Historical comparisons show evolutionary changes in drought responses in European plant species after two decades of climate change

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Abstract

Plants must continuously respond to environmental changes, and a timely question is whether and how populations respond to ongoing global warming and increased drought frequencies and intensities. Plants can either respond through migration or through phenotypic plasticity or their populations can adapt evolutionarily, which encompasses the evolution of trait means and of trait plasticity. One way to detect such evolutionary changes within plant populations is through historical comparisons where plants grown from seeds collected in the past ("ancestors") are compared to freshly collected seeds from the same populations ("descendants") in common garden experiments. We used 21- to 26-year-old seeds stored in seed banks for two multi-species experiments that investigated changes in phenotypic traits and their plasticity conferring drought tolerance in early life stages of European plant species. In the first experiment, we used seedlings of four Mediterranean species, ceased watering and recorded their day of mortality. In the second experiment, we studied phenotypic responses to drought in juvenile plants of nine species originating from temperate regions in Europe. In one of four species in the first experiment, descendants survived significantly longer without watering and were smaller than their ancestors. In the second experiment, descendant plants were generally taller under well-watered conditions but smaller under drought than their ancestors, thus showing stronger plasticity. Our historical comparisons suggest that some populations have likely evolved through changes in trait means and plasticity in ways consistent with adaptation to increased drought. Using seed bank material for historical comparisons has several weaknesses, such as unknown sampling protocols or invisible fractions. However, we show how accurately sampled and stored seed

bank collections can be used similar to the resurrection approach for investigating rapid evolutionary processes in early life stages of plants under climate change.

Introduction

Climate change has increased dramatically over the last several decades (IPCC, 2018), and plant populations are already responding (Peñuelas & Filella, 2001; Parmesan & Yohe, 2003). Projections for Europe forecast that, during the 21st century, annual precipitation sums will further increase in the north and decrease in the south (IPCC, 2013). For central and western Europe, precipitation is expected to increase in the winter and decrease in the summer (IPCC, 2013), leading to more droughts in the growing season. Moreover, the higher temperatures will lead to higher evapotranspiration (Feng & Fu, 2013). These changes in environmental conditions will likely increase the frequency, duration and geographic extent of drought events in Southern and Central Europe (Ruosteenoja et al., 2018; Samaniego et al., 2018; Spinoni et al., 2018).

Changes in water availability and more frequent droughts are strong stressors for plants (Jaleel et al., 2009; Fleta-Soriano & Munné-Bosch, 2016), and many plant populations may not be adapted to these novel conditions (Anderson et al., 2012; Shaw & Etterson, 2012). To avoid extinction, some plant populations may migrate to track suitable conditions whereas others may respond through phenotypic plasticity or adaptive evolution (Holt, 1990; Hoffmann & Sgrò, 2011). Such evolutionary adjustments could be the result of selection of better adapted phenotypes and might involve reduced growth, reduced evapotranspiration (Kusaka et al., 2005, Borrell et al., 2014), or increased root-shoot ratio (Sharp & LeNoble, 2002; Aroca, 2012) to promote water uptake. In environments that become generally drier, constitutive changes in such traits may be adaptive. However, drought events are often periodic, which would render the ability to change functional trait values through phenotypic plasticity a better strategy than to evolve constitutive changes in mean traits (Sultan & Spencer, 2002; Alpert & Simms, 2002; Gianoli & Valladares, 2012), especially in environments with strong climatic variability (Scheepens et al., 2018). Still, studies on the effects of climate change on plant populations often only consider changes in mean climate conditions (Bertrand et al., 2011), despite the strong evidence for increased climatic variability both among and within years (IPCC, 2013; Gherardi & Sala, 2019), specifically more heavy rain events followed by longer dry periods in many regions (Kharin et al., 2007).

Phenotypic plasticity itself can also evolve and is thought to be selected for particularly in spatially or temporally variable environments (Ackerly et al., 2000; Richards et al., 2006). For example, Lázaro-Nogal et al., (2015) showed in a common garden study with *Senna candolleana* that populations from environments with stronger interannual precipitation variation had a higher plasticity in growth traits. A similar observation was made by Gianoli and González-Teuber, 2005 who showed that plasticity in leaf area, leaf shape, leaf area ratio, and foliar trichome density in *Convolvulus chilensis* was highest for plants from the population with the highest interannual variation in precipitation. Thus, increased climatic variability appears to be associated with systematic, and presumably adaptive, changes in phenotypic plasticity of plants. The fate of plant populations will thus depend on their ability to adapt to altered climatic variability and increased drought intensities through evolution of plasticity and/or constitutive adaptation to drought.

A powerful method to test for recent evolution − whether in trait means or in their plasticity − is to compare ancestors with their descendants by using stored propagules such as seeds (Franks et al., 2007; Orsini et al., 2013; Merilä & Hendry, 2014; Franks et al., 2018). If ancestors can be revived, the resulting plants can be compared to contemporary individuals raised from propagules sampled from the same population. Growing ancestors and descendants together under common conditions then allows for direct tests for heritable trait differentiation among temporally separated populations (Franks et al., 2007, 2008). Understanding how populations and species responded evolutionarily in the past is extremely valuable for making predictions for future population and species responses to environmental change (Orsini et al., 2013; Franks et al., 2018).

