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Improved demethylation in ecological epigenetic experiments: Testing a simple and harmless foliar demethylation application

RNDr. Thesis

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Annotation:

Spray application of 5-azacytidine on established plant seedlings was tested for its demethylating efficiency. It represents a novel method for plant experimental demethylation with a potentially lower negative impact on plant development compared to the traditional application of the demethylating agent through germination of seeds in its solution. Further, the 5-azacytidine spray application was used in practice to erase the epigenetic memory in offspring of *Taraxacum brevicorniculatum* plants from different competitive conditions. The impact of parental competition on the juvenile phenotype was estimated by measuring growth related traits, while the experimental demethylation allowed for evaluating the significance of DNA methylation marks in bioticaly induced transgenerational effects in *T. brevirorniculatum*.

Declaration [in Czech]:

Prohlašuji, že svoji rigorózní práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Hana Dvořáková

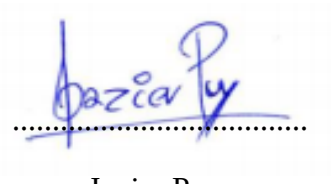
Declaration of co-author:

Hereby I declare that Hana Dvořáková made a significant contribution to the following article:

Puy J, Dvořáková H, Carmona CP, de Bello F, Hiiesalu I, Latzel V. Improved demethylation in ecological epigenetic experiments: Testing a simple and harmless foliar demethylation application. *Methods Ecol Evol.* 2018; 9:744–753.

I agree that the order of the article authors corresponds with the amount of work spent in its preparation. Hana Dvořáková conducted the Experiment 1 of the paper (testing an improved demethylation method), including participation on processing the samples by methods of molecular biology. Hana also participated significantly on writing the manuscript and literature searching.

In summary, I consider my and Hana's efforts on this article to be almost equal.



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Javier Puy

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RESEARCH ARTICLE

Methods in Ecology and Evolution BRITISH ECOLOGICAL SOCIETY

Improved demethylation in ecological epigenetic experiments: Testing a simple and harmless foliar demethylation application

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Abstract

1. Experimental demethylation of plant DNA enables testing for epigenetic effects in a simple and straightforward way without the use of expensive and laborious DNA sequencing. Plants are commonly demethylated during their germination with the application of agents such as 5-azacytidine (5-azaC). However, this approach can cause unwanted effects such as underdeveloped root systems and high mortality of treated plants, hindering a full comparison with untreated plants, and can be applied only on plant reproducing by seeds. Here we test a simple alternative method of plant demethylation designed to overcome the shortcomings of the germinating method.
2. We compared a novel method of demethylating plants, based on periodical spraying of 5-azaC aqueous solution on established seedlings, with the previous method in which seeds were germinated directly in 5-azaC solution. We quantified the amount of methylated DNA and measured various aspects of plant performance. Also, we demonstrated its applicability in ecological epigenetic experiments by testing transgenerational effects of plant–plant competition.
3. We found that the spray application had similar DNA-demethylating efficiency than the germination method, particularly in the earlier phases of plant development, but without unwanted effects. The spray application method did not reduce plant growth and performance compared to untreated plants, as opposed to the traditional method which showed reduced growth. Also, the spray application method equalized the epigenetically modified plant features of seedlings coming from plants grown under competition and plants growing without competition, demonstrating its application in ecological epigenetic experiments.
4. We conclude that regular spraying of 5-azaC solution onto established seedlings surpassed the germination-in-solution method in terms of vigour and fitness of treated plants. This novel method could thus be better suited for experimental studies seeking valuable insights into ecological epigenetics. Furthermore, the spray method can be suitable for clonal species reproducing asexually, and most importantly, it opens the possibility of community-level experimental demethylation of plants.

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KEYWORDS
5-azacytidine, competition, demethylation, DNA methylation, ecological epigenetics, ecology, plant memory, plant performance, toxicity, transgenerational effects

1 | INTRODUCTION

A growing body of evidence suggests that heritable epigenetic variation is of crucial importance for the ecological and evolutionary processes of plants (Bossdorf, Richards, & Pigliucci, 2007). Epigenetic variation is caused by various DNA modifications, including DNA methylation, which is known to occur in response to environmental factors (Gonzalez et al., 2016; Herman & Sultan, 2016). Direct quantification of epigenetic variation often requires using highly sophisticated and computationally demanding molecular methods, including real-time PCR (Pecinka et al., 2009), methylation-sensitive amplified fragment length polymorphism (MS-AFLP; Foust et al., 2016; Herrera & Bazaga, 2010; Paun et al., 2010; Preite et al., 2015), whole-genome bisulphite sequencing (Becker et al., 2011; Colicchio, Monnahan, Kelly, & Hileman, 2015; Keller, Lasky, & Yi, 2016) or reduced representation bisulphite sequencing (RRBS; Trucchi et al., 2016; van Gurp et al., 2016). Except for RRBS, a full reference genome of the study plant is a prerequisite for analysing the obtained DNA methylation profiles. However, full genome information is scarce for non-model plants from natural ecosystems (Ellegren, 2014). Consequently, research on ecological epigenetics remains daunting to most plant ecologists, which hinders the process of unravelling ecological and evolutionary consequences of epigenetic variation in plants.

An alternative approach to test the ecological role of epigenetic variation is to alter the epigenetic status of the study plants (e.g. Bossdorf, Arcuri, Richards, & Pigliucci, 2010; Johannes et al., 2009). Altering their epigenetic status generally involves changing the level of cytosine methylation of DNA. Cytosine methylation can be experimentally reduced via the application of demethylating agents such as 5-azacytidine (5-azaC) or zebularine (Bossdorf et al., 2010; Herman & Sultan, 2016; Liu et al., 2015; Verhoeven & van Gurp, 2012). Demethylating agents are small biomolecules which interfere with gene expression by inhibiting DNA methyltransferase—an enzyme responsible for incorporating methyl groups into DNA. The result is partial demethylation or hemi-demethylation of the genome (e.g. Jones, 1985; Burn, Bagnall, Metzger, Dennis, & Peacock, 1993; Tatra, Miranda, Chinnappa, & Reid, 2000). Experimental demethylation represents a simple yet elegant technique for testing the ecological role of epigenetic variation, as it is designed to remove epigenetic marks related to abiotic or biotic factors experienced by the offspring or previous parental generations (Bossdorf et al., 2010; Herman & Sultan, 2016; Verhoeven, Jansen, Van Dijk, & Biere, 2010). Therefore, comparing treated vs. untreated plants enables testing of the importance of past environmental interactions, or the so-called "epigenetic memory," on plant performance (Gonzalez et al., 2016; Herman & Sultan, 2016). As a result, experimental demethylation of DNA has advanced our knowledge on the effect of epigenetic variation in plant phenotypic plasticity (Bossdorf et al., 2010), including flowering phenology (Fieldes & Amyot, 1999; Kondo, Miura, Wada, & Takeno, 2007), the importance of transgenerational adaptation to stress (Boyko et al., 2010; Herman & Sultan, 2016; Herrera, Pozo, & Bazaga, 2012) and in the control of plant inbreeding depression (Vergeer, Wagemaker, & Ouborg, 2012).

