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# Characterisation of fructan accumulation in grasses and its implication for cold adaptation and overwintering

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Lektorutdanning i realfag

**Abstract**

Fructans are fructose-based oligo- and polysaccharides that occur in many plants in temperate climates. Beside its main function as storage carbohydrate, fructan has been suggested to play a role in temperate adaptations, e.g., as cryoprotectant or by increasing photosynthesis efficiency at low temperatures. Here, I investigate the role of fructans in winter survival and global distribution of perennial temperate grasses by conducting a common garden experiment with 35 phylogenetically diverse species. Sampling of crown material was performed once a month from 30th October 2019 to 29th April 2020 and fructan content in the samples was analysed and quantified with high performance anion exchange chromatography coupled with pulsed amperometric detection. The results were combined with a phylogenetic tree to investigate the correlation of fructan accumulation with regrowth in spring as well as global distribution data. I identified several new fructan accumulators in the Pooideae subfamily and my results reveal a large variety in fructan types among Pooideae species. The observed phylogenetic pattern of fructan types suggests several independent origins of FTs and FEHs in the subfamily. Furthermore, fructan amount throughout the winter showed a clear correlation with regrowth ability in spring, a measure of frost tolerance and winter endurance. A reciprocal relationship was found between the bioclimatic variable isothermality and amount of fructan throughout the winter months. A phylogenetic analysis of variance with global distribution data revealed a striking pattern of fructan accumulators being able to survive in areas with longer winters than non-fructan accumulators.

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Eirin Landsem Ås, 31th May

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## Abbreviations

| | | | |
|---|---|---|---|
| **1-FFT** | Fructan:Fructan 1- fructosyltransferase | **Glc** | Glucose |
| **1-K** | 1-Kestose | **HPAEC-PAD** | high-performance anion-exchange chromatography with pulsed amperometric detection |
| **1-SST** | Sucrose:Sucrose 1-fructosyltransferase | **ML** | Maximum likelihood |
| **6G-FFT** | Fructan:Fructan 6G- fructosyltransferase | **MRCA** | Most recent common ancestor |
| **6-K** | 6-Kestose | **myr** | million years |
| **6-SFT** | Sucrose:Fructan 6- fructosyltransferase | **NK** | Neokestose |
| **6-SST** | Sucrose:Sucrose 6-fructosyltransferase | **Nys** | Nystose |
| **AH** | Acid hydrolysis | **PGLS** | Phylogenetic generalised least squares |
| **AUC** | Area under the curve | **PH** | Post hydrolysis |
| **BH** | before hydrolysis | **pPCA** | Phylogenetic Principal component analysis |
| **CA** | Cold acclimation | **Raf** | Raffinose |
| **DP** | degree of polymerisation | **RFO** | Raffinose family oligosaccharides |
| **DW** | dry weight | **ROS** | Reactive oxygen species |
| **FEH** | Fructan exohydrolase | **Sta** | Stachyose |
| **Fru** | Fructose | **Suc** | Sucrose |
| **FT** | Fructosyl transferase (enzyme) | **TF** | Total fructan content |
| ***FT*** | Fructosyl transferase (gene) | **WSC** | Water-soluble carbohydrates |

# 1 Introduction

## 1.1 Surviving in long, cold darkness

To survive in temperate and cold climates, overwintering plants (hereafter plants) need to endure severe abiotic stresses such as longer periods of short days, frost, and snow cover. To cope with such stresses, plants have evolved the ability to undergo a process called cold acclimation (CA) (Panter et al.; Xin & Browse, 2000; Körner, 2016; Nievola et al., 2017; Ritonga & Chen, 2020). Cold acclimation occurs under low, non-freezing temperatures and increase the frost tolerance in plants. Cold acclimating plants undergo multiple physiological and biochemical changes, such as the accumulation of carbohydrates and other osmoprotectants, changes in membrane composition and reduction in growth which are regulated trough changes in gene transcription and hormonal balance (Xin & Browse, 2000; Valluru & Van den Ende, 2008; Tarkowski & Van den Ende, 2015). When the temperature falls below zero degrees, sub-zero acclimation, or "second phase hardening", occurs in some cold adapted plants (Livingston & Henson, 1998; Livingston et al., 2009). In this process, increased activity of hydrolysing enzymes, and thus increased hydrolysis of storage carbohydrates, leads to an increased level of low molecular weight carbohydrates. Sub-zero acclimation has been shown to increase the freezing tolerance of plants even further (Livingston & Henson, 1998; Livingston et al., 2009).

A big challenge for plants at high latitudes is the length of the winter and hence, a long period in which they cannot photosynthesise. The initial chilling stress in early autumn slows down metabolism and inhibits growth, which leads to a build-up of sucrose (Suc) and other photosynthetic metabolites (Versluys et al., 2018). In turn, increased mono- and disaccharide levels initiate the synthesis of storage carbohydrates (Versluys et al., 2018). For overwintering plants, a large carbohydrate storage is vital to maintain the cellular functions in their dormant organs and meristems, and to kickstart regrowth early in the next spring (Rohde & Bhalerao, 2007). To manage this, plants must store enough carbohydrates in autumn before temperatures drop to a point where photosynthesis ceases and photosynthetic tissue wilts.

When temperatures reach the freezing point, another major challenge for plants emerges, i.e., ice formation in plant tissues (Panter et al.; Xin & Browse, 2000; Valluru & Van den Ende, 2008; Ritonga & Chen, 2020). The formation of ice crystals is induced in the extracellular spaces and cell walls of plants, because of a high concentration of nucleation agents and low

concentration of osmoregulatory metabolites (Xin & Browse, 2000; Ritonga & Chen, 2020). Due to the low water potential of ice, the freezing of extracellular spaces leads to a difference in water potential between the extracellular and intracellular space, which in turn leads to loss of water from the cytoplasm (Panter et al.; Xin & Browse, 2000; Körner, 2016; Ritonga & Chen, 2020). This cellular dehydration also occurs under salt and drought stress and is the reason why several of the mechanisms involved in freeze-, drought- and salt tolerance are similar. The amount of water loss depends on the cytoplasmic solute concentration and the freezing temperature (Xin & Browse, 2000). Despite the accumulation of water soluble carbohydrates and other osmoregulatory metabolites during CA, the resulting freezing point depression is too small to bridge the gap in water potential between the extracellular and intracellular space (Körner, 2016). However, it has been shown that carbohydrates accumulate in specific regions of the apoplast which may lead to high local concentrations and thus adhesion prevention in critical interfaces (Livingston & Henson, 1998).

The plasma membrane is considered the primary site of freezing injury and can be affected in several ways (Panter et al.; Xin & Browse, 2000; Valluru & Van den Ende, 2008; Ritonga & Chen, 2020). As a result of the reduction in intracellular liquid, parts of the cellular membrane may bud off and the cell thus bursts upon thawing (Panter et al.; Xin & Browse, 2000; Ritonga & Chen, 2020). Furthermore, proximity of different cellular membranes may lead to phase separation and hence disruption of the bilayers (Panter et al.; Xin & Browse, 2000; Ritonga & Chen, 2020). Therefore, stabilisation and protection of the plasma membrane during frost is vital for winter survival of plants.

Reactive oxygen species (ROS) production has also been shown to increase drastically in response to abiotic stresses, such as low temperatures (Huang et al., 2019). ROS can cause major damage to plants by e.g., irreversible DNA damage, modification of cellular components and cell death (Huang et al., 2019). Thus, temperate plants must possess effective antioxidating mechanisms to counteract oxidative stress.

Deep snow covers have an insulating and protective effect on overwintering plant tissues (Kawakami & Yoshida, 2012). However, the resulting dark and humid conditions increase the risk of infections with snow moulds which can be lethal for plants (Kawakami & Yoshida, 2012). It has been shown that accumulation of water-soluble carbohydrates during CA increase the resistance to such infections (Kawakami & Yoshida, 2012).

## 1.2 Multifunctional water-soluble carbohydrates

The main function of water-soluble carbohydrate (WSC) accumulation is energy storage. However, research on some types of carbohydrates has shown that WSCs may serve other important functions in CA and provide solutions to the challenges described above (Livingston & Henson, 1998; Vijn & Smeekens, 1999; Hisano et al., 2008; Valluru & Van den Ende, 2008; Livingston et al., 2009; Kawakami & Yoshida, 2012; Yoshida & Kawakami, 2012; Elsayed et al., 2013; Tarkowski & Van den Ende, 2015; Versluys et al., 2018; Tarkowski et al., 2019)

Fructans and raffinose family oligosaccharides (RFOs) are two of the most important classes of WSCs and have been reported to be associated with stress responses in plants (Van den Ende, 2013). RFOs are a group of non-structural, WSCs that consist of a Suc residue with an α1,6-linkage to one or more galactosyl residues (Van den Ende, 2013; Sengupta et al., 2015). RFO accumulators have been found in Cucurbitaceae, Lamiaceae, Leguminosae, Oleaceae, Vitaceae, Pinus and Poaceae (Elsayed et al., 2013). The smallest RFO, raffinose (Raf), is ubiquitous in plants whereas stachyose (Sta) and higher degree of polymerisation (DP), i.e., the number of carbohydrates monomer units in the molecule, RFOs mostly occur in true RFO accumulators (Van den Ende, 2013). So called "Alternative" RFOs do not contain the terminal fructosyl unit, as shown in Figure 1.

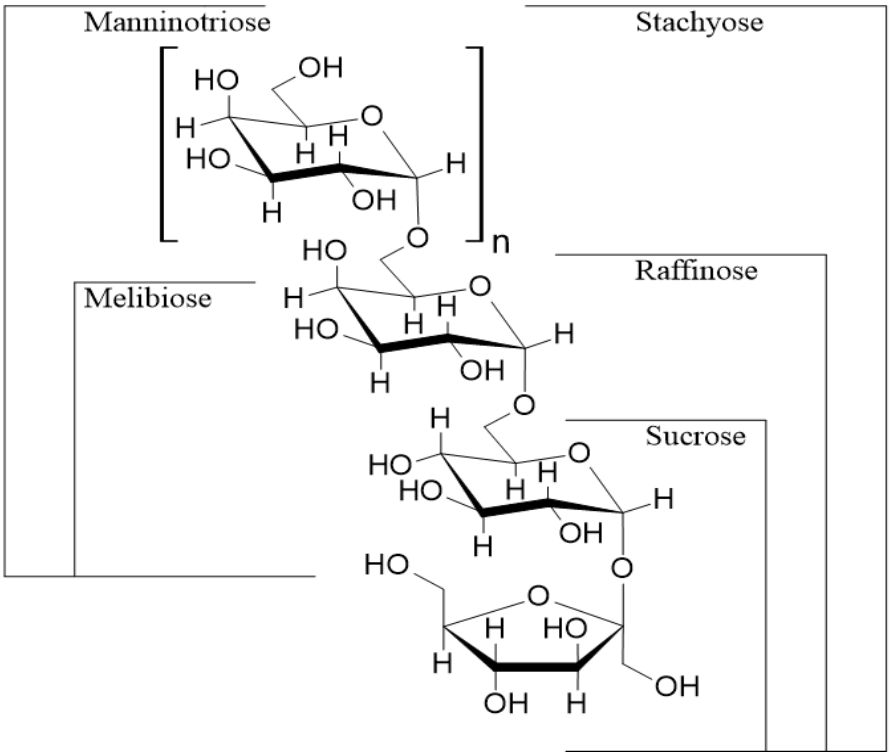


**Figure 1.** Low DP RFO's and "Alternative" RFOs edited after Obendorf et al. (2012)

Fructans are fructose (Fru)-based, non-structural oligo- and polysaccharides found across several biological kingdoms (Livingston et al., 2009; Van den Ende, 2013; Versluys et al., 2018). Fructans are synthesised by different fructosyltransferases (FTs) which utilise Suc or precursor fructan oligomers as substrate (Van den Ende, 2013). Six classes of fructan types have been distinguished in plants, where each class is characterised by the type of fructosyl-linkage to the starter Suc unit. These classes are levan series fructans, graminan series fructans, levan neoseries fructans, inulin neoseries fructans, inulin series fructans and agavins (Vijn & Smeekens, 1999; Tarkowski et al., 2019). Agavins are fructan types only found in the Agavaceae (Versluys et al., 2018). The biosynthetic pathway of the different fructan types begins with a Suc molecule being converted to either 1-kestose (1-K) or 6-kestose (6-K) by sucrose:sucrose 1-fructosyltransferase (1-SST) or sucrose:sucrose 6-fructosyltransferase (6-SST), respectively (see Figure 2).

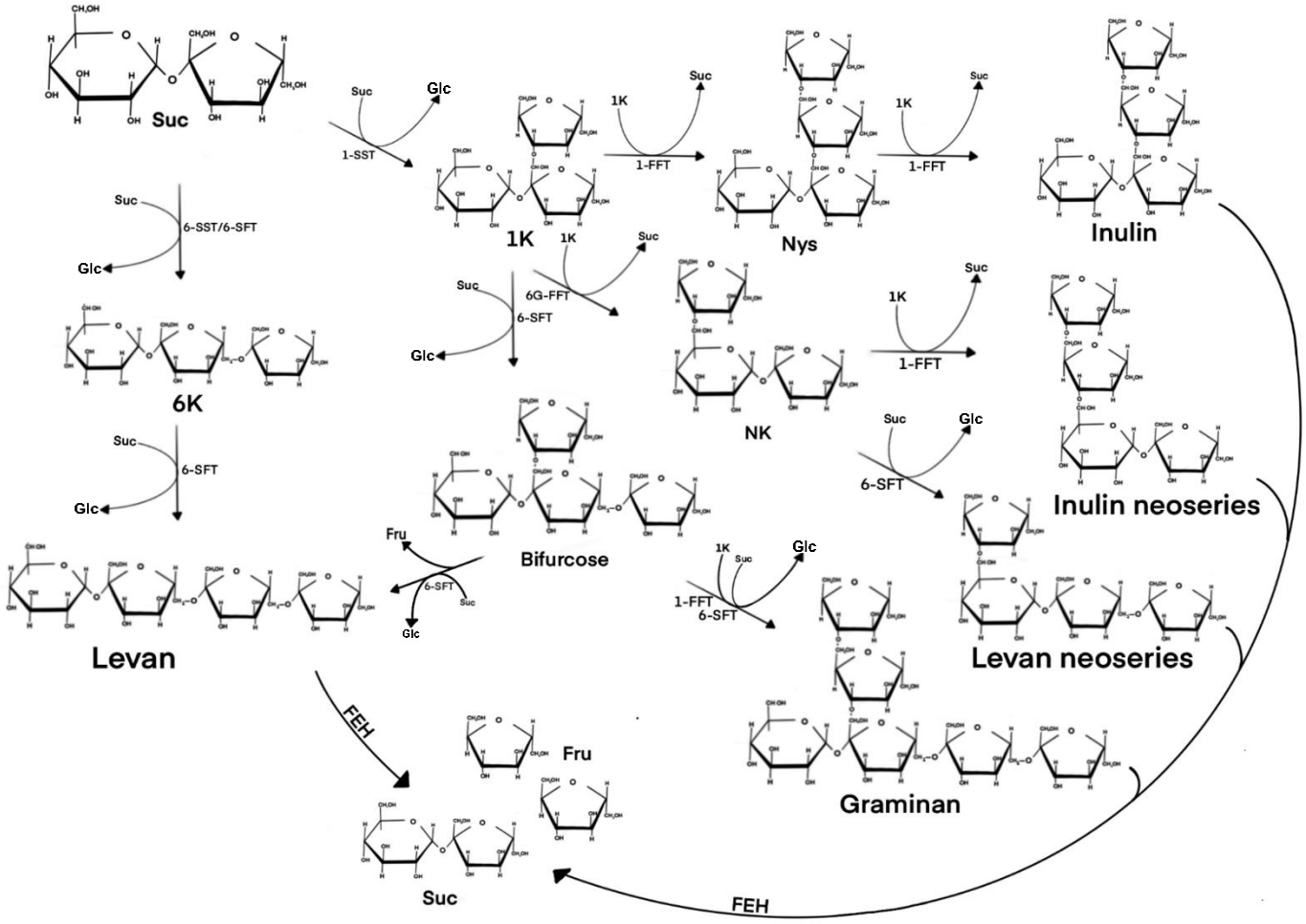


**Figure 2.** The biosynthetic pathway of fructan synthesis. Edited after Tarkowski et al. (2019). 1-FFT: Fructan:Fructan 1-fructosyltransferase, 1-SST: Sucrose:Sucrose 1-fructosyltransferase, 6G-FFT: Fructan:Fructan 6G- fructosyltransferase, 6-SFT: Sucrose:Fructan 6- fructosyltransferase, 6-SST: Sucrose:Sucrose 6-fructosyltransferase, 1K: 1-Kestose, 6K:6-Kestose, NK: Neokestose, Nys: Nystose, Suc: Sucrose, Glc: Glucose

Fructans are degraded by Fructan exohydrolases (FEHs). FTs and FEHs have evolved from vacuolar invertases and cell wall invertases, respectively (Ritsema & Smeekens, 2003; Valluru & Van den Ende, 2008). Most fructan accumulators have so far been identified in Asteraceae, Campanulaceae, Poaceae and Liliaceae (Versluys et al., 2018).

RFOs and fructans share many similarities regarding physiological functions in response to abiotic stress in plants (Van den Ende, 2013). They both primarily function as carbohydrate storage. However, they are thought to be localised in different compartments of the plant cell. RFOs are believed to be synthesised in the cytosol whereas fructans are synthesised in the vacuole (Van den Ende, 2013). Several studies have shown that both oligo RFOs and oligo fructans can be localised in other compartments in the cell where they may be utilised for protective purposes, signalling and energy source. (Valluru & Van den Ende, 2008; Livingston et al., 2009; Elsayed et al., 2013; Van den Ende, 2013; Tarkowski & Van den Ende, 2015; Versluys et al., 2018).

Both RFOs and fructans have been proposed to be involved in ROS scavenging during abiotic stress responses (Elsayed et al., 2013; Van den Ende, 2013; Tarkowski & Van den Ende, 2015). Furthermore, they have been found to have stabilising effects on cellular membranes (Livingston & Henson, 1998; Ritsema & Smeekens, 2003; Valluru & Van den Ende, 2008; Livingston et al., 2009; Elsayed et al., 2013; Van den Ende, 2013; Sengupta et al., 2015; Tarkowski & Van den Ende, 2015; Versluys et al., 2018). However, research has indicated that only fructans can interact with the tonoplast and the cell membrane, whereas RFOs are restricted to the thylakoid membrane (Tarkowski & Van den Ende, 2015). Fructan polysaccharides are the only class of polysaccharides that have been observed to interact with lipid headgroups in a similar way as oligosaccharides like RFOs (Valluru & Van den Ende, 2008; Livingston et al., 2009). A proposed explanation to this unique property lies in the biochemical structure of the fructans. In contrast to several other carbohydrate types e.g., starch, fructans mainly consists of flexible furanose rings with a -O-$CH_2$ glycosidic linkage (Vereyken et al., 2003; Livingston et al., 2009; Versluys et al., 2018). A high DP fructan may thus avoid negative steric effects by having flexible conformations (Livingston et al., 2009).

In experiments with liposomes as membrane model systems, different carbohydrates' ability to stabilise membranes and protection against fusion were investigated (Livingston et al., 2009). Here it was shown that high DP chicory inulin, despite its large size, had protective effects under freeze-drying by decreasing the phase-transition temperature ($T_m$) in dry membranes as well as hydrogen bonding to the lipid P=O groups. However, during slow air drying it had no

protective effects. Comparisons between the different fructan types showed varied abilities in membrane stabilisation under different drying treatments. Fructans with lower DP did not precipitate during the air drying and thus had a protective effect. Levan with a DP ~125 also showed protective effects during air-drying despite having a higher DP than the chicory inulin. This can be explained by the higher solubility of levan (Livingston et al., 2009). Thus, the protective effects of fructans on membranes is dependant upon the fructan type and DP.

Fructan accumulating species appear to be more common in dry and cold environments and less common in tropical and warmer regions where starch accumulators dominate (Versluys et al., 2018). Several potential advantages of using fructan as the main storage carbohydrate over starch in temperate and cold climates have been investigated (Vijn & Smeekens, 1999; Araya et al., 2006; Valluru & Van den Ende, 2008; Livingston et al., 2009; Versluys et al., 2018). It has been suggested that excess accumulation of starch in chloroplasts may lead to the deformation and repression of photosynthetic activity (Araya et al., 2006). Fructans on the other hand, are synthesised and stored in the vacuole and, thus, would not have the same inhibiting effect on the photosynthetic apparatus. Furthermore, it has been speculated that the relative simplicity of fructan synthesis as compared to starch may allow fructan accumulators to faster remobilise carbon in response to temperate stressor cues (Van den Ende, 2013).

Another, potentially great, advantage for plants in temperate regions is the fact that fructan synthesis can continue at low temperatures, whereas starch synthesis decreases drastically at temperatures below 10°C (Hendry, 1987; Van den Ende, 2013). The decrease in starch synthesis leads to accumulation of glucose (Glc) and Suc, which have been found to repress photosynthetic activity (Araya et al., 2006). Therefore, fructan accumulating perennials might be able to synthesise fructans longer in autumn to accumulate a greater energy storage when entering the winter period. These plants may also be able to start regrowth earlier in spring because of excess carbohydrate storage. This could effectively lengthen the growth period for fructan accumulating plants in cold and temperate climates.

