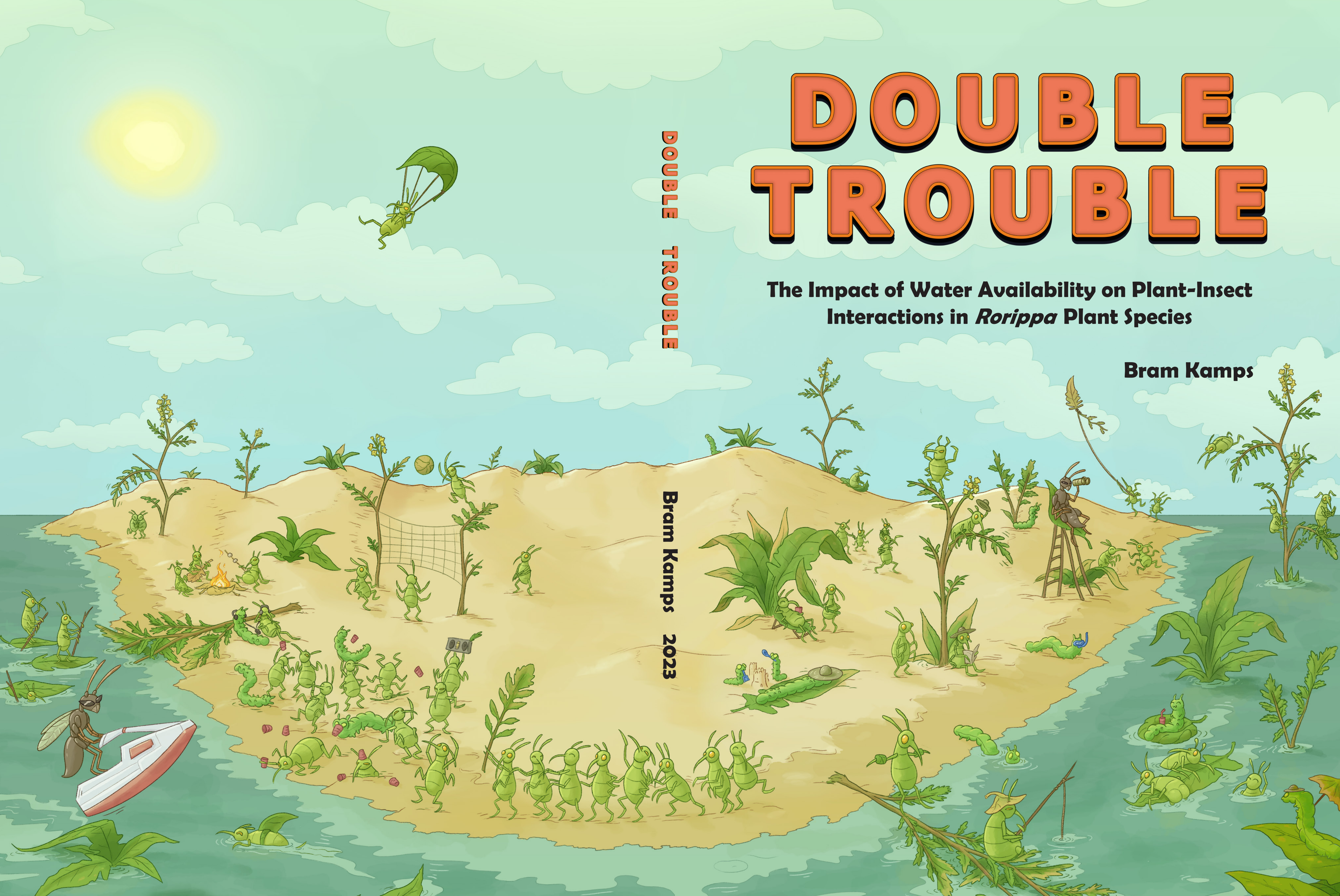

DOUBLE TROUBLE

Bram Kamps 2023

DOUBLE TROUBLE

The Impact of Water Availability on Plant-Insect Interactions in *Rorippa* Plant Species

Bram Kamps

Propositions

1. Studying adaptations of wild plants to complex environments is pivotal for improving resilience in agriculture.
(this thesis)

2. Deciphering the response signature of plants to insect herbivory requires investigations across a spectrum of abiotic conditions.
(this thesis)

3. Not supplementing the salaries of PhD candidates who arrive with a small scholarship leads to marginalization of their career perspective and social security.

4. The current PhD system causes a brain drain in academia.

5. Results oriented evaluation in science diminishes the value of proper scientific practices.

6. The effectiveness of a democratic system depends on the education of its participants.

7. Transitioning to worker-owned cooperatives increases economic and environmental sustainability.

Propositions belonging to the thesis, entitled:
Double Trouble: The Impact of Water Availability on Plant-Insect Interactions in *Rorippa* Plant Species

Bram B.J. Kamps

Wageningen, 8 December 2023

Double Trouble

The Impact of Water Availability on Plant-Insect Interactions in *Rorippa* Plant Species

Bram B.J. Kamps

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This research was conducted under the auspices of the Graduate School Experimental Plant Sciences.

Double Trouble

The Impact of Water Availability on Plant-Insect Interactions in *Rorippa* Plant Species

Bram B.J. Kamps

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Chapter 1

General introduction

In the past decades, there has been a notable increase in global weather extremes, leading to more frequent and severe instances of water-stress-induced crop damage (Lee et al. 2023; Zhao and Running 2010). This water stress arises from both excessive rainfall and prolonged periods of drought. Concurrently, insect herbivores are a significant additional cause of yield loss in crops (Deutsch et al. 2018; Lehmann et al. 2020). In response to sustainable agriculture initiatives, the use of insecticides for controlling these pests has been reduced, resulting in a growing demand for insect-resistant crop varieties (van der Werf and Bianchi 2022; Wagner et al. 2021). Water stress and insect herbivory represent prominent threats to plant survival and productivity, with their combined effects possibly exacerbating the overall damage. Hence the plant might find itself entangled in a situation of **double trouble**, grappling with both water stress and insect herbivory simultaneously, intensifying its struggles. The effective management of insect pests in an environment with escalating occurrences of water stress poses a major challenge to modern agriculture. Water stress not only directly impacts plant performance, but it may also influence plant resistance to insect herbivores (Leybourne et al. 2021; Pineda et al. 2016). Historically, research efforts have primarily concentrated on understanding the responses of plants to either biotic or abiotic stresses in isolation, disregarding the intricate interplay between these factors. Yet, in reality, these stressors frequently occur concurrently. For the development of resilient crops, it is imperative to gain a comprehensive understanding of how plants have evolved to adapt and respond to complex environments characterized by the simultaneous occurrence of biotic and abiotic stressors i.e. double trouble. By deciphering the mechanisms that enable wild plants to thrive in such challenging conditions, we can enhance crop breeding strategies and develop more robust agricultural systems capable of withstanding combined stress factors. Thus, in my study I aim to explore the intertwined effects of water stress and insect herbivores on plants.

The world's lush greenery owes its existence to the remarkable adaptations of wild plants, enabling them to withstand the double trouble posed by water stress and insect herbivory. These adaptations highlight the extraordinary defense mechanisms that plants have evolved to ensure their survival in the presence of herbivores (War et al. 2012; Wilkinson and Sherratt 2016). The coexistence of plants and herbivores within natural ecosystems is attributed to the plants' ability to employ a combination of direct and indirect defenses, such as physical barriers and toxic compounds, as well as attracting natural enemies of herbivores to mitigate the pressure of herbivory (Belete 2018; War et al. 2012). Moreover, in the context of water stress, wild plants demonstrate adaptive traits and mechanisms that strike a delicate balance between conserving water and using resources to grow and defending against herbivores (Karabourniotis et al. 2014). These sophisticated adaptations reflect the dynamic interplay between ecological factors and evolutionary pressures, imperative for survival in challenging environments. Understanding these adaptations and the underlying mechanisms behind them offers valuable insights into the interactions between plants and insects under varying water availability conditions. By leveraging this knowledge, we can explore innovative strategies to enhance crop resilience and sustainability amid changing environmental conditions.

Water is crucial for plants

Water, an indispensable resource, plays a pivotal role in the growth, survival, and reproductive success of plants. As primary producers, plants rely heavily on water for vital physiological processes, including photosynthesis, nutrient uptake, and transpiration. Water serves as a critical medium for transporting essential nutrients and minerals throughout the plant, supporting crucial metabolic activities. Moreover, it provides structural support to plant cells, maintaining their turgidity and preventing wilting (Wahab et al. 2022; Wang et al. 2003). Furthermore, water availability profoundly influences plant morphology, impacting leaf size, root development, and overall plant architecture (Grubb 1986). The significance of water in plant biology and ecosystem dynamics cannot be overstated, as it is the foundation for plant functioning and productivity. However, both excess and scarcity of water can pose significant challenges and potential harm to plants.

How plants respond to drought

Drought stress, characterized by insufficient water availability, poses a significant environmental challenge with profound implications for plant growth, development, and productivity (Anjum et al. 2011; Showler 2013). Prolonged or severe drought stress can result in detrimental effects, including wilting, stunted growth, leaf abscission, and the accumulation of reactive oxygen species (ROS), ultimately leading the death of the plant and to reduced crop yield (Kumaraswamy and Shetty 2016; Liliane and Charles 2020). In response to water scarcity, plants undergo a series of intricate physiological, biochemical, and molecular changes (Anjum et al. 2011; Chaves et al. 2003). Drought stress triggers a diverse array of adaptive mechanisms aimed at minimizing water loss, optimizing water use efficiency, and maintaining cellular homeostasis. At the cellular and molecular levels, plants engage a complex network of signaling pathways and gene expression to regulate water transport, osmotic adjustment, antioxidant defense, and the synthesis of protective compounds (Anjum et al. 2011; Bi et al. 2017; Chaves et al. 2003; Leybourne et al. 2022; Lin et al. 2021). Central to the regulation of drought responses is the upregulation of phytohormones such as Abscisic acid (ABA), Ethylene (ET), Jasmonic acid (JA), and Salicylic acid (SA) (Arraes et al. 2015; Nakashima et al. 2014; Orellana et al. 2010). These responses

encompass a range of adaptations, including 1) morphological adjustments like enhanced root growth, leaf rolling, and reduced leaf area (Bi et al. 2017; Hanley et al. 2007), 2) chemical modifications like the production and release of osmolytes to maintain cell turgor (Camisón et al. 2020; Wahab et al. 2022) or an increase in antioxidants to mitigate oxidative stress (Ahmad et al. 2010; Sharma et al. 2012), and 3) physiological changes like stomatal closure to limit water loss through transpiration (Miyashita et al. 2005; Murtaza et al. 2016). However, stomatal closure also restricts the plant's photosynthetic capabilities. Consequently, plants have to strike a balance between water conservation and photosynthesis. While drought can exert detrimental effects on plants, plants possess a repertoire of adaptive strategies to limit the damage caused by water scarcity.

How plants respond to waterlogging

Excessive water can lead to a condition known as waterlogging, in which plant roots suffer from oxygen deprivation, resulting in root damage, disrupted nutrient uptake, and ultimately, plant death (Liliane and Charles 2020; Parent et al. 2008; Tian et al. 2021). The responses of plants to excessive water conditions exhibit striking similarities with their responses to drought stress. Waterlogging impairs root health due to the lack of oxygen, leading to diminished water and nutrient absorption. This, in turn, induces osmotic and oxidative stress, causing nutrient deficits that impede plant growth (Muhammad 2012; Pan et al. 2021; Parent et al. 2008). In response to oxidative stress, plants up-regulate the production of antioxidants to counteract harmful reactive oxygen species (Sharma et al. 2012). Moreover, the synthesis and release of osmo-protective compounds aids in water retention. These processes, reminiscent of drought stress responses, are under the influence of the same phytohormones ABA, ET, JA, and SA (Arbona and Gómez-Cadenas 2008; Tamang et al. 2021). Interestingly, the same hormones in the context of waterlogging elicit distinctly different morphological changes. For instance, certain plants undergo elongation as a preparatory mechanism for potential flooding subsequent to waterlogging, utilizing their aboveground parts as a snorkel to facilitate oxygen transport to their submerged roots (Koramutla et al. 2022; Laan et al. 1989). In contrast, other plants adopt a survival strategy of minimizing metabolism and patiently waiting for the water to recede (Akman et al. 2012). These divergent responses underscore the plethora of adaptations that plants have evolved to cope with the problems of waterlogging.

How plants respond to insect herbivory

Plants exhibit complex responses to not only their abiotic environment, but also biotic factors, including interactions with herbivorous insects. This process begins with the recognition of the herbivore's presence even before they start feeding (Arimura et al. 2011; Bown et al. 2002; Hilker and Fatouros 2015). As the herbivore inflicts damage on the plant, the plant releases molecules that it can perceive as damage signals, known as Damage-Associated Molecular Patterns (DAMPs) (Zebelo and Maffei 2015). Additionally, plants can recognize herbivore specific molecules, such as those present in the saliva of the insect, termed Herbivore-Associated Molecular Patterns (HAMPs) (Gandhi et al. 2020). This recognition initiates a cascade of general and specific responses aimed at resisting the herbivore's attack, occurring both locally and systemically. These responses involve the induced biosynthesis of phytohormones, including Jasmonic acid (JA) and Salicylic acid (SA) (Ryan and Moura 2002). Initiated by these phytohormones, plants can activate direct defenses, involving morphological or biochemical adaptations that deter the herbivore. Morphological responses may include increasing trichome density (Handley et al. 2005; Peters and Berry 1980; Tian et al. 2012). Induced biochemical responses entail the production and release of compounds toxic to the herbivore, like the well-studied glucosinolates in many brassicaceous plants (Hopkins et al. 2009). Moreover, plants can employ indirect defense strategies by releasing Volatile Organic Compounds (VOCs) upon being attacked by an herbivore. These VOCs attract natural enemies of the herbivore, assisting them in locating and eliminating the plant's attacker (Turlings and Erb 2018). The co-evolutionary arms race between plants and herbivores has led to the development of a wide array of defense responses, which may vary in specificity and effectiveness depending on the prevailing circumstances. As plants continue to evolve defenses against herbivores, these herbivores in turn may adopt strategies to overcome these defenses. The resulting diversity of defense mechanisms allows plants to effectively respond to different herbivore challenges and illustrates the intricate and dynamic nature of plant-herbivore interactions.

Double trouble

In a natural environment, the occurrence of a water deficit or surplus can coincide with insect herbivory. It is plausible that the response to one stressor will intricately influence the interaction that the plant establishes with the other stressor. For example, the reduction of biomass caused by suboptimal water conditions may render plants less tolerant to herbivore damage. Conversely, this reduced biomass might make the plant less attractive or apparent to herbivores, potentially causing them to seek other, more suitable hosts (Smilanich et al. 2016; Strauss et al. 2015). At a morphological level, changes in leaf shape or leaf cuticle, as a response to drought, could affect insects that feed on those leaves (Chaves et al. 2003; Grubb 1986; Hanley et al. 2007). Similarly, waterlogging-induced formation of aerenchyma in certain plants (Koramutla et al. 2022; Laan et al. 1989; Yamauchi et al. 2018) may impact how aphids and other piercing/sucking herbivores can navigate their stylets to reach the

phloem for feeding. These dynamic interactions between plant responses to water stress and insect herbivory illustrate that even before responses to the two stressors start interfering, a response to one might already have a substantial impact on the perception of the other.

Furthermore, in response to water stress, many plant species exhibit adaptive responses involving the release of stored energy in the form of free amino acids and soluble sugars. These compounds serve as osmo-protectants, maintaining cell turgidity, or are utilized by the plant to cope with the stress (Krasensky and Jonak 2012; Parida et al. 2018). Studies have demonstrated significant alterations in sucrose concentrations within leaves of water-stressed plants, along with changes in amino acid levels, particularly asparagine, leucine, and proline (Barber and Müller 2021; Khan et al. 2010; Lin et al. 2021). Interestingly, these water-stress-induced responses in plants can have direct implications for insect herbivores, as these sugars and amino acids are crucial food sources for herbivores (Bouchebti et al. 2022; Bursell 1981; Noor-ul-Ane and Jung 2022; Sacktor and Childress 1967; Stec 2018; Teulier et al. 2016). Consequently, the suitability of a plant as a host for specific herbivores can be influenced by these alterations in nutrient availability and composition (Leybourne et al. 2021; Mewis et al. 2012; Mezgebe and Azerefegne 2021; Pompon et al. 2011). The changes in plant chemistry under water stress may impact the nutritional value of the plant for herbivores, potentially affecting their feeding behavior and overall fitness.

As previously mentioned, the responses of plants to water stress and herbivory are regulated by the biosynthesis of phytohormones, initiating signaling cascades to elicit appropriate reactions. The same phytohormones that regulate defense against herbivore attacks also play crucial roles in plant responses to water stress (Kessler et al. 2004; Nguyen et al. 2016a; Nguyen et al. 2016b; Per et al. 2018; Ullah et al. 2018; Wu and Baldwin 2009). Plants utilize various combinations of phytohormones to tailor their responses to specific stressors. Notably, the balance between jasmonic acid (JA) and salicylic acid (SA) is essential for the response to drought, waterlogging and insect herbivory. When the phytohormonal signals of two stressors coincide, the response to one stressor might prime the plant to react more effectively to the other (Mittler 2006; Nakashima et al. 2014). However, mismatches and antagonisms in phytohormonal signals could have detrimental effects on the plant's response to either stressor (Nakashima et al. 2014). Crosstalk between phytohormones is a widely proposed mechanism by which these stress responses interact (Arbona and Gómez-Cadenas 2008; Hickman et al. 2019; Thaler et al. 2012; Zhang et al. 2015). This crosstalk is facilitated by the antagonistic or synergistic relationships between different phytohormones. For instance, jasmonic acid (JA) and salicylic acid (SA) are believed to exhibit antagonism, while ethylene (ET) can amplify JA signaling in certain contexts (Leon-Reyes et al. 2009; Stam et al. 2014). Such crosstalk enables plants to integrate and fine-tune their responses, but it also provides a potential avenue for abiotic stress to influence plant responses to biotic stress and vice versa (Nguyen et al. 2016a). Moreover, since plants have evolved diverse responses to water stress involving distinct signaling pathways, the influence of water stress on phytohormones also varies. Consequently, the outcomes of this intricate network of phytohormonal interactions will depend on the specific plant and insect species under consideration. Understanding the dynamics of phytohormonal signaling and crosstalk in response to combined abiotic and biotic stresses is essential for unraveling the complexities of plant stress responses.

When signals in response to different stressors interact, their combined effects can significantly impact the plant's defenses against pests. One such impact is the change in concentrations of biochemical defenses. In specific cases waterlogging stress induces elevated concentrations of defensive compounds in leaves, subsequently leading to a reduced aphid growth rate (Mezgebe and Azerefegne 2021). However, the interaction between stressors is not always straightforward, as different studies have reported varying effects on defensive compound concentrations. For instance, the impact of waterlogging and drought on glucosinolate concentrations can depend on the specific plant species and glucosinolate type examined. In some cases, waterlogging and drought may have similar effects on defensive compound levels, while in others, they had opposing effects (Barber and Müller 2021; Khan et al. 2010; Teixeira et al. 2020). Exactly how plants are adapted and thus respond to a shortage or surplus of water will determine how water affects the direct defenses of those plants.

Moreover, the impact of water availability extends beyond direct defenses and can also influence indirect defenses. Abiotic conditions, including water availability, have been shown to affect the quantity and composition of plant volatiles (Jardine et al. 2015; Lou and Baldwin 2004; Takabayashi et al. 1994; Vázquez-González et al. 2022; Vivaldo et al. 2017; Zhou et al. 2018). One mechanism through which water conditions can modulate volatile organic compound (VOC) emission is by regulating stomatal closure. In sub-optimal water conditions, plants tend to close their stomata to reduce water loss, consequently limiting gas exchange and potentially reducing VOC release (Muhammad 2012; Murtaza et al. 2016; Parent et al. 2008; Xu et al. 2010), which plays a crucial role in attracting natural enemies of herbivores such as parasitoids. Furthermore, the phytohormones involved in plant responses to water stress are also essential for the regulation of indirect defenses against insect herbivory (Kessler et al. 2004; Nguyen et al. 2016a; Per et al. 2018; Wu and Baldwin 2009). This overlap and crosstalk between phytohormonal signals could potentially affect VOC production and subsequently impact parasitoid attraction. Consequently, variation in water availability has the potential to influence the indirect defense system against insect

herbivores (Copolovici et al. 2014; Kansman et al. 2021; Martini and Stelinski 2017; Salerno et al. 2017; Weldegergis et al. 2015). Understanding these intricate relationships between water availability, plant defenses, and herbivore interactions is essential for unraveling the full complexity of the effects of water availability on plant-insect interactions and their ecological consequences.

Not only do plant responses to different stressors interact and interfere with each other, plants have evolved specific adaptations tailored to cope with combinations of stress. Recent studies have shed light on the complex nature of plant responses to combined stress, revealing that the transcriptomic response is not merely an additive of individual stress responses. Instead, the plant's molecular response involves the activation of unique genes that are specific to particular stress combinations (Mittler 2006; Rasmussen et al. 2013; Rizhsky et al. 2004; Zhang et al. 2019). This highlights the intricate and sophisticated ways in which plants have evolved to tackle multiple stressors simultaneously. Moreover, some plant responses seem to exhibit multifunctionality, serving as adaptive strategies to address both water stress and herbivory at the same time (Ali et al. 2021; Bi et al. 2017; Erb and Kliebenstein 2020; Karabourniotis et al. 2014; Kosma and Jenks 2007). For example, certain glucosinolates, known for their role as antifeedants against herbivores, can also act as osmo-protectants during drought stress, helping the plant to maintain turgidity and survive water scarcity (del Carmen Martínez-Ballesta et al. 2013; Salehin et al. 2019). Such dual-purpose responses represent an ingenious way by which plants optimize their defenses and resource allocation in challenging environments. It is crucial to acknowledge that wild plants have evolved and acclimated to thrive in complex environments where they must confront and endure multiple stressors. Consequently, studying what makes plants resilient under adverse circumstances requires a comprehensive understanding of their inducible responses within the context of complex environments. By delving into the molecular and physiological mechanisms underlying responses to double trouble, we can gain insights into how plants have fine-tuned their defense strategies over time. This knowledge is vital not only for advancing our understanding of plant biology but also for developing effective strategies to enhance crop resilience in the face of environmental challenges.

This thesis

Few studies have investigated the impact of water stress on plant responses to insect herbivory, and these studies have been primarily focused on drought stress. As a result, our understanding of how plant responses to herbivory are influenced by water availability remains limited. Moreover, the existing research has produced conflicting results, possibly due to variations in the severity of water stress applied, the plant species studied, and the specific insect herbivores involved (Leybourne et al. 2021; Pineda et al. 2016). Furthermore, the effects of water stress on predatory insects, which play a crucial role in controlling herbivore populations, have received little attention (Kansman et al. 2021; Weldegergis et al. 2015). Consequently, the ecological consequences and underlying mechanisms of the interaction between plant responses to water stress and herbivory remain poorly understood. To address these knowledge gaps, this thesis aims to elucidate how water availability influences plant defenses against herbivory. The research goes beyond the direct effects of drought and waterlogging and investigates their broader ecological implications. By examining the intricate interplay between water availability and responses to herbivory, this study seeks to shed light on the mechanisms driving plant-insect interactions under variable water conditions. Such insights are crucial for advancing our understanding of plant resilience and adaptability in complex environments.

Furthermore, studying stressors in isolation has revealed that plants can exhibit diverse adaptations and responses to stress conditions (Anjum et al. 2011; Muhammad 2012; War et al. 2012). As a result, it is crucial to consider the variation in plant adaptations to water stress when investigating the effects of water availability on plant-insect interactions. To address this, I adopted a comparative approach, focusing on a selection of closely related wild plant species from the *Rorippa* genus that exhibit varying adaptations over a water gradient. By studying differently adapted plants in diverse water conditions ranging from drought to waterlogging, I aimed to link plant adaptations to water stress, to how water conditions can influence plant-insect interactions.

To this end, I took a multidisciplinary approach that integrated experiments on transcriptomics and metabolomics with insect performance and behavior. I was able to elucidate how differently adapted plants are affected in their response to herbivory under different water regimes. By using this combination of approaches, I aimed to ascertain how both direct and indirect defenses against herbivory were affected, what the transcriptomic basis of this effect was and how these effects translate to an ecological field setting. In my thesis, I aimed to attain fundamental knowledge on how plants integrate responses to water stress and insect herbivory that is necessary to predict and avert the ecological and agronomical effects of climate change.

Study system

In my research to identify how plants respond to the double trouble of water stress and herbivory, I leveraged the variation in plant adaptations to water stress. To gain a comprehensive understanding of nature's solutions to double trouble, I studied multiple

closely related plant species from the *Rorippa* genus, namely *Rorippa amphibia*, *Rorippa palustris*, *Rorippa austriaca*, and *Rorippa sylvestris*. These species are differently adapted to grow along a water gradient, ranging from semi-aquatic habitats (*R. amphibia*) to marshes and floodplains (*R. palustris*) and finally to drier sandy fields (*R. austriaca* and *R. sylvestris*) (Fig. 1). Each species has evolved distinct strategies to cope with water stress. For example, previous studies on *R. amphibia* and *R. sylvestris* have shown that they employ different approaches to deal with flooding. *Rorippa amphibia* responds with increased shoot growth, while *R. sylvestris* adopts a quiescent state (Akman et al. 2012). The intriguing aspect of this research lies in uncovering how these diverse coping strategies of the four *Rorippa* species affect their interactions with insect herbivores under different water conditions. Furthermore, *R. palustris* and *R. sylvestris* are annual plants while *R. amphibia* and *R. austriaca* are dominantly biennial. Despite their diverse adaptations to water stress, these four species share a similar insect herbivore community, which they also have in common with crops from the Brassicaceae family, including cabbage and mustard. Moreover, the recent sequencing of the closely related *Rorippa islandica* has proven to be a valuable resource, providing us with genetic tools to study our *Rorippa* plants (Schoch et al. 2020). Additionally, the successful utilization of microarrays based on model species *Arabidopsis thaliana* in previous studies to explore the *Rorippa* transcriptome has demonstrated the feasibility of incorporating tools from the *Arabidopsis* toolbox in our study (Sasidharan et al. 2013). By employing this study system, my aim is to investigate the intricate dynamics between water availability and plant-insect interactions in a variety of plant species. This research has the potential to provide valuable insights into the resilience and adaptability of wild plants in response to changing environmental conditions.

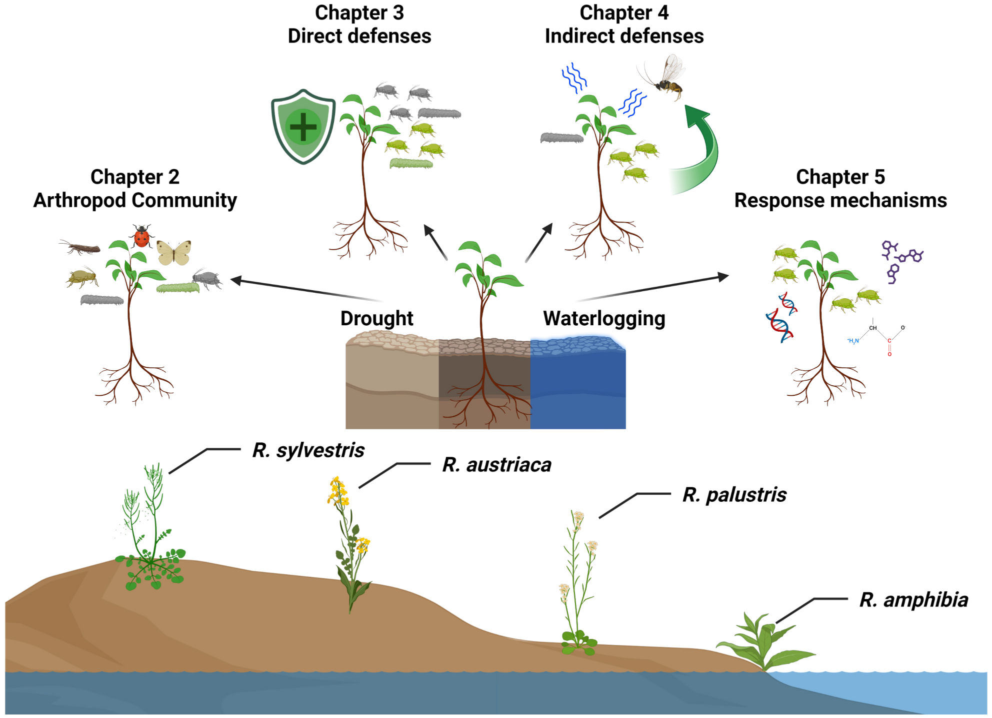

Figure 1. Project overview showing the study system consisting of four different *Rorippa* plant species with their habitats spread over a water gradient. With this study system I explore the effects of drought and waterlogging on: **Ch. 2** Plant-associated arthropod community, **Ch. 3** direct defenses against insect herbivores, **Ch. 4** indirect defenses against insect herbivores and **Ch. 5** transcriptomic and metabolomic responses to herbivory. (Created with BioRender.com)

Thesis outline

In **Chapter 2**, I explore the effects of waterlogging and drought on the arthropod community composition of four different *Rorippa* plant species in the field. To achieve this, I closely monitored the phenotype of the plants and the arthropods naturally arriving on them as they are subjected to different watering regimes. This approach allowed me to investigate how different plant species are influenced phenotypically by different watering regimes and how these changes, in turn, affect the arthropod community through plant-mediated interactions. I hypothesized that plants subjected to water regimes that differ significantly from their natural habitat will experience more pronounced phenotypic effects compared to those in a well-watered condition. Phenotypic changes induced by water availability can have various effects on particular arthropod species. As water stress alters the apparency or defensive capabilities of the plants for certain arthropod species, it might create opportunities for other arthropod species to better exploit these newly created niches. Additionally, these effects can cascade to higher trophic levels, influencing predatory species and resulting in far-reaching ecological consequences on arthropod community dynamics in natural field settings. This study aids in understanding the intricate relationships between water availability, plant phenotypes, and arthropod communities and highlights the complex and interconnected nature of ecological interactions in response to changing environmental conditions.

In **Chapter 3**, I look more specifically into what might cause the differences in communities found in Chapter 2. Here, I aimed to elucidate how plant resistance against herbivores of different feeding guilds is affected when plants are subjected to different water conditions. Given their unique feeding mode, I hypothesized that different feeding guilds would be uniquely affected by water-stress-induced changes in the plant. To comprehensively investigate these interactions, I conducted experiments involving four herbivores and three different plant species growing under three distinct watering regimes, ranging from drought to waterlogging. The herbivores tested included the chewers *Pieris brassicae* (large cabbage white) and *Plutella xylostella* (diamondback moth), and piercer/suckers *Myzus persicae* (green peach aphid) and *Lipaphis erysimi* (mustard aphid), all well-known pests of Brassicaceae plants. By studying the responses of these herbivores on different plant species under various water conditions, I investigate if water stress influences plant resistance against specific herbivores and whether this differed between herbivore and plant species.

In **Chapter 4**, I continue with two of these previously used herbivores to identify how well plants are able to recruit natural enemies to control these herbivores under different water conditions. Specifically, I investigated whether plants infested with *Plutella xylostella* can still effectively recruit the parasitoid *Diadegma semiclausum*, and whether plants infested with *Myzus persicae* can still attract the parasitoid *Aphidius ervi* under various levels of water availability. In addition, I also collected headspace volatiles emitted by these plants to measure the effect of water availability in combination with herbivory on the volatile blend plants release. I anticipated that more stressed plants would have reduced stomatal openings, resulting in lower volatile emissions, and potentially limiting their ability to attract natural enemies. Furthermore, given their distinct feeding guilds, I hypothesized that the impact of water scarcity or surplus on the production of volatiles would depend on the herbivore that infested the plant. This way I hoped to uncover whether higher trophic levels are also influenced through plant-mediated changes caused by water availability. Additionally, this revealed whether direct defenses are differently affected by water availability than indirect defenses or if they are both affected in a similar direction, further uncovering the actual ecological impact of double trouble.

In **Chapter 5** of my thesis, I studied the differences in underlying mechanisms by which closely related plant species from different habitats respond to water stress and herbivory. Given the strongest effects of water availability on plant defenses were found for plants infested by the aphid *Myzus persicae* in previous experiments, I chose to focus on this herbivore for further investigation. Additionally, I selected two plant species, the semi-aquatic *Rorippa amphibia* and the terrestrial *Rorippa sylvestris*, characterized by the strongest dissimilarity in habitat among the plants that I tested. To understand in what way water stress influences *Myzus persicae* aphids, I measured their feeding behavior under different water conditions. By observing their feeding behavior, I intended to determine if they encountered more difficulties in feeding depending on the water availability. I also collected and analyzed phloem exudates from the plants infested with aphids to examine the composition of their food source and determine if it changed under different water conditions. This analysis provides insights into how water availability affects the nutritional quality of the phloem sap and, consequently, the performance of the aphids. Furthermore, I conducted a transcriptomic analysis to study how these plant species respond to stressors in isolation and how their responses differ when facing different combinations of water treatments and insect herbivory. By combining data on aphid feeding behavior, phloem exudates, and transcriptomics, I aimed to gain a comprehensive understanding of the physiological responses of differently adapted wild plants to the simultaneous stresses of double trouble.

In **Chapter 6**, I integrate results from different chapters and emphasize the multifaceted effects of water availability on plant-insect interactions. I discuss the importance of studying how wild plants have thrived in their native habitats to study how plants have evolved adaptations to multi-stress environments. Understanding these adaptations and their ecological consequences could allow us to enhance the plant resilience by inducing plants to bend rather than break under intricate multi-stress scenarios.

“In the whimsical landscapes of nature’s realm, wild plants, wizened with time, flourish in the embrace of complex environments, gracefully swaying in response to a cacophony of environmental challenges. It is within this realm that we embark on a journey to uncover the secrets of their tenacious spirits, thriving amidst the trials and tribulations that color life's grand tapestry.”- Kamps 2023

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Chapter 2

Water conditions affect the composition and structure of plant-associated arthropod communities differently in four closely related *Rorippa* plant species

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Abstract

Water availability plays a fundamental role in shaping plant physiology and phenotype, consequently influencing the plant's interactions with its associated arthropod community. Furthermore, given that different plant species have specialized adaptations to specific habitats, they have developed unique strategies to respond to fluctuating water levels, potentially impacting their interactions with arthropod communities. We investigated how four closely related *Rorippa* plant species (Brassicaceae), that are adapted to distinct water conditions, respond to changes in water availability and how these responses affect arthropod community structure. In two years of study, we replicated a common-garden field experiment, manipulating water availability from drought to waterlogging and monitored plant phenotype and arthropod community structure on the four *Rorippa* species under these conditions. Water availability influenced plant phenotype, with drought generally leading to smaller plants for all four plant species, while waterlogging did not. Interestingly, both drought and waterlogging generally reduced arthropod species richness, indicating that plant size alone did not account for changes in arthropod biodiversity. Water availability influenced the overall structure of arthropod communities in three out of the four plants tested. Furthermore, the impact of drought and waterlogging on the arthropod community depended on the plant species. Although drought and waterlogging reduced the presence of some insect herbivores, other herbivore species were more abundant on certain plant species under suboptimal water conditions. The effects on herbivores cascaded into effects on prevalence of higher trophic level organisms. Overall, this study shows that variation in water availability impacts both plant development and the plant's interactions with its associated arthropod community, emphasizing the existence of complex and species-specific interactions among water availability, plant adaptations, and arthropod communities. Unraveling how water availability affects the community dynamics of a plant's associated arthropod community can provide insights into the ecological consequences of changing water regimes, ultimately enhancing our ability to predict and manage the impacts of environmental change on ecological communities in natural and agricultural ecosystems.

Introduction

In their habitat, plants are exposed to a myriad of challenges that impact their fitness. Often the response to one challenge affects the potential to deal with another. Moreover, the occurrence of these challenges are typically linked with one challenge affecting the likelihood of the occurrence of another (Mertens et al. 2021a). For example, abiotic factors like water availability significantly affect plant physiology, and with that, the ability of plants to defend themselves against arthropod herbivory as well as host plant acceptance by arthropods (Kamps and Poelman 2023; Leybourne et al. 2022; Pineda et al. 2016). The notion of responding to multiple stressors may be especially relevant under conditions of climate change, which is projected to bring more frequent and intense droughts as well as more intense precipitation events, altering water regimes in many ecosystems. Understanding the intricate interplay between water availability and plant-arthropod interactions is therefore crucial in improving our ability to predict and manage the impacts of climate change on ecological communities in both natural and agricultural ecosystems (Barton and Ives 2014; Kansman et al. 2021a; Torode et al. 2016).

The impact of water availability on plants can have complex and multi-faceted effects on arthropods (Barton and Ives 2014). Water stress can influence the morphology and architecture of the plant (Chaves et al. 2003; Mattson and Haack 1987; Zhong et al. 2020). The response to a water shortage or surplus can, for example, affect the number of leaves or leaf shape and texture, which are important plant traits that shape arthropod community assembly on plants (Langellotto and Denno 2004; Nalam et al. 2019). Additionally, several studies have shown a relation between water availability and the nutritional quality of plant tissues. As plants react to water shortage or surplus, they mobilize nutrients to utilize them in their response to water stress conditions. This causes variation in free amino acids and sugars to be available for herbivores, altering the quality of the plant as a host for arthropods (Khan et al. 2011; Leybourne et al. 2021; Mewis et al. 2012; Mezgebe and Azerefegne 2021; Pons et al. 2020; Showler and Castro 2010).

The relationship between water availability and arthropod communities is further shaped by the impact of water stress on plant defenses against herbivorous arthropods. Plants can actively respond to herbivory by producing defensive compounds that deter or inhibit herbivores from feeding on plant tissues. However, under water stress, plants may prioritize water conservation over the production of defensive compounds, compromising their ability to fend off herbivorous arthropods effectively. Consequently, herbivores may experience reduced resistance from their host plants. In this way water stress can facilitate

herbivores, increasing their population size (Kamps and Poelman 2023; Mewis et al. 2012; Pineda et al. 2016). In contrast, some studies have also found that water stress can lead to cross-resistance against herbivores. Since the same compounds that protect the plant from water stress can also have an anti-feedant effect on herbivores, this could negatively affect herbivore performance (Leybourne et al. 2021; Teixeira et al. 2020).

By changing the community dynamics of herbivores, water scarcity or surplus might also indirectly alter communities of a higher trophic level (Genung et al. 2012; Haddad et al. 2009; Kondoh and Williams 2001). For example, water stress may influence the production of volatile organic compounds (VOCs) emitted by plants (Copolovici et al. 2014; Salerno et al. 2017; Weldegergis et al. 2015). VOCs play a crucial role in mediating plant-insect interactions by attracting or repelling specific arthropod species. Some studies have shown that water-stressed plants emit different blends of VOCs compared to well-watered plants, leading to changes in the attraction or repellence of herbivores, pollinators, or natural enemies (Burkle and Runyon 2016; Kansman et al. 2021b; Salerno et al. 2017; Weldegergis et al. 2015). These changes in VOC emissions can have cascading effects on the entire community structure and dynamics.

While an increasing number of studies show that water stress can shape plant-associated herbivore and predator communities (Kansman et al. 2021a; Leybourne et al. 2021), the effects are likely intricately linked to the plant's adaptations to varying water conditions. Depending on the plant's specific response, water stress could have positive or negative effects on its quality as a host plant for all associated herbivore species or specific members of the herbivore community, and thus result in plant-specific effects of water conditions on arthropod community assembly (Pineda et al. 2016). However, the dependency of the effects of water stress on plant adaptations remains poorly understood and calls for a comparative study among closely related but differently adapted plant species across a gradient of water stress.

We conducted a common-garden field study involving four closely related plant species belonging to the *Rorippa* plant genus within the Brassicaceae that are adapted to different water conditions. We compared arthropod communities on the amphibious *Rorippa amphibia,* the floodplain inhabiting *Rorippa palustris* and the terrestrial *Rorippa sylvestris* and *Rorippa austriaca*. By manipulating water availability from drought to waterlogging, we investigated how inter-species variation in adaptation to water conditions influences the arthropod communities associated with each plant species when growing under these different watering regimes. We predicted that the arthropod community composition would be most affected when plants species were grown under watering conditions that were most dissimilar from the conditions they were adapted to. For the floodplain species (*R. palustris*) that experiences drought spells as well as flooding events, we hypothesized that it would be less affected by variation in the level of water stress (drought or waterlogging). By collecting plant growth parameters in addition to arthropod community composition in two years of study, we provide insights into how plant performance under drought, well-watered and waterlogging conditions correspond with arthropod community assembly. The comparative approach of this study further allows us to unravel the complex interactions among water availability, plant adaptations, and arthropod communities and provides insight into the ecological consequences of changing water regimes as projected under conditions of climate change.

Methods

Plants

Root cuttings from *R. amphibia* and seeds from *R. sylvestris, R. palustris, R. austriaca* were collected around the city of Wageningen, the Netherlands. Root cuttings were planted, and seeds were germinated in potting soil (Lentse Potgrond B.V.) under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). One week after sprouting, plants were transplanted into peat soil cubes (Lentse Potgrond B.V.). Two-week-old plants were then transported under a roofed shelter to acclimatize to outside conditions. Four-week-old plants were planted in the field and used for experiments.

Field study – common garden experiment

A common garden experiment was set up on the organic arable farm of Unifarm, Wageningen, the Netherlands (51°59'22.7"N 5°39'55.7"E) where 36 plots of 4 plants each were planted. Plants in each plot were planted 50 cm from the border of the plot and with a planting distance of 100 cm between plants (Fig. 1). Plots were installed 2 m apart from each other in two rows of 18 plots. The four plant species were each assigned to 9 of the 36 plots (Fig. 1). To allow experimental regulation of the amount of water each plot gets, the two rows of 18 plots were each covered by one of two transparent foil tunnel shelters (Rovero Systems B.V.) with open sides to a height of 1.5m to keep rainwater off the plants but allow arthropods to access the plants (Fig. 1). Additionally, a 3 m x 3 m piece of pond liner was dug in 60 cm below and around each plot to prevent rainwater from seeping in through the soil and to contain water given in the plot (Fig. 1). After plants had one week to acclimatize to the field and were watered regularly, plots were assigned one of three watering treatments: drought treated plants received 250 ml – 750 ml water per week depending on evaporation rates. We adjusted the water regime to maintain plants close to their wilting point. Well-watered

Figure 1. Field setup showing plots of four plants planted in a square with 2 m in between each plot. The plots are covered by a transparent foil tunnel to prevent rainwater from landing on the plots but opened on either side to a height of 1.5 m to allow arthropods to pass under. Plots were also lined with pond liner up to a depth of 60 cm to prevent water from seeping into the plot and to contain the water provided.

plants were watered regularly to approximately 1500 ml per week to ensure that plants kept leaf turgidity. Waterlogged plots were filled with water until the soil was completely saturated and a layer of 2 cm of water was visible on top of the soil. Waterlogged plots were refilled every week or every other week depending on evaporation rates. In total, this amounts to three replicated plots of each of the four plant species per water treatment (drought, well-watered and waterlogged). The experiment was repeated for two years. In 2019 it ran from the 8th of July until the 14th of October while in 2020 it ran from the 2nd of June until the 24th of September.

Monitoring plant performance and arthropod communities

All plants were monitored weekly to bi-weekly. In 2019 all plants were monitored five times while in 2020 plants were monitored 11-12 times. *Rorippa palustris* plants could only be monitored six times, because it completed its lifecycle faster than the other plants. Monitored plants were thoroughly inspected for insects and other arthropods, which were identified to the highest taxonomic level possible and counted. A classification of all organisms monitored is given in table 1. Furthermore, plant height and width were measured in each monitoring round, as well as the length of the longest leaf, the number of leaves and the number of leaves that show arthropod damage. Additionally, we assessed the number of flowering branches and seed axes for the annuals *R. sylvestris* and *R. palustris*. *Rorippa amphibia* and *R. austriaca* are perennials that did not flower during the experiments. Seeds of *R. sylvestris* and *R. palustris* were harvested when they started to ripen. An approximation of the number of seeds was made by weighing the total number of seeds of a plant and dividing that by the weight of exactly 500 seeds.

Statistical Analysis

We used Generalized Linear Mixed Models (GLMM) to analyze all measured phenotypic plant parameters. Water treatment, plant species, and the experimental year were used as explanatory variables. To account for dependency of plants measured in the same plot, the plot in which the plant was situated was included as a random factor. The choice of probability distribution was determined by comparing the Akaike Information Criterion (AIC) scores of models that were specified using a Gaussian, Poisson, negative binomial, or gamma distribution. Subsequently, a post-hoc analysis with Tukey HSD correction for multiple testing was conducted to identify pairwise differences among differently treated plants belonging to the same plant species within each of the two experimental years separately.

To investigate the impact of water stress on plant-associated arthropod communities, the species richness and the Shannon-Wiener biodiversity index were calculated for each individual plant, considering the community data collected throughout the entire growing season. Both indices were analyzed similarly to plant traits using a GLMM with water treatment, plant species and experimental year as explanatory variables while accounting for dependencies of plants measured in the same plot. Type II Wald chi-square test was used to estimate the effect size of each factor in the full-factorial design. Subsequently, a post-hoc analysis with Tukey HSD correction for multiple testing was conducted to identify pairwise differences within plant species among differently treated plants within an experimental year.

To assess the dissimilarity of arthropod communities among plant species, water treatment and experimental year, a Nonmetric Multidimensional Scaling (NMDS) ordination was performed based on the Bray-Curtis dissimilarity matrix of relative abundance calculated as log(summed abundance of arthropod species on individual plant/times a plant was measured), accompanied by a Permutational Multivariate Analysis of Variance (PERMANOVA) to statistically test for the differences in community composition illustrated by NMDS (Anderson 2001). The statistical significance of all PERMANOVA analyses was assessed by Monte Carlo permutation testing using 9999 random permutations while grouping individual plants within the same plot to account for dependencies within plot. First, we tested the full dataset for differences in the communities associated with plant species. However, our analysis revealed a significant interaction between the plant species and the year in which the field season was conducted shaping the arthropod communities. Hence, we proceeded to analyze the differences in communities associated with different plant species for the two years separately. We further accounted for the variation among the arthropod communities caused by water treatment by constraining the permutation of the observed communities only to plants that received the same water treatment. Second, we tested the effects of water treatment for each of the plant species separately. Our analysis shows that the effects of water treatment on the arthropod community were consistent across the two years. To make optimal use of the number of independent samples, we combined the data of two years while accounting for the variation in arthropod communities structured by the year in our permutation design. Post-hoc pairwise PERMANOVA analyses were performed to identify specific communities that exhibited significant dissimilarities, adjusting P values for multiple testing using a false discovery rate method. To unravel the key species associated with the differentiation of distinct communities, a redundancy analysis (RDA) was employed, allowing for the identification of the most influential species within the community. Finally, the abundance of specific influential species was analyzed using a GLMM in which water treatment, plant species and experimental year were used as explanatory factors while accounting for dependencies of the plot in which the plants were planted and for repeated observations on the same plant by including timepoint and plant ID as additional random intercepts. Subsequently, a post-hoc analysis with Tukey HSD correction for multiple testing was conducted to identify pairwise differences within plant species among differently treated plants within an experimental year. Analyses were carried out in R (R Core Team 2013), using the glmmTMB (Magnusson et al. 2017), emmeans (Lenth et al. 2019) and vegan (Oksanen et al. 2013) packages.

Results

Plant phenotype

Overall, water availability significantly affected plant development (Fig. 2, Supplementary Fig. 1). Plant traits that responded to water treatment varied across plant species and year (Fig. 2, Supplementary Fig. 1). For example, all four plant species produced more leaves under waterlogged conditions than under drought conditions in at least one of the two experimental years, indicating a higher biomass under waterlogged conditions than under drought conditions (Supplementary Fig. 1). *Rorippa amphibia* in 2020 and *R. austriaca* in both years displayed elongation under waterlogging conditions. Under drought conditions *R. amphibia* in 2019 and *R. palustris* in 2020 exhibited reduced maximum height compared to plants in well-watered environments (Fig. 2A). Plant radius was not significantly affected by water availability in 2019 for any of the four plant species, whereas the maximum radius of the plants in 2020 was smaller under drought conditions in most species, except for *R. amphibia* (Fig. 2B). Waterlogging had no significant influence on maximum plant radius compared to the well-watered treatment in either year for any of the plant species. The proportion of leaves showing signs of herbivory was significantly higher in drought-treated *R. palustris* in both years and drought-treated *R. austriaca* in 2020. *Rorippa sylvestris* had a significantly lower proportion of damaged leaves under well-watered conditions than under both drought and waterlogged conditions, indicating that herbivores fed more from water stressed plants (Supplementary Fig. 1). Due to differences in life history, only the annuals *R. palustris* and *R. sylvestris* produced seeds, whereas the perennials *R. amphibia* and *R. austriaca* did not flower during the experiments. Compared to plants under well-watered conditions, *Rorippa palustris* had a lower number of seeds under drought in both years, while *R. sylvestris* had a higher number of seeds under waterlogged conditions (Supplementary Fig. 1). This shows that plant phenotype was affected by the water conditions in which they grew. However, these effects depended on the plant species and, the year the experiment took place.

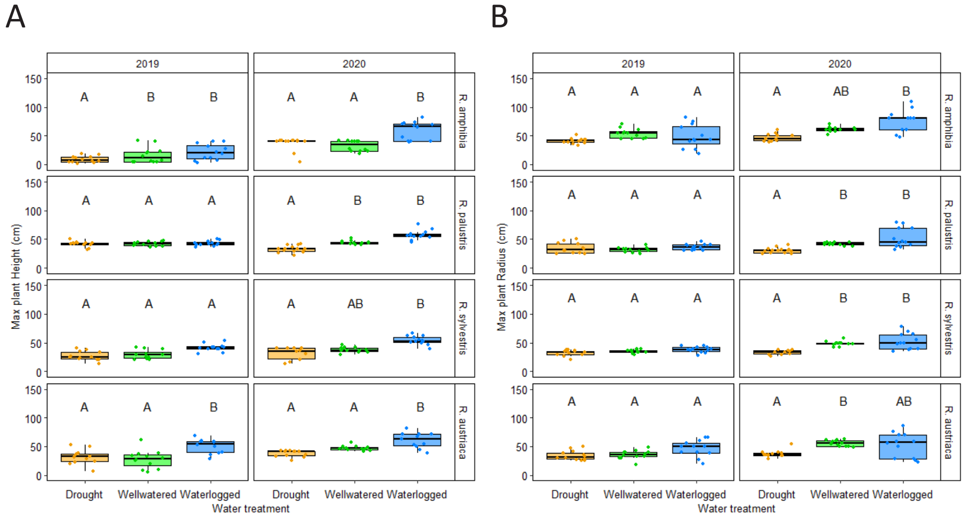

Figure 2. A: Maximum plant height (cm) **B:** Maximum plant radius (cm), that plants reached over the growing season. Separated per year and per plant species on plants subjected to one of three watering treatments. Different letters indicate a significant difference between watering treatments on a specific plant species within a specific year (GLMM; α=0.05).