An increasing number of studies have used this "resurrection approach" to examine rapid evolution to increased drought. Some of these studies convincingly showed that plants adapted their phenology towards an earlier flowering in order to avoid drought (Franks et al., 2007; Nevo et al., 2012; Vigouroux et al., 2011; Thomann et al., 2015). For growth traits, results appear to be more species-specific. For example, in an experiment with *Mimulus laciniatus* by Dickman (2016) the descendants were better adapted to drought and grew larger, whereas Vigouroux et al., (2011) found the opposite results in a study with *Pennisetum glaucum* where descendant populations that experienced drier climates

during 27 years grew smaller. Thus, although species may vary in their evolutionary responses to drought, some traits show consistent evolution across species. Multi-species resurrection experiments can elucidate such commonalities, and therefore improve our ability to forecast future evolution under climate change. Nevertheless, such studies are still rare because they require seed collections specifically compiled for this purpose (Franks et al., 2018). In order to use untapped resources for environmental change research, seed collections stored in seed banks can be used in a similar way as resurrection studies. To conduct such historical comparisons, the amount of stored seeds should be high and information on the sampling locality, the number of collected individuals and the genetic diversity should be available.

Here, we used seed material from seed banks in historical comparisons to investigate whether single populations of multiple plant species from Mediterranean and temperate regions of Europe have evolved their drought tolerance over the last decades in response to more frequent and longer drought events (Met Office, 2011; DWD, 2018; IRM, 2020). To investigate this, we conducted two complementary common garden experiments in which we applied drought treatments to plants raised from seeds stored for at least 21 years in three different seed banks (ancestors) and from seeds that we collected from the same populations in 2018 (descendants). Seedling establishment is a key process for population survival (Grubb, 1977), and seedlings are especially susceptible to drought (Moles & Westoby, 2004). Therefore, the drought resistance of seedlings should be under high selection pressure in increasingly dry and more variable environments (Schupp, 1995; Fenner & Kitajima, 1999). In 2019, Dickman and colleagues already published a resurrection study with *Mimulus laciniatus* showing that contemporary populations, which experienced droughts during the last years, germinated earlier (Dickman et al., 2019). However, studies on evolution of drought resistance in early life stages are generally still scarce and multi-species experiments using watering treatments are missing. This is why we examined differences between ancestors and descendants in their responses to drought treatments at early life stages for multiple species.

As seedlings are generally very sensitive to dehydration, especially in environments with large fluctuations in water availability and a high probability of drought events (Padilla & Pugnaire, 2007), we expect evolutionary change in drought tolerance when drought regimes change. We used four herbaceous Mediterranean species in the first experiment to test whether seedlings of the descendants survived longer without watering than the seedlings of their ancestors ("seedling survival experiment"). In the second experiment ("watering response experiment") we worked with juvenile (i.e. establishing, non-flowering) plants from nine temperate European species which experience the lowest precipitation during early growth between April and June (data from the Climatic Research Unit; Camarillo-Naranjo et al., 2019; Harris et al., 2020). We subjected ancestors and descendants

to well-watered vs. dry conditions and compared their growth responses within the first weeks after germination to test the hypothesis that populations evolved phenotypic traits, and/or their plasticities, to cope with increased droughts.

Materials and methods

Seed collection

For the seedling survival experiment, we obtained seeds of four species - *Anthemis maritima* L., *Matthiola tricuspidata* (L.) R.Br., *Medicago marina* L. and *Plantago subulata* L. - from the seed bank at the Conservatoire Botanique National Méditerranéen de Porquerolles (CBNMed, Hyères, France). For the watering response experiment investigating juvenile plants, the seed material of eight species - *Centaurium erythraea* Rafn., *Clinopodium vulgare* L., *Digitalis lutea* L., *Leontodon hispidus* L., *Melica ciliata* L., *Pimpinella saxifraga* L., *Sedum album* L. and *Teucrium chamaedrys* L. - was provided by the seed bank of Meise Botanic Garden (Belgium) and of one species - *Dianthus carthusianorum* L. - by the Botanical Garden of the University of Osnabrück (Germany). For both experiments, we only used seeds which had precise sampling dates and location records, which occurred in nature protection areas, and which had been stored for at least 21 years. We selected species with a short life cycle as they were expected to respond more quickly to selection and were therefore more likely to show rapid evolution. We confirmed under greenhouse conditions that all chosen species started to reproduce at least in the second year of growth (see Appendix A: Table 1). To reduce the chance that the sampled populations were strongly influenced by gene flow from other populations, we specifically chose seed material from populations of origin that were relatively isolated (but sufficiently large).

Franks and colleagues recommend the following criteria for seed sampling in resurrections studies: at least two time points for sampling, each time collecting >30 plants on each sampling occasion while keeping maternal lines separated (Franks et al., 2018). These criteria safeguard that genetic diversity within a population is captured sufficiently and that the original genetic structure is kept largely intact. As the materials from the seed banks were not originally collected with the aim to conduct resurrection experiments (e.g. the number of sampled individuals is often unknown, and all sampled seeds were bulked) our study does not fulfil these strict criteria. However, with two types of further information, we are convinced that seed bank material can be used in a similar way to the resurrection approach, and that historical comparisons based on it are meaningful.

The first type of evidence is information on sampling. All species occur rather abundantly in their original habitat, the amount of seeds within the stored lots was high (see Appendix A: Table 1) and the collectors tried to maximize the

number of sampled individuals. Thus, we are confident that the genetic diversity of seed bank collections we used is representative of what was present at the time of sampling.

