

Plant stress memory within and across generations

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AOX	Antioxidant System
APS	Ammonium persulfate
APX	Ascorbate Peroxidases
BADH	Glycine Betaine Aldehyde Dehydrogenase
BSA	Bovine Serum Albumin
Car	Carotenoids
CAT	Catalase
CBB	Coomassie brilliant blue
Chl <i>a</i>	Chlorophyll a
Chl <i>b</i>	Chlorophyll b
DHAR	Dehydroascorbate Reductase
DMF	N, N dimethylformamide
EDTA	Ethylenediaminetetraacetic Acid
GPOX	Guaiacol Peroxidase
GPX	Glutathione peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GST	Glutathione S-Transferase
HSP	Heat Shock Proteins
LMA	Leaf Mass per Area ratio
MAE	Maternal Adaptive Effects
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate Reductase
Na Pi	Sodium-potassium buffer
NBT	Nitro Blue Tetrazolium
PAGE	Polyacrylamide Gel Electrophoresis
PMSF	Phenylmethylsulphonyl Fluoride
POX	Peroxidase
PPO	Phenol oxidase
PRX	Peroxiredoxins
PSI	Photosystem I

PSII	Photosystem II
PVP	Polyvinylpyrrolidone
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
RuBP	Ribulose-1,5-bisphosphate
RWC	Relative Water Content
SDS	Sodium Dodecyl Sulphate
SLA	Specific Leaf Area
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TCA	Trichloroacetic acid
TE	Transgenerational effects
TEMED	Tetraacetylenediamine
TRX	Thioredoxin
WALD	Wald analytical long-distance dispersal model

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SUMMARY

Under climate change, drought and waterlogging stresses will become more frequent and intense in many parts of the world. These stresses depress plant growth by inhibiting photosynthesis and causing oxidative cell damage. One of the promising strategies of plants to cope with such harmful conditions is plant stress memory. Plant stress memory is the capacity of plants to store previous stress information to trigger an improvement in plant performance as a response to repeated stress in the same generation (within-generation memory) or in the next generation (transgenerational memory). Plants also have cross-stress memory; they can tolerate one type of stress while previously exposed to another. Additionally, plant stress memory could be stronger in species and individuals with dispersal limitation since dispersal limitation could enlarge congruence between offspring and their mothers. Mechanisms underlying drought and waterlogging memory and possible cross-stress memory in single or offspring generations are currently unclear. In addition, studies testing the strength of plant stress memory are rare.

In this PhD thesis, I hypothesised that drought and waterlogging stress lead to a formation of stress memory that improves plant performance and causes changes in morphological, photosynthetic and antioxidative parameters under recurring drought within the same generation. To test this hypothesis, I repeatedly subjected *Alopecurus pratensis* grass plants to waterlogging and drought over two years. In the third year, plants were well-watered for three weeks to recover and then subjected to drought stress for two weeks. My results confirmed the first hypothesis that plants pre-exposed to drought showed less tissue damage, higher levels of *Rubisco* content, antioxidative enzymes (POX and SOD) and chlorophyll *b* after repeated drought stress in the same generation. I thus show for the first time that grasses can show a long-term drought stress memory lasting over several weeks.

Further, I hypothesised that offspring experiencing stress performed better when mothers had experienced stress before, irrespective of the type of stress. To test this, I conducted a full-factorial pot experiment over two generations, subjecting maternal and offspring plants to waterlogging and drought. In line with my second hypothesis, maternal waterlogging and drought experience in four perennial species increased biomass and reproductive output in offspring experiencing the same stress conditions as their mothers (transgenerational adaptive effects). This transgenerational memory was linked to changes in the antioxidative system of one species, reducing oxidative damage through the upregulation of protective enzymes in offspring experiencing the same conditions as their mothers. However, I detected only same-stress but no cross-stress memory in the experiment.

Lastly, I hypothesised that the strength of adaptive maternal effects increases in species or individual seeds with lower seed dispersal ability. To test the third hypothesis, seed heads were collected from the lower and upper parts of each mother plant to test the strength of the transgenerational memory and used for dispersal distance calculation. Offspring from the maternal lower and upper parts were exposed to the same water treatments as mothers. In the third experiment, maternal plants could not prepare their offspring for upcoming water stresses. Also, a maternal stress experience *per se* did not pre-condition offspring to other types of stress. Further, opposite to my expectations, seeds with a longer expected dispersal distance showed a stronger maternal adaptive effect when exposed to the same water stress as their mothers.

My research provides evidence for within-generational and transgenerational drought memory and links this to underlying photosynthetic and redox-related mechanisms. In addition, transgenerational waterlogging was linked to a similar mechanism. This might contribute to the rapid responses of plants to environmental change. However, as I showed in this thesis, plant stress memory is not present in all plant species. In the future, it will be important to investigate the cause of the ecologically significant discrepancies in maternal effects among species. Different maternal effects among species could increase biodiversity. A broader understanding of transgenerational memory could help to adjust crop improvement strategies for economically and ecologically important species.

ZUSAMMENFASSUNG

Im Zuge des Klimawandels werden Trockenheit und Staunässe in vielen Teilen der Welt immer häufiger und intensiver auftreten. Diese Stressfaktoren beeinträchtigen das Pflanzenwachstum, indem sie die Photosynthese hemmen und oxidative Zellschäden verursachen. Eine vielversprechende Strategie von Pflanzen, um mit solchen schädlichen Bedingungen fertig zu werden, ist das pflanzliche Stressgedächtnis. Dies ist die Fähigkeit von Pflanzen, frühere Stressinformationen zu speichern, um als Reaktion auf wiederholten Stress in derselben Generation (Gedächtnis innerhalb einer Generation) oder in der nächsten Generation (transgeneracionales Gedächtnis) eine Verbesserung der Pflanzenleistung auszulösen. Pflanzen verfügen auch über ein Cross-Stress-Gedächtnis; die Konfrontation mit einer Art von Stress, erhöht die Toleranz gegenüber einer anderen Stressart. Darüber hinaus könnte das Stressgedächtnis von Pflanzen bei Arten und Individuen mit eingeschränkter Ausbreitung stärker ausgeprägt sein, da eine eingeschränkte Ausbreitung die Kongruenz zwischen der Umgebung der Mutter und ihrer Nachkommen vergrößern könnte. Die Mechanismen, die dem Gedächtnis für Trockenheit und Staunässe und dem möglichen Cross-Stress-Gedächtnis innerhalb einzelner Generationen oder bei Nachkommen zugrunde liegen, sind derzeit unklar. Außerdem gibt es nur wenige Studien, die die Stärke des Stressgedächtnisses von Pflanzen untersuchen.

In dieser Doktorarbeit stellte ich die Hypothese auf, dass Trockenheit und Staunässe zur Bildung eines Stressgedächtnisses führen, das die Leistung der Pflanzen verbessert und Veränderungen der morphologischen, photosynthetischen und antioxidativen Parameter bei wiederholter Trockenheit innerhalb derselben Generation bewirkt. Um diese Hypothese zu testen, habe ich *Alopecurus pratensis* über zwei Jahre hinweg wiederholt Staunässe und Trockenheit ausgesetzt. Im dritten Jahr wurden die Pflanzen ann zwei Wochen lang Trockenstress ausgesetzt. Meine Ergebnisse bestätigten die erste Hypothese, dass Pflanzen, die zuvor Trockenheit ausgesetzt waren, nach wiederholtem Trockenstress in derselben Generation weniger Gewebeschäden und einen höheren Rubisco-Gehalt, und Gehalt an antioxidativen Enzymen (POX und SOD) und Chlorophyll b aufwiesen. Gräser weisen ein langfristiges Trockenstressgedächtnis über mehrere Wochen hinweg auf und dies hängt mit dem antioxidativen System zusammen.

Darüber hinaus stellte ich die Hypothese auf, dass Nachkommen, die Stress erfahren, besser abschneiden, wenn die Mütter zuvor Stress erfahren haben, unabhängig von der Art des Stresses. Um dies zu testen, führte ich ein voll-faktorielles Topfexperiment über zwei

Generationen durch, bei dem Mutter- und Nachkommenpflanzen Staunässe und Trockenheit ausgesetzt wurden. Im Einklang mit meiner zweiten Hypothese erhöhte die Erfahrung der Mutter mit Staunässe und Trockenheit bei vier mehrjährigen Arten die Biomasse und die Reproduktionsleistung der Nachkommen, die denselben Stressbedingungen ausgesetzt waren wie ihre Mütter. Dieses transgenerationale Gedächtnis wurde mit Veränderungen im antioxidativen System einer Art in Verbindung gebracht, die oxidative Schäden durch die Hochregulierung von schützenden Enzymen bei den Nachkommen verringerten, die denselben Bedingungen ausgesetzt waren wie ihre Mütter. Allerdings konnte ich in dem Experiment nur eine Erinnerung an die gleiche Belastung, aber keine Erinnerung an eine andere Belastung feststellen (keine Cross-Stress-Toleranz).

Schließlich stellte ich die Hypothese auf, dass die Stärke der adaptiven mütterlichen Effekte bei Arten oder einzelnen Samen mit geringerer Fähigkeit zur Samenverbreitung zunimmt. Um die dritte Hypothese zu prüfen, wurden von den unteren und oberen Teilen jeder Mutterpflanze Samenköpfe gesammelt, um die Stärke des transgenerationalen Gedächtnisses zu testen, und für die Berechnung der Ausbreitungsdistanz verwendet. Die Nachkommen der unteren und oberen Teile der Mutterpflanzen wurden denselben Wasserbehandlungen ausgesetzt wie die Mütter. Im dritten Experiment konnten die Mutterpflanzen ihre Nachkommen nicht auf den bevorstehenden Wasserstress vorbereiten. Auch eine mütterliche Stresserfahrung als solche konditionierte die Nachkommen nicht auf andere Arten von Stress. Außerdem zeigten Samen mit einer längeren erwarteten Ausbreitungsdistanz entgegen meinen Erwartungen einen stärkeren mütterlichen Anpassungseffekt, wenn sie demselben Wasserstress ausgesetzt waren wie ihre Mütter.

Meine Forschung liefert Beweise für ein Trockenheitsgedächtnis innerhalb von Generationen und über Generationen hinweg und stellt eine Verbindung zu den zugrunde liegenden photosynthetischen und redoxbezogenen Mechanismen her. Darüber hinaus wurde die transgenerationale Staunässe mit einem ähnlichen Mechanismus in Verbindung gebracht. Dies könnte zu den schnellen Reaktionen der Pflanzen auf Umweltveränderungen beitragen. Wie ich jedoch in dieser Arbeit gezeigt habe, ist das pflanzliche Stressgedächtnis nicht bei allen Pflanzenarten vorhanden. In Zukunft wird es wichtig sein, die Ursache für die ökologisch bedeutsamen Diskrepanzen bei den mütterlichen Effekten zwischen den Arten zu untersuchen. Unterschiedliche mütterliche Effekte zwischen den Arten könnten die Artenvielfalt erhöhen. Ein umfassenderes Verständnis des transgenerationalen Gedächtnisses könnte dazu beitragen, die Strategien zur Verbesserung von Pflanzen für wirtschaftlich und ökologisch wichtige Arten anzupassen.

Chapter 1

GENERAL INTRODUCTION

Increases in the frequency of extreme events are caused mainly by global warming (Jiang et al., 2018). Being sessile, plants cannot easily evade stress environmental stress conditions. Environmental changes and stresses can occur episodically or in a cyclic way over days, seasons and years (de Freitas Guedes et al., 2019; IPCC, 2021). The climate models predict higher inter-annual and intra-annual variability (Kreuzwieser and Gessler, 2010). On the one hand, frequent and extended periods of summer drought events; on the other hand, changes in precipitation patterns and increased rainfall will alter the risk of drought and flooding in Central and Northern Europe (Kreuzwieser and Gessler, 2010). Such an extreme event may cause more severe effects on plants, populations, communities, and ecosystems than gradual changes (Backhaus et al., 2014). Depending on the plant's tolerance to reduced water availability, on the one side, and the oxygen depletion caused by flooding, on the other side, plants' growth, reproduction and competition abilities could be remarkably affected (Kreuzwieser and Gessler, 2010).

Drought and waterlogging stress are major abiotic stresses. Drought stress causes changes in plants: reduced water content, leaf water potential and turgor loss, causing stomata closure and diminishing cell enlargement and growth. This stress can decrease net photosynthesis due to a low leaf area, increased senescence, and a higher resistance for gas exchange in stomata (Jaleel et al., 2008; Zare et al., 2011; Shao et al., 2008). Consequently, a reduction in plant size, aboveground plant biomass and reproductive output will be noted (Zare et al., 2011). On the other hand, drought causes expansion in the plant root system, which can improve water uptake capacity (Shao et al., 2008).

On the other hand, waterlogging and flooding induce various changes in soil properties, such as pH, redox potential and oxygen level (Ashraf, 2012). Due to oxygen deprivation, roots are not aerated during waterlogging, and gas diffusion is reserved. Consequently, plants are forced to change from aerobic to anaerobic metabolism, which could be severe for plant growth, development and survival (Onyekachi et al., 2019). Both drought and waterlogging stress lead to morphological (reduced plant biomass, plant height, number of leaves, germination), physiological (reduced photosynthetic activity, pigment content, membrane integrity), biochemical (accumulation of osmoprotectants and antioxidants), and molecular (expression of stress-related genes) changes (Farooq et al., 2012; Fahad et al., 2017).

In order to adapt to changing climates, plants have to find suitable ways to respond to recurrent stresses. Pre-exposure to mild abiotic or biotic stress can preadapt plants to the following severe stress exposure (Walter et al., 2011). This phenomenon is known as plant stress memory, a promising way of improving stress tolerance (Bruce et al., 2007). Generally,

stress memory represents genetic, epigenetic and physiological changes in plants under stress conditions that modify responses to repeated stress within one generation or in the next generation (also known as transgenerational memory). Drought-induced and waterlogging-induced acclimation responses and stress memory in plants could be crucial strategies for coping with the harmful effects of global warming (Li and Liu, 2016; Feng et al., 2022). In recent years, plant stress memory has received much attention since it might help plants to cope rapidly and effectively with future stressful events (Liu et al., 2022; Agualongo et al., 2022; Mollard et al., 2022). Plant stress memory could be a promising strategy for future research and applications in agriculture and breeding selection processes. However, it is still unknown how widespread waterlogging plant stress memory is and – more generally – which mechanisms cause plant stress memory. A better understanding of these mechanisms will contribute to a better understanding of ecological processes and more precise predictions and strategies to cope with the consequences of climate change. This PhD thesis thus tries to close existing knowledge gaps in plant stress memory, plant stress tolerance and generally ecology.

1.1. Within-generation plant stress memory (Priming)

Pre-exposure to stress can induce a stress memory with the consequence that plants show a faster and improved reaction to a second stress event. This phenomenon is also known as priming and is considered one possible way to improve stress resistance. It relates to plant stress memory (Bruce et al., 2007). Priming has three phases: pre-challenge priming, challenge priming and transgenerational priming state (Conrath et al., 2002; 2006; Gamir et al., 2014). This can be applied to different developmental plant stages, from seeds to adult plants (Lal et al., 2018). Both abiotic and biotic stresses can induce priming. Different abiotic stresses can induce the same type of signalling cascades on the molecular, cellular and physiological levels; therefore, a mild pre-exposure could enhance tolerance to a second, stronger stress. Priming can either be “cis-priming”, for which the stimuli and second stress are the same, or “trans-priming”, where the stimuli and the second stress are not the same (Hilker et al., 2016).

The main abiotic priming stimuli studied are high temperatures, cold, drought, salinity, and chemical compounds (Fig. 1.1). For instance, short-term heat priming can cause thermo-tolerance to later recurred heat stress, improving osmoregulation, detoxification, stress protein synthesis (Wang et al., 2014a; Fan et al., 2018), grain yield and quality (Wang et al., 2014b). A

higher rate of photosynthesis followed by redox homeostasis was observed in heat-primed compared to non-primed plants (Wang et al., 2014a). Another factor that affects plant growth and development is cold. Pre-exposure to cold stress induces osmoprotectants accumulation, increased membrane stability (Iba, 2002), higher photosynthetic performance, and reproductive development of plants after the second cold exposure (Cvetkovic et al., 2002).

In water-limited areas, short-term drought priming could be a promising strategy to induce drought tolerance in water-limited areas. Drought tolerance generated during drought-priming is based on a higher photosynthesis rate, maintenance of reactive oxygen species (ROS) homeostasis, and accumulation of proline and sugars (Ben Abdallah et al., 2017). Nevertheless, adaptive effects are not guaranteed; the efficiency of priming may depend on the plant species, threshold effects of extreme conditions, plant life, exposure time etc. Many studies have reported priming effects that lasted for several days, and this kind of memory may last longer than this (e. g. Wang et al., 2014a, b; Fan et al., 2018). However, studies showing long-term (over several weeks) drought priming are largely missing. Also, the mechanisms by which plants store information from previous stress exposure are still unclear. In this thesis, I tried to fill this gap.

1.2. Transgenerational plant stress memory

Transgenerational plant stress memory means that environmental conditions experienced by previous generations affect the phenotype and plant performance of the offspring beyond the effect of transmitted genes (Mousseau and Fox, 1998). Transgenerational plant stress memory contributes to phenotypic variation (Lal et al., 2015). As such, transgenerational plant stress memory can affect plant fitness and population dynamics. Numerous types of environmental information can be transmitted through these mechanisms of transgenerational memory, such as paternal exposure to drought (Sultan et al., 2009; Herman et al., 2012; Herman and Sultan, 2016; Wang et al., 2019), temperature changes (Suter and Widmer, 2013; Xin et al., 2016), heavy metal exposure (Reza Rahavi et al., 2011), salt (Suter and Widmer, 2013) and UV-C irradiation (Ćuk et al., 2010) (Table 1.1).

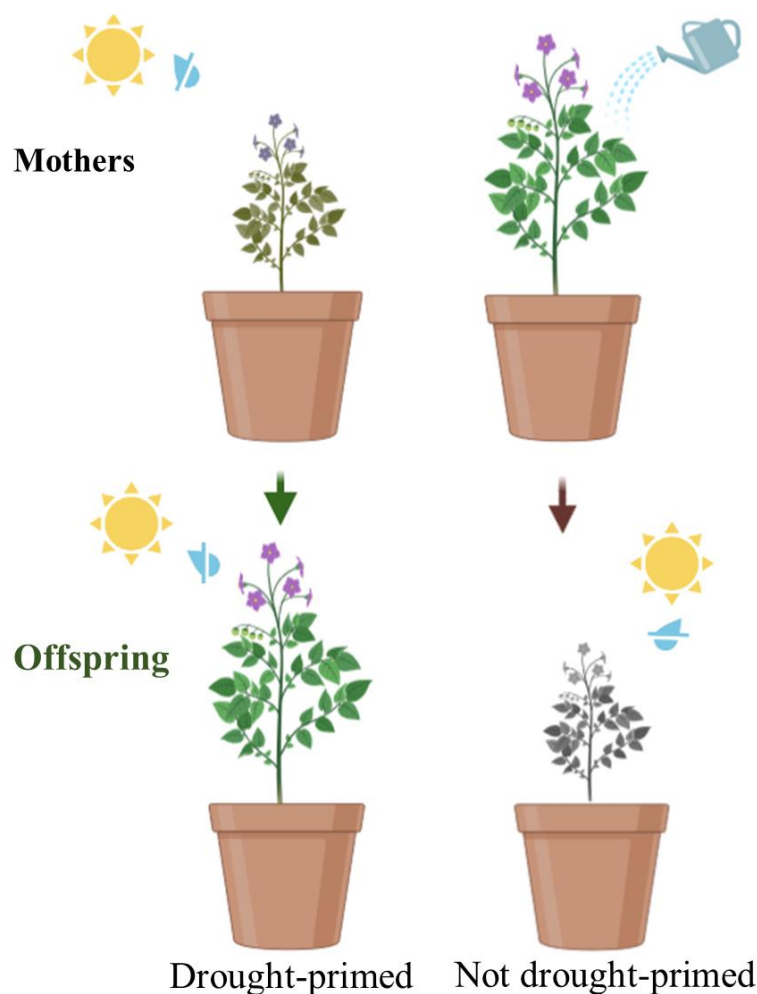


Fig. 1.1 Drought priming triggering plant tolerance.

Table 1.1 Examples of transgenerational memory. An overview of different species, parental environment and transgenerational responses are given.

Species	Parental environment	Transgenerational responses	References
<i>Arabidopsis thaliana</i>	UV-C irradiation	↑ Peroxidase, HSP70 proteins ↓ Catalase, Ascorbate peroxidase	Čuk et al., 2010
<i>Polygonum persicaria</i>	Drought	↑ Plant performance, aboveground biomass	Sultan et al., 2009
<i>Trifolium repens</i>	Drought Copper	↓ aboveground biomass ↑ aboveground biomass	Rendina González et al., 2018
<i>Lycopersicon esculentum</i>	Cold/H ₂ O ₂	↑ oxidative stress response, amelioration of chilling injury, and activation of antioxidant enzymes	İşeri et al., 2013; Zhang et al., 2013
<i>Brassica napus</i>	Cold/heat/drought	↑ Heat/drought resistance, increased growth and yield	Liu et al., 2017; Hatzig et al., 2018
<i>Medicago sativa</i>	Drought	↑ growth, photosynthetic efficiency, leaf area, plant height, K ⁺ accumulation	Mouradi et al., 2016
<i>Hordeum vulgare</i>	Drought/	↓ shoot/root ratio, the number of thick roots in the progeny ↑ growth of thin roots	Nosalewicz et al., 2016
<i>Arabidopsis thaliana</i>	Mild drought	↑ phenotypic and DNA methylation plasticity ↓ Transgenerational plasticity	Van Dooren et al., 2018
<i>Glycine max</i>	Drought	↓ germination, seedling vigour, and seed quality ↑ Transgenerational effect	Wijewardana et al., 2019
<i>Triticum aestivum</i>	Drought	↑ Reproductive output, water retention, osmolytes accumulation, oxidative stress responses	Tabassum et al., 2017; 2018
<i>Triticum aestivum</i>	Drought	↑ grain yield, photosynthetic leaf rate and antioxidant capacity, osmolytes accumulation, oxidative stress responses	Wang et al., 2019
<i>Triticum aestivum</i>	Heat	↑ grain yield, leaf photosynthesis, activities of antioxidation enzymes, histone demethylation, global DNA methylation ↓ cell membrane damage	Wang et al., 2016a

Maternal effect

Transgenerational plant stress memory can be in the form of maternal and paternal effects. It has been shown that maternal effects are stronger and more predictable than paternal effects since environmental heterogeneity within a population is often on a larger scale than seed dispersal distances but smaller than pollen movement (Galloway, 2004; Galloway and Etterson, 2007). Therefore, the offspring's environment will be similar to the maternal environment but less likely to the father's. Indeed, maternal adaptive effects are a special case of transgenerational plant stress memory. It means that the maternal environment positively affects offspring fitness and performance (Fig. 1.2b). In other words, it offers a strategy for plants to maximize the fitness of their offspring. However, transgenerational plant stress memory can also be maladaptive or neutral (Fig. 1.2a, c).

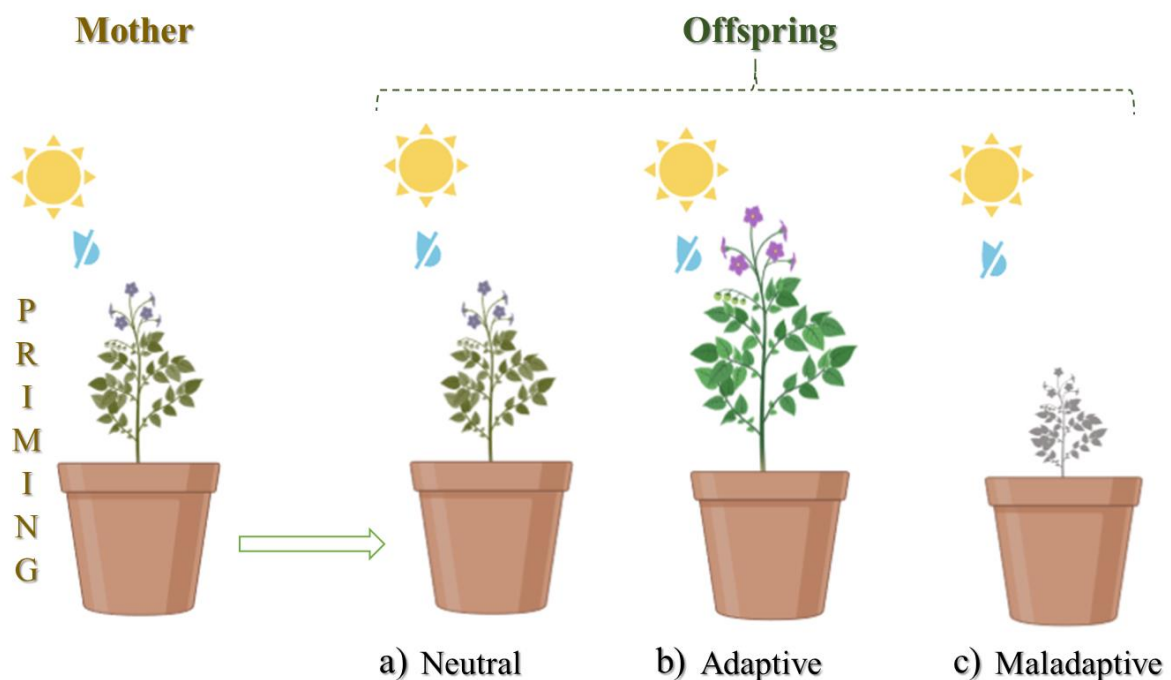


Fig. 1.2 Different types of maternal effects, neutral, adaptive and maladaptive, when mothers and offspring are exposed to drought.

Marshall and Uller (2007) suggests that maternally-induced changes are adaptive when:

- (1) there is an environmental change between generations;
- (2) offspring environment can be predicted from maternal phenotype;
- (3) the costs of maternal cue information (detection and transmission) are low;
- (4) parent-offspring conflict is on a low level.

Based on this, the authors (Marshall and Uller, 2007) introduced three different types of maternal effects:

- (1) **Selfish maternal effects:** increased maternal performance, which is a drawback for their offspring. This type of maternal effect occurs when the maternal effect is too costly for the mother, when mothers have to reproduce rapidly because of selection pressure or when mothers have an opportunity for reproduction in a “better” environment.
- (2) **Bet-hedging maternal effects (maternal adaptive effect):** reduce variance in maternal fitness by producing offspring with a range of phenotypes and modifying offspring traits to enhance the performance in a changeable environment. Mothers, in this case, not only adjust the offspring's performance but also produce offspring with highly diverse phenotypes that are immensely beneficial under unpredictable changeable environments.
- (3) **Transmissive maternal effects:** maternal and offspring fitness reduction. For instance, when plants are exposed to toxic substances, they produce offspring with little or no survival potential (Wiklund and Sundelin 2001).

Maternal and grandmaternal drought in *Polygonum persicaria* plants improved offspring seed-provision, growth and root systems when exposed to the same type of stress as their mothers and grandmothers (Herman et al., 2012). These developmental adjustments were mediated by hormonal and/or epigenetic modifications (Sultan et al., 2009). In addition, the large survivorship of plants in drought shows that adaptive transgenerational effects were persistent over a few generations and can impact realized fitness in nature (Herman and Sultan, 2011).

Costs of transgenerational plant stress memory include direct allocation costs, which are directly related to the biosynthesis of defensive proteins and secondary metabolites (Züst et al., 2011). Additionally, plants developed erasing and resetting mechanisms that can cause metabolic costs if maintained for an extended period (Cool and De Veylder, 2009; Choudhary et al., 2021). Indeed, many transcripts, stress-related proteins and metabolites would be in the prestress state (Crisp et al., 2016).

1.3 Cross-stress memory

Plants are constantly exposed to a broad range of environmental stress factors that occur either simultaneously or successively. They must find a way to adapt to the changeable nature and prevent an attack of other stresses. Consequently, plants may develop a cross-stress memory, where they can tolerate one type of stress while previously exposed to another. Many stresses act similarly on a molecular and physiological level, such as cold, drought, heat etc. Cross-stress memory is a complex process involving physiological, genetic, and morphological changes (Munné-Bosch and Alegre, 2013). This type of plant stress memory can be inherited or induced (Llorens et al., 2020). The first type of cross-stress memory represents the capacity of plants to resist multiple stresses because of their genetic background. Inherited cross-stress memory is correlated with changes in signal transduction, detoxification processes, and genetic changes (Poland et al., 2009). On the other hand, induced cross-stress memory is defined as the activation of systemic plant tolerance due to previous exposure to another kind of stress.

Cross-stress memory has been reported in different stress types (Steinberg, 2012). Many studies showed that cold and heat-induced stress could lead to a cross-stress memory of other abiotic stresses (Şekara et al., 2016; Hossein et al., 2018). For instance, different species showed cross-stress memory (*Arabidopsis thaliana*, *Triticum aestivum*, *Avena sativa* and *Fragaria vesca*) where drought-stressed plants, besides drought tolerance, induced cold tolerance (Rajashekar and Panda 2014). One explanation for cross-stress memory is the plant response on the cellular and transcriptional level, in which mechanisms of plant stress memory are highly connected (Mahajan and Tuteja, 2005). But also, in the opposite direction, Blödner et al. (2005) reported that cold-priming in *Picea abies* could increase drought tolerance. *Camelia sinensis* plants exposed to cold stress showed drought-induced leaf senescence because of enhanced antioxidative enzyme activity (Zheng et al., 2016). Similar results were reported in *Coffea spp.* plants by Ramalho et al. (2018), where water deficit in the cold season improved cold cross-tolerance mainly due to an increase in antioxidative adaptation. Heat-induced stress in maize plants can also enhance plant tolerance to cold, chilling, drought and salt (Alexieva et al., 2001).

In addition, biotic stresses could enhance abiotic cross-tolerance and the opposite (Llorens et al., 2020). For instance, *Arabidopsis thaliana* plants exposed to the soil fungal pathogen *Verticillium longisporum* increased drought tolerance through *de novo* xylem formation (Reusche et al., 2012).

The mechanisms of cross-stress memory are still not completely clear; however, some studies showed that plant single stress responses (biotic and abiotic) are mediated by common signals: reactive oxygen (ROS) and nitrogen (RNS) species (Turkan, 2018), calcium signalling (Ranty et al., 2016), heat shock proteins (HSP) (Swindell et al., 2007), as well as phytohormones, especially salicylic, jasmonic and abscisic acids. An interplay between molecules could be the key to cross-stress tolerance (Bartoli et al., 2013).

A similar mechanism on the cell level could be an advantage for plants that experienced drought in one season and waterlogging in the next season and in reverse. Still, drought/waterlogging cross-stress memory remains unstudied.

1.4 Maternal characteristics affecting maternal effects

Detecting the maternal characteristics influencing the existence and strength of maternal effects is crucial. Studies relating maternal plant traits to the prevalence or strength of maternal effects are rare. One of the most important maternal attributes is the range of seed dispersal. It is expected that maternal seed dispersal distance impacts maternal effects. In theory, mother plants with long-distance seed dispersal will not be a good predictor of their offspring environment. Consequently, maternal effects that transmit information about specific environments are less likely to evolve. On the other hand, in plants where short-distance seed dispersal is common, offspring are more likely to encounter the same conditions as their mothers, and this is mainly when an environment has temporal constancy and high spatial heterogeneity. In theory, dispersal limitation could enlarge congruence between offspring and their mothers, indicating that maternal effect strongly depends on their dispersal distance (Sultan et al., 2009; Herman and Sultan, 2011; McNamara et al., 2016; Yin et al., 2019). Generally, when the scale of seed dispersal is less than the extent of habitat patches, offspring and maternal environment will be compatible, contributing to the evolution of adaptive maternal effects (Galloway, 2004).

In contrast, we can expect genetic adaptation when environmental variation is larger than the seed movement. This phenomenon is known as local adaptation (Linhart and Grant, 1996). Pollen dispersal among habitats will favour adaptive plasticity evolution (Sultan and Spencer, 2002), but if the plant fitness in the habitat is increased by its own or maternal experience, then the plants have phenotypic adaptation. Hence, a heterogeneous environment,

limited seed dispersal, and habitat patches should favour maternal effects to obtain phenotypic adaptation to local environmental conditions (Galloway, 2005).

In addition, maternal effects are seed-dimorphism dependent. Seed dimorphism and heteromorphism are common phenomena in plants (Imbert, 2002; Wang et al., 2010) when one plant simultaneously produces two or more seed types. Maternal plants with dimorphic or heteromorphic seeds are likely to store a different amount of nutrients and phytohormones in divergent seeds (Liu et al., 2018; Arshad et al., 2019; Gan et al., 2022). Therefore, maternal effects and cues to the same environment in the dimorphic seed might differ (Gan et al., 2022). Wang et al. (2022) showed that maternal effects differed among heteromorphic seeds from a single mother exposed to salinity stress. The same pattern was confirmed in the recently published study by Gan et al. (2022), where authors showed that transgenerational effects were dimorphism-dependent, also showing a significant divergence in drought tolerance among offspring. Authors suggested that divergent effects on offspring had differences in abscisic acid and soluble sugar concentration.

1.5 Mechanisms of plant memory

Plant stress memory enables plants to remember previous stress experiences (related to metabolic change and gene expression) and to store them for future stress events (Bruce et al., 2007; Galis et al., 2009; Ramírez et al., 2015). The mechanism of stress memory could involve modification on different levels, including morphological, physiological, transcriptions, translational and epigenetic (Kinoshita and Seki, 2014; Sun et al., 2018).

