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Transgenerational effect in *Taraxacum brevicorniculatum*: test of a novel method of experimental plant DNA demethylation and its practical application in exploring the impact of maternal competition on progeny phenotype

Master thesis

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Annotation:

Spray application of 5-azacytidine on established plant seedlings was tested for its demethylating efficiency, as 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 demethytaling 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*.

I hereby declare that this master thesis is entirely the result of my own work except where otherwise indicated. I have only used the resources given in the list of references.

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Hana Dvořáková

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Contents

1. Introduction

Epigenetic effects and its importance as a means of transgenerational inheritance in plants has been a highly discussed topic in the field of plant ecology and evolution recently. There has already been a lot of research conducted to demonstrate the ability of plants to pass some of their qualities acquired in response to environmental conditions on their direct progeny through so called transgenerational or parental effects (Roach & Wulff, 1987a; Rossiter, 1996; Weiner *et al.*, 1997; Agrawal *et al.*, 1999; Galloway, 2005; Holeski *et al.*, 2012). Some of the heritable characteristics can even act adaptively to increase the fitness of the following generation (Mousseau & Fox, 1998; Agrawal, 2002; Dyer *et al.*, 2010; Latzel *et al.*, 2010; Colicchio *et al.*, 2015b; Herman & Sultan, 2016; Rendina González *et al.*, 2016a). Nevertheless, the exact way of the transmission of such heritable information from one generation to another is still not very clear. Epigenetic effects, which are able to alter DNA expression without DNA sequence modification, seem to be a hot candidate for this role. One of such DNA modification, which is known to occur in response to environmental factors, is DNA methylation.

However, direct quantification of epigenetic processes often requires using highly sophisticated, computationally demanding and expensive molecular methods including a full reference genome of the study plant, which makes it daunting to most plant ecologists. To overcome this obstacle, alternative approaches have been developed (Johannes *et al.*, 2009; Bossdorf *et al.*, 2010). They are based on artificial altering of the epigenetic status of the study plant by increasing or decreasing its level of cytosine methylation. One of the possibilities is using a demethylation agent, a biomolecule inhibiting function of DNA methyltransferase - an enzyme responsible for incorporating methyl groups into DNA (Cubas *et al.*, 1999; Bossdorf *et al.*, 2010; Verhoeven & van Gurp, 2012; Liu *et al.*, 2015; Herman & Sultan, 2016). Comparison of treated vs. untreated plant samples of the same genetic and epigenetic background then allows for estimation of the range and importance of plant 'epigenetic memory' (Herman & Sultan, 2016; Rendina González *et al.*, 2016b).

Although the experimental demethylation represents an easy and elegant tool of epigenetic research, it also has some serious limitations. 5-azacytidine, a commonly used demethylation agent with a high demethylating efficiency, was also shown to have significantly negative effects on plant development by causing various growth aberrations, especially in the

root part, and thus reducing plant fitness and viability (Akimoto *et al.*, 2007; Kondo *et al.*, 2007; Bossdorf *et al.*, 2010; Amoah *et al.*, 2012). This fact unfortunately substantially challenges the practical applicability of this approach and the ecological conclusions derived from those experiments.

Based on the findings of so far conducted studies on the role of epigenetics in plant ecology and evolution, this thesis aimed to contribute to improvement of the experimental demethylation methods by testing the efficiency of a newly proposed spray application of the demethylation agent 5-azacytidine, and to enhance the range of research on bioticaly induced trangenerational plasticity by a two generation competition experiment with practical usage of the novel demethylation method.

1.1. Transgenerational effects

Transgenerational effects can be defined as modifications of offspring phenotype induced by environmental conditions experienced by its parents, independently of the DNA sequence (Roach & Wulff, 1987a; Jablonka & Lamb, 1995; Rossiter, 1996; Mousseau & Fox, 1998; Galloway, 2005). In the past they were often referred to as maternal effects since it had been thought they were mediated solely by the additional egg material, i. e. via cytoplasmic genetics (plastids and mitochondria), endosperm or via phenotypic based structure of the maternal tissues immediately surrounding the developing embryo and endosperm (Roach & Wulff, 1987a; Rossiter, 1996; Mousseau & Fox, 1998). Maternal effects were recognized in 1909 (Correns, 1909) and for a long time they had been considered a ‘troublesome’ source of error as they violated the rules Mendelian system and so reduced the precision of genetic studies (Roach & Wulff, 1987a). Not earlier than in the late 80's an increased attention started to be paid to this important source of non-genetic inheritance that can have a substantial impact on plant phenotype. Since then the potential of maternal effects for an adaptive environmental response of the offspring has been increasingly recognized (Roach & Wulff, 1987a; Dudley, 1991; Mousseau & Fox, 1998; Wolf *et al.*, 1998; Agrawal *et al.*, 1999; Agrawal, 2002).

With the increasing amount of research concentrated on the significance of maternal effects in plant ecology and evolution, it has been revealed that an adaptive response of progeny is able to persist for several generations (Fieldes & Amyot, 1999; Akimoto *et al.*, 2007; Whittle

et al., 2009; Kou *et al.*, 2011; Herman *et al.*, 2012). Such long-term persistence could not be explained by simple egg provisioning any more as such transmission can only influence the direct progeny. Transmission of acquired phenotypic characteristics over several generations required mechanisms, which would be highly plastic but would also dispose of some stability (Herman & Sultan, 2016). This function can be provided by mechanisms able to modulate DNA expression while maintaining DNA sequence unchanged. Such mechanisms are altogether called epigenetic processes.

Once understood that maternal provisioning is not the only source of transgenerational change, the term maternal effects started to be substituted by the term parental or even better transgenerational effects, especially in studies of sexually reproducing plants, where the paternal influence can not be excluded.

1.1.1. Adaptive significance of transgenerational effects

Probably the most important property of transgenerational effects for plant ecology and evolution is its adaptive potential. The ability to pass information about the biotic or abiotic conditions the parent lived in onto the following generation to mediate their increased fitness or competitive ability opens a possibility of directed microevolution resonating with the Lamarckian idea of evolution, which seemed inconceivable from the genetical point of view (Wolf *et al.*, 1998; Akimoto *et al.*, 2007; Richards *et al.*, 2010; Hauser *et al.*, 2011; Zhang *et al.*, 2013; Zuppinger-Dingley *et al.*, 2014).

Of course, it is not reasonable to attribute these processes to some mysterious information that telepathically travels between the parent and the progeny telling it to create novel phenotypic traits to prosper better. The process of adaptation works in two phases. The first phase is commonly known and not shocking – it is the ability of plants to adapt to abiotic conditions such as amount of light, moisture, nutrients etc. and biotic conditions such as herbivory or competition by adjusting their phenotypic traits. These changes develop gradually during plant ontogenesis in response to direct environmental stressors, increasing the chance of the individual to survive and compensate for possible losses (Dyer *et al.*, 2010). Nevertheless, these changes have no chance to incorporate into the DNA sequence and so they were thought to be reset by the process of meiosis (Feng *et al.*, 2011; Paszkowski & Grossniklaus, 2011).

Surprisingly, it has been shown that the phenotypic adjustments acquired during ontogeny can endure the process of reproduction and be passed on the progeny making them pre-adapted for the environmental conditions experienced by their parents (Jablonka & Lamb., 1989; Donohue & Schmitt, 1998; Dyer *et al.*, 2010). Such transgenerational effects represent the second phase of adaptation. They are considered adaptive in cases in which offspring have higher fitness in environments resembling parental conditions (Galloway, 2005; Galloway & Etterson, 2007; Chen *et al.*, 2014; Latzel *et al.*, 2014).

1.1.2. Functional traits as a response to abiotic and biotic stressors

Phenotypic functional traits are a key mechanism for estimating plant fitness, adaptation and transgenerational inheritance. They represent measurable plant characteristics referring to morphological, physiological and life-history properties of plants that can directly or indirectly influence their fitness and competitive ability (Violle *et al.*, 2007), which made them an essential tool of most ecological studies. Following plant phenotypic development in response to biotic and abiotic condition throughout several generations by measuring more or less complex parental and offspring functional traits pushes the research on transgenerational plant adaptation forward. By the means of measuring various growth-related functional traits, heritable plastic response to numerous stressors was repeatedly demonstrated. For example, previous studies have shown transgenerational phenotypic response to disturbance and nutrients in perennial *Plantago* species (Latzel & Klimešová, 2010; Latzel *et al.*, 2010), to drought and nutrients in an annual *Arabidopsis thaliana* (Zhang *et al.*, 2013), to drought stress alone in an annual *Polygonum persicaria* (Herman & Sultan, 2016), impact of drought and herbivory on growth-related traits of ramets in clonal *Trifolium repens* (Rendina González *et al.*, 2016a,b), trangeneratrionally induced resistence to herbivory in an annual *Raphanus raphanistrum* (Agrawal, 2002), transgenerational phenotypic response to hormones providing defence against herbivory and pathogens in *Arabidopsis thaliana* (Latzel *et al.*, 2012), increased leaf trichome density in response to parental leaf damage in both annual and perennial populations of *Mimulus guttatus* (Holeski, 2007) or heritability over several generations of as complex trait as flowering initiation time in *Arabidopsis thaliana* (Johannes *et al.*, 2009).

It is quite obvious that, except for the herbivory defence, studies of abiotic stressors are much more broadly represented than studies on bioticaly induced transgenerational effects,

particularly studies considering maternal competition. Nevertheless, the importance of competition in transgenerational inheritance of plant communities was well demonstrated in the study by Zuppinger-Dingley *et al.* (2014). The study consisted of experimental plant communities of 12 grassland species that were grown either in monocultures or in mixtures over several generations. Progeny of those communities were then planted in control conditions in both mixture or monoculture conditions and their biomass and functional traits were measured. Increased character displacement between species and higher net biodiversity effect was observed in test communities of mixture types (progeny of mixed communities) indicating niche differentiation and, thus, pre-preparation for competitive conditions. Contrarily, test communities of monoculture types (progeny of monoculture communities) exhibited increased intraspecific trait variation indicating pre-preparation for better prosperity in monocultures due to broader spread of phenotypes. Both mixture and monoculture types showed better performance when grown in the parental conditions, suggesting transgenerational adaptive response to parental competitive conditions (Zuppinger-Dingley *et al.*, 2014)

1.2. Epigenetic processes

Growing body of evidence suggests that transgenerational phenotypic variability might not only be conditioned genetically and modulated through ovule or seed provisioning but it can also be induced epigenetically. Epigenetic processes are a suit of molecular mechanisms able to alter gene expression and function without changes in DNA sequence (Richards, 2006; Bird, 2007; Zhang *et al.*, 2013) and so provide a plastic response to the environment during plant ontology with a potential to be transmitted to the next generation (Akimoto *et al.*, 2007; Bossdorf *et al.*, 2008; Jablonka & Raz, 2009; Johannes *et al.*, 2009; Amoah *et al.*, 2012). These processes affect chromatine structure by epigenetic marks superimposed on the DNA sequence of eukaryote chromosomes and include histone modification, RNA interference and DNA methylation (Bird, 2007; Bossdorf *et al.*, 2010; Amoah *et al.*, 2012).

