

Transgenerational herbivory effects on performance of clonal offspring of the invasive plant *Alternanthera philoxeroides*

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Abstract

Interactions between alien plants and the newly emerging enemies in introduced ranges may determine plant invasion success. However, little is known about whether herbivory-induced responses are transmitted across vegetative generations of plants, and whether epigenetic changes are involved during this process. In a greenhouse experiment, we examined the effects of current, parental and/or grand-parental herbivory by the generalist herbivore *Spodoptera litura* on the growth, physiology, and biomass allocation, and the status of DNA methylation of the first-, second- and/or third-generation clonal offspring of the invasive plant *Alternanthera philoxeroides*, which were derived from two types of root fragments with different branching orders (i.e., the primary- or secondary-root fragments of taproots). Parental herbivory promoted the growth of the second-generation plants sprouted from the secondary-root fragments, but had a neutral or negative effect on the growth of the second-generation plants from the primary-root fragments. The growth of the third-generation plants was significantly reduced by current herbivory, but not affected by grand-parental herbivory. The first-generation plants exhibited a higher level of DNA methylation when they were grazed by herbivores than when they were not, while neither the second-generation nor the third-generation plants showed the herbivory-mediated changes in DNA methylation. Overall, the herbivory-induced growth response within one generation may represent rapid acclimatization of *A. philoxeroides* to the unpredictable generalist herbivores in the introduced ranges. Herbivory-induced trans-generational effects may be transient for clonal offspring of *A. philoxeroides*, which can be influenced by the branching order of taproots, but be less characterized by DNA methylation.

Introduction

Biological interactions such as plant-herbivore relationships play a crucial role in the invasion success of at least some alien plants (Mitchell et al. 2006; Enders et al. 2020; Dai et al. 2020). The enemy release hypothesis (ERH) postulates that the rapid adaptation and competitive advantage of many alien species in invasive ranges can be attributed to the absence of coevolved native enemies, which greatly contributes to their invasion potential (Keane and Crawley 2002). This hypothesis, despite some supports (e.g., Lucero et al. 2019; Xu et al. 2021; Costan et al. 2022), has also been challenged (Colautti et al. 2004; Mitchell et al. 2006; Liu and Stiling 2006; Meijer et al. 2016). For instance, the performance of alien species may still be heavily influenced by local herbivores in the invasive ranges after new plant-herbivore relationships have been established (Colautti et al. 2004; Chun et al. 2010). It is, therefore, worthwhile to explore the biological interactions between alien plants and the newly emerging enemies in invasive ranges, to obtain further insight into the mechanism of plant invasion.

As a common type of local enemies, aboveground insect herbivores often cause direct or indirect harm to plant organs, thereby restricting plant growth and development (Schoonhoven et al. 2005). Chewing insects (e.g., grasshoppers) can severely destroy the physical and physiological structure of plant organs, thereby blocking the mobilization of resources between different organs, while sucking insects (e.g., aphids) can cause the accelerated senescence of damaged organs through excessive absorption of nutrients from plants (Schoonhoven et al. 2005). To alleviate herbivory pressure, resource allocation often becomes the primary strategy for damaged plants during their growth and development (Strauss and Agrawal 1999; Stowe et al. 2000; Boege and Marquis 2005). Internal resources (e.g., non-structural carbohydrates) could be mobilized from aboveground damaged plant organs (e.g., leaves and stems) and re-distributed to the underground organs (Dong et al. 2018, 2019). Allocation of plant biomass to roots may thus correspondingly increase in damaged plants to prepare for the compensatory growth after damage. As an alternative of herbivory-induced responses, the secondary metabolites (e.g., phenolic compounds) can be selectively released in damaged organs of some species (Schoonhoven et al. 2005; Agrawal and Weber 2015; War et al. 2018). The release of chemicals may effectively retard the herbivore feeding rate by either deterring herbivores or reducing the nutritive value of plant tissues (Pearse and Hipp 2009; Tanentzap et al. 2011; Huang et al. 2014). Consequently, herbivory-induced responses (including changes in both primary and secondary metabolites) may better represent the strategies adopted by alien plants in response to insect herbivores.

Herbivory-induced responses of plants may not only persist within one generation, but also transmit across two or more generations. A growing body of two-generation (i.e., parent-offspring pairs) studies has documented that herbivory-induced parental effects could be vital to fitness for both clonal and non-clonal offspring of plants (Steets and Ashman 2010; Rasmann et al. 2012; Dong et al. 2017, 2018, 2019; Kellenberger et al. 2018; Kafle and Wurst 2019; Nihraz et al. 2019, 2020; Sobral et al. 2021). In particular, the parental effect is often considered adaptive in sexually or clonally produced offspring, since the similarity between parental and offspring environments is predicted to allow offspring to pre-adapt to the predictable environments that parent plants have encountered (Uller et al. 2013; Engqvist and Reinhold 2016; Yin et al. 2019). However, there is still uncertainty regarding the persistence of herbivory-induced effects over more than two generations of plants, i.e., whether they continue to persist across three generations or even longer (Bell and Hellmann 2019).

DNA methylation is an epigenetic mechanism that drives transgenerational effects in plants (Richards et al. 2017; Alonso et al. 2019). DNA methylation can regulate the gene expression, by altering the addition of methyl groups to the nucleotides without changes in the DNA sequences (Richards 2006). When environment-induced DNA methylation becomes relatively stable over generations and is less dependent on genetic variation, it can enable offspring to adapt to predictable conditions with some specific strategies (Bossdorf et al. 2008; Jablonka and Raz 2009; Herman et al. 2014; Richards et al. 2017; Thiebaut et al. 2019). While previous studies have highlighted the role of DNA methylation in the transgenerational effects on shaping defense against and/or tolerance to herbivory in sexually reproducing plants (e.g., Herrera and Bazaga 2011; Kellenberger et al. 2016), such knowledge in asexually reproducing (clonal) plants is still fragmentary (Latzel and Klimešová 2010; Douhovnikoff and Dodd 2015).

