RESEARCH PAPER

Drought stress triggers differential survival and functional trait responses in the establishment of Arnica montana seedlings

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ABSTRACT

- The establishment and survival of seedlings are critical stages in the life cycle of plants and therefore usually well timed to humid and favourable conditions. Climate projections suggest that the threatened mountain grassland species *Arnica montana* may be increasingly exposed to drought stress. However, studies that focus on the species' early development are missing. We evaluated impacts of drought-induced stress on *A. montana* seedlings in their early establishment phase and identified traits that could cause the species' fitness to decline.

- In a greenhouse experiment, we tested the response of *A. montana* seedlings to different drought levels (moderate, strong, extreme). To assess their fitness under increasing drought, we evaluated survival of the seedlings based on four senescence stages and measured the performance of above- and belowground morphological and physiological functional traits.

- *Arnica montana* seedlings showed high resistance to drought. Senescence accelerated and survival declined only under strong and extreme drought conditions. However, the seedlings' vegetative performance decreased even with moderate drought, as indicated by smaller values of most leaf traits and some root traits. Physiological trait response was less sensitive.

- Drought stress hinders the establishment and survival of *A. montana* seedlings. Following the functional trait responses to drought and their association with survival, we suggest declining leaf length, leaf width, and leaf number are sensitive traits that can lead to a decline in performance.

INTRODUCTION

Effects of climate change have been identified as major threats to the biodiversity of ecosystems around the world (Sala *et al.*, 2000; Bellard *et al.*, 2012). However, how these changes manifest themselves differs regionally. Mountain regions with their important role for biodiversity and their diversity of montane plants and mountain grasslands, are disproportionally exposed to climate change, with reduced precipitation being a major component in Central Europe (Engler *et al.*, 2011; Perrigo *et al.*, 2020). For example, changes in distribution ranges, plant phenology or functional traits have been observed as part of the climate change response of mountain grassland plants (Gottfried *et al.*, 2012; Gritsch *et al.*, 2016). Even if several studies have documented such effects, there are still considerable knowledge gaps about how species will cope with climate changes, and drought specifically. In this context, studies are needed that investigate trait–environment interactions, shedding light on early life stages under the most realistic climate change projections (Gibson & Newman, 2019).

Early life stages, such as germination, emergence and establishment of seedlings, are considered to be critical for population growth of plants (James *et al.*, 2011). Many perennial plants have developed mechanisms, such as a persistent soil seed bank or bud dormancy, as adaptive ecological responses to bridge unfavourable periods, *e.g.* dry periods, until germination conditions become favourable (Baskin & Baskin, 2014). Another key transition is seedling survival under stressful conditions because seedlings are exceptionally vulnerable (*e.g.* to drought stress), but cannot escape this stress by reversing the one-way germination process (James *et al.*, 2011). Moreover, after germination in the humid early spring, droughts are becoming more frequent in late spring and summer, which are the most critical times for seedlings. However, while survival is an important fitness component, it requires functional traits and their relation to fitness under simulated drought conditions to understand the plant response. Therefore, linking functional traits to drought on an experimental basis is essential to predict how drought is likely to affect a species' future development and population structure, and could improve predictions of ecosystem sensitivity to climate change (Ehrlén *et al.*, 2016).

During seedling development, plant functional traits can respond rapidly to altered environments (*e.g.* Lampei, 2019). For example, plants can develop thicker and smaller leaves, resulting in a lower specific leaf area (SLA) that will reduce transpiration and provide improved heat protection (Hameed *et al.*, 2012). Another adaptive response is a shift in seedling

above- and belowground biomass allocation following environmental changes (Mašková & Herben, 2018). Increased belowground allocation is frequently observed in connection with drought as plants are forced to extract more water from the soil (Eziz *et al.*, 2017; Sandner & Matthies, 2018). Hence, functional trait performance, the combined effects of functional traits on plant vigour (cf. Stanik *et al.*, 2020), can provide insights into the actual threats to seedling survival well before a species starts to disappear from a site. Therefore, knowledge about the ecological response of seedlings to drought is of high ecological relevance to obtain a comprehensive assessment of drought impacts on species.

The threatened target species, *Arnica montana*, is, like many other mountain grassland species, considerably exposed to the impacts of climate change, such as more frequent and prolonged droughts. Current field observations suggest that the species reproduces predominantly by rhizomes from the mother rosette (Stanik *et al.*, 2018). Consequently, many small- or medium-sized *A. montana* populations consist of only a few different genotypes, which leads to inbreeding depression and results in reduced fitness (Kahmen & Poschlod, 2000). *A. montana* depends, like other perennials plants, on sexual reproduction to adapt to future environmental changes (Eckert, 2001; Stöcklin *et al.*, 2009). This species relies for its generative reproduction on bare soil patches in grassland swards, which provide both low competition for germination sites and a beneficial microclimate for seedling establishment (Schwabe, 1990; Kahmen & Poschlod, 1998), which are increasingly rare due to changes in habitat structures and current management of mountain grasslands (Hollmann *et al.*, 2020; Peppler-Lisbach *et al.*, 2020). Moreover, recent field observations from *in situ* reintroduction projects suggest increased pressure of drought on *A. montana* seedlings in the early establishment phase after successful germination (Blachnik & Saller, 2015). Therefore, it remains unclear to what extent *A. montana* will be influenced by drought stress in the context of climate change and how this will affect survival and functional trait responses.

In this study, we used a greenhouse experiment to assess the impacts of reduced soil moisture, and thereby drought-induced stress on seedlings of *A. montana* in their early establishment phase. In order to approach more realistic climate change conditions and to gain a more robust species response to future climate, we used drought scenarios from regional climate change projections [Intergovernmental Panel on Climate Change (IPCC), 2014; PIK/Potsdam Institute for Climate Impact Research, 2017]. This approach addresses the recent call for more realistic climate change experiments to reduce the mismatch between experimental manipulation and climate change projection (Korell *et al.*, 2019). Our aim is to evaluate whether and to what extent different drought stress levels affect the survival and performance of functional traits of *A. montana* seedlings in their establishment phase. Therefore, we addressed the following questions: (i) does drought stress have a significant negative effect on survival and functional trait performance of *A. montana*; and (ii) which functional traits respond rapidly to water reduction and are well correlated with survival under increased drought stress? The results provide important insights on the ecological response of a threatened target species of biodiverse mountain grasslands in the critical transition phase of seedling establishment at different drought stress levels.

