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Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation, volume II

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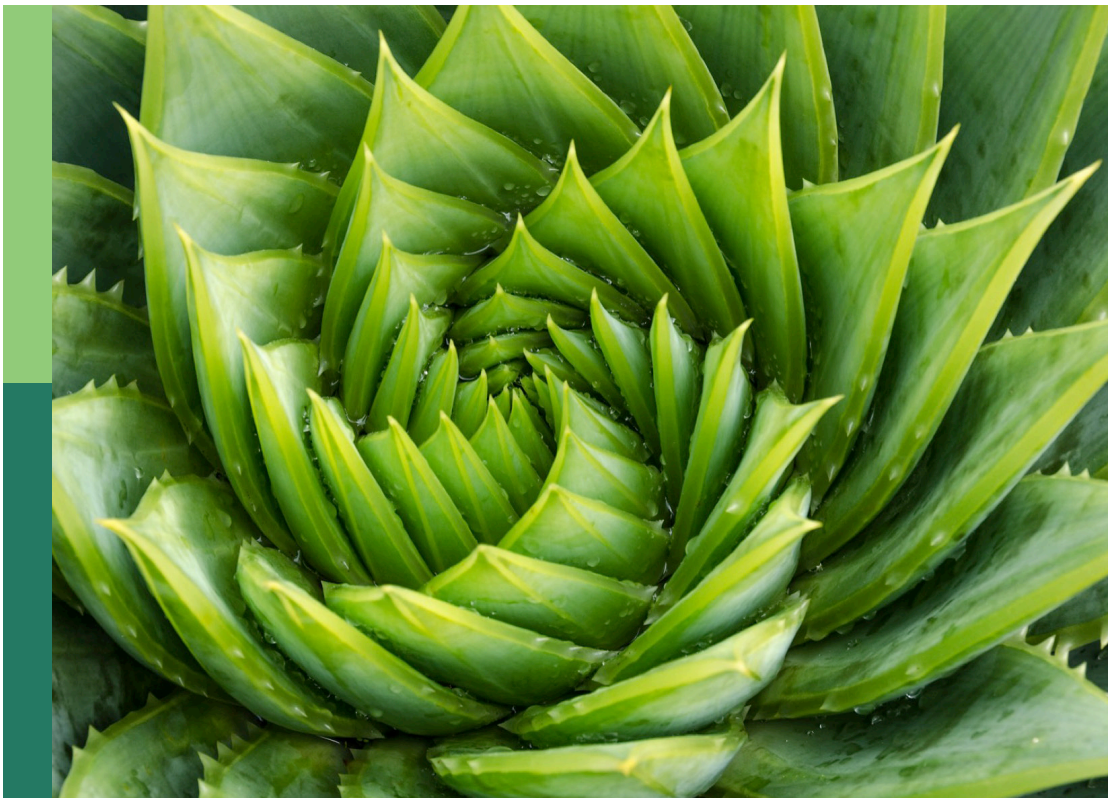
Published in
Frontiers in Plant Science



ISSN 1664-8714
ISBN 978-2-83251-445-0
DOI 10.3389/978-2-83251-445-0

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Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation, volume II

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Citation

Dong, B.-C., Roiloa, S., Yu, F.-H., Xue, W., eds. (2023). *Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-445-0

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frontiers | Frontiers in Plant Science

TYPE Editorial
PUBLISHED 11 January 2023
DOI 10.3389/fpls.2022.1126610

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

RECEIVED 18 December 2022
ACCEPTED 28 December 2022
PUBLISHED 11 January 2023

CITATION
Dong B-C, Roiloa SR, Xue W and Yu F-H (2023) Editorial: Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation, Volume II. *Front. Plant Sci.* 13:1126610. doi: 10.3389/fpls.2022.1126610

Editorial: Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation, Volume II

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KEYWORDS

clonal growth, environmental change, epigenetics, parental effect, resource provisioning, transgenerational plasticity, inbreeding plants

The Editorial on the Research Topic

Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation, Volume II

Accelerating environmental changes at the local, regional, and global scales are likely to favor species that can rapidly adapt to new environmental conditions. Long-lived clonal plants, in which reproduction is mainly asexual and clonal growth plays a central role in their population spread and maintenance, have long been thought to possess low genetic variation so that the potential for genetics-based adaptation to environmental changes may be limited. Thus, epigenetic variation may be particularly important for these clonal plants to adapt to rapid environmental changes (Latzel and Klimešová, 2010; Dodd and Douhovnikoff; Mounger et al., 2021).

Recent work suggests that the performance of an individual (ramet) of clonal plants is influenced by not only its current environmental condition but also the environmental condition of its parents (Huber et al., 2021; Xue et al., 2022). At least three mechanisms can explain such transgenerational (parental or maternal) effects (Herman and Sultan; Luo et al., 2022). First, parental environments could directly influence the performance of clonal offspring by altering the provisioning of carbohydrates and nutrients in vegetative propagules (e.g., fragmented stolons, rhizomes, or storage roots) (Dong et al., 2019). Second, environmental stress could also induce non-provisioning effects between clonal generations, *via* modifying the allocation of defensive chemicals and/or defence-inducing hormones to clonal offspring (Herman and Sultan). Third, parental environments could

trigger epigenetic changes in parent plants (e.g., the methylation of DNA and modifications of histones), to facilitate and optimize phenotype variation of clonal offspring in response to environmental change (Douhovnikoff and Dodd, 2015). This Research Topic consists of 11 articles, most of which explore the ecological significance of transgenerational effects and epigenetic variation in clonal plants.

Three papers focus on the relationship between epigenetic regulation and local adaptation of clonal plants under variable environmental stress. By experimental demethylation in natural conditions across different regions of Europe, Sammarco et al. found that the local adaptation mediated by epigenetic variation allowed the stoloniferous plant *Fragaria vesca* to better respond to changing climatic conditions. They suggest that epigenetic-based local adaptation may provide clonal plants with sufficient time to tackle the ongoing environmental crisis and to genetically adapt to it afterwards. In a field experiment, Campoy et al. compared variations in DNA methylation and phenotypic traits between native and introduced populations of the clonal succulent species *Carpobrotus edulis* under a climate change scenario, showing that phenotypic plasticity and global DNA methylation might be related to its rapid adaptation to new habitats. Wang et al. grew experimental populations of the creeping plant *Hydrocotyle vulgaris*, consisting of the same genotype, in two flood regimes and found significant phenotypic differences and associated DNA methylation differentiation between the two types of populations. They suggest that DNA methylation was involved in plant responses to environmental variation.

Four papers consider clonal transgenerational effects on growth, stress tolerance, and competitive ability of clonal offspring. Calibrated with data from two experiments, Wang et al. developed a model to test the transgenerational nitrogen effects on the summed and the mean performance of clonal offspring of the creeping clonal plant *Alternanthera philoxeroides*. They found that transgenerational effects at the whole-generation scale could be jointly influenced by multiple plant inherent characteristics (e.g., the survival rate, the number and the size distribution of clonal propagules), and the magnitude of transgenerational effects could also be obscured by developmental constraints. Zhang et al. tested transgenerational nitrogen effects on the fitness of three generations of the floating clonal plant *Pistia stratiotes*. They found that resource provisioning can increase the initial establishment of clonal offspring in favourable conditions, but this effect may not always be beneficial to their subsequent growth. Yu et al. showed that transgenerational effects could regulate interspecific competition between *P. stratiotes* and *Eichhornia crassipes* by altering the competitive ability of *P. stratiotes*, *via* changes in resource provisioning and/or DNA methylation. Guo et al. tested transgenerational ultraviolet-B (UV-B) effects on the fitness of clonal offspring of the stoloniferous plant *Glechoma longituba*. They found that transgenerational effects could promote the increase in the biomass allocation to aboveground parts in clonal offspring under similar UV-B stress, as well as their defence substances (e.g., flavonoid and anthocyanin), suggesting that the anticipatory transgenerational effects were likely to improve the UV-B resistance.

Two papers examine the effects of population differentiation on the offspring performance of widely-distributed species. Chen et al. examined whether parental environments (i.e., plants were collected from the high and low elevations in the hydro-fluctuation belt of the *Three Gorges Reservoir* region) and the early exposure of offspring of *Polygonum hydropiper* to flooding (accompanied with or without eutrophication) became as a positive or stressful cue on the subsequent growth of these offspring. They found that offspring produced by parental plants in the low elevation might have high adaptability in response to this "predictable" periodic flooding stress. Liu et al. tested the effects of populations with different introduction histories on growth traits of an invasive herb *Erigeron annuus* both in the wild and in common garden experiments. They found that there was parallel genetic and phenotypic differentiation among different invasive populations and that the populations that were introduced earlier had higher genetic diversity and higher growth dominance.

Two papers report within-generation responses of clonal species to stressful environments. Qi et al. tested the interaction effects between arbuscular mycorrhizal fungi (AMF) and soil phosphorus availability on the uptake ability and allocation strategies of an invasive clonal herb *Solidago canadensis*. They found that AMF were able to facilitate phosphorous acquisition by *S. canadensis* in insoluble phosphorous conditions, and also contribute to the invasiveness of *S. canadensis* in the resource-deficient environment. Jing et al. tested the effects of submergence depths on the growth responses of the clonal herb *A. philoxeroides*. They found that *A. philoxeroides* switched from "escape" to "quiescence" strategies in response to increasing submergence depths, and that morphological plasticity such as stem elongation could be essential for the acclimatization of *A. philoxeroides* to water-level fluctuations.

Transgenerational effects in clonal plants have drawn increasing attention during the last few years (Luo et al., 2022). However, there is still a long way to explore in this exciting field. Thus, the knowledge of the mechanisms that relate transgenerational environmental effects to epigenetic inheritance in clonal plants, the correlation between epigenetic variation with genetic and phenotypic variation in wild plant populations, the ecological and evolutionary role of transgenerational effects at different scales (e.g., the individual, population and community levels) have rarely been investigated so far. With the publications on this topic, we hope to further fill in the knowledge gap and stimulate more research on this important issue in the future.

Author contributions

All authors listed have made substantial and direct intellectual contributions to the work and approved it for publication.

Funding

Research was supported by grants from the National Natural Science Foundation of China (32071527) to F-HY, and the National Natural Science Foundation of China (31500331) to B-CD.

Conflict of interest

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ORIGINAL RESEARCH
published: 28 February 2022
doi: 10.3389/fpls.2022.827166

DNA Methylation Can Mediate Local Adaptation and Response to Climate Change in the Clonal Plant *Fragaria vesca*: Evidence From a European-Scale Reciprocal Transplant Experiment

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OPEN ACCESS

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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: 01 December 2021
Accepted: 07 February 2022
Published: 28 February 2022

Citation:
Sammarco I, Münzbergová Z and Latzel V (2022) DNA Methylation Can Mediate Local Adaptation and Response to Climate Change in the Clonal Plant Fragaria vesca: Evidence From a European-Scale Reciprocal Transplant Experiment. Front. Plant Sci. 13:827166. doi: 10.3389/fpls.2022.827166

The ongoing climate crisis represents a growing threat for plants and other organisms. However, how and if plants will be able to adapt to future environmental conditions is still debated. One of the most powerful mechanisms allowing plants to tackle the changing climate is phenotypic plasticity, which can be regulated by epigenetic mechanisms. Environmentally induced epigenetic variation mediating phenotypic plasticity might be heritable across (a)sexual generations, thus potentially enabling rapid adaptation to climate change. Here, we assessed whether epigenetic mechanisms, DNA methylation in particular, enable for local adaptation and response to increased and/or decreased temperature of natural populations of a clonal plant, *Fragaria vesca* (wild strawberry). We collected ramets from three populations along a temperature gradient in each of three countries covering the southern (Italy), central (Czechia), and northern (Norway) edges of the native European range of *F. vesca*. After clonal propagation and alteration of DNA methylation status of half of the plants *via* 5-azacytidine, we reciprocally transplanted clones to their home locality and to the other two climatically distinct localities within the country of their origin. At the end of the growing season, we recorded survival and aboveground biomass as fitness estimates. We found evidence for local adaptation in intermediate and cold populations in Italy and maladaptation of plants of the warmest populations in all countries. Plants treated with 5-azacytidine showed either better or worse performance in their local conditions than untreated plants. Application of 5-azacytidine also affected plant response to changed climatic conditions when transplanted to the colder or warmer locality than was their origin, and the response was, however, country-specific. We conclude that the increasing temperature will probably be the limiting factor determining *F. vesca* survival and distribution. DNA methylation may contribute to local adaptation and response to climatic change in natural ecosystems;

however, its role may depend on the specific environmental conditions. Since adaptation mediated by epigenetic variation may occur faster than *via* natural selection on genetic variants, epigenetic adaptation might to some degree help plants in keeping up with the ongoing environmental crisis.

Keywords: adaptation, survival, 5-azacytidine, climate change, latitudinal gradient, clonal plant, epigenetics

INTRODUCTION

Understanding the basis of plants ability to cope with rapidly changing environment is crucial in predicting and mitigating the consequences of the ongoing climate crisis. Plants face rapid climatic and environmental change with three commonly accepted mechanisms: by moving to more favorable conditions, by genetically adapting to the changed environment, or by adjusting their phenotypes to the changed environment. Considering that the sessile lifestyle limits plant movement to novel environments, the "escape" strategy can have only limited effect (Loarie et al., 2009). Furthermore, natural selection may not be quick enough to enable plant populations to adapt to changing environment (Visser, 2008; Hoffmann and Sgrò, 2011; Merilä, 2012; Chevin et al., 2013; Carlson et al., 2014; Botero et al., 2015). Phenotypic plasticity can be thus on the forefront as the most powerful mechanism of plants to tackle the changing climate (Nicotra et al., 2010; Hoffmann and Sgrò, 2011).

Phenotypic plasticity can be mediated by epigenetic mechanisms, such as DNA methylation, which affect phenotypes by regulating gene activity and that may result in locally adapted phenotypes (Merilä and Hendry, 2014). Epigenetic variation can be triggered by environmental variation, arise stochastically (i.e., epimutations) or can be driven by genetic variants (Ahmed et al., 2011; Zhang et al., 2013, 2018; Johannes and Schmitz, 2019). Genetically induced epigenetic variants are completely under genetic control and arise slowly within a population because of the low rate of genetic changes (Richards, 2006). On the contrary, by acting independently of genetic variants, environmentally and stochastically induced epigenetic variants can quickly create novel phenotypes. Importantly, epigenetic variation can be heritable across multiple generations, thus representing another source of heritable variation on which natural selection can operate (Richards, 2006; Hauser et al., 2011; McNamara et al., 2016; Sobral et al., 2021).

Heritable epigenetic variation triggered either by the environment or originating by stochastic epimutations can play a crucial role in the adaptation of clonal populations (Latzel and Klimešová, 2010; Richards et al., 2012; Verhoeven and Preite, 2014; Dodd and Douhovnikoff, 2016; Latzel et al., 2016; Münzbergová et al., 2019; Shi et al., 2019). In fact, clonal species usually form populations with limited standing genetic variation, which can remarkably slow down their adaptation to the rapidly changing environment (Dodd and Douhovnikoff, 2016). As heritability of epigenetic variants seems to be more prominent across clonal than sexual generations (reviewed in Feng et al., 2010; Anastasiadi et al., 2021), heritable epigenetic variation can compensate the lack of standing genetic variation in clonal populations (Dodd and Douhovnikoff, 2016; González et al., 2016; Rendina González et al., 2018; Zhang et al., 2018).

Recent studies suggested that mechanisms enabling epigenetic adaptation can depend on environmental conditions (Fan et al., 2013; Ci et al., 2015; Li et al., 2016; Hossain et al., 2017; Tang et al., 2018). For example, in many plant species, heat but not cold stress is accompanied by a global reduction of DNA methylation (i.e., hypomethylation; Ci et al., 2015; Li et al., 2016; Hossain et al., 2017), suggesting that the role of DNA methylation in response to different temperatures can vary.

However, despite DNA methylation seems to be a potent mechanism enabling rapid adaptation to climate change, solid evidence of its importance in plant adaptation in natural ecosystems is still missing. In fact, studies have traditionally attempted to provide evidence of the role of DNA methylation in local adaptation only indirectly, by associating patterns of epigenetic variation of natural populations to local conditions (Dubin et al., 2015; Platt et al., 2015). However, this approach can provide ambiguous evidence of the role of DNA methylation in local adaptation, as the observed epigenetic patterns might be linked to genetic variation (i.e., genetic rather than epigenetic adaptation), or might not be heritable and thus evolutionarily relevant. Such limitations can be bypassed by using reciprocal transplant experiments involving experimental alteration of plant epigenomes before transplantation to their home and away environments. Comparing the fitness of plants with altered DNA methylation with those with natural DNA methylation can serve as a test of epigenetically driven local adaptation (e.g., Herden et al., 2019). Reciprocal transplant experiments are indeed traditional tests for the study of local adaptation (e.g., Ågren and Schemske, 2012). When including the experimental alteration of epigenomes of clones of the same plants, reciprocal transplant experiments can provide a direct evidence of the role of epigenetic mechanisms in local adaptation and/or response to changing climate, without the confounding effect of underlying genetic variation. However, these studies are currently very rare, and to our knowledge, there is only one other study employing such an approach in field conditions (Herden et al., 2019).

In our study, we asked whether epigenetic mechanisms, DNA methylation in particular, enable for local adaptation of natural populations of a clonal species, *Fragaria vesca* (wild strawberry). We also tested whether potential DNA methylation driven by local conditions alters plant response to climate change, to increased and/or decreased temperature, respectively. We chose *F. vesca* as it occurs in broadly heterogeneous habitats, it has wide geographic distribution and extensive clonal propagation. To fulfil our aims, we collected plants (ramets) from three sites along a temperature gradient in each of three countries covering the southern (Italy), central (Czechia), and northern (Norway) edges of

the native European range of *F. vesca*. We moved the ramets to a common garden where we let them clonally propagate for one season. We used two ramets from each clone and planted them individually in a greenhouse and let them to produce several offspring ramets. In half of these ramets (hereafter plants), we altered the DNA methylation status by using the demethylating agent 5-azacytidine (5-azaC), employing the foliar application method described by Puy et al. (2018). In the following spring 2019, we transplanted plants to their home locality and to the two other localities (away) within the country of their origin. Three months later, we recorded their survival, their leaf number, and size and damage due to herbivory.

We asked three specific questions to address three hypotheses: (1) Is there evidence of local adaptation of *F. vesca* populations? We hypothesize that our populations show evidence of local adaptation, that is, that the local plants will have higher survival and/or biomass, and/or reduced herbivory damage than the non-local plants. (2) Is local adaptation mediated by DNA methylation? We hypothesize that local adaptation is under DNA methylation control, that is, that the local plants with altered DNA methylation will have reduced survival and/or biomass, and/or increased herbivory damage than the local plants with natural DNA methylation. (3) Is adaptation to warm conditions mediated differently than to cold conditions? We hypothesize that DNA methylation plays different roles in adaptation to warm and/or cold conditions (e.g., Pan et al., 2011; Ci et al., 2015; see above), that is, that effects of altered DNA methylation on survival, biomass, and/or herbivory damage will be influenced by climatic conditions and/or regions (countries) of plant origin.

MATERIALS AND METHODS

Study Species

Fragaria vesca L., Rosaceae, is an herbaceous perennial species growing in disturbed and degraded forests, forest edges, and meadows. It has a wide geographic distribution: it occurs throughout Europe, northern Asia, North America, and northern Africa (Darrow, 1966). It is able to reproduce both sexually through seeds and clonally by producing stolons although its sexual reproduction is very rare in natural conditions (Schulze et al., 2012).

Sites Selection

We conducted the study at nine sites across three European countries: Italy, Czechia, and Norway (**Table 1**). We selected the countries to include populations from the southern (Italy) and northern (Norway) limits of the native range of *F. vesca* distribution and populations from the core of its distribution range (Czechia) in Europe. Within each country, we selected three sites to be distributed along a climatic gradient ranging from warmest to coldest mean annual temperatures (defined as: warm, intermediate, and cold sites), usually from lowland to mountain regions. Therefore, hereafter we use term "temperature of origin" of plants, although we are aware that selected populations differed also in other environmental factors. The sizes of the selected populations ranged from 12 to 800 m^2 (**Table 1**).

Climatic Data

We sourced climatic data from the European gridded dataset E-OBS, available through the C3S Climate Data Store (CDS) website (https://cds.climate.copernicus.eu/cdsapp#!/home; Cornes et al., 2018). We retrieved the mean of daily values of temperature over the years 2011–2018, with a horizontal resolution at $0.1 \times 0.1°$ (v20.0e).

Plant Collection, Cultivation, and 5-azaC Treatment

We collected 5 to 7 ramets from each of the nine populations (sites) between May and July 2018 and transported them to the common garden of the Institute of Botany of the Czech Academy of Sciences in Průhonice, Czechia (49.994°N, 14.566°E). The collected ramets were wrapped in wet paper cloth, placed in plastic bags, and transported in a refrigerating box at 8°C. Individual ramets were planted 1 to 10 days after collection in $70 \times 40 \times 20$ cm trays filled with a commercial mixture of compost and sand, under a shading coverage (reduction of light for 50% to simulate natural light levels at most of the localities). In October 2018, we collected two comparable offspring ramets (F1 clonal offspring, connected with original maternal ramet *via* stolon) from each of the maternal plants and planted them individually in separate $35 \times 22 \times 5$ cm trays placed in a greenhouse tempered at 20/15°C (day/night), 14 h photoperiod to precultivate plant material for the transplant experiment, see later. We sprayed half of the plants twice a week with an aqueous solution of 5-azaC (50 μM in the first 3 weeks of the treatment, and 100 μM in the following months) and a surfactant (1 ml Silwet Star—AgroBio Opava s.r.o./ 1 l solution), from the beginning of February 2019 to late May 2019. This approach allows to obtain similar demethylation effects as the original method based on the germination of seeds in a 5-azaC solution, however avoiding the unwanted side effects usually observed in the original method (e.g., underdeveloped root systems and high mortality of treated plants; Puy et al., 2018). In order to control for potential unknown effects of the surfactant, we sprayed the other half of the plants only with water and surfactant (1 ml Silwet Star/1 l solution). One month prior to the transplant experiment (April 2019), we moved all plants back to the common garden. For transplantation to the field sites, we preferred using the youngest ramets, that is, ramets developed after the start of the demethylation treatment. Moreover, a global DNA methylation analysis confirmed overall demethylating effect of 5-azaC on treated plants (see later).

We also determined the genetic relatedness of a random subset of transplanted plants using whole-genome SNP data to test whether the effect of 5-azaC differed between genotypes and to determine genetic diversities of the populations (see later; Sammarco et al., unpublished).

TABLE 1 | Temperature range, mean annual temperature, elevation, population size (m^2), and location of selected populations in the three European countries.

Country (distribution range)	Temperature range	2011–2018 mean T (°C)	Elevation (m)	Population size (m^2)	Location
Italy (southern edge)	Warm	10.34	468	45	46.471°N, 11.343°E
	Intermediate	3.55	1,436	156	46.725°N, 11.422°E
	Cold	2.45	1905	42	46.337°N, 11.790°E
Czechia (center)	Warm	10.62	201	140	50.399°N, 14.412°E
	Intermediate	9.49	306	400	50.459°N, 14.785°E
	Cold	5.59	875	800	50.811°N, 15.359°E
Norway (northern edge)	Warm	2.88	597	160	60.895°N, 7.349°E
	Intermediate	1.50	818	12	61.036°N, 9.079°E
	Cold	1.25	323	60	60.821°N, 8.706°E

Global DNA Methylation Quantification

We quantified global DNA methylation level of a subset of the 5-azaC-treated plants ($N = 11$) and of the respective ramets with natural DNA methylation ($N = 11$), that is, not treated plants by 5-azaC, with the MethylFlash Methylated DNA Quantification Kit-Colorimetric (Epigentek) following the manufacturer's instructions. Briefly, we extracted genomic DNA using the Qiagen DNeasy Plant Mini Kit and used between 50 and 200 ng of input DNA per reaction. After binding the DNA to the strip wells, we incubated the reaction with capture and detection antibodies allowing the quantification of global DNA methylation through an ELISA-like reaction at 450 nm.

We then normalized the DNA methylation level of 5-azaC plants ($5mC\%_{5azaC}$) with that of ramets with natural methylation ($5mC\%_{Ctrl}$), following the "Relative Quantification" method as in the manufacturer's instructions, and using the following formula:

$$\text{Normalized } 5mC\%_{5azaC} = \frac{5mC\%_{5azaC}}{5mC\%_{Ctrl}} \times 100\%$$

Reciprocal Transplant Experiment

Between late May and early June 2019, we collected individual rooting ramets from the precultivated plants. We separated them, wrapped in wet paper cloth, placed in plastic bags, and transported in a refrigerating box at 8°C to the target localities within 1–7 days. We standardized all ramets to consist of two leaves, to avoid different plant sizes at the transplantation time. The transplantation sites included the home locality of the maternal plants and the other two away sites within the country of their origin, for example, plants from the warm localities were transplanted to the warm localities (home sites), intermediate and cold localities (away sites) within each country (**Supporting Information**, **Supplementary Table S1**). Accordingly, we planted ramets of local plants and ramets of other two populations from the same country in each site. We distinguish a target site (site where the plants were transplanted) and a site of origin (site where the original ramet was collected). We consider plants transplanted back to their site of origin as growing in their *home* environment (i.e., origin site = target site), whereas plants transplanted to different sites as growing in *away* sites (i.e., origin site ≠ target site). In each target site, we planted between 49 and 116 plants. The number of transplanted plants depended on the availability of plant material at the collection time (the specific number of transplanted ramets is presented in **Supplementary Table S1**). Across all target sites, we evenly distributed 2 to 4 ramets originated from the same maternal plant. However, in minority of cases we transplanted only 1 ramet for a specific site (according to the availability of plant material). In order to control for the unknown transplantation effect and precultivation of plants in the common garden of the Institute, we also replanted between 8 and 10 local plants of *F. vesca* population found at the transplantation site at each locality except for the cold locality in Czechia, where we could not find any local plants at the time of transplantation. We dug out the local plants, standardized them to consist of two leaves (i.e., similar to the precultivated ramets), and replanted them immediately back to the same locality. Thus, these plants had not been transplanted across different localities and had not been precultivated in the common garden (hereafter referred to as *replanted local plants*). We planted the ramets at least 10 cm apart from each other in a randomized grid. Each ramet was labelled with a unique and anonymized code. We watered all plants immediately after planting, but did not provide any additional treatment later during the growing season. Together, we transplanted 801 ramets across all localities.

Measurements

In September 2019, that is, 3 months after planting, we recorded survival of transplanted ramets, number of leaves, length of the longest leaf (cm), herbivory damage (5 categories: "0" no damage, "1" 1–5% of leaf area removed, "2" 6–25%, "3" 26–50%, "4" 51–75%, "5" >75%), number of flowers and fruits, and number of stolons and ramets. For each plant, we estimated its biomass as the total number of leaves of transplanted (maternal) ramet multiplied by the length of its longest leaf. Since only few plants flowered, fruited, or produced stolons and offspring ramets (<4%), we did not analyze these data.

Data Analysis

To test for evidence of local adaptation of *F. vesca* populations and for the role of DNA methylation in local adaptation, we tested the effects of country, temperature of origin, home/away, and 5-azaC treatment on plant survival, biomass, and herbivory damage. To test for the role of DNA methylation in plants from different climatic conditions and/or countries, we replaced both temperature of origin and home/away with the temperature distance between origin and target sites. We calculated temperature

distance as the difference between the long-term mean average temperature of the site of origin (over the years 2011–2017) and the short-term mean average temperature of the target site (i.e., after the transplantation time, 2019). We calculated all the temperature averages using only the months included in the growing season (from June to August). We also repeated the same test replacing temperature distance with precipitation distance, and we found similar significant interactions as for the temperature distance (**Supplementary Note**).

We could not test the effects of both temperature and precipitation distances together since we found these variables to be highly correlated ($p = 0.033$, $R = -0.41$). We then chose to show temperature as climatic variable for the above-mentioned test in the main text since the field sites were selected primarily along a temperature gradient.

We used survival, herbivory damage, and biomass as dependent variables. We log-transformed biomass to fit the assumptions of normality. We coded herbivory as 0 and 1 (respectively, 0–5 and > 5% of area removed) as the data had strongly bimodal distribution. We analyzed survival data using the complete dataset, while we used only the surviving plants when analyzing biomass and herbivory damage.

To analyze the data, we used mixed effect models with maternal plant code as random factor. To account for the effect of plant biomass on herbivory, we included biomass as a covariate in the models testing herbivory damage.

We also tested for possible unknown side effects induced by the transplantation and precultivation of the plants in the common garden, by comparing survival and biomass of plants with natural DNA methylation to those of replanted local plants (**Supplementary Note**).

We tested the binary variables (survival and herbivory damage) with Generalized linear mixed models (GLMMs; binomial distribution and logit link function), using the LME4 package for R (Bates et al., 2015). We tested the biomass index using Linear mixed models (LMMs) with the LMERTEST package (Kuznetsova et al., 2017). We performed all analyses in RStudio, using R 3.6.2 (R Core Team, 2017).

To test whether the effect of 5-azaC differed between genotypes, we partly modified the approach used in Münzbergová et al. (2019). We calculated the genetic relationship matrix for each individual plant from whole-genome SNP data (Sammarco et al., unpublished) with the SNPRelate package for R (Zheng et al., 2012). For each genotype and separately for each locality, we calculated average proportional change for both survival and biomass ("Trait") after removal of DNA methylation compared to plants with natural DNA methylation, as:

$$\text{Proportional change} = \frac{\text{Trait}_{Ctrl} - \text{Trait}_{5azaC}}{\text{Trait}_{Ctrl} + \text{Trait}_{5azaC}}$$

We calculated pairwise differences in response to removal of DNA methylation of each genotype in each target locality using Euclidean distance. We tested the correlation between the genetic relationship matrix and matrix of distances in response to removal of DNA methylation using the vegan R package (Oksanen et al., 2020). We also repeated the tests with partial Mantel tests using four different matrices as covariates, accounting for the temperature distance of either the origin or target sites, and for origin or target site (calculated as: "0" when the two genotypes had the same origin or target site, respectively; "1" when their origin or target sites were different). The tests showed no significant results in neither case and are thus only shown in (**Supporting Information**, **Supplementary Table S2**).

RESULTS

Reduced Global DNA Methylation in 5-azaC-Treated Plants

In order to assess the actual alteration of DNA methylation in plants treated with 5-azaC, we quantified global DNA methylation level of a random subset of the 5-azaC-treated plants ($N = 11$) and of the same genotypes with natural DNA methylation ($N = 11$). The mean methylation level of 5-azaC-treated plants was 26.85% lower (SE ± 8.00) than control plants ($t = -3.352$, $p < 0.001$), after excluding four outliers whose DNA methylation level exceeded 100% methylation change compared to the control plants.

Is There Evidence of Local Adaptation of *F. vesca* Populations? (Hypothesis 1)

To test for evidence for local adaptation of *F. vesca* populations and for the role of DNA methylation in local adaptation, we tested the effects of country, temperature of origin, home/away, and 5-azaC treatment on plant survival, biomass, and herbivory damage (**Table 2**).

Survival and biomass of plants transplanted to their home environment significantly differed according to the country and temperature of origin (Country × Temperature. Origin × Home, **Table 2A**). For survival, we found evidence of local maladaptation for the warm populations in all countries (**Figure 1A**). In the warm sites, survival of plants was consistently lower for plants transplanted to their home than away sites. On the other hand, in the intermediate sites, survival was higher for the populations in their home environment both in Italy and Norway, while it did not differ in Czechia. Lastly, survival was higher for the home population in the cold sites in Italy and Czechia, but did not differ in Norway.

Biomass of surviving plants from the warm populations did not significantly differ among home and away plants (**Figure 1B**). Plants from the intermediate sites tended to have higher biomass when transplanted to their home than away sites in all three countries. Finally, plants from the cold sites tended to have lower biomass when transplanted to their home than away sites in all countries but significantly only in Italy.

Is Local Adaptation Mediated by DNA Methylation? (Hypothesis 2)

We found a significant interaction of 5-azaC with home-away effects for biomass and marginally significant for survival (Temperature.Origin × Home × 5-azaC, **Table 2B**). In the warm sites, both survival and biomass consistently decreased in

TABLE 2 | Test of local adaptation (Hypothesis 1; A) and role of DNA methylation in local adaptation (Hypothesis 2; B).

	Survival			Biomass			Herbivory damage		
	d.f.	**F**	***P***	**d.f.**	**F**	***P***	**d.f.**	**F**	***P***
A									
Country (C)	2	**4.30**	**<0.001**	2	**3.81**	**0.024**	2	0.11	0.537
Temperature (T)	2	0.37	0.331	2	1.57	0.210	2	0.69	0.221
Home/Away (H/A)	1	0.00	0.950	1	0.49	0.483	1	0.36	0.145
C × T	4	0.22	0.419	4	1.84	0.125	4	0.66	0.298
C × H/A	2	6.78	0.293	2	1.56	0.212	2	0.22	0.716
T × H/A	2	25.10	0.081	2	**12.48**	**<0.001**	2	4.31	0.848
C × T × H/A	4	**1.04**	**0.004**	4	**3.08**	**0.016**	4	1.70	0.089
B									
5-azaC	1	1.04	0.271	1	0.27	0.605	1	**5.81**	**0.005**
C × 5-azaC	2	**1.99**	**<0.001**	2	1.64	0.195	2	**0.02**	**0.004**
T × 5-azaC	2	0.83	0.255	2	1.36	0.258	2	0.33	0.694
H/A × 5-azaC	1	1.78	0.417	1	0.53	0.467	1	2.11	0.278
C × T × 5-azaC	4	0.25	0.557	4	1.48	0.207	4	0.99	0.139
C × H/A × 5-azaC	2	0.36	0.160	2	2.90	0.056	2	0.13	0.580
T × H/A × 5-azaC	2	2.44	0.064	2	**6.44**	**0.002**	2	1.31	0.086
C × T × H/A × 5-azaC	4	0.08	0.433	2	2.11	0.123	2	0.90	0.399

*Effects of country (Country, C), temperature of origin (Temperature, T), home/away (Home/Away, H/A), 5-azaC treatment (5-azaC), and their interactions on plant survival, biomass, and herbivory damage. N = 730 (survival), N = 403 (biomass, herbivory damage). D.f.: degrees of freedom. Significant values ($p \leq 0.05$) are shown in bold. Estimates of the effects can be found in (***Supporting Information***, **Supplementary Table S3***).*

5-azaC-treated plants transplanted in their home sites compared to plants with natural DNA methylation (**Figures 2A**,**B**). In the other temperature sites, 5-azaC treatment had mostly no effect on plant survival and biomass, except for the populations from the intermediate sites transplanted in their home sites, in which biomass of 5-azaC-treated plants was higher than survival of plants with natural DNA methylation (**Figures 2A**,**B**).

For herbivory damage, we found significant effect of 5-azaC treatment alone and in combination with country (5-azaC; Country × 5-azaC, **Table 2B**), but no significant interaction of application of 5-azaC with home-away effects. Specifically, plants treated with 5-azaC showed increased levels of herbivory than control plants (mean ± SE, Ctrl = 0.36 ± 0.03, 5-azaC = 0.53 ± 0.04), and such a difference was weaker in Norway than in the other two countries (**Supporting Information**, **Supplementary Figure S1**).

Is Adaptation to Warm Conditions Mediated Differently Than to Cold Conditions? (Hypothesis 3)

To test for the role of DNA methylation in response of transplantation to different climatic conditions in the three countries, we tested the effects of 5-azaC treatment in interaction with country and temperature distance between origin and target sites on plant survival, biomass and herbivory damage.

We found no significant interactions of 5-azaC treatment with temperature distance and/or country on either plant biomass or herbivory damage (**Table 3**). However, we found a significant effect on survival of 5-azaC treatment in interaction with both country and temperature distance (Country x Temperature.Distance x 5-azaC, **Table 3**). Specifically, in all the countries, survival consistently increased for plants transplanted from warmer to colder sites, while the effect of the 5-azaC treatment was country-specific (**Figure 3**). For both Italy and Czechia, the correlation between survival and temperature distance was stronger in plants with natural DNA methylation than 5-azaC plants, while in Norway it was stronger in 5-azaC than plants with natural DNA methylation.

DISCUSSION

The ongoing climate crisis that is threatening plants and other organisms triggered an intense debate on whether and how plants will be able to adapt to future environmental conditions. By using a reciprocal transplant experiment, we tested whether *Fragaria vesca* is adapted to local conditions in three European countries across a climatic gradient and if it performs better or worse to increasing temperatures. We measured survival, biomass, and herbivory damage of transplanted ramets, and we consider survival as the main proxy of performance since this is the ultimate measure of plant fitness. Based on survival, we detected local adaptation only for the intermediate and cold populations in Italy. On the other hand, we found evidence for maladaptation of *F. vesca* to warm temperature in all countries (**Figure 1A**). Moreover, based on biomass, we found evidence for maladaptation for the cold population in Italy (**Figure 1B**). By experimental alteration of DNA methylation of selected plants, we also tested whether local adaptation can be under DNA methylation control. Ramets with altered DNA methylation (5-azaC, about 27% reduced overall DNA methylation in comparison to controls) showed worse performance (significant only for survival) in their local conditions than untreated plants, but only in the warm sites,

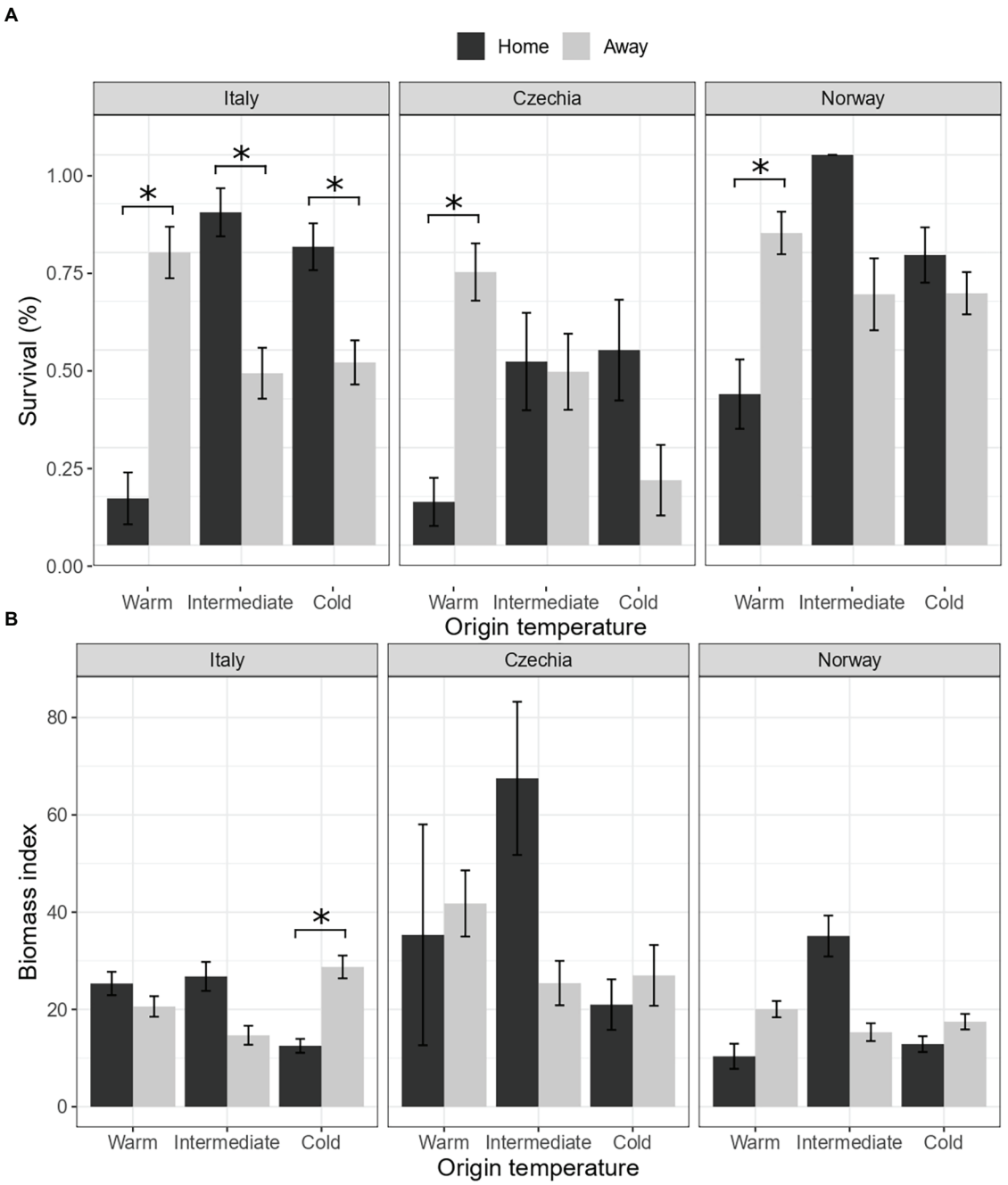

FIGURE 1 | Is there evidence of local adaptation of *F. vesca* populations (Hypothesis 1)? Effects of country, origin temperature and home/away site on survival **(A)** and biomass **(B)**. Home: plants in their home site, away: plants in away sites. Values represent the means ± 1 standard error (SE). Significance level $p < 0.05$ (*).

suggesting that DNA methylation plays particularly a role in response to warmer temperatures (**Figure 2**). Finally, we tested whether DNA methylation plays a distinct role in adaptation to contrasting environments (warm/cold conditions). Both plants with natural DNA methylation and plants with altered DNA methylation showed a positive correlation between survival and temperature distance between the origin and target sites, but the effect of the 5-azaC treatment was country-specific (**Figure 3**).

Local Adaptation and Response to Climate Change

Regarding the survival of transplanted ramets, our data revealed that all populations from the warm localities were maladapted to their home environment. On the other hand, we found no evidence of maladaptation to intermediate or cold temperature conditions in all countries, suggesting that the limiting factor for the survival of *F. vesca* were high summer temperatures. The maladaptation observed in the warm populations might

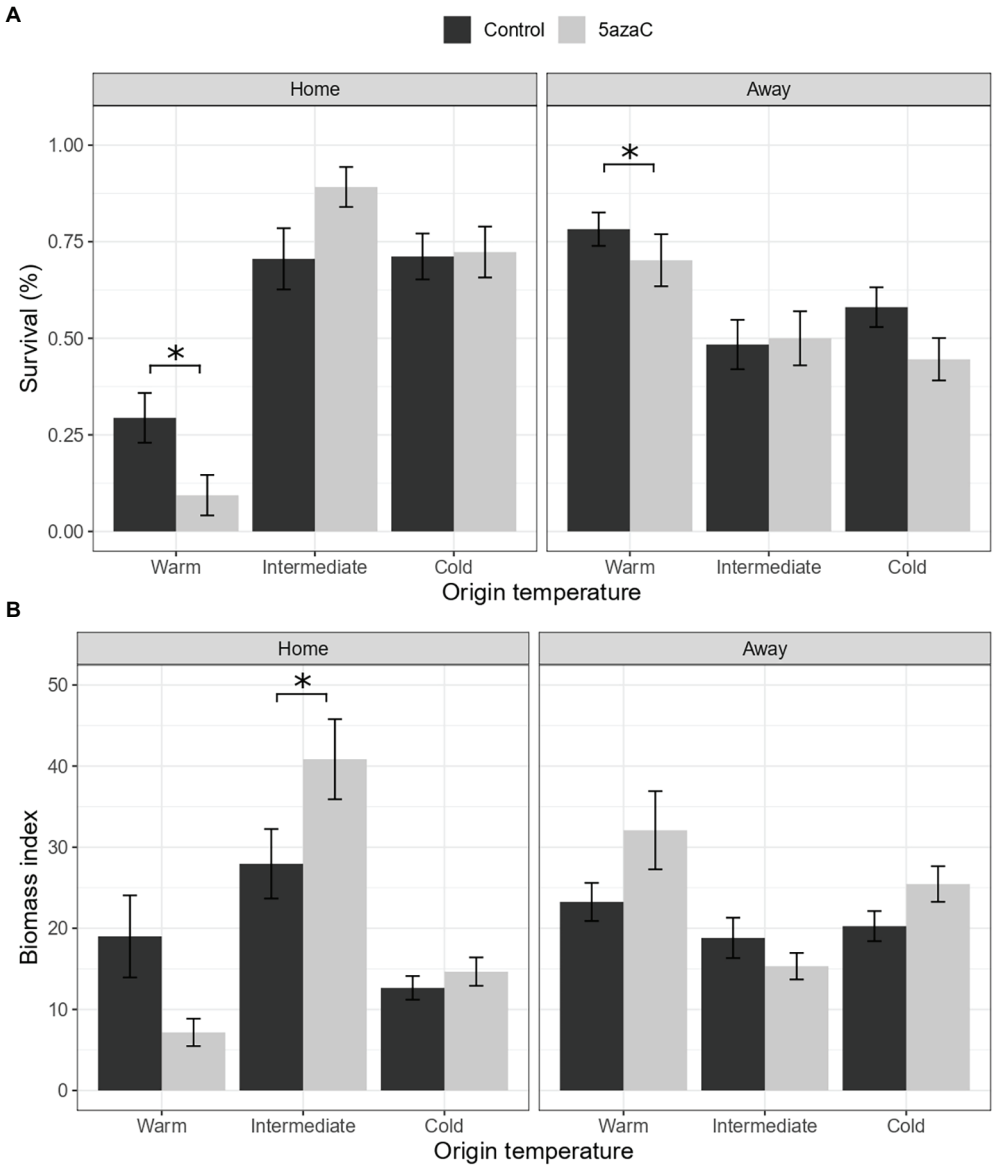

FIGURE 2 | Is local adaptation mediated by DNA methylation (Hypothesis 2)? Effects of origin temperature, home/away site and 5-azaC on plant survival **(A)** and biomass **(B)**. Home: plants in their home site, away: plants in away sites. Control: plants with natural DNA methylation, 5azaC: plants treated with 5-azaC. Values represent the means ± 1 standard error (SE). Significance level $p < 0.05$ (*).

be due to the exceptionally high summer temperatures that our populations experienced in the transplantation year, which might have crossed physiological limits enabling them to survive. Interestingly, the cold Italian population showed local adaptation if survival is considered but maladaptation in the case of biomass. We have no clear explanation for such contrasting response. It is possible that lower biomass could be ascribed to higher survival rate of smaller plants. However, more research would be needed to uncover the cause of this discrepancy.

It is also important to acknowledge that *F. vesca* usually forms large systems of interconnected ramets that can exchange resources as well as information, which was not possible to address in our study. In theory, plants in warm and/or dry localities might benefit from among-ramets resource sharing more than plants from cold and/or wet localities due to potential water reallocation. Individual ramets can be specialized for water acquisition due to division of labor, which can enhance performance of the whole clone (Alpert and Stuefer, 1997). Therefore, the observed ramet maladaptation in warm localities could be mitigated or even absent in situations when clones consist of more interconnected ramets. This possibility remains to be elucidated in future studies.

The lack of local adaptation found in Czechia and Norway may stem from several reasons. First, we could miss the evidence

TABLE 3 | Test of role of DNA methylation in adaptation to warm and/or cold conditions (Hypothesis 3).

		Survival		Biomass		Herbivory damage	
	d.f.	**F**	***P***	**F**	***P***	**F**	***P***
Country (C)	2	0.94	0.364	1.01	0.411	0.25	0.808
Temperature.Distance (T)	1	0.79	0.206	0.17	0.692	2.25	0.237
5-azaC	1	2.45	0.102	0.16	0.693	**10.22**	**0.001**
C × T	2	**2.55**	**0.035**	0.32	0.740	1.70	0.211
C × 5-azaC	2	**2.96**	**0.048**	0.91	0.402	0.03	0.998
T × 5-azaC	1	0.01	0.979	0.04	0.837	0.84	0.357
C × T × 5-azaC	2	**4.43**	**0.008**	0.38	0.681	0.61	0.542

*Effects of country of origin (Country, C), temperature distance (Temperature.Distance, T), 5-azaC treatment (5-azaC), and their interactions on plant survival, biomass, and herbivory damage. N = 730 (survival), N = 403 (biomass, herbivory damage). D.f.: degrees of freedom. Significant values ($p \leq 0.05$) are shown in bold. Estimates of the effects can be found in (***Supporting Information***, ***Supplementary Table S4***).*

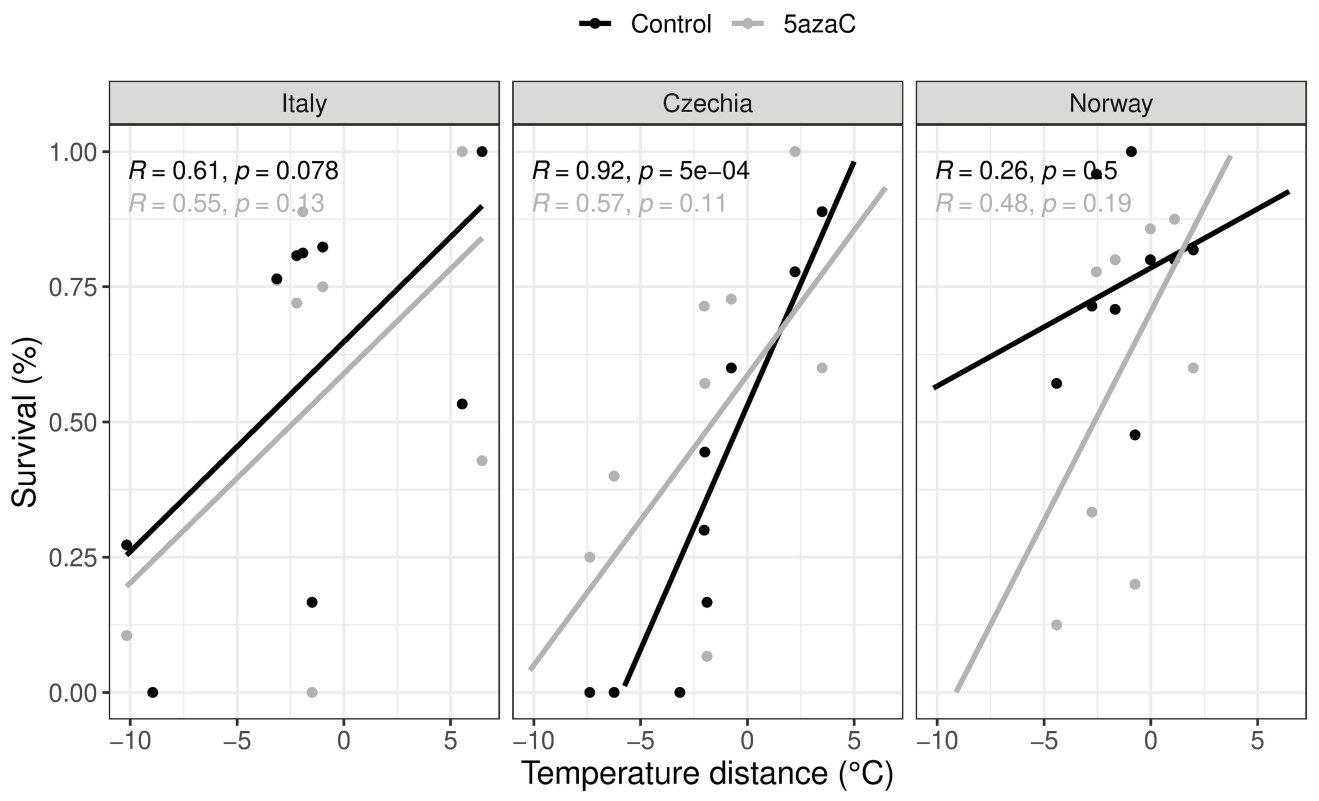

FIGURE 3 | Is adaptation to warm and/or cold conditions mediated by DNA methylation (Hypothesis 3)? Effects of country, temperature distance and 5-azaC on plant survival. Positive values represent shift to colder sites, negative values to warmer sites. Control: plants with natural DNA methylation, 5azaC: plants treated with 5-azaC. R: Pearson correlation, *p*: *p*-value.

of local adaptation at other developmental stages, for example at the level of reproduction of transplanted plants as only very few plants flowered in the experiment. Local adaptation could be also better expressed over longer period, typically during overwintering, which was not a part of our study. According to Germino et al. (2019), proper testing of local adaptation requires a longer time to become evident, sometimes even decades. The absence of local adaptation could be attributed to the relatively low standing genetic variation of our populations, visible from low allelic richness ranging from 1.52 to 1.74 and observed heterozygosity lower than the expected under Hardy–Weinberg equilibrium, and same genotypic diversity among populations (Shannon–Wiener Index of MLG diversity; Sammarco et al., unpublished). Low genetic variability, inbreeding depression and genetic drift can in fact hinder local adaptation of small populations (<1,000 flowering individuals; Leimu and Fischer, 2008). Finally, the lacking evidence for local adaptation was found also in many other studies (e.g., Ebeling et al., 2011; Tíscar et al., 2018; Anderson and Wadgymar, 2020), as was evidence for maladaptation (reviewed in Brady et al., 2019),

suggesting that local adaptation might be surprisingly rare to find in the wild.

In general, survival decreased in plants transplanted to warmer localities and increased when these were transplanted to colder localities. The negative effect of increased temperature on plant survival is perhaps a more general pattern as this phenomenon has been found for other plant species as well (e.g., Birami et al., 2018; Hammer et al., 2018). However, it is worth stressing that such a pattern does not seem to be universal. For example, in a meta-analysis of several mountain species including forbs, graminoids, and trees, the authors found no difference in survival for individuals transplanted at elevations lower than the site of origin, but lower survival for individuals transplanted at higher elevations (Midolo and Wellstein, 2020). In rare cases, plants can even thrive better in warmer sites than was their origin, and this can be ascribed to competitive release (Lenoir et al., 2010) or other climate-related factors than to the increased temperature *per se* (Dobrowski et al., 2013; Rapacciuolo et al., 2014; Putnam and Reich, 2017). Thus, plant response to climatic shifts may be species- or life-history-specific, and/or depend on the specific environmental conditions.

It is not only temperature but also the change in precipitations that can contribute to the success or failure of plants in climatically different environments (Midolo and Wellstein, 2020). Plant response to climatic shifts might also depend on the species' distribution optima. In fact, colonization success has been shown to increase for species with warmer distribution optima than the target site, and to decrease for species with colder distribution optima (Reich et al., 2015; Liu et al., 2018; Lynn et al., 2021). We are not able to completely disentangle the temperature and precipitations effects on plant survival due to the correlation between the two factors. Nonetheless, in our study, the precipitation change did not explain survival of transplanted ramets better than the temperature suggesting that the primary driving factor for survival was the temperature change, rather than the precipitation change.

Epigenetic Variation in Local Adaptation

In the populations from the warm sites, both survival and biomass significantly decreased in 5-azaC-treated plants when transplanted to their home environment (**Figure 2**). Better survival and biomass of plants that were not treated with 5-azaC in warm localities may be at least partly explained by contribution of DNA methylation to adaptation to warm conditions that was interfered by 5-azaC application. Considering that we did not observe such a pattern in other temperature conditions, we speculate that DNA methylation played different roles in adaptation to warmer and colder climatic conditions. In agreement with our findings, heat and cold stresses can affect plant epigenome differently. In many plant species, heat stress induces a global reduction in methylation level of DNA (i.e., hypomethylation; Ci et al., 2015; Li et al., 2016; Hossain et al., 2017), while cold stress causes a global increase in methylation level of DNA (i.e., hypermethylation; Pan et al., 2011; Ci et al., 2015). However, in species such as upland cotton (*Gossypium hirsutum*) or rubber trees (*Hevea brasiliensis*), cold treatment induces demethylation of genes involved in cold tolerance (Fan et al., 2013; Tang et al., 2018). Despite that the epigenetic response to heat and cold stresses seems to be species-specific, these results together with our study suggest that warm and cold climatic conditions shape plant epigenomes differently, meaning that the role of DNA methylation in response to different temperatures can vary.

To our knowledge, there is currently only one other study, Herden et al. (2019), investigating the effect of DNA methylation in local adaptation *via* experimental modification of plant methylome. The authors did not find evidence of local adaptation in several plant species but they also found no evidence of the role of DNA methylation in local adaptation. However, in the study of Herden et al. (2019), plants with experimentally altered methylomes were always smaller than control plants, suggesting negative side effects of methylation alteration during germination of the plants in the demethylating solution. Indeed, the significant reduction in plant growth has been already observed in other studies employing a similar approach for altering plant methylome (e.g., Ruiz-García et al., 2005; Akimoto et al., 2007; Kondo et al., 2007; Bossdorf et al., 2010). Thus, the lack of evidence of the role of DNA methylation in local adaptation found in Herden et al. (2019) might be due to the negative side effects associated with the demethylation approach, rather than by an actual lack of importance of DNA methylation in plant local adaptation. Instead, in our study, we can exclude the negative association between demethylation treatment and local adaptation, since we did not observe any negative side effects of application of 5-azaC on plant biomass. On the contrary, we have shown that the 5-azaC plants were even on average bigger than the plants with natural DNA methylation (**Supplementary Note**). This is in line with other studies using foliar application of 5-azaC for altering DNA methylation level of plants (e.g., González et al., 2016; Puy et al., 2018; Rendina González et al., 2018; Münzbergová et al., 2019). Our findings thus support the foliar application of 5-azaC over using it during seed germination, which provides a big advantage for plant ecological epigenetics studies (Puy et al., 2018). As the foliar application approach does not require growing plants from seeds, it allows alteration of DNA methylation level even on fully developed plants, thus enabling to work for example with a genetically uniform background in case of clonal plants.

Epigenetics in Response to Climate Change

Climatic changes affect epigenetic variation in many organisms, which might help them adapt to rapid climatic changes (e.g., Gugger et al., 2016; Chano et al., 2021; reviewed in Thiebaut et al., 2019). In our study, however, experimental alteration of DNA methylation had inconsistent effects on the plant response to climate change in the three countries. While 5-azaC had virtually no effect on plant survival in response to temperature change in Italy, 5-azaC had contrasting effect on climate change in Czechia and Norway. Compared to plants with natural DNA methylation, application of 5-azaC reduced plant survival if plants were moved to warmer

conditions in Norway but increased survival of plants moved to warmer conditions in Czechia. This might imply that removal of epigenetic memory on the original environment changed plant's ability to survive their shift to climatically different localities. The contrasting effect of 5-azaC among Czech and Norwegian populations and the lack of effect on Italian populations on survival in changing climate suggests that the effect of DNA methylation is dependent on the local environmental conditions and/or on specific characteristics of the populations. For example, clones consisting of fewer ramets might have less division of labor, with each ramet expressing more generalist methylomes able to better respond to transplantations. This could be accompanied by variable effects of 5-azaC on plant's methylome. We know that the methylome of treated plants was highly variable across localities (Sammarco et al., unpublished), suggesting that 5-azaC might have different effects in different localities. Nevertheless, the speculation needs to be tested in further studies. Alternatively, the different effect of 5-azaC might be due to genetic differences of plant populations between localities and, even more, between regions (5-azaC effect can be genotype-specific, Münzbergová et al., 2019). Furthermore, demethylation is also random and can be accompanied by activation of epigenetically silenced genes (Feng et al., 2010) or transposons (Griffin et al., 2016; Boonjing et al., 2020), which can together and in an unpredictable way affect behavior and thus also survival of 5-azaC-treated plants, which might be at least partly responsible for the different effect of 5-azaC in different regions of Europe.

Future Outlooks

In order to provide unambiguous and strong data for generalization, we need studies similar to this one encompassing more species and populations over longer time periods. The studies should be also accompanied by sophisticated molecular methods such as whole-genome bisulfite sequencing. These can provide insights into the epigenetic variation of the plants. Experimental demethylation is however still crucial in such studies as molecular methods provide only indirect evidence of the role of epigenetic variation in local adaptation. Thus, even if the alteration of DNA methylation by 5-azaC occurs randomly and varies among individuals, it is still an important practical approach to provide direct evidence of the role of epigenetic variation in local adaptation when coupled with reciprocal transplant experiments. In fact, random demethylation of 5-azaC reduces the likelihood of identifying significant effects of 5-azaC treatment. Despite this potential for demethylation noise, our study observed significant effects of 5-azaC, suggesting a strong regulatory role played by DNA methylation. Finally, it is also likely that the role of epigenetic variation plays different roles at the level of individual ramet and whole clone. It is known that epigenetic variation can be greatly variable even within individual non-clonal plants (e.g., Herrera and Bazaga, 2013; Herrera et al., 2021). Considering clonal plants, it was proposed that differences in DNA methylation among communicating ramets could enhance whole genet functioning (Latzel et al., 2016), suggesting that the observed responses of single ramets may not be scalable to whole plant generalizations. Therefore, future studies should try incorporate larger parts of individual clones.

CONCLUSION

Our study is among the first testing the role of DNA methylation in local adaptation by employing experimental demethylation in natural conditions across a broad scale of local climatic conditions. It provides evidence that epigenetic variation may contribute to adaptation to local conditions in natural ecosystems. Results of our study also suggest that the increasing temperature will be highly probably the limiting factor determining *F. vesca* survival, which can alter the distribution of the species in case of further temperature increases. By experimental alteration of DNA methylation, we also provided one of the first evidence that epigenetic variation can alter plant response to changing climatic conditions. Since adaptation mediated by epigenetic variation may occur faster than *via* random genetic processes, epigenetic adaptation might provide clonal plants with the necessary time to tackle ongoing environmental crisis and genetically adapt to it afterwards.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

The study was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 764965, the Czech Science Foundation (GACR 20-00871S) and partly by institutional research project RVO 67985939.

ACKNOWLEDGMENTS

We thank members of the EpiDiverse consortium (www.epidiverse.eu) for valuable inputs during preparation and execution of the study, and reviewers for useful comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.827166/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 March 2022
doi: 10.3389/fpls.2022.831175

DNA Methylation Correlates With Responses of Experimental *Hydrocotyle vulgaris* Populations to Different Flood Regimes

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Epigenetic mechanisms such as DNA methylation are considered as an important pathway responsible for phenotypic responses and rapid acclimation of plants to different environments. To search for empirical evidence that DNA methylation is implicated in stress-responses of non-model species, we exposed genetically uniform, experimental populations of the wetland clonal plant *Hydrocotyle vulgaris* to two manipulated flood regimes, i.e., semi-submergence vs. submergence, measured phenotypic traits, and quantified different types of DNA methylation using MSAP (methylation-sensitive amplified polymorphism). We found different epi-phenotypes and significant epigenetic differentiation between semi-submerged and submerged populations. Compared to subepiloci (denoting DNA methylation conditions) for the CG-methylated state, unmethylation and CHG-hemimethylation subepiloci types contribute more prominently to the epigenetic structure of experimental populations. Moreover, we detected some epimarker outliers potentially facilitate population divergence between two flood regimes. Some phenotypic variation was associated with flood-induced DNA methylation variation through different types of subepiloci. Our study provides the indication that DNA methylation might be involved in plant responses to environmental variation without altering DNA sequences.

Keywords: artificial populations, epigenetic variation, clonal plant, phenotypes, *Hydrocotyle vulgaris*, flooding

OPEN ACCESS

Edited by:
Mangal Singh Rathore, Council of Scientific and Industrial Research (CSIR), India

Reviewed by:
Vinod Singh Gour, Amity University Jaipur, India
Michael James Wilkinson, Aberystwyth University, United Kingdom

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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: *08 December 2021*
Accepted: *11 February 2022*
Published: *07 March 2022*

Citation:
Wang M-Z, Li H-L, Tang M and Yu F-H (2022) DNA Methylation Correlates With Responses of Experimental Hydrocotyle vulgaris Populations to Different Flood Regimes. Front. Plant Sci. 13:831175. doi: 10.3389/fpls.2022.831175

INTRODUCTION

Plants exposed to environmental changes often exhibit plastic phenotypes (Putnam et al., 2016; Colicchio et al., 2018). The classical view advocates that an individual phenotype is determined by its environment, genotype, and their interaction, including both plasticity and evolutionary adaptation (Richards et al., 2010; Bian et al., 2013). However, epigenetic regulation (e.g., DNA methylation, histone modifications, chromatin remodeling, and expression of non-coding RNAs) without changing DNA sequence has been widely considered as another candidate mechanism accounting for plant phenotypic variation (Bossdorf et al., 2008; Marfil et al., 2009). Nowadays, the best-studied hallmark of epigenetic modification is DNA methylation, which is mainly the addition of a methyl group to the C5 position of a cytosine residue in three different sequence contexts (CG, CHG, and CHH sites, H = A, C, T), *via* catalysis of several DNA methyltransferase enzymes (Bossdorf et al., 2008; Zoldoš et al., 2018).

