Target Site Resistance Mechanism of Protoporphyrinogen Oxidase Inhibiting Herbicides in *Eleusine indica*

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
In partial fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 7, 2021

Key words: *Eleusine indica*, goosegrass, PPO, Protoporphyrinogen Oxidase, Oxadiazon

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Abstract

Goosegrass is considered as one of the five most troublesome weeds in the world. Protoporphyrinogen oxidase (PPO) with two isoforms, chloroplast targeted (PPO1) and mitochondrial targeted (PPO2), is a step in the biosynthesis of chlorophyll and heme. PPO1 and *PPO2* are herbicide target sites of PPO inhibitors. Target-site mutations reported to date conferring resistance to PPO inhibitors have all been in PPO2. Oxadiazon is a unique PPO inhibitor utilized for goosegrass control in field courses and crop management. Studies were conducted to better understand target site resistance mechanism of PPO inhibitors.

First, two previously confirmed oxadiazon preemergence resistant and susceptible goosegrass biotypes were evaluated to different PPO inhibitors. Two goosegrass biotypes were confirmed to be resistant to oxadiazon, but not to other structurally unrelated PPO inhibitors, such as lactofen, flumioxazin and sulfentrazone. A novel mutation A212T was identified in PPO1, conferring resistance to oxadiazon in *in vitro* enzyme and *Escherichia coli* functional expression system. Computational structural modeling provided a mechanistic explanation for reduced herbicide binding to the variant protein: the presence of a methyl group of Threonine 212 changes PPO1 active site and produces repulsive electrostatic interactions that repel oxadiazon from the binding pocket.

Secondly, A binary goosegrass populations collected from different locations were submitted to the Herbicide Resistance Diagnostic Lab at Auburn University to evaluate for resistance to oxadiazon at 1.12 kg ha^{-1}. Eleven of the 21 suspected populations were diagnosed as R based on postemergence screen. PCR sequencing results showed that there are 9 new resistant populations encoded a nonsynonymous SNP resulting in A212T amino acid substitution in PPO1, except for one population. A dCAPs primer and leaf chlorophyll fluorescense can be

effectively utilized as a combination assay to detect the A212T mutation of all populations containing the mutation without sequencing. There is no other reported mutations occurred either in PPO1 or PPO2 of any populations. The target-site mutation A212T in PPO1 have been further substantiated as the primary mechanism of oxadiazon resistance. However, this does not preclude other non-target site mechanisms conferring resistance to oxadiazon in Sandestin or in future populations.

At last, three different PPO isoforms from susceptible goosegrass plants (S-PPO1, S-PPO2) and resistant goosegrass plants (R-PPO1) were extracted and transformed to hemG mutant E. coli strain to evaluate the sensitivity response with three PPO inhibitors: oxadiazon, lactofen and sulfentrazone. The results showed that oxadiazon inhibits the PPO1 isoform greater than PPO2, lactofen inhibits the PPO2 isoform greater than PPO1, and sulfentrazone inhibits both PPO1 and PPO2 similarly, indicating that oxadiazon may prefer to binding chloroplast PPO1 isoform.

Acknowledgments

My PhD journey at Auburn has been an educational and incredible experience for me, with highs and lows. There are many people who have helped me throughout my study, and I would like to say 'thank you' to all of them.

First of all, I would like to express the greatest appreciation to my advisor, Dr. Scott McElroy. I feel fortunate to be under his patient mentorship and am extremely grateful for his strong encouragement and support. Without his advice and support, none of my research in the past five years would be done. Because of him, I have a very good and interesting project and a very good committee. The critical thinking, hypothesis development, effective presentation and time management will benefit my career and life.

Secondly, I would like to thank all the committee members, Dr. Steve Li, Dr. Jeffrey Coleman, Dr. Andrew Price, and my University Reader Dr. Sushan Ru, from the department of Horticulture, for the guidance and continued support of my PhD research and dissertation. Their suggestions make the dissertation more logical and deepen my understanding of how to be a good researcher.

Thirdly, I wish to thank all my lab members for their help and guidance, including Dr. Hui Zhang, Dr. Jinesh Patel, Mr. Austin Brown, Mr. John Peppers, Mr. Eli Russel, Ms. Claudia Ann Rutland and Ms. Suma Basak.

Finally, but not least, I would like to express my appreciation to my family in China, for their love, support, and encouragement while I am here in Unite States.

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PPO	Protoporphyrinogen oxidase
ALS	Acetolactate synthase
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ACCase	Acetyl CoA carboxylase
PSII	photosystem II
PEP	phosphoenolpyruvate
P450	cytochrome P450 monooxygenase
GST	Glutathione-s-transferase
dCAPS	derived Cleaved Amplified Polymorphic Sequence
SNPs	single nucleotide polymorphisms

Chapter I Literature reviews

1.1 **Goosegrass**

Goosegrass biology. Goosegrass (*Eluesine indica* (L.) Gaertn) is a native perennial summer annual grass (Bryson and DeFelice, 2009, McCarty, 2001, Murphy, 2004), which is mainly classified as an environmental and agricultural weed (Randall, 2017) and is known as one of the world's five most problematic weeds. Goosegrass is commonly found growing in compacted and/or droughty soil conditions where moisture and oxygen levels are low (Jones and Waddington, 1992). When temperatures stayed constant at 20, 25, and 35 C, goosegrass germination was less than 10%, but increased to 99% with frequent fluctuating regimes of 20 C for 16 h and 35 C for 8 h with the addition of light (Nishimoto and McCarty, 1997). With higher, alternating temperature regimes of 30/20 and 35/25 C compared to 25/15 C, Chauhan and Johnson (2008) have recorded higher percentages of goosegrass germination (Chauhan and Johnson, 2008). There is also an effect of goosegrass germination on planting depth. Holm (1979) stated that as the burial depth increased, goosegrass seedling germination decreased. Two resistant and susceptible populations were seeded, 5 days after planting, at a depth of 0 cm, and for seeds planted at a depth of 2 cm, the seeds started to germinate 6 day. Researches showed that goosegrass seed emergence declined at depths greater than 4 cm and no emergence was reported at depths greater than 8 cm. In hot climates where the soil surface is always moist and warm, much of goosegrass seed germinate continuously (Wiecko, 2000).

Goosegrass resistance to herbicide. According to the recently International Survey of Herbicide Resistant Weeds (Heap, 2020), a total of 36 resistance cases have been reported in goosegrass, which are from 12 countries and 10 US states, across 8 unique mode of actions, and in over 15 different cropping systems (Heap 2020). Goosegrass has evolved resistance to more

than 15 herbicides including glyphosate, paraquat, metribuzin, trifluralin, pendimethalin, glufosinate, fluazifop, propaquizafop, imazapyr, sethoxydim, cyhalofop, fenoxaprop, clethodim, haloxyfop and oxadiazon (Heap, 2020). At least two goosegrass biotypes are reported as resistant to two sites of action, acetyl CoA carboxylase (ACCase) and enolpyruvyl shikimate-3-phosphate synthase (EPSPS) (Heap, 2020), as well as photosystem I and glutamine synthesis inhibition (Seng et al., 2010). There have been numerous documentations, however, of cross-resistance within goosegrass biotypes (Marshall et al., 1994, Vaughn et al., 1990). Goosegrass also ranks 4th out of all resistant weed species for total number of sites of action with eight, same rank with annual bluegrass (*Poa annua*), Common waterhemp (*Amaranthus palmeri*), and rigid ryegrass (*Lolium rigidum*) ranks 1st (14 total sites of action) (Heap, 2020). To date, more glyphosate and paraquat cases of goosegrass have been found around the world than any other site of actions (Heap, 2020).

Glyphosate Resistance. Glyphosate-resistant goosegrass has been reported in nine countries and within ten cropping systems (Heap, 2020). A goosegrass biotype in Malaysia was found to be 8 to 12-fold resistant to glyphosate when treated with 5.76 kg ai ha^{-1} comparing to susceptible plants (Lee and Ngim, 2000). Glyphosate at rates of 210 g ae ha^{-1} or higher controlled a glyphosate-susceptible population in Tennessee greater than 90%, 21 days after treatment (DAT) (Mueller et al., 2011); however, a glyphosate-resistant population was controlled only 12% at the same rates (Mueller et al. 2011). I_{50} values associated with the resistant population were three to ten times greater than those of the resistant population in regards to fresh weight of goosegrass harvested 21 days after treatment (DAT) (Mueller et al. 2011). A Pro106 point mutation to Ser (P106S) was found to confer glyphosate resistance in a goosegrass biotype in Davao, Mindanao Island, Philippines (Kaundun et al., 2008). Extensive

testing of the two biotypes at different goosegrass growth stages and glyphosate rates produced a strong correlation ($P < 0.001$) between presence of the mutated P106S in EPSP synthase and the resistant plants (Kaundun et al., 2008). No glyphosate absorption or translocation patterns were observed between all genotypes of resistant and susceptible goosegrass populations at that location, indicating the resistance was due to an altered binding site (Kaundun et al. 2008). Other studies have indicated a significant mechanism of glyphosate resistance in goosegrass to be an altered EPSPS with a cytosine to thymine replacement in the resistant allele (Baerson et al., 2002).

Dinitroaniline Resistance. A goosegrass population was found to be unaffected by trifluralin in cotton fields near the southeastern portion of North Carolina and northeastern South Carolina, which is the first report case of goosegrass resistant to dinitroaniline resistance. (Heap, 2020). It is also believed that goosegrass biotypes resistant to one dinitroaniline herbicide such as trifluralin are likely cross-resistant to other members of the dinitroaniline family (Powles and Yu, 2010). High levels of dinitroaniline resistance have been observed in goosegrass biotypes with an α-tubulin gene mutation and Thr-239-Ile substitution (Mudge et al., 1984). This particular substitution also enables cross-resistance to phosphoroamidate and pyridine herbicides but negative cross-resistance to some carbamates (Powles and Yu 2010). A Met-268-Thr mutation was found to endow lower-level dinitroaniline resistance in goosegrass biotypes (Yamamoto et al., 1998). Regardless of resistance level, target site dinitroaniline resistance is inherited as a recessive gene (Jasieniuk et al., 1994, Wang et al., 1996, Zeng and Baird, 1997) and only homozygous plants survive herbicide treatment at labeled rates. When evaluated under noncompetitive conditions and compared to dinitroaniline-susceptible biotypes, a fitness cost did

not seem to be associated with dinitroaniline-resistant goosegrass (Harris et al. 1995; Murphy et al. 1986).

Paraquat Resistance. A 30-fold, paraquat-resistant biotype of goosegrass was confirmed in Manatee County, Florida after years of repeated applications in tomato fields (Buker et al., 2002). In addition, a goosegrass population growing in a bitter gourd field in Malaysia was reported to be 3.4- to 3.6-fold more resistant to treatments of glufosinate and paraquat than the susceptible biotype after a minimum of six sequential applications of these herbicides over the previous four years (Seng et al., 2010). These results were the first to demonstrate multiple resistances of goosegrass to two non-selective herbicides in field conditions (Seng et al. 2010).

Oxadiazon resistance. Oxadiazon has become one of the most frequently used preemergence herbicides for goosegrass management in bermudagrass turf (Bingham and Hall 1985; Bingham and Shaver 1981; Busey 1999; Johnson 1976). A first case of goosegrass evolved resistance to oxadiazon was from the golf courses in VA and NC (McElroy et al., 2017). When treatment with oxadiazon at rates less than 2.24 kg ha^{-1} did not prevent the emergence of two goosegrass resistant biotypes. Based on the I90 values, seedling emergence data of two resistant biotypes was predicted 7.9 and 3.0 times greater than susceptible biotypes (McElroy et al., 2017).

1.2 Protoporphyrinogen Oxidase Inhibitors.

Protoporphyrinogen oxidase (PPO) is a critical enzyme in the common biosynthesis pathway of chlorophyll and heme, which are needed for photosynthesis and electron transfer chains, respectively. PPO catalyzes the oxidation of Protogen IX to Proto IX (Duke et al., 1991). The inhibition of PPO activity will accumulate the substrate Protogen IX, which leaks from the chloroplast or mitochondria into the cytoplasm (Duke et al., 1991). The oxidation product, Proto

IX, will be produced in the cytoplasm and generate highly reactive singlet oxygen (O2) when light occurs, which inducing rapid peroxidation of the cell membrane (Jacobs et al., 1991). There are a total of seven different PPO-inhibiting herbicides chemical families using in the United States, including diphenyl ethers, phenylpyrazoles, triazolinones, oxadiazoles, pyrimidinediones, N-phenylphthalimides thiadiazoles, and oxazolidinedione. These herbicides families are structurally unrelated chemical classes and can be used to control weeds in field crops, vegetables, lawns, industry, etc.

Fomesafen (trade name: Reflex) is a diphenyl ether PPO-inhibiting herbicide which widely used to control broadleaf weeds (Senseman, 2007, Montgomery et al., 2018). Fomesafen can be used as Early POST or POST directly in soybean and cotton to various broadleaf weeds such as Palmer amaranth. There is a study conducted to observe fomesafen with another herbicide modes of action to control two glyphosate-resistant and one glyphosate-susceptible Palmer amaranth populations in glufosinate-resistant cotton (Norsworthy et al., 2008). The results suggested that fomesafen applied with S-metolachlor and pendimethalin would provide 100% control to all the populations (Norsworthy et al., 2008). Some studies have showed that fomesafen effectively control Palmer amaranth which have evolved resistance to dinitroaniline herbicides when utilized as PRE compared to POST (Troxler et al., 2002, Stephenson IV et al., 2004). There are three other diphenyl ether PPO inhibitors using to weed control in soybean and cotton, such as acifluorfen (trade name: Blazer), lactofen (trade name: Cobra) and oxyflurofen (trade name: Goal). Fomesafen is considered to be the less injurious to the crops than others, and lactofen is generally considered to be the “hottest” of the others. So far, there have been 9 species have evolved resistance to diphenyl ethers (Heap, 2020), which ranks the first one compared to the other PPO inhibitors. Most of these resistant species, such as common

waterhemp and Palmer Amaranth, can be multiple resistant to the inhibitors from same class, and lightly resistant to other families, like sulfentrazone and flumioxazin (Heap, 2020). However, no diphenyl ethers resistant species evolved resistant to oxadiazon, another PPO inhibitor in the oxadiazoles families.

Sulfentrazone (trade name: Dismiss) is a PPO-inhibiting herbicide from the triazolinone herbicide chemical family (Theodoridis et al., 1992). Sulfentrazone can be applied as both PRE and POST control of broadleaf weeds in soybean and turf courses (Dayan, 1995, Dayan et al., 1997). Sulfentrazone is also can be applied as POST goosegrass control in turf courses (McCullough et al., 2012). Sulfentrazone always used to weed control in a combination with other herbicides which has other mode of action, such as pyroxasulfone, an acetolactate synthase (ALS) inhibitors (Tidemann et al., 2014). Researchers suggested that application of sulfentrazone alone achieved less than 35% control of goosegrass. However, when applied with the addition of nicosulfuron, another ALS inhibitors utilized mainly in maize, can improve goosegrass control to around 60% (McCullough et al., 2012). There is no single case reported to sulfentrazone resistance. However, sulfentrazone was tested to be resistant in some diphenyl ethers resistant species. There is a common waterhemp population which had been treated with acifluorfen for decades conferring 34, 82, 8 times resistance to acifluorfen, lactofen, fomesafen. This common waterhemp population also had been confirmed 4 times more resistant to sulfentrazone than susceptible (Shoup et al., 2003).

Flumioxazin (trade name: Sureguard) is a PPO-inhibiting herbicide from N-phenylphthalimide chemical family. Flumioxazin always be applied as both PRE and POST control effective in problematic weeds, like common ragweed (*Ambrosia artemisiifolia*)(Glomski and Netherland, 2013), and annual grass such as annual bluegrass (*Poa annua*) in lawn and

landscape (Flessner et al., 2013). Generally flumioxazin has predominates PRE activity. When PRE applied with flumioxazin, it can control 79-83% common ragweed (Niekamp and Johnson, 2001). While the efficacy of POST application of flumioxazin will be influenced by plant maturity. It is reported that flumioxazin at 0.43 kg ha-1 controlled annual bluegrass ≥ 95% at a 2-tillers growth stage, but only 50% control at 4 to 6 tillers (Flessner et al., 2013). Moreover, there have been no single reports to flumioxazin resistance, but same as sulfentrazone, there is a common ragweed biotype multiple resistance to ALS inhibitors and PPO inhibitors, including flumioxazin (Rousonelos et al., 2012). This biotypes was identified 10-times resistant to PRE flumioxazin, compared with 80- times to fomesafen, 24- times to acifluorfen and 20-times to oxyfluorfen (Rousonelos et al., 2012). This common ragweed biotype was also a little resistant (3-times) to carfentrazone, another triazolinones PPO inhibitors same as sulfentrazone (Rousonelos et al., 2012).

Oxadiazon (trade name: Ronstar) is a PPO-inhibiting herbicide belongs to the oxadiazole group (Duke et al., 1997). Oxadiazon is mainly applied as PRE to control of annual grasses such as goosegrass, and broadleaves such as carpetweed (*Mollugo verticillata*.) (Johnson, 1976, Derr, 1994). A single PRE application of oxadiazon provided excellent goosegrass control (> 90%) which are resistant to dinitroaniline herbicides, while sulfentrazone with PRE only inhibited less than 60% (McCullough et al., 2013). Lack of goosegrass control was noted when oxadiazon was applied at rates of 3.4 to 4.5 kg ai/ha. It is unknown whether this biotype was an isolated incident or if there are other resistant populations of goosegrass in the area (Askew 2013).

Saflufenacil (trade name: Sharpen) is a new PPO-inhibiting herbicide in the pyrimidinedione chemical family, which is introduced in 2009 and typically PRE applied for the control of grass and broadleaf weeds in corn, soybean and other major crops (Grossmann et al.,

2011). Saflufenacil also has POST applied activity where it is used as a burn-down and pre harvest desiccant in the crop. It is reported that saflufenacil applied as PRE at a ranging rate of 6 to 30 g ha^{-1} reduced the biomass of Palmer amaranth biomass along with two other Amaranthus species 82 to 98% (Geier et al., 2009). Because of the low use rates and effective control of weeds which are resistant to other herbicide modes of action, saflufenacil has been widely used to control grass in turf (Soltani et al., 2009). There is no single case reported to evolve resistant to saflufenacil, however, a common ragweed biotype which is resistant to fomesafen was also 22-fold resistant to saflufenacil, a little higher resistant comparing with 10-fold resistant to flumioxazin (Rousonelos et al., 2012).

1.3 Herbicide resistance mechanism.

The mechanisms by which weeds develop resistance to herbicides can be summarized into two categories, one is the target site resistance (TSR) mechanism and the other is the non-target site resistance (NTSR) mechanism.

Target site resistance. The TSR mainly refers to the mutation of the target site genes or the overexpression of the target enzymes. After the target gene is mutated, the structure of the target enzyme is changed, resulting in weakening or hindering the binding of the herbicide to the target enzyme, leading to the herbicide resistance. The target resistance mechanism of weeds mainly occurs in six major herbicides, including ALS inhibitors, triazine herbicides, ACCase inhibitors, dinitroaniline herbicides, organophosphorus herbicides, and PPO inhibitors.

ALS inhibitors have been widely used worldwide since the 1980s. Due to continuous and large-scale use of the same type of herbicides, weeds have been evolving increasingly resistance to ALS inhibitors. In 1987, the first case of ALS herbicide-resistant weed was discovered in prickly lettuce (*Lactuca serriola*) (Mallory-Smith et al., 1990). From 1987 to 2017, 159 weed

biotypes around the world have developed resistance to ALS inhibitors (Heap, 2020). Studies have shown that the target resistance mechanism of weeds to ALS inhibitors is mainly the eight amino acid positions on the ALS gene (Ala l22, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, Gly654) (Powles and Yu, 2010).

Triazine herbicides are photosystem II inhibitors, and their target is the D1 protein in photosystem II (PSII). Herbicides bind to D1 protein to change the spatial structure of membrane proteins and block electrons transmission in PSII, leading to the destruction of the optical system. As early as the 1950s, triazine herbicides have been widely used in corn areas around the world. From the first report of weed resistance to triazine herbicides in 1970 (Ryan, 1970), so far, there have 74 weeds species been developed resistance to triazine herbicides. The resistance mechanism to triazine herbicides is mainly caused by target mutations. The Ser-264-Gly mutation on the psbA gene encoding the D1 protein reduces the affinity between the herbicide and the protein, resulting in the resistance to photosystem II inhibitors. At present, the Ser-264-Gly mutation on the psbA gene have developed rapidly around the world. The Ser-264-Gly mutation reduces the affinity of the herbicide to the D1 protein, but the binding of PQ to the D1 protein is also affected to a certain extent, resulting in photosynthesis. The weakening of the role has resulted in a certain fitness cost (Holt and Thill, 1994, Vila-Aiub et al., 2009). The Ser-264-Gly mutation only confers resistance to triazine herbicides, but not to other photosystem II inhibitors. It has been reported that Val-219-Ile (Mengistu et al., 2005), Ala-251-Val (Mechant et al., 2008), Phe-255-Ile (Perez-Jones et al., 2009), Asn-266-Thr (Park and Mallory-Smith, 2006) can be resistant to other Photosystem II inhibitors.

ACCase inhibitors can target the enzyme Acetyl-CoA carboxylase, which inhibits the synthesis of fatty acids in plants. There are three main chemical types of such herbicides:

Aryloxyphenoxypropionate (FOPs), cyclohexanedione (DIMs), and phenylpyrazolin (DENs). From the first reported case of ryegrass resistance to ACCase inhibitors (Heap and Knight, 1982), 48 weed species have developed resistance to ACCase inhibitors. Studies have shown that there are 11 different amino acid substitutions at 7 amino acid positions in the ACCase gene related to the resistance, including Ile-1781-Leu, Ile-1781-Val, Trp-1999-Cys, Trp-1999-Leu, Trp-2027-Cys, Ile-2041-Asn, Ile-2041-Val, Asp-2078-Gly, Cys-2088-Arg, Gly-2096-Ala and Gly-2096-Ser (Beckie and Tardif, 2012).

The target of dinitroaniline herbicides is tubulin. Herbicides bind to tubulin to inhibit the polymerization of microtubules and cause damage to microtubules. Microtubules are important cytoskeleton polymerizations for cell mitosis, conduction and motility. Herbicides can break the microtubules and affect cell mitosis by exerting herbicidal activity. As early as 1994, studies found that tubulin was still polymerized normally in the resistant plants when treated with dinitroaniline herbicides (Yamamoto et al., 1998). There were reports confirming the presence of the amino acid substitution Thr-239-Ile in the tubulin gene produced high resistance to dinitroaniline herbicides, and cross-resistance to phosphoramidates and pyrenes, but negative cross-resistance to carbamates in 1998 (Yamamoto et al., 1998). Later, some other mutations were found in the tubulin genes which endows resistance to dinitroaniline herbicides: Met-268-Thr (Yamamoto et al., 1998), Leu-136-Phe (Délye et al., 2004), Arg-243-Lys/Met (Chu et al., 2018) and Val-202-Phe (Chen et al., 2020).

PPO is a key enzyme in the process of chlorophyll and heme biosynthesis. After it is inhibited by herbicides, cell peroxidation damages cell membranes, hinders chlorophyll synthesis, and ultimately causes plant death. In plants, there are two kinds of PPO enzymes, PPO1 and PPO2. One is in the chloroplast and is encoded by the PPO1 gene; the other is in the

mitochondria and is encoded by the PPO2 gene. A total of five target-site mutations at three different amino acid residues of PPO2 have been confirmed to confer resistance in PPO inhibitor in weed species. The first weed species discovered with increased resistance to PPO inhibitors was common waterhemp in 2001 (Shoup et al., 2003), and to date there are total 35 cases which are from thirteen plant species confirmed resistance to PPO inhibitors, compared to 48, 160, and 43 species for ACCase, ALS, and EPSPS inhibitors, respectively. A glycine deletion at position 210 (referred to as ΔG210) in the PPO2 confers resistance to PPO inhibitors (fomesafen and lactofen) in common waterhemp (Patzoldt et al., 2006). An amino acid substitution (R128L) in PPO2 has been reported to evolve resistance to PPO inhibitors in common ragweed, and two other amino acids substitutions at the same site (R128G and R128M) also conferred resistance in Palmer amaranth (Giacomini et al., 2017). Most recently, a glycine substituted to alanine at 399 (G399A) in PPO2 has been proved to evolve resistance in a field-selected Palmer amaranth population (Rangani et al., 2019).

