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Transgenerational phenotypic responses to herbicide stress are more rapid than to shade and simulated herbivory in Arabidopsis

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SUMMARY

Weeds in agricultural settings continually adapt to stresses from ecological and anthropogenic sources, in some cases leading to resistant populations. However, consequences of repeated sub-lethal exposure of these stressors on fitness and stress "memory" over generations remain poorly understood. We measured plant performance over a transgenerational experiment with *Arabidopsis thaliana* where plants were exposed to sub-lethal stress induced by the herbicides glyphosate or trifloxysulfuron, stresses from clipping or shading in either one (G1) or four successive generations (G1–G4), and control plants that never received stress. We found that fourth-generation (G4) plants that had been subjected to three generations of glyphosate or trifloxysulfuron stress produced higher post-stress biomass, seed weight, and rosette area as compared to that produced by plants that experienced stress only in the first generation (G1). By the same measure, clipping and shade were more influential on floral development time (shade) and seed weight (clipping) but did not show responsive phenotypes for vegetative metrics after multiple generations. Overall, we found that plants exhibited more rapid transgenerational vegetative "stress memory" to herbicides while reproductive plasticity was stressor dependent and similar between clipping/shade and anthropogenic stressors. Our study suggests that maternal plant stress memory aids next-generation plants to respond and survive better under the same stressors.

Keywords: glyphosate, shade, simulated herbivory, sub-lethal dose, transgenerational stress, trifloxysulfuron.

INTRODUCTION

Phenotypic plasticity is the ability of an organism to shift morphology or physiological processes to acclimate to environmental stressors in the absence of concomitant changes in the genotype. Plants often cope with environmental and anthropogenic stressors through plastic changes to growth habit, or the production of defensive compounds (Stotz et al., 2021). The degree to which these changes occur varies among species and stressors, but these changes are commonly observed through genotype by environment interactions within a population (Sultan, 2003). Importantly, these changes can happen within and across generations – i.e., transgenerational plasticity or transgenerational effects (Wadgymar et al., 2018). Transgenerational effects result when plant phenotypes in progeny are influenced by the environmental conditions faced by previous generation(s) (Alvarez et al., 2020; Groot et al., 2016, 2017), and can be beneficial when parent and progeny environments are similar (Lampei, 2019). The inheritance of this "primed state" in the progeny is called transgenerational memory or stress memory (Murgia et al., 2015).

Transgenerational plasticity can be inherited due to epigenetic mechanisms, which are non-DNA-sequence-based modifications such as DNA methylation, histone modifications, or non-coding small RNAs (Holeski et al., 2012; Schlichting & Wund, 2014). For instance, Boyko et al. (2010) showed that transgenerational adaptation of Arabidopsis to cold, heat, salt, ultraviolet C, and flood requires DNA methylation and smRNA silencing pathways. Similarly, there is empirical evidence of transgenerational phenotypic change in Arabidopsis due to salt (Groot et al., 2017), heat stress (Suter & Widmer, 2013), herbivory (Rasmann et al., 2012), and pathogen infection (Luna et al., 2012; Slaughter et al., 2012). For salt stress, imposition of 50 mM NaCl over three generations resulted in bigger leaves and larger rosette diameter. Heat stress (40°C, 2 h for 3 days) over three generations in *Arabidopsis thaliana* caused accelerated flowering (Suter & Widmer, 2013). Thus, offspring inherited the parental phenotype that developed in response to stressful conditions and maintained improved fitness in subsequent generations.

Weeds in agricultural environments face both typical environmental stressors (e.g., temperature, moisture, etc.), along with anthropogenic stressors such as herbicides (Sharma et al., 2021). Modern agriculture relies heavily on herbicides to control weeds, applying 2.2 billion Kg in 2019 across the globe (fao.org/faostat/en/#data/RP/visualize). However, repeated use of herbicides with the same site of action has led to weeds developing widespread resistance (Jasieniuk et al., 1996). Herbicide resistance falls into two broad categories: target site resistance (TSR), which includes structural changes to herbicide binding sites or increased expression of the target gene (Délye, 2013), and non-target site resistance (NTSR), which includes mechanisms through which weeds minimize the herbicide concentration at its site(s) of action to non-toxic levels (Busi & Powles, 2009). TSR is much better understood, but the impact of NTSR herbicide-resistant weeds can be particularly consequential as it can confer cross resistance to herbicides with other modes of action, including those not yet developed (Petit et al., 2010). Recent studies by Benedetti et al. (2021) and Vieira et al. (2020) showed that a sublethal dose of herbicide stress for three consecutive generations can cause reduction of herbicide sensitivity. Herbicide-resistant weeds present an important threat to agriculture globally, creating an urgent need to better understand how weeds adapt to herbicide stress over generations.

Here we sought to evaluate the transgenerational phenotypic responses of *A. thaliana* to transient (single generation) and recurrent (over multiple generations) stresses induced by sub-lethal exposure to two common herbicides (glyphosate and trifloxysulfuron) and two common non-chemical "ecological" stressors, namely shading and clipping. Here we are defining "ecological" stresses as common stresses a weed would experience that are non-chemical. This study will help to understand the heritability of "stress memory" induced by different types of stresses. We focus on comparing the phenotypic responses following recurrent sub-lethal doses of herbicides and clipping/-shade stresses in *A. thaliana*, which varied in the number of generations (0, 1, or 4) they received each stress. This design allowed us to address three key questions: (i) What are the transgenerational effects of herbicide and ecological stresses on the phenotypes of offspring? (ii) What are the differences between herbicide and ecological stress responses? (iii) How quickly do plants recover after a single generation of stress exposure? Results from this study will help to better understand the evolution of stress responses, including herbicide resistance, and how these responses differ from those of non-chemical stresses experienced by weeds.