Alteration of epigenetic markers mainly originate from genetic variation, environmental induction, or spontaneous epimutations (Dubin et al., 2015; Trucchi et al., 2016; Richards et al., 2017). According to dependence degree on genetic context, epigenetic variation could be classified into three categories: obligate (fully dependent), facilitated (semi-independent), or pure (completely independent) (Richards, 2006; Robertson and Richards, 2015). Unlike genetic variation, DNA methylation patterns are sensitive to changing environments and commonly possess a much higher variation rate (Schulz et al., 2014; Jueterbock et al., 2020). Such variation can be reversibly transient within one generation or stably heritable to several generations (Angers et al., 2010; Paun et al., 2010; Colicchio et al., 2018). DNA methylation alters gene expression through transcriptional repression or remodeling chromatin, further affecting plant phenotypes (Boyko and Kovalchuk, 2010; Grativol et al., 2012; Griffin et al., 2016; Colicchio and Herman, 2020). Therefore, environment-induced epigenetic regulation could not only offer a rapid pathway for phenotypic plasticity, but also underlie plant adaptive evolution when across-generational plasticity confers fitness benefits in predictable environments (Putnam et al., 2016; Huang et al., 2017; Groot et al., 2018; Colicchio and Herman, 2020).

Depending on sequence context, different DNA methylation types (i.e., CG/CHG/CHH) vary in their responses to environmental factors, associations with genetic variation, or functions in gene expression, etc. (Schulz et al., 2014; Colicchio et al., 2018). For instance, in *Arabidopsis thaliana* accessions, CHH methylation of transposable elements (TEs) was sensitive to growth temperature and under *cis*- and *trans*-acting genetic control, whereas CG methylation on the gene coding regions was independent of genetic effects and instead strongly correlated with the latitude of origin (Dubin et al., 2015). In general, CG methylation in gene bodies (GbM) usually activates gene expression, while that in promoters and TEs is associated with gene silencing (Dubin et al., 2015; Colicchio et al., 2018; Jueterbock et al., 2020). Non-CG methylation (i.e., CHH or CHG methylation) mostly occurring in transposons or repeat regions seems to regulate transcriptional repression through chromatin remodeling (Grativol et al., 2012; Schulz et al., 2013; Dubin et al., 2015; Colicchio et al., 2018).

To explore ecological and evolutionary significance of DNA methylation, the first step is to find evidence that at least part of the epigenome changes correlate with plant stress-responses. In recent years, there are accumulating ecological studies exploring different DNA methylation types responding to environmental stresses and/or their relations to plant phenotypic characteristics. Most of these studies focused on plants with the genome reference at the individual level (e.g., Colicchio et al., 2018; Jueterbock et al., 2020). However, roles of epigenetic variation in plastic responses of natural populations to specific environment changes is still largely unknown, especially for non-traditional model organisms lacking the genome reference (Paun et al., 2010; Abratowska et al., 2012; Rico et al., 2014; Watson et al., 2018).

Given the sensitivity of DNA methylation to environmental variation, a direct test for the epigenetic contribution in natural systems could be confounded by complex and dynamic natural conditions (Schulz et al., 2014). Moreover, some previous studies used genetically diverse plant materials, which might hardly disclose the pure epigenetic effects with the presence of genetic variation (Lira-Medeiros et al., 2010; Schulz et al., 2014; Robertson et al., 2017). Therefore, manipulated experimental populations without any genetic variation, such as those consisting of genetically identical asexual individuals (ramets) vegetatively propagated by a single genet (clone) of clonal plants, are better materials to strictly assess roles of epigenetic variation in plastic responses (Rapp and Wendel, 2005; Verhoeven et al., 2010; Verhoeven and Preite, 2014; Huang et al., 2017).

The wetland clonal plant *Hydrocotyle vulgaris* L. (Araliaceae) is considered potentially invasive in China due to high phenotypic plasticity, rapid clonal growth, strong adaptability, and exclusion of other native species (Miao et al., 2011; Liu et al., 2014; Dong et al., 2015). Our previous study showed that the natural *H. vulgaris* populations in southern China possessed low genetic variation but high epigenetic variation, and that their phenotypic variation was largely correlated with epigenetic variation rather than genetic variation (Wang et al., 2020). *H. vulgaris* often experiences water depth changes, which may represent a strong selective force for its population diversification (Wang et al., 2020). In this study, we explored roles of different DNA methylation types in phenotypic responses of *H. vulgaris* to flood variation by exposing its genetically uniform experimental populations to two different flood regimes and by evaluating phenotypic and DNA methylation consequences using MSAP (methylation-sensitive amplified polymorphism). Specifically, we addressed the following questions. (1) What are the phenotypic responses of experimental *H. vulgaris* populations to different flood regimes? (2) What DNA methylation patterns can be generated in different flood regimes? (3) Are environmentally induced alterations in different methylation types related to phenotypic variation?

MATERIALS AND METHODS

Material Propagation

From June to August 2016, 128 plants of *H. vulgaris* were collected from 10 natural populations in southern China (Wang et al., 2020). Using AFLP (amplified fragment length polymorphism), we distinguished 20 genotypes from the 128 individuals, among which a single wide spread genotype accounted for 82% of the total samples and dominated in all 10 populations (Wang et al., 2020). Plants of the most dominant genotype were mixed cultivated and vegetatively propagated under the same condition in a greenhouse at Taizhou University. In early July 2017, we selected more than 576 newly generated similar-sized ramets at the same developmental stage. Each ramet consisted of one node, one leaf and some adventitious roots (petiole length: 22.5 $\pm$ 0.2 cm, mean $\pm$ SE, n = 30). To ensure that all ramets are epigenetically uniform at the start of the experiment, thirty ramets of them were randomly selected for detection of DNA methylation patterns by MSAP, and were identified to be assigned to the same epigenotype.

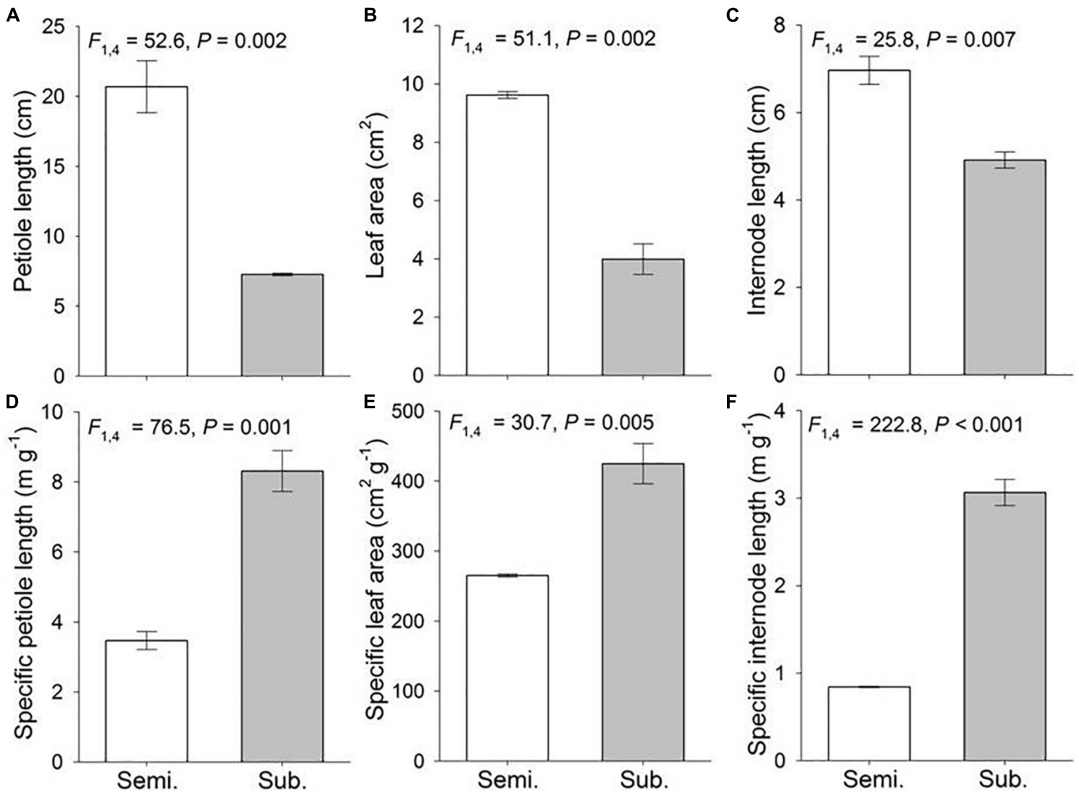

FIGURE 1 | Phenotypic traits of the *Hydrocotyle vulgaris* at population level under semi-submergence (Semi.) and submergence (Sub.). Phenotypic traits are **(A)** petiole length, **(B)** leaf area, **(C)** internode length, **(D)** specific petiole length, **(E)** specific leaf are, and **(F)** specific internode length. Mean ± SE are shown (n = 3). F, P, and degree of freedom of nested ANOVAs are also given.

Experimental Design

The remaining 546 ramets were used for the experiment described below. We constructed experimental populations of *H. vulgaris* in six big plastic tanks (1.38 m in bottom diameter, 1.60 m in top diameter and 0.89 m in height) filled with a 30-cm-deep mixture of sand and local soil at a 1:1 volume ratio. In each tank, the 91 similar-sized, genetically identical ramets were evenly planted in the range of a 50-cm-edged hexagon from the center point of the soil surface, with two adjacent ramets spacing 10 cm apart (**Supplementary Appendix 1A**). The soil in the tanks was always kept moist after planting. After 20 days recovery of the six established experimental populations, two flooding treatments were applied. No ramet died before the flooding treatments.

The two flooding treatments were semi-submergence and submergence, each with three replicate tanks (experimental populations). For the semi-submergence treatment, the tank was filled with tap water to a depth of 10 cm above the soil surface, so that the ramets could protrude from the water surface. By contrast, for the submergence treatment, the water level in the tank was maintained 30 cm above the soil surface, so that the ramets were submerged (under the water surface). The experiment was conducted in an open area at Taizhou University, and all the six tanks were placed closely and randomly to avoid potential confounding effects of micro-environmental differences.

Harvest and Measurements

The experiment lasted from 10 September to 20 December 2017. At harvest, we uniformly set 19 sampling points in each tank, similar to the planting approach, and the difference was that the sampling points were at 25 cm intervals (**Supplementary Appendix 1B**). At each sampling point, we took two connected mature ramets with fully expanded leaves: one ramet was randomly selected for phenotypic measurement and the other for epigenetic analysis.

For epigenetic analysis, the leaf of the ramet was dried in silica gel. Total genomic DNA from 30 mg of the dry leaf was extracted using Dingguo Plant Genomic DNA Kit (Beijing, China), and quantified spectrophotometrically. After verifying integrity and purity by 1% agarose gel electrophoresis, DNA was diluted to 20 ng/μL as the starting material for epigenetic analysis. The MSAP protocol and scoring method were exactly the same as our previous study, with five selective primer combinations, i.e., E-AGT/H-TAT, E-AGT/H-TTC, E-ATC/H-TGA, E-AAC/H-TCG, and E-ATG/H-TGA (Wang et al., 2020). The error rates for *Hpa*II and *Msp*I scores were about 0.65 and 0.46%, respectively (Wang et al., 2020). Only the repeatable markers were involved in the following molecular analyses.

To quantify phenotypic responses, we first measured leaf petiole length, leaf area and stem internode length of the ramet. Then, the petiole, leaf blade and stem internode were dried at

90°C for 48 h and weighed. Specific petiole length was calculated as petiole length per unit petiole dry mass, specific leaf area as leaf area per unit leaf dry mass, and specific internode length as internode length per unit internode dry mass.

Data Analysis

We used nested ANOVA to test the effect of flooding treatments on each of the six phenotypic traits (petiole length, specific petiole length, leaf area, specific leaf area, internode length, and specific internode length) at the population level. Experimental populations were nested within the treatment. Before analyses, specific petiole length, internode length and leaf area were log-transformed to improve homoscedasticity (**Supplementary Appendix 2**).

For MSAP data, the presence or absence of the bands from specific isoschizomer digestions (*Eco*RI/*Hpa*II and *Eco*RI/*Msp*I) results in four conditions of a particular fragment: (I) bands present in both enzyme combinations (1/1), indicating an unmethylated state; (II) bands absent in both enzyme combinations (0/0), indicating an uninformative state; (III) bands present only in *Eco*RI/*Msp*I profiles (0/1), indicating hemi- or fully methylated CG-sites; (IV) bands present only in *Eco*RI/*Hpa*II profiles (1/0), indicating hemimethylated CHG-sites. Due to the fact that different methylation states participate in different regulating processes, considering them separately would give the most comprehensive picture of DNA methylation (Schulz et al., 2013). Therefore, we used the "Mixed-Scoring 2" approach implemented in R script "MSAP_calc.r" (Schulz et al., 2013) to transform the three discernible methylation status represented by combination of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I banding patterns at each epilocus into binary matrices of different types of subepiloci. Thus, for each epilocus, up to three subepiloci can be generated: u-subepilocus (denoting the unmethylated loci where type I is scored as 1 and other types were scored as 0), m-subepilocus (denoting the CG-methylated loci where type III is scored as 1), and h-subepilocus (denoting the CHG-hemimethylated loci where type IV is scored as 1) (Schulz et al., 2014).

Based on the binary matrices of all MSAP subepiloci and each subepiloci type, we conducted the following analyses. Epigenetic diversity of each experimental population in terms of the percentage of polymorphic loci (*PLP*) and Shannon's information index (*H*) were assessed by "MSAP_calc.r" (Schulz et al., 2013). To visualize population epigenetic structure, principal coordinate analyses (PCoA) were performed with GenALEx 6.5 based on the matrix of Nei's distances (Peakall and Smouse, 2012). Epigenetic differentiation at different hierarchical components, that is, between treatments (ϕ_{RT}), among experimental populations within treatments (ϕ_{PR}) and within experimental populations (ϕ_{PT}), was calculated using analysis of molecular variance (AMOVA) with Genalex 6.5. Significance levels were determined after 9,999 permutations.

To identify putatively adaptive epiloci that may facilitate shaping population epigenetic responses to flood variation, we performed outlier detection based on individuals from different experimental populations by using the BayeScan 2.1 (Foll and Gaggiotti, 2008) for estimating the posterior odds (PO) of each epilocus. The analyses were run for 100,000 iterations, with a burn-in of 50,000 iterations, a sample size of 5,000 and a thinning interval of 10. An additional burn-in was carried out by 20 short pilot runs of 5,000 iterations. Only loci exceeding a "strong" detection level [$\log_{10}$ (PO) > 1] were considered as putative outliers.

To establish the relationships between environmental, epigenetic and phenotypic variation, structural equation modeling (SEM) was conducted in AMOS 24.0, by relating flood regime and epigenetic variation on phenotypic variation. For each subepiloci type, we examined the direct effects of methylation variation (first three PCoA axis for corresponding subepiloci) and the environmental factor (two flood regimes; semi-submergence was coded as "0," while submergence treatment was coded as "1") on phenotypic variation (six phenotypic traits), and indirect effects of flood regimes on phenotypic traits through methylation variation.

RESULTS

Phenotypic Responses to Different Flood Regimes

Flooding significantly affected phenotypic traits of *H. vulgaris* (**Figure 1**). Submerged populations exhibited significantly shorter petiole length, internode length and smaller leaf area, but higher specific petiole length, specific internode length and specific leaf area than semi-submerged populations (**Figure 1**).

Epigenetic Responses to Different Flood Regimes

Epigenetic Diversity

The MSAP analysis resulted in 345 scorable epiloci, of which 75 (21.7%) were polymorphic. Mixed scoring 2 detected 144

TABLE 1 | Epigenetic diversity of the six clonally propagated populations of *Hydrocotyle vulgaris* under semi-submergence (Semi.) and submergence (Sub.) as quantified by **(A)** percentage of polymorphic loci and **(B)** Shannon's information index based on all subepiloci and u-, m-, and h-subepiloci of MSAP.

	All subepiloci		u-subepiloci		m-subepiloci		h-subepiloci	
	Semi.	**Sub.**	**Semi.**	**Sub.**	**Semi.**	**Sub.**	**Semi.**	**Sub.**
(A) Percentage of polymorphic loci (*PLP*)								
Replicate 1	9.03	45.14	4.55	62.12	11.90	42.86	13.89	16.67
Replicate2	10.42	51.39	6.06	39.39	14.29	61.90	13.89	61.11
Replicate3	16.67	38.19	12.12	46.97	21.43	35.71	19.44	25.00
Mean	**12.04**	**44.91**	**7.58**	**49.49**	**15.87**	**46.82**	15.74	34.26
(B) Shannon's information index (*H*)								
Replicate 1	0.049	0.282	0.024	0.391	0.077	0.269	0.061	0.098
Replicate 2	0.069	0.296	0.039	0.255	0.106	0.372	0.079	0.281
Replicate 3	0.101	0.237	0.087	0.326	0.122	0.200	0.103	0.116
Mean	**0.073**	**0.271**	**0.050**	**0.324**	**0.102**	**0.280**	0.081	0.165

Each treatment has three replicate populations. Significant differences ($P < 0.05$) of the mean values between treatments are shown in bold (by t-tests).

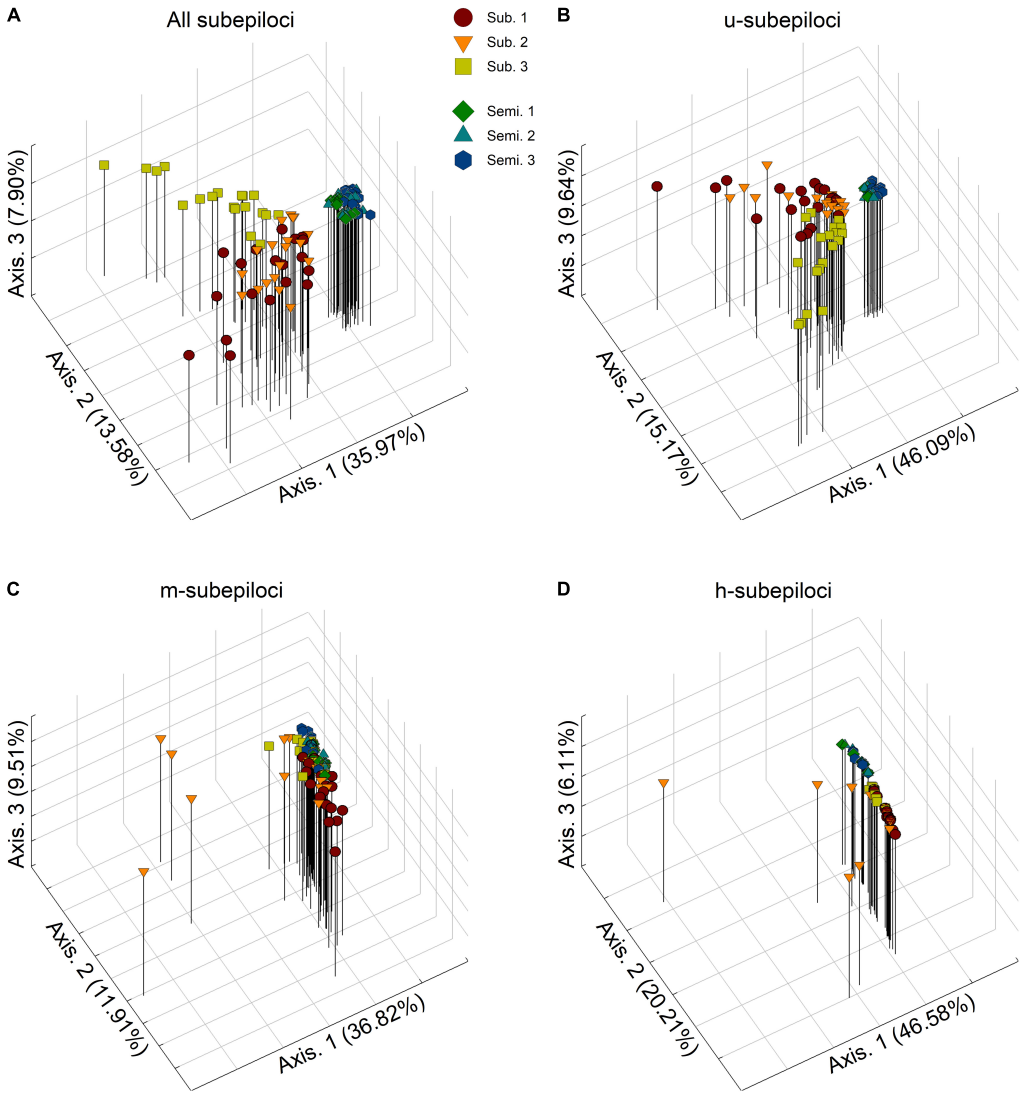

FIGURE 2 | Results of principal coordinate analyses (PCoA) based on **(A)** all subepiloci, and **(B)** u-, **(C)** m-, and **(D)** h-subepiloci of MSAP. Semi. and Sub. stand for the semi-submergence and submergence treatment, respectively.

polymorphic subepiloci, including 66 u-, 42 m-, and 36 h-subepiloci. Epigenetic diversity of submerged populations was significantly higher than that of semi-submerged populations, as quantified by percentage of polymorphic loci and Shannon's information index based on all subepiloci, u-subepiloci and m-subepiloci (**Table 1** and **Supplementary Appendix 3**).

Epigenetic Structure

Principal coordinates analysis revealed that the epigenetic structure of experimental populations differed among different types of subepiloci (**Figure 2**). Based on all MSAP subepiloci, epigenetic distances separated semi-submergence and submergence treatments along the first axis, forming two separated clusters (**Figure 2A**). Moreover, the experimental populations 1 and 3 in the submergence treatment fell apart, whereas the experimental populations in the semi-submergence treatment were much closer, with higher convergence degree. A similar differentiation pattern was also found in u-subepiloci, with individuals more clumped in each treatment (**Figure 2B**). For m-subepiloci, most individuals from different flood regimes grouped together without clear population differentiation, and only some individuals of the submerged experimental population 2 scattered from the cluster (**Figure 2C**). For h-subepiloci, the two treatments were mainly separated along the second coordinate, forming two clusters, with several individuals separated from the group of the submerged populations (**Figure 2D**).

For the combined epigenetic dataset (**Table 2**), AMOVA showed that 36% of epigenetic variance occurred between treatments, 50% within experimental populations, and only 14% among experimental populations within treatments. Similarly, for u-subepiloci and h-subepiloci, most variance occurred between treatments and within experimental populations (for

TABLE 2 | Results of hierarchical AMOVA based on **(A)** all subepiloci and **(B)** u-, **(C)** m-, and **(D)** h-subepiloci of MSAP.

	Variance	%	ϕ	*P*
(A) All subepiloci				
Between treatments	4.131	36	0.363	<0.001
Among populations within treatments	1.568	14	0.216	<0.001
Within populations	5.689	50	0.500	<0.001
(B) u-subepiloci				
Between treatments	3.254	46	0.458	<0.001
Among populations within treatments	0.964	14	0.250	<0.001
Within populations	2.895	41	0.593	<0.001
(C) m-subepiloci				
Between treatments	0.213	8	0.083	<0.001
Among populations within treatments	0.489	19	0.209	<0.001
Within populations	1.853	73	0.275	<0.001
(D) h-subepiloci				
Between treatments	0.664	39	0.386	<0.001
Among populations within treatments	0.114	7	0.108	<0.001
Within populations	0.942	55	0.453	<0.001

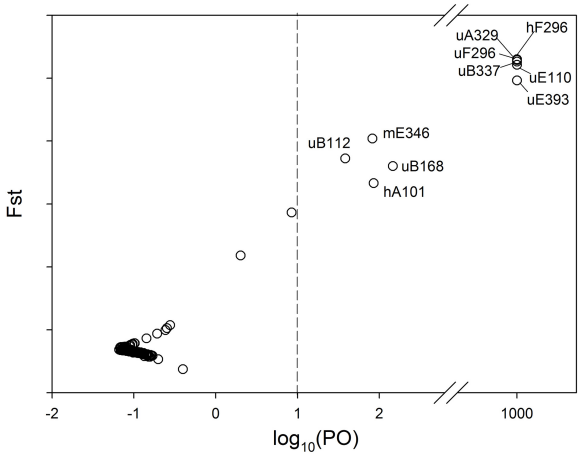

FIGURE 3 | MSAP outliers identified by Bayescan. The posterior odds (PO) for a locus to be under divergent selection are shown on a log scale on the *x*-axis. The vertical dash line indicates the threshold for strong evidence for selection. Outliers on the right side of the vertical line are marked with their names.

u-subepiloci, 46 and 41%, respectively; for h-subepiloci, 39 and 55%, respectively). However, for m-subepiloci, variation mainly existed within experimental populations (73%).

Outlier Detection

For the complete set of the 144 MSAP subepiloci, BayeScan identified 10 (6.9%) outliers (**Figure 3**), among which seven were u-subepiloci, one was m-subepiloci and two were h-subepiloci, accounting for 10.61, 2.38, and 5.56% of the corresponding type of outliers, respectively. Based on PCoA analysis, outliers clearly separated the semi-submergence and submergence populations along the first axis, while there was no clear differentiation between the two treatments for neutral subepiloci (**Supplementary Appendix 4**).

Relationships Among Environmental, Epigenetic and Phenotypic Variation

The SEM linked the two flood treatments, variation of the different types of subepiloci and the six measured phenotypic traits. The treatments directly affected all traits; however, petiole length and internode length were only significantly correlated with different flood regimes, with no relationship with subepiloci variation (**Figure 4**). Leaf area and specific leaf area were related to flood-independent u-subepiloci variation (**Figure 4**), which may arise from spontaneous epimutation. Flood-induced epigenetic variation affected leaf area by m-subepiloci, specific petiole length by all subepiloci types, specific internode length and specific leaf area by h-subepiloci (**Figure 4**).

DISCUSSION

Phenotypic Responses to Different Flood Regimes

Submergence inhibited growth of *H. vulgaris*, possibly due to that decreased irradiance, sediment anoxia, and osmotic stress in this severe environment restrained plant carbohydrate storage, oxygen transport, and nutrient acquisition (Vretare et al., 2001; Santamaría, 2002). However, in response to semi-submergence, *H. vulgaris* may develop flood-tolerant responses and soil-oxygen deficiency resistance, such as elongating stout petiole to extend above the water surface and enlarging thick leaf to capture light and increase gas exchange (Vretare et al., 2001; Luo and Xie, 2009). Moreover, the oxygen transported to the node may drive the length extension of internode for further dispersal (Vretare et al., 2001). These changes could confer a fitness benefit for *H. vulgaris* under semi-submergence, with significant higher aboveground biomass and population density [for ramet aboveground biomass (mean ± SE), submergence = 0.038 ± 0.005 g, semi-submergence = 0.188 ± 0.009 g, $F_{1,4} = 160.791$, $P < 0.001$).

Epigenetic Responses to Different Flood Regimes

Principal coordinate analyses showed a clear epigenetic differentiation between the semi-submergence and the submergence experimental populations of *H. vulgaris*, indicating that environmental conditions could shape DNA methylation patterns of plant populations (Note that if DNA methylation changes largely arise from random epimutation, the presence/absence of private bands would be observed in many loci and such epiloci could be neutral so that the treatment-induced epigenetic differentiation would not occur) (Boyko and Kovalchuk, 2010; Schulz et al., 2014; Zhang et al., 2016). Consistent to previous studies (e.g., Gao et al., 2010; Lira-Medeiros et al., 2010; Li et al., 2013; Zoldoš et al., 2018), our results also suggest that experimental *H. vulgaris* populations can not only respond differently in phenotypic traits, but also undergo a genome-wide epigenetic reprogramming under divergent pressures from contrasting treatments. Therefore, such environment-directed DNA methylation mechanism may be

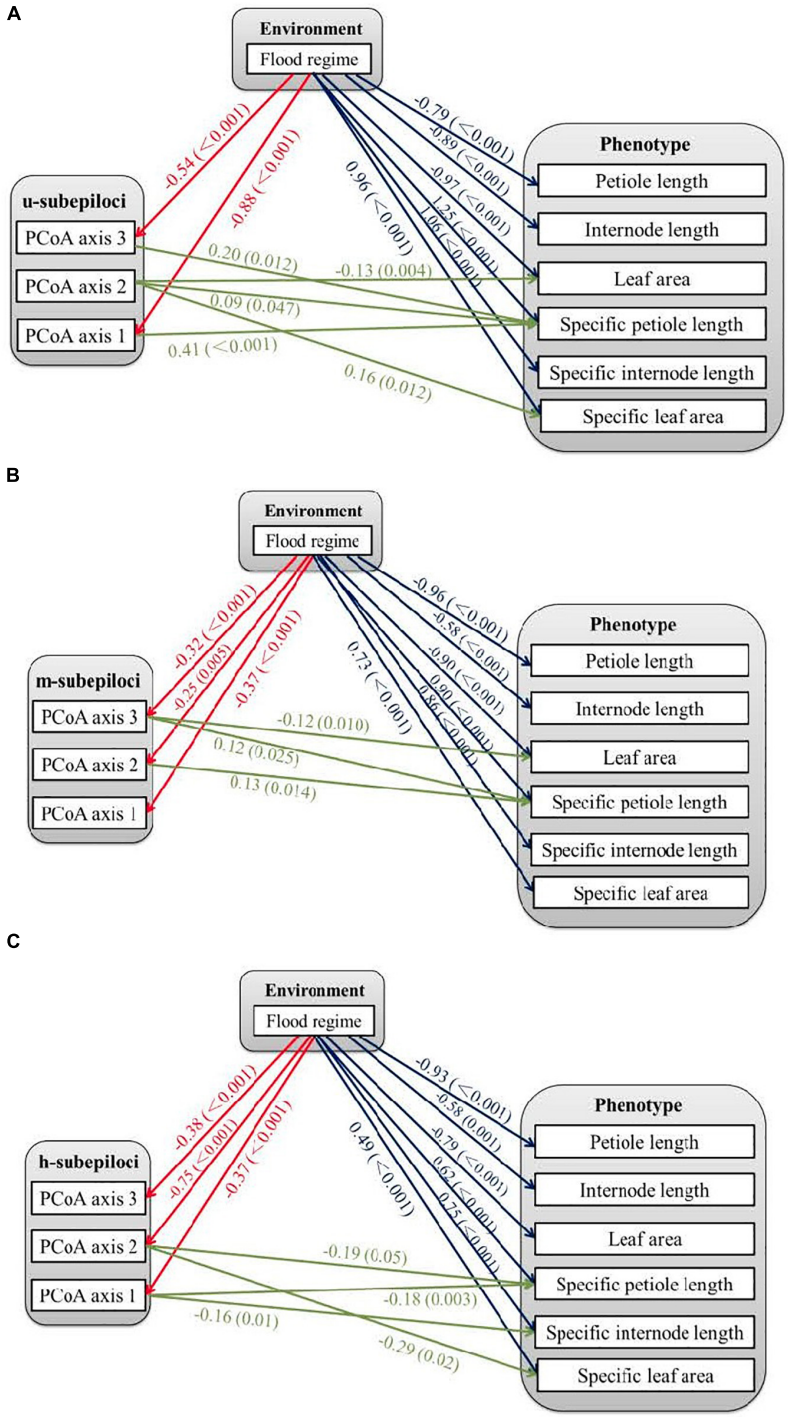

FIGURE 4 | Relationships among environmental, epigenetic and phenotypic variation of *Hydrocotyle vulgaris*. The epigenetic variables are the first three axes from principal coordinate analyses (PCoA) of different types of subepiloci, i.e., **(A)** u-subepiloci, **(B)** m-subepiloci, and **(C)** h-subepiloci. The lines with arrows indicate significant correlations between variables, with coefficients and *P*-values showing near the lines.

involved in plant adaptation to stress (Boyko and Kovalchuk, 2010; González et al., 2016).

Moreover, the submerged experimental populations of *H. vulgaris* exhibited greater epigenetic diversity and differentiation than the semi-submerged populations. Some of previous studies reported that severe stress could trigger epigenome variability, providing a possible mechanism for fine-tuning short-term adaptive benefits (Boyko et al., 2010;

Verhoeven and Preite, 2014; Colicchio et al., 2018). However, long periods of constant stress can fix the allelic variant that confers tolerance to stress *via* strong directional selection, leading to the constrained epigenetic diversity and differentiation (Lira-Medeiros et al., 2010; Grativol et al., 2012; Rico et al., 2014).

Population epigenetic differentiation based on u-subepiloci was highly similar to that based on all MSAP subepiloci. Also, h-subepiloci revealed the semi-submergence and the submergence population cluster. This could indicate a functional difference of subepiloci types, with the additive contribution of u-subepiloci and h-subepiloci to population divergence between the two flood regimes. Moreover, AMOVA results showed that variation mainly existed between treatments and within experimental populations based on both u- and h-subepiloci, similar to that based on all subepiloci, whereas most variation existed only within experimental populations based on m-subepiloci. Therefore, the hemimethylation or demethylation in the CHG-context may play a more important role in habitat adjustment in plants than changes of CG-context. Several previous studies have revealed that u-subepiloci and m-subepiloci are more significant in shaping epigenetic structure of natural populations from different habitats (e.g., Schulz et al., 2014; Zoldoš et al., 2018). Such inconsistency suggests that the function of CG- and CHG-methylated states in response to environmental factors is species- and/or environment-specific (Rico et al., 2014; Putnam et al., 2016). We identified ten outlier epiloci facilitated separation of *H. vulgaris* experimental populations between semi-submergence and submergence (**Supplementary Appendixes 4**, **5**), which may contribute to plastic responses of populations to the flood variation.

Relationships Among Environmental, Epigenetic and Phenotypic Variation

Structural equation modeling analyses showed that petiole length and internode length of *H. vulgaris* were only significantly correlated with flood, but not with epiloci variation. These results may arise from effects of nutritional or physiological activities, or the low-resolution of MSAP technique (Zhang et al., 2016). Leaf area and specific leaf area are partially affected by u-subepiloci without environmental induction, possibly due to the spontaneous epigenetic variation, arising from imperfect action of enzymes that ensure proper maintenance of epigenetic information through cell division (Verhoeven and Preite, 2014). Stochastic DNA methylation variation is a source for phenotypic diversity in plants, which may mediate phenotypes for several generations that could affect subsequent selection and contribute to adaptive processes (Verhoeven and Preite, 2014; van der Graaf et al., 2015; Groot et al., 2018).

Some phenotypic variation was associated with environment-induced DNA methylation variation through different types of subepiloci, possibly due to their functional differences in regulating gene expression. However, it provides no direct causal information about the region or gene influenced by DNA methylation, as MSAP epiloci are anonymous markers. Our results support the emerging three-way link among flood regimes, DNA methylation and phenotypic changes, suggesting that epigenetic variation might be involved in plastic responses to environmental variation (Wu et al., 2013; Putnam et al., 2016).

CONCLUSIONS

We conclude that plants can exhibit significant phenotypic differences between flood regimes, with clear DNA methylation differentiation associated with phenotypes. Moreover, by using the mixed scoring approach, we find the different contributions of methylation types to epigenetic processes in habitat-related responses. Our study potentially adds to the knowledge base of DNA methylation-environmental interactions. However, we did not demonstrate heritability of the epigenetic changes in later-generation and their long-term adaptive and evolutionary implications. Moreover, information on the mechanistic link between methylation and phenotype is still limited. Therefore, more profound studies are needed to deeply uncover the epigenetic role in plant ecological and evolutionary processes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

M-ZW and F-HY designed the research. M-ZW and H-LL performed the research. M-ZW contributed new reagents or analytical tools, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (32071527, 31870610, and 31761123001), Ten Thousand Talent Program of Zhejiang Province (2018R52016), and the Joint Fund of Zhejiang Provincial Natural Science Foundation (LTZ20C030001).

ACKNOWLEDGMENTS

We thank Jing-Xia Shen and Qian Zhang for assistance with the experiment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.831175/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 March 2022
doi: 10.3389/fpls.2022.831654

Arbuscular Mycorrhizal Fungi Contribute to Phosphorous Uptake and Allocation Strategies of *Solidago canadensis* in a Phosphorous-Deficient Environment

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OPEN ACCESS

Edited by:
Bi-Cheng Dong, Beijing Forestry University, China

Reviewed by:
Yong-Jian Wang, Huazhong Agricultural University, China
Fang-Li Luo, Beijing Forestry University, China

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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: *08 December 2021*
Accepted: *21 February 2022*
Published: *24 March 2022*

Citation:
Qi S, Wang J, Wan L, Dai Z, da Silva Matos DM, Du D, Egan S, Bonser SP, Thomas T and Moles AT (2022) Arbuscular Mycorrhizal Fungi Contribute to Phosphorous Uptake and Allocation Strategies of Solidago canadensis in a Phosphorous-Deficient Environment. Front. Plant Sci. 13:831654. doi: 10.3389/fpls.2022.831654

Arbuscular mycorrhizal fungi (AMF) can facilitate the uptake of limiting or inaccessible nutrients by plants. However, the importance of AMF for invasive plants under phosphorus (P) limitation is poorly well understood because of the presence of non-focal microorganisms, such as endophytes or rhizosphere bacteria. In this study, we investigated how an invasive clonal plant *Solidago canadensis* benefits from the AMF *Glomus intraradices* by using a completely sterile culturing system, which is composed of aseptic seedlings, a pure AMF strain, and a sterile growth environment. We found that the colonization rate, abundance, and spore production of AMF in the insoluble P treatment was more than twice as much as in the available P treatment. Plant above-ground growth was enhanced almost 50% by AMF in the insoluble P treatment. Importantly, AMF were able to facilitate P acquisition by the plant in insoluble P conditions, allowing plants to have lower investment into below-ground biomass and higher benefit/return for above-ground biomass. This study demonstrated the important contribution that AMF make to plants in phosphate-deficient environments eliminating interference from non-focal microorganisms. Our results also suggest that interaction with AMF could contribute to the invasiveness of clonal plant *S. canadensis* in a resource-deficient environment.

Keywords: arbuscular mycorrhizal fungi, invasive clonal plant, nutrient limitation, phosphorus uptake, Canada goldenrod, sterile culture system

INTRODUCTION

Phosphorus (P) is crucial for normal plant growth and development (Luo et al., 2019) and is often present in the soil in relatively large amounts but with low bioavailability due to the complexation with iron, calcium, and aluminum (Smith et al., 2011). Terrestrial plants have evolved two specialized strategies to increase the uptake of inorganic P from soils

(Schachtman et al., 1998; Luo et al., 2019). The first is to directly take up soluble P *via* root epidermal cells and root hairs. This strategy often involves the alteration of root architecture to increase root-to-shoot ratios (Péret et al., 2014) as well as the production of organic acids, phosphatases, and P transporters to solubilize bio-unavailable P (Raghothama and Karthikeyan, 2005). The second strategy employs mutualistic symbionts, such as mycorrhizae and phosphorus-solubilizing bacteria, to increase the absorptive surface area of the root system (Smith et al., 2004; Amaya-Carpio et al., 2009; Priyadharsini and Muthukumar, 2017). Direct uptake of P through the roots requires a larger investment of plant resources than symbiont-driven P acquisition (Smith et al., 2003).

Approximately 75% of land plant species are colonized by and have mutualistic relationships with arbuscular mycorrhizal fungi (AMF) of the phylum Glomeromycota (Gutjahr et al., 2015). AMF contribute to the growth and health of their host plants by increasing nutrient acquisition, drought and salt tolerance, and also by increasing the biotic resistance to pathogens and herbivores (Kula et al., 2005; Yooyongwech et al., 2016; Lin et al., 2017). In return, AMF draw organic nutrients and photosynthates from plants (Smith and Smith, 2011). AMF can form a network with plant roots to increase inorganic phosphorus acquisition by producing organic acids and phosphatases (Lee et al., 2014; Majewska et al., 2017). Recent studies also revealed that AMF possess various key genes involved in the phosphate response signal transduction pathway (Salvioli et al., 2016; Venice et al., 2020). However, molecular mechanisms of phosphate transport and metabolism are still need further study (Xie et al., 2022).

Invasive plants cause both huge economic losses and severe ecological problems (Vila et al., 2011). Successful invasive plants often possess rapid growth abilities and have strong survival under adversity (Dai et al., 2016a; Chen et al., 2019). Invasive plants are also often influenced by mutualistic interactions with AMF (Majewska et al., 2015; van Kleunen et al., 2018; Chen et al., 2019), which increase their competitive abilities and facilitate invasion of new habitats (Callaway et al., 2001; Yuan et al., 2014; Zhang et al., 2017). For example, Dong et al. (2021) found that invasive plants grew larger and with lower competitive suppression with AMF colonization. However, Majewska et al. (2017) observed that the effects of AMF on the growth of two invasive plants, *Rudbeckia laciniata* and *Solidago gigantea*, were different depending on various AMF species and soil types.

Our focus in this study is on the invasive clonal plant *Solidago canadensis* L. (Asteraceae), a North American plant that has successfully invaded Europe, Asia, and Oceania (Jin et al., 2004). *S. canadensis* often forms monocultures in its invaded ranges, likely due to having both sexual and asexual clonal reproduction and allelopathic impacts on competitors (Dong et al., 2006). *S. canadensis* has become a notorious weed in various habitats in East China, including roadsides, abandoned and agricultural fields, and even open barren areas (Wan et al., 2018b; Ren et al., 2019). Wan et al. (2018b) found that, in its invaded ranges, *S. canadensis* tends to be found in areas with nutrient-poor soil, where available P is low because of the loss of organic material. *S. canadensis* is known to have strong performance even under very low P availability (Yu et al., 2016; Wan et al., 2018a). One possibility is that *S. canadensis* achieves success under low P conditions through its association with AMF, such as *G. intraradices*. Although *G. intraradices* can be found in almost all soils and is a generalist fungus that associates with many plant taxa (van der Heijden et al., 2015), there is no existing evidence that *G. intraradices* forms a mutualistic relationship with *S. canadensis*. Thus, *G. intraradices* was chosen as a representative AMF species in this work to study the effects on the growth of *S. canadensis*.

Our aim is therefore to determine how AMF affects invasive clonal plant *S. canadensis* to achieve high performance in phosphorus-deficient soils. We predicted that associations with AMF would increase the ability of plants to absorb phosphorus and allow them to change their resource allocation strategy to favor increased above-ground biomass (Berta et al., 1993; Vance et al., 2003). Our study extends previous work in this field by using axenic conditions to avoid potential confounding factors caused by the presence of non-focal microorganisms, such as endophytes or rhizosphere bacteria, that are known to affect plant growth and nutrient uptake (Chen et al., 2006; Rout et al., 2013; Afkhami and Strauss, 2016; Dai et al., 2016b; McLeod et al., 2016; Priyadharsini and Muthukumar, 2017). We used pure cultures of the AMF *G. intraradices* with aseptic seedlings of *S. canadensis* grown under completely sterile culture conditions to determine:

(1) Does *G. intraradices* form a mutualistic relationship with *S. canadensis*?
(2) Does the relationship between *G. intraradices* and *S. canadensis* vary with nutrient availability?
(3) Do P availability and colonization by *G. intraradices* affect the growth of *S. canadensis*?
(4) Does *G. intraradices* increase phosphate uptake of *S. canadensis*?
(5) What effect does *G. intraradices* have on the resource allocation strategy of *S. canadensis*?

MATERIALS AND METHODS

We began by asking whether the AMF *G. intraradices* forms a mutualistic relationship with *S. canadensis* seedlings by growing aseptic seedlings with a monoxenic culture of AMF. Aseptic seedlings (**Supplementary Figure 1**) were produced from fresh shoots of *S. canadensis* according to the method by Dai et al. (2016b). Briefly, fresh apical buds of *S. canadensis* were surface-sterilized with 75% ethanol for 1 min and 5% sodium hypochlorite solution for 10 min, and then washed five times with sterilized distilled water. These apical buds were then put into sterilized Murashige and Skoog (MS) solid medium supplemented with 0.8 $mg{\cdot}L^{-1}$ 6-benzylaminopurine, 0.1 $mg{\cdot}L^{-1}$ 1-naphthaleneacetic acid, and 0.8 $mg{\cdot}L^{-1}$ silver nitrate. After clusters of axillary buds proliferated (∼50 days of culturing), the aseptic shoots were cut and maintained in MS medium for about 5 days to obtain seedlings with roots for further treatments. The absence of contaminant microorganisms in the seedlings (**Supplementary Figures 2**, **4**) was assessed using both

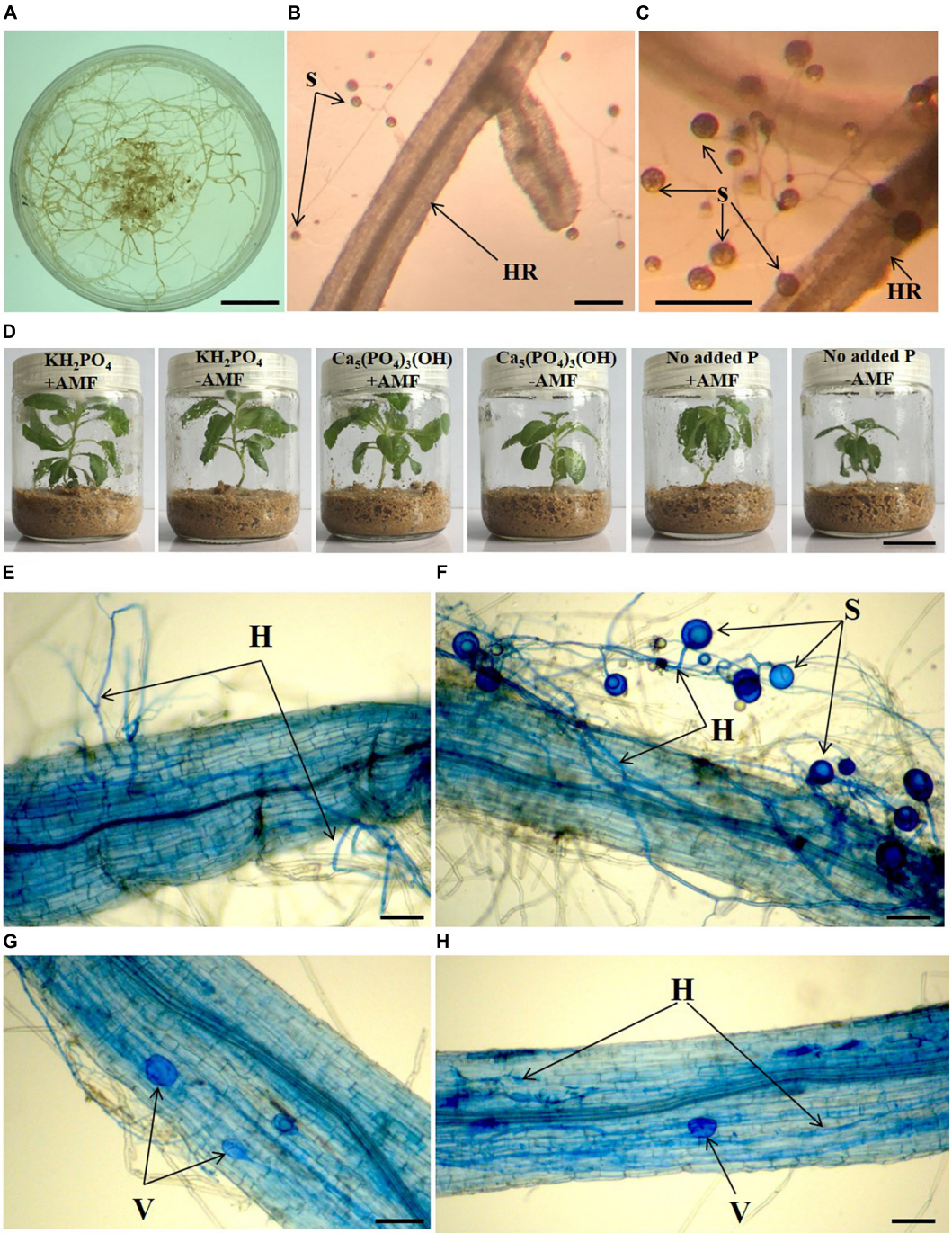

FIGURE 1 | Monoxenic culture system of *Glomus intraradices* **(A–C)**, aseptic growth conditions **(D)** of *Solidago canadensis* grown in different phosphorus treatments with or without arbuscular mycorrhizal fungi (AMF) colonization, and mycorrhizal root colonization of *S. canadensis* in aseptic seedling culture system **(E–H)**. HR, hairy root, H, hyphae, S, spore, V, vesicles, +AMF- with AMF colonization, -AMF- without AMF colonization. Bars in **(A,D)** = 2 cm, bars in **(B,C)** = 100 μm, bars in **(E–H)** = 25 μm.

the coating plate method and 16S/18S rRNA gene amplification (Dai et al., 2016b).

To generate a monoxenic AMF culture, we used carrot (*Daucus carota* L.) roots transformed with the T-DNA from a tumor-inducing plasmid as the host for the pure AMF strain *G. intraradices* (**Figures 1A–C**). The pure AMF strain and aseptic hair-root system (available from the Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese

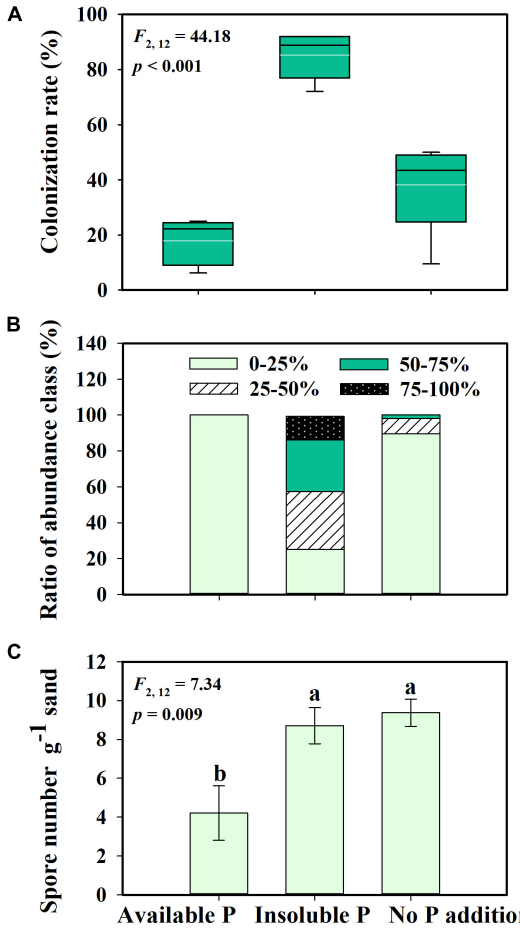

FIGURE 2 | Mycorrhizal colonization rate **(A)**, hyphae abundance class **(B)**, and spore number in sand **(C)** of *Glomus intraradices* interacting with *Solidago canadensis* under different phosphorus (P) treatments. The white lines are means, the black lines are medians, the boxes show 25 to 75% quantiles in panel **(A)**. Available P, P was added as soluble KH_2PO_4; Insoluble P, P was added as $Ca_5(PO_4)_3(OH)$; No P addition, no P was added. Error bars are the S.E. (n = 5). Different letters indicate a significant difference at $p < 0.05$.

Academy of Sciences) was used to obtain aseptic spores. Spores of *G. intraradices* were isolated and applied to the aseptic plant seedlings. The colonized roots from the Minimal Medium (Bécard and Fortin, 2010) which contains spores, were placed in a sterile flask. A 10-mM sodium citrate (10 times volume of medium) solution was added, and then the mixture was stirred for 10 min to separate spores from hyphae. Spores were harvested from the hairy roots of *D. carota via* initial filtration using sterile gauze, and subsequently sterile spores were collected through 0.45-μm syringe filters. Spores were washed three times using sterile water to remove the remaining sodium citrate before being suspended in sterile water.

To provide a sterile growth environment, we used glass tissue culture flasks (Dai et al., 2016b) for the different types of P supplementation to *S. canadensis* seedlings in April 2017 (**Figure 1D**). Washed sand (90 g) was put into each flask, sterilized at 121°C for 2 h, and then cooled to room temperature. For each flask, 25 mL modified 0.5 × Hoagland (Dai et al., 2016a) without P was added to the sand. A sterile *S. canadensis* seedling was transferred into each flask. Seedlings were grown in an incubator at 28°C and light for 16 h a day at 360 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 8 h darkness. For AMF inoculation, 1 mL *G. intraradices* spores (+ AMF) suspension (approximately 770 ± 84 spores) was added into the flasks. No spore was added as the control treatment (−AMF).

To address question 1, we measured the hyphal colonization rate and spore production in sand media in inoculated vs. non-inoculated treatments after 45 days. Plants and sand media were harvested from the flask. Roots were pulled out and the sand was shaken off and collected to count the number of spores. Roots of *S. canadensis* were sampled for hyphal staining of AMF to determine root colonization. There were five replicates for each treatment. Hyphal staining was assessed following the procedure described in Phillips and Hayman (1970). Briefly, root samples for each treatment were gently washed with distilled sterile water and cut into 2-cm pieces before being externally cleaned in 10% KOH and then acidified with 1% HCl. The surface of the root samples was then stained with 0.05% trypan blue in lactophenol before the microscopic observation of mycorrhizal colonization rate and the abundance of arbuscules in the roots (Yang et al., 2014). The abundance of colonization was classified into four classes: 0–25% colonized, 25–50% colonized, 50–75% colonized, and 75–100% colonized. No colonization or spores of *G. intraradices* were detected in the non-inoculated seedlings, confirming the lack of contamination in our experiments.

To determine whether the mutualistic relationship varied with nutrient availability (question 2), we grew *S. canadensis* seedlings in media as above, but with three different nutrient levels. Sterile conditions were established as above, and 30 $mg \cdot kg^{-1}$ P (approximately P content in Hoagland nutrient solution) was added either as soluble KH_2PO_4 (hereafter referred to as "Available P") or insoluble $Ca_5(PO_4)_3(OH)$ (hereafter referred to as "Insoluble P") to a flask and mixed evenly. No P was added to the controls (hereafter referred to as "No P addition"). Five replicates of each treatment were set up for a total of 30 flasks.

To quantify *S. canadensis*' performance in P-deficient soil and to determine whether AMF could contribute to the growth of *S. canadensis* (question 3), treatments of available and unavailable P were set up as above. We measured a suite of traits associated with growth and resource allocation: leaf number, maximum leaf area, maximum leaf dry mass, shoot length, shoot dry mass (i.e., aboveground dry mass), root length, root dry mass, root dry mass/shoot dry mass, and specific leaf area (SLA, the ratio of maximum leaf area/dry mass). These traits were measured and calculated following Dai et al. (2016b).

To quantify the impact of AMF on phosphate uptake of *S. canadensis* (question 4), root phosphatase activity was determined following Zalamea et al. (2016). Briefly, roots were placed in a glass vial with 25 mL of 0.2 M sodium acetate–acetic acid buffer (pH 5.0) and shaken in a water bath at 28°C. The assay was initiated by adding 2.5 mL substrate (50 mM *para*-nitrophenyl phosphate, *p*NPP) and incubated for 30 min. The

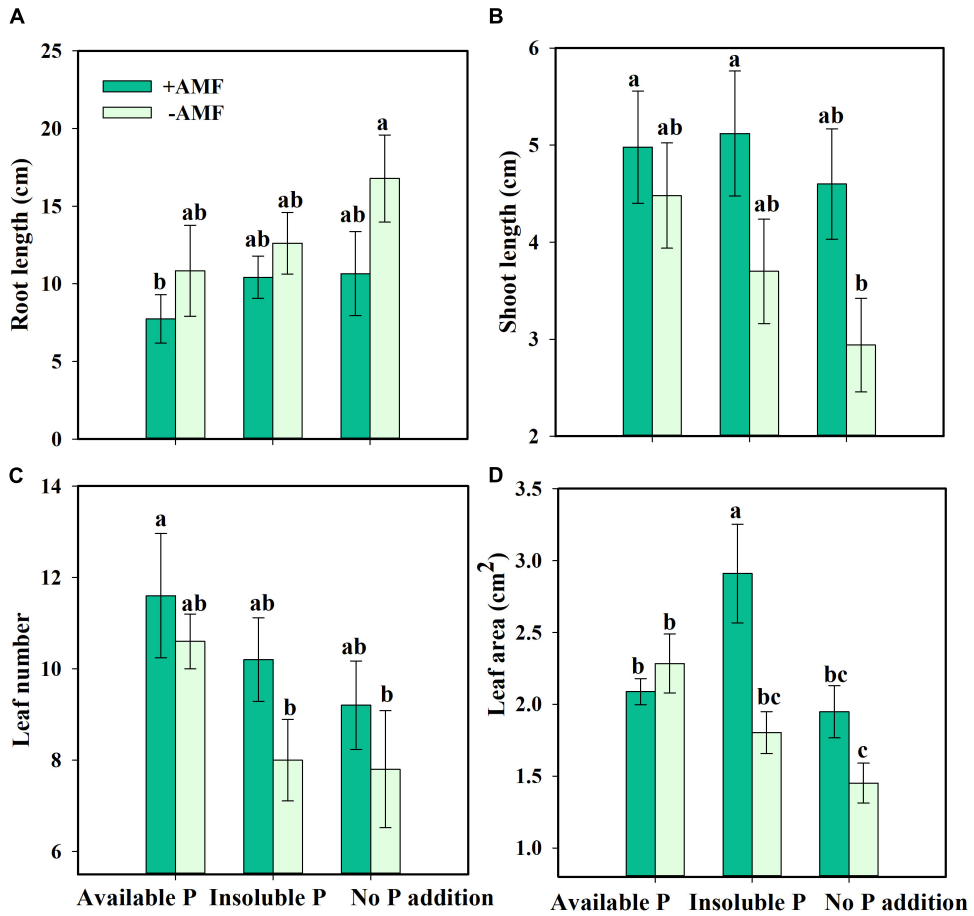

FIGURE 3 | Root length **(A)**, shoot length **(B)**, leaf number **(C)**, and leaf area **(D)** of *Solidago canadensis* in different phosphorus (P) treatments. Available P, P was added as KH_2PO_4; Insoluble P, P was added as $Ca_5(PO_4)_3(OH)$; No P addition, no P was added. +AMF, with arbuscular mycorrhizal fungi (AMF) colonization; -AMF, without AMF colonization. Error bars are the S.E. (n = 5). Different letters indicate a significant difference at $p < 0.05$.

reaction was terminated by removing 0.5 mL of buffer solution and adding it to 4.5 mL of terminator solution (0.11 M NaOH) in a glass test tube. After vortexing, the absorption was measured at 405 nm against *para*-nitrophenol (*p*NP). Phosphatase activity was expressed in mmol *p*NP g^{-1} h^{-1} produced from *p*NPP through hydrolysis by phosphatase.

All seedlings were ground and analyzed for total P content (Fujita et al., 2010). After a digestion procedure (Kjeldahl digestion method; 1 h at 200°C and 2 h at 340°C in a mixture of concentrated sulfuric acid and 30% hydrogen peroxide), the seedlings were cooled and diluted with deionized water to 45 ml. The P content was determined colorimetrically using a UV-1200 spectrophotometer (MAPADA, Shanghai, China) following Wan et al. (2018a).

To address the effects of AMF on the resource allocation strategy of *S. canadensis* (question 5), the effective resource allocation (ERA) was calculated using the following equation (Grace et al., 2009):

$$\mathrm{ERA}\ (\%) = \frac{V_{+AMF} - V_{-AMF}}{V_{-AMF}} \quad (1)$$

where V_{+AMF} and V_{-AMF} is the value of phenotypic and physiological indicators (i.e., above- and below-ground biomass, phosphatase activity, or phosphorus content) with and without AMF inoculation, respectively.

Duncan's multiple range tests were conducted to find if there were significant differences in colonization rate and spore number among treatments. We quantified the effects of different phosphorus treatments on the growth of *S. canadensis* with or without AMF colonization using the two-way analysis of variance (ANOVA). Duncan's multiple range tests were also performed to determine the growth of *S. canadensis* with different treatments and also used to compare the differences of the effect of AMF on plant resource allocation. All statistical analyses were performed with the SAS statistical software 9.1, and figures were drawn with SigmaPlot 12.0 software.

RESULTS

Glomus intraradices did form a mutualistic interaction with *S. canadensis* (**Figures 1E–H**). Consistent with our second

hypothesis, the proportion of roots colonized by *G. intraradices* varied with P availability (F = 44.18; $p < 0.001$). More than 80% of roots in inoculated treatments were colonized in the Insoluble P treatment, which was significantly higher than the colonization in either the No P addition (38% colonized) or the Available P treatments (17% colonized; **Figure 2A**; $p < 0.001$). The abundance of *G. intraradices* on colonized roots was also higher in the Insoluble P treatment than in the Available P or No P addition treatments (**Figure 2B**). All roots in the Available P treatment and 90% of the roots in the No P addition treatment had below 25% colonization by *G. intraradices*, while 75% of the roots in the Insoluble P treatment were more than 25% colonized. Finally, spore numbers in the sand beneath the No P addition and Insoluble P treatments were more than twice as high as in sand from the Available P treatment (F = 7.34; p = 0.009; **Figure 2C**).

Next, we asked how nutrient treatment and AMF inoculation affected the growth and functional traits of *S. canadensis* (question 3). We found that roots tended to be longer in the treatment with No P addition or AMF inoculation than in the treatment with available P and with AMF inoculation (**Figure 3A**). In contrast, shoot length, leaf number, and leaf area tended to increase with AMF inoculation (**Figures 3B–D**). Some of these effects were substantial. For example, leaf area in the Insoluble P treatment was 61% higher with AMF inoculation than in the non-inoculated treatment (1.8 vs. 2.9 cm^2; **Figure 3D** and **Table 1**). In the Insoluble P treatment, the total dry mass tended to be greater than in the other two P treatments (**Supplementary Figure 5**). Compared to the non-inoculated treatment, AMF inoculation decreased both root dry mass and root to shoot ratio of plants grown in the insoluble P condition, but did not affect these traits of plants grown in the available P and no P conditions (**Figures 4A,C** and **Table 1**). On the other hand, compared to the non-inoculated treatment, the AMF inoculation increased both shoot dry mass and specific leaf area of plants grown in the insoluble P condition, but imposed no effect on these traits of plants grown in the available P or no P conditions (**Figures 4B,D** and **Table 1**). In summary, plants grown in soil with low P availability tended to shift resources from below-ground to above-ground tissues in the presence of AMF.

For the fourth question, we found that AMF inoculation changed phosphatase activity and phosphate uptake of *S. canadensis*. AMF inoculation significantly decreased phosphatase activity in the Insoluble P treatment but did not affect the phosphatase activity in the Available P or No P addition treatments (**Figure 5A**). In the Insoluble P treatment, the AMF inoculation increased P concentration by 107% compared to the non-inoculated treatment (**Figure 5B**). That is, *G. intraradices* significantly promoted phosphate uptake and decreased phosphatase activity of *S. canadensis* in conditions of insoluble P (**Figure 5**).

Finally, we found that *S. canadensis* changed its resource allocation when it was colonized by *G. intraradices*. In insoluble P conditions, colonized plants allocated 47.5% more resources to above-ground growth while decreasing the biomass allocation to below-ground (**Figure 6A** and **Table 2**). Plants with AMF achieved a higher P content despite allocating fewer resources to below-ground growth and having decreased phosphatase activity (**Figure 6B** and **Table 2**). The effective resource allocation was higher in the No P addition treatment than in the Available P treatment (**Figure 6B**).