Glyphosate is a typical representative of organophosphorus herbicides. It is the most widely used herbicide in the world. The target of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), by competitively inhibiting the combination of phosphoenolpyruvate (PEP) and EPSPS, blocks the reaction of PEP and S3P to produce EPSP, leading to accumulation of shikimic acid and affecting the biosynthesis of aromatic amino acids and ultimately leads to the death of plants. By 1996, glyphosate had been used for more than 20 years, but there has been no report on weed resistance to glyphosate. Therefore, it was once thought that it was impossible to evolve weeds resistant to glyphosate under field conditions. However, with the large-scale planting of glyphosate-resistant genetically modified crops, glyphosate-resistant weeds began to be used in a single large amount with high intensity. Under

high selective pressure, glyphosate-resistant weeds began to develop rapidly. To date, there are 36 hybrid species grass has evolved resistance to glyphosate. A Pro-106-Ser substitution was found in the EPSPS gene of glyphosate-resistant goosegrass, which is first report target site resistance to glyphosate (Lee and Ngim, 2000). Ng et.al (2003) found A Pro-106-Thr substitution in the EPSPS gene of glyphosate resistant goosegrass (Ng et al., 2003). Yu et al. (2007) found the Pro-106-Ala amino acid substitution in the EPSPS gene on ryegrass (Yu et al., 2007). At present, the three amino acid substitutions at the Pro-106 position of the EPSPS gene are found in resistant goosegrass and ryegrass in different parts of the world (Kaundun et al., 2008, Preston et al., 2009).

NTSR mechanism. Refers to other resistance mechanisms in addition to the target resistance mechanism, mainly including the reduction in the absorption and conduction of weeds to herbicides, the enhancement of herbicide metabolism and detoxification, and the shielding of herbicides Isolation and so on. Studies on the mechanism of non-target site resistance of weeds have found that improved metabolic detoxification (metabolic resistance) is the main part of non-target resistance, while the absorption and conduction of herbicides is reduced and the shielding and isolation effects of herbicides are only a few. Found in resistant weeds. Metabolic resistance mainly involves cytochrome P450 monooxygenase (CYP450), glutathione-S-transferase (GSTs), glycosyltransferase (GT), peroxidase (POD) and ABC transporter (Siminszky, 2006, Yuan et al., 2007, Yu and Powles, 2014).

Cytochrome P450 monooxygenases are a supergene family that play a very important role in the metabolic resistance of weeds to herbicides. Existing studies have found that the resistant smoot pigweed (*Amaranthus hybridus*), black grass (*Alopecurus myosuroides*), wild oats (*Avena sterilis*), ryegrass , cheatgrass (*Bromus tectorum*) (Manley et al., 1999, Park et al.,

2004, Letouzé and Gasquez, 2003, Maneechote et al., 1997, Tardif and Powles, 1999). A large number of studies have shown that the metabolic resistance of weeds to herbicides is related to P450 enzymes, but the identification of P450 genes related to metabolic resistance has been slow. Iwakamie (2014a) found two P450 enzyme genes (CYP71AK2 and the overexpression of CYP72A) is related to the resistance of rice barnyardgrass (*Echinochloa phyllopogon*) to bispyribac-sodium (Iwakami et al., 2014b). In addition, when the CYP81A12 and CYP81A21 genes are expressed in Arabidopsis thaliana, they develop resistance to jedsulfuron and penoxsulam (Iwakami et al., 2014a).With the rapid development of gene technology, transcriptome sequencing (RNA-Seq) has begun to be applied in various aspects. RNA-Seq technology can be used to study the resistance-related genes of weed species. Gaines et al (2014) adopted RNA-Seq technical research found that in nine resistant Swiss ryegrass populations, four genes (two cytochrome P450, one 2-nitropropane-dioxygenase, and one glycosyltransferase) were highly expressed compared to the sensitive populations. RNA-Seq sequencing of ryegrass and sensitive populations resistant to sulfentrazone confirmed the excess expression of two cytochrome P450s, one glycosyltransferase and one glutathione-S-transferase is related to its resistance (Duhoux et al., 2015). Gardin *et al* (2015) found that highly expression of encoded genes of three cytochromes P450 (CYP71A, CYP71B and CYP81D), one peroxidase and one disease resistance protein in black-grass can be NTSR related to ALS herbicides resistance (Gardin et al., 2015).

Glutathione-s-transferase (GST) is a family of multifunctional enzymes, which catalyze the binding of glutathione to various electrophilic and hydrophobic substrates. The protective mechanism of GSTs against oxidative stress is to catalyze the co-rolling reaction between glutathione and reactive oxygen species, thereby detoxifying the toxic substances entering the

plant. Corn has high resistance to triazine herbicides, mainly because the highly active GSTs in the body can catalyze the co-morphing effect of herbicides and glutathione, which makes the herbicides inactive. Anderson et al. (1991) and Gronwald et al.(1989) reported that the resistance to atrazine is mainly due to the enhancement of GSTs catalytic activity and atrazine detoxification in resistant velvetleaf (*Abutilon theophrasti*) (Anderson and Gronwald, 1991, Gronwald et al., 1989). Bakkali et al. (2007) found that the glutathione conjugation rate in resistant barnyardgrass is faster than that of sensitive populations, which is the main reason for its resistance to ACCase inhibiting herbicides Fenoxaprop-p-ethyl (Bakkali et al., 2007). Cummins et al. (1999) found that black-grass is resistant to a variety of herbicides with different mode of actions. The resistance mechanism is mainly caused by the increased expression of GST gene (AmGST2) in resistant populations (Cummins et al., 1999).

The detoxification effect of the ABC transporter on the herbicide is by separating the herbicide and its metabolites (Rea, 2007). Peng et al (2010) used the RNA-Seq method to counteract the non-toxicity of glyphosate resistant horseweed (Peng et al., 2010). The target resistance genes were analyzed, and the study found that 17 ABC transporter genes in the resistant horseweed are up-regulated and related to their resistance to glyphosate. The study of glyphosate resistant horseweed also found that the target enzyme EPSPS Pro 106 did not have mutations, and also was not overexpressed, while the ABC transporter genes (M10 and M1l) has a significant increase (Peng et al., 2010).

1.4 Dissertation Objectives

The objectives of this research were to (1) characterize two PPO isoforms genes in goosegrass and decipher the mechanism conferring resistance to oxadiazon, (2) further identify the A212T mutation in oxadiazon resistant populations and rapid detect the oxadiazon resistance

with a combination assay, and (3) compare the different sensitivity among three different structural-unrelated PPO inhibitors with *in vitro* functional assay.

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Chapter II. A Novel Mutation A212T in Chloroplast Protoporphyrinogen Oxidase (PPO1) Confers Resistance to PPO inhibitor Oxadiazon in *Eleusine indica*

This chapter has been published in *Pest Management Science* (2020)

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Abstract:

BACKGROUND: Protoporphyrinogen oxidase (PPO) with two isoforms, chloroplast-targeted (PPO1) and mitochondrial-targeted(PPO2), catalyzes a step in the biosynthesis of chlorophyll and heme. PPO1 and PPO2 are herbicide target sites of PPO-inhibiting herbicides. Target-site mutations conferring resistance to PPO inhibitors have all thus far been in PPO2. Oxadiazon is a unique PPO inhibitor utilized for preemergence Eleusine indica control. In this research, we evaluated the response of two previously confirmed oxadiazon-resistant and susceptible *E. indica* biotypes to other PPO inhibitors and identified the resistance mechanism in two oxadiazon resistant *E. indica* biotypes.

RESULTS: Two *E. indica* biotypes were resistant to oxadiazon, but not to other structurally unrelated PPO inhibitors, such as lactofen, flumioxazin and sulfentrazone. A novel mutation A212T was identified in the chloroplast-targeted PPO1, conferring resistance to oxadiazon in a heterologous expression system. Computational structural modeling provided a mechanistic explanation for reduced herbicide binding to the variant protein: the presence of a methyl group of threonine 212 changes the PPO1 active site and produces repulsive electrostatic interactions that repel oxadiazon from the binding pocket.

CONCLUSION: The novel A212T mutation in PPO1 conferring resistance specifically to PPO inhibitor oxadiazon was characterized. This is the first evidence of the direct role of PPO1 in the PPO mode of action, and the first evidence of evolved resistance in PPO1.

Keywords: *Eleusine indica*, protoporphyrinogen oxidase, oxadiazon, PPO inhibitors, resistance mechanism, PPO1

2.1 Introduction

Protoporphyrinogen IX oxidase (PPO) (EC 1.3.3.4) is an oxygen-dependent enzyme which plays a role in the biosynthesis of chlorophyll and heme, catalyzing the oxidation of protoporphyrinogen IX to protoporphyrin IX (Poulson and Polglase, 1975, Duke et al., 1991, Hao et al., 2009). In plants, PPO is one of the common herbicide targets. When PPO is inhibited, the substrate protoporphyrinogen IX accumulates and is exported into the cytoplasm, and the catalytic product of PPO, protoporphyrin IX, accumulates in the cytoplasm (Duke et al., 1991, Jacobs and Jacobs, 1993). Protoporphyrin IX induces the formation of singlet oxygen in the presence of light, causing lipid peroxidation and cell membrane leakage(Duke et al., 1991). PPO-inhibiting herbicides classified in Herbicide Resistance Action Committee (HRAC) mode of action class E that include diphenyl ethers, phenylpyrazoles, triazolinones, thiadiazoles, oxadiazoles, pyrimidinediones, oxazolidinedione, and N-phenylphthalimides classes, all of which are structurally unrelated herbicide chemical families (Halling et al., 1994, Theodoridis, 1997, Hiraki et al., 2001, Gitsopoulos and Froud-Williams, 2004). Oxadiazon is a PPO inhibitor in the oxadiazole chemical family, which is uniquely utilized as a preemergence-applied herbicide to control annual grasses, such as *Eleusine indica* (L.) Gaertn (goosegrass), and broadleaf weeds, such as *Oxalis stricta* L. (yellow wood sorrel) in turfgrass (Duke et al., 1997, Derr, 1994, Johnson, 1976, Rahman et al., 2005).

Although PPO inhibitors have been commercialized for more than forty years (since the 1980s), resistance to these compounds has evolved relatively slowly. The first weed discovered with increased resistance to PPO inhibitors was *Amaranthus tuberculatus* (Moq.) J. D. Sauer (common waterhemp) in *Glycine max* (L.) Merr. (soybean) fields in 2001 (Shoup et al., 2003), and to date there are only thirteen plant species with confirmed resistance to PPO inhibitors,

compared to 48, 160, and 43 species for acetyl-CoA caroboxylase (ACCase), acetolactate (ALS) synthase, and 5-enolpyruvyl shikimate 3-phospate (EPSP) synthase inhibitors, respectively (Heap, 2020). There are two isoforms of PPO encoded by two nuclear genes targeted by PPO inhibitors, plastid PPO1 and mitochondrial PPO2, where PPO1 is located in the envelope membranes of chloroplast, while PPO2 is located on the outer surface of the inner mitochondrial membrane (Duke et al., 1991, Hao et al., 2011). In some prokaryote and plant species, the PPO2 can dual-target to both organelles, chloroplast and mitochondria, which translate to larger and smaller products (Watanabe et al., 2001, Jung et al., 2004). The slow rate of field-evolved resistant species to PPO inhibitors evolution is partially attributed to the two target sites rather than one target site for previously mentioned herbicide modes of action.

Thus, both PPO1 and PPO2 are the targets for PPO inhibitors even though located in different organelles (Lermontova et al., 1997). However, all the target-site mutations conferring resistance to PPO-inhibitors were in the mitochondrial-targeted PPO2. Five amino acid target-site mutations at three sites of PPO2 have been confirmed to confer resistance in PPO inhibitor in various resistant weed species. A glycine deletion at position 210 (referred to as ΔG210) in the target site of the PPO2 confers resistance to PPO inhibitors (fomesafen and lactofen) in *A. tuberculatus* (Patzoldt et al., 2006). An amino acid substitution (R128L) in PPO2 confers resistance to PPO inhibitors in *Ambrosia artemisiifolia* L. (common ragweed), and two mutations at the same site (R128G and R128M) also conferred resistance in *Amaranthus palmeri* S. Wats. (Palmer amaranth) (Giacomini et al., 2017). The latest mutation conferring resistance to PPO inhibitors is a PPO2 substitution at position 399 (G399A) in a field-selected *A. palmeri* population (Rangani et al., 2019). So far, there have been no reported target-site mutations occurring in chloroplast-targeted PPO1 conferring resistance to PPO-inhibitors in field-evolved

resistant weed species. In all cases, these reported PPO2 target-site mutations did not provide complete prophylaxis against injury but did allow for greater survival for individuals carrying the mutation.

In 2017, we confirmed two *E. indica* biotypes from Virginia and North Carolina resistant to the PPO-inhibitor oxadiazon (McElroy et al., 2017), which was the first confirmed resistance to PPO inhibitor in grass species, but no information on the resistance mechanism was provided. Here, we conducted experiments to: (1) evaluate the response of two *E. indica* biotypes to the applications of four different PPO inhibitors-flumioxazin, sulfentrazone, lactofen and oxadiazon, which are from different herbicide chemical families, (2) and elucidate the resistance mechanism conferring resistance to the PPO inhibitor oxadiazon in the *E. indica* biotypes.

2.2 Materials and methods

2.2.*1 E. indica* Biotypes and Growth Condition.

Three *E. indica* biotypes from different locations were used in this study. The R1 biotype was collected in Country Club of Virginia, Richmond, VA, U.S.A and the R2 biotype was from River Bend Golf, New Bern, NC. These two biotypes were previous confirmed resistant to preemergence application of oxadiazon, but have not been screened to other PPO inhibitors. The S biotype was collected from the Alabama Agricultural Experiment Station, Plant Breeding Unit, Tallassee, AL, which was confirmed susceptible to PPO inhibitors. Seeds of the mature plants were harvested, air dried and stored in the 4℃ freezer until planted in the greenhouse.

Greenhouse conditions were 30±3℃ at day/night temperature and ~70% average relative humidity. The *E. indica* seeds were placed on the soil surface and lightly covered with sand in 28 cm * 20 cm flats and watered as needed daily to ensure germination. Two weeks after emergence, seedlings were separated and transplanted to in individual 10-cm pots (volumes = 0.5

L). Pots were filled with surface horizon of Marvyn sandy loam (fine-loamy, kaolinitic, thermic Typic Kanhapludults) soil with pH 6.4 and 1.2% organic matter. The plants were irrigated three times daily for 2 min with overhead irrigation and fertilized once at one week after transplanting at approximately 50 kg N ha-1 with Scott's Miracle-Gro All-Purpose fertilizer (The Scotts Miracle-Gro Co. Marysville, OH). Plants were 5-10 cm in height with 3-5 tillers in size at the time of herbicide treatment.

2.2.2 Herbicide Treatment and Data Analysis.

Herbicide treatments were foliar-applied at 280 L ha-1 using an enclosed spray chamber with a single 8002E nozzle (TeeJet Spray Systems Co, Wheaton, IL) at 32PSI. Four herbicides were selected for the experiment: oxadiazon (Ronstar FLO, Bayer Environmental Sci., Research Triangle Park, NC), sulfentrazone (Dismiss, FMC Corporation, Philadelphia, PA), flumioxazin (Sureguard, Valent U.S.A. Corp., Walnut Creek, CA) and lactofen (Cobra, Valent U.S.A. Corp., Walnut Creek, CA). All the herbicides are from different herbicide chemical families of PPO inhibitors: oxadiazoles, triazolinone, N-phenylphthalimide and diphenyl ether, respectively. Herbicides rates were applied at 7 different rates based on each herbicide label rate: oxadiazon ranging from 0.14 to 8.96 kg ha^{-1}, sulfentrazone from 0.07 to 4.50 kg ha^{-1}, flumioxazin from 0.08 to 5.70 kg ha^{-1}, and lactofen from 0.029 to 1.75 kg ha^{-1}. A non-treated control (0 kg ha^{-1}) was included. 192 plants of each biotype were tested and experiments were conducted as completely random design, three replications for two runs.

The visual injury rating scores per plant at 14 d after treatment (DAT) were recorded, where the visual injury rating scores were based on a 0 to 100 scale, which 0 is equated to no phytotoxicity and 100 is equated to complete control. Data subjected to ANOVA analysis at a significance level of $P < 0.05$ using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc.,

Cary, NC). All the herbicide rates were log transformed to make equal spacing between the herbicide treatments in order to facilitate regression analysis. The non-treated control was transformed to equal spacing based on the log rates of each herbicide, respectively. Data were fitted to a sigmoidal model using SigmaPlot 10.2 (Systat Software Inc., London, UK) using a sigmoidal function (Equation 1).

$$y=a/(1+e^{(-((x-x0)/b))))} \quad (1)$$

In this fit sigmoidal model, where y represents *E. indica* visual damage relative to non-treated control (%), x represents the log-transformed herbicide rates (kg ha^{-1}), three parameters (a, b, x0) represents the y intercept. This sigmoidal equation was used to calculate the inhibition rate at 50% (I_{50}) and 90% (I_{90}) relative to the non-treated control of each herbicide for each biotype, and the 95% confidence intervals ($\alpha = 0.05$) were calculated for regression parameters (Seefeldt et al., 1995).

2.2.3 RNA Extraction.

Total RNA of three *E. indica* biotypes (R1, R2 and S) were extracted from fresh leaves using the RNeasy plant kit (QIAGEN, Aarhus, Denmark). Leaves of each biotypes were taken from three well-growth plants. The quality and quantity of the total RNA was assessed by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Co., Waltham, MA) and determined with gel electrophoresis before RNA_Seq analysis. cDNA was synthesized from high-quality total RNA using the ProtoScript first strand cDNA synthesis kit (New England Biolabs Inc. Ipswich, MA).

2.2.4 Transcriptome Assembly and Protein Alignment.

RNA-Seq libraries R1 and R2 *E. indica* biotypes was generated at the Genomic Service Laboratory at the Hudson Alpha Institute for Biotechnology (Cummings Research Park,

Huntsville, AL). The raw sequencing reads of R1 and R2 *E. indica* biotypes have been submitted in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database as accession nos. SAMN10817169, SAMN10817194, respectively. The RNA-Seq dataset of the S biotype was acquired from the NCBI-SRA database under the accession number: SRR 1560465 (Chen et al., 2015). Similarly, a previously published draft genome assembly of the S biotype was downloaded from NCBI as accession number: SAMN09001275 (Zhang et al., 2019)25. Raw RNA-Seq reads R1, R2 and S were assembled using the following pipeline. Adaptor contamination and unqualified reads were removed via Trimmomatic-0.32, then the trimmed reads were quality checked with FastQC and de novo assembly with Trinity 2014-04-13pl (Grabherr et al., 2011). Three assembly datasets were annotated with the NCBI nonredundant (Nr) protein database (https://blast.ncbi.nlm.nih.gov) with ncbi-blast-2.2+. The Nr blast results were processed to identify and compare to reference PPO1 and PPO2 downloaded from the NCBI database (list in Table 2.1). Reads extractions and mapping to identify single nucleotide polymorphisms and other related mutations, all the contigs reads of the blast PPO genes were extracted using bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/) and samtools (http://samtools.sourceforge.net/), and compared with the draft genome annotation scaffold in the CLC Genomics Workbench 6.5.2 (QIAGEN, Aarhus, Denmark). The protein alignment of PPO1 and PPO2 were using clustalX2 and ENDscript 3.0 server (Robert and Gouet, 2014) .

2.2.5 cDNA Sequencing.

Two pairs of oligonucleotide primers were designed based on the sequences of PPO1 and PPO2, respectively. The primers for PPO1 are 5′-ATGGTCGCCACGCCCGCAAT-3′ (chlF) and 5′-CTTGTAGGCGTACTTGGTCAAG-3′ (chlR) and 1587bp PCR product. The primers for PPO2 are 5′-ATGGCGGGCTCCGACGACAC-3′ (mitF) and 5′-

ATGTGAACTGTCATGCTTTGTGC-3′ (mitR), and 1533bp PCR product. The PCR reaction system contained up to 1 μg cDNA, 200 nM of the forward and reverse primers, 200 μM dNTPs and 1.0 U of Taq polymerase (New England Biolabs Inc., Ipswich, MA) with a 1× concentration of standard Taq buffer in a final volume of 25 μL. After initial denaturation of the cDNA at 95°C for 1 min; there were 35 cycles of 30 s at 95°C, 1 min at 58°C and 2 min at 68°C; then a final extension at 68°C for 10 min. PCR products were extracted by gel electrophoresis, sequenced, and analysis conducted using the CLC Genomics Workbench 6.5.2 (QIAGEN, Aarhus, Denmark).

2.2.6 *E.coli* Functional Assay.

Two putative PPO-inhibitor resistant (R-) and susceptible (S-) plasmids were created to test the role of the chloroplast-targeted PPO1 in the *E. indica* biotypes. The PPO1 from R1, R2 and S *E. indica* biotypes were cloned into the pBAD-TOPO expression vector using the pBAD TOPOTM TA Expression kit (Invitrogen, Carlsbad, CA), respectively. The PPO1 product was amplified using the same PCR primers and PCR reaction system as cDNA sequencing, so that the PPO1 translation began at the ATG start codon. Three different pBAD-TOPO PPO1 constructs were created and sequenced to confirm they were identical with the PPO1 gene from R1, R2 and S *E. indica* biotypes and there were no other nucleotide polymorphisms in the cloning experiment. R- and S- PPO1 plasmids were used to transform a hemG mutant Escherichia coli strain SASX38 by electroporation. The SASX38 mutant strain was grown on LB medium supplemented with 10 $\mu g \cdot mL^{-1}$ hematin. Expression of the PPO1 in the transformed colonies of the SASX38 mutant strain were induced on LB medium with 2% L-arabinose. Growth and survival of the transformed colonies of *E. coli* with PPO1 from R1, R2 and S *E. indica* biotypes (marked as: R1, R2 and S, respectively) and a non-transformed control strain

(NT), were tested on three different media: LB alone, LB medium supplied with 10 μg·mL^{-1} hematin, or with the PPO inhibitor oxadiazon from 10 μM to 200 μM, and incubated at 37°C for 20 h.

2.2.7 Recombinant Expression, Purification, and *in vitro* Inhibition Studies of PPO1.

Effects of A212T substitution were studied using the *E. indica* backbone. The wild-type *E. indica* PPO1 and the *E. indica* PPO1 A212T variants were synthesized de novo and subcloned into pRSetB plasmid (Invitrogen, Carlsbad, CA). The complete description of expression and purification of *E. indica* PPO1 and *E. indica* PPO1 A212T variant proteins were referenced to the method described for PPO2 by Rangani et al. (Rangani et al., 2019). Six PPO inhibitors, belonged to five different chemical families, were evaluated to the PPO enzyme activity at a concentrations ranging from 5.00×10^{-5}M to 5.12×10^{-12}M. Oxadiazon, sulfentrazone, saflufenacil and lactofen are from the class of oxadiazole, triazolinone, pyrimidinedione, and diphenyl ether, respectively. Flumioxazin and trifludimoxazin are from the same chemical family, N-phenylphthalimide. The concentration of the wild-type PPO1 activity and variant PPO1 A212T activity 50% (IC_{50} values) reduced by the inhibitors was estimated using non-linear regression procedures, based on each inhibitor. The assay was replicated twice.

2.2.8 Computational Modeling.

A homology model of wild-type *E. indica* PPO1 (S-PPO1 model) was built using the workflow of Schrödinger's Prime (Schrödinger Release 2019-1: Prime, Schrödinger, LLC, New York, NY) using. Default settings and protein preparation settings were applied. As reference structure, an in-house protein crystal structure of *Amaranthus tuberculatus* PPO2 was selected. The sequence similarity between *E. indica* PPO1 and A. tuberculatus PPO2 is 29.2% in total, and 46.4% within the binding site (all residues within 5 Å to the modeled ligand). Oxadiazon was

modeled into the binding site using binding mode information of known in-house co-crystal structures and docking functionality of the modeling program Molecular Operating Environment (MOE, 2019.01: Chemical Computing Group, Montreal, QC, Canada). The predicted poses were refined by local minimization of the ligand and the receptor structure. To examine the effect of the A212T mutation on oxadiazon binding, the homology model of *E. indica* PPO1 was modified into a second model (R-PPO1 model), where Ala212 was virtually mutated to Thr212.

2.3 Results

2.3.1 Herbicide Rate Screening.

Herbicide rate responses focus on comparison of oxadiazon, lactofen, flumioxazin and sulfentrazone on the S and R biotypes. The resistant and susceptible biotypes are not obviously different before herbicide screening. A dose response curve was developed to model the individual biotype response to each tested herbicide (Figure 2.1). Labelled rate applications of flumioxazin (0.357 kg ha^{-1}), sulfentrazone (0.28 kg ha^{-1}), and lactofen (0.22 kg ha^{-1}) controlled the resistant biotypes (R1 and R2) approximately 70 to 100% while the labelled rate application of oxadiazon (2.24 kg ha^{-1}) provided less than 10% control. Little to no difference was observed between the R1, R2 and S *E. indica* biotype response to flumioxazin, lactofen and sulfentrazone at these labelled rates. However, a significant difference was observed between the resistant (R1 and R2), and susceptible (S) response to oxadiazon. Oxadiazon at 2.24 kg ha^{-1} controlled the S biotype > 80% compared to < 20% for the R1 and R2 biotypes. Difference in the R1 and R2 compared to the S response to oxadiazon was consistent for all oxadiazon concentrations tested, while little difference was observed between R1 and R2 compared to S at lower rates of lactofen and sulfentrazone, 0.055 kg ha^{-1} and 0.14 kg ha^{-1}, respectively, were observed in dose response curves. Such a response at lower rates may indicate a slight resistance to lower rates in the

oxadiazon-resistant R1 and R2 *E. indica* biotypes. No such differences between R1, R2, and S were observed with respect to the flumioxazin rates.

