plant biology

Plant Biology ISSN 1435-8603

RESEARCH ARTICLE

A dynamic epigenetic perspective on above- and belowground phenotypic responses to drought: Insights from global DNA methylation in *Erodium cicutarium*

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Keywords

5-azacytidine; development; DNA methylation; Geraniaceae; Mediterranean; plant stress adaptation; root biomass; water stress.

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Editor

M. Bitterlich

Received: 6 March 2025;
Accepted: 4 August 2025

doi:10.1111/plb.70110

ABSTRACT

- There is mounting evidence that plant responses to environmental stress are mediated by epigenetic factors, including DNA methylation. Understanding relationships between DNA methylation, plant development and individual fitness in contrasting environments is key to uncover potential impacts of epigenetic regulation on plant adaptation. Here, we used an experimental approach combining controlled alteration of epigenetic features with exposure to stress.
- Two provenances of *Erodium cicutarium* were exposed to a demethylating agent (5-azacytidine) and recurrent drought, and effects on above- and belowground phenotypic traits related to early development, phenology and fitness assessed.
- Application of 5-azacytidine significantly reduced DNA methylation in leaf and root tissues. This slowed plant development, delayed flowering, and reduced the number of inflorescences produced, independent of water regime. Recurrent drought reduced final above- and belowground biomass and inflorescence production, regardless of the 5-azacytidine exposure. Increased fruit and seed-set were the only adaptations to drought in *E. cicutarium*, together with an increased number of flowers per inflorescence in water-stressed plants previously treated with 5-azacytidine.
- Epigenetic effects can desynchronize plant growth, flowering and senescence in both favourable and adverse environments. Future studies should focus on understanding intraspecific variation in ability to change the plant methylome in response to stress, and transgenerational transmission of such responses.

INTRODUCTION

Understanding how plant phenotype and fitness change under stressful conditions is fundamental in evolutionary ecology, with potential applications in agronomy and biodiversity conservation. Elements considered to explain phenotypic variations include genotype (i.e. clones, siblings, varieties or populations), environment (i.e. level of stress), and genotype-by-environment interaction, which evaluates the response of a genotype to changing environments (Bradshaw 1965; Schlichting 1986). More recently, epigenetic factors, such as DNA methylation, histone modifications and small RNAs, have been shown to participate in plant phenotypic variation and response to stress (e.g. Gutzat & Mittelsten Scheid 2012; Gallusci *et al.* 2023; Masuelli *et al.* 2025), which are related to phenotype plasticity, i.e. plasticity of a genotype in different environments (e.g. Herman *et al.* 2014; Kooke *et al.* 2015; Schneider 2022). Epigenetic variants (unlike conventional genetic mutations) are reversible, more frequent, and can be induced by environmental factors (Lloyd & Lister 2022; Gallusci *et al.* 2023). Hence, epigenetics might explain the rapid rise in phenotypic plasticity in changing environments (Jablonka 2013; Zhang *et al.* 2013; Herman *et al.* 2014; Kooke *et al.* 2015).

Drought is a major environmental stress that limits plant growth and productivity. Plants have evolved distinct strategies to reduce deleterious effects according to the extent of water deficit and life history (Chaves *et al.* 2002; Kooyers 2015; Marin de la Rosa *et al.* 2019). The genetic basis for phenotypic responses to drought, e.g. changes in root growth and water use efficiency, together with genetic variation in drought resistance into natural populations have been documented (reviewed in Kooyers 2015). Furthermore, epigenetic changes after drought stress are frequently observed in experiments with crop and model species (e.g. Bossdorf *et al.* 2010; Neves *et al.* 2017; Zheng *et al.* 2017), although consequences for individual fitness are less frequently reported (but see Zhang *et al.* 2013; Van Dooren *et al.* 2020). Modification of DNA cytosine methylation can be used to evaluate the impact of epigenetic mechanisms on phenotypic traits, development and individual fitness in contrasting environments (e.g. Herman & Sultan 2016; Rendina González *et al.* 2016; Münzbergová *et al.* 2018). Inhibitors of DNA methyltransferases, such as 5-azacytidine, 5-aza-2′-deoxycytidine or zebularine, modify DNA cytosine methylation in plants, which can increase natural epigenetic variation while controlling for genetic relatedness (Dvořák Tomaštíková & Pecinka 2024, and references therein).

Spatially heterogeneous and unpredictable environments favour phenotypic plasticity and epigenetic responses to stress, while the direction and strength of selection can vary in space and/or time (Bradshaw 1965; Hendry 2016). Mediterranean mountains are a good example of a heterogeneous and

unpredictable environment, with variations in seasonal and interannual rainfall associated to topography (see e.g., Cowling *et al.* 2005; Cook *et al.* 2016). Annual plants frequently adopt an escape-avoidance strategy (i.e., early flowering to complete the lifecycle before drought becomes severe) and/or changes in root growth and water use efficiency to reduce fitness costs (Chaves *et al.* 2002; Franks 2011; Kooyers 2015). We investigated the potential role of DNA methylation on phenotypic responses to recurrent drought in *Erodium cicutarium* (Geraniaceae), a native annual in the Mediterranean Basin that flowers in spring when rainfall is unpredictable (Cowling *et al.* 2005), currently naturalized worldwide and invasive in several regions (see e.g., Blackshaw & Harker 1998; Francis *et al.* 2012; Kimball *et al.* 2014).

Using a 2 × 2 factorial design, in the first 48 h of seed imbibition, half of the seeds were exposed to a low concentration of 5-azacytidine to alter DNA cytosine methylation. Sibling seedlings were subsequently grown under two water regimes. A recurrent drought with recovery periods was applied because it elicits distinct and stronger epigenomic signals than single extreme events and is expected to promote stress memory and tolerance to subsequent stress (Walter *et al.* 2011; Fleta-Soriano & Munne-Bosch 2016). We first checked whether the demethylation treatment effectively reduced cytosine methylation at the seedling stage without leading to anomalous growth (Alonso *et al.* 2017), and that recurrent drought modified global DNA methylation in leaf and root tissues at the adult stage. Although roots are essential to overcome drought (Kooyers 2015), changes in root DNA methylation have received less attention (see e.g., Chwialkowska *et al.* 2016; Abid *et al.* 2017) and, to the best of our knowledge, never studied in adult plants experiencing recurrent stress.

