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Transgenerational effects of plant biotic interactions

Ph.D. Thesis

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Annotation

This thesis focuses on the transgenerational effects triggered by plant biotic interactions and explores their relavance on ecological and evolutionary processes. The following sections document novel results that show their important consequences on different aspects. Primarily, we stablished the necessary methodology to be able to explore these questions and to disentangle the mechanisms originating the transgenerational plasticity by validating a demethylation method. Then, we checked wether the biotic interactions alter the phenotype via within-generation and transgenerational plasticity, examining the magnitude and direction of the response on each specific “response traits”. Lastly, the potencial role of transgenerational plasticity for adaptation, species coexistence, creating biodiversity and population and ecosystem functioning is tested.

Declaration [in Czech]

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

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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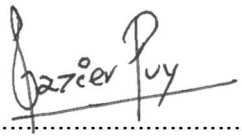

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Javier Puy

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The thesis is based on the following papers:

I. **Puy J**.*, Dvořáková H.*, Carmona C.P., de Bello F., Hiiesalu I., Latzel V. (2018) Improved demethylation in ecological epigenetic experiments: a simple and harmless foliar demethylation application. *Methods in Ecology and Evolution* 9: 744-753 https://doi.org/10.1111/2041-210X.12903 (IF = 7.09; citations = 4)

**These authors contributed equally. 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 with substantial input of J.P. All authors contributed substantially to revisions and gave final approval for publication.*

II. **Puy, J.**, de Bello, F., Dvořáková, H., Medina, N. G., Latzel, V., Carmona, C. P. Competition-induced transgenerational plasticity feedback on the offspring's competitive interactions and leaf decomposition. (Submitted manuscript)

All authors designed the research; J.P., N.G.M., C.P.C. and H.D. performed the experiment; J.P., F.d.B. and C.P.C. analysed the data; J.P. wrote the main manuscript. All authors contributed substantially to revisions and gave final approval for publication.

III. **Puy, J**., Carmona, C.P., Hiiesalu, I., Öpik, M., de Bello, F., Moora, M. Mycorrhizal symbiosis alleviates plant drought stress within and across plant generations via plasticity. (Submitted manuscript)

All authors designed the research; J.P. performed the experiment; J.P. and C.P.C. analysed the data; J.P. wrote the main manuscript. All authors contributed substantially to revisions and gave final approval for publication.

IV. **Puy, J.**, Dvořáková, H., Carmona, C. P., Latzel, V., de Bello, F. Parental diversity mitigates negative effect of intraspecific competition on productivity of *Arabidopsis*' populations by increasing phenotypic variation. (Manuscript)

All authors designed the research; H.D. and J.P imprint the plant material meanwhile J.P. performed the main experiment; J.P. analysed the data; J.P. wrote the main manuscript. All authors contributed substantially to revisions and gave final approval for publication.

Table of Contents

General Introduction

Understanding the processes involved in the assembly of natural communities and what promotes species coexistence and biodiversity is one of the oldest questions in ecological research (Diamond 1975). In the last decades, a growing concern about the loss and homogeneization of diversity an the potential impacts on the ecosystem functioning due to global change has further ignited an interest on the consequences of community assembly on the functions and services provided by ecosystems (Hooper *et al.* 2005).

Plant community composition is the result of the filtering of species from a regional species pool, so that only those species that are able to disperse (Hubbell 2001), and tolerate the prevailing abiotic environment (Weiher & Keddy 1999) and biotic interactions (Ackerly 2003; Lortie *et al.* 2004; Mitchell *et al.* 2009; Fort *et al.* 2014) are found in a site. Such filtering implies that different factors (dispersal, abiotic and biotic filters) influence the chance of species to establish, grow and reproduce in a given location. The major effects of these three mechanisms are expected to be prevaling on different spatial scales: from dispersal acting on broader scales (e.g. regional scale) to biotic interactions on the very fine local scales (Bello *et al.* 2013). Hence it is expected that these processes act as a series of hierarchical “filters” through which only individuals

with suitable abilities and characteristics (so called functional traits; Violle *et al.* 2007), are filtered into locally coexisting communities (de Bello *et al.* 2012; Vellend 2016). Since variation in traits happen both at the between-species and the within-species levels, the trait-based filtering process occurs at these two levels, selecting the individuals which better adapted traits. Thus, functional traits determine organisms' abilities to live in given ecological conditions and coexist with other organisms (Götzenberger *et al.* 2012; Kraft *et al.* 2015) both between but also within species (Violle *et al.* 2012). The general aim of this thesis is assessing the importance of local filters, particularly biotic interactions, on trait filtering within species, and their potential consequences for the functioning of local communities (Fig. 1).

1. Intraspecific phenotypic variation: why is important in community ecology?

Individuals in natural settings are not identical. This is so regardless of whether they belong to the same population of the same species and undergo exactly the same environmental conditions. In other words, individuals from the same species present variable phenotypes; differences in observable traits between organisms, such as colour, shape, sex, etc., emerging from the differential expression of their genes. Sometimes, traits can even vary subindividually within the same organism (Herrera 2017). Despite of the origin of these trait differences (see below), recent work is increasingly acknowledging the importance of this intraspecific variability, suggesting its significance for ecology and evolution (Bolnick *et al.* 2011; Violle *et al.* 2012).

Despite this, theoretical and applied ecology have typically focused on predicting the dynamics of communities and species' abundances without considering the variation in individual phenotypes. This approach, referred as "mean fiel theory", assumes that all individuals within a species are identical, or rather that intraspecific trait variability is negligible compared to interspecific one (Bolnick *et al.* 2011; Violle *et al.* 2012). This approach overlooks the exist ence of individuals with different traits and considers only the mean trait values

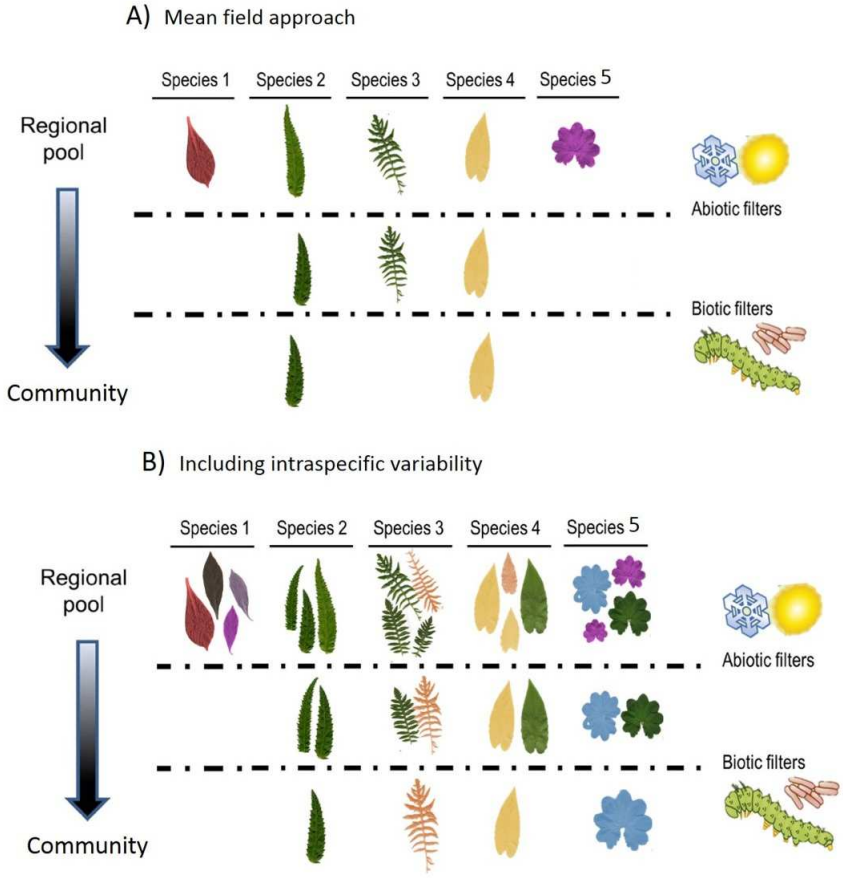

Figure 1: Community assembly theory a) under the classical approach where only mean trait values are considered for species of the regional pool; and b) incorporating intraspecific variability. Each leaf shape represents a species and each color represents a given trait value within a species. Dashed lines represent abiotic and biotic filters. Species enter in the community if their trait values match with the abiotic conditions (environmental filtering excluding red, pink and black phenotypes). Then, the biotic filters exludes organisms that possess trait values that are too similar (limiting similarity hypothesis). Models that incorporate intraspecific variability are better able to predict the species that will pass biotic and abiotic filters. Figured modified from Violle *et al.* (2012).

of each species (Fig. 1). In doing so, estimations of species' realized niches and ecological strategies are reduced, which in turn can result into underestimations of the ability of species to endure different ecological conditions, use different niches and overlap between coexisting individuals. In short, using mean traits may lead to critical misinterpretations and reduce the predictive ability of

community ecology. Indeed, phenotypic variation within species is generally lower than among species. However, the extent of intraspecific trait variability is non-negligible and critically affects community assembly at local scales (Siefert *et al.* 2015; Des Roches *et al.* 2018), specially in communities where within species phenotypic variation is as large or larger than the observed among species (Hughes *et al.* 2008; Jung *et al.* 2010; Messier *et al.* 2010).

Further, ecological theory has ignored that the ability of species to adapt to a particular environment does not only operate via selection of the fittest phenotypes (Barrett & Schluter 2008), but it also depends on species' phenotypic plasticity (Price *et al.* 2003). Phenotypic plasticity is the ability of an organism to adjust its traits in response to the environment (Price *et al.* 2003). Trait adjustments to the environment can in turn affect ecological interactions by altering their strength and outcome (Gross *et al.* 2009; Kraft *et al.* 2015; Carmona *et al.* 2019). In case of high heritability of the traits filtered by selection, trait plasticity can promote rapid adaptive evolution on the population.

As we have seen, since environmental factors filter species as a function of their traits, some functional traits seem to be associated and respond to environmental conditions. Some examples of these "response traits" are the typical characteristics of xerophytic or fire-tolerant plants. However, functional traits could also be "effect traits" if they impact on ecosystem processes and functioning (Cornelissen & Thompson 1997; de Bello *et al.* 2010). Some example of those are the ones found in fire-promoting species or nitrogen fixing plants. The "response–effect" framework developed by Lavorel & Garnier (2002) brings these two concepts together, and recognizes that traits can simultaneously explain responses to biotic and abiotic factors and effects on ecosystems (Fig. 2). Under this framework, plants can respond to the environmental factors and potentially affect ecosystem properties and services. Thus, trait plasticity could promote the ability of organisms to shape the environment where they live in, affecting ecological interactions and ecosystem functioning (van der Putten *et al.* 2013; Semchenko *et al.* 2017).

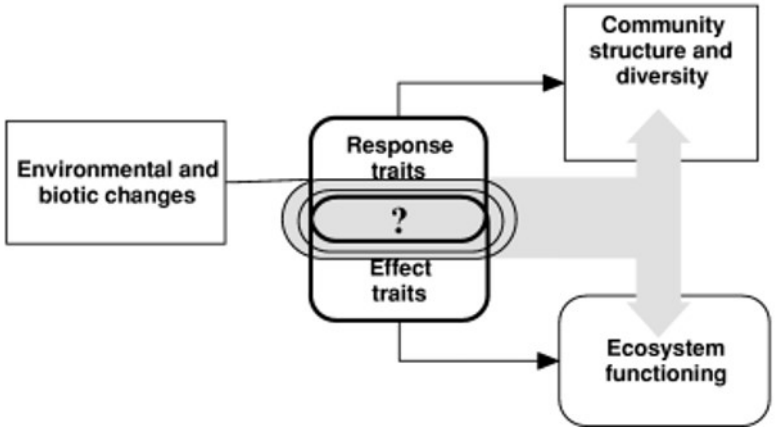

Figure 2: Representation of the conceptual framework proposed by Lavorel & Garnier (2002) for the effects of environmental changes on plant community structure or biodiversity and ecosystem functioning recognizing the overlap between response and effect traits.

1.1 Sources of variation

Although this thesis is mainly focused on the effects of phenotypic variation and not on the mechanisms responsible of this variation, it is important to understand the different origins of intraspecific variation. There are two sources of intraspecific phenotypic variation: genetic and/or epigenetic, wich have alternative ecological and evolutionary importance.

Genetic variation refers to the diversity in genotypes of the organisms (i.e. differences in the DNA sequence). Genetic variation is triggered, fundamentally, by mutations, but also by genetic recombinations produced during sexual reproduction (Foust *et al.* 2016). These changes are frequently neutral, but in some instances the new alleles can be favoured by natural selection or genetic drift, leading to the evolution of the species. A good example of this process is the well-known Darwin's finches (Darwin 1859). Because genetic variation has been more thoroughly studied for its relevance in ecological genetics and evolutionary processes, within-species phenotypic variation has been most often attributed to genetic variation, overlooking the contribution of epigenetic variation until very recently (Hughes *et al.* 2008; Latzel *et al.* 2013).

Although partially genetically controlled, epigenetic variation is any difference in the DNA expression caused whitout modifying the underlying sequence (Richards 2006; Bird 2007; Zhang *et al.* 2013). This is produced by means of various mechanisms that affect the chromatine structure; including histone modification, RNA interference and DNA methylation (Bird 2007; Bossdorf *et al.* 2010; Amoah *et al.* 2012). Among them, DNA methylation is the most studied, best understood and possibly even the most significant one (Akimoto *et al.* 2007; Reinders *et al.* 2009; Bossdorf *et al.* 2010; Zhang *et al.* 2013; Kanchanaketu & Hongtrakul 2015). Although DNA methylation appears to be relatively stable within an individual, it exhibits predictable plastic responses to environmental stimuli (Tatra *et al.* 2000; Bond & Baulcombe 2014; Preite *et al.* 2018), which, together with its transgenerational heritable potential (Richards 2006; Hauser *et al.* 2011), makes DNA methylation a excelent mediator for transgenerational inheritance (Chinnusamy & Zhu 2009; Herman *et al.* 2014; Colicchio *et al.* 2015a).

Epigenetic variation is known to occur in response to environmental factors (Herman & Sultan 2016; Richards *et al.* 2017), and to cause phenotypic variability (Cubas *et al.* 1999; Latzel *et al.* 2012; Zhang *et al.* 2013). Thus, it provides a plastic response of the organism to the environment during plant life, that could also be potentially transmitted to the next generation (Akimoto *et al.* 2007; Bossdorf *et al.* 2008; Jablonka & Raz 2009; Johannes *et al.* 2009; Amoah *et al.* 2012). Whereas within-generation variation is caused when the environment triggers phenotype modifications on the individual (normally referred as “plasticity” or “acclimatation”; Fig. 3), transgenerational plasticity (explained in more depth in the following section) occurs when the individual phenotype of the progeny is affected by the parental environment via heritable epigenetic modifications (denominated as “transgenerational” or “parental effect”; Fig. 3) (Jablonka & Raz 2009; Herman & Sultan 2011; Herrera *et al.* 2012; Herman *et al.* 2014).

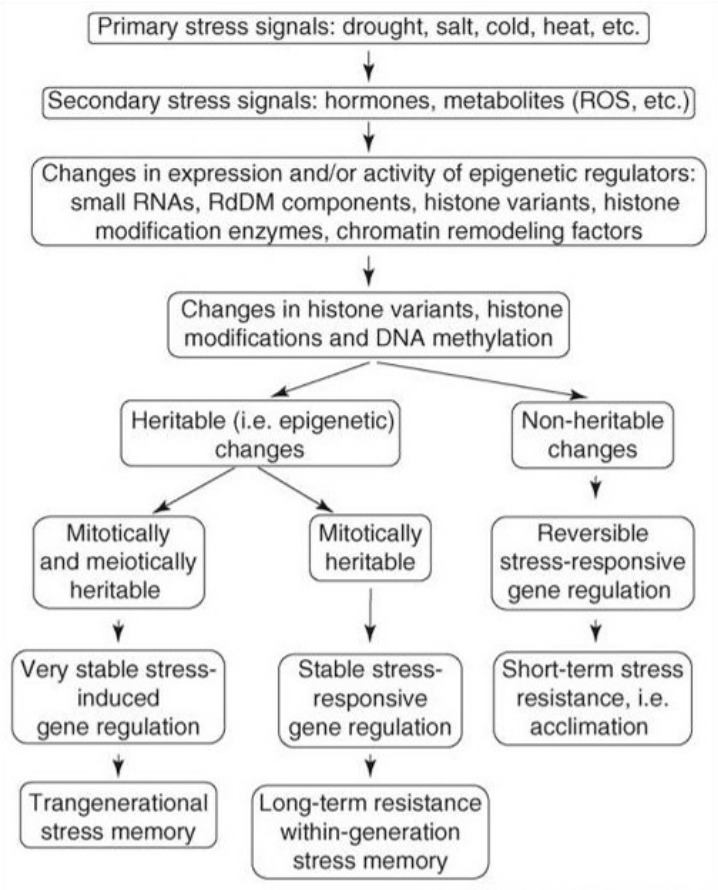

Figure 3: Regulation of environmentally induced epigenetic mechanisms and their role on stress tolerance and "memory". While some epigenetic modifications are transient, mediating plasticity and acclimatation response, others are heritable epigenetic modifications that provide within-generation and transgenerational plasticity leading to adaptive "stress memory". Extracted from Chinnusamy & Zhu (2009).

1.2 Transgenerational effects

Transgenerational effects can be defined as modifications of offspring phenotype induced by environmental conditions experienced by the parents, without changes in DNA sequence (Roach & Wulff 1987; Jablonka & Lamb 1995; Mousseau & Fox 1998; Galloway 2005). In plants, mechanisms underlying transgenerational effects can mainly be categorized as seed modification (Roach & Wulff 1987) or epigenetic variation (Fig. 3) (Boyko *et al.* 2010).

In the past, it was thought that seed modification, often referred as "maternal effect" or "seed mass effect", was solely mediating the phenotypic variation of the offspring by creating differences in seed provisioning, seed quality (i.e nutritional quality), or hormonal balance stocked up by the maternal plants or the in embryos (Roach & Wulff 1987; Herman & Sultan 2011). Transgenerational effects originated by embryo modification could play a significant role during early stages of the development, but tend to fade away with time when ongoing environmental factors outweigh them (Latzel *et al.* 2010). In contrast, the effects originated by mechanisms of epigenetic variation could have more substantial impact since the modification could last the individuals' entire lives and be transmitted to several generations (Herman & Sultan 2011; Dechaine *et al.* 2015; Germain *et al.* 2019)

The role of epigenetic transgenerational effects as a possible mechanism for stress "memory" in plants due to its potential adaptive environmental response has received increasing attention since the 80's (Roach & Wulff 1987; Mousseau & Fox 1998). Epigenetic variation can enable plants to store information about their past environmental interactions for several generations, and to modify their development according to expected conditions (Shemesh *et al.* 2010; Novoplansky 2016) maximizing the progeny's fitness, especially during the juvenile stage (Mousseau & Fox 1998). Recent research, especially focused on the response to abiotic conditions, has recognized the role of epigenetic transgenerational effects in adaptation (Roach & Wulff 1987; Mousseau & Fox 1998; Sultan *et al.* 2009; Latzel *et al.* 2010, 2014; Dechaine *et al.* 2015), opening up the possibility of directed microevolution, resonating with Lamarckian notions of evolution which had previously seemed inconceivable from the genetical point of view.

1.3 Methodological approaches

Heritable epigenetic variation has been studied using highly sophisticated molecular methods (e.g. Pecinka *et al.* 2009a; Becker *et al.* 2011; Colicchio *et al.* 2015b). Consequently, research on ecological epigenetics remains somehow inaccessible to most biologists, which obviously slows the process of unravelling

the full ecological and evolutionary aspects of epigenetic variation in plants. Alternative approaches, such as the alteration of the epigenetic status of plants, can be applied to indirectly test the ecological role of epigenetic variation (Johannes *et al.* 2009; Bossdorf *et al.* 2010).

Typically, the alteration of the epigenetic status has been based on reducing DNA methylation. This reduction can be achieved either by working with plants derived from mutants (like in epiRILs; Johannes *et al.* 2009; Reinders *et al.* 2009; Latzel *et al.* 2012, 2013; Zhang *et al.* 2013) or by using demethylating agents such as 5-azacytidine or zebularine (Cubas *et al.* 1999; Bossdorf *et al.* 2010; Liu *et al.* 2015). Demethylating agents inhibite the methyltransferase enzyme during DNA replications, which results in partial demethylation of the genome (e.g. Jones 1985; Burn *et al.* 1993; Tatra *et al.* 2000). In other words, demethylation 'removes' the epigenetic "memory" of the abiotic and biotic conditions in which plants originated. Experimental demethylation of DNA has greatly helped to discover that epigenetic variation is involved in plant phenotypic plasticity (Bossdorf *et al.* 2010) such as flowering phenology of plants (Fieldes & Amyot 1999; Kondo *et al.* 2007), and transgenerational adaptation of plants to stress (Boyko & Kovalchuk 2011; Herrera *et al.* 2012).

Despite the potential of demethylation to reveal epigenetic effects on plant development, existing methods might fail to meet the requirements of researchers applying them. The main limitation of the demethylation agents is their toxicity on seedlings even under low concentrations (Akimoto *et al.* 2007). Consequent growth of such treated plants often express various aberrations, with treated plants being usually smaller (Kondo *et al.* 2007; Amoah *et al.* 2008; Bossdorf *et al.* 2010) and with reduced survival than controls (Akimoto *et al.* 2007; Amoah *et al.* 2012). Hence, the ecological relevance and realism of studies using demethylation agents can be questioned.

Other important point to remark under the methodological section is the election of species to work with. The selection of model plants like *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays* is necessary for understanding the mechanisms and dynamics of epigenetic variation where further molecular and

genetic analysis need to be made (Verhoeven *et al.* 2016; Richards *et al.* 2017). However, to test the ecological role of epigenetic variation, other non-model plants can, and probably should, be selected. One clever attempt to minimize the possibilities for genetic control and reduce genetic variability of the study, is working with clonal or completely inbred study species, or alternatively, using statistical approaches to uncover patterns of epigenetic variation that are not predictable from patterns of genetic variation (Foust *et al.* 2016; Herrera *et al.* 2016; Verhoeven *et al.* 2016). To study the effects of epigenetic variation in the nonmodel systems, experimental DNA demethylation is the only currently available tool in order to have the proper control (Verhoeven *et al.* 2016), despite the potential undesired side effects.

In this thesis, the role of epigenetic transgenerational effects was tested by means of a series of pot experiments where two generations were grown. In the first generation (i.e. parental generation), the plants were grown under different conditions to trigger potential transgenerational effects in the offspring. Thus, seeds from this first generation were collected and then used for the experiment of the second generation. Two alternative designs were used in the second-generation experiments. In the first design, the collected seeds were grown under identical control conditions, so that any differences between the offspring's phenotype should be due to transgenerational effects. In the second design, the different seeds were grown undergoing the same or distinct conditions than the parental generation. This last factorial design is needed in order to test if the transgenerational effects are adaptive. Further, prior to the parental generation experiments, plants were grown for one generation in a common environment, to even out possible unknown transgenerational effects of previous cultivations (Latzel 2015).

Two different species were used in the different experiments composing this thesis: the non-model species *Taraxacum brevicorniculatum* Korol., which is an obligate apomictic polycarpic perennial plant (Kirschner *et al.* 2013), and the model species *Arabidopsis thaliana* L. which is a predominantly self-fertilizating annual (rarely biennial) plant. Since *T. brevicorniculatum* is an obligate apomictic species (i.e. all seeds produced by a plant are effectively

clones) and *A. thaliana's* outcrossing rate is very low, genetic variation between progeny could be assumed to be negligible. These characteristics of the selected species enable to focus on the study of the effects of plasticity within and across generations. Since transgenerational plasticity, besides the epigenetic origin, could be also ascribed to seed modifications, the effect of seed characteristics was experimentally controlled by incorporiating it into the statistical models, and when possible, by altering the epigenetic status of the plants by *in vivo* experimental DNA demethylation.

2. Biotic interactions controls on species coexistence and biodiversity maintenance

Species coexistence is determined by many processes that operate on different scales: from evolutionary scales, like speciation and historical constraints, to ecological scales, like dispersal influences (Chesson 2000; Wilson 2011; HilleRisLambers *et al.* 2012). This thesis focuses on the processes involved in the coexistence at the local scale where prevailing biotic interactions are the leading factors driving assembly, biodiversity maintenance and ecosystem functioning (van der Putten *et al.* 2013; Kraft *et al.* 2015; Valladares *et al.* 2015).

2.1 Competition and coexistence

The role of competition in plant communities' assembly is based on the common assumption that competitive interactions increase with increasing trait similarity between interacting individuals (Fig. 4). This means that species with similar and overlapping ecological niches (i.e. generally possessing more similar traits), will compete more intensely for resources (Darwin 1859; Gause 2003; Cahill *et al.* 2008; Rosindell *et al.* 2011). Consequently, according to the "limiting similarity" principle (Macarthur & Levins 1967), it is expected that competition for resources would lead to co-occuring species having different ecological niches and therefore more different traits. This is usually tested by assessing if trait differences between organisms are greater (divergence) or smaller (convergence)

than expected by chance using null-models (Mason & Wilson 2006; Mason *et al.* 2008).

Although limiting similarity should lead to divergence, it is increasingly acknowledged that competition can also lead to an alternative convergence pattern, which can be produced depending on the nature of the trait(s) considered (Grime *et al.* 1997; Cahill *et al.* 2008; Kraft *et al.* 2008, 2014; Violle *et al.* 2012; Adler *et al.* 2013). When the trait is related to the competitive ability (i.e. there is a specific phenotype competitively superior on fitness or on the ability to obtain the limiting resource), the intensity of competition increases with increasing trait dissimilarity. This is stated by the "limiting dissimilarity" principle (Fig. 4), and it leads to a phenotypic convergence of the individuals in the community as result of the exclusion of the species with lower competitive ability (Mayfield & Levine 2010; Kunstler *et al.* 2012). Thus, there are two limits for coexistence: one limit to similarity in resource utilization, i.e. niche differences; and one limit to dissimilarity in competitive ability, i.e. fitness differences (Fig. 4). In other words, species can avoid exclusion either by being sufficiently different in their demands for a resource or, if they have similar demands, by being sufficiently similar in their skills to compete for this resource (Adler *et al.* 2010; Mayfield & Levine 2010).

Contemporary coexistence theory emphasizes that coexistence depends on niche and fitness differences between co-occuring individuals. The mechanisms that allow coexistence can be divided in two types depending on how they affect to the competitive-dominance relationships between species (Chesson 2000). First, equalizing mechanisms are those that reduce the fitness differences between the most abundant species and the less competitive ones, making competition less assymmetric. If we express the mechanism based in functional traits, when equalizing mechanisms prevail, individuals would tend to have similar trait values that minimize fitness differences (i.e. limiting dissimilarity) in the community. Some examples of equalizing mechanisms include the intermediate disturbance hypothesis (Connell 1978), and temporal or spatial heterogenity (Adler *et al.* 2013).

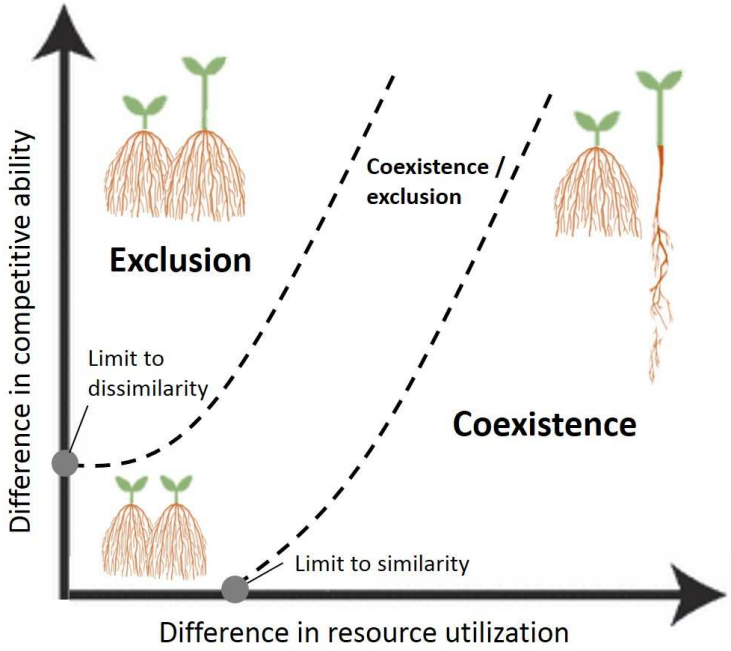

Figure 4: Regions of coexistence and exclusion depending on the niche differences (resource utilization) and fitness differences (competitive ability) between co-occuring individuals. While height variation is presented as a competitive ability difference, root structure variation is presented as a niche difference. The area of the middle indicates where exclusion was predicted from classical theory, but by including stochasticity, Agren & Fagerstrom (1984) predicted coexistence explaining the special case where ecologically identical species can coexist stably. Individuals can coexist when they are relatively similar and little niche differences overcome small competitive ability differences, and when large niche differences overcome large competitive ability differences. Combining Agren & Fagerstrom (1984) and Mayfield & Levine (2010).

Second, stabilizing mechanisms are those that favor rare species by reducing competition intensity when they are rare and increasing the spatial-temporal niche differences with the most abundant species. Stabilizing mechanisms will lead to a limiting similarity, with individuals having different trait values (Mayfield & Levine 2010; Le Bagousse-Pinguet *et al.* 2014). Examples of stabilizing mechanisms include differentiation in resource use and frequency/density dependent predation (also herbivory or pathogen infection), known as the Janzen-Connel hypothesis (Janzen 1970; Connell 1971). While only stabilizing mechanisms can lead to permanent stable species coexistence, equalizing mechanisms just delay competitive exclusion.

These hypotheses are further complicated when we integrate intraspecific variability. It should be recalled that altough the original paper on limiting similarity of Macarthur & Levins (1967) considers intraspecific trait variability, many following studied did not. This is particuylarly important because trait plasticity mediated by biotic interactions can in turn feedback the strength and outcome of the interactions by changing trait hierarchies and species dissimilarities (Gross *et al.* 2009; McGill 2010; Violle *et al.* 2012; Kraft *et al.* 2015; Bennett *et al.* 2016; Hart *et al.* 2016; Carmona *et al.* 2019). Trait plasticity could theoretically promote species coexistence via stabilizing mechanisms (Clark 2010), but it very much depends on the responding trait as well as the direction of the change (Kraft *et al.* 2015). There is the possibility that intrasecific plasticity can reduce competitive hierarchies promoting species coexistence via equalizing mechanism (Kraft *et al.* 2015; Carmona *et al.* 2019). However, theoretical studies also suggest that plasticity can make coexistence more difficult (Hart *et al.* 2016). One example where trait variability within species could be important is intraspecific competition, where the competitive ability and the niche between the two individuals are the same. Although other factors like kin recognition can also reduce competition and competitive exclusion (Dudley & File 2007; Cahill *et al.* 2010; Cahill & McNickle 2011). Yet, very little is known on the importance of the intraspecific differences on the coexistence of species, and the effect of transgenerational trait adjustments have not yet been teased apart (Zuppinger-Dingley *et al.* 2014).

