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Responses to waterlogging and drought of timothy and related *Phleum* species: phenotype and transcriptome diversity

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Cover: Illustration of timothy (*P. pratense*) under waterlogging and drought stress, drawn by Elena Raposo.

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Responses to waterlogging and drought of timothy and related *Phleum* species: phenotype and transcriptome diversity

Abstract

Timothy (*Phleum pratense*) is a cool-season perennial forage grass commonly used in temperate regions for silage, hay and grazing. Climate change is causing abiotic stresses on agriculture and there is an increasing need for tolerant cultivars. To expand the gene pool for breeding stress-tolerant cultivars, the diversity in natural populations can be exploited. One goal of this study was to understand the diversity of plant traits among a set of *Phleum* accessions in response to waterlogging and drought stress. A second goal was to identify accessions with putative beneficial traits based on their responses in growth, development, anatomy, and transcriptome. Diversity in biomass and development was evaluated in the field and in a greenhouse for a large collection of 244 wild and domesticated accessions of timothy and two related species, turf timothy (*P. nodosum*) and alpine timothy (*P. alpinum*), from different locations and habitats in Europe. Large diversity was found between species and accessions. The groups of wild accessions and cultivars of *P. pratense* differed in development, but not in biomass. The same pattern was found for *P. nodosum*. A subset of 19 wild accessions and cultivars from the large collection, were studied in a greenhouse for physiological responses to 21 days of waterlogging, and to 28 days of drought followed by a recovery period. The *Phleum* species and accessions differed in their response to waterlogging and drought and in their ability to recover. Under waterlogging, some accessions had beneficial adaptations in root growth and aerenchyma formation in roots that were positive for shoot biomass production. In drought, shoot growth was severely affected, but *P. nodosum* had a greater ability to produce tillers than *P. pratense*. Based on indexes for drought stress tolerance and resilience, some wild accessions appeared to be more tolerant and to have better recovery. Interestingly, one wild *P. pratense* accession appeared to have tolerance to both waterlogging and drought. Eight accessions were further investigated for transcriptional responses to waterlogging and drought. Clear differences in the expression pattern due to stress were observed, and abundant tissue-specific (leaf or root) expression to stress was found. Differentially expressed genes (DEGs) associated with stress responses, transport, hormone biosynthesis were found in response to waterlogging and drought. In all three species, DEGs were mainly accession specific. Interestingly, in *P. pratense*, a wild accession and an old cultivar shared 56 DEGs under drought and showed a similar phenotypic response. The knowledge gained on the diversity of trait responses and transcriptome, together with the identified accessions, are valuable resources for further pre-breeding and development of stress-resilient cultivars.

Keywords: aerenchyma, breeding, cultivar, flooding, forage, grass, growth allocation, perennial, root, shoot, stress, wild accession

Respons på vattenmättnad och torka hos timotej och besläktade arter: diversitet i fenotyp och transkriptom

Abstract

Timotej *(Phleum pratense*) är ett flerårigt vallgräs som odlas i tempererade områden för ensilage, hö och bete. Jordbruket utsätts för stora påfrestningar av klimat-förändringarna och grödor och sorter toleranta mot abiotisk stress behövs. För att utöka genpoolen för växtförädling av stress-tolerans kan naturliga populationer nyttjas. Ett mål med denna studie var att förstå diversiteten av växt-egenskaper bland accessioner från släktet *Phleum* vid vattenmättnad och torkstress. Ytterligare ett mål var att identifiera accessioner med bra egenskaper för tolerans baserat på tillväxt, utveckling, anatomi och transkriptom. Diversitet i biomassa-produktion och utveckling utvärderades i fält och i växthus för en stor grupp accessioner. Den bestod av 244 vilda och domesticerade accessioner av timotej och två besläktade arter, vildtimotej *(P. nodosum*) och fjälltimotej *(P. alpinum*), insamlade från olika platser och livsmiljöer i Europa. Diversiteten var stor bland dessa arter och accessioner. Vilda accessioner och sorter av *P. pratense* skiljde sig åt i utveckling, men inte i biomassa-produktion. Samma mönster upptäcktes hos *P. nodosum*. Nitton accessioner studerades vidare i växthus för påverkan i fysiologiska egenskaper orsakad av 21 dagars vattenmättnad och av 28 dagars torka följd av återhämtning. Bland *Phleum*-arterna och accessionerna fanns skillnader i påverkan av vatten-mättnad och torka, och i återhämtning. Vid vattenmättnad hade vissa accessioner fördelaktiga anpassningar i rottillväxt och aerenkym-bildning i rötterna vilket var positivt för skottets tillväxt. Vid torka påverkades skottillväxten kraftigt, men *P. nodosum* hade större förmåga att producera skott än *P. pratense*. Baserat på index för tolerans och resiliens mot torkstress var vissa vilda accessioner mer toleranta och hade bättre återhämtningsförmåga. Intressant är att en vild *P. pratense*-accession hade tolerans mot både vattenmättnad och torka. Åtta accessioner undersöktes ytterligare och visade stora skillnader i genuttryck vid vattenmättnad och torka genom studier av transkriptom. Även stora skillnader för vävnadsspecifikt (blad eller rot) uttryck vid stress hittades. Differentiellt uttryckta gener (DEG) associerade med stress-respons, transport och hormon-biosyntes hittades vid vattenmättnad och torka. I alla tre arterna var DEG i huvudsak accessions-specifika. Intressant är att en vild accession och en gammal sort av *P. pratense*, hade 56 gemensamma DEG vid torka och liknande fenotypisk respons. Kunskap om diversitet i påverkan av stress och transkriptom, samt de identifierade accessionerna, är värdefulla resurser för utveckling av stress-tåliga sorter.

Nyckelord: aerenkym, perenn, rot, skott, stress, tillväxt-allokering, vallgräs, vild accession, växtförädling, översvämning

Dedication

To my parents, Patricio and Maria Elena.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Rahimi, Y., Bedada, G., **Moreno, S.**, Gustavsson, A.-M., Ingvarsson, P. K., & Westerbergh, A. (2023). Phenotypic diversity in domesticated and wild timothy grass, and closely related species for forage breeding. *Plants*, 12, 3494. doi:10.3390/plants12193494

II. **Moreno S.**, Bedada G., Rahimi Y., Ingvarsson P. K., Westerbergh A., Lundquist P-O. (2023). Response to waterlogging stress in wild and domesticated accessions of timothy (*Phleum pratense*) and its relatives *P. alpinum* and *P. nodosum*. *Plants*, 12, 4033. doi.org/10.3390/plants12234033

III. **Moreno S.**, Bedada G., Ingvarsson P. K., Westerbergh A., Lundquist P-O. Diversity in phenotypic response to drought stress in wild and domesticated accessions of timothy (*Phleum pratense*) and related species *P. alpinum* and *P. nodosum*. (manuscript)

IV. **Moreno S.**, Bedada G., Lundquist P-O., Westerbergh A., Ingvarsson P. K. Transcriptional and phenotypic responses of *Phleum* species to waterlogging and drought stresses. (manuscript)

Papers I and II are reproduced with the permission of the publishers.

Publications produced during the study period, but not included in this thesis

V. Hu J, Bettembourg M, **Moreno S.**, Zhang A., Schnürer A., Sun C., Sundström J., Jin Y. (2023). Characterisation of a low methane emission rice cultivar suitable for cultivation in high latitude light and temperature conditions*. Environmental Science and Pollution Research*, 30, 92950-92962. doi: 10.1007/s11356-023-28985-w

The contribution of Silvana Moreno to the papers included in this thesis was as follows:

I. Involved in the data collection and the reviewing of the manuscript.

II. Planned the study together with the co-authors, performed all experiments, highly involved in the data analysis, and the writing and editing of the manuscript.

III. Planned the study together with the co-authors, performed all experiments, highly involved in the data analysis and the writing of the manuscript.

IV. Planned the study together with the co-authors, performed all experiments, highly involved in the data and bioinformatics analyses, and the writing of the manuscript.

List of figures

Introduction

Farming is directly exposed to climate change. Shifting weather patterns with rising temperatures and increased frequency of extreme weather events with heavy rainfall or drought lead to soil erosion (Eekhout & de Vente, 2022), plant diseases outbreaks (Singh et al., 2023), crop losses, and reductions in yield and quality (Fu et al., 2022) affecting crop production and food security (FAO, 2023). In northern Europe, climate change is associated with both potential benefits as well as notable challenges (Wiréhn, 2018). Warmer temperatures and longer growing seasons could potentially increase the number of harvests per year and allow for the cultivation of new crops. However, heavy precipitation is expected to increase throughout the year (SMHI, 2022). In addition, there is an increased risk of pests and diseases, and difficulties in planning the timing of planting and harvesting.

To mitigate the detrimental effects of climate changes, it is essential to change agricultural practices and develop new resilient varieties and crops. Wild plant populations are a valuable source of genetic material for crop improvement, as they offer high genetic diversity and are adapted to different habitats, abiotic and biotic stresses (Peeters, 2004). In addition, perennial crops may be important resources for improvement of food security in stressed environments and in the long-term resources for mitigating climate changes. With their extensive root systems and year-round ground cover they provide important ecological benefits including higher soil carbon sequestration, reduced nutrient leaching and improved soil health (Gomiero et al., 2011; Soto-Gómez & Pérez-Rodríguez, 2022). The perennial root system improves nutrient and water uptake, which may also make them more tolerant to abiotic stresses such as drought.

In Sweden and other regions in northern Europe, the perennial forage crop timothy (*Phleum pratense* L. subsp. *pratense*) is widely cultivated for livestock grazing and for the production of hay and silage with high nutritive value and digestibility (Bélanger et al., 2001; Jing et al., 2013). Outside agricultural fields, wild undomesticated populations of timothy can be found on meadows, roadsides, fields, and grasslands (Peeters, 2004). *P. pratense* is closely related to the perennial species turf timothy (*P. nodosum,* syn. *P. pratense L*. subsp. *bertolonii* (DC.) Bornm, and *P. bertolonii* (DC.) Bornm) and alpine timothy *P. alpinum* (Stewart et al., 2009 ; Stewart et al., 2011). Timothy can be crossed with *P. nodosum* and *P. alpinum* and produce offspring (wide-hybridization)(Gregor & Sansone, 1930; Kula & Grabowska-Joachimiak, 2009; Muntzing, 1935; Nordenskiöld, 1937). These species, together with wild *P. pratense* populations, may therefore serve as potential genetic resources for timothy breeding.

As the world struggles with the consequences of climate change, the focus on understanding the physiological and genetic responses of crops to a changing climate and abiotic and biotic stresses has never been more important for the development of stress-tolerant cultivars and crops. The work presented in this thesis covers the phenotypic diversity among wild and domesticated accessions of *P. pratense*, *P. nodosum* and *P. alpinum*, their physiological responses to waterlogging and drought and differences in gene expression patterns between stress and non-stressed conditions. It also explores traits of interest for selecting abiotic stress-tolerant genotypes in pre-breeding research and as genetic resources in breeding programmes.

The following sections (1.1-1.9) serve as an introduction and background to my PhD studies.

1.1 Perennial crops in agriculture

To meet the growing global demand for food, natural landscapes are increasingly converted into agricultural lands, often changing complex ecosystems of diverse perennial plants into simplified annual plant communities grown in monocultures (Crews et al., 2016). This transformation leads to significant ecological changes, including reduced carbon input into ecosystems and altered soil microbial activities, which may potentially increase greenhouse gas emissions and impair soil health (Means et al., 2022; J. Wang et al., 2020). Maintaining plant diversity is essential for ecosystem functioning as it enhances biodiversity, improves soil structure, and provides more stable production over time (Yang et al., 2021). Agricultural land is primary divided into two categories: land that is covered with permanent crops, for example perennial forage crops or tree crops, and arable land covered by annual crops such as cereals, oil crops and legumes. In 2019, around 1.4 billion hectares was used as arable land, while only 170 million hectares where used for permanent crops (FAO, 2021).

The main characteristic of perennial crops is their ability to regrow after harvest, eliminating the need for replanting. Consequently, field management and production costs are reduced. Additionally, perennials provide numerous environmental benefits, including enhancing soil diversity, soil health, and carbon sequestration (Gomiero et al., 2011; Soto-Gómez & Pérez-Rodríguez, 2022). Perennial species typically have deeper root systems than annual crops, enabling them to more efficiently utilise subterranean water and nutrients while reducing the risk of soil erosion. Although, perennial forage grasses and legumes have been cultivated for many decades by farmers, perennial grain crops are at an early stage of domestication and breeding (Cox et al., 2018; DeHaan et al., 2018; Westerbergh et al., 2018).

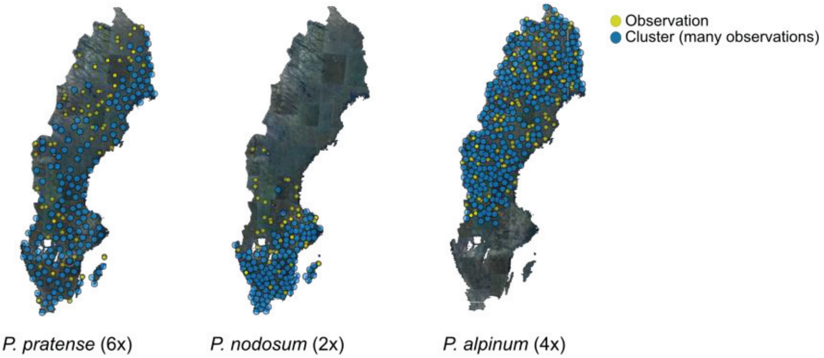
Perennial forage crops are predominantly outcrossing, which results in high heterozygosity within individuals and high genetic diversity within breeding populations. Although this broad genetic base is an advantage in breeding, high levels of heterozygosity and the long generation time in perennials may prolong selection cycles and reduce the genetic gain per cycle compared to annual crops (McClure et al., 2014). This will increase the costs associated with breeding programmes of perennial crops. However, modern genetic tools such as genomic selection, marker-assisted selection and gene editing, together with new technologies such as speed breeding and high-throughput phenotyping, can help shorten the breeding cycles and improve breeding programmes.

1.2 *Phleum* species

The genus *Phleum* belongs to the Poaceae family and it has 8 annual and 6 perennial species (Joachimiak & Kula, 1997; Stewart et al., 2011). The genus is native to Asia, Europe, North Africa, South America and North America (Stewart et al., 2011). In the genus *Phleum*, molecular studies have identified an Asian origin of the genus and at least two separate migration events have introduced the genus into Europe (Stewart & Ellison, 2016).

Cytological and karyotype analysis have shown that *Phleum* species have a basic chromosome number of seven, except *P. echinatum* that has five chromosomes (Joachimiak, 2005). The species have ploidy levels ranging from diploid (2n=14) to octoploid (2n=56) and they differ in their distribution (Stewart et al., 2011). In Sweden, wild and domesticated *P. pratense* can be found in most of the regions, while *P. nodosum* is restricted to the south and *P. alpinum* to the north of Sweden (Figure 1).

Previous studies have shown that *P. pratense*, *P. nodosum* and *P. alpinum* are closely related (Cai et al., 2003; Stewart et al., 2009 ; Stewart et al., 2011). *Phleum* species have only rarely been crossed with species outside the genus (Stewart et al., 2011). However, it has been shown that the three species can be crossed and produce fertile offspring (Gregor & Sansone, 1930; Muntzing, 1935; Nordenskiöld, 1937).



P. pratense (6x) *P. nodosum* (2x) *P. alpinum* (4x)

Figure 1. Distribution of *P. pratense*, *P. nodosum* and *P. alpinum* in Sweden. Source: SLU Artdatabanken (2024). Artportalen. https://www.artportalen.se [2024-04-02]

1.2.1 Timothy (*Phleum pratense*)

Timothy (*Phleum pratense* L. subsp. *pratense*) is a hexaploid (2n = 6x = 42) cool-season, cross-pollinated perennial grass widely grown for silage and hay production in cool regions of Europe, North America, North Africa and Asia (Bélanger et al., 2001). It has a high value as a forage crop due to its high palatability and digestibility for cattle and horses and its nutritional content (Bélanger et al., 2001; Berg et al., 1996). Timothy grows in pastures and meadows, and wild populations can be found in roadsides, fields, grassland and forests close to cultivation areas (Peeters, 2004; Stubbendieck et al., 2003). Timothy is well adapted to cool, moist climates but does not tolerate extended periods of flooding and it is not suited to dry or warm conditions (Berg et al., 1996; Casler & Undersander, 2019; Charlton & Stewart, 2000). Timothy is cultivated either in pure stands or in mixtures with other perennial forage grasses, such as meadow fescue, and perennial legumes, including red and white clover. In Sweden, about 40% of the agricultural land is used to grow fodder crops, with hexaploid timothy being the dominant fodder grass due to its winter hardiness. The average forage yield in Sweden is 5.2 t/ha. The county with the highest yield production is Halland County with 7.1 t/ha, and the county with the lowest yield production is Stockholm County with 3.9 t/ha (Swedish Agency for Agriculture, 2023).

The heads of timothy are cylindrical and green or purple. The leaves are flat or loosely involute and their length can vary from 5 to 30 cm and the width from 3 to 8 mm. The sheaths are round and open, hairless and distinctly veined, and often purplish at the base. The tillers are erect and between 50 to 120 cm tall, occasionally bending at the base. While most grass species have vegetative and generative tillers, timothy has a third type of tiller called vegetative elongating tiller (Charlton & Stewart, 2000; Virkajärvi et al., 2010). These tillers have a true stem with palpable nodes but they do not produce inflorescences, thus they are able to produce new leaf primordia (Gustavsson, 2011; Virkajärvi et al., 2012). The development of timothy and other grasses can be described in different developmental stages such as vegetative, stem elongation, booting, heading, flowering, development of fruit, ripening and senescence (Gustavsson, 2011). The transition from vegetative stage to stem elongation occurs when one internode begins to elongate and the inflorescence becomes palpable. The booting stage is reached when the tip of the inflorescence can be palpable in the flag leaf sheath. The plants reach heading when the tip of the head is visible above the flag leaf and flowering when a node on the stem is visible between the collar of the flag leaf and the inflorescence. The kernels then swell and pass through various stages, ending with late milk. This is followed by the ripening stage, which is characterised by the grain's transition from a soft to a hard dough state. In the last phase, ripening and senescence phase, the grains are hard, most of the seeds have been shed and the plant eventually collapses (Gustavsson, 2011).

Tillers at different stages are usually found in the spring growth. In regrowth after the first harvest (cut), a majority of the tillers remain in the vegetative stage. Flowering tillers can, however, be found in regrowth after the different cuts (Gustavsson, 2011; Virkajärvi et al., 2012). The majority of new timothy cultivars are harvested two to four times per season. Timothy can flower without vernalization. However, flowering is stimulated by vernalization, especially in northern accessions (Fiil et al., 2011; Jokela et al., 2015; Jokela et al., 2014; Seppänen et al., 2010). In addition, a longer photoperiod stimulates flowering and stem elongation (Fiil et al., 2011; Heide, 1982; Heide, 1994; Jokela et al., 2014; Seppänen et al., 2010).

1.2.2 P. nodosum

P. nodosum (syn. *P. pratense* L. *subsp. bertolonii* (DC.) Bornm. and *P. bertolonii* (DC.) Bornm.), commonly called turf timothy, is diploid (2n = 2x = 14). Outside Sweden, it is found in other countries in northern Europe, and in Spain, Portugal, Greece, the Balkans and northern Africa (Stewart et al., 2011). In northern Europe, cultivars of *P. nodosum* are often used in mixtures with other turf grasses (Stewart & Ellison, 2016; Tamaki et al., 2010). It has evolved from the diploid *P. alpinum* subsp. *rhaeticum* (2n = 2x = 14) adapted to higher elevations. During this process *P. alpinum* subsp. *rhaeticum* colonized habitats at lower elevations, which resulted in a differentiated into two species (Joachimiak, 2005; Stewart et al., 2009).

The stems of *P. nodosum* are at least 8 cm long and the base is swollen. The leaf blades are more than 12 cm long, and the leaf sheath is not inflated. (El-Gazzar et al., 2016).

1.2.3 P. alpinum

P. alpinum L. (syn. *P. commutatum* Gaudin)*,* commonly called alpine timothy is tetraploid (2n=4x=28). Wild populations can be found in the northern hemisphere and in southern South America and South Georgia (Callaghan, 1974; Castellaro et al., 2021; Lavrinenko et al. 2024; Nordenskiold, 1945). It grows in mountainous regions, and rocky habitats and it is not commonly cultivated for agricultural purposes. However, *P. alpinum* has been used for high-altitude revegetation purposes (Stewart & Ellison, 2016).

P. alpinum has few, short tillers with short, broad (6 mm width) and glabrous leaves. Spikes are compact, short and cylindrical (10-50 mm), and they have brownish purple or dark-blue color (Walker, 2014). To induce flowering, *P. alpinum* requires a dual induction by low temperature and/or short days, while a combination of long days and high temperature is required to enhance heading development (Heide, 1994; Heide & Solhaug, 2001).

1.3 Domestication and breeding of timothy

Domestication is a dynamic evolutionary process that occurs when wild populations are made cultivatable by humans. This process starts with recognition of the value of a wild species and leads to the domestication of a new species. Domestication of cereal grain crops has resulted in a number of important agronomical traits such as high-seed yield, reduced seed shattering, high degree of selfing, synchronized flowering and seed maturity (Boelt & Studer, 2010; Doebley et al., 2006), while the domestication of forage grasses have been towards higher forage biomass (Casler et al., 1996).

The first domesticated forage grass was Italian ryegrass (*Lolium multiflorum* Lam.) was used in northern Italy as early as the 12th century (Peeters, 2004). The domestication of new crops is followed by breeding and development of new varieties of the crop for cultivation in different growing conditions and climates. The breeding of annual grain crops usually involves a combination of traditionally methods (phenotypic selection and crossing) and modern genetic (marker-assisted selection) and/or genomic methods (genomic selection), while genomic selection has more recently been implemented in the breeding of perennial crops (Bajgain et al., 2022; Crain et al., 2020; Pembleton et al., 2018).

P. pratense was introduced to United States in 1720 under the name of "Timothy" and then exported to England in 1760 (Peeters, 2004). Timothy has been bred for over 100 years in many regions of the world and breeding efforts have largely focused on forage yield production (Bélanger et al., 2001), traits related to early and late flowering (Hays, 1892; Rahimi et al., 2023), forage digestibility and quality traits (Surprenant et al., 1990), resistance to diseases such as stem rust (Webber, 1912; Witte, 1919), purple spot and leaf streak (Ueda, 1990), and traits related to plant vigour (Casler, 2001).

Ongoing climate changes underline the importance of broadening the future breeding objectives towards tolerance to various abiotic stresses (Moreno et al., 2023). The phenotypic diversity in forage yield and development among wild and domesticated accessions of timothy and its related species *P. nodosum* and *P. alpinum* are discussed in Paper I. Additionally, responses to abiotic stresses and traits of interest for selecting waterlogging and drought tolerant accessions as genetic resources for forage breeding is also discussed in Papers II-IV.

1.4 Climate changes in Northern Europe

In Northern Europe, climate change has a dual impact on agriculture, with potential benefits and significant challenges (Wiréhn, 2018). Some of the expected benefits for agriculture are the prolonged growing season that may increase the number of harvests per year (Bindi & Olesen, 2011; Höglind et al., 2013; Rognli et al., 2021; Uleberg et al., 2014). This will favour perennial crop production (Fogelfors et al., 2009; Olesen & Bindi, 2002), crop expansion in the north (Elsgaard et al., 2012; Thorsen & Höglind, 2010), and increase opportunities to grow C3 plants (Fogelfors et al., 2009; Maracchi et al., 2005). However, prolonged wet and dry periods and rising temperatures are expected to lead to harvest losses, difficulties in soil management, complicated harvesting conditions, and the need for increased use of crop protection products (herbicides and pesticides). Negative effects in Northern Europe are associated with summer droughts (Eckersten et al., 2007), increased autumn temperature and precipitation (de Toro et al., 2015; Hakala et al., 2012; Rötter et al., 2013; Uleberg et al., 2014), milder winters with reduced snow cover and frequent freeze-thaw events (Fogelfors et al., 2009; Marttila et al., 2005; Sharif et al., 2017; Thorsen & Höglind, 2010), and spring flooding (Olesen et al., 2012; Thorsen & Höglind, 2010). In 2018, persisting heatwaves over Scandinavia and other parts of Europe had drastic impacts on agriculture. For example, in Sweden, the drought periods of 2018 lead to a shortage of water, which affected the production of forage and other crops (Wilcke et al., 2020). Many farmers were forced to slaughter animals due to the lack of fodder, thus impacting also beef production.

Agriculture must adapt to the negative effects of the ongoing climate changes. These challenges could be addressed by modifying agricultural practices and using new technologies. However, new crops and varieties adapted to these changes are crucial keys and prerequisites for a sustainable food and feed production. A deeper understanding of their physiological responses to stresses, as well as identification of traits related to tolerance and potential genetic resources will have a great impact on the breeding of climate-adapted varieties. In papers II-IV we provided valuable knowledge related to the physiological and genetic responses to waterlogging and drought of wild and domesticated accessions of *Phleum* species, which may be used for further pre-breeding research and in future breeding programs.

1.5 Waterlogging stress

Intense periods of rainfall, flooded lakes and rivers, and poor soil drainage can lead to waterlogged and submerged soils. Unlike aquatic plants, a majority of the terrestrial plant species are susceptible to flooding. Some crops are, however, adapted to growth in flooded fields, such as paddy rice and taro, where their root systems and most of their above ground tissues are submerged under water for extended periods of time. Long-term submergence and waterlogging of soils lead to anaerobic conditions impairing photosynthesis and respiration, and disrupting energy metabolism of the plant. The effect of waterlogging stress and oxygen deficiency in perennial grasses has been studied in e.g. ryegrass and tall fescue (Mcfarlane et al., 2003; Mui et al., 2021), and recently timothy and related *Phleum* species discussed in this thesis (Paper II-IV). Among the visual symptoms of waterlogging stress on plants are reduced shoot and root growth, delayed seed development, leaf chlorosis, leaf drop and wilting, premature senescence and root tip death (Ashraf & Yasmin, 1991; Ploschuk et al., 2017; Setter & Belford, 1990). While some plants are severely affected by waterlogging, others are able to adapt by altering different morphological traits. Some plants are able to form adventitious roots and aerenchyma (air-filled cavities) in the root cortex, as seen for example in timothy (Figure 2, Paper II, IV). Adventitious roots develop from aerial tissues, for example at the base of the stem, which promotes gas exchange and facilitates the absorption of water and nutrients. Aerenchyma is formed as a result of programmed cell death and degradation of the cortical cells caused by oxygen deficiency (Evans, 2004). These anatomical structures facilitate the transport of oxygen, carbon dioxide and toxic volatiles from the roots (Pan et al., 2021; Yamauchi et al., 2013). Other plants can adapt by elongating their apical meristems, or by forming a gas film on the surface on their leaves, and thereby enabling aerobic respiration and photosynthesis (Kurokawa et al., 2018; Pedersen et al., 2017; Steffens & Rasmussen, 2016; Winkel et al., 2017).

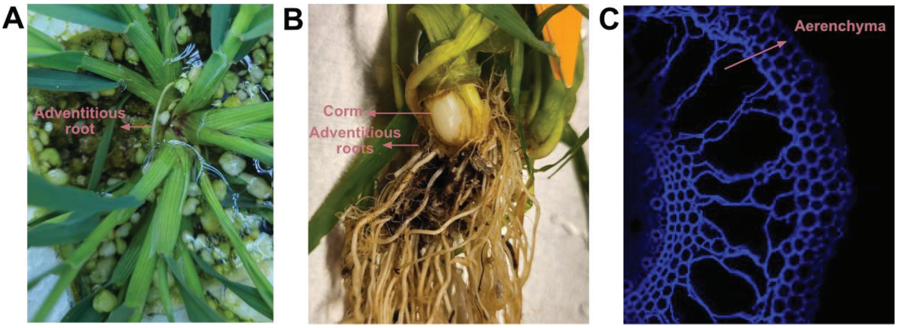


Figure 2. Adventitious roots of timothy (A, B) and aerenchyma in the root cortex (C) formed in response to waterlogging.

The current knowledge of tolerance responses and adaptations to abiotic stresses are mainly based on studies involving annual plants, while studies on perennial plants are less common. This thesis aims to narrow this knowledge gap.

1.6 Drought stress

Drought limits water and nutrient uptake by plants and a major consequence of this is reduced leaf turgor (Salehi-Lisar & Bakhshayeshan-Agdam, 2016). In addition, when roots sense water deficit, hormone signalling triggers the closure of stomata (microscopic pores in the leaf epidermis) to prevent transpirational water loss (Agurla et al., 2018; Chaves et al., 2002; Cruz de Carvalho, 2008; Kaur & Asthir, 2017). Stomatal closure results in lower CO_2 uptake and thereby lower photosynthetic rates and chlorophyll production (Ghannoum, 2009; He et al., 2020; Zhang et al., 2021). During prolonged drought stress and stomatal closure, the absorbed light energy becomes higher than the energy used for photosynthesis (Chaves et al., 2009). This results in high production of reactive oxygen species (ROS), which causes damages to chloroplasts (Cruz de Carvalho, 2008). The reduced rate of photosynthesis and lower uptake of water and nutrients have negative effects on plant growth and survival, with reduced leaf and tiller formation in grasses and early senescence (Kramer & Boyer, 1995; Pirnajmedin et al., 2017; Seleiman et al., 2021; Staniak & Kocoń, 2015; Xu et al., 2010). Plants may prioritize their resource allocation to enable root growth during drought, which affects the root-shoot ratio (Becker et al., 2015). Extended root growth allows plants to sustain water uptake to maintain water influx during drought (Bacher et al., 2021).

Plants have developed various strategies and mechanisms to adapt to water deficit. One strategy is to escape drought by shortening the plants life cycle through rapid development, early flowering and seed set (Havrlentová et al., 2021; Zhang et al., 2020). In perennials, buds, rhizomes and other meristems can temporarily become dormant under prolonged drought (Gillespie & Volaire, 2017; Keep et al., 2021). The drought avoidance strategy relies on plants increasing their water uptake through a well-established root system and maintaining water potential by closing stomata (Seleiman et al., 2021).

Plants using the drought tolerance strategy adjust their morphology, for example by reducing leaf area, limiting new leaf expansion, increasing leaf trichome and cuticula formation, and increasing root growth and proliferation, resulting in increased root length and root to shoot ratio. At the physiological and biochemical levels, drought tolerance strategies include reduced transpiration through lower stomatal conductance, osmotic adjustment, and synthesis of protective proteins and other metabolites (Salehi-Lisar & Bakhshayeshan-Agdam, 2016; Seleiman et al., 2021).

The ability to recover after a period of drought depends on the species and variety, and intensity and duration of the drought period (Taleb et al., 2023). Plants can recover partially when the stress leads to tissue damage and plants need to redirect energy and carbon from other biological pathways to repair the damage tissue, potentially limiting growth or other functions (Xu et al., 2010). Plants can show a complete recovery when physiological processes are downregulated during the drought stress without any actual damage to tissues or supply systems (Xu et al., 2010). Upon relief from stress, these processes can be swiftly reactivated. A compensatory growth after drought is a common phenomenon in grassland and implies the ability of plants to restore organic functionality and maintain their original growth after a period of stress (Zhou et al., 2022). Mechanisms facilitating the ability of perennial grasses to exhibit post-drought recovery have been studied only in a few species such as orchard grass (*Dactylis glomerata*) (Pirnajmedin et al., 2018; Saeidnia, Majidi, Mirlohi, et al., 2020), smooth bromegrass (*Bromus inermis*) (Saeidnia, Majidi, Bakhtiari, et al., 2020), fescues (*Festuca* spp.) and ryegrass (*Lolium* spp.) (Taleb et al., 2023).

1.7 Genes and metabolic pathways involved in waterlogging and drought stress responses

Responses to waterlogging and drought stress involve many molecular mechanisms, signalling pathways, genes, proteins and metabolites that are part of complex regulatory networks. Due to the lack of oxygen caused by waterlogging, plant cells respond by activating fermentation pathways. With the limitation of mitochondrial ATP production due to scarce oxygen, plants rely on ATP generated through glycolysis. This process leads to the accumulation of NADH, which must be converted back to NAD+ to sustain glycolytic activity. While lactic acid fermentation contributes to this conversion, it is primarily achieved through ethanolic fermentation, involving enzymes such as alcohol dehydrogenase and pyruvate decarboxylase (Mustroph, 2018). In addition, transcription factors belonging to group VII ethylene response factors (VII-ERFs) regulate the transcription of genes encoding fermentative enzymes, metabolic and regulatory proteins (Klaas et al., 2019; Mustroph, 2018). During drought, plants accumulate peroxides due to oxidative damage and counteract this with enzymatic antioxidants such as superoxide dismutase, catalase and peroxidase, and non-enzymatic antioxidants such as ascorbic acid and glutathione (Cheng et al., 2022). To maintain cell turgor and osmotic adjustment, plants accumulate osmolytes such as proline and trehalose, as well as inorganic ions like K+, Na+, and Cl- (Cheng et al., 2022). In addition, plants produce organic compounds such as polyamines (spermidine, spermine and putrescine) as a protective response and adaptive mechanism to reduce oxidative damage. Spermidine has been associated to drought tolerance in maize (*Zea mays L.*) (Li et al., 2018), white clover (*Trifolium repens L.*) (Li et al., 2016), foxtail barley (*Hordeum jubatum L.*) (Tian et al., 2022) and creeping bentgrass (*Agrostis stolonifera L.*) (Tan et al., 2022). Similarly, a higher accumulation of putrescine is associated to drought tolerance in maize (Ahangir et al., 2020), creeping bentgrass (Tan et al., 2022) and sugar beet (*Beta vulgaris L.*) (Islam et al., 2022) among other plants. Genes associated with osmoregulation, detoxification, stress response, molecule transport, and biosynthesis of chlorophyll and proline, along with ethylene biosynthesis and signalling pathways are critical components of the plant's response to both drought and waterlogging stress.

Transcriptome analysis provides a comprehensive view of gene expression across the genome, providing valuable insights into the regulatory networks that control plant adaptability and stress tolerance at the transcriptional level (X. Wang et al., 2020). Waterlogging is well studied in cereals such as maize, wheat and barley, and some data of *Lolium perenne L.* are available (Mustroph, 2018). Few studies have focused on the transcriptional responses of perennial grasses to waterlogging and drought. This thesis is one of the first reports on transcriptional responses to waterlogging and drought in *P. pratense*, *P. nodosum* and *P. alpinum*.

2. Aim of the study

The overall aim of this thesis is to understand the diversity of the responses to waterlogging and drought in *P. pratense*, *P. nodosum* and *P. alpinum* through physiological and transcriptomic analyses of accessions with different geographical and habitat origins. To accomplish this, the specific goals were as follow

- To explore the diversity in a large collection of wild and domesticated accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field and greenhouse and to select candidates for abiotic stress response studies.