1.2.1. DNA methylation

Among known epigenetic processes, DNA methylation is the most studied, best understood and possibly even the most important one (Akimoto *et al.*, 2007; Pavlopoulou & Kossida, 2007; Reinders *et al.*, 2009; Bossdorf *et al.*, 2010; Zhang *et al.*, 2013; Kanchanaketu & Hongtrakul,

2015). Methylation of cytosine at the 5 position of the pyrimidine ring has been found in most eukaryotes and plays a role in the gene repression and in overall genome stability through controlling transposable elements (Akimoto *et al.*, 2007; Pavlopoulou & Kossida, 2007; Johannes *et al.*, 2009; Hauser *et al.*, 2011; Moricová *et al.*, 2013; Colicchio *et al.*, 2015a; Kanchanaketu & Hongtrakul, 2015). While the level of cytosine methylation ranges from 3 to 8 % in vertebrates, its prevalence in plants can be several times higher, ranging from 6 to 30 or even 40 % of methylated cytosines (Gruenbaum *et al.*, 1981; Klaas *et al.*, 1989; Finnegan E. J. *et al.*, 1998; Chen & Li, 2004). High level of cytosine methylation in plants is attributed to the fact that while in animal DNA only CpG (cytosine-phosphate-guanine) dinucleotides can be methylated, in plants cytosine methylation is possible in both CpG dinucleotides and CpNpG or CpNpNp trinucleotides, where N stands for any nucleotide (Gruenbaum *et al.*, 1981). Moreover, unlike animal, plant methylation paterns in symmetric CpC and CpNpG sequences can be inherited over several generations (Kakutani, 2002; Jablonka & Raz, 2009; Feil & Fraga, 2012). Methylation of asymmetric CpNpNp trinucleotides is usually considered nonheritable (Jones *et al.*, 2001).

Although the methylome appears to be relatively stable within an individual, it exhibits predictable plastic responses to environmental stimuli (Kinoshita & Jacobsen, 2012; Bond & Baulcombe, 2014). Various biotic and abiotic environmental factors are associated with cytosine methylation induction and establishment of epiallels (Tatra *et al.*, 2000; Hauser *et al.*, 2011; Dowen *et al.*, 2012; Herman & Sultan, 2016). Such environmentally induced change in methylation can vary by genotype (Dubin *et al.*, 2015) and can mediate an adaptive response to stressors in natural populations (Tatra *et al.*, 2000). Together with its multi-generational heritable potential, this makes DNA methylation a prime candidate for transgenerational inheritance (Colicchio *et al.*, 2015a).

1.2.2. Spontaneous cytosine methylation in natural populations

Recent studies have demonstrated that spontaneous DNA methylation occurs in natural plant populations and such naturally emerged epialleles can have phenotypic consequences (Cubas *et al.*, 1999; Bossdorf *et al.*, 2010; Becker *et al.*, 2011; Zhang *et al.*, 2013) and influence a range of characters such as flower shape or plant pigmentation (Cubas *et al.*, 1999; Manning *et al.*, 2006). Spontaneous epimutations appear to accumulate gradually like genetic mutation but with

many orders of magnitude higher frequency and with an evidence of recurrent cycles of forward and reverse mutations fluctuating over relatively short timescales (Bossdorf *et al.*, 2010; Ossowski *et al.*, 2010; Becker *et al.*, 2011). Nevertheless, not all DNA regions are equally prone to epigenetic mutations. Methylation of regulatory regions and of transposable elements exhibits much higher stability compared to gene-bodies methylation likely because mutations in those regions would have much stronger and riskier impact on plant development (Zhang *et al.*, 2006; Vaughn *et al.*, 2007; Lisch, 2009; Ossowski *et al.*, 2010; Becker *et al.*, 2011). In *Arabidopsis thaliana*, CpG methylation in regulatory sequences has been demonstrated to correlate negatively with gene expression (Zhang *et al.*, 2006; Zilberman *et al.*, 2007), presumably through limiting promoter accessibility (Colicchio *et al.*, 2015a). Gene body CpG methylation, on the contrary, had much softer consequences being found in moderate to highly expressed genes (Gruenbaum *et al.*, 1981; Zilberman *et al.*, 2007; Li *et al.*, 2012). Similarly, DNA methylation has an important role in inhibiting movements of transposable elements, DNA sequences able to change their position within the genome causing possibly mutagenic alternation of DNA chain. Naturally emerged differential DNA methylation therefore tends to occur farther from transposable elements, implying that the density and distribution of transposable elements, which can differ greatly even between closely related species, affect epigenetic variation throughout the genome (Becker *et al.*, 2011; Hollister *et al.*, 2011). However, in studies using experimental demethylation generated by demethylation agent application, it is not possible to rule out the option that at least part of the observed phenotypic variation can result from DNA sequence changes induced by movement of transposable elements (Fieldes & Amyot, 2000; Johannes *et al.*, 2009; Bossdorf *et al.*, 2010; Zhang *et al.*, 2013).

1.2.3. Methyltransferases and their functions

DNA methylation is enabled by the functioning of methyltransferases, group of enzymes responsible for recognision of a specific DNA sequence and for catalyses of the transfer of a methyl group from the cofactor S-adenosyl-L-methionine to carbon 5 in the pyrimidine ring of cytosine residue (Kumar *et al.*, 1994; Pavlopoulou & Kossida, 2007). Plant genome methylation consists of two separate activities, which together create the methylome of an individual plant during its onthogeny, reflecting both transgenerational epigenetic inheritance and present

environmental conditions. They are 'maintenance' methylation, providing preservation of existing methylation patterns after DNA replication, and *'de novo'* methylation allowing for emergence of novel epialleles by methylation of previously unmethylated sites (Chen & Li, 2004; Pavlopoulou & Kossida, 2007). So far identified plant C5 methyltransferases are classified into four main families. Domains–rearranged methyltransferases accomplish the function of 'de novo' methylation, while methyltrasferases and chromomethyltransferases presumably procure the 'maintenance' of GpG and CpNpG methylation, respectively. The role of the last family, DNA methyltransferase homologue 2, in DNA methylation remains largely unexplained (Bartee & Bender, 2001; Cao *et al.*, 2003; Pavlopoulou & Kossida, 2007).

1.3. Methods to explore the significance of epigenetic processes

The proportion of phenotypic variation that is brought about by epigenetics alone is hard to assess (Johannes *et al.*, 2009), as some relationship between genetics and epigenetics exists and different genotypes often respond differently to environmental conditions (Bossdorf *et al.*, 2010; Richards *et al.*, 2010; Zhang *et al.*, 2013; Herman & Sultan, 2016). To avoid this problem in epigenetic studies, several research methods were developed. There are advanced molecular methods allowing for direct quantification of epigenetic marks, which require sophisticated and computationally demanding techniques such as real-time PCR (Pecinka *et al.*, 2009), methylation-sensitive amplified fragment length polymorphism (MS-AFLP; Herrera & Bazaga, 2010; Paun *et al.*, 2010; Preite *et al.*, 2015; Foust *et al.*, 2016), whole-genome bisulphite sequencing (WGBS; Becker *et al.*, 2011; Colicchio *et al.*, 2015a; Keller *et al.*, 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 pre-requisite for analysing the obtained DNA methylation profiles. However, full genome information is scarce for non-model plants from natural ecosystems (Ellegren, 2014).

Therefore, alternative approaches have been developed to facilitate the research on ecological epigenetics. They are mostly based on experimental altering of the epigenetic status of the study plant (Johannes *et al.*, 2009; Bossdorf *et al.*, 2010). One of the possibilities is growing epigenetic recombinant inbred lines (epiRILs), created through artificial crossing resulting in populations of nearly identical genetic background but highly variable at the epigenetic level (Johannes *et al.*, 2009; Reinders *et al.*, 2009; Latzel *et al.*, 2012; Zhang *et al.*,

2013). Another method is focused on artificial changing of the DNA cytosine methylation level using chemicals called demethylation agents. Demethylation agents are small biomolecules interacting with methyltransferases, ezymes responsible for incorporating methyl residuals into DNA, and inhibiting their function. Such treatment results in partial demethylation or hemimethylation of the genome (e.g. Jones, 1985; Burn *et al.*, 1993; Tatra *et al.*, 2000). Agents commonly used for experimental reduction of cytosine methylation include zebularine (Baubec *et al.*, 2009; Verhoeven & van Gurp, 2012; Liu *et al.*, 2015; Herman & Sultan, 2016) and 5-azycytidine (Tatra *et al.*, 2000; Bossdorf *et al.*, 2010; Amoah *et al.*, 2012; Vergeer *et al.*, 2012; Rendina González *et al.*, 2016a,b). Comparing treated vs. untreated plants enables testing of the importance of past environmental interactions, or the so-called 'epigenetic memory', on plant phenotypic plasticity (Herman & Sultan, 2016; Rendina González *et al.*, 2016a,b).