I_{50} and I_{90} values of the different PPO-inhibitors for each *E. indica* biotype were calculated based on the model for the curve and the best fit equation (Table 2.2 and Table 2.3). The I_{50} values of the S biotype for oxadiazon was 0.32 kg ha^{-1}, while I_{50} value of the R1 biotype and R2 biotype for oxadiazon was 8.15 kg ha^{-1} and 8.88 kg ha^{-1}, respectively. The I_{90} values of the R1 biotype and the R2 biotype for oxadiazon was 14.60 kg ha^{-1} and 18.29 kg ha^{-1}, respectively, while the I_{90} value of the S biotype for oxadiazon was 1.56 kg ha^{-1}. This indicates that the previously confirmed preemergence oxadiazon resistant *E. indica* biotypes still displayed up to 20-fold increased resistance than the susceptible biotype when postemergence applied with oxadiazon. No significant differences in response to flumioxazin, sulfentrazone and lactofen were observed for I_{50} and I_{90} values between the R1, R2 and S biotypes (Table 2.3). These two previous confirmed oxadiazon resistant *E. indica* biotypes had no significant cross-resistance to other PPO inhibitors except to oxadiazon.

2.3.2 PPO Gene Isolation and Target Site Assessment.

Two related genes in *E. indica*, PPO1 and PPO2, were isolated based on the transcriptome analysis and cDNA sequencing. In the S biotype, the related gene reads of PPO1 and PPO2 were extracted and mapped with the *E. indica* assembly draft genome (Table 2.4, Figure 2.2). The genomic DNA (gDNA) of chloroplast-targeted PPO1 of *E. indica* has 3816 bp, containing 9 exons and 8 introns (Table 2.5); the mitochondrial-targeted PPO2 gDNA has 6043 bp, including 17 exons and 16 introns (Table 2.6). Comparison of the translated sequences of the PPO1 and PPO2 of the S *E. indica* biotype reveal 23.35% amino acid identity and 38.50% similarity (Figure 2.3). These results showed that there are much difference between PPO1 and

PPO2 in *E. indica*, indicating that these two nuclear genes may target different organelles, chloroplast and mitochondria, respectively, and have different protein structure. The gDNA and cDNA of PPO1 and PPO2 in S biotype have been submitted to NCBI GenBank database as accession number: MK573537, MK040459, MK573539, MK573538, respectively.

2.3.3 A Novel Single Amino Acid Substitution A212T in PPO1 as the Hypothesis of Oxadiazon Resistance in the *E. indica* Biotypes.

The sequence of the two unique genes of the three biotypes (S, R1 and R2) were aligned and mapped to the *E. indica* genomic DNA to identify any possible single nucleotide polymorphisms (SNPs) (Figure 2.2, Figure 2.4). There were no nonsynonymous substitutions in the R1 biotype PPO2 gene, but two amino acid substitutions, V207I and T303A, were identified only in the R2 biotype (Figure 2.3). Protein alignments of *E. indica* PPO2 with the A. tuberculatus PPO2 indicated that Val207 (V235 in A. tuberculatus) and Thr303 (Ser324 in A. tuberculatus) are not part of the catalytic domain (Figure 2.3), suggesting that these two substitutions in R2 biotype mitochondrial PPO2 maybe not confer resistance. However, some SNP nonsynonymous substitutions were identified in the PPO1 when comparing S, R1 and R2 *E. indica* (GenBank accession nos. MK040459, MK040460, MK040461, respectively). There was a single amino acid substitution, A212T, identified in both R1 and R2 *E. indica* biotypes (Table 2.7), and two other amino acid substitutions only in R2 biotype, T283A and K366M (Table 2.7). Resequencing of this locus confirmed the presence of the SNP and the resulting amino acid substitution (Figure 2.5a). Protein alignment of the amino acid sequences of PPO1 and PPO2 indicates that alanine 212 in PPO1 is highly conserved in other species (Figure 2.5b), and it is synonymous with glycine 210 in PPO2, which was also highly conserved in other species and previously confirmed as the causal resistance mechanism to PPO inhibitors in *A. tuberculatus*

when lacking G210. This indicated that the substitution A212T in *E. indica* PPO1 as the possible mutation conferring resistance to PPO inhibitor oxadiazon. The cDNA of PPO1 and PPO2 in R1 and R2 biotypes were submitted to NCBI GenBank database as accession numbers: MK040460, MK040461, MN256107, MN256106, respectively.

2.3.4 The R-PPO1 Confers Resistance to Oxadiazon in hemG mutant *E. coli* Complementation Assay.

An *E. coli* functional assay using a mutant of the bacterial protoporphyrinogen IX oxidase-deficient, hemG, was implemented to compare the function of PPO1 from the R1, R2 and S biotypes in the presence of oxadiazon (Patzoldt et al., 2006). The SASX38 mutant strain can grow when supplemented with exogenous heme (hematin) or an alternative source of PPO. The SASX38 *E. coli* strain was transformed with plasmids expressing the PPO1 genes of R1, R2 and S. All the transformed SASX38 strains were able to grow on LB medium without being supplemented with hematin, while the non-transformed SASX38 strain (NT) was unable to grow unless supplied with exogenous hematin (Figure 2.6a), indicating that all the PPO1 genes encoded a functional protein. Five increasing oxadiazon concentrations (0, 10, 50, 100, and 200 μM) were evaluated, and the medium with 50 μM oxadiazon inhibited the growth of the *E. coli* transformed with the S PPO1 alleles, but the *E. coli* transformed with the PPO1 from the R1 and R2 *E. indica* alleles were able to grow at a concentration of up to 200 μM oxadiazon (Figure 2.6b). This is sufficient evidence to conclude that the R1 and R2 PPO1 remains functional in the presence of oxadiazon and the A212T mutation is the most likely mechanism of resistance to oxadiazon in R1 and R2 *E. indica* biotypes since it is the only common amino acid change in PPO1, which suggest that the single amino acid change in PPO1 causes resistance to oxadiazon.

2.3.5 *E. indica* PPO1 A212T is Highly Resistant to Oxadiazon but not to Saflufenacil, Sulfentrazone, Lactofen, Flumioxazin and Trifludimoxazin.

The above described greenhouse experiments, complementation assay and sequencing data suggest that the R1 and R2 alleles carry a mutation that endows resistance specifically to oxadiazon. Interestingly, both R1 and R2 alleles contain the A212T substitution. To test whether A212T in *E. indica* PPO1 is the main cause of the observed oxadiazon resistance, we used an in vitro activity assay to determine the inhibition potency (IC_{50}) of oxadiazon towards recombinant wild-type *E. indica* PPO1 and the mutant A212T PPO1 enzymes. In addition, to test whether A212T leads to cross-resistance to other PPO-inhibiting herbicides in vitro, the assay was also performed with other PPO-inhibitors: saflufenacil, sulfentrazone, lactofen, flumioxazin and trifludimoxazin.

Oxadiazon strongly inhibited wild-type *E. indica* PPO1 exhibiting the classical dose response curve and an IC_{50} of 2.47×10^{-8} M (Table 2.8, Figure 2.7). At the highest concentration of oxadiazon (1.00×10^{-5} M) more than 90 % of the enzyme activity was inhibited (Table 2.8). In contrast, the A212T mutant *E. indica* PPO1 was so poorly inhibited, that no IC_{50} could be determined ($>1.00 \times 10^{-5}$ M) (Table 2.8). At the highest concentration of oxadiazon only 16% of the A212T mutant *E. indica* PPO1 enzyme activity was inhibited. This result indicates that A212T confers high resistance to oxadiazon in vitro. Saflufenacil, lactofen, flumioxazin and trifludimoxazin strongly inhibited both the wild-type *E. indica* PPO1 and the A212T mutant *E. indica* PPO1 enzymes (Table 2.8). At the highest herbicide concentration, more than 90% of inhibition was achieved for both recombinant enzymes (Table 2.8). Sulfentrazone inhibition potency was slightly less towards the A212T mutant *E. indica* PPO1 when compared to the wild-type *E. indica* PPO1, exhibiting an IC_{50} of 1.87×10^{-6} M and 2.75×10^{-7} M, respectively. Among

the tested PPO-inhibiting herbicides trifludimoxazin and flumioxazin were the most potent towards the A212T mutant *E. indica* PPO1 (Table 2.8).

2.3.6 Computational Modeling of the A212T Mutation in PPO1.

An in-house high resolution X-ray crystal structure of A. tuberculatus PPO2 as a template to model the consequence of the A212T mutation in *E. indica* PPO1. The modeled oxadiazon binding pose fits well into the binding site of the *E. indica* PPO1 wild-type homology model, forming many favorable Van der Waals interactions. In the homology model with the A212T mutation, this was not the case. The resulting visualization (Figure 2.8) suggests the following hypothesis for A212T-induce resistance to oxadiazon. The threonine 212 can form a hydrogen bond with the carbonyl backbone of the neighboring tyrosine 211. This causes the C-gamma methyl group (-CH3) to be very restrained in an orientation facing the oxadiazon binding. Due to clashes between the threonine C-gamma methyl (-CH3) and tert-butyl group (−C4H9) of oxadiazon, thus the inhibitor, oxadiazon, is pushed out of the binding site (Figure 2.8). Therefore, these repulsive interactions reduce the strength of enzyme-ligand binding energy, weakening the inhibition of PPO1 by oxadiazon.

2.4 Discussion

De novo resistance to PPO inhibitors in species previously susceptible to PPO-inhibiting herbicides was not discovered until 2001. The first case of PPO inhibitors resistance was reported in A. tuberculatus from Kansas in 2001(Shoup et al., 2003). The first report of an annual grass resistant to the PPO-inhibitor oxadiazon was *E. indica* collected from golf courses in Virginia and North Carolina (McElroy et al., 2017). McElroy et al. (2017) previously tested the three different *E. indica* biotypes presented in this research with preemergence-applied oxadiazon. The two resistant *E. indica* biotypes were 7.9 and 3.0 times more resistant to

preemergence-applied oxadiazon than the susceptible biotype based on the I90 values, while both resistant biotypes were up to 20 times more resistant to postemergence applications (Table 2.2). This research confirms that resistant and susceptible biotypes can be separated via a postemergence screen, however the magnitude of response may differ in these two screening methods.

Such differences in preemergence and postemergence response to PPO-inhibiting herbicides has been observed in the past. Shoup and Al-Khatib (Shoup and Al-Khatib, 2004) stated that postemergence applications of acifluorfen and lactofen inhibited PPO-resistant A. tuberculatus 40%, while preemergence applications of sulfentrazone and flumioxazin inhibited the resistant population greater than 85%. Another case showed that preemergence applications of flumioxazin and sulfentrazone controlled a resistant A. tuberculatus biotype 90%; however, postemergence applications of other PPO inhibitors controlled less than 60% (Harder et al., 2012). Researchers have also noted that different resistant levels varies between different PPO inhibitor chemical families. Shoup et al. (2003) found that sulfentrazone slightly injured lactofen-resistant A. tuberculatus biotype. No difference between acifluorfen and lactofen was observed, which indicated that the selection pressure for the R biotype at the site was with acifluorfen or lactofen. In 2005, Patzoldt et al. found that a A. tuberculatus biotype with multiple resistance against herbicides with three mode of action had higher resistance to lactofen, and slight resistance to sulfentrazone and flumioxazin (Patzoldt et al., 2005).

PPO inhibiting herbicides encompass multiple chemically distinct families. Despite the chemical diversity of PPO inhibitors, they are largely assumed to inhibited PPO similar fashion (Dayan et al., 2014, Hao et al., 2011). Recent research has demonstrated variability in cross-resistance depending on the target-site mutation that occurs in PPO2, specifically ΔG210 induces

resistance to pyrimidinedione and diphenyl ether families, but lower resistance in thiadiazole and oxadiazole families (Rangani et al., 2019). Similarly, the recently reported G399A PPO2 mutation induced high level resistance in diphenyl ether and lower level resistance in pyrimidinedione and triazolinone (Rangani et al., 2019). Recent research on the new PPO inhibiting herbicide trifludimoxazin indicated variability in PPO2 inhibition depending on the target site change at ΔG210 or R128 (Lillie et al., 2019).

Mitochondrial-targeted PPO2 from *Nicotiana tabacum* (mtPPO2) has three domains: a FAD-binding domain, a membrane-binding domain and a substrate-binding domain (Koch et al., 2004). The homology similar amino acid sequence indicates that the crystal structure of PPO1 would resemble the structure of PPO2 in higher plants. Based on the crystal structural of mtPPO2, Gly210 is not located in the catalytic site, however, it plays an important role in stabilizing the α-8 helix of PPO2 (Koch et al., 2004, Dayan et al., 2010). The deletion of Gly210 destabilizes the α-8 helix capping region and unravels the last turn of the helix, which causes ~ 50% magnification of the catalytic site cavity (Dayan et al., 2010). This enlargement of the substrate binding pocket would reduce the affinity of PPO inhibitors for PPO2. The substitution R128L in PPO2 caused cross-resistance because R128 is in the pocket between the substrate entryway and the herbicide active site (Hao et al., 2013, Hao et al., 2014, Dayan et al., 2018), which is essential for stabilizing the substrate in the catalytic domain. Computational modeling indicated that the mutation of R128G removed the important hydrogen-bonding interactions with acifluorfen and sulfentrazone, reducing the binding of these PPO inhibitors (Hao et al., 2013). Another mutations at same site, the replacement of arginine to a hydrophobic leucine inducing a radical change in the enzyme conformation. However, this mutation R128L/G does not impact resistance to oxadiazole (Rangani et al., 2019). A reason is that there is no hydrogen bond

formed between R128 with oxadiazole, inducing no effect on oxadiazole and plants remains sensitive to this herbicide chemical family. The computational modeling on the recently discovered mutation G399A in PPO2 in *A. palmeri*, conferring resistance to diphenyl ether and N-phenylphthalimide, indicated that the additional methyl group of Ala399 makes the binding pocket smaller, reducing the magnitude of the enzyme-ligand binding energy and weakening the inhibition of the PPO2 activity (Rangani et al., 2019).

The novel mutation A212T in PPO1 confers specific resistance to the PPO inhibitor oxadiazon, while causes no cross-resistance to other herbicides evaluated. A212T is a simple substitution and has never been reported neither in PPO1 nor in PPO2 in other PPO inhibitor resistant species. It is a novel mutation which inhibits herbicides through the conjugate exclusion, in spite of being in the catalytic domain, compared with the current reported mutations. Based on the discovery of a new target-site mutation that confers resistance to oxadiazon the authors would like to pose a question -- if PPO-inhibiting herbicides dually target both PPO1 and PPO2, why have all mutations reported until this point been only associated with PPO2? Further, why did A212T mutation in PPO1 impact resistance in oxadiazon only and not the other PPO inhibitors? In our opinion, questions remain regarding single or dual inhibition of chloroplast-targeted PPO1 and mitochondrial-targeted PPO2 by PPO inhibitors. In Amaranthus species, the PPO2 is dual targeted to chloroplast and mitochondria, which suggested that the current mutations reported in PPO2 can protect PPO activity not only in the mitochondria, but also in the chloroplast (Patzoldt et al., 2006). Nonetheless, the computational modeling of previous reported mutation sites in PPO2 and our novel mutation A212T in PPO1 suggest the hypotheses that not all the PPO-inhibiting herbicides are acting the same (at the same site of action or with the same affinity) and some PPO-inhibiting herbicides may have preferences for

one or the other target isoform are worthy of future exploration. Such differences could be attributed to translocation differences between structurally different herbicide chemistries or differences in affinity for the PPO isoforms. These hypotheses will be the focus of future research.

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Table 1

Table 2.1 List of PPO1 and PPO2 in other species and accession numbers downloaded from NCBI database.

Abbreviations	Scientific Name	Gene Name	NCBI accession number
At_PPO1	*Arabidopsis thaliana*	*PPO1*	NP_192078
Nt_PPO1	*Nicotiana tabacum*	*PPO1*	BAA34713
Si_PPO1	*Setaria italica*	*PPO1*	XP_004967639
Sb_PPO1	*Sorghum bicolor*	*PPO1*	XP_002455484
AmT_PPO1	*Amaranthus tuberculatus*	*PPO1*	ABD52324
At_PPO2	*Arabidopsis thaliana*	*PPO2*	NP_001190307
AmT__r_PPO2	*Amaranthus tuberculatus* (resistant)	*PPO2*	ABD52328
AmT__s_PPO2	*Amaranthus tuberculatus* (susceptible)	*PPO2*	ABD52326
Si_PPO2	*Setaria italica*	*PPO2*	XP_004976030
Sb_PPO2	*Sorghum bicolor*	*PPO2*	XP_002446710
Ap_PPO2	*Amaranthus palmeri*	*PPO2*	ATE88443
St_PPO2	*Solanum tuberosum*	*PPO2*	XP_006356026
Gm_PPO2	*Glycine max*	*PPO2*	NP_001236376
Nt_PPO2	*Nicotiana tabacum*	*PPO2*	NP_001312887

Table 2

Table 2.2 Predictive model with sigmoidal equation for percent injury in response to increasing rates of four different PPO inhibitors relative to a non-treated control within three *E. indica* biotypes. Parameter estimate and parameter estimate 95% confidence interval (CI) are presented as means of model comparison.

Herbicides	Biotypes [a]	Equation[b]	R^2	Parameter estimates and confidence interval					
		y = a/(1+exp(-(x-x0)/b))		a	95% CI	b	95% CI	x0	95% CI
Oxadiazon	S	y = 91.25/(1+exp(-(x-(-0.54))/0.25))	0.97	91.25	（81.65, 100.65）	0.25	(0.13, 0.37)	-0.54	(-0.86, -0.41)
	R1	y = 9925.40/(1+exp(-(x-3.75)/0.53))	0.99	9925.40	(9747.04, 10103.76)	0.53	(0.26, 0.80)	3.75	(-93.36, 100.87)
	R2	y = 64371.40/(1+exp(-(x-3.99)/0.43))	0.99	64371.40	(64167.56, 64575.24)	0.43	(0.15,0.70)	3.99	(-590.17, 598.15)
Sulfentrazone	S	y = 98.21/(1+exp(-(x-(-1.10))/0.16))	0.98	98.21	(91.88, 104.55)	0.16	(0.09, 0.23)	-1.10	(-1.18, -1.02)
	R1	y = 95.12/(1+exp(-(x-(-0.84))/0.12))	0.99	95.12	(91.13, 99.10)	0.12	(0.08, 0.16)	-0.84	(-1.03, -0.65)
	R2	y = 100.90/(1+exp(-(x-(-0.73))/0.25))	0.98	100.90	(92.45, 109.34)	0.25	(0.16, 0.33)	-0.73	(-0.83, -0.63)
Flumioxazin	S	y = 98.00/(1+exp(-(x-(-1.09))/0.02))	0.99	98.00	(95.41, 100.59)	0.02	(-0.11, 0.14)	-1.09	(-1.28, -0.90)
	R1	y = 96.53/(1+exp(-(x-(-1.11))/0.04))	0.99	96.53	(93.75, 99.31)	0.04	(-0.08, 0.17)	-1.11	(-1.30, -0.92)
	R2	y = 96.81/(1+exp(-(x-(-1.11))/0.04))	0.99	96.81	(94.23, 99.39)	0.04	(-0.16, 0.23)	-1.11	(-1.41, -0.81)
Lactofen	S	y = 90.29/(1+exp(-(x-(-1.52))/0.13))	0.97	90.29	(84.29, 96.29)	0.13	(0.06, 0.20)	-1.52	(-1.60, -1.45)
	R1	y = 84.65/(1+exp(-(x-(-1.44))/0.29))	0.86	84.65	(68.30, 101.00)	0.29	(0.02, 0.57)	-1.44	(-1.71, -1.17)
	R2	y = 89.20/(1+exp(-(x-(-1.31))/0.30))	0.95	89.20	(77.82, 100.58)	0.30	(0.14, 0.46)	-1.31	(-1.48, -1.14)

[a] biotype abbreviations: S, known oxadiazon susceptible *E. indica* wild-type; R1, oxadiazon resistant *E. indica* biotype from Country Club of Virginia, Richmond, VA; R2, oxadiazon resistant *E. indica* biotype from River Bend Golf Course, New Bern, NC.

[b] In the sigmoidal equation, x is herbicide rate and y in the response variable of percent injury relative to the non-treated control.

Table 3

Table 2.3 Estimated rate of different PPO inhibiting herbicides required to reduce *E. indica* biotype by 50% (I_{50}) and 90% (I_{90}) based on the injury scores collected 14 days after treatment. 95% confidence intervals (CI) at I_{50} and I_{90} values are provided as means of comparison.

Herbicides	Biotype[a]	Visual Control/Injury [b]			
		I_{50} (kg ha^{-1})	95% CI	I_{90} (kg ha^{-1})	95% CI
Oxadiazon	S	0.32	(0.05, 1.18)	1.56	(1.13, 3.24)
	R1	8.15	(7.18, 9.12)	14.60	(13.63, 15.57)
	R2	8.88	(8.13, 9.64)	18.29	(17.53, 19.05)
Sulfentrazone	S	0.08	(0.00, 0.34)	0.19	(0.00, 0.45)
	R1	0.15	(0.00, 0.44)	0.32	(0.03, 0.61)
	R2	0.18	(0.00, 0.51)	0.63	(0.31, 0.95)
Flumioxazin	S	0.08	(0.04, 0.12)	0.09	(0.04, 0.13)
	R1	0.08	(0.04, 0.12)	0.10	(0.06, 0.14)
	R2	0.08	(0.04, 0.13)	0.10	(0.05, 0.15)
Lactofen	S	0.03	(0.00, 0.12)	0.17	(0.08, 0.26)
	R1	0.04	(0.00, 0.18)	0.51	(0.36, 0.66)
	R2	0.06	(0.00, 0.21)	0.50	(0.35, 0.65)

[a]biotype abbreviations: S, known oxadiazon susceptible *E. indica* wild-type; R1, oxadiazon resistant *E. indica* biotype from Country Club of Virginia, Richmond, VA; R2, oxadiazon resistant *E. indica* biotype from River Bend Golf Course, New Bern, NC.

[b] I50 and I90 values of the percent injury were calculated based on sigmoidal fit equation.

Table 4

Table 2.4 The reads and gene assembly of PPO1 and PPO2 of the RNA datasets of *E. indica* biotypes.

Biotype[a]	Reads	Trimmed Read	Trimming	*PPO1*[b]					*PPO2*[b]				
				Reads Extractions		Exons	Length	Amino Acid	Reads Extraction		Exons	Length	Amino Acid
S	136513042	113353352	83.03%	——	——	——	——	——	——	——	——	——	——
R1	38990558	33333028	85.49%	111352	0.33%	9	3816 bp	529	8864	0.02%	17	6043 bp	511
R2	59063288	51089744	86.50%	105520	0.21%	——	——	——	7216	0.01%	——	——	——

[a] biotype abbreviations: S, known oxadiazon susceptible *E. indica* wild-type; R1, oxadiazon resistant *E. indica* biotype from Country Club of Virginia, Richmond, VA; R2, oxadiazon resistant *E. indica* biotype from River Bend Golf Course, New Bern, NC.
b PPO1, chloroplast-targeted PPO gene, PPO2, mitochondria-targeted PPO gene.

Table 5

Table 2.5 The structure of the goosegrass chloroplast-targeted PPO1 gene for the exons and introns based on the mapping results analysis.

Exon[a]	Location	Size (bp)	% (G+C)	Intron	Location	Size (bp)	% (G+C)	5' donor & 3' acceptor seq
exon1	1- 354	354	74.85	intron1	355-435	81	54.32	CC/GTACGC…….GCAG/GT
exon2	436-621	186	67.20	intron2	622-783	162	44.09	CA/GTTCGT…….TCAG/GG
exon3	784-868	85	62.35	intron3	869-963	95	33.68	AG/GTGCTT…….TAAG/GT
exon4	964-1111	148	47.30	intron4	1112-1183	72	31.94	CC/GTAAGA…….CCAG/CC
exon5	1184-1264	81	54.32	intron5	1265-1690	426	34.74	AG/GTTTAT…….ACAG/GT
exon6	1691-1862	172	43.02	intron6	1863-2930	1068	35.54	CA/GTAAGT…….ACAG/AG
exon7	2931-3090	160	45.00	intron7	3091-3236	146	26.71	AG/GTAAAT…….AAAG/GA
exon8	3237-3340	104	45.19	intron8	3341-3516	176	33.52	AG/GTTCTA…….GCAG/AG
exon9	3517-3816	300	48.67					

[a] The exons begin at the start codon, and end at the stop codon.

Table 6

Table 2.6 The structure of the goosegrass mitochondria-targeted PPO2 gene for the exons and introns based on the mapping results analysis.

