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Short-term drought and long-term climate legacy affect production of chemical defenses among plant ecotypes

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ABSTRACT

Long and short-term climatic variation affect the ability of plants to simultaneously cope with increasing abiotic stress and biotic interactions. Specifically, ecotypes adapted to different climatic conditions (i.e., long-term legacy) may have to adjust their allocation to chemical defenses against enemies under acute drought (i.e., short-term response). Although several studies have addressed drought effects on chemical defense production, little is known about their intraspecific variation along resource gradients. Studying intraspecific variation is important for understanding how different environments select for defense strategies and how these may be affected directly and indirectly by changing climatic conditions.

We conducted greenhouse experiments with the annual *Biscutella didyma* (Brassicaceae) to test the effects of long-term climatic legacy *versus* short-term drought stress on the concentrations of defense compounds (glucosinolates). To this aim, four ecotypes originating from a steep aridity gradient were exposed to contrasting water treatments. Concentrations of chemical defenses were measured separately in leaves of young (8 weeks) and old (14 weeks) plants, respectively. For young plants, ecotypes from the wettest climate (long-term legacy) as well as plants receiving high water treatments (short-term response) were better defended. A marginally significant interaction suggested that wetter ecotypes experienced a larger shift in defense production across water treatments. Older plants contained much lower glucosinolate concentrations and showed no differences between ecotypes and water treatments. Our results indicate that younger plants invest more resources into chemical defenses, possibly due to higher vulnerability to tissue loss compared to older plants. We propose that the strong response of wet ecotypes to water availability may be explained by a less pronounced adaptation to drought.

1. Introduction

Plant traits and strategies vary along environmental gradients as a result of abiotic and/or biotic filters that shape plant responses (Reich and Oleksyn, 2004; Johnson and Rasmann, 2011). How abiotic factors, such as water availability, affect traits fundamental to plant fitness and to the ability to cope with biotic stressors, is a key question in ecological research (Chaves et al., 2003; Halpern et al., 2010; Williams et al., 2015). In particular, increasing efforts have been devoted to studying how allocation of resources to plant defense strategies varies within species distributed along environmental gradients. One of the major assumptions regarding such allocations (Resource availability hypothesis, Coley et al., 1985; Sampedro et al., 2011) postulates that plants living in adverse environments, where replacement of tissues incurs high metabolic costs, will invest more into the production of secondary metabolites (Mailer and Cornish 1987; Haugen et al., 2008; Schreiner et al., 2009). Another prominent hypothesis (plant vigor hypothesis) suggests that bigger and thus more vigorous plants growing in productive environments are highly nutritious and thus preferred by herbivores (Price, 1991) compared to plants growing in low-resource environments (Bouchereau et al., 1996; Gutbrodt et al., 2011; Mewis et al., 2012). However, most empirical tests of these hypotheses were performed employing many plant species across geographic ranges that encompassed different habitats and underlying abiotic conditions. Although this allowed for addressing the role of macroevolutionary processes on plant defense strategies, selection of specific plant defense

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traits could not be unequivocally tested due to confounding effects with species identity. Accordingly, we do not yet know much about whether or not variation in plant defense is caused by long-term evolutionary processes or plastic responses or both, and whether long-term and short-term responses would be in the same direction.

Studies using intraspecific comparisons may uncover how variation in plant traits is constrained by abiotic and biotic factors (Woods et al., 2012; Pratt and Mooney 2013) that are often neglected in large-scale multispecies comparisons (Abdala-Roberts et al., 2016). Plant ecotypes that have evolved under different climates may have adapted their defense strategies to the local biotic and abiotic constraints according to one of the above hypotheses. Such strategies may result from the direct influence of climate on plant traits (i.e., long-term climatic legacy) or from indirect effects. For example, climate may select for different levels of herbivore abundance (Haddad et al., 2001; Pearse and Hipp 2012; Abdala-Roberts et al., 2016) to which plants will respond with increased or decreased defense production. In a recent review, Hahn and Maron (2016) illustrated how the well-known trade-off between growth and defense is not necessarily corroborated for intraspecific comparisons. In high resource environments, plants are exposed to higher herbivore load, but can allocate more resources to both growth and defense due to their larger "energy budget" (Pennings et al., 2009; Pellissier et al., 2014). Conversely, conspecifics constrained by limited resources invest little in both traits. Therefore, positive rather than negative trait correlations may be observed when comparing different populations that occur or have evolved in different environments (van Noordwijk and de Jong, 1986). This suggests that while trade-offs between growth and defense may still be observed, the allocation of resources between these traits within individuals may be highly context-dependent. Thus, the likelihood of observing trade-offs may depend on resource availability, species-specific traits, abiotic and biotic factors and their interaction.