Studies suggested that remembering and recalling stored information could be possible due to cell signalling and adaptive development. Signalling molecules, such as jasmonic, salicylic and abscisic acids, could play a crucial role in drought memory-enhancing drought tolerance (Bruce, 2014; Farooq et al., 2012). This establishment and maintenance of plant memory could occur through a well-organised network from the genome and cells to the whole plant level. However, the mechanism, on a molecular and physiological level, are still largely unknown. It is crucial to understand the maintenance and mechanisms of stress memory, especially on the physiological and transcriptional levels (Ding et al., 2012, 2014; Virilouvet and Fromm, 2015) (Fig. 1.3).

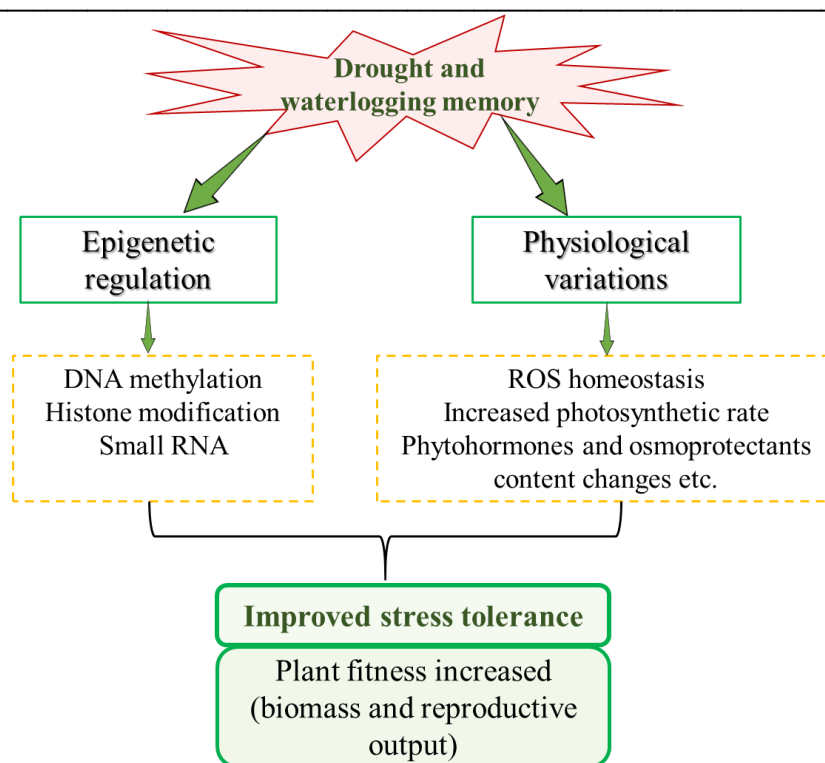


Fig. 1.3 An overview of drought and waterlogging plant stress memory and possible mechanisms to improve plant tolerance

1.5.1 Molecular mechanisms

Epigenetic regulations of the genome are one of the likely mechanisms which could explain transgenerational plant stress memory in plants. Studies in drought stress memory indicate that molecular mechanisms regulating the maintenance and transmission of memory differ from plant responses to the single stress events (Avramova, 2015). DNA methylation, histone modification and small RNAs inherited through mitosis could be essential in regulating gene expression and transgenerational plant stress memory (Kinoshita and Seki, 2014) (Fig. 1.3).

DNA methylation. A specific CpG dinucleotides could be a target for *de novo* methylation of the DNA. Enzymes DNA methyltransferase 3 and/or DNA methyltransferase 1 are in charge of DNA methylation proliferation. Indeed, earlier studies indicated that methylation pattern changes are strongly related to the variation of environmental factors. DNA methylation influences gene expression by changes in transcription and regulating transposon silencing and chromatin interactions (Zhang et al., 2018). Methylation can be maintained through DNA replication in meiosis and mitosis (Takeda and Paszkowski, 2006; Richards,

2009). The state of DNA methylation can be adjusted in response to biotic and abiotic stress during vegetation growth, and it could be transmitted from one to the next generation (Richards, 2011). As an illustration, Cortijo et al. (2014) reported that DNA methylation could cause heritable changes in root length and flowering timing in the absence of DNA sequence diversity in *Arabidopsis thaliana* plants. In addition, exposing *Arabidopsis thaliana* maternal plants to different kinds of abiotic stresses, such as salt, UV, flooding, heat and cold stress, resulted in the upregulation of global genome methylation, where offspring developed higher tolerance to the abiotic stresses (Boyko et al. 2010; Pecinka et al. 2009). Indeed, hypermethylation as a response to drought-induced transgenerational plant stress memory was reported in *Gossypium hirsutum* plants (Lu et al., 2017). Testing transgenerational plant stress memory in *Oryza sativa* plants, Zheng et al. (2017) showed that drought-induced epimutations and altered DNA methylation are advanced in the next generations. Moreover, genes related to these changes directly participate in stress-responsive pathways and their DNA methylation patterns were affected by transgenerational drought.

Histone modification. Nucleic DNA is tightly packed around globular proteins and histones, which undergo various types of post-translational changes, such as **acetylation** (addition of an acetyl group to lysine residues) (Kouzarides, 2007), **methylation** (addition of a methyl group to lysine and arginine residues), **phosphorylation** (adding negatively charged phosphate group on serine, threonine or tyrosine), and **ubiquitylation** (ubiquitin to the histone core proteins), could potentially transmit maternal effects. The combination of DNA and histones makes chromatin which can enable or disable DNA transcription. Consequently, chromatin structure can positively and negatively affect gene expression (Rapp and Wendel, 2005). Besides that, histones play an important role in DNA repair, replication, and stemness processes. For instance, prolonged heat stress could generate transgenerational plant stress memory in *Arabidopsis thaliana* plants, mediated by histone demethylases, heat shock transcription factors and activating small interfering RNA (siRNA) (Zhong et al., 2013; Liu et al., 2019).

Small RNA. Non-coding RNAs, such as microRNA (miRNA) and siRNA, are short RNA molecules that receive much attention as they could be a part of the transgenerational plant stress memory. Indeed, these molecules alter gene expression through mRNA degradation of targeted nucleotides or methylation induction on a complementary DNA chain. Consequently, these molecules can trigger epigenetic inheritance and transgenerational plant

stress memory because they are able to transmit experience-dependent information from parent to offspring (Rodgers et al., 2015).

1.5.2 Physiological mechanisms

Stress-induced plants invest their energy into producing metabolites, such as amino acids, organic acids, phenols, and lipids, that could suppress injuries caused by stress, acting such as osmolytes, antioxidants, and stress phytohormones and different kinds of defence components (Rivas-Ubach et al., 2012).

Since both drought and adverse waterlogging conditions enhance ROS generation (such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2)), and testing redox changes in the context of transgenerational plants stress memory is indispensable. Excessive ROS generation can trigger oxidative-induced damages, such as protein oxidation, cytotoxicity, destruction of the cellular membrane and ion leakage, triggering the programmed cell death pathways, and even DNA abandonment (such as DNA hypomethylation), hence threatening the cellular viability (Sharma et al., 2012; Soares et al., 2019). Besides that, enzymes involved in the Calvin cycle are susceptible to a high level of hydrogen peroxide, which can also reduce the assimilation of CO_2 (Farooq et al., 2012).

Lipide peroxidation is a cascade of reactions in which ROS react with unsaturated fatty acids compromising membrane integrity, selectivity and fluidity (Gill and Tuteja, 2010; Yadav et al., 2018). This process is frequently estimated through membrane ion leakage and by the quantification of lipid peroxidation sub-products. Indeed, malondialdehyde (MDA) is one of the final byproducts of polyunsaturated fatty acids in cell membranes. Overproduction of MDA, caused by excessive ROS, is a commonly known marker of oxidative stress. Redox status and redox balance in cells control nearly every aspect of the cellular process, such as gene expression, metabolism, growth, development, and adaptation to different abiotic or biotic stresses in plants (Locato et al., 2018).

Antioxidants. To maintain redox homeostasis in cells, plants use a powerful and multifaceted antioxidant system (AOX) in the form of enzymatic and nonenzymatic machinery involved in the elimination, detoxification or neutralisation of ROS (Gratão et al., 2005; Liebthal et al., 2018). AOX enzymes, such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and ascorbate peroxidase (APX) (Table 1.2), maintain ROS homeostasis to avoid oxidative-induced damage and improve plant development (Gratão et al., 2015; Mittler

2017). Together with the enzymatic components of AOX machinery, nonenzymatic components, such as phenols, glutathione, and carotenoids (Table 1.2), are mostly low-mass metabolites able to neutralise, remove or transform ROS to maintain redox homeostasis under stress (Gratão et al., 2005; Mittler, 2017). Upregulation of the AOX machinery to achieve the cellular redox balance under stress conditions could be a promising mechanism behind plant priming. However, this mechanism is only shown in a few studies, testing heat, drought, and cold, but not waterlogging plant memory.

Drought priming and tolerance of plants to this kind of stress seem to be correlated with the higher activity of antioxidant enzymes produced by the first drought exposure and maintained after that in maize seedlings (Virlouvet et al., 2018). An enhancement in the activity of SOD, CAT and APX and a decline in the oxidative stress parameters, such as MDA content, was highlighted as one of the main mechanisms of drought memory in *Moringa oleifera* plants (Rivas et al., 2013). In addition, drought priming enhanced grain yield, photosynthesis, and APX activity in drought-primed *Triticum aestivum* plants (Wang et al., 2014b). Earlier findings have shown an increase in the activities and gene expression of SOD, APX and CAT and enhanced ROS scavenging capacity in one-generation drought (Wang et al., 2014b), waterlogging (Wang et al., 2016b), heat (Wang et al., 2014a), and cold (Li et al., 2014; Baier et al., 2018) priming.

In addition, adjusting the plant metabolism and enhancing the antioxidant machinery could also be a mechanism of transgenerational memory. For instance, multigeneration drought priming in wheat plants maintained better redox homeostasis and higher SOD, APX and CAT, and HSP activity, contributing to the lower superoxide radical and H₂O₂ production and MDA content (Wang et al., 2019). Čuk et al. (2010) reported that the upregulation of antioxidant enzymes, such as CAT and APX, in *Arabidopsis thaliana* offspring proves UV-C irradiation tolerance obtained through transgenerational memory.

This mechanism could also be involved in cross-stress memory, where heat priming of *Brassica campestris* seedlings prior to drought increased the activity of enzymes involved in the ascorbate-glutathione cycle (APX, dehydroascorbate reductase (DHAR) and glutathione reductase (GR)), and enzymes involved in detoxification of damaging cell components (glutathione-S transferase (GSH) and glyoxalase) (Hossain et al., 2013). An increase in SOD and POX activity, followed by lower lipid peroxidation, oxidative damage and less cell membrane damage rate, was observed in plant exposed to heat (pre-exposure) and drought (the second event) (Zhang et al., 2016). The same effect was highlighted in the opposite direction,

where drought priming prior to heat significantly increased the activity of SOD, POX, and APX (Peng et al., 2012). However, it is also necessary to investigate the role of ROS and antioxidative system in drought/waterlogging stress memory.

Table 1.2 Enzymatic and nonenzymatic antioxidants (Hasanuzzaman et al., 2020)

	Antioxidants	Reactions
Enzymatic antioxidants	Superoxide dismutase (SOD) EC 1.15.1.1	$2\text{O}_2^{\cdot-} + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$
	Catalase (CAT) EC 1.11.1.6	$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
	Peroxidase (POX) EC 1.11.1.7	$2\text{PhOH} + \text{H}_2\text{O}_2 \rightarrow 2\text{PhO}^{\cdot} + 2\text{H}_2\text{O}$ $\text{PhO}^{\cdot} + \text{Asc} \rightarrow \text{PhOH} + \text{MDHA}$ $\text{PhO}^{\cdot} + \text{MDHA} \rightarrow \text{PhOH} + \text{DHA}$
	Phenol oxidase (PPO) EC 1.14.18.1	$\text{PhOH} + \text{O}_2 \rightarrow \text{Catechol}$ $\text{Catechol} + \text{O}_2 \rightarrow \text{Q} + \text{H}_2\text{O}$
	Ascorbate peroxidase (APX) EC 1.11.1.11	$\text{H}_2\text{O}_2 + \text{AA} \rightarrow 2\text{H}_2\text{O} + \text{MDHA}$
	Monodehydroascorbate reductase (MDHAR) EC 1.6.5.4	$\text{MDHA} + \text{NAD(P)H} \rightarrow \text{AA} + \text{NAD(P)}^+$
	Dehydroascorbate reductase (DHAR) EC 1.8.5.1	$2\text{GSH} + \text{DHA} \rightarrow \text{GSSG} + \text{AA}$
	Glutathione reductase (GR) EC 1.6.4.2	$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{GSH} + \text{NADP}^+$
	Glutathione peroxidase (GPX) EC 1.11.1.9	$\text{H}_2\text{O}_2 + \text{GSH} \rightarrow \text{H}_2\text{O} + \text{GSSG}$
	Glutathione S-transferase (GSH) EC 2.5.1.18	$\text{R} - \text{X} + \text{GSH} \rightarrow \text{GS} - \text{R} + \text{H} - \text{R}$
	Peroxioredoxins (PRX) EC 1.11.1.15	$\text{H}_2\text{O}_2 + \text{PRX} - \text{S}^- \rightarrow \text{OH}^- + \text{PRX} - \text{SOH}$ $\text{PRX} - \text{SOH} + \text{GSH} \rightarrow \text{PRX} - \text{SSG} + \text{H}_2\text{O}$ $\text{PRX} - \text{SSG} + \text{GSH} \rightarrow \text{PRX} - \text{S}^- + \text{GSSG}$
	Thioredoxin (TRX) EC 1.8.1.9	$\text{TRX} - \text{RS}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{TRX} - \text{R}(\text{SH})_2 + \text{NADP}^+$
Nonenzymatic antioxidants	Ascorbic acid (AA)	Remove $\text{O}_2^{\cdot-}$, $\text{OH}^{\cdot}$, H_2O_2 and $^1\text{O}_2$
	Glutathione (GSH)	Remove $\text{OH}^{\cdot}$, H_2O_2 and $^1\text{O}_2$
	Tocopherol	Remove $\text{OH}^{\cdot}$, $\text{ROO}^{\cdot}$, ROOH and $^1\text{O}_2$
	Carotenoids (Car)	Remove $^1\text{O}_2$
	Flavonoids	Remove $\text{O}_2^{\cdot-}$, H_2O_2 and $^1\text{O}_2$
	Phenols	Remove $\text{O}_2^{\cdot-}$, $\text{OH}^{\cdot}$, H_2O_2 , $\text{ROO}^{\cdot}$ and ONOO^-
	Alkaloids	Remove $\text{O}_2^{\cdot-}$, $\text{OH}^{\cdot}$, H_2O_2 and $^1\text{O}_2$
	Nonprotein amino acids	Remove $\text{O}_2^{\cdot-}$, H_2O_2 and $^1\text{O}_2$

AA – ascorbic acid; MDHA – monodehydroascorbate; DHA – dehydroascorbate; GSSG – glutathione disulphide.

Phytohormones. The plant responses to stress caused by drought and waterlogging also include activating protective compounds and hormones (Charlton et al., 2008). Drought stress memory and drought tolerance could be correlated with the accumulation of plant hormones, such as auxins, brassinosteroids, cytokinin, ethylene, gibberellic acid, as well as abscisic, jasmonic and salicylic acids (Fig. 1.3). Phytohormones such as brassinosteroids, ethylene, jasmonic, and salicylic acids are often secondary messengers responding to various abiotic stresses, which lead to the activation of different physiological processes caused by water stress (Ullah et al., 2018; Sharma et al., 2019).

Absciscic acid is a stress phytohormone involved in many aspects of plant development, from seed germination to modulation of cellular compartments, such as membrane and maintenance of turgor. Studies showed that abscisic acid leads to stomata closure, accumulation of dehydrins and late embryogenesis of abundant proteins (Borovskii et al., 2002), osmolytes (sugars and proline), as well as increases in photosynthesis (Huang et al., 2015). In addition, abscisic acid is one of the crucial signalling hormones which can induce the accumulation of various proteins involved in stress tolerance (Wani et al., 2016). For instance, Wang et al. (2015) indicated a significant increase in abscisic acid concentration in the leaves of drought-primed wheat plants. The high abscisic acid content in reproductive organs could significantly increase grain yield (Wang et al., 2015). Moreover, abscisic acid accumulation can also increase root biomass in heat-primed plants compared to non-primed (Kuromori et al., 2018).

Salicylic acid is a very important stress phytohormone involved in plant growth and development regulation. In addition, salicylic acid has a vital role in abiotic and biotic stress tolerance (Ding et al., 2016). Indeed, the regulation of antioxidant enzymes during abiotic stresses in barely (Mutlu et al., 2016) and banana (Kang et al., 2003) plants was noted after exogenous salicylic acid application. Salicylic acid stimulates proline accumulation and upregulations of antioxidant enzymes in heavy metal-induced mung bean seedlings (Ali, 2017).

Jasmonic acid plays a role in alternating net photosynthesis, grain yield and developing drought tolerance in maize, soybean, and banana plants (Anjum et al., 2016; Yu et al., 2018). Jasmonic acid promoted drought tolerance can be detected through the accumulation of osmoprotectants, upregulations of antioxidative activity, pigment content, and improved water-use efficiency (Xiong et al., 2020).

Osmoregulation. Osmoprotectants are small soluble, electrically neutral molecules that improve the osmotic balance between cytosol and cell surrounding, helping the plant adapt to

a changeable environment (McNeil et al., 1999). In order to maintain osmotic balance and membrane stability during drought stress, drought-tolerant plants generate a higher concentration of osmoprotectants such as amino acids (e. g. proline), glycine betaine, as well as polyols and sugars (e.g. mannitol and trehalose) (Conde et al., 2011; Zandalinas et al., 2018). For instance, glycine betaine can be involved in antioxidative protection and signalling pathways to maintain redox homeostasis (Hossain et al., 2018). Drought priming increased the concentration of proline, soluble sugars in *Poa pratensis* plants (Hossain et al., 2018). In addition, an increase in the proline content was recorded in drought-primed rice (Samota et al., 2017) and wheat (Wang et al., 2019) plants, which obtained improved water status, and higher photosynthesis grain yield and lower oxidative stress damage.

1.6 Ecological and evolutionary perspective

Plant stress memory can be studied from physiological, ecological and evolutionary perspectives. Climate change and water availability fluctuations have immensely affected plant species' phenology, abundance and distribution and, consequently, plant communities and composition (Walther et al., 2002). Water availability problems could cause habitat fragmentation, soil degradation and desertification. Recurring disturbance patterns cause selective pressure on life history affecting population spread and survival (Keeley et al., 2011).

No unique framework adequately explains the role of plant stress memory in evolution because it is unclear whether plant stress memory protects genotypes from selection or causes novel opportunities for selection (Ghalambor et al., 2007). Plant stress memory may be advantageous when it generates phenotypes with broader tolerance to environmental conditions and altered fitness across different environments (Pigliucci, 2001). Adaptive stress memory allows establishment and persistence in novel habitats, but the selection type will depend on how close the new phenotype generated from stress memory is to the favoured phenotypic optimum in that environment. For instance, the population could experience stabilizing selection when adaptive stress memory generates a perfect match with the phenotype that is optimal in a new habitat (Price et al., 2003). Consequently, adaptive stress memory would slow genetic differentiation between populations (Ghalambor et al., 2007). On the other side, a new phenotype generated by adaptive stress memory could also be below the optimum, and in such cases, the population would experience directional selection for extreme phenotypes, promoting adaptation (Price et al., 2003). By diminishing the cost of directional selection and permitting

enough time for population establishment, adaptive stress memory could be an essential first step in plant adaptation to novel environments (Pigliucci, 2001). In this way, stress memory could diminish the probability of extinction in upcoming novel conditions (Amarillo-Suarez and Fox, 2006). In addition, environmentally induced variation that is not heritable could later be genetically “assimilated” and accelerate the process of adaptive evolution (Schlichting, 2004; Badyaev, 2005). The second type of adaptive stress memory is more common, resulting in rapid adaptive differentiation between populations due to reduced extinction and directional selection (Price et al., 2003).

On the other hand, maladaptive or non-adaptive stress memory could generate phenotypes that are further away from the optimum phenotype. In non-adaptive stress memory, the offspring phenotype will have reduced fitness and be further away from the adaptive peak. Also, non-adaptive stress memory could alter the variance of phenotypes expressing cryptic genetic variation (Ghalambor et al., 2007). Phenotypes created due to non-adaptive plasticity are likely to be quickly eliminated by selection in novel stressful habitats, eliminating deleterious phenotypes (Ghalambor et al., 2007).

Adaptive stress memory will likely diminish population extinction by facilitating adaptation (Amarillo-Suarez and Fox 2006). This could be immensely important in invading populations with a small number of individuals when plasticity could play a crucial role in survival during the initial phases of invasion (Strass et al., 2006). Plant stress memory is not an evolutionary mechanism (de Jong, 2005), but rather it could be the first step for adaptation which is further dependent on new mutations (Ghalambor et al., 2007). Theory indicates that offspring could colonise novel environments, opening the way for diversification (Snell-Rood and Ehlman, 2021).

A prolonged drought or waterlogging may act as a strong selection force in a population, and selection should favour the genotypes with improved drought-tolerant or waterlogging-tolerant phenotypes. Moreover, transgenerational plant stress memory could be immensely beneficial in plants with limited dispersal reducing local extinctions (Galloway and Etterson, 2007). For instance, if local conditions are detrimental, mothers could produce more dispersive progeny to colonise more beneficial habitats (Krug and Zimmer, 2000). Also, mothers could produce larger and more competitive offspring if competition increases (Plaistow et al., 2007). Transgenerational plant stress memory has been proposed to significantly impact competitive interspecific interactions and plant community dynamics (Day and Bonduriansky, 2011). Also,

plant stress memory and cross-stress tolerance could be an environmental-friendly technique for alleviating the adverse effects of drought and waterlogging stress.

1.7 Research questions and hypotheses

Plant stress memory is a promising strategy for application in agriculture and breed selection processes. However, it is still unknown how widespread within-generation and transgenerational plant stress memory is, especially waterlogging memory, and which mechanisms cause plant stress memory. The role of the antioxidative system drought and waterlogging stress memory and a potential cross-stress memory within a generation or in the next generation is still missing. A better understanding of these mechanisms will contribute to a better understanding of ecological processes and more precise predictions and strategies to cope with the consequences of climate change. Also, missing studies are testing the magnitude of maternal adaptive effects in species with divergent dispersal.

The objective of this thesis was to close the existing research gaps. This thesis investigates how drought and waterlogging stress lead to single and next-generation plant stress memory formation under repeated drought and waterlogging conditions. In addition, this thesis explores drought/waterlogging cross-stress memory as well. The mechanisms behind plant stress memory, particularly the role of the antioxidative system, was also examined. Further, I aimed to examine the magnitude of adaptive transgenerational plant stress memory and to answer whether transgenerational water stress memory depends on the seed dispersal distance.

The following research questions and hypotheses were addressed in this thesis:

- (1) How does drought pre-conditioning influence plant performance after the second drought exposure? I hypothesized that drought-primed plants would have higher plant biomass, increased photosynthesis and antioxidant capacity.
- (2) How do extreme water conditions (drought and waterlogging) experienced by maternal plants affect plant growth, reproduction, and photosynthesis in the next generation? I hypothesized that plants that experienced the same type of stress (drought or waterlogging) or irrespective of the type of stress (cross-stress memory) in both generations would have higher aboveground and reproductive biomass, as well as a higher concentration of photosynthetic pigments.
- (3) How do drought and waterlogging, experienced by mothers, shape offspring's oxidative and antioxidant activity? I hypothesized that plants exposed to the same type of stress

(drought and waterlogging) or irrespective of the type of stress (cross-stress memory) in the maternal and offspring generation would decrease oxidative damage and upregulate the antioxidant system.

- (4) How do maternal plants with short-distance and long-distance dispersed seeds on individual and on species levels exposed to water extremes (drought and waterlogging) shape the fitness of their offspring? I hypothesized that species and individual seeds with short-distance seed dispersed would have higher fitness when exposed to repeated drought or waterlogging during maternal and offspring generation than species with long-distance seed dispersal.

I planned and conducted three multi-species common garden experiments to address these aims. For these experiments, I used the species presented in Fig. 1.4.



Fig. 1.4 Study species used in this thesis: *Hypochoeris glabra* (A), *Hypochoeris radicata* (B), *Capsella bursa-pastoris* (C), *Crepis sancta* (D), *Jacobea vulgaris* (E), *Bellis perennis* (F), *Plantago lanceolata* (G), *Atriplex hortensis* (H), *Alopecurus pratensis* (I), *Trifolium pratense* (J).

1.7.1 Outlines

The present PhD thesis contains the project's outcomes in three main articles (Chapter 2 to Chapter 4), which are the body of the dissertation.

Chapter 2: The paper from this chapter was published in *Environmental and Experimental Botany* entitled “Plant stress memory is linked to high levels of antioxidative enzymes over several weeks.”. It describes the outcomes of a single-generation plant stress memory formation under repeated drought conditions, answering the first research question.

Chapter 3: The paper from this chapter was published in *Environmental and Experimental Botany* entitled “Transgenerational stress memory in plants is mediated by upregulation of the antioxidative system”. In this chapter, I explore how hydrological conditions experienced by mother plants influence their offspring's growth and reproductive biomass, photosynthetic- and redox-related performance, answering the second and the third research question.

Chapter 4: The paper from this chapter was submitted to *Oikos* entitled “Effects of maternal environment and dispersal ability on transgenerational plasticity in plants”. In this chapter, I examined whether the maternal effect is stronger in species with short-distance seed dispersal since they are more likely to experience the same stress as mothers, answering the second and the fourth research question.

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Chapter 2

Plant stress memory is linked to high levels of anti-oxidative enzymes over several weeks*

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Abstract

Drought stress is one of the major stressors for plants under climate change, depressing growth through inhibition of photosynthesis and causing oxidative cell damage. It has been shown that plants can form a drought stress memory, improving their performance under recurring drought stress after being primed by drought before. Mechanisms underlying such a drought stress memory and possible cross-stress tolerance (improved performance to drought after plants have been stressed by other stressors) are currently unclear. We aimed to test whether previous waterlogging stress and drought stress leads to improved performance and changes in morphological, photosynthetic and antioxidative parameters under recurring drought. Therefore, we repeatedly subjected *Alopecurus pratensis* grass plants to waterlogging and drought over two years. In the third year, plants were well-watered for three weeks to recover and then subjected to drought stress for two weeks. Plants primed with drought before showed less tissue damage and higher levels of *Rubisco* content, antioxidative enzymes (POX, SOD) and chlorophyll *b* after the recovery and the drought period. We show for the first time a long-term drought stress memory in a grass species, lasting over several weeks. Our results indicate that drought priming enhances the activity of antioxidative enzymes, which is key for depressing oxidative damage and for improving tolerance to subsequent drought stress in *A. pratensis*.

Keywords: drought stress memory, priming, grass, antioxidative defence, *Rubisco*, photosynthetic pigments.

2.1 Introduction

Climate change leads to changes in rainfall patterns, which will cause extended dry periods, but also an increase in the occurrence and intensity of heavy rain spells (IPCC, 2014). Therefore, plants will have to cope more often with environmental stress in the future, which they can potentially cope with through their high plasticity and different protection mechanisms. Also, it has been shown in recent years that plants are able to form a stress memory, helping them to cope better with repeated stress. Stress memory has been defined as genetic, epigenetic and physiological changes in plants under stress conditions that modify responses to reoccurring stress in the same generation (within-generation, priming) or in the next generation (trans-generation) (Law and Jacobsen, 2010; Boyko and Kovalchuk, 2011; Walter et al., 2013; Bej and Basak, 2017).

Drought stress is one of the major abiotic stresses for plants worldwide (Li et al., 2014; Muscolo et al., 2015), affecting all levels of plant organisation, from cell metabolism to morphology (Pallardy and Kozlowski, 2008; Aroca, 2012; Khan and Khan, 2017). Root/shoot biomass ratio, the number of leaves, leaf area, leaf mass per area ratio (LMA), leaf size and the activity of photosynthetic enzymes (mostly *Rubisco* activity) (Bota et al., 2004; Pallardy and Kozlowski, 2008; Aroca, 2012; Fathi and Tari, 2016) may be changed during drought stress. Drought stress causes an imbalance between the production of reactive oxygen species (ROS) and their scavenging, leading to oxidative stress in plant cells. This includes the formation of superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), hyperoxide radical ($HO_2^{\cdot}$), alkoxy radicals ($RO^{\cdot}$) and singlet oxygen ($^1O_2$) (Munné-Bosch and Penuelas, 2003). ROS can damage proteins, DNA and lipids, impairing the normal functions of cells (Foyer and Fletcher, 2001). However, plants possess an efficient antioxidant defence system to cope with ROS-induced oxidative stress (Anjum et al., 2011; Hernández et al., 2012; Ashraf et al., 2015). Antioxidant enzymes such as catalase (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC. 1.15.1.1) and Class III peroxidases (POX, EC 1.11.1.7) play a key role in the antioxidant defence. Plant secondary metabolites, e.g. phenols, are also involved in the antioxidative metabolism (Ashraf, 2009). Many studies provide evidence for a drought stress memory in plants (Molinier et al., 2006; Pecinka et al., 2009; Walter et al., 2011; Thellier and Luttge, 2012; Guan et al., 2012; Wang et al., 2014; Hu et al., 2015 a, b; Berry and Dean, 2015; Avramova, 2015; Abid et al., 2016; Abid et al., 2017). Plants primed by drought stress have shown higher rates of photosynthesis, higher grain yield and a higher leaf water status, due to higher contents of proline and glycine betaine aldehyde dehydrogenase (BADH) (Wang et al.,

2014; 2015a; Li et al., 2015). Recent research suggests that the antioxidative defence systems have an influential part in stress memory. Drought primed plants showed higher antioxidative capacity (lower O_2^- rate and content of H_2O_2 and MDA) two, three and five days after drought exposure (Wang et al., 2014). No study to date investigated if the antioxidative system might be involved in a longer-termed drought stress memory over several weeks in a grass species.

Another abiotic stress likely to occur more often and severe under climate change is hypoxia or anoxia, caused by waterlogging or an increase in precipitation. Lack of oxygen causes increased anaerobic metabolism in roots and ultimately reduced root growth (lack of ATP) and root damage as a consequence of acidosis and the formation of toxic soil compounds (Chang et al., 2000; Bailey-Serres and Voesenek, 2008). Therefore, hypoxia or anoxia resembles drought stress in plants, including the formation of ROS, during oxygen deprivation, but also after re-oxygenation (Yan et al., 1996; Blokhina et al., 1999; Biemelt et al., 2000; Blokhina et al., 2001; Fukso and Bailey-Serres, 2004).

Plants are frequently exposed to more than one type of stress or a combination of abiotic and biotic stresses. Cross-stress memory means that the formation of a stress memory to one kind of stress also prevents damage by other stressors, providing cross-stress memory and tolerance (Blodner et al., 2005). For example, frost, heat, drought and waterlogging stress all involve cell dehydration, and therefore acclimation mechanisms are very similar (Beck et al., 2007).

Drought stress can induce increased cold tolerance in different plant species such as *Arabidopsis thaliana*, *Triticum vulgare*, *Avena sativa*, *Secale cereale*, *Lolium perenne* and *Pinus sp.* (Hoffman et al., 2012; Kreyling et al., 2012; Rajashekar and Panda, 2014). The same promotor-binding proteins are induced in both drought and low-temperature tolerance in *Arabidopsis thaliana* plants (Pastori and Foyer, 2002), providing cross-stress memory. Studies of Hossain et al. (2013) showed that induced oxidative stress tolerance after salt and drought stress modulates the antioxidative and glyoxalase systems. Also, Li et al. (2015) described how drought priming enhanced antioxidant capacity and photosynthetic performance of wheat re-exposed to cold stress and short-term low-temperature stress. No study to date investigated whether waterlogging stress can induce priming to reveal cross-stress tolerance under drought stress.

The objective of this study was to close existing research gaps by investigating the role of the antioxidative system for a long-term drought stress memory and a potential cross-stress memory between waterlogging and drought stress.

We subjected a grass species that was previously exposed for two years to drought or waterlogging to drought conditions after they had recovered. The following hypotheses were tested: (1) drought and waterlogging stress lead to a formation of stress memory that improves their performance under repeated drought stress, (2) the antioxidative system plays a crucial role in forming a plant stress drought and waterlogging memory via changes in the activity pattern of antioxidative enzymes (SOD and POD) and non-enzymatic antioxidative defence; (3) morphological changes (shoot-root ratio, size, leaf form) will also play a role for enhancing the performance of plants under repeated stress.

2.2 Material and methods

2.2.1 Experimental design

The experiment was carried out at the agricultural field station (48.712433°N, 9.189879°E) of the University of Hohenheim in Stuttgart, Germany. In 2016, the first year of the study, June was exceptionally wet (115 mm rainfall) and was followed by a dry period from July to September (117 mm in the three months). 2017 was a rather moist year, with almost 90 mm more rainfall than the long-term 30-year average (1981-2010) during the growing season. 2018 had a record-breaking dry and hot summer (+ 2.3 K and 90 mm less precipitation than the long-term average from April until November) (Wetter-BW.de, 2019).