The second important information comes from a molecular analysis using ddRAD-SNP marker data for all species. We assessed pairwise genomic relatedness among samples within ancestral and descendant populations using two estimators, genomic relatedness G (Yang et al., 2010) and the kinship estimator r^{β} (Goudet et al., 2018), which were applied to ancestral and descendant individuals in one population. In addition, we assessed genomic diversity within ancestral and descendant populations as allelic richness, Ar (El Mousadik & Petit, 1996). We show that the relatedness of plants is similar within ancestors and descendants for 12 out of 13 species, providing further support for similar sampling procedures and that sufficient seeds were sampled during both periods, avoiding biased sampling of particular mother plants. Furthermore, allelic richness was similar for 10 out of 13 species, indicating low influence of bottlenecks or gene flow (see Appendix A: Fig. 2 , Durka et al., unpublished data).

For the seedling survival experiment, we used seeds of four Mediterranean species: *A. maritima, M. tricuspidata, M. marina* and *P. subulata*. The seeds of these four species had been collected in the same area close to Hyères, Southern France, between 1992 and 1997. Data from the Climatic Research Unit (Camarillo-Naranjo et al., 2019, Harris et al., 2020) show that average temperatures between March and July have increased and precipitation decreased during the last 30 years in comparison to the long-term means from 1900-1999. Combining both environmental variables, we calculated the "De Martonne aridity index" (IDM, Pellicone et al 2019), which demonstrated soaring drought during the last three decades ($IDM_{1988-2018}$ = 21.3) in comparison to the long-term mean ($IDM_{1900-1999}$ = 25.5). In addition, precipitation variability (CV) during 2009-2018 was 35% larger than during 1988-1997 (Camarillo-Naranjo et al., 2019, Harris et al., 2020). After the collection of ancestor seeds, the seeds of *A. maritima, M. tricuspidata* and *M. marina* were cleaned, dried and stored at 5°C, whereas seeds of *P. subulata* were ultra-desiccated and stored at 17°C at the CBNMed until November 2018.

For the watering response experiment, we used seeds of nine temperate species: *C. erythraea, C. vulgare, D. carthusianorum, D. lutea, L. hispidus, M. ciliata, P. saxifraga, S. album* and *T. chamaedrys*. The seeds of these nine species had been collected between 1992 and 1995 in Belgium (two different regions) and close to Osnabrück (Germany). Comparing the last 30 years with 1900-1999, the average temperatures between March and July have also increased and precipitation has decreased, which led to lower values of the "De Martonne aridity index" indicating a drier environment in all three regions. For *D. carthusianorum,* close to Osnabrück we calculated $IDM_{1988-2018}$ = 31.4 compared to $IDM_{1900-1999}$ 34, for *L. hispidus* $IDM_{1988-2018}$ = 37.2 compared to $IDM_{1900-1999}$ = 40, and for the remaining species in Belgium $D_{1988-2018}$ = 36.1 compared to $IDM_{1900-1999}$ = 37.4. In addition, precipitation variability (CV) during 2009-2018 compared to 1988-1997 was decreased by 45% for *D. carthusianorum*, did not change for *L. hispidus* and increased by 25% for the remaining species in Belgium. After ancestor seed collection, all seeds had been cleaned, dried at 15% relative humidity and then stored at -20°C at Meise Botanic Garden and the Botanical Garden of the University of Osnabrück until we received the seed materials in November 2018.

To obtain the descendants, seeds of all species were collected from the same populations in spring (Mediterranean species) and summer (temperate species) of 2018. To be sure about the resampling of the same population, the exact location of all populations was identified either by precise coordinates of the target population, or by relocation of the same collector as 30 years ago. In each population, we aimed to sample at least 30 individuals with different height and life stage to account for temporal variation in fruit ripening. The realized sample size ranged from 15 to 103 (see Appendix A: Table 1) with only 15 individuals sampled from *M. tricuspidata* and 21 individuals from *M. ciliata* because population size of these species was only 30 and 25 individuals, respectively. We then bulked all seeds to have a comparable seed mix as for the ancestors.

Seedling survival experiment

For the seedling survival experiment with the Mediterranean plants, we initially sowed 100 seeds per temporal origin (i.e. ancestors and descendants) of each species. To break physical seed dormancy of *M. marina*, we scarified the seeds of this species by softly scrubbing them with sandpaper (Royal Botanic Gardens Kew, 2020), and to reduce the growth of microbes during germination, we surface-sterilized all seeds for ten minutes with 3% sodium hypochlorite (NaOCl) and two drops of Tween20 per 200 ml solution, and washed them three times with sterilized water. We germinated all seeds on 1% water agar in 90 mm Petri dishes. After one week of cold-dark stratification at 5°C we transferred the Petri dishes to a walk-in growth chamber (light intensity = 230 μmol•m^{-2}•s^{-1}, 50% relative humidity) with a light/dark cycle of 8/16 hours and temperatures of 23/18°C and recorded germination success every second day. The germination rates were similar for ancestor and descendant seeds of *A. maritima* and *M. tricuspidata* but they differed for *M. marina* and *P. subulata* (see Appendix A: Table 1).