Roots can be the storage and regeneration organ for many clonal plants (Klimešová and Klimes 2008; Klimešová et al. 2018; Ott et al. 2019). Root fragments may provide the foundation of clonal offspring when roots can form adventitious buds that will develop into shoots (Klimešová and Klimes 2008; Martínková and Klimešová 2016). Since roots are a highly branched system organized hierarchically from lower-order distal roots to higher-order basal roots, roots with different orders may differ greatly in structure and function (McCormack et al. 2015; Kong et al. 2021; Guo et al. 2008). One expectation is that the differentiated root vigor, anatomical structure, and chemicals (e.g., non-structural carbohydrates) may remain even after root fragmentation (McCormack et al. 2015), and thereby modify the (grand-)parental effects on clonal offspring originating from root fragments of different orders. However, no study has tested the effects of root fragments with different branching orders on (grand-)parental effects in clonal plants.

We conducted a three-generation experiment on a well-studied, invasive clonal plant *Alternanthera philoxeroides*. We examined the effects of current, parental and/or grand-parental herbivory by the generalist herbivore *Spodoptera litura* on the growth, physiology, and biomass allocation of the first-, second- and/or third-generation clonal offspring of *A. philoxeroides*, which were derived from two types of root fragments with different branching orders (i.e., the primary- or secondary-root fragments of the first-generation plants). Additionally, we compared the levels of methylated DNA in leaves of different generations between the herbivory treatments. Specially, we tested the following hypotheses: (1) Herbivory effects persist across multiple vegetative generations. In particular, herbivory-induced grand-parental effects will influence the growth, physiology and biomass allocation of grand offspring. (2) Herbivory-induced transgenerational effects are context-dependent. In particular, grand-parental effects will be beneficial to the grand offspring that experienced the similar herbivory as their grand-parent plants did. (3) Herbivory-induced transgenerational effects differ between clonal offspring sprouted from the root fragments of different orders. (4) Herbivory-induced transgenerational effects are regulated by DNA methylation.

Materials And Methods

Study species

Alternanthera philoxeroides (Mart.) Griseb., native to South America, is a creeping perennial herb of the Amaranthaceae family (Schmid et al. 1998). The species was intentionally introduced into China as livestock feed in the 1930s and is now considered one of the most noxious invasive weeds (Lu et al. 2015). The genetic diversity of *A. philoxeroides* is extremely low in China, and most individuals are recruited by clonal growth (Ye et al. 2003; Wang et al. 2005). *Spodoptera litura* Fabricius belongs to the Noctuidae family, which is distributed all over the world. The species is an omnivore agricultural pest and is also reported as a generalist herbivore of *A. philoxeroides* in the field (Liu et al. 2020, Liu et al. 2021).

In May 2011, plants of *A. philoxeroides* were collected from a riparian agricultural area (28.87°N, 121.01°E) in Taizhou, Zhejiang Province, China. They were vegetatively propagated in the greenhouse at Forest Science Co., Ltd., at Beijing Forestry University. Larvae of *S. litura* were obtained from Henan Jiyuan Baiyun Industrial Co., Ltd. (<http://www.keyunnpv.cn/>).

Experimental design

Three clonal generations of *A. philoxeroides* were used in the experiment series (Fig. 1). On 13 May 2018, 840 stem fragments, each with one stem node, two internodes of 6 cm long, and two opposite leaves, were cut off from the stock plants of *A. philoxeroides*. They were grown in 50 × 50 × 8 cm containers (long × wide × high) filled with a mixture of commercial nutrient solution (Scotts Miracle-Gro All Purpose; Scotts Miracle-Gro Company, Wuhan, China) and tap water at a ratio of 1:16 for one month. During the 40-day cultivation, a shoot started to come out from one axillary bud of the stem node and then roots started to develop (hereafter, the rooted stem node with a shoot is called a plant).

First-generation experiment: On 21 June 2018, a subset of 48 plants with uniform sizes were transplanted into plastic pots (14 cm in diameter and 12 cm deep) and cultivated for two months. The pots were filled with a mixture of clay soil and peat (Pindstrup Seedling; Pindstrup Mosebrug A/S, Pindstrup, Denmark) at a volume ratio of 1:1 (total P in average: 0.47 mg/g; total N: 1.62 mg/g). On 23 August 2018, these plants were randomly assigned to one of two herbivory treatments, i.e., no herbivory (control) or herbivory by *S. litura*. Each treatment was replicated 24 times. In the herbivory treatment, eight fourth-instar larvae of *S. litura* were released on the leaf surface of each plant, grazed the plant for two weeks, and then removed. After two weeks of recovery, another eight fourth-instar larvae of *S. litura* were released on the leaf surface of each plant, grazed the plant for another two weeks, and then were removed. Two weeks after completely stopping grazing, on 4 October 2018, five replicates of the plants were randomly selected and then harvested to measure biomass and offspring ramet production. For the remaining 19 replicates of the plants, primary and secondary root fragments with 6-cm length were harvested, and used for the second-generation experiment. The initial fresh masses of the primary and secondary root fragments produced by the first-generation plants without herbivory were, respectively, 1.37 ± 0.23 mg and 0.86 ± 0.15 mg (mean $\pm$ SE); the initial fresh masses of the primary and secondary root fragments produced by the first-generation plants with herbivory were 1.24 ± 0.06 mg and 0.68 ± 0.07 mg, respectively.