MATERIAL AND METHODS

Study species and plant material

The study species, *A. montana* L. (Asteraceae), is characteristic of nutrient-poor acidic grassland and heathland habitats, which has experienced a strong decline during the past few decades across Europe and is now listed as endangered in Germany (Peppler-Lisbach & Petersen, 2001; Metzing *et al.*, 2018). The perennial species forms long-lived rhizomes with rosettes for vegetative reproduction (Luijten *et al.*, 2000). *A. montana* also produces wind-dispersed seeds (achenes) and is self-incompatible, thus preventing inbreeding (Luijten *et al.*, 2002). The species has no persistent soil seed bank, thus seeds germinate either directly in autumn after fruiting or in the following spring under moist conditions (Kahmen & Poschlod, 1998). In 2018, we collected *A. montana* seeds from randomly chosen inflorescences of 60 plants from a population in the German core distribution area (Rhön accession, Hesse, Germany; 50°30′23.8″ N, 9°57′28.4″ E) and stored them dry at 5 °C in a refrigerator until the experiment established in 2019. The sampled population is at an elevation of 815 m a.s.l., with a population size of approximately 1,000 individuals. This location represents the study species in its typical environment in the Central European mountain range (Fig. S1). The monthly mean temperature during the experiment at that site is 8.3 °C in May, 11.3 °C in June, 13.1 °C in July, and 13.0 °C in August (DWD Climate Data Center, 2020). The monthly precipitation values correspond to the precipitation amounts for the control treatment (T0) in the experiment (Table 1).

Experimental design and conditions

We set up a greenhouse experiment with three drought treatments, each replicated 14 times, in a fully randomised design. We applied treatments to *A. montana* seedlings in standard Mitscherlich pots (soil volume: 6.2 l, pot diameter: 20 cm; $N = 56$), filled with undisturbed soil cores taken from the site where the seeds were collected. Hence, soil chemistry and mycorrhiza reflect natural site conditions of the seed origin. The greenhouse is located in Kassel, 96 km northwest of the population's origin in the Rhön Mountains. Measured mean topsoil pH (H_2O) of the soil cores was 5.21 (± 0.12 SD), thus potential negative effects of aluminium toxicity on seedling roots from low soil pH can be ruled out (Abedi *et al.*, 2013). The soil was 5.9 $\pm$ 1.1% sand, 47.1 $\pm$ 2.1% silt, 47 $\pm$ 2.6% clay with a mean organic matter content of 60.3 $\pm$ 3.8%. Field capacity was determined using the method of Wolkewitz (1964).

The experiment lasted 100 days (6 May to 13 August 2019) to fully cover the early development stage of seedlings after germination in spring and to consider increased summer drought risk under climate change. In each pot, we sowed 12 *A. montana* seeds into bare soil 1 month before the start of the experiment for germination (indicated by fully expanded cotyledons) under standard water supply. Five days before the experiment start, we thinned the seedlings of all pots to three evenly developed and separated individuals per pot. From this day until the experiment start, initial amounts of water matching the experiment's reference treatment were supplied three times to all pots to level out potential differences in soil moisture.

–>Precipitation amounts [monthly sum (mm)].

Table 1. Precipitation amounts [monthly sum (mm)].

month	precipitation (mm) of T0	precipitation (mm) of T1	precipitation (mm) of T2	precipitation (mm) of T3
May	96.46	67.52	38.58	18.09
June	114.55	80.19	45.82	0
July	109.42	76.59	43.77	0
August	37.52	26.26	15.01	0

Control (T0) is current precipitation at the population origin; treatments T1 and T2 represent moderate and strong precipitation declines, respectively, under climate change for the population origin. For August, the supplied water is relative to the whole month because of the experiment ended on 13 August. Treatment T3 only received water in May. All other treatments received three waterings with the amount of T0 to remove short-term soil moisture differences among treatments. Monthly precipitation amounts for control (T0) were calculated based on measurements at the weather station Wasserkuppe in the reference period (1961–1990) (DWD Climate Data Center, 2020).

To simulate different drought levels, treatments were approximated to projected future monthly precipitation regimes from May to August under different climate change trajectories for the high altitude of the Rhön Mountains compared to the reference period (1961–1990) based on the RCP 4.5 scenario (IPCC, 2014; PIK/Potsdam Institute for Climate Impact Research, 2017). Four treatments were applied (Table 1):

- Control (T0) corresponding to the monthly precipitation of the climate reference period in the Rhön Mountains,
- Moderately reduced water supply (−30% compared to control, T1),
- Strongly reduced water supply (−60% compared to control, T2),
- Extreme drought (T3) where no water was supplied (except initial three water applications mentioned above).

Mean air temperature increased over the course of the experiment: $14.8 \pm 3.64\,°C$ in May, $23.60 \pm 3.32\,°C$ in June, $21.7 \pm 4.60\,°C$ in July, $22.10 \pm 1.47\,°C$ for the 13 days of August until the experiment ended. In the same time period, mean monthly humidity was fairly stable: $62.5 \pm 7.89\%$ in May, $57.10 \pm 7.68\%$ in June, $59.4 \pm 11.1\%$ in July, $61.90 \pm 4.77\%$ in August (Fig. S2). Based on the temperature and precipitation regimes, the treatments differed in their whole-month mean aridity (after Martonne, 1926) between May and August: treatment T0 aridity 3.51 ± 0.23, T1 2.43 ± 0.19 and T2 1.39 ± 0.11. The aridity of T0 and T1 thus represent current climates in Central European sites at elevations of approximately 500 and 200 m a.s.l., respectively, (Stanik et al., 2020).