For each species we filled one seedling tray (96-cell QuickPot®, 3.8 × 3.8 cm cells) with a standard peat-free potting soil (Einheitserde®, BioLine, Topfsubstrat Öko torf-frei) and planted seedlings (see Appendix A: Table 1 for the numbers of individuals) into every other cell so that the seedlings did not grow directly next to each other. We planted the ancestors and descendants in an alternating

pattern. To identify the seedlings, we noted their positions but did not use any labels in order to reduce observer bias. The trays were placed in a walk-in growth chamber with a light/dark cycle of 12/12 hours and 23/18°C (light intensity = 230 μmol•m^{-2}•s^{-1}, 50% relative humidity). The seedlings were watered regularly for 2-3 weeks (depending on the species) to allow their establishment. After that, we stopped watering to simulate drought. We recorded mortality due to desiccation at least every second day. A seedling was scored as dead when it was completely dry and all leaves had lost their green colour. We cut each dead seedling 1 mm above ground, dried it at 60°C for 72 h, and weighed it.

Watering response experiment

For the watering response experiment with temperate plant species, we germinated 100-1000 seeds per temporal origin (see Appendix A: Table 1 for precise numbers) in trays filled with a standard potting soil (Einheitserde®, BioLine, Pikiersubstrat), with a separate tray for each origin. To break seed dormancy, we kept the soil moist and cold-dark stratified the seeds at 5°C for two months. In March 2019, we transferred the trays to the greenhouse and allowed the seeds to germinate at 20°C under a natural spring daylight regime. We kept the seedlings in these trays for three months before the start of the experiment

For the main experiment, we filled 9 × 9 cm pots with a 3:1 mixture of peat-free potting soil (Einheitserde®, BioL-ine, Topfsubstrat Öko torffrei) and sand (0-2 mm play sand, WECO GmbH). In early June 2019, we transplanted each seedling into its own pot while making sure that we transplanted pairs of ancestors and descendants that were approximately of equal size. Right after transplantation, we measured shoot length or rosette diameter (henceforth referred to as plant size) as well as, depending on the species, the number of leaves or shoots (see Appendix A: Table 1). After two weeks, we split all juvenile plants into a well-watered control group and a drought group, with 7-12 replicates per temporal origin and species (see Appendix A: Table 1). When five of the pots of a species had a dry soil surface, all plants of that species were watered, with control plants receiving 60 mL and drought plants receiving 30 mL water at each watering. We re-randomized all pots in the greenhouse weekly. After eight weeks we repeated the growth trait measurements and then harvested all plants and determined their aboveground biomass after oven-drying at 60°C for three days.

Statistical analyses

In both experiments, we used linear models to examine differences between the temporal origins (ancestors vs. descendants). We analysed data from the seedling survival experiment with models testing for effects on the number of days of survival (i.e. time between start of the drought treatment and death) and the aboveground biomass as response variable. In both models, we included species identity and its interaction with temporal origin as an additional explanatory variable to account for species differences. For the analysis of number of days of survival we also included the number of days between transplanting and start of the experiment as a covariate, and for the analyses of aboveground biomass we included the total lifespan of the seedling as a covariate. In addition to these multi-species models we also analysed the data separately for each species, using the same models but excluding species identity. Finally, we used linear models to test whether the aboveground biomass of a plant predicted its number of days of survival, while correcting for the total lifespan of the seedling by including it as a covariate.

We analysed the data from the watering response experiment with juvenile plants with models testing for effects of the drought treatment on plant size, number of leaves of shoots, and aboveground biomass. We first square-root-transformed the number of leaves and aboveground biomass to normalise model residuals. In order to be able to compare different measurements across the nine species, we standardised all data per trait to a mean of 0 and a standard deviation of 1. We then analysed the variation in plant size, number of leaves or shoots, and aboveground biomass with linear models that included temporal origin (ancestors vs. descendants), treatment (drought vs. control) and species, and all possible interactions, as explanatory variables. A two-way interaction between temporal origin and treatment would suggest that plants had evolved a different response to drought, and a three-way interaction between temporal origin, treatment and species would suggest that species vary in their evolutionary responses to drought. In addition to the multi-species analyses, we also analysed the data for each species separately, using linear models that included only temporal origin, treatment and their interaction. As the sizes of the transplanted seedlings differed, we corrected for this by including the initial size measurements as a covariate in all our models.

For all models, we visually checked the residuals for normality and heteroscedasticity. All analyses were done in R (Version 4.0.2) using the package *plyr* for data management (Wickham, 2011) and the *lm()* function to run linear models. Data is accessible as a supplement in Tables S2 and S3.

