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Research Article

A population genetics approach for the study of fluridone resistance in hydrilla

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Abstract

Fluridone-resistant hydrilla was first suspected in Florida in 1999 and was confirmed using molecular genetics techniques in 2003. Although the vast majority of species that evolve resistance to herbicides or other stressors do so through the genetic mutations that occur during sexual reproduction, all hydrilla in Florida is of the dioecious pistillate ("female") biotype and all reproduction and spread is via vegetative means. The Hardy-Weinberg principle of constant allele frequencies (i.e., $p + q = 1$), used to predict allelic frequency shifts within populations due to selection, is based on a number of assumptions that are violated by species that reproduce asexually. In this paper, we address the assumptions of the model in the context of the clonally propagated species hydrilla and compare theoretical model predictions to the likely timeline of actual events that occurred in many bodies of water in Florida. The generational shifts in within-population allele frequencies from almost exclusively fluridone-susceptible to almost exclusively fluridone-resistant track well with the actual development of fluridone-resistant populations of hydrilla in Florida when considering fitness differences among fluridone resistance alleles after fluridone treatments. The present study illustrates how the Hardy-Weinberg principle of constant allele frequencies can be used as an exploratory tool to model resistance evolution in asexually reproducing species such as hydrilla.

Key words: Hardy-Weinberg, herbicide resistance, asexual reproduction, modeling, evolution, allele

Introduction

Hydrilla (*Hydrilla verticillata* (L.f.) Royle, 1839) is a Hydrocharitaceae, monocotyledonous perennial submersed plant that Langeland (1996) referred to as "the perfect aquatic weed". The species is cosmopolitan in distribution. Although hydrilla is most common in tropical regions, it has invaded waters throughout the world. There are two distinct biotypes of hydrilla; the dioecious biotype is native to India and bears pistillate ("female") and staminate ("male") flowers on separate plants, whereas the monoecious biotype is native to Korea and produces pistillate and staminate flowers on the same plant (Cook and Lüönd 1982; Madeira et al. 1997). Hydrilla is considered a noxious weed virtually everywhere outside its native range. It is a federally listed noxious weed in the United States and is listed as one of the ten worst aquatic weeds globally (ICID 2002).

Hydrilla was first introduced to Florida in the 1950s by aquarium plant growers on the east and west coasts, who intentionally planted hydrilla in canals to provide a ready source of plant material to ship to their customers. The species escaped cultivation and was found in Miami and Crystal River in 1960, although hydrilla was originally misidentified as the Florida native elodea (*Elodea canadensis* Michx.). By the time the species was correctly classified in 1967, it had already infested more than 14,000 ha of Florida's waters (Blackburn et al. 1969). Hydrilla had become established in the primary water bodies of all drainage basins in the state by the early 1970s and was present in over 20,000 ha of Florida's waters by the late 1980s (Schardt and Nall 1988).

The herbicide fluridone was labeled for aquatic use in 1986. Fluridone acts by inhibiting the enzyme phytoene desaturase (PDS) encoded by the *pds* gene, which plants use to synthesize carotenoids – pigments that help stabilize photosynthesis by protecting and preventing the breakdown of chlorophyll (Michel et al. 2004). Although the fluridone label allows resource managers to apply up to 150 ppb (μg L^{-1}) annually, rates as low as 12 ppb result in a good selectivity profile with acceptable levels of damage to many non-target plants and can yield several seasons of control, provided static exposure of 60 or more days is maintained (Netherland et al. 1993).

Its high efficacy and selectivity spectrum led Florida's resource managers to begin field evaluation of fluridone for hydrilla control soon after it was labeled and most applications employed low use rates – between 12 to 15 ppb (University of Florida IFAS Center for Aquatic and Invasive Plants 2014). Several years of trial and error were required to determine optimal concentrations and contact exposure times before fluridone was widely adopted as the primary tool for hydrilla control in Florida. However, funding for aquatic weed control in Florida was drastically reduced in the early 1990s, so all aquatic weed management strategies – including herbicide usage – were curtailed, which allowed hydrilla to invade around 80% of public waters and resulted in coverage of more than 40,000 ha by the mid-1990s (Florida Fish and Wildlife Conservation Commission 2013). Once consistent and adequate funding was made available, large-scale fluridone treatments were expanded, and fluridone became the primary herbicide used for hydrilla control.