Despite the potential of experimental demethylation to reveal epigenetic effects on plant development and adaptation, existing methods have critical limitations. Experimental demethylation of plants has been achieved mostly by the germination of seeds in water solution with various concentrations of 5-azaC (e.g. Ruiz-García, Cervera, & Martínez-Zapater, 2005). Although this approach is very efficient in inhibiting DNA methylation, it also has some fundamental disadvantages, which negatively affect its applicability and the ecological conclusions derived from those experiments. The main limitation of the 5-azaC treatment is its known toxicity on germinating seeds, even at low concentrations (Akimoto et al. 2007; Amoah et al., 2012). Plants grown from seeds germinated in 5-azaC solution often express various aberrations, such as dwarfism (Akimoto et al. 2007; Bossdorf et al., 2010; Kondo et al., 2007), and reduced vigour and survival compared to untreated individuals (Akimoto et al. 2007; Amoah et al., 2012). The reduced performance of plants germinated in 5-azaC solution can be partly explained by the limited development of their root system (Kanchanaketu & Hongtrakul, 2015). Due to the confounding effects of 5-azaC treatment, estimating the net effect of epigenetic change on plant performance is complicated, because changes in phenotypes might not be only due to demethylation but also due to the side effect of its application. Moreover, the method can only be applied to plants establishing from seeds. Thus, already established or clonal plants cannot be considered using this approach. Hence, the application of 5-azaC solution to germinating seeds is questionable in terms of ecological relevance and realism.

Recently, a study by Gonzalez et al. (2016) applied a different demethylation method that consists in periodical spray of 5-azaC solution onto plant leaves of clonal offspring of *Trifolium repens*. This promising approach could potentially solve problems with germinating seeds on 5-azaC and it could be applied also to already established or non-commonly reproducing by seed plants like clonal species, which was the primary motivation of Gonzalez et al. (2016). Unfortunately, while these authors applied this method, they do not compare it to the traditional approach of germinating seeds on 5-azaC, nor they test whether the approach has some side effects on plant growth as the traditional approach has. Although they demonstrate a 4.5% decrease in global methylation, the extent of demethylation was not compared to the one obtained with the germinating approach, which is considered as a reference. This promising approach therefore lacks a proper validation, specifically testing if the foliar application method has similar DNA-demethylating efficiency than the traditional method, and if

the differences between treated and untreated plants are not result of the toxic and unwanted effects of the 5-azaC.

Here, we test a demethylation by spraying method that aims to overcome the limits of the demethylation by germinating seeds in the solution, while maintaining demethylation efficiency. In order to compare the spraying method to the previous method of germinating seeds directly in 5-azaC solution on filter paper, we quantified genome-wide DNA methylation as well as various aspects of plant performance. Also, we demonstrated its application for ecological epigenetic experiments, testing transgenerational effects of plant–plant competition, by applying it to seedlings coming from parental plants that either experienced competition or not.

2 | MATERIALS AND METHODS

2.1 | Study species and seed material

To test the method, we chose a clone of *Taraxacum brevicorniculatum* Korol. as our model species. *Taraxacum brevicorniculatum* is a triploid obligate apomictic species (Kirschner, Štěpánek, Černý, De Heer, & van Dijk, 2013). Genetically identical seeds (collected and genetically identified by Kirschner et al., 2013) were collected from a greenhouse-grown population of plants experiencing equal conditions for five generations. This strategy reduces the effect of genetic and epigenetic variation in the experimental samples.

2.2 | Growth chamber experiments

The spray application was tested by means of two experiments.

2.2.1 | Experiment 1

The aim of this experiment was to compare the demethylation efficiency and possible deleterious effects of the spray application vs. the germination method. Seeds of *T. brevicorniculatum* were thoroughly mixed, and 300 seeds were randomly selected and divided into three treatment groups: germination, spraying and control treatments. One hundred seeds received the germinating treatment (G treatment), where seeds were germinated on filter paper with 5-azaC solution in Petri dishes of 8 cm diameter (Bossdorf et al., 2010; Vergeer et al., 2012; Yang et al., 2010). The filter paper was saturated daily with a 50 μM aqueous solution of 5-azaC (Sigma-Aldrich, Prague, Czech Republic) for 10 days. Thirty-three successfully germinated seeds were picked randomly and subsequently grown in individual pots (square-shaped pots of 7 × 7 cm and 18 cm depth) without further 5-azaC addition. For the spraying approach (S treatment), 100 seeds were first germinated on filter paper in Petri dishes saturated with water for 10 days. Thirty-three of these seedlings were then transferred into individual pots, where they received the demethylation treatment in which 5-azaC solution was sprayed onto the leaves. Specifically, each seedling in the S treatment was sprayed with a 50 μM aqueous solution of 5-azaC on a daily basis until the end of the experiment. For the control group (C treatment), 100 seeds were germinated in water for 10 days (as described for the S treatment) and then 33 seedlings were transplanted into individual pots and grown without any application of the demethylation solution.

It should be noted that a drop of surfactant (in the form of liquid soap) was added to the 5-azaC solution in the spraying method for lowering surface tension, ensuring an even layer of the demethylation agent on the leaf surface. The same amount of surfactant and water solution was also sprayed daily onto the plants of the other two treatments (G and C) to exclude possible confounding effects of the surfactant. The daily addition of 5-azaC is required due to the fast degradation of the 5-azaC at room temperature (Walker, Charbonneau, Law, & Earle, 2012). Sand was used as the potting substrate in all cases to facilitate root removal during the harvest. Plants were grown in a growth chamber for 3 weeks with a 12 hr (20°C)/12 hr (10°C) light/darkness and temperature regime, and watered regularly to keep the substrate moist. The position of all 99 pots in the chamber was randomized to ensure uniform growing conditions.

2.2.2 | Experiment 2

The aim of this experiment was to test if spraying of 5-azaC affected plant morphology and methylation on longer term basis, as well as to demonstrate its applicability in ecological epigenetic research. For this experiment, seeds of *T. brevicorniculatum* of two different origins were used. The origin of the first set of seeds was the same as in the previous experiment, i.e. seeds coming from plants experiencing no competition during previous generations. The second set of seeds came from plants grown under competition with *Plantago media* L. for one generation. Seeds coming from plants grown with competition could develop different phenotypes via environmentally induced transgenerational changes.

Twenty plants of each origin were grown under similar conditions as in Experiment 1 but for 6 weeks. Half of these plants received the spraying method (i.e. 5-azaC solution daily sprayed onto the leaves), while the other half received the control treatment as explained above. The application of the demethylation agent should remove potential transgenerational effects derived from the competition experienced by the parental plants. This way, demethylated plants should be similar in their traits, regardless of their origin.