## 1.3 Fructan in the Pooideae subfamily

The Pooideae subfamily is the largest subfamily in the grass family (Poaceae) and consists of 3968 species classified in 15 tribes (Soreng et al., 2017). The tribes in this subfamily can be divided into core Pooideae and non-core Pooideae. The core Pooideae consist of the tribes Poeae, Avenaeae and Triticeae as defined by Davis and Soreng (1993) and contain many economically important cereals, forage grasses and turf grasses, such as *Hordeum vulgare* (barley), *Triticum aestivum* (bread wheat), *Avena sativa* (oat) and *Lolium perenne* (perennial ryegrass). In contrast to other Poaceae subfamilies, Pooideae species (hereafter pooids) have successfully diverged into temperate climate regions and are of great importance in many temperate and cold ecosystems (Hartley & Slater, 1960; Sandve et al., 2011). There is strong evidence that Pooideae evolved from a tropical ancestor (Bouchenak-Khelladi et al., 2010; Edwards & Smith, 2010; Strömberg, 2011). Hence, in the evolutionary history of Pooideae a niche transition from tropical to temperate environments has occurred.

Many species in Pooideae, especially core Pooideae, have managed to disperse into the coldest, most extreme and polar regions. As shown in Figure 3, the genera *Poa* L. and *Agrostis L.* from the core tribes Poeae and Avenae, respectively, occur in the Köppen-Geiger zones “Dfc” and “ET” (see Figure 4). “Dfc” is classified as a cold climate without a dry season and cold summers. In this climate zone the average temperature in the hottest month does not increase above 22°C, the average temperature of the coldest month does not decrease below -38°C and the number of months that have a mean temperature above 10°C are 4 or less (i.e., very long winters). “ET” is a polar tundra climate zone where temperatures are never above 10°C or are on average below 0°C.

The non-core genus *Melica* L. for example, does not appear to be able to occupy these extreme zones to the same degree (Fig. 4c). This confinement to less extreme climate zones becomes even clearer in the genus *Stipa* L. (Fig. 4d). The distinct, geographical line where *Stipa* species cease to occur, coincides with the “Dfb”-“Dfc” border. The Köppen-Geiger climate class “Dfb” describes a cold climate zone without dry seasons and with warm summers. Interestingly, the “Dfb” class is solely separated from ”Dfc” by the length of winter. Additionally, extreme cold does not seem to be a limiting factor for the distribution of *Stipa* species due to several occurrences of *Stipa capillata* L. in southern Siberia (GBIF Backbone Taxonomy, *Stipa capillata* L. in GBIF Secretariat (2021)). It is however unclear, if this geographical pattern is influenced by adaptations to long “Dfc“ winters, that specifically evolved in core Pooideae, such as *Poa* and *Agrostis* but not in non-core lineages like *Stipa* or *Melica*.

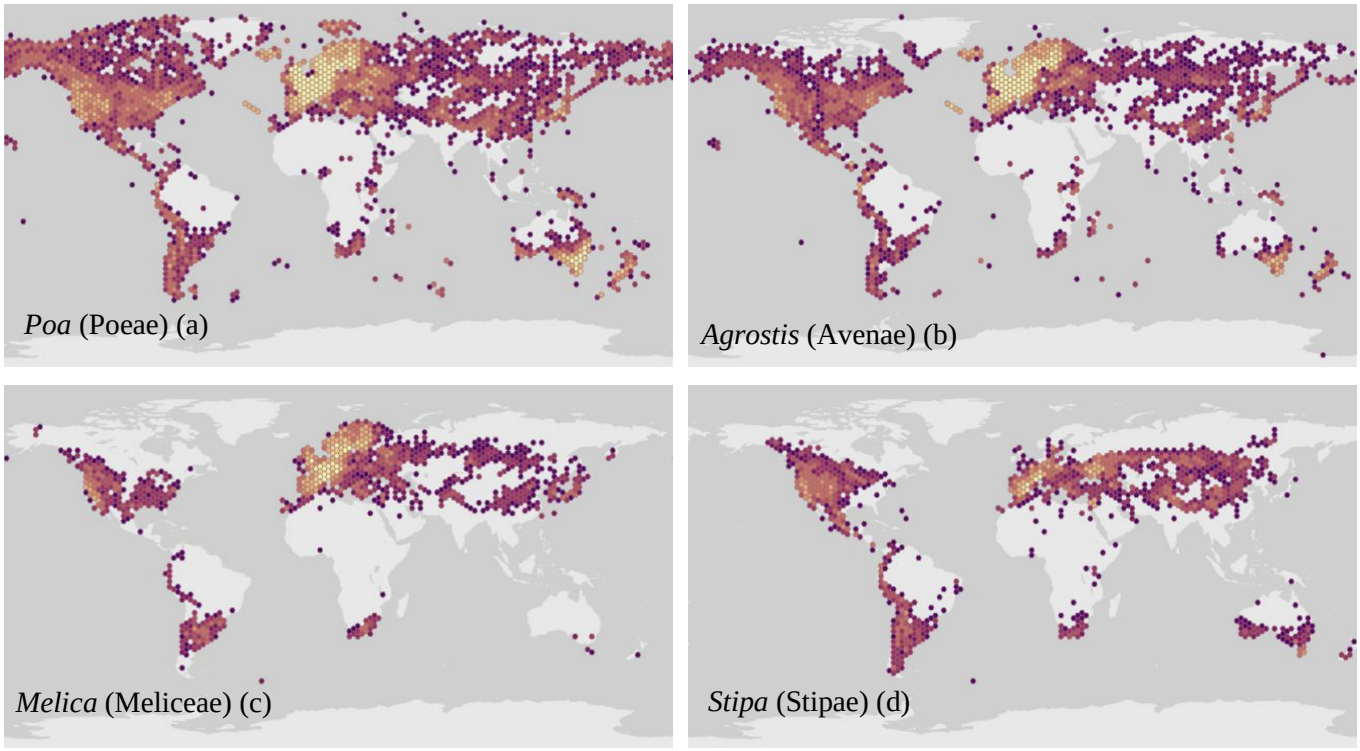


**Figure 3.** Occurrence data from four Pooideae Genera. Tribes in parentheses. Data from gbif (GBIF Backbone Taxonomy, *Agrostis L.* in GBIF Secretariat (2021); GBIF Backbone Taxonomy., *Melica* L. in GBIF Secretariat, 2021; GBIF Backbone Taxonomy., *Poa* L. in GBIF Secretariat, 2021; GBIF Backbone Taxonomy., *Stipa L.* in GBIF Secretariat, 2021)

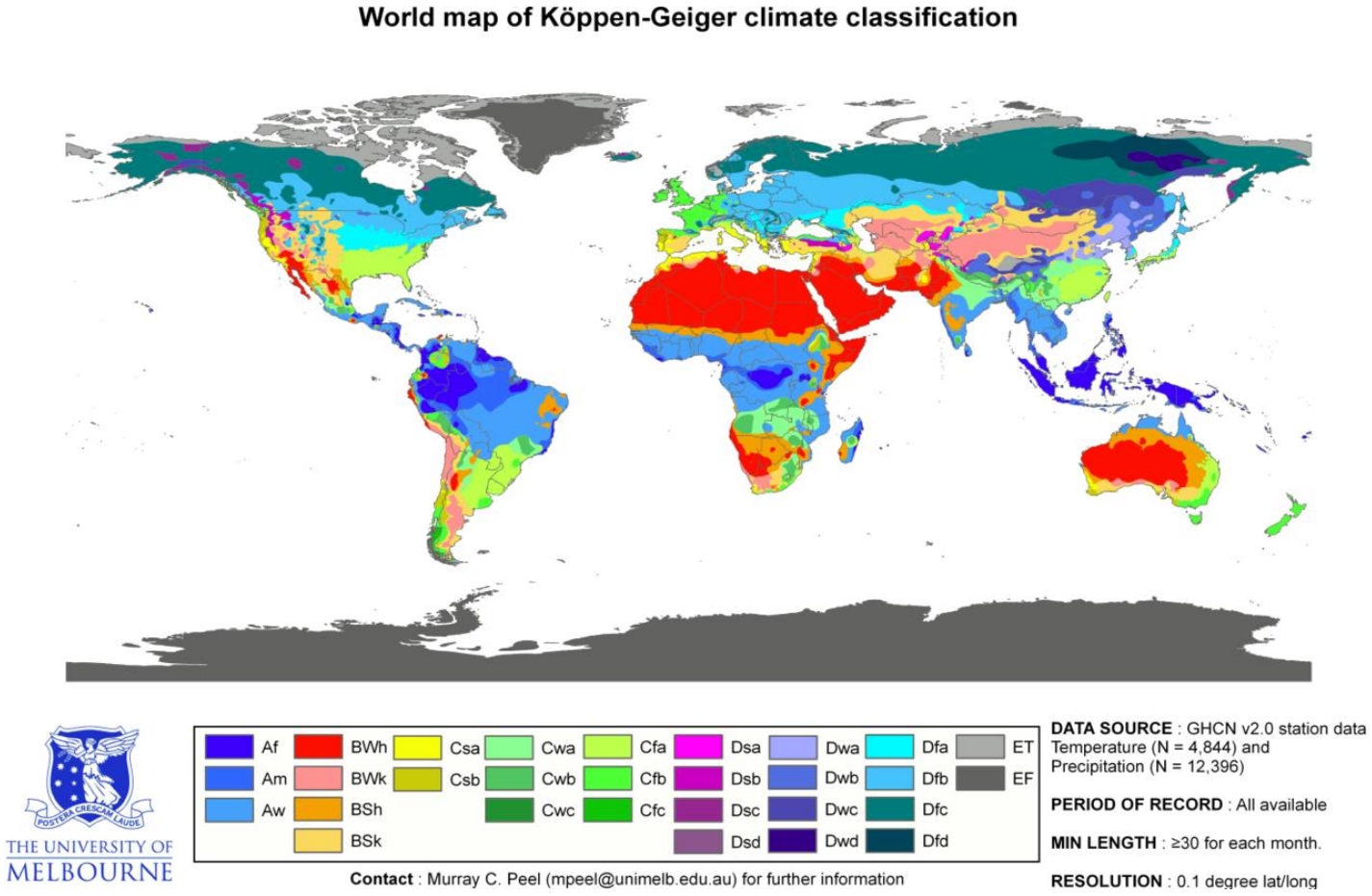


**Figure 4.** The Köppen-Geiger climate classification. See publication for detailed description of data collection and the different climate zones (Peel et al., 2007).

It has been known for a long time that some pooids accumulate fructan in response to chilling temperatures (Hartley & Slater, 1960; Pollock & Jones, 1979; Hendry, 1987; Hendry, 1993). Pollock and Jones (1979) investigated the seasonal patterns of fructan metabolism in the forage grasses *Festuca pratensis*, *Lolium perenne* and *Phleum pratense.* They conducted a field experiment in England and sampled material for carbohydrate analyses throughout a whole year. The resulting fructan patterns showed a fructan accumulation peak in December followed by a rapid decline coinciding with a rise in low molecular weight carbohydrates. The same pattern was observed in Phleum pratense by Tamura et al. (2014), who linked the increase and decrease of fructan concentration to expression patterns of FTs and FEHs.

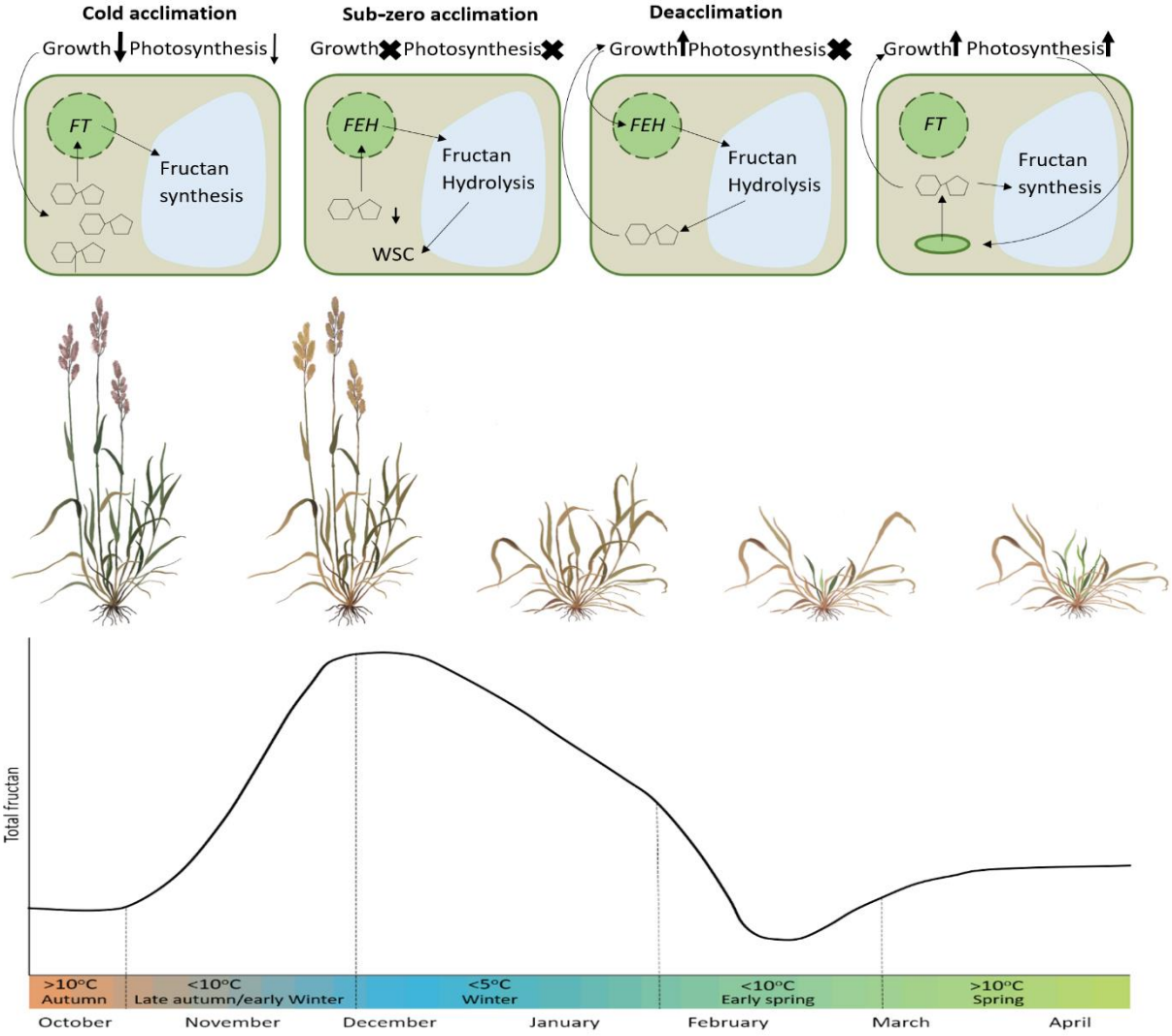


**Figure 5.** Schematic fructan accumulation pattern in Pooideae species based on previous experiments and theory described above (Pollock & Jones, 1979; Bergjord Olsen & Skjelvåg, 2011; Tamura et al., 2014). When the temperatures drops in late autumn, cold acclimation starts, and excess Suc triggers the accumulation of fructan in the vacuole by activating transcription factors (TaMYB13 in wheat) (Tarkowski & Van den Ende, 2015). When the temperature decreases further, sub-zero acclimations occur where fructan is broken down to low DP oligomers and mono-/dimers. In the dormant plant, the fructan storage is used throughout the winter for cell maintenance. As environmental ques break the dormancy in early spring, the remaining fructan storage is hydrolysed by FEH and used for growth. When enough photosynthetic activity has been restored, fructan is again synthesised in the vacuole from excess photosynthetic products.

The effect of fructan on membrane stability and frost tolerance has been functionally proven in Pooideae. Hisano et al. (2004) transformed *L. perenne* with 1-SST and 6-SFT from *T. aestivum* and observed that an increase in fructan oligomer synthesis reduced the electrolytic leakage during freezing stress, which is indicative of less membrane damage. Furthermore, Kawakami et al. (2008) showed that genetically engineered *Oryza sativa* expressing 1-SST and 6-SFT, also from *T. aestivum,* had a significantly higher chilling tolerance than the wild type.

The evolutionary history of *FT* genes in Pooideae is gradually being unravelled. Schubert et al. (2019a) inferred that the most recent common ancestor (MRCA) of Pooideae had a Cretaceous-Palaeocene origin and is about 69 million years (myr) old. They performed an ancestral state reconstruction and the results suggested that the Pooideae ancestor experienced periodic frost, whilst severe winters (i.e., coldest quarter mean below 2-4°C) were only experienced by later diverged descendants. The diversification of the Pooideae tribes was inferred to coincide with falling temperatures throughout the Eocene and increase even further throughout the cool Oligocene and Miocene. In a study by Sandve and Fjellheim (2010) expansion of the *FT* gene family was inferred to occur in core Pooideae after the divergence from non-core lineages. This was corroborated by Schubert et al. (2019b) who further found that cold responsive genes are positively assosiated with gene family size in Pooideae. Their results indicated that gene family expansion, including the expansion of the *TF* gene family, was an important way of adapting to falling temperatures and new expanding temperate niches.

There have been several studies investigating the carbohydrate composition during the winter months in pooids (Pollock & Jones, 1979; Chatterton et al., 1989; Bush et al., 2000; Dionne et al., 2001; Dionne et al., 2010; Bergjord Olsen & Skjelvåg, 2011; Tamura et al., 2014). However, most of these experiments have focused on a single or few species, especially the ones of economic interest from the core Pooideae. To date, there are no multi-species studies that have investigated the fructan composition and accumulation ability in connection to winter survival. Previous studies has classified the main fructan types in the lower organs of some core pooids. For example, in the Triticieae species *Triticum aestivum* and *Hordeum vulgare* (i.e., wheat and barley) graminan and levan type fructans have been detected (Sprenger et al., 1997; Verspreet et al., 2013; Veenstra et al., 2017), wheras in *Avena sativa* (i.e., oats, Aveneae) neoseries fructans have been found (Henson & Livingston, 1996). However, although fructan has been reported in several core pooids, less is known about the carbohydrate types synthesised by non-core pooids. Additionally, no study has investigated the correlation between fructan accumulation ability in Pooideae with the climatic distribution of species. Thus, it is not known

whether fructans might have played a role in shaping the geographical distribution patterns within the Pooideae subfamily. To get a better understanding of the importance of fructan accumulation abilities in regard to winter survival and geographical distribution in Pooideae, as well as investigating it in an evolutionary context, more research is needed.

In this study, a common garden experiment with 35 phylogenetically diverse species was designed and conducted to investigate the fructan concentration patterns throughout the winter months, as well as fructan types, in the Pooideae. Because of the inferred timing of gene family expansion of FT in Pooideae in relation to the divergence of the tribes, as well as previously detected fructan types, a diverse pattern of fructan accumulating abilities and types is expected between the tribes. To test this hypothesis, type of fructan accumulated by each species was determined using high performance anion exchange chromatography coupled with pulsed amperometric detection. A phylogeny of the species included in the experiment was constructed and type of fructan was plotted on the phylogeny. Based on the presented knowledge, grasses that accumulate fructan are expected to be more tolerant to winter stress and exhibit an increased regrowth ability in spring compared to grasses that do not accumulate fructan. I investigated if fructan is correlated with winter survival across the Pooideae, by testing whether fructan accumulating species exhibited a higher regrowth score than species that did not accumulate fructan over winter. Because the fructan storage is gradually being used throughout the winter months for cell maintenance, I expect that species heading into winter with more fructan storage will have an advantage over the ones with less storage. Species that do not completely deplete fructan during winter will be able to use the remaining fructan storage as a boost for regrowth after the winter period.

I further investigated whether fructan accumulating species can endure longer winters than non-fructan accumulating species, by extracting climatic variables to geographical distribution data and analysed the variance between the groups. Because of the putative ability of fructan accumulators to shorten the dormant winter period, fructan accumulation ability might be beneficial in climates with long winters. I hence expect that fructan accumulating species occur in areas with longer winters than non-fructan accumulating species.

# 2 Materials and Methods

## 2.1 Plant material and experimental design

In total, 35 Poaceae species were chosen for the experiment (supplementary 1), of which 33 belong to eight different Pooideae tribes and two species, *Danthonia decumbens* and *Ehrharta calycina*, are part of the subfamilies Danthonioideae and Oryzoideae, respectively. When possible, pooids were chosen to form related species triplets, including one dominantly temperate, one dominantly subtropical and one tropical alpine species.

Seeds were sown in turf soil at Vollebekk research station (NMBU) 15th of April 2019. They were stratified after the following protocol: Pots were kept humid in total darkness in four days at 4 °C. The fifth day the temperature was raised to 25 °C, still in darkness. After stratification, the pots were transferred to a glasshouse for germination at 17 °C and a daylength of 16 hours. Natural light was supplemented with extra lighting. Plants, 945 in total, were pricked out into single pots when they had reached an appropriate size. When the plants started growing, they were watered by hand with fertilising water.

To expose plants for natural growing conditions, trays were transferred outside at the 23rd of May 2019. At 5th August, the plants were removed from their pots and planted in a field at Vollebekk (59°39'43.7"N 10°44'58.4"E). The experiment had a common garden experimental design with seven blocks, one for each sampling month. One individual from each species was randomly placed in each column of the individual blocks. 20 additional blocks were used to classify the regrowth of each individual plant in the spring of 2020 as described by Rapacz et al. (2004).

### 2.1.1 Sample preparations

Sampling of crown material was performed at the end of each month from 30th October 2019 to 29th April 2020. In total, 245 tissue samples were collected. No snow cover occurred throughout the experiment period. Figure 6 shows the sampling dates as black dots in the experimental timeline, as well as the minimum temperature per hour at grass level.