Managers began to notice reduced fluridone efficacy in some systems around 2000. Isolated events of herbicide failure can be attributed to a number of factors, including operator error and insufficient contact exposure time due to flow, but as widespread reports of ineffective treatments rapidly increased (WT Haller, UF/IFAS Center for Aquatic and Invasive Plants, *personal communication*) it became clear that something else was responsible for this sudden apparent resistance of hydrilla to fluridone. Because Florida hydrilla reproduces exclusively by vegetative means, it is unlikely that the species had evolved resistance to fluridone as a result of a

mutation in a gamete during sexual reproduction (Hill 1982; Maxwell and Mortimer 1994). Nonetheless, some type of genetic change was the most plausible explanation for this troubling phenomenon. Although some have suggested that fluridone-resistant hydrilla evolved after 15 years of constant exposure to the herbicide, this is not an entirely accurate assessment. Fluridone was labeled for aquatic use in 1986, but a number of years between then and the evolution of resistance were "lost" due to time spent determining use patterns and a hiatus in the use of this herbicide due to government budget cuts in the mid-1990s. As a result, large-scale fluridone treatments did not become the norm until almost a decade after fluridone was labeled, and the development of resistance occurred in a shorter timeframe than the 15-year estimate when more aggressive fluridone use (i.e., higher rates and multiple applications per year) was implemented. This premise is supported by reports of rapid population shifts from susceptible to resistant hydrilla in Florida's aquatic systems. For example, Hoyer et al. (2005) indicated that susceptible hydrilla in the 1,619 ha Lake Cypress became resistant to fluridone in 2000 – only four years after the first large-scale treatment was applied in 1996. Also, Netherland and Jones (2015) reported that hydrilla in the 28,500 ha Kissimmee Chain of Lakes, where fluridone treatments were initiated in 1993, had shifted to the resistant biotype by 2000 and that fluridone use in this system was finally discontinued by 2004. Interestingly, Netherland and Jones surveyed this system in 2012 and found susceptible, moderately resistant and highly resistant biotypes, but 80 to 90% of the hydrilla sampled was of a fluridone-resistant biotype, despite eight years without exposure to fluridone.

A large-scale sampling program was instituted in Florida waters in 2001 and 2002 to evaluate the extent of fluridone-resistant hydrilla in public and private systems (Michel et al. 2004). Briefly, two hundred Florida lakes were evaluated, with between 25 and 100 hydrilla samples collected from each lake. Plant material was cultured in water containing fluridone at concentrations ranging from 0.75 to 48 ppb; after two weeks of exposure, samples were tested for dry biomass, chlorophyll, phytoene and β-carotene content. Based on the results of these experiments and genetic analyses that included sequencing of the *pds* gene, Michel et al. (2004) determined that three distinct mutations in the form of single-base substitutions were present in the plant material and that each conferred a different level of resistance to fluridone, the highest being an almost six-fold increase in fluridone resistance compared to susceptible plants. All mutations occurred in codon 304 of the *pds* gene; wild-type (susceptible) samples had the sequence coding for arginine (Arg), whereas mutants had sequences coding for serine (Ser), cysteine (Cys) or histidine (His). All resistant plants were heterozygous (Michel et al. 2004).

An additional factor that likely contributed to the shift from fluridone-susceptible to fluridone-resistant populations is hydrilla's explosive growth

and colonization potential. Conventional wisdom suggests that clonal populations are presumably genetically identical to one another and to the parent/donor source material, and these plants lack the genetic diversity necessary to survive changing environments (Stoltenberg 2004). However, in several weed species, low genetic diversity is not necessarily a limitation for survival in perturbed systems (Horak and Holt 1986; Wilen et al. 1995). Gould (1995) pointed out that "clonal" populations of asexually reproducing plants actually have significant levels of genetic variation and hypothesized that somatic mutations could be an especially important source of genetic variation in these species. Cullis (1999) stated that clonally propagated species frequently undergo spontaneous genomic mutation that can serve as a source of genetic diversity, and Jiang et al. (2011) provided evidence of this phenomenon in clonal populations of *Arabidopsis*. In bermudagrass (*Cynodon* spp.), the appearance of off-types that cause contamination in turfgrass scenarios has been associated with somatic mutations that are propagated vegetatively (Caetano-Anolles 1998; Harris-Shultz et al. 2011). Reasor et al. (2016) indicated that, in bermudagrass, somatic mutations can exceed three phenotypically different off-types per ha per year in nurseries. Therefore, although the rate of somatic mutation is considerably lower than mutations caused during sexual reproduction, the former might be enough to generate the genetic diversity that makes fluridone resistance possible.