Results

Seedling survival experiment

Across species, seedlings from descendants survived on average almost two days longer than seedlings from ancestors (Fig. 1A, $F_{1,208}$ = 12.99, p < 0.001). The studied
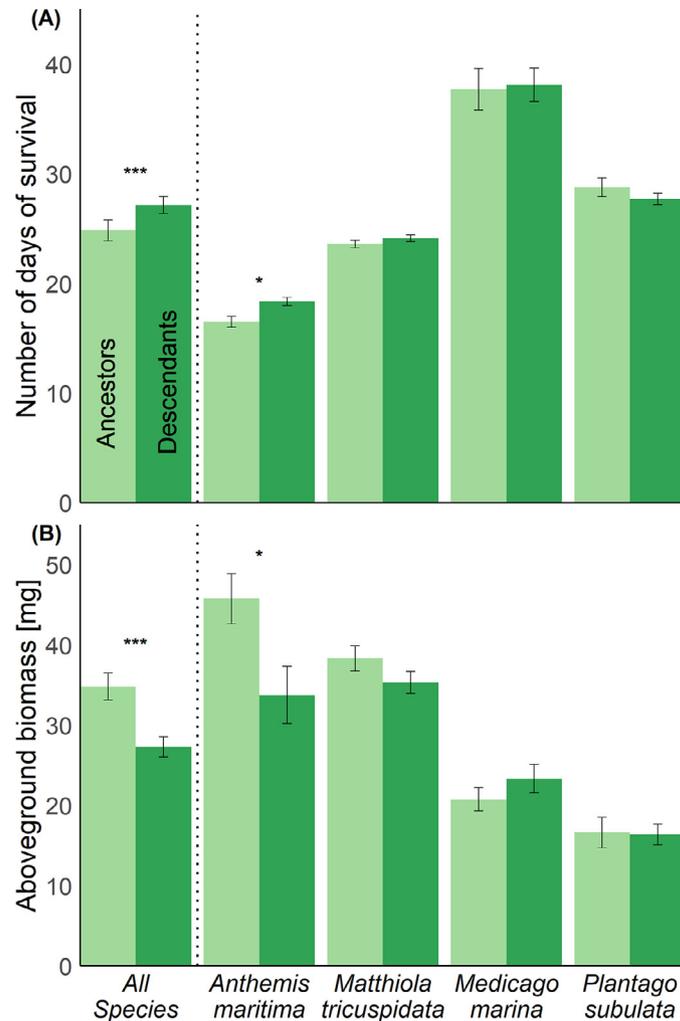
Fig. 1. Mean number of days of survival after watering ceased (A) and aboveground biomass at harvest (B) of seedlings of four Mediterranean species - *Anthemis maritima, Matthiola tricuspidata, Medicago marina, Plantago subulata* - from two different temporal origins (ancestors vs. descendants). The bars show means and standard errors. * = p<0.05, *** = p<0.001

species also differed in mean survival ability (Fig. 1A, $F_{3,208}$ = 255.21, p < 0.001) and we found an interaction between species and the temporal origins (Fig. 1A, $F_{3,208}$ = 2.74, p = 0.04). Although descendants of *Matthiola tricuspidata* and *Medicago marina* also survived slightly longer, the overall effect of temporal origin was mainly driven by one of the species, *Anthemis maritima*, since only descendants of this species showed a significantly longer survival than their ancestors in the individual-species analyses ($F_{1,60}$ = 6.01, p = 0.017). Across species, seedlings from descendants had a significantly lower biomass than those from ancestors (Fig. 1B, $F_{1,204}$ = 19.92, p < 0.001). Again, there was an interaction between species and temporal origin (Fig. 1B, $F_{3,204}$ = 3.57, p = 0.015), with the overall effect largely driven by *A. maritima* as only this species showed a significant biomass difference between temporal origins in individual-species analyses (Fig. 1B, $F_{1,59}$ = 6.08, p = 0.016). Across species, plants with a lower biomass generally survived longer ($F_{1,200}$ = 12.43, p = <0.001, r^2=0.46).

However, at the species level we observed a significant negative correlation between biomass and survival only for *A. maritima* ($F_{4,58}$ = 4.03, p = 0.006, r^2=0.16), whereas for *M. tricuspidata* ($F_{3,59}$ = 4.01, p = 0.012, r^2=0.13) and *M. marina* ($F_{4,46}$ = 7.71, p <0.001, r^2=0.35) there were positive correlations, i.e. larger plants survived longer.

Watering response experiment

The drought treatment had a significant effect on all three measured growth traits. Across all nine species, plants grown under drought conditions were smaller, produced fewer branches or leaves and had a lower aboveground biomass (Fig. 2A-C, Table 1). These observations were also consistent at the species level: in all species where a significant effect occurred, drought decreased plant growth (Table 2). Seven out of the nine tested species were affected in at least one of the measured traits. The temporal origin
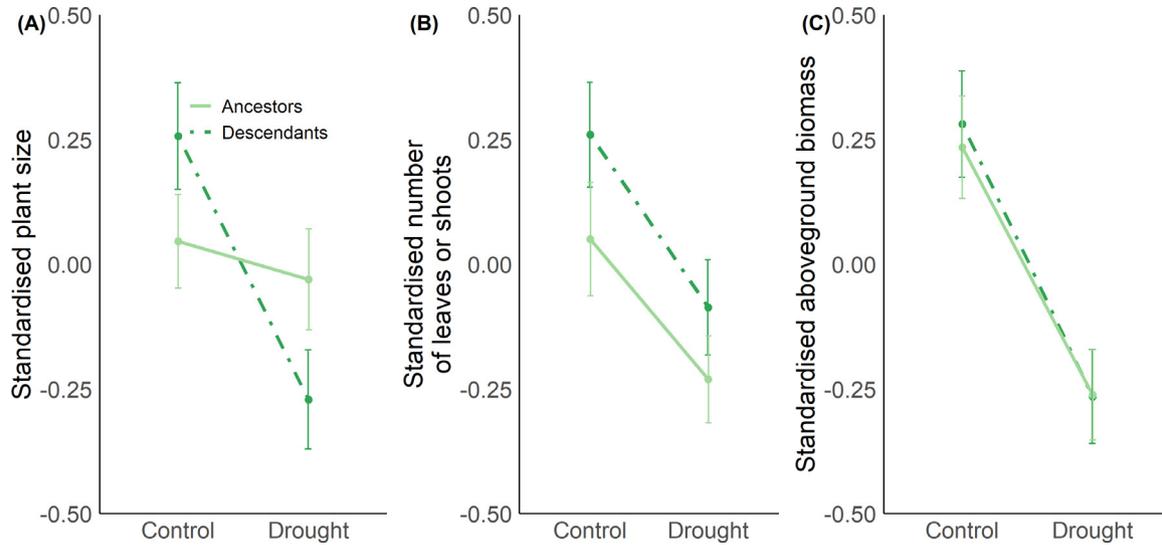
Fig. 2. Reaction norm plots of plant size (A), number of leaves or shoots (B) and aboveground biomass (C) in the watering response experiment. The data are transformed and averaged across all nine species from two temporal origins (ancestors vs. descendants). Error bars show standard errors