RESULTS

Although all four stresses substantially injured treated Arabidopsis plants, all plants were able to recover as expected and produced progeny that exhibited changes to plant growth and reproduction compared to control plants, and these changes were transient over subsequent generations. All four stresses reduced Arabidopsis aboveground biomass, total seed weight, and rosette area in G1 relative to the untreated plants (Figures 1, 2 and 3a–c), while the effect on flowering time varied among stressors (Figure 3d). When stressors were either repeated in each generation or not repeated, the subsequent G1–G4 generations responded with various phenotypic changes, ranging from a shift in flowering time to changes in biomass and seed weight (Figures 2 and 3).

A single application of stress in the first generation

Plants treated only in the first generation and then grown without repeating the experimental stress for the following three generations (G2–G4) were defined as "Unigenerational stress." In the generation following stress (G2), the progeny of unigenerational-stressed plants grew similar to pre-stress or control plants for aboveground biomass, total seed weight, and rosette area (Figures 1 and 3; Table S1). However, in the case of flowering time, we found that unigenerational-shade-stressed plants flowered ~2 days earlier than control plants in the G2 generation (Figure 3d). By the G3 generation, all phenotypic metrics measured from progeny of unigenerational-shade-treated plants were equivalent to those of the control.

Repeated stress over four generations

Another set of plants were repeatedly stressed in each of four generations (G1–G4). Plants treated with herbicides for multiple generations compensated for these stresses after G1 and achieved the same biomass as untreated controls, although the number of generations needed to respond to the stresses varied (Figures 1 and 3; Table S1). For example, the biomass of glyphosate-treated plants was the same as the untreated control by G3 (Figure 3a), whereas trifloxysulfuron-treated plants achieved equivalent levels of biomass as untreated controls in G4. Herbicide-treated plants had 128% (trifloxysulfuron) and 99% (glyphosate) greater biomass in G4 compared to G1, respectively (Figures 1, 3a and 4–6).

In contrast, clipped and shade-stressed plants did not demonstrate the same degree of transgenerational stress response as the herbicide-stressed plants, showing just 30 and 8% greater biomass in G4 compared to G1, respectively (Figures 1, 3a and 4a). Overall, comparing transgenerational recovery of biomass for ecological (clipping and shading) and herbicide (glyphosate and trifloxysulfuron)-stressed

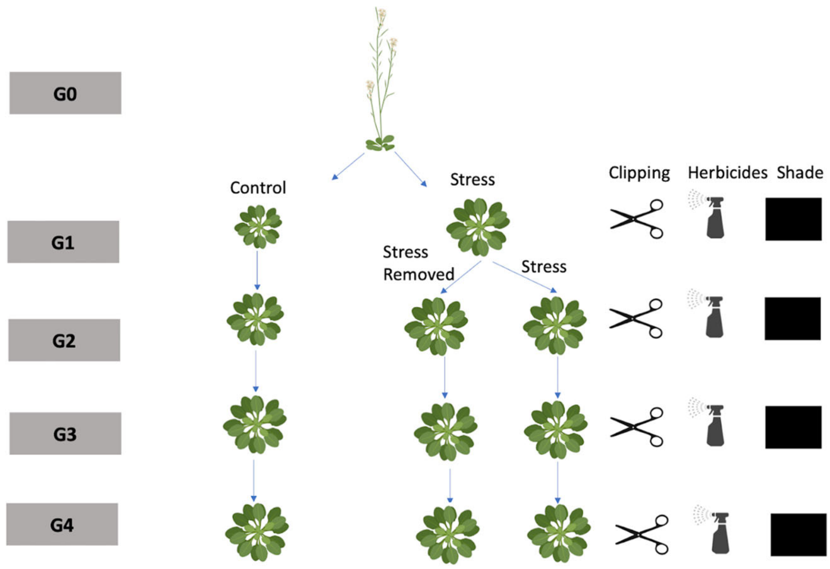

Figure 1. Schematic representation of the treatments across the four experimental generations G1–G4 all derived from a single seed mother plant at G0. Arabidopsis plants in each generation were exposed to a sub-lethal dose of herbicides, clipping, or shading as well as untreated controls. Stress removed = Plants from G1 stress progeny did not receive any stress in subsequent generations.

plants, we found that herbicide-treated plants showed significantly greater biomass in G4 compared to G1, while ecologically stressed plants exhibited similar biomass in G4 and G1 (Figures 1 and 4a).

Plants in G4 had higher seed weight than plants in G1 across all four treatments (Figures 1, 3b and 4b). Trifloxysulfuron-treated plants showed 432% greater seed weight in G4 compared to G1 (Figures 3b and 4b–d). Similarly, shade-, glyphosate-, and clipping-treated plants had 342, 67, and 60% higher seed weight, respectively, in G4 when compared to G1. Whereas we found that herbicide-stressed plants have 366% greater seed weight compared to ecological stresses in G4 (Figures 1 and 4b; Table S1).

For rosette area, we found 45% greater area for glyphosate-treated plants and 105% higher in trifloxysulfuron-treated plants between G4 and G1 plants. In contrast, shade-treated and clipped plants showed similar rosette areas in G1 and G4 (Figures 3c and 4c). These results are in accordance with total seed weight and aboveground biomass.