During sampling, whole plants were retrieved from the appropriate block at the field, and roots were washed in water to remove soil. Crown material were put in 15 mL Falcon tubes, flash-frozen in liquid nitrogen and stored in -80°C before they were freeze-dried using a Lyoalfa 15 machine from Telstar Technologies with a chamber temperature of -80°C for 24 hours.

The freeze-dried samples were manually cut into smaller pieces before being homogenised to a fine powder with a TissueLyser II bead mill using two 3 mm tungsten carbide beads (QIAGEN, Hilden, Germany). The samples were then sent to the laboratory of Prof. Wim van den Ende (KU Leuven, Belgium) for carbohydrate extraction and analysis.

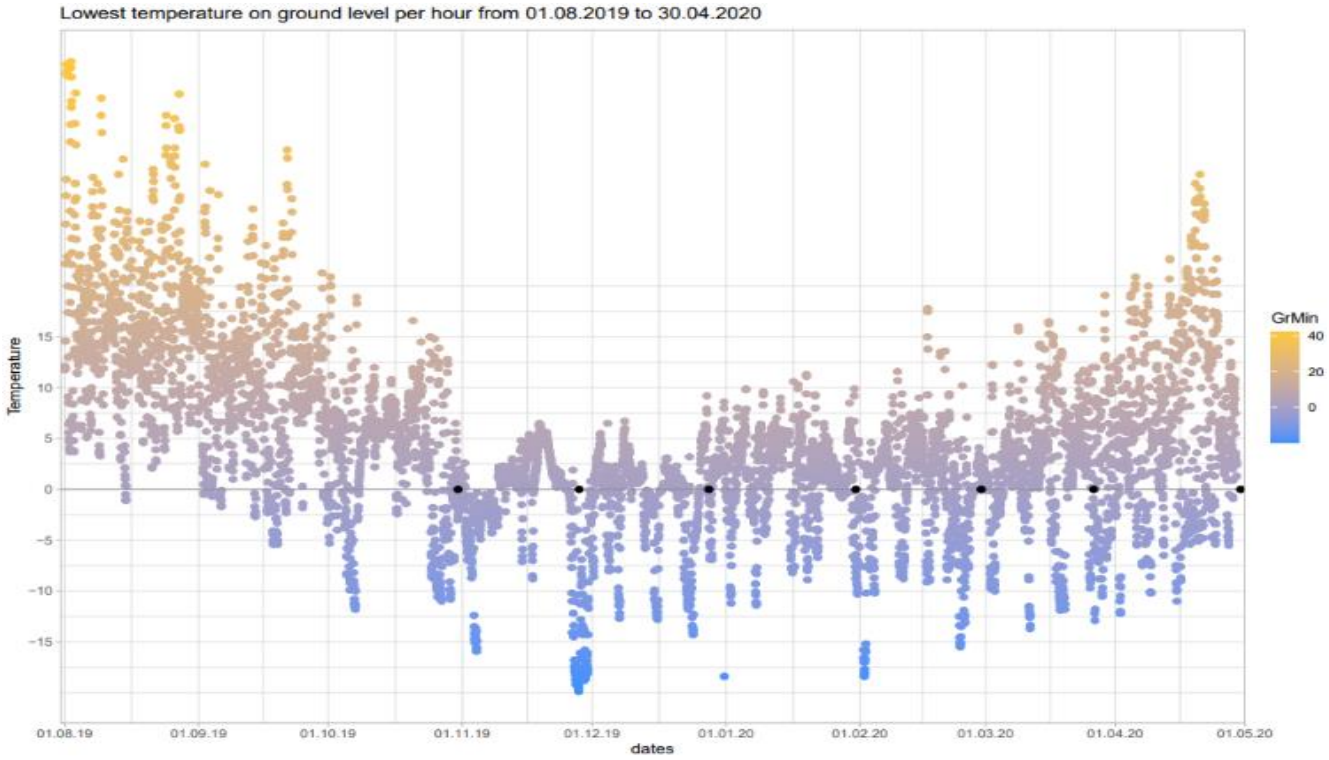


**Figure 6.** Minimum temperature measured every hour at grass level throughout the period the grasses were in the field. Black dots are sampling dates. The measurements were caried out at Søråsjordet, around 500 m from the field experiment (Wolff & Grimenes, 2019 – 2020). The plot shows that the grasses experienced chilling and frost stress before the first sampling.

## 2.2 Carbohydrate extraction, acid hydrolysis and high performance anion exchange chromatography coupled with pulsed amperometric detection

### 2.2.1 Extraction

10 mg of freeze-dried crown tissue were transferred to an Eppendorf tube. 400 µL of preheated 40% EtOH-solution (50°C) was added, and the mixture was vortexed. The mixture was then heated to 82°C and centrifuged at 14000 rpm for 5 minutes. The supernatant was transferred to a new tube. The previous step was repeated two more times. The supernatant was dried using a vacuum-centrifuge. 500 µL $H_2O$ was added and the mixture was vortexed thoroughly. 200 µL of the resulting sample was added to a mixed-bed ion-exchange column prepared with 300 µL Dowex Acetate-resin and 300 µL Dowex $H^+$-resin. The column was rinsed 6 times with 200 µL milli-Q water. The collected eluent was vortexed and centrifuged at 14000 rpm for 5 minutes and stored at -20°C until carbohydrate and total fructan content (TF) analysis.

### 2.2.2 Carbohydrate analysis

To detect and quantify specific carbohydrate content in the carbohydrate extracts, the frozen eluate was thawed and analysed with a Dionex$^{TM}$ ICS-5000$^{+}$ HPAEC-PAD (Thermo Scientific) system. 10 µL of each sample was diluted in 90 µL rhamnose before injecting 6 µL of the dilution on a Dionex CarboPac PA100 column (2x250 mm analytical column with a 2x50 mm guard column) operated at 32°C, with a flow rate of 0.25 ml/min. Reference standards with known concentrations for Glc, Fru, Suc, rhamnose (internal standard), kestoses, Nys, Raf, Sta, blastose and maltose were used to quantify the content of these carbohydrates in the samples. Chicory and onion were used for qualitative comparison.

### 2.2.3 Acid hydrolysis and total fructan content quantification

To obtain a measure of the total amount of Glc and Fru originating from fructan oligo- and polymers, the samples were hydrolysed using acid hydrolysis. 200 µL of the eluent was transferred to a new tube and 2 µL 6M HCl was added. The solution was heated to 70°C for 2 hours. 6 µL $Na_2CO_3$ was added to neutralise the solution and the sample was analysed with HPAEC-PAD. The TF and average DP for each sampling month was calculated as described in supplementary 7.6.

### 2.2.4 Correcting for hydrolysis of RFOs

Under the previously described hydrolysis conditions, raffinose and stachyose will form melibiose and manninotriose, respectively (Ispiryan et al., 2019). This leads to a free Fru molecule as the Glcα1, 2βFru glycosidic bond is hydrolysed (see Figure 1). As a result of a significant amount of RFO in several of the samples after extraction (as seen in the chromatograms in Supplementary 7.2), the amount of Fru originating from the hydrolysis of RFOs needed to be corrected for in the calculations of TF and the average DP. This was done by calculating the mg Fru per g dry weight of sample (DW) from RFO origin using the concentrations of raffinose and stachyose measured in the carbohydrate analysis and subtracting this from the post hydrolysis Fru concentration as shown in supplementary 7.6.

## 2.3 Phylogenetic comparative analyses

### 2.3.1 Phylogenetic reconstruction

To determine the phylogenetic relationship between the species and produce an ultrametric, phylogenetic tree, the evolutionary history of the used species was reconstructed. Coding sequences for the barcode genes *mat*K, *ndh*F and *rbc*L were either obtained from the NCBI GenBank (Clark et al., 2016), or sequenced using Sanger sequencing. Electropherograms of the

sequenced genes were inspected and manually adjustments were conducted. The genes were aligned separately with MAFFT v7 (Katoh & Standley, 2013; Madeira et al., 2019), manually adjusted and finally concatenated into one fasta file using MEGAX v10.2.4 (Kumar et al., 2018).

The fit of nucleotide substitution models for the three gene partitions was determined using the function 'modelTest' from phangorn v2.5.5 (Schliep, 2011) in R. The best model was selected based on the Akaike Information Criterion with a small sample size correction (AICc). For *mat*K the model with the lowest AICc value was GTR+G+I, for *ndh*F it was GTR+G and for *rbc*L it was HKY+ G+ I. Phylogenetic trees were reconstructed by maximum likelihood (ML) and Bayesian inference (BI) methods.

The ML analysis was performed in RAxML v1.0.0. (Kozlov et al., 2019). A partitioning approach was used in which the multiple sequence alignment was separated into three partitions and the evolutionary history of the different genes was reconstructed under their respective nucleotide substitution models.

Bayesian inference was performed with BEAST v 1.10.4 (Suchard et al., 2018), unlinking the site models to allowing each gene partition to evolve under their own nucleotide substitution model, choosing the model priors as suggested by 'modelTest'. The clock model and partition tree were kept linked and pooids were constraint to form a monophyly. An uncorrelated relaxed clock prior with a lognormal distribution was chosen assuming a Yule speciation process and starting with a random tree. The Pooideae's crown node age prior was set to a normal distribution with a mean of 68.7 myr and a standard deviation of 4 myr, in accordance with estimations by Schubert et al. (2019a).

Two replicate Markov chain Monte Carlo were run for 100mill generations and parameters were logged every 1000 generations. Log and tree files from both chains were combined with LogCombinerv1.10.4 (Suchard et al., 2018) discarding the first 10mill generations as burn-in. The combined log file was analysed with Tracer v.1.7.1 (Rambaut et al., 2018) to ensure that chains had converged and ESS values were above 200. The posterior tree distribution was summarised by constructing a maximum clade credibility (MCC) tree using TreeAnnotator v1.10.4 (Suchard et al., 2018) setting node heights to the mean node hight for each of the clades from the respective node distribution. The ultrametric MCC tree from BEAST was used as input for all subsequent comparative analyses.

### 2.3.2 Classification of true fructan accumulators, 1-K accumulators, putative RFO accumulators and fructan type determination

The resulting carbohydrate profiles seen in the HPAEC-PAD chromatograms (supplementary 7.2), quantitative data of fructan related oligomers in mg/g DW and the TF quantified in the acid hydrolysis (mg/g DW) were used to determine types of fructans in the tissue samples for each species (supplementary 7.3). The data was further used to classify species into four different groups: true fructan accumulators, 1-K accumulators, putative RFO accumulators and non-fructan accumulators. To be classified as a true fructan accumulator the species data had to fulfil three requirements:

1. One or more fructan oligomers throughout the months > 1mg/g DW

2. Area under the curve of TF throughout the sampling months > 100mg/g DW

3. Higher DP fructan peaks in chromatogram and in more than one month (one might be a sampling artifact)

These requirements were set based on biochemical reasoning as well as the observation from already known true fructan accumulators such as *H. lanatus*. Vacuolar invertases have an intrinsic promiscuity and can convert low amount of Suc to 1-K when Suc concentrations are high (Ritsema et al., 2006). Thus, detection of low levels (i.e., <1mg/g DW) of 1-K in some species, but none of the other precursor oligosaccharides in the fructan synthesis pathway, does not indicate an evolved 1-SST in that species. If species had a significant amount of 1-K but no high DP peaks, they were classified as 1-K accumulators. The remaining species were classified as non-fructan accumulators.

Putative RFO accumulators had to fulfil two requirements:

1. Must contain both raffinose and stachyose.

2. Raffinose and stachyose > 1 mg/g DW

These requirements were based on that raffinose is ubiquitous in plants whereas stachyose and higher DP RFOs mostly occur in true RFO accumulators (Van den Ende, 2013).

The types of fructan oligomers detected in the true fructan accumulators were used to determine the fructan types. This was done by assessing which biosynthetic pathway the species has enzymes to enable. For example, *L. perenne* has significant levels of 1K, NK and Nys. This

means it has 1-SST, 6G-FFT and 1-FFT. Thus, *L. perenne* can synthesise neoseries- and inulin type fructan.

### 2.3.3 Data preparation and exploration

For calculating variables and conducting analysis the programming environment R was used (R Core Team, 2020). The dependant variable, regrowth, was calculated per species by estimating the mean of the 20 regrowth scores from each plant. To incorporate the change in the amount of TF (mg/g DW) over the winter months into the PGLS, several variables were derived from the functional data. The variables were first inspected by plotting them against the dependant variable and log transformations were performed when necessary. The variables were further evaluated against the dependant variable regrowth with several phylogenetic principal component analyses (pPCA). The pPCAs was performed using the 'phyl.pc'a function from phytools v0.7-70 (Revell, 2012) and a biplot was created using 'ggbiplot' from ggfortify v0.4.11(Tang et al., 2016).

The area under the curves (AUC) for all carbohydrate functions was calculated with the 'sintegral' function from Bolstad2 v1.0-28 (Curran, 2013) in R and the "month zero" variable for each carbohydrate was retrieved by conditionally formatting the data in excel (all values below 3 mg/g DW coloured red) and visually inspecting which month were below the cut-off and stayed below for the following sampling months. The latter variable represents the sampling month where the level of the respective carbohydrate type was depleted. This was further transformed into another binary (yes/no) variable termed "Deplete". Additionally, temperature data was retrieved from the field station for bioclimatic studies at NMBU at Søråsfeltet in Ås to assess the temperatures the plants were exposed to throughout the sampling period (Wolff & Grimenes, 2019 – 2020).

### 2.3.4 Analysis of total fructan content and regrowth

PGLS is a common method to investigate the relationship between a response variable, in this case regrowth, and one or several predictor variables in a phylogenetic framework (Symonds & Blomberg, 2014). Because of the shared evolutionary history between species, it can be expected that closer relatives share more similar traits than more distant ones. Thus, the measurements of traits across a phylogeny may violate the assumption of independent residuals which is one of the fundamental assumptions in many statistical analyses and hence needs to be corrected for (Symonds & Blomberg, 2014). This is done by estimating the expected covariance between the residuals for different species based on their relatedness described by the branch length in the phylogeny (Symonds & Blomberg, 2014).

To test the relationship between the amount of fructan over the winter months and regrowth in spring a PGLS analysis was performed. The logarithm of the area under the curve of total TF throughout the sampling months (hereafter AUCTF) was chosen as the independent variable for the PGLS as it showed a strong correlation with the dependant variable in the pPCA analysis. Furthermore, this variable was negatively correlated with the "deplete" and "no fructan" variables and positively correlated with the "month zero of total fructan" variable and thus incorporated the essential information from the functional data.

The PGLS analysis of the logarithm of regrowth with a pseudocount = 0.5 versus AUCTF was conducted using the 'phylolm' function from the package phylolm (Ho & Ane, 2014). Before the analysis, the distribution of the dependant and independent variable was checked for outliers and "skewness" as recommended by Roger Mundry (Mundry, 2014).

Six different models were tested (see table 1). The best fitting model was chosen based on AICc values calculated using the 'AICc' function from MuMIn v1.43.17 (Barton, 2020). Checking of model assumptions was done by creating diagnostics plots as seen in supplementary 7.5. $R^2_{pred}$, which indicates how much of the variance in the data is explained by a model, was estimated with the 'R2' function from the rr2 package (Ives, 2018; Ives & Li, 2018).

### 2.3.5 Analysis of fructan accumulation abilities in relation to winter length

#### 2.3.5.1 PGLS of isothermality and total fructan

In the first "winter length vs fructan accumulating abilities analysis" the Bioclimatic variable "BIO3" called Isothermality was used. This variable equals mean monthly diurnal range divided by annual temperature range (Max temperature of warmest month-Min temperature of coldest month). In other words, isothermality quantifies how large the day-to-night temperatures oscillate relative to the summer-to-winter oscillations (O'Donnell & A. Ignizio, 2012). This variable is a proxy for the length of winter in geographic regions. For example, in the tropics, the difference between daily and nightly temperature may be very large, whereas the annual difference is not. This would result in a high BIO3 value (short/no winter). In temperate regions, the difference between day and night may not be so large, whereas the annual temperature variation is, resulting in a low BIO3 value (i.e., long winter) (see Schubert et al. (2019a), figure 3, for BIO3 variable plotted on the world map).

To obtain the data for the analysis, occurrence coordinate data for each species was downloaded from gbif (GBIF.org, 10 May 2021). BIOclim data, which are ecological meaningful variables derived from the monthly temperature data from WorldClim (Fick & Hijmans, 2017), was

downloaded using the ‘getData’ function from the raster package (Hijmans, 2020). A spatial resolution of 2,5 arcminutes was chosen. Thereafter, BIOclim variables was extracted for species coordinates using the ‘extract’ function (Hijmans, 2020). Subsequently, coordinates with above two decimal points were extracted and further filtered using the ‘clean_coordinates’ function from CoordinateCleaner v2.0-18 to remove implausible coordinates (e.g., sea coordinates, centroids, capitals) (Zizka et al., 2019). A normalisation step was performed to obtain a single observation for the same species in each geographic square. Variables for the following PGLS analysis was calculated by taking the mean. The PGLS analysis was performed following the same procedure as described above (2.3.4).

#### 2.3.5.2 pANOVA

To investigate more concretely whether fructan accumulators have the physiological ability to endure longer winters than non-fructan accumulating species in Pooideae, historical maximum temperature ($T_{max}$) data for each month was downloaded from Worldclim in a resolution of 2,5 arcminute. Climate data was linked to occurrence coordinates, normalised and filtered as described above.

For each species and month, 20% of the occurrences with the lowest $T_{max}$ were extracted using the ‘group_by’ and ‘slice_min’ functions from dplyr and monthly means were estimated (Wickham et al., 2021). All monthly mean values were divided by 10 and for each species and new variables were created by counting months with $T_{max}$ below x. The data was combined with the fructan accumulation data as described in 2.3.5.1. Phylogenetic Analysis of variance (pANOVA) was conducted with the function ‘phylANOVA’ from the phytools package (nsim = 10000, posthoc= TRUE and p.adj = “holm”) (Garland et al., 1993; Harmon et al., 2007; Revell, 2012).

# 3 Results

## 3.1 Investigation of fructan type in Pooideae

Carbohydrate profiles were obtained for the 35 species from the HPAEC-PAD analysis as shown in supplementary 7.2. Three out of the 245 samples were not tested: *P. bulbosa* (March), *M. nutans* (October) and *F. abyssinica* (December) due to too small amounts of sample. The subsequent evaluation of carbohydrate profiles described in 2.3.2 revealed a wide variation of fructan types among pooids, especially between tribes (Figure 7 ).

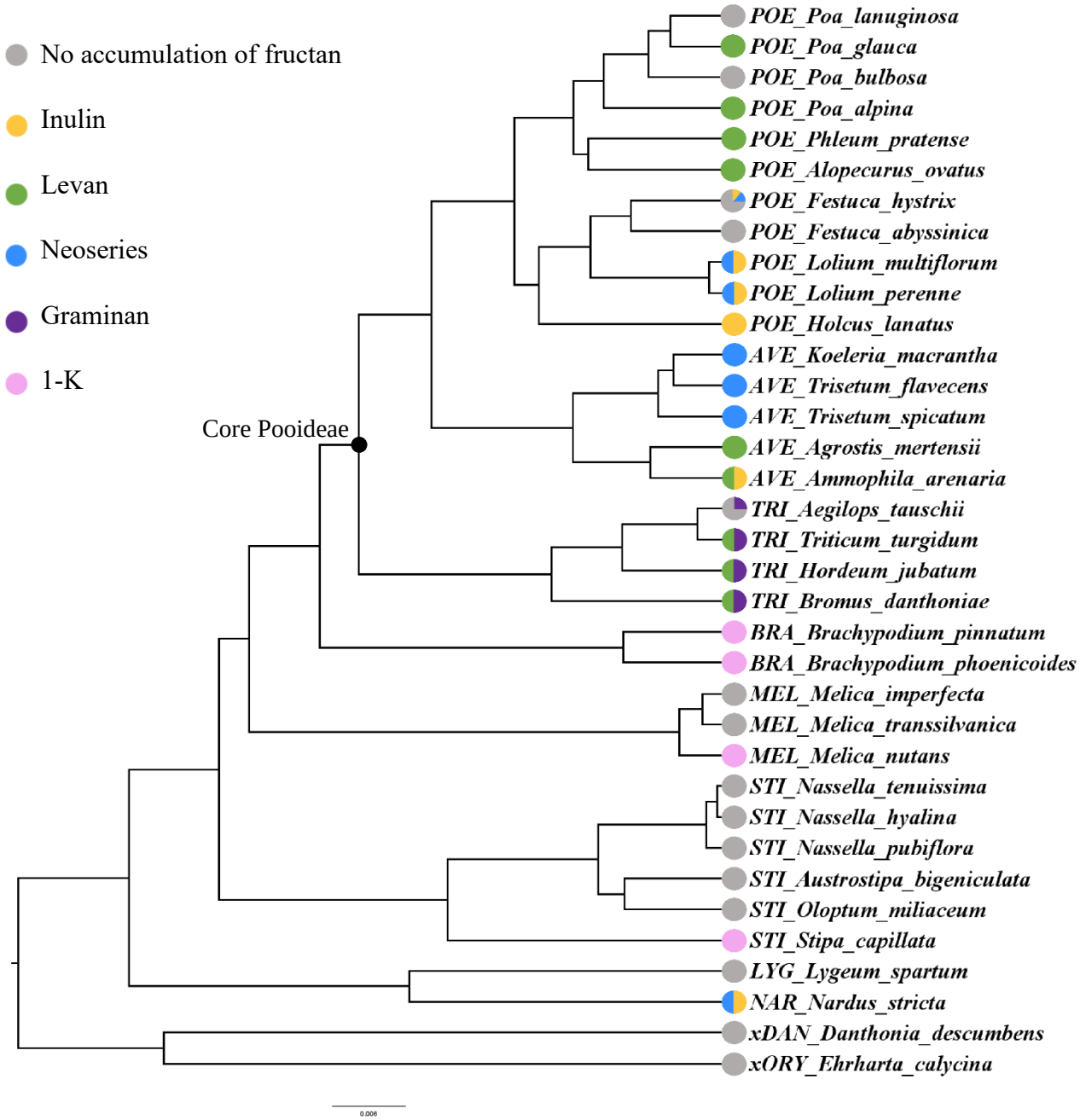


**Figure 7.** Type of fructan plotted on the maximum clade credibility tree. Several species had two types of fructan which is indicated by two colours. Some species only had substantial fructan oligomers in some months which is indicated by smaller coloured parts of the circle (not classified as true fructan accumulators).

