group residing upstream of the UGT75D1 protein, allowing for induction and elevated expression (fig 6).

Conclusion

Quinclorac response and evolved herbicide resistance is a complex process involving multiple biological pathways. In the susceptible accession this research validates the previous literature on quinclorac response and expands the description to further asses the ABA mitigated responses. We were able conclude that the interaction of quinclorac with its target is rapid and the response occurs within 24 hours (Fig 7). The necrosis and cell death which occurs after this time is directly linked to this immediate activity. In response to the herbicide, *E. colona* enters an unstable stress response that results in the induction of several disease, abiotic, and metabolic related genes to reduce the impact of the herbicide. Several metabolism genes are induced with auxin hormone activity but their specificity and quantity does not appear to relieve the stress.

ECO-R is a unique population with multiple-resistance and an extremely high level of quinclorac resistance. Without herbicide treatment, this population is well adapted to abiotic stress and is predisposed to tolerate a number of harsh conditions, including some herbicides give the enhanced gene set. The enrichment of the trehalose pathway has not been deeply investigated in weed species but appears to play a pivotal role in the evolved processes in this population. Not only would the presence of high trehalose concentrations aid in stress response and potentially mitigate the negative effects of the herbicide, but the presence of the pathway

may aid the functioning of the potential glucosyltransferase resistance mechanism. Traditional RNA-sequencing analysis in weed science uses a R and S sample from the same population for characterization of the specific resistance mechanism, which is a shortcoming of our research [57]. However, by using the methodology described in this research we were able to better evaluate the herbicide response in a susceptible population and differentiate the underlying potential biological frameworks which contribute to the resistant phenotype. The results of this experiment and the proposed pathway need to be validated using biochemistry and molecular biology techniques. If validated, these results are the first characterized resistance mechanism that utilizes UGT75D1 for resistance and also has evolved an interconnected mechanism that would aid in general abiotic stress tolerance.

Materials and Methods

Plant Materials

Beginning in 2010, through the 2016 cropping cycle, *Echinochloa spp.* from throughout the rice-producing counties of Arkansas, USA, were sampled for a survey of the current status of herbicide resistance. Seed were bulk sampled from plants that had matured in rice and soybean production fields, which had survived at least one herbicide application, were collected and sent to the University of Arkansas Altheimer Laboratory in Fayetteville for characterization and evaluation of herbicide resistance to common rice herbicides. Results from this screen can be found in Rouse et al. [68] in which the method for characterization and results of the profiling are presented. From this screening program, two populations of *E. colona* were selected for use in this experiment. ECO-R is a multiple-resistant population from Lawrence County, Arkansas, characterized with resistance to three rice herbicides- cyhalofop, propanil, and quinclorac; as well as one soybean herbicide- glufosinate. This population has been further characterized with a

high level of resistance to propanil (>8x field dose) and quinclorac (>32X field dose); the cyhalofop and glufosinate resistance is low comparatively (~2X field dose) (data not shown). The second population, ECO-S, was selected as a susceptible standard for contrasting with ECO-R. ECO-S is characterized as susceptible to the aforementioned herbicides, however for propanil, tolerance is observed to approximately twice the recommended field dose. To establish inbred and homozygous accessions for the experiment, a single plant, verified as resistant/susceptible of ECO-R and ECO-S were grown in isolation to produce seed for further experiments. Due to its low outcrossing rate, a single generation was enough to achieve the desired genetic purity.

Pure-line generated seed of each accession were germinated in pots containing potting soil within a temperature/light controlled growth chamber set to a 14-hr day length, 33° C day temperature, and 24° C night time to simulate environmental conditions early in the rice growing season. A single plant was maintained in each of the pots and used for the treatments, two pots were used as individual biological replicates. Table S4 provides a treatment list including all relevant information for the treatments used in this experiment. At the two-leaf growth stage, in which two collars on the plant are visible approximately two weeks after planting, the plants were moved inside to an air propelled mechanized spray chamber for herbicide application; the sprayer was calibrated to deliver 187 L ha^{-1} using a 250-mL tank volume. Pots for each of the respective treatments were labeled as either ECO-R/S-T for the treated samples and ECO-R/S-N for the non-treated counterpart. To minimize the effect that the sprayer may play in the application, both plants of the ECO-R and ECO-S were treated at the same time. After approximately 30 minutes, allowing for the plants to dry, the treated and non-treated plants of both accessions were moved back into the growth chamber. Precisely 24-hours after application, the aboveground portion of each of the plants were removed and immediately frozen in liquid

nitrogen to cease all biological function. Samples were then transferred to RNAlater™-ICE (Invitrogen, Carlsbad CA, USA) for shipment to the Clemson University Genomics Institute (CUGI), in Clemson, South Carolina.

RNA Extraction, Processing, and Sequencing

RNA was extracted from the young leaf tissues of both replications for the ECO-R and ECO-S, T and N samples, at Clemson University. Total RNA was extracted with a kit according to the manufactures instructions. The extracted RNA was treated with DNase (Invitrogen, Calsbad, CA, USA) to remove any DNA contamination prior to further processing. The samples were prepared for sequencing by CUGI. For library preparation, the TruSeq Stranded Total RNA kit (Illumina Inc., San Diego, CA, USA) was used according to the instructions provided by the manufacturer to produce a paired-end library for sequencing. Ribosomal RNA was removed using target-specific oligonucleotides paired with rRNA removal beads, removing all cytoplasmic and mitochondrial rRNA that may result in poor quality results. RNA was fragmented and reverse transcribed to cDNA using random primers, followed by a second strand cDNA synthesis. Each fragment is then ligated with an additional 'A' and an adapter for sequencing. The PCR enriched product is then used to create the final cDNA library. All samples were sequenced on an Illumina Hiseq 2500 platform housed in the Holdings Cancer Center at the Medical University of South Carolina, Charleston, SC, USA. Samples, regardless of treatments or replication were ran across three lanes to reduce sequencing errors from the equipment. The resulting data were processed by CUGI.

Transcriptome Assembly and Annotation

A *de novo* transcriptome was assembled from the treatments described for this experiment as well as several treatments which included herbicides profiled in ECO-R. The

treatments were applied to both ECO-R and ECO-S samples with two replications as described previously. In total, 20 individual plants, from both the ECO-R and ECO-S accessions, as well as T and NT samples, were used for the assembly. The transcriptome was assembled using the Trinity RNA-Seq pipeline (Broad Institute, Cambridge, MA, USA). Raw data were assessed for quality using FastQC (Babraham Institute, Cambridge, UK) and then processed to remove adapter sequences and low quality bases using a sliding window method [69]. The processed data were then rerun using FastQC to ensure high quality reads. Using the TrinityRNASeq 2.2.0 software, the samples were normalized, by replication, using a coverage size of 100 and kmer of 32. The normalized reads were then assembled as transcripts and genes using Trinity with the stranded library set as the default. Transdecoder 3.0.1 (Broad Institute) was used to scan the transcriptome for one open reading frame based on homology from the blastP database and to identify existing proteins using HMM Scan against pfam; transcripts matching both criteria were retained. CD-HIT-EST (Sanforn Burnham Prebys Medical Discovery Institute, San Diego, CA, USA) was used to cluster the transcripts based on sequence identity, sequences with 98% or greater similarity were retained. The transcriptome was assessed for transcriptome completeness using BUSCO (University of Geneva, Geneva, CH). Following assembly, the Trinotate 3.0 suite of software (https://trinotate.github.io/) was used for functional annotation of the transcriptome via homology to BLAST+ and Swissprot databases to produce protein identification information based on HMMER and PFAM as well as generate information for the primary annotation databases including eggNOG, GO, and KEGG.

Gene Ontology Analysis

Gene ontology enrichment/depletion analysis was used to describe the functional components associated with herbicide response and resistance. Using the Trinotate output, the

goseq package from Bioconductor was used to assign GO terms to the transcripts from the transcriptome. The analysis of enrichment/depletion was performed on transcripts which had been expressed or depressed at a log2 fold-change of ≤-2 or ≥2 and a p-value of ≤0.01. The results of the analysis were visually assessed using REVIGO (http://revigo.irb.hr/) to best characterize the resulting ontological terms and describe interconnected pathways within the treatments. For description of the gene ontology terms and functions of the terms EggNOG [70] and GO Consortium [71] databases.

Differential Gene Expression

Using the *de novo* transcriptome as a reference, differential gene expression was quantified by comparing several pairwise orthogonal sets of treatments. The multi-dimensional scaling (MDS) plot was generated to assess the disparity of the replications for each treatment and accession. The second replication for ECO-R-N was excluded as it did not fit within an acceptable distance on the MDS plot to the other samples used for the analysis, all other treatments were retained for analysis. A GTF file of the transcripts was generated as a boundary for comparing each sample to the reference transcriptome. Feature counts were generated using the Subread package (http://subread.sourceforge.net/), allowing for quantification of the differentially expressed transcripts with each replication which were paired concordantly. The Bioconductor (https://www.bioconductor.org/) package- edgeR, developed for use within the R statistical software program (https://www.r-project.org/), was used to quantify the filtered raw counts produced from the RNA-sequencing [72,73]. Standard normalization using trimmed mean of M-values (TMM) was applied to the counts. The counts were fit using a GLM model for determination of significance ($p \leq 0.01$) and a likelihood ratio test for specific comparisons of interest in the experiment. The resulting analysis was then evaluated using a false discovery rate

for p-value correction to reduce the error in the results. Volcano plots, for visual assessment of gene expression, and a table of log2-fold changes with respective genes within the comparisons of interest were generated from the analysis. These results were then used in subsequent descriptive analysis to describe the patterns of expression within the tested conditions. In order to reduce the number of potential genes used in describing the expression patterns, categories or groupings were assigned to the sets of differentially expressed genes. Based on a review of the literature, enzymes which may be involved in one of the four phases of chemical detoxification were assigned into one of eight categories: ABC transporters, aminotrasferases, amylase, cytochrome P450s, glutathione-S-transferases, glucosyltransferase, hydrolases, and peptidases [18,74,75]. The description of the genes and pathways are based on the data on the Uniprot [76] and KEGG databases [77].

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Tables and Figures

Table 1. Expression summary of detoxifying gene families and subsequent genes involved in xenobiotic detoxification differentially expressed in ECO-R compared to ECO-S

Gene Family	Gene	Fold Change
ABC Transporter	B family member 11	-5
	B family member 6	2
	G family member 53	2
	F family member 1	4
	D family member 2	4
	F family member 4	5
	G family member 48	8
Aminotransferase	Alanine--glyoxylate aminotransferase 2 homolog 3, mitochondrial	3
	Aspartate aminotransferase, mitochondrial	5
	Probable alanine aminotransferase, mitochondrial	5
Amylase	Beta-amylase 3, chloroplastic	2
	Alpha-amylase isozyme 3D	4
Cytochrome P450	72A14	-9
	72A15	-8
	89A9	-8
	76C1	-4
	71A1	-3
	90D2	4
	94C1	5
	71A21	6
GST	Glutathione S-transferase 1	-3
	Glutathione S-transferase T3	11
GT	UDP-glycosyltransferase 74D1	3
	UDP-glycosyltransferase 83A1	3
	UDP-glycosyltransferase 73C2	4
	UDP-glycosyltransferase 75C1	4
	UDP-glycosyltransferase 73C1	4
	UDP-glycosyltransferase 90A1	6
	UDP-glycosyltransferase 73E1	6
Hydrolase	Ubiquitin carboxyl-terminal hydrolase 27	-7
	Uncharacterized abhydrolase domain-containing protein	-5
	Nudix hydrolase 21, chloroplastic	-3
	Haloacid dehalogenase-like hydrolase domain-containing protein 3	-2
	Probable xyloglucan endotransglucosylase/hydrolase protein 30	2
	Putative aminoacrylate hydrolase RutD	3
	Xyloglucan endotransglucosylase/hydrolase protein 22	3

Table 1 (Cont.)

Gene Family	Gene	Fold Change
	Hydrolase C26A3.11	4
	IAA-amino acid hydrolase ILR1-like 4	4
	Pyrimidine-specific ribonucleoside hydrolase RihA	4
	Ubiquitin carboxyl-terminal hydrolase 6	4
	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	9
Peptidase	Serine carboxypeptidase-like 27	3
	Serine carboxypeptidase-like 18	3
	Aspartyl aminopeptidase	3
	Leucine aminopeptidase 2, chloroplastic	4
	Carboxypeptidase 1	4
	Mitochondrial-processing peptidase subunit alpha	4
	Serine carboxypeptidase-like 49	4
	Probable cytosol aminopeptidase	4
	Prolyl endopeptidase	4
	Mitochondrial-processing peptidase subunit beta	4
	Puromycin-sensitive aminopeptidase	5
	Methionine aminopeptidase 2	5
	Mitochondrial intermediate peptidase	5
	Thimet oligopeptidase	5
	Probable aminopeptidase NPEPL1	5
	Dipeptidyl aminopeptidase BI	5
	Cytosolic non-specific dipeptidase	6

Table 2. Enhanced gene ontology terms and the respective frequencies identified in ECO-S following quinclorac application.

GO Type	GO Term	Frequency[1]
Biological Process	protein phosphorylation	4.14%
	detection of hypoxia	0.00%
	response to hypoxia	0.05%
	anaerobic respiration	0.05%
	hydrogen peroxide catabolic process	0.09%
	nitrate assimilation	0.09%
	oxidation-reduction process	15.06%
	cell surface receptor signaling pathway	0.92%
	auxin catabolic process	0.00%
	salicylic acid catabolic process	0.00%
	peptidyl-cysteine oxidation	0.00%
	response to bacterium	0.15%
	defense response to bacterium	0.10%
	'de novo' CTP biosynthetic process	0.07%
	pyrimidine nucleobase biosynthetic process	0.24%
	abscisic acid-activated signaling pathway	0.01%
	regulation of salicylic acid mediated signaling pathway	0.01%
	response to jasmonic acid	0.01%
	defense response	0.57%
	abscisic acid catabolic process	0.00%
	protein autophosphorylation	0.08%
	response to oxidative stress	0.58%
	defense response to oomycetes	0.00%
	response to oomycetes	0.00%
	ethylene-activated signaling pathway	0.01%
Molecular Function	transcription factor activity, sequence-specific DNA binding	4.22%
	protein kinase activity	3.39%
	protein serine/threonine kinase activity	1.00%
	symporter activity	0.29%
	polysaccharide binding	0.10%
	CTP synthase activity	0.04%
	peroxidase activity	0.38%
	heme binding	1.36%
	sequence-specific DNA binding	2.22%
	ATP binding	14.13%
	(+)-abscisic acid 8'-hydroxylase activity	0.00%
	alcohol dehydrogenase (NAD) activity	0.04%

Table 2 (Cont.)

GO Type	GO Term	Frequency[1]
	oxidoreductase activity	0.18%
	inositol oxygenase activity	0.01%
	oligopeptide transmembrane transporter activity	0.01%
	cysteine dioxygenase activity	0.00%

[1] Frequency is the percentage of proteins in UniProt which were annotated with terms in the underlying GOA database, lower frequency indicates very specific terms while higher values indicate more general terms.

Table 3. Differentially expressed genes involved in xenobiotic detoxification expressed in ECO-R-T compared to ECO-S-T 24-hr after quinclorac application.

Gene Family	Gene	Fold Change
Aldolase	Fructose-bisphosphate aldolase, chloroplastic	4
Aminotransferase	Branched-chain-amino-acid aminotransferase 5, chloroplastic	4
	Aspartate aminotransferase, chloroplastic	4
	Alanine aminotransferase 2	4
	Alanine aminotransferase 2	4
	Alanine aminotransferase 2	4
Amylase	Beta-amylase 1, chloroplastic	4
Cytochrome P450	CYP89A2	-10
	CYP72A15	-7
	CYP71A9	-6
	CYP71A1	3
	CYP71A1	3
	CYP71A8	4
	CYP71A1	5
	CYP71A4	6
	CYP709B2	6
	CYP71A1	6
	CYP709B2	7
	CYP709B2	11
GST	GSTU20	6
GT	UGT74D1	-4
	UGT88A1	4
	UGT73E1	5
	UGT73D1	8
	UGT75D1	9
Hydrolase	Pyrimidine-specific ribonucleoside hydrolase RihA	-9
	IAA-amino acid hydrolase ILR1-like 4	-6
	Probable xyloglucan endotransglucosylase/hydrolase protein 28	-4
	Nudix hydrolase 21, chloroplastic	3
	Nudix hydrolase 21, chloroplastic	4
	Haloacid dehalogenase-like hydrolase domain-containing protein 3	4
Peptidase	Serine carboxypeptidase-like 42	-5
	Serine carboxypeptidase-like 42	-5
	Desumoylating isopeptidase 1	4
	Prolyl endopeptidase	8
	Prolyl endopeptidase	9
	Prolyl endopeptidase	15

Figure 1. Depiction of the quinclorac activated physiological pathway in *E. colona* following treatment including the attachment of quinclorac to the TIR-1 DNA repressor complex and the activation of the 1-aminocyclopropane-carboxylase (ACC) synthase enzyme leading to the build-up of ethylene and hydrogen cyanide in the plant.

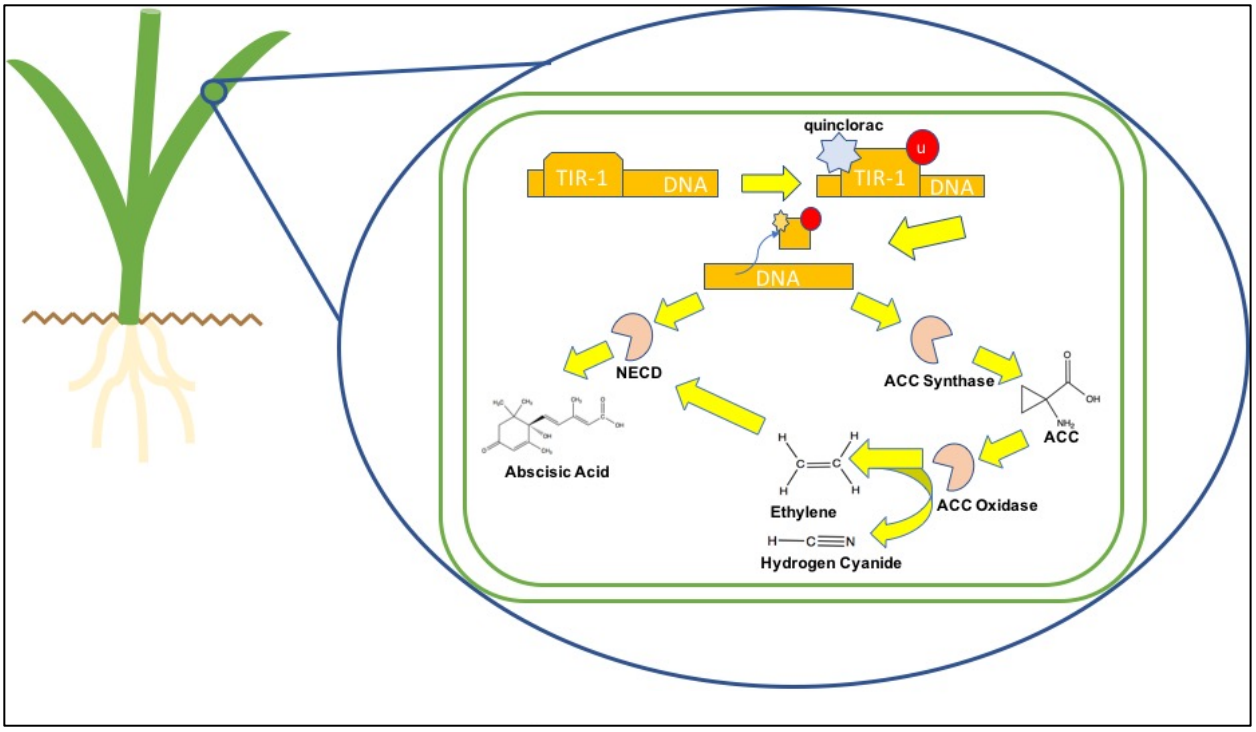
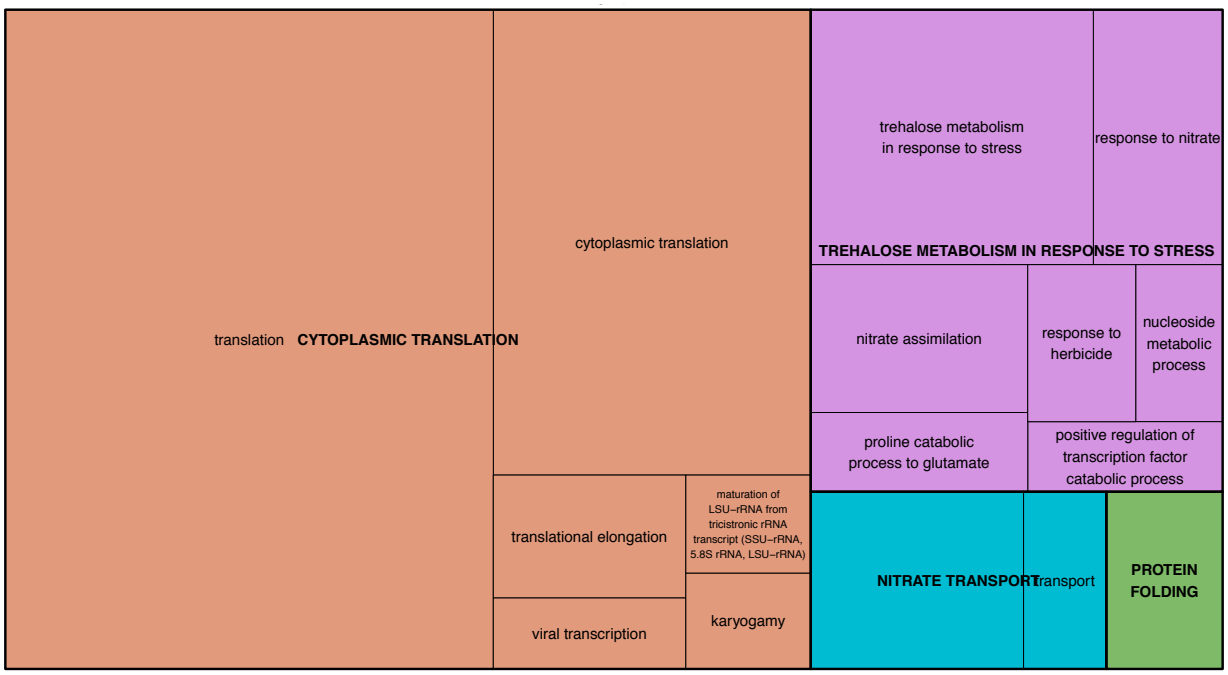
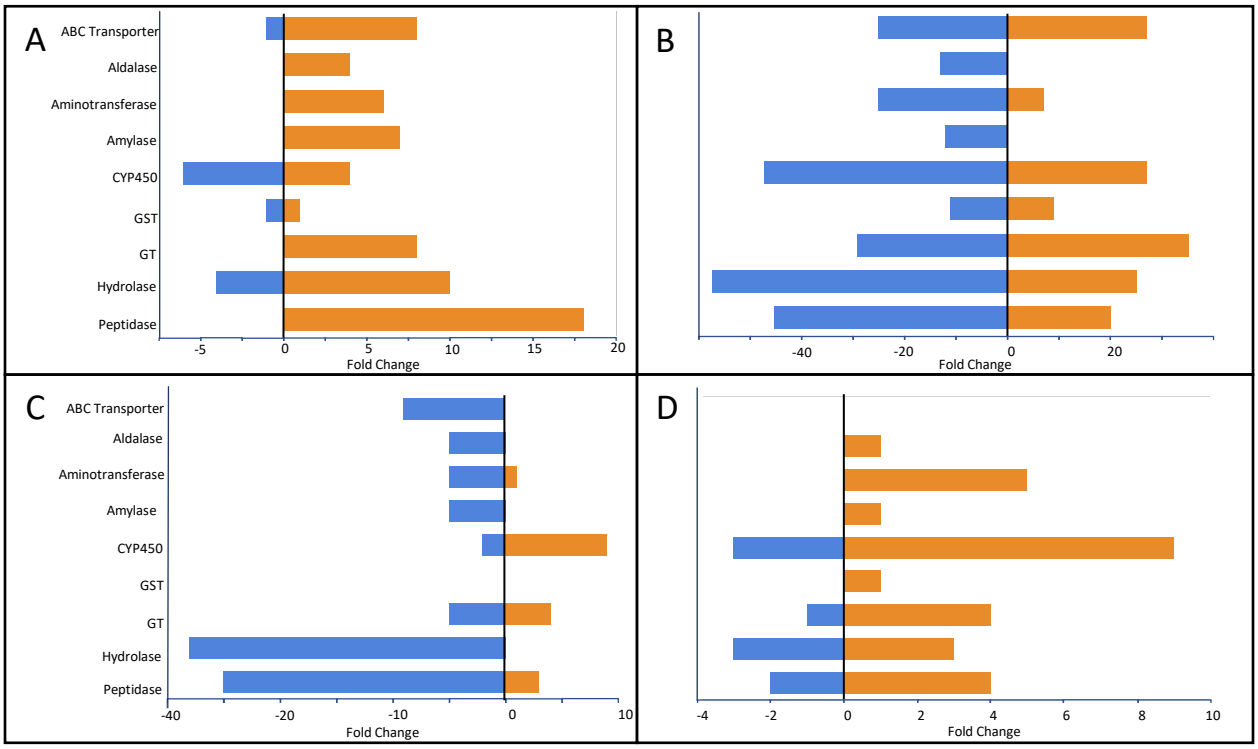