2.3 | Plant morphological measurements

In Experiment 1, the effect of the G treatment (germination on filter paper saturated with 5-azaC solution) on seedling morphology in early stages of development was assessed by measuring total root length and leaf area of 25 randomly selected 10-day-old seedlings (out of the 67 not used for transplanting, see above). These seedlings were compared to 50 of those germinated in pure water (25 from the S and 25 from the C treatment, which were virtually equivalent up to that point because they had not been sprayed yet). Total root length (cm) and leaf area (mm^2) were estimated based on scanned images of the seedlings. The seedlings used for these

measurements were not transplanted to pots afterwards. The seedlings transplanted into pots (33 per treatments) were harvested after 3 weeks. The plant material was dried at 60°C and the total biomass was weighted.

In Experiment 2, the 40 plants were grown for 6 weeks in pots. During that time, we measured the diameter of the rosette every 2 days and used these measurements to estimate growth rate (change in diameter of the rosette between the transplantation and harvest; mm/day). After 6 weeks, two leaves from each plant were collected, and their area, water-saturated fresh mass and dry mass were estimated. We used these measurements to estimate leaf dry matter content (LDMC; the ratio of leaf dry mass to leaf fresh mass, mg/g) and specific leaf area (SLA; the ratio of leaf area to leaf dry mass, mm^2/mg). Furthermore, we separated the aerial and root systems and measured their biomass after drying at 60°C. The specific root length (SRL; the ratio of total root length to root dry mass; m/g) was estimated based on the scanned images by using the image analysis software WinRHIZO Pro, 2008 (Regent Instruments Inc., Quebec, Canada).

2.4 | DNA extraction and genome-wide DNA methylation

We assessed differences in genome-wide DNA methylation between treatments in Experiment 1 by extracting DNA from the plants that were transplanted and grown for 3 weeks. We combined both shoots and roots for the DNA extraction, as plants were still small at the time of harvest (but we tested the effect of this combination in Experiment 2, see below). Plant material was pulverized with 2-mm stainless steel beads in a Mixer Mill MM400 (Retch GmbH, Haan, Germany) and the DNA was extracted using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The amount of DNA was evaluated using Qubit Fluorimeter and Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Finally, genome-wide DNA methylation was quantified by measuring the amount of 5-methylcytosine (5-mC) from the DNA extracts using the Colorimetric MethylFlash Methylated DNA Quantification Kit (Epigentek Group Inc., Farmingdale, NY, USA), measured on the Infinite® F200 microplate reader (Tecan Trading AG, Männedorf, Switzerland). We quantified the absolute amount of genome-wide methylated DNA by first generating a standard curve, following the manufacturer's instructions (i.e. six 5-mC concentration points including a zero point); the slope of that curve was then used to estimate the percentage of methylated DNA. This percentage was estimated in two independent replicates of each sample.

In Experiment 2, we first assessed the efficiency of the spraying application of 5-azaC in older plants, as well as differences in demethylation efficiency between different parts of the plant. For this, we only assessed differences in genome-wide DNA methylation in the plants with the same origin as in Experiment 1, i.e. seeds coming from plants experiencing no competition during previous generations, and using the same procedure as before. The essential difference between experiments is that the quantification was done on older plants and, independently, in roots and aerial parts of each plant. This last distinction was possible in this case because of the bigger size of the 6-week-old plants. For both experiments, we estimated an "error rate" of the quantification technique as the difference in percentage of methylated DNA between the two replicates per sample divided by total number of comparisons. This error rate was 0.13% in the first experiment and 0.03% in the second one.

2.5 | Statistical analyses

In Experiment 1, the effect of the treatments on the percentage of methylated DNA was analysed taking into consideration the two replicates of each individual, by means of a generalized mixed effects model with binomial errors. The identity of the individual was used as a random factor. In addition, we performed an ANOVA to analyse the effects of the treatments on the total biomass of the seedlings. In both cases, we performed a post hoc Tukey test to see whether pairs of treatments differed significantly ($p < .05$). Finally, the differences between 10-day-old seedlings traits in different treatments (G vs. C treatments; seedling root length and leaf area) were evaluated by means of *t* tests (root length was log-transformed to achieve normality). It should be noticed that with the G treatment, a limited number of individuals provided enough amount of DNA to meet the requirement of the Methylated DNA Quantification Kit (Epigentek Group Inc., Farmingdale, NY, USA), thus reducing the number of observation for this treatment (see Figure 1).

For Experiment 2, the effect of the treatment and the part of the plant on the percentage of methylated DNA were analysed as described above for Experiment 1, using generalized mixed effects model with the identity of the individual as random factor. The difference in this case was that we included in the model, the interaction between the demethylation treatment (Control vs. S treatment) and plant part (aerial vs. root system). We performed ANOVAs to analyse the effects of the treatment and the origin on the plant traits (growth rate, root and aerial biomass, LDMC, SLA and SRL). Again, whenever we found a significant result in the model, we performed a post hoc Tukey test to see which combinations differed significantly ($p < .05$). All analyses were conducted using R v3.2.3 (R Core Team, 2016).

3 | RESULTS

3.1 | Experiment 1

The treatments affected the percentage of methylated DNA ($\chi^2 = 10.99$, $df = 2$, $p = .004$). Compared to the control treatment (4.7 ± 1.9% methylated DNA, $n = 61$), we found significantly reduced DNA methylation in both treatments using the 5-azaC demethylation agent, both for the germination treatment (1.6% decrease in methylated DNA to 3.1 ± 1.4% methylated DNA, $n = 16$, i.e. 34% relative reduction; Tukey post hoc test germinating treatment vs. control, G vs. C, $p = .005$), and in the spraying treatment (1% decrease in methylated DNA to 3.7 ± 1.5% methylated DNA, $n = 61$, i.e. 21% relative reduction; spraying vs. control, S vs. C, $p = .041$). Most importantly,

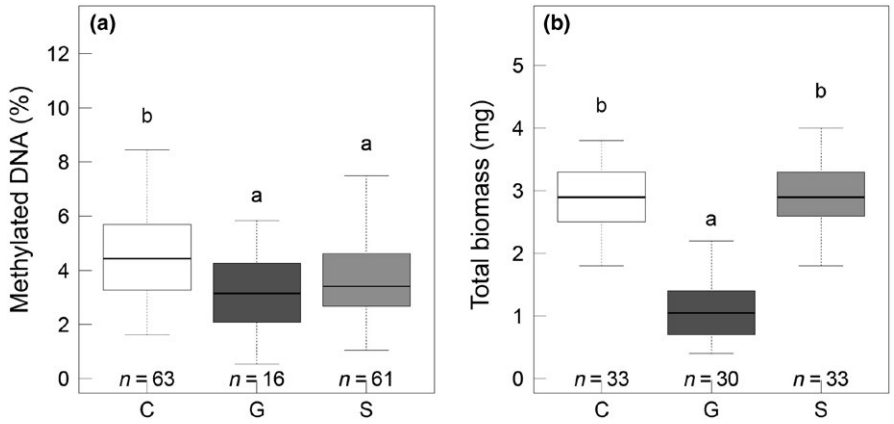

FIGURE 1 Differences between experimental treatments in the 3-week-old seedlings. (a) Effects of the treatments (C, control; G, germinating method; S, spraying method) on the level of genome-wide DNA methylation and (b) on the dry weight total biomass of the plants at the end of the 3-week experiment. The bottom and top of the boxes are the 25th and 75th percentiles respectively, the centred band is the median and the whiskers represent the maximum or minimum observation. Different letters within each panel indicate significant differences between treatments (post hoc Tukey test, $p = .05$)