did not affect plant size in any of the studied species, but we found a significant difference in the number of leaves or shoots and in aboveground biomass between the ancestors and descendants of two and three species, respectively. In *Centaurium erythraea* and *Melica ciliata,* descendants produced significantly more leaves or shoots and biomass, but in *Dianthus carthusianorum* descendants produced less biomass (Table 2). Across species, there was a significant drought-by-temporal origin interaction for plant size (Fig. 2A, Table 1). While ancestral plants showed only a slight decrease of plant size in response to drought, the descendants strongly decreased plant size under drought. This observation is consistent across species, as there was no significant three-way interaction among the watering treatment, temporal origin and species in our model (Table 1). However, none of the individual-species models showed a significant treatment by temporal origin interaction for plant size (Table 2).

Discussion

We used seed material stored in seed banks and contemporary seeds collected from the same populations a couple of decades later to investigate rapid evolution. Specifically, to test for recent evolutionary responses of plants in early life stages to climate change, we compared the drought tolerance of ancestral and descendent plants of several Mediterranean (seedling survival) and temperate plant species (juvenile plants). In the species' regions of origin, drought is a particularly important stress during the investigated life stages.

Seedling survival experiment

In our seedling survival experiment with Mediterranean plant species, we found that in one of the four studied

Table 1. *F*- and p-values from cross-species linear models of the watering response experiment, each testing for effects of species, treatment (drought vs. control), temporal origin (ancestors vs. descendants), and their interactions. The arrows indicate the direction of a significant effect ($\downarrow$/$\uparrow$ = transformed values of the drought treatment are smaller/larger). Significant results are bold marked. Degrees of freedom (d.f.) are shown for tested variables and vary for the residuals.

	d.f.	Plant size		Number of leaves or shoots		Aboveground biomass	
		F-value	p-value	F-value	p-value	F-value	p-value
Species	8	**19.92**	**<0.001**	**3.08**	**0.01**	**13.71**	**<0.001**
Treatment	1	**14.88 $\downarrow$**	**<0.001**	**10.56 $\downarrow$**	**<0.001**	**41.90 $\downarrow$**	**<0.001**
Origin	1	<0.01	0.97	3.60	0.06	0.25	0.62
S × O	8	0.71	0.68	**2.38**	**0.02**	**4.27**	**<0.001**
S × T	8	1.91	0.06	1.28	0.26	**2.63**	**0.01**
T × O	1	**6.16**	**0.01**	0.05	0.83	0.01	0.91
S × T × O	1	0.48	0.87	0.82	0.58	1.47	0.33

Table 2. *F-* and p-values from linear models of the watering response experiment, each testing for effects of treatment (T; drought vs. control), temporal origin (O: ancestors vs. descendants), and their interaction (T × O). The arrows indicate the direction of a significant effect (↓/↑ = transformed values for descendants or drought, respectively, are smaller/larger). Significant results are in bold. Degrees of freedom for all tested effects was 1 and varies for the residuals.

Species	Plant size						Number of leaves or shoots						Aboveground biomass					
	T		O		T × O		T		O		T × O		T		O		T × O	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Centaurium erythraea	7.91↓	**0.01**	3.69	0.06	4.04	0.05	0.01	0.94	4.22↑	**0.04**	0.17	0.68	5.50↓	**0.02**	15.40↑	**<0.001**	2.87	0.14
Clinopodium vulgare	0.28	0.60	0.05	0.82	0.67	0.42	1.07	0.31	0.62	0.44	0.61	0.44	0.65	0.43	0.09	0.77	0.67	0.42
Dianthus carthusianorum	0.25	0.62	0.11	0.75	0.47	0.47	1.19	0.29	0.51	0.48	0.51	0.48	0.07	0.79	10.23↓	**0.01**	4.92	**0.04**
Digitalis lutea	0.73	0.40	0.04	0.85	0.04	0.85	7.38↓	**0.01**	2.74	0.11	0.39	0.54	5.12↓	**0.03**	<0.01	0.95	0.21	0.65
Leontodon hispidus	20.25↓	**<0.001**	1.64	0.21	0.86	0.36	7.26↓	**0.01**	3.13	0.08	<0.01	0.98	29.44↓	**<0.001**	3.86	0.06	0.81	0.37
Melica ciliata	0.36	0.56	0.19	0.67	2.43	0.13	1.55	0.23	9.58↑	**0.01**	0.56	0.46	2.77	0.11	23.24↑	**<0.001**	0.60	0.45
Pimpinella saxifraga	1.50	0.23	2.54	0.12	0.48	0.50	5.54↓	**0.03**	1.55	0.22	0.10	0.76	4.12	0.05	0.16	0.69	2.24	0.15
Sedum album	5.01↓	**0.03**	0.04	0.84	0.65	0.43	12.23↓	**<0.001**	0.61	0.44	0.90	0.35	37.07↓	**<0.001**	0.23	0.63	0.73	0.40
Teucrium chamaedrys	1.75	0.19	0.39	0.53	0.97	0.33	1.34	0.25	1.62	0.21	0.92	0.34	3.02	0.09	2.63	0.11	0.88	0.35

species, *Anthemis maritima*, descendant seedlings survived longer under drought than their ancestors and produced less aboveground biomass (Fig. 1 A,B). Although our experimental approach cannot assert whether observed evolutionary changes are adaptive, our observations are consistent with what would be expected if adaptation to drought had occurred in the studied population during the last decades.