1.3.1. Demethylating agent 5-azacytidine

5-azacytidine (5-azaC) is a chemical analogue of cytidine, a nucleoside in RNA and DNA. It was originally developed as a drug for cancer treatment (Čihák, 1974; Jones, 1985). At low doses, it inhibits DNA methyltransferases, which can be used in non-clinical studies for *in vitro* removal of methyl groups from DNA (Martens, 2014). 5-azaC incorporates into DNA in place of cytidine nucleoside during replication; there it forms covalent complexes with methylteransferase group MET1 and thereby inhibit its function, which results in overall DNA hypomethylation (Santi *et al.*, 1983; Burn *et al.*, 1993; Cheng *et al.*, 2005; Baubec *et al.*, 2009; Bossdorf *et al.*, 2010; Kanchanaketu & Hongtrakul, 2015). Specific interference of 5-azaC with MET1 gives it a special desirable quality. MET1 is known to mediate 'maintenance' methylation of CpG site, which are associated with exonic DNA in plant (Finnegan, 1996; Saze *et al.*, 2003; Reinders *et al.*, 2009; Amoah *et al.*, 2012). Thus, demethylation by 5-azaC can provide a selective targeting of coding-sequence (Amoah *et al.*, 2012), moreover, deleting originally present methylation patterns in gene bodies. Another obvious advantage of using a demethylation agent for experimental demethylation is the possibility to create and compare different epigenetic variants of the same genotype. Thanks to relatively simple applicability and processability of such demethylation method it allows for analyzes of a solid amount of samples and so for generalization across different genetic backgrounds as well as for multi-factorial experiments (Bossdorf *et al.*, 2010).

Nevertheless, usage of 5-azaC is joined with several important negatives. The most crucial limitation of the demethylating agent is its toxicity to seeds germinated even in low concentrations of the chemical (Akimoto *et al.*, 2007; Amoah *et al.*, 2012). Plants grown from treated seeds exhibit various grow-related aberrations including dwarfism and radical reduction of root development leading to decreased fitness and viability of plant (Akimoto *et al.*, 2007; Kondo *et al.*, 2007; Bossdorf *et al.*, 2010; Amoah *et al.*, 2012; Kanchanaketu & Hongtrakul, 2015). There has also been demonstrated a substantial influence of 5-azaC on flowering time initiation (Burn *et al.*, 1993; Fieldes & Amyot, 1999; Kondo *et al.*, 2007). All these side effect of 5-azaC complicate its applicability in ecological epigenetic research, as they can confound the conclusions driven from observed phenotypic differences between treated and untreated plants. The other problematic quality to be mentioned is the toxicity of the chemical for human., especially negative effect on male germ cells have been reported (Doerksen & Trasler, 1996; Doerksen *et al.*, 2000; Tunc & Tremellen, 2009). Therefore, the chemical should be treated with utmost caution, particularly because it requires daily preparation of fresh solution due to its instability in water environment.

1.4. Aims of the thesis

The first aim of this thesis was to test the efficiency of a novel method of experimental demethylation with potentially less disturbing effects on the study plant development. While according to the traditional approach plant seeds are soaked in the water solution of 5-azaC during 10 days of germination (Vergeer *et al.*, 2012), the novel method is based on daily spraying of the 5-azaC solution of the same concentration on plant seedlings after 10 days of germination in pure water. It was hypothesized that later application of the chemical after establishment of the principal organs could reduce the developmental damage, particularly allow for normal root development, which was the crucial limitation of survival in the germinating method (Kanchanaketu & Hongtrakul, 2015). A climabox experiment was designed to demonstrate that the spray application method is able to overcome the growth-related limitations of the germinating method while maintaining demethylating efficiency.

The second aim was to search for heritable epigenetic effects induced by maternal competition in *Taraxacum brevicorniculatum* by measuring growth related traits, which appear to be particularly important during early stages of offspring development (e.g. Metz *et al.*, 2015;

Vu *et al.*, 2015; Walter *et al.*, 2016). In this experiment an interspecific and intraspecific competition was used as a biotic environmental factor affecting the development of the parental population of *T. brevicorniculatum* in the greenhouse. Collected seeds were germinated and grown in control conditions of a growing chamber, where a half of the germinating seedlings was experimentally demethylated using the novel spraying application of 5-azaC solution. Differences in functional traits in early stages of development were evaluated among the progeny of different parental treatments and between progeny of the same parental treatment but with different methylation status (treated vs. untreated with 5-azaC) to demonstrate the impact of the strength of maternal competition on the early development of progeny and the ability of 5-azaC spray application to remove the plant 'epigenetic memory', respectively.

2. Methods

Two separate experiments were conducted (1) to compare the effectiveness and favourableness of the proposed novel method of experimental DNA demethylation via 5-azacytidine spray application with the traditionally used germinating method and (2) to test for the effect of parental plant competition on early developments of progeny through heritable DNA methylation.

2.1. Study species and seed material

Taraxacum brevicorniculatum Korol. was used as the focal species in both experiments. *T. brevicorniculatum* is a Central Asian species, whose all available *ex situ* seed collections were recognised to belong to a single triploid apomictic clone (Kirschner *et al.*, 2013). Genetically identical seeds collected from a greenhouse-grown population of plants experiencing equal conditions for several generations and genetically identified by Kirschner *et al.*, 2013 were obtained for these experiments. This strategy reduced the effect of genetic and epigenetic variation in the experimental seed samples and the obligate apomixes of the species allowed for further control of the genetic uniformity during the experiments. It should be noticed that, although *T. brevicorniculatum*, is not itself typical from Central European flora, it owns to the group of *T. ruderalia*, being functionally similar to species present in the Central European

flora. Its advantage over local species is that all individuals are genetically identical, so that it can be better employed for studies on maternal effects.

2.2. Experiment 1 – comparison of spraying vs. germinating treatment for experimental demethylation

2.2.1. Experimental design

Seeds used in this experiment were the first generation progeny of the seeds obtained from Kirschner *et al.*, 2013, that were grown in a greenhouse without competition in the NC treatment of the Experiment 2 (see below). Seeds were thoroughly mixed, and 300 of them 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; Yang *et al.*, 2010; Vergeer *et al.*, 2012). 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) for another three weeks 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. 33 of these seedlings were then transferred into individual pots, where they received the novel 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 for three weeks. 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 for three weeks.

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. Sand was used as the potting substrate in all cases to facilitate root removal during the harvest. Plants were grown in a chamber with a 12 h (20 °C) /

12 h (10 °C) light/darkness and temperature regime, and watered regularly to keep the substrate moist. The initial position of all 99 pots in the chamber was randomized to ensure uniform growing conditions.

2.2.2. Plant morphological measurements

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 shoot and root traits of 25 randomly selected 10-day-old seedlings. 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). 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.

After transplantation into pots, those plants that were transplanted into pots (n = 99) were grown for three weeks and then 96 were harvested (3 individuals in the G treatment died). The plant material was dried at 60 °C and the aboveground biomass weighted.

In addition, 10 C-S plant pairs form the progeny generation of the NC treatment (no competition) in the Experiment 2 were used to show impact of the spray treatment on the vegetative traits after 6 weeks of its daily application. These seedlings were grown from seeds of the same harvest and in the same growth chamber conditions as those previously mentioned. Two leaves from each plant were collected, and their water-saturated fresh mass and dry mass estimated to compute leaf dry matter content (LDMC; the ratio of leaf dry mass to leaf fresh mass, mg/g). Further, the aerial and root biomass of each of these plants was separated, and their final biomass estimated after drying at 60 °C.

2.2.3. DNA extraction and genome-wide DNA methylation estimation

To assess differences in genome-wide DNA methylation between treatments, DNA was extracted from the plants from the three-week experiment. We used both shoots and roots for the DNA extraction, as plants were still small at the time of harvest. 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). The absorbance of the ELISA-like assay (ELISA - enzyme-linked immunosorbent assay) was 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, and the measurement error was calculated by dividing the difference in methylation between the two replicates by the sum of the two methylation values. The percentage of measurement variation was averaged over all the data.

2.2.4. Statistical analyses

The effect of the treatments on the percentage of methylated DNA was analysed taking into consideration the two estimates for each individual, by means of a mixed effects model with binomial errors. In this model the identity of the individual was used as a random factor. An ANOVA was used to analyse the effects of the treatments on the final aboveground biomass of the seedlings. In both cases, a post hoc Tukey test was performed to see whether pairs of treatments differed significantly ($P < 0.05$).

Differences between plant traits in different treatments were evaluated both in the 10-day-old seedlings (G vs. C treatments; seedling root length and leaf area) and in the plants grown for 6 weeks in pots (S vs. C treatments; root and aerial biomass, and LDMC). These differences were analysed by means of t-tests in the case of the seedlings (root length was log-transformed to achieve normality), and paired t-tests in the case of the potted plants. All analyses were conducted using R v3.2.3 (R Core Team 2016).

2.3. Experiment 2 – test of the effect of parental competition on progeny's early development

2.3.1. Experimental design – maternal generation

For the purpose of growing a maternal population of the study plant *Taraxacum brevicorniculatum* experiencing various levels and kinds of competition a greenhouse experiment was established. 10 common Central European meadow species from 4 different functional groups were chosen for competitors. Functional group of tall forbs was represented by *Achillea millefolium, Leontodon hispidus* and *Plantago lanceolata*, short forbs were represented by *Dianthus deltoids, Plantago media* and *Prunella vulgaris*, legumes were exemplified by *Lotus corniculatus* and *Trifolium pratense*, and finally *Alopecurus praten*sis with *Holcus lanatus* stood for grasses. Genetically identical *Taraxacum* seeds of uniform environmental background were obtained from Kirschner *et al.*, 2013. Seeds of the competitor plants were ordered from a seed company (Planta naturalis, Markvartice u Sobotky, Czech Republic). All seeds were germinated in water in a growing chamber (12 h (20 °C) / 12 h (10 °C) light/darkness and temperature regime) for 2 weeks and then transplanted into round pots of 2 liter volume and situated in a greenhouse. Mixture of commercial lawn soil and sand in the ratio 1:1 was used as the growing substrate. Plants were grown in following combinations and replications per combination: zero competition conditions (1 *Taraxacum* plant in the center of the pot; no competition treatment – NC) in 16 replicates, intraspecific competition (1 central *Taraxacum* surrounded by 6 other *Taraxacum* plants; intraspecific treatment – Intra) in 8 replicates, interspecific competition with one species competitor (1 central *Taraxacum* surrounded by 6 individuals of one of the competitor species; single competitor treatment - SC) in 8 replicates for the 10 possible competitors and interspecific competition with 6 species competitors (1 central *Taraxacum* surrounded by 6 competitors of 6 different species; functional diversity treatment - FD) in 5 replicates for each of the 8 designed combinations of species (Tab. I.). Combinations of competitors were selected with respect to their functional distance. Plants were watered in the trays with frequency depending on the weather conditions, to minimize water stress. The maternal generation was grown in the greenhouse for 9 weeks from late May till the end of July; temperature or light conditions were not regulated in the

greenhouse. Pots were distributed randomly to balance spatial heterogeneity of growing conditions.