Figure 2. Treemap of the enriched gene ontology terms for ECO-R without herbicide treatment compared to ECO-S without herbicide treatment.



[1] Each box represents an ontological term and the size of the box depicts the p-value for the terms based on the gene ontology analysis. The colors signify superclusters of loosely associated terms related via semantic analysis and identified by the description that is capitalized in bold.

Figure 3. Total fold change, both increasing and decreasing, for the gene families of xenobiotic detoxification enzymes categorized in the analysis for the differential gene expression analysis.



[1]Figure Legend: (A) ECO-S-N vs ECO-R-N, (B) ECO-S-N vs ECO-S-T, (C) ECO-R-N vs ECO-R-T, (D) ECO-S-T vs ECO-R-T.

Figure 4. Venn diagram for the differential gene expression analysis with each oval representing the number of repressed (A/B) or induced (C/D) genes within the comparisons of ECO-S-N vs ECO-R-N (A/C) and ECO-R-N vs ECO-R-T (B/D).

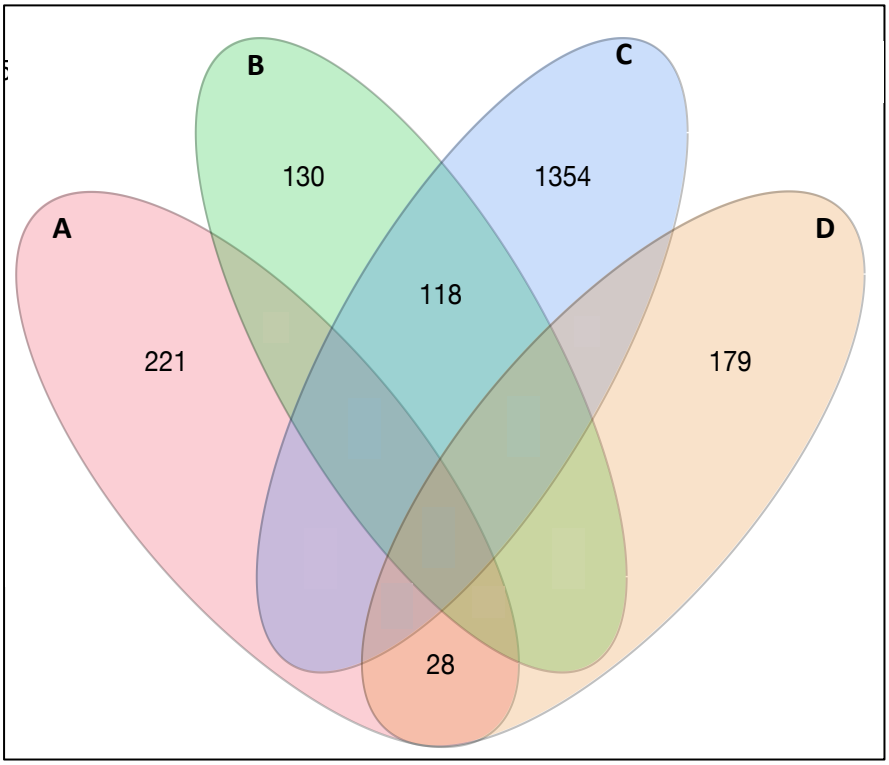
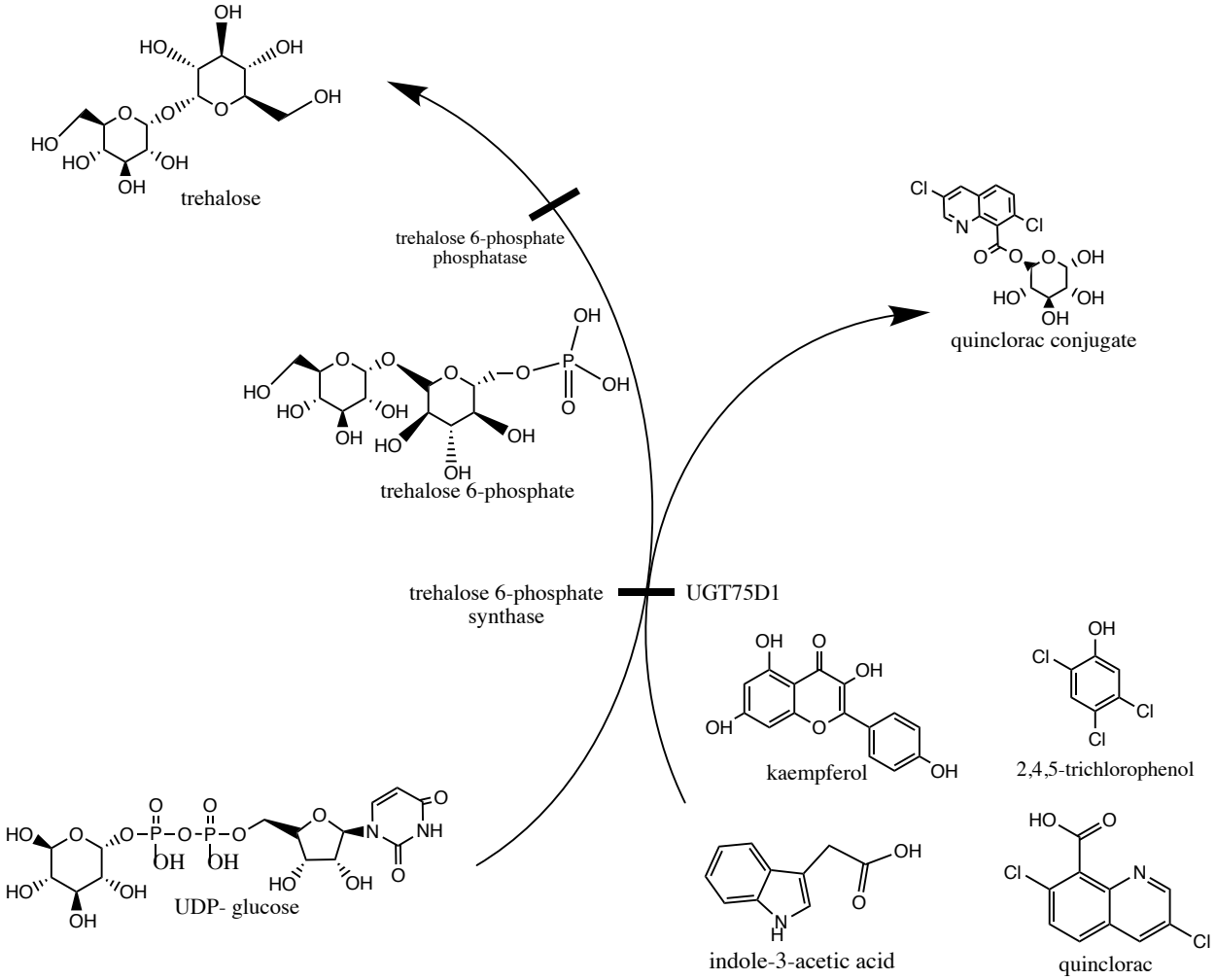


Figure 5. Diagram depicting the proposed biological pathway for the conjugation of quinclorac via UGT75D1 to the UDP-glucose molecule which is a component of the trehalose biosynthetic process.



[1] The alternative substrates for the UGT75D1 enzyme- the endogenous molecule indole-3-acetic acid and exogenous xenobiotics kaempferol and 2,4,5-trichlorophenol, are presented as structural comparisons to quinclorac.

Figure 6. Proposed interconnected pathways describing the potential activities of ALPL1 and UGT75D1 which work in concert to endow the quinclorac-resistant phenotype in ECO-R.

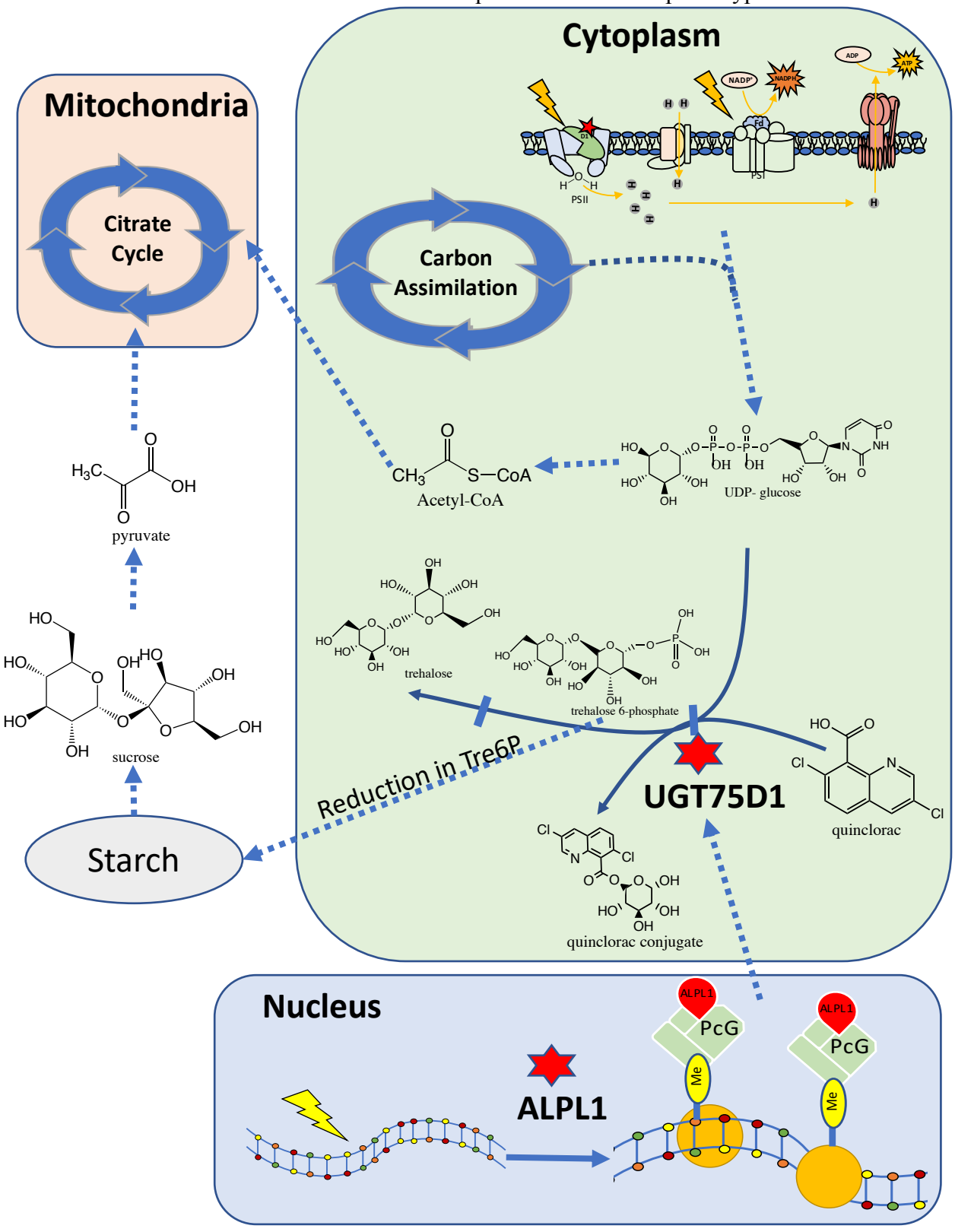
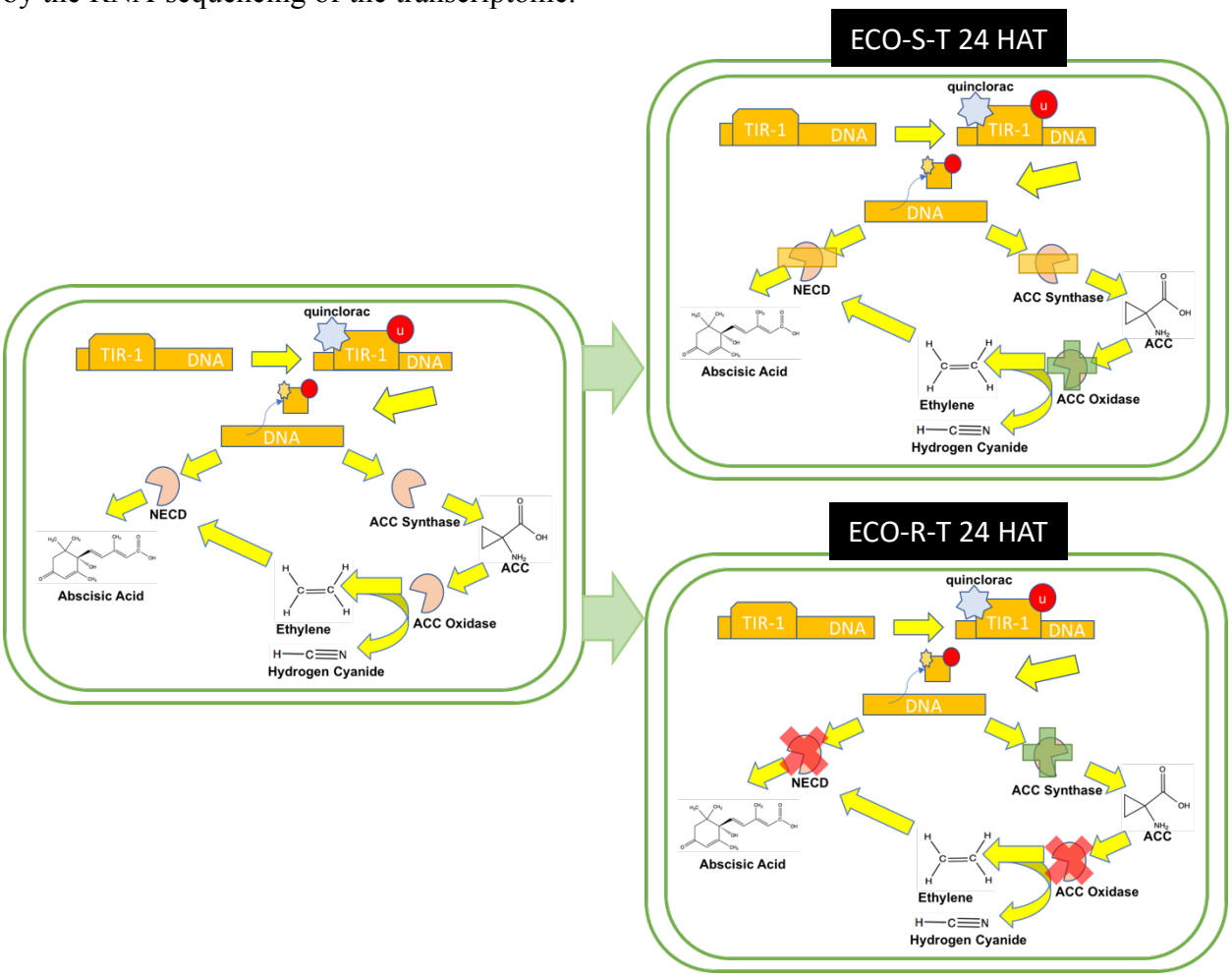


Figure 7. Diagram depicting the quinclorac activated physiological pathway as explained by the literature and the response of ECO-S and ECO-R, 24-hours after treatment (HAT), as explained by the RNA-sequencing of the transcriptome.



[1] Orange negative (-) symbols indicate enzyme transcripts which were depressed, green plus symbols (+) indicate enzyme transcripts that were induced, and red crosses (X) indicate enzyme transcripts which were not present in the differential gene expresion analysis.

Appendix

Appendix Table 1. Summary of the reults of the *de novo* transcriptome assembly analysis.

Transcriptome Component	Size/ Length
Illumina raw read pairs	544,870,782
Number of Transcripts	109,539
Annotated sequences (blastX)	66,448
Assembled genes	60,530
Number of bases	250
Read Length (n)	125

Appendix Table 2. Summary of the gene anotation for assembly of the *de novo* transciptome for the comparisons between ECO-R and ECO-S.

Annotation	Expression	ECO-S-N vs ECO-R-N	ECO-S-T vs ECO-S-T	ECO-R-T vs ECO-R-T	ECO-S-T vs ECO-R-T
Non-Annotated	Decrease	99	1248	2677	78
	Increase	655	1259	18	62
	Total	754	2507	2695	140
Annotated	Decrease	249	4010	2634	248
	Increase	1472	2667	56	207
	Total	1721	6677	2690	455
Total	Decrease	348	5258	5311	326
	Increase	2127	3926	74	269
	Total	2475	9184	5385	595

Appendix Table 3. Summary of the repression or induction of genes in different fold change categories from the differential gene expression analysis for the comparisons between ECO-R and ECO-S

Fold Change Category	ECO-S-NT vs ECO-R-NT		ECO-S-NT vs ECO-S-T		ECO-R-NT vs ECO-R-T		ECO-S-T vs ECO-R-T	
	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase
1-2	56	166	203	1344	8	6	0	0
3-4	141	1201	2853	1569	25	30	115	103
5-6	42	554	807	696	287	17	124	73
7-8	79	178	1208	276	3480	18	41	46
9-10	26	25	162	39	1243	3	33	18
≥11	4	3	24	2	268	0	13	28

Appendix Table 4. Treatments for ECO-R and ECO-S including the application rate and adjuvant used for RNA-sequencing.

