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Variation in DNA methylation and response to short-term herbivory in *Thlaspi arvense*

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ABSTRACT

Plant metabolic pathways and gene networks involved in the response to herbivory are well-established, but the impact of epigenetic factors as modulators of those responses is less understood. Here, we use the demethylating agent 5-azacytidine to uncover the role of DNA cytosine methylation on phenotypic responses after short-term herbivory in *Thlaspi arvense* plants that came from two European populations with contrasting flowering phenotypes expected to differ in the response to experimental demethylation. The experimental design followed a 2 × 3 factorial design, that was replicated for each flowering-type. First, half the seeds were submerged in a water solution of 5-azacytidine and the other half only in water, as controls. Then, we assigned control and demethylated plants to three herbivory categories (i) insect herbivory, (ii) artificial herbivory, and (iii) undamaged plants. The effects of the demethylation and herbivory treatments were assessed by quantifying genome-wide global DNA cytosine methylation, concentration of leaf glucosinolates, final stem biomass, fruit and seed production, and seed size. For most of the plant traits analysed, individuals from the two flowering-types responded differently. In late-flowering plants, global DNA methylation did not differ between control and demethylated plants but it was significantly reduced by herbivory. Conversely, in early-flowering plants, demethylation at seed stage was still evident in leaf DNA of reproductive individuals whereas herbivory did not affect their global DNA methylation. In late-flowering plants, artificial herbivory imposed a stronger reduction than insect herbivory in global DNA methylation and final stem biomass, and induced higher concentration of aliphatic glucosinolates. In early-flowering plants, the effects of herbivory were non-significant for the same traits. Finally, the effect of herbivory on reproductive parameters varied with the level of demethylation and the plant flowering-type. Although further investigations with more populations and families are required to confirm our results, they suggest that the genetic background of experimental plants and timing of damage can affect the response to herbivory and point towards multifaceted genetic-epigenetic interactions in determining herbivory-induced phenotypic plasticity.

1. Introduction

Interactions between plants and herbivorous insects are ubiquitous and these associations are diverse among biomes, communities and species (see e.g., Fornoni et al., 2004; Moreira et al., 2018). Plants have evolved multiple traits to reduce herbivory, including mechanical and chemical defences and poor nutritional quality (Carmona et al., 2011). Some plant species also show the capacity to respond to herbivory through phenotypic plasticity. Such responses involve the activation of defense signaling pathways regulated by plant hormones such as jasmonic acid (JA), salicylic acid and ethylene (Pieterse and Dicke, 2007) and vary with timing, salivary factors associated to certain consumers, and amount of damage (see e.g. Agrawal, 2000a; Bossdorf et al., 2004; Züst and Agrawal, 2017). In order for a response to become evolutionarily successful, it should reduce the impact on fitness even when incurring some costs (Cipollini et al., 2003; Douma et al., 2017). The magnitude of plastic phenotypic change varies across and within species and such variation could stem from both genetic and environmental factors (Josephs, 2018; Ogran et al., 2020; Wagner and Mitchell-Olds, 2018).

Besides genetic and environmental components, epigenetic factors such as DNA methylation, small RNAs and post-translational histone

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modifications have emerged as relevant modulators of plant responses to biotic challenges (Herrera et al., 2018; Ramos-Cruz et al., 2021). DNA methylation is the best studied epigenetic mechanism in plants and experimental evidence suggests its link to phenotypic plasticity. Experimental approaches to uncover the role of DNA methylation on ecologically relevant plant traits have often used DNA demethylating agents such as 5-azacytidine or zebularine (Alonso et al., 2017; Fieldes et al., 2005; Latzel et al., 2020; Puy et al., 2018; Richards et al., 2010; Verhoeven and van Gurp, 2012). A combination of manipulation of DNA methylation with controlled herbivory experiments can be a fruitful strategy to elucidate the contribution of DNA methylation to specific plant responses after herbivory (Alonso et al., 2019). Although we focused on DNA methylation, it might be worth to indicate that 5-azacytidine is able to reduce cytosine methylation also in RNA. This aspect is out of our scope but it might contribute to plant response to stress (see e.g., Tang et al., 2020).

Here, we studied the effects of 5-azacytidine and controlled herbivory on the performance and defense of the annual plant *Thlaspi arvense* L. (Brassicaceae) to evaluate the relationships between herbivory, phenotypic plasticity and epigenetic changes in plants. We selected this fast growing and widespread annual plant because many studies contributing to the molecular understanding of inducible defenses have been performed in the Brassicaceae (e.g., Rasmann et al., 2012; Lucas-Barbosa et al., 2017). This plant family is well characterized by the presence of specific specialized (or secondary) compounds, the glucosinolates (Halkier, 2016). These compounds are classified as aliphatic, indole and aromatic and, together with their breakdown products that are released upon tissue disruption, play an active role in plant defense (Wittstock et al., 2016; Züst et al., 2012). In particular, aliphatic glucosinolates are found in higher concentrations after herbivory or JA application, have a role in resistance against pest insects (Guo et al., 2013; Mikkelsen et al., 2003; Textor and Gershenzon, 2009) and their relative abundance can vary geographically within a species (e.g., Züst et al., 2012). Furthermore, *T. arvense* has agronomic value as potential biofuel crop and it shows contrasting phenotypic traits associated to geographic origin and length of life-cycle that may be relevant to better understand variation in induced responses (Claver et al., 2017; Dorn et al., 2015; McIntyre and Best, 1978; Moser et al., 2009; Royo-Esnal et al., 2015).

Our experimental approach included demethylation with 5-azacytidine at seed stage (Alonso et al., 2017) and three different herbivory treatments: artificial leaf damage combined with JA-spraying, consumption by caterpillars of *Pieris brassicae* (Lepidoptera: Pieridae) and undamaged controls. The two herbivory treatments were expected to elicit analogous responses by dint of JA addition and the artificial one aimed to mimic consumption by any insect that could provide insight on generality of the observed responses (Züst and Agrawal, 2017). We estimated the genome-wide global DNA methylation level in leaves of reproductive adult plants and measured concentration of leaf glucosinolates, final stem biomass, fruit and seed production, and seed mass to test whether seed-stage demethylation influenced plant responses to short-term herbivory stress. As we were uncertain which plant-type would be more responsive to herbivory, we used seeds collected from two European populations that exhibited contrasting phenotypes, which roughly correspond to two formerly described strains and commercial varieties, namely early- and late-flowering types that differ in foliar and ecological traits (Best and McIntyre, 1975; McIntyre and Best 1978; Moser et al., 2009), and belonged to two different genetic clusters (Galanti et al., 2022). Our specific postulations were: i. Experimental demethylation at seed germination will reduce leaf DNA methylation levels of reproductive adult plants of *T. arvense*, at least in late flowering phenotypes (Burn et al., 1993) ii. Under controlled conditions, short-term herbivory will increase glucosinolates and can reduce plant fitness or not depending on tolerance (Núñez-Farfán et al., 2007; Textor and Gershenzon, 2009). iii. Also, herbivory can alter DNA methylation, although the magnitude and sign of this latest effect is uncertain iv. Altering DNA methylation will impair plant inducible defences (Latzel et al., 2020) and modify at least some of the plant responses after herbivory.