Interestingly, it has been suggested that plants relying exclusively or primarily on asexual reproduction are more susceptible to somatic mutations that occur during mitosis. For example, Jiang et al. (2011) reported elevated mutation rates (e.g., substitutions, insertions and deletions) in *Arabidopsis* clones, which they attributed to reduced activity of DNA repair mechanisms. Other researchers have found that somatic mutation rates may increase in relation to a species' reproductive strategy – i.e., the greater the rate of asexual reproduction, the higher the incidence of somatic mutation. Reusch and Boström (2011) stated that genetic mosaicism in the marine eelgrass *Zostera marina* L. increased as clonality increased and postulated that genetic mosaics resulting from somatic mutation are more common at the limits of a species' distribution where sexual reproduction is rare or absent. Genetic mosaicism has been documented in hydrilla as well. Puri et al. (2007) found variations in ploidy level (diploid ($2n$ = 16), triploid ($2n$ = 24) and tetraploid ($2n$ = 32)) among different hydrilla biotypes, plants within each biotype, and within shoot tissues of the same plant. Tetraploidy was rare but triploidy was predominant in all biotypes. Diploid plants, which were represented in resistant biotypes only, showed evidence of endoreduplication, but endoreduplication was not noted in triploid or tetraploid plants.

The phenomenon of fluridone resistance in the clonally reproducing species hydrilla has been well-documented and is attributed to somatic mutation. Individual plants of hydrilla are now understood to be genetic

mosaics due to somatic mutation, and individuals within or among populations likely differ from one another due to somaclonal variation (Michel et al. 2004). Amino acid substitutions that confer resistance to enzyme-inhibiting herbicides are not a new phenomenon. For example, the first ALS (acetolactate synthase)-inhibiting herbicide was introduced in 1982 and the first case of resistance was reported a mere five years later (Tranel and Wright 2002). Resistance to ALS herbicides is more common than any other type of herbicide resistance and has thus far been documented in 159 species (Heap 2017); the extraordinary number of cases may be due to the fact that resistance is conferred by a single dominant gene that is encoded in the nucleus (Tranel and Wright 2002). In contrast, resistance to fluridone and other carotenoid biosynthesis inhibitors in Group F1 of the Herbicide Resistance Action Committee (HRAC) or Group 12 of the Weed Science Society of America (WSSA) is much less common. Heap (2017) reports that only four species exist with documented resistance to these types of herbicides; of these, three species reproduce sexually. Only the vegetatively propagated hydrilla has been reported as resistant to fluridone (Dayan et al. 2014). This low frequency of resistance evolution cases could be partially due to the lower selection pressure Group F1 herbicides exert in comparison to other more commonly used herbicides such as ALS inhibitors and glyphosate (Dayan et al. 2014), while hydrilla has been more consistently exposed to fluridone due to its original high efficacy.

Herbicide resistance (HR) evolutionary models that are based on demographic and genetic parameters allow predicting how soon HR will be noticed (Gressel and Segel 1990; Maxwell et al. 1990), and more importantly, how variation in weed control strategies might delay increasing frequencies of the HR trait within a population (Gressel and Segel 1990; Neve 2008; Neve et al. 2011). Most models have been developed for monogenic dominant HR traits in diploid species (Jasieniuk et al. 1996). These models explicitly or implicitly rely on the Hardy-Weinberg equation (Diggle and Neve 2001; Jasieniuk et al. 1996; Maxwell et al. 1990; Neve 2008), a hallmark of population genetics, which is often used to predict allele and genotype frequencies within a population and to track changes in these frequencies from one generation to the next (Hardy 1908; Weinberg 1908). The Hardy-Weinberg equation is based on the Hardy-Weinberg principle that allele frequencies remain constant over time (Waples 2015). However, this principle is based on the following set of assumptions:

1) No selective forces are acting on the population;
2) No new alleles are introduced to the population as a result of mutation;
3) There is no migration into or out of the population;
4) The population size is infinitely large; and
5) Random mating and equal production of offspring occurs among all individuals in the population (Hartl and Clark 1997; Waples 2015).

Deviations from Hardy-Weinberg predictions indicate that factors such as selection and drift might be affecting the frequency of a given allele, but Hardy-Weinberg allelic changes will still be proportional to the number of alleles present in the population. Thus, for a two-allele system, $p + q = 1$, where p and q are two alleles of a given gene or locus. Although the mechanisms of fluridone resistance in Florida populations of hydrilla are understood, predicting the evolution of herbicide resistance—or any heritable trait—in populations of asexually reproducing species can be challenging because presumably the frequencies of the resistant and wild-type (WT) alleles are independent from each other.

We hypothesized that because of the dominant nature of fluridone resistance alleles (i.e., homozygous and heterozygous individuals both exhibit high levels of resistance) and the stem fragmentation-based propagation of hydrilla, the evolutionary rate of fluridone resistance in the species could be modeled based on the Hardy-Weinberg principle of constant allele frequencies. The Hardy-Weinberg genetic model is geared entirely toward sexually reproducing populations, and hydrilla clearly violates the assumptions associated with it, but we considered that when properly accounting for fitness differences among genotypes after fluridone exposure, changes in the frequencies of WT and resistant alleles might still be used to predict changes in resistance over time using the Hardy-Weinberg principle of constant allele frequencies. Therefore, our objective was to determine whether a binomial approach to allele frequencies (i.e., $p + q = 1$) based on this principle could be used to model shifts in genotype frequencies within populations of a species that reproduces only by asexual means and to predict the effects of fluridone selection on population structure over time.