Testing intraspecific trait variation along resource gradients also allows for separating ecotypic differentiation in response to short-term (i.e., sudden drought) from long-term (i.e., mean annual rainfall) climatic effects. For example, ecotypes from variable climates may respond differently to acute climatic variation (short-term) than ecotypes from stable climates (Penuelas et al., 2004; Carvajal et al., 2015). It has been suggested that plants from more variable and unpredictable environments should exhibit larger plasticity in response to environmental variation (Sultan, 1987; Pratt and Mooney, 2013). Acute climatic variation may increase fitness costs associated with herbivore damage either directly by causing a shift in resource allocation to growth (Kleine and Müller 2014; Kozlov et al., 2015) or indirectly through a shift in biotic or abiotic factors that are linked to chemical defense production (Leimu et al., 2012; Tariq et al., 2013). For example, increased glucosinolate production mediated by drought caused a shift in the strength of the interactions between different herbivore guilds (Tariq et al., 2013). Similarly, Gutbrodt et al. (2011) showed that under drought stress concentrations of nitrogen-containing compounds in leaves increase, therefore increasing food quality for herbivores. Indirect effects of short-term climatic variation can have an important role in plant response, but estimating shifts in herbivore and pathogen defense associated to drought can be challenging as the trade-off between growth and defense is context-dependent (Bode and Kessler, 2012). Furthermore, investment in defense against antagonists can vary across life stages and environments (Boege et al., 2007; Barton and Koricheva, 2010; Sampedro et al., 2011). Generally, younger plants are more vulnerable to herbivores and are expected to produce relatively more chemical defenses, however this varies considerably across taxa (Barton and Koricheva, 2010).

Glucosinolates are a class of chemical defenses, produced mainly by Brassicaceae, that are expressed constitutively and are also inducible (Halkier and Gershenzon, 2006; Hopkins et al., 2009). They have been well studied in an ecological context for their role in defense against herbivores and pathogens (Beekwilder et al., 2008; Bidart-Bouzat and Kliebenstein, 2008) and are thus well suited to address the above questions. Previous studies indicated that glucosinolate concentrations vary in response to acute drought (Milford and Evans 1991; Haugen et al., 2008), however no significant differences were found in plants exposed to a medium-term rainfall manipulation study (Metz et al., 2014).

We carried out two experiments to test how glucosinolate concentrations vary in response to i) long-term aridity, ii) short-term drought and iii) plant age. We predicted that short-term response to artificial drought would have similar effects on glucosinolate production as long-term climate conditions. Secondly, we predicted that plants inhabiting more variable climates would exhibit a larger plasticity in response to artificial drought. We tested these predictions, in two separate experiments, and we predicted that young plants would show a larger response to drought with respect to glucosinolate production than old plants.

2. Methods

2.1. Seed origins

For both our experiments, we used *Biscutella didyma* L. (Brassicaceae), a winter annual species widespread in the Eastern Mediterranean region (Feinbrun-Dothan, 1986). Seeds of 400 individuals in total were collected in 2012 from four sites along a gradient of decreasing rainfall and increasing climate variability in Israel. At each site, sampling was done over an area of approximately 1 ha and sampled individuals were at least 1 m distant from each other. The sites were characterized by equal aspect, slope, elevation and mean annual temperature, but differed in rainfall regime (Tielbörger et al., 2014). They represent mesic-Mediterranean (MM, 780 mm/year rainfall), Mediterranean (M, 540 mm/year rainfall), semi-arid (SA, 270 mm/year rainfall) and arid (A, 90 mm/year rainfall) conditions, respectively. Decreasing mean annual rainfall is coupled with increasingly variable and unpredictable rainfall patterns towards the drier sites (Holzapfel et al., 2006; Siewert and Tielbörger 2010). In order to remove maternal effects from field-collected seeds, plants were grown for one generation in a greenhouse where they received standardized conditions of natural light, regular watering, and temperatures ranging between approx. 15–18 °C (night) and 18–25 °C (day). These conditions had been successfully applied in previous studies and approximated field conditions (e.g. Metz et al., 2014). Our focal species is predominantly selfing, and wrapping individual plants in transparent, light fabric (organza) prevented cross-pollination in the greenhouse. The seeds produced by the first generation of plants in the greenhouse were used for both our experiments conducted in 2014 (experiment 1- older plants) and 2015 (experiment 2 − younger plants).