Further, we explored the consequences of seed demethylation and recurrent drought on plant development (first leaf length, time to first flower, changes in leaf and inflorescence number) and final (static) phenotype (reproductive output, above- and belowground biomass). We predicted that: (1) reduced DNA methylation at germination will impact plant development, phenology and fitness; (2) recurrent drought will change DNA methylation, development, phenology and/or fitness; (3) the magnitude of response will differ above- and belowground; and (4) if DNA methylation is involved in drought-induced plasticity, demethylation will impair phenotype responses to drought.

MATERIAL AND METHODS

Study species and plant material

Erodium cicutarium (L.) L'Hér. (Geraniaceae) is an annual native to Mediterranean Europe, North Africa and western Asia, common in temperate areas with hot summers in both hemispheres (Fiz-Palacios *et al.* 2010). Rapid completion of the lifecycle allows fruiting before seasonal summer droughts, and autonomous (i.e., vectorless) self-pollination has contributed to naturalization in diverse habitats, where it can be invasive (Blackshaw & Harker 1998; Francis *et al.* 2012; Kimball *et al.* 2014).

Mature fruits from adult plants of two *E. cicutarium* populations in the Cazorla mountains (Jaén province, SE Spain), Puerto del Tejo (PT, 1590 m a.s.l.) and Nava de las Correhuelas (CH, 1625 m a.s.l.) were collected in spring 2015 and stored in darkness at room temperature. In autumn, seeds were removed from fruits, scarified, germinated on a universal substrate (COMPO SANA®) mixed 3:1 with perlite ("substrate" hereafter), and emerged seedlings subsequently transferred to 1 L pots with the same substrate (16 h light; 25–20°C). Plants from the two populations were grouped in trays and periodically rotated within the greenhouse. After establishment, half of the trays were watered twice per week, and the other half were watered once every 10–11 days until the end of reproduction (ca. 6 months). Autonomously self-pollinated fruits were collected in paper bags and stored at room temperature.

Experimental conditions and trait data collection

A detailed description of experimental conditions and measured traits is provided in Supplementary Material. We analysed effects of two factors, seed demethylation and recurrent drought, on plant traits (see below) and global DNA cytosine methylation in the genomes of leaf and root tissues following a 2 × 2 factorial design.

Plants were the second generation of greenhouse-grown individuals; thus, the maternal environment was also considered. In October 2016, offspring of six F1 mothers from each of the two water regimes and populations were selected (N = 24 families: 2 populations × 2 water maternal regimes × 6 F1 mothers), with each F1 mother having a sib in the other water regime (i.e., the same six plants per population provided seeds for all treatment levels). Seeds were scarified with sandpaper and, for each maternal line (line, hereafter), a demethylation treatment was applied to half of the seeds before planting. Seeds were submerged in either Control (water with DMSO 97:3, v:v) or a 0.5 mM solution of 5-azacytidine (Sigma A2385-100 mg; 5azaC hereafter) for 48 h at 4°C. Although some side effects after exposure to 5azaC might interfere with DNA replication, they are usually dose-dependent (Dvořák Tomaštíková & Pecinka 2024) and not evident at the low dose used here (Alonso *et al.* 2017). 20 days after sowing, individuals were transplanted into 1 L pots. The recurrent drought treatment started 3 weeks after transplanting (6-week-old seedlings). For each line, half of the offspring were watered to field capacity twice per week, and half were watered to field capacity once every 10–11 days until the end of the experiment. The experiment ended when plants were 17 weeks old and showing signs of senescence. The full design was a 2 × 2 factorial with three replicates per line (N = 288 individuals), 16 h light at 25–20°C.

For each F2 plant, we recorded development at different times (ontogenic approach) and final fitness-related traits (static approach). The ontogenic approach characterized initial size (first leaf length), flowering phenology (time to first flower) and growth (maximum leaf length before transplant, number of leaves, inflorescences at 4-week intervals). The static approach assessed reproductive fitness (total number of inflorescences produced, number of flowers per inflorescence, proportion of flowers that set fruit, proportion of seeds per ovule) and final individual size (above- and below-ground biomass).

Sample processing and laboratory methods

Once an F2 individual reached flowering stage, 2–3 fully grown undamaged leaves from each individual were collected, placed

in labelled paper bags and dried at ambient temperature in sealed containers with silica gel. For a subsample ($N = 96$ plants), at the end of experiment, roots were removed from soil, washed in water, excess water wiped off using absorbent paper, and a sample of fine roots (avoiding the primary thickest root) collected for DNA analyses, placed in a labelled paper bag and dried at ambient temperature in sealed containers with silica gel. Dried leaf and root tissues were homogenized to fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted using Bioline ISOLATE II Plant DNA Kit and digested with DNA Degradase PlusTM (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. We estimated percentage total cytosines methylated using reverse phase HPLC with spectrofluorimetric detection, obtaining integrated areas under peaks for 5-methyl-2′-deoxycytidine (5mdC) and 2′-deoxycytidine (dC), using the formula $100 \times 5\ \text{mdC}/(5\ \text{mdC} + \text{dC})$. Two independent replicates of digested DNA were processed per sample. This method was selected as it does not require a reference genome and can be applied to a large number of samples at a reasonable cost and is suitable to evaluate global effects of our study treatments (see Alonso *et al.* 2016 for further details).

Statistical analyses

All statistical analyses used the R environment (R Development Core Team 2019; R v. 3.6.3). All data were visually inspected and, for some variables, obvious outliers ($N \leq 3$) were excluded. For variables measured in several parts of each plant (e.g. number of flowers per inflorescence) or estimated twice (e.g. global cytosine methylation percentage), an average value was calculated and individual data used as response variable. For reproductive success (i.e. fruit and seed set), which have highly skewed distributions because many individuals had 100% success with median value for each proportion at 0.91 for fruit set and 0.83 for seed set, we used the standardized variable (scaled to mean 0 and SD 1) as response because this transformation improved model adjustment and normality of residuals.