Materials and methods

Modelling changes in population structure in this exercise requires making a set of assumptions. For example, the precise rate of mutation in the 304 codon that is responsible for fluridone resistance in hydrilla is unknown, so we modeled fluridone resistance evolution for several initial frequencies of mutant individuals in the population ranging from one in one million to one in one billion. This latter value has been used for models of resistance evolution for herbicides with lower mutation rates such as the gene encoding EPSPS for glyphosate resistance (Neve 2008; Neve et al. 2011). Therefore, our model assumes that a given population with a mutation rate of one in one billion would have initial genotypic frequencies of q = f(mutant) = 0.000000001 and p = f(wild-type) = $1-q$ = 0.999999999 prior to selection. This model was run for hypothetical resistant mutations conferring 90, 95, and 98% survival and 10% survival of WT after fluridone treatment (Netherland and Schearer 1996). We also assumed that mutations conferring resistance to fluridone are neutral under non-selection conditions

(i.e., the mutation does not reduce fitness in plants that are not being treated with fluridone). Hydrilla does not reproduce sexually and lacks a true "generation", but the species is a perennial that grows almost continually in Florida. Treatments with fluridone typically occur on an annual basis, so in these exercises we assume that one year is roughly equivalent to one generation (i.e., cycle of selection).

With these assumptions in mind, we predicted population shifts from susceptible (predominantly WT plants with a single rare mutant) to resistant (predominantly mutants with rare WT plants) using the Hardy-Weinberg principle of constant allele frequencies and the equation $p + q = 1$. Additionally, because mixed populations comprising susceptible, slightly resistant, moderately resistant and highly resistant biotypes of hydrilla have been reported (Netherland and Jones 2015), we evaluated changes in the frequency of each mutation for independent populations, and then we modeled how fluridone resistance would evolve when all three types of mutation are present in a given population. Fitness coefficients after fluridone treatment were developed for each genotype based on biomass accumulation compared to untreated plants; these corresponded to 0.10, 0.38, 0.58 and 0.72 for wild-type, Ser304, Cys304 and His304 mutants, respectively, after treatment with 4 ppb of fluridone (Michel et al. 2004). As with any population undergoing selection, genotype frequencies were adjusted after each cycle of selection by dividing each frequency by the total frequency of individuals remaining after selection. For example, consider an unselected population comprising the wild-type genotype at a frequency of $p = 0.999999999$ and the Ser304 mutant at a frequency of $q = 0.000000001$. Fitness values of the wild-type and Ser304 genotypes are 0.10 and 0.38, respectively, so after a single cycle of selection the remaining genotypes would be $p = (0.999999999)(0.10) = 0.0999999999$ and $q = 0.000000001(0.38) = 0.00000000038$. Total population size would be $0.0999999999 + 0.00000000038 = 0.10000000028$, so the frequency of genotypes after a single cycle of selection would be $p = (0.0999999999/0.10000000028) = 0.9999999962$ and $q = (0.00000000038/0.10000000028) = 0.0000000038$. These frequencies were then used to model the second cycle of selection and so on until a shift from almost completely susceptible to almost completely resistant was achieved. When modelling populations with all three resistant biotypes, the proportion of surviving plants was adjusted at the end of each cycle of selection by the relative frequency of each biotype based on their fitness coefficient. In this way, we were able to model changes in frequency for each resistant allele under the assumption that biomass production within the population for each biotype is directly related to their fitness after fluridone treatment and no uneven competitive relationships were present among biotypes. The number of generations under continuous fluridone exposure in which a population shift to a resistant biotype occurred (i.e., frequency of resistant mutation ≥ 0.5 and ≥ 15% survival

Table 1. Effects of different selection intensities and initial mutant frequencies on the development of fluridone resistance in hydrilla; shift (noticeable loss of efficacy): 15% alive after selection (in most cases: also when resistant allele frequency > 50%).