Accession	Treatment	Herbicide	Application Rate	Adjuvant	Adjuvant Concentration
ECO-R	T	Quinclorac	560 g ha^{-1}	Crop oil	1% v/v
	NT	None	-	-	-
ECO-S	T	Quinclorac	560 g ha^{-1}	Crop oil	1% v/v
	NT	None	-	-	-

Concerted action of abiotic stress responsive genes may impart high resistance to propanil in multiple-resistant *Echinochloa colona*

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Abstract

Propanil is amongst the oldest herbicide compounds used for selective control of *Echinochloa spp.* in rice production in the mid-south, USA. Research with a multiple-resistant *E. colona* (junglerice) from Arkansas, USA, identified that an unknown metabolic enzyme is potentially allowing for high levels of resistance to propanil in this population. Physiological assays were able to conclude that the pattern of response was similar to inactivation of the propanil molecule by the aryl acylamidase enzyme, but further investigation was required. An RNA-sequencing experiment was conducted on the ECO-R population and a susceptible counterpart (ECO-S), to describe the response patterns following propanil treatment and elucidate the potential herbicide resistance mechanism of ECO-R based on differential gene expression. Using the *de novo* transcriptome produced by our research group previously, differential gene expression in ECO-S following propanil treatment indicates that 1,765 genes were repressed and 1,775 were induced. In general, the stress response elucidated by ECO-S indicates perception of both abiotic and biotic stressors leading to the induction of abscisic acid and jasmonic acid metabolism. Several glucosinolate producing enzymes and hypersensitive response enzymes related to diseases were also induced. The propanil application induced trehalose biosynthesis. For ECO-R following propanil treatment, only 152 genes were induced but a number of similar processes including both abiotic and biotic stress perception were the same as ECO-S. The differential gene expression analysis revealed two cytochrome P450 enzymes- CYP709B2 (>8-fold induction) and CYP72A14 (~3-fold induction) that have the potential to hydroxylate the propanil molecule in phase one degradation. The profile also shows induction of several glutathione-S-transferase and glycosyltransferase genes that may be involved in phase II conjugation of the 3,4-dichloroanaline and propionic acid molecules. This is

the first such characterization of abiotic and biotic signal perception following propanil application using the transcriptome of multiple-resistant *E colona*.

Introduction

Echinochloa spp. are weeds of global importance and widespread influence through the upland and lowland agricultural production systems of the world [1,2]. Current research in Arkansas, and throughout the USA mid-south production regions, positions the *Echinochloa genus* as the number one most common and troublesome weeds impacting rice production and among the top ten in soybean and cotton production [3]. Its sphere of influence is largely due to its biology and morphology which allows for significant adaptive evolution under imposed stress in the agriculture landscape [4]. This adaptability under diverse agricultural systems may be indicative of its early co-domestication with rice as a millet crop over 10,0000 years ago, and may have had long term implications for its ability to mimic rice today [5,6]. Within USA rice production, *Echinochloa crus-galli,* has historically been among the topmost researched species. First characterized in Arkansas rice in 1968, it has since become a dominate factor in reducing crop yields in rice, second only to weedy rice in terms of threat to productivity [7,8]. One plant per square meter imposing season-long interference can reduce yield as much as 65 kg ha^{-1} and competition of approximately 50 plants in 0.1 m^2 up to 37 days can reduce rice crop yields by 20% [7,9]. While *Echinochloa crus-galli* has been widely accepted in the literature as the major species of importance, recent research indicates that *E. colona* is the primary species impacting Arkansas rice producers, and that complexes of *E. colona, E. muricata, and E. crus-gallli* co-exist within single rice fields [10].Throughout the southern USA, *Echinochloa spp.* have been widely misidentified. Recent research indicates *E. colona* are the more dominate species throughout southern production fields [11] and complexes of *E. colona* and *E. crus-galli* are present throughout most of Arkansas [12]. Due to this fact, we considered that both the *E. colona*

and *E. crus-galli* have been colloquially referred to as barnyardgrass in the literature and thus are presented *as Echinochloa spp.* for data prior to 2017 in the USA.

Herbicides have been a long-standing component of *Echinochloa spp.* management in rice and other cropping systems. In 1959, propanil was the among first commercially available herbicides for selective control of *Echinochloa* in rice [13]. As innovations continued through the 1990s, several other herbicides including quinclorac, fenoxaprop, clomazone, and cyhalofop were introduced. Later in the early 2000s, imidazolinone herbicides with the Clearfield rice system® were registered for use in rice. Propanil is a photosystem II (PSII) inhibitor (WSSA group 7), which irreversibly binds to the D1 protein blocking the interaction between plastoquinone and PSII, blocking electron flow through the complex [14]. The limitation in photosynthetic activity leads to reductions in carbon assimilation but the free energy build-up leads to the secondary effects of herbicide action which is reactive oxygen species (ROS) and hydrogen peroxide production. These highly reactive molecules are capable of destroying cells. Rice is highly tolerant to propanil because of an elevated production of hydroxylating enzyme, aryl acylamidase, which is capable of detoxifying propanil into two metabolites: 3,4 dichloroanaline and propionic acid [15,16]. Due to the overreliance on propanil, resistant *Echinochloa spp.* have become a widespread problem. First documented in *E. crus-galli* in 1986 from populations in Greece, propanil-resistant *Echinochloa spp.* have evolved in 14 countries across the globe [17]. In the USA mid-south, all of the states that make up the region- Arkansas, Mississippi, Missouri, Louisiana, and Texas contain resistant populations. In Arkansas, 50% of the *Echinochloa spp.* populations are resistant to propanil, while 12% of all populations are multiple resistant to propanil and quinclorac [10]. This is a major concern considering the USA

mid-south alone accounts for 65% of the USA rice production, leaving producers with concerns for the role resistance plays in crop management [18].

Herbicide resistance is an evolved survival trait as a consequence of sustained herbicide selection pressure, especially under prolonged mono-cropping systems with a lack of herbicide diversity or weed control methods [19]. Mechanisms of resistance are broadly categorized as target-site (TSR) or non-target-site (NTSR). TSR involves structural modification of the herbicide target protein, lowering the binding efficiency of the herbicide, and consequently reducing its efficacy [20] . These monogenic changes can be selected by continuous high-dose selection, eventually causing a shift toward a resistant population [19]. NTSR is a more complex polygenic mechanism that involves a network of abiotic stress response mechanisms that attempt to reduce the uptake of, modify, or redistribute the herbicide, to limit its availability at the site of action [22]. These processes include several phases of the xenobiotic detoxification process, employed by plants to mitigate the harmful effects of exogenous compounds [23]. This mechanism evolves slowly and often results from low dose selection over the course of several years; specifically, as the plant accumulates the necessary genetic components to persist through herbicide action. *Echinochloa spp.* have evolved resistance to seven herbicide modes of action involving both TSR and NTSR mechanisms [17]. TSR to acetolactate synthase-inhibiting herbicides [24], atrazine [25], and glyphosate [26,27] has been identified in *Echinochloa spp.* around the world. NTSR has also been identified involving several herbicide modes of action including clomazone [13,28], fenoxaprop [29] and quinclorac [30]. Resistance to propanil in *Echinochloa spp.*is also a NTSR mechanism which involves detoxification via the aryl acylamidase, the same enzyme employed by rice [16,31–33].

To evaluate the underlying causes of herbicide resistance, researchers have historically used physiological, biochemical, or molecule biology approaches. Until recently, genomic approaches were limited by lack of resources to investigate non-model organisms and a lack of genomic assemblies for comparative analysis [34,35]. Recently, several transcriptomes have been published for weedy species including one for *E. crus-galli* [36] and *E. colona* [37]. Results from these experiments have produced repositories of genes for further research and led to the identification of novel resistance mechanisms and biological pathways involved in herbicide response. Using genomics to assess herbicide resistance evolution in weedy species has led to the identification of several herbicide target genes [38], advanced phylogenetic analysis of herbicide targets, and identification of previously unknown herbicide detoxifying genes [39]. This research is considered novel to the weed science discipline and presents new information that may be of value in the future of weed management and understanding weed biology.

Propanil resistance in *Echinochloa spp.* is a long-studied topic, but the recent research on the distribution of resistance in Arkansas is alarming. Using RNA-sequencing of a multiple-resistant *Echinochloa colona,* we utilized the transcriptome to describe the patterns of gene expression following propanil treatment and identify candidate genes involved in propanil resistance. To date, the definitive enzyme endowing resistance has been the aryl acylamidase protein, which has activities in both rice and *Echinochloa spp.* This research identifies two cytochrome P450 enzymes, capable of detoxifying propanil, in the absence of an elevated aryl acylamidase gene.

Results

Biological framework and gene expression response to propanil action in susceptible E. colona populations

Gene network analysis reveals complex stress induced responses. The susceptible accession, ECO-S-T, expresses a series of abiotic stress proteins following treatment. Gene ontology or biological network characterization revealed several biological processes centered around inositol catabolism that were enriched in ECO-S-T (Fig 1). The sub-cluster-trehalose metabolism was enriched, which is a general plant response to stress. The 'abscisic acid (ABA) metabolism' supercluster, which has a role in stomatal closure and stress response signaling, was enriched. This contains several ontological terms including 'ABA metabolic process', 'ABA catabolic process', and 'ABA-activated signaling'. Starch and salicylic acid catabolic processes were also significantly enriched. This is related to the 'responses to water deprivation' and 'nitrate transport and assimilation' terms that were also enriched. The remaining biological processes, were involved in ethylene-activated signaling and DNA transcription regulation, including 'transcription factor catabolism'. 'Inositol oxygenase activity', 'ABA hydroxylase activity', and 'trehalose metabolism' enzyme activities were all significantly enriched.

Growth regulation and maintenance genes are impacted by propanil. Twenty-four hours following treatment with propanil, 3,539 transcripts were differentially expressed; 1765 were repressed and 1771 were induced (Table 1). Several genes associated with photosynthesis were repressed including ATP synthase alpha subunits and plastocyanin, both of which are critical in the electron transfer process. However, three forms of ferredoxin-6 were induced 3.8- to 4.8-fold. In the mitochondria, the majority of transcripts were repressed 24 hours following propanil treatment including several ribosomal proteins, ATP synthase subunits, ADP/ATP carrier

proteins, and many transferase type proteins. A vast number of DNA and RNA polymerase-related transcripts were repressed by as much as -8.7-fold. A similar number of proteins associated with DNA were enhanced, 45 of which were transcription factors, 8 chaperone proteins, and several other DNA binding proteins. Key genes relevant to carbon metabolism, phosphoenolypyruvate carboxylase (-2.1 to -3.4), and pyruvate dehydrogenase (-5.6 to -6.2) were also repressed. These are essential to the breakdown and utilization of stored energy reserves and the assimilation of new carbon products. Acetyl-CoA carboxylase (ACCase) and ACCase 2 were repressed up to -6.9-fold, indicating reduced fatty acid production. In terms of biological and physiological processes, a large number of genes related to sugar metabolism were induced by propanil treatment. Three sugar metabolism genes were repressed including those coding for proteins associated with sugar transport, indicating reduced, or no transmembrane movement. The vast majority of the induced genes (1.5- to 7.4-fold), were transcripts coding for various forms of enzymes in the trehalose biosynthetic pathway, including both the trehalose phosphate synthase (TPS) and trehalose-phosphate phosphatase (TPP) enzymes. The sugar transporter ERD6-like 6 was induced 2.3-fold, supporting the elevated status of trehalose synthesis, which would need to be transported across membranes to fulfill demand in various organelles and tissues.

Abiotic and biotic stress-responsive genes. Stress-induced genes are key not only in mediating the stressor but also lead to downstream signaling of the stressed state required for defense and tolerance to the stressor. Following treatment, ECO-S-T exhibited the repression of heat stress transcription factors and proteins. Several MYB (myeloblastosis) and MYC proteins were differentially expressed. MYB-related genes, specifically MYB44 (>3-fold change), act as transcription factor. Overexpression of MYB44 results in stomatal closure in the absence of

ABA under drought, low temperature, or salinity stress [40]. MYC3 was induced 3.4-fold following treatment, indicating stimulation of jasmonic acid production. Jasmonic acid is another stress-signaling hormone. MYC3 can interact with MYB to regulate glucosinolate biosynthesis; compounds which are responsive to herbivory and form toxic compounds to insects [41]. Multiple disease response/ resistance genes were repressed including- RPP13 like protein 4, RPM1, RGA4 and several putative resistance genes. Of note is the repression of the RGA4, which is one of a four-gene family residing at the same locus. RGA2 (>7.5-fold) and RGA3 (1.7) were induced following treatment. While all four members of the RGA family contain avirulence proteins, only RGA2 induces a resistant response to *Pseudomonas infestans*, restricting growth of the pathogen. To reduce the damaging build-up of hydrogen peroxide, several peroxidase genes-1, 2, 4, 15, 54, 52, 57, and multiple forms of these peroxidase transcripts were induced 2.8- to 7-fold. This is an expected response given the mode of action previously described, but these specific genes have not been described in response to propanil. Ethylene induction is a major component of stress signaling. However, the precursor enzymes for ethylene production [1-aminocycloproane-1-carboxylate (ACC) synthase (-2.7) and multiple ACC oxidase genes (-1.8 to -2.2)] were repressed 24 h after propanil treatment. This means that at this time, ethylene was not produced. It is possible that ethylene induction by herbicide stress occurred earlier, as indicated by the presence of multiple ethylene responsive transcription factors (ERF).

Induction of potential herbicide detoxifying enzymes. Xenobiotic detoxification genes were also investigated. Fifty-two transcripts were repressed while 30 were induced 24 h following treatment. Among the induced genes were members of five large gene families including ABC transporters, acetyltransferases, cytochrome P450s, glutathione-S-transferases, and glycosyltransferases (Table 2). Of these, the largest families are the cytochrome P450 (CYP),

glutathione-S-transferase (GST), and glucosyltransferase (GT) proteins, all of which have been previously characterized in response to herbicide action. Four of the CYP genes are from the CYP71 family, which have not been characterized except for being similar to other members in this family. CYP71A1 is a component of the flavonoid biosynthetic process, induced by ethylene. CYP94C1 is associated with the jasmonic acid-mediated signaling pathway. The four propanil-induced GST genes have roles in endogenous and exogenous chemical glutathionylation, including herbicides, based on their similarity to previously described genes in the Uniprot database. GSTU1 and GSTU6 are members of Tau family of GST's which have known roles in xenobiotic detoxification [42]. UGT73B4 (3.8 & 5.7) have quercetin 3- and 7-O-glucosyltranferase activities but are also able to detoxify 2,4,6-trinitrotoluene (TNT), the explosive compound in dynamite [43]. UGT74D1 glycosylates jasmonate derivatives as well as IAA, and several components of the flavonoid biosynthetic process.

Concerted repression of gene expression and biological networks in the response of herbicide-resistant *E. colon*a

Plant growth and maintenance gene response. Herbicide action resulted in a significant repression in plant activities. Only three ontological terms were enriched, one biological process term- flavonoid glucoridination, and two molecular function terms- quercetin 7 and 3-O-glucosyltransferase activity. In terms of gene expression, a total of 5,639 genes were repressed and only 153 induced genes (Table 1). Photosynthetic complex proteins were all repressed 24 h following treatment. This includes reduction of cytochrome c1, ferredoxin thioredoxin reductase, ATP synthase subunits (<-10), ferredoxin 6 (<-2.4), NADH-cytochrome b5 reductase proteins (<-7.4), and ubiquinol cytochrome-c reductase complex core protein (<-8.6). This followed a similar pattern of gene repression for carbon metabolism and nitrogen metabolism. Carbon

assimilation genes including malate dehydrogenase (-10.8), pyruvate phosphate dikinase (-12), NADP-dependent malic enzyme (-9.3), pyruvate dehydrogenase E1 subunit (<-10.4), ADP sugar pyrophosphatase (-7.2), fructose-1,6-bisphosphatase (-9.3), and several other compounds modifying proteins were repressed. Glutamine synthetase was also repressed (-10.5-fold) following treatment. Similar to ECO-S-T, most genes associated with DNA and subsequent translation and transcription were repressed including many polymerase and topoisomerase proteins. The remaining TF transcripts have general functions in cis-acting DNA activation. In terms of sugar metabolism, again no transcripts were induced. However, TPS6, which was induced following treatment in ECO-S was repressed 6.1-fold. Two other trehalose transcripts, both for trehalose phosphorylase were repressed. These have a role in the catabolism of trehalose[44]. Two sugar transporters, ERD6-like 8 and SWEET2a, were also repressed (4.2- and 3.8-fold, respectively). ACCase (-9.7) and ACCase 2 (-10.2), as well as acetyl-coenzyme A synthetase (-8.4), and phenolic glucoside malonyltransferase 1 (-3.5) were all repressed. Based on the broad-spectrum repression of key metabolic genes across all major biochemical pathways, ECO-R seemed to be at a quiescent physiological state 24 h after treatment with propanil.

Stress-responsive gene expression. In terms of stress response, the results were similar to the plant growth genes previously described. A general repression in stress-induced genes occurred following propanil application, with only two transcripts induced. Similar to MYC3 induced in ECO-S-T, MYC2 is a transcriptional activator involved in jasmonic acid regulation and can complex with MYB proteins to regulate glucosinolate biosynthesis. It has a secondary role of regulating ABA response under drought conditions, inducing rd22 a gene responsible for alleviating drought stress and induced by ABA [45]. Several heat shock proteins were repressed by as much as -9.9-fold. While no peroxidase genes were induced, one was repressed-

peroxidase 5 (-3.1). ACC oxidase is also repressed (-4.5) and given ethylene's role in stress response signaling, this indicates a contrast to ECO-S-T. However, three ERFs were induced- ERF060 (2.1 & 2.1) and ERF7 (6.7), providing some indication of ethylene biosynthesis early in the response process. Both proteins interact with the GCC-pathogenesis promoter sequence but ERF060 is an activator while ERF7 is a repressor. Stress enhanced protein 2 (2.5) and disease resistance protein RPM1 (7.1) were two of the induced stress- response genes. RPM1, induces a hypersensitive response following recognition of *P. syringae* avirulence proteins. Stress enhanced protein 2 (SEP2), is a unique protein believed to act as a photo-oxidative protectant, against ROS and cellular degradation [46].

Xenobiotic detoxification genes in response to propanil. Other stress-induced genes, which are potentially involved in xenobiotic detoxification for ECO-R-T are listed in Table 3. Only one gene, UGT73C2, was induced in both ECO-R-T and ECO-S-T; the remaining transcripts were different. CYP709B2 and CYP72A15 are of interest because of the multiple transcript variants, and high level of induction following treatment compared to the other cytochrome P450 genes. GSTU17 is also of note given its role in light signaling and morphogenesis which utilizes phytohormone signals to direct developmental changes [47]. The remaining detoxifying genes require further research to elucidate the impact they have on herbicide resistance.

Comparative overall response of ECO-S and ECO-R to propanil

Gene ontology analysis revealed no significant enrichment of terms in ECO-R-T, but several were enriched in ECO-S-T (Fig 3). A supercluster identified as 'trehalose metabolism in response to stress' was formed, which was composed of overexpressed terms related to response to nitrate, response to herbicide, nitrate assimilation, ABA metabolism, and response to water deprivation. Of the multiple enriched terms, the trehalose-metabolism-in-response–to-stress

term, was the most over-represented, as observed in the induction of genes in ECO-S-T described previously.

For comparatively repressed genes in ECO-R-T, 846 transcripts were repressed in ECO-R-T compared to ECO-S-T (Table 1). In terms of biological functions necessary for growth activities including photosynthesis, carbohydrate metabolism, sugar metabolism, and nitrogen metabolism, the majority of the genes were at a lesser abundance in ECO-R-T. These include transcripts for ferredoxin-6 (-6.5), nitrate reductase (<-6), nitrate transporters (<-4), and several trehalose biosynthesis enzymes (<-3). In terms of ABA action, several transcripts for ABA 8'-hydroxylase 1 (<-6.5) and two ABA receptors PYL5 (-4.8) and PYL8 (-3.7) were significantly repressed in ECO-R-T. Several ethylene-responsive transcription factors were comparatively repressed, however, only one ACC oxidase homolog was repressed in ECO-R-T. Finally, in terms of herbicide detoxification several GT and GST enzymes were repressed, indicating their reduced if not ineffective role in herbicide resistance. One cytochrome P450- CYP704C1, was significantly repressed (-7.9).

A total of 281 transcripts were elevated in ECO-R-T more so than ECO-S-T. Several transcription factors, mostly from the WRKY family which are elicitor-responsive proteins that interact with the W box segment of DNA, were induced. DNA-directed RNA polymerase 1 subunit RPA112 III (10.3) and polymerase III subunit 2 (10.8) were also enhanced. The latter, functions in the synthesis of small RNA's, regulatory RNA fragments which may be beneficial for coordinating the abiotic stress response. Phosphoenolpyruvate carboxylase (PEPc) kinase 1 was induced (>5). This is a protein essential for the activation of (PEPc) for the production of oxaloacetate, a primary component of plant metabolism. Activation of the ethylene biosynthetic pathway was also observed. Several ACC oxidase transcripts (3.3 to 4.1-fold) and an ACC

synthase transcript (8.6) were present at a much higher level in ECO-R-T. In turn, three ERF- 4 (4.3), 7 (8), and 11 (3.4), were induced; all are transcriptional repressors of the GCC-box pathogenesis promoter. Xenobiotic detoxifying enzymes were present in significantly higher concentrations in ECO-R-T. Transcripts for CYP704C1 (7.7), CYP71A1 (3.6), CYP72A15 (6.6 & 11.3), and CYP709B2 (3 & 10.4) were significantly induced following herbicide treatment. Also, GSTU17 (10.9), GST1 (3.9), and GST4 (>5.3) were all at a greater abundance. Three GT enzymes were induced including UGT88A1 (3.2), UGT88F3(5.9), and UGT73D1(7.3). Beta-glucosidase 22 (5.9) was also present.