Linear models were used to analyse the fixed effect of demethylation (control vs. 5azaC) and recurrent drought (watered vs. drought), and their interaction on all study traits (except early phenotype traits measured before the recurrent drought, where only demethylation was included). The lme function from the nlme package (Pinheiro *et al.* 2017) was used to fit mixed-effect models with Restricted Maximum Likelihood (REML), including maternal family as random effect (Bolker 2015). Analysis of the possible effect of maternal water regime was included as a random grouping factor in which maternal family was nested. This procedure corrected for non-independence of data so that family heterogeneity in genetic and environment backgrounds was adequately accounted for (i.e., blocked; Mead 1988). The effect of provenance (PT vs. CH) was considered fixed as the two provenances are known to differ in consequences of demethylation treatment for DNA global methylation of seedling roots (Alonso *et al.* 2017). When provenance had significant interactions with either of the two experimental factors, partial models for each population were run separately to better reveal effects of the focal factors. In the case of percentage cytosine methylation, the full model included tissue (leaf vs. root), demethylation, recurrent drought, provenance, and all interactions as fixed factors. Partial models were subsequently applied independently to leaf and root tissue to better interpret the sign of observed interactions. Finally, the effect of maternal water regime was investigated. As transgenerational transmission should be impaired by demethylation, only plants not exposed to seed demethylation ($N = 144$) were included. We applied linear models without random effects (function lm, stats), given the reduced replication within each combination of treatment levels, and because the design was balanced to include progeny from all mothers in the two water regimes. Further, as power to obtain a significant 3-way interaction was limited, we only ran the analysis for the subset of traits most responsive to drought in the F2 experiment. If the 3-way effect was not significant, only 2-way interactions were retained.

Plant development was characterized by changes with time in numbers of leaves and inflorescences per individual. These two variables were analysed as repeated measures with the lmer function in the lme4 package (Bates *et al.* 2015), modelling time as an ordered fixed factor with three levels, including individual as a categorical random factor, and explicitly analysing interactions between time and the three experimental factors [i.e., response variable time × provenance + time × demethylation + time × drought + (1 | individual)].

For every fitted model, inspection of residuals and goodness-of-fit assessment was conducted using 'check_model' in the Performance package (Lüdecke *et al.* 2021). In all models, residuals were continuous, with no sign of non-linearity and close to normal distribution. Significance of fixed effects and interactions were obtained using the ANOVA function from the car package (Fox & Weisberg 2018). Model-adjusted marginal averages for fixed main effects used the emmeans function in the emmeans package (Lenth 2016).

RESULTS

Global DNA methylation in adults

To test efficacy of the seed demethylation treatment, we examined its specific effect only in well-watered plants. In this subset, the effect of seed demethylation was statistically significant ($\chi^2 = 24.6$, df = 1, $P < 0.0001$), similar for both provenances ($\chi^2 = 1.35$, df = 1, $P = 0.24$), and more evident in root than in leaf DNA ($\chi^2 = 3.2$, df = 1, $P = 0.07$, for demethylation × tissue interaction; Fig. 1). At this adult stage, DNA cytosines were more methylated in roots than leaves ($\chi^2 = 41.9$, df = 1, $P < 0.0001$; 22.8 ± 0.3% vs. 20.9 ± 0.3%, for root and leaf tissue, respectively).

In the full experimental design (i.e. including well-watered and drought plants), global DNA methylation in leaves was poorly explained by the fixed factors (Table 1A). In contrast, for root DNA there were multiple significant effects. Both demethylation and drought significantly reduced global DNA methylation, which was also lower in roots of PT plants (Table 1B). There were also significant interactions between drought and provenance, and demethylation and provenance, indicating that the effect of drought was stronger in PT plants and, interestingly, the effect of 5azaC was lost in plants experiencing recurrent drought (Table 1B, Fig. S1).

Moreover, DNA cytosine methylation in adult leaves and roots was positively related in well-watered plants ($r = 0.33$, $P = 0.02$, $N = 48$). In contrast, in recurrent droughted plants,

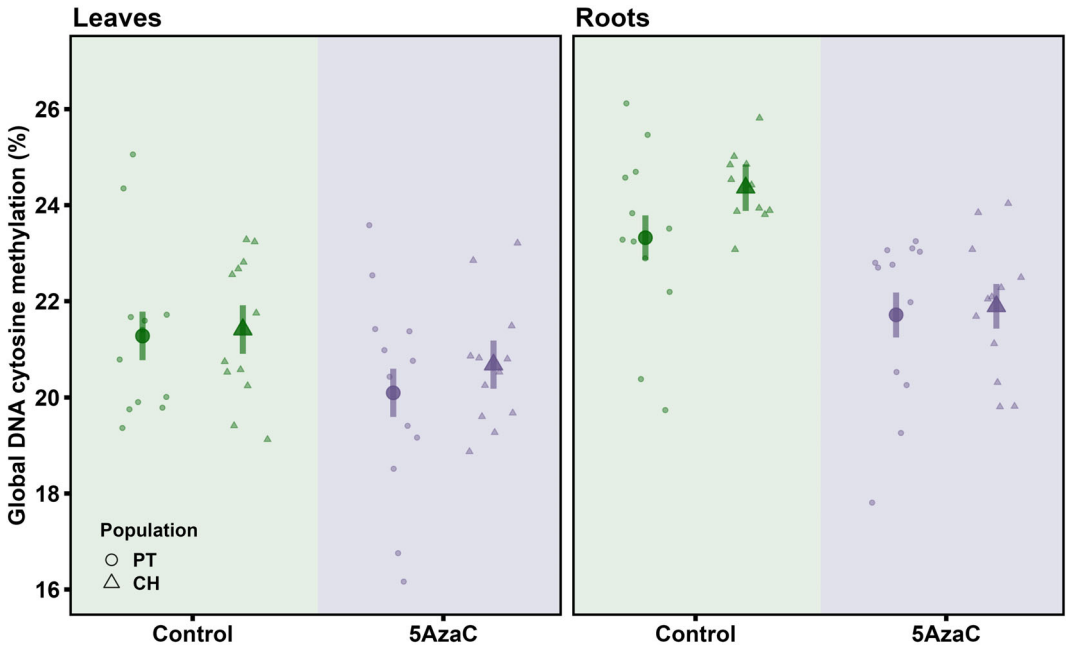

Fig. 1. Effects of seed demethylation treatment as global percentage of methylated cytosines in DNA from adult leaves and roots of *Erodium cicutarium* plants without drought during development. Small circles and triangles denote individuals from PT and CH populations, respectively. Large symbols and bars represent least-squared means and 95% confidence level, respectively.