2. Materials and methods

2.1. Study system

Thlaspi arvense L. (Brassicaceae) is a diploid annual species with a small genome size (1C = 539 Mb) (Johnston et al., 2005) and has a high degree of ecotypic variation, including variance in its life-cycle phenology and the need of previous vernalization to induce flowering (Burn et al., 1993). The wild populations exhibit significant genetic diversity, phenotypic differentiation (Frels et al., 2019; Sedbrook et al., 2014) and epigenetic variation associated to environmental drivers (Galanti et al., 2022). Plants initially grow as a vegetative rosette, then bolt and produce racemes of flowers at the apices of the terminal and axillary branches. Plants of the early-flowering spring-type produce only a few leaves before internodes begin to elongate and individuals switch to reproductive growth, whereas the late-flowering winter-type forms a rosette of large leaves and requires vernalization to start flowering (Best and McIntyre, 1975; Burn et al., 1993; Moser et al., 2009). The two flowering types have genetic and ecological differences (Dorn et al., 2018; McIntyre and Best, 1978). The species mainly self-pollinates and produces abundant siliques, each containing 10–20 seeds.

We used plants collected from two European populations with contrasting flowering phenotypes (Supplementary Material S1) and genetic backgrounds (Galanti et al., 2022). Plants collected near Uppsala (59°49′N, 17°39′E, 26 m a.s.l., in central Sweden) were late-flowering SE winter-type. Plants collected from Bossdorf (52°00′ N 12°35′E, 151 m a.s.l., in north Germany) were early-flowering DE spring-type. The two populations occurred in roadsides and field margins, with a soil depth of >50 cm. In the field, all the mature fruits from available individuals (N = 12–15) were collected from each population in July-August 2018 and stored in darkness for 3–4 months at room temperature. In the lab, we counted fruits and seeds of each sampled individuals and randomly selected three fruiting individuals per population, each having at least 30 mature fruits containing dark-brown seeds to obtain enough seeds for our experimental design (see below).

Insect herbivory assays were conducted by larvae of *Pieris brassicae* (L.), a specialist herbivore that feeds only on plants in the Brassicaceae (Lucas-Barbosa., 2016). We obtained L2 instar larvae from a commercial supplier (www.lombricesdecalifornia.com), reared them on leaf cabbage and 1–2 days before the beginning of our experiments they were fed with leaves of *T. arvense*.

2.2. Experimental design, growing conditions, and treatments

We investigated the effects of experimental demethylation with 5-azacytidine at seed stage and leaf herbivory on plant performance, concentration of leaf glucosinolates, and epigenetic features of *T. arvense* plants using an experimental design in which the two factors were crossed. More specifically, the leaf herbivory treatment had three levels: insect herbivory (hereafter named "INSH"), artificial herbivory ("ARTH") and undamaged controls ("CONH") and it was performed on young plants grown from seeds that were previously assigned to each of the two levels of the demethylation treatment: control (hereafter named "CON") and azacytidine-treated ("AZA").

Germination and plant growth were conducted in a growth chamber (Aralab CLIMAPLUS 400) at Doñana Biological Station, with long-day (LD) conditions: 16 h of light at 22 ºC, and 8 h of darkness at 18 ºC, and 55% humidity. Chamber shelves were at a short distance (< 35 cm) to a combination of fluorescent cool-white and purple light tubes. Plants were regularly watered every 2 days during the germination period (the first 3 weeks), and every 3–5 days during the rest of the experiment. No fertilizer was applied during the whole experiment. In total, plants were grown during 14 weeks and first herbivory experiments were performed

when plants were between 7 and 8 weeks old, closely before flowering for late-flowering SE and at the time of flowering onset for early-flowering DE.

Seed demethylation treatment. We selected 48 well-formed brown seeds per study plant (hereafter families). In total, 240 seeds were used to begin this experiment (2 population x 2–3 mother per population x 48 seeds per mother family). Seeds were surface-sterilized with a 5% bleach solution, washed with distilled water, individually scarified using clean sandpaper and placed in distilled water for 48 h at room temperature. For the demethylation treatment a 100 mM stock solution of 5-azacytidine (Sigma A2385–100 mg) in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) was prepared, stored at −20 °C, and diluted in water to 0.25 mM just before treatment (AZA). This nucleoside analogue incorporates into the genome of proliferating cells during DNA synthesis and traps DNA methyltransferases, targeting them for degradation and resulting in genome-wide demethylation (Lopez et al., 2016). In late-flowering SE *T. arvense*, this concentration has been reported as optimum to induce changes in methylation levels without other nonspecific toxic effects (Burn et al., 1993). As a control, a mock solution of DMSO in water (3:97; v:v) was used (CON). Half of the seeds of each family were immersed, respectively in AZA and CON solutions during 48 h at 4 ºC in darkness.

Seeds were then individually sown on commercial soil (Sustrato Universal El Clavel de Martínez SL) on 10 cm diameter pots. Groups of 14–15 pots were placed in trays and were randomized by the provenance of the seeds (families and populations). After three weeks, all emerged seedlings were subjected to vernalization at 5 °C during 21 d, with short day conditions (6 h of light and 16 h of darkness, watering once a week), to shorten the period of vegetative growth and synchronize flowering. We applied this protocol to all study plants because vernalization was known to reduce DNA methylation in *A. thaliana* (Burn et al., 1993). Thus, if applied only to late-flowering plants we could blur the consequences of vernalization and demethylation treatments applied to different sets of plants. For not having enough seeds per family, we could not include further replicated study factors. After vernalization, all the seedlings were re-transplanted into different pots using an enriched soil brand (Sustrato Universal Gramoflor Blumenerde) because of unsuitable quality of the previous one. Seedlings were allowed to grow during 2–3 weeks under LD conditions before further treatment.

Herbivory treatments. When plants were 7–8 weeks old, we selected up to 12 similar sized plants from each family and demethylation treatment, and randomly assigned four of them to each of the three herbivory treatments. Due to reduced germination rates such design was barely possible for two families in late-flowering SE and three families in early-flowering DE. Altogether, we began this second part of the experiment with a total of 113 plants (46 from SE and 67 from DE, allocating 3–4 replicates per family in every group of treatment). In plants assigned to the ARTH treatment, simulated herbivory was induced by punching holes in two similar sized and well-developed leaves, and spraying a JA (Sigma J2500- 100MG) solution all over the leaves. JA was solubilized in ethanol, then diluted in distilled water to a 1 mM JA solution, and 0.1% triton-x 100 was added as a surfactant to increase penetration through the cuticle (Arnold and Schultz, 2002). In the INSH treatment, we selected two well-developed leaves of the plant and used small nylon mesh bags to individually encage two L4 instar larvae on each leaf. We let the larvae consume leaves for approximately 1–2 days, and then all the larvae were removed when 60–80% of each leaf was consumed. In the control treatment, two well-developed leaves were selected in each plant and sprayed with a control solution with the same composition as the JA solution except that it contained no JA. To ensure that all plants received a comparable amount of solution, each treated leaf was first sprayed with two pumps of a mechanical sprayer, and then the third pump was sprayed over the whole plant. After 10 days, a second bout of the same herbivory treatment was conducted on each plant to enable a priming effect and finally get a stronger and/or faster response (Rasmann et al., 2012; Mauch-Mani et al., 2017). Treated leaves of this second herbivory treatment were selected as close as possible to the first ones and from the same developed stage. During all the treatment phases, all plants including controls were covered with individual nylon mesh bags that were removed 48 h after the end of the treatment. Also, to avoid any interference of volatile compounds between plants with different treatments, plants subjected to either INSH or ARTH treatments were stored in two separate chambers with identical conditions as controls (germinator MiniDiGiTII, Rabider) in LD conditions for 48 h.