Second-generation experiment

Offspring plants developed from the primary root and the secondary root fragments produced by the first-generation plants subjected to herbivory by *S. litura* or not (Appendix Fig. 1) were grown isolated from herbivory during the second-generation experiment. Thus, the second-generation experiment consisted of two factors, i.e., parental root order (primary vs. secondary root fragments produced by the first-generation plant), and parental herbivory (the first-generation plant was subjected to herbivory by *S. litura* or not).

These primary and second root fragments were planted in pots of the same size and filled with the same soil mixture as described in the first-generation experiment. The second-generation experiment was initiated shortly after the first-generation experiment was harvested, and lasted for about eight months. On 22–23 June 2019. Five replicates of plants sprouted from the root fragments of each of the four types were harvested. For the remaining 14 replicates of the offspring plants, primary root fragments with 6-cm length were harvested, and used for the third-generation experiment.

Third-generation experiment

Grand-offspring plants developed from the root fragments produced by the offspring plants of each of the four types were again subjected to herbivory by *S. litura* or not (Appendix Fig. 1). Thus, the third-generation experiment consisted of three factors, i.e., grand-parental root order (primary vs. secondary root fragments produced by the first-generation plant), grand-parental herbivory (the first-generation plant was subjected to herbivory by *S. litura* or not), and current herbivory (the grand-offspring plant was subjected to herbivory by *S. litura* or not).

Again, the root fragments of the offspring plants were planted in pots of the same size and filled with the same soil mixture as described in the first-generation experiment. The third-generation experiment was also initiated shortly after the second-generation experiment was harvested. The root fragments were allowed to germinate and produce shoots (i.e., grand-parent offspring) without herbivory in the first two months. On 26 August 2019, the grand-offspring plants were randomly assigned to one of two herbivory treatments, i.e., no herbivory (control) or herbivory by *S. litura*. Herbivory treatment followed the same procedure as in the first-generation experiment. Seven replicates of grand-offspring plants per each of the third-generation treatment were harvested. The mean air temperature in the greenhouse during the experiment series was 21.28 ± 0.36 °C (mean $\pm$ SE), as measured with HOBO Pendant Temperature/Light Data Logger (UA-002-64; Onset Computer, Bourne, MA, USA).

Measurements

For three clonal generations of *A. philoxeroides*, the number of stem nodes, the number of leaves, and the stolon length were measured after each generation harvest. Four youngest fresh leaves for plants in each generation were stored in plastic bags filled with silica gel for the measurement of DNA methylation levels. Unfortunately, the youngest leaves of the third-generation plants with current herbivory were not collected for the further measurement of DNA methylation levels, due to the severe destruction of the youngest leaves caused by herbivory of *S. litura*. The remaining leaves, stems, and roots of each plant were separated, dried in an oven at 70°C for 48 hours, and weighted.

Leaves of *A. philoxeroides* dried in the oven were finely ground using a Retsch MM400 Mixer Mill at a frequency of 4000 Hz for 10 min (Retsch GmbH, Haan, Germany), and used for the measurement of physiological traits. The concentrations of non-structural carbohydrate compounds in leaves (including water-soluble sugars and starch) were measured using the perchloric acid/anthrone method, and the concentration of total phenolic in leaves was measured with the Folin-Ciocalteu method (see details in Dong et al. 2017, 2019). Physiological measurements were replicated five times for the first-generation and the third-generation plants, and four times for the second-generation plants.

For the measurement of DNA methylation levels, the total genome DNA from each silica gel-dried leaf sample was first isolated following the manual of the TIANGAN Plant Genomic DNA kit (DP350; Tiangen Biotech, Beijing, China). The global DNA methylation levels in leaves were then quantified with the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (AP1030; Epigentek Group Inc., Farmingdale, NY, USA). The MethylFlash kit uses an enzyme-linked immunosorbent assay (ELISA) to estimate the global DNA methylation (%5-mc), by measuring the amount of 5-methylcytosine (5-mC) from the DNA extracts. This method of global DNA methylation quantification has been increasingly used in plant species (Puy et al. 2018; Atighi et al. 2020; Ruocco et al. 2021). DNA methylation measurements were replicated five times for the first-generation and the third-generation plants without herbivory, and four times for the first-generation plants with herbivory and the second-generation plants.

Data analysis

For the grand-parent plants (i.e., the first-generation plants), the independent t-tests were employed to test the effects of herbivory on plant growth (i.e., total mass, leaf mass, stem mass, root mass, number of stem nodes, and number of leaves), biomass allocation (root to shoot ratio), and physiological traits [the concentrations of water-soluble sugar, starch, total non-structural carbohydrates (the summed concentration of water-soluble sugar and starch), and total phenolic], and DNA methylation level. For the offspring plants (i.e., the second-generation plants), two-way ANOVAs were used to test the effects of the parental herbivory, parental root order, and their interaction on plant growth, biomass allocation, physiological traits and DNA methylation level. For the grand-offspring plants (i.e., the third-generation plants), three-way ANOVAs were used to test the effects of the grand-parental herbivory, grand-parental root order, current herbivory, and their interactions on plant growth, biomass allocation, and physiological traits. For lack of the youngest leaves of the grand-offspring plants with herbivory, we only used the two-way ANOVA to examine the effects of the grand-parental herbivory, grand-parental root order, and their interaction on DNA methylation level of the grand-offspring plants without herbivory. If the data did not meet the assumptions of normality and homogeneity of variance, they were square-root transformed. All analyses were conducted using R v 4.0.5 (R Core Team 2021).