Trifloxysulfuron and shading caused a significant delay in flowering of Arabidopsis plants. In G1 trifloxysulfuron-treated plants flowered 61 days after sowing (DAS) and shaded plants flowered 53 DAS (Figures 1 and 3c; Table S1). Whereas in G4, we found trifloxysulfuron-treated plants flowered 38 DAS and shade-treated plants flowered 35 DAS (Figure 3d). Additionally, we did not find any change in flowering time for clipping and glyphosate between G1 and G4 generations, 33–35 DAS.

We also compared plant performance after stress at each generation (G1, G2, G3, and G4) against control levels, helping us to pin-point at which generation recovery occurred and if that change was stable in subsequent generations. Glyphosate-treated plants showed similar performance to control levels in G3, which persisted to G4 (Figure 3a). However, for trifloxysulfuron-treated plants, a return to control levels in both biomass and seed weight occurred in G2 (Figure 3a). Similarly, the change in the seed weight of clipping-treated plants occurred in G4 and significantly shorter flowering time for shade-treated plants occurred in G3, which remained the same in G4.

To better understand the difference between the response of one generation and four generations of stress, we conducted a series of contrasts between plants in G4 exposed to one generation of stress (unigenerational stress) and those that were exposed to stressors at each of four generations (stress at all generations). Trifloxysulfuron- and glyphosate-treated plants showed similar aboveground biomass in G4 whether stressed for one or four generations (Figures 3a and 4d). Whereas shade- and clipping-treated plants in G4 that were stressed for four generations had significantly higher biomass as compared to plants which were stressed for one generation (Figures 3a and 4d). Similarly, when we compared the total seed weight of plants at G4, we found that shade-treated plants had significantly less seed weight after four generations of stress while all other treatments showed similar seed weight (Figures 3b and 4e).