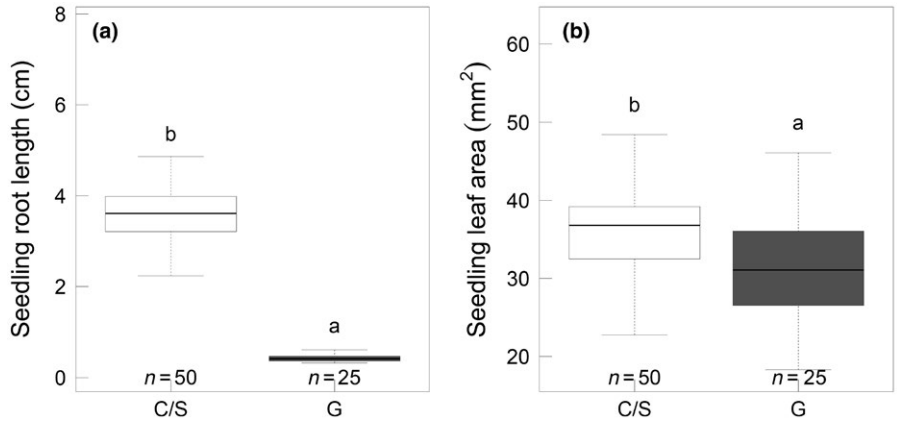

FIGURE 2 Differences between 10-day-old seedlings germinated either in water (C/S, which were virtually equivalent up to that point because they had not been sprayed yet) or a 50 μM water solution of 5-azaC (G) in (a) root length and (b) leaf area. The bottom and top of the boxes are the 25th and 75th percentiles respectively, the centred band is the median and the whiskers represent the maximum or minimum observation. Different letters within each panel indicate significant differences between treatments (*T* test, $p = .05$)

we found no differences in the levels of DNA methylation between the germinating and the spraying demethylation approaches (S vs. G, $p = .257$; Figure 1a).

We found no significant differences in the total plant biomass between the spraying treatment and the control (S vs. C; Figure 1b). The germinating treatment (G), on the contrary, substantially decreased plant performance in terms of total biomass ($p < .001$; Figure 1b), both in relation to the control and to the spraying treatment. Seedlings whose seeds germinated in 5-azaC solution developed roots remarkably smaller than seedlings that germinated in water (C vs. G *t* test: $t = 43.967$, $df = 65.63$, $p < .001$; Figures 2a and 3), as well as smaller leaves ($t = 2.228$, $df = 44.86$, $p = .031$; Figures 2b and 3).

3.2 | Experiment 2

Genome-wide DNA methylation in control plants was higher in roots than in aerial parts (roots = 5.3 ± 4% methylated DNA, $n = 17$; aerial part = 3.8 ± 1.4% methylated DNA, $n = 20$), although such difference was not found to be significant. Neither were any significant differences in the demethylation effect of the spraying treatment between roots and aerial part (0.9% decrease to 4.3 ± 1.5% methylated DNA in roots, $n = 17$, and 0.5% decrease to 3.2 ± 1.3% methylated DNA in leaves, $n = 20$; i.e. 17 and 14% relative reduction respectively), being in average a 0.7% methylated DNA reduction comparing sprayed treatment and control (S treatment = 3.7 ± 1.5% methylated DNA, $n = 37$; C treatment = 4.4 ± 2.9% methylated DNA, $n = 37$, i.e. 16% relative reduction). Thus, we did not detect a significant effect of any of the predictors in the model (treatment, $p = .92$; plant part, $p = .86$; and their interaction $p = .98$) in the percentage of methylated DNA (Figure S1).

Differences in competition in the parental generation resulted in morphological differences between seedlings. Tukey post hoc tests revealed that untreated offspring of parents from competition conditions (Competition-C treatment) were significantly smaller than

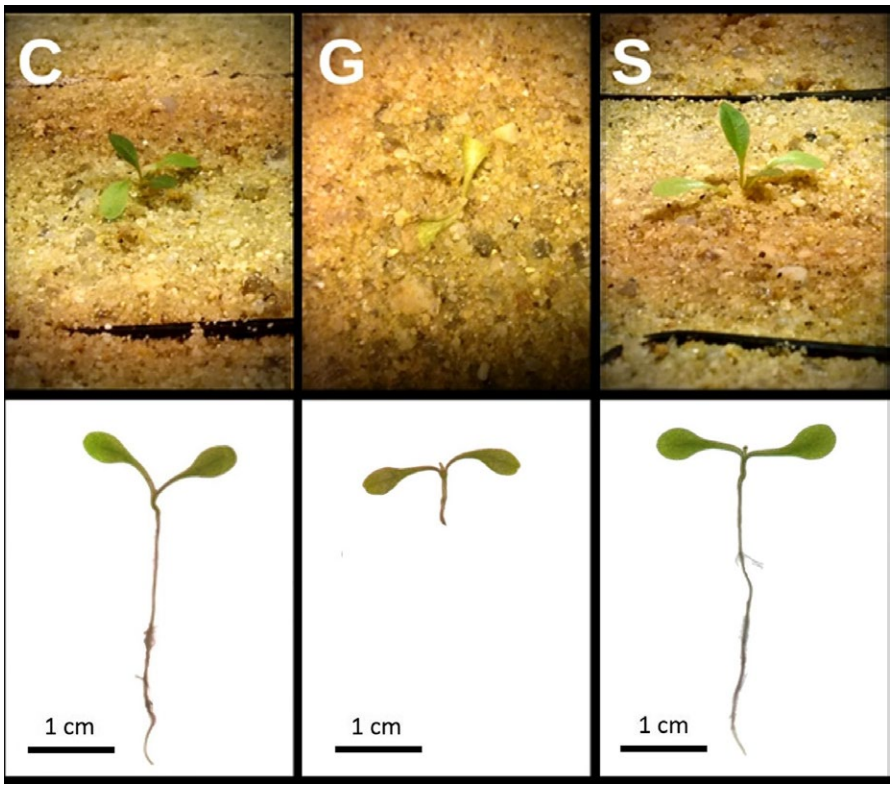

FIGURE 3 Details of the differences in early development of plants between the three treatments (C, control; G, germinating method; S, spraying method). Upper row shows seedlings in the pots 2 weeks after transplanting, whereas the lower row displays some of the images of 10-day-old seedlings that were used to estimate root length and leaf area

offspring of parents from non-competition conditions, both considering shoots (Figure 4a; $p = .02$ for comparison with No competition-C treatment, and $p = .03$ with No competition-S treatment) and roots (Figure 4b; $p = .03$ with No competition-C treatment, and $p < .01$ with No competition-S treatment), and had higher SLA (Figure 4e; $p = .03$ with No competition-C treatment, and $p < .01$ with No competition-S treatment). However, these differences in offspring morphology ceased to be significant after the application of the spraying treatment (Tukey post hoc test for Competition-S treatment vs. No competition treatments in shoots, Figure 4a: $p = .08$ with C treatment and $p = .09$ with S treatment; in roots, Figure 4b: $p = .27$ with C treatment and $p = .03$ with S treatment; and in SLA, Figure 4e: $p = 1$ with C treatment and $p = .63$ with S treatment). Moreover, the treatment did not have any effect on the traits of the seedlings coming from plants that did not experience competition in the previous generations (Tukey post hoc test for No Competition Control treatment vs. No Competition Sprayed treatment: $p > .05$). In other words, the application of 5-azaC did not alter the traits in the non-competition origin, therefore not inducing unwanted phenotypic variation in plants (Figure 4).