Results

Performance of grand-parent plants

Total mass, stem mass, number of nodes, number of leaves and stolon length of grand-parent plants were significantly lower in the herbivory treatment than in the control treatment (Appendix Fig. 2). By contrast, the methylated DNA level was significantly higher in the grand-parent plants with herbivory than those without herbivory (Fig. 4A). Otherwise, the other growth (i.e., leaf mass or root mass) and physiological traits (i.e., the concentration of soluble sugars, starch, total NSC, or total phenolic), as well as biomass allocation (i.e., root to shoot ratio), were not affected by herbivory (Appendix Figs. 2, 3).

Performance of offspring plants

There were significant interaction effects between the parental herbivory and parental root order on total mass, leaf mass, stem mass, and stolon length of the offspring plants (Appendix Table 1). The parental herbivory strongly depended on the root order of parent plants (Appendix Table 1). The negative effects of parental herbivory on total mass, leaf mass, stem mass, and stolon length were weak and not significant in the offspring plants taken from primary-root fragments of parent plants, while the positive effects of parental herbivory were significantly detected in the offspring plants taken from secondary-root fragments (Appendix Fig. 4A-C, G).

Table 1

Results of ANOVAs testing effects of grand-parental herbivory (control vs. herbivory in the first generation), grand-parental root order (primary vs. secondary root order of the first generation), and current herbivory (control vs. herbivory in the third generation) on growth, biomass allocation, and physiological traits of the grand-offspring plants of *Alternanthera philoxeroides*. Total mass, leaf mass, and stem mass were square-root transformed. Values for which $P < 0.05$ are in bold.

Variable	Grand-parental herbivory (GH)		Grand-parental root order (GR)		Current herbivory (CH)		GH × GR		GH × CH		GR × CH		GH × GR × CH	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Growth														
Total mass	3.18	0.081	0.26	0.614	25.94	< 0.001	2.96	0.092	2.78	0.102	0.79	0.380	0.93	0.33
Leaf mass	3.12	0.084	0.51	0.479	49.52	< 0.001	1.85	0.180	2.08	0.156	0.95	0.334	0.46	0.50
Stem mass	1.52	0.223	0.19	0.665	26.46	< 0.001	1.99	0.165	3.50	0.067	1.53	0.222	0.55	0.48
Root mass	5.10	0.029	0.26	0.610	1.40	0.243	4.30	0.044	2.14	0.150	0.05	0.816	2.20	0.14
Number of nodes	0.80	0.376	0.08	0.776	41.13	< 0.001	1.22	0.275	1.85	0.180	0.22	0.645	0.20	0.65
Number of leaves	1.96	0.168	0.02	0.896	38.56	< 0.001	1.72	0.195	2.24	0.141	0.93	0.340	0.26	0.61
Stolon length	1.16	0.288	0.33	0.567	27.90	< 0.001	0.75	0.392	3.27	0.077	0.14	0.713	0.59	0.44
Biomass allocation														
Root to shoot ratio	1.37	0.248	0.05	0.833	28.50	< 0.001	0.10	0.756	1.85	0.180	0.28	0.602	0.15	0.70
Physiology														
Total phenolic	0.27	0.606	2.78	0.105	0.11	0.737	0.01	0.911	0.79	0.381	0.07	0.791	< 0.01	0.91
water-soluble sugar	1.79	0.191	2.13	0.154	0.12	0.736	0.04	0.850	0.01	0.908	0.35	0.556	0.13	0.72
Starch	0.15	0.706	0.15	0.698	16.29	< 0.001	< 0.01	0.985	1.55	0.222	1.23	0.276	0.82	0.37
Total NSC	0.47	0.498	0.57	0.457	15.26	< 0.001	0.02	0.884	1.48	0.233	0.85	0.363	0.40	0.53

The root to shoot ratio and the concentration of total phenolic were independently affected by parental herbivory (Appendix Table 1). Compared to the offspring produced by the parent plants without herbivory, the offspring produced by the parent plants with herbivory had a lower root to shoot ratio, and a higher concentration of total phenolic (Appendix Fig. 4H). Otherwise, the other growth (i.e., root mass, number of nodes, or number of leaves), physiological traits (i.e., the concentrations of water-soluble sugars, starch, or total NSC), or methylated DNA levels were influenced by neither the parental herbivory nor parental root order (Fig. 4B, Appendix Table 1, Appendix Figs. 4, 5).

Performance of grand-offspring plants

Being consistent with the effects of herbivory on the grand-parent plants, most of the growth (i.e., total mass, leaf mass, stem mass, number of nodes, number of leaves and stolon length), physiological (i.e., the concentrations of starch and total NSC) traits and biomass allocation (i.e., root to shoot ratio) of grand-offspring plants were strongly influenced by current herbivory (Table 1). Total mass, leaf mass, stem mass, number of nodes, number of leaves, stolon length, and the concentrations of starch and total NSC were significantly lower in the grand-offspring plants with current herbivory than without current herbivory (Figs. 2,3). On the contrary, root to shoot ratio was significantly higher in the grand-offspring plants with current herbivory than without current herbivory (Fig. 2H).