2.2 Arbuscular Mycorrhizas

Relentless and fierce competition for resources between and within species is a widely observed phenomenon in nature but it is not the only existing strategy for survival. Cooperation and beneficial interactions between contrasting organisms are also prevalent in nature and influence coexistence. However they are often overlooked (Gross *et al.* 2015; Peay 2016). Plants interact with a multitude of organisms, but one of the most important interactions is with arbuscular mycorrhizal fungi.

Arbuscular mycorrhizal symbiosis is a widespread mutualistic association between plant roots and fungi from the subphylum Glomeromycotina (Smith & Read 2008; Spatafora *et al.* 2016). This association is considered mutually beneficial, since, in exchange for photosynthetic carbon, the arbuscular mycorrhizal fungi provide host plants with soil nutrients (mainly phosphates), mitigate abiotic stress (e.g. drought) and increase resistance to biotic stress, including pathogens (Lu & Koide 1994; Smith & Read 2008). Thus, AM fungi determine and potentially expand the realized niches of the plant species by enabling plants to access otherwise unavailable nutrients (van der Heijden *et al.* 2003; Peay 2016; Gerz *et al.* 2018). In this case, mycorrhiza could act as a stabilizing mechanism (Chesson 2000). Due to this property, mycorrhizas are known to provide ecosystem resistance and resilience against stresses or disturbances (Martínez-García *et al.* 2017).

Beyond the advantage that this symbiosis implies for plants, arbuscular mycorrhizal fungi are known to alter plant-plant competition (Grime *et al.* 1987; O'Connor *et al.* 2002; Veresoglou *et al.* 2017). Mycorrhiza influence plant coexistence by altering fitness differences across plant species. The symbiosis could act as an equalizing mechanism if they reduce fitness differences between species (Chesson 2000; Wagg *et al.* 2011). Conversely, the symbiosis could promote exclusion by exhacerbating the dominance of some species. Thus, mycorrhizas play a key role in ecosystem processes and properties by controlling the establishment and successional change of plant communities (García de León *et al.* 2016), and by promoting plant biodiversity and plant productivity (van der Heijden *et al.* 1998; Smith & Read 2008)

The fitness benefits of plants with arbuscular mycorrhizal symbiosis are well known (Lu & Koide 1994; Smith & Read 2008). However it is unclear whether these benefits could partly operate through the phenotypic plasticity that mycorrhizas mediate, such as root architecture (Nuortila *et al.* 2004; Goh *et al.* 2013; Fusconi 2014). Moreover, it remains unclear whether AM symbiosis of the parental generation triggers phenotypic changes in their offspring (i.e. transgenerational effects) that provide benefits to the offspring generation (Koide 2010; Varga *et al.* 2013). Most of the existing evidence demonstrates that having

mycorrhizal parents can be beneficial during the early stages of development of the offspring (Heppell *et al.* 1998; Koide 2010). However, the relative effect of epigenetic mechanisms on these transgenerational effects has been rarely considered (with the exception of Varga *et al.*, 2013).

2.3 Diversity and ecosystem functioning

As we have seen, species interact in multiple ways, from negative interactions when one species reduces the performance of another, to positive ones where the presence of a species facilitates others through provisioning of resources or amelioration of stresses (Chesson 2000; Mayfield & Levine 2010; Gross *et al.* 2015; Peay 2016). Such interactions significantly determine the identity and abundance of the species present in the communities and thus also alter the resulting biodiversity patterns (Diaz & Cabido 2001; Araújo & Luoto 2007).

It is generally recognised that biodiversity – which includes taxonomical, functional, and genetic and epigenetic diversity (Balvanera *et al.* 2006; Hughes *et al.* 2008; Marquard *et al.* 2009; Latzel *et al.* 2013) – drives ecosystems functioning and processes, which ultimately provides ecosystem services (Tilman *et al.* 1997; Hooper *et al.* 2005). It is important to note that biodiversity includes different components, i.e. taxonomical, functional, and genetic and epigenetic diversity (Balvanera *et al.* 2006; Hughes *et al.* 2008; Marquard *et al.* 2009; Latzel *et al.* 2013). The positive relationship between diversity and functioning has been demonstrated repeatedly in many observational and experimental studies. This body of research has generally found that more diverse communities are generally more productive, more stable and more resistant to disturbances/stresses than less diverse ones (Balvanera *et al.* 2006; Marquard *et al.* 2009).

The positive biodiversity effects on ecosystem processes could be driven by two not mutually exclusive mechanisms: complementarity and selection (Loreau & Hector 2001; Marquard *et al.* 2009; Tobner *et al.* 2016). Selection operates when a specific competitively superior species is dominant in the mixtures and drives disproportionately the functioning of the community (Loreau

& Hector 2001). By contrast, complementarity takes place when niche differences between coexisting species result in a more efficient use of resources by the community (Loreau & Hector 2001). Because fitness and niche differences can be directly measured and explained with plant functional traits, both mechanisms can be also approximated from a trait-based perspective. In this case, when selection is the main mechanism, we should expect to observe a dominance of particular traits or less trait variance in the trait associated to the competitive/fitness advantage (Cadotte 2017). On the other hand divergence in traits related to resource foraging between individuals of the community would reflect that complementarity is the main mechanism driving the positive effect of diversity (Loreau & Hector 2001; Cadotte 2017).

While most research has commonly measured biodiversity at the community level as interspecific diversity (taxonomic or functional diversity) (Marquard *et al.* 2009; Hector *et al.* 2010), the effect of intraspecific diversity at the population level has been overlooked. However, intraspecific diversity effects on population and ecosystem functioning can be of comparable magnitude to those of intraspecific diversity (Crutsinger *et al.* 2006; Hughes *et al.* 2008; Latzel *et al.* 2013). Some studies have reported a positive effect of intraspecific diversity in ecosystems functioning (Hughes *et al.* 2008; Latzel *et al.* 2013; Zuppinger-Dingley *et al.* 2014). However this effect has been most often attributed to genetic variation (Zhu *et al.* 2000; Booth & Grime 2003; Reusch *et al.* 2005; Crutsinger *et al.* 2006; Hughes *et al.* 2008; Kotowska *et al.* 2010; Moore 2015; Cook-Patton *et al.* 2016), overlooking the relative effect of epigenetic variation (Latzel *et al.* 2013), both within and across generations, despite its importance for responding to the environment. While existing phenotypic variation of the community should promote positive biodiversity effects by increasing complementarity (Clark 2010; Roscher *et al.* 2015), phenotypic plasticity could either decrease or increase trait dissimilarities. Depending on the trait and the direction of the change, this could enhance or decrease selection and complementarity effects (Roscher *et al.* 2015). Thus, it is necessary to disentangle the relative effect of genetic diversity from that of epigenetic diversity on population functioning, as well testing for within- and transgenerational plasticity separately.

Thesis scope and framework

In this thesis, I focused on exploring the existence of transgenerational effects triggered by plant biotic interaction and the relevance of their role on plant adaptation, species coexistence, and population and ecosystem functioning. To face these aims I used a general conceptual framework (Fig. 5), in which starting from the environmental induced phenotypic variation caused by heritable epigenetic modifications, I evaluated all the possible consequences relevant from an ecological and evolutionary perspective.

First, in **Chapter I**, I aimed to validate the demethylation efficiency and suitability for ecological research of a novel method of experimental plant demethylation. This method, because of its potentially fewer disturbing effects on plant development, could overcome the traditional methodology and could discriminate the phenotypic changes caused by the DNA-methylation from the side effects of the demethylation agent. In case of validation, this methodology will be used in the following research question and will check for the epigenetic basis of the transgenerational effects.

Then, I explored the transgenerational effects triggered by biotic interactions. First, in **Chapter II,** I examined the transgenerational effects triggered by plant–plant competition as a representative of a negative interaction. With the four coordinated experiments presented in this chapter, I tested the existence of transgenerational effects and, also, their feedback on offspring's competitive interactions, and their possible consequences on adaptation, promoting coexistence and affecting ecosystem processes.

Similarly, in **Chapter III,** I investigated the transgenerational effects triggered by arbuscular mycorrhizas, as a representative of positive interactions across trophic levels. Since arbuscular mycorrhizas are known to benefit plants by mitigating drought stress, I focused on the benefits that could be partly explained by the plant phenotypic plasticity that they mediate, with especial attention on the heritable plasticity.

Last, in **Chapter IV**, the focus is on the effects of intraspecific phenotypic variability, rather than on the causes. In this chapter, I assesed the role of genetic diversity and environmentally induced heritable epigenetic diversity on generating phenotypic variability and on affecting productivity and resistance against stress of plant populations.

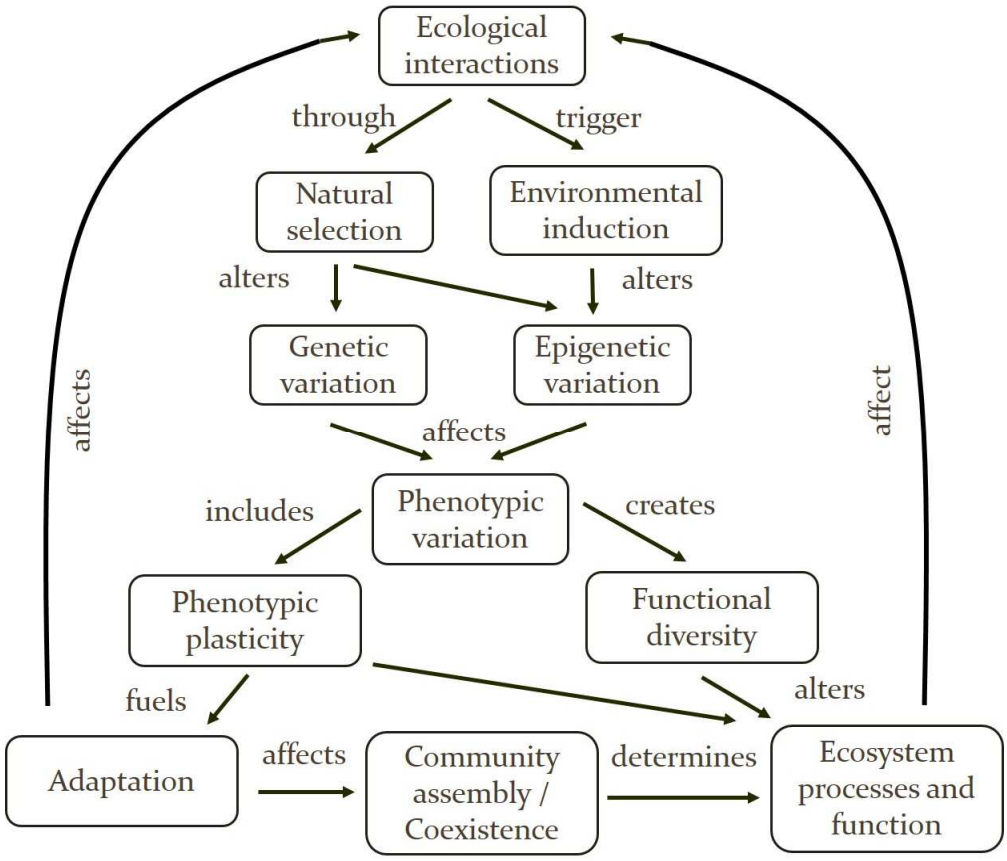

Figure 5: Research framework followed in the thesis.

Chapter I

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

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Abstract

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. 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. 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. We conclude that regular spraying of 5-azaC solution onto established seedlings surpassed the germination-in-solution method in terms of vigor 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.

Introduction

A growing body of evidence suggests that heritable epigenetic variation is of crucial importance for the ecological and evolutionary processes of plants (Bossdorf *et al.* 2008). Epigenetic variation is caused by various DNA modifications, including DNA methylation, which is known to occur in response to environmental factors (González *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.* 2009b), 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.* 2015; 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). 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. Johannes *et al.* 2009; Bossdorf *et al.* 2010). 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; Verhoeven & van Gurp 2012; Liu *et al.* 2015; Herman & Sultan 2016). 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. Experimental demethylation represents a simple yet elegant technique for testing the ecological role of epigenetic variation, since 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; Verhoeven *et al.* 2010; Herman & Sultan 2016) Therefore, comparing treated vs. untreated plants enables testing of the importance of past environmental interactions, or the so-called "epigenetic memory", on plant performance (González *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 *et al.* 2007), the importance of transgenerational adaptation to stress (Boyko *et al.* 2010; Herrera *et al.* 2012; Herman & Sultan 2016), and in the control of plant inbreeding depression (Vergeer *et al.* 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 *et al.* 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; Kondo *et al.* 2007; Bossdorf *et al.* 2010), 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 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 González *et al.* (2016) applied a different demethylation method that consists in periodical spray of 5-azaC solution onto plants 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 seeds plants like clonal species, which was the primary motivation of González *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, by testing transgenerational effects of plant-plant competition applying it to seedlings coming from parental plants that either experienced competition or not.

Material and methods

Study species and seed material

To test the method, we chose a clone of *Taraxacum brevicorniculatum* Korol. as our model species. *T. brevicorniculatum* is a triploid obligate apomictic species (Kirschner *et al.* 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.

Growth chamber experiment

The spray application was tested by means of two experiments.

Experiment 1. The aim of this experiment was to compare the demethylation efficiency and possible deleterious effects of the spray application versus 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; 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 x 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. 5-azaC addition.

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 *et al.* 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 three weeks with a 12 h (20 °C) / 12 h (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.

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 six 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.

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 three weeks. The plant material was dried at 60 °C and the total biomass weighted.

In Experiment 2 the 40 plants were grown for six weeks in pots. During that time, we measured the diameter of the rosette every two days and used these measurements to estimate growth rate (change in diameter of the rosette between the transplantation and harvest; $mm \times day^{-1}$). After six weeks, two leaves from each plant were collected, and their area, water-saturated fresh mass and dry mass estimated. We used these measurements to estimate leaf dry matter content (LDMC; the ratio of leaf dry mass to leaf fresh mass, $mg \times g^{-1}$), and specific leaf area (SLA; the ratio of leaf area to leaf dry mass, $mm^2 \times mg^{-1}$). Further, 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; $m \times g^{-1}$) was estimated based on the scanned images by using the image analysis software WinRHIZO Pro, 2008 (Regent Instruments Inc., Quebec, Canada).

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 three 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 aza-5C 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-olds 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.

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 ($P < 0.05$). 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 Fig. 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 ($P < 0.05$). All analyses were conducted using R v3.2.3 (R Core team 2016).

Results

Experiment 1. The treatments affected the percentage of methylated DNA (Chi-square = 10.99, df = 2, P = 0.004). 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 = 0.005), 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 = 0.041). Most importantly, we found

no differences in the levels of DNA methylation between the germinating and the spraying demethylation approaches (S vs G, P = 0.257; Fig. 1a).

We found no significant differences in the total 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 total biomass (P < 0.001; Fig. 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 < 0.001; Fig. 2a and Fig. 3), as well as smaller leaves (t = 2.228, df = 44.86, P = 0.031; Fig. 2b and Fig. 3).

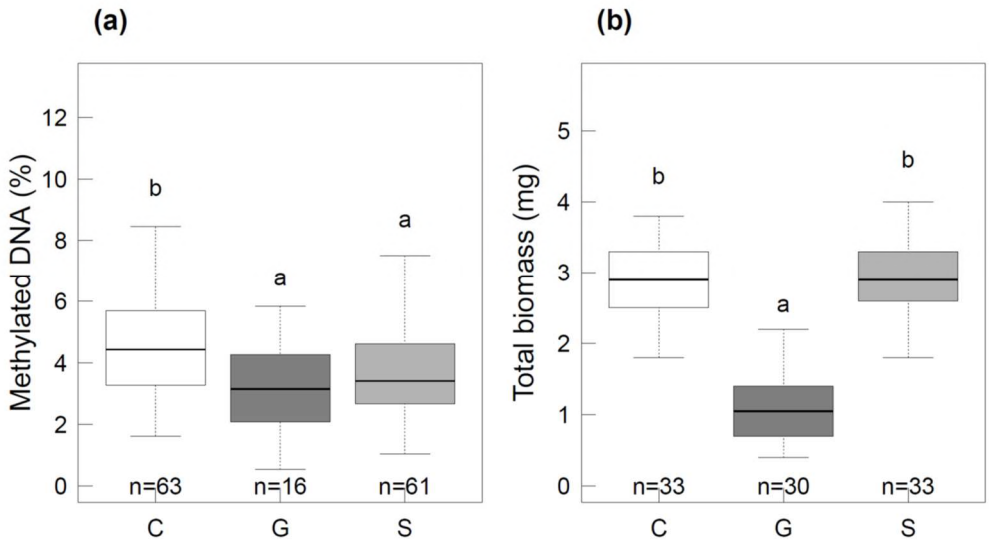

Figure 1: Differences between experimental treatments in the three-week-old seedlings. (a) effects of the treatments (C - control, G - germinating method, S - spraying method) on the level of genome-wide DNA methylation and (b) on the dry weight total biomass of the plants at the end of the three-week 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 = 0.05).

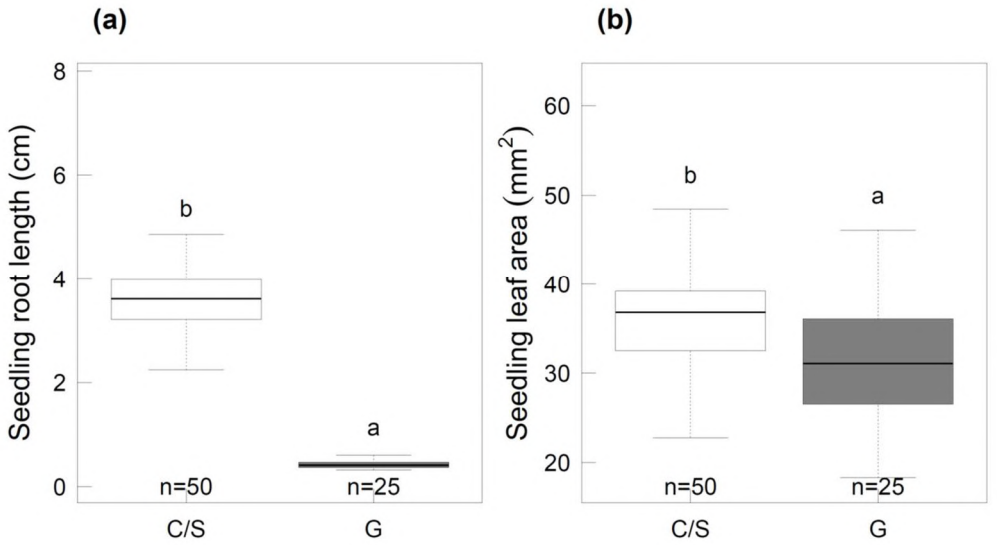

Figure 2: Differences between 10-day-old seedlings germinated either in water (C/S, which were virtually equivalent up to that point because they had not been sprayed yet) or a 50 μM water solution of 5-azaC (G) in (c) root length and (d) leaf area. The bottom and top of the boxes are the 25th and 75th 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 = 0.05).

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 and14% 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 = 0.92; plant part, P = 0.86; and their interaction P = 0.98) in the percentage of methylated DNA (Fig. S1).

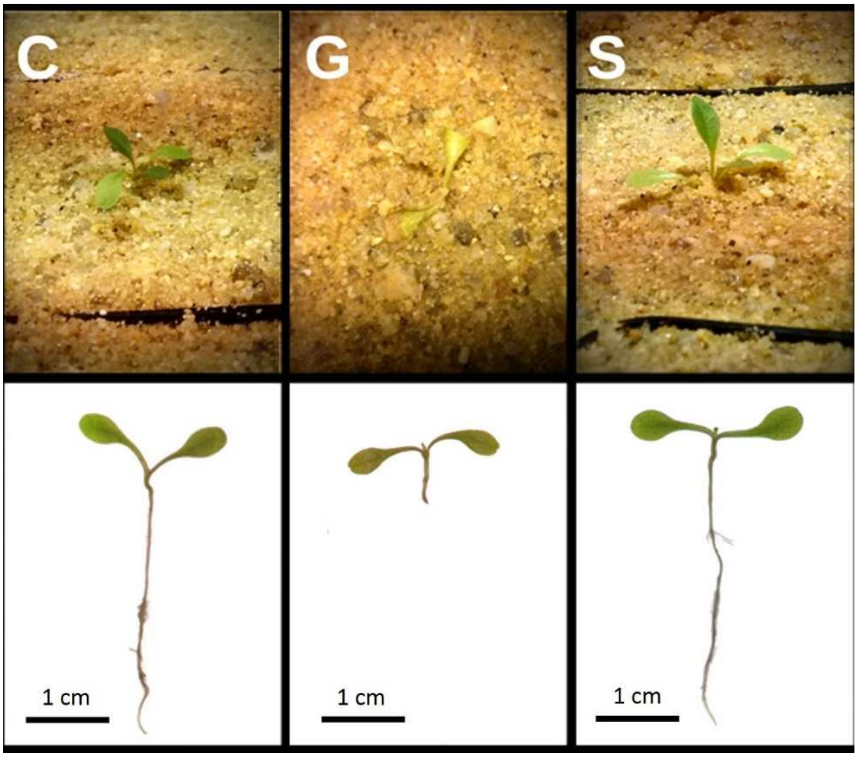

Figure 3: Details of the differences in early development of plants between the three treatments (C - control, G - germinating method, S - spraying method). Upper row shows seedlings in the pots two weeks after transplanting, whereas the lower row displays some of the images of 10-day-old seedlings that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

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 offspring of parents from non-competition conditions, both considering shoots (Fig. 4a; $P = 0.02$ for comparison with No competition-C treatment, and $P=0.03$ with No competition-S treatment) and roots (Fig. 4b; $P = 0.03$ with No competition-C treatment, and $P<0.01$ with No competition-S treatment), and had higher SLA (Fig. 4e; $P = 0.03$ with No competition-C treatment, and $P<0.01$ 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, Fig. 4a: $P = 0.08$ with C

treatment and P = 0.09 with S treatment; in roots, Fig. 4b: P = 0.27 with C treatment and P = 0.03 with S treatment; and in SLA, Fig. 4e: P = 1 with C treatment and P = 0.63 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 > 0.05). 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 (Fig. 4).

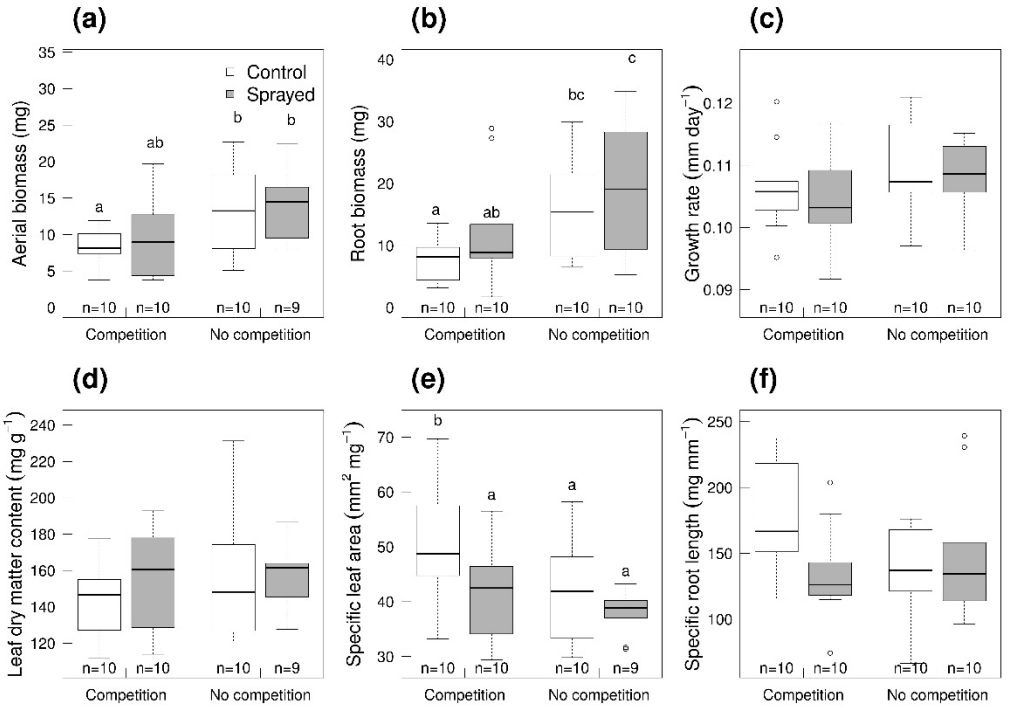

Figure 4: Effect of the demethylation treatment (C – control in white, S - spraying method in grey) on morphological and performance measurements of plants grown under competition during last generation (left), and on plants with no competition in previous generations(right). Differences (a) on the aerial biomass, (b) on the root biomass, (c) on the growth rate, and (c) on the leaf dry matter content, (d) specific leaf area, and (e) 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 absence of outliers. Different letters within each panel indicate significant differences between treatments (post hoc Tukey test, P = 0.05).

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; Verhoeven & van Gurp 2012; Herman & Sultan 2016). 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. Finnegan *et al.* 1996; Akimoto *et al.* 2007; Bossdorf *et al.* 2010). 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 (Fig. 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 (Fig. 2). These undesired effects of 5-azaC have previously been reported by other studies (Finnegan *et al.* 1996; Akimoto *et al.* 2007; Bossdorf *et al.* 2010; 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), since differences were already 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 (Fig, 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 (Fig. 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 six weeks of growing in pots (Experiment 2; Fig. 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 (Fig. 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 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 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 'equalised' 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 González 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. Whereas 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; Fig. 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 using plants coming from seeds and when assessing transgenerational effects *sensu stricto*. Also, it is a clear demonstration that competition can cause transgenerational effects on offspring phenotypes. In González *et al.* 2016, the clonal offspring of *Trifolium 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 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. 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 are not feasible, such an *in vivo* demethylation agent is currently the only tool readily available for experimental manipulation of non-model species (Verhoeven *et al.* 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 & Trasler 1996; Doerksen *et al.* 2000; 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 has been applied without clear standardized approaches, causing heterogeneity even in the application of the ‘traditional’ approach of germinating seeds in 5-azaC solution, and possibly adding

uncertainly in 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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Data accessibility

Data used in the analyses is available in the Dryad repository: http://doi:10.5061/dryad.k9f51 (Puy et al., **2017**).

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Supplementary material

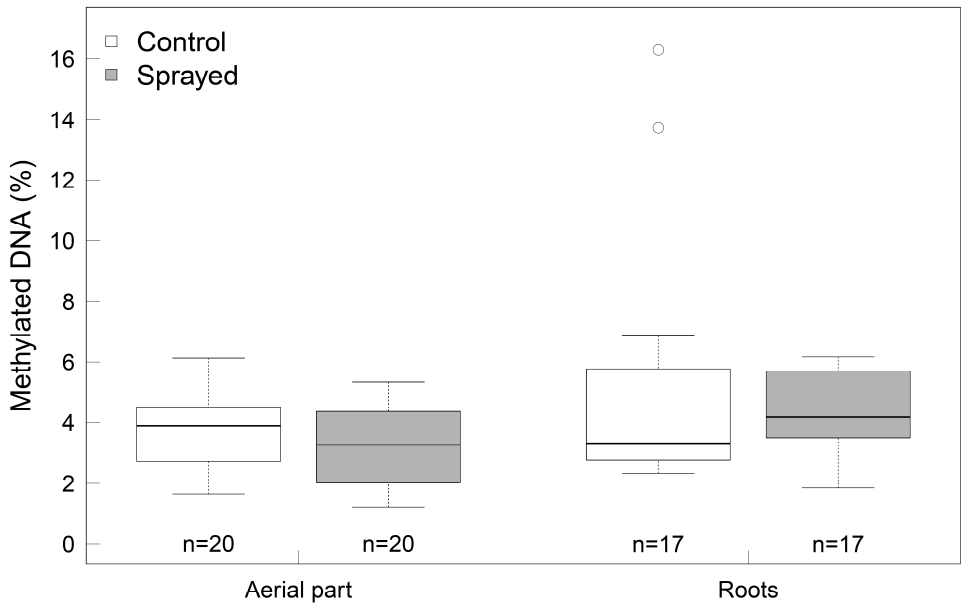

Figure S1: Differences between experimental treatments in the six-week-old seedlings (C – control in white, S - spraying method in grey) on the level of genome-wide DNA methylation in aerial part (left), and roots (right) of the plant. The bottom and top of the boxes are the 25th and 75th percentiles 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.

Chapter II

Competition-induced transgenerational plasticity feedbacks on the offspring's competitive interactions and leaf decomposition

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Manuscript submitted: Puy J., de Bello F., Dvořáková H., Medina N.G., Latzel V., Carmona C.P. Competition-induced transgenerational plasticity feedback on the offspring's competitive interactions and leaf decomposition. *New Phytologist*

Abstract

Phenotypic adjustments resulting from epigenetic plasticity can drive the adaptive responses of organisms to the environment without modifying the underlying DNA. These adjustments can be heritable via transgenerational effects, thus promoting fast adaptation of populations. Empirical studies have, so far, mainly focused on transgenerational effects in response to abiotic factors, but the response to species competition is still unknown. We tested for within- and across-generational plant plasticity triggered by different plant–plant competition intensities, using the perennial apomictic herb *Taraxacum brevicorniculatum* in four coordinated experiments. Also, we tested the role of phenotypic plasticity on promoting rapid adaptation and feedbacks on the competitive interactions, and on affecting ecosystem processes such decomposition. We found that, by promoting differences in DNA methylation, offspring from plants under stronger competition developed faster and presented more resource-conservative phenotypes. Further, these adjustments associated with a less degradable phenotypes that, in turn, might favour plants with more conservative traits creating a positive plant–soil feedback. Competition in the parental generation can thus reduce the intensity of competition and induce changes in decomposition, subsequently affecting competitive interactions. Our results demonstrate that competition-induced transgenerational effects can promote rapid adaptations and species coexistence, and feedback on biodiversity assembly and nutrient cycling.