Exon[a]	Location	Size (bp)	% (G+C)	Intron	Location	Size (bp)	% (G+C)	5' donor & 3' acceptor seq
exon 1	1-68	68	79.41	intron 1	69-217	149	58.39	AG/GTGAGT…….GCAG/TG
exon 2	218-353	136	64.71	intron 2	354-681	328	42.99	TG/GTGAGC…….GCAG/AC
exon 3	682-747	66	40.91	intron 3	748-850	103	34.95	AT/GTATGT…….TCAG/CC
exon 4	851-901	51	47.06	intron 4	902-1005	104	36.54	TG/GTAATA…….TCAG/AT
exon 5	1006-1062	57	31.58	intron 5	1063-1395	333	31.53	AG/GTATGT…….GCAG/TT
exon 6	1396-1481	86	36.05	intron 6	1482-1828	347	35.16	AG/GTGAGT…….GCAG/TG
exon 7	1829-1865	37	48.65	intron 7	1866-1983	118	29.66	AG/GTGAGT…….ACAG/GT
exon 8	1984-2049	66	42.42	intron 8	2050-2218	169	37.87	CT/GTGAGT…….GCAG/AT
exon 9	2219-2259	41	36.59	intron 9	2260-2534	275	28.36	AA/GTAAGT…….CCAG/GT
exon 10	2535-2667	133	42.86	intron 10	2668-4322	1655	40.06	AG/GTAACC…….TCAG/TC
exon 11	4323-4511	189	42.33	intron 11	4512-4605	94	37.23	CA/GTAAGG…….TCAG/GC
exon 12	4606-4680	75	41.33	intron 12	4681-4907	227	33.04	AG/GTCAGG…….GCAG/GT
exon 13	4908-5034	127	45.67	intron 13	5035-5204	170	36.47	TG/GTAGGT…….ATAG/GT
exon 14	5205-5313	109	45.87	intron 14	5314-5392	79	29.11	AC/GTATAT…….GCAG/GA
exon 15	5393-5467	75	41.33	intron 15	5458-5618	151	34.44	AA/GTAAGT…….TCAG/GC
exon 16	5619-5725	107	49.53	intron 16	5726-5930	205	37.56	AG/GCAAGC…….ATAG/GA
exon 17	5931-6043	113	45.13					

Table 7

Table 2.7 Identification of nucleotide polymorphisms using read mapping to assembled chloroplast PPO1 contigs of both resistant and susceptible *E. indica* biotypes.

Nucleotide position	Amino acid position	S*		R1*		R2*	
		Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
634	212	G	Ala	A	Thr	A	Thr
847	283	A	Thr	A	Thr	G	Ala
1097	366	A	Lys	A	Lys	T	Met

Table 8

Table 2.8 Effects of PPO inhibitors on in vitro enzyme activity of recombinant *E. indica* wild-type PPO1 and variant A212T PPO1 enzyme.

PPO chemical family	Herbicides	Wild-type *E. indica* PPO1 sensitivity [IC50] (M)	Variant *E. indica* PPO1 A212T sensitivity [IC50] (M)	Wild-type *E. indica* PPO1 % inhibition†	Variant *E. indica* PPO1 A212T % inhibition†
Oxadiazoles	Oxadiazon	2.47×10-8	>1.00×10-5	>90%	16%
Triazolinones	Sulfentrazone	2.75×10-7	1.87×10-6	>90%	76%
Pyrimidinediones	Saflufenacil	1.94×10-8	4.49×10-7	>90%	>90%
Diphenyl ethers	Lactofen	2.64×10-8	3.62×10-7	>90%	>90%
N-Phenyl-phthalimides	Flumioxazin	1.67×10-8	1.34×10-8	>90%	>90%
	Trifludomoxazin	1.71×10-8	2.65×10-8	>90%	>90%

† %inhibition with the rate of the highest herbicide concentration 10^{-5}M.

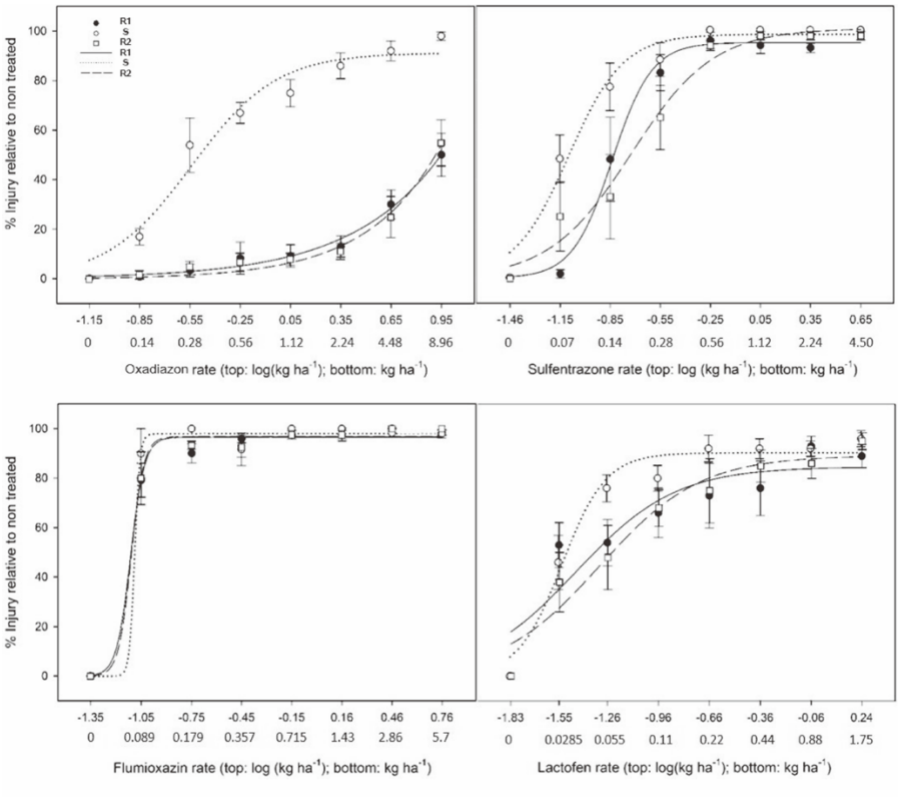


Figure 2.1 Percent injury response relative to the nontreated control of three *E. indica* biotypes 14 days after treatment with increasing rates of four different PPO inhibitors. Response was modeled based on the log rate of the herbicides to create equal spacing between rates using sigmoidal regression of percent injury relative to the nontreated control. Non-log transformed herbicide rates are presented for reference. Means are represented by differing symbols for each biotype and regression equation models are represented by differing line type for each biotype. Vertical bars represent standard errors (P = 0.05). *E. indica* biotype abbreviations: S, known susceptible wild-type ; R1, oxadiazon resistant biotype from Country Club of Virginia, Richmond, VA; R2, oxadiazon resistant biotype from River Bend Golf Course, New Bern, NC.

Figure 1

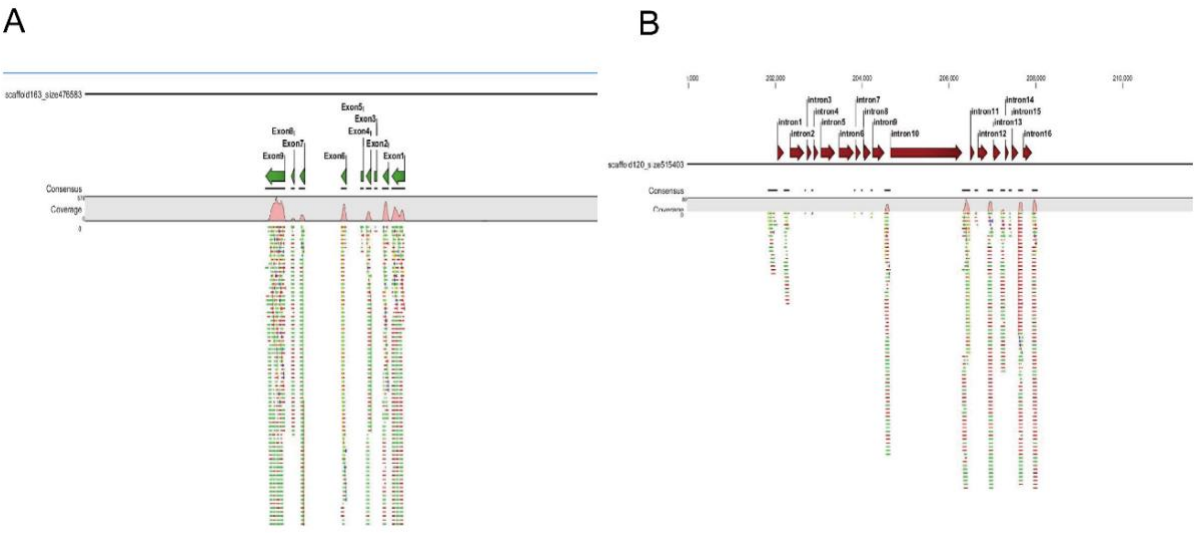
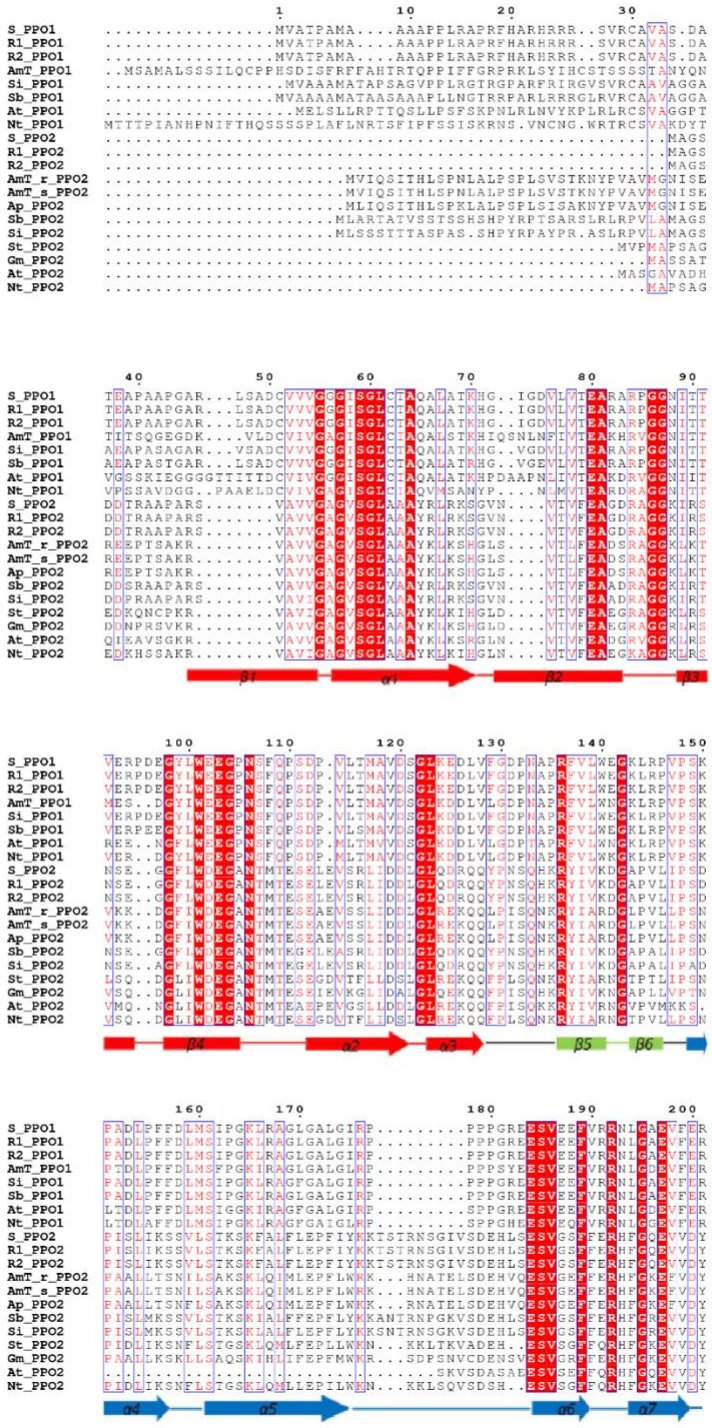


Figure 2.2. Reads mapping of *E. indica* PPO1 and PPO2 gene referenced with genome DNA scaffold.**(A)** Reads extraction of chloroplast-targeted PPO1. The annotated bar showed the exons numbers and locations. **(B)** Reads extraction of mitochondrial-targeted PPO2. The annotated bar showed the introns numbers and locations

Figure 2


S_PPO1
R1_PPO1
R2_PPO1
AmT_PPO1
Si_PPO1
Sb_PPO1
At_PPO1
Nt_PPO1
S_PPO2
R1_PPO2
R2_PPO2
AmT_r_PPO2
AmT_s_PPO2
Ap_PPO2
Sb_PPO2
Si_PPO2
St_PPO2
Gm_PPO2
At_PPO2
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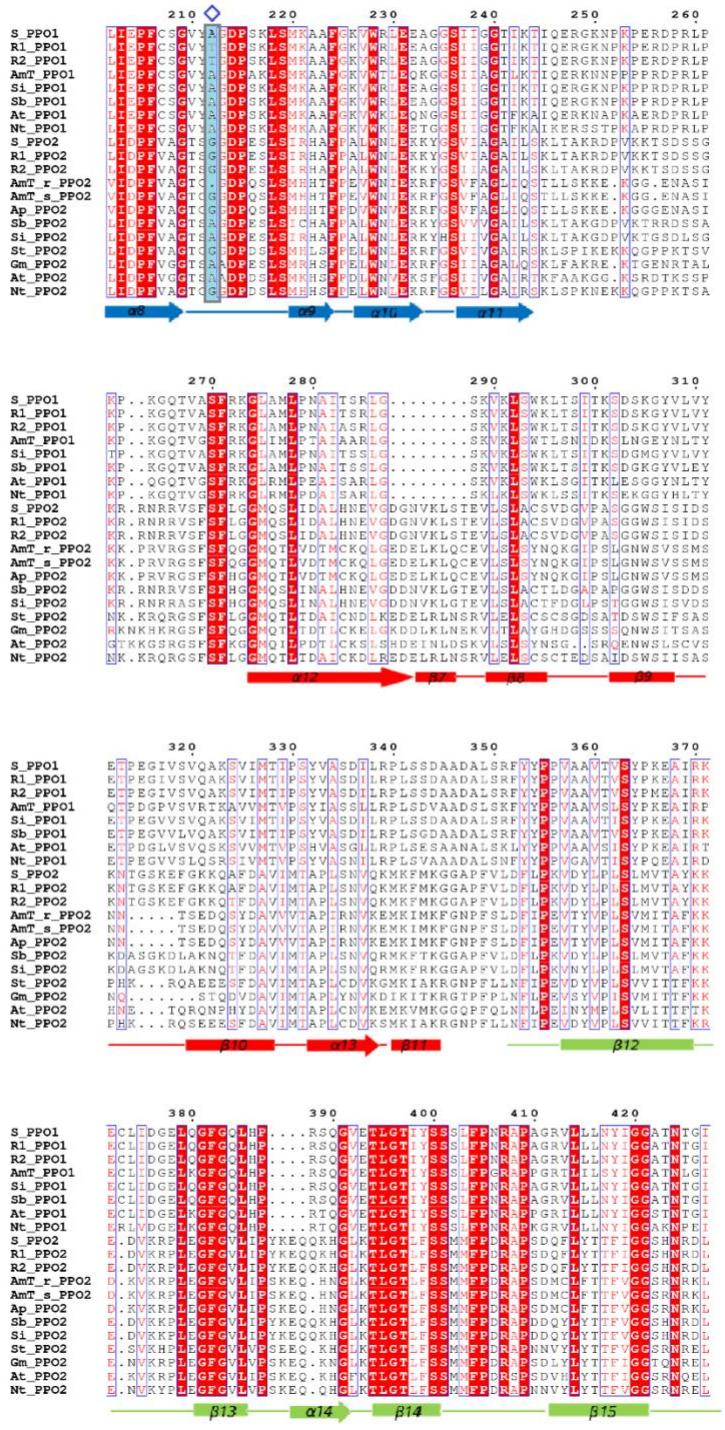
α3
β5
β6
α4
α5
α6
α7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R1_PPO1
R2_PPO1
AmT_PPO1
Si_PPO1
Sb_PPO1
At_PPO1
Nt_PPO1
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R1_PPO2
R2_PPO2
AmT_r_PPO2
AmT_s_PPO2
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Gm_PPO2
At_PPO2
Nt_PPO2

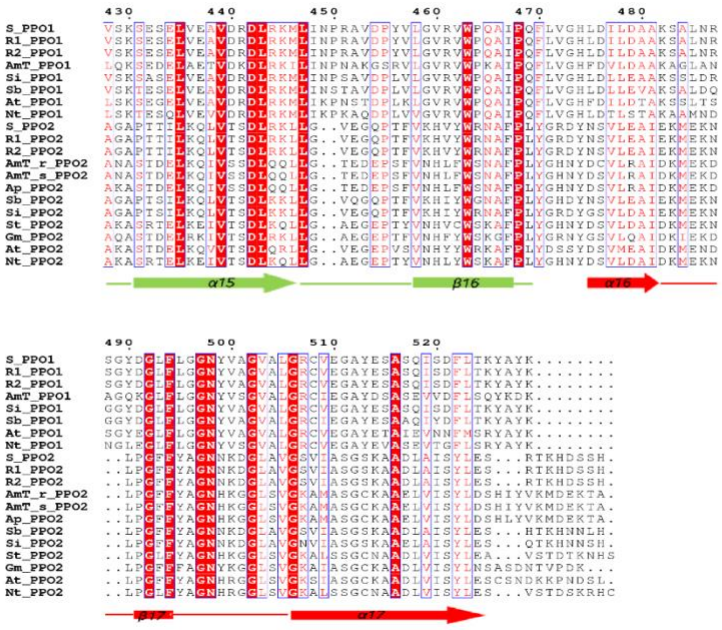


Figure 2.3. Protein alignment with other species of PPO1 and PPO2.The protein structure on the bottom is modeled as the template of mitochondrial PPO2 in Nicotiana tabacum (mtPPO2, PDB entry: 1SEZ). The ‘right arrow’ showed the α-helix secondary structure, the ‘rectangle’ showed the β-sheet secondary structure. The red color showed the FAD binding domain, the green color showed the substrate-binding domain, the blue color showed the membrane-binding domain. The codon containing PPO1 A212T substitution is indicated by a blue rhombus. Abbreviations: S, known susceptible *E. indica* wild-type, R1, oxadiazon resistant *E. indica* biotype from Country Club of Virginia, Richmond, VA; R2, oxadiazon resistant *E. indica* biotype from River Bend Golf Course, New Bern, NC. PPO1

Figure 3

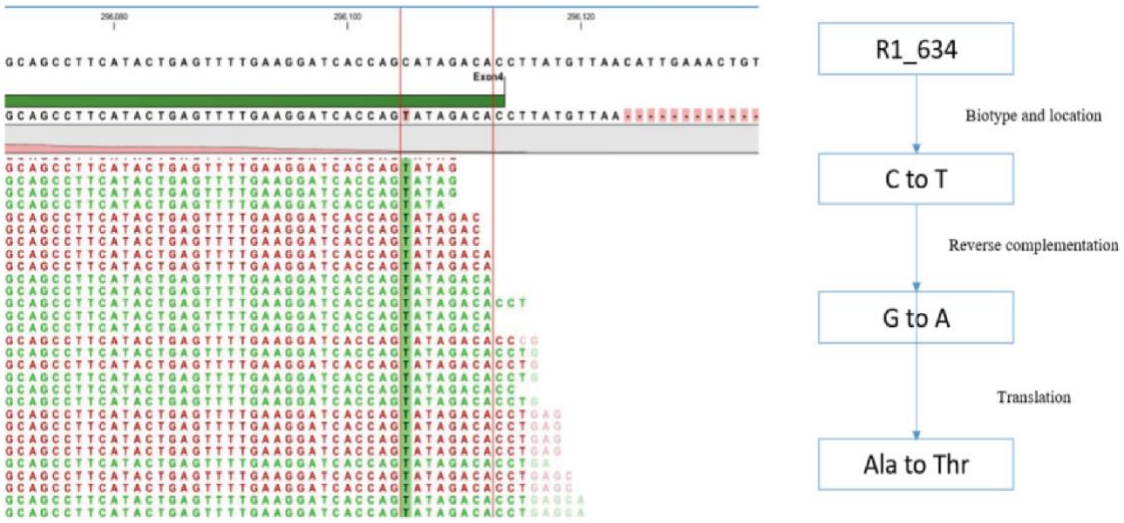


Figure 2.4 Reads mapping to the mutation site PPO1 A212T in the R1 *E. indica* biotype referenced with genome DNA scaffold. The reads showed the nucleotide in transcriptome reads at position 634 is thymine, while in genome DNA is cytosine. When translated to amino acid, in R1 *E. indica* biotype is threonine 212, while alanine 212 in S *E. indica* biotype.

Figure 4

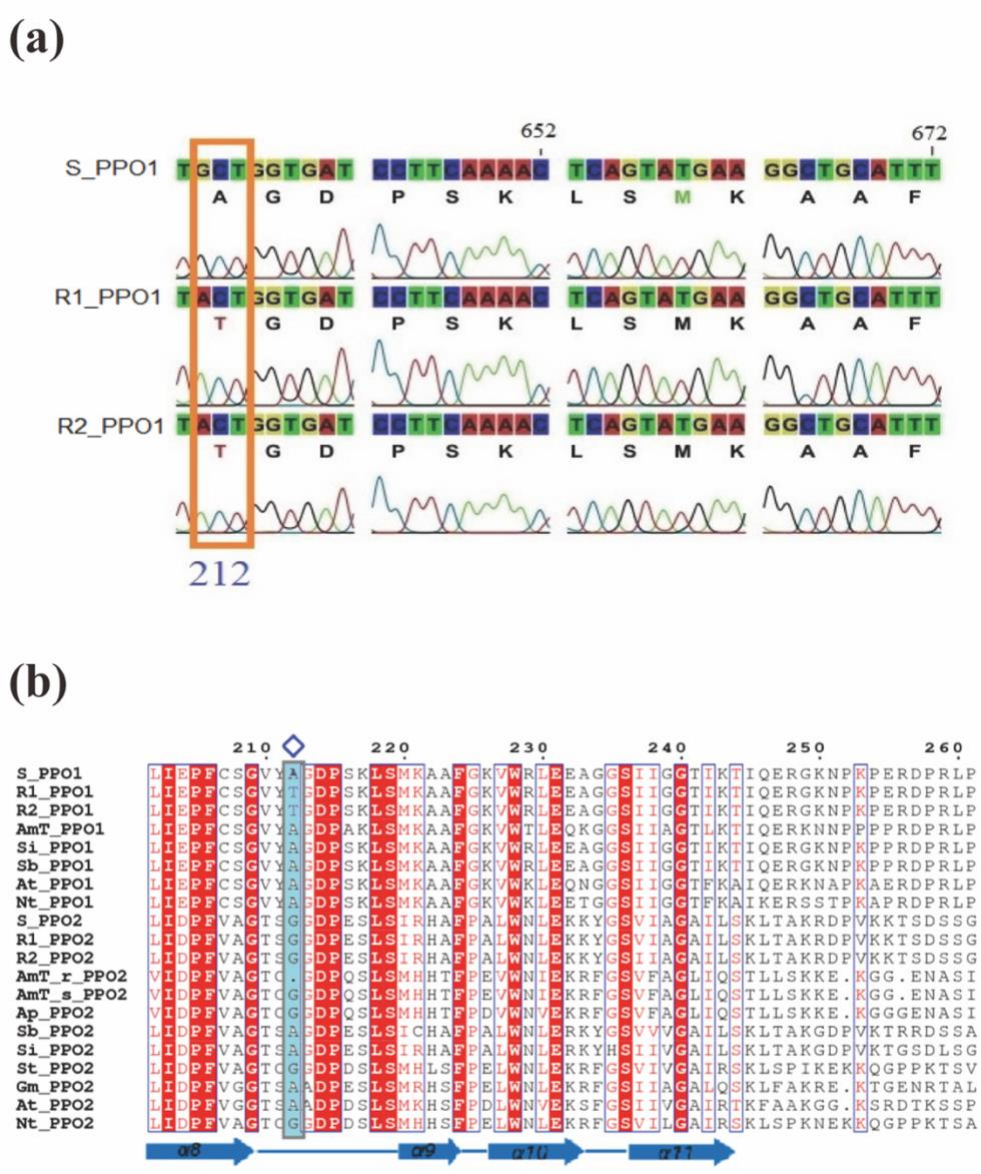


Figure 2.5 Protein alignment of PPO1 and PPO2.

(a) Protein alignment of PPO1 among three *E. indica* biotypes. The codon containing the single nucleotide polymorphism for the A212T mutation is depicted in the orange rectangle. **(b)** Amino acid sequence conservation of PPO1 and PPO2 in plant species (partial). A highly conserved amino acid residual at position 212 in PPO1 is indicated by a blue rhombus.