Only total mass, leaf mass, and root mass of the grand-offspring plants tended to be or was significantly influenced by the grand-parental herbivory (Table 1). Total mass, leaf mass, and root mass were relatively higher in the grand-offspring plants derived from the grand-parent plants without herbivory than the ones from the grand-parent plants with herbivory (Fig. 2A, B, H). There were no significant main effects caused by the root order of grand-parent plants (Table 1).

However, the negative effects of grand-parental herbivory on root mass were significant in the grand-offspring plants derived from the secondary-root fragments of the grand-parent plants, but weakened in those from the primary-root fragments of the grand-parent plants (Table 1, Fig. 2D).

Apart from root mass, there were no other two-way or three-way interaction effects on growth and physiological traits, and biomass allocation (Table 1). Also, there were no significant main and interaction effects on the concentrations of total phenolic and soluble sugars of the grand-offspring plants (Table 1, Fig. 3), or no effects on methylated DNA levels of the grand-offspring plants without current herbivory (Fig. 4C, Appendix Table 2).

Discussion

Direct effects of herbivory

Irrespective of the grand-parent plants and the grand-offspring plants, the aboveground herbivory caused by larvae of *S. litura* could directly postpone and retard the development of the leaf organ, thereby leading to a dramatic reduction in the aboveground growth and overall fitness of the damaged plants of *A. philoxeroides* (Fig. 2 and Appendix Fig. 2). In contrast, the aboveground herbivory had no direct impact on the root growth of the damaged plants, so a relatively high proportion of biomass was allocated to the roots of damaged plants (Dong et al. 2018; Yu and Fan 2018; Hu and Dong 2019). Also, the aboveground herbivory had little impact on the concentrations of primary and secondary metabolites of leaves in the damaged plants, with the exception of the decreased concentration of leaf starch and non-structural carbohydrates in the grand-offspring plants. The combined results of growth and physiological traits suggested that *A. philoxeroides* did not appear to directly defend against the attack of aboveground herbivores such as *S. litura* with the release of leaf chemicals, but instead tolerant to the aboveground herbivory via the mobilization and maintenance of internal resources in roots (Dong et al. 2018, 2019). The latter strategy might better facilitate the rapid regrowth and vegetative reproduction of plants after herbivore damage, to compensate for herbivory loss (Wilson et al. 2007; Jia et al. 2009; Heinze 2020). Meanwhile, the selection of tolerance traits might greatly contribute to the potential invasion of *A. philoxeroides* in the invasive ranges, especially when they are confronted with the unpredictable generalist herbivores in the new habitats (Ashton and Lerdau 2008; Huang et al. 2010; Liu et al. 2020).

Parental and grand-parental effects of herbivory

For the offspring plants, the root order of parent plants (i.e., primary and secondary roots) were included in the analysis. Interestingly, the parental experience of being grazed could positively influence the aboveground performance of clonal offspring originating from secondary-root fragments of the parent plants, but had neutral impacts on the performance of the offspring originating from primary-root fragments. In detail, compared to the clonal offspring taken from the secondary-root fragments of parent plants without herbivory, the clonal offspring taken from the secondary-root fragments of parent plants with herbivory, had better aboveground growth and stolon expansion ability (Appendix Table 1). On the other hand, regardless of the root order of the parent plants, the parent plants that experienced herbivory both allowed clonal offspring to have the capacity to accumulate more phenolic in leaves and maintain a high biomass allocation to roots. These results supported the first hypotheses, suggesting that herbivory-induced parental effects via vegetative reproduction could be transmitted between two vegetative generations, which contributed to the establishment of tolerance traits in clonal offspring.

Also, as predicted by the third hypothesis, such parental effects on aboveground performance could also depend on the root order of parent plants, from which clonal offspring are produced. However, because the initial size of primary and secondary root fragments produced by parent plants failed to match their final performance in the second generation (see the details about the initial mass of root fragments in the Material and Methods section), we suggested that the difference in parental effects caused by root order might not be simply due to the variance in the provisioning of clonal propagule (Dong et al. 2019). One plausible reason is that the quality difference between two types of root fragments may be responsible for the differentiated parental effects, i.e., the clonal offspring sprouted from lower-order distal roots (i.e., secondary roots) might be more vigorous and also more effective at utilizing stored resources to support the subsequent development of photosynthetic tissues and the establishment of juvenile individuals (Guo et al. 2008; McCormack et al. 2015). To the best of our knowledge, this may be the first to report that the root order of clonal plants plays a role in the cause of parental effects.

For the grand-offspring plants, the grand-parental herbivory effects on plant characteristics related to growth, physiology and biomass allocation were mostly neutral, and they did not interact with the current herbivory environment. Only the root growth of the grand-offspring plants derived from the secondary fragments of grand-parent plants, was negatively affected due to the grand-parental experience of being grazed. The overall results were not inconsistent with the second hypothesis, and indicated that the persistence of herbivory-induced responses across vegetative generations was not stable for *A. philoxeroides*. One reason is possible that herbivory-induced traits tend to function as a local acclimation (not an adaptation) of *A. philoxeroides* to the newly emerging generalist enemies in the introduced ranges, resulting in local phenotypic plasticity as opposed to transgenerational inheritance (Geng et al. 2007; Rodríguez et al. 2021). In turn, the historical biological interactions between invasive plants and their native coevolved enemies may be the foundation for the adaptive herbivory-induced responses that pass across generations (Strauss and Agrawal 1999; Karban 2011). If so, some additional studies will have to be undertaken to determine whether transgenerational effects differ in clonal plants, when they encounter generalist and specialist herbivores.