Arthropod communities

When examining cumulative arthropod community species richness over the growing season, a significant effect of water treatment was observed (GLMM, df = 2, χ^2=27.2, P<0.001). Generally, drought-treated and waterlogged plants exhibited lower species richness compared to well-watered plants. Plant species and the year of measurement also had significant effects on species richness (Plant species: GLMM, df=3, χ^2=46.0, P<0.001; Year: GLMM, df=1, χ^2=105.1, P<0.001). There was a significant interaction between plant species and year (GLMM, df=3, χ^2=11.3, P=0.010). Waterlogging led to a lower species richness on *R. amphibia* and *R. sylvestris* in 2019 and on *R. austriaca* in 2020. Drought led to a lower species richness in *R. amphibia* in 2020 (Fig. 3A).

Also the Shannon-Wiener diversity index was significantly affected by water treatment (GLMM df=2, χ^2=14.4, P<0.001). Generally, drought and waterlogging led to a lower Shannon-Wiener diversity index compared to well-watered plants. However, the index was only significantly lower in waterlogged *R. palustris* plants (Fig. 3B). Opposite to species richness, neither plant species nor experimental year significantly affected the Shannon-Wiener diversity of the arthropod community on plants (Plant species: GLMM df=3, χ2= 2.3, P=0.521; Year: GLMM df=1, χ^2=0.5, P=0.490).

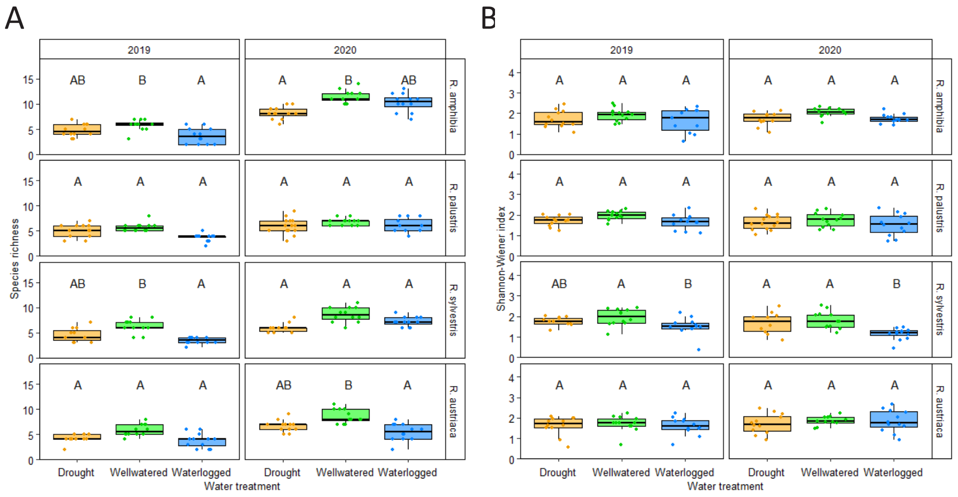

Figure 3. A: Arthropod species richness as total number of species measured **B:** Shannon-Wiener biodiversity index, calculated over the entire observed arthropod community on an individual plant throughout the growing season. Separated per year and per plant species on plants subjected to one of three watering treatments. Different letters indicate a significantly different biodiversity between watering treatments on a specific plant species within a specific year (GLMM; α=0.05).

PERMANOVA analyses revealed that the arthropod community composition was significantly affected by the year in which the experiment was done (PERMANOVA, R^2=0.045, P<0.001) (Supplementary Fig. 3). This was primarily driven by a higher overall abundance of arthropods in 2020 compared to 2019 (Supplementary Fig. 4). Additionally, since there was a significant interaction between the effect of year and plant species, the two years were further analyzed separately. In both years, plant species explained a significant proportion of the variation in arthropod community composition, although the effect was stronger in 2020 than in 2019 (2019: Permanova, R^2=0.035, P=0.019; 2020: Permanova, R^2=0.105, P<0.001) (Supplementary Fig. 3).

The arthropod community of three out of four plant species was significantly structured by the water treatment they received. Only for *R. palustris*, water conditions did not affect arthropod community composition (Permanova: R^2=0.039, P=0.211) (Fig. 4). Although main effects of water treatment for each of the three other species were significant (*R. amphibia*: Permanova: R^2=0.069, P=0.004; *R. sylvestris*: Permanova: R^2=0.049, P=0.032; *R. austriaca*: Permanova: R^2=0.047, P=0.047), none of the post-hoc analyses could separate individual treatments. Even though no significant pairwise difference could be found between specific treatments, drought and waterlogged plants seem most differentiated in *R. amphibia* (P=0.058). This difference was primarily driven by a different relative abundance of the flea beetle *Phyllotreta undulata*, and the aphid *Lipaphis erysimi* (Fig. 4A). For *R. sylvestris*, visual inspection of the NMDS shows that drought-treated plants had the most distinct communities compared to the other two treatments. The main drivers were a difference in the relative abundance of plant galls and cocoons of *Cotesia glomerata* parasitoids that are associated with *Pieris* caterpillars (Fig. 4C). The community of *R. austriaca* was most distinctly different on drought-treated plants compared to the two other treatments. The difference in community composition compared to well-watered and waterlogged plants can mainly be attributed to a difference in the relative abundance of *P. undulata*, *Plutella xylostella* caterpillars and Chrysopidae (Supplementary Fig. 5).

To highlight the effects of water treatment on specific arthropod species we also analyzed the abundance of four specific arthropods separately. The abundance of the flea beetle *P. undulata* was significantly affected by water treatment (GLMM df=2, χ^2=18.0, P<0.001). Furthermore, there is a significant interaction between water treatment and plant species (GLMM df=6, χ^2=19.1, P=0.004). In 2020, more *P. undulata* were observed on waterlogged than on drought-treated *R. amphibia* and *R. austriaca* plants. In contrast, in 2019 fewer *P. undulata* were observed on waterlogged than on well-watered *R. palustris* plants. Water treatment did not affect *P. undulata* on *Rorippa sylvestris* plants (Fig. 5A). The abundance of caterpillars of *Plutella xylostella* was significantly affected by water treatment (GLMM df=2, χ^2=30.6, P<0.001) and water treatment significantly interacted with plant species (GLMM df=6, χ^2=16.5, P=0.011). In 2019 *P. xylostella* abundance was negatively affected by waterlogging in *R. sylvestris* and negatively affected by drought in *R. austriaca*. These patterns stayed the same in 2020, however abundance also dropped in waterlogged *R. amphibia* and *R. austriaca* as compared to well-watered plants. Water treatment did not affect abundance of *Plutella xylostella* on *R. palustris* (Fig. 5B). Abundance of the plant-feeding bug *Lygus hesperus* was also significantly influenced by water treatment (GLMM χ^2=13.7, df=2, P=0.001). Waterlogged *R. palustris* had fewer *L. hesperus* compared to drought and well-watered plants in 2020 (Fig. 5C). The abundance of natural enemies of herbivores such as predatory *Orius* bugs was also significantly affected by water treatment (GLMM χ^2=23.4, df=3, P<0.001). *Orius* abundance was negatively affected by waterlogging in *R. palustris* in both 2019 and 2020 and in *R. sylvestris* in 2020 (Fig. 5D). Collectively, this shows that certain insects are susceptible to the impacts of drought and waterlogging. Furthermore, these effects are dependent on the plant species involved, highlighting the significant role of plant-mediated influences on the associated arthropod community in response to varying water conditions.

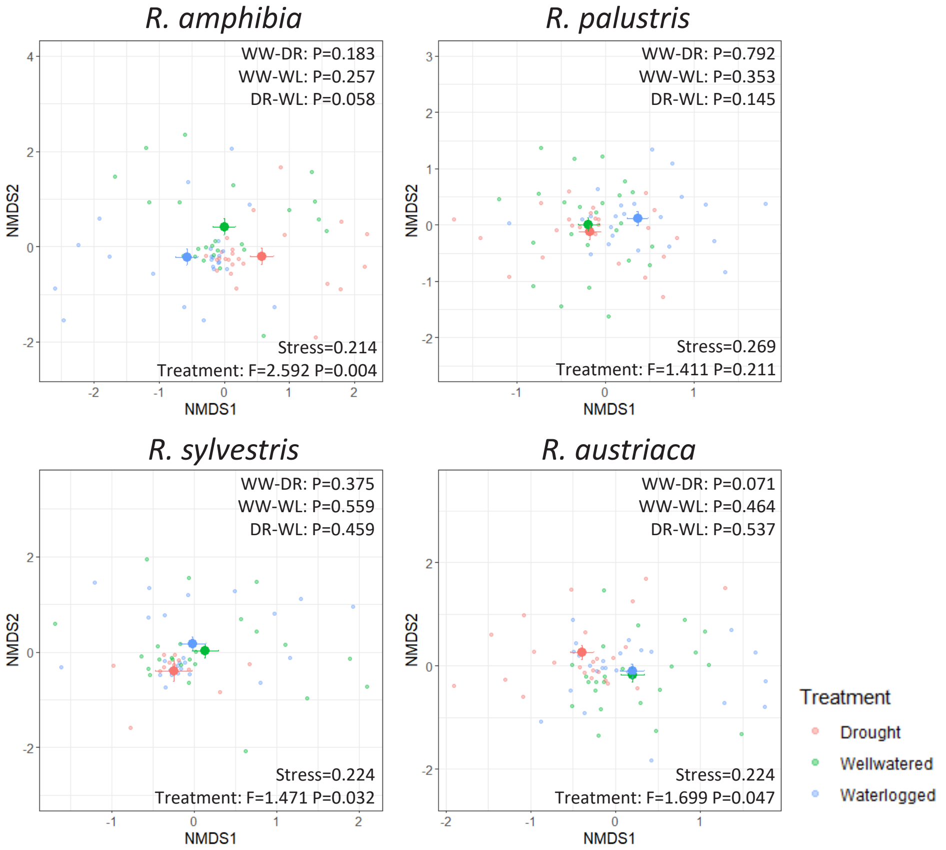

Figure 4. Ordination of arthropod community composition expressed as log(summed abundance of arthropod species on individual plant/times a plant was measured) for the first two NMDS ordination axes with accompanying stress value. Small dots depict the community composition of individual plants while large dots depict centroids of communities for plants that received a specific water treatment represented by colors. Error bars represent standard errors of communities in multivariate space. Additionally, a PERMANOVA analysis was done to calculate the main effect of water treatment. Pair-wise differences between water treatments were also analyzes using a PERMANOVA (WW=Well-watered, DR=Drought, WL=Waterlogged).

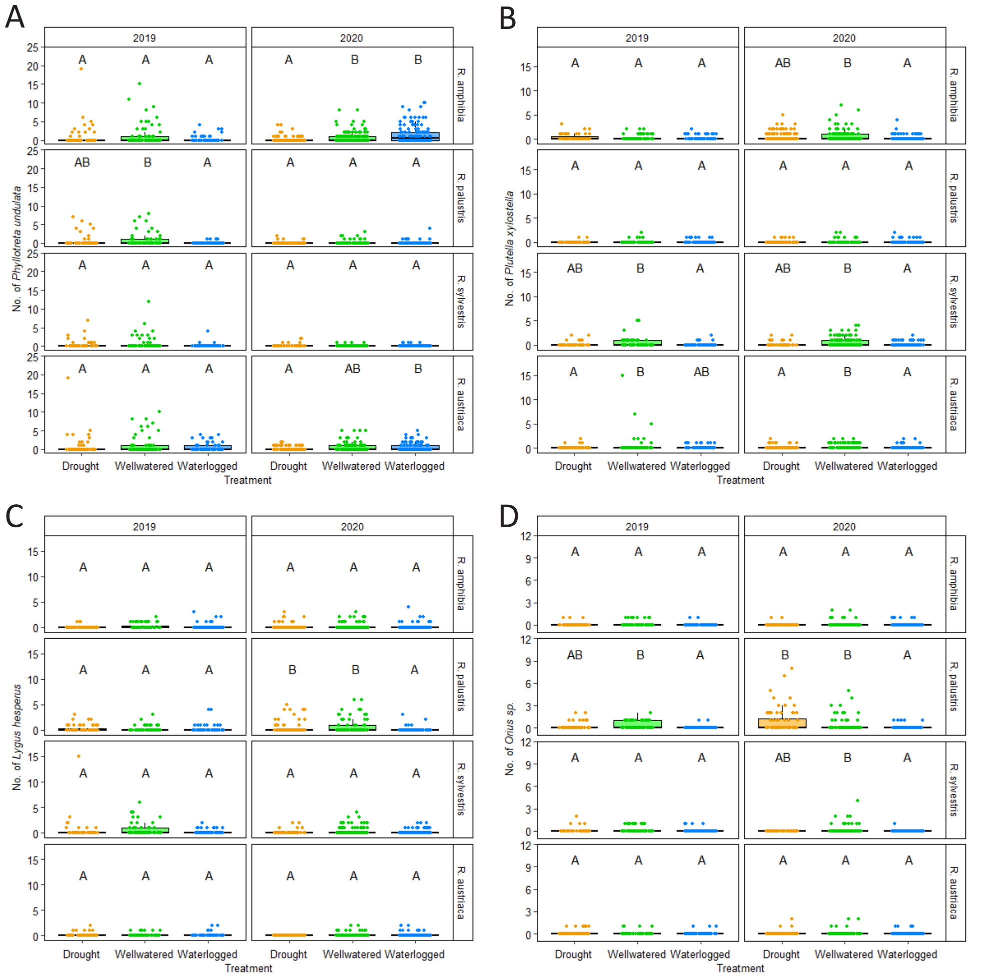

Figure 5. Abundance of **A** *Phyllotreta undulata* **B** *Plutella xylostella* **C** *Lygus hesperus* **D** *Orius sp.* bugs per observation separated by experimental year and per plant species on plants subjected to one of three watering treatments. Different letters indicate a significant difference in abundance between watering treatments on a specific plant species within a specific year (GLMM; α=0.05).

Table 1. Average ± st. dev. abundance of arthropod species found during a single observation on the four different plant species monitored under three different watering treatments. Colors depict a lower (blue) or higher (red) relative abundance compared to the wellwatered plants within a plant species.

		R. amphibia			*R. palustris*			*R. sylvestris*			*R. austriaca*		
Chewers		Drought	Wellwatered	Waterlogged	Drought	Wellwatered	Waterlogged	Drought	Wellwatered	Waterlogged	Drought	Wellwatered	Waterlogged
Coleoptera	*Ceutorhynchus assimilis*	0±0.07	0±0.07	0±0.07	0.01±0.08	0.03±0.17	0±0	0.01±0.08	0.03±0.16	0.01±0.1	0±0	0.01±0.07	0.02±0.13
Coleoptera	*Meligethes aeneus*	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0.01±0.07	0.02±0.12	0±0	0±0	0.01±0.07
Coleoptera	*Phyllotreta atra*	0.17±0.46	0.6±1.48	0.55±1.45	0.14±0.54	0.17±0.61	0.06±0.24	0.05±0.24	0.15±0.44	0.08±0.31	0.18±0.47	0.37±0.7	0.24±0.54
Coleoptera	*Phyllotreta undulata*	0.4±1.6	1.01±2.02	1.17±2.02	0.28±1.04	0.52±1.36	0.11±0.44	0.31±0.97	0.27±1.15	0.06±0.34	0.41±1.6	0.76±1.69	0.56±1.04
Coleoptera	*Phaedon cochleariae*	0±0.07	0±0.07	0±0	0±0	0±0	0±0	0±0	0.01±0.1	0.01±0.07	0±0	0.01±0.07	0.01±0.07
Hymenoptera	*Athalia rosae*	0.17±0.88	0.23±0.6	0.22±0.76	0.32±0.94	0.67±1.97	0.27±0.65	0.16±0.56	0.45±1.13	0.31±0.89	0.04±0.23	0.18±0.53	0.18±0.71
Lepidoptera	*Autographa gamma*	0.05±0.23	0.09±0.37	0.05±0.26	0.03±0.16	0.03±0.17	0.02±0.12	0.03±0.17	0.06±0.25	0.02±0.12	0.07±0.33	0.07±0.27	0.07±0.25
Lepidoptera	*Evergestis extimalis*	0±0	0±0.07	0.03±0.49	0.06±0.28	0.05±0.27	0.27±1.58	0.01±0.08	0.01±0.1	0±0	0±0	0±0	0.01±0.1
Lepidoptera	*Evergestis forficalis*	0.06±0.34	0.12±0.54	0.14±0.59	0.03±0.26	0.03±0.17	0.06±0.37	0.01±0.11	0.02±0.12	0±0	0±0	0.01±0.11	0.05±0.32
Lepidoptera	*Lacanobia suasa*	0.01±0.16	0.01±0.1	0±0	0.01±0.12	0.01±0.09	0±0	0±0	0.01±0.1	0.01±0.07	0±0	0±0	0±0
Lepidoptera	*Mamestra brassicae*	0.03±0.21	0.02±0.18	0.18±1.71	0.01±0.12	0±0	0.01±0.09	0.04±0.47	0.03±0.16	0.02±0.12	0.02±0.22	0.08±0.91	0.01±0.07
Lepidoptera	Noctuidae sp.(looper)	0.01±0.1	0.02±0.16	0±0	0.01±0.08	0±0	0±0	0.01±0.11	0.02±0.12	0.01±0.07	0.04±0.3	0±0	0±0
Lepidoptera	*Pieris brassicae*	0.96±6.99	2.17±11.09	0.6±4.5	0.37±2.73	0.08±0.71	2.15±10.16	0.15±1.65	0.41±3.42	0±0	0.87±8.86	1.26±7.61	0.3±2.71
Lepidoptera	*Pieris rapae*	0.59±0.96	0.84±1.35	0.59±1.1	0.24±0.49	0.4±0.7	0.15±0.4	0.16±0.44	0.23±0.58	0.07±0.26	0.39±0.85	0.79±1.55	0.16±0.46
Lepidoptera	*Plutella xylostella*	0.38±0.78	0.48±1	0.21±0.49	0.06±0.23	0.12±0.39	0.14±0.37	0.17±0.45	0.51±0.93	0.11±0.34	0.09±0.32	0.37±1.35	0.11±0.35
Lepidoptera	*Spodoptera frugiperda*	0±0	0.01±0.12	0±0	0±0	0±0	0±0	0±0	0.01±0.07	0±0	0.01±0.07	0±0	0±0
	Leaf miner	0.43±0.84	0.57±1.11	0.3±0.77	0.28±0.58	0.64±0.96	0.67±1.22	0.07±0.26	0.17±0.5	0.03±0.16	0.31±0.66	0.34±0.94	0.13±0.49
Piercers													
Hemiptera	*Aleyrodes proletella*	0.13±0.79	0.7±3.06	0.08±0.51	0±0	0.04±0.36	0.07±0.54	0±0	0±0	0±0	0±0	0.02±0.13	0.01±0.07
Hemiptera	Auchenorrhyncha sp.	0.09±0.35	0.05±0.27	0.09±0.44	0.03±0.18	0.05±0.22	0.01±0.09	0.03±0.17	0.04±0.2	0.04±0.21	0.05±0.24	0.03±0.26	0.03±0.18
Hemiptera	*Brevicoryne brassicae*	0.01±0.1	0.01±0.1	0±0.07	0.02±0.19	0±0	0±0	0.01±0.08	0.01±0.07	0.28±3.83	0±0	0.01±0.07	0±0
Hemiptera	*Lipaphis erysimi*	0.08±0.31	0.09±0.6	8.68±45.08	0.08±0.84	0.07±0.25	0.02±0.12	0.01±0.08	0.1±1.16	0.98±8.09	0.02±0.24	0.27±3.06	0.02±0.23
Hemiptera	*Lygus hesperus*	0.1±0.43	0.19±0.49	0.14±0.5	0.52±1.3	0.55±1.16	0.19±0.63	0.27±1.28	0.42±0.93	0.15±0.41	0.04±0.22	0.1±0.34	0.1±0.36
Hemiptera	*Myzus persicae*	2.41±7.64	3.34±9.11	3.48±18.8	0.54±3.61	0.48±3.61	0.55±3.65	0.42±3.23	0.23±0.7	1.66±9.43	0.84±5.36	1.56±6.55	0.36±3.16
Hemiptera	*Myzus persicae Nicotiana*	0.02±0.22	0±0.07	0±0	0±0	0±0	0.01±0.09	0±0	0±0	0±0	0.01±0.11	0±0	0±0
Hemiptera	Planococcus sp.	0.02±0.14	0.01±0.1	0±0	0.01±0.12	0±0	0±0	0.01±0.08	0.01±0.07	0.01±0.07	0.01±0.11	0.01±0.07	0±0
Pentatomidae	Pentatomidae sp.	0±0	0.01±0.12	0±0.07	0±0	0.03±0.21	0±0	0±0	0.01±0.1	0±0	0.11±1.06	0.02±0.13	0.02±0.15
Thysanoptera	*Thrips tabaci*	0.28±0.73	0.4±0.71	0.13±0.44	0.33±0.94	0.45±1.41	0.45±1.36	0.29±0.72	0.39±0.85	0.35±0.84	0.17±0.46	0.33±0.6	0.2±0.53
Predators													
Araneae	Spider	0.13±0.43	0.14±0.41	0.16±0.51	0.03±0.22	0.05±0.22	0.02±0.15	0.04±0.23	0.2±0.5	0.1±0.32	0.02±0.18	0.06±0.29	0.06±0.24
Coleoptera	Coccinellidae	0.04±0.22	0.11±0.41	0.68±3	0.17±0.96	0.27±0.64	0.35±0.65	0.11±0.33	0.28±0.73	0.32±0.67	0.07±0.27	0.13±0.37	0.12±0.37
Hemiptera	Orius sp.	0.02±0.16	0.09±0.32	0.05±0.22	0.58±1.29	0.39±0.82	0.05±0.22	0.13±0.43	0.18±0.49	0.03±0.17	0.06±0.25	0.07±0.29	0.04±0.19
Hymenoptera	*Cotesia glomerata* cocoon	0.03±0.18	0.06±0.26	0.07±0.28	0.08±0.42	0.05±0.22	0.11±0.6	0.07±0.33	0.12±0.37	0.06±0.24	0.07±0.28	0.14±0.46	0.08±0.34
Neuroptera	Chrysopidae	0.17±0.8	0.33±0.76	0.36±0.99	0.08±0.28	0.08±0.35	0.02±0.15	0.07±0.28	0.13±0.46	0.04±0.21	0.31±1.05	0.32±0.81	0.16±0.46
	Parasitized aphids (mummies)	0.04±0.21	0.27±2.89	0.1±0.87	0.02±0.14	0.02±0.15	0.02±0.15	0.03±0.17	0.04±0.19	0.41±3.43	0.04±0.23	0.14±0.42	0.02±0.15
Miscellaneous													
Collembola	Collembola sp.	0.65±3.38	0.8±3.39	0.01±0.16	0.44±2.33	1.33±5.5	0.03±0.17	0.85±4.72	1.08±4.96	0.01±0.07	0.88±3.14	1.36±6.01	0.01±0.07
	Gals	0±0	0±0	0±0	0.44±3.6	0.42±3.65	0.11±0.47	0.92±4.65	3.92±9.71	5.71±13.43	0±0	0.01±0.07	0.28±2.37

Discussion

Our findings reveal the significant and highly species-specific influence of water availability on the composition of arthropod communities across multiple plant species. The impact of water availability on plant phenotype and arthropod community dynamics could not be readily predicted based on the adaptations exhibited by the individual plant species based on their natural habitat. For instance, despite *R. sylvestris* being the most adapted to drier conditions among the four species studied, its community was still more profoundly affected by drought rather than waterlogging. Similarly, *R. amphibia*, which displays adaptation to waterlogged environments, exhibited distinct changes in its arthropod community when subjected to waterlogging. These findings underscore the notion that regardless of whether a plant species is adapted to specific conditions, altered abiotic conditions influence the arthropod community associated with that plant. This may result from an intricate interplay between the effect of water *per se* on arthropod communities, the effect of water regime on plant biomass as well as its nutritional and defensive status on herbivorous and predatory arthropods, emphasizing complex species-specific relationships among water availability, plant traits, and arthropod communities.

When plants are exposed to waterlogged environments, the semi-aquatic environment may affect the arthropod community simply by posing a physical barrier between the plant and arthropods. This physical barrier may discourage certain arthropods from accessing the plant, while simultaneously providing a more favorable habitat and refuge from competition or natural enemies for arthropods that are capable of reaching the plant (Elderd and Doak 2006; Sipura et al. 2002). Our results show that species richness is lower on waterlogged plants than on well-watered plants in all plant species except for *R. palustris* in at least one of the two experimental years. Looking into what arthropod species have lower abundances on waterlogged plants we find that, for example, *Plutella xylostella* caterpillar abundance was significantly lower on waterlogged than on well-watered *R. amphibia*, *R. sylvestris* and *R. austriaca* plants in 2020. In addition to *P. xylostella* many other arthropods like *Lygus hesperus* and predatory *Orius* bugs, which all have the ability to fly, still show lower abundances on waterlogged plants of at least one of the four plant species. This indicates that even when being able to reach the plant by flying, waterlogging still affected the host-plant choice of some arthropods. Furthermore, our observations indicate that waterlogging had contrasting effects on different plant species. For instance, the flea beetle *Phyllotreta undulata* avoided waterlogged *R. palustris*, but not waterlogged *R. amphibia* and *R. austriaca* plants. These findings suggest that the effects of waterlogging cannot be solely attributed to the physical barrier effect, but rather to the specific response of the plant to waterlogging.
Our findings reveal distinct effects of water availability on plant phenotype, with drought and

waterlogging yielding contrasting outcomes. Drought conditions frequently resulted in reduced plant height and radius, whereas waterlogging exhibited no significant effect or led to elongation in *R. amphibia* and *R. austriaca*. Plant elongation in response to waterlogging has also been observed in other plants growing in flood-prone habitats, indicating an adaptive strategy to gain a head start in outgrowing potential floods (Hattori et al. 2009; Kuroha et al. 2018; Pan et al. 2021). Notably, despite these variations in phenotype, both drought and waterlogging generally exerted a negative influence on arthropod species richness and abundance. This indicates that increased plant size does not necessarily translate into a more diverse arthropod community. This is contradicting with other studies in which plant biomass largely correlates with a richer or more abundant arthropod community (Haddad et al. 2001; Marques et al. 2000). Plants with more biomass are generally more apparent for arthropods. However, apparency is not solely related to plant size, other factors like plant architecture, leaf shape, plant smell and direct surroundings can all influence a plant's apparency (Smilanich et al. 2016; Strauss et al. 2015). All these factors are potentially affected by the water conditions of the plant and can thus shape the arthropod community composition (Chaves et al. 2003; Grubb 1986; Hanley et al. 2007).

Furthermore, the nutritional quality and defensive capabilities of host plants, critical determinants of their suitability for herbivorous arthropods, are intricately influenced by the plant's response to water availability (Barber and Müller 2021; Kamps and Poelman 2023; Teixeira et al. 2020). Water stress may lead to changes in the amount and composition of sugars and amino acids in leaves and phloem sap (Mewis et al. 2012; Stallmann et al. 2020). This can be associated with differences in herbivore performance on these plants (Leybourne et al. 2021; Mewis et al. 2012; Mezgebe and Azerefegne 2021; Stallmann et al. 2020). Additionally, plants employ a complex network of signaling molecules and pathways to communicate adverse conditions and initiate responses to mitigate the effects of stress. Interestingly, some of these signaling components involved in water-stress responses overlap and crosstalk with those associated with defense against insect herbivory (Arbona and Gómez-Cadenas 2008; Hickman et al. 2019; Leybourne et al. 2022; Mezgebe and Azerefegne 2021; Thaler et al. 2012; Zhang et al. 2015). Through this crosstalk, the response to water stress can hamper the response to damage by certain herbivores while facilitating the defense against others (Barber and Müller 2021; Huberty and Denno 2004; Khan et al. 2010; Pineda et al. 2016; Teixeira et al. 2020). Additionally, each herbivore species possesses specific dietary requirements and constraints that guide their selection of a suitable host plant. The impact of water stress on herbivores can vary depending on how the plant's response aligns with the herbivore's dietary needs and constraints, leading to both positive and negative effects under different water levels (Khan et al. 2011; Mewis et al. 2012; Pompon et al. 2011; Pons et al. 2020). Furthermore, certain specialized herbivores even utilize plant defense traits to recognize their host plant and might therefore prefer a plant with heightened defenses (Mertens et al. 2021b; Sun et al. 2009).

Water stress not only impacts herbivores but also has implications for higher trophic levels within the plant's arthropod community. Specifically, we observe a decreased abundance of *Orius* predatory bugs on waterlogged *R. palustris* and *R. sylvestris*, and in 2020 a decreased abundance of *Cotesia glomerata* cocoons in waterlogged *R. austriaca* and *R. sylvestris* indicating that water availability influences the presence of natural enemies. Previous studies have similarly documented the effects of water stress on natural enemies of herbivores through changes in the volatile organic compounds emitted by water-stressed plants (Kansman et al. 2021b; Weldegergis et al. 2015). These alterations in plant chemistry can either enhance or reduce the attractiveness of the plant to natural enemies, thereby potentially creating enemy-free zones or increasing attractiveness to natural enemies, consequently affecting herbivores (Denno et al. 2002; Hunter and Price 1992).

Future research

This study unveils the compelling influence of water conditions on the dynamics of plant-associated arthropod communities, mediated through plant-induced modifications. The intricate interplay between plant adaptations to water environments and the direct impact of water barriers plays a pivotal role in shaping these arthropod communities. Noteworthy are the plant's responses to the compounded stressors of abiotic (e.g., water availability) and biotic (e.g., herbivores and predators) factors, which prompt distinctive species-specific effects on arthropod community composition under different water conditions. In the context of climate change, it becomes evident that plant-species-specific adaptations determine the manner in which plants respond to alterations in water conditions. However, precisely predicting the ramifications of these adaptations on arthropod community dynamics remains a formidable challenge. Future research should investigate direct and indirect resistance to unravel the complex interactions between plants, herbivores, and natural enemies under specific water conditions. Furthermore, how plant species differ in their response to water conditions on a physiological level should be measured, including nutrient quality and defensive compounds to find a causal relationship between plant response to water stress and its quality as a host for arthropods. While climate change is predicted to bring about increased drought and heavy precipitation, it is also important to take a broader perspective and consider the effects of other factors such as elevated CO_2 levels and temperature on plant mediated interactions. Furthermore, it is essential to assess the direct effects of these climatic conditions on arthropod communities, independently of plant-mediated effects. By addressing these research gaps, we can gain a comprehensive understanding of the intricate relationships between water stress, plants, and associated arthropod communities in the context of climate change.

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Supplementary Information

Supplementary Table 1. Results of GLMM analysis on phenotypic plant traits using Plant species, Water condition and Experimental year as explanatory factors in a full factorial design.

Trait and distribution used for analysis	Factor	χ2	Df	P
Max. plant height (cm)	Plant species	65.4	3	**3.99E-14**
Negative binomial (log link)	Water condition	56.7	2	**4.86E-13**
	Year	61.3	1	**4.76E-15**
	Plant species:Water condition	10.4	6	0.108
	Plant species:Year	42.2	3	**3.49E-09**
	Water condition:Year	1.7	2	0.419
	Plant species:Water condition:Year	11.5	6	0.074
Max. plant radius (cm)	Plant species	46.4	3	**4.48E-10**
Poisson (log link)	Water condition	37.3	2	**7.65E-09**
	Year	27.2	1	**1.80E-07**
	Plant species:Water condition	1.5	6	0.956
	Plant species:Year	0.6	3	0.893
	Water condition:Year	10.0	2	**0.006**
	Plant species:Water condition:Year	8.6	6	0.196
Max. length longest leaf (cm)	Plant species	49.3	3	**1.08E-10**
Negative binomial (log link)	Water condition	6.8	2	**3.18E-02**
	Year	28.3	1	**1.00E-07**
	Plant species:Water condition	2.1	6	0.901
	Plant species:Year	13.0	3	**0.004**
	Water condition:Year	2.5	2	0.279
	Plant species:Water condition:Year	12.1	6	0.058
Max. number of leaves on a plant (#)	Plant species	38.1	3	**2.59E-08**
Negative binomial (log link)	Water condition	54.2	2	**1.66E-12**
	Year	45.5	1	**1.47E-11**
	Plant species:Water condition	2.2	6	0.895
	Plant species:Year	48.9	3	**1.33E-10**
	Water condition:Year	10.4	2	**0.005**
	Plant species:Water condition:Year	6.7	6	0.340

Supplementary Table 1 continued.

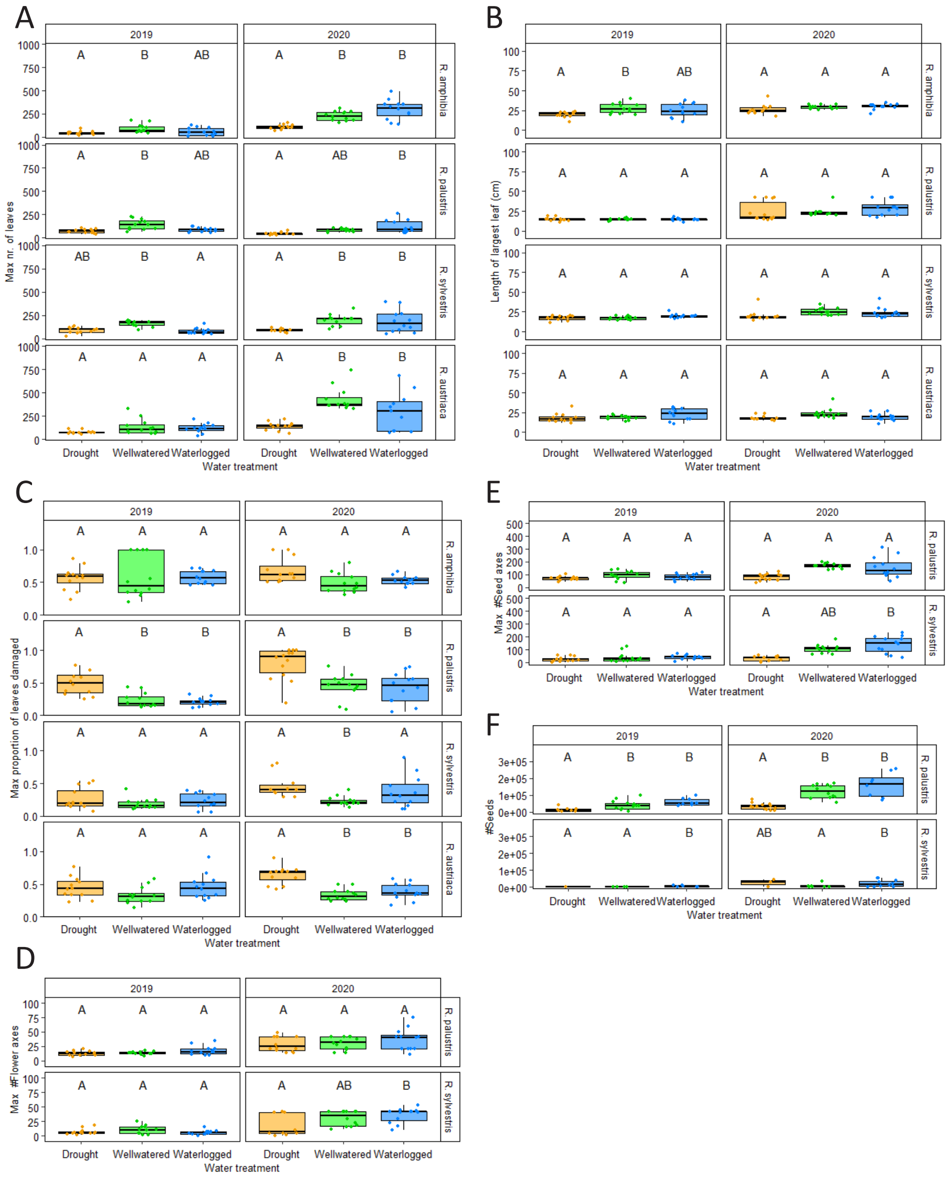
Trait and distribution used for analysis	Factor	χ2	Df	P
Max. proportion of leaves showing damage	Plant species	69.0	3	**6.89E-15**
(Nr. damaged leaves/nr. leaves)	Water condition	41.6	2	**8.81E-10**
Gamma (inverse link)	Year	16.4	1	**5.04E-05**
	Plant species:Water condition	19.5	6	**0.003**
	Plant species:Year	21.3	3	**8.76E-05**
	Water condition:Year	0.1	2	0.910
	Plant species:Water condition:Year	10.7	6	0.095
Max. number of flower axes (#)	Plant species	144.7	10	**2.20E-16**
Negative binomial (log link)	Water condition	10.3	8	**2.42E-01**
	Year	90.4	10	**4.38E-15**
	Plant species:Water condition	10.7	10	0.377
	Plant species:Year	21.9	7	**2.56E-03**
	Water condition:Year	6.5	6	0.362
	Plant species:Water condition:Year	6.2	6	0.395
Max. number of seed axes (#)	Plant species	217.6	7	**2.20E-16**
Negative binomial (log link)	Water condition	15.8	8	**4.46E-02**
	Year	47.3	5	**4.80E-09**
	Plant species:Water condition	9.8	8	0.276
	Plant species:Year	20.1	5	**1.18E-03**
	Water condition:Year	9.5	4	**0.048**
	Plant species:Water condition:Year	6.1	6	0.404
Number of seeds harvested	Plant species	187.1	1	**2.20E-16**
Negative binomial (log link)	Water condition	53.1	2	**2.85E-12**
	Year	59.8	1	**1.02E-14**
	Water condition:Plant species	11.5	2	**3.06E-03**
	Year:Plant species	2.3	1	0.122
	Water condition:Year	0.1	2	0.914
	Water condition:Year:Plant species	7.0	2	**0.029**

Supplementary Table 2. GLMM analysis with Poisson distribution (log link) of Species Richness (number of arthropod species found on a plant over its lifecycle) for both experimental years with explanatory factors, Plant Species, Water condition and Experimental year accounting for dependencies by included Plot as random factor. Because there was a significant effect of year. We also analyzed each year separately with only plant species and water condition as explanatory factors.

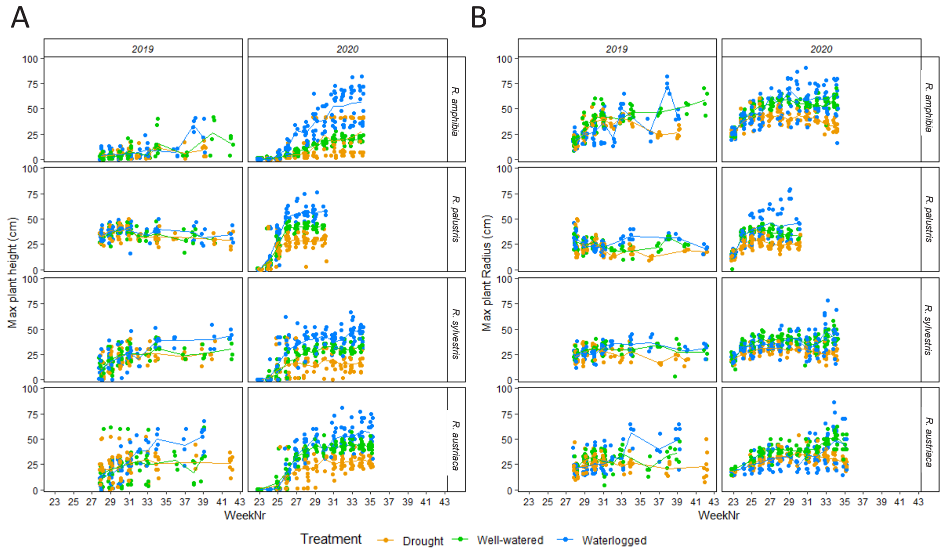
Year	Factor	χ2	Df	P
2019+2020	Plant species	46.0	3	**5.59E-10**
	Water condition	27.2	2	**1.22E-06**
	Year	105.1	1	**2.20E-16**
	Plant species:Water condition	4.7	6	0.574
	Plant species:Year	11.3	3	**0.01013**
	Water condition:Year	0.8	2	0.666
	Plant species:Water condition:Year	2.5	6	0.868
2019	Plant species	6.2	3	0.101
	Water condition	15.3	2	**0.000471**
	Plant species:Water condition	5.3	6	0.497
2020	Plant species	70.8	3	**2.87E-15**
	Water condition	17.2	2	**0.001**
	Plant species:Water condition	2.9	6	0.813

Supplementary Table 3. GLMM analysis with gamma distribution (inverse link) of Shannon-Wiener index calculated over all arthropods measured per plant for both experimental years with explanatory factors, Plant Species, Water condition and Experimental year accounting for dependencies by included Plot as random factor. Because there was a significant effect of year, we also analyzed each year separately.

Year	Factor	χ2	Df	P
2019+2020	Plant species	2.2559	3	**5.21E-01**
	Water condition	14.4306	2	**7.35E-04**
	Year	0.4853	1	**4.86E-01**
	Plant species:Water condition	10.169	6	0.117
	Plant species:Year	2.8882	3	0.409
	Water condition:Year	1.3193	2	0.517
	Plant species:Water condition:Year	1.0129	6	0.985
2019	Plant species	1.8219	3	0.610
	Water condition	2.9318	2	**0.230**
	Plant species:Water condition	4.3324	6	0.631
2020	Plant species	3.5783	3	**3.11E-01**
	Water condition	15.9761	2	**0.001**
	Plant species:Water condition	7.7233	6	0.259



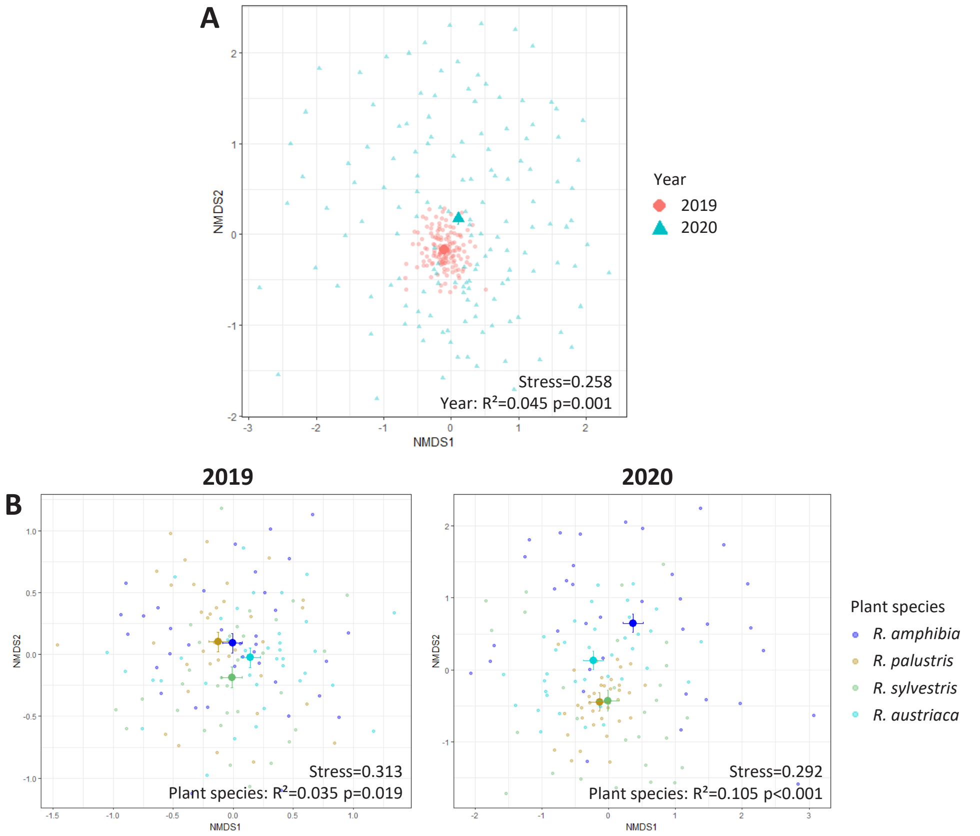
Supplementary Figure 1. Measured plant traits separated by experimental year and per plant species on plants subjected to one of three watering treatments. Different letters indicate a significantly difference between watering treatments on a specific plant species within a specific year (GLMM; α=0.05).



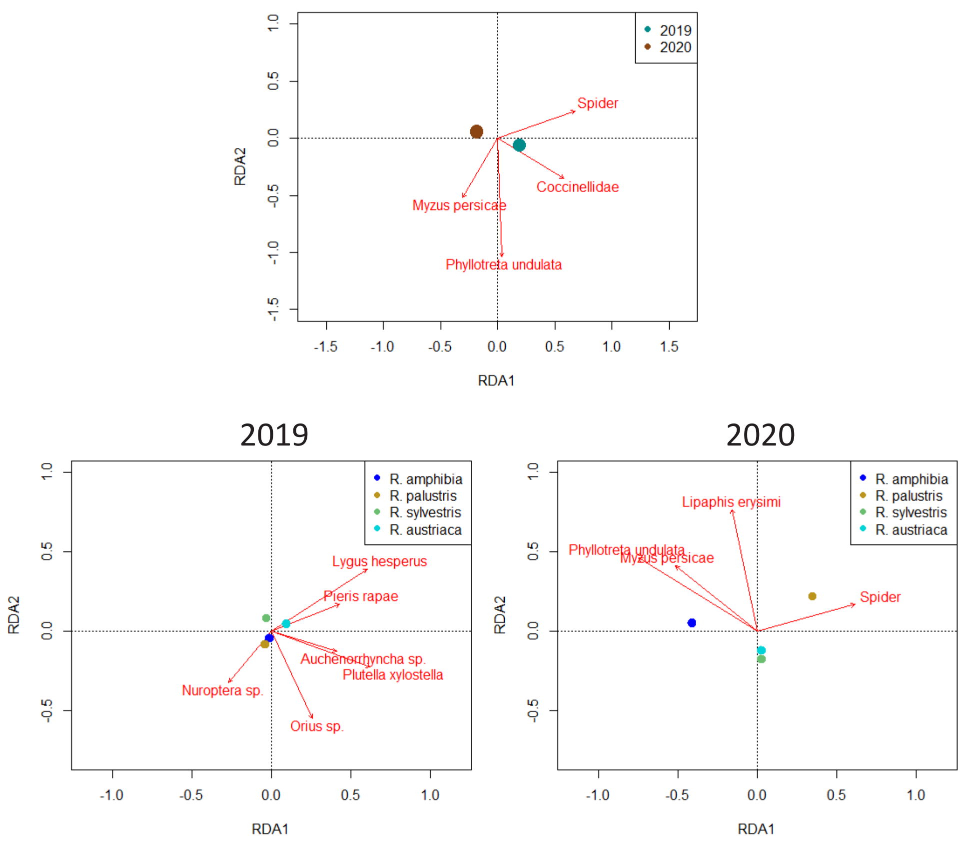
Supplementary Figure 2. Measured plant traits **A:** plant height **B:** plant radius over time separated by experimental year and per plant species on plants subjected to one of three watering treatments.

Supplementary Table 4. Results of the PERMANOVA analysis on the average arthropod community per plant over time, expressed as log(summed abundance of arthropod species on plant/number of times that plant was monitored). As no significant interaction was found between Water conditions and other factors this was moved to a block factor in the next analysis. As Experimental year and Plant species showed a significant interaction, both years were analyzed separately. In all permutation designs we controlled for dependencies of plants growing in the same Plot. Significant P values (P<0.05) are indicated in bold.

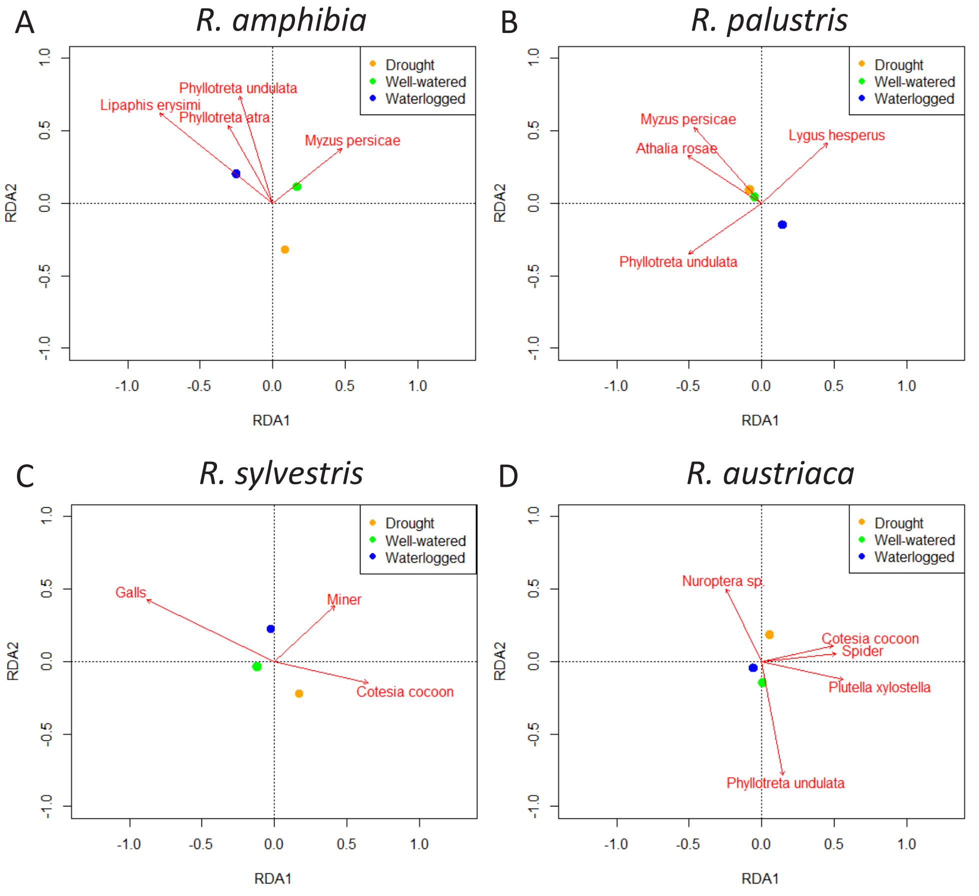
Model	Factor	DF	R^2	Pseudo-F	P
full factorial	Plant species	3	0.041	4.13	**<0.001**
	Year	1	0.045	14.598	**<0.001**
	Water condition	2	0.019	3.201	**0.007**
	Plant species * Year	3	0.042	4.544	**<0.001**
	Plant species * Water condition	6	0.030	1.623	0.656
	Year * Water condition	2	0.013	2.118	0.183
	Plant species * Year * Water condition	6	0.032	1.718	0.502
Treatment as block effect	Plant species	3	0.041	4.184	**<0.001**
	Year	1	0.045	13.839	**<0.001**
	Plant species * Year	3	0.042	4.302	**<0.001**
Treatment as block effect, analysis separated per experimental year					
2019	Plant species	3	0.035	1.620	**0.019**
2020	Plant species	3	0.105	5.152	**<0.001**



Supplementary Figure 3. Ordination of arthropod community composition expressed as log (summed abundance of species/times a plant was measured) for the first two NMDS ordination axes with accompanying stress value. Small dots depict the community composition of individual plants while large dots depict centroids of communities for plants **A:** Between experimental years. **B:** Between plant species for each experimental year separately. Error bars represent standard errors of communities in multivariate space. Additionally, a PERMANOVA analysis was done to calculate the main effect of water treatment as well as determine pair-wise differences between water treatments.