- To characterize and elucidate the phenotypic responses to waterlogging and drought of selected accessions of *P. pratense*, *P. nodosum* and *P. alpinum* to compare species and accessions.

- To investigate the transcriptional responses to waterlogging and drought of selected accessions of *P. pratense*, *P. nodosum* and *P. alpinum*.

- To identify traits, candidate genes and accessions for further pre-breeding research of tolerance to abiotic stress in *Phleum* species.

3. Results and Discussion

The results obtained during this work are detailed in the research articles and manuscripts. This section is intended to summarise and synthesise the results and discussion.

3.1 Growth and development of *Phleum* species and selection of wild and domesticated accessions for abiotic stress studies (Paper I)

To study the phenotypic diversity in wild (W) and domesticated (D) accessions of *P. pratense* (212), *P. nodosum* (14) and *P. alpinum* (18), a total of 244 *Phleum* accessions were evaluated in a farmer's field north of Uppsala, Central Sweden (60°00'N, 17°42'E) and in a greenhouse. Accessions are described in Paper I, Table S7. Eight plants (genotypes) per accession were grown for two months. Four genotypes were then randomly selected and cloned. Six clones per genotype were used for the field and greenhouse studies. In the field, four genotypes per accession and four clones of each genotype were planted in a randomized complete block design with one clone of each genotype in each of the four blocks. This gave a total of 3904 plants. The other two clones of each genotype were grown in the greenhouse for two more weeks and vernalized for six weeks to stimulate flowering. After the vernalization treatment, the plants were transferred to the greenhouse and arranged in two complete randomized blocks with one replicate of each genotype per block as described in Paper I.

We evaluated growth and developmental traits such as shoot fresh and dry weight, plant height, days to stem elongation, days to booting and days to heading as described in Paper I.

In the field, *P. pratense* accessions had a significantly higher shoot fresh and dry weight and were taller than *P. nodosum* and *P. alpinum*. Cultivars of *P. pratense* and *P. nodosum* reached stem elongation, booting and heading earlier than their wild accessions. Moreover, *P. nodosum* cultivars developed faster than *P. pratense* cultivars, while wild accessions of these two species showed no differences in the days to reach the different developmental stages. Wild accessions of *P. alpinum* reached booting and heading earlier than the wild accessions of *P. pratense*.

In the greenhouse, shoot fresh and dry weight was lower than in the field for all three species. However, *P. nodosum* showed a similar dry weight to *P. pratense* in the greenhouse. Regarding developmental stages, *P. pratense* cultivars reached stem elongation, booting and heading earlier than wild accessions as seen in the field. Interestingly, *P. nodosum* cultivars reached booting and heading later than wild accessions in the greenhouse, but showed the opposite pattern in the field. Most of the accessions of *P. alpinum* remained in vegetative stage in the greenhouse.

Differences in biomass production between the environments could be related to the differences in photoperiod, temperature, humidity, water and nutrient content in the soil. Furthermore, the difference in the developmental stage could also be influenced by the length of vernalization, as in the greenhouse, plants were given a cold treatment for six weeks while in the field they were exposed to a longer cold period. Plants differ in their need for vernalization to flower, some perennial grasses such as ryegrass and tall fescue require vernalization (Fjellheim et al., 2014; Heide, 1994; MacMillan et al., 2005), while *P. pratense* can flower without vernalization. However, in accessions from northern Scandinavia, flowering is stimulated by vernalization, whereas in southern accessions, the heading date does not change in response to vernalization (Fiil et al., 2011; Jokela et al., 2015; Jokela et al., 2014; Seppänen et al., 2010). Moreover, *P. alpinum* requires low temperature and/or short days to induce flowering and a combination of long days and high temperatures to stimulate heading and inflorescence development (Heide, 1994; Heide & Solhaug, 2001).

To explore the diversity in growth and development between wild and domesticated accessions of the three species, principal component analysis (PCA) was performed on the field and greenhouse data. The first two principal components explained 91.7% and 84.5% of the variation in the field and greenhouse data, respectively (Figure 3). *P. alpinum* showed a higher diversity than *P. nodosum* and *P. pratense* in both environments. In the greenhouse, *P. pratense* and *P. nodosum* showed overlapping distributions while in the field the two species were clearly separated.

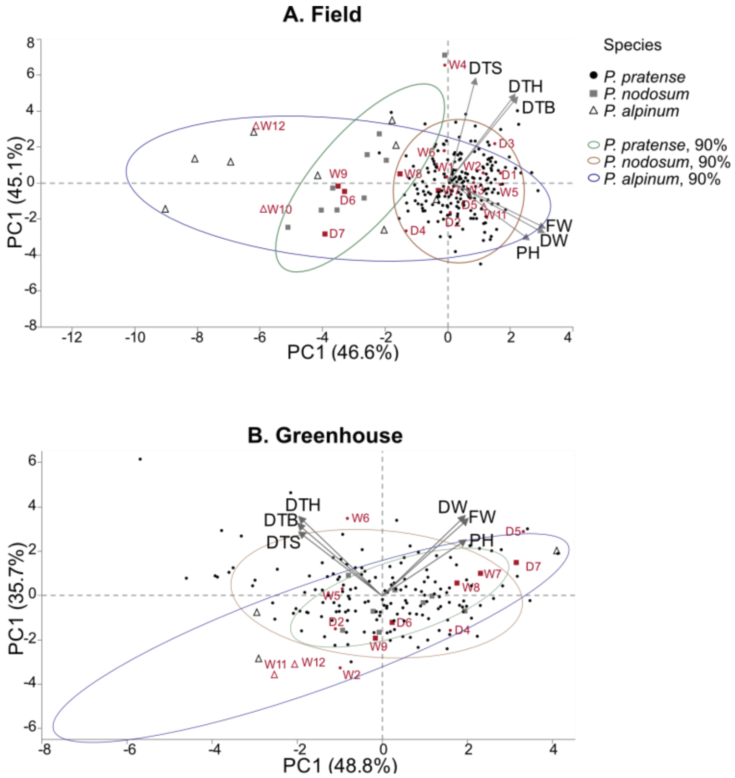
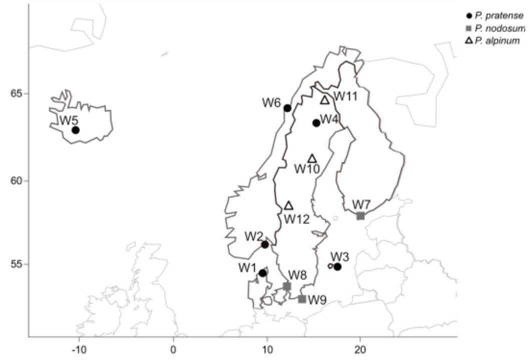


Figure 3. Diversity in accessions of *P. pratense*, *P. nodosum and P. alpinum* based on growth and developmental traits used for selection of accessions for Paper II to IV. A) Field. B) Greenhouse. Ellipses represent the 90% confidence interval. Abbreviations: fresh weight (FW), dry weight (DW), plant height (PH), days to stem elongation (DTS), days to booting (DTB), days to heading (DTH).

From this large collection of accessions, a set of 19 accessions (Figure 3) were selected for studying the response to waterlogging and drought stress at the species and accession level. These accessions were selected to represent the diversity among accessions of each species, and comprised six wild accessions and five cultivars of *P. pratense*, three wild accessions and two cultivars of *P. nodosum* and two wild accessions and one breeding line of *P. alpinum* These accessions differ in the biomass production, the time taken to reach different developmental stages and the location of the original collection site (Figure 4).



Species	Domesticated accessions	Country of origin
P. pratense	D1	Sweden
	D2	Denmark
	D3	Sweden
	D4	Sweden
	D5	Sweden
P. nodosum	D6	Sweden
	D7	Denmark
P. alpinum	D8	Iceland

Figure 4. Collection site of the wild accessions of *P. pratense*, *P. nodosum* and *P. alpinum* and country of origin of the domesticated accessions used in the response to stress studies.

3.2 Phenotypic responses of *Phleum* species to waterlogging and drought (Paper II and Paper III)

Experiments were laid out in a randomized complete block design with four blocks. For each of the selected accessions of *P. pratense*, *P. nodosum* and *P. alpinum,* plants, i.e. genotypes, were grown in a greenhouse to investigate their response to 21 days of waterlogging, 28 days of drought, and 28 days of drought followed by 18 days of rehydration. Each experiment had one plant per accession under well-watered conditions, and another plant per accession exposed to waterlogging or drought in each block as described in Paper II and Paper III. The experiments made it possible to present the results by species and to make some general conclusions about traits at the species level.

3.2.1 Shoot and root growth allocation of *Phleum* species under non-stressed conditions (Paper II and III)

In this section, the growth and morphology of the plants under well-watered conditions in the greenhouse experiments is compared.

Differences in growth of the three species were observed among experiments. These differences could be due to plant age and environmental conditions such as temperature and light. In one of the experiments, we harvested 10-weeks old plants and it was perform in the spring, with an average maximum and minimum temperature of 25°C and 17°C, respectively. In the other two experiments, we harvested 10 and 13-weeks old plants and they were conducted during the summer where the maximum and minimum average temperature were 28°C and 19°C, respectively. Studies on the effect of temperature on biomass production in *P. pratense* have reported that the optimal temperatures for growing *P. pratense* are 18ºC - 22ºC with night temperatures above 10ºC (Bertrand et al., 2008; Smith, 1972). Nevertheless, there is a similar pattern in growth among experiments (Figure 5). For example, *P. pratense* and *P. nodosum* produced a significantly higher shoot dry weight than *P. alpinum* in all three experiments. *P. nodosum* had a higher numbers of tillers per shoot dry weight and a higher number of leaves per shoot dry weight than *P. pratense* in all the experiments. *P. alpinum* showed a higher percentage of root dry weight than the other two species, while *P. pratense* and *P. nodosum* had similar percentage of root dry weight.

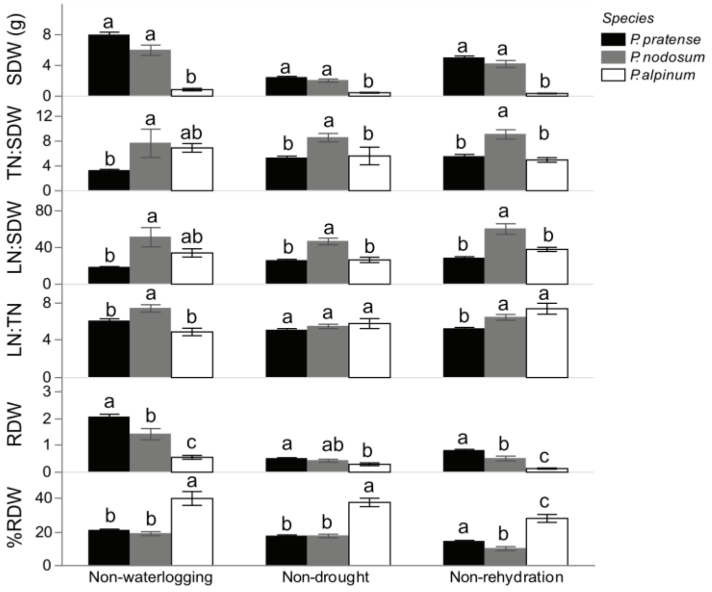


Figure 5. Growth traits of *P. pratense*, *P. nodosum* and *P. alpinum* under well-watered conditions in the greenhouse experiments. Bar represent mean ± SE. Species within the same treatment that do not share the same letter are significantly different from each other ($p < 0.05$ ANOVA, Tukey's test). Abbreviations: SDW, shoot dry weight; TN:SDW, numbers of tillers per shoot dry weight; LN:SDW, numbers of tillers per shoot dry weight; LN:TN, number of leaves per tiller; RDW, root dry weight; %RDW, percentage of root dry weight.

The development of *P. pratense* was similar in the different experiments. *P. pratense* showed a similar distribution of plants at different developmental stages (Figure 6). In the second and third experiments, *P. nodosum* plants developed further than the first experiment. For the older plants of the third experiment, all plants produce generative tillers. There was variation in the developmental stages of *P. nodosum* among the experiments. In line with the results seen in the greenhouse experiment described in Paper I, all accessions of *P. alpinum* remained in the vegetative stage.

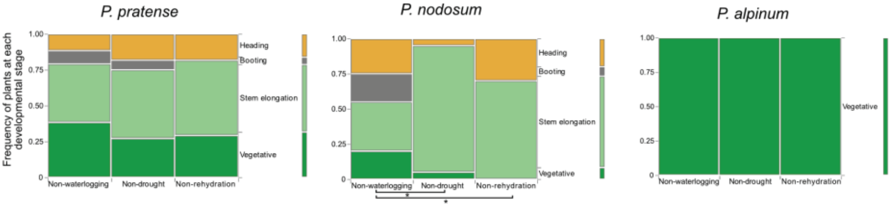


Figure 6. Distribution of the developmental stages in *P. pratense*, *P. nodosum* and *P. alpinum* under well-watered conditions in the greenhouse experiments. (*) Significant difference according to Fisher's exact test.

3.2.2 *Phleum* species response to waterlogging, drought and post-drought rehydration (Paper II and III)

Response to abiotic stress was calculated as the proportional or absolute difference between the treatment and the control in each experiment. Under waterlogging conditions, the shoot biomass and the growth rate of leaves and tillers were not affected in the three *Phleum* species (Figure 7). *P. pratense* was the only species that showed a significantly lower shoot water content under waterlogging conditions. The water content in shoots of *P. pratense* may have decreased due to a restricted root uptake capacity, resulting from oxygen deprivation caused by waterlogging (Setter et al., 2009; Zhang et al., 2019). Moreover, waterlogging clearly affected the root development. Under waterlogging, the three species showed lower root biomass and other morphological adaptations, such as the formation of adventitious roots and aerenchyma in the root cortex (Paper II). In annual monocots, such as barley and wheat, even short periods of waterlogging have long-term effects on shoot biomass, leaf appearance and flowering (Borrego-Benjumea et al., 2021; de San Celedonio et al., 2015; Malik et al., 2001). These types of responses were not observed in the *Phleum* species under waterlogging. Similar to our results, waterlogging-tolerant varieties of other perennial grasses such as cocksfoot, tall fescue and perennial ryegrass have shown no difference in shoot biomass (Mcfarlane et al., 2003; Mui et al., 2021). These results suggest that the three *Phleum* species are able to tolerate extended periods of waterlogging of at least 21 days.

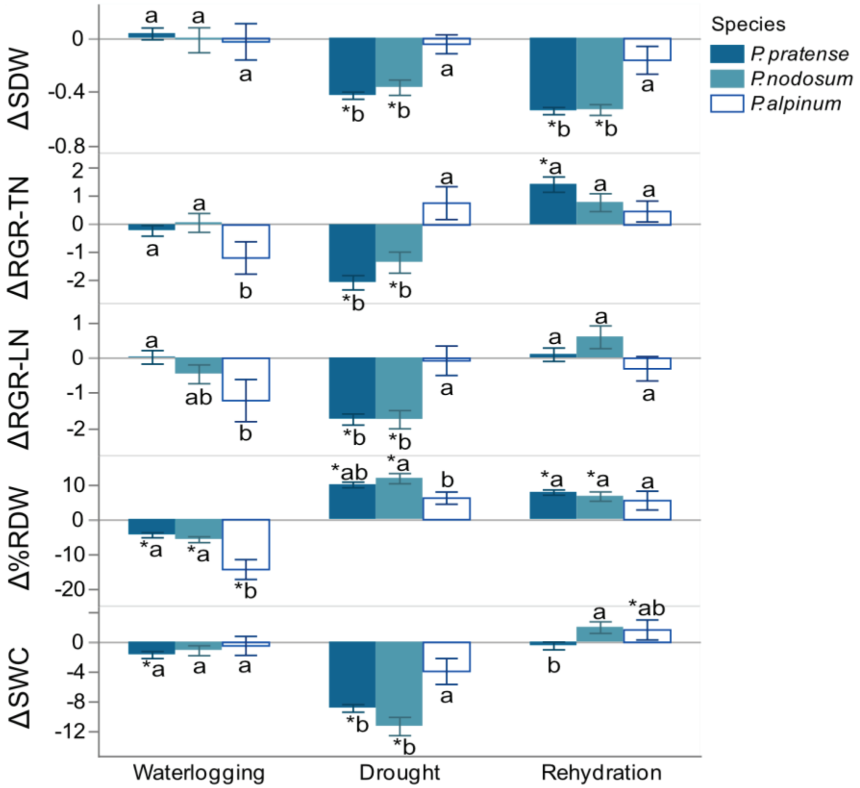


Figure 7. Response to waterlogging, drought and rehydration after drought of *P. pratense*, *P. nodosum* and *P. alpinum* shown as differences between treated and non-treated plants. Species within the same treatment that do not share the same letter are significantly different from each other ($p < 0.05$ ANOVA, Tukey's test). Stars (*) represent significant difference between the control and treatment ($p < 0.05$ ANOVA). Abbreviations: Shoot dry weight (SDW), relative growth rate of tillers (RGR-TN) and leaves (RGR-LN), percentage of root dry weight (%RDW), and shoot water content (SWC)

Under waterlogging conditions, the reduction in root biomass is associated with accelerated root senescence and deficiencies in oxygen, energy and nutrients. This is a common response in both annual crops (Arduini et al., 2019; Marti et al., 2015; Steffens et al., 2005; Wua et al., 2018) and perennial forages (Enkhbat et al., 2021; Real et al., 2008). However, some plants are able to respond to waterlogging by forming more adventitious roots, as seen in the three *Phleum* species, which help them to improve the exchange of gases and absorption of nutrients (Steffens & Rasmussen, 2016). Other anatomical traits, such as thick roots, aerenchyma formation and large cortex to stele areas, have been also associated to waterlogging tolerance (Pedersen et al., 2021; Yamauchi, Noshita, et al., 2021; Yamauchi, Pedersen, et al., 2021). In the present study, *Phleum* accessions were found to form aerenchyma as part of their normal development, however, the aerenchyma area increased significantly under waterlogging. Moreover, we found a positive correlation (r=0.72) between the shoot dry weight and the percentage of aerenchyma in the cortex in waterlogging conditions. Our results suggest that the three *Phleum* species are able to tolerate waterlogging by producing adventitious roots and aerenchyma in the root cortex (Paper II).

Under drought, the shoot biomass and the growth rates of leaf and tiller of *P. pratense* and *P. nodosum* were significantly affected (Figure 7). The effects of drought in *P. pratense* and *P. nodosum* were seen as reductions in production of tillers from 14 days of treatment, when the substrate moisture had dropped to approximately 40%. The leaf number was significantly lower in the two species from 21 days of drought. Also, as expected, at the end of the drought the water content in shoots was significantly lower due to the low substrate moisture. The reduction in shoot growth minimizes water loss through transpiration. Our results show that *P. pratense* and *P. nodosum* prioritize the production of leaves over the tillers. Leaves play a key role in photosynthesis and energy production, necessary for basic cell functions (Farooq et al., 2010) and growth, and minimize water loss by closing their stomata. Reducing tiller production allows the plants to allocate limited resources towards maintaining leaf function. These suggest that *P. pratense* and *P. nodosum* use a survival strategy by reducing shoot growth.

As in the waterlogging experiment, differences in the root system between drought and non-drought treatments were observed. *P. pratense* and *P. nodosum* showed a higher percentage of root dry weight under drought conditions, which is a common response by plants to drought (Becker, et al., 2015). The increase in root growth allow plants to explore a larger volume of soil to enhance water and nutrient absorption (Fernández & Reynolds, 2000). Under drought conditions, more individuals of *P. pratense* remained in vegetative stages, while for the other species there was no difference between treatments. In contrast, under waterlogging, the three *Phleum* species showed no difference in the developmental stage between conditions. Under stress conditions, annual crops tend to prioritize seed production, while perennials use different mechanisms to enhance survival (Sustek-Sánchez et al., 2023). For instance, drought tolerant perennial grasses survive by reducing shoot growth and maintaining the plant crowns vigorous to be able to recover under adequate rainfall. *P. alpinum* was not affected by drought, which might be related to its size and slower growth compared to the other two species (Geller & Smith, 1982). Moreover, *P. alpinum* is adapted to harsh, mountainous environment (Hultén & Fries, 1986), and may be a potential source of genes for adaptation to stressful growing condition.

After 28 days of drought, plants were rehydrated to study their recovery response. After rehydration, *P. pratense* produced tillers more rapidly and less leaves than *P. nodosum*. Despite the increase in leaf and tiller growth rate after re-watering, the shoot dry weight did not reach the level of the well-watered conditions. A desirable trait for perennial crops is the ability to regrow. Drought-tolerant species use dehydration tolerance and summer dormancy strategies to survive (Balachowski et al., 2016). Our results suggest that although *P. pratense* and *P. nodosum* are sensitive to drought, they are able to resume growth as soon as water is again available (Paper III).

3.2.3 Abiotic stress tolerant accessions of *P. pratense*, *P. nodosum* and *P. alpinum (*Paper II and III)

At the accession level, we observed diversity in response to waterlogging and drought (Figure 8). Under waterlogging, the cultivars *P. pratense* D1 and *P. nodosum* D6 showed a lower number of leaves than the non-waterlogging conditions. In contrast, accessions *P. pratense* W1 and *P. alpinum* W11 produced more leaves per tiller, and for W1, the growth rate of leaves was higher in waterlogging (Paper II). Interestingly, the wild accessions *P. pratense* W3 and W4, *P. nodosum* W8 and W9 and *P. alpinum* W10 showed a decrease in the proportion of root biomass, which perhaps makes them less tolerant (Figure 8). In contrast, accessions *P. pratense* W6 had a larger root system, while *P. pratense* W1 and *P. nodosum* W7 showed no difference between the treatments. The increase in root dry weight could be the result of the higher formation of adventitious roots, less root senescence and continued root growth. From a plant breeding perspective, a waterlogging tolerant plant should be able to maintain shoot and root growth and have a robust root system able to form adventitious roots and aerenchyma, and roots with high cortex to stele ratio (Paper II). Wild accessions W1, W6, W7 and the domesticated D3 might be consider more tolerant than other accessions also due to their ability to adapt to waterlogging by forming a higher percentage of aerenchyma in the roots, higher cortex area to stele area and by maintaining shoot and root growth (Paper II; Yamauchi, Noshita, et al., 2021; Yamauchi, Pedersen, et al., 2021).

For a faster recovery, a larger root system, as seen in accessions W6 and W7 showed might be advantageous under waterlogging as it can provide physical stability and improve water, nutrients and oxygen uptake. In contrast, cultivar D5 (Tryggve) had a smaller root system, and it showed the lowest percentage of aerenchyma in the cortex among *P. pratense* accessions, suggesting that D5 may be less tolerant to waterlogging. Based on the shoot and root traits, we suggest including the wild accessions of *P. pratense*, W1 and W6, and *P. nodosum* W7 in pre-breeding studies for waterlogging tolerance.

Under drought, we found a variation in growth in response to drought among accessions. *P. pratense* accessions W5, D1 and D5 had a lower shoot dry weight and produced less leaves and tillers, suggesting that they were more sensitive than the other accessions. Whereas, *P. pratense* accessions W1, W3 and D3 and *P. nodosum* accessions W9 and D6 were the less affected as they showed no difference in the shoot dry weight and the formation of leaves and tillers. Accession *P. pratense* W1 had a higher root dry weight and a higher proportion of root dry weight of total dry weight (Figure 8). The percentage of root dry weight increased in all *P. pratense* and *P. nodosum* accessions except W3 and W4. Accessions of *P. alpinum* showed no difference in shoot traits between the treatments which might be related to their small size and slow growth rate.

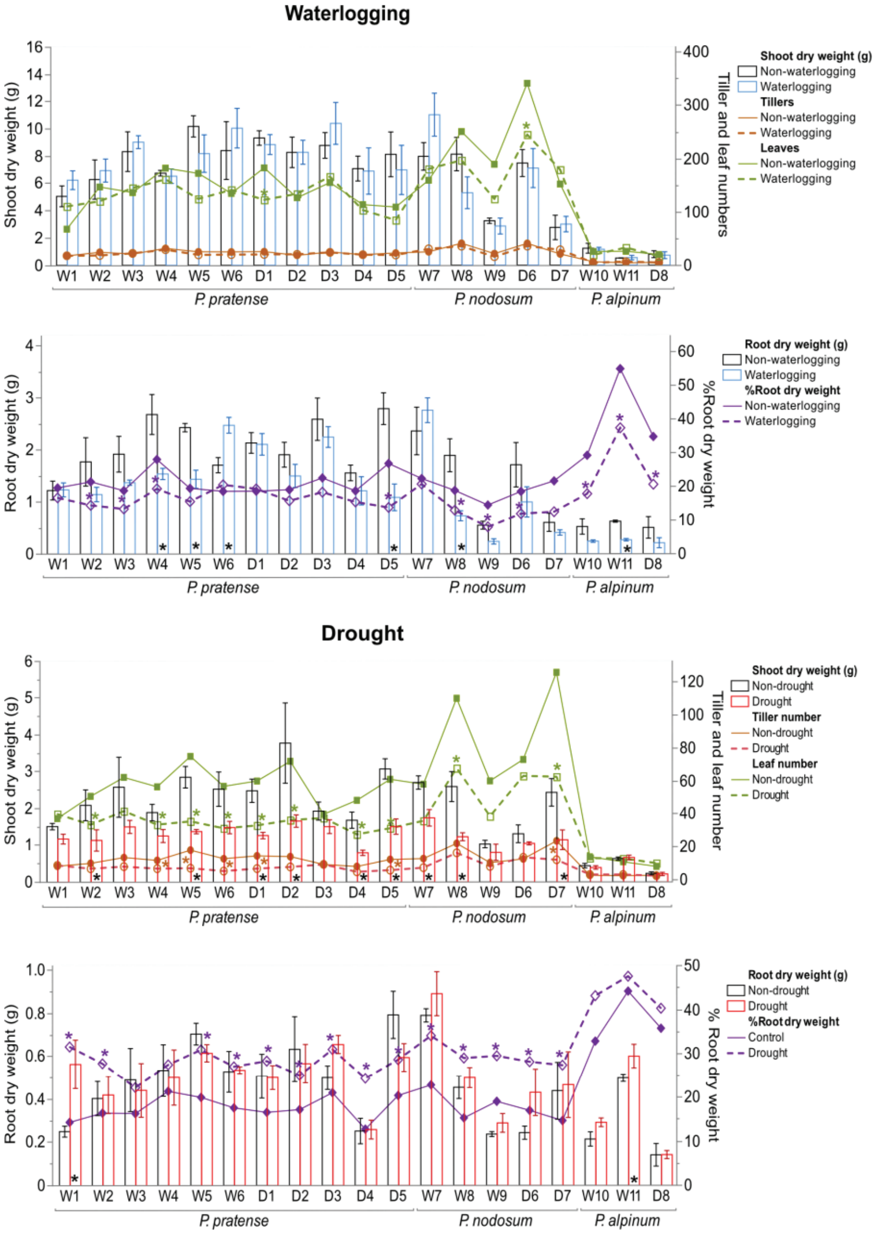


Figure 8. Growth traits of wild (W) and domesticated (D) accessions of *P. pratense*, *P. nodosum* and *P. alpinum* under waterlogging and drought. Significant difference in traits represented by stars (*) and colours according to the trait ($p < 0.05$, ANOVA).

After 28 days of drought, plants were rehydrated to study their recovery response. Water status in the shoots was fully recovered in all accessions. Accessions *P. pratense* W2, W3, W6 and D2 and *P. nodosum* accessions W7, D6 and D7 recovered by producing new leaves and tillers, reaching the level of the well-watered conditions. Accessions *P. pratense* W4, D3 and *P. nodosum* W8 and W9 recovered the total number of tillers per plant, but not the number of leaves per plant. Only accession W2 had an increase in shoot biomass with no significant difference between treatments. Accessions W5 and D1 had a higher growth rate than the other accessions, however, the shoot traits did not reach the level of the control.

Drought tolerance and recovery are different processes and depend on different physiological mechanisms and metabolic processes. When comparing different traits and accessions, indexes that can describe how resistant and also how resilient a plant is could be very useful. Resistance and resilient indexes were used in a principal component analysis (PCA) to explore the diversity among accessions and identify groups of accessions that have common traits (Paper III). Accessions W5, D1, D4 and D5 had lower resistance and high resilience index and clustered together in the PCA, in other words, they were affected by drought but were able to recover by producing new leaves and tillers. Another group of accessions (W1, D3 and D6) were less affected by drought but also low resilience. The selection of accessions for further research could be based on the groups and different traits of interest. Under natural conditions, plants are exposed to multiple stresses and different traits are beneficial in different habitats which can explain why the response to drought and recovery varied among accessions. This type of trade-offs between drought tolerance, high yield and recovery, can be a dilemma for breeding. Interestingly, there are accessions that performed fairly well under both drought and waterlogging stress for example the *P. pratense* accession W1.

3.2.4 Transcriptional responses to waterlogging and drought stresses (Paper IV)

For the transcriptome analysis, eight accessions were selected based on the results from the greenhouse experiments. Two wild accessions (W1 and W6) and three cultivars of *P. pratense* (D3, D4 and D5), two wild *P. nodosum* accessions (W7 and W9) and two wild *P. alpinum* accession (W10 and W12) were selected to study gene expression during waterlogging and drought stress. Young plants at tillering stage (35 days after sowing) were exposed to 21 days of waterlogging and 14 days of drought under controlled conditions (22°C/18°C, 60% humidity, 350 µmolm^{-2}s^{-1} photon flux density and 16h light/8h dark photoperiod). Phenotypic traits including tiller and leaf production, shoot and root weights, plant height and stomatal density were evaluated. Chlorophyll and proline contents were measured in mature leaves in both waterlogged and drought treatments, while adventitious root anatomy was only examined in the waterlogged experiment. Young leaves and adventitious roots samples were used for transcriptional analysis as described in Paper IV.

Consistent with the results of the greenhouse experiments, the three species adapted to waterlogging stress by changes in root morphology, including the formation of adventitious roots and aerenchyma in the cortex, while shoot dry weight increased or remained similar to the control. On the other hand, under drought stress, plants reduced shoot growth but prioritize root growth, with developmental differences in some accessions, including fewer reproductive tillers. Responses to stress in plants are regulated by changes at the physiological (Paper II and III), and transcriptional levels (Paper IV) driven by changes in gene expression. In our experiments, exposure to waterlogging and drought stresses induced changes in gene expression which was reflected by a clear separation of the stress and non-stress conditions in the PCA plots of the leaf and root transcriptomes of *P. pratense, P. nodosum* and *P. alpinum* (Figure S1, Paper IV).

Under waterlogging, a smaller root system is associated with a lack of oxygen, energy and nutrients and with accelerated root senescence (Tong et al., 2021). During prolonged waterlogging, the ability of roots to take up water is impaired due to reduced root hydraulic conductivity. Plasma membrane proteins called aquaporins act as a functional water channels that enhance water uptake (Holbrook & Zwieniecki, 2003). Under hypoxia, some aquaporins may also contribute to the influx of oxygen and the efflux of carbon dioxide, lactic acid, nitric oxide, and the signal transduction of hydrogen peroxide (Tan et al., 2018). The transcription and posttranslational modification of aquaporins may be regulated by plant hormones such as ethylene and abscisic acid (ABA) (Tan et al., 2018). In response to low oxygen, ethylene accumulates in flooded tissues and acts both as a local and long-range signal during soil flooding and re-oxygenation (Voesenek et al., 1993). Ethylene also mediates the formation of adventitious roots and aerenchyma (Leeggangers et al., 2023; Yamauchi et al., 2018). The *AP2/ERF* (*APETALA2*/ethylene-responsive element binding factors) superfamily related to ethylene response plays a key role in responses to hypoxia. Members of the *AP2/ERF* superfamily positively regulate waterlogging stress response by decreasing the oxidative damage and enhancing waterlogging tolerance (He et al., 2023) and are also implicated in the regulation of aquaporins (Shivaraj et al., 2021). Accordingly, genes related to root senescence (*P. pratense*, *P. alpinum*), aquaporins (*P. pratense* and *P. nodosum*), and ethylene biosynthesis and signalling (*P. pratense*, *P. nodosum* and *P. alpinum*) were expressed in roots and/or leaves of *Phleum* species under waterlogging stress. Similar to our results, genes and transcription factors related to ethylene biosynthesis and signalling pathway have been identified in wheat (Shen et al., 2020), rice (Minami et al., 2018), reed canary grass (*Phalaris arundinacea*) and cocksfoot (*Dactylis glomerata*) (Klaas et al., 2019) exposed to waterlogging.

Plants under waterlogging conditions exhibited similar or higher shoot dry weight compared to those grown under well-watered conditions. Interestingly, a negative correlation was observed between chlorophyll content and shoot dry weight in *P. pratense* (r = -0.62) and *P. nodosum* (r = -0.71). This suggests that although plants can maintain or increase shoot growth under waterlogging stress, nitrogen could be recycled within the plant to allow growth but photosynthesis might be compromised as indicated by reduced chlorophyll content. In *P. nodosum*, the GO term (GO:0016168) associated with chlorophyll binding may indicate a potential adaptive or stress response mechanism involving changes in chlorophyll-binding proteins.