Figure 5

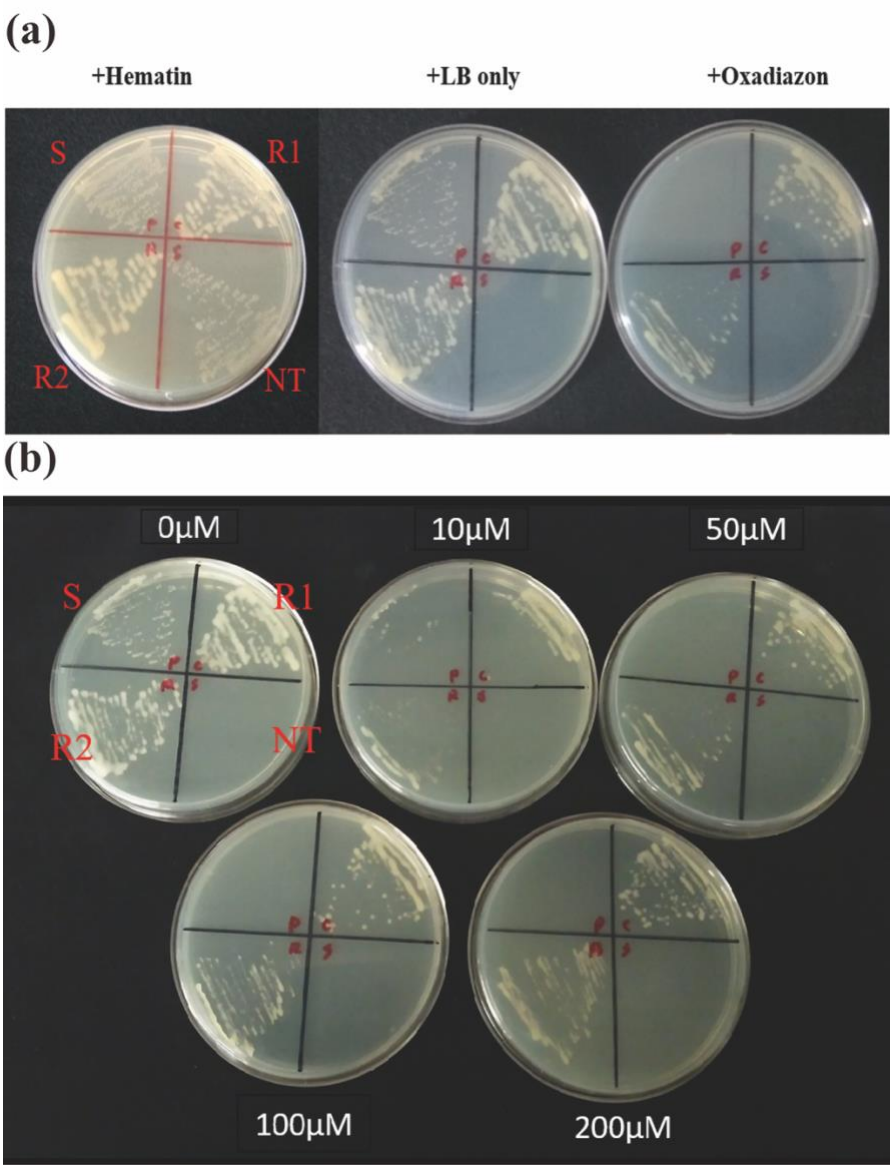


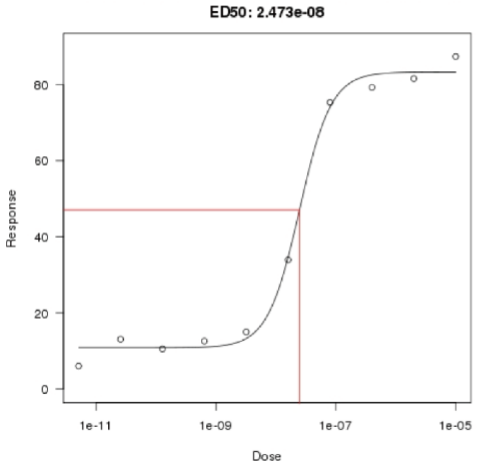
Figure 2.6 Protein expression of PPO1 alleles from three goosegrass biotypes transformed with the hemG mutant *E. coli* strain SASX38.

(A) The transformed and non-transformed *E. coli* strain were grown on LB media alone, or supplemented with hematin (10 μg·mL^{-1}), or in presence of oxadiazon (50 μM). Only R1 and R2 PPO1 alleles with A212T grow on media with oxadiazon, all alleles function for heme biosynthesis, and all grow on hematin supplemented media. **(B)** The transformed and non-

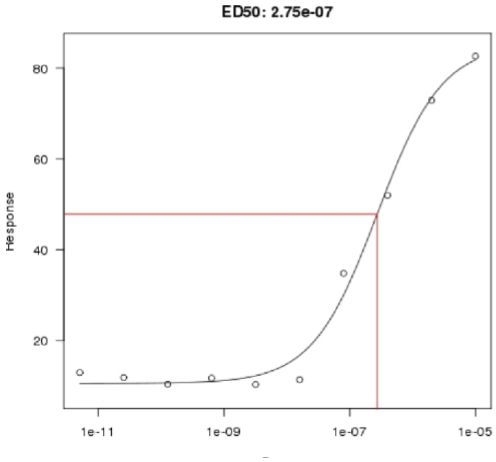
transformed *E. coli* strain were grown on LB media containing with increasing concentration oxadiazon (0, 10, 50, 100, 200 μM). All the transformed *E. coli* strain (S, R1, R2) can grow on the media with lower concentration oxadiazon (10 μM), while only R1 and R2 alleles with A212T grow on media with the highest concentration oxadiazon (200 μM). Abbreviations: NT: non-transformed hemG mutant *E. coli* strain SASX38; S: hemG mutant *E. coli* strain SASX38 transformed with a vector encoding S *E. indica* biotype PPO1 with Ala212; R1: hemG mutant *E. coli* strain SASX38 transformed with a vector encoding R1 *E. indica* biotype PPO1 with A212T; R2: hemG mutant *E. coli* strain SASX38 transformed with a vector encoding R2 *E. indica* biotype PPO1 with A212T.

Figure 6

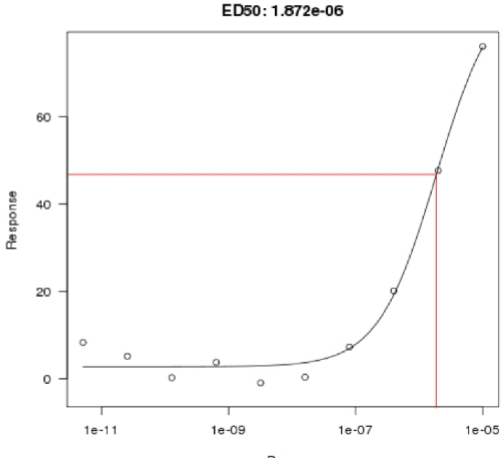
A.1. IC50 Wildtype *E. indica* PPO1 for Oxadiazon



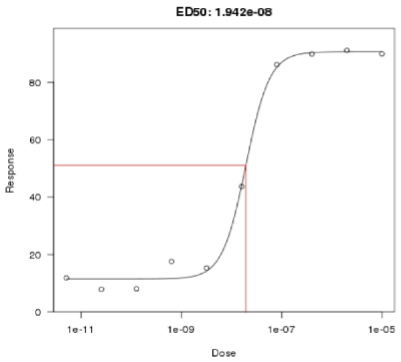
B.1. IC50 Wildtype *E. indica* PPO1 for Sulfentrazone



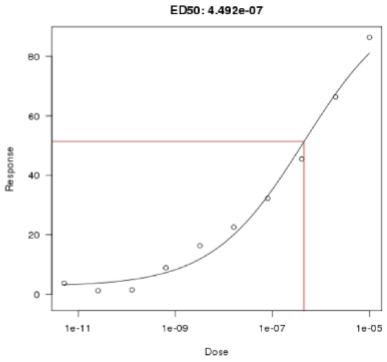
B.2. IC50 Variant *E. indica* PPO1 A212T for Sulfentrazone



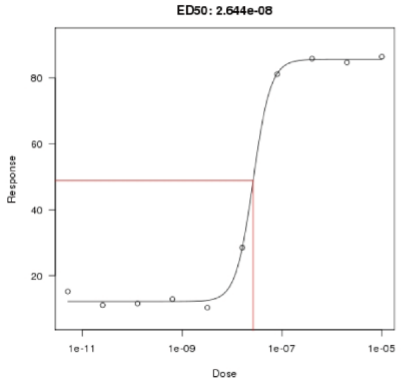
C.1. IC50 Wildtype *E. indica* PPO1 for Saflufenacil



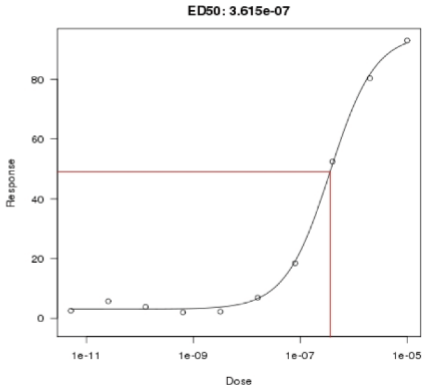
C.2. IC50 Variant *E. indica* PPO1 A212T for Saflufenacil



D.1. IC50 Wildtype *E. indica* PPO1 for Lactofen



D.2. IC50 Variant *E. indica* PPO1 A212T for Lactofen



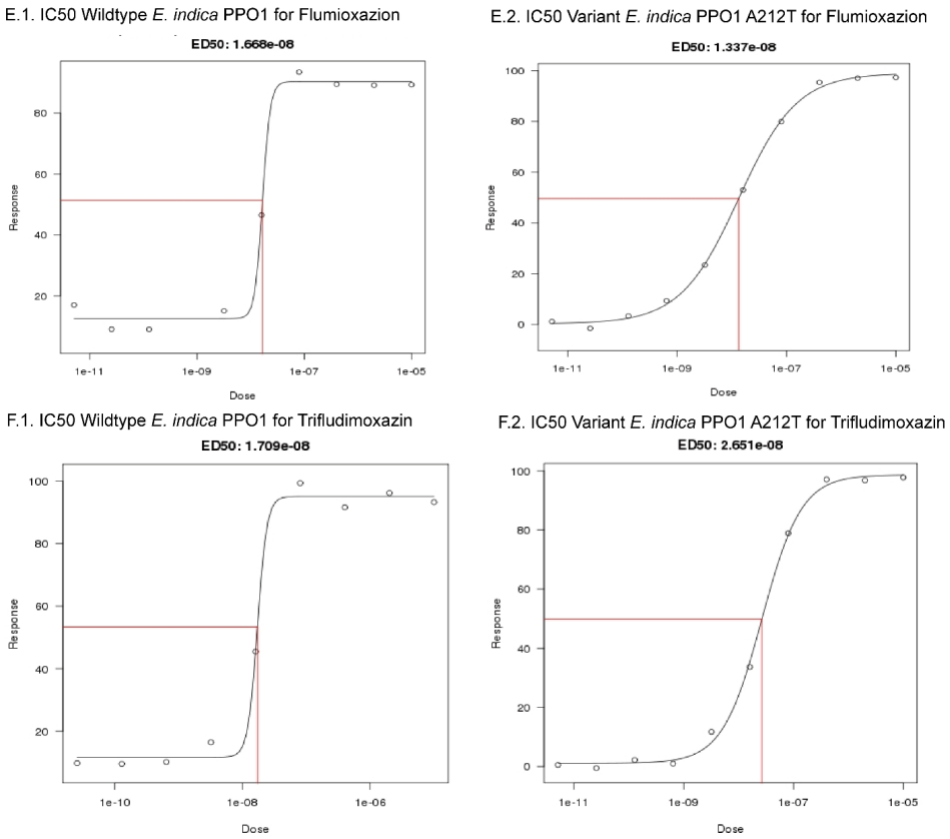


Figure 2.7 Percent effects of PPO inhibitors on in vitro enzyme activity of *E. indica* wild-type PPO1 and PPO1 A212T variants.

Six different PPO inhibitors belong to five different structurally unrelated chemical families. The unit of dose rate is mole (M). ***(A.1)*** IC_{50} wild-type *E. indica PPO1* for oxadiazon, while no IC_{50} results for variant *E. indica PPO1* A212T because of completely resistant. *(B.1)* IC_{50} wild-type *E. indica PPO1* for sulfentrazone; ***(B.2)*** IC_{50} variant *E. indica PPO1* A212T for sulfentrazone**.** ***(C.1)*** IC_{50} wild-type *E. indica PPO1* for saflufenacil; ***(C.2)*** IC_{50} variant *E. indica PPO1* A212T for saflufenacil**.** ***(D.1)*** IC_{50} wild-type *E. indica PPO1* for lactofen; ***(D.2)*** IC_{50} variant *E. indica PPO1* A212T for lactofen**.** ***(E.1)*** IC_{50} wild-type *E. indica PPO1* for flumioxazin; *(E.2)* IC_{50}

variant *E. indica PPO1* A212T for flumioxazin. *(F.1)* IC_{50} wild-type *E. indica PPO1* for trifludimoxazin; ***(F.2)*** IC_{50} variant *E. indica PPO1* A212T for trifludimoxazin.

Figure 7

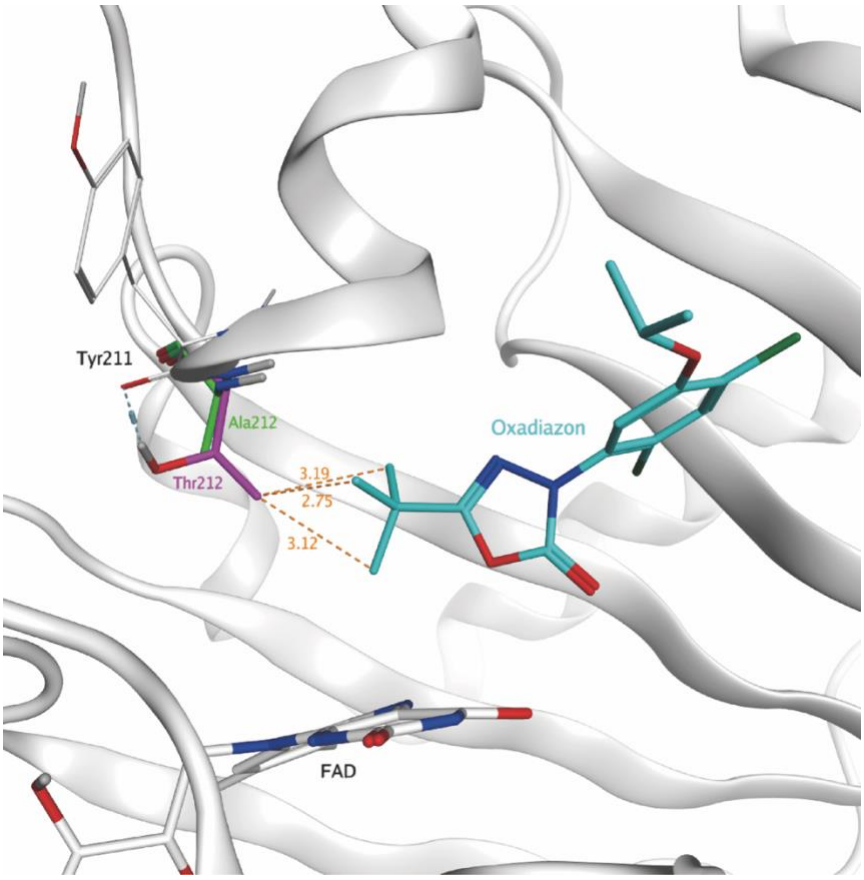


Figure 2.8 Position of the A212T mutation relative to the predicted binding mode of oxadiazon. Oxadiazon (cyan sticks) was modeled into the binding-site of the homology model (gray with secondary structure elements in cartoon style) of E. indica PPO1 protein. Alanine 212 (depicted as green sticks) is present in the wild type model, Threonine 212 (magenta sticks) is present in A212T mutation model. The hydroxyl group of Thr212 can form an intramolecular hydrogen bond to the carbonyl backbone of Tyrosine 211. As a result, it creates close several steric contacts (2.75Å-3.19Å depicted as orange dashed lines) with the modeled ligand oxadiazon. The FAD cofactor (gray) is partially visible. Other amino acid side chains are not shown to improve clarity.

Figure 8

Chapter III. Detection and Improved Identification of A212T as the Primary Mechanism of Resistance to Oxadiazon in goosegrass (*Eleusine indica*)

Abstract: Goosegrass *(Eleusine indica* L.) is one of the most problematic weeds in turfgrass and cropping systems. Oxadiazon, a protoporphyrinogen oxidase (PPO) inhibiting herbicides, is an effective preemergence treatment for goosegrass control and eventual resistance has developed. An A212T substitution in PPO1 was recently identified as a causal mechanism of oxadiazon resistance in goosegrass. Research was conducted to evaluate new populations for resistance to oxadiazon, determine if resistance can be detected using chlorophyll florescence, and develop a dCAPs assay for detecting the A212T mutation. 21 new suspected resistant populations were submitted to the Herbicide Resistance Diagnostic Lab at Auburn University with suspected resistance to oxadiazon. Plants treated with oxadiazon 1.12 kg ha^{-1} were rated on a binary scale of resistant (R) or susceptible (S) in comparison to previously diagnosed R and S biotypes. Eleven of the 21 suspected R biotypes were diagnosed as R based on postemergence screen. PCR sequencing results showed that there are 9 new resistant populations encoded a nonsynonymous SNP resulting in A212T amino acid substitution in PPO1, except for one population. A dCAPs primer effectively detected the A212T mutation of all populations containing the mutation without sequencing. F_v/F_m was evaluated as a diagnostic tool for fast detecting oxadiazon resistance. I_{50} values of F_v/F_m at 72 h after treatment were 0.62, 9.15 and 3.33 kg ha^{-1} oxadiazon for PBU (known susceptible), RB (known resistant) and Currituck (suspected resistant), respectively, indicating the newly diagnosed resistant population Currituck could be diagnosed with chlorophyll florescence; however, unknown variation exists between the two resistant populations. No other reported mutations occurred either in PPO1 or PPO2 of any populations. The target-site mutation A212T in PPO1 have been further substantiated as the primary

mechanism of oxadiazon resistance. However, this does not preclude other non-target site mechanisms conferring resistance to oxadiazon in Sandestin or in future populations.

Nomenclature: goosegrass, *Eleusine indica* L.; oxadiazon

Keywords: goosegrass, leaf chlorophyll fluorescense, dCAPs, PPO1, oxadiazon

3.1 Introduction

Goosegrass (*Eleusine indica* (L.) Gaertn.) is a summer grassy weed species in turfgrass and agricultural crops and is considered one of the five most problematic weeds in the world (Bryson and DeFelice, 2009; McCarty, 2001). Goosegrass has C4 carboxylation and is usually considered as annual, but can perennialize in tropical conditions (Buker et al., 2002). Goosegrass control is difficult because of the wide spread of herbicide resistance. According to the International Survey of Herbicide Resistant Weeds, a total of 36 goosegrass herbicide resistance cases have been reported, which are from 12 countries and 10 US states, across 8 unique mode of actions, and in over 15 different cropping systems (Heap 2020).

Oxadiazon is a protoporphyrinogen oxidase (PPO) inhibiting herbicide belongs to the oxadiazole chemical family, mainly applied as a preemergence to control of annual grasses such as goosegrass, and broadleaves such as green carpetweed (*Mollugo verticillata* L.) (Derr, 1994; Duke et al., 1997; Johnson, 1976). Oxadiazon is thought to be more effective than dinitroaniline herbicides since the resistance to mitotic inhibitors is exacerbated by populations (Johnson, 1976; Mudge et al., 1984). In recent years, two goosegrass populations have evolved resistance to oxadiazon in Virginia and North Carolina (McElroy et al., 2017).There are two nuclear PPO genes, PPO1 and PPO2, which encode chloroplast- and mitochondrial- targeted PPO isozymes, respectively (Duke et al., 1997; Hao et al., 2011). In addition, an Alanine substitution to Threonine at the 212th amino acid residue (A212T) has been first discovered as the target site mutation in PPO1 conferring resistance to oxadiazon only (Bi et al., 2020).Three target site mutations in PPO2, G210 deletion, R128G or R128M and G399A, have been deciphered to conferring cross resistance to some PPO inhibiting herbicides in *Amaranthus* species, such as

diphenyl ethers, saflufenacil, flumioxazin and carfentrazone (Giacomini et al., 2017; Patzoldt et al., 2006; Rangani et al., 2019).

Chlorophyll fluorescence has been previously used to diagnose activity of light active herbicides, such as Photosystem (PS)-II inhibitors (Ahrens et al., 1981; Norsworthy et al., 1998; Van Oorschot and Van Leeuwen, 1992), phytoene desaturase (PDS) inhibitors (Kaňa et al., 2004), Acetyl-CoA carboxylase (ACCase) (Dayan and Watson, 2011) and Acetolactate synthase (ALS) inhibitors (Zhang et al., 2016). To our knowledge, measurement of leaf chlorophyll fluorescence has not been utilized to diagnose resistance in response to PPO inhibitors in goosegrass, like oxadiazon. However, based on previous experience, the factor of light-dependent PPO inhibitor can be related to the degradation of the photosynthetic apparatus associated with the rapid peroxidation of the cellular membranes (Dayan and Duke, 1997), affecting the chlorophyll biosynthesis and resulting to the indirect change of chlorophyll fluorescence induction (Dayan and Zaccaro, 2012; Duke et al., 1991). Therefore, chlorophyll fluorescence can be potential utilization as a rapid method for resistance diagnosis of PPO inhibitor.

Target-site mutations conferring resistance are commonly detected using PCR product for capillary sequencing (Délye et al., 2008; Giacomini et al., 2017; Sanger et al., 1977). Sequencing, normally conducted by an external lab requires additional cost for shipping samples, a delay of 2-3 days to receive sequencing, and a time for sequence interpretation. Derived Cleaved Amplified Polymorphic sequence (dCAPs) is a molecular marker technology for rapid detection of known mutations without sequencing. dCAPs inserts a restriction endonuclease site when a known single nucleotide polymorphism (SNP) is present. Amplicons are then cleaved via restriction endonucleases and visualized via gel electrophoresis. The dCAPs method has been

widely reported to detect the TSR mutation in weed species, such as resistance to ALS inhibitors in green amaranth (*Amaranthus powellii* S.Wats.) (Ashigh et al., 2009), and ryegrass (*Lolium rigidum* Gaud.) (Yu et al., 2008); or mutation conferring resistance to ACCase inhibitors from 29 grass species (Delye et al., 2011).

Twenty-one new goosegrass populations from different locations were submitted to the Herbicide Resistance Diagnostic Lab (http://resistancelab.org) in summer 2020. To develop a reliable and high efficacy approach to rapid diagnose the resistance and detect the mutation has become more and more essential for goosegrass control. Here, our research were conducted to : (1) evaluate the resistance to oxadiazon in new suspected resistant goosegrass populations and further validate the A212T mutation; (2) develop the dCAPs assay to direct detect the A212T mutation without sequencing; (3) develop and determine if leaf chlorophyll fluorescence assay can rapid diagnose the resistance to oxadiazon.

3.2 Materials and Methods

Plant materials and herbicide treatment. Twenty-one goosegrass populations were submitted to the Auburn University Herbicide Resistance Diagnostic Lab (http://resistancelab.org) for evaluation of suspected oxadiazon resistance in summer 2020. Sixteen to 23 plants of each population were submitted for evaluation. Each population was identified by a single facility name (Table 1). Two known resistant (R) populations, CCV and RB, and one susceptible (S) population, PBU, were included for comparison. PBU was collected from the EV Smith – Plant Breeding Unit in Tallassee, AL, RB was collected from River Bend Golf Course in New Bern, NC, and CCV was collected from the Country Club of Virginia in Richmond, VA. All three populations were previously evaluated for oxadiazon resistance (Bi et al., 2020; McElroy et al., 2017).

Received and known resistant and susceptible biotypes were propagated in a greenhouse environment prior to utilization for research. Greenhouse temperature conditions were 30/25 (± 2C) at day/night temperature. Plastic pots (10 cm diameter) were filled with Marvyn loamy sand (Fine-loamy, kaolinitic, Thermic Typic Kanhapludults) soil with a pH of 6.5 and 0.5% humid matter. Seeds were planted at a 2-cm depth in 28 x 20-cm flats and were hand watered as needed daily to ensure germination. Once the seedlings emerged they were separated and planted in individual 4-cm pots, one seedling per pot approximately 4 cm in height. The plants were irrigated three times daily for 2 min with overhead irrigation and treated 7 days after final potting.

***Oxadiazon resistance evaluation*.** Research was conducted to evaluate sensitivity of 21 new goosegrass populations to PPO inhibitor oxadiazon compared to known resistant and susceptible. Oxadiazon (Ronstar FLO, Bayer Environmental Science, Cary, NC) was applied at a single recommended rate 1.12 kg ai ha^{-1}. Populations were divided into tiller from received plants or from greenhouse propagated plants with 20 tillers divided into four different flats. Three flats (60 tillered plants) were treated whereas one flat (20 tillered plants) were not treated. Herbicide treatments were foliar application made with a CO2-pressurized handheld TeeJet TP8003 VS four-nozzles boom calibrated to deliver 280 L ha^{-1} at 32 psi with 25cm spacing (Spraying Systems CO., Wheaton, IL). Goosegrass populations were visually assessed as dead or alive at 21 d after treatment (DAT). Plants were considered as alive when conspicuous green tissue was observed, whereas completely withered plants were considered as dead.