Biotype	Selection intensity resistant	Selection intensity wild type	Initial resistant allele frequency	% alive at shift	Allele frequencies at shift	Number of selection cycles at shift
Ser304	0.38	0.1	1 in 1 billion	20.12%	0.407wt:0.593Ser304	16
	0.38	0.1	1 in 100 million	19.90%	0.477wt:0.523Ser304	14
	0.38	0.1	1 in 10 million	15.96%	0.554wt:0.446Ser304	12
	0.38	0.1	1 in 1 million	21.33%	0.343wt:0.657Ser304	11
Cys304	0.58	0.1	1 in 1 billion	19.60%	0.408wt:0.592Cys304	12
	0.58	0.1	1 in 100 million	24.45%	0.286wt:0.714Cys304	11
	0.58	0.1	1 in 10 million	15.45%	0.574wt:0.426Cys304	9
	0.58	0.1	1 in 1 million	18.68%	0.438wt:0.562Cys304	8
His304	0.72	0.1	1 in 1 billion	26.89%	0.271wt:0.729His304	11
	0.72	0.1	1 in 100 million	31.21%	0.211wt:0.789His304	10
	0.72	0.1	1 in 10 million	15.65%	0.581wt:0.419His304	8
	0.72	0.1	1 in 1 million	17.58%	0.500wt:0.500His304	7
Any mutant	0.90	0.1	1 in 1 billion	32.34%	0.222wt:0.777mut	10
	0.90	0.1	1 in 100 million	34.07%	0.205wt:0.795mut	9
	0.90	0.1	1 in 10 million	35.88%	0.189wt:0.811mut	8
	0.90	0.1	1 in 1 million	37.76%	0.173wt:0.827mut	6
Any mutant	0.95	0.1	1 in 1 billion	15.29%	0.613wt:0.387mut	9
	0.95	0.1	1 in 100 million	15.55%	0.601wt:0.399mut	8
	0.95	0.1	1 in 10 million	15.82%	0.589wt:0.411mut	7
	0.95	0.1	1 in 1 million	16.10%	0.576wt:0.424mut	6
Any mutant	0.98	0.1	1 in 1 billion	16.90%	0.545wt:0.455mut	9
	0.98	0.1	1 in 100 million	17.03%	0.540wt:0.460mut	8
	0.98	0.1	1 in 10 million	17.16%	0.535wt:0.465mut	7
	0.98	0.1	1 in 1 million	17.30%	0.530wt:0.470mut	6

after fluridone treatment) was used to compare the predictions of the models with reports by managers about noticeable reductions in fluridone efficacy under field conditions in Florida.

Results and discussion

Using the simplest model, which assumes no fitness penalties, fluridone resistance was predicted after ten cycles of continuous selection when the original mutation rate was 1.0×10^{-9} and six cycles of selection with a mutation rate of 1.0×10^{-6} regardless of a survival rate between 90 and 98% (Table 1). These predictions represent a much faster evolution rate than the *ca.* 15 cycles of selection reported by managers. Although it is likely that there was a lag period between the actual population shift for resistance and when managers noticed it, it is unlikely that five to nine years passed without managers noticing such a dramatic change. Therefore, it is possible that the mutation rate is considerably lower than 1.0×10^{-9} and/or other factors might be modulating resistance evolution.

Although initial mutation rate is important for assessing changes in resistance over time, genetic models have shown that the combination of selection intensity, population size, and fitness changes are more important for determining changes in mutation rates in finite asexual populations (Andre and Godelle 2006). In order to improve the accuracy of the model, we assigned fitness values after fluridone treatment to the mutations conferring resistance to fluridone. Michel et al. (2004) found that biomass