Table 1. Summary of results of linear mixed models analysing effects of seed demethylation, recurrent water stress, plant provenance and all interactions on the observed variation in global cytosine DNA methylation percentage of adult *Erodium cicutarium* grown in a greenhouse.

response	predictor	estimated parameter	± SE	chi-squared	*P*
(A) Leaf DNA methylation	Intercept	20.68	0.54		
	Demethylation (AZA)	0.73	0.57	2.72	*0.099*
	Drought (WAT)	0.09	0.57	0.74	0.39
	Provenance (POP)	−0.59	0.74	0.04	0.85
	AZA:WAT	−0.63	0.81	2.82	*0.093*
	AZA:POP	0.45	0.81	0.04	0.84
	WAT:POP	1.27	0.81	2.64	*0.10*
	AZA:WAT:POP	−0.67	1.15	0.34	0.56
(B) Root DNA methylation	Intercept	21.89	0.55		
	Demethylation (AZA)	2.47	0.61	24.65	**<0.0001**
	Drought (WAT)	−1.16	0.61	58.00	**<0.0001**
	Provenance (POP)	−0.18	0.61	23.39	**<0.0001**
	AZA:WAT	−0.62	0.87	2.75	*0.097*
	AZA:POP	−0.87	0.87	4.22	**0.040**
	WAT:POP	−1.36	0.87	8.13	**0.004**
	AZA:WAT:POP	−0.80	1.23	0.42	0.51

(A) Leaf tissue. (B) Root tissue. Each individual plant is average value of two to four replicates per tissue (N = 96 individuals). Significance of effects are highlighted in bold when $P < 0.05$ and in italic when $0.05 < P \leq 0.10$.

global DNA methylation in both tissues was not significantly correlated ($r = -0.15$, $P = 0.31$, $N = 48$; Fig. S2).

Phenotypic traits prior to recurrent drought

For germination, 42.7% of individuals developed a radicle during the 48-h imbibition, and 95% of seedlings developed cotyledons within 48 h after sowing in soil. The first true leaf was shorter in individuals previously treated with 5azaC (58.1 ± 2.2 mm and 84.0 ± 2.2 mm, treated and control, respectively; $\chi^2 = 103.3$, df = 1, $P < 0.0001$; Fig. S3). There was a significant interaction between demethylation and provenance ($\chi^2 = 6.5$, df = 1, $P = 0.01$), with a larger reduction in leaf length after 5azaC treatment in CH plants (Fig. S3). 11 days after sowing, seedlings produced 0 to 8 true leaves, while most of those treated with 5azaC produced less leaves than untreated plants (treated: 3.7 ± 0.1 leaves, control: 4.9 ± 0.1 leaves; $\chi^2 = 51.2$, df = 1, $P < 0.0001$). Again, the

effect of 5azaC was stronger in CH plants, as suggested by the marginally significant interaction between demethylation and provenance ($\chi^2 = 3.7$, df = 1, $P = 0.05$).

In the 4 weeks between the first count and initiation of drought treatment, individual plants produced 2–39 leaves. This difference was related to a reduced leaf production in plants treated with 5azaC, which, on average, produced 7.4 fewer leaves than the untreated plants ($\chi^2 = 91.9$, df = 1, $P < 0.0001$; Fig. S3). In addition, a significant interaction between demethylation and provenance indicated a stronger reduction in CH plants ($\chi^2 = 12.4$, df = 1, $P = 0.0004$; Fig. S3). The length of the longest leaf at this time was between 33 and 279 mm. This range was explained by the combined effects of demethylation and provenance. Plants treated with 5azaC had shorter leaves ($\chi^2 = 64.05$, df = 1, $P < 0.0001$), while plants from the CH population had leaves that were 12.5 mm longer than those of the PT population ($\chi^2 = 10.9$, df = 1, $P = 0.0009$), and experienced a stronger reduction in length after seed demethylation ($\chi^2 = 4.95$, df = 1, $P = 0.026$).

Development traits: Flowering phenology and vegetative growth

Both demethylation and provenance had significant effects on time required to reach flowering, with no significant interaction between factors (Fig. 2). On average, PT plants started to flower after 2 months (60.6 ± 1.7 days), whereas those from the CH population started flowering 10.6 days later ($\chi^2 = 19.9$, df = 1, $P < 0.0001$; Fig. 2). Further, demethylated plants flowered 5.1 ± 1.3 days later than untreated plants ($\chi^2 = 27.7$, df = 1, $P < 0.0001$; Fig. 2). The effect of recurrent drought was not statistically significant for this trait ($\chi^2 = 1.5$, df = 1, $P = 0.22$), and no two-way interactions were significant ($P > 0.1$ in all cases).

As regards plant growth, production of new leaves changed with time, with a linear positive relationship (effect size: 8.21 ± 0.22) and a negative quadratic term (effect size: −6.17 ± 0.22) during the first 9 weeks of growth. Demethylation treatment interacted significantly with the quadratic term (effect size: −3.08 ± 0.42; Fig. S4A) indicating that control plants tended to produce fewer new leaves at bolting time (W9), whereas demethylated plants had more stable leaf production at this stage.