In this study, we used the grass species *Alopecurus pratensis* L. (meadow foxtail; Poaceae), which is an important perennial forage grass. Seeds were germinated in small pots in the greenhouse in April 2016 and seedlings were transferred to larger pots (20 L; height 28 cm, diameter 35 cm) filled with sandy loam (14% clay, 70% sand, 16 % silt; pH 7.88). All pots were transported outside in May 2016 and were randomly arranged in large 275 L pools (only one pot of this species per pool to avoid pseudo-replication). These pools were used to create different water levels, ranging from extremely wet to extremely dry conditions by creating overflows at different heights, beginning in June 2016 (pre-treatment). In the waterlogged treatment, the water table was kept at – 5 cm below the soil surface (hereafter W; n=3), according to e.g. Striker (2012), defining waterlogging as inundation of the root system, but not the shoot. To account for differences in precipitation amount in different years of our study, we included two control treatments, with a water table at – 15 cm and at -25 cm. We did not find any significant differences between these two water levels, so both were pooled and are referred to as control treatments hereafter (hereafter M; n=5). In the dry treatment, pools were

completely empty, and pots received natural rainfall, while chronic dry treatment was covered with PVC stripes mounted on a wood construction to reduce 30% of precipitation. To study pulsed stress, we applied pulsed drought (three weeks under chronic drought treatment) leaving them under a transparent PVC rain-out shelter to prevent any precipitation, from June to July. Because all dry treatments did not differ in the performance parameters assessed, these were pooled for statistical analyses as well (hereafter D; n=7). Higher or lower water levels than desired were prevented by making holes in the pools at the respective height and pools were refilled with water twice per week. In 2018, the year of the study, all plants were kept under mesic conditions for three weeks, until no obvious signs of stress were apparent (no differences in % of wilting) (23 July - 13 August 2018) (Fig. 2.1). On August 13, the first data collection was conducted (recovery). Afterwards, all plants were subjected to drought conditions for two weeks (13 – 27 August 2018) (Fig. 2.1, Supplementary Fig. 2.1, second stress exposure) (WD = plant that had been subjected to waterlogging in previous years, DD = plant that had been subjected to drought in previous years and CD = plants that had been kept under well-watered control conditions previously). Plants were placed under a transparent PVC rain-out shelter to prevent any precipitation. On August 27, the second data acquisition was conducted, after plants had been exposed to two weeks of drought stress. The second stress lasted until 50% of *A. pratensis* plants were wilted. The percentage of soil moisture in the pot was measured once per month using a TDR probe with 20 cm rods from 2016 to 2018, as well as during the second stress exposure (Supplementary Fig. 2.2).

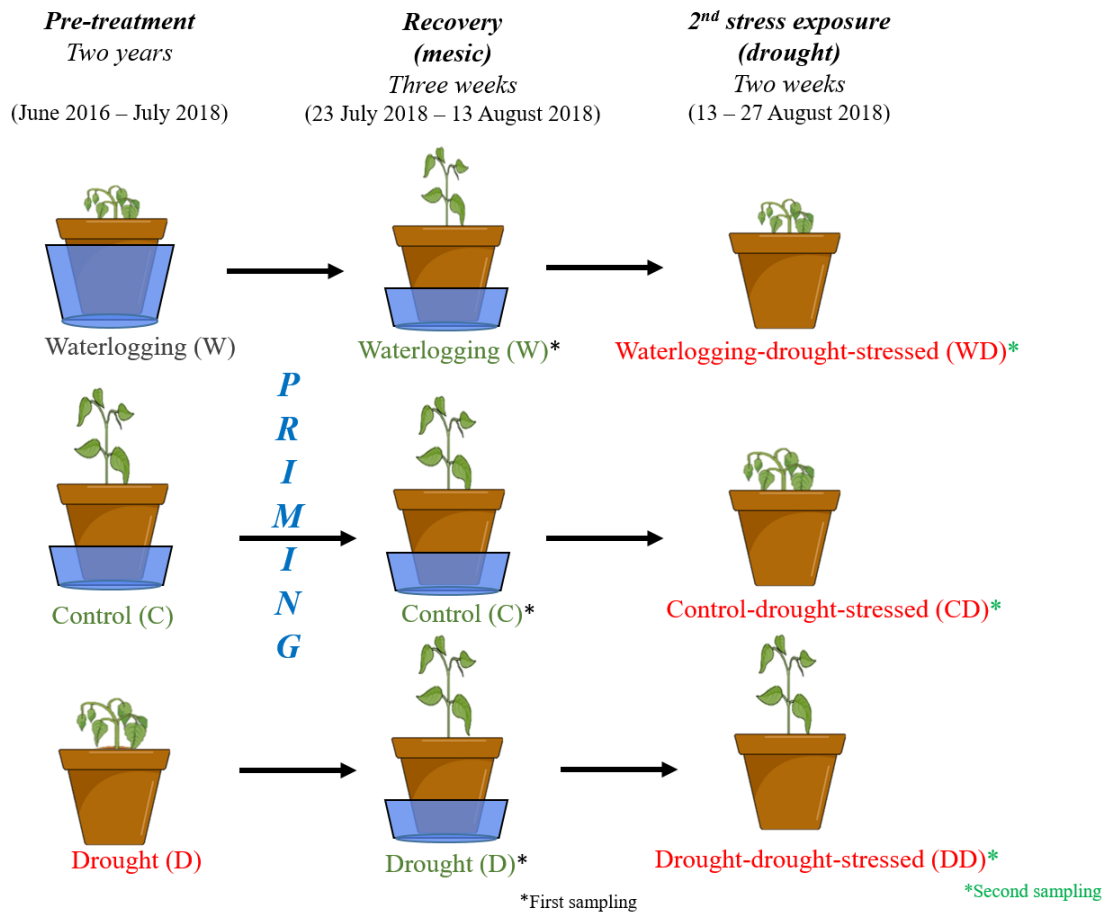


Fig. 2.1 Diagram of the experimental design. Priming was applied for two years (drought and waterlogging stress). After that plants were recovered for three weeks staying under mesic conditions (W – plants from the previous waterlogging stress, C – control, plants from normal mesic conditions and D – plants from drought stress). Afterwards, all plants were subjected to drought conditions for two weeks (WD – plants with waterlogging priming subjected to drought stress, CD – control plants subjected to drought, DD – plants with drought priming subjected to drought stress). The small stars indicate the time of sampling.

2.2.2 Growth-related response variables

To quantify the effects of pre-treatments (2016-2018), plants were harvested twice per year in October and June, starting in October 2016. Biomass was dried at 70°C for four days and then weighed.

Before and after re-exposure to drought stress (13th and 27th of August 2018), two leaves from each individual were collected to determine the Specific Leaf Area (SLA), using Image J software (Schneider et al., 2013). Each leaf (including petiole) was gently rubbed dry before

measurement and was scanned with a flatbed scanner. The height of the plants, diameter, and the length of the youngest, fully opened leaf, were also measured after recovery (13 August 2018) and after the second exposure to drought stress (27 August 2018).

At the end of the second exposure period (27 August 2019), all plants were harvested and separated into root biomass and dead (brown and wilted) and living (green) aboveground biomass. Plant material was dried at 70° C for four days and then weighed.

2.2.3 Biochemical response variables

For measurements of all biochemical parameters, we used evenly aged leaves.

Protein extraction and determination of total protein content

From each plant, we collected 8-10 evenly aged leaves after the recovery period (13 August 2018) and after stress re-exposure (27 August 2019). Collected leaves were powdered in liquid nitrogen and stored at -70° C until further analyses.

For protein extraction, plant tissue was extracted with 0.1 mM Na-phosphate pH 6.4 extraction buffer containing 1 mM PMSF (phenylmethyl sulphonyl fluoride) and 0.2% TWEEN (1: 4 (w/v)). The homogenate was then centrifuged for 10 min at 10000g (Domet Centric 200R), and the supernatant was used for further analysis of total protein and enzyme activities. Total protein concentration was determined by the Lowry method (Lowry et al. 1951) for three repetitions per sample, using bovine serum albumin (BSA) as a standard

Chlorophyll a, b and carotenoids content

For analyses of photosynthetic pigment concentration, leaves were homogenised with 100% DMF (N, N dimethylformamide). The homogenate was centrifuged at 10 000 g for 10 min at 8°C, and the supernatant was used for further analyses. Absorbance was measured at 480 nm (carotenoids; Car), 647 nm (chlorophyll b; Chl b) and 664 nm (chlorophyll a; Chl a) using a spectrophotometer UV-VIS 1800 Shimadzu. Based on the measured absorbance, the concentration of photosynthetic pigments was calculated according to Wellburn (1994).

Determination of total phenolic content

For the determination of total phenolic and flavonoid content, extraction of leaf tissues was performed in methanol (0.5 mg of plant tissues was extracted in 4 mL of methanol). The homogenate was incubated 30 min at 37° C and then centrifuged at 10 000 g for 10 min at 4°C. The supernatant was mixed with 10% Folin-Ciocalteu reagent (diluted with distilled water in the ratio 1:3) and 1M sodium carbonate (Singleton and Rossi, 1965). Absorbance was measured at 724 nm. The phenol concentration in the samples was calculated based on the standard curve for gallic acid and expressed as mg g⁻¹ FW.

Total peroxidase activity

Total POX activity in leaf fractions was measured as absorbance increase at 430 nm with pyrogallol as the hydrogen donor. The reaction mixture consisted of 30 mM pyrogallol, 1.3 mM H₂O₂ and 100 mM Na-phosphate buffer (pH 6.4). Absorbance was measured three times per sample (Kukavica et al., 2013).

Native electrophoresis

Native electrophoresis (BioRad Mini-PROTEAN Tetra Cell), the method developed by Schagger and Jagow (1991), was performed on 5% stacking and 10% running gel, with a reservoir buffer consisting of 0.025 M TRIS and 0.192 M Glycine (pH 8.3), at 24 mA for 120 min. Samples (15 µg of proteins) were diluted in loading buffer to final concentrations of 62.5 mM TRIS-HCl, 10% (w/v) glycerol, and 0.002% (w/v) bromophenol blue.

To determine POX activity, the gel was incubated with 0,01% 4-chloro-naphthol and 3% H₂O₂ in 100 mM Na-phosphate buffer (pH 6.4) (Kukavica et al., 2013). The determination of SOD activity on the gels was performed according to Beauchamp and Fridovich (1971). After incubation in the reaction mixture (0.1 M EDTA, 0.098 mM nitroblue tetrazolium, 0.03 mM riboflavin, and 2 mM TEMED in Na-phosphate buffer, pH 7.8) for 30 min in the dark, the gel was washed in distilled water and illuminated.

Total Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content

For protein separation, SDS PAGE was performed with 12% polyacrylamide gels, as described by Laemmli (1970). The gels were soaked in the solution Coomassie brilliant blue (CBB) and molecular weights of visualised bands were obtained comparing these with SeeBlue® Plus2 Pre-Stained protein standard with a molecular mass in the range 198 -3 kDa

was used. The protein band with MW of 55 kDa, which represents the Rubisco content, has been analysed densitometrically using Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA).

2.2.4 Statistical analysis

For biochemical and physiological analyses, all samples were measured three times, and the values of three repetitions per sample were averaged and used for further statistical analyses. One-way ANOVA (Analysis of variance) was applied to analyse the effects of water (pre) treatments on plant performance. In case of a significant main effect of water treatment, Tukey HSD post-hoc tests were conducted. Prior to all analyses, it was tested whether the assumptions of ANOVA, homogeneity of variances and normality, could be met (Faraway, 2006). If this was not the case, data were log-transformed (shoot/root ratio data) or arcsin-transformed (percentage of dead biomass) accordingly. For non-independent data (total aboveground biomass of 2017 and 2018) mixed models were fitted, with water treatment as a fixed, and year as a random effect (packages lme4 and lmerTest). Pearson correlation coefficients were used to detect the relationship between the activity of POX and total phenolic content. All statistical analyses were performed using R 3.6.1. Relative band intensities for the 1D electrophoresis gels were estimated, measuring density with Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA).

2.3 Results

2.3.1 Pre-treatment

Water pre-treatment significantly affected biomass production over the years ($F_{2,10}=3.48$, $p=0.004$). Drought plants (D) yielded significantly less biomass over two years compared to the control (C) (-30%, post-hoc $p=0.04$) while waterlogged (W) plants yielded more biomass compared to control (+38%) and drought-stressed plants (D) (+57%, post-hoc $p=0.003$) (Fig. 2.2).

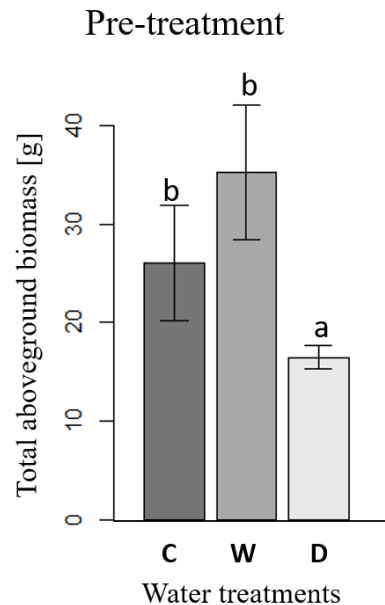


Fig. 2.2 Effects of waterlogging (W), drought (D) and well-watered control conditions (C) on total aboveground biomass over two years (2017 and 2018). Means $\pm$ 1 SE are shown; different letters indicate significant differences between the treatments ($P \leq 0.05$).

2.3.2 Recovery period

Plants were completely recovered at the end of the recovery period after three weeks under well-watered conditions, as could be seen from the low percentage of wilted biomass, that did not differ between pre-treatments (under 2% in all treatments) (Fig. 2.3A).

Plant height, leaf diameter, and leaf length were not affected by drought or waterlogging compared to the control after recovery (Table 2.1). On the other hand, SLA was significantly increased after pre-exposure to drought stress ($F_{2,11}=5.952$, $p=0.01$) compared with control (+16%, post-hoc $p=0.04$) and waterlogged plants (+19%, post-hoc $p=0.04$) (Table 2.1).

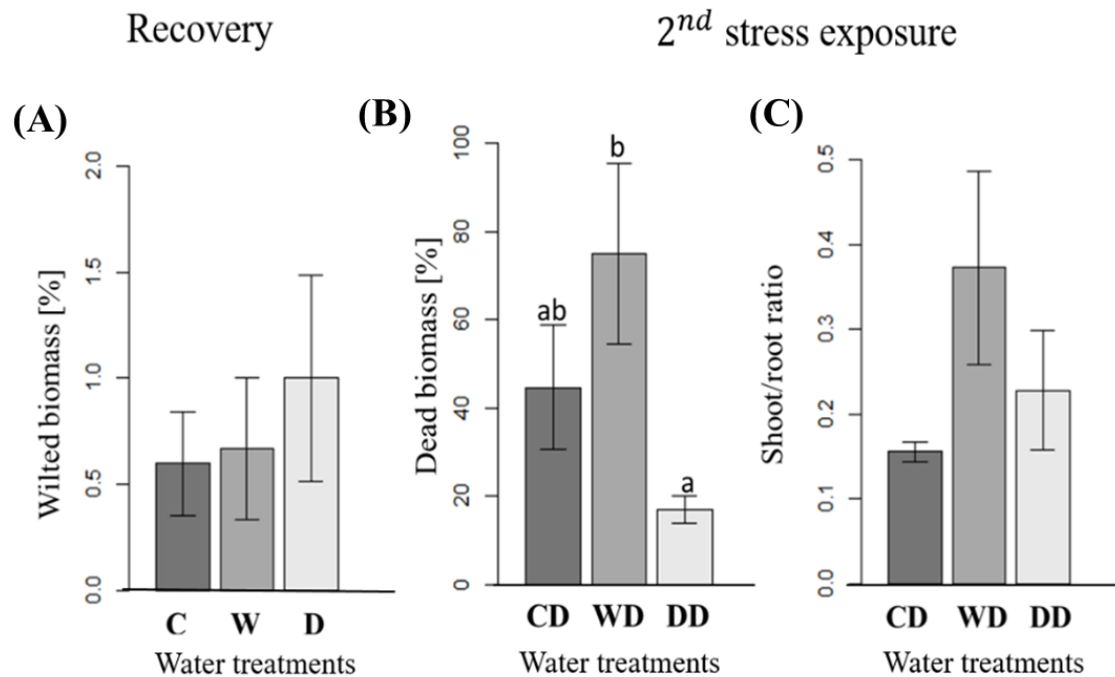


Fig. 2.3 Morphological parameters: estimated percentage of wilted biomass after recovery period (A), percentage of dead biomass (B), shoot/root ratio (C). For the recovery period, C (dark grey) = plants kept under well-watered control conditions before the recovery period, W (grey) = plants subjected to waterlogging before the recovery period, and D (light grey) = plants subjected to drought before the recovery period. For the second stress exposure, CD (dark grey) = plants kept under well-watered control conditions previously subjected to drought, WD (grey) = plants subjected to waterlogging in previous years subjected to drought, and DD (light grey) = plant subjected to drought in previous years. Means $\pm$ 1 SE are shown; different letters indicate significant differences between the treatments ($P \leq 0.05$). Parameters without letters did not have any significant difference.

Total proteins content

After the recovery period, the total protein content of *A. pratensis* leaves was significantly lower in previously drought-stressed plants (D) compared to waterlogged plants (W, -37%) and marginally significant lower in drought-stressed plants (compared with control plants (C) (-22%, post-hoc $p=0.09$) (Table 2.1).

Photosynthetic parameters

Total photosynthetic pigments content (total chlorophyll (Table 2.1) and carotenoids content (Fig. 2.4E), Chl a content (Fig. 2.4A), Chl a/b ratio (Table 2.1) and the total Rubisco content (Fig. 2.4G) of *A. pratense* leaves were not affected by the previous drought or waterlogging stress compared with control after the recovery period. However, at the end of the recovery period Chl b content was significantly higher in drought-stressed plants (D) ($F_{2,11}=10.29$; $p=0.003$) compared with control (C) and waterlogging (W) plants (Fig. 2.4C).

The content of non-enzymatic antioxidants

Pre-treatments did not affect the content of non-enzymes involved in ROS scavenging. Total phenol content was significantly affected by the pre-treatments at the end of the recovery period (Table 2.1).

The activities of antioxidant enzymes

Antioxidative enzymes reflect the ability of the plant to reduce harmful intracellular ROS concentration. Water pre-treatments significantly affected SOD ($F_{2,11}=4.677$, $p=0.04$) and POX ($F_{2,11}=2.953$, $p=0.09$) activity at the end of the recovery period. SOD activity was increased in formerly drought-stressed plants when compared to both waterlogged and control plants (D, post-hoc $p=0.05$) (Fig. 2.5A). Peroxidase (POX) activity was significantly upregulated in previously drought-stressed plants at the end of the recovery period compared to former control and waterlogged plants (Fig. 2.5C), with no significant differences between waterlogged and control plants. On the other hand, the isoenzyme profiles of SOD (Fig. 2.6A) and POX (Fig. 2.6C) were not affected by pre-treatment.

2.3.3 Second stress exposure

Most importantly, the percentage of dead biomass was significantly reduced under repeated drought stress in drought-drought plants when compared to waterlogging-drought plants (-77%; $F_{2,9}=4.275$, $p=0.04$) (Fig. 2.3B). The percentage of dead biomass in DD plants was only 17%. Plants that were previously under control conditions showed intermediate levels of tissue die-back (68% more than in previously drought exposed plants and 40% less than in previously waterlogged plants), with no significant differences to either of those.

Moreover, pre-treatment did not significantly affect the shoot-root ratio of plants (Fig. 2.3C), plant height, leaf diameter, leaf length, as well as SLA, assessed after repeated stress exposure (Table 2.1).

Total proteins content

Proteins were affected by pre-treatment under repeated drought stress ($F_{2,11}=4.426$; $p=0.04$) (Table 2.1). Total protein concentration was significantly higher in WD plants compared with DD plants (+43%, post-hoc $p=0.03$).

Photosynthetic parameters

Pre-treatment significantly affected pigment contents after the second stress-exposure. Total chlorophyll content was significantly higher in drought-drought plants (DD) compared to control-drought (CD) and waterlogging-drought plants (WD) (Table 2.1). This was mostly related to differences in Chl b content ($F_{2,11}=11.29$; 0.002). Drought-drought stressed plants (DD) showed significantly higher content of Chl b, compared with the controls-drought (CD) (+57%, post-hoc $p=0.003$) and waterlogging-drought stressed (WD) (+54%, post-hoc $p=0.01$), with no significant difference between waterlogging-drought (WD) and control-drought stressed (CD) plants (Fig. 2.4D). On the other hand, Chl a content did not differ between treatments (Fig. 2.4B). Likewise, Chl a/b ratio in drought-drought plants (DD) plants was significantly lower compared to control-drought plants (CD; -39 %), post-hoc $p=0.008$) and waterlogging-drought plants (WD) ($F_{2,11}=0.008$). Carotenoid content increased sharply during the drought period but did not differ significantly between pre-treatments, although carotenoid content was highest in DD plants (Fig. 2.4F).

Priming significantly increased the Rubisco content ($p=0.001$) (Fig. 2.4H), which was significantly higher in drought-drought plants (DD) compared with control-drought (CD) (+75%, post-hoc $p=0.00006$) and waterlogging-drought stressed plants (WD) (+79%, post-hoc $p=0.00005$).

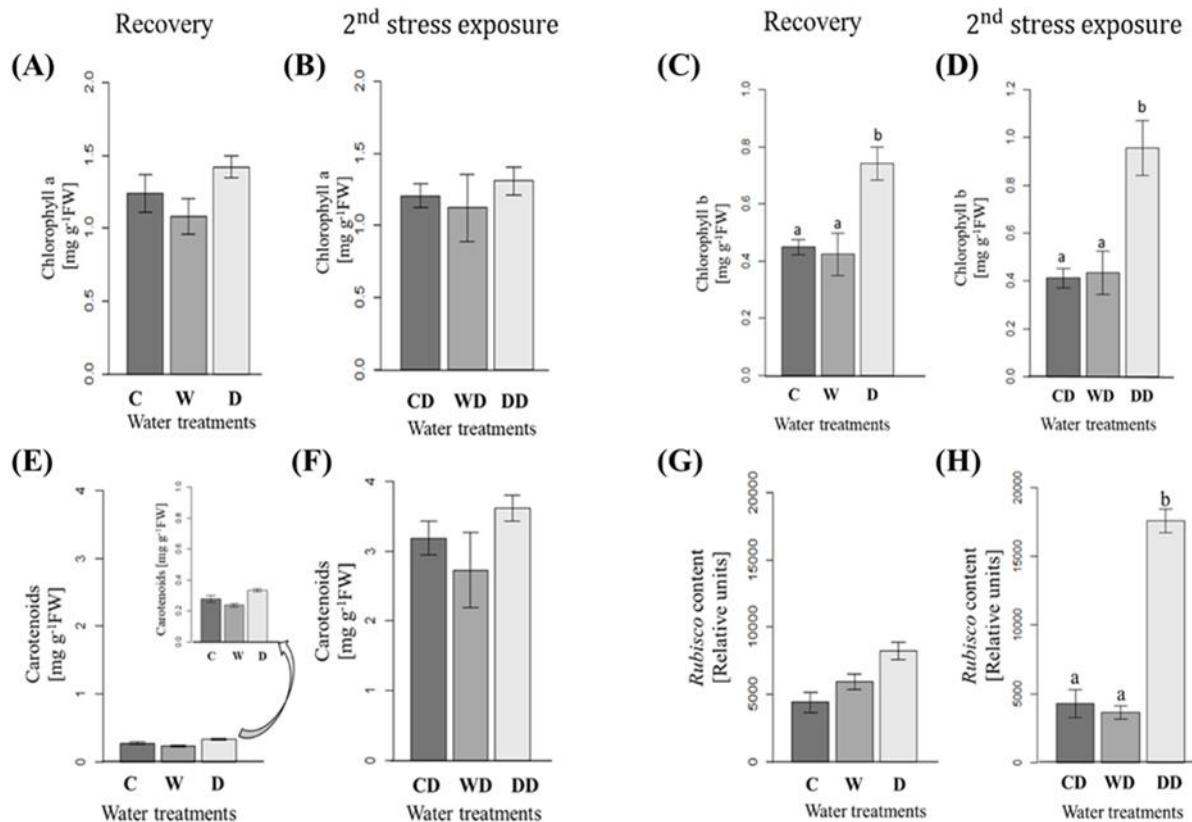


Fig. 2.4 Photosynthetic parameters at the end of the recovery period in which all plants were kept under well-watered conditions and at the end of the second exposure to drought stress. The concentration of chlorophyll a after recovery period (A) and after the end of the second stress exposure (B), the concentration of chlorophyll b after recovery period (C) and after the end of the second stress exposure (D), the concentration of carotenoids after recovery period (E) and after the second exposure (F), the concentration of Rubisco after recovery period (G) and after the second exposure (H) measured after three weeks of the recovery period and two weeks of the second exposure. For the recovery period: C (dark grey) = plants kept under well-watered control conditions before the recovery period, W (grey) = plants subjected to waterlogging before the recovery period, and D (light grey) = plants subjected to drought before the recovery period. For the second exposure: CD (dark grey) = plants kept under well-watered control conditions previously subjected to drought WD (grey) = plants subjected to waterlogging in previous years subjected to drought, and DD (light grey) = plant subjected to drought in previous years. Means ± 1 SE are shown; different letters indicate significant differences between the treatments ($P \leq 0.05$). Parameters without letters did not have any significant difference.

The content of non-enzymatic antioxidants

Priming affected the total phenol content only marginally significantly after second stress exposure ($F_{2,11}=2.797$, $p=0.1$). The concentration of total phenol compounds marginally significantly decreased in drought-drought stressed plants compared to control-drought (+31%, post-hoc $p=0.09$) and waterlogging-drought stressed plants (Table 2.1).

The activities of antioxidative enzymes

Priming significantly affected activity of antioxidative enzymes SOD ($F_{2,11}=8.106$; 0.007) and POX ($F_{2,11}=14.73$, $p=0.0008$). SOD activity was significantly higher in previously drought-stressed plants under repeated drought (DD) compared to WD and CD plants (Fig. 2.5B, post-hoc $p=0.009$). Isoenzyme profiles of SOD did not differ between pre-treatments (Fig. 2.6B). We detected four SOD isoforms in all plants (SOD1 (Rf=0.465), SOD2 (Rf=0.656), SOD3 (Rf=0.699) and SOD4 (Rf=0.739)).

A similar pattern was observed regarding POX activity, where drought priming increased the activity of peroxidase in drought-drought stressed plants in relation to the control-drought (CD; +47%, post-hoc $p=0.002$) and waterlogging-drought stressed (WD) plants (+50%, post-hoc $p=0.003$) (Fig. 2.5D), with no differences between CD and WD plants. Isoenzyme profiles of POX were unaffected after the second exposure to drought stress (Fig. 2.6D). We detected five POX isoforms (POX1 (Rf=0.134), POX2 (Rf=0.227), POX3 (Rf=0.268), POX4 (Rf=0.289), POX5 (Rf=0.432)). Another POX isoform was detected in one control plant labelled as POX6 (Rf=0.470).

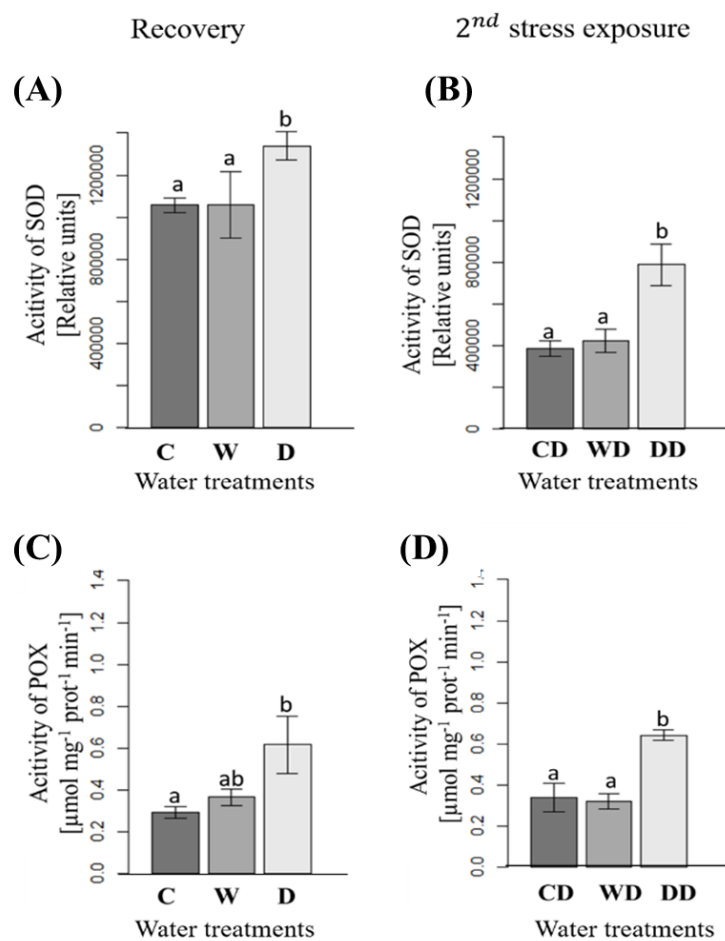


Fig. 2.5 Activity of antioxidative enzymes at the end of the recovery period in which all plants were kept under well-watered conditions and after the end of the second exposure to the drought stress. The activity of superoxide dismutase after the recovery period (A) and after the second stress exposure (B), the activity of peroxidase (POX) (C) and after the second stress exposure (D). For the recovery period: C (dark grey) = plants that had been kept under well-watered control conditions before the recovery period, W (grey) = plants that had been subjected to waterlogging before the recovery period, and D (light grey) = plants that had been subjected to drought before the recovery period. For the second stress exposure: CD (dark grey) = plants that had been kept under well-watered control conditions previously that had been subjected to drought WD (grey) = plants that had been subjected to waterlogging in previous years that had been subjected to drought, and DD (light grey) = plant that had been subjected to drought in previous years. Means $\pm$ 1 SE are shown; different letters indicate significant differences between the treatments ($P \leq 0.05$).

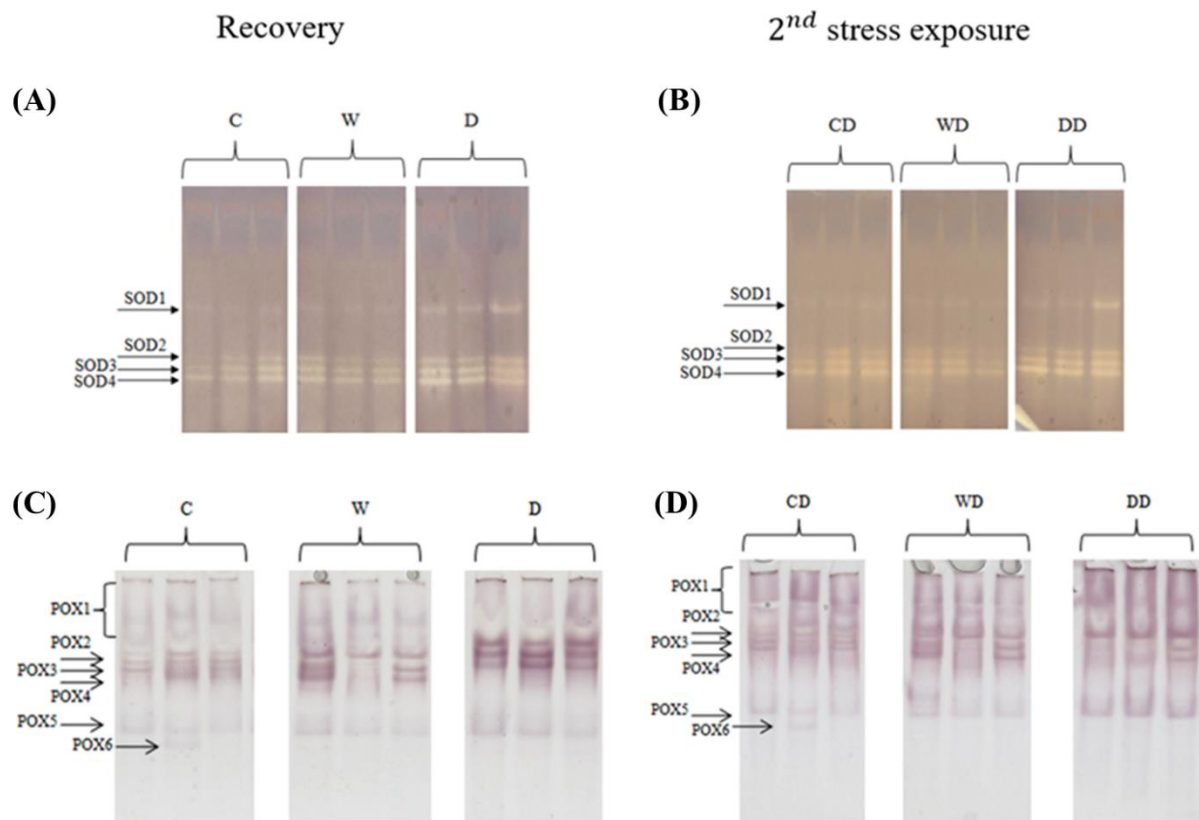


Fig. 2.6 Isoenzyme profiles of superoxide dismutase (SOD) after recovery period (A) and after the second exposure (B), and peroxidase (POX) after recovery period (C) and after the second exposure (D), at the end of the recovery and the second exposure period. For the recovery period, C = plants that had been kept under well-watered control conditions before the recovery period, W = plants that had been subjected to waterlogging before the start of the recovery period, and D = plants that had been subjected to drought before the recovery period. For the second exposure, CD = plants that had been kept under well-watered control conditions previously that had been subjected to drought WD = plants that had been subjected to waterlogging in previous years that had been subjected to drought, and DD = plant that had been subjected to drought in previous years. Arrows indicate different SOD and POD isoforms.