The water in T0 and in T1 and T2 was applied every second day, resulting in 16 single applications per month (Table S3). Additionally, we applied a low amount of fertiliser to each plant five times to provide a regular basic nutrient supply (15 ml, Substral universal fertiliser, 18-14-18 NPK at $1.5\,g\,l^{-1}$). During the experiment, we measured volumetric soil water content (VWC) (Vol-%, scale 0–1) and soil temperature (°C) between the watering days (ECH$_2$O 5 TE-sensor; METER Group Europe, Mettlacher Straße, München, Germany). Air temperature and humidity in the greenhouse were recorded hourly using an EasyLog EL-USB-2 data logger (Lascar Electronics, Module House, Salisbury, Wiltshire, UK) during the experiment and 5 days prior to experiment start to cover the whole month of May. We shuffled the pots on a weekly basis to balance potential differences in light.

Evaluation of plant performance and survival

Violle et al. (2007: 882) state 'in its simplest definition, a trait is a surrogate of organismal performance'. In this sense, we used the functional trait approach to evaluate trait performance under simulated drought scenarios. This approach implies a direct or indirect connection of a functional trait with fitness of a plant. As some traits have a more direct link with fitness than other traits, we use this difference in fitness correlation of traits to our advantage. While a fitness surrogate, such as survival or biomass, provides complete *post hoc* information on plant performance under drought, a trait less closely connected with fitness, *e.g.* leaf length, may be more predictive. This means that the strength of the trait–fitness correlation is key to our functional trait concept. Here, we evaluated a set of morphological and physiological above- and belowground functional traits commonly used as proxies for species growth and size and which indicate drought stress (Pérez-Harguindeguy et al., 2013).

We measured vegetative height, leaf number, length, width and dry mass of the latest fully expanded leaf, and leaf area; collectively referred to as 'leaf traits'. These leaf traits are related to growth and aboveground productivity (Younginger et al., 2017). Moreover, the number of side shoots and length of the longest side shoot were measured to account for the start of vegetative reproduction of individuals. We measured rooting depth, root length, root dry mass, and root mean diameter; collectively hereafter 'root traits'. These root traits relate to water uptake and belowground resource acquisition (Kramer-Walter et al., 2016; McCormack et al., 2017). For functional traits that represent the economic spectrum of a plant, we determined SLA and leaf dry matter content (LDMC), as well as specific root length (SLR) and root dry matter content (RDMC) (Wright et al., 2004; Reich, 2014). Finally, total aboveground dry biomass (including side shoots), belowground dry biomass, total biomass (dry), and the root/shoot ratio of each individual were calculated to account for overall growth and aboveground/belowground biomass allocation of

the plants. The measurement, sampling, and further processing of trait samples were performed according to Pérez-Harguindeguy *et al.* (2013). Leaf area was determined by using ImageJ2 (Rueden *et al.*, 2017) and the size-related root traits using WinRHIZO™ (version 2016a) (Regent Instruments, Québec, Canada). We measured traits at the end of the experiment on seedlings that survived the treatments, *i.e.* those that did not reach senescence stage 3. However, in accordance with Pérez-Harguindeguy *et al.* (2013), we did not measure leaf area, leaf dry mass, SLA, and LDMC for plants in treatment T3 as leaves often showed signs of senescence.

Plant senescence development and consequent survival was assessed every second day based on a four-stage visual quantification key (0–3, where 0 is 'completely undamaged', 1 'slightly wilted and slightly damaged', 2 'moderately wilted and dried, partly damaged', and 3 'completely dried out and brittle') (Malyshev *et al.*, 2016). In the survival analysis, each senescence stage (1–3) was evaluated separately to account for ongoing senescence. Together with the measured functional traits, senescence stages allow adequate evaluation of *Arnica* fitness.

Data analysis

All statistical analyses were conducted in R 3.6.3 (R Core Team, 2020). The development of soil moisture and soil temperature during the experiment and differences among treatment groups were analysed using linear mixed models and *post hoc* ANOVA comparisons with the R package' lmerTest' (Kuznetsova *et al.*, 2017). The models contained VWC and soil temperature as dependent variables and treatment and experiment month (the latter as proxy for progressing over time) as independent variables with the pot ID and experiment month as crossed random factors to account for repeated measurements.

Measured trait data assessment used the protocol described in Zuur *et al.* (2010). To obtain or improve normality, we log+1-transformed SLR, LDMC, and RDMC. In the overall dataset, leaf length ($r_s = 0.89$, $P < 0.001$), leaf width ($r_s = 0.83$, $P < 0.001$), leaf dry mass ($r_s = 0.91$, $P < 0.001$), total aboveground dry biomass ($r_s = 0.99$, $P < 0.001$), and root dry mass ($r_s = 0.97$, $P < 0.001$) showed strong collinearity with total biomass. However, only leaf dry mass, total aboveground dry biomass, and root dry mass were excluded from further trait model analyses because leaf length and leaf width are informative traits due to their non-destructive measurement. We fitted mixed effects models to evaluate trait performance differences between control and drought treatments and to investigate specific trait relationships to drought levels. We used linear mixed effects models with a Gaussian error distribution for all traits except number of side shoots, which was analysed using a generalized mixed effects model with Poisson distribution. Each model contained the individual trait as dependent variable, drought treatment as independent variable and pot ID as random intercept. Where T3 measurement of traits was not applicable, models were fitted with a data subset containing only three factor levels (T0, T1, T2). This was also the case in the models for number of side shoots and side shoot length because otherwise the parameters could not be uniquely determined. We fitted all mixed effects models using Restricted Maximum Likelihood with the package 'lme4' (Bates *et al.*, 2015) and conducted *post hoc* multi-comparison tests with Benjamini–Hochberg correction for multiple comparisons to

identify differences among treatment groups with the package 'multicomp' (Benjamini & Hochberg, 1995; Hothorn *et al.*, 2008).

Plant senescence stages and final survival were assessed in response to drought. We fitted Kaplan-Meier survival curves and used log-rank tests to identify differences in median time to reach each senescence stage and subsequent survival time at senescence stage 3 (Zwiener *et al.*, 2011). For survival analyses, we used the packages 'survival' and 'survminer' (Therneau, 2015; Kassambara *et al.*, 2019). To test relation of single traits to start of senescence, we fitted generalised linear mixed effects models with binominal distribution. The models contained occurrence of senescence stage 1 as dependent variable, standardized values of selected traits as independent variable and pot ID as random intercept with a data subset containing only the individuals of treatments T2 and T3 because of the different number of events in the treatments.