Introduction

Phenotypic adjustments resulting from epigenetic plasticity can drive the adaptive responses of organisms to the environment without modifying the underlying DNA. These adjustments can be heritable via transgenerational effects, thus promoting fast adaptation of populations facing environmental change. Empirical studies have, so far, mainly focused on transgenerational effects in response to abiotic factors, but the response to species competition is still unknown. Here we show, for the first time, transgenerational effects triggered by plant–plant competition with feedback on competitive interactions and ecosystem processes such decomposition. We found that, by promoting differences in DNA methylation, stronger parental competition induced more competitive, resource-conservative, and less degradable offspring phenotypes. Competition in the parental generation can thus reduce the intensity of competition and induce changes in decomposition, subsequently affecting competitive interactions. Our results demonstrate that competition-induced transgenerational effects can promote rapid adaptations and feedback on biodiversity assembly and nutrient cycling.

Functional traits determine organisms' abilities to live in given ecological conditions and coexist with other species (Götzenberger *et al.* 2012; Kraft *et al.* 2015). Further, traits also shape the environment organisms live in by affecting ecosystem processes, such as nutrient cycling (Cornelissen & Thompson 1997; de Bello *et al.* 2010). These ideas have been formalized within the field of functional ecology by the "response–effect" framework (Lavorel & Garnier 2002). Recent studies show that adaptive responses of organisms to the environment do not only operate via selection of the fittest genotypes (Barrett & Schluter 2008), but also by phenotypic adjustments via plasticity (Price *et al.* 2003; Des Roches *et al.* 2018). Plasticity drives responses in traits linked to an organism's performance within its life cycle without any modifications to the underlying DNA sequence, i.e. via epigenetic mechanisms like DNA methylation (Bossdorf *et al.* 2008; Verhoeven *et al.* 2016; Richards *et al.* 2017). Furthermore, plasticity can be transmitted to the following generations (i.e. transgenerational

plasticity) (Bossdorf *et al.* 2008; Jablonka & Raz 2009; Herman & Sultan 2011; Verhoeven *et al.* 2016).

Transgenerational plasticity should promote fast, functional adaptations of populations towards environmental change and, by doing this, it can theoretically feed back to the functioning of the ecosystem (Bossdorf *et al.* 2008; Jablonka & Raz 2009; Herman & Sultan 2011; Richards *et al.* 2017). Thus, the response–effect framework could be theoretically applied also in the case of transgenerational plasticity. However, it remains unclear if transgenerational plasticity can feed back to key ecosystem functions (Latzel *et al.* 2013; Metz *et al.* 2015b; Richards *et al.* 2017). Furthermore, the great majority of existing studies focus on transgenerational responses to abiotic factors (Galloway & Etterson 2007; Latzel *et al.* 2014; Auge *et al.* 2017; Bej & Basak 2017), have overlooked the role of biotic interactions (Alonso *et al.* 2019a), such as competition between organisms, despite being leading factors for controlling species coexistence, biodiversity maintenance and ecosystem functioning (van der Putten *et al.* 2013; Kraft *et al.* 2015; Valladares *et al.* 2015).

Phenotypic changes towards more conservative phenotypes are frequently found in response to plant–plant competitive interactions (Gross *et al.* 2009; Kraft *et al.* 2015; Carmona *et al.* 2019). In the plant economics spectrum, a conservative phenotype is characterized by low SLA, high LDMC, high allocation to the roots and low SRL; and is associated with longer lifespan and better nutrient-use conservation (Díaz *et al.* 2016).

We thus hypothesize that coexistence-driven plant–plant interactions can (1) cause phenotypic plasticity toward more conservative strategies, which can also (2) be transmitted to the following generations (i.e. transgenerational plasticity), and both plasticity, in turn, can (3) affect the competitive interactions and decomposition processes, therefore creating a potential feedback-loop on the communities. Here, we summarize the results of four coordinated experiments (for one parental generation, two offspring generations, and one decomposition experiment; Fig. 1) using genetically identical individuals of *Taraxacum brevicorniculatum* Korol. to fully test the existence of transgenerational effects

triggered by plant–plant competition and their feedback on ecosystem functioning.

Material and methods

Study material

Taraxacum brevicorniculatum Korol. is an obligate apomictic polycarpic perennial species (Kirschner *et al.* 2013), ecologically similar to any other *Taraxacum sect. Ruderalia*. The genetically identical seeds used in this study were collected from a greenhouse-grown population of plants experiencing equal conditions for several generations (collected and genetically identified by Kirschner *et al.* (2013). This strategy ensured homogeneous genetic and epigenetic variation in the plant material. We ran four experiments using *T. brevicorniculatum*: a parental generation, two offspring generations, and a decomposition experiment (Fig. 1). Since *T. brevicorniculatum* is an obligate apomictic species, all plants in all experiments were genetically identical, and after experiencing different competition levels during the parental generations, the offspring only differed in non-genetic information they inherited. Thus, any differences in the offspring generation must be due to competition-related transgenerational effects.

Experimental setup

Parental generation. To induce competition-related transgenerational effects, we conducted a two-month greenhouse-pot experiment (May–July 2015) where genetically identical individuals of *T. brevicorniculatum* were grown with or without competition until flowering. For pots with competition we planted one individual of the focal species surrounded by six other individuals. The six surrounding individuals could be either monospecific (i.e. only one species from either *T. brevicorniculatum* itself or other ten different species, replicated eight times per combination; see Table S1) or a mixture of six different species (eight different combinations, replicated five times, see Table S1). This resulted in 19 competition levels. Further, a no competition treatment (replicated eight times)

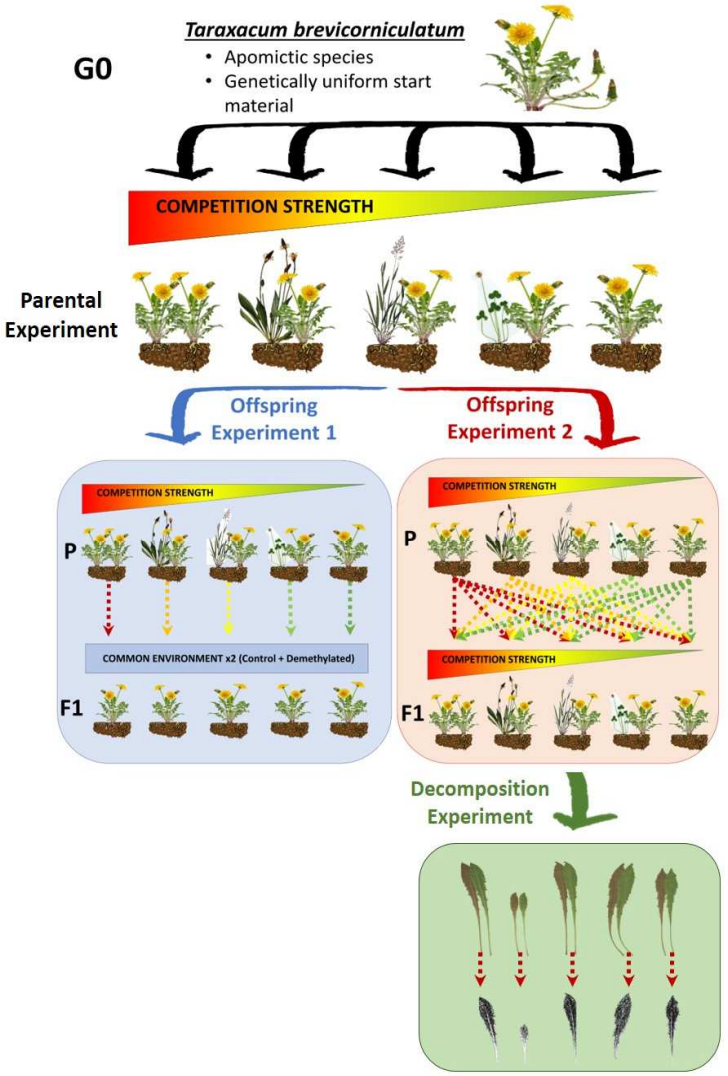

Figure 1: Schematic representation of the experiments considered.

was performed, where only the focal *T. brevicorniculatum* was planted in the pot; this gave a total of 20 different competition levels. All combinations were planted after germinating the seeds separately in Petri dishes and then transplanting the seedlings into round pots with a volume of 2 l filled with a 1:1 mixture of sand and commercial soil. Throughout the entire experiment, plants were watered regularly from the bottom ensuring the pot surface was wet.

We estimated the intensity of the competition experienced by the focal *T. brevicorniculatum* with the relative interaction intensity (RII) index, which reflects the effect of competition by comparing the aboveground biomass observed when growing with competitors with the biomass achieved growing in absence of interaction, following the formula outlined in Armas *et al.* (2004). The more negative the RII value is, the stronger is the reduction in biomass experienced by the focal plant relative to the biomass without competition. Consequently, in subsequent experiments, we used the average RII across all pots from each of the 20 competition treatments of the parental experiment to express the competition intensities experienced by the parental generation as a continuous variable (see Table S1).

At the end of the parental generation experiment, seeds of each focal plant were collected. After measuring the average seed mass per competition level, seeds were stored in the cold (2–4 ºC).

Offspring experiment 1. The aims of this experiment were to test for transgenerational effects on the performance of juvenile offspring, and to test whether these effects were transmitted via DNA methylation. For this purpose, we used seeds coming from individuals that experienced monospecific competition during the previous competition experiment. Seedlings from these seeds were grown individually, and without competition, in a growth chamber until they reached the juvenile stage. Plants were grown with a 12 h (20°C) / 12 h (10°C) light/darkness and temperature regime and watered regularly. From each monospecific parental competition level, we established 20 pots (7 x 7 cm square-shaped and 18 cm depth), and for half of them we altered the epigenetic status by DNA-demethylation with 5-azacytidine (5-azaC). Experimental demethylation is a well-established method that by removing, heritable or not, epigenetic marks; allows to test whether the variation in plant phenotypic traits was mediated by epigenetic mechanisms (Richards *et al.* 2017; Puy *et al.* 2018; Alonso *et al.* 2019a).

To measure germination, six seeds were placed in each pot and after 11 days, when all the pots contained at least one individual with a true leaf (i.e.

excluding cotyledons), the emerged seedlings were thinned until only the biggest one remained in each pot. At the same time (after 11 days), we started to apply the demethylation treatment, which involved spraying a 50 μM aqueous solution of 5-azaC onto the leaves daily for six weeks (following Puy *et al.* 2018). To remove any potential effect of non-uniform growing conditions from our design, we distributed the replicates in 10 blocks, each of them including two replicates of each of the 11 monospecific competition levels, one with and one without the demethylation treatment. Thus, the final design comprised 10 blocks x 11 competition levels x 2 demethylation treatments = 220 plants in total. The position of the replicates for each competition level was randomized between the blocks but maintained between demethylation treatments within blocks. Sand was used as the potting substrate in all cases to facilitate root extraction during the harvest.

Offspring experiment 2. The aims of this experiment were to test for transgenerational effects on the offspring during their adult stage, meanwhile they undergo similar or distinct competition intensity than their parents. We consider transgenerational effects to be adaptive when the offspring living under the same conditions as their parents perform better in those conditions (e.g. higher biomass) than plants with a different origin. In this experiment, seeds from six of the 20 parental competition levels were selected to attain a manageable experimental size – see below. The six levels included: two intense competition levels (one from the monospecific and another from the mixture combination), two weak competition levels (one from the monospecific and another from the mixture combination), intraspecific competition, and no competition (see Table S1). For this, after germinating the seeds in Petri dishes, we transplanted and grew the offspring under the six competition levels experienced by the parental generation using a full factorial design. This design considered all six competition levels (6 parental competition levels x 6 offspring competition levels = 36 combinations). Following the same experimental set-up as in the parental generation, we conducted a two-month greenhouse-pot experiment (May–July 2016) where 12 replicates per parental and offspring condition combination were randomly placed in the greenhouse, for a total of 432 pots. The pots, substrate

and watering regime were the same as in the parental experiment to ensure the most similar conditions.

Decomposition experiment. We aimed to test whether transgenerational plasticity may not only affect the life of offspring individuals, but also their "afterlives", by analysing the decomposability of leaves and litter-senescent material. For this purpose, we incubated five replicates per treatment of fresh leaves from offspring experiment 2 and, as a reference, one replicate of senescent material. The plant material was collected during the harvest of offspring experiment 2 and oven-dried at 60ºC. The samples were incubated in 18 x 18 cm nylon bags with a 1 mm mesh on the bottom and a 4 mm mesh on the top to avoid loss of litter material and, at the same time, allow macrofauna access to the litter. Each litterbag contained 0.36 g of biomass. The litterbags were placed in a purpose-built outdoor incubation bed, located in an open area of the botanical garden of the Institute of Botany in Třeboň, Czech Republic (N 49°00′ 20″, E 14°46′25″). To maintain homogeneous microenvironmental conditions, the incubation bed was cleaned from vegetation and covered with sand. For the same reason, the litterbags were covered with 1 cm of sand. Extra samples of all the treatments were incubated and checked every two weeks to monitor the speed of the decomposition and terminate the experiment when the samples reached on average 50% biomass loss (Pérez-Harguindeguy *et al.* 2013). Incubation started on 19th September and was terminated on 21st October when the samples had lost ca. 65% of biomass.

Measured variables

Parental generation. At the time of harvest, we measured seed output (i.e. number of seeds), total dry biomass (radicular and aerial) per plant, and aboveground vegetative traits. For each focal plant, two leaves were collected, the leaf area scanned, and then they were weighed by fresh mass and dry mass after drying at 60ºC (48 h). We used these measurements to estimate specific leaf area (SLA; leaf area per unit dry mass, mm^2/mg) and leaf dry matter content (LDMC; the ratio of leaf dry mass to leaf fresh mass, mg/mg). As mentioned above, the intensity of the competition experienced by the focal individual was

estimated using the RII index based on the aboveground biomass. Using other indicators to measure RII (e.g. total biomass or seed production) gave similar results due to their high correlation (0.97 and 0.79 Pearson's coefficient respectively). We transformed the 20 competition levels into a continuous variable reflecting the competition gradient by assigning to each level the average RII of the focal plants at that same competitive level (see Table S1). This allowed us to characterize each plant in the offspring experiments by a "parental competition" RII.

Offspring experiment 1. The number of germinated seeds per pot was counted five times (4, 6, 8, 10 and 11 days after sowing, always before applying the demethylation treatment). Total germination percentage was calculated as the final cumulative germination of the six sown seeds. We also calculated T_{50}, i.e. the time at which half of the total germination percentage was reached in each pot, following (Coolbear *et al.* 1984). Every fourth day, starting four days after the beginning of the demethylation treatment until the end of the experiment (six weeks), we measured the maximum diameter of the rosette (cm) and the total number of leaves. We used this information to estimate growth rates for the plants; for this, in each pot, we regressed the diameter of the rosette and number of leaves against time (in days), using linear and Poisson regressions, respectively. We used the slopes of these regressions in each pot as indicators of the growth rates in these two parameters, with greater slopes indicating faster growth.

Epigenetic parental effects are likely to fade away with time (Dechaine *et al.* 2015). We checked this by estimating the growth rates described above several times in each pot; the first growth rates were estimated considering only the first four measurements (i.e. 4, 8,12 and 16 days after the beginning of the demethylation treatment), and then we estimated an extra growth rate for each day added (each time including all the measurements until that time). Thus, we had seven measurements of growth rate from the first 16 days, until the 42nd day, every four days.

At the end of the experiment, plants were harvested and above- and belowground vegetative traits and total biomass were measured. For each plant, SLA and LDMC were measured. In addition, roots were carefully extracted by digging up the whole root system, washing it, scanning it and weighing it as both fresh mass and dry mass after drying at 60ºC (48 h). Total root length, average root diameter (mm), and distribution of root length in different diameter classes were determined using the image analysis software WinRHIZO Pro, 2008 (Regent Instruments Inc., Quebec, Canada). We used these measurements to estimate specific root length (SRL; root length per unit dry mass, m/g), root dry matter content (RDMC; the ratio of root dry mass to root fresh mass, mg/mg) and percentage of fine roots (ratio of root length with a diameter < 0.5mm by the total root length). Further, we estimated root mass factor (RMF; ratio of root biomass per total biomass, g/g) after drying the remaining aerial plant parts at 60ºC (48 h).

Offspring experiment 2. Seed output per plant and biomass were measured at the time of harvest, as for the parental generation. In addition, for each plant we measured SLA, LDMC, SRL, fine root percentage and RMF, following the protocols described above. Additionally, for five replicates per parental and offspring condition we measured C, N and P content of leaves, as well as storage-carbohydrates content of taproots. Total C and N concentrations were determined by dry combustion using an elemental analyser (CHNS Elemental Analyzer vario MICRO cube, Elementar Analysensysteme GmbH, Germany). Total P was determined by flow injection analysis (FIA), and storage-carbohydrates content was measured using a total starch assay procedure (Megazyme, Bray, Ireland) following the amyloglucosidase/alpha-amylase method.

Decomposition experiment. Biomass loss was estimated as the proportion of initial *vs.* remaining biomass. Given that the samples were difficult to separate from the sand, the remaining biomass was measured after burning the samples in a specifically designed oven at 575ºC for four hours. Thus, the remaining biomass after decomposition was calculated as the difference between the initial weight before burning and the final weight after ashes were removed in which only inorganic material remained.

Statistical analysis

All analyses were carried out using R v3.2.3 (R Core Team 2016) with $\alpha = 0.05$ as the significance threshold. Because parental competition could generate differences in seed quality and resources of the offspring that could mask the effect of transgenerational plasticity in its performance and phenotype (Herman & Sultan 2011; Dechaine *et al.* 2015; Germain *et al.* 2019), we included seed mass as a covariate in all analysis when significant.

Offspring experiment 1. We tested the effect of the competition experienced by the parental generation (RII computed from the parental experiment) on germination (T_{50} and germination percentage) and growth rate (rosette diameter increase rate and leaf production rate: from the first 16 days until the 42nd day, every four days) in the offspring experiment. This was analysed using mixed effects models where parental competition was used as fixed factor, including seed mass as covariable, and the experimental blocks as a random factor. Demethylated and control individuals were analysed separately, except for the germination-related parameters because the demethylation treatment had not been applied yet.

To account for the effect on functional traits, we analysed the effect of parental competition on single traits and also on the combination of traits. The latter was approached via a principal component analysis (PCA) on the different traits, performed in order to reduce the multi-trait space to a single main axis as in Kraft *et al.* (2014). We fitted a mixed effects model similar to the one described above, separating demethylated and control plants. However, we did not include seed mass as a covariate, due to its lack of significance in the models.

Offspring experiment 2 and decomposition experiment. The parental and offspring competition experienced by the plants were characterized with the RII measured in the parental competition experiment (see above, i.e. average of the treatment level RII values). In other words, we assigned a competition strength value (RII measured in the parental generation) to each of the identities of the competitors (no matter they are from the parental or offspring generation). For

example, let us consider an offspring plant coming from a parental plant that competed with *Leontodon* in the parental experiment (parental competition RII = -0.59; Table S1). This offspring plant could grow with the same competitor or with a different one in offspring experiment 2. If the plant in question grows with the same competitor, the expected offspring competition of the offspring would be the same as in the parental experiment, so that the two RII values would be the same. If the plant in question grows with a different competitor, then the expected offspring competition would correspond to the RII of the corresponding competition level measured in the parental experiment (e.g. if competing with *Plantago media* during offspring experiment 2, the expected offspring competition would be equal to -0.24, reflecting the lower competitive impact of *P. media*).

The effect of parental and offspring competition on plant traits (single traits and also on a combination using PCA) and leaf decomposition was analysed using mixed effects models with parental and offspring competition and their interaction (when significant) as fixed factors and taking into consideration the seed mass as a covariable (also when significant). The location where the individual was placed in the greenhouse was used as a random factor to account for potential effects of spatial heterogeneity.

Results

Offspring experiment 1

We found that juvenile offspring coming from parents experiencing more intense competition had faster germination (i.e. lower T50; $F = 6.76$, $df = 208$, $P = 0.010$; Fig. 2a), without differences in the overall germination percentage (z value = -0.008, $P = 0.994$) and faster growth (measured as leaf creation rate; $F = 7.42$, $df = 98.09$, $P = 0.008$; Fig. 2b). The competition experienced by parents also affected the phenotypic characteristics of the offspring.

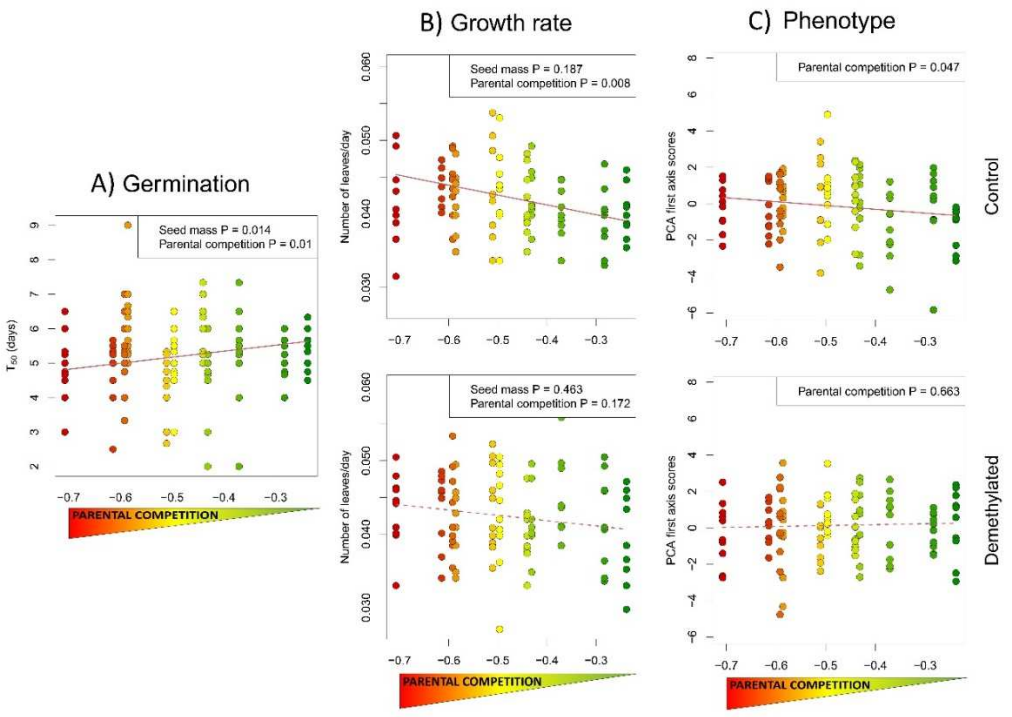

Figure 2: Effect of the competition experienced by the parents on a) offspring germination, b) growth rate over 42 days for the control treatment (top row) and demethylated treatment (bottom row), and c) multi-trait variation for the control treatment (top row) and demethylated treatment (bottom row). The different colours of the points, from green to red tones, represent the gradient of competition experienced by the parents from low to high. The significance values of the fixed factors included in each model are shown in the boxes.

In the PCA based on the traits measured, the first axis which absorbed 46% of the variation reflected the resource-use strategy gradient between individuals: from higher values reflecting plants with a conservative strategy (higher LDMC, RMF and root diameter) to lower values for individuals with a more acquisitive strategy (higher SLA, SRL and percentage of fine roots) (Fig. S1). Based on PCA scores, offspring's phenotype became more conservative resource-use phenotype with stronger parental competition ($F = 4.05$, $df = 98.02$, $P = 0.047$; Fig. 2c).

Further, we confirmed that these effects were controlled epigenetically. This was demonstrated by making these effects disappear when we removed the epigenetic signature of the individuals by application of a demethylation agent (Puy *et al.* 2018) (growth rate: $F = 1.90$, $df = 98.08$, $P = 0.172$; Fig. 2b; phenotype: $F = 0.19$, $df = 96.90$, $P = 0.663$; Fig. 2c) suggesting that they were associated to different DNA methylation patterns induced by the parental competition.

Offspring experiment 2

Offspring functional traits were strongly affected by the offspring competitive environment and, towards more conservative phenotypes (Fig. 3). Additionally, for some of the traits (SLA, RMF and storage-carbohydrate allocation, Fig. 3) we found that transgenerational effects further reinforced the conservative phenotype when the offspring came from parents experiencing strong competition. These transgenerational effects were concordant with the plastic response to the offspring competition environment (lower SLA, high RMF; Fig. 3a, 3b) or operated regardless of the offspring conditions (allocating more storage-carbohydrates; Fig. 3d). Offspring from parents that suffered non or little competition became smaller when growing with strong competition, whereas the offspring from parents under strong competition showed the opposite pattern, becoming taller when they had a competitive environment (Fig. 3c).

Decomposition experiment

We showed that increasing levels of both offspring ($F = 24.44$, $df = 192.44$, $P < 0.001$) and parental competition ($F = 8.35$ $df = 192.40$, $P = 0.004$) resulted in reduced leaf decomposition rates (Fig. 4), consistent with the shift in more conservative traits shown above. The effect of parental competition on decomposition was mediated by changes in the leaf traits that regulate these processes; decomposition rates were positively correlated with SLA and leaf P content, and negatively with LDMC and leaf C:N content ratio (Fig. S2). The litter decomposed following the same decomposition pattern as the fresh leaves (Table S2, Fig. S3).

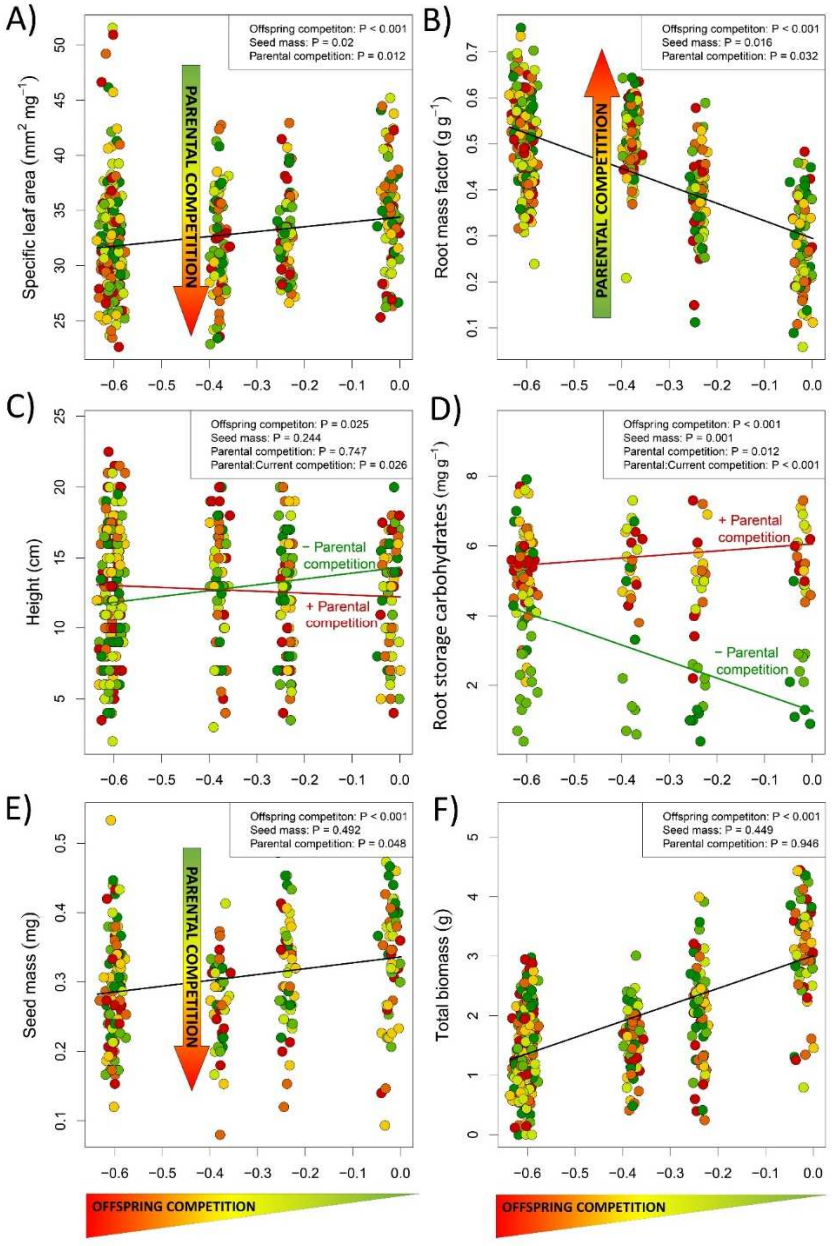

Figure 3: Effect of the offspring and parental competition on different adult phenotype characteristics of the offspring: a) Specific leaf area, b) root mass factor, c) vegetative height, d) root storage-carbohydrates content, e) seed mass and f) total dry biomass. The different colours of the points, from green to red tones, represent the gradient of competition experienced by the parents from low to high. The significance values of the fixed factors included in each model are shown in the boxes.

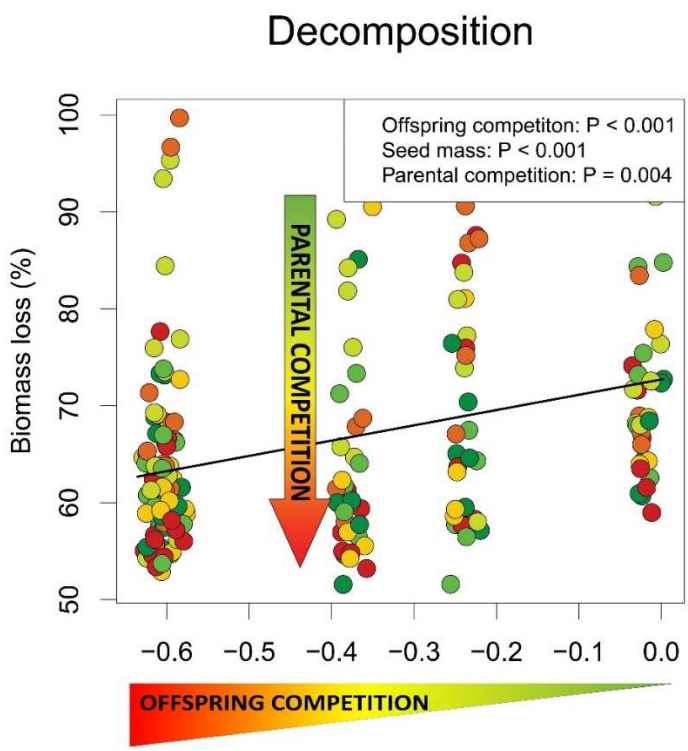

Figure 4: Effect of the offspring and parental competition in the leaf decomposability of the offspring. The different colours of the points, from green to red tones, represent the gradient of competition experienced by the parents from low to high. The significance values of the fixed factors included in each model are shown in the boxes.

Discussion

To the best of our knowledge this is the first empirical evidence that demonstrate the importance of parental competition affecting the competition and functioning of following generations via transgenerational trait plasticity. We found that stronger competition triggered plastic modifications towards a more competitive resource-conservative phenotype. We found that the offspring from plants under stronger competition had also more resource-conservative phenotypes and faster development, affecting back the competitive interactions. Further, we demonstrated that these transgenerational changes are controlled by DNA-methylation mechanisms. Moreover, we test whether these transgenerational effects can feedback on ecosystem processes by means of a leaf decomposition

experiment finding that stronger parental competition resulted in less decomposable leaves.