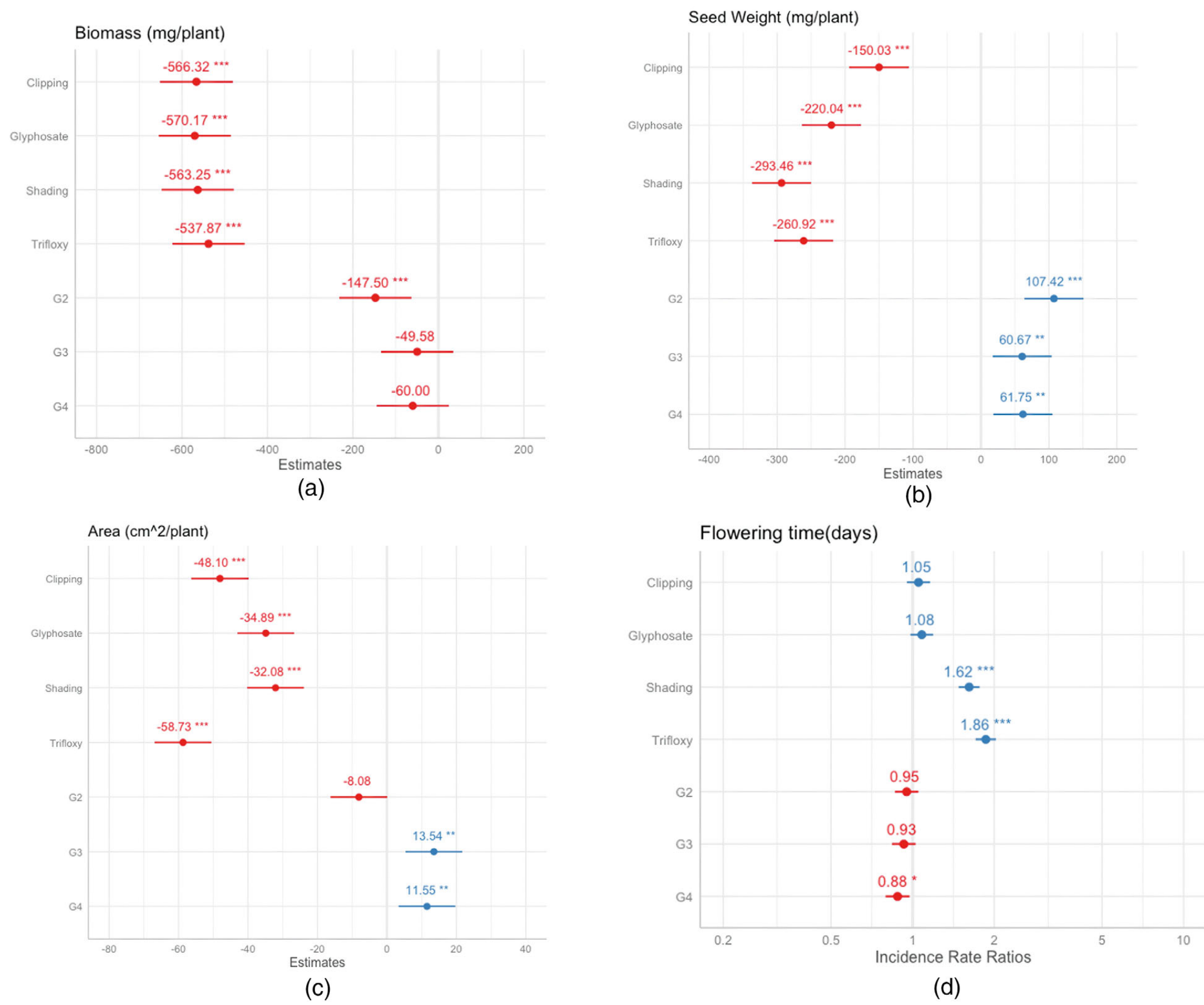

Figure 2. Generalized linear model results for the effects of treatment, generation, and their interactions on Arabidopsis.
(a) Aboveground biomass, (b) seed weight, (c) rosette area, and (d) flowering time. These are results are from the model (Response variable ~ Treatment + Generation + Treatment × Generation). Values represent mean estimates, whereas asterisk signs represent significance at *** = 0.001, ** = 0.01, * = 0.05, Ordinary Least Squares regression was used for biomass, area, and seed weight whereas Poisson distribution was used for flowering time.

DISCUSSION

Parentally experienced stress responses can be passed on to progeny therein increasing performance and fitness of subsequent generations, but this has not been compared across different stress categories. Our study was designed to compare phenotypic responses between single and multiple generations of sub-lethal doses of four common stresses experienced by weeds in agroecosystems. We found that Arabidopsis plants faced with repeated stress over four generations responded to attain biomass and total seed weights at levels similar to untreated control plants, despite the continued presence of stress. Importantly, this only occurred when stressors were applied over successive generations, while we detected no response after unigenerational stress treatments. Further, transgenerational responses to herbicide stress occurred in fewer generations than either clipping or shading treatments in Arabidopsis (Figure 3).

Herbicide stresses

Glyphosate is the world's most used herbicide with 55 weed species having evolved resistance globally (Heap, 2022). Here we showed that after an initial large reduction in performance after exposure to a single sub-lethal dose of glyphosate, Arabidopsis quickly responds in the next generation. Repeated exposure to glyphosate resulted in Arabidopsis growing as large and producing nearly as many seeds (by weight) as untreated control plants by G2, in spite of the herbicide application. It is worth mentioning that glyphosate

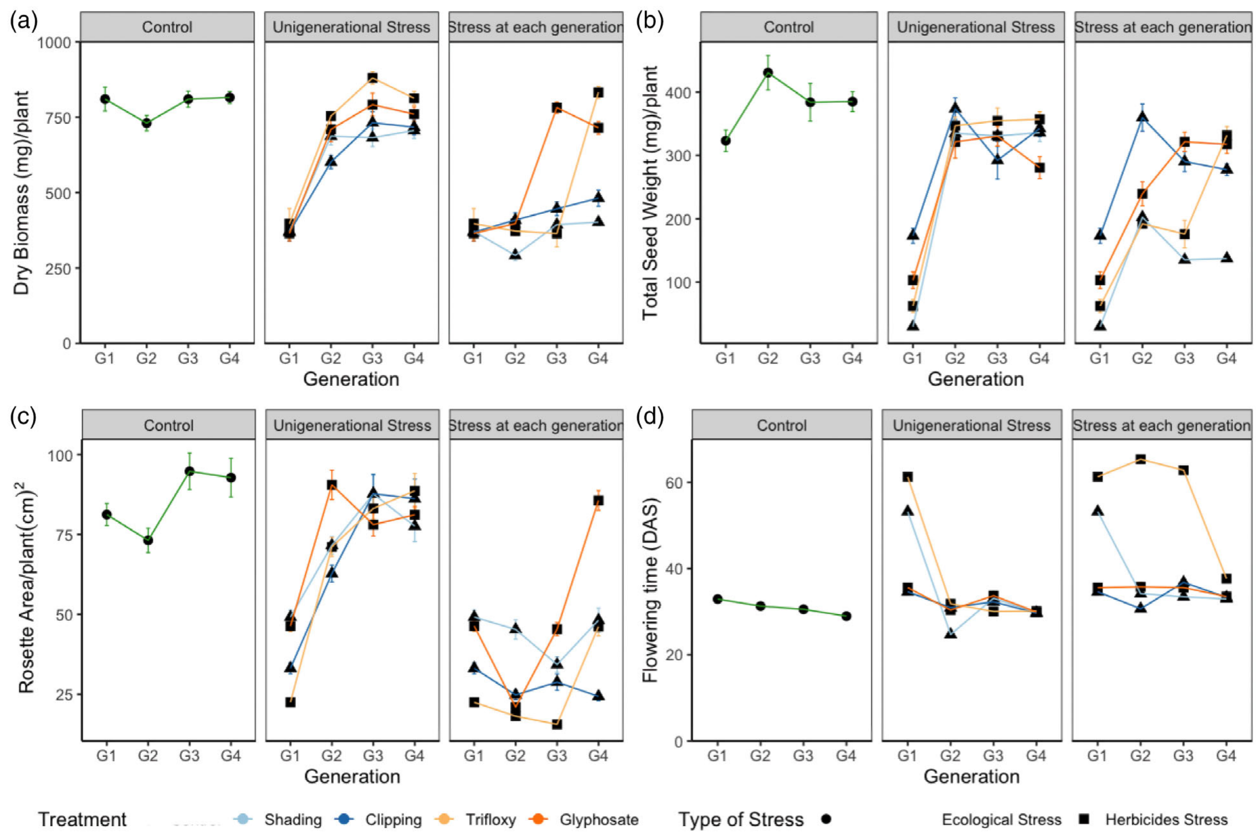

Figure 3. Phenotypic responses (mean ± SE) of Arabidopsis.
(a) Aboveground biomass, (b) total seed weight, (c) rosette area, and (d) flowering time (days after sowing) in response to the herbicide's glyphosate and trifloxysulfuron and the ecological stressors shade and clipping, as well as a non-treated control. The first panel is for untreated control plants in all four generations. The second panel shows plants that received stress only at G1, while the third panel shows plants that experienced stress at each generation (G1–G4). These graphs are based on using both the models mentioned in material and methods (Tables S1 and S2).