2.2. Experimental set up

In both experiments, we used seeds of 13 randomly selected individuals from each origin grown in the greenhouse. Plants were grown under greenhouse conditions similar to those experienced by the mother plants. In order to account for individual genetic differences, 15 seeds of each maternal sibship were planted in one pot per water treatment in both experiments. Following a standard protocol, pots (10 cm x 10 cm x 10 cm) were filled with a mixture of potting soil and sand (1:1) that had been previously enriched with a total of 100 mg Osmocote® slow release fertilizer (14-14-14NPK; Scotts Deutschland GmbH, Nordhorn, Germany). In both experiments, all pots were watered equally to saturation on the first and third day of the experiment to ensure seed germination. After germination was completed (approx. 10 days), seedlings in each pot were thinned to one. Water treatments started after seven days and subsequently pots were irrigated every 2-3 days, i.e., when plants of the lowest water level showed clear signs of water stress (Fig. 1). During the first experiment, conducted in 2014,

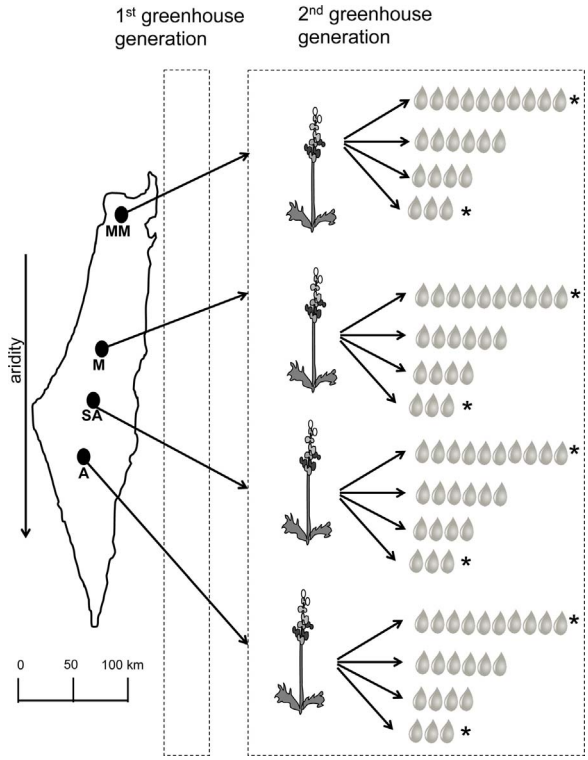

Fig 1. Scheme of the experimental set up. Seeds of the Brassicaceae *Biscutella didyma* were sampled along a steep aridity gradient (spanning 240 km) in Israel in 2012, and then grown for one year under controlled greenhouse conditions. Inbred seeds produced by this generation were exposed to different water treatments and leaves of adult plants were analyzed for glucosinolates. The first experiment (2014) involved four water treatments (15 ml, 20 ml, 30 ml, 50 ml) and leaf sampling occurred 14 weeks after germination. The second experiment (2015) involved two water treatments (30 ml, 90 ml) and leaves were sampled 8 weeks after germination. In the second experiment the difference between water treatments mimicked the difference between the lowest and the highest water treatments adopted in the first experiment (indicated by asterisks in the figure).

plants were exposed to four water treatments (50 ml, 30 ml, 20 ml, 15 ml per irrigation event) resulting in a total of 208 pots. Fourteen weeks after germination, one young leaf of each plant was sampled. Leaves were flash frozen in liquid nitrogen and subsequently analyzed for glucosinolate concentrations. At the time of leaf sampling, approximately one fifth (46/208) of the plants had started flowering. During the second experiment, conducted in 2015, two water treatments were applied (90 ml, 30 ml). This set up resulted in 104 pots. Leaf sampling was conducted 8 weeks after germination. In addition, plant biomass was harvested. This measure was not possible for the first experiment in 2014 as biomass was sampled after seed ripening for the purpose of another experiment and was thus not representative for the time of leaf sampling. The water volumes in the first experiment were based on successful protocols from preceding experiments on various aspects of drought effects conducted with the same target species (e.g. Lampei and Tielbörger 2010; Metz et al., 2015; Lampei et al., 2017). They covered the entire gradient from severe water stress to water saturated soil. For reasons of feasibility, we had to simplify the second experiment. Increasing the water volumes allowed us to reach very similar contrasts in soil moisture as in the first experiment (i.e. fully saturated soil *vs.* severe drought stress) by using fewer irrigation events. Therefore, the relative contrast between the highest and lowest water treatment was comparable, yet slightly smaller in the second experiment than in the first (factor 3.0 *vs.* factor 3.33).

2.3. Glucosinolate analyses

Glucosinolates were analyzed following the same procedure as described in Metz et al. (2014). After lyophilization, leaf samples were weighed and homogenized. Samples were extracted three times in 80% methanol, adding *p*-hydroxybenzyl glucosinolate (Phytoplan Diehm & Neuberger, Heidelberg, Germany) as an internal standard at the first extraction. Supernatants were applied on ion-exchange columns, washed and incubated with a sulfatase overnight. Desulfoglucosinolates were washed from the columns and analyzed by high performance liquid chromatography (1200 Series, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a reversed phase column (Supelcosil LC 18, 150 mm × 3 mm, 3 µm, Supelco, Bellefonte, PA, USA) and a diode array detector (for details see Metz et al., 2014). Desulfoglucosinolates were quantified by integrating the peaks at 229 nm, and relating them to the peak area of the internal standard, including response factors as described by Opitz et al. (2010) and relating them to the dry weight of the sample. Identification of compounds was done based on the characteristic UV spectra and retention times, comparison to standards (where available), and ultra-HPLC coupled with a time of flight mass spectrometer (1290 Infinity UHPLC and 6210 ToF-MS Agilent, Technologies, Santa Clara, CA, USA). We used the same conditions as those described in Kutyniok et al. (2014).