Number of new inflorescences produced every 4 weeks throughout the reproductive period (W9–W17) had a linear positive relationship with time (effect size: 17.92 ± 0.47). Demethylation treatment interacted significantly with time (effect size: −1.89 ± 0.88; Fig. S4B), indicating that control plants produced fewer inflorescences at late stage, whereas demethylated plants did not change the rate of inflorescence production during this second period. Effects of recurrent drought did not interact with rate of production of leaves or inflorescences.

Final individual size and fitness

Only recurrent drought had a significant and negative effect on aboveground biomass ($\chi^2 = 20$, df = 1, $P < 0.0001$). Plants from the two provenances reached similar final sizes (3.89 ± 0.12 g and 3.74 ± 0.14 g for CH and PT plants, respectively; $\chi^2 = 0.66$, df = 1, $P = 0.42$). Neither demethylation nor any interactions between study factors were statistically significant ($P > 0.4$).

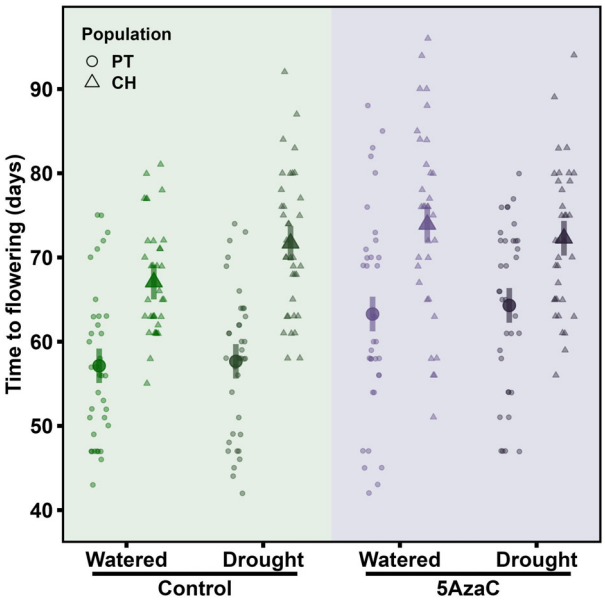

Fig. 2. Effects of seed demethylation and recurrent drought treatments as time required to start flowering in *Erodium cicutarium* grown under controlled greenhouse conditions. Small circles and triangles denote individuals from PT and CH populations, respectively. Darker colour is individuals experiencing recurrent drought. Large symbols and bars are least-squared means and 95% confidence level, respectively.

Root biomass was always less than aerial biomass, and these two traits were positively correlated across well-watered plants ($r_s = 0.30$, $P = 0.037$, $N = 48$) but not in those experiencing recurrent droughts ($r_s = 0.03$, $P = 0.84$, $N = 48$). Recurrent drought had a significant and negative effect on root biomass ($\chi^2 = 33.5$, df = 1, $P < 0.0001$). Moreover, root biomass was larger for CH plants (1.16 ± 0.06 g) than for PT plants (0.91 ± 0.06 g; $\chi^2 = 7.9$, df = 1, $P = 0.005$). Neither demethylation nor any interactions between study factors were statistically significant in explaining variations in final root biomass ($P > 0.4$). The PT plant range of shoot:root biomass (1.7–7.8; excluding an outlier = 10.9) increased in water-stressed plants ($\chi^2 = 10.1$, df = 1, $P < 0.0015$), whereas this biomass ratio was less variable in CH plants (2.1–6.1; excluding an outlier = 10.4) and it did not change under drought stress ($\chi^2 = 0.21$, df = 1, $P = 0.6$).

The total number of inflorescences per plant by the end of the experiment averaged 42.7 (range 15–94; three outliers were excluded with 118, 121 and 122 inflorescences). Recurrent drought had a significant and negative effect on total inflorescence production ($\chi^2 = 13.6$, df = 1, $P = 0.0002$). The effect of seed demethylation was also statistically significant ($\chi^2 = 4.5$, df = 1, $P = 0.033$), with plants grown from 5azaC-treated seeds producing 3.8 ± 2.6 fewer inflorescences than untreated plants (Fig. 3A). Furthermore, CH plants had 5.0 ± 3.2 fewer inflorescences than PT plants ($\chi^2 = 5.9$, df = 1, $P = 0.015$). No interactions between study factors were statistically significant ($P > 0.19$).

Investment in reproduction also depended on the number of flowers produced per inflorescence and ranged between 1 and 13. This parameter was again significantly lower in CH plants