Sample collection. Unwounded leaves from all individuals were collected and immediately frozen in liquid nitrogen 24 h after second artificial herbivory or larvae removal. Vials were kept at −80 °C until further processing. The experiment was finished in total 14 weeks after seed sowing, when fruits were mature, and individuals started to become senescent. All the aboveground biomass of each plant, including senescent leaves, fruits and stems, was collected in individually labelled paper bags and placed in well-aerated room until measuring stem biomass, and fruit and seed numbers.

2.3. Data collection and sample processing

2.3.1. Global DNA cytosine methylation

For each individual an aliquot of 30 mg leaf frozen material was homogenized to a fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted using Bioline ISOLATE II Plant DNA Kit, which contain RNAse A to remove RNA from the samples, and quantified using a Qubit fluorometer 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). 100-ng aliquot of DNA extract was digested with 3 U of DNA Degradase PlusTM (Zymo Research, Irvine, CA, USA), a nuclease mix that degrades DNA to its individual nucleoside components. Digestion was carried out in a 40 μl volume at 37 °C for 3 h, and terminated by heat inactivation at 70 °C for 20 min. Three independent replicates of digested DNA per sample were initially processed to estimate global cytosine methylation more precisely; the number of replicates was increased for some individual samples to reduce inconsistencies; altogether 376 vials were processed in randomized order. Selective derivatization of cytosine moieties with 2-bromoacetophenone under anhydrous conditions and subsequent reverse phase high performance liquid chromatograph (HPLC) with spectrofluorimetric detection were conducted. The percentage of total cytosine methylation on each replicate was estimated as 100×5mdC/(5mdC + dC), where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2′-deoxycytidine and 2′-deoxycytidine, respectively (see Alonso et al., 2016 for further details).

2.3.2. Germination, seedling emergence and early developmental traits

Germination of field collected seeds from the two populations was monitored after planting for every two days during three weeks. After that time, germination was extremely rare. For each individual seed we recorded the dates of germination (radicle visible, i.e., at least 1 mm long), seedling emergence (both cotyledons fully opened) and appearance of the first two leaves (completely expanded). Time to seedling emergence and appearance of the first leaf for each seedling was calculated from date of sowing. Germination probability was calculated as the total number of seeds germinated from the total number of seeds planted after three weeks of sowing. Flowering phenology was monitored with two censuses per week during 8–9 weeks, after that all plants were in bloom. Plant height (in cm) at the onset of flowering was measured as the length from cotyledon insertion to the apex of the main inflorescence.

2.3.3. Individual size and fecundity

After harvesting, each plant was carefully separated into three different components: stems, leaves and reproductive organs (including fruits, seeds, perianth segments and bracts). Dry weight of each

component was separately determined after oven-drying plants to a constant mass during at least 48 h at 40 °C using a digital balance to the nearest 0.01 mg. The stem biomass constituted the biggest portion of total biomass and was selected as the most informative trait because some old dry leaves and dry fruits were eventually detached from plants at harvesting.

Fruit production was estimated by taking each raceme individually and all mesocarps and bare pedicels attached to the plant were counted as fruits. Additionally, ten fully developed and mature fruits from each plant were randomly selected, including always fruits produced both in the main flowering stem and in its lateral branches. For each fruit we counted the number of sound seeds (fully developed and well-shaped) and unripe seeds (shrunken and markedly smaller in size) and then mean seed number per fruit was calculated. The total mass of all the sound seeds produced per fruit was weighed collectively in a digital balance to the nearest 0.01 mg and the average seed mass for each fruit was then estimated as group mass / no. seeds.

2.3.4. Glucosinolates

For glucosinolate (GLS) analyses, leaf material of two individuals per group of treatment and mother ($N = 49$) was shock-frozen in liquid nitrogen and lyophilized. The material was weighed and used for extraction of GLS, following the protocol by Abdalsamee and Müller (2012). The dried material was extracted threefold with 80% methanol, adding *p*-hydroxybenzyl GLS (glucosinalbin, Phytoplan, Heidelberg, Germany) as internal standard at the first extraction. After centrifugation, supernatants were applied onto ion-exchange columns containing diethylaminoethyl (DEAE) Sephadex A25 (Sigma Aldrich, St.Louis, MO, USA) in 0.5 M acetic acid buffer, pH 5. Purified sulfatase was added to convert GSs to desulfoGLSs overnight. DesulfoGLSs were eluted in water and analyzed on a HPLC coupled to a DAD detector (HPLC-1200 Series, Agilent Technologies,Inc., Santa Clara, CA, USA). A gradient of water to methanol was used to elute desulfoGSs from a Supelcosil LC 18 column (3 μm, 150×3 mm, Supelco, Bellefonte, PA, USA). The gradient started at 5% methanol, which was kept for 6 min and then increased from 5 to 95% within 13 min with a hold at 95% for 2 min, followed by column cleaning. GLSs were identified based on their retention times and UV spectra in comparison to respective standards (Fahey et al., 2001). Peak areas were integrated at 229 nm and the concentration of each of the four identified GLS compounds was calculated in relation to sample dry mass, using the following glucosinolates response factors: 1 for 2-propenyl-glucosinolate (sinigrin), 0.95 for benzyl-glucosinolate (glucotropaeolin), and 0.26 for both indol-3-ylmethyl-glucosinolate (glucobnsicin) and 4-methoxy-indole-3-yl glucosinolate (4-methoxyglucobrassicin). Total GLS concentration was estimated by summing all the concentrations for the four measured GLS compounds.

2.4. Data analyses

All statistical analyses were carried out using the R environment (R Development Core Team, 2020). Data distributions were visually inspected and absence of obvious outliers was confirmed. As a rule, we used linear or generalized linear mixed models to assess the sign, magnitude and statistical significance of the effects of demethylation and herbivory (fixed factors), accounting for the appropriate grouping random effects as defined in lmer and glmer functions of the lme4 library (Bates et al., 2015). Although a three-way factorial analysis that included population as a fixed factor would have been statistically more robust, we split the dataset by population to improve resolution because two reasons: firstly, graphical exploration of data distributions and model outputs suggested contrasting patterns that were not detected as significant interactions due to large variances and reduced sample sizes within groups, constraining greatly our statistical power; and secondly, some of the variables were far from a continuous normal distribution, actually they were nearly bimodal or had important discontinuities between populations, and therefore splitting the data improve models adjustment. Below we describe the models applied to each response variable type, which passed model diagnostic tests and were selected according to lower AIC (Bolker, 2015).

The model for the replicated global DNA cytosine methylation data was applied using *lmer*, it included demethylation, herbivory (with three levels) and their interaction as fixed effects, and plant as a random effect to correctly identify all replicates of the same sample. For germination analyses, every germinated seed was coded as 1 and every non-germinated seed coded as 0. Germination probability was modelled in *glmer* as a binomial process using logit as the link function, the model included demethylation as the only fixed factor, and families (seeds coming from the same mother plant) as a random effect, which ensured that any possible influence of family heterogeneity in genetic background were adequately accounted for (i.e., blocked; Mead, 1988). Count variables such as days to seedling emergence and days to appearance of the first leaf were modelled as Poisson processes in *glmer* with logit link function. As these variables were measured before herbivory, models included only demethylation as fixed factor and families as random effect. Total fruit number was modelled also as a Poisson process with demethylation and herbivory as fixed factors and families as random effect using logit link function. Stem biomass, average seed number and average seed mass were modelled as Gaussian response variables in *lmer* including the same fixed and random factors.