Out of the 35 species, 16 species were classified as true fructan accumulators, four species were classified as 1-K accumulators, and the remaining 15 species were classified as non-fructan accumulators. One exception from the requirements was made for *P. pratense*, where true fructan accumulator requirement one was not met but the AUCTF was 862,2 mg/g DW and high DP fructan peaks matching the ones in the *Poa* species were observed (supplementary 7.2). Besides *Nardus stricta*, all true fructan accumulators were found in core Pooideae. Five Putative RFO accumulators were classified. These were *T. spicatum*, *M. transsilvanica*, *D. decumbens*, *A. ovatus* and *A. mertensii*. Out of the five, *M. transsilvanica* and *D. decumbens* were not classified as true fructan accumulators and had a regrowth score of 6.16 and 5.85, respectively.

Several Triticeae and Poeae species (Figure 7) showed significant values of fructan precursor oligosaccharides in different fructan type pathways, as well as high DP peaks (supplementary 7.2). Thus, these species must possess specific enzymes to synthesise two types of fructans.Figure 7 *F. hystrix* and *A. tauschii* showed significant peaks indicating high DP fructan in two months, but no peaks indicating either fructan precursor oligosaccharides or high DP fructan for any of the remaining months. These were thus classified as non-fructan accumulators as they did not fulfil the requirements described in 2.3.2.

After correcting for RFOs (Raf and Sta), TF data obtained from the acid hydrolysis, and subsequent HPAEC-PAD analysis, was plotted against the sampling months (supplementary

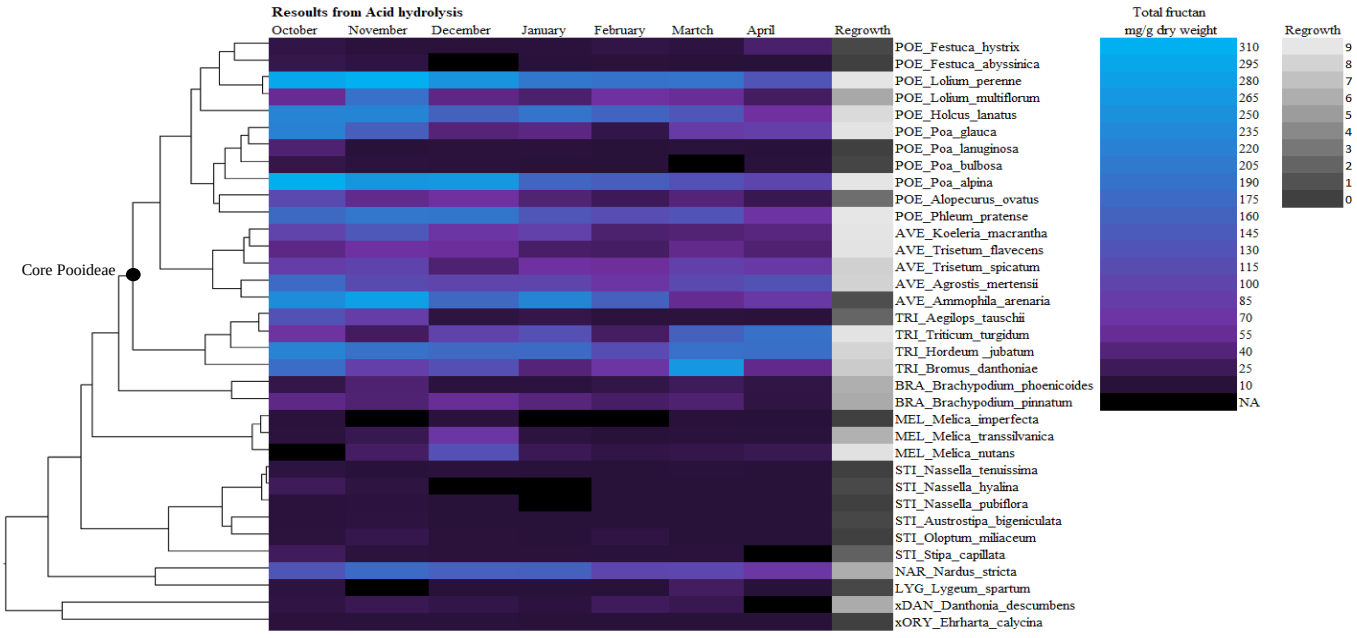


**Figure 8.** Heatmap ordered after the phylogeny for TF detected in each species over the sampling months and regrowth scores. Na values are values that were negative or missing.

7.4). For several species, fructan amount peaked in October or November, and decreased the following months. An increase of fructan in spring was only observed in *A. mertensii, H. jubatum, T. turgidum* and *P. glauca*. The highest fructan concentrations (around 300 mg/g DW) were observed in the two core pooids *L. perenne* and *P. alpina* in October. Species with no or low levels of fructan throughout the winter months had in general low regrowth scores whereas the opposite was true for species with high fructan levels (Figure 8).

Despite the measures taken to correct for hydrolysis of Raf and Sta, some non-fructan accumulators, i.e., *D. decumbens* and *M. transsilvanica* showed positive values for TF in certain months. This may be the result of correcting with Raf and Sta values from a separate HPAEC-PAD run i.e., the initial carbohydrate analysis, where the fraction of Raf and Sta may have been smaller than in the hydrolysed samples.

The patterns of average DP of the fructan molecules for each species throughout the winter months (Figure 9) overlap to some degree with the pattern observed for TF (Figure 8), however, a larger monthly variation was observed for average DP. 1-K accumulators, such as *B. phoenicoides* and *B. pinnatum* exhibited an average DP around three each month, which is indicative of the DP for 1-K. True fructan accumulators, i.e., most core pooids, have a DP over four, which is the minimum DP of fructan molecules from the distinct types (see Figure 2).

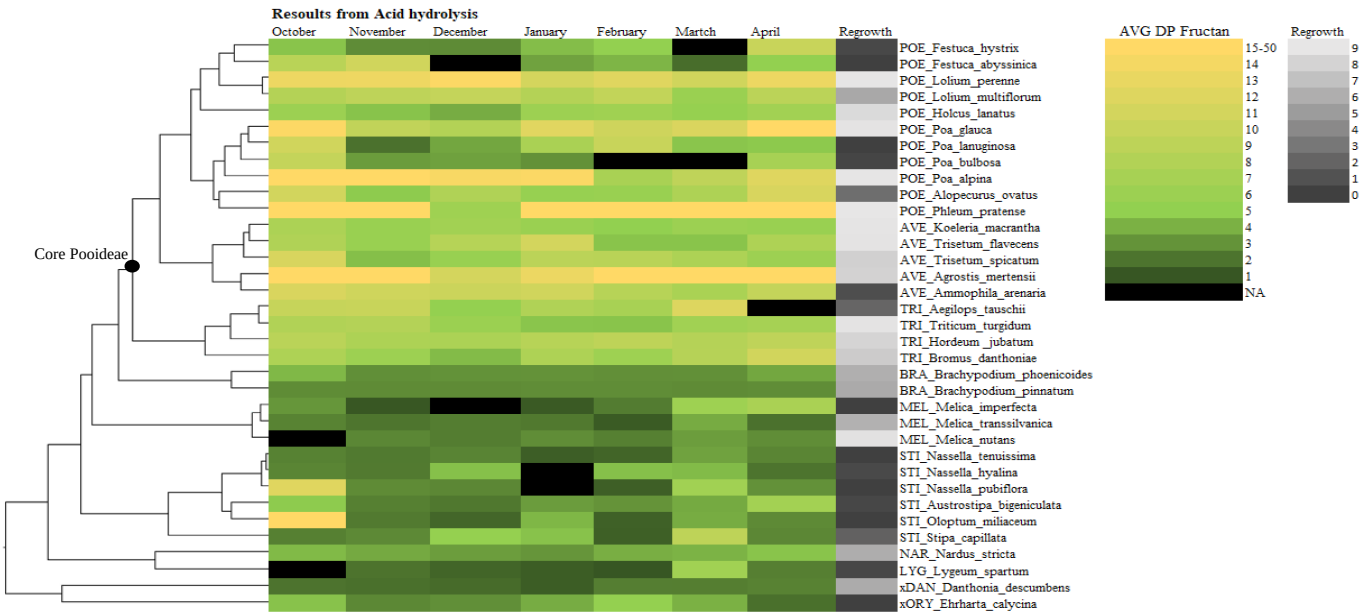


**Figure 9.** Heatmap ordered after the phylogeny for average degree of polymerisation detected in each species over the sampling months and regrowth scores. Na values are values that were negative or missing.

Some values of TF and average DP became negative (Na values in Figure 8 and Figure 9). This occurs especially in samples with low fructan content and is the result of either relative high starch residue in the extract, or a large concentration of mono- and di saccharides, resulting in

higher values of Suc, Glc and/or Fru before AH than the values after because of no, or low amounts, of hydrolysed fructan.

## 3.2 Fructan and regrowth


**Figure 10.** Biplot from phylogenetic PCA for fructan and RFO related variables. The two first principal components are shown, which together explains ~68% of the variance. Black dots are samples, arrows are loadings. Green arrows indicate variables positively correlated with the test variable Regrowth, colour strength indicate strength of correlation. Orange arrows indicate negatively correlated variables with Regrowth. See 2.3.3 for explanation of variable abbreviations.

The pPCA revealed a very strong correlation between the dependant test variable regrowth and the AUCTF (Figure 4). The derived binary variables “Deplete” and “No fructan” were negatively correlated with the AUCTF whereas the “mz total fructan” variable showed collinearity. The placement of *D. decumbens* is a result of the high RFO content in this species relative to all others. No strong correlation was observed between the average AUC DP Fructan variable nor the log of this variable (not shown).

The PGLS model with lowest AICc score was the Ornstein-Uhlenbeck (OU) model (Table 1). The difference between the AIC scores for OU and Pagels λ was less than 2. Hence, the latter model also has substantial support (Burnham & Anderson, 2004). The ML estimate for Pagels λ was ~0, corresponding to no phylogenetic signal in the residuals. The estimated parameters

in the two best fitting models were similar to the estimates from a regular ordinary least squares (OLS) analysis (results not shown).

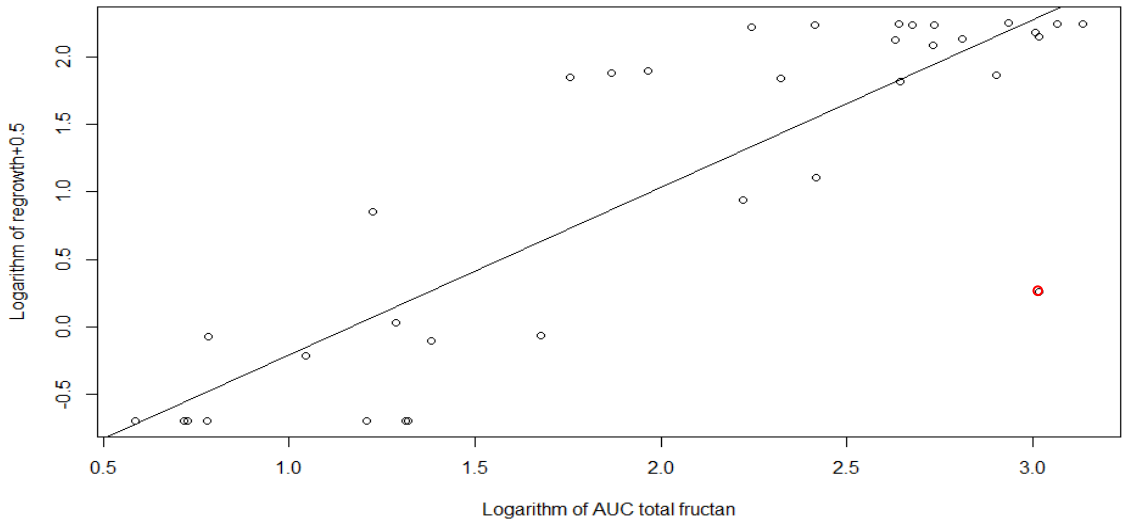


**Figure 11.** The fitted OU model (randomroot, Formula= logregrowth~log AUCTF, pseudocount for logregrowth= 0.5), added on a scatterplot for the log-transformed species data (dots). Red Outlier is Ammophila arenaria.

**Table 1.** Parameters for the fitted PGLS models for regrowth and fructan accumulation (Formula= logregrowth~log AUCTF). $\sigma^2$ is the estimated variance of the trait and reflects the stochastic rate at which the trait evolves over time.

| Model | parameters | ML estimate | $\beta_1$ | P-value | $\sigma^2$ | $R^2_{pred}$ | AICc |
|---|---|---|---|---|---|---|---|
| OU (randomroot) | α | 155.97 | 1.30 | 9.74e-11 | 124.71 | 0.86 | 73.66 |
| Pagels λ | λ | 1.45e-08 | 1.24 | 5.78e-11 | 6.48 | 0.86 | 74.69 |
| Pagels δ | δ | 3.00 | 1.52 | 7.38e-12 | 13.59 | 0.84 | 79.55 |
| Pagels κ | κ | 0.38 | 1.50 | 9.78e-11 | 1.33 | 0.84 | 78.40 |
| Brownian motion | - | - | 1.58 | 2.18e-12 | 32.95 | 0.84 | 81.62 |
| Early Burst | rate | 0 | 1.58 | 2.18e-12 | 32.95 | 0.84 | 83.25 |

The diagnostic plots for the analysis showed that the assumptions of normality in the residuals as well as homoscedasticity were met (supplementary 7.5). However, all the diagnostic plots show that *A. arenaria* is a clear outlier (red circle in Figure 11). A model stability test showed that removing *A. arenaria* increased the $\beta_1$ estimate by 0.23. Both, the predictor and independent variable were bimodally distributed (Figure 11 and the diagnostics plot in supplementary 7.5).

## 3.3 Fructan and winter length

A reciprocal relationship was observed between the logarithm of Isothermality and the logarithm of AUCTF. The model fitted was thus logIsothermality~1/logarithm of AUTF The model with the lowest AICc score was the Pagels λ model as shown in Table 2. λ was estimated to ~0, again showing no phylogenetic signal in the residuals and thus equivalent to a regular OLS.

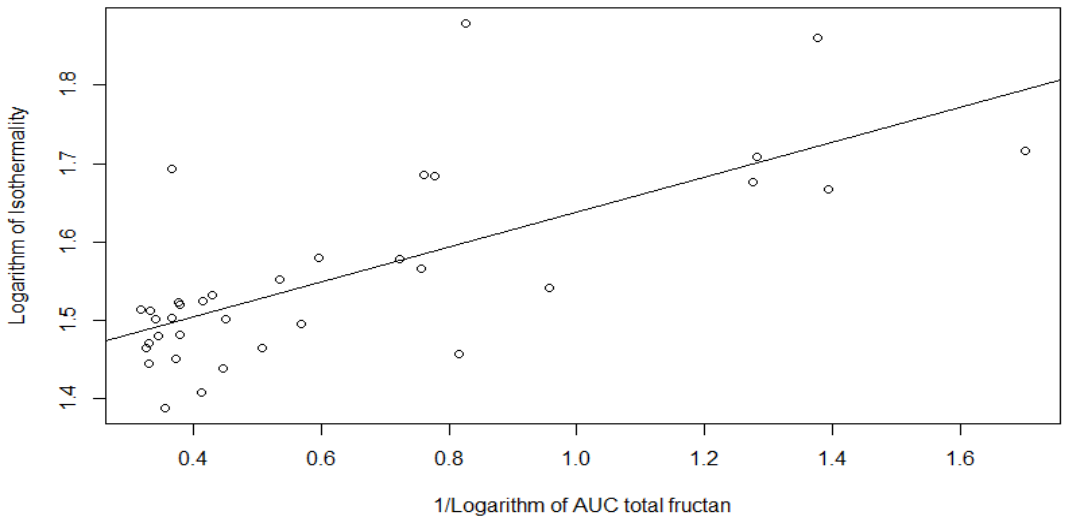


**Figure 12.** The fitted Pagels λ model (randomroot, Formula= logbio3~1/log AUCTF,) added on a scatterplot for the log-transformed species data (dots).

**Table 2.** Parameters for the fitted PGLS models for isothermality and fructan accumulation (Formula= logbio3~1/log AUCTF) $\sigma^2$ is the estimated variance of the trait and reflects the stochastic rate at which the trait evolves over time.

| Model | parameters | ML estimate | $\beta_1$ | P-value | $\sigma^2$ | $R^2_{pred}$ | AICc |
|---|---|---|---|---|---|---|---|
| Pagels λ | λ | 6.36e-09 | 0.23 | 1.61e-06 | 0.12 | 0.69 | -66.34 |
| OU (randomRoot) | α | 854.62 | 0.22 | 3.10e-06 | 11.69 | 0.69 | -65.88 |
| Pagels κ | κ | 1.30-08 | 0.25 | 4.96e-06 | 0.004 | 0.60 | -54.53 |
| Pagels δ | δ | 3.00 | 0.19 | 4.14e-04 | 0.44 | 0.60 | -40.32 |
| BM | - | - | 0.18 | 6.66e-04 | 1.21 | 0.58 | -31.55 |
| Early Burst | rate | 0 | 0.18 | 6.66e-04 | 1.21 | 0.58 | -28.99 |

The diagnostics plots for the PGLS analysis showed some deviation from normal distribution of residuals (supplementary 7.5), revealing a tendency of heteroscedasticity, likely due to an overrepresentation of fructan accumulating species with low BIO3 (supplementary 7.5). The range of $\beta_1$ obtained in the stability test was 0.202-0.254 and 1.400-1.425 for $\beta_0$, indicating no inordinate influential cases and a stable positive correlation. λ was estimated to ~0 for all excluded taxa.

Determining the number of months with a $T_{max}$ below 10°C revealed that several true fructan accumulators occur in areas with long periods (over six months) where the temperature never reaches 10°C. Non-fructan accumulators in contrast, never occur in such climates. Areas experiencing at least six months with $T_{max}$ below 10°C closely corresponded with the border between the Köppen-Geiger zones Dfc and Dfb (orange line in Figure 13). The pANOVA'analyses showed that the number of months with $T_{max}$ below 10-3°C differed significantly ($p<0.05$) between true fructan accumulators and non-fructan accumulators (supplementary 7.9) Neither true fructan accumulators nor non-fructan accumulators were significantly different from the 1-K accumulator group. The lowest point in the $T_{max}$ below 10°C plot in the true fructan accumulator group is *T. turgidum*, and the three highest points in the non-fructan accumulating species group are *M. transsilvanica*, *F. abyssinica* and *D. decumbens*.

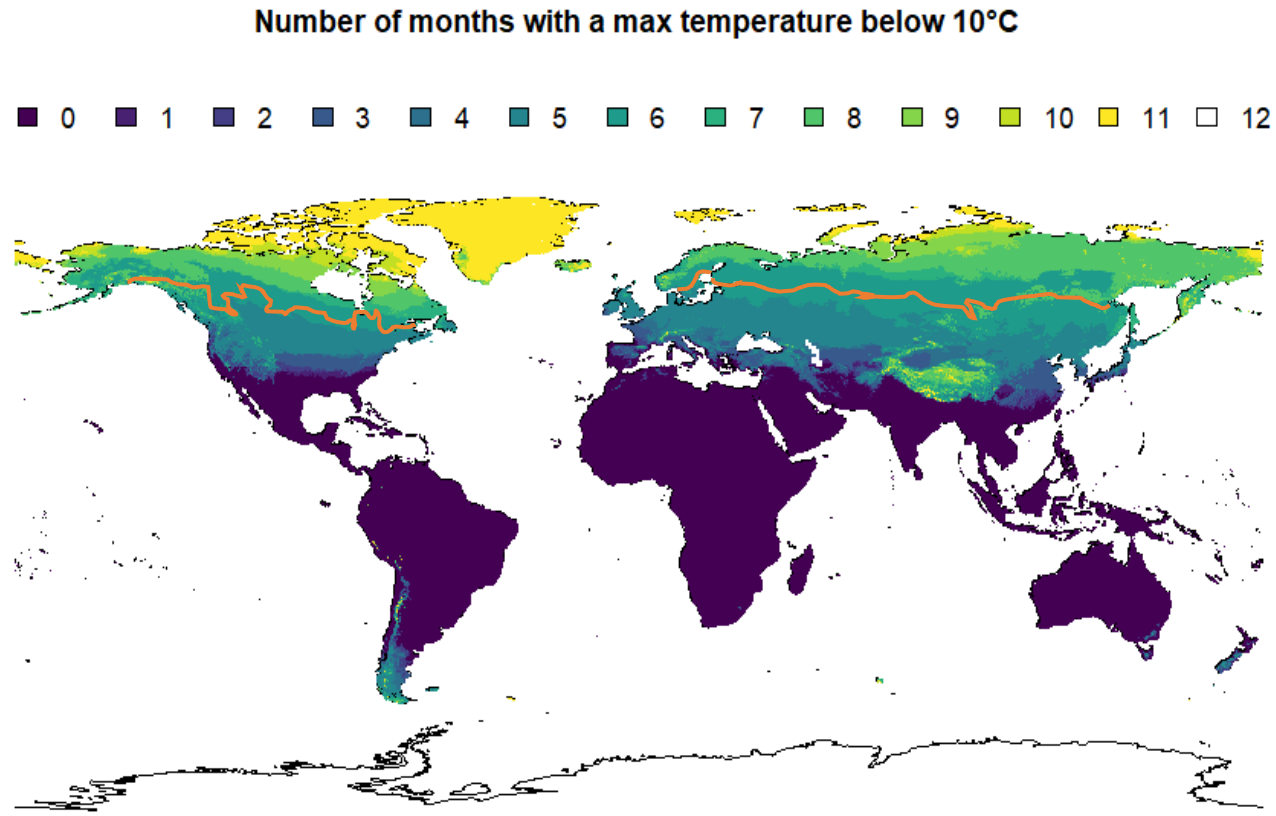


**Figure 13.** Number of months with a $T_{max}$ below 10°C plotted on the world map. Data was collected from Worldclim and has a spatial resolution of 5 archminutes. Orange line is the border between the Köppen-Geiger zones Dfc and Dfb which overlap with the 6 months with max below 10°C region.