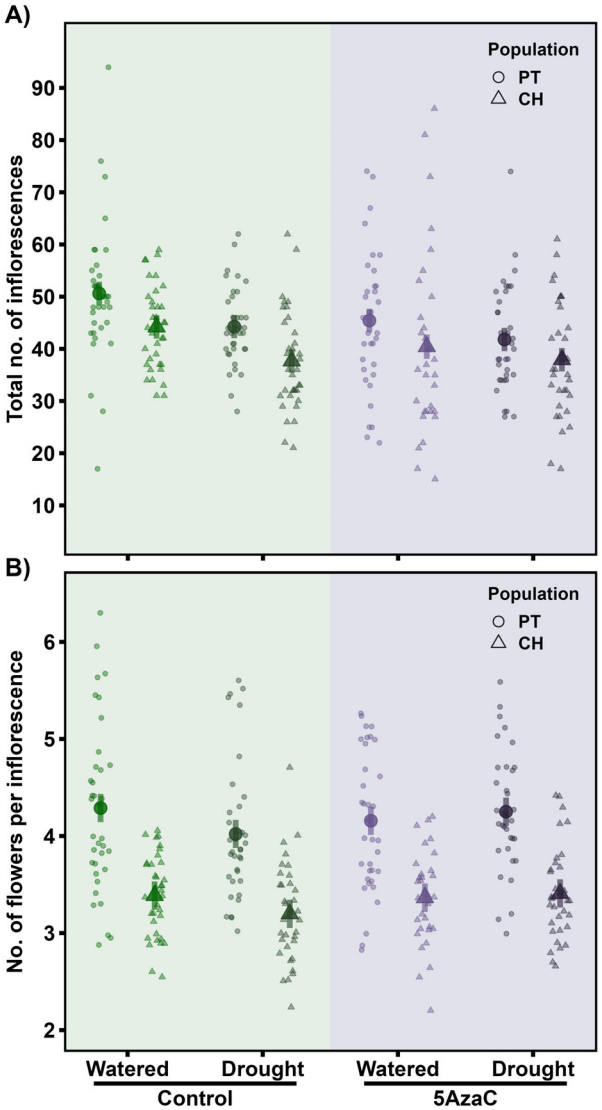

Fig. 3. Effects of seed demethylation and recurrent drought treatments on flower investment of *Erodium cicutarium*. (A) Total number of inflorescences produced. (B) Average number of flowers per inflorescence. Small circles and triangles denote individuals from PT and CH populations, respectively. Darker colour is individuals experiencing recurrent drought. Large symbols and bars are least-squared means and 95% confidence level, respectively.

(4.18 ± 0.13 for PT; 3.34 ± 0.13 for CH plants; $\chi^2 = 21.0$, df = 1, $P < 0.0001$; Fig. 3B) indicating that PT plants invested more in reproduction. There was a significant interaction between demethylation and drought ($\chi^2 = 7.5$, df = 1, $P = 0.006$), indicating that recurrent drought induced higher flower production per inflorescence only when plants had been previously exposed to 5azaC treatment (Fig. 3B), i.e. when the number of inflorescences produced was lower. No other interactions were significant ($P > 0.5$).

Plants grown from 5azaC-treated seeds had lower reproductive success (Fig. S5). The effect of seed demethylation on fruit set probability was negative and significant ($\chi^2 = 12.08$, df = 1, $P = 0.0005$) and significantly interacted with provenance ($\chi^2 = 6.5$, df = 1, $P = 0.01$), indicating a stronger effect of 5azaC in CH plants. Further, fruiting success was higher in plants that experienced recurrent drought, although only marginally significant ($\chi^2 = 3.1$, df = 1, $P = 0.08$). Neither provenance nor any other interaction was statistically significant ($P > 0.28$). Similar results were found for seed set (Fig. S5B). The effect of seed demethylation was negative and statistically significant ($\chi^2 = 5.1$, df = 1, $P = 0.02$), with a significant interaction with provenance ($\chi^2 = 7.3$, df = 1, $P = 0.007$), indicating that the effect of 5azaC was stronger in CH plants. Recurrent drought increased production of seeds per ovule ($\chi^2 = 5.7$, df = 1, $P = 0.017$). Neither provenance nor any other interaction was statistically significant ($P > 0.19$).

Transgenerational effects

Table 2 shows results of models for early size (no. leaves before onset of drought), flowering time, total inflorescence production, and final root biomass. Overall, the effect of maternal water regime was either variable between the two provenances or mild, with no effect on final root biomass (Table 2). Plants from PT population whose mothers were stressed had more leaves at week 4 (20.0 ± 0.8 vs. 18.8 ± 0.8 leaves; for F1-stressed vs. F1-control, respectively), whereas the opposite was true for CH plants (21.9 ± 0.8 vs. 23.5 ± 0.8), suggesting that maternal stress was detrimental only for CH. Similarly, inflorescence production was not affected by maternal stress in PT plants (47.7 ± 1.6 vs. 47.1 ± 1.6), but maternal stress had a negative fitness effect in CH plants (37.6 ± 1.7 vs. 43.9 ± 1.6; for F1-stressed vs. F1-control, respectively). Further, maternal stress delayed flowering although, again, the effect was restricted to CH plants (71.5 ± 1.4 vs. 67.5 ± 1.4 days to flower) and absent in PT plants (57.6 ± 1.4 vs. 57.2 ± 1.4).

Phenotypic variability associated with actual variation in global DNA methylation

Finally, we analysed continuous linear and quadratic relationships between phenotypic traits and global DNA methylation in leaf and root tissue (Table S1). Global cytosine methylation in leaf DNA was more related to ontogeny-related traits. Plants with lower global cytosine methylation in leaf DNA produced shorter first leaves, had fewer leaves at early stage (11 days), and required more time to start flowering, compared to those with intermediate and high global leaf DNA methylation percentage.

Variation in global cytosine methylation in root DNA was linearly and positively related to the number of leaves in plants at mid- and late-vegetative stages, and more significantly to final root mass (Table S1).

DISCUSSION

Plant DNA cytosine methylation is a dynamic epigenomic feature that can change in response to developmental and environmental cues (Finnegan 2010; Brautigam & Cronk 2018). Analysing how these changes can be interrelated is key to understanding epigenetic contributions in plant response to stress that might lead, or not, to phenotypic plasticity and priming, depending on timing (Brautigam & Cronk 2018; Cooper & Ton 2022). Experimental demethylation at seed stage increased variance in global DNA methylation and could

Table 2. Results of linear models analysing effects of maternal water regime (F1-treat), recurrent water stress, plant provenance and all two-way interactions on variations in phenotypic traits.

response	predictor	estimated parameter	± SE	*t*-value	*P*
(A) No. leaves before drought	Intercept	23.5	0.77		
	Maternal (F1-treat)	−1.64	1.09	−1.51	0.13
	Provenance (POP)	−4.75	1.08	−4.39	**<0.0001**
	F1-treat:POP	2.89	1.54	1.88	*0.062*
(B) Time to flowering (days)	Intercept	65.3	1.82		
	Maternal (F1-treat)	4.08	2.40	1.70	*0.092*
	Drought (WAT)	4.40	2.38	1.85	*0.067*
	Provenance (POP)	−8.32	2.38	−3.50	**0.0006**
	F1-treat:WAT	−0.04	2.76	−0.01	0.989
	F1-treat:POP	−3.70	2.76	−1.34	0.181
	WAT:POP	−3.85	2.76	−1.40	0.165
(C) No. inflorescences produced	Intercept	46.1	2.14		
	Maternal (F1-treat)	−4.14	2.83	−1.46	0.146
	Drought (WAT)	−4.41	2.80	−1.57	0.118
	Provenance (POP)	3.04	2.83	1.07	0.286
	F1-treat:WAT	−4.32	3.26	−1.32	0.188
	F1-treat:POP	6.94	3.26	2.13	**0.035**
	WAT:POP	0.32	3.26	0.099	0.921
(D) Root biomass (mg)	Intercept	1.41	0.11		
	Maternal (F1-treat)	−0.09	0.14	−0.60	0.552
	Drought (WAT)	−0.37	0.14	−2.56	**0.014**
	Provenance (POP)	−0.46	0.14	−3.17	**0.003**
	F1-treat:WAT	0.19	0.17	1.12	0.267
	F1-treat:POP	0.28	0.17	1.36	0.182
	WAT:POP	0.06	0.17	0.34	0.73