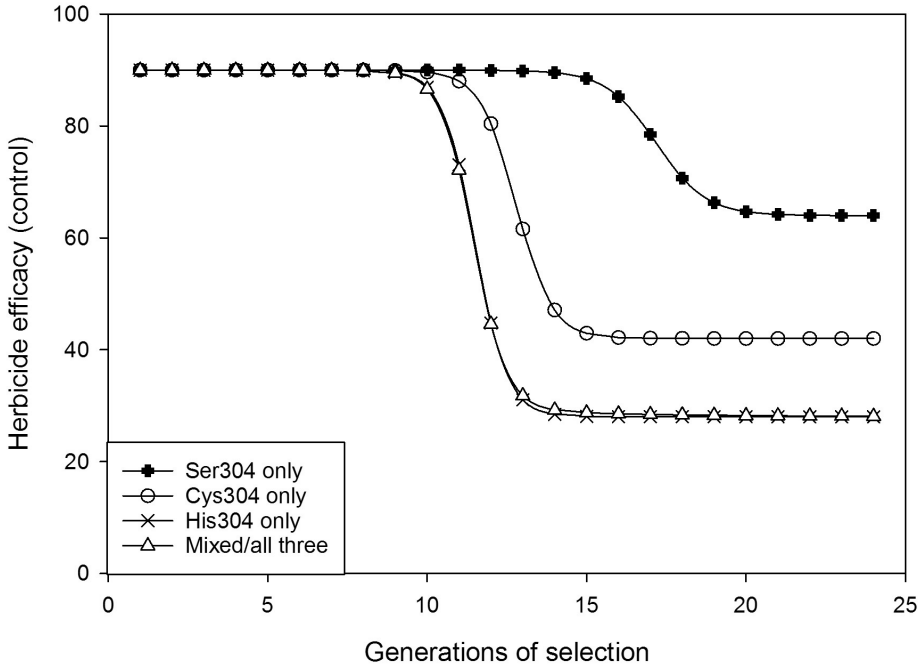

Figure 1. Predicted reductions in fluridone efficacy on Florida populations of hydrilla in response to changes in genotype frequencies resulting from selection. The model assumes 1) that the initial population is predominantly wild-type (susceptible); 2) one year is roughly equivalent to one cycle of selection/generation; 3) the mutation conferring resistance to fluridone is neutral under non-selection conditions; and 4) absolute fitness values (based on biomass accumulation compared to untreated plants) of wild-type, Ser304, Cys304 and His304 mutants are 0.10, 0.38, 0.58 and 0.72, respectively. Populations coded as "Ser304 only", "Cys304 only" and "His304 only" are assumed to initially host a single plant with a single resistance mutation. The population coded as "Mixed/all three" is assumed to initially host a single plant of each of the three mutant biotypes.

accumulation differed among susceptible wild-type and the three fluridone-resistant mutants. Wild-type susceptible plants treated with 12 nM (4 ppb) fluridone produced 90% less biomass than untreated plants, whereas Ser304, Cys304 and His304 mutants generated 62, 42 and 28% less biomass, respectively, than untreated plants from the same respective mutant populations. In other words, susceptible plants produced 10% as much biomass as untreated plants and Ser304, Cys304 and His304 mutants achieved 38, 58 and 72% of the biomass of untreated plants. In our model, fitness was considered equivalent to the probability of survivorship after fluridone treatment, so these biomass data can be viewed as a proxy for fitness. In the case of hydrilla, this corresponds to absolute fitness as opposed to relative fitness, as survivability of each genotype is not influenced by survivability of other genotypes assuming no asymmetric competition among genotypes.

Based on this model, a population hosting a single Ser304 mutant (low level of resistance) would be expected to require 16 cycles of selection (i.e., annual applications) before an appreciable loss in herbicide efficacy was noticeable (20.1% of the population remaining after treatment *vs.* 10% remaining in a pure-stand wild-type population) (Figure 1). Allele frequencies at this point would be 0.407 wild-type and 0.593 Ser304 (Figure 2). Efficacy would continue to decline until reaching a stable state after 20 cycles of

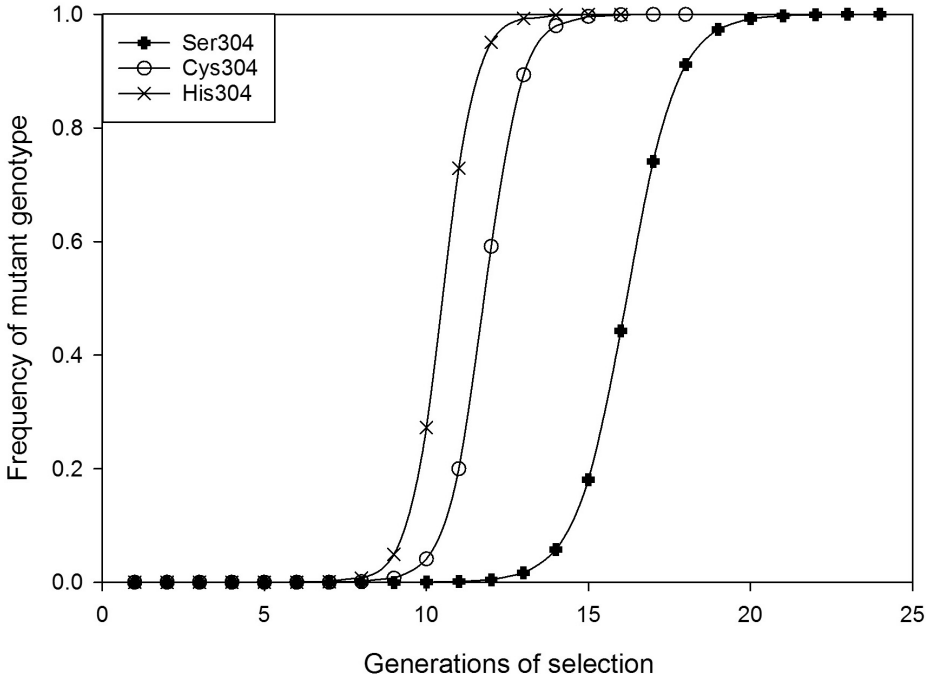

Figure 2. Predicted changes in the frequency of mutant genotypes in Florida populations of hydrilla in response to selection with fluridone. The model assumes 1) that the initial population is predominantly wild-type (susceptible) and hosts a single plant with a single resistance mutation; 2) one year is roughly equivalent to one generation; 3) the mutation conferring resistance to fluridone is neutral under non-selection conditions; 4) absolute fitness values (based on biomass accumulation compared to untreated plants) of wild-type, Ser304, Cys304 and His304 mutants are 0.10, 0.38, 0.58 and 0.72, respectively.