Under drought conditions, mechanisms such as reduced shoot growth and root expansion are common in plants (Fernández & Reynolds, 2000). Also in drought ethylene plays an important role. It has been linked to inhibition of shoot growth, stomatal closure, decrease in chlorophyll content, leaf senescence, and activation of defence mechanisms (Pérez-Llorca et al., 2023). Adaptations in the root system include root growth, which allows the plants to explore a larger volume of soil, and increase water and nutrient uptake. The flux of water within and across plant cells is regulated by the activity and abundance of aquaporins (Shivaraj et al., 2021). In addition to regulating water balance, some aquaporins can also facilitate the transport of reactive oxygen species (ROS) (Phukan et al., 2016). ROS have a dual role in plants. On one hand, ROS acts as signalling molecules that regulate various pathways but they also represent a toxic by-product of stress metabolism that cause plant death (Choudhury et al., 2017). Plants use ROS levels to monitor their intracellular stress status. However, ROS levels need to be tightly regulated to avoid accumulation of ROS to prevent oxidative damage (Cruz de Carvalho, 2008). ROS also act as a signalling component linked to hormonal signalling pathways to generate antioxidants leading to an increase in stress tolerance (Cruz de Carvalho, 2008). Among the roles of ethylene mentioned above, this hormone also regulates proline accumulation. Proline is an amino acid that acts as a metal chelator and as an antioxidant defence and signalling molecule (Hayat et al., 2012). In barley, proline accumulation in response to drought stress has been associated with drought resistance (Rai, 2002). In the present study, the results of the GO enrichment analysis showed enriched GO terms associated with water transport (*P. pratense* and *P. alpinum*), proline biosynthesis (*P. nodosum* and *P. pratense*), ABA (*P. pratense* and *P. nodosum*) and ethylene (*P. nodosum*) signalling pathways, chlorophyll degradation pathway (P. alpinum), and chlorophyll binding (*P. nodosum*) in leaves and/or roots of *Phleum* species under drought. In addition, *P. alpinum* showed a positive correlation (r=0.64) between chlorophyll content and shoot dry weight. In *P. nodosum,* less dehydrated plants showed a higher chlorophyll content, seen as a positive correlation (r=0.54) between chlorophyll content and shoot water content. A negative correlation between proline content and soil moisture was observed in both *P. pratense* (r = -0.45) and *P. nodosum* (r = -0.50). In particular, *P. pratense* showed a strong negative correlation (r = -0.64) between shoot water content (%SWC) and proline. Drought stress causes dehydration of the plant tissues leading to proline accumulation in the leaves of *P. pratense* and *P. nodosum,* with W1, D3 and W7 being the accessions that shown the higher proline content under drought conditions. In contrast, proline content in *P. alpinum* was lower under drought. Proline content was not correlated with shoot dry weight or number of tillers and leaves in all species under drought. In *P. alpinum* proline negatively correlated to the number of leaves per tiller under drought (*r*=-0.52). Interestingly, two enriched GO terms related to spermidine (GO:1903711) and putrescine (GO: 0015847) transport were identified in roots of drought-treated *P. alpinum.* These two polyamines have been associated to responses to environmental stress as they serve as protectants (Bouchereau et al., 1999). Previous studies in alfalfa (*Medicago sativa*) (Bouchereau et al., 1999), maize (Li et al., 2018), white clover (Li et al., 2016), foxtail barley (Tian et al., 2022) and creeping bentgrass (Tan et al., 2022) have shown that the accumulation of spermidine improves drought tolerance by triggering protective responses and adaptive mechanisms to reduce oxidative damage. Similarly, a higher accumulation of putrescine is associated to drought tolerance in maize (Ahangir et al., 2020), creeping bentgrass (Tan et al., 2022) and sugar beet (Islam et al., 2022). These results in *P. nodosum* and *P. pratense* suggest that proline content might be useful diagnostic tool for effective selection of drought-tolerant genotypes, as has been suggested in wheat (Mwadzingeni et al., 2016) , tobacco (Van Rensburg et al., 1993), soybean (Mwenye et al., 2016), tall fescue (Man et al., 2011), among others. On the other hand, *P. alpinum* under drought stress may use metabolites or physiological adaptations other than proline, such as spermidine and putrescine.

Similar to the wild W1, the cultivar D3 (Bottnia II) responded to drought with reduced shoot size, increased root system, no difference in the number of reproductive tillers and increased proline accumulation in leaves. Bottnia II was released in 1955 and withdrawn in 2011, so there have been no further breeding efforts including this cultivar, which may explain the similarities with the wild accession. Interestingly, at the molecular level, 56 (17%) DEGs in roots were shared between W1 and D3.

Transcriptional research in timothy remains limited. To the best of my knowledge, there appears to be a notable gap in the literature concerning transcriptional studies focused on drought and waterlogging stress in *Phleum* species. Furthermore, this study is among the pioneering efforts to elucidate the transcriptional responses of *P. pratense*, *P. alpinum* and *P. nodosum* to stress, marking a significant contribution to our understanding of these species' adaptive mechanisms.

4. Conclusions

This thesis provides knowledge of physiological and transcriptional responses to abiotic stress, and diversity in traits among *Phleum* species and accessions, essential for further evaluation in field conditions and use in future breeding of abiotic-stress tolerant cultivars. Specific findings include:

- Large diversity was found between species and accessions. The groups of wild accessions and cultivars of *P. pratense* differed in development, but not in biomass. The same pattern was found for *P. nodosum.*
- *Phleum* species responded to waterlogging with changes in their root morphology. Some accessions had beneficial adaptations in root growth and aerenchyma formation in roots, which were positive for shoot biomass production.
- *P. pratense* and *P. nodosum* were affected by drought seen as a reduction in shoot growth and an increase in the proportion of root biomass. While most accessions were affected by drought, a few accessions showed shoot growth similar to non-drought-treated plants.
- *P. pratense* and *P. nodosum* were able to recover from drought by forming new leaves and tillers during rehydration. Some accessions fully recovered the number of leaves and tillers.
- Differentially expressed genes associated with stress responses, transport, hormone biosynthesis were found in response to waterlogging and drought.
- Some accessions of *P. pratense* and *P. nodosum* accumulated proline in leaves under drought, which was reflected in the gene expression related to proline biosynthesis. Genes related to spermidine and putrescine were differentially expressed in roots of *P. alpinum.*

5. Future perspectives

Climate change is altering precipitation patterns worldwide, leading to more frequent droughts and floods that threaten crop productivity. These challenges, combined with growing demand for feed, highlight the urgent need to breed climate-resilient varieties of key forages such as timothy (*P. pratense*). However, there is still a significant gap in our understanding, particularly concerning the genetic underpinnings and adaptive mechanisms that timothy and *Phleum* species employ to withstand abiotic stress. Stress responses are complex and influenced by plant developmental stage, duration of stress exposure, environmental conditions, and others.

To address this knowledge gap, conducting further studies at different plant age, developmental stage and duration of drought and waterlogging stresses could provide valuable insights. For instance, it would be worthwhile to investigate the time taken by *Phleum* species to develop adventitious roots and aerenchyma in response to waterlogging, and the influence of plant age on these responses. It would also be beneficial to examine the recovery process after waterlogging to understand the effect on yield. While controlled experimental conditions are necessary for dissecting the molecular and physiological aspects of stress responses, it is important to recognise that these findings may not always be directly applicable to field conditions. Therefore, it is important to evaluate the performance of the selected accessions under real-world agricultural settings and monitor their performance and the impact of stress over successive years. In this study, we identified material that might be suitable for use in pre-breeding programmes, which offers valuable prospects for breeding and domesticating new forage crops. This information, combined with improved agricultural practices, is fundamental for a sustainable agriculture.

As agriculture moves towards sustainability and resilience in the face of climate change, perennial crops are expected to transform agricultural landscapes with their deep root systems that significantly reduce soil erosion, improve water retention and enhance soil health through carbon sequestration. Although timothy is a short-lived perennial, it has the potential to provide environmental benefits, particularly in enhancing soil health, decreasing nutrient leakage, and preserving biodiversity. However, there is a need for more comprehensive research into perennials to decode the genetic and biochemical pathways that underpin plant growth, physiology, and responses to both biotic and abiotic stresses.

The data obtained from the RNA-sequencing offers significant potential for enhancing our understanding of *Phleum* species. We have used the data to study responses of the three species to drought and waterlogging. Additionally, the transcriptomic data can be used to identify differences between timothy type (wild and cultivated), tissues (root and leaves), and between species under well-watered conditions. Further experiments could be conducted to validate the expression patterns of the genes to confirm the results using the same samples or different samples. Integrating these insights into breeding programs presents a significant opportunity to enhance the stress tolerance of timothy grass.

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Popular science summary

Timothy (*Phleum pratense*) is an important forage grass cultivated for grazing, silage and hay. Farmers grow timothy in mixed stands with legumes, including clover and alfalfa. With global warming, climate patterns are changing worldwide, resulting in intense rainfall and longer periods of drought that affect agricultural productivity and cause significant economic losses to farmers. Therefore, it is essential to understand how timothy responds to environmental factors such as drought and waterlogging (flooding). For this study, we selected a total of 244 wild and domesticated accessions of timothy and two closely related species, turf timothy (*P. nodosum*) and alpine timothy (*P. alpinum*) from different European countries. The growth of these accessions was evaluated in the field and greenhouse. The results showed that wild accessions and cultivars of timothy and turf timothy did not differ in biomass, but they did differ in flowering time. Based on these results, we selected 19 wild accessions and cultivars of the three *Phleum* species that represented the diversity of our collection. Plants of the selected accessions were exposed to waterlogging by submerging them in a bucket full of water, and to drought by not watering them. We then studied their phenotypic (observable characteristics) response to the stress. Based on these results, we narrowed down and selected 8 accessions to study their physiological response and changes in gene expression to abiotic stress in leaves and roots.

Plants are unable to move from stress due to their sessile nature. Therefore, they employ various physiological and genetic mechanisms to adapt to stress. Waterlogging leads to a lack of oxygen, causing hypoxia within roots. Interestingly, we found that the three *Phleum* species exposed to waterlogging had similar dry weights than their respective control plants. Belowground, due to the accumulation of ethylene (plant hormone), plants responded to waterlogging by developing adventitious roots that grow from the crown, and also developed air-filled cavities in the roots called aerenchyma. Adventitious roots and aerenchyma facilitate the diffusion of oxygen into roots, and carbon dioxide, and toxic volatiles out of the roots. Most of the accessions showed a smaller root system in waterlogging conditions. However, some accessions were able to maintain root growth, indicating that they might be more tolerant. A large root system is crucial for achieving high yields. Aerenchyma and a large root system could be used as selection criteria for waterlogging-tolerant accessions.

Drought can limit the uptake of water and nutrients, which negatively impacts plant growth. In response to drought, *Phleum* species increased the size of their root systems while reducing shoot growth. This larger root system allows the plant to explore the soil more effectively in search of water and nutrients. The production and accumulation of metabolites, amino acids, hormones and other compounds was also triggered by drought. For example, two timothy accessions and one turf timothy produce proline, an amino acid that helps protect plants against osmotic drought and oxidative damage. On the other hand, alpine timothy seems to produce two polyamines, putrescine and spermidine, which are involved in drought tolerance, rather than proline. After the drought period, the plants were re-watered to observe their response to rehydration. Both timothy and turf timothy are sensitive to drought, but they responded to re-watering by producing more tillers and leaves. For breeding purposes, traits such as increased rooting volume, ability to maintain turgor and growth rate, and fast recovery could be used to select material.

The several wild accessions and the old and new cultivars used in these studies showed that there is a large diversity in adaptation to waterlogging and drought. The diversity in how they grow, and in their transcriptomes and metabolites show that they have traits that makes them interesting for further use.

This is possibly one of the first reports of transcriptional responses to drought and waterlogging in *Phleum* species. These findings provide valuable information for breeding timothy. Additionally, this study represents a pioneering effort to elucidate the transcriptional responses of alpine timothy and turf timothy to stress, making a significant contribution to our understanding of *Phleum* species' adaptive mechanisms.

Populärvetenskaplig sammanfattning

Timotej *(Phleum pratense*) är ett viktigt vallgräs som odlas för bete, ensilage och hö. Det odlas ofta i blandade bestånd med baljväxter, inklusive klöver och lusern. Med den globala uppvärmningen förändras klimatmönstren över hela världen, vilket leder till intensiva regn och längre perioder av torka. Det är mycket negativt för jordbrukets produktivitet och orsakar betydande ekonomiska förluster för jordbrukarna. Därför är det viktigt att förstå hur timotej reagerar på miljöfaktorer som torka och vattenmättnad (översvämning). För den här studien valdes totalt 244 vilda och domesticerade exemplar av timotej och två närbesläktade arter, vildtimotej *(P. nodosum*) och fjälltimotej *(P. alpinum*) från olika europeiska länder. Utvecklingsstadiet, höjden på plantorna och biomassan för dessa sorter utvärderades både i fält och i växthus. Resultaten visade att vilda accessioner och sorter av timotej inte skiljde sig åt i biomassa, men de skiljde sig åt i blomningstid. Samma mönster upptäcktes hos vildtimotej. Baserat på dessa resultat valde vi ut 19 vilda accessioner och kultivarer av de tre *Phleum-arterna* som representerade diversiteten i vår samling. Dessa utvalda accessioner utsattes för vattenmättnad och torka i ett växthus och vi studerade deras fysiologiska respons. Från växthusstudierna valde vi sedan ut 8 accessioner för att mer i detalj studera deras fysiologiska respons och respons i gen-uttryck på abiotisk stress i blad och rötter. Växter kan inte förflytta sig från stress eftersom de växer på en bestämd plats. Därför använder de olika fysiologiska och genetiska mekanismer för att anpassa sig till stress. Vattenmättnad leder till syrebrist och orsakar syrebrist i rötterna. Intressant nog fann vi att de tre *Phleum*-arterna som utsattes för vattenmättnad hade liknande torrvikter på skotten som sina respektive kontroll-plantor. På grund av ackumuleringen av etylen reagerade växterna på vattenmättnaden genom att bilda mera adventivrötter som växer från kronan och i de rötterna bildade de även luftfyllda hålrum som kallas aerenkym. Adventivrötter och aerenkym underlättar diffusionen av syre in i rötterna och diffusionen av koldioxid och giftiga flyktiga ämnen ut från rötterna. De flesta accessionerna uppvisade en minskning av rotsystemet. Vissa sorter kunde dock bibehålla rottillväxten, vilket tyder på att de kan vara mer toleranta. Ett starkt rotsystem är avgörande för att uppnå hög avkastning. Därför skulle aerenkym och ett längre rotsystem kunna användas som urvalskriterier för vattenmättnadstoleranta sorter.

Torka begränsar upptaget av vatten och näringsämnen, vilket är mycket negativt för växters tillväxt. Som svar på torkan ökade *Phleum*-arterna storleken på sina rotsystem samtidigt som skott-tillväxten minskade. Det större rotsystemet gör att växten kan utforska jorden mer effektivt i sökandet av vatten och näringsämnen. Produktion och ackumulering av metaboliter, aminosyror, hormoner och andra föreningar stimulerades också av torka. Till exempel producerar två timotej-accessioner och en vildtimotej-accession prolin, en aminosyra som hjälper till att skydda växter mot osmotisk torka och oxidativa skador. Däremot verkar fjälltimotej producera två polyaminer, putrescin och spermidin, som är involverade i torktolerans, snarare än prolin. Efter torkperioden gavs vatten till växterna igen för att studera hur de reagerade på god tillgång på vatten. Både timotej och vildtimotej är känsliga för torka, men de reagerade på återvattning genom att producera fler strån och blad. För förädlingsändamål kan egenskaper som ökad rot-volym, förmåga att bibehålla turgor och tillväxthastighet, samt snabb återhämtning användas för att välja ut material.

De många vilda accessionerna och de gamla och nya sorterna som användes i dessa studier visade att det finns en stor diversitet i anpassning till vattenmättnad och torka. Diversiteten i hur de växer och i deras transkriptom och metaboliter visar att de har egenskaper som gör dem intressanta för vidare användning.

Detta är möjligen en av de första rapporterna om reaktioner på torka och vattenmättnad på transkriptions-nivå hos *Phleum*-arter. Dessa resultat ger värdefull information för förädling av timotej. Dessutom representerar denna studie en banbrytande insats för att klargöra transkriptions-svaren hos alpin timotej och vildtimotej på stress, vilket ger ett betydande bidrag till vår förståelse av *Phleum*-arternas anpassningsmekanismer.

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Phenotypic Diversity in Domesticated and Wild Timothy Grass, and Closely Related Species for Forage Breeding

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Abstract: Timothy grass (*Phleum pratense* L.) is one of the most important forage crops in temperate regions. Forage production, however, faces many challenges, and new cultivars adapted to a changing climate are needed. Wild populations and relatives of timothy may serve as valuable genetic resources in the breeding of improved cultivars. The aim of our study is to provide knowledge about the phenotypic diversity in domesticated (cultivars, breeding lines and landraces) and wild timothy and two closely related species, *P. nodosum* (lowland species) and *P. alpinum*, (high altitude species) to identify potential genetic resources. A total of 244 accessions of timothy and the two related species were studied for growth (plant height, fresh and dry weight) and plant development (days to stem elongation, days to booting and days to heading) in the field and in a greenhouse. We found a large diversity in development and growth between the three *Phleum* species, as well as between the accessions within each species. Timothy showed the highest growth, but no significant difference was found between wild accessions and cultivars of timothy in fresh and dry weight. However, these two groups of accessions showed significant differences in plant development, where timothy cultivars as a group reached flowering earlier than the wild accessions. This suggests that there has not been a strong directional selection towards increased yield during the domestication and breeding of timothy; rather, timothy has been changed for other traits such as earlier heading. Principal component analysis and cluster analysis based on all traits revealed distinct clusters. Accessions falling within the same cluster showed similarities in the development and growth rather than the type of accession. The large diversity found in this study shows the potential of using timothy accessions as genetic resources in crosses with existing cultivars. Also, accessions of *P. nodosum* with favorable traits can be candidates for the domestication of a novel forage crop, and the high-altitude relative *P. alpinum* may be a source of genes for the development of more cold and stresstolerant cultivars.

Keywords: biomass; development; forage crop; genetic resources; perennial; *Phleum alpinum*; *Phleum nodosum*; *Phleum pratense*; wild relatives

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1. Introduction

Timothy (*Phleum pratense* L. subsp. *pratense*) is one of the most important forage crops in temperate regions. It is an outcrossing, short-lived perennial with shallow roots and, due to its winter hardiness, it is the preferred species for forage grass cultivation at higher latitudes in Northern Europe, East Asia and North America [1]. Timothy is a leafy and tall-growing grass with high biomass production, as well as high nutritive value and digestibility compared to many other forage grasses [2]. The forage quality of timothy is regulated by the developmental stage of the plant and is usually highest during the early growth of the crop when most of the tillers are in the vegetative stage [3,4].

Biomass production increases with age when the tillers elongate and reach the flowering stage. In contrast to some other forage crops such as *Lolium perenne* L. and *Festuca arundinacea* Schreb., timothy can form elongated and heading tillers without vernalization (cold treatment) [5,6]. However, flowering is stimulated by vernalization, especially in northern accessions [6–9]. Flowering and stem elongation are also stimulated by a longer photoperiod [5,8–10].

New tillers are formed from buds in leaf sheaths at the base of the stem. The stem bases (corms) are swollen and can serve as energy reserves [11]. With its rapid growth and formation of tillers, timothy can be harvested two to four times per season. However, the buds and corms in the crown are sensitive to trampling and grazing by cattle, which decreases the production of tillers. Another limitation for its persistence in pure stands or in mixtures with perennial legumes and other forage grasses is its shallow root system and its sensitivity to drought [12].

Timothy, together with fourteen perennial and annual species as well as several subspecies, form the genus *Phleum* [13,14]. Besides timothy, several other of these species are domesticated and cultivated as forage crops. The *Phleum* species vary in ploidy level from diploid to octoploid [13,14]. Even different ploidy forms are described within the same taxa. The polyploid forms are presumed to be the result of hybridization events and subsequent polyploidizations. The most common form of timothy is hexaploid, with 42 chromosomes ($2n = 6x = 42$). Hexaploid timothy is native to Europe, except for the Mediterranean areas, and the cultivated crop was domesticated from hexaploid wild populations in Northern Europe. Wild timothy populations grow in lowland areas throughout Europe.

The genetic relationship between the *Phleum* species and the origin of hexaploid timothy have puzzled scientists over the years and are still not fully understood. Based on genetic analysis, the similarity between hexaploid timothy and the diploid relative *P. nodosum* (syn. *P. pratense* L. subsp. *bertolonii (DC.) Bornm.* and *P. bertolonii (DC.) Bornm.*, $2n = 2x = 14$) was found in chloroplast trnL intron DNA sequences [14]. Genetic similarities were also found between hexaploid timothy and the diploid *P. alpinum* subsp. *rhaeticum* Humphries ($2n = 2x = 14$). A hybridization is suggested to have occurred between the two diploid species *P. nodosum* and *P. alpinum* subsp. *rhaeticum* in the Italian Alps, which resulted in an allotetraploid *Phleum*. Moreover, cytological studies suggest the presence of two genomes of *P. nodosum* in hexaploid timothy [15,16]. Thus, the allotetraploid may have hybridized with northern European populations of *P. nodosum*, and with subsequent polyploidization, formed the hexaploid timothy, *P. pratense* subsp. *pratense*.

Wild populations of the different *Phleum* species originate from various geographical regions and are adapted to different habitats such as meadows and grasslands. Hybridization and polyploidy events in the evolution of *Phleum* are likely to have played an important role in the geographical and ecological patterns as well as in the diversification of the species. Also, variation in the direction and intensity of natural selection may have resulted in locally adapted populations within the species. Due to the strong human selection during crop domestication, the species may have undergone large bottlenecks, leaving much of the traits and genes behind in the wild populations [17–19]. These traits may be of large interest for the improvement of crops [20]. The wild populations of timothy and the related *Phleum* species are therefore potential genetic resources for the development of new high-yielding timothy cultivars adapted to a changing climate.

In this study, we investigated the development and growth in domesticated and wild hexaploid timothy and the two closely related species, the diploid *P. nodosum* and the tetraploid *P. alpinum* ($2n = 4x = 28$), growing in the Nordic countries. Taxonomically, the tetraploid *P. alpinum* belongs to the same species as the diploid *P. alpinum* subsp. *rhaeticum* [13,14]. The wild populations of *P. alpinum* commonly grow on meadows, riverbanks, roadsides and in birch forests at high elevations. At northern latitudes *P. alpinum* is found in Iceland, Norway, northern Sweden and northern Finland. Wild populations of *P. nodosum* are commonly found on meadows at low elevations. At northern latitudes, it has a more southern distribution than *P. alpinum*. Wild populations of timothy are found throughout the Nordic countries and grow at low elevations on meadows and in human-impacted and disturbed soils. The wild and domesticated accessions were studied both in the greenhouse and in the field to address the following questions: Do the three *Phleum* species differ in growth and development? Does the pattern of phenotypic diversity vary within and between the *Phleum* species? Do the wild and domesticated accessions of timothy differ in growth and development? Do some accessions show traits of interest for future breeding, and are therefore important genetic resources?

2. Results

2.1. Survival in the Field

The survival after the first winter was high in all groups of timothy accessions comprising wild, semi-wild, landraces, breeding lines and cultivars (100% of the accessions and from 93% to 96% of the plants; Table 1). The survival was also high in the cultivars and in the wild accessions of *P. nodosum* (100% of the accessions and from 92% to 96% of the plants). However, in *P. alpinum,* only 67% of the wild accessions and 66% of the plants survived the first winter.

Table 1. Number of accessions and number of plants planted in the field and the survival of accessions and plants after the first winter for different groups of accessions.

Category	Species	Group	Planted (Number)	Survived (Number)	Survival Rate (%)
Accessions	*P. pratense*	Cultivar	56	56	100.0
		Breeding line	10	10	100.0
		Landrace	55	55	100.0
		Wild	61	61	100.0
		Semi-wild	30	30	100.0
	P. nodosum	Cultivar	7	7	100.0
		Wild	7	7	100.0
	P. alpinum	Wild	18	12	66.6
Plants	*P. pratense*	Cultivar	823	769	93.4
		Breeding line	151	145	96.0
		Landrace	779	741	95.1
		Wild	928	876	94.4
		Semi-wild	440	415	94.3
	P. nodosum	Cultivar	90	86	95.6
		Wild	106	97	91.5
	P. alpinum	Wild	29	19	65.5

2.2. Phenotypic Differences between Species

The ANOVA results demonstrated significant differences between the three *Phleum* species for all the studied growth traits and developmental stages under both field and greenhouse conditions (Tables S1 and S2). In the field, we observed significant block effects but failed to detect such effects in the greenhouse experiment. *P. nodosum* and *P. alpinum* showed similar days to booting (DTB) and days to heading (DTH) in the field, while timothy reached booting and heading later (Tukey HSD, $p < 0.05$; Table S3). However, in the greenhouse, *P. alpinum* and timothy showed similar development and reached booting and heading later than *P. nodosum* (Tukey HSD, $p < 0.05$; Table S4). In the field, the highest fresh weight (FW), dry weight (DW) and plant height (PH) were found in timothy (Tukey HSD, $p < 0.05$; Table S3), while in the greenhouse *P. nodosum* had the highest means of FW, DW and PH (Tukey HSD, $p < 0.05$; Table S4).

2.3. Variation in Growth Traits among Accessions

2.3.1. Field Trial

The mean FW and DW were about one-third higher in almost all groups of accessions of timothy compared to the cultivars and wild accessions of *P. nodosum,* and almost twice as high compared to the wild accessions of *P. alpinum* (Figure 1a,b). Interestingly, no significant difference was found between cultivars and wild accessions of timothy in FW or in DW (Tukey HSD, $p > 0.05$), while the semi-wild accessions had a significantly lower FW and DW. Similarly, no significant difference was observed between cultivars and wild accessions of *P. nodosum* (Student's *t*-test, $p > 0.05$).

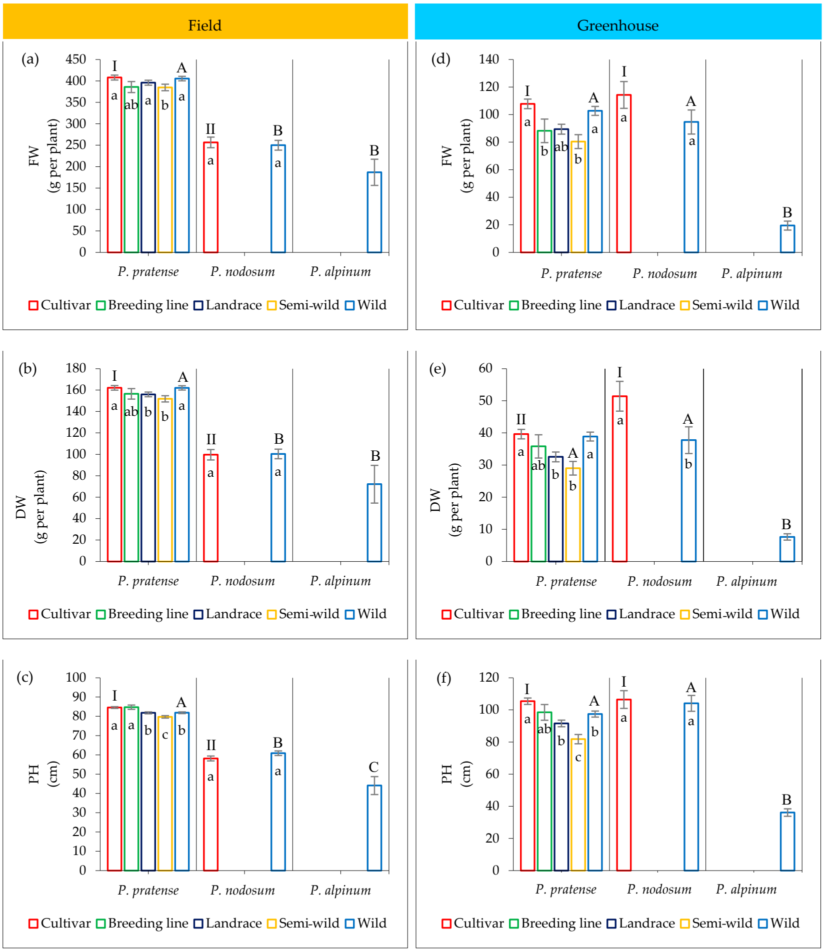


Figure 1. Mean and SD of fresh weight (FW), dry weight (DW) and plant height (PH) in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field (**a**–**c**) and in the greenhouse (**d**–**f**). Mean values that do not share the same letter are significantly different among accessions according to Student's *t*-test, $p < 0.05$ for comparing two groups of accessions, and Tukey HSD, $p < 0.05$ for comparing more than two groups. Letters inside the bars show comparisons between groups of accessions within each species and capital letters outside the bars show comparisons between the groups of wild accessions of the three species, and Roman letters show comparisons between groups of cultivars of *P. pratense* and *P. nodosum*.

A similar pattern was also found for PH. All groups of timothy accessions showed about one-third higher mean than the *P. nodosum* cultivars and wild accessions, and more than twice the mean of *P. alpinum* (Figure 1c). There was no significant difference in PH between wild and cultivated accessions of *P. nodosum* (Student's *t*-test, $p > 0.05$). However, within timothy the wild and semi-wild accessions as well as the landraces showed significantly lower PH than the breeding lines and cultivars (Tukey HSD, $p < 0.05$).

2.3.2. Greenhouse Trial

The mean FW and DW of all groups of accessions in all species were significantly lower in the greenhouse than in the field (Figure 1d,e). For timothy, the FW and DW were about one-fourth of the yield in the field, and for *P. alpinum* they were only one-fifth of the yield in the field. However, in *P. nodosum* the yield was only reduced by half in the greenhouse compared to the field.

Even though the FW and DW were lower in the greenhouse, the pattern of variation among groups of timothy accessions was similar to the pattern found in the field, where no significant difference was found between the cultivars and the wild accessions (Tukey HSD, $p > 0.05$). In contrast to the field trial, the DW was significantly higher in the cultivars than in the wild accessions of *P. nodosum* (Student's *t*-test, $p < 0.05$).

The mean PH was higher in the greenhouse than in the field for all groups of timothy accessions, except for the semi-wilds (Figure 1f), but the pattern of variation was similar to that found in the field.

2.4. Variation in Development among Accessions

2.4.1. Field Trial

A partially deviant pattern of variation was found when comparing the development within and between the three *Phleum* species compared to the pattern of variation for the growth traits. In timothy, the mean of DTB and DTH was significantly lower in the cultivars compared to the other groups of accessions, while for days to stem elongation (DTS) it was only significantly different from the landraces (Tukey HSD, $p < 0.05$; Figure 2a–c). In *P. nodosum*, the cultivars showed a significantly lower mean than the wild accessions for all developmental stages. In other words, the cultivars of both timothy and *P. nodosum* reached the three developmental stages earlier than their wild accessions did. Moreover, the *P. nodosum* cultivars reached these developmental stages earlier than the timothy cultivars. The wild accessions of timothy and *P. nodosum* did not differ significantly in DTB or DTH, while the wild accessions of *P. alpinum* reached booting earlier than timothy and *P. nodosum*, and reached heading earlier than the wild timothy accessions.

2.4.2. Greenhouse Trial

In timothy, two wild accessions, four semi-wild accessions, four landraces, one breeding line and two cultivars only formed vegetative tillers at the end of the greenhouse trial about six months after the vernalization treatment. In addition, twelve wild accessions, nine semi-wild accessions, nine landraces, two breeding lines and four cultivars remained in the elongated stage at the end of the trial. For the plants that reached heading, the results from the greenhouse trial showed a pattern similar to what was found in the field for the developmental traits (Figure 2d,f), where cultivars had a significantly lower mean of DTS compared to the other groups of timothy accessions (Tukey HSD, $p < 0.05$). The cultivars also showed a significantly lower DTB and DTH compared to the landraces and the wild accessions of timothy (Tukey HSD, $p < 0.05$).

The pattern of variation was, however, different for *P. nodosum*. In contrast to the field trial, the cultivars reached booting and heading later than the wild accessions. Moreover, the development of the wild accessions of *P. alpinum* was rather different in the greenhouse than in the field since 12 out of the 18 accessions (86%) did not form elongated or heading tillers in the greenhouse.

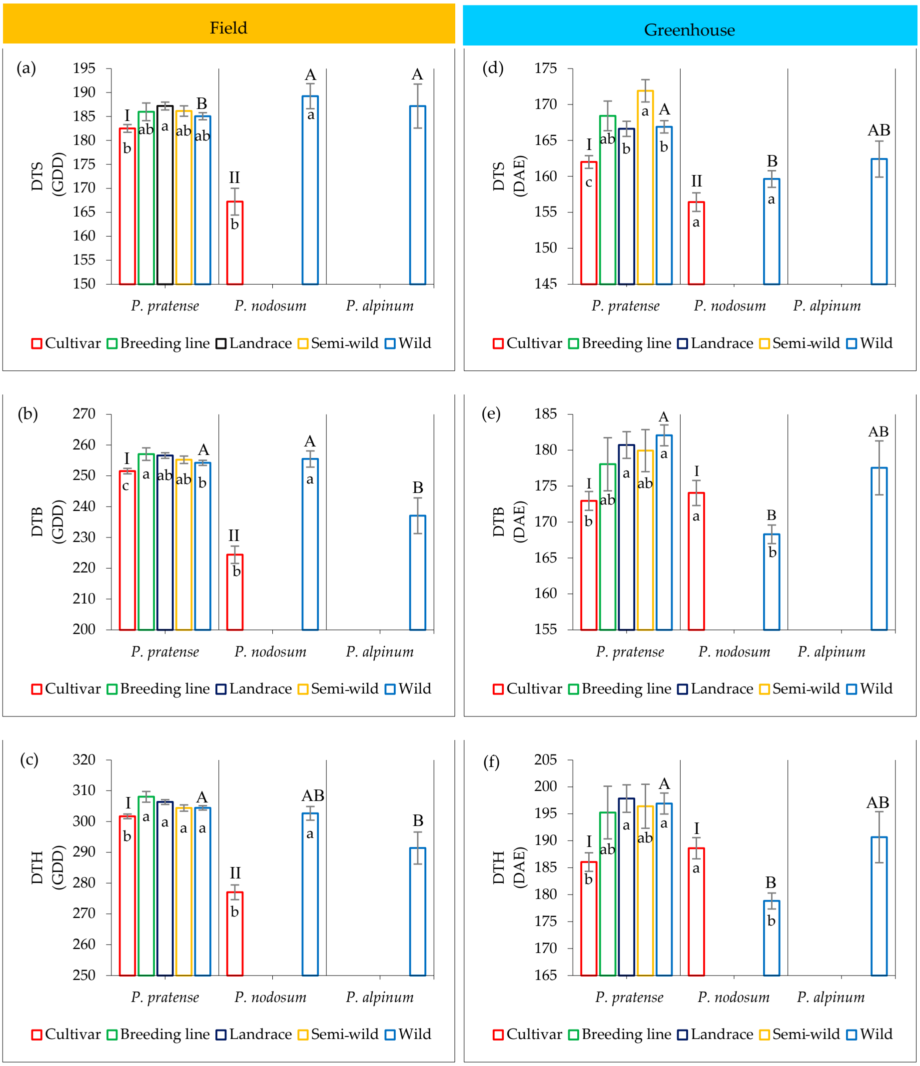


Figure 2. Mean and SD of days to stem elongation (DTS), days to booting (DTB) and days to heading (DTH) in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field based on growing degree days (GDD, **a**–**c**) and in the greenhouse based on days after emergence (DAE, **d**–**f**). Mean values that do not share the same letter are significantly different among accessions according to Student's *t*-test, $p < 0.05$ for comparing two groups of accessions, and Tukey HSD, $p < 0.05$ for comparing more than two groups. Letters inside the bars show comparisons between groups of accessions within each species and capital letters outside the bars show comparisons between the groups of wild accessions of three species, and Roman letters show comparisons between groups of cultivars of *P. pratense* and *P. nodosum*.