2.4. Statistical analyses

For both experiments, linear mixed models were applied using the concentrations of aliphatic, indole and total glucosinolates as separate response variables. Origin, water treatment and their interaction were used as fixed categorical variables, and identity of mother individuals was fitted as random factor. In order to meet model assumptions, the concentration of aliphatic and total glucosinolates was square root transformed and the concentration of indole glucosinolates was log transformed. Furthermore, plant biomass was included as a covariate in the analyses of experiment 2. This allowed for assessing the effect of plant size on glucosinolate production (in line with the plant vigor hypothesis) independently of potential effects of origin and water treatment (in line with the resource availability hypothesis). Post-hoc tests were then performed using Tukey HD tests.

In order to assess whether the relative proportion of individual glucosinolates varied across origins and water treatments, we performed Redundancy Analyses ((RDA), Legendre et al., 2011) in the R package 'vegan' (Oksanen et al., 2015). Redundancy analysis is a multivariate modeling and hypothesis-testing method that is used to explicitly explain ecological patterns in one set of variables (i.e. glucosinolate concentrations) as a function of another set of variables (i.e. water treatment and origin) (Legendre and Anderson 1999; Kenkel et al., 2002; Legendre and Legendre 2012). The data was standardized by sample and log transformed (Anderson et al., 2006). Significance of ANOVA models including origin, water treatment, and their interaction as explanatory factors were tested by permutation (Legendre and Anderson, 1999), which offers an unbiased test for evaluating the significance of the different axes generated by the RDA (Legendre et al., 2011). For graphical representation of the multivariate analysis we extrapolated scores of the first two RDA axes. We represented site scores (weighted average scores), which project the original data onto RDA axes, and linear combinations of environmental variables (linear combination scores), which project the fitted values of the multiple regressions (Legendre and Legendre, 1998). All analyses were carried out with the software R.3.1.0 (R Development Core Team, 2014).

3. Results

In each sample, aliphatic glucosinolates comprised approx. 98% of total glucosinolates and behaved very similarly to the total glucosinolates. Because aliphatic and indole glucosinolates can differ in their biological activity due to distinct hydrolysis products (Agerbirk and Olsen, 2012), results for both groups are presented separately (for results of total glucosinolates see Appendix A). In total, we found 10

Table 1
Results of linear mixed models (ANOVA table) on glucosinolate concentrations (total, aliphatic and indole glucosinolates) of leaves of 14-weeks old *Biscutella didyma* plants (Experiment 1) in response to origin, water treatments and their interaction, using identity of maternal individuals as a random factor. In this experiment, performed in 2014, plants from four origins were exposed to four different water treatments (15 ml, 20 ml, 30 ml, 50 ml).

Aliphatic glucosinolates	num DF	error DF	F-value	p-value
origin	3	45	0.855	0.470
water treatment	3	36	1.284	0.282
origin:water treatment	9	36	0.697	0.710
Indole glucosinolates	**num DF**	**error DF**	**F-value**	**p-value**
origin	3	50	0.113	0.952
water treatment	3	137	1.116	0.355
origin:water treatment	9	137	0.735	0.674
Total glucosinolates	**num DF**	**error DF**	**F-value**	**p-value**
origin	3	50	0.911	0.442
water treatment	3	137	1.298	0.277
origin:water treatment	9	137	0.685	0.722

Table 2
Results of linear mixed models (ANCOVA table) applied to glucosinolate concentrations (total, aliphatic and indole glucosinolates) of leaves of 8-weeks old *Biscutella didyma* plants (Experiment 2) in response to origin, water treatments and their interaction, and plant biomass, identity of maternal individuals was used as a random factor. In this experiment, plants from 4 different origins were exposed to two different levels of watering (30 ml and 90 ml).

Aliphatic glucosinolates	num DF	error DF	F-value	p-value
origin	3	47	3.932	0.014
water treatment	1	44	15.658	0.0003
biomass	1	44	16.33	**0.0002**
origin:water treatment	3	44	2.658	**0.059**
Indole glucosinolates	**num DF**	**error DF**	**F-value**	**p-value**
origin	3	47	17.635	< **0.0001**
water treatment	1	44	26.495	< **0.0001**
biomass	1	44	0.063	0.803
origin:water treatment	3	44	1.602	0.202
Total glucosinolates	**num DF**	**error DF**	**F-value**	**p-value**
origin	3	47	4.292	**0.009**
water treatment	1	44	15.675	**0.0003**
biomass	1	44	16.239	**0.0002**
origin:water treatment	3	44	2.610	0.063

glucosinolates; 7-methylsulfinylheptyl glucosinolate (7MSOH), 8-methylsulfinyl-octyl glucosinolate (8MSOO), 8-methylthiooctyl glucosinolate (8MTO), two indole glucosinolates [indol-3-ylmethyl glucosinolate (I3 M) and 4-methoxy-indol-3- ylmethyl glucosinolate (4MOI3 M), and five minor unidentified glucosinolates.