Survival under drought can be enhanced by a small plant size, as we observed for *A. maritima*. In a multi-species approach Harrison and LaForgia (2019) compared seedling survival of ten grassland herbs under different water availabilities. They showed that the survival rate of smaller seedlings was higher under dry conditions. A possible explanation for this is reduced evapotranspiration through decreased leaf number, leaf size and branching and lower plant biomass (Aroca 2012). These observations also fit to the observation that plants in dry conditions often decrease aboveground biomass production and allocate more biomass to roots, leading to a higher root:shoot ratio (Martin & Stephens, 2006; Villagra & Cavagnaro, 2006; Erice et al., 2007). However, increased seedling drought tolerance can also be mediated by other traits such as root structures (e.g. hypocotyl hairs; Aronne & De Micco, 2004). However, it is also possible that the observed reduction in plant size was the result of passive stress responses instead of the above-mentioned active responses of plants to droughts.

Our main research question was to test for evolutionary changes between ancestors and descendants, and our historical comparison, a somewhat less strict version of the resurrection approach, has some weaknesses here, particularly when interpreting biomass results. First, we did not grow a 'refresher generation' of ancestors and descendants prior to our main experiment, because part of the study species only started to reproduce in the second year. We therefore cannot exclude that storage or maternal effects influenced the results (Franks et al., 2018). Second, if stored seeds have low germination rates, there is a possibility of "invisible fractions" (Weis, 2018), with germinating individuals representing only a subset of the stored phenotypes. In *A. maritima* the germination rate for the ancestors was only 25%, so we cannot rule out such invisible fraction effects. A third potential drawback of such historical comparisons with seed bank material not designed for these purposes is that sampling efforts can be very different for seeds from different periods. Fortunately, our molecular analysis found similar levels of relatedness among ancestors and descendants, indicating that the sampling probably had been conducted in a similar way and that sampling effort was sufficiently high.

While descendants of *A. maritima* showed improved drought resistance compared to their ancestors, we did not find similar patterns for three other species (Fig. 1A,B). Possible reasons for this could be that these species did not evolve due to lack of genetic variation or other evolutionary constraints (e.g. trade-offs) preventing evolution in specific phenotypes. Alternatively, it is also possible that these species evolved different (phenological) strategies to cope with

drought during the seedling stage which we did not explicitly study.

In summary, we show that seedling survival under drought has likely evolved in the last decades through adjustments of phenology and growth strategy in one out of four studied Mediterranean plant species. To disentangle evolution by means of natural selection from random evolutionary processes, i.e. mutation, drift and gene flow, quantitative genetic differentiation (Q_{ST}) can be compared with neutral molecular differentiation (F_{ST}) (Merilä & Crnokrak 2001, McKay & Latta 2002). Unfortunately, our design, comparing one ancestral with one descendant population per species, is suboptimal for such comparisons. However, our molecular data suggests low gene flow (see Appendix A) and population sizes for three of four species are sufficiently high (see Appendix A: Table 1), reducing the influence of random genetic drift. Furthermore, future experiments could exclude potential influences such as maternal or storage effects by growing refresher generations (Franks et al., 2018).

Watering response experiment

In our watering response experiment with nine species from temperate Europe, we subjected juvenile plants to drought that generally led to decreased plant size, number of leaves or shoots and aboveground biomass. Across species, we found no differences in mean traits between ancestors and descendants, but there was an overall difference between ancestors and descendants in the plasticity of plant size in response to drought, with a much stronger decrease of size in the descendant plants (Table 1). Since precipitation variability has increased for most of the studied species during the last decades this observation could corroborate predictions that such conditions favour the evolution of increased phenotypic plasticity (Sultan & Spencer, 2002; Alpert & Simms, 2002; Gianoli & Valladares, 2012).

In none of the single-species analyses did we find an interaction between treatment and temporal origin for plant size, which is probably partly explained by the moderate replicate numbers per species. Nevertheless, seven out of nine species showed the same trend for plant size as the cross-species analyses (see Appendix A: Fig. 1), and the three-way-interaction with species, temporal origin and treatment was insignificant, indicating similar cross-species patterns (Table 1). Since plant biomass and number of leaves or shoots were unaffected, this stronger shift in plant size under dry conditions could be accompanied by changes to other functional traits we did not measure in our study such as leaf thickness or leaf shape, which are known to be highly plastic (Gianoli & González-Teuber, 2005; Lázaro-Nogal et al., 2015). A reduction of leaf area accompanied by increasing leaf thickness and/or more pubescent leaves may reduce evapotranspiration (Gianoli & González-Teuber, 2005) and can therefore be a successful strategy under drought (Riva et al., 2016).

Plant responses to drought are generally complex and may even differ between closely related species (Bouzid et al., 2019), as drought affects plants at various developmental stages and in different tissues (Yordanov et al., 2000). Our experiment does not allow us to − but future studies should − identify the processes underlying the observed patterns that may include increased resource allocation to roots (Martin & Stephens, 2006; Villagra & Cavagnaro, 2006; Erice et al., 2007), changes in stomatal density (Liu et al., 2015) and reduced evapotranspiration (Aroca, 2012), or a combination of these and other factors.