Supplementary Figure 4. Redundancy analysis (RDA) of arthropod community composition expressed as log(abundance of species/times a plant was measured) for the first two RDA ordination axes 1. Between experimental years. 2. Between plant species for each experimental year separately. Coloured dots depict centroids of communities belonging to the explanatory variables. Named arrows depict arthropod species explaining most of the variation between communities.



Supplementary Figure 5. Redundancy analysis (RDA) of arthropod community composition expressed as log(abundance of species/times a plant was measured) for the first two RDA ordination axes for each plant species separately. Colored dots depict centroids of communities of plants under a specific watering treatment. Named arrows depict arthropod species explaining most of the variation between communities.

Supplementary Table 5. Results of PERMANOVA analysis on the average arthropod community per plant over time, expressed as log(summed abundance of arthropod species/ number of times that plant was monitored) separated per plant species. Water condition and Experimental year were modeled as explanatory factor. In all permutation designs we controlled for dependencies of plants growing in the same Plot. As no significant interaction term was found between Water condition and Experimental year, we run our final analysis with Experimental year as block factor (supplementary Table 6).

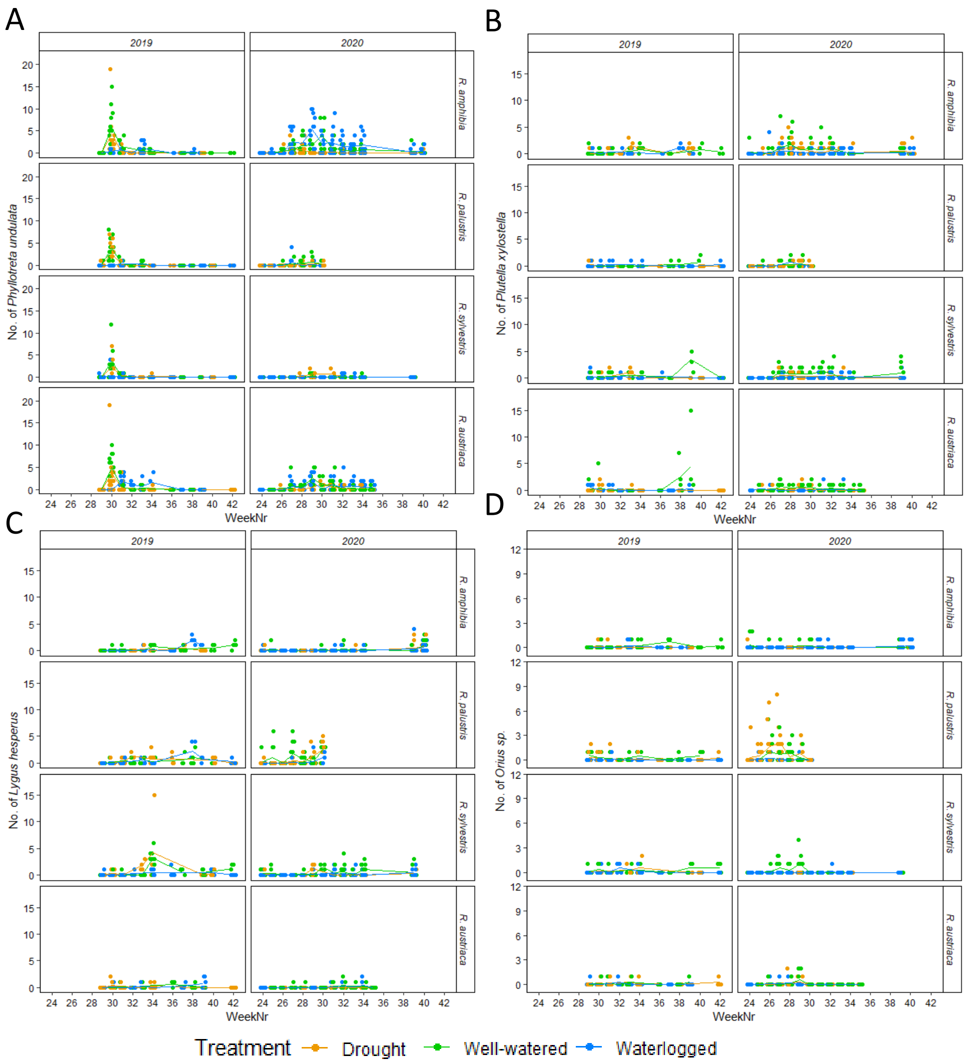
Plant species	Factor	DF	R^2	Pseudo-F	P
R. amphibia	Water condition	2	0.069	2.790	0.064
	Year	1	0.098	8.351	**<0.001**
	Water condition*Year	2	0.058	2.485	0.183
R. palustris	Water condition	2	0.039	1.466	0.363
	Year	1	0.046	3.401	**<0.001**
	Water condition*Year	2	0.031	1.140	0.794
R. sylvestris	Water condition	2	0.049	1.691	0.358
	Year	1	0.117	8.081	**<0.001**
	Water condition*Year	2	0.050	1.739	0.342
R. austriaca	Water condition	2	0.047	1.861	0.206
	Year	1	0.081	6.448	**<0.001**
	Water condition*Year	2	0.039	1.557	0.422

Supplementary Table 6. Results of PERMANOVA analysis on the average arthropod community per plant over time, expressed as log(summed abundance of arthropod species/ number of times that plant was monitored) with Water condition as explanatory factor, Experimental year as block factor and Plot number as random effect to account for dependencies. Each plant species was analyzed separately. Afterwards, a pairwise PERMANOVA was performed on subsets of water conditions (WW=Well-watered, D=Drought, WL=Waterlogged). P-values were adjusted for multiple testing using an FDR (false discovery rate) adjustment.

Plant species	DF	R^2	Pseudo-F	P	pairwise comparison	Pseudo-F	P	FDR
R. amphibia	2	0.069	2,592	**0.004**	WW-D	2.334	0.183	0.257
					WW-WL	2.288	0.257	0.257
					D-WL	3.185	0.058	0.174
R. palustris	2	0.039	1,411	0.211	WW-D	0.974	0.792	0.792
					WW-WL	1.571	0.353	0.515
					D-WL	1.709	0.145	0.434
R. sylvestris	2	0.049	1,471	**0.032**	WW-D	1.598	0.375	0.559
					WW-WL	1.311	0.559	0.559
					D-WL	1.586	0.459	0.559
R. austriaca	2	0.047	1,699	**0.047**	WW-D	2.321	0.071	0.255
					WW-WL	1.427	0.464	0.537
					D-WL	1.356	0.537	0.537

Supplementary Table 7. GLMM analysis with gamma distribution (log link) of abundance of specific arthropod species with explanatory factor, Plant Species, Water condition and experimental year, accounting for dependencies by including Plot as random factor and Timepoint and Plant ID as random intercepts.

Arthropod species	Factor	χ^2	DF	P
Plutella xylostella	Year	0	1	0.997
	Water condition	30.5	2	**2.27E-07**
	Plant species	35.3	3	**1.01E-07**
	Year:Water condition	2.6	2	0.266
	Year:Plant species	5.8	3	0.120
	Water condition:Plant species	16.5	6	**0.011**
	Year:Water condition:Plant species	1.4	6	0.962
Phyllotreta undulata	Year	7.3	1	**0.006**
	Water condition	18.0	2	**0.001**
	Plant species	58.1	3	**1.47E-12**
	Year:Water condition	9.3	2	**0.009**
	Year:Plant species	12.1	3	**0.006**
	Water condition:Plant species	19.1	6	**0.003**
	Year:Water condition:Plant species	3.9	6	0.681
Orius sp.	Year	5.3	3	0.149
	Water condition	23.4	3	**3.31E-05**
	Plant species	44.0	5	**2.3E-08**
	Year:Water condition	3.9	3	0.262
	Year:Plant species	11.9	4	**0.017**
	Water condition:Plant species	19.1	7	**0.007**
	Year:Water condition:Plant species	8.8	6	0.181
Lygus hesperus	Year	13.8	1	**0.001**
	Water condition	13.7	2	**0.001**
	Plant species	43.3	4	**8.82E-09**
	Year:Water condition	0.9	2	0.633
	Year:Plant species	7.9	3	**0.047**
	Water condition:Plant species	5.6	6	0.464
	Year:Water condition:Plant species	7.1	6	0.310



Supplementary Figure 6. Abundance of **A:** *Phyllotreta undulata* **B:** *Plutella xylostella* **C:** *Lygus hesperus* **D:** *Orius sp.* bugs per observation over time separated by experimental year and per plant species on plants subjected to one of three watering treatments.

Chapter 3

Adaptations to water gradient in three *Rorippa* plant species correspond with plant resistance against insect herbivory under drought and waterlogged conditions

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Abstract

Plants live in environments where they are constantly, and often simultaneously, exposed to different types of biotic and abiotic stress, such as insect herbivory and water availability. How plants are adapted to abiotic conditions may determine how a surplus or shortage of water affects plant resistance to insect herbivory. Moreover, this effect may vary depending on the feeding mode of the herbivore.

We explored how three closely related *Rorippa* plant species that vary in adaptations to different water levels, resist herbivory by four different insects (aphids: *Myzus persicae*, *Lipaphis erysimi*, and caterpillars: *Pieris brassicae*, *Plutella xylostella*) under waterlogging or drought conditions. We hypothesized that plants that are differently adapted to water availability will be disparately affected by water availability in their resistance to insect herbivory.

On the semi-aquatic plant species *R. amphibia*, both aphid species reached a larger colony size under drought conditions. This indicates that *R. amphibia* was compromised in resistance to aphid feeding when under drought conditions, to which it is less well adapted. Water conditions did not affect aphid performance on the flood-plain species *R. palustris*. On the terrestrial plant species *R. sylvestris*, aphids performed worse on waterlogged than drought treated plants. Neither caterpillar species was significantly affected by the water availability of their food plant. Our findings suggest that water availability can have distinct effects on plant-insect interactions. We propose that plant adaptations to water conditions can be a major predictor toward explaining the variation of effects that water availability can have on plant-insect interactions.

Introduction

Plants live in environments where they are often exposed to several types of biotic and abiotic stress simultaneously. Two devastating stressors are sub-optimal water availability and insect herbivory (Boyer et al. 2013; Liliane and Charles 2020; Raderschall et al. 2021; Tian et al. 2021). The spectrum of water availability may range from flooding, to waterlogging, to drought periods. The intensity of insect herbivory may vary among plant organs and the type of damage inflicted by the feeding mode of the herbivore, such as sap-feeding aphids and leaf-chewing caterpillars. To maximize its fitness, a plant must optimize its responses to deal with this range of stress conditions (Ben Rejeb et al. 2014; Fernández de Bobadilla et al. 2022). Depending on the plant's habitat, the availability of water may be highly variable. To increase their plasticity and resilience toward variabilities in water availability, plant species have evolved adaptations to navigate changes in water availability (Akman et al. 2012; Colmer and Voesenek 2009). Plants can, for example, mitigate the drought by investing resources in deeper roots to reach new water sources (Kuster et al. 2013; Sponchiado et al. 1989; Uga et al. 2013) or they can endure drought by slowing down their metabolism and reducing water loss to a minimum (Tamang et al. 2021; Zhao et al. 2015). Adaptations to flooding may include enhancing gas transport to flooded organs (Akman et al. 2014; Laan et al. 1989; Sasidharan and Voesenek 2015). Importantly, responses or adaptations to sub-optimal water conditions can have consequences for plant defense responses to insect herbivory (Lin et al., 2021).

Several studies have shown that water availability can affect plant-insect interactions (Khan et al. 2011; Leybourne et al. 2021; Lin et al. 2021; Mewis et al. 2012; Mody et al. 2009; Pineda et al. 2016; Pons et al. 2020). Responding to simultaneous sub-optimal water availability and insect herbivory can have different consequences for the plant and is captured by two contrasting hypotheses. The "plant stress hypothesis" states that water stress has a positive effect on insect herbivores due to increased nutrient concentration (Mattson and Haack 1987), whereas the "plant vigor hypothesis" states that plant performance is enhanced under well-watered conditions and provides higher quality food for insect herbivores (Price 1991). However, these hypotheses are not framed in the context of plant adaptations to sub-optimal water availability. A major knowledge gap is how plant adaptations to water availability in its habitat correspond with their resistance to insect attack under various water regimes.

Plant species widely differ in their adaptations to sub-optimal water availability. In some plant species, drought has been shown to lead to smaller but thicker leaves to reduce evaporation (Chaves et al. 2003). Because more sturdy thicker leaves enhance

morphological resistance against leaf-chewing insect herbivory, this adaptation indirectly leads to better protection against certain insect herbivores (Grubb 1986; Hanley et al. 2007). Under waterlogging conditions, some plants have evolved the ability to create aerenchymous tissue allowing better gas exchange to the roots (Akman et al. 2014; Laan et al. 1989; Sasidharan and Voesenek 2015). The changes in cell-layer composition may affect how an aphid can navigate its stylets to reach the phloem. Moreover, plants regulate responses to abiotic and biotic stress through signaling cascades that involve the same phytohormones (Ullah et al. 2018). The hormone ethylene, for example, builds up in waterlogged roots and is a signal for the plant that it is waterlogged (Sasidharan and Voesenek 2015; Voesenek and Sasidharan 2013). Ethylene also plays a role in regulating defense responses to leaf-chewing herbivores (Winz and Baldwin 2001). Similarly, jasmonic acid and salicylic acid regulate the signaling of both plant responses to sub-optimal water availability and herbivory-induced responses (Koramutla et al. 2022; Riemann et al. 2015; Smith et al. 2009). The signal-transduction routes regulated by these phytohormones cross talk (Hickman et al. 2019; Thaler et al. 2012; Zhang et al. 2015). This crosstalk allows plants to integrate and finetune responses to biotic and abiotic stress separately but might also provide a way for abiotic stress to affect how plants deal with biotic stress (Nguyen et al. 2016). Additionally, sub-optimal water availability is known to have a big metabolomic impact on the plant. Under sub-optimal water availability, some plant species start mobilizing stored resources to respond to the stress (Krasensky and Jonak 2012; Mewis et al. 2012). These resources can then be utilized by insect herbivores, increasing their performance (Barber and Müller 2021; Irfan et al. 2010; Lothier et al. 2020). In *Brassica oleracea,* waterlogging has been correlated with a decrease in plant defense compounds (Barber and Müller 2021). Contrarily, other studies have found that sub-optimal water availability causes an increase in defensive compounds which can hamper insect herbivore performance (Schreiner et al. 2009). This demonstrates the complexity of predicting the effect of sub-optimal water availability on plant-insect interactions.

A comparative study on related plant species may reveal how plants that are differently adapted to abiotic conditions such as water availability, are disparately affected in their resistance against insect herbivory under various levels of water availability. In this study we characterize how well three closely related *Rorippa* plants adapted to different water gradients resist insect herbivory under drought, well-watered, and waterlogged conditions. *Rorippa amphibia* grows in semi-aquatic conditions along the edges of lakes and swamps. It is adapted to waterlogging and we, therefore, hypothesize it will be able to resist insect herbivory to a similar degree in waterlogged and moderate water conditions. As being semi-aquatic, drought conditions are hypothesized to result in more severe stress that may interfere with the plant's resistance against insect herbivores. *Rorippa palustris* grows in wetlands and floodplains and is often exposed to large variation in water conditions by flooding and drought. To escape large fluctuations in water availability, its life-history strategy is to flower and set seeds fast to complete its life cycle before stresses get too severe. We hypothesize that prioritizing resources to reproduction comes at the cost of defense against herbivory (Lind et al. 2013; Lucas-Barbosa et al. 2013). Since its defensive capabilities are expected to be limited, the effect of watering regime on these defensive capabilities might also be limited. *Rorippa sylvestris* grows in drier habitats than the other two plant species, in sandy, disturbed soils and may thus better cope with drought conditions. It is known to adopt an enduring strategy under flooding conditions where it lowers its metabolism to the minimum while waiting for conditions to improve (Akman et al. 2012). Therefore, we hypothesize that this strategy might interfere with mounting a defense response against insect herbivory under sub-optimal water availability. This will cause insect herbivores to perform better on waterlogged *R. sylvestris* plants while remaining unaffected by drought conditions. How plants deal with insect herbivory under different water regimes may also depend on the feeding mode of the herbivore. Aphids that feed on phloem sap are likely affected by changes in the nutritional value of phloem sap after drought or waterlogged conditions. Drought stressed plants have a lower water potential (Mewis et al. 2012). This lower water potential might lead to an increase in concentration of nutrients in the phloem. Therefore, we hypothesize aphids will perform better on drought stressed plants (but see Pompon et al. (2011) for an alternative hypothesis that osmotic pressure may reduce phloem intake by aphids). At the same time, plant responses to aphids are predominantly regulated through Salicylic Acid, whereas the responses to caterpillar attacks are predominantly regulated by Jasmonic Acid (Erb et al. 2012; Koornneef and Pieterse 2008). Since abiotic stress by sub-optimal water availability induces Abscisic Acid that often enhances Jasmonic Acid signaling, sub-optimal water availability might positively affect the plant's resistance against caterpillars while not affecting the plant's resistance against aphids (Erb et al. 2012; Howe and Jander 2008; Marquis et al. 2020; Vos et al. 2013).

In this study, we discuss how plant adaptations to water conditions can help predict their capacity to deal with aphid or caterpillar attacks under different watering regimes.

Methods

Plants and insects

We selected three closely related plant species of the *Rorippa* genus that are well studied for their difference in adaptation to water conditions (Akman et al. 2012; Sasidharan et al. 2013). We explicitly test whether, for these three plant species, their adaptations

correspond with differences in how water availability affects insect herbivory. Seeds of the terrestrial *Rorippa sylvestris*, the floodplain inhabiting *Rorippa palustris,* and rhizomes of the semi-aquatic *Rorippa amphibia* were collected around Wageningen, the Netherlands (51°57'38.2"N 5°39'41.9"E). Seeds were sown and rhizomes were planted in trays with Arabidopsis potting soil (Lentse Potgrond B.V.), watered, and kept under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). Five weeks after sowing the seeds and two weeks after planting the rhizomes, plants were transplanted into pots (ø 12 cm, 1 Liter) containing 1:1 Arabidopsis potting soil and sand (Lentse Potgrond B.V.). Plants were allowed to acclimatize and grow in pots for one week prior to the watering regime and subsequent insect infestation.

The insect species for which performance was assessed were the phloem feeders *Myzus persicae* (green peach aphid), *Lipaphis erysimi* (wild crucifer aphid), and the leaf-chewing caterpillars of *Plutella xylostella* (diamondback moth) and *Pieris brassicae* (large cabbage white). In our own additional field experiments not reported here, all insect herbivores have been confirmed to naturally occur on the plant species tested (Kamps unpublished data; (Mertens et al. 2021)). The insects were reared under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). The phloem feeders *M. persicae* and *L. erysimi* were both reared on *Raphanus sativus* (Raddish) plants. The leaf chewers *P. xylostella* and *P. brassicae* were reared on *Brassica oleracea* (Brussels sprout) plants. The four insect species were originally collected from cabbage in the same experimental field location around Wageningen. Each of the cultures has been routinely reared for more than three years at the Laboratory of Entomology of Wageningen University.

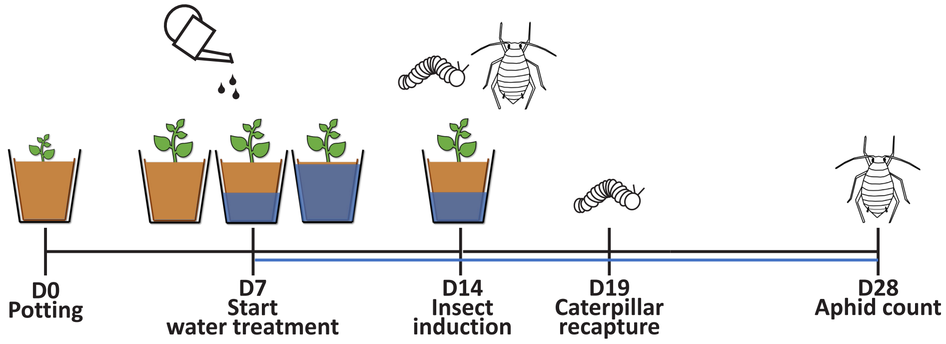

Figure 1. Workflow of experiments. On day 0 (D0) plants of all three plant species (*R. amphibia, R. palustris, R. sylvestris*) were potted. Pots were placed in buckets to retain all water of the water treatment. Day 7 marks the start of the water treatment (drought, well-watered, waterlogged). This water treatment was maintained for the rest of the experiment. On day 14, plants were infested with one of four insect species (*M. persicae, L. erysimi, P. brassicae, P. xylostella*). On day 19 caterpillars were recaptured and weighed. On day 28 aphid population size was measured.

Experimental setup

To assess how different water regimes affect resistance to insect attack for three *Rorippa* plant species an experiment was conducted in a climate-controlled greenhouse (22 ± 2 °C, 60-70% relative humidity, 16L:8D). Plants were first randomly appointed to one of three water treatments: drought, well-watered, and waterlogged. These water treatments approximate 8 ± 4%, 20 ± 5% soil moisture content, and a submerged waterlogged soil respectively. The well-watered treatment is an intermediate water condition under which all three plant species show no signs of water stress in their growth or morphology. To achieve drought stress based on soil water content measurement, we assessed in pilot experiments that 8% soil water content is close to the permanent wilting point of all three plant species (Supplementary Information, Fig. S1, Table S1). The water content was maintained by measuring soil water content daily with an electronic water potential meter (Extech MO750) and adding water accordingly. The probe was inserted at the side of the pot on a new spot every day to most accurately measure soil moisture content. To achieve the water regimes, all pots were placed inside buckets (ø 21 cm, 2.75 Liter) and for the waterlogged treatments these buckets were filled with water up to the soil line (Fig. 1). The water treatments were maintained for the entirety of the experiment.

One week after the onset of the water treatment, plants were infested with one of four insect herbivores (Fig. 1): The aphids *M. persicae*, or *L. erysimi,* or the caterpillars *P. xylostella* or *P. brassicae*. Each of the four herbivores was tested in separate experiments in the same greenhouse where plants and treatments were completely randomized over the greenhouse space. To assess aphid performance, per aphid species 90 plants of each of the three *Rorippa* species were potted. These plants were equally divided over the three water treatments (drought, well-watered, waterlogged). This resulted in a total of 30 plants per plant species, water treatment and aphid combination. Per plant, five adult aphids were placed on a young fully expanded leaf, and the plant was covered with a mesh bag to prevent aphids from wandering to other plants. After two weeks the number of aphids was counted for each plant as a proxy for plant resistance to aphid attack. In two weeks time, the aphid populations have grown exponentially and treatment effects on population growth become visible.

To assess caterpillar performance, per caterpillar species 45 plants of each of the three *Rorippa* were potted. These plants were equally divided over the three water treatments (drought, well-watered, waterlogged). This resulted in a total of 15 plants per plant species, water treatment and caterpillar combination. Per plant five freshly hatched (L1) caterpillars of similar size were placed on a young fully expanded leaf and the plant was covered with a mesh bag to prevent caterpillars from wandering to other plants. After five days the caterpillars were recaptured and weighed. Their weight was used as a proxy for how well a plant was able to resist the specific caterpillar. We selected a five-day growth period to avoid that the fastest developing species (*P. xylostella*) would reach pupation which coincides with mass reduction.

Statistical analysis

All statistical analyses were performed using RStudio (Allaire 2012) under R 3.6.3 (R Core Team 2013), with packages *lme4* (Bates et al. 2015) *emmeans* (Lenth et al. 2019), and *ggplot2* (Wickham 2016). Aphid colony size data was analyzed using a Generalized Linear Model (GLM) with gamma distribution including the full factorial interaction for plant species and water treatment followed by a Tukey's HSD post-hoc test for each factor. Since multiple caterpillars were measured per plant, caterpillar weight data was analyzed using a Generalized Linear Mixed Model (GLMM) adding plant ID as a random factor to accommodate for the variance in both the caterpillars and replicated plants (Pineda et al. 2016; Walter et al. 2012) and including the full factorial interaction of plant species and water treatment followed by a Tukey's HSD post-hoc test for each factor. Performance of each herbivore was analyzed with a separate model because experiments were conducted during different moments in time.

Results

Aphid performance

The population development of the aphid species *Myzus persicae* and *Lipaphis erysimi* after 14 days of feeding, was significantly affected by the water conditions their food plants were exposed to (*M. persicae*: GLM, χ2=47.9, df=2, P<0.001 and *L. erysimi*: χ2=41.7, df=2, P<0.001). Both aphid species generally performed better on plants growing under drought conditions than on waterlogged plants (Fig. 2). Moreover, the plant species affected the performance of the two aphid species (*M. persicae*: GLM, χ2=212.6, df=2, P<0.001 and *L. erysimi*: GLM, χ2=432.7, df=2, P<0.001). Both aphid species had the smallest population growth on the semi-aquatic *R. amphibia* plants. The poor performance on this plant species was accompanied by high mortality of *L. erysimi,* with only 11 well-watered and 16 waterlogged *R. amphibia* plants out of 30 supporting an aphid population. The performance of *M. persicae* was similar on the flood-plain species *R. palustris* and the terrestrial *R. sylvestris*, whereas *L. erysimi* performed better on *R. palustris* than on *R. sylvestris* (Fig. 2). The effect of the water condition interacted with plant species for the performance of both aphid species (*M. persicae*: GLM, χ2=24.8, df=4, P<0.001 and *L. erysimi*: GLM, χ2=37.6, df=4, P<0.001). On the semi-aquatic species *R. amphibia*, both aphid species reached a larger colony size under drought conditions than on well-watered and waterlogged plants. Performance of *M. persicae* was similar on well-watered and waterlogged *R. amphibia,* whereas the performance of *L. erysimi* was poorer on waterlogged than on well-watered plants. Water conditions did not affect aphid colony size on the flood-plain species *R. palustris*. On the terrestrial species *R. sylvestris*, aphid colony size of *M. persicae* and *L. erysimi* was similar on drought-exposed and well-watered plants, whereas the population size reached was smallest on waterlogged plants but not significantly different from well-watered plants for both aphid species (Fig. 2).

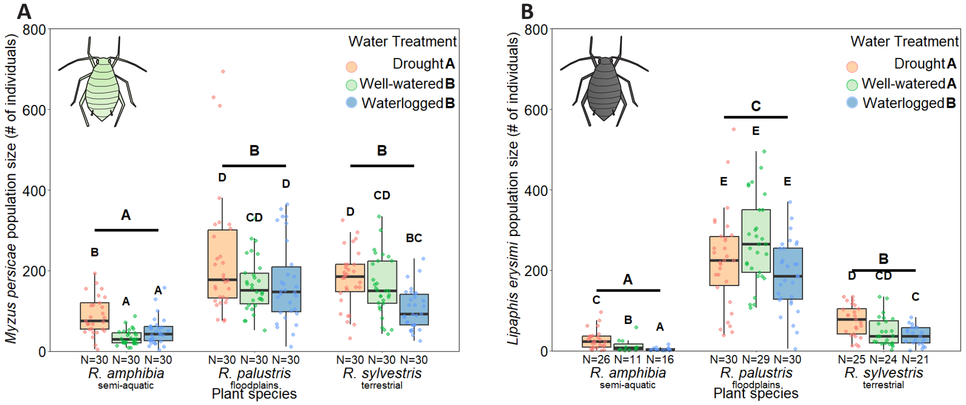

Figure 2. Population size of the aphids **(A)** Myzus persicae and **(B)** *Lipaphis erysimi* after feeding for 14 days on one of three plant species from the genus *Rorippa* that were subjected to different water treatments (drought, well-watered and waterlogged). The three plant species (*R. amphibia, R. palustris and R. sylvestris*) are ordered on the x-axis for their habitat along the water gradient from semi-aquatic to fully terrestrial. Letters in the legend show main effects of water treatment, letters above each plant species show main effects of plant species and letters above each bar show significant differences among each treatment for the performance of aphids (GLM, Gamma distribution, α = 0.05).

Caterpillar performance

Pieris brassicae caterpillar weight after five days of feeding was not significantly affected by the water conditions their food plants were exposed to (GLMM, χ2=3.2, df=2, P=0.19) (Fig. 3A). Contrarily, *Plutella xylostella* caterpillar weight was significantly affected by water treatment (GLMM, χ2= 14.3, df=2, P<0.001) (Fig. 3B). Similar to the aphids, *P. xylostella* performed slightly better when feeding on drought treated plants than on well-watered or waterlogged plants. While *P. brassicae* performance was unaffected by the water conditions of its food plant, *P. brassicae* performance was affected by plant species (Fig. 3A). It grew bigger on the flood-plain species *R. palustris* than on the semi-aquatic *R. amphibia* or the terrestrial *R. sylvestris* (GLMM, χ2=27.0, df=2, P<0.001). On the other hand, *P. xylostella* performance was not significantly different between the three plant species (GLMM, χ2=3.5, df=2, P=0.17) (Fig. 3B). There was no significant interaction between water regime and plant species on caterpillar performance (*P. brassicae* GLMM, χ2=4.3, df=4, P=0.366 and *P. xylostella*: GLMM, χ2=5.2, df=4, P=0.257).

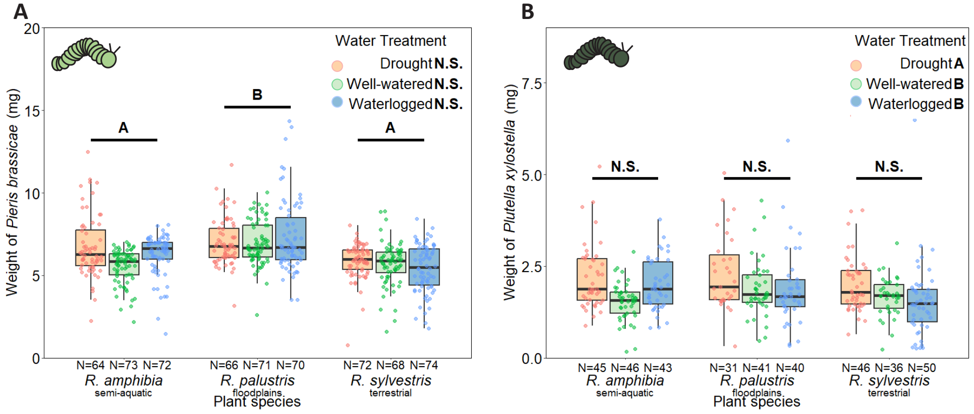

Figure 3. weight (mg) of **(A)** *Pieris brassicae* **(B)** *Plutella xylostella* after feeding for 5 days on one of three plant species from the genus *Rorippa* that were subjected to different water treatments (drought, well-watered, waterlogged). The three plant species (*R. amphibia, R. palustris and R. sylvestris*) are ordered on the x-axis for their habitat along the water gradient from semi-aquatic to fully terrestrial. Letters in the legend show main effects of water treatment, letters above each plant species show main effects of plant species. No significant effects were found between water treatments within a single plant species (GLMM, Gamma distribution, α = 0.05).

Discussion

Our results show that plant species are differently affected by watering regime in their resistance to herbivore attack. We demonstrate that the more terrestrial plant species *R. sylvestris* is not hampered in its resistance against insect herbivores when faced with drought. In contrast, the waterlogging adapted plant species *R. amphibia* was not affected in its resistance against herbivore attack under waterlogging conditions. Both aphid species *M. persicae* and *L. erysimi* generally performed worse on waterlogged plants and better on drought-treated plants. A surplus or shortage of water had less effect on the caterpillar species *P. brassicae* and *P. xylostella*. However, similar to aphids, *P. xylostella* performed better on drought treated plants.

The better performance of aphids under drought conditions contrasts with the observations of other studies of poorer performance under drought (Huberty and Denno 2004; Leybourne et al. 2021; Simpson et al. 2012). Generally, poorer aphid performance under drought stress is correlated with a reduction in plant vigor and an increase in chemical defense in drought-stressed plants (Beetge and Krüger 2019; Inbar et al. 2001; Simpson et al. 2012; Xie et al. 2020). Yet ours is not the only study that shows a better performance of aphids on drought-stressed plants (Mewis et al. 2012; Oswald and Brewer 1997). Better aphid performance on drought-stressed plants may be explained by increased concentration of amino acids in the plant (Barber and Müller 2021; Mewis et al. 2012; Xie et al. 2020). Additionally, drought can interrupt the production of defensive compounds when plants are attacked by aphids, as was found in *Brassica oleracea* var. *italica* and *Arabidopsis thaliana* (Khan et al. 2010; Mewis et al. 2012). Moreover, aphids may benefit from increases in phloem sugar concentrations under drought stress as identified in the plant species *Triticum aestivum* and *A. thaliana* (Mewis et al. 2012; Xie et al. 2020). At the other end of the water-stress spectrum, waterlogging of plants in our study reduced aphid performance. This pattern is more consistent across the few studies on plant responses to waterlogging and may be explained by the effects of waterlogging on primary and secondary metabolites in the phloem sap (Khan et al. 2010; Lin et al. 2021; Mewis et al. 2012). In *A. thaliana,* waterlogging led to lower total amino acid and sugar concentration in the phloem sap compared to drought (Mewis et al., 2012). Additionally, concentrations of defensive compounds after waterlogging in *A. thaliana* or *B. oleracea* were not significantly different from well-watered plants and in some cases even increased, in contrast to drought which decreased the concentration of defensive compounds in phloem sap (Mewis et al., 2012, Khan et al., 2010). Another explanation might be the fact that some plant species produce aerenchymous tissue under waterlogged conditions (Akman et al., 2012). This tissue might hamper aphids in reaching the phloem, reducing their performance. However, to our knowledge, no studies have yet been done to investigate the effect of aerenchyma on aphid feeding behavior. Additionally, leaf water content in waterlogged *A. thaliana* was even lower than in drought-stressed plants (Mewis et al., 2012). Possibly the water content of the plant can deplete to a level where aphids will have difficulty feeding. Even the severity of the stress, such as the duration of drought or waterlogging as well as the stress pattern, i.e., continuous versus pulsed, may affect the outcome of water stress on aphid performance. For example, a moderate level of water stress might increase amino acid concentrations and decrease defensive compounds while a severe level of stress might lower the water content of the leaf so much that aphids are struggling to feed (Kansman et al. 2020; Mody et al. 2009; Rai et al. 2018; Sconiers and Eubanks 2017).

Caterpillars that are feeding on the leaf tissues were not significantly affected by suboptimal water conditions such as drought or waterlogging in our study. Other studies have found both positive and negative effects of suboptimal water conditions on caterpillar performance (Faustino et al. 2021; Gutbrodt et al. 2011; Pineda et al. 2016; Rai et al. 2018; Walter et al. 2012). This seems to correlate with the level of defensive compounds under drought or waterlogged conditions. The increase in defensive compounds mainly affected generalist caterpillars negatively while it had no or a positive effect on specialists (Rai et al., 2018, Pineda et al., 2016, Gutbrodt et al., 2011). Both caterpillar species investigated in this study are specialists on Brassicaceae. They are therefore adapted to cope with the defensive compounds produced by the plants (Ratzka et al. 2002; Smallegange et al. 2007). Any alterations in defensive compounds might thus not have a significant effect on the performance of these caterpillars compared to generalist caterpillars.

Our results show that plant species differ in how they cope with insect herbivory under various water conditions. For these three plant species, the adaptations to their habitat corresponded with their capability to maintain resistance to insect herbivory under suboptimal water conditions. This emphasizes that taking into account plant evolutionary differences may be imperative in explaining the variation of outcomes of herbivore performance on plants with water stress. These evolutionary differences may govern whether the host quality of a plant to insect herbivores changes under different water levels. We show that in the semi-aquatic plant species *R. amphibia,* waterlogging had little effect on herbivore performance compared to well-watered plants. *R. amphibia* is well adapted to waterlogging and is known to quickly attempt to escape the stress caused by flooding (Akman et al., 2012, Sasidharan et al., 2013). Since plants were already waterlogged for seven days before herbivores were added, the *R. amphibia* plants might have already resolved the stress and returned to a normal physiological state. This could explain why herbivore performance on waterlogged plants was similar to well-watered plants for most insect species tested. Drought on the other hand might be particularly stressful for the semi-aquatic *R. amphibia* and therefore had a significant effect on the performance of both *M. persicae* and *L. erysimi* aphids. Which of the plethora of responses to drought causes the increase in aphid performance, however, requires further research. In contrast, on the terrestrial plant species *R. sylvestris*, aphids performed similarly on drought-treated and well-watered plants. *Rorippa sylvestris* is more accustomed to drier environments than *R. amphibia* and could thus be less challenged by drier conditions (Stift et al. 2008). Performance on drought-treated *R. sylvestris* could therefore be indistinguishable from well-watered plants. Waterlogging on the other hand significantly reduced the performance of both aphid species tested. *Rorippa sylvestris* is known to reduce its metabolism to a minimum when faced with flooding (Akman et al., 2012, Sasidharan et al., 2013). This way it endures rather than escapes the stress. Perhaps by reducing its metabolism it also reduces the sap stream and amount of mobile nutrients reducing its quality as a host for aphids. However, further research into the phloem composition is necessary to confirm this. On the floodplain-inhabiting plant species *R. palustris*, both aphid species, and the caterpillar *P. brassicae* generally performed well regardless of water treatment. This could also be explained by the plant's adaptations. *Rorippa palustris* has a much shorter life cycle than the other two species. *Rorippa palustris* plants were already flowering and setting seeds weeks before the other two *Rorippa* plant species. Perhaps this takes resources away from defense to reallocate them for reproduction allowing herbivores to perform better. This strategy, named "reproductive escape", is already observed in other plant species (Lucas-Barbosa et al., 2013, Lind et al., 2013). By investing in reproduction under stress, the plant can escape the stress by completing its life cycle before stresses get too severe.

Our study contributes to the understanding of the effects of abiotic stress on plant-insect interactions by showing that plant evolutionary differences correspond with their capacity to deal with variation in combinations of water stress and herbivore attack. Our study thereby suggests that the two major hypotheses predicting herbivore performance on water stressed plants, i.e., the "plant stress hypothesis" and "plant vigor hypothesis", may align with the context of plant evolutionary variation due to water availability in their habitat. Comparing closely related plant species that differ in adaptation to abiotic conditions for their plasticity in responses to combinations of abiotic and biotic stress, provides a strong tool to further unravel how plants adapt to managing stress combinations. To draw strong conclusions on how to generalize these relationships, broader sampling across plant families as well as more in-depth phylogenetic comparisons for the role of plant adaptations will be imperative. At the same time, we should understand how the ecological context of plant interactions with insects is altered by the water availability in the environment. Soil water content available to plants not only affects the oviposition preference of herbivores (Helmberger et al. 2016; Showler and Castro 2010), but also the recruitment of natural enemies that reduce the impact of herbivore attack on plants (Kansman et al. 2021; Martini and Stelinski 2017; Salerno et al. 2017; Weldegergis et al. 2015). Water availability may thus have profound effects on the insect community assembly on plants. Results of water regime on plant-insect interactions under controlled greenhouse conditions should therefore be further explored with field experiments to understand how these interactions are shaped under more natural conditions. We report on the effects of watering regime on plant-insect interaction in the field for the *Rorippa* plant species studied here in forthcoming publications. Moreover, unravelling the plant physiological responses to water availability is a key aspect of understanding insect herbivore responses to plant water stress and the consequences for insect ecology (Ben Rejeb et al. 2014). Understanding how different plant species have evolved to simultaneously respond to abiotic and biotic stress will aid us in predicting how the ecological context matches physiological adaptations to manage multi-stress situations. These insights will provide important guidelines for plant breeding to develop crops that are resilient to more extreme and frequent combinations of abiotic stress and insect herbivore attack in agroecosystems under predicted climate change.

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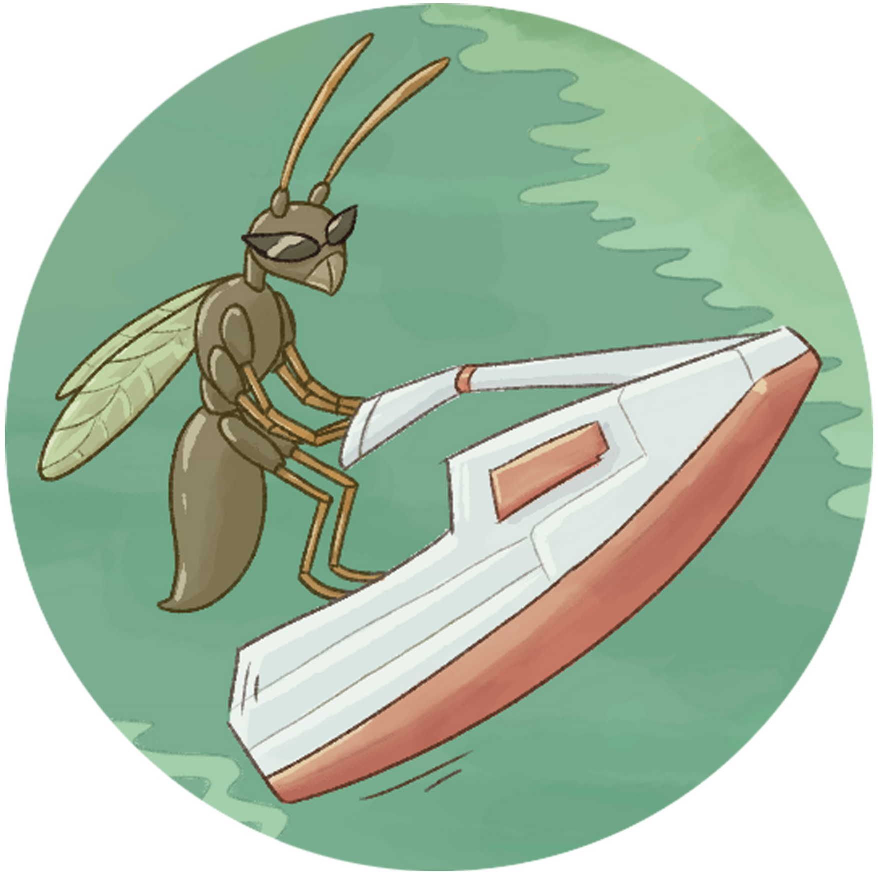

Chapter 4

Water availability affects parasitoid recruitment and plant volatile profile of three *Rorippa* plant species

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Abstract

Plants possess several defense mechanisms against insect herbivore attacks, including attracting the natural enemies of herbivores by emitting volatile organic compounds (VOCs). This indirect defense system might, however, be affected by sub-optimal water conditions like drought and waterlogging that could alter the plant's VOC emission.

We examined how drought and waterlogging affect parasitoid attraction and VOC profiles in three closely related *Rorippa* plant species that naturally occur in diverse water conditions, from terrestrial to semi-aquatic habitats. Water availability significantly influenced parasitoid recruitment. *Aphidius ervi* parasitoids showed no preference between VOCs from drought or waterlogged, herbivore-stressed *R. amphibia* or *R. sylvestris* plants. However, drought-treated *R. palustris* attracted fewer *A. ervi* parasitoids compared to well-watered conspecifics. *Diadegma semiclausum* parasitoids did not prefer caterpillar-induced VOCs over those from drought-treated or waterlogged *R. amphibia* plants. Waterlogging made caterpillar-induced *R. sylvestris* plants more attractive to *D. semiclausum*, while *R. palustris* plants were less attractive under waterlogging compared to well-watered caterpillar-induced plants. VOC profiles were primarily affected by water availability and secondarily by herbivore induction. Changes in VOC profiles due to water availability overlapped with those induced by herbivory, potentially explaining parasitoids' indiscriminate response to herbivore damage or VOCs induced by water conditions. Our study shows that drought and waterlogging can alter plant defenses against herbivores by modifying their attractiveness to parasitoids through changes in VOC profiles. The specific effects depend on the plant and herbivore species studied, providing insights into herbivore suppression in a changing climate with more frequent drought and waterlogging events.

Introduction

A vital means for plants to defend themselves against insect herbivores is to attract natural enemies of the herbivores (Abdala-Roberts et al. 2019; Turlings and Erb 2018). When a plant is attacked by a herbivore, it emits volatile organic compounds (VOCs) that provide natural enemies with reliable and detectable information on the presence of their prey or host (Dicke and Baldwin 2010; Douma et al. 2019). Natural enemies like parasitoids often have a limited range of hosts they can parasitize, comprised of one or a few closely related species (Rossinelli and Bacher 2015). Consequently, the reliability of plant volatile cues is dependent on the information they contain about the specific insect herbivore species attacking the plant in order to attract the parasitoid that uses the herbivore as its host (De Moraes et al. 1998; Dicke 1999). This information is often embodied by the composition of the VOC blend emitted rather than by specific compounds (Arimura et al. 2009; Bernasconi Ockroy et al. 2001). Plants achieve this specificity by perceiving and recognizing the type of damage they are receiving. This starts by perceiving the general damage pattern the herbivore inflicts but is fine-tuned by recognizing elicitors that are present in e.g., the saliva of a specific herbivore species (Hall et al. 2004; Huffaker et al. 2013; Roda et al. 2004). Upon recognition, these elicitors activate a signaling cascade inducing, among others, the phytohormones jasmonic acid (JA) and salicylic acid (SA), which govern the induced release of a specific VOC pattern (Bodenhausen and Reymond 2007; Kessler et al. 2004; Wu and Baldwin 2009).

However, the volatile profile of a plant is shaped by more than just insect herbivory. Abiotic conditions are known to affect the amount and composition of plant volatiles (Jardine et al. 2015; Lou and Baldwin 2004; Takabayashi et al. 1994; Vázquez-González et al. 2022; Vivaldo et al. 2017; Zhou et al. 2018). As such, the indirect defense system against insect herbivores may be affected by variations in abiotic conditions such as water availability e.g., waterlogging and drought (Copolovici et al. 2014; Kansman et al. 2021; Martini and Stelinski 2017; Salerno et al. 2017; Weldegergis et al. 2015). One way water conditions can influence VOC emission is through the closure of stomata. Stomata allow gas exchange between the plant and the outside world. They also allow water to evaporate out of the plant. Thus, to reduce water loss in sub-optimal water conditions, plants tend to close their stomata, reducing gas exchange in the process (Muhammad 2012; Murtaza et al. 2016; Parent et al. 2008; Xu et al. 2010). This can limit the amount of VOCs released by the plant to attract parasitoids. Furthermore, the same phytohormones involved in the regulation of defense after herbivore attack also act in the response of plants to water-stress (Kessler et al. 2004; Nguyen et al. 2016a; Nguyen et al. 2016b; Per et al. 2018; Sasidharan and Voesenek 2015; Ullah et al. 2018; Wahab et al. 2022; Wu and Baldwin 2009). This overlap and crosstalk between phytohormonal signals could cause drought and waterlogging to affect VOC

production, and consequently parasitoid attraction. Because phloem-feeding herbivores often elicit plant responses dominated by SA and leaf-chewing herbivores induce responses dominated by JA (Bodenhausen and Reymond 2007; Li et al. 2016), abiotic conditions may have a different impact on recruitment of parasitoids associated with aphids or caterpillars. Moreover, plant species may differ in their adaptations to water conditions and respond differently to insect herbivory and drought or waterlogging (Kamps and Poelman 2023).

Therefore, we explored how three closely related *Rorippa* plant species that grow in different habitats over a water gradient are affected in their recruitment of parasitoids under drought and waterlogging conditions. The three species include the semi-aquatic *Rorippa amphibia*, the flood plain residing *Rorippa palustris* and the terrestrial species *Rorippa sylvestris*.

We examined the effect of two herbivores belonging to two different feeding guilds: 1) the sap-feeding aphid *Myzus persicae* and its parasitoid *Aphidius ervi* and 2) the leaf chewing caterpillar *Plutella xylostella* and its parasitoid *Diadegma semiclausum*. We hypothesized that the response to the aphid *M. persicae* will be especially affected by drought and waterlogging due to the discrepancy and crosstalk in phytohormonal signaling between water stress and aphid feeding (Kamps and Poelman 2023; Kansman et al. 2021; Per et al. 2018). Since the phytohormonal signaling to caterpillar damage and water stress is more similar, we expect the caterpillar-induced response to be less affected by water stress (Ben Rejeb et al. 2014; Kamps and Poelman 2023; Per et al. 2018; Weldegergis et al. 2015). We analyzed the VOC profile of the three plant species to illustrate how the combination of water availability and herbivory affect VOC production. We discuss the importance of considering plant adaptations as well as herbivore species that attack the plant in unravelling plant responses to simultaneous biotic and abiotic stress.