4 | DISCUSSION

Experimental demethylation via demethylation agent application is a simple and affordable, yet powerful technique for gaining essential mechanistic insights into the relatively new field of ecological epigenetics. In vivo treatment with 5-azaC is expected to remove methylation marks of plants, including those inherited from previous generations, making it an ideal tool for studying various ecological and evolutionary questions (Bossdorf et al., 2010; Herman & Sultan, 2016; Verhoeven & van Gurp, 2012). Nevertheless, previous approaches include serious development- and survival-related problems connected with the application of 5-azaC, particularly during the germination of seeds (e.g. Akimoto et al. 2007; Bossdorf et al., 2010; Finnegan, Peacock, & Dennis, 1996). The deleterious effects of the most common demethylation method (germination-in-solution) on the early development of seedlings impede a proper evaluation of the net role of epigenetic change in the performance of demethylated plants compared to control ones. We demonstrated these deleterious effects in our experiment, i.e. in terms of reduced biomass, root length and leaf area, where the germination of seeds in 5-azaC created unwanted phenotypic variation and generally decreased plant performance (Figures 1 and 2). We show that the alternative method (foliar application of the common demethylation agent 5-azaC on already germinated seedlings) does not affect plant performance, thus providing ecological insight on transgenerational effects, and generally providing DNA demethylation levels comparable to those achieved by the traditional germination of seeds in 5-azaC solution (21 and 16% relative reduction in methylation in our case).

Germinating seeds directly in 5-azaC solution affected the development of the seedlings and hindered the formation of a functional root system, ultimately affecting the growth of the whole plant (Figure 2). These undesired effects of 5-azaC have previously been reported by other studies (Akimoto et al. 2007; Bossdorf et al., 2010; Finnegan et al., 1996; Kanchanaketu & Hongtrakul, 2015). We point out that we only measured the root length of the 10-day-old seedlings for the germinating technique (i.e. at the point in time when the S and C treatments were virtually identical), as differences were already

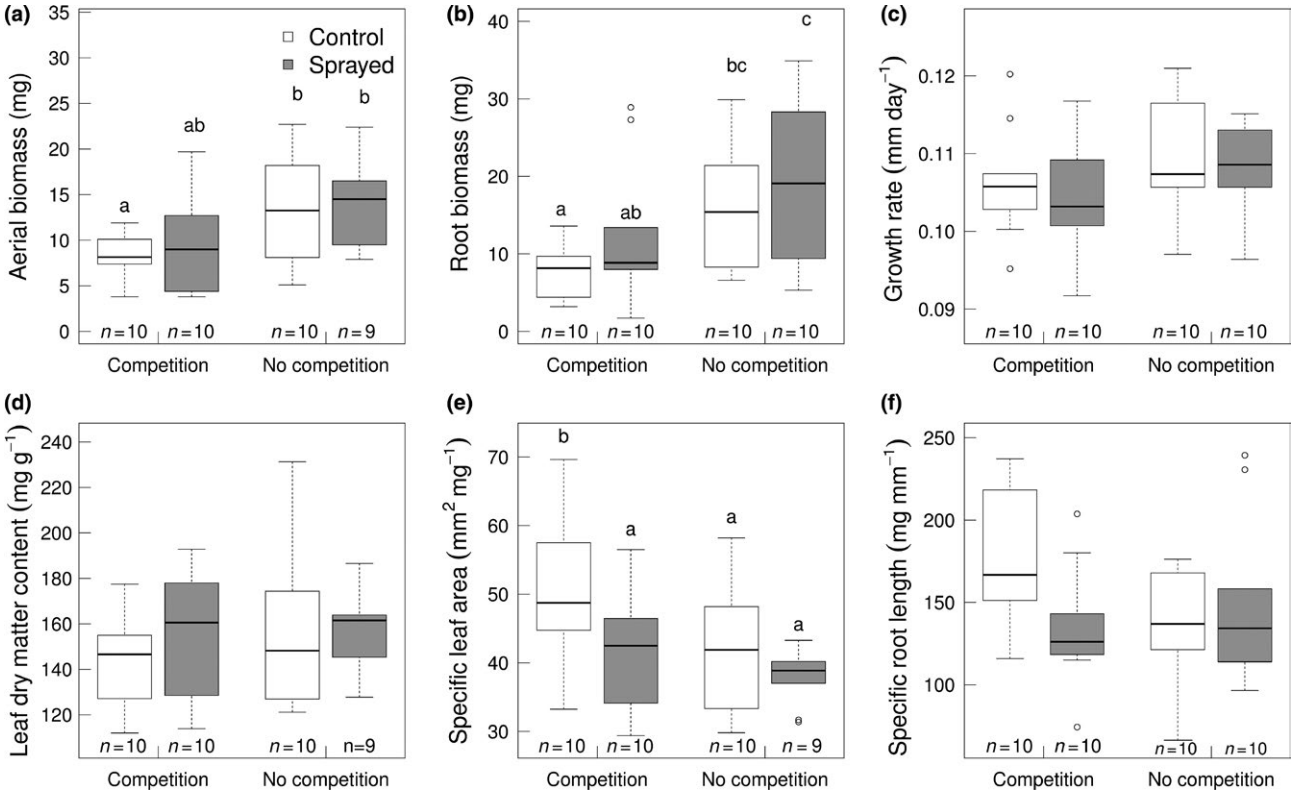

FIGURE 4 Effect of the demethylation treatment (C, control in white; S, spraying method in grey) on morphological and performance measurements of plants grown under competition during last generation (left), and on plants with no competition in previous generations (right). Differences (a) on the aerial biomass, (b) on the root biomass, (c) on the growth rate, and (d) on the leaf dry matter content, (e) specific leaf area and (f) specific root length. The bottom and top of the boxes are the 25th and 75th percentiles respectively, the centred band is the median and the whiskers represent 1.5 times the length of the box further from the box limits or the maximum or minimum observation in the absence of outliers. Different letters within each panel indicate significant differences between treatments (post hoc Tukey test, $p = .05$)
[Correction added on 22 December 2017, after first online publication: The figure was corrected and now reflects the results reported in the text.]

considerable at that stage. Our results clearly show that the G treatment was extremely harmful for root development, to the extent that the roots were barely present at the point of transplanting seedlings into pots (Figure 2). Not surprisingly, these plants achieved a much smaller size in later stages, as shown by the great differences in total biomass of G vs. S and C treatments (Figure 1). Remarkably, this was not the case in plants sprayed by 5-azaC solution (S treatment), which reached a final size similar to the control plants, despite the relatively intense level of demethylation. Moreover, the lack of morphological differences in the non-competition origin between the sprayed plants and the control ones after 6 weeks of growing in pots (Experiment 2; Figure 4) further confirms the lack of undesirable secondary effects related to the spraying treatment on plants in the longer term.