Several studies in recent years have shown the importance of trait plasticity for the assembly and functioning of populations and communities (Price *et al.* 2003; Valladares *et al.* 2015; Des Roches *et al.* 2018). In response to plant–plant competitive interactions, intraspecific adjustments towards more conservative phenotypes are frequently found (Gross *et al.* 2009; Kraft *et al.* 2015; Carmona *et al.* 2019). In our case, during the parental generation we found the same pattern, where stronger competition triggered plastic modifications towards a more conservative phenotype (i.e. higher LDMC and RMF; Fig. S4). This plasticity can lead to adaptation when there is a competitive hierarchy dominated by more conservative-strategy phenotypes (Kraft *et al.* 2015), and can promote coexistence by reducing trait hierarchies and competition's intensity (Carmona *et al.* 2019). We then hypothesized that if these phenotypic changes were passed to the offspring through transgenerational effects, this could in turn modify the competitive interactions in the next generation. This is the first work reporting that competitive interactions trigger transgenerational plasticity, which affects not only the early performance of the offspring, but also their adult life stage and their "afterlives".

We found that juvenile offspring coming from parents experiencing more intense competition achieved greater competitive performance. These benefits included faster germination and faster growth, which provide greater performance and a competitive advantage (Seiwa 2000; Afonso *et al.* 2014; Gioria *et al.* 2018). Further, the offspring from parents under intense competition displayed a more conservative resource-use phenotype (i.e. higher LDMC, RMF and root diameter), maintaining the same pattern as the parental generation. Parental competition can affect offspring performance and phenotype through two mechanisms: by generating differences in seed quality and resources, or by transgenerational plasticity (Herman & Sultan 2011; Dechaine *et al.* 2015; Germain *et al.* 2019). In our case, stronger parental competition produced smaller seeds. However, the effects of parental competition remained significant even after including seed mass as a covariate. This suggests that seed resources were

not the only mechanism driving our observed transgenerational effects and points to other mechanisms such as heritable epigenetic plasticity or hormonal balance in embryos (Herman & Sultan 2011; Rottstock *et al.* 2017). Also, even the parental effects are likely to fade away with time (Dechaine *et al.* 2015), the effects associated to differences in seed mass seem to fade away faster. Meanwhile the effect of seed mass on growth rate lasted until the 24th day (i.e. 35 days old plants), the transgenerational effects persisted been detectable until the end of the experiment (Fig. S5). In our case, when we applied the demethylation agent that removed the epigenetic signature of the plants (Puy *et al.* 2018), the differences in performance and phenotype of the individuals disappeared; strongly suggesting that the observed adaptive transgenerational effects was controlled epigenetically, and at least partially enabled by DNA cytosine methylation.

We found that the transgenerational effects also extended during the adult stage. At that stage, transgenerational effects further reinforced the conservative phenotype when the offspring came from parents experiencing strong competition (Fig 3a-b). While we did not find that offspring that re-experienced the exact condition as their parents had higher biomass (which could reflect an adaptive inheritance of characteristics, Fig. 3f), offspring grew taller when they had the same competitive environment as their parents (Fig. 3c). Altogether these results confirm broad phenotypic modification due to parental coexistence conditions that are maintained in the offspring generation.

Finally, we found that these transgenerational effects affect the "afterlives" of the individuals, showing for the first time that transgenerational effects can extend on larger scales, affecting ecosystem processes like decomposition. Increasing levels of offspring and parental competition resulted in more conservative leaf traits (like LDMC and leaf C:N), that are related to more structural and slower degradable organic matter in leaves, which takes longer to be returned to the soil (Cornelissen & Thompson 1997). Interestingly, slower degradation might in turn favor those plants with a more resource-use-conservative phenotype, which have lower rates of nutrient uptake, subsequently affecting the plant–plant competitive interactions (van der Putten *et al.* 2013;

Semchenko *et al.* 2017). This opens a new field of research on the potential positive plant–soil feedback triggered by plant–plant competition.

Thus, our results suggest that transgenerational plasticity can promote rapid adaptation with feedback on plant–plant competitive interactions (Gross *et al.* 2009; Kraft *et al.* 2015; Carmona *et al.* 2019), and ecosystem functioning (van der Putten *et al.* 2013; Semchenko *et al.* 2017). In a context where the importance of intraspecific variability for populations and communities is increasingly acknowledged, our study adds transgenerational plasticity to this as both a consequence and a driver of coexistence between species.

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Supplementary material

Table S1: Summary of the competitive strength associated with each of the 20 different competition levels, measured during the parental experiment. The table presents the competition levels that were grown during offspring experiment 1 (only monospecific competition; highlighted in grey) and the competition levels that were grown during offspring experiment 2 (two intense competition levels: one from the monospecific and another from the mixture combination, two weak competition levels: one from the monospecific and another from the mixture combination, intraspecific competition, and no competition; in bold face).

Competition levels	Competitors identity	Associated RII
No competition	**—**	**-0.018**
Monospecific: Interspecific	*Achillea millefolium*	-0.586
	Alopecurus pratensis	-0.431
	Dianthus deltoides	-0.283
	Holcus lanatus	-0.511
	Leontodon hispidus	**-0.592**
	Lotus corniculatus	-0.371
	Plantago lanceolata	-0.707
	Plantago media	**-0.238**
	Prunella vulgaris	-0.496
	Trifolium pratense	-0.440
Monospecific: Intraspecific	***Taraxacum brevicorniculatum***	**-0.614**
Mixture	*A. millefolium, A. pratensis, D. deltoides, L. hispidus, P. lanceolata, P. media*	-0.409
	A. pratensis, D. deltoides, P. lanceolata, P. media, P. vulgaris, T. pratense	-0.447
	A. millefolium, A. pratensis, D. deltoides, H. lanatus, P. media, P. vulgaris	-0.621
	A. millefolium, D. deltoides, L. hispidus, P. media, P. vulgaris, T. pratense	-0.378
	H. lanatus, L. hispidus, L. corniculatus, P. lanceolata, P. media, T. pratense	-0.579
	A. millefolium, L. corniculatus, P. lanceolata, P. media, P. vulgaris, T. pratense	**-0.603**
	A. pratensis, L. hispidus, L. corniculatus, P. lanceolata, P. vulgaris, T. pratense	-0.343
	A. pratensis, D. deltoides, H. lanatus, L. corniculatus, P. vulgaris, T. pratense	**-0.378**

Table S2: Summary of the model in which offspring and parental competition explain litter-senescence material. Non-significant values are caused by the absence of replicates.

Variable	**Estimate**	**t value**	***P* (>*F*)**
Offspring competition	14.79	1.86	0.07
Parental competition	8.70	1.09	0.28

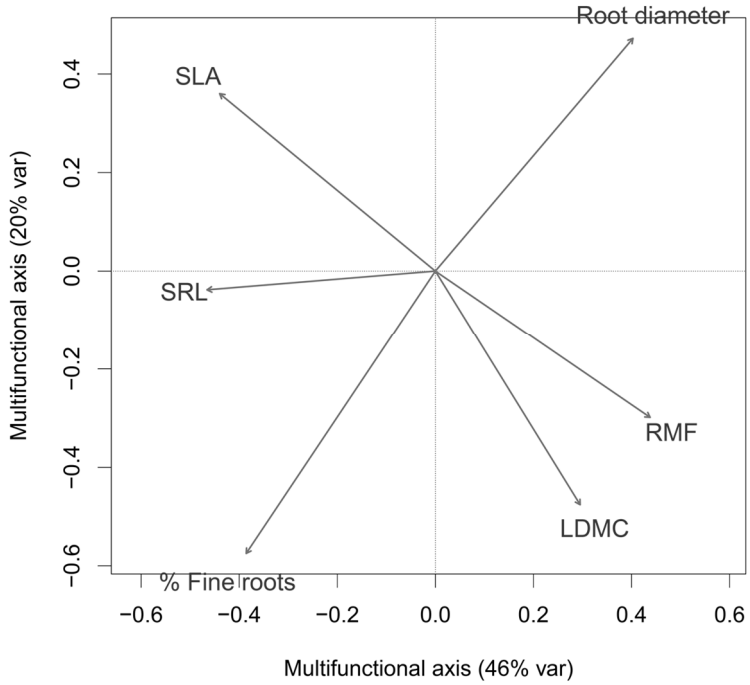


Figure S1: Principal component analysis (PCA) showing relationships among morphological traits of the offspring generation (offspring experiment 1). Arrows represent the traits used to build the principal component, i.e. specific leaf area (SLA), specific root length (SRL), root mass factor (RMF), leaf dry matter content (LDMC), average root diameter (Root diameter) and percentage of fine roots (% Fine roots).

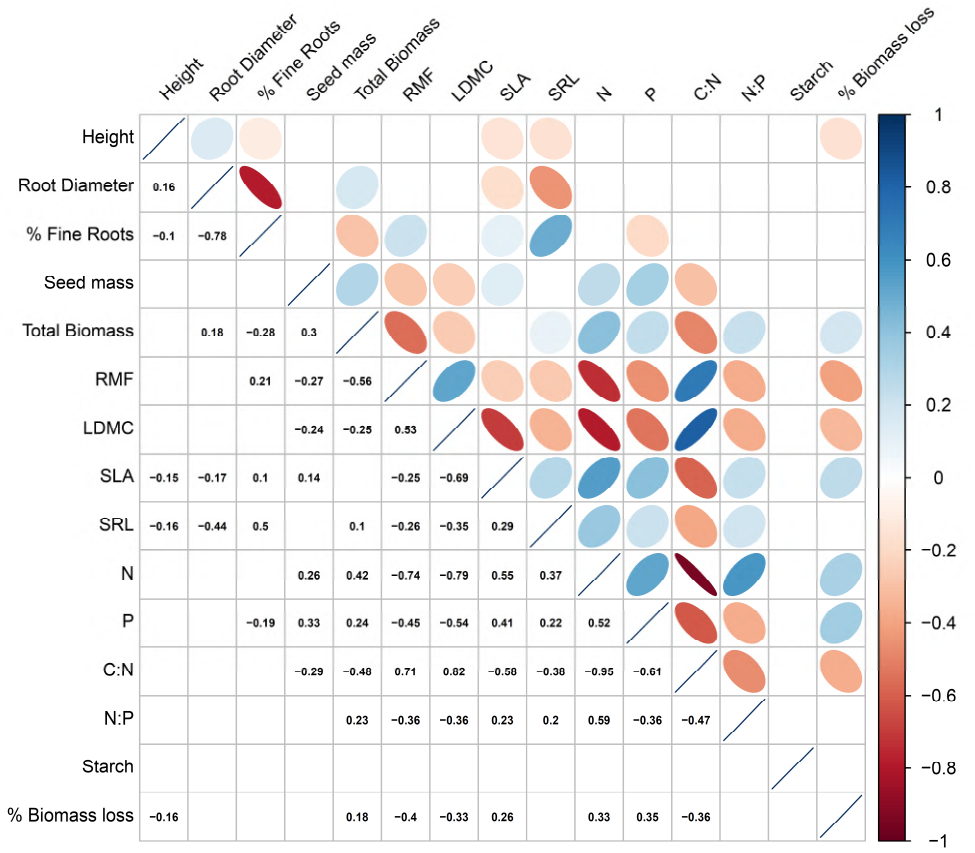


Figure S2: Correlation between pairs of traits measured in offspring experiment 2 and the decomposition rate of the decomposition experiment. Only significant correlations are represented. Below the diagonal, the numerical Pearson coefficients are displayed, while above the diagonal the coefficients are represented by coloured ellipses: blue is positive, red is negative, and the intensity of the colour represents the strength of the coefficient.

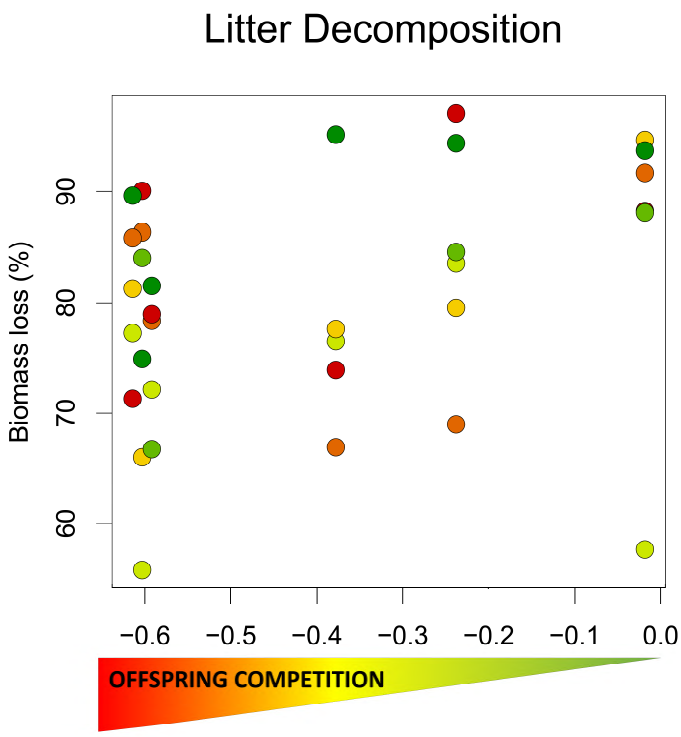


Figure S3: Effect of the offspring and parental competition on the litter-senescence decomposability of the offspring. The different colours of the points, from green to red tones, represent the gradient of competition experienced by the parents from low to high.

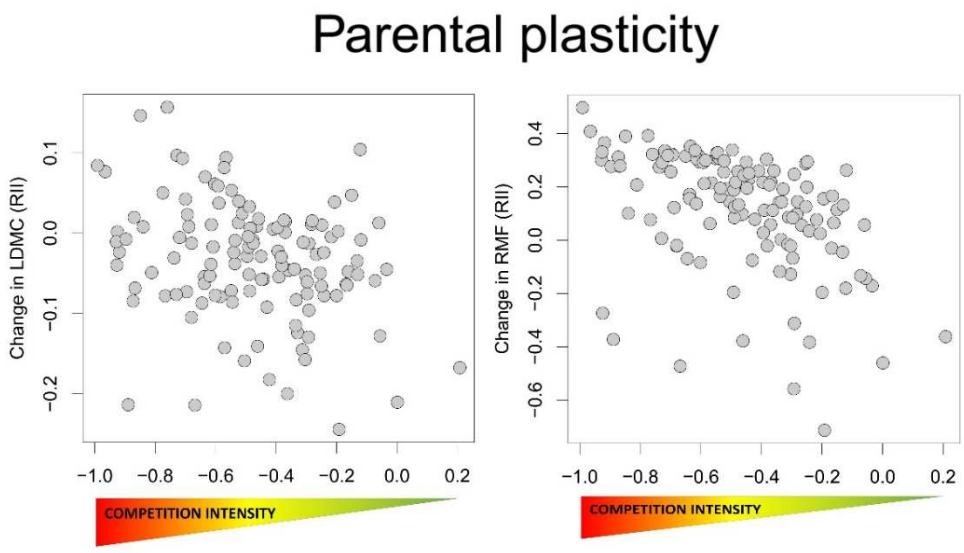


Figure S4: Effect of competition on trait plasticity of the parental generation measured with leaf dry matter content (LDMC, on the left), and root mass factor (RMF, on the right); both are traits indicative of a conservative phenotype. Both traits, are expressed with the relative interaction intensity (RII) index, which reflects the trait plasticity by comparing the trait value measured when growing with competitors with the value achieved growing in absence of interaction. The more negative the RII values are, the more the focal plant reduces its LDMC or RMF compared with the value of the plant without competition. comparing the aboveground biomass observed when growing with competitors with the biomass achieved growing in absence of interaction.

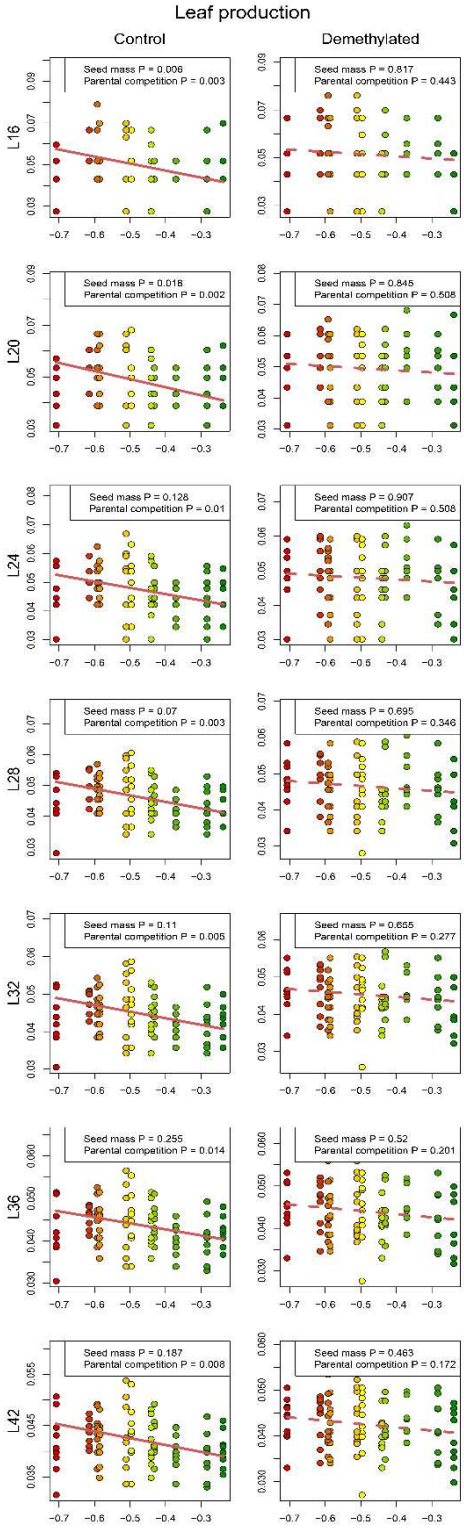
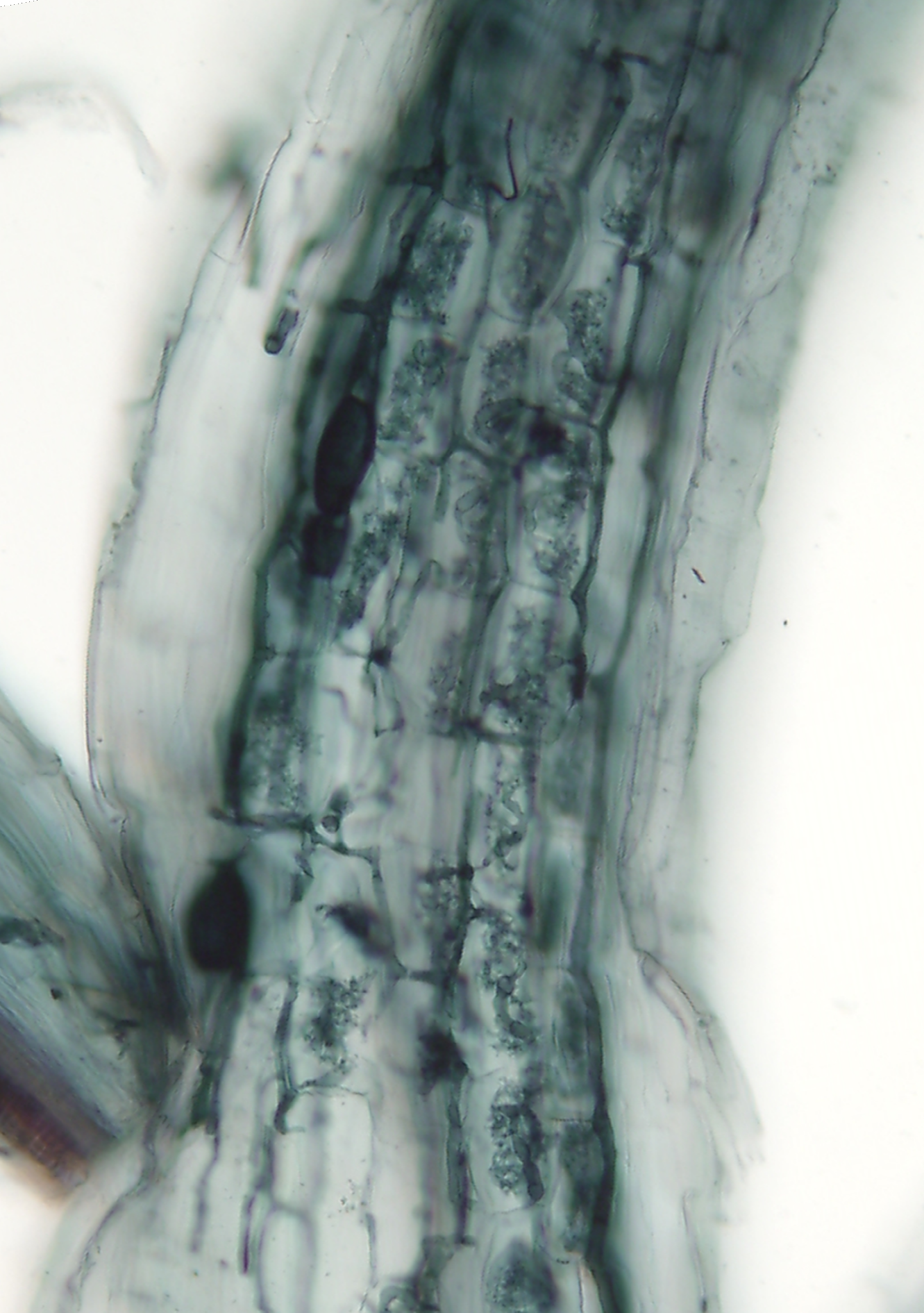


Figure S5: Effect of the competition experienced by the parents on leaf production rate from the first 16 days (upper row) until the 42nd day (lower row), for the control treatment (left column) and demethylated treatment (right column). The first P value is the effect of seed mass, and second one is the effect of the competition experienced by the parents.



Chapter III

Mycorrhizal symbiosis alleviates plant drought stress within and across plant generations via plasticity

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Abstract

Phenotypic plasticity is essential for organisms to adapt to local ecological conditions. Little is known about how mutualistic interactions, such as arbuscular mycorrhizal (AM) symbiosis, mediate plant phenotypic plasticity and to what extent this plasticity may be heritable (i.e. transgenerational effects). We tested for within- and across-generational plant plasticity in response to AM symbiosis and varying water availability in a full factorial experiment over two generations, using the perennial apomictic herb *Taraxacum brevicorniculatum*. We examined changes in phenotype, performance and AM fungal colonization of the offspring throughout plant development. AM symbiosis and water availability triggered phenotypic changes during the life cycle of plants. Moreover, AM triggered adaptive transgenerational effects, especially detectable during the juvenile stage. Drought stress and absence of AM fungi triggered concordant plant phenotypic modifications towards a "stress-coping phenotype", both within- and across-generations. Additionally, transgenerational effects influenced AM fungal colonization, in turn affecting the mutualistic interaction. AM symbiosis can trigger transgenerational effects, including changes in the AM fungal colonization of offspring and their functional traits related to resource-use acquisition. Thus, the transgenerational effects of mycorrhizal symbiosis are not limited to plant fitness, but also improve plants' ability to cope with environmental stress.

Introduction

Abiotic processes and prevailing biotic interactions select for the best-adapted individuals within and across species (de Bello *et al.* 2012; Vellend 2016). The ability of a species to adapt to a particular environment may depend on the pressure of natural selection, heritable genetic variability, but also on its phenotypic plasticity (Price *et al.* 2003). Phenotypic plasticity is the ability of an organism to modify its performance in response to the environment (Price *et al.* 2003). Epigenetic mechanisms have been proposed as key in phenotypic plasticity because they lead to changes in an organism's performance within its life cycle, without any modifications in the underlying genomic DNA sequence (Bossdorf *et al.* 2008; Verhoeven *et al.* 2016). These changes may also be transmitted to the following generations via transgenerational plasticity, i.e. the abiotic and biotic environment experienced by the parental generation can influence the phenotype of the offspring (Jablonka & Raz 2009; Herman & Sultan 2011). Thus, transgenerational effects could play a key role in the adaptation of organisms, particularly during juvenile stages, and have proven essential for adaptation to predictable environmental conditions (Latzel *et al.* 2014; Dechaine *et al.* 2015). However, little is known about the relative effect of transgenerational effects triggered by biotic conditions (Alonso *et al.* 2019b), and even less about how they interact with abiotic factors.

Together with species' adaptations to environmental conditions in a site, biotic interactions are considered key drivers of plant community assembly (de Bello *et al.* 2012). Among these, positive interactions such as mycorrhizal symbiosis are essential in determining, and potentially expanding, the realized niches of species (van der Heijden *et al.* 2003; Peay 2016; Gerz *et al.* 2018). Arbuscular mycorrhizal (AM) symbiosis is a widespread mutualistic association between plant roots and fungi from the subphylum Glomeromycotina (Smith & Read 2008; Spatafora *et al.* 2016). This association is considered mutually beneficial, since, in exchange for photosynthetic carbon, the AM fungi provide host plants with soil nutrients (mainly phosphates), mitigate abiotic stress (e.g., drought) and increase resistance to biotic stress, including pathogens (Lu & Koide 1994; Smith & Read 2008). The AM establishment, activity, and the final

outcome of the interaction (from positive to negative) can depend on multiple factors (Johnson *et al.* 1997; Hoeksema *et al.* 2010). These factors include the genotype of both partners, plant developmental stage (Jones & Smith 2004), and environmental factors such as soil nutrient and water availability (Pozo *et al.* 2015). Phosphorus, nitrogen or water deficiency in plants generally stimulates AM symbiosis and influences the proportion of AM structures (i.e. arbuscules, vesicles, etc.) (Martínez-García *et al.* 2012; Pozo *et al.* 2015). However, it is not known whether the environmental stress experienced by the parental generation also affects the AM symbiosis of the offspring (De Long *et al.* 2019).

The fitness benefits of plants in AM symbiosis are well known (Lu & Koide 1994; Smith & Read 2008). However it is unclear whether these benefits could partly operate through adaptive phenotypic plasticity leading to changes in plant morphological traits, such as root architecture (Nuortila *et al.* 2004; Goh *et al.* 2013; Fusconi 2014). Moreover, it remains unclear whether AM symbiosis of the parental generation triggers phenotypic changes in their offspring (i.e. transgenerational effects) that provide benefits to the offspring generation (Koide 2010; Varga *et al.* 2013). Most of the existing evidence demonstrates that having mycorrhizal parents can be beneficial during the early stages of development of the offspring, i.e. increasing biomass, survival, growth rate, nutrient content, and seed production (Heppell *et al.* 1998; Koide 2010). These differences can be due to epigenetic heritable phenotypic plasticity, but also due to differences in seed provisioning, where nutritional reserves are stocked up by the maternal plants (Herman & Sultan 2011). However, the latter has rarely been considered when testing for transgenerational effects of AM symbiosis (with the exception of Varga *et al.*, 2013). Furthermore, it is not known whether transgenerational effects persist to the adult stage of the offspring. Finally, the relative effect of combined biotic and abiotic drivers on transgenerational effects has been very rarely assessed (Metz *et al.* 2015a; González *et al.* 2017), yet biotic drivers can potentially modulate the effect of environmental stress via phenotypic plasticity.

Here, we conducted a two-generation experiment to test for within- and across-generation plant plasticity (i.e. transgenerational effects) in response to AM symbiosis using the perennial apomictic herb *Taraxacum*

brevicorniculatum. Further, in order to test whether this plasticity differs under abiotic stress conditions, we included a drought stress treatment. Importantly, we tested whether these changes were adaptive, resulting in an improved ability to cope with drought stress. We then evaluated the persistence of the transgenerational effects throughout offspring development by measuring phenotypic traits, performance and AM fungal colonization on juvenile and adult offspring.

Material and methods

Study material

Taraxacum brevicorniculatum Korol. is an obligate apomictic polycarpic perennial plant (Kirschner *et al.* 2013). Like most species of the genus *Taraxacum*, it has a wide ecological niche, accepting all types of soils, pH and moisture levels (Luo & Cardina 2012), and forms an active symbiosis with AM fungi (J. Puy, personal obs.). In this study we used genetically identical seeds collected from a population of plants grown under the same glasshouse conditions for several generations (collected and genetically identified by Kirschner *et al.* 2013). This strategy ensured homogeneous genetic and epigenetic variation in the plant material. Since *T. brevicorniculatum* is an obligate apomictic species, all seeds produced by a plant are effectively clones, thus enabling the study of plasticity within and across generations (Puy *et al.* 2018). In other words, all plants in the experiments were genetically identical, and after experiencing different conditions during the parental generation, their epigenetic status differed in the offspring generation.

Experimental setup

Parental generation. In order to induce the potential transgenerational effects related to mycorrhizal symbiosis and water availability, we conducted a three-month glasshouse experiment (April-July 2017). We grew 364 genetically identical individuals of *T. brevicorniculatum* in individual pots (7 x 7 x 18 cm), half inoculated with AM fungi (AM) and the other half without (NM). The

substrate consisted of 2:1 mixture of sterilized sand and natural soil collected from a mesic meadow 30 km southeast of Tabor, 660 m a.s.l. (Vysočina region, Czech Republic, 49.331N, 15.003E), where *Taraxacum sect. Ruderalia* was present. For the AM treatment the natural soil containing indigenous AM fungi was used,; whereas for the NM treatment the same soil was sterilized via γ irradiation (>25kGy dose) and a microbial wash added (McNamara *et al.* 2003; Liang *et al.* 2015). We obtained the microbial wash by blending 5kg of non-sterilized soil in 10 l water and filtering the solution through 20µm pore-size filter paper (Whatman® quantitative filter paper, Grade 41) following van der Heijden *et al.*, (1998), with slight modifications. Gamma-sterilization did not change the chemical composition of the soil compared to the non-sterilized soil (Fig. S1).

Additionally, these AM and NM treatments were factorially combined with two levels of water availability. Half of the individuals were subjected to cycles of drought stress (Drought stress; W-), while the other half were watered regularly for creating control conditions (Control; W+). The drought stress treatment included watering only when 50% of the individuals had wilted leaves followed by one-week recovery in control conditions. By the end of the experiment, the drought stress treatment comprised two drought pulses (the first started 12th of May and the second 15th of June) that lasted three weeks each.

Prior to the establishment of the experiment, seeds were surface sterilised by immersion in 0.5% sodium hypochlorite solution (commercial bleach) for 20 minutes to avoid inoculation via seeds, and then germinated in Petri dishes. After 10 days of germination, the seedlings were transplanted individually into the pots specified above, with 91 replicates per treatment. After three months we harvested all the plants except 15 plants per treatment that were maintained for four more months in ambient conditions (water control condition) to promote seed production. Then, seeds of each plant were collected, and after measuring the average seed mass per plant, were stored in cold (2-4 ºC).