selection; allele frequencies in the population would be 0.003 wild-type and 0.997 Ser304 and *ca*. 38% of the population would be expected to survive the herbicide treatment.

Herbicide efficacy in populations with a single Cys304 mutant (moderate resistance) would be reduced more quickly. Twelve cycles of selection would result in noticeable loss of efficacy, with ca. 20% of the population remaining after treatment vs. 10% remaining in a pure-stand wild-type population (Figure 1), and allele frequencies of 0.408 wild-type and 0.592 Cys304 (Figure 2). Efficacy would reach a stable state after 16 cycles of selection; allele frequencies would be 0.001 wild-type and 0.999 Cys304, and 58% of the population would be expected to survive treatment.

Herbicide efficacy in populations with a single His304 mutant (high resistance) would be reduced the most quickly. Eleven cycles of selection would result in noticeable loss of efficacy, with ca. 27% of the population remaining after treatment vs. 10% remaining in a pure-stand wild-type population (Figure 1), and allele frequencies of 0.271 wild-type and 0.729 His304 (Figure 2). Reductions in efficacy would stabilize after 14 cycles of selection; allele frequencies would be 0.001 wild-type and 0.999 His304, and 72% of the population would be expected to survive treatment.

The predicted allele frequencies described above correspond well with observations of Florida populations of hydrilla between the time fluridone was introduced in 1986 and the first reductions in efficacy were noted ca.

Table 2. Mixed population with all 3 mutants each with an initial allele frequency = 1 in a billion and an initial frequency for the wild type allele = 999,999,997 in a billion.

Biotype	Initial frequency	Selection intensity	% alive at shift	Allele frequency at shift	Number of selection cycles at shift
wt	0.999999997	0.1	27.84%	0.2533014896	11
Ser304	0.000000001	0.38		0.0006043066	
Cys304	0.000000001	0.58		0.0632915416	
His304	0.000000001	0.72		0.6828026622	

15 years later, since the model predicted that fluridone would become noticeably less effective in 11, 12 or 16 years in populations hosting a single His304, Cys304 or Ser304 mutant, respectively (Figure 1). However, these estimates might be more applicable to larger areas comprising a wide range of conditions and might not be accurate for specific situations in which large-scale fluridone treatments were less common (i.e., before the mid-1990s) or where selection pressure was stronger due to repeated applications or higher fluridone rates. For example, resistance was reported in as little as 4 years in one population (Hoyer et al. 2005). This latter case might be closer to the scenario in our model with high selection pressure (> 0.98) and a relatively high initial frequency of the resistant allele (1×10^{-6}) that predicted a shift after 6 selection cycles (Table 1).

Our model assumes the presence of a single mutant plant in a population before selection, but in reality, it is much more likely that such populations actually host multiple mutants with varying degrees of resistance, which would cause populations to shift from susceptible to resistant more quickly than predicted. Therefore, we modeled fluridone resistance evolution in a population carrying the three resistant mutations assuming they occurred at the same time and at the same original frequency (Figure 1, Table 2). The results showed that the population shift occurred after 11 cycles of selection, so the presence of multiple alleles did not accelerate resistance rate. Thus, it seems that His304, which was the mutation with the lowest fitness penalty after fluridone treatment, was the main driver of the population shift towards fluridone resistance.

The results suggest that the Hardy-Weinberg principle of constant allele frequencies appears to predict changes in the genetic structure of populations of hydrilla that, like other asexually reproducing species, violates some or all of the assumptions associated with this principle, particularly when the species is being managed to reduce invasions. For example:

1) The assumption that no selective forces are acting on the population is violated when herbicide treatments are applied; in the case of hydrilla, fluridone applications selectively eliminate wild-type susceptible members from the population
2) The assumption that no new alleles are introduced to the population as a result of mutation is violated, as somatic mutation is known to be responsible for fluridone resistance in hydrilla