We visualized glucosinolates profiles as nonmetric multidimensional scaling (NMDS) plots using Bray-Curtis dissimilarity index matrices. Difference in total glucosinolate profiles was analysed for herbivory and demethylation treatment using ANOSIM (function adonis2) with 9999 permutations in *vegan* (Oksanen et al., 2020). Further, variance in concentration of total glucosinolates, sinigrin and 4-methoxyglucobrassicin was analysed with *lmer*, models included demethylation and herbivory as fixed factors, and families as a random effect.

For each analysis, significance of fixed factors and their interaction was tested using the function Anova (package car; Fox and Weisberg, 2018), with type II sum of squares and the Kenward-Roger approximation to calculate the residual degrees of freedom. Estimated marginal means and associated confidence intervals for the response variable at each factor level were obtained with the 'emmeans' function of the 'emmeans' library (Lenth, 2018). Post hoc analyses were done by conducting multiple pairwise comparisons of the estimated marginal means with Tukey adjustment. Marginal means from generalized linear models were back transformed to the original scale of measurement. Mean ± SE will be shown unless otherwise stated.

3. Results

3.1. Global DNA cytosine methylation

The percentage of cytosine methylation in leaf DNA of untreated *T. arvense* adult plants averaged 15.1% (± 0.20, standard error). DNA methylation ranged between 13.8 and 16.5% in control individuals ($N = 15$) and between 13.0 and 16.6% in the full dataset ($N = 91$). The results of the full factorial ANOVA for global DNA methylation, stem biomass, reproductive traits and chemical defences are provided in the supplementary material Table S1.

The effect of demethylation and herbivory treatments on leaf DNA methylation at adult stage varied among the two study populations (Fig. 1). In late-flowering SE, leaf DNA methylation did not differ between CON and AZA treated plants. In contrast, leaf DNA methylation was significantly affected by herbivory ($\chi^2 = 6.52$, df = 2, $P = 0.038$), methylation being higher in CONH plants (15.3% ± 0.2) and more significantly reduced in plants assigned to ARTH (14.5% ± 0.2) than those consumed by insect herbivores (14.8% ± 0.2). In early-flowering DE, leaf DNA of AZA treated plants had slightly lower methylation levels than the CON plants (14.4% ± 0.2 *vs.* 14.9% ± 0.2, respectively; $\chi^2 = 3.48$, df = 1, $P = 0.06$), whereas herbivory did not affect DNA methylation levels of collected leaves.

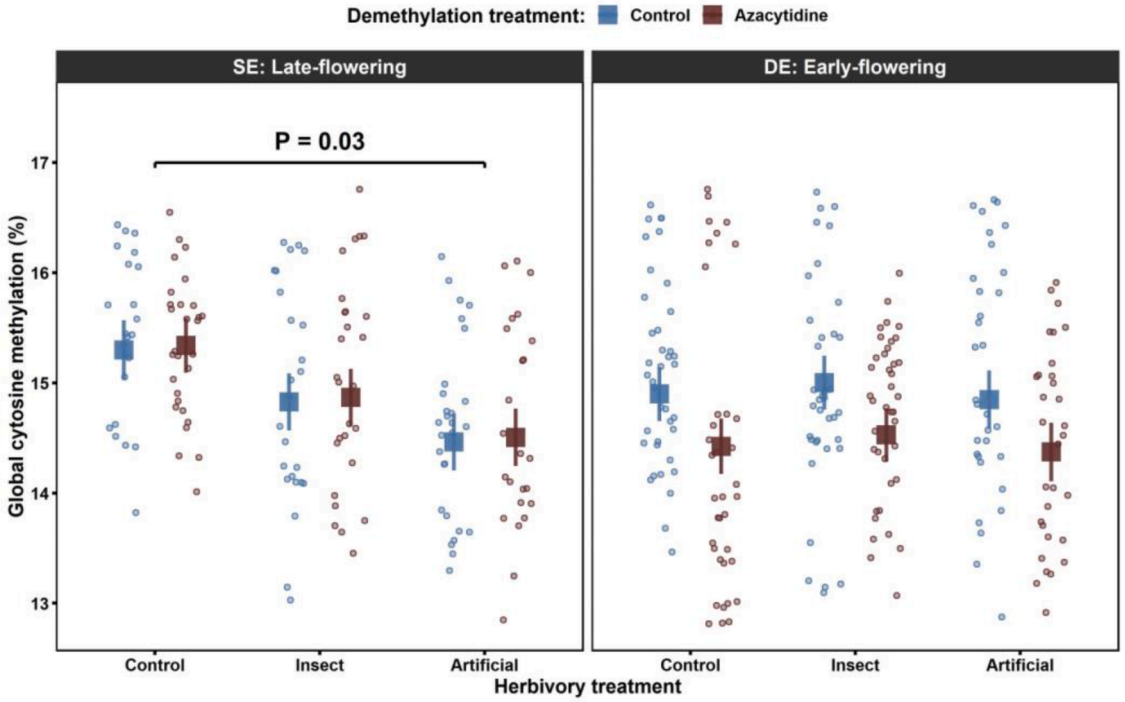

Fig. 1. Variation in global cytosine methylation levels for plants ($N = 374$ data points, 3–6 replicates in 91 plants) of the two study populations (late-flowering SE and early-flowering DE), the three levels of our herbivory treatment (control, insect and artificial herbivory) and the two levels of our demethylation treatment (controls –in dark blue-, and azacytidine –in dark red-). Solid squares with bars show estimated marginal means and standard errors (from linear mixed-effects models, with the interaction "herbivory×demethylation" effect included, and "plant" as a random effect) across each level of the two treatments in the two study populations. The only contrast with significant *P*-value obtained for the comparison between artificial herbivory and control plants in late-flowering SE population, is shown. In early-flowering DE population, the contrasts obtained for every azacytidine vs control comparison were marginally significant ($P = 0.06$).

3.2. Germination, seedling emergence and early developmental traits

Germination probability was similar in the two study populations (0.732 ± 0.06 and 0.764 ± 0.05, for SE and DE, respectively). Demethylation treatment did not affect it (SE: $\chi^2 = 0.21$, df = 1, $P = 0.646$; DE: $\chi^2 = 1.01$, df = 1, $P = 0.314$).

On average, seedlings treated with AZA emerged one day later and needed 2.5 days more to produce their first leaf than CON seedlings (Table 1). But, the effect of the demethylation treatment was slightly different in the two populations. The effect of AZA was not statistically significant for seedling emergence of the late-flowering SE plants ($\chi^2 = 0.75$, df = 1, $P = 0.39$) and showed only a near significant effect on first leaf development time ($\chi^2 = 3.15$, df = 1, $P = 0.060$). Whereas, in early-flowering DE plants the delay was statistically significant for both seedling emergence ($\chi^2 = 5.36$, df = 1, $P = 0.020$) and the appearance of the first leaf ($\chi^2 = 9.76$, df = 1, $P = 0.001$).

At the onset of flowering, just before herbivory treatment, again only in early-flowering DE plants the demethylation treatment produced a significant effect, being AZA treated plants almost 2 cm shorter than control ones ($\chi^2 = 4.45$, df = 1, $P = 0.04$). The same treatment had no effect on plant height of late-flowering SE plants (Table 1).

Table 1
Time (in days) from seed sown to seedling emergence and to the appearance of the first two leaves (first leaf emergence), and height (in cm) of plants at the onset of flowering (height at flowering) from control and 5-azacytidine treated seeds of the two *T. arvense* populations studied (late-flowering SE and early-flowering DE). Values shown are model estimated marginal means and their standard errors (in brackets). Only significant differences ($P < 0.05$) between controls and azacytidine treated plants are shown (*P* values).