Role of DNA methylation

DNA methylation can be modulated by environmental stress, and plays an important role in the phenotypic plasticity to environmental changes (Bossdorf et al. 2008; Jablonka and Raz 2009; Herman et al. 2014; Richards et al. 2017; Thiebaut et al. 2019; Mounger et al. 2021). In our work, for the grand-parent plants, the aboveground herbivory triggered a dramatic increase (about three times higher) in the level of methylated DNA in the damaged leaves (Fig. 4A). This may imply that the herbivory-induced growth responses within one generation could be closely associated with some chromatin modifications (i.e., DNA methylation). In a recent study of the association between insect herbivory and DNA methylation, it was also documented that the defense traits (including physical and chemical traits) of the perennial herbaceous species *Raphanus sativus* induced by aboveground herbivory of caterpillars could correspond to the increase in the methylation probabilities at the genetic loci of the damaged plants (Sobral et al. 2021). Inconsistent with the fourth hypothesis, as with no

obvious herbivory-induced transgenerational effects in the (grand-)offspring generations (with the exception of the offspring plants sprouted from the secondary roots of parent plants), herbivory-induced methylation of DNA may easily drop to an average level (between 2.2% and 4.0%) when vegetative generations are continuously established. These results further underscored that herbivory-induced transgenerational plasticity might not be a promising strategy for *A. philoxeroides* to adapt to the emerging enemies in the introduced ranges.

Conclusions

The presence of generalist herbivores such as *S. litura* significantly inhibited the performance of *A. philoxeroides* in the invasive ranges. To reduce herbivory loss, tolerance traits (e.g., biomass allocation to roots), rather than defense traits (e.g., the release of phenolic), became the primary strategy of the introduced *A. philoxeroides*. These herbivory-induced responses may further benefit the development of clonally produced offspring, but these effects are closely related to the root order of parent plants. It is also worth noting that the herbivory-induced responses may not always be stable across generations, and most of them could terminate in more than two generations. This decline of transgenerational effects also corresponds to the stabilized DNA methylation levels in the second and the third generation. This suggests that within-generation phenotypic plasticity, rather than transgenerational inheritance, may provide a more efficient means for *A. philoxeroides* to acclimate to the unpredictable herbivory environments by generalist enemies in the invasive ranges.

Declarations

Data availability statements

The raw data and the codes of the main analyses will be permanently archived online upon the acceptance of the manuscript.

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Authors' contributions

BCD designed the experiment; QYF, CLY, and RD performed the experiment; QYF and CLY analyzed the data; QYF and CLY wrote the paper, with major inputs from BCD and FHY, and further inputs from JS, FLL, and JQG.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