2.3.2. Seed collection and biomass yield

During the last week of the greenhouse experiment, all mature seeds of the central *Taraxacum* plants were collected daily. Finally, seeds of all replicates of each treatment were mixed together, 25 seeds of each treatment were randomly picked 10 times, weighed and used for the estimation of the average seed weight of each treatment. Seeds were stored in a fridge to be used for later planting of progeny generation of known parental competitive conditions. Dry biomass yields (80 °C / 48 h) of the focal *Taraxacum* plants were assessed at the time of harvest to estimate the intensity of competition experienced by the focal *Taraxacum*.

2.3.3. Experimental design – offspring generation

Seeds of each of the 20 maternal competitive conditions (10×SC+1×Intra+1×NC+8×FD; see Tab. I.) were thoroughly mixed, several seeds (to ensure establishment of at least one individual) of the same maternal treatment were sown in the middle of an individual pot (square-shaped pots of 7 × 7 cm and 18 cm depth) and placed in a growing chamber (12 h (20 °C) / 12 h (10 °C) light/darkness and temperature regime). After germination of the first seed in a pot, this seedling was noted and further watched, all additional sprouts were removed to set standardized non-competition conditions with one individual per pot. Pots were organized in 10 blocks to assure equal growing condition. Each block included 20 pots with seedlings of all maternal treatments (Tab. I) arranged randomly within one tray and another tray with 20 pots copying the treatment positions of the previous one. One of the trays in each block was daily demethylated by the means of spray application of 50 μM 5-azaC (+ surfactant), the other tray was only sprayed with water solution of surfactant to exclude possible confounding effects of the surfactant. The 5-azaC treatment started to be applied after germination initiated in all the pots. Sand was used as the potting substrate to facilitate the root removal. Plants were watered regularly (every second day) to keep the substrate moist. The offspring generation all together counted for 400 pots with one individual per pot (20 maternal treatments by 2 in 10 blocks).

2.3.4. Measurements of the early development traits of the progeny

Two growth related traits, number of leaves and rosette diameter, started to be measured on the day of the first application of 5-azaC (day 0) and continued to be measured every 4 days until the day 36 and then for the last time on the day 42. At the time of the harvest, various above- and belowground vegetative traits were estimated. Root and aerial biomass and LDMC estimates of NC maternal treatment progeny was used for the demonstration of resetting plant memory by 5-azaC spray application in Experiment 1. Analyzes of the other final traits are not included in this thesis.

2.3.5. Computations and statistical analyses

Dry biomass of the maternal *Taraxacum* generation was used to assess the strength of competition experienced by the focal plant in different treatments through computing the relative interaction index (RII; Eq. 1) for mean biomass yield of each treatment proposed by Armas *et al.* (2004):

$$\mathrm{RII} = \frac{\mathrm{B_w} - \mathrm{B_o}}{\mathrm{B_w} - \mathrm{B_o}}$$

Equation 1

In the equation, B_o represents a trait value of a target plant growing in absence of inter- or intraspecific competition (mean biomass of *Taraxacum* from NC treatment in this case), while B_w represents a trait value observed when a target individual is growing in interaction with other plants (mean biomass of *Taraxacum* from Intra, SC1-10 and FD1-8 treatments successively). Values of the RII range from -1 to 1, being positive in predominance of facilitation and negative in predominance of competition.

Two different growth rates were estimated in each pot, including number of leaves and rosette diameter, respectively. In order to get an indication of the temporal evolution of these rates, they were estimated considering different time periods, always starting on the day 0 (first day of 5-azaC application) and finishing on days 16, 20, 24, 28, 32, 36 and 42. This way, the growth rates estimated on the day 16 (D16 in Fig. 4) included the data from days 0, 4, 8, 12 and 16, whereas the growth rates estimated on the day 42 (D42 in Fig. 4) included all the available

data (from day 0 to day 42 measured in periods of 4 days, except of the last measurement conducted with 6 day distance). Growth rate in the rosette diameter in each pot was then estimated as the slope of a linear regression where diameter was the response variable and time in days the explanatory variable. Leafing rate was calculated in a similar way, but using a regression with a Poisson distribution, since number of leaves can only have positive integer values.

Then, the relationship between the level of competition experienced by the mother plant (RII) and the growth rates estimated at the different times periods was analyzed. On that account, for each considered day of measurement each type of growth rate was regressed on RII by the means of a mixed model, where RII was used as a fixed effects variable and the block identity as a random factor. The relationship was considered significant, when $P < 0.05$. Models were run separately for plants with and without 5-azaC treatment.

Since the level of competition can also affect the size of the seeds produced by the mother plant, which in turn can potentially have an effect on the development of seedlings, the seed mass was included as a covariate in all the models. All analyses were conducted using R v3.2.3 (R Core Team 2016).

3. Results

3.1. Experiment 1 – comparison of spraying vs. germinating treatment for experimental demethylation

Compared to the C treatment, DNA methylation was significantly reduced in both treatments using the 5-azaC demethylation agent, either during germination (Tukey post hoc test germinating treatment vs. control, G vs. C, $P = 0.005$), or by spraying leaves of established seedlings (spraying vs. control, S vs. C, $P = 0.041$). Most importantly, no difference was found in the levels of DNA methylation between the germinating and the spraying demethylation approaches (S vs. G, $P = 0.257$; Fig. 1a). The measurement error in the estimations of DNA methylation was 6.04 %, which does not question the detected decline of DNA methylation counting for about 20 % in both demethylating treatments.

There were no significant differences in the final aboveground plant biomass between the spraying treatment and the control (S vs. C; Fig. 1b). The germinating treatment (G), on the contrary, substantially decreased plant performance in terms of final aboveground biomass ($P < 0.001$; Fig. 1b), both in relation to the control and to the spraying treatment. Seedlings that germinated in 5-azaC solution developed roots remarkably smaller than seeds that germinated in water (C vs. G t-test: $t = 43.967$, $df = 65.63$, $P < 0.001$; Fig. 1c and Fig. 2), as well as smaller leaves ($t = 2.228$, $df = 44.86$, $P = 0.031$; Fig. 1d and Fig. 2).

Finally, no difference were detected in subsequent development between the spraying treatment and the control, either in final aboveground biomass (paired t-test C vs. S: $t = -1.235$, $df = 9$, $P = 0.248$; Fig. 3a), in final belowground biomass ($t = -1.543$, $df = 9$, $P = 0.157$; Fig. 3b), or in LDMC ($t = 0.467$, $df = 9$, $P = 0.652$; Fig. 3c).

3.2. Experiment 2 – test of the effect of parental competition on progeny's early development

The relationship between the strength of maternal competition (RII) and the growth rate estimated with leafing was found significant in normally methylated plants, but not in demethylated ones. Leafing rate of plants without 5-azaC treatment increased in response to declining RII in all considered time periods of 16 – 42 days ($P_1 = 0.012$, 0.008, 0.049, 0.012, 0.03, 0.046 and 0.032, respectively; Fig. 4a, untreated). On the contrary, no significant effect was found between RII and leafing rate in demethylated plants ($P_1 = 0.466$, 0.326, 0.397, 0.327, 0.272, 0.309 and 0.305, respectively; Fig. 4a, 5-azaC treated). When seed mass was used to explain the variability in leafing rate of untreated plants, positive significant correlation occurred in time periods of 16, 20 and 28 days ($P_2 = 0.019$, 0.048 and 0.049, respectively; Fig. 4a, untreated). Time periods of 24, 32, 36 and 42 days showed no correlation between seed mass and leafing rate ($P_2 = 0.12$, 0.122, 0.2 and 0.154, respectively; Fig. 4a, untreated). Similarly, no significant effect of seed mass on leafing was found in the demethylated plants in all time periods ($P_2 = 0.218$, 0.122, 0.16, 0.09, 0.103, 0.133, 0.156, respectively; Fig. 4a, 5-azaC treated).

In terms of rosette diameter, significant effects were found neither in response to RII ($P_1 = 0.644$, 0.57, 0.538, 0.583, 0.577, 0.539 and 0.482, respectively; Fig. 4b, untreated) nor in

response to seed mass (P_2 = 0.084, 0.113, 0.136, 0.145, 0.205, 0.341 and 0.592, respectively; Fig. 4b, untreated) in normally methylated plants. Accordingly, there were no correlations with any explanatory variable in demethylation treatment, either (P_1 = 0.425, 0.434, 0.444, 0.47, 0.662, 0.687 and 0.817, P_2 = 0.202, 0.204, 0.208, 0.218, 0.15, 0.194 and 0.292, respectively; Fig. 4b, 5-azaC treated).

4. Discussion

4.1. Relevance of the 5-azaC spray application to recent ecological epigenetic research

Studying the epigenetic background of transgenerational effects is of growing importance in the field of plant ecology and evolution, as it can reveal the role of intraspecific trait plasticity in heritable plant adaptation to certain environment. Epigenome is relatively plastic compared to DNA sequence; epigenetic changes can be induced by biotic and abiotic factors and stay heritable over several generations. Observing the presence of epigenetic marks, therefore, introduces a great potential for research on adaptation and microevolution of plants. The experimental alteration of the level of DNA methylation in the offspring of plants from different environmental conditions appears to be a favourable method to explore the significance of epigenetic processes, with DNA methylation being one of the most common epigenetic marks. For that reason, demethylation agents such as 5-azacytidine are frequently used, being easy to apply, efficient in removing methylation marks, allowing for reasonable control and for generalization over various genotypes (Bossdorf *et al.*, 2010). Traditionally, seeds of the target plant are germinated in water solution of 5-azaC for several days. Nevertheless, most studies using this method of application recorded serious development aberrations leading to lowered biomass yield and viability of the treated plants, which can substantially bias trait measurements and conclusions drawn from them (Akimoto *et al.*, 2007; Kondo *et al.*, 2007; Bossdorf *et al.*, 2010; Amoah *et al.*, 2012; Kanchanaketu & Hongtrakul, 2015). Substantial part of the decline in fitness can be explained by especially poor development of roots (Kanchanaketu & Hongtrakul, 2015). For the purpose of overcoming the side effects of otherwise efficient and practical research method, a novel way of 5-azaC application was proposed and tested in this study. The novel application consists of spraying 5-azaC on already established seedlings previously

germinated in pure water for 10 days. As 5-azaC causes stochastic demethylation of random loci throughout the whole genome, some of the impaired sites can have crucial effect on the establishment of basic organs at the very beginning of the development (Akimoto *et al.*, 2007). Thereby, application after the initiation of a viable seedling could potentially prevent the fundamental growth limitations.