Differences in plant growth between the 5-azaC application by germination-in-solution and by spraying (Figure 1) can have several explanations. Application of a demethylation agent alters gene expression, and this effect is probably much more crucial during the initial stages of seedling development, i.e. germination, compared to already established seedlings (Akimoto et al. 2007). Furthermore, morphological changes in plants germinated in 5-azaC could be ascribed to indirect effects of 5-azaC on other factors such as transposable elements, which are known to alter gene expression and thus cause abnormal seedling development (Kanchanaketu & Hongtrakul, 2015). Finally, we cannot rule out the possibility that the observed morphological changes in the G treatment, as opposed to S treatment, were the result of mutations caused by 5-azaC in the primary sequence of DNA (Fieldes & Amyot, 2000). However, this is highly unlikely as an absorbance-based ELISA-like assay showed notable and comparable hypomethylation levels in both the demethylation treatments, not only in the G treatment where the growth aberrations occurred. More in-depth molecular methods such as AFLP and MS-AFLP could be employed to disentangle the effects of 5-azaC, both on the underlying DNA sequence and its methylation patterns.

The notion that the demethylation agent alters methylation stronger during the early stages of seedling development was also partially confirmed by the second experiment. In our second experiment, where the duration of the 5-azaC spray application was applied for 6 weeks, we observed an almost similar reduction in genome-wide methylated DNA compared to the first experiment (21% average relative reduction of 5-mC in roots and shoots in the first experiment and 16% in the second) but the reduction was not found statistically significant. While the lack of a significant effect might also partially be due to the smaller number of replicates in Experiment 2, the percentage reduction was also slightly lower. This reinforces the idea that the demethylation is more effective during first stages of the plant development.

Further research is needed to understand how methylation patterns vary depending on the plant life stage.

Despite the non-significant effect of the demethylation in the second experiment, we demonstrate the applicability of the 5-azaC spray approach for ecological epigenetic experiments. We showed that the application of demethylation agent generally "equalized" the phenotype of plants with different parental origin. In other words, competition in the parental generation triggered offspring with different phenotypes, and spraying with 5-azaC deleted this transgenerational effect, by making the sprayed offspring whose mothers experienced competition more similar to those that did not experience it. Most importantly, this was achieved without causing any change or deleterious effect for the control plants (i.e. from no competition origin). It is important also to notice that, in the second experiment, the effect of 5-azaC spraying in equalizing phenotypic differences was effective even though we did not observe statistically significant reduction of methylation with the spraying approach. The degree of demethylation can be less marked than its actual ecological effect. This further reinforces the idea that even though the absolute number of demethylation efficacy seems to be low, it is enough to promote biological variations, in a magnitude possible to discern and observe ecological relevant changes. In a study by Gonzalez et al. (2016), even 4.5% relative reduction in global DNA methylation was enough to reset some transgenerational memories. As such the spraying approach offers a feasible way to directly manipulate the epigenetic status of plants and is therefore useful in experiments investigating the ecological and evolutionary potential of epigenetic variation.

In addition, the bigger size of the plants in Experiment 2 allowed us to examine differences in methylation between aerial and root systems. Even though the difference was not statistically significant, we found that root tissues had more methylated DNA that aerial tissues. This difference could account for the slightly higher (but not statistically significant) demethylation efficiency of the G treatment observed in Experiment 1. Although the material used for the quantification of DNA methylation in Experiment 1 included both roots and aerial parts in the S treatment, it did not include a considerable amount of roots in the G treatment (because roots did not develop well; Figure 1). This suggests that the S treatment could have a higher demethylation power than it seems from Experiment 1, and that the reduction reported here is a conservative estimation. As such the lack of significant difference in demethylation between spraying and germination treatment provides an even stronger test of the viability of the spraying approach compared to the germination approach. Finally, the lack of interaction between the part of the plant and the treatment shows that the spraying treatment systematically demethylates the whole plant. Both roots and shoots were demethylated equally (17% and 14% reduction in DNA methylation respectively), even though the spraying of 5-azaC was only applied onto the leaf surface.

To the best of our knowledge, this is the first demonstration of the ecological applicability of 5-azaC spraying on plants coming from seeds to asses transgenerational effects sensu stricto. Also, it is a clear demonstration that competition can cause transgenerational effects on offspring phenotypes. In Gonzalez et al. (2016), the clonal offspring of *T. repens* "remembered" drought events experienced by parental plants, and this memory was erased by spraying parental plants with 5-azaC. However, such experiment was done on a ramet of the same plant of *T. repens* not undergoing sexual reproduction and causing artificial clonal splitting. Otherwise, it is important to stress that both these studies were conducted on broad-leaved herb species (*T. brevicorniculatum* and *T. repens*) which may absorb 5-azaC solution through leaves more easily than species with needle-like leaves and/or leaves with thick cuticles, which may prevent absorbance of the solution. We therefore recommend, in the case of using some potentially problematic species, to verify the most adequate demethylation technique with a pilot study.

Finally, a few studies indicate that the effects of demethylation agents can be transient as DNA methylation marks could be restored in somatic tissues formed after cessation of the treatment (Baubec, Pecinka, Rozhon, & Mittelsten Scheid, 2009; Kumpatla & Hall, 1998). In this case, applying 5-azaC solution only during the germination of seeds might not be enough to ensure the stable status of DNA demethylation in long-lasting experiments. Even in our case, the efficiency of the demethylation seems to decrease; the method of spraying 5-azaC solution onto the plants throughout the whole duration of the experiment will likely guarantee more stable and potentially inheritable demethylation effects.