DISCUSSION

Our findings provide the first direct proof that AMF increase P uptake, change plant resource allocation strategy, and promote the growth of *S. canadensis* under conditions deficient in inorganic phosphorus. The efficient use of limited resources facilitated by mutualism with AMF contributes to the rapid growth of invasive weed*S. canadensis* and may facilitate the invasion of new habitats.

The Importance of Axenic Systems in Arbuscular Mycorrhizal Fungi Studies

The AMF colonization rate in our study (up to 83%) was substantially higher than in most previous studies, where they ranged from 21 to 73% (Li et al., 2006; Balzergue et al., 2011; Yuan et al., 2014; Hack et al., 2019). One reason for this could be that the axenic systems eliminate interference

TABLE 1 | Two-way ANOVAs of the effects of different phosphorus (P) treatments on the growth and functional traits of *Solidago canadensis* with/without arbuscular mycorrhizal fungi (AMF).

Growth traits	Source	*d.f.*	*F*	*p-value*
Root length	AMF	1	4.07	*0.055*
	P	2	1.84	0.181
	AMF × P	2	0.39	0.678
Shoot length	AMF	1	6.79	**0.016**
	P	2	1.52	0.239
	AMF × P	2	0.60	0.559
Leaf number	AMF	1	3.29	*0.082*
	P	2	3.45	**0.048**
	AMF × P	2	0.17	0.841
Leaf area	AMF	1	8.20	**0.009**
	P	2	5.77	**0.009**
	AMF × P	2	5.28	**0.013**
Root dry mass	AMF	1	2.85	0.104
	P	2	13.68	**0.001**
	AMF × P	2	1.13	0.339
Shoot dry mass	AMF	1	14.42	**0.001**
	P	2	10.90	**0.001**
	AMF × P	2	5.90	**0.008**
Root to shoot ratio	AMF	1	8.13	**0.009**
	P	2	13.00	**0.001**
	AMF × P	2	3.39	**0.050**
Specific leaf area	AMF	1	16.31	**0.001**
	P	2	9.55	**0.001**
	AMF × P	2	0.30	0.746

Values of $p < 0.05$ are in bold.Values are in italics where $0.05 < p < 0.1$.

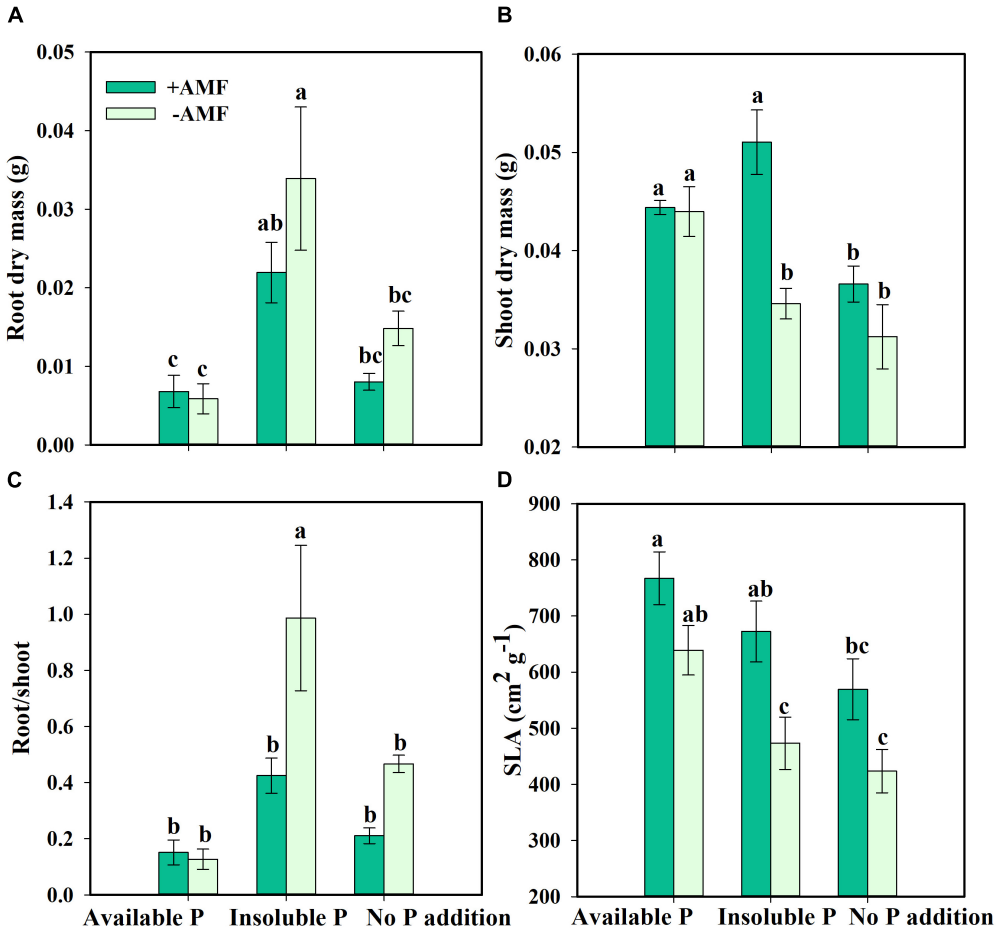

FIGURE 4 | Root dry mass **(A)**, shoot dry mass **(B)**, root/shoot **(C)**, and specific leaf area [SLA, **(D)**] of *Solidago canadensis* in different phosphorus (P) treatments. Available P, P was added as KH_2PO_4; Insoluble P, P was added as $Ca_5(PO_4)_3(OH)$; No P addition, no P was added. +AMF, with arbuscular mycorrhizal fungi (AMF) colonization; -AMF, without AMF colonization. Error bars are the S.E. ($n = 5$). Different letters indicate a significant difference at $p < 0.05$.

from other phosphorus solubilizing bacteria (Spagnoletti et al., 2017). Plants may need to invest more resources in maintaining a mutualistic relationship with AMF in the absence of these bacteria.

In non-axenic systems, the presence of other endophytes or rhizosphere bacteria has been found to promote both above- and below-ground growth (Calonne-Salmon et al., 2018; Zhan et al., 2018). In contrast, we found that AMF allow plants to reduce the amount of resources used to construct a root system and allocate more resources to above-ground structures in this study (**Figure 4**). This difference in results suggests that the presence of other microorganisms in non-axenic systems can obscure the true effects of AMF.

Arbuscular Mycorrhizal Fungi Contribute to Phosphorus Uptake in Low Nutrient Conditions

Arbuscular mycorrhizal fungi can play significant roles in plant nutrient absorption, especially in nutrient-poor soil (Mikkelsen et al., 2008; Eissenstat et al., 2015). In our study, *S. canadensis* in the Insoluble P treatment accumulated more than twice as much P in the presence of AMF compared to their absence (**Figure 5B**). This finding is consistent with previous work by Li et al. (2006) and Yang et al. (2012), who found that over half of the P uptake by plants was due to AMF in soils with low P bioavailability. The mechanism underpinning this process is relatively well-understood. AMF secrete organic acids, phosphatases, and inorganic phosphorus transporters that contribute to the solubilization of insoluble P and the release of orthophosphate, which enhances P uptake and facilitates plant growth (Joner et al., 2000; Koide and Kabir, 2000; Bagyaraj et al., 2015). *S. canadensis* in a P-deficient environment secretes only half the amount of phosphatase with AMF colonization compared to no AMF colonization (**Figure 5A**). These findings were consistent with the idea that plants profit more from AMF whose hyphae would secrete phosphatases when insoluble P is available, allowing plants to decrease the resources allocated to phosphatase activity (Priyadharsini and Muthukumar, 2017). Evidence suggests that association with

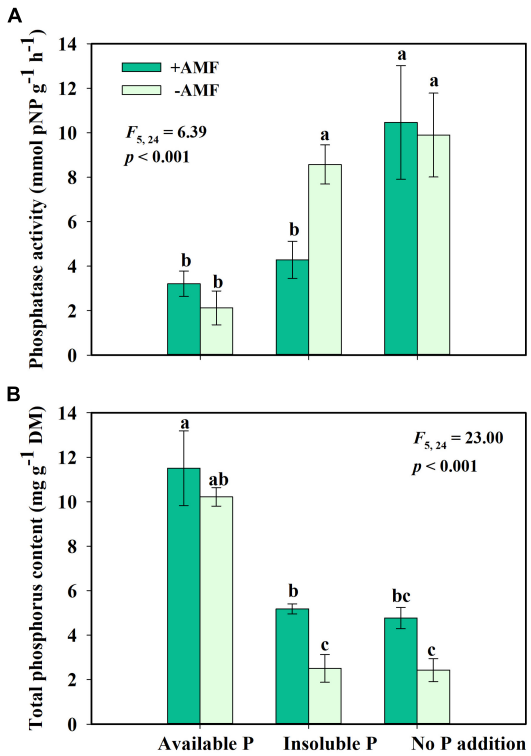

FIGURE 5 | Phosphatase activity **(A)** and phosphorus content **(B)** of *Solidago canadensis* grown on three phosphorus (P) treatments with or without arbuscular mycorrhizal fungi (AMF) colonization. Available P, P was added as KH_2PO_4; Insoluble P, P was added as $Ca_5(PO_4)_3(OH)$; No P addition, no P was added. +AMF, with AMF colonization; -AMF, without AMF colonization. Error bars are the S.E. (n = 5). Different letters indicate a significant difference at $p < 0.05$.

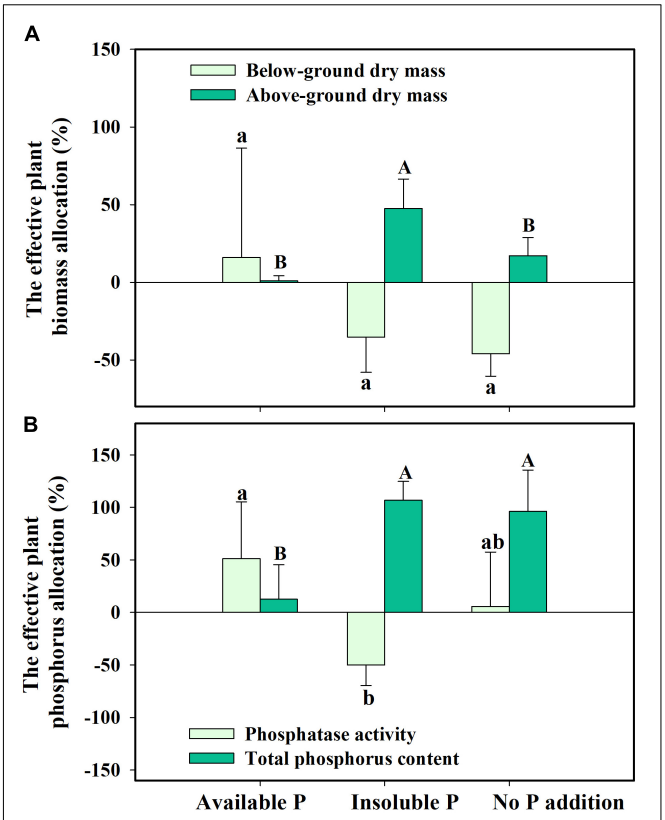

FIGURE 6 | The effective resource allocation (ERA) on plant biomass allocation **(A)** and phosphorus allocation **(B)** in *Solidago canadensis* forming mutualism with arbuscular mycorrhizal fungi (AMF). Available P, P was added as KH_2PO_4; Insoluble P, P was added as $Ca_5(PO_4)_3(OH)$; No P addition, no P was added. Error bars are the S.E. (n = 5). Different letters indicate a significant difference at $p < 0.05$.

AMF leads to increase plant growth (**Figures 3**, **4**, **6**; Delavaux et al., 2017).

Effects of Arbuscular Mycorrhizal Fungi in the Allocation Strategies in Invasive Plants

There is still some debate as to whether invasive plants receive a greater benefit from mutualism with AMF than native species. Bunn et al. (2015) showed that native and invasive plants did not respond differently to AMF, but invasive plants had a higher level of AMF colonization when grown in competition with native plants. However, Menzel et al. (2017) showed that a mycorrhizal mutualism could promote the invasion success of neophyte plant species. The presence of arbuscular mycorrhiza has also been closely linked with plant invasions through facilitating nutrient cycling (Jo et al., 2018). Consistent with this, we found that AMF facilitate the growth of invasive plant *S. canadensis*. In nutrient-limited soil, *S. canadensis* allocates fewer resources to above-ground growth but more resource to below-ground growth for foraging more nutrients without AMF colonization. However, due to the presence of AMF, *S. canadensis* changes its resource allocation strategy and is able to allocate more resources to above-ground growth and facilitate phosphorus (P) uptake by the plant, allowing plants to have lower investment into below-ground biomass, and higher benefit/return for above-ground biomass (**Figure 6**, and schematic on the right in **Figure 7**). This is consistent with what Zhang et al. (2015) found, i.e., the colonization of AMF led to higher allocation to shoot biomass in rice. Shen et al. (2020) found that AMF increased P acquisition of the invasive *Eupatorium adenophorum*, and the P acquisition in above-ground was higher than in roots, which is consistent with our results.

TABLE 2 | Analyses of variance due to the effects of different phosphorus (P) treatments on the effective resource allocation of *Solidago canadensis*.

Measures	*F*2, 12	*p*-value
Below-ground dry mass	2.32	0.141
Above-ground dry mass	13.21	**0.001**
Phosphatase activity	5.19	**0.024**
Total phosphorus content	10.80	**0.002**

Values of $p < 0.05$ are in bold.

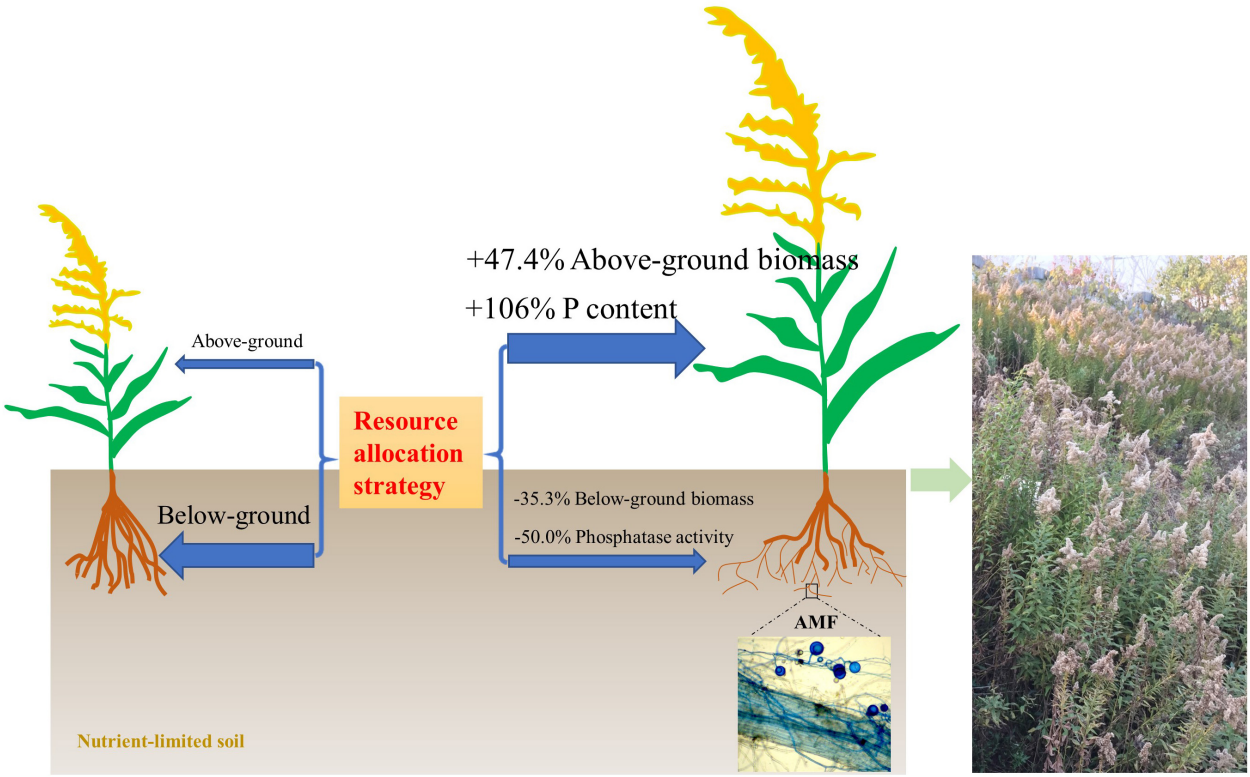

FIGURE 7 | A model of the resource allocation strategy with/without arbuscular mycorrhizal fungi (AMF) on the growth of invasive weed *Solidago canadensis* in nutrient-limited soil.

In conclusion, we have shown that colonization by AMF is associated with changes in P uptake and increased growth in *S. canadensis*. With the contribution of AMF, the clonal plant *S. canadensis* is able to allocate more resources to above-ground growth, which might also affect its clonal performance. As a consequence, association with AMF likely contributes to *S. canadensis*' success as an invasive clonal species, particularly in nutrient-limited habitats.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SQ and ZD designed the experiment, analyzed the data, and wrote the manuscript. SQ, JW, and LW performed the experiment. DS, DD, SE, SB, TT, and AM commented on the details of the manuscript drafts. All authors contributed critically to the drafts and gave final approval for publication.

FUNDING

This study was funded by the National Natural Science Foundation of China (32171509 and 32071521), Jiangsu Planned Projects for Postdoctoral Research Funds (2021K160B), and the Natural Science Foundation of Jiangsu (BK20211321). DS acknowledges the CNPq (Fellowship of Research productivity 307839/2014-1), AM acknowledges ARC grant (DP190100243), and TT also acknowledges the support by the Australian Research Council. Part of the funding for this research was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

ACKNOWLEDGMENTS

We thank Xiang Xiao for kindly providing the pure AMF strain *Glomus intraradices* and the aseptic carrot hair-root system.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.831654/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Plant Science

ORIGINAL RESEARCH
published: 02 June 2022
doi: 10.3389/fpls.2022.883800

Differential Growth Responses of *Alternanthera philoxeroides* as Affected by Submergence Depths

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Global climate change has resulted in an increase in intensity and frequency of flooding, plants living in lowlands, and shore areas have to confront submergence caused by flooding, submergence-tolerant plants usually respond by adopting either escape or quiescence strategies. While certain plants exhibit a changeover from escape strategy upon partial submergence to quiescence strategy under complete shallow submergence, it remains unknown whether plants completely submerged at different water depths would adjust their strategies to cope with the change in submergence depth. *Alternanthera philoxeroides* is an ideal species to explore this adjustment as it is widely distributed in flood-disturbed habitats and exhibits an escape strategy when completely submerged in shallow waters. We investigated the responses of *A. philoxeroides* in terms of morphology, anatomy, and non-structural carbohydrate metabolism by conducting experiments using a series of submergence depths (0, 2, 5, and 9 m). During the submergence treatment, environmental factors such as light, dissolved oxygen, and temperature for submerged plants were kept constant. The results showed that *A. philoxeroides* plants submerged at depth of 2 m presented an escape strategy *via* fast stem elongation, extensive pith cavity development, and small biomass loss. However, the retarded stem elongation, reduced pith cavity transverse area, and increased biomass loss along the water depth gradient indicated that *A. philoxeroides* altered its growth response as water depth increased from 2 to 9 m. It is found that the changeover of response strategies occurred at higher submergence depths (5–9 m). Based on the results of our experiments, we demonstrated that water depth played an important role in driving the change in strategy. The water-depth-dependent growth performance of *A. philoxeroides* would benefit the species in habit exploration and exploitation. Further studies should focus on the performances of plants when submerged at varied water depths with different light climates and dissolved oxygen content, and how water depths drive the response behaviors of the submerged plants.

Keywords: adaptive strategy, submergence-tolerant plants, hydrostatic pressure, flood-prone habitats, submergence depths

OPEN ACCESS

Edited by:
Bi-Cheng Dong,
Beijing Forestry University, China

Reviewed by:
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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: *25 February 2022*
Accepted: *27 April 2022*
Published: *02 June 2022*

Citation:
Jing S, Zhang X, Niu H, Lin F, Ayi Q, Wan B, Ren X, Su X, Shi S, Liu S and Zeng B (2022) Differential Growth Responses of Alternanthera philoxeroides as Affected by Submergence Depths. Front. Plant Sci. 13:883800. doi: 10.3389/fpls.2022.883800

INTRODUCTION

Global climate change has increased the intensity and frequency of flood events, and this trend is predicted to continue in the future (Milly et al., 2002; Blöschl et al., 2019). River engineering has considerably altered the hydrological regimes of rivers worldwide (Dynesius and Nilsson, 1994; Huang et al., 2016). These changes mainly include the transition from shallow floods to deep floods (Belt, 1975; Brock et al., 1987; Sparks et al., 1998; Bertola et al., 2020). The construction of more than 50,000 dams higher than 15 m worldwide (Lehner et al., 2011) has resulted in huge drawdown zones. For example, the Three Gorges Reservoir in China has a drawdown zone of ∼350 km^2 with a maximum submergence depth of 30 m, the water level of which fluctuates annually between 145 and 175 m above sea level (Lei et al., 2017). Such deep submergence has detrimental effects on the growth and survival of plants (Lei et al., 2014; Bejarano et al., 2018; Huang et al., 2021), especially on those completely submerged (Vervuren et al., 2003; Fukao et al., 2019; Striker et al., 2019). As a consequence, plants have to strive to cope with submergence in flood-prone habitats.

One of the major problems that terrestrial plants face when submerged is the energy crisis, induced by low gas partial pressure and light intensity in water (Bailey-Serres et al., 2012; Huber et al., 2012; Pedersen et al., 2017). Gas (e.g., oxygen, carbon dioxide, and ethylene) exchange between completely submerged plants and water column significantly slows as gas diffusion is severely restricted underwater (Jackson, 1985; Voesenek et al., 2006; Voesenek and Bailey-Serres, 2015). Due to the low oxygen content in the water column, the aerobic respiration of completely submerged plants decreases, leading to reduced ATP production and even plant death (Voesenek et al., 2006; Bailey-Serres and Voesenek, 2008). Compared to the atmosphere, water bodies have relatively low levels of CO_2, moreover, the photosynthetically active radiation (PAR) in water bodies sharply declines from the water surface downwards (Voesenek et al., 2006; Voesenek and Bailey-Serres, 2015). This further limits underwater photosynthesis and increases the energy crisis of plants when submerged.

The escape and quiescence strategies are two major strategies adopted by plants in flood disturbed habitats (van Veen et al., 2014). Generally, the escape strategy enables plants to cope with shallow but prolonged submergence, whereas the quiescence strategy is favored to withstand deeper and short-term submergence (Bailey-Serres and Voesenek, 2008; Manzur et al., 2009; Striker et al., 2017). The "escape strategy" syndrome includes fast elongation of shoot organs (Sauter et al., 1993; Müller et al., 2019) or leaf petioles (Groeneveld and Voesenek, 2003; Pierik et al., 2009) to restore air contact rapidly, enhanced adventitious root formation to improve oxygen uptake in water (Ayi et al., 2016; Zhang et al., 2017), and increased formation of internal aerenchyma tissue to efficiently transport gas (Manzur et al., 2009; Striker et al., 2019). These morph-physiological behaviors require carbohydrates for cell division and new cell production (Sauter, 2000; Voesenek et al., 2006; Luo et al., 2011). Therefore, one side effect of the "escape strategy" would be fast carbohydrate depletion (Bailey-Serres and Voesenek, 2008; Manzur et al., 2009; Akman et al., 2012), which is lethal for submerged terrestrial plants if they cannot protrude from the water surface. Under deep submergence conditions (where water depths are usually deeper than 2 m and light climate is poor), plants with a quiescence strategy will be more successful. They suppress energy consumption by only running basic metabolism with little or no organ elongation and new tissue formation, thus conserving carbohydrate reserves to prolong survival time in deep water while waiting for the water level to recede (Manzur et al., 2009; Pierik et al., 2009; Luo et al., 2011; Akman et al., 2012; Striker et al., 2017). Theoretically, it is likely that plants would survive and distribute from low to high elevations in the river riparian zones (or the reservoir drawdown zones and other flood disturbed habitats) if they are able to alter their response strategy at different submergence depths. It was reported that the wetland plant *Lotus tenuis* chose to escape from partial submergence by shoot elongation but adopted a non-elongating quiescent strategy when completely immersed in shallow water (Manzur et al., 2009). Nevertheless, the literature on whether terrestrial plants can alter their response strategies along a gradient of submergence depths is still scarce.

Besides gas and light, which induce plant responses to submergence, submergence depth—especially extremely deep submergence—may strongly affect plant performance (Vervuren et al., 2003). Submergence depth is a primary physical factor that varies along elevational gradients in many riparian regions (Howard and Mendelssohn, 1995). The effects of submergence depth on plant metabolism and growth can be direct or indirect (increasing hydrostatic pressure, increasing soil oxygen consumption, and changing temperature, which indirectly affects plant performance in water) (Grace, 1989; Howard and Mendelssohn, 1995; Casanova and Brock, 2000; Bejarano et al., 2018; Meng et al., 2022). Some studies have demonstrated the negative effect of increased submergence depth on plant survival; the median lethal time (LT_{50}) of Rhine riparian plants at a depth of 1.6 m was approximately half of that at 0.4 m (Vervuren et al., 2003), similar to the effects observed in rice cultivars (Adkins et al., 1990). So far, the published literatures primarily reported the studies focused on the influences of submergence depths <2 m on terrestrial plants. The absence of studies investigating how deeper water affects plant performance might limit our understanding of plant distribution patterns in flood-prone habitats.

Alternanthera philoxeroides (Mart.) Griseb., a terrestrial perennial herbaceous plant belonging to the Amaranthaceae family, originates from South America and has spread to many parts of the world. It is considered an invasive species in the United States, Australia, New Zealand, Thailand, and China, and able to survive nicely in flood-disturbed habitats (Zhang et al., 2015a; Dong et al., 2018). *A. philoxeroides* exhibits an escape strategy in shallow submergence (Luo et al., 2009, 2011; Ayi et al., 2016), with quickly elongated shoots, increased adventitious roots formation, and widened aerenchyma channels conducive to enhance gas transport (Ayi et al., 2019). In addition, the species lowers its metabolic rate to substantially reduce carbohydrate consumption in water (Ye et al., 2016). *A. philoxeroides* not only distributes in shallow wetlands but also well exists in areas

experiencing submergence with a maximum depth of 20 m for up to 4 months (Zheng et al., 2021). Therefore, this species is an ideal species for investigating how plants respond to submergence depth gradient. Considering the side effects of escape strategy and the wide distribution of *A. philoxeroides* in areas with varied submergence depths, we hypothesize that *A. philoxeroides* is likely to change its growth strategy when submergence depth differs.

In this study, we aimed to explore how *A. philoxeroides* plants respond to a gradient of submergence depths in terms of morphology, anatomy, biomass, and carbohydrate metabolism by conducting submergence experiments with varied water depths. Physical conditions of the water body including light, dissolved oxygen, pH, and temperature were kept constant in the experiments. The study may help understand the mechanisms of plant tolerance to extreme flooding and explain why *A. philoxeroides* remains highly invasive in regions where submergence depths are varied (e.g., the drawdown zones of large reservoirs). The findings may also provide insight into the effective management of this species.

MATERIALS AND METHODS

Plant Material and Cultivation

Alternanthera philoxeroides is a herbaceous perennial plant, under normal conditions, it can spread quickly *via* clonal growth (Luo et al., 2009; Ayi et al., 2016), growing to a height of 50–120 cm, with a long single or sparsely branched stem. The stem has several internodes of different lengths, and its maturity degree gradually decreases from the base to the top, making it an ideal organ type to study the adaptation and response of *A. philoxeroides* to different submergence depths.

To reduce the influence of environmental conditions on mother plants, *A. philoxeroides* plants used in this experiment were cultivated from cuttings obtained from plants naturally growing on the banks of the Jialing River in Chongqing, Southwest China (29°49′N, 106°25′E). In May 2020, unbranched plants with a stem length of ∼30 cm were selected and cut at the stem base. The cuttings were transported to the laboratory immediately, and healthy and vigorous cuttings were selected for subsequent treatments, all trimmed to have five internodes and six leaves. Each selected cutting was planted in a plastic pot (diameter and depth were both 13 cm) containing riparian soil from the Jialing River banks, two stem nodes of the cutting (hereafter referred to as "plant") were buried in soil for rooting. All plants were cultivated under the same conditions and placed in an open field of the experimental garden affiliated to the Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education) at Southwest University, Chongqing. The temperature, relative humidity, daily maximum light (PAR) intensity, and water provision were maintained at 10–15°C, 75–85%, 600–800 μmol m^{-2} s^{-1}, and ∼80–90% of soil water-holding capacity, respectively. Plants were watered daily. Lateral buds, if produced, were removed to ensure that the main stem of plants grew without branching. Plants were kept growing upright by the support of thin bamboo sticks. After ∼1 month of cultivation, plants with ∼45 cm in height and 12 internodes were selected for submergence treatments.

Submergence Treatments

Four submergence treatments were applied in a fully randomized design using selected plants (20 replicates per treatment). Submergence depth of 0 m was set as control: unsubmerged plants were placed under dark conditions and watered normally to keep soil water at field capacity. Additionally, three groups of plants were submerged in a water-filled concrete reservoir (length × width × depth = 2.3 × 1.9 × 10 m), with the top of plants 2, 5, and 9 m beneath the water surface. The plants in pots were suspended at planned water depths (**Figure 1A**). Pilot experiments showed that the stem tips of plants started to die (characterized by becoming flaccid) on the 7th day following submergence at depth of 9 m. Therefore, the treatment duration was set at six days for all submergence depths to ensure tested plants kept vigorous during this period.

Water Environment Management

To investigate the effects of submergence depth on plants, the physico-chemical status of water (light, dissolved oxygen (DO), pH, and temperature) in the concrete reservoir were kept constant at any depths. The water was air-saturated by pumping air every day through an air pipe with vent holes installed at the bottom of the reservoir, ensuring the adequate and uniform supply of oxygen and carbon dioxide at various water depths. The temperature of the whole water body was kept constant by applying an electric heating system equipped at the bottom of the reservoir. The reservoir was covered with black sun-shading nets to eliminate the influence of light. The control treatment was conducted in darkness under the same temperature as the submergence treatments. DO concentration, photosynthetically active radiation (PAR) intensity, temperature, and pH of the water column at different depths in the reservoir were checked twice per day (morning and evening) using a multi-parameter water quality analyzer (Hydrolab DS5, Hach, United States). During the experiment, no significant difference in these factors was found among different water depths (**Table 1**).

Growth Measurements

Each plant had ∼12 stem internodes at the start of treatments. From the stem base upwards, the 1st to 6th internodes were mature and the 7th to 12th internodes were immature (**Figure 1B**). We marked non-destructively immature internodes so as to distinguish between the mature, immature internodes formed before treatment and the new internodes produced during treatment. At the beginning of treatments, for each plant, 26 leaves were retained on the upper stem by trimming other leaves from the stem base upwards. The length of all internodes of each plant was measured twice (at the beginning and the end of treatments). We compared the elongation of mature, immature, and newly produced internodes at the end of the treatments to assess the effects of submergence depths on the growth of internodes of varying maturity degrees. The elongation of mature internodes, immature internodes, and newly produced internodes was, respectively,

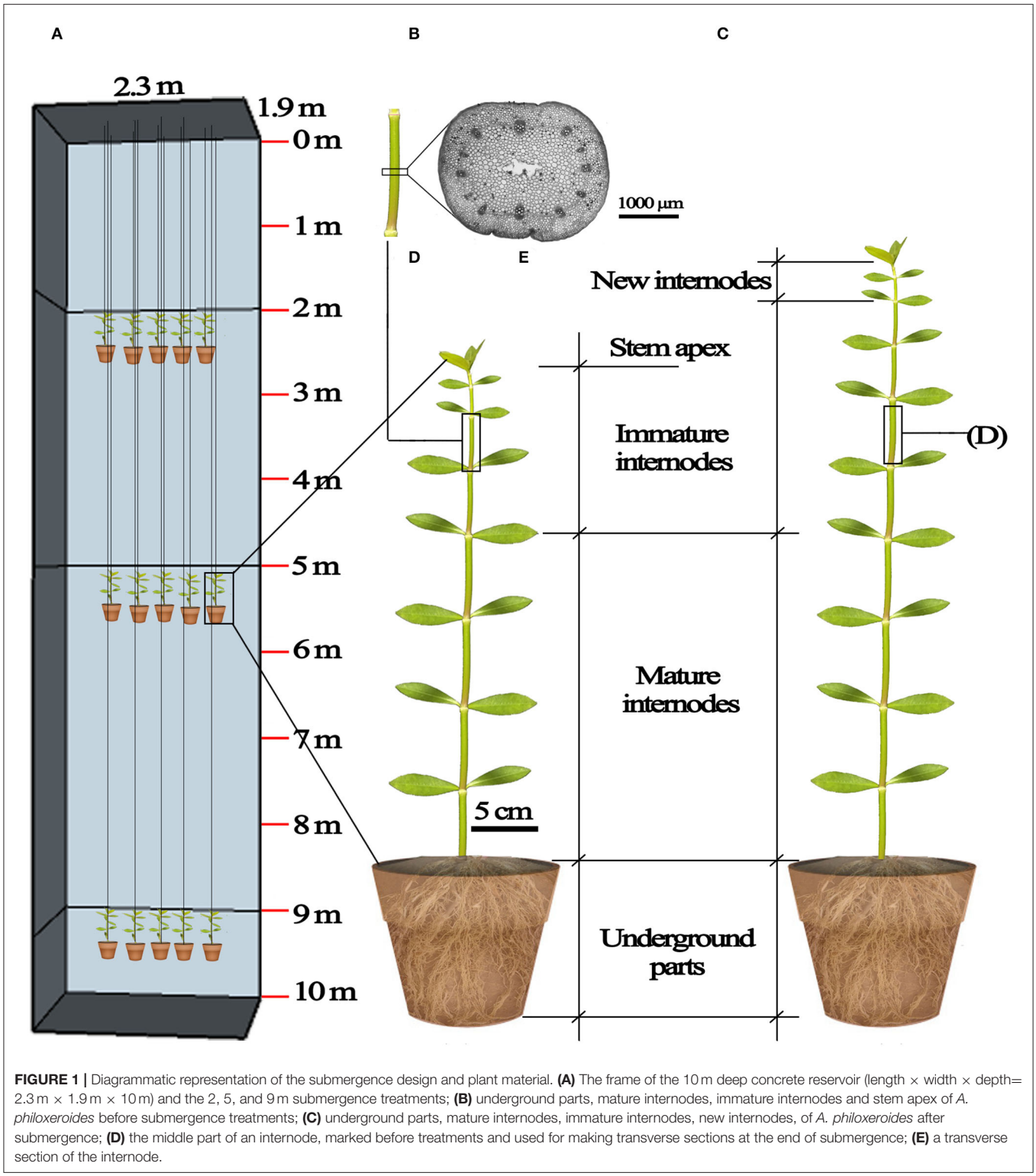

FIGURE 1 | Diagrammatic representation of the submergence design and plant material. **(A)** The frame of the 10 m deep concrete reservoir (length × width × depth= 2.3 m × 1.9 m × 10 m) and the 2, 5, and 9 m submergence treatments; **(B)** underground parts, mature internodes, immature internodes and stem apex of *A. philoxeroides* before submergence treatments; **(C)** underground parts, mature internodes, immature internodes, new internodes, of *A. philoxeroides* after submergence; **(D)** the middle part of an internode, marked before treatments and used for making transverse sections at the end of submergence; **(E)** a transverse section of the internode.

calculated as the sum of elongation from the 1st to 6th internodes, the sum of elongation from the 7th to 12th internodes, and the length sum of all newly produced internodes (**Figure 1C**). The number of newly produced internodes was also counted. Afterward, the leaves, stem (viz. the aboveground stem part), and underground part (including the underground stem part which can be regarded as rhizome, and roots) of each plant were oven-dried to constant weights

TABLE 1 | Physico-chemical properties of water in submergence reservoir.

Submergence depth(m)	Dissolved oxygen concentration (mg L^{-1})	Temperature (°C)	pH	PAR (μmol m^{-2} s^{-1})
0	n.a.	23.49 ± 0.13 a	n.a.	0 a
2	9.21 ± 0.16 a	23.45 ± 0.05 a	7.02 ± 0.02 a	0 a
5	9.19 ± 0.18 a	23.38 ± 0.05 a	7.03 ± 0.01 a	0 a
9	9.12 ± 0.09 a	23.28 ± 0.07 a	7.04 ± 0.00 a	0 a

The dissolved oxygen, temperature, photosynthetically active radiation (PAR), and pH of the water were checked at different depths twice per day (in the morning and evening) using a multi-parameter water quality analyzer (Hydrolab DS5, Hach, USA) during the experiments (mean ± S.E.; n = 13); n.a. indicates no data. Same lower-case letter indicates no significant difference (p > 0.05) between submergence depths.

at 75°C, and their dry mass was determined (BSA124S, Sartorius, Germany).

No adventitious roots were formed on the aboveground stem nodes of *A. philoxeroides* before the submergence treatments. Any formation of aquatic adventitious roots on the aboveground stem nodes was recorded at the end of the treatments.

Transverse Section and Pith Cavity of Internodes

Because the pilot experiment showed that the 10th stem internode had relatively large elongation during treatments (**Supplementary Figure S1**), and its pith cavity had not been formed at the start of the treatments, we marked the 10th internode before treatments to investigate the effects of submergence depths on the development of internodal pith cavity. Transverse sections were made at the middle of the internodes immediately when treatments terminated (**Figures 1D,E**). Sections were observed and photographed using a stereomicroscope (SMZ25, Nikon, Japan). The pith cavity cross-area of each section was measured using the NIS-elements imaging software (version 4.30).

Non-Structural Carbohydrate Analysis

Soluble sugars and starch of the dried stems and underground parts of plants were measured by using fine powder samples prepared with a ball mill (WS-MM200, Retsch, Haan, Germany). Soluble sugars and starch were extracted and determined using a modified method based on the traditional anthrone-sulfuric acid method (Zhang, 2003; Lei et al., 2014). Ethanol-soluble sugars of 0.01 g powder sample soaked in 80% (v/v) ethanol solution were extracted in the water bath at 80°C for 40 min. The extraction of each sample was repeated twice and the extracts were mixed and diluted with ultrapure water to 50 ml. Subsequently, the residue of each sample was soaked in 5 ml ultrapure water and extracted for soluble but ethanol-insoluble sugars in the water bath at 80°C for 40 min, the extraction of each sample was also repeated twice and the extracts were mixed and diluted with ultrapure water to 50 ml. The starch of the residue was hydrolyzed to soluble sugars using 6 mol L^{-1} HCl, the hydrolyte solution was filtered into a flask and diluted with ultrapure water to 100 ml. A total of 1 ml of the above-mentioned 50, 50, and 100 ml diluted extracts was respectively added with 5 ml anthrone-sulfuric acid reagent (0.1 g anthrone dissolved in 100 ml 75% (v/v) sulfuric acid solution) and heated for 10 min at 100°C, and the light absorbance at 625 nm was measured (UV-2700, Shimadzu, Japan) to determine the concentration of ethanol-soluble sugars, soluble but ethanol-insoluble sugars, and starch based on a glucose calibration curve. The concentration of total soluble sugars in a plant sample was the concentration sum of ethanol-soluble sugars plus soluble but ethanol-insoluble sugars. Since the starch was hydrolyzed to soluble sugars, starch concentration was therefore calculated by multiplying the concentration of hydrolytic soluble sugars with a hydrolysis coefficient (0.9). The sum of ethanol-soluble sugars, soluble but ethanol-insoluble sugars, and starch was regarded as non-structural carbohydrates in plant samples.

Data Analyses

The effects of treatments (control and submergence at different depths) on stem and internode elongation, plant and organ biomass, number and length of newly produced internodes, internodal pith cavity cross area, number of nodes forming adventitious roots, and contents of non-structural carbohydrates (soluble sugars and starch) were checked using one-way ANOVA. Logarithm transformation of data was performed to equalize variance if necessary. Differences between treatments were detected using Duncan's multiple range test, and the significance level was set at $p = 0.05$. All the analyses were conducted using SPSS 22 (SPSS Inc., Chicago).

RESULTS

Stem Elongation and new Internode Production

A. philoxeroides plants subjected to four treatments [water depths of 0 m (control), 2, 5, and 9 m] all elongated their stems during the experiment; however, stem elongation significantly decreased with increasing submergence depth after 6 days of treatment (**Figure 2A**). The stem elongation of plants subjected to submergence at a water depth of 0 (control), 2, 5, and 9 was 19.79, 28.56, 23.21, and 11.99 cm, respectively. Plants submerged at water depths of 2 and 5 m presented significantly larger stem elongation than control plants, but plants submerged at water depth of 9 m presented much less stem elongation than control plants (**Figure 2A**). The contribution of mature and immature internodes produced before treatments and new internodes produced during treatment to the stem elongation differed significantly, immature internodes comparatively made the largest contribution to plant stem elongation (**Figure 2B**).

The elongation responses to submergence depths differed between mature, immature, and newly produced internodes.

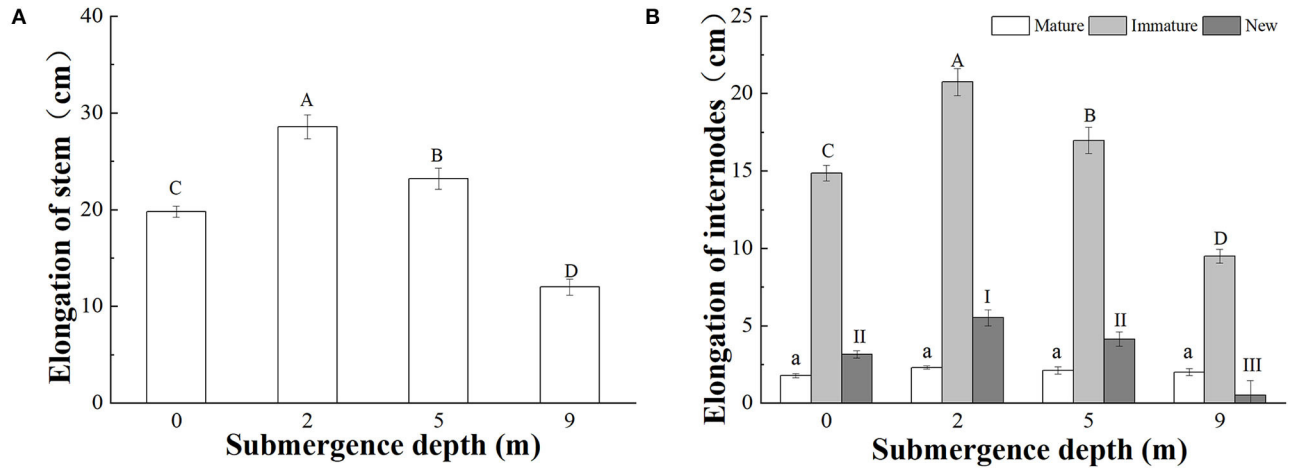

FIGURE 2 | Stem elongation of **(A)** the whole stem; **(B)** mature and immature internodes before the treatments, and new internodes produced during the treatments, of *A. philoxeroides* at the end of treatments at different submergence depths (mean ± S.E; $n = 20$). Different lower-case letters indicate a significant difference ($p < 0.05$) in the elongation of mature internodes between treatments. Different upper-case letters indicate significant differences ($p < 0.05$) in elongation of the whole stem **(A)** and immature internodes **(B)** between treatments. Different roman letters indicate significant differences ($p < 0.05$) in the elongation of new internodes between treatments.

TABLE 2 | Internode numbers at the start and the end of treatments (means ± S.E. $n = 20$).

Submergence depth (m)	Internode numbers		New internode numbers
	Before submergence	After submergence	
0	12.50 ± 0.21a	14.40 ± 0.22a	1.85 ± 0.08b
2	12.30 ± 0.25a	14.60 ± 0.29a	2.40 ± 0.11a
5	12.20 ± 0.25a	14.00 ± 0.25a	1.85 ± 0.13b
9	12.40 ± 0.22a	12.75 ± 0.27b	0.30 ± 0.11c

Different lower-case letters indicate significant differences ($p < 0.05$) between submergence depths.

The total elongation of mature internodes was not affected by submergence depths, but the total elongation of immature internodes and newly produced internodes differed significantly between submergence depths (**Figure 2B**). The total elongation of immature internodes were 14.86, 20.75, 16.95, and 9.48 cm in plants submerged at water depths of 0 (control), 2, 5, and 9 m, respectively (**Figure 2B**).

Very few new internodes were produced during submergence at water depth of 9 m, and 1.85, 2.40, and 1.85 new internodes on average were produced at water depths of 0, 2, and 5 m, respectively (**Table 2**). The total length of new internodes was significantly different between the treatments, with the largest length realized in plants submerged at depth of 2 m (**Figure 2B**).

Based on the results of this study, it was found that the stem elongation and internode production of submerged *A. philoxeroides* decreased with increasing submergence depths, with the largest stem elongation and new internode production showing under the shallowest submergence (2 m depth) and the smallest stem elongation and nearly no new internode production under the deepest submergence (9 m depth). However, in comparison with plants unsubmerged, the stem elongation and internode production of plants were enhanced by submergence at depths of 2 and 5 m.

Internodal Pith Cavity and Adventitious Root Formation

The internodal pith cavity developed during the experiment varied among four treatments (**Figure 3**). Plants submerged at depths of 2 and 9 m had the broadest and the narrowest internodal pith cavity, respectively. Plants submerged at depth of 5 m did not differ from unsubmerged plants in size of internodal pith cavity (**Figure 3**).

The number of stem nodes forming adventitious roots upon submergence decreased significantly with increasing submergence depth (**Figure 4**). Approximately 4–9 nodes (an average of 6.5) on plants submerged at depth of 2 m formed adventitious roots, and 0–5 nodes (an average of 1) on plants at a submergence depth of 5 m formed adventitious roots, only 1 node of 1 plant (20 plants in total) formed adventitious roots when submerged at depth of 9 m (**Figure 4**).

It was obvious that increasing water depth impeded the pith cavity development and adventitious root formation of submerged *A. philoxeroides* plants.

Biomass and Carbohydrates

At the end of the experiment, the total biomass of submerged *A. philoxeroides* plants decreased gradually with increasing submergence depth, however, unsubmerged plants and plants submerged at a depth 2 m did not differ in total biomass (**Figure 5A**). The total leaf mass of plants submerged at a depth 9 m was the smallest among all treatments, but the total leaf mass did not differ between plants submerged at

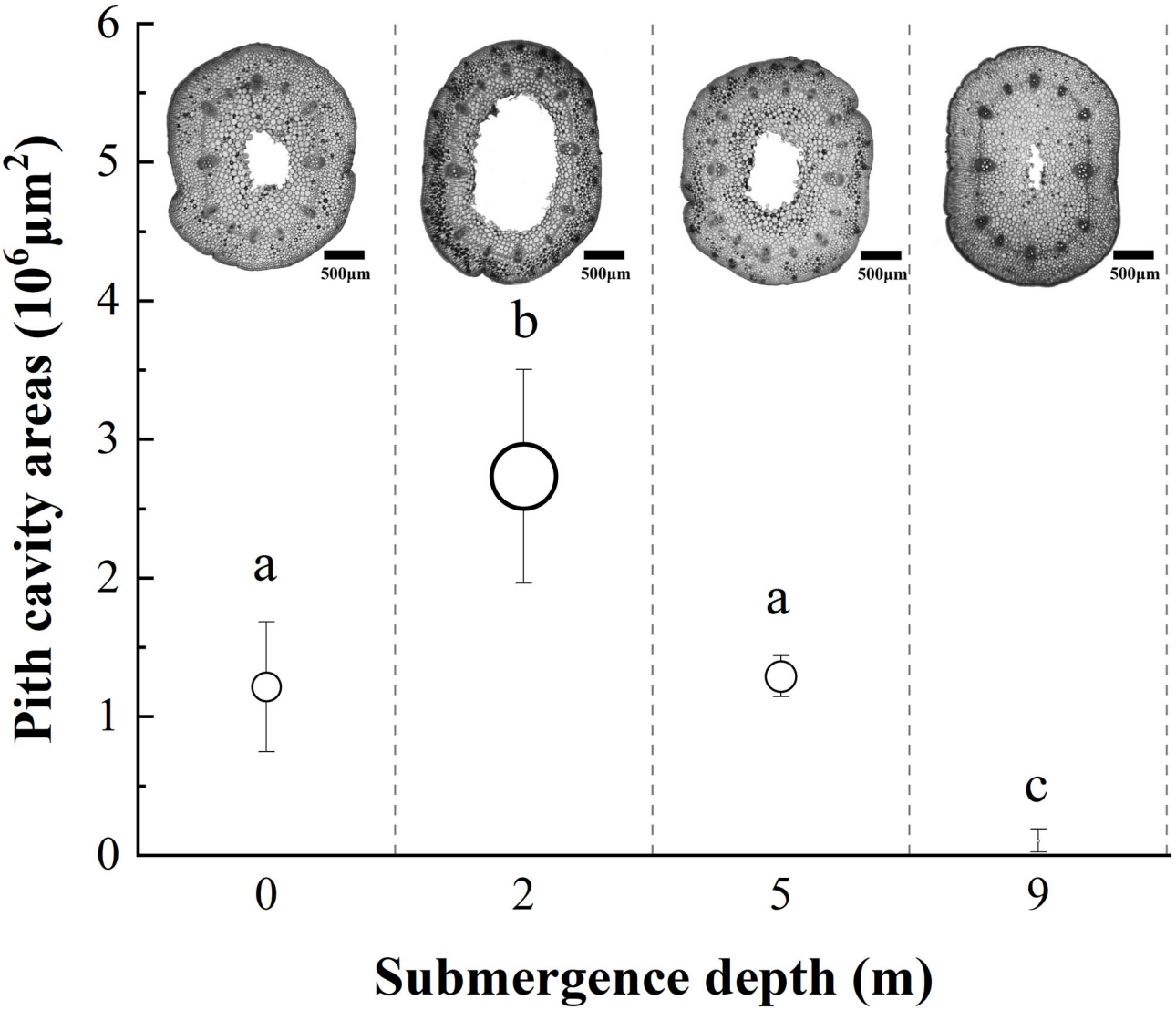

FIGURE 3 | The pith cavity transverse areas of immature internodes of *A. philoxeroides* at the end of treatment at different submergence depths (mean ± S.E; $n = 3$). Sections were made at the middle position of internodes. Different lower-case letters indicate significant differences ($p < 0.05$ using one-way ANOVA) between treatments.

depths of 2 and 5 m, and plants unsubmerged. The largest and the smallest stem mass presented respectively in plants submerged at depths of 2 and 9 m, and plants submerged at depth of 5 m had similar stem mass to control plants (**Figure 5B**). Unlike the responses of stem and total leaf mass to treatments, the underground mass of plants declined with increasing submergence depths, plants unsubmerged tended to achieve the largest underground mass, and plants submerged at a depth 9 m had the smallest underground mass (**Figure 5B**).

The non-structural carbohydrates of *A. philoxeroides* plants were mainly composed of soluble sugars. At the end of the experiment, the concentration of soluble sugars in both stems and underground parts of submerged plants decreased with increasing submergence depth (**Figures 6A,B**). As to the concentration of soluble sugars in stems, unsubmerged plants were not different from plants submerged at depth of 5 m but were lower than plants submerged at depth of 2 m and higher than plants submerged at depth of 9 m (**Figure 6A**). Unsubmerged plants were similar to plants submerged at depth of 2 m in the concentration of soluble sugars in the underground plant parts but had a higher concentration of soluble sugars than plants submerged at depth of 5 and 9 m (**Figure 6B**).

No significant difference was found between treatments in starch concentration in stems of *A. philoxeroides* (**Figure 6C**). But starch concentration in underground plant parts tended to increase with increasing submergence depth, plants submerged at depth of 9 m had the highest concentration of starch (**Figure 6D**).

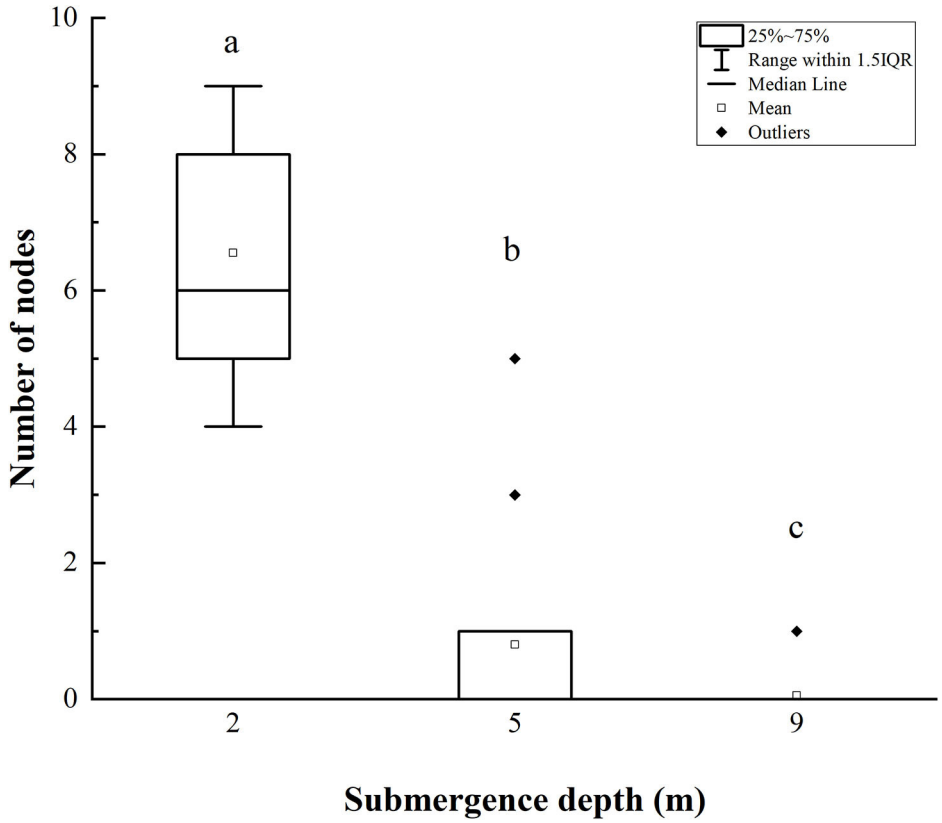

FIGURE 4 | The number of stem nodes forming adventitious roots of *A. philoxeroides* at the end of submergence treatments (mean ± S.E.; $n = 20$). Different lower-case letters indicate significant differences ($p < 0.05$ using one-way ANOVA) between treatments.

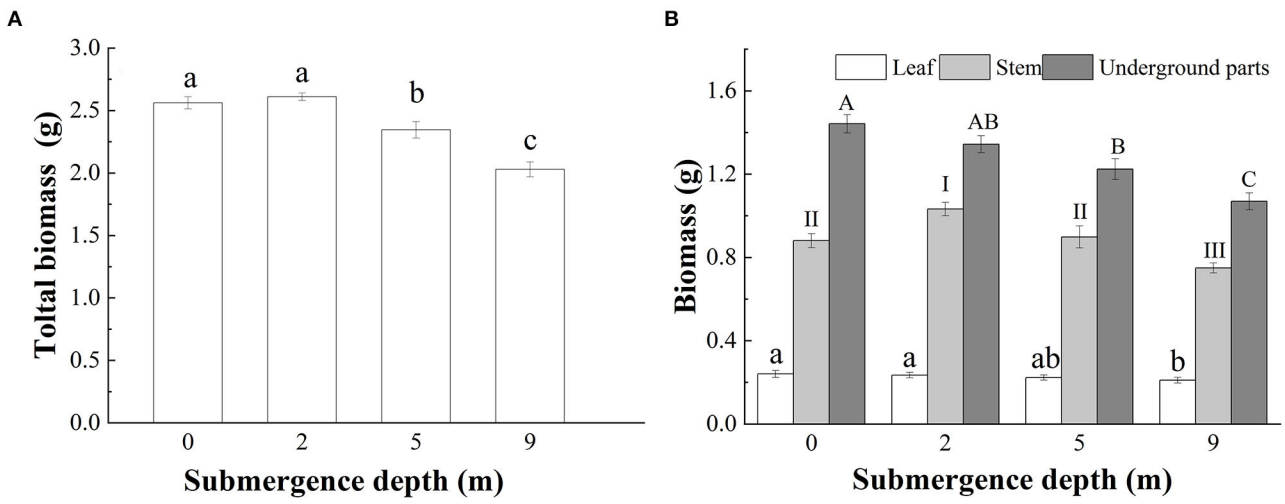

FIGURE 5 | Biomass of **(A)** whole plant; and **(B)** leaves, stems, and underground parts of *A. philoxeroides* at the end of submergence treatments (Mean ± S.E, $n = 20$). Different lower-case letters indicate significant differences ($p < 0.05$) in the total plant mass **(A)** and the leaf mass **(B)** between treatments, different roman letters, and upper-case letters, respectively indicate significant differences ($p < 0.05$) in stem mass and underground plant mass between treatments.

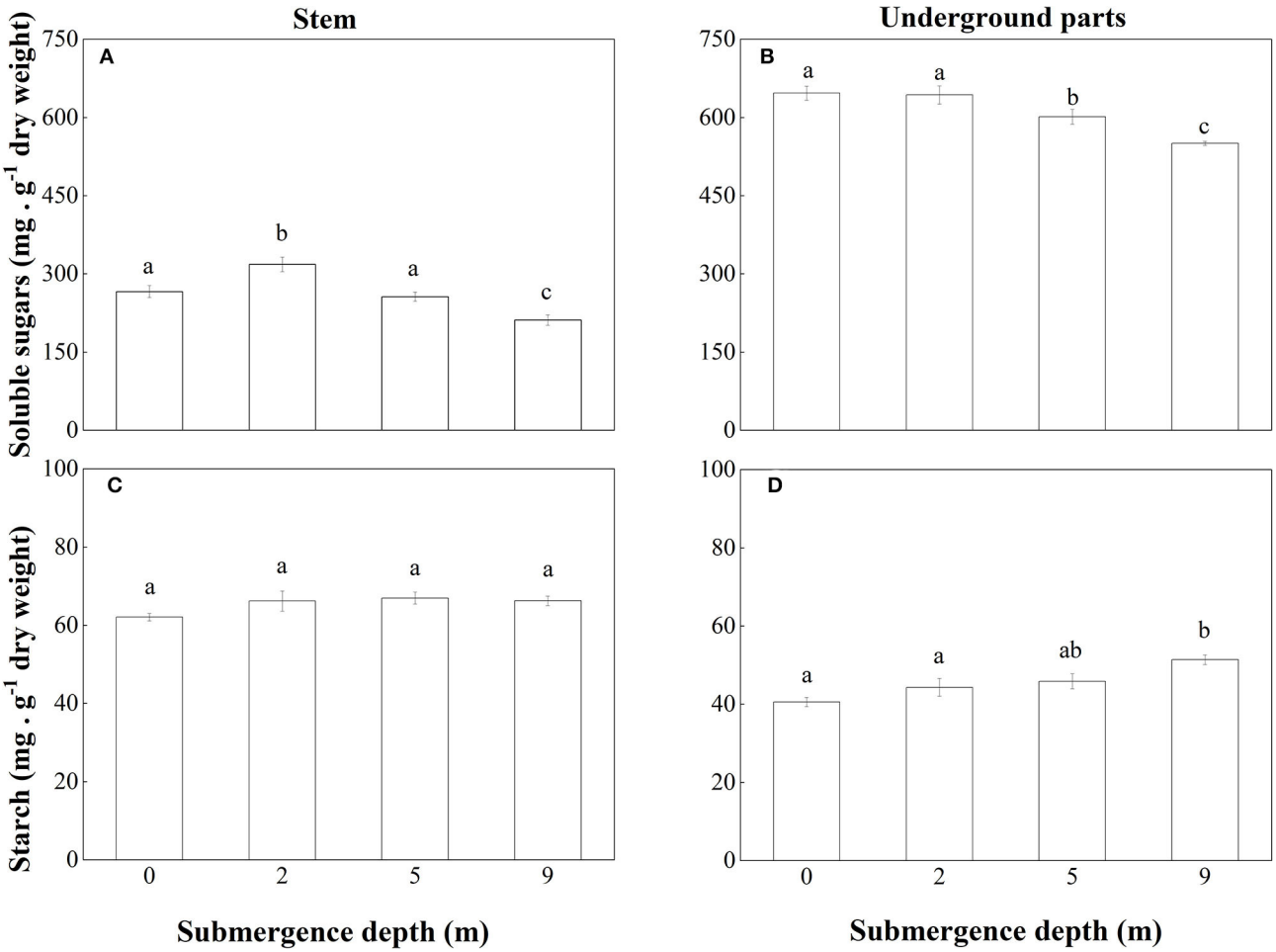

FIGURE 6 | Concentrations of soluble sugars in **(A)** stems and **(B)** underground parts; starch in **(C)** stems and **(D)** underground parts of *A. philoxeroides* at the end of the submergence treatments (Mean ± S.E; $n = 5$). Different lower-case letters indicate significant differences ($p < 0.05$ using one-way ANOVA) between treatments.

DISCUSSION

This study aimed to investigate the responses of terrestrial plants to alteration in submergence depth. In some previous studies, the escape or quiescence strategies selected by submerged plants were extensively discussed (Bailey-Serres and Voesenek, 2008; Voesenek and Bailey-Serres, 2015). The strategy selection by plant species was directly linked to where the species is able to distribute in habitats prone to flooding at various depths (Parolin, 2002). However, whether species having wide distribution in flood-prone habitats can alter their response strategy to cope with various submergence depths is rarely discussed. Using *A. philoxeroides* as a model species, this study demonstrated that strategy change as a response to submergence depths alteration was possible in flood-tolerant plants.

Escape Strategy at Submergence Depth of 2 m

A. philoxeroides exhibited a typical "escape strategy" when submerged at depth of 2 m *via* fast shoot elongation by producing new internodes and elongating its immature internodes (**Figure 2**, **Supplementary Figure S1**, **Table 2**). Although previous studies showed that escape strategy was mainly presented in plants submerged partially or in waters shallower than 2 m depth (Bailey-Serres and Voesenek, 2008; Manzur et al., 2009; Akman et al., 2012), our present work indicated that escape strategy can also be selected by *A. philoxeroides* under submergence depths equal or larger than 2 m, which implies that *A. philoxeroides* can escape from the complete submergence with a water depth of 2 m or even deeper. Theoretically, the fast elongation of stems under submergence at depth of 2 m might be lethal to plants because of the substantial energy consumption and consequent energy crisis. However, our experimental results showed that the biomass and carbohydrate consumption of plants at 2 m submergence depth did not differ from those of unsubmerged plants at the end of the experiment (**Figures 5**, **6**). This might be due to the morpho-physiological behaviors the plant took to help mitigate possible damages, especially energy crisis. First, adventitious root formation was enhanced in *A. philoxeroides* plants submerged at depth of 2 m

(**Figure 4**), which was beneficial for plants to improve the supply of oxygen and mineral nutrients under submergence stress, because adventitious roots are able to absorb oxygen and mineral nutrients from water (Ayi et al., 2016, 2019). Second, the plants submerged at depth of 2 m developed the widest internodal pith cavity (**Figure 3**), which surely facilitated plants' internal gas transport (Jackson and Armstrong, 1999; Pedersen et al., 2021). Therefore, our results indicated that *A. philoxeroides* was able to resist submergence of 2 m depth by adopting an escape strategy *via* stem elongation, pith cavity development, and adventitious roots formation.