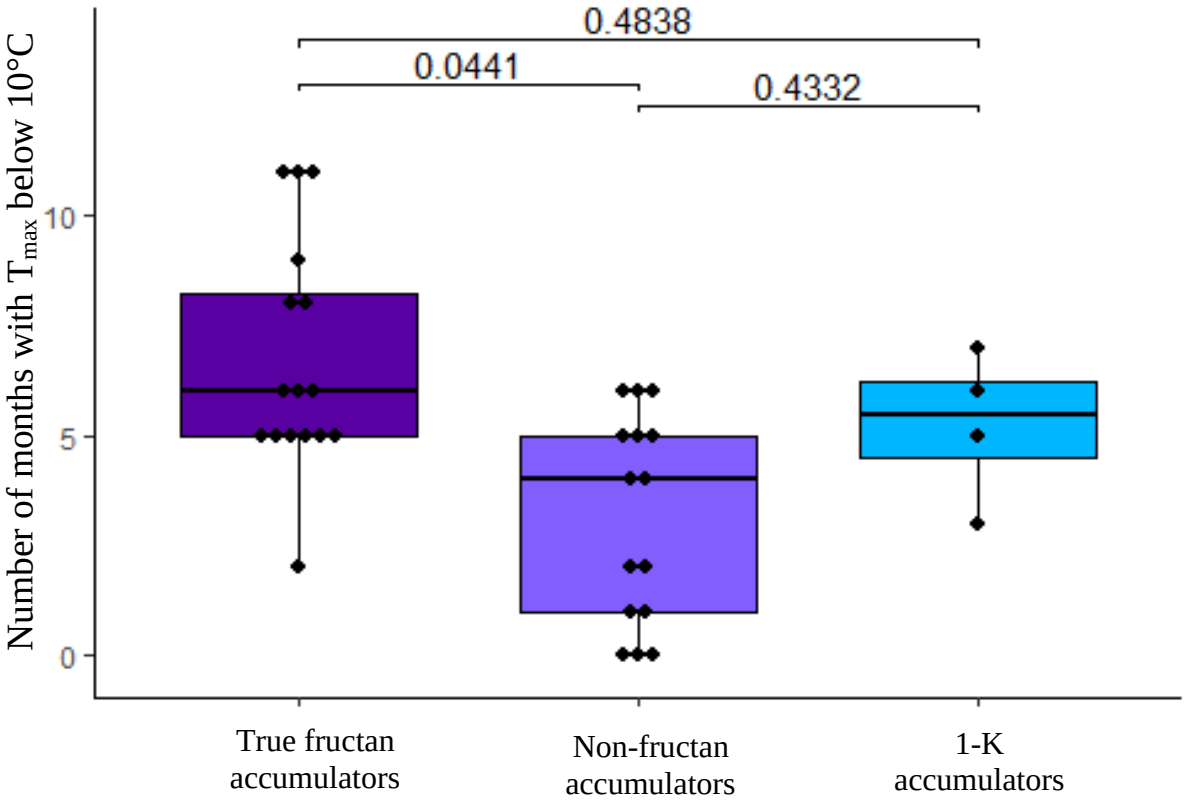


**Figure 14.** Phylogenetic ANOVA results for number of months with $T_{max}$ below 10°C versus the fructan accumulator classification groups. For plot with species labels see supplementary 7.7.

# 4 Discussion

## 4.1 High diversity of fructan types in Pooideae

The results from this project reveal a large diversity of fructan types across the Pooideae subfamily (Figure 7). Further, the obeserved fructan types are clustered within clades, following, to a certain extent, the major core Pooideae tribes. This pattern is in agreement with main fructan types determined in the lower organs of other core pooids from earlier studies (Henson & Livingston, 1996; Sprenger et al., 1997; Verspreet et al., 2013).

The phylogentically clustered pattern of fructan types is in accordance with the evolutionary history of Pooideae (Sandve & Fjellheim, 2010; Schubert et al., 2019a; Schubert et al., 2019b) Within Poeae and Aveneae there are two separate clades, respectively, that show different fructan types. The divergence of these clades was inferred to occur in late Eocene and Eocene-Oligocene transition, respectively, just prior to the super-cooling period (Schubert et al., 2019a). The clades that synthesise levan must have evolved genes encoding 6-SFT from vacuolar invertases, as well as 1-SST if the alternative pathway occur (Figure 2) (Ritsema & Smeekens, 2003; Valluru & Van den Ende, 2008). Inulin synthesising species must have evolved 1-SST and 1-FFT, whereas the ones synthesising neoseries must have evolved 6G-FFT. Furthermore, all true fructan accumulators must have evolved several types of FEHs from cell wall invertases to be able to hydrolyse the different fructan types. This illustrates the complex evolutionary trajectory of FTs within the core Pooideae, which is likely the result of extensive gene duplication and neofunctionalisation coinciding with the expansion of temperate climates.

In addition to previously described true fructan accumulating core pooids (3.1), I identified the non-core pooid *Nardus stricta* as a true fructan accumulator. Zhong et al. (2018) inferred that many cold responsive genes evolved prior to the Pooideae MRCA, but that a subgroup of genes evolved *de novo*. The number of diverse fructan accumulators in the core Pooideae suggests that the MRCA of these tribes possessed some fructan synthesising abilities or was, at least, predisposed to evolve this ability. This was corroborated by Schubert et al. (2019b) where the FT gene family was inferred to originate at the base of core Pooideae. If the Pooideae MRCA would have possessed true FTs, many losses appear to have occurred within non-core Pooideae tribes, which seems implausible. Thus, it is likely that FT encoding genes evolved *de novo* from invertases multiple times. The tendency of convergent evolution towards fructan accumulation in several pooid tribes suggests an adaptive advantage.

Interestingly, some core Pooids, i.e., *P. lanuginosa, P. bulbosa, F. abyssinica, A. tauschii* and *F. hystrix,* did accumulate verly low or even no amounts of fructan in my experiment. Because of the phylogenetically clustered fructan type pattern, it is most parsimonious to assume that these species have either lost their ability to synthesise fructan or the ability to initiate fructan synthesis in response to low temperatures at some point in their evolutionary history. Multiple losses of fructan accumulation ability (in response to cold) may suggest that there is no longer an adaptive advantage for these species to maintain this function. Which may further imply, that the cost of fructan accumulation overules its benefits.

In general, at least *F. hystrix* and *A. tauschii* seem to be physiologically able to synthesise fructan, due to small amounts of neoseries and inulin in *F. hystrix* and graminan in *A. tauschii*. These species are predominantly found in Spain and the middle east, respectively, and are thus most likely adapted to drought and heat stress. Interestingly, fructan has also been linked to drought and heat tolerance in previous studies, probably as a result of membranes beeing a critical point of injury under such stress as well as cellular dehydration and the formation of ROS (Livingston et al., 2009; Nievola et al., 2017). It would therefore be enlightening to analyse fructan content and type in Pooideae species in response to different stresses.

The non-core pooids *B. phoenicoides, B. pinnatum, M. nutans* and *S. capillata* were classified as 1-K accumulators and likely possess 1-SST enzymes but not additional FTs that enable biosynthesis of higher DP fructans. Relatively small amounts of Nys in *B. pinnatum* and *B. phoenicoides* further suggest that both species possess 1-FFTs. Alternatively, this may also indicate a high promiscuity of their 1-SST enzymes (Ritsema et al., 2006). In the case of the two *Brachypodium* species, it is plausible that their *1-SST* genes share a common lineage which goes back to the Brachypodieae MRCA, possibly even with the MRCA of Brachypodieae and core Pooideae. However, it has been found that relatively few mutations in the Suc binding domain of vacuolar invertases are needed to obtain an enzyme with 1-SST activity (Schroeven et al., 2008). For this reason, the ability to synthesise fructan has long been acknowledged as a polyphyletic trait with multiple independent origins across the plant kingdom (Hendry, 1993).

### 4.1.1 Regrowth and its connection with fructan concentration and degree of polymerisation

Because the plants in my experiment had experienced a period of chilling and freezing prior to the first sampling (Figure 6), I expected that they had already started to cold acclimate and accumulate fructan (Versluys et al., 2018). This was corroborated by the curves obtained from the experimental data (supplementary 7.4). Due to the use of accessions based on wild

populations, it is reasonable to expect variable fructan concentrations within species. In addition, the fact that different individuals were sampled each month, without biological replicates, may be another reason for the deviation of the TF curves from the theoretical curve (Figure 5). Despite the intraspecific variation, there is a clear trend of species maintaining a certain amount of fructan throughout winter having a greater regrowth ability and less frost damage in spring. This observation is in accordance with my expectations. The three species with the highest regrowth score (*L. perenne*, *P. alpina* and *P. pratense*) had around 100 mg/g DW fructan in the last sampling in April. All three species accelerated fructan breakdown between late December and late January, coinciding with a slight temperature increase (figure 6), which may have triggered fructan hydrolysis providing energy for regrowth.

In *F. hystrix* and *A. tauschii*, which did not fulfil all the true fructan accumulator requirements, considerable amounts of oligo- and high DP fructans in two months were detected (supplementary 7.2). *A. tauschii* appears to deplete its fructan storage rapidly in the start of winter, whereas *F. hystrix* appears to start the fructan accumulation in mid-winter before depleting it. These results were unexpected and deviate from all other patterns observed in this experiment, as well as previous experiments (Pollock & Jones, 1979; Bergjord Olsen & Skjelvåg, 2011; Tamura, Sanada et al., 2014). *F. hystrix* obtained a regrowth score of 0.44, indicating dead plants. *A. tauschii* obtained a slightly higher regrowth score of 2.06, which still indicates dead plants, however.

*Phleum pratense* was the species with the highest fructan DP (average DP of 30) and, interestingly, also the one with the highest regrowth score. *Poa alpina*, *P. glauca*, *L. perenne* and *A. mertensii* had average DPs varying between 10-20 and exhibited high regrowth scores as well. However, several species with high regrowth scores had average DPs in the 5-10 range. Hence, no strong correlation between average DP and winter survival was observed in either the pPCA (Figure 10) or a PGLS analysis (results not shown). Liposome experiments, as well as molecular dynamics studies, have shown that the DP influences the stabilising effects of different types of fructan on membranes in a rather complex, and not fully understood, manner (Vereyken et al., 2003; Livingston et al., 2009) which might explain the lack of clear connection between DP and winter survival.

## 4.2 Strong correlation of regrowth and fructan accumulation

The result of the PGLS analysis revealed a strong correlation between the amount of fructan throughout the winter months and regrowth score in spring. This result is in accordance with

my hypothesis based on the research on fructan's putative role in cold adaptation. Several core Pooideae species did not accumulate fructan, which disrupts the phylogenetic pattern in fructan accumulating abilities and thus explains the lack of phylogenetic signal in the residuals. This lack of phylogenetic signal became very apparent between the four species from the *Poa* genus. The distribution of both the dependant and independent variable (supplementary 7.5) indicates bimodal distributions. This is expected, as only few species showed an intermediate regrowth score as well as an intermediate fructan accumulation ability. However, there were some species in this intermediate range which appeared to follow the same trend as the two extremes, I thus concluded that enough of the spectra was covered to conduct a valid regression. The outlier *Ammophila arenaria*, with a regrowth score of 0.8 and a AUCTF value of 1040.78, is native to Europe and north Africa where it is most often found in sand dunes along the coastline (Huiskes, 1979). Its low regrowth score might be explained by its biology, since *A. arenaria* naturally grows in tight tussocks, which it did not form in this experiment.

## 4.3 Analysis of fructan and distribution pattern in Pooideae

Despite the five outliers in the qq- plot (supplementary 7.5), a clear, reciprocal trend between Isothermality and TF was observed. In other words, species which accumulated high amounts of fructan tend to occur in regions with low isothermality, i.e., long winters. However, because isothermality is only a proxy for winter length, a more direct investigation was conducted (2.3.5.2).

The pANOVA revealed that true fructan accumulators occur in regions with longer winters than non-fructan accumulators. Starch synthesis efficiency has been found to decrease drastically below 10°C, whereas fructan synthesis may continue at lower temperatures (Hendry, 1987; Van den Ende, 2013). The decrease in starch synthesis leads to accumulation of Glc and Suc, which have been shown to repress photosynthetic activity (Araya et al., 2006). Thus, number of months with a $T_{max}$ below 10°C seems to be a biologically meaningful measure of length of winter, since it likely poses an ecological obstacle for non-fructan accumulators, that cannot photosynthesise efficiently below this temperature. However, as the temperatures from Worldclim are most likely not measured at grass level, and the 5 arcminutes resolution equals rather large geographical tiles, a gradient of $T_{max}$ from below 10-0°C was explored (supplementary 7.9). Mainly fructan accumulating pooids appear to have been able to disperse above the Dfc/Dfb border (supplementary 7.9 and Figure 13). The five core Pooideae species that did not accumulate fructan in my experiment are distributed below this border, indicating that adaptations to frost and specifically long winters might be less important for their

ecological success. Thus, maintaining fructan accumulation ability in response to cold might be too costly for these species in regard the adaptive benefits. The 1-K accumulators appear to have an intermediate climate distribution, i.e., one of them occurs at higher latitudes of the western part of the Dfc zone (Supplementary 7.8; *M. nutans*), two of them occur in low latitudes of the Dfc zone (Supplementary 7.8; *S. capillata* and *B. pinnatum*), whereas one does not occur in this zone (Supplementary 7.8;*B. phoenicoides*). It is, however, important to note that the 1-K accumulators are only represented by four species in this experiment. It would be interesting to repeat my pANOVA with more Pooideae species from this and other categories.

Two of the non-fructan accumulating species that endure relatively long winters, i.e., *D. decumbens* and *M. transsilvanica,* fulfilled the requirements to be classified as putative RFO accumulators (supplementary 7.7 and 7.3). Like fructans, RFOs possess many abilities to protect against frost stress (Van den Ende, 2013). However, these putative RFO accumulators seem not to be able to endure the long winters of the Dfc zone. This was also evident in my growth experiment since both species showed clear signs of winter and frost damage (regrowth scores around 6). This indicates that fructan accumulators may have some advantage over RFO accumulators when it comes to protection against frost damage. One advantage might be the ability of fructans to interact with the tonoplast and cellular membranes whereas RFOs are restricted to thylakoid membranes (Tarkowski & Van den Ende, 2015).

When plotting species of different fructan types against their $T_{max}$ below 10°C, there is a clear pattern of winter length separating fructan types (Figure 15). Only species of the levan type group, as well as one of the neoseries type group, appear to occur in regions with very long winters. Despite few species in each category, the observed pattern may indicate that certain fructan types are more advantageous than others in regions with long, cold winters. This finding underlines the importance of analysing the *type* of fructan, in addition to the amount, when conducting comparative studies of fructan accumulating abilities.

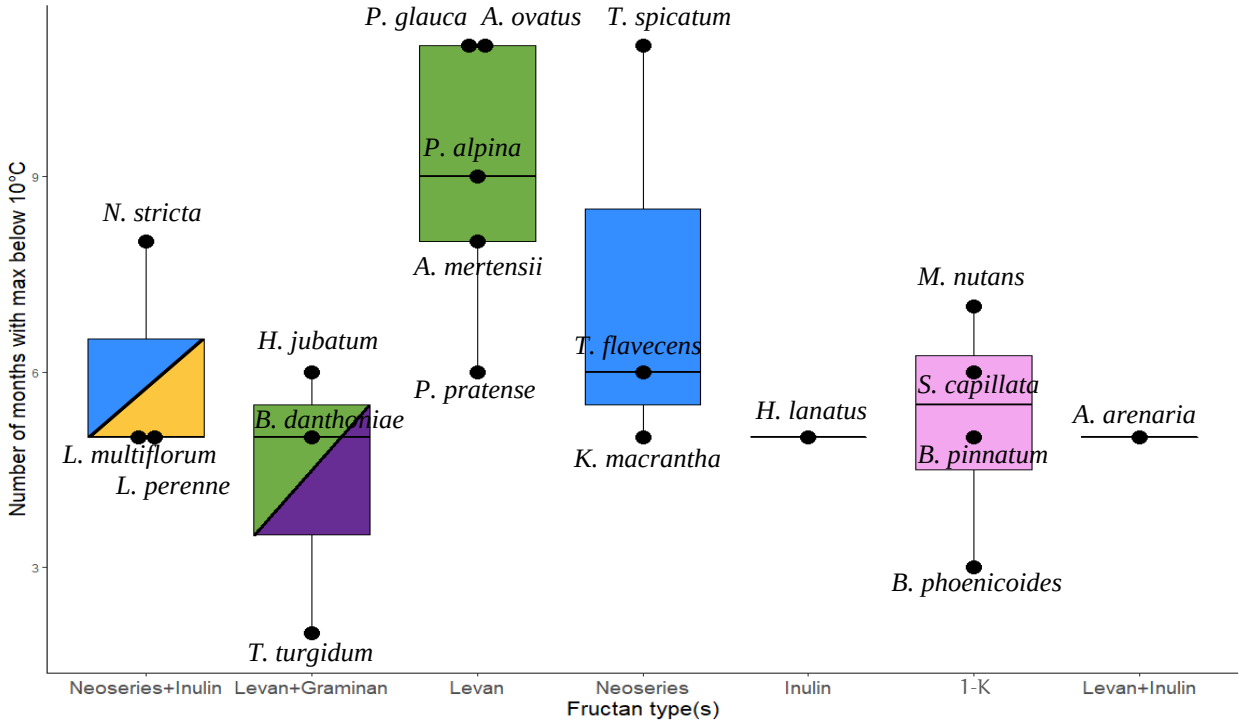


**Figure 15.** Fructan types plotted against number of months below 10°C. Colours match the fructan type plotted on the phylogeny in **Figure 7**.

Finally, CA and the ability to endure a long winter requires the development of an array of physiological responses. Many morphological features, such as small stature and "cuddliness", are also important for adaptation to temperate and cold climates (Körner, 2016). The ability to synthesise fructans and maintain a certain amount during winter does therefore not alone explain the full picture of cold adaptation and endurance in climate regions with long winters. However, the results from my study indicate that fructan accumulation in response to cold has played a crucial role in the successful dispersal of Pooideae into cold temperate climates.

# 5 Conclusion and prospects

My results reveal a large variety in fructan types in the Pooideae subfamily and I discovered several previously unknown fructan accumulators. The observed phylogenetic pattern of fructan types suggests several independent origins of FTs and FEHs in the subfamily, which is in accordance with the acknowledged polyphyletic nature of the fructan accumulation trait. It would be interesting to further investigate this by molecular evolution studies on the *FT* and *FEH* genes. An increasing body of evidence correlates the ability to synthesise fructan and adaptation to extreme environments. My results corroborate this as fructan amount throughout the winter showed a clear correlation with regrowth ability in spring in phylogenetically diverse Pooideae species. Moreover, the analysis of variance with global distribution data revealed a trend of fructan accumulators being able to survive in areas with longer winters than non-fructan accumulators. Further research in this field should seek to investigate whether different types of fructan have distinct adaptational advantages in different climates. New insights in the adaptive role of fructan can potentially be used to breed crop species with higher drought and frost tolerance, which is important in the light of a changing climate.