RESULTS

Soil moisture and temperature among treatments

Over the course of the experiment, VWC differed among treatments (Fig. S4.1). While VWC was initially rather even, for T0 at 0.31 ± 0.02 and for T3 at 0.28 ± 0.02, values differed significantly at the end of the experiment (T0 = 0.347 ± 0.04; T1 = 0.283 ± 0.04; T2 = 0.218 ± 0.05; T3 = 0.097 ± 0.08; ANOVA: $F_{(3,224)} = 5.15$, $P < 0.01$). Moreover, VWC of T0 remained steady around the upper boundary of field capacity, but T1 reached mean field capacity and T2 dropped to a lower limit after 50 days. T3 dropped below the lower field capacity limit after 20 days, resulting in a significant main effect of factor 'experiment month' in the drought treatments (ANOVA: $F_{(1,230)} = 395.86$, $P < 0.001$) but not in the control ($\beta = -0.005$, $P = 0.113$). Soil temperature in pots was also influenced by experiment month following the natural increase in air temperature during the experiment ($\beta = 1.11$, $P < 0.001$). Neither treatment nor interaction of treatment × experiment month influenced soil temperatures (Table S4.2).

Seedling survival under drought stress

The drought treatments significantly affected *A. montana* seedling survival. All plants survived throughout the experiment in control (T0) and moderate drought treatment (T1). This shows the overall survival was high when the plants had sufficient water supply. Conversely, in the strong (T2) and extreme (T3) drought treatments, seedlings had a probability of reaching senescence stage 3 of $14.3 \pm 0.05\%$ and $81.2 \pm 0.05\%$, respectively (Fig. 1a). Kaplan-Meier curves differed significantly among treatments (log-rank test: $\chi^2 = 158$, $df = 3$, $P < 0.001$).

To further assess sensitivity of survival ability of *A. montana*, we additionally classified the plants as moderately damaged (senescence stage 2) and slightly damaged (senescence stage 1). Again, only seedlings of T2 and T3 reached senescence stage 2 (Fig. 1b), although with higher probabilities of $16.8 \pm 0.05\%$ and $92.2 \pm 0.03\%$, respectively. Kaplan-Meier curves differed significantly among treatments (log-rank test: $\chi^2 = 208$, $df = 3$, $P < 0.001$). Even senescence stage 1 was only reached in the strong (T2) and extreme (T3) drought treatments (Fig. 1c); the probabilities of reaching senescence stage 1 were $23.8 \pm 0.06\%$
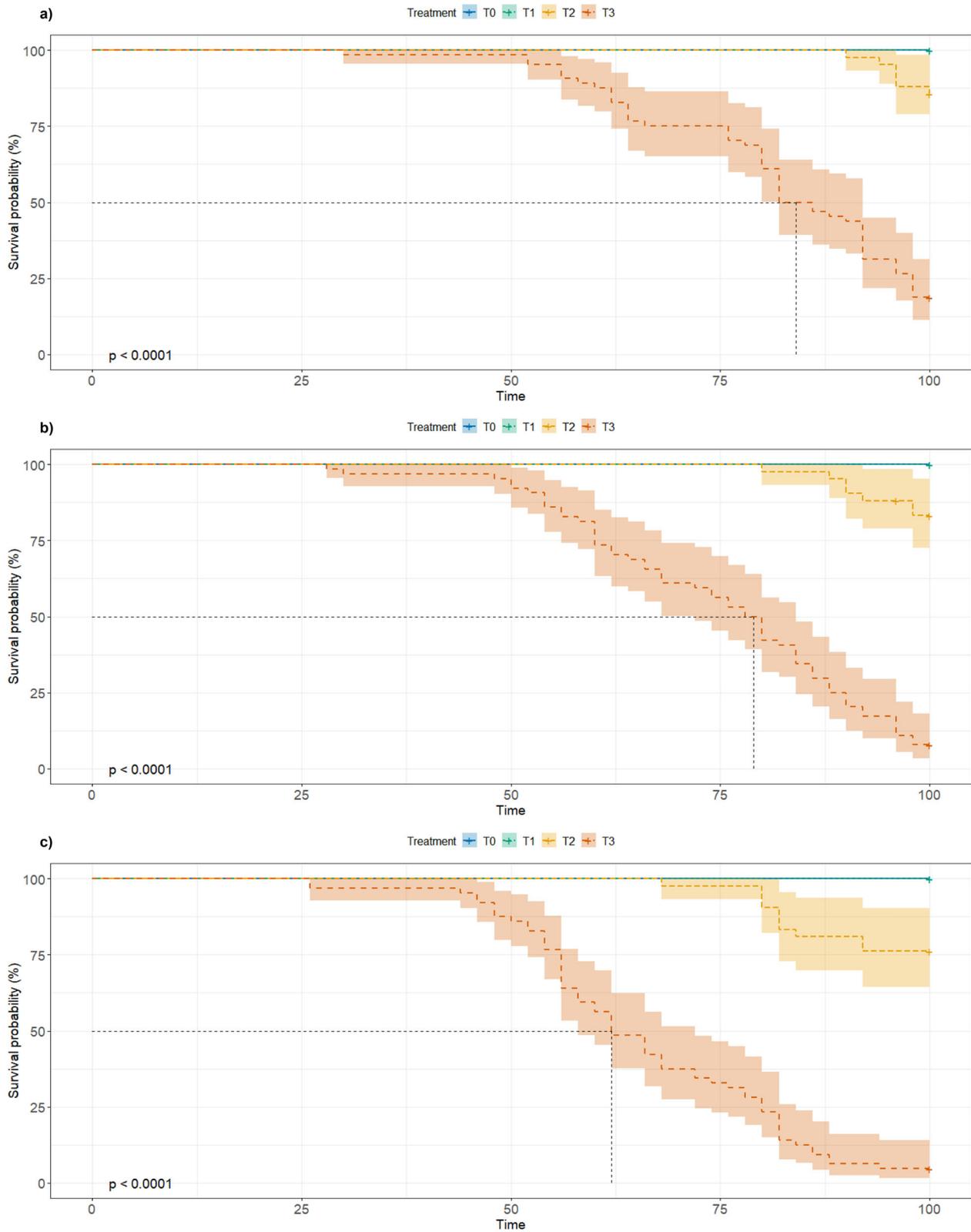
Fig. 1. Kaplan-Meier survival curves with confidence intervals for the *Arnica montana* individuals that reached (a) senescence stage 3 (total damage/dead, (b) senescence stage 2 (moderate damage), and (c) senescence stage 1 (slight damage).

and 95.3 ± 0.02%, respectively. Like stages 3 and 2, also for stage 1 the Kaplan-Meier curves differed significantly between treatments (log-rank test: $\chi^2 = 219$, $df = 3$, $P < 0.001$).