Quiescence Strategy at Submergence Depth of 9 m

When submerged at depth of 9 m, *A. philoxeroides* adopted a quiescence strategy by minimizing its growth, including almost no new internode production (**Table 2**) and new internode elongation (**Figure 2**), suppressed pith cavity development (**Figure 3**) and adventitious root formation (**Figure 4**). This quiescence had a "two-edged sword" effect. On one hand, the weakened growth of new tissues and organs led to low consumption of substances including carbohydrates; on the other hand, suppressed pith cavity development and adventitious roots formation decreased the utilization efficiency of carbohydrates under deep submergence conditions. The former would reduce the carbohydrate requirement of submerged plants and enhance their submergence tolerance; the latter may lead to fast carbohydrate consumption, which would be lethal under prolonged submergence. Our results showed that the loss of total biomass in plants submerged at depth of 9 m was significantly higher than that in unsubmerged plants (**Figure 5**), and the concentration of soluble sugars was lower in plants submerged at depth of 9 m than in unsubmerged plants (**Figures 6A,B**). Nevertheless, all plants submerged at depth of 9 m survived with no tissue corruption, indicating the high submergence tolerance of *A. philoxeroides*.

Strategy Shift at Intermediate Submergence Depth

The responses of *A. philoxeroides* to submergence at depth of 5 m indicated that the plant was probably in the process of strategy shift from escape to quiescence. Plants submerged at depth of 5 m presented larger stem elongation than unsubmerged controls (**Figure 2**), which was an obvious sign of escape strategy. However, the reduced adventitious root formation, decreased total plant biomass, and lowered soluble sugars concentration in the underground parts of plants submerged at depth of 5 m (**Figures 4–6**), as compared to those of unsubmerged controls, revealed quiescence strategy was also adopted by plants submerged at depth of 5 m. Thus, we inferred that *A. philoxeroides* may gradually switch its strategy from "escape" to "quiescence" as a response to submergence with increasing depth. It was reported that *Lotus tenuis* could quickly switch from an escape strategy under partial submergence to a quiescence strategy when confronted with complete shallow submergence (Manzur et al., 2009). Obviously, *A. philoxeroides* did not take this quick strategy switchover but performed gradual change in strategy when submergence depth altered.

Previous studies have pointed out that *A. philoxeroides* is a representative species that adopts an escape strategy in response to submergence (Luo et al., 2009, 2011; Ayi et al., 2016). This study supported this argument. However, it was also found in this study that *A. philoxeroides* was able to change its strategy from "escape" to "quiescence" under deep submergence (deeper than 5 m in this study), an intriguing phenomenon that was not observed in previous studies. Based on our experimental results, two questions need to be more focused on in the following studies are raised: (1) do all species that typically adopt escape strategy automatically alter their strategy with increasing submergence depth and (2) what role does the submergence depth play in the strategy switchover?

Role of Hydrostatic Pressure

Low light, whether in terrestrial or in aquatic habitats, is an inducing factor for plants to adopt an escape strategy (via fast shoot and petiole elongation) due to shade avoidance (Mommer et al., 2005; Pierik et al., 2011; Sasidharan et al., 2014). In this study, the stem elongation of *A. philoxeroides* submerged at depths of 0, 2, and 5 m in darkness substantiated this statement (**Table 2**; **Figure 2**); however, the retarded growth of *A. philoxeroides* submerged at depth of 9 m in the same darkness (**Table 2**; **Figure 2**) suggested that the light availability can not sufficiently explain the strategy change along a gradient of submergence depths. If the stem elongation upon submergence were induced by low light or darkness due to phototaxis of plants, plants submerged at any depths in darkness should present similar stem elongation.

Generally, the escape and quiescence strategies represent the syndrome that plants exhibit as a response to low oxygen levels in water (Voesenek and Bailey-Serres, 2013, 2015). Oxygen shortage might induce the production of reactive oxygen species (Paradiso et al., 2016; Sasidharan et al., 2018), nitric oxide (Mugnai et al., 2012; Paradiso et al., 2016), ethylene (Fukao and Bailey-Serres, 2008; Sasidharan et al., 2018), and other signaling molecules (Sasidharan et al., 2018), as well as activate a series of phytohormone-synthesizing molecules (Cox et al., 2004) to induce different strategies among species. In plant species adopting an escape strategy, the low oxygen level not only stimulates the elongation of shoots and petioles, but also induces aerenchyma formation, *via* increasing porosity in the roots or stem (Parlanti et al., 2011; Pedersen et al., 2021), widening the pith cavity (Steffens et al., 2011), and forming adventitious roots (Zhang et al., 2015b; Ayi et al., 2016). However, in a water body with dissolved oxygen saturated (like the water environment *A. philoxeroides* experienced in this study), it is hard to attribute the strategy shift of plants submerged at different water depths to oxygen availability.

In our experimental system, all factors except water depth were kept constant (**Table 1**). Therefore, it is logical to infer that water depth was linked to the strategy changeover of *A. philoxeroides*. It was found in previous studies that water depth affected plant distribution *via* hydrostatic pressure (Dale, 1981;

Makarov, 2011). Some studies suggested that low hydrostatic pressure could accelerate physiological activities and stimulate plant growth, whereas high hydrostatic pressure may affect the stabilization of cells or enzymes, depress physiological activities, and impede cell division or elongation (Adkins et al., 1990; Vervuren et al., 2003; Yi et al., 2016; Bejarano et al., 2018). The strategy changeover of *A. philoxeroides* along submergence depth gradient in this study was very probably caused by the change in hydrostatic pressure, but how hydrostatic pressure induces strategy changeover needs to be clarified.

CONCLUSION

This study revealed that submerged *A. philoxeroides* can gradually change its response strategy from "escape strategy" to "quiescence strategy" when water depth was increasing. Notably, this changeover took place under deep submergence (deeper than 5 m), which implies that water depth plays an important role in the strategy selection of plants in response to submergence. According to our observations, we found the morphological responses of *A. philoxeroides* to submergence of different depths were chiefly the outcome of acclimatization, because the plants presenting strong stem elongation under shallow submergence did not exhibit stem elongation when transferred to deep submergence, and plants presenting no stem elongation under deep submergence can still show strong stem elongation upon shallow submergence. Undoubtedly, this acclimatization enhances greatly the capability of *A. philoxeroides* in coping with floods with unpredictable depths. Further studies should focus on understanding the driving mechanism of strategy changeover of plants submerged at different water depths, especially the driving effects of hydrostatic pressure.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BZ and XZ conceived the original research plan, designed, and supervised the experiments. SJ and HN performed most of the experiments. BW, XR, XS, and SS provided assistance for some experiments. SJ, XZ, QA, and BZ analyzed the data and wrote the article with contributions from SL and FL. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Grant Numbers 31400480, 31800331, and 31770465) and Chongqing Talents Program (Grant Number cstc2021ycjh-bgzxm0316).

ACKNOWLEDGMENTS

The authors thank Xingrong Wu, Senrui Zhang, Dongdong Ji, Meng Wang, Jine Huang, Zhao Zhang, and all the people who helped with the fieldwork over the course of the experiments. We thank Xiaoqi Ye for helping amend the article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.883800/full#supplementary-material

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ORIGINAL RESEARCH
published: 17 June 2022
doi: 10.3389/fpls.2022.888391

Epigenetic and Phenotypic Responses to Experimental Climate Change of Native and Invasive *Carpobrotus edulis*

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OPEN ACCESS

Edited by:
Wei Xue,
Taizhou University, China

Reviewed by:
Fang-Li Luo,
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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: *02 March 2022*
Accepted: *28 April 2022*
Published: *17 June 2022*

Citation:
Campoy JG, Sobral M, Carro B, Lema M, Barreiro R and Retuerto R (2022) Epigenetic and Phenotypic Responses to Experimental Climate Change of Native and Invasive Carpobrotus edulis. Front. Plant Sci. 13:888391. doi: 10.3389/fpls.2022.888391

Despite the recent discoveries on how DNA methylation could help plants to adapt to changing environments, the relationship between epigenetics and climate change or invasion in new areas is still poorly known. Here, we investigated, through a field experiment, how the new expected climate scenarios for Southern Europe, i.e., increased temperature and decreased rainfall, might affect global DNA methylation in relation to phenotypic variation in individuals of clonal plant, *Carpobrotus edulis*, from its native (Southern African) and invaded (northwestern Iberian Peninsula) area. Our results showed that changes in temperature and rainfall induced phenotypic but not global DNA methylation differences among plants, and the climatic effects were similar for plants coming from the native or invaded areas. The individuals from the Iberian Peninsula showed higher levels of global methylation than their native counterparts from South Africa. We also observed differences between natives and invasive phenotypes in traits related to the pattern of biomass partitioning and to the strategies for water uptake and use and found an epigenetic contribution to phenotypic changes in some leaf traits, especially on the nitrogen isotopic composition. We conclude that the increased temperature and decreased rainfall projected for Southern Europe during the course of the twenty-first century may foster phenotypic changes in *C. edulis*, possibly endowing this species with a higher ability to successful cope the rapid environmental shifts. The epigenetic and phenotypic divergence that we observed between native and invasive plants suggests an intraspecific functional variation during the process of invasion. This result could indicate that phenotypic plasticity and global DNA methylation are related to the colonization of new habitats. Our findings reinforce the importance of epigenetic plasticity on rapid adaptation of invasive clonal plants.

Keywords: Aizoaceae, adaptation, DNA methylation, environmental change, ice plant, invasive species, phenotype, trait

INTRODUCTION

Invasion of natural areas by exotic species is a major cause of biodiversity loss (Mack et al., 2000; McGeoch et al., 2010), a situation that could be particularly aggravated by the ongoing climate change (Walther et al., 2009; Bellard et al., 2013; IPCC, 2014). It is therefore essential to increase our understanding of the mechanisms that underlie the rapid responses of invasive plants to environmental shifts to efficiently manage current and future invasions.

The mechanisms that have been attributed to the huge success of some clonal plant invaders include an effective ecophysiological adaptation to the new environment and major genomic events, such as hybridization and polyploidization. These processes may lead to different patterns of gene expression re-programming and epigenetic modifications, which, in turn, can contribute to phenotypic novelty and plasticity (Verhoeven and Preite, 2014; Fenollosa et al., 2016; Richards et al., 2017; Mounger et al., 2021). Phenotypic plasticity could be especially advantageous for sessile organisms such as plants in a rapidly changing climate by allowing a genotype to express different phenotypes under the influence of different environments (Bradshaw, 1965; Nicotra et al., 2010; Henn et al., 2018). Likewise, it is well established that genetic variation can interact with the environment to alter phenotype and that this interaction may be determinant for evolutionary changes and adaptation (Goldstein and Ehrenreich, 2021). However, current evidence also supports that, even in the absence of genetic variation, phenotypic variation can be triggered by epigenetic modifications, such as DNA methylation (Bossdorf et al., 2010; Verhoeven et al., 2010). This is because epigenetic changes of the genome can alter gene expression and affect how the genotype translates into the phenotype without changing the DNA sequence (Jablonka and Raz, 2009; Verhoeven et al., 2010; Erdmann and Picard, 2020). These epigenetic changes can be related to a variety of ecologically relevant traits (e.g., Bossdorf et al., 2010) that may be inherited to some extent by future generations (e.g., Zhang et al., 2013; Sobral et al., 2021a, 2021b). Thereby, epigenetic mechanisms can contribute to plant adaptation (Schmid et al., 2018) and may provide an alternative source of phenotypic and functional diversity (e.g., Medrano et al., 2014) which could be especially important for clonal plants and plant invaders with low genetic diversity (Verhoeven and Preite, 2014; Mounger et al., 2021).

Furthermore, epigenetic variation may be altered by ecological interactions, providing thereby an additional pathway for evolutionary change (Bossdorf et al., 2008). Plants can alter their epigenetic marks to adjust their responses to abiotic factors such as temperature, drought, salt and nutrient stress (Verhoeven et al., 2010; Nicotra et al., 2015; González et al., 2017), elevated atmospheric CO_2 concentration (Saban et al., 2020), or heavy metals (Shafiq et al., 2019). It has also been demonstrated that epigenetic mechanisms may be relevant for biotic interactions (see Alonso et al., 2019 for a review), for adaptation to different environments and habitat conditions (Lira-Medeiros et al., 2010; Richards et al., 2012; Foust et al., 2016), for phenotypic variation between introduced and native populations (Banerjee et al., 2019, and references therein), and for helping plants to colonize new habitats (Zhang et al., 2016; Liu et al., 2018). Thereby, because epigenetic variation may contribute to fast plant adaptation to novel environments, this process may be an important mechanism for invasive success. However, despite these recent discoveries on epigenetic regulation in plants, the relationship between epigenetics and responses to climate change or invasion of new areas is still poorly known. Progress on this matter requires research combining the study of plant epigenetic variation and their ecologically relevant phenotypic effects over a range of environmental conditions and regions of origin.

Alien invasive species, such as *Carpobrotus edulis* (L.) N.E. Br., native to the South African, often face sudden environmental changes when they colonize new territories. This offers a good opportunity to examine how these species cope with novel environments and whether this process influences the phenotype and/or the epigenotype. In this study, we used a set of ecophysiological measurements and methylation-sensitive amplified fragment length polymorphism markers (MSAP) to assess how the increased temperature and decreased rainfall predicted for Southern Europe affect global DNA methylation in relation to the phenotypic variation of *C. edulis*. To this end, and considering the temperature and rainfall projections for Southern Europe over the twenty-first century (IPCC, 2014; EEA, 2017), we studied the responses of *C. edulis* individuals from the native (South Africa) and the invaded region (northwestern Iberian Peninsula) to simulated climatic scenarios in a field experiment.

Specifically, in this work we addressed two questions: (1) might the increased temperature and decreased rainfall predicted for Southern Europe induce phenotypic and epigenetic changes in *C. edulis?* In a previous research we showed that the traits considered in this study have functional consequences for the responses of *C. edulis* to climate change (Campoy et al., 2021). Moreover, because epigenetic regulation may be an important source of phenotypic plasticity and thus can contribute to increase the existing phenotypic and functional biodiversity, we expect that the new climate scenarios will foster rapid phenotypic and epigenetic changes in this species. (2) Are there phenotypic and epigenetic differences among *C. edulis* from the native and invaded areas*?* Considering the potential role of epigenetics in plant invasion (Mounger et al., 2021), we expect high phenotypic and epigenetic plasticity in individuals from the invaded regions.

MATERIALS AND METHODS

Study Plant

Carpobrotus edulis (L.) N.E. Br. (Aizoaceae) is a succulent perennial plant native from Southern Africa (Wisura and Glen, 1993). It was introduced in the five worldwide Mediterranean-type ecosystems more than 100 years ago, and since then, this species has become invasive in many coastal habitats (review by Campoy et al., 2018). The combination of sexual and asexual (clonal) reproduction in *C. edulis*, together with the high hybridization potential of the species (Vilà and D'Antonio, 1998; Suehs et al., 2004), explains its worldwide successful propagation.

The high plant invasiveness under a wide range of stressful conditions (e.g., drought, warming, or strong irradiance) has also been related to a high morphological and ecophysiological plasticity in growth, biochemical, and physiological traits (Campoy et al., 2017, 2021; Fenollosa et al., 2017) and to the ability of the species to use and modify soil resources (e.g., Fenollosa et al., 2016; Vieites-Blanco and González-Prieto, 2018).

Sampled Sites and Experimental Design

The experimental plant material was sampled between January and April 2015 from eight sites, four from the native area (Cape Region, South Africa) and four from the invaded area (northwest of the Iberian Peninsula, Southern Europe; **Supplementary Table 1**). To have a more comprehensive illustration of the variability in each region, we collected, within each site, plants separated at least 25 m from the others. After collection, plants were transported in polystyrene trays from the original sites to the glasshouse of the University of Santiago. Then, plants were transplanted into individual 2.5-L pots filled with dune sand until the beginning of the experiment. To prevent hydric stress, plants were watered according to their requirements (approximately once or twice per week).

The study was conducted on a permanent field plot (21 m long × 12 m wide) located over a secondary dune (IGME, 2014) on the island of Sálvora (42°28′44″N, 9°0′34″W; northwest Iberian Peninsula; **Supplementary Figure 1**). Inside this plot, we experimentally manipulated the climate in 32 subplots of 1.27 m^2 to establish a full factorial experimental design with three factors: region of plant origin (native vs. invaded), temperature (control vs. increased), and rainfall (control vs. reduced). To modify temperature conditions, we installed methacrylate open top chambers (OTCs) as those typically employed in warming experiments (e.g., Hollister and Webber, 2000; Yahdjian and Sala, 2002; Maestre et al., 2013), in eight of the subplots. Throughout the study period (September 2015–November 2016), the OTCs increased the air temperature by 2.0°C, on average (Campoy et al., 2021), a realistic scenario for the study area according to the predictions of the PROMES regional atmospheric model (Castro et al., 1993). To achieved the rainfall reduction predicted in the study area (~33%), we placed methacrylate rainfall collectors as those commonly used in manipulative climatic experiments (e.g., Yahdjian and Sala, 2002), in another eight subplots. To examine the combined effects of increased temperature and reduced rainfall, we installed an OTC below the rainfall collectors in eight other subplots. Finally, the eight remaining subplots were not modified to represent the current climatic conditions (C). The island's climate is "Mediterranean sub-humid of Atlantic tendency" (Allué, 1966) or "Warm temperate, with dry and warm summers" (*Csb*), under the Köppen–Geiger climate classification (Kottek et al., 2006). The mean annual rainfall is 1,193 L/m^2 and the mean temperature of the warmest (August) and the coldest (December) month is 20°C and 10°C, respectively.[1] For a more detailed information of experimental chambers and the different sensors used to monitor the climatic conditions in the field plot (see Campoy et al., 2021).

[1]www.meteogalicia.es

In September 2015, we carefully removed the natural vegetation growing in the 32 subplots, where we transplanted into the soil a total of 64 plants, eight from each of the sites of the invaded and the native area. Specifically, we haphazardly assigned two plants to each subplot: one from the invaded area and one from the native area. Thus, 8 plants per region (two per site) were grown under each of the four climatic treatment (eight subplots per treatment). Because we lost two replicates during the progress of the experiment due to non-demonic intrusion (*sensu* Hurlbert, 1984), the number of individuals considered in the final design was reduced to 48 plants (24 from the native and 24 from the invaded regions), i.e., six replicates for each treatment combination. These replicates came from the four sites in their respective region, so all sites were included in our final design, although they were not equally represented.

Phenotypic Traits

To provide an estimate of the radiation use efficiency and the photosynthetic pigment contents of leaves, we measured the reflectance spectra (from 300 to 1,000 nm) in all plants at intervals of ~2 months (between November 2015 and November 2016), with a portable spectrometer (Unispec, PP Systems Haverhill, MA, United States). Reflectance data were processed using AVICOL v.6 software (Gomez, 2006), and the structural independent pigment index [SIPI = $(R_{800} - R_{445})/(R_{800} - R_{680})$] and the photochemical reflectance index [$PRI_{531} = (R_{531} - R_{570})/(R_{570} + R_{531})$] were calculated. The former provides a semi-empirical estimation of the carotenoid-to chlorophyll *a* ratio (Peñuelas et al., 1995b), and the latter is directly correlated with radiation use efficiency (RUE, mol CO_2• mol^{-1} photons; Gamon et al., 1992; Peñuelas et al., 1995a) and inversely correlated with the dissipation of excess radiation energy as heat (Guo and Trotter, 2004). Due to the repeated measure nature of leaf reflectance data (SIPI and PRI indices), for this study we calculated the mean values of the eight set of measurements performed throughout the 14 months of the duration of the experiment.

To determine the proportion of biomass allocated to roots, we used the root-to-shoot ratio (RSR), calculated as RSR = root dry mass/shoot dry mass. To assess plant growth under the experimental conditions, we calculated the relative growth rate (RGR) as follows, RGR = [ln (dry weight t_2) − ln (dry weight t_1)]/($t_2 - t_1$), where t_2 and t_1 are the final time and the initial time, respectively (Villar et al., 2004).

For determination of C and N percentages of dry mass (C/N ratio) and the molar $^{15}N/^{14}N$ ($\delta^{15}N$) and $^{13}C/^{12}C$ ($\delta^{13}C$) ratios, a composite sample of leaves (~2–3 mg dry wt.) from the three apical-most ramets in each plant was ground in a ball mill (Mixer Mill 400 Retsch GMBH, Haan, Germany) and then analyzed at the Research Support Services of the University of A Coruña (Spain). Carbon and N isotope ratios were expressed relative to the composition of a standard (Pee Dee belemnite [PDB] $CaCO_3$ for C, and atmospheric N for N). The δ values (‰) were calculated as $[(R_{sam}/R_{std}) - 1] \times 1{,}000$, where R refers to the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio in the plant sample and standard, respectively. Polyethylene (International

Atomic Energy Agency [IAEA C6]) and $(NH_4)_2SO_4$ (IAEA N1) were used as secondary international isotope standards for C and N, respectively. The $\delta^{13}C$ values were transformed into $\Delta^{13}C$ values by using the following expression: $\Delta^{13}C = (\delta^{13}C_{air} - \delta^{13}C_{plant})/(1 + \delta^{13}C_{plant})$, assuming a $\delta^{13}C$ air value of −8‰ on the PDB scale (Farquhar et al., 1989). Plant $^{13}C/^{12}C$ and the $^{15}N/^{14}N$ isotopic signatures provide valuable and integrated information on the water use efficiency (WUE), and the source, absorption, and assimilation of nitrogen, respectively (Robinson, 2001; Dawson et al., 2002).

Epigenetic Analyses

For epigenetic analyses and for testing its reproducibility, we collected two opposite, fully developed, and healthy leaves from each plant ($n = 48$). To guarantee that all leaves had a similar development stage, samples were taken from the apical-most ramets of the main shoots (stolons).

DNA Extraction and MSAP Reactions

From each plant, we extracted the total genomic DNA from 20 mg fresh leaf tissue using the Maxwell® 16 LEV Plant DNA extraction Kit (PROMEGA) following manufacturer instructions. To avoid cross-contamination, each leaf was dissected using disposable tools and/or flame-sterilized material. The integrity of extracted DNA was verified by electrophoresis on 1.5% agarose gels. After DNA quantification using a Tecan Genios Microplate reader and the Quant-iT dsDNA Assay Kit, High-Sensitivity (HS; Thermo Fisher Scientific), samples were normalized to 5 ng·µl^{-1} and stored at −20°C.

The methylation-sensitive amplified polymorphism (MSAP) reactions were performed following the general steps described by (Vos et al., 1995) for the Amplified Fragment Length Polymorphism (AFLP), with some modifications. We used the same *Eco*RI endonuclease as rare cutter and replaced the frequent cutter *Mse*I in two parallel runs by the methylation-sensitive restriction enzymes *Hpa*II and *Msp*I. The two isoschizomers recognize and cleave the same tetranucleotide sequence 5-CCGG but differ in their sensitivity to the methylation state of cytosine (Pérez-Figueroa, 2013; Schulz et al., 2013).

Briefly, the MSAP protocol was performed as follows. For each sample, 10 µl of genomic DNA (*ca.* 50 ng) was restricted in a total volume of 20 µl containing 10X CutSmart Buffer (BioLabs), 2.5 units of *EcoRI* (BioLabs), and 2.5 units of *Hpa*II or *Msp*I (BioLabs) for 3 h at 37°C and 20 min at 70°C. After incubation, 20 µl of digested fragments was added, in parallel reactions, to 6 µl of a ligation solution containing 0.1 µM *EcoRI*-adapters (Eurofins MWG Operon), and 1 µM *Hpa*II/*Msp*I-adapters (Macrogen; see **Supplementary Table 2** for adaptor sequences), 0.52 units of T4 DNA ligase (Fermentas) and 10X ligation buffer (Fermentas). The ligation was carried out for 3 h at 37°C. Ligation products were diluted 10-fold in Milli-Q H_2O (Millipore Co.), and 10 µl was used for a pre-selective amplification with 0.3 µM *EcoRI*-primer (Macrogen), 0.3 µM *HpaII*/*MspI*-primers (Macrogen; see **Supplementary Table 2** for primer sequences), 2.5 mM $MgCl_2$, 10X PCR buffer, 0.04 µgµl^{-1} BSA, 0.2 µM dNTPs, and 0.4 U of *AmpliTaq Gold* polymerase (Applied Biosystems) in a final volume of 20 µl. The thermocycler protocol was 2 min at 72°C, 2 min at 94°C, 20 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C, followed by a final extension of 30 min at 60°C. Two microliters from the 8-fold diluted products of the pre-amplification was finally used for selective amplifications with 0.6 µM of each *Eco*RI, *Hpa*II/*Msp*I selective primers (Macrogen), 0.8 µM dNTPs, 2.5 mM $MgCl_2$, 0.04µgµl^{-1} BSA, 10X PCR buffer, and 0.4 units of *HotStartTaq Gold* polymerase (Applied Biosystems) in a final volume of 10 µl. Thermocycler conditions for selective amplification were: 4 min at 95°C, 12 cycles of 30 s at 94°C, 30 s at 65°C (first cycle, then decreasing 0.7°C for each one of the last 11 cycles), and 2 min at 72°C; 26 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C, followed by a final extension of 30 min at 72°C. Preamplification was performed immediately after ligation, whereas the products of the other reactions were kept overnight at −20°C. PCRs were performed in a Hybaid thermocycler model PxE (Thermo Fisher Scientific Inc., Waltham, MA, United States). Reactants were always mixed in a laminar flow cabin; DNA and PCR product solutions were always added using filter tips to minimize the risk of cross-contamination.

After an initial screening of 28 primer combinations, we choose six selective primer combinations (1: *Eco*RI + AA/ *Hpa*II/*Msp*I + AC; 2: *Eco*RI + AT/ *Hpa*II/*Msp*I + AA; 3: *Eco*RI + AT/ *Hpa*II/*Msp*I + AC; 4: *Eco*RI + TA/ *Hpa*II/*Msp*I + AA; 5. *Eco*RI + TC/ *Hpa*II/*Msp*I + AT; 6. *Eco*RI + TG/*Hpa*II/*Msp*I + AC; see **Supplementary Table 3** for sequences). The 5′ end of the selective *E*-primers was labeled with FAM, HEX, or NED fluorochromes. PCR fragments were separated on an ABI 3130xl Genetic Analyzer (Applied Biosystems Foster City, United States) with automated DNA (Applied Biosystems) sequencer with HD-500 as size standard (Applied Biosystems). The selected paired primer combinations for MSAP analyses yielding the highest levels of polymorphism were tested again on new, independent DNA extractions of 48 replicate samples (100%) to assess reproducibility. The overall scoring error rate (3.89%; see also **Supplementary Table 3** for scoring error rate by primer combination) was consistent with former studies (e.g., Herrera and Bazaga, 2010).

Fingerprint patterns of MSAP profiles were processed with the software GeneMarker v1.70 (Softgenetics LLC, State College, PA, United States). The scoring was blindly done by the same person (JGC) following common recommendations for AFLP markers (Whitlock et al., 2008), with minor modifications. Peak height data were exported and for each fragment a specific peak height threshold was manually determined based on the peak height distribution which allowed scoring presence (1) and absence (0) of the fragments.

MSAP Scoring

To determine the DNA methylation status of every locus from the presence/absence scores of both *Eco*RI-*Msp*I and *Hpa*II/*Msp*I reactions, we used the R script *MSAP_calc* provided by (Schulz et al., 2013), following the methylation transformation scheme described by (Herrera and Bazaga, 2010). Under this approach, for every individual and particular fragment, it was first determined whether the fragment was: (1) present in both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I profiles, indicating a non-methylated

state (condition I, pattern 1/1); (2) present only in either *Eco*RI/*Msp*I or in *Eco*RI/*Hpa*II profiles, denoting, respectively, a methylated state of ^{HMe}CG- or ^{Me}CG-sites (condition II, pattern 0/1) or a methylated state of ^{HMe}CCG-sites (condition III, pattern 1/0); and (3) absent from both *Eco*RI/*Msp*I profiles (condition IV, pattern 0/0), representing an uninformative state as it can have multiple and equivocal reasons (Pérez-Figueroa, 2013; Schulz et al., 2013). Methylation-susceptible fragments were then scored as 0, for the non-methylated state (condition I); 1, for the methylated state (conditions II and III); and unknown (i.e., score missing) for uninformative condition IV (Herrera and Bazaga, 2010; Morán and Pérez-Figueroa, 2011; Schulz et al., 2013). For the purpose of this study, we only used the "methylation-susceptible loci" obtained with a fixed threshold of 5% as performed by (Schulz et al., 2013). Based on the selecting scoring strategy (Herrera and Bazaga, 2010), we obtained a total of 223 fragments (**Supplementary Table 3**), from which we calculated the percentage of global DNA methylation.

Statistical Analyses

The effect of treatments on global DNA methylation was analyzed using a linear mixed model (LMM) with region of origin (native vs. invaded), temperature (control vs. increased temperature), and rainfall (control vs. reduced rainfall) as fixed factors, and the sampling sites of the populations as a random effect nested within region of origin.

To analyze the effect of treatments and global DNA methylation on the phenotype of *C. edulis* plants from both native and invaded regions, we first summarized the seven selected phenotypic traits (RGR, RSR, C/N, $\Delta^{13}C$, $\delta^{15}N$, SIPI, and PRI_{531} indices) using principal component analysis. The selection of phenotypic traits was based on previous findings on the ecophysiological responses of native and invasive genotypes of *C. edulis* to climate change (Campoy et al., 2021). The first three principal components, jointly explaining approx. 70% of the variation, were included in the following analyses as response variables in three different LMM models to test the effects of region, temperature, rainfall, and global DNA methylation, on these three principal components. Site nested within region was also included as a random effect in these models. We performed Pearson correlations to assess to which extent each principal component was linearly related to global DNA methylation. All variables fitted a normal distribution, and no transformations were required. LMM parameters were estimated using a restricted maximum likelihood (REML) approach. To examine the effects of treatments on variables, *p*-values were estimated using Satterthwaite degrees of freedom. Following the AICc criterion, interaction terms were included or excluded in the models when appropriate (Burnham and Anderson, 2002). Statistical analyses were made in IBM SPSS Statistics v25. A p-value ≤ 0.05 was considered as statistically significant.

Finally, to analyze the simultaneous relationships of region of plant origin, rainfall, and temperature, with global DNA methylation and the phenotype (represented by the three principal components of the PCA), we performed structural equation models (SEM). For the SEM analyses, all variables were standardized, and tested models were based on our previous knowledge of the species (Campoy et al., 2021). Different models (see **Supplementary Figures 2, 3** for some examples) were analyzed. Model selection was made by testing the goodness of fit of the models using the means of maximum likelihood estimation on the variance–covariance matrix. A non-significant goodness of fit test indicates that the model is a good description of the observed covariance among the variables (Grace, 2006). Structural equation modeling was performed with SEPATH procedure in STATISTICA (StatSoft Inc., 2011).

RESULTS

Differences in Methylation Between *Carpobrotus edulis* From the Native and Invaded Areas

The *C. edulis* plants from the native and the invaded region differed in their global DNA methylation, which was significantly higher in plants from the invaded region (**Table 1**; **Figure 1A**). We did not detect any significant effect of temperature or rainfall treatments on the global DNA methylation (**Table 1**; **Figures 1E,I**).

Phenotypic Variation of *Carpobrotus edulis* in Function of Plant Origin, Climatic Treatment, and Global DNA Methylation

Seventy percent of the total variation of the seven phenotypic traits measured on *C. edulis* plants was explained by the first three principal components, which accounted for 35.1%, 21.2%, and 14.5% of the total variance, respectively (**Supplementary Table 4**).

Results of LMM showed that temperature significantly decreased the PC 1 values (**Table 2**; **Figure 1F**). We did not detect significant effects of rainfall on PC1 (**Table 2**; **Figure 1J**). The PC 2 was the only component that was different between regions of origin (**Table 2**; **Figures 1B–D**), with plants from the invaded region showing lower values than plants from the native region (**Figure 1C**). We did not detect significant effects of temperature or rainfall on PC 2 (**Table 2**; **Figures 1G,K**). LMM also showed that reduced rainfall significantly decreased the PC 3 values of plants (**Table 2**; **Figure 1L**), but the magnitude of this effect marginally depended on temperature (**Table 2**; **Supplementary Figure 4**). This result means that the effect of reduced rainfall in decreasing $\delta^{15}N$ was only manifested in plants under the control temperature (**Supplementary Figure 4C**). Interestingly, only the PC 3 was significantly correlated with methylation (Pearson's $r = -0.312$; $p = 0.031$; **Table 2**; **Figure 2**), showing lower values with increasing values of methylation (**Figure 2C**).

Integrating the Relationships Between Phenotypic and Epigenetic Variation of *Carpobrotus edulis*

As shown by the SEM, neither temperature nor rainfall significantly influenced the epigenotype (global DNA methylation), but the epigenotype was different in function of plant origin (higher global methylation in plants from the invaded region; **Figure 3**).

TABLE 1 | Results of linear mixed model examining the effect of region, temperature, and rainfall on the global DNA methylation of *Carpobrotus edulis* plants.

			Methylation (%)			
Effects	**Variance**	**SE**	**Estimate**	**df**	***F***	***p***
Nested random						
Site(region)	0.370	1.708				
Fixed						
Region			−3.061	1, 6	6.496	**0.044**
Temperature			−1.359	1, 40	1.474	0.232
Rainfall			−0.276	1, 40	0.061	0.807

Site nested within region was included as a random effect in the model (estimate of residual ± SE = 14.992 ± 3.417). Values of $p < 0.05$ are highlighted in bold. See **Figure 1** *for significant differences for main effects (n = 48).*

Region of origin was not associated with PC1 (PRI_{531} and SIPI indices, RGR, and C/N), but it explained variation in PC 2 (i.e., on the $\Delta^{13}C$ and RSR; **Figure 3**). Also, our results showed that region of origin indirectly influences PC 3 (mainly the $\delta^{15}N$), through its effect on methylation (**Figure 3**). The PC 3 was the only principal component affected by methylation and by rainfall (**Figure 3**). Finally, the SEM showed that PC 1 variables were affected by temperature (**Figure 3**; see also **Supplementary Figures 2, 3**).

DISCUSSION

Current and future global change scenarios challenge the capacity of plants to respond to the rapid shifts in environmental conditions. By testing the connection between epigenetic mechanisms and phenotypic responses in native and invasive populations of *C. edulis*, this study showed that plants can adapt to shifts in environmental conditions through phenotypic plasticity. In addition, environmental-induced epigenetic modifications can contribute to extending phenotypic and functional diversity, which may also help plants to cope with these changing environments and to colonize new habitats.

Climatic Effects on Epigenetic and Phenotypic Responses and Relationship Between DNA Methylation and Phenotypes

Our findings support our hypothesis that the new climate scenarios predicted for Southern Europe may foster rapid phenotypic changes in *C. edulis,* but, contrary to our predictions, we did not detect any significant variation in global DNA methylation in response to experimental climate change.

The increase in temperature primarily affected phenotypic traits (PC 1 = 35.1% of the total variance) of *C. edulis* related to some relevant physiological and plant growth characteristic (**Table 2**; **Supplementary Table 4**). Specifically, loadings for traits associated with this principal component (RGR, PRI_{531} and SIPI indices, and C/N) indicate that higher temperatures increased the relative growth rate and the photochemical efficiency of plants whereas decreased the proportion of carotenoids to chlorophyll *a* and the ratio of carbon to nitrogen. Previous studies have demonstrated that PRI is inversely correlated with the dissipation of excess radiation energy as heat, and with the SIPI index (i.e., with the ratio of carotenoids to chlorophylls *a;* Peñuelas et al., 1995b; Guo and Trotter, 2004), and directly correlated with the net CO_2 uptake and photosynthetic radiation-use efficiency (RUE, mol CO_2• mol^{-1} photons; Gamon et al., 1992; Peñuelas et al., 1995a). The observed higher values of the PRI index and lower values of the SIPI index at elevated temperature therefore suggest that native and invasive plants can protect photosynthesis from high temperatures with a low investment in photoprotection, thus allowing plants to obtain a high photochemical efficiency and, consequently, a high RGR. These results are consistent with findings of our previous research in which we showed that the highly plastic response of *C. edulis* to global warming in terms of growth, physiology, and biochemistry may increase the expansion of the species under warmer climates (Campoy et al., 2021). These outcomes also reinforce current evidence that high plasticity levels may enable plants to better tolerate the rapid shifts in environmental conditions and the idea that plasticity could play an important role in invasions (Richards et al., 2006; Nicotra et al., 2015; Henn et al., 2018).

Regarding foliar chemistry and stoichiometry of plants, in a recent meta-analysis of experimental field studies, (Xu et al., 2020) examined shifts in foliar ratios in response to several global change drivers, and their results showed that variation in foliar C/N was mostly explained by shifts in atmospheric nitrogen deposition (i.e., N addition) but not by experimental warming. This result contrasts somewhat with our observation that increased temperature decreased leaf C/N ratio of plants. This change in the C/N ratio could be related to an increase leaf transpiration rate under warming conditions. A higher transpiration rate would imply higher water requirement and greater nutrient translocation from the soil to the plant, which could lead to an increase in N concentration and, consequently, a decrease in the C/N ratio (Jauregui et al., 2015; Wang et al., 2019). Because the leaf C/N ratio plays an important function in ecosystem energy and nutrient dynamics (Zhou et al., 2019; Xu et al., 2020), our findings suggest that the observed shift in this foliar trait of *C. edulis* in response to warming may have a large impact upon ecosystem function at invaded sites.

Our study also revealed that the effect of reduced rainfall in decreasing PC 3 values under the control temperature, may affect phenotypic aspects of *C. edulis* (PC 3 = 14.5% of the total variance; **Table 2**; **Supplementary Figure 2C**) mainly

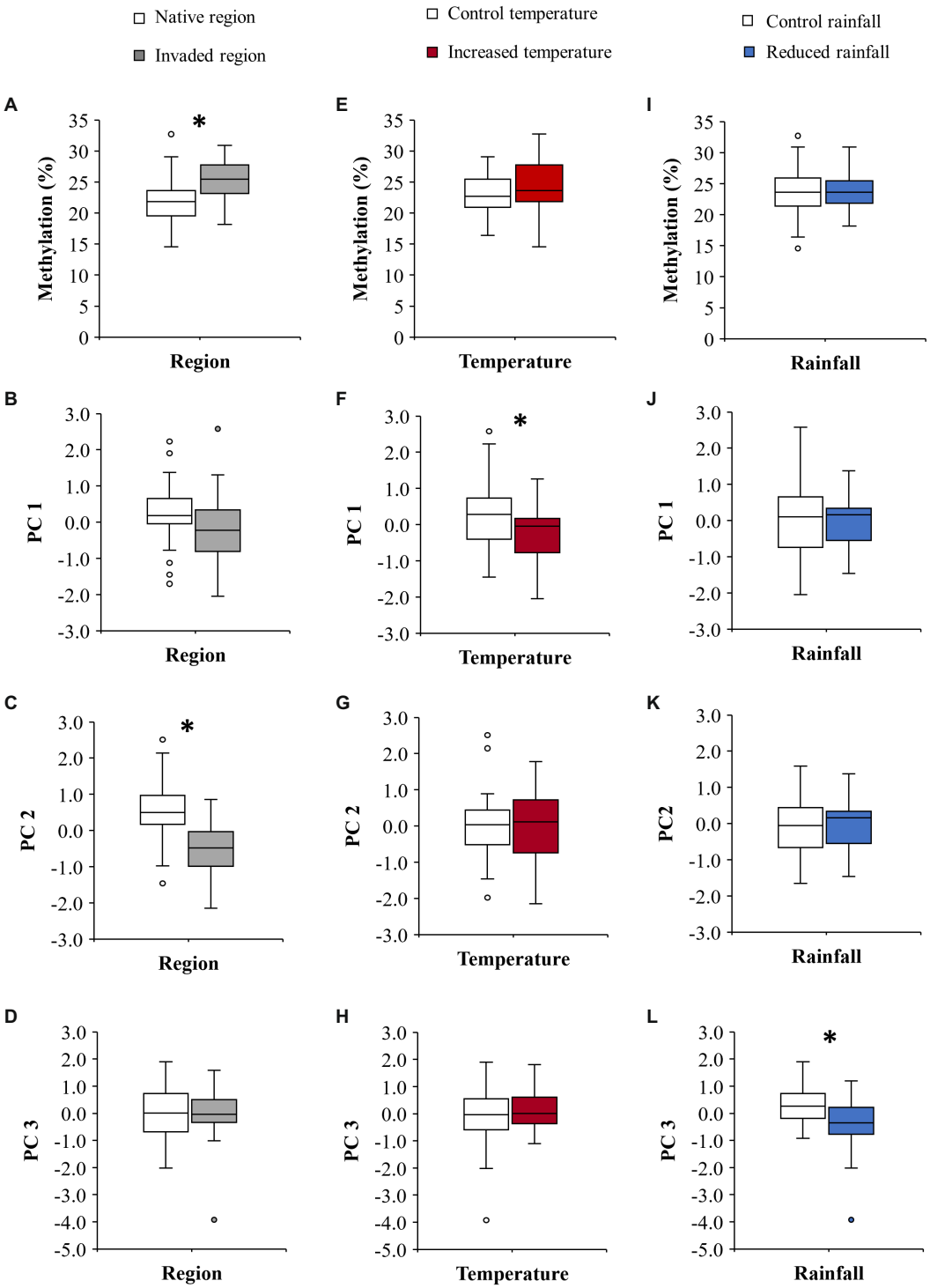

FIGURE 1 | Effect of region (native vs. invaded) on the global DNA methylation **(A)**, the first principal component (PC 1 = 35.1% variance; **B**), the second principal component (PC 2 = 21.2% variance; **C**), and the third principal component (PC 3 = 14.5% variance; **D**) of *C. edulis* phenotype. **(E–H)** show the effect of temperature (control vs. increased) on global methylation **(E)**, PC 1 **(F)**, PC 2 **(G)**, and PC 3 **(H)**. **(I–L)** show the effect of rainfall (control vs. reduced) on global methylation **(I)**, PC 1**(J)**, PC 2 **(K)**, and PC 3 **(L)**. The boxplots show the median, interquartile range, minimum, maximum, and the outliers; $n = 24$. Significant differences are denoted by *. See text for statistics.

related to the ^{14}N:^{15}N isotope ratio of plant tissues (i.e., $\delta^{15}N$; **Supplementary Table 4**). (Campoy et al., 2021) demonstrated a similar relationship between temperature and water availability and leaf $\delta^{15}N$ in *C. edulis*, but these findings contrast with

TABLE 2 | Results of linear mixed models examining the effects of region (Re), temperature (T), rainfall (R), and genome global methylation, on the three principal components (PC 1, PC 2, PC 3) of the PCA performed on seven phenotypic traits (RGR, RSR, C/N, $\Delta^{13}C$, $\delta^{15}N$, SIPI, and PRI_{531} indices) of *Carpobrotus edulis*.

Effects	PC 1 (=35.1% variance)					PC 2 (=21.2% variance)					PC 3 (=14.5% variance)				
	Variance	SE	Estimate	df	*F*	*P*	Variance	s.e	Estimate	df	*F*	*P*	Variance	s.e	Estimate	df	*F*	*P*
Nested random																		
Site(region)	0.258	0.222					0.070	0.124					0.001	0.091				
Fixed																		
Region			−0.065	1, 7	0.169	0.694			1.392	1, 6	12.009	**0.014**			−0.843	1, 5	0.001	0.979
Temperature			0.137	1, 34	4.560	**0.040**			0.451	1, 34	0.124	0.727			−1.242	1, 34	1.156	0.290
Rainfall			−0.523	1, 34	0.267	0.609			0.375	1, 33	0.515	0.478			−0.289	1, 34	9.751	**0.004**
Methylation			−0.035	1, 37	0.947	0.337			0.019	1, 38	0.320	0.575			−0.087	1, 39	6.729	**0.013**
Re x T			0.096	1, 34	0.018	0.895			0.070	1, 33	0.282	0.599			0.964	1, 34	1.131	0.295
Re x R			0.573	1, 34	0.635	0.431			−0.296	1, 34	1.649	0.208			1.146	1, 34	2.027	0.164
T x R			0.900	1, 35	2.042	0.162			−0.473	1, 35	2.717	0.108			1.417	1, 36	3.892	*0.056*
Re x T x R			−0.327	1, 34	0.101	0.752			−0.651	1, 35	0.454	0.505			−0.875	1, 36	0.781	0.383

*Site nested within region was included as a random effect in the models (estimates of residuals ± SE = 0.766 ± 0.187 for the PC 1, 0.687 ± 0.171 for the PC 2, and 0.734 ± 0.183 for the PC 3). Values of p < 0.05 are highlighted in bold, and values of p marginally significant are highlighted in italics. See **Figure 1** and **Supplementary Figure 4**, for significant differences.*

the commonly reported patterns showing that plant $\delta^{15}N$ usually decreases with increasing mean annual precipitation and with decreasing mean annual temperature (Amundson et al., 2003). Nitrogen isotopes have been widely applied in ecological studies because plant variation in $\delta^{15}N$ is strongly associated with many important biogeochemical processes including N mineralization, ammonia volatilization, nitrification, and denitrification (Makarov, 2009; Chen et al., 2018). However, comparison across studies and the identification of the precise N processes that can be affected are highly complex because the variation in $\delta^{15}N$ in plants can be determined by the combined effect of several interrelated factors (Makarov, 2009; Wang and Liu, 2011; Chen et al., 2018; Henn et al., 2018). In fact, another remarkable finding of our research is that the variation of the PC 3, and thus $\delta^{15}N$, was not only significantly correlated with climatic treatments, but also with global DNA methylation, showing lower values with increasing values of methylation (**Table 2**; **Figure 2C**). These results would indicate that epigenotype and climatic treatments did not affect the whole phenotype of *C. edulis* but affected specific trait combinations.

The contribution of DNA methylation to phenotypes has been previously documented using both model and non-model species (review in Richards et al., 2017). For instance, Bossdorf et al. (2010) used *Arabidopsis thaliana* (L.) Heynh to demonstrate that experimental alteration of DNA methylation can cause major shifts in plant phenotypes affecting not only means and variability of growth, fitness, and phenological traits, but also their phenotypic plasticity. Likewise, Zhang et al. (2013) provided evidence of heritable variation among epigenetic recombinant inbred lines of *A. thaliana* in root allocation and in the plasticity to drought and nutrient levels. Other studies using wild plant populations of some species such as *Ilex aquifolium* L., *Viola cazorlensis* Gand., and *Helleborus foetidus* L. also found correlations between anonymous MSAP markers and ecologically important leaf traits (Herrera and Bazaga, 2013), flower morphology (Herrera and Bazaga, 2010), and fitness-related traits (Medrano et al., 2014).

Furthermore, several studies have documented changes in DNA methylation with exposure to different environmental stresses (Verhoeven et al., 2010; Nicotra et al., 2015; González et al., 2017; Shafiq et al., 2019; Saban et al., 2020), which can be transgenerational (Verhoeven et al., 2010; Sobral et al., 2021a). However, in our experiment, we did not detect any significant changes in global DNA methylation between control plants and plants grown under the experimental climatic conditions for 14 months. This interesting finding provides evidence that DNA methylation at the whole-genome level in *C. edulis* does not vary in response to increase in temperature, reduction in rainfall, or by the combined effects of both stressful climatic factors, suggesting that the observed phenotypic variation in response to our experimental climatic conditions was not related to changes in the epigenotype of the species. The high plasticity for morphological and ecophysiological traits of *C. edulis*, which includes a facultative C3-CAM physiology (Campoy et al., 2018), explains its tolerance to a wide range of ecological conditions and could contribute to understand how the species can display successful phenotypic responses to our climatic treatments without epigenetic regulation. Thereby, this result reinforces the current knowledge that the

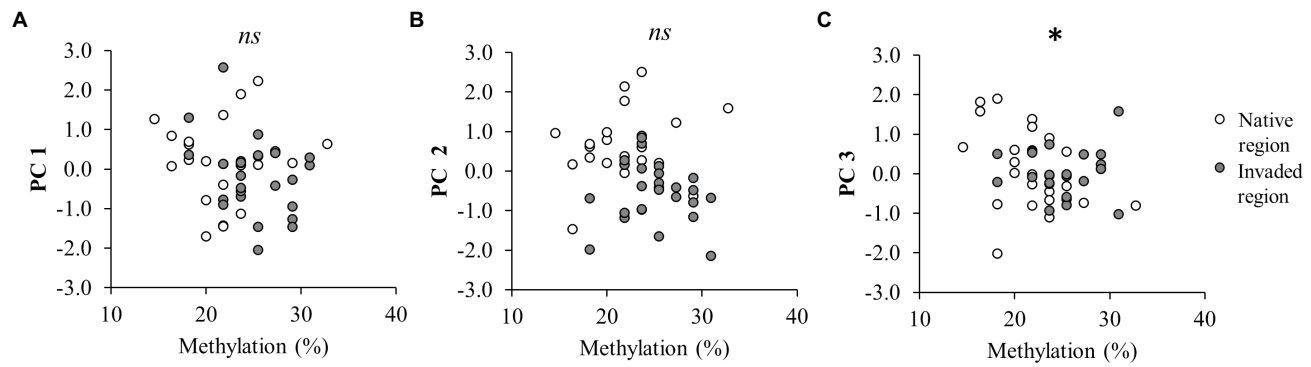

FIGURE 2 | Effect of global DNA methylation on the first principal component (PC 1 = 35.1% variance; $F_{1,37} = 0.95$; $p = 0.337$; **A**), the second principal component (PC 2 = 21.2% variance; $F_{1,38} = 0.32$; $p = 0.575$; **B**), and the third principal component (PC 3 = 14.5% variance; $F_{1,38.9} = 6.73$; $p = 0.013$; **C**) of *C. edulis* phenotype ($n = 48$). Significant correlation between methylation and principal components is denoted by *, whereas non-significant correlation is denoted by *ns*.

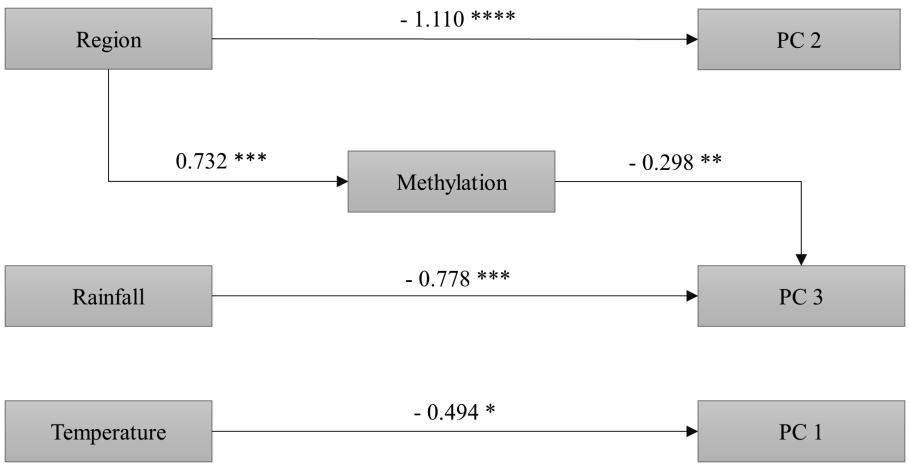

FIGURE 3 | Results of the structural equation model analyzing the relationship of region of plant origin (native and invaded region), rainfall (control and reduced rainfall), and temperature (control and increased temperature), with global DNA methylation and the three principal components of the PCA performed on seven phenotypic traits (RGR, RSR, C/N, $\Delta^{13}C$, $\delta^{15}N$, SIPI, and PRI_{531} indices) of the invasive plant *C. edulis*. We show coefficients for each significant path. *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$, ****$p < 0.001$. $N = 48$ plants. Model properly fitted the data as shown by the non-significant Chi-squares values (χ^2: 4.015; df: 12; p: 0.983). Initial model and other alternative models can be found in **Supplementary Figures 2**, **3**.

magnitude of the epigenetic effects depends on several factors, including the specific environmental stress, the exposure time of plants to stress, or the species under consideration (e.g., Verhoeven et al., 2010; Nicotra et al., 2015; Huang et al., 2017).

Phenotypic and Epigenetic Differences Between Native and Invasive *Carpobrotus edulis*

Given that the environmental changes faced by invasive plants may be much greater and/or more rapid than those experienced by species under natural conditions, it has been suggested that adaptive plasticity and epigenetic variation may be important mechanisms by which invasive populations can successfully adapt to these novel environments (Richards et al., 2006; Estoup et al., 2016; Mounger et al., 2021).

In this study, we found a divergence between South African and Iberian Peninsula phenotypes and epigenotypes during the process of invasion, regardless of climatic treatments. As inferred from the PCA, the region of origin influenced phenotypic traits (PC 2 = 35.1% of the total variance) of *C. edulis* related to carbon isotope discrimination and biomass partitioning (i.e., on the $\Delta^{13}C$ and RSR), with plants from the invaded region showing lower values than plants from the native region (**Table 2**; **Figure 1C**; **Supplementary Table 4**). The positive linear relationship between carbon isotopic discrimination and water use efficiency (WUE; Farquhar et al., 1989) indicates a higher efficiency for the use of water in the Iberian Peninsula plants (i.e., lower $\Delta^{13}C$ values) than in the South African plants, which is consistent with the relatively lower below-ground biomass allocation (i.e., lower RSRs ratios) observed in the invasive Iberian Peninsula plants. Because shifts in these morphological and physiological traits may lead to invasive plants to use water and light more efficiently, this phenotypic divergence can be considered as an adaptive strategy for successful expansion in the introduce range. Other studies

have demonstrated rapid genetic shifts in important ecological traits between native and invasive populations after introduction in new territories (Zou et al., 2007; Caño et al., 2008; Matesanz et al., 2012; Roiloa et al., 2016; Portela et al., 2019; Campoy et al., 2021), and also suggest that intraspecific variability in relevant functional traits may play a crucial role in plant invasion. Moreover, another interesting finding of this work is that some phenotypic traits (i.e., PC 3 mainly related to $\delta^{15}N$) of *C. edulis* differs between regions of origin, partly through its effect on methylation (**Figure 3**). This indicates that global DNA methylation may also provide an additional source of intraspecific variation in leaf $\delta^{15}N$ of *C. edulis* that could significantly affect the N dynamics of invaded coastal ecosystems.

One of the most important findings of this study is that in addition to the phenotypic differentiation between plants from the different regions, we provide evidence for epigenetic differences between native and invasive *C. edulis*, with the invasive individuals from the Iberian Peninsula showing higher levels of global DNA methylation compared to their native counterparts from South Africa (**Table 1**; **Figure 1A**). This significantly higher level of methylation in invaders than in natives suggests that the invasion process may have selected plants with a greater capacity for epigenetic control. Previous studies have documented higher epigenetic variation levels in introduced compared to native populations for several species (e.g., Spens and Douhovnikoff, 2016), and changes in DNA methylation of some clonal plants with exposure to different habitat types (Richards et al., 2012) and with climate of origin (Zhang et al., 2016), what suggests that epigenetically regulated phenotypic variation may be crucial for the establishment, spread and invasion success of an invasive population, especially in the absence or with low levels of genetic variation (Mounger et al., 2021). In fact, recent literature has highlighted the potential importance of epigenetic variation for the success of clonal invaders based on two reasons. First, because these processes may provide a non-genetic source of heritable variation that, when related to individual fitness, can contribute to generate adaptive phenotypes through rapid selective changes. Second, because clonal reproduction does not reset epigenetic effects that is thought to occur through meiosis (Verhoeven and Preite, 2014; Mounger et al., 2021). Thus, epigenetic changes in asexual organism could be more stably inherited to the progeny (although they can be also inherited through sexual reproduction as well, see for example Sobral et al., 2021a).

The epigenetic differences that we observed in global DNA methylation between native and invasive plants and the relationship between epigenetic features and phenotypic traits of *C. edulis*, also suggest that epigenetic modifications may be a source of intraspecific functional diversity in this clonal plant, and it may also contribute to the successful and rapid adaptation of this species to new habitats. However, we cannot exclude other factors (i.e., multiple introductions from different source populations and the combination of different reproductive strategies, including hybridization; reviewed by Campoy et al., 2018) that could also contribute to explain the epigenetic differences observed. Thereby, further investigations are needed to unravel the relative importance of genetic vs. epigenetic variation in determining the ecological and evolutionary consequences of invasion.

CONCLUSION

We found epigenetic (i.e., global DNA methylation) and phenotypic differences (i.e., biomass partitioning pattern and water take up and use) between individuals from the native (South Africa) and the invaded area (Iberian Peninsula) of *C. edulis*. This divergence between native and invasive populations evidences an intraspecific functional variation during the process of invasion and suggests that phenotypic plasticity and global DNA methylation may be related to the successful and rapid adaptation of this species to new habitats. Moreover, our findings strongly suggest that the fractionating processes in the N cycle might be affected by the changing conditions in temperature and rainfall, by methylation and by region of origin, which in turn could impact the N dynamics in coastal ecosystems invaded by *C. edulis*.

With this work, we also showed that the new climate scenarios projected for Southern Europe (i.e., increased temperature and reduced rainfall) might foster rapid changes in functional traits of *C. edulis,* but, interestingly, we also demonstrated that these phenotypic changes seem to be independent of epigenetic ones. Finally, this study highlights that phenotypic plasticity might improve species fitness in new climatic scenarios and adds to the current evidence of the important role of epigenetic mechanisms for the adaptive success of invasive species to new areas.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RR designed and conceived the work and together with RB and MS get fundings for the study. RR, JC, and ML conducted field sampling, set up the experiment, and collected data. RB, BC, and JC carried out the multistage study to select primer combinations for the MSAP analyses. MS provided the statistical approach and contributed substantially to analyze data and writing the manuscript. JC led the analysis, manuscript writing, and submission. All authors commented on and reviewed the final draft. All authors contributed to the article and approved the submitted version.

FUNDING

Funding for this study was provided by the Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund (ERDF; grant Ref. CGL2013-48885-C2-2-R and Ref. CGL2017-87294-C3-1P awarded to RR) and by the

Autonomous Government of Galicia (grant ref. I2CB awarded to MS).

ACKNOWLEDGMENTS

The authors thank the authorities of the Parque Nacional Illas Atlánticas (Atlantic Islands National Park), especially J. A. Fernández Bouzas and M. Martínez Morán, for permission to work at the study site, and to the park keepers, Roberto, Pablo, José, and Rosa for logistical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.888391/full#supplementary-material

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frontiers | Frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 June 2022
doi: 10.3389/fpls.2022.890309

Clonal Parental Effects on Offspring Growth of Different Vegetative Generations in the Aquatic Plant *Pistia stratiotes*

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Parental (environmental) effects can modify the growth of offspring, which may play an essential role in their adaptation to environmental variation. While numerous studies have tested parental effects on offspring growth, most have considered offspring growth of only one generation and very few have considered offspring growth of different generations. We conducted a greenhouse experiment with an aquatic clonal plant *Pistia stratiotes*. We grew a single ramet of *P. stratiotes* under low or high nutrients, the initial (parent) ramets produced three different generations of offspring ramets, and these offspring ramets were also subjected to the same two nutrient levels. High nutrients currently experienced by the offspring increased biomass accumulation and ramet number of all three offspring generations of *P. stratiotes*. However, these positive effects on biomass were greater when the offspring ramets originated from the parent ramets grown under low nutrients than when they were produced by the parent ramets grown under high nutrients. These results suggest that parental effects can impact the performance of different offspring generations of clonal plants. However, heavier offspring ramets produced under high nutrients in parental conditions did not increase the subsequent growth of the offspring generations. This finding indicates that parental provisioning in favorable conditions may not always increase offspring growth, partly depending on root allocation but not ramet size such as ramet biomass.

Keywords: clonal plant, nutrients, provisioning effect, ramet size, trans-generational effects

OPEN ACCESS

Edited by:
Noemi Tel Zur, Ben-Gurion University of the Negev, Israel

Reviewed by:
Jiaxin Quan, Northwest University, China
Yupeng Geng, Yunnan University, China

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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: *05 March 2022*
Accepted: *30 May 2022*
Published: *27 June 2022*

Citation:
Zhang L-M, Roiloa SR, Zhang J-F, Yu W-H, Qiu C-Y, Wang D-H and Yu F-H (2022) Clonal Parental Effects on Offspring Growth of Different Vegetative Generations in the Aquatic Plant Pistia stratiotes. Front. Plant Sci. 13:890309. doi: 10.3389/fpls.2022.890309

INTRODUCTION

The phenotype of a plant individual can be influenced by the environmental condition experienced by its parent, a phenomenon called parental effects or trans-generational effects (Agrawal, 2002; Mousseau et al., 2009; González et al., 2016; Waterman and Sultan, 2021). Parental effects can influence offspring not only *via* seeds produced through sexual reproduction (Bossdorf et al., 2010; Mondoni et al., 2014; Baker et al., 2019; Veselá et al., 2021) but also *via* ramets (asexual individuals) produced through clonal growth or vegetative reproduction (Latzel and Klimešov, 2010; Dong et al., 2018a; González et al., 2018; Portela et al., 2020).

Both sexual and clonal parental effects have important ecological and evolutionary implications as they can potentially influence plant fitness, intraspecific and interspecific interactions, population dynamics, and community structure (Badyaev and Uller, 2009; Droste et al., 2010; Geng et al., 2016; Herman and Sultan, 2016; Pérez-Ramos et al., 2019).

Increasing evidence shows that parental effects may be adaptive, enhancing the fitness of the offspring when established in an environment similar to their parents (Herman et al., 2012; Rasmann et al., 2012; Latzel et al., 2014; Dong et al., 2017; Baker et al., 2018). For instance, when offspring of *Polygonum persicaria* grow in shade, offspring produced by shaded parents perform better than offspring produced by parents under sunlight (Baker et al., 2018). Adaptive parental effects have been reported in several plant species in response to a variety of biotic and abiotic factors (Lacey and Herr, 2000; Whittle et al., 2009; Dong et al., 2017; Waterman and Sultan, 2021). As a result, parental effects are recognized as an important source of phenotypic variation that may have an essential role in local adaptations (Herman and Sultan, 2011; Holeski et al., 2012; Dong et al., 2018a).

Many studies have shown that parental effects caused by differences in the quality of resources provisioned to offspring may be more likely to be adaptive because they can persist through the life cycle (Roach and Wulff, 1987; Herman and Sultan, 2011; Germain et al., 2013; Zas et al., 2013). For example, larger seedlings of *Pinus pinaster* can come from larger seeds that are produced in favorable parental environments (Zas et al., 2013). Compared to sexual propagules (e.g., seeds), vegetative propagules (e.g., ramets) of clonal plants are larger in size and mass, and thus their potential for resource provisioning may be relatively high (Dong et al., 2019). For instance, González et al. (2017) found that parent ramets of *Trifolium repens* grown in better conditions produced larger/heavier vegetative propagules that enabled offspring to grow better. Dong et al. (2018a, 2019) and Portela et al. (2020) showed that clonal fragments of *Alternanthera philoxeroides* produced in favorable parental environments benefited the subsequent growth of clonal offspring. These studies suggest that resource provisioning is one of the most important mechanisms underlying clonal parental effects (González et al., 2017; Dong et al., 2018a, 2019; Portela et al., 2020).

A high proportion of aquatic species are clonal and capable of rapid vegetative reproduction (Sosnová et al., 2010; Wang et al., 2016; Zhang et al., 2019; Adomako et al., 2021). A clone often consists of a large network of interconnected ramets belonging to different vegetative generations (Dong, 2011). However, previous studies testing clonal parental effects on offspring performance have involved clonal offspring of only a single vegetative generation (Dong et al., 2017, 2019; González et al., 2017; Portela et al., 2020), and have not considered the potential differences among offspring ramets of different vegetative generations. As the size and biomass of offspring ramets commonly become smaller with increasing vegetative generation (Wang et al., 2014), their ability of resource provisioning may become weaker. Therefore, the magnitude of clonal parental effects on offspring performance may differ when offspring of different vegetative generations are considered.