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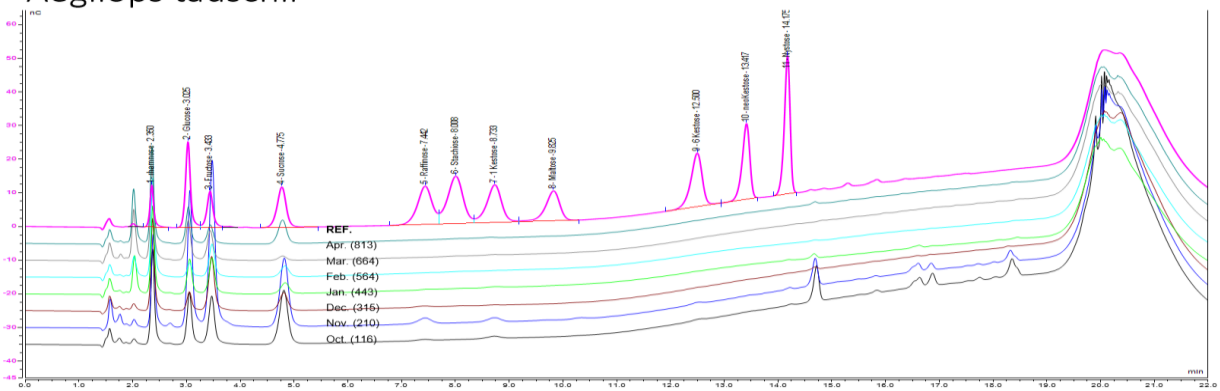
# 7 Supplementary information

## 7.1 Species in the experiment

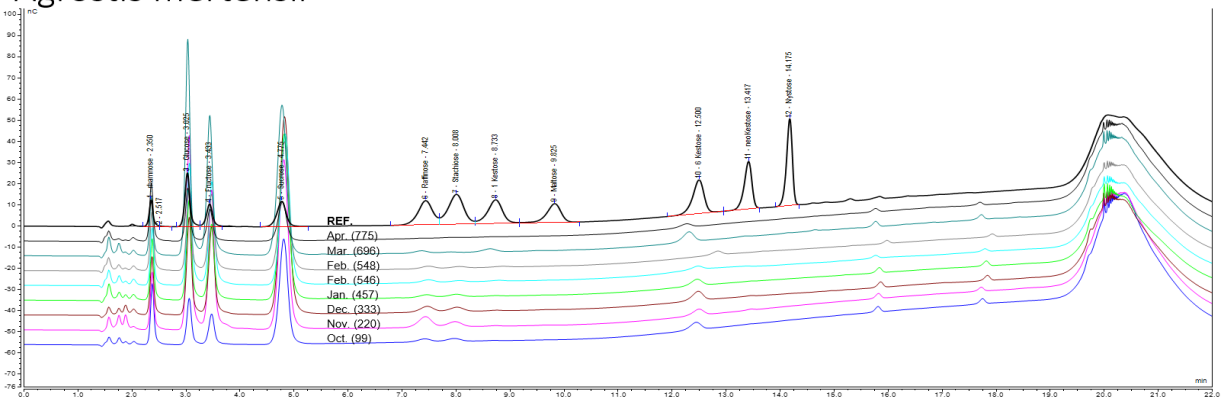
| Species | Tribe | regrowth |
|---|---|---|
| Aegilops_tauschii | TRI | 2,06 |
| Agrostis_mertensii | AVE | 7,95 |
| Alopecurus_ovatus | POE | 2,53 |
| Ammophila_arenaria | AVE | 0,8 |
| Austrostipa_bigeniculata | STI | 0,43 |
| Brachypodium_phoenicoides | BRA | 6,05 |
| Brachypodium_pinnatum | BRA | 5,79 |
| Bromus_danthoniae | TRI | 7,55 |
| Danthonia_decumbens | DAN | 5,85 |
| Ehrharta_calycina | EHR | 0 |
| Festuca_abyssinica | POE | 0 |
| Festuca_hystrix | POE | 0,44 |
| Holcus_lanatus | POE | 8,35 |
| Hordeum_jubatum | TRI | 8,05 |
| Koeleria_macrantha | AVE | 8,9 |
| Lolium_multiflorum | POE | 5,67 |
| Lolium_perenne | POE | 8,95 |
| Lygeum_spartum | LYG | 0,4 |
| Melica_imperfecta | MEL | 0 |
| Melica_nutans | MEL | 8,74 |
| Melica_transsilvanica | MEL | 6,16 |
| Nardus_stricta | NAR | 5,95 |
| Nassella_hyalina | STI | 0,53 |
| Nassella_pubiflora | STI | 0 |
| Nassella_tenuissima | STI | 0 |
| Oloptum_milliaceum | STI | 0 |
| Phleum_pratense | POE | 9 |
| Poa_alpina | POE | 8,95 |
| Poa_bulbosa | POE | 0,31 |
| Poa_glauca | POE | 8,89 |
| Poa_lanuginosa | POE | 0 |
| Stipa_capillata | STI | 1,84 |
| Trisetum_flavescens | AVE | 8,89 |
| Trisetum_spicatum | AVE | 7,85 |
| Triticum_turgidum | TRI | 8,84 |