***RNA extraction and cDNA synthesis*.** Approximately 1 g of fresh leaf tissues were collected from the 21 new goosegrass populations from greenhouse. Total RNA extraction were performed using the RNeasy plant kit (QIAGEN, Aarhus, Denmark) described by Gehrig *et al.*(2000). The RNA concentrations were assessed by a NanoDrop 2000 Spectrophotometer

(Thermo Fisher Scientific Co., Waltham, MA) and RNA qualities were determined with gel electrophoresis. First-strand cDNA synthesis was made using the ProtoScript first strand cDNA synthesis kit (New England Biolabs Inc. Ipswich, MA).

PPO1 and PPO2 sequencing. Two pairs of PCR primers were designed based on the full-length cDNA sequences of PPO1 and PPO2 previously isolated in goosegrass (Bi et al., 2020). The primers for PPO1 and PPO2 amplification are including: chlF: 5′-ATGGTCGCCACGCCCGCAAT-3′, chlR: 5′-CTTGTAGGCGTACTTGGTCAAG-3′, mitF: 5′-ATGGCGGGCTCCGACGACAC-3′, mitR: 5′-ATGTGAACTGTCATGCTTTGTGC-3′. The PCR reaction system contained up to 1 μg cDNA, 500 nM forward and reverse primers, 200 μM dNTPs and 1U of Phusion DNA polymerase (New England Biolabs Inc., Ipswich, MA) with a 1× concentration of Phusion HF buffer in a final volume of 50 μL. After initial denaturation of the cDNA at 98°C for 30s; 35 cycles of 30 s at 98°C, 1 min at 60°C and 1 min at 72°C; then a final extension at 72°C for 10 min. The PCR products were visualized with a 1.2% agarose gel electrophoresis and purified with E.Z.N.A Gel Extraction Kit (Omega Bio-tek, Inc. Norcross, GA). The sequencing results and nucleotide polymorphisms were analyzed using the CLC Genomics Workbench 20 (QIAGEN, Aarhus, Denmark).

***dCAPs assay and A212T specific confirmation*.** dCAPs primers for S-PPO1 and R-PPO1 were designed by the dCAPs Finder 2.0 (http://helix.wustl.edu/dcaps/) (Neff et al., 2002). Since there is only one substitution A212T in PPO1 conferring resistance to oxadiazon, rapid genotyping of goosegrass plants for presence or absence of the PPO1 A212T mutations was accomplished by developing a dCAPs assay. Two dCAPs primers were designed to detected the susceptible and resistant biotype, respectively (Figure 5). The A212T mutation was detected using the dCAPs primers with one mismatch nucleotide R212-F (5′ -

AGCCTTTCTGCTCAGGTGTGTAT-3′) and PPO1-R (5′- TTTAGCCTGGACTGAGACG-3′). The wild susceptible A212 biotype was detected using the dCAPS primers with one mismatch nucleotide S212-F (5′-AGCCTTTCTGCTCAGGTGTCTAAGCTGGTGA-3′) and the same reverse primer as PPO1-R. The resulting PCR cleanup products were mixed with 1 unit of restriction endonuclease either *AluI* or *AccI* (New England Biolabs Inc., Ipswich, MA) and incubated at 37C for 5h. After digestion, 10 μL of the reaction was analyzed for DNA fragment length by gel electrophoresis. Any fully (or partial) digested products by restriction enzyme *AluI* or *AccI* were recognized as wildtype or resistant biotype, respectively. The fully non-digested products by both *AluI* and *AccI* were scored as new nucleotide polymorphisms.

Chlorophyll fluorescence assay and data analysis. This assay was performed to test if chlorophyll fluorescence can be utilized to diagnose resistance to oxadiazon in goosegrass populations. Three different goosegrass populations, PBU (known S), RB (known R) and Currituck (newly diagnosed R) were treated with foliar application oxadiazon (Ronstar FLO) in greenhouse at four different rates: 1.12, 2.24, 4.48 and 8.96 kg ha^{-1}. A non-treated control (0 kg ha^{-1}) was included. 15 to 20 plants of each populations were tested and experiments were conducted as random design and three replications. Following oxadiazon applications, leaf chlorophyll fluorescence was measured using a hand-held chlorophyll fluorometer (OS-FL1, Opti-Sciences, Inc., Hudson, NH) from the newest fully formed leaf surface. Based on the classical fluorescence parameters, the F_v/F_m value $[(F_m-F_0)/F_m]$ was recorded at 2, 4, 8, 16, 32, 48, 64, 72, 96, 120, 144 and 168 h after oxadiazon application. A total of 60 points measurement and three replications were made for each population.

In order to reduce the measure time and simplify procedure, another experiments was conducted simultaneously with petri dishes. Two goosegrass populations, PBU and RB, were

tested in this experiments. When the plants were at 5-10 cm height, approximately 3cm × 1cm fresh leaf was cut and put into the Corning™ Bio-Agricultural petri dishes (Thermo Fisher Scientific Co., Waltham, MA) with Phosphate Buffer (PH =7.4), adding with oxadiazon at two different concentrations: 100 μM and 2000 μM. The oxadiazon solution was dissolved with 1% DMSO. A non-treated control was included as well. Then the petri dishes were placed into the growth chamber which was set up 20h/4h day/night at 25C. Leaf chlorophyll fluorescence was measured at 2, 4, 6, 8, 12, 16, 24, 30, 36 and 48h with the hand-held chlorophyll fluorometer. A total of 30 measurement points was recorded and three replications for each population.

All data obtained from chlorophyll fluorescence assay were subjected ANOVA using the GLM procedure in SAS (SAS, v9.4). As the ANOVA showed significant treatment effects ($P<0.05$), the data were subjected to subsequent nonlinear regression analysis. The herbicide doses causing 50% inhibition of F_v/F_m (I_{50}) (in the greenhouse leaf chlorophyll fluorescence assay) were estimated by nonlinear regression analysis by fitting the data to four-parameter log-logistic dose–response model (Streibig, 1980).

$$Y=a+[(b-a)/(1+(X/C)^{\wedge d})] \qquad \text{[Equation 1]}$$

where Y is the F_v/F_m value expressed as a percentage of the nontreated control at the rate X (log-transformed), a is the lower limit, b is the upper limit, and d is the slope of the curve through the C (I_{50} value).

3.3 Results and Discussion

Oxadiazon resistance evaluation of different goosegrass populations. New suspected-resistant goosegrass response at oxadiazon rate 1.12 kg ha^{-1} were evaluated based on previous research (Bi et al., 2019). Suspected resistant populations were compared with the known susceptible population PBU and two known resistant population CCV and RB. A total 11 of 21

new goosegrass populations were ranked as susceptible due to 95 to 100% mortality from oxadiazon after 21 days. Populations ranked as susceptible were received from six different states: Texas, Alabama, Georgia, Florida, Tennessee, South Carolina (Table 3.1, Figure 3.1). In contrast, 10 of 21 new goosegrass populations were ranked as resistant due to <5% mortality from oxadiazon after 21 days. These oxadiazon resistant populations (including the known resistant population RB and CCV) were submitted from golf courses from six southeast states: Virginia, Tennessee, North Carolina, South Carolina, Alabama and Florida (Table 3.1; Figure 3.1). These observations indicated resistance to oxadiazon is present throughout the southeastern United States.

Identification and specific detection of A212T substitution in PPO1. In consideration of the resistance mechanism, two related target-site genes PPO1 and PPO2 of all the new goosegrass populations were sequenced for reported target-site mutations validation. There have 9 populations been identified a A212T substitution which can evolve resistance to oxadiazon. These populations were from Mid-Pines, Currituck, Furman University, GNRTJ, Lebanon, Pine Lakes, USF, Verdae and Wild Dunes, which have been newly confirmed as resistant to oxadiazon (Table3.1, Figure 3.2). However, there is no A212T substitution recognized in the resistant population Sandestin. In order to identify if there have any other single nucleotide polymorphisms (SNPs) except for A212T mutations related to oxadiazon resistance, both PPO1 and PPO2 were also aligned with known susceptible population. In both PPO1 and PPO2 , the results showed highly amino acid identity and there have only 5 SNPs in PPO1 and 4 SNPs in PPO2. Four of the SNPs were synonymous, whereas a total 5 amino acid replacements were observed in all sequencing plants, including: A212T, T283A, K366M in PPO1 and V207I, A303T in PPO2 (Table 3.2). These results indicated that the A212T in PPO1 is the primary

target site mechanism (11 of 12 populations) conferring resistance to PPO inhibitor oxadiazon in goosegrass. In other words, the goosegrass plants will be evolved resistance to oxadiazon once there carries a substitution A212T in PPO1. This might be good knowledge in goosegrass control.

In the genetic detection of single goosegrass plants, a dCAPS assay was successfully developed to confirm the relation between the 212th amino acid residue mutation and oxadiazon resistance. Digestion with two different restriction endonucleases allowed the positive identification of the wild type alanine or mutant threonine alleles. Typical dCAPS profiles as resolved on 2% agarose gel electrophoresis are provided in Figures 6. PCR generated a 359 bp fragment with the dCAPS primer S212-F/PPO1-R and R212-F/PPO1-R in all goosegrass plants. When digested with the restriction enzyme *AluI*, the wild goosegrass biotype showed a single restricted band of 335 bp and 24 bp fragment not visible of 2% agarose gel, however, goosegrass biotypes that are resistant to oxadiazon with the T212 allele displayed undigested (Figure 3.3). In contrast, when digested with the enzyme AccI, the resistant biotype with A212T substitution can be digested to a single restricted band of 338bp (the 21bp fragment was not visible either), but the susceptible biotype showed fully non-digested (Figure 3.4). The dCAPS and sequencing results what we have sequenced before for the goosegrass plants were totally correlated demonstrating the accuracy of the dCAPS assay.

The dCAPS method can identify SNPs by restriction endonuclease digestion of specific PCR products. The well-known specificity of a restriction endonuclease for its recognition site is exploited by this approach and can be used to detect any SNPs. The technique of dCAPs utilizes cheap technology and reagents common to most laboratories. It only includes PCR, digestion restriction and fundamental gel electrophoresis. It has also been shown to be a very accurate

technique and to determine wild and mutant alleles precisely. Since more genes identified by phenotype are cloned and multiple alleles of these genes are identified, the dCAPS has become to be very popular to detect target site gene mutations in herbicide resistant weed species. dCAPS assay can be used to test all the target site mutations conferring resistance to ACCase, ALS and PPO inhibitors. Delye et al. (2011) developed molecular assays using the dCAPS method to detect amino acids replacements in seven ACCase codons (1781, 1999, 2027, 2041, 2078, 2088 and 2096) and two ALS codons (197 and 574) in any weed species without prior knowledge of the ACCase or ALS sequence of the species. It is readily transferable from one weed to another and has been validated with minimal efforts and changes. dCAPs method was also used to detected for the presence of two amino acid mutations in PPO2 (R128G/M) in Palmer Pigweed species (Giacomini et al., 2017), and then successfully in identifying the distribution of heterozygous and homozygous mutants for both R98 substitutions (Copeland et al., 2018). The dCAPS methods detect all the amino acid replacements at the targeted codon of ACCase or ALS known to confer herbicide resistance, there is some other one to five additional substitutions, which might have a role in resistance but have never been investigated (Delye et al., 2011). A sequencing followed by the dose-response assay or whole-plant assay, or any other methods, will be precisely to validate this resistance. Consequently , in order to detect the target site resistance of oxadiazon in goosegrass populations, we performed combination assays of leaf chlorophyll fluorescence and dCAPS, and it can be readily established for any other species.

Chlorophyll fluorescence response to oxadiazon in goosegrass. In the greenhouse leaf chlorophyll fluorescense assay, the F_v/F_m values continuously decreased with time lapse after oxadiazon treatment at a full registered rate 1.12 kg ha^{-1} (Figure 3.5A). There is no significant difference of the F_v/F_m values within 64 HAT between PBU, RB and Currituck, however, an

obvious difference in F_v/F_m values reduction was observed at 72 h after treatment (HAT). The F_v/F_m values of PBU decreased rapidly, while F_v/F_m values of RB and Currituck still declined slowly. In the case of rapid diagnose the oxadiazon resistance in the greenhouse, the F_v/F_m values in leaf chlorophyll fluorescence assay at 72 HAT significantly ($P<0.05$) decreased with an increasing oxadiazon rates, presenting that the log-logistic dose-response model described the data appropriately to diagnose the oxadiazon resistance in goosegrass (Figure 3.5B). Furthermore, the R^2 showed a goodness of fit to the models for leaf chlorophyll fluorescence assay for each population (Table 3.3). The I_{50} of F_v/F_m values were estimated by the fitted model. The I_{50} values at 72 HAT were 0. 62, 9.15, and 3.33 kg ha^{-1} for PBU, RB and Currituck, respectively, resulting in an R/S ratio of 14.75 for RB, and 5.37 for Currituck, indicating that the leaf chlorophyll fluorescence assay can rapid detect the oxadiazon resistance in goosegrass.

To simplify the measurement procedure, another experiment with petri dishes was conducted. No difference of F_v/F_m values was observed with non-treated control within 48 HAT (Figure 3.6). When the buffer added with oxadiazon solution, the F_v/F_m values of PBU continually decreased. When the buffer with 100 uM oxadiazon, F_v/F_m values of PBU decreased after putting in the growth chamber at 20 HAT, and had visually colorless damage at 30 HAT, while the F_v/F_m values of RB had no significant change and visual damage (Figure 3.7). When the oxadiazon concentration was 2000 uM, the F_v/F_m values of PBU decreased sharply at 12 HAT, and the significant colorless damage or 'burn down' was observed at 30 HAT (Figure 3.8), however, the RB populations showed slowly reduction trend of F_v/F_m values and no colorless visual damage at 30 HAT, indicating the simplified leaf chlorophyll fluorescence assay with petri dishes can be determined to rapid detect the resistance to oxadiazon in goosegrass as well, which can reduce the time within 24 hours.

Leaf chlorophyll fluorescence assay has been used a good biomarker to rapid detect the resistance or stress. (Zhang et al., 2016) had measured the *Fv/Fm* values between the resistant and susceptible *Echinochloa* species with the applications of cyhalofop-butyl, penoxsulam and propanil, which belongs to ACCase inhibitor, ALS inhibitor and PS-II inhibitors, respectively. It showed that the leaf chlorophyll fluorescence assay were consistent with the whole plant assay. Furthermore, the discrimination time can be shorten to 64h, 168h and 192h after the application PS-II, ACCase, ALS inhibitor, respectively (Zhang et al., 2016). Dayan *et al*. (2012) demonstrated that chlorophyll fluorescence as a marker to test different mechanisms of action, the reason for the changes in chlorophyll fluorescence induction was interpreted as those herbicides with different modes of action meditating or inhibiting a certain process in chlorophyll synthesis. Although the leaf chlorophyll fluorescense assay has not been used to detect the resistance to PPO inhibitor, measurement of leaf chlorophyll fluorescence changes still can be an efficient potential method to detect resistance of PPO inhibitors since PPO is an essential enzyme in the biosynthesis of chlorophyll. In addition, consistent with this light-dependent mode of action, PPO inhibitors may have no effect of photosynthetic electron transporting during the period the dark incubation, while caused the rapid reduction in the presence of light. Therefore, in our research, we have simplified the chlorophyll fluorescence assay integrated with a petri dish to collect the leaf tissues and to incubate in the growth chamber which had long-day/light compared with performing in the greenhouse. The results has completely proved that the resistant and susceptible goosegrass populations can be discriminated with both two types of chlorophyll fluorescence assay effectively and accurately in the early stage and significant save time within a couple of days.

Further implication. The increasing number of goosegrass population with resistance to PPO inhibitors oxadiazon is big problem in turf courses. After years of continuous use of oxadiazon, more and more goosegrass populations from different states have been evolved resistance, which limits herbicide options to control. Since the first two case of resistance to oxadiazon reported in Virginia and North Carolina (McElroy et al., 2017), more than 10 new populations which distributed in six southeastern states have been evolved resistance to oxadiazon in natural selection. Therefore, rapidly and effectively diagnosis resistant plants will be more and more important to control weeds and slow down the resistance evolution. Furthermore, the majority of the oxadiazon resistant goosegrass individuals carried the A212T substitution in PPO1. No other reported mutations, such as Gly-210 deletion, R128G/M in PPO2, were investigated in these populations. Although our research showed the A212T mutation is the only target site mechanism to oxadiazon resistance, the additional data of resistance level to oxadiazon or other PPO inhibitors are needed to qualify the impact of this substitution on the resistance. Furthermore, we have not ruled out the possibility of other resistance mechanism. The population Sandestin from Florida evolved resistance to oxadiazon while no A212T occurred in PPO1, indicating that there may be other mechanism can confer resistance to oxadiazon.

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Table 9

Table 3.1 Course facilities, locations, GPS coordinates, response to oxadiazon and A212T validation for goosegrass populations.

Population	Course Facility	Locations	GPS Coordinate	Response to Oxadiazon	A212T validation
Sandestin	Sandestin Golf Resort	Miramar Beach, FL	30.3751, -86.3634	R*	N*
Mid-Pines	Mid-Pines Inn and Golf Club	Southern Pines, NC	35.1856, -79.4026	R	Y
Currituck	The Currituck Club	Corolla, NC	36.3847,-75.8286	R	Y
CCV	Country Club of Virginia	Reston, VA	37.5727,-77.6137	R	Y
Furman University	Furman University Golf Club	Greenville, SC	34.9429,-82.4415	R	Y
GNRTJ	Grand National Golf Course	Opelika, AL	32.6769,-85.4252	R	Y
Lebanon	Lebanon Golf and Country Club	Lebanon, TN	36.2251,-86.3082	R	Y
Pine Lakes	Pine Lakes International Country Club	Myrtle Beach, SC	33.7336,-78.8488	R	Y
RB	River Bend Country Club	New Bern, NC	35.1084,-77.0441	R	Y
USF	University of South Florida	Tampa, FL	28.0599,-82.4138	R	Y
Verdae	Verdae Green Golf Club	Greenville, SC	34.8258,-82.3275	R	Y
Wild Dunes	Wild Dunes Resort	Isle of Palms, SC	32.8008,-79.7541	R	Y
PBU	Plant Breeding Unit	Tallassee, AL	32.5368,-85.8987	S	N
Surf Club	Surf Club	North Myrtle Beach, SC	33.8276,-78.6557	S	N
BallenIsles	BallenIsles Country Club	Palm Beach Gardens, FL	26.8281,-80.1178	S	N
Clanton	Clanton Country Club	Clanton, AL	32.8273,-86.6054	S	N
Innsbrook	Innsbruck Golf Club	Helen, GA	34.7009,-83.7294	S	N
Magnolia	Magnolia Golf Course	Houston, TX	29.4835,-95.1469	S	N
Monarch	Monarch Golf Club	Leesburg, FL	28.7931,-81.9144	S	N
Naples	Country Club of Naples	Naples, FL	26.1380,-81.7040	S	N
Paris MT	Paris Mountain Country Club	Greenville, SC	34.9327,-82.4146	S	N
Ridges	The Ridges Golf and Country Club	Jonesborough, TN	36.2946,-82.4796	S	N
Troy	Troy Country Club	Troy, AL	31.8088,-85.9699	S	N
Vanderbilt	Vanderbilt Legends Club	Franklin, TN	35.9252,-86.8689	S	N

* resistant, no A212T mutation observed.

Table 10

Table 3.2 Identification of SNPs of both PPO1 and PPO2 in 24 goosegrass populations.

Population	nonsynonymous SNPs in PPO1						nonsynonymous SNPs in PPO2				Resistance
	Nucleotide-643	Amino Acid-212	Nucleotide-847	Amino Acid-283	Nucleotide-1097	Amino Acid-366	Nucleotide-619	Amino Acid-207	Nucleotide-907	Amino Acid-303	
Sandestin	Guanine	A	Guanine	A	Adenine	K	Adenine	I	Adenine	T	R
Mid-Pines	Adenine	T	Adenine	T	Adenine	K	Adenine	I	Adenine	T	R
Currituck	Adenine	T	Guanine	A	Thymine	M	Guanine	V	Guanine	A	R
CCV	Adenine	T	Adenine	T	Adenine	K	Guanine	V	Adenine	T	R
Furman University	Adenine	T	Adenine	T	Adenine	K	Adenine	I	Adenine	T	R
GNRTJ	Adenine	T	Adenine	T	Adenine	K	Guanine	V	Adenine	T	R
Lebanon	Adenine	T	Guanine	A	Thymine	M	Guanine	V	Adenine	T	R
Pine Lakes	Adenine	T	Adenine	T	Adenine	K	Adenine	I	Adenine	T	R
RB	Adenine	T	Guanine	A	Thymine	M	Guanine	V	Adenine	T	R
USF	Adenine	T	Adenine	T	Adenine	K	Adenine	I	Guanine	A	R
Verdae	Adenine	T	Guanine	A	Adenine	K	Guanine	V	Adenine	T	R
Wild Dunes	Adenine	T	Adenine	T	Adenine	K	Adenine	I	Guanine	A	R
PBU	Guanine	A	Adenine	T	Adenine	K	Guanine	V	Guanine	A	S
Surf Club	Guanine	A	Guanine	A	Thymine	M	Guanine	V	Adenine	T	S
BallenIsles	Guanine	A	Guanine	A	Thymine	M	Guanine	V	Guanine	A	S
Clanton	Guanine	A	Adenine	T	Adenine	K	Guanine	V	Guanine	A	S
Innsbrook	Guanine	A	Guanine	A	Adenine	K	Guanine	V	Adenine	T	S
Magnolia	Guanine	A	Adenine	T	Adenine	K	Guanine	V	Guanine	A	S
Monarch	Guanine	A	Adenine	T	Adenine	K	Guanine	V	Guanine	A	S
Naples	Guanine	A	Guanine	A	Adenine	K	Adenine	I	Guanine	A	S
Paris MT	Guanine	A	Adenine	T	Thymine	M	Adenine	I	Adenine	T	S
Ridges	Guanine	A	Adenine	T	Thymine	M	Adenine	I	Adenine	T	S
Troy	Guanine	A	Adenine	T	Adenine	K	Guanine	V	Adenine	T	S
Vanderbilt	Guanine	A	Guanine	A	Thymine	M	Guanine	V	Adenine	T	S

Table 11

Table 3.3 Summary of parameter estimates for the log-logistic model of F_v/F_m (I_{50}) in the leaf chlorophyll fluorescence assay at 72 HAT in greenhouse experiment.

Biotype	Parameter estimates				R/S ratio	R^2	P
	a	b	d	I_{50} (kg ai ha^{-1}) (95% CI)			
PBU	0.19	103.3	-1.58	0.62 (0.35, 0.76)		0.9969	<0.05
RB	12.07	99.21	-1.52	9.15 (3.92,12.51)	14.75	0.9786	<0.05
Currituck	6.05	92.42	-3.11	3.33 (2.79, 4.52)	5.37	0.9742	<0.05

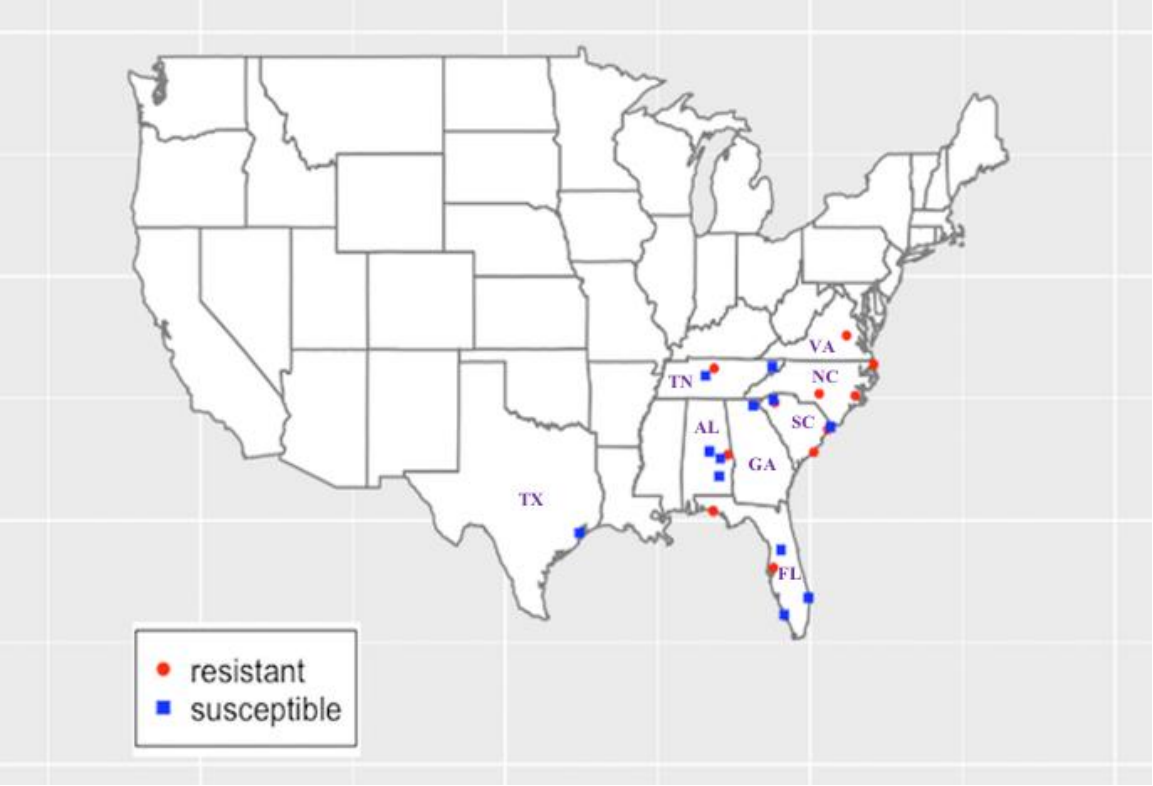

Figure 3.1 Field locations in different states where 24 goosegrass population collected.