To test how clonal parental effects influence the offspring growth of different vegetative generations, with a focus on provisioning as a possible mechanism, we conducted a greenhouse experiment on an aquatic clonal plant *Pistia stratiotes*. We grew a single ramet of *P. stratiotes* under low or high nutrients, and its offspring ramets of three different generations, i.e., primary, secondary, and tertiary offspring ramets were also subjected to low or high nutrients. Specifically, we tested three hypotheses: (1) clonal parental effects would impact offspring growth of all three vegetative generations of *P. stratiotes*; (2) for all three vegetative generations, offspring produced by the parent ramet under high nutrients would perform better than offspring produced by the parent ramet under low nutrients, because providing high nutrients to the parent ramet may allow them to produce high-quality clonal offspring; and (3) the magnitude of clonal parental effects on offspring performance would become smaller with increasing the vegetative generation, i.e., parental effects would be the highest on the growth of the primary ramets, the lowest on that of the tertiary ramets, and in between on that of the secondary ramets.

MATERIALS AND METHODS

Study Species and Material Preparation

Pistia stratiotes L. (water lettuce, Araceae) is an aquatic, free-floating, stoloniferous clonal plant (Pettet and Pettet, 1970). This species is native to South America and is now widely spread in tropical and subtropical regions of the world (Yang et al., 2014; Galal et al., 2019). Rosette leaves come out from nodes of the highly compressed stems and adventitious roots arise at the base of the rosette. *Pistia stratiotes* are capable of rapid clonal growth (Adomako et al., 2021) and stolons grow out from the leaf axils to form offspring ramets (Odjegba and Fasidi, 2004). The species is listed as an invasive species in the Global Invasive Species Database (http://issg.org/database/welcome/) because they can form extensive floating mats that block the air-water interface, reduce oxygen levels in the water, and decrease biodiversity (Adebayo et al., 2011; Galal et al., 2019). However, *P. stratiotes* have been shown to have the potential for the management of water quality due to their ability to accumulate heavy metals from water (Hanks et al., 2015; Adomako et al., 2020).

On June 12, 2020, ramets of *P. stratiotes* were collected from Yongning River (28°40′3″N, 121°23′4″E) in Taizhou, Zhejiang Province, China, and brought to a tank (95 cm in diameter × 60 cm in height) in a greenhouse at Taizhou University for propagation. After 3 weeks, all ramets had produced new offspring ramets. On July 3, 2020, 30 new offspring ramets of similar size were selected and their stolons, if any, were removed. Of the 30 ramets, ten were randomly selected, dried at 70°C for 48 h, and weighed to measure initial dry mass (mean ± SE: 1.95 ± 0.19 g). The remaining 20 ramets (hereafter referred to as parent ramets) were used for the experiment described below.

Experimental Design

The experiment consisted of two phases. In the first phase, parent ramets (F0) were randomly subjected to two nutrient levels, and the primary (F1) offspring ramets (i.e., daughter ramets of the

parent ramets), secondary (F2) offspring ramets (i.e., daughter ramets of the F1 ramets or granddaughter ramets of the parent ramets), and tertiary (F3) offspring ramets (i.e., daughter ramets of the F2 ramets or granddaughter ramets of the F1 ramets or grand-granddaughter ramets of the parent ramets) from each parent ramet were harvested (**Figure 1**). In the second phase, the F1, F2, and F3 ramets originating from the F0 ramets grown at each nutrient level in the first phase of the experiment were subjected to the same two nutrient levels (**Figure 1**).

The first phase of the experiment started on July 3, 2020. We randomly assigned the 20 parent ramets to two nutrient levels (high vs. low), and each treatment had ten replicates. For the high and low nutrient levels, the parent ramets were grown individually in containers (65 cm in diameter × 44 cm in height) filled with 70 L of 30% and 3% Hoagland solution, respectively. The Hoagland solution contained 945 mg/L of $Ca(NO_3)_2{\cdot}4H_2O$, 506 mg/L of KNO_3, 80 mg/L of NH_4NO_3, 136 mg/L of KH_2PO_4, 493 mg/L of $MgSO_4$, 13.9 mg/L of $FeSO_4{\cdot}7H_2O$, and 18.7 mg/L of EDTA·2Na. Every week, 2 L of 30% or 3% Hoagland solution was added to each container to compensate for nutrient depletion by the plants and, if needed, additional water was added to each container to compensate for water loss due to evaporation. The experiment was conducted in an open area on the campus of Taizhou University. The first phase of the experiment ended on July 24, 2020 and lasted for 3 weeks. At harvest, each parent ramet had produced 12–29 F1 offspring ramets, 20–121 F2 offspring ramets, and 2–135 F3 offspring ramets.

The second phase of the experiment began on July 24, 2020. We selected two F1, two F2, and two F3 ramets from each container (produced by each parent ramet) at the end of the first phase of the experiment, and, thus, obtained 20 offspring ramets of each generation (F1, F2, and F3) from each of the two nutrient levels (high vs. low). Of the 20 offspring ramets of each generation from each nutrient level, 16 were randomly selected and assigned to the two nutrient treatments as described in the first phase, and the remaining four, which were not used in the second phase, were dried at 70°C for 48 h and weighed to measure initial biomass. Initial biomass of the F1, F2,and F3 ramet were 0.723 ± 0.133 g, 0.249 ± 0.055 g, 0.026 ± 0.004 g (mean ± SE), respectively, from the low nutrient level, and 1.328 ± 0.317 g, 0.454 ± 0.057 g, and 0.101 ± 0.016 g, respectively, from the high nutrient level. We selected heavier ramets under high nutrients than under low nutrients of *P. stratiotes* because a previous study found that high nutrients increased biomass per ramet of *P. stratiotes* (Adomako et al., 2021).

In the second phase, each treatment had eight replicates, resulting in a total of 96 containers (36 cm in diameter × 32 cm in height). Containers filled with 15 L of 30% and 3% Hoagland solution for the high and the low nutrient treatment, respectively. Every week, 1 L of 30% or 3% Hoagland solution was added to each container to compensate for nutrient depletion by the plants and, if needed, additional water was added to each container to compensate for water loss due to evaporation.

The second phase of the experiment ended after 3 weeks, on August 14, 2020, when plants in most treatments had occupied the whole water surface in the containers. Photosynthetic photon flux density at the water surface at noon was 632–1,806 μmol m^{-2} s^{-1}, as measured weekly with a quantum sensor (LI-250 A; LI-COR Biosciences). The daily mean air temperature was 29.1°C, and the mean relative humidity was 80.3%, each measured hourly with Hygrochron temperature loggers (iButton DS1923; Maxim Integrated Products, USA).

Harvest and Measurements

At the end of the first phase of the experiment, after the selection of the two F1, F2, and F3 ramets for the second phase of the experiment, the remaining parts of the plants in each container were harvested. We recorded the number of the F1, F2, and F3 ramets separately for each container. Then, the parent ramet, as well as the F1, F2, and F3 offspring ramets were separately dried at 70°C for 72 h and weighed to obtain biomass. Biomass per ramet (i.e., final total dry mass/number of ramets) was calculated for F1, F2, and F3 offspring ramets, respectively. At the end of the second phase of the experiment, we counted the number of all ramets in each container. Then, the plants in each container were dried at 70°C for 72 h and weighed to measure biomass.

Data Analysis

For the first phase of the experiment, we used a *t*-test to examine the differences in biomass and number of the F1, F2, and F3 ramets, as well as biomass per F1, F2, and F3 ramet of *P. stratiotes*. For the second phase of the experiment, we employed two-way ANOVAs to test the effects of parental nutrient level, offspring nutrient level, and their interaction on biomass and the number of ramets of *P. stratiotes* produced by the offspring ramets of each generation, i.e., the F1, F2, and F3 offspring ramets produced by the parent ramets in the first phase of the experiment. Before analysis, biomass and number of ramets derived from the F2 offspring ramets and number of ramets derived from the F3 offspring ramets were ln-transformed to remove heteroscedasticity and to increase normality; figures show untransformed data. Statistical analyses were carried out with SPSS 22.0 (IBM Corp., Armonk, New York, USA). Plants in three containers (one F1 ramet produced by the parent ramet under low nutrients in the first phase of the experiment and grown under low nutrients in the second phase, one F2 ramet produced by the parent ramet under high nutrients in the first phase and grown under high nutrients in the second phase, and one F3 ramet produced by the parent ramet under high nutrients in the first phase and grown under high nutrients in the second phase) were damaged by herbivores during the experiment and were thus excluded from harvest and analysis.

RESULTS

Effects of Nutrients on Offspring Performance in the First Phase

Compared to low nutrients, high nutrients significantly increased biomass per F1 ramet of *P. stratiotes* but had little effect on biomass per F2 ramet and biomass per F3 ramet (**Figure 2**). Total biomass and number of the F1, F2, and F3 offspring ramets were significantly greater under high than under low nutrients (**Supplementary Figure S1**).

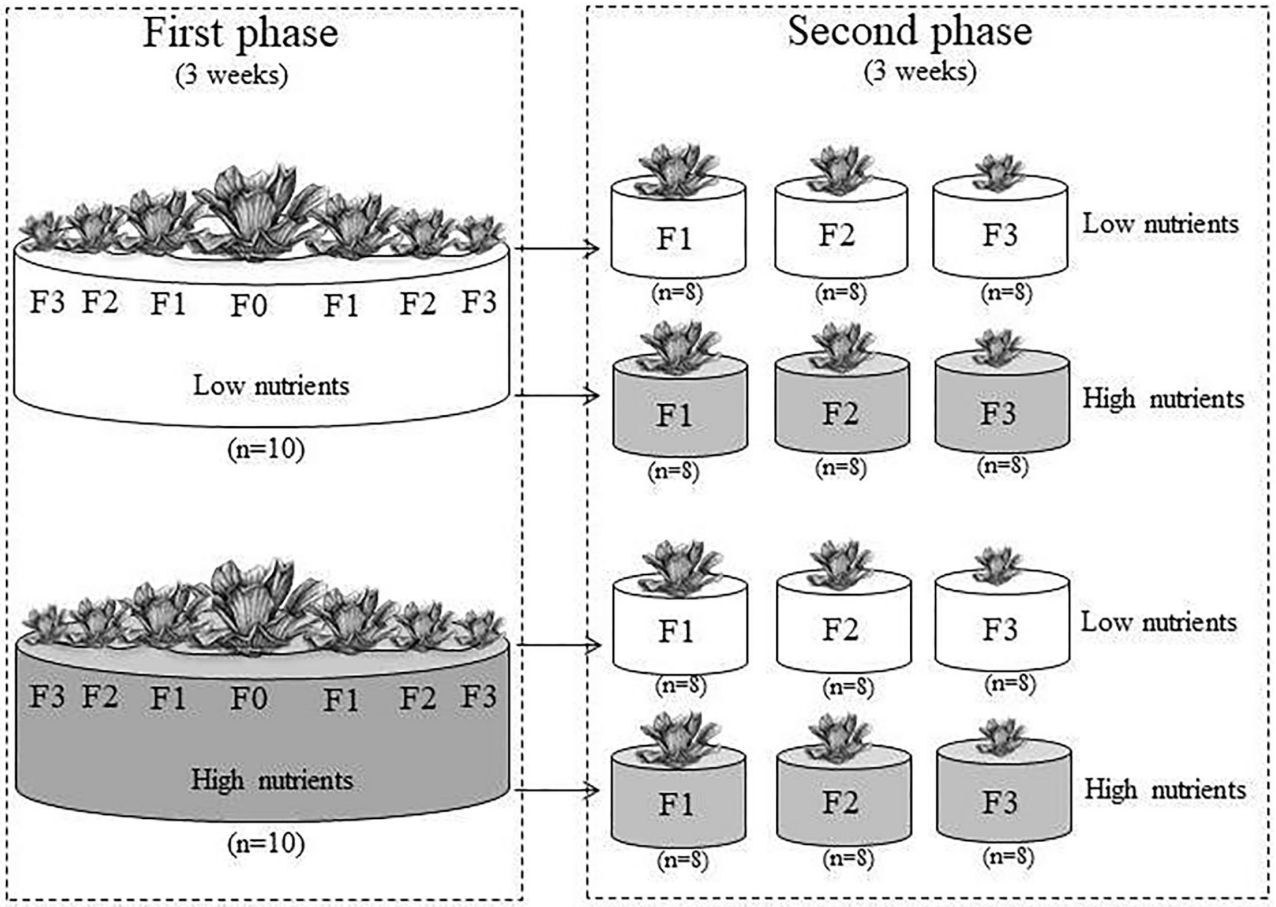

FIGURE 1 | Scheme of the experimental design. In the first phase, parent ramets (F0) produced F1, F2, and F3 offspring ramets under low or high nutrient levels. In the second phase, the F1, F2, and F3 offspring ramets from the first phase were also subjected to the same two nutrient levels.

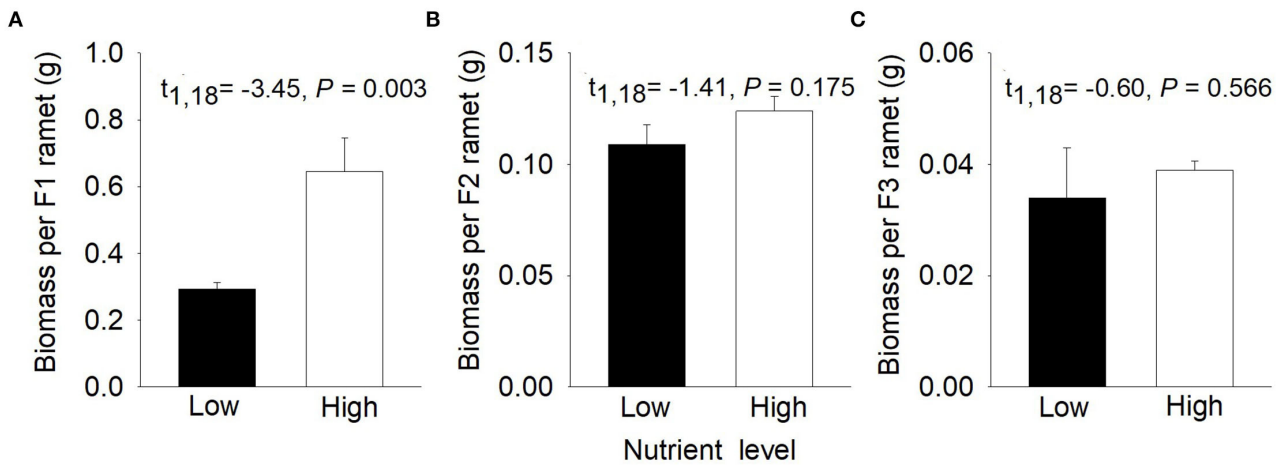

FIGURE 2 | Effects of nutrient level (low vs. high) on **(A)** biomass per F1 ramet, **(B)** biomass per F2 ramet, and **(C)** biomass per F3 ramet of *Pistia stratiotes* in the first phase. Bars show means + SE ($n = 10$).

Effects of Parental and Offspring Nutrients on Offspring Performance in the Second Phase

Compared to low nutrients currently experienced by offspring, offspring high nutrients significantly increased total biomass and the number of F1 offspring ramets of *P. stratiotes* in the second phase (**Table 1**, **Figure 3**). However, such an effect on total biomass was much greater when the F1 offspring ramets were produced by the parent ramets grown under low nutrients than when they were produced by the parent ramets grown under high nutrients in the first phase (**Figure 3A**), as indicated by the significant interactive effect of parental nutrient level × offspring nutrient level (**Table 1**). Also, compared to parental low nutrients, parental high nutrients tended to increase total biomass (by 23.7%) of *P. stratiotes* under offspring low nutrients but markedly decreased it (by 43.5%) under offspring high nutrients (**Figure 3A**, **Table 1**). The parental nutrient level had no significant effect on the number of F1 offspring ramets of *P. stratiotes* in the second phase (**Figure 3B**, **Table 1**).

Compared to offspring low nutrients, offspring high nutrients increased total biomass and the number of the F2 offspring ramets of *P. stratiotes* in the second phase (**Table 1**, **Figure 4**). However, such an effect on total mass and number of ramets was much greater when the F2 offspring ramets were produced by the parent ramet grown under low nutrients than when they were produced by the parent ramets grown under high nutrients in the first phase (**Figure 4**), as indicated by the significant interactive effect of parental nutrient level × offspring nutrient level (**Table 1**). Also, under offspring's low nutrients, the parental nutrient level had little effect on total biomass of *P. stratiotes*, but under offspring high nutrients, parental high nutrients markedly decreased it (by 51.4%) compared to parental low nutrients (**Figure 4A**, **Table 1**). Parental high nutrients increased the number of the F2 offspring ramets (by 92.1%) under offspring low nutrients but decreased it (by 25.9%) under offspring high nutrients (**Figure 4B**, **Table 1**).

Offspring high nutrients increased total biomass and the number of the F3 offspring ramets of *P. stratiotes* in the second phase (**Table 1**, **Figure 5**). However, such an effect on total biomass was much higher under parental low nutrients than under parental high nutrients (significant interactive effect of parental nutrient level × offspring nutrient level in **Table 1**, **Figure 5A**). The parental nutrient level did not significantly affect the number of the F3 offspring ramets in the second phase (**Table 1**, **Figure 5B**). Parental high nutrients increased total biomass (by 82.9%) under offspring low nutrients but decreased it (by 52.2%) under offspring high nutrients (**Figure 5A**, **Table 1**).

DISCUSSION

As expected, both in the first and the second phase, current high nutrients increased the production of biomass and the number of new offspring ramets of *P. stratiotes*, agreeing with many previous findings on other aquatic plants (Zhao et al., 2006; Jampeetong and Brix, 2009; Zhang et al., 2020). Besides the direct positive effect of high nutrients on the growth of offspring ramets, the benefit was also from physiological integration from their parent ramets growing under the favorable conditions of high nutrient availability. Physiological integration can allow acropetal transport of resources from well-established ramets to developing offspring. Benefits from physiological integration have been repetitively reported by various previous studies (e.g., Slade and Hutchings, 1987; Saitoh et al., 2002; Roiloa and Retuerto, 2006; Dong et al., 2015; Elgersma et al., 2015; Portela et al., 2021; Wang et al., 2021). In our study, it is logical to anticipate that the benefit gained by offspring connected to parents with high nutrient availability was due to the support received from their parents growing under favorable conditions.

High nutrients increased ramet number of different offspring generations but had different effects on ramet weight (i.e., biomass per ramet). Previous studies found that plants produced lighter and likely smaller ramets in response to high-density environments and produced heavier and, likely, also bigger ramets in response to low-density environments (Wang et al., 2014; Adomako et al., 2021). Therefore, the relationship between ramet biomass and ramet number was caused by the different responses to plant density (Wang et al., 2014). In our study, high nutrients increased the biomass of the F1 offspring ramets but had little effect on the biomass of the F2 and the F3 ramets. This is mainly because plants did not occupy the whole surface of containers when producing the F1 ramets, so high nutrients increased both ramet biomass and number under the low-density environment. However, the high nutrients increased the number of the F2 and the F3 ramets, resulting in increased plant density and intensity of intraspecific competition, which may limit the growth of the F2 and the F3 ramets. Thus, ramet biomass of the F2 and the F3 ramets was not significantly greater under high nutrients than in low nutrients.

The interaction of parental nutrient level and offspring nutrient level significantly affected the growth of different offspring generations. Compared to low nutrients experienced by the parent ramet, parental high nutrients inhibited subsequent growth of offspring in high nutrients for all three offspring generations but increased growth of the F3 offspring ramets in low nutrients. These results support the first hypothesis that parental effects could impact offspring growth of different vegetative generations of clonal plants. Similarly, previous studies have shown that parental environment effects can persist across vegetative generations in clonal plants (e.g., González et al., 2017; Dong et al., 2018b; Portela et al., 2020; Zhang et al., 2021). Our results suggest that paternal effects are fast in clonal plants as the vegetative reproduction is rapid.

The selected offspring ramets from the first phase that grew under high nutrients were relatively bigger and had nearly two times greater initial mass than did offspring taken from the first phase grew under low nutrients. However, such a sizeable advantage of ramets inhibited subsequent growth of offspring in high nutrients for all three vegetative generations. Contrary to the second prediction, the benefit obtained by offspring generations (F1, F2, and F3) growing in high nutrients

TABLE 1 | ANOVA results for effects of parental nutrient level, offspring nutrient level, and their interaction on total biomass and total number of ramets of *Pistia stratiotes* produced by the (A) F1, (B) F2, and (C) F3 offspring ramets.

	Total biomass		Total number of ramets	
	$F_{1,27}$	*P*	$F_{1,27}$	*P*
(A) F1 offspring ramets				
Parental nutrient level (P)	5.66	**0.025**	0.52	0.479
Offspring nutrient level (O)	29.45	**<0.001**	42.86	**< 0.001**
P × O	11.88	**0.002**	1.70	0.203
(B) F2 offspring ramets				
Parental nutrient level (P)	9.73	**0.004**	0.03	0.877
Offspring nutrient level (O)	25.48	**<0.001**	70.52	**<0.001**
P × O	4.75	**0.038**	9.13	**0.005**
(C) F3 offspring ramets				
Parental nutrient level (P)	1.55	0.224	1.48	0.234
Offspring nutrient level (O)	18.84	**<0.001**	34.11	**<0.001**
P × O	4.92	**0.035**	0.36	0.555

The bold values indicates P < 0.05.

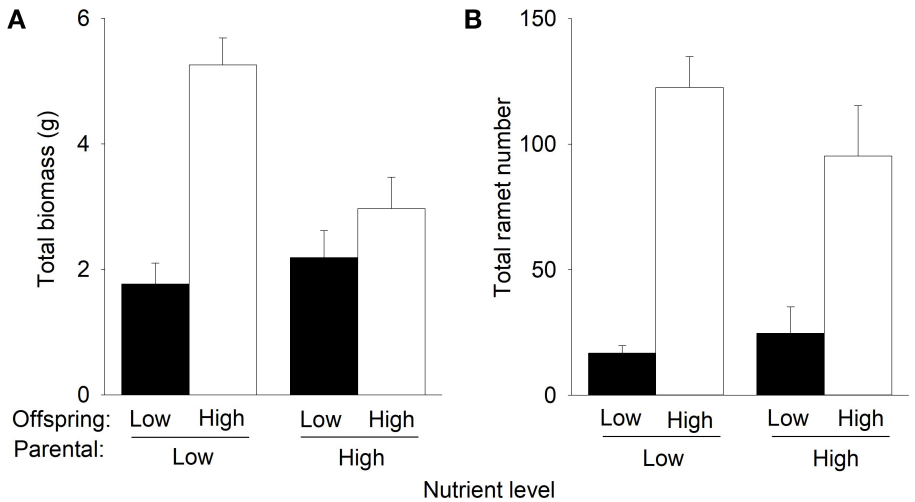

FIGURE 3 | Effects of parental nutrient level and offspring nutrient level on **(A)** total biomass and **(B)** total ramet number of *Pistia stratiotes* produced by the F1 offspring ramets in the second phase of the experiment. Bars show means + SE.

was significantly higher when their parent ramets grew in low nutrients. A plausible explanation for this unexpected result could be based on biomass partitioning responses. As predicted by the optimal partitioning theory (Bloom et al., 1985; Hilbert, 1990; Gleeson and Tilman, 1992), an increase in the proportional biomass allocated to roots would be expected in parents growing under low nutrient conditions. Furthermore, trans-generational plasticity predicts that the plastic response experienced by the parental generation could be transferred to their offspring generations, as a mechanism to facilitate offspring establishment (Latzel et al., 2014; Dong et al., 2017, 2018a). Therefore, it is reasonable to predict that parent ramets of *P. stratiotes* growing under low nutrients would have increased the root production, and this plastic response would have been transmitted to the subsequent offspring generations (F1, F2, and F3). In this situation, offspring ramets having a higher proportion of roots and growing under high nutrients would be able to make better use of them and achieve maximum nutrient acquisition efficiency, which translates into the highest growth. Unfortunately, leaf, stem, and root of *P. stratiotes* were harvested together, and biomass allocation ratios were not available in our study. Therefore, to truly test this plausible explanation, studies should consider not only parental effects in terms of biomass production but also in terms of biomass allocation ratio.

Our finding was opposite to previous studies showing that the high-quality offspring produced in favorable parental environments benefited subsequent growth of offspring (Zas et al., 2013; Dong et al., 2018a, 2019; Portela et al., 2020), as stated by the "silver-spoon" effect, where parent plant growing in favorable conditions can provide more resources to their

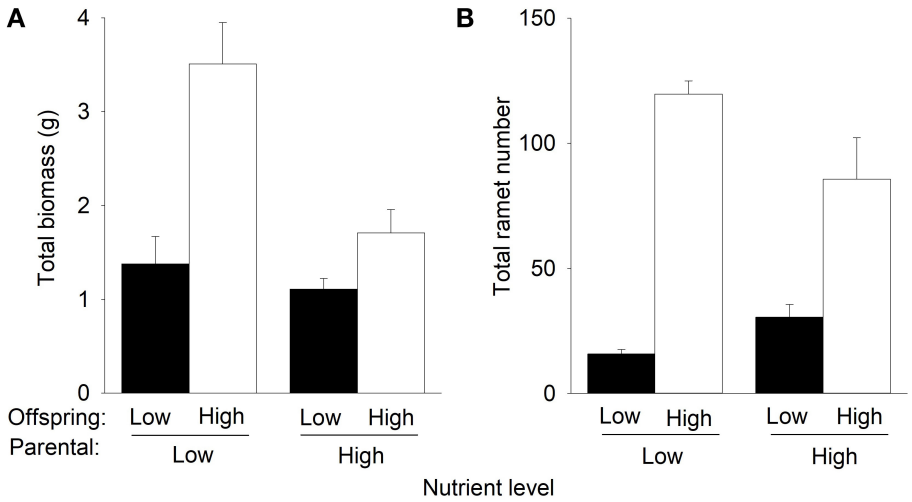

FIGURE 4 | Effects of parental nutrient level and offspring nutrient level on **(A)** total biomass and **(B)** total ramet number of *Pistia stratiotes* produced by the F2 offspring ramets in the second phase of the experiment. Bars show means + SE.

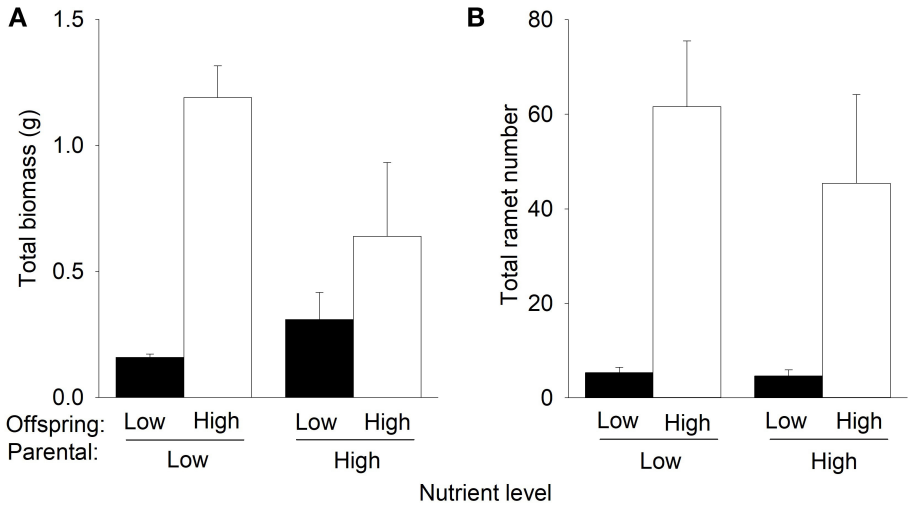

FIGURE 5 | Effects of parental nutrient level and offspring nutrient level on **(A)** total biomass and **(B)** total ramet number of *Pistia stratiotes* produced by the F3 offspring ramets in the second phase of the experiment. Bars show means + SE.

offspring (Roach and Wulff, 1987). In addition, our results also do not fit with another potential benefit of parental effects, which states that offspring generations could gain an advantage when establishing in the same or similar conditions to those experienced by their parents (Dong et al., 2017, 2018a).

The "silver-spoon" effect was detected when the F3 offspring ramets of *P. stratiotes* were grown under low nutrients but was absent when the F1 and F2 offspring ramets were grown under low nutrients. Also, the magnitude of parental nutrient effects on the subsequent growth of the F1, F2, and F3 offspring ramets in high nutrients were similar (**Figures 3–5**). These results do not support the third hypothesis that the magnitude of parental effects would decrease with increasing vegetative offspring generations. This is very likely because the difference in the size advantage of offspring of different generations cannot transmit to their growth benefits, as discussed above.

CONCLUSIONS

We conclude that nutrient-induced clonal parental effects can influence offspring growth of different vegetative generations, suggesting that clonal parental effects can transmit fast. However, heavier and, likely, bigger ramets produced under high nutrients in the parental generation could not increase the subsequent growth of offspring. Thus, parental provisioning in favorable conditions may not always increase the growth of their offspring, partly depending on root allocation but not ramet biomass.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

L-MZ and F-HY designed the experiment. L-MZ, J-FZ, W-HY, C-YQ, and D-HW performed the experiment and collected data. L-MZ and J-FZ analyzed the data. L-MZ wrote the first draft of the manuscript. F-HY and SR contributed substantially to the revisions. All authors contributed to the article and approved the submitted version.

FUNDING

The research was supported by the National Natural Science Foundation of China (Grants 32071527 and 32101261).

ACKNOWLEDGMENTS

We thank Si-Mei Yao and Yu Jin for their assistance with harvesting.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.890309/full#supplementary-material

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TYPE Original Research
PUBLISHED 22 July 2022
DOI 10.3389/fpls.2022.924001

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

RECEIVED 20 April 2022
ACCEPTED 30 June 2022
PUBLISHED 22 July 2022

CITATION
Yu W-H, Zhang L-M, Luo F-L, Yu F-H and Li M-H (2022) Roles of clonal parental effects in regulating interspecific competition between two floating plants.
Front. Plant Sci. 13:924001.
doi: 10.3389/fpls.2022.924001

Roles of clonal parental effects in regulating interspecific competition between two floating plants

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Parental effects can influence offspring fitness, which may further impact interspecific competition. However, few studies have tested the role of clonal parental effects in regulating interspecific interactions and examined the underlying mechanisms. We conducted two consecutive experiments with two clonal plants (*Pistia stratiotes* and *Eichhornia crassipes*). In the first experiment, the mother ramet of *P. stratiotes* and *E. crassipes* were grown in two nutrient levels and treated with a DNA demethylation reagent (5-azacytidine) or not. In the second experiment, the offspring ramets from each of the four treatments in the first experiment were grown alone (no competition) or with a heterospecific neighbor (with interspecific competition). We found no parental nutrient effect on the competitive ability of *E. crassipes*, but a significant parental nutrient effect of both *E. crassipes* and *P. stratiotes* on the competitive ability of *P. stratiotes*. Furthermore, the parental nutrient effect of *P. stratiotes* on the competitive ability of *P. stratiotes* varied depending on the DNA methylation status of both *P. stratiotes* and *E. crassipes*. These clonal parental effects were related to resource provisioning and/or DNA methylation. We conclude that clonal parental nutrient effects can regulate interspecific competition between *P. stratiotes* and *E. crassipes* by altering the competitive ability of *P. stratiotes*. Both resource provisioning and epigenetic mechanisms can be involved in these clonal parental effects. By regulating interspecific competition, clonal parental effects may further influence species coexistence, community structure, and ecosystem functioning.

KEYWORDS

clonal plants, DNA demethylation, epigenetic inheritance, maternal effect, nutrients, transgenerational plasticity

Introduction

The environmental condition of a parent can influence the phenotype of their offspring (Wulff and Roach, 1987; Badyaev and Uller, 2009). Such a parental (environmental) effect can be transmitted to offspring generations *via* sexual propagules such as seeds (sexual parental effects) or clonal propagules such as offspring ramets (clonal parental effects) (Miao et al., 1991a; Agrawal, 2002; Dong et al., 2017; González et al., 2018; Baker et al., 2019; Luo et al., 2022). A large body of evidence shows that parental effects can influence fitness measures (e.g., growth and production) of offspring (Galloway, 2005; Latzel et al., 2014; Dong et al., 2018a,b), which may cascade to impact population- and community-level patterns and processes (Miao et al., 1991b; Bossdorf et al., 2009; Castro et al., 2013; Baker et al., 2019; Luo et al., 2022).

In nature, most plants do not grow alone, and interactions with heterospecific neighbors (i.e., interspecific interactions) are common (Grace and Tilman, 1990; Du et al., 2004). The significant role of parental effects in regulating fitness measures of offspring may further affect their interspecific interactions with heterospecific neighbors (Miao et al., 1991b; Bossdorf et al., 2009), thereby generating profound impacts on population dynamics, species coexistence, biodiversity maintenance, and ecosystem functions and services (Zou and Xu, 1998; Kraft et al., 2015; Valladares et al., 2015). If, for instance, parental effects can improve the growth of offspring (Latzel et al., 2014; Dong et al., 2017, 2018a, 2019b), then they may enhance their competitive ability (Miao et al., 1991a; Bossdorf et al., 2009). On the contrary, if parental effects can reduce the growth of offspring, e.g., in some cases due to phenotypic changes (Galloway, 2005), then they may weaken their competitive ability (Bossdorf et al., 2009). However, studies on the roles of parental effects in regulating interspecific interactions are still limited (Bossdorf et al., 2009), and the few existing studies in this field focused mostly on the role of sexual parental effects in non-clonal plants (Miao et al., 1991a,b; Bossdorf et al., 2009). No studies have considered clonal parental effects on interspecific interactions (Luo et al., 2022).

Compared with non-clonal plants, clonal plants can avoid genetic variation due to meiosis, so that the epigenetic information of environmental interactions experienced by parents can be transmitted to offspring more effectively (Latzel and Klimešová, 2010; Verhoeven and Preite, 2014; Luo et al., 2022). As clonal propagules (e.g., ramets) are larger in size and mass than sexual propagules (e.g., seeds), the potential for resource provisioning might be relatively high in clonal than in non-clonal plants (Dong et al., 2019a). Therefore, parental effects may be very important for clonal plants (Verhoeven and Preite, 2014; Dodd and Douhovnikoff, 2016), particularly for those with a low ability of sexual reproduction. Thus, testing clonal parental effects on interspecific competition can deepen our understanding of the community-level roles of parental effects in clonal plants (Luo et al., 2022).

Epigenetic inheritance is an important mechanism underlying clonal parental effects (Jablonka and Raz, 2009; Latzel and Klimešová, 2010; Richards et al., 2017; González et al., 2018), and DNA methylation is one of the most important epigenetic modifications (Schulz et al., 2014). Even if the environment of offspring is different from their mother's and the original stimulus of DNA methylation disappears, the offspring can still retain the previous methylation imprint after DNA replication (Rico et al., 2014). This epigenetic mechanism enables plants to remember past environmental experience, predict and overcome future environmental stress, and carry adaptive information through reliable transmission and selection over multiple generations (Schulz et al., 2014). By applying DNA demethylation reagent (e.g., 5-azacytidine) to plants, it was found that DNA methylation plays an important role in clonal parental effects on the morphology and growth of offspring (González et al., 2016, 2017; Portela et al., 2020). Consequently, DNA methylation may also regulate clonal parental effects on interspecific interactions. However, the epigenetic mechanism of parental effects on interspecific interactions has not been confirmed.

We conducted two consecutive experiments with two clonal floating plants (*Pistia stratiotes* and *Eichhornia crassipes*) to explore how clonal parental effects regulate interspecific interactions and the role of DNA methylation. We chose these two species because they frequently co-occur and compete with each other, and also because they can spread quickly by clonal growth. In the first experiment, the mother ramet of *P. stratiotes* and *E. crassipes* were grown in two nutrient levels and treated with a DNA demethylation reagent (5-azacytidine) or not. In the second experiment, the offspring ramets from each of the four treatments in the first experiment were grown alone (no competition) or with a heterospecific neighbor (with interspecific competition). Specifically, we tested two hypotheses: (1) clonal parental effects can alter interspecific interactions by influencing DNA methylation level; (2) the offspring produced by a mother ramet under the high nutrient condition were more competitive than those produced by a mother under the low nutrient condition, because parents under the high nutrient condition can produce offspring of higher quality.

Materials and methods

Study species

Pistia stratiotes L. (water lettuce, Araceae) is a stoloniferous floating rosette herb (Pettet and Pettet, 1970; Adomako et al., 2020, 2021). The main (i.e., vertical) stem is short with highly compressed internodes so that leaves are clustered (Odjegba and Fasidi, 2004; Adomako et al., 2021, 2022). Stolons come out from leaf axils, and new ramets are produced from stolon

tips (Odjegba and Fasidi, 2004). This species is native to South America, and are now widely distributed in tropical and subtropical regions around the world, including China (Evans, 2013; Hussner et al., 2014). As one of the wetland weeds, a massive accumulation of *P. stratiotes* leads to the decline of biodiversity in local ecosystems; therefore, it has been included in the Global Invasive Species Database, and are also listed as one of the most dangerous invasive species in China (Wang et al., 2014; Galal et al., 2019).

Eichhornia crassipes (Mart.) Solms (water hyacinth, Pontederiaceae) is a stoloniferous floating rosette herb with a similar morphology and distribution of *P. stratiotes* (Wang et al., 2016). This species is also listed as an aggressive invasive species in many counties, including China (Wang et al., 2016), because it can quickly spread by clonal growth to form a dense mat on water surface and rapidly displace local species (Zhao, 2006). *Eichhornia crassipes* and *P. stratiotes* share similar niches and occur in lakes, rivers, ponds, and diches (Wang et al., 2016).

Sampling and cultivation

On July 5, 2020, a clone of ramets of both *P. stratiotes* and *E. crassipes* were collected from Yongning River (28°40′3″N, 121°23′4″E) in Taizhou, Zhejiang Province, China. They were brought to a greenhouse at Taizhou University, where they were vegetatively propagated in tanks (95 cm in diameter × 60 cm in height) filled with water. On July 26, 2020, 74 newly produced offspring ramets with similar size were selected for both *P. stratiotes* and *E. crassipes* (148 ramets in total), and the stolons attached to these ramets, if any, were removed. For each species, ten of the 74 ramets were randomly selected and dried for 48 h at 70°C and weighed to measure the initial dry mass (1.08 ± 0.04 g for *P. stratiotes* and 1.32 ± 0.03 g for *E. crassipes*; mean ± SE). The remaining 64 ramets of both *P. stratiotes* and *E. crassipes* were used in the experiments described below.

Experiment design

The study consisted of two consecutive experiments. The first experiment was launched on July 26, 2020. We randomly subjected the 64 ramets (thereafter referred to as the mother ramets) of both *P. stratiotes* and *E. crassipes* to two nutrient levels (high and low) and two DNA demethylation treatments (ramets were treated with a DNA demethylation agent or not). Thus, for both species, each of the four treatments was replicated 16 times (with 16 mother ramets). Each mother ramet was grown in a bucket (65 cm in diameter × 44 cm in height) filled with 13 L of either a high or a low nutrient solution (30 and 6% Hoagland solution, respectively). The Hoagland solution contained 945 mg/L Ca $(NO_3)_2{\cdot}4H_2O$, 506 mg/L KNO_3, 80 mg/L NH_4NO_3, 136 mg/L KH_2PO_4, 493 mg/L $MgSO_4$, 13.9 mg/L $FeSO_4{\cdot}7H_2O$, and 18.7 mg/L EDTA·2Na. We chose these two nutrient levels because the nitrogen and phosphorous concentrations are within the gradient of nutrient levels found in water body in China (Zhang et al., 2017) and also because their difference is likely to induce a significant effect on plant growth. For DNA demethylation, 10 ml of 50 μM 5-azacitidine (5-azaC) solution was sprayed to each plant once every 3 days. For the treatment without DNA demethylation, 10 ml distilled water was sprayed.

To supplement for evapotranspiration and nutrient loss due to plant uptake, 1 L of 30% or 6% Hoagland nutrient solution was added weekly to each bucket. The first experiment lasted for 3 weeks and ended on August 16, 2020. At the end of the experiment, 2–3 offspring ramets in each bucket (produced by each mother ramet) were selected as the materials for the second experiment, and the remaining parts in each bucket were harvested to measure growth traits.

The second experiment started on 16 August 2020. For each species, the offspring ramets produced by the mother ramets grown in each of the four conditions (i.e., high and low nutrients with and without DNA demethylation) in the first experiment were randomly assigned to one of five treatments: the target ramet was grown alone (no competition, one treatment), with a ramet of a different species whose mother ramet was grown in the same condition (interspecific competition from ramets with the same parental effects; one treatment) and with a ramet of a different species whose mother ramet was grown in a different condition (interspecific competition from ramets with different parental effects; three treatments). The 16 treatments with interspecific competition were shared by the two species, resulting in a total of 24 treatments (eight treatments without competition and 16 treatments with competition).

Ramets were grown in buckets (65 cm in diameter × 44 cm in height) filled with 30% Hoagland nutrient solution. Each treatment was replicated seven times, making a total of 168 buckets with a total of 280 ramets. To compensate for water loss and nutrient consumption, 1 L of 30% Hoagland nutrient solution was added weekly to each bucket. The second experiment lasted for 3 weeks and ended on September 6, 2020. The mean temperature was 29.2°C and the mean relative humidity was 79.8% (measured hourly with a Hygrochron temperature loggers; iButton DS1923; Maxim Integrated Products, United States). At noon, the photosynthetic photon flux density at water surface was 632–1806 μmol m^{-2} s^{-1} (Li-250A; LI–COR Biosciences, United States).

Harvest and measurements

At the end of the first experiment, we counted, for each species, the number of offspring ramets in each bucket, and measured biomass after oven-drying them at 70°C for 72 h. At the end of the second experiment, we measured biomass of each species in each bucket after drying them at

70°C for 72 h. Biomass per ramet was calculated as total biomass/number of ramets.

Data analysis

Two-way ANOVA was used to test the effects of the nutrient level (high and low) and DNA demethylation (treated with 5-azaC or not) on total biomass, number of ramets and biomass per ramet of *P. stratiotes* and *E. crassipes* separately for the first experiment. To analyze the data from the second experiment, we first quantified the competitive response of a target plant by calculating log response ratio of biomass (LnRR), i.e., LnRR = ln(total biomass of a target ramet grown with a competing ramet/average biomass of the target ramet grown alone across the seven replicates) (Goldberg et al., 1999; Hedges et al., 1999). This calculation was carried out for each type of ramets of each species. One-sample *t*-test was used to analyze whether the mean value of the competitive response of each treatment was significantly different from zero. Then we used four-way ANOVA to examine the effects of the nutrient level and DNA demethylation of the target's mother ramet and the nutrient level and DNA demethylation of the competitor's mother ramet on the competition response (LnRR) of the target plant.

Before analysis, data on biomass, number of ramet and biomass per ramet were checked for normality (by Kolmogorov–Smirnov test) and homogeneity of variance (by Levene's test). During the first experiment, one replicate of *P. stratiotes* in the low nutrient level and not treated with 5-azaC, one replicate of *E. crassipes* in high nutrient level and treated with 5-azaC and one replicate of *E. crassipes* in low nutrient level and treated with 5-azaC were completely destroyed by herbivores. During the second experiment, plants in two buckets (one with a ramet of *P. stratiotes* whose mother was grown in the high nutrient level and treated with 5-azaC and a ramet of *E. crassipes* whose mother was grown in the low level and not treated with 5-azaC, and one with a ramet of *P. stratiotes* whose mother was grown in the high nutrients level and not treated with 5-azaC and a ramet of *E. crassipes* whose mother was grown in the low nutrient level and treated with 5-azaC) were also dead. Statistical analyses were carried out with SPSS 22.0 (IBM Corp., Armonk, NY, United States).

Results

Effects of nutrients and DNA demethylation on performance in the first experiment

Compared with the low nutrient level, the high nutrient level significantly increased both biomass (by 30 and 14%) and number of ramets (by 70 and 126%) of *P. stratiotes* and *E. crassipes* (**Table 1** and **Figures 1A,B,D,E**). However, the final mean size of the ramets, as measured by biomass per ramet, was significantly higher in the low than in the high nutrient level for both species (**Table 1**): biomass per ramet of *P. stratiotes* was about 1.3 times larger under the low than under the high nutrient level (**Figure 1C**), and biomass per ramet of *E. crassipes* was about 2.0 times larger (**Figure 1F**). Compared to the control (no 5-azaC), the application of 5-azaC significantly reduced total biomass (by 32%) and biomass per ramet (by 37%) of *P. stratiotes*, but had no effect on ramet production (**Table 1A** and **Figures 1A–C**). The application of 5-azaC did not significantly affect total biomass, number of ramets and biomass per ramet of *E. crassipes* (**Table 1B** and **Figures 1D–F**).

Parental effects on interspecific interactions in the second experiment

The nutrient level of the target's mother ramet, the nutrient level of competitor's mother ramet and the demethylation of the competitor's mother ramet had no significant effect on the competitive response of the target plant of *P. stratiotes* (**Table 2B**; Appendix **Figure 1**). For both *E. crassipes* and *P. stratiotes*, the application of the 5-azaC to the mother ramet of the target plant significantly decreased the competitive response of the target (**Table 2** and **Figures 2**, **3A**). The competitive response of *P. stratiotes* became less negative when the competitor's mother had been grown under the high nutrient level than when it had been grown under the low nutrient level (**Table 2A** and **Figure 3B**). We observed a significant interactive effect between the nutrient level of the target's mother ramet and DNA demethylation of the competitor's mother ramet and between the nutrient level of the target's mother ramet and DNA

TABLE 1 ANOVA results for effects of nutrient level and DNA demethylation on the growth of (A) *Pistia stratiotes* and (B) *Eichhornia crassipes* in the first experiment.

	Total biomass		No. of ramets		Biomass per ramet	
Effect	***F***	***P***	***F***	***P***	***F***	***P***
(A) *P. stratiotes*						
Nutrient level (N)	**23.75**	**<0.001**	**96.48**	**<0.001**	**14.00**	**<0.001**
Demethylation (D)	**53.31**	**<0.001**	2.56	0.115	**48.62**	**<0.001**
N × D	2.14	0.149	0.63	0.43	0.15	0.702
(B) *E. crassipes*						
Nutrient level (N)	**11.54**	**<0.001**	**259.85**	**<0.001**	**89.44**	**<0.001**
Demethylation (D)	0.073	0.789	1.59	0.212	0.03	0.867
N × D	0.485	0.489	3.305	0.074	0.52	0.473

Degree of freedom is 1, 59 for all effects of *P. stratiotes* and 1, 58 for all effects of *E. crassipes*. Values are in bold when $P < 0.05$.

demethylation of the target's mother ramet on the competitive response of the target plant of *P. stratiote* (Table 2A). When the competitor' mother was not treated with 5-azaC, the competitive response of the target plant of *P. stratiote* was not significantly different from zero if the target' mother had been grown under the low nutrient level, but was highly significantly negative when it had been grown under the high nutrient level; however, when the target's mother was treated with 5-azaC, a reverse pattern was observed (Figure 4). When the target' mother was not treated with 5-azaC, the competitive response of the target plant of *P. stratiote* was significantly negative if the target' mother had been grown under the low nutrient level, but was close to zero when it had been grown under the high nutrient level; however, when the target's mother was treated with 5-azaC, a reverse pattern was observed (Figure 5).

Discussion

Effects of nutrient availability and 5-azaC on offspring growth

As expected, high nutrient availability of the mother ramets promoted total biomass and number of offspring ramets of both

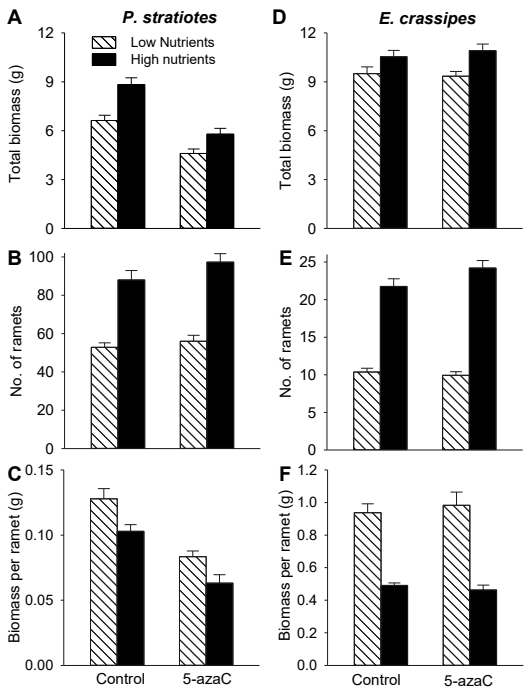

FIGURE 1
Effects of nutrient level (high and low) and DNA demethylation (control and 5-azaC) on the growth of offspring ramets of **(A–C)** *Pistia stratiotes* and **(D–F)** *Eichhornia crassipes*. Bars show means + SE.

TABLE 2 Effects of nutrient level and DNA demethylation of the target's mother and the competitor's mother on the interspecific competitive response (LnRR) of the target plant of (A) *Pistia stratiotes* and (B) *Eichhornia crassipes* in the second experiment.

	(A) *P. stratiotes*		(B) *E. crassipes*	
Effect	***F***	***P***	***F***	***P***
Nutrient level of target's mother (TN)	1.72	0.193	0.28	0.596
Demethylation of target's mother (TD)	**7.59**	**0.007**	**9.98**	**0.002**
Nutrient level of competitor's mother (CN)	**8.99**	**0.003**	0.26	0.612
Demethylation of competitor's mother (CD)	0.10	0.754	1.16	0.284
TN × TD	**28.35**	**<0.001**	0.421	0.518
TN × CN	1.52	0.220	0.08	0.779
TN × CD	**7.76**	**0.006**	0.02	0.901
TD × CN	<0.01	0.975	2.12	0.149
TD × CD	0.434	0.512	1.06	0.306
CN × CD	1.33	0.252	0.06	0.812
TN × TD × CN	0.09	0.764	0.20	0.655
TN × TD × CD	0.95	0.334	0.39	0.534
TN × CN × CD	2.40	0.125	0.141	0.708
TD × CN × CD	0.01	0.909	0.10	0.758
TN × TD × CN × CD	1.06	0.305	1.47	0.228

Degree of freedom is 1, 94 for all effects and for both species. Values are in bold when $P < 0.05$.

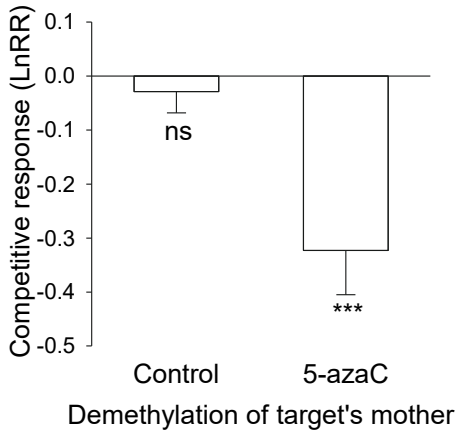

FIGURE 2
The effect of DNA demethylation of the target's mother on the competitive response of the target plant of *Eichhornia crassipes*. Bars show means + SE. Symbols (***$P < 0.001$) indicate significant difference from zero (by one-sample *t*-test).

P. stratiotes and *E. crassipes* (Figure 1), which is consistent with previous findings on other aquatic plants (Zhao, 2006; Jampeetong and Brix, 2008; Zhang et al., 2020). This effect is

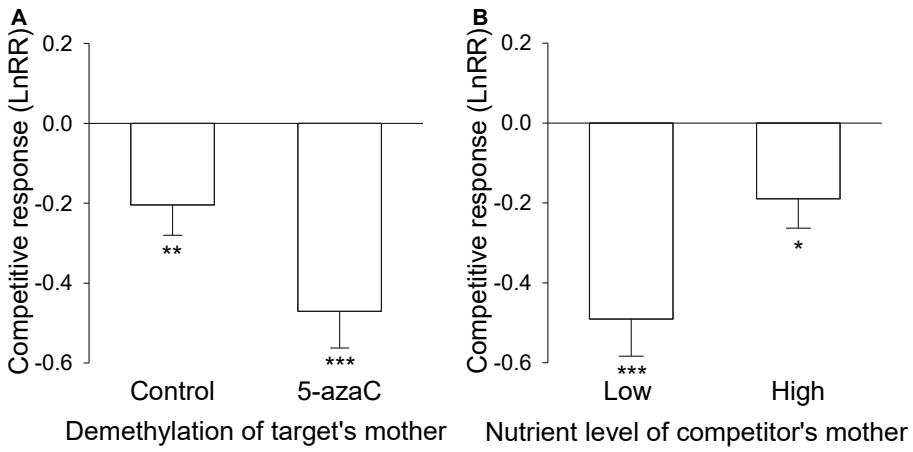

FIGURE 3
(A) The effect of DNA demethylation of the target's mother and **(B)** the effect of the nutrient level of the competitor's mother on the competitive response of the target plant of *Pistia stratiotes*. Bars show means + SE. Symbols (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$) indicate significant difference from zero (by one-sample *t*-test).

related to physiological integration as mother ramets growing under higher nutrient availability can transfer more resources (photo-assimilates and nutrients) to newly produced offspring ramets that remain connected to them (Slade and Hutchings, 1987a,b; Wang et al., 2017, 2021; Portela et al., 2021). However, the magnitude of this growth promotion was stronger for number of ramets than for total biomass so that biomass per offspring ramet was significantly smaller under high than under low nutrient availability (**Figures 1C,F**). This result suggests that the favorable nutrient environment experienced by the mother ramets can increase the fitness of the whole offspring population, but may not necessarily promote the competitive ability of individual offspring in their subsequent growth and development.

We observed that application DNA demethylation agent 5-azaC decreased biomass per offspring ramet of *P. stratiotes* as it decreased total biomass of all offspring ramets but had no effect on their number. This finding indicates that, in addition to DNA demethylation, 5-azaC was toxic to the growth of *P. stratiotes*. A detrimental effect of 5-azaC on plant growth and development has also been reported in other species (Fieldes, 1994; Finnegan et al., 1996; Bossdorf et al., 2010), which should be considered when parental effects are subsequently considered. However, 5-azaC application had no effect on the growth of offspring ramets of *E. crassipes*, suggesting no side effect of 5-azaC on the growth of *E. crassipes*.

Parental nutrient effects on interspecific interactions

Parental effects were found to frequently influence fitness measures of offspring (Latzel and Klimešová, 2010; González et al., 2016; Dong et al., 2019a), and thus may further influence their competitive ability when they grow with heterospecific neighbors (Miao et al., 1991a; Baker et al., 2019). For examples, parental effects improved the growth and thus competitive ability of *Polygonum persicaria* offspring in shade conditions (Baker et al., 2019). If two co-existing species both benefit from parental effects but the degree of the benefit is different (Li et al., 2021), or if parental effects can influence fitness measures of one species but had no impact on the other (Miao et al., 1991a), then parental effects can modify the competitive interactions of these co-occurring species. We found no clonal parental nutrient effects on the competitive ability of *E. crassipes* (**Table 2B**), but significant clonal parental nutrient effects on that of *P. stratiotes* (**Table 2A** and **Figures 3B**, **4**, **5**). Consequently, clonal parental nutrient effects significantly influenced the competitive interaction between *E. crassipes* and *P. stratiotes*. In a previous study, Miao et al. (1991a) reported signifiant sexual parental nutrient effects on the competitive ability of two *Plantago* species, showing the competitive ability of *Plantago major* depended on the period of parental nutrient pulse, while that of *Plantago rugelii* depended on maternal background nutrient levels. If plant communities are highly niche-differentiated, then parental effects may facilitate species coexistence (Li et al., 2021).

We observed significant parental nutrient effects of the competitor *E. crassipes* on the competitive ability of the target *P. stratiotes* (**Figure 3B**). The competitive ability of the offspring ramet of *P. stratiotes* became much lower when it competed with the offspring ramet of *E. crassipes* produced by the mother ramet growing under low than under high nutrient availability. It is well-known that the size of a plant is commonly positively related to its competitive ability (Goldberg and Fleetwood, 1987; Gaudet and Keddy, 1988). Because the mother ramet

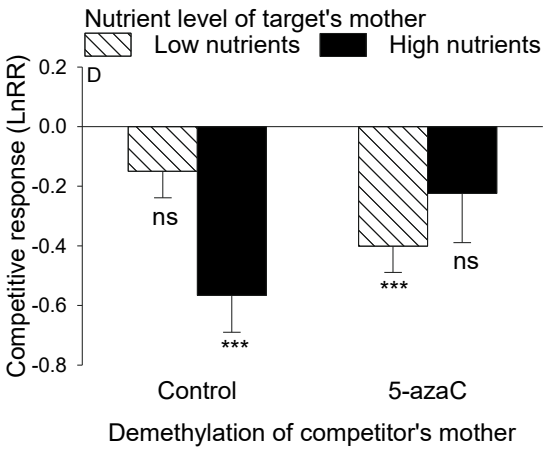

FIGURE 4
The interactive effect between DNA demethylation of the competitor's mother and the nutrient level of the target's mother on the competitive response of the target plant of *Pistia stratiotes*. Bars show means + SE. Symbols (***$P < 0.001$) indicate significant difference from zero (by one-sample *t*-test).

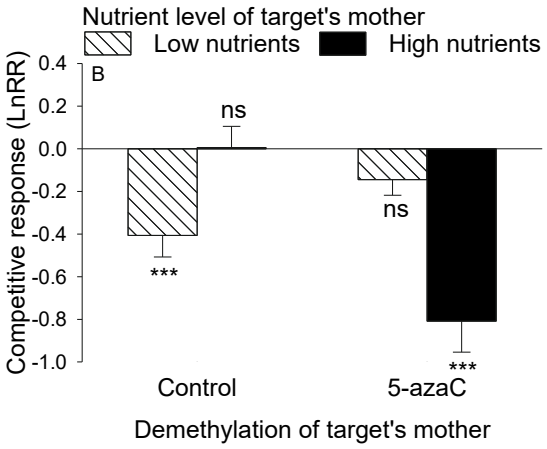

FIGURE 5
The interactive effect between nutrient level and DNA demethylation of the target's mother on the competitive response of the target plant of *Pistia stratiotes*. Bars show means + SE. Symbols (***$P < 0.001$) indicate significant difference from zero (by one-sample *t*-test).

of *E. crassipes* produced much larger offspring ramets under low than under high nutrient availability (**Figure 1F**), the competitive ability of the offspring ramets originated from the mother ramet growing under low nutrients would show a much greater competitive ability when competing with *P. stratiotes*. Consequently, the parental low nutrient effect of *E. crassipes* greatly reduced the competitive ability of the offspring ramet of *P. stratiotes*. As the size of the offspring (biomass per offspring ramet) is closely related to their ability of resource provisioning (Dong et al., 2019a), the parental nutrient effect of *E. crassipes* on the competitive ability of *P. stratiotes* was likely due to resource provisioning, as reported before (Wulff and Roach, 1987; Herman and Sultan, 2011; Zas et al., 2013). However, this parental nutrient effect of *E. crassipes* was not affected by the application of 5-azaC, suggesting that DNA methylation played little role during this process (Griffin et al., 2016).

We also observed a parental nutrient effect of *P. stratiotes* on the competitive ability of its offspring ramet, but such an effect varied depending on the DNA methylation status of the mother ramet of the competitor *E. crassipes* (**Figure 4**). Without application of 5-azaC to the mother ramet of *E. crassipes*, the competitive ability of the offspring ramet of *P. stratiotes* was much smaller when its mother ramet had been grown under the high than under the low nutrient level. This parental nutrient effect can also be explained by resource provisioning (Wulff and Roach, 1987; Herman and Sultan, 2011; Germain et al., 2013; Zas et al., 2013) as the size of the offspring ramet of *P. stratiotes* was significantly smaller when its mother ramet had been grown under the high than under the low nutrient level (**Figure 1C**). However, with application of 5-azaC to the mother ramet of *E. crassipes*, the nutrient level of the mother ramet of *P. stratiotes* had no significant effect on the competitive ability of its offspring ramet, suggesting that DNA demethylation of the competitor's mother can alter the parental nutrient effect of the target plant. The underlying mechanism for this observation is not clear and deserves further studies.

The parental nutrient effect of *P. stratiotes* on the competitive ability of its offspring ramet also varied depending on DNA demethylation status of mother ramet (**Figure 5**). Without application of 5-azaC to the mother ramet of *P. stratiotes*, competition from *E. crassipes* resulted in a significantly negative competitive response of the offspring ramet of *P. stratiotes* when its mother had been grown under the low nutrient level, but had no significant negative effect on the offspring ramet of *P. stratiotes* when its mother ramet had been grown under the high nutrient level. This result cannot be explained by resource provisioning (Cheplick, 1997) as the size of the offspring ramet of *P. stratiotes* was significantly smaller when its mother ramet had been grown under the high than under the low nutrient level (**Figure 1C**). When the mother ramet of *P. stratiotes* was treated with 5-azaC, an opposite pattern was observed (**Figure 5**). These results suggest that epigenetic mechanisms such as DNA methylation must have played a role in mediating the parental nutrient effect, as reported also in other studies (Latzel and Klimešová, 2010; Verhoeven and Preite, 2014; González et al., 2016).

Parental 5-azaC effects on interspecific interactions

Average across all other treatments, application of 5-azaC to the mother ramet of *P. stratiotes* markedly decreased the

competitive ability of its offspring ramets when competing with *E. crassipes* (**Figure 3A**), suggesting a parental effect of 5-azaC application. This parental effect was likely caused by resource provisioning as the size of the offspring ramet was significantly reduced when the mother ramet was treated with 5-azaC (**Figure 1C**) so that the offspring contained less energy for their subsequent growth and competition (Goldberg and Fleetwood, 1987; Gaudet and Keddy, 1988).

Surprisingly, application of 5-azaC to the mother ramet of *E. crassipes* greatly decreased the competitive ability of its offspring ramet (**Figure 2**), despite the fact that it did not influence the size of the offspring (**Figure 1F**). Thus, resource provisioning cannot explain this clonal parental effect and DNA methylation might have played a role.

Conclusions

This appears the first study testing the role of clonal parental effects in shaping interspecific competition between plants. Our findings suggest that clonal parental nutrient effects can regulate interspecific competition between *P. stratiotes* and *E. crassipes* by altering the competitive ability of *P. stratiotes* in different ways. Both resource provisioning and epigenetic mechanisms can be involved in these clonal parental effects. By regulating interspecific competition, clonal parental effects may further influence species coexistence, community structure, and ecosystem functioning.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

W-HY conducted the experiment, analyzed the data, and drafted the manuscript. L-MZ assisted with data analysis and contributed substantially to manuscript revision. F-LL and M-HL contributed substantially to manuscript revision. F-HY designed the experiment and contributed substantially to manuscript revision. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the National Natural Science Foundation of China (grants: 32071527, 3210126, and 32071525) and National College Students' Innovation and Entrepreneurship Training Program (202010350031).