## 7.2 HPAEC-PAD chromatograms

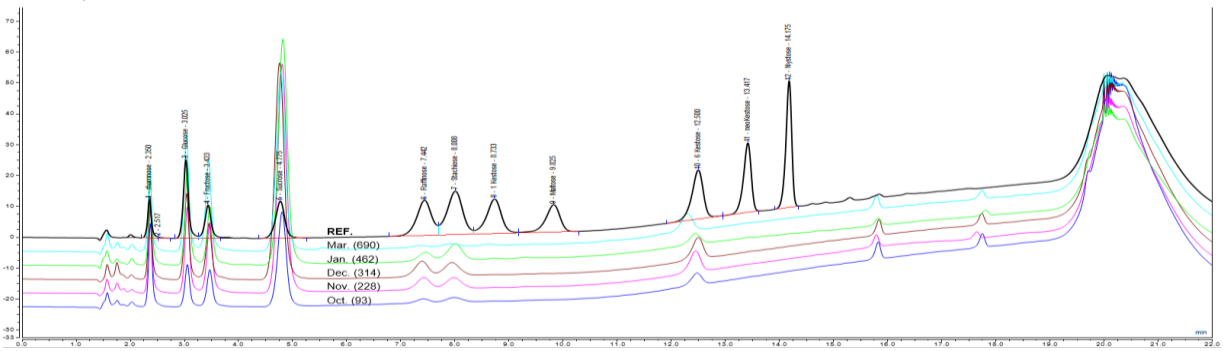
Aegilops tauschii



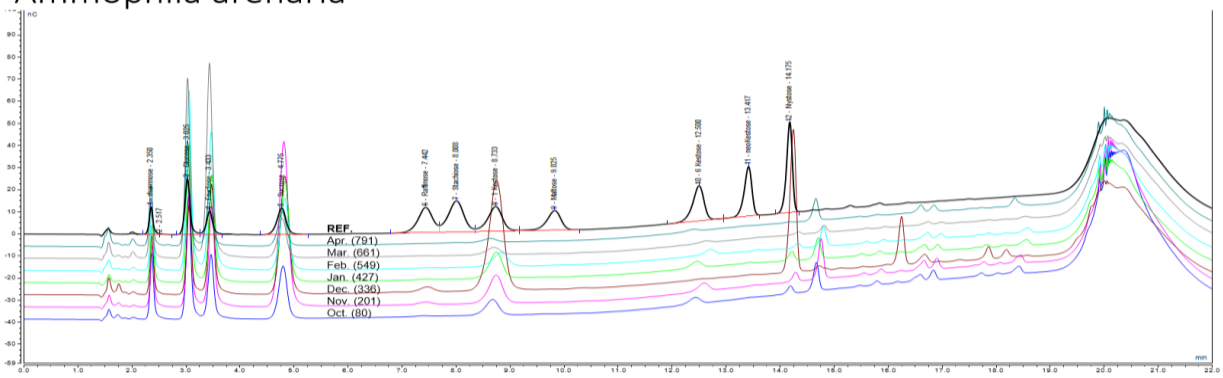

Agrostis mertensii



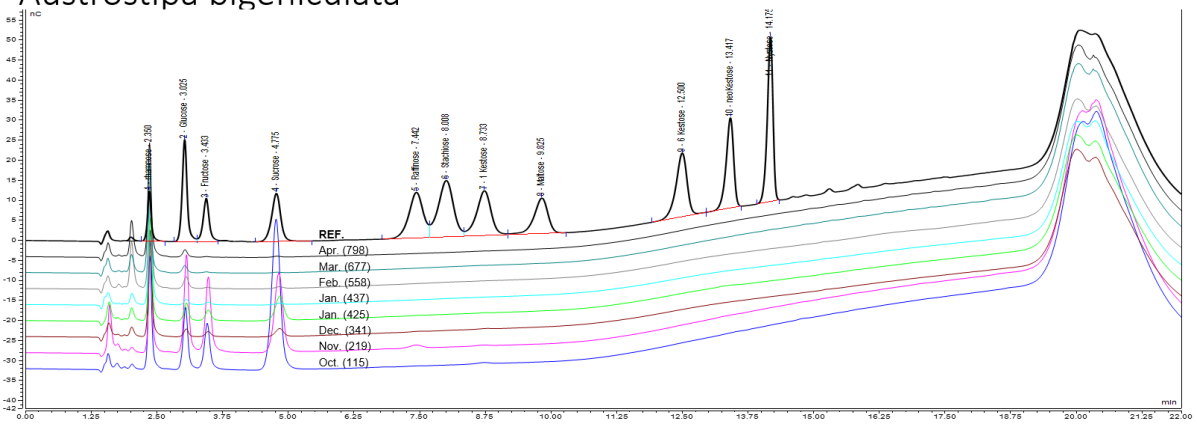

Alopecurus ovatus



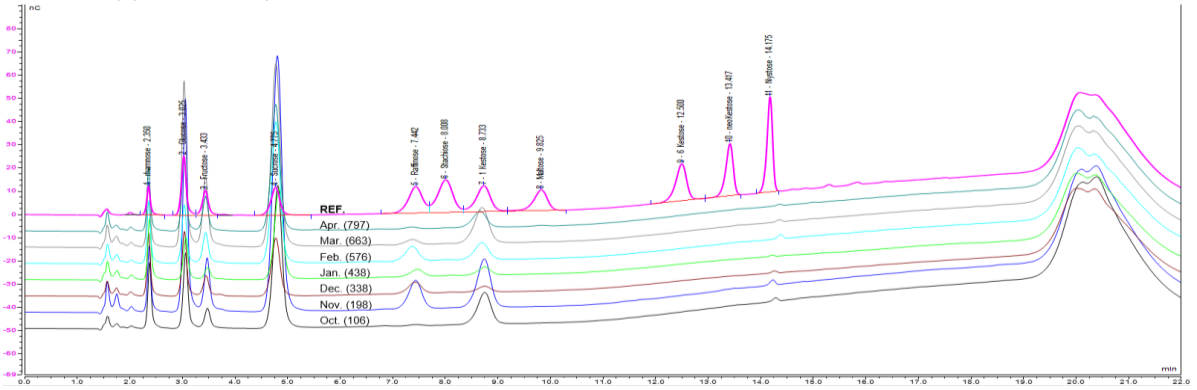

Ammophila arenaria



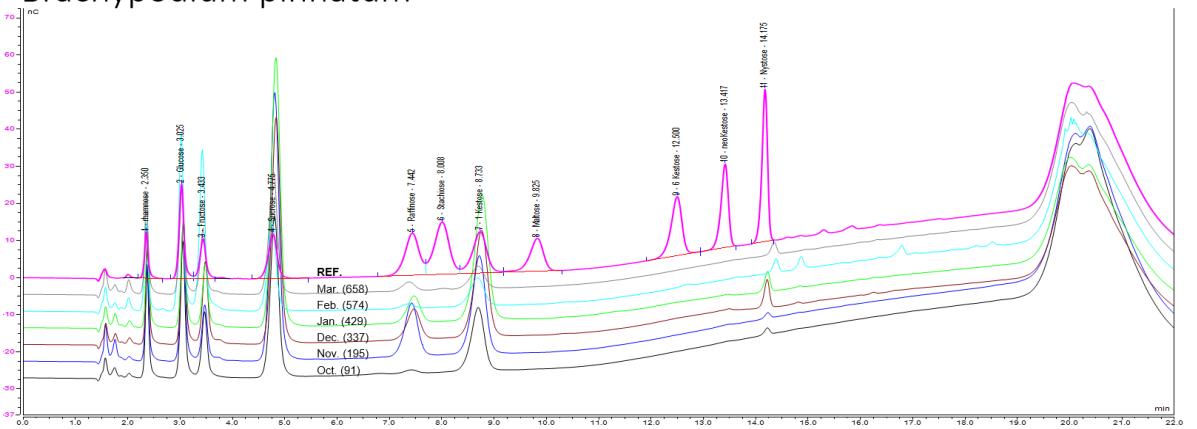
## Austrostipa bigeniculata



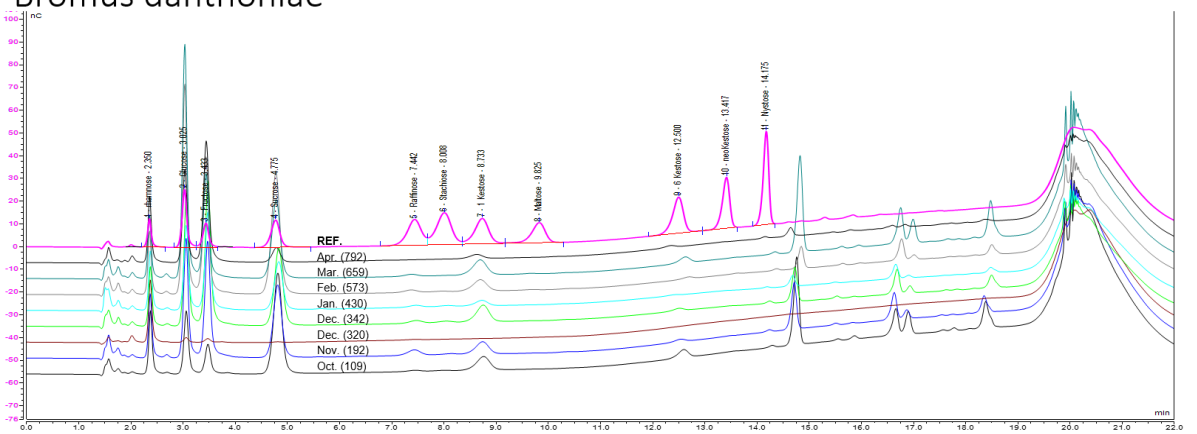

## Brachypodium phoenicoide



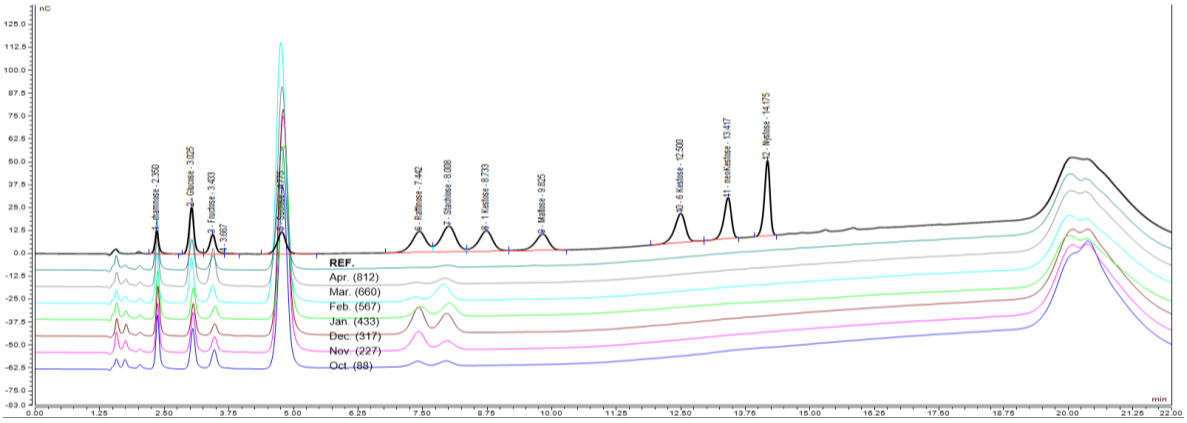

## Brachypodium pinnatum



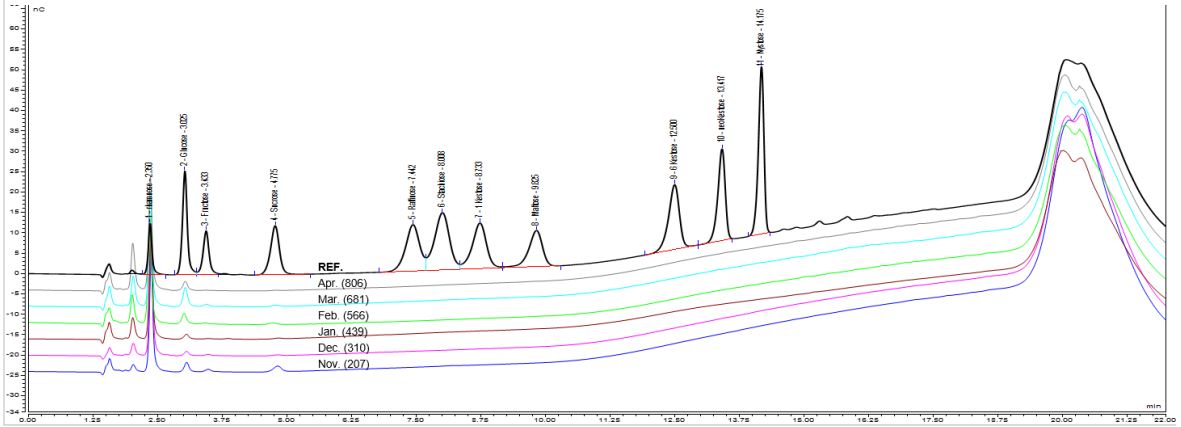

## Bromus danthoniae



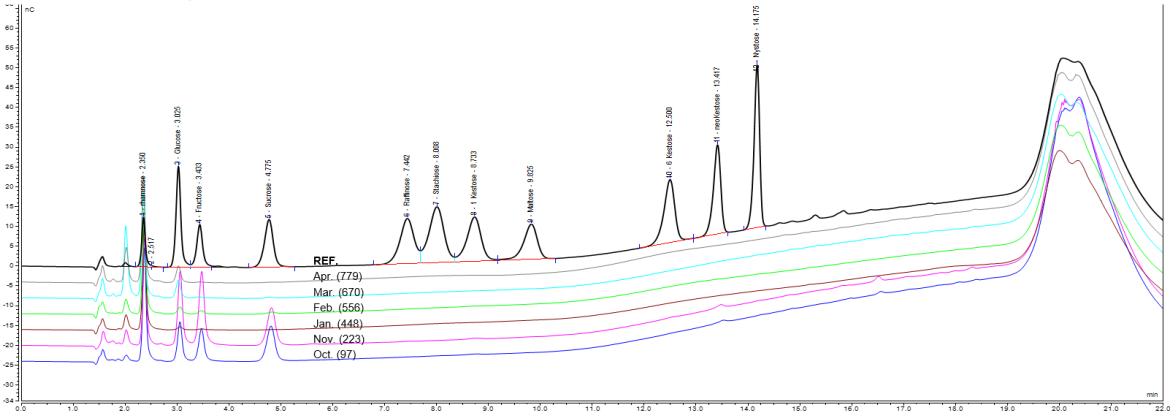
## Danthonia descumbens



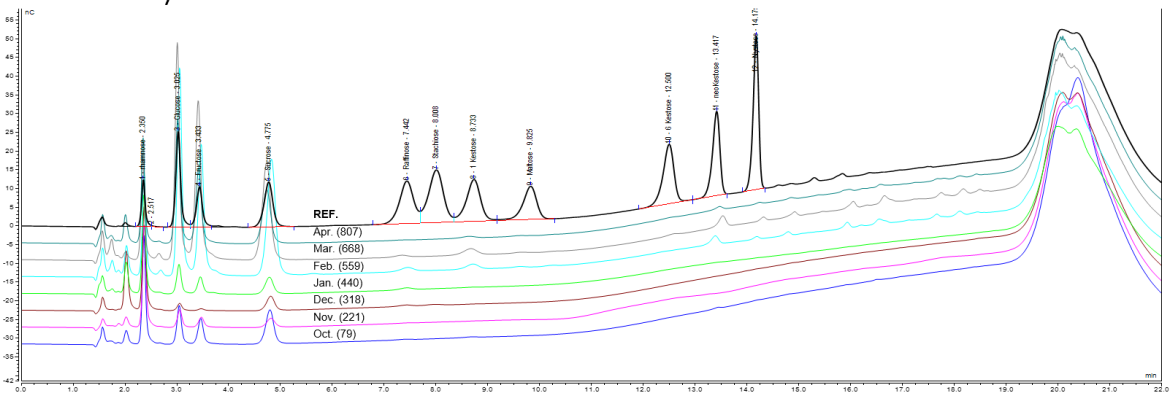

## Ehrharta calycina



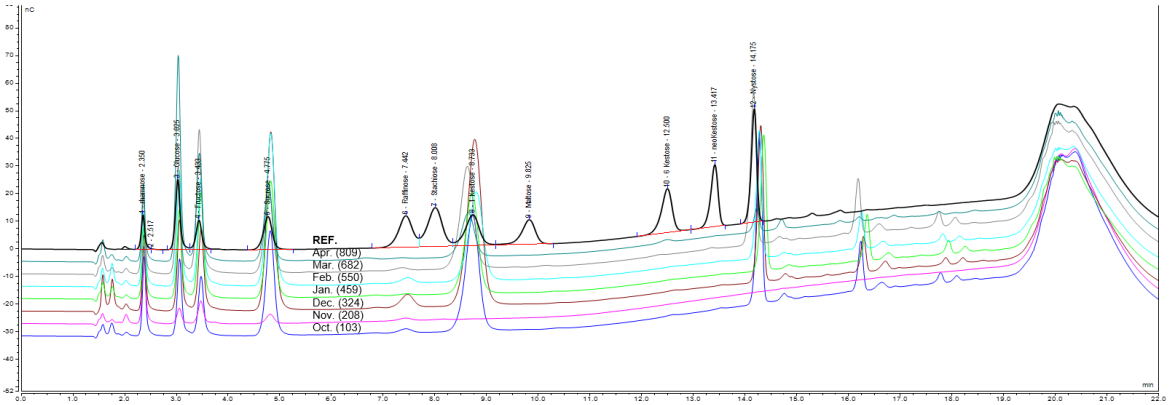

## Festuca abyssinica



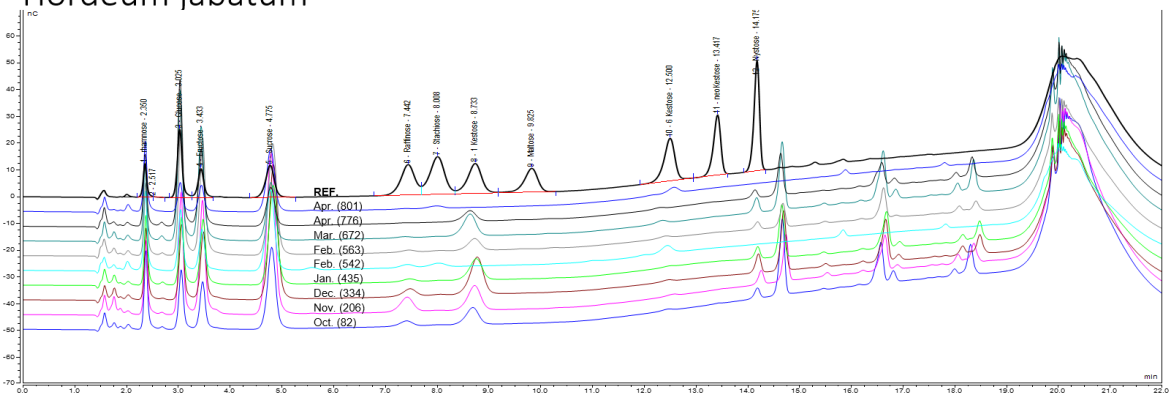

## Festuca hystrix



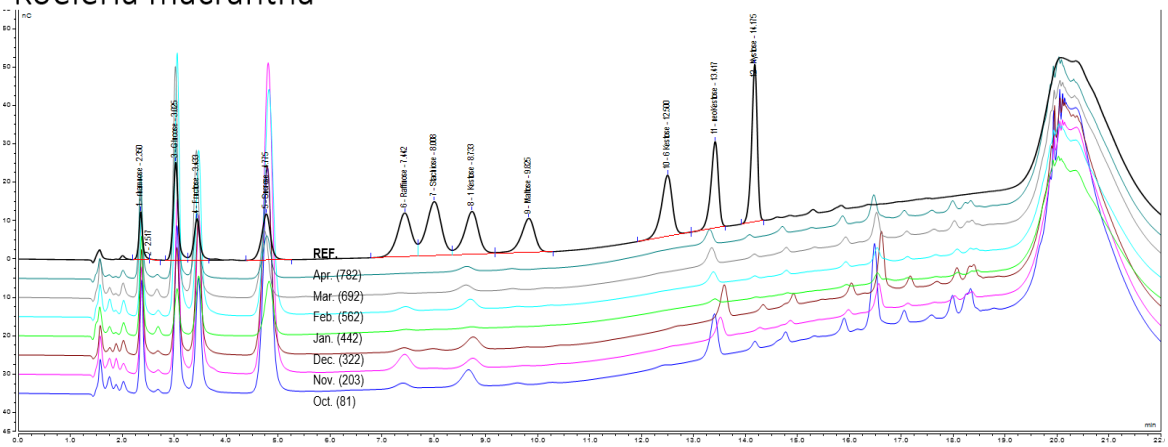
## Holcus lanatus



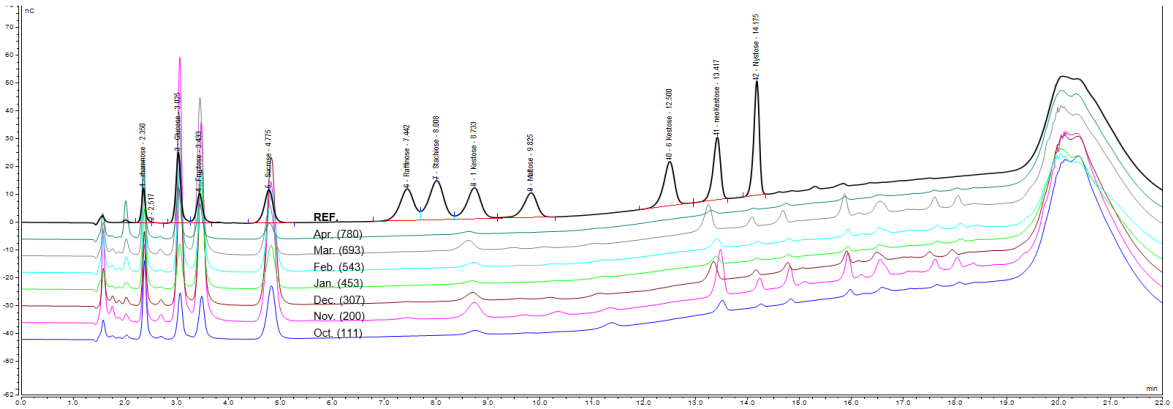

## Hordeum jabatum



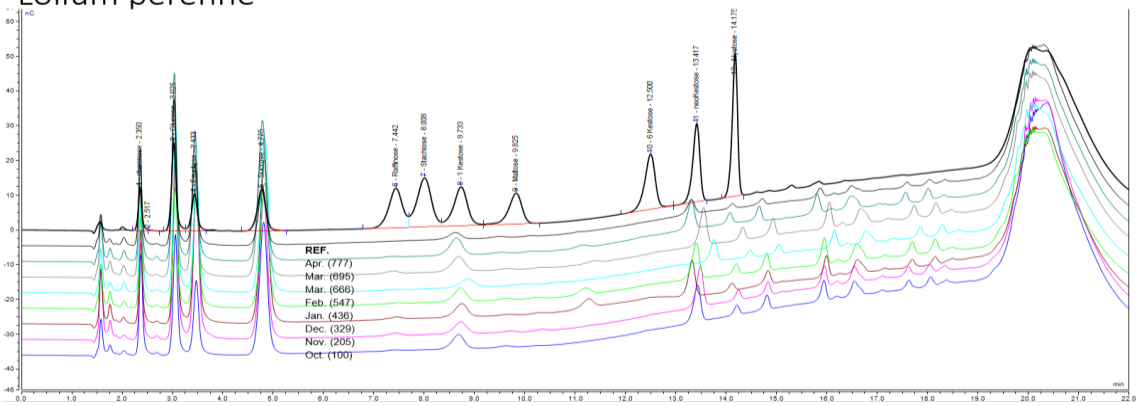

## Koeleria macrantha



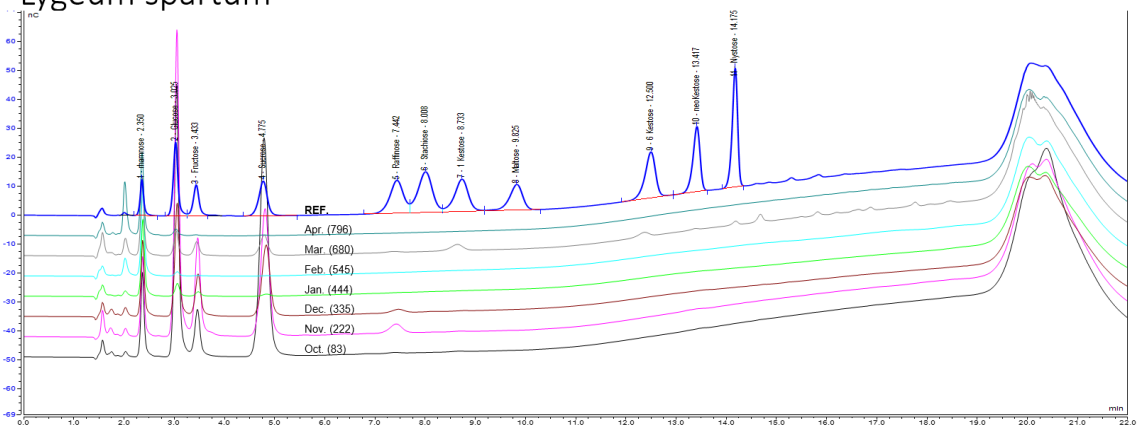

## Lolium multiflorum



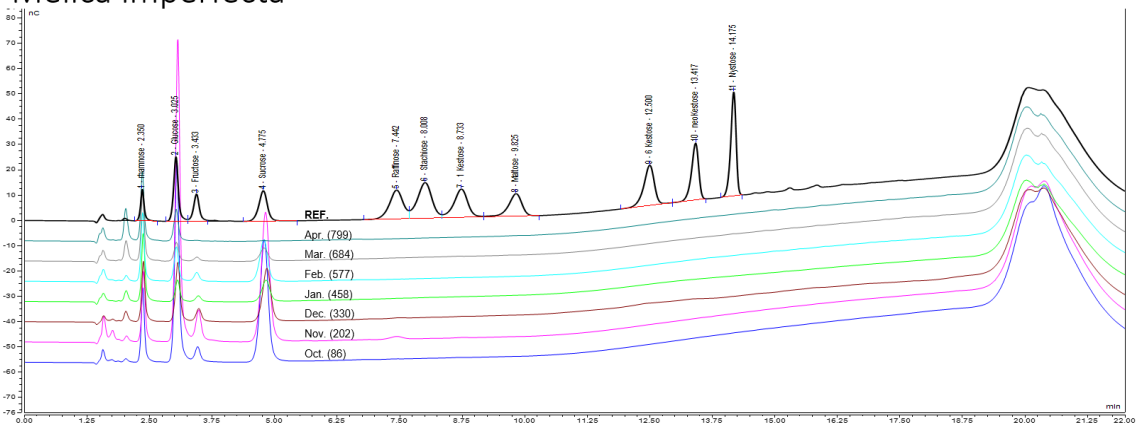
## Lolium perenne



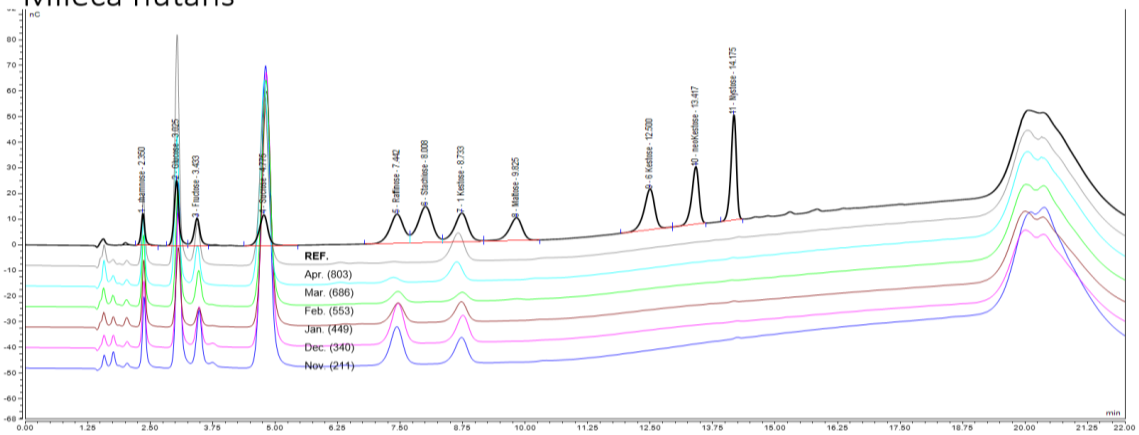

## Lygeum spartum



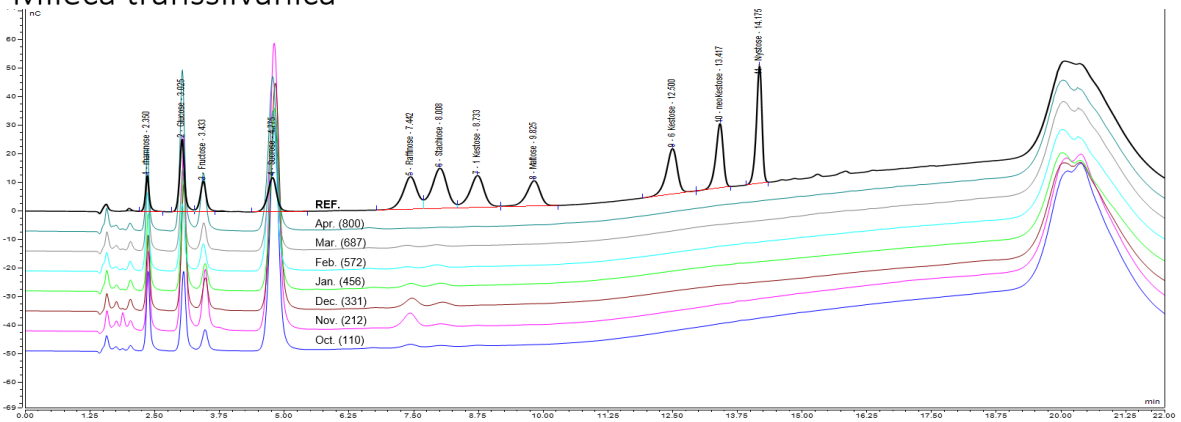

## Melica imperfecta



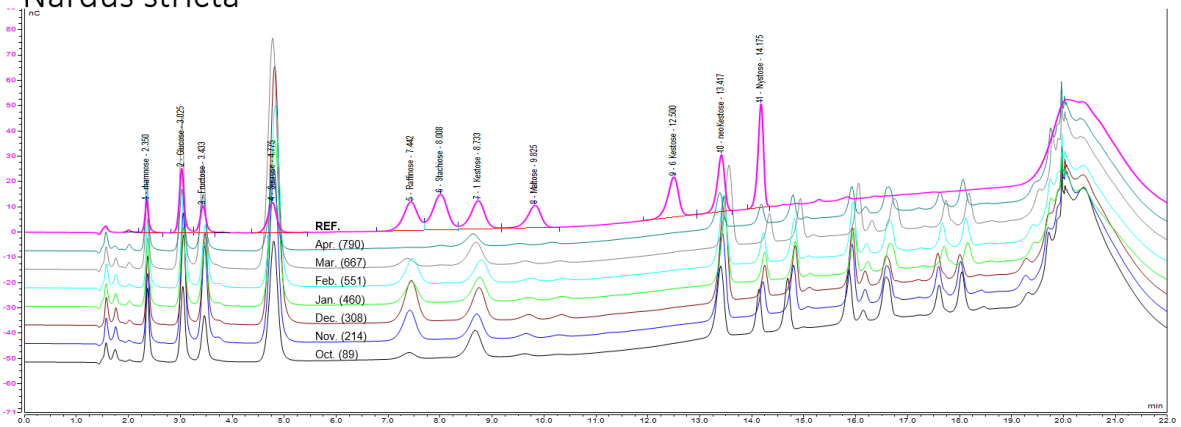

## Mileca nutans



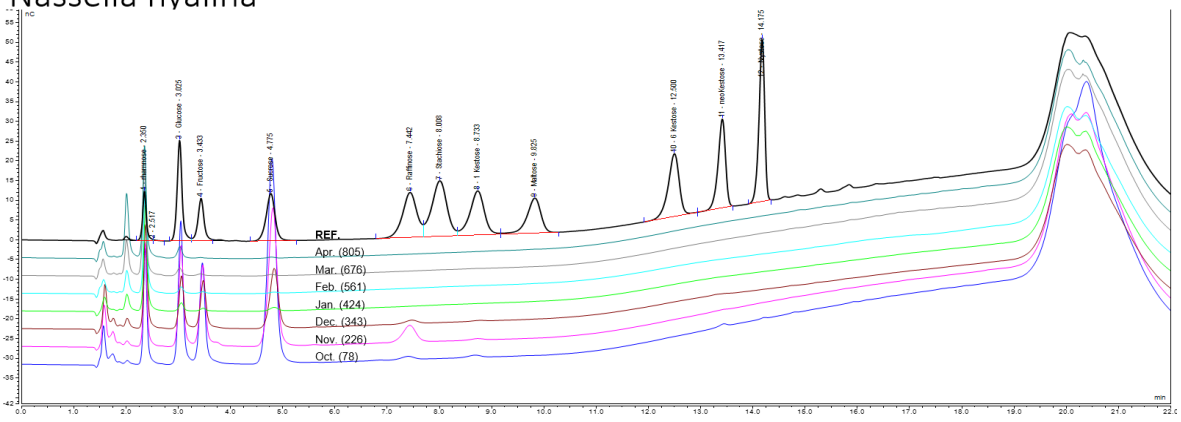
## Mileca transsilvanica



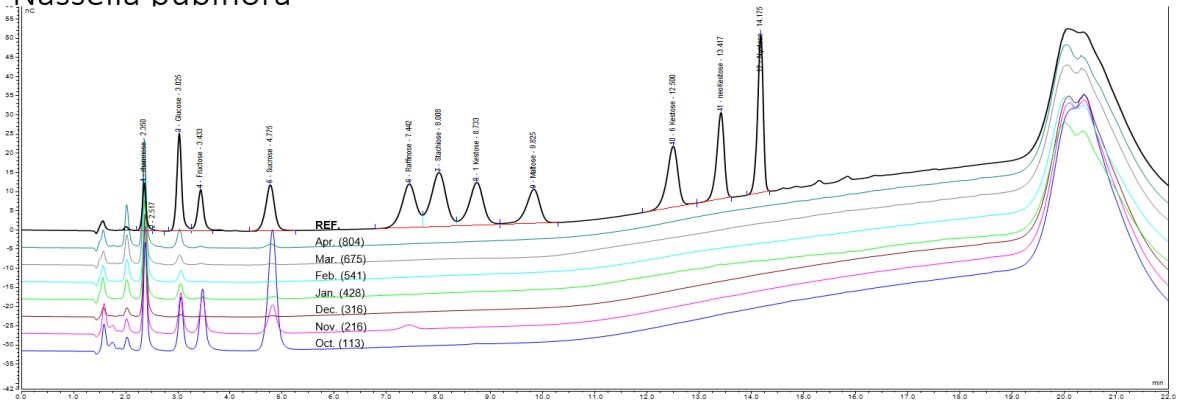

## Nardus stricta



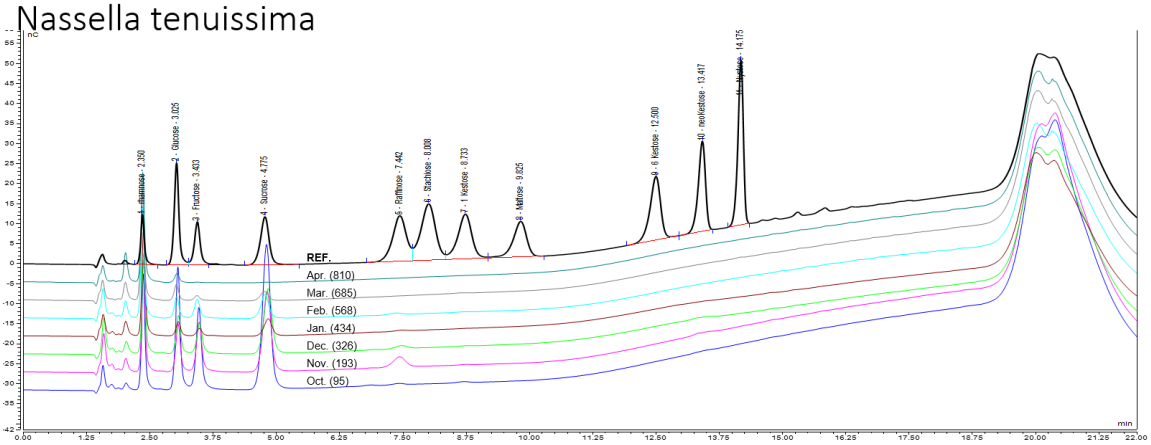

## Nassella hyalina



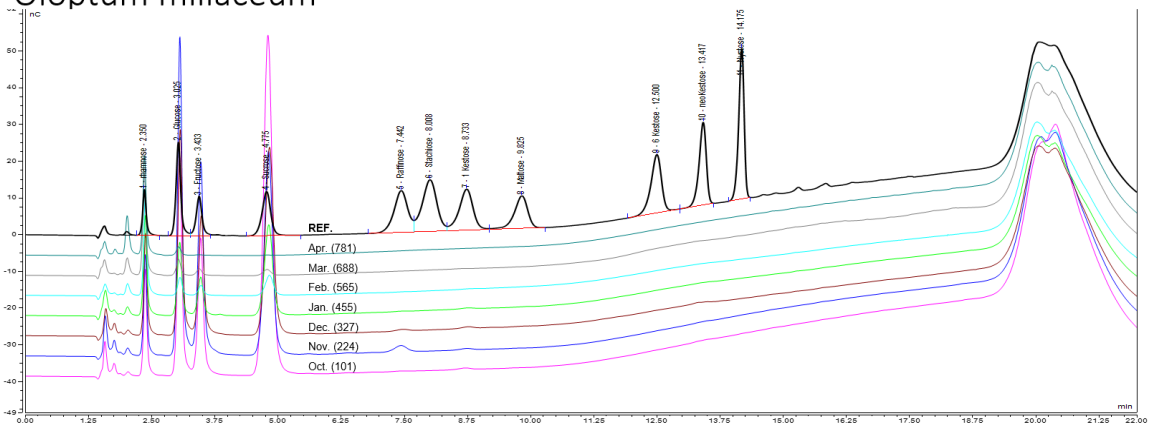

## Nassella pubiflora



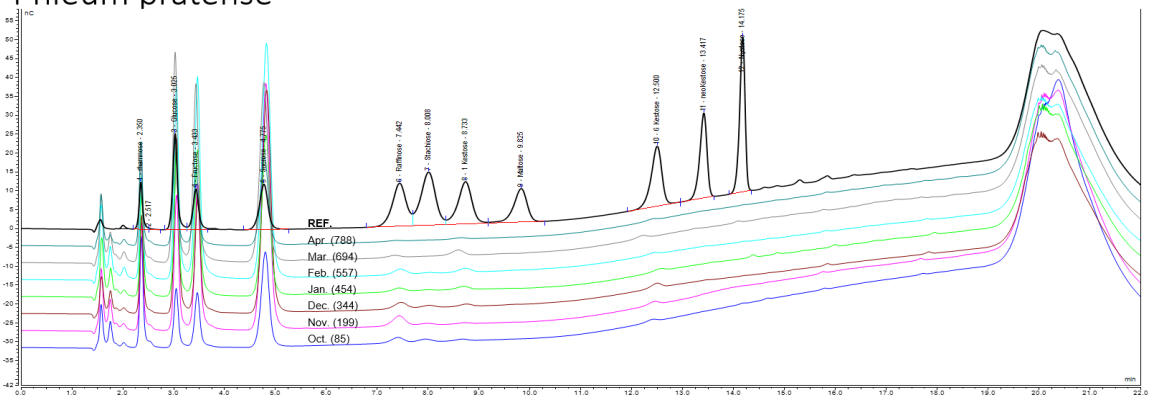
## Nassella tenuissima



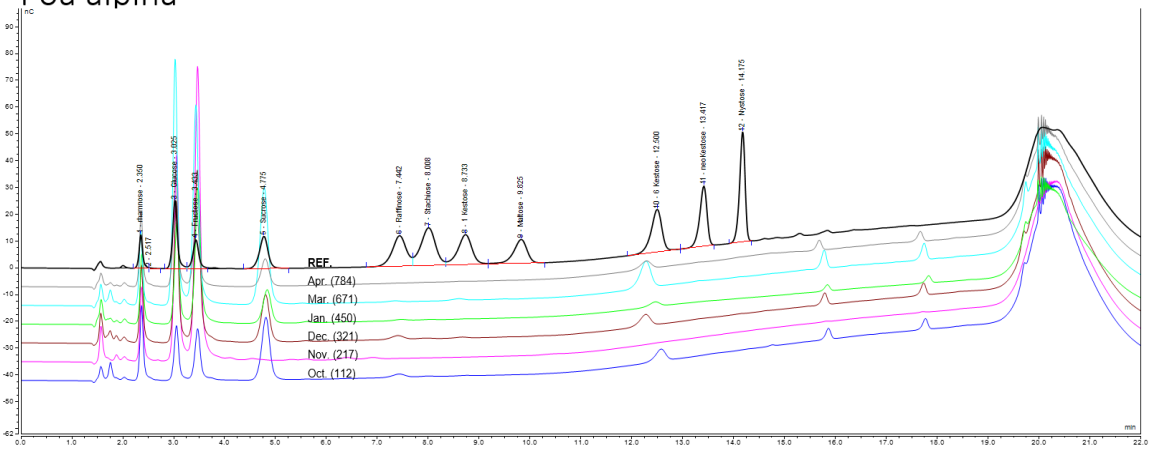

## Oloptum miliaceum



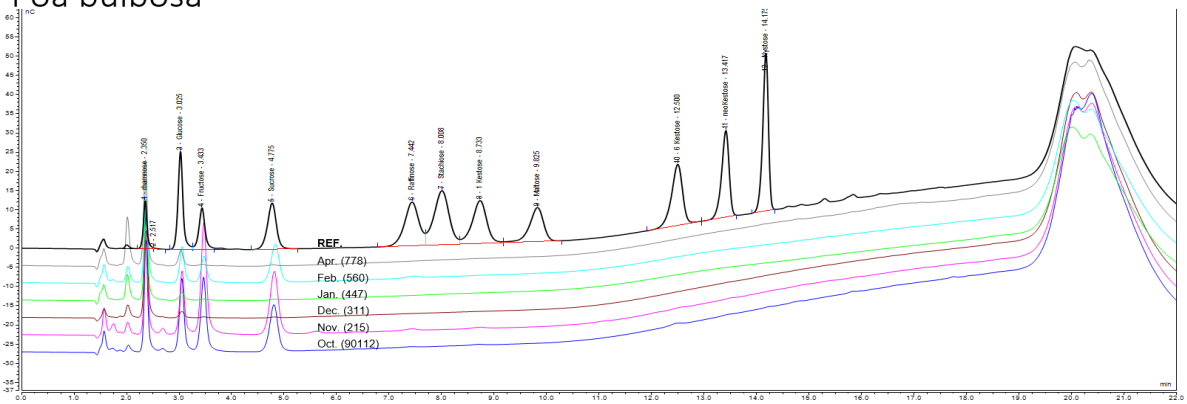

## Phleum pratense



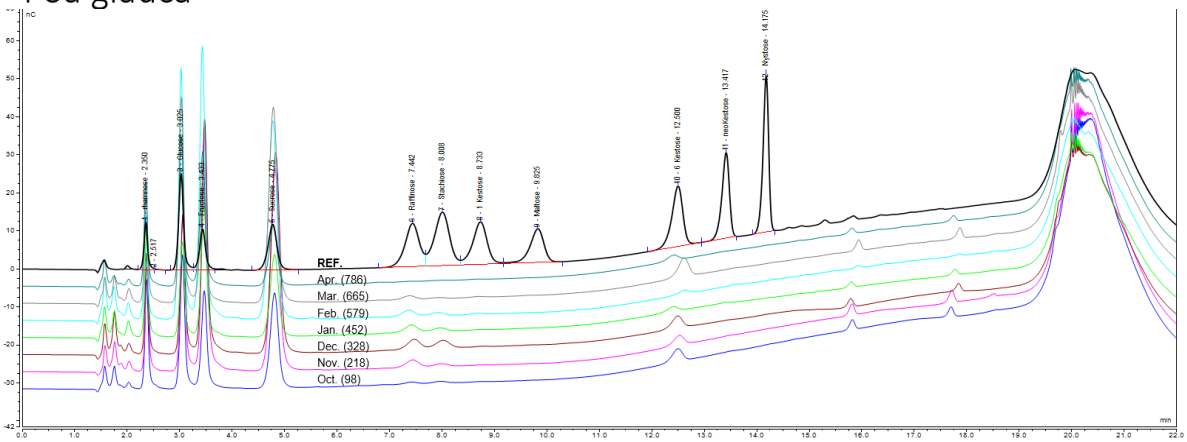

## Poa alpina



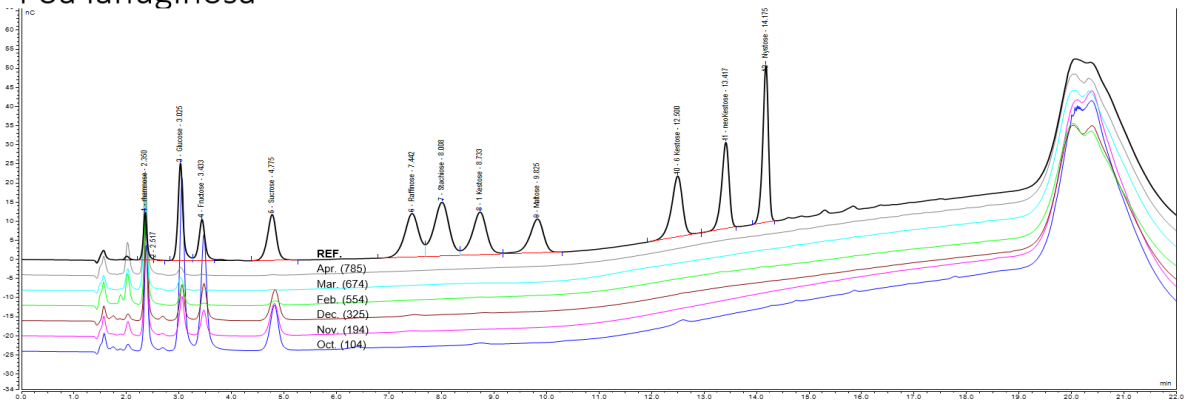
## Poa bulbosa



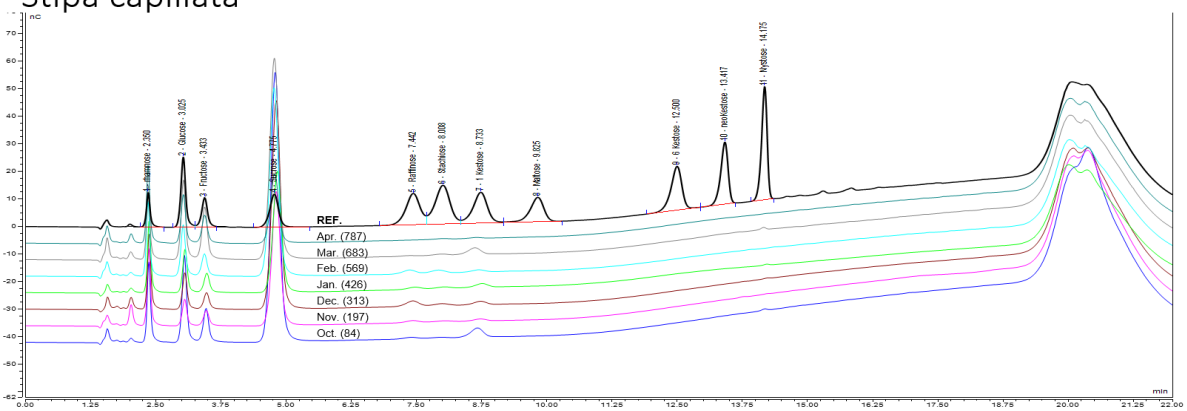

## Poa glauca



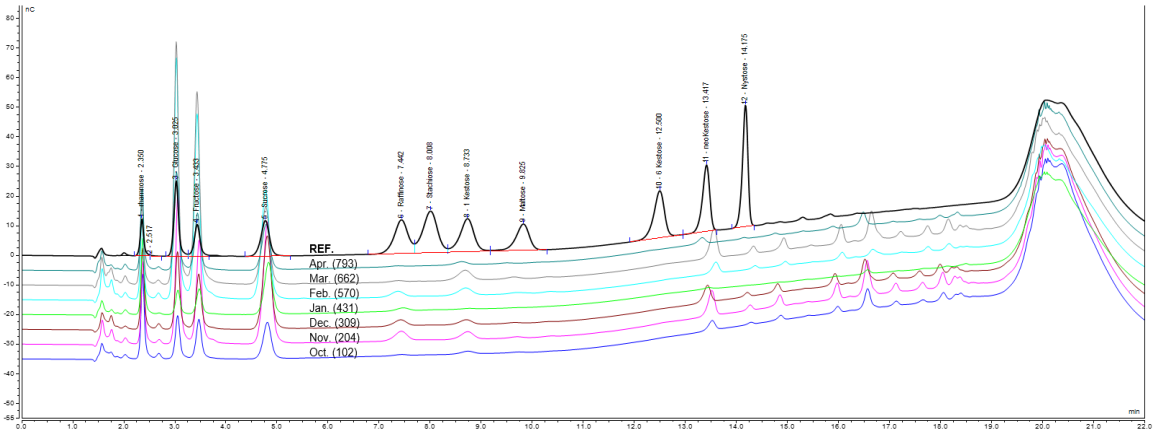