Materials and Methods

Plants

We collected rhizomes from the semi-aquatic *Rorippa amphibia* and seeds from flood plain residing *Rorippa palustris* and terrestrial *Rorippa sylvestris* around Wageningen, the Netherlands (51°57'38.2"N 5°39'41.9"E). The rhizomes were planted, and seeds were sown in trays containing Arabidopsis potting soil (Lentse Potgrond B.V.). These trays were watered daily and kept under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). Two weeks after planting the rhizomes and five weeks after sowing the seeds, we transplanted plantlets into pots (ø 12 cm, 1 Liter) with a 1:1 mixture of Arabidopsis potting soil and sand. We allowed plants to grow and acclimatize for one week in the pots before being subjected to further experiments.

Insects

We used two herbivore species with their associated parasitoid: 1) *Myzus persicae* and the parasitoid *Aphidius ervi*. The *M. persicae* aphids were multiplied on *Raphanus sativus* (radish) plants under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). *Aphidius ervi* parasitoids were ordered from Koppert Biological Systems B.V. in the form of aphid mummies. These were allowed to egress from the mummies under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D) and supplied with honey. 2) *Plutella xylostella* and its parasitoid *Diadegma semiclausum*. The *P. xylostella* caterpillars were reared on *Brassica oleracea* (Brussels sprouts) plants under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). While the *D. semiclausum* parasitoids were reared on *P. xylostella* caterpillars under similar greenhouse conditions and the adult wasps were supplied with honey. Three-to-seven-day old naive female wasps were used for experiments to ensure that they had had ample time to mate.

Plant preparation

After acclimatizing for one week in the pot, plants were assigned to one of six treatment groups. Plants were subjected to one of three water regimes (drought, well-watered, and waterlogged) and were either induced with insect herbivore feeding (aphids or caterpillars) or left undamaged. The three water treatments: drought, well-watered and waterlogged were administered for the entirety of the experiment (Fig. 1). These treatments represented a soil water content of 8±4%, 20±5% and +100%, respectively. Soil water content was sustained by measuring each pot daily with an electronic water potential meter (Extech MO750) and adding water where needed. To reach the water level required for waterlogging, all pots were placed in buckets (ø 21 cm, 2.75 Liter). For the waterlogging treatment, these buckets were filled with water up to the soil line (Fig. 1) (Kamps and Poelman 2023).

To study host location by the aphid parasitoid *A. ervi,* plants were induced with 10 adult wingless *M. persicae* aphids seven days after the onset of the water treatment or left undamaged (Fig. 1) (Li et al. 2017). The aphids were then allowed to feed and reproduce for one week before the plants were used for further experiments. To study host location by the caterpillar parasitoid *D. semiclausum,* plants were induced with 10 L1 *P. xylostella* caterpillars 12 days into the water stress treatment or were left undamaged. Caterpillars were allowed to feed for 48 hours before the plants were used in experiments (Fig. 1) (Weldegergis et al. 2015).

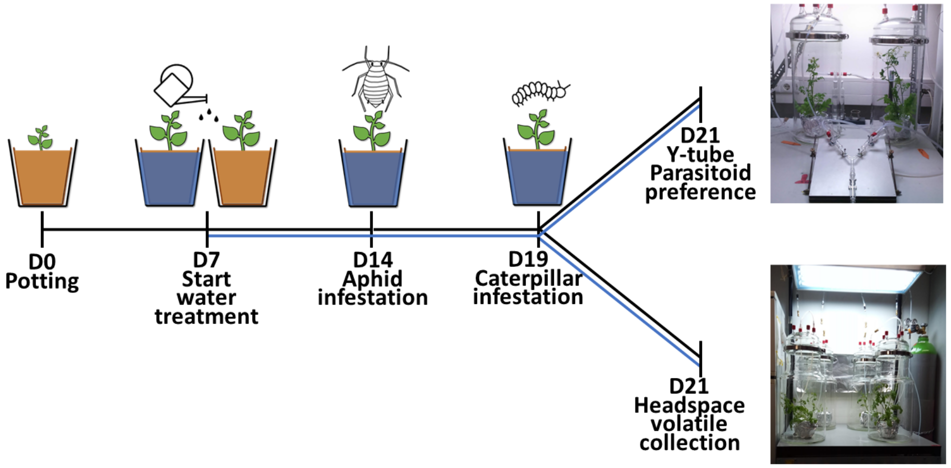

Figure 1. Workflow of plant preparation. On day 0 plantlets from all three plant species were potted (*R. amphibia, R. pallustris, R. sylvestris*). pots were placed in buckets to retain all water from the water treatments. Day 7 marks the start of the three different water treatments (drought, well-watered, waterlogged). This water treatment was maintained for the entire experiment. On day 14, a subset of plants was induced with 10 adult wingless *M. persicae* aphids. On day 19, a subset of plants was induced with 10 L1 *P. xylostella* caterpillars. On day 21, plants were used to assess parasitoid preference in the y-tube, or to collect headspace volatiles.

Parasitoid preference

To test the relative attractiveness of the plant treatments to parasitoids, we tested plants that received different treatments pairwise in a Y-tube olfactometer bioassay (Fig. 1). We investigated whether water availability affected parasitoid recruitment by offering parasitoids a choice between undamaged and herbivore-damaged plants grown in similar water conditions (drought, well-watered, waterlogged). In addition, we studied how water conditions affected relative parasitoid recruitment to herbivore-damaged plants by offering a choice between herbivore-damaged plants that differed in water conditions (Table 1). Shortly before plants were placed in the olfactometer, insect herbivores were removed from the plants. Next, the pots containing the plants were wrapped in aluminium foil to limit the release of volatiles from the plastic pots and the soil. Plants were then placed in one of two airtight glass jars that were closed off, to allow compressed air that passed through a charcoal filter into the system at 200 mL min-1 (Fig. 1). The air was led through the jars carrying plant odors into the Y-tube. Plants were left to acclimatize for 10 minutes before testing commenced. To test parasitoid preference, we released female wasps individually in the bottom end of the Y-tube and recorded their choice when the wasp stayed in one arm of the olfactometer for 10 seconds. Eight to ten wasps were individually tested for each plant pair. After four to five wasps were tested, the odor sources were swapped to control for a positional bias. In total, 70 to 80 wasps were tested per treatment over the course of seven to ten days. A total of six treatment pairs were tested every day in a randomized order to reduce the effect of the time of the day. Every combination of plant species and herbivore-parasitoid group was tested in a separate experiment.

Table 1. Pairs of treatments tested in the y-tube for each combination of plant species and insect herbivore

Treatment 1		Treatment 2	Reasoning
Well-watered	vs	Well-watered + Herbivory	Positive control: Can the plant attract parasitoids in response to herbivore attack?
Well-watered + Herbivory	vs	Drought + Herbivory	Knock-on effects of drought on parasitoid attraction
Well-watered + Herbivory	vs	Waterlogged + Herbivory	Knock-on effects of waterlogging on parasitoid attraction
Drought	vs	Drought + Herbivory	Direct effects of drought on parasitoid attraction
Waterlogged	vs	Waterlogged + Herbivory	Direct effects of waterlogging on parasitoid attraction
Drought + Herbivory	vs	Waterlogged + Herbivory	Differential effects of drought or waterlogging on parasitoid attraction

Volatile collection and analysis

To assess the effect of different water conditions on plant volatiles, we collected the headspace composition of all three plant species after being subjected to one of three watering regimes and induced with insect herbivores or left undamaged (Fig. 1). We prepared new sets of plants similar to those tested on the Y-tube following the same protocol. Dynamic headspace sampling of volatiles was conducted in a climate-controlled room at 20 ± 1 °C. Volatiles were collected from 10 replicates per treatment. Shortly prior to volatile collection, insect herbivores were removed and waterlogged plants were allowed to drain to reduce moisture during volatile collection. The pots were carefully wrapped with aluminium foil to reduce the contribution from volatiles that were not plant derived. The plants were then individually placed into a 30 L glass jar connected to a closed air system. Synthetic air (Air Synthetic 4.0 Monitoring from Linde Gas, Schiedam, The Netherlands) was supplied at 230 mL min^{-1} to each jar. Plants were left for 30 minutes to acclimate before volatile collection began. Volatile collection started by drawing air out of the glass jars at a rate of 200 mL min^{-1} through a stainless-steel tube filled with 200 mg Tenax TA adsorbent (20/35 mesh; Markes, Llantrisant, UK) for two hours. To control for the contribution of non-plant derived volatiles such as from the collection set-up, the adsorbent material and the analytical system, volatiles from potted soil without a plant were collected at regular intervals during the scheme of volatile collection and used as background samples. The Tenax TA cartridges with volatile samples were dry-purged under a stream of helium or nitrogen (50 mL min^{-1}) for 15 minutes at room temperature (21±2 °C) to remove moisture from the samples.

The collected volatiles were thermally released from the Tenax TA adsorbent using an Ultra 50:50 thermal desorption unit (Markes, Llantrisant, Glamorgan, UK) at 250°C for 10 min under a helium flow of 20 mL min^{-1}, whilst simultaneously re-collecting the volatiles in a thermally cooled universal solvent trap: Unity (Markes, Llantrisant, Glamorgan, UK) at 0°C. Once the desorption process was completed, volatile compounds were released from the cold trap by ballistic heating at 40°C sec^{-1} to 280°C, which was then kept for 10 min, whilst all the volatiles were transferred to a 30 mL x 0.25 mm ID x 1mm F.T. ZB-5MS analytical column for *R. amphibia* samples and 30 mL x 0.25 mm ID x 1mm F.T. DB-5MS analytical column for *R. palustris* and *R. sylvestris* samples (Phenomenex, Torrance, CA, USA) and placed inside the oven of a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) or of a GC (Agilent Technologies) at a split ratio of 3:1 for further separation of the plant volatiles. The gas chromatograph (GC) oven temperature was initially held at 40°C for 2 min and was then raised at a rate of 6°C min^{-1} to a final temperature of 280°C, where it was kept for 5 min under a constant helium flow of 1-1.2 mL min^{-1}. For the detection of volatiles, a Thermo Trace DSQ quadrupole MS (Thermo Fisher Scientific) or Q-TOF MS (Agilent Technologies) coupled to the GC was operated in an electron impact ionization (EI) mode at 70 eV in a full scan with a mass range of 35–400 m z^{-1} at 4.70-5.00 scans sec^{-1}. The MS transfer line and ion source were set at 275 and 250°C or 280 and 230°C, respectively.

Automated baseline correction, peak selection (S/N > 3) and alignments of all extracted mass signals of the raw data were processed following an untargeted metabolomic workflow using MetAlign software, producing detailed information on the relative abundance of mass signals representing the available metabolites (Lommen 2009). This was followed by reconstructing the extracted mass features into potential compounds using the MSClust software through data reduction employing unsupervised clustering and extraction of putative metabolite mass spectra (Tikunov et al. 2012). Tentative identification of volatile metabolites was based on a comparison of the reconstructed mass spectra with those in the NIST 2014 and Wageningen Mass Spectral Database of Natural Products MS libraries, as well as experimentally obtained linear retention indices (LRIs).

Statistical analyses

Data from the parasitoid choice assay were analyzed using RStudio (Allaire 2012) under R 3.6.3 (R Core Team 2013), with packages *lme4* (Bates et al. 2015) *emmeans* (Lenth et al. 2019) and *ggplot2* (Wickham 2016). The parasitoid choice data was analyzed using a two-tailed Binomial test. The volatile emission data as peak heights were imported to SIMCA-P 17 statistical software (Umetrics, Umea, Sweden), followed by log-transformation, mean-centring, and unit-variance scaling before subjecting the data to multivariate data analysis. Supervised partial least squares-discriminant analyses (PLS-DA) and its extension orthogonal partial least squares-discriminant analyses (OPLS-DA), were used where appropriate as a tool to compare and correlate treatment groups. R2 and Q2 metrics, which describe the explained variation within the data set and the model's predictability, respectively, were calculated based on the averages of the sevenfold cross-validation. A CV-ANOVA analysis was done to confirm the model's reliability.

Results

Attraction of the aphid parasitoid *Aphidius ervi*

The aphid parasitoid *Aphidius ervi* significantly preferred VOCs emitted by well-watered aphid-induced plants over well-watered undamaged plants for all three plant species. This shows that all three plant species alter their VOC profile in response to herbivory by *Myzus persicae* and provide *A. ervi* parasitoids with information on host presence (Fig. 2). On plants of the semi-aquatic *R. amphibia* plants, *A. ervi* parasitoids show no preference for VOCs emitted by well-watered aphid-induced plants over drought-treated or waterlogged aphid-induced plants (P=1.000 and P=0.282 respectively). This indicates that water conditions did not change *R. amphibia's* ability to attract *A. ervi* parasitoids (Fig. 2A). However, the parasitoid had no preference for VOCs emitted by a drought-treated or waterlogged undamaged plant, over drought-treated or waterlogged aphid-induced plants (P=0.720 and P=0.712 respectively). This suggests that *A. ervi* was unable to perceive aphid infestation among plants that were water-stressed (Fig 2A).

On plants of the floodplain species *R. palustris*, *A. ervi* parasitoids preferred the VOCs of well-watered aphid-induced plants over drought-treated, aphid-induced plants (P=0.006). A similar preference for aphid-induced well-watered plants over aphid-induced waterlogged plants was also visible but not significant (P=0.072). This indicates that in contrast to *R. amphibia* plants, drought and waterlogging had a negative effect on *R. palustris'* ability to attract *A. ervi* parasitoids (Fig. 2B). This is further supported by the fact that drought-treated

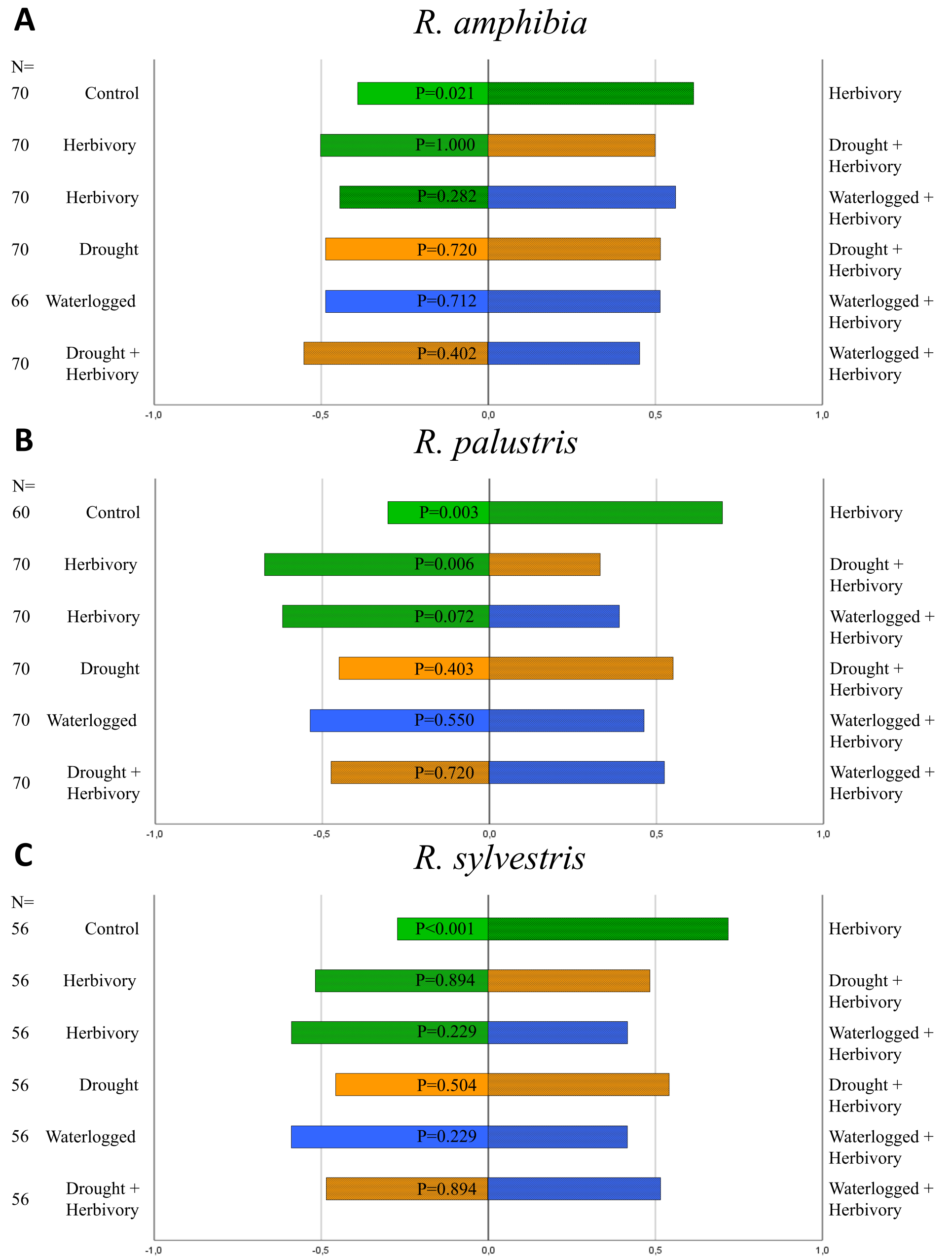

Figure 2. Choice of aphid parasitoid *A. ervi* in a Y-tube olfactometer to odours emitted by **A:** *R. amphibia* **B:** *R. palustris* **C:** *R. sylvestris* plants subjected to different water treatments and herbivory by *M. persicae.* Significance of discrepancy in the choice distribution of the parasitoids over the two differently treated plants calculated by two-tailed binomial tests.

or waterlogged, aphid-induced plants were not preferred by *A. ervi* over undamaged drought-treated or waterlogged plants (P=0.403 and P=0.550 respectively).

Finally, on plants of the terrestrial species *R. sylvestris*, *A. ervi* parasitoids had no preference for well-watered, aphid-induced plants over drought-treated or waterlogged, aphid-induced plants (P=0.894 and P=0.229, respectively). This indicates that in *R. sylvestris*, similar to *R. amphibia,* drought and waterlogging did not have a negative effect on the attraction of *A. ervi* parasitoids (Fig. 2C). Additionally, VOCs released by aphid-induced, drought-treated, and waterlogged plants were as attractive as undamaged drought-treated and waterlogged plants (P=0.504 and P=0.229, respectively). This shows that, similar to *R. amphibia*, parasitoids were not differentially attracted to stressed *R. sylvestris* plants, regardless of whether this stress originated from sub-optimal water conditions or herbivory by aphids (Fig. 2C).

Attraction of the caterpillar parasitoid *Diadegma semiclausum*

The caterpillar parasitoid *Diadegma semiclausum* was significantly attracted to VOCs emitted by well-watered plants induced by feeding of its host caterpillars over undamaged well-watered plants on all three plant species. All three plant species changed their VOC profile in response to herbivory by *Plutella xylostella* and attracted *D. semiclausum* parasitoids (Fig. 3).

On *R. amphibia* plants, similar to the aphid parasitoid *A. ervi,* the parasitoid *D. semiclausum* had no preference for well-watered herbivore induced plants over drought or waterlogged herbivore induced plants (P=1.000 and P=1.000, respectively). This indicates that water conditions did not affect parasitoid attraction in *R. amphibia* (Fig. 3A). Furthermore, *D. semiclausum* parasitoids did not prefer caterpillar-induced drought-treated or waterlogged plants over undamaged drought-treated or waterlogged plants (P=0.609 and P=0.155, respectively). This further characterizes that all stressed plants were attractive to *D. semiclausum* regardless of whether this stress originated from caterpillar herbivory, sub-optimal water levels, or a combination of both (Fig. 3A).

On *R. palustris* plants, *D. semiclausum* parasitoids showed no preference for well-watered caterpillar-induced plants over drought-treated caterpillar-induced plants (P=0.572). Furthermore, parasitoids showed no preference for drought-treated caterpillar-induced plants over drought-treated undamaged plants (P=0.720) (Fig. 3B). Waterlogged caterpillar induced plants were less attractive to *D. semiclausum* than well-watered caterpillar induced plants (P=0.031), indicating that waterlogging had a disruptive effect on the attraction of *D. semiclausum* (Fig. 3B). This is further supported by the fact that parasitoids preferred drought-treated caterpillar-induced *R. palustris* plants over waterlogged caterpillar-induced plants (P=0.017).

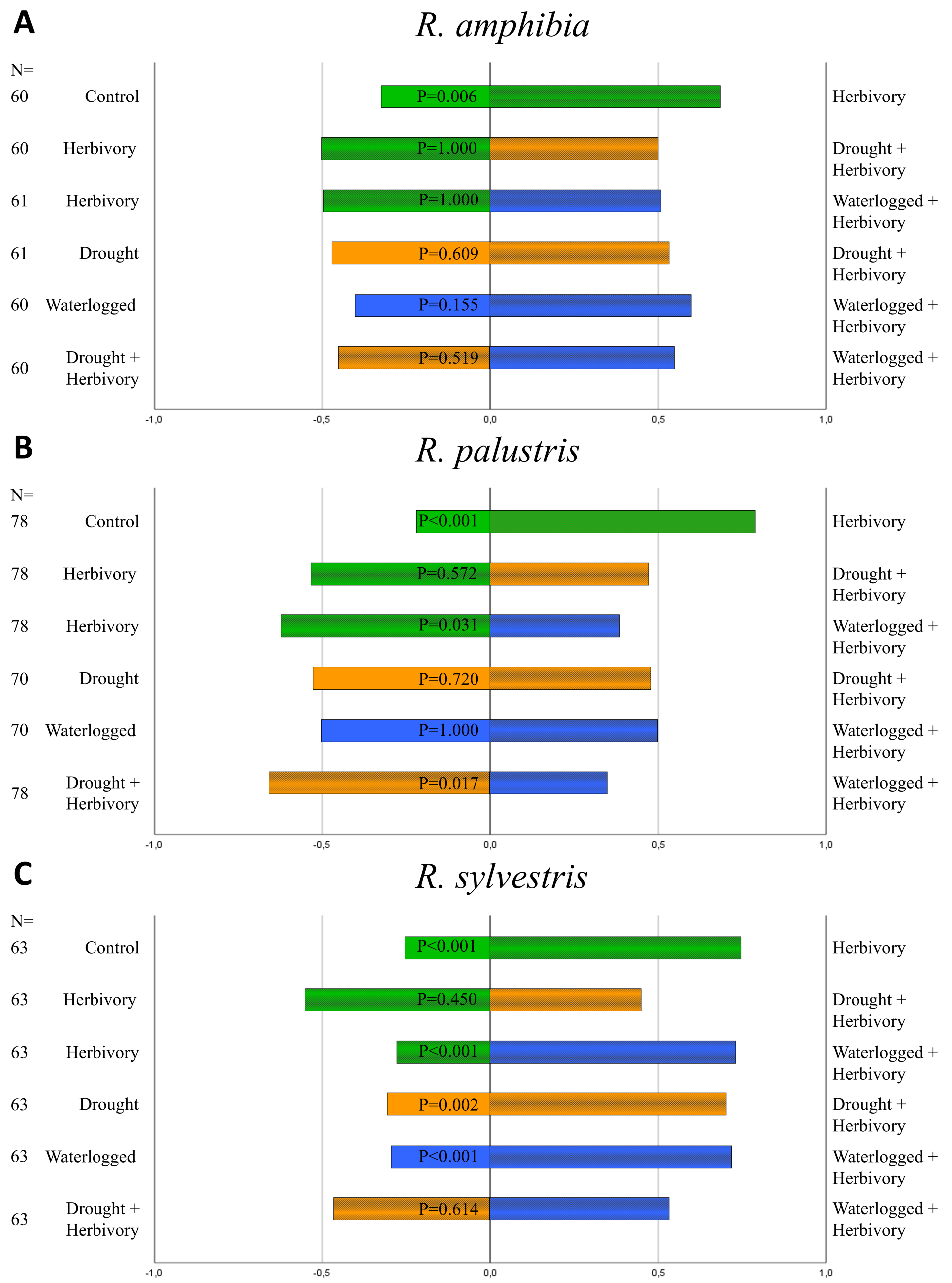

Figure 3. Choice of caterpillar parasitoid *D. semiclausum* in a Y-tube olfactometer to odours emitted by **A:** *R. amphibia* **B:** *R. palustris* **C:** *R. sylvestris* plants subjected to different water treatments and herbivory by P. *xylostella*. Significance of discrepancy in the choice distribution of the parasitoids over the two differently treated plants calculated by two-tailed binomial tests.

On *R. sylvestris* plants, *D. semiclausum* parasitoids showed no preference when offered drought-treated or well-watered caterpillar-induced plants (P=0.450). Furthermore, *D. semiclausum* parasitoids preferred volatiles from drought-treated caterpillar-induced plants over undamaged drought-treated plants (P=0.002). This shows that drought did not significantly affect the attraction of *D. semiclausum* parasitoids in *R. sylvestris* plants (Fig. 3C). In contrast, waterlogged caterpillar-induced plants were significantly more attractive than well-watered caterpillar-induced plants (P<0.001). This demonstrates that waterlogging had an additive attracting effect to *D. semiclausum* parasitoids (Fig. 3C). Waterlogged undamaged plants were less attractive than waterlogged caterpillar-induced plants (P<0.001).

Overall, these data show that drought and waterlogging can affect the attraction of parasitoids. However, the direction of the effect differs between plant species and the tri-trophic interaction of the herbivore, and the parasitoid species studied.

Changes in volatile profiles under different water conditions

All three plant species were predominantly affected in their overall VOC profile by drought conditions, while waterlogging had less pronounced effects. Similarly, feeding by *P. xylostella* caterpillars also affected the VOC profile of *R. amphibia* and *R. sylvestris*, while induced responses to feeding by *M. persicae* aphids were less distinct on all three plant species. Based on the low variation explained by the first two PLS-DA axes, the VOC profile of *R. palustris* was least affected by our treatments (Fig. 4).

The VOC profile of *R. amphibia* was affected by drought, separating the VOC profile from that of waterlogging but not significantly from that of well-watered plants (Supplementary Table 4A). Waterlogging significantly altered the VOC profile of *R. amphibia* compared to well-watered plants (Supplementary Table 4A). VOC profiles of aphid-induced plants of all three water treatments were never significantly distinguishable from their uninduced counterparts (Supplementary Table 4A). Caterpillar induction on the other hand did lead to significant changes in the VOC profile in well-watered and waterlogged plants but not in drought-treated plants (Supplementary Table 4A).

Volatile compounds cucumene, β-sesquiphellandrene, 3-butenenitrile, α-zingiberene, (*Z*)-3-hexen-1-ol and (*E*)-β-ocimene were mainly responsible for the difference between well-watered and drought stressed *R. amphibia* plants (Supplementary Table 1). While volatile compounds (*E*)-β-ocimene, myrcene, (*E*)-β-farnesene, β-sesquiphellandrene, cucumene, 2-methylpropanenitrile were responsible for the difference between well-watered and waterlogged *R. amphibia* plants. Caterpillar induction is characterized by changes in

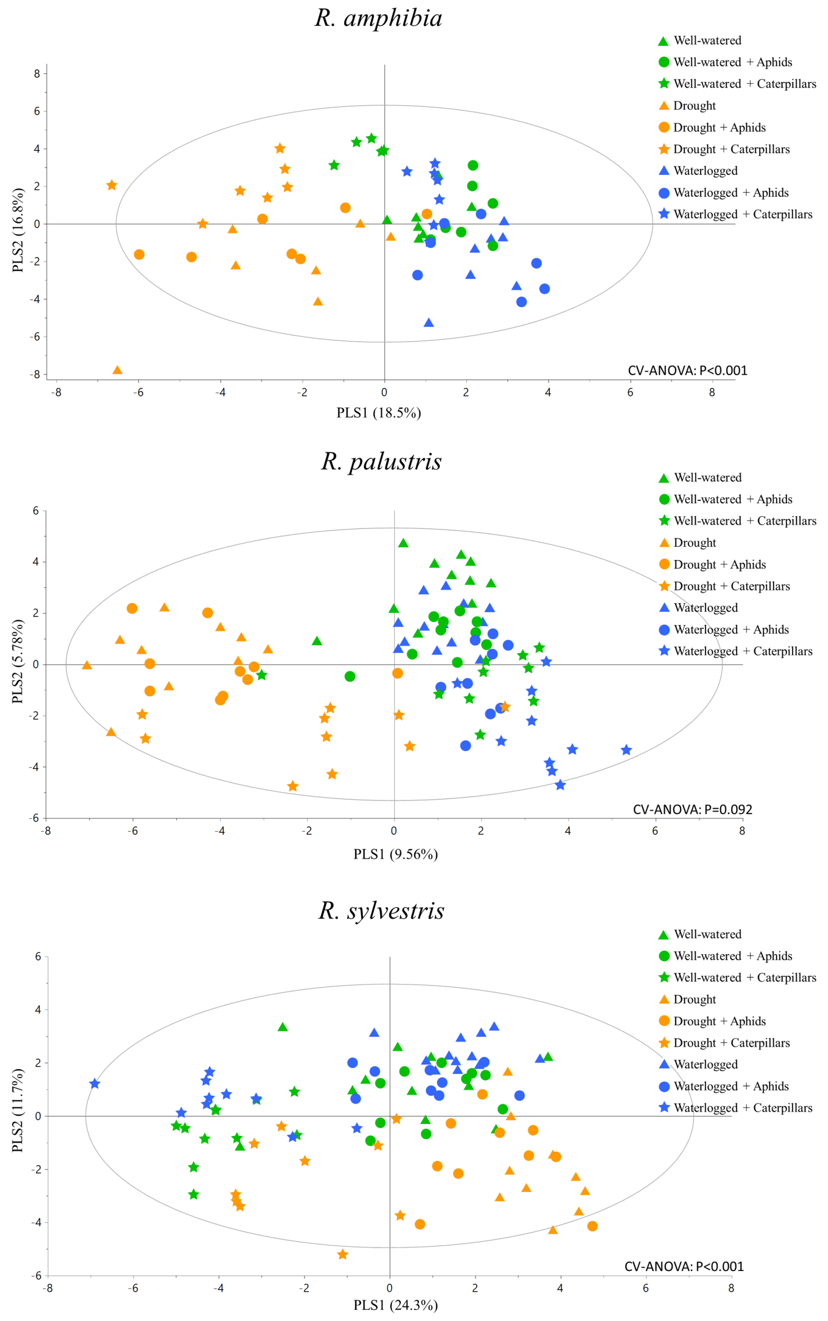

Figure 4. PLS-DA comparison of the effect of water treatment (green: well-watered, orange: drought treated, blue: waterlogged) and herbivory by aphid *Myzus persicae* (circles) or caterpillar *Plutella xylostella* (stars) on the volatile profile of three different *Rorippa* plant species.

volatile compounds (*E*)-4,8-Dimethyl-1,3,7-nonatriene ((*E*)-DMNT), 2-methylbutanenitrile, (*E*)-β-farnesene, 3-butenenitrile, 2-butenenitrile, (*E*)-β-ocimene, 2-methylpropanenitrile, 3-methyl-3-butenenitrile (Supplementary table 1). Since the concentrations of (*E*)-β-ocimene, 3-butenenitrile, (*E*)-β-farnesene and 2-methylpropanenitrile were similarly affected by water stress and herbivory, they could therefore be part of a general stress signal affecting parasitoid recruitment.

The VOC profile of *R. palustris* was strongly affected by drought, separating from waterlogging and well-watered plants in multivariate analyses (Supplementary Table 4B). Waterlogging did not significantly alter the VOC profile of *R. palustris* compared to well-watered plants. Like in *R. amphibia* plants, VOC profiles of aphid or caterpillar-induced plants of all three water treatments were never significantly distinguishable from their uninduced counterparts (Supplementary Table 4B). Similar to *R. amphibia* this did not lead to a difference in attraction between waterlogged induced and undamaged *R. palustris* plants.

Volatile compounds cadina-3,9-diene, α-neocallitropsenem, methyl thiocyanate, terpinolene, β-pinene, γ-terpinene, cyclobazzanene, β-patchoulene, acoradiene, (*E*)-DMNT, β-chamigrene, α-bulnesene and (*E*)-β-ocimene were mainly responsible for the difference between well-watered and drought-stressed *R. palustris* plants (Supplementary Table 2). While volatile compounds (*E*)-DMNT, (*E*)-β-ocimene, linalool, p-mentha-1,3,8-triene, (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene ((*E*,*E*)-TMTT), methyl salicylate, β-patchoulene, isomenthol, 4-acetyl-α-cedrene, isomenthone, 3-pentanone, (*E*)-α-bergamotene, β-acoradiene, α-cuprenene and α-barbatene concentrations varied most between undamaged and caterpillar induced *R. palustris* plants (Supplementary Table 2). Similar to *R. amphibia,* certain compounds like (*E*)-β-ocimene, (*E*)-DMNT and β-patchoulene, that were affected by drought overlap with those affected by caterpillar damage.

The VOC profile of *R. sylvestris* was strongly affected by drought and waterlogging, separating them from well-watered plants (Supplementary Table 4C). Additionally, the VOC profile from *R. sylvestris* changed in different directions for both waterlogging and drought making them significantly different from each other (Supplementary Table 4C). Like the other two plant species, aphid induction did not lead to a significant change in VOC profile of *R. sylvestris* regardless of the prior water treatment (Supplementary Table 4C). Caterpillar induction however led to a significantly different VOC profile regardless of prior water treatment (Supplementary Table 4C).

Volatile compounds 3,3,5-trimethyl cyclohexanol, cucumene, benzyl cyanide, 3-(2-methylpropyl) cyclohexene, 2-methylbutanenitrle, isocurcumenol, (*E*)-β-ocimene, (7a-isopropenyl-4,5-dimethyloctahydroinden-4-yl) methanol were mainly responsible for the difference between well-watered and drought-treated *R. sylvestris* plants (Supplementary Table 3). Volatile compounds 3-(2-methylpropyl) cyclohexene, 1-octen-3-ol, (*Z*)-3-hexen-1-ol acetate, (*Z*)-3-hexen-1-ol butanoate and methyl salicylate were mainly responsible for the difference between well-watered and waterlogged *R. sylvestris* plants (Supplementary Table 3). Caterpillars on the other hand induced the production of (*E*)-β-farnesene, (*E*)-β-ocimene (*E*,*E*)-α-farnesene, (*E*)-DMNT, (*E*)-β-bergamotene, (*Z*)-β-ocimene, (*Z*)-2-penten-1-ol acetate, indole, (*E*,*E*)-TMTT (Supplementary Table 3). Similar to the other two plant species there is an overlap in the compounds that are enhanced between herbivory and water conditions. In *R. sylvestris* this overlap is mainly attributed to (*E*)-β-ocimene and (*Z*)-2-penten-1-ol acetate.

Discussion

Water availability interacts with herbivore-induced plant responses and directly affects the VOC profile of all three *Rorippa* plant species. Our data demonstrate that in various situations, parasitoids were attracted to stressed plants regardless of whether this stress originated from herbivory, sub-optimal water conditions, or a combination of both. However, exceptions to this trend were also found in specific combinations of plant species and water treatments. This shows that the effect of water stress on indirect defenses can be plant species specific and vary greatly among closely related plant species. Analyzing the VOC profile of these plants confirmed our hypothesis that water availability had a significant effect on the blend of VOCs produced by all three plant species. Furthermore, these changes often overlapped with the changes caused by herbivory. This makes it a plausible link as to why, in most cases, parasitoids showed no preference for induced plants over uninduced plants when the plant was waterlogged, or drought treated.

Interestingly, the semi-aquatic plant species *R. amphibia* was affected in the recruitment of parasitoids under waterlogging conditions to which it is well adapted (Akman et al. 2014). Under waterlogging and drought conditions both *A. ervi* and *D. semiclausum* parasitoids showed no preference between *R. amphibia* plants with or without herbivore hosts. In contrast, in a previous study waterlogging did not significantly affect the direct resistance of *R. amphibia* against *M. persicae* aphids (Kamps and Poelman 2023). This shows that abiotic conditions affect both direct and indirect plant defenses. Furthermore, even though a plant is adapted to certain environmental conditions, this may not predict how abiotic conditions affect indirect defenses. In contrast, our previous work suggests that plant adaptations to environmental conditions predict how abiotic conditions affect a plant’s direct defenses (Kamps and Poelman 2023).

Moreover, the quantities of volatile compounds (*E*)-β-farnesene, (*E*)-β-ocimene, 3-butenenitrile and 2-methylpropanenitrile were positively correlated with both drought-treated, waterlogged and caterpillar-induced *R. amphibia* plants. (*E*)-β-farnesene is a known attractant for natural enemies of herbivores in several studies (Acar et al. 2001; Francis et al. 2004; Zhu et al. 1999). (E)-β-Ocimene is a proven attractant to other insects and might thus also be attractive to parasitoids (Farré-Armengol et al. 2017; Pecetti et al. 2002). 3-Butenenitrile induces JA and SA signaling and can therefore also play a role in the crosstalk of responses to herbivory and water stress (Ting et al. 2020). These compounds could therefore attribute to the fact that both parasitoids tested showed no preference for herbivory-induced plants over undamaged waterlogged or drought-treated plants.

R. palustris plants attracted fewer *A. ervi* parasitoids when drought-treated or waterlogged. Additionally, *R. palustris* plants were hampered in the attraction of caterpillar parasitoid *D. semiclausum* under waterlogged conditions. This may be explained by the fact that *R. palustris* is a fast-cycling plant species compared to the terrestrial *R. sylvestris* and semi-aquatic *R. amphibia*. Hence, *R. palustris* invests many resources in reproduction, perhaps at the cost of a response to insect herbivory. The plant might thus invest in evading the stress through a “reproductive escape” (Lucas-Barbosa et al. 2013).

In general, the VOC profile of *R. palustris* was less affected by water stress or herbivory compared to the other two plant species studied. The biggest differences could be attributed to drought and overlapped with changes caused by caterpillar damage, which may overshadow the changes caused by herbivory. This might hamper the parasitoid in distinguishing drought-treated plants with or without herbivores. Other studies have also found that drought can hamper a parasitoid’s ability to locate a host as it causes VOC changes comparable to insect herbivory (Martini and Stelinski 2017; Salerno et al. 2017). Interestingly, waterlogging had no significant effect on the VOC profile of *R. palustris* which could explain why *D. semiclausum* parasitoids still prefer waterlogged caterpillar-induced plants over waterlogged undamaged plants.

Remarkably, the terrestrial plant species *R. sylvestris* was even more attractive to the caterpillar parasitoid *D. semiclausum* when waterlogged than when well-watered, indicating a synergistic effect of waterlogging and herbivory. This finding is surprising because *R. sylvestris* generally grows in a drier environment than the other two species. Additionally, it has a quiescence strategy under flooding conditions, during which metabolism slows down to a minimum while the plant waits for the water to retreat (Akman et al. 2012; Akman et al. 2014; Sasidharan et al. 2013). We therefore expected that waterlogged *R. sylvestris* plants would be compromised in their ability to respond to herbivory while the opposite was found.

Indeed, VOC data on *R. sylvestris* show that waterlogging and caterpillar damage change the VOC profile in a similar direction. This indicates that overlap in stress responses can lead to an additive effect in the VOC production. Additionally, a previous study showed that direct resistance of *R. sylvestris* against *M. persicae* aphids was also increased under waterlogging conditions (Kamps and Poelman 2023). This further illustrates that there can be a synergy in responses to abiotic and biotic stresses where a plant can intensify its response against one stress while being subjected to another.

Comparing all three plant species shows that in most cases, parasitoids showed no preference between waterlogged, or drought stressed plants with or without host-herbivore. Plants use an interplay between different phytohormones like SA and JA to fine-tune their response to both herbivory and water stress (Bodenhausen and Reymond 2007; de Ollas et al. 2013; Pan et al. 2021; van Poecke and Dicke 2002). This overlap in signaling responses to water stress and herbivory is a potential way in which water stress affects a plant's recruitment of parasitoids. Ultimately, specific adaptations of the plant to water stress determine when and how a plant responds to changes in water availability (Arbona et al. 2010; Urano et al. 2017). As is evident from this study, these species-specific responses can have consequences for how indirect defenses are affected by water availability. For example, upon attack by caterpillars, plants respond with an increase in JA which is crucial for the defense response (Bodenhausen and Reymond 2007), while to respond to waterlogging, JA is associated with reduced growth and metabolism typical for the quiescence strategy known from *R. sylvestris* plants (Akman et al. 2014; Kamal and Komatsu 2016; Pan et al. 2021). Because of this overlap in JA signaling, the stress response of *R. sylvestris* to waterlogging might have an additive effect to the response to caterpillar damage by *P. xylostella,* which increases the attraction of *D. semiclausum* parasitoids by waterlogged plants. Whether this additive effect aids or hampers the parasitoid in distinguishing host-induced from undamaged plants, however, depends on the plant and herbivore-parasitoid combination considered.

Furthermore, drought generally had a bigger overall effect on VOC profile than herbivory in all three plant species. Similarly, other studies have found that drought can hamper a parasitoid's ability to locate a host as it causes VOC changes comparable to insect herbivory (Martini and Stelinski 2017; Salerno et al. 2017). In contrast, more parasitoid activity was measured on mildly drought-stressed *Triticum aestivum* plants than on well-watered plants (Kansman et al. 2021). Yet another study with *Brassica oleracea* measured no effect of drought on parasitoid attraction (Weldegergis et al. 2015). Waterlogging is less well studied but plants close their stomata to create a barrier to reduce radial oxygen loss as response to waterlogging (Muhammad 2012; Parent et al. 2008; Pedersen et al. 2021). These responses might produce a physical barrier for volatile compounds to leave the plant, which can hamper the attraction of parasitoids. Additionally, the overlap in stress responses between drought, waterlogging and herbivory might explain why parasitoids in this study showed no preference between waterlogged, drought-treated, or herbivore stressed plants (Bodenhausen and Reymond 2007; Pan et al. 2021; Weldegergis et al. 2015). This supports the notion that plant-specific responses to drought or waterlogging can have knock-on effects on the attraction of parasitoids. Unlike our initial hypothesis, drought and waterlogging rarely hinder parasitoid foraging by reducing the plant's herbivore-induced volatile emission, but it does do so through the overlap in VOCs induced by abiotic and biotic stress that hamper parasitoids in discriminating between plants with and without host herbivores.

Contrary to our hypothesis, both caterpillar- and aphid-induced responses were affected by water availability in a similar fashion. This shows that regardless of the different phytohormonal signals at the base of the response to each herbivore (JA for caterpillars and SA for aphids), water stress affects defense response to both herbivores. This is also in contrast with a previous study with *Rorippa* plants, where direct defenses against *P. xylostella* caterpillars were not affected while direct defenses against *M. persicae* were affected by water availability (Kamps and Poelman 2023). This is indicative of the fact that a plant has multiple lines of defense against herbivory which are regulated in their own unique way. Only by considering all these lines of defense can we fully understand the interactions between plants and herbivores.

Additionally, caterpillar induction generally led to a stronger change in VOC profile than aphid induction. This could be explained by the fact that aphids navigate their stylet in between plant cells with great care, generally causing relatively little damage and thus inducing a weaker damage-related response (Turlings et al. 1998). Some aphid species may have been able to suppress the plant response through compounds in their saliva (Wang et al. 2020; Will et al. 2007). In contrast, *P. xylostella* caterpillars are especially adapted to the defensive compounds of their host. They, therefore, do not need to suppress the response they induce, leading to a stronger induced response (Li et al. 2000; Li et al. 2016; Ratzka et al. 2002; Sun et al. 2019). A strong induced VOC profile change, however, did not necessarily lead to a strong attraction. This is exemplified by the fact that the VOC profile of drought-treated *R. sylvestris* plants with and without caterpillar feeding were significantly different but did not lead to the attraction of *D. semiclausum*. In contrast, well-watered *R. sylvestris* plants with and without aphids did not have a significantly different volatile profile but *A. ervi* parasitoids were significantly more attracted to the plants with aphids. Various studies have shown the immense sensitivity of parasitoids and their ability to pick up minute changes in volatile blends (Bukovinszky et al. 2005; Pareja et al. 2007). Slight changes in VOC profile might thus be picked up on while the overall variability of VOC profile as a whole is disregarded.

In conclusion, both drought and waterlogging can affect a plant's ability to indirectly defend itself against insect pests by attracting parasitoids. The exact effect, however, depends on the plant species and their adaptations to these stresses. Further research is needed to link specific plant adaptations to water availability with their response to simultaneous herbivory and water stress. By understanding these adaptations, we might better predict how water stress affects the responses to herbivory for different plant species. We show that volatiles released by plants under drought or waterlogged conditions can hamper the parasitoid's innate ability to find its host. This might have severe consequences for pest suppression under a changing climate in both natural and agricultural ecosystems. Future studies should, however, consider the strong learning capabilities of parasitoids (Dukas and Duan 2000; Giunti et al. 2015; Meiners et al. 2003). Parasitoids have the ability to learn associatively and might thus be able to distinguish drought or waterlogging from herbivory after an initial learning period.

Furthermore, we show that responses to drought or waterlogging can interact with responses to herbivory. Studying how plants mechanistically interweave responses to multiple types of stress will enhance our understanding of why there is such variation, even between closely related plant species, in how they react to combinations of stress. Additionally, to accurately predict parasitoid behavior, future studies might consider the complexity of the environment in which parasitoids need to find their host. In these complex systems, parasitoids are faced with multiple hosts and non-hosts on a field with different plant species under varying abiotic conditions (de Rijk et al. 2013; Gouinguené and Turlings 2002; Lou and Baldwin 2004). The current study adds to this complex framework by exploring the effects of drought and waterlogging on indirect defense in different plant species.

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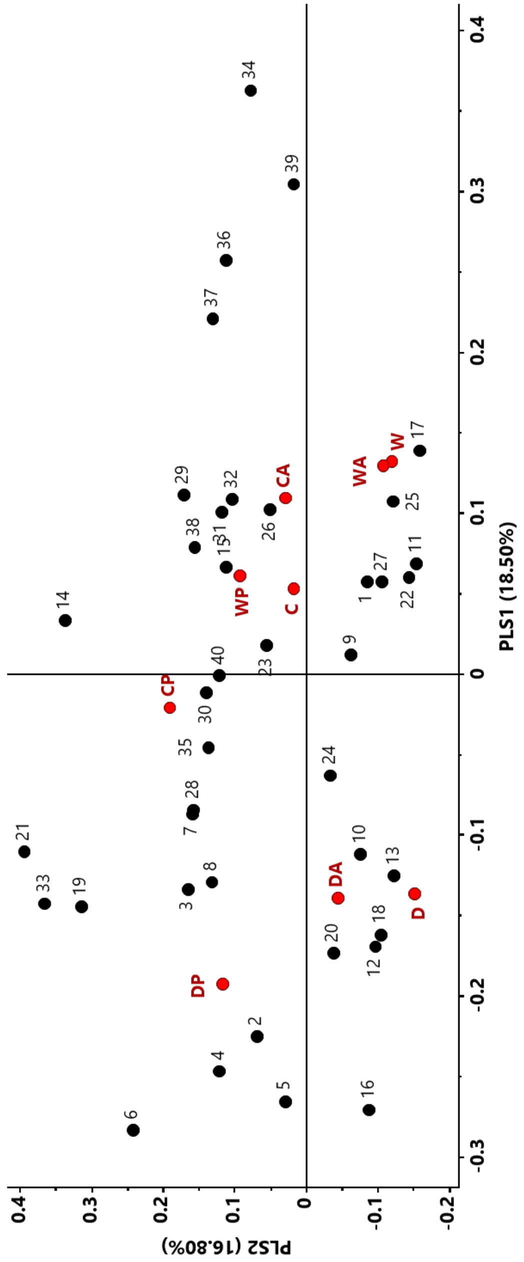
Supplementary Information



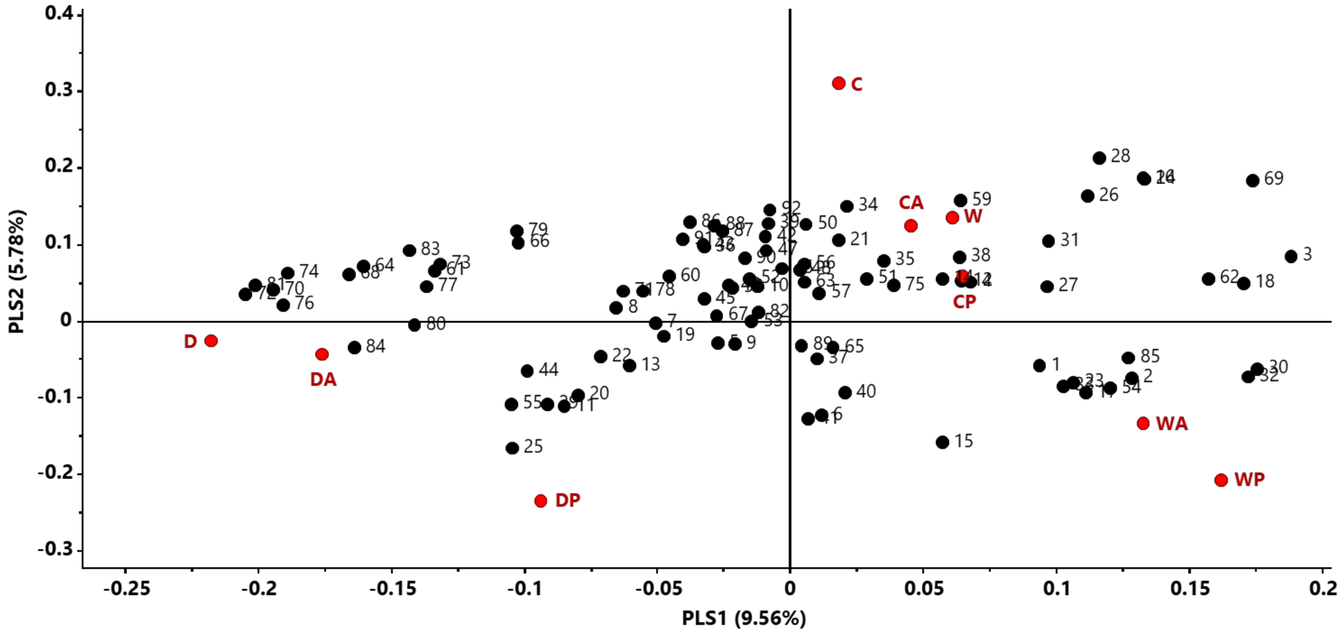
Supplementary Figure 1. Loading plot from PLS-DA of VOC data of *R. amphibia* plants. Numbers indicate specific compounds specified in, Supplementary Table 1. Red dots depict plant treatments as loading variables: C=well-watered undamaged, CA= well-watered + aphid induced, CP=well-watered+ caterpillar induced, D=drought, DA= drought + aphid induced, DP= drought + caterpillar induced, W=waterlogged, WA=waterlogged + aphid induced, WP=waterlogged + caterpillar induced.