2.5. Correlation between Traits

2.5.1. Field Trial

A strong positive correlation was found between the variation in all developmental stages (DTS, DTB and DTH) in all three species in the field (Figure 3a–c). FW and DW were also strongly correlated in all three species, while FW and PH, as well as DW and PH, showed a weaker correlation in both timothy and *P. nodosum*. The variation in the growth traits was not strongly correlated with the variation in DTS, DTB and DTH in all three species. In fact, we found no correlation between most of the growth and developmental traits in *P. nodosum* and *P. alpinum*. In timothy, no correlations or weak correlations were found between the two types of traits.

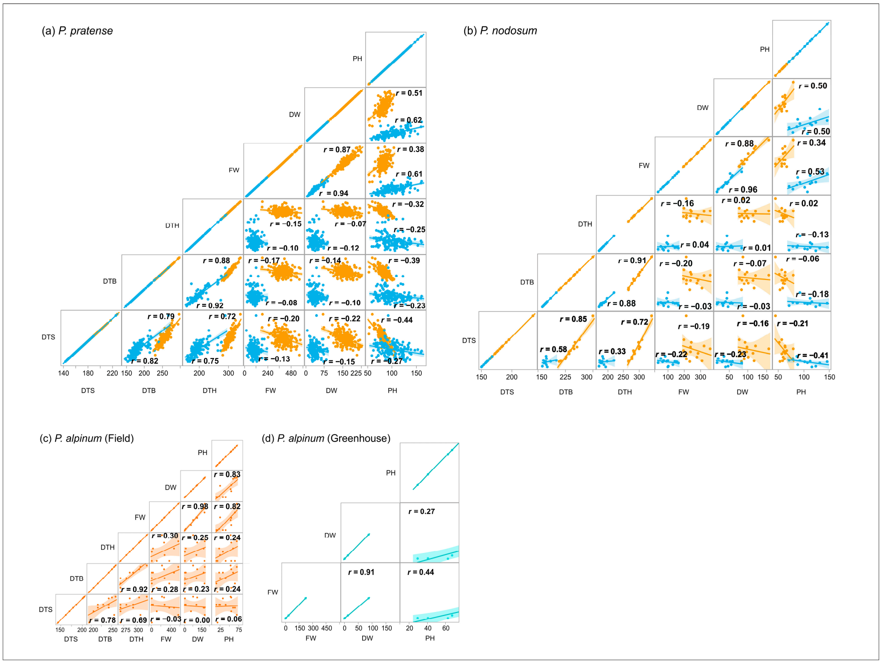


Figure 3. Correlation analysis between the studied traits, days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field (brown) and in the greenhouse (blue). (**a**) *P. pratense*, (**b**) *P. nodosum* and (**c**) *P. alpinum* in the field and (**d**) *P. alpinum* in the greenhouse.

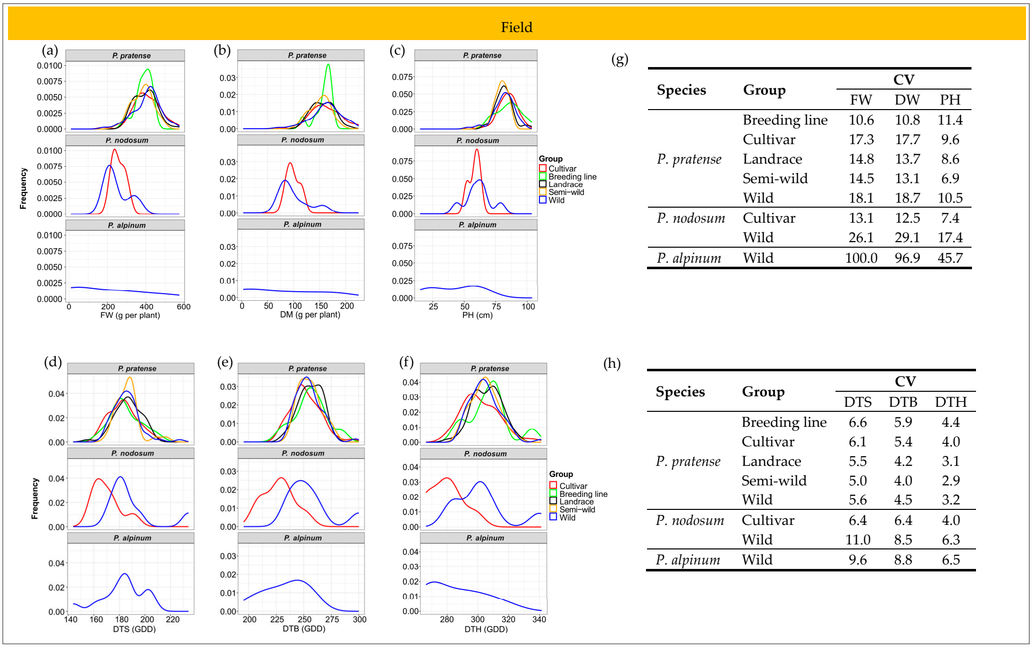
2.5.2. Greenhouse Trial

Only the accessions that developed stem elongation, booting or heading in the greenhouse trial were included in the correlation studies. In timothy, a similar pattern of correlation between traits as seen in the field was also found in the greenhouse (Figure 3a). This was also true for *P. nodosum*, except for the weaker correlation between DTS and DTB, and between DTS and DTH, and the stronger correlation between FW and PH found in the greenhouse (Figure 3b). Moreover, *P. alpinum* plants showed a weaker correlation between FW and PH, and between DW and PH in the greenhouse (Figure 3d). Due to the low number of *P. alpinum* plants that formed elongated and flowering tillers, we did not perform a correlation test between the three developmental stages or between them and the growth traits.

2.6. Patterns of Phenotypic Diversity

2.6.1. Field Trial

The distribution of the studied traits varied between species and between groups of accessions within the species (Figure 4a–f). Coefficient of variation (CV) was calculated for each trait to evaluate the phenotypic diversity among the species and groups of accessions. *P. alpinum* showed a much higher CV than timothy and *P. nodosum* for all growth traits in the field (Figure 4g). In timothy, the wild accessions and cultivars showed a similar diversity and the highest CV for FW and DW, while the breeding lines had the highest CV for PH. However, in *P. nodosum*, the wild accessions had a much higher CV than the cultivars for all growth traits. The highest diversity in the developmental traits was found in *P. alpinum* and the wild accessions of *P. nodosum* (Figure 4h). In timothy, the cultivars and breeding lines showed larger diversity in DTS, DTB and DTH than the other groups of accessions.



(g)

Species	Group	CV		
		FW	DW	PH
P. pratense	Breeding line	10.6	10.8	11.4
	Cultivar	17.3	17.7	9.6
	Landrace	14.8	13.7	8.6
	Semi-wild	14.5	13.1	6.9
	Wild	18.1	18.7	10.5
P. nodosum	Cultivar	13.1	12.5	7.4
	Wild	26.1	29.1	17.4
P. alpinum	Wild	100.0	96.9	45.7

(h)

Species	Group	CV		
		DTS	DTB	DTH
P. pratense	Breeding line	6.6	5.9	4.4
	Cultivar	6.1	5.4	4.0
	Landrace	5.5	4.2	3.1
	Semi-wild	5.0	4.0	2.9
	Wild	5.6	4.5	3.2
P. nodosum	Cultivar	6.4	6.4	4.0
	Wild	11.0	8.5	6.3
P. alpinum	Wild	9.6	8.8	6.5

Figure 4. Distribution of studied traits in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the field. (**a**) Fresh weight (FW), (**b**) dry weight (DW), (**c**) plant height (PH), (**d**) days to stem elongation (DTS), (**e**) days to booting (DTB), (**f**) days to heading (DTH), (**g**) coefficient of variation (CV) of growth traits, (**h**) CV of developmental stages.

A principal component analysis (PCA) based on the variation in all growth traits and the developmental stages studied in the field showed a large phenotypic diversity among timothy accessions and formed six clusters (Figure 5a). In general, the accessions did not cluster according to the type of accessions (cultivar, breeding line, landrace, semi-wild and wild).

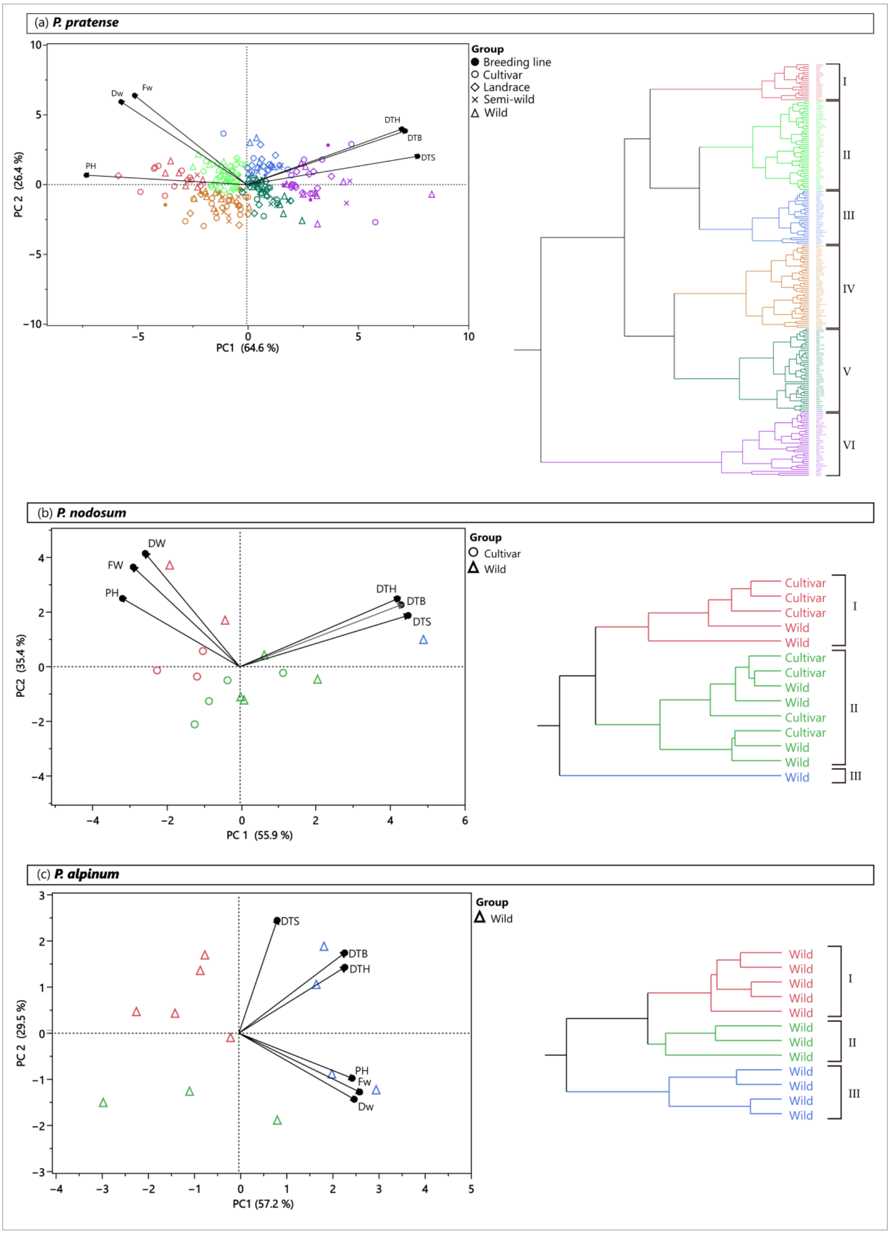


Figure 5. Principal component analysis (PCA) and cluster analysis based on all studied traits, days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field for (**a**) *P. pratense*, (**b**) *P. nodosum* and (**c**) *P. alpinum*. Different colors in the PCA correspond to the clusters of accessions defined in the cluster analysis.

Timothy accessions falling within the same cluster in the PCA showed similarities in the development and growth traits rather than the type of accession. Accessions in cluster I (highlighted in red) had on average the highest PH, FW and DW, and reached the three developmental stages (DTS, DTB and DTH) earlier than the accessions in the other clusters (Figure 5a and Table 2). On the contrary, the accessions in cluster VI (highlighted in purple) showed on average the lowest yield and PH, and reached elongation, booting and heading later than any of the other clusters. The accessions in cluster II (highlighted in light green) and cluster III (highlighted in blue) had similar and relatively high FW and DW, but accessions in cluster II reached the three developmental stages earlier than those in cluster III. Both cluster IV (highlighted in brown) and cluster V (highlighted in dark green) consisted of relatively low-yielding accessions. The two clusters differed, however, in DTS, DTB and DTH. The total number of accessions of each group of accessions is shown in Table S5.

Table 2. Mean of each cluster for each studied trait in *P. pratense*, *P. nodosum* and *P. alpinum* in the field.

Species	**Cluster**	**Number of Accessions**	**Trait**					
			DTS (GDD)	**DTB (GDD)**	**DTH (GDD)**	**FW (g per Plant)**	**DW (g per Plant)**	**PH (cm)**
P. pratense	I	19	170.1	239.1	291.9	480.9	196.9	94.0
	II	46	182.1	252.0	302.1	448.2	176.7	85.6
	III	28	190.8	266.0	314.4	442.9	173.7	81.8
	IV	43	176.7	243.1	293.9	364.8	148.4	86.7
	V	43	187.5	255.5	305.7	364.0	142.4	78.7
	VI	33	201.4	270.6	318.5	339.3	133.2	71.1
P. nodosum	I	5	173.0	233.9	285.6	309.8	121.5	64.5
	II	8	177.1	239.0	288.2	221.9	87.2	58.7
	III	1	234.1	300.1	340.0	212.3	86.6	44.2
P. alpinum	I	5	188.9	232.4	279.5	69.0	25.4	27.0
	II	3	164.2	205.9	268.5	168.3	80.7	46.0
	III	4	187.0	252.7	307.0	386.5	136.9	59.6

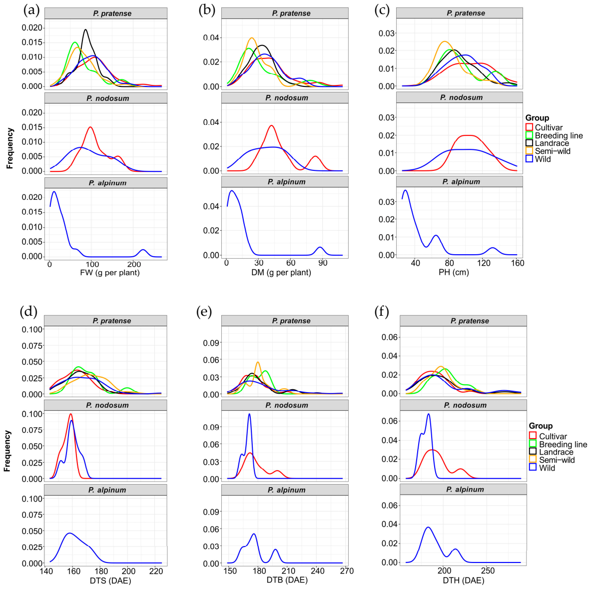
For *P. nodosum*, two clusters were formed with wild accessions and cultivars in both clusters (Figure 5b). In addition, a single wild accession did not cluster with any other accession. Cluster I (highlighted in red) consisted of accessions with, on average, the tallest plants as well as the highest FW and DW (Table 2). In contrast, the single accession (highlighted in blue) reached heading very late and had the lowest PH, FW and DW. The accessions in cluster II (highlighted in green) showed a phenotype in between the accessions in cluster I and the single accession.

The wild accessions of *P. alpinum* were divided into three clusters (Figure 5c). Cluster I (highlighted in red) was represented by accessions with very low PH, FW and DW, while cluster III (highlighted in blue) showed the highest yield (Table 2). Cluster II (highlighted in green) consisted of very early booting and heading accessions.

2.6.2. Greenhouse Trial

Differences in the distribution of the growth traits and the developmental stages were also found between species and between groups of accessions within species in the greenhouse trial (Figure 6a–f). However, the distribution pattern in the greenhouse differed partly from the pattern found in the field. For example, the variation in the distribution of PH was larger between groups of timothy accessions in the greenhouse than in the field (Figures 4c and 6c) and the variation in the developmental traits was larger between the wild accessions and the cultivars of *P. nodosum* in the field (Figures 4d–f and 6d–f).

Greenhouse



(g)

Species	Group	CV		
		FW	DW	PH
P. pratense	Breeding line	48.3	62.0	26.5
	Cultivar	41.7	48.2	23.9
	Landrace	29.4	35.1	22.3
	Semi-wild	41.9	43.6	22.4
	Wild	36.7	40.7	20.4
P. nodosum	Cultivar	30.5	37.1	15.1
	Wild	44.9	41.6	25.7
P. alpinum	Wild	162.3	157.4	61.9

(h)

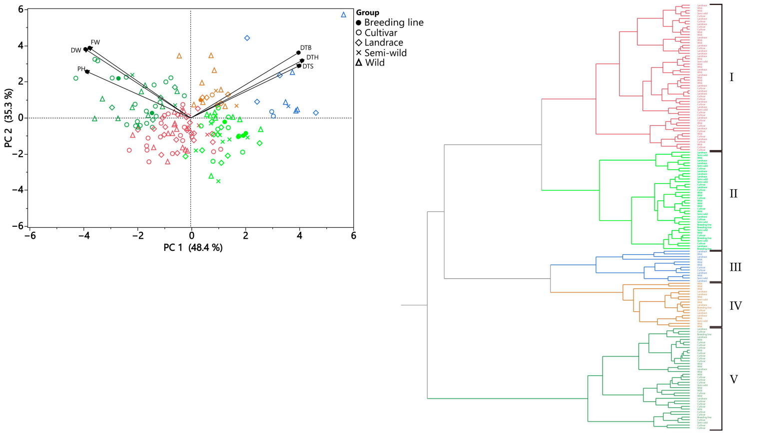
Species	Group	CV		
		DTS	DTB	DTH
P. pratense	Breeding line	7.3	4.7	7.6
	Cultivar	6.2	7.4	8.4
	Landrace	6.6	10.0	12.5
	Semi-wild	6.8	7.0	8.0
	Wild	8.5	12.7	15.6
P. nodosum	Cultivar	2.5	6.9	7.8
	Wild	3.3	2.5	2.9
P. alpinum	Wild	4.7	7.3	7.7

Figure 6. Distribution of studied traits in different groups of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. (**a**) Fresh weight (FW), (**b**) dry weight (DW), (**c**) plant height (PH), (**d**) days to stem elongation (DTS), (**e**) days to booting (DTB), (**f**) days to heading (DTH), (**g**) coefficient of variation (CV) of growth traits, (**h**) CV of developmental stages.

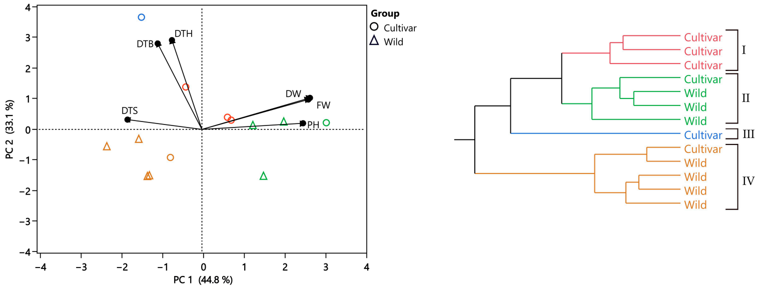
Similar to what was found in the field trial, *P. alpinum* showed a much higher diversity for the growth traits than the other two species in the greenhouse (Figure 6g). Also, the wild accessions of *P. nodosum* had a higher CV than the *P. nodosum* cultivars for FW, DW and PH. While the wild accessions and cultivars had the highest diversity for FW and DW in the field, the breeding lines of timothy showed the highest CV for the growth traits in the greenhouse. The highest diversity for the developmental traits was found in the wild accessions of timothy, while the wild accessions of *P. nodosum* showed the lowest CV for DTB and DTH (Figure 6h).

A PCA based on all studied traits in the greenhouse also showed a large diversity among accessions in all the three *Phleum* species (Figure 7). Among the timothy accessions that reached heading, five clusters were formed. Both domesticated and wild accessions were found in all clusters. Accessions in cluster V (highlighted in dark green) had the highest yield and PH and reached the three developmental stages early (Figure 7a and Table 3). Cluster II (highlighted in light green) and III (highlighted in blue) included both low-yielding accessions, but the accessions in cluster III reached heading much later than the accessions in cluster II. Mid-early accessions were found in cluster IV (highlighted in brown). These accessions showed high FW and DW. The total number of accessions of each group of accessions is shown in Table S6.

(a) ***P. pratense***



(b) ***P. nodosum***



(c) ***P. alpinum***

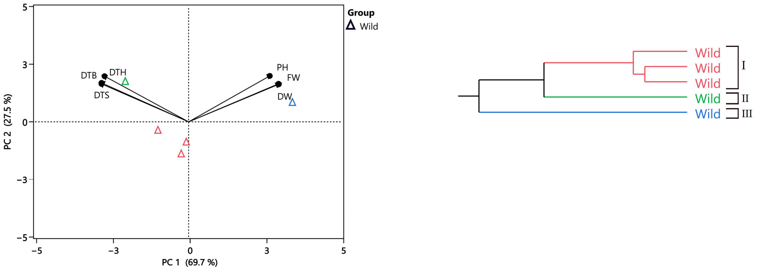


Figure 7. Principal component analysis (PCA) and cluster analysis based on all studied traits, days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the greenhouse for (**a**) *P. nodosum*, (**b**) *P. alpinum* and (**c**) *P. pratense*. Different colors in the PCA correspond to the clusters of accessions defined in the cluster analysis.

Table 3. Mean of each cluster for each studied trait in *P. pratense, P. nodosum* and *P. alpinum* in the greenhouse.

Species	Cluster	Number of Accessions	Trait					
			DTS (DAE)	DTB (DAE)	DTH (DAE)	FW (g per Plant)	DW (g per Plant)	PH (cm)
P. pratense	I	55	159.4	170.2	183.4	92.6	34.2	103.2
	II	37	169.3	180.9	195.6	70.0	25.1	80.2
	III	12	183.7	224.2	252.3	71.4	24.9	87.6
	IV	17	175.1	196.1	217.1	123.1	46.0	96.6
	V	38	160.2	174.5	184.9	143.9	56.9	125.7
P. nodosum	I	3	154.7	177.4	193.2	108.3	46.2	105.2
	II	4	156.7	167.5	179.6	141.5	60.1	128.0
	III	1	161.3	200.0	220.0	100.7	43.8	92.1
	IV	5	161.9	170.0	179.9	65.4	27.4	88.8
P. alpinum	I	3	161.2	174.7	187.2	3.1	1.6	44.1
	II	1	173.0	198.0	214.0	33.8	12.8	67.1
	III	1	154.0	163.5	176.5	223.6	87.5	132.0

The accessions of *P. nodosum* were grouped into three clusters (one more cluster than in the field) and a single cultivar that did not cluster with any other accession (Figure 7b). Cluster I (highlighted in red), which comprised only cultivars, cluster II (highlighted in green) and cluster III (highlighted in blue) had all high and similar mean FW and DW, while cluster IV (highlighted in brown) showed a low yield (Table 3). All clusters reached stem elongation early, but differed in DTB and DTH, where accessions in cluster III were very late.

P. alpinum formed one cluster with three accessions (highlighted in red, Figure 7c). The other two accessions did not cluster with each other or any other *P. alpinum* accession. The mean FW and DW in the accessions were very low in cluster I and one of the single accessions (highlighted in green) compared to *P. nodosum* and timothy (Table 3). Cluster I and this single accession (highlighted in green) also showed late booting and heading, while the other single accession (highlighted in blue) was early and high yielding.

2.7. Heritability

The broad-sense heritability (H^2) was lower in the field than in the greenhouse for all traits, except for PH which showed similar H^2 in both trials (Table 4). The largest difference was found for the developmental stages where H^2 was as high as 0.95 for both DTB and DTH in the greenhouse, while it was 0.37 for both traits in the field. In the greenhouse, the growth traits showed much lower H^2 compared to the developmental stages. However, in the field, the heritability for the growth traits and the developmental stages was more similar.

Table 4. Genotypic variance (V_g), environmental variance (V_e), phenotypic variance (V_p) and broad-sense heritability (H^2) of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field and in the greenhouse for *P. pratense*.

Trial	Trait	V_g	V_e	V_p	H^2
Field	DTS	0.9	5.6	2.3	0.40
	DTB	0.6	3.8	1.5	0.37
	DTH	0.5	3.2	1.3	0.37
	FW	2449.5	24,289.1	8521.7	0.29
	DW	401.0	3708.0	1328.0	0.30
	PH	48.7	160.6	88.9	0.55

Table 4. *Cont.*

Trial	Trait	V_g	V_e	V_p	H^2
Greenhouse	DTS	99.2	111.6	127.1	0.78
	DTB	286.4	60.1	301.5	0.95
	DTH	505.0	98.9	529.7	0.95
	FW	745.9	3204.1	1546.9	0.48
	DW	125.2	559.9	265.1	0.47
	PH	315.3	1092.8	588.4	0.54

3. Discussion

Agriculture is challenged by an increased demand for food for a growing human population and thereby an increased demand for animal feed during a changing climate. To respond to this challenge, high-yielding cultivars adapted to grow in a changing environment need to be developed. In this context, wild populations and closely related species to the crop may serve as valuable genetic resources for the development of improved cultivars. The aim of our study was to provide knowledge about the phenotypic diversity in the forage crop timothy and two of its closely related species, *P. nodosum* and *P. alpinum*. We studied different growth and plant developmental traits in domesticated and wild accessions and identified potential genetic resources for timothy breeding.

The three *Phleum* species showed variation in growth. All groups of timothy accessions had a significantly higher FW, DW and PH than *P. nodosum* and *P. alpinum* in the field. Moreover, *P. nodosum* formed taller tillers than *P. alpinum*. The greater growth of timothy was expected as it is one of the most important forage crops in temperate regions and cultivated for its high biomass production. Cultivars of *P. nodosum* and *P. alpinum* are developed, but to the best of our knowledge not used in mixtures with other forage crops. However, in the greenhouse *P. nodosum* showed similar growth to timothy, while the plants of *P. alpinum* were very small and most of them did not produce elongated tillers. The cultivation of plants under different growing conditions in the greenhouse compared to in the field, such as differences in temperature, photoperiod and water and nutrient content in the soil, most likely contributed to the overall lower FW and DW in the greenhouse. For example, a longer photoperiod has been shown to stimulate growth and biomass production in *P. alpinum* [21]. The variation in the growing conditions seems to have also affected the heritability of the studied traits in timothy, where the heritability was higher in the greenhouse than in the field for almost all traits.

P. nodosum was, however, less affected by the different growing conditions in the field and the greenhouse than timothy and *P. alpinum*. The adaptation to different habitats may explain the difference in performance among the species in the two trials. *P. nodosum* is characterized as a lowland species with rapid growth and spread, while *P. alpinum* is primarily found at higher altitudes adapted to harsh mountainous environments [22]. The diploid *P. nodosum* and the tetraploid *P. alpinum* carry different genomes [13,14]. The genomic form of *P. nodosum* is described as B_NB_N and the genomic form of *P. alpinum* as R_ER_EXX. Our result suggests that the B_N genome carries genes for good and robust growth in different growing environments. In addition, cytological studies indicate that the hexaploid timothy carries two genomes of *P. nodosum* [15,16]. The polyploidization and duplication of the B_N genome in timothy is likely to have contributed to its large growth and biomass production. In fact, polyploidy has been shown to increase plant growth in wild plants and domesticated crops [23–25]. Polyploidization is an important tool in forage crop breeding, and the effect of genome duplication on biomass production and other traits would be of interest to explore.

A large phenotypic diversity was found within each *Phleum* species and the different groups of accessions. However, even though the diversity was high within the wild accessions and the cultivars of timothy, these two groups did not differ significantly in FW and DW in the field or in the greenhouse. Moreover, we did not find significant difference in spring growth between wild accessions and cultivars of *P. nodosum*. The similarity in the phenotype of wild accessions and cultivars as well as the large diversity among cultivars found in this study suggest that there has not been a strong directional selection towards increased yield during the domestication and breeding of timothy and *P. nodosum.* A strong directional selection towards a higher seed yield and other domestication traits such as resistance to seed shattering and apical dominance is found in annual grain crops [17–19,26], while in perennial forage crops, a high biomass production and forage quality are desirable [27–30]. The large changes in phenotypic traits in seed crops are a result of strong selection during many breeding cycles, while most of the forage crops have gone through relatively few cycles of selection. The human selection may also have been counteracted by cross-pollination in self-incompatible grasses such as timothy, increasing the diversity within the species.

In timothy, the undomesticated wild accessions showed a vigorous growth and high FW and DW, which are desirable traits for a forage crop. Our results suggest that the timothy cultivars have rather been selected and improved for other traits. We found a significant difference in plant development between the wild accessions and the cultivars of timothy both in the field and in the greenhouse. The group of timothy cultivars reached stem elongation, booting and heading earlier than the wild accessions. This shows that the breeding has favored a rapid plant development in spring growth, and thereby made repeated harvests of timothy possible during the same season. Also, in *P. nodosum,* the cultivars showed earlier stem elongation, booting and heading than the wild accessions in the field. The difference between the wild accessions and the cultivars in plant development was more pronounced in *P. nodosum* than in timothy, and in the field the *P. nodosum* cultivars reached heading much earlier than the timothy cultivars. The contrary was, however, found in the greenhouse, where the *P. nodosum* cultivars showed booting and heading later than the wild accessions, and reached heading at about the same time as the timothy cultivars. Differences in performance in the two growing environments were also found in timothy and *P. alpinum* accessions. Many domesticated and undomesticated accessions of timothy and a majority of the wild *P. alpinum* accessions did not flower in the greenhouse. The difference in the ability to flower in *P. alpinum* and timothy, and the opposite performance of the *P. nodosum* cultivars and wild accessions in the two growing environments, may be influenced by the difference in photoperiod. Also, the difference in length of vernalization between the six-week cold treatment in the greenhouse and the longer cold period in the field may have affected the development and ability to flower. Plants differ in their need for vernalization to flower, and winter-type plants of annual cereals and some perennial grasses such as ryegrass and tall fescue require vernalization [5,31,32].

The transition to the reproductive stage may also be affected by the photoperiod. In timothy, an increasing temperature and a longer photoperiod stimulate stem elongation during spring growth [5,8–10]. The need for vernalization for the transition to reproductive tillers differs between timothy accessions from different geographical origin [6–9]. While accessions in northern Scandinavia require vernalization to flower, more southern-growing accessions are not affected by the vernalization. This adaptation to the climate (e.g., photoperiod and temperature) at the cultivation and growing sites may explain the variation in heading time that we have found among accessions, and the different performance of some accessions in the field and the greenhouse. A weak correlation was found between the latitude of the geographical origin of the wild accessions of timothy and the heading time (Figure S1). A correlation between heading time and geographical origin in timothy has also been shown in other studies [7,10].

The large diversity that we have found in this study is of value for further pre-breeding studies. It also shows the potential to identify genetic resources for developing new improved timothy cultivars. The variation in days to stem elongation and flowering indicates the difference in response to photoperiod, where some accessions required a shorter photoperiod to reach flowering compared to other accessions. These accessions may therefore serve as important germplasm for development of improved cultivars for cultivation at lower latitudes. Accessions that respond to longer days for flowering could be used as germplasm for the development of new cultivars for northern latitudes. However, early cultivars having a vigorous growth and a high biomass production may have a lower nutritional value and forage quality than cultivars flowering later due to their faster production of elongated tillers [3,4]. This negative relationship between high yield and high forage quality is challenging in the breeding of forage grasses. Deviant accessions with high yield and delayed and suppressed flowering would therefore be of great interest for breeding. Interestingly, no strong correlation was found between the growth and plant developmental traits in our study, which indicates that these traits can be selected independently of each other. Timothy accessions with favorable traits could either be crossed with existing cultivars to transfer these traits into the crop or domesticated to make them adapted to grow on agricultural land.

Moreover, the relatively high yield of *P. nodosum* and its adaptation to low altitudes make this species an interesting candidate for the development of new forage crops. Selected accessions could be domesticated as a forage crop or crossed with already domesticated *P. nodosum* cultivars. On the other hand, *P. alpinum* may also be a potential source of genes for adaptation to colder and more stressful growing conditions. Genes from the wild relatives may also be transferred into the timothy forage crop through wide hybridization since Nordenskiöld [33] found that crosses between *P. nodosum* and *P. pratense*, and between *P. pratense* and *P. alpinum*, could give fertile hybrids.

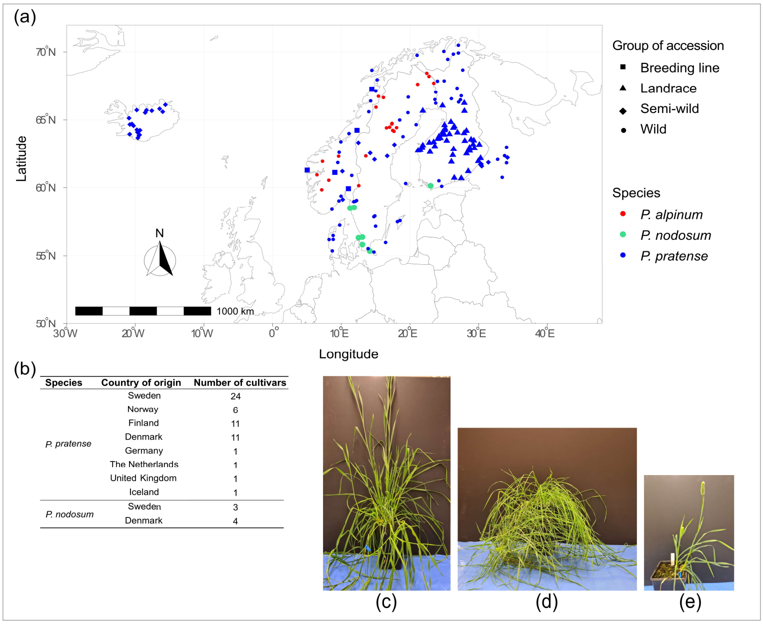
4. Materials and Methods

4.1. Plant Material

Clonal plants from 212 accessions of timothy (*P. pratense*), 14 accessions of *P. nodosum* and 18 accessions of *P. alpinum* originating from the Nordic countries, UK, Germany, the Netherlands and Russia were planted in the field (Table S7 and Figure 8). The same clonal plants, except four accessions (two timothy, one *P. nodosum* and one *P. alpinum*), were also studied in a greenhouse at the Plant Cultivation Facility, Uppsala BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden. The seeds were provided by the genebank NordGen, Alnarp, Sweden. In the Nordic Baltic Genebanks Information System, *Phleum* accessions are described as wild, semi-wild (from populations nearby cultivated fields with potential gene flow between populations), landrace (from a cultivated variety adapted to a certain ecogeographical area), breeding line (group of related genotypes under evaluation in a breeding program) or cultivar (Table S7).