Discussion

E. colona response to propanil involves the abiotic stress response pathway driven by ABA flux

ABA is a critical phytohormone necessary for the activation and downstream signaling of multiple abiotic stress responses, particularly in response to water deprivation [48]. Given its role in various activities, the signaling pathway and its implications have not been described in terms of signaling herbicide-induced response. While ABA itself functions to reduce stomatal conductance, it also directs several activities via calcium-dependent channels in the plant that lead to responses to abiotic stress [49]. High ABA concentrations alone can limit photosynthesis which in itself is detrimental to carbon assimilation and leads to cessation of grown, cellular disruption and even potentially plant death [50]. The response of ECO-S-T and ECO-R-T to propanil highlights the involvement of ABA in plant response herbicide (at least with propanil). The ECO-S-T transcriptome was greatly enriched with terms indicative of an ABA-mediated response, including ABA metabolism, response to nitrate, and response to water deprivation. Several genes were induced following treatment including two ABA receptors- PYL5 and PYL8

enzymes, signaling the potential presence of elevated ABA concentrations in the plant that might lead to stomatal closure. Induction of PYL5 and PYL8 have been shown to enhance resistance to drought via stomatal closure [51]. This is indicative of the plant attempting to slow photosynthesis in the presence of a photosynthesis inhibitor, such as propanil. MYB44, as well as other MYB-like proteins, were also expressed in ECO-S-T, which in the absence of ABA would reinforce stomatal closure, serving as a secondary factor that limits photosynthetic activity [45,40]. In terms of calcium transport directed by ABA, several calcium exchanger proteins, calcium-dependent protein kinases, and a calcium binding protein CLM36 were induced. This further implicates ABA-directed activities following treatment. The presence of ABA hydroxylase genes at the magnitude of induction observed in ECO-S-T is indicative of the high levels of ABA present 24 HAT. These proteins were not induced in ECO-R-T. Instead, the ABA hydroxylase and PYL5 and PYL8 genes were constitutively enhanced in ECO-R compared to ECO-S (data not shown). Treatment with propanil did not illicit further increase in expression of these genes, indicating that the native levels were sufficient to signal herbicide effects and initiate mitigation processes.

***E. colona* response to propanil is also tightly linked with biotic stress responses**

Jasmonic acid-mediated response. Biotic response characterization is as important as characterization of the abiotic stress signaling and response pathway. The jasmonic acid pathway as well as the general plant defense response were activated by propanil in both ECO-S-T and ECO-R-T. This was indicated by the induction of transcription factors MYC3 and MYC2 in ECO-S-T and ECO-R-T, respectively. MYC2 is directed by the action of ABA, complexing with MYB to impart ABA directed drought tolerance [45]. This interaction is also capable of producing glucosinolates, which are compounds toxic to insects and deter herbivory [41]. While

these glucosinolates and the jasmonic acid activity may not be involved in herbicide resistance, the signal transduction allows USA to connect the abiotic stress response to biotic stress response. Specifically, the the ABA activities are apparently inducing a wider whole-plant response that overlaps the jasmonic acid pathway, mediated through the MYC proteins [52]. While the induction of ABA related genes is much lesser in ECO-R-T, the high basal production of ABA would have stimulatory effects on the jasmonic acid pathway following treatment given these connections.

Disease resistance response and the implications for resistance. Disease resistance transcripts were also rampant across both the ECO-S-T and ECO-R-T responses, interestingly they often did not have similar transcript expression patterns. Important to general disease or pathogen infection is the induced hypersensitivity response to the avirulence proteins of certain pathogens. The hypersensitive response is characterized by an intermittent burst of hydrogen peroxide that results in cell death at the site of infection, limiting the movement of the pathogen out of the infected area [53]. The four-member RGA family of disease resistance proteins were differentially expressed across ECO-S-T. RGA4 transcripts were repressed in ECO-S-T while RGA2 and RGA1 were induced following treatment. While these genes act in concert, because of their similar positions on the locus, RGA2 is the only protein which recognizes and responds to the avirulence protein. Transcripts of disease resistance protein RPM1 were more prevalent in ECO-R-T but also observed in ECO-S-T. RPM1 is another avirulence recognition protein that incites an oxidative response that leads to a hypersensitive reaction [54]. These among several other non-specific disease resistance proteins were all expressed. These proteins may contribute to herbicide response, and eventual resistance, by restricting the movement of the herbicide following treatment; such as observed in glyphosate-resistant *Ambrosia trifida* populations [55].

In this case, rapid cell death caused by ROS imparts a high level of resistance to glyphosate [56]. Within 24 hours of treatment, propanil action in R and S populations results in lesions on the leaf surface, often described as necrosis or leaf burn. In some instances including with ECO-R, resistant plants would appear completely necrotic and dead within one week of treatment, but regenerate to a healthy plant by three weeks, as described by so-called 'phoenix-resistance' [personal observation, 56] . This response may be imparted by these hypersensitive pathogen-response genes. The difference in response between S and R may be based on the presence or absence of these avirulence protein genes and their action. More importantly, the hypersensitive type response may be imparted as a abiotic stress avoidance mechanism. Multiple peroxidase genes were greatly induced in ECO-S-T, while none were induced in ECO-R-T. While the action of the peroxidase genes helps to alleviate the oxidative stress, this may allow the herbicide to move and become more destructive, resulting in prolonged exposure to propanil, leading to death. The 'hypersensitive-response' in ECO-R-T may contribute to the resistance mechanism of the plant. This would require biochemical validation and physiological assessment.

Trehalose biosynthesis has a role in ECO-S response to herbicide and ECO-R predisposition to tolerate the herbicide

Trehalose is a non-reducing sugar that has been implicated in abiotic stress tolerance in several plant and bacterial species [58,59]. This sugar can impart several properties to the plant including tolerance to dehydration, enhancement of photosynthesis, and scavenging ROS [60,61]. Its intermediate, trehalose-6-phosphate (Tre6P), is also a major constituent in sucrose signaling and starch to sucrose conversion in the plant, capable of coordinating many growth processes. This would result in larger plants [62]. Trehalose induction was noted in ECO-S-T in both the network assessment and the gene expression profile whereby both of the precursory

enzymes TPS and TPP were induced, but a significant induction was not observed in ECO-R-T. This provides evidence that no significant change in the regulation has occurred. Unlike ECO-S-N, ECO-R both constitutively upregulates both TPS and TPP without and following herbicide treatment, as implicated by both the gene ontology clusters and the differential gene expression profiles. This means that the trehalose is present at the time of application and the onset of the abiotic stress response. The induction of trehalose biosynthesis in ECO-S-T is of note as this is the first observation of trehalose biosynthesis involvement in herbicide response. Trehalose may provide several benefits to the plant following herbicide application; however, the concomitant decrease in photosynthesis and carbon assimilation processes may mean this response is transient. The lack of trehalose supply would limit its activity.

In contrast, ECO-R-N has constitutive enhancement of the genes necessary to produce trehalose as well as elevated carbon assimilation activities compared to ECO-S-N (data not presented). Free trehalose and Tre6P may have active roles in the potential for the plant to survive treatment with a photosynthesis inhibitor when paired with upregulation of a detoxifying enzyme. Trehalose may be acting as an integral membrane stabilizer to protect not only against oxidative damage caused by ROS but also those produced in the hypersensitive response, described previously. The sugar moiety is able to stabilize protein membranes by connecting itself with the polar heads of the lipid bilayer, forming hydrogen bonds, subsequently stabilizing the membrane by preventing phase transition and leakage [59,60,63]. The trehalose may also serve as an osmolyte that allows for the hypersensitive response to occur in the leaf tissues and the plant to regenerate from the meristematic zone of the grass [61]. This would make it a somewhat unique feature to grasses, as the meristematic regions are at or below the soil surface, making them less affected by the photo-oxidative damage occurring in the leaves. The build-up

and storage of trehalose in the plant may be the necessary source for prolonged growth following severe photo-oxidative damage. Finally, another key component of survival for the ECO-R population may be the presence of transcripts for trehalose phosphorylase. This is a key enzyme involved in the catabolism of trehalose into glucose 6-phosphate and beta-D-glucose 1-phosphate, two compounds with active roles in several biological processes [44]. Under severe stress the plant would require both compounds, glucose 6-phopshate in particular, to not only regenerate by serving as an energy source, but as a substrate in several metabolic processes. This not only provides a basic framework for the role of trehalose in protection against herbicides but implicates it as a pivotal compound in the prolonged activities of plants following herbicide treatment.

Herbicide resistance in ECO-R is driven by coordinated induction of cytochrome P450 hydroxylation and glutathione conjugation

Initial investigation into the herbicide resistance mechanism included a search or the aforementioned aryl-acylamidase protein transcript but it was not present within the transcriptome response profile. Based on the transcriptome profiles of ECO-R-T before and after propanil treatment and the comparative analysis between ECO-R-T and ECO-S-T, it is possible that we have identified both the primary and secondary mechanisms of propanil detoxification (Fig 4). First, the oxidative step may involve either CYP709B2 and/or CYP72A15. Both have near identical expression profiles and would provide the necessary hydroxylation to reduce propanil into the two products- 3,4 dichloroaniline and propionic acid; as observed with the aryl-acylamidase protein [31]. The shear abundance of their transcripts and expression profiles across differential expression analysis implicate the role of these enzymes in propanil detoxification. CYP72A15 has not been described in the literature while some research on CYP709B2 has been

conducted. A sister gene in the family-CYP709B3, has been investigated for its role in ABA- and salt stress response; increasing ABA levels do result in the induction of CYP709B2 but serve no function in alleviating the stressor [64]. Members of the CYP709 subfamily are stimulated in response to IAA which is also induced under stress [65]. The secondary step in the detoxification pathway is the conjugation of metabolites to an endogenous moiety usually sugar (GT) or glutathione (GST) [23]. While several GT enzymes were induced, they serve various roles in the plant that are less associated with the detoxification process. However, given their affinity for carboxylic acid side chains, one of the GT enzymes may act on the propionic acid derivative [66]. Several of the GST enzymes are members of the tau family of GST's, with known roles in exogenous xenobiotic detoxification including herbicides [42]. One in particular GSTU17, has been investigated due to its response to light and plant hormones including ABA [47]. This study also investigated its affinity to the substrate 1-chloro-2,4-dinitrobenzene, a compound similar in structure to propanil and the 3,4-dichloroanaline metabolite, containing both a chloride and nitro group. The chloride side group serves as an indicator of potential GST activity whereby a nucleophilic substitution can occur and the thiol group of glutathione can form a bond to the benzene ring [67]. Given the gene expression profile and known substrates for the enzyme, this is most likely the secondary step in the xenobiotic detoxification process for propanil in ECO-R. This requires further validation in bacterial and plant systems to verify the hypothesized interactions.

Conclusions

Herbicide resistance is a complex polygenic response to the imposed abiotic stress from herbicide action. Continuous selection pressure imposed not just by herbicides but general management, selects for a variety of traits that can be classified as both domesticated but also

weedy. The weed management process allows for the adaptive evolution in the face of adversity, and the traits evolved for specific tolerances or resistance cannot simply be identified in isolation and considered as monogenic. This is best exemplified by the propanil response and herbicide resistant transcriptome outline here. By contrasting the low-tolerance phenotype of ECO-S to that of the multiple and highly propanil-resistant ECO-R, with and without treatment, we conclude that three pathways were responsive to propanil: abscisic acid, jasmonic acid, and the trehalose biosynthetic pathways. While the responses are not identical, key features such as the altered regulation of the phytohormones, the impact of hypersensitive responses, and the use of trehalose to mediate the negative effects or secondary or tertiary herbicide activity are present. Using the transcriptome and the comparative analysis between gene ontology and expression profiles, we are able to provide a novel herbicide resistance pathway that may be employed by ECO-R. Two cytochrome P450 genes CYP709B2 and CYP72A15, with the ability to detoxify propanil into its hydroxylated substrates, clearly take action in response to the herbicide as indicated by their respective expression profiles. Given the presence of multiple GT and GST enzymes transcripts the potential for secondary interaction with the products 3,4 dichloroanaline and propionic acid is also highlighted. Collectively, this research provides the first holistic understanding and documentation of stress-responsive genes affected by propanil in a multiple-resistant *E. colona* population. This work not only demonstrates the utility of transcriptomics in understanding weed biology and physiology, but provides gene expression and literature support for the findings.

Materials and Methods

Plant materials

Accession source and population profiling. From 2010 to 2016, the University of Arkansas weed physiology group has been collecting putative herbicide-resistant *Echinochloa* populations from rice production fields throughout the state of Arkansas. These accessions survived late into the season following early herbicide applications for management. Collections took place in the late summer to early fall prior to rice harvest; these accessions were bulk sampled by in-field location and farm, with field histories collected when possible. All of the samples were submitted to the University of Arkansas Altheimer Laboratory for assessment of their resistance profile and characterized according to their species. Results for the herbicide resistance screening and species abundance, including, the methodology used for resistance profiling are presented in Rouse et al. [10]. Populations with unique profiles and of interest for further research were then grown in isolation to produce pureline seed. Due to the low outcrossing rate of *E. colona*, a single generation has been determined as adequate for production of homozygous individuals. *E. colona resistant* (ECO-R) was collected in 2010 from Lawrence County, Arkansas, and selected for this research due to its unique multiple-resistant profile to three rice herbicides- cyhalofop (~2x field dose), propanil (>8x field dose), and quinclorac (>32x dose), and one soybean herbicide- glufosinate (~2x field dose) (data not presented). Another accession, *E. colona susceptible* (ECO-S), was also selected and grown in isolation. This accession was selected because of its similar cropping history and geographic location, as well as its high level of susceptibility to the herbicides of interest. Propanil tolerance is observed at approximately a 3x field dose, which is common to the state of Arkansas but a sufficient contrast to the ECO-R phenotype.

Plant Treatment and Processing for RNA- sequencing. Pure-line generated seed of both the ECO-R and ECO-S accessions were germinated in square pots with commercial potting soil in a growth chamber set to 14-hr day length, 33C day temperature, and 24C night temperature. Each pot was replicated twice to provide two biological replications of each herbicide by accession combination. Within each pot, a single plant was maintained prior to herbicide application for approximately two weeks. When the plants reached the two fully expanded leaf growth state, they were treated with propanil (4.5 kg ha^{-1} + nonionic surfactant at 0.25%v/v). The plants designated to receive the treatment were treated simultaneously in a motorized spray chamber calibrated to deliver 187 L ha^{-1} from a 250-mL tank volume. After the plants were allowed to dry, approximately 30 mins, both the treated and nontreated counterpart were labeled as either ECO-S/R-N (for nontreated) or ECO-S/R-T (for the treated), and moved back into the growth chamber. Exactly 24 hours after application, the above ground portion of the plants, including both the shoot and leaf tissues, were harvested and immediately submerged in liquid nitrogen. The samples were then transferred to RNAlater™-ICE (Invitrogen, Carlsbad, CA, USA) for shipment to the Clemson University Genomics Institute, in Clemson (CUGI), South Carolina.

RNA Extraction, Transcriptome Assembly, and Annotation

The processes and methodology for the RNA extraction, RNA-sequencing, transcriptome assembly, and transcriptome annotation is outline in Rouse et al. [37]. A brief summary is included here to provide a cohesive understanding of the research and analysis pipeline. Total RNA was extracted according to the manufacturer's instructions for the commercially available kit. The paired-end library was prepared using the TruSeq Stranded Total RNA kit (Illumina Inc., SandDiego, CA, USA). Cytoplasmic and mitochondrial rRNA was removed to improve the quality of results. Following RNA fragmentation, the RNA was reverse-transcribed into cDNA

using random primers and then second strand of cDNA was synthetized based on the cDNA template. These fragments were then tagged with an additional 'A' and the adapter for sequencing. The final cDNA library was prepared from PCR enriched and tagged sequences. All of the samples were submitted to the Holdings Cancer Center at the Medical University of South Carolina, Charleston, SC, USA where they were sequenced on an Illumina Hiseq 2500 platform. The *de novo* transcriptome was assembled from the sequenced results. The data files were assembled using the Trinity RNA-Seq pipeline (Babraham Institute, Cambridge, UK). Following data quality checks and processing to normalize the samples using the TrinityRNASeq 2.2.0 software, the transcriptome was assembled using Trinity and Transdecoder 3.0.1 (Broad Institute. Following assembly, the Trinotate 2.0 software package was used for the functional annotation of the transcriptome by homology to BLAST+ and Swissprot data bases. Both the HMER and PFAM were used to generate the necessary information for the primary annotation databases which included eggNOG, GO, and KEGG.

Differential gene expression analysis

The aforementioned transcriptome was used in all subsequent analysis for a description of the treatments of interest. A GTF file was generated for comparing each of the samples, T and N, to the reference transcriptome. Feature counts were generated using the Subread package (http://subread.sourceforge.net/), which were used to quantify the differentially expressed transcripts for both replications that were paired concordantly, ensuring proper analysis given the differences between the two replications. Using the R statistical software (https://www.r-project.org/), the edgeR package developed by Bioconductor (https://www.bioconductor.org/), quantified the filtered raw counts from the RNA sequencing with standard normalization performed using the trimmed mean of M-values applied to the counts[68,69]. Each set of counts

were fit using a GLM model for determination of significance ($p \leq 0.01$). For each comparison of interest, including the nontreated and treated conditions, a likelihood ratio test (LRT) was performed to identify the fold differences. The analysis was then further evaluated using a false discovery rate for p-value correction to reduce the error in the results.

The raw output from these results were used to generate a table of annotated data for the comparisons of interest as well as the statistical values and log2-fold changes for each of the transcripts of interest. This information was further qualified manually to categorize important genes into functionally relevant categories including: carbon assimilation, photosynthesis, sugar synthesis, fatty acid synthesis, stress signaling, ethylene biosynthesis, nitrogen metabolism, and herbicide detoxification. Several subcategories were assembled for each. For herbicide detoxification, a review of literature revealed several key categories that were investigated in the analysis including ABC transporters, aminotransferases, cytochrome P450s, glutathione-S-transferase, glucosyltransferases, and glucosidases [23,70,71]. To describe each of the genes and pathways for the associated genes, both the Uniprot [72] and KEGG [73]databases were used for basic descriptions. To process the large quantities of data for characterization and to identify various overlapping gene profiles, JMP Pro 13 (SAS institute, Cary, NC) with the Venn Diagram add on package was used.

Gene ontology analysis

The Trinotate output was used with the 'goseq' package from Bioconductor to assign GO terms to the transcripts from the transcriptome. The enrichment analysis was performed on the transcripts which have been expressed at a log2 fold change of ≤ -2 or ≥ 2 and a p-value of ≤ 0.01. This generated an over represented p-value which was used to assess the significance of each term. The results of the analysis were visually assessed using REVIGO (http://revigo.irb.hr/) to

generate superclusters that share overlapping terminologies based on semantic similarity. The output from this clustering was then visualized with the Cytoscape Network Analysis software (http://www.cytoscape.org/). For description of the gene ontology terms and functions of the terms EggNOG [74] and the GO Consortium [75] databases were used.

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Table and Figures

Table 1. Summary of the repression and induction of genes from the differential gene expression analysis of each of the comparisons of interest.

	Expression	ECO-S-NT vs ECO-R-NT	ECO-S-NT vs ECO-S-T	ECO-R-NT vs ECO-R-T	ECO-S-T vs ECO-R-T
Total Genes	Decrease	348	1765	5639	846
	Increase	2127	1774	153	281
	Total	2475	3539	5792	1127

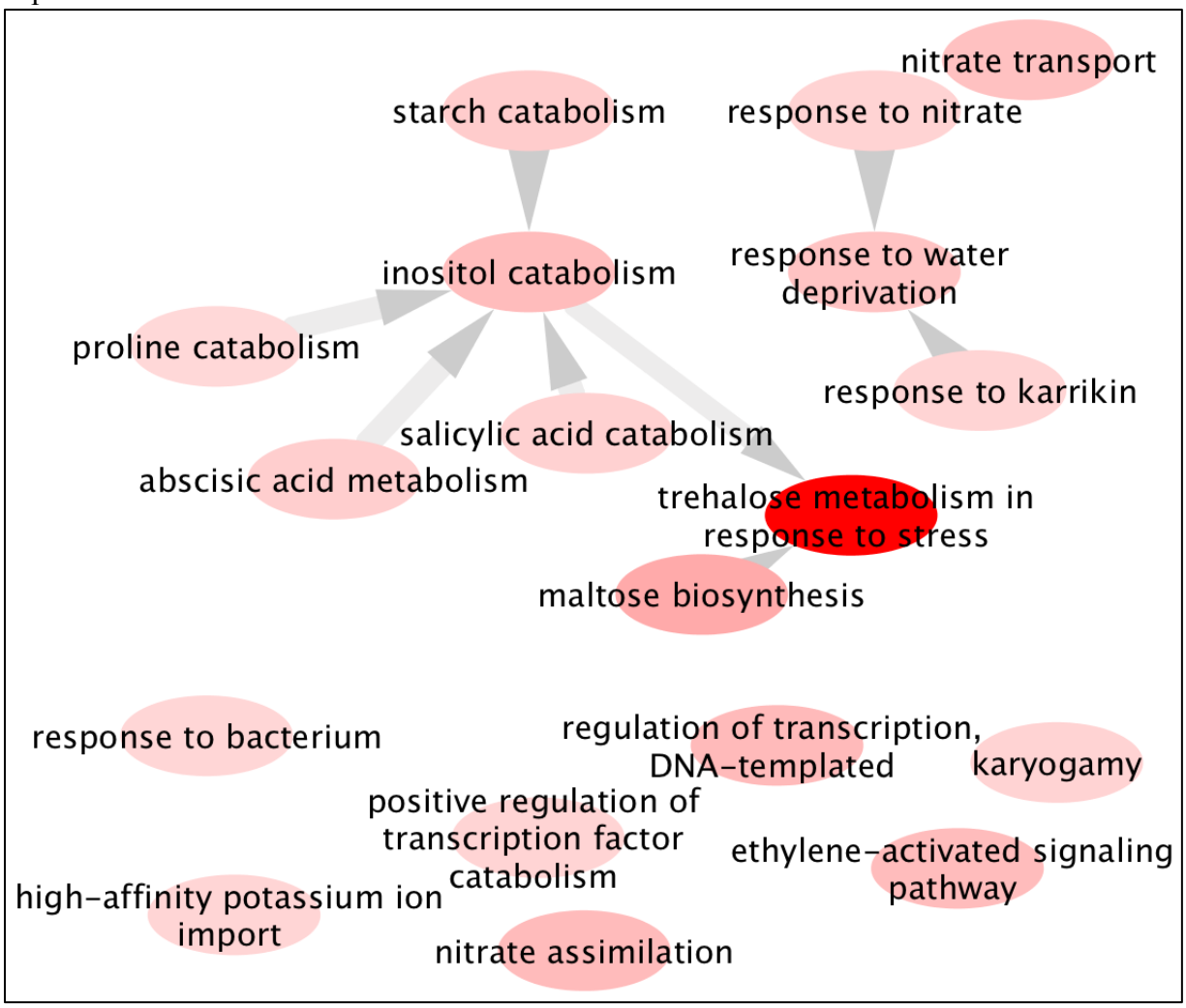
Table 2. Summary of the induction of xenobiotic detoxification genes and gene families with the corresponding fold change induced within ECO-S following propanil treatment.