In treatment T3, all wilting stages were scored frequently enough to compare the median event time, which was not evenly distributed across the experiment. Despite the exceedingly low soil water content in T3, the median event time was not reached before 62 days (CI: 58–72) for senescence stage 1, 79 days (CI: 72–84) for senescence stage 2, and 84 days (CI: 82–92) for senescence stage 3. This means that it took plants 17 days from emergence of early wilting signals (stage 1) to reaching a state of serious damage (stage 2), which was a median of only 5 days before the plants died. This shows that the response of *A. montana* in senescence development at all stages is not linear with the decrease in soil moisture in the extreme drought treatment. Instead, plants could withstand drought for quite a long time, followed by a rather abrupt die off.

Trait performance differences under drought

Generally, the overall performance of *A. montana*, *i.e.* combined information of multiple functional traits on vigour of an individual, decreased with increasing drought, as indicated by significant negative responses of almost all traits to drought treatments. Unlike the senescence stages, many of the measured traits responded significantly to moderate drought treatment (T1), including total biomass (Fig. 2a), which is also a very good fitness surrogate for perennial plants. Compared to the control, total biomass (above- and belowground) showed a slight decrease under moderate drought ($\beta = -0.38$, $P = 0.012$), which intensified in the strong and extreme drought treatments ($\beta = -1.24$ and -3.14, $P < 0.001$, respectively). Further, all leaf traits decreased more or less steadily with increasing drought level (Fig. 2, Table S5.1). Only leaf number did not respond significantly to moderate drought ($\beta = 0.46$, $P = 0.51$), but decreased markedly in the strong (T2) and extreme (T3) drought treatment ($\beta = -2.98$ and -7.81, $P < 0.001$, respectively). For vegetative reproduction, a higher number of individuals in the control (T0) developed side shoots compared to T1 and T2. Plants in T3 did not develop side shoots (Table S5.2). In addition, the side shoots were considerably shorter under strong drought ($\beta = -51.62$, $P = 0.001$) than under control conditions, but not under moderate drought ($\beta = -8.44$, $P = 0.517$).

Similar to leaf traits, most root traits had a performance decrease with increased drought (Fig. 3). However, roots tended to be less sensitive to moderate drought (T1). For example, while both root length and rooting depth showed a statistically significant decrease in the strong and extreme drought treatments, rooting depth did not decline significantly in T1 (Table S5.3).
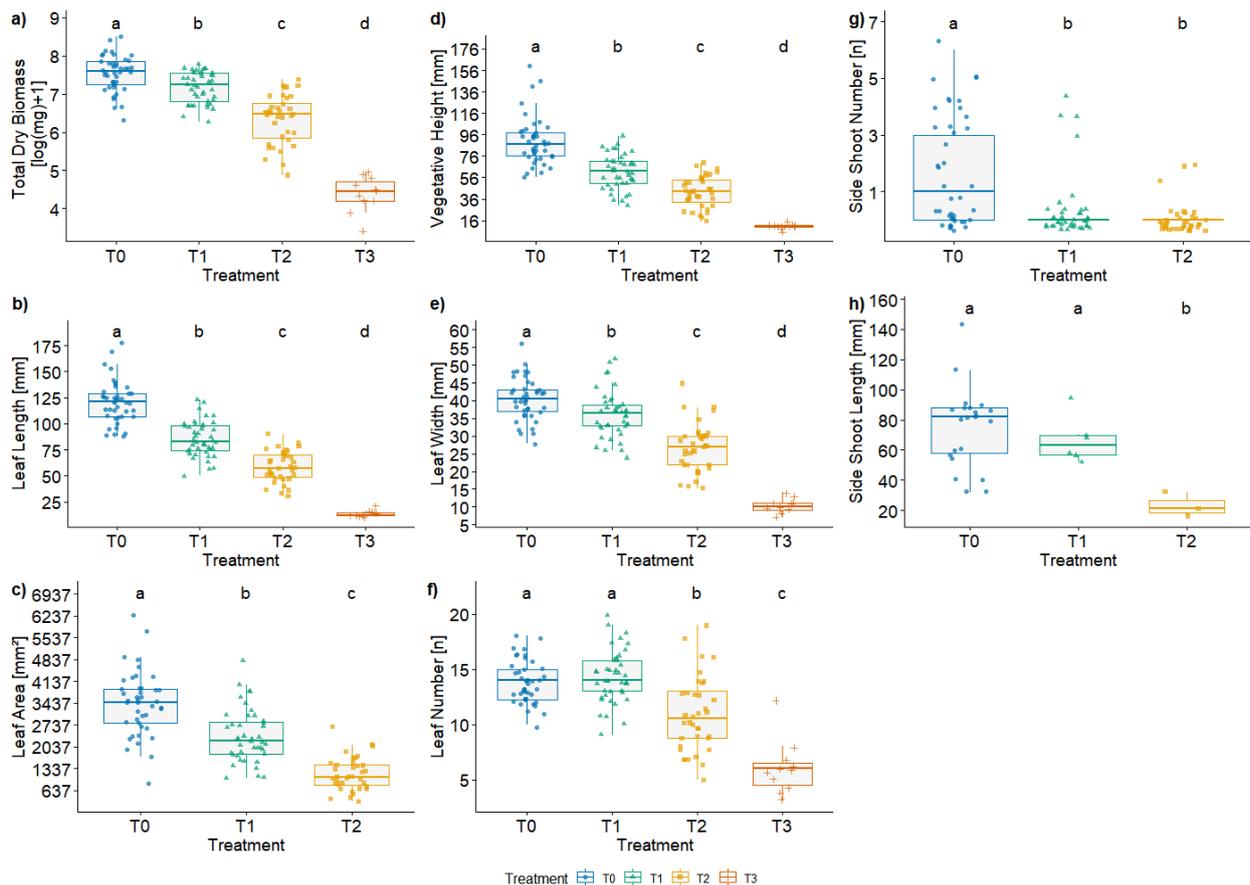