Trait	Population	Control	Azacytidine	*P* value
Seedling emergence (d)	SE	5.85 (0.72)	6.50 (0.76)	
	DE	5.00 (0.51)	6.35 (0.60)	0.02
First leaf emergence (d)	SE	12.25 (0.78)	14.34 (0.79)	
	DE	14.96 (0.81)	18.20 (0.92)	0.002
Height at flowering (cm)	SE	13.00 (0.67)	12.80 (0.67)	
	DE	14.60 (0.83)	13.00 (0.82)	0.04

3.3. Final stem biomass

Final stem biomass in untreated (non-demethylated and undamaged) plants of late-flowering SE was on average two-fold higher than those of early-flowering DE (1.06 *g* ± 0.04 and 0.46 *g* ± 0.05, for SE and DE, respectively), with almost no overlapping in figures obtained for each population (Fig. 2), and this difference was statistically significant (*t*-test: $t = 8.03$, df = 18, $P < 0.001$).

In late-flowering SE, variance in the final stem biomass was explained by a significant effect of herbivory ($\chi^2 = 10.25$, df = 2, $P = 0.005$), there was no effect of demethylation and the interaction between main factors was also not significant (Table S1). Specifically, ARTH treated plants had a strong and significant reduction in the stem biomass when compared to CONH and INSH plants, regardless of their initial seed demethylation treatment (Fig. 2). Further, stem biomass was higher in AZA treated plants without herbivory but lower in those which were subjected to insect herbivory.

In early-flowering DE, there was no significant effect of demethylation or herbivory treatment or their interaction on the final stem biomass (Fig. 2; Table S1).

3.4. Total fruit number

Thlaspi arvense plants grown in pots produced between 57 and 176 fruits for untreated plants ($N = 20$) and ranged between 23 and 198 in the full data set ($N = 112$). Total fruit number in untreated plants of late-flowering SE was on average higher by ten fruits than those of early-flowering DE (108.75 ± 35.59 and 98.66 ± 29.15, for SE and DE,

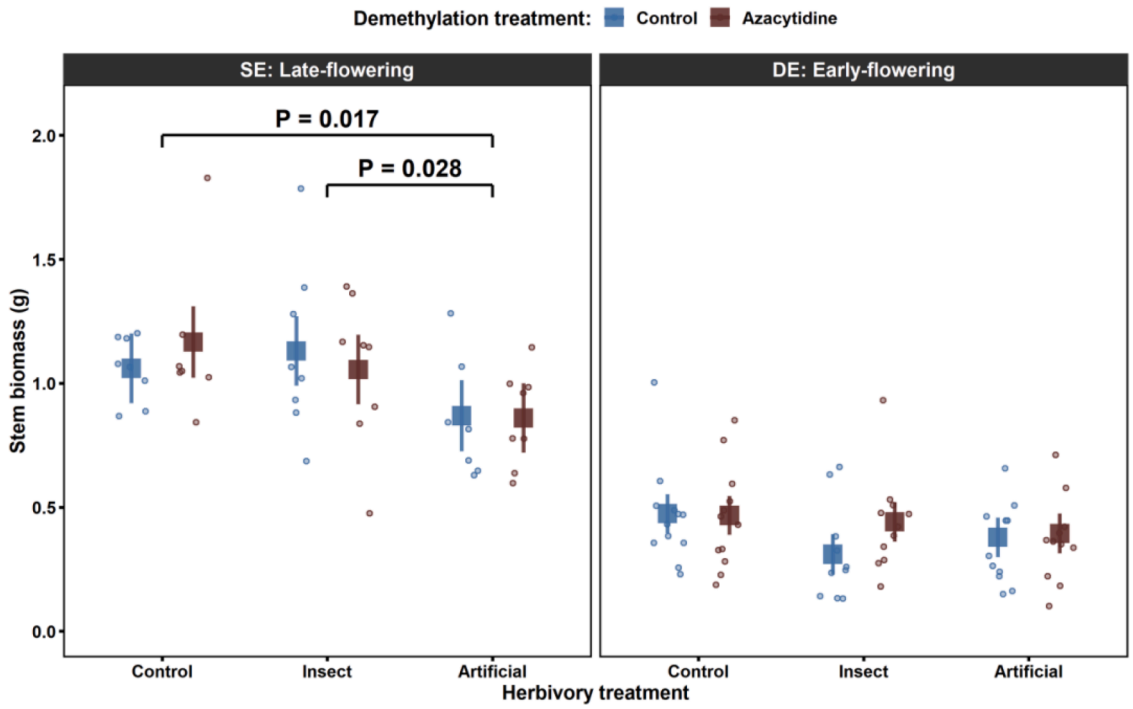

Fig. 2. Variation in stem biomass for plants ($N = 113$ individual plants) of the two study populations (late-flowering SE and early-flowering DE), the three levels of our herbivory treatment (control, insect and artificial herbivory) and the two levels of our demethylation treatment (controls –in dark blue-, and azacytidine –in dark red-). Solid squares with bars show estimated marginal means and standard errors (from gaussian general linear mixed-effects models, with the interaction "herbivory×demethylation" effect included, and "plant" as a random effect) across each level of the two treatments in the two study populations. Contrasts with significant *P*-values ($P < 0.05$), obtained only for control vs artificial and insect vs artificial comparisons in late-flowering SE population, are also shown.

respectively).

In late-flowering SE, variance in total fruit number was explained by a significant effect of herbivory treatments ($\chi^2 = 13.36$, df = 2, $P < 0.001$). Demethylation and the interaction between the two factors were not significant (Table S1). In particular, plants that experienced INSH produced a significantly lower number of fruits than plants that experienced no herbivory and ARTH, although these differences seem to be significant only for plants without AZA treatment (CON) (Table 2).

In early-flowering DE, total fruit production differed for demethylation ($\chi^2 = 9.56$, df = 1, $P < 0.001$) and herbivory treatments ($\chi^2 = 5.89$, df = 2, $P = 0.052$). The AZA treated plants produced more fruits, the impact being stronger in both ARTH and INSH plants (Table 2).

3.5. Average seed number per fruit

The average number of seeds per fruit ranged between 4.5 and 11.6 for untreated individuals ($N = 20$) and it was between 3.1 and 11.7 in the full data set ($N = 112$). Untreated late-flowering SE plants produced more seeds per fruit than early-flowering DE plants (9.81 ± 1.87 and 6.89 ± 1.05, for SE and DE, respectively; *t*-test: $t = 3.99$, df = 9.96, $P = 0.002$).

In late-flowering SE, the demethylation treatment had a significant effect on average seed number per fruit ($\chi^2 = 7.45$, df = 1, $P = 0.006$). Herbivory and the interaction between the two factors were not significant (Table S1). AZA treated plants produced significantly lesser number of seeds per fruit and this effect was particularly large in the group of undamaged controls (Table 2).

In early-flowering DE, average seed number per fruit varied significantly with demethylation ($\chi^2 = 12.45$, df = 1, $P<0.001$), herbivory treatment ($\chi^2 = 7.88$, df = 2, $P = 0.02$) and there was no significant interaction between the two experimental factors (Table S1). In this case, AZA treated plants produced higher number of seeds per fruit than their controls in all the three herbivory treatments but the effect was particularly large in artificially damaged individuals (Table 2).