In conclusion, the findings of this study are especially relevant as this is the first formal comparison of the foliar demethylation application method against the commonly used germination one. The demethylation method based on daily spraying of 5-azaC solution onto the leaf surface of established seedlings reduced methylation comparably to the treatment of germinating seeds in 5-azaC solution, but surpassed it in terms of viability and healthy early development of treated plants. Also, we demonstrated its applicability in ecological epigenetic experiments to remove transgenerational effects, in this case, caused by plant–plant competition. In cases where the use of elaborate and frequently expensive molecular techniques is not feasible, such an in vivo demethylation agent is currently the only tool readily available for experimental manipulation of non-model species (Verhoeven, VonHoldt, & Sork, 2016). Its application is easy and fast; however, as in the case of the germinating method, handling 5-azaC following adequate safety procedures is recommended due to its potential risks to human health (Doerksen, Benoit, & Trasler, 2000; Doerksen & Trasler, 1996; Gaudet et al., 2003; Tunc & Tremellen, 2009). Although the novel spraying method presented here should be tested on more plant species and on different life stages, it allows more credible ecological epigenetic studies to be conducted with a proper control. Up to now, demethylation approaches have been applied without clear standardized methods, causing heterogeneity even in the application of the "traditional" technique of germinating seeds in 5-azaC solution, and possibly adding uncertainty to the results. It is thus premature to provide a universal methodological framework without further large-scale validation. Our study shows, however, that the alternative approach, by regular spraying 5-azaC solution, can provide a feasible approach which can be applied, and further tested, on a broad scale. Experiments using this method will

potentially create a better and ecologically more robust link between epigenetic variation and changes in plant phenotype, behaviour or response to environmental stress. Furthermore, the sprayed method can be applied directly to seedlings or established plants, making it suitable for clonal species reproducing asexually. And, most importantly, it opens the possibility of community-level experimental demethylation of plants.

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AUTHORS' CONTRIBUTIONS

All authors designed the research; J.P. and H.D. performed the experiment; J.P., H.D. and C.P.C. analysed the data; H.D. wrote a first version of the manuscript. All authors contributed substantially to revisions and gave final approval for publication.

DATA ACCESSIBILITY

Data used in the analyses are available in the Dryad Digital Repository https://doi.org/10.5061/dryad.k9f51 (Puy et al., 2017).

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REFERENCES

Akimoto, K., Katakami, H., Kim, H.-J., Ogawa, E., Sano, C. M., Wada, Y., & Sano, H. (2007). Epigenetic inheritance in rice plants. *Annals of Botany*, *100*, 205–217.

Amoah, S., Kurup, S., Rodriguez Lopez, C. M., Welham, S. J., Powers, S. J., Hopkins, C. J., … King, G. J. (2012). A hypomethylated population of *Brassica rapa* for forward and reverse epi-genetics. *BMC Plant Biology*, *12*, 193.

Baubec, T., Pecinka, A., Rozhon, W., & Mittelsten Scheid, O. (2009). Effective, homogeneous and transient interference with cytosine methylation in plant genomic DNA by zebularine. *Plant Journal*, *57*, 542–554.

Becker, C., Hagmann, J., Müller, J., Koenig, D., Stegle, O., Borgwardt, K., & Weigel, D. (2011). Spontaneous epigenetic variation in the *Arabidopsis thaliana* methylome. *Nature*, *480*, 245–249.

Bossdorf, O., Arcuri, D., Richards, C. L., & Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology*, *24*, 541–553.

Bossdorf, O., Richards, C. L., & Pigliucci, M. (2007). Epigenetics for ecologists. *Ecology Letters*, *11*, 106–115.

Boyko, A., Blevins, T., Yao, Y., Golubov, A., Bilichak, A., Ilnytskyy, Y., … Kovalchuk, I. (2010). Transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of dicer-like proteins. *PLoS ONE*, *5*, e9514.

Burn, J. E., Bagnall, D. J., Metzger, J. D., Dennis, E. S., & Peacock, W. J. (1993). DNA methylation, vernalization, and the initiation of flowering. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 287–291.

Colicchio, J. M., Monnahan, P. J., Kelly, J. K., & Hileman, L. C. (2015). Gene expression plasticity resulting from parental leaf damage in *Mimulus guttatus*. *New Phytologist*, *205*, 894–906.

Doerksen, T., Benoit, G., & Trasler, J. M. (2000). Deoxyribonucleic acid hypomethylation of male germ cells by mitotic and meiotic exposure to 5-azacytidine is associated with altered testicular histology. *Endocrinology*, *141*, 3235–3244.

Doerksen, T., & Trasler, J. M. (1996). Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats. *Biology of Reproduction*, *55*, 1155–1162.

Ellegren, H. (2014). Genome sequencing and population genomics in non-model organisms. *Trends in Ecology and Evolution*, *29*, 51–63.

Fieldes, M. A., & Amyot, L. M. (1999). Epigenetic control of early flowering in flax lines induced by 5- azacytidine applied to germinating seed. *Journal of Heredity*, *90*, 199–206.

Fieldes, M. A., & Amyot, L. M. (2000). Evaluating the potential of using 5-azacytidine as an epimutagen. *Canadian Journal of Botany*, *77*, 1617–1622.

Finnegan, E. J., Peacock, W. J., & Dennis, E. S. (1996). Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proceedings of the National Academy of Sciences of the United States of America*, *93*, 8449–8454.

Foust, C. M., Preite, V., Schrey, A. W., Alvarez, M., Robertson, M. H., Verhoeven, K. J. F., & Richards, C. L. (2016). Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. *Molecular Ecology*, *25*, 1639–1652.

Gaudet, F., Hodgson, J. G., Eden, A., Jackson-grusby, L., Dausman, J., Gray, J. W., … Jaenisch, R. (2003). Induction of tumors in mice by genomic hypomethylation. *Science*, *300*, 489–492.

Gonzalez, A. P. R., Chrtek, J., Dobrev, P. I., Dumalasova, V., Fehrer, J., Mraz, P., & Latzel, V. (2016). Stress-induced memory alters growth of clonal offspring of white clover (*Trifolium repens*). *American Journal of Botany*, *103*, 1567–1574.

Herman, J. J., & Sultan, S. E. (2016). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 20160988.

Herrera, C. M., & Bazaga, P. (2010). Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis*. *New Phytologist*, *187*, 867–876.

Herrera, C. M., Pozo, M. I., & Bazaga, P. (2012). Jack of all nectars, master of most: DNA methylation and the epigenetic basis of niche width in a flower-living yeast. *Molecular Ecology*, *21*, 2602–2616.

Johannes, F., Porcher, E., Teixeira, F. K., Saliba-Colombani, V., Simon, M., Agier, N., … Colot, V. (2009). Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS Genetics*, *5*, e1000530.

Jones, P. A. (1985). Altering gene expression with 5-azacytidine. *Cell*, *40*, 485–486.

Kanchanaketu, T., & Hongtrakul, V. (2015). Treatment of 5-azacytidine as DNA demethylating agent in *Jatropha curcas* L. *Kasetsart Journal (Natural Science)*, *49*, 524–535.

Keller, T. E., Lasky, J. R., & Yi, S. V. (2016). The multivariate association between genomewide DNA methylation and climate across the range of *Arabidopsis thaliana*. *Molecular Ecology*, *25*, 1823–1837.

Kirschner, J., Štěpánek, J., Černý, T., De Heer, P., & van Dijk, P. J. (2013). Available ex situ germplasm of the potential rubber crop *Taraxacum koksaghyz* belongs to a poor rubber producer, *T. brevicorniculatum* (Compositae-Crepidinae). *Genetic Resources and Crop Evolution*, *60*, 455–471.