The present study tested for both efficiency and growth related effects of the spray application demethylation method. The reduction of the DNA methylation after application of 5-azaC showed comparable for the traditional germinating method and the novel spraying method (about 20% reduction), demonstrating equal efficiency of both methods (Fig. 1a). However, significant differences were detected in terms of prosperity of the seedlings. After 10 days of germination in 5-azaC solution (G treatment) vs. pure water (C and S treatment), the roots of the G treatment were markedly impaired in comparison to the rest of the plants (C and S treatments did not differ at his stage of the experiment, Fig. 2). The leaf area was also negatively affected after 10 days of germination in 5-azaC (Fig. 1d), referring to deficiency even in aboveground growth. The differences between treatments remained evident during the further development in pots; plants of the G treatment struggled to establish properly in the substrate and eventually started dying after 3 weeks. Contrastingly, the S treatment did not exhibit any visible differences compared to the control, which was confirmed by no significant distinction between the final biomass in S and C. G treatment, on the contrary, substantially decreased final plant biomass relative to both C and S (Fig. 1b). No negative effect of S treatment on plant functional traits was found even during the 'long-term' growth. There was no significant divergence in aerial biomass, root biomass or LDMC between S treatment and the control in 6 weeks old plants (Fig. 3), implying that the spray application of demethylation agent could allow normal growth of treated plants, while providing an efficient demethylation of their genome.

Differences between germinating and spray application of 5-azaC can have several explanation. DNA methylation marks can newly alter gene expression but also control the integrity of genome and stable expression of methylated sites over generations. Violation of the epigenome by experimental demethylation can have crucial consequences for the initial organ establishment in germinating seed. Furthermore, DNA methylation stabilizes the position of

nearby transposable elements, whose mobilization leads to induced change in DNA sequence (Akimoto *et al.*, 2007; Kondo *et al.*, 2007; Bossdorf *et al.*, 2010; Amoah *et al.*, 2012; Kanchanaketu & Hongtrakul, 2015). Finally, the possibility of classical mutations to primary DNA sequence induced by 5-azaC influence cannot be excluded as source of morphological differences between treatments (Fieldes & Amyot, 2000). However, this is highly unlikely since an absorbance-based ELISA-like assay showed notable and comparable hypomethylation levels in both of 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 spray application of 5-azaC is a practical and feasible way of *in vivo* alternation of plant epigenetic status, which makes it a very promising method for manipulation studies of ecological and evolutionary potential of epigenetic variation. It is especially valuable for experimental manipulation of non-model species (Verhoeven *et al.*, 2016), where the genomic sequence is not available, and of clonal plants (Rendina González *et al.*, 2016b), where the epiRILs cannot be created. However, it is important to stress that the method was only tested on broad-leaved herb species *T. brevicorniculatum*, 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. Therefore, in the case of using some potentially problematic species, a pilot study should be designed to verify the most adequate demethylation technique. Finally, a few studies indicate that the effects of demethylation agents can be transient since DNA methylation marks could be restored in somatic tissues formed after cessation of the treatment (Kumpatla & Hall, 1998; Baubec *et al.*, 2009). 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. Therefore, the proposed method of spraying 5-azaC solution onto the plants throughout the duration of the experiment will likely guarantee more stable and potentially inheritable demethylation effects.

The present experiment provides the first proper test of the spray application of 5-azaC on already established seedlings. Nevertheless, previous to this validation, the spray method was already successfully used in the study by Rendina González *et al.* (2016b) for resetting plant

memory in clonal species *Trifolium repens*. Rendina González *et al.* demonstrated that offspring ramets can 'remember' parental stress conditions (different drought levels and intensities) and reflect them by differences in growth parameters after transplanting to control conditions. The study further proved that this parentaly induced variation of offspring growth was erased when the parental plant was sprayed by 5-azaC preceding the ramet detachment. The second experiment included in this thesis also focused on transgenerational memory of maternal conditions and its resetting by 5-azaC application. However, in this case the apomictic species *T. brevicorniculatum* was used as the focal plant with controlled genetic background and different kinds and levels of competition represented the parental stress conditions.

4.2. The role of parental competitive conditions in progeny's early development

Competition is one of the poorly explored yet important biotic factors inducing transgenerational effects, as shown by Zuppinger-Dingley *et al.* (2014). Their study clearly demonstrated that parental competitive conditions meaningfully modulate the phenotype representation of individual species in future generations of the community. However, in the multi-generational field experiment by Zuppinger-Dingley *et al.* (2014), it was not possible to disentangle the role of selection effects (i.e. the individuals with less fit genotypes being excluded from a community) and potential transgenerational effects in response to plant-plant interactions. For this reason, the experiment 2 in this thesis was designed only to assess the latter.

In case of this thesis experiment, an effect of maternal competition on early development of the direct progeny was evaluated. Some studies indicate, that the transgenerational effects are particularly important in the early stages of development (Metz *et al.*, 2015; Vu *et al.*, 2015; Walter *et al.*, 2016). It can be explained, for example, by the lack of own environmental experience that would overweight the parental one, nevertheless, this should not happen in the control conditions. Transgenerational effect that are not supported by experiencing similar environmental conditions to the parental ones can shade off with time but there are studies demonstrating that these effects are rather persistent even over several generations of control conditions (Fieldes & Amyot, 1999; Akimoto *et al.*, 2007; Johannes *et al.*, 2009). Most importantly, some authors imply that strong transgenerational effects in the juvenile stages are caused by variances in maternal seed provisioning (Roach & Wulff, 1987b; Weiner *et al.*, 1997;

Latzel & Klimešová, 2010). Seed size is known to influence seed germination, seedling size and seedling competitive ability meaningfully (Roach & Wulff, 1987b). However, the correlation between seed mass and parental competitive conditions in not clear. Older studies argue that the importance of seed provisioning on seedling development should be increased in presence of competition (Wulff, 1986; Kromer & Gross, 1987; Aarssen & Burton, 1990). Weiner *et al.* (1997) tested for this effect of parental competition on seed provisioning and subsequent seed development. They confirmed positive correlation between seed mass and initial growth of seedling, nevertheless, this effect was not accelerated by competition. Moreover, they found no significant relationship between seed mass and different competitive treatments. The effects of seed provisioning on seedling development decreased over time. Similar result was demonstrated in Latzel & Klimešová (2010), who established a long-term study to follow the significance of seed quality and epigenetics for the ecology of perennial species. They suggest that seed provisioning might be of significant importance during the juvenile development but gradually disappears. Meanwhile, environmental effects pronounced by epigenetic marks outweigh the seed influence after some time and remain important even over several generations (Latzel & Klimešová, 2010).

The study presented in this thesis demonstrated significant influence of both maternal competition and seed mass on the rate of leaf initiation during first 6 weeks of established seedling development. These explanatory variables were evaluated together and separately, showing that the effect of maternal competition is not overridden by the effect of seed mass. The strength of maternal competition affected the leafing rate positively, i. e. the rougher competitive conditions (the more negative RII) the faster development of leaves (Fig. 4a, untreated). Such result indicates the tendency of seedlings coming from harsher competition to grow faster in order to gain size advantage before the plant interactions start to limit them. In terms of seed mass, negative correlation with the level of maternal competition was found, i. e. mothers exposed to stronger competition produced lighter seeds. This correlation in inconsistent with Weiner *et al.* (1997), who found no interaction between seed mass and experienced competition and was suggesting that reproductive output can only response through the number of seeds. However, it is also inconsistent with the preceding studies predicting mutually reinforcing effects of seed mass and competition (Wulff, 1986; Kromer & Gross, 1987; Aarssen & Burton, 1990). Hereby presented relationship between these two factors imply that effects of

seed mass and competition on the seedling development have opposing effects that actually weaken each other during the first weeks of development. Nevertheless, in agreement with both Latzel & Klimešová (2010) and Weiner *et al.* (1997), influence of the seed mass was found to decrease with time, being significant in the 1st, 2nd and 4th time period, appearing insignificant in the 3rd time period and remaining consistently insignificant throughout the 5th, 6th and 7th time period (Fig. 4a, untreated). The effect of the level of competition alone, on the contrary, exhibited significantly positive correlation with the leafing rate through all the 6 weeks of the experiment, suggesting that the biotic effect of competition affects plant development also in later stages, when seed provisioning influence has already disappeared (Latzel & Klimešová, 2010).

Further, the same analysis of the relationship between leafing rate and competition level and leafing rate and seed mass was applied on plants treated with the novel demethylation method, spray application of 5-azaC. Plants with removed methylation mark, however, exhibited no significant relationship between leafing rate and any of the two biotic factors (Fig. 4a, 5-azaC treated). This result indicates that demethylation managed to delete substantial part of the plant memory, supporting the idea that meaningful part of adaptive transgenerational effects works on epigenetic bases.

The absence of significant results for rosette diameter as a growth rate trait suggests that it may not be a good characteristic of the response to parental competition during early development of progeny. It cannot be excluded that response of this trait would express in later stages of development. However, it is also possible that the horizontal space limitations were of minor importance in the parental generation conditions.

Only a minor part of the second experiment was presented in this thesis, there were obtained additional data for later consideration and further analyses. This first outcome rather gives the first insight in a broader study of competitively induced transgenerational effects, which could enhance the ecological and evolutionary research with more relevant analyses in the future.

5. Conclusions

1) The novel method of spray application of 5-azaC solution on established seedling outstripped the traditional approach of germinating seeds in 5-azaC solution owing to elimination of negative effect on seedling growth related traits frequently associated with the germinating method. At the same time, the spray method proved equal to the germinating one in terms on demethylation efficiency. Therefore, the spray method of experimental demethylation can be recommended for future ecological epigenetic studies. Experiments using this method can potentially create ecologically more robust link between epigenetic variation and changes in plant phenotype, behaviour, or response to environmental stress.