Table 2.1 Growth-related and biochemical parameters measured after the recovery period (left-hand side) and after the second exposure to stress (right-hand side). Chl a = chlorophyll a concentration; Chl b = chlorophyll b concentration, SLA = Specific Leaf Area. Means and SE are shown; different letters indicate significant differences between the treatments within one period (e.g. recovery period or the second exposure period) ($p \leq 0.05$). Parameters without letters did not have any significant difference.

Treatments	Recovery period			The second stress exposure		
	Waterlogging (W)	Control (C)	Drought (D)	Waterlogging-drought (WD)	Control-drought (CD)	Drought-drought (DD)
Growth-related parameters						
Height (cm)	33.0±1.56	31.7±3.36	28.4±1.24	30.1±0.47	28.6±1.66	29.3±1.69
SLA	180.2±4.97 ^a	186.9±12.43 ^a	223.7±7.62 ^b	176.8±4.32	186.9±12.42	249.7±29.58
Leaf diameter (cm)	0.4±0.10	0.3±0.04	0.4±0.07	0.17±0.03	0.2±0.05	0.3±0.04
Leaf length (cm)	21.9±3.61	24.3±3.68	18.2±1.18	23.8±1.37	24.1±1.39	24.1±1.86
Biochemical parameters						
Concentration of proteins (mg g ⁻¹ FW)	16.0±1.20 ^b	12.8±0.83 ^b	10.0±0.82 ^a	21.4±1.00 ^b	16.7±1.26 ^{ab}	12.2±1.26 ^a
Chl a/b (mg g ⁻¹ FW)	2.6±0.36	2.8±0.85	2.0±0.39	2.6±0.95 ^{ab}	3.1±0.95 ^b	1.9±0.73 ^a
Total chlorophyll content (mg g ⁻¹ FW)	1.5±0.33	1.7±0.28	2.1±0.25	1.6±0.55 ^a	1.6±0.20 ^a	2.2±0.33 ^b
Phenols (mg g ⁻¹ FW)	2.7±0.13	2.0±0.13	2.2±0.23	3.7±0.25 ^b	3.0±0.40 ^{ab}	2.5±0.23 ^a

2.4 Discussion

This study aimed to investigate drought stress memory and cross-stress memory at the level of morphological and physiological/biochemical parameters in *A. pratensis* plants that were primed by drought and waterlogging stress over two years.

2.4.1 Effects of water treatments and recovery period

Expectedly and according to its range, *A. pratensis* suffered most under drought. That *A. pratensis* generally performed best under waterlogged conditions indicates that for *A. pratensis* the water table height was not extreme enough, as the species is known to like moist conditions (Ellenberg's indicator value F6; Ellenberg and Leuschner, 2010). Other species growing under the same conditions for studying different questions showed reductions in biomass production under waterlogging in the same experimental set-up (Rai et al., 2018; Walter, 2020).

In the present study, higher SLA was recorded in drought-stressed plants compared to control. Similar results were given by Wellstein et al. (2017), who showed an increase in SLA induced by drought stress in sub-mediterranean grasses. Higher SLA might enhance the allocation of resources in case of continued drought stress, as has been shown for *Sesleria nitida* (Wellstein et al., 2013; Puglielli et al., 2015). Higher SLA can increase photosynthesis and root foraging ability for water under drought (Kleyer and Minden, 2015; Wellstein et al., 2017).

Plants recovered quickly from drought stress, showing no more differences in wilted biomass, the height of the plants, leaf length and diameter as well as in non-enzymatic antioxidative parameters, such as the total amount of phenols and flavonoids. On the other hand, levels of pigment concentration and antioxidative enzymes remained upregulated over the whole recovery period. Similar to our study, Parihar and Soni (2019) have shown that rehydration after drought stress in *Triticum aestivum* and *Glycine max* significantly induced activity of CAT and SOD and removed generated ROS completely.

2.4.2 Stress memory and potential underlying mechanisms

Plants are altering their physiology and metabolism in response to prior experience. It is well accepted that drought-induced stress memory enhances the performance of primed plants and can increase grain yield (Yang et al., 2011; Zhang et al., 2013; Bachkhaus et al., 2014). Other studies suggested that an increase in root growth under drought leads to improved performance of drought-primed plants (Abdallah et al., 2017). We could not confirm this in our study, because neither root biomass, nor root/shoot ratio changed significantly in response to

pre-stress treatment. However, our results demonstrated better protection of drought primed plants, as they had less tissue die-back under drought compared with non-primed plants.

We demonstrate that drought primed plants were able to acclimate to recurrent drought episodes by altering leaf physiology and biochemistry. One of the critical primary processes that present an integrative physiological status of the plants is photosynthesis (Menezes-Silva et al., 2017). Besides, photosynthesis is one of the most sensitive processes affected by drought stress (Reddy et al., 2004). Here we clearly demonstrated that priming induced increases in the concentration of Chl b as well as levels of total Rubisco content. The observed higher concentrations of Chl b, as well as total chlorophyll content, could potentially lead to an increase in the transfer of excitation energy to the PSII and a higher capacity of light-harvesting, which directly leads to a higher photosynthetic rate. It is now well-established that photosynthetic yield is positively related to Chl b content and total chlorophyll content in plants (Aaeide and Zabihe-Mahmoodabad, 2009). Besides, a decrease in Chl a/b ratio in drought-primed plants can potentially increase productivity and adaptability to environmental conditions (Fedulov and Podushin, 2009; Terletskeya et al., 2017). Similar to our results, Abid et al. (2017) showed an increase in photosynthetic pigments in drought primed plants. Moreover, enhancement of leaf photosynthetic efficiency under drought in drought-primed *Triticum aestivum* plants has been reported by Wang et al. (2015b).

Furthermore, the photosynthesis in plants depends also on the activity and synthesis of Rubisco (Chaitanya et al., 2002; Parry et al., 2002). Drought stress triggers a decline in Rubisco synthesis via a remarkable decrease in the abundance of Rubisco small subunit, which indicated its decreased synthesis (Vu et al., 1999) in non-primed plants. However, a significant increase in the abundance of Rubisco small subunit as well as Rubisco activity and content has been measured in drought-primed plants (Wang et al., 2014). Nevertheless, increases in Rubisco content in our primed plants could lead to a decrease in oxidative pressure in the chloroplast leading to less ROS production (Cruz de Carvalho, 2008; Foyer and Shigeoka, 2011; Li et al., 2014; Wang et al., 2014; Menezes-Silva et al., 2017).

To control the ROS amount in the stress cells, plants have evolved an antioxidative protecting system (Foyer and Fletcher, 2001). SOD, as an endogenous first line antioxidant enzyme, has a crucial role in the elimination of O_2^- producing H_2O_2 , less hazardous ROS, scavenged by POX and CAT (Asada, 2006). Likewise, POX is essential antioxidative enzymes localised in the vacuole, cell wall, and cytosol which scavenging H_2O_2 oxidising a wide range of plant phenolic components (Asada, 1992; Ferreres et al., 2011). A very wide range of Km

values (0.04 – 15 mM) for H₂O₂ and a large number of POX isoforms puts POX in an essential place in maintaining plant plasticity in response to different types of stress (Jovanović et al., 2018). In addition to being involved in the removal of H₂O₂ as peroxidase substrates, phenolic components can also remove ROS in direct interactions (Rice-Evans et al., 1997). Our results indicate that phenolic components are consumed in the peroxidase reaction since their concentration in DD plants decreases significantly compared to CD and at the same time POX activity increases, followed by a strong negative correlation between these parameters (Pearsons correlation coefficient $r = -0.667$, $p = 0.009$) (Supplementary Fig. 2.3B). These results indicate that jointly POX and phenols could potentially maintain the H₂O₂ levels in the priming plants.

Besides, our results indicated that underlying mechanisms of better performance were linked to antioxidative enzymes. Obtained results indicate that the levels of POX and SOD significantly increased in drought primed plants and remain increased over weeks, possibly reducing oxidative damage by ROS. Other studies suggest as well that the enhancement of the antioxidative system is involved in short-termed drought stress memory (Sairam and Saxena, 2000; Selote and Khanna-Chopra, 2006; Wang et al., 2014; Menezes-Silva et al., 2017). However, only one study showed that antioxidative enzymes (SOD, POX, CAT, and APX) could be upregulated even over several weeks (Abid et al., 2017). Such an alert state, by accelerating and maximising the defence responses would trigger significantly higher drought tolerance of plants to cope with future stress (Kim et al., 2012; Menezes-Silva et al., 2017). Further, other studies showed that especially the enhancement of the SOD and POX activity in leaves under drought stress was correlated with drought-tolerant crops (Singh and Usha, 2003; Wang et al., 2005; Wang et al., 2014; Zhang et al., 2016; Wang et al., 2019). Drought priming reduced lipid peroxidation and concentration of H₂O₂, via enhancement of SOD and POX activity in winter *Triticum aestivum* plants, showed by Wang et al. (2015b) and Zhang et al. (2016). Further, higher SOD activity recorded in drought primed plants improved redox defence status against lipid peroxidation of cell membrane caused by ROS (Abid et al., 2016). Accordingly, enhancement of the antioxidative cellular system, associated with oxidative stress control, provide enormous metabolic flexibility in plants under water stress (Li et al., 2014; Wang et al., 2014).

In summary, the plant's priming drought response mechanism contains an upregulation of antioxidative enzymes overlapping with higher chlorophyll and Rubisco content providing high stimulus-induced plasticity and flexibility to plants. This plasticity allows plants to

modulate their physiological and developmental phenotype to prior experience (Sultan, 2000; Agrawal, 2001; West-Eberhard, 2003; Hilker et al., 2016), which prepares plants for enhanced response to upcoming environmental challenges.

However, further research is needed to identify other potential underlying mechanisms, such as epigenetic modifications, and to investigate a water-related cross-stress memory. Moreover, it remains to be tested, whether a transgenerational drought stress memory, acting via maternal priming, can enhance the antioxidative system in offspring plants as well.

2.5 Conclusion

Our study confirms that plants possess a “drought stress memory” enabling them to cope with repeated drought stress. Repeatedly stressed plants showed less tissue senescence. We show that photosynthetic pigments, Rubisco and antioxidative enzymes are involved in a long-term stress memory, lasting over several weeks. On the other hand, we do not find consistent anatomical or morphological changes that are linked to the drought stress memory. Surprisingly, *A. pratensis* was not stressed by waterlogging, so we could not test a potential cross-stress memory. However, we show that different stress levels also lead to different “levels of stress memory”, with the least stressed plants suffering most under drought. Our study provides further evidence for a drought stress memory and that this is linked to the antioxidative metabolism. Unlike most other studies, we show that such a memory and an upregulation of antioxidants is kept over several weeks. This might shape plant responses towards hydrological stress under on-going climatic change and needs to be considered when estimating future plant response scenarios. Drought priming can be a strategy for improving plant performance towards recurrent abiotic stress.

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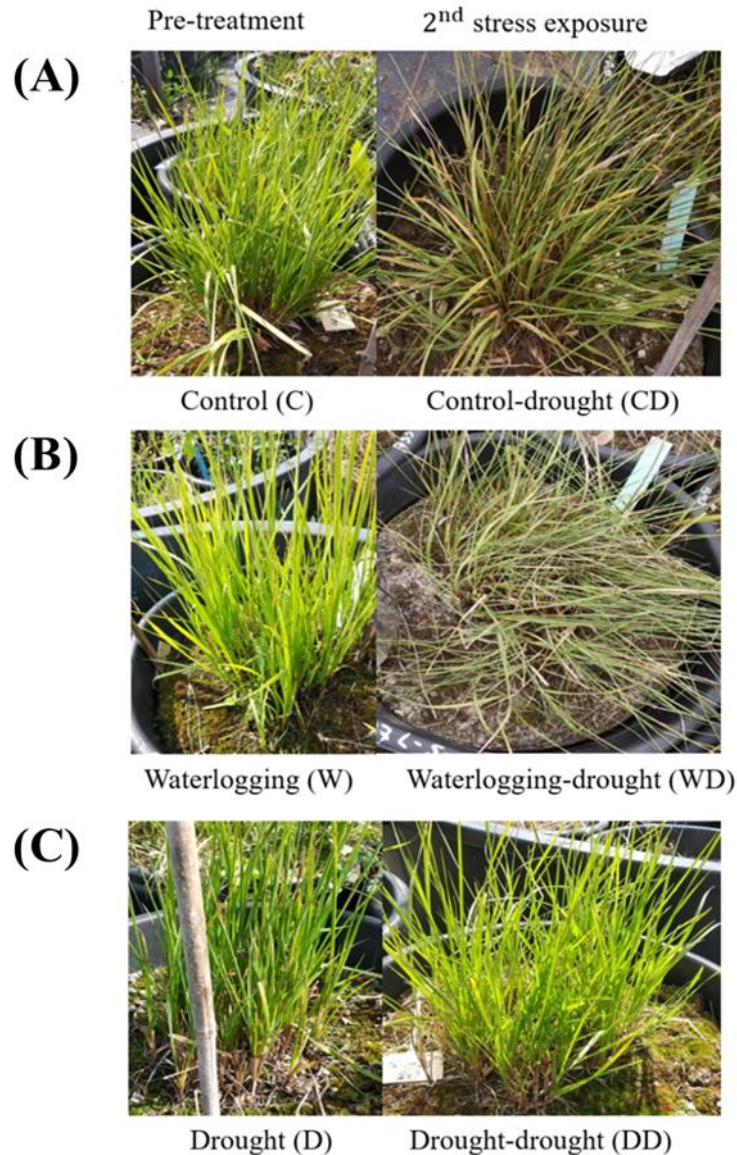
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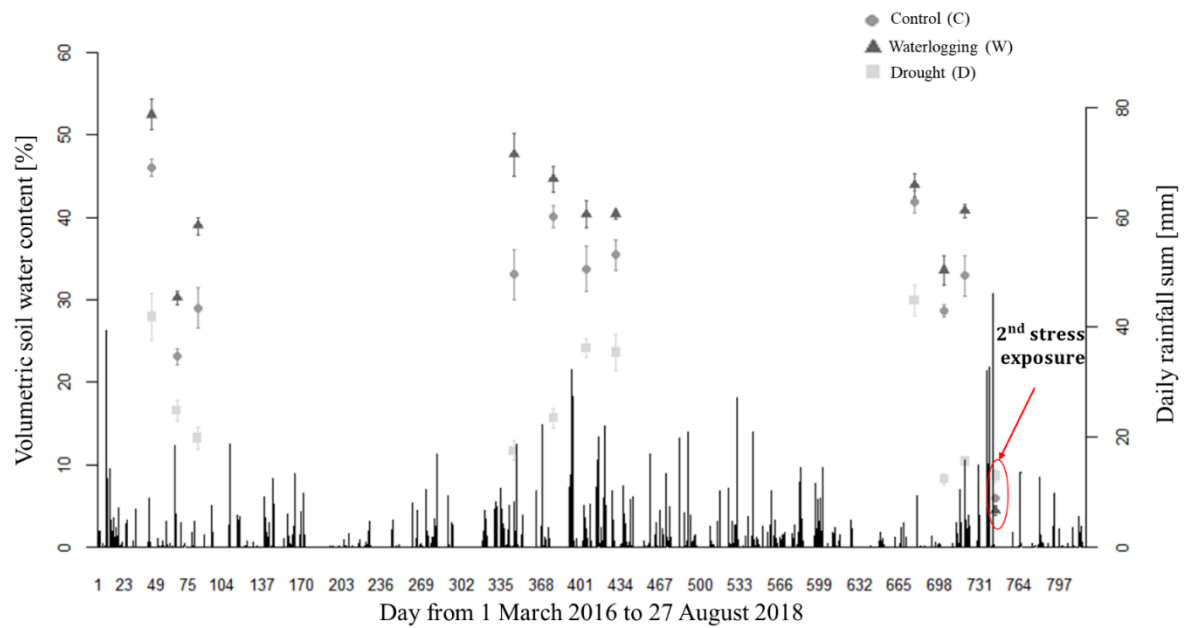
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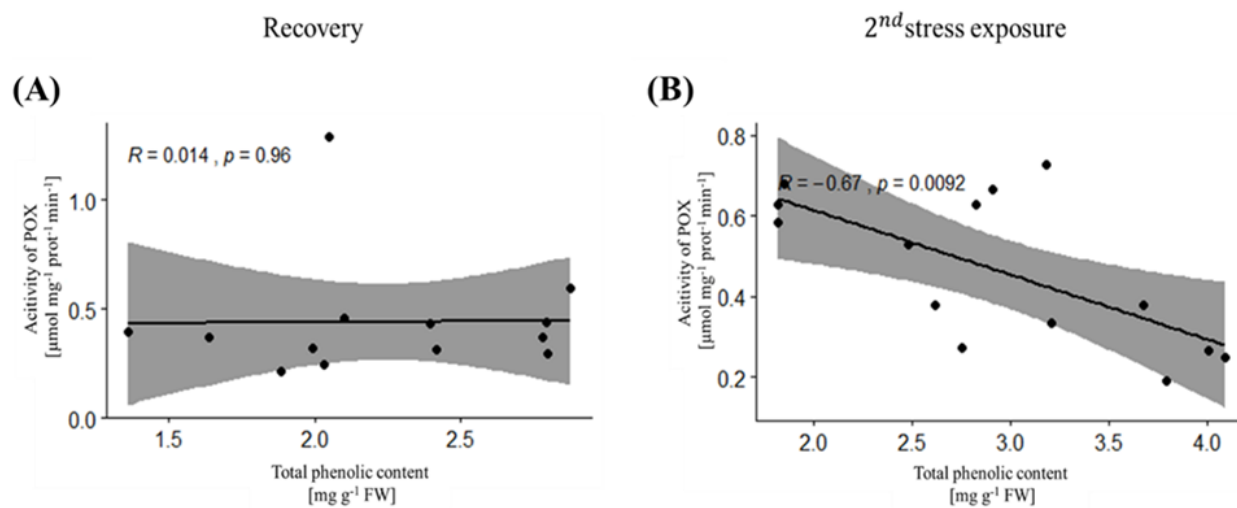
2.7 Supplementary information



Supplementary Fig. S2.1 *Alopecurus pratensis* plants subjected to waterlogging (B), drought (C) and control (A) conditions for two years priming (pre-treatment). Further, plants were subjected for two weeks in the second drought stress from previous waterlogging (E), drought (F), and control conditions (D). For pre-treatment (A), C = plants that had been kept under well-watered control conditions before the recovery period, W = plants that had been subjected to waterlogging before the start of the recovery period, and D = plants that had been subjected to drought before the recovery period. For the second exposure (B), CD = plants that had been kept under well-watered control conditions previously that had been subjected to drought WD = plants that had been subjected to waterlogging in previous years that had been subjected to drought, and DD = plant that had been subjected to drought in previous years.



Supplementary Fig. S2.2 Water treatment data, pre-treatment precipitation and soil moisture from 2016 to 2018 measured in single plant pots as well as soil moisture data measured during the second stress exposure (red circle and arrow). Symbols show mean ± 1 SE of the different water conditions.



Supplementary Fig. S2.3 Pearson correlation between the activity of POX and total phenolic content measured in *A. pratensis* leaves after recovery (A) and the second stress exposure (B).

Transgenerational stress memory in plants is mediated by upregulation of the antioxidative system²

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Abstract

Transgenerational stress memory transferred from parents to offspring may enable plants to respond to changing environments rapidly. Yet transgenerational adaptive effects and underlying mechanisms in response to waterlogging and drought are still unclear. Furthermore, transgenerational cross-stress tolerance, pre-adapting offspring to any stress when maternal plants experienced stress, is currently understudied. We conducted a full-factorial pot experiment over two generations, using four perennial plant species and subjecting maternal and offspring plants to waterlogging and drought. We hypothesized that offspring experiencing stress performed better when mothers had experienced stress before, irrespective of the type of stress. We found increased biomass and reproductive output in offspring experiencing the same stress conditions as their mothers (transgenerational adaptive effects). However, a maternal stress experience per se did not pre-condition offspring to other types of stress. Transgenerational adaptive effects were linked to changes in the antioxidative system of one species, reducing oxidative damage through upregulation of protective enzymes in offspring experiencing the same conditions as their mothers. Our research provides evidence for transgenerational waterlogging and drought memory and links this to underlying photosynthetic and redox-related mechanisms. Transgenerational adaptive effects acting on the antioxidative system might therefore contribute to the rapid responses of plants to environmental change.

Keywords: antioxidative enzymes, drought, maternal adaptive effect, ROS, stress memory, transgenerational adaptive effects, waterlogging

3.1 Introduction

Under climate change, drought and waterlogging stresses will become more frequent and intense in many parts of the world (IPCC, 2021). Plants evolved different strategies to cope with such stress (Silvertown et al., 1999; 2015). Drought and waterlogging stress lead to diverse morphological (plant growth), physiological (reduced net photosynthetic activity, pigment content, membrane integrity), biochemical (changes in metabolism), and molecular (expression of stress-related genes) changes (Farooq et al., 2012; Fahad et al., 2017). Drought mainly alters plant-water relations on cellular and entire-plant levels, reducing leaf water potential and stomatal conductance (Neumann, 2008). Waterlogging leads to chlorophyll degradation, which ends with limitations in photosynthesis and decreases in energy production due to the transition from aerobic to anaerobic metabolism (Li et al., 2011; Lukić et al., 2021).

Both drought and waterlogging induce oxidative stress along with the formation and accumulations of reactive oxygen species (ROS: singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2)), which are detrimental to all macromolecules (Deng et al., 2012). Understanding these processes is crucial, since ROS alters cells' redox status and balance, controls almost any cellular process, including gene expression, post-translational modification of proteins, epigenetic modifications (DNA methylation, histone acetylation and methylation), and regulates miRNA biogenesis, metabolism, growth, development, and plant plastic responses to abiotic or biotic stresses (Locato et al., 2018; Ramakrishnan et al., 2022). ROS generation can cause oxidative-induced damage during drought and waterlogging stress, threatening cellular viability (Sharma et al., 2012; Carvalho et al., 2018) and causing plant death. Oxidative stress, as an outcome of the disbalance between antioxidants and pro-oxidants, is an essential component of life-history trade-offs among growth, reproduction and defence (Morales and Munné-Bosch, 2016). To protect cellular and subcellular organelles from damage and to maintain the redox homeostasis in cells, plants use a powerful and multifaceted antioxidant system in the form of an enzymatic (mainly including superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX), and Class III peroxidases (POX, EC, 1.11.1.7)) and a non-enzymatic (glutathione, ascorbic acid, tocopherol, phenolic compounds and proline) machinery, involved in elimination, detoxification or neutralisation of ROS (Gratão et al., 2005; Liebthal et. al., 2018). Upregulation of these antioxidative enzymes helps plant cells to maintain ROS homeostasis and protects the photosynthetic apparatus, proteins, lipids, and nucleic acids (Lisar et al., 2012;

Bhargava and Sawant, 2013). In addition, as secondary metabolites, phenolic compounds with large antioxidative capacity can eliminate ROS (H_2O_2), decreasing the lipid peroxidation process and consequently reducing membrane fluidity (Rice-Evans et al., 1996). An important antioxidant role of phenolic compounds is the ability to chelate redox-active metals (Fe and Cu) and thus prevent the formation of extremely reactive hydroxyl radicals through the Fenton reaction (Halliwell and Gutteridge, 1988; Rice-Evans et al., 1996).

Besides acclimation or avoidance, plant stress memory is one strategy to cope with such harmful conditions (Fleta-Soriano et al., 2015). Plant stress memory is defined as the capacity of plants to store previous stress information to trigger an improvement in plant performance as a response to repeated stress (Walter et al., 2011; Fleta-Soriano et al., 2015). The existence of within-generation plant stress memory is well documented (Abid et al., 2017; Wang et al., 2019; Lukić et al., 2020). Nevertheless, transgenerational stress memory has more seldomly been studied (Zhang et al., 2016; Nosalewicz et al., 2016; Wang et al., 2018). Transgenerational memory means that the experience of a parent influences offspring performance via mechanisms other than the transmission of genes (Crean and Bpnduriansky, 2014). Transgenerational effects (TE) on offspring can be adaptive, neutral or maladaptive (Walter et al., 2016; Marshal and Uller, 2007). As a specific case of TE, maternal effects are defined as the influence of the mothers' phenotype or environment on the offspring's phenotype (Marshall and Uller, 2007). Adaptive TE adjust offspring phenotypes to match the conditions their parents had experienced, thus increasing offspring fitness when growing under the same conditions their parents had experienced (Nosalewicz et al., 2016; Hatzig et al., 2018; Mojzes et al., 2021). By enabling phenotypic changes that are more rapid than those resulting from evolutionary adaptation, adaptive TE could play an essential role in facilitating plant population establishment in novel, stressful environments (Plaistow et al., 2006; Räsänen and Kruuk, 2007).

Recent studies show that within-generation drought stress memory is strongly positively correlated with upregulation of the antioxidative machinery (Wang et al., 2019; Lukić et al., 2020). In addition, waterlogging priming in wheat during one vegetation season enhanced waterlogging tolerance indicated by increased net photosynthesis, stomatal conductance, transpiration and upregulation of SOD, APX and CAT activity ending with significantly improved vegetation growth and reproductive output (Li et al., 2011). However, the underlying mechanisms of TE are still largely unknown. Possible strategies for conveying adaptive TE could be similar to those in within-generational memory, such as adjustments in growth,

photosynthesis, membrane integrity or reproduction of offspring (Galloway and Etterson, 2007; Latzel et al., 2009; Sultan *et al.*, 2009; Letzel and Klimešová, 2010).

Drought-induced TE have been documented (Zheng et al., 2017; Wang et al., 2018; Mojzes et al., 2021). However, it is unclear whether plants show adaptive TE in response to waterlogging and whether they cross-stress transgenerational memory. Such adaptive cross-stress memory results if the experience of one type of stress alters responses to another type of stress (Munné-Bosch and Alegre, 2013). This phenomenon is essential because plants are exposed to various stress factors simultaneously or at different times during their lifespan. For instance, cross-stress memory between drought and waterlogging could result because these two stress types, as well as other abiotic stresses (frost, heat, salinity), have similar impacts on leaf growth, stomatal closure, net photosynthesis and ROS production (Li et al., 2014). Particularly, it is proposed that ROS metabolism has a key role in the abiotic cross-stress tolerance (Suzuki et al., 2011; Bartoli et al., 2013).

To our knowledge, this is one of the first studies to test for adaptive TE of drought and waterlogging and to investigate possible cross-stress adaptive TE. To this end, we conducted an experiment in which we measured the performance of four common European perennial plant species in reciprocal, cross-generational transplants between drought, waterlogging and control treatments. For one of these species (*Plantago lanceolata*), we additionally quantified the anti-oxidative and photosynthetic parameters. We hypothesized that maternal stress improves offspring performance under the same type of stress (1) or irrespective of the type of stress (2; cross-stress memory) and that this improved performance is partly based on an increase in the activity of antioxidative enzymes and the concentration of non-enzymatic antioxidants (3).

3.2 Material and methods

The experiment was conducted from March 2016 to October 2019 at the agricultural field station (48.712433 °N, 9.189879 °E) of the University of Hohenheim in Stuttgart, Germany. The mean annual temperature from 2016 to 2019 was 10.5 °C, and the mean annual precipitation was 732.9 mm (range: 649.7 mm - 856.5 mm) (wetter-bw.de).

We chose four perennial common European species: *Plantago lanceolata* L. (ribwort plantain; Plantaginaceae), *Bellis perennis* L. (common daisy; Asteraceae), *Alopecurus pratense* L. (meadow foxtail; Poaceae) and *Trifolium pratense* L. (red clover; Fabaceae) as study species. All species occur in mesic to moderately moist grasslands (Ellenberg indicator values 5-6 or indifferent) and do not indicate strongly variable moisture conditions (Ellenberg and Leuschner, 2010). *P. lanceolata*, a model system of plant population ecology (Smith et al., 2020; Villellas et al., 2021; Morales et al., 2021), was used to determination of the mechanism behind plant stress memory.

3.2.1 Maternal generation

Maternal plants (Fig. 1) from which seeds were collected for offspring plants were germinated in the glasshouse in April 2016 in small quick pots (7 cm diameter). Seedlings were transplanted into larger pots (20 L; height 28 cm, diameter 35 cm) filled with sandy loam (14 % clay, 70 % sand, 16 % silt; pH 7.9, which had a rather small water holding capacity). Mother plants were growing alone, without competition or with competition (Supplementary Table S3.1). All pots were transported outside and placed in separate large pools (275 L) in May 2016. The pools were arranged randomly, and they were used to create different water availability treatments from June 2016 to 2018. Mother plants were subjected to three different treatments: maternal control (C; water table height at - 22 – 25 cm below the soil surface), maternal waterlogging (L; water table height at - 5 cm below the soil surface) and maternal drought (D; pools were completely empty, plants received only natural precipitation) (Fig. 3.1) (Lukić et al., 2020). Overflow-holes maintained the water table in the pools at a specific height, and pools were refilled with water twice per week. Under these conditions, mother plants were grown over three years (2016-2018). Pools were emptied from November until March to avoid freezing damage. Maternal aboveground biomass was cut at 2-4 cm aboveground, dried at 70°C for 72 h, and weighed at the end of October 2016, 2017 and 2018. Soil moisture was measured once per month during the season from 2016 to 2019 (presented in Supplementary information, Fig. S3.5) using a TDR probe (TDR 100) with 20 cm rods (Walter, 2020; Lukić et al., 2020).

3.2.2 Offspring generation

Seeds were sampled separately from each mother plant and stored at room temperature in paper bags. They were germinated in small pots (0.6 L, 9 cm square pots) in the glasshouse in spring 2019 and irrigated twice per week. Seedlings originating from each maternal water treatment were then transplanted outside into each of the three offspring water treatments (D - drought, C – control and L – waterlogging) (Fig. 3.1). In the following, we characterize the resulting nine treatment combination with two letters where the first letter denotes the maternal and the second letter denotes the offspring treatment (Fig. 3.1). Offspring plants growing under the same water conditions as mother plants were replicated five times (however, some treatment combinations were missed, we did not have waterlogging-primed *B. perennis* and drought-primed *A. pratense* plants). Offspring were transplanted into the same type of soil, pots and pools as the maternal generation. In each pot, we planted one central and three marginal plants (5 cm from the pot margin). Marginal plants were collected monthly (to assess juveniles' root : shoot ratio), and central plants grew in the pots over the entire season. Soil moisture was also measured in the offspring season.

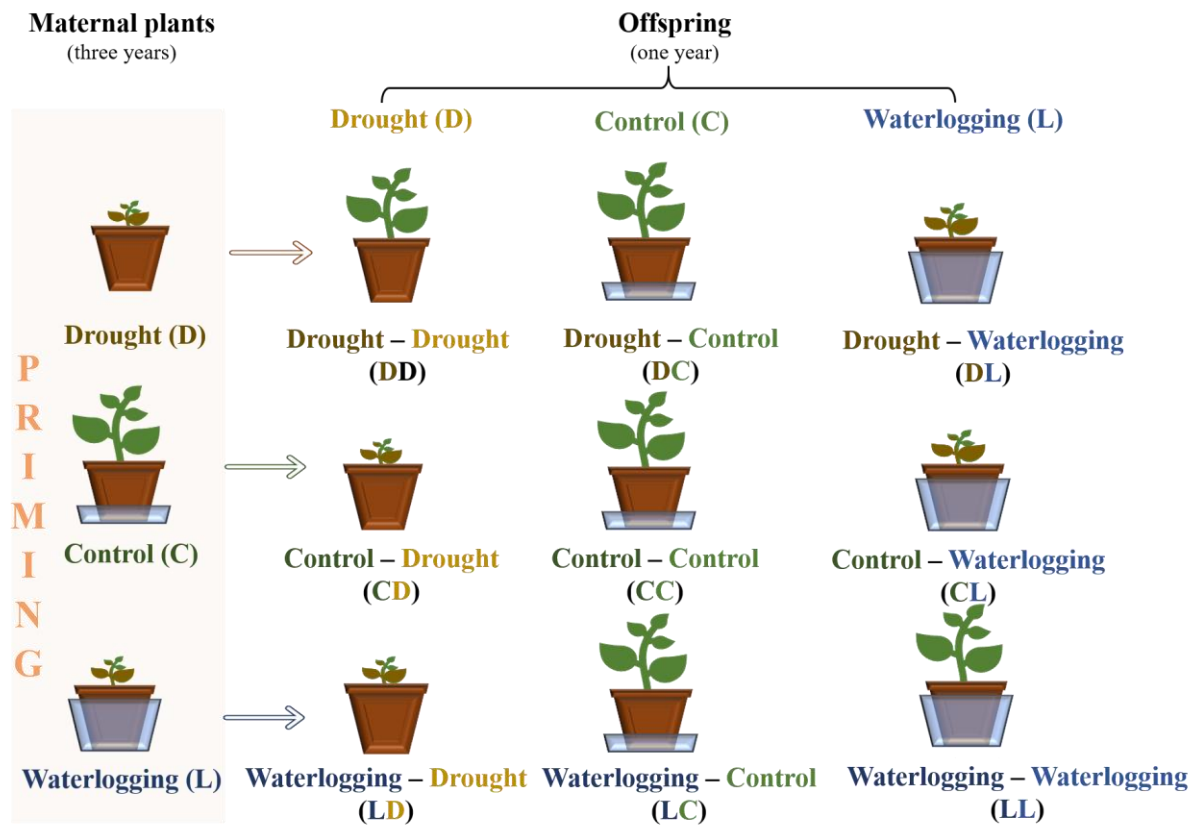


Fig. 3.1 Design of the experiment used to investigate transgenerational stress memory. Mothers were subjected to waterlogging or drought stress for three years. Offspring were subjected to drought and waterlogging conditions for one year. D - drought; C - control; L - waterlogging. The first letter signifies the maternal treatment and the second offspring conditions.