4.2. Pre-Cultivation and Cloning

Eight seeds of each accession were sown in low nutrient commercial potting soil (S-jord, Hasselfors Garden, Örebro, Sweden, Figure S2) in a climate chamber at the Plant Cultivation Facility, BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden, with 16/8 h day/night photoperiod (350 µmol m^{-2} s^{-1} light), 22/17 °C day/night temperature and 65 percent humidity. After two months of growth, four randomly selected plants (genotypes) of each accession were cloned. Since the *Phleum* species are outcrossing, we considered each of the four plants within an accession genetically different. Vegetative tillers were gently separated from each other, and six of the tillers from each plant were individually transplanted into pots (15 cm × 15 cm × 20 cm) with commercial potting soil (P-jord, Hasselfors Garden, Örebro, Sweden). The cloned plants (totally six plants per genotype) were then cultivated in the greenhouse with a 16/8 h day/night photoperiod.



Species	Country of origin	Number of cultivars
P. pratense	Sweden	24
	Norway	6
	Finland	11
	Denmark	11
	Germany	1
	The Netherlands	1
	United Kingdom	1
	Iceland	1
P. nodosum	Sweden	3
	Denmark	4

Figure 8. (**a**) Collection sites of wild and semi-wild accessions, landraces and breeding lines of *P. pratense*, *P. nodosum* and *P. alpinum*, and (**b**) country of origin of developed cultivars based on passport data from the genebank NordGen, Alnarp, Sweden. Two months old plants of (**c**) *P. pratense*, (**d**) *P. nodosum* and (**e**) *P. alpinum* grown in a greenhouse.

4.3. Field Trial

In July 2020, four plants of each genotype were transplanted in clay soil in a farmer's field north of Uppsala, Central Sweden (60°00′ N, 17°42′ E). The field was located at an organic farm, and a low level of animal manure fertilizer was added. Manual weeding was carried out, and no herbicides were applied. In total, 244 *Phleum* accessions with 4 genotypes of each accession and 4 plants of each genotype were planted in a randomized complete block design with one plant of each genotype in each of the 4 blocks. The distance between the plants was 1 m within and between rows. The development and growth were studied during the season 2021, one year after planting, until the harvest in June the same year.

4.4. Greenhouse Trial

The other two plants of each genotype were grown in the greenhouse for two weeks and then given a vernalization treatment for six weeks at 4 °C under an 8 h photoperiod at 100 µmol m^{-2} s^{-1}, to stimulate flowering. After the vernalization, the plants were transferred back to the greenhouse (16/8 h; 22/17 °C) and placed in two blocks with one replicate of each genotype randomized within each block. In total, 240 *Phleum* accessions with 4 genotypes of each accession and 2 plants of each genotype were evaluated from September 2020 until the last plant was harvested in March 2021. During the experiment, the plants were given nutrient solution (N: 102 mg/L (NH_4: 40 mg/L, NO_3: 62 mg/L), P: 20 mg/L, K: 86 mg/L, S: 8 mg/L, Ca: 6 mg/L, Mg: 8 mg/L, Fe: 0.34 mg/L, Mn: 0.4 mg/L, B: 0.2 mg/L, Zn: 0.06 mg/L, Cu: 0.03 mg/L, Mo: 0.08 mg/L; Wallco Miljöcenter AB, Arlöv, Sweden) twice a month.

4.5. Evaluated Traits

The clonal plants grown in the greenhouse and in the field were individually studied for the same growth traits and developmental stages (Table 5). Development was evaluated by recording the number of days to stem elongation (DTS), days to booting (DTB) and days to heading (DTH). We considered a plant to have reached stem elongation when the first tiller internode started to elongate, and the inflorescence was palpable at least 1 cm above the stem base [34] in about one-fourth of the total number of tillers (vegetative and elongated tillers). A plant was considered to have reached booting when the tip of the inflorescence was palpable in the flag leaf sheath and heading when the tip of the head was visible above the flag leaf in about one-fourth of the total number of tillers (vegetative, elongated and heading tillers). In the greenhouse, DTS, DTB and DTH were measured by recording the number of days after emergence of the coleoptile, including the time of vernalization.

Table 5. Studied traits in individual plants of *P. pratense*, *P. nodosum* and *P. alpinum* in the field and in the greenhouse.

Trait	Abbreviation	Description
Fresh weight	FW	Fresh weight (g) of tillers cut at 3 cm above the soil surface
Dry weight	DW	Dry weight (g) of tillers cut at 3 cm above the soil surface
Plant height	PH	The average length of 5 tillers (cm) in the field and the average length of 9 tillers (cm) in the greenhouse
Days to stem elongation	DTS	Number of days from emergence of coleoptile until the first tiller internode started to elongate, and the inflorescence was palpable at least 1 cm above the stem base in about one-fourth of the total number of tillers
Days to booting	DTB	Number of days from emergence of coleoptile until the tip of the inflorescence was palpable in the flag leaf sheath below the flag leaf base in about one fourth of the total number of tillers
Days to heading	DTH	Number of days from emergence of coleoptile until the head was visible above the flag leaf base in about one-fourth of the total number of tillers

In the field, the number of days to reach a specific developmental stage was recorded according to the day-of-year calendar. Based on that, DTS, DTB and DTH were calculated as accumulated growing degree days (GDD)

$$GDD = \frac{T_{max} + T_{min}}{2} - T_{base} \quad (1)$$

where T_{max} is the daily maximum temperature, T_{min} is the daily minimum temperature and T_{base} (the base temperature) is the minimum temperature at which growth can occur for a plant species. The base temperature of 5 °C is used for timothy. Daily temperatures were recorded from January 2021 to December 2021 at the Swedish Metrological and Hydrological Institute's weather station 458, Uppsala, 59°90′ N, 17°59′ E. The accumulated growing degree days was calculated from the start of the meteorological growth that is defined as the first five consecutive days with a daily mean temperature above 5 °C in spring 2021 (Figure S3).

The plants in the greenhouse were harvested when the peduncle turned yellow below at least one head of the plant. However, 14 accessions of timothy and 12 accessions of *P. alpinum* remained in the vegetative stage in the greenhouse and did not form elongated or heading tillers. In addition, 36 accessions of timothy reached only the stem elongation stage. The 26 accessions with only vegetative tillers and the 36 accessions with elongated tillers were harvested at the end of the experiment, about 6 months after the vernalization treatment. All tillers were cut 3 cm above the soil surface. The length of 3 randomly selected vegetative tillers, 3 elongated tillers and 3 tillers with heads were then evaluated and the plant height (PH) was estimated as the average of the 9 tillers. For the plants that did not form elongated and heading tillers, the length of 9 vegetative tillers were measured. In addition, the fresh weight (FW) of all tillers was weighed. The plant material was then dried at 60 °C for 48 h and the dry weight (DW) was measured.

In the field, the plants were harvested when 30–50 percent of the tillers on a plant had reached the booting to heading stage. At harvest, the height of five elongated tillers (2 tall, 1 medium height and 2 short tillers) of each plant was measured from soil surface and PH was estimated as the average of these tillers. The tillers were cut 3 cm above the soil surface, and the FW and DW of the plants were recorded. Plants within the same block were harvested within 3 days and the whole experiment was harvested within 12 days.

4.6. Data Analysis

Datasets were analyzed using the software RStudio [35] and JMP ver. 15 SAS Institute Inc., Cary, NC, USA. All traits showed normally distributed residuals and were therefore analyzed with parametric Analysis of Variance (ANOVA) tests. Since the number of accessions differed among the three *Phleum* species, and the number of accessions differed within each group of timothy accessions (wild, semi-wild, landraces, breeding lines and cultivars), an unbalanced nested design was used in the ANOVA to compare species initially. In this model, genotype was nested within accession, and accession was nested within species. Genotypes within accession and species were considered as random effect in the nested model. A normal ANOVA was then used for comparisons of different types of accessions within each species. In addition, the LSMeans Differences Tukey HSD test was performed to study the relationship among groups of accessions within species. Student's *t*-test was used for pairwise comparisons. Phenotypic diversity within each group of accessions was evaluated by calculating the coefficient of variation (CV)

$$CV = \frac{\delta}{\mu} \times 100 \tag{2}$$

where δ is the standard deviation and μ is the mean of the trait within the group.

To analyze and display relations among accessions based on all studied growth and developmental traits, we used the multivariate analyses principal component analysis (PCA) and cluster analysis. Moreover, Pearson correlation was used to evaluate and estimate the pattern and level of association between traits. The broad-sense heritability (H^2) was estimated for each trait using the R packages "inti" and "variability" in RStudio

$$H^2 = \frac{\delta_g^2}{\delta_p^2} \tag{3}$$

where, δ_g^2 is the genotypic variance, δ_p^2 is the phenotypic variance and $\delta_p^2 = \delta_g^2 + \frac{\delta_e^2}{r}$ in which r is the number of replicates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12193494/s1. Figure S1: Correlation between days to heading (DTH) in the field and latitude coordinate of the collection site of wild *P. pratense* accessions. Figure S2: Cloning of *Phleum* genotypes planted in the field and in the greenhouse. Figure S3: Accumulated growing degree days (GDD) calculated from meteorological growth start (defined as the first of five consecutive days with daily mean temperature above 5 °C) for 2021 in Uppsala, Sweden based on the climate data from the Swedish Meteorological and Hydrological Institute, Norrköping, Sweden. Table S1: Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$. Table S2: Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the greenhouse. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$. Table S3: Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the field. GDD: Growing degree days. Mean values that do not share the same letter are significantly different among species according to Tukey HSD, $p < 0.05$. Table S4: Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. DAE: days after coleoptile emergence. Mean values that do not share the same letter are significantly different among species accessions according to Tukey HSD, $p < 0.05$. Table S5: Number of accessions of each group in each cluster in *P. pratense*, *P. nodosum* and *P. alpinum* in the field. Table S6: Number of accessions of each group in each cluster in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. Table S7: Information of the studied accessions of *P. pratense*, *P. nodosum* and *P. alpinum* from the genebank NordGen, Alnarp, Sweden.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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Supplementary materials to:

Phenotypic Diversity in Domesticated and Wild Timothy Grass, and Closely Related Species for Forage Breeding

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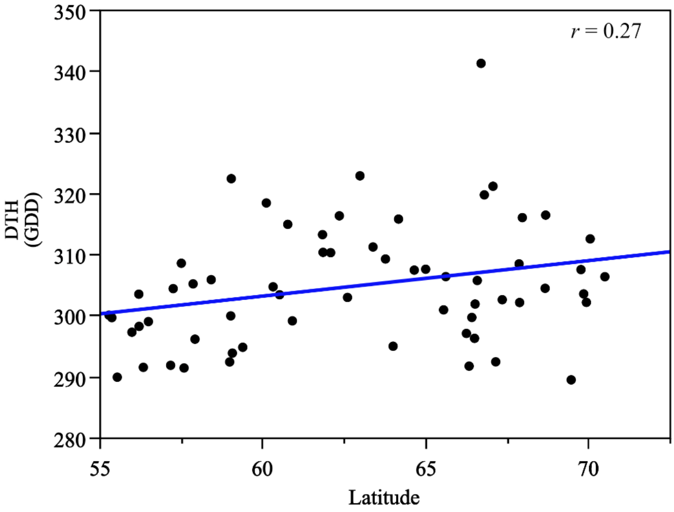

Figure S1. Correlation between days to heading (DTH) in the field and latitude coordinate of the collection site of wild *P. pratense* accessions.

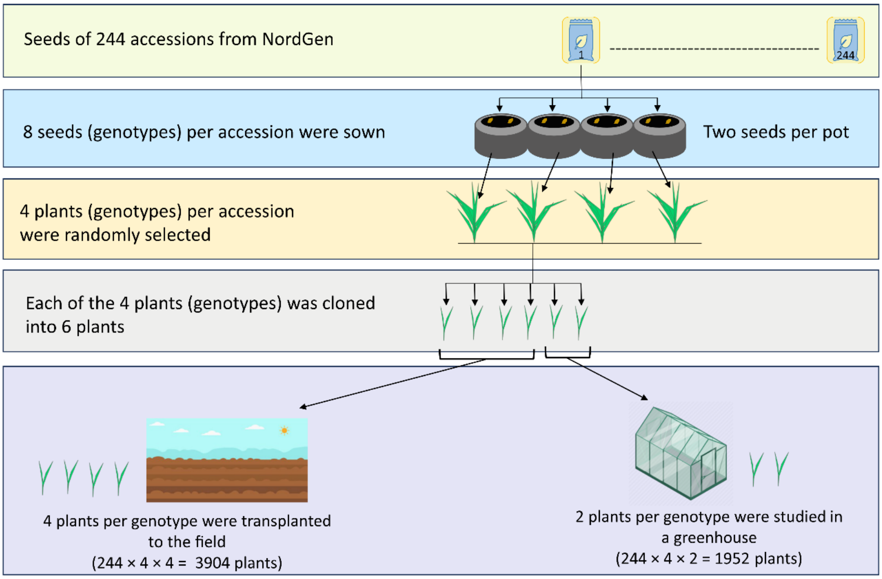

Figure S2. Cloning of *Phleum* genotypes planted in the field and in the greenhouse.

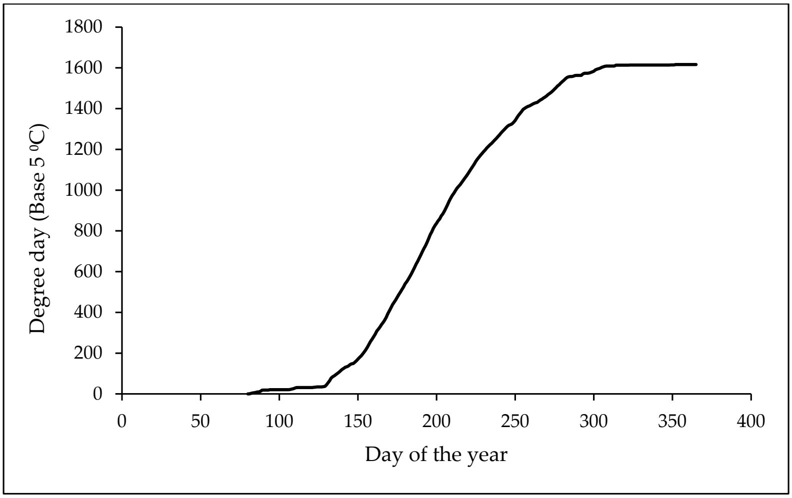

Figure S3. Accumulated growing degree days (GDD) calculated from meteorological growth start (defined as the first of five consecutive days with daily mean temperature above 5 °C) for 2021 in Uppsala, Sweden based on the climate data from the Swedish Meteorological and Hydrological Institute, Norrköping, Sweden.

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Table S1. Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the field. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$.

Source of Variation	**DF**	**Mean Square**					
		DTS	**DTB**	**DTH**	**FW**	**DW**	**PH**
Block	3	9566.1**	54169.1**	82409.2**	1033059.0**	399403.0**	25279.9**
Species	2	2429.6*	16319.5**	16604.1**	1764388.0**	277685.0**	44013.9**
Accession (Species)	236	1698.5**	2200.5**	1701.2**	54460.4**	8847.4**	830.7**
Genotype (Accession(Species))&Random	671	930.2	1240.5	957.3	38146.5	5405.4	359.6
RSquare Adj	-	0.4	0.5	0.5	0.3	0.4	0.7

Table S2. Unbalanced-nested analysis of variance (ANOVA) for the components block, species, accession and genotype for the studied traits days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in the greenhouse. DF: degrees of freedom, ns: non-significant, *: $p < 0.05$, **: $p < 0.01$.

Source of Variation	**DF**	**Mean Square**					
		DTS	**DTB**	**DTH**	**FW**	**DM**	**PH**
Block	1	19.2 ^{ns}	17.7 ^{ns}	23.3^{ns}	420.4^{ns}	44.6 ^{ns}	206.9 ^{ns}
Species	2	2297.8**	872.8**	1531.2**	82984.3**	13030.8**	53878.2**
Accession (Species)	210	445.1**	673.0**	1085.0**	7157.8**	1248.3**	2513.2**
Genotype (Accession(Species))&Random	359	153.0	104.8	144.6	4164.1	782.6	1438.4
RSquare Adj	-	0.8	0.9	0.9	0.5	0.5	0.6

Table S3. Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the field. GDD: Growing degree days. Mean values that do not share the same letter are significantly different among species according to Tukey HSD, $p < 0.05$.

Species	**DTS (GDD)**	**DTB (GDD)**	**DTH (GDD)**	**FW (g per plant)**	**DW (g per plant)**	**PH (cm)**
P. pratense	185.1±0.5^{ab}	254.4±0.6^{a}	304.4±0.5^{a}	400.2±3.4^{a}	158.9±1.3^{a}	82.4±0.3^{a}
P. nodosum	178.9±2.2^{b}	240.9±2.5^{b}	290.6±2.1^{b}	254.5±14.5^{b}	100.6±5.5^{b}	60.4±1.3^{b}
P. alpinum	187.2±6.5^{a}	237.1±7.1^{b}	291.2±6.4^{b}	186.7±43.6^{b}	72.2±16.4^{b}	44.1±3.9^{c}

Table S4. Mean and SD of days to stem elongation (DTS), days to booting (DTB), days to heading (DTH), fresh weight (FW), dry weight (DW) and plant height (PH) in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse. DAE: days after coleoptile emergence. Mean values that do not share the same letter are significantly different among species accessions according to Tukey HSD, $p < 0.05$.

Species	**DTS (DAE)**	**DTB (DAE)**	**DTH (DAE)**	**FW (g per plant)**	**DW (g per plant)**	**PH (cm)**
P. pratense	165.9±0.5^{a}	178.0±0.6^{a}	192.7±0.7^{a}	97.2±2.0^{a}	36.1±0.9^{b}	96.1±1.6^{a}
P. nodosum	158.2±1.4^{b}	170.4±1.6^{b}	182.4±1.9^{b}	103.4±6.8^{a}	43.8±2.9^{a}	105.1±4.0^{a}
P. alpinum	162.9 ±4.8^{ab}	178.0±4.0^{a}	191.0±4.7^{a}	18.8±11.6^{b}	7.5±5.0^{c}	36.2±6.8^{b}

Table S5. Number of accessions of each group in each cluster in *P. pratense, P. nodosum* and *P. alpinum* in the field.

Species	**Cluster**	**Total number of accessions**	**Number of accessions in each group**				
			Cultivar	**Breeding line**	**Landrace**	**Semi-wild**	**Wild**
P. pratense	I	19	9	-	3	1	6
	II	46	9	2	15	4	16
	III	28	7	1	9	4	7
	IV	43	17	4	6	7	9
	V	43	7	-	11	11	14
	VI	33	7	3	11	3	9
P. nodosum	I	5	3	-	-	-	2
	II	8	4	-	-	-	4
	III	1	-	-	-	-	1
P. alpinum	I	5	-	-	-	-	5
	II	3	-	-	-	-	3
	III	4	-	-	-	-	4

Table S6. Number of accessions of each group in each cluster in *P. pratense*, *P. nodosum* and *P. alpinum* in the greenhouse.

Species	Cluster	Total number of accessions	Number of accessions in each group				
			Cultivar	Breeding line	Landrace	Semi-wild	Wild
P. pratense	I	55	18	-	18	3	16
	II	37	8	4	9	8	8
	III	12	2	4	-	1	5
	IV	17	1	1	5	2	8
	V	38	20	2	4	2	10
P. nodosum	I	3	3	-	-	-	-
	II	4	1	-	-	-	3
	III	1	1	-	-	-	-
	IV	5	1	-	-	-	4
P. alpinum	I	3	-	-	-	-	3
	II	1	-	-	-	-	1
	III	1	-	-	-	-	1

Table S7. Information of the studied accessions of *P. pratense* , *P. nodosum* and *P. alpinum* from the genebank NordGen, Alnarp, Sweden.

Species	Accession number	Country of origin	Type	Latitude	Longitude
Phleum pratense	NGB10779	Norway	Wild	59.025	9.658
Phleum pratense	NGB10784	Norway	Wild	60.308	10.608
Phleum pratense	NGB1096	Finland	Landrace	64.608	25.042
Phleum pratense	NGB1097	Finland	Landrace	63.942	25.758
Phleum pratense	NGB1098	Finland	Landrace	63.108	21.875
Phleum pratense	NGB1102	Finland	Landrace	64.508	26.458
Phleum pratense	NGB1104	Finland	Landrace	63.958	24.792
Phleum pratense	NGB1111	Finland	Landrace	64.408	26.325
Phleum pratense	NGB1114	Finland	Landrace	64.242	25.058
Phleum pratense	NGB1115	Finland	Landrace	64.342	25.125
Phleum pratense	NGB11156	Norway	Cultivar		
Phleum pratense	NGB1117	Finland	Landrace	66.058	24.742
Phleum pratense	NGB1120	Finland	Landrace	63.442	27.992
Phleum pratense	NGB1121	Finland	Landrace	62.842	28.625
Phleum pratense	NGB1122	Finland	Landrace	62.508	28.242
Phleum pratense	NGB1127	Finland	Landrace	62.825	26.358
Phleum pratense	NGB11427	Sweden	Cultivar		
Phleum pratense	NGB11428	Sweden	Cultivar		
Phleum pratense	NGB11429	Sweden	Cultivar		
Phleum pratense	NGB11430	Sweden	Cultivar		
Phleum pratense	NGB11431	Sweden	Cultivar		
Phleum pratense	NGB11677	Denmark	Cultivar		
Phleum pratense	NGB132	Finland	Landrace	63.475	29.308
Phleum pratense	NGB13218	Sweden	Cultivar		
Phleum pratense	NGB13219	Sweden	Cultivar		
Phleum pratense	NGB13220	Sweden	Cultivar		
Phleum pratense	NGB13221	Sweden	Cultivar		
Phleum pratense	NGB13222	Sweden	Cultivar		
Phleum pratense	NGB13223	Sweden	Cultivar		
Phleum pratense	NGB13224	Sweden	Cultivar		
Phleum pratense	NGB13226	Sweden	Cultivar		
Phleum pratense	NGB13227	Finland	Cultivar		
Phleum pratense	NGB13228	Sweden	Cultivar		
Phleum pratense	NGB1324	Sweden	Wild	64.158	17.675
Phleum pratense	NGB1331	Sweden	Wild	63.758	18.992
Phleum pratense	NGB135	Finland	Landrace	61.208	28.492
Phleum pratense	NGB13524	Sweden	Cultivar		
Phleum pratense	NGB138	Finland	Landrace		
Phleum pratense	NGB13884	Sweden	Breeding line		
Phleum pratense	NGB13894	Finland	Cultivar		
Phleum pratense	NGB13976	Norway	Landrace		
Phleum pratense	NGB14008	Norway	Landrace		
Phleum pratense	NGB14009	Norway	Landrace		
Phleum pratense	NGB14174	Sweden	Cultivar		
Phleum pratense	NGB14175	Sweden	Cultivar		
Phleum pratense	NGB143	Finland	Landrace	63.542	28.908
Phleum pratense	NGB14328	Finland	Landrace	63.772	24.234
Phleum pratense	NGB14336	Finland	Landrace	63.903	25.001
Phleum pratense	NGB14375	Finland	Cultivar	61.234	26.185
Phleum pratense	NGB14378	Finland	Cultivar	63.812	26.424
Phleum pratense	NGB14416	Finland	Wild	60.508	24.221
Phleum pratense	NGB14417	Finland	Wild	60.101	24.627
Phleum pratense	NGB14467	Finland	Landrace	63.650	24.434
Phleum pratense	NGB14469	Finland	Landrace	61.903	26.042
Phleum pratense	NGB14470	Finland	Landrace	64.101	24.782
Phleum pratense	NGB145	Finland	Landrace	62.892	29.392
Phleum pratense	NGB14618	Finland	Landrace	63.592	24.058
Phleum pratense	NGB147	Finland	Landrace	63.175	29.208
Phleum pratense	NGB15094	Denmark	Cultivar		
Phleum pratense	NGB151	Finland	Landrace	62.708	28.708
Phleum pratense	NGB1526	Sweden	Wild	57.575	18.558
Phleum pratense	NGB153	Finland	Landrace		
Phleum pratense	NGB1537	Sweden	Wild	57.492	18.175
Phleum pratense	NGB15461	Denmark	Wild	56.196	8.826

Species	**Accession number**	**Country of origin**	**Type**	**Latitude**	**Longitude**
Phleum pratense	NGB15477	Denmark	Wild	56.481	8.815
Phleum pratense	NGB15487	Denmark	Wild	56.187	8.230
Phleum pratense	NGB15502	Norway	Cultivar	60.292	12.075
Phleum pratense	NGB15556	Norway	Semi-wild	61.208	10.158
Phleum pratense	NGB156	Finland	Landrace	60.692	27.475
Phleum pratense	NGB157	Finland	Landrace	63.158	30.108
Phleum pratense	NGB158	Finland	Landrace	62.158	30.292
Phleum pratense	NGB159	Finland	Landrace	61.942	29.958
Phleum pratense	NGB16264	Sweden	Wild	57.911	14.890
Phleum pratense	NGB16268	Sweden	Wild	57.853	14.749
Phleum pratense	NGB16271	Sweden	Wild	57.163	14.999
Phleum pratense	NGB1671	Denmark	Cultivar		
Phleum pratense	NGB1672	Denmark	Cultivar		
Phleum pratense	NGB1673	Denmark	Cultivar		
Phleum pratense	NGB1674	Denmark	Cultivar		
Phleum pratense	NGB1675	Denmark	Cultivar		
Phleum pratense	NGB1676	Denmark	Cultivar		
Phleum pratense	NGB1677	Denmark	Cultivar		
Phleum pratense	NGB16790	Finland	Landrace		
Phleum pratense	NGB17194	Norway	Wild	70.492	27.058
Phleum pratense	NGB17200	Norway	Wild	69.458	25.508
Phleum pratense	NGB17202	Norway	Wild	70.042	24.942
Phleum pratense	NGB17203	Norway	Wild	69.758	21.042
Phleum pratense	NGB18354	Finland	Landrace	65.664	27.939
Phleum pratense	NGB18357	Finland	Landrace	66.253	27.916
Phleum pratense	NGB18367	Finland	Landrace	64.188	28.230
Phleum pratense	NGB18462	Sweden	Semi-wild	62.325	16.692
Phleum pratense	NGB18489	Sweden	Semi-wild	62.075	14.842
Phleum pratense	NGB18498	Sweden	Semi-wild	62.542	14.208
Phleum pratense	NGB18511	Sweden	Semi-wild	63.308	12.492
Phleum pratense	NGB18566	Sweden	Semi-wild	63.158	17.742
Phleum pratense	NGB20365	Finland	Landrace	64.059	26.924
Phleum pratense	NGB20369	Finland	Landrace	64.018	27.886
Phleum pratense	NGB20791	Iceland	Semi-wild	64.238	19.321
Phleum pratense	NGB20792	Iceland	Semi-wild	64.271	19.771
Phleum pratense	NGB20793	Iceland	Semi-wild	64.090	19.686
Phleum pratense	NGB20794	Iceland	Semi-wild	63.895	19.380
Phleum pratense	NGB20795	Iceland	Semi-wild	63.856	19.468
Phleum pratense	NGB20796	Iceland	Semi-wild	63.672	19.625
Phleum pratense	NGB20798	Iceland	Semi-wild	63.945	20.815
Phleum pratense	NGB20799	Iceland	Semi-wild	64.566	20.259
Phleum pratense	NGB20801	Iceland	Semi-wild	64.664	20.787
Phleum pratense	NGB20802	Iceland	Semi-wild	64.696	20.487
Phleum pratense	NGB20803	Iceland	Semi-wild	65.129	20.919
Phleum pratense	NGB20804	Iceland	Semi-wild	65.728	19.792
Phleum pratense	NGB20805	Iceland	Semi-wild	65.708	18.379
Phleum pratense	NGB20806	Iceland	Semi-wild	65.538	18.559
Phleum pratense	NGB20807	Iceland	Semi-wild	66.121	15.613
Phleum pratense	NGB20808	Iceland	Semi-wild	65.820	16.458
Phleum pratense	NGB20809	Iceland	Semi-wild	65.603	16.012
Phleum pratense	NGB20810	Iceland	Semi-wild	65.678	17.597
Phleum pratense	NGB21082	Norway	Semi-wild	59.125	10.208
Phleum pratense	NGB2166	Norway	Landrace		
Phleum pratense	NGB2167	Norway	Breeding line		
Phleum pratense	NGB2169	Norway	Cultivar		
Phleum pratense	NGB2180	Norway	Cultivar		
Phleum pratense	NGB2181	Norway	Cultivar		
Phleum pratense	NGB22615	Denmark	Wild	55.272	14.756
Phleum pratense	NGB2355	Sweden	Cultivar		
Phleum pratense	NGB2356	Sweden	Cultivar		
Phleum pratense	NGB24047	Norway	Breeding line	61.120	9.060
Phleum pratense	NGB24050	Norway	Breeding line	61.120	9.060
Phleum pratense	NGB24053	Norway	Breeding line	59.930	11.040
Phleum pratense	NGB24056	Norway	Breeding line	67.280	14.450
Phleum pratense	NGB24061	Norway	Breeding line	61.290	5.040
Phleum pratense	NGB24064	Norway	Breeding line	64.230	12.290
Phleum pratense	NGB24163	Finland	Landrace	64.842	25.975
Phleum pratense	NGB24165	Finland	Landrace	62.942	28.692

Species	**Accession number**	**Country of origin**	**Type**	**Latitude**	**Longitude**
Phleum pratense	NGB24169	Russian Federation	Wild	60.758	33.442
Phleum pratense	NGB24184	Russian Federation	Semi-wild	61.575	30.458
Phleum pratense	NGB24185	Russian Federation	Semi-wild	61.692	30.275
Phleum pratense	NGB24192	Russian Federation	Semi-wild	61.725	30.392
Phleum pratense	NGB24205	Russian Federation	Semi-wild	61.858	31.425
Phleum pratense	NGB24214	Russian Federation	Wild	62.075	32.775
Phleum pratense	NGB24215	Russian Federation	Wild	61.825	34.075
Phleum pratense	NGB24221	Russian Federation	Semi-wild	62.208	34.208
Phleum pratense	NGB24235	Russian Federation	Wild	62.342	33.792
Phleum pratense	NGB24247	Russian Federation	Wild	62.975	34.075
Phleum pratense	NGB2528	Sweden	Wild	58.975	11.825
Phleum pratense	NGB2530	Sweden	Wild	59.008	12.075
Phleum pratense	NGB2532	Sweden	Wild	59.058	12.225
Phleum pratense	NGB2597	Sweden	Breeding line		
Phleum pratense	NGB2755	Sweden	Cultivar		
Phleum pratense	NGB2756	Sweden	Cultivar		
Phleum pratense	NGB2757	Sweden	Cultivar		
Phleum pratense	NGB2776	Finland	Landrace	60.725	26.458
Phleum pratense	NGB2777	Finland	Landrace	63.042	23.625
Phleum pratense	NGB2795	Finland	Landrace	62.958	21.775
Phleum pratense	NGB2796	Finland	Landrace	62.758	21.475
Phleum pratense	NGB2798	Finland	Landrace	62.908	21.725
Phleum pratense	NGB2799	Finland	Landrace	62.725	21.192
Phleum pratense	NGB2816	Finland	Landrace	63.308	22.825
Phleum pratense	NGB2817	Finland	Landrace	63.358	23.458
Phleum pratense	NGB2823	Finland	Landrace	62.625	22.925
Phleum pratense	NGB2836	Finland	Landrace	62.392	26.242
Phleum pratense	NGB2850	Finland	Landrace	63.458	25.358
Phleum pratense	NGB2933	Norway	Wild	59.377	9.967
Phleum pratense	NGB4053	Denmark	Wild	57.242	9.775
Phleum pratense	NGB4066	Finland	Cultivar		
Phleum pratense	NGB4067	Finland	Cultivar		
Phleum pratense	NGB4070	Finland	Cultivar		
Phleum pratense	NGB4122	Denmark	Cultivar		
Phleum pratense	NGB4141	Iceland	Cultivar		
Phleum pratense	NGB4349	Sweden	Wild	55.525	13.908
Phleum pratense	NGB4351	Sweden	Wild	56.325	12.525
Phleum pratense	NGB4509	Norway	Wild	60.900	11.527
Phleum pratense	NGB4511	Norway	Wild	61.842	9.492
Phleum pratense	NGB4520	Norway	Wild	58.408	8.642
Phleum pratense	NGB4534	Norway	Breeding line		
Phleum pratense	NGB4548	Denmark	Wild	55.358	8.692
Phleum pratense	NGB6779	Sweden	Wild	55.975	13.392
Phleum pratense	NGB7154	Norway	Wild	68.658	14.458
Phleum pratense	NGB7159	Norway	Wild	66.408	14.275
Phleum pratense	NGB717	Finland	Wild	66.242	23.758
Phleum pratense	NGB719	Sweden	Wild	66.508	23.642
Phleum pratense	NGB722	Sweden	Wild	66.692	19.758
Phleum pratense	NGB728	Sweden	Wild	65.542	19.708
Phleum pratense	NGB729	Sweden	Wild	64.992	18.508
Phleum pratense	NGB732	Sweden	Wild	64.642	20.908
Phleum pratense	NGB733	Sweden	Landrace	65.675	21.875
Phleum pratense	NGB739	Finland	Wild	67.058	23.758
Phleum pratense	NGB742	Finland	Wild	67.875	24.992
Phleum pratense	NGB743	Finland	Wild	67.858	24.008
Phleum pratense	NGB744	Finland	Wild	68.675	27.692
Phleum pratense	NGB747	Finland	Wild	69.842	26.325
Phleum pratense	NGB748	Finland	Wild	69.925	27.058
Phleum pratense	NGB752	Finland	Wild	67.342	26.875
Phleum pratense	NGB756	Finland	Wild	66.492	27.358
Phleum pratense	NGB7566	Norway	Wild	62.592	9.692
Phleum pratense	NGB7572	Norway	Wild	63.992	11.125
Phleum pratense	NGB7577	Norway	Wild	63.375	9.942
Phleum pratense	NGB758	Finland	Wild	66.792	27.475
Phleum pratense	NGB759	Finland	Wild	66.325	27.092
Phleum pratense	NGB763	Finland	Wild	66.575	26.042
Phleum pratense	NGB7707	Norway	Wild	67.958	15.225
Phleum pratense	NGB7710	Norway	Wild	67.142	15.025

Species	**Accession number**	**Country of origin**	**Type**	**Latitude**	**Longitude**
Phleum pratense	NGB7717	Norway	Wild	65.608	14.008
Phleum pratense	NGB7785	Norway	Cultivar		
Phleum pratense	NGB8363	Finland	Cultivar		
Phleum pratense	NGB8381	Denmark	Cultivar		
Phleum pratense	NGB8418	Finland	Cultivar		
Phleum pratense	NGB9285	Finland	Cultivar		
Phleum pratense	NGB9290	Finland	Cultivar		
Phleum pratense	NGB9301	United Kingdom	Cultivar		
Phleum pratense	NGB9302	Germany	Cultivar		
Phleum pratense	NGB9924	Netherlands	Cultivar		
Phleum pratense	Switch	Sweden	Cultivar		
Phleum pratense	Tryggve	Sweden	Cultivar		
Phleum nodosum	NGB13229	Sweden	Cultivar		
Phleum nodosum	NGB13230	Denmark	Cultivar		
Phleum nodosum	NGB13231	Sweden	Cultivar		
Phleum nodosum	NGB14477	Finland	Wild	60.154	23.088
Phleum nodosum	NGB1678	Denmark	Cultivar		
Phleum nodosum	NGB16972	Sweden	Wild	55.812	13.042
Phleum nodosum	NGB17008	Sweden	Wild	58.537	11.282
Phleum nodosum	NGB17010	Sweden	Wild	58.496	11.301
Phleum nodosum	NGB17012	Sweden	Wild	56.370	12.824
Phleum nodosum	NGB1724	Denmark	Cultivar		
Phleum nodosum	NGB1725	Denmark	Cultivar		
Phleum nodosum	NGB2378	Sweden	Cultivar		
Phleum nodosum	NGB4352	Sweden	Wild	55.392	14.158
Phleum nodosum	NGB4364	Sweden	Wild	56.325	12.525
Phleum alpinum	NGB1335	Sweden	Wild	64.742	17.392
Phleum alpinum	NGB1336	Sweden	Wild	64.692	17.358
Phleum alpinum	NGB1337	Sweden	Wild	64.408	16.592
Phleum alpinum	NGB1338	Sweden	Wild	64.458	17.042
Phleum alpinum	NGB1340	Sweden	Wild	64.425	18.058
Phleum alpinum	NGB1341	Sweden	Wild	64.225	17.492
Phleum alpinum	NGB18474	Sweden	Wild	62.325	13.575
Phleum alpinum	NGB31885	Norway	Wild	60.547	8.185
Phleum alpinum	NGB31886	Norway	Wild	59.843	7.156
Phleum alpinum	NGB31887	Norway	Wild	62.300	9.617
Phleum alpinum	NGB770	Sweden	Wild	67.625	21.125
Phleum alpinum	NGB772	Sweden	Wild	68.208	22.775

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Article

Response to Waterlogging Stress in Wild and Domesticated Accessions of Timothy (*Phleum pratense*) and Its Relatives *P. alpinum* and *P. nodosum*

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Abstract: Timothy (*Phleum pratense*) is a cool-season perennial forage grass widely grown for silage and hay production in northern regions. Climate change scenarios predict an increase in extreme weather events with fluctuating periods of high rainfall, requiring new varieties adapted to waterlogging (WL). Wild accessions could serve as germplasm for breeding, and we evaluated the responses of 11 wild and 8 domesticated accessions of timothy, *P. nodosum* and *P. alpinum* from different locations in northern Europe. Young plants at tillering stage were exposed to WL for 21 days in a greenhouse, and responses in growth allocation and root anatomy were studied. All accessions produced adventitious roots and changed allocation of growth between shoot and root as a response to WL, but the magnitude of these responses varied among species and among accessions. *P. pratense* responded less in these traits in response to WL than the other two species. The ability to form aerenchyma in the root cortex in response to WL was found for all species and also varied among species and among accessions, with the highest induction in *P. pratense*. Interestingly, some accessions were able to maintain and even increase root growth, producing more leaves and tillers, while others showed a reduction in the root system. Shoot dry weight (SDW) was not significantly affected by WL, but some accessions showed different and significant responses in the rate of production of leaves and tillers. Overall correlations between SDW and aerenchyma and between SDW and adventitious root formation were found. This study identified two wild timothy accessions and one wild *P. nodosum* accession based on shoot and root system growth, aerenchyma formation and having a root anatomy considered to be favorable for WL tolerance. These accessions are interesting genetic resources and candidates for development of climate-resilient timothy varieties.