2) Positive correlation between the strength of maternal competition (RII) and the rate of leaf initiation in early stages of *T. brevicorniculatum* development demonstrated transgenerational link between competition-induced stress in maternal generation and phenotypic change in progeny generation.

3) The seed mass correlated negatively with the strength of maternal competition, reducing the effect of RII on the leafing rate of progeny by advantaging seedlings coming from less competitive treatments. The effect of the seed mass disappeared after about 4 weeks of the measurement, while the effect of RII on the leafing rate persisted over all 6 weeks of the experiment. This suggests the maternal competition to be a longer-term biotic effect than the seed mass.

4) The spray application of 5-azaC managed to delete the 'epigenetic memory' in the *T. brevicorniculatum* seedlings, which showed no significant correlation between the leafing rate and RII or the seed mass under the demethylation treatment. It confirms that DNA methylation is one of the key epigenetic marks involved in transgenerational effects.

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7. Appendix – figures and tables

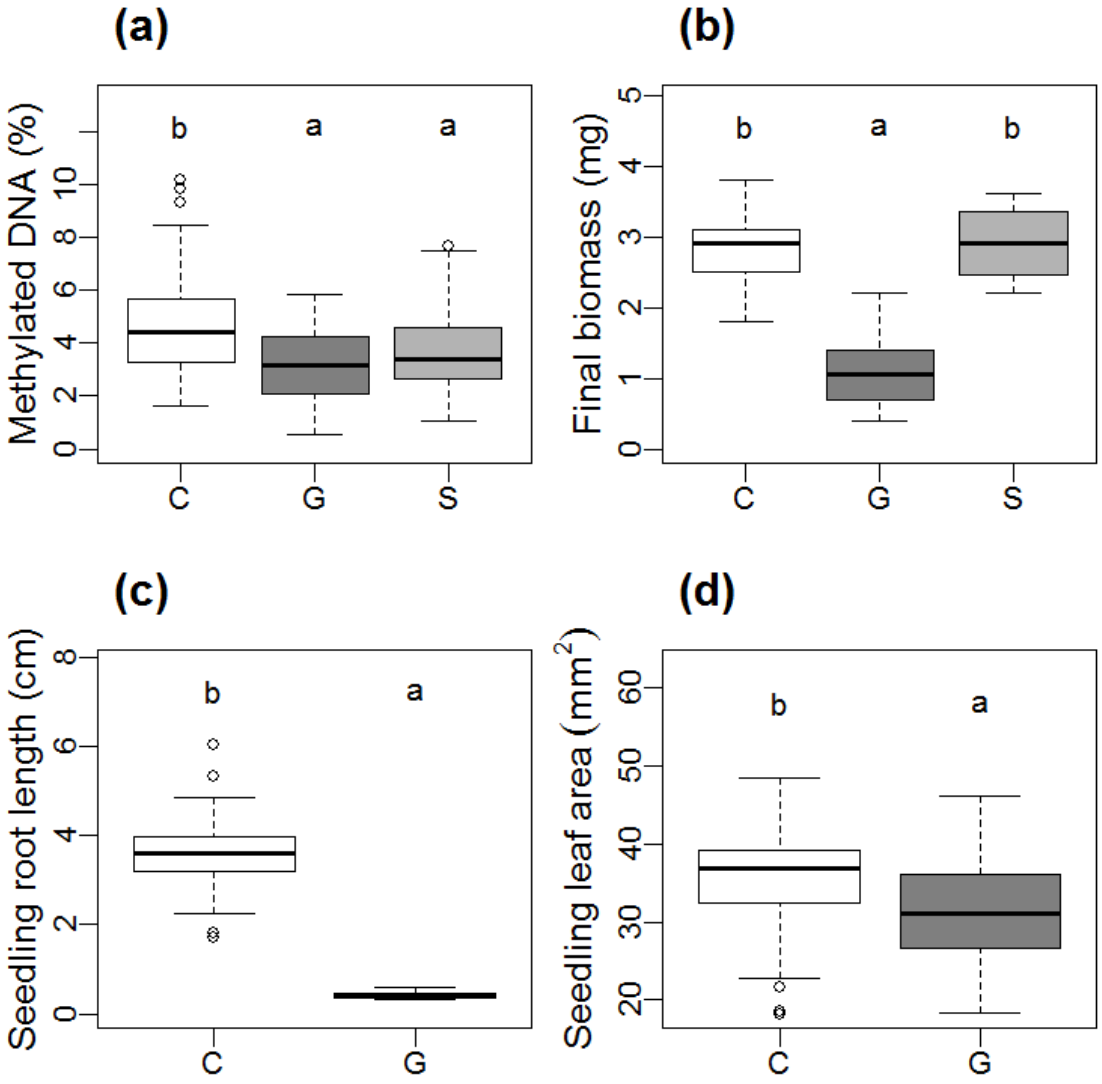


Figure 1: Differences between experimental treatments in the three-week-old seedlings (Experiment 1). (a) effects of the treatments (C - control, G - germinating method, S - spraying method) on the level of genome-wide DNA methylation and (b) on the aboveground biomass of the plants at the end of the three-week experiment. Lower panels show differences between 10-day-old seedlings germinated either in water (C) or a 50 μM water solution of 5-azaC (G) in (c) root length and (d) leaf area. Different letters within each panel indicate significant differences between treatments (P = 0.05).

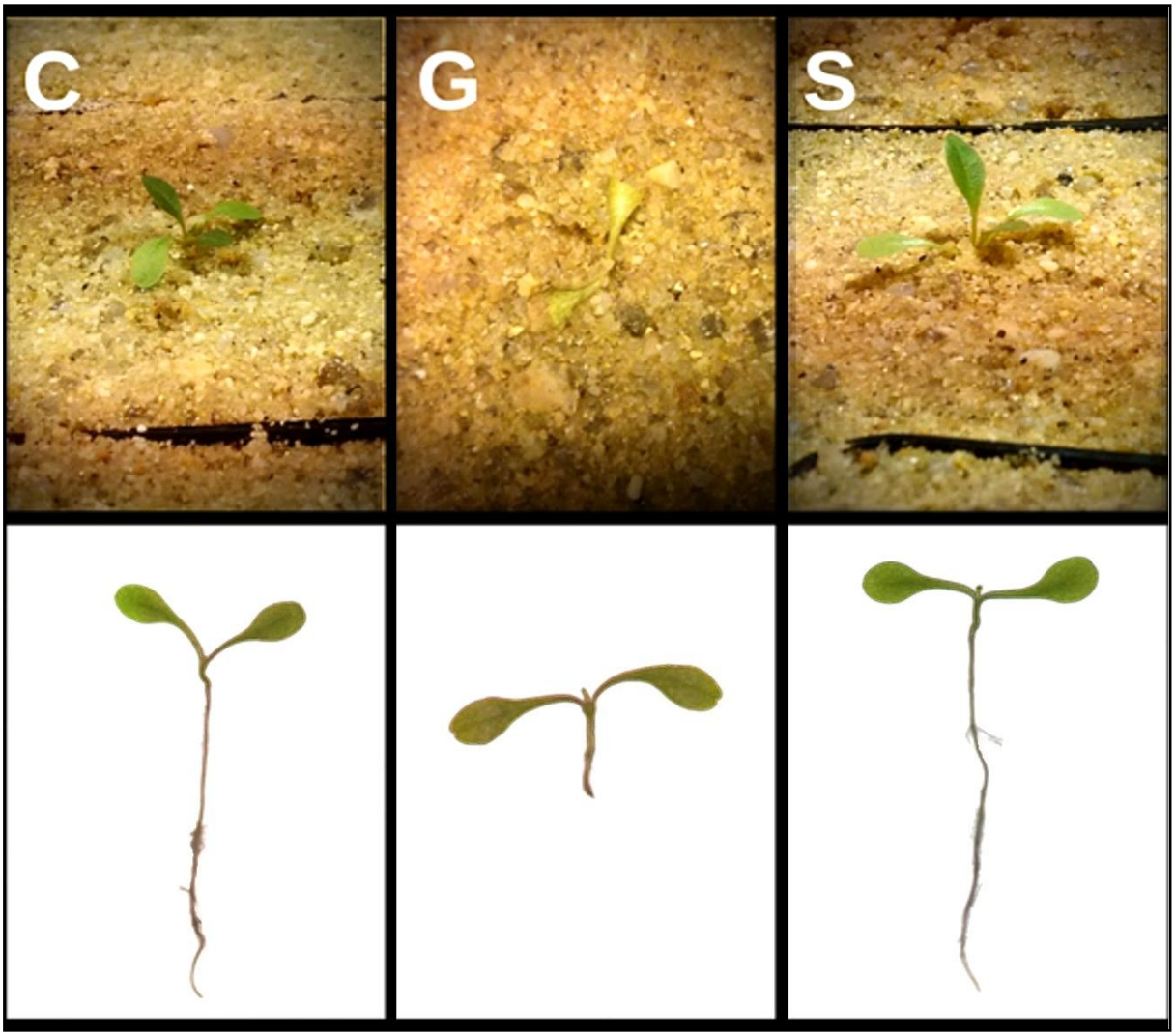


Figure 2: Details of the differences in early development of plants between the three treatments (C - control, G - germinating method, S - spraying method) in experiment 1. Upper row shows seedlings in the pots two weeks after transplanting, whereas the lower row displays some of the images of 10-day-old seedlings that were used to estimate root length and leaf area.