3.1. Experiment 1 – year 2014 (older plants)

We did not find any significant difference in absolute leaf concentrations of glucosinolates between *B. didyma* plants from different origins or water treatments. This result was consistent among glucosinolate groups (Table 1, Fig. 2A and 3A, Appendix A Fig. A1A). Origin, water treatments and their interaction explained 4.5% of the variation in composition of the glucosinolates (df = 15, F = 1.63, p = 0.001). Origins were strongly correlated with RDA axis 1, and water treatments was strongly correlated with RDA axis 2 (Appendix B Fig. A2). ANOVA showed that glucosinolate composition was significantly different across origins (2.6% explained variation, df = 3, F = 2.74, p = 0.001) after correcting for any effect of water treatment and interactions. However, water treatments (df = 3, F = 1.24, p = 0.21) and the origin x water treatment interaction did not affect glucosinolate composition (df = 9, F = 0.91, p = 0.66) after correcting for origin.

3.2. Experiment 2 – year 2015 (younger plants)

The highest glucosinolate concentrations (total, aliphatic and indole) were found in plants from the wettest (MM) origin and in plants exposed to high water treatments (Table 2, Figs. 2B and 3B , Appendix A Fig. A1B). A marginally significant (p = 0.059) origin x water treatment interaction (Table 2, Fig. 2B) indicated a larger effect of water treatment on aliphatic and total glucosinolate concentrations in plants from MM origins (Fig. 2B, Appendix A, Fig. A1B). We found a positive significant correlation between total plant biomass and aliphatic glucosinolates, as well as total glucosinolates (Table 2). Overall, glucosinolate concentrations were approximately 3–6 times higher in younger (experiment 2) than in older (experiment 1) plants (Figs. 2 and 3, Appendix A Fig. A1).

Origin, water treatments and their interaction explained 14.2% of the variation in glucosinolate composition (df = 7, F = 3.34, p = 0.001). Origins were strongly correlated with RDA axis 1, and water treatment was strongly correlated with RDA axis 2 (Fig. 4). ANOVA results indicated that glucosinolate composition was significantly different across origins (df = 3, F = 5.92, p = 0.001), and water treatments (df = 1, F = 3.12, p = 0.009) after correcting for other factors in the model. The interaction origin x water treatment did not affect glucosinolate composition (df = 3, F = 0.84, p = 0.63).

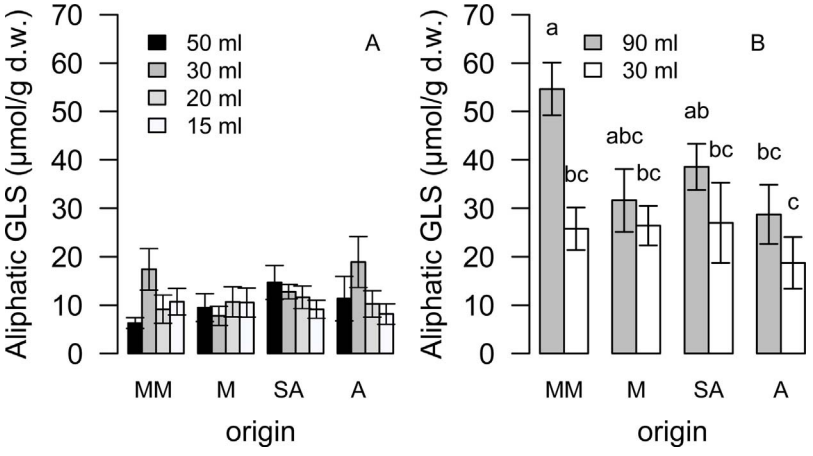

Fig. 2. Mean concentrations (± standard error; N = 13 per treatment) of aliphatic glucosinolates (GLS) of leaves of *Biscutella didyma* plants from different origins (MM = mesic Mediterranean, M = Mediterranean, SA = semi-arid, A = arid) exposed to different water treatments A) for experiment 1 (14-weeks old plants) which used four water treatments (no significant differences were found across origins and water treatments) and B) for experiment 2 (8-weeks old plants) which used two water treatments.