Supplementary Table 1. Relative amount of VOCs (mean ± st. dev.) in the headspace composition of *R. amphibia* plants. Differences between treatments were calculated using a Kruskal Wallis test using a pairwise Dunn test with Bonferroni correction (P<0.05). VIP scores corresponding to plot presented in Figure 4A and Supplementary Figure 1.

ID	Compound	Class	Drought	Drought + Aphids	Drought + Caterpillars	Waterlogged	Waterlogged + Aphids	Waterlogged + Caterpillars	Well-watered	Well-watered + Aphids	Well-watered + Caterpillars	P-value K-W	VIP-score
1	(Z)-1,3-Pentadiene	Alkene	134240 ± 134096	120699 ± 150087	117645 ± 186858	613578 ± 1355295	101919 ± 111282	248547 ± 313068	88670 ± 82691	75023 ± 83377	54505 ± 54000	0.912	0.420
2	2-Methylpropanenitrile	Nitrogen and/or sulphur containing compounds	2361915 ± 3644541 a	1134809 ± 600727 a	1653433 ± 954608 a	366393 ± 106692 bc	307382 ± 80321 b	694541 ± 142123 abc	550158 ± 136567 abc	606835 ± 617492 abc	1124683 ± 646883 c	**<0.001**	1.074
3	2-Butenenitrile	Nitrogen and/or sulphur containing compounds	246752 ± 513186	88140 ± 123795	79231 ± 110982	485 ± 8	6599 ± 16196	80545 ± 88645	21706 ± 36582	9249 ± 23237	137640 ± 46572	0.074	0.883
4	3-Butenenitrile	Nitrogen and/or sulphur containing compounds	5849734 ± 10796472 ab	1576913 ± 1931353 ab	1989169 ± 1873285 a	738095 ± 1677054 b	123626 ± 126195 b	630222 ± 486661 ab	232288 ± 126924 ab	161570 ± 65243 b	1000416 ± 491703 ab	**<0.001**	1.218
5	Methyl thiocyanate	Nitrogen and/or sulphur containing compounds	1240733 ± 585753 a	1090751 ± 754791 ab	1822450 ± 1560711 a	462932 ± 328306 ab	355299 ± 123106 b	701219 ± 755142 ab	1622828 ± 3359604 ab	408158 ± 113878 ab	611432 ± 167885 ab	**<0.001**	1.251
6	2-Methylbutanenitrile	Nitrogen and/or sulphur containing compounds	21771568 ± 39294187 abc	7334197 ± 5156136 abc	19992600 ± 11596445 a	2035400 ± 495175 bd	1291048 ± 358095 d	7654688 ± 3315797 abc	2932589 ± 1034853 abcd	3700258 ± 4122790 bcd	16645649 ± 7123785 ac	**<0.001**	1.585
7	3-Methylbutanenitrile	Nitrogen and/or sulphur containing compounds	665101 ± 1028151 ab	389519 ± 244930 ab	630011 ± 336053 ab	424713 ± 330813 ab	200235 ± 107769 a	580819 ± 88265 b	491064 ± 162588 ab	393769 ± 164934 ab	646669 ± 232029 b	0.005	0.743
8	3-Methyl-3-butenenitrile	Nitrogen and/or sulphur containing compounds	395550 ± 521783 ab	225717 ± 326062 ab	392359 ± 343976 a	213568 ± 476518 ab	51912 ± 59643 ab	253165 ± 121068 ab	39449 ± 50948 b	42400 ± 23747 ab	308074 ± 49963 ab	**<0.001**	0.773
9	(Z)-3-Hexen-1-ol	Alcohol	15091876 ± 20493056	2104841 ± 2345265	4516909 ± 9581723	4756771 ± 4310373	3823299 ± 5352809	15572322 ± 31918231	2651391 ± 2394587	2959862 ± 1585676	1084151 ± 756902	0.125	0.262
10	Allyl isothiocyanate	Nitrogen and/or sulphur containing compounds	3020132 ± 2957311	4194296 ± 7651749	5120823 ± 7936307	1122213 ± 1343524	1757037 ± 1773205	1801844 ± 1207665	798191 ± 1190165	681914 ± 341318	623010 ± 296313	0.319	0.651
11	(Z)-2-Penten-1-ol, acetate	Ester	251388 ± 316319	51582 ± 53126	308971 ± 772495	4761361 ± 12199636	45193 ± 47606	564481 ± 1334171	45395 ± 42454	69474 ± 56534	25353 ± 27153	0.073	0.690
12	(E,E)-2,4-Hexadienal	Aldehyde	2132753 ± 4837753	1564785 ± 3413866	1543847 ± 2469233	1478359 ± 3910155	37312 ± 65948	155834 ± 295838	19302 ± 49839	16877 ± 43415	19286 ± 42069	0.086	0.947
13	(E)-4-Oxo-2-hexenal	Aldehyde	6332394 ± 7448944	2064341 ± 2491350	3059369 ± 4260969	527473 ± 606166	781494 ± 943029	1478119 ± 2372105	1137575 ± 2836933	937513 ± 743643	333949 ± 745656	0.143	0.837
14	Myrcene	Monoterpene	389858 ± 116433 a	503536 ± 171180 ab	833976 ± 363986 abc	536101 ± 153355 abc	460321 ± 198622 ab	727527 ± 214854 abc	902090 ± 528120 abc	908780 ± 214869 bc	1220400 ± 223245 c	**<0.001**	1.448
15	6-Methyl-5-hepten-2-ol	Alcohol	273681 ± 169462 a	944659 ± 676481 ab	566125 ± 273355 ab	639523 ± 411797 ab	1694526 ± 1308147 b	902668 ± 545101 ab	378665 ± 132351 a	1019991 ± 407943 b	741142 ± 266651 ab	**<0.001**	0.604
16	(E,E)-2,4-Heptadienal	Aldehyde	1567105 ± 3359098 ab	2983456 ± 6822094 ab	1499330 ± 2330435 a	479 ± 12 ab	32927 ± 59844 ab	469 ± 16 ab	14978 ± 38378 ab	462 ± 11 b	492 ± 7 ab	**0.001**	1.382
17	(Z)-3-Hexen-1-ol, acetate	Ester	33244086 ± 41827248 a	6997306 ± 7967846 a	8431457 ± 15545591 a	71623567 ± 125965165 a	8055661 ± 4920885 a	57445140 ± 121886425 a	8351579 ± 4036309 a	15387013 ± 12943854 a	4577453 ± 3215153 a	**0.005**	0.874
18	Unknown	unknown	205055 ± 306177	107128 ± 113948	179919 ± 244163	237525 ± 531859	5695 ± 13786	11138 ± 28227	79390 ± 110496	24248 ± 62914	6139 ± 12669	0.097	0.934
19	(E)-beta-Ocimene	Monoterpene	127345 ± 95829 abc	146106 ± 86531 abc	611609 ± 580788 ad	59553 ± 41806 bc	41219 ± 46369 b	302645 ± 164148 abcd	447147 ± 461493 acd	309197 ± 112917 acd	956484 ± 383295 d	**<0.001**	1.415
20	Isophorone	Norisoprene	1083621 ± 2229628	922477 ± 1822712	1140268 ± 1919090	43171 ± 40882	92584 ± 110745	44496 ± 50030	47806 ± 107631	487 ± 7	42446 ± 41596	0.223	0.858

Supplementary Table 1 continued.

ID	Compound	Class	Drought	Drought + Aphids	Drought + Caterpillars	Waterlogged	Waterlogged + Aphids	Waterlogged + Caterpillars	Well-watered	Well-watered + Aphids	Well-watered + Caterpillars	P-value K-W	VIP-score
21	(E)-4,8-Dimethyl-1,3,7-nonatriene	Homoterpene	834410 ± 810763 a	3302886 ± 5312838 ab	73557996 ± 66498584 b	448829 ± 486641 a	638136 ± 576383 a	46889238 ± 32263659 b	1541724 ± 619968 ab	1702747 ± 909714 ab	110550801 ± 77651585 b	**<0.001**	1.682
22	(Z)-3-Hexen-1-ol, isobutyrate	Ester	1701581 ± 3117872	123358 ± 182082	233184 ± 615666	9450299 ± 23835523	92991 ± 147027	1824159 ± 4387253	225315 ± 468580	253504 ± 380287	29122 ± 64067	0.125	0.635
23	Benzyl nitrile	Nitrogen and/or sulphur containing compounds	163114 ± 156397	196601 ± 183180	261540 ± 294397	208547 ± 126766	125403 ± 64776	148172 ± 60110	85503 ± 40614	129480 ± 25170	119787 ± 26708	0.234	0.256
24	4-Oxoisophorone	Norisoprene	397968 ± 768761	310585 ± 637825	427110 ± 643090	78088 ± 80230	99237 ± 106909	40867 ± 51265	61725 ± 80284	44011 ± 60489	48814 ± 69280	0.713	0.345
25	(Z)-3-Hexen-1-ol, butanoate	Ester	2649934 ± 3343219 a	388479 ± 433879 a	673121 ± 1258054 a	7661198 ± 17828233 a	404874 ± 311126 a	5070075 ± 11584817 a	1056676 ± 1903251 a	713894 ± 424408 a	237267 ± 183427 a	**0.048**	0.675
26	(Z)-3-Hexen-1-ol, 2-methylbutanoate	Ester	2206304 ± 4629237	449701 ± 447890	203728 ± 148761	10004391 ± 24657044	491688 ± 448265	814694 ± 498003	878517 ± 1516045	1645115 ± 1722998	340250 ± 126841	0.093	0.553
27	(Z)-3-Hexen-1-ol, 3-methylbutanoate	Ester	3139830 ± 3104225	1340128 ± 1254272	2475215 ± 3725979	2884167 ± 5248315	864911 ± 1228564	1128971 ± 1628073	336384 ± 214387	606088 ± 421195	368881 ± 438994	0.14	0.493
28	Indole	Nitrogen and/or sulphur containing compounds	13645 ± 34804	91172 ± 108546	177216 ± 101611	748183 ± 1956213	35135 ± 47734	44291 ± 72042	198988 ± 502589	42557 ± 30714	160584 ± 192208	0.056	0.734
29	alpha-Cubebene	Sesquiterpene	134631 ± 71882 a	170343 ± 56988 a	164891 ± 33144 a	184425 ± 64426 a	177795 ± 42119 a	261078 ± 125576 a	246780 ± 49672 a	283319 ± 128205 a	276559 ± 79941 a	**0.012**	0.951
30	beta-Cubebene	Sesquiterpene	458979 ± 280059	377936 ± 192930	557082 ± 148513	396957 ± 159771	447008 ± 172685	451916 ± 127771	493443 ± 101085	478964 ± 196685	487565 ± 68039	0.605	0.582
31	gamma-Elemene	Sesquiterpene	305937 ± 197890	288413 ± 169650	297454 ± 114670	307018 ± 122721	344271 ± 92127	351249 ± 71241	408303 ± 97475	452348 ± 196931	357807 ± 59344	0.563	0.733
32	beta-Caryophyllene	Sesquiterpene	116635 ± 81159	102445 ± 54451	117343 ± 27174	133386 ± 30414	118604 ± 42644	155309 ± 18746	132396 ± 32428	151125 ± 52200	131265 ± 30123	0.342	0.717
33	(E)-beta-Farnesene	Sesquiterpene	3047907 ± 3070263 ab	3624208 ± 4377662 ab	10338412 ± 9774975 a	1316662 ± 1273032 b	1205467 ± 1145495 b	7982781 ± 4443203 a	3131016 ± 2154163 ab	2830583 ± 846820 ab	14401380 ± 4070452 a	**<0.001**	1.604
34	Cucumene	Sesquiterpene	620910 ± 296709 a	591611 ± 239252 a	652580 ± 247133 a	2474589 ± 674925 b	2030942 ± 495637 ab	2776017 ± 447853 b	1671638 ± 764564 ab	2116583 ± 667411 ab	1743952 ± 128916 ab	**<0.001**	1.794
35	(E)-beta-Ionone	Norisoprene	389315 ± 456093	787273 ± 1150503	638752 ± 427209	316939 ± 352995	325004 ± 324852	765505 ± 1198562	173494 ± 181351	322529 ± 455612	427945 ± 213169	0.403	0.591
36	alpha-Zingiberene	Sesquiterpene	731042 ± 505420 a	576570 ± 477895 a	1088228 ± 693508 ab	5249129 ± 3057147 c	4751078 ± 2850425 bc	6040006 ± 1572935 c	3133036 ± 1539008 abc	3734616 ± 1710839 abc	3217132 ± 171917 abc	**<0.001**	1.366
37	(E,E)-alpha-Farnesene	Sesquiterpene	16183 ± 41562 a	477 ± 12 a	68063 ± 125052 a	211776 ± 184746 ab	218043 ± 158773 ab	297446 ± 81792 b	90728 ± 118451 ab	134547 ± 136446 ab	293665 ± 96775 ab	**<0.001**	1.247
38	alpha-Cadinene	Sesquiterpene	398562 ± 191783	392175 ± 154672	436863 ± 85449	412064 ± 98520	419037 ± 114448	495836 ± 76918	468240 ± 128729	523185 ± 150789	476937 ± 49979	0.568	0.797
39	beta-Sesquiphellandrene	Sesquiterpene	285532 ± 143656 ab	233664 ± 122351 a	297667 ± 217724 ab	1583714 ± 757362 c	1371786 ± 678723 bc	1739862 ± 427259 c	902356 ± 457707 abc	1130619 ± 438144 abc	961674 ± 82311 abc	**<0.001**	1.456
40	Calamenene	Sesquiterpene	246040 ± 144549	240198 ± 86739	235449 ± 32919	202985 ± 29073	212472 ± 41265	255301 ± 56495	236372 ± 65399	276274 ± 81257	244493 ± 31058	0.613	0.513



Supplementary Figure 2. Loading plot from PLS-DA of VOC data of VOC data of *R. palustris* plants. Numbers indicate specific compounds specified in the table below. Red dots depict plant treatments as loading variables: C=well-watered undamaged, CA= well-watered + aphid induced, CP=well-watered+ caterpillar induced, D=drought, DA= drought + aphid induced, DP= drought + caterpillar induced, W=waterlogged, WA=waterlogged + aphid induced, WP=waterlogged + caterpillar induced.

Supplementary Table 2. Relative amount of VOCs (mean ± st. dev.) in the headspace composition of *R. palustris* plants. Differences between treatments were calculated using a Kruskal Wallis test using a pairwise Dunn test with Bonferroni correction (P<0.05). VIP scores corresponding to plot presented in Figure 4B and Supplementary Figure 2.

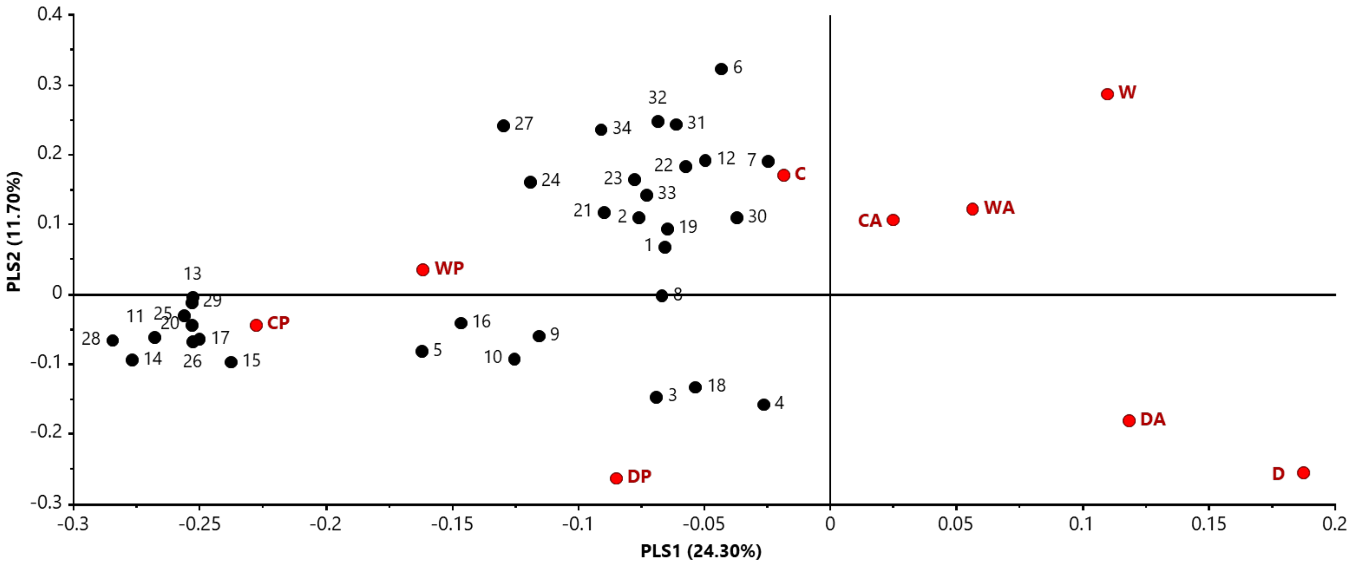
ID	Compound	Class	Drought	Drought + Aphids	Drought + Caterpillars	Waterlogged	Waterlogged + Aphids	Waterlogged + Caterpillars	Well-watered	Well-watered + Aphids	Well-watered + Caterpillars	P-value K-W	VIP-score
1	1-Penten-3-ol	Alcohol	4669279 ± 3481394	4222621 ± 1081251	7739968 ± 6678448	7956873 ± 5984605	7707552 ± 2990629	10328294 ± 5233837	6736030 ± 3385735	6633847 ± 5836313	4891164 ± 2948170	0.034	1.009
2	3-Pentanone	Ketone	25266 ± 19744 a	24548 ± 9782 a	41881 ± 29254 ab	67211 ± 52032 ab	66359 ± 44156 ab	104991 ± 56489 b	41054 ± 23397 ab	32339 ± 27657 a	26145 ± 16044 a	**<0.001**	1.118
3	Methyl thiocyanate	Nitrogen and/or sulphur containing compounds	1196914 ± 639584 b	1978891 ± 852023 abc	1676441 ± 821522 bc	2071230 ± 813416 bc	2850363 ± 631876 ac	2191548 ± 551228 abc	2600450 ± 1247936 abc	3262659 ± 775684 a	2316822 ± 443452 abc	**<0.001**	1.162
4	2-Methylbutanenitrile	Nitrogen and/or sulphur containing compounds	60546 ± 60662	123858 ± 190305	41862 ± 17655	133318 ± 171004	337058 ± 826218	133123 ± 191879	176614 ± 215044	543070 ± 1574474	99513 ± 86225	0.138	0.569
5	Dimethyl disulfide	Nitrogen and/or sulphur containing compounds	1116234 ± 939657	758831 ± 96098	1011993 ± 405377	788807 ± 443815	895846 ± 306577	738079 ± 328223	1057848 ± 502168	945010 ± 382315	906336 ± 594358	0.137	0.301
6	(E)-2-Penten-1-ol	Alcohol	102969 ± 124412	57176 ± 30116	208919 ± 223230	111038 ± 148024	111255 ± 74927	161881 ± 134911	68679 ± 56977	82733 ± 111606	44165 ± 32686	0.112	1.103
7	(Z)-3-Hexenal	Aldehyde	52590 ± 50587	32781 ± 26705	100728 ± 151816	88918 ± 215971	36589 ± 43828	78011 ± 93243	53597 ± 66815	52556 ± 101749	23021 ± 16612	0.779	1.004
8	5-Hexen-3-ol	Alcohol	448977 ± 618512	599748 ± 1350183	147835 ± 275091	101823 ± 134865	179010 ± 299370	109980 ± 217624	62158 ± 93485	116934 ± 227918	74431 ± 126253	0.317	0.645
9	(E)-3-Hexen-1-ol	Alcohol	57966 ± 105041	35568 ± 17529	125332 ± 143262	43636 ± 45351	38076 ± 16120	44004 ± 32505	47479 ± 47338	51763 ± 52840	37342 ± 29291	0.751	0.918
10	(Z)-3-Hexen-1-ol	Alcohol	2245217 ± 2924069	1920902 ± 1228271	4025511 ± 4557829	2325453 ± 3313996	2332514 ± 1796799	2864176 ± 3291817	2853374 ± 2474779	2608416 ± 3604822	1921285 ± 2011217	0.983	0.844
11	1,2,4,4-Tetramethylcyclo-pentene	Cycloalkene	49568 ± 69673	72060 ± 51733	77807 ± 149039	38210 ± 13094	33419 ± 10985	36881 ± 14037	30850 ± 13858	28996 ± 17236	27912 ± 13537	0.172	0.865
12	(Z)-2-Penten-1-ol, acetate	Ester	7974 ± 11906	6772 ± 5672	32217 ± 46801	14894 ± 23281	10504 ± 8988	22929 ± 26427	22898 ± 21157	13433 ± 14241	14372 ± 15473	0.281	0.976
13	(E,E)-2,4-Hexadienal	Aldehyde	587158 ± 949896	278235 ± 147507	907664 ± 1731933	424688 ± 716323	226703 ± 178679	466628 ± 537004	340695 ± 218265	273990 ± 286059	285038 ± 214279	0.767	0.993
14	2,3-Dimethylpyrazine	Nitrogen and/or sulphur containing compounds	81130 ± 59359	96683 ± 45148	72914 ± 53347	130176 ± 91101	139486 ± 70714	126749 ± 89073	125966 ± 99226	97013 ± 46499	125327 ± 101508	0.329	0.761
15	1-Octen-3-ol	Alcohol	45324 ± 47895	34637 ± 13393	62090 ± 90082	53422 ± 54772	92717 ± 75098	55322 ± 45003	32207 ± 22857	42051 ± 51832	31926 ± 20813	0.072	1.095
16	beta-Pinene	Monoterpene	572748 ± 119311 c	607316 ± 60603 bc	648206 ± 317662 bc	1470736 ± 651229 a	1138206 ± 810892 abc	1218666 ± 873452 ab	1423921 ± 701953 a	1306131 ± 859380 ab	901911 ± 304834 abc	**<0.001**	1.251
17	3-Octanone	ketone	27398 ± 39273	19483 ± 31630	42609 ± 41752	77656 ± 144304	80658 ± 43279	64790 ± 40142	34803 ± 45066	43454 ± 53628	36190 ± 26825	0.083	0.780
18	6-Methyl-5-hepten-2-ol	Alcohol	55682 ± 32741 a	103965 ± 50144 abc	90072 ± 72714 ab	142947 ± 37476 b	166902 ± 74398 b	127758 ± 73806 ab	91572 ± 47500 ab	144112 ± 94805 ab	118952 ± 51793 abc	**0.004**	1.099
19	2,6-Dimethylheptan-2-ol	Alcohol	132444 ± 74010	105655 ± 85723	118809 ± 59064	90814 ± 52576	97356 ± 70810	71224 ± 24666	75410 ± 40792	78630 ± 54835	72219 ± 43743	0.278	0.453
20	(E,E)-2,4-Heptadienal	Aldehyde	573849 ± 1270635 a	185879 ± 136307 a	805309 ± 1898353 a	176178 ± 124616 a	221402 ± 148597 a	242766 ± 242619 a	112652 ± 83529 a	104095 ± 64951 a	97886 ± 66065 a	**0.008**	1.034
21	(Z)-3-Hexen-1-ol, acetate	Ester	1786444 ± 2217619	1868769 ± 1280055	4523536 ± 5278013	3156708 ± 4374156	2670461 ± 2223348	4619217 ± 5083598	4535969 ± 3342730	3097952 ± 3319979	3709583 ± 3337286	0.533	1.03859
22	3,3,6-Trimethyl-1,5-heptadien-4-one	Ketone	113517 ± 134996	69828 ± 36932	151006 ± 174539	96925 ± 118341	62543 ± 53803	82864 ± 48499	73982 ± 65616	55099 ± 44390	43624 ± 30225	**0.319**	0.907
23	(E)-beta-Ocimene	Monoterpene	143373 ± 101562 b	215701 ± 160508 b	2660376 ± 2476952 ac	265708 ± 64890 bc	258022 ± 137802 b	2474482 ± 1648422 a	462589 ± 518476 abc	1866770 ± 4866904 abc	2812586 ± 1609812 a	**<0.001**	1.804

Supplementary Table 2 continued.

ID	Compound	Class	Drought	Drought + Aphids	Drought + Caterpillars	Waterlogged	Waterlogged + Aphids	Waterlogged + Caterpillars	Well-watered	Well-watered + Aphids	Well-watered + Caterpillars	P-value K-W	VIP-score
24	gamma-Terpinene	Monoterpene	16187 ± 4891 c	19323 ± 6082 abc	17584 ± 8404 bc	31248 ± 8919 a	25374 ± 13974 abc	28613 ± 11130 abc	35123 ± 15209 a	30067 ± 10803 ab	26302 ± 7963 abc	**<0.001**	1.323
25	2,6-Dimethyl-5-heptenal	Alcohol	102751 ± 116998	96882 ± 53998	158279 ± 298426	62926 ± 22648	63850 ± 26013	65727 ± 16331	59452 ± 25945	54006 ± 29255	58771 ± 26256	0.158	1.104
26	(E)-4-Thujanol	Monoterpene	12857 ± 4367 b	15372 ± 5012 ab	12809 ± 5172 ab	20185 ± 3961 a	18524 ± 6307 ab	19319 ± 6278 ab	17326 ± 4460 ab	20792 ± 4785 a	15401 ± 3485 ab	**0.002**	1.225
27	p-Cymenene	Monoterpene	5629 ± 5796	7863 ± 7421	3402 ± 4102	7366 ± 5802	7049 ± 3585	9384 ± 9046	8824 ± 6087	9314 ± 6062	7139 ± 9713	0.431	0.937
28	Terpinolene	Monoterpene	95514 ± 39895 b	105861 ± 33134 b	104155 ± 76950 b	198254 ± 52730 a	167080 ± 116783 ab	183608 ± 91402 ab	229821 ± 97157 a	195756 ± 91784 ab	173350 ± 32502 ab	**<0.001**	1.483
29	2-(2-Propenyl) bicyclo[2.2.1]heptane	Monoterpene	176949 ± 186591	124914 ± 56767	498453 ± 1216669	105011 ± 51944	81403 ± 24057	91555 ± 16038	96655 ± 29625	106416 ± 52152	90751 ± 25597	0.621	0.863
30	Linalool	Monoterpene	42175 ± 28960 a	35272 ± 10347 a	69502 ± 46161 ab	63780 ± 14656 ab	62278 ± 21645 ab	100726 ± 25962 b	47530 ± 22309 a	44239 ± 24866 a	69624 ± 37086 ab	**<0.001**	1.098
31	2,2-Dimethylocta-3,4-dienal	Aldehyde	367835 ± 300800	313001 ± 222098	374272 ± 429659	626106 ± 978760	412469 ± 284111	485806 ± 494602	843299 ± 1611334	414362 ± 198778	644751 ± 1086373	0.865	0.854
32	(E)-DMNT	Homoterpene	165559 ± 140097 a	475945 ± 645003 ab	2158928 ± 2376629 bcd	350133 ± 112632 ab	476147 ± 201411 abcd	2607823 ± 1608925 cd	466508 ± 167912 abc	558444 ± 154012 abcd	3564740 ± 2064156 d	**<0.001**	1.638
33	p-Mentha-1,3,8-triene	Monoterpene	2763 ± 1944 b	5255 ± 4247 b	48477 ± 47473 a	5664 ± 2212 b	6121 ± 1906 b	53248 ± 39002 a	8840 ± 6808 ab	32426 ± 77629 ab	45067 ± 21223 a	**<0.001**	1.779
34	6-Camphenol	Monoterpene	204759 ± 106591	156390 ± 67563	158525 ± 67815	177465 ± 47173	151284 ± 42298	176698 ± 59735	198071 ± 79397	182273 ± 34474	168559 ± 68971	0.698	0.948
35	(Z)-3-Hexen-1-ol, isobutyrate	Ester	172836 ± 479698	32365 ± 25444	218678 ± 284998	121299 ± 207032	71933 ± 66840	133786 ± 168638	160841 ± 156557	78378 ± 90934	95998 ± 101931	0.345	1.143
36	p-Cymen-8-ol	Monoterpene	107716 ± 59638	96205 ± 82815	107103 ± 65459	93383 ± 52323	79158 ± 45686	90132 ± 65312	89505 ± 65975	73671 ± 35968	75691 ± 53620	0.862	0.912
37	Isomenthone	Monoterpene	9484 ± 8972	10437 ± 8158	42528 ± 95889	8711 ± 7132	44310 ± 96830	34580 ± 68275	7170 ± 4606	46504 ± 111153	10129 ± 8698	0.913	0.732
38	(Z)-3-Hexen-1-ol, butanoate	Ester	485396 ± 470533	405661 ± 142648	1377458 ± 1941483	1719904 ± 2792675	1340592 ± 1095527	1915147 ± 2156984	1835177 ± 1622236	1198962 ± 2034609	1299748 ± 1461248	0.188	0.927
39	alpha-Pinene oxide	Monoterpene	67797 ± 42514	51313 ± 41636	48374 ± 27592	57360 ± 29961	40109 ± 16418	50580 ± 39119	56343 ± 35196	44145 ± 15570	52936 ± 37973	0.809	0.886
40	Isomenthol	Monoterpene	80292 ± 51706	84790 ± 43979	305887 ± 642772	70173 ± 34332	232949 ± 434896	228205 ± 410191	58559 ± 19689	262952 ± 575719	73402 ± 43856	0.561	0.719
41	Methyl salicylate	Ester	88064 ± 70264 ab	143439 ± 123063 ab	208635 ± 166458 b	69094 ± 23572 ab	107689 ± 91068 ab	106638 ± 55960 ab	60898 ± 35888 a	120730 ± 158750 ab	111976 ± 83689 ab	0.0419	1.001
42	D-Verbenone	Monoterpene	68357 ± 12315	63951 ± 18128	69819 ± 23616	78519 ± 29669	68154 ± 17246	72962 ± 27119	77916 ± 32641	76483 ± 17743	62511 ± 9891	0.843	0.943
43	(Z)-3-Hexen-1-ol, 2-methylbutanoate	Ester	826814 ± 1803708	278663 ± 157511	1721531 ± 2498977	551439 ± 820863	315582 ± 176110	697582 ± 1130297	1090927 ± 1289680	475722 ± 212751	584202 ± 592580	0.287	1.205
44	beta-Cyclocitral	Norisoprene	20813 ± 25461	20327 ± 15990	36625 ± 71969	14053 ± 4450	12978 ± 3894	15585 ± 6649	10509 ± 4351	9607 ± 4547	11881 ± 5492	**0.009**	1.025
45	(E)-Ocimenone	Monoterpene	104760 ± 45332	84468 ± 54672	106106 ± 56765	81347 ± 35449	73677 ± 29404	83929 ± 45477	83223 ± 74143	87340 ± 40089	71784 ± 26679	0.729	0.838
46	Benzothiazole	Nitrogen and/or sulphur containing compounds	3129835 ± 874116	3550313 ± 844949	3050736 ± 829194	3397467 ± 968968	3411924 ± 504063	3170570 ± 791910	3454500 ± 878526	3179703 ± 524731	3560060 ± 929015	0.814	0.613
47	Butyl 4-oxopentanoate	Ester	11536 ± 6680	9782 ± 7081	10799 ± 5801	10250 ± 4140	9516 ± 5802	8472 ± 7522	12215 ± 6745	8796 ± 4370	10693 ± 7275	0.869	0.514
48	11,12,13-Trinor-5-eudesmene	Cycloalkene	41425 ± 11950	37768 ± 10990	36141 ± 9450	41940 ± 8847	43079 ± 8640	44522 ± 31960	37598 ± 11443	37587 ± 4149	31750 ± 7527	0.272	1.051
49	Isoquinoline	Nitrogen and/or sulphur containing compounds	47034 ± 23901	41606 ± 20283	44388 ± 13822	41820 ± 16219	40709 ± 16843	33569 ± 13406	43074 ± 23256	39855 ± 12411	39612 ± 5897	0.938	0.577
50	2,6,6-Trimethylcyclohex-2-ene-1-methanol	Norisoprene	31775 ± 20494	25941 ± 18869	29949 ± 21492	31586 ± 20878	31243 ± 23065	25336 ± 23735	44238 ± 35391	27643 ± 15810	36399 ± 38005	0.846	0.665
51	Dihydrocarveol acetate	Monoterpene	13342 ± 10361	8660 ± 5015	15545 ± 15305	14219 ± 9312	15316 ± 14061	26810 ± 53213	11926 ± 14840	10408 ± 12120	9328 ± 2306	0.861	0.556
52	1,2,8,9-Diepoxy-limonene	Monoterpene	160065 ± 75861	115015 ± 53316	143021 ± 73528	118795 ± 57495	97215 ± 60535	122441 ± 67803	123058 ± 93478	119525 ± 52346	103477 ± 59412	0.676	0.74402
53	Anethole	Ether	32979 ± 30900	122582 ± 230688	579921 ± 1789258	48475 ± 57374	378322 ± 1029513	344743 ± 990286	98344 ± 220572	322220 ± 882568	129637 ± 217455	0.9	0.498
54	Indole	Nitrogen and/or sulphur containing compounds	116666 ± 88209 a	214689 ± 212111 a	367143 ± 281490 a	214869 ± 178039 a	337427 ± 197790 a	330395 ± 201867 a	749138 ± 1935962 a	199040 ± 147143 a	344208 ± 184565 a	**0.025**	0.949
55	2-(2-Methylpropylidene) cycloheptanone	Ketone	10708 ± 17097 ab	9483 ± 7144 b	15044 ± 28770 ab	5147 ± 1306 ab	5257 ± 927 ab	4827 ± 1277 ab	3943 ± 1956 a	4101 ± 1179 a	3912 ± 2422 a	**<0.001**	0.946
56	alpha,alpha-Dimethylphenethyl acetate	Ester	28578 ± 11989	34896 ± 22101	30320 ± 19811	25507 ± 8454	29942 ± 11044	31737 ± 20672	24996 ± 10336	25516 ± 4117	24492 ± 10505	0.957	0.849
57	Terpinen-4-ol acetate	Monoterpene	10033 ± 12378	7044 ± 10093	16782 ± 20799	9202 ± 8947	6925 ± 8157	10055 ± 13006	14452 ± 24554	19603 ± 26967	6733 ± 6509	0.988	0.456
58	Isodauca-6,9-diene	Sesquiterpene	41915 ± 23530	41737 ± 14813	40348 ± 19142	42195 ± 22848	34079 ± 13747	41053 ± 21968	40455 ± 17486	43485 ± 18608	43316 ± 18925	0.964	0.805
59	beta-Patchoulene	Sesquiterpene	2323 ± 4278 ab	4524 ± 4529 ab	3473 ± 8193 b	8734 ± 4126 ab	4967 ± 7452 ab	4721 ± 4611 ab	10309 ± 5932 a	7277 ± 4523 ab	7284 ± 4341 ab	**0.004**	1.024
60	beta-Longipinene	Sesquiterpene	9820 ± 7892	7635 ± 5413	5291 ± 3770	7544 ± 6386	3921 ± 2679	4839 ± 3658	9692 ± 11421	5385 ± 2770	8684 ± 6429	0.23	0.6948
61	(E)-alpha-Bergamotene	Sesquiterpene	266089 ± 61565	318986 ± 117945	228137 ± 88613	247925 ± 81485	193528 ± 61665	186022 ± 75819	204314 ± 91836	207397 ± 69468	189831 ± 57350	**0.013**	1.158
62	Geosmin	Alcohol	6058 ± 6480 a	6190 ± 6578 a	4025 ± 5477 a	20161 ± 7143 b	24266 ± 13045 b	57421 ± 130773 b	11123 ± 7307 ab	11395 ± 5037 ab	10765 ± 7192 ab	**<0.001**	1.162
63	Geranylacetone	Norisoprene	254899 ± 198143	235638 ± 142315	270958 ± 190928	187915 ± 113659	218658 ± 84373	177686 ± 120206	231017 ± 247471	408549 ± 420914	248709 ± 86373	0.565	0.699
64	alpha-Barbatene	Sesquiterpene	5001003 ± 952654	6061555 ± 2617114	4403309 ± 1752230	4040766 ± 988778	3321756 ± 1585294	3403228 ± 1180440	4452946 ± 1098439	4220476 ± 1268251	4135486 ± 1310677	**0.018**	1.258
65	(E)-beta-Caryophyllene	Sesquiterpene	53471 ± 38385 ac	1073636 ± 1601435 b	255614 ± 491591 abc	260412 ± 593468 ac	702521 ± 827910 bc	57536 ± 38184 ac	72274 ± 173122 a	1161073 ± 1622294 b	153374 ± 185647 abc	**<0.001**	0.400
66	(E)-beta-Bergamotene	Sesquiterpene	69276 ± 14610	90004 ± 30663	66398 ± 32475	59616 ± 16695	54301 ± 18344	54278 ± 19323	71400 ± 32738	80592 ± 43590	61724 ± 22204	0.062	1.054
67	1-(4-tert-Butylphenyl) propan-2-one	Ketone	37850 ± 19313	33279 ± 24326	26819 ± 23114	25426 ± 17012	28927 ± 16707	22398 ± 15164	20174 ± 18869	28121 ± 18295	22221 ± 14787	0.503	0.425
68	beta-Acoradiene	Sesquiterpene	965375 ± 504675 a	1458842 ± 1107199 a	806703 ± 533910 a	592910 ± 132738 a	512372 ± 378342 a	509973 ± 214912 a	725946 ± 263402 a	730591 ± 469108 a	705567 ± 475574 a	**0.026**	1.210
69	Cadina-3,9-diene	Sesquiterpene	80027 ± 46255 c	124961 ± 54813 bc	110074 ± 100716 bc	250923 ± 54166 a	210574 ± 93072 abc	208633 ± 74465 abc	260512 ± 86936 a	220329 ± 41378 ab	226511 ± 84194 ab	**<0.001**	1.448
70	alpha-Bulnesene	Sesquiterpene	49074 ± 8151 a	63251 ± 31312 a	45078 ± 20115 ab	35869 ± 8920 ab	28882 ± 14963 b	30922 ± 13115 ab	38488 ± 11632 ab	36481 ± 15651 ab	35640 ± 14792 ab	**0.003**	1.303
71	alpha-Isomethylionone	Norisoprene	170000 ± 130816	146091 ± 132545	103938 ± 65640	91237 ± 56906	121661 ± 125314	74649 ± 28514	94228 ± 69039	151671 ± 144207	91704 ± 53184	0.608	0.713

Supplementary Table 2 continued.

ID	Compound	Class	Drought	Drought + Aphids	Drought + Caterpillars	Waterlogged	Waterlogged + Aphids	Waterlogged + Caterpillars	Well-watered	Well-watered + Aphids	Well-watered + Caterpillars	P-value K-W	VIP-score
72	alpha-Neocallitropsene	Sesquiterpene	66028 ± 9185 a	83431 ± 35757 a	56967 ± 26068 ab	44476 ± 12221 ab	37724 ± 18659 b	37730 ± 16717 b	45447 ± 15472 ab	44609 ± 18015 ab	42707 ± 19649 ab	**<0.001**	1.336
73	5beta,7beta,10alpha-Selina-4(15),11-diene	Sesquiterpene	7114 ± 1251 a	9006 ± 4025 a	6299 ± 2501 ab	5410 ± 1319 ab	5176 ± 2040 ab	3891 ± 2486 b	5967 ± 1741 ab	5814 ± 2140 ab	4968 ± 2520 ab	**0.008**	0.929
74	Acoradiene	Sesquiterpene	82756 ± 13030 ab	100947 ± 43416 a	69335 ± 31628 abc	58382 ± 15707 abc	48334 ± 20709 bc	47991 ± 20953 c	61234 ± 21011 abc	56117 ± 20109 abc	55789 ± 23963 abc	**0.001**	1.326
75	(E,E)-alpha-Farnesene	Sesquiterpene	122813 ± 70552 a	148102 ± 91420 a	298714 ± 252854 a	265680 ± 175697 a	241778 ± 162015 a	328893 ± 226925 a	359895 ± 254687 a	411338 ± 275571 a	480908 ± 332223 a	**0.016**	0.937
76	beta-Chamigrene	Sesquiterpene	670486 ± 80087 a	1064906 ± 629325 a	709782 ± 335694 ab	550041 ± 91951 ab	453288 ± 137458 b	491921 ± 134704 b	558100 ± 132916 ab	542571 ± 155799 ab	574397 ± 248336 ab	**<0.001**	1.282
77	Unknown sesquiterpene	Sesquiterpene	14719 ± 3396 a	17622 ± 7951 a	13485 ± 6090 a	10783 ± 3402 a	9913 ± 4606 a	9623 ± 3975 a	11286 ± 4595 a	10713 ± 3212 a	10889 ± 3812 a	**0.035**	1.210
78	alpha-Irone	Norisoprene	58710 ± 51647	59931 ± 58773	41063 ± 36464	33954 ± 35147	48639 ± 68243	26175 ± 22079	27179 ± 25884	54669 ± 64445	37330 ± 21937	0.752	0.513
79	alpha-Cuprenene	Sesquiterpene	225511 ± 153674	313068 ± 225647	175191 ± 143399	179520 ± 63257	126073 ± 125176	137186 ± 114226	203172 ± 127694	163313 ± 105333	168574 ± 98511	0.239	1.1985
80	Cuparene	Sesquiterpene	2648033 ± 712017	3072069 ± 781994	2587515 ± 1261771	2130340 ± 729269	1786855 ± 777157	2141964 ± 723590	2055521 ± 849829	2215037 ± 817830	2119136 ± 748916	0.061	1.074
81	Cyclobazzanene	Sesquiterpene	46788 ± 10409 a	57651 ± 27623 a	40224 ± 19739 ab	30653 ± 7815 ab	26237 ± 11560 b	26012 ± 11395 b	32910 ± 11764 ab	31437 ± 13492 ab	30059 ± 12920 ab	**<0.001**	1.349
82	Dihydroactinidiolide	Norisoprene	33399 ± 29106	48207 ± 28861	47457 ± 22340	42579 ± 19480	38329 ± 23055	32000 ± 19213	36775 ± 20935	38634 ± 25207	37497 ± 11971	0.336	0.620
83	gamma-Cuprenene	Sesquiterpene	93994 ± 39221	141273 ± 123983	69529 ± 28540	63553 ± 17735	58398 ± 27236	54588 ± 27365	80108 ± 47488	61988 ± 27168	62903 ± 32489	0.077	1.203
84	Unknown sesquiterpene	Sesquiterpene	28367 ± 19808 a	43149 ± 23927 a	28416 ± 25913 a	10441 ± 14087 a	8545 ± 17838 a	7761 ± 16336 a	11376 ± 16766 a	14015 ± 18444 a	8121 ± 17504 a	**0.031**	1.002
85	(E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	Homoterpene	5157 ± 16262 b	19987 ± 44807 b	125664 ± 111570 ab	23970 ± 38753 b	45291 ± 36391 ab	94376 ± 78941 ab	31117 ± 46765 ab	99350 ± 119398 ab	134591 ± 81274 a	**<0.001**	1.247
86	Bulnesol	Sesquiterpene	64260 ± 85299	31340 ± 27073	24389 ± 24952	37930 ± 41139	17379 ± 10390	25202 ± 26030	46788 ± 70336	31115 ± 26051	36998 ± 30249	0.557	0.936
87	(7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	Sesquiterpene	466059 ± 200540	498190 ± 480560	384368 ± 197794	411015 ± 187137	327084 ± 182971	323980 ± 205210	501828 ± 514979	539243 ± 457109	412807 ± 166496	0.813	0.850
88	2-(4a,8-Dimethyl-6-oxo-1,2,3,4,4a,5,6,8a-octahydro-naphthalen-2-yl)-propionaldehyde	Sesquiterpene	164046 ± 76687	149408 ± 123910	135211 ± 76155	138876 ± 63634	111352 ± 59184	114198 ± 73087	155223 ± 115530	164876 ± 108422	137921 ± 59456	0.8	0.908
89	Unknown	Unknown	87623 ± 58345	72151 ± 73940	73326 ± 56876	55483 ± 51848	56820 ± 39836	55966 ± 46353	66941 ± 87669	94238 ± 75116	64624 ± 42433	0.76	0.363
90	(Z)-alpha-Hexylcinnamaldehyde	Aldehyde	104818 ± 46924	104006 ± 89629	121287 ± 69107	99339 ± 47706	109392 ± 79093	69033 ± 40675	122722 ± 121880	116584 ± 69094	98513 ± 33541	0.78	0.766
91	4-Acetyl-alpha-cedrene	Sesquiterpene	50767 ± 30024	57190 ± 43970	47755 ± 30673	55094 ± 30414	46773 ± 31874	35041 ± 27213	48062 ± 26926	48543 ± 24789	63046 ± 27614	0.765	0.725
92	Galaxolide	Ether	177303 ± 73693	217469 ± 217628	192323 ± 133800	215614 ± 144096	202165 ± 162457	162178 ± 163929	213870 ± 140870	219632 ± 136212	198107 ± 106167	0.847	0.934



Supplementary Figure 3. Loading plot from PLS-DA of VOC data of *R. sylvestris* plants. Numbers indicate specific compounds specified in the table below. Red dots depict plant treatments as loading variables: C=well-watered undamaged, CA= well-watered + aphid induced, CP=well-watered+ caterpillar induced, D=drought, DA= drought + aphid induced, DP= drought + caterpillar induced, W=waterlogged, WA=waterlogged + aphid induced, WP=waterlogged + caterpillar induced.

Supplementary Table 3. Relative amount of VOCs (mean ± st. dev.) in the headspace composition of *R. sylvestris* plants. Differences between treatments were calculated using a Kruskal Wallis test using a pairwise Dunn test with Bonferroni correction (P<0.05). VIP scores corresponding to plot presented in Figure 4C and Supplementary Figure 3.