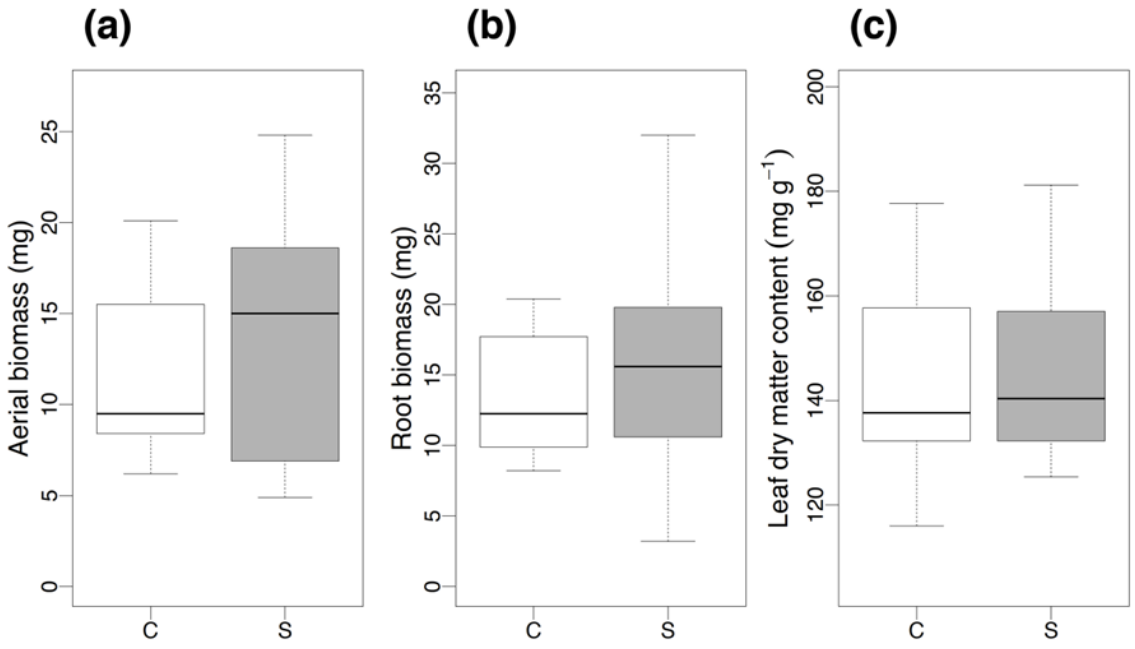
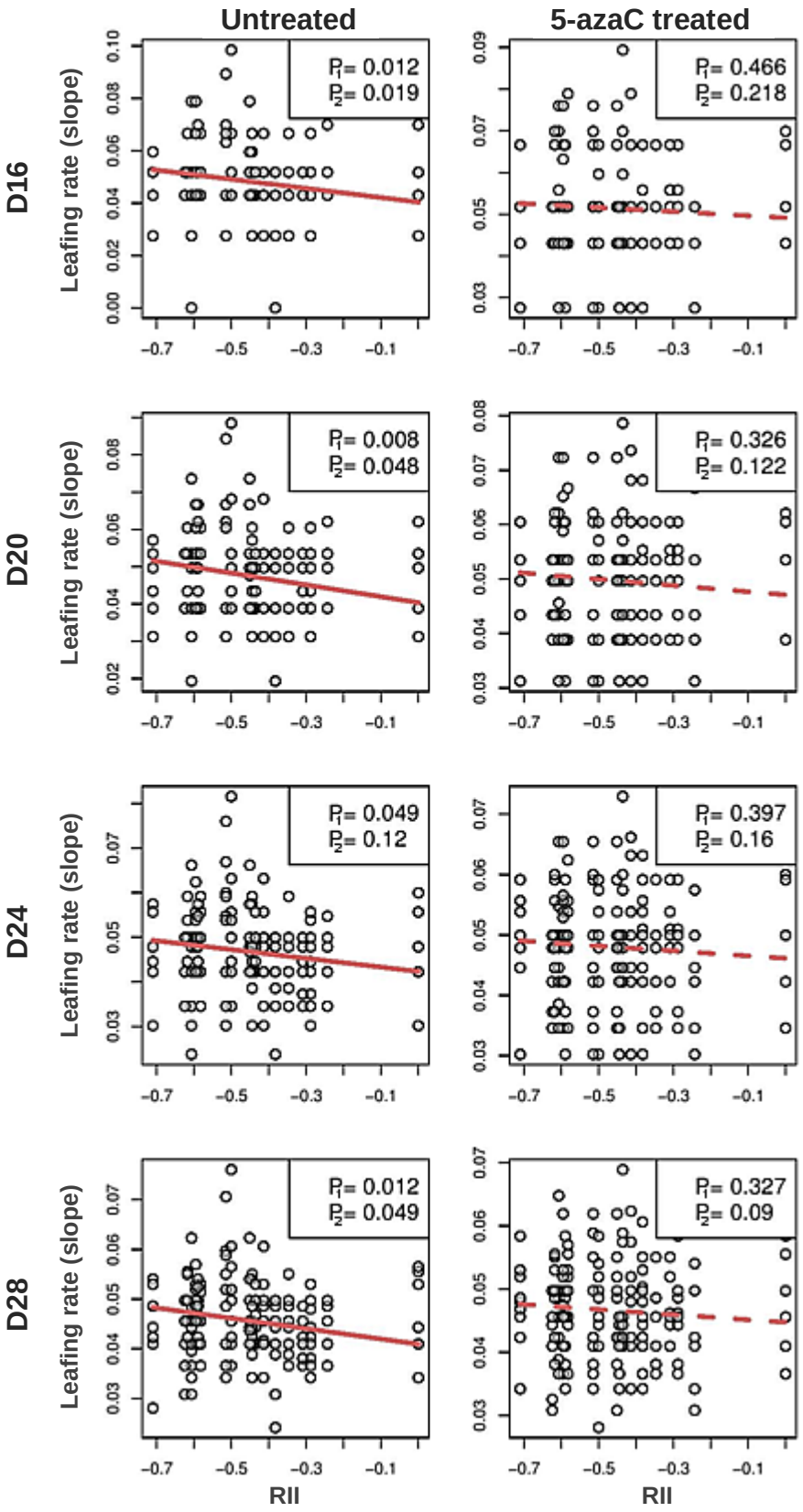


Figure 3: Effects of the experimental treatments in plants grown for six weeks (data from experiment 2 analyzed in experiment 1). (a) effect of the treatments (C – control, S - spraying method) on the aerial biomass, (b) on the root biomass, and (c) on the leaf dry matter content of the plants at the end of the six-week experiment.

Table I.: Overview of all the maternal competitive conditions used in experiment 2 with the numbers of replicates for each treatment: NC – no competition treatment, Intra – intraspecific treatment, SC(1-10) – single competitor treatment, FD(1-8) – functional diversity treatment.

treatment	**NC**	**Intra**	**SC1**	**SC2**	**SC3**	**SC4**
focal species	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*
competitor 1-6	-	*Taraxacum*	*Achillea*	*Alopecurus*	*Dianthus*	*Holcus*
no. of pots	16	8	8	8	8	8
treatment	**SC5**	**SC6**	**SC7**	**SC8**	**SC9**	**SC10**
focal species	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*
competitor 1-6	*Leontodon*	*Lotus*	*Plantago l.*	*Plantago m*	*Prunella*	*Trifolium*
no. of pots	8	8	8	8	8	8
treatment	**FD1**	**FD2**	**FD3**	**FD4**		
focal species	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*		
competitor 1	*Achillea*	*Alopecurus*	*Achillea*	*Achillea*		
competitor 2	*Alopecurus*	*Dianthus*	*Alopecurus*	*Dianthus*		
competitor 3	*Dinathus*	*Plantago l.*	*Dianthus*	*Leontodon*		
competitor 4	*Leontodon*	*Plantago m.*	*Holcus*	*Plantago m.*		
competitor 5	*Plantago l.*	*Prinella*	*Plantago m.*	*Prunella*		
competitor 6	*Plantago m.*	*Trifolium*	*Prunella*	*Trifolium*		
no. of pots	5	5	5	5		
treatment	**FD5**	**FD6**	**FD7**	**FD8**		
focal species	*Taraxacum*	*Taraxacum*	*Taraxacum*	*Taraxacum*		
competitor 1	*Holcus*	*Achillea*	*Alopecurus*	*Alopecurus*		
competitor 2	*Leontodon*	*Lotus*	*Leontodon*	*Dianthus*		
competitor 3	*Lotus*	*Plantago l.*	*Lotus*	*Holcus*		
competitor 4	*Plantago l.*	*Plantago m.*	*Plantago l.*	*Lotus*		
competitor 5	*Plantago m.*	*Prunella*	*Prunella*	*Prunella*		
competitor 6	*Trifolium*	*Trifolium*	*Trifolium*	*Trifolium*		
no. of pots	5	5	5	5		
total no. of pots						144