Fig. 2. Boxplots showing differences in total dry biomass (a) and aboveground vegetative traits (b–h) among treatments. Significant differences among treatments are based on *post hoc* multiple comparison tests of mixed effects models and are indicated by small letters with a significance threshold of $P < 0.05$. The leaf area could not be measured in plants of treatment T3.
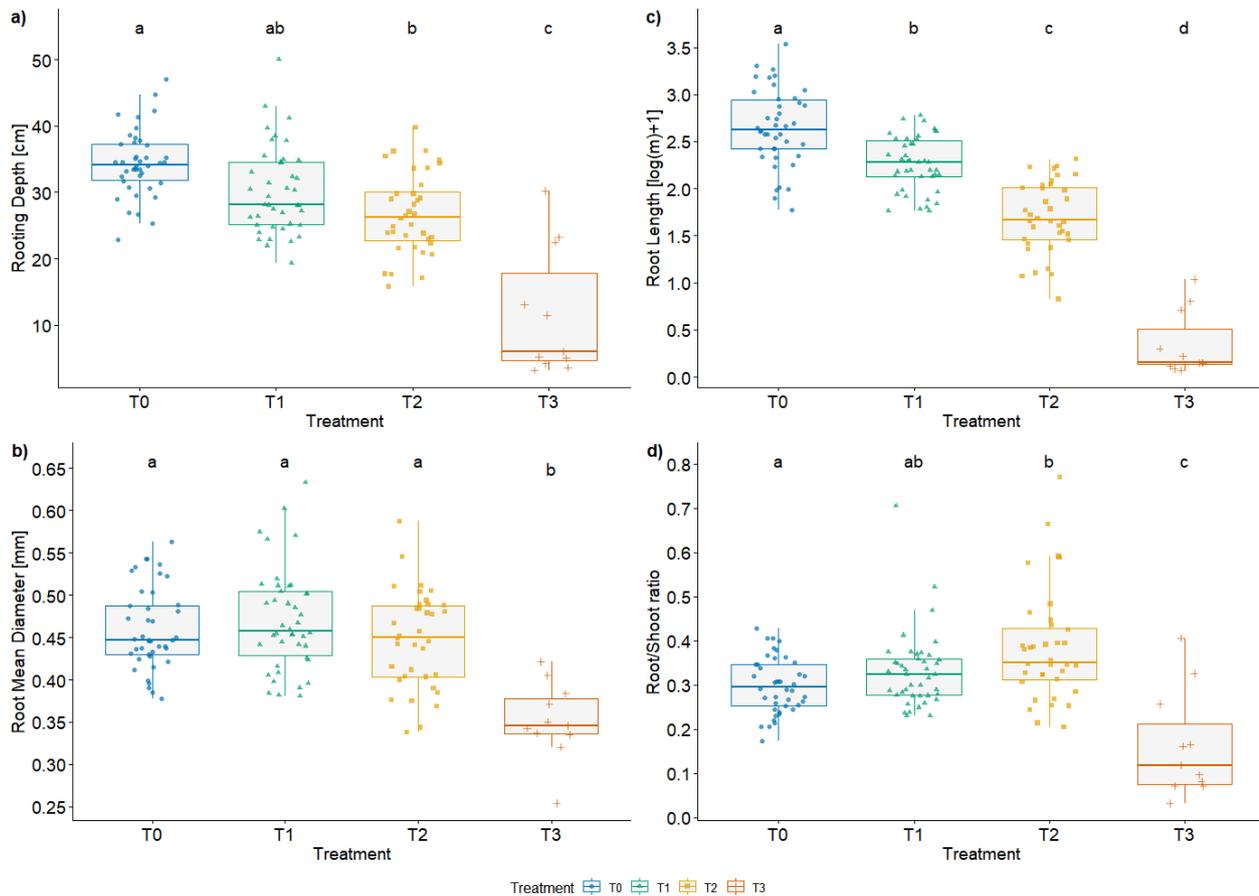
Fig. 3. Boxplots showing differences in belowground vegetative root traits (a–c) and root/shoot ratio (d) among treatments. Significant differences among treatments are based on *post hoc* multiple comparison tests of mixed effects models and are indicated by small letters with a significance threshold of $P < 0.05$.

Similarly, root mean diameter only significantly decreased after extreme drought ($\beta = -0.11$, $P < 0.001$). Moderate drought resulted in a slight, but not significant, increase in root/shoot ratio, which increased in response to strong drought ($\beta = 0.08$, $P = 0.002$), *i.e.* towards lower aboveground biomass; however, root/shoot ratio decreased considerably in the extreme drought treatment and shifted towards a higher proportion of aboveground biomass ($\beta = -0.14$, $P < 0.001$).

Physiological traits of the above- and belowground parts, in contrast, had few associations with increased drought (Fig. 4, Table S5.4). Neither SRL nor SLA showed a linear response to drought. However, SLA was largest under moderate drought (T1) and significantly reduced under strong drought (Fig. 4c). RDMC increased in response to moderate and strong drought ($\beta = 0.08$, $P < 0.01$), and was most pronounced in the extreme drought treatment ($\beta = 0.58$, $P < 0.001$). LDMC increased only in the extreme drought treatment ($\beta = 0.19$, $P < 0.001$).