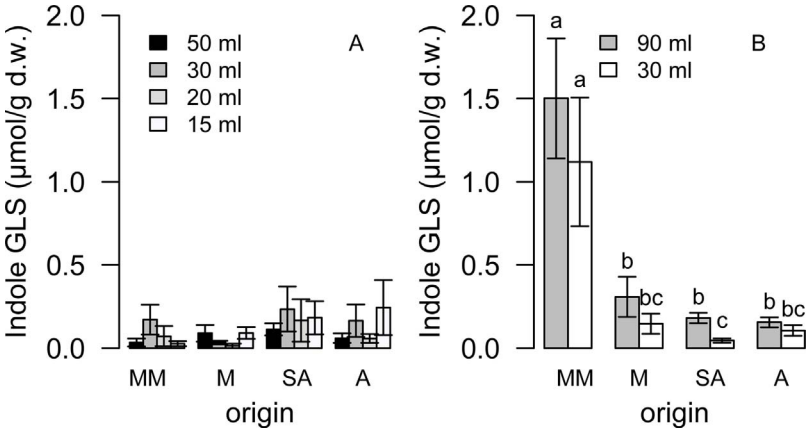

Fig. 3. Mean concentrations (± standard error; N = 13 per treatment) of indole glucosinolates (GLS) of leaves of *Biscutella didyma* plants from different origins (MM = mesic Mediterranean, M = Mediterranean, SA = semi-arid, A = arid) and water treatments A) for experiment 1 (14-weeks old plants) which used four water treatments, in this first experiment we did not find significant differences across origins and water treatments and B) for the experiment 2 (8-weeks old plants), which used two water treatments.

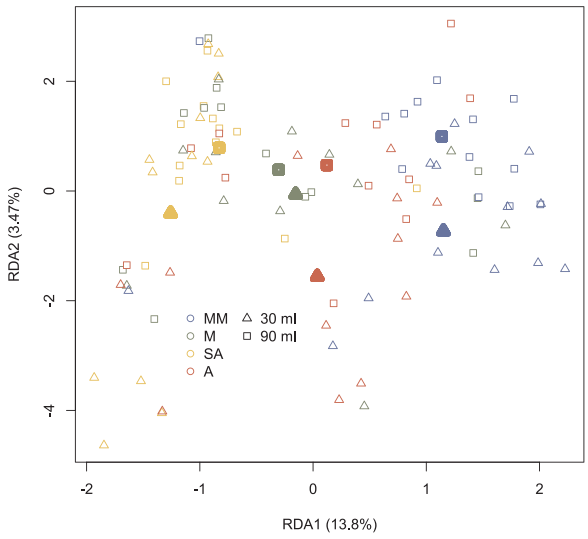

Fig. 4. RDA projection of glucosinolates concentrations in leaves of *Biscutella didyma* plants from different origins (MM, M, SA, A) and water treatments (30 ml, 90 ml) in experiment 2 (8-weeks old plants). Centroids with heavier outline represent the linear combination scores of the environmental variables (i.e. origin and water treatment). Symbols with thin outline represent weighted average scores (i.e. a projection of raw data on RDA axis). Different symbols correspond to water treatments, and different colors are associated to each origin.

Biomass production (Appendix C Table A1, Fig. A3) did not differ significantly across origins, however, high water treatments led to significantly higher biomass. A marginally significant interaction indicated that such increase was larger for plants originating from the most arid site (A).

4. Discussion

Our overall findings suggest strong ecotypic differentiation in the amount and type of chemical defenses produced by plants from different origins. Namely, ecotypes from the wettest origin produced higher concentrations of glucosinolates compared to ecotypes from drier origins, at least in leaves of young plants, indicating long-term adaptation to different climatic conditions. In addition, we found that young plants grown under wet conditions produced higher glucosinolate concentrations in leaves than plants exposed to drought. Adaptation to long-term climate and response to short-term drought thus followed the same direction. Younger plants had higher leaf defense concentrations and responded more strongly to water treatments than older plants. Because significant differences across origins and water treatments were observed only for younger plants, in the following we will focus on these findings.

The higher glucosinolate production in leaves of plants from wetter origins suggested an adaptation to long-term climate at the origin sites. This result is in line with a previous greenhouse study, where ten-week old plants grown from field-collected seeds of our target species, *B. didyma,* showed higher leaf glucosinolate concentrations towards wetter origins (Metz et al., 2014). The consistent results across both studies indicate that differences in glucosinolate concentrations among origins (i.e., long-term climatic legacy) persisted for at least two generations and were not resulting from maternal effects. We can also rule out an interaction between genotype and the environment (namely water treatment), as our experiment was genetically controlled, with seeds produced by each mother plant being exposed to all water treatments (Fig. 1). Patterns of chemical defenses among origins appear thus driven by a strong genetic component (Arany et al., 2009).