Figure 9

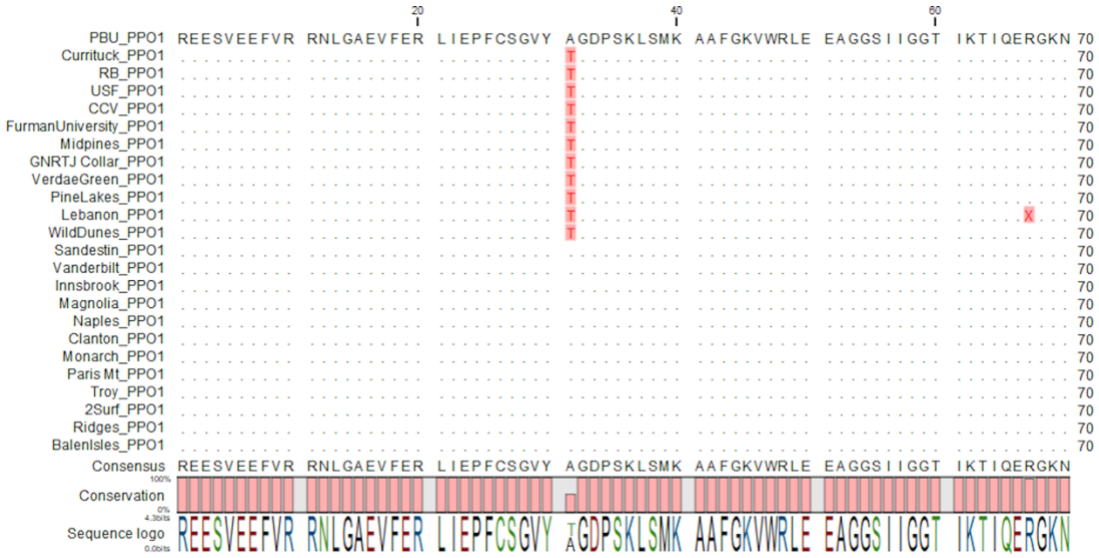

Figure 3.2 Sequence alignment of PPO1 in 24 goosegrass populations. The A212T mutation was marked with red background color.

Figure 10

(A) The AluI dCAPs assay:

S-PPO1 AGCCTTTCTGCTCAGGTGTCTATGCTGGTGA

R-PPO1 AGCCTTTCTGCTCAGGTGTCTATACTGGTGA

AluI AGCT

The S-212dCAP primer:

CTGCTCAGGTGTCTAAGCTGGTGA

AluI digestion:

S-PPO1 AGCCTTTCTGCTCAGGTGTCTAAG / CTGGTGA

R-PPO1 AGCCTTTCTGCTCAGGTGTCTAAACTGGTGA

(B) The AccI dCAPs assay:

S-PPO1 AGCCTTTCTGCTCAGGTGTCTATGCTGGTGA

R-PPO1 AGCCTTTCTGCTCAGGTGTCTATACTGGTGA

AccI GTMKAC

The R-212dCAP primer:

AGCCTTTCTGCTCAGGTGTGTAT

AccI digestion:

S-PPO1 AGCCTTTCTGCTCAGGTGTGTATCTGGTGA

R-PPO1 AGCCTTTCTGCTCAGGTGTGT / ATACTGGTGA

Figure 3.3 The A212TdCAP primer for the detection of oxadiazon susceptible and resistant biotype. (A) A212 alleles wildtype in PPO1 by digesting the PCR product with the restriction endonuclease AluI. (B) The A212T mutation can be detected after digestion with the restriction endonuclease AccI.

Figure 11

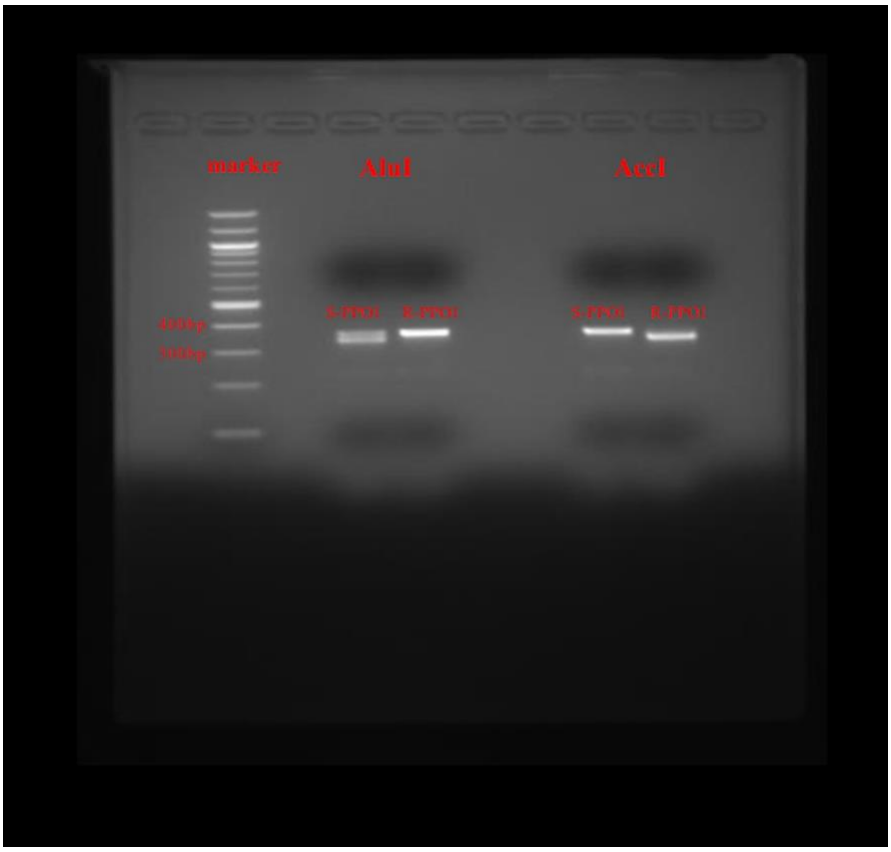

Figure 3.4 Restriction analysis of A212TdCAPs. S-PPO1 represent the A212 alleles wildtype by digesting the PCR product with the restriction endonuclease AluI. R-PPO1 represents the A212T mutation by digesting with the restriction endonuclease AccI.

Figure 12

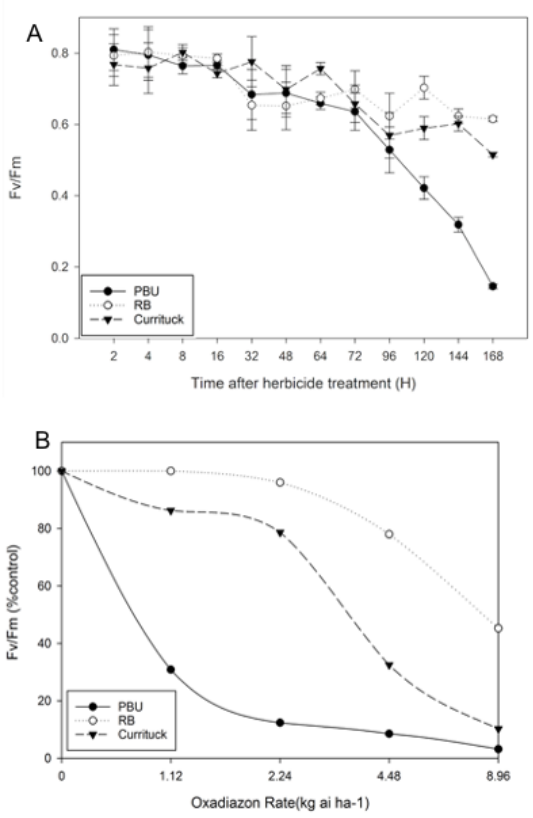

Figure 3.5 F_v/F_m values with time lapse and oxadiazon rate. (A) F_v/F_m with time lapse after oxadiazon treatment at rate 1.12 kg ai ha^{-1} ; (B) F_v/F_m values (% of control) at 72 HAT in the greenhouse.

Figure 13

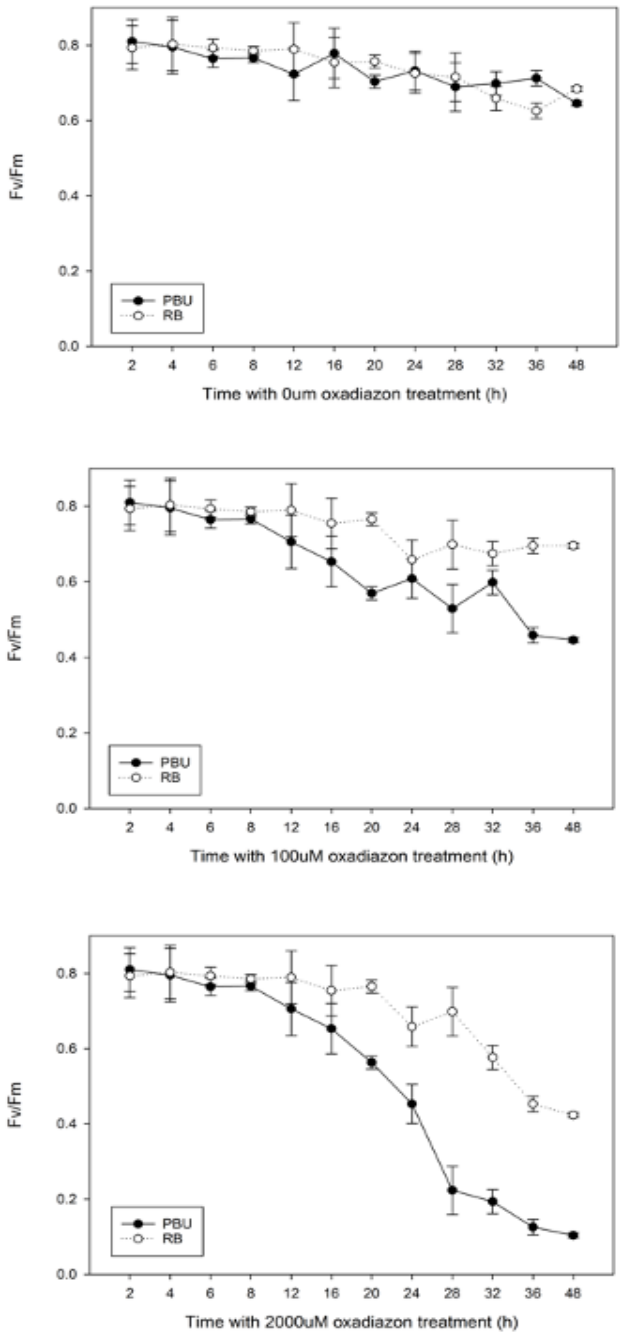

Figure 3.6. *Fv/Fm* values with time lapse after oxadiazon treatment with concentration of 0, 100uM, 2000uM in petri dishes.

Figure 14

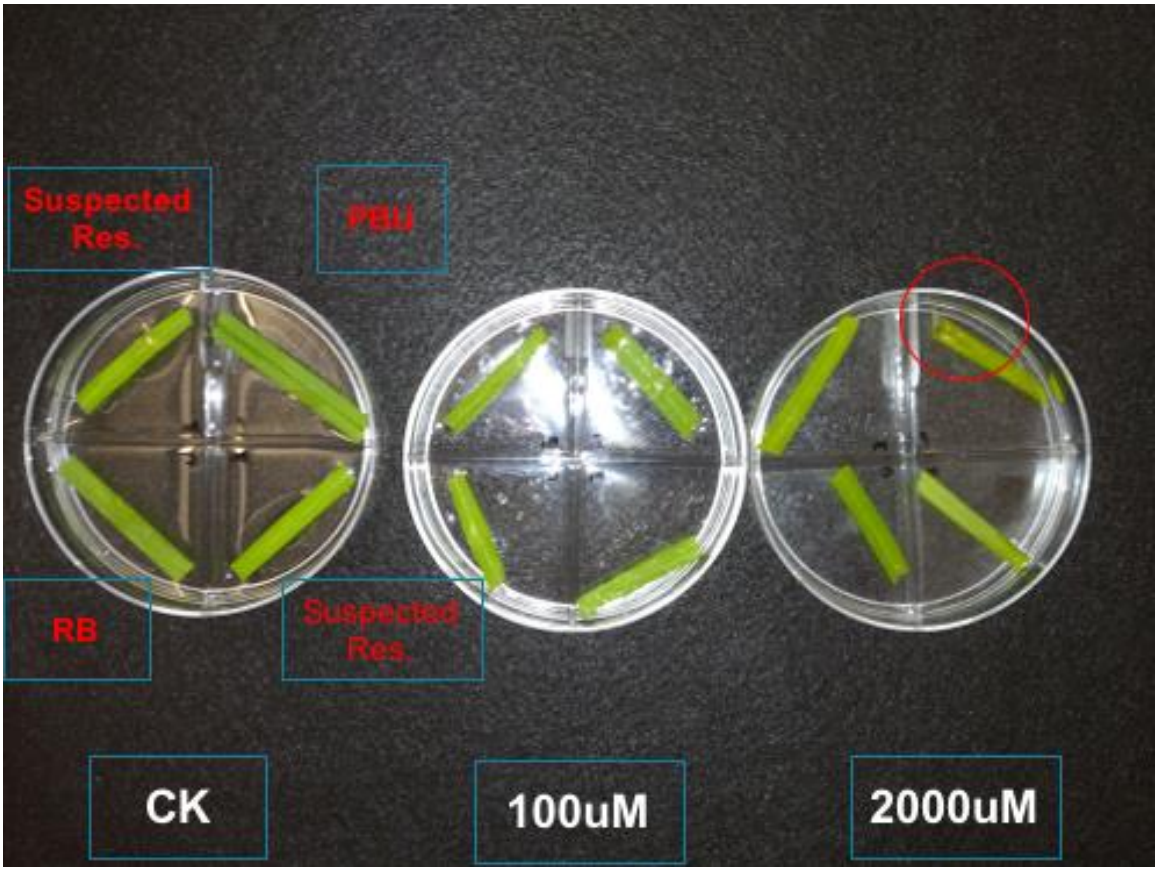

Figure 3.7 The susceptible leaf tissue can be observed ‘colorless’ or ‘burn down’ damage at 20 HAT when oxadiazon concentration adding to 2000 uM in petri dish leaf chlorophyll fluorescence assay.

Figure 15

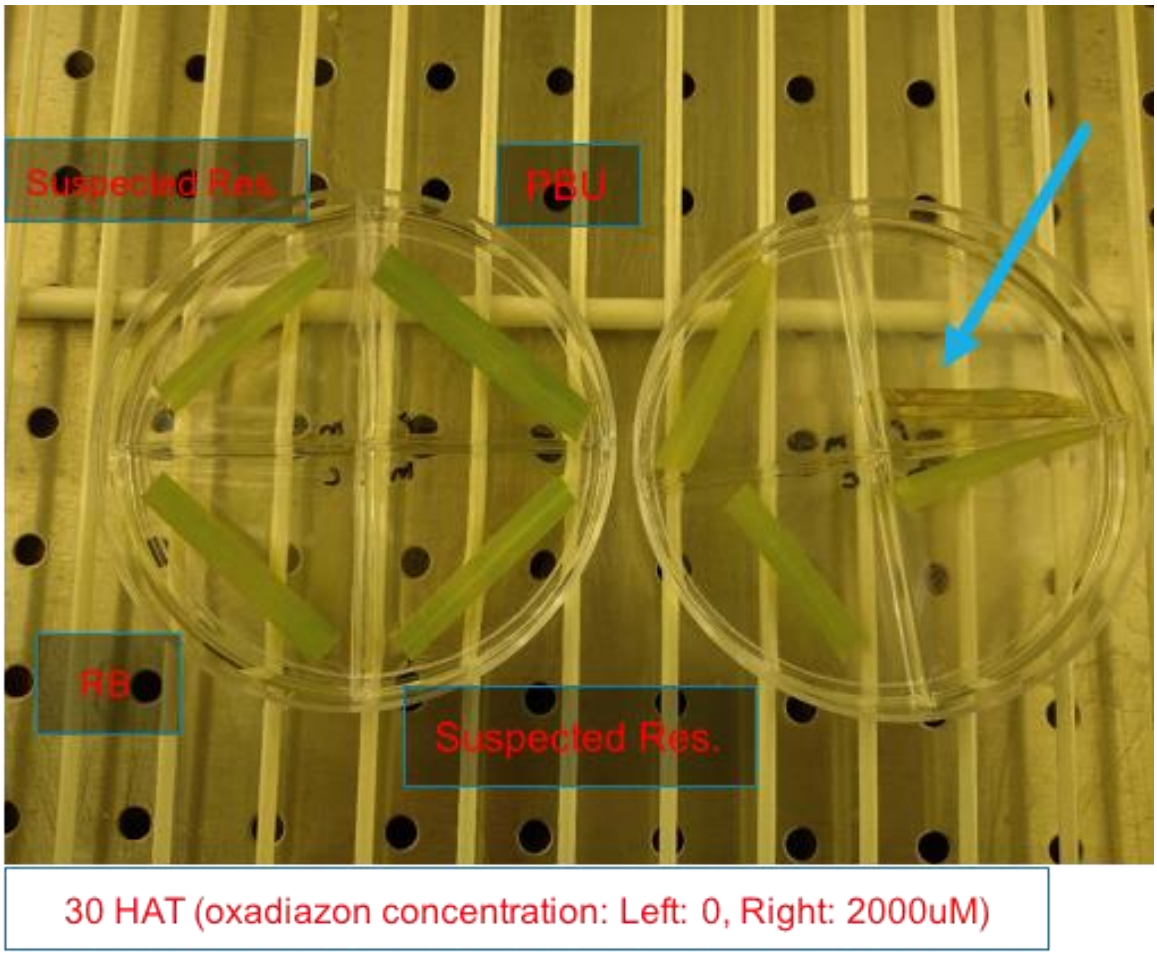

Figure 3.8 Petri dish leaf chlorophyll fluorescence assay at 30 HAT.

Figure 16

Chapter IV. Differential sensitivity of *Eleusine indica* PPO isoforms to PPO-inhibiting herbicides

Abstract: Protoporphyrinogen oxidase (PPO) is an essential enzyme in the biosynthesis of chlorophyll and heme. There are two isoforms localized in chloroplast and mitochondria in plants. Two nuclear genes, PPO1 and PPO2, were isolated to encode the chloroplast-targeted PPO1 and mitochondrial-targeted PPO2, respectively. Both two isozymes can be active sites of PPO inhibitors. There are more than eight different PPO inhibitor structural-unrelated chemical families. The relationship and the sensitivity between the different herbicide chemical families and the PPO isoforms are not clear. In this research, an *in vitro Escherichia coli* functional assay was conducted to test the sensitivity of three PPO isoform, wild-type PPO1 and PPO2 with no known TSR to PPO-inhibiting herbicides (S-PPO1 and S-PPO2, respectively) and oxadiazon-resistant PPO1 containing an A212T amino acid substitution (R-PPO1). *E. coli* growth varied in response to each herbicide depending on PPO isoform based on predicted IC_{50} values. Oxadiazon inhibited S-PPO1 transformed *E. coli* greater thanS-PPO2 and R-PPO1. S-PPO2 was inhibited more by lactofen than R-PPO1 and S-PPO1. Lactofen inhibited S-PPO2 (28.19 μM) greater than S-PPO1 and R-PPO1 (94.18 and 108.8 μM, respectively). Sulfentrazone inhibited S-PPO1 (57.91 uM) greater than S-PPO2 (152.1 μM), but no difference was observed between S-PPO2 and R-PPO1 (97.3 μM) or S-PPO1 and R-PPO1. Based on these results, we conclude that oxadiazon inhibits the PPO1 isoform greater than PPO2, lactofen inhibits the PPO2 isoform greater than PPO1, and sulfentrazone inhibits both PPO1 and PPO2 similarly. These results only analyzed PPO isoforms from *Eleusine indica* and future results should evaluate PPO isoforms from other species.

Nomenclature: *Escherichia coli*, *Eleusine indica*, oxadiazon, sulfentrazone, lactofen

Keywords: protoporphyrinogen oxidase, PPO, oxadiazon, sulfentrazone, lactofen

4.1 Introduction

Protoporphyrinogen oxidase (PPO, E.C. 1.3.3.4) is a key enzyme in the biosynthesis of heme and chlorophyll, catalyzing the substrate of the biosynthesis, protoporphyrinogen IX (Protogen IX), to protoporphyrin IX (Proto IX). In the next step, the product of the pathway, Proto IX, can bind to the ferro chelatase and magnesium chelatase, continue to the following synthesis of heme and chlorophyll (Duke et al., 1991). PPO has two different isozymes and localized into different organelles chloroplast and mitochondria, respectively (Hao et al., 2014). Both chloroplast-targeted PPO1 and mitochondria-targeted PPO2 are active sites of PPO inhibiting herbicides (HRAC Group E/14). When PPO enzyme is inhibited by the herbicides, the substrate of PPO enzyme, Protogen IX, will accumulate and escape to the cytoplasm of the cells from chloroplast or mitochondria. When Protogen IX accumulates enough, it can be auto-oxidized to Proto IX in the presence of oxygen and light. Proto IX is a light-active pigment and it can produce singlet oxygens in the occurrence of light, resulting in cell membrane leakage and plant death (Duke et al., 1991).

PPO inhibitors are a diverse group of chemicals from eight different herbicide families: diphenyl ethers, N-phenylphthalimides, oxadiazoles, oxazolidinediones, Phenylpyrazoles, pyrimidindiones, thiadiazoles, triazolinones and others. PPO inhibitors are used as both preemergence and postemergence herbicides, however most except oxadiazon are used as postemergence. All PPO inhibitors induce rapid, photo-active phytotoxicity when applied foliar to green tissue. Oxadiazon is a PPO-inhibiting herbicide belongs to the oxadiazole group (Duke et al., 1997). Oxadiazon is mainly applied as preemergence to control of annual grasses such as goosegrass, and broadleaves such as carpetweed (Derr, 1994; Johnson, 1976). Two goosegrass biotypes have been evolved resistance to oxadiazon in turf which were not cross resistant to three

other PPO inhibiting herbicides: lactofen, sulfentrazone and flumioxazin (Bi et al., 2020). There is no single case reported to sulfentrazone resistance, but some diphenyl ethers resistant species were also tested cross-resistance to sulfentrazone (Rangani et al., 2019). Lactofen is one of diphenyl ethers PPO- inhibiting herbicides which was widely used to postemergence control weed species. There have total 9 weed species been evolved resistance to diphenyl ethers chemicals (Giacomini et al., 2017; Rangani et al., 2019).

There are two nuclear genes, PPO1 and PPO2, encoding the chloroplast-targeted PPO1 and mitochondrial-targeted PPO2, respectively. Some target-site mutations in these two genes were deciphered to be the mechanism conferring resistance to PPO inhibiting herbicides. A glycine deletion at position 210 in PPO2 (ΔG210) was the first reported mechanism responsible for fomesafen and lactofen in common waterhemp (*Amaranthus tuberculatus*) (Patzoldt et al., 2006). An amino acid residue arginine substituted to glycine or methionine at position 128 in Palmer amaranth PPO2 (*A. palmeri*) (R128G/M, homologous to R98 mutation in *Ambrosia artemifiifolia*) evolved resistance to PPO inhibiting herbicides (Giacomini et al., 2017). A single site mutation of glycine to alanine at position 399 (G399A) were detected resistance to PPO inhibitors in Palmer amaranth (Rangani et al., 2019). A recently amino acid substitution arginine to leucine at 128 in PPO2 (R128L) has been evolved resistance to PPO inhibiting herbicides in wild poinsettia (*Euphorbia heterophylla*) (Mendes et al., 2020). The amino acid substitution of alanine to threonine at position 212 (A212T) in goosegrass PPO1 was determined to be the causal mechanism of oxadiazon resistance, which was the only known mutation reported in PPO1 as mechanism conferring resistance to PPO inhibitors in grass species (Bi et al., 2020). In consideration of the cross resistance of these target site mutations, ΔG210 and G399A has been detected high resistance to PPO inhibitors from Diphenyl ethers, pyrimidinedione, and N-

phenylphthalimide, less resistance to oxadiazole and triazolinone (Rangani et al., 2019). R128G/L/M has been evolved resistance to Diphenyl ethers, sulfentrazone, and flumioxazin, while no resistance to oxadiazon (Mendes et al., 2020; Rangani et al., 2019). The A212T mutation in PPO1 was only resistant to oxadiazon in goosegrass, and no cross resistance to other PPO inhibitors (Bi et al., 2020). Thus, the relationship and the sensitivity between the herbicide chemical families and the PPO isoforms are not clearly.