Offspring performance

Offspring aboveground biomass was cut at 2-4 cm in October 2019. Subsequently, the entire aboveground biomass of central plants was sorted into reproductive biomass (seeds and flowers) and vegetative biomass. Seeds from the central plants were collected over the whole growing season to obtain the total reproductive output (data not available for *B. perennis* and *A. pratensis*) at the end of the year. We divided each marginal plant into above- and below-ground parts to assess the root : shoot ratio (data not available for *T. pratense* plants). All plant material was dried at 70°C for 72 h before weighing. The plant's maximum height and the number of leaves were measured thrice. The proportion of total and reproductive biomass and root : shoot ratio was calculated based on the overall mean value.

Biochemical and physiological measurements

For biochemical parameters, we collected evenly aged fresh leaves of *Plantago lanceolata* central offspring plants (five replicates per treatment combination) harvested on August 12th 2019. This species was used because it produced enough biomass for both growth-related and biochemical parameters. Plant material was immediately frozen and later homogenised in liquid nitrogen and kept at –80 °C until further analyses.

In order to detect the level of oxidative stress, we measured the concentration of H₂O₂, following the procedure by Sergiev et al. (1997) and malondialdehyde using Heath and Packer's protocol (1968). In addition, to quantify the antioxidative responses against oxidative stress, we measured the activity of antioxidative enzymes (SOD (Beauchamp and Fridovich, (1971), CAT (Aebi, 1974), APX (Nakano and Asada, 1981) and POX (Kukavica et al., 2009)) and the concentrations of non-enzymes (total phenolic compounds (Singleton and Rossi, 1965)). Also, we detected ferrous ion-chelating ability, following the procedure described by Nićiforović et al. (2010), as well as the concentration of total soluble proteins (following the method described by Lowry et al. (1951)) and proline (following the protocol described by Carillo and Gibon (2011)). The concentration of photosynthetic pigments was assessed according to the method described by Holm (1954). Detailed protocols are given in Supplementary information Protocol S1.

3.2.3 Statistical analyses

Statistical analyses were performed using linear mixed-effect models (package *lme4*; Bates et al., 2015) in R 4.01 (R Core Team, 2020). To test fitness-related parameters (total aboveground biomass, reproductive biomass, root : shoot ratio plant height, and the number of leaves) and our first hypothesis, we fitted the full and same-stress models (Table 3.1). Using offspring and maternal water conditions as fixed effects allow us to analyse the interaction between offspring and maternal treatment (full model), providing evidence for TE (the effect of the offspring treatment depends on the maternal treatment). In the full model, we used maternal pot and pool, species, seeds coming from plants growing without or with competition, and month (the height and number of leaves were recorded monthly) as random effects. To directly determine whether transgenerational effects were adaptive for the main fitness parameters, we fitted another model contrasting the performance of offspring experiencing the same condition as their mothers (henceforth „same as maternal conditions“) to the performance of offspring experiencing conditions different from their mothers („different from maternal conditions“) (same-stress model). To test our second hypothesis, we used the cross-stress

model, in which we compared treatments where both mothers and offspring experienced stress (DD, DL, LD and LL) to the other treatments (CC, CD, CL, DC and LC). In addition to the models mentioned above, we fitted the null model, considering only offspring treatment as a fixed factor. We identified the best model with the lowest small sample size Akaike Information Criterion (AICc) (*MuMin* R package (Barton and Barton, 2015)) (Table 3.1). We used the same random effects described in the full model for all other models.

Linear mixed-effects models were used for biochemical and physiological measures (third hypothesis), using Satterthwaite correction for degrees of freedom (Kuznetsova et al., 2017). In this model, we used the same random factors as in the above-mentioned full model, except species effect. In case of a significant offspring–maternal interaction, Tukey HSD post-hoc tests were applied (*lsmeans* R package (Lenth, 2016)). All response variables (fitness-related, biochemical and physiological) were log-transformed except root : shoot ratio.

Table 3.1 An overview of the four different models for total aboveground, reproductive biomass, root : shoot ratio, plant height and the number of leaves that have been used in this study. The delta AICc and degree of freedom (df) value (difference between the respective model and the model with the lowest AICc for each response variable) are given.

Models	df	Total biomass	Reproductive biomass	Root : shoot ratio	Height	Number of leaves
		Δ AICc	Δ AICc	Δ AICc	Δ AICc	Δ AICc
Full model: offspring x maternal treatment interaction	13	0	0	22.15	0	0
Same-stress model	8	14.67	11.79	0	22.97	19.88
Cross-stress model	8	51.29	32.89	106.18	14.81	25.00
Null model	7	28.29	26.71	99.24	15.74	6.08

3.3 Results

3.3.1 Effects of maternal and offspring environment on fitness proxies

Key proxies for offspring fitness in the four-study species responded significantly to interactions between maternal and offspring environment, indicating TE (Fig. 3.2). This held for total aboveground biomass (likelihood-ratio test $\chi^2_{4df} = 18.96$; $P < 0.0001$), reproductive biomass ($\chi^2_{4df} = 11.66$; $P < 0.0001$), plant height ($\chi^2_{4df} = 4.15$; $P = 0.002$) and number of leaves ($\chi^2_{4df} = 7.70$; $P < 0.0001$). Figures of individual species are given in Supplementary information (Fig. S3.1-3.4).

The observed responses of these fitness proxies to hydrological treatments are consistent with adaptive transgenerational effects: offspring experiencing the same conditions as their mothers had higher aboveground ($\chi^2_{1df} = 41.56$; $P < 0.0001$; Fig. 3.2A, B) and reproductive biomass ($\chi^2_{1df} = 22.18$; $P < 0.0001$; Fig. 2C, D), grew taller ($\chi^2_{1df} = 12.93$; $P = 0.0003$) and produced more leaves ($\chi^2_{1df} = 26.33$; $P < 0.0001$). While this indicates the existence of same-stress memory, we found no evidence for cross-stress tolerance and memory. For all fitness proxies, the cross-stress model performed worse than the same-stress model (Table S3.1). Moreover, the cross-stress model indicated that total aboveground biomass ($\chi^2_{1df} = 2.25$; $P = 0.13$) and reproductive biomass ($\chi^2_{1df} = 0.74$; $P = 0.39$), plant height ($\chi^2_{1df} = 1.71$; $P = 0.91$) and number of leaves ($\chi^2_{1df} = 1.10$; $P = 0.29$) were not significantly higher in cross-stressed offspring (DD, DL, LD and LL) than in offspring where plants did not experience stress or experienced stress only in one generation (CC, CD, CL, DC and LC) (Fig. 3.2A, B).

The detected same-stress transgenerational memory could be due to the effects of the maternal environment on root : shoot ratio as a key whole-plant trait determining responses to hydrological conditions. However, we detected no significant effect of the maternal environment on the root : shoot ratio of marginal offspring plants ($\chi^2_{1df} = 0.034$; $P = 0.85$; Fig. 3.2E, F). Hence, transgenerational effects are likely mediated at the cellular level (see next section).

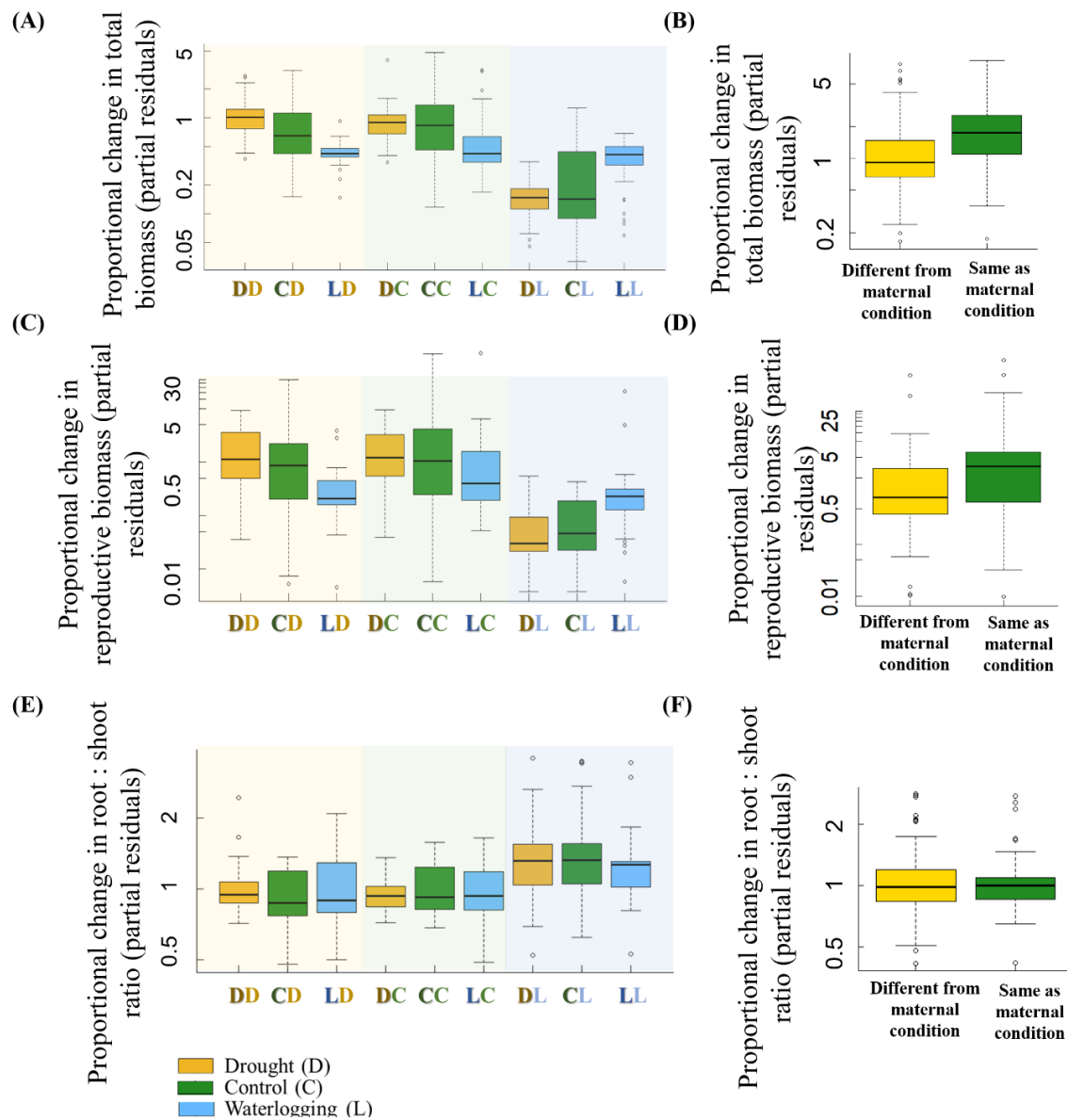


Fig. 3.2 Responses of offspring performance to stress conditions experienced by the offspring themselves and by their mothers. Boxplots show proportional changes in (A, B) total aboveground biomass, (C, D) reproductive biomass, and (E, F) root : shoot ratio of marginal plants in offspring experiencing the same or different stress as their mothers. D - drought; C - control; L - waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition. Same as maternal condition - CC, DD, and LL, Different from maternal condition - CD, LD, DC, LC, DL, and CL. All species' data are shown together (*Plantago lanceolata* L., *Bellis perennis* L., *Alopecurus pratense* L. and *Trifolium pratense* L.).

3.3.2 Biochemical and physiological responses to hydrological stress

In order to obtain a deeper understanding of the underlying mechanisms behind adaptive transgenerational effects, we conducted photosynthetic- and redox-related analyses using *P. lanceolata* leaves (Fig. 3.3-3.5). Both proportional changes in total aboveground and reproductive biomass for *P. lanceolata* plants showed adaptive TE (Supplementary Fig. S3.1A-D). Adaptive TE are primarily visible in LL plants (Supplementary Fig. S3.1A-D).

Results (*P*-values) obtained from offspring, and maternal interaction model and post-hoc tests of physiological and biochemical parameters determined in *P. lanceolata* offspring are given in in Tables 3.2 and 3.3.

We found that offspring exposed to drought had increased the concentration of photosynthetic pigments when they originated from drought-primed mothers as opposed to mothers from control and waterlogging treatments (DD vs LD and CD; Fig. 3.3).

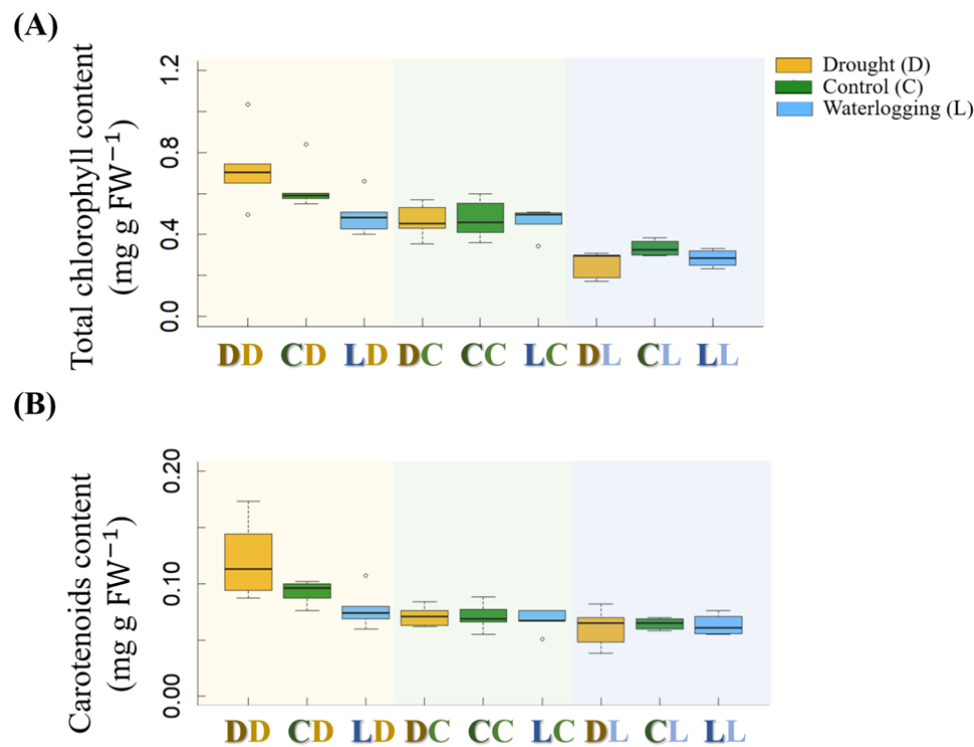


Fig. 3.3 Responses of offspring photosynthetic pigments to stress conditions experienced by the offspring themselves and by their mothers. Boxplots show (A) the concentration of total chlorophyll and (B) carotenoids in *Plantago lanceolata* offspring experiencing the same or different stress as their mothers. D - drought; C – control; L – waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition.

This seems to reflect the fact that drought-exposed offspring experienced lower oxidative stress (indicated by H_2O_2 and malondialdehyde concentrations) when their mothers were also exposed to drought (Fig. 3.4). Reduced H_2O_2 concentrations in drought-exposed offspring of drought-exposed mothers appears to result from increased activity of CAT (Fig. 3.5D) and POX (Fig. 3.5B), which converts H_2O_2 to oxygen and water. In addition, upregulation of SOD activity and elimination of superoxide anion radicals in drought-exposed offspring of drought-exposed mothers (DD) consequently led to a decline in oxidative stress levels (Fig. 3.5A). Reduced malondialdehyde concentrations under transgenerational drought priming seem to be caused by increased chelation of hazardous ferrous ions (Fig. 3.5F), which initiate lipid peroxidation (Lin et al., 2006) and malondialdehyde formation. However, we did not detect a significant accumulation of osmoprotectants (indicated by proline concentration) in drought-exposed offspring of drought-exposed mothers (DD; Fig. 3.5G).

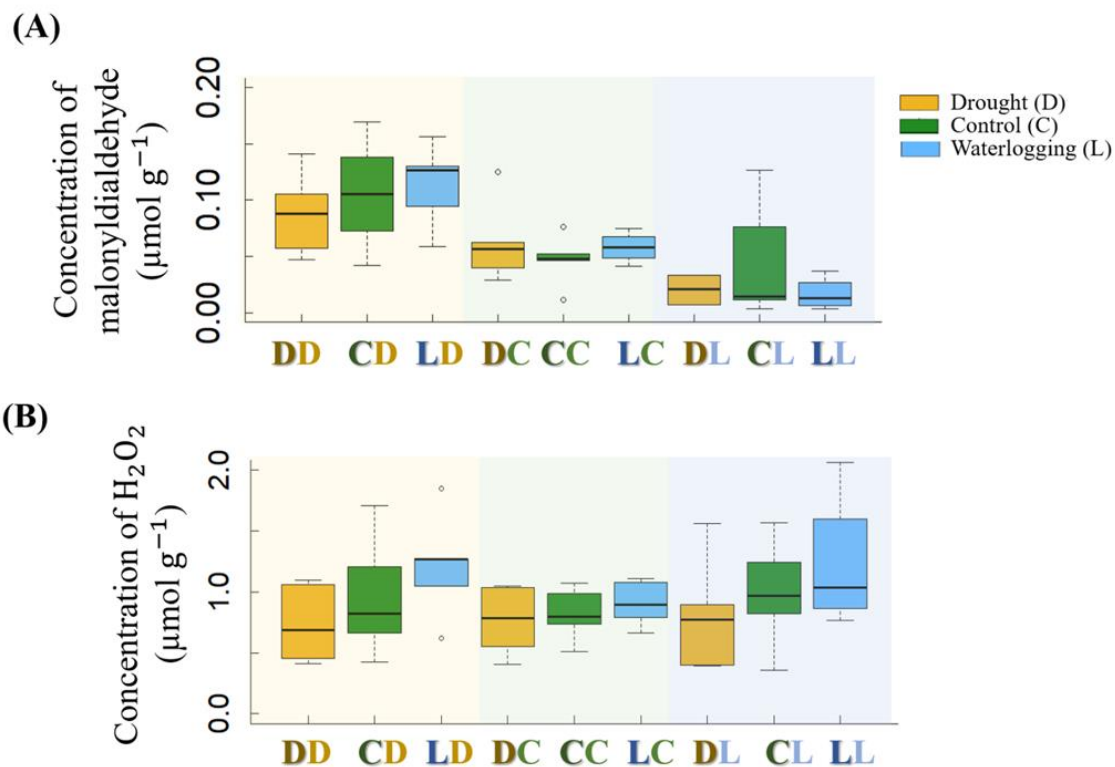


Fig. 3.4 Offspring oxidative stress parameters due to stress conditions experienced by the offspring themselves and their mothers. Boxplots show (A) the concentration of malondialdehyde and (B) H_2O_2 in *Plantago lanceolata* offspring plants. D - drought; C - mesic (control); L - waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition.

The mechanism of waterlogging memory had slightly different pathways. Upregulation of SOD activity recorded in waterlogging-exposed offspring of waterlogging-exposed mothers (LL) could contribute to superoxide anion radicals scavenging (Fig. 3.5A). We found that offspring exposed to waterlogging had strongly increased the concentration of phenolic compounds when they originated from waterlogging-primed mothers as opposed to mothers from control and drought treatments (LL vs CL and DL; Fig. 3.5E). However, waterlogging-primed offspring did not significantly change the concentration of the photosynthetic pigments (Fig. 3.3) nor the oxidative stress level (indicated by H_2O_2 and malondialdehyde concentrations; Fig. 3.4).

Biochemical analyses also indicate why we found no adaptive cross-stress transgenerational memory (Fig. 3.2). Comparing cross-stress primed offspring to offspring from ‘control mothers’ in the same environment (LD vs CD and DL vs CL, respectively), we found no evidence for increased photosynthetic capacity (Fig. 3.3) or decreased oxidative stress (Fig. 3.4) and generally also no evidence for upregulated stress responses (Fig. 3.5). A notable exception is SOD activity, which increased in offspring of mothers experiencing either drought or waterlogging compared to the offspring of mothers from control conditions (Fig. 3.5A). A similar but weaker trend was found for phenolic compounds concentration (Fig. 3.5E).

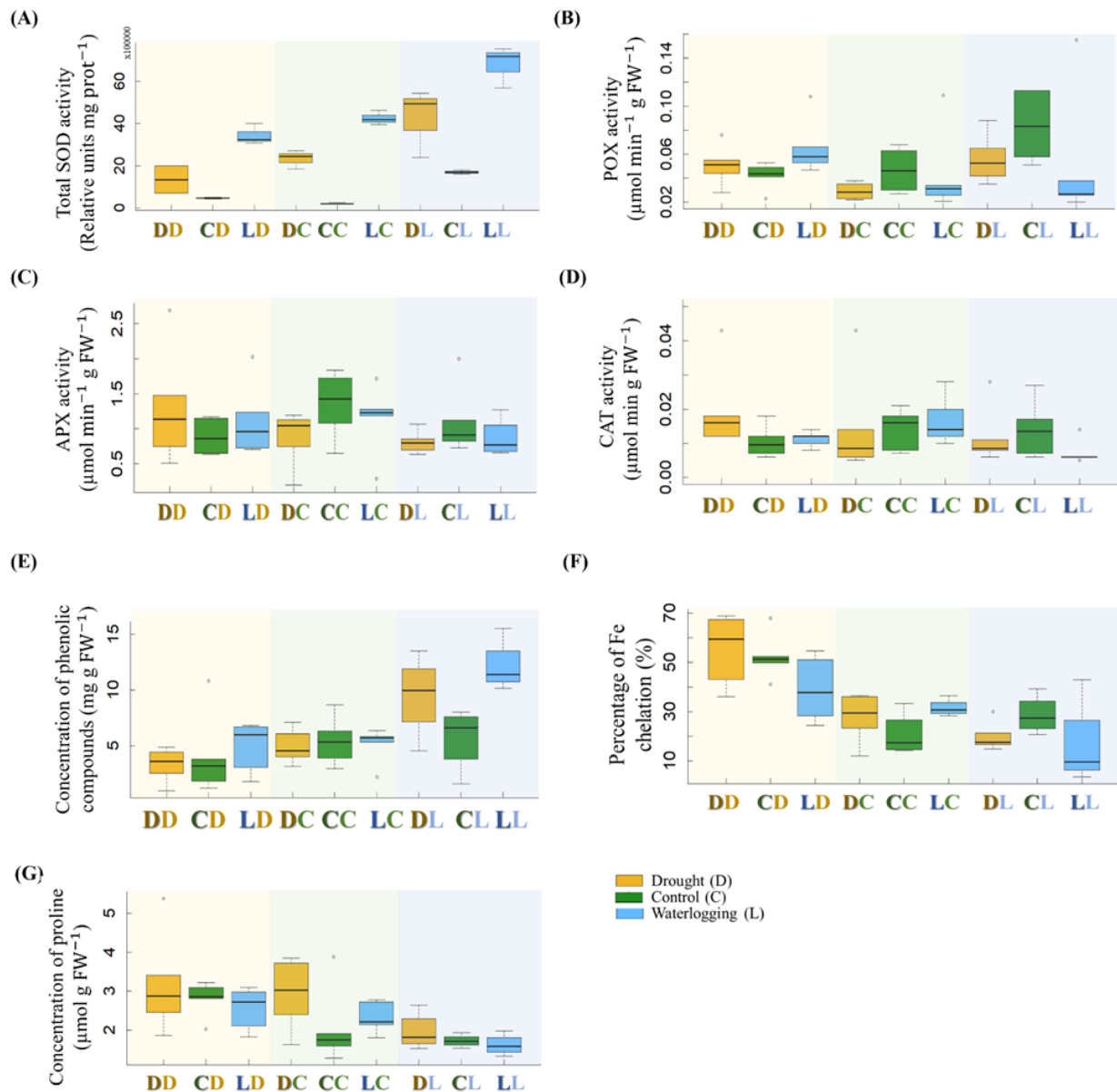


Fig. 3.5 Responses of offspring antioxidative enzymatic and non-enzymatic machinery and proline to stress conditions experienced by the offspring themselves and by their mothers. Boxplots show the activity of (A) total superoxide dismutase (SOD), (B) non-specific peroxidase Class III (POX), (C) ascorbate peroxidase (APX), (D) catalase (CAT), (E) the concentration of phenols, (F) percentage of Fe chelation and (G) the concentration of proline in *Plantago lanceolata* offspring plants. D - drought; C – control; L – waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition.

Table 3.2 An overview of degree of freedom (df), *F*-value and *P*-values obtained from offspring and maternal interaction model measured in offspring *Plantago lanceolata* plants.

		MDA content	H ₂ O ₂ content	SOD activity	CAT activity	POX activity	APX activity	Proline content	Total protein content	Phenolic compounds content	Percentage of Fe chelation	Total Chl content	Car content
Maternal treatments	df	2	2	2	2	2	2	2	2	2	2	2	2
	<i>F</i> -value	0.09	3.79	30.35	0.33	1.60	0.30	0.88	0.09	2.20	1.72	1.52	1.86
	<i>P</i> -value	0.92	0.03*	0.02*	0.72	0.24	0.73	0.53	0.91	0.31	0.19	0.39	0.35
Offspring treatments	df	2	2	2	2	2	2	2	2	2	2	2	2
	<i>F</i> -value	23.02	0.38	62.68	1.77	5.15	0.75	11.64	3.35	16.21	19.19	54.62	20.29
	<i>P</i> -value	<0.0001 ***	0.69	<0.0001 ***	0.18	0.01*	0.48	0.0001 ***	0.04*	<0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***
Offspring x maternal treatment interaction	df	4	4	4	4	4	4	4	4	4	4	4	4
	<i>F</i> -value	0.64	0.24	13.11	2.36	11.06	1.29	0.91	1.24	2.77	2.90	2.95	2.52
	<i>P</i> -value	0.06.	0.08.	<0.0001 ***	0.06.	0.01*	0.30	0.47	0.31	0.04*	0.04*	0.03*	0.05.

MDA – malondialdehyde; SOD – superoxide dismutase; CAT – catalase; POX – peroxidase; APX – ascorbate peroxidase, Chl – chlorophyll; Car – carotenoids.

Table 3.3 An overview of *P*-values obtained from offspring and maternal Tukey post-hoc test comparisons in offspring *Plantago lanceolata* plants.

	DD vs CD	DD vs LD	LL vs CL	LL vs DL
Total chlorophyll content	0.04*	0.02*	0.54	0.61
Carotenoids content	0.01*	0.01*	0.98	0.88
Malondialdehyde content	0.06.	0.59	0.68	0.84
H ₂ O ₂ content	0.06.	0.16	0.66	0.27
Catalase (CAT)	0.04*	0.16	0.18	0.44
Peroxidase (POX) activity	0.06.	0.37	0.08	0.06.
Ascorbate peroxidase (APX) activity	0.64	0.97	0.56	0.97
Superoxide dismutase (SOD) activity	0.05*	0.04*	< 0.0001***	0.01*
Total proteins content	0.39	0.25	0.99	0.98
Proline content	0.46	0.48	0.51	0.30
Phenolic compounds	0.10	0.62	0.06.	0.65
Fe chelation	0.06.	0.09	0.12	0.36

D - drought; C – mesic (control); L – waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition.

3.4 Discussion

This study shows that adaptive TE play an essential role in the rapid acclimation of plants toward water stress. Offspring showed an increase in proportional change of total and reproductive biomass in plants experiencing the same type of stress as their mothers (LL and DD) (hypothesis 1), which can be linked to redox- and photosynthetic-related adjustments (hypothesis 3). However, contrary to our hypothesis 2, we did not detect cross-stress adaptive TE because maternal stress *per se* did not increase offspring performance under stress compared to offspring whose mothers did not experience stress.

This study gave a comprehensive overview of adaptive TE (drought and waterlogging) related to plant fitness, plant redox status, and photosynthesis. Also, only a few studies have addressed the mechanism of adaptive TE of drought based on seed provisioning (Sultan et al., 2009; Germain et al., 2013) and DNA methylation (Herman and Sultan, 2016). Plants' sensitive and systemic physiological and biochemical changes are essential for phenotypic responses to a changeable environment (Ledón-Rettig and Ragsdale, 2021).

In line with our first hypothesis, we showed that waterlogging and drought in the maternal environment altered the offspring's overall plant performance (total aboveground biomass, reproductive biomass, height and number of leaves). Similar to our study, a recent study of adaptive TE showed that the offspring of drought-treated mothers, *Secale sylvestre*, had higher shoot biomass and seed production under drought than the offspring of control mothers (Mojzes et al., 2021). In addition, offspring plants whose parents were drought-stressed were significantly more drought-tolerant than offspring without drought history (Alsdurf et al., 2013). However, this is the first study showing transgenerational waterlogging memory.

One of the mechanisms behind transgenerational plant drought memory could be to improve offspring photosynthetic function. *P. lanceolata* plants experiencing drought in offspring and maternal generation (DD) had a higher photosynthetic pigment concentration and upregulation of enzymatic and non-enzymatic antioxidative machinery than plants experiencing stress only in the offspring generation (CD and LD), which is in line with our third hypothesis. Testing within-generational plant stress memory, in our previous paper, we showed an increase in chlorophyll and carotenoid content followed by higher Rubisco content in *Alopecurus pratense* drought-primed plants was strongly linked to improvement in plant biomass and overall plant performance (Lukić et al., 2020). Apparently, transgenerational plant drought memory relies on a similar mechanism, enhancing photosynthetic pigment concentration. This

adaptive TE mechanism could improve *P. lanceolata* capability to protect and improve photosynthetic function and net photosynthesis. In addition, an increase in carotenoid content could decrease oxidative stress levels due to their ability to quench singlet oxygen and minimise its formation by receiving energy from the excited chlorophyll (Seifermann-Harms, 1987).

Improving cell signalling and antioxidative system are critical mechanisms for developing stress-resistant plants (Ledón-Rettig and Ragsdale, 2021). A recent study (Morales et al., 2021) showed that an increase in mortality on the whole-organismal level in *Plantago lanceolata* is associated with a plant's inability to enhance oxidative stress protection generated under abiotic stress conditions. Our results reveal that *P. lanceolata* plants experiencing drought and waterlogging in offspring and maternal generation (DD and LL, respectively) enhanced antioxidative machinery through increased total SOD activity compared to drought- and waterlogging-exposed offspring of “control mothers” (CD and CL, respectively). Upregulation of SOD in *P. lanceolata* plants will ensure the elimination of superoxide anions, and as metal-binding proteins prevent transition metal (especially iron and copper) ions from facilitating the production of the highly reactive hydroxyl radical from hydrogen peroxide in DD and LL offspring. Its overexpression can be an efficient tool to increase drought and waterlogging stress tolerance. In addition, an increase in both CAT and POX activity was recorded only in *P. lanceolata* plants experiencing drought in offspring and maternal generation (DD) compared to drought-exposed offspring of “control mothers” (CD). This result suggests that DD plants redirected their resources in mass scavenging of H_2O_2 synergically by CAT and POX. Our results are in accordance with Wang et al. (2018), who found that multigeneration drought priming in wheat maternal plants generation maintained better redox homeostasis and higher SOD and CAT activity in offspring exposed to drought stress. Additionally, our results illuminated that *P. lanceolata* plants experiencing drought in offspring and maternal generation (DD; compared to CD) had a strong ability to chelate ferrous ions, which will disable hazardous ferrous ions to initiate lipid peroxidation (Lin et al., 2006) and generate hydroxyl radicals (Halliwell and Gutteridge, 1988).

A slightly different mechanism was recorded in waterlogging TE. Waterlogging mothers invested more energy in activating SOD and in the non-enzymatic part of the ROS-scavenging system. A remarkable increase in phenolic compound content in the *P. lanceolata* plants experiencing waterlogging in offspring and maternal generation (LL) increased tolerance to waterlogging stress. Increased accumulation of phenolic compounds under waterlogging stress has been correlated with the higher antioxidative capacity of *P. lanceolata* plants,

consequently adapting to novel conditions (Bansal and Srivastavam, 2012; Jiménez et al., 2015; Lukić et al., 2021). A preventive increase in phenolic compound content and SOD activity, instrumented by waterlogging-exposed mothers, consequently minimising ROS generation under repeated stress, could be a good strategy for waterlogging adaptive TE to maximise growth and reproduction under stressful conditions.

Our results suggest that adaptive TE help plants to overcome trade-offs between growth, reproduction and defence. Adaptive TE improved not only offspring growth and reproduction but also increased plant survival under drought and waterlogging. Adaptive TE could thus help plants to maintain high fitness in variable and rapidly changing water conditions and to gain dominance over individuals and species that do not show adaptive TE. Moreover, adaptive TE should increase population persistence under environmental change even in the absence of evolutionary adaptation involving genetic changes (Ghalambor et al., 2007; Chevin et al., 2010; Chown et al., 2010). We expect plants which experienced reduced soil water (drought) or oxygen (waterlogging) in both offspring and maternal generations to be favourable for the niche width and persistence of populations (Snell-Rood and Ehlman, 2021). Moreover, offspring pre-adapted to extreme water conditions may more easily colonise novel environments, thus opening the way for diversification (Snell-Rood and Ehlman, 2021). Moreover, adaptive TE could be immensely beneficial in limited dispersal areas, especially in vulnerable early life cycle stages, reducing local extinctions (Galloway and Etterson, 2007). However, we cannot completely rule out a selection, but it is quite unlikely because we did not find an indication that only the fittest mother plants survived stressful conditions.