ID	Compound	Group	Well-watered	Well-watered + Aphids	Well-watered + Caterpillars	Drought	Drought + Aphids	Drought + Caterpillars	Waterlogged	Waterlogged + Aphids	Waterlogged + Caterpillars	P-value K-W	VIP-scores
1	2-Methylbutanenitrile	Nitrogen and/or sulphur containing compounds	82536 ± 75628 a	179812 ± 406562 a	164569 ± 385296 a	24364 ± 5221 b	37118 ± 26029 ab	41086 ± 20549 ab	102985 ± 239274 ab	68573 ± 75127 ab	81737 ± 139131 ab	**0.002**	0.435
2	Dimethyl disulfide	Nitrogen and/or sulphur containing compounds	177758 ± 177758 ab	142613 ± 142613 ab	157440 ± 157440 ab	182618 ± 182618 ab	128786 ± 128786 ab	90011 ± 90011 a	155000 ± 155000 ab	142239 ± 142239 ab	274068 ± 274068 b	**0.016**	0.591
3	(E)-3-Hexen-1-ol	Alcohol	7158 ± 7752	12065 ± 15535	11866 ± 6852	42522 ± 82137	183015 ± 419716	8795 ± 8946	4245 ± 5820	9892 ± 9506	9190 ± 6057	0.527	0.663
4	(Z)-3-Hexen-1-ol	Alcohol	352275 ± 330428	563028 ± 719462	546237 ± 415069	796017 ± 1117539	2338093 ± 4797703	298580 ± 260694	195409 ± 199302	337723 ± 288397	261742 ± 191139	0.223	0.844
5	(Z)-2-Penten-1-ol, acetate	Ester	6968 ± 10044 ab	6877 ± 11771 ab	14469 ± 11242 a	2204 ± 4047 b	16961 ± 39844 ab	4776 ± 6428 ab	1948 ± 2485 b	4108 ± 5317 ab	6657 ± 6520 ab	**0.033**	0.865
6	3-(2-Methylpropyl) cyclohexene	Cycloalkenes	18936 ± 12701 ab	19052 ± 8672 abc	16872 ± 10101 ab	3626 ± 5585 a	5242 ± 7151 a	3854 ± 5059 a	450896 ± 275069 c	339381 ± 276602 bc	228788 ± 292732 bc	**<0.001**	1.359
7	1-Octen-3-ol	Alcohol	599 ± 1300 ab	354 ± 1075 a	808 ± 1777 ab	958 ± 2141 ab	782 ± 2432 a	510 ± 1565 ab	21658 ± 26111 b	22307 ± 21280 ab	18661 ± 15045 b	**<0.001**	1.167
8	4-Penten-1-ol, 3-methyl-, acetate	Ester	4324 ± 4407	4708 ± 6120	6510 ± 5871	2999 ± 5569	28416 ± 68157	2855 ± 5203	2332 ± 2604	2073 ± 3547	5102 ± 5084	0.236	0.561
9	(Z)-3-Hexen-1-ol, acetate	Ester	2407175 ± 2660111 ab	2481411 ± 3366944 ab	3508156 ± 3290924 a	1253767 ± 1517309 ab	3885694 ± 7278577 ab	1440313 ± 1640582 ab	691853 ± 652415 b	1245270 ± 1432104 ab	1331336 ± 1016725 ab	**0.043**	0.991
10	(E)-2-Hexen-1-ol, acetate	Ester	8004 ± 11977 ab	9934 ± 14556 a	16631 ± 16486 a	3031 ± 6910 ab	24971 ± 59282 ab	9993 ± 22206 ab	15 ± 2 b	2794 ± 6069 ab	5691 ± 7616 ab	**0.015**	0.837
11	(Z)-beta-Ocimene	Monoterpene	9864 ± 21917 a	15 ± 2 a	244861 ± 188846 b	16 ± 3 ac	436 ± 1332 a	65363 ± 100743 abc	15 ± 2 a	14 ± 2 a	202394 ± 260466 bc	**<0.001**	1.215
12	3,3,5-Trimethylcyclo-hexanol	Norisoprene	24297 ± 16669 ab	14991 ± 6489 abc	21973 ± 11156 a	8627 ± 4092 c	9854 ± 6748 bc	11484 ± 10208 abc	17532 ± 11270 abc	16167 ± 13820 abc	12388 ± 9234 abc	**<0.001**	0.865
13	(E)-beta-Ocimene	Monoterpene	16815 ± 14395 abc	15162 ± 18532 ab	336102 ± 317894 c	3355 ± 5203 a	4133 ± 2106 a	97738 ± 164429 bc	7136 ± 5607 ab	10570 ± 11529 ab	420756 ± 800651 c	**<0.001**	1.115
14	(E)-4,8-Dimethyl-1,3,7-nonatriene	Homoterpene	14266 ± 44288 a	1064 ± 2240 ab	349925 ± 226465 c	14 ± 2 a	16 ± 2 ab	171338 ± 145838 bc	14 ± 2 a	5919 ± 14154 ab	311294 ± 323398 c	**<0.001**	1.293
15	Benzyl cyanide	Nitrogen and/or sulphur containing compounds	19531 ± 6616 abc	20696 ± 16384 ab	56924 ± 37109 ac	11302 ± 1514 b	15041 ± 5692 b	71840 ± 44511 c	17190 ± 16368 b	17722 ± 6287 abc	43346 ± 18300 ac	**<0.001**	1.112
16	(Z)-3-Hexen-1-ol, butanoate	Ester	175908 ± 203162	138492 ± 180168	173190 ± 134344	26965 ± 45313	244833 ± 493982	58482 ± 74351	24435 ± 49349	87300 ± 140179	99553 ± 79177	0.06	0.893
17	Methyl salicylate	Ester	483173 ± 1109480 abc	145878 ± 163458 ab	1261608 ± 1507120 ac	69777 ± 44052 b	56008 ± 23367 b	571527 ± 433264 ac	76998 ± 61845 b	313217 ± 299544 abc	1887207 ± 2161823 c	**<0.001**	1.155
18	(Z)-3-Hexen-1-ol, 2-methylbutanoate	Ester	123931 ± 126500	100416 ± 149102	174984 ± 146129	582085 ± 1081195	1971559 ± 4400665	72518 ± 37171	136876 ± 171764	116475 ± 101907	93333 ± 65972	0.773	0.700
19	Benzothiazole	Nitrogen and/or sulphur containing compounds	2726052 ± 716259	2451171 ± 757002	2696472 ± 808213	1917126 ± 705001	1924463 ± 569982	2374278 ± 692734	2228852 ± 887834	1954110 ± 733046	1940109 ± 428696	**0.049**	0.767
20	Indole	Nitrogen and/or sulphur containing compounds	18405 ± 14871 ab	18254 ± 17812 ab	115356 ± 94196 a	6289 ± 2095 b	7671 ± 4504 b	156078 ± 166439 a	15524 ± 21053 b	21269 ± 24863 ab	178731 ± 288924 a	**<0.001**	1.166
21	4-(3-Cyclohexen-1-yl)-3-buten-2-one	Monoterpene	50900 ± 43308	42664 ± 29682	39063 ± 34772	35111 ± 56605	22243 ± 29685	36015 ± 22689	50826 ± 30987	35513 ± 27098	45142 ± 25081	0.218	0.618
22	alpha-Longipinene	Sesquiterpene	186504 ± 462004 ab	3201 ± 3837 ab	17073 ± 31523 ab	742 ± 1552 a	5068 ± 15981 ab	1009 ± 2038 ab	9292 ± 10703 ab	21122 ± 28351 ab	32786 ± 43132 b	**0.03**	0.810
23	Geosmin	Alcohol	12618 ± 24022 ab	5916 ± 4521 ab	7578 ± 6844 ab	2586 ± 4541 a	4290 ± 5111 ab	4715 ± 3589 ab	65243 ± 177586 b	17547 ± 16055 b	12885 ± 13808 ab	**0.002**	0.833
24	Geranylacetone	Norisoprene	110859 ± 68842	100879 ± 51325	112008 ± 46346	60518 ± 39061	78138 ± 68559	94871 ± 47098	105361 ± 56502	74780 ± 56190	101728 ± 72038	0.258	0.983
25	(E)-beta-Farnesene	Sesquiterpene	277147 ± 554049 ab	107821 ± 58806 ab	3055992 ± 2626581 c	79656 ± 181144 a	70403 ± 64938 a	1069155 ± 767910 bc	48730 ± 62101 a	115483 ± 127035 ab	4563105 ± 4539063 c	**<0.001**	1.138
26	(E)-beta-Bergamotene	Sesquiterpene	11193 ± 26141 ab	3500 ± 4683 ab	149038 ± 138805 c	3787 ± 8992 a	3888 ± 5400 ab	51974 ± 38483 bc	4739 ± 8004 ab	4585 ± 6965 ab	242441 ± 263177 c	**<0.001**	1.163
27	Cucumene	Sesquiterpene	73373 ± 124671 abc	34615 ± 24381 abc	70260 ± 138444 abc	5962 ± 3108 d	8817 ± 5262 ad	11004 ± 5044 abd	42027 ± 29024 bc	23079 ± 19062 abcd	69124 ± 49295 c	**<0.001**	1.104
28	(E,E)-alpha-Farnesene	Sesquiterpene	5979 ± 10660 ab	2249 ± 4719 a	82780 ± 61438 c	15 ± 2 a	403 ± 1230 a	31827 ± 28798 abc	895 ± 3045 a	3757 ± 8281 a	121526 ± 189797 bc	**<0.001**	1.292
29	(E,E)-TMTT	Homoterpene	41017 ± 101106 abc	5909 ± 18638 a	142143 ± 115310 bc	15 ± 6 a	17293 ± 37463 abc	40063 ± 41796 abc	6220 ± 17599 ab	8558 ± 20345 abc	263835 ± 408259 c	**<0.001**	1.125
30	Bulnesol	Sesquiterpene	40410 ± 39299	27403 ± 14456	28169 ± 13361	28737 ± 40962	36826 ± 30984	24556 ± 10194	30635 ± 15704	21992 ± 12009	29409 ± 15185	0.534	0.769
31	(7a-Isopropenyl-4,5-di-methyloctahydroin-den-4-yl)methanol	Sesquiterpene	356952 ± 240450 a	297229 ± 175530 ab	261744 ± 88431 ab	192286 ± 206556 b	215052 ± 134453 ab	205857 ± 66843 ab	267915 ± 101883 ab	211758 ± 85825 ab	227478 ± 70840 ab	**0.026**	1.240
32	Isocurcumenol	Sesquiterpene	106592 ± 67452 a	90356 ± 50397 ab	79183 ± 26008 ab	56593 ± 63856 b	61258 ± 36168 ab	61602 ± 19788 ab	78374 ± 27148 ab	60833 ± 30162 ab	67894 ± 19199 ab	**0.01**	1.274
33	2-(4a,8-Dimethyl-6-oxo-1,2,3,4,4a,5,6,8a-octahydro-naphthalen-2-yl)-propionaldehyde	Sesquiterpene	52899 ± 32900	39851 ± 32212	38290 ± 13249	24622 ± 29053	27802 ± 22676	28065 ± 13456	37975 ± 19974	30844 ± 16313	31190 ± 13630	0.072	0.836
34	alpha-Hexylcinnamaldehyde	Aldehyde	46443 ± 19592	43804 ± 20707	40305 ± 8929	26864 ± 11927	29558 ± 11931	33719 ± 10835	40280 ± 15996	30853 ± 13308	36876 ± 12000	0.055	1.195

Supplementary Table 4A. Volatile profile comparisons between treatments using OPLS-DA analyzed by CV-ANOVA of *R. amphibia* plants.

No	Treatment 1	vs	Treatment 2	CV-ANOVA P-value	F	R2cum	Q2cum
1	Well-watered	vs	Drought	0.062	3.61	0.547	0.396
2	Well-watered	vs	Waterlogged	0.021	5.619	0.961	0.801
3	Drought	vs	Waterlogged	0.01	0.019	0.972	0.766
4	Well-watered	vs	Well-watered + Aphids	0.723	0.521		
5	Drought	vs	Drought + Aphids	1	0		
6	Waterlogged	vs	Waterlogged + Aphids	0.121	2.453		
7	Well-watered + Aphids	vs	Drought + Aphids	0.032	4.987	0.966	0.758
8	Well-watered + Aphids	vs	Waterlogged + Aphids	0.009	6.817	0.985	0.81
9	Drought + Aphids	vs	Waterlogged + Aphids	0.039	4.29	0.995	0.786
10	Well-watered	vs	Well-watered + Caterpillars	0.007	8.718	0.978	0.833
11	Drought	vs	Drought + Caterpillars	0.226	1.82		
12	Waterlogged	vs	Waterlogged + Caterpillars	0.027	5.08	0.683	0.48
13	Well-watered + Caterpillars	vs	Drought + Caterpillars	0.02	6.236	0.769	0.581
14	Well-watered + Caterpillars	vs	Waterlogged + Caterpillars	0.042	4.433	0.927	0.722
15	Drought + Caterpillars	vs	Waterlogged + Caterpillars	0.001	14.863	0.998	0.927
16	Well-watered	vs	Drought + Aphids	0.059	3.694	0.648	0.402
17	Well-watered	vs	Drought + Caterpillars	0.0003	16.319	0.952	0.879
18	Well-watered	vs	Waterlogged + Aphids	0.028	5.008	0.732	0.477
19	Well-watered	vs	Waterlogged + Caterpillars	0.038	4.123	0.962	0.72

No	Treatment 1	vs	Treatment 2	vs	Treatment 3	CV-ANOVA P-value	F	R2cum	Q2cum
20	Well-watered	vs	Drought	vs	Waterlogged	0.051	2.233	0.577	0.424
21	Well-watered	vs	Well-watered + Aphids	vs	Well-watered + Caterpillars	0.46	1.0224		
22	Drought	vs	Drought + Aphids	vs	Drought + Caterpillars	0.628	0.654		
23	Waterlogged	vs	Waterlogged + Aphids	vs	Waterlogged + Caterpillars	0.487	0.984		
24	Well-watered + Aphids	vs	Drought + Aphids	vs	Waterlogged + Aphids	0.0022	2.505	0.768	0.548
25	Well-watered + Caterpillars	vs	Drought + Caterpillars	vs	Waterlogged + Caterpillars	0.053	2.25	0.679	0.479

Supplementary Table 4B. Volatile profile comparisons between treatments using OPLS-DA analyzed by CV-ANOVA of *R. palustris* plants.

No	Treatment 1	vs	Treatment 2	CV-ANOVA P-value	F	R2cum	Q2cum
1	Well-watered	vs	Drought	0.003	5853	0.942	0.715
2	Well-watered	vs	Waterlogged	1	0		
3	Drought	vs	Waterlogged	<0.001	14796	0.985	0.847
4	Well-watered	vs	Well-watered + Aphids	1	0		
5	Drought	vs	Drought + Aphids	1	0		
6	Waterlogged	vs	Waterlogged + Aphids	1	0		
7	Well-watered + Aphids	vs	Drought + Aphids	0.001	8061	0.902	0.683
8	Well-watered + Aphids	vs	Waterlogged + Aphids	0.965	0.263		
9	Drought + Aphids	vs	Waterlogged + Aphids	<0.001	10006	0.922	0.741
10	Well-watered	vs	Well-watered + Caterpillars	0.399	1121		
11	Drought	vs	Drought + Caterpillars	1	0		
12	Waterlogged	vs	Waterlogged + Caterpillars	0.066	2669	0.83	0.372
13	Well-watered + Caterpillars	vs	Drought + Caterpillars	0.588	0.798		
14	Well-watered + Caterpillars	vs	Waterlogged + Caterpillars	0.201	1705		
15	Drought + Caterpillars	vs	Waterlogged + Caterpillars	0.532	0.887		
16	Well-watered	vs	Drought + Aphids	<0.001	10240	0.883	0.719
17	Well-watered	vs	Drought + Caterpillars	0.006	5472	0.877	0.578
18	Well-watered	vs	Waterlogged + Aphids	0.427	1159		
19	Well-watered	vs	Waterlogged + Caterpillars	0.005	5413	0.99	0.816

No	Treatment 1	vs	Treatment 2	vs	Treatment 3	CV-ANOVA P-value	F	R2cum	Q2cum
20	Well-watered	vs	Drought	vs	Waterlogged	0.011	2269	0.88	0.465
21	Well-watered	vs	Well-watered + Aphids	vs	Well-watered + Caterpillars	0.992	0.184	0.407	0.143
22	Drought	vs	Drought + Aphids	vs	Drought + Caterpillars	1	0		
23	Waterlogged	vs	Waterlogged + Aphids	vs	Waterlogged + Caterpillars	1	0		
24	Well-watered + Aphids	vs	Drought + Aphids	vs	Waterlogged + Aphids	0.004	3022	0.732	0.405
25	Well-watered + Caterpillars	vs	Drought + Caterpillars	vs	Waterlogged + Caterpillars	0.22	1339		

Supplementary Table 4C Volatile profile comparisons between treatments using OPLS-DA analyzed by CV-ANOVA of *R. sylvestris* plants.

No	Treatment 1	vs	Treatment 2	CV-ANOVA P-value	F	R2cum	Q2cum
1	Well-watered	vs	Drought	0.0004	12.389	0.713	0.579
2	Well-watered	vs	Waterlogged	0.008	6.036	0.584	0.376
3	Drought	vs	Waterlogged	0.003	6.014	0.925	0.586
4	Well-watered	vs	Well-watered + Aphids	1			
5	Drought	vs	Drought + Aphids	1			
6	Waterlogged	vs	Waterlogged + Aphids	1			
7	Well-watered + Aphids	vs	Drought + Aphids	0.062	2.838	0.848	0.431
8	Well-watered + Aphids	vs	Waterlogged + Aphids	0.435	1.01		
9	Drought + Aphids	vs	Waterlogged + Aphids	0.004	6.496	0.923	0.65
10	Well-watered	vs	Well-watered + Caterpillars	0.004	5.659	0.852	0.571
11	Drought	vs	Drought + Caterpillars	<0.001	15.369	0.916	0.804
12	Waterlogged	vs	Waterlogged + Caterpillars	<0.001	18.908	0.951	0.816
13	Well-watered + Caterpillars	vs	Drought + Caterpillars	0.001	7.9	0.886	0.664
14	Well-watered + Caterpillars	vs	Waterlogged + Caterpillars	0.007	5.274	0.878	0.569
15	Drought + Caterpillars	vs	Waterlogged + Caterpillars	0.007	5.076	0.952	0.701
16	Well-watered	vs	Drought + Aphids	0.048	3.625	0.598	0.287
17	Well-watered	vs	Drought + Caterpillars	0.005	5.726	0.814	0.589
18	Well-watered	vs	Waterlogged + Aphids	0.244	1.535		
19	Well-watered	vs	Waterlogged + Caterpillars	<0.001	13.228	0.923	0.768

No	Treatment 1	vs	Treatment 2	vs	Treatment 3	CV-ANOVA P-value	F	R2cum	Q2cum
20	Well-watered	vs	Drought	vs	Waterlogged	<0.001	6.661	0.609	0.453
21	Well-watered	vs	Well-watered + Aphids	vs	Well-watered + Caterpillars	0.058	2.427	0.348	0.302
22	Drought	vs	Drought + Aphids	vs	Drought + Caterpillars	0.028	2.955	0.427	0.38
23	Waterlogged	vs	Waterlogged + Aphids	vs	Waterlogged + Caterpillars	0.01	3.643	0.442	0.405
24	Well-watered + Aphids	vs	Drought + Aphids	vs	Waterlogged + Aphids	0.037	2.288	0.436	0.273
25	Well-watered + Caterpillars	vs	Drought + Caterpillars	vs	Waterlogged + Caterpillars	0.002	3.649	0.644	0.427

Chapter 5

How plants react to double trouble - Waterlogging and drought stress differentially affect responses to aphids in two closely related *Rorippa* species

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Abstract

Agriculture is challenged by climate change which brings about more extreme weather events and increased pest pressure. Understanding the adaptations of wild plants to complex environments with multiple simultaneous stressors can help develop resilient agricultural systems. Water stress, such as drought and waterlogging, affects various aspects of plant physiology. These changes can have contrasting effects on a plant's response to insect herbivores and may depend on plant adaptations to water stress.

Here, we compared a semi-aquatic and a terrestrial *Rorippa* plant species in how they deal with aphid attack under drought, well-watered and waterlogged conditions. We assessed how water conditions affect resistance to aphid feeding by measuring aphid performance and feeding behavior. To gain insight into the underlying physiological processes of coping with simultaneous water and aphid stress, we characterized the transcriptomic response and phloem metabolomics to aphid attack for plants under drought, well-watered and waterlogged conditions.

Aphid population growth on the semi-aquatic *Rorippa amphibia* was increased under drought conditions, whereas it was less affected by waterlogged conditions compared to well-watered conditions. Under drought conditions *R. amphibia* responded less vigorously to aphid attack apparent by reduced defense-related gene activity. In contrast, the terrestrial *R. sylvestris* that is adapted to drier soils, exhibited less pronounced effects of drought on responses to aphid attack. Aphid performance on *R. sylvestris* was reduced under waterlogged conditions, possibly because of an increase in reactive oxygen species associated with the response to waterlogging. Water conditions did not profoundly alter aphid feeding behavior on either plant species. Relative concentrations of measured primary metabolites differed between plant species. Other than a relative increase in proline in drought-treated plants, relative concentrations of primary metabolites and amino acids were not strongly affected by water availability.

This comparative approach highlights species-specific responses to combined water stress and aphid attack, influenced by aphid-induced responses and plant-specific water regime responses. By unraveling the intricate dynamics of plant responses to water stress and insect herbivory, we can gain valuable insights into the resilience of natural ecosystems and devise effective strategies for mitigating the impacts of climate change on agricultural ecosystems.

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Introduction

In the face of escalating climate change, agriculture faces an unprecedented challenge to ensure sustainable food production and the resilience of farming systems. An increased frequency of extreme weather events will expose crops to more severe drought spells as well as more frequent events of waterlogged conditions. These conditions may coincide with more frequent insect outbreaks. Warmer climates especially enhance population growth of aphids, that are prominent pests of a wide range of crops (Hulle et al. 2010; Yamamura and Kiritani 1998). Aphid damage has a bimodal impact, involving both a significant reduction in crop productivity due to stunting caused by their feeding, and acting as vectors for numerous plant viruses, detrimental to overall plant health (Capinera 2001; Kennedy et al. 1962; Petitt and Smilowitz 1982). To tackle these challenges, it is essential to understand and harness the remarkable adaptations observed in wild plants, which have evolved to thrive in diverse, often harsh, environmental conditions (Akman et al. 2012; Pierik and Testerink 2014; van Veen et al. 2013). In these environments, just like in agroecosystems, plants must respond to multiple types of abiotic and biotic stress simultaneously. Wild plants provide valuable lessons through their adaptations to multiple abiotic and biotic stressors. These adaptations demonstrate that the response to combined stressors is not merely additive but a unique interaction (Mittler 2006). Studying these adaptations can uncover genetic traits and physiological mechanisms that enhance crop resilience to complex environments.

One of the primary effects of water stress, both drought and waterlogging, is a reduction in water uptake (Blom 1999; Mewis et al. 2012). As a response, plants can alter their leaf morphology (Chaves et al. 2003). Leaves can get thinner due to a reduction in water potential, or rather thicker or tougher in response to water loss depending on the adaptations of the specific plant species (Grubb 1986; Hanley et al. 2007). Additionally, the epicuticular wax layer, which plays an important role in protecting plants against both biotic and abiotic stressors, grows thicker during drought in some plant species (Zhong et al. 2020). Both changes could affect host-plant quality for insect herbivores (Nalam et al. 2019). Furthermore, water stress can also lead to the mobilization of sugars and amino acids in order for the plant to utilize them in its response to water stress (Camisón et al. 2020; Mewis et al. 2012). These nutrients will end up in the phloem sap and will thus be taken up by aphids, which might increase their performance (Leybourne et al. 2021; Mewis et al. 2012; Mezgebe and Azerefegne 2021; Pompon et al. 2011). Phloem exudation of amino acids like asparagine, leucine and in particular proline were affected by water stress (Mewis et al. 2012; Stallmann et al. 2022). However, the extent and direction of these changes depend highly on the plant species and degree of stress the plants are subjected to. Furthermore, these effects could also modulate concentrations of defensive compounds like glucosinolates.

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Some studies have found a higher concentration of defensive compounds in leaves under waterlogging stress linked to a lower aphid growth rate (Mezgebe and Azerefegne 2021). Other studies have found similar or opposite effects of waterlogging and drought on leaf glucosinolate concentrations depending on the specific plant species and glucosinolate examined (Barber and Müller 2021; Khan et al. 2010; Teixeira et al. 2020). Whether this also translates to changes in the phloem sap remains to be investigated. This exemplifies that plant adaptations in response to water stress can have contrasting consequences for the effects of water stress on plant-insect interactions.

Additionally, these stressors can already interact at a transcription/signal transduction level. Recent studies found that stress combinations led to unique transcriptome profiles specific for the combination of stressors (Coolen et al. 2016; Mittler 2006; Nguyen et al. 2016; Rizhsky et al. 2004). This proves that the combination of stresses doesn't just lead to a simple sum of the two stress responses, but that it is clearly an integrated plant response to combinations of stress. An often-proposed mechanism by which responses to these stressors interact is through the crosstalk between phytohormones (Arbona and Gómez-Cadenas 2008; Hickman et al. 2019; Thaler et al. 2012; Zhang et al. 2015). Regulation of the plant stress response to drought and waterlogging is predominantly organized through abscisic acid (ABA) and ethylene (ET) (Fukao et al. 2011; Nakashima et al. 2014; Orellana et al. 2010; Voesenek and Bailey-Serres 2015). Regulation of responses to herbivory, on the other hand, is organized through jasmonic acid (JA) and salicylic acid (SA) (Moran and Thompson 2001; Riemann et al. 2015; Smith et al. 2009; Wu and Baldwin 2009). In general, SA is associated with a better defense against aphids while JA is associated with a better defense against chewers (Erb et al. 2012). A balance between JA and SA is however needed to fine tune responses against stressors (Beckers and Spoel 2006; Koornneef and Pieterse 2008). Interestingly, ABA and ET can modulate plant responses to an increase in the herbivory-associated phytohormone JA (Howe and Jander 2008; Marquis et al. 2020; Vos et al. 2013). Furthermore, some plants require SA together with ET and ABA to fully respond to waterlogging stress (Pan et al. 2021). SA can also increase drought tolerance by upregulating the biosynthesis of the osmo-protectant proline (La et al. 2019). This complex interactive network of phytohormonal synergists and antagonists might explain why certain plants are more resistant and others are less resistant to herbivory under water stress.

In this study we explored how two closely related plant species belonging to the *Rorippa* genus (Brassicaceae) that have distinct adaptive responses to water stress (Akman et al. 2012; Sasidharan et al. 2013) are affected in resistance to aphids under drought or waterlogged conditions. The semi-aquatic *Rorippa amphibia* grows along edges of lakes and swamps and is tolerant to regular waterlogging. In contrast, the terrestrial *Rorippa sylvestris* grows on drier, sandy soils, and is more adapted to drought. We hypothesize that these distinct adaptations to water stress predict how plants respond to a multi-stress environment of suboptimal water conditions and aphid attack. By taking a comparative approach we aim to uncover how variations in plant responses to water availability can affect plant-insect interactions in two differently adapted plant species. We measured the performance of *Myzus persicae* aphids on the two *Rorippa* plant species under a hydrological gradient ranging from drought, well-watered to waterlogged conditions. To explain aphid performance on the two plant species exposed to different water conditions, we measured differences in aphid feeding behavior on these plants using Electrical Penetration Graph (EPG) recording. We characterize the modulation of the physiological response to aphids under different water conditions by a plant transcriptome analysis after aphid feeding. Finally, we measured the metabolic composition of phloem exudates to link aphid performance to the composition of their food. Our data highlight the importance of considering plant adaptations to abiotic conditions in understanding how plants deal with combined abiotic and biotic stress.

Materials and Methods

Plants and Insects

Two closely related plant species of the *Rorippa* genus (Brassicaceae) were used. Seeds of the terrestrial *Rorippa sylvestris*, and rhizomes of the semi-aquatic *Rorippa amphibia* were collected around Wageningen, the Netherlands (51°57'38.2"N 5°39'41.9"E). Seeds were sown and rhizomes were planted in trays with Arabidopsis potting soil (Lentse Potgrond B.V., the Netherlands), watered, and kept under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D). Five weeks after sowing the seeds and two weeks after planting the rhizomes, plants reached a similar size and were transplanted into pots (ø 12 cm, 1 L) containing a 1:1 mix of Arabidopsis potting soil and sand (Lentse Potgrond B.V.). Plants were allowed to acclimatize in pots for one week prior to being subjected to their experimental treatments. The generalist *Myzus persicae* aphids were reared on *Raphanus sativus* (Brassicaceae) plants for generations under greenhouse conditions (22 ± 2 °C, 60-70% relative humidity, 16L:8D), except for the EPG experiment in which they were reared on *R. sylvestris* or *R. amphibia* plants for four weeks to ease their transition to the experiment.

Water treatments

To assess how different water regimes affect resistance to insect attack, plants were first randomly appointed to one of three water treatments: drought, well-watered, and waterlogged. These water treatments corresponded to 8 ± 4%, 20 ± 5% soil moisture content, and a submerged waterlogged soil respectively. The water levels were maintained by measuring soil water content daily with an electronic water potential meter (Extech MO750) and adding water accordingly. To achieve the water regimes, all pots were placed inside buckets (ø 21 cm, 2.75 L) and for the waterlogged treatments these buckets were filled with water up to the soil line (Fig. 1). The water treatments were maintained for the entirety of the experiments.

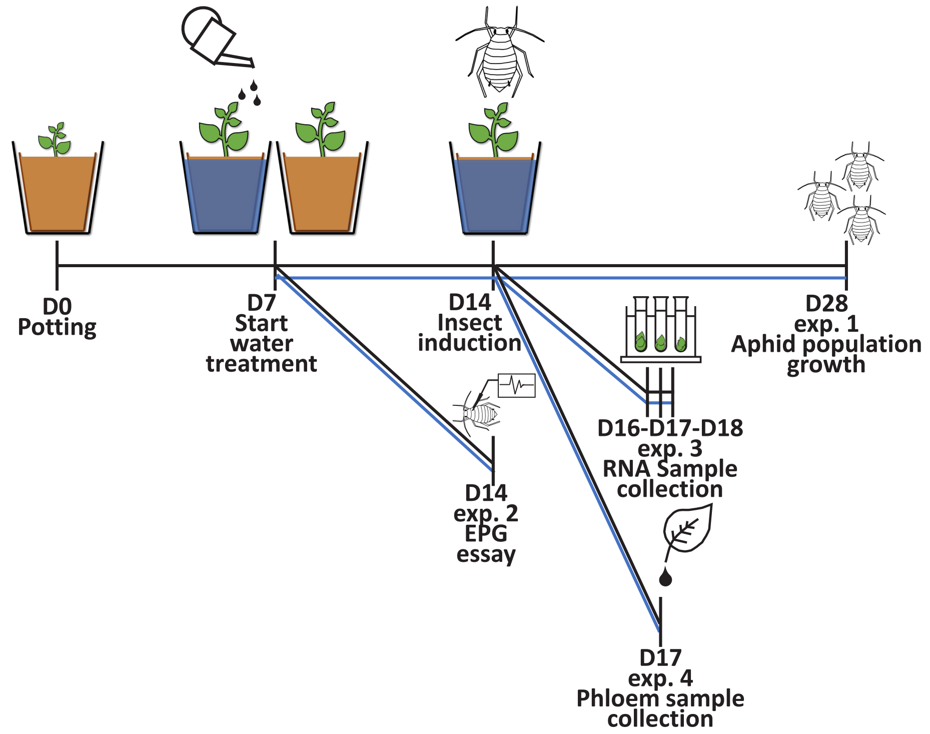


Figure 1. Timeline of each experiment of plant responses to waterlogging or drought stress and attack by the aphid *Myzus persicae*. Water treatments started 7 days after potting and continued throughout all experiments that measured (1) aphid performance, (2) aphid feeding behavior, (3) plant responses to aphid attack, (4) phloem chemical composition.

Aphid performance

Seven days after the onset of the water treatment, plants were infested with five adult wingless *M. persicae* aphids on a young, fully expanded leaf. The plants were then covered in a mesh bag to prevent aphids from moving to other plants. To assess aphid performance, 30 plants of each of the two *Rorippa* plant species were used for each water treatment. After 14 days the aphid population size was assessed by counting all aphids for each plant as a proxy for plant resistance to aphids.

The data were analyzed using RStudio (Allaire 2012) with R version 3.6.3 (R Core Team 2013), packages lme4 (Bates et al. 2015) lme4 emmeans (Lenth et al. 2019), and ggplot2 (Wickham 2016). A Generalized Linear Model (GLM) with gamma distribution including the full factorial interaction for plant species and water treatment followed by a Tukey's HSD post-hoc test was applied. To ascertain main effects between water treatments a separate model was run using only water treatments as explanatory factor followed by a Tukey's HSD post-hoc test.

Aphid feeding behavior

We measured feeding behavior of *M. persicae* aphids on plants seven days after the onset of the water treatments using Electrical Penetration Graph (EPG) recordings (Fig. 1). This was done by making the insect and plant part of an electrical circuit by attaching a gold wire (Ø 18 µm) to the dorsum of the insect using conductive water-based silver glue, and inserting an electrode into the soil (Tjallingii 1988). Each wired aphid was then moved to the youngest fully developed leaf of an individual plant which had received a specific water treatment (Fig. 1). EPG signals were recorded for 8 hours with a Direct Current Giga-8 EPG system (www.epgsystems.eu) and were performed at room temperature with a light intensity of 120 µmol m^{-2} s^{-2} (Philips TL5 HO 39W/840). Waveforms were annotated using EPG Stylet+ software (www.epgsystems.eu) (Tjallingii and Esch 1993). A total of 25 recordings were collected per plant species and water treatment combination. The calculation of behavioral variables was done using RStudio (Allaire 2012) and R version 3.6.3 (R Core Team 2013), according to Kloth et al. (2021). Waveforms that did not occur, were (1) considered zero for the calculation of the occurrence, number and total duration of behavioral variables, were (2) considered as missing data for the mean and maximum duration and proportion of behavioral variables and were (3) considered as the end of the recording for latency variables. Events that were interrupted by the end of the recording were included in all calculations, the duration of those events are thus truncated. To test for significant differences between treatments and plant species GLMs followed by a Tukey's HSD post-hoc test using R and the packages described above were applied.

Transcriptomic response in leaves

Seven days after the onset of their water treatment the youngest fully developed leaf per plant was bagged with a mesh bag and was either infested with 20 adult wingless aphids or left un-infested. After 48, 72 or 96 hours a single leaf sample was collected per plant from the bagged leaf. Aphids were first removed from the leaf using a soft brush. Un-infested leaves were also brushed to equalize treatments. For *R. amphibia* plants an augur (Ø 1 cm) was used to punch out three leaf discs. Since the leaf shape of *R. sylvestris* plants is not fit for an augur, we used scissors to cut off three similarly sized leaflets. Leaf samples were taken on or as close to aphid feeding sites as possible. All equipment was rinsed with RNaseZap (Thermofisher Scientific) before harvesting each sample. The leaf material of three plants from the same treatment was pooled into one biological replicate and flash frozen in liquid nitrogen. A total of five biological replicates were collected. All samples were stored at -80°C.

We extracted RNA from *R. sylvestris* samples using a BioLine ISOLATE II Kit (Meridian Bioscience) in accordance with the manufacturer's instructions. As RNA yields were too low for *R. amphibia* using only this kit, we used TRIzol (Thermofisher Scientific) for the RNA extraction and the BioLine ISOLATE II Kit (Meridian Bioscience) for clean-up. A detailed protocol can be found in the supplementary (Supplementary Methods 1). All samples were checked for purity and quantity using a BIOANALYZER (Agilent Technologies) with RNA chip (Agilent RNA 6000 Nano) in accordance with the manufacturer's instructions.

To determine the effect of the water treatments on the response of both plant species to aphids over time we measured transcription levels of two related genes, i.e. WRKY DNA-binding protein 70 (WRKY70) and pathogenesis-related protein 1 (PR1) that are characteristic for plant responses to aphid feeding (Kroes et al. 2015; Li et al. 2004) using qPCR at different time points (48, 72, 96 h). We used beta-tubulin (TUB) and actin 2 (ACT2) as reference genes (Nicot et al. 2005). Both WRKY70 and PR1 showed the most interesting differences between treatments 72 hours after aphid induction (Supplementary Fig. 10). We therefore sent out the samples that were taken 72 hours after aphid induction for RNA sequencing (BGI Tech Solutions).

RNA samples were sequenced paired end, 150 bp, with a minimum sequencing depth of 30 million read pairs. Adapter sequence on low quality base calls were removed with Trimmomatic and library quality was assessed using FASTQC (Andrews 2017). For each species a *de novo* transcriptome was assembled strand specifically with Trinity (Haas et al. 2013), using a Kmer length of 31 and a minimal Kmer abundance of 2. Transcriptomes were annotated and assessed for completeness by blast search against a known genome of the closely related *Rorippa islandica* (NCBI:txid157092)(Schoch et al. 2020) and *Arabidopsis thaliana* (TAIR10)(Lamesch et al. 2012) as well as transcript coverage by the sequenced libraries. Transcripts with a raw read count of less than 10 were filtered out. Differential expression and transcript abundance were estimated with DEseq2 (Love et al. 2014).

To identify orthologs between the transcriptomes of the two *Rorippa* species we performed an all-vs-all blast, which also included *R. islandica*. Groups of orthologous sequences were subsequently identified with Orthofinder (Emms and Kelly 2015). Here the inflation parameter was optimized to obtain the highest number of groups with orthologs from all three species, in this case the inflation was 1.6.

Transcript abundance in our samples was assessed by mapping the reads to the corresponding *de novo* transcriptome with Kallisto (Bray et al. 2016). By comparing each treatment pairwise to the well-watered un-infested treatment of the same plant species we calculated differentially expressed genes (DEGs) with DEseq2 at gene level per species or orthogroup when comparing both species. Z-scores were then calculated based on the Log$_2$ fold changes (LFC) of all DEGs. We then performed a hierarchical clustering on the Z-scores. Clusters were annotated by performing a gene ontology enrichment analysis using GOseq (Young et al. 2012).

Metabolomics of phloem exudates

For each plant, the youngest fully developed leaf was bagged seven days after the onset of the water treatments and was either infested with 20 adult wingless aphids or left un-infested. Per treatment group (i.e., plant species * water treatment * herbivory treatment), there were *n* = 8 biological replicates (pooled from three plants each, see below). Three days after the aphid infestation had started, phloem exudates of the leaves that had been bagged (either with or without aphids) were collected and analyzed according to Stallmann et al. (2022). with some modifications. To avoid the contamination of the samples with aphid honeydew, cotton wool was attached at the bottom of all bags during aphid infestation. The leaves of three different plant individuals of the same treatment group were pooled. Aphids were removed with a brush from the leaves, with control plants (no aphids) being likewise treated with a brush. The leaves were cut at the base of their petioles, re-cut in ethylenediaminetetraacetic acid solution (EDTA; Sigma-Aldrich; 8 mM, pH = 7) and incubated with their cutting edges in 50 mL Falcon tubes with 1 mL 8 mM EDTA solution to prevent sieve tube plugging (2 h, in the dark at 22 ± 2 °C, 60-70% relative humidity). Afterwards, the EDTA was washed off with Milli-Q water and leaves were further incubated in 1 mL Milli-Q water (2 h, in the dark at 22 ± 2 °C, 60-70% relative humidity) to collect phloem exudates. To correct for background compounds and contaminants, eight blanks were included from this step onwards. Then, aliquots (300 µL for each analytical platform, see below) were frozen in liquid nitrogen, stored at -80 °C and lyophilized.

For the analysis of carbohydrates, organic acids and the cyclic polyol *myo*-inositol, metabolites were extracted in 80% methanol, derivatized (methoximation, silylation) and measured by gas chromatography coupled to mass spectrometry (GC-MS). Amino acids were analysed via high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD)

with pre-column derivatization after the samples had been extracted in 80% methanol (for detailed methods, see Supplementary Methods 2).

GC-MS data were analyzed using GCMS Postrun Analysis (GCMSsolution 4.45; Shimadzu), while for the analysis of the HPLC-FLD data OpenLab ChemStation C.01.07 (Agilent Technologies) was used. Some samples had to be excluded from the data set, as they were lost due to problems during sample collection or technical issues with the GC-MS or HPLC-FLD. The final sample sizes per treatment were *n* = 6-8 biological replicates. Analytes that were measured via GC-MS were identified based on Kóvats retention indices (Kovats 1958), as well as on mass spectra, comparing both to reference substances measured under the same conditions and to an in-house database as well as to entries in the Golm metabolome database (Kopka et al. 2005). Amino acids were identified by comparing retention times with those of reference standards. For both analytical platforms, peak areas (for GC-MS: based on total ion currents) were used for quantification, including only chromatographically well-separated peaks. Peak areas were normalized by dividing them by the peak area of the corresponding internal standard. For the amino acids, calibration response factors (relative to the internal standards) were applied in addition. Blank subtraction was done, using average peak areas found in the blanks. For metabolites measured by GC-MS and showing more than one analyte (i.e., fructose and glucose), the corresponding peak areas were summed. Only metabolites were left in the data set, which occurred in at least half of the replicates of at least one treatment group. For further data analyses, percent data (0-100%) were used, because the phloem exudation rate may have differed between the plant species and treatment groups and thus absolute concentrations could not be used for comparing samples. For the amino acids, the data are given on a molar basis (mol%) based on the application of calibration response factors. Nonmetric multidimensional scaling analyses were applied for GC-MS as well as for HPLC-FLD data in R 4.2.1 (R Core Team 2013) using the package *vegan* (Oksanen et al. 2013), both across and within plant species. For this, Wisconsin double standardization of square root-transformed data and Kulczynski distances were used. Proportional differences between treatments in the abundance of specific metabolites were determined by using a Mann-Whitney U test. Amino acids were categorized as essential *versus* non-essential based on (Douglas 2006), with the following ones being considered as essential: histidine, threonine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine and lysine.

Results

Aphid performance is affected by water availability

The population size of *M. persicae* after 14 days was significantly affected by plant species (GLM, χ^2=139.646, df=1, P<0.001), by water treatment of their host plant (χ^2=41.254, df=2, P<0.001) as well as by the interaction of these factors (χ^2=23.555, df=2, P<0.001), indicating an effect of water availability on plant resistance to aphids (Fig. 2). Aphids generally performed better on *R. sylvestris* than on *R. amphibia* plants. On the semi-aquatic *R. amphibia*, aphids performed better on drought-treated plants than on well-watered or waterlogged plants, indicating a lowered resistance of *R. amphibia* under drought conditions in the face of aphid herbivory. In contrast, on the terrestrial *R. sylvestris*, aphid performance was not significantly altered by drought but was instead negatively affected by waterlogging, indicating a heightened resistance under waterlogged conditions. Aphid feeding behavior differed between plant species but not water treatment. EPG data revealed profound differences in aphid feeding behavior between the two plant species (Supplementary Table 1). Aphids spent a significantly longer time ingesting phloem sap on *R. sylvestris* than on *R. amphibia* (GLM: χ^2=21.6, df=1, P=0.003) (Fig. 3A). Furthermore, 91% of the aphids performed one or more phloem sap feeding events on *R. sylvestris* within the 8-hour recording, opposed to only 42% of the aphids on *R. amphibia*. These differences correlate with an overall lower aphid population growth rate on *R. amphibia* (Fig. 2). Interestingly, all aphids were able to reach the sieve tube in *R. amphibia*, as they all

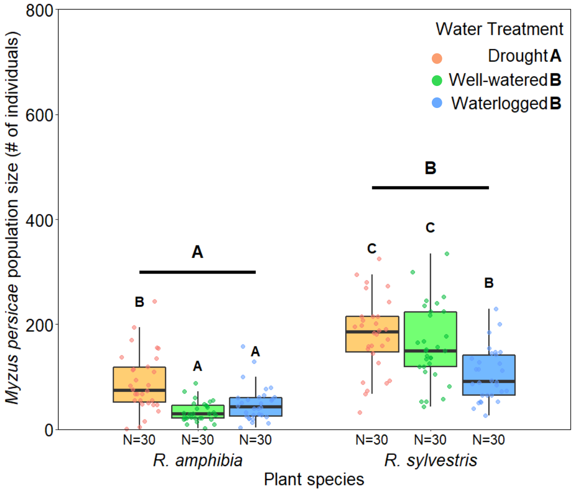


Figure 2. Population size of the *Myzus persicae* aphids after 14 days on *R. amphibia* or *R. sylvestris* plants that were subjected to different water treatments (drought, well-watered and waterlogged). Letters in the legend show main effects of water treatment, letters above the plant species show main effects of plant species and letters above each bar show significant differences among treatments both within and between species (GLM with Tukey's HSD post hoc, Gamma distribution, α = 0.05).

salivated in the phloem sap at least once and showed comparable latency between the first pathway probe and the first salivation in the phloem as aphids feeding on *R. sylvestris* (GLM: χ^2=1.54, df=1, P=0.214) (Supplementary Table 1). This illustrates that localization of the phloem was equally successful on both species, but phloem sap ingestion was more problematic in *R. amphibia*. The total time spent drinking xylem showed the opposite pattern. Aphids spent significantly more time ingesting xylem sap on *R. amphibia* than on *R. sylvestris* (GLM: χ^2=8.68, df=1, P=0.003) (Fig. 3B), most likely due to the unsuccessful phloem-sap feeding and need for alternative water and nutrient resources.

Water treatments imposed only minor effects on feeding behavior in a host plant-specific manner. No significant difference was found in the total time ingesting phloem or xylem sap between water treatments (GLM: χ^2=3.36, df=2, P=0.187) (GLM: χ^2=4.97, df=2, P=0.083). Likewise, water treatments did not significantly alter the latency between the first probe and salivating in the phloem (GLM: χ^2=1.20, df=2, P=0.549) (Supplementary Table 1).

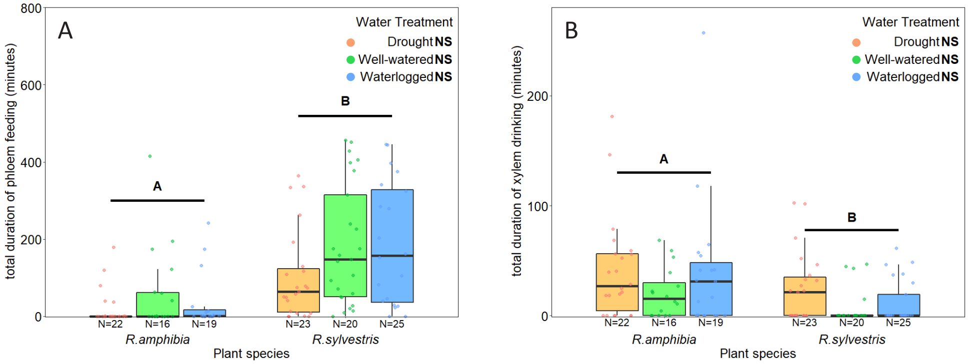


Figure 3. Traits from EPG recordings of the behavior of *Myzus persicae* on *Rorippa amphibia* or on *Rorippa sylvestris* plants that were subjected to different water treatments (drought, well-watered or waterlogged). EPG recordings were done for 8 hours. The **A** total duration spent feeding from phloem, **B** total duration spent drinking from xylem. Letters above treatments depict significant differences between species treatments. Main effects of water treatment were not significant (NS) (GLM, gamma distribution, α = 0.05).

Leaf transcriptome shows distinct responses to double trouble

De novo transcriptomes for *R. amphibia* and *R. sylvestris* consisted of 22826 and 39036 pseudogenes to characterize responses to feeding by the aphid *M. persicae* under waterlogged, well-watered and drought conditions. 18443 orthogroups were identified as common in all three species by blasting the transcriptomes against each other and against the known genome of the closely related *R. islandica* (NCBI:txid157092)(Schoch et al. 2020). Of these orthogroups, 16729 had more than 10 counts and were used to calculate differentially expressed genes (DEGs), comparing all other treatment groups to well-watered un-infested plants within each plant species.

Leaf transciptome responses to the different water regimes and aphid feeding were highly plant species-specific. When comparing the responses to drought versus waterlogging, the majority of the DEGs reacting to water stress could be attributed to drought in *R. amphibia* (436 regulated by drought, 37 by waterlogging) and to waterlogging in *R. sylvestris* (527 regulated by waterlogging, 8 by drought) (Fig. 4). Furthermore, in *R. sylvestris* only 6 DEGs were identified in response to herbivory under well-watered conditions as opposed to 1358 (820 up- + 538 down-regulated) DEGs in *R. amphibia* under similar conditions. This indicates that *R. sylvestris* responds less vigorously to aphid infestation under well-watered conditions than *R. amphibia*. However, 1344 unique DEGs were recorded for the combination of waterlogging and herbivory, suggesting *R. sylvestris* responds more strongly to the aphids when it is waterlogged (Fig. 4). Furthermore, many DEGs in both plant species were unique to a combination of water stress and herbivory (*R. amphibia*: 1280 *R. sylvestris*: 1358), revealing that the response to simultaneous stress is more than a simple sum of the response to both stresses individually (Fig. 4).

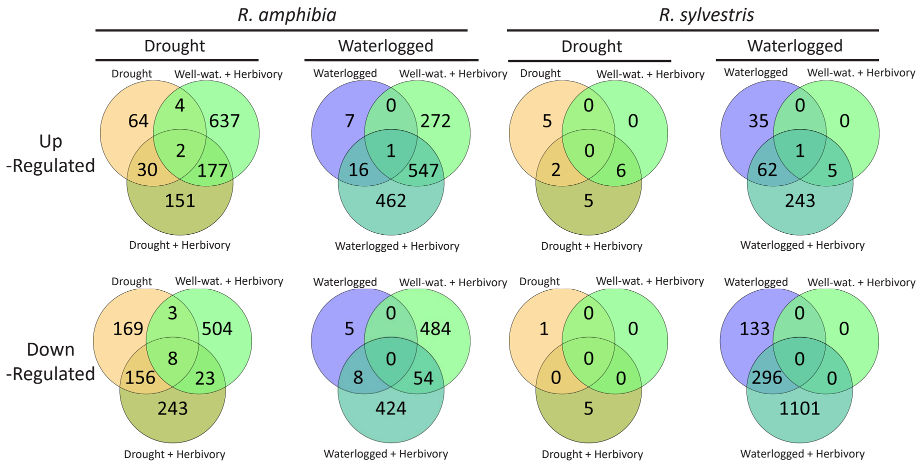


Figure 4. Venn diagrams showing the number of DEGs in *Rorippa amphibia* and *Rorippa sylvestris* leaves of plants that were subjected to different water treatments (drought, Well-wat. = well-watered and waterlogged) and to different herbivory treatments (un-infested or infested with *Myzus persicae* aphids). DEGs were calculated by comparing all treatment groups to well-watered plants without herbivores separately for R. amphibia and R. sylvestris plants and divided in up- and down- regulated DEGs. Overlapping areas indicate DEGs that were differentially expressed in two or three treatments compared to the common control group. The following cut-off values for DEGs were set: adjusted P value < 0.01 and $\log_2$ fold change > 0.5.

Hierarchical clustering of the DEGs confirms that *R. amphibia* responded more strongly to drought in both up- and down regulation of genes, while *R. sylvestris* plants were more responsive to waterlogging (Fig. 5). The overall response to herbivory was much stronger for *R. amphibia* than for *R. sylvestris*. Additionally, for the two plant species, different clusters of genes respond to simultaneous water stress and herbivory. While both plants are impacted by double trouble in their SA response, the specific genes involved seem to be located in different clusters. In *R. amphibia* drought + herbivory caused a different reaction compared to drought or herbivory in clusters 3 and 5. In *R. sylvestris* waterlogging + herbivory caused a different reaction compared to waterlogging or herbivory in cluster 7. This indicates that each species has its own unique mechanism of responding to combinations of stress.

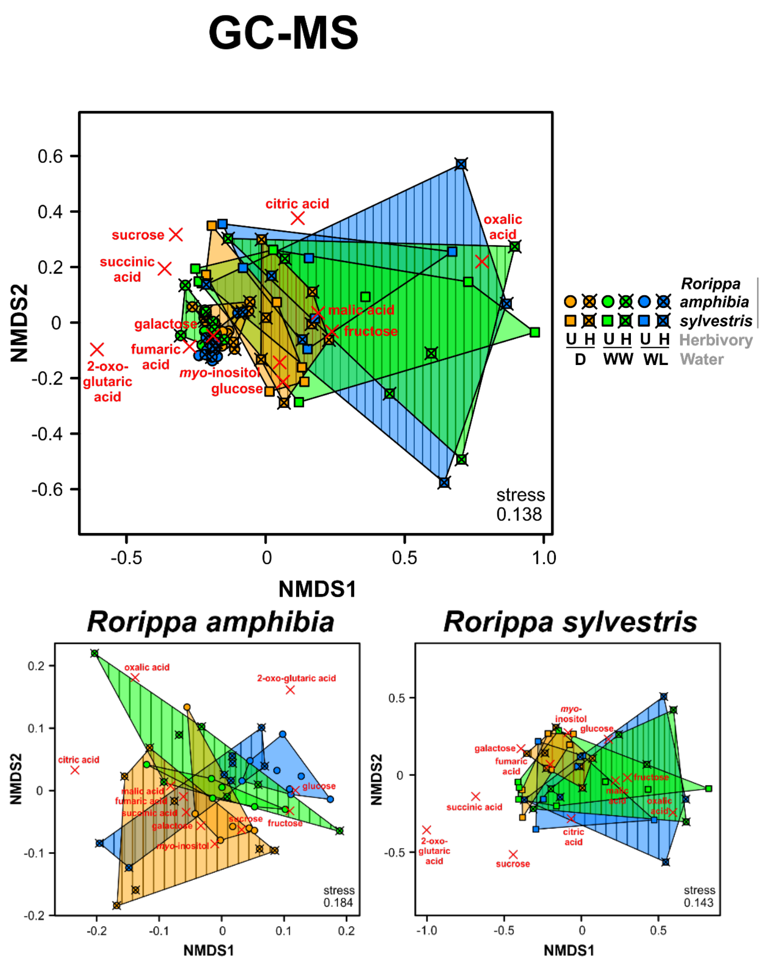
Figure 5. Heatmap of hierarchical clustering of Z-scores of all DEGs from *Rorippa amphibia* and *Rorippa sylvestris* plants under different water (drought, well-watered, waterlogged) and herbivory (un-infested or infested with *Myzus persicae* aphids) treatments. GO enrichment analyses were performed on each cluster and overrepresented GO terms are presented on the right. Cut-offs were set at a $\log_2$ fold change > 1 and an adjusted P-value < 0.001. Number of DEGs in cluster 1=110, 2=287, 3=215, 4=45, 5=122, 6=159, 7=125, 8=84, 9=27, 10=51.