We hypothesize that PPO isoforms in *Eleusine indica* may have different sensitivity to PPO inhibiting herbicides. Differential targeting of PPO isoforms by PPO-inhibiting herbicides could potentially explain why only oxadiazon has selected for PPO1 target-site resistance and others have selected for PPO2 target-site resistance.

4.2 Materials and Methods

Research was conducted to evaluate susceptible (S) and resistant (R) PPO1 and PPO2 expressed in heme-deficient *Escherichia coli* exposed to PPO-inhibiting herbicides. S biotype was previously referred to as PBU (Bi et al., 2020; McElroy et al., 2017) and has no known resistance to any herbicide or mutations in PPO1 or PPO2 previously identified to confer resistance to PPO herbicides. R biotype was previously referred to as RB and is confirmed resistant to oxadiazon due to a A212T mutation in PPO1 (Bi et al., 2020; McElroy et al., 2017).

E. coli transformation. Methodology for *E. coli* transformation was identical to that used in Bi *et al.* (2020). SASX38 hemG mutant *E. coli* strain deficient in heme, commonly used in PPO-inhibiting herbicide TSR functional assays was utilized. The *PPO1* and *PPO2* genes from susceptible (S) and resistant (R) goosegrass population were amplified and cloned into the pBAD-TOPO expression vector using the pBAD TOPO^{TM} TA Expression kit (Invitrogen, Carlsbad, CA). The *PPO1* and *PPO2* gene products were amplified using the same PCR primers

and PCR reaction system as previously described in Bi *et al.* (2020) , so that the *PPO1* and *PPO2* translation began at the ATG start codon. Three different pBAD-TOPO plasmids were created and sequenced to confirm the identities with the target site *PPO1* and *PPO2* gene from S and R goosegrass populations (marked as S-PPO1, S-PPO2 and R-PPO1, respectively). The R-PPO1 has an A212T substitution compared with S-PPO1, and there were no any nucleotide polymorphisms of PPO2 between S and R populations in the cloning experiment. Three different plasmids were then used to transform a *hemG* mutant *E. coli* strain SASX38 by electroporation.

In vitro E. coli functional complementary assay. To test the *E. coli* cells response to three different PPO inhibitors, a petri dishes assay and a microplate assay were conducted separately. Petri dish assays were conducted to determine if transformation was successful with a functional protein and provide a qualitative measure of transformed E. coli growth in response to herbicides, while microplate assays were conducted to collected quantitative data for predictive modeling.

In the petri dishes assay, expression of the PPO1 and PPO2 in three different transformed colonies of the SASX38 mutant strain were induced on LB medium with 2% L-arabinose and grown on LB medium supplied with three different PPO inhibitors: lactofen, oxadiazon, and sulfentrazone, at different concentrations from 0, 10, 100, 500 μM, and dark incubated at 37°C for 20h. After that, the growth of different *E. coli* transformed strains were visual observed.

In the microplate assay, each 96-well microplate supported the first row of wells containing LB medium alone and contained the transformed 1:2 serial dilutions transformed SASX38 mutant *E. coli* cells (bacterial controls). Following the sterility control row, each row of wells contained the same dilutions *E. coli* cells and different concentrations of PPO inhibitors has been added (lactofen, oxadiazon, sulfentrazone). The PPO inhibitors concentrations were:

10, 20, 50, 100, 200, 500, 1000 μM. Following incubation for 22 h, optical density (OD) at 600 nm was recorded and replicated three times.

Statistical analysis. The optical density was transformed to growth percentage relative to control, [OD of test well/mean OD of bacterial control wells]×100, where 100% means herbicides did not inhibit the growth of *E. coli* transformed strain, and 0 % means herbicides can complete inhibit the growth. The data were subjected to analysis of variance at a significance level of $P < 0.05$ using the PROC GLM procedure of SAS v9.4 (SAS Institute Inc., Cary, NC) to analyze main effects and interactions of herbicide, herbicide concentration, PPO isoforms, and replications. The data were fit and modeled with GraphPad Prism (Windows version 9.0, GraphPad Software, San Diego ,California) using a modified sigmoidal model.

Y = 100/(1+10EXP(X-LogIC50)) [Equation 1],

In this model, Y represents the growth percentage relative to the control (%) of the *E. coli* strains transformed with different PPO isoforms, X represents the Log10-transformed herbicides concentrations (uM). IC_{50} is the concentration of the herbicide resulting in 50% inhibition of the growth. Growth relative to the control (%) versus different herbicide concentration curves were plotted from the predicted growth values using GraphPad Prism (Windows version 9.0, GraphPad Software, San Diego, California).

4.3 Results and Discussion

Response to different PPO inhibitors in petri dish assay. The SASX38 mutant strain transformed with the plasmids expressing R-PPO1, S-PPO1, and S-PPO2 from plants grew on the LB medium alone, indicating functional proteins were expressed. Images of transformed *E. coli* growth on different herbicide media is provided for qualitative inhibition assessment. Oxadiazon at 10 μM inhibited growth of *E. coli* transformed with S-PPO1 alleles, but growth of

R-PPO1 transformed *E. coli* was not inhibiting with oxadiazon at 500 μM (Figure 4.1). S-PPO2 transformed *E. coli* was not inhibited by oxadiazon at 500 μM as well. These data indicate that the oxadiazon did not inhibit S-PPO2 or R-PPO1.

Comparing different sulfentrazone concentrations (Figure 4.2), both S-PPO1 and R-PPO1 *E. coli* strains were inhibited by 100 μM sulfentrazone, while S-PPO2 was inhibited minimally with 500 μM sulfentrazone. This indicates that the R-PPO1 with the A212T substitution is not resistant to sulfentrazone, and sulfentrazone can target to both PPO1 and PPO2 genes. However, S-PPO1 is inhibited to a greater degree by sulfentrazone than S-PPO2.

Comparing different lactofen concentrations, S-PPO2 transformed *E. coli* was inhibited at 10 μM, while R-PPO1 and S-PPO1 transformed *E. coli* strains were not inhibited at this concentration. Lactofen at 100 and 500 μM inhibited all three *E. coli* strains (Figure 4.3)

***Response to different PPO inhibitors in microplate assay*.** Percent growth relative to the non-treated of the three *E. coli* transformations were modeled against herbicide concentrations in Figure 5.4. Significant differences were observed between *E. coli* transformations response to herbicides and herbicide concentrations. Oxadiazon at 100 μM completely inhibited S-PPO1 transformed *E. coli,* but inhibited S-PPO2 and R-PPO1 less than 50% at concentrations less than 1000 μM (Figure 4.4). Sulfentrazone at 200 μM inhibited S-PPO1 and R-PPO1 transformed *E. coli* > 80% (Figure 4.4). Sulfentrazone at 500 μM inhibited S-PPO2 transformed *E. coli* greater than lower concentrations and near complete inhibition at 1000 μM; thus, demonstrating that the sulfentrazone targets both PPO1 and PPO2 gene in goosegrass, albeit to varying degrees. Lactofen at 100 μM completely inhibited S-PPO2 transformed *E. coli,* however, S-PPO1 and R-PPO1 were not complete inhibited until lactofen at 500 μM (Figure 4.4).

IC_{50} values of the different *E. coli* strain response to oxadiazon, sulfentrazone and lactofen were estimated based on the model for the curve and the modified fit equation (Table 4.1). When tested with oxadiazon, the IC_{50} value of the *E. coli* strain transformed with S-PPO1 isoform was 7.92 μM, however, the IC_{50} value of the *E. coli* strain transformed with S-PPO2 and R-PPO1 isoforms were 450.9 μM and 610.8 μM, respectively, indicating the S-PPO1 protein was highly sensitivity to PPO inhibitor oxadiazon, while S-PPO2 and R-PPO1 protein were not significant inhibited by this herbicide. Comparing with test of sulfentrazone, IC_{50} value of S-PPO1, S-PPO2 and R-PPO1 were 57.91 μM, 152.1 μM and 97.63 μM, respectively, showing that sulfentrazone may inhibit PPO1 and PPO2 protein. However, IC_{50} values of the S-PPO2 *E. coli* strains was 28.19 μM, much more sensitivity to PPO inhibitor lactofen compared with S-PPO1 (94.18 μM) and R-PPO1 (108.8 μM), indicating that herbicides from diphenyl ethers chemical families can inhibit PPO2 proteins more significant than PPO1 proteins.

The sensitivity results indicated that oxadiazon only inhibit PPO1 gene in goosegrass, while sulfentrazone and lactofen may inhibit both PPO1 and PPO2 at the higher concentration. However, when at lower concentration, sulfentrazone inhibits PPO1 greater than PPO2 and lactofen mainly inhibit PPO2 greater than PPO1.

***Discussion and conclusion*.** PPO inhibiting herbicides encompass more than nine chemically families. Here we only compared three of them, which are widely used in controlling weeds. PPO has two different target-site organelles, chloroplast or mitochondria, in most of the plants, however, in spinach (*Spinacia oleracea* L.) and waterhemp, PPO2 encodes a dual-targeting peptide that directs PPO2 to both chloroplast and mitochondria (Bi et al., 2020; Dayan et al., 2018; Patzoldt et al., 2006; Watanabe et al., 2001), which means that the mutation occurred in dual-targeting PPO2 may confer resistance in both organelles. Recent research has

demonstrated variability in cross-resistance depending on the target-site mutation that occurs in PPO2, ΔG210 evolved resistance to pyrimidinedione and diphenyl ether families, but lower resistance in thiadiazole and oxadiazole families (Rangani et al., 2019).There is an amino acid change G399A in PPO2 induced high level resistance in diphenyl ether and lower level resistance in pyrimidinedione and triazolinone in Palmer pigweed (Rangani et al., 2019). Most recently research reported that an amino acid substitution R128L can evolve cross resistance to at least three different chemical groups of PPO inhibitors: diphenyl ether, pyrimidinedione, and N-phenylphthalimide in Wild poinsettia (Mendes et al., 2020). This substitution R128L in PPO2 was reported to confer high level resistance to diphenyl ethers and pyrimidinedione, lower resistance to phenylpyrazole in common ragweed (Giacomini et al., 2017). The *in vitro E. coli* functional assay proved that the R128L mutations confers resistance to PPO inhibitors (Rangani et al., 2019; Rousonelos et al., 2012). Some other amino acid polymorphisms in R128, such as R128G and R128M, conferring resistance to fomesafen (Giacomini et al., 2017). According to all the mutations in PPO2 conferring cross resistance to other PPO inhibitors, few cases showed resistance to oxadiazole families. It is possible that some herbicides prefer one of the target organelles, for instance, the oxadiazon from oxadiazole families may only target to PPO1.

Based on these data we theorize that PPO inhibiting herbicide may be subdivided into primarily PPO1-inhibitors, primarily PPO2-inhibitors, and indiscriminate PPO-inhibitors. Oxadiazon would be classified as a primarily PPO1 inhibitor, lactofen would be classified as primarily a PPO2-inhibitor, and Sulfentrazone would be an indiscriminate PPO-inhibitor. However, we acknowledge that these data could be complete specific for goosegrass and may not extrapolate to other species. If so, reclassification of PPO-inhibiting herbicides into

subclasses may not be possible and species will need to be evaluated on a case by case basis to determine which isoform is inhibited in each species.

In conclusion, we tested the response of the chloroplast-targeted PPO1 and mitochondrial-targeted PPO2 to three different chemical groups of PPO inhibitors *in vitro*. The oxadiazon may only binding to the PPO1 isoform, and lactofen and sulfentrazone may both have binding sites in PPO1 and PPO2. However, it cannot be the direct evidence to prove which organelles the chemical groups prefer. To better understanding the relationship between herbicide chemical groups and target-site PPO genes, we need test more PPO genes containing with different mutation sites from other PPO resistant species.

4.4 References

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Table 12

Table 4.1. IC_{50} values of differential sensitivity between *E. coli* of different PPO isoforms and different PPO inhibitors.

PPO chemical families	Inhibitors	*E. coli* strains	LogIC50	IC50 (uM)	95% CI of IC50 (uM)	R2
Oxadiazoles	Oxadiazon	S-PPO1	-5.101	7.92	(4.733,12.25)	0.9619
		S-PPO2	-3.346	450.9	(283.88,731.2)	0.9072
		R-PPO1	-3.214	610.8	(380.4,1011)	0.9182
Triazolinone	Sulfentrazone	S-PPO1	-4.237	57.91	(37.22,89.12)	0.9493
		S-PPO2	-3.818	152.1	(89.59,260.8)	0.8764
		R-PPO1	-4.01	97.63	(62.21,152.2	0.9466
Diphenyl ether	Lactofen	S-PPO1	-4.026	94.18	(75.25,117.9)	0.9846
		S-PPO2	-4.55	28.19	(19.14,41.11)	0.9618
		R-PPO1	-3.964	108.8	(85.54,138.2)	0.9814

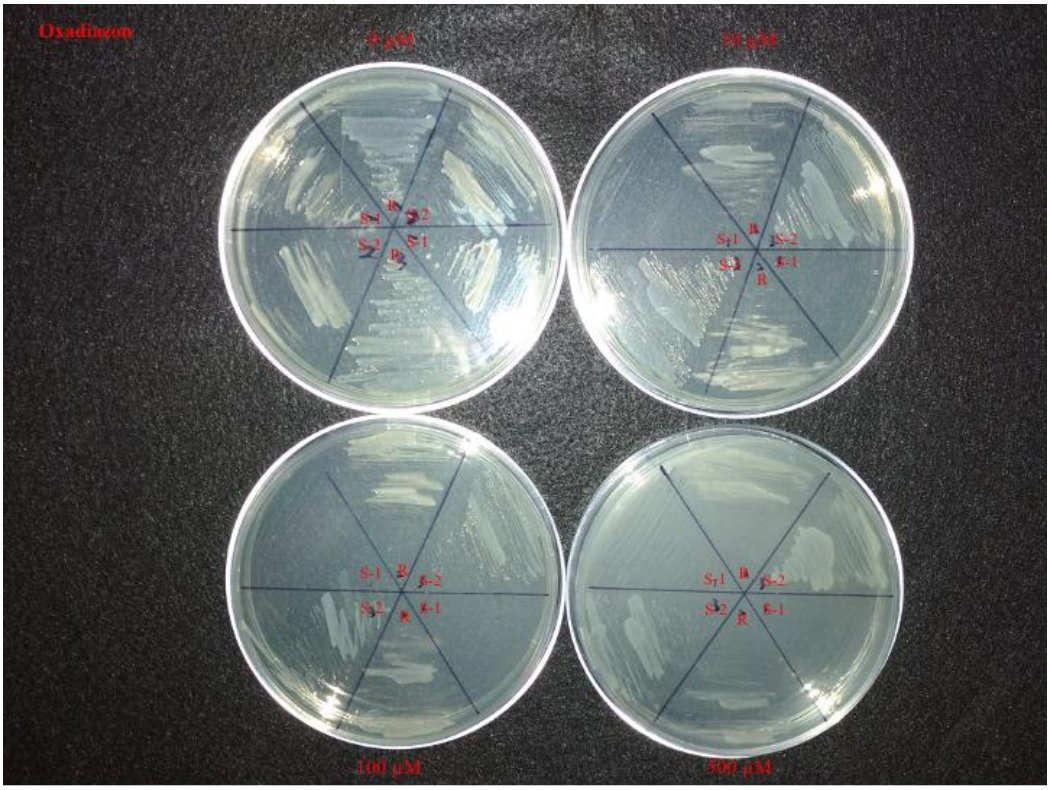

Figure 4.1 Detection of HemG mutant *E. coli* stain transformed with PPO1 and PPO2 with Oxadiazon. S-PPO1: *E. coli* strain transformed with PPO1 from susceptible goosegrass plant. R: same as R-PPO1, *E. coli* strain transformed with PPO1 from resistant goosegrass plant. S-PPO2: *E. coli* strain transformed with PPO2 from susceptible goosegrass plant.

Figure 17

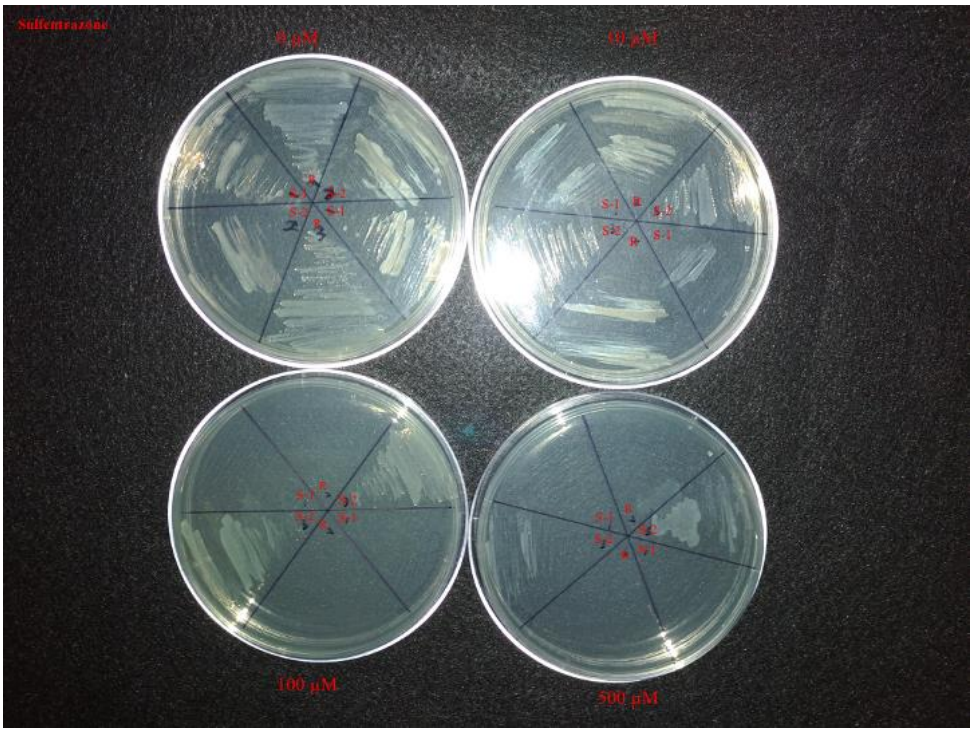

Figure 4.2 Detection of HemG mutant *E. coli* stain transformed with PPO1 and PPO2 with Sulfentrazone. S-PPO1: *E. coli* strain transformed with PPO1 from susceptible goosegrass plant. R: same as R-PPO1, *E. coli* strain transformed with PPO1 from resistant goosegrass plant. S-PPO2: *E. coli* strain transformed with PPO2 from susceptible goosegrass plant.

Figure 18

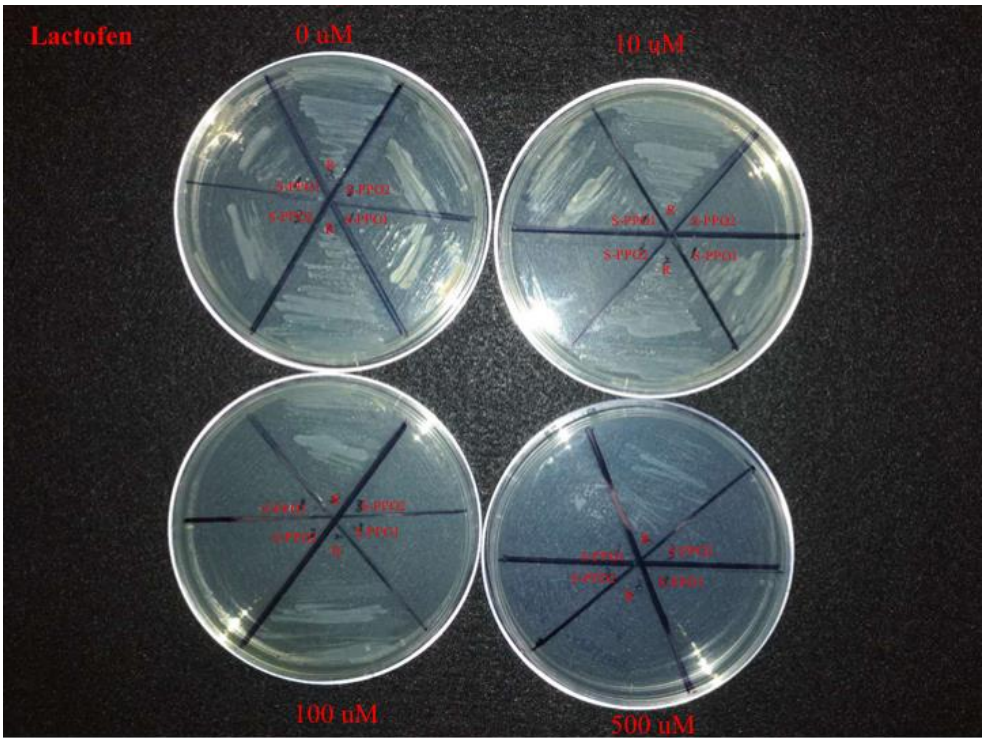

Figure 4.3 Detection of HemG mutant *E. coli* stain transformed with PPO1 and PPO2 with lactofen. S-PPO1: *E. coli* strain transformed with PPO1 from susceptible goosegrass plant. R: same as R-PPO1, *E. coli* strain transformed with PPO1 from resistant goosegrass plant. S-PPO2: *E. coli* strain transformed with PPO2 from susceptible goosegrass plant.

Figure 19

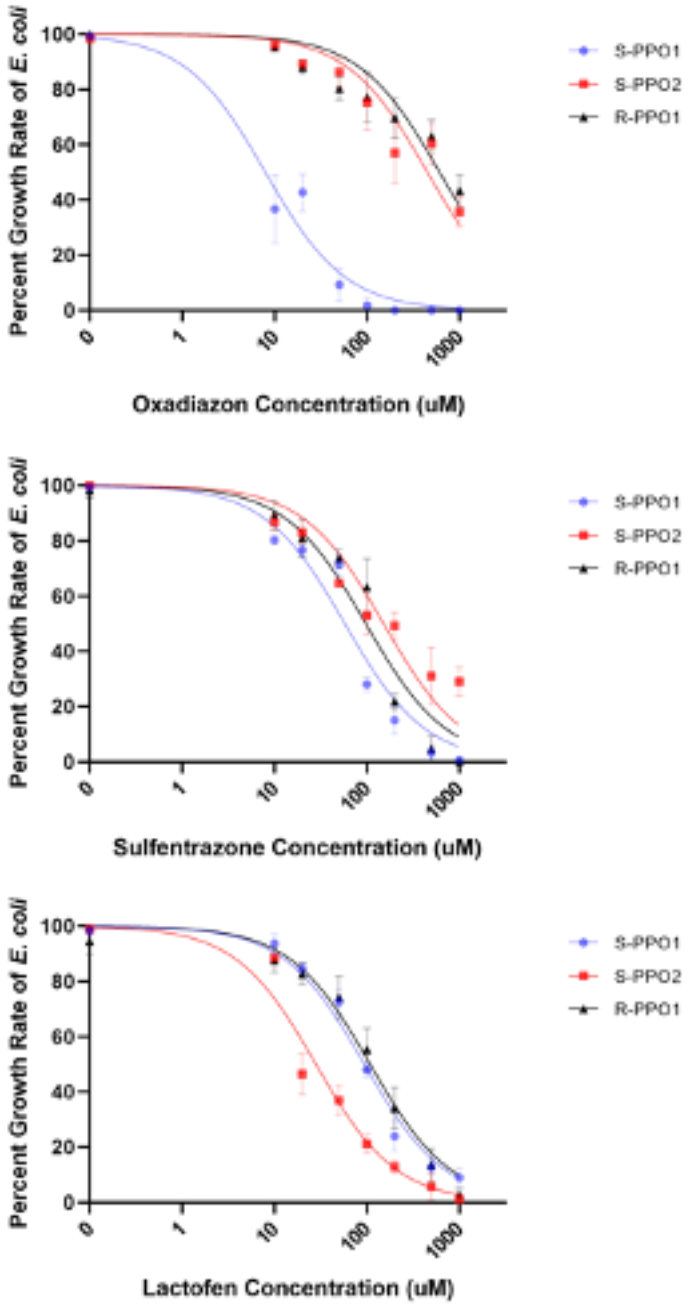

Figure 4.4 *E. coli* strain growth rate response to different inhibitors at different concentration

Figure 20