These results indicate that total biomass, vegetative height, leaf length, leaf width, and leaf area were highly sensitive even to moderate drought and showed an increasingly negative response to higher drought stress. Although side shoot number responded in a similar direction, there was no difference between the moderate and strong drought treatments. Leaf number and side shoot length were less sensitive and only significantly reduced under strong drought. As survival is the best

fitness surrogate for this drought experiment, we also investigated the relationship between performance of previously identified traits and senescence stage 1, the survival indicator for which we had most observations (Table S6). Plants that reached senescence stage 1 had significantly lower total biomass ($\beta = -1.33$, $P = 0.005$) and significantly higher leaf width ($\beta = 1.01$, $P < 0.001$) compared to undamaged plants. However, vegetative height ($\beta = -0.12$, $P = 0.841$), leaf length ($\beta = -0.005$, $P = 0.993$) and leaf number ($\beta = -0.88$, $P = 0.066$) did not differ between plants starting to wilt and unwilted plants. Furthermore, among individuals, leaf width and total biomass were positively correlated ($r_s = 0.86$, $P < 0.001$), suggesting that leaf width may be directly linked to plant fitness under drought.

DISCUSSION

By applying an experimental approach to multi-level drought effects on seedling development and survival of the threatened mountain grassland species *A. montana*, our results demonstrate that many functional traits, especially in the morphological aboveground spectrum, decreased long before fitness (in sense of survival) declined. For example, leaf length and width were highly sensitive before dramatic reductions in plant fitness were visible.
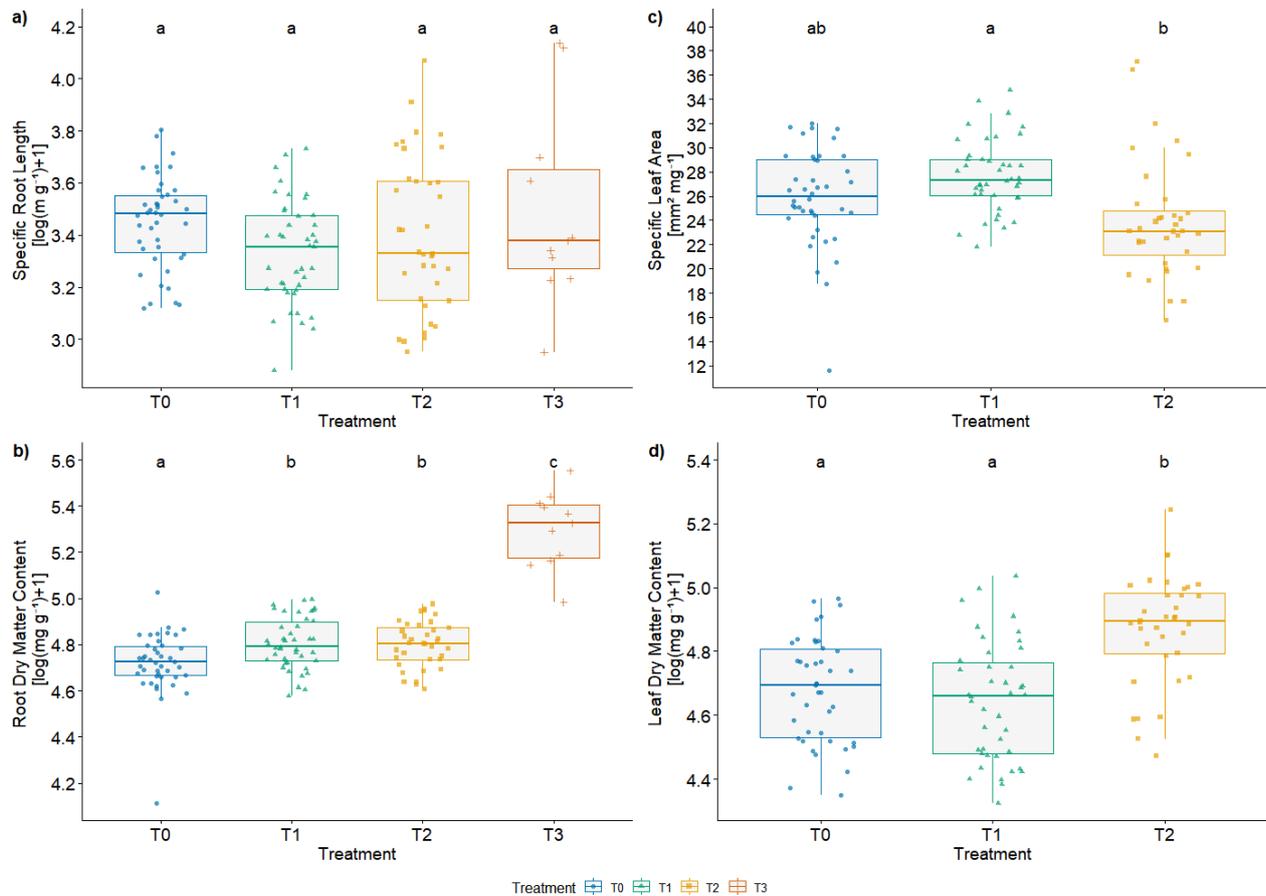
Fig. 4. Boxplots showing differences in physiological above- and belowground traits (a–d) among treatments. Significant differences among treatments are based on *post hoc* multiple comparison tests of mixed effects models and are indicated by small letters with a significance threshold of $P < 0.05$. Specific leaf area and leaf dry matter content could not be measured for plants of treatment T3.

Plant survival was not affected with sufficient water supply (T0) or even moderate drought (T1) but was markedly reduced by strong (T2) and extreme (T3) drought. This means that our experiment successfully simulated a range of growing conditions and could therefore resolve which traits were strongly associated with emerging drought stress. Moreover, the experiment showed that excluding all other potential threats to survival under natural conditions, *A. montana* juveniles from Central European mountains are resistant to moderate drought but threatened under the strong drought conditions. Notably, senescence and mortality strongly lagged behind falls in soil moisture below the permanent wilting point under extreme drought. This suggests that *A. montana* can withstand adverse environmental conditions over a rather long period before a fitness decline is observed.

Many of the traits declined steadily with increased drought even in the moderate drought treatment (T1), particularly total biomass and leaf traits. These plastic responses of *A. montana* to drought corroborate findings from studies of other grassland species, in which there was a reduced performance in relevant functional traits to drought stress (*e.g.* Weißhuhn *et al.*, 2011; Larson & Funk, 2016; Ludewig *et al.*, 2018). It is noteworthy that these responses to drought also correspond to earlier observations on adult *A. montana* along an aridity gradient, where plants in drier sites had smaller trait values (Stanik *et al.*,

2020). In that study, reproductive capacity declined at dry sites, while in the current study vegetative reproduction of juveniles was reduced with increased drought. This indicates that under strong drought *A. montana* was no longer able to invest resources into reproduction, resulting in a reduction in their fitness. Conversely, traits in the leaf and root economics spectrum were less sensitive across drought treatments, similar to results for temperate forbs that maintain SLA under drought (Wellstein *et al.*, 2017). Overall, *A. montana* seedlings showed plastic responses with declines in most above- and belowground vegetative traits in response to increased drought: plants became shorter, developed fewer and smaller leaves and had shorter roots.