Keywords: accessions; aerenchyma; anatomy; forage grass; perennial; root

check for updates

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1. Introduction

Phleum pratense L. is a perennial grass growing at higher latitudes in Northern Europe, East Asia and North America [1]. Its domesticated form is known as timothy and is widely cultivated for grazing and production of hay and silage. It is highly palatable for cattle and horses, having a high nutritive value and digestibility [2,3]. In Scandinavia, timothy is the dominant forage grass due to its winter hardiness, rapid growth and high biomass production. It is grown in pure stands or in mixtures with other perennial forage grasses such as *Festuca pratensis*, as well as perennial legumes such as red and white clover. Forage crops are cultivated on about 40% (>1 million hectares) of the agricultural land in Sweden, which is about the same size as the area used for the cultivation of annual cereal crops [4].

Timothy is an outcrossing species. High polymorphism and large genetic diversity have been found within cultivars [5]. Although timothy is perennial, it is short-lived. It has a shallow root system, and new tillers are formed from buds in the crown and swollen stem bases. These superficially lying meristems are easily damaged by cattle tramping and grazing, particularly in wet soils. Timothy is cultivated in both mesic and drier meadows but is considered to be sensitive to waterlogged soils, drought and heat [6–8].

The production of timothy is challenged by the ongoing climate change. Fluctuations in precipitation intensity with longer extreme wet and dry periods are expected to be more frequent [9–11], and increased rainfall and snowmelt in winter will cause stressful conditions for overwintering perennial plants [12]. Intense precipitation increases the risk of water saturation in the soil, leading to oxygen (O_2) deficiency. The deficiency of O_2 limits root and microorganism respiration and nutrient cycling, leading to reduced uptake of mineral nutrients by the plant roots [13–15], which negatively influences root and shoot growth [16]. Due to reduced N uptake and photosynthetic capacity and degradation of chlorophyll, responses by plants to waterlogging (WL) are also associated with leaf chlorosis and senescence [17,18]. Some plants have the ability to form extended gas-filled cavities in the shoots, called lacunae, and in the roots, called aerenchyma, under WL. These anatomical structures facilitate the diffusion of oxygen to organs that suffer from O_2 deficiency, as well as transport CO_2 and toxic volatiles from the roots [19–22].

Aerenchyma is formed specifically in the root cortex, but not in other root tissues such as the epidermis, hypodermis, endodermis or stele. It is formed through programmed cell death and the degradation of cortical cells, particularly in adventitious roots. The formation of adventitious roots increases the exchange of gases and absorption of nutrients and is also an adaptation to WL stress [23]. Certain plant species form aerenchyma as part of their normal development in drained soil [24], but its formation is particularly promoted when plants are exposed to flooding conditions [25–27]. A greater and faster capacity to form aerenchyma can enhance flooding tolerance [28,29]. Other anatomical root traits, such as a narrow stele and a large root diameter, may also contribute to improving WL tolerance [30,31]. The stele may be the first region at which hypoxia is sensed [22,32]. The stele of thin roots is the pathway of water movement into the central vascular tissues. A narrower stele and larger cortex have been found to be associated with an increased development of aerenchyma [31].

Tolerant plants can develop additional morphological changes, such as barriers to radial O_2 loss (ROL), created by suberization and/or lignification of the cell walls in the outer cell layers of the roots [33–37]. Physiological adaptations, such as changes in photosynthesis and biochemical mechanisms involving endogenous hormone signals, play a central role in WL tolerance [38–41]. Knowledge about adaptations and mechanisms in WL tolerance in monocots is mostly known from studies of annual cereals and less from perennial forage grasses. For timothy, waterlogging tolerance has been addressed in relation to hardening and freezing tolerance [42,43], but there are no studies to date on the phenotypic diversity among accessions and possible tolerance mechanisms.

Although changes in crop and soil management may help to reduce yield losses caused by WL, the development of tolerant crops is urgently needed to achieve a highly stable crop production and sustainable agriculture. Wild relatives of crops make up a potential gene pool of traits and genes that are important for the development of climate-resilient cultivars. While crops are shaped by human selection to produce high yields in homogenous agricultural environments, their wild relatives are adapted to various habitats and geographical regions across the species range. Possible fluctuations in the intensity and direction of natural selection over time and across habitats and geographical sites have shaped the genetic structure of wild populations, and large genetic and phenotypic diversity may be found within wild crop relatives [44–47]. Moreover, intense directional selection during the domestication process has reduced the genetic diversity in domesticated crops, and traits and genes have been left behind in the wild relatives [48,49]. We hypothesize that there is variation in response to WL among wild and among domesticated accessions of *P. pratense* and related species, and that tolerant accessions can be found in wild populations and serve as genetic resources for improving WL tolerance in timothy.

P. pratense and its related species turf timothy, *P. nodosum* (syn. P. pratense L. subsp. bertolonii (DC.) Bornm. and P. bertolonii (DC.) Bornm.), and alpine timothy, *P. alpinum* L., commonly grow in the Nordic countries. Wild *P. pratense* is adapted to meadows and human-impacted and disturbed soils at low elevations throughout the Nordic countries. *P. alpinum* is commonly found at high elevations on meadows, riverbanks and roadsides, and in birch forests in Iceland, Norway, northern Sweden and northern Finland. *P. nodosum* has a more restricted and southern distribution than *P. alpinum* and is found in human-impacted habitats at low elevations. These species differ in ploidy level. *P. pratense* is hexaploid (2n = 6x = 42), *P. nodosum* is diploid (2n = 2x = 14) and *P. alpinum* L. is tetraploid (2n = 4x = 28). The phylogenetic relationships between these three species and other *Phleum* species are not yet fully understood. However, similarities have been found between two of the genomes of *P. pratense* and the diploid *P. nodosum* genome. Genetic similarities have also been found between *P. pratense* and *P. alpinum* [50,51]. *P. nodosum* and *P. alpinum* may therefore have had a direct or indirect role in the evolution of *P. pratense*, and it is therefore relevant to study the diversity of phenotypic responses among accessions of these related species.

We investigated the response to WL in domesticated and wild accessions from different habitats and latitudes. To identify tolerant accessions, we compared the performance of the same accessions in WL and non-waterlogging (NWL) cultivation by studying their shoot and root growth, their root anatomy and their ability to form aerenchyma.

2. Results

2.1. Plant Morphology and Growth among Phleum Species in NWL

An overall comparison of the three species in NWL showed that *P. pratense* and *P. nodosum* had higher growth than *P. alpinum. P. pratense* had a similar shoot dry weight (SDW) to *P. nodosum* but a significantly higher SDW than *P. alpinum* at the end of the experiment ($p < 0.05$, ANOVA, Table 1). The morphology/growth habit differed significantly among the species since, for both tiller number (TN) and leaf number (LN) per plant, *P. nodosum* showed the highest values, followed by *P. pratense* and *P. alpinum* (Figure S1). To further investigate the morphology and growth habit, several ratios between SDW, TN and LN as well as the exponential growth rate constants for the relative growth rates of TN and LN (RGR-TN, RGR-LN) were calculated.

Table 1. Shoot traits in *P. pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) treatment. Values are means of plants within each species and standard error (±SE). Differences among species in NWL are indicated by different letters (a, b or c) when significantly different according to ANOVA and Tukey's method, $p < 0.05$. No significant differences were found between treatments within each species ($p < 0.05$, *t*-test). Abbreviations of traits: shoot dry weight (SDW), tiller number (TN), leaf number (LN), ratio of the tiller number to shoot dry weight (TN:SDW), ratio of the leaf number to shoot dry weight (LN:SDW), leaf number per tiller (LN:TN), growth rates of the tiller number (RGR-TN) and leaf number (RGR-LN).

Species	***P. pratense***		***P. nodosum***		***P. alpinum***	
Traits	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**
SDW (g)	7.88 ± 0.38^{a}	8.07 ± 0.37	6.23 ± 0.62^{a}	6.02 ± 0.86	0.85 ± 0.17^{b}	0.82 ± 0.12
TN (#plant^{-1})	23.34 ± 0.99^{a}	21.05 ± 0.89	30.79 ± 3.05^{b}	29.21 ± 2.32	5.09 ± 0.79^{c}	5.18 ± 0.42
LN (#plant^{-1})	138.89 ± 7.23^{a}	129.14 ± 6.01	227.16 ± 21.12^{b}	185.37 ± 13.5	24.0 ± 2.95^{c}	23.27 ± 2.72
TN:SDW (#plant × g^{-1})	3.24 ± 0.21^{b}	2.76 ± 0.13	5.40 ± 0.44^{a}	6.31 ± 0.79	6.89 ± 0.69^{ab}	10.02 ± 3.11
LN:SDW (#plant × g^{-1})	18.26 ± 0.84^{b}	16.57 ± 0.65	41.47 ± 4.59^{a}	39.47 ± 4.03	33.93 ± 4.48^{ab}	46.92 ± 17.38
LN:TN	6.06 ± 0.25^{b}	6.20 ± 0.19	7.57 ± 0.39^{a}	6.7 ± 0.40	4.9 ± 0.40^{b}	4.42 ± 0.30
RGR-TN (#day $^{-1}$)	0.052 ± 0.002^{b}	0.050 ± 0.002	0.061 ± 0.002^{a}	0.062 ± 0.004	0.036 ± 0.006^{c}	0.027 ± 0.004
RGR-LN (#day $^{-1}$)	0.072 ± 0.002^{b}	0.072 ± 0.002	0.092 ± 0.003^{a}	0.087 ± 0.003	0.047 ± 0.004^{c}	0.041 ± 0.004

TN:SDW and LN:SDW showed that the values for *P. nodosum* were 2-fold and 3-fold higher than those of *P. pratense*, respectively. *P. nodosum* formed a higher number of leaves per tiller (LN:TN) than the other species.

RGR-TN and RGR-LN were highest in *P. nodosum* and lowest in *P. alpinum* in NWL ($p < 0.05$, ANOVA, Table 1). *P. alpinum* had the lowest TN and LN at the start of the experiment, and it also showed the smallest increase in numbers of tillers and leaves in NWL during the experiment (ΔTN and ΔLN), as well as the increase expressed as percentage (%TN and %LN) (Figure S2). Plant growth allocation to root biomass was similar in *P. pratense* and *P. nodosum*, but higher in *P. alpinum* (Figure 1B). The species also differed in root anatomy, where *P. pratense* had a significantly higher root cross section area (RA) and ratio of area of cortex including epidermisto RA (CEA:RA), and lower ratio of stele to RA (SA:RA) than *P. nodosum* and *P. alpinum* (Figure 1D–I). Also, *P. pratense* had higher ratios of aerenchyma area to RA (AA:RA), AA:CEA and CEA:SA than *P. nodosum*, where most of the accessions did not form aerenchyma in NWL (Table S5).

2.2. Effects of WL on Growth Traits

2.2.1. Comparisons at the Species Level

Comparisons at the species level showed that WL resulted in lower TN:SDW for *P. pratense* but not for the other two species (Table 1). None of the other shoot traits were significantly different in response to WL for any species. The percentage of growth allocation to roots (%RDW) decreased for all species in WL (Figure 1B), *P. alpinum* being the most affected (Table S6). Interestingly, the proportion of root biomass in the upper 5 cm part of the root system (5RDW:RDW) was ca. 30% in NWL for all species, while in WL, it was significantly higher at 45%, 65% and 71% for *P. pratense*, *P. nodosum* and *P. alpinum*, respectively ($p < 0.05$, ANOVA, Figure 1C). *P. pratense* responded significantly less to WL in the trait 5RDW:RDW compared to *P. nodosum* and *P. alpinum* (Table S6).

None of the accessions in any species showed chlorotic leaves in WL. Generative tillers were produced in both NWL and WL conditions (61% and 74% of all plants, respectively) and by all accessions except one for *P. pratense* (Table S3). All accessions of *P. nodosum* formed generative tillers in NWL and WL (75% and 80% of all plants, respectively), while none of the *P. alpinum* accessions had generative tillers.

2.2.2. *P. pratense* Accessions

There was a large variation among accessions for SDW, ΔTN, ΔLN, RGR-TN, RGR-LN, TN and LN, as well as the ratios TN:SDW, LN:SDW and LN:TN, with 1.5- to 3-fold differences between the highest and lowest values in all traits ($p < 0.05$, ANOVA, Tables 2, S2 and S3) for both treatments. TN:SDW was the only shoot trait that showed a significant difference in response to WL ($p < 0.05$, ANOVA, Table 2).

The variation among accessions was not related to the type of group of accession (wild and domesticated) for the shoot traits ($p > 0.05$, ANOVA, Table 2). Between the two groups of accessions, there was a significant difference in response to WL in TN:SDW and LN:SDW ($p < 0.05$, ANOVA, Table 2). The accession W1 differed from the other *P. pratense* accessions by having a significantly higher number of leaves in WL, seen as LN:TN, as well as higher RGR-LN in WL ($p < 0.05$, Tables S2 and S3). Also, accession D1 produced significantly fewer leaves in WL.

From a production perspective, the shoot biomass based on leaf and tiller numbers is crucial. Different growth and morphology of accessions and their responses to WL taken together could highlight possible traits relevant for sustainable yields. Therefore, to further analyze responses by all accessions of *P. pratense* and *P. nodosum*, we performed a principal component analysis (PCA) on the difference between the WL and NWL of all measured and calculated shoot traits (Figure 2). This showed that accessions grouped based on the shoot dry weight, tiller and leaf production. Accessions W1, W6 and D3 can be suggested to be more tolerant to waterlogging based on the correlation for the shoot traits stimulated by waterlogging. In contrast, the accessions W5 and D1 are more affected by WL.

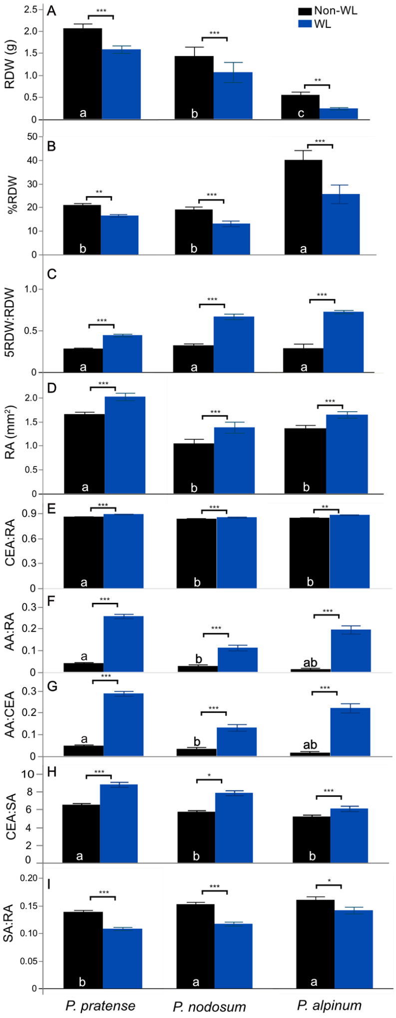

Figure 1. Root traits in *P. pratense*, *P. nodosum* and *P. alpinum* at 21 days in waterlogging treatment. (**A**) Root dry weight (RDW). (**B**) Percent root weight of total plant dry weight (%RDW). (**C**) Ratio of dry weight of the upper 5 cm of the root system and the dry weight of the total root (5RDW:RDW). The root anatomy traits: (**D**) root cross section area (RA). (**E**) Ratio of the cortex area to root cross section area (CEA:RA). (**F**) Ratio of aerenchyma to root cross section area (AA:RA). (**G**) Ratio of aerenchyma to cortex area (AA:CEA). (**H**) Ratio of the cortex to stele (CEA:SA). (**I**) Ratio of the stele area to root cross section area (SA:RA). Values are means and error bars are standard error (± SE). Significant differences between treatments within each species are represented by * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ according to ANOVA. Differences among species in NWL are indicated by different letters when significantly different according to ANOVA and Tukey's method, $p < 0.05$.

Table 2. Two-way ANOVA results showing the effect of accession, treatment and their interaction in the different *Phleum* species, as well as the effect of type of accession (wild (W) or domesticated (D)), treatment and their interaction in *P. pratense* in non-waterlogging (NWL) and waterlogging (WL) conditions. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Abbreviations: shoot dry weight (SDW), tiller number (TN), leaf number (LN), ratio of the tiller number to shoot dry weight (TN:SDW), ratio of the leaf number to shoot dry weight (LN:SDW), leaf number per tiller (LN:TN), tiller number produced during the treatment (ΔTN), leaf number produced during the treatment (ΔLN), growth rates of the tiller number (RGR-TN) and the leaf number (RGR-LN), root dry weight (RDW), percent root weight of total plant dry weight (%RDW), ratio of dry weight of the upper 5 cm of the root system and the dry weight of the total root (5RDW:RDW), root cross section area (RA), ratio of cortex area to root cross section area (CEA:RA), ratio of aerenchyma to cortex area (AA:CEA), ratio of cortex to stele area (CEA:SA), ratio of stele area to root cross section area (SA:RA).

Source	SDW	TN	LN	TN:SDW	LN:SDW	LN:TN	ΔTN	ΔLN	RGR-TN	RGR-LN	RDW	%RDW	5RDW:RDW	RA	CEA:RA	AA:CEA	CE:SA	SA:RA
P. pratense																		
Accessions	2.5 **	2.5 **	3.7 ***	3.6 ***	5.3 ***	2.9 ***	2.3 *	3.7 ***	2.2 *	2.4 **	4.4 **	2.6 **	2.2*	4.2 ***	9.2 ***	3.8 ***	6.5 ***	9.1 ***
Treatment	0.1	3.4	1.6	5.3 *	3.8	0.2	4	1.6	1.1	0	21.2 ***	32.0 ***	85.9 ***	17.2 ***	78.6 ***	565.2 ***	66.0 ***	76.8 ***
Accessions x Treatment	0.5	0.5	1.2	1	1.3	1.5	0.4	1.3	0.9	1.5	3.6 ***	2.5 **	0.9	2.6 ***	2.4 **	5.7 ***	2.2 **	2.4 **
P. nodosum																		
Accessions	13.0 ***	6.9 ***	6.8 ***	2.1	4.3 ***	4.0 ***	5.6 **	6.4 ***	5.9 **	11.5 *	19.4 ***	6.0 ***	6.22 ***	50.3 ***	4.8 **	23.2 ***	2.3	4.1 **
Treatment	0	0	2.7	0.2	1	2.3	0	3.3	0.2	1.1	4.9 *	18.9 ***	126.1 ***	22.3 ***	55.5 ***	229.2 ***	5.7 *	53.9 ***
Accessions x Treatment	1.8	1	1.5	0.5	0.6	0.3	1.2	1.6	2	1.8	2.3	0.8	1.9	0.8	1.4	9.4 ***	1	2.4
P. alpinum																		
Accessions	6.5 **	0.1	3.2	2.6	4.7 ***	22.2 ***	0.2	2.7	0.5	0.3	0.4	13.0 ***	0.6	1	3.5 **	0.3	4.1 **	3.4 *
Treatment	0.1	0	0.1	1.1	0.6	2.7	0	0.3	0.1	1.1	14.2 **	12.7 ***	50.4 ***	10.9 ***	4.4 **	29.2 ***	53.9 ***	4.9 *
Accessions x Treatment	0.1	2.1	1.8	1.6	1.1	1.7	1.4	2.6	0.4	2.6	0.1	0.2	1.2	1.2	1.5	0.4	2.4	0.3
W and D of P. pratense																		
Group	1.5	2.7	1.1	3.9	2.6	0.1	0.8	0.6	1	0	1.7	0	1.1	14.5 ***	36.6 ***	394.4 ***	54.1 ***	54.1 ***
Treatment	0.1	0.9	0.5	5.5*	6.7 **	0.1	3.5	1.1	0.8	0.7	12.0 ***	22.1 ***	75.1 ***	0.4	65.3 ***	0.6	19.8 ***	19.8 ***
Group x Treatment	0.1	0.3	0.2	1.8	0.1	3.2	0.1	0.2	0	0.5	0.2	0	0.6	1.8	3.1	5.5 *	0.5	0.5

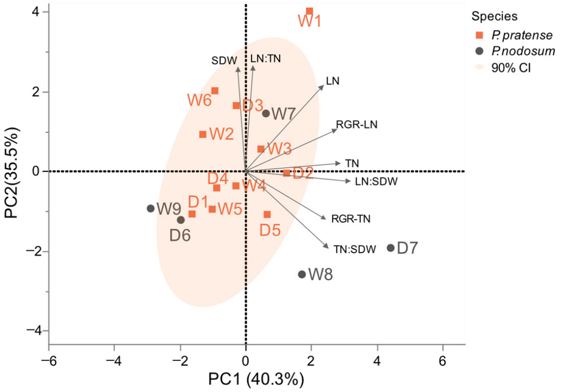

Figure 2. PCA based on the shoot dry weight, tiller and leaf production and growth rates of accessions of *P. pratense* and *P. nodosum*. *P. alpinum* was excluded due to no development of generative tillers. Data points represent the difference between WL and NWL. The loadings are shown for the variables of the shoot dry weight (SDW), tiller number (TN), leaf number (LN), the ratios of the tiller number to shoot dry weight (TN:SDW), the leaf number to shoot dry weight (LN:SDW), the leaf number per tiller (LN:TN) and the growth rates of the tiller number (RGR-TN) and leaf number (RGR-LN). The ellipse corresponds to the 90% confidence interval.

For the root traits, there was a significant difference among accessions and between treatments in RDW, %RDW and 5RDW:RDW ($p < 0.05$, ANOVA, Table 2). There was also an interaction between accessions and treatments in RDW and %RDW. For traits that showed significant effects of WL based on ANOVA, we tried to resolve and display differences among accessions by calculating absolute or proportional differences (Figure 3A–C). For the absolute difference in %RDW, accessions W2, W3, W4 and D5 showed a significantly lower response to WL ($p < 0.05$, Figure 3A, Table S4). Root biomass was higher in the upper part of the root system in WL for W2, W3, W4, W5, W6, D1, D2 and D5, seen as a significant absolute difference in 5RDW:RDW ($p < 0.05$, Figure 3C, Table S4). This increased between 1.3- and 1.9-fold.

2.2.3. *P. nodosum* Accessions

For *P. nodosum*, the response pattern for the accessions was similar to what was found for *P. pratense*. There was a significant difference among accession for all the traits, except for the TN:SDW ($p < 0.05$, ANOVA, Tables 2 and S2), and no significant response to WL for all shoot traits ($p > 0.05$). There was a variation in shoot traits of 1.5- to 3-fold differences between the highest and lowest values ($p < 0.05$, ANOVA, Tables 2 and S3) irrespective of the treatment. Accession D6 showed a significantly lower number of leaves (LN and ΔLN) in response to WL ($p < 0.05$, ANOVA, Tables S2 and S3).

The results of the PCA showed that accession W7 exhibited indications of tolerance to waterlogging due to the positive correlation with shoot traits that are enhanced by waterlogging (Figure 2). On the contrary, accessions W9 and D6 displayed greater susceptibility. Notably, the *P. nodosum* accessions W8 and D7 can be considered tolerant based on tiller traits stimulated by waterlogging.

For the root traits, there was a significant difference among accessions and between treatments in RDW, %RDW and 5RDW:RDW ($p < 0.05$, ANOVA, Table 2). The accessions W8, W9 and D6 allocated less biomass to the root system (%RDW) in WL ($p < 0.05$, Figure 3A, Table S4). All accessions of *P. nodosum* showed an increase in growth on the upper part of the root system in response to WL, since the absolute difference in 5RDW:RDW was significantly higher ($p < 0.05$, Figure 3C, Table S4).

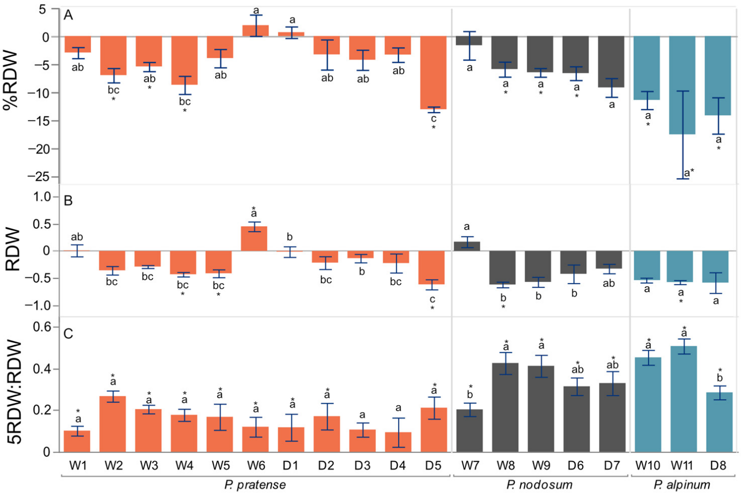

Figure 3. Effect of waterlogging on root traits of accessions. (**A**) Absolute difference between WL and NWL in percent root weight of total plant dry weight (%RDW). (**B**) Proportional difference in total root dry weight (RDW). (**C**) Absolute difference in ratio of the dry weight of the upper 5 cm of the root and the dry weight of the total root (5RDW:RDW) in wild (W) and domesticated (D) accessions of *P. pratense*, *P. alpinum* and *P. nodosum*. Values are means and error bars are ±SE. Significant differences between WL and NWL according to *t*-test are shown with *. Mean values that do not share the same letter are significantly different among accessions within each species according to ANOVA and Tukey's method, $p < 0.05$.

2.2.4. *P. alpinum* Accessions

For the *P. alpinum* accessions, SDW, LN:SDW and LN:TN, there were significant differences among accessions ($p < 0.05$, ANOVA, Table 2). In WL, the number of leaves per tiller increased for accession W11 ($p < 0.05$, ANOVA, Table S2) and the RGR-LN decreased for accession W10 ($p < 0.05$, ANOVA, Table S3).

In WL, all accessions allocated less biomass to the root system (%RDW) ($p < 0.05$, Figure 3A, Table S4) and showed increased growth in the upper part of the root system (5RDW:RDW) ($p < 0.05$, Figure 3C, Table S4).

2.3. *Effects of WL on Root Anatomy*

2.3.1. Comparisons at the Species Level

The anatomy of the roots was also affected by the WL treatment (Figure 4). Significant differences between treatments were observed in RA, CEA:RA, AA:CEA, SA:RA and CEA:SA for all species ($p < 0.05$, ANOVA, Table 2). Interestingly, the formation of aerenchyma was several-fold higher in WL, as seen in the increase in the AA:RA and AA:CEA (Figure 1F,G). The CEA:SA was 1.2- to 1.4-fold higher in WL.

2.3.2. *P. pratense* Accessions

RA and SA varied among accessions and in response to WL (Figure 5A,E, Table S5). Accessions W5, W6, D3 and D4 showed a significant increase in the RA in WL. Accession W6 had a significantly bigger SA, while accession D1 had a significantly smaller SA in WL. All accessions of *P. pratense* formed aerenchyma as part of their normal development in NWL. They showed a strong increase in response to WL since the AA:CEA was significantly higher in WL in all accessions except D5 (Figure 5C, Table S5). Accessions W1, W2, W3, W4, D1, D2, D3 and D4 had a slightly higher CEA:RA and lower SA:RA in WL.

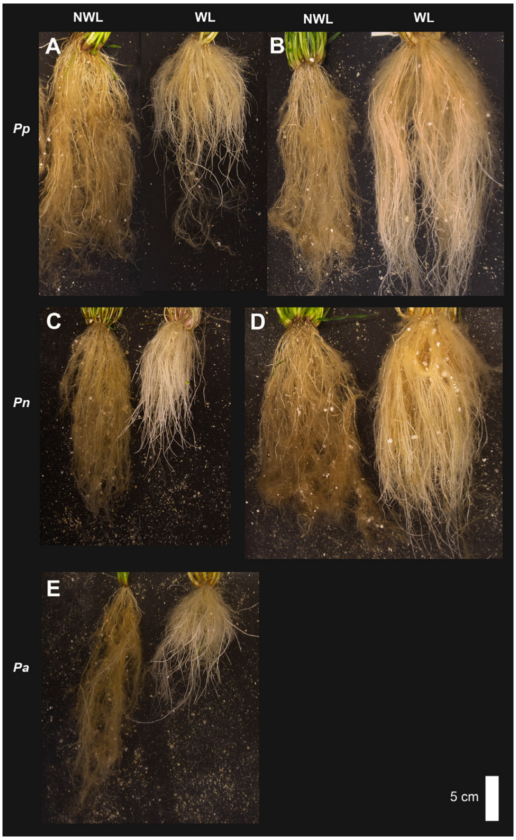

Figure 4. Root systems of representative plants showing different responses in non-waterlogging conditions (NWL, plants to the left side) and in waterlogging conditions (WL, plants to the right side). (**A**) *P. pratense*, accession D4, (**B**) *P. pratense*, accession W6, (**C**) *P. nodosum*, accession W9, (**D**) *P. nodosum*, accession W7 and (**E**) *P. alpinum*, accession W10. Bar is 5 cm.

There was a significant difference between the two groups of accessions (wild and domesticated) and a significant interaction between treatments and groups for AA:CEA ($p > 0.05$, ANOVA, Table 2). Between the two groups, there were significant differences in response to WL in RA, CEA:RA, AA:RA, AA:CEA and SA:RA (*t*-test $p < 0.05$, Figure S5).

2.3.3. *P. nodosum* Accessions

Accession W7 of *P. nodosum* formed aerenchyma in NWL and WL conditions at a similar level as *P. pratense*, while the remaining accessions only formed aerenchyma in WL (Figure 5A–E, Table S5). Accession W7 had a 2-fold higher RA and SA than other *P. nodosum* accessions in WL and NWL. The absolute difference in response to WL in CEA:RA and SA:RA was significant for all accessions and followed a pattern similar to that of *P. pratense*.

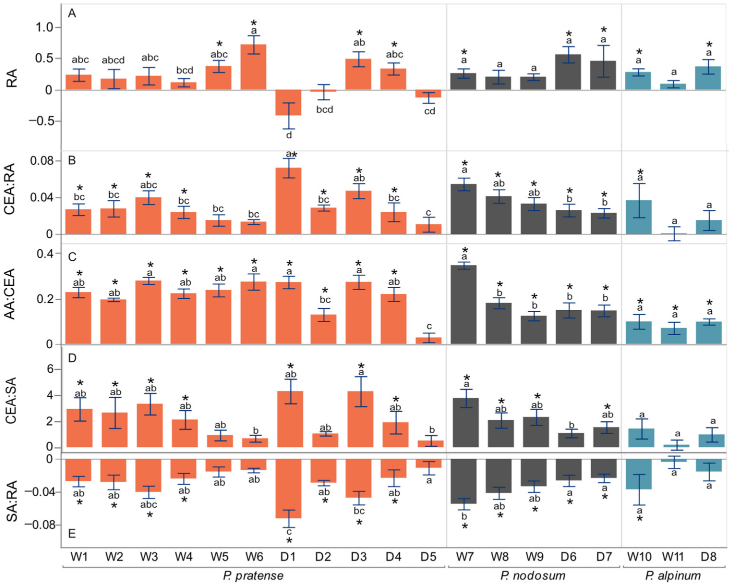

Figure 5. Effect of waterlogging on root anatomy in wild (W) and domesticated (D) accessions of *P. pratense*, *P. nodosum* and *P. alpinum*. (**A**) Proportional difference between WL and NWL in the root cross section area (RA). (**B**) Absolute difference in the ratio of the cortex area to root cross section area (CEA:RA) (**C**) Absolute difference in the ratio of the aerenchyma to cortex area (AA:CEA). (**D**) Absolute difference in the cortex to stele area (CEA:SA). (**E**) Absolute difference in the ratio of the stele to root cross section area (SA:RA). Values are means and error bars are ±SE. Significant differences between WL and NWL according to *t*-test are shown with *. Mean values that do not share the same letter are significantly different among accessions within each species according to ANOVA and Tukey's method, $p < 0.05$.