Gene Family	Transcript ID	Fold Change
ABC Transporter	ABC transporter G family member 42	1.5
	ABC transporter G family member 5	2.8
	ABC transporter G family member 53	2.3
	ABC transporter G family member 53	4.7
Acetyltransferase	Heparan-alpha-glucosaminide N-acetyltransferase	2.5
	Uncharacterized acetyltransferase At3g50280	2.5
	Uncharacterized acetyltransferase At3g50280	3.5
Cytochrome P450	CYP716B1	3.0
	CYP71A1	2.9
	CYP71A21	5.1
	CYP71D7	5.9
	CYP94C1	3.7
	CYP94C1	5.4
	CYP94C1	5.5
Glutathione-S-transferase	Probable glutathione S-transferase	1.8
	GSTU1	2.5
	GSTU1	2.7
	GSTU6	2.3
	GSTU6	4.1
	MSR-1	2.1
Glycosyltransferase	UGT73B4	3.8
	UGT73B4	5.7
	UGT73C1	3.1
	UGT73C2	3.3
	UGT74D1	2.2
	UGT75C1	2.9
	UGT83A1	2.0
	UGT83A1	3.6
	UGT83A1	6.0

Table 3. Summary of the xenobiotic detoxification genes and gene families with the corresponding fold change induced within ECO-R following propanil treatment.

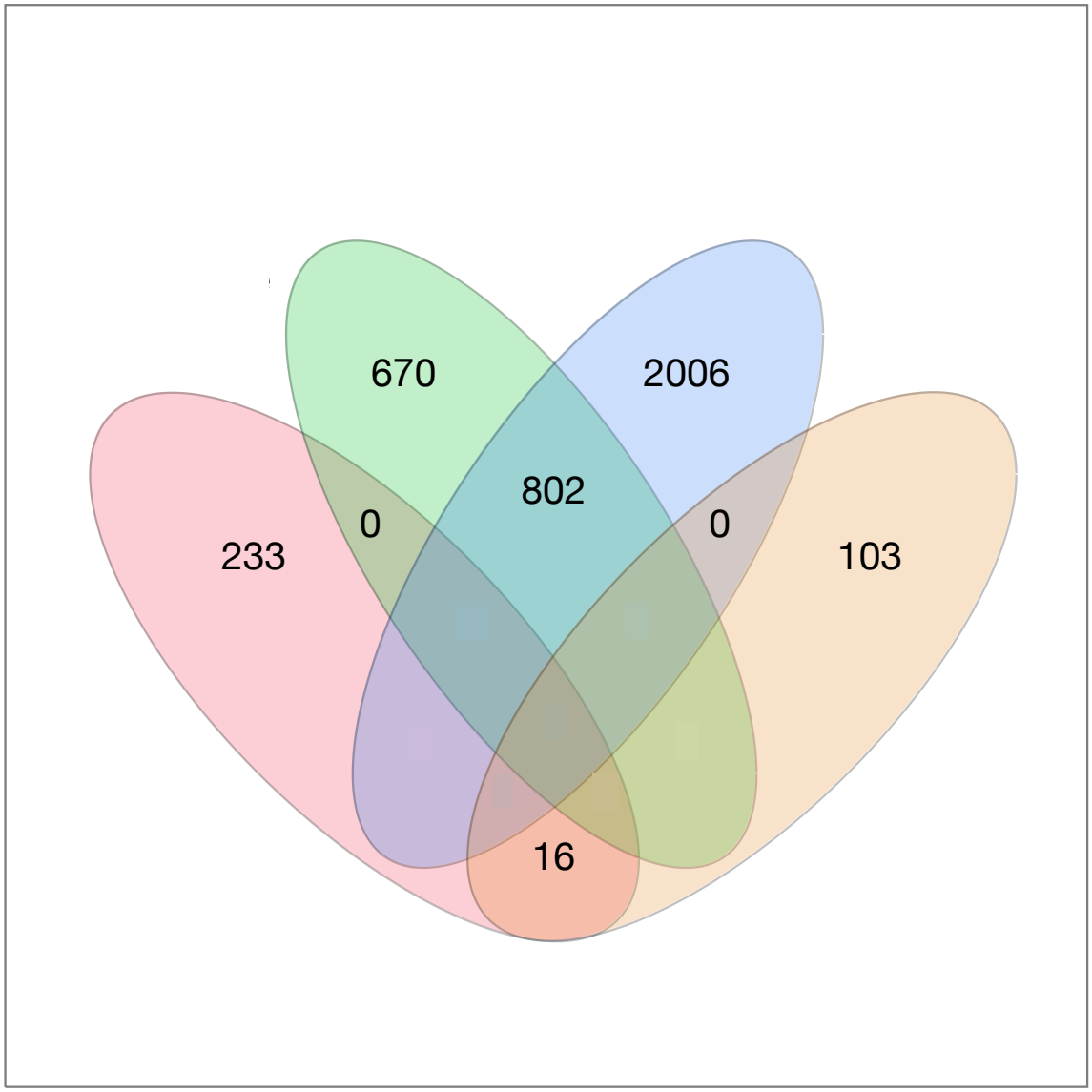
Gene Family	Transcript ID	Fold Change
ABC Transporter	ABC transporter C family member 10	7.4
Cytochrome P450	CYP72A11	3.1
	CYP89A2	2.5
	CYP89A2	3.8
	CYP89A2	3.6
	CYP709B2	8.6
	CYP709B2	3.9
	CYP72A13	3.8
	CYP72A15	2.6
	CYP72A15	3.4
	CYP72A15	2.9
	CYP709B2	4.7
	CYP709B1	7.0
Glucosidase	Beta-glucosidase 22	3.6
Glutathione-S-transferase	GSTU17	7.9
	GST23	6.7
Glycosyltransferase	UGT73E1	3.0
	UGT73C2	4.7
	UGT83A1	2.3
	UGT74G1	4.6
	UGT74G1	4.5
	UGT73D1	5.3
	UGT73D1	6.9
	UGT73D1	4.8
	UGT75D1	7.4

Figure 1. Diagram of the significantly enriched gene ontology terms in ECO-S following propanil treatment.



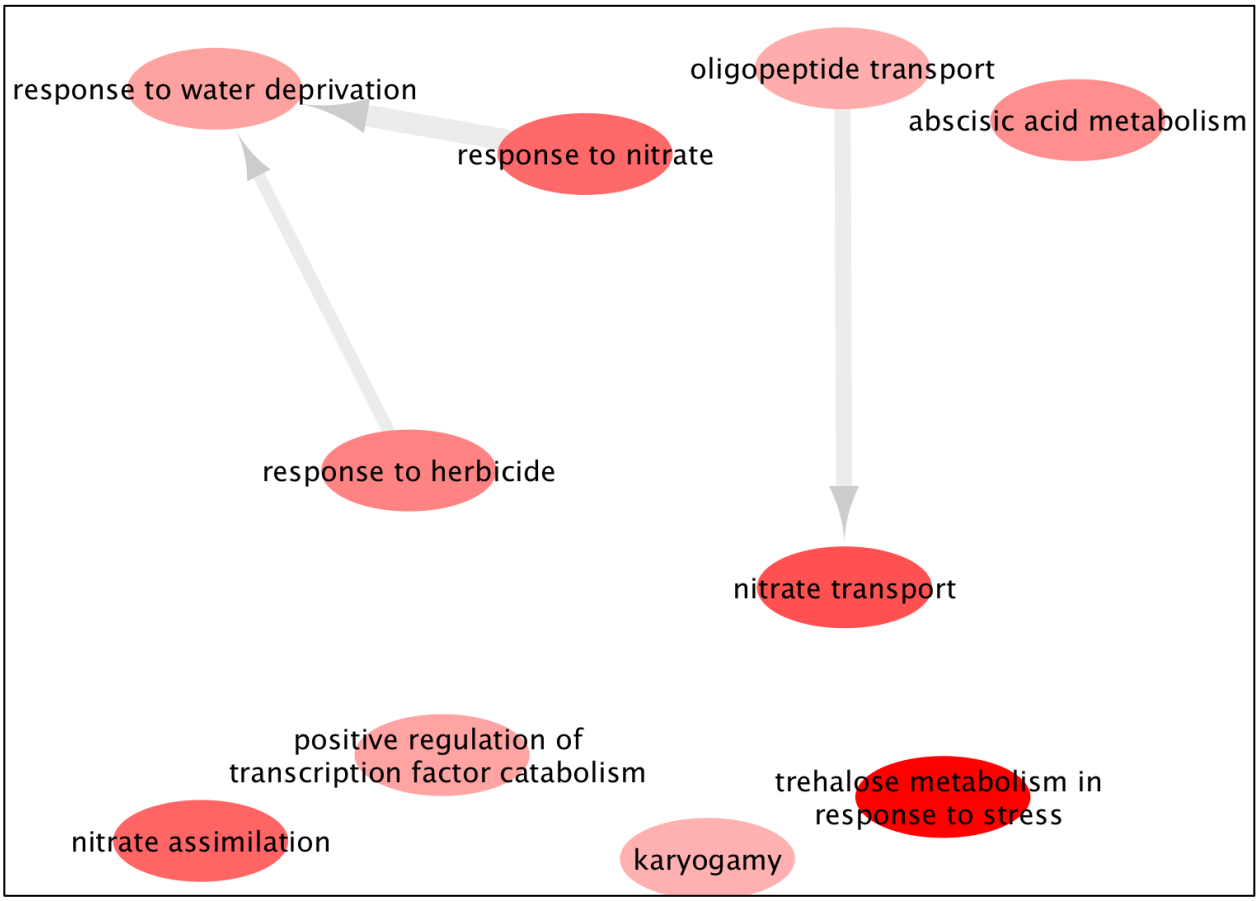
[1] The intensity of the red color for each oval indicated the significance of the p-values based on the gene ontology analysis, with the arrows signifying the relationship between each of the onotlogical terms. The location and relation of each oval within the cluster signifies the relationships to the terms in semantic, 2-dimensional space.

Figure 2. Venn diagram for the differential gene expression analysis with each oval representing the number of repressed (A/C) or induced (B/D) of genes within the comparisons of ECO-S-N vs ECO-R-N (A/B) and ECO-R-N vs ECO-R-T (C/D).



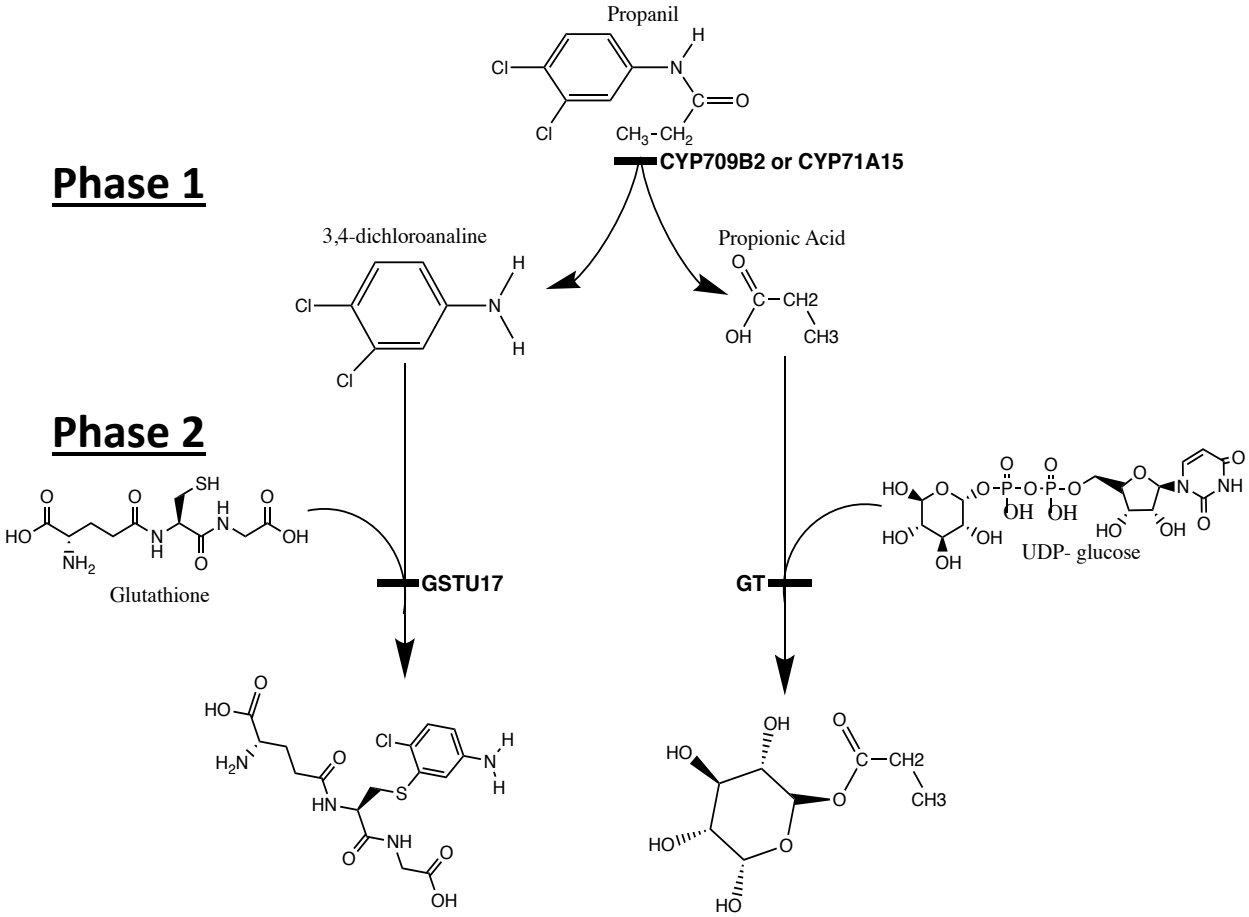
[1]Overlapping ovals signify that specific genes are shared across the comparisons.

Figure 3. Diagram of the significantly enriched gene ontology terms in ECO-R following propanil treatment.



[1] The intensity of the red color for each oval indicated the significance of the p-values based on the gene ontology analysis, with the arrows signifying the relationship between each of the onotlogical terms. The location and relation of each oval within the cluster signifies the relationships to the terms in semantic, 2-dimensional space.

Figure 4. Proposed two pahse detoxification pathway for propanil via phase 1 hydroxylation by CYP709B2 and/or CYP71A15 and phase II conjugation of glutathione and UDP-glucose to the substrates 3,4-dichloroanaline and propionic acid, respectively.



Multiple Herbicide Resistance in *Echinochloa colona*: A multi-herbicide comparative transcriptome analysis

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Abstract

The *Echinochloa* genera are among the most problematic weeds in upland and lowland agricultural environments throughout the world. A history of co-evolution and management with major crops, in particular rice, have led them to their modern prominence. It is their ability to adapt to both abiotic and biotic stressors that allow them to persist and accumulate the necessary genomic and physiological components to persist in dynamic agricultural environments. Our research into a multiple-resistant *E. colona* population from Arkansas (ECO-R) has led to the identification of several potential genomic components and physiological factors that endow high levels of resistance to propanil and quinclorac. The following research provides the first multi-herbicide comparison of the response of *E. colona* to cyhalofop, glufosinate, propanil, and quinclorac to describe the global transcriptional patterns. Initial investigation into the responses of ECO-R following cyhalofop and glufosinate treatment revealed the constitutive induction of both herbicide targets-acetyl CoA carboxylase and glutamine synthetase, respectively. Cross response comparisons between the herbicides of interest in susceptible *E. colona* (ECO-S) indicate that the abiotic stress response pathway, specifically actions mediated by abscisic acid, are involved in the herbicide response. Biotic stress signaling is also key to the response by ECO-S as the accumulation of several enzymes responsible for reducing disease or pathogen infection are induced. ECO-R is very different in that it enters a state of static action following treatment, with very few genes induced across all of the herbicide responses. UGT75D1 is the only gene expressed across all of the herbicides of interest. Given its's actions as a glycosyltransferase it is possible it can interact with the four herbicides. This research validates previously held conceptions that there are shared responses following herbicide action with both

abiotic and biotic stress responses. It also provides insight into the potential of a shared herbicide resistance mechanism endowing multiple-resistance.

Introduction

Weeds are the most problematic biotic factors that impact crop production and threaten sustainability of modern agriculture. Crop competition with weedy species account for 34% yield loss across agricultural systems world-wide and without chemical control may result in as much 74% and 82% yield loss in major commodities such as corn and soybean, respectively, in North America [1–3]. To manage weeds in agroecosystems, herbicides are the most efficient, cost-effective tools. The efficiency and low cost of herbicides have resulted in overdependence on them as primary means for weed control [4]. The shift away from integrated approaches to a system of heavy reliance on herbicides has led to rampant herbicide resistance evolution across 91 cropping systems in over 259 weedy species [5,6]. Investigation into the underlying mechanisms of resistance has been a major topic of weed research since the late 1990s [7]. Technology advancements have expanded the capabilities of investigators and explorations on genomic approaches to understand weedy traits, including herbicide resistance, has increased significantly [4,8,9].

Weedy species can adapt to adverse conditions. Domestication of weedy species as crops has positioned several weeds to be less responsive to management imposed in the cropping systems that they infest. Barnyardgrass (*Echinochloa crus-galli*), a major rice weed, was grown and processed alongside rice 10,000 years ago in China [10]. Selection may be imposed by management strategies including tillage, modified cultural practices such as crop rotations, and herbicides which all can result in rapid evolutionary change [7,11]. The selection may lead to weedy populations arising from cultivated crops. De-domestication has occurred in California rice production, resulting in a resurgence of several weedy rice populations [12]. This evolutionary ‘escape-to-ferality’ poses a significant threat to the crop due to a lack of adequate

control measures for the evolved weedy relative[12]. Upland weeds such as Palmer amaranth (*Amaranthus palmeri S. Wats*) also may exhibit morphological changes which aid in reproduction, driven by the cropping system for which it is grown [13]. This has led to dramatic shifts in its reproductive potential in diverse cropping conditions and expansion of the geographic range it may impact. Adaptation to control and environment have led to crop mimicry which has been observed within the *Echinochloa* genus [14]. Several populations of *Echinochloa* have been unintentionally selected for which have morphological and biological similarity to rice, making hand weeding and eradication impossible.

The *Echinochloa* species complex is a global concern impacting many agricultural commodities, particularly those in lowland agriculture production such as rice. These species also impact upland cropping systems with populations throughout North America observed in several grain crops, soybeans, vegetables, and perennial fruits [15]. *Echinochloa* is composed of several weedy species including *E. colona* (junglerice), *E. crus-galli* (barnyardgrass), *E. oryzoides* (early-watergrass), and *E. phyllopogon* (late-watergrass). In Arkansas, and throughout the Mid-south USA, the dominant species is *E. colona* with *E. crus-galli* and *E. muricata* also being present and growing within the same production areas [16]. While the frequency of herbicide resistance among this populations does not appear to shift in favor of one species over another, their underlying genetics and biology make the *Echinochloa* species adaptive and problematic. *Echinochloa* species range in ploidy from 4x to 6x, amplifying the complexity of the genome. Management of this species has historically been a combination of cultural management and herbicides [17]. Propanil, a photosystem II inhibitor, and quinclorac, a plant growth regulator, have long been standards for *Echinochloa* management in the Mid-south where they have been used on an extensive number of acres. Since the early 1990s, herbicide resistance

in *Echinochloa* in the USA has been a problem with populations resistant to all major rice herbicides including propanil, quinclorac, cyhalofop, clomazone, and imazethapyr and non-rice herbicides such as glyphosate [6]. *Echinochloa* is described as one of the "worst herbicide-resistant" weeds in the world due to its high genetic variability, partially imparted by its ploidy [18]. This is exemplified by *E. colona* with 25 reported cases of resistance to 6 herbicide modes of action in 14 countries [6]. Multiple resistance is also a concern with as much as 27% of Arkansas populations exhibiting resistance to two or more herbicides, and increasing in recent years [16]. Research into the mechanisms of resistance has been limited to traditional physiological and biochemical assessment focused on single resistance mechanisms, but genomic characterization is limited. Given its unique physiology, genetics, and ability to adapt to adversity, *Echinochloa* should be considered as a valuable resource for information on weedy traits and herbicide resistance mechanisms.