Interestingly, we found consistency between the response of plants to long-term climatic legacy and short-term drought, suggesting that water availability may be correlated with glucosinolate production. Results of both long and short-term treatments are thus consistent with previous literature (Pennings et al., 2009; Pellissier et al., 2014; Wang et al., 2016), suggesting that larger and more vigorous plants may attract more herbivores, and due to high resource availability, may increase both biomass production and investment in chemical defense (Noordwijk and Jong, 1986; Hahn and Maron, 2016). Variation in defense production may be explained in two non-mutually exclusive ways. Under natural field conditions, plants from the wettest origin (MM) generally grow bigger than those from arid (A) origins (Petrů et al., 2006), and they may also invest more in glucosinolate production as a result of their larger size. Although, we did record larger biomass for plants exposed to high water treatment, we did not find differences across origins. Alternatively, plants from MM origins may produce more glucosinolates due to higher herbivore or pathogen load in the field. A parallel study conducted along several gradients in the region indicated a trend towards increasing herbivore damage in Brassicaceae (including our study species) in wetter sites (Gibson-Forty, pers. comm.), a similar trend was observed in the tropics (Brenes-Arguedas et al., 2009). The higher production of glucosinolates in plants from wetter origins may thus be due to an adaptation to larger herbivore and/or pathogen pressure. It is possible that adaptations to antagonists drive differences in defense production across origins (long-term), whereas short-term variation in resources may drive changes in biomass and defense production.

A weak interaction between origin and water treatment indicated that the response of aliphatic glucosinolates to short-term drought was stronger for the wettest ecotypes (MM origin). This partly contradicts a study, conducted on the same ecotypes of *B. didyma*, that suggested lower plasticity of MM ecotypes in various morphological traits (Petrů et al., 2006). Although we need to be cautious in drawing conclusions from this marginally significant result, a potential explanation may be the different adaptation to drought among ecotypes. As ecotypes have

evolved in and adapted to different degrees of intra-annual rainfall variation, ecotypes from drier origins have become better adapted to sudden droughts than ecotypes from wetter origins. Therefore, the same absolute level of drought stress may be perceived differently among ecotypes and thus trigger different responses in terms of defense production (Orrock et al., 2015). Namely, our low water treatment may represent a much stronger cue for MM ecotypes adapted to high water availability and climate stability than for drier ecotypes adapted to large rainfall fluctuations.

The leaf concentration of glucosinolates in younger plants harvested after 8 weeks was approx. 3–6 times higher compared to that of older plants harvested after 14 weeks. This result supports previous findings indicating higher investment in chemical defense in younger plants (Brown et al., 2003; Boege and Marquis, 2005; Velasco et al., 2007). This also indicates that young plants may be more plastic than older plants in their response to drought, due to the larger risk associated with herbivore attack during the early life stages as opposed to mature plants (Coley and Barone 1996; Ochoa-López et al., 2015; Wiggins et al., 2016). Our multivariate analyses indicated a strong origin-specific signature in glucosinolate composition in both younger and older plants. However, there was a larger amount of unexplained variation in the multivariate analyses of older plant leaf glucosinolate composition, suggesting that other factors than origin and water treatments may affect the relative abundance of different types of glucosinolates. This difference in glucosinolate profiles across ontogenetic stages suggest that not only the quantitative, but also the qualitative profile of plant defenses changes (Petersen et al., 2002; Cook et al., 2016). Besides indicating potentially varying levels of plasticity across a plant's life cycle, our findings point to the importance of taking into consideration plant life stages in studies of local adaptation and ecotypic differentiation in general. Although our two experiments were conducted under identical greenhouse conditions and used the same seed material, they were not carried out in parallel. Thus, we cannot exclude that other factors than plant age affected the outcome of the experiment and possibly confounded our results. Nevertheless, the difference among experiments was not only expressed in absolute glucosinolate concentrations but also in the magnitude of response to the drought treatments.

5. Conclusions

Our study adds to a still very limited body of research addressing intraspecific variation in secondary metabolites in response to resource availability, and provides sound data on long-term (evolutionary) and short-term (plastic) responses in defense (Hahn and Maron 2016; Smilanich et al., 2016). Our study, together with previous research, suggests that multiple factors have contributed to evolving contrasting strategies among different origins along steep rainfall gradients. Therefore, different ecotypes may respond differently to the same cue due to their different adaptations. Disentangling the effects of these factors while accounting for adaptations of each ecotype may allow us to assess how plant defense strategies might evolve under climate change.

Statement of authorship

KT, CM, JM planned the experimental set up, LH, JM and ST performed the experiments and collected the data, CM and KD performed the glucosinolate analyses, CM evaluated the glucosinolate data, ST and CB analyzed the data statistically, ST wrote the first draft. All authors contributed to revisions.

Declaration of interest

All authors declare that they do not have any conflict of interest.