Offspring experiment. A similar glasshouse experiment to the one described above was repeated the following year (April-August 2018) with the seeds produced by the parental generation. The aim of the offspring experiment was to

test for adaptive transgenerational effects of AM symbiosis and water availability on the offspring at their juvenile and the adult stages. We tested this with a full factorial design where the offspring plants from each of the four parental treatments were exposed again to the four possible conditions (AM W+,AM W-, NM W+, NM W-). Thus, the offspring experimental design resulted in 16 combinations: two parental mycorrhizal inoculations (Par. M) x two parental water availability levels (Par. W) x two offspring mycorrhizal inoculations (Off. M) x two offspring water availability levels (Off. W) x 40 seedlings = 640 pots (Fig. S2)**.** Since the seed mass of AM parents was on average lower than that of NM parents (Fig. S3, Table S1), and seed provisioning is a potential mechanism of transgenerational effects (Herman & Sultan 2011), we controlled for it by classifying seeds from all parental treatments into 5 size categories. Then, we took the same number of seeds from each size-group in each parental treatment, resulting in a similar distribution of seed sizes between parental treatments.

Plants were harvested at two different developmental stages. Half of the offspring plants were harvested 1.5 months after planting, at their juvenile stage; and the rest of the replicants were harvested five months after planting, at their adult stage. Pots, substrate and watering regime were the same as in the parental experiment to ensure the most similar conditions. However, the first drought stress pulse of the offspring generation lasted four weeks instead of three (first one started the 25th of April and the second one, the 1st of June) to ensure comparable effects on plants response (i.e. % of plants with wilted leaves). In order to facilitate the application of the treatments, the replicates were distributed in blocks, placing four replicates of a parental treatment in parallel, one in each offspring treatment (Fig. S2).

Measured traits

In each of the generations we measured plant traits. For each plant in the parental generation, at the time of harvest, we measured survival, seed output (i.e. number of seeds), total dry biomass (aerial plus root biomass), and several above- and belowground vegetative traits. For each plant, two leaves were scanned for leaf area and weighed for fresh mass and dry mass after drying at 60º C (48h). We

used these measurements to estimate specific leaf area (SLA; leaf area per dry mass, mm^2/mg) and leaf dry matter content (LDMC; leaf dry mass per leaf fresh mass, mg/mg). In addition, roots were carefully extracted, washed and a subsample of roots (6 cm^2) was scanned at 600 dpi with an Epson Perfection 4990 scanner. From the scans, total root length, average root diameter (mm), and distribution of root length in different diameter classes were determined by using the image analysis software WinRHIZO Pro, 2008 (Regent Instruments Inc., Quebec, Canada). After scanning, the root subsample and the rest of the root system were dried for 48 h at 60 °C and weighed. We used these measurements to estimate specific root length (SRL; root length per dry mass, m/g), and fine roots percentage (root length with a diameter < 0.5mm per total root length). Further, we estimated root biomass allocation (i.e. root mass factor; RMF; root biomass per total biomass, g/g) after drying the remaining radicular part at 60º C (48h). Additionally, we measured seed C, N and P content of five randomly chosen plants per treatment. Total C and N content were determined by dry combustion using an elemental analyser (CHNS Elemental Analyzer vario MICRO cube, Elementar Analysensysteme GmbH, Germany). Total P was determined by flow injection analysis.

For each plant in the offspring generation, at the time of the respective harvest (i.e. juvenile and adult offspring harvest), we measured total dry biomass (aerial plus root biomass), and the same above- and belowground vegetative traits as described above. Additionally, we analyzed the content of C, N and P in the leaves of two randomly chosen plants from the juvenile stage and eight plants from the adult stage per treatment, following the methods described above. The root subsamples were stained with Chlorazol Black according to the protocol by Štajerová *et al.* (2009). We quantified the AM fungal colonization by measuring the percentage of root length colonized (%RLC) by AM fungal structures (arbuscules, vesicles and hyphae). Magnified intersection method (McGonigle *et al.* 1990) was used with 400 x magnification using a light microscope and observing at least 100 intersections per root sample. We further calculated the arbuscule:vesicle ratio (relative abundance of arbuscules per vesicles), suggested as an indicator of the fungal activity status and the relative cost or benefit of the fungus to the host plant (Braunberger *et al.* 1991; Titus & Lepš 2000).

Statistical analysis

All analyses were carried out using R v3.2.3 (R Core Team 2016) with α=0.05 as significance level. In the parental generation, the effects of the mycorrhizal inoculation treatments, the parental water availability treatment, and their interaction were analysed by using linear effects models. In the offspring generation, individuals were grouped into sixteen different treatments (coming from the combination of four factors with two levels each) depending on the parental background and the current conditions. Two of the factors corresponded to parental conditions: mycorrhizal inoculation treatment (Par. M), and water availability treatment (Par. W). The other two factors corresponded to offspring conditions: mycorrhizal inoculation treatment (Off. M) and water availability treatment (Off. W). We analysed the effects of parental and offspring conditions on plant traits of the offspring using linear mixed-effects models (lmer, library lme4), where the four experimental factors (two parental, two offspring conditions) and all their interactions were used as fixed effects, and the experimental blocks as a random effect. We controlled for differences in seed provisioning (Herman & Sultan 2011) by including seed mass as a covariate (i.e. in the fixed effects part of the model). For the analysis of the effect of parental and offspring treatments on AM fungal colonization and arbuscule:vesicle ratio of the offspring, we used identical models, but excluding the offspring mycorrhizal inoculation factor (Off. M) from the model due to the lack of AM fungal colonization in the NM plants.

Results

Parental generation

In the parental generation, AM fungal inoculation increased *T. brevicorniculatum* growth, drought tolerance, survival and productivity (Fig. S3, Table S1). Drought stress decreased the total plant biomass and survival only in NM plants, with no effect on AM plants (Fig. S3a,b; Table S1). Additionally, AM plants started flowering earlier and flowered for longer, but produced lower average seed mass

per plant. However, AM and NM plants did not differ in total number of seeds or seed P content and C:N ratio (Fig. S3, Table S1).

Offspring plant traits

In the juvenile offspring, both offspring conditions (offspring mycorrhizal inoculation treatment, Off. M; and offspring water availability treatment, Off. W) had generally strong effects on most of the measured plant traits, except for SRL and average root diameter that only were affected by the water treatment (Off. W; Fig. 1 and Table S2). In general, plants under drought stress and absence of AM symbiosis had higher RMF, higher LDMC and lower SRL (Fig. 1b,c,f and Table S2).

Additionally, we found transgenerational effects (i.e. where offspring plants were affected by the conditions experienced by their parents; parental mycorrhizal inoculation, Par. M; and water availability treatments, Par. W) in seven out of the nine traits. These effects were either direct or, more commonly, interacting with the offspring conditions (Table S2). From the seven offspring traits, three (total biomass, average root diameter and percentage of fine roots) were affected by both parental conditions (Table S2; Par. M and Par. W), whereas for the other four traits only a single parental condition triggered transgenerational effects. Offspring of parents under water stress (Par. W) had in general lower SRL, whereas offspring of mycorrhizal parents (Par. M) had in general lower RMF, higher LDMC, and higher leaf P content (Fig. 1b,c,d and Table S2). Except for LDMC, the transgenerational effects (Par. M and Par. W) were concordant in the direction of the plastic response to the conditions experienced during their life cycle (Off. M and Off. W). For example, mycorrhizal offspring showed lower RMF, but this reduction was even more pronounced if the parent was mycorrhizal (Fig. 1b and Table S2).

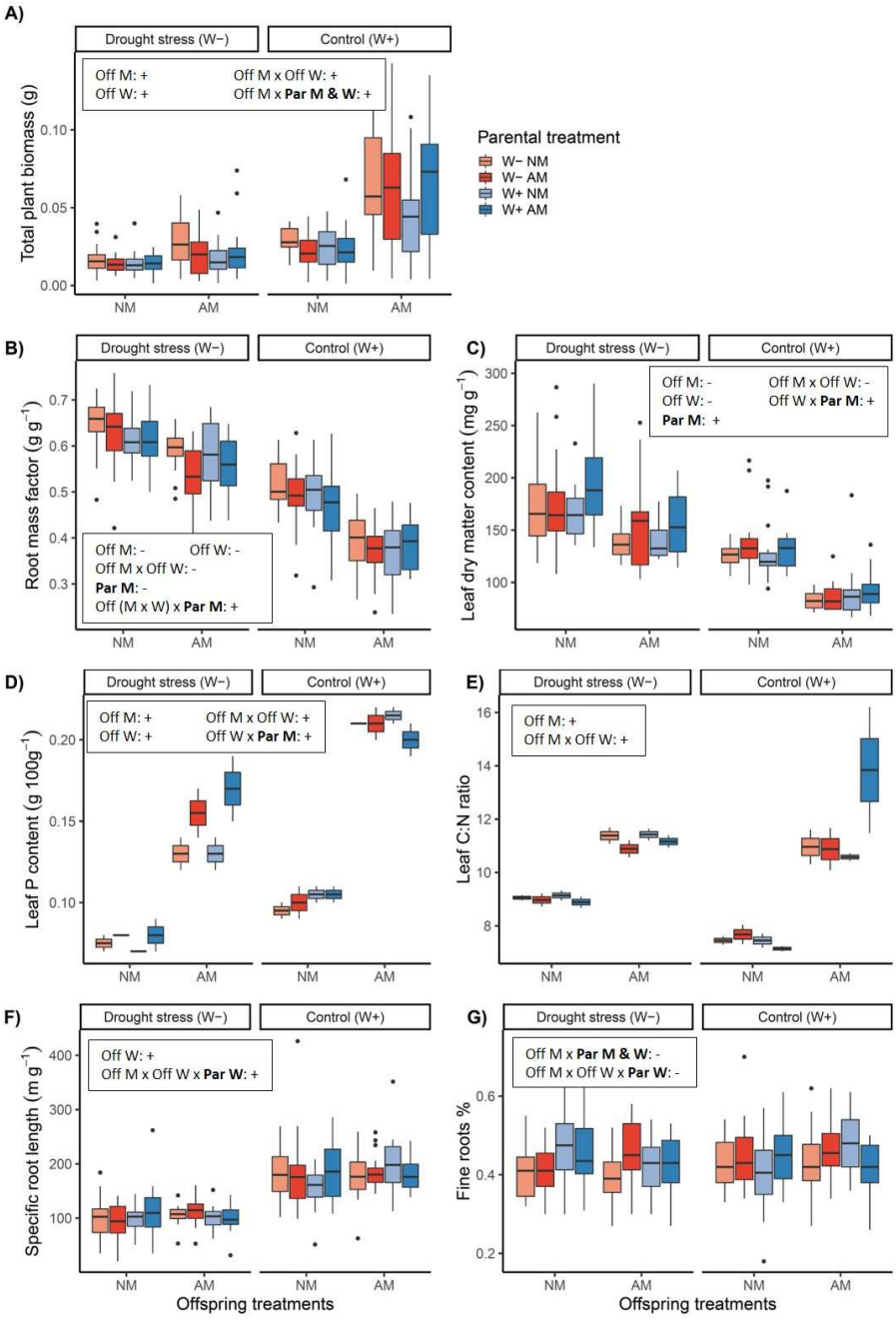

Figure 1: Effect of the offspring and parental treatments on plant phenotype characteristics of the juvenile offspring. a) total plant biomass, b) root mass factor, c) leaf dry matter content, d) leaf P content, e) leaf C:N ratio, f) specific root length and g) fine roots percentage. The significant factors of each model with the directionality of each effect are shown in the boxes. The factors corresponded to the offspring conditions:

mycorrhizal inoculation treatment, Off. M; and water availability treatment Off. W; and the parental conditions (also highlighted in bold face): mycorrhizal inoculation treatment, Par. M; and water availability treatment, Par. W. Colour coding indicates the parental treatments: red - offspring of water-stressed parents, blue - offspring of parents that experienced water control conditions; intense colour - offspring of mycorrhizal parents, light colour - offspring of non-mycorrhizal parents. 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.

At the adult stage of offspring plants, we did not detect significant transgenerational effects except for LDMC (Fig. 2c and Table S3; Off. W x Par. M), since only the offspring rather than the parental conditions (Off. M and Off. W) were the main drivers of plant plasticity (Fig. 2 and Table S3). The direction of the plasticity in response to offspring conditions (Off. M and Off. W) reversed compared with the juvenile stage, with the exception of leaf P content and total biomass (Fig. 2 and Table S3). For example, offspring with AM fungi and water availability had lower RMF during the juvenile stage, but higher RMF during the adult stage (Fig. 1b and Fig. 2b). Additionally, in the adult stage, more traits responded to the offspring mycorrhizal inoculation treatments (Off. M) compared with the juvenile stage. These additional traits included SRL and percentage of fine roots (Fig. 2f,g and Table S3), which was larger in non-mycorrhizal plants.

Offspring's AM fungal colonization

In the juvenile offspring, water availability (Off. W) generally had a strong effect on AM fungal colonization: drought-stressed plants had lower %RLC, and higher arbuscule:vesicle ratio than control plants (Fig. 3a,b and Table S2).

Additionally, the AM fungal colonization of the offspring was affected by the conditions experienced by their parents. From the two parental treatments (Par. M and Par. W), only the water conditions experienced by the parents influenced the %RLC of the offspring and only in the case of offspring plants under drought stress (Fig. 3a,b and Table S2; Off. W x Par. W). Drought-stressed offspring plants had higher %RLC when their parents had experienced water

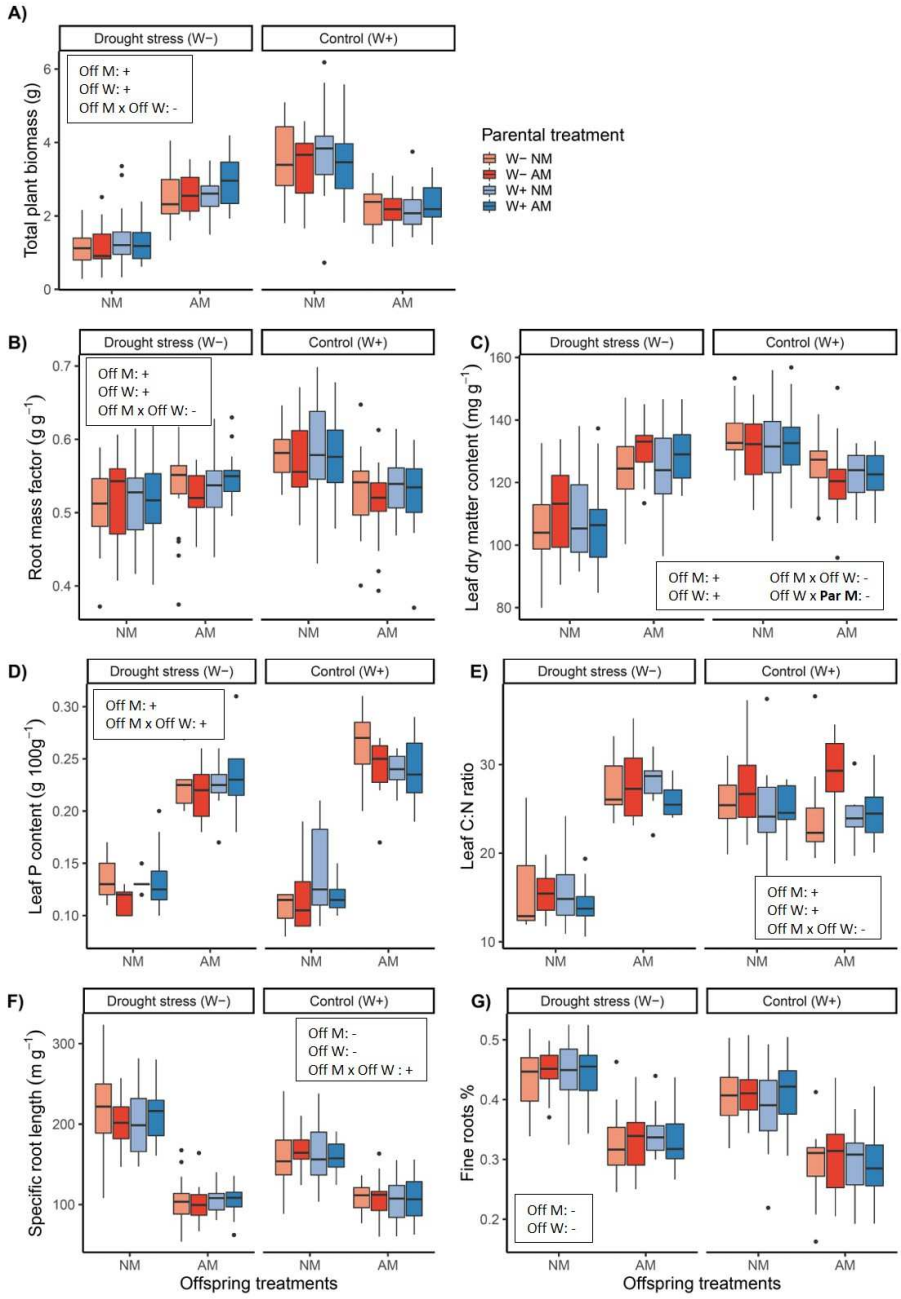

Figure 2: Effect of the offspring and parental treatments on plant phenotype characteristics of the adult offspring. a) total plant biomass, b) root mass factor, c) leaf dry matter content, d) leaf P content, e) leaf C:N ratio, f) specific root length and g) fine roots percentage. The significant factors of each model with the directionality of each effect are shown in the boxes.

control conditions (Fig. 3a,b, Table S2 and Fig. S4; Off. W x Par. W). For the arbuscule:vesicle ratio we did not detect significant transgenerational effects.

During the adult stage, we found no significant difference in %RLC between the water availability conditions (Off. W), although there was a higher arbuscule:vesicle ratio in the plants experiencing control water availability (Fig. 3c,d, Table S3 and Fig. S4). Additionally, %RLC of the offspring in the adult stage was affected by the parental mycorrhizal inoculation treatment (Fig. 3c; Table S3; Off. W x Par. M). Offspring of mycorrhizal parents had higher %RLC if drought-stressed, but lower %RLC in water-control conditions (Fig. 3c, Table S3 and Fig. S4).

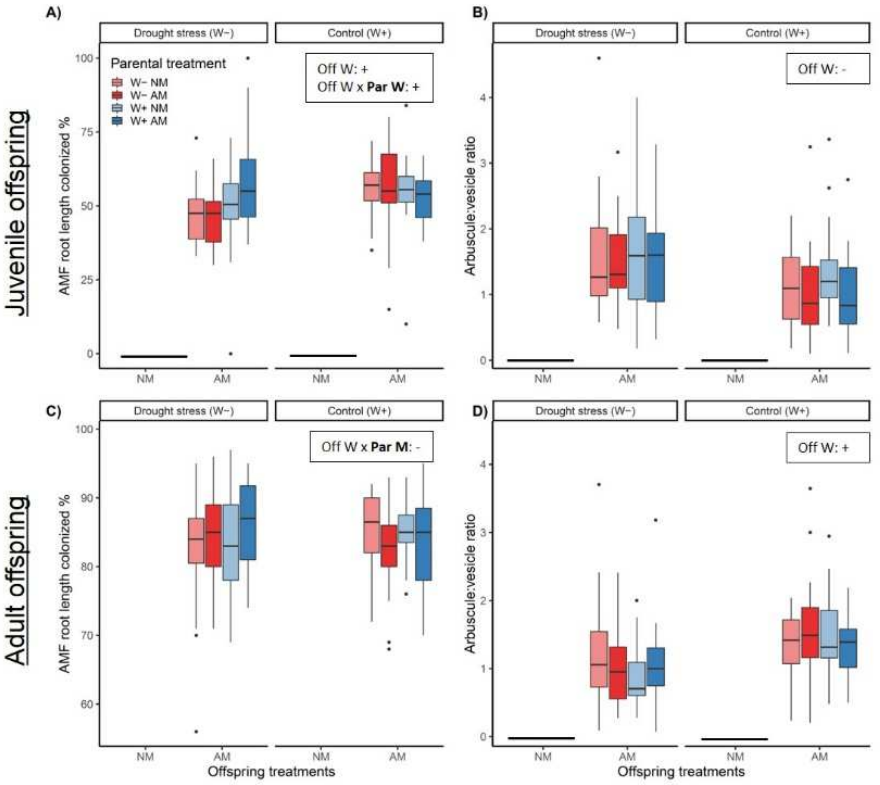

Figure 3: Effect of the offspring and parental treatments on AM fungal root colonisation in juvenile stage (upper row) and adult stage (lower row): a) and c) percentage of root length colonized by AM fungi; b) and d) arbuscule:vesicle ratio. The significant factors of each model with the directionality of each effect are shown in the boxes. The nonmycorrhizal offspring treatment plants were not colonized by AM fungi.

Discussion

This study expands the knowledge on the importance of AM symbiosis in triggering phenotypic changes in plants during their life cycle that improve their ability to cope with environmental stress and likely increase the species' realized niche. Earlier it was known that mycorrhizal symbiosis influences plant fitness-related performance. Here we show that mycorrhizal symbiosis also affects plant functional traits related to resource use and acquisition strategies. Additionally, we demonstrate adaptive transgenerational mycorrhizal effects in plant performance and phenotype. Further, we provide evidence of transgenerational effects on AM fungal colonization. Finally, we found that transgenerational effects could persist through offspring development. Importantly, we show that the transgenerational effects of mycorrhizal symbiosis and water availability could have been transmitted via heritable epigenetic mechanisms, such as DNA methylation, because these effects were not caused by differences in the quality and resources provided in the seed (Herman & Sultan 2011).

Within-generational plasticity on offspring traits is development–specific

The strong response of plants of *T. brevicorniculatum* to the conditions experienced during their life cycle (mycorrhizal inoculation and water availability) shows the high level of plasticity of this species. However, we found that the response of the performance and phenotype to these conditions differed in juvenile and adult phases, suggesting specific plant plasticity at different developmental stages (Coleman *et al.* 1994).

As expected, measurements of different fitness-related characteristics suggest that AM symbiosis improved plant performance and mitigated water stress. However, the biomass increase in offspring was even more pronounced in adults than in juveniles (Fig. 2a vs Fig. 1a), probably because during the juvenile stage the cost/benefit ratio of the symbiosis is still high (Johnson *et al.* 1997). Mycorrhizal fungal inoculation dramatically increased leaf P content (Fig. 1f and Fig. 2f) at both developmental stages of the offspring plants. This result reflects

that, even in drought-stressed conditions, AM fungi were able to mitigate water stress and provide important nutrients, such as P, to the host (Lu & Koide 1994).

In both developmental stages, mycorrhizal inoculation in combination with water availability induced significant changes in multiple plant phenotypic traits, including both root and aboveground traits (Nuortila *et al.* 2004; Goh *et al.* 2013; Fusconi 2014). During the juvenile stage the effects on trait plasticity triggered by water availability and by the mycorrhizal fungal colonization were additive and in the same direction (Table S2). Similar to findings of Shumway & Koide (1994), plant traits shifted towards a more conservative phenotype under drought stress and in the absence of AM symbiosis (i.e. higher allocation of biomass in the roots; higher leaf dry matter content; and lower specific root length; Fig. 1). A conservative phenotype refers to a conservative resource use and exploitation strategy of the plant, based on the plant economics spectrum framework (Díaz *et al.* 2016). It is associated with longer lifespan, better resource-use conservation, and generally is the strategy selected in plants from resource-poor environments (Díaz *et al.* 2016). Thus, the plastic response towards a conservative phenotype could improve *T. brevicorniculatum* ability to cope with drought stress.

During the adult stage, the direction of plasticity changed, and more traits plastically responded to the mycorrhizal fungal inoculation treatments compared with the juvenile stage. Plants increased their specific root length and percentage of fine roots in response to the absence of mycorrhizal fungi, reflecting an adaptive plasticity that improved resource uptake, thereby compensating for the lack of AM symbiosis (Goh *et al.* 2013; Fusconi 2014; Pozo *et al.* 2015). The diversity of plant responses depending on developmental stage could explain why previous studies found a variable effect of mycorrhizal fungi on plant phenotype (Nuortila *et al.* 2004; Johnson *et al.* 2012).

Transgenerational effects on offspring performance and phenotype

Most studies have shown that having mycorrhizal parents is beneficial for the performance of the offspring, reflected in higher biomass, survival, growth rate,

and seed production (Heppell *et al.* 1998; Koide 2010; Varga *et al.* 2013). However, we did not observe any benefit of having mycorrhizal parents in terms of biomass, nor from growing in the same environmental conditions as the parental generation (Fig. 1a). This contrasting result is probably because we experimentally removed the differences in quality and resources provided in the seed, aiming to identify possible underlying epigenetic mechanisms in transgenerational effects (Herman & Sultan 2011). Nonetheless, we found that offspring of mycorrhizal parents had higher leaf P content than those of non-mycorrhizal parents (Fig. 1d), suggesting that in addition to directly providing soil nutrients to host plants, mycorrhizal symbiosis increase their offspring's nutrient uptake via epigenetic transgenerational effects. One way to confirm that these effects were epigenetically controlled would be to modify the epigenetic signature of the plants via application of a demethylation agent (Puy *et al.* 2018). However, it should be first tested whether the demethylation application also affects AM fungi.

Moreover, we found adaptive transgenerational effects in offspring phenotypes and traits linked with the resource use and exploitation strategy of the plant (Díaz *et al.* 2016). In general, offspring transgenerational plasticity had concordant responses to the within-generational response to treatments. As discussed above, plant traits shifted towards a "stress-coping phenotype" under drought stress and absence of AM symbiosis experienced during their life cycle (i.e. more conservative phenotype: increased the RMF and decrease SRL). Additionally, offspring became even more conservative when their parents were under those stressful conditions (i.e. drought stress and absence of AM symbiosis; Fig. 1). Thus, the transgenerational effects further reinforced trait plasticity in the same direction, reflecting an adaptive epigenetic "stress memory" that could improve the ability of plants to cope with the predicted environment.

Even though both abiotic and biotic parental environments seemed to trigger transgenerational effects, we found that mycorrhizal inoculation affected plant traits more than the water availability treatments (RMF, LDMC and leaf P content, versus SRL; Fig. 1 and Table S2). Although this may have changed if we had measured another set of phenotypic traits, it is important to note that

T. brevicorniculatum has a wide ecological niche, accepting all types of moisture conditions (Luo & Cardina 2012). Consequently, it is likely that water is a less crucial stressor and the transgenerational effects due to water were not evolutionary relevant for this species (Rendina González *et al.* 2018). Also, it is important to emphasize that we found that the transgenerational effects on traits were expressed early on the ontogeny (Fig. 1), fading away over offspring life development (Fig. 2). This result reinforces the idea of transgenerational effects as an important factor promoting adaptation to repeated ecological conditions, especially during juvenile stages and establishment of communities (Latzel *et al.* 2014; Dechaine *et al.* 2015).

Within- and trans-generational effect on AM fungal colonization

As expected, the environmental factors experienced by offspring during their life cycle affected the offspring AM fungal colonization (Martínez-García *et al.* 2012; Pozo *et al.* 2015). However, although water deficiency did not stimulate the root AM fungal colonization (%RLC decreased), the proportion of arbuscules:vesicles increased in water-stressed offspring, showing that water deficiency stimulated an increase in the proportion of arbuscules, that are the structures where resource exchange takes place.

We found that AM fungal colonization and AM symbiosis activity could be influenced by the conditions experienced by the parental generation. To our knowledge, this is the first study that shows that epigenetic transgenerational effects influence the AM fungal colonization. The offspring of parents in water-control conditions had higher AM fungal colonization and proportion of arbuscules, but only if they were under drought stress. While this appears to contradict our initial hypothesis, relative to the total root biomass of the plant (i.e. root biomass x %RLC), offspring from water-stressed and NM parents had more total root colonized and a greater number of arbuscules per individual than offspring from parents under control water conditions because they had larger root systems (see Fig S5). Thus, the result supports that transgenerational effects modify offspring towards the “stress-coping phenotype” stimulating the establishment and activity of the AM symbiosis.

Surprisingly, and contrary to the general pattern found in plant traits, the conditions experienced by the parental generation still influenced AM fungal colonization during the adult stage. This suggests that epigenetic transgenerational effects influence plant–AM fungi relationship, and not only during the establishment and early stages of the symbiosis. Moreover, at the adult stage, %RLC was affected by the parental mycorrhizal status, so that offspring from mycorrhizal parents had higher %RLC under drought stress. These results suggest that mycorrhizal symbiosis could be promoted in the offspring when the parental generation has experienced mycorrhizal symbiosis.

Conclusions

We found transgenerational effects of mycorrhizal symbiosis, in combination with water availability, on offspring performance, phenotype, and root AM fungal colonization. Importantly, we show that transgenerational effects of mycorrhizal symbiosis and water availability were mainly transmitted via heritable epigenetic mechanisms, such as DNA methylation because the effects were not related to larger seed mass or seed resource provisioning (Herman & Sultan 2011). Drought stress and absence of AM fungi triggered concordant plant phenotypic plasticity (towards a "stress-coping phenotype") both within- and across generations. This reflects an adaptive epigenetic mechanism that promotes rapid adaptation, and probably improves the ability of the species to cope with drought stress. In a context where the importance of individual and intraspecific variation of mycorrhizal plants and fungi in ecosystems is increasingly acknowledged (Johnson *et al.* 2012), our paper adds a new mechanism of variation that has been ignored so far: transgenerational plasticity. These plastic changes confer competitive advantages to the next generation.

Acknowledgments

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Supplementary material

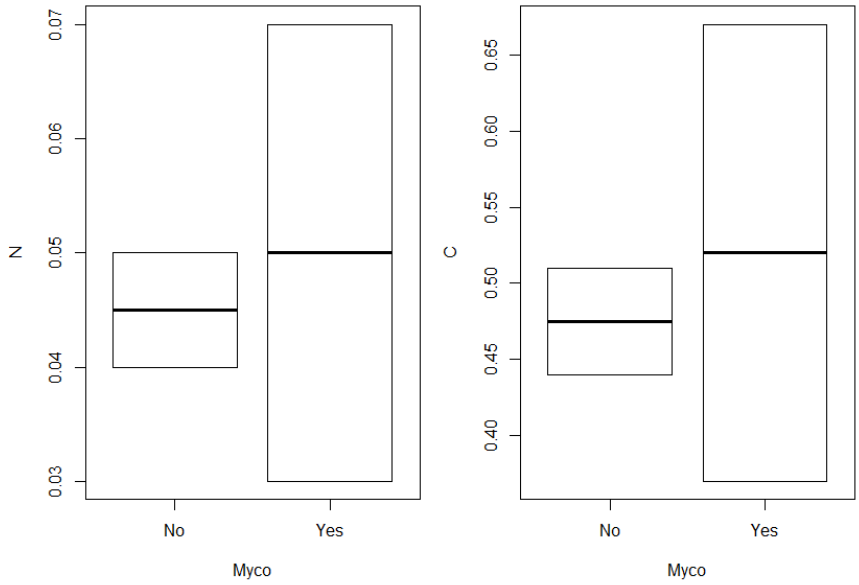

Figure S1: N and C content of the substrate (AMF+ and AMF-).