3.6. Average seed mass

In *T. arvense,* average seed mass ranged between 0.65 mg and 1.11 mg in untreated individuals ($N = 20$) and it ranged between 0.09 mg and 1.32 mg for entire data set after removing individuals with outlier values ($N = 113$). Average seed mass in untreated plants was similar in the two study populations (0.82 ± 0.08 mg and 0.90 ± 0.15 mg, for SE and DE, respectively).

In late-flowering SE, a significant demethylation treatment effect was observed ($\chi^2 = 3.88$, df = 1, $P = 0.048$). But herbivory treatment or interaction between two factors had no significant effect on average seed mass (Table S1). Initial demethylation reduced the average seed mass per fruit in all the levels of our herbivory treatment, this effect was particularly large in insect consumed plants (Table 2).

In early-flowering DE, a significant and stronger effect of demethylation ($\chi^2 = 10.81$, df = 1, $P < 0.001$) and a near significant interaction between the two experimental factors ($\chi^2 = 5.61$, df = 2, $P = 0.06$) were found. In contrast to SE, the AZA treated plants produced heavier seeds than their controls, and again this effect was stronger for insect consumed plants (Table 2).

3.7. Leaf glucosinolates

Total glucosinolate concentration in unwounded leaves collected 24 h after the second herbivory event varied between 1.2 μmol.g−1 d.w. to 1.7 μmol.g−1 d.w. in untreated individuals ($N = 8$) and it ranged between 1.2 μmol.g−1 d.w. to 39.4 μmol.g−1 d.w. for the entire data set ($N = 49$). The glucosinolate profiles were studied by NMDS using the concentrations of the two indole and two aliphatic compounds that were most abundant (Fig. 3). The ANOSIM test showed that herbivory treatments exhibited a significant effect for late-flowering SE plants ($P < 0.005$) and not significant for early-floweirng DE plants ($P = 0.07$). The effect of demethylation was not statistically significant to explain variance in multidimensional glucosinolate profile. In late-flowering SE,

Table 2
Total fruit number, average seed number per fruit and average seed mass (mg) produced by control and 5-azacytidine treated plants after each of the three levels of herbivory treatment (undamaged control, insect herbivory, artificial herbivory) of the two *T. arvense* populations (late-flowering SE and early-flowering DE). Values shown are model estimated marginal means and their standard errors (in brackets). Only significant differences ($P < 0.05$) for each of the contrasts between controls and 5-azacytidine treated plants are shown (*P* values). Values with different letters indicate significant differences ($P < 0.05$) between the three levels of the herbivory treatment for each population and each level of demethylation treatment.

Trait	Population	Herbivory	Control	Azacytidine	*P* value
Total fruit number	SE	Control	106.75 (15.03)[a]	103.35 (14.62)	
		Insect	88.59 (12.55)[b]	95.83 (13.54)	
		Artificial	100.62 (14.25)[a,b]	94.97 (13.42)	
	DE	Control	99.85 (7.76)	104.30 (8.15)	
		Insect	90.37 (7.12)	99.58 (7.87)	0.032
		Artificial	95.06 (7.49)	104.67 (8.18)	0.027
Average seed number	SE	Control	9.81 (0.78)	7.01 (0.84)	0.018
		Insect	8.74 (0.78)	7.00 (0.78)	
		Artificial	9.00 (0.84)	8.21 (0.78)	
	DE	Control	6.89 (0.39)	7.58 (0.39)	
		Insect	5.69 (0.45)	6.63 (0.41)	
		Artificial	6.04 (0.41)	7.84 (0.41)	0.002
Average seed mass (mg)	SE	Control	0.82 (0.07)	0.70 (0.08)	
		Insect	0.92 (0.07)	0.74 (0.07)	
		Artificial	0.76 (0.08)	0.70 (0.07)	
	DE	Control	0.90 (0.08)[a]	0.99 (0.08)	
		Insect	0.71 (0.08)[b]	1.02 (0.08)	0.0003
		Artificial	0.87 (0.08)[a]	0.94 (0.08)	

variance in total GLS concentration was significantly explained by demethylation ($\chi^2 = 5.14$, df = 1, $P = 0.02$), herbivory ($\chi^2 = 40.37$, df = 2, $P < 0.001$) and interaction ($\chi^2 = 7.73$, df = 2, $P = 0.02$). The total GLS concentration was higher in leaves of ARTH plants, intermediate in INSH and significantly lower in undamaged CONH plants, specifically for plants which had undergone seed-stage AZA treatment (Table 3). In early-flowering DE, no significant effect of herbivory or demethylation was observed

The aliphatic GLS sinigrin was predominant and accounted for more than 98% of total GLS amount. The effect of experimental treatments on concentration of sinigrin, and the most abundant indole glucosinolate, 4-methoxyglucobrassicin, were also analyzed. In late-flowering SE, variance in leaf sinigrin concentration among individual plants was significantly explained by herbivory ($\chi^2 = 38.95$, df = 2, $P < 0.001$), demethylation ($\chi^2 = 5.06$, df = 1, $P = 0.02$), and the interaction between the two experimental factors ($\chi^2 = 7.77$, df = 2, $P = 0.02$). In regards of herbivory, we found that the sinigrin concentration in leaves of ARTH plants were on average 3–10 fold higher than in leaves of plants assigned to INSH and CONH (Table 3). The AZA treatment led to a higher concentration of sinigrin, although the difference was only significant in leaves of ARTH plants (Table 3). In early-flowering DE, no significant effect of herbivory or demethylation was observed.

Variation in concentration of the most abundant indole GLS, 4-methoxyglucobrassicin was only explained by the experimental treatments in SE population. The effects of herbivory treatment ($\chi^2 = 6.20$, df = 2, $P = 0.04$) and interaction of demethylation and herbivory ($\chi^2 = 6.18$, df = 2, $P = 0.04$) were statistically significant in late-flowering SE (Table 3). Leaves of AZA treated plants had a significantly lower concentration of 4-methoxyglucobrassicin after artificial herbivory but a higher concentration in those plants consumed by insects (Table 3). In early-flowring DE, similar to the response of other GLS, the effects of experimental treatments did not significantly explained variance in concentration of 4-methoxyglucobrassicin (Table 3).

4. Discussion

In this study, we combined experimental herbivory with experimental demethylation in *T. arvense* aiming to elucidate the role of epigenetic variation on short-term defense and plant performance of this annual herb under controlled conditions. This approach has been successfully applied to other study systems although mainly to analyze the responses to abiotic stress (see e.g., Latzel et al., 2012; Herman and Sultan, 2016). To better interpret the obtained results it is important to emphasize that the two study populations exhibited contrasting phenotypes (Supplementary material, S1) and belonged to different genetic clusters within Europe (Galanti et al., 2022). Phenotypic differences were reduced because all individuals were vernalized as seedlings and, thus, SE plants flowered earlier than usual for the late-flowering morph (at week 8–9 *vs*. 18–21 weeks reported by Moser et al., 2009), although still about two weeks later than the DE plants. As expected, early-flowering DE plants had longer stems at flowering, reached lower final size (stem biomass) and tended to produce less fruits and seeds. Thus, although our results are preliminary and need to be confirmed with further investigations including more populations of the two flowering types and more families within populations, they suggest that the genetic background of the assayed plants and even variance between individuals of the same family can affect the responses to experimental treatments (see also Herman and Sultan, 2016). In the following paragraphs, we discuss the main observed effects of the two treatments on the two provenances and further steps required to better interpret their contrasting responses.