2.3.4. *P. alpinum* Accessions

Accessions of *P. alpinum* had between 3- and 6-fold more aerenchyma in the cortex than in NWL (Figure 5C, Table S5). Accessions W10 and D8 showed significantly thicker roots (Figure 5A, Table S5). In addition, W10 showed a significantly larger cortex (CEA:RA) and smaller stele (SA:RA) in WL (Figure 5B,E, Table S5).

2.4. Relations of Root Anatomy and Growth Traits

To try to understand which traits can be beneficial for tolerance to waterlogging and provide a basis to identify potential tolerant accessions, we analyzed the data for correlations among traits based on all data. Pearson correlation analysis showed that all shoot traits had strong positive correlations between each other in both treatments (Table 3). The 5RDW:RDW was negatively correlated to SDW (Table 3, Figure S6) and RDW in WL, suggesting that plants allocate resources to the production of more adventitious roots, which also has an effect on shoot development.

Table 3. Pearson correlation coefficient (r) for shoot and root traits for all accessions in non-waterlogging (NWL) and waterlogging (WL, in grey) conditions. Abbreviations: shoot dry weight (lnSDW), tiller number (lnTN), leaf number (lnLN), root dry weight (lnRDW), percent root weight of total plant dry weight (%RDW), ratio of dry weight of the upper 5 cm of the root system and the dry weight of the total root (5RDW:RDW), root cross section area (RA), ratio of cortex area to root cross section area (CEA:RA), ratio of aerenchyma to root cross section area (AA:RA), ratio of aerenchyma to cortex area (AA:CEA), ratio of cortex area to stele area (CEA:SA) and ratio of stele area to root cross section area (SA:RA).

WL NWL	ln(SDW)	ln(TN)	ln(LN)	ln(RDW)	%RDW	5RDW:RDW	RA	CEA:RA	AA:RA	AA:CEA	CEA:SA	SA:RA
ln(SDW)		0.83	0.83	0.91	−0.47	−0.75	0.41	0.61	0.72	0.72	0.57	−0.61
ln(TN)	0.91		0.98	0.68	−0.49	−0.42	0.08	0.55	0.48	0.48	0.48	−0.55
ln(LN)	0.84	0.97		0.67	−0.48	−0.4	0.08	0.59	0.50	0.50	0.52	−0.59
ln(RDW)	0.87	0.72	0.65		−0.08	−0.88	0.59	0.58	0.83	0.83	0.56	−0.58
%RDW	−0.78	−0.78	−0.73	−0.4		−0.03	0.22	−0.24	0.01	0.01	−0.18	0.24
5RDW:RDW	−0.15	−0.02	−0.07	−0.41	−0.28		−0.69	−0.53	−0.79	−0.80	−0.53	0.53
RA	0.36	0.03	−0.06	0.64	0.08	−0.5		0.37	0.72	0.72	0.39	−0.37
CEA:RA	0.22	0.25	0.18	0.20	−0.12	0.08	0.11		0.74	0.72	0.98	−1.00
AA:RA	0.31	−0.03	−0.09	0.54	0.09	−0.47	0.90	0.08		1.00	0.73	−0.74
AA:CEA	0.30	−0.03	−0.09	0.54	0.09	−0.47	0.90	0.05	1.00		0.71	−0.72
CEA:SA	0.25	0.29	0.19	0.24	−0.14	0.05	0.12	0.97	0.05	0.02		−0.97
SA:RA	−0.24	−0.27	−0.19	−0.21	0.15	−0.09	−0.12	−1.00	−0.08	−0.06	−0.98	

There was no correlation between the shoot and the root anatomical traits in NWL, while in WL the CEA:RA, AA:RA and AA:CEA were positively correlated to the shoot traits and RDW. In WL, the RA and the proportions of aerenchyma (AA:RA, AA:CEA) and cortex (CEA:RA) were negatively correlated to 5RDW:RDW, suggesting that plants having high formation of aerenchyma or a thick cortex, allocated less growth to the upper part of the root system.

The SA:RA was negatively correlated to the shoot traits in WL but not in NWL, suggesting that a smaller stele is beneficial for tolerance to waterlogging. The SA:RA was positively correlated to 5RDW:RDW, suggesting that plants that have a smaller stele area allocate their growth to the upper part of the root system. RA did not correlate to shoot traits but to RDW in both treatments.

To explore interactions of root anatomical traits and accession diversity, we performed a principal component analysis (PCA) on the areas and ratios of the root tissues (Figure S3). The results showed that the two components (PC1 and PC2) explained 94.4% of the variation. AA:CEA, CEA:SA, and SA:RA contributed strongly to PC1, while RA and SA contributed strongly to PC2. Most of the *P. pratense* accessions in WL correlated to AA:CEA and CEA:SA and separated from the NWL (Figure S3A). In WL, the domesticated accessions showed a large diversity in response, while the wild accessions showed a more similar response in root anatomy (Figure S3B). In NWL, the accessions of domesticated and wild *P. pratense* formed two partially overlapping clusters. In NWL, four out of five *P. nodosum* accessions were different from *P. pratense* and *P. alpinum*, and distributed with a negative correlation to SA and RA. The fifth *P. nodosum* accession (W7) clustered together with the *P. pratense* accessions in both NWL and WL.

To display variation in tolerance among accessions, we performed a PCA and an HCA on the proportional or absolute difference in response to WL for shoot and root traits, growth rates of leaf and tiller, and root anatomy traits (Figure 6). SDW, % RDW, AA:CEA and CEA:SA contributed positively, and 5RDW:RDW contributed strongly and negatively to PC1 (36.1%). TN, LN and the growth rates of LN (RGR-LN) and TN (RGR-TN) contributed to PC2 (22.9%) in the PCA (Figure 6A). The response to WL of the accessions W1, W6, W7 and D3 correlated to the SDW, % RDW, AA:CEA and CEA:SA and this distribution was supported by the HCA (Figure 6B). Accession D5 (Tryggve) was strongly affected by 5RDW:RDW and negatively correlated to the other anatomy traits.

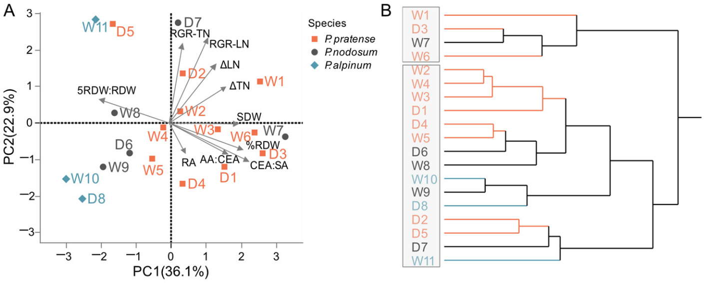

Figure 6. Distribution of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* based on differences between WL and NWL in the shoot and root traits and the root anatomy traits. (**A**) PCA where data points represent an accession. For measured traits (SDW, ΔTN, ΔLN and RA), proportional differences between WL and NWL were used, and for the remaining calculated traits (RGR-TN, RGR-LN, %RDW, 5RDW:RDW, AA:CAE, CAE:SA), the absolute differences were used. (**B**) HCA based on the same traits as for the PCA. Abbreviations can be found in Table 2.

To analyze possible differences in the timing of response to WL, we performed a PCA on weekly growth rates of tiller and leaf numbers of all accessions (Figure S4). Accessions showing a positive correlation with the loading variable had a higher growth rate in WL. Some accessions were grouped depending on the first week response to WL (R-LN7, R-TN7), while others grouped along the axis influenced by the second (R-LN14, R-TN14) or third (R-LN21, R-TN21) week variables.

R-LN7 and R-TN7 contributed strongly and positively to PC1. A high positive value along the first week variables showed that growth in WL was higher than in NWL. In contrast, those accessions placed in the opposite direction of these variables were negatively affected by the WL condition in the first week. The direction of R-LN21 and R-TN21 variables corresponded to a higher rate of formation of tillers and leaves in WL. Accessions W1, W4, W5, W8, W11 and D4 were more affected during the first week; however, their growth increased in the last week of WL conditions.

3. Discussion

Timothy is one of the most cultivated forage crops in northern Europe, and new cultivars adapted to a changing climate are needed to secure harvest yields. While drought is predicted to be the main abiotic stress for plants in central and southern Europe, more frequent and heavy rainfalls, with flooded and waterlogged soils as a result, are predicted to be the main challenge in northern Europe. The effect of WL stress on annual cereals such as barley and wheat has been well studied, but few studies have focused on perennial forage grasses in general and timothy and other *Phleum* species in particular.

Our aim was to evaluate timothy and two related species for their responses to WL and to investigate tolerance mechanisms, which have not been previously described in *Phleum* species. By evaluating a large set of accessions, we discovered variation in growth allocation, root growth and root anatomy, suggesting possible mechanisms of tolerance to waterlogging and how they vary among accessions and species. These results provide valuable knowledge for the selection of WL-tolerant accessions as possible inclusions in breeding based on their phenotypic responses and as candidates for further genetic studies.

To compare species and accessions, it is valuable to know their morphologies and growth rates under a normal irrigation regime. The results show that the three species differ clearly in growth habit and morphology, e.g., number of tillers and leaves per plant (Table S3, Figure S1). Interestingly, one *P. nodosum* accession appeared to be closer to *P. pratense* in these shoot traits (Figure S1), as well as in root anatomy traits (Figure S3). The taxonomy of *Phleum* is challenging, with many species and subspecies [51], and the phenotypic relatedness of this *P. nodosum* accession to *P. pratense* points out the importance of studying the diversity of *Phleum* gene bank collections.

Both *P. pratense* and *P. nodosum* developed reproductive tillers in both WL and NWL. In future studies on the effects of WL under field conditions, forage quality and seed production should be investigated, as it has been shown that WL has a negative effect on these traits in annual cereals [52–54] and forage crops [55–57]. *P. alpinum* differed from the other species since all accessions remained in the vegetative stage under these growth conditions (Table S3). This may be related to the high temperatures in the greenhouse and partly to the daylength, since *P. alpinum* has been shown to require a dual induction by low temperature and/or short day to induce flowering, as well as a combination of long days and high temperatures to enhance heading and inflorescence development [58,59].

The response to WL with regards to SDW, compared at the species level with data from all accessions combined, showed no significant differences between waterlogged and non-waterlogged plants in the three species (Table 1). Previous studies in perennial grasses, using a similar length of WL treatment as in this study, have reported tolerant varieties of cocksfoot, tall fescue and perennial ryegrass that showed no significant difference in SDW in WL and NWL [60,61]. In annual cereals, such as wheat and barley, exposure to WL affected the leaf appearance, shoot biomass and flowering [62–64]. Even short periods of WL in young wheat plants have significant long-term effects and cause reduced plant growth and development [65]. It is clear from our experiment that the negative effects of WL on shoot biomass seen in annual monocots were not observed in any of the three *Phleum* species. This suggests that *P. pratense*, *P. nodosum* and *P. alpinum* are relatively tolerant to WL compared to annuals and have evolved mechanisms to cope with this stress. To compare the three species, WL response indexes for several traits were calculated (Table S6), and the indexes for %RDW and for formation of root biomass in the upper part of the root system (5RDW:RDW) suggest that *P. pratense* responded less to WL than the other two species.

A major objective of this study was to investigate the diversity among *Phleum* accessions in how they respond to WL. Interestingly, we found that among the several accessions in this study, there was a variation in WL tolerance (Table 2), as well as between type of accession and between species. In some accessions, growth was negatively affected by WL (*P. pratense* D1, *P. nodosum* D6, *P. alpinum* W10); interestingly, in contrast, some other accessions responded by producing more leaves per tiller (*P. pratense* W1, *P. alpinum* W11), and for W1, there was a higher growth rate in leaf formation (RGR-LN). The timing of the response in the growth rate of tillers and leaves also appeared to vary among accessions, with some being affected in the first week and others in the second or third week of WL (Figure S4).

Root development and growth were clearly affected by WL (Figure 4), and in general, the three species showed a decrease in root biomass. This is a common response in plants, as shown for annual cereals [52,66–68], annual dicot crops [69,70], perennial forage grasses [61,71] and perennial forage legumes [72,73]. Root growth decreases due to a deficiency of oxygen, energy, nutrients and accelerated root senescence [74].

Interestingly, among the accessions, there seem to be different types of responses in distribution of the root and shoot biomass (Figure 4). Some accessions, perhaps less tolerant, showed a decrease in the proportion of root biomass (%RDW) in response to WL (*P. pratense* W3 and W4, *P. nodosum* W8 and W9, *P. alpinum* W10). In contrast, accessions *P. pratense* W6 and *P. nodosum* W7 had an increase in RDW or no effect on RDW, as for *P. pratense* W1. These accessions showed no significant effect on %RDW (Figure 3A), consistent with the fact that shoot growth was not negatively affected (Figure 2). The response by W1, W6 and W7 could be part of a mechanism to escape WL by the root as well as the shoot. It may resemble the response to escape WL through shoot elongation, which is a common flood and submergence response of many wetland plants and crops, where it serves to restore contact with the atmosphere and sustain internal aeration [75,76].

The increase in RDW in WL for the two accessions W6 and W7 could be the result of continued root growth, less senescence and higher formation of adventitious roots. In all accessions of the three species, the root abundance in the 5 cm section below the root:shoot interface (5RDW:RDW) increased in WL due to the formation of adventitious roots (Figure 3C). The formation of new adventitious roots with aerenchyma can increase oxygen availability in the roots and nutrient uptake from the soil, enabling roots to grow [21,77]. Anatomical adaptations such as aerenchyma formation, thick roots, large cortex to stele areas and the growth of adventitious roots improve WL tolerance [78,79]. All *P. pratense* and *P. alpinum* accessions and one accession of *P. nodosum* formed aerenchyma in a low percent of the root cortex in NWL conditions. Formation of aerenchyma in NWL conditions has been also reported in other perennial grasses, e.g., roughstalk bluegrass, tall fescue and cocksfoot [60,80]. However, it can be increased in hypoxic conditions, as we observed in most accessions. Some accessions showed the ability to form more aerenchyma than others (Figure 5C). We found a high positive correlation between the shoot dry weight (SDW) and the ratio between aerenchyma and cortex (AA:CEA) in WL (Table 3), suggesting the importance of aerenchyma formation for WL tolerance in *Phleum* also.

Another important objective was to test the hypothesis that there are differences between accessions in WL tolerance and, if possible, suggest new germplasm for breeding. A tolerant plant that is desirable from a breeding perspective should have a high capacity to form adventitious roots and aerenchyma, a high cortex to stele ratio and the ability to maintain shoot and root growth. Combining shoot and root traits (Figure 6), accessions W1, W6, W7 and D3 may be considered more tolerant than the other accessions due to a higher production of aerenchyma in the cortex (AA:CEA), higher CEA:SA and maintained shoot and root dry weight. A maintained and even increased root growth, as seen in accessions W6 and W7, might be advantageous for a rapid recovery after WL, as was also suggested for WL-tolerant species [81]. In contrast, accession D5 had a smaller root system with a higher proportion of roots in the upper 5 cm region (5RDW:RDW) and produced aerenchyma at the same low level as in NWL, suggesting that D5 (cv. Tryggve, common cultivar in Sweden) may be less tolerant to WL under the experimental conditions. The response of producing higher 5RDW:RDW could be in line with an increased production of the number of adventitious roots in WL as an adaptation to WL, which was shown for cocksfoot (*Dactylis glomerata*) [54]. For further pre-breeding studies, we suggest including the wild accessions of *P. pratense* W1 and W6 and *P. nodosum* W7.

WL is an important ecological factor, and species adapted to flooded habitats commonly form aerenchyma in response to WL [22]. Root anatomies with high CA:SA and AA:CA were also suggested to be advantageous for WL tolerance [41]. The accession passport information, available for most of the wild accessions in the present study (Table S1), shows that, e.g., some of the less tolerant accessions, such as W3 and W9, were collected from areas with comparatively less precipitation (www.smhi.se) and habitats described as meadow and grassland. In contrast, two of the more tolerant accessions, W1 and W6, were collected from habitats described as bog and marsh, and these accessions have a root anatomy favorable for WL tolerance. Differential strategies within a species to tolerate flooding depending on the habitat from where the populations originate has been found for other plant species (e.g., [82,83]), supporting the importance of WL as a selective ecological factor.

Comparing wild and domesticated accessions of *P. pratense* made it possible to test the hypothesis that there are differences between these two groups of accessions. In NWL, there is a difference in the cortex (CEA:RA, CEA:SA) and stele (SA:RA) areas between the domesticated and wild groups of accessions. Interestingly, among the wild accessions, the accessions collected in wetlands, W1 and W6, had the smallest root cross-sectional and stele areas, as well as the highest CEA:SA. High cortex to stele area is one of the root anatomical factors that explain the adaptation of wild grasses to high soil water content [84].

Since a two-way ANOVA showed significant differences in root anatomical traits (Table 2) between the two groups, the root anatomical trait data were further tested for absolute or proportional differences between WL and NWL plants (Figure S5). For the response to WL in RA, CEA:RA, AA:RA, AA:CEA and SA:RA, the wild and domesticated accessions were significantly different (*t*-test, $p > 0.05$). The significant differences for some of these traits were largely due to accession D5. When D5 was excluded from the *t*-test analyses, the significant difference between the wild and domesticated groups remained in the response in the CEA:RA and SA:RA traits. Our results showed that SA:RA was significantly lower in WL for both wild and domesticated accessions, but the reduction during WL was lower for the wilds (Figure S5). Also, in NWL, the group of wild accessions had lower SA:RA compared to the domesticated accessions. The xylem makes up a large proportion of the stele (SA), and it has been found that for plants adapted to wet soils, the xylem to the whole root area is generally lower [84].

WL results in low O_2 availability for the root system and thus affects respiration, metabolism and transport of mineral nutrients and water. Tolerance to WL is therefore a complex trait needed for sustained growth and depends on several plant adaptations in physiology, metabolism and gene expression. Our study has demonstrated a diversity in phenotypic responses of growth and root anatomy to WL in young plants of *P. pratense*, *P. nodosum* and *P. alpinum* at the tillering stage. A reduction in root system and a production of adventitious roots was observed in most of the accessions, while a few accessions also maintained root growth. Based on plant growth traits, aerenchyma formation and other root anatomical traits, we could identify wild accessions of *P. pratense* and *P. nodosum* that could be tested further for use in breeding programs. In screening for WL-tolerant timothy, the studied root morphology and anatomy traits could be valuable when identifying tolerant offspring. Further studies are required to elucidate the molecular mechanisms underpinning WL tolerance in *Phleum* species.

4. Materials and Methods

4.1. Plant Material

The performance of 244 wild and domesticated accessions of *P. pratense*, *P. nodosum* and *P. alpinum* was evaluated in the field and greenhouse in Uppsala, Sweden [85]. A total of 19 accessions that represented the variation within species were selected for the present study of the response to WL. Six wild accessions and five cultivars of *Phleum pratense* (timothy), three wild accessions and two cultivars of *P. nodosum*, and two wild accessions and one breeding line of *P. alpinum* from the Nordic countries were used (Table S1). Seeds were obtained from the gene bank NordGen, Alnarp, Sweden, except for the cultivars Tryggve and Switch, provided by Lantmännen, Svalöv, Sweden.

4.2. Pre-Cultivation

Seeds of each accession were sown in 2 L pots with a mixture of vermiculite and perlite (1:1) in a greenhouse at the Plant Cultivation Facility, Uppsala BioCenter, SLU, Uppsala, Sweden, with a 16/8 hrs day/night photoperiod with supplementary light from metal halogen lamps. The experiment was carried out from February to April 2022, at a maximum temperature of 24 °C and a minimum of 16 °C. For each accession, three seeds per pot were sown in eight pots. Plants were given a complete nutrient solution (N:102 mg/L (NH_4^+: 40 mg/L, NO_3^-: 62 mg/L), P: 20 mg/L, K: 86 mg/L, S: 8 mg/L, Ca: 6 mg/L, Mg: 8 mg/L, Fe: 0.34 mg/L, Mn: 0.4 mg/L, B: 0.2 mg/L, Zn: 0.06 mg/L, Cu: 0.03 mg/L, Mo: 0.08 mg/L; Wallco Miljöcenter AB, Arlöv, Sweden) every third day. Three weeks after sowing, eight plants of each accession of similar size were selected by removing the other plants in each pot.

4.3. WL Experiment

After seven weeks in pre-cultivation, four of the eight plants of each accession were exposed to WL. All plants were at vegetative stage at the start of the experiment. Each pot was placed in a 5 L bucket. Deionized water and 200 mL of the complete nutrient solution were added up to the surface of the vermiculite and perlite mixture. The water level was maintained 1 cm above the soil line by gently adding deionized water. The other four plants were grown in NWL and were watered every third day with 300 mL of the complete nutrient solution. The plants were divided into four blocks, where one plant in WL and one plant in NWL of each accession were randomized in each block. The experiment was conducted for 21 days.

4.4. Studied and Calculated Traits

The tiller number (TN) and leaf number (LN) of each plant (Figure 7) were counted at the start of the experiment and at 7, 14 and 21 days of WL. The number of tillers and leaves produced during the experiment (ΔTN and ΔLN) were calculated by subtracting the number of tillers and leaves at day 0. The TN and LN values for the time points 0, 7, 14 and 21 were used to calculate the growth rates by linear regression based on their natural logarithmic values (ln), where the slopes ln(TN) day^{-1} and ln(LN) day^{-1} represent the relative growth rates RGR-TN and RGR-LN, respectively. For weekly growth rates, the difference between time points was used.

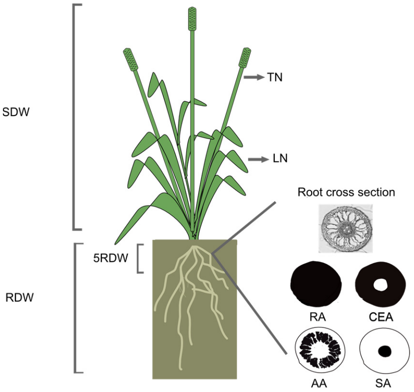

Figure 7. Drawing of the studied shoot and root traits and their abbreviations; shoot dry weight (SDW), number of tillers (TN) and number of leaves (LN) per plant, root dry weight (RDW), dry weight of the upper 5 cm part of the root system (5RDW), area of the root cross section (RA), stele area (SA), cortex area including epidermis (CEA) and aerenchyma area (AA). Measured tissue areas in the root anatomy are indicated in black.

At the end of the experiment, each plant was taken out from the pot and the roots were gently rinsed with tap water to remove the vermiculate and perlite substrate. We then divided the shoot and the root by cutting the plant at the interface between the two tissues. The shoot was dried at 65 °C for 48 h and the shoot dry weight (SDW) was then measured for each plant.

At harvest, two adventitious roots emerging from the crown were randomly selected from each plant and stored in 70% ethanol at 4 °C for anatomical analysis. Cross sections of the maturation zone of the roots (10–30 mm from the root base) were cut by hand. These root sections were visualized at 10× magnification using a bright-field Zeiss Axio Scope A1 microscope, and images were taken by an AxioCam ICc5 camera. The area of the root cross section (RA), the stele area (SA), the cortex area including epidermis (CEA) and the aerenchyma area (AA) were measured in ImageJ v. 1.52t (National Institute of Health, Bethesda, MD, USA) using the free hand tool.

After sampling for root anatomy studies, the root systems were cut 5 cm below the interface between the shoots and the roots. The two root parts were kept separately and dried at 65 °C for 48 h. Then, the dry weight of the upper 5 cm of the root (5RDW) and the total dry weight of the root were measured. The percentage of allocation of plant growth to roots, %RDW, was calculated as 100 × RDW:(RDW + SDW).

4.5. Analysis of Phenotypic Responses to WL

This study comprised wild and domesticated accessions of three different *Phleum* species. The non-treated accessions showed large diversity in shoot and root traits both within and between the species (Tables S2 and S3). To be able to compare the responses of different accessions, we calculated the absolute difference between WL and NWL conditions as

$$\overline{x}_{WL} - \overline{x}_{NWL} = \frac{1}{n}\sum_{i=1}^{n}(x_i - \overline{x}_{NWL})$$

and the proportional difference as

$$\frac{\overline{x}_{WL} - \overline{x}_{NWL}}{\overline{x}_{NWL}} = \frac{1}{n}\sum_{i=1}^{n}(x_i - \overline{x}_{NWL}) / \ \overline{x}_{NWL}$$

where $\overline{x}_{WL}$ is the average in WL, $\overline{x}_{NWL}$ is the average in the NWL and x_i is the value for the individual i in WL.

The absolute difference was calculated for %RDW and the ratios LN:SDW, TN:SDW, LN:TN, RGR-TN, RGR-LN, 5RDW:RDW, CEA:RA, AA:RA, AA:CEA, CEA:SA and SA:RA. For the SDW, RDW, ΔTN, ΔLN and RA, the proportional difference was used.

4.6. Statistical Analysis

All traits showed normally distributed residuals and were therefore analyzed with the parametric test Analysis of Variance (ANOVA) using a Mixed Effects Model approach. The mixed model analysis was performed using restricted maximum likelihood estimation (REML) and unbounded variance components. For each *Phleum* species, the fixed effects included accessions and treatments (WL and NWL) and the interaction between them, while block was considered as random effects. For comparison of the wild and domesticated groups of *P. pratense* accessions, the fixed effects included group, treatment and the interaction between treatment and group. For comparisons between WL and NWL by accession, a subdesign ANOVA was performed considering the same fixed and random effects as the initial model. To compare the response to WL among accessions, a Tukey test was performed. Moreover, Pearson correlation analysis was applied to evaluate the linear association between traits. Data of growth traits used for Pearson correlation analysis were natural logarithm transformed. The multivariate analysis principal component analysis (PCA) and hierarchical cluster analysis (HCA) using Ward's method were used to analyze and display the variation in response among accessions based on all studied shoot and root traits. Statistical significance was determined at $p < 0.05$. All statistical analyses were performed using JMP Pro 16.0.

5. Conclusions

WL of soil leads to reduced agricultural plant productivity and is expected to increase due to more frequent extreme weather conditions caused by climate change. This study has demonstrated substantial diversity in the response to WL of a range of wild and domesticated accessions of *P. pratense*, *P. nodosum* and *P. alpinum*. The identification of specific traits, including the ability to maintain root growth, the development of adventitious roots and aerenchyma formation, suggests the potential for these features to serve as valuable screening criteria for identifying tolerant accessions. Moreover, the study has pinpointed two wild *P. pratense* and one wild *P. nodosum* accessions with traits that could be useful as genetic resources for improving cultivars in the future. WL tolerance has not historically been a conscious target of breeding programs for timothy, which instead focused on traits such as early heading. Our findings offer a pathway for future breeding efforts to enhance the resilience of timothy to waterlogged conditions, further underscoring the importance of continued research in this area.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/plants12234033/s1. Figure S1. Relations among accessions of *P. pratense*, *P. nodosum* and *P. alpinum* based on morphological traits in non-waterlogging conditions (NWL) after 70 days of growth in greenhouse conditions. Figure S2. Growth of plants of *P. pratense*, *P. nodosum* and *P. alpinum* accessions shown as production of leaves and tillers during the waterlogging (WL) and non-waterlogging (NWL) treatments. Figure S3. Distribution and response of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* to NWL and WL conditions analyzed using principal component analysis based on root anatomy traits. Figure S4. Diversity in effects of WL on weekly growth rates of tiller and leaf numbers. Figure S5. Effect of WL on root anatomy traits of domesticated and wild accessions of *P. pratense*. Figure S6. Correlations between shoot dry weight (SDW) and aerenchyma area to cortex–epidermis area (AA:CEA) and between SDW and proportion of dry biomass of upper 5 cm part of the root system (5RDW:RDW) of *Phleum* accessions in NWL and WL conditions. Table S1. Studied accessions and their passport data. Table S2. Shoot traits of *P. pratense*, *P. nodosum* and *P. alpinum* in NWL and WL at the end of the 21-day treatment. Table S3. Growth rates and generative tillers. Table S4. Root traits of *P. pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) at the end of the 21-day treatment. Table S5. Root anatomy traits of accessions of *P. pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) conditions at the end of the 21-day treatment. Table S6. WL response indexes of *P. pratense*, *P. nodosum* and *P. alpinum*, calculated as the proportional difference between the WL and NWL.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary materials to:

Response to waterlogging stress in wild and domesticated accessions of timothy (*Phleum pratense*) and its relatives *P. alpinum* and *P. nodosum*

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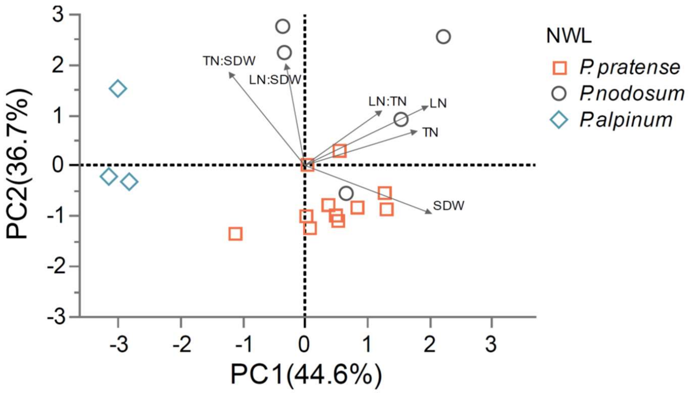

Figure S1. Relations among accessions of *Phleum pratense*, *P. nodosum* and *P. alpinum* based on morphological traits in non-waterlogging conditions (NWL) after 70 days of growth in greenhouse conditions. The data used for the principal component analysis is shoot dry weight (SDW), tiller number (TN) and leaf number (LN) per plant and their ratios.

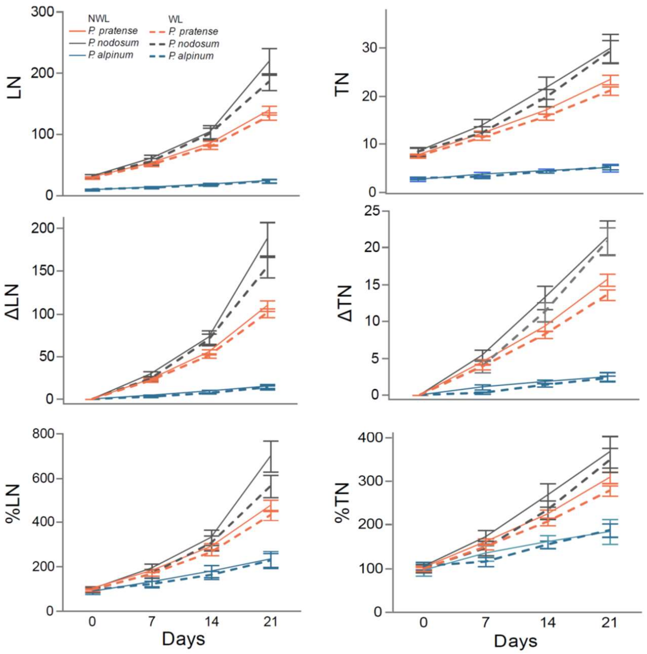

Figure S2. Growth of plants of *Phleum pratense*, *P. nodosum* and *P. alpinum* accessions shown as production of leaves and tillers during the waterlogging (WL) and non-waterlogging (NWL) treatments. The increase in number of leaves and tillers is shown as absolute number or as percentage increase in relation to number at the start of the experiment. The treatment started 49 days after germination. Data shown is averages for 11, 5 and 3 accessions for the three species respectively, with 4 replicate plants per accession. Error bars are standard errors.

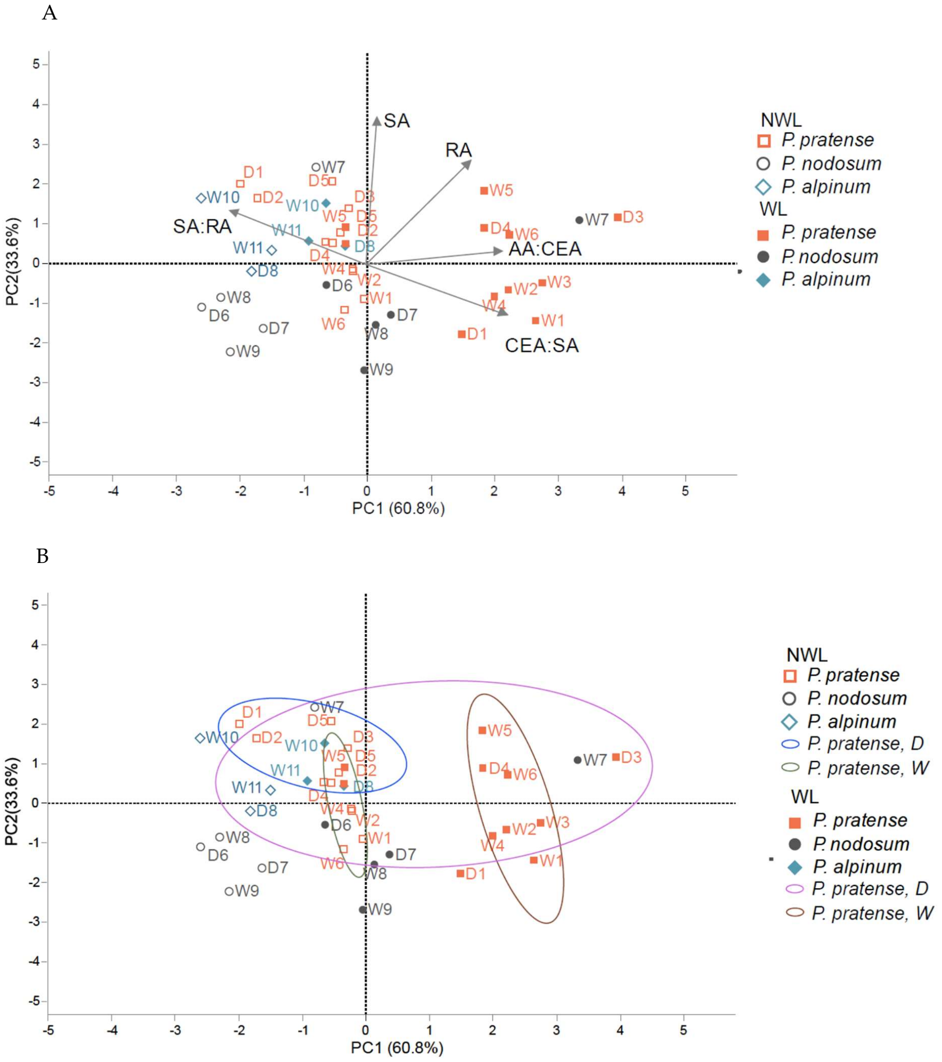

Figure S3. Distribution and response of accessions of *Phleum pratense*, *P. nodosum* and *P. alpinum* to non-waterlogging (NWL) and waterlogging (WL) conditions analyzed by principal component analysis based on root anatomy traits. Figure A shows the loadings of each anatomy variable (RA, root area; SA, stele area; and the ratios SA:RA; CEA:SA, cortex-epidermis area to stele area; and AA:CEA, aerenchyma area to cortex-epidermis area, together with the scores representing the accessions. Figure B shows the wild and domesticated accessions of *P. pratense* in WL and NWL where each group is circled by 90% confidence intervals.