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Appendix A

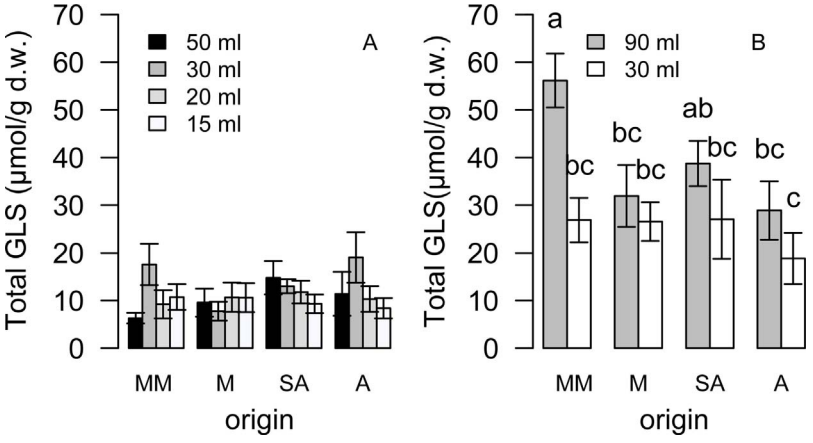

Fig. A1. Mean concentrations (± standard errors; N = 13 per treatment) of total glucosinolates (GLS) of leaves of *Biscutella didyma* from different origins (MM = mesic Mediterranean, M = Mediterranean, SA = semi-arid, A = arid) exposed to water treatments A) for experiment 1 (14-weeks old plants) which used four water treatments (no significant differences were found between origins and water treatments), and B) for experiment 2 (8-weeks old plants) which used two water treatments.

Appendix B

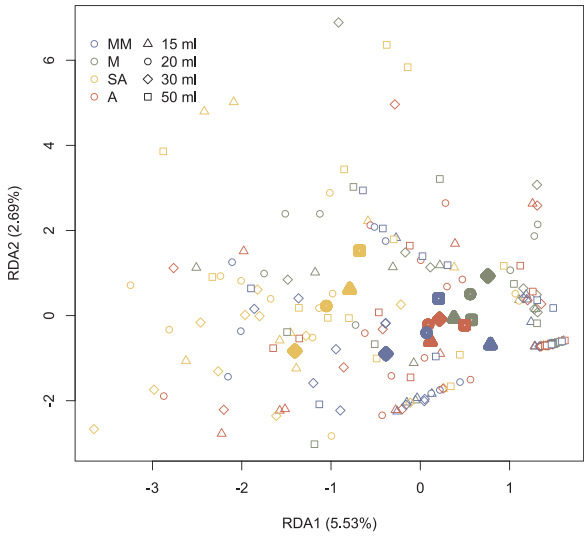

Fig. A2. Fig. 3: RDA projection of glucosinolate concentrations in leaves of *Biscutella didyma* plants from different origins (MM, M, SA, A) and water treatments (15 ml, 20 ml, 30 ml, 50 ml) in experiment 1 (14-weeks old plants). Centroids with heavier outline represent the linear combination scores of the environmental variables (i.e. origin and water treatment). Symbols with thin outline represent weighted average scores (i.e. a projection of raw data on RDA axis). Different symbols correspond to water treatments, and different colors are associated to each origin.

Appendix C

Table A1
Results of linear mixed models (ANOVA table) applied to total biomass of 8-weeks old *Biscutella didyma* plants in response to origin, water treatments and their interaction.

Effect	num DF	error DF	F-value	p-value
origin	3	47	2.008	0.1256
water treatment	3	45	21.376	< **0.0001**
origin: water treatment	3	45	2.678	**0.0583**

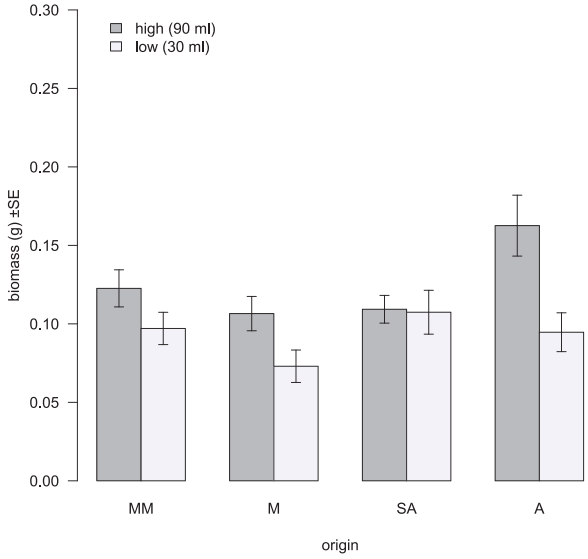

Fig. A3. Mean total biomass (± standard error; N = 13 per treatment) produced by *Biscutella didyma* plants from different origins (MM = mesic Mediterranean, M = Mediterranean, SA = semi-arid, A = arid) and exposed to two water treatments in experiment 2 (8 week old plants).

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