Mapping of the regulated transcripts to GO terms revealed that the transcripts relate to genes with diverse functions. Responses of *R. amphibia* to attack by *M. persicae* were characterized by upregulation of genes involved in SA responses, indole glucosinolate biosynthesis, and systemic acquired resistance (WRKY38, WRKY50, WRKY51, WRKY62, RKS1, ACS6, EVR, ERD6, XBAT34). Particularly genes involved in phytoalexin and camalexin biosynthesis were upregulated after aphid feeding (GSTU4, CRT3). Interestingly, these responses were weaker when the plants were drought stressed (Cluster 3 and 5, Fig. 5). Additionally, genes involved in flavonoid biosynthesis were downregulated in drought treated, aphid-infested *R. amphibia* plants. This indicates that the plant's chemical defense was suppressed by drought stress, which matches with the increased performance of *M. persicae* on drought-treated *R. amphibia* (Fig. 2). Remarkably, this response was already visible in plants that were solely drought stressed without aphids but was in many cases amplified by the presence of aphids on the plant (Fig. 5).

R. sylvestris, on the other hand, did not respond strongly to 72 hours of aphid feeding. Only when waterlogged does *R. sylvestris* show a slight upregulation of phytoalexin and camalexin biosynthesis genes in response to aphids. In contrast to *R. amphibia*, the response of *R. sylvestris* to aphid feeding is characterized by a distinct SA response that is associated with a hypersensitive response (Cluster 6 and 7, Fig. 5). This coincides with a downregulation of genes involved in ROS scavenging and antioxidants (ALDH12A1, MDH2, ATHNIR). Interestingly, it only shows this response when the plant is waterlogged and corresponds with the decreased performance of aphids on waterlogged *R. sylvestris* plants (Fig. 2). Similar to *R. amphibia*, this response was already visible in plants that are waterlogged without aphids but was amplified by the presence of aphids on the plant.

Phloem metabolomics

In total, four carbohydrates, six organic acids and one polyol were retained in the GC-MS data set of phloem exudates, while the HPCL-FLD data set comprised 20 amino acids, including the secondary amino acid proline as well as 19 primary amino acids, of which nine are considered to be essential for aphids (Fig. 7) (Douglas 2006). Compared to the essential amino acids, the non-essential amino acids were dominant. The two plant species largely



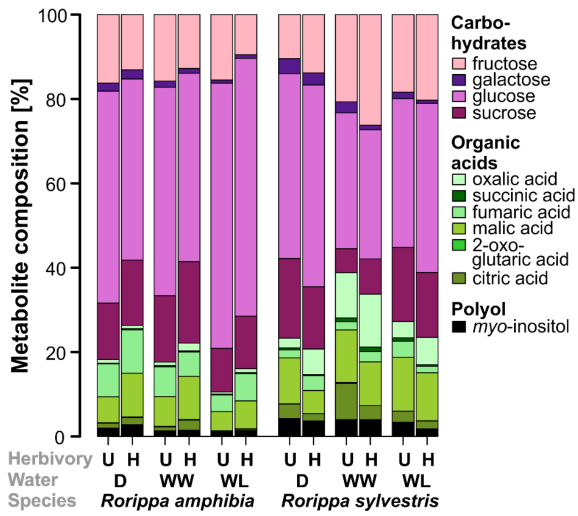
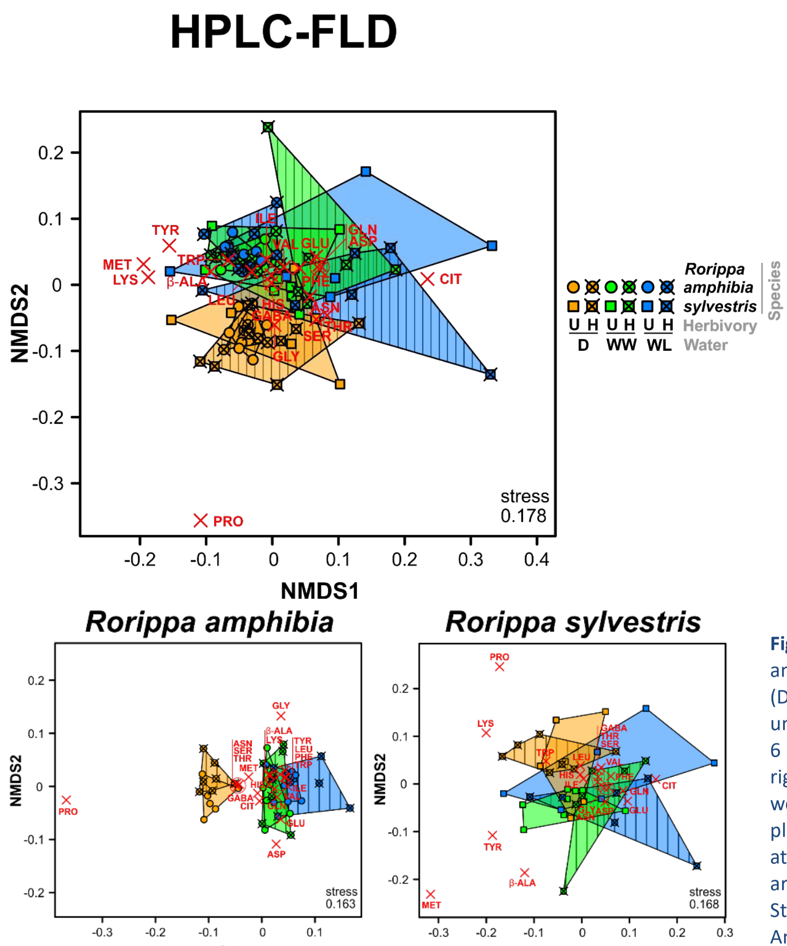


Figure 6. Metabolite profiles of phloem exudates of *Rorippa amphibia* and *Rorippa sylvestris* plants that were subjected to different water (D, drought; WW, well watered; WL, waterlogged) and herbivory (U, un infested; H, infested with *Myzus persicae* aphids) treatments n = 6 8). Metabolites measured by GC MS. Relative concentrations (0 100%) of the metabolites were used for the analyses. **A:** Non metric multidimensional scaling plots across both species and within species, with stress values given at the bottom, treatment groups surrounded by convex hulls (hatched areas: herbivory treatment) and metabolite loadings as red crosses. **B:** Stacked bar plots showing the mean proportions of the metabolites.



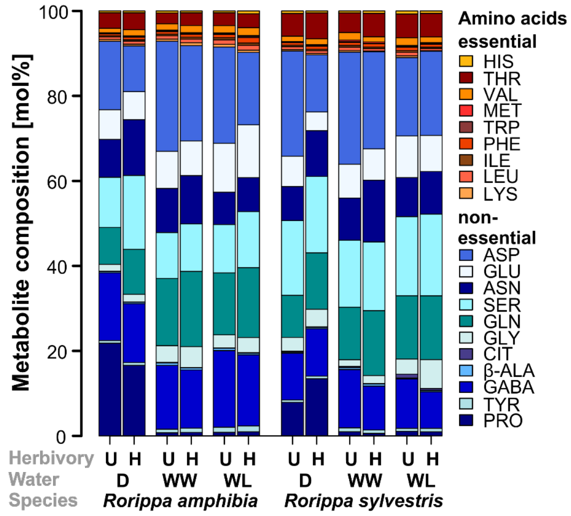


Figure 7. Metabolite profiles of phloem exudates of *Rorippa amphibia* and *Rorippa sylvestris* plants that were subjected to different water (D, drought; WW, well watered; WL, waterlogged) and herbivory (U, un infested; H, infested with *Myzus persicae* aphids) treatments n = 6 8). Metabolites measured by HPLC FLD are shown on the left and right, respectively. Relative concentrations (0 100%) of the metabolites were used for the analyses. **A:** Non metric multidimensional scaling plots across both species and within species, with stress values given at the bottom, treatment groups surrounded by convex hulls (hatched areas: herbivory treatment) and metabolite loadings as red crosses. **B:** Stacked bar plots showing the mean proportions of the metabolites. Amino acids are given in the common three letter code; CIT, citrulline; GABA, y aminobutyric acid.

differed in the composition of the metabolites measured via GC-MS, but less in the amino acid profiles (Fig. 6 and Fig. 7). the primary metabolite profiles measured with GC-MS of *R. amphibia* showed less variation than those of *R. sylvestris*. There were several metabolites that were differently abundant when comparing well-watered un-infested plants of the two species. The proportion of oxalic acid was on average 13 times higher in *R. sylvestris* than in *R. amphibia* (U=1, P=0.001), and the proportion of the essential amino acid threonine was on average 1.8 times higher in *R. sylvestris* than in *R. amphibia* (U=0, P<0.001) (Supplementary Tables 2&3). Furthermore, the proportion of essential amino acids was 1.8 times higher in *R. sylvestris* than in *R. amphibia* (U=5, P=0.013) (Supplementary Table 3). In un-infested plants, the effects of different water treatments were most obvious in *R. amphibia.* Mainly the amino-acid profile of the drought-treated groups formed a distinct cluster in NMDS analyses (Fig. 7). This difference could mainly be attributed to a higher proportion of proline and a lower proportion of aspartic acid and glutamine in drought-treated plants (PRO: U=0, P=0.002, ASP: U=35, P=0.004, GLN: U=36, P=0.002). Waterlogging on the other hand had a bigger impact on GC-MS-measured metabolites where the proportion of citric acid (U=47, P=0.001) and galactose (U=47, P=0.001) was lower in waterlogged than in well-watered *R. amphibia* (Supplementary Table 2) (Fig. 6).

Discussion

The two closely related plant species, *Rorippa amphibia* and *Rorippa sylvestris*, exhibit distinct responses to variation in water regime and aphid infestation. These species-specific adaptations to water availability are reflected in their induced responses to aphid feeding and subsequent effects on aphid performance. *Rorippa amphibia*, which is better adapted to waterlogging, displays minimal alterations in its response to herbivory under waterlogged conditions compared to well-watered plants. However, when subjected to drought treatment, aphid populations exhibit significantly accelerated growth. Transcriptomic analysis reveals a decline in the activity of defense-related genes during drought stress, potentially accounting for the enhanced aphid performance. On the other hand, *R. sylvestris*, thriving in drier soils, experiences less pronounced impacts during drought compared to *R. amphibia*. In contrast to their performance on *R. amphibia*, aphids exhibit diminished performance on waterlogged *R. sylvestris* plants. This observation aligns with the transcriptomic profile of *R. sylvestris*, which indicates limited aphid-induced responses except under waterlogged conditions. These findings exemplify how species-specific responses to the combined stressors of water regime and aphid attack can be attributed to both the overall response to aphid feeding and the specificity of response to different water regimes, with or without aphid infestation.

Drought reduced the resistance of *R. amphibia* to aphids while having no significant impact on resistance of *R. sylvestris*. No significant differences were found in the feeding behavior of aphids on drought-treated *R. amphibia* plants. This suggests the phloem sap, rather than the path leading up to it, causes aphids to perform better on drought-treated *R. amphibia*. This finding contrasts with observations from other studies, which typically report poorer aphid performance under drought stress on various plant species (Huberty and Denno 2004; Leybourne et al. 2021; Simpson et al. 2012). Generally, reduced aphid performance under drought stress is associated with decreased plant vigor and increased chemical defenses in drought-stressed plants (Beetge and Krüger 2019; Inbar et al. 2001; Simpson et al. 2012; Xie et al. 2020). However, other studies report a lower chemical defense after drought (Barber and Müller 2021; Mewis et al. 2012). In line with these observations, our transcriptomic data reveal a lower expression of genes related to chemical defenses in drought-treated *R. amphibia* plants. Furthermore, drought-treated plants were characterized by a higher proportion of proline in their phloem exudates, which is a common response of plants to drought (Hayat et al. 2012; Stallmann et al. 2022). Proline is a known osmo-protectant in many plant species, aiding plants in water retention under drought (Dar et al. 2016; Hayat et al. 2012). Interestingly, proline was found to be an important energy source for several hymenopteran, dipteran, coleopteran and orthopteran insects (Bouchebti et al. 2022; Bursell 1981; Noor-ul-Ane and Jung 2022; Sacktor and Childress 1967; Stec 2018; Teulier et al. 2016). These insects show the capability to quickly and efficiently convert proline to energy predominantly used for long flights (Bursell 1981). Levels of proline in the plants have even been found to influence host-plant choice by two orthopteran insect species (Behmer and Joern 1994). The effects of proline on aphid performance are not well-studied. However, increased proline content might have a positive effect on aphid growth rates. Apart from proline, we did not observe significant differences in phloem exudate composition after drought treatment. Yet, we cannot exclude that absolute concentrations did change. Other studies have reported higher concentrations of amino acids in the drought-treated compared to waterlogged plants associated with an increase in aphid performance (Mewis et al. 2012). However, this was measured in leaf tissue and not in phloem directly. Furthermore, increased nutrient concentrations can also lead to higher osmolarity in the phloem, which may negatively impact aphids (Pompon et al. 2011). Further research is needed to investigate how these water-condition-induced changes in phloem sap relate to the dietary requirements of aphids.

The performance of *M. persicae* aphids on *R. sylvestris* plants was negatively impacted when the plant was waterlogged. Although responses to waterlogging are less well studied than responses to drought, the consistent pattern of reduced aphid performance suggests a potential link between waterlogging and alterations in primary and secondary metabolites

present in the phloem exudates (Khan et al. 2010; Lin et al. 2021; Mewis et al. 2012). In our study, the proportions of different phloem exudates in *R. sylvestris* were not significantly influenced by waterlogging. We cannot, however, exclude that absolute concentrations of metabolites were affected by waterlogging. In another study with *A. thaliana,* waterlogging resulted in lower total amino acid and sugar concentration in the phloem exudates compared to drought (Mewis et al. 2012). This could explain the lower performance of aphids on waterlogged *R. sylvestris* plants.

Additionally, transcriptome analysis revealed that *R. sylvestris* exhibited a strong response to waterlogging. However, *R. sylvestris* seems to hardly react to aphid herbivory. Apart from a slight increase in phytoalexin biosynthesis genes, when the plant experiences simultaneous waterlogging and herbivory, no other signals indicative of a direct chemical defense were observed. Other studies have shown that concentrations of defensive compounds were affected by waterlogging. In *A. thaliana* indole glucosinolate concentrations increased compared to drought while in *B. oleracea* glucosinolate concentrations decreased after waterlogging (Barber and Müller 2021; Khan et al. 2010; Mewis et al. 2012). Interestingly, *R. sylvestris* displayed an enrichment of upregulated genes involved in a hypersensitivity response rather than chemical defense. Although hypersensitivity responses are typically associated with resistance against plant pathogens, previous studies have linked hypersensitivity responses at aphid feeding sites with reduced aphid survival (Belefant-Miller et al. 1994; Fernandes 1990; Goggin 2007; Lyth 1985). While no visible necrosis was observed, localized cell death may interfere with aphid feeding. Our EPG data on aphid feeding behavior did not provide evidence of this interference. However, we only measured aphid feeding behavior for the first eight hours. It is possible that this hypersensitivity response takes longer to manifest or requires stronger induction to become initiated. Additionally, *R. sylvestris* exhibited an enrichment of downregulated genes involved in scavenging reactive oxygen species (ROS). ROS are important signaling molecules in plant responses to abiotic stress and hypersensitivity responses (Foyer et al. 2016; Sharma et al. 2012; Woźniak et al. 2019). Moreover, other studies have found a correlation between strong and fast ROS production upon aphid feeding and resistance against aphids (Botha et al. 2014; Goggin 2007; Kerchev et al. 2012; Qi et al. 2020). Although the mechanistic details are not yet fully understood, this illustrates another way in which abiotic stress can influence plant-insect interactions. Furthermore, in the waterlogging-adapted *R. amphibia*, minimal differential gene expression was observed under similar waterlogging conditions. This further demonstrates that the specific adaptations of plant species to particular abiotic conditions determine the manner in which abiotic factors impact the plant's response to insect herbivory.

Future perspectives

This study contributes to our understanding of how plants respond mechanistically to simultaneous stressors, shedding light on their implications for plant-herbivore interactions. We demonstrate that plants can exhibit either impaired or enhanced responses to aphid herbivory depending on the water levels they experience and their adaptation to different water conditions. Comprehending the ecological consequences of altered water availability necessitates further investigating plants within a broader community context, encompassing other plant and insect species. This includes understanding how water availability can affect herbivore community assembly on plants as well as multitrophic interactions that plant-aphid interactions are part of. Water availability can affect the oviposition preference of herbivores (Helmberger et al. 2016; Showler and Castro 2010), and the recruitment of natural enemies that reduce the impact of herbivore attack on plants (Kansman et al. 2021; Martini and Stelinski 2017; Salerno et al. 2017; Weldegergis et al. 2015). Our work highlights that it is essential to recognize that responses to multiple stressors are highly plastic and vary among plant species, as evident from the contrasting effects observed in our study involving two closely related plant species. Understanding how wild plants have evolved diverse responses to multi-stress situations can provide valuable insights into the resilience and adaptability of plants in complex environments. By breeding for resilience and inducibility we can better prepare our crops and food systems to a changing climate, characterized by more frequent weather extremes and increased insect herbivory resulting from insecticide resistance.

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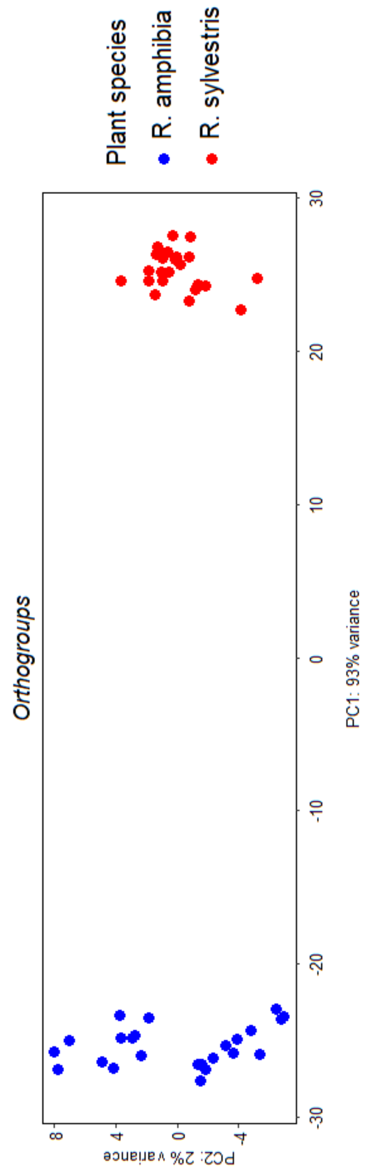
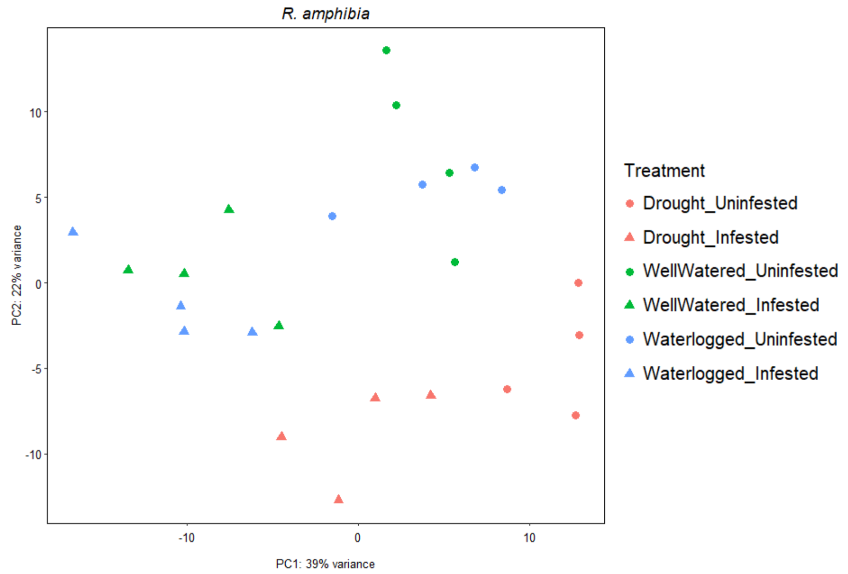
Supplementary Information

Supplementary Table 1. Mean ± st. dev. of several EPG measured traits on two plant species *R. amphibia* and *R. sylvestris* tested for significant differences using a Generalized Linear Model with Gamma distribution using Plant species * Water treatment as explanatory factors (GLM, *P*<0.05)

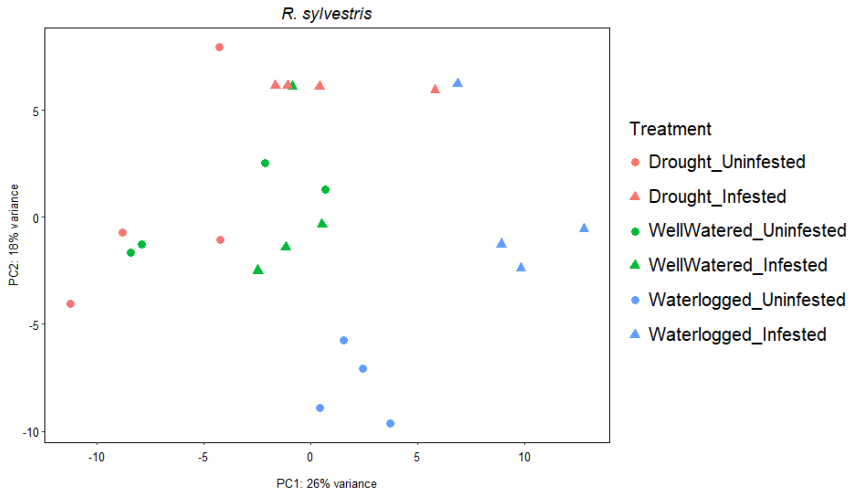
		R. amphibia			***R. sylvestris***			**Main effects**		
Factor	**Discription**	**Well-watered**	**Drought**	**Waterlogged**	**Well-watered**	**Drought**	**Waterlogged**	**Plant-species**	**Water-treatment**	**Interaction term**
sum.Np	sum of time non-probing (min)	35.4 ± 29.1	47.4 ± 29.1	41.0 ± 30.5	50.7 ± 46.7	52.3 ± 28.4	50.7 ± 44.6	0.104	0.641	0.721
lat.C	Latency to first pathyway from start of recording (min)	4 ± 13	7.7 ± 28	0.7 ± 2.5	13.1 ± 46.2	2.5 ± 9	1.2 ± 3.9	0.850	**0.019**	0.264
mean.C	mean time spent on pathway (min)	13.1 ± 6.2	10.9 ± 5.7	10.1 ± 6.9	9.1 ± 7.6	9.4 ± 5	10.8 ± 5.4	0.215	0.869	0.294
lat.e1p	Latency to first salivation in phloem from start of first probe (min)	119.2 ± 118.6	121.9 ± 100.2	81.2 ± 106.6	73.3 ± 63.9	94.2 ± 65.0	85.6 ± 91.6	0.214	0.550	0.465
sum.e1	Total duration salivation in phloem (min)	62.3 ± 37.8	58.3 ± 34.3	69.7 ± 36.4	27.6 ± 23.8	45.1 ± 38.7	31.9 ± 30.8	**0.013**	0.388	0.173
mean. e1	Mean duration salivation in phloem (min)	11 ± 10.7	9.5 ± 8.5	8.6 ± 5.7	3.8 ± 2	4.4 ± 4.1	5.2 ± 4	**<0.001**	0.909	0.299
occ. sing.e1	Does insect perform salivation in phloem? (1=yes, 0=no)	0.9 ± 0.3	1 ± 0.2	1 ± 0	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.298	0.738	0.241
occ.e2	Does insect perform phloem feeding (1=yes, 0=no)	0.6 ± 0.5	0.3 ± 0.5	0.4 ± 0.5	0.9 ± 0.3	0.9 ± 0.3	1 ± 0.2	**<0.001**	0.298	0.540
occ.e2s	Does insect perform sustained sustained phloem feeding (>10 min)? (1=yes, 0=no)	0.3 ± 0.5	0.2 ± 0.4	0.4 ± 0.5	0.9 ± 0.3	0.7 ± 0.4	0.9 ± 0.3	**<0.001**	0.145	0.748
lat.e2	Latency to first phloem feeding from start of recording (min)	386.8 ± 116.8	403.3 ± 134.1	374.9 ± 154.8	196.4 ± 151.1	216.2 ± 137.6	198 ± 147.2	**<0.001**	0.778	0.978
lat.e2p	Latency to first phloem feeding from start of first probe (min)	382.8 ± 115	395.6 ± 133	374.2 ± 154.4	183.3 ± 143.7	213.7 ± 140	196.7 ± 148.2	**<0.001**	0.742	0.896
sum.e2	Total duration of phloem feeding (min)	37.7 ± 75.1	20.8 ± 46.8	56.4 ± 106.7	187.9 ± 159.3	102.9 ± 115.7	184 ± 156.4	**0.003**	0.187	0.249
mean. e2	Mean duration of phloem feeding (min)	52.3 ± 69.4	55.2 ± 68.2	105.1 ± 139.4	140.2 ± 150.3	74.8 ± 105.3	146.3 ± 135.8	0.075	0.112	0.546
occ.f	Does insect show derailed stylet mechanics? (1=yes, 0=no)	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	0.2 ± 0.4	0.3 ± 0.5	0.802	0.916	0.705
sum.f	Total duration derailed stylet (min)	16.9 ± 35.5	41 ± 72	19.5 ± 47.9	32 ± 64.1	24.8 ± 61	22.1 ± 44.2	**<0.001**	0.170	0.851
mean.f	Mean durationderailed stylet (min)	33 ± 33.8	67.2 ± 25.5	26.7 ± 22.1	69.7 ± 84.6	37.3 ± 23.6	28 ± 18.7	0.846	0.071	0.163
occ.g	Does insect perform xylem drinking? (1=yes, 0=no)	0.7 ± 0.5	0.7 ± 0.5	0.7 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.3 ± 0.5	**0.047**	0.146	0.412
sum.g	Total duration xylem drinking (min)	21.1 ± 22.8	41.4 ± 46.9	40.6 ± 61	7.5 ± 16.5	24.8 ± 31.9	11.2 ± 19.6	**0.003**	0.083	0.151
mean.g	Mean duration xylem drinking (min)	17 ± 9.1	39.4 ± 34.4	36.5 ± 32.4	26.5 ± 14.1	40.2 ± 22	25.4 ± 10.1	0.999	**0.012**	0.308



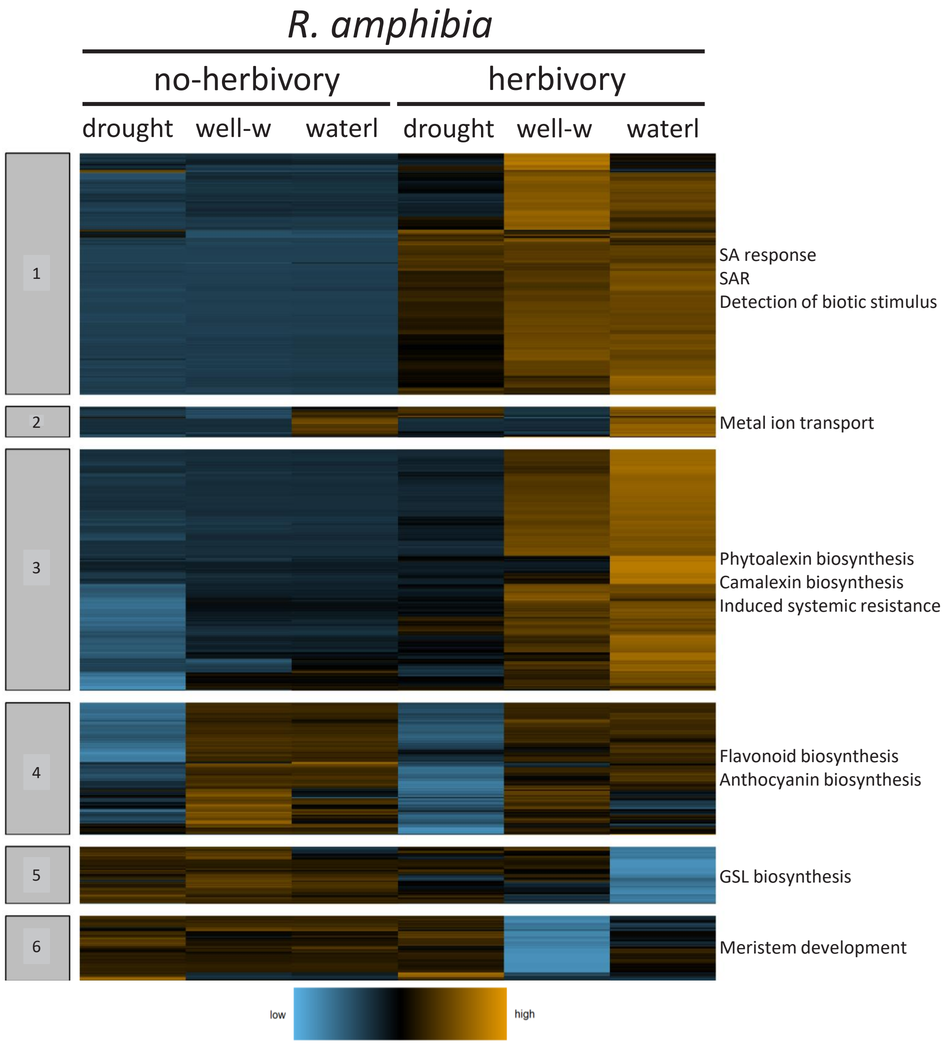
Supplementary Figure 1. PCA of transcript counts after variance stabilizing transformation of all orthogroups. This shows the two species clearly have their own unique transcriptomic fingerprint.



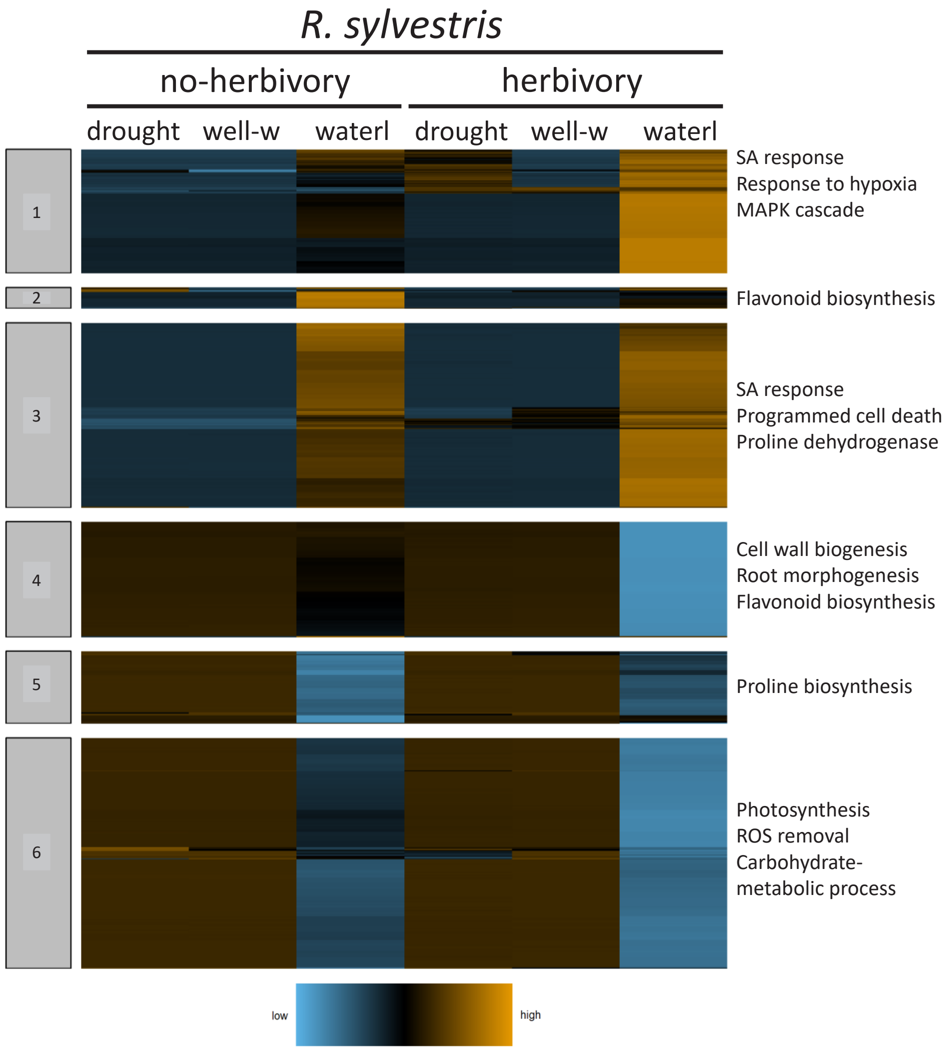
Supplementary Figure 2. PCA of transcript counts of *Rorippa amphibia* plants under different water treaments either infested with *Myzus persicae* aphids or left un-infested. This shows that drought-treated plants are separated from the other two water treatments. Additionally, infested and un-infested plants are separated.



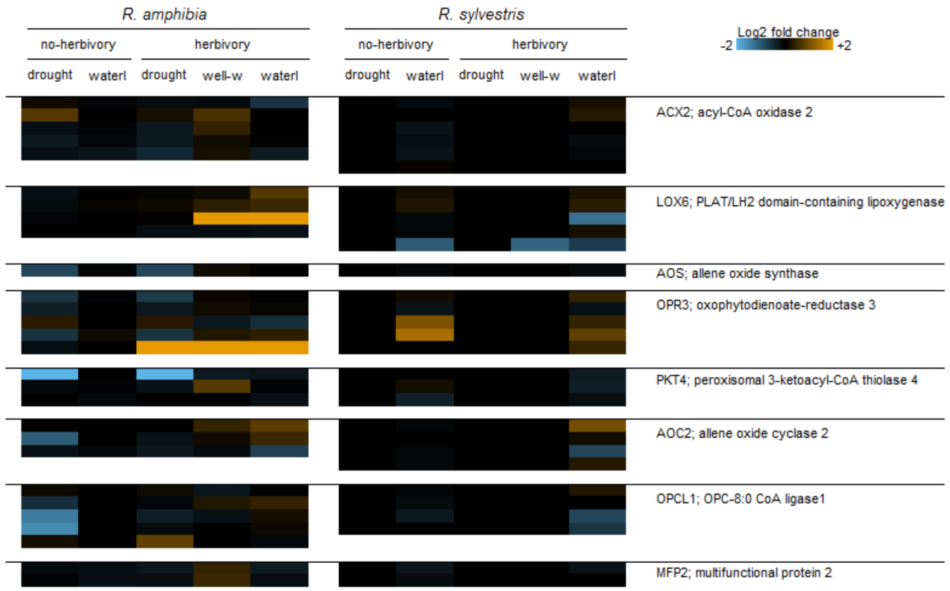
Supplementary Figure 3. PCA of counts of *Rorippa sylvestris* transcripts. This shows waterlogged plants are separated from the other two water treatments. Furthermore, *M. persicae*-infested plants are only separated from un-infested plants in cases where the plant was waterlogged.



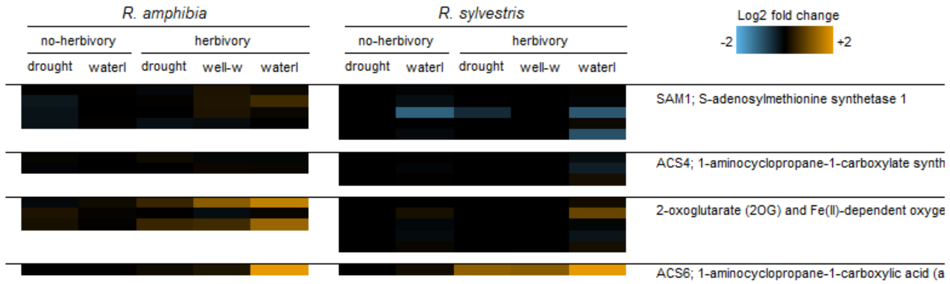
Supplementary Figure 4. Heatmap of hierarchical clustering of Z-score of all DEGs from *R. amphibia* plants under different watering regimes and un-infested or infested with aphids. GO enrichment was performed on each cluster and overrepresented GO terms are presented on the right. Cut-offs were set at a log2 fold change > 1 and an adjusted P-value < 0.001. DEGs per cluster: 1=300, 2=38, 3=300, 4=16, 5=71, 6=81.



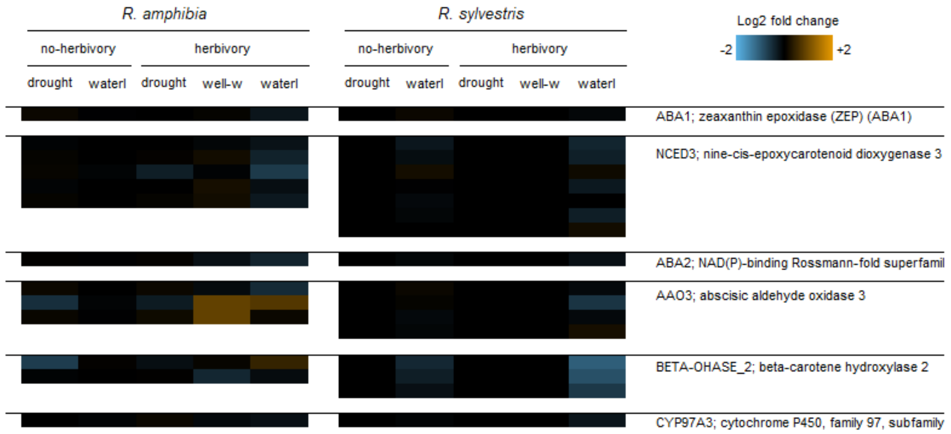
Supplementary Figure 5. Heatmap of hierarchical clustering of Z-score of all DEGs from *R. sylvestris* plants under different watering regimes and un-infested or infested with aphids. GO enrichment was performed on each cluster and overrepresented GO terms are presented on the right. Cut-offs were set at a log2 fold change > 1 and an adjusted P-value < 0.001. DEGs per cluster: 1=130, 2=22, 3=194, 4=121, 5=76, 6=242.



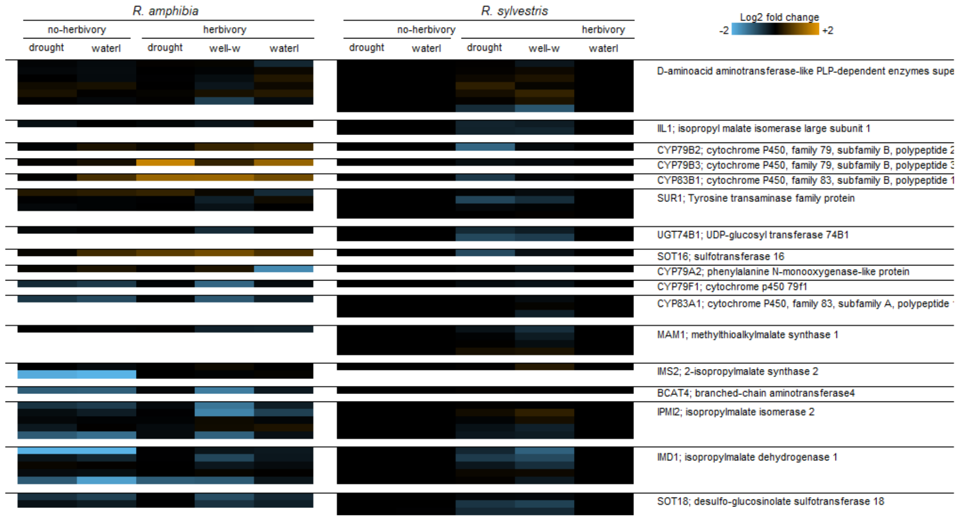
Supplementary Figure 6. Heatmap showing log2 fold change compared to un-infested well-watered plants of that plant species of genes associated with Jasmonic acid biosynthesis (KEGG module M00113); well-w = well-watered, waterl = waterlogged.



Supplementary Figure 7. Heatmap showing log2 fold change compared to un-infested well-watered plants of that plant species of genes associated with Ethylene biosynthesis. well-w = well-watered, waterl = waterlogged.



Supplementary Figure 8. Heatmap showing log2 fold change compared to un-infested well-watered plants of that plant species of genes associated with Abscisic acid biosynthesis. well-w = well-watered, waterl = waterlogged.



Supplementary Figure 9. Heatmap showing log2 fold change compared to un-infested well-watered plants of that plant species of genes associated with glucosinolate biosynthesis. well-w = well-watered, waterl = waterlogged.

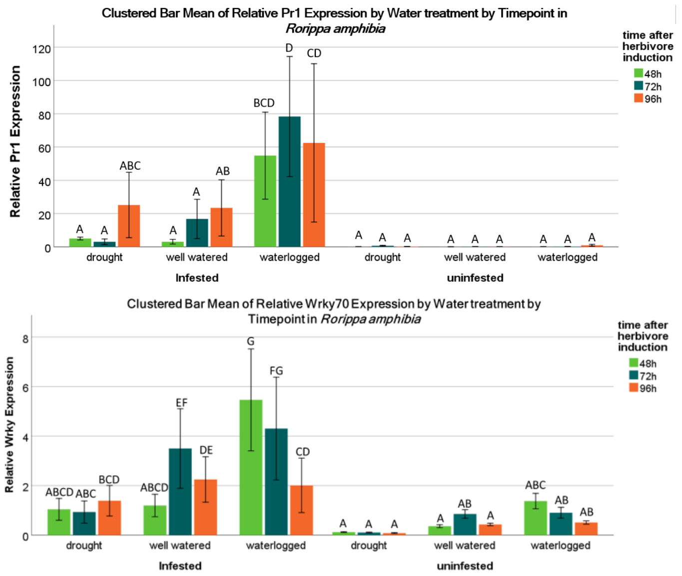
Supplementary Table 2. Results from a pairwise Mann-Whitney U analysis comparing the relative amount of organic acids and sugars under drought, waterlogging or herbivory to well-watered un-infested plants of that species (*R. amphibia* or *R. sylvestris*). Fold changes (FC) were calculated as (B-A)/A where A is the relative concentration in well-watered un-infested plants and B in the specified treatment. Finally, also the comparison was made between well-watered plants of both plant species.

	R. amphibia									*R. sylvestris*									plant species		
	Drought			Waterlogging			Herbivory			Drought			Waterlogging			Herbivory			Well-watered		
Phloem metabolite	FC	U	P	FC	U	P	FC	U	P	FC	U	P	FC	U	P	FC	U	P	FC	U	P
Oxalic acid	0.09	20	0.818	-0.39	33	0.282	1.23	28	0.366	-0.79	37	0.108	-0.64	34	0.220	0.17	30	0.491	12.19	1	**0.001**
Succinic_acid	-0.39	31	**0.041**	-0.26	31	0.414	-0.20	13	0.295	-0.46	33	0.262	-0.11	30	0.502	0.23	22	0.836	1.82	18	0.476
Fumaric acid	0.10	16	0.818	-0.44	44	**0.008**	-0.18	13	0.295	-0.03	14	0.215	0.94	15	0.262	0.25	25	0.945	-0.72	41	**0.031**
Malic_acid	-0.13	23	0.485	-0.36	40	**0.043**	0.45	25	0.628	-0.13	31	0.414	0.02	23	0.950	-0.17	20	0.662	0.77	11	0.108
2_oxo_glutaric_acid	-0.17	27	0.180	-0.24	38	0.081	0.15	27	0.445	-0.63	31	0.332	-1.00	33	0.127	-1.00	15	0.127	0.20	30	0.468
Citric acid	0.21	12	0.394	-0.77	47	**0.001**	1.51	23	0.836	-0.60	35	0.218	-0.69	36	0.137	-0.61	12	0.129	8.13	12	0.137
Galactose	0.30	10	0.240	-0.50	47	**0.001**	-0.23	12	0.234	0.39	14	0.220	-0.39	33	0.270	-0.60	12	0.129	0.79	13	0.175
Myo_inositol	0.51	2	**0.009**	-0.24	45	**0.005**	0.12	25	0.628	0.06	25	0.950	-0.16	28	0.698	0.00	26	0.897	2.05	6	**0.020**
Sucrose	-0.15	20	0.818	-0.34	32	0.345	0.23	24	0.731	2.33	15	0.262	2.10	22	0.836	0.47	18	0.408	-0.64	39	0.058
Fructose	0.03	16	0.818	-0.02	24	1.000	-0.19	9	0.101	-0.50	44	**0.008**	-0.11	28	0.662	0.27	35	0.181	0.31	16	0.345
Glucose	0.02	23	0.485	0.27	5	**0.013**	-0.10	19	0.836	0.36	14	0.228	0.09	24	1.000	-0.05	19	0.573	-0.35	39	0.059

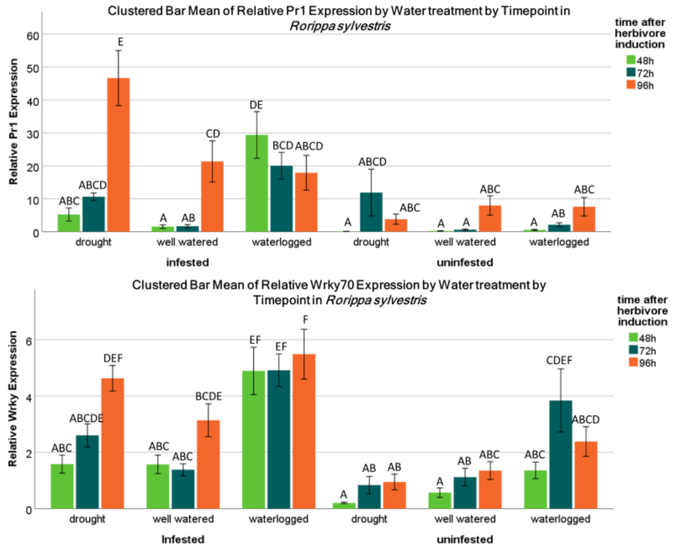
Supplementary Table 3. Results from a pairwise Mann-Whitney U analysis comparing the relative amount of amino acids under drought, waterlogging or herbivory to well-watered un-infested plants of that species (*R. amphibia* or *R. sylvestris*). Fold changes (FC) were calculated as (B-A)/A where A is the relative concentration in well-watered un-infested plants and B in the specified treatment. Finally, also the comparison was made between well-watered plants of both plant species.

	R. amphibia									*R. sylvestris*									plant species		
	Drought			Waterlogging			Herbivory			Drought			Waterlogging			Herbivory			Well-watered		
Amino acid	FC	U	P	FC	U	P	FC	U	P	FC	U	P	FC	U	P	FC	U	P	FC	U	P
ASP	-0.38	35	**0.004**	-0.13	32	0.345	-0.13	16	0.534	-0.06	27	0.755	-0.30	43	0.013	-0.14	13	0.181	0.02	20	0.662
GLU	-0.20	31	**0.041**	0.32	8	**0.043**	-0.07	17	0.628	-0.10	26	0.852	0.23	19	0.573	-0.07	19	0.573	-0.09	27	0.755
ASN	-0.14	26	0.240	-0.27	48	**<0.001**	0.09	23	0.838	-0.19	36	0.142	-0.07	27	0.755	0.46	45	**0.005**	-0.04	27	0.755
SER	0.09	11	0.310	0.05	19	0.573	0.04	25	0.628	0.12	16	0.345	0.18	17	0.414	0.02	22	0.852	0.46	4	**0.008**
GLN	-0.45	36	**0.002**	-0.08	30	0.491	0.12	33	0.101	-0.20	36	0.142	0.20	11	0.108	0.24	35	0.181	-0.22	42	**0.020**
HIS	-0.15	23	0.485	-0.13	31	0.414	-0.09	18	0.731	0.27	25	0.950	0.53	21	0.755	0.21	34	0.228	-0.04	23	0.950
GLY	-0.59	29	0.093	-0.22	25	0.950	0.26	21	1.000	1.07	26	0.852	1.29	13	0.181	0.20	24	1.000	-0.60	40	**0.043**
THR	0.46	0	**0.002**	0.20	11	0.108	0.19	29	0.295	0.16	11	0.108	0.22	4	**0.008**	0.20	44	**0.008**	0.83	0	**<0.001**
CIT	-0.35	24	0.394	-0.58	36	0.142	-0.44	13	0.295	-0.31	30	0.491	2.06	19	0.573	-0.61	8	0.043	1.42	7	**0.029**
bALA	-0.53	36	**0.002**	-0.04	24	1.000	-0.20	16	0.534	-0.46	36	0.142	-0.58	37	0.108	0.10	24	1.000	-0.24	37	0.108
GABA	0.06	15	0.699	0.20	11	0.108	-0.09	17	0.628	-0.20	29	0.573	-0.15	29	0.573	-0.25	14	0.228	-0.09	36	0.142
TYR	-0.23	28	0.132	0.45	8	**0.043**	0.22	27	0.445	-0.40	35	0.181	-0.22	30	0.477	-0.12	19	0.573	0.10	28	0.662
VAL	-0.31	32	**0.026**	0.15	16	0.345	0.11	26	0.534	-0.28	36	0.142	0.02	26	0.852	-0.32	11	0.108	0.18	16	0.345
MET	-0.16	24	0.394	-0.07	25	0.950	0.08	26	0.534	-0.68	44	**0.012**	-0.55	36	0.137	-0.12	10	0.081	0.85	10	0.081
TRP	-0.30	29	0.093	0.21	18	0.491	0.07	24	0.731	-0.33	38	0.081	0.28	29	0.573	-0.35	10	0.081	0.17	21	0.755
PHE	-0.26	32	**0.026**	0.27	17	0.414	0.12	29	0.295	-0.17	32	0.345	0.14	16	0.345	0.04	25	0.950	0.19	14	0.228
ILE	-0.33	29	0.093	0.31	10	0.081	0.16	26	0.534	-0.10	30	0.491	0.10	23	0.950	-0.10	20	0.662	0.00	21	0.755
LEU	-0.22	29	0.093	0.38	10	0.081	0.14	25	0.628	-0.14	30	0.491	0.00	25	0.950	-0.27	16	0.345	0.05	24	1.000
LYS	-0.19	27	0.180	0.19	14	0.228	0.29	27	0.445	-0.03	21	0.755	0.14	21	0.698	-0.42	19	0.573	-0.33	42	**0.020**
PRO	28.13	0	**0.002**	0.12	20	0.662	0.08	24	0.731	7.04	8	**0.043**	0.08	15	0.282	-0.41	10	0.081	0.31	22	0.852
Essential amino acids	0.00	18	1.000	0.19	11	0.108	0.14	26	0.534	-0.03	25	0.950	0.13	14	0.228	0.00	25	0.950	0.35	5	**0.013**

R. amphibia



R. sylvestris



Supplementary Figure 10. Mean calibrated normalized relative quantities (CNRQ) of the PR1 and WRKY70 genes in *Rorippa sylvestris* and *Rorippa amphibia* plants with different water treatments: drought, well-watered and waterlogged, either with or without the presence of *M. persicae* aphids: infested, un-infested. The colors represent the different timepoints of harvesting (48 hours, 72 hours, and 96 hours after aphid induction and 9 days 11 days or 13 days after the onset of drought or waterlogging). Significant differences are indicated by different letters above the bars (GLM: Bonferroni, P <0.05).