Since climate change has been predicted to accelerate arid land expansion (Huang et al., 2016), with an estimation that drylands will cover more than half of the total terrestrial surface by the end of this century (Pillet et al., 2022), drought adaptive TE mechanism could be a tremendous advantageous for plants in upcoming conditions. In addition, an increase in variability of precipitation patterns could be defeated by waterlogging adaptive TE. On the other hand, we detected same-stress but no cross-stress memory; in this case, adaptive TE should help plants adjust to a directional change in hydrological conditions (that become either drier or more waterlogged) but not to increased variability of hydrological conditions is predicted for many parts of the world. TE do not appear to be beneficial in the future interchange between arid and wet conditions.

However, this study has two main limitations. The first limitation is that we used only one species (*P. lanceolata*) for redox- and photosynthetic-related parameters measurements,

and we could not generalize for all four species. Finally, we cannot completely rule out natural selection. Still, study focused on the ecological and physiological perspective of TE are urgently needed. However, transgenerational plant stress memory associated with the cell's redox status also requires in-depth studies. A broader understanding of TE could adjust crop improvement strategies for economically and ecologically important crops and tree species.

In summary, plants with drought and waterlogging memory will have less oxidative stress and tolerate stress conditions more effectively. Due to this, adaptive TE played a crucial role in plant fitness improvement and acclimation to drought and waterlogging stress. These adaptive TE showed significant improvement in overcoming trade-offs among plant growth, reproduction and defence. However, pre-adaptation to drought and waterlogging stress could not help plants tolerate cross-stress conditions.

Author contribution

This paper was drafted by NL, based on conversations with JW and FS. JW obtained the funding for the research. JW, FS and NL designed the study, and JW and NL performed the pot experiments and collected the data. NL, BK and TT conducted the biochemical analyses. NL, FS and JW analysed the data. All authors contributed critically to the drafts and gave final approval for publication.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.6 Supplementary information

Protocol S3.1: Biochemical and physiological measurements

Oxidative stress parameters

Concentration of hydrogen peroxide (H₂O₂). H₂O₂ content was examined by following the procedure by Sergiev et al. (1997). Briefly, 1 g of fresh leaf tissue was homogenised with 7 mL TCA (0.1 %). This mixture was centrifuged at 10 000 rpm for 20 min, and 0.3 mL of supernatant was mixed with 0.3 mL 10 mM potassium phosphate buffer (NaPi) pH 6.8 and 0.625 mL 1 M KI. After that, absorbance was measured at 390 nm using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). Results are expressed in $\mu\text{mol g}^{-1}\text{FW}$.

Malondialdehyde content. Powdered leaves (1 g) were homogenised with 7 mL of 0.1 % TCA solution. Afterwards, the homogenate was centrifuged at 10 000 rpm for 20 min. After centrifugation, the supernatant was used to analyse the malondialdehyde using Heath and Packer's protocol (1968). The reaction mixture containing 1 mL 0.5% of 2-thiobarbituric acid (TBA) in 20% TCA, a 0.1 mL supernatant and a 0.4 mL of 0.1% TCA was heated in a hot water bath at 95°C for 30 minutes. Afterwards, the mixture was cooled in ice, centrifuged at 10 000 rpm for 10 min, and absorbance was measured at 532 nm using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). Malondialdehyde content was calculated using the extinction coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$, and the results are expressed in $\mu\text{mol g}^{-1}\text{FW}$.

Antioxidative responses

Powdered leaves (0.5 g) were homogenised with 4 mL of cold 0.1 mM Na-Pi buffer pH 6.4 containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2% TWEEN and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10 000 rpm for 20 min at 4°C, and the supernatant was used for assays of the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase Class III (POX).

Superoxide dismutase activity. SOD activity was determined using native electrophoresis (BioRad Mini-PROTEAN Tetra Cell). Native electrophoresis was performed on 5% stacking and 10% running gel. A reservoir buffer consisted of 0.025 M TRIS and 0.192 M Glycine (pH 8.3). Native electrophoresis ran at 160 V for 120 min at room temperature. Samples were mixed with loading buffer (62.5 mM TRIS-HCl, 10% (w/v) glycerol, and

0.002% (w/v) bromophenol blue) in a 2:1 ratio, and gel 10 µg of proteins/well were applied on a gel. SOD activity on the gel was determined according to Beauchamp and Fridovich (1971). After incubation of native gel in the reaction mixture (0.1M EDTA, 0.098 mM nitro blue tetrazolium (NBT), 0.03 mM riboflavin, and 2 mM TEMED in Na-phosphate buffer, pH 7.8) for 30 min in the dark, the gel was washed in distilled water and illuminated. Total SOD activity was obtained by measuring density with Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA). Total SOD was expressed in relative units per mg of soluble proteins.

Catalase activity. CAT activity was determined following the oxidation of H₂O₂ at 240 nm over a 60 s period in the presence of 10 mM NaPi (pH 6.8) containing 3% H₂O₂ (Aebi, 1974). Absorbance was measured using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). CAT activity was calculated according to the H₂O₂ molar extinction coefficient (40 mM⁻¹ cm⁻¹), and the results are expressed in µmol H₂O₂ g⁻¹FW min⁻¹.

Peroxidase Class III activity. The POX activity was determined following the method described by Kukavica *et al.* (2009). Total POX activity in leaf fractions was measured as absorbance increase at 430 nm (using UV-VIS spectrophotometer (Shimadzu UV-160, Kyoto, Japan)) with pyrogallol as a hydrogen donor. The reaction mixture contained 30 mM pyrogallol, 1.3 mM H₂O₂, 100 mM Na-Pi (pH 6.4), and 30 µL of the plant extract. The activity was calculated using the extinction coefficient for purpurogallin ($\epsilon_{430} = 12 \text{ mM}^{-1} \text{ cm}^{-1}$). Results are expressed in mol min⁻¹ g FW⁻¹.

Ascorbate peroxidase (APX) activity. APX activity was detected following the method described by Nakano and Asada (1981) with slight modifications. Leaves (0.5 g) were homogenised with 4 mL of cold and freshly prepared 90 mM Na-Pi buffer pH 7.8 containing 1 mM PMSF, 1 mM EDTA, 8% glycerol, and 5 mM ascorbate. The homogenate was centrifuged at 10 000 rpm for 20 min at 4°C. The supernatant was used for assays of the APX activities. The decrease in absorbance at 290 nm for 60 s was monitored using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). Reaction buffer contained 935 µL 50 mM NaPi pH 7.0, 50 µL 0.5 mM ascorbate, 0.03% H₂O₂ and 100 µL leaf extract. APX activity was calculated according to the ascorbate extinction coefficient (2.8 mM⁻¹ cm⁻¹), and the results are expressed in µmol ascorbate g⁻¹FW min⁻¹.

Concentration of phenolic compounds. Phenolic concentration was determined following the Folin-Ciocalteu method described by Singleton and Rossi (1965). Leaf tissue (1

g) was mixed with 7 mL of ethanol (95%). The homogenate was incubated 2 h at 37°C and centrifuged at 10 000 rpm for 10 min at 8°C. The supernatant was mixed with 10% Folin-Ciocalteu reagent (diluted with distilled water in the ratio 1:2) and 1 M sodium carbonate. The mixture was vortexed for 15 s and incubated for 1 h at room temperature in the dark for colour appearance. After incubation, the mixture was centrifuged for 10 min at 10 000 rpm. Absorbance was measured at 724 nm using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). The phenolic compounds content is expressed in mg of GAE (gallic acid) equivalents per gram of sample (FW).

Ferrous ion-chelating ability assay. We followed the procedure described by Nićiforović *et al.* (2010). It is based on reducing a ferric-tripyridyl triazine complex to its ferrous, coloured form in the presence of antioxidants. Leaves (1 g) were homogenised with 7 mL 95% ethanol, incubated for 2h at 37°C and centrifuged for 10 min at 10 000 rpm. Briefly, we mixed 3.75 mL 95% ethanol, 0.05 mL 2 mM FeSO₄, 0.2 mL 5 mM ferrozine with 0.2 mL of supernatant. After 10 min incubation at room temperature, the absorbance was measured at 562 nm, and percentage inhibition of ferrozine-Fe²⁺ complex was calculated: [(Absorbance of control – Absorbance of the sample)/Absorbance of control] × 100.

Concentration of soluble proteins. Total protein content was determined following the method described in Lowry *et al.* (1951). Leaves (0.5 g) were extracted with 4 mL 0.1 mM Na-Pi pH 6.4 extraction buffer containing 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.2% TWEEN and 1% polyvinylpyrrolidone (PVP). The mixture was centrifuged at 10 000 rpm for 20 min at 4°C. As standard bovine serum albumin (BSA) ranging from 0.1-1 mg was used. The supernatant's optical density was measured at 550 nm using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). All results are expressed in mg g⁻¹ FW.

Concentration of proline. Proline was determined as described by Carillo and Gibon (2011). Leaves (1 g) were homogenised with a 7 mL mixture of ethanol-water (70:30). The mixture was incubated at 37 °C for 2 h in a water bath and centrifuged for 10 minutes at 10 000 rpm. After incubation, 0.5 mL of supernatant was mixed with 1 mL of reaction mixture (1% ninhydrin (w/v), 60% glacial acetic acid (v/v) and 20% ethanol (98%) (v/v)), heated at 95 °C for 20 min and centrifuged for 5 min at 10 000 rpm. Absorbance was measured at 520 nm using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). Proline concentration was calculated according to the calibration curve using L-proline as the standard. Results are expressed in µmol proline g⁻¹ FW.

Concentration of pigments. The concentration of photosynthetic pigments was assessed according to the method described by Holm (1954). Briefly, 1 g leaf sample was placed in 7 mL 95% acetone (v/v), incubated in a water bath for 2h at 37°C, and the extract was centrifuged at 10 000 rpm for 10 minutes at 8°C. The optical density of the extract was measured at 665, 649 and 470 nm for chlorophyll *a*, *b* and carotenoids using a UV-Vis spectrophotometer (Shimadzu UV-160, Kyoto, Japan). The concentration of pigments was calculated using the formulas provided by Lichtenthaler and Wellburn (1983).

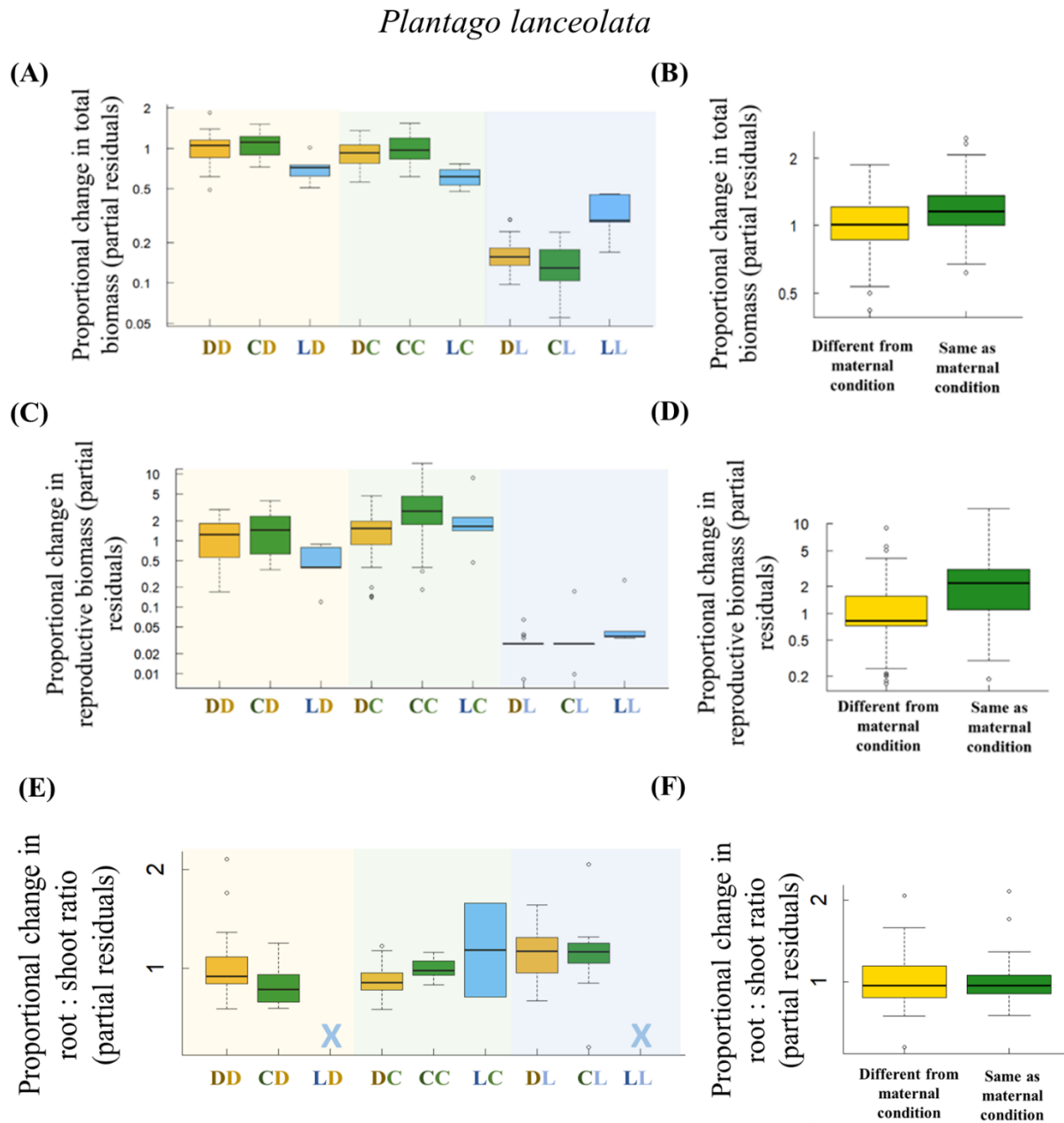
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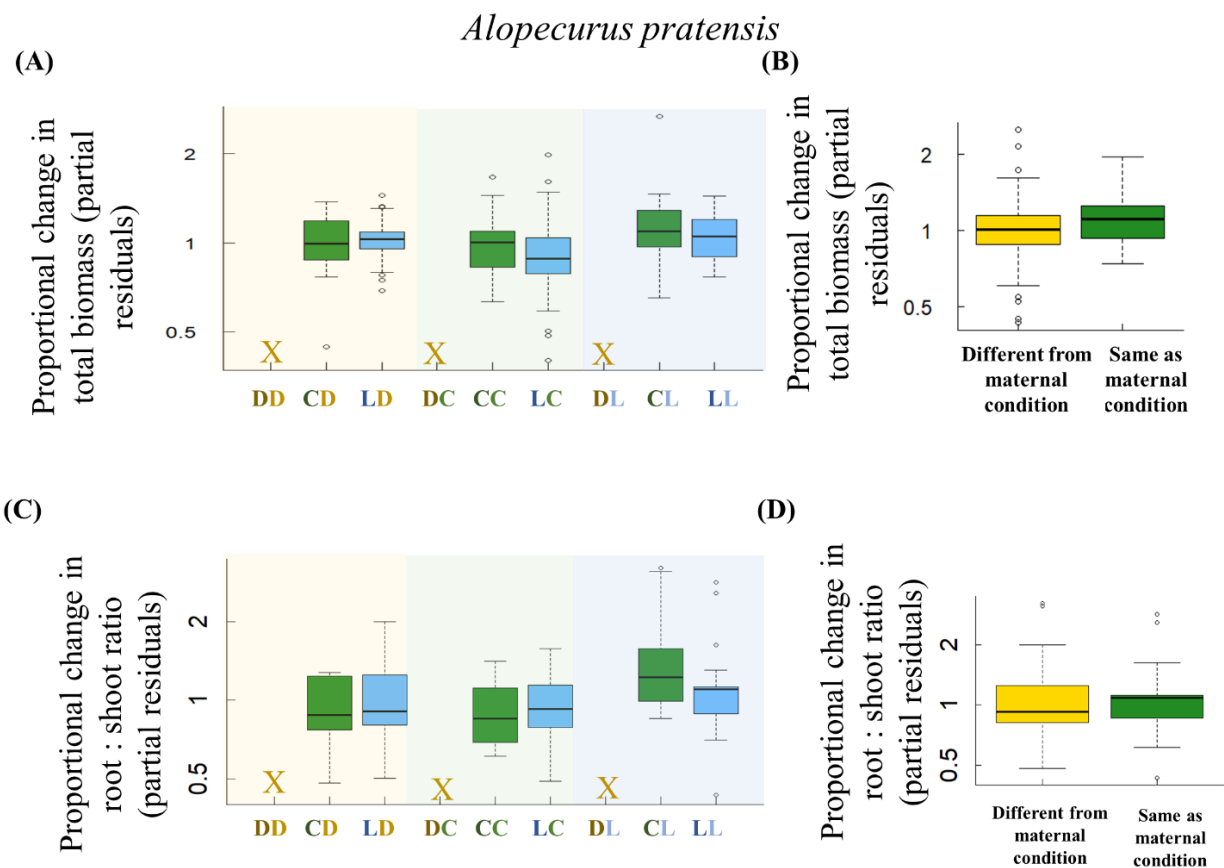
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Supplementary Table S3.1 The number of maternal plants (tested species were growing with or without competition) exposed to waterlogging, control or drought conditions, used for testing transgenerational adaptive effect.

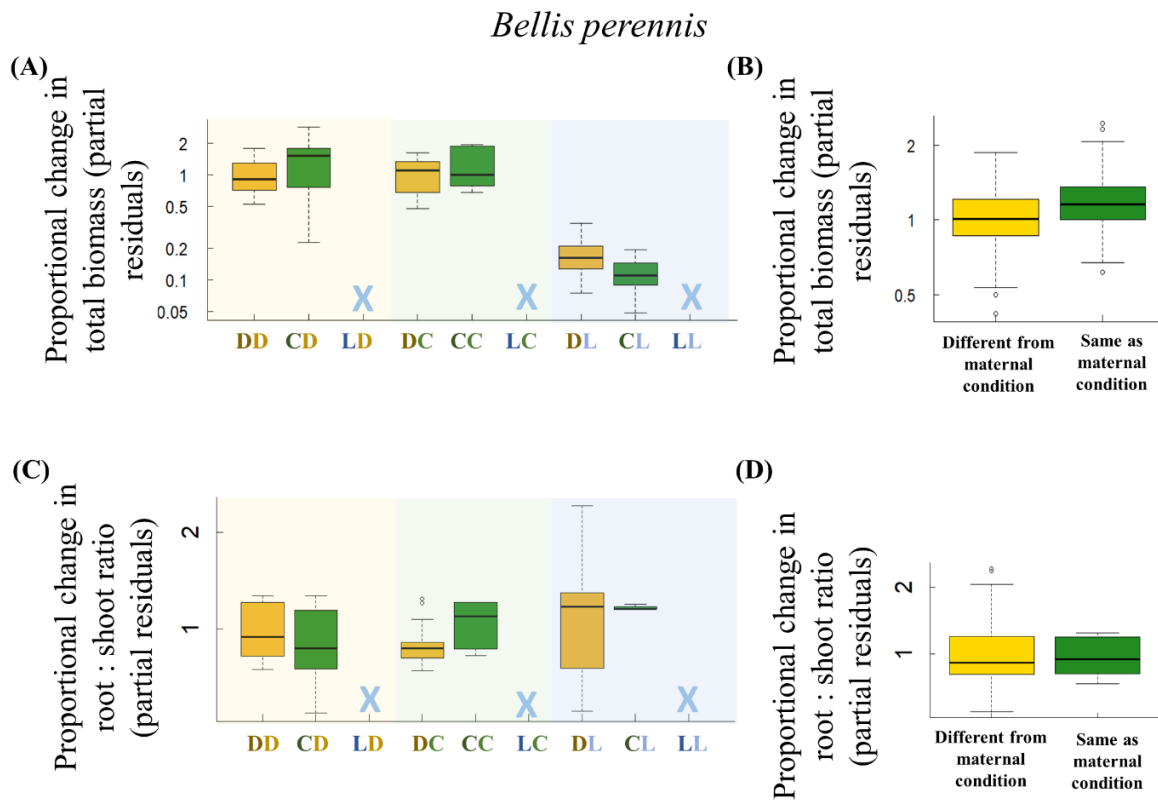
Species	Maternal plants	Maternal water treatments		
		Waterlogging	Control	Drought
<i>Alopecurus pratensis</i>	Without competition	2	2	0
	With competition	9	3	0
<i>Plantago lanceolata</i>	Without competition	3	5	5
	With competition	0	0	3
<i>Trifolium pratense</i>	Without competition	2	5	3
	With competition	5	6	2
<i>Bellis perennis</i>	Without competition	0	5	5
	With competition	0	0	2



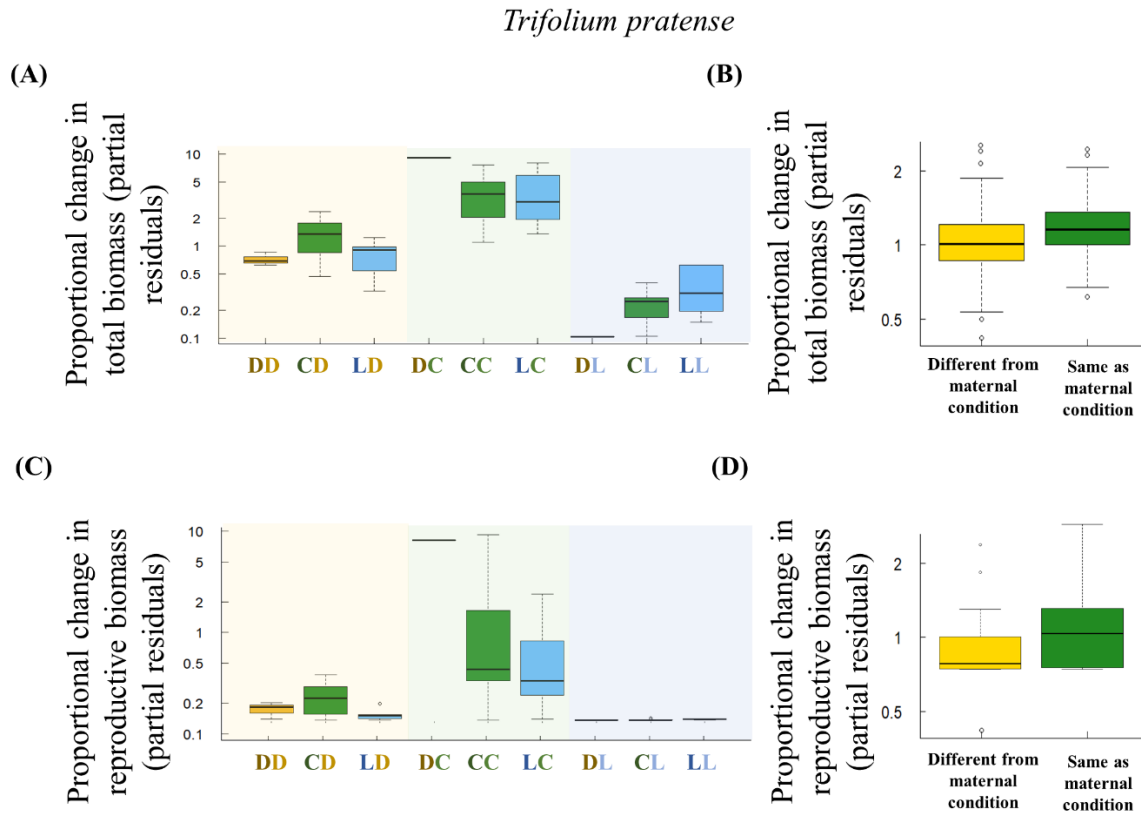
Supplementary Fig. S3.1 Responses of offspring *Plantago lanceolata* total and reproductive biomass and root : shoot ratio to stress conditions experienced by the offspring themselves and by their mothers. Proportional change in (A, B) total aboveground biomass and (C, D) reproductive biomass and (E, F) root : shoot ratio of *Plantago lanceolata* offspring plants. D - drought; C – control; L – waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition. Same as maternal condition – CC, DD, and LL, Different from the maternal condition – CD, LD, DC, LC, DL, CL. Crosses indicate that data was not available in the respective treatments.



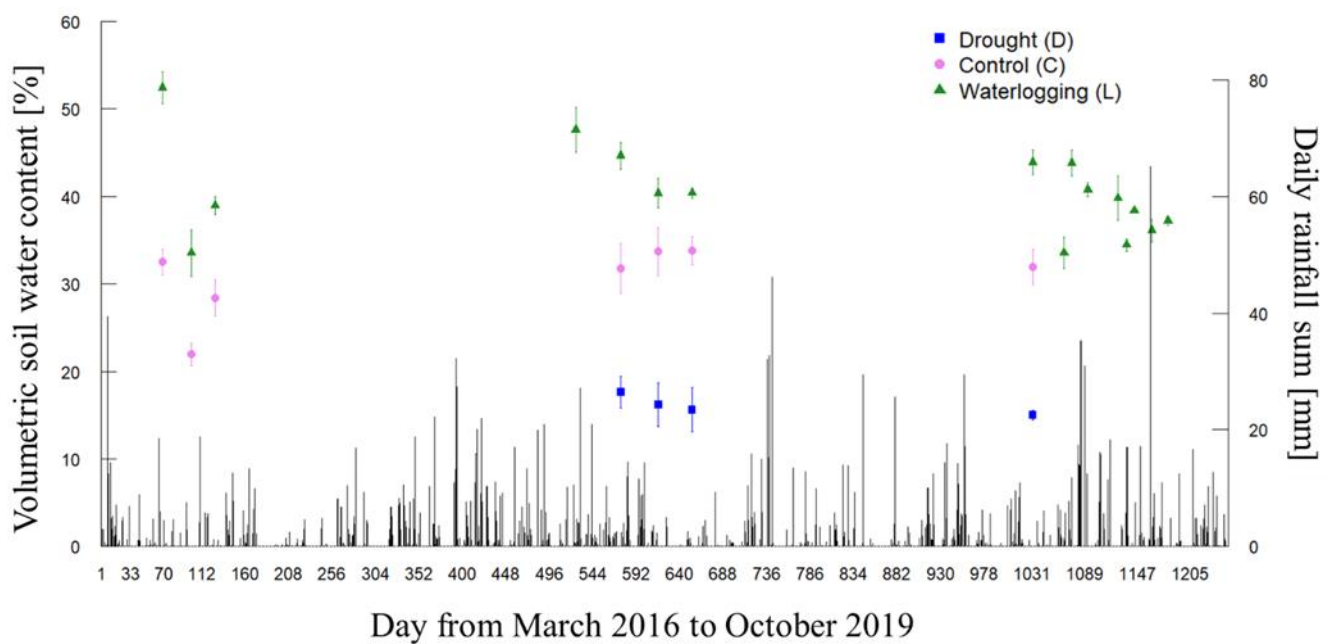
Supplementary Fig. S3.2 Responses of offspring *Alopecurus pratensis* total biomass and root : shoot ratio to stress conditions experienced by the offspring themselves and by their mothers. Proportional change in (A, B) total aboveground biomass and (C, D) root : shoot ratio of *Alopecurus pratensis* offspring plants. D - drought; C - control; L - waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition. Same as maternal condition - CC, DD, and LL, Different from the maternal condition - CD, LD, DC, LC, DL, CL. Crosses indicate that data was not available in the respective treatments.



Supplementary Fig. S3.3 Responses of offspring *Bellis perennis* total biomass and root : shoot ratio to stress conditions experienced by the offspring themselves and by their mothers. Proportional change in (A, B) total aboveground biomass and (C, D) root : shoot ratio of *Bellis perennis* offspring plants. D - drought; C – control; L – waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition. Same as maternal condition – CC, DD, and LL, Different from the maternal condition – CD, LD, DC, LC, DL, CL. Crosses indicate that data was not available in the respective treatments.



Supplementary Fig. S3.4 Responses of offspring *Trifolium pratense* total biomass and root : shoot ratio to stress conditions experienced by the offspring themselves and by their mothers. Proportional change in (A, B) total aboveground biomass and (C, D) root : shoot ratio of *Trifolium pratense* offspring plants. D - drought; C – control; L – waterlogging. The first letter represents maternal water condition, and the second letter represents offspring water condition. Same as maternal condition – CC, DD, and LL, Different from the maternal condition – CD, LD, DC, LC, DL, CL.



Supplementary Fig. S3.5 Water treatment data, precipitation and soil moisture from 2016 to 2019 measured monthly over the whole experiment. Symbols show mean ± 1 SE of the different water conditions.

Chapter 4

Effects of maternal environment and dispersal ability on transgenerational plasticity in plants[‡]

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Abstract

Transgenerational effects enable the transmission of environmental cues from parents to offspring. Adaptive maternal effects are expected to evolve if the maternal (or parental) environment contains information about the environment experienced by offspring. This correlation between maternal and offspring environments should be strongest in plant species with reduced dispersal ability. However, studies relating dispersal ability to the strength of maternal effects are rare. This study aimed to explore whether and how the dispersal distance of species and individuals affects offspring plant performance. Using seven common European plant species, we conducted a multi-year common garden experiment exposing maternal plants to three different water conditions (mesic, drought and waterlogging). At the end of the season in the first year, seed heads were collected from the lower and upper parts of each mother plant and used for dispersal distance calculation. Offspring coming from the maternal lower and upper parts were exposed to the same water treatments as mothers. Contrasting our hypothesis, we found that maternal water experience and species' dispersal abilities did not influence offspring performance (plant aboveground, belowground, reproductive and dead biomass). We did not detect maternal effects, meaning that offspring plants with the same water conditions as their mothers had the same fitness as offspring with different water conditions. However, opposite to our expectations, the longer dispersal distance of individual seeds ensured a stronger maternal effect when exposed to the same water stress as their mothers. Consequently, a stressful environment would select for long-distance dispersal.

Keywords: dispersal distance, drought, fitness-related parameters, maternal effect, multi-species study, seed dispersal, transgenerational memory

4.1 Introduction

Plants have evolved sophisticated mechanisms to acclimate to different stressors since they are incapable of moving from unfavourable conditions. A plant's phenotype can respond to variations in water, nutrients, solar radiation, temperature, photoperiod, salinity and many other environmental factors (Cayuela et al., 2019). This so-called phenotypic plasticity is the ability of a single genotype to produce multiple divergent phenotypes under different growth environments (Cayuela et al., 2019).

Plastic responses of parental plants to a new environment can subsequently alter offspring phenotypes, a phenomenon known as transgenerational phenotypic plasticity (Crean and Bpnduriansky, 2014). Transgenerational phenotypic plasticity is thus a mechanism by which the parental environment can alter offspring phenotypes without altering allele frequencies in the population. Transgenerational effects can be transmitted to their offspring through non-genetic and epigenetic mechanisms, such as seed provisioning (allocation of nutrients), DNA methylation, histone modification or small interfering RNA (Richards et al., 2017) but also through an alternation of proteins and secondary metabolites (Lukić et al., 2022).

Maternal effects, a special case of transgenerational phenotypic plasticity, represent an influence of the maternal environment on offspring plant performance (Marshall and Uller, 2007). They are adaptive when they enhance maternal and/or offspring fitness independently of genetic inheritance (Herman and Sultan, 2011). Maternal effects can only be adaptive and enhance offspring fitness and/or mothers when the maternal environment contains information about the offspring's environment. The offspring's environment should be similar to the mother's, e. g. when water stress conditions (drought and waterlogging) persist among generations (Galloway and Etterson, 2007). However, transgenerational phenotypic plasticity/maternal effects can also be neutral or maladaptive (Galloway and Etterson, 2007; Guillaume et al., 2016).

Seed dispersal distance is expected to alter the fitness consequences of maternal effects, as offspring with short-distance seed dispersal are more likely to encounter the same conditions as their mothers (Fenesi et al., 2014; McNamara et al., 2016; Yin et al., 2019). On the other hand, for species with frequent long-distance seed dispersal, the maternal environment may be a poor predictor of the offspring's environment (Galloway, 2005). Evaluating the dispersal

limitation on the strength of maternal effects can help to identify their ecological and evolutionary consequences. However, studies dealing with this issue are largely missing.

Finally, in wind-dispersed species, seeds starting from higher up on the mother plant are expected to disperse further (e.g. Thomson et al., 2011) and may thus show less (adaptive) maternal effects. For instance, a recent study (Gan et al., 2022) showed that the strength of the maternal effects could also be different in seeds coming from the same individual. Some species can simultaneously produce two (dimorphism) or more (heteromorphism) kinds of seeds differing in morphology, chemical contents or physiology (Imbert, 2002), with different dispersal abilities. These divergent seeds from a single plant could contain different maternal information (Gan et al., 2022). The authors (Gan et al., 2022) confirmed that offspring that came from short-dispersed seeds had a stronger maternal effect. However, multi-species studies testing the magnitude of maternal adaptive effects in species with divergent dispersal traits are rare. Little attention has been paid to species with short-distance versus long-distance seed dispersal and also to seeds with different dispersal traits/distances within a single species.

To examine the existence and the strength of maternal effects, we conducted a common garden experiment using multiple wind-dispersed species and the mechanistic Wald analytical long-distance dispersal (WALD) model (Katul et al., 2005) to test the maternal effects of drought and waterlogging on offspring performance. We further tested whether such effects depended on the seed dispersal ability of species or individual seeds. We hypothesised that maternal hydrological conditions (drought and waterlogging) improve offspring fitness under the same type of stress (H_1). In addition, the strength of adaptive maternal effects increases in species (H_2) or individual seeds (H_3) with lower seed dispersal ability.