Herbicide resistance is an adaptive response to abiotic stress. The rate of resistance evolution is dictated by several traits including fitness, fecundity, frequency of herbicide resistance genes, and the total number of individuals treated over time [18]. The underlying mechanisms of resistance, target-site or non-target-site, evolve as response to the herbicide dose. Target-site resistance is a monogenic trait resulting from mutations in the genetic code that substantially alter the herbicide target protein and reduce the herbicide activity, evolutionarily driven by high dose selection [19]. Conversely non-target-site resistance, a polygenic trait, is a result of continuous low dose selection and involves enzymes or proteins that have a role in physiological response to stress that reduce the activity or concentration of the herbicide at its target [19–21]. These processes may involve restricting the movement of, redistributing, modifying, or sequestering the herbicide. The basis for these activities are a function of the

physiological processes of xenobiotic detoxification which include the four phase degradation process involving the breakdown, conjugation, transportation, and inactivation of compounds [22]. Non-target-site resistance is a complex trait that is widespread but less understood. Multiple resistance arising from one, or a combination of TSR and NTSR is the largest concern for weed management. Multiple resistance is the evolved resistance to more than one herbicide mode of action within a single plant. Non-target-site resistance has the potential to impart multiple resistance via a single mechanism which limits the options available for weed management [23].

Genomic assessment of weedy species is limited because of a lack of resources to evaluate non-model organisms. However, as the cost of 'omics' technologies has declined with time, a call for more genomic resources in weed science has been made [8,24–26]. Next-generation-sequencing and bioinformatics facilitate assembly of databases that contain useful genes for various research needs and comparative analysis. The *de novo* transcriptome constructed from Illinois *Amaranthus tuberculatus* (waterhemp) populations provided the first set of herbicide-target genes for this genera and also allowed for phylogenetic assessment of other weedy species from this genus [27]. Using transcriptomics, non-target-site resistance markers for *Lolium sp,* have been characterized related to acetolactate-synthase (ALS) inhibitor resistance [28]. *Alopecurus myosuroides* (black-grass) sequencing has also led to the development of a database of non-target-site alleles for investigation into novel resistance traits [29]. This research, and others like it, have led to the development of a list of candidate genes or gene families involved in herbicide resistance including: ABC transporter, cytochrome P450 enzymes, glucosyltransferases (GT), glutathione-s-transferases (GST), among several other degradative genes [30]. RNA-sequencing was used to probe *Lolium rigidum* populations from Australia to elucidate potential herbicide resistance mechanisms [31]. This research identified

four candidate genes- two cytochrome P450s, a nitronate monoxygenase, and one GT enzyme, with active roles in the resistant phenotype. We recently released the first *de novo* transcriptome of *E. colona* and used this to characterize herbicide response and identify potential genes involved in resistance to quinclorac and propanil [32,33]. These are the first such characterization and global genetic network characterization of the potential resistance mechanisms and co-evolved abiotic stress responsive genes.

To date, the global molecular response to herbicides in *E. colona* has not yet been explored. Most especially, a comparative analysis of molecular response to different herbicides has not yet been done. This research presents a comparative analysis of the transcriptome profiles of multiple-resistant *E. colona* in response to four herbicides. The goal is to utilize the multiple-herbicide-response-transcriptome to resolve the underlying mechanisms that could impart resistance to multiple herbicides in *E. colona*, or other weeds. In this study, we aimed to identify candidate genes, gene networks, and biochemical pathway modifications in a multiple-resistant *E. colona* that are specifically or universally responsive to cyhalofop, glufosinate, propanil, and quinclorac. The outcomes of this research will provide potential genes for non-target-site resistance and also indicate future research avenues to preemptively manage weedy populations and identify weediness traits.

Results

Unique transcriptomic profiles for the constitutive- and herbicide response differences amongst ECO-R and ECO-S

Constitutive gene expression differences between ECO-R and ECO-S without herbicide application. Constitutive expression of gene networks and specific genes unique to ECO-R and ECO-S are presented in Rouse et al. [32]. This analysis revealed several traits unique to ECO-R

that potentially predispose the population to tolerate or avoid herbicide action. The trehalose metabolism pathway was enriched in ECO-R. The constitutively upregulated processes include response to nitrate, proline catabolism to glutamate, ethylene activated signaling, response to herbicides, and trehalose metabolism in response to stress. Nitrate metabolism was also enriched, indicated by the assimilation and transport of nitrate within the plant. Among these terms were multiple highly enriched terms relating to galactinol-galactosyltransferase activity, cytoplasmic translation, transcription, transcriptional elongation and protein folding. When paired with the cellular component terms related to ribosomes, it is apparent that ECO-R exhibits elevated biological function without the addition of abiotic stress.

Using ontological terms for probing into specific genes of importance within the nontreated ECO-R treatments (ECO-R-N) revealed that ECO-R is possesses several traits that make it a more vigorous plant when compared with ECO-S-N. The comparative profile for ECO-R-N included induction of transcripts for photosynthetic apparatus proteins such as ferredoxin, ATP synthase subunits, and photosystem II core complex proteins. Enhanced photosynthetic capacity results in the potential build-up of energy sources for physiological functions. Carbon assimilation gene transcripts were also enhanced. Transcriptome profiling identified key components such as malate dehydrogenase, phosphoenolpyruvate carboxylase kinase, and pyruvate dehydrogenase subunits that were enhanced in ECO-R-N. Fatty acid synthesis, via the acetyl-CoA carboxylase transcripts for the respective proteins, was also enhanced. The constitutive induction of these genes supports many processes including trehalose biosynthesis. Gene transcripts for proteins including the synthase and phosphatase genes necessary for UDP-glucose conversion to trehalose were enhanced in ECO-R-N. This is highly relevant to this research because of the role of trehalose sugars in abiotic stress mediation. The analysis also

identified several xenobiotic detoxification genes in the cytochrome P450, glutathione-S-transferase (GST), and glucosyltransferase (GT) families of proteins that may have a role in reducing exogenous compounds, like herbicides. Collectively, the induction of these genes and processes indicates that ECO-R-N may have the traits necessary, prior to herbicide action, to tolerate adversity following herbicide treatment.

Cyhalofop transcriptome response following treatment. Cyhalofop elicited the same transcriptomic response in ECO-S and ECO-R (data not presented). For ECO-S, several superclusters for biological process terms composed of response to high light intensity, polysaccharide metabolism, and carbohydrate metabolism were enriched. Molecular functions including oxidoreductase activity, alternative oxidase activity, and indole acetic acid (IAA) carboxyl methyltransferase activity was also enriched. Unique to ECO-R response were 38 terms for biological processes containing superclusters for 'response to cytokinin', containing the terms response to stress, nitric oxide, and salt stress. Superclusters of terms relating to chitin catabolism, chaperone-mediated protein folding, and ribosomal small subunit assembly were also enriched. In terms of gene expression, regardless of phenotype, a large number of plant growth activities were repressed including several for photosynthetic components, fatty acid metabolism, and nitrogen metabolism. In ECO-S, respiration related gene transcripts were induced including succinate dehydrogenase subunits, cytochrome c oxidase subunits, and cytochrome b-c1 complex subunits. In terms of stress response, aminocyclopropane-1-carboxylate (ACC) synthase and oxidase enzymes were induced following treatment, leading to stress induced ethylene production. A total of 240 xenobiotic-modifying genes were induced in both ECO-S and ECO-R which included cytochrome P450s, glucosidases, GSTs, and GTs; most of which were common

in both accessions. Without any comparison to the other herbicides of interest there appears to be no significant induction of genes that leads to the resistance profile to cyhalofop.

Glufosinate transcriptome response following treatment. Glufosinate response in ECO-S was primarily grouped into several ontological responses including response to fungus, flavonoid biosynthesis, amino acid import, oxalate metabolism, and aromatic compound metabolism. The glufosinate response in ECO-R was also similar to ECO-S but included several superclusters formed for the biological processes- response to xenobiotic stimulus, oxalate metabolism, glutathione metabolism, amino acid transport, hydrogen peroxide catabolism, auxin biosynthesis, and protein phosphorylation. In general, photosynthesis was repressed given the number of transcripts for various components of the process. Carbon metabolism genes were largely repressed in ECO-S but induced in ECO-R. Fatty acid biosynthesis was also repressed as indicated by the decrease in transcripts for acetyl-CoA carboxylase. Glutamine synthetase, the target for glufosinate, was repressed in ECO-S. In ECO-R, one form of glutamine synthetase (7.5) and glutamine synthetase cytosolic isozyme 1-3 (8.2) were induced, indicating that ECO-R may be able to express the necessary enzyme for normal function even in the presence of the herbicide. Nitrate reductase, in multiple forms, was repressed in ECO-R, but not observed in response to glufosinate in ECO-S. As observed in ECO-S and ECO-R following cyhalofop treatment, a high number of xenobiotic detoxifying genes (666) were expressed within both accessions. Clearly, both ECO-S and ECO-R are attempting to reduce the activity of glufosinate through detoxification but no single gene can be considered the primary enzyme endowing the resistance. Further assessment using the comparative analysis is required.

Propanil transcriptome response following treatment. A detailed analysis of the propanil response transcriptome can be found in Rouse et al. [33]. Propanil enriched biological processes

included response to water deprivation, abscisic acid (ABA) metabolism, maltose biosynthesis, high affinity potassium ion transport activity, and positive regulation of transcription factor catabolism. Molecular function terms were categorized as beta-amylase activity, transcription factor activity, hydroperoxide dehydratase activity, and galactinol-sucrose galactosyltransferase activity. Only three terms were enriched within ECO-R-P, one biological process- flavonoid biosynthesis and two molecular function terms quercetin O-glucosyltransferases. Following propanil application ECO-S induces several abiotic and biotic stress responses in an attempt to mediate the herbicide action. This includes several gene transcripts for ABA induction and metabolism. In terms of biotic responses, the jasmonic acid pathway is induced in both ECO-S and ECO-R, leading to a down-stream build-up in glucosinolates that would not have action on herbicides, only insects. Several genes associated with hypersensitive response were induced in ECO-R which have the potential to restrict the movement and immediate action of the herbicide. This was related to the aforementioned potential build-up of trehalose from constitutive gene expression. The trehalose sugar would be beneficial following the hypersensitive response to regenerate the plant. Following treatment, induction of trehalose biosynthesis genes were also observed, further implicating the potential abiotic stress alleviation imparted by trehalose. The primary mechanisms believe to endow propanil resistance involves a two-phase process. First hydroxylation via two cytochrome P450 enzymes- CYP709B2 and CYP72A15, followed by the conjugation of the two products via the GSTU17 and an undetermined glycosyltransferase.

Quinclorac transcriptome response following treatment. A detailed characterization of ECO-S and ECO-R transcriptome following quinclorac treatment is described in Rouse et al. [32]. Gene ontology analysis for ECO-S-Q response was composed of several terms coined as auxin catabolism, protein auto-phosphorylation, and aerobic respiration. No terms were enriched

following quinclorac treatment in ECO-R. In both ECO-S and ECO-R, the transcriptome response validated previous research implicating the ethylene biosynthetic pathway induction following treatment [34]. However, based on the transcriptome of ECO-S, the high expression of genes in this pathway might have occurred much earlier and by 24-hours after treatment these processes were already being repressed. This was related to the presence of ABA hydroxylase genes which were induced in response to the concomitant induction of ABA from this same pathway. The enzyme responsible for ABA induction, 9-cis-epoxycarotenoid dioxygenase (NECD), was also being repressed 24 h after treatment due to feedback inhibition from the high ABA concentration. In ECO-R, induction of the ethylene biosynthetic pathway had stopped by 24-hours after treatment, indicating that the herbicide reached its target enzyme but did not cause rampant ethylene production. Several xenobiotic detoxification genes were induced in ECO-R-Q. One gene in particular, UGT75D1, was identified that is potentially involved in conjugating the active quinclorac molecule. Another gene of interest in the ECO-R-Q transcriptome was one that codes for ALPL1 protein, a potential epigenetic factor that antagonizes polycomb group proteins that repress DNA transcription. ALPL1 may be what is allowing the high expression of UGT75D1 in ECO-R. Again, the role of constitutive induction of trehalose biosynthesis was also described to play a large role in mitigating the secondary or tertiary effects of quinclorac.

Functional characterization of the herbicide response transcriptome of ECO-S

Gene ontology enrichment in ECO-S across herbicide treatments. A comparative analysis for enriched terms related to herbicide response across all four herbicides was conducted (Fig 2a). A total of 188 terms were enriched following herbicide treatment in ECO-S. Unique terms to each herbicide include 26 for cyhalofop, 68 for glufosinate, 19 for propanil, and 14 for quinclorac. Sixty-one enriched processes across all four herbicides were shared amongst two or more of the

transcriptome profiles (Fig 2a). Only one, inositol oxygenase induction, was shared by all four herbicide response profiles. Two enriched terms were shared between cyhalofop, glufosinate, and propanil treatments in the susceptible phenotype (ECO-S-C/G/P): inositol catabolic process and response to karrikin (Fig 3a). Nitrate assimilation and salicylic acid catabolic processes were enriched across glufosinate, propanil, and quinclorac (ECO-S-G/P/Q). The terms shared between cyhalofop, glufosinate, and quinclorac (ECO-S-C/G/Q) included several biological processes categorized as oxidation-reduction process, response to oxidative stress, and hydrogen peroxide metabolism as well as the molecular function terms heme-binding and peroxidase activity. Responses shared only between cyhalofop and glufosinate (ECO-S-C/G) included the biological processes categorized collectively as response to ABA, killing of cells of other organisms, toxin catabolism, glutathione metabolism, and ABA biosynthesis. Three biological process and three molecular function terms were shared between glufosinate (ECO-S-G) and propanil (ECO-S-P) responses; all were related to nitrate transport and trehalose biosynthesis. Given the sites of action and biological pathways associated with propanil and quinclorac (Fig 1) several shared terms related to ABA catabolism and signaling, as well as ethylene-activated signaling were enriched as expected. Responses common between glufosinate and quinclorac (ECO-S-G/Q) included cell surface receptor signaling, defense response to oomycetes, and response to bacterium, categorized as response to jasmonic acid. Several molecular function terms were also enriched- ATP binding, oxidoreductase activity, polysaccharide binding, symporter activity, and protein kinase activity. Collectively, regardless of the herbicide used, it is apparent that the susceptible plants perceived the abiotic stress caused by the herbicides and responded accordingly within 24 hours. While each herbicide has a unique physiological effect, the superclusters of terms affected by all four herbicides were related to only a few endogenous

hormones (ABA, salycilic acid, auxin, jasmonic acid) or compounds (sugars or carbohydrates). It was also clear that signaling to specific biological pathways was active; hence the enrichment or expression of several similar processes across the four herbicide treatments.

Expression of genes related to plant growth and maintenance proteins. A total of 22,761 transcripts were differentially expressed in ECO-S amongst the four herbicide treatments (Fig 5a and 5b). For the respective transcriptome profiles, the numbers of differentially expressed transcripts were: 5,395 with cyhalofop, 8,105 with glufosinate, 2,583 with propanil, and 6,678 with quinclorac. In general, the number of repressed genes was similar to the number induced by the herbicides. Five hundred and fifty transcripts were repressed and shared amongst all four herbicides of interest in ECO-S (Figure 5a). The repressed genes are primarily associated with a reduction in biological activity and a shift to abiotic stress response. Collectively, these genes which include ribosomal proteins, kinases, cytoskeletal related proteins, elongation factors, RNA polymerases, and several ATP related enzymes are involved in maintenance and plant growth and development. Thus, the plant's initial response is to repress growth proteins and produce only what is necessary for sustaining minimum-level processes under abiotic stress.

Expression of genes related to abiotic and biotic stress response characterization. The induction of 102 transcripts, common to four herbicide treatments, suggested a shift from plant growth to a state of survival. Several abiotic stress-induced genes were expressed (Fig 5b). ABA receptor PYL5, was induced (2.6 to 5.3) conferring perception of elevated ABA, leading to stomatal closure [35]. The transcription factor MYB44 is also induced by the four herbicides because of the ABA induction and enhances the abiotic stress tolerance via the action of stomatal closure[36]. While stomatal closure helps to alleviate immediate stress it also results in the production of reactive oxygen species (ROS). This is caused an increase in the electrons in the

transfer chain without the necessary CO_2 concentrations to complete the assimilation process. Three peroxidase genes with known ROS reductive properties were induced following treatment across herbicides: PER15, PER54, and PER57. Stress induced genes in the ethylene biosynthetic pathway were not expressed; however, shared downstream responses indicate the presence of ethylene. Three ethylene responsive transcription factors (ERTF) were expressed ERF073, ERF113, and RAP2-1. These three are all involved in transcriptional activation and bind to the GCC-box pathogenesis promoter; ERF113 is known to be induced by wounding and waterlogging [37]. These, along with several pathogen-response-related genes, were also induced by the herbicide treatments. Forty-eight forms of indole-3-acetaldehyde oxidase transcripts, the majority in the cyhalofop (21) and glufosinate (22) responses, were induced. In response to pathogen infection, indole-3-acetaldehyde oxidase can produce auxin and hydrogen peroxide- a stress signal, protective, and potentially harmful molecule [38,39]. Mitogen-activated protein (MAP) kinase 8 and MAP kinase kinase 5 were induced. Both are essential for host-immune response in the pathogen defense pathway, but also induced by oxidative stress and high light intensity, resulting in reduction of ROS [40–42]. Finally, two genes that could potentially interact with herbicides were also induced- ABCG53 and UGT74D1. ABCG53 is listed as a possible defense protein. UGT74D1 conjugates IAA rendering it inactive; this may serve as a feedback response to the IAA oxidase which forms the active IAA molecule. Given the susceptibility of this population to the herbicides, the induction of these detoxifying enzymes does not impart resistance to herbicides and may be produces to mitigate some of the secondary herbicide effects on the plant physiology.

Functional characterization of the herbicide response transcriptome in ECO-R

Gene Ontology enrichment in ECO-R following herbicide treatment. Enrichment of ontological terms following treatment in ECO-R was much lower than in the ECO-S population. In total, 108 terms were significantly enriched, with only 18 shared between ECO-R-C and ECO-R-G responses (Fig 2b). For the 18 shared terms between ECO-R-C and ECO-R-G, the majority of the biological processes were linked to RNA translation and protein synthesis, similar to the molecular function terms. While the data for gene ontology depletion is not presented, no depletion in response to propanil nor quinclorac were observed. Only a few terms were depleted by cyhalofop and glufosinate treatment and were mostly related to protein synthesis. These ontological enrichment profiles were a stark contrast to the ECO-S population. ECO-R appears unresponsive to the herbicide treatments. Another factor of note in the response, is the relatively low enrichment and lack of depletion in ECO-R before and after treatment. This would indicate that the ontology terms that were constitutively enriched in ECO-R, were also present to some degree following treatment. No changes in carbohydrate partitioning nor enhanced metabolism are evident given the herbicide treatment responses in ECO-R.

Repression of major plant growth and maintenance gene transcripts. The differential expression of transcripts in ECO-R across the herbicide responses was similar to ECO-S with a total of 21,791 transcripts characterized (Fig 5c and 5d). However, unlike ECO-S, the vast majority (73%) of differentially expressed transcripts in ECO-R were repressed following herbicide treatment (Fig 5c). Collectively 2,591 transcripts were shared across the four herbicide responses; 2,588 of these were repressed while only 3 were induced. As observed in ECO-S, a number of growth related genes are repressed following treatment including a large number of ribosomal proteins-40S and 60S, limiting protein synthesis. DNA-related proteins including

several DNA repair proteins, topoisomerases, and polymerases enzymes and proteins were also repressed. This occurred along with the depression in endoplasmic reticulum (ER)- related genes ER lectin 1, ER oxidoreuctin-1 and ER-golgi intermediate compartment 3. Several ATP-related enzymes including mitochondrial and chloroplastidic ATP synthase subunits, ATP-binding cassettes, ATP-dependent RNA helicases, ADP/ATP carrier proteins were repressed. Other repressed purine-related enzymes include AMP deaminase, ADP ribosylation factor-like proteins, and ADP-sugar pyrophosphatase. Examination of genes associated with the constitutively enhanced pathways revealed repression of most genes related to carbon metabolism. These include aspartate aminotransferase, PEP carboxylase, malate dehydrogenase, and NADP-dependent malic enzyme. At the same time, several sugar-metabolism-related genes including GDP-L-fucose synthase, GDP-mannose 4,6 dehydratase 2, GDP-mannose transporter, and several glucose transporters- 1E and 2A were repressed. None of the trehalose- related enzymes were repressed across all four herbicide treatments. However, alpha-alpha trehalose phosphorylase, which is responsible for the breakdown of trehalose into D-glucose, was repressed approximately seven-fold across all responses. A putative reduction in fatty acid synthesis was observed based on reduction in transcripts for acyl-CoA related enzymes including a dehydrogenase, desaturase, synthetase and binding domains. Acetyl-coenzyme A synthetase is required for the formation of acetyl-CoA, this enzyme was repressed following treatment. The ACCase and ACCase 2, which were constitutively enhanced, were repressed across the four herbicide treatments along with several very long chain fatty acid elongation proteins 2 and 6. The ACCase enzymes were not the only herbicide-response related genes repressed across treatments. Several forms of the glutamine synthetase enzyme, inhibited by glufosinate, were repressed by as much as 10.5-fold across all herbicide treatments. In general, repression of most

genes was also related to plant growth and development as observed in ECO-S. Several critical pathways were repressed including the carbon metabolism, energy relations, nitrate assimilation, and fatty acid synthesis pathways, providing evidence that ECO-R is preserving previously formed energy sources and minimizing the destructive impact of herbicides or abiotic stressors.