appears to be the stressor to which Arabidopsis has shown the most rapid response. For example, *Echinochloa colona* (Benedetti et al., 2021) and *Amaranthus* spp. (Vieira et al., 2020) showed reduced sensitivity to sub-lethal doses of glyphosate after three and two generations, respectively.

Likewise, Rahavi et al. (2011) showed that Arabidopsis plants returned to "normal" or nearly normal levels (in terms of root growth and recombination frequency) when plants were propagated without heavy metal salt (Ni^{2+}, Cd^{2+}, and Cu^{2+}) stress for one or two generations. We did not however observe any changes to flowering time in response to single or continual exposure to glyphosate, which is in contrast with a previously reported study where they used two low glyphosate rates (5 and 10%) for one generation on seven different *Brassica* spp. and observed delaying flowering in six species (Londo et al., 2014). This difference could be due to higher rates of glyphosate compared to our study or due to differences in the genetic makeup of *Brassica* spp. (*B. juncea* and *B. nigra*) and Arabidopsis.

Acetolactate synthase (ALS)-inhibiting mode-of-action herbicides, which include trifloxysulfuron, have the highest number (169) of resistant weed species globally (Heap, 2022). Trifloxysulfuron-treated plants showed dramatically reduced performance when exposed to stress in each generation—delayed flowering, lower seed production, reduced aboveground biomass, and smaller rosette, though they become resistant to the sub-lethal dose by G4. Benedetti et al. (2021) reported reduced herbicide sensitivity in *E. colona* after three generations of recurrent selection with a combination of a sub-lethal dose of ALS-inhibiting herbicide and salt stress. Babineau et al. (2017) reported an ALS-resistant biotype of loose silky bentgrass (*Apera spica-venti*) flowered earlier compared to a susceptible biotype. Additionally, early flowering has also been reported in ALS-resistant wild oats (*Avena fatua*) (Lehnhoff et al., 2013).

Ecological stresses

Clipping had less of a negative impact on Arabidopsis growth than either herbicide. There was no significant