Acknowledgments

We thank Jia-Fan Zhang, Chen-Yan Qiu, Dan-Hao Wang, Si-Mei Yao, and Yu Jin for assistance with harvesting.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.924001/full#supplementary-material

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ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fpls.2022.919409

Nutrient Inputs Alleviate Negative Effects of Early and Subsequent Flooding on Growth of *Polygonum hydropiper* With the Aid of Adventitious Roots

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Edited by:
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Specialty section:
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

Received: 13 April 2022
Accepted: 21 June 2022
Published: 22 July 2022

Citation:
Chen Y-H, Wei G-W, Cui Y and Luo F-L (2022) Nutrient Inputs Alleviate Negative Effects of Early and Subsequent Flooding on Growth of Polygonum hydropiper With the Aid of Adventitious Roots. Front. Plant Sci. 13:919409. doi: 10.3389/fpls.2022.919409

Riparian plants are exposed to harmful stress induced by flooding, which is often accompanied by eutrophication in the Three Gorges Reservoir Region. The phenomenon is mainly caused by domestic sewage discharges, slow water flow, and agricultural fertilizer pollution. Simulating abiotic stress, such as flooding at the initial period, can act as a signal and induce positive responses of plants to subsequent severe stress. In addition, eutrophication supplies nutrients, provides a favorable environment in the early stages of plant, and facilitates good performance in later development. However, whether early flooding (with or without eutrophication) acts as positive cue or as stress on plants at different developmental stages remains unclear. To address this question, seeds of *Polygonum hydropiper* were collected from low and high elevations in the hydro-fluctuation belt of the Three Gorges Reservoir Region. Plants germinated from these seeds were subjected to shallower and shorter early flooding treatments with or without eutrophication. Subsequently, plants were subjected to deeper and longer flooding treatments with or without eutrophication. Early flooding and eutrophic flooding significantly induced generation of adventitious roots, suggesting morphological adaptation to flooding. Although early flooding and eutrophic flooding treatments did not increase plant biomass in subsequent treatments compared with control, stem length, length and width of the 1st fully expanded leaf, and biomass of plants in the early eutrophic treatment were higher than these of the early flooding treatment plants. These results suggest a negative lag-effect of early flooding, and also indicate that nutrient inputs can alleviate such effects. Similarly, subsequent eutrophic flooding also enhanced plant growth compared with subsequent flooding, showing significantly higher values of leaf traits and adventitious root number. Plants originated from low elevation had significantly higher functional leaf length and stem biomass compared with those from high elevation. These results suggest that nutrient inputs can alleviate negative effects of early and subsequent flooding on growth of *P. hydropiper* with the generation of adventitious roots.

Keywords: adventitious root, early flooding, eutrophication, submergence, Three Gorges Reservoir Region

INTRODUCTION

Flooding is one of the detrimental stresses for riparian plants (Ryser et al., 2011; Ayi et al., 2019). Hypoxia and low light intensity because of partial or complete coverage with water can limit photosynthesis and respiration in plants. This may result in growth retardation or even plant death (Striker et al., 2012, 2014; Kaspary et al., 2020; Kolton et al., 2020; Hartman et al., 2021). However, it has been shown that the stimulation of mild flooding at an early period can act as a signal and induce positive plant responses to subsequent severe flooding stress (Yin et al., 2014; Li et al., 2020a). When plants were subjected to flooding, both direct and indirect oxygen sensing mechanisms can quickly react to domestication responses. The resulting changes can improve the flood-tolerance of plants (Bailey-Serres et al., 2012; Yang, 2014; Herzog et al., 2016; Striker and Colmer, 2017; Striker et al., 2019). In plants, these stimulation effects of flooding are often reflected by the formation of adventitious roots within several days or even hours after flooding (Iturralde Elortegui et al., 2020; Joshi and Ginzberg, 2021). Under hypoxic stress conditions, wheat seedlings form lysigenous aerenchyma in roots and develop further, which induce advanced adaptation before the environment becomes hypoxic (Yamauchi et al., 2014; Jia et al., 2021). In addition, in response to low oxygen levels caused by shallow and prolonged flooding, plants can elongate shoots to restore contact with the atmosphere, and thus overcome flooding stress (Striker et al., 2012; Chen et al., 2019). However, negative impacts of early flooding on growth and metabolism of plant seedlings have also been shown (Lim et al., 2015; Zhang et al., 2015; Yeap et al., 2019). Whether early flooding acts as a positive induction or stress on plant seedlings and on plants at their later developmental stage remains unclear.

The Three Gorges Reservoir (TGR) in China is one of the largest reservoirs in the world (Yang et al., 2012). The water level of the TGR fluctuates repeatedly over the year from an elevation of 145 m in summer to 175 m in winter, forming a hydro-fluctuation belt (HFB) with an area of ~349 km^2 (Lin et al., 2020). This causes plants that are naturally distributed at low and high elevations of the HFB to experience different flooding conditions and nutrient levels (Zhang et al., 2013; Liu et al., 2019). Generally, plants distributed at high elevation experience flooding at a lower frequency and for a shorter duration, while plants distributed at low elevation experience flooding at a higher frequency and for a longer duration (Schreiber et al., 2011; Chen et al., 2019). Plants at low and high elevations show different trait responses to flooding, which are particularly reflected in their leaf trait responses (Chen et al., 2009; Wei et al., 2020). Because the water level of the TGR rises gradually, early shallow flooding may induce growth responses in certain riparian species, but these responses may differ between low and high elevation plants. Moreover, in low and high elevation plants, the formation of "plastic memory" (environmentally induced phenotypic plasticity can sometimes be heritable; Portela et al., 2020) may be induced after stimulation by different levels of flooding stress for an extended period (Wei et al., 2021). This may be transmitted to the offspring through seeds produced by sexual reproduction, thus affecting the growth performance of offspring plants such as germination, biomass accumulation, and flowering. Consequently, offspring plants may adjust phenotypic strategies regulated by plastic memory to adapt to the environment that is similar to their maternal environment (Latzel et al., 2010; Alvarez et al., 2021; Jiang et al., 2021; Sánchez et al., 2021).

Eutrophication is very common in wetland ecosystems, including certain basins in the TGR region (Tercero et al., 2015; Tian et al., 2017; Huang et al., 2020; Wang et al., 2020). After the first impoundment of the TGR region in 2003, eutrophication often occurs in major tributaries (Li et al., 2020b). The effect of nutrient inputs on plant fitness at the early stage of plant development is important (Huber et al., 2012). Plant individuals that experience nourishing nutritional resources at the early stage of development often have higher phenotypic plasticity and lasting adaptability than plants that experience adverse conditions, i.e., see the silver-spoon effect (Hopwood et al., 2014). Therefore, eutrophication at an early developmental stage may alleviate flooding pressure on riparian plants (Qiu et al., 2020). In addition, the environment that parental plants experienced, or the environment offspring plants experienced early in their development may be similar to the environment adult plants will likely be exposed to. Thus, offspring plants would have higher fitness in the predicted environment at a later developmental stage (Auge et al., 2017). However, if phenotypes can respond to the parental environment and their own current environment, intragenerational plasticity is considered to evolve more easily than parental effects. The reason is that the offspring environment is more useful for predicting the future selective offspring environment than the parental environment (Alvarez et al., 2021).

The riparian plant *Polygonum hydropiper* is naturally distributed at both low and high elevations in the HFB in the TGR. This species has high phenotypic plasticity in response to flooding, and especially, plasticity in specific leaf area differs significantly between low and high elevation plants (Wei et al., 2020, 2021). Flooding significantly reduces growth, but induces adventitious root formation within hours after flooding (Wei et al., 2021). Therefore, the formation of adventitious roots can be considered as one of the most important developmental processes plants to employ when sensing flooding. In the lowest elevation (145 m) area of the TGR zone, it is difficult for plants to survive flooding, leading to low coverage and density of vegetation (Wang et al., 2009; Ye et al., 2013; Zhu et al., 2020). *P. hydropiper* is a native dominant species that naturally grows in this zone (Wang et al., 2009; Sun et al., 2011). Moreover, this species has a low demand for soil nutrients and a low nutrient release by its shoots even after extended soaking and decomposition time. Therefore, it is considered a suitable species for ecological restoration in this zone (Xiao et al., 2017). In this study, plants of *P. hydropiper* germinated from seeds collected from both low and high elevations were selected to address the following questions: (1) Does early flooding/eutrophic flooding affect the growth of plant seedlings? (2) Does early flooding/eutrophic flooding act as positive induction or stress on plant growth at a later developmental

stage? and (3) Are plants from low and high elevations responding differently to early and subsequent flooding?

MATERIALS AND METHODS

Study Material

Polygonum hydropiper L. (Polygonaceae) is an annual herb, which is a common and dominant species across different types of wetlands, such as rivers and lakes, in temperate Asia, Australia, Europe, and North America (Chen et al., 2019). It has branched stems, enlarged nodes, and lanceolate leaves. This species bears axillary spikelike racemes, blooms from May to September, and generates seeds from June to October (Wei et al., 2021). This species is common and distributed across all water level gradients of the TGR, and has a high percentage of importance value index in plant communities and soil seed banks (Zhang et al., 2013, 2017; Chen et al., 2020a; Zhu et al., 2020). With the water level of the TGR rises gradually, plants of *P. hydropiper* distributed at the HFB of the TGR often suffer from shallower flooding at the early developmental stage to deeper flooding at the later developmental stage (Wei et al., 2020).

Experimental Design

Seedlings were propagated from seeds collected from three pairs of low elevation [150–155 m above sea level (a.s.l.)] and high elevation (165–175 m a.s.l.) populations in the HFB of the TGR in September 2016. The low and high elevation populations are located in Beibei District (E106°26′58.3′, N29°40′59.9′; E106°26′53.9′, N29°41′01.7′), Fuling District (E107°11′23.3′, N29°39′59.5′; E107°11′26.0′, N29°39′57.8′), and Yunyang County (E108°42′58.2′, N31°00′04.8′; E108°42′59.2′, N31°00′03.6′) in Chongqing City, China. From each population, seeds were collected from nine randomly selected plants separated by at least 2 m. All seeds were cleaned, air dried, and stored at room temperature. For the experiment, fully matured and healthy seeds with uniform size were selected from each population.

In June 2019, enough seeds for each population were germinated on seedling trays at the Beijing Sheng Fang greenhouse (N40°0′27.9′, E116°20′19.5′). After germination, they were transplanted into plastic pots (11 cm upper diameter, 8 cm bottom diameter, and 9.6 cm height), one seedling per pot. After most seedlings had grown to a height of 15 cm, 195 seedlings with uniform size from each population were selected for the experiment, with 1,170 seedlings in total for six populations. The substrate in pots was a quartz sand and vermiculite mixture (v:v, 1:3), containing nutrients of 1.52 ± 0.05 mg total N g^{-1} and 1.46 ± 0.07 mg total P g^{-1}. In August 2019, early treatments were conducted, including three early flooding treatments × two elevations (low and high) × 65 replicates × three districts (**Figure 1**). The three early treatments lasted for 3 days, including early control (no flooding, keeping the soil surface slightly moist), early flooding (using a floodwater depth of 1 cm above the soil surface), and early eutrophic flooding (using a eutrophic floodwater depth of 1 cm above the soil surface). All water used in the early control and early flooding treatments was deionized water, and N and P concentrations in the early eutrophic flooding treatment were 2.0 and 0.15 mg L^{-1}, respectively, which were set in reference to the eutrophication level of water in the TGR (Huang et al., 2014; Tang et al., 2015; Xiang et al., 2021). Three days later, most flooded plants (more than 95%) had generated adventitious roots. The morphologic traits of all plants were measured, including stem length, length and width of 1st fully expanded leaf (thereafter named functional leaf), total leaf number, and adventitious root number. A total of 90 plants were harvested (three early flooding treatments × two elevations × five replicates × three districts). Plants were separated into leaves, stems, and roots (both belowground roots and adventitious roots), dried, and the biomass was measured. The remaining plants that underwent early treatments were subjected to subsequent treatments resulting in three early flooding treatments × three subsequent flooding treatments × two elevations × 20 replicates × three districts. The three subsequent treatments lasted for 20 days and included: control (no flooding), flooding (where the floodwater depth was 7 cm above the soil surface, and nearly at half of the plant height), and eutrophic flooding (where the eutrophic floodwater depth was 7 cm above the soil surface). The plants subjected to subsequent treatments were subjected to three early treatments. All water used in the subsequent control and subsequent flooding treatments was deionized water, the N and P concentrations in the eutrophic flooding treatment were 2.0 and 0.15 mg L^{-1}, respectively. Starting at the seed germination, the experiment lasted for 3 months. The air temperature in the greenhouse ranged from 28°C to 35°C and the relative air humidity ranged from 40% to 60% at noon.

Growth Measurements

The time of harvesting of these 90 plants after 3 days of induction treatments was defined as day 0 of subsequent treatments. During subsequent treatments, the adventitious root number was measured on days 1, 3, 5, 7, 10, 13, 16, and 20. A total of 270 plants (three early flooding treatments × two elevations × five replicates × three subsequent flooding treatments × three districts) were harvested every 5 days for four times and dried in an oven at 70°C for at least 72 h to measure root biomass on days 5, 10, and 15, and leaf, stem, root, and adventitious root biomass on day 20. Then, the root to shoot ratio was calculated.

Data Analyses

Before analyses, data were assessed for normality and homogeneity of variance. For early treatments, two-way ANOVA was used to test for effects of early treatments (fixed effect) and elevation (fixed effect) on stem length, functional leaf length and width, total leaf number, leaf biomass, stem biomass, root biomass, total biomass, root to shoot ratio, and adventitious root number. For subsequent treatments, three-way ANOVA was used to test for effects of early treatments (fixed effect), subsequent treatments (fixed effect), and elevation (fixed effect) on stem length, functional leaf length and width, total leaf

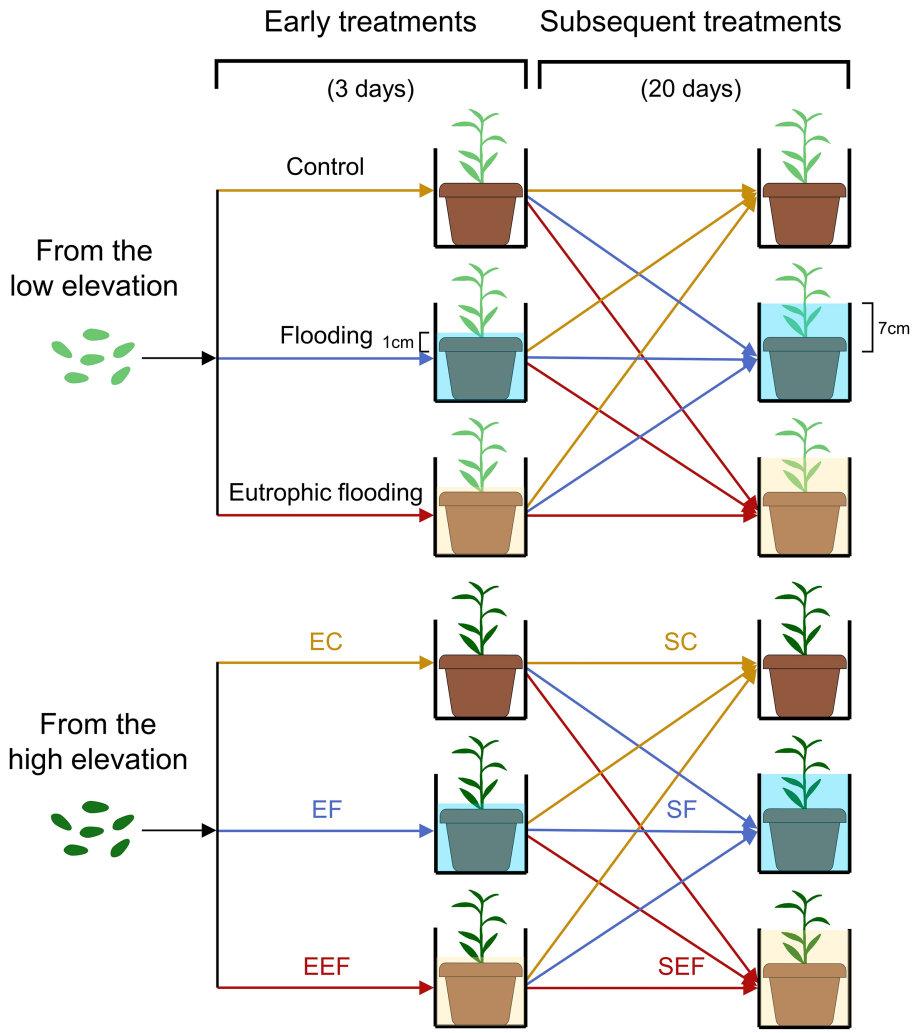

FIGURE 1 | Schematic representation of the experimental design. Seeds of *Polygonum hydropiper* were collected from three populations each at low and high elevations in the Three Gorges Reservoir (TGR) region. Early treatments include early control (no flooding, EC), early flooding (using a floodwater depth of 1 cm above the soil surface, EF), and early eutrophic flooding treatments (using a eutrophic floodwater depth of 1 cm above the soil surface, EEF). After early treatments, plants were subjected to three subsequent flooding treatments: control (no flooding, SC), flooding (using a floodwater depth of 7 cm above the soil surface, SF), and eutrophic flooding (using a eutrophic floodwater depth of 7 cm above the soil surface, SEF). The N and P concentrations of eutrophic water were 2 and 0.15 mg L^{-1}, respectively, which were set in reference to the eutrophication level of water in the TGR.

number, leaf biomass, stem biomass, root biomass, total biomass, root to shoot ratio, adventitious root number, and adventitious root biomass. Plant height on day 1 of the subsequent treatments was set as covariate for all growth variables. Analyses were conducted using R, version 4.1.1 (R Core Team, 2021).

RESULTS

Effects of Early Treatments on Plant Performance

Early treatments had significant effects on adventitious root number but did not significantly affect other growth variables (**Table 1**). The generation of adventitious roots in both early flooding and eutrophic flooding treatments started on day 1 and retained a high generation rate until day 7, but no adventitious roots were found in early control treatment (**Supplementary Figures 1A–C**). Early flooding and eutrophic flooding treatments did not show significant differences in adventitious root number (**Figure 2**). The elevation and interaction of elevation and early treatments had no significant effect on any growth variable (**Table 1**).

Early treatments had significant effects on all growth variables except for root to shoot ratio and adventitious root biomass of plants at the end of subsequent treatments (**Table 2**). The stem length, functional leaf length and width, leaf biomass, stem biomass, root biomass, and total biomass of plants that had been subjected to early control treatment showed no significant differences compared with early eutrophic treatment, but were significantly higher than early flooding treatment (**Figure 3**).

TABLE 1 | ANOVA results for effects of early treatments (ET: early control, early flooding, and early eutrophic flooding) and elevations (E: low and high) on stem length, functional leaf length and width, total leaf number, leaf biomass, stem biomass, root biomass, total biomass, root to shoot ratio, and adventitious root number in *Polygonum hydropiper* at the end of early treatments.

Trait	Early treatments (ET)		Elevation (E)		ET × E	
	$F_{2,84}$	*p*	$F_{1,84}$	*p*	$F_{2,84}$	*p*
Stem length	0.06	0.944	0.01	0.909	0.42	0.660
Functional leaf length	0.20	0.818	1.10	0.297	0.48	0.623
Functional leaf width	0.91	0.409	0.35	0.556	2.59	0.081
Total leaf number	0.09	0.919	1.33	0.252	1.95	0.149
Leaf biomass	0.04	0.957	0.45	0.504	2.12	0.126
Stem biomass	0.12	0.886	3.30	0.073	1.61	0.206
Root biomass	1.30	0.279	1.15	0.287	1.76	0.179
Total biomass	0.07	0.929	1.50	0.223	1.98	0.145
Root to shoot ratio	1.35	0.263	1.71	0.195	0.23	0.798
Adventitious root number	76.95	**<0.001**	0.85	0.361	0.24	0.790

A p-value smaller than 0.05 is formatted in bold.

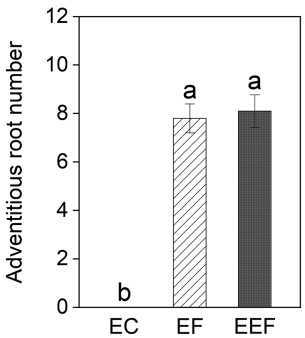

FIGURE 2 | Adventitious root number of *P. hydropiper* subjected to early control (EC), early flooding (EF), and early eutrophic flooding (EEF) at the end of early treatments. Different lowercase letters represent significant differences among the three early treatments.

Effects of Subsequent Treatments on Plant Performance

Subsequent treatments had significant effects on all growth variables except for stem length, functional leaf length, and stem biomass of plants on day 20 (**Table 2**). The leaf width, leaf number, and leaf biomass of plants in the subsequent control and eutrophic flooding treatments were significantly higher than in the subsequent flooding treatment (**Figures 4B–D**). The leaf length, stem biomass, total biomass, and root to shoot ratio of plants in the subsequent control treatment were not significantly different compared with subsequent eutrophic treatment. However, these variables were significantly higher than in subsequent flooding treatment (**Figures 4A,E,G,H**). Both subsequent flooding and eutrophic flooding treatments stimulated the generation of adventitious roots, with a higher number in subsequent eutrophic flooding; however, these two treatments significantly suppressed root growth (**Figures 4F,I,J**; **Supplementary Figures 1D–F**). Elevation had significant effects on functional leaf length and width, stem biomass, and total biomass (**Table 2**). Plants that originated from the low elevation had significantly higher functional leaf length and stem biomass compared with plants from the high elevation (**Figure 5**).

DISCUSSION

Growth Responses of *Polygonum hydropiper* to Early Flooding Treatments

The generation of adventitious roots appeared quicker than other changes in shoot traits during early flooding treatments (Panozzo et al., 2019; Joshi and Ginzberg, 2021). Early flooding and eutrophic flooding treatments quickly generated adventitious roots but did not affect other growth variables (**Supplementary Figure 1**). Fast generation of adventitious roots may facilitate oxygen diffusion and improve aeration of flooded organs (Ayi et al., 2016; Park et al., 2020), which has been proved to be caused by ethylene accumulating because of limited gas diffusion in floodwater (Voesenek and Bailey-Serres, 2015; Herzog et al., 2016; Joshi and Ginzberg, 2021). In response to flooding, plants often prioritize activities related to survive such as aeration and nutrient absorption, with faster responses in roots than in shoots (Striker et al., 2014; Vidoz et al., 2016; Kaspary et al., 2020).

Effects of Early Treatments on Growth of *Polygonum hydropiper* at Later Developmental Stage

Studies have shown that when plants experience abiotic stresses at the early developmental stage, this experience can stimulate responses enabling plants to better cope with subsequently experienced severer stress (Harb et al., 2010; Hatzig et al., 2018; Nosalewicz et al., 2018; Avramova, 2019; Mantoan et al., 2020; Li et al., 2020a, 2022; Zamorano et al., 2021). Some studies have also found no significant effects that early stresses have

TABLE 2 | ANOVA results for effects of early treatments (ET: early control, early flooding, and early eutrophic flooding), subsequent treatments (ST: control, flooding, and eutrophic flooding), and elevation (E: low and high) on stem length, functional leaf length and width, total leaf number, leaf biomass, stem biomass, root biomass, total biomass, root to shoot ratio, adventitious root number, and adventitious root biomass in *P. hydropiper* at the end of subsequent treatments on day 20.

Trait	Early treatments (ET)	Subsequent treatments (ST)	Elevation (E)	ET × ST	ET × E	ST × E	ET × ST × E
	$F_{2,251}$	$F_{2,251}$	$F_{1,251}$	$F_{4,251}$	$F_{2,251}$	$F_{2,251}$	$F_{4,251}$
Stem length	**24.38*****	0.65	0.16	0.41	0.29	0.17	1.43
FLL	**16.22*****	1.26	**22.69*****	1.95	0.38	0.00	0.10
FLW	**8.93*****	**4.35***	**4.86***	0.32	1.21	0.12	1.33
Total leaf number	**3.36***	**30.77*****	0.55	1.02	0.75	0.02	1.34
Leaf biomass	**13.58*****	**6.78****	2.72	1.86	2.31	0.08	1.13
Stem biomass	**29.02*****	2.33	**13.19*****	1.22	1.62	0.07	0.60
Root biomass	**20.63*****	**147.87*****	1.56	1.66	1.30	0.43	0.92
Total biomass	**23.22*****	**3.49***	**7.29****	1.75	2.12	0.05	0.84
Root to shoot ratio	0.55	**4.95****	0.94	1.35	1.80	0.24	0.29
ARN	**4.55***	**501.68*****	1.96	1.24	0.53	0.31	0.34
ARB	1.69	**101.50*****	0.24	1.10	0.62	0.29	0.40

*FLL, functional leaf length; FLW, functional leaf width; ARN, adventitious root number; ARB, adventitious root biomass. p-values smaller than 0.05 are formatted in bold. No symbols p > 0.05. ***p < 0.001; **p < 0.01; *p < 0.05.*

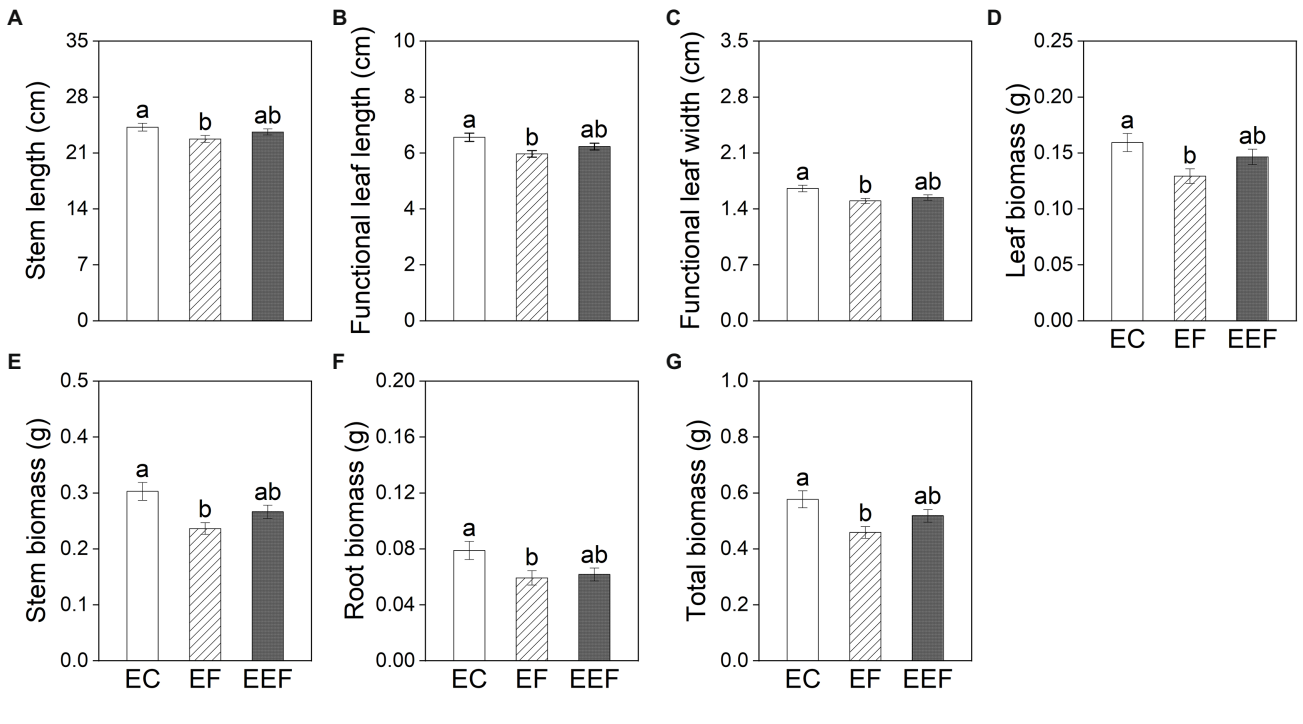

FIGURE 3 | Stem length **(A)**, functional leaf length **(B)** and width **(C)**, leaf biomass **(D)**, stem biomass **(E)**, root biomass **(F)**, and total biomass **(G)** of *P. hydropiper* subjected early control (EC), early flooding (EF), and early eutrophic flooding (EEF) at the end of subsequent treatments on day 20. Different lowercase letters represent significant differences among early treatments.

on plant performance (Ploschuk et al., 2020; Tang et al., 2022). The results of the present study showed that short early flooding induced morphological adaptation to flooding, manifesting as the generation of adventitious roots. However, it did not improve growth responses to subsequent flooding, and a significant decrease in growth variables was observed. In comparison, plants that had experienced early eutrophic flooding treatment did not show a significant decrease in growth variables compared with plants in the control treatment at the end of subsequent treatments. These results suggest negative lag effects of early flooding, further indicating that nutrient inputs can alleviate such effects.

Flooding has detrimental effects on the growth of many riparian plants; however, under conditions of nutrient enrichment, plants can absorb and utilize nutrients to offset the negative effects of flooding (Qiu et al., 2020). Plant individuals growing in favorable habitats (e.g., with high nutrient availability) at the early seedling stage often have higher fitness at their adult stage (Engqvist and Reinhold, 2016). Especially, when the conditions

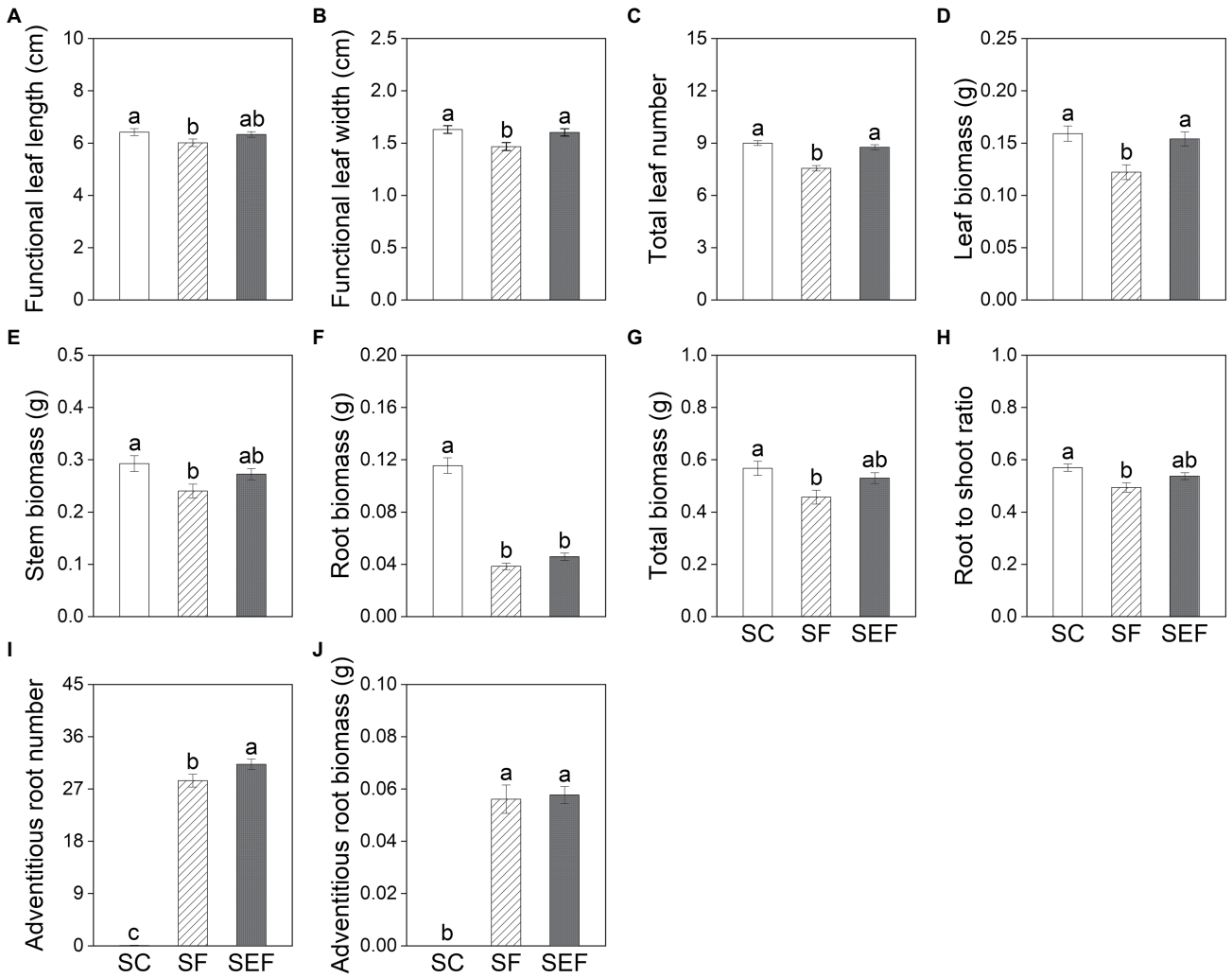

FIGURE 4 | Functional leaf length **(A)** and width **(B)**, total leaf number **(C)**, leaf biomass **(D)**, stem biomass **(E)**, root biomass **(F)**, total biomass **(G)**, root to shoot ratio **(H)**, adventitious root number **(I)** and biomass **(J)** of *P. hydropiper* subjected to subsequent treatments of control (SC), flooding (SF), and eutrophic flooding (SEF) at the end of subsequent treatments on day 20. Different lowercase letters represent significant differences among subsequent treatments.

at later developmental stages are comparatively hostile, such an effect becomes more important (Portela et al., 2020). Favorable resource conditions such as light and nutrient availability at the early stage also determine the subsequent phenotypic responses of *Rumex palustris* and may produce different adaptive strategies in response to spatially or temporally heterogeneous conditions (Chen et al., 2011; Huber et al., 2012). Early eutrophic conditions were beneficial for plants in coping with flooding but did not significantly increase plant growth compared with control at the end of subsequent treatments. The possible reason could be that the positive effects of nutrient inputs are not sufficiently strong to offset negative effects of flooding, resulting in a negative effect overall (Nguyen et al., 2018b). Alternatively, several studies on the silver spoon effects have found a time-lag effect (Germain and Gilbert, 2014; van Allen et al., 2021; Luo et al., 2022).

Subsequent eutrophic flooding also improved plant growth compared with subsequent flooding, resulting in significantly higher growth of leaves and adventitious roots (**Figure 4**). These results indicate a higher adaptation of plants to subsequent eutrophic flooding, suggesting that the effects of nutrient inputs are beneficial for plant flood-tolerance (Joshi and Ginzberg, 2021). In this study, nitrate was added which reportedly plays a beneficial role in maintaining photosynthetic metabolism during short-term flooding (Nguyen et al., 2018a; Borella et al., 2019; Posso et al., 2020; Da-Silva et al., 2021). Upon flooding, because of the inhibited growth of belowground roots, plants of *P. hydropiper* may prioritize the allocation of resources obtained from eutrophic water to expand leaf lamina, and elongate leaves and adventitious roots.

Different Responses of *Polygonum hydropiper* Plants Originated From Low and High Elevations

The compensation for biomass loss of offspring plants under stress is not only driven by the environment offspring plants experience, but it can also be driven by their parents (Latzel et al., 2010; Quan et al., 2021). The environmental stress

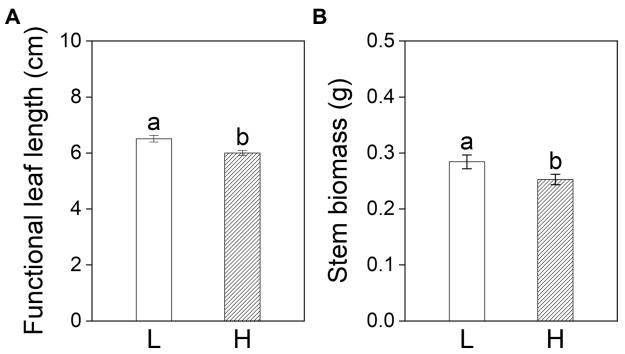

FIGURE 5 | Functional leaf length **(A)** and stem biomass **(B)** of *P. hydropiper* from low (L) and high (H) elevations on day 20. Different lowercase letters represent significant differences between two elevations.

experienced by parental plants can form a kind of "memory," which affects seeds and the growth performance of offspring plants (Bossdorf et al., 2009; Li et al., 2021; Waterman and Sultan, 2021). Compared with plants from high elevation, plants from low elevation had significantly higher stem biomass and functional leaf length. For the maternal generation, compared to populations from the low elevation, *P. hydropiper* populations that originated from the high elevation are often flooded less frequently and covering water is shallower. The maternal experience of high-elevation plants may weaken the ability of their offspring plants to respond to sudden flooding stress. In contrast, offspring plants that originated from the low elevation may have higher adaptability in response to this "predictable" periodic stress (Herman et al., 2012; van Dooren et al., 2020). Our former studies also reported such different strategies of *P. hydropiper* populations in low and high elevations (Chen et al., 2020b; Wei et al., 2020, 2021). The differences in flooding regimes of low and high elevations may also induce epigenetic variation in the maternal generation, which can be transmitted to the offspring, leading to phenotypic variations (González et al., 2016, 2018; Benson et al., 2021; Sánchez et al., 2021).

CONCLUSION

The results clearly showed that early flooding and eutrophic flooding treatments quickly induced the generation of adventitious roots in *P. hydropiper*. Early flooding exerted a negative lag-effect on plant growth at the end of subsequent flooding, which could be alleviated by nutrient inputs. Moreover, nutrient inputs also alleviated negative effects of subsequent flooding. Therefore, nutrient inputs can alleviate negative effects of early and subsequent flooding because of the quick induction of new adventitious roots. However, if plants do not generate adventitious roots upon flooding, the effect of nutrient inputs may not be apparent, which will be further studied in the future. Furthermore, the growth of riparian flood-tolerant species could be promoted under eutrophic conditions in the reservoir area.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Y-HC, G-WW, and F-LL conceived and designed the study. G-WW participated in the field work and provided study materials. Y-HC carried out greenhouse experiments, analyzed the data, and wrote the manuscript. YC and F-LL participated in the structuring and editing of the manuscript. F-LL wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Fundamental Research Funds for the Central Universities (grant number 2021ZY90) and the National Natural Science Foundation of China (grant number 32071525).

ACKNOWLEDGMENTS

We sincerely thank Xin-Sheng Sun for his assistance in conducting greenhouse experiments, plant harvesting, and index determination.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.919409/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 04 August 2022
DOI 10.3389/fpls.2022.949752

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Systems and Synthetic Biology, a section of the journal Frontiers in Plant Science

RECEIVED 23 May 2022
ACCEPTED 04 July 2022
PUBLISHED 04 August 2022

CITATION
Guo Y, Quan J, Wang X, Zhang Z, Liu X, Zhang R and Yue M (2022) Predictability of parental ultraviolet-B environment shapes the growth strategies of clonal *Glechoma longituba*. *Front. Plant Sci.* 13:949752. doi: 10.3389/fpls.2022.949752

Predictability of parental ultraviolet-B environment shapes the growth strategies of clonal *Glechoma longituba*

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Although there is an increasing debate about ecological consequences of environmental predictability for plant phenotype and fitness, the effect of predictability of parental environments on the offspring is still indefinite. To clarify the role of environmental predictability in maternal effects and the growth strategy of clonal offspring, a greenhouse experiment was conducted with *Glechoma longituba*. The parental ramets were arranged in three ultraviolet-B (UV-B) conditions, representing two predictable environments (regular and enhanced UV-B) and an unpredictable environment (random UV-B), respectively. The offspring environments were the same as their parent or not (without UV-B). At the end of experiment, the growth parameters of offspring were analyzed. The results showed that maternal effects and offspring growth were regulated by environmental predictability. Offspring of unpredictable environmental parents invested more resources in improving defense components rather than in rapid growth. Although offspring of predictable parents combined two processes of defense and growth, there were still some differences in the strategies between the two offspring, and the offspring of regular parent increased the biomass allocation to roots (0.069 g of control vs. 0.092 g of regular), but that of enhanced parent changed the resource allocation of nitrogen in roots and phosphorus in blade. Moreover, when UV-B environments of parent and offspring were matched, it seemed that maternal effects were not adaptive, while the growth inhibition in the predictable environment was weaker than that in unpredictable environment. In the predictable environment, the recovered R/S and the increased defense substances (flavonoid and anthocyanin) contributed to improving offspring fitness. In addition, when UV-B environments of parent and offspring were mismatched, offspring growth was restored or improved to some extent. The offspring performance in mismatched environments was controlled by both transgenerational effect and within-generational plasticity. In summary, the maternal effects affected growth strategies of offspring, and the differences of

strategies depended on the predictability of parental UV-B environments, the clone improved chemical defense to cope with unpredictable environments, while the growth and defense could be balanced in predictable environments. The anticipatory maternal effects were likely to improve the UV-B resistance.

KEYWORDS

environmental predictability, phenotypic plasticity, clonal plants, UV-B radiation, maternal effects, transgenerational effect, within-generational plasticity

Introduction

Variability is an intrinsic character of natural environment, and the magnitude and frequency of environmental variability are predicted to increase due to anthropogenic environmental change (Kochanek et al., 2011; Smale et al., 2019; Bintanja et al., 2020). Although plants can perceive the changes in environment and adapt to new conditions by adjusting their phenotype (Bornette and Puijalon, 2011; Li et al., 2021), variability and predictability of environment complicated the growth of plants. Plant phenotyping is a consequence of the interaction between genotype and environment (Walter et al., 2015; Pratap et al., 2019). Phenotypic plasticity is the main mechanism for plants to respond to changing environments and fast-changing climates (Sultan, 2001; Matesanz and Milla, 2017; Khodadadi et al., 2021). Phenotypes of offspring are determined not only by the genetic inheritance of causative alleles, but also by non-genetic influences of their environments and the environments experienced by parental generations (Auge et al., 2017a). Whether the environments of parent and offspring can be a cue to accurately predict the selective environment experienced by offspring, which will have different effects on the offspring phenotypes. In addition, the accuracy of this prediction will bring the offspring phenotype nearer to or further away from the optimum phenotype in the new environment (Sultan et al., 2009; Herman and Sultan, 2011; Auge et al., 2017a).

Maternal effects occur when the environment experienced by the mother influences the offspring phenotype over and above the direct effect of transmitted genes (Marshall and Uller, 2007). Despite the importance of maternal effects has been confirmed by many studies (Galloway and Etterson, 2007; Marshall and Uller, 2007; Auge et al., 2017b; Lyu et al., 2017; Donelson et al., 2018; Zettlemoyer and Lau, 2021; Zhou et al., 2021), the adaptive significance of maternal effects is often controversial. Some studies have found that adaptive maternal effects are widespread, allowing offspring to cope with rapidly changing environments or even increase fitness (Yin et al., 2019; Donelan et al., 2020). However, others showed weak evidence for adaptive maternal effects, and maternal effects may even reduce offspring fitness in these studies (Marshall and Uller, 2007; Uller et al., 2013). The contrasting results may be caused by the variability of the environment experienced by the parents.

Clonal plants are dominant species in many habitat types (Song et al., 2001). Compared with non-clonal plants, clonal offspring are thought to have a stronger ability to store the environmental information of parent for their asexual reproduction properties (Thellier and Lüttge, 2013; Vít et al., 2016; Richards et al., 2017). Although there have been some reports about the effect of parental environment on adaptability of clonal plants (Herman and Sultan, 2016; González et al., 2018; Baker et al., 2019; Dong et al., 2019), few studies consider the influence of environmental predictability of parent on the growth strategies of clonal offspring.

As an intrinsic part of the solar spectrum, ultraviolet-B (UV-B, 280–315 nm) light has many effects on the growth and development of plants (Liu et al., 2015; Quan et al., 2021). The effect of UV-B radiation on plants is comprehensive, and low-intensity UV-B radiation acts as a specific regulator for plants, while high-intensity UV-B radiation plays a negative effect on the growth and development of plants (Willing et al., 2016; Yin and Ulm, 2017). Low-intensity UV-B radiation regulates plant photomorphogenesis and thermomorphogenesis *via* UVR8 photoreceptor (Parihar et al., 2015; Hayes et al., 2017). Nevertheless, relatively high intensities of UV-B irradiation can damage macromolecules, inhibit photosynthesis, depress leaf expansion, reduce plant height, decrease biomass, and consequently affect plant growth, development, and morphology (Hectors et al., 2007; Berli et al., 2010; Liu et al., 2011). Therefore, variation of UV-B intensity in nature, such as the light environment under the forest, complicates the growth of plants (Bais et al., 2015; Robson et al., 2015).

In this study, parental ramets of clonal plant *Glechoma longituba* were assigned to three UV-B conditions, which represented two predictable environments (regular and enhanced UV-B) and an unpredictable environment (random UV-B), respectively. The offspring ramets were divided into two groups: One grew in the same UV-B environment as their parents, and the other was in an ambient light condition. The growth parameters of offspring were explored to evaluate (1)

the difference of maternal effects caused by parental UV-B environment on the phenotypes and growth strategies of clonal offspring; (2) whether maternal effects help to improve the offspring adaptability when parental–offspring environments matched; and (3) the effect of offspring environments on its performance. We hypothesized that (1) both maternal effects and offspring environments significantly affect the phenotypes and growth strategies of clonal offspring, and the difference of growth strategies depends on environmental predictability, and (2) maternal effects contributed to improve offspring adaptation when the parental and offspring environments were identical.

Materials and methods

Plant material and propagation

Glechoma longituba (Nakai) Kuprian, a perennial clonal plant of the Lamiaceae family, was used in this experiment. This species produces long stolons with ramets on its nodes and is commonly employed in clonal plant research due to its high phenotypic plasticity (Liu et al., 2015). The *G. longituba* in our experiment was collected from Jiwozi in the Qinling Mountains, Shaanxi, China. The plant materials were collected from a genet to ensure the uniform of the genotypes and then were vegetatively propagated for at least 4 months in a greenhouse at Northwest University in Xi'an (34.3°N, 108.9°E; altitude 397 m a.s.l.) to reduce the impact of the previous environment through acclimatization.

The experiment was conducted in our greenhouse from May to August 2021. A total of 56 healthy ramets of similar size were selected as parental ramets and transplanted individually to plastic pots (7 cm length × 7 cm width × 7.8 cm depth) filled with nutrient soil (peat soil, perlite, vermiculite, and coconut bran). During the experiment, the culture conditions of the greenhouse were a 24/20°C day/night temperature cycle and a 13/11-h light/dark cycle, the mean irradiance was 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and the relative humidity was maintained at 40%. Ramets were watered every 3 days to prevent water stress.

Experimental design

The experiment was carried out in two stages: The first was the growth stage of parental ramets, and the second was that of offspring ramets. In the first stage, 56 ramets of similar size were selected as parents and divided into four groups randomly. One group was used as a control treatment, which grew in the ambient environment of the greenhouse. There was only a very low intensity of UV-B radiation (0.2 $\mu W \cdot cm^{-2}$) in the greenhouse. The other three groups were treated with UV-B radiations in different ways: random, regular, and enhanced UV-B radiation, which represented two predictable environments (regular and enhanced UV-B) and an unpredictable environment (random UV-B), respectively. This unpredictable random UV-B radiation was presented with the random treatment of UV-B intensity and frequency. The detail of UV-B treatments was described in the section of "Ultraviolet-B radiation treatments." The control group was designed with eight replicates, and the UV-B treatment groups were designed with 16 replicates.

After 27 days of growth, parental ramets had grown about eight offspring ramets. According to our previous study, epigenetic variation caused by maternal UV-B environment can maintain in the third offspring ramets (Zhang et al., 2021). Therefore, the third offspring ramet was removed and replanted as the material of the second stage experiment. To avoid confusion, we described this third offspring ramet as the initial ramet of the second stage. In the second stage, half of the initial ramets in each treatment were placed in the same environment as their parents, and the other half were in the ambient environment as control groups. The offspring also grew for 27 days, the same time as their parents in the first stage.

Therefore, there were seven treatments in the second stage experiment: CK-CK, Ra-Ra, Ra-CK, Re-Re, Re-CK, En-En, and En-CK. The details are described in **Figure 1** and **Table 1**. In this experiment, there were three matched parental–offspring environments: Both parental ramets and initial ramets were grown under UV-B environments (Ra-Ra, Re-Re, and En-En). Meanwhile, three types of mismatched parental–offspring environments were selected: Parental ramets were grown under the UV-B conditions, and initial ramets were transplanted in an ambient condition (Ra-CK, Re-CK, and En-CK). In the whole process of the experiment, for the UV-B radiation treatments, only the parental ramets in the first stage experiment and the initial ramets of the second stage experiment were irradiated with UV-B, and other newborn ramets grew in an ambient greenhouse environment without additional UV-B treatment.

Ultraviolet-B radiation treatments

There were three UV-B radiation treatments involved in our experiment (random radiation, regular radiation, and enhanced radiation). In all treatments, the UV-B lamps were suspended above the plants, and the intensity of UV-B radiation was adjusted by modifying the distance between the lamps and the canopy. The differences among treatments were the intensity and frequency of UV-B radiation. The random UV-B radiation was controlled by turning off the lamp 4–6 times during 9:00 a.m.–17:00 p.m. per day randomly. To ensure the randomicity of random UV-B radiation, the frequency and duration of UV-B radiation were designed with the "dplyr" package of R software (RStudio, Auckland, New Zealand). The regular UV-B radiation treatment was conducted with UV-B radiation regularly, which means UV-B radiation (5 $\mu W \cdot cm^{-2}$) lasted 8 h (from 9:00 a.m.

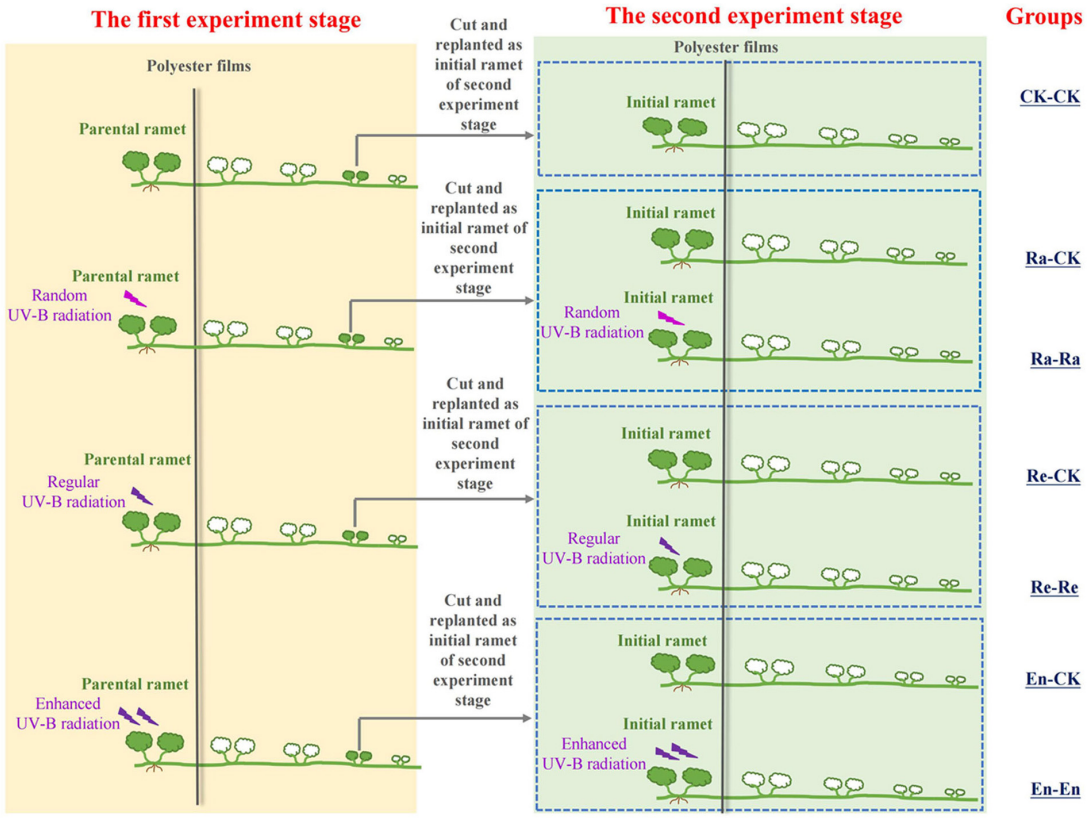

FIGURE 1
Schematic of the experiment. The experimental design consisted of seven treatments according to the condition of the offspring ramet in the second stage.

TABLE 1 Different treatments conducted in the study.

Treatments	Interpretation
CK-CK	Control group, all the ramets grew in the ambient environment of greenhouse during experiment, whatever that were in the first or second stage.
Ra-Ra	Only the parent in the first stage and the initial ramet in the second stage were treated with UV-B radiation randomly. Other ramets grew in the ambient environment of greenhouse. UV-B radiation (5 $\mu W \cdot cm^{-2}$) was controlled by turning off the UV-B lamps 4–6 times for 8 h radiation (9:00 a.m.–17:00 p.m.) per day, and the duration of interruption was 5–20 min each time.
Ra-CK	Except for the parental ramets of the first stage were treated with random UV-B radiation, other ramets all grew in the ambient environment of greenhouse.
Re-Re	Only the parent in the first stage and the initial ramet in the second stage were treated with UV-B radiation regularly, that means UV-B radiation (5 $\mu W \cdot cm^{-2}$) lasted 8 h (from 9:00 a.m. to 17:00 p.m.) per day. Other ramets all grew in the ambient environment of greenhouse.
Re-CK	Except for the parent of the first stage were treated with regular UV-B radiation, other ramets all grew in ambient light of greenhouse.
En-En	Only the parent in the first stage and the initial ramet in the second stage were treated with enhanced UV-B radiation. That means, the intensity of UV-B radiation was enhanced every 3 days (5, 6, 7, 8, 8.5, 9, 9.5, 10, and 10.5 $\mu W \cdot cm^{-2}$, respectively) and the radiation duration was 8 h (from 9:00 a.m. to 17:00 p.m.) per day. Other ramets all grew in ambient light of greenhouse.
En-CK	Except for the parent of the first stage were treated with enhanced UV-B radiation, other ramets all grew in ambient light of greenhouse.

to 17:00 p.m.) per day. The plants of enhanced UV-B treatment were exposed to the increased UV-B radiation, the intensity of UV-B was improved every three days (5, 6, 7, 8, 8.5, 9, 9.5, 10, and 10.5 $\mu W \cdot cm^{-2}$, respectively), and the radiation duration was 8 h (from 9:00 a.m. to 17:00 p.m.) per day.

Supplementary UV-B radiation was artificially supplied by square-wave UV-B fluorescent lamps (36 W, Beijing Lighting Research Institute, Beijing, China) according to the method of Zhang et al. (2021). The maximum output wavelength of these lamps was 313 nm. During the experiment, these lamps were wrapped with either 0.13-mm cellulose acetate film (Grafix Plastics, Cleveland, OH, transmission down to 290 nm) for the supplemental UV-B radiation groups or with 0.13-mm polyester plastic film (absorbs radiation below 320 nm, Grafix Plastics)

for the control group. Thus, the spectral difference between the control and UV-B groups is the presence or absence of UV-B. The cellulose acetate and polyester plastic films were replaced every 5 days. Furthermore, during the experiment, to avoid the interference from maternal UV-B radiation on the newborn ramets, the transparent polyester film (0.3 mm, Dongguan Linuo Plastic Insulation Material Co. Ltd., China) was placed vertically on both sides of the parental ramets in the first stage and the initial ramets of the second stage separately to ensure that the bottom of the films did not affect the growth of the newborn ramets. The intensity of UV-B radiation of different treatments was measured with an UV radiometer (Handy, Beijing, China).

Measurement of parameters

At the beginning of the experiment, each treatment was repeated eight times; however, during the experiment, some plants died. At the end of the experiment, the ramets with healthy growth state were selected for parameter measurement and statistical analysis, and each treatment at least had three replicates. The initial ramets and whole clones of the second stage were harvested carefully according to the needs of measurement and analysis. The following growth parameters of initial ramet were measured: biomass of leaf, blade, petiole, node and roots, petiole length, blade area, flavonoid and anthocyanin content of blades, total carbon (TC) and total nitrogen (TN) of roots, total phosphorus (TP), nitrate-nitrogen, ammonium nitrogen, and organic carbon (OC) of blade. Moreover, the clones were harvested, and length of the longest stolon, number of ramets, branching intensity, the biomass of leaf, stolon, and roots were measured.

Growth parameters

The leaf, blade, petiole, and roots of ramets, and the leaf, stolon, and roots of clones were collected for biomass measurement. These samples were dried at 75°C for 48 h to a constant weight, and biomass was measured immediately with an electronic balance (Sartorius BT25S, Beijing, China). Root–shoot ratio (R/S) was calculated by the ratio of root biomass to aboveground biomass; aboveground biomass of ramets was calculated as the sum of the biomass of blade, petiole, and node, while aboveground biomass of clone was calculated as the sum of the biomass of stolon and leaf; total biomass was obtained by summing aboveground and root biomass.

The fresh blades and petioles of the initial ramets and the longest stolons of clones were collected for the measurement of specific leaf area (SLA), specific petiole length (SPL), and specific stolon length (SSL) according to the method of Liu et al. (2015) and Zhu et al. (2018). Fresh blades were scanned with a scanner (Perfection V19, EPSON, China); then, blade area was calculated with Motic software (Motic Images Plus 2.0. Ink, Motic, China). Petiole length and stolon length were measured with a vernier caliper. SLA was calculated as the ratio of blade area to blade biomass. SPL was calculated as the ratio of petiole length to petiole biomass, and the ratio of stolon length to stolon biomass was calculated as SSL.

Ultraviolet-B absorbing compound concentration

The UV-B absorbing compound content of fresh blades was measured as described by Liu et al. (2015). Blade disks were soaked in 4-ml centrifuge tubes containing methanol, HCl, and distilled H_2O (79:1:20 volume) for 48 h in darkness. The concentration of UV-B absorbing compounds, mainly flavonoids and anthocyanins, was estimated by measuring the absorbance at 300 and 530 nm with a multimode microplate reader (Infinite 200 PRO NanoQuant, TECAN, Switzerland). The absorbance was used as an index of the relative concentration of UV-B absorbing compounds.

Resource allocation

After drying treatment, the collected blades and roots of the initial ramets in the second experiment stage were crushed with a high-flux tissue grinder (Scientz-48, Ningbo Xinzhi, China), and dried powdered samples were used for resource allocation analysis. The contents of TC and TN in the roots and the contents of OC, ammonium nitrogen, nitrate-nitrogen, and TP in the blades were determined. The contents of ammonium nitrogen, nitrate-nitrogen, and TP in blades were digested by H_2SO_4-H_2O_2 and then determined by continuous flow analyzer (SEAL AutoAnalyzer3), ultraviolet and visible spectrophotometry, and Mo-Sb antispetrophotography method. The OC content in blades was determined by potassium dichromate external heating method. The contents of TC and TN in roots were determined by a German element analyzer (vario MACRO cube). TN in blades was obtained from ammonium nitrogen plus nitrate-nitrogen. Then, the N:P in blades and the C:N in roots were calculated.

Statistical analysis

Before the statistical analyses, data were checked for normality and homoscedasticity using Shapiro–Wilk and Levene's tests, and Blom transformation (Zhang et al., 2015) was used to achieve normality for non-normal data in SPSS Statistics 24.0 software (IBM, United States). Then, considering the slight difference among the initial biomass of initial

ramets, analysis of covariance (ANCOVA) was used to test the effects of parental environment and current environment of offspring on the growth characters (biomass, leaf parameters, defensive substances, and growth architecture) and resource allocation of offspring. Duncan's test was chosen as the method of multiple comparisons to test the significance among different treatments, and the significance level was set at the $P < 0.05$ level. All ANCOVA analyses were performed with STATISTICA 6.0 software (StatSoft, Tulsa, OK, United States). In addition, the data of ramet number and branching intensity still did not accord with the normal distribution after Blom transformation, so they were analyzed by the Kruskal–Wallis test of non-parametric test to determine the effect of different environments on the growth architecture of whole clones. Analytical mapping was performed using Origin Pro 8.0 software (OriginLab, United States).

Results

The influence of parental environment on the growth of offspring

Mismatched parental–offspring environments

To study the maternal effect triggered by parental environment on offspring performance, the offspring growth in ambient environments was analyzed, and the parents of these offspring were exposed to different UV-B radiation (Tables 2, 3). Compared with the CK-CK, the total biomass of ramet decreased significantly in the Ra-CK (0.336 g of CK-CK vs. 0.292 g of Ra-CK; $P < 0.05$), which was caused by the overall decrease in aboveground and underground biomass. In addition, the decrease in aboveground biomass was mainly caused by the decrease in blades biomass. There was no significant change in R/S between the CK-CK and Ra-CK (0.249 of CK-CK vs. 0.244 of Ra-CK; $P > 0.05$). There were no significant differences in ramet biomass (total, aboveground, leaf, and petiole biomass) among the CK-CK, Re-CK, and En-CK treatments ($P > 0.05$), but the changing trend of root biomass was different, and the root biomass in Re-CK increased significantly (0.092 vs. 0.069 g of control; $P < 0.05$), which led to the increase in R/S (0.379 vs. 0.249 of CK-CK; $P < 0.05$). Although R/S also increased (0.321 of En-CK vs. 0.249 of CK-CK; $P < 0.05$), there was no significant difference in root biomass between the CK-CK and En-CK ($P > 0.05$), and the highest increase in R/S was displayed in Re-CK.

There was no significant difference in petiole length, blade area, and SLA between the CK-CK and Ra-CK ($P > 0.05$), but the petiole became slender in the Ra-CK (411.962 cm/g of SPL vs. 371.739 cm/g of SPL in CK-CK; $P < 0.05$). Compared with CK-CK, the petiole in Re-CK and En-CK also became thin, and SLA was increased, but no difference was found in blade area. Furthermore, the petiole length increased only in the Re-CK by comparing with the CK-CK (4.246 vs. 3.906 cm of CK-CK; $P < 0.05$).

Concerning the content of defensive substances, the flavonoid and anthocyanin values of offspring in the CK-CK were 0.681 and 0.030. Compared with the CK-CK treatment, flavonoids were increased significantly in Ra-CK, Re-CK, and En-CK groups, while anthocyanins were increased in Ra-CK and Re-CK, but no difference was found in En-CK. The maximum value of flavonoid and anthocyanin both appeared in the Ra-CK (1.680 of flavonoid and 0.075 of anthocyanin).

There was no significant difference in clone biomass and biomass allocation between the CK-CK and Ra-CK ($P > 0.05$). The biomass of all parts of clone (biomass of stolon, leaf, and aboveground, and total biomass) and stolon biomass allocation (stolon biomass/total biomass) increased in the Re-CK and En-CK. But the leaf biomass allocation in the Re-CK group (leaf biomass/total biomass) decreased (0.620 vs. 0.649 of CK-CK; $P < 0.05$) and R/S increased (0.088 vs. 0.079 of CK-CK; $P < 0.05$). These two parameters had no differences between the CK-CK and En-CK (Table 2).

No significant difference in the number of ramets was found among the four treatments ($P > 0.05$). There were also no significant differences in branching intensity among the CK-CK, Ra-CK, and En-CK treatments. The increase in branching intensity was only observed in the Re-CK. The clone in the En-CK had longer stolon lengths (49.710 cm) by comparing with the Ra-CK (45.025 cm). Compared with the CK-CK (378.115 cm/g), the SSL increased in Ra-CK (412.741 cm/g) and decreased in both Re-CK (343.379 cm/g) and En-CK (344.731 cm/g). The longest stolon of the offspring in the Ra-CK became slender, but it became thicker in the Re-CK and En-CK (Table 2).

The value of nitrate-nitrogen of blade, TC, and TN of roots in Ra-CK was the highest in all groups. The TN level of roots increased in En-CK (24.36 g/kg vs. 22.73 g/kg of CK-CK; $P < 0.05$), but C:N in roots of En-CK was decreased (17.56 vs. 18.68 of CK-CK; $P < 0.05$). These parameters of resource allocation in Re-CK all did not display significant differences with CK-CK (Table 3).

Matched parental–offspring environments

In order to clarify whether maternal effects helped to improve the offspring adaptability when parental–offspring environments matched, the growth of offspring, which grew in the same UV-B environments as their parent, was compared (Tables 4, 5). The changing trends of initial ramet biomass were similar, showing a significant decrease in total, aboveground, root, leaf, blade, and petiole biomass. The minimum value of root biomass and petiole biomass both appeared in the Ra-Ra (0.022 g of root and 0.010 g of petiole). The R/S decreased significantly in the Ra-Ra (0.182 vs. 0.249 of CK-CK; $P < 0.05$). Compared with CK-CK, there was no significant difference in R/S in

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TABLE 2 Growth parameters of offspring in the ambient environment.