The analyses were conducted for a subset of F2 plants that were not demethylated ($N = 143$ for all traits, except root biomass that was estimated in $N = 48$ plants). Significant effects are highlighted in bold when $P < 0.05$ and italics when $0.05 < P \leq 0.10$.

elucidate epigenetic and phenotypic outcomes of stress exposure both above- and belowground. In this study, an initial short treatment of *E. cicutarium* seeds with 5azaC was still detectable in roots and leaves as lower global cytosine methylation in DNA when plants reach reproductive stage, supporting use of this protocol to alter individual methylation profiles (see Balao *et al.* 2024, for an in-depth molecular study, showing a higher reduction of methylation in CG sequences and a similar number of methylation gains and losses in cytosines in other sequence contexts). Ontogenetic phenotypic characterization showed a delay in development and growth after seed demethylation, while the final phenotypic characterization showed a reduction in size and inflorescence production after recurrent drought, with partial compensation of seed output through increased autonomous reproductive success. Below we discuss in more detail the consequences of seed demethylation and recurrent drought in terms of global DNA methylation, plant development, individual final size and fitness-related traits.

Dynamic analysis of global DNA methylation

Global DNA methylation increased in leaf tissue from seedling to adult age in *Arabidopsis thaliana* (Baubec *et al.* 2009) and a few other species (e.g., Alves *et al.* 2020; Perrin *et al.* 2020). Furthermore, DNA methylation can change among tissues and vary across growth stages (or dates) and tissues, depending on environmental conditions (Demeulemeester *et al.* 1999; Seymour *et al.* 2014; Lloyd & Lister 2022). In well-watered – control – plants of *E. cicutarium*, global DNA methylation varied between leaf and root tissues and increased from seedling to adult stage, the age difference being stronger in root tissue (from 13.0% to 22.8%) than in leaf tissue (from 17.7% to 20.9%). Therefore, under benign conditions, DNA was more methylated in leaves than roots at seedling stage (Alonso *et al.* 2017), whereas the opposite was true at the adult stage, as reported here.

Cytosine methylation in DNA from adult leaves and roots was positively correlated in well-watered plants. However, in individuals experiencing recurrent drought throughout their life, global DNA methylation in these two tissues was decoupled, suggesting that epigenetic processes above- and belowground might be regulated independently (see Abid *et al.* 2017 for evidence in seedlings of *Vicia faba*). The absence of a correlation most likely reflects that recurrent water stress consistently and largely reduced methylation in adult root DNA, whereas in leaves the reduction in DNA methylation during development was milder and its relationship to water regime more stochastic. At molecular level, in leaf DNA there were similar numbers of cytosines that gain or loose methylation in response to water stress, mainly located in CHH sequences and associated with non-coding regions, and recorded methylation changes were much less than in response to 5azaC (see Balao *et al.* (2024) for sequence analyses in a subsample of this experiment). Further, the complexity of interaction effects between seed demethylation and recurrent drought revealed eight patterns of methylation change in cytosines in

different fragments of leaf DNA. In many cases, 5azaC and drought induced methylation changes to different cytosines, and the combination of these two treatments re-established methylation level to that in control plants (see Balao *et al.* 2024 for further details). Ongoing analyses of molecular changes in root DNA will shed light on the extent to which epigenetic regulation in response to seed demethylation and other external factors, such as drought, differ between above- and belowground tissues (Lloyd & Lister 2022).

Developmental and fitness consequences of seed treatment with 5azaC

The short seed exposure to 5azaC used here did not affect early seedling mortality or cause abnormal morphologies in young seedlings that might indicate substantial toxicity, which is typical with higher doses (Alonso *et al.* 2017 and references therein). Furthermore, transcriptome analyses of leaf and root tissues confirmed that the number of genes differentially expressed after 5azaC treatment in any tissue was less than that characterizing divergence in gene expression between tissues, which contradicts a generalized toxic effect and supports restricted significance of the treatment (unpubl. data). The seed demethylation treatment slowed individual growth, delayed flowering and leaf production, and had a negative impact on fitness by reducing both inflorescence production and reproductive success. These results therefore confirm that artificially altering DNA methylation has phenotypic consequences and, therefore, epigenetic modifications can desynchronize plant growth, flowering and senescence among individual plants in both favourable and adverse environments (see Herrera *et al.* 2019 for within-plant effect). Early studies on the effects of 5azaC highlighted its ability to simulate vernalisation, establishing links between DNA methylation and flowering transition (Fieldes & Amyot 1999; Kondo *et al.* 2007). At population level, both flowering time and flowering synchrony impact fitness, particularly in annual plants and temperate climates (Munguía-Rosas *et al.* 2011). Epigenetic regulation of flowering time could be particularly beneficial in unpredictable environments and in response to climate change (Franks & Hoffmann 2012). For example, in Mediterranean mountains, shorter growing periods were associated with earlier and hotter dry summers and could favour early blooming individuals in some years but not in others (Giménez-Benavides *et al.* 2010) and, thus, reversible epigenetic changes could be particularly advantageous. Future studies combining analysis of the methylome and transcriptome effects of 5azaC in different plant species or ecotypes could help to better characterize pathways linking DNA methylation and flowering.