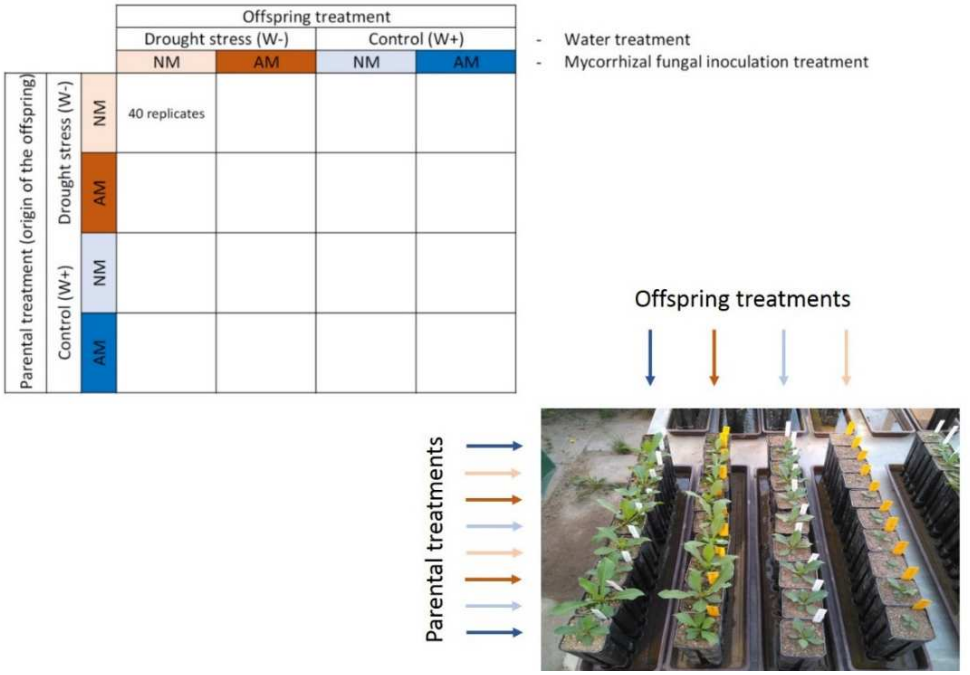

Figure S2: Schematic representation of the design of the offspring experiment, and detailed representation of the experimental blocks.

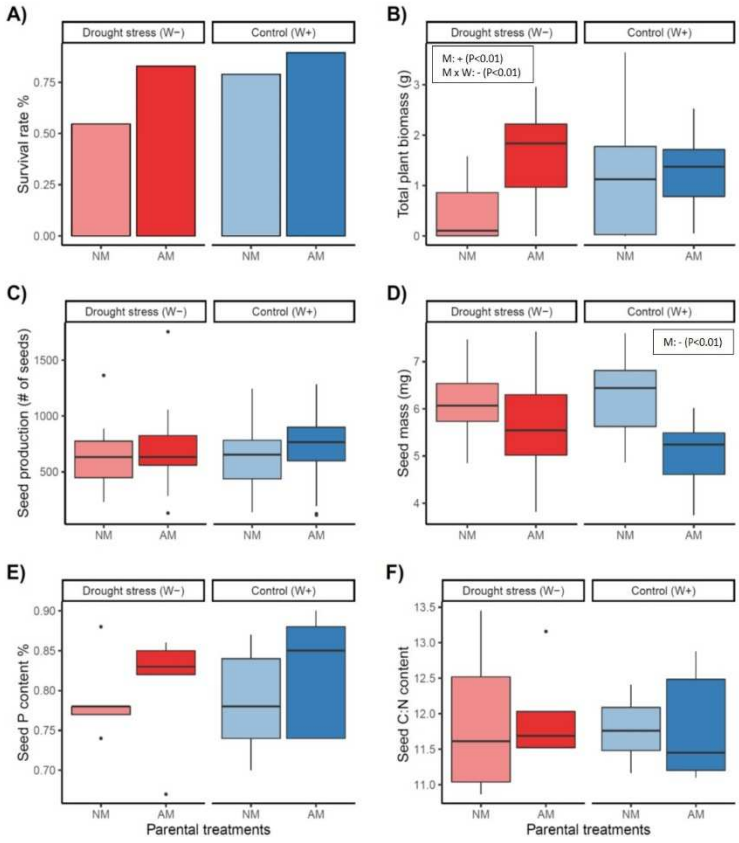

Figure S3: Effect of the treatments on parental generation: a) survival rate, b) total plant biomass, c) number of seeds produced, d) average seed mass, e) seed P content and f) seed C:N ratio. The significant factors of each model with the directionality of each effect are shown in the boxes. The different coloured boxplots represent the treatments. 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.

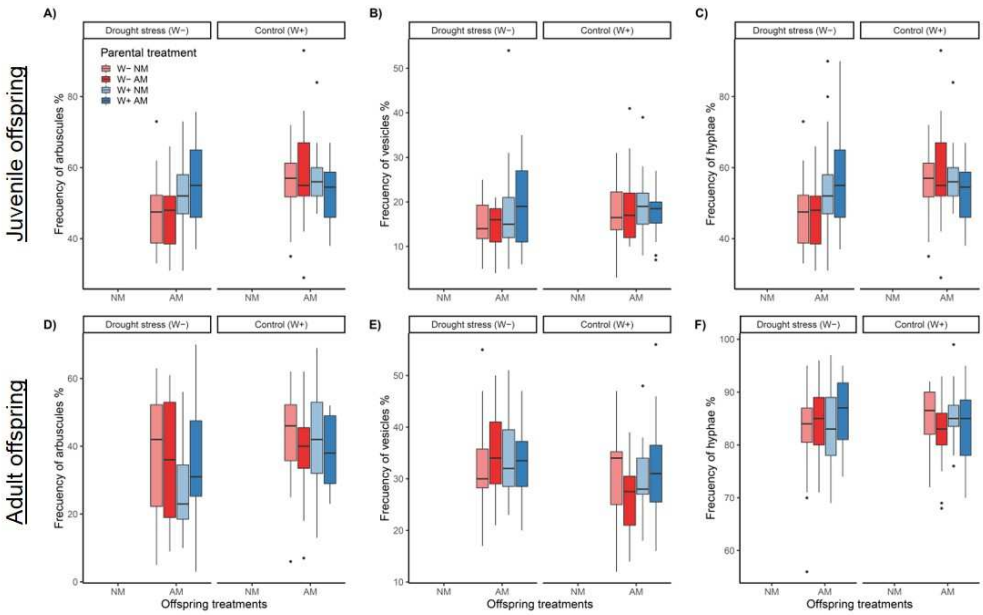

Figure S4: Effect of the offspring and parental treatments on other AM symbiosis characteristics of the juvenile offspring (upper row) and adult offspring (lower row): a) and d) frequency of arbuscules b) and e) vesicles and c) and f) hyphae. In each of the offspring treatments, the different coloured boxplots represent the parental treatments: offspring of water-stressed parents in red and offspring of parents that experienced water control conditions in blue; and offspring of mycorrhizal parents more intense coloured, and offspring of non-mycorrhizal parents with a lighter colour. The nonmycorrhizal offspring treatment plants were not AMF colonized. 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.

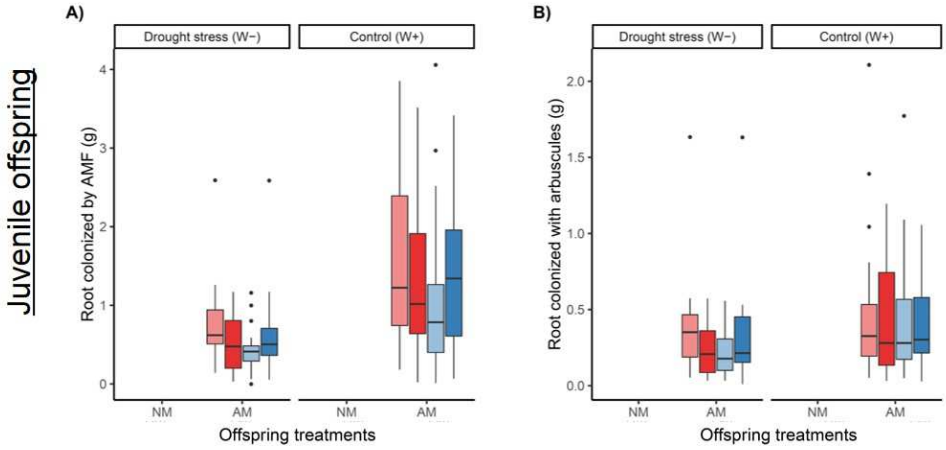

Figure S5: Effect of the offspring and parental treatments on total AM fungal root colonisation in juvenile stage relativized by the individual root biomass: a) total mass of root colonized by AM fungi; and b) total mass of root colonized with arbuscules. There are no significant effects of the parental factors.

Table S1: Summary of the linear mixed-effect model for main and interaction effects of the treatments in the parental generation. Degrees of freedom followed by F values and P values are given for all the effects analysed. Significant results are shown in bold face, and the colour indicates the direction of the effect (positive in green, negative in red)

Source of variation	df	Total biomass		Number of seeds		Seed mass		Seed P content		Seed C:N ratio	
		F	P	F	P	F	P	F	P	F	P
Mycorrhizas inoculation treatment	1	19.7	**<0.01**	0.4	0.51	20.5	**<0.01**	0.7	0.42	0.0	0.86
Water availability treatment	1	1.2	0.27	0.0	0.99	1.9	0.17	0.0	0.85	0.2	0.7
Mycorrhizas x Water	1	14.6	**<0.01**	0.0	0.92	1.9	0.17	0.1	0.75	0.0	0.95

Table S2: Summary of the linear mixed-effect model for main and interaction effects of offspring and parental treatments (the latter highlighted in bold) on the juvenile offspring. Degrees of freedom followed by $\chi 2$ values and P values are given for all the effects analysed. Significant results are shown in bold, and the colour indicates the direction of the effect (positive in green, negative in red)

		Plant traits																			
		Whole-plant traits				Leaf traits								Root traits						AMF colonization			
Source of variation	df	Total biomass		Root mass factor (RMF)		Leaf dry matter content (LDMC)		Specific leaf area (SLA)		Leaf P content		Leaf C:N content		Specific root length (SRL)		Average root diameter		% Finc roots		Root length colonized by AMF (%RLC)		Arbuscules: vesicles ratio	
		$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P
Seed mass	1	2.7	0.102	3.2	0.073	0.0	0.973	3.1	0.078	0.2	0.663	0.0	0.865	2.9	0.088	1.4	0.238	0.2	0.636	0.6	0.448	2.4	0.123
Offspring Mycorrhizal inoculation (Off. M)	1	68.2	**<0.01**	123.6	**<0.01**	136.1	**<0.01**	335.3	**<0.01**	354.5	**<0.01**	82.9	**<0.01**	0.4	0.503	5.6	**0.018**	0.0	0.967				
Offspring Water availability (Off. W)	1	137.7	**<0.01**	401.0	**<0.01**	320.3	**<0.01**	286.4	**<0.01**	86.1	**<0.01**	3.1	0.076	226.1	**<0.01**	57.8	**<0.01**	0.3	0.594	4.6	**0.032**	11.5	**<0.01**
Parental Mycorrhizal inoculation (Par. M)	1	1.1	0.301	7.5	**<0.01**	9.5	**<0.01**	1.3	0.246	2.7	0.102	0.2	0.690	0.1	0.708	0.5	0.498	1.3	0.260	0.3	0.594	1.5	0.221
Parental Water availability (Par. W)	1	1.3	0.262	0.5	0.464	0.6	0.444	2.4	0.121	0.1	0.776	0.7	0.403	0.3	0.615	0.4	0.552	0.4	0.522	1.6	0.209	1.5	0.225
Off. M x Off. W	1	21.7	**<0.01**	10.1	**<0.01**	5.9	**0.015**	58.8	**<0.01**	15.8	**<0.01**	7.7	**<0.01**	0.8	0.365	11.0	**<0.01**	2.2	0.141				
Par. M x Par. W	1	1.1	0.301	0.4	0.521	0.5	0.480	0.1	0.760	0.1	0.759	0.4	0.537	0.0	0.854	0.9	0.338	3.2	0.076	0.1	0.785	1.4	0.243
Off. M x **Par. M**	1	2.9	0.091	0.2	0.687	0.0	0.990	0.7	0.403	0.6	0.426	1.0	0.309	0.5	0.497	0.0	0.965	0.5	0.475				
Off. M x **Par. W**	1	0.2	0.618	0.7	0.419	0.1	0.759	1.6	0.208	0.0	1.000	1.5	0.217	0.1	0.749	0.0	0.879	0.6	0.427				
Off. W x **Par. M**	1	0.1	0.700	1.9	0.170	4.6	**0.032**	0.1	0.808	5.7	**0.017**	2.3	0.133	0.4	0.514	0.4	0.535	0.0	0.961	0.5	0.478	0.1	0.793
Off. W x **Par. W**	1	0.2	0.656	0.0	0.852	0.2	0.623	1.2	0.277	0.0	1.000	0.4	0.534	0.4	0.547	4.1	**0.043**	2.7	0.102	6.3	**0.012**	0.1	0.773
Off. M x **Par. M** x **Par. W**	1	6.0	**0.014**	0.2	0.679	0.2	0.650	0.3	0.617	0.0	1.000	2.4	0.124	2.9	0.088	8.7	**<0.01**	7.2	**<0.01**				
Off. W x **Par. M** x **Par. W**	1	0.2	0.648	0.0	0.842	2.6	0.105	0.2	0.683	1.1	0.289	1.0	0.324	0.0	0.968	1.2	0.267	0.4	0.532	1.9	0.169	0.1	0.718
Off. M x Off. W x **Par. M**	1	2.1	0.145	3.9	**0.048**	0.6	0.426	0.2	0.632	3.4	0.063	1.7	0.186	2.5	0.115	5.6	**0.018**	4.3	**0.038**				
Off. M x Off. W x **Par. W**	1	0.0	0.885	0.1	0.796	1.2	0.267	0.9	0.352	1.1	0.289	1.0	0.313	4.9	**0.026**	1.7	0.199	2.6	0.105				
Off. (M x W) x **Par. (MxW)**	1	0.6	0.429	1.2	0.281	1.2	0.269	0.2	0.650	0.3	0.596	1.6	0.212	1.1	0.288	0.6	0.432	1.4	0.238				

Table S3: Summary of the linear mixed-effect model for main and interaction effects of offspring and parental treatments (the latest highlighted in bold) on adult offspring. Degrees of freedom followed by χ2 values and P values are given for all the effects analysed. Significant results are shown in bold, and the colour indicates the direction of the effect (positive in green, negative in red)

		Plant traits																AMF colonization			
		Whole-plant traits				Leaf traits								Root traits							
Source of variation	df	Total biomass		Root mass factor (RMF)		Leaf dry matter content (LDMC)		Specific leaf area (SLA)		Leaf P content		Leaf C:N ratio		Specific root length (SRL)		Average root diameter		% Fine roots		Root length colonized by AMF (%RLC)		Arbuscules: vesicles ratio	
		χ2	P	χ2	P	χ2	P	χ2	P	χ2	P	χ2	P	χ2	P	χ2	P	χ2	P	χ2	P	χ2	P
Seed mass	1	0	0.99	0.1	0.8	0	0.85	1	0.32	1.1	0.29	2.7	0.10	3.3	0.07	2.6	0.10	0.1	0.80	5.5	**0.029**	0	0.986
Offspring Mycorrhizal inoculation (Off. M)	1	23.9	**<0.01**	7.9	**<0.01**	15.5	**<0.01**	0	0.918	471.5	**<0.01**	63.8	**<0.01**	581.8	**<0.01**	143.1	**<0.01**	301.7	**<0.01**				
Offspring Water availability (Off. W)	1	127.6	**<0.01**	27.4	**<0.01**	69.6	**<0.01**	56.4	**<0.01**	1.9	0.17	34.7	**<0.01**	41.4	**<0.01**	8.1	**<0.01**	30.3	**<0.01**	0	0.938	13.1	**<0.01**
Parental Mycorrhizal inoculation (Par. M)	1	0.6	0.422	0.4	0.506	0.1	0.747	0.8	0.366	1.5	0.225	0.3	0.586	0	0.983	0.3	0.598	1.8	0.178	0	0.868	0.1	0.731
Parental Water availability (Par. W)	1	1.7	0.19	0.2	0.665	0.9	0.336	0.4	0.548	2	0.156	4.0	0.046	1	0.313	0.5	0.464	1.2	0.272	0.3	0.587	1.3	0.248
Off. M x Off. W	1	254.7	**<0.01**	44.6	**<0.01**	145	**<0.01**	122.8	**<0.01**	7.9	**<0.01**	67.7	**<0.01**	51.6	**<0.01**	0	0.895	0	0.897				
Par. M x Par. W	1	0.4	0.506	0.8	0.378	0.2	0.651	0.1	0.76	0.1	0.709	1.3	0.252	1.3	0.262	2	0.16	0.6	0.443	0.6	0.447	0.3	0.588
Off. M x **Par. M**	1	0.6	0.439	0	0.845	0.6	0.441	0.6	0.453	0	0.84	0.4	0.510	0	0.827	1.5	0.228	1	0.307				
Off. M x **Par. W**	1	0.2	0.69	0.5	0.495	0.1	0.707	0.1	0.722	2.3	0.127	0.1	0.750	0.8	0.358	0.6	0.445	0.2	0.641				
Off. W x **Par. M**	1	1	0.322	1.3	0.26	5.0	**0.026**	2.5	0.111	0.4	0.509	3.1	0.078	0	0.891	0.1	0.803	0.3	0.57	5.8	**0.016**	0.1	0.789
Off. W x **Par. W**	1	1.3	0.256	0.1	0.795	0.2	0.642	0	0.942	0.3	0.591	0.8	0.378	0.1	0.732	0.9	0.331	0	0.966	0	0.933	0	0.981
Off. M x **Par. M** x **Par. W**	1	0.5	0.485	1.1	0.305	0.2	0.69	0.2	0.694	1.6	0.211	0.2	0.672	0.2	0.642	0.3	0.616	0.6	0.433				
Off. W x **Par. M** x **Par. W**	1	0	0.879	0	0.882	3.7	0.056	2.3	0.128	1.5	0.224	0.1	0.750	1.6	0.204	0.2	0.674	0.4	0.536	0	0.861	2	0.153
Off. M x Off. W x **Par. M**	1	0	0.969	0.4	0.545	1.5	0.214	1.8	0.182	0.5	0.487	0.1	0.765	0	0.996	0.9	0.353	0	0.942				
Off. M x Off. W x **Par. W**	1	0	0.924	0	0.893	0.7	0.404	0.2	0.632	2.1	0.151	0.0	0.894	0.4	0.504	0.3	0.602	0	0.969				
Off. (M x W) x **Par. (M x W)**	1	0.2	0.675	1.1	0.301	0.2	0.629	0.1	0.717	2.5	0.113	0.0	0.901	1.3	0.253	0	0.849	0.1	0.776				


G3
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M1

Chapter IV

Parental diversity mitigates negative effects of intraspecific competition on productivity of *Arabidopsis'* populations by increasing phenotypic variation

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Abstract

The commonly founded positive diversity effect on ecosystem and community functioning it has been scarcely studied in the population scale. Intraspecific phenotypic variability, apart from expressing the underlying genetic variability of the population, can also be generated via epigenetic variation: within- and among-generations. We test the role of the role of parental diversity (transgenerational effects) and genetic diversity on creating phenotypic diversity and on affecting assemblage and functioning of the populations, examining their productivity and resistance against stress. Parental environment triggered epigenetic phenotypical differences on the offspring, translated into more functional diverse populations when the different origins were brought together in mixtures. In general, the increase on diversity had null effect on populations' productivity and resistance to stress. However, when the epigenetic variation was removed via demethylation, the effect of diversity became negative because an increasing of the competition intensity generated by the reduction of niche differences between origins. Thus, heritable epigenetic diversity seems to ameliorate the negative effect of competition between different origins by increasing phenotypic differences between them. This is the first empirical demonstration of the effect of parental diversity or diversity of environmentally induced transgenerational effects on productivity and resistance to stress of the populations.

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Introduction

Positive relationships between biodiversity and ecosystem functioning have been demonstrated repeatedly in many observational and experimental studies. This body of research has found that more diverse communities are generally more productive, more stable and more resistant to disturbances/stresses than less diverse ones (Balvanera *et al.*, 2006; Marquard *et al.*, 2009). While most of the research has commonly measured biodiversity at the community level as interspecific diversity (taxonomic or functional diversity) (Marquard *et al.*, 2009; Hector *et al.*, 2010), the effect of intraspecific diversity at the population level has been overlooked. However, intraspecific diversity effects on population and ecosystem functioning can be of comparable magnitude to those of intraspecific diversity (Crutsinger *et al.*, 2006; Hughes *et al.*, 2008), and phenotypic variation within species is sometimes as large or larger than that observed among species (Hughes *et al.*, 2008).

Positive biodiversity effects on ecosystem processes could be driven by two not mutually exclusive mechanisms: complementarity and selection (Loreau & Hector, 2001; Marquard *et al.*, 2009; Tobner *et al.*, 2016). Selection operates when a specific competitively superior individual/species is dominant in mixtures and drives disproportionately the functioning of the community (Loreau & Hector, 2001). By contrast, complementarity takes place when niche differences between coexisting species result in a more efficient use of resources by the community and a better functioning of the community (Loreau & Hector, 2001). Because fitness and niche differences can be directly measured and explained with plant functional traits, both mechanisms can be also approximated from a trait-based perspective. In this case, when selection is the main mechanism, we should expect to observe a dominance of particular traits and/or convergence to trait values associated to the competitive/fitness advantage (e.g. tall stature). On the other hand divergence in traits related to resource foraging between individuals of the community would reflect that complementarity is the main mechanism driving the positive effect of diversity (Loreau & Hector, 2001; Cadotte, 2017). While an increase in phenotypic diversity should enhance net biodiversity effects by increasing complementarity, phenotypic plasticity could

either decrease or increase trait dissimilarities; thus, enhancing selection or complementarity effect respectively (Roscher *et al.*, 2015).

Some studies have reported a positive effect of intraspecific diversity in productivity and stability and resistance to disturbances/stresses of populations (Bolnick *et al.*, 2011; Latzel *et al.*, 2013; Zuppinger-Dingley *et al.*, 2014). Within-species phenotypic variation has been most often attributed to genetic variation. As a result, experiments have mostly manipulated the genetic diversity of the populations by modifying the number of genotypes of the populations (Zhu *et al.*, 2000; Booth & Grime, 2003; Reusch *et al.*, 2005; Crutsinger *et al.*, 2006; Hughes *et al.*, 2008; Kotowska *et al.*, 2010; Moore, 2015; Cook-Patton *et al.*, 2016). However, within-species differences, and thus potentially functional biodiversity, can also be generated by epigenetic variation (Zhang *et al.*, 2013; Richards *et al.*, 2017). Epigenetic variation could be enabled by various mechanisms, including DNA methylation that modifies the expression of the DNA without modifying its underlying sequence. Epigenetic variation is known to occur also in response to environmental factors (Herman & Sultan, 2016; Richards *et al.*, 2017), and to cause phenotypic variation (Cubas *et al.*, 1999; Latzel *et al.*, 2012; Zhang *et al.*, 2013). Epigenetic variation could include individual plasticity both within- or among-generations: within-generations variation is caused when the environment triggers phenotype modifications on the individual; transgenerational plasticity occurs when the individual phenotype is affected by the parental environment where epigenetic modifications transmitted to the progeny are one of the mechanisms underlying parental effects (Herrera *et al.*, 2012; Herman *et al.*, 2014).

The seminal work by Latzel *et al.* (2013) showed that epigenetic diversity increased the productivity and stability of plant populations. Nevertheless, authors suggested further steps from their proof-of-principle study to improve our general knowledge on the role of epigenetic diversity in population/ecosystem functioning. First, decomposing biodiversity effects into its selection and complementarity components with a design that controls the origin of all the individuals. They also propose, comparing the effects of epigenetic versus genetic diversity on populations functioning. Last but not least,

they point out on the importance of doing "more realistic studies" operating with epigenetic variation that is realistic for natural populations, i.e. induced by the environment. In case of Latzel *et al.* experiment, authors manipulated the epigenetic diversity by creating populations of differing number of epigenetic recombinant inbred lines (epiRILs) of *Arabidopsis thaliana* L. with highly variable DNA methylation originated by artificial crossings of Columbia wild type and mutants with decreased genome-wide DNA methylation. Now, with new and tested techniques such as experimental demethylation of plant material (Puy *et al.*, 2018), the effect of "natural" epigenetic diversity on populations could be more easily tested, and the relative effect of within and trans-generational plasticity potentially assessed.

Here, we summarize the results of a two-generation experiment on *A. thaliana* to test the role of parental diversity, i.e. populations consisting of individuals with different parental origin, and genetic diversity on 1) creating phenotypic variation and on 2) affecting plant assemblage and productivity and resistance against stress of plant populations. By experimental alterations of DNA methylation statutes of a subset of populations we were able linking parental diversity effects to the effects of epigenetic diversity. Further we control the role of within-generational plasticity on modulating those effects. We hypothesize that, compared to the effect of genetic diversity, the effect of parental diversity (further referred to as epigenetic diversity) effects on population functioning will be weaker, but still important, and that within-generational plasticity can partially compensate for both trans-generational and genetic diversity effects. Also, we test whether traits (average and variance of the trait in the populations) explain these diversity effects.

Material and methods

Plant material and environmental imprinting

For this experiment, four different ecotypes or accessions of *A. thaliana* were selected and provided by the Nottingham Arabidopsis Stock Centre (NASC): Col-1, Gue-0, Mer-6 and Vav-0. Because the natural predominance of this species for self-fertilization, ecotypes are genetically adapted to specific environments and show unique ecologies (Alonso-Blanco & Koornneef, 2000). The Col-1 ecotype was chosen because of its widely use in genetic studies. The other three ecotypes were selected from populations of the Iberian peninsula with differing moisture and fertility preferences due to their selection history and phenotypic variation (Picó *et al.*, 2008; Méndez-Vigo *et al.*, 2013): Gue-0 from north and oceanic influenced part of the peninsula, and Mer-6 and Vav-0 from southern and more continental part (NASC). Mer-6 and Vav-0 habitats differed in their fertilization influence: while the first population was located on a sandy area, the second was on a farm (NASC).

To ensure sufficient seed stock of the ecotypes, and to maintain homogeneous genetic variation and evening out possible unknown transgenerational effects of previous cultivation in NASC, we grew all the ecotypes in controlled conditions for one generation in populations with conspecifics of the same ecotype. The ecotypes from the Iberian Peninsula were grown all in identical control condition to avoid any epigenetic variation in the seed material. However, the Col-1 ecotype was grown under three different conditions (control, fertilization and waterlogging), to trigger transgenerational effects in the offspring and potentially generate material with heritable epigenetic variation. The control treatment meant watering only when plants needed. The fertilization treatment comprised the same watering regime as the control treatment but with an addition of fertilizer (KRISTALON; NPK 15-5-30+3Mg+5S) at the concentration of 300ppm in each watering day. The waterlogging stress treatment consisted in constantly watered plants ensuring wet surface of the soil. All the treatments started 7 days after the transplanting to ensure the good establishment of the populations and lasted until all the plants

produced seeds. Each population was established by transplanting 30 seedlings of the same ecotype in 9 cm square plant pots with a volume of 0,5 l filled with a 2:1 mixture of sand and commercial soil, creating dense populations (similar to Latzel *et al.*, 2013) with realistic population structure, allowing interactions between individuals. The 30 individuals within a pot were placed in a regular distribution, covering the whole surface with 6 columns x 5 rows of plants with equal space between individuals. Seeds were germinated in sterilized plotting mix, after one-week stratification at 4º C.

Experimental design (Fig S1)

After collecting seeds from the different plant material (previous section) we run a one-month diversity experiment where we stablished two different types of populations of *A. thaliana* in pots. As in the previous generation, each population was established by transplanting 30 seedlings which could have same or different origin. Transplanting allowed us to spatially arrange the populations maximizing interactions between dissimilar individuals in mixtures (Fig. S1) and to know the origin of each of one.

The first type of populations included varying levels of genetic diversity achieved by sowing individuals from one genotype (monocultures), and mixtures of the three genotypes (Mer-6, Gue-0, Var-0). Monocultures were replicated five times, and the mixtures replicated 15 times.

The second type of populations included varying levels of parental diversity and/or potential epigenetic diversity achieved by sowing individuals from single ecotype (Col-1), but either from one parental origin (i.e. experienced one specific environmental condition; monocultures) or mixing individuals from the three parental origins. In this case, monocultures were replicated seven times, and the mixtures replicated 21 times. We specify "potential" epigenetic diversity because, apart from epigenetic heritable modifications, the parental origins could trigger transgenerational effects through other mechanisms like generating differences in seed quality and hormonal balance (Herman & Sultan, 2011). One way to confirm that the parental diversity was indeed epigenetic diversity was to

modify the epigenetic status of the plants via application of a demethylation agent (Puy *et al.*, 2018). Thus, another set of populations with the same amount of pots (3 monocultures replicated 7 times and mixtures replicated 21 times) were demethylated with 5-azacytidine suppressing their epigenetic-parental status (Puy *et al.*, 2018). With this approach we aimed at creating population types which did not differ in genetic diversity and had reduced epigenetic diversity if compared to non-demethylated populations.

All the combinations mentioned above were replicated 3 times, to grow the populations under three different environmental conditions (control, waterlogging and fertilization; described in the previous section). Thus, the final set-up finally comprised (5+7+7) x 3 monocultures and 15+21+21 mixtures x 3 environmental conditions = 342 experimental populations and 10,260 individuals of *A. thaliana*. Pot size, substrate, population density and the environmental conditions were the same as in the previous generation to ensure the most similar conditions.

Measured traits

At the time of harvest, we measured survival and total dry biomass of the 18 individuals of the edge of each pot, and survival and individual total dry biomass (radicular and aerial) for each of the 12 central plants. Additionally, we estimated the specific leaf area (SLA; leaf area per dry mass, mm^2/mg) of the central plants by scanning the area of one to three leaves per plant and weighting their dry mass (after drying at 60º C for 48h). In each pot, we estimated the SLA of four individuals of each origin: i.e. the twelve central individuals in case of the mixtures, and four individuals in the monocultures (always choosing the ones situated in fixed positions to avoid subjective election from the researcher).