Traits	CK-CK	Ra-CK	Re-CK	En-CK
Ramets biomass				
Total (g)	0.336 ± 0.009 a	0.292 ± 0.010 b	0.341 ± 0.018 a	0.321 ± 0.013 ab
Aboveground (g)	0.266 ± 0.007 a	0.231 ± 0.007 b	0.250 ± 0.016 ab	0.244 ± 0.008 ab
Root (g)	0.069 ± 0.002 b	0.057 ± 0.002 c	0.092 ± 0.003 a	0.074 ± 0.004 b
R/S	0.249 ± 0.008 c	0.244 ± 0.008 c	0.379 ± 0.012 a	0.321 ± 0.010 b
Leaf (g)	0.257 ± 0.006 a	0.224 ± 0.007 b	0.241 ± 0.016 ab	0.239 ± 0.008 ab
Blade (g)	0.236 ± 0.006 a	0.204 ± 0.006 b	0.213 ± 0.014 b	0.219 ± 0.008 ab
Petiole (g)	0.021 ± 0.001 a	0.020 ± 0.001 a	0.021 ± 0.001 a	0.020 ± 0.001 a
Leaf parameters				
Petiole length (cm)	3.906 ± 0.093 b	4.050 ± 0.107 ab	4.246 ± 0.094 a	4.125 ± 0.088 ab
SPL (cm/g)	371.739 ± 8.789 b	411.962 ± 9.300 a	411.783 ± 15.766 a	416.197 ± 17.491 a
Blade area (cm^2)	58.237 ± 1.099 a	53.756 ± 1.758 a	60.185 ± 3.915 a	59.682 ± 3.440 a
SLA (cm^2/g)	248.486 ± 5.853 b	265.085 ± 7.287 ab	276.506 ± 8.191 a	279.906 ± 9.153 a
Defensive substances				
Flavonoid (OD_{300})	0.681 ± 0.038 c	1.680 ± 0.071 a	1.335 ± 0.038 b	1.205 ± 0.043 b
Anthocyanin (OD_{530})	0.030 ± 0.001 c	0.075 ± 0.003 a	0.045 ± 0.006 b	0.035 ± 0.005 bc
Clone biomass				
Total (g)	0.923 ± 0.027 b	0.861 ± 0.064 b	1.188 ± 0.026 a	1.124 ± 0.038 a
Aboveground (g)	0.856 ± 0.025 b	0.838 ± 0.049 b	1.097 ± 0.023 a	1.043 ± 0.029 a
Leaf (g)	0.591 ± 0.016 b	0.526 ± 0.038 b	0.727 ± 0.023 a	0.708 ± 0.018 a
Stolon (g)	0.244 ± 0.006 b	0.237 ± 0.021 b	0.345 ± 0.011 a	0.330 ± 0.016 a
Leaf biomass/total biomass	0.649 ± 0.008 a	0.653 ± 0.007 a	0.620 ± 0.008 b	0.636 ± 0.009 ab
Stolon biomass/total biomass	0.269 ± 0.006 b	0.270 ± 0.007 b	0.289 ± 0.006 a	0.291 ± 0.006 a
R/S	0.079 ± 0.003 b	0.076 ± 0.002 b	0.088 ± 0.003a	0.077 ± 0.004 b
Growth architecture				
Number of ramets	6.167 ± 0.146 a	6.333 ± 0.188 a	6.500 ± 0.151 a	6.583 ± 0.149 a
Branching intensity	2.500 ± 0.167 b	3.000 ± 0.211 ab	3.667 ± 0.142 a	3.125 ± 0.125 ab
Length of the longest stolon (cm)	47.536 ± 1.017 ab	45.025 ± 1.251 b	49.180 ± 0.963 ab	49.710 ± 1.530 a
SSL (cm/g)	378.115 ± 8.586 b	412.741 ± 14.801 a	343.379 ± 10.633 c	344.731 ± 10.067 c

Values with different letters were significantly different among four treatments, whereas the same letter indicates no significant difference among four treatments ($P < 0.05$). Data were mean ± SE ($n \geq 3$). R/S, root–shoot ratio; SPL, specific petiole length; SLA, specific leaf area; SSL, specific stolon length.

TABLE 3 Resource allocation of initial ramet in the second experiment stage under ambient condition.

Organ	Parameters	CK-CK	Ra-CK	Re-CK	En-CK
Blades	Total phosphorus (g/kg)	8.04 ± 0.09 a	7.54 ± 0.19 ab	8.01 ± 0.17 a	7.24 ± 0.32 b
	Nitrate nitrogen (g/kg)	2.28 ± 0.06 b	2.69 ± 0.06 a	2.17 ± 0.05 b	2.09 ± 0.04 b
	Ammonium nitrogen (g/kg)	28.66 ± 0.20 a	28.61 ± 0.22 a	28.5 ± 0.66 a	27.81 ± 0.28 a
	Organic carbon (g/kg)	397.89 ± 6.50 a	399.64 ± 2.90 a	402.9 ± 2.87 a	406.81 ± 0.84 a
	Total nitrogen (g/kg)	30.94 ± 0.22 ab	31.3 ± 0.17 a	30.68 ± 0.61 ab	29.91 ± 0.29 b
	N:P	3.85 ± 0.07 a	4.16 ± 0.09 a	3.83 ± 0.03 a	4.15 ± 0.23 a
Roots	Total carbon (g/mg)	424.54 ± 2.04 b	439.54 ± 3.92 a	424.35 ± 1.61 b	427.78 ± 3.06 b
	Total nitrogen (g/kg)	22.73 ± 0.12 c	24.92 ± 0.21 a	22.61 ± 0.17 c	24.36 ± 0.08 b
	C:N	18.68 ± 0.02 a	17.64 ± 0.29 b	18.77 ± 0.21 a	17.56 ± 0.17 b

Values with different letters were significantly different among four treatments, whereas, the same letter indicates no significant difference among four treatments ($P < 0.05$). Data were mean ± SE ($n \geq 3$). N:P, total nitrogen/total phosphorus (blades); C:N, total carbon/total nitrogen (roots).

the Re-Re and En-En, but a larger increase in R/S was presented in En-En (0.227 of Re-Re vs. 0.273 of En-En; $P < 0.05$).

After the initial ramets experienced the matched parental–offspring UV-B radiation, the changing trend of leaf parameters was similar. The petiole became short and slender, the blade

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TABLE 4 Growth parameters of offspring in the different ultraviolet-B (UV-B) environments.

Traits	CK-CK	Ra-Ra	Re-Re	En-En
Ramets biomass				
Total (g)	0.336 ± 0.009 a	0.143 ± 0.011 c	0.203 ± 0.010 b	0.164 ± 0.012 bc
Aboveground (g)	0.266 ± 0.007 a	0.121 ± 0.009 c	0.169 ± 0.007 b	0.127 ± 0.008 c
Roots (g)	0.069 ± 0.002 a	0.022 ± 0.001 c	0.033 ± 0.003 b	0.033 ± 0.004 b
R/S	0.249 ± 0.008 ab	0.182 ± 0.012 c	0.227 ± 0.015 b	0.273 ± 0.011 a
Leaf (g)	0.257 ± 0.006 a	0.114 ± 0.009 c	0.163 ± 0.007 b	0.122 ± 0.009 c
Blade (g)	0.236 ± 0.006 a	0.104 ± 0.008 c	0.146 ± 0.008 b	0.112 ± 0.013 c
Petiole (g)	0.021 ± 0.001 a	0.010 ± 0.001 c	0.013 ± 0.001 b	0.012 ± 0.001 b
Leaf parameters				
Petiole length (cm)	3.906 ± 0.093 a	2.736 ± 0.112 c	3.161 ± 0.094 b	3.168 ± 0.089 b
SPL (cm/g)	371.739 ± 8.789 d	592.877 ± 24.937 a	483.448 ± 15.364 c	531.260 ± 25.509 b
Blade area (cm^2)	58.237 ± 1.099 a	35.836 ± 1.502 bc	42.894 ± 2.237 b	31.189 ± 2.109 c
SLA (cm^2/g)	248.486 ± 5.853 c	328.156 ± 7.964 a	293.605 ± 6.700 b	304.989 ± 8.320 b
Defensive substances				
Flavonoid (OD_{300})	0.681 ± 0.038 b	1.781 ± 0.075 a	1.930 ± 0.075 a	1.860 ± 0.074 a
Anthocyanin (OD_{530})	0.030 ± 0.001 c	0.023 ± 0.004 c	0.075 ± 0.003 a	0.049 ± 0.007 b
Clone biomass				
Total (g)	0.923 ± 0.027 a	0.399 ± 0.016 b	0.461 ± 0.025 b	0.512 ± 0.052 b
Aboveground (g)	0.856 ± 0.025 a	0.384 ± 0.017 b	0.442 ± 0.032 b	0.484 ± 0.048 b
Leaf (g)	0.591 ± 0.016 a	0.295 ± 0.010 c	0.340 ± 0.018 b	0.360 ± 0.032 b
Stolon (g)	0.244 ± 0.006 a	0.081 ± 0.005 c	0.112 ± 0.008 b	0.116 ± 0.013 b
Leaf biomass/total biomass	0.649 ± 0.008 c	0.736 ± 0.014 a	0.697 ± 0.011 b	0.688 ± 0.006 b
Stolon biomass/total biomass	0.269 ± 0.006 a	0.200 ± 0.006 c	0.223 ± 0.009 b	0.232 ± 0.006 b
R/S	0.079 ± 0.003 a	0.058 ± 0.002 b	0.078 ± 0.002 a	0.076 ± 0.002 a
Growth architecture				
Number of ramets	6.167 ± 0.146 a	5.444 ± 0.176 a	6.000 ± 0.289 a	6.000 ± 0.191 a
Branching intensity	2.500 ± 0.167 ab	2.000 ± 0.000 b	3.000 ± 0.236 a	3.286 ± 0.184 a
Length of the longest stolon (cm)	47.536 ± 1.017 a	29.957 ± 1.008 bc	28.700 ± 0.836 c	34.620 ± 1.649 b
SSL (cm/g)	378.115 ± 8.586 c	591.822 ± 38.130 a	523.747 ± 29.775 ab	483.232 ± 22.129 b

Values with different letters were significantly different among four treatments, whereas the same letter indicates no significant difference among four treatments ($P < 0.05$). Data were mean ± SE ($n \geq 3$). R/S, root–shoot ratio; SPL, specific petiole length; SLA, specific leaf area; SSL, specific stolon length.

TABLE 5 Resource allocation of initial ramet in the second experiment stage under the different ultraviolet-B (UV-B) conditions.

Organ	Parameters	CK-CK	Ra-Ra	Re-Re	En-En
Blades	Total phosphorus (TP) (g/kg)	8.04 ± 0.09 a	3.99 ± 0.09 c	4.58 ± 0.15 b	4.79 ± 0.13 b
	Nitrate nitrogen (g/kg)	2.28 ± 0.06 c	3.21 ± 0.05 a	2.63 ± 0.06 b	2.55 ± 0.10 b
	Ammonium nitrogen (g/kg)	28.66 ± 0.20 a	23.47 ± 0.15 b	22.45 ± 0.11 c	23.75 ± 0.49 b
	Organic carbon (OC) (g/kg)	397.89 ± 6.50 c	427.21 ± 2.15 ab	424.20 ± 0.60 b	430.14 ± 1.56 a
	Total nitrogen (TN) (g/kg)	30.94 ± 0.22 a	26.68 ± 0.11 b	25.08 ± 0.16 c	26.31 ± 0.44 b
	N:P	3.85 ± 0.07 c	6.68 ± 0.17 a	5.49 ± 0.20 b	5.50 ± 0.06 b
Roots	Total carbon (TC) (g/mg)	424.54 ± 2.04 ab	412.78 ± 4.46 c	425.41 ± 1.36 a	414.23 ± 3.10 bc
	Total nitrogen (TN) (g/kg)	22.73 ± 0.11 a	19.55 ± 0.19 c	19.25 ± 0.21 c	21.39 ± 0.23 b
	C:N	18.68 ± 0.02 c	21.13 ± 0.41 b	22.10 ± 0.29 a	19.37 ± 0.07 c

Values with different letters were significantly different among four treatments, whereas, the same letter indicates no significant difference among four treatments ($P < 0.05$). Data were mean ± SE ($n \geq 3$). N:P, total nitrogen/total phosphorus (blades); C:N, total carbon/total nitrogen (roots).

area decreased, and the SLA increased in the UV-B radiation groups by comparing with the CK-CK. In the Ra-Ra treatment, the petiole was the shortest and the slenderest, and the SLA was the largest. In addition, the SPL of the En-En was larger than that of the Re-Re (531.260 cm/g of En-En vs. 483.448 cm/g of Re-Re; $P < 0.05$), and the blade area of the En-En was

smaller than that of the Re-Re (31.189 cm^2 of En-En vs. 42.894 cm^2 of Re-Re; $P < 0.05$). There was no significant difference in petiole length (3.161 vs. 3.168 cm; $P > 0.05$) and SLA (293.605 cm^2/g vs. 304.989 cm^2/g; $P > 0.05$) between Re-Re and En-En.

Flavonoids increased significantly in the radiation environments, and there was no significant difference among the three different UV-B treatments (0.681 of CK-CK vs. 1.781 of Ra-Ra, 1.930 of Re-Re, and 1.860 of En-En). There was no significant difference in anthocyanin content between the CK-CK and Ra-Ra (0.030 vs. 0.023; $P > 0.05$), but the content of anthocyanin in the Re-Re and En-En was increased significantly (0.075 of Re-Re; 0.049 of En-En) and the maximum value of anthocyanin was shown in the Re-Re.

The biomass of all parts of the clone (biomass of stolon, leaf, and aboveground, and total biomass) in the matched parental–offspring UV-B environments decreased significantly. The minimum value of the leaf (0.295 g) and stolon (0.081 g) biomass appeared in the Ra-Ra. In the UV-B treatments, the leaf biomass allocation increased significantly, the stolon biomass allocation decreased significantly, but the larger increase or decrease in biomass allocation was presented in Ra-Ra. There was no significant difference in clone biomass and biomass allocation between the Re-Re and En-En. The R/S decreased in the Ra-Ra group (0.058 vs. 0.079 of CK-CK; $P < 0.05$), but had no significant difference among the CK-CK, Re-Re, and En-En treatments (Table 4).

There was no difference among the different treatments in the number of ramets. The branching intensity in the Re-Re and En-En was more than that in the Ra-Ra. The longest stolon became short and slender after plants experienced UV-B radiation, and the maximum value of SSL was presented in the Ra-Ra group (591.822 cm/g). The longest stolon in the En-En (34.620 cm) was longer than that in the Re-Re (28.700 cm). The SSL indicated no significant difference between the Re-Re and En-En (523.747 cm/g vs. 483.232 cm/g; $P > 0.05$).

After UV-B radiation, the TP, ammonium nitrogen, and TN in blades decreased significantly, and nitrate-nitrogen, OC, and N:P increased significantly. Besides, the content of TN in root decreased significantly in UV-B treatments by comparing with the CK-CK. Compared with other treatments, the TP of blades in the Ra-Ra (3.99 g/kg) was the lowest, while nitrate-nitrogen (3.21 g/kg) and N:P (6.68) were the largest. There was no significant difference in TP, nitrate-nitrogen, and N:P between the Re-Re and En-En. The larger value in ammonium nitrogen (23.75 vs. 22.45 g/kg of Re-Re), OC (430.14 vs. 424.20 g/kg of Re-Re), TN of blade (26.31 vs. 25.08 g/kg of Re-Re), and TN of root (21.39 vs. 19.25 g/kg of Re-Re) was presented in En-En treatment, but the larger value in TC (425.41 vs. 414.23 g/mg of En-En) and C:N (22.10 vs. 19.37 of En-En) of root appeared in Re-Re (Table 5).

The influence of offspring environment on their growth

To study the effects of offspring current environment on offspring growth, the difference of offspring in ambient condition and UV-B environment was analyzed (Figures 2–6 and Table 6). When the offspring grew in the mismatched parental–offspring environments, lots of growth parameters, such as total biomass, aboveground and root biomass, root–shoot ratio, biomass of blade, leaf and petiole, blade area, and petiole length, were all increased significantly and the SPL was decreased, while the decreased SLA was only observed in Ra-CK (Figures 2, 3).

There was no significant difference between the anthocyanin content of En-En and En-CK (0.049 vs. 0.035; $P > 0.05$) and between the flavonoid level of Ra-CK and Ra-Ra (1.680 vs. 1.781; $P > 0.05$), but flavonoid (1.930 vs. 1.335; $P < 0.001$) and anthocyanin (0.075 vs. 0.045; $P < 0.01$) level of Re-Re was higher than that of Re-CK, and the content of anthocyanin in Ra-CK was also higher than that of Ra-Ra (0.075 vs. 0.023; $P < 0.001$) (Figure 4).

The difference in TC of root was not significant in the predictable environments, but the TC of root decreased significantly in the Ra-Ra treatment by comparing with the Ra-CK (412.78 g/mg vs. 439.54 g/mg of Ra-CK; $P < 0.05$). There were significant differences in other indexes in the different offspring environments. TP, ammonium nitrogen, TN in blades, and TN in root decreased significantly after UV-B radiation, while nitrate-nitrogen, OC, N:P in blade, and C:N in root increased significantly under UV-B radiation (Table 6).

The biomass of all parts of the clone (biomass of stolon, leaf, and aboveground, and total biomass) decreased significantly after the offspring were exposed to the matched parental–offspring environments. Compared with offspring of the mismatched environment, the biomass allocation to leaf increased significantly, while the biomass allocation to stolon decreased significantly in the matched UV-B environments. The R/S decreased in the Ra-Ra (0.058 vs. 0.076 of Ra-CK; $P < 0.001$) and Re-Re (0.078 vs. 0.088 of Re-CK; $P < 0.01$), but there was no significant difference in the R/S between the En-CK and En-En (0.077 vs. 0.076; $P > 0.05$) (Figure 5).

In the Ra-Ra treatment, the number of ramets (5.4 vs. 6.3 of Ra-CK; $P < 0.01$) and branching intensity (2 vs. 3 of Ra-CK; $P < 0.001$) decreased significantly by comparing with the Ra-CK. There was no significant difference in the number of ramets between the Re-CK and Re-Re (6.5 vs. 6; $P > 0.05$). Compared with the Re-CK, the branching intensity decreased significantly in the Re-Re (3 vs. 3.67 of Re-CK; $P < 0.05$). In the En-En treatment, the number of ramets decreased significantly (6 vs. 6.6 of En-CK; $P < 0.05$), and the branching intensity had no significant change (3.29 vs. 3.13 of En-CK; $P > 0.05$) by comparing the En-CK. The longest stolons of the offspring

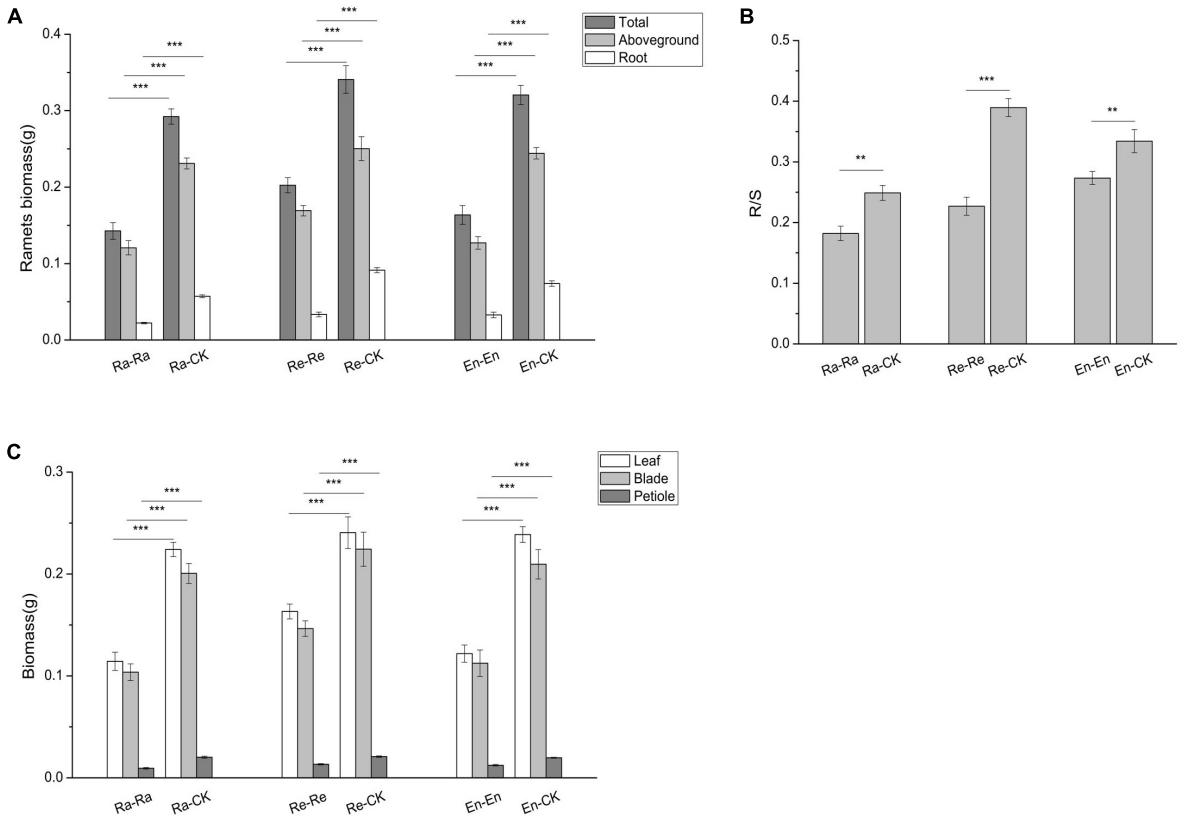

FIGURE 2
Biomass and its allocation of the initial ramet in the second experiment stage under different offspring conditions. **(A)** Ramets biomass; **(B)** root biomass/shoot biomass (R/S); **(C)** biomass of leaf, blade, and petiole. Different numbers of asterisks indicate significant differences of the same parameter between two groups, with "**" indicated $p < 0.01$, and "***" indicated $p < 0.001$. Error bars showed ± SE ($n \geq 3$).

became short and slender when they were exposed to UV-B radiation (**Figure 6**).

Discussion

The maternal effects on the growth strategy of clonal offspring

Maternal effects have become an important field of study in ecology, and there is an ongoing debate regarding their adaptive significance for offspring fitness (Marshall and Uller, 2007). Maternal effects can be either adaptive if they increase offspring fitness or not if they are neutral or harmful to the fitness of offspring (Galloway and Etterson, 2007; Donelson et al., 2018; Zhou et al., 2021). In this study, the clonal offspring of different UV-B radiated parents displayed various performances, despite they were in an unirradiated environment (**Tables 2**, **3**). This difference of offspring was elicited by the maternal effects of parental environment. The maternal effects are regarded as highly contingent on the environmental variations. The environments with slowly and predictably changing select positive maternal effects, while, the environments with rapidly and unpredictably changing select negative maternal effects (Uller, 2008; Ezard et al., 2014). In our study, random UV-B radiation could be regarded as an unpredictable environment and regular and enhanced UV-B as two predictable environments. Obviously, diverse variations of parental environment triggered different maternal effects, which regulated different growth strategies of offspring. For offspring whose parent grow in unpredictable environments (random UV-B radiation), the content of defensive substances in blade was improved obviously to effectively reduce the damage of UV-B radiation to plant tissues. In addition, more carbon and nitrogen resources were allocated to roots. The offspring biomass was maintained (in clone) or even fully decreased (in initial ramets). Above all, rapid growth to increase biomass was not the goal of these offspring, and they invested more resources in enhancing defense process to resist the unpredictable UV-B stress. However, for the offspring of parents growing in predictable environment (regular and enhanced UV-B), their growth strategy was to combine both defense and growth processes. For instance, the contents of

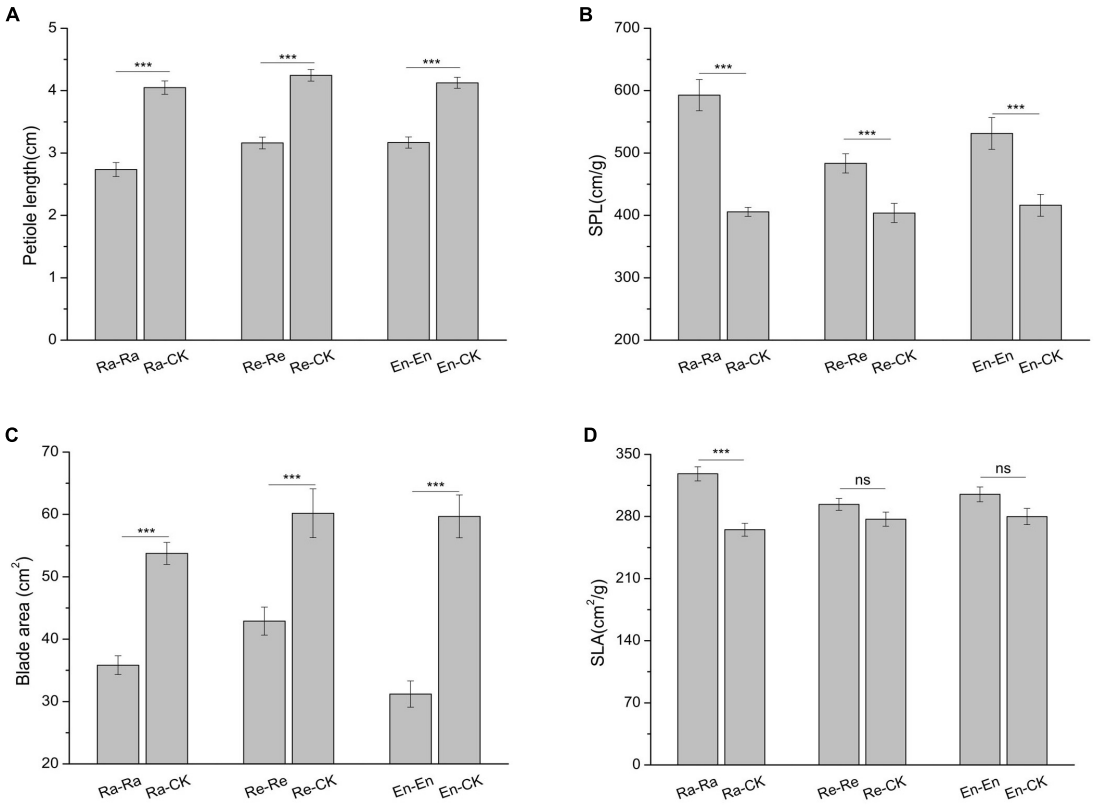

FIGURE 3
Leaf parameters of initial ramet in the second experiment stage under different offspring conditions. **(A)** Petiole length; **(B)** specific petiole length (SPL); **(C)** blade area; **(D)** specific leaf area (SLA). Different numbers of asterisks indicate significant differences of the same parameter between two groups, with "***" indicated $p < 0.001$, and "ns" indicated $p > 0.05$. Error bars showed ± SE ($n \geq 3$).

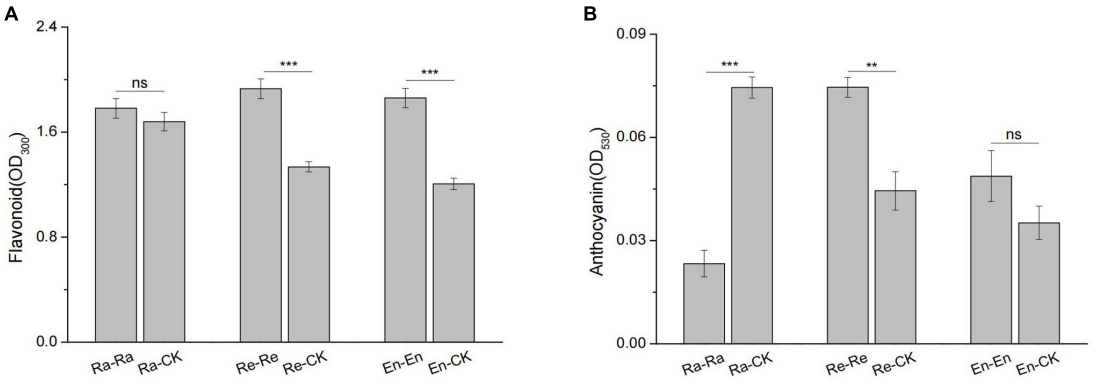

FIGURE 4
Defensive substances of initial ramet in the second experiment stage under different offspring conditions. **(A)** Flavonoid; **(B)** anthocyanin. Different numbers of asterisks indicate significant differences of the same parameter between two groups, with "**" indicated $p < 0.01$, "***" indicated $p < 0.001$, and "ns" indicated $p > 0.05$. Error bars showed ± SE ($n \geq 3$).

defensive compounds were also improved significantly, but the level was lower than that of offspring of unpredictable environmental parents. Moreover, the offspring growth was not influenced by the negative effects of parental UV-B. Of course, there were some growth differences between two kinds of offspring, such as the change of root biomass and resource allocation pattern. The offspring of regular radiated parent exhibited increased root biomass, but another

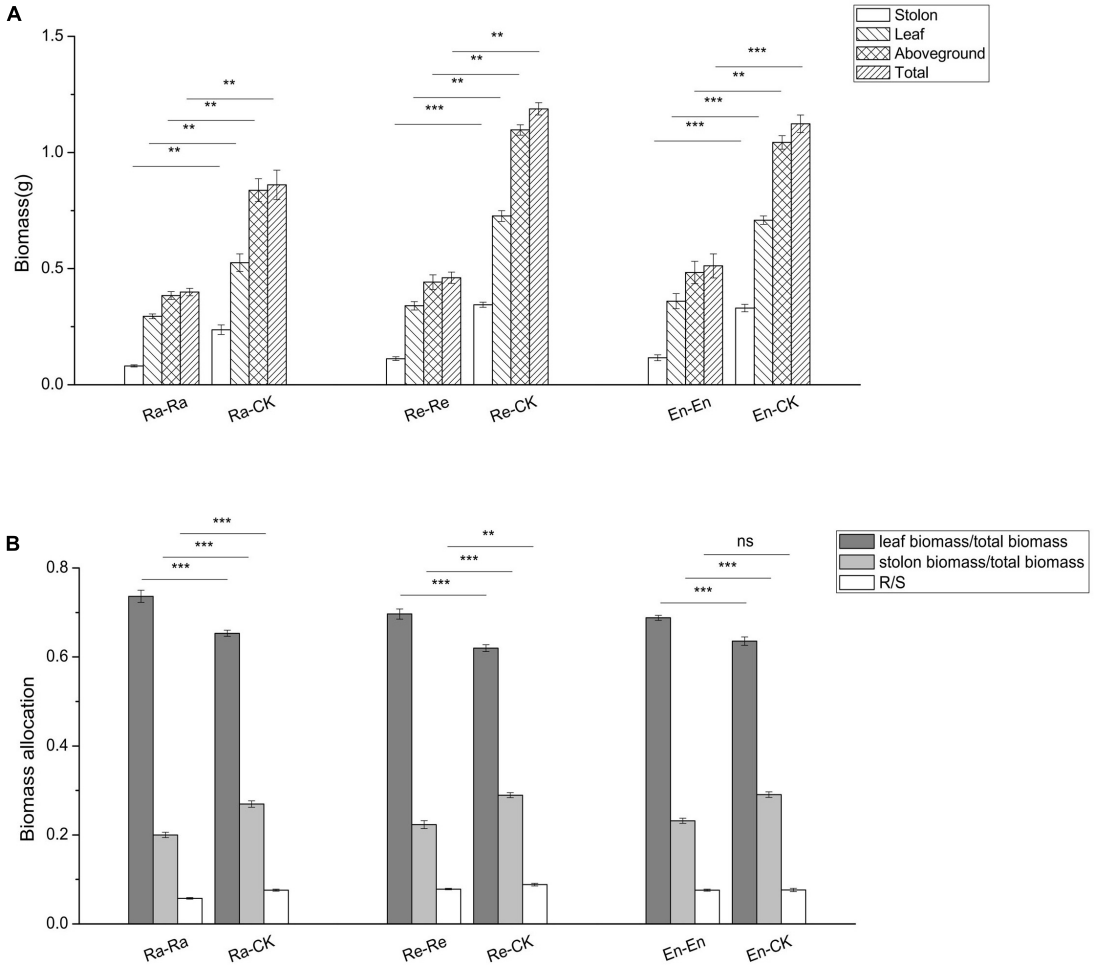

FIGURE 5
Biomass and its allocation of the clone in the second experiment stage under different offspring conditions. **(A)** Biomass; **(B)** biomass allocation. Different numbers of asterisks indicate significant differences of the same parameter between two groups, with "**" indicated $p < 0.01$, "***" indicated $p < 0.001$, and "ns" indicated $p > 0.05$. Error bars showed ± SE ($n \geq 3$).

offspring displayed regulated the allocation of nitrogen and phosphorus resources.

These diverse growth strategies induced by maternal UV-B effects were achieved by transgenerational plasticity, which had advantages in the corresponding environment. Transgenerational plasticity in response to maternal environments was common in plants (Galloway and Etterson, 2007). It could be found from these results that some traits showed high transgenerational plasticity, such as UV-B absorbing components, aboveground and underground biomass, petiole length, stolon length, and so on. While transgenerational plasticity was not observed in other traits (petiole biomass, blade area, ramets number, the level of N:P, ammonium nitrogen, and organic carbon).

In addition, the growth differences between predictable or unpredictable environments were attributed to both the changing intensity and frequency of UV-B environment experienced by the parental plants. In our study, the variation of environmental predictability contained the change of radiation frequency and/or intensity. The treatment of random UV-B radiation was to imitate the change of UV-B in nature, which included inconstant intensity and frequency of radiation. Meanwhile, only intensity changes were included in the enhanced UV-B radiation group. These variations among UV-B environments induced various effects and ultimately led to different growth performances.

The influence of maternal effects on the adaptability of clonal offspring in ultraviolet-B environment

For the *G. longituba* in this study, when offspring grew in matched maternal–offspring UV-B environments, their

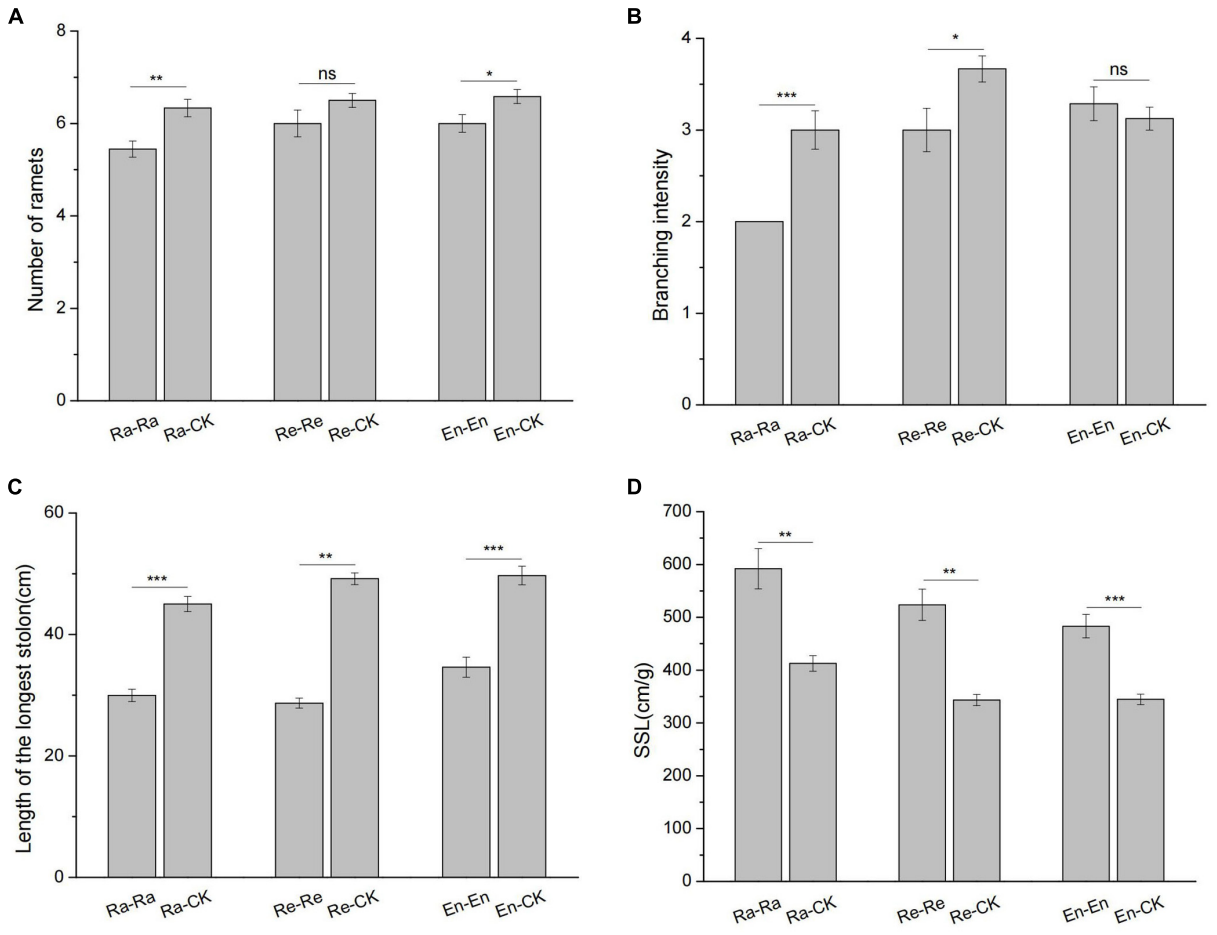

FIGURE 6
Growth architecture of clone in the second experiment stage under different offspring conditions. **(A)** The number of ramets; **(B)** branching intensity; **(C)** length of the longest stolon; **(D)** specific stolon length (SSL). Different numbers of asterisks indicate significant differences of the same parameter between two groups, with "*" indicated $p < 0.05$, "**" indicated $p < 0.01$, "***" indicated $p < 0.001$, and "ns" indicated $p > 0.05$. Error bars showed ± SE ($n \geq 3$).

TABLE 6 Resource allocation of initial ramet in the second experiment stage under different offspring conditions.

Organ	Parameters	Ra-Ra	Ra-CK	Re-Re	Re-CK	En-En	En-CK
Blades	Total phosphorus (TP) (g/kg)	3.99 ± 0.09 b	7.54 ± 0.18 a	4.58 ± 0.15 b	8.01 ± 0.17 a	4.79 ± 0.13 b	7.24 ± 0.32 a
	Nitrate nitrogen (g/kg)	3.21 ± 0.05 a	2.69 ± 0.06 b	2.63 ± 0.06 a	2.17 ± 0.05 b	2.55 ± 0.10 a	2.09 ± 0.04 b
	Ammonium nitrogen (g/kg)	23.47 ± 0.15 b	28.61 ± 0.22 a	22.45 ± 0.11 b	28.50 ± 0.66 a	23.75 ± 0.49 b	27.81 ± 0.28 a
	Organic carbon (OC) (g/kg)	427.21 ± 2.15 a	399.64 ± 2.89 b	424.20 ± 0.60 a	402.90 ± 2.87 b	430.14 ± 1.56 a	406.81 ± 0.84 b
	Total nitrogen (TN) (g/kg)	26.68 ± 0.11 b	31.30 ± 0.17 a	25.08 ± 0.16 b	30.68 ± 0.61 a	26.31 ± 0.44 b	29.91 ± 0.29 a
	N:P	6.68 ± 0.17 a	4.16 ± 0.09 b	5.49 ± 0.20 a	3.83 ± 0.03 b	5.50 ± 0.06 a	4.15 ± 0.23 b
Roots	Total carbon (TC) (g/mg)	412.78 ± 4.46 b	439.54 ± 3.92 a	425.41 ± 1.36 a	424.34 ± 1.61 a	414.23 ± 3.10 a	427.78 ± 3.06 a
	Total nitrogen (TN) (g/kg)	19.55 ± 0.19 b	24.92 ± 0.20 a	19.25 ± 0.21 b	22.61 ± 0.17 a	21.39 ± 0.23 b	24.36 ± 0.08 a
	C:N	21.13 ± 0.41 a	17.64 ± 0.29 b	22.10 ± 0.29 a	18.77 ± 0.21 b	19.37 ± 0.07 a	17.56 ± 0.17 b

Values with different letters were significantly different between different offspring environments, whereas, the same letter indicates no significant differences ($P < 0.05$). Error bars showed ± SE ($n \geq 3$). N:P, total nitrogen/total phosphorus (blades); C:N, total carbon/total nitrogen (roots).

growth was depressed. Among treatments, the ramets in the unpredictable environment as their mother ramets, the inhibition of growth was the strongest, while ramets in predictable environment accumulated more defensive (flavonoid and anthocyanin) components to resist UV-B radiation (Tables 2, 3). It seemed that maternal effects in unpredictable habitats were maladaptive, while some transgenerational effects of predictable environments

were partially beneficial to improve the offspring fitness. Although some studies suggested that when the maternal environment is an accurate predictor of the environment that offspring will encounter, beneficial maternal effects are expected to promote adaptive shifts (Marshall and Uller, 2007; Ezard et al., 2014). However, whether the transgenerational effects in phenotype could be adaptive is difficult to interpret, as many of these fitness-linked traits responded in opposite directions, suggesting the potential for complex trade-offs among traits. The decrease in growth does not necessarily mean that maternal effect is completely unadaptable, and resources may be more devoted to production of chemical or physical defense. A trade-off between plant growth and defense to maintain optimal fitness has been reported in much research (Bazzaz et al., 1987; Züst and Agrawal, 2017; Guo et al., 2018; Dwivedi et al., 2021). According to the acclimatory response hypothesis, activating defenses is an adaptive response. Plants will reconfigure their metabolic and allocation strategies to optimize the use of potentially limiting resources (Ballaré and Austin, 2019). Another hypothesis for the growth-defense trade-off is that growth, at least in some ways, is not suitable in certain stresses (Díaz et al., 2001; Ballaré and Austin, 2019). As such, it was beneficial for the ramets of *G. longituba* to reduce growth to minimize exposure to UV-B radiation. They put more energy into chemical defense in predictable UV-B environments, which is an economically feasible strategy. It is pointed out that plants will avoid excessive growth or defense through a negative feedback-regulatory loop and achieve balance in response to adverse environments (Li et al., 2019). In brief, predictable maternal conditions experienced by clonal *G. longituba* can affect growth of offspring, and some phenotypes induced by the transgenerational effects may be adaptive. These responses ultimately allow offspring to tolerate the stress conditions they currently experienced.

The influence of offspring environment on their performance

In our study, if UV-B radiation was released, the growth of offspring became better, and it could be observed from the recovery of all organ biomass, which was most significant in offspring of predictable radiated parent (**Figures 2**, **5**). The performance of offspring was related to the within-generation plasticity. Within-generation plasticity relies on the current environmental information (Auge et al., 2017a). This compensation of growth under no UV-B condition was attributed to the recovery of leaf, the enlargement of blade area contributed to absorbing more light for photosynthesis, and more photosynthate transferred to roots, helping to increase the root biomass. However, the maternal effects still played the role in offspring growth, and it could be found from the increased flavonoid content. The defenses induced in parents can be inherited by offspring, allowing progenies to deploy stronger defenses had been discovered (Karasov et al., 2017). It had been suggested that the growth and behavior of clonal plants might be significantly modified by environments that the parent had experienced but are no longer present, and the methylation variations of offspring inherited from the parents make the effects on their phenotype (Chinnusamy and Zhu, 2009; Verhoeven et al., 2010; Huber et al., 2014). Of course, the maternal effects might reduce, and the limitation of transgenerational transmission in response to stress has also been suggested in some studies, which may also contribute to traits recovery (Boyko et al., 2010; Zhang et al., 2021). In short, in our study, the performance of clonal offspring was affected by the combination of within- and transgenerational plasticity.

Conclusion

In our study, maternal effects play an important role in shaping the growth strategies of clonal offspring, and the effects were regulated by predictability of parental environment. Maternal effects induced by unpredictable environment affected offspring growth by investing more resources in defense than rapid growth, while that by predictable environment regulated the growth of offspring via combining the two processes of defense and growth. However, there were still some differences between the offspring of regular and enhanced radiated parents. In addition, when the offspring were exposed to the matched parental–offspring UV-B environments, the adaptability of maternal effects varied with different predictability cues. It seemed that transgenerational effects of predictable environments were beneficial in improving the adaption of offspring partly. Clonal offspring in predictable habitats devoted more resources to chemical defense while also trying to maintain growth. Besides, when the offspring were transplanted to the non-UV-B environment, their growth was significantly recovered for the within-generational and transgenerational plasticity. Our findings suggest that environmental predictability plays an important role in the trade-off between plant growth and defense.

Data availability statement

The data presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Author contributions

XL conceived and designed the experiments. YG, JQ, and XW performed the experiments. YG wrote the manuscript. JQ, XW, ZZ, XL, RZ, and MY provided data analysis and editorial advice. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Fourth National Survey of Traditional Chinese Medicine Resources (2018-43).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 07 September 2022
DOI 10.3389/fpls.2022.872065

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

RECEIVED 09 February 2022
ACCEPTED 02 August 2022
PUBLISHED 07 September 2022

CITATION
Wang L-H, Si J, Luo F-L, Dong B-C and Yu F-H (2022) Parental effects driven by resource provisioning in *Alternanthera philoxeroides*—A simulation case study.
Front. Plant Sci. 13:872065.
doi: 10.3389/fpls.2022.872065

Parental effects driven by resource provisioning in *Alternanthera philoxeroides*—A simulation case study

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Parental environmental effects can be a rapid and effective means for clonal plants in response to temporally or spatially varying environments. However, few studies have quantitatively measured the ecological significance of parental effects in aquatic clonal plants. In this study, we developed a two-generation (parent-offspring) growth model to examine the parental effects of nitrogen (N) conditions on summed and mean performance of clonal offspring of one wetland species *Alternanthera philoxeroides*. We also examined the role of survival status and developmental stage of clonal offspring in the consequence of parental effects in aquatic clonal plants. Our results indicated direct evidence that (1) there were significant non-linear correlations between the performance of parental plants and initial status of clonal offspring (i.e., the mass and number of clonal propagules); (2) parental N effects on the summed performance of clonal offspring were content-dependent (i.e., there were significant interactions between parental and offspring N effects), while parental effects on the mean performance of offspring were independent of offspring conditions; (3) parental effects mainly occurred at the early development stage of clonal offspring, and then gradually declined at the late stage; (4) the context-dependent parental effects on the summed performance of clonal offspring gradually strengthened when offspring survival was high. The mathematical models derived from the experimental data may help researchers to not only deeply explore the ecological significance of parental environmental effects in aquatic clonal plants, but also to reveal the importance of potential factors that have been often neglected in empirical studies.

KEYWORDS

***Alternanthera philoxeroides*, clonal plant, individual and population scales, mathematical modeling, parental N effects**

Introduction

Clonal plants, that spontaneously produce offspring *via* vegetative reproduction, are widespread in nature (de Kroon and van Groenendael, 1997). During the entire life cycle of clonal plants, clonal offspring ramets are repeatedly produced by parental ramets, so the performance of offspring ramets is greatly influenced by the environments that parental ramets have encountered (Latzel and Klimešová, 2010; Douhovnikoff and Dodd, 2015). An increasing body of evidence has documented that parental environments may to some extent regulate the survival, early development, and subsequent growth of clonal offspring across vegetative generations, and also adjust the life-history strategy of clonal offspring to pre-adapt to future environments (González et al., 2016, 2017; Münzbergová and Hadincová, 2017; Dong et al., 2019a,b; DuBois et al., 2020; Huber et al., 2021). Compared to sexually reproducing plants, such parental environmental effects are especially important for clonal plants with a low potential for adaptation through genetically based natural selection.

There are two commonly known types of mechanisms that mediate parental environmental effects in clonal plants, including either the epigenetic-based mechanism such as DNA methylation and histones modification (Latzel and Klimešová, 2010; Douhovnikoff and Dodd, 2015), or provisioning of resources in vegetative propagules such as carbohydrate- and/or nitrogen-based compounds (Dong et al., 2019a; DuBois et al., 2020). For clonal plants, the epigenetic-based mechanism may allow clonal offspring to maintain a long-term and stable phenotype in response to the predictable environments that parental plants have experienced, *via* the accumulation of gene expressions across multiple clonal generations (Douhovnikoff and Dodd, 2015). Alternatively, the changing in provisioning of resources in vegetative propagules caused by parental environments is considered a direct means for clonal offspring in response to the changing environments. Also, the impact of provisioning of resources is expected to become more prominent within several or few clonal generations, because it can directly influence the initial status of vegetative propagules and the sequential growth trajectory of clonal offspring (Dong et al., 2019a; DuBois et al., 2020). From the perspective of mathematical modeling, the parental effects regulated by the provisioning of resources appear to be easily parameterized and accurately estimated in the mathematical models, compared to the parental effects through epigenetic inheritance. However, to our knowledge, studies of quantifying such parental effects have been very scarce.

Several potential factors may influence the magnitude of parental effects in the next clonal generations. The primary factor is the resource level of environments that clonal offspring experienced (Engqvist and Reinhold, 2016; Bonduriansky and Crean, 2018; Yin et al., 2019). One likely scenario is that parental effects will be favorable when the environmental predictability between parent and offspring environments prevails, thereby being often adaptive for clonal offspring (Latzel and Klimešová, 2010; Douhovnikoff and Dodd, 2015). A secondary scenario is that parental effects may interact with offspring environments, but not shift their direction, i.e., the benefits from parental favorable environments may amplify or dwindle with the increased suitability of offspring environments. Such phenomena have been reported especially in the abiotic conditions that offspring experienced, such as drought (González et al., 2016), light (Dong et al., 2019b) and nutrient availability (Dong et al., 2018a). Also, in some cases, parental effects on offspring performance are parallel with the effects caused by offspring environments (Schwaegerle et al., 2000; Dong et al., 2018b).

In addition, the magnitude of parental effects may fluctuate at the different developmental stages of clonal offspring (Schwaegerle et al., 2000; Huber et al., 2021). Provided that parental effects are mainly regulated by the provisioning of resources, such kind of parental effects are predicted to play a key role for clonal offspring at the early developmental stage than at the late stage (Schwaegerle et al., 2000). This speculation may be reasonable that the sufficient supply of resources from vegetative propagules not only guarantees the early normal growth of clonal offspring, but also supports the new development of nutrient absorbing organs such as leaves and roots (Stuefer and Huber, 1999; Dong et al., 2010, 2011; Song et al., 2013). Such early-stage advantage will be gradually weakened at the late developmental stage, especially when the resource supply for plant growth begins to shift from the provisioning of resources from storage organs to the acquisition of the resources from external environments. However, to our knowledge, the changes in the strength of parental effects at different developmental stages have rarely been explored.

Furthermore, the magnitude of parental effects may vary with the study scale of clonal offspring (Dong et al., 2018a). Compared to the performance of individual offspring ramet, the performance of one offspring generation appears to be a complex process, which is closely associated with the survival status, initial-size distribution and number of clonal offspring within one offspring population (Dong et al., 2018a). Given that the performance of the offspring generation is jointly determined by the initial size and number of the surviving offspring, the pattern of parental effects at the offspring-generation level become unpredictable, compared to the performance at the individual level. Previous published studies, indicate that the parental nutrient effect in *Alternanthera philoxeroides* was independent of offspring conditions at the individual level, but also became context-dependent (i.e., the parental nutrient effects interacted with offspring nutrient condition) at the offspring-generation level

(Dong et al., 2018a). In an opposite example, because of the trade-off between offspring size and number within one offspring population of the perennial sedge *Scirpus maritimus*, the consequence of parental effects at the individual level was concealed when the overall fitness of clonal offspring are considered (Charpentier et al., 2012). Therefore, it is worth systematically examining the magnitude of parental effects at different study scales.

To provide an explicit test for the parental environmental effects with the provisioning of resources as a potential mechanism, we developed a mathematical model based on the empirical data from two separated greenhouse experiments on the well-studied, amphibious clonal species *A. philoxeroides*. We manipulated N availability as a key external factor that can significantly increase the provisioning of resources in vegetative propagules of clonal plants. Our study especially focused on the following questions: (1) whether parental effects are mediated by the provisioning of resources in clonal propagules, so that they can be predictable *via* modeling? (2) whether parental effects are influenced by the N conditions that clonal offspring experienced? (3) whether parental effects are influenced by the developmental stages of clonal offspring? (4) whether parental effects are influenced by other status of clonal offspring, such as offspring survival?

Materials and methods

Plant species

Alternanthera philoxeroides (Mart.) Griseb. is a creeping perennial herb of the Amaranthaceae family, native to South America (Holm et al., 1997). The species is considered one of the most noxious invasive weeds in China. This species can rapidly disperse and colonize both aquatic and terrestrial habitats, thereby causing severe ecological and environmental problems (Wu et al., 2016, 2017). In southern China, the invasive populations of *A. philoxeroides* have been reported to belong to the same genotype (Xu et al., 2003; Ye et al., 2003; Wang et al., 2005). *A. philoxeroides* also mainly relies on clonal growth by producing stem and/or root fragments to achieve offspring recruitment (Jia et al., 2009; Dong et al., 2012, 2019a). Each stem node of *A. philoxeroides* can be naturally and/or incidentally fragmented, and become a physiologically independent unit with the potential to develop into ramets (Dong et al., 2012, 2018a).

In this study, plants of *A. philoxeroides* were collected from populations in a riparian agricultural area in Taizhou, Zhengjiang, China (28.87°N, 121.01°E), on 18–19 May 2011. They were vegetatively propagated for more than 3 years in a greenhouse at Beijing Forestry University.

Experimental design

Greenhouse experiment on growth trajectory

To simulate the growth trajectory of plants of *A. philoxeroides*, a greenhouse experiment was conducted for 75 days, from 12th June to 24th September 2015. The average air temperature and humidity during the first experiment, measured by HOBO UX100-003 data loggers (Onset Computer Corporation, Bourne, MA, United States), was 25.00 ± 0.31°C and 77.80 ± 1.07% (mean ± SE), respectively.

For this experiment, 80 clonal fragments of *A. philoxeroides*, each consisting of a single stem of about 10 cm long with two nodes and an apex, were used for the first experiment. Twenty-four additional, similar fragments were selected and dried to estimate the initial dry mass, which was 22.38 ± 0.90 mg (mean ± SE).

Plants were grown in 500 ml plastic plots (9.5 cm in diameter, 13.2 cm deep) fertilized with a modified Hoagland solution containing 10, 20, 40, and 60 mg N L^{-1} supplied as $Ca(NO_3)_2$, to mimic four different nitrogen conditions that cover a range from limiting to non-limiting amounts for plant growth of *A. philoxeroides* (Wang et al., 2017). Because we added $Ca(NO_3)_2$ to different Hoagland solutions to vary the concentrations of N, the concertation of Ca^{2+} changed accordingly. To maintain the same Ca^{2+} concentration in different solutions, we need to add $CaSO_4$ to compensate for the missing Ca^{2+} in the solutions with high-level N. Finally, only SO_4^{2-} was always supplied in surplus, since it was expected to impose a negligible impact on plant growth (Wang et al., 2017; Dong et al., 2019c). The gradients of the modified Hoagland solution can be found in **Supplementary Table 1**. Nutrient solutions were completely refreshed every 4 days to minimize any cumulative buildup or depletion of nutrients. There were four replicates for each N treatment at each harvest.

Plants were separately harvested at 30, 45, 60, and 75 days after the starting of the experiment. At each harvest, plants were washed with distilled water and divided into leaves, stems and roots. Dry masses of each plant part were weighted after oven-drying at 70°C for 48 h.

Greenhouse experiment on size distribution

To parameterize the size distribution of clonal propagules in *A. philoxeroides*, a second experiment was conducted for 45 days, from 22nd July to 4th September 2018. The average air temperature and humidity was 30.43 ± 0.40°C and 35.90 ± 0.99% (mean ± SE), respectively.

For this experiment, 28 clonal fragments of *A. philoxeroides* of similar size as in the first experiment, were used. Plants were also grown in 500 ml plastic plots filled with the modified Hoagland solution containing 10, 20, 40, and 60 mg N L^{-1}. There were seven replicates of plants for each N treatment. After the 45-day cultivation, stem nodes per plant were counted, and the aboveground part per plant was then subdivided into

single-node stem fragments (i.e., clonal offspring) attached with two opposite leaves and half of both the proximal and distal internodes, irrespectively. Each single-node stem fragment (hereafter referred to as clonal propagules) was then stored in individual envelopes. All clonal propagules of each plant and its roots were oven-dried at 70°C for 48 h, and weighed.

Simulation models—Variable selection

Using the data from the first experiment, we compared the following six classic growth models: linear, exponential, power, monomolecular, three-parameter logistic, and Gompertz models (see **Supplementary Appendix Table 1**). We then selected the best-fitted model to simulate the growth trajectory of *A. philoxeroides* (Paine et al., 2012). The criterion of model selection was based on the goodness-of-fit (AICs) *via* the "nls" function of the "stats" package. In the growth models, the N levels of external conditions were included as the proxy of the environmental capacity *K*. Also, we examined the correlation between the total mass of each parental plant and the mean mass of the clonal propagules of each parental plant (Eq. 1), and the one between the total mass of each parental plant and the total number of clonal propagules (Eq. 2).

$$N_{propagule} = \alpha_1 * \mathrm{TM}_{parent}{}^{\beta_1} \quad (1)$$

$$MM_{propagule} = \alpha_2 * \mathrm{TM}_{parent}{}^{\beta_2} \quad (2)$$

In which $N_{propagule}$ is the number of clonal propagules produced by one parental plant, $MM_{propagule}$ is the mean mass of clonal propagules, TM_{parent} is the total mass of one parental plant, $\alpha_{(1,\,2)}$ and $\beta_{(1,\,2)}$ are parameters of each correlation equation, respectively.

Using the data from the second experiment, we compared four candidate size distribution models (i.e., the Normal, Log-Normal, Gamma and Weibull distribution) for the size distribution of the clonal propagules produced by one parental plant. The best-fitted model was selected to simulate the size distribution of the clonal propagules of *A. philoxeroides*. The criterion of the best-fitted distribution model was based on the result from the Cramer-von Mises test with the "gofstat" function of the "fitdistrplus" package (Delignette-Muller and Dutang, 2015). Also, we examined the correlations with the mean mass of clonal propagules and each of the parameters from the size distribution model of clonal propagules (Eq. 3).

$$Pars = \alpha_{(3,\,4)} * MM_{propagule} + \beta_{(3,\,4)} \quad (3)$$

In which *Pars* are parameters of the size distribution of clonal propagules, $MM_{\mathrm{propagules}}$ is the mean mass of clonal propagules, $\alpha_{(3,\,4)}$ and $\beta_{(3,\,4)}$ is the parameter of each correlation equation, respectively.

Simulation models—Model construction

By integrating the growth, size distribution models and the correlations (Eqs. 1–3) as mentioned above, we constructed a two-generation (parent-offspring) growth model (**Figure 1**). In the model, we assumed that (1) both the parental plants and clonal offspring would follow the growth trajectory as simulated by the same growth model; (2) in the offspring generation, clonal propagules would be totally fragmented and physically independent of one another in homogenous N conditions (i.e., no occurrence of resource sharing and competition between clonal offspring); (3) the size of clonal propagules would be randomly generated by the size distribution model, in which the parameters of the size distribution are regulated by the mean mass of clonal propagules; (4) the offspring derived from clonal propagules with different size would share the same survival rate. In particular, the second assumption seems to be more true for aquatic clonal plants than for terrestrial ones, since aquatic environments are more open and allow for less competition, and resource distribution is also more uniform in aquatic environments. Also, this model derived from the data of stolon fragments may be more valid for the aquatic form of *A. philoxeroides*, which often lacks the formation of taproot system in aquatic environments.

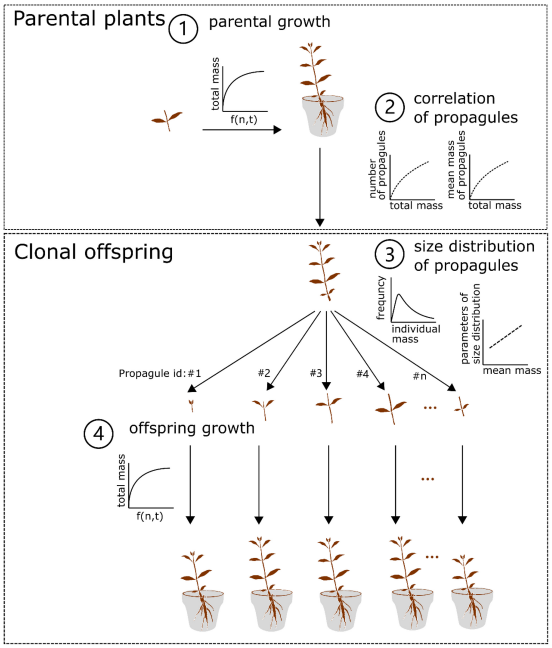

FIGURE 1
Conceptual diagram of the two-generation growth model of *Alternanthera philoxeroides*. In brief, the parental plant grows following the setup of the three-parameter logistic model with N levels and time included (1), and the final (total) mass of the parental plant is non-linearly correlated with the number and mean mass of its clonal propagules (2). In addition, the size of the surviving clonal propagules produced by the parental plant followed the Weibull distribution, in which the scale and shape values are linearly correlated with the mean mass of the surviving clonal propagules (3). The surviving offspring continue to grow following the setup of the growth model for the parental plant (4). See details in the Material and Method section.

Simulation experiment

We used the empirical results from two greenhouse experiments to parameterize the two-generation (parent-offspring) growth model of *A. philoxeroides* (see the conceptual diagram in Table 1). To explore the effect of parental N environments on the performance of clonal offspring at both individual and offspring-generation scales, the parental and offspring N environments were both set along one gradient of N levels (i.e., 10, 20, 30, 40, 50, and 60 mg/L). To explore the parental effects on clonal offspring that experienced different survival status, the survival rates of clonal propagules were separately set as 25, 50, 75, and 100%. One of our interests was to see if the parental effects on the performance of the remaining clonal offspring generation changed, when a number of clonal offspring died due to some other external factors, such as herbivory stress or mechanical removal. It is worth noting that the order of clonal propagules was not considered in the model. To explore the parental effects on clonal offspring at different developmental stages, the growth time of parental plants was constant as 75 days, but the simulated growth time of clonal offspring were separately set as 30, 45, 60, 75, 150, and 300 days. The simulation process was repeated five times. ANOVAs were followed to test the effects of parental N levels, offspring N levels, survival rate, developmental time, and their interactions on the summed and mean performance (i.e., mass) of the surviving clonal offspring produced by one parental plant. All analyses and simulation processes were conducted using R v. 4.0.2 (R Core Team, 2020).

TABLE 1 Parameter values used in the simulation experiment.

Parameter/ Function	Definition	Values
$\frac{M_0 K}{M_0 + (K - M_0)e^{-rt}}$	Three-parameter logistic model (the best-fitted growth model); M_0 as the initial mass of one plant; r as the relative growth rate; t as the time of plant growth K as the environmental capacity.	*Parental plants*: M_0 = 22.38 mg; r = 0.103; t = 75 days. *Clonal offspring*: r = 0.103; t = 30, 45, 60, 75, 150, and 300 days.
$K = \gamma \times N_{level} + \delta$	K in the growth model as a function of N levels.	*Parental plants and clonal offspring*: N_{level} = 10, 20, 30, 40, 50, and 60 mg/L; γ = 219.642; δ = 1965.396.
Eq. 1	Number of clonal propagules as a function of total mass of one parental plant.	α_1 = 0.253; β_1 = 0.684.
Eq. 2	Mean mass of clonal propagules as a function of total mass of one parental plant.	α_2 = 2.727; β_2 = 0.354.
Weibull distribution	The best-fitted model for the size distribution of clonal propagules produced by one parental plant.	
Eq. 3	Shape value of Weibull distribution as a function of mean mass of clonal propagules.	α_3 = 0.015; β_3 = 0.674.
	Scale value of Weibull distribution as a function of mean mass of clonal propagules.	α_4 = 1.183; β_4 = −5.083.
Survival rate		25, 50, 75, and 100%.