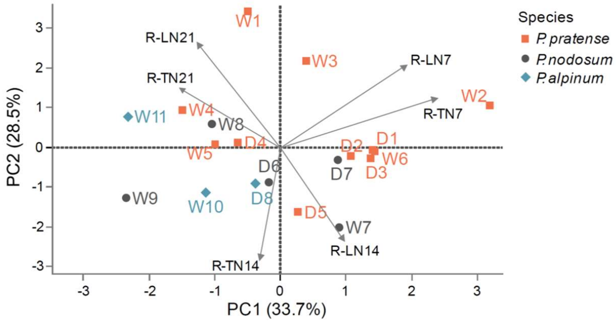

Figure S4. Diversity in effects of WL on weekly growth rates of tiller and leaf numbers. The calculated variables used for the principal component analysis are the differences in growth rate of tiller number (TN) and leaf number per plant (LN) for each accession between plants in WL and NWL during each of the first 7 days (7), the days 8-14 (14) and the days 15-21 (21) of the experiment. Accessions projected to be positively correlated to the loading of a variable had a higher growth rate in WL during that time period, and conversely, accessions negatively correlated to a variable loading had higher growth rate in NWL during that time period.

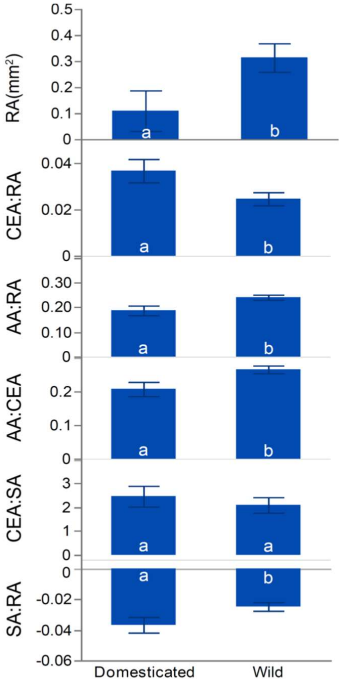

Figure S5. Effect of WL on root anatomy traits of domesticated and wild accessions of *Phleum pratense*. The effect was calculated as a proportional difference between plants in WL and NWL for root area (RA) and for absolute differences for the ratio variables. Domesticated and wild accessions indicated with different letters are significantly different ($p < 0.05$). Data shown is averages for 5 domesticated and 6 wild accessions, with 4 replicate plants per accession. Error bars are standard errors.

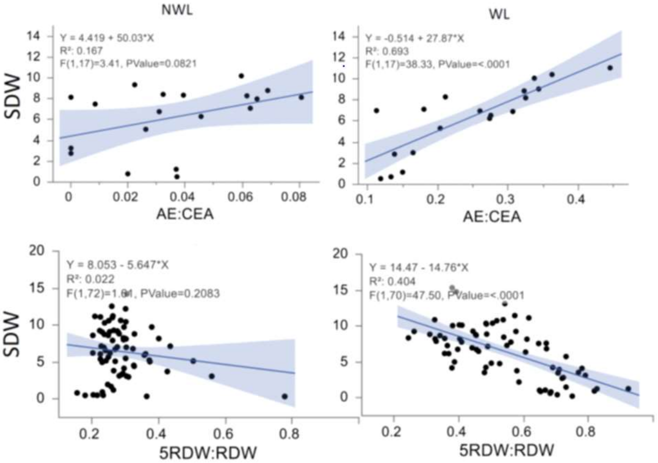

Figure S6. Correlations between shoot dry weight (SDW) and aerenchyma area to cortex-epidermis area (AA:CEA) and between SDW and the proportion of dry biomass of upper 5 cm part of the root system (5RDW:RDW) of *P. pratense*, *P. nodosum* and *P. alpinum* accessions in NWL and in WL conditions at the end of the 21 days treatment.

Table S1. Studied *Phleum* sp. accessions and their passport data.

Accession ID-SLU	Species	Accession number or cultiva	Type of accession	Habitat	Country of origin	Coordinates	Latitude	Longitude	Collection date
W1	*Phleum pratense*	NGB4053	Wild	Bog	Denmark	57.2417, 9.7750	57° 14' 30.12"	9° 46' 30"	08/26/1983
W2		NGB21082	Wild	Field margin	Norway	59.1158, 10.1971	59° 6' 56.88"	10° 11' 49.56"	8/21/2009
W3		NGB1537	Wild	Meadow	Sweden	57.4917, 18.1750	57° 29' 30.12"	18° 10' 30"	8/9/1985
W4		NGB722	Wild	Grassland	Sweden	66.6917, 19.7583	66° 41' 30.1194"	19° 45' 29.8794"	8/25/1979
W5		NGB20801	Wild	Field	Sweden	64.6644, -21.2133	64° 39' 51.84"	-21° 12' 47.88"	5/28/2008
W6		NGB7707	Wild	Marsh	Norway	67.9432, 15.2253	67° 56' 35.52"	15° 13' 31.08"	1/1/1976
D1		NGB13524	Domesticated		Sweden				
D2		NGB11677	Domesticated		Denmark				
D3		NGB13221	Domesticated		Sweden				
D4		Cultivar Switch	Domesticated		Sweden				
D5		Cultivar Tryggve	Domesticated		Sweden				
W7	*Phleum nodosum**	NGB14477	Wild	Meadow/Road/Field/Ditch	Finland	60.1539, 23.0878	60° 9' 14.04"	23° 5' 16.08"	8/16/1998
W8		NGB4364	Wild	Grassland	Sweden	56.3250, 12.5250	56° 19' 30"	12° 31' 30"	9/2/1983
W9		NGB4352	Wild	Grassland	Sweden	55.3917, 14.1583	55° 23' 30.12"	14° 9' 29.88"	8/17/1983
D6		NGB2378	Domesticated		Sweden				
D7		NGB1725	Domesticated		Denmark				
W10	*Phleum alpinum*	NGB1340	Wild	Grassland	Sweden	64.4250, 18.0583	64° 25' 29.9994"	18° 3' 29.8794"	9/15/1980
W11		NGB772	Wild	Grassland	Sweden	68.2083, 22.7750	68° 12' 29.8794"	22° 46' 29.9994"	8/24/1979
D8		NGB4143	Domesticated		Iceland				

**P. nodosum* (syn. *P. pratense* L. subsp. *bertolonii* (DC.) Bornm. and *P. bertolonii* (DC.) Bornm.).

Table S2. Shoot traits of *P. pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) at the end of the 21 days treatment. Shoot dry weight (SDW), number of tillers (TN), number of leaves (LN), the ratios of the number of tillers to shoot dry weight (TN:SDW), the number of leaves to shoot dry weight (LN:SDW), the number of leaves to the number of tillers (LN:TN). Values are means and standard error (± SE) of plants within each accession.

Accessions	**SDW (g)**		**TN (#plant^{-1})**		**LN (#plant^{-1})**		**TN:SDW (#plant x g^{-1})**		**LN:SDW (#plant x g^{-1})**		**LN:TN**	
	NWL	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**
P. pratense												
W1	5.07 ± 0.76	6.25 ± 0.69	17.75 ± 2.93	18.00 ± 2.04	68.00 ± 9.68	110.50 ± 13.36	3.62 ± 0.56	2.88 ± 0.02	13.80 ± 1.67	17.89 ± 1.55	**3.90 ± 0.21**	**6.23 ± 0.57**
W2	6.30 ± 1.44	6.96 ± 0.85	24.50 ± 2.33	18.50 ± 2.50	147.00 ± 16.04	120.25 ± 19.54	**4.53 ± 1.12**	**2.71 ± 0.30**	**25.26 ± 2.91**	**17.51 ± 2.14**	6.14 ± 0.79	6.45 ± 0.25
W3	8.34 ± 1.46	9.04 ± 0.47	21.25 ± 2.29	22.50 ± 1.89	136.50 ± 26.73	145.25 ± 19.61	2.72 ± 0.38	2.51 ± 0.26	16.79 ± 2.04	16.22 ± 2.39	6.28 ± 0.56	6.39 ± 0.35
W4	6.76 ± 0.21	6.54 ± 0.53	31.75 ± 4.87	29.25 ± 3.12	182.25 ± 16.91	161.25 ± 19.60	4.66 ± 0.64	4.44 ± 0.20	26.86 ± 2.06	24.51 ± 1.89	5.92 ± 0.42	5.51 ± 0.28
W5	10.19 ± 0.78	8.20 ± 1.38	25.25 ± 1.03	19.50 ± 3.48	172.50 ± 20.07	124.50 ± 20.11	2.51 ± 0.17	2.56 ± 0.48	17.02 ± 1.84	15.47 ± 1.60	6.91 ± 1.01	6.73 ± 1.27
W6	8.42 ± 2.12	10.06 ± 1.45	25.00 ± 2.04	20.00 ± 1.87	134.75 ± 11.98	141.50 ± 16.15	**3.69 ± 1.12**	**2.06 ± 0.19**	18.01 ± 2.66	14.40 ± 1.09	5.55 ± 0.75	7.05 ± 0.41
D1	9.34 ± 0.53	8.86 ± 0.73	25.50 ± 2.66	20.25 ± 1.65	**182.75 ± 32.79**	**122.75 ± 14.35**	2.72 ± 0.21	2.29 ± 0.05	19.35 ± 2.65	13.80 ± 0.75	7.34 ± 1.38	6.02 ± 0.25
D2	8.28 ± 1.12	8.29 ± 0.88	18.50 ± 3.01	20.50 ± 1.44	126.50 ± 15.77	134.00 ± 10.27	2.26 ± 0.31	2.54 ± 0.26	15.39 ± 0.59	16.59 ± 1.67	7.10 ± 0.73	6.55 ± 0.28
D3	8.79 ± 0.94	10.41 ± 1.54	24.75 ± 4.37	24.00 ± 5.76	155.00 ± 21.00	166.25 ± 31.60	2.75 ± 0.24	2.23 ± 0.31	17.53 ± 1.27	15.71 ± 1.23	6.45 ± 0.46	7.28 ± 0.61
D4	7.09 ± 0.91	6.91 ± 1.70	19.75 ± 3.35	19.67 ± 3.18	113.50 ± 10.18	103.33 ± 15.07	2.91 ± 0.58	3.07 ± 0.47	16.80 ± 2.53	16.18 ± 2.50	6.01 ± 0.52	5.32 ± 0.50
D5	8.13 ± 1.64	6.99 ± 1.81	22.75 ± 2.81	19.00 ± 1.78	109.00 ± 22.01	84.50 ± 8.76	3.24 ± 0.91	3.14 ± 0.57	14.10 ± 2.50	13.86 ± 2.42	5.06 ± 1.06	4.44 ± 0.14
P. nodosum												
W7	7.98 ± 1.01	11.04 ± 1.58	25.00 ± 4.34	30.50 ± 5.24	159.25 ± 26.89	180.50 ± 15.99	3.15 ± 0.36	2.85 ± 0.51	19.78 ± 1.56	17.37 ± 3.06	6.41 ± 0.44	6.27 ± 0.81
W8	8.15 ± 1.23	5.31 ± 1.17	41.00 ± 7.43	35.50 ± 1.76	250.50 ± 40.51	196.75 ± 10.96	**5.06 ± 0.61**	**8.04 ± 2.08**	30.54 ± 0.57	41.85 ± 7.39	6.29 ± 0.70	5.62 ± 0.55
W9	3.26 ± 0.19	2.87 ± 0.58	21.50 ± 2.60	15.75 ± 2.14	189.00 ± 35.42	124.25 ± 16.53	6.79 ± 1.07	6.10 ± 1.06	59.91 ± 13.48	48.42 ± 9.87	8.76 ± 1.02	8.10 ± 1.02
D6	7.49 ± 0.99	7.12 ± 1.43	40.75 ± 7.70	35.50 ± 3.95	**341.00 ± 44.20**	**244.75 ± 35.17**	5.69 ± 1.05	5.63 ± 1.44	47.81 ± 7.37	35.63 ± 2.15	8.68 ± 0.85	7.08 ± 0.98
D7	3.61 ± 0.41	3.01 ± 0.57	24.00 ± 3.05	28.67 ± 5.49	185.66 ± 19.46	179.00 ± 40.58	6.64 ± 0.31	9.81 ± 1.81	51.91 ± 4.80	58.97 ± 3.28	7.79 ± 0.43	6.33 ± 0.88
P. alpinum												
W10	1.24 ± 0.36	1.16 ± 0.14	6.25 ± 1.93	4.50 ± 0.65	25.50 ± 6.76	19.00 ± 3.03	6.12 ± 1.79	4.14 ± 0.85	24.20 ± 5.29	16.91 ± 2.73	4.21 ± 0.35	4.29 ± 0.44
W11	0.52 ± 0.02	0.55 ± 0.16	4.00 ± 0.00	6.25 ± 0.48	25.75 ± 0.95	32.75 ± 1.93	7.79 ± 0.22	17.30 ± 7.42	50.28 ± 3.14	91.90 ± 40.86	**6.44 ± 0.24**	**5.29 ± 0.30**
D8	0.80 ± 0.25	0.72 ± 0.25	5.00 ± 1.53	4.67 ± 0.88	19.67 ± 7.31	16.33 ± 3.67	6.73 ± 1.16	8.15 ± 2.45	25.12 ± 3.55	26.97 ± 6.17	3.78 ± 0.26	3.44 ± 0.29

Data in bold indicates significant difference between NWL and WL ($p < 0.05$, sub-design ANOVA).

Table S3. Growth rates and generative tillers. Growth rates were calculated as produced number of tillers (ΔTN) and leaves (ΔLN) during the treatment, and exponential growth rates of the number of tillers (RGR-TN) and leaves (RGR-LN) in *P. pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) treatment. Values are means and standard error (± SE) of plants within each accession. Number of plants with generative tillers of the total number of plants per accession.

Accessions	**ΔTN (# plant^{-1})**		**ΔLN (# plant^{-1})**		**RGR-TN (# day^{-1})**		**RGR-LN (# day^{-1})**		**Number of plants with generative tillers**	
	NWL	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**
P. pratense										
W1	12.25 ± 2.29	11.25 ± 1.65	47.50 ± 7.90	89.00 ± 11.19	0.057 ± 0.003	0.046 ± 0.006	**0.057 ± 0.003**	**0.076 ± 0.005**	3/4	4/4
W2	16.75 ± 2.50	12.75 ± 2.06	119.25 ± 13.75	98.00 ± 17.13	0.056 ± 0.005	0.057 ± 0.009	0.077 ± 0.001	0.081 ± 0.005	2/4	4/4
W3	12.75 ± 1.03	14.50 ± 2.25	106.75 ± 20.56	113.50 ± 21.70	0.046 ± 0.004	0.047 ± 0.006	0.073 ± 0.004	0.070 ± 0.010	4/4	4/4
W4	22.75 ± 4.23	20.75 ± 2.95	144.50 ± 15.55	124.75 ± 15.25	0.061 ± 0.005	0.062 ± 0.007	0.076 ± 0.004	0.072 ± 0.005	0/4	0/4
W5	17.00 ± 1.41	12.25 ± 2.32	137.50 ± 14.01	98.00 ± 16.70	0.053 ± 0.006	0.048 ± 0.005	0.077 ± 0.006	0.072 ± 0.003	1/4	2/4
W6	15.75 ± 1.80	11.75 ± 2.10	104.25 ± 9.99	108.50 ± 12.98	0.046 ± 0.003	0.044 ± 0.007	0.071 ± 0.002	0.068 ± 0.003	2/4	2/4
D1	16.50 ± 2.33	12.00 ± 1.08	**147.75 ± 30.08**	**94.50 ± 10.20**	0.048 ± 0.004	0.043 ± 0.002	0.076 ± 0.004	0.069 ± 0.002	4/4	2/4
D2	12.00 ± 2.38	13.50 ± 1.32	98.75 ± 15.17	105.00 ± 6.75	0.046 ± 0.003	0.049 ± 0.004	0.070 ± 0.005	0.074 ± 0.003	3/4	3/4
D3	16.75 ± 3.33	14.75 ± 4.75	123.50 ± 16.18	132.50 ± 27.76	0.051 ± 0.003	0.041 ± 0.008	0.076 ± 0.004	0.075 ± 0.007	1/4	4/4
D4	14.75 ± 3.09	12.67 ± 1.33	93.50 ± 10.18	77.00 ± 12.66	0.063 ± 0.006	0.051 ± 0.005	0.081 ± 0.004	0.067 ± 0.009	4/4	3/3
D5	14.75 ± 2.72	13.00 ± 0.71	75.75 ± 18.08	60.75 ± 5.31	0.046 ± 0.005	0.059 ± 0.004	0.055 ± 0.004	0.063 ± 0.005	3/4	4/4
P. nodosum										
W7	16.25 ± 3.04	19.25 ± 3.57	126.75 ± 20.87	136.00 ± 13.95	0.049 ± 0.001	0.049 ± 0.006	0.074 ± 0.002	0.070 ± 0.006	1/4	3/4
W8	28.50 ± 5.52	25.50 ± 1.32	205.25 ± 34.99	162.00 ± 8.80	0.060 ± 0.007	0.063 ± 0.003	0.084 ± 0.006	0.083 ± 0.003	3/4	2/4
W9	16.25 ± 2.46	11.00 ± 2.20	170.25 ± 36.48	106.25 ± 15.27	0.067 ± 0.005	0.058 ± 0.008	**0.106 ± 0.010**	**0.091 ± 0.004**	3/4	3/4
D6	30.25 ± 6.42	25.25 ± 3.86	**298.75 ± 38.51**	**205.75 ± 30.71**	0.064 ± 0.003	0.059 ± 0.006	0.097 ± 0.002	0.087 ± 0.001	4/4	4/4
D7	18.00 ± 2.08	24.00 ± 5.51	164.67 ± 19.78	162.33 ± 39.35	**0.066 ± 0.001**	**0.086 ± 0.009**	0.101 ± 0.006	0.110 ± 0.007	3/3	3/3
P. alpinum										
W10	3.50 ± 1.55	1.75 ± 0.63	17.00 ± 5.37	9.00 ± 2.74	0.038 ± 0.011	0.025 ± 0.009	**0.053 ± 0.006**	**0.031 ± 0.006**	0/4	0/4
W11	1.50 ± 0.50	2.75 ± 0.75	15.25 ± 0.48	21.75 ± 2.32	0.026 ± 0.011	0.028 ± 0.008	0.043 ± 0.005	0.050 ± 0.005	0/4	0/4
D8	2.33 ± 0.88	2.33 ± 0.33	12.00 ± 5.51	8.67 ± 1.45	0.043 ± 0.012	0.029 ± 0.003	0.046 ± 0.012	0.042 ± 0.008	0/3	0/3

Data in bold indicates significant difference between NWL and WL ($p < 0.05$, sub-design ANOVA).

Table S4. Root traits of *P. pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) at the end of the 21 days treatment. **.** Root dry weight (RDW), percent root weight of total plant dry weight (%RDW) and ratio of dry weight of the upper 5cm of the root system to the dry weight of the total root (5RDW:RDW). Values are means and standard error (± SE) of plants within an accession.

		RDW (g)		%RDW		5RDW:RDW	
Acc. ID-SLU	**Species**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**
W1	*P. pratense*	1.22 ± 0.18	1.24 ± 0.13	19.55 ± 1.06	16.60 ± 0.99	**0.38 ± 0.03**	**0.48 ± 0.02**
W2	*P. pratense*	1.77 ± 0.47	1.15 ± 0.14	**21.34 ± 2.74**	**14.35 ± 1.29**	**0.30 ± 0.02**	**0.56 ± 0.03**
W3	*P. pratense*	1.92 ± 0.34	1.37 ± 0.05	**18.71 ± 0.60**	**13.27 ± 0.82**	**0.25 ± 0.00**	**0.45 ± 0.02**
W4	*P. pratense*	**2.68 ± 0.38**	**1.54 ± 0.11**	**27.99 ± 2.43**	**19.27 ± 1.62**	**0.25 ± 0.01**	**0.42 ± 0.03**
W5	*P. pratense*	**2.43 ± 0.08**	**1.44 ± 0.17**	19.40 ± 0.76	15.45 ± 1.64	**0.27 ± 0.01**	**0.44 ± 0.06**
W6	*P. pratense*	**1.71 ± 0.15**	**2.48 ± 0.15**	18.50 ± 2.78	20.43 ± 1.91	**0.28 ± 0.02**	**0.40 ± 0.05**
D1	*P. pratense*	2.14 ± 0.20	2.11 ± 0.21	18.53 ± 0.74	19.23 ± 1.03	**0.29 ± 0.03**	**0.41 ± 0.06**
D2	*P. pratense*	1.91 ± 0.24	1.50 ± 0.22	18.96 ± 1.63	15.67 ± 2.69	**0.29 ± 0.03**	**0.46 ± 0.06**
D3	*P. pratense*	2.59 ± 0.41	2.25 ± 0.20	22.47 ± 1.25	18.23 ± 1.81	0.28 ± 0.02	0.39 ± 0.03
D4	*P. pratense*	1.56 ± 0.15	1.21 ± 0.27	18.62 ± 2.67	15.31 ± 1.30	0.25 ± 0.02	0.34 ± 0.07
D5	*P. pratense*	**2.79 ± 0.31**	**1.09 ± 0.26**	**26.76 ± 3.83**	**13.66 ± 0.50**	**0.30 ± 0.07**	**0.51 ± 0.05**
Mean ± SE		2.07 ± 0.11	1.59 ± 0.09	20.98 ± 0.75	16.52 ± 0.55	0.29 ± 0.01	0.45 ± 0.02
W7	*P. nodosum*	2.36 ± 0.46	2.76 ± 0.24	22.27 ± 1.85	20.63 ± 2.55	**0.24 ± 0.02**	**0.45 ± 0.03**
W8	*P. nodosum*	**1.90 ± 0.32**	**0.73 ± 0.10**	**18.73 ± 1.17**	**12.83 ± 1.34**	**0.27 ± 0.02**	**0.70 ± 0.05**
W9	*P. nodosum*	0.55 ± 0.08	0.24 ± 0.05	**14.38 ± 1.53**	**7.90 ± 0.77**	**0.35 ± 0.07**	**0.77 ± 0.05**
D6	*P. nodosum*	1.71 ± 0.43	1.00 ± 0.29	**18.42 ± 2.97**	**11.79 ± 1.23**	**0.34 ± 0.03**	**0.66 ± 0.04**
D7	*P. nodosum*	0.77 ± 0.08	0.41 ± 0.05	17.62 ± 0.27	12.37 ± 1.67	**0.31 ± 0.01**	**0.69 ± 0.06**
Mean ± SE		1.43 ± 0.21	1.06 ± 0.23	18.32 ± 0.97	13.14 ± 1.19	0.30 ± 0.02	0.65 ± 0.03
W10	*P. alpinum*	0.53 ± 0.15	0.25 ± 0.02	**29.16 ± 2.51**	**17.73 ± 1.61**	**0.27 ± 0.03**	**0.73 ± 0.04**
W11	*P. alpinum*	**0.63 ± 0.02**	**0.27 ± 0.02**	**54.91 ± 1.37**	**37.33 ± 7.85**	**0.21 ± 0.01**	**0.71 ± 0.04**
D8	*P. alpinum*	0.51 ± 0.21	0.21 ± 0.10	**34.72 ± 7.37**	**20.53 ± 3.25**	**0.40 ± 0.19**	**0.68 ± 0.03**
Mean ± SE		0.56 ± 0.07	0.25 ± 0.03	40.04 ± 4.12	25.62 ± 3.94	0.28 ± 0.05	0.71 ± 0.02

Data in bold indicates significant difference between NWL and WL ($p < 0.05$, ANOVA)

Table S5. Root anatomy traits of accessions of *Phleum pratense*, *P. nodosum* and *P. alpinum* in non-waterlogging (NWL) and waterlogging (WL) conditions at the end of the 21 days treatment. The traits shown are root cross section area (RA), stele area (SA), ratios of the cortex area to root cross section area (CEA:RA), the aerenchyma to cortex area (AA:CEA), the cortex to stele area (CEA:SA) and the stele area to root cross section area (SA:RA). Values are means and standard error (± SE) of plants within an accession.

		RA (mm^2)		SA (mm^2)		CEA:RA		AA:CEA		CEA:SA		SA:RA	
Acc. ID-SLU	**Species**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**	**NWL**	**WL**
W1	*P. pratense*	1.47 ±0.19	1.82 ± 0.15	0.17 ± 0.03	0.16 ±0.01	**0.89 ±0.01**	**0.91 ±0.01**	**0.026 ±0.010**	**0.27 ± 0.03**	**8.0±0.6**	**10.9±0.9**	**0.11 ±0.01**	**0.09 ±0.01**
W2	*P. pratense*	1.64 ±0.14	1.92 ± 0.25	0.20 ± 0.02	0.19 ±0.04	**0.88 ±0.01**	**0.90 ±0.01**	**0.046 ±0.012**	**0.26 ± 0.01**	**7.2±0.5**	**9.9±1.2**	**0.13 ±0.01**	**0.10 ±0.01**
W3	*P. pratense*	1.69 ±0.20	2.07 ± 0.24	0.23 ± 0.03	0.19 ±0.02	**0.87 ±0.01**	**0.91 ±0.01**	**0.039 ±0.017**	**0.34 ± 0.02**	**6.7±0.5**	**10±0.8**	**0.14 ±0.01**	**0.10 ±0.01**
W4	*P. pratense*	1.60 ±0.11	1.78 ± 0.11	0.20 ± 0.03	0.18 ±0.02	**0.88 ±0.01**	**0.90 ±0.01**	**0.031 ±0.015**	**0.28 ± 0.02**	**7.4±0.5**	**9.5±0.7**	**0.12 ±0.01**	**0.10 ±0.01**
W5	*P. pratense*	**1.78 ±0.12**	**2.45 ± 0.17**	0.24 ± 0.02	0.29 ±0.01	0.86 ±0.01	0.88 ±0.01	**0.060 ±0.013**	**0.33 ± 0.03**	6.5±0.4	7.5±0.4	0.14 ±0.01	0.12 ±0.01
W6	*P. pratense*	**1.32 ±0.14**	**2.26 ± 0.19**	**0.15 ± 0.02**	**0.24 ±0.02**	0.88 ±0.01	0.89 ±0.00	**0.032 ±0.016**	**0.34 ± 0.04**	7.7±0.8	8.4±0.3	0.12 ±0.01	0.11 ±0.00
D1	*P. pratense*	1.65 ±0.11	1.31 ± 0.45	**0.29 ± 0.02**	**0.13 ±0.04**	**0.82 ±0.01**	**0.90 ±0.01**	**0.022 ±0.009**	**0.32 ± 0.03**	**4.8±0.3**	**9.1±0.9**	**0.18 ±0.01**	**0.11 ±0.01**
D2	*P. pratense*	1.59 ±0.15	1.54 ± 0.19	0.27 ± 0.03	0.22 ±0.03	**0.83 ±0.01**	**0.86 ±0.00**	**0.062 ±0.020**	**0.21 ± 0.03**	5.0±0.4	6.0±0.2	**0.17 ±0.01**	**0.14 ±0.00**
D3	*P. pratense*	**1.99 ±0.20**	**2.96 ± 0.24**	0.27 ± 0.02	0.26 ±0.02	**0.86 ±0.01**	**0.91 ±0.01**	**0.069 ±0.020**	**0.36 ± 0.03**	**6.4±0.5**	**10.7±1.2**	**0.14 ±0.01**	**0.09 ±0.01**
D4	*P. pratense*	**1.64 ±0.13**	**2.20 ± 0.16**	0.23 ± 0.02	0.25 ±0.03	**0.86 ±0.01**	**0.89 ±0.01**	**0.063 ±0.017**	**0.31 ± 0.03**	**6.3±0.3**	**8.2±0.9**	**0.14 ±0.01**	**0.12 ±0.01**
D5	*P. pratense*	2.03 ±0.20	1.78 ± 0.17	0.30 ± 0.04	0.25 ±0.03	0.85 ±0.01	0.86 ±0.01	0.081 ±0.029	0.11 ± 0.03	5.8±0.5	6.4±0.4	0.15 ±0.01	0.14 ±0.01
Mean ± SE		1.66 ±0.05	2.03 ± 0.08	0.23 ± 0.009	0.21 ±0.009	0.861 ±0.003	0.892 ±0.003	0.047 ±0.005	0.288 ±0.011	6.5±0.2	8.8±0.3	0.139 ±0.003	0.108 ±0.003
W7	*P.nodosum*	**2.05 ±0.11**	**2.59 ± 0.15**	**0.32 ± 0.02**	**0.26 ±0.02**	**0.85 ±0.00**	**0.90 ±0.01**	**0.065 ±0.026**	**0.45 ± 0.02**	**5.5±0.2**	**9.3±0.7**	**0.16 ±0.00**	**0.10 ±0.01**
W8	*P.nodosum*	0.92 ±0.06	1.13 ± 0.11	0.15 ± 0.01	0.13 ±0.01	**0.84 ±0.01**	**0.88 ±0.01**	**0.000 ±0.000**	**0.20 ± 0.03**	**5.6±0.6**	**7.6±0.6**	**0.16 ±0.01**	**0.12 ±0.01**
W9	*P.nodosum*	0.59 ±0.06	0.79 ± 0.05	0.09 ± 0.01	0.09 ±0.01	**0.86 ±0.01**	**0.89 ±0.01**	**0.000 ±0.000**	**0.14 ± 0.02**	**6.0±0.3**	**8.4±0.6**	**0.14 ±0.01**	**0.11 ±0.01**
D6	*P.nodosum*	**0.78 ±0.12**	**1.22 ± 0.10**	**0.13 ± 0.02**	**0.17 ±0.02**	**0.84 ±0.01**	**0.86 ±0.01**	**0.008 ±0.008**	**0.18 ± 0.04**	5.2±0.4	6.3±0.3	**0.17 ±0.01**	**0.14 ±0.01**
D7	*P.nodosum*	**0.88 ±0.08**	**1.34 ± 0.25**	0.12 ± 0.01	0.15 ±0.02	**0.86 ±0.01**	**0.89 ±0.01**	**0.000 ±0.000**	**0.17 ± 0.03**	**6.4±0.3**	**7.9±0.5**	**0.14 ±0.01**	**0.11 ±0.01**
Mean ± SE		1.05 ±0.09	1.38 ± 0.12	0.16 ± 0.015	0.16 ±0.011	0.848 ±0.004	0.883 ±0.004	0.015 ±0.007	0.221 ±0.021	5.2±0.2	6.1±0.3	0.152 ±0.004	0.117 ±0.004
W10	*P. alpinum*	**1.38 ±0.12**	**1.77 ± 0.08**	0.26 ± 0.02	0.27 ±0.03	**0.81 ±0.01**	**0.85 ±0.02**	**0.037 ±0.014**	**0.15 ± 0.04**	4.3±0.3	5.8±0.8	**0.19 ±0.01**	**0.15 ±0.02**
W11	*P. alpinum*	1.38 ±0.09	1.51 ± 0.08	0.21 ± 0.02	0.22 ±0.01	0.85 ±0.01	0.85 ±0.01	**0.037 ±0.015**	**0.12 ± 0.03**	5.7±0.3	5.9±0.4	0.15 ±0.01	0.15 ±0.01
D8	*P. alpinum*	**1.19 ±0.16**	**1.63 ± 0.14**	0.18 ± 0.02	0.22 ±0.02	0.85 ±0.01	0.86 ±0.01	**0.020 ±0.013**	**0.13 ± 0.02**	5.5±0.2	6.5±0.6	0.15 ±0.01	0.14 ±0.01
Mean ± SE		1.33 ±0.07	1.61 ± 0.07	0.22 ± 0.013	0.23 ±0.011	0.836 ±0.006	0.854 ±0.006	0.033 ±0.008	0.131 ±0.015	5.7±0.2	7.9±0.3	0.165 ±0.006	0.146 ±0.006

Data in bold indicates significant difference between NWL and WL ($p < 0.05$, ANOVA).

Table S6. WL response index of *P. pratense*, *P. nodosum* and *P. alpinum*, calculated as the proportional difference between the WL and NWL.

Traits	*P. pratense*	*P. nodosum*	*P. alpinum*
SDW	0.034^{a} ± 0,043	-0.014^{a} ± 0,093	-0.025^{a} ± 0,135
RGR-LN	0.016^{a} ± 0,028	-0.048^{a} ± 0,029	-0.106^{a} ± 0,103
RGR-TN	-0.034^{a} ± 0,037	0.013^{a} ± 0,053	0.028^{a} ± 0,144
%RDW	-0.198^{a} ± 0,032	-0.320^{ab} ± 0,045	-0.370^{b} ± 0,056
5RDW:RDW	0.573^{a} ± 0,056	1.062^{b} ± 0,093	1.717^{c} ± 0,259
AA:CEA	6.337^{b} ± 0,436	15.62^{a*} ± 2,678	3.77^{b} ± 0,605
CEA:SA	0.358^{a} ± 0,043	0.374^{a} ± 0,049	0.152^{a} ± 0,062

*Based on two accession that formed AA in NWL conditions. Three accessions have no AA in NWL.

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Climate change causes abiotic stresses on agriculture and tolerant crops are needed. This thesis explores the diversity of wild and domesticated accessions of the important forage grass timothy (*Phleum pratense*) and two closely related species, *P. nodosum* and *P. alpinum*. Large variation in response of plant traits and transcriptome to waterlogging and drought made it possible to identify traits and accessions important for stress tolerance. The findings are valuable as resources for further pre-breeding and development of cultivars.

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