4.2 Material and methods

A common garden experiment was carried out at the Heidfeldhof field station (48.712433 °N, 9.189879 °E) from March to October 2019, and in a greenhouse of the University of Hohenheim in Stuttgart, Germany, from March to September 2020. The mean annual temperature in 2019 was 10.57 °C, and the mean annual precipitation was 856.5 mm (www.wetter-bw.de). May was the wettest month in the year, and the mean monthly precipitation was 156.5 mm. The summer, however, was dry and hot (June - August, mean temperature 19.7 °C and precipitation 85.2 mm) (www.wetter-bw.de).

We used seven common European species for this experiment: *Crepis sancta* (L.) Bornm. (Asteraceae), *Hypochaeris glabra* L. (Asteraceae), *Hypochaeris radicata* L. (Asteraceae), *Bellis perennis* (Asteraceae), *Jacobaea vulgaris* L. (Asteraceae), *Atriplex hortensis* L. (Amaranthaceae) and *Capsella bursa-pastoris* (L.) Medik. (Brassicaceae). All species, except *C. bursa-pastoris* and *B. perennis*, have some form of diaspore appendages (pappus or membrane) (Table S4.1). Seed dimorphism is common in *A. hortensis*, *H. glabra* and *C. sancta* (Table S4.1). We used *H. glabra* and *C. sancta* seeds with a pappus from the central part of the seed head, and brown seeds of *A. hortensis* covered by a pale-yellow membrane. Additional information about the study species is given in Table S4.1.

4.2.1 Maternal generation

The mother plants (obtained from Rieger-Hofmann and Constance botanical garden) were sown and germinated in small pots (7 cm diameter) in a greenhouse from March to May 2019. Large pots (20 L; height 28 cm, diameter 35 cm) filled with sandy loam (14 % clay, 70 % sand, 16 % silt; pH 7.88) were used for seedlings transplantation (one seedling in the middle of the pot). Large pots were randomly arranged in large pools (275 L; randomly arranged in the field) in May 2019. We evaded potential pseudo-replication by placing all pots from the same species in different pools. Maternal plants were exposed to three different water-table treatments: mesic (M; water table depth (wtd) at -25 cm below the soil surface), waterlogging (L; wtd at -5 cm below the soil surface) and drought (D; empty pools) (Fig. S4.1; Lukić et al., 2020). Overflow holes maintained the specific water level in the pools. We used ten replicates for each species in each treatment. Maternal plants were grown under these conditions for one season (from March to October 2019). Seed heads were collected from the lower and upper

parts of each plant and used to determine the seed release height (H_r) and terminal velocity (V_t) (Fig. S4.1). Two species (*B. perennis* and *J. vulgaris*) were excluded from this part because these species had seed heads only on the lower or upper part of mother plants. We used these parameters to calculate seed dispersal distance. Plant height, percentage of wilted biomass and aboveground biomass were assessed at the end of October 2019. Aboveground biomass was cut at 2 cm, dried at 65°C for 72 h, and weighed.

4.2.2 Offspring generation

Seeds collected from the lower and upper parts of the mother plant were stored in paper bags at room temperature. Offspring treatments were conducted in the greenhouse from March to September 2020 (due to the SARS-CoV2 pandemic, we could not use the field station). In spring 2020, seeds from the lower and upper parts of the mother plants were separately germinated in the greenhouse in small pots (0.6 L, 9 cm diameter) (20 seeds per pot) filled with sandy loam (14 % clay, 70 % sand, 16 % silt; pH 7.88). Initially, we sowed 20 seeds per pot to determine the germination rate. Plants were irrigated twice per week. After one month (April 2020), all seedlings were removed (roots and shoots), except one target plant, which grew in the middle of the pot until the end of the season. Pots were randomly arranged using another word here (15 L, 60l x 40w x 6.4h cm, randomly arranged in the greenhouse). Offspring plants were also exposed to mesic (M; -6 cm wtd below the soil surface), waterlogging (L; -2 cm below the soil surface) and drought (D; empty pools) (Fig. S4.1). Pools were refilled twice per week to maintain the water table in control and waterlogging treatment. Soil moisture was determined in maternal and offspring generation once per month using a TDR probe (TDR 100) with 20 and 4 cm rods (Lukić et al., 2020).

Life-history traits

Aboveground biomass of offspring plants was harvested at three different points in time according to species phenologies and ripening: at the end of July (*C. bursa-pastoris*, *H. radicata*, and *A. hortensis*), in August (*H. glabra* and *C. sancta*) and in September (*B. perennis* and *J. vulgaris*). Total aboveground biomass was divided into vegetative, green parts (living biomass), wilted, brown parts (dead biomass) and reproductive biomass (seed and flower heads), dried at 65°C for 72 h and weighed. The proportion of dead biomass was determined as

the ratio of dead biomass to the total aboveground biomass. Besides that, reproductive biomass was collected over the whole season to obtain the total reproductive output (except *A. hortensis* and *B. perennis*, because offspring did not produce seed in that generation). We determined root biomass in *A. hortensis* and *C. bursa-pastoris* by washing roots over a fine sieve, drying them at 65°C for 72 h and weighing them. The number of leaves, the plant height and the percentage of wilted biomass were assessed monthly.

Dispersal traits

Upon fruit dehiscence, H_r was measured as the vertical distance between the soil surface and the mature seed head (capitulum). After each H_r measurement, the corresponding seed head was cut off and stored in a cylindrical paper bag to keep the seed appendages intact. This procedure was repeated, and H_r values and seed heads were collected from the upper and lower parts of the target plant. Seed terminal velocity (V_t) was estimated using the method described by Zhu et al. (2022)

Seed dispersal distance

We simulated seed dispersal distances of maternal and offspring seeds using the WALD mechanistic model (Katul et al., 2005).

$$p(x) = \left(\frac{\lambda}{2\pi x^3} \right)^{1/2} \exp \left[-\frac{\lambda(x - \mu)^2}{2\mu^2 x} \right]$$

where x is dispersal distance, the location parameter $\mu = H_r U / V_t$, the scale parameter $\lambda = (H_r / \sigma)^2$, U is the horizontal wind speed at seed release height, σ is a turbulent flow parameter reflecting wind speed variation. U was drawn from a Weibull distribution fitted to wind measurements with an eddy covariance station at the Heidfeldhof research station (Wulfmeyer et al., 2018). H_r and V_t were simulated with linear mixed-effects models where H_r and V_t are response variables, hydrological conditions, species identity and their interaction are fixed effects, and pot identity nested in pool identity are random effects. σ was estimated following Katul et al. (2005) (Supporting Information Appendix S1). To calculate the mean dispersal distance, we simulated 10^6 dispersal events for each plant species by randomly drawing a unique combination of parameters for U , H_r , V_t , and σ for each dispersal event (Travis et al., 2011; Zhu et al., 2021).

4.2.3 Statistical analyses

All statistical analyses were performed using R 4.01 (R Core Team, 2020). To test our first hypothesis, linear mixed-effect models were fitted for total aboveground, reproductive, root biomass, the proportion of dead biomass, and shoot/root ratio of offspring generation, using the *lme4* R package (Bates et al., 2015). Maternal and offspring water treatments were fixed effects, and pool and maternal pot (for repeated measurements) and species were random effects. To determine whether maternal effects were adaptive or not, we compared the performance of offspring that had experienced the same hydrological condition as their mothers (hereafter "S - same ", DD, MM, LL) with the performance of offspring that had experienced different hydrological conditions from their mothers (hereafter "O - other ", LM, LD, ML, MD, DL, DM). All response variables were log-transformed (Quinn and Keough, 2002). The fitted model was checked with the function `testDispersion()` in the package DHARMA (Hartig and Lohse, 2021), and no underdispersion or overdispersion was detected.

For further analyses (Hypotheses 2 & 3), we calculated fitness benefit as the proportion of total aboveground and reproductive biomass of offspring that experienced the same water stress conditions as their mothers (DD and LL) and offspring with different water conditions from their mothers (DL, LD, MD and ML). In order to determine the strength of maternal effects, we included only offspring under the same stress as their mothers (DD and LL), and we excluded offspring that did not experience stress in any generation. To test the second hypothesis, linear regression was fitted for fitness benefits of total aboveground and reproductive biomass and mean dispersal ability at the species level. For our third hypothesis, we fitted linear mixed-effect models where the response variable was fitness benefits (of total aboveground and reproductive biomass), dispersal ability at the seed level was the fixed effect, and species was a random effect. All response variables were log-transformed.

4.3 Results

Offspring fitness-related traits of the seven study species did not respond significantly to interactions between maternal and offspring environment (Fig. 4.1-4.2; S4.2-S4.3) (Hypothesis 1 rejected). Namely, offspring plants exposed to the same hydrological conditions as their mothers (S) did not significantly increase either the total aboveground biomass (likelihood-ratio test: $\chi^2_{1df} = 0.05$, $P = 0.824$; Fig. 4.1), reproductive biomass ($\chi^2_{1df} = 1.52$, $P = 0.217$; Fig. 4.2), the proportion of dead biomass ($\chi^2_{1df} = 0.01$, $P = 0.972$; Fig. S4.2) or root biomass ($\chi^2_{1df} = 1.99$, $P = 0.161$; Fig. S4.3A, B) compared with offspring growing under different conditions from their mothers. Moreover, there was no significant influence of maternal hydrological conditions on offspring shoot/root ratio ($\chi^2_{1df} = 0.26$, $P = 0.611$; Fig. S4.3C, D). Generally, there was no evidence for any maternal adaptive effect (Fig. 4.1-2; S4.2-S3) in the sense that offspring under the same conditions as their mothers performed better in any way.

Species dispersal abilities did not play a considerable role in the fitness benefits of either total aboveground biomass ($R^2 = 0.28$; $F_{1,5} = 1.95$, $P = 0.222$, Fig. 4.3) or reproductive biomass ($R^2 = 0.58$; $F_{1,3} = 4.14$, $P = 0.135$, Fig. 4.3) when offspring plants experienced the same water stress conditions as their mothers compared with offspring growing under different water conditions from their mothers. Obtained results did not support our second hypothesis.

Maternal seed position (lower and upper) played a considerable role in the fitness of their offspring, but a lower position did not lead to enhanced maternal effects (Fig. 4.4). Namely, the dispersal abilities of individual seeds significantly impacted the fitness benefits of both total aboveground ($\chi^2_{1df} = 4.06$, $P = 0.043$; Fig. 4.4A) and reproductive ($\chi^2_{1df} = 5.42$, $P = 0.02$; Fig. 4.4B) biomass in offspring that experienced the same water stress as their mothers compared with offspring growing under different conditions from their mothers. However, obtained results are contrary to our third hypothesis since we detected an increase in reproductive and total biomass of offspring coming from a maternal upper part that experienced the same stress condition as their mothers.

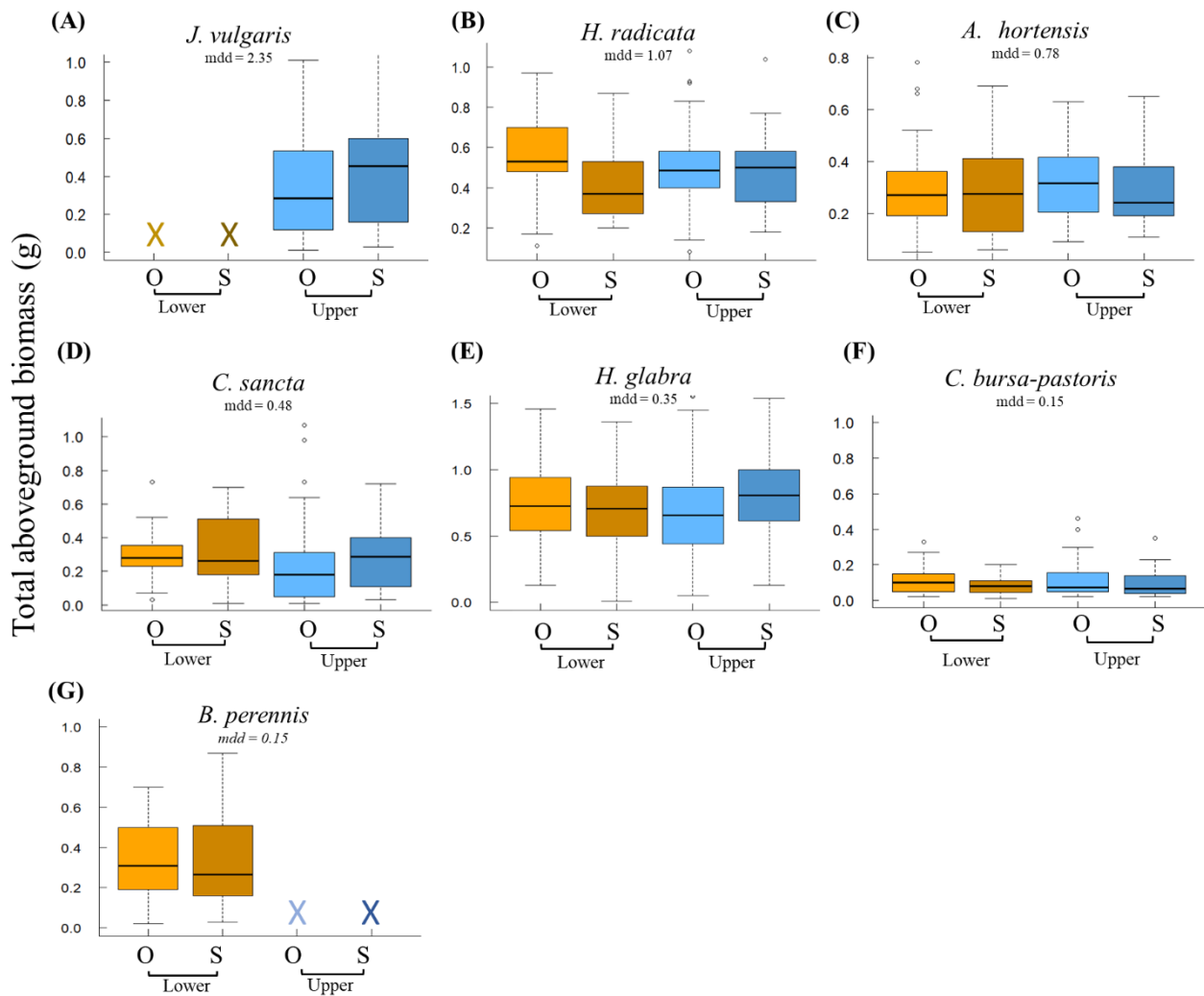


Fig. 4.1. Effects of different hydrological conditions on aboveground dry biomass of (A) *Jacobaea vulgaris*, (B) *H. radicata*, (C) *Atriplex hortensis*, (D) *Crepis sancta*, (E) *Hypochaeris glabra*, (F) *Capsela bursa-pastoris* and (G) *Bellis perennis*. S - the same (DD, LL and MM), O - other (DL, DM, LD, LM, MD and ML) hydrological conditions (D – drought, M – mesic, L – waterlogging) applied in offspring and maternal plants. mdd – mean dispersal distance (in m) on species level. Crosses indicate that data was not available in the respective treatments.

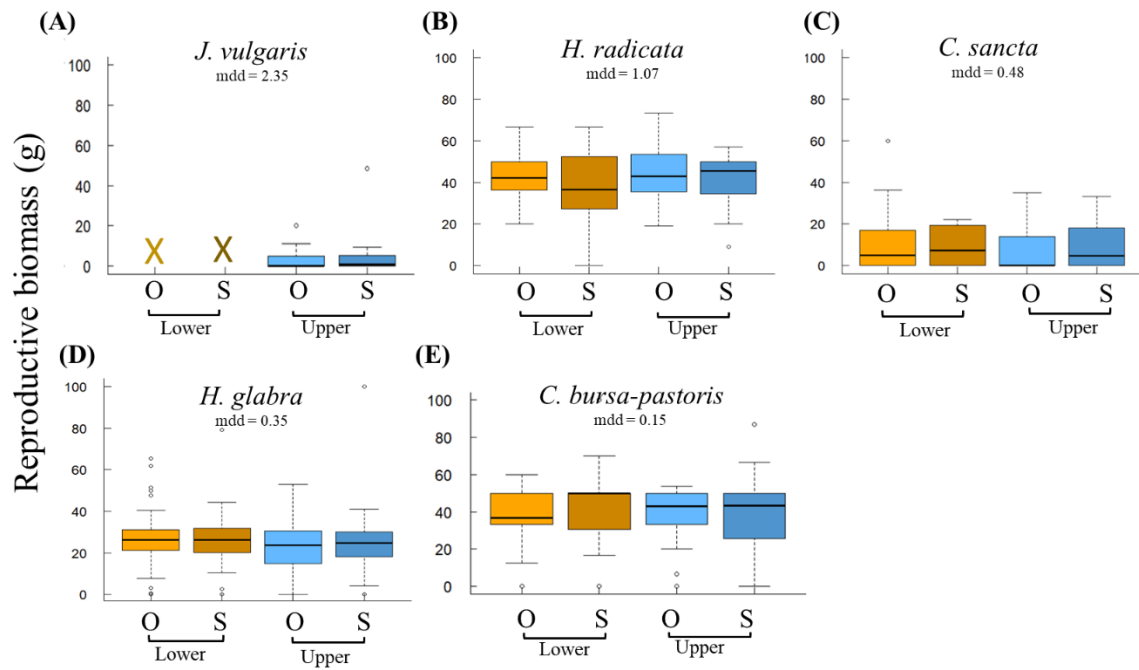


Fig. 4.2. Effects of different hydrological conditions on reproductive biomass of (A) *Jacobaea vulgaris*, (B) *Atriplex hortensis*, (C) *Crepis sancta*, (D) *Hypochaeris glabra* and (E) *Capsela bursa-pastoris*. S - the same (DD, LL and MM), O - other (DL, DM, LD, LM, MD and ML) hydrological conditions (D – drought, M – mesic, L – waterlogging) applied in offspring and maternal plants. mdd – mean dispersal distance (in m) on species level. Data for *Atriplex hortensis* and *B. perennis* reproductive biomass are not available. Crosses indicate that data was not available in the respective treatments.

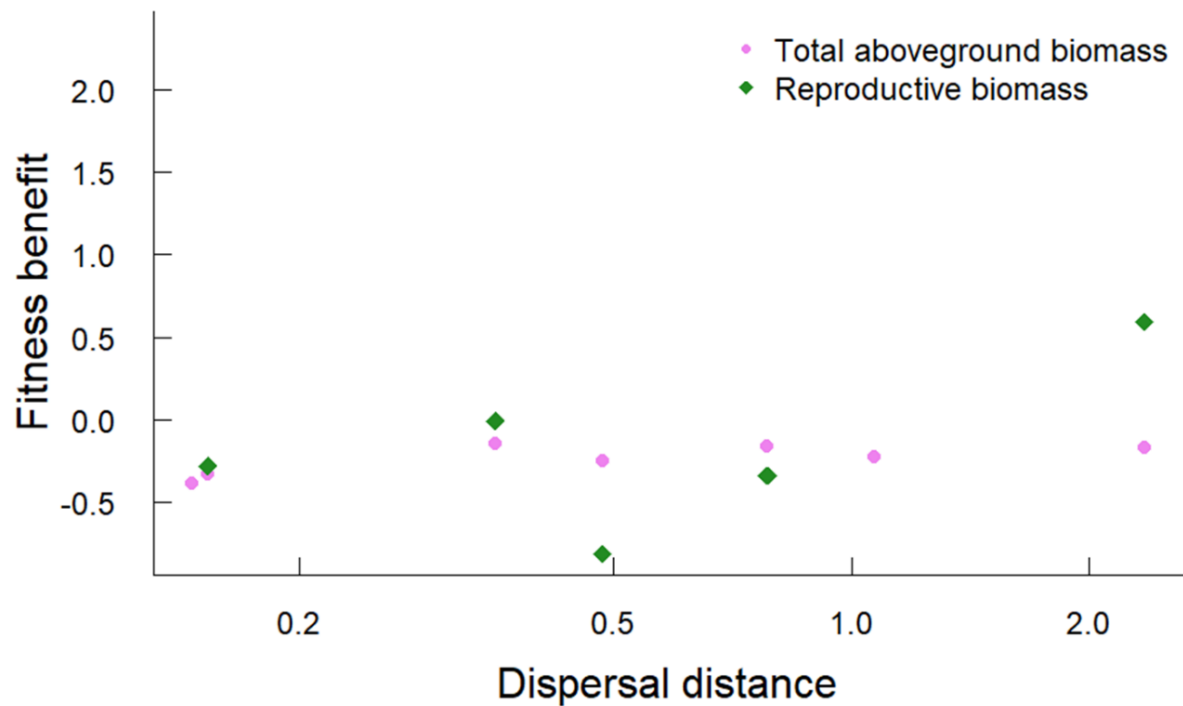


Fig. 4.3. Fitness benefits as a function of the expected mean seed dispersal distance of seven plant species. Fitness benefits were calculated as the proportion of total aboveground and reproductive biomass of offspring that experienced the same water stress conditions as their mothers (DD and LL) and offspring with different water conditions from their mothers (DL, LD, MD and ML). Data for *Atriplex hortensis* and *Bellis perennis* reproductive biomass are not available.

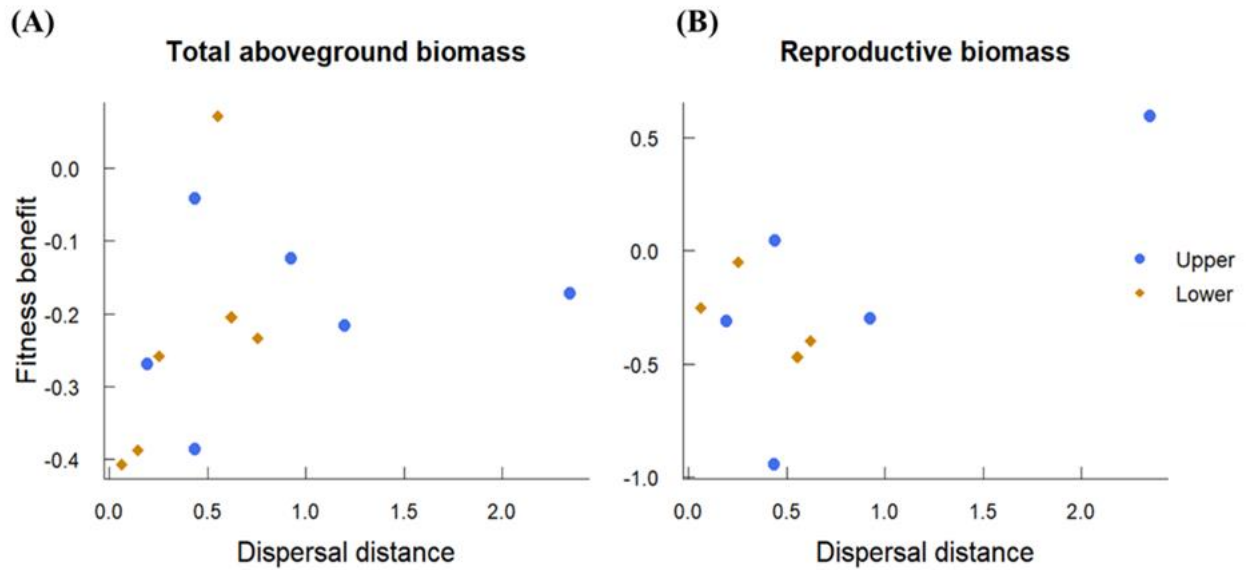


Fig. 4.4. Fitness benefits as a function of the expected mean seed dispersal distance of seven plant species from the lower and upper parts of mother plants. Fitness benefits were calculated for (A) total aboveground and (B) reproductive biomass. Fitness benefit were calculated as the proportion of total aboveground and reproductive biomass of offspring that experienced the same water stress conditions as their mothers (DD and LL) and offspring with different water conditions from their mothers (DL, LD, MD and ML). Data for *Bellis perennis* upper and *Jacobea vulgaris* lower parts, neither in total nor reproductive mass, are available. Also, data for *Atriplex hortensis* and *B. perennis* reproductive biomass are unavailable.

4.4 Discussion

To our knowledge, this is the first study to evaluate the impact of maternal effects and dispersal abilities on offspring plant performance during drought and waterlogging stress. Our results did not support our first hypothesis (H_1); offspring plants exposed to the same stress conditions as their mothers did not improve plant performance. Likewise, our second hypothesis was not supported. Moreover, opposite to our third hypothesis (H_3), individual seeds with longer dispersal distances increased the fitness benefits of aboveground and reproductive biomass.

Plant biomass and reproductive output are the main indicators of drought and waterlogging stress, as they integrate various molecular and physiological mechanisms involved in plant responses to repeated stress. We did not find any hints towards adaptive transgenerational effects in offspring. This suggests that droughted or waterlogged mothers may not transfer adaptive maternal effects to their offspring. This could be because abiotic stress is highly unpredictable in their natural environment (Metz et al., 2015), especially drought or excess waterlogging, which might differ between years. Still, many studies show transgenerational effects of drought (Zheng et al., 2017; Wang et al., 2018; Mojzes et al., 2021).

Our results are opposite to our H_3 hypotheses due to the fact that maternal effects are stronger in long-dispersed seeds. Our findings do not support the theory that cross-generational information transmission can be largely strengthened by dispersal limitation (Nathan and Muller-Landau, 2000) since dispersal limitation could increase the similarity between the offspring and maternal hydrological conditions. On the contrary, species that experienced the same stress as their mother could improve their fitness when they are far away from their maternal condition. Wang et al. (2022) suggest that seed dispersal could both strengthen or weaken transgenerational effects since different seed types represent different dispersal abilities (Yang et al., 2015; Wang et al., 2022). Contrary to our results, a recent study found that double drought-stressed offspring that originated from short-dispersed seeds transmit stronger adaptive transgenerational effects (Gan et al., 2022). This phenomenon could be common in annual species since the authors used *Synedrella nodiflora*, species with a very short life cycle that grows and reproduce very quickly. However, in our study, we mostly used perennial or biannual species, which could be one of the reasons for getting different results. Recently, Yin et al. (2019), in their meta-analysis, showed that transgenerational effects could be more common for short-lived annual species and less common for perennial species since they could have more

time for adaptation and do not have to rely on a single year reproductive output. In addition, to test hypothesis 3, we used only five species, and it is essential to include more species and height groups (not only upper and lower) in such a study to exceed limited statistical power.

There could be several reasons for the absence of a maternal adaptive effect and the presence of maladaptive or neutral effects in our plants. First, maternal effects depend not only on maternal phenotype but also on offspring genotype (Wolf and Wade, 2009). In addition, the maternal environmental experience could be reduced throughout offspring development, causing maternal cues to decline (Auge et al., 2017). As a result, offspring could respond to cues from their own environments irrespectively to the maternal information, whereas within-generation plasticity could mask maternal effects on offspring traits. It is suggested that within-generational plasticity would be advantageous if there is a high environmental fluctuation frequency (Kuijper and Hoyle, 2015; Auge et al., 2017). For instance, leaf morphology in *Claytonia perfoliata* plants responded to shade experienced by offspring but not by their mothers (McIntyre and Strauss, 2014). Secondly, offspring plasticity could also be masked when mother plants experience extreme environments. Backhaus et al. (2014), after applying severe and mild drought stress to two perennial species, reported that recurrent mild drought applied over a few years triggered plant stress memory, improving offspring drought resistance of plant communities and species. The authors suggested that maternal stress that triggers transgenerational effects does not have to be extreme. In our experiment, extreme water conditions, such as waterlogging and drought, could mask offspring plasticity. Thirdly, improvement in offspring performance could be costly to maternal fitness. In this case, a parent-offspring conflict can lead to suboptimal offspring traits (Uller, 2008). This parent-offspring conflict could lead to antagonistic responses in mother and offspring generations, particularly in perennials. Perennials might tend to conserve maternal fitness, which would be more important as they could lose only one offspring generation and invest in later generations.

In stressful environments, maternal effects can either increase offspring dispersal to enable them to escape stressful habitats (Teller et al., 2014) or increase offspring stress tolerance in the current stressful conditions (Mondoni et al., 2014). In our study, the absence of maternal effects in short-dispersed seeds is a disadvantage for their offspring, and it could have larger consequences on the local plant population, leading to local population extinction at a larger time scale. The absence of maternal effect in the local population could be detrimental to the offspring of endangered plant species. On the other hand, favouring long-distance dispersal seeds in a population and having higher fitness in stressful environments might promote species

coexistence at a larger scale. Avoiding local intraspecific competition and increased tolerance to water stress conditions in offspring with longer dispersal distances could speed up population spread. For the conservation of endangered species, such maternal effects could be very beneficial.

Species-specific patterns of transgenerational effects are a less understood aspect of ecological diversity (Sultan et al., 2009), and it remains largely unknown which maternal attributes and environment strengthen offspring performance. Further research should focus on the importance of plastic mechanisms in biodiversity response to environmental change. In addition, multi-generational experiments are essential to reveal the stability and strength of the transgenerational effects. Specifically, the effects of life-history and dispersal strategies on the strength and evolution of transgenerational effects are an exciting challenge for future studies.

4.5 Conclusion

Using multiple plant species of different life forms in controlled growing environments, this study provides mounting evidence demonstrating that adaptive transgenerational effects in response to drought and waterlogging may not be that frequent in plants, especially not in perennials. Maternal hydrological conditions and dispersal abilities on the species level did not significantly influence offspring plant performances, and this may decrease long-term population drought and waterlogging tolerance. We showed that dispersal limitation on seed level did not ensure a stronger maternal effect. We anticipate that the maternal-influenced dispersal process should considerably accelerate the selective advantage in stressful conditions.

Author contribution

This paper was drafted by NL based on conversations with JZ, JW and FS. JW obtained the funding for the research. JW, FS and NL designed the study, and NL performed the pot experiments and collected the data. All the authors together analysed the data, contributed critically to the drafts, and gave final approval for publication.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4.7 Supplementary information

Simulation of dispersal distance with the WALD mechanistic model

The WALD model is an analytical mechanistic model derived from a simplified 3-D stochastic dispersion model that retains the essential physics contained within the more computationally intensive coupled Eulerian-Lagrangian closure (CELC) model (Nathan et al., 2002). The analytical model reduces to the Wald (or inverse Gaussian) distribution (eqn 5b in Katul et al., 2005) that describes the probability density of dispersal distances x

$$p(x) = \left(\frac{\lambda}{2\pi x^3} \right)^{1/2} \exp \left[-\frac{\lambda(x-\mu)^2}{2\mu^2 x} \right] \quad (1)$$

where μ and λ are dispersal kernel parameters, and $\mu = H_r * U / V_t$, $\lambda = (H_r / \sigma)^2$, H_r is seed release height, U is the horizontal wind speed, V_t is seed terminal velocity, and σ is a turbulent flow parameter reflecting wind speed variation (Katul et al., 2005, Skarpaas and Shea, 2007).

$$\sigma^2 = \frac{4\sigma_w^4}{C_0 \varepsilon U} \quad (2)$$

where σ_w is the vertical turbulence, C_0 is Kolmogorov constant (3.125), and ε is the dissipation rate. While estimating ε is challenging within the canopy (Katul et al. 2005), we are mainly concerned with turbulent flow above the canopy, so we assume $\varepsilon = u_*^3 / K(z-d)$ and the constant $A_w \equiv \sigma_w / u_* \approx 1.3$ following Skarpaas and Shea (2007), where K is von Kármán's constant (0.41), z is height above ground, d is zero-plane displacement distance. Based on these assumptions and equation (2), we obtain

$$\sigma = 2A_w^2 \sqrt{\frac{K(z-d)u_*}{C_0 U}} \quad (3)$$

In the WALD simulation, each seed released was dispersed by randomly drawing a distance from the probability distribution function generated by the WALD model with parameters U , H_r and V_t . U was drawn from a Weibull distribution fitted to wind measurements with an eddy covariance station at the Heidfeldhof research station (Wulfmeyer et al., 2018), University of Hohenheim, Germany. H_r and V_t were simulated with linear mixed-effects models where H_r and V_t are response variables, hydrological conditions, species identity and their interaction are fixed effects, and pot identity nested in pool identity are random effects.

Dispersal environments

For the biological dispersal environment, the leaf area index (LAI) was estimated from the total aboveground biomass. For the physical dispersal environment, mean horizontal wind speed, friction velocity, zero-plane displacement distance and aerodynamic roughness length were measured with an eddy covariance station at the reference height of 2.63 m with high temporal resolution (0.1 Hz) throughout the dispersal season from June to September 2019 and 2020 (Wulfmeyer et al., 2018). A logarithmic wind profile was used to calculate the horizontal wind speed at any height of interest,

$$u = \frac{u_*}{k} \ln \frac{z-d}{z_0} \quad (4)$$

where u is the mean horizontal wind speed at the height z ; u_* is the friction velocity; k is von Kármán's constant (0.41); d is zero-plane displacement distance; and z_0 is aerodynamic roughness length (Monteith & Unsworth, 2013).