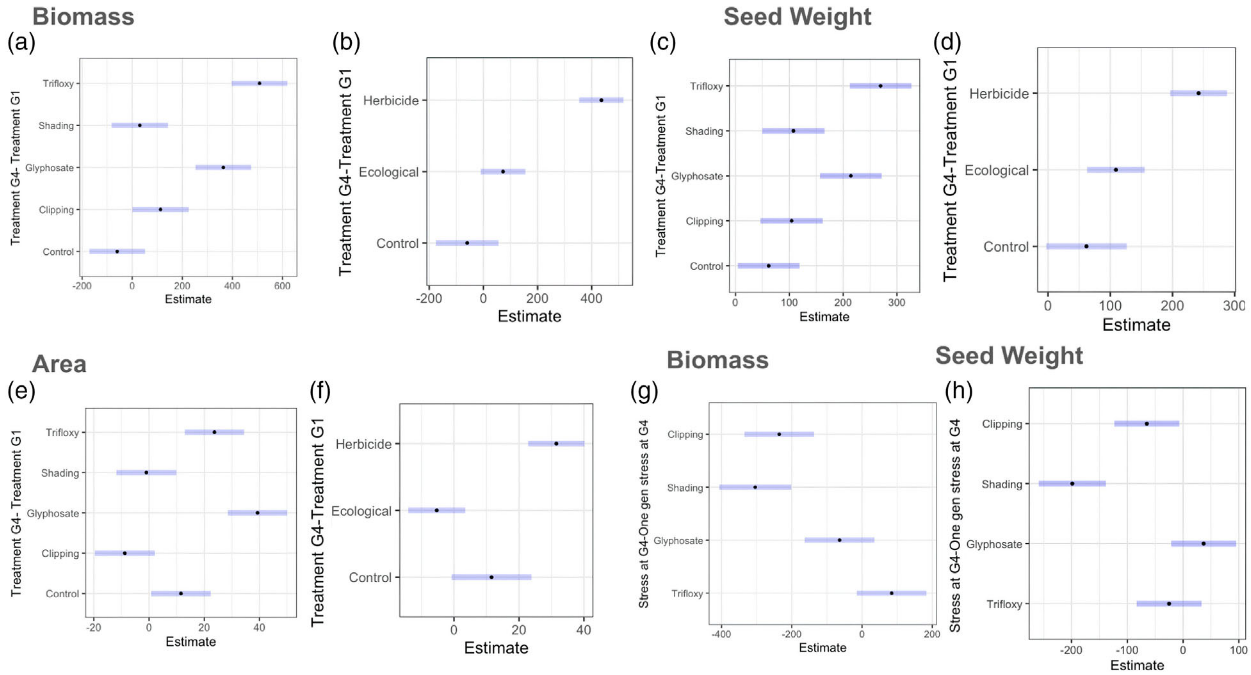

Figure 4. The blue bars on the plot are the confidence intervals (difference between the contrasts) and black dot represents the mean value.
If blue bar arrows overlap zero, then that contrast is not significantly different. Panels (a, c, e) are contrasts between stress at each generation at G4 and G1 for biomass, seed weight, and rosette area for individual treatments, which were based on the model (response ~ treatment + generation + treatment × generation).
Panels (b, d, f) are contrasts between stress at each generation at G4 and G1 for biomass, seed weight, and area for control; ecological and herbicide stresses were based on the model (response ~ treatment + generation + stress level + treatment × generation × stress level).
Panels (g, h) are contrasts between stress at each generation and one generation stress at G4 for biomass and seed weight, were based on model (response ~ treatment + generation + stress level + treatment × generation × stress level) The units for *x*-axis for (a–d, g, h) is mg/plant and for (e, f) is cm^2/plant.

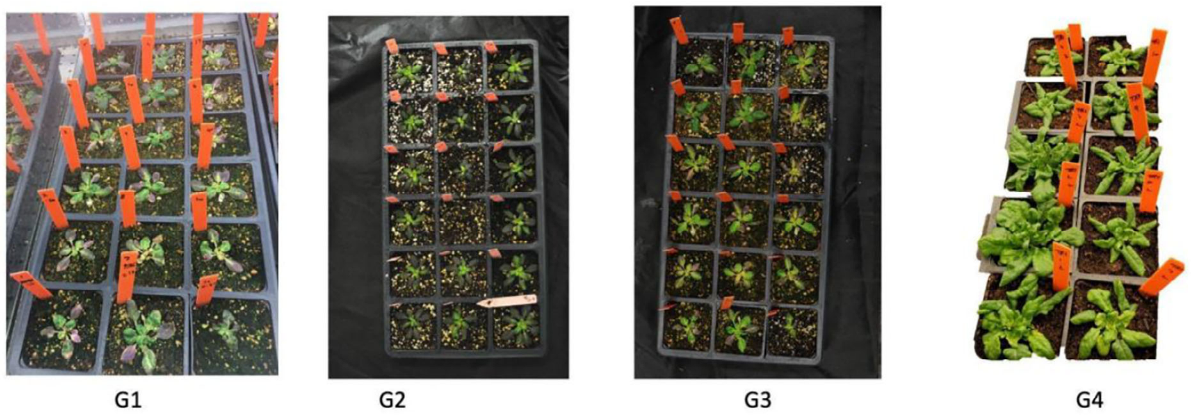

Figure 5. Trifloxysulfuron-treated plants at each generation at 21 days after the herbicide application. G, generation.

change in biomass as compared to the stress at G1 and G4 generation, but it showed higher total seed weight after G1 generation (stress at every generation). Agrawal (2001) reported that herbivory in wild radish seedlings can influence seed mass, which may mediate effects of competitive ability of offspring and the ability to protect from herbivory. In our case, clipping is acting as a proxy for herbivory/mowing and offspring are responding by increasing seed weight – we did not count seeds; therefore, it is not clear if the mechanism was an increased maternal investment in each seed or the production of more seed. In our study, Arabidopsis seed biomass quickly responded to repeated clippings each generation, which others have observed as well (Lampei, 2019). Thus, plants

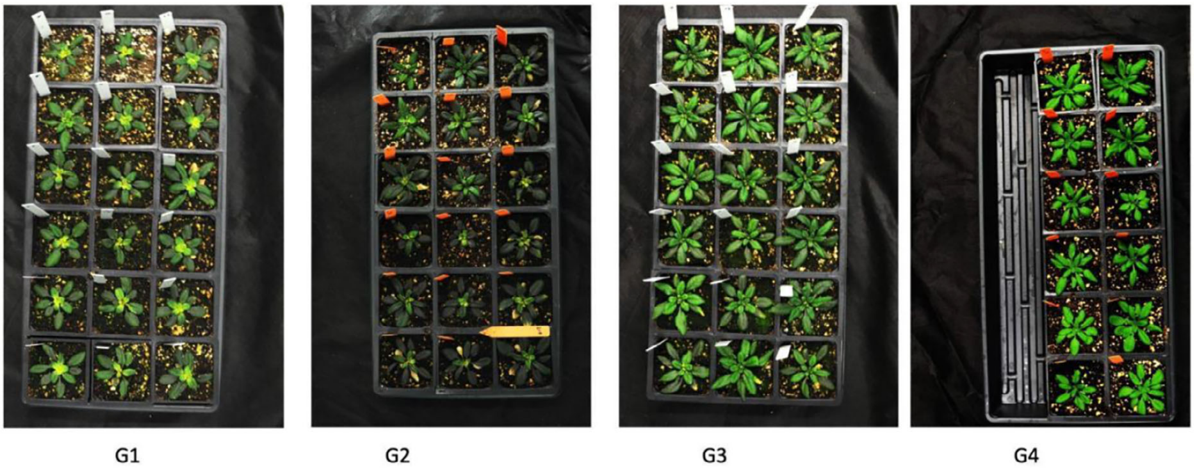

Figure 6. Glyphosate-treated plants at each generation at 21 days after the herbicide application. G, generation.

under clipping stress allocated more resources to seed production.