We also found significantly larger plant sizes but not higher aboveground biomasses in the control treatment for the descendants compared to ancestors across species. This may be an adaptation of the species' life cycles: As flowering onset is often related to plant size (Vile et al., 2006; Sun & Frelich, 2011), we argue that plants grow and develop fast when water supply is sufficient to escape potential drought stress later in their life cycle (Grene et al., 2011). When interpreting the results of our study we should keep in mind though that our drought treatment was simplified, with water applied at constant low versus constant normal levels. In nature, patterns of water availability may be more variable, and we do not know how our plants would have, for example, responded to drought after a period of sufficient watering. This is important given that under ongoing climate change, not only mean precipitation but also temporal patterns are changing.

Greater environmental heterogeneity in space or time, when perceived within the organism's − or its immediate descendants' − lifetime, is generally expected to favour greater phenotypic plasticity (Alpert & Simms, 2002; Bradshaw & Holzapfel, 2006; Matesanz et al., 2010). In the regions of origin of most of the study species, drought frequency has increased over the last 20 years (Spinoni et al., 2018), and environmental conditions have thus become more unpredictable (Altvater et al., 2011). This could have favoured evolution of stronger plasticity through natural selection for more plastic genotypes (Ackerly et al., 2000; Richards et al., 2006). To test whether the observed greater plasticity in plant size of the descendant plants is an adaptive change requires further experiments that include longer-term measurements of plant fitness (Richards et al., 2006). Ideally, such experiments should take place at the species' sites of origin and incorporate a large number of populations which experienced different rates of climate change, and in particular increased precipitation variability, during the past decades.

Although our results may be influenced by other factors, we are confident that we observed a true evolutionary pattern here that is common in nature: greater plasticity of descendants − as a trend − was consistent for seven out of the nine studied species (see Appendix A: Fig. 1), which is very unlikely if part or all of these patterns were due to chance or unintentional selection during sampling and storage or due to maternal effects on each species separately

(see discussion on seedling survival above). Furthermore, germination rates in most species were high, and there were no relationships between germination rate and plasticity, suggesting that variation in germination rates did not affect other traits. However, we cannot completely exclude potential storage effects or hidden fractions, especially for *Leontodon hispidus*, for which germination rates differed strongly between ancestors and descendants (see Appendix A: Table 1). Random evolutionary processes, such as drift or gene flow, as well as unintentional selection are unlikely to have stronger effects than those exerted by the drought treatments, which pose strong selection pressures on seedling recruitment and drought responses (Schupp, 1995; Fenner & Kitajima, 1999).

Using seed bank material for historical comparisons

Resurrection studies are a powerful tool for studying recent evolution, but the appropriate genetic resources are rarely available. Large-scale long-term efforts have recently been set up to conduct powerful resurrection studies in the future (e.g. *Project Baseline*, Etterson et al., 2016). However, if material from regular seed banks could be used for similar before-after comparisons, it would open up a vast resource for environmental change research. Although seed banks often lack population replicates within species, multi-species approaches can make studies more powerful by testing for common evolutionary patterns across taxa.

Despite previously mentioned shortcomings of our study, we show that it is possible to use seed bank material, not explicitly collected for resurrection studies, for similar historical comparisons. In our study, genomic relatedness analyses indicated that the ancestor and descendant seed pools were similar, and that seed sampling had been conducted in a comparable way. Our molecular data also suggests that the genetic diversity of seed bank collections and newly collected seeds was sufficiently large for conducting the experiments. We are therefore reasonably confident that the use of seed bank material in our study was meaningful. Our approach opens up a new avenue for studies on recent plant evolution and may be a useful complement to other approaches that study contemporary populations or use other stored materials such as herbarium specimen (Lang et al., 2019; DeLeo et al., 2020).

Conclusions

Ongoing climate change is expected to influence the evolution of plant populations, but so far experimental tests of this are rare. Our multi-species historical comparisons using taxa from two different biogeographic regions in Europe, investigating drought responses of plants in early life stages,

indicate that plants have evolved within the last decades, possibly in response to increased drought frequencies. We observed evolutionary changes in several, but not all, species, in both trait means and trait plasticity in response to experimental drought. Given the increased occurrence of drought events in most of the populations of origin, our results suggest that climate change may have already influenced the evolutionary trajectory of many plant species in different regions of Europe. Our study also demonstrates that historical comparisons similar to the resurrection approach can be made using plants from seed bank collections, and are a powerful tool for studying rapid evolution in plants. There is great potential for future studies to make use of the wealth of seed bank collections for investigating rapid adaptation to recent environmental changes. Replicated populations of the same species may be scarce in seed banks, which is why a multi-species approach is generally advantageous. Ideally, seeds from a refresher generation should be used to minimize possible maternal effects. To disentangle adaptive from non-adaptive and maladaptive responses to recent climate change, future experiments should incorporate fitness measures, comparative transplantations of descendants and ancestors into their original habitat. In addition, Q_{ST}-F_{ST} comparisons might help to infer the relative roles of selection and random evolutionary processes for population differentiation.

Data accessibility

Raw data of the ddRAD-SNP sequencing have been deposited in the Europoean Nucleotide Archive (ENA) at EMBL under accession 409 number PRJEB47887 (https://www.ebi.ac.uk/ena/browser/view/PRJEB47887).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j. baae.2021.11.003.

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