Results

Greenhouse experiments

Within six candidate growth models, the three-parameter logistic model with N levels included was determined as the best-fitted model and used in the following simulation (Supplementary Appendix Table 2 and Supplementary Appendix Figure 1). The corresponding parameter values for the growth model were obtained: r = 0.103, γ = 219.642, and δ = 1965.396 (Supplementary Appendix Table 2). In addition, the non-linear regression in Eqs. 1 and 2 fitted the data very well (R^2 = 0.91 and 0.77, respectively; Eqs. 4 and 5 and Supplementary Appendix Figure 2).

$$N_{propagule} = 0.253 * \mathrm{TM}_{parent}^{0.684} \quad (4)$$

$$MM_{propagule} = 2.727 * \mathrm{TM}_{parent}^{0.354} \quad (5)$$

Within four candidate distribution models, the Weibull distribution provided the best-fitting result for the size distribution of clonal propagules in 19 out of the 28 individual parental plants with almost the lowest AICs, thereby being used in the simulation experiment. The scale value of Weibull distribution was well-fitting to the mean mass of clonal propagules by a linear correlation (R^2 = 0.98 and $P < 0.001$; Eq. 6 and Supplementary Appendix Figure 3), and the shape value was marginally significantly linearly related to the mean

TABLE 2 Effects of parental N levels and the survival rate of clonal offspring on the total and mean mass, and the number of the surviving clonal propagules produced by one parental plant.

Variables		Total mass of propagules		Number of propagules		Mean mass of propagules	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Parental N (PN)	1, 116	**7,098**	**<0.001**	**8,549**	**<0.001**	**348.0**	**<0.001**
Survival rate (SR)	1, 116	**11,261**	**<0.001**	**30,129**	**<0.001**	3.1	0.079
PN × SR	1, 116	**1,277**	**<0.001**	**1,643**	**<0.001**	1.1	0.293

Degrees of freedom *(df)*, F- and P-values are given. Significance is highlighted in bold.

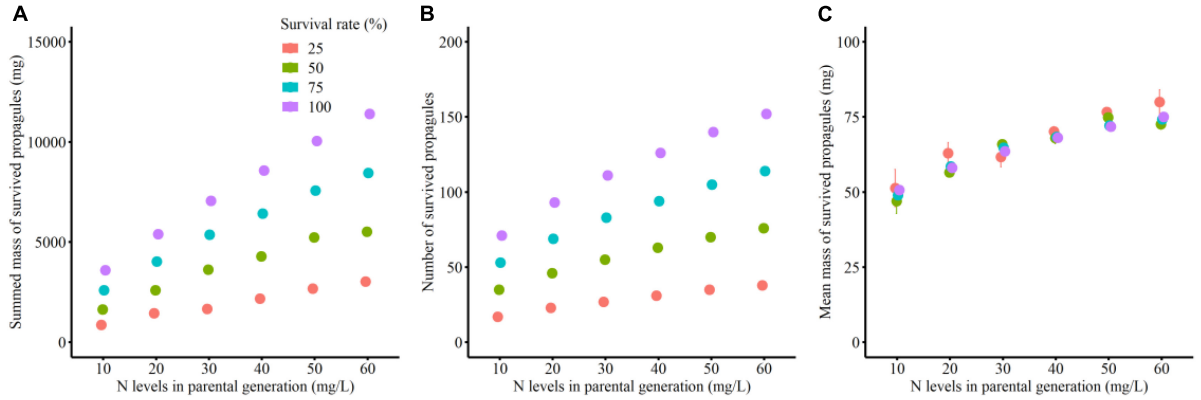

FIGURE 2
Summed mass **(A)**, mean mass **(B)** and number **(C)** of the surviving clonal propagules produced by each parental plant in the simulation experiment.

mass of clonal propagules (R^2 = 0.16 and P = 0.088; Eq. 7 and Supplementary Appendix Figure 3).

$$Scale = 1.183 * MM_{propagule} - 5.083 \quad (6)$$

$$Shape = 0.015 * MM_{propagule} + 0.674 \quad (7)$$

Simulation experiment

Number and mean mass of clonal propagules

Total mass and number of the surviving clonal propagules per parental plant were significantly influenced by parental N levels, survival status of clonal offspring and their interaction (Table 2 and Figures 2A,B). The total mass and number of the surviving clonal propagules were significantly elevated with the increased N levels, and the positive effect of parental N levels was more remarkable when the survival rate of clonal propagules was higher (Table 2 and Figures 2A,B). By contrast, the mean mass of the surviving propagules was independently affected by offspring N levels, rather than by the survival status of clonal propagules and their interaction. The mean mass of the surviving propagules was significantly elevated with the increased N levels, but the positive effect of N levels only tended to be improved when the survival rate of clonal propagules became higher (Table 2 and Figure 2C).

Growth performance of clonal offspring

The summed mass of clonal offspring was significantly affected by parental N level, offspring N level, survival status, developmental time of clonal offspring, and their interactions (Table 3). Summed performance of clonal offspring significantly improved with the increased N levels in both parental and offspring environments (Figure 3 and Supplementary Figures 1–4). In particular, the benefits from parental high N levels to the performance of clonal offspring became more profound with increased offspring N levels (PN × ON in Table 3 and Supplementary Figures 1–4). The interaction effect between parental and offspring N levels on the summed performance of clonal offspring gradually strengthened with the extended developmental time of plants (PN × ON × T in Table 3 and Supplementary Figures 1–4). In addition, the interaction effect between parental and offspring N levels on the summed performance of clonal offspring gradually strengthened when the survival rate of clonal propagules was maintained at a

TABLE 3 Effects of parental *N* levels, offspring *N* levels, the survival rate of offspring, and developmental time of offspring on summed and mean performance of clonal offspring produced by one parental plant.

Variables		Summed mass		Mean mass	
	df	*F*	*P*	*F*	*P*
Parental N (PN)	1, 4304	**1605.7**	**<0.001**	**17.1**	**<0.001**
Offspring N (ON)	1, 4304	**2754.3**	**<0.001**	**3481.1**	**<0.001**
Survival rate (SR)	1, 4304	**4800.7**	**<0.001**	<0.1	0.753
Time (T)	1, 4304	**2823.3**	**<0.001**	**3670.9**	**<0.001**
PN × ON	1, 4304	**189.8**	**<0.001**	2.5	0.111
PN × SR	1, 4304	**305.1**	**<0.001**	<0.1	0.831
ON × SR	1, 4304	**555.7**	**<0.001**	<0.1	0.904
PN × T	1, 4304	**118.2**	**<0.001**	**6.3**	**0.012**
ON × T	1, 4304	**645.1**	**<0.001**	**834.9**	**<0.001**
SR × T	1, 4304	**577.1**	**<0.001**	<0.1	0.847
PN × ON × SR	1, 4304	**36.0**	**<0.001**	<0.1	0.933
PN × ON × T	1, 4304	**29.6**	**<0.001**	0.8	0.382
PN × SR × T	1, 4304	**23.3**	**<0.001**	<0.1	0.894
ON × SR × T	1, 4304	**131.6**	**<0.001**	<0.1	0.945
PN × ON × SR × T	1, 4304	**5.8**	**0.016**	<0.1	0.962

Degrees of freedom *(df)*, F- and P-values are given. Significance is highlighted in bold.

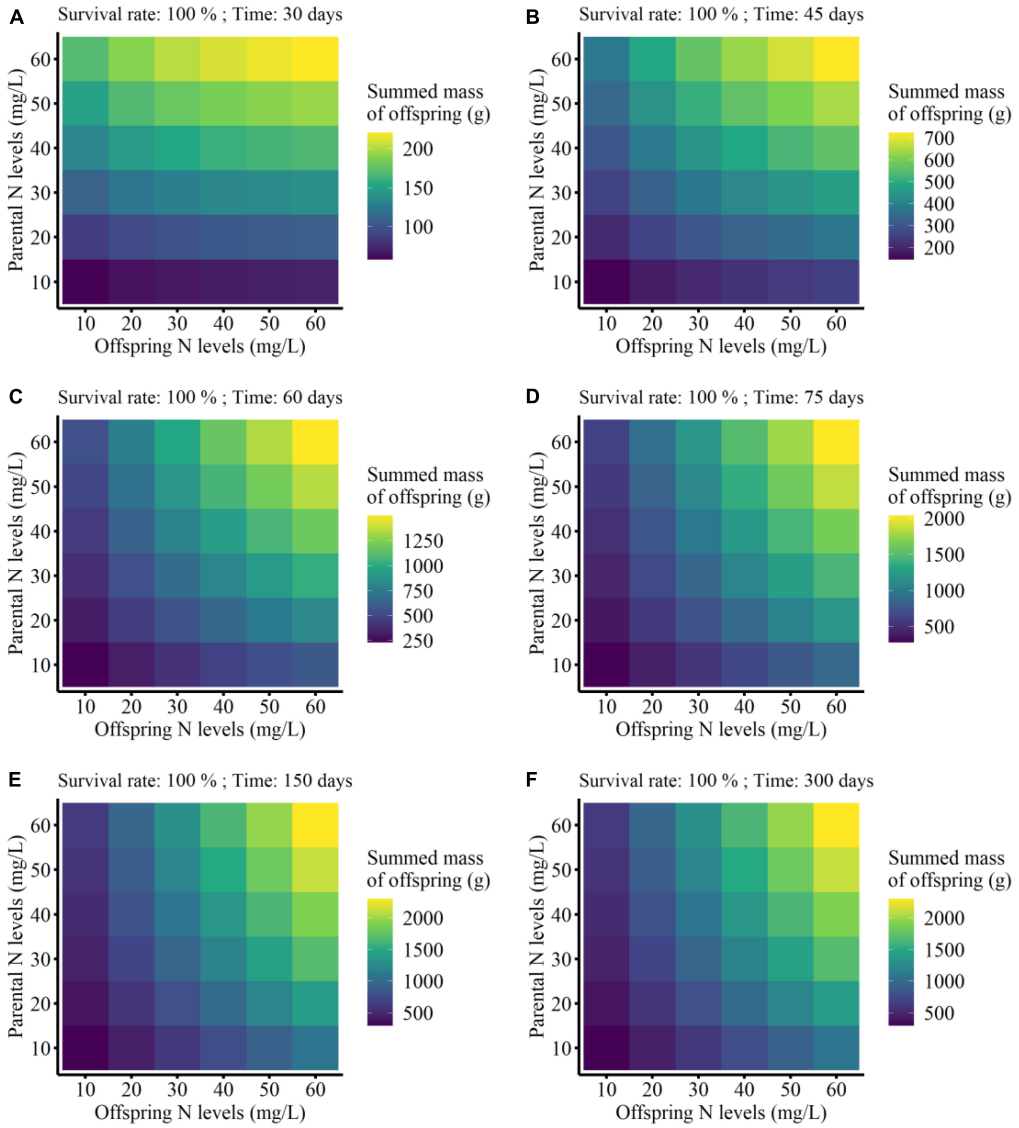

FIGURE 3
Summed finial mass of the offspring grown from the surviving clonal propagules produced by each parental plant at the different developmental time (from 30 to 300 days; **A–F**) in the simulation experiment. The summed performance of clonal offspring with 100% survival rate were shown here.

higher level (PN × ON × SR in Table 3 and Supplementary Figures 1–4).

On the other hand, the mean mass of the surviving clonal offspring was only affected by parental N level, offspring N level, and developmental time of clonal offspring, but not affected by the survival rate of clonal offspring (Table 3). Also, there were no interaction effects between survival rate and the other three factors (Table 3). As predicted, the mean performance of clonal offspring improved in the high N levels in both parental and offspring environments. However, the impact of parental N levels mainly occurred at the early stage of plant growth, and then gradually declined and even vanished at the late stage (PN × T in Table 3, Figure 4, and Supplementary Figures 5–8). On the contrary, the impact of offspring N levels persisted at all the stages of plant growth, and then gradually amplified with the time of plant growth increased (ON × T in Table 3, Figure 4, and Supplementary Figures 5–8).

Discussion

Parental environmental effects are considered a rapid and effective means for clonal offspring to respond to future predictable (stressful and benign) environments that parental plants have often encountered (Latzel and Klimešová, 2010;

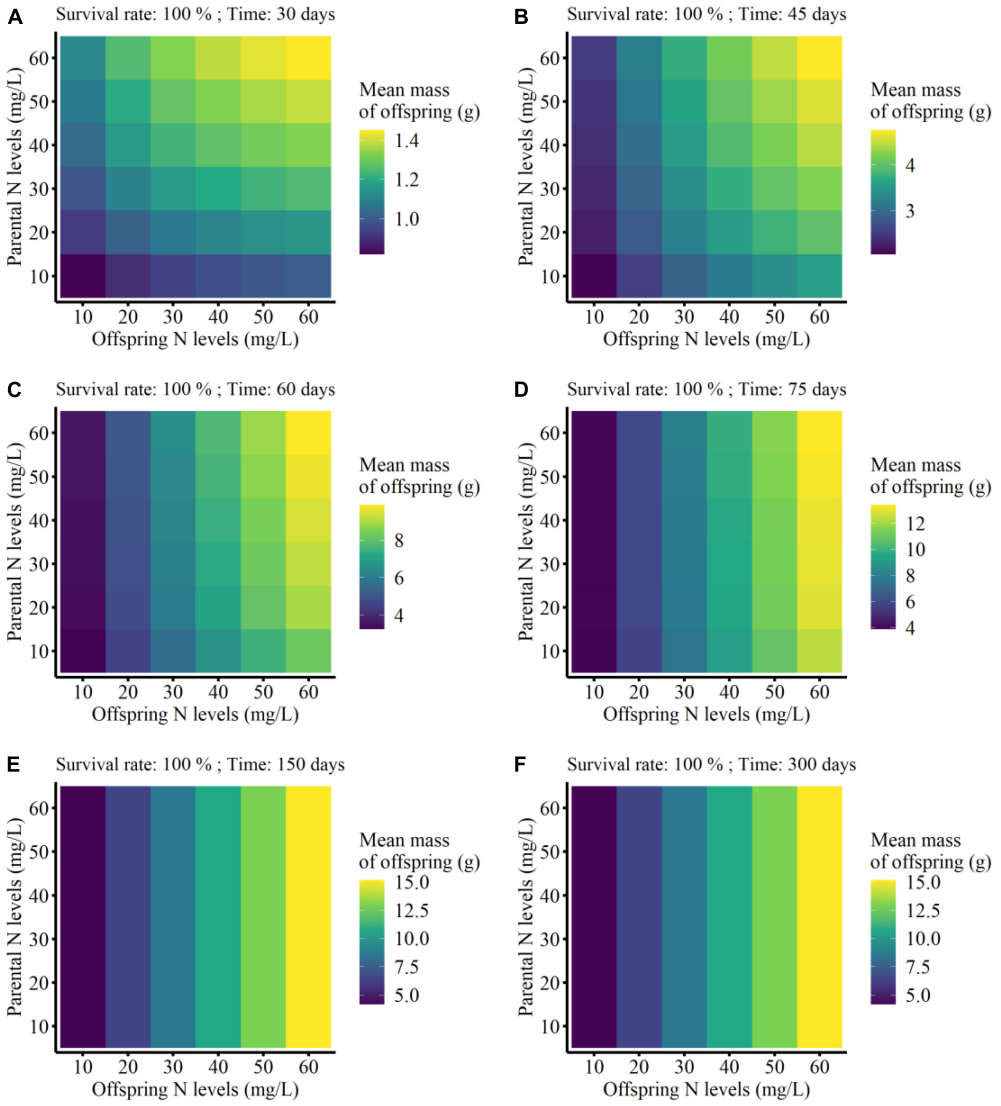

FIGURE 4
Mean finial mass of the offspring grown from the surviving clonal propagules of each parental plant at the different developmental time (from 30 to 300 days; **A–F**) in the simulation experiment. The mean performance of clonal offspring with 100% survival rate were shown here.

Douhovnikoff and Dodd, 2015). In this study, we constructed a two-generation growth model to quantify parental N effects mediated by the provisioning of resources. These results from the non-linear correlations between the total mass of parental plants and the mean mass and/or the number of clonal propagules, clearly indicated that there might be a direct relationship between the performance of parental plants and the initial status of clonal offspring (Dong et al., 2019a; DuBois et al., 2020). We speculated that parental effects mediated by the provisioning of resources might be one term of condition-transfer effects, rather than one of anticipating effects (Bonduriansky and Crean, 2018). Such parental effects allowed parental plants to modify the initial status of clonal offspring in response to the ongoing environments, *via* changing the resource investment to vegetative propagule. Such kind of mechanism appears to be very common in the clonal offspring subject to parental abiotic conditions such as temperature, light and nutrient availability (Dong et al., 2018a, 2019b; DuBois et al., 2020), and also some biotic conditions such as insect herbivory (Dong et al., 2019a).

Our model has further shown that the outcome of parental effects due to N conditions could interact with the offspring environment. In the present simulation, the positive effect of the parental high-N level was gradually amplified with the increased N levels that clonal offspring experienced. It is also possible for these effects to be some kind of "silver-spoon effect" (Grafen, 1988; Uller et al., 2013; Yin et al., 2019). These parental effects have been previously reported in *A. philoxeroides* (Dong

et al., 2018a). The results implied that parental effect could allow clonal offspring to accumulate the size advantage over previous generations in favorable habitats, thereby contributing to the abundance and invasiveness of *A. philoxeroides* in the environments where resource availability is relatively high, e.g., crop fields and irrigation ditches (Pan et al., 2006; Wu et al., 2017).

Besides, the magnitude of parental effects also varied at two study scales (Dong et al., 2018b). The context-dependent parental effect (i.e., the significant interaction effects between parental and offspring N conditions) in *A. philoxeroides* was detected at the offspring-generation scale, but it did not occur in individual performance. These results implied that the context-dependent parental effects might be attributed to some other underlying factors of clonal offspring that was often unrevealed at the individual scale (Dong et al., 2018a). In the simulation experiment, the offspring-size distribution of *A. philoxeroides* followed the rule of the Weibull distribution, in which the shape and scale values of this distribution tended to or strongly correlated with the mean size of clonal propagules. We thus speculated that the parental environments to some degree transformed the size distribution of clonal propagules (e.g., the increased N levels induced clonal offspring to possess a more platykurtic (flat) and symmetrical size distribution with greater mean initial size), so that the majority of clonal offspring within one generation shared the relatively uniform and higher fitness (Dong et al., 2018a). On the one hand, the pattern of skewed offspring-size distribution may strengthen the consequence of parental N effects, and optimize the final performance of the whole offspring-generation especially when the future habitats were further improved (Dong et al., 2018a). On the other hand, the pattern of skewed offspring-size distribution allowed clonal offspring to respond better to temporally or spatially unpredictable environments (Charpentier et al., 2012). In brief, the advantage of skewed offspring-size distribution is not only that more small-sized clonal offspring can grow under favorable conditions, but also that large-sized clonal offspring will keep a strong competitive ability regardless of adverse conditions. In another previous study, the clonal sedge *S. maritimus* also employed the variable-size strategy (i.e., the distribution of tuber size followed the log-normal distribution), to adapt to the temporal variation in water levels that characterized its natural Mediterranean environment (Charpentier et al., 2012). Therefore, the selection for the size distribution of clonal propagules may become the often neglected but key factor underlying the consequence of parental effects on the population growth of clonal plants.

The magnitude of the parental N effect varied at different developmental stages of clonal offspring. In the simulation experiment, the early performance of clonal offspring is more susceptible to the parental N conditions, compared to that to the condition that clonal offspring experienced, e.g., the parental N effects were found to be weakened at the late growth period of plants (Schwaegerle et al., 2000). This may be because that the early development of clonal offspring that lacked the mature root system, strongly relied on the provisioning of resources in plant storage organs of parental plants (Dong et al., 2019a; DuBois et al., 2020). When the absorbing organs of clonal offspring (i.e., new leaves and roots) were produced, the clonal offspring may not continue to depend on storage but could attain sufficient resources through assimilation by newly regenerated tissues, so their late development began to be regulated by the ongoing N condition. Therefore, the developmental constraints of clonal plants may play a key role in consequence of parental effects (Yin et al., 2019).

Furthermore, the magnitude of parental N effect at the individual and whole-generation scales was differently influenced by the survival status of clonal propagules. At the offspring-generation scale, the content-dependent parental effects (i.e., parental effects depended on offspring N conditions) interacted with the survival status of clonal propagules. In detail, when the survival rate of clonal propagules within one offspring generation dramatically decreases, the context-dependent parental effects may be to some extent obscured. By contrast, there was no direct association between offspring survival and parental effects at the individual scale. The results further suggested that the reduction in the survival rate of clonal propagules did not only decrease the number of clonal offspring within one population, but also weakened generation expansion of *A. philoxeroides*, especially in resource-rich habitats.

Conclusion

Our study attempted to quantitatively measure the importance of parental environmental effects in the clonal plants, from the perspectives of individual performance and whole-generation growth. There are several novel findings that have been often neglected in previous studies. First, parental environmental effects could be quantified based on the initial status of clonal offspring (i.e., size of clonal propagules), in the premise that parental effects are mainly regulated by the provisioning of resources, rather than by the genetic and/or epigenetic inheritance. Second, parental environmental effects at the whole-generation scale may be influenced by multiple inherent characteristics of plants (e.g., the survival rate, the number and the size distribution of clonal propagules). Consequently, parental effects differ at the whole-generational level from those at the individual level, as is reflected by the classical paradigm of population ecology. Third, the magnitude of parental effects is to some degree obscured by the developmental constraints of clonal plants. Overall, mathematical models derived from field and/or experimental data may provide

some novel perspectives to assist researchers in understanding parental environmental effects in clonal plants.

Data availability statement

The raw data and main codes required for the analyses are available on GitHub at https://github.com/bichengdong/parental_effect_model.git.

Author contributions

L-HW and B-CD designed the experiment, did the statistical analysis and the model simulation, and wrote the first draft of the manuscript. JS and B-CD performed the experiment. L-HW, B-CD, F-LL, and F-HY contributed substantially to the revisions. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Development Research Center of State Grassland and Forestry Administration of China (2021-D12 and 2021-D6) and the National Natural Science Foundation of China (grant nos. 31500331 and 32071525).

Acknowledgments

We thank Prof. Daniel Taylor for language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.872065/full#supplementary-material

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TYPE Original Research
PUBLISHED 04 January 2023
DOI 10.3389/fpls.2022.994367

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Plant Systematics and Evolution, a section of the journal Frontiers in Plant Science

RECEIVED 14 July 2022
ACCEPTED 03 November 2022
PUBLISHED 04 January 2023

CITATION
Liu Y-Y, Yang Q-F, Li Z, Zhou Z-X, Shi X-P and Wang Y-J (2023) Parallel genetic and phenotypic differentiation of *Erigeron annuus* invasion in China. *Front. Plant Sci.* 13:994367. doi: 10.3389/fpls.2022.994367

Parallel genetic and phenotypic differentiation of *Erigeron annuus* invasion in China

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Introduction: The factors that determine the growth and spread advantages of an alien plant during the invasion process remain open to debate. The genetic diversity and differentiation of an invasive plant population might be closely related to its growth adaptation and spread in the introduced range. However, little is known about whether phenotypic and genetic variation in invasive plant populations covary during the invasion process along invaded geographic distances.

Methods: In a wild experiment, we examined the genetic variation in populations of the aggressively invasive species *Erigeron annuus* at different geographical distances from the first recorded point of introduction (FRPI) in China. We also measured growth traits in the wild and common garden experiments, and the coefficient of variation (CV) of populations in the common garden experiments.

Results and discussion: We found that *E. annuus* populations had better growth performance (i.e., height and biomass) and genetic diversity, and less trait variation, in the long-term introduced region (east) than in the short-term introduced region (west). Furthermore, population growth performance was significantly positively or negatively correlated with genetic diversity or genetic variation. Our results indicate that there was parallel genetic and phenotypic differentiation along the invaded geographic distance in response to adaptation and spread, and populations that entered introduced regions earlier had consistently high genetic diversity and high growth dominance. Growth and reproduction traits can be used as reliable predictors of the adaptation and genetic variation of invasive plants.

KEYWORDS

geographical population, growth performance, coefficient of variation, genetic diversity and variation, dominance ecotype

Introduction

Alien plant invasions are considered to be a major threat to native biodiversity and ecosystems worldwide (Richardson and Pyšek, 2006; Richardson et al., 2007; van Kleunen et al., 2015; Pyšek et al., 2020; Diagne et al., 2021). The factors that determine the growth and spread advantages of an alien plant during the long-term invasion process remain open to debate (Bossdorf et al., 2005; Beerli and Palczewski, 2010; Wang et al., 2017; Xu et al., 2019; Sun et al., 2020). Once alien plant species emerge in a new range, their genetic diversity and differentiation are likely to be closely related to population growth adaptation during the invasion process (Colautti et al., 2010; Qiao et al., 2019; Zhang et al., 2021). Moreover, as predicted by the evolution of the increased competitive ability hypothesis, alien plants often have the potential for evolutionary adaptations to new habitats, which may allow them to spread and invade successfully (Bossdorf et al., 2005; Sax et al., 2007; Prentis et al., 2008; LaForgia et al., 2020; Wang et al., 2022). Plant invaders might often undergo multiple evolutionary adaptations to different environmental stresses and selection pressures in the introduced range (Lee, 2002; Richards et al., 2006; Colautti et al., 2010; Hendry, 2016), as was the case with the aggressive invaders *Mimulus guttatus* (in Europe) and *Tamarix ramosissima* and *Helianthus tuberosus* (North America) (Sexton et al., 2002; Bock et al., 2018; Querns et al., 2022). Evolutionary adaptations promote the spread of invasive plants due to increasing competitiveness (such as accumulating more biomass, growing taller, or producing more flowers and seeds) through genetic variation (Lee, 2002; Lambrinos, 2004; Sax et al., 2007; Prentis et al., 2008). However, few studies have tested the relationship between population-level genetic and phenotypic variation of invasive plants and rapid adaptation in long-term introduction processes.

Regional expansion of invasive plant populations along geographical distance gradients provides opportunities for genetic variance and the potential for evolutionary adaptation of populations to strong selection (Lee, 2002; Xu et al., 2010). The processes leading to population-level genetic variation directly affect survival, reproduction, and other fitness-related phenotypes of invading plants (Garcría-Ramos and Rodríguez, 2002; Ohadi et al., 2016). Once invasive plants spread in large geographic areas, genetic variation (such as allelic diversity) within populations might be reduced and result in high genetic differentiation based on the dominant population effect (Walker et al., 2009). Indeed, the alternative genetic variability of invading populations is commonly believed to be driven by both natural selection and genetic drift, which might modify their tolerance or behavior (Hall and Willis, 2006; Sambatti and Rice, 2006; Stroup, 2015). On the other hand, recent studies have shown that environmental variation along geographic gradients can also lead to the growth adaptation differentiation of invasive plants (Colautti and Barrett 2010, Colautti and Barrett, 2013). Geographic gradients or geographic distance in different regions not only reflected growth and resource competition between alien and native plants but also exhibited the invasion process (i.e., invading periods of earlier invasion or more recent invasion) of alien plants and their phenotypic adaptation and differentiation (Griffith and Watson, 2006; Eckert et al., 2008). Thus, whether an alien plant species can spread and invade on a larger scale is largely dependent on growth advantage and phenotypic adaptation, and finally, through environmental selection and self-evolution, a dominating genotype with low genetic variation is formed (Alpert et al., 2000; Li et al., 2014). Evolutionary adaptation in invasive species arose in response to shifts in environmental conditions and genetic variation. However, the effects of environmental conditions (i.e., different geographic populations) in different regions and genetic variation, and their correlation with phenotypic variation in invasive plant species, have rarely been discussed.

Previous studies showed that the genetic diversity of invasive plants was very different between populations with different geographic distances (Wang et al., 2012; Havrdová et al., 2015; Zhang et al., 2016; Qiao et al., 2019), and might lead to significantly adaptive traits along geographic distances (i.e., as a result of invading time, Griffith and Watson, 2006; Eckert et al., 2008). Invasive plants commonly suffered long-term adaptation and multiple or single evolutions at the point of introduction (FRPI, i.e., the first collected location). Thus, in the earlier invasion ranges, i.e., FRPI, the invader was likely to have high genetic diversity and high growth dominance (i.e., grew taller and produced more biomass) than the later or recent invasion ranges (Sultan et al., 2013; Jeschke and Heger, 2018; Egbon et al., 2020; Querns et al., 2022). Genotypes of invasive plants have dramatic effects on performance during introduction and colonization (Taylor and Keller, 2007; Keller et al., 2012). Therefore, the variation in genetic diversity and differentiation of invasive plants with invasion time or geographic distance can be used to understand the adaptation and invasion mechanism of alien plant species.

Invasive plants respond to evolving adaptations not only through phenotypic changes in parents but also in offspring. The transgenerational effects allow organisms to conserve long-term environmental adaptation between generations and enhance offspring performance (Dong et al., 2019). By removing complex field environmental effects and underlining population evolutionary adaptation in offspring, common garden experiments can characterize the genetic variation in plant phenotypic performance, e.g., plant height (Andalo et al., 2005; Wei et al., 2019; Depardieu et al., 2020). Therefore, a combination of field and common garden experiments can provide novel insights into how plant phenotypes react to genetic influences over time.

Erigeron annuus L. (Asteraceae) is an annual or biennial plant. It is an apomictic plant, producing large numbers of minute seeds that are genetically identical to the mother plant. Thus, it can maintain the dominant performance of the mother

plant for a long time, and its genetic diversity is reduced within populations; however, most populations contain several dominant genotypes, suggesting that sexual reproduction does occur occasionally (Edwards et al., 2006; Trtikova et al., 2011; Ma and Li, 2018). Owing to its strong apomictic reproductive ability and rapid dispersal, it can invade local ecosystems across broad anthropogenic habitats in China, particularly grasslands and farmlands (Wang et al., 2010; Liu et al., 2022). Several studies have investigated the ecological adaptability, interspecific competition, reproductive and biological characteristics, and phenotypic plasticity of *E. annuus*, as well as its genetic diversity and genotypic differentiation (Edwards et al., 2006; Trtikova et al., 2010; Trtikova et al., 2011). However, whether phenotypic and genetic variation in *E. annuus* populations covary in the invasion process in China along the geographic distance of the invasion remains unclear.

In a wild experiment, we examined the genetic variation of *E. annuus* populations at different geographical distances to the first recorded point of introduction (FRPI, i.e., the first collected invasive specimen of *E. annuus*) in China. We also measured the growth traits in the wild and in a common garden experiment, and analyzed the coefficient of variation (CV) of growth traits in nine geographical populations. Furthermore, we examined both the genetic and phenotypic differentiation and their relationships with the distance to the FRPI. We specifically addressed the following questions: (1) Do growth traits and their CV differ among populations at different geographical distances in wild or common garden conditions, and respond to the geographical distances from the FRPI? (2) Do genetic diversity and variation decrease and differentiation increase in populations with distance from the FRPI? (3) Do phenotypic and genetic variation of populations covary in response to adaptation and spread, and can phenotypic traits be used as significant predictors of adaptation and genetic variation in invasive plants?

Materials and methods

Plant species

Individuals of *E. annuus* are invasive and widely distributed in anthropogenically disturbed habitats, including roadsides, grasslands, and farmlands (Wang et al., 2010). The first collected invasive specimen of *E. annuus* in China was from Shanghai in 1886, and the species now covers a wide range, with longitudes ranging from 95°E to 123°E (Wang et al., 2010; Ma and Li, 2018).

In our study, we chose the populations according to geographic distance to the first recorded point of introduction (Shanghai in 1886, i.e., the zero point of geographic distance, FRPI), as we were interested in testing for variations between different geographic distances and invasion times. Therefore, the nine selected geographic populations had different distances (from 30 km to 1425 km) from Shanghai (FRPI), which were located in different cities: Jiaxing (JX, 30 km), Hangzhou (HZ, 133 km) and Wenzhou (WZ, 340 km) in the eastern region, Xianning (XN, 660 km), Wuhan (WH, 685 km), and Xiangyang (XY, 874 km) in the central region, and Chengkou (CK, 1230 km), Nanchuan (NC, 1373 km), and the main districts of Chongqing (CQ, 1425 km) in the western region (Supplementary Table 1; Supplementary Figure 1). In total, we chose three geographic populations (i.e., sites) in each region and selected four populations within each geographic population or site. For each population, we collected plants and seeds from four locations >500 m apart to increase the likelihood of sampling plants from different genotypes. The habitats of the populations were chosen on habitat-similar roadside farmlands. In total, we had 36 populations.

Field survey and seeds collection

From July to August 2012, within each of four selected populations in each geographic population, five typical mother plants (the distance between two sample plants was at least 10 m) were selected, and the heights were measured during the flowering period. Then, we collected two to three fully developed and undamaged leaves from each plant in 50 ml tubes placed on ice for DNA extraction. The sampled leaves were air-dried and used for leaf biomass calculation of each sampled mother plant. Then, whole mother plants were harvested and brought to the laboratory, separated into envelopes, on the basis of whether they were root, stem, leaf, branch, and flower, and dried at 80°C for 72 h in an oven. The total biomass of the plants was the sum of the biomasses of the five parts. To compare the complex genetic difference and maternal effect in offspring between different *E. annuus* populations, the CV in traits was included in the analysis. The CV was calculated using the following formula:

$$CV = \text{the standard deviation} / \text{the mean value for each morphological trait.}$$

We also selected five other typical mother plants and sampled fresh leaves for DNA extraction. Then, we collected seeds from the top inflorescence of the plants at the mature seed stage. Each mature seed sample was taken directly from each mother plant and then brought back to the laboratory for preservation. All seeds were cleaned, air-dried, and stored at 4°C. These seeds were used in the common garden experiment. There were 360 samples in total (ten [i.e., five from mother plants related to growth traits and five from other mother plants related to seed collection and for common garden experiment] × four selected populations × nine geographic populations) for DNA extraction and microsatellite analyses.

Sampling and DNA extraction

For each collected leaf sample, total genomic DNA was extracted from 50-100 mg of leaf material using either the CTAB method (Doyle and Doyle, 1987) or a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The leaves were ground with a glass bead in 2 ml tubes for 3 min at an amplitude of 80 using a vibration mill (Retsch MM 2000). The ground material mixed with 300 µl of CTAB buffer with 5% 2-mercaptoethanol was incubated at 65°C for 30 min, extracted twice with 300 µl chloroform-isoamyl alcohol (24:1), precipitated with 100 µl of isopropanol, and washed with 250 µl of 70% ethanol. Finally, DNA was suspended in 50 µl of double-distilled water. DNA quality was assessed by electrophoresis on a 1% agarose gel. All extracted samples were stored at -20°C.

Microsatellite analysis

Expressed sequence tag-microsatellite sequence (EST-SSR) markers developed for *E. annuus* were screened for transferability and polymorphism in *E. annuus*. Three of these were found to be sufficiently polymorphic (Supplementary Table 2). The polymorphic loci have been cross-amplified in related species (*Dendranthema morifolium* (Asteraceae) and *Lactuca sativa* (Asteraceae)). The markers were able to amplify corresponding DNA in the other two related species. Similar high cross-amplification was also observed in EST-SSR markers developed from *Solidago virgaurea* (Asteraceae), which were successfully transferred to the invasive species *Solidago canadensis* (Asteraceae) and *Solidago hispida* (Asteraceae) (Sakaguchi and Ito, 2014). PCRs were carried out in a total volume of 20 µl, consisting of approximately 50 ng/µl template DNA (4.0 µl), 10 × PCR buffer (2.0 µl), 2 mM dNTP mixture (2.0 µl), 100 pM primer pair (0.2 µl of each), and 2.5 U/µl Blend Taq (0.5 µl) (TOYOBO, Osaka, Japan). The PCR amplification conditions included initial denaturation at 94°C for 5 min; 40 cycles of 95°C for 50 s, 55°C for 30 s, and 72°C for 60 s; and 72°C for 8 min as the last elongation step. Fragment size analysis was conducted using GeneMarker (Softgenetics, State College, PA, USA) and then corrected using the FlexiBin Excel macro (Amos et al., 2010).

EST-SSR data were examined for typographic errors, scoring errors (e.g., allele drop-out, and stuttering), and estimates of null alleles with Micro-Checker (Van Oosterhout et al., 2004). Parameters of diversity were as follows: the proportion of distinguishable genotypes (PD) (Ellstrand and Roose, 1987) was measured as I (*G*/*N*), where G is the number of genets (distinct genotypes) and N is the total number of individuals sampled; for each population, the number of alleles, the percentage of polymorphic loci (*PPL*), *Nei*'s gene diversity (*Nei*), observed (H_o), and expected (H_e) heterozygosities were calculated using GenALEx 6.5.1 (Peakall and Smouse, 2006; Peakall and Smouse, 2012). The inbreeding coefficient (F_{IS}) and allelic richness (R_s) were calculated using FSTAT 2.9.3 (Goudet, 2001). All indices were adjusted for the frequency of null alleles (Van Oosterhout et al., 2004). An analysis of molecular variance (AMOVA) was performed, and the genetic differentiation index (F_{ST}) was determined using Arlequin version 3.0, with significance tests based on 1,000 permutations (AMOVA, Excoffier et al., 2007).

Common garden experiment

In the common garden experiment, we used the collected seeds of mother plants from the same populations in the wild to test the real growth and phenotypic differentiation among geographic populations and the response to genetic differentiation. For each of the nine geographic populations, we used the seeds from five collected mother plants within each of four populations. The experiment was carried out in a greenhouse at Huazhong Agricultural University, Wuhan, China (114°21' E, 30°27' N). Hubei has a subtropical monsoon climate, the average annual temperature is 16.4°C, and the average annual precipitation is approximately 1269 mm. From 17 December 2012 to 13 January 2013, we sowed the collected seeds in plastic trays (19.5 cm × 14.6 cm × 6.5 cm) filled with peat moss as substrate (Pindstrup Plus, Pindstrup Mossebrug A/S, Denmark). Because the time required for germination and the germination rate varied between the different populations, we sowed them on different dates to ensure that there were enough seedlings and that all the seedlings were in similar developmental stages at the start of the experiment. We placed all the trays with seeds in a greenhouse under natural light conditions, with a temperature between 20 and 26 °C.

On 25 February 2013, we transplanted similar-sized seedlings of each population into 5 L square plastic pots (24 cm × 24 cm × 15 cm) filled with field soil. The field soil included a total N of 0.78 ± 0.03 g kg^{-1}, a total P of 0.58± 0.04 g kg^{-1}, and a total K of 24.27 ± 1.15 g kg^{-1} (mean ± SE, n = 8). We transplanted one seedling of *E. annuus* in the center of each pot. After transplanting, we randomly assigned all pots in the greenhouse under natural light conditions, with a temperature between 20 and 28°C. We rerandomized the positions of all the pots every 4 weeks. There was a total of 180 pots (9 geographic populations × 4 populations × 5 mother plants = 180 pots).

On 12 June 2014 (i.e., fifteen weeks after transplanting, during the flowering period), we first calculated the plant height and then harvested the aboveground and underground biomass of the plants in all pots. Then, they were brought to the laboratory, separated into envelopes according to root (underground), stem, leaf, branch, and flower tissue, and dried at 80°C for 72 h in an oven. The total biomass of plants is the sum of aboveground biomass and underground biomass. The CV for each phenotypic trait was calculated.

Data analysis

All statistical analyses were performed using R 4.0.3 (R Core Team, 2020). As geographic distances ranged from 30 km to 1425 km for the populations, the regions were defined as east (0-500 km), center (500-1000 km), or west (1000-1500 km). We then analyzed the effects of geographic region on growth traits (i.e., height, total biomass, flower biomass, leaf biomass, stem biomass, branch biomass, and root biomass) and their CV of *E. annuus* populations using a linear mixed-effects model with the 'lme' function in the 'nlme' package (Pinheiro et al., 2015). In these models, Region (east vs. center vs. west) was included as a fixed term. To account for the nonindependence of geographic populations (i.e., site) in a region, and populations within a geographic population, geographic population and population (nested within geographic populations) were included as random terms (Wang et al., 2019). We also included random structure to allow for variance between regions using the 'varIdent' function in the R package 'nlm' (Pinheiro et al., 2020). To meet the assumptions of normality of variance, plant height was sqrt-root transformed, flower biomass and total biomass were natural-log transformed, and the CV of growth was logit transformed (Table 1; Supplementary Tables 3, 4). In the linear mixed-effect models described above, we assessed the significance of fixed-effect-independent variables using likelihood-ratio tests (Zuur et al., 2009). A multiple post-hoc Tukey's HSD test was used to compare the means for the phenotypic traits and their CV of *E. annuus* geographic populations.

Linear regression models were used to analyze the relationship between the growth of geographic populations in the wild experiment or in the common garden experiment and the genetic diversity or variation of geographic populations in the wild experiment, and geographic distance to the FRPI. We also analyzed the correlation between key traits (height, total biomass, and flower biomass) of geographic populations in the wild and their genetic diversity. We used ANCOVA to compare the difference between slopes of the linear regression between the growth of the wild experiment or of the common garden experiment and geographic distance to the FRPI.

Results

Growth and phenotype of *E. annuus* populations in wild and common garden experiments with geographic distance

The plant height and biomass of the *E. annuus* populations in both the wild and common garden experiments exhibited great differences between the three regions in China (Table 2). Specifically, height, total biomass, and flower biomass in the eastern region (i.e., short geographic distance to the FRPI and long-term introduced) were significantly greater than in the center and western (i.e., long geographic distance to FRPI, and short-term introduced) regions (Figure 1). The plant phenotype also exhibited a clump ecotype in the eastern region and a scatter ecotype in the central and western regions in both the wild and common garden experiments. Height and total biomass among geographic populations in the eastern region also varied greatly, with the greatest values in the Hangzhou (HZ) population and the lowest values in the Wenzhou (WZ) population (Figure 1). Leaf biomass, stem biomass, branch biomass, and root biomass showed similar patterns to plant height and total biomass (Supplementary Table 3; Supplementary Figure 2). Furthermore, the height, total biomass, and flower biomass of *E. annuus* populations in both the wild and common garden experiments decreased with geographic distance to the FRPI (Supplementary Figure 4), indicating the trait advantage of long-term introduced populations to invasion success. However, their negative correlations were steeper under

TABLE 1 Statistical comparisons of genetic diversity indices for E. annuus in wild population. Distance, distance to the first recorded point of introduction; N, number of samples; Nei, Nei' gene diversity; PPL, the percentage of polymorphic loci; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient; R_s, allelic richness and F_{ST}, genetic differentiation index.

Region	Population	Distance (km)	N	*Nei*	*PPL* (%)	H_o	H_e	F_{IS}	R_s	F_{ST}
East	JX	30	40	0.293	59.61	0.638	0.702	0.084	3.926	0.042
East	HZ	133	40	0.391	64.76	0.677	0.754	0.095	4.012	0.029
East	WZ	340	40	0.339	59.27	0.605	0.676	0.081	3.869	0.053
Center	XN	660	40	0.112	24.02	0.457	0.466	0.059	2.521	0.148
Center	WH	685	40	0.14	35.36	0.538	0.561	0.062	2.783	0.139
Center	XY	874	40	0.111	24.1	0.494	0.513	0.065	2.616	0.167
West	CK	1230	40	0.069	10.94	0.398	0.439	0.141	3.328	0.204
West	NC	1373	40	0.067	6.11	0.213	0.267	0.201	2.349	0.264
West	CQ	1425	40	0.068	8.14	0.312	0.368	0.117	2.483	0.235

Indices were calculated from allele frequencies of three microsatellite markers and adjusted for frequency of null alleles.
JX, Jiaxing; HZ, Hangzhou; WZ, Wenzhou; XN, Xianning; WH, Wuhan; XY, Xiangyang; CK, Chengkou; NC, Nanchuan; CQ, Chongqing.

wild conditions than under common garden conditions (slopes of the linear regression between the two experiments were x and y: height, ANCOVA, $F = 2.474$, $P = 0.013$; total biomass, ANCOVA, $F = 4.731$, $P < 0.001$; flower biomass, ANCOVA, $F = 5.337$, $P < 0.001$).

Coefficient of variation of growth of *E. annuus* populations in the common garden experiment

In the common garden experiment, the CVs of height and total biomass were lowest in the eastern region, showing great stability in growth traits in the long-term introduced populations (Figure 2). Moreover, the CV of flower biomass was not different between the three different regions in the common garden experiment, indicating a low variation range of reproductive traits between the different populations.

Genetic diversity or variation in wild *E. annuus* populations with geographic distance

Within regions, the percentage of polymorphic loci (*PPL*), Nei's gene diversity (*Nei*), the proportion of distinguishable genotypes (*G/N*), observed heterozygosity (H_o), expected heterozygosity (H_e), and allelic richness (R_s) were higher in the eastern region and lowest in the western region (especially in Hangzhou [HZ] and Nanchuan (NC)) (Table 1; Supplementary Table 5). The H_o, H_e, and R_s of *E. annuus* wild populations were significantly negatively related to geographic distance to the FRPI (Figure 3A; Supplementary Figures 5A-C). These results indicate that genetic diversity decreased from long-term to short-term introduced wild populations. The genetic differentiation index (F_{ST}) and inbreeding coefficient (F_{IS}) were significantly positively related to geographic distance to the FRPI (Table 1; Figure 3B; Supplementary Figure 5D). AMOVA showed a definite geographic trend of genetic differentiation in *E. annuus* populations in China, with 23% variation between regions, 38% between populations, and 39% within populations (Table 3; Figure 3).

Relationships between the growth and genetic variation of *E. annuus* populations

The height, total biomass, and flower biomass of *E. annuus* populations were significantly positively correlated with H_o (Figures 4A–C), and significantly negatively correlated with F_{ST} (Figures 4D–F). Furthermore, greater height and biomass were associated with higher H_o or lower F_{ST} in the eastern populations than in the central and western populations.

TABLE 2 Results of linear mixed-effects models testing the effects of region (East vs. Center vs. West) on height, total biomass, flower biomass in wild or common garden experiments in China.

Wild experiment		**Height (square root transformed)**		**Total biomass (square root transformed)**		**Flower biomass (square root transformed)**	
Fixed effects	df	X^2	p	X^2	p	X^2	p
Region	2	**18.82**	**<0.001**	**20.72**	**<0.001**	**22.49**	**<0.001**
Random effects		SD		SD		SD	
Geographic population		0.55		0.79		0.27	
population		0.73		0.76		0.34	
Residual		0.72		0.66		0.43	
		R^2m	R^2c	R^2m	R^2c	R^2m	R^2c
R^2 of the model		0.63	0.86	0.73	0.89	0.69	0.85
Common garden experiment		**Height (square root transformed)**		**Total biomass (log root transformed)**		**Flower biomass (log root transformed)**	
Fixed effects	df	X^2	p	X^2	p	X^2	p
Region	2	**19.45**	**<0.001**	**22.65**	**<0.001**	**19.01**	**<0.001**
Random effects		SD		SD		SD	
Geographic population		0.48		0.2		0.24	
*p*opulation		0.38		0.29		0.31	
Residual		0.53		0.2		0.21	
		R^2m	R^2c	R^2m	R^2c	R^2m	R^2c
R^2 of the model		0.69	0.87	0.75	0.94	0.67	0.92

R^2m: Marginal R^2; R^2c: Conditional R^2; Significant effects ($p < 0.05$) are in bold.

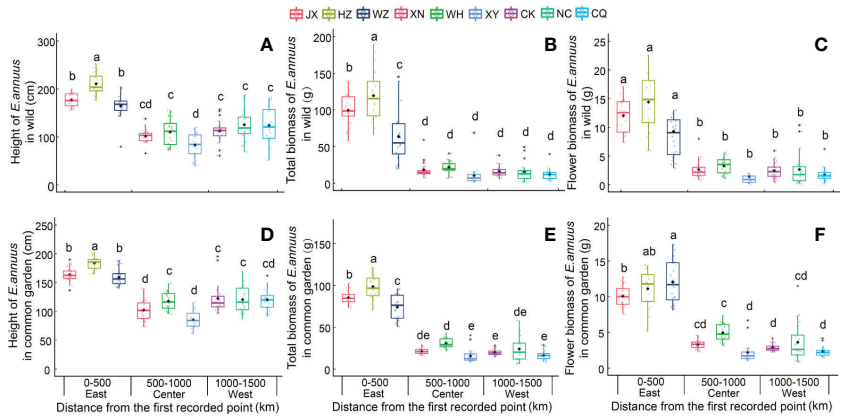

FIGURE 1
Height **(A, D)**, total biomass **(B, E)** and flower biomass **(C, F)** of E. annuus geographic populations in different regions in wild and common garden experiments. Mean ± SE were given. Different lowercase letters mean statistically significant differences (P < 0.05) JX, Jiaxing; HZ, Hangzhou; WZ, Wenzhou; XN, Xianning; WH, Wuhan, XY, Xiangyang; CK, Chengkou; NC, Nanchuan; CQ, Chongqing.

Discussion

We found that *E. annuus* populations had better growth performance (i.e., height and biomass) and genetic diversity, and less variation of traits and genetic differentiation in the long-term introduced region (i.e., east) than in the short-term introduced (west) region in China. Furthermore, within populations, genetic diversity showed a similar pattern to growth performance, which indicated significantly positive or negative correlations between growth performance and genetic diversity or genetic differentiation, especially for common garden experiments. These results indicate that growth traits and genetic variation of populations covary in response to regional adaptation and spread, and populations that entered earlier have parallel high genetic diversity and high growth dominance in introduced environments.

Growth and phenotypic differentiation in *E. annuus* populations

Our results showed that in *E. annuus* populations within wild and common garden conditions, growth traits were significantly more varied, whereas their CVs were significantly lower in the eastern region than in the central and western regions, indicating a decrease in growth and an increase in phenotypic differentiation with invasion time. The *E. annuus* populations had a better growth advantage in the long-term introduced region. This is consistent with previous findings that populations of some invasive plant species in the earlier introduced ranges grew taller and produced larger biomass than those in the later ranges (Sultan et al., 2013; Jeschke and Heger, 2018; Egbon et al., 2020; Querns et al., 2022). Although previous report demonstrated that plant growth and functional

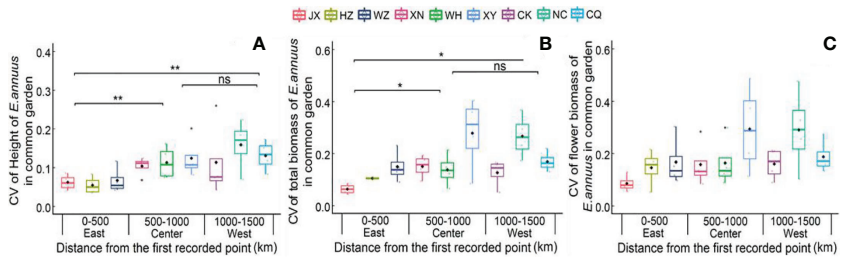

FIGURE 2
Coefficient of variation (CV) of height **(A)**, total biomass **(B)** and flower biomass **(C)** of E. annuus geographic populations of common garden experiments in different regions. Mean ± SE were given. The significance levels are: **p < 0.01, *p < 0.05, and ns, p > 0.05. JX, Jiaxing; HZ, Hangzhou; WZ, Wenzhou; N, Xianning; WH, Wuhan; XY, Xiangyang; CK, Chengkou; NC, Nanchuan; CQ, Chongqing.

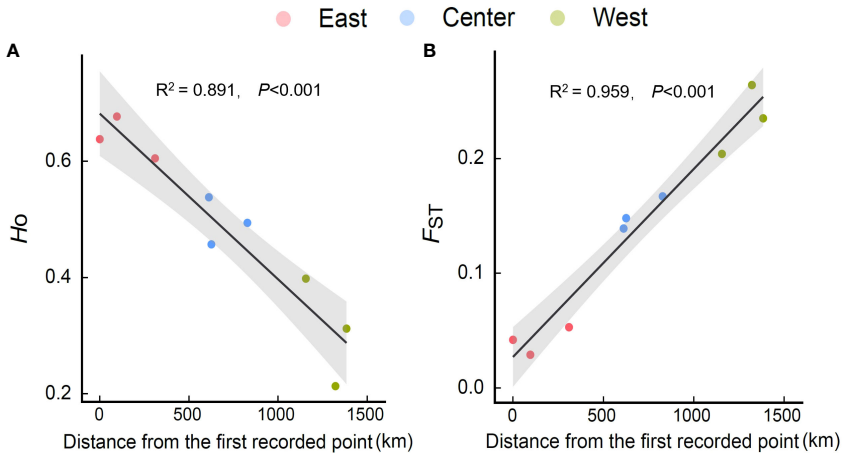

FIGURE 3
Relationships of H_o [**(A)** observed heterozygosity] and F_{ST} [**(B)** fixation index] of *E. annuus* geographic populations with the distance to the first recorded point of introduction (FRPI). Similar symbols are different sites within the same region.

traits are affected by soil nutrients and spatial gradients in the field (Wang et al., 2017), our results from common garden experiments showed convincing findings similar to those of wild experiments, indicating that growth and phenotypic differentiation are probably related to the genetic variation of *E. annuus*. A potential mechanism is likely to be that invasive plants have greater competitive traits under new abiotic and biotic environments after being introduced into a new area (Sultan, 1995; Prentis et al., 2008; Sultan et al., 2013; Jeschke and Heger, 2018). We also found that *E. annuus* exhibited both clump and scatter ecotypes (but clump dominated) in the eastern region, and only the scatter ecotype in the western region, in both the wild and common garden experiments. The clump ecotype had more branches and total biomass, which may be an adaptive advantage to interferences in farmland habitats (i.e., herbicide, herbivory, and trampling) through risk sharing between multiple branches (our experimental observation). This is supported by experimental evidence showing that dominant ecotypes of *Imperata cylindrica* can be maintained in the early stages of invasion and can invade a wider area with more dominant traits (common ecotype [C-type] and early flowering ecotype [E-type] are found scattered in the Japanese Islands) (Matumura and Yukimura, 1980; Maeda et al., 2009). Similarly, dominant ecotypes (hexaploid and octoploid) of *Fallopia sachalinensis* prevail across the invaded area but not in the native range (Mandák et al., 2003). In our study, the high consistency of growth and phenotype of *E. annuus* populations in long-term (different geographical distance gradients) introduced regions in wild and common garden conditions indicated largely genetic effects. This result is in line with the variable performance of many invasive plants (*Mimulus guttatus*, *Polygonum cespitosum*, *Triadica sebifera*, and *Catorhintha schaffneri*) in the regions they were introduced, indicating that adaptive evolution of invasive plants occurs in the new region (Kawecki and Ebert, 2004; Huang et al., 2013; Sultan et al., 2013; Huang et al., 2015; Egbon et al., 2020; Querns et al., 2022).

Genetic diversity and genetic differentiation in *E. annuus* populations

We found that the genetic diversity of *E. annuus* significantly decreased with geographic distance, with the same pattern as growth, i.e., highest in the long-term introduced region and lowest in the short-term introduction region. The rapid genetic decline of *E. annuus* with geographic distance to the FRPI is consistent with previous studies showing that alien species may decrease their genetic diversity during the invasion process and maintain population genetics depending on changes in selection,

TABLE 3 Analysis of molecular variance (AMOVA) results of *E. annuus* populations based on microsatellite markers.

Source of variation	DF	Sum of squares	Variance component	Percentage of variation (%)	*P* value
Among regions	2	347.12	166.28	23	**<0.01**
Among populations	33	1987.45	67.59	38	**<0.01**
Within populations	324	1083.22	4.87	39	**<0.01**

Significant effects ($p < 0.05$) are in bold.

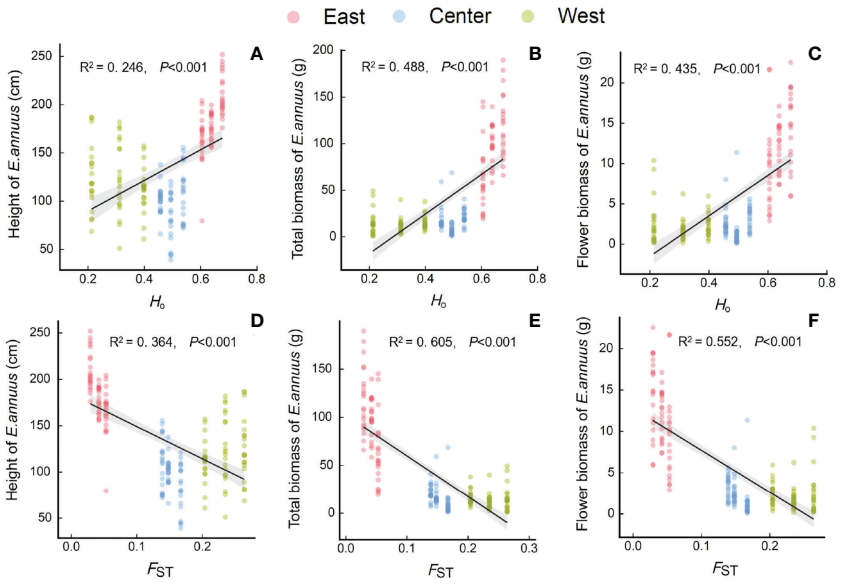

FIGURE 4
Relationships between genetic diversity and differentiation [**(A-C)** H_o, observed heterozygosity; **(D-F)**, F_{ST}, genetic differentiation index] with height **(A, D)**, total biomass **(B, E)** and flower biomass **(C, F)** of *E. annuus* geographic populations in different regions in China. Similar symbols are different sites within the same region.

genetic drift, and gene flow (Broennimann et al., 2012; Dlugosch et al., 2015). Ohadi et al. (2016) found high rates of gene variation among *Cakile maritima* populations occupying long-term invaded regions. Moreover, genetic diversity was significantly positively correlated with height and biomass in *E. annuus* with geographic distance, suggesting that long-term introduced populations had higher genetic diversity and variation, as well as parallelly higher growth adaptability and higher vitality than short-term introduced populations. Previous studies have shown that genetic diversity and variation in *E. annuus* may reflect a variety of genotype frequencies and apomictic reproduction in long-distance dispersal (Ellstrand and Roose, 1987; Edwards et al., 2006; Trtikova et al., 2011). Apomixis is typically a reproductive advantage for *E. annuus*, allowing better preservation of the dominant traits (i.e., height and biomass) of the mother plant and more successful spread, but reducing genetic diversity. Thus, *E. annuus* can maintain the dominant performance of the mother plant in the offspring. Therefore, eastern geographic populations had more dominant genotypes, i.e., clump ecotype, which have been well preserved for a long time (Trtikova et al., 2011; Sultan et al., 2013; Querns et al., 2022). However, there were fewer dominant genotypes in the western region due to increasing gene diversity. Furthermore, the CVs of height, total biomass, and flower biomass in wild geographical populations were also higher in the western region, suggesting that relatively short-term introduced populations had greater variation and instability of growth performance, and were more influenced by regional environments. In our common garden experiment, the CV of growth traits was generally consistent with the wild experiments influenced by environmental conditions. From our common garden experiment, the transgeneration presented long-term environmental adaptation and characterized the variation of plant traits with genetic diversity and variation (Depardieu et al., 2020). The parallel high variability in growth traits and high genetic differentiation among western geographical populations is likely to depend on the limitation of seed dispersal and adaptive time. In contrast, eastern geographical populations had high levels of genotypes based on high gene diversity in long-term evolution. These genotypes also exhibited similar growth dominance, consistent with the lowest growth variation and high stability. Therefore, the consistency of high genetic diversity and high growth dominance in *E. annuus* populations have allowed them to easily invade the eastern range.

Coefficient of variation in common garden conditions

Although we found a greater decrease in growth with geographic distance in wild conditions than in common garden conditions, there was no significant environmental effect on population growth, suggesting that the different growth performances of *E. annuus* populations with geographical distance were attributed to genetic variation.

Similarly, previous studies showed that variation in the growth fitness of *E. annuus* did not depend on the patterns of environmental variation (Stratton, 1992; Stratton, 1994). However, we observed that the CV of flower biomass between different regions was not different under common garden conditions but under wild conditions. This may have resulted from environmental changes (i.e., availability resources and stress) rather than genetic variation, which is in line with the finding that reproductive (i.e., flower biomass) fitness in the invasive plant *Polygonum cespitosum* altered in its introduced range (Alpert and Simms, 2002; Richardson and Pyšek, 2006; Sultan et al., 2013; Sultan and Matesanz, 2015). Additionally, the success of invasive grass *Pennisetum setaceum* populations in their invasive ranges resulted in high environmental adaptation to reproduction (Williams et al., 1995; Poulin et al., 2005; Poulin et al., 2007). On the other hand, the different growth and reproduction patterns of *E. annuus* in long- or short-term introduced regions might result from naturally selected dominant genotypes and genetic variation. *E. annuus* is an apomictic species in which nondominant genotypes are eliminated in the long-distance spread process (Noyes, 2000; Trtikova et al., 2010; Trtikova et al., 2011). Moreover, the reproductive stage of *E. annuus* might provide more high-quality seed production and the generation of new genotypes under different environmental conditions (Baker, 1965; Noyes, 2000; Trtikova et al., 2011).

Our experiment did not test the distinguishing genotypes among populations, and it would be difficult to translate the results to evaluate genotype differences with geographic distance. Additionally, the correlations between growth traits and genetic variation at the population level might not demonstrate evolutionary adaptation for *E. annuus*. However, the integration of wild and common garden experiments can largely reveal genotypic differences between different regions in China.

Conclusions

Our results indicate that the growth traits and genetic variation of *E. annuus* populations covary in response to adaptation and spread, and populations that entered introduced regions earlier have consistently high genetic diversity and high growth dominance. However, the short-term nature of our experiments with the population of *E. annuus* meant that our investigation was somewhat limited. Therefore, longer-term field research is needed to test whether different performances among populations occur at different geographical distances. Future studies should also test the genetic structure and invasion history at broader geographic distances in China or worldwide. However, we conclude that parallel genetic and phenotypic variation with invaded geographical distance, growth, and reproductive traits can be used as important predictors of the adaptation and genetic variation of invasive plants.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

Y-JW set up the experiment. Y-JW, X-PS and ZL conducted field sampling. X-PS and Y-YL conducted molecular analysis. Q-FY and Y-YL carry out the statistical analysis. Y-YL. wrote the first draft of the manuscript. Y-JW, Z-XZ, X-PS and Y-YL contributed substantially to the revisions. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the National Natural Science Foundation of China (32171510, 31770449, 32071527), by Fundamental Research Funds for the Central Universities (2662020YLPY016).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.994367/full#supplementary-material

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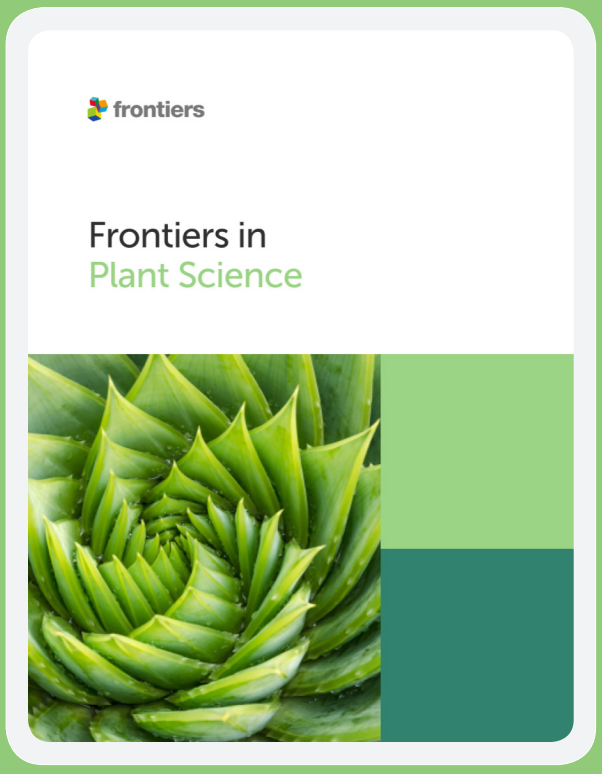

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