One of the major phenotypic changes for shade-treated plants was flowering time. Plants in G2 (stress at each generation) flowered in half the time compared to G1. This phenological change indicates that parental effects were responsive but not environment-specific until G2 and demonstrates a potential role of stress memory. Previous studies have shown the role of light in fitness and stress memory of plants (Galloway & Etterson, 2007; Müller-Xing et al., 2014). Baker et al. (2019) showed that inbred progeny of *Polygonum persicaria* after five generations of shade stress had faster reproductive onset and higher reproductive output in shade. Whereas progeny of plants that underwent five generations of stress in sunny conditions showed delayed reproductive onset and decreased reproductive output. This shows that plant stress memory to light stress can be adaptive or maladaptive to progeny, depending on environmental conditions. Our results showed a response flowering phenology after four generations of shade stress in Arabidopsis.

Plants have developed different strategies against herbivory/mechanical injury such as producing chemical toxins, structural barriers, and indirect defenses (Mortensen et al., 2013). Similarly, plants respond to shade either through shade avoidance or shade tolerance both of which consist of various biochemical and physiological pathways. Shade avoidance is triggered by a reduction in the ratio of far-red (R:FR) wavelengths caused by phytochromes, cytochromes, and UVR8 (Sessa et al., 2018). Whereas shade tolerance causes thinner leaves, reduced chlorophyll a: b ratio, and increased branching in plants (Xu et al., 2021).

Overall, we found that sub-lethal exposure to both herbicides and ecological stresses elicits immediate strong decreases in growth and reproduction. However, Arabidopsis plants descended from these stressed plants quickly returned to normal growth if the stress was not repeated or may eventually adapt to show normal growth even if the stress is repeated in each generation. We observed differences in both the magnitude and rate of response to herbicides versus ecological stressors. Quick recovery of biomass, seed weight, and rosette area increases the chances of successful establishment of the offspring in subsequent generations. Whereas earlier flowering acts as a mechanism to avoid stressful conditions that would otherwise increase mortality before the start of reproduction.

One-generation stress versus multiple-generation stress

Our results suggest that plants under multiple generations of herbicide stress showed a responsive phenotype, whereas plants under unigenerational stress quickly revert to the control phenotype. Similarly, shade and clipped plants after one generation of stress did not show any unique phenotype. This suggests that the parental effects acted like a memory that was observed only upon repeated exposure to the treatments. This is a very efficient strategy given the high uncertainty of predicting the offspring environment.

Others have shown that recurrent selection with low sub-lethal doses of herbicides can rapidly result in resistance across a range of major agricultural weeds—*A. fatua*, *Chenopodium album*, *Lolium rigidum* (Belz, 2020; Busi et al., 2016; Manalil et al., 2011; Neve & Powles, 2005). In contrast, Lagator et al. (2013) showed that herbicide mixtures can inhibit or slow the evolution of resistance in an experimentally induced resistant population of *Chlamydomonas reinhardtii*. It seems clear that understanding the impacts of low sub-lethal stress to weed stress evolution and the mechanisms conferring this resistance is important to combating resistance evolution.

One possible mechanism for transgenerational stress memory could be epigenetic inheritance, which is mediated by changes in DNA methylation, histone modifications, or small RNA. DNA methylation changes in herbicide stress have been reported in *A. thaliana* (Kim et al., 2017). Furthermore, epigenetic modifications can evolve much faster than genetic mutations and may be especially relevant when rapid adaptation is required (Bossdorf et al., 2008; Rando & Verstrepen, 2007). Exploring epigenetic changes over generations would be a natural next step to identifying mechanisms of stress memory.

CONCLUSION

This study provides insight into transgenerational phenotypic responses to herbicide and ecological stressors in Arabidopsis. We showed that maternal environment and number of generations that were exposed to stresses played an important role in the development of a responsive and lasting phenotype. We also showed that plants respond to herbicide stressors more rapidly than ecological stressors; this could potentially be attributed to the targeted nature of herbicides as opposed to the more general stressors of clipping and shade. Transgenerational effects commonly occur and have impact on offspring fitness and are one of the important ways for plants to respond to stress. The possible mechanism that would serve as possible next steps would be that stress changes the epigenome leading to differential metabolism conferring enhanced, and in some cases, lasting stress tolerance.

MATERIALS AND METHODS

Anthropogenic and ecological stresses

We used four different stressors that are commonly encountered by weeds in agricultural settings. Two of these were herbicide stressors, and the other two we broadly classified as ecological (non-chemical) stressors. We used glyphosate, an inhibitor of aromatic amino acid biosynthesis and the most widely used herbicide globally, and trifloxysulfuron, an inhibitor of branched-chain amino acid biosynthesis and a member of the herbicide mode of action group that has the greatest number of reported resistance cases (Heap, 2022). The ecological stressors were clipping, the physical removal of leaf tissue that also mimics the mechanical damage from mowing in an agricultural setting or herbivory in any setting, and shading, which most plants experience growing in competition with other plants.

We chose to use Arabidopsis for this study because as a companion study we also sequenced the methylome, which necessitated using a model system and not more common agricultural weeds. Seeds of *A. thaliana* ecotype Columbia that were inbred and used as untreated controls in a previous study (Kim et al., 2017) were soaked in 0.1% agarose media for 5 days in the dark at 4°C and were then sown in Sunshine® Mix # 1 media (Sun Gro® Horticulture) and grown in a Conviron® growth chamber with a 16 h light cycle, 22°C/20°C day/night temperature, and light intensity of 90 μmol m^{-2} sec^{-1}. Plants were transplanted to 9 cm × 9 cm cells 5–7 days after germination. All treatments were imposed on 24 randomly selected plants at the pre-floral rosette stage (~25–30 DAS), before flowering initiation.