Induction of xenobiotic-related gene transcripts. Unlike ECO-S, only three transcripts were induced across all four herbicides in ECO-R: protein ALP-1 like protein, secologanin synthase, and UGT75D1 (Fig 5b). Across the four responses no large-scale shift toward stabilizing or maintenance proteins occurred that would indicate a state of significant abiotic stress as seen in ECO-S. Multiple herbicide detoxification genes were commonly induced across three herbicide treatments. GSTU17, GST23, and disease resistance protein RPM1 were all expressed across transcriptomes of ECO-R-C/G/P. For ECO-R-G/P/Q comparisons- CYP89A2, CYP709B2, CYP709B1, UGT73E1, and UGT73D1were induced. Finally, ECO-R-C/G/Q had no herbicide detoxification genes shared across the responses. Only CYP709B1 was not expressed in ECO-S following any one of the four treatments meaning this enzyme may have implications for resistance. CYP709B2 was however induced across ECO-S-C/G/Q, but not in ECO-S-P, further supporting its potential role in herbicide detoxification, except with propanil. None of these CYP genes were expressed in ECO-S-P even though they were induced in at least one other herbicide response in ECO-S. Overall, this shift in favor of repression in ECO-R following treatment is opposite of what occurred in ECO-S whereby the reduction in transcripts was paired with an almost equal induction of stress-responsive genes. Far fewer genes were induced across all herbicide treatments in ECO-R. The resistant plant appeared to be in a stasis condition with minimal biological activity and far less response to abiotic stress stimuli compared to ECO-S.

Comparative differential gene response and functional characterization between ECO-S and ECO-R following herbicide application

A total of 2004 transcripts, 1281 repressed and 723 enhanced, were differentially expressed in ECO-R across the four herbicides than in ECO-S. Only 44 were shared across the four herbicide responses. For the repressed transcripts, 40 were shared across the herbicides and were primarily characterized as transporters and integral membrane proteins associated with movement of solutes and other compounds into and out of the cell. Two aquaporin proteins TIP4-1 and TIP4-2, two oligopeptide transporters 2 and 4, and two nitrate transporters NPF6.2 and NPF6.3 were comparatively repressed in ECO-R. Multiple stress responsive genes were repressed in ECO-R including ABA related proteins. ABA 8'-hydroxylase 1 and ABA receptor PYL5 were both repressed in response to the treatments. E3 ubiquitin-protein ligase MIEL1 and XB3 and VQ motif-containing protein 25 [43], all responses to abiotic and biotic stress perception and signaling were significantly repressed. In terms of pathogen/disease response, which was significantly induced in ECO-S, two genes ERF073 and pathogen-related protein were depressed in ECO-R compared with ECO-S. Collectively the repressed genes that signify the comparative response between ECO-S and ECO-R validate the described differences in the state of the plants following treatment. ECO-S devotes a number of resources to moving solutes around the plant to supply the needed substrates for the elevated enzymatic reactions. ECO-R is not perceiving an elevated abiotic stressed state and thus requires few resources.

Only three genes were enhanced across the four herbicides: nudix hydrolase 21, ERF4, and two forms of Protein ALP1-like. Nudix hydrolase 21 is a general-purpose enzyme involved in the hydrolysis of nucleoside diphosphate derivative capable of producing orthophosphate. ERF4 is a transcriptional repressor of the aforementioned GCC-box pathogenesis promoter

element. Finally, protein ALP1-like, also mentioned previously, may be a stress induced antagonist of the polycomb group of genes associated with chromatin modifications in the form of transcriptional repression [44]. For cyhalofop response, only 39 transcripts were unique and among these were only a few genes potentially related to herbicide detoxification: CYP71A1, CYP71A35, CYP72A14, and CYP87A3. Glufosinate response elucidated enhancement in 81 transcripts for ECO-R; in terms of xenobiotic interactions only CYP71A1 CYP76M5 had elevated expression. Propanil response was much higher with as many as 129 unique transcripts. CYP704C1, CYP709B2, CYP72A15, GST1, GST4, GSTU17, UGT73D1, UGT88A1, and UGT88F3 were all enhanced to a greater number following treatment in ECO-R. Finally, ECO-R-Q, elicited 122 unique transcripts, including CYP709B2, CYP45071A1, CYP71A4, CYP71A8, GSTU20, UGT73D1, UGT75D1, and UGT88A1. Few genes, were shared amongst the various profiles. In general, several shared responses did have stress induced proteins including heat stress proteins, ERF's, and ABC transporters. However, there were no unique profiles that significantly distinguished themselves as having a causative role in resistance or stress mitigation.

Discussion

Unique herbicide responses have a role in the mitigation of herbicide action to cyhalofop and glufosinate

The resistance to cyhalofop and glufosinate in ECO-R is marginal in ECO-R compared with ECO-S. However, the transcriptome does reveal several elements which may contribute to the observed level of resistance and may indicate the early evolutionary period of resistance in this population. The constitutive enhancement of ACCase in ECO-R implies that when treated with an ACCase inhibitor (cyhalofop), the putatively higher amount of enzymes present would

reduce the inhibitory effects of the herbicide. At the plant level, this was exhibited as the ability to recover from phytotoxic effects of cyhalofop as observed in previous experiments with ECO-R [45]. After cyhalofop treatment, these are greatly repressed indicating there is still an interaction between the herbicide and its site of action. For glufosinate, the target enzyme glutamine synthetase was also enhanced prior to treatment and repressed following application, but another form of glutamine synthetase was induced It is possible that the overall mechanism driving the enhanced activities described previously, preempts the effects these herbicides have. This paired with the effects that the trehalose metabolism may have on abiotic stress tolerance may be the causal agents in reducing the effects of these herbicides.

Abiotic stress inducible response is a primary action in susceptible *E. colona*

Within 24 hours of the herbicide application, a series of physiological events including perception, signaling, and transduction of abiotic stress occur. In general, the transcriptome profile indicates that the plant induces a number of the growth and maintenance processes associated with early development to aid in the stress response. The response to each herbicide were in accordance to what is known about their respective modes of action. The transcriptome data provided mechanistic details of how certain responses to herbicides come about. A common response to all four herbicides was increased ABA perception and signaling, which was indicative of increased ABA production. ABA signaling is a key component in abiotic stress response and results in the closing of leaf stomata, limiting water transpiration and increasing free radical production [46]. ABA concentrations in the cell have downstream effects on calcium ion redistribution via the induction of calcium permeable channels, which aid in the mediation of abiotic stresses [47,48]. The concerted signals enabled by the ABA molecule and calcium ion help to mitigate the negative impact of abiotic stresses (i.e. drought, cold, heat) have on the plant.

However, long-term stomatal closure due to ABA directly represses the photosynthetic process [49] and leads to the build-up of reactive oxygen species with cell membrane damaging properties.

Biotic stress mitigation is also central to the action of susceptible *E. colona*

ABA action does provide an immediate stabilization effect, it is clear that ECO-S still induces additional biotic stress mitigating proteins and enzymes to prevent prolonged negative impacts that lead to plant death. This includes the increased expression of several transcription factor proteins associated with host-pathogen responses, hydrogen peroxide forming enzymes, and MAP kinase proteins. Collectively these processes would not be capable of detoxifying herbicides or reducing the secondary damaging effects of their action. The production of hydrogen peroxide is helpful in the host-immune response to biotic pathogens and has been described to have a potential role in propanil response. But when paired with the reactive oxygen species (ROS) evolved from the buildup of free energy in the photosynthetic electron transport chain the compounding effects may be harmful. This response to herbicide has been characterized for aiding in the suppression of *Sclerotenia* stem rot infection in soybeans [50]. Lactofen, a protoporphyrinogen IX inhibiting herbicide applied to soybean, is believed to induce a hypersensitive response similar to most plant defense to pathogen activity. Peroxidase genes and isoforms of these genes, capable of neutralizing the activity of ROS, were induced by as much as 11-fold in the responses to all of the herbicides. The production of indole-3-acetyhyde oxidase is also of note in the response. This leads to the production of hydrogen peroxide and is a potential response to pathogen infection, it also indicates a demand for auxin production and a build-up of the IAA precursor. Auxin, the intercellular signal molecule, is necessary to direct general plant growth [51]. However, under the stress induced state, elevated auxin production and

uncontrolled accumulation of free auxin alone could result in reduced plant growth and lead to plant death as observed with auxin-type herbicides [52]. The concomitant increase in hydrogen peroxide also produced via this auxin catabolic pathway also has negative effects on the health of the plant. Finally, the repressed genes across all of the responses indicates that the shift to stress response comes at a significant cost to the developmental potential of ECO-S. The multitude of proteins reduced were seemingly related to the production of maintenance proteins necessary for cell elongation and division, as well as cytoskeletal development. Given this profile, it is clear that the survival state following treatment is a high energy demanding process that on its own limits the production potential of ECO-S. This comes at a dramatic cost to the plant which may not be recoverable if ECO-S were to recover.

Multiple-herbicide resistance may be an adaptive evolutionary response to herbicides and abiotic stress

Adaptive evolution may be the single most advantageous process employed by weedy species in agro-environmental landscapes. The genomic plasticity results in weedy populations existing in a middle-ground state of highly advantageous domesticated traits and strong genomic resources for exploitation of weediness [53]. ECO-R is a unique population, highly resistant to propanil and quinclorac with low level resistance to cyhalofop and glufosinate, with a tremendous ability to tolerate adversity and produce high levels of biomass (date not shown). Given the heightened state of abiotic stress exhibited by ECO-S and the repression in the plant growth proteins, it is clear that a latent effect of herbicide action is a depletion in energy reserves. This depletion paired with the continuous inhibition of key enzymes by the herbicides lead to plant death. Any process that can supply and protect the cellular structure under this stress, would benefit the plant and assist in overcoming the secondary effects of herbicide stress.

Analysis of the constitutive difference between the contrasting ECO-S and ECO-R populations revealed a litany of biological processes that are functioning at a greater level in ECO-R compared with ECO-S. Of the enhanced processes, were major proteins related to photosynthesis, carbon assimilation, fatty acid metabolism, and sugar metabolism and transport. Specifically, within these processes were a significant number of transcripts associated with the trehalose biosynthetic process- the trehalose phosphate synthase (TPS) and the trehalose phosphate phosphatase (TPP) enzymes. This has not been proposed as a potential pathway to mitigate herbicide effects previously but our research has explained the means to which this may occur. The trehalose pathway has been described in the literature due to its overwhelming positive effect on abiotic stress response and adaptive ability to oxidative and drought stressed conditions [54,55]. The presence of these key enzymes, paired with the elevated activity dedicated to growth, and the ontological enrichment of terms related to trehalose response to stress, provide overwhelming support for the role of trehalose in mitigating the herbicide stressors. This complex pathway would allow the plant to persist under the conditions imposed by herbicide activity of a variety of compounds more than just those investigated in this research. This has the potential to mitigate the harsh effects caused by several herbicides that effect tolerance to photosynthesis inhibiting herbicides (WSSA Group 5/6/7), protoporphyrinogen IX inhibitors (WSSA Group 14), cell membrane disruptors (WSSA group 22), phytoene desaturase inhibitors (WSSA Group 12), diterpene biosynthesis inhibitors (WSSA Group 13), and HPPD inhibitors (WSSA Group 27). Based on this information, it is plausible that only propanil and quinclorac are being actively metabolized, and the cyhalofop and glufosinate resistance is imparted by the effects of this trehalose biosynthesis.

Only three stress induced genes were shared across the herbicide responses. While UGT75D1 may have be active on the herbicides, it does not appear to impart a high level of resistance to cyhalofop, glufosinate, ore propanil alone. Given their structures it is possible that UGT 75D1 may still conjugate cyhalofop and propanil in a similar manner to quinclorac but not completely inactive the compounds (Figure 7). This indicates that our hypothesis of a shared resistance mechanism is not present in ECO-R. However, the continued presence of the ALPL1 protein across the various responses is of interest. The literature describes this protein as potentially stress induced [56,57] and given that it is present in all of the response profiles, it is clearly induced within ECO-R in response to herbicide stress. The elevated presence of this protein may have a number of effects on the multiple herbicide-resistant phenotype that would require further validation. While we posed that there is a single mechanism endowing the multiple resistance through detoxification, it is possible that the role of this epigenetic repressor antagonist may be more important. This would support the idea of adaptive co-evolution of abiotic stress resistance proteins and the shared role they may have in herbicide resistance. It may also indicate that ALPL1 has an active role in expressing certain genes which may be beneficial to the plant under high stress conditions or herbicide application.

Conclusions

Herbicide activity in plants results in a complex and genetically diverse response to abiotic stress as observed in the characterization of the ECO-S and ECO-R transcriptome presented in this research. However, using constitutive gene expression of contrasting phenotypes and supplementing this information with the transcriptomic response following herbicide application reveals a great deal of information on multiple herbicide resistance. Given the profile for ECO-S, it is apparent that herbicide stress is perceived very similarly to both

biotic and abiotic stressors. The initial cascade of responses, 24 hours after treatment, relate to processes associated with reducing the effects of drought stress as well as several key components in host pathogen response, to disrupt or control a pest/pathogen. However, the induction of a variety of these genes result in the secondary or tertiary effects of herbicide action, most notably hydrogen peroxide and ROS formation. ECO-R is a much different population that has evolved to not only metabolically reduce herbicide action via xenobiotic detoxification, but has evolved to compensate these mechanisms through and enhanced carbohydrate assimilation pathway. This is the first such description of the trehalose biosynthetic process imparting tolerance to herbicides and the subsequent effects it has on mitigating the abiotic stress effects caused by herbicide action. This will require further research into the role it plays in weedy species, specifically *Echinochloa*, and quantifying the effects it has on abiotic stress and potentially herbicide resistance. It is also of interest that this ECO-R population is capable of shifting into a near sedentary state following herbicide application, as indicated by the vast repression of genes following treatment. This implies that by reducing the activities of the plant there can be less secondary effects which were describe in the ECO-S population. This may be an example of a weedy species reducing biological activities to allow for the herbicide detoxification to occur, prolonging the period in which the enzymes may act and reducing secondary herbicide effects.

Materials and Methods

Plant Materials

Echinochloa colona samples were selected from the Arkansas state-wide sampling program conducted at the University of Arkansas between 2010 and 2011 based on their profiling in the surveys presented in Rouse et al. [16]. The herbicide susceptible (ECO-S) and

multiple-resistant (ECO-R) populations were profiled previously for their respective herbicide resistance profiles and potential physiological mechanisms of resistance [45]. For the RNA-sequencing experiments pureline generated seed of both ECO-R and ECO-S were grown in isolation in a growth chamber set to a 14-hour day length, 33C day temperature and 24C night temperature. Approximately one week after planting the plants were thinned to a single plant per pot, with two plants per accession serving as two biological replicates. When the plants reached the 2- to 3-leaf, one collar stage, they were treated with the four respective herbicides at the field application rates listed in table 1. An identical set of plants was prepared and left untreated to serve as a nontreated control for the experiment. Twenty-four hours after treatment the shoots from all plants were frozen in liquid nitrogen and stored in -80C for further processing. The tissues were transferred into individual tubes containing RNAlater^{TM}-Ice for shipping to the Clemson University Genomics Institute (CUGI) for RNA extraction and library preparation.

RNA-sequencing, Transcriptome Assembly, and Functional Annotation

The process for RNA extraction, sequencing, transcriptome assembly, and functional annotation are detailed in Rouse et al. [32]. A summary of these processes is presented here. Total RNA was extracted using a commercially available kit by CUGI. The prepared RNA samples for all treatments were fragmented and reverse transcribe into cDNA using random primers for library assembly. The fragments were then annealed with an additional ‘A’ and the adapter sequence for high-throughput sequencing. Following enrichment via PCR, the cDNA library was submitted to the Holdings Cancer Center at the Medical University of South Carolina, Charleston SC, USA for sequencing. The samples were arranged in three lanes on the Illumina Hiseq 2500 platform and analyzed using paired end reads.

The *de novo* transcriptome was assembled using all of the treatments for both the ECO-R and ECO-S accessions. The transcriptome was assembled using the Trinity RNA-Seq pipeline (Broad Institute, Cambride, MA, USA). Following the primary raw data processing the normalized reads wre assembled using Trinity with the stranded library set as the default. Transdecoder 3.0.1 (Broad Institute) was used to identify open reading frames in the transcriptome and assign proteins to the gene sequences based on homology to the blastP database and HMM scan against pfam. Transcripts matching both criteria were retained for analysis. After quality assessment of the transcriptome, the Trinotate 3.0 suite of software (https://trinotate.github.io/) utilized the BLAST+ and Swissprot databases to generated functional annotation of the proteins. It also produced output for the eggnog, GO, and KEGG databases for each of the annotated proteins.

Comparative assessment

Gene ontology (GO) enrichment and differential gene expression analysis was conducted for the paired treatments of interest. Several treatment conditions were paired to assess the responses of interest including: nontreated ECO-S with nontreated ECO-R, nontreated ECO-S/R with their respective herbicide treatments, and the four-herbicide treatments for ECO-S and the counterpart ECO-R treatments. For the gene ontology analysis, the Trinotate output was analyzed using the 'goseq' package from Bioconductor (https://www.bioconductor.org/) to assign GO terms to the transcripts from the transcriptome. The analysis of enriched terms was performed only on transcripts which were expressed or depressed at a log2 fold-change of $\leq$-2 or $\geq$2 and a p-value of $\leq$0.01. The results of the analysis were visually assessed based on the p-value of the GO analysis using REVIGO (http://revigo.irb.hr/) to best characterize the results and identify representative subsets and superclusters of the terms using clustering algorithms based

on semantic similarity[58]. Cytoscape (Cytoscape Consortium, San Diego, CA, USA) software (http://cytoscape.org/) was used to visualize the output from REVIGO to produce relevant graphics of the results.

Differential gene expression was conducted using the R statistical software program (https://www.r-project.org/) with the Bioconductor package - edgeR [59,60]. EdgeR was used to quantify the filtered raw counts from the RNA-sequencing experiment. Standard normalization using the trimmed mean of M-value was applied to the counts and the counts were fit using a GLM model for the determination of significance. A log-fold change was determine based on these results and used to describe the expression change under the various treatment conditions. The data were visualized using volcano plots and used for the follow-up descriptive analysis to identify patterns of gene expression. Further manual processing of the differential gene expression data applied categories and gene families to the results. The JMP® Pro 13.1 (SAS Institute, Cary, North Carolina, USA) software was used to summarize the results and the venn diagram add-in package was used to compare the various treatments and produce relevant graphs. Descriptions of the genes and the physiological pathways for which they function are based on the data in the Uniprot [61] and KEGG [62] databases.

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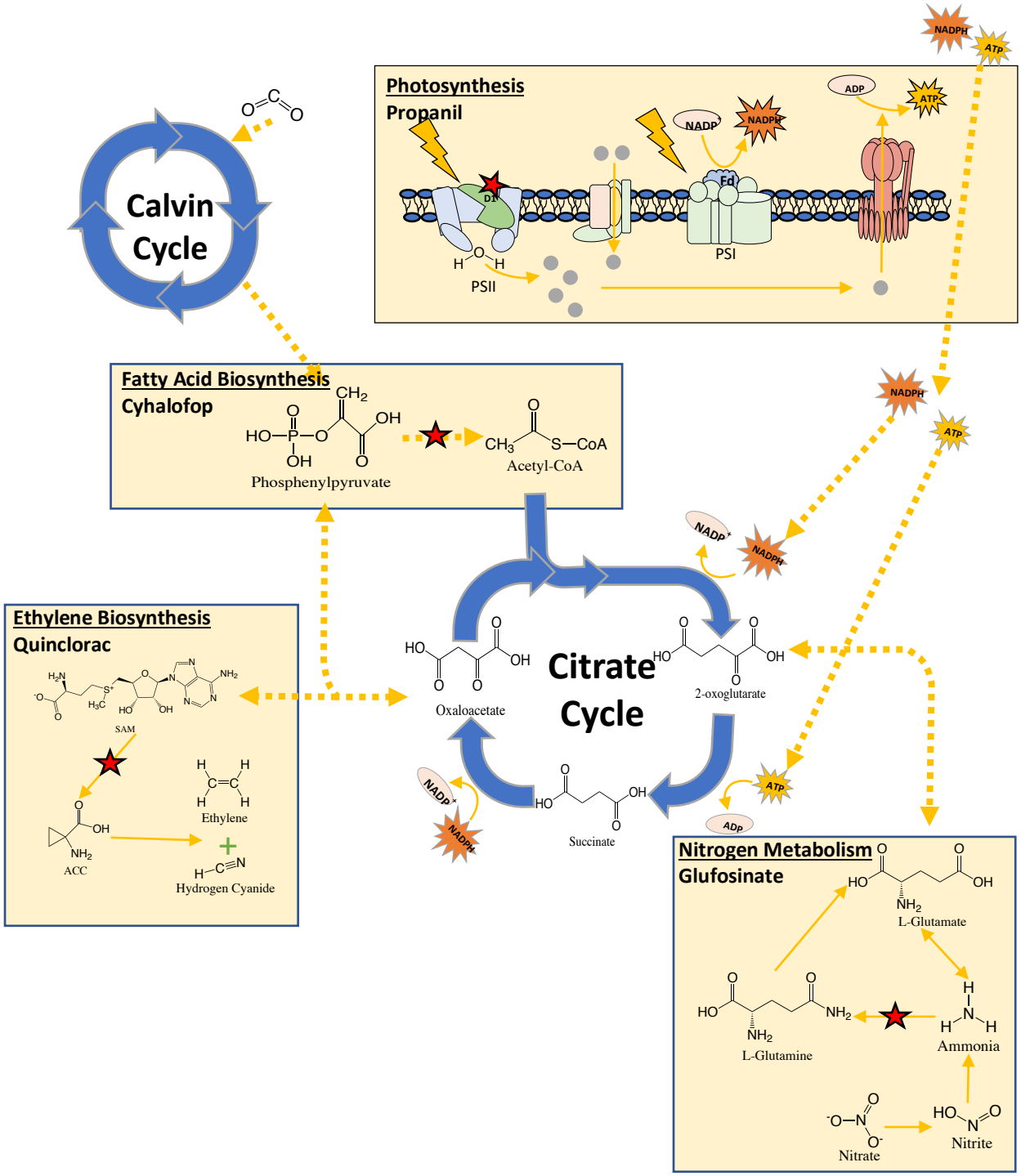
Tables and Figures

Table 1. Herbicides, trade names, application rate and adjuvant with rate used for the RNA-sequencing experiments conducted on ECO-R and ECO-S.