(a) Leafing rate



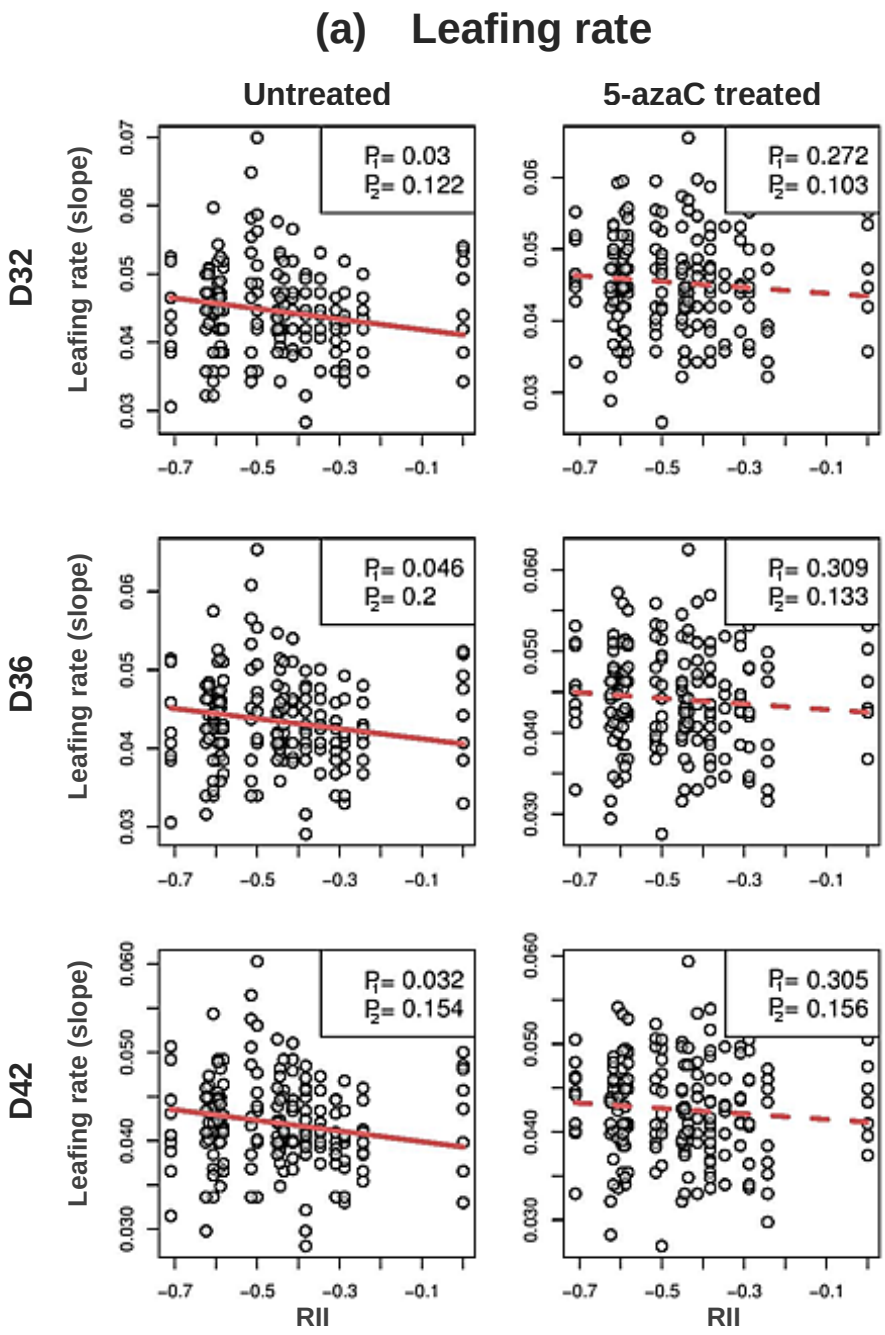
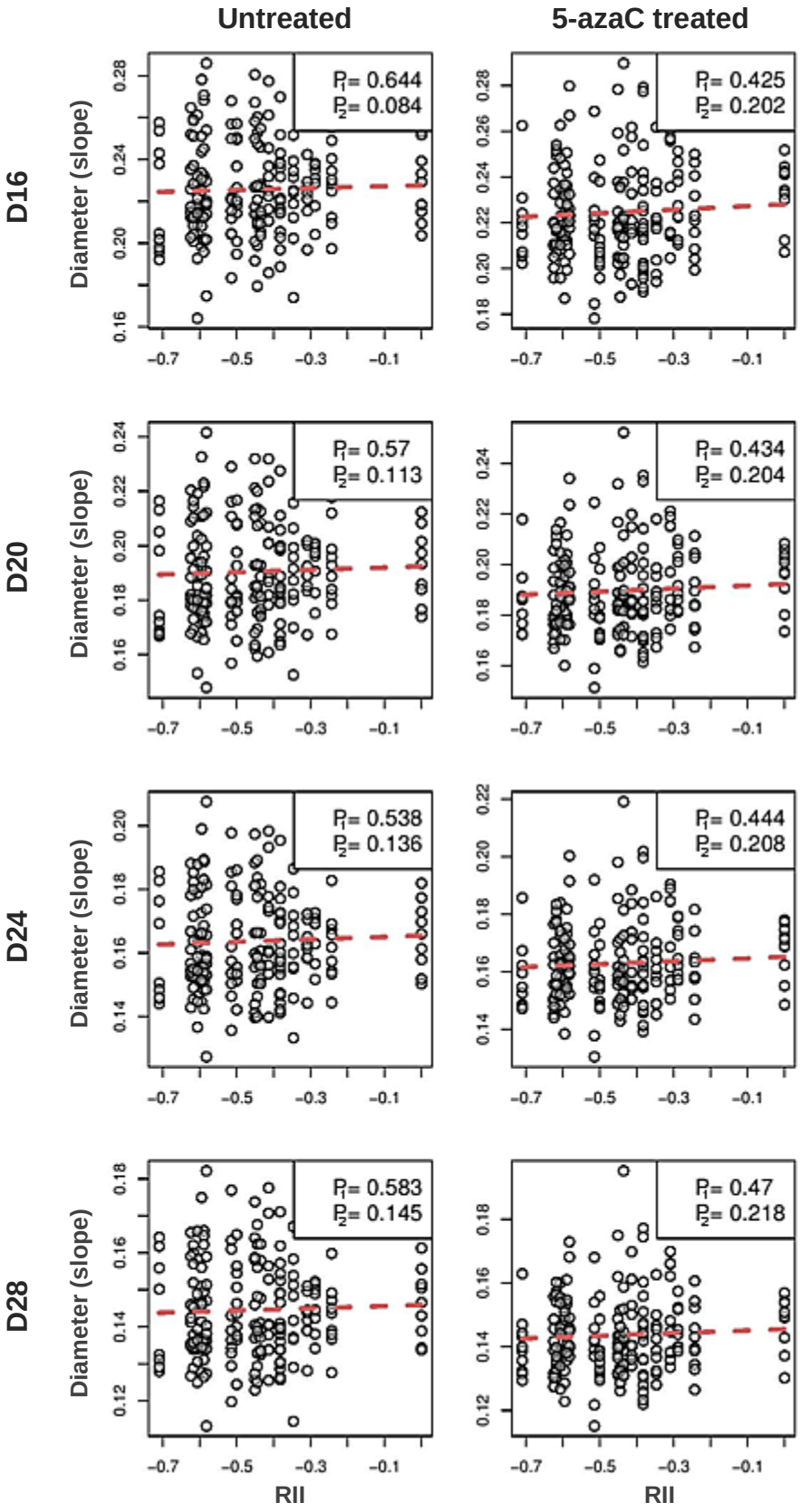


Figure 4 – (a) Leafing rate: Effects of the strength of maternal competition (RII) on the offspring leafing rate (represented by the slope of its linear regression of relationship with time in Poisson distribution) in different time periods (on measurement days: D16, D20, D24, D28, D32, D36, D42) of the six week long experiment (Experiment 2). Left column shows results for plants with normal methylation. Right slope shows results for experimentally demethylated plants. Significance (P = 0.05) of the relationship between the leafing rate and RII is expressed by P_1. Effect of the seed mass on the leafing rate is demonstrated by P_2.

(b) Rosette diameter



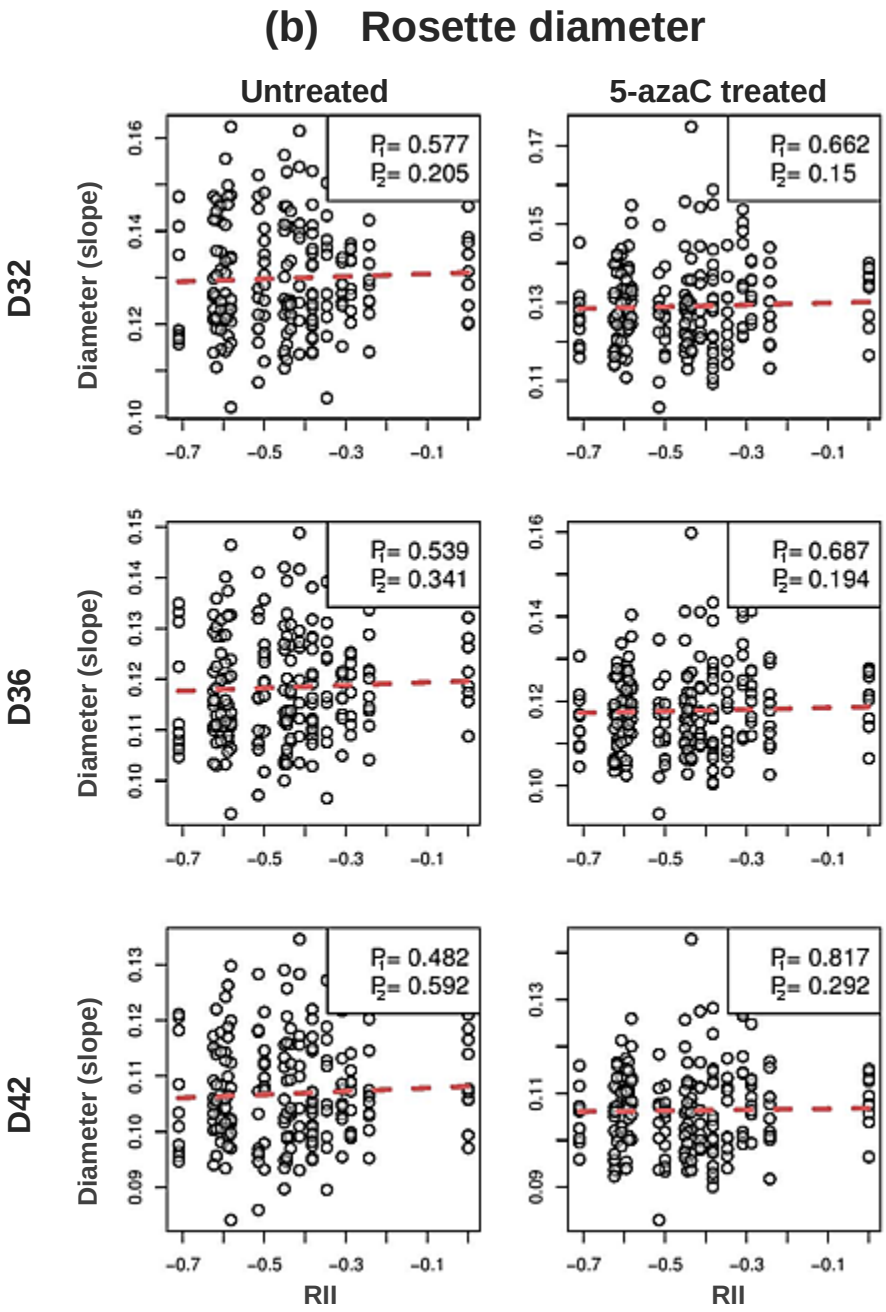


Figure 4 – (b) Rosette diameter: Effects of the strength of maternal competition (RII) on the offspring rosette diameter (represented by the slope of its linear regression in relationship with time) in different time periods (on measurement days: D16, D20, D24, D28, D32, D36, D42) of the six week long experiment (Experiment 2). Left column shows results for plants with normal methylation. Right slope shows results for experimentally demethylated plants. Significance (P = 0.05) of the relationship between the rosette diameter and RII is expressed by P_1. Effect of the seed mass on the rosette diameter is demonstrated by P_2.