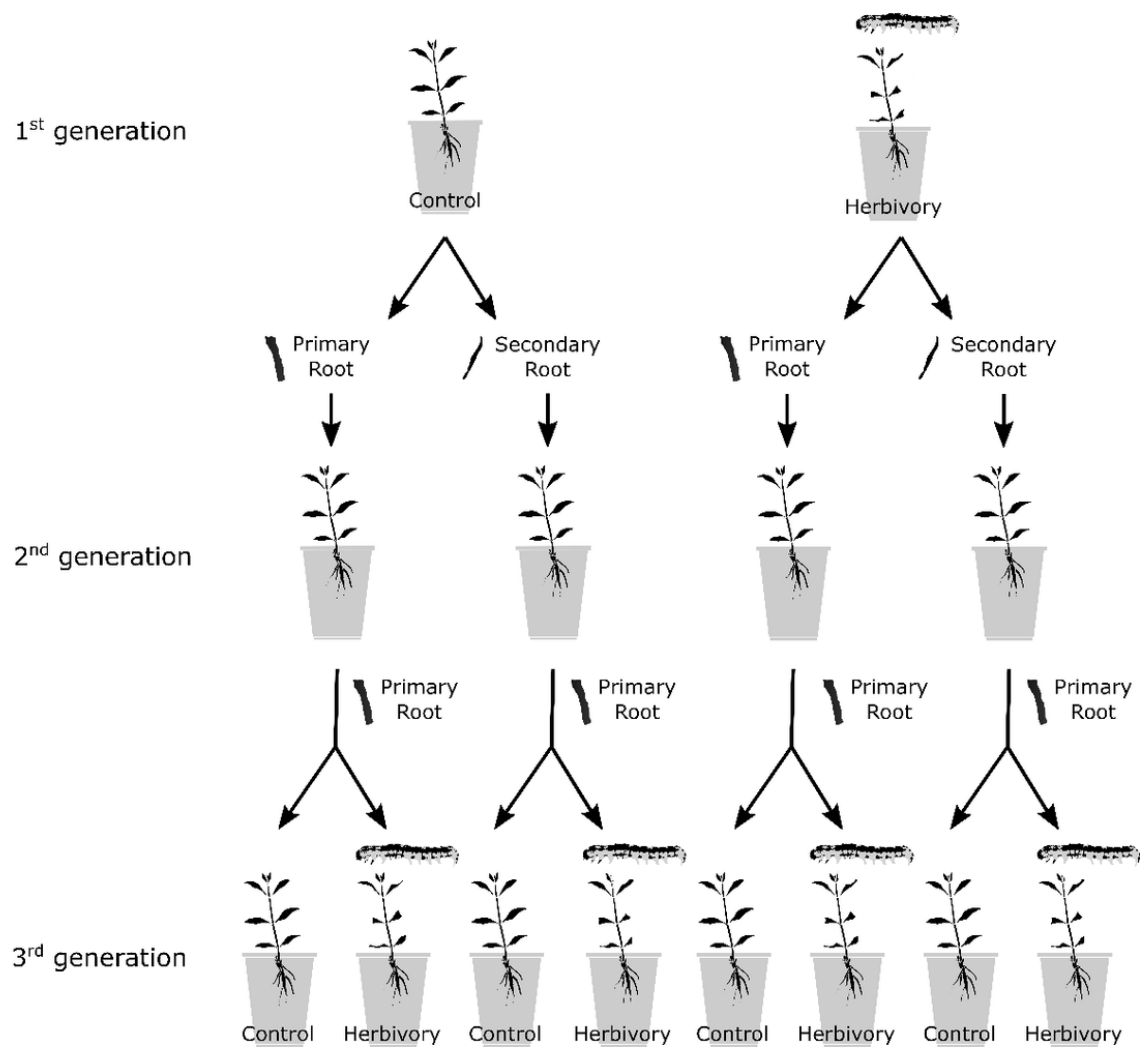


Figure 1

The diagram of the experimental design. Three clonal generations of *Alternanthera philoxeroides* were used in the experiment. In the first generation, plants were grown with or without herbivory (control vs. herbivory by *Spodoptera litura*). In the second generation, the offspring plants were subjected to two-way factorial treatments, including the parental herbivory (i.e., control vs. herbivory in the first generation) and the parental root order (i.e., primary or secondary roots of the first generation). In the third generation, the grand-offspring plants were subjected to three-way factorial treatments, including grand-parental herbivory, grand-parental root order, and current herbivory (i.e., control vs. herbivory in the third generation).

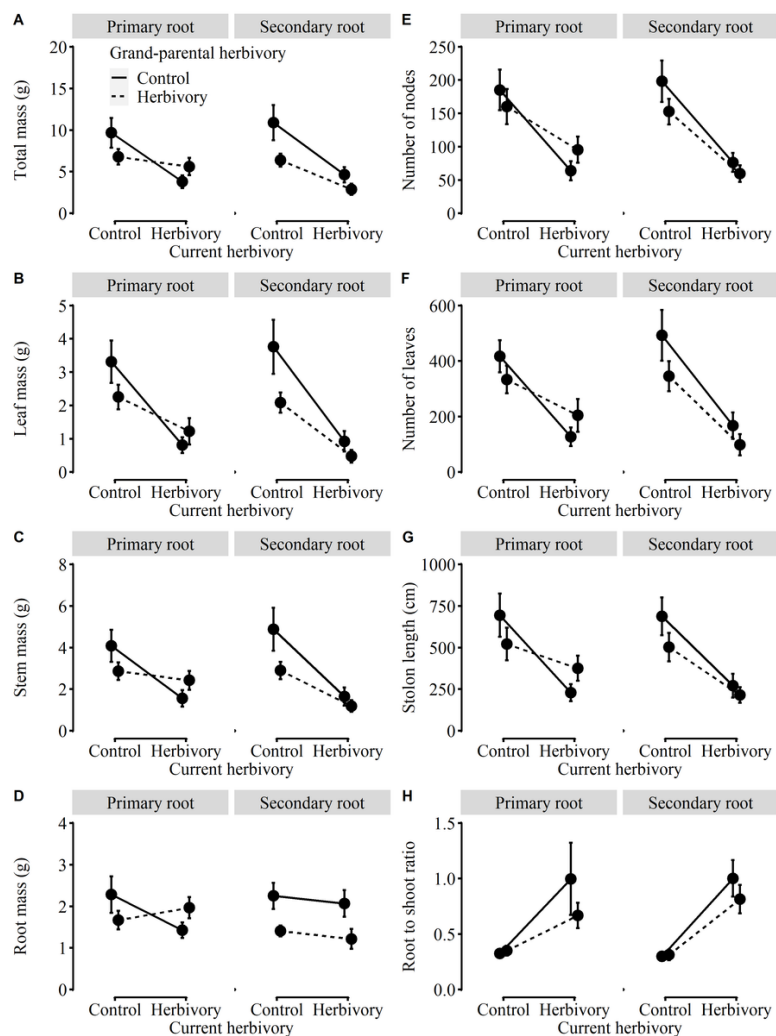


Figure 2

Effects of grand-parental herbivory (control vs. herbivory in the first generation), grand-parental root order (primary vs. secondary roots of grand-parent plants), and current herbivory (control vs. herbivory in the third generation) on (A) total mass, (B) leaf mass, (C) stem mass, (D) root mass, (E) number of nodes, (F) number of leaves, (G) stolon length, and (H) root to shoot ratio of the grand-offspring plants of *Alternanthera philoxeroides*.