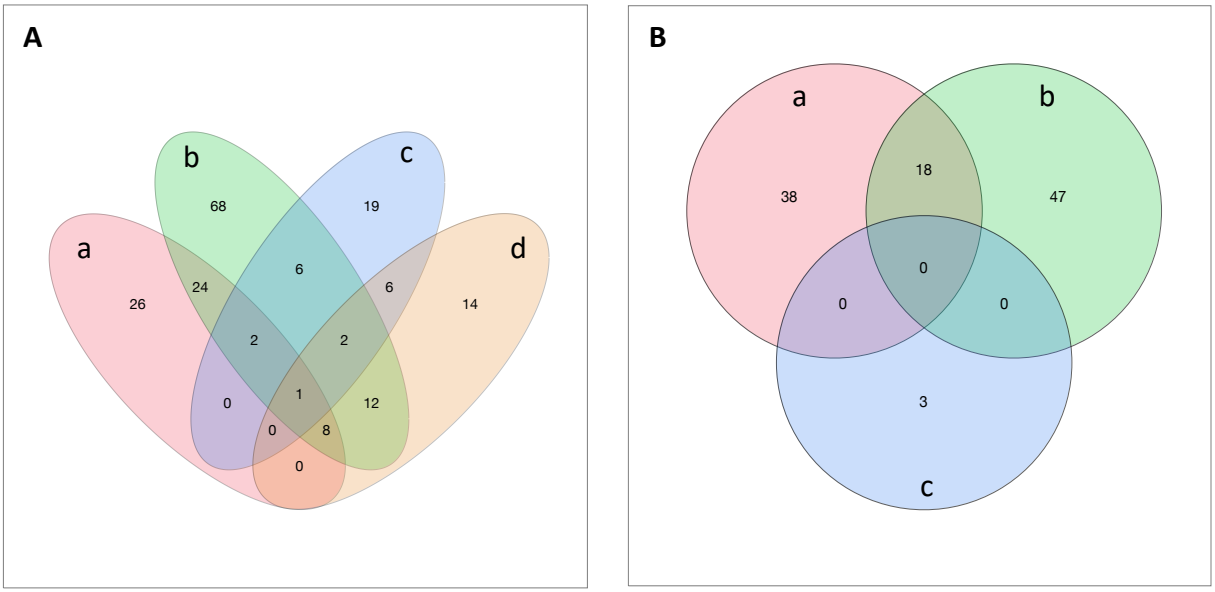
Herbicide	Trade Name	Application Rate	Adjuvant
		kg ha^{-1}	%
cyhalofop	Clincher®	315	1% COC
glufosinate	Liberty®	590	0.25% NIS
propanil	Stam®	4500	0.25% NIS
quinclorac	FacetL®	560	1% COC

Figure 1. Diagram of the physiological pathways for which cyahalofop, glufosinate, propanil, and quinclorac inhibit and the means through which they interconnect within the plant.



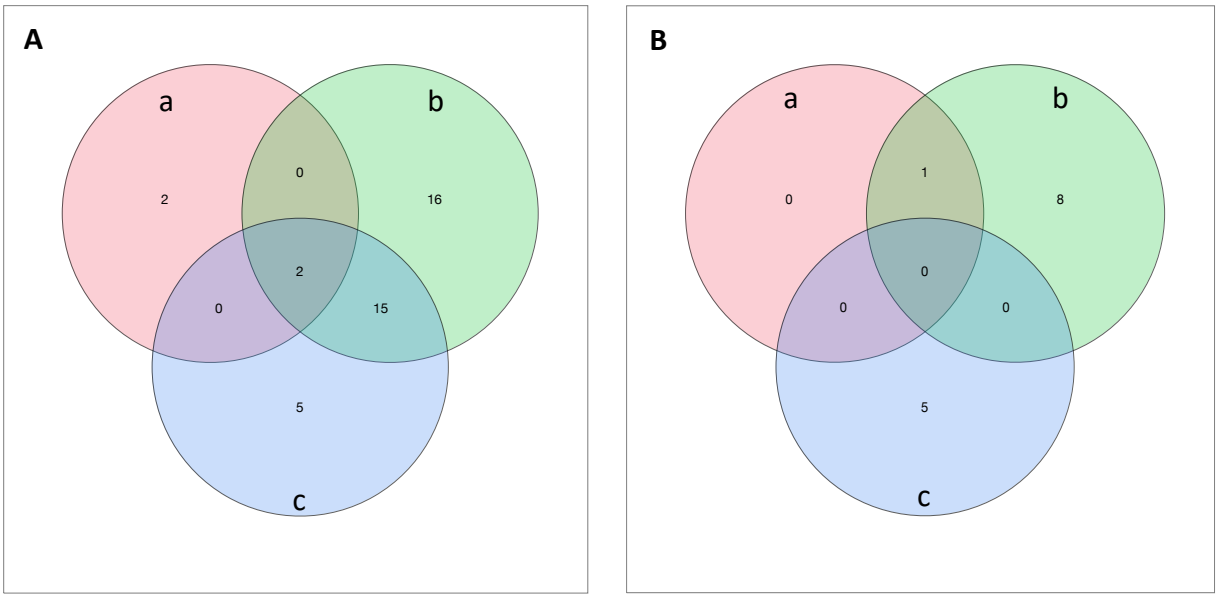
[1]Red stars within each of the labeled boxes indicate the site or enzyme the herbicides inhibit.

Figure 2. Venn diagrams depicting the number of shared and contrasting gene ontology terms that were enriched following herbicide application within either ECO-S (A) or ECO-R (B).



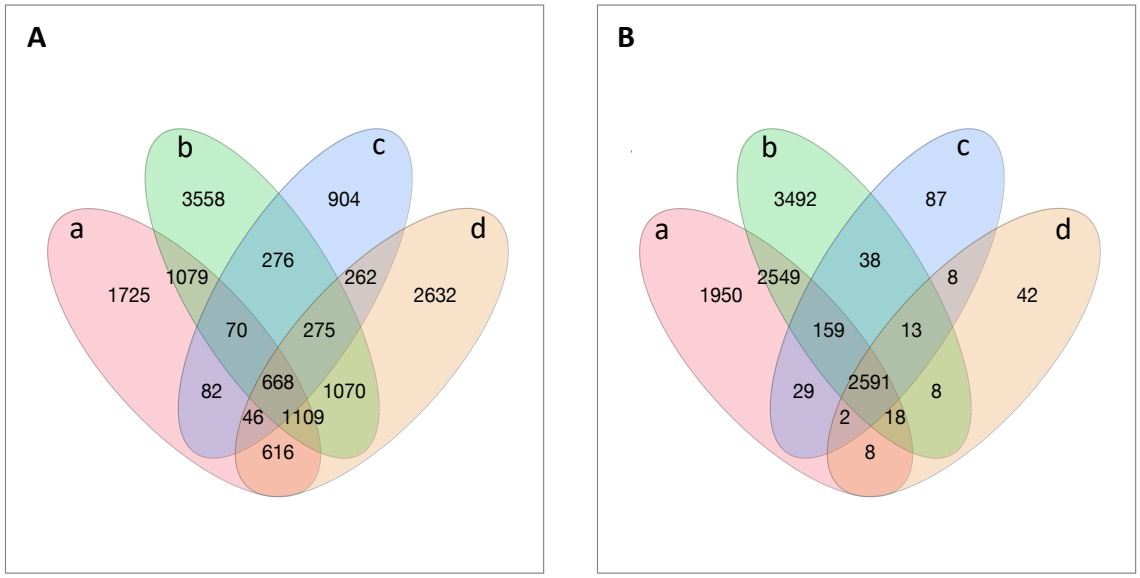
[1]Each oval represents a single herbicide response for the respective accession: cyhalofop (a), glufosinate (b), propanil (c), or quinclorac (d).

Figure 3. Venn diagrams depicting the number of shared and contrasting gene ontology terms that were enriched in the comparative herbicide responses in ECO-S (A) and ECO-R (B).



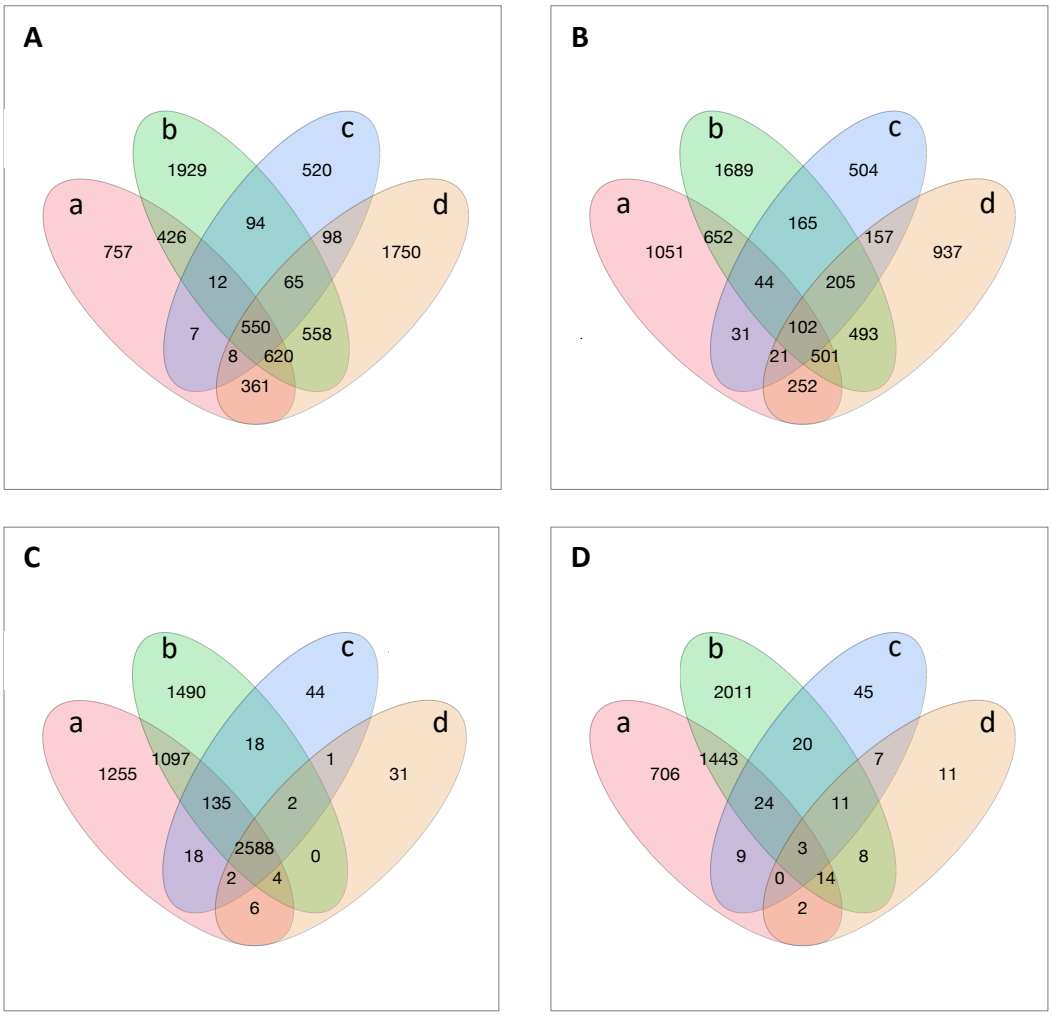
[1] Each oval represents a single herbicide: cyhalofop (a), glufosinate (b), propanil (c), or quinclorac (d).

Figure 4. Venn diagrams for the results of the differential gene expression analysis for both induced and reppressed gens in ECO-S (A) and ECO-R (B) following cyhalofop (a), glufosinate (b), propanil (c), and quinclorac (d) application.



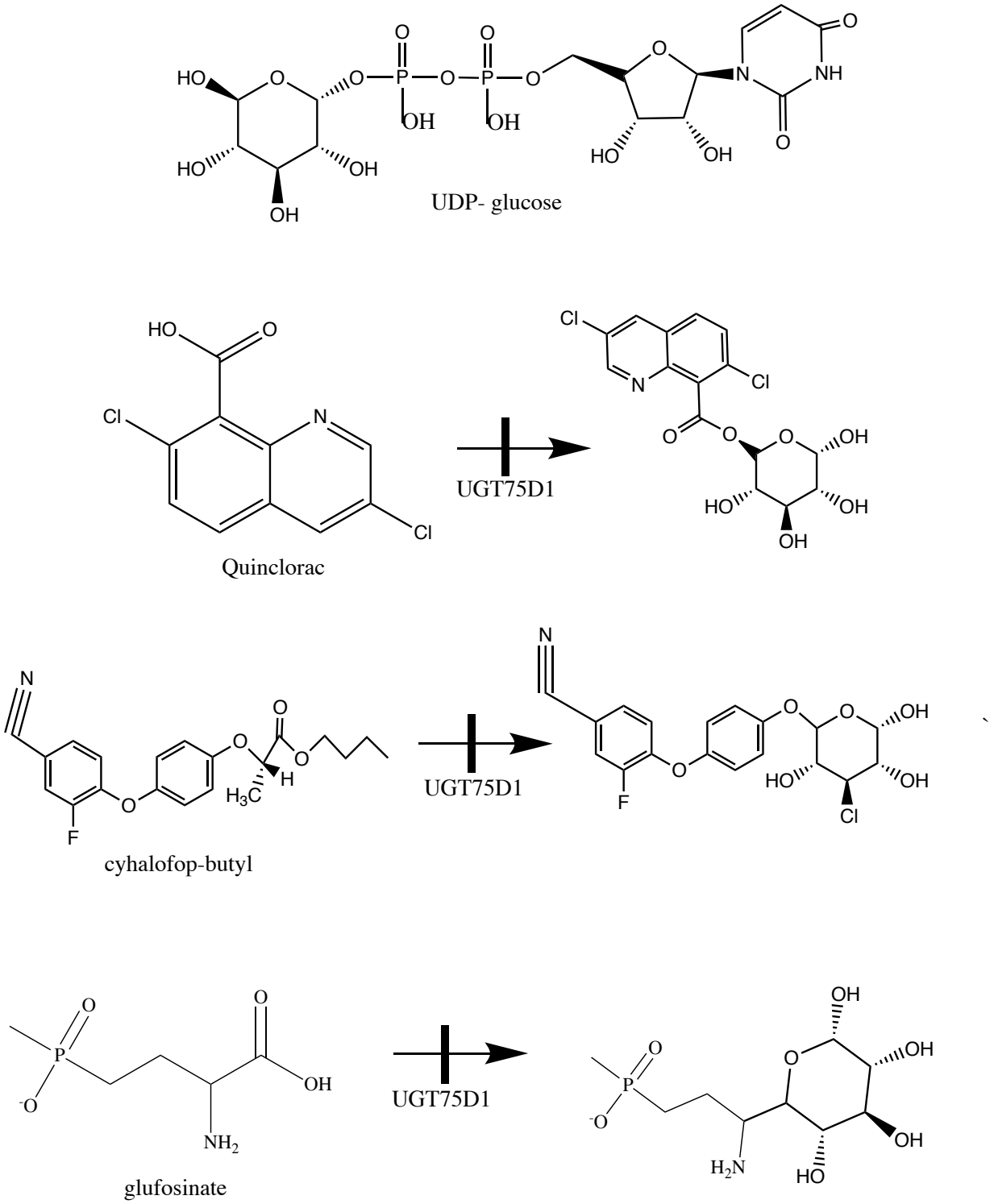
[1] Each oval represents a single herbicide: cyhalofop (a), glufosinate (b), propanil (c), or quinclorac (d) response.

Figure 5. Shared and unique genes for the repressed and induced genes in ECO-S and ECO-R following the differential gene expression analysis.



[1]Figures 5A and 5B are ECO-S repressed and induced genes, respectively; Figures 5C and 5D are ECO-R repressed and induced genes, respectively.
[2] Each oval represents a single herbicide: cyhalofop (a), glufosinate (b), propanil (c), or quinclorac (d).

Figure 6. Diagram for the potential conjugation of the UDP-glucose molecule to quinclorac, cyhalofop, and glufosinate via the UGT75D1 based on the functional side groups of each molecule and the preferential conjugation of these sites by glycosyltransferase enzymes.



Conclusion

Multiple herbicide resistance in *Echinochloa colona* is increasing in frequency and distribution throughout Arkansas rice producing regions. The long-utilized herbicides propanil and quinclorac have become the primary selectors for resistance. Approximately 50% of Arkansas *Echinochloa spp.* are resistant to propanil while 23% are resistant to quinclorac. Cyhalofop (3%) and imazethapyr (13%) resistance is increasing in prevalence over recent years but well behind propanil and quinclorac resistance. Multiple resistance has increased over the past ten years, with propanil + quinclorac resistance (12%) being the most observed amongst the various profiles. However, this research has identified resistance to three (4.7%) and even four (0.9%) modes of action within a single population in the state. The threat posed by multiple resistance is of great concern for future weed management strategies. Limiting the utility of future control options.

Non-target-site resistance mechanisms are non-selective, making them more responsive to a greater number of xenobiotics or herbicides. The initial investigation into ECO-R revealed high levels of resistance to propanil and quinclorac, with some tolerance to cyhalofop and glufosinate. The use of known xenobiotic detoxification inhibitors prior to propanil application identified synergism between these compounds. Indicating that at least one detoxification enzyme has a role in the resistance mechanism to propanil. Radiolabeled herbicide absorption, translocation, and metabolism provided insights into the cause of quinclorac resistance. Translocation from the treated leaf into the new leaf tissues was greater in ECO-R. This information paired with the presence of two unknown quinclorac metabolites, implicated a separate enzyme in the resistance mechanisms to quinclorac. RNA-sequencing and functional gene annotation was then used to better identify and explain the response to these two herbicides.

This revealed several potential genes that may be the causal mechanisms of resistance. For quinclorac, UGT75D1 possesses known interactions with molecules similar in structure and activity as quinclorac. It is present in high abundance following quinclorac treatment and in response to the other herbicides of interest. The induction of this enzyme may be influenced by the presence of ALPL1, an antagonist to epigenetic silencing protein complexes, stimulated by abiotic stress and upregulated in ECO-R without and with herbicide treatment. The transcriptome analysis also revealed that trehalose biosynthesis may have a role in the resistance mechanisms. While this process does not have an active role in resistance, it may provide a source of UDP-glucose for conjugation of the herbicides. The trehalose sugar could also endow several abiotic stress mitigating features that allow the plant to survive under severe stress that herbicides impose. These processes were induced in response to propanil, further implicating them as a potential mediator of herbicide stress. Propanil resistance may be endowed by one or two cytochrome P450 enzymes- CYP709B2 and/or CYP72A125. Both have the potential to hydroxylate propanil into the product 3,4-dichloroanaline and propionic acid. The propanil response profile also identified several glutathione-S-transferase and glycosyltransferase genes that may conjugate these substrates. This complete assessment of the physiological and genomic aspects of resistance have not been profiled previously for this species. This research provides significant information on novel resistance mechanisms and the means to which *E. colona* evolve through modifications in the abiotic and/or biotic stress response pathways.

Herbicide resistance is more complex than previously thought. Following treatment, any number of abiotic or biotic responsive genes may be induced to respond to the primary or secondary activities of the herbicide. It is the exploitation of these processes by weeds that allow for non-target-site resistance evolution to occur. Our research provides the best evidence to date

of this phenomena. The physiological cascades following treatment that we have highlighted are not random occurrences. These are processes that act independently to mitigate stress, but through recurrent selection may evolve to become fierce herbicide resistance mechanisms. Compensatory evolution of multiple physiological pathways selected along with herbicide resistance is as much of a threat to weed control as the mechanism itself. These processes which support and/or function independently of these mechanisms have the potential to endow increased fitness and vigor or even greater competitive abilities. Our research allows for a better understanding of weedy traits and provides detailed information on the processes which occur in response to herbicides in *E. colona*. We have provided significant evidence that two independent mechanisms can endow high levels of resistance to propanil and quinclorac. However, we have also provided plausible connections between these two mechanisms that link to the ability of ECO-R to better tolerate abiotic stressors, making it a more competitive weed under adverse environmental conditions. This lays the foundation for future research into multiple-resistant *E. colona* and for further investigation into the role these responses may have in other problematic weedy species.