Supplementary Methods 1.

Adapted RNA extraction protocol for *R. amphibia*

1. **Trizol treatment**
    a. . Lyse and homogenize samples in TRIzol™ Reagent according to your starting material. Add 1 mL of TRIzol™ Reagent per 50–100 mg of tissue to the sample and homogenize using a homogenizer.
    b. (Optional) If samples have a high fat content, centrifuge the lysate for 5 minutes at 12,000 × g at 4–10°C, then transfer the clear supernatant to a new tube.
    c. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.
    d. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, then securely cap the tube.
    e. Incubate for 2–3 minutes.
    f. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C. The mixture separates into a lower red phenol-chloroform, and interphase, and a colourless upper aqueous phase.
    g. Discard the aqueous phase containing the RNA, then proceed directly to the next section with the interphase containing the DNA.
2. **Precipitate RNA**
    a. Remove any remaining aqueous phase overlying the interphase.
    This is critical for the quality of the isolated DNA.
    b. Add 0.3 mL of 100% ethanol per 1 mL of TRIzol™ Reagent used for lysis.
    c. Cap the tube, mix by inverting the tube several times.
    d. Incubate for 2–3 minutes.
3. **Bind RNA**
    - Place one ISOLATE II RNA Plant Column (blue) in a 2 mL Collection Tube and load the lysate.
    - Centrifuge for 1min at 11.000 x g
    - Place the column in a new 2 mL Collection tube.
4. **Desalt silica membrane**
    - Add 350 uL Membrane Desalting Buffer (MEM) and centrifuge at 11.000 x g for 2 min to dry the membrane.
5. **Digest DNA**
    - Prepare DNase I reaction mixture in a sterile 1.5 mL microcentrifuge tube
    - Add 10 uL reconstituted DNase I to 90 uL reaction Buffer for DNase I (RDN).
    - Mix gently by flicking the tube.
    - Apply 95 uL DNase I reaction mixture directly onto the centre of the silica membrane of the column.
    - Incubate at room temp for 15 min.
6. **Wash and dry silica membrane**
    - 1st wash
        - Add 200 uL Wash Buffer RW1 to the ISOLATE II RNA Plant column (to inactivate DNase).
        - Centrifuge for 1 min at 11.000 x g.
        - Place the column into a new Collection Tube (2mL).
    - 2nd wash
        - Add 600 uL Wash Buffer RW2 to the ISOLATE II RNA Plant column.
        - Centrifuge for 1 min at 11.000 x g.
        - Discard flow trough and place column back into the collection tube.
    - 3rd wash
        - Add 250 uL Wash Buffer RW2 to the ISOLATE II RNA Plant column.
        - Centrifuge for 2 min at 11.000 x g to dry membrane completely.
        - Place column into a nuclease-free 1.5 mL Collection Tube.
7. **Elute RNA**
    - Elute the RNA in 50 uL RNase-free water.
    - Centrifuge for 1 min at 11.000 x g.
    - Reapply the filtrate on top of the column and spin again for 1 min at 11.000 x g.
    - Put samples on ice immediately and let cool for 5 minutes before further use.
8. **Quantification with UV spectroscopy**
    - Load 1uL of sample into the spectrophotometer to determine the concentration of RNA in the sample.

Supplementary Methods 2.

Complete phloem exudates analysis protocol

For the analysis of carbohydrates, organic acids and the cyclic polyol *myo*-inositol, metabolites were re-dissolved in 100 µL 80% (v:v) methanol (LC-MS grade; Fisher Scientific, Loughborough, UK) containing the internal standard ribitol (AppliChem, Darmstadt, Germany; 0.0115 g L^{-1}) and 80 µL of the supernatants were dried under nitrogen. Then, a two-step derivatisation was performed. First, a methoximation was done using 55 µL *O*-methylhydroxylamine hydrochloride (> 98%; Alfa Aesar, Kandel, Germany) in pyridine (> 99%, extra pure; Fisher Scientific; 20 mg mL^{-1}) at 37 °C for 90 min. Then, a silylation was performed (37 °C, 30 min) with 55 µL *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (Macherey-Nagel, Düren, Germany). The samples were subjected to gas chromatography coupled to mass spectrometry (GC-MS; GC-2010 Plus, QP2020; Shimadzu, Kyoto, Japan). Samples (1 µL) were injected at 225 °C with a split ratio of 1:10. Analytes were separated on a VF-5ms column (30 m x 0.25 mm i.d., with guard column; Varian, Palo Alto, CA, USA) with a helium (carrier gas) flow of 1.14 mL min^{-1} and a temperature gradient: 80 °C for 3 min, then ramped with 5 °C min^{-1} to 310 °C, held for 2 min, increase (15 °C min^{-1}) to 325 °C, held for 3 min. The interface (transfer line) temperature was set to 250 °C. The mass spectrometer was operated in electron impact positive ionisation mode at 70 eV with an ion source temperature of 230 °C, detecting ions with mass-to-charge (*m/z*) ratios of 40 to 600. *n*-alkanes (C$_7$-C$_{40}$; Sigma-Aldrich, St. Louis, MO, USA) were measured to allow metabolite identifications.

Amino acids were analysed via high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD; 1260/1290 Infinity; Agilent Technologies, Santa Clara, CA, USA). Samples were extracted with 50 µL 80% methanol, which contained L-norvaline and sarcosine (each 50 pmol µL^{-1}; Agilent Technologies, Waldbronn, Germany) as internal standards for primary and secondary amino acids, respectively. A pre-column derivatisation was performed in the autosampler at 6 °C. For this, samples were incubated with borate buffer (0.4 M, pH = 10.2; Agilent Technologies), ortho-phthalaldehyde (OPA, for derivatisation of primary amino acids; 10 mg mL^{-1} each of OPA and 3-mercaptoproprionic acid in 0.4 M borate puffer; Agilent Technologies), 9-fluorenyl-methyl chloroformate (FMOC, for derivatisation of secondary amino acids; 2.5 mg mL^{-1} in acetonitrile; Agilent Technologies) and injection diluent [mobile phase A (see below) and 85% phosphoric acid (AppliChem) in a ratio of 1:0.004 (v:v)]. Derivatised amino acids were separated on a ZORBAX Eclipse Plus C18 column (250 x 4.6 mm i.d., 5 µm, with guard column; Agilent Technologies). A gradient from mobile phase A [1.4 g Na_2HPO_4 (Roth, Karlsruhe, Germany), 3.8 g $Na_2B_4O_7 \cdot 10\ H_2O$ (Sigma-Aldrich) and 32 mg NaN_3 (Roth) in 1 L Millipore water, pH = 8.2, filtered through 0.45 µm membrane] to mobile phase B [4.5:4.5:1 mixture (v:v:v) of methanol, acetonitrile (LC-MS grade; Fisher Scientific) and Millipore water] was applied: 2% B for 0.84 min, followed by a ramp to 57% B (reached at 68.4 min) and by column cleaning and equilibration. Separation was done at a flow rate of 1.5 mL min^{-1} and at 40 °C. For fluorescence detection, excitation and emission wavelengths of 340 nm and 450 nm (OPA-derivatised primary amino acids) and 260 nm and 325 nm (FMOC-derivatised secondary amino acids), respectively, were used.

Chapter 6

General discussion

General discussion

Plants in natural environments face the double trouble of defending against insect attacks while coping with suboptimal water conditions, such as water scarcity or water surplus. The influence of water conditions on plant-insect interactions has been acknowledged, but a comprehensive understanding of the underlying mechanisms and the impact of species-specific adaptations to double trouble remains limited (Leybourne et al. 2021; Lin et al. 2023; Pineda et al. 2016). Advancements in our knowledge have uncovered that plants can respond to stressors in isolation but also have to integrate responses to more intricate and realistic environments with multiple stressors (Mittler 2006; Rasmussen et al. 2013; Rizhsky et al. 2004; Zhang et al. 2019). By studying plants within a natural context, where they have evolved and adapted in the face of multiple challenging environmental factors, valuable insights can be gained on how plants integrate responses to multiple stressors and remain resilient to suboptimal conditions. Thereby the study of wild plants offers a wealth of knowledge that can be leveraged for the development of resilient farming strategies in the face of climate change. One of our societal challenges is to secure food production under the more extreme weather events such as drought and flooding that will happen as a result of climate change and maintain crop resistance to insect herbivory. Therefore, it is imperative to explore and learn from adaptations found in different plants, unraveling the specific ways in which water availability affects plant-insect interactions.

Insights from studying wild *Rorippa* plants

This study utilized a comparative approach of several wild plant species to gain insight in how they solve the double trouble of water stress and herbivory. Initially, I started out with four closely related *Rorippa* plant species that naturally grow in different habitats over a water gradient. I used the semi-aquatic *R. amphibia*, the floodplain inhabiting *R. palustris*, and the more terrestrial *R. sylvestris* and *R. austriaca*. I intensively studied these plant species under different water conditions ranging from drought to waterlogging. I found that the arthropod community in three of the four species was significantly shaped by the water treatment they received. Drought-treated plants were generally smaller than well-watered or waterlogged plants even for plant species most adapted to drier habitats. Interestingly, the arthropod species richness was generally lower on both drought-treated and waterlogged plants, indicating that plant size alone doesn’t explain how arthropod communities are affected by water availability, but rather other plant-mediated factors are influencing arthropod community composition and structure. Additionally, predatory arthropods were also impacted, warranting further investigation into plant-mediated effects of water conditions on higher trophic levels (**Chapter 2**).

Continuing with three plant species I measured both the direct and indirect defensive capabilities of these plants under different watering regimes. Here I found that, for two out of three plant species, direct defenses against aphids were affected by water availability. Furthermore, this seemed to link to the adaptations of water availability in the natural habitat of the plant species. The semi-aquatic plant *R. amphibia* was negatively affected in its resistance against aphids when drought treated, while drought had no effect on the level of resistance on the other two plant species. Meanwhile, the terrestrial plant *R. sylvestris* was most affected by waterlogging. Interestingly, waterlogging led to an increased resistance as opposed to the decrease found in *R. amphibia*. This indicates that different water conditions might be differentially experienced as stressful for plant species and that the response to stress can have opposing plant-mediated effects on plant-insect interactions (**Chapter 3**).

Indirect defenses were more uniformly affected by water availability. Both drought and waterlogging affected parasitoid recruitment in all three plant species. Generally, water conditions did not disrupt volatile production but rather also induced changes in volatile blends making it difficult for parasitoids to distinguish plants with and without insect host. This demonstrates that indirect defenses can be very differently affected than direct defenses and only by considering both, a more complete picture of the ecological impact of changes in water availability can be obtained (**Chapter 4**).

Continuing with two plant species, I unraveled how the responses to herbivory were mechanistically affected by water availability. Transcriptomic analysis identified a strong species-specific effect correlating with the direct defenses measured previously (**Chapter 5**). *Rorippa amphibia*, adapted to waterlogging, showed minimal changes in response to aphid feeding during waterlogging but exhibited accelerated aphid population growth under drought, likely due to reduced defense-related gene activity. In contrast*, R. sylvestris*, adapted to drier soils, exhibited less pronounced effects during drought and had reduced aphid performance during waterlogging, possibly through an increase in reactive oxygen species associated with the response to waterlogging having a negative effect on aphids. In conclusion, this study on wild *Rorippa* plants revealed that water availability significantly influences plant-insect interactions in many ways, having far reaching ecological consequences. Furthermore, by comparing several *Rorippa* plant species I started describing the variation in strategies deployed by plant species reacting to double trouble. I identify that plant adaptations to water availability in their habitat should be prominently considered in understanding how plants deal with combinations of biotic and abiotic stress, which may inspire breeding for stress resilient crops.

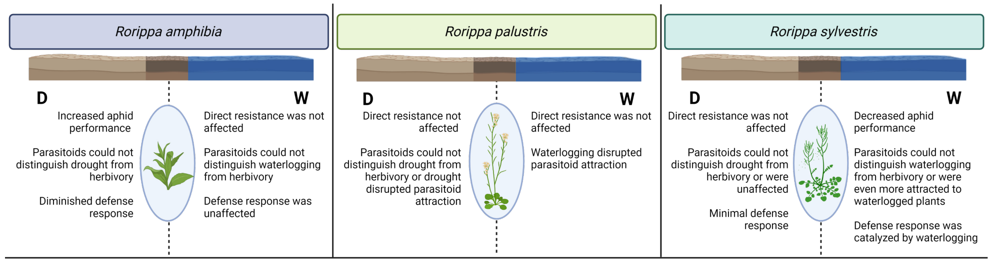


Figure 1. Important findings on three *Rorippa* plant species studied separated by the effects of drought (D) or waterlogging (W) on plant-insect interactions. Effects of water conditions were distinct for the three plant species and effects differed for direct and indirect defenses. (Created with BioRender.com)

The ecological significance of water availability on plant-insect interactions

Water as direct barrier

Water availability plays a crucial role in plant-insect interactions, impacting various ecological factors. Under extreme water conditions, such as waterlogging, a physical barrier arises between plants and insects. This affects various aspects of the insects' foraging behavior, limiting their ability to reach their target plants. Firstly, waterlogged soil becomes compacted and anaerobic, resulting in limited oxygen availability for soil dwelling arthropods (Plum 2005). Additionally, the excess water hampers the locomotion of insect herbivores, making it nigh impossible for non-flying insects to reach the plant. As a result, insect herbivores may face substantial challenges in locating and feeding on their preferred plant hosts, ultimately impacting their survival and reproductive success. This can, in turn, provide herbivores who do brave the journey to reach the plant with less competition from other herbivores as well as less risk of predation (Schowalter 2012; Sipura et al. 2002). Indeed, when monitoring arthropod communities, I found a general lower abundance of arthropods on waterlogged and drought-treated plants (**Chapter 2**). The physical barrier posed by waterlogging serves as a significant ecological factor influencing the distribution and abundance of insect herbivores in waterlogged environments. However, I found that the physical barrier caused by waterlogging doesn’t explain the full impact of water availability on plant-insect interactions as the effect of waterlogging wasn’t uniform among the four plant species I investigated. This suggests a plant-mediated effect of waterlogging that was species-specific affecting plant-insect interactions.

Water availability affects plant-mediated interactions with insects

Water availability can affect how plants interact with their environment in many ways. One such plant-mediated effect can be the influence of water availability on plant morphology, consequently affecting plant-insect interactions. Under waterlogged or drought conditions, plants often exhibit morphological adaptations aimed at conserving water and reducing transpiration. These adaptations can include changes in leaf morphology, thicker cuticles, and altered root architecture (Blom 1999; Chaves et al. 2003). Such morphological changes can have direct implications for plant-insect interactions (Hanley et al. 2007). For instance, reduced leaf surface area may result in decreased resource availability for herbivorous insects, potentially leading to altered feeding behaviors and reduced fitness (Marques et al. 2000; Price 1991). During my study, I measured reduced plant size after drought correlating with a lower biodiversity and the abundance of certain insects (**Chapter 2**). However, waterlogging did not lead to a significant reduction of plant size but did lead to a lower biodiversity of the plant-associated arthropod community, indicating that a larger plant doesn’t necessarily equal a larger arthropod community (**Chapter 2**). Additionally, the thicker cuticles of plants can act as a barrier, making it more challenging for insects to penetrate plant tissues for feeding or oviposition (Chaves et al. 2003; Lin et al. 2021; Zhong et al. 2020). In a study on wheat, *Rhopalosiphum padi* aphids spent 2.8 to 6.1 times longer to reach the phloem on drought-treated than well-watered or waterlogged plants (Lin et al. 2021). Yet, another study found no differences in aphid feeding behavior between drought-treated and well-watered wheat. It did, however, find aphids ingest sap at a lower rate on drought-treated plants (Hale et al. 2003). In my research, I did not find an effect of water condition on aphid feeding behavior (**Chapter 5**). This illustrates that the morphology of different plant species with their own adaptations is uniquely affected by water conditions that can affect the associated arthropod community.

Furthermore, water conditions can exert effects that extend beyond the external morphological changes observed in plants. It also exerts considerable influence on the metabolomics and nutrient mobilization processes within plants (Anjum et al. 2011; Barber and Müller 2021). As a consequence, these intricate mechanisms directly influence the suitability of plants as viable hosts for insect herbivores. In fact, the study system of this thesis, the *Rorippa* plant genus, was originally chosen because in two plant species previous research identified distinct strategies of coping with flooding stress which might impact the metabolomic profile of plant tissues insect herbivores eat. *Rorippa amphibia* has an escape strategy under flooding in which it mobilizes stored reserves to outgrow the stress. *Rorippa sylvestris* on the other hand has a quiescence strategy in which it lowered metabolism under flooding to a minimum preserving its energy for the flooding to subside (Akman et al. 2012; Sasidharan et al. 2013).

My hypothesis was that this would lead to distinct metabolomic profiles of these plants under different water conditions. During my studies I could, however, not find strong effects on relative levels of amino acids or sugars in phloem exudates under drought or waterlogging apart from an increase in proline levels under drought (**Chapter 5**). The increase of proline levels is a common response to drought. Proline has known Osmo-protectant properties, aiding the plant in water retention (Szabados and Savouré 2010). Interestingly, proline was also found to be an important energy source for several insect species (Bouchebti et al. 2022; Noor-ul-Ane and Jung 2022; Sacktor and Childress 1967; Stec 2018; Teulier et al. 2016). These insects show the capability to quickly and efficiently convert proline to energy, predominantly used for long flights (Mantilla et al. 2017; Sacktor and Childress 1967; Stec 2018), and levels of proline can even influence host plant choice (Behmer and Joern 1994). I could not measure absolute proline concentrations, but this might be a pathway through which drought can influence plant-insect interactions. As plants respond to water stress with the mobilization of solutes in the phloem, this can result in the increase of phloem osmolarity (Sevanto 2014; Xiong and Zhu 2002). A high osmotic pressure can be detrimental for aphids as they have to actively counteract this pressure. An increase in solutes might therefore not always be beneficial for insect herbivores. In response to increased phloem osmolarity, it has been hypothesized that aphids mix xylem and phloem feeding as a form of osmoregulation (Pompon et al. 2011). During my study, I did find that aphids drink more xylem on *R. amphibia* plants than *R. sylvestris* plants which might be connected to osmoregulation. However, no effect of drought or waterlogging was found (**Chapter 5**). Water availability thus plays a critical role in the availability and distribution of nutrients within plants and affects plant-aphid interactions. In conditions of water scarcity or surplus, plants may experience reduced nutrient uptake and transport, leading to alterations in nutrient composition and distribution in plant tissues (Mewis et al. 2012; Pons et al. 2020; Stallmann et al. 2020). Interestingly, I only found effects of water availability on the performance of the aphids *Myzus persicae* and *Lipaphis erysimi*. No effects were found on the performance of the two caterpillars *Pieris brassicae* and *Plutella xylostella*. This could be explained by their feeding mode. As chewers, they are less intimately linked with nutrient concentrations of the phloem and might be able to compensate for changes in nutrient concentrations. Furthermore, their specialization to feed from brassicaceous plants might allow them to be less affected by subtle changes in nutritional value and defense of their host plant caused by different water conditions. As a consequence, the host-plant quality for herbivores changes in herbivore-specific ways which ultimately affect their population dynamics.

Effects of water on direct plant defenses

Additionally, the complex interplay between water availability, metabolomics, and nutrient mobilization can also influence plant defenses (Beetge and Krüger 2019; Huberty and Denno 2004; Inbar et al. 2001; Leybourne et al. 2021; Pineda et al. 2016; Simpson et al. 2012; Xie et al. 2020). Water-stressed plants may exhibit altered patterns of resource allocation, diverting resources away from defensive mechanisms and compromising their ability to mount effective defenses against herbivory (Khan et al. 2010; Mewis et al. 2012; Oswald and Brewer 1997; Pons et al. 2020). Consequently, insect herbivores may encounter reduced resistance from water-stressed plants, facilitating their feeding and potentially leading to increased herbivory rates. Indeed, I found a higher population growth of *Myzus persicae* aphids on *R. amphibia* plants that were drought treated (**Chapter 3**). Interestingly, this is contrary to most other studies on the effects of drought on aphid performance (Huberty and Denno 2004; Leybourne et al. 2021). In most other studies there is a strong correlation between decreased plant vigor and aphid performance under drought (Beetge and Krüger 2019; Cornelissen et al. 2008; Hatier et al. 2014; Inbar et al. 2001; Simpson et al. 2012; Xie et al. 2020). The fact that my results tell a different story once again strengthens the notion that plant species-specific adaptations to water can have far reaching ecological consequences. Digging deeper, transcriptomic analysis revealed that genes involved in defensive responses such as the production of the phytoalexin camalexin were less strongly upregulated in drought-treated *R. amphibia* plants (**Chapter 5**). Camalexin is a known strong anti-feedant for *M. persicae* aphids (Kettles et al. 2013). One possible explanation could be that drought caused an increase in the phytohormone jasmonic acid (JA) (de Ollas et al. 2013; Fang et al. 2016; Wang et al. 2021). As this phytohormone can, under circumstances, interfere with the signals that are dominated by salicylic acid (SA), like the defense response to aphids, drought could alter the defensive capabilities of *R. amphibia* (Erb et al. 2012; Hickman et al. 2019; Koornneef and Pieterse 2008; Thaler et al. 2012; Wu and Baldwin 2009). Waterlogging, to which *R. amphibia* is better adapted, had no effect on its resistance to insect herbivory (**Chapter 3**). *Rorippa sylvestris*, which is better adapted to drier conditions, was not affected in its direct defenses upon being drought treated (**Chapter 3**). Interestingly, waterlogging increased *R. sylvestris*' resistance to *M. persicae* aphids (**Chapter 3**). Perhaps, as waterlogging increased SA-related signaling, this synergized with the response the plant had to aphids, boosting its defenses (Arbona and Gómez-Cadenas 2008; Erb et al. 2012; Wu and Baldwin 2009). Transcriptomic analysis revealed a large transcriptomic change in *R. sylvestris* upon being waterlogged. Mainly genes involved in a hypersensitivity response were upregulated and genes involved in the scavenging of reactive oxygen species were downregulated (**Chapter 5**). A response often seen in plants in reaction to abiotic stress (Ahmad et al. 2010; Apel and Hirt 2004). Although no hypersensitivity response was visible with the naked eye, other studies have found negative effects of hypersensitivity responses and reactive oxygen species on aphid performance (Botha et al. 2014; Goggin 2007; Goggin and Fischer 2022; Kerchev et al. 2012; Qi et al. 2020). ROS can even be so detrimental that certain aphids carry facultative endosymbionts that disrupt ROS accumulation in the plant, allowing aphid species to colonize their host plant (Wang et al. 2020). Interestingly, high amounts of ROS are also damaging to the plant itself and require detoxification by anti-oxidants (Noctor and Foyer 1998; Wang et al. 2003). This might also mean that crops bred for a higher constitutive antioxidant content, like vitamin C, cope better with the oxidative stress caused by abiotic stress but are more susceptible for aphids, granting a unique interaction between water availability and insect herbivory (Kerchev et al. 2012; Venkatesh and Park 2014). This variation in responses beautifully emphasizes that each plant species has its distinctly different defensive mechanism and that these mechanisms were differently affected by water availability. Understanding these mechanisms is necessary to predict the outcomes of plant–insect interactions under a changing climate.

Effects of water on multi-trophic interactions

Water availability exerts effects that extend beyond direct defenses against insect herbivores, encompassing influences on higher trophic levels and indirect defense mechanisms. Specifically, water stress has the potential to impact the production of volatile organic compounds (VOCs) emitted by plants (Copolovici et al. 2014; Salerno et al. 2017; Weldegergis et al. 2015). VOCs play a critical role in mediating plant-insect interactions by attracting or repelling specific arthropod species. In my research, I found that both drought and waterlogging affected parasitoid recruitment. Generally, this was not caused by disrupting volatile production but rather by inducing changes in volatile blends with similarities to those induced by herbivory. This hindered the parasitoids in distinguishing plants with and without insect hosts (**Chapter 4**). As the effects on parasitoid recruitment were often similar for both drought and waterlogging, there was no apparent link to be made with the plant's adaptations to water conditions in their original habitat. This contrasts with the findings of such a relationship between adaptation to habitat and direct defenses (**Chapter 3**). Surprisingly, in the singular case of *R. sylvestris*, combined waterlogging and herbivory by *Plutella xylostella* made the plant significantly more attractive to *Diadegma semiclausum* parasitoids. Transcriptomic analyses revealed that waterlogging was akin to a catalyst for *R. sylvestris* to respond to *Myzus persicae* herbivory (**Chapter 5**). Perhaps waterlogging similarly enhanced the indirect defense response of *R. sylvestris* against *P. xylostella*. The absence of this link in other plant species in relation to their measured direct defenses (**Chapter 3**) remains puzzling. This highlights that water does not uniformly affect direct and indirect defenses. Similarly, other research reported positive, neutral, or negative effects of water scarcity or surplus on host localization, choice, and pest suppression by natural enemies (Kansman et al. 2021; Salerno et al. 2017; Weldegergis et al. 2015). This poses challenges in accurately predicting the exact impact on biological pest control in the face of a changing climate. Further studies have looked into how well parasitoids are able to locate their hosts in environments with increasing complexity, increasing for example the number of plant species and adding non-host herbivores. These studies also show that increased complexity can initially interfere with host localization of parasitoids (Andow and Prokrym 1990; Ponzio et al. 2016; Wäschke et al. 2013). Hence environmental complexity might be detrimental for the biological control of pests by parasitoids. However, in natural, and arguably far more complex environments, parasitoids are also able to locate their host. The ability of associative learning by natural enemies may allow natural enemies to more effectively forage in complex environments (Giunti et al. 2015). Many studies, including my own, use naïve natural enemies to study innate responses to volatile cues. However, natural enemies might well be able to learn to distinguish the volatile profile of water stressed plants with and without herbivore host after an initial learning period. Once natural enemies acquire the ability to distinguish volatile profiles of herbivore-infested plants in a background of water-stressed plants, their capacity to locate hosts accurately may be maintained even under the added environmental complexity of abiotic stress. Fluctuations in water availability can thus have cascading effects on the chemical signaling and communication processes between plants and insects, potentially influencing the efficacy of biological pest suppression in both natural and agricultural ecosystems. These findings further emphasize the profound and wide-ranging consequences of climatic conditions on the intricate dynamics of plant-insect interactions.

> **"Nothing in biology makes sense except in the light of evolution"**
>
> **-Dobzhansky 1972**

In summary, water availability exerts multifaceted effects on plant-insect interactions by influencing the morphology, metabolomics, nutrient dynamics, and signaling processes of plants. These effects can modulate plants traits, collectively shaping the quality of plants as hosts for insects. Understanding the intricate interplay between water availability and plant-insect interactions is crucial for unraveling the complex mechanisms underlying ecological dynamics in an environment with a surplus or scarcity of water. However, as becomes apparent from the comparative approach I took, closely related species already show vastly diverse effects from water availability on their interactions with insects. This demonstrates that the impact of water on plant-insect interactions is highly species-specific and should be placed into context.

The context that is largely missing in the research to unravel plant resilience in complex environments is that of evolutionary adaptations. Through natural selection, different plant species have undergone adaptive changes that enable them to thrive in specific ecological niches. Notably, these adaptations include the ability to cope with varying water levels, which influences their responses to fluctuations in water availability. Consequently, it becomes evident that the impact of an excess or deficiency of water on plant-insect interactions will be affected by the plant's intrinsic adaptations developed in response to changing water conditions. During my research, I have indeed found links between the natural habitat of the plant species and the water levels at which they were most affected (**Chapters 3&5**). Interestingly, the direction of the effect was less predictable. Furthermore, despite being highly adapted to their respective water conditions, plants still exhibit responses to changes in water availability, albeit to varying degrees or even utilizing different sets of genes (Sasidharan et al. 2013; van Veen et al. 2013). Although *R. amphibia* plants are well adapted to waterlogged conditions, transitioning to being waterlogged had its impact on plant phenotype, volatile profile and arthropod community. Similarly, *R. sylvestris,* growing in the driest habitat of all plant species studied in this project, was most significantly affected by drought in its phenotype, volatile profile and arthropod community (**Chapters 2&4**). In fact, an adaptation can be that the plant reacts rigorously and quickly as soon as it perceives a change (Pierik and Testerink 2014; Sasidharan et al. 2013; van Veen et al. 2013). Consequently, even plant species that are specifically adapted to particular water levels can be influenced by fluctuations in water availability, exerting effects on plant-insect interactions. By acknowledging the fundamental role of evolutionary processes in shaping plant characteristics and understanding that plants have evolved in complex multi-stress environments we can elucidate the underlying adaptations to multi-stress environments that govern the intricate dynamics between plants and insects in relation to abiotic factors like water availability.

Intriguingly, recent studies, including my own investigations detailed in **Chapter 5**, have revealed that the responses of plants to the combined challenges of water stress and insect attacks are not simply additive or overlapping. Instead, plants exhibit unique transcriptomic profiles that are specific to this interaction. These responses may involve the activation of distinct sets of genes and the modulation of signaling pathways that differ from those triggered by individual stressors alone (Mittler 2006; Rasmussen et al. 2013; Rizhsky et al. 2004; Zhang et al. 2019). This indicates that wild plant species, which have evolved in complex environments, must have evolved to maximize their fitness while coping with a wide range of biotic and abiotic stressors simultaneously instead of responding to each stressor individually and therefore possess unique strategies to effectively navigate double trouble (Fernández de Bobadilla et al. 2022). The multifaceted adaptability of plants highlights the complexity of their responses to the combined challenges of water stress and insect attacks. These responses likely represent an integrated defense mechanism aimed at minimizing the negative impacts of both water stress and insect attack. Understanding these specialized responses is crucial for unraveling the intricate dynamics of plant survival strategies and resilience in natural environments where multiple stress factors are the norm.

One such remarkable adaptation that is gaining more attention is multifunctionality of certain metabolites in plants. Metabolites may serve multiple roles, fulfilling essential functions in response to diverse stressors. For instance, waxy compounds in the leaf cuticle not only act as physical barriers against herbivores but also play a role in regulating water loss through transpiration (Ali et al. 2021; Bi et al. 2017; Kosma and Jenks 2007). Additionally, metabolites can be multifunctional in the sense that the same compound can function as a signaling compound for abiotic stress, as chemical defense against herbivory and as storage for later reintegration into primary metabolites (Erb and Kliebenstein 2020). For example, certain glucosinolates, well studied antifeedants for insect herbivores in Brassicaceae plants, have been found to also have osmo-protectant properties or play a role in stomatal regulation, aiding the plant in water retention during drought (del Carmen Martínez-Ballesta et al. 2013; Salehin et al. 2019). This multifunctionality allows plants to allocate resources efficiently and optimize their response to concurrent challenges. Furthermore, the production and accumulation of specific metabolites can be regulated by signaling pathways that integrate information from both water availability and insect-induced stress signals. In this way, a response to a change in water availability might lead to priming or in extreme cases even cross resistance between abiotic and biotic stressors as I saw in the transcriptomic data for *R. sylvestris* under waterlogged conditions (**Chapter 5**). The identification and characterization of these multifunctional metabolites and the elucidation of their underlying regulatory mechanisms hold great promise for enhancing plant resilience in complex environments. Continued research in this area will contribute to our understanding of the intricate interplay between plant metabolism, stress adaptation, and ecological interactions in the face of double trouble.

Future perspectives

Moving forward, it is important to explore the role of plant phylogeny to understand the general principles that link evolutionary adaptations to the impact of water stress to plant-insect interactions. By investigating the phylogenetic relationships among plant species and examining their evolutionary history as well as their life-history and habitat, we can gain insights into the shared traits, strategies, and adaptations that have evolved in response to multiple environmental challenges (Liu et al. 2020; Woldesemayat et al.

2018). One avenue for future research is to conduct comparative studies across plant lineages to identify common patterns or trends in the responses of plants and their associated insect communities to water stress. By elucidating common patterns and their underlying mechanisms, we can begin to unravel the fundamental rules governing evolutionary adaptations to multi-stress environments.

Furthermore, integrating genomic and transcriptomic approaches can provide a deeper understanding of the molecular basis underlying these adaptations. Comparative analyses of transcriptomic profiles across different plant lineages can identify conserved molecular pathways or gene families that play key roles in mediating plant-insect interactions under water stress conditions, and other multi-stress responses. This information can shed light on the genetic basis of plant resilience to double trouble and the potential trade-offs or synergies between adaptations to different stressors. These insights can form the basis for targeted plant-breeding programs aimed at developing crop varieties that are better adapted to the challenges posed by complex and changing environmental conditions.

In this study, I focused primarily on the fact that climate change will bring with it longer droughts and more extreme precipitation events leading to waterlogging. However, to gain a comprehensive understanding of the impact of climate change on ecosystems, it is essential to consider all facets of climate change. For instance, rising temperatures due to climate change can induce heat stress in plants, which in turn can have implications for plant-insect interactions (Hamann et al. 2021; Sentis et al. 2013). Additionally, the rising levels of atmospheric carbon dioxide (CO_2) associated with climate change demand exploration. Plants have the ability to utilize increased CO_2 concentrations through enhanced photosynthesis, potentially altering their interactions with insects (Lincoln et al. 1986; Pincebourde et al. 2017; Robinson et al. 2012). Furthermore, climate change also impacts insects directly. With increasing temperatures associated with climate change, insect metabolism is likely to be influenced, given their ectothermic nature (Bale et al. 2002). Additionally, climate change causes a shift in communities. As habitats move, insect species can be differently affected (Devictor et al. 2012). This creates new community dynamics as certain species leave and others arrive. Understanding how temperature changes affect insect development rates, population dynamics, and interactions with host plants will provide valuable insights into the potential shifts in pest pressure and distribution patterns. Only when considering all aspects of climate change together can we start to predict the true impact of a changing climate on natural and agricultural ecosystems.

In conclusion, water availability can significantly shape plant-insect interactions, manifested through myriad direct and indirect mechanisms. Wild plants also exhibit great adaptability when confronted with the combined challenges of water stress and insect attacks. Understanding the distinct and specialized responses of plants to these multi-stress environments is pivotal for comprehending their resilience and survival strategies. Further exploration of these strategies holds promise for sustainable agriculture, as it can inform the development of crop varieties that display inducible traits which allow plants to bend rather than break under multi-stress environments.

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Summary

Summary

Plants face a myriad of biotic and abiotic stress factors. Among these stressors, water stress, caused by either a scarcity or surplus of water, and herbivory by insects are prominent threats to plants. In recent years, global weather extremes have resulted in amplified occurrences of water-stress-induced crop damage due to excessive precipitation and prolonged droughts. Concurrently, insect herbivory contributes significantly to diminished crop yields. The push for reduced insecticide application due to insecticide resistance and sustainable agricultural practices has underscored the need for pest-resistant crop varieties. While water stress and insect herbivory individually pose significant threats to plant survival, their co-occurrence can exacerbate this challenge. This simultaneous challenge, or "double trouble", introduces complexities in how plants should respond to their environment. Notably, water stress not only directly impacts plant performance but could also influence resistance to insect herbivores. Previous research has frequently studied plant responses to either biotic or abiotic stress in isolation, neglecting their interconnectedness. Given that the response to one stressor can interact with the response to the other, understanding the evolutionary adaptations of plants to cope with environments featuring concurrent biotic and abiotic stress is paramount.

The aim of this thesis was to elucidate the influence of water availability on plant responses to insect herbivory, extending beyond direct impacts to encompass broader ecological consequences. To achieve this, a comparative approach was adopted, focusing on closely related wild plant species within the *Rorippa* genus that grow in different habitats along a water gradient. This approach aimed to establish connections between plant adaptations to- and the impact of water conditions on interactions between plants and insects. A diverse array of techniques, including transcriptomics, metabolomics, analysis of insect behavior and performance, was employed to unveil the distinct responses of differently adapted plants to herbivory under varying water conditions. By delving into the repercussions of double trouble on diverse wild plants, this study offers crucial fundamental insights into how wild plants thrive in complex, multi-stress, environments. Such understanding is crucial for predicting the repercussions of climate change on ecosystems and bolstering agricultural resilience.

In **chapter 2** I delved into the effects of waterlogging and drought on the plant-associated arthropod community composition of four different *Rorippa* plant species in the field. To achieve this, I closely monitored plant phenotype and the arthropods naturally arriving on the plants under different watering regimes. Drought had the biggest negative effect on plant size, even on plant species most adapted to drier climates. Still, both drought and waterlogging exerted an adverse effect on the richness and composition of the plant-associated arthropod community. The arthropod community was significantly affected by water conditions in three out of four species tested, exemplifying the species-specific consequences of water conditions on plant-arthropod interactions. Furthermore, these effects extend to higher trophic levels, influencing the abundance of predatory insects. This confirms the capacity of water conditions to modulate the apparency or defensive capabilities of the plants for certain arthropod species, thereby inducing far-reaching ecological consequences on arthropod community dynamics in field settings.

In **chapter 3** I investigated the impact of water availability on plant resistance to insect herbivory. To comprehensively explore this relationship, I conducted experiments involving three distinct plant species and four herbivore species. These experiments encompassed three watering regimes, spanning from drought to waterlogging. The herbivores tested included the chewers *Pieris brassicae* (large cabbage white) and *Plutella xylostella* (diamondback moth), as well as the piercer/suckers *Myzus persicae* (green peach aphid) and *Lipaphis erysimi* (mustard aphid), all of which are recognized pests of Brassicaceae plants. The semi-aquatic plant *Rorippa amphibia* exhibited decreased resistance against aphids under drought conditions, while the resistance of the other two plant species remained unaffected. The terrestrial plant *Rorippa sylvestris* showed heightened vulnerability to herbivory from aphids under waterlogged conditions, contrary to the resistance patterns observed in *R. amphibia*. Notably, resistance against caterpillars remained consistently unaffected by water conditions. The third plant species, *Rorippa palustris*, consistently maintained its resistance levels across varying water conditions. Furthermore, herbivores demonstrated greater success on *R. palustris*, potentially attributed to a shorter life history compared to the other two plant species. These findings suggest that different water conditions may elicit species-specific responses in plant species that may correspond with variation in their adaptations to water conditions in their habitat.

In addition to direct resistance, plants can also defend themselves against herbivores by releasing volatiles that attract natural enemies of the herbivores. Therefore, in **chapter 4**, I elucidate how well plants are able to recruit natural enemies to control herbivores under different water conditions. Specifically, I examined whether plants infested with *Plutella xylostella* caterpillars can still effectively recruit the parasitoid *Diadegma semiclausum*, and whether plants infested with *Myzus persicae* aphids can still attract the parasitoid *Aphidius ervi* under various water conditions. In addition, I measured the effect of water availability in combination with herbivory on the volatile blend that plants release. I found that both drought and waterlogging altered the volatile profile of *Rorippa* plant species. In most cases, this resulted in the inability of both parasitoid species to differentiate between water-stressed and herbivore-stressed plants, although the strength of effect on these interactions differed for plant species. As a consequence of water stress hindering the parasitoid's ability to locate its host, this finding has considerable implications for the efficacy of biological pest control under fluctuating abiotic conditions. Furthermore, comparing the direct defenses studied in **chapter 3** and the indirect defenses explored in **chapter 4** shows that water availability can have distinct effects on direct and indirect defenses and that these patterns differed for closely related plant species.

Chapter 5 of my thesis was dedicated to investigating how closely related plants integrate their responses to water stress and herbivory. Building upon the prominent influence of water availability on plant defenses observed in previous experiments, I concentrated on the aphid *Myzus persicae* for further exploration. Additionally, I selected two plant species, namely the semi-aquatic *Rorippa amphibia* and the terrestrial *Rorippa sylvestris*. To unravel the underlying physiological processes governing the simultaneous stress responses to water and aphid infestation, I characterized the transcriptomic and phloem metabolomic reactions triggered by aphid herbivory as well as aphid feeding behavior itself under three different water conditions ranging from drought, well-watered to waterlogging. In the case of the semi-aquatic *R. amphibia*, drought conditions were found to hamper its response to aphid herbivory, manifesting as a reduction in defense-related gene activity. This aligns with the diminished resistance observed in **Chapter 3**, suggesting that drought impairs the defense mechanism of *R. amphibia* in the face of aphid infestation. In contrast, the terrestrial *R. sylvestris* demonstrated minimal change to drought but displayed a strong response to waterlogging. Waterlogged conditions yielded heightened resistance in *R. sylvestris*, potentially attributable to an increase in reactive oxygen species induced by the waterlogging response, which adversely affected aphid performance but had minimal effects on their feeding behavior. This comparative approach highlights plant-specific effects of water conditions on the plant response to aphid feeding, likely influenced by plant-specific adaptations to water conditions.

In **chapter 6** I integrate results from different chapters and emphasize the multifaceted effects of water availability on plant-insect interactions. I discuss the value of studying how wild plants have thrived in their native habitats to understand how plants have evolved adaptations to multi-stress environments. In conclusion, this study on wild *Rorippa* plants revealed significant ecological consequences of water availability on plant-insect dynamics. Furthermore, by comparing several *Rorippa* plant species we started describing the variation in strategies deployed by plant species reacting to double trouble. These fundamental insights may guide plant breeding in the development of crops that are resilient to the double trouble of water tress and insect attack.

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About the author

Bram B.J. Kamps was born on 14 February 1993 in 's-Hertogenbosch, Noord-Brabant, the Netherlands. After obtaining a propaedeutic degree at the HAS University of Applied Sciences Den Bosch in Applied Biology, Bram moved to Wageningen to attain a BSc degree in Biology. During his studies Bram focussed on the ecology and biological control of pest insects, and graduated with a thesis on mosquito host preference. After that he decided to pursue an MSc degree with double specialization in bio-interactions and developmental biology. For this he completed two major theses. In the first one Bram looked at plant-mediated interactions between above and belowground organisms. For the second thesis Bram travelled to Sierra Leone to study insecticide resistance in malaria mosquitoes. Torn between studying insects as human or plant pests Bram decided on the latter. Bram went on to write his PhD project proposal in collaboration with Prof. Dr Erik H. Poelman on the effect of water availability on plant-insect interactions. This proposal got funded by the Graduate Programme grant from the graduate school Experimental Plant Sciences.

In April 2019, he began his PhD journey at the Laboratory of Entomology, where his research covered a wide range of methodologies, from field ecology to transcriptomic studies. During his time as a PhD candidate, Bram had the opportunity to work together with many students and fellow researchers from other institutes in the Netherlands and Germany. These collaborations truly broadened his knowledge and mind which contributed to the creation of the book you are holding in your hands right now.

List of publications

Poelman, E.H., Bourne, M.E., Croijmans, L., Cuny, M.A., Delamore, Z., Joachim, G., Kalisvaart, S.N., **Kamps, B.B.J.**, Longuemare, M., Suijkerbuijk, H.A.C. and Zhang, N.X., 2023. Bringing fundamental insights of induced resistance to agricultural management of herbivore pests. In press *Journal of Chemical Ecology*.

Kamps B.B.J., Mertens D., Poelman E.H., Water conditions change the composition and structure of plant-associated arthropod communities differently in four closely related *Rorippa* plant species. In preparation **(Chapter 2 of this thesis)**

Kamps B.B.J., Poelman E.H., Adaptations to water gradient in three *Rorippa* plant species correspond with plant resistance against insect herbivory under drought and waterlogged conditions. In press *Ecological Entomology* **(Chapter 3 of this thesis)**

Kamps B.B.J., Gelens L.P.J., Weldegergis B.T., Poelman E.H., Water availability affects parasitoid recruitment and plant volatile profile of three *Rorippa* plant species. Under review **(Chapter 4 of this thesis)**

Kamps B.B.J., van Veen H., Schweiger R., Vitiello A., de Haan J., Verrijp I., Kloth K.J., Sasidharan R., Müller C., Poelman E.H., How plants react to double trouble - Strategies to cope with waterlogging and drought stress affect responses to aphids in two closely related *Rorippa* species. In preparation **(Chapter 5 of this thesis)**

Mertens D, Douma J.C., **Kamps B.B.J.**, Zhu Y, Zwartsenberg S.A., Poelman E.H., Priority effects in herbivore communities vary in effect on plant development and reproduction in four Brassicaceae plant species. Under review

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Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School
EXPERIMENTAL
PLANT
SCIENCES

Issued to:	**Bram B.J. Kamps**
Date:	**08 December 2023**
Group:	**Laboratory of Entomology**
University:	**Wageningen University & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project**		
The double trouble of insect attacks and water stress	03 Jun 2019	1,5
Subtotal Start-Up Phase		1,5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD days**		
EPS Get2Gether 2020	10-11 Feb 2020	0,6
EPS Get2Gether 2021 (online)	01-02 Feb 2021	0,4
EPS Get2Gether 2022	03-04 May 2022	0,6
► **EPS theme symposia**		
Theme 2 symposium “Interaction between Plants and Biotic Agents” (online)	09 Feb 2021	0,2
Theme 2 symposium “Interaction between Plants and Biotic Agents” (online)	08 Feb 2022	0,2
Theme 2 symposium “Interaction between Plants and Biotic Agents”	19 Jan 2023	0,3
► **National platform meetings**		
Annual meeting of the Netherlands Entomological Society (NEV)	13 Dec 2019	0,3
Netherlands Annual Ecology Meeting (NAEM)	12 Feb 2020	0,3
Netherlands Annual Ecology Meeting (NAEM)	20-21 Sep 2022	0,6
Annual Meeting Experimental Plant Sciences	11-12 Apr 2022	0,6
► **Seminars (series), workshops and symposia**		
Ento-Seminar Yoshihiro Yamada: Caste-fate determination in paper wasps	07 Oct 2019	0,1
Ento-Seminar Chantal Vogels: Does arbovirus evolution matter?	24 Feb 2020	0,1
NIOO-seminar Ted Turlings: Can herbivore-induced plant volatiles be exploited for the real-time detection of crop pests?	09 Mar 2020	0,1
Plant-Insect Interactions Workshop 2019	14 Nov 2019	0,3
Plant-Insect Interactions Workshop 2021	21 Oct 2021	0,3
Yearly Entomology Research Exchange Meeting	07 Jun 2019	0,3
Yearly Entomology Research Exchange Meeting	13 May 2022	0,3
Entosymposium	20 Feb 2023	0,1
► **International symposia and congresses**		
Symposium on insect-plant interactions (SIP) (online)	25-30 Jul 2021	1,5
Gordon Research Symposium & Conference on herbivore-plant interactions, Ventura, USA	25 Feb - 03 Mar 2023	1,8
► **Presentations**		
Poster presentation, Symposium on insect-plant interactions (SIP), poster prize	25-30 Jul 2021	1,0
Poster presentation, Gordon Research conference on herbivore-plant interactions, Ventura,USA	26 Feb - 03 Mar 2023	1,0
Oral presentation, Universität Bielefeld, Chemical Ecology group	10 Feb 2020	1,0
Oral presentation, Gordon Research symposium on herbivore-plant interactions, Ventura, USA	26 Feb 2023	1,0
Oral presentation, Entosymposium	20-2-2023	1,0
Oral presentation, EPS Theme 2 symposium “Interaction between Plants and Biotic Agents”	19 Jan 2023	1,0
► **Excursions**		
Lab visit, Universität Bielefeld, Chemical Ecology group, Prof. Dr. Caroline Müller	09-10 Feb 2020	0,6
Subtotal Scientific Exposure		15,6

3) In-Depth Studies	date	cp
▶ **Advanced scientific courses & workshops**		
HarvardX course Introduction to Bioconductor (online)	Jul 2020	0,6
PE&RC course Chemical communication	02-06 Feb 2020	1,5
University of Cambridge course Analysis of bulk RNA-seq data (online)	22-24 Mar 2021	0,9
Subtotal In-Depth Studies		3,0

4) Personal Development	date	cp
▶ **General skill training courses**		
EPS Introduction Course	11 Jun 2019	0,3
WGS course Supervising BSc & MSc Students	14 Jun 2022	0,6
WGS course Scientific Writing	23 May - 11 Jul 2022	1,8
▶ **Organisation of scientific meetings, PhD courses or outreach activities**		
Thesis markets 2021 -2023	2021-2023	0,6
Organisation of bi-weekly PhD discussions/workshops at Entomology	2021	1,5
▶ **Membership of EPS PhD Council**		
EPS PhD Council member	2021 & 2022	1,4
Organisation of EPS Get2Gether 2022 & EPS Get2Gether 2023	2021 & 2022	1,5
Setting up a buddy system for EPS PhD candidates	2022	0,7
Organisation of a career event connecting EPS alumni with current PhD candidates	2021	0,8
Subtotal Personal Development		9,2

5) Teaching & Supervision Duties	date	cp
▶ **Courses**		3,0
Ecology 1	2019 & 2022	
Ecological Aspects of Biointeractions	2019 - 2021	
Insect Plant Interactions	2021	
▶ **Supervision of BSc/MSc projects**		3,0
BSc thesis 'Biotic and abiotic stresses in plants: How Rorippa species defend themselves against chewing herbivores while facing water stress'	2019	
BSc thesis 'Drought and waterlogging changes the Composition of the Insect community on *Rorippa amphibia* and *Rorippa sylvestris'*	2020	
BSc thesis 'An overview and meta-analysis on the effects of water stress on the plant-insect (herbivore) interactions'	2021	
MSc thesis 'How water stress affects defenses against insect herbivory in Rorippa plant species'	2020	
MSc thesis 'The double trouble of insect attack and water stress; Can plants still attract natural enemies while subjected to water stress?'	2021	
MSc thesis 'Quantifying plant defence against aphids in two Rorippa species exposed to water stress'	2021	
MSc thesis '*Myzus persicae* performance on Waterlogged and drought stressed *Rorrippa amphibia* and *Rorrippa sylvestris'*	2021	
Subtotal Teaching & Supervision Duties		6,0

TOTAL NUMBER OF CREDIT POINTS*	**35,3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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