We calculated the average value and coefficient of variation (CV) of the traits (biomass and SLA) per pot (i.e. all origins together), as well the average mean and CV of each identity/origin in each pot separately (one pair of mean and CV per pot in monocultures, and three pairs in mixtures). This segmentation allowed us to estimate the net effect of diversity on productivity and its

components – selection effect and complementarity effects –, an analysis that was not possible in the design by Latzel *et al.* (2013) because they did not identify the origins of the individuals. The calculations of the diversity net effect were made following the additive partitioning method (Loreau & Hector, 2001), based on the difference between the observed yield in the mixture compared to the yield of the monocultures for each origin in the same experimental treatment.

Statistical analysis

Epigenetic diversity effect. The effects of environmental condition (control, waterlogging, fertilization), diversity (monocultures vs mixtures) and demethylated treatment (yes vs no), and all possible interactions between these variables (Table 1a) on *Arabidopsis* populations' total mortality, total biomass and average and variation in SLA were analysed with linear models. When interactions were significant, the effect of diversity was tested within the other treatments.

Epigenetic diversity effect vs genetic diversity effect. The effects of environmental condition (control, waterlogging, fertilization), diversity (monocultures vs mixtures) and source of variation (epigenetic vs genetic), and all possible interactions between these variables (Table 1b) on *Arabidopsis* populations' total mortality, total biomass and average and variation in SLA were analysed with linear models. When interactions were significant, the effect of diversity was tested within the other treatments.

Biodiversity effects on productivity. As before, differences on the biodiversity effects were tested with separate linear models for epigenetic vs. demethylated treatments (Table 2a), and epigenetic vs. genetic diversity (Table 2b). The environmental conditions (control, waterlogging, fertilization), and the interaction were also included as fixed factors (Table 2a, b). When the interaction was significant, the effect of the source of diversity was tested within the different environments.

Traits' influence on biodiversity effects. To see whether traits (i.e. average and CV in SLA) influence the biodiversity effects (net diversity effect and its components: selection and complementarity effect), linear models were used; including the source of variation (genetic, epigenetic and demethylated treatments) and the environmental conditions and their interactions as fixed factors (Table S1a). When the interactions were significant, the effect of the trait on biodiversity effects was tested within the different treatments separately (Table 3).

All analyses were carried out using R v3.2.3 (R Core Team 2016) with α=0.05 as significance level.

Results

We found no effect of diversity on *Arabidopsis* populations' mortality in response to the disturbance/stress (Table 1a, 1b; Fig. S2). Mortality was similar between monocultures and mixtures, and between environmental conditions (Fig. S2). However, the sources of variation affected mortality. Populations with manipulated genetic variation showing lower mortality than epigenetic ones (Table 1a; Fig. S2). Further, demethylated populations had an overall lower mortality than populations with environmentally induced epigenetic variation (Table 1b; Fig. S2).

The waterlogging treatment caused a general decrease in the productivity of the *Arabidopsis* populations (Table 1a, 1b; Fig. 1). Also, we detected an overall lower biomass of populations with manipulated epigenetic variation compared to the demethylated populations and to the populations with manipulated genetic variation (Table 1a, 1b; Fig. 1). Additionally, we detected no overall significant effect of diversity on productivity (Table 1a and b; Fig. 1). Total biomass was similar between mixtures and monocultures. However, diversity had a more negative effect in demethylated populations (Fig. 1). Under fertilization, demethylated mixtures produced significantly lower biomass compared with monocultures (Fig. 1).

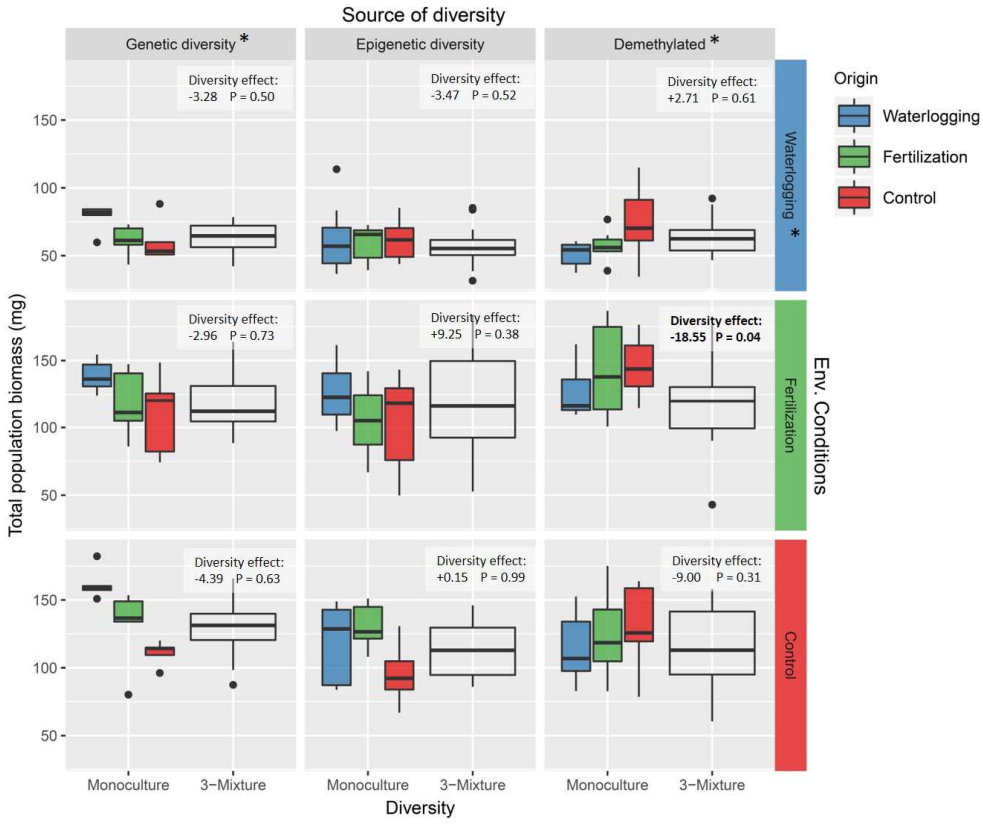

Figure 1: Productivity of experimental populations of *Arabidopsis*: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control. The direction and magnitude of the effect of increasing diversity in each experimental treatment are shown in the boxes. Asterisks in the environmental conditions indicates the treatment significantly different from the others. Asterisks in the source of variation treatments indicate significant overall differences of the marked population compared with the population with manipulated epigenetic diversity (i.e. genetic vs. epigenetic; epigenetic vs. demethylated).

Populations under waterlogging stress had more phenotypic variability (i.e. CV of SLA) than under other environmental conditions (Table 1a, 1b; Fig 2). Also, the populations with manipulated genetic variation had lower CV of SLA than ones with manipulated epigenetic variation (Table 1b; Fig 2). In general, diversity increased phenotypic variability (i.e. genetic and epigenetic

Table 1: Effects of environmental conditions, diversity, and source of variation, and their interactions on *Arabidopsis* populations' mortality, total productivity, and variation and average SLA, testing separately the effect of source of variation: A) Epigenetic vs. Demethylated; B) Genetic vs. Epigenetic. Result of the full factorial linear models including, as fixed factors. In bold the significant effects of the factors.

A) Epigenetic Vs. Demethylated		Mortality			Total biomass			CV SLA			Average SLA		
	d.f.	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value
Env. condition	2	0.76	0.26	0.77	94281	147.39	**<0.01**	0.429	63.51	**<0.01**	45356	179.48	**<0.01**
Diversity	1	2.28	0.79	0.38	622	0.97	0.32	0.100	14.80	**<0.01**	290	1.23	0.27
Source of Variation	1	16.25	5.6	**0.02**	3656	5.71	**0.02**	0.000	0.05	0.83	2439	10.33	**<0.01**
E x D	2	4.33	1.49	0.23	127	0.20	0.82	0.066	9.70	**<0.01**	535	2.27	0.11
E x V	2	1.53	0.53	0.59	545	0.85	0.43	0.001	0.10	0.9	889	3.77	**0.02**
D x V	1	0.68	0.93	0.34	1608	2.51	0.11	0.008	1.18	0.27	26	0.11	0.74
E x D x V	2	2.2	0.76	0.47	1481	2.31	0.10	0.005	0.69	0.50	48	0.20	0.81
Residuals	233*	2.9			640			0.006			236		

E = Environmental condition; V = Source of variation; D = Diversity; d.f. = degrees of freedom; MS = means square; F = variance ratio; P = error probability. *For mortality d.f.= 240

B) Genetic Vs. Epigenetic		Mortality			Total biomass			CV SLA			Average SLA		
	d.f.	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value
Env. condition	2	1.87	0.63	0.53	78204	138.96	**<0.01**	0.183	28.54	**<0.01**	29392	188.89	**<0.01**
Diversity	2	4.17	1.41	0.24	8	0.01	0.91	0.093	14.54	**<0.01**	61	0.39	0.53
Source of Variation	1	41.87	14.14	**<0.01**	5170	9.19	**<0.01**	0.031	4.78	**0.03**	50550	324.86	**<0.01**
E x D	2	3.18	1.08	0.34	264	0.47	0.63	0.013	2.03	0.13	208	1.33	0.27
E x V	2	0.47	0.16	0.85	1165	2.07	0.13	0.049	7.67	**<0.01**	307	1.97	0.14
D x V	1	1.07	0.36	0.55	390	0.69	0.40	0.005	0.79	0.37	217	1.40	0.24
E x D x V	2	2.11	0.71	0.49	168	0.30	0.74	0.008	1.31	0.27	271	1.74	0.18
Residuals	197*	2.95			563			0.006			156		

E = Environmental condition; V = Source of variation; D = Diversity; d.f. = degrees of freedom; MS = means square; F = variance ratio; P = error probability. *For mortality d.f.= 204

mixtures had higher CV of SLA than monocultures; Table 1a, 1b), although only in the latest (epigenetic diverse mixtures), was significantly higher (Fig. 2). In contrast, in the demethylated populations this effect disappeared, and phenotypic variability decreased in mixtures (Fig. 2). The higher CV of SLA of mixtures was explained by the differences between origins of the mixtures and no because the different origins increased their CV of SLA from monocultures to mixtures (Fig. S4). It suggests that phenotypic variability of the mixtures was explained by transgenerational plasticity with no within-generation plasticity involved.

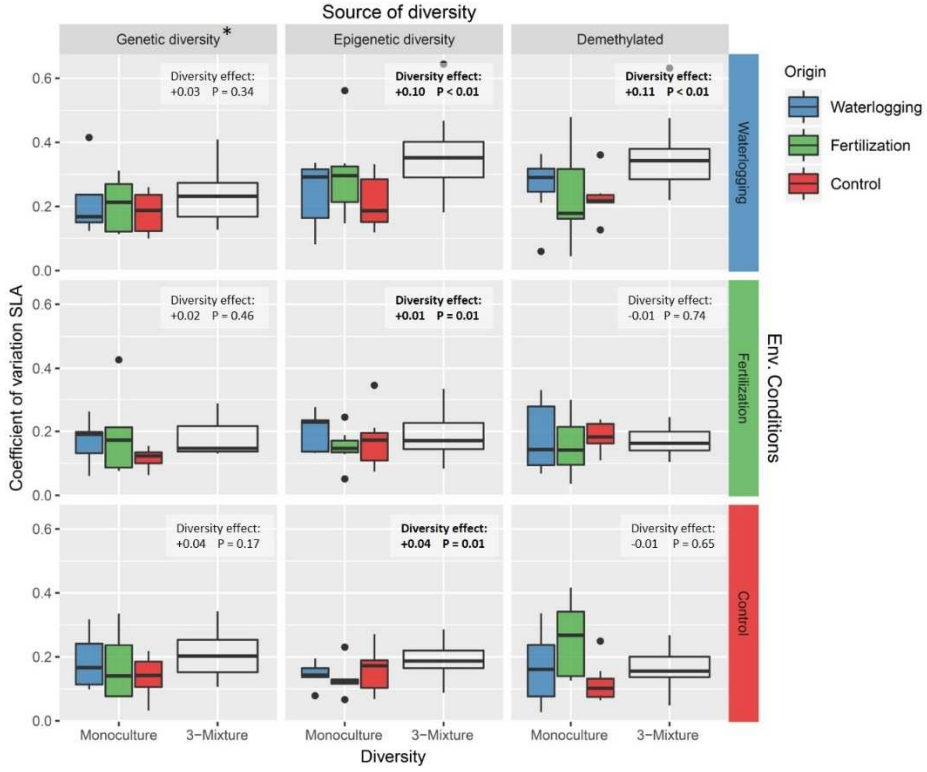

Figure 2: Functional diversity measured as coefficient of variation of SLA within each experimental population of *Arabidopsis*: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control. The direction and magnitude of the effect of increasing diversity in each experimental treatment are shown in the boxes. Asterisks in the source of variation treatments indicate significant overall differences of the marked population compared with the population with manipulated epigenetic diversity (i.e. genetic vs. epigenetic; epigenetic vs. demethylated).

At the same time populations' average values of SLA differed depending on the environmental condition and the source of variation (Table 1a, ab). We found lower SLA in all *Arabidopsis* populations under waterlogging stress (Fig. 3). Also, populations with manipulated genetic variation had in general lower SLA, and demethylated populations higher SLA than the populations with manipulated epigenetic diversity (Fig. 3). In general, we found no differences in SLA values between monocultures and mixtures with the exception of genetic diverse mixtures in the fertilization treatment with significant lower SLA than the respective monoculture. As we found with the CV of SLA, the average SLA of the different origins were no changing between monoculture and mixture (Fig. S5), suggesting no within-generation phenotypic plasticity triggered by the population structure.

In accordance to the previous results on populations' biomass, we found a general null net diversity effect, as well as for its components: selection and complementarity on genetic and epigenetic diverse populations (Table 2a; Fig. 4). Only we detected a significantly higher selection effect of the epigenetic diverse mixtures compared to the genetic diverse ones, in the fertilization treatment (Fig. 4b). This increase in productivity of the mixture was associated to a greater productivity of the waterlogging origin (Fig. S3). Nevertheless, we found greater differences between the diversity effects on epigenetic and demethylated mixtures, although they were specific for each environmental condition (Table 2a). We found a negative net effect in the demethylated population under fertilization (Table 2a; Fig. 3a), which was mostly explained by a negative complementarity effect (Fig. 4c), meaning that all the different origins were less productive in mixtures than in monocultures (Fig. S3). Although the effect of selection compared with complementarity was relatively small (± 3 mg vs. ± 50 mg; Fig. 4b and 4c), we found more positive selection effect of the epigenetic diverse mixtures compared with the demethylated ones in the waterlogging treatment (Table 2a, Fig 4b). This difference did not contribute to generate any differences on the overall net effect.

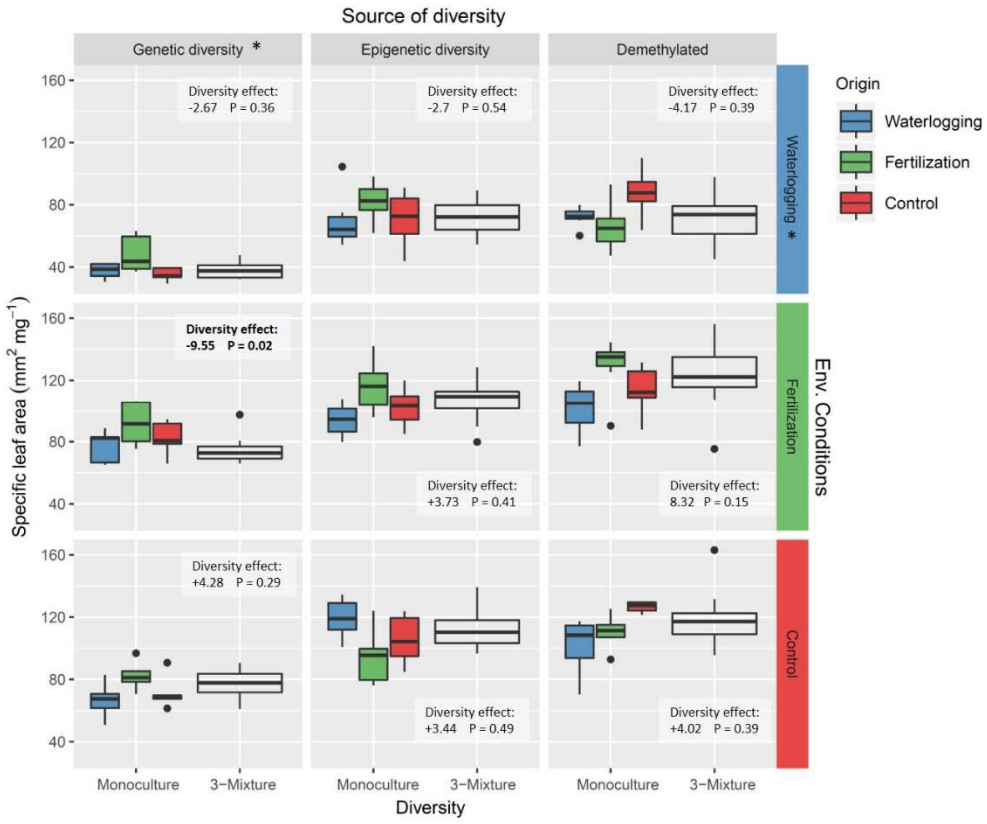

Figure 3: Average SLA of each experimental population of *Arabidopsis*: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control. The direction and magnitude of the effect of increasing diversity in each experimental treatment are shown in the boxes. Asterisks in the environmental conditions indicates the treatment significantly different from the others. Asterisks in the source of variation treatments indicate significant overall differences of the marked population compared with the population with manipulated epigenetic diversity (i.e. genetic vs. epigenetic; epigenetic vs. demethylated).

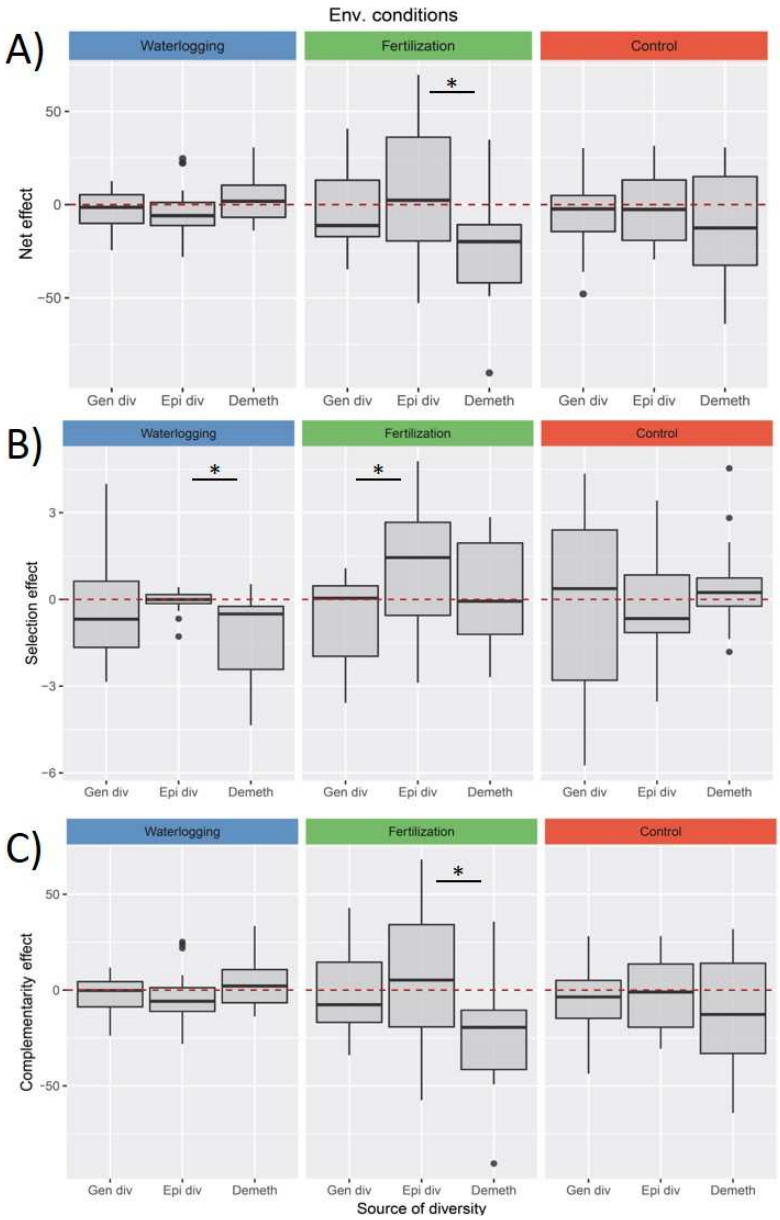

Figure 4: A) Net biodiversity effect, and its two components B) selection effect and C) complementarity effect in the mixtures of *Arabidopsis* with differing source of diversity: epigenetic, genetic and absent (demethylated). Each column corresponds to the different environmental conditions: waterlogging, fertilization and control. Asterisks indicate significant differences of the mixtures compared with epigenetic diverse mixtures (i.e. genetic vs. epigenetic; epigenetic vs. demethylated).

Table 2: Effects of environmental conditions and source of variation and their interactions on diversity net effect, selection effect and complementarity effect on *Arabidopsis* populations' biomass, testing separately the effect of source of variation: A) Epigenetic vs. Demethylated; B) Genetic vs. Epigenetic. Result of the full factorial linear models including, as fixed factors. In bold the significant effects of the factors.

A) Epigenetic Vs. Demethylated		Net effect			Selection effect			Complementarity effect		
	d.f.	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value
Env. condition	2	548	0.92	0.40	17.50	6.82	**<0.01**	741	1.28	0.28
Source of Variation	1	3196	5.37	**0.02**	7.05	2.75	0.10	2903	5.01	**0.03**
E x V	2	3040	5.10	**<0.01**	10.26	4.00	**0.02**	3088	5.32	**<0.01**
Residuals	115	595			2.56			580		

B) Genetic Vs. Epigenetic		Net effect			Selection effect			Complementarity effect		
	d.f.	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value
Env. condition	2	591	1.22	0.30	2.70	0.70	0.50	513	1.10	0.34
Source of Variation	1	490	1.01	0.32	11.08	2.86	0.09	354	0.76	0.39
E x V	2	399	0.62	0.54	7.31	1.89	0.16	220	0.47	0.63
Residuals	97	486			3.87			466		

When we use traits to explain the net diversity effect and its two components, we found a general significant effect of average SLA on increasing net effect and complementarity, plus an effect of CVSLA dependent of the environmental conditions and source of variation (i.e. interaction E x V x CVSLA; Table S1a). In order to characterize these effects correctly we explore the relationship within the populations under the same environmental condition (i.e. waterlogging, fertilization and control). Only in waterlogging and control treatment, we found that traits could explain the diversity effects (Table S1b). In mixtures undergoing waterlogging, a positive net effect (i.e. more productive mixtures beyond the predicted by the monocultures) was characterized by populations with low CVSLA and high values of SLA (Table S1b). In control conditions the effect of CVSLA and SLA was dependent of the source of variation (i.e. interaction V x CVSLA/SLA; Table S1b), so we further segmented the treatments. By doing that, in control conditions, we found a positive effect of SLA in the selection effect of epigenetic diverse mixtures, and a positive effect of CVSLA on net effect and complementarity in demethylated mixtures (Table 3)

Discussion

This study expands the knowledge on the role of within and transgenerational plasticity as a potential mechanism of coexistence and functioning of communities. Specifically, this is the first empirical demonstration of the effect of "parental diversity" or diversity of environmentally induced transgenerational effects on productivity and resistance to stress of the populations. In general, we found a null diversity effect on the productivity and resistance to stress, except in demethylated populations where we found a negative effect. This result strongly suggests, first, that the transgenerational effects were controlled epigenetically, and at least partially enabled by DNA cytosine methylation. And second, that epigenetic diversity seems to ameliorate the negative effects of intraspecific competition on productivity of *Arabidopsis* populations. The higher phenotypic variation found in the epigenetic diverse mixtures compared to the

Table 3: Influence of average SLA and CVSLA on diversity net effect, and its additive components: selection and complementarity effect on *Arabidopsis* mixtures. Regression standardized coefficients of each trait predictors of the linear models made in each experimental treatment separately (i.e. mixtures with different source of variation and under different environmental condition separately). In bold the significant effects of the factors.

Environmental condition	Trait predictor	Source of Variation								
		Genetic variation			Epigenetic Variation			Demethylated		
		Net	Sel	Comp	Net	Sel	Comp	Net	Sel	Comp
Waterlogging	SLA	28.00	-0.93	**28.93[a]**	12.76	0.26	12.49	**13.87***	0.88	**12.99***
	CVSLA	-4.47	0.47	-4.95	-3.22	0.05	-3.27	1.24	0.65	0.59
Fertilization	SLA	-6.79	**-2.57[a]**	4.22	10.02	-0.14	10.16	18.15	0.22	17.93
	CVSLA	4.37	-0.91	5.28	-19.52	-0.33	-19.19	2.49	-0.65	3.14
Control	SLA	17.57	-2.81	20.28	**21.29[a]**	**2.69***	18.60	9.00	-0.29	9.29
	CVSLA	-11.45	-1.19	-10.26	-5.88	-1.08	-4.81	**29.88****	-0.20	**30.08****

Net = Net diversity effect; Sel = Selection effect; Comp = Complementarity. Significance: [a] ($0.05 \leq P < 0.1$); * ($0.01 \leq P < 0.05$); ** ($P < 0.01$)

demethylated suggests that the reduction of competition could be partially caused by the increase of niche differences between origins

We show that the waterlogging treatment was the most stressful condition for the populations of *Arabidopsis*. Although it did not cause higher mortality, it decreased at least 50% of the biomass production. However, we did not find that diversity increased population resistance again the stress. The strong decrease on the size of the individuals could have avoid a real physical interaction between individuals, thus, not letting any other potential property of the population to emerge from these interactions. Similarly, the stronger diversity effects founded in the fertilization treatment compared with the other environmental conditions, could be caused by the greater size of the individuals that increased the intensity of the interactions. The relatively low selection effect founded in our results could be promoted by the low mortality/exclusion occurred in the experiment.

We show that the three different conditions (control, fertilization and waterlogging) imprinted on the Col-1 ecotype triggered phenotypical differences between the offspring (i.e. transgenerational effects), which consequently translated into more functional diverse populations (i.e. higher CV SLA) when the different origins were brought together in mixtures. Interestingly, when we applied the demethylation treatment that partially or fully removed the epigenetic signature of the individuals, the functional diversity of the mixtures disappeared. The lack of phenotypical variation of the demethylated mixtures strongly suggests that the "potential" epigenetic diversity treatment was indeed reflecting epigenetic variation, and at least partially enabled by DNA cytosine methylation. If we would partition the variance; we would detect that the higher CV of SLA of the mixtures was due to differences between origins and no because an increase of the variation within origins from monocultures to mixtures (Fig. S4). So, it suggests that transgenerational rather than within generational phenotypic plasticity was driving the phenotypic variability of the mixtures, thus, promoting niche/resource partitioning and potentially increasing complementarity.

However, the more functional diversity of the epigenetic diverse populations, did not translate in any positive effect on productivity or resistance. By increasing divergence in traits between individuals, we expected reduce competition between origins, enhancing niche segregation and complementarity, and thus promoting a positive diversity net effect (Fridley, 2001). However, we found no positive, nor negative diversity effects on epigenetic diverse mixtures. On the contrary, in demethylated mixtures, where we removed the epigenetic diversity and its relative functional diversity, we found a negative diversity effect, due to a negative complementarity effect, only significant under fertilization. Although demethylation, by removing the epigenetic trait differences, could have probably reduced niche differences between origins and increased the competition intensity between origins; it does not only explain the negative values of net diversity effect. If so, it was expected that the biomass produced in demethylated mixtures was similar to the demethylated monocultures. However, we found higher average biomass of the different origins in monocultures than in mixtures, meaning that the competition was less intense among conspecifics of the same origin than between origins; indicating that other mechanism of origin-specific cooperative behaviour should have been also involved for reducing the competition between conspecifics of the same origin (also found in Semchenko *et al.*, 2014). Anyhow, epigenetic diversity seems to ameliorate the negative effect of competition between different origins.

Although we expected genetic diverse populations to have the highest phenotypic variability related to resource foraging compared to the rest of populations, we found a lack of functional diversity on the mixtures compared with the monocultures. It surprises that the mixture of the three selected ecotypes with different selection history, which are described to differ in phenotypic traits like root system and flowering period (Picó *et al.*, 2008; Méndez-Vigo *et al.*, 2013); did not differ in SLA which is a trait related to resource foraging strategy of the plant. Probably the absence of deep morphological variability in SLA of these ecotypes compared to Col-1 ecotype suggests that the low SLA is a selected plant life-history trait that contributed to their success under Mediterranean climates (Wright *et al.*, 2005; Blonder *et al.*, 2015). Nevertheless, by not finding a negative diversity effect in these populations (like what we found in

demethylated populations), indicates that, indeed, there is functional phenotypic variability.

When we tested the relative importance of trait values and variation for explaining the biodiversity effects, we found that SLA average values rather than trait variance (i.e. CVSLA) of the populations drive the productivity of the mixtures. We found that, in general, the net diversity effects were higher when mixtures contained individuals with high SLA (i.e. higher average SLA), and were also associated with lower variation in SLA (i.e. negative standard coefficients of CVSLA; Table 3). Meaning that populations of individuals with high SLA supplied more function than more diverse assemblages with lower SLA. Although probably, measuring SLA alone could have be insufficient for characterizing organisms' niche differences (Kraft *et al.*, 2015; Kunstler *et al.*, 2016; Cadotte, 2017), this result suggests that the increased in functioning was not driven only by niche differences and average traits provided better explanation for the function (Kunstler *et al.*, 2016). This does not surprise because, besides being a trait related to resource foraging strategy of the plant (i.e. niche segregation), SLA is a hierarchical trait linked to the fitness or competitive ability of the individuals (Roscher *et al.*, 2012; Kraft *et al.*, 2014, 2015). Species tend to achieve greater biomass when have higher SLA (Kraft *et al.*, 2014). However, and interestingly only in demethylated mixtures, we found a more positive effect of niche differences (i.e. CVSLA) on the functioning, in some cases even stronger that the effect of trait average (Table 3). This suggests that when the phenotypic variation is reduced, and consequently the competitive intensity increased, the importance of phenotypic variation and niche differentiation gets more important.