4.1. Effects of seed demethylation treatment

Use of 5-azacytidine in earlier studies showed impaired plant growth (see e.g., Finnegan et al., 1996; Fieldes and Amyot, 1999; Kondo et al., 2006). Our 48 h treatment did not affect seed germination and was applied with a moderate concentration to avoid survival-related problems and any serious developmental effects (see also, Burn et al., 1993; Akimoto et al., 2007; Bossdorf et al., 2010). Importantly, the treatment induced a moderate, statistically significant reduction in global DNA cytosine methylation only in plants from the early-flowering DE population and regardless of subsequent herbivory treatment. Such results were somehow unexpected according to previous studies conducted only in late-flowering plants (Burn et al., 1993). The novelty of this result stands in showing that the effect of seed-stage experimental demethylation can last to adulthood in short-lived plants, and not only in seedling leaf DNA as assessed previously (Alonso et al., 2017; Griffin et al., 2016; Puy et al., 2018). Also, the magnitude of the effect varied with seed provenance and this might be due to small differences in permeability of seed coat and the physiological status of *T. arvense* seeds from different populations that would condition penetrance of 5-azacytidine during the short period of seeds imbibition we applied (see also Burn et al., 1993). Alternatively, we cannot discard that restoration of methylation marks in adult plants after 12–14 weeks since

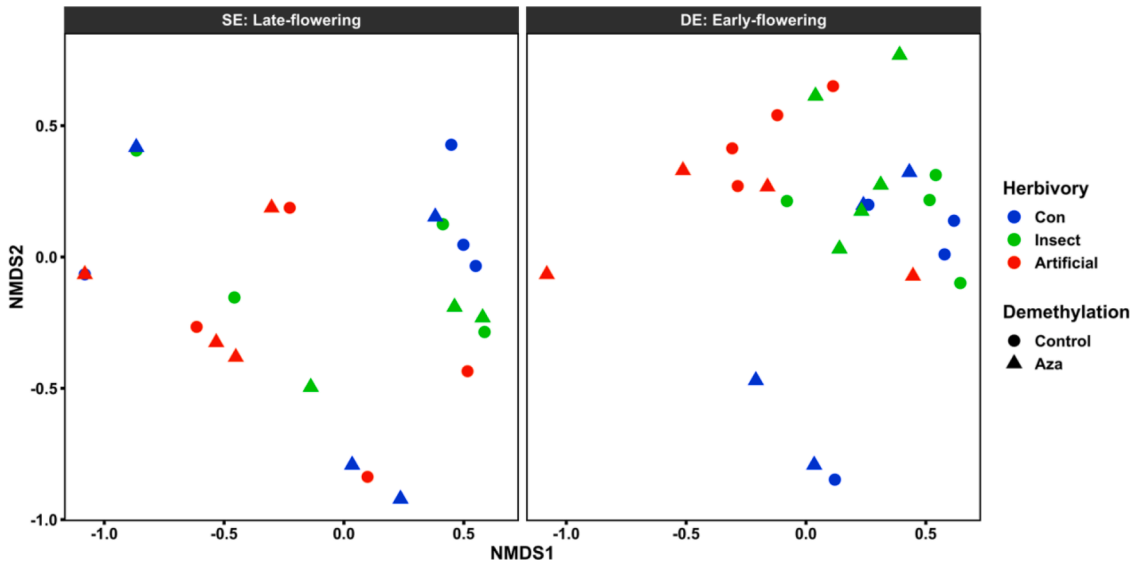

Fig. 3. Nonmetric multidimensional scaling (NMDS) plots for glucosinolate profiles in *Thlaspi arvense* leaves in the two study populations (late-flowering SE and early flowering DE, $N = 49$ individual plants). Colors denote herbivory treatments (controls -in blue-, insect herbivory -in green-, and artificial herbivory -in red-) and shapes denote demethylation (CON -triangle- and AZA -round-). The composition and concentration of the main four glucosinolate were analysed to distinguish their clustering patterns among herbivory and demethylation treatments.

Table 3
Concentrations (expressed in μmol/g of dry weight) of total glucosinolates (Total GLS), 2-propenyl-glucosinolate (sinigrin), and 4-methoxy-indole-3-yl glucosinolate (4-methoxyglucobrassicin) detected in samples collected from leaves of control and 5-azacytidine treated plants of the two *T. arvense* populations (late-flowering SE and early-flowering DE) 24 h after the end of our herbivory trial. Values shown are model estimated marginal means and their standard errors (in brackets). Only significant differences ($P < 0.05$) for each of the contrasts between controls and 5-azacytidine treated plants are shown (P values). Values with different letters indicate significant differences ($P < 0.05$) between the three levels of the herbivory treatment for each population and each level of demethylation treatment.

Trait	Population	Herbivory	Control	Azacytidine	*P* value
Total GLS	SE	Control	1.68 (2.08)^{a,b}	1.55 (2.08)^{a}	
		Insect	0.97 (2.26)^{a}	3.20 (2.08)^{a}	
		Artificial	7.35 (2.26)^{b}	16.28 (2.52)^{b}	0.004
	DE	Control	1.24 (4.32)	2.04 (4.32)	
		Insect	5.82 (4.32)	4.80 (4.07)	
		Artificial	7.82 (4.75)	10.87 (4.75)	
Sinigrin	SE	Control	1.64 (2.14)	1.50 (2.14)^{a}	
		Insect	0.88 (2.31)	3.07 (2.14)^{a}	
		Artificial	7.18 (2.31)	16.25 (2.58)^{b}	0.004
	DE	Control	1.20 (4.32)	2.03 (4.32)	
		Insect	5.77 (4.32)	4.78 (4.07)	
		Artificial	7.81 (4.75)	10.82 (4.75)	
4-methoxyglucobrassicin	SE	Control	0.03 (0.04)	0.04 (0.04)	
		Insect	0.06 (0.04)	0.13 (0.04)	
		Artificial	0.09 (0.04)	0.04 (0.04)	
	DE	Control	0.03 (0.01)	0.01 (0.01)	
		Insect	0.03 (0.01)	0.02 (0.01)	
		Artificial	0.01 (0.03)	0.04 (0.02)	

demethylation might change with provenance (Fieldes and Amyot, 1999; Kumpatla and Hall, 1998). Advanced methylome analyses based on deep sequencing after bisulfite conversion (see e.g., Becker et al., 2011; Colicchio et al., 2018) would be required to fully understand the observed differences, e.g. a null change in global methylation can arise from similar frequency of additions and drops of methyl groups to cytosines in different genomic locations.

Immediately after demethylation, time to first leaf appearance delayed similarly for azacytidine treated plants from the two plant-types supporting that the treatment indeed altered the initial development in all of them (see also Burn et al., 1993; Finnegan et al., 1996; Kondo et al., 2006). The phenotypic response of the two populations to azacytidine treatment diverged after 7–8 weeks shortly after vernalization. Azacytidine reduced early growth more strongly in early-flowering DE plants, that were shorter than their control relatives immediately before herbivory treatments started. However, at flowering onset, the effect of azacytidine did not significantly reduce plant height in the late-flowering SE plants, partially due to their different plant architecture of large rosette, but was still evident in early-flowering DE plants. Such finding suggests that late-flowering plants were able to recover faster from the initial delayed growth.