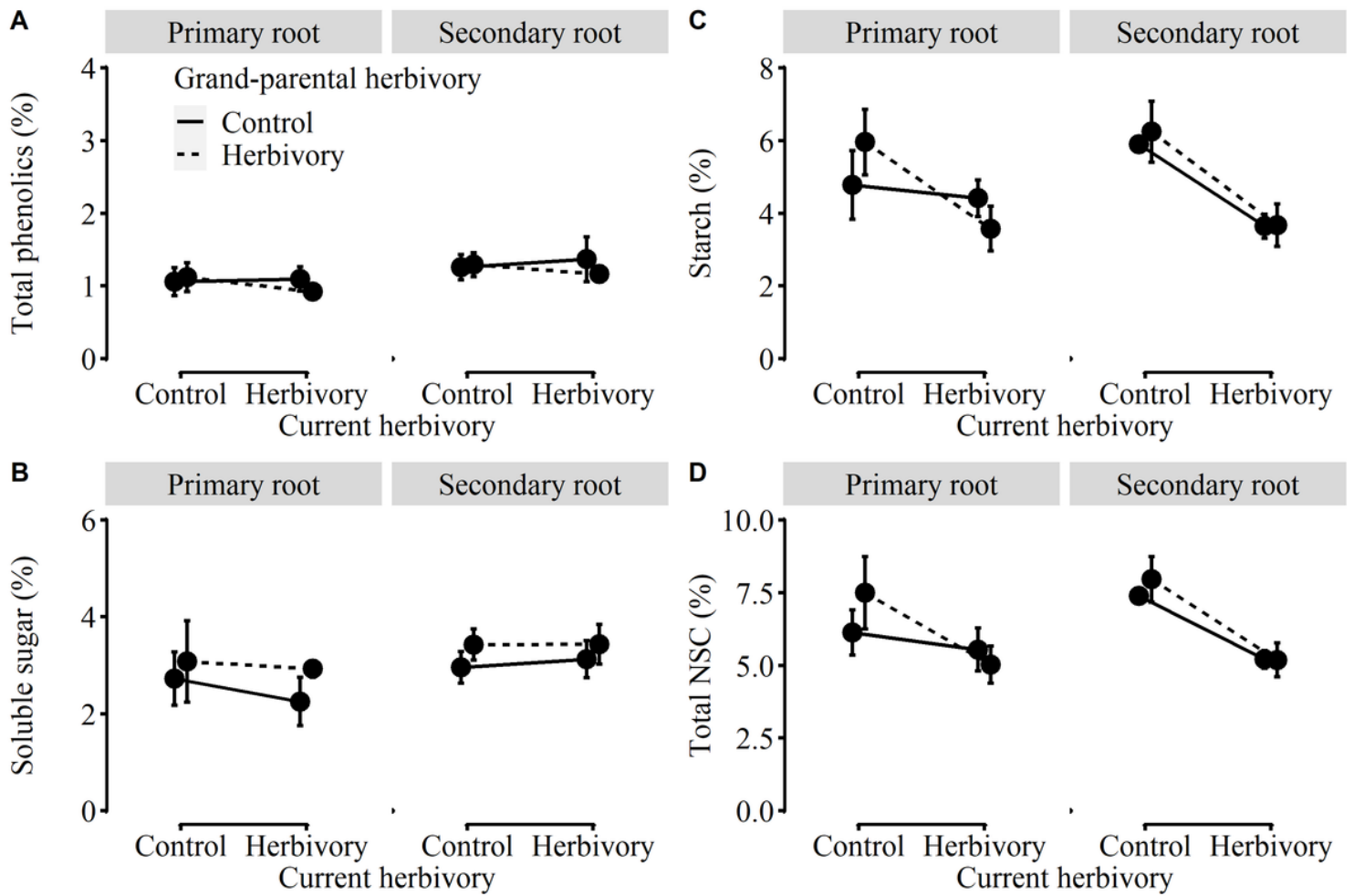


Figure 3

Effects of grand-parental herbivory (control vs. herbivory in the first generation), grand-parental root order (primary vs. secondary roots of grand-parent plants), and current herbivory (control vs. herbivory in the third generation) on the concentrations of (A) total phenolic, (B) water-soluble sugar, (C) starch, and (D) total NSC of the grand-offspring plants of *Alternanthera philoxeroides*.

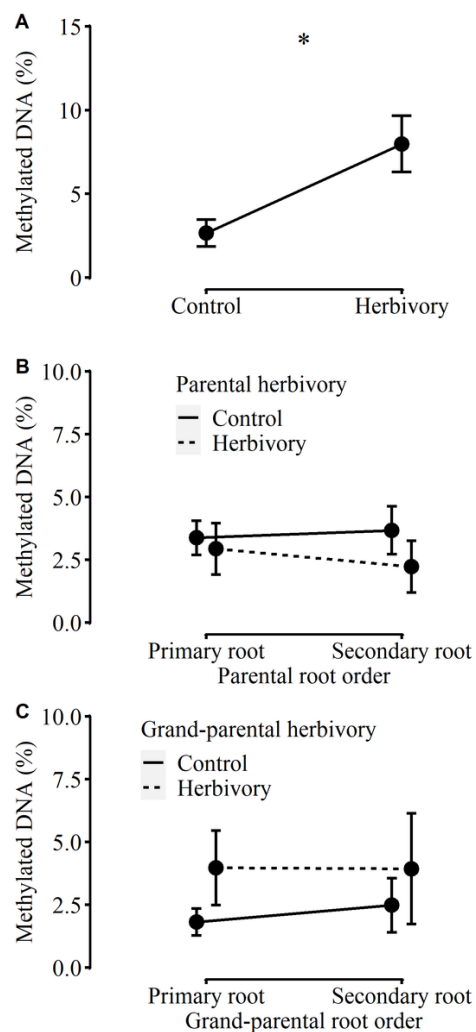


Figure 4

The methylated DNA levels in three clonal generations of *Alternanthera philoxeroides*, including (A) the methylated DNA levels in the grand-parent plants (i.e., the first generation); (B) the methylated DNA levels in the offspring plants (i.e., the second generation); (C) the methylated DNA levels in the grand-offspring plants (i.e., the third generation) without current herbivory. In (B) and (C), the points and error bars connected by dash lines presented the performance of clonal offspring taken from the (grand-)parent plants that experienced herbivory; the points and error bars connected by solid lines presented the performance of clonal offspring taken from the (grand-)parent plants that did not experience herbivory.

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