Treatment rates were identified via a series of preliminary experiments to determine the maximum stress the plants could survive that still allowed recovery and reproduction. Arabidopsis plants 25–30 days old, and still at the vegetative rosette stage, were found to be impacted by, yet survive and produce seed, the following four treatment rates: (i) glyphosate at 7% of the label rate (0.80 kg acid equivalency ha^{-1} of RoundUp Pro Concentrate); (ii) trifloxysulfuron at 2.8% of the label rate (0.02 kg active ingredient ha^{-1} of Monument 75WG); (iii) clipping such that 90% of aboveground biomass was removed by cutting three times at 7 day intervals, or (iv) shading by placing plants under neutral shade cloth such that they received 10% of ambient light. An equal number of untreated control plants were grown under the same conditions as non-shaded treated plants. All herbicide treatments were applied at 187 L ha^{-1} in a spray chamber, at a speed of 2.09 kph, equipped with the TeeJet 8001 EVS. Following the herbicide treatment application, all plants were put back in the growth chamber.

Transgenerational experimental design

Plants in the initial set of treatments were considered generation one (G1). Plants in the G1 were treated as described above with one of the stresses (herbicides, clipping, and shade) along with untreated controls. In generations two through four (G2–G4) there were three different sets of plants (Figure 1): (i) untreated controls (i.e., never received any stress treatment in any generation); (ii) plants that were the progeny of plants stressed in G1, but which did not receive any stress in G2, G3, or G4 (i.e., labeled as "unigenerational stress" in figures); and (iii) plants that received a stressor in all generations (i.e., labeled as "stress at all generations" in figures). Seeds from each plant were collected using Arasystem (Betatech BVBA, New-Yorkstraat 4, Gent, Belgium) and stored at 4°C. The G1 generation had a total of 120 plants (5 treatments [including controls] × 24 replicates), whereas the G2–G4 generations included a total of 216 plants (5 treatments × 24 replicates + 4 lines of G1 stressed progeny × 24 reps) in each generation. All plants in all generations were grown in the same growth chamber under the conditions described above.

Phenotypic data collection

We collected rosette area and leaf chlorophyll fluorescence at 7, 14, and 21 days after the first exposure to the stress treatment and recorded the presence of the first inflorescence as flowering time, which was recorded as DAS. Chlorophyll fluorescence is the ratio of fluorescence or fluorescence emission at 735 nm/700 nm and was measured using CCM-300 by Opti-Sciences. Total seed weight and aboveground dry biomass were collected after plant senescence.

Statistical analyses

The data were divided into two subsets to understand the impacts of multigenerational versus unigenerational stress on plant phenotypes. Phenotypic responses (biomass, seed weight, area of rosette) of plants subjected to multigenerational stress were compared to that of control plants at each generation via a linear model consisting of main effects and two-way interaction of treatment by generation:

$$(\text{Response variable} \sim \text{Treatment} + \text{Generation} + \text{Treatment} \times \text{Generation})$$

Treatment includes clipping, shading, glyphosate, and trifloxy; Generation has four levels which includes from G1 to G4.

For flowering time, we fit the same model structure using a generalized linear model (Poisson distribution).

Phenotypic responses to unigenerational stress were compared to that of multigenerational stress at each of the four generations using a three-way, linear interaction model of treatment, generation, and stress frequency (uni- versus multigenerational).

$$\text{Response variable} \sim \text{Treatment} + \text{Generation} + \text{Stress Level} + \text{Treatment} \times \text{Generation} \times \text{Stress Level}$$

Treatment includes clipping, shading, glyphosate, and trifloxy; Generation has four levels which includes from G1 to G4 and two levels of stress (unigenerational and multigenerational stress).

Total seed weight and area of the rosette were $\log_{10}$ transformed to meet the assumptions of the linear model. Several *a priori* contrasts were tested using linear models to answer specific questions. To account for multiple testing, an experiment-wide false discovery rate of 0.05 was established and all *P*-values from the same contrast analysis were evaluated against this FDR. Contrast analysis plots were prepared using the "emmeans" package. All the data were analyzed in R 4.1.2 (R Core Team, 2020) and visualized in R Studio (RStudio Team, 2020).

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data associated with this study is available upon request from the first author Gourav Sharma who can be contacted through the Corresponding Author.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Analysis of variance (ANOVA) for biomass, seed weight, area and flowering time based on the multigenerational stress (response ~ treatment + generation + treatment × generation).

Table S2. List of linear contrasts represented in figure 4, it consists of estimates, parameter (biomass, seed weight, area) std error and respected p-value set 0.05 for each contrast. The treatments here showed the level at which contrasts were compared. Contrasts, which were performed on the stresses were based on the model (response ~ treatment + generation + treatment * generation), whereas contrasts which were between stress and no-stress were based on model (repsonse~treatment + generation + stress level + treatment * generation * stress level).

Table S3. List of linear contrasts represented in Figure 4, it consists of estimates, parameter (biomass, seed weight, area) std error and respected *P*-value set 0.05 for each contrast.

Figure S1. (a) Comparison between the control plants and clipped plants 21 days after the first treatment, (b) Shade treated plants and control plants 21 days after treatment.

Figure S2. Left to right (four generation stress) (a) clipping, (b) trifloxy, (c) Glyphosate, (d) shading and (e) control at 21 days after treatment.

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