4.2. *Effects of herbivory treatment and interactions with seed demethylation*

Our herbivory treatments were repeated twice to prime plants and elicit a stronger and fast defense response (Mauch-Mani et al., 2017; Sobral et al., 2021). However, we did not analyze the priming effect itself. We searched for the molecular systemic consequences in unwounded leaves collected after 24 h of the second event that could be

detected as changes in DNA global methylation levels (Kellenberger et al., 2016), increased glucosinolate concentration (Textor and Gershenzon, 2009) or both. The herbivory simulation treatment included JA-spraying, a plant hormone involved in regulation of plant growth and defense (Züst and Agrawal, 2017) whose exogenous application is able to increase glucosinolates concentration in Brassicaceae plants and reduce subsequent insect consumption (Fritz et al., 2010; Jeschke et al., 2017; Kellenberger et al., 2016; Textor and Gershenzon, 2009). Accordingly, our artificial herbivory treatment increased sinigrin concentration. Further, it decreased final size (stem biomass) of late-flowering SE plants supporting a negative impact on plant performance that has not been frequently reported in studies that used just hormone application in other Brassicaceae species (Van Dam et al., 2004). This indicates that JA application together with a mild defoliation, induced the jasmonate cascade and changed the growth–defense prioritization more strongly than *P. brassicae* consumption in late-flowering SE plants (Züst and Agrawal, 2017). In regards to reproductive output, previous studies reported larger effects on seed mass and seed production after mechanical leaf defoliation conducted at flowering time compared to earlier and later treatments (Akiyama and Ågren, 2012; Barto and Cipollini, 2005). In our study, artificial herbivory did not alter fruit or seed number likely due to lower defoliated surface and performance at an earlier developmental stage than the referred studies.

Interestingly the magnitude of the effects of insect and artificial herbivory on leaf DNA methylation and glucosinolate concentration, and their interaction with previous seed demethylation treatment varied with plant-type and were not significant in the early-flowering type. In the late-flowering SE population, that did not show differences in global DNA cytosine methylation after AZA treatment, DNA global methylation level of artificially damaged and insect-consumed plants were reduced significantly compared to undamaged control plants. Furthermore, an overall upsurge of glucosinolates was observed after herbivory in this population, although the effect was somehow herbivore-type specific and varied with demethylation treatment. Sinigrin, the most abundant aliphatic glucosinolate, increased more in artificially damaged plants, whereas insect eaten plants got higher concentration of indole glucosinolates such as 4-methoxyglucobrassicin, when treated with AZA at seed-stage. Furthermore, seed stage demethylated individuals undergoing artificial herbivory treatment had the highest sinigrin concentration, suggesting that DNA demethylation can regulate the jasmonate signaling cascade towards sinigrin biosynthesis (Textor and Gershenzon, 2009). This is relevant for commercial purposes because sinigrin is a precursor of mustard oil glucoside in seeds of *T. arvense* (Warwick et al., 2002) and we found surge in sinigrin after demethylation and artificial herbivory is better predicted for late-flowering SE plants. In the early-flowering DE plants, global DNA methylation did not differ between any of the two herbivory treatments and controls. On average, artificial but not insect herbivory led to higher leaf glucosinolates concentration, despite the observed large variance among treated individuals from this population. Again, advanced methylome analyses could help to elucidate whether the observed population specific responses are mainly due to genetic divergence (see e.g., Aller et al., 2018 for glucosinolate production), and/or epigenetic variation among them (see e.g., Latzel et al., 2012 for response to JA).

As regards plant performance, previous herbivory studies on the Brassicaceae family have showed contrasting results for artificial defoliation, hormone application and insect herbivore damage (Agrawal, 2000b and references therein; Kellenberger et al., 2016; Sotelo et al., 2014; Tucker and Avila-Sakar, 2010). Some earlier studies also showed reduction in seed production when insects consume leaf of young plants and suggested that tolerance levels increase from earlier to later developmental stages in crops and Brassicaceae plants (Boege et al., 2007; Sobral et al., 2021; Tucker and Avila-Sakar, 2010). Here, we have found contrasting responses to herbivory between the two study plant-types, similar to previous studies conducted with several accessions or provenances in other Brassicaceae (Manzaneda et al., 2010; Tucker and Avila-Sakar, 2010). Although further studies with more populations and families are needed before more robust conclusions can be drawn, our findings suggest that the two flowering ecotypes of *T. arvense* may have evolved different anti-herbivore strategies. We hypothesize that the early-flowering ecotype, that has a fast-growing cycle, may be more tolerant against herbivores since after damage plants did not alter their chemical defenses but tended to increase reproductive output. Whereas, plants of the late-flowering ecotype, that need much more time to complete its growing-cycle, could be more resistant and less tolerant to herbivory after damage as they tended to invest more resources in increasing their chemical defenses while reducing seriously their growth and reproduction.

In our study, we can speculate also that despite being conducted on the same dates, both the priming and herbivory treatments reached plants at different stage of their life-cycle, more close to bolting in early-flowering DE type, and that could reduce the impact of insect herbivory (see Sobral et al., 2021 for an analysis of age effect). Such finding emphasizes the relevance of using multiple provenances to gain generalization in understanding plant responses to herbivory, and the lack of studies addressing so. Moreover, seed-stage demethylation treatment altered fruit production and seed mass after herbivory suggesting that the two treatments had contrary effects on the two plant-types. In particular, late-flowering SE plants that were azacytidine treated produced significantly less number of seeds per fruit and smaller seeds. In early-flowering DE, plants treated with 5-azacytidine at seed-stage tend to produce more fruits, more seeds per fruit, and heavier seeds and some of the differences become even larger in plants experiencing herbivory. As long as late-flowering winter type of *T. arvense* is currently emerging as a new winter biofuel crop (Sedbrook et al., 2014; Dorn et al., 2015, 2018; García Navarrete et al., 2022), our findings could be relevant somehow for future research towards improvement of seed yield and reduction of glucosinolate content in potential new crop varieties.

5. Conclusions

Overall, this study illustrates the importance of DNA methylation variation in plant performance and short-term chemical defense after herbivory, supports experimental demethylation as a useful approach to investigate epigenetic regulation of plant-herbivory interactions and reveals the value of including different modes of herbivory and plant provenances to avoid oversimplification. According to our initial predictions we can conclude: i. Seed-stage demethylation is suitable to alter DNA methylation levels in leaves of reproductive adult plants of *T. arvense,* although the magnitude of the effect can vary between populations of origin and flowering ecotypes. A longer treatment could perhaps produce stronger effects. ii. Herbivory had different effects depending on provenance. In SE late-flowering plants it increased glucosinolates concentration, and reduced final size and fruit production. However, the effects were non-significant in DE early-flowering type except for seed production. iii. Herbivory reduced DNA methylation only in plants of late-flowering SE type, the effect being stronger for the artificial treatment. iv. For early-flowering DE type demethylation increased reproductive output mainly in plants experiencing herbivory somehow reducing its detrimental effect (i.e., increased tolerance). Such effect was not observed, however, in late-flowering plants, in which the two treatments reduced reproductive output. Altogether, such findings indicated that variation in DNA methylation had subtle interactions with plant response to short-term herbivory and the responses depend largely on plant ecotype associated to geographic origin, genetic background and the life-cycle phenology. Deeper methylome and transcriptome analyses need to be conducted for a more comprehensive understanding of molecular epigenetic mechanisms that regulate plant responses to herbivory.

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CRediT authorship contribution statement

A. Niloya Troyee: Methodology, Data curation, Writing – original draft, Visualization, Investigation. **Mónica Medrano:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Caroline Müller:** Data curation, Formal analysis, Writing – review & editing. **Conchita Alonso:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing 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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.flora.2022.152106.

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