Table S4.1. Additional information about study species

Species	Family	Plant height (cm)	Ellenberg moisture indicator (F-value)	Diaspore appendages	Pollination	Habitat	Life forme
<i>Atriplex hortensis</i> L.	Amaranthaceae	50 - 120	5	seed dimorphism: brown seeds – covered by a pale-yellow membrane black seed – without appendages	outcrossing and selfing ⁶	Roadsides, field edges	Annual therophyte
<i>Bellis perennis</i> L.	Asteraceae	10 – 15	5	none	outcrossing and selfing ⁶	Well-drained and moderately moist meadows, pastures, lawns	Perennial hemicryptophyte
<i>Capsella bursa-pastoris</i> (L.) Medik.	Brassicaceae	5 – 80	5	none	outcrossing and selfing (predominately) ⁵	Roadsides, fields, wasteland	Annual or short-lived perennial monocarpic hemicryptophyte, therophyte
<i>Crepis sancta</i> (L.) Bornm.	Asteraceae	30 – 50	2	seed dimorphism: pappus: 4-5 mm long seed without pappus	outcrossing and selfing ³	Roadsides, vineyards, railroad areas, warm locations	Annual therophyte
<i>Hypochaeris glabra</i> L.	Asteraceae	5 – 50	3	seed dimorphism: pappus: central 9.5-15 mm long; peripheral 3-4 mm long	outcrossing and selfing (predominately) ¹	Roadsides	Annual/perennial therophyte
<i>Hypochaeris radicata</i> L.	Asteraceae	20 – 30	4	pappus: 9-10 mm long	outcrossing, self-incompatible ^{1,2}	Meadows	Perennial hemicryptophyte
<i>Jacobaea vulgaris</i> L.	Asteraceae	30 – 100	4	pappus: 4 mm long	outcrossing (predominately) and selfing ⁴	Pastures, woods and roadsides	Perennial hemicryptophyte, annual, biennial or short-lived perennial

¹Ortiz et al., 2006; ²Pico et al., 2004; ³Cheptou et al., 2001; ⁴Andersson, 1996; ⁵Douglas et al., 2015; ⁶Walter, 2020.

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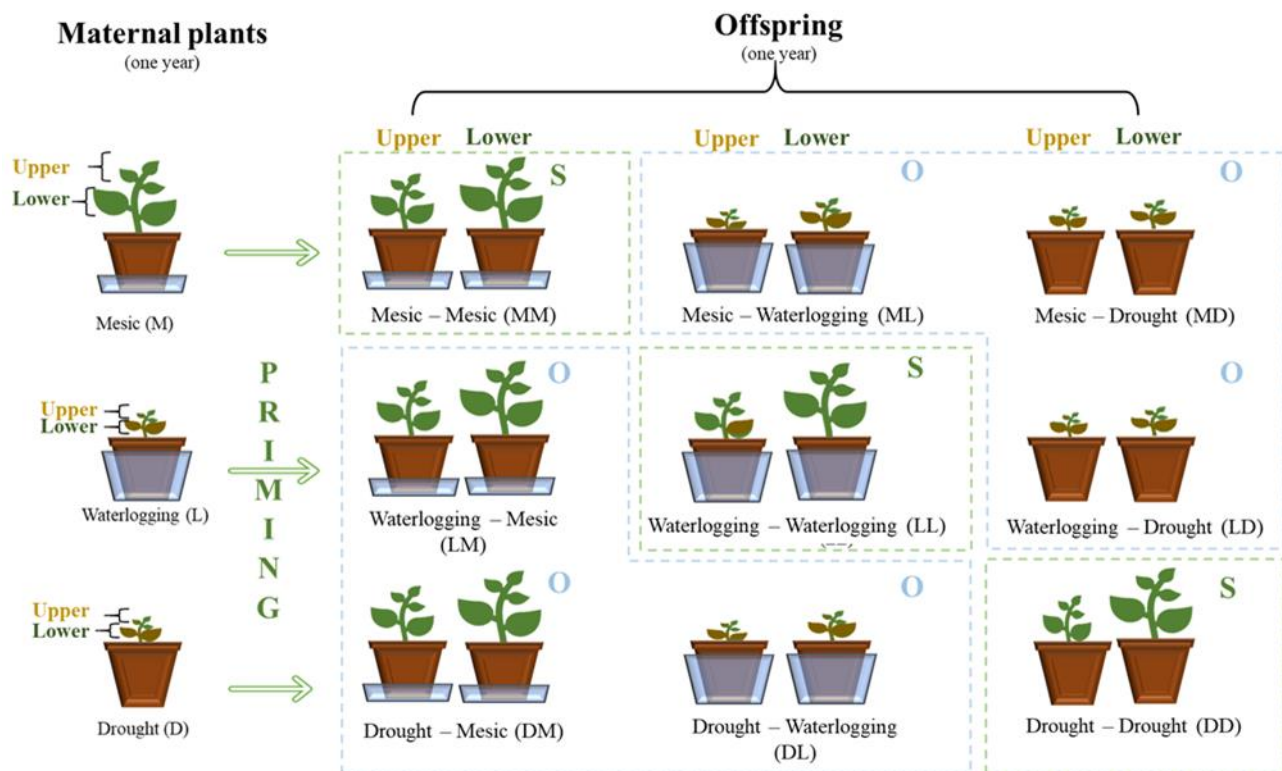


Fig. S1. Design of the experiment used to investigate the strength of transgenerational stress memory. Mothers and offspring were subjected to waterlogging or drought stress for one year. Seeds were collected from lower and upper parts of mother plants and sowed separately in offspring generation. D - drought; M - mesic; L – waterlogging. The first letter signifies the maternal treatment and the second offspring conditions. S – the same (DD, LL and MM), O – other (DL, DM, LD, LM, MD and ML) hydrological conditions applied in offspring and maternal plants.

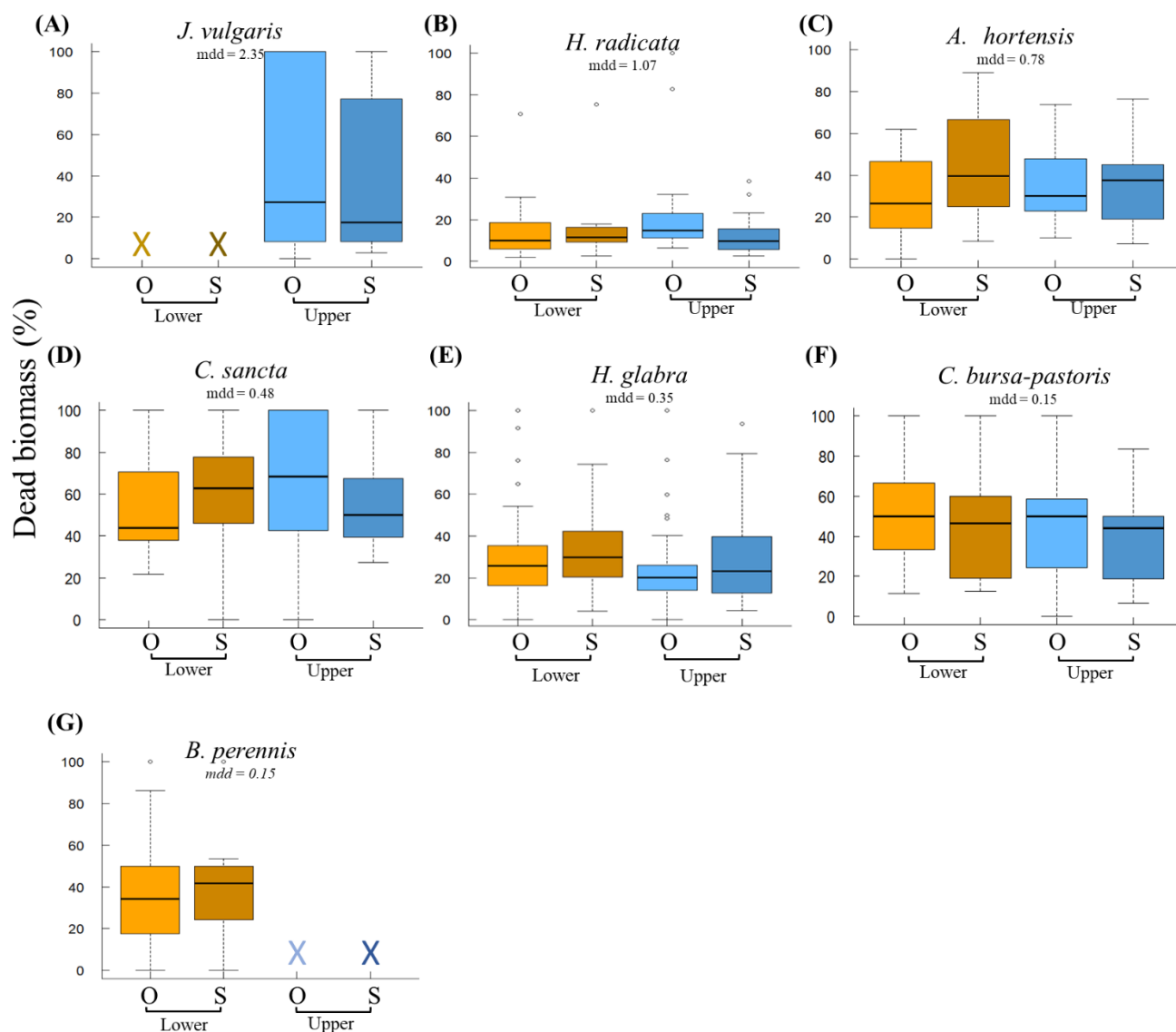


Fig. S4.2. Effects of different hydrological conditions on the proportion of dead biomass of (a) *Jacobaea vulgaris*, (b) *H. radicata*, (c) *Atriplex hortensis*, (d) *Crepis sancta*, (e) *Hypochaeris glabra*, (f) *Capsela bursa-pastoris* and (g) *Bellis perennis*. S – the same (DD, LL and MM), O – other (DL, DM, LD, LM, MD and ML) hydrological conditions (D – drought, M – mesic, L – waterlogging) applied in offspring and maternal plants. mdd – mean dispersal distance (in m) on species level. Crosses indicate that data was not available in the respective treatments.

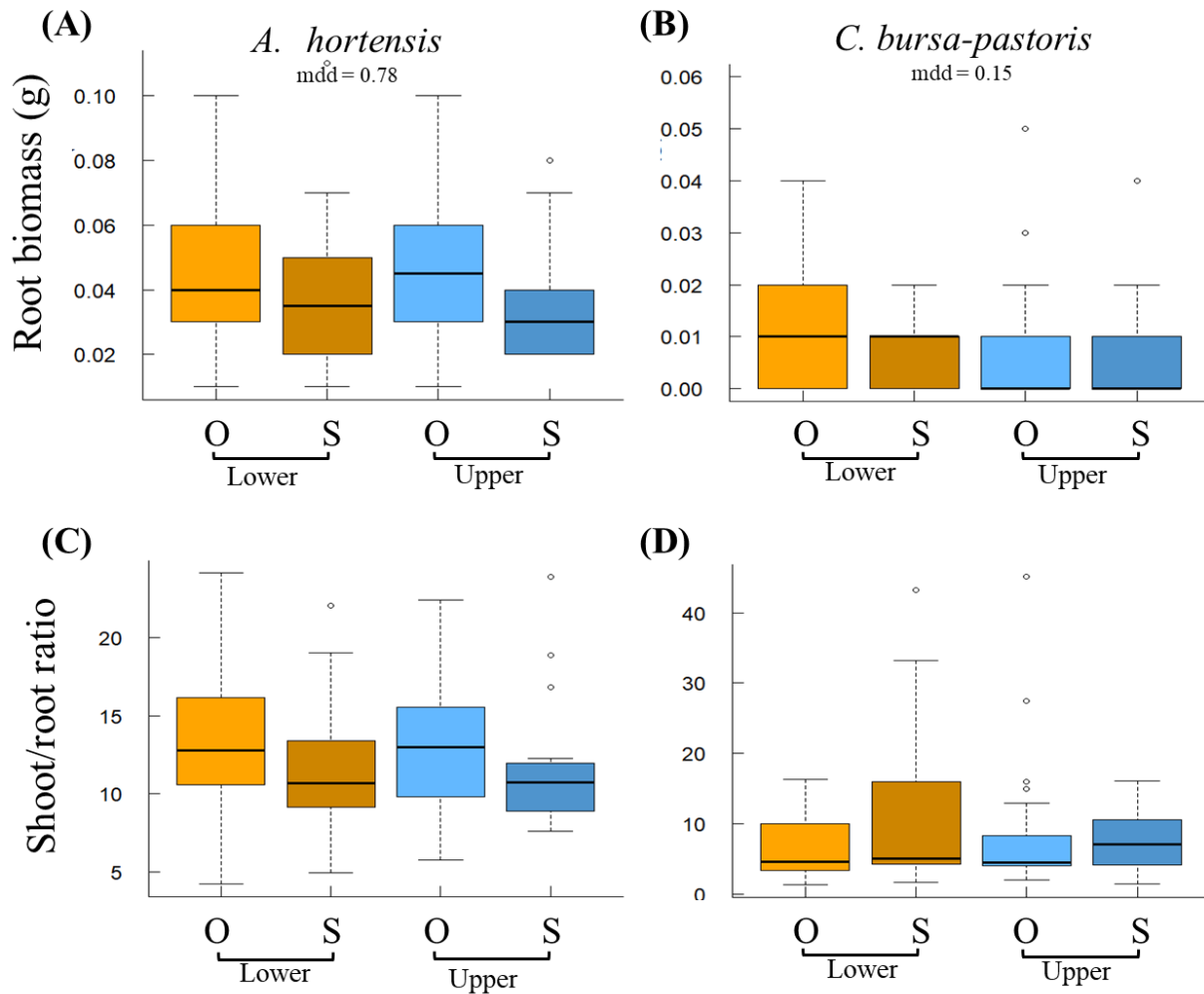


Fig. S4.3. Effects of different hydrological conditions on root biomass and shoot/root ratio of (a, c, respectively) *Atriplex hortensis* and (b, d, respectively) *Capsela bursa-pastoris*. S - the same (DD, LL and MM), O - other (DL, DM, LD, LM, MD and ML) hydrological conditions (D – drought, M – mesic, L – waterlogging) applied in offspring and maternal plants. mdd – mean dispersal distance (in m) on species level.

Chapter 5

GENERAL DISCUSSION

In the final part of my thesis, I summarise the previous chapters' essential findings and discuss the importance of plant stress memory for drought and waterlogging periods expected under climate change. Finally, I discuss potential directions for further research.

Drought and waterlogging are one of the most severe forms of plant stress and will become more frequent in the future under ongoing climate change. Tolerance to these stresses is a crucial solution for terrestrial plants to survive (Farrant and Moor, 2011). Plant stress memory could be one tool for improving tolerance (García-Campa et al., 2022).

In this thesis, I presented the influence of drought stress memory for several weeks on plant fitness and physiology. Drought-primed plants showed faster and stronger responses to the antioxidative defence system within-generation than a plant that did not experience drought stress before (Chapter 2; Hypothesis 1).

Chapter 3 (Hypothesis 2) shows that transgenerational plant stress memory plays an essential role in the rapid acclimation of plants toward water (drought and waterlogging) stress. Offspring showed an increase in plants' fitness when experiencing the same stress as their mothers, which can be linked to redox- and photosynthetic-related adjustments (Hypothesis 3). However, I did not detect cross-stress transgenerational memory because maternal stress *per se* did not increase offspring performance under stress compared to offspring whose mothers did not experience stress.

The influence of maternal seed position and maternal seed dispersal ability on offspring performance was examined under water stress conditions (Chapter 4; Hypothesis 4). However, in contrast to the findings of Chapter 3, transgenerational plant stress memory was not always adaptive for plants; I showed that in response to environmental stress such as drought and waterlogging, transgenerational memory was neutral for seven species studied in Chapter 4. In addition, maternal hydrological conditions and dispersal abilities did not significantly change offspring plant performance (Chapter 4; Hypothesis 5).

In the following, I will discuss drought, waterlogging, and cross-stress plant memory, the strength of transgenerational memory, and give implications and potential directions for further research.

5.1 Drought stress memory

Plant memory from previously experienced environmental cues could last several days or be translated to the next generation. These kinds of cues can influence subsequent plant stress responses and development. Drought-induced stress memory identified in *A. pratensis* lasted several weeks (Chapter 2). Plant responses to previous drought events affected plants on a morphological, physiological and biochemical level, improving plant tolerance to subsequent stress. In line with hypothesis 1 of this thesis, plants exposed to double drought stress minimised the percentage of dead biomass, ROS generation, upregulation of antioxidative enzymes (SOD and POX) and alternation of photosynthesis (Chapter 2). In accordance with my results, Wang et al. (2014) reported that drought priming upregulated the abundance of Rubisco small units and the activity of Rubisco. This has been proposed as a remarkable indicator of drought tolerance (Xiao et al., 2017). Similar results were shown when *A. thaliana* plants were exposed to the second, third and fourth drought stress (Ding et al., 2012). Further, Feleta-Soriano and Munné-Bosch (2016) showed that plants significantly decreased water loss rate, maintained ROS homeostasis and altered photosynthetic rate when plants were submitted for the second time to drought.

Environmental cues can be transmitted from parents to the next generation. My study revealed adaptive transgenerational effects only in some plant species (*P. lanceolata*, *A. pratensis*, *T. pratense*, *B. perennis*; Chapter 3), but not in species used for Chapter 4. Transgenerational plant stress memory played a crucial role in plant fitness improvement and acclimation to drought stress when offspring and mothers were exposed to drought stress (Chapter 3, hypothesis 2). *P. lanceolata* offspring with drought memory had less oxidative stress and tolerate drought stress conditions more effectively. Transgenerational drought memory relies on a similar mechanism as within-generation plant stress memory, enhancing photosynthetic pigment concentration; as a result improving the total photosynthetic outcome (Chapter 3). My results reveal that *P. lanceolata* plants experiencing drought in both offspring and maternal generation (DD) enhanced antioxidative machinery through increased total SOD, CAT and POX activity compared to drought-exposed offspring of "control mothers" (CD) (Chapter 3).

During drought and cell dehydration, intensive production of ROS will induce oxidative stress. Consequently, this could damage plant cell proteins, nucleic acids and cause lipid peroxidation (Mittler et al., 2004; Liu & Chan, 2015). An increase in antioxidative enzymes or non-enzymes can obtain ROS homeostasis and increase plant tolerance to drought stress.

Drought-priming within-generation in *A. pratensis* (Chapter 2) and *P. lanceolata* transgenerational priming (Chapter 3) increased the activity of main antioxidative enzymes SOD and POX in charge of elimination of superoxide anions and H_2O_2 (respectively). Additionally, *P. lanceolata* (Chapter 3), experiencing drought in both generations, also had a strong ability to deactivate ferrous ions, disabling lipid peroxidation (Lin et al., 2006) and generation of hydroxyl radicals (Halliwell and Gutteridge, 1988). Similar to my results, a recent study (García-Campa et al., 2022) showed that transgenerational priming helped plants quickly face repeated stress events, activating and rearranging secondary metabolism, discarding damaged lipids, decreasing photooxidation, and promoting redox homeostasis and respiration. Wang et al. (2018) showed that multigenerational drought priming maintained redox homeostasis in offspring. Also, Abdallah et al. (2017) showed that drought priming improved drought tolerance of drought-sensitive trees through an accumulation of proline and sugar content, leading to better water retention, activation of the antioxidative system, and maintenance of ROS homeostasis, increase in photosynthetic rate and biomass. My results suggest that adaptive transgenerational effects can partly overcome trade-offs between growth, reproduction and plant stress tolerance. However, further studies are needed to confirm these findings, since effect size, for redox- and photosynthesis-related parameters were small, and I used only *P. lanceolata* plants for this purpose.

Apart from being adaptive, transgenerational effects can also be neutral or maladaptive. Indeed, I did not detect the transgenerational memory in the plant species studied in Chapter 4. Namely, mothers did not show any adaptive maternal effect (Chapter 4). In this experiment, offspring grew in a glasshouse, where drought stress was more severe than in the maternal environment. Maternal plants grew in drought field conditions over the whole season for one year. Differences in the severity of drought could be one of the potential reasons for not detecting maternal effects in this study, even though it was suggested that events that trigger drought tolerance do not have to be extreme themselves (Backhaus et al., 2014). The authors (Backhaus et al., 2014) showed that mild drought stress improved plant resistance and adaptation in extreme drought events.

In Chapter 3, maternal and offspring plants grew in field conditions and experienced quite similar drought conditions in both generations. Another possible reason could be that improvement in offspring performance could be costly to maternal fitness (explained in Introduction section 1.2). In this case, a parent-offspring conflict can lead to suboptimal

offspring traits (Uller, 2008). This parent-offspring conflict could lead to negative responses in mother and offspring generations.

5.2 Waterlogging stress memory

In line with my second hypothesis, I demonstrated for the first time that waterlogging maternal effects increased offspring fitness in four perennial species when exposed to the same stress as their mother (Chapter 3). Plants that experienced waterlogging conditions in maternal and offspring generation produced more reproductive and vegetative biomass grew higher and produced a higher number of leaves than offspring experiencing different water conditions than the mother plant (Chapter 3).

Chapter 3 suggests a slightly different mechanism in waterlogging transgenerational memory compared to drought transgenerational memory. Waterlogged mothers invested more energy in activating SOD and the non-enzymatic part of the ROS-scavenging system. The antioxidative system is a crucial protective mechanism dealing with waterlogging stress in crop plants (Watkins et al., 2014). Indeed, *P. lanceolata* lineages experiencing waterlogging in both offspring and maternal generation (LL) enhanced antioxidative machinery through increased total SOD activity compared to waterlogging-exposed offspring of "control mothers" (CL) (Chapter 3). Moreover, a remarkable increase in phenolic compound content in the *P. lanceolata* plants experiencing waterlogging in offspring and maternal generation (LL) increased tolerance to waterlogging stress. Increased accumulation of phenolic compounds under waterlogging stress has been correlated with the higher antioxidative capacity of *P. lanceolata* plants, consequently adapting to novel conditions (Bansal and Srivastavam, 2012; Jiménez et al., 2015; Lukić et al., 2021). A preventive increase in phenolic compound content and SOD activity, instrumented by waterlogging-exposed mothers, consequently minimising ROS generation under repeated stress, could be a good strategy for waterlogging adaptive transgenerational effects to maximise growth and reproduction under stressful conditions.

Similar to transgenerational waterlogging memory presented in *P. lanceolata* in Chapter 3 of this PhD thesis, Li et al. (2011) and Wang et al. (2016) found that within-generation priming also alleviated the tolerance of wheat plants to waterlogging events after anthesis. Likewise, plants exposed to waterlogging in the vegetative growth phase increased net photosynthesis, stomata conductance, transpiration and activities of antioxidative enzymes SOD, CAT and APX and significantly increased reproductive output in the generative growth stage (Li et al., 2011). Waterlogging priming promoted stomata opening through an increase in

leaf photosynthesis and exogenous ethylene, indicating that energy metabolism and stress resistance were remarkably induced by waterlogging priming (Wang et al., 2016). Additionally, waterlogging priming remarkably induced enzymatic and non-enzymatic processes involved in the ascorbic-glutathione cycle and a higher aerenchyma formation rate in roots (Wang et al., Xiao et al., 2017). Waterlogging within-generation priming induced glycolysis, adventitious root development and ethylene biosynthesis in cucumber (*Cucumis sativus*) plants (Kęska et al., 2021).

However, similar to drought transgenerational memory, I did not detect the same pattern in plant species used for Chapter 4. Namely, transgenerational waterlogging memory was not adaptive in species used for this Chapter. A deeper understanding of the mechanism of plant stress memory is still lacking. Also, epigenetic regulatory pathways of waterlogging memory are mainly unknown.

5.3 Cross-stress memory

Cross-stress memory means that the acclimation towards one type of stress (e. g. drought) improves performance to a subsequent different stress type (e. g. waterlogging) (Huang et al., 2016). In the meantime, an increase drought periods as well as the frequency and amount of precipitation in some regions has also been predicted (IPCC, 2021). As a result, plants could face drought, waterlogging, or flooding during a single rainy season (Jaiphong et al., 2016). Exposure of plants to these two water stresses may be significantly more harmful than single stress (Grzesiak et al., 2016). The interchange between these stresses could be overcome by drought/waterlogging cross-stress memory. However, my study (Chapter 3) tested this kind of cross-stress memory for the first time and detected only the same-stress but no cross-stress memory. In this case, adaptive transgenerational memory should help plants adjust to a directional change in hydrological conditions (that become either drier or more waterlogged) but not to increased variability of hydrological conditions predicted for many parts of the world. Transgenerational memory thus does not appear to be beneficial in the future frequent changes between arid and wet conditions. Likewise, Quan et al. (2021) reported that long-lasting transgenerational memory could even be maladaptive when maternal-offspring environments differ.

Abiotic and biotic stresses could have a synergistic effect on plants (Mittler, 2006), where a number of studies showing cross-stress memory between drought and cold and heat, or even between abiotic and biotic stresses among different plant species (Rajashekar and Panda

2014; Wang et al. 2015; Hossain et al., 2018). Also, abiotic stresses could have an antagonistic effect (Mittler, 2006). In general, adaptive effects of drought and waterlogging stress should share the same metabolomic signature, having similar responses to stress: the maintenance of ROS homeostasis, activation of antioxidant enzymatic system in chloroplasts and mitochondria, as well as modulation in C metabolism and plant photosynthesis (Li et al., 2014). ROS homeostasis is one of the key factors contributing to cross-stress tolerance (Liu et al., 2022). A combination of these stresses during a single growing season could create divergent responses in plant metabolism. For instance, plants that first faced drought and then flooding decreased CO₂ assimilation, closing their stomata and leaf area (Jaiphong et al., 2016). On the other hand, flooding followed by drought conditions did not show any effect on CO₂ assimilation and plant leaf area (Jaiphong et al., 2016). The antagonistic effect of cross-stress memory was also shown when plants were exposed to heat/drought cross-stress (Rizhsky et al., 2002). Authors (Rizhsky et al., 2002) reported that a combination of heat and drought stress caused stomata closure; consequently, leaves could not be cooled. Also, high light and drought stress may limit CO₂ availability and enlarge O₂ generation, leading to ROS production (Mittler, 2002). Plant responses to stress combinations could create unique acclimation responses compared to single stress responses (Pandey et al., 2017).

However, there is still a limited understanding of the complex phenomenon of cross-stress memory, given that Chapter 3 is the first paper testing for drought/waterlogging cross-stress memory. Further research should focus on a more profound understanding of the signalling network involved in cross-stress tolerance and its influence on natural populations and communities.

5.4 The importance and strength of plant stress memory

In theory, within-generation plant stress memory is favourable when a) plants live in special heterogeneous habitats (Berrigan and Scheiner, 2004), b) environmental cues are relevant (Reed et al., 2010; Auld et al., 2010; Ernande and Dieckmann, 2004). Similarly, transgenerational memory (maternal effects) favours patchy environments and limited costs (Marshall and Uller, 2007; Uller, 2008). Within-generation memory cues will directly reflect the environment (Berrigan and Schener, 2004); on the contrary, maternal effects rely on maternal phenotype as the source of information about the maternal environment (English et al., 2015). Consequently, the maternal phenotype needs to be correlated with the maternal environment and the offspring environment (Kuijem and Hoyle, 2015). Transgenerational and

within-generation plant stress memory can have reinforcing, antagonistic, or interactive effects on offspring fitness (Auge et al., 2017; Vayda et al., 2018; Wadgyamar et al., 2018). In some cases, within-generation memory may override transgenerational memory to let offspring react to more accurate environmental cues from their own environment, even if transgenerational effects are adaptive. However, transgenerational memory could be an advantage when environmental variation in the maternal generation interacts with the offspring's environment during early ontogeny, tuning early development to the expected conditions (Wadgyamar et al., 2018). In general, plants with the highest fitness in a given environment will be selected, but when plants have different performances among habitats, a different individual with specific traits will be selected in different environments. The strength of plant stress memory will depend on the ability of plants in the same generation or maternal plants to produce diverse phenotypes prepared for the upcoming environments.

Dispersal limitation is considered one of the important mechanisms that could increase the strength of transgenerational plant stress memory. It is assumed that species with short-distance seed dispersal are more likely to encounter the same conditions between mother and offspring plants. However, my findings do not support the theory (and my fourth hypothesis) that cross-generational information transmission can be largely strengthened by dispersal limitation (Nathan and Muller-Landau, 2000) since maternal effects (drought and waterlogging) were not stronger in short-dispersed plant species or seeds (Chapter 4). To the contrary, I ultimately rejected my fourth hypothesis since I detected maladaptive maternal effects. Namely, species and individual seeds of offspring with longer dispersal distance, coming from the same stress conditions as their mothers, had a higher fitness. On the other hand, a recent study on *Synedrella nodiflora* showed that maternal effects could be dimorphism-dependent; the authors found that drought causes offspring (*Synedrella nodiflora*) originating from short-dispersed seeds have a stronger adaptive transgenerational effect (Gan et al., 2022). This discrepancy to my results could come from the fact that *S. nodiflora* are annual plants with a very short life cycle; they grow and reproduce very quickly; on the contrary, I tested mostly perennial species.

Plant stress memory, within the same or in the next generation, can affect the strength and direction of selection. Natural selection would change a population's frequency distribution of phenotypes. Further, standing genetic variations or developing new mutations with phenotyping effects can promote stress tolerance and adaptation to a novel environment (Fordyce, 2006). Additionally, it is proposed that plant stress memory could help in "buying time" for a population to survive and evolve when faced with novel environmental conditions

(Diamond and Martin, 2021). Plant memory could speed up this process, especially when memory costs are low and environmental changes are predictable. However, the conditions under which plant stress memory seems to be able to rescue the population and buy time for evolutionary adaptation appear to be limited (Diamond and Martin, 2021). The limited magnitude of the plastic responses that ultimately cope with novel environmental conditions could be one of the potential reasons. Another reason is that plant memories are not always beneficial in novel environments; they can also be detrimental or neutral, as shown in Chapter 4. Such non-adaptive or detrimental effects can significantly influence the strength and direction of ecological and evolutionary interactions and shift selection pressure across generations.

Finally, plant stress memory could lead to change in community patterns and extend the evolutionary potential of species (Fordyce, 2006).

5.5 Implications and potential directions for further research

Plant stress memory, as a term, has been well-established in plant ecology, but further studies on clarifying this phenomenon are urgently needed. Plant and seed priming in one or multiple generations could be an efficient, low-cost approach to increase plant reproductive output and enlarge plant tolerance to water stresses. Also, stress memory could be a supporting tool for plant populations to adapt to an uncertain climate.

However, it is essential to remember that plant stress memory is not a universal mechanism due to adaptive transgenerational effects that are not present in all plant species, as shown in Chapter 4. Investigating the ecologically significant discrepancies in maternal effects among species would be vital. Different maternal effects among species could increase biodiversity. Also, not having the same magnitude of stress in the maternal and offspring generations could lead to the absence of plant stress memory. Due to this, testing the magnitude of maternal/offspring stress and the engagement of temporal dynamics in stress memory is urgently needed.

Future studies should provide a better idea about the role of abiotic stresses, their intensity and duration on stress memory persistence. Nevertheless, it is important to detect traits that are linked to transgenerational plant stress memory. Additionally, upcoming research testing the role of temporal dynamics in stress memory is urgently needed (Quan et al., 2021). Future studies should focus on clarifying the persistence of plant stress memory and the duration

of adaptive effects, both within-generation and in the next generation. For instance, if plant memory is maintained over an extended period, but stress events are not that frequent or completely absent, it could become maladaptive (Quan et al., 2021). The role of temporal dynamics in stress memory will provide a better understanding of plant stress memory's ecological and evolutionary aspects. These investigations will also give answers needed for accurately predicting plant responses to changing climate.

Moreover, studying transgenerational effects in an ecological context is crucial to reveal their adaptiveness. Further, different plant population attributes will impact the likelihood that parental effects will get as a source of phenotypic adaptation to local habitats. So, it is essential to detect which attributes of the parental plant will be the best information source for predicting the offspring's environment. Generally, for future studies to understand plant stress memory, it is necessary to consider the ecological context in which transgenerational plasticity are expressed. For instance, it is important to detect the patterns of environmental heterogeneity in populations and their responses to it.

A better insight into the molecular (epigenetic) and biochemical mechanisms involved in within-generation or transgenerational stress memory will improve understanding of memory mechanisms and changes in plant populations. Indeed, illuminating the precise mechanism, especially behind transgenerational memory, will contribute to a deeper understanding of the evolutionary mechanisms and might be an innovative approach to improving crop resistance to abiotic stresses (Liu et al., 2022; García-Campa et al., 2022). Also, responses of grasslands to climate change and potential improvement in crop tolerance to abiotic factors are very important for farmers. Moreover, it is also vital to ascertain the ways of priming applications on a large scale.

5.6 Conclusions

This thesis makes novel contributions to understanding how plants respond to upcoming water stresses either as single stress or when applied in combination for one or two generations. Particular emphasis is placed on the strength of maternal effects related to dispersal abilities. Plant stress memory within-generation and in the maternal generation showed an increase in plant fitness, which can be linked to redox- and photosynthetic-related adjustments (Chapters 2&3). Indeed, the response of plant stress memory depends on the underlying physiological processes that natural selection could impact. Antioxidative enzymes and non-enzymes could improve the resistance of plants to abiotic stresses that could be applied for crop improvement in the future. Nevertheless, transgenerational plant stress memory associated with the cell's redox status also requires further studies since I used only studied it for one plant species (*P. lanceolata*). However, a combination of stresses and cross-stress transgenerational memory did not increase offspring performance under stress compared to offspring whose mothers did not experience stress (Chapter 3).

In general, drought and waterlogging memory can be a promising strategy for improving plant performance towards recurrent abiotic stress and possibly could "buy time" for a population to evolve and be a part of rapid evolution. This might shape plant responses towards hydrological stress under ongoing climatic change and needs to be considered when estimating future plant response scenarios. However, as I showed in this thesis, plant stress memory is not present in all tested plant species. A broader understanding of transgenerational memory could adjust crop improvement strategies for economically and ecologically important crops and tree species.

5.7 References

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Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Plant stress memory within and across generations

.....
is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart, 15.11.2022

Place, Date

Natasa Lugo

Signature