Kondo, H., Miura, T., Wada, K. C., & Takeno, K. (2007). Induction of flowering by 5-azacytidine in some plant species: Relationship between the stability of photoperiodically induced flowering and flower-inducing effect of DNA demethylation. *Physiologia Plantarum*, *131*, 462–469.

Kumpatla, S. P., & Hall, T. C. (1998). Longevity of 5-azacytidine-mediated gene expression and re-establishment of silencing in transgenic rice. *Plant Molecular Biology*, *38*, 1113–1122.

Liu, C.-H., Finke, A., Díaz, M., Rozhon, W., Poppenberger, B., Baubec, T., & Pecinka, A. (2015). Repair of DNA damage induced by the cytidine analog zebularine requires ATR and ATM in *Arabidopsis*. *The Plant Cell*, *27*, 1788–1800.

Paun, O., Bateman, R. M., Fay, M. F., Hedrén, M., Civeyrel, L., & Chase, M. W. (2010). Stable epigenetic effects impact adaptation in allopolyploid orchids (Dactylorhiza: Orchidaceae). *Molecular Biology and Evolution*, *27*, 2465–2473.

Pecinka, A., Rosa, M., Schikora, A., Berlinger, M., Hirt, H., Luschnig, C., & Scheid, O. M. (2009). Transgenerational stress memory is not a general response in *Arabidopsis*. *PLoS ONE*, *4*, e5202.

Preite, V., Snoek, L. B., Oplaat, C., Biere, A., Van Der Putten, W. H., & Verhoeven, K. J. F. (2015). The epigenetic footprint of poleward range-expanding plants in apomictic dandelions. *Molecular Ecology*, *24*, 4406–4418.

Puy, J., Dvořáková, H., Carmona, C. P., de Bello, F., Hiiesalu, I., & Latzel, V. (2017). Data from: Improved demethylation in ecological epigenetic experiments: Testing a simple and harmless foliar demethylation. *Dryad Digital Repository*, https://doi.org/10.5061/dryad.k9f51

R Core Team. (2016). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. 55, 275–286. ISBN 3-900051-07-0, Retrieved from: http://www.R-project.org/

Ruiz-García, L., Cervera, M. T., & Martínez-Zapater, J. M. (2005). DNA methylation increases throughout *Arabidopsis* development. *Planta*, *222*, 301–306.

Tatra, G. S., Miranda, J., Chinnappa, C. C., & Reid, D. M. (2000). Effect of light quality and 5-azacytidine on genomic methylation and stem elongation in two ecotypes of *Stellaria longipes*. *Physiologia Plantarum*, *109*, 313–321.

Trucchi, E., Mazzarella, A. B., Gilfillan, G. D., Lorenzo, M. T., Schönswetter, P., & Paun, O. (2016). BsRADseq: Screening DNA methylation in natural populations of non-model species. *Molecular Ecology*, *25*, 1697–1713.

Tunc, O., & Tremellen, K. (2009). Oxidative DNA damage impairs global sperm DNA methylation in infertile men. *Journal of Assisted Reproduction and Genetics*, *26*, 537–544.

van Gurp, T. P., Wagemaker, N. C. A. M., Wouters, B., Vergeer, P., Ouborg, J. N. J., & Verhoeven, K. J. F. (2016). epiGBS: Reference-free reduced representation bisulfite sequencing. *Nature Methods*, *13*, 322–324.

Vergeer, P., Wagemaker, N., & Ouborg, N. J. (2012). Evidence for an epigenetic role in inbreeding depression. *Biology Letters*, *8*, 798–801.

Verhoeven, K. J. F., Jansen, J. J., Van Dijk, P. J., & Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytologist*, *185*, 1108–1118.

Verhoeven, K. J. F., & van Gurp, T. P. (2012). Transgenerational effects of stress exposure on offspring phenotypes in apomictic dandelion. *PLoS ONE*, *7*, e38605.

Verhoeven, K. J. F., VonHoldt, B. M., & Sork, V. L. (2016). Epigenetics in ecology and evolution: What we know and what we need to know. *Molecular Ecology*, *25*, 1631–1638.

Walker, S. E., Charbonneau, L. F., Law, S., & Earle, C. (2012). Stability of azacitidine in sterile water for injection. *The Canadian Journal of Hospital Pharmacy*, *65*, 352–359.

Yang, F., Zhang, L., Li, J., Huang, J., Wen, R., Ma, L., … Li, L. (2010). Trichostatin A and 5-azacytidine both cause an increase in global histone H4 acetylation and a decrease in global DNA and H3K9 methylation during mitosis in maize. *BMC Plant Biology*, *10*, 178.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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SUPPORTING INFORMATION

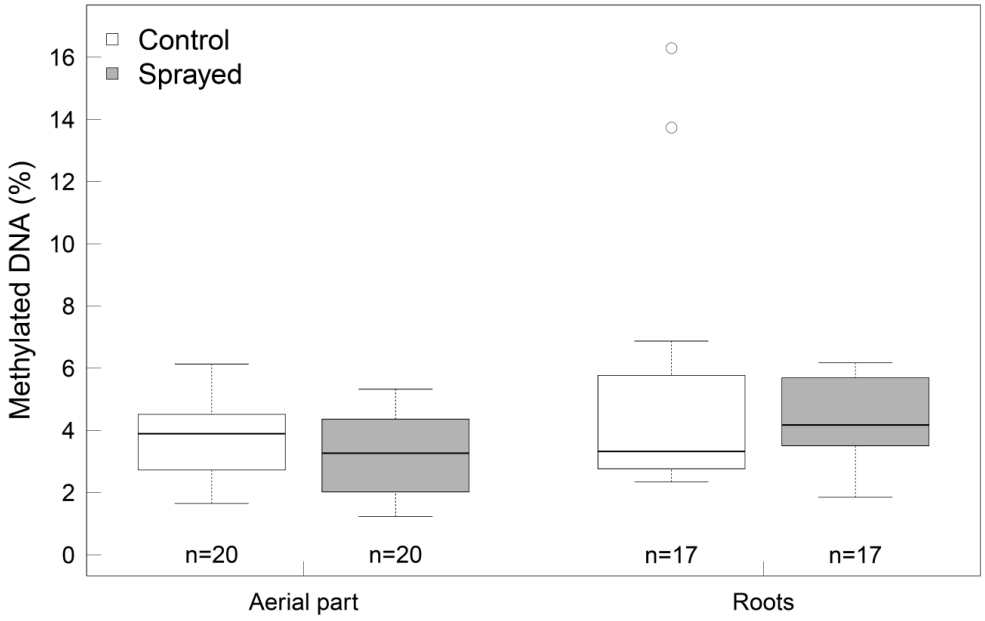

FIGURE S1 Differences between experimental treatments in the six-week-old seedlings (C – control in white, S - spraying method in grey) on the level of genome-wide DNA methylation in aerial part (left), and roots (right) of the plant. The bottom and top of the boxes are the 25th and 75th percentiles respectively, the centred band is the median and the whiskers represent 1.5 times the length of the box further from the box limits or the maximum or minimum observation in absence of outliers.