Changes in final size and fitness induced by recurrent drought and interaction with 5azaC

Erodium cicutarium could not fully compensate for negative impacts of recurrent water shortage, with smaller plant size and inflorescence production and non significant effects on phenology or growth-related traits. Interestingly, stressed plants could partially compensate their reproductive output. First, recurrent drought induced higher flower production per inflorescence only if plants that had been previously exposed to 5azaC treatment. As regards seed production, fruit set and seed set increased under recurrent drought but were negatively affected by seed demethylation. These findings suggested that allocation of resources to reproduction can be upregulated by stress and mediated by changes in DNA methylation, likely through their combined effects on hormone metabolism (Fleta-Soriano & Munne-Bosch 2016; Gallusci *et al.* 2023).

Heterogeneous response of seed provenances: From experimental nuisance to transgenerational effects and ecological relevance

The delay in flowering and growth induced by seed demethylation and decreased final reproductive output induced by recurrent drought varied in magnitude between the two seed provenances, despite the short distance between the two source populations (ca. 10 km). This unexpected result suggest that not only intraspecific variability associated with broad geographic scales and climatic gradients may determine the magnitude of plant phenotypic plasticity mediated by DNA methylation changes (e.g. Bossdorf *et al.* 2010; Münzbergová *et al.* 2018; Sammarco *et al.* 2022). Short-distance environmental heterogeneity emerged as another fundamental scale leading to intraspecific epigenetic variability (e.g. Herrera & Bazaga 2016; Herrera *et al.* 2017; Valverde *et al.* 2024) whose relevance for plant adaptation to stress deserves further analysis. First, the magnitude of reduction in DNA methylation of leaves and roots after seed treatment with 5azaC was not homogeneous between provenances (see e.g. Troyee *et al.* 2022 for heterogenous response in two ecotypes of *Thlaspi arvense*; Browne *et al.* 2020 for heterogenous response in gene transcription between families of *Quercus lobata*). As a practical consequence, the predicted interaction between our two fixed factors was weakened by enlarged within-class variance, and the statistical power for our three-way interaction model was reduced. Second, some of phenotypic traits that changed in response to water stress had variable transgenerational effects between the two provenances. Maternal stress had negative consequences for early growth and inflorescence production in CH plants but had either no or positive effects in PT plants. These findings suggest transgenerational transmission which is not necessarily adaptive (see Sultan *et al.* 2009 for similar findings for a comparison between congeners).

Finally, individuals from PT flowered earlier and, according to our expectations for the escape-avoidance, were less affected by drought, producing more flowers per inflorescence and higher fruit- and seed-set probabilities. Interestingly, they were also individuals in which DNA methylation in leaves and roots was decoupled after recurrent drought, suggesting that a more responsive methylome might improve fitness under stress and benefit from maternal stress, with the side-effect of blurred consequences of seed treatment with 5azaC.

CONCLUSIONS

Investigations of changes in global DNA methylation in leaves and roots of *E. cicutarium* found heterogeneous phenotypic responses to water availability within and across individuals. Within individuals, there was some independence for epigenetic regulation above- and belowground during plant development, and how drought elicited stronger phenotypic and molecular changes in roots. Across individuals, there was

intraspecific variation in ability to change the plant methylome in response to drought, supporting that an escape-avoidance strategy could be mediated by changes in the plant methylome.

AUTHOR CONTRIBUTIONS

All authors designed the research; CA and MM conducted greenhouse experiments, data collection and data analyses; CMH and CA provided economic resources; CA led the writing; MM prepared all figures; all authors contributed to refining the final manuscript.

ACKNOWLEDGEMENTS

The authors thank Maciej Barczyk, Laura Cabral, Esmeralda López, Daisy Johnson, Pablo Martín, Alejandro Mira, Ricardo Pérez, Elena Villa and Noelia Zarza, for assistance in field, greenhouse and laboratory work, and Javier Puy and three anonymous referees for suggestions on earlier versions of the manuscript. Financial support was provided by grants EPIECOL-CGL2013-43352-P, EPIENDEM-CGL2016-76605-P, EPINTER-PID2019-104365GB-I00 and DISTEPIC-PID2022-141530NB-C22 (Ministerio de Ciencia e Innovación, Spanish Government). We acknowledge support for the publication fee from the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The dataset used here, including global cytosine methylation estimates and individual phenotypic data, can be found in the CSIC public institutional repository, and should be cited as Alonso, C., M. Medrano, and C. M. Herrera 2025. Above- and below-ground phenotypic traits and global DNA methylation in *Erodium cicutarium* experimentally exposed to either seed demethylation, recurrent drought or both [Dataset]; DIGITAL.CSIC; https://doi.org/10.20350/digitalCSIC/17419.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Detailed description of experimental procedures.

Table S1. Summary of the results of the linear mixed models conducted to analyze the continuous relationships between phenotypic traits and global DNA methylation in (A) leaf tissue and (B) root tissue.

Fig S1. Effects of seed demethylation and recurrent drought treatments in global DNA cytosine methylation percentage of *Erodium cicutarium* plants. (A) DNA obtained from root tissue. (B) DNA obtained from leaf tissue.

Fig S2. Graphical representation of the relationship between global percent of methylated cytosines in DNA obtained from leaves and roots of individual adult plants according to the level of water availability experienced along their lifetime.

Fig S3. Effects of seed demethylation treatment in early growth traits of *Erodium cicutarium*. (A) Length of the first true leaf (mm). (B) Number of leaves produced after five weeks of sowing.

Fig S4. Effect of seed demethylation treatment in developmental phenology of *Erodium cicutarium* plants (A) Number of full-grown leaves per individual recorded on different weeks (W1-W9). (B) Number of inflorescences produced on different weeks (W9-W17).

Fig S5. Effects of seed demethylation and recurrent drought treatments in reproductive success of *Erodium cicutarium* plants. (A) Average proportion of flowers setting fruits. (B) Average proportion of ovules setting seeds within fruits.

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