Are if some of these plastic responses adaptive, in the sense that they adjust the plant to perform better when water is limited? By definition, an adaptive plastic response must be closely correlated to a positive change in fitness (Dechaine *et al.*, 2007). However, some responses may be part of a complex plastic adjustment to limited water conditions (Volaire, 2018). For example, despite a reduction in root length, the root/shoot ratio increased under strong drought towards increased belowground biomass, which likely represents increased water acquisition and reduced transpiration due to a decrease in leaf area and SLA (Wang *et al.*, 2020). However, this capacity was limited under extreme drought (T3) when biomass allocation

halted, leading to a markedly higher proportion of above-ground biomass produced in the early ontogenetic stage. The lower root mean diameter under extreme drought might indicate that these plants were 'trapped' in an early growth state and were not able to develop thicker roots. Hence, these plants could not adjust belowground growth for higher water acquisition or drought avoidance under increased drought stress (Balachowski et al., 2016; Larson & Funk, 2016). Overall, we observed some belowground adjustments to strong drought but not to extreme drought.

Another potentially adaptive adjustment is regulation of leaf traits to reduce transpiration when water is limited (e.g. Stropp et al., 2017). Despite the observed responses of morphological leaf traits, typical candidate traits for functional drought adaptation in the leaf economics spectrum, such as SLA or LDMC, responded only at high levels of drought. Similar to the findings from subalpine grasslands of Jung et al. (2014), A. montana under strong drought responded by decreasing SLA and increasing LDMC. Both responses are related to higher investment in structural tissues in order to maintain leaf turgor and enhance water-use efficiency under drought, both of which prolong the leaf life span (Chaves et al., 2002; Yan et al., 2019). Hence, the observed leaf trait responses of A. montana to different drought levels may constitute a strategy of phenotypic adjustment to cope with drought stress (Albert et al., 2010; Wellstein et al., 2017).

Based on the determined survival and functional trait responses of A. montana, we identified sensitive response traits that can indicate a decline in performance and have predictive power for plant survival. Most promising are leaf traits, such as leaf length or width, because they respond sensitively even to moderate drought. Of these functional traits, leaf width is of special interest because individuals that senesced had, after removal of the treatment effect, a high leaf width, which leads to increased transpiration. In addition, leaf width was negatively associated with total biomass. This suggests that leaf width could be a target of direct selection under drought.

In the present study, limitations of the experimental approach to evaluate drought effects on seedlings can arise from the experimental set up, even when the experimental drought treatments reflect current climate conditions for A. montana lowland populations in Central Europe (Stanik et al., 2020). The experiment considered a competition-free environment, characterised by edge effects, e.g. a reduced soil volume for potted plants. Nonetheless, this may also be present for the species under inter- and intraspecific competition in its natural habitat. Moreover, under natural conditions additional threats, such as herbivory or shallow soil, may further reduce plant viability (Scheidel & Bruelheide, 2005; Ford & HilleRisLambers, 2020). Here, we conducted a drought experiment to examine the effects of drought as one of the main climate change challenges to A. montana. We applied several drought treatments designed to match the expected drought forecasts in climate change projections, which makes our results relevant for projecting the future of this threatened plant (Parolo et al., 2008). Despite higher monthly air temperatures in the experiment compared to those in the native population, no negative effects of temperature were observed, e.g. reduced survival of plants in the control treatment. This supports the view that the observed response is largely drought- but not temperature-driven (Kreyling et al., 2017). A potential confounding side effect of the applied drought treatments was that it contained a selection pressure that may have led to a survivorship bias in the results towards possible increased frequency of drought-resistant genotypes in the tested population (Gienapp et al., 2008). Due to methodological requirements in the trait sampling, dead seedlings from the extreme drought treatment (T3) could not be incorporated into the results to cover all individuals in the experiment. However, measurements in the other treatments were unbiased and directly linked seedling survival to the drought-induced response of traits and, therefore, could link the observed trait differences to the species' plasticity.

To conclude, drought stress increasingly affects the performances of functional traits and hinders subsequent establishment and survival of seedlings of the threatened mountain grassland species A. montana. The species sensitive response to drought starts with a decrease in most functional traits but clear signs of senescence only occur comparatively late, which suggests relatively high stress tolerance to moderate drought, which limits only reached under severe drought conditions. The identified leaf traits of A. montana thus indicate a fitness decline prior to a substantial increase in mortality. Future research may be needed to investigate the potential of the identified trait–survival relationships and their use in a trait-based drought stress assessment and monitoring of A. montana and other threatened grassland species (Baer et al., 2019).

AUTHOR CONTRIBUTIONS

NS and GR conceived the idea, NS designed and conducted the experiment; NS and CL analysed the data. All authors contributed to interpretation and discussion of the results. NS led the writing of the manuscript, GR and CL provided critical comments and revisions. All authors gave their final approval for submission and publication.

DATA AVAILABILITY STATEMENT

The functional trait and senescence data of A. montana and data about the experimental conditions are published at Zenodo.org at https://doi.org/10.5281/zenodo.5018814.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Climate characterization of the seed origin Arnica montana population.

Figure S2. Climate conditions in the greenhouse over the course of the experiment.

Table S1. Water amounts applied in each treatment.

Table S2. (a) Development of soil water content over the course of the experiment and (b, c) detailed mixed effects model results for soil water and soil temperature differences in the experiment treatments.

Table S3. Detailed mixed effects model results of the functional trait performance per drought treatment. (a) Aboveground vegetative traits, (b) whole plant vegetative traits, (c) belowground traits, performance of physiological traits. Results of the generalized binominal mixed effects models on the events of senescence stage 1 and selected functional trait performances.

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