3) The assumption that there is no migration into or out of the population may be met, but may be violated if fragments from geographically discrete populations are introduced via contaminated boats or other watercraft
4) The assumption that population size is infinitely large is rarely met (even by sexually reproducing species), so violation of this assumption occurs when evaluating most populations
5) The assumption that random mating and equal production of offspring occurs among all individuals in the population is violated because hydrilla in Florida is of the dioecious pistillate biotype; no pollen source is present, so the species reproduces strictly by asexual means

In this case, assumptions 1 to 3 are not critical because we are using the Hardy-Weinberg principle to determine the change in allele frequencies over time caused by selection, and we know that the resistance allele is present either as a result of a new mutation or immigration. Assumptions 4 and 5 are more important because they determine the stability of the allele in the population by minimizing the effect of genetic drift and ensuring its "random" distribution within the population. In other words, the probability of the allele being passed on to the next generation is not limited to the survival of a single or very few individuals. We think that a somatic mutation that generates an individual that is resistant to fluridone is, by itself, not enough to explain our results without considering what this individual represents for the size of the population. Instead, for practical purposes, this resistant individual likely behaves more like a group of individuals due to the aggressive growth of this species and the fact that shoot fragmentation multiplies the trait within a generation. Thus, this rapid clonal growth acts as a force that counterbalances reductions in population size resulting from fluridone applications and avoids dramatic deviations from assumption 4. Furthermore, because this propagating process is presumably the same among fluridone resistance and WT alleles, the chances of these alleles being present in the next generation are likely the same in the absence of the herbicide (assumption 5).

As shown in Table 1, using values of survival based on traditional estimates of control overestimates the rate of HR evolution. Conversely, when used in conjunction with appropriate fitness coefficients to adjust for plant/population growth after fluridone treatment, the model based on Hardy-Weinberg principle of constant allele frequencies provided HR evolution rates that were closer to those observed in Florida. By considering biomass production after fluridone treatment, it is possible to better account for changes in population size between cycles of selection and at the same time allele frequency. Furthermore, this biomass-based fitness estimate compensates for differences in heritability rates between WT and resistance alleles (i.e., violation of assumption 5) caused by

fluridone treatment. Michel et al. (2004) proposed that biomass production is likely a key component for the development of fluridone-resistant populations of hydrilla in Florida's waters. Thus, they outlined several factors that might be involved:

- **The target site** for fluridone activity is the PDS enzyme encoded by the *pds* gene. Recall that carotenoids from PDS ultimately serve a protective role and prevent the degradation of chlorophyll and cellular membranes by reactive oxygen species; as a result, fluridone is most effective in the upper strata of the water column, where plants are exposed to high light intensities and need sufficient amounts of carotenoids from PDS in order to prevent necrosis. Hydrilla has an extremely low light compensation point and can grow in 1% sunlight (Van et al. 1976); thus, susceptible plants in the lower strata of the water column are less likely to suffer necrosis associated with a deficit of PDS because light intensities are low. Resistant plants near the surface of the water carrying the *pds* gene might be injured but they are able to survive exposure to fluridone by receiving resources from plant material in lower levels of the water column
- **The release from apical dominance** due to destruction of apical meristems results in a higher chance for somatic mutations in axillary meristems. Apical meristems near the surface of the water (where fluridone is most effective) are destroyed, which allows development of secondary meristems that may house cells with *pds* mutations
- **Vegetative reproduction** is the sole means of propagation and spread in Florida populations of hydrilla because plants are dioecious and produce only pistillate flowers. Individual plants of hydrilla are understood to be genetic mosaics (Michel et al. 2004; Puri et al. 2007) and single-node fragments of hydrilla can produce an entire plant, so a mutation in any part of the plant—including stem and root fragments, tubers and turions—would be transmitted to new plants produced vegetatively. Because of this particular chimeric situation, mortality of individual plants does not accurately represent the demographic dynamics driving fluridone resistance evolution in hydrilla.

Conclusions

The present study illustrates the value of considering the use of the Hardy-Weinberg principle of constant allele frequencies (i.e., $p + q = 1$) for modeling herbicide resistance rate in populations of asexually propagated plant species, especially when the herbicide resistance trait is controlled by a single dominant allele and population growth is high enough that the risk of genetic drift is largely diminished. Although this modeling approach relies on multiple assumptions that might limit its accuracy, our results indicate that it might be a useful exploratory tool to approximate the

timeframe in which herbicide resistance evolution could occur. Additionally, the results showed that because there is a direct relation between biomass production and the frequency of the resistance allele, the accuracy of the herbicide resistance evolution model can be increased by taking into account how biomass production is affected by variations in the impact of the resistance allele on fitness and on herbicide injury. More research is needed to determine how fluridone rates affect the prediction of the model because it is likely that biomass production will vary for each biotype depending on this factor.

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