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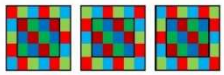
Supplementary material

Experimental design

Genetic diversity			
Population	Drought	Water	Fertilization
Monocultures	5	5	5
Monocultures	5	5	5
Monocultures	5	5	5
Mixture	15	15	15

Parental/epigenetic diversity			
Population	Drought	Water	Fertilization
Monocultures	7	7	7
Monocultures	7	7	7
Monocultures	7	7	7
Mixture	21	21	21

Demethylated (no genetic, no epigenetic)			
Population	Drought	Water	Fertilization
Monocultures	7	7	7
Monocultures	7	7	7
Monocultures	7	7	7
Mixture	21	21	21



Mixture populations design

Figure S1: Schematic representation of the experimental design and the distribution of the different individuals in polycultures (mixtures).

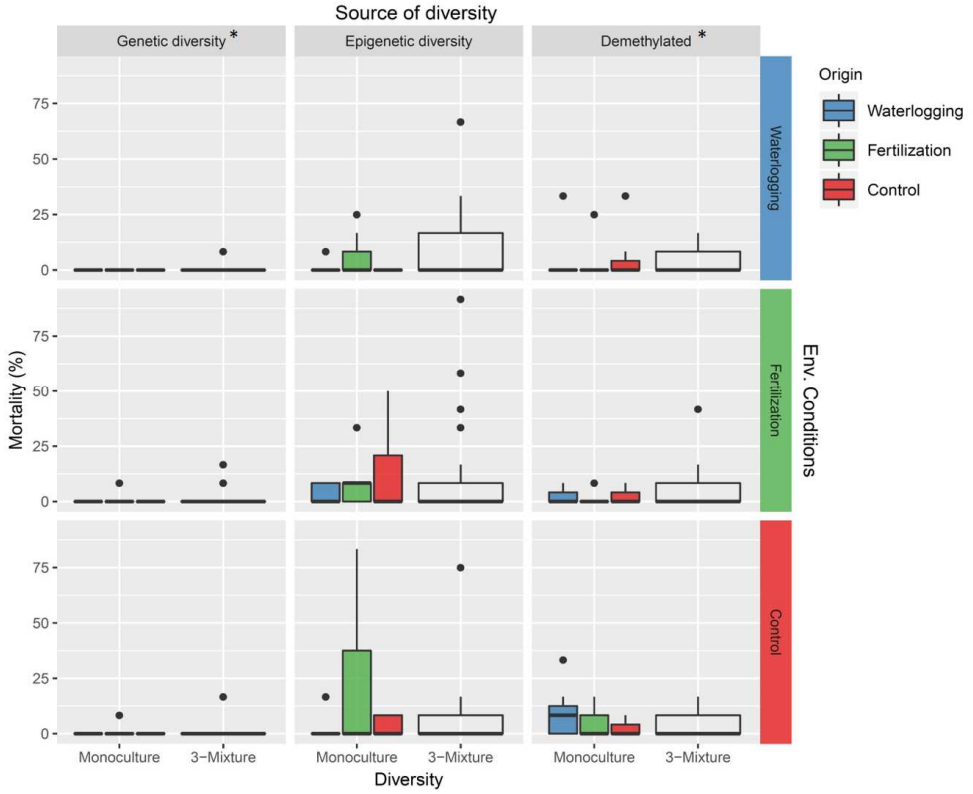


Figure S2: Mortality within each experimental population of *Arabidopsis*: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control. Asterisks in the source of variation treatments indicate significant overall differences of the marked population compared with the population with manipulated epigenetic diversity (i.e. genetic vs. epigenetic; epigenetic vs. demethylated).

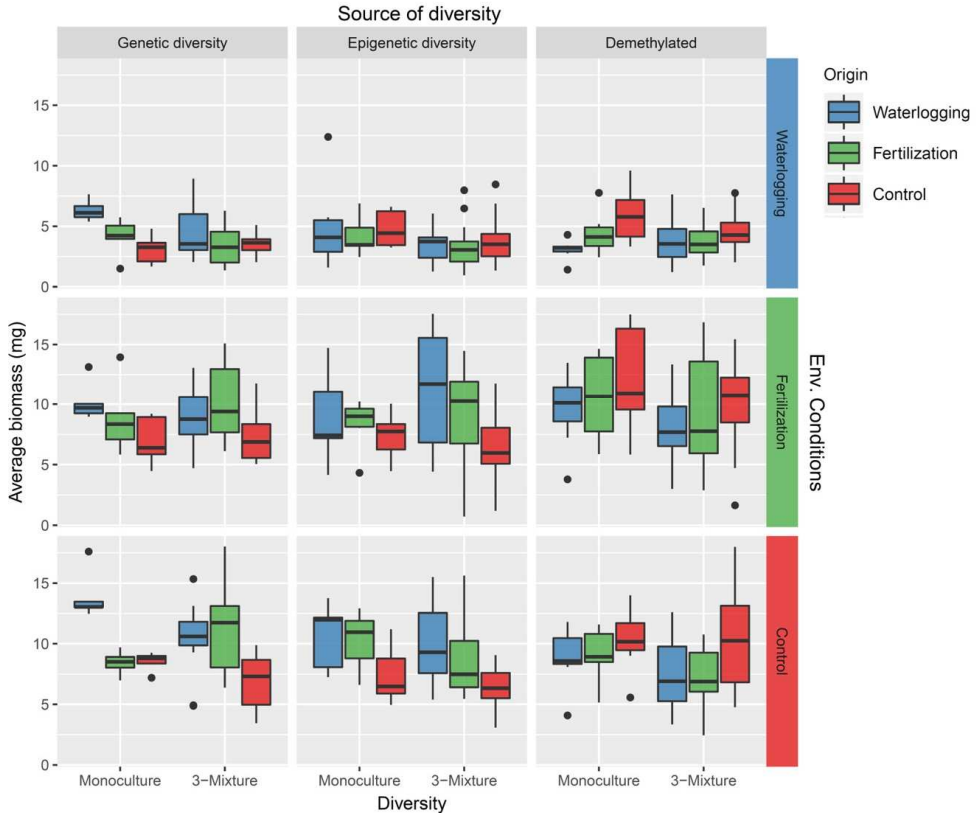


Figure S3: Average biomass of each origin in each experimental populations of *Arabidopsis*: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control.

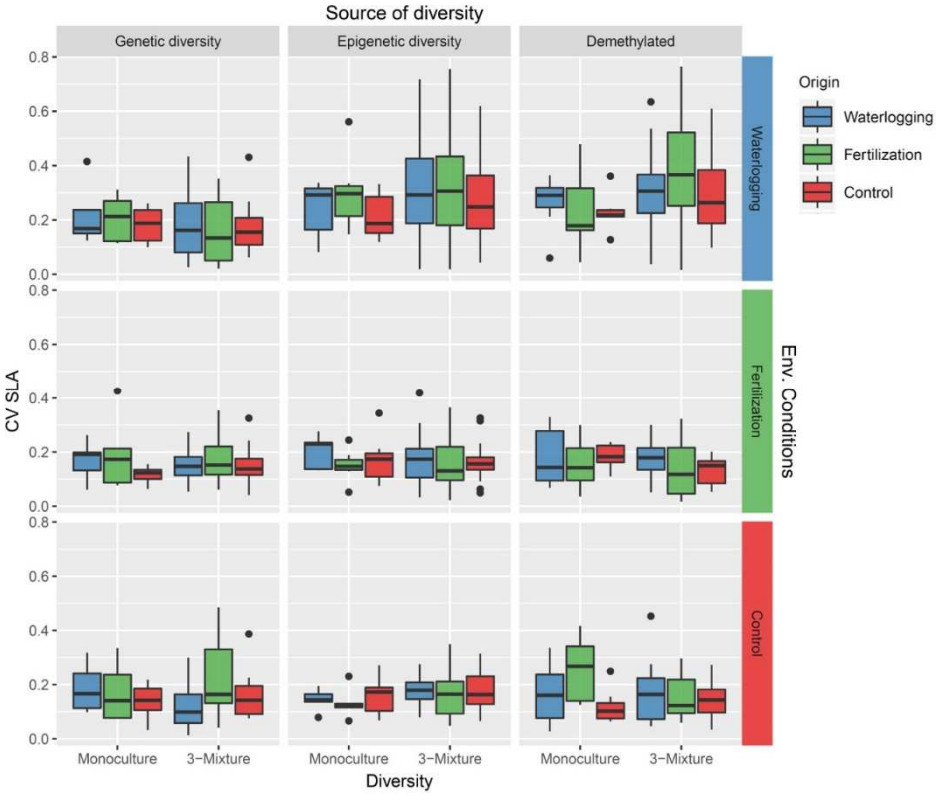


Figure S4: Coefficient of variation of SLA within each origin in each experimental populations of *Arabidopsis*.: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control.

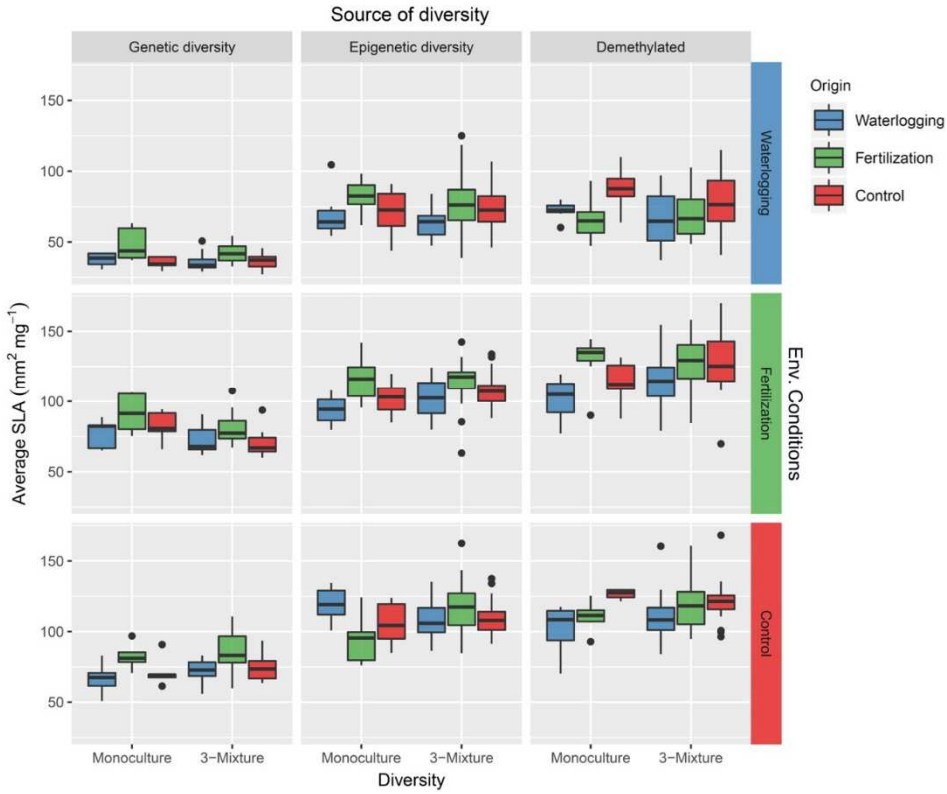


Figure S5: Average of SLA of each origin in each experimental populations of *Arabidopsis*: monocultures vs. mixtures. Each column corresponds to the differing source of diversity: genetic, epigenetic and demethylated; and each column to the different environmental conditions waterlogging, fertilization and control.

Table S1: Influence of traits: average SLA and CVSLA; plus environmental conditions and source of variation, and their interactions on diversity net effect, and its additive components: selection and complementarity effect on *Arabidopsis* mixtures. A) General model, B) Segmented by environmental condition (i.e. mixtures under different environmental condition separately)**.** Result of the full factorial linear models. Significant effects of the factors are shown in bold, and the colour indicates the direction of the effect (positive in green, negative in red).

A) General model		Net effect			Selection effect			Complementarity		
	d.f.	MS	F ratio	P value	MS	F ratio	P value	MS	F ratio	P value
Environmental condition	2	426.41	0.89	0.41	11.33	3.50	**0.03**	564.13	1.21	0.30
Source of Variation	2	1611.33	3.36	**0.04**	6.2	1.91	0.15	1480.27	3.18	**0.04**
CVSLA	1	370.88	0.77	0.38	6.63	2.05	0.15	278.29	0.60	0.44
SLA	1	2866.72	5.97	**0.02**	0.59	0.18	0.67	2785.12	5.98	**0.02**
E x V	4	2365.74	4.63	**<0.01**	7.51	2.33	0.06	2422.58	5.21	**<0.01**
E x CVSLA	2	414.58	0.86	0.42	11.13	3.45	**0.03**	483.02	1.04	0.36
E x SLA	2	89.75	0.19	0.83	1.26	0.39	0.68	101.14	0.22	0.20
V x CVSLA	2	1581.68	3.3	**0.04**	1.92	0.59	0.55	1473.81	3.17	**0.04**
V x SLA	2	107.61	0.22	0.8	10.78	3.34	**0.04**	50.38	0.11	0.90
E x V x CVSLA	4	1210.32	2.52	**0.04**	1.53	0.47	0.75	1191.99	2.56	**0.04**
E x V x SLA	4	219.77	0.46	0.77	3.53	1.09	0.36	191.33	0.41	0.80
Residuals	139	479.9			3.23			465.36		

E = Environmental condition; V = Source of variation; D = Diversity; d.f. = degrees of freedom; MS = means square; F = variance ratio; P = error probability.

B) Segmented by Environmental conditions		d.f.	Net effect MS	Net effect F ratio	Net effect P value	Selection effect MS	Selection effect F ratio	Selection effect P value	Complementarity MS	Complementarity F ratio	Complementarity P value
Waterlogging	Source of Variation	2	273.29	1.89	0.16	8.2	4.65	**0.01**	374.99	2.52	0.09
	CVSLA	1	686.81	4.73	**0.03**	3.14	1.78	0.19	780.65	5.54	**0.03**
	SLA	1	1358.63	9.39	**<0.01**	2.77	1.57	0.22	1238.73	8.31	**<0.01**
	V x CVSLA	2	121.88	0.8426	0.44	1.05	0.59	0.56	108.36	0.73	0.48
	V x SLA	2	40.42	0.28	0.76	0.78	0.44	0.65	49.14	0.33	0.72
	Residuals	46	144.65			1.77			149.02		
Fertilization	Source of Variation	2	4051.1	4.68	**0.01**	12.64	3.61	**0.03**	3859.10	4.56	**0.02**
	CVSLA	1	1419.7	1.64	0.2	3.81	1.09	0.30	1276.40	1.51	0.23
	SLA	1	2825.1	3.27	0.08	0.25	0.07	0.79	2878.30	3.40	0.07
	V x CVSLA	2	858.4	0.99	0.37	0.3	0.09	0.92	890.90	1.05	0.36
	V x SLA	2	293.1	0.34	0.71	3.39	0.97	0.39	235.60	0.28	0.76
	Residuals	46	865.2			3.5			845.50		
Control	Source of Variation	2	471.32	1.09	0.32	2.75	0.62	0.54	544.28	1.35	0.27
	CVSLA	1	176.5	0.41	0.52	17.11	3.89	0.05	303.51	0.75	0.39
	SLA	1	875.67	2.03	0.16	0.21	0.05	0.82	848.26	2.01	0.15
	V x CVSLA	2	3101.68	7.2	**<0.01**	3.23	0.74	0.48	2904.79	7.21	**<0.01**
	V x SLA	2	134.03	0.31	0.73	14.06	3.2	**0.05**	101.99	0.25	0.78
	Residuals	46	430.91			4.4			402.93		

E = Environmental condition; V = Source of variation; D = Diversity; d.f. = degrees of freedom; MS = means square; F = variance ratio; P = error probability.

General Discussion & Conclusions

In a context where the importance of intraspecific variation for community assembly and ecosystem functioning is increasingly acknowledged, this thesis brings some light on understanding how it affects the processes involved in the assembly of natural communities and promotes species coexistence and biodiversity. Particularly, this thesis explores a mechanism of variation that has been generally ignored: transgenerational plasticity (i.e. transgenerational epigenetic inheritance). Although increasing research has recognized the role of epigenetic transgenerational effects in adaptation, the great majority of existing studies have focused on transgenerational responses to abiotic factors, overlooking the role of biotic interactions. This point of view ignores that biotic interactions are leading factors for controlling species coexistence, biodiversity maintenance and ecosystem functioning. This thesis fills the gap of knowledge and adds empirical evidence focused on the plasticity triggered by plant biotic interaction. Moreover, besides exploring the potential adaptative role of transgenerational plasticity, this thesis moves forward, expanding the scope of consequences that transgenerational plasticity could have in ecology. Under the "response-effect" framework, we studied how transgenerational trait plasticity

could also promote the ability of organisms to shape the environment where they live in and affect ecological interactions. These changes could promote coexistence and enhance aspects of ecosystem functioning such as biodiversity assembly and nutrient cycling. Therefore, this thesis shows that transgenerational plasticity has consequences that go beyond the scale of the individual plants.

Exploring the consequences in ecological and evolutionary processes of transgenerational plasticity required the examination of different aspects. Primarily, stablishing the necessary methodology to be able to explore these questions and to disentangle the mechanisms originating the transgenerational plasticity. Then, checking wether the biotic interactions alter the phenotype, examining the magnitude and direction of the response on each specific "response traits". And finally, dilucidating the potencial role of these modifications as "effect traits", checking their relevance for adaptation, species coexistence, creating biodiversity and population and ecosystem functioning. In the remaining of this section the main findings of this thesis are summarised and discussed, and some lines for future research are proposed.

First, I have showed the strong ability of plants to respond to the biotic and abiotic conditions experienced during their life cycle. This response was examined in terms of differences on fitness-related characteristics, such as individual biomass or seed production depending on wether the conditions were stressful or mild. Further, I also examined responses considering multiple plant phenotypic traits, including both root and aboveground systems. If the responding traits were aggregated under the umbrella of the plant economics spectrum framework (Díaz *et al.* 2016), in general, the more stressful ecological interactions presented in this thesis, i.e. high plant-plant competition intensity, absence of arbuscular mycorrhizal symbiosis, and waterlogging and water drought stress, triggered a conservative phenotype on the plants, characterized by lower SLA, higher LDMC, higher allocation to the roots and lower SRL. Conservative phenotypes are associated with longer lifespan and better resource-use conservation. Although I did not test the adaptive role of this within-generation plasticity, considering that conservative strategies are generally

selected in plants from resource-poor environments (Díaz *et al.* 2016) and that conservative-strategy phenotypes are normally dominating the competitive hierarchy in pairwise competition experiments (Kraft *et al.* 2015; Carmona *et al.* 2019; Puy, unpublished obs.), the plastic response towards a conservative phenotype seems to be adaptive and to improve organism fitness by reducing the negative intensity of ecological interactions or increasing the ability to cope with stress.

Moreover, I found that also the different ecological interactions presented in this thesis triggered transgenerational plasticity. This was evidenced by the fact that the phenotypes of the offspring were affected by the environmental conditions that parents experienced. Despite species identity or the environmental condition, transgenerational plasticity had generally concordant responses with the within-generational plasticity previously described. Hence, offspring became more conservative when the parents experienced more stressful ecological interactions (high competition intensity, absence of AMF, etc.). Therefore, in case that offspring experienced the same conditions that the parents, the transgenerational plasticity further reinforced trait plasticity in the same direction. Hence, although transgenerational plasticity has a much lower effect than the plasticity expressed during the life cycle of a plant, its effects were far from being negligible.

There are two types of responses between within- and across-generation plasticity: concordant that drives progeny phenotypes to a distant optimum, and opposing responses, which stabilize phenotypes in an intermediate optima (Auge *et al.* 2017). Concordant responses can accelerate adaptation to the environment as long as the selective environment persists, so that the environment experienced by the progeny matches with that of the parents (Herman *et al.* 2014). By contrast, concordant and persistent plasticity may become less adaptive, or even maladaptive once that optimum is achieved (Auge *et al.* 2017). Although transgenerational plasticity is especially strong during juvenile stages of the progeny, it can also persist during plant development. In any case, within-generation plasticity seems to be stronger than across-generation plasticity. Therefore, although transgenerational plasticity acts like an adaptive "stress

memory" that improves the ability of the offspring to cope with the predicted environment, within-generation plasticity could override across-generation plasticity to let progeny respond more accurately to the cues of its own environment.

Along this thesis I have generally point to heritable epigenetic mechanisms, such as DNA methylation, as responsibles for the induced transgenerational effects. However, besides the epigenetic origin, transgenerational plasticity could have been mediated by differences in seed provisioning, seed quality (i.e nutritional quality) and hormonal balance stocked up by the maternal plants (Roach & Wulff 1987). In order to disentangle both mechanisms and to focus on the epigenetic mechanims, I have tried to experimentally control the effect of the seed characteristics by incorporing them into the models. Further, whenever possible, the epigenetic status of the plants was altered by *in vivo* experimental DNA demethylation. The selection of the later approach could not have been possible without the proper validation of the method for suitability in ecological research presented in Chapter I. This method, based on daily spraying onto the leaves of the plants of a solution of a demethylation agent (i.e. azacytidine), removes methylation marks of plants, including those inherited from previous generations. The new method has the same demethylating efficiency as the traditional methodology, but without the adverse side effects that affected plant survival and development. Thus, this tool is ideal for ecological epigenetics since it allows to reliably discriminate the phenotypic changes caused by the DNA-methylation from the side effects of the demethylation agent. Furthermore, this methodology is suitable for clonal species reproducing asexually, and opens the possibility for community-level experimental demethylation of plants.

Besides exploring the potential adaptative role of transgenerational plasticity, this thesis moves forward and expands the scope of consequences that transgenerational plasticity could have in ecology on larger scales. I showed that transgenerational plasticity allows organisms to shape their environment, in which their progeny will live. Paticularly, in Chapter II, I showed that transgenerational plasticity can affect ecosystem processes like decomposition.

Increasing levels of parental competition resulted in more conservative phenotypes (e.g. higher LDMC and leaf C:N), that are related to more structural and slower degradable organic matter in leaves, which takes longer to be returned to the soil (Cornelissen & Thompson 1997). Interestingly, this slower degradation might in turn favor those plants with a more resource-use-conservative phenotype, which have lower rates of nutrient uptake, subsequently affecting the plant–plant competitive interactions (van der Putten *et al.* 2013; Semchenko *et al.* 2017). This opens a new field of research on the potential positive plant–soil feedback triggered by transgenerational plasticity.

Last but not least, in Chapter IV, I demostrated that populations of individuals from diverse parental environmental origins are, indeed, more phenotypic and functional diverse, probably on account of heritable epigenetic mechanims. Therefore, the different parental environments induced transgenerational effects that increased trait differences between origins. This increase in differences did not seem to provide any positive consequences on populations' productivity or resistance against stress by increasing complementarity between origins. However, trait differences seemed to increase niche differences between origins and decrease the intensity of intraspecific competition, ameliorating its negative effect on productivity. Thus, transgenerational plasticity can play a role on the coexistence and functioning of the communities. Probably, testing these results in longer experiments, in natural communities is needed. Besides promoting rapid evolution, the accumulation of concordant plasticity across the years, could for example explain the increase in complementarity founded in biodiversity experiments over the time (e.g. Meyer *et al.* 2016).

Conclusions

- More stressful ecological interactions triggered plant plasticity towards conservative phenotypes. These adjustments seem to improve fitness of the organism by reducing the negative intensity of the ecological interactions and to increase the ability to cope with stress, thus promoting coexistence.
- The ecological interactions also triggered transgenerational plasticity in the same direction that within-generation plasticity, further reinforcing the phenotypes towards a conservative phenotype. Although transgenerational plasticity is especially strong during juvenile stages of the progeny, it can also persist during plant development.
- Transgenerational effects were generally removed when experimental demethylation was applied, strongly suggesting that the transgenerational plasticity was controlled epigenetically, and at least partially enabled by DNA cytosine methylation.
- Transgenerational plasticity acted like an adaptive "stress memory" that improves the ability of the offspring to cope with the predicted environment. As long as the selective ecological interaction persists, the concordant response between within and across-generation plasticity could promote rapid evolution and drive species phenotypes to a distant optimum.
- Transgenerational plasticity allowed organisms to shape their environment, and where their progeny will live by affecting ecosystem processes like decomposition. Subsequently, transgenerational plasticity feedbacks on the ecological interactions.
- Diversity of parental origins in a population created phenotypic and functional variation via transgenerational effects that, as any other source of biodiversity, could have a positive effect on population and ecosystem functionality.
- Different parental environments increased trait differences between origins. This increase of niche differences between origins decreased the intensity of intraspecific competition, ameliorating its negative effect on productivity. Thus, transgenerational plasticity can play a role on the coexistence and functioning of the communities.

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EDUCATION

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2013-2015: **MSc in Ecology** *(cum laude) Autonomous University of Madrid & Complutense University of Madrid (ES)*
2009-2013: **BS in Biological sciences** *(with honours) University of Navarra, Faculty of Sciences, Department of Environmental Biology (ES)*

RESEARCHER EXPERIENCE & INTERNSHIPS

February 2019 – July 2019: International PhD stay. Cristina Branquinho – Centre for Ecology, Evolution and Environmental Change, University of Lisboa (PT)
2016–2018 (8 months in total): International PhD stay. Mari Moora – Department of Botany, University of Tartu (EE)
February 2015 – August 2015: Research assistant. Courtney Murren – Department of Biology, College of Charleston (USA)
January 2015 – December 2016: Internship. Fernando Valladares – Biogeographic and Global Change Department, Spanish Research Council (ES)
September 2012 – August 2013: Research & lab. assistant. Ricardo Ibañez – Department of Environmental Biology, University of Navarra (ES)

PUBLICATIONS

Puy J., Dvořáková H., Carmona C.P., de Bello F., Hiiesalu I., Latzel V. (2018) Improved demethylation in ecological epigenetic experiments: a simple and harmless foliar demethylation application. *Methods in ecology and evolution* 9: 744-753 doi: 10.1111/2041-210X.12903

Galland T., Adeux G., Dvořáková H., E-Vojtkó A., Orbán I., Lussu M., **Puy J.**, Blažek P., Lanta V., Lepš J., de Bello F., Carmona C.P., Valencia E., Götzenberger L. (2019) Colonization resistance and establishment success along gradients of functional and phylogenetic diversity in experimental plant communities. *Journal of Ecology* 107 (5): 2090-2104. https://doi.org/10.1111/1365-2745.13246

Puy J., de Bello F., Dvořáková H., Medina N.G., Latzel V., Carmona C.P. Competition-induced transgenerational plasticity feedback on the offspring's competitive interactions and leaf decomposition. *New Phytologist* (**Submitted**)

Puy J., Carmona C.P., Hiiesalu I., Öpik M., de Bello F., Moora M. Mycorrhizal symbiosis alleviates plant drought stress within and across plant generations via plasticity. *Journal of Ecology* (**Submitted**)

Martínková J. & **Puy J.** Effect of disturbance on clonal versus non-clonal herbs: the cost of clonal growth revealed**.** *Perspectives in Plant Ecology, Evolution and Systematics* (**Minor revision**)

Murren C.J., **Puy J.,** Kohler C., Traba J., Malo J.E., Peco B. Sancho G. Root variation in common gardens: Divergent responses in native and non-native field sites of an annual ruderal Mediterranean plant. *International Journal of Plant Science* (**Submitted**)

Valerio-Galán M., Ibáñez R., **Puy J.**, Gazol A. The assembly of understory communities in temperate forests is mostly influenced by abiotic filtering processes. *Journal of Ecology* (**Submitted**)

Puy J., Dvořáková H., Carmona C.P., Latzel V., de Bello F. Parental diversity mitigates negative effects of intraspecific competition on productivity of Arabidopsis' populations by increasing phenotypic variation. (**In preparation**)

Puy J., Peco B.: Vegetative functional traits and moisture gradients in semiarid grasslands establishment stages demethylation in ecological epigenetic experiments: a simple and harmless foliar demethylation application. (**In preparation**)

G. Medina N., de Bello F., **Puy J.**, Galland T., Hájek T., Skuhrovec J., Dvořáková H., Cornelissen J. H., Latzel V. From memory to after-life: impacts of current and past environments on trait plasticity and decomposability of the clonal *Trifolium repens*. (**In preparation**)

CONFERENCE PRESENTATIONS (Only as first author)

December 2017: BES, GFÖ, NecoV & EEF Annual meeting – Ghent (BEL) Poster: "Transgenerational effects in plant interactions: does parental competition improves offspring adaptability? effects in plant competition"

Sept 2017: 40th New Phytologist Symposium – Vienna (AU) Poster: "Transgenerational effects in plant interactions: does parental competition improves offspring adaptability?"

Sept 2017: 40th New Phytologist Symposium – Vienna (AU) Poster: "Improved demethylation in ecological epigenetic experiments: Testing a simple and harmless foliar demethylation"

June 2017: 60th IAVS annual Symposium – Palermo (IT) Talk: "Transgenerational effects in plant interactions: does parental competition improves offspring adaptability?"

December 2016: BES Annual meeting – Liverpool (UK) Talk: "Transgenerational effects in plant competition"

May 2015: PopBio – Trebon (CZ) Talk: "Functional trait dissimilarity decreasing the effect of competitive hierarchies in plant interactions"

Other selected conferences (as coauthor):

October 2018: International conference of the French Ecological Society –Rennes, (FR) Talk: "Resistance is futile! or is it? A study on natural colonisation resistance and colonisation success in experimental plant communities along functional and phylogenetic diversity gradients"

August 2018: Evolution 2018 – Montpellier (FRA) Poster: "Natural selection and phenotypic integration of above and belowground traits"

July 2018: 12th Clonal Plant Symposium – Maine, (USA) Talk: "Do clonal herbs cope with disturbance better than non-clonal ones?"

August 2014: ESA Annual meeting – Baltimore (USA) Poster: "Ecological genetics of root architecture variation in *Arabidopsis thaliana* in native and non-native environments"

PROFESSIONAL ACTIVITIES & AWARDS

Reviewer in: *New Phytologist, Annals of Botany, Perspectives in Plant Ecology, Evolution and Systematics, Journal Vegetation Science,* and *Fungal ecology*

2015-2019: Scholarship as PhD student at Faculty of Science, University of South Bohemia, Czech Republic

2019: Awarded 1st place for best oral presentation by the students and 3rd by the staff at the Ph.D. student seminar of the Department of Botany of University of South Bohemia

2019: Erasmus + grant for visiting PhD students – University of Lisboa (PT) (3.000€)

2018: Awarded 2nd place for best oral presentation at the Ph.D. student seminar of the Department of Botany of University of South Bohemia, Czech Republic.

2018: Dora plus grant for visiting PhD students – University of Tartu (EE) (1.800€)

2017: Dora plus grant for visiting PhD students – University of Tartu (EE) (1.100€)

2013-2015: Exina Manuel Álvarez López grant– UCM

2013: Nomination for BS Extraordinary Award

2009-2013: Banco Popular grant for excellence

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