## Poa lanuginosa



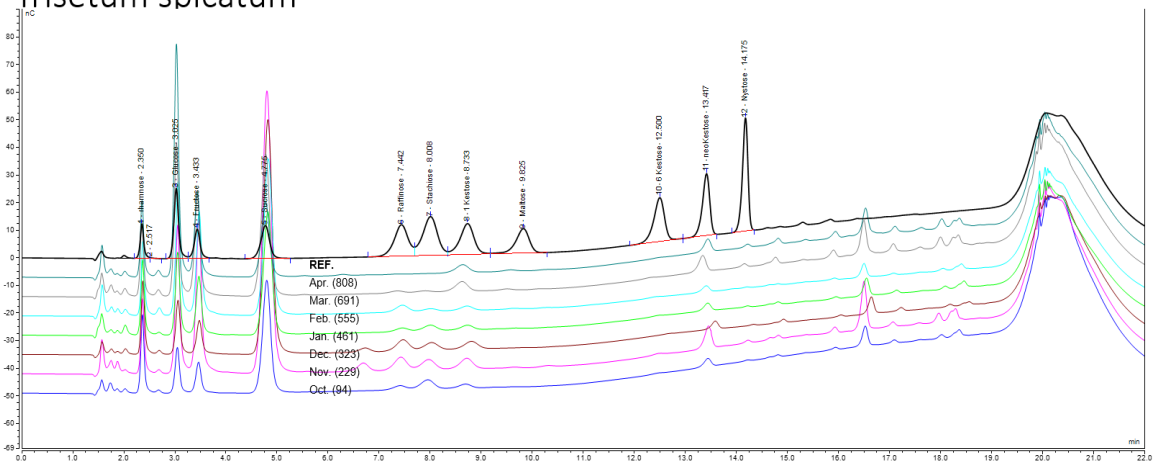

## Stipa capillata



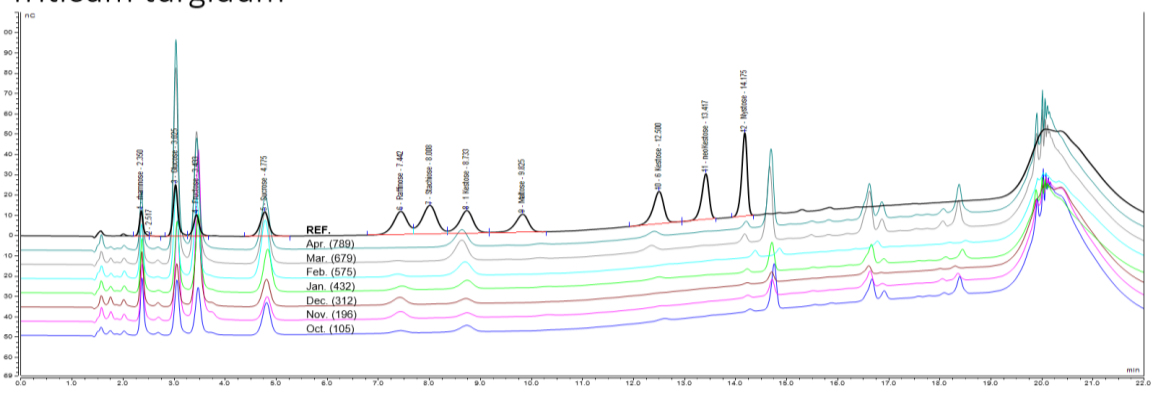
## Trisetum flavescens




## Trisetum spicatum




## Triticum turgidum

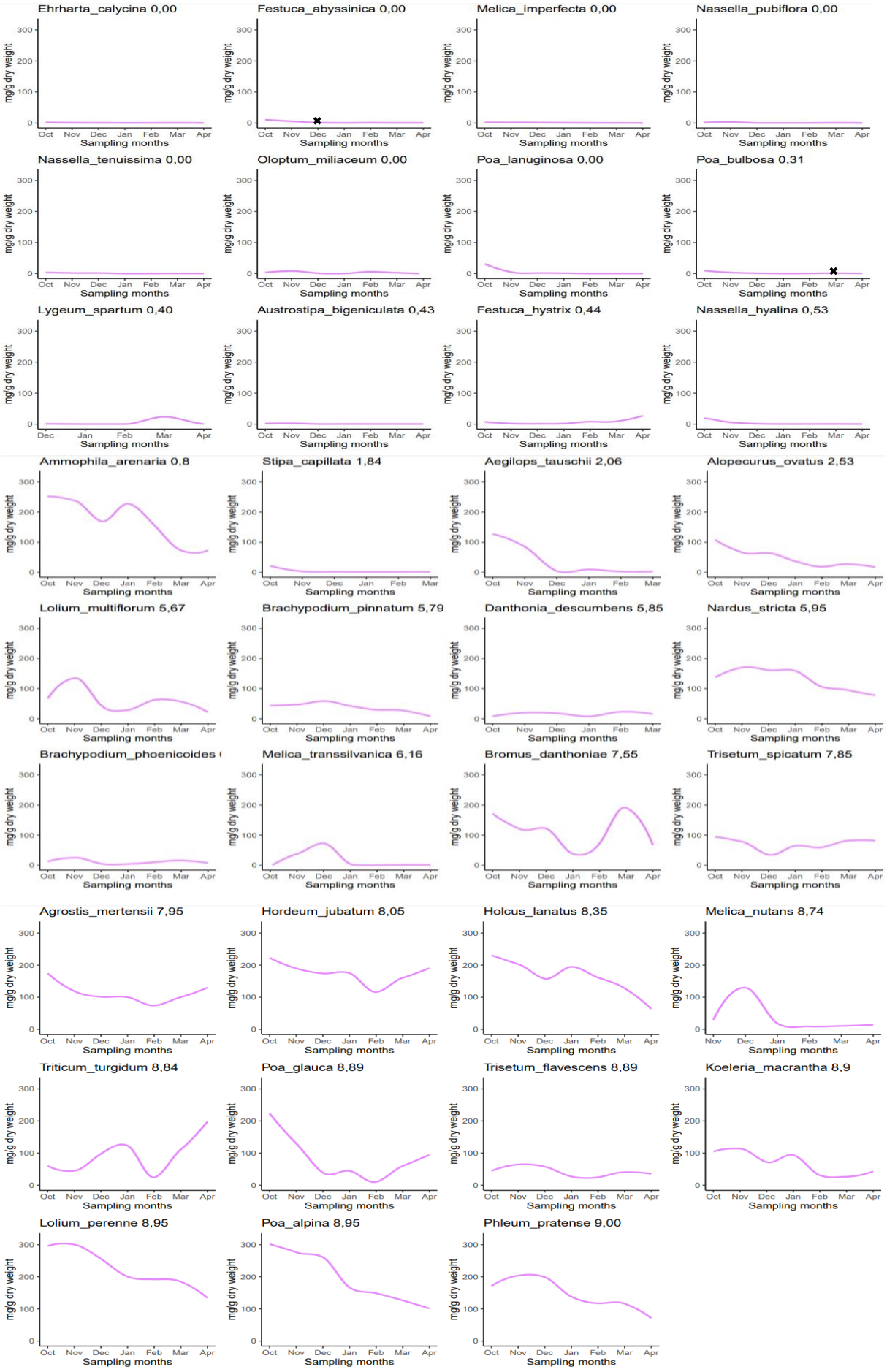


## 7.3 Table of values used for fructan classification.

| Species | AUCTOT-FRUCTAN | Raf | Sta | P RFO accum? | 1K | 6K | NK | NYS | TF accum? | Fructan type 1 | Fructan type 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aegilops tauschii* | 165,8243967 | 0,37 | 0,05 | NO | 0,38 | 0,12 | 0,03 | 0,08 | NO | Graminan? | No fructan |
| *Agrostis mertensii* | 645,5549164 | 1,98 | 1,32 | YES | 0,45 | 2,52 | 0,12 | 0,02 | YES | Levan | No fructan |
| *Alopecurus ovatus* | 261,5644905 | 2,35 | 3,10 | YES | 0,18 | 3,74 | 0,04 | 0,03 | YES | Levan | No fructan |
| *Ammophila arenaria* | 1040,779302 | 0,57 | 0,06 | NO | 11,44 | 2,07 | 0,18 | 1,34 | YES | Levan | Inulin |
| *Austrostipa bigeniculata* | 6,087026458 | 0,15 | 0,01 | NO | 0,10 | 0,00 | 0,01 | 0,01 | NO | No fructan | No fructan |
| *Brachypodium phoenicoides* | 73,91400332 | 4,41 | 0,15 | NO | 11,80 | 0,01 | 0,07 | 0,46 | PRI | 1-K | No fructan |
| *Brachypodium pinnatum* | 210,5468915 | 8,00 | 0,32 | NO | 29,26 | 0,00 | 0,15 | 1,77 | PRI | 1-K | No fructan |
| *Bromus danthoniae* | 540,7421942 | 1,08 | 0,39 | NO | 5,83 | 1,15 | 0,07 | 0,36 | YES | Levan | Graminan |
| *Danthonia decumbens* | 56,85266432 | 5,84 | 6,64 | YES | 0,01 | 0,00 | 0,03 | 0,01 | NO | No fructan | No fructan |
| *Ehrharta calycina* | 3,870427952 | 0,00 | 0,06 | NO | 0,02 | 0,00 | 0,00 | 0,01 | NO | No fructan | No fructan |
| *Festuca abyssinica* | 16,210895 | 0,04 | 0,02 | NO | 0,08 | 0,02 | 0,14 | 0,03 | NO | No fructan | No fructan |
| *Festuca hystrix* | 47,40933742 | 0,36 | 0,04 | NO | 0,57 | 0,06 | 0,54 | 0,13 | NO | Neoseries | Inulin |
| *Holcus lanatus* | 1013,294027 | 1,94 | 0,00 | NO | 50,57 | 0,26 | 0,16 | 23,50 | YES | Inulin | No fructan |
| *Hordeum jubatum* | 1040,660607 | 1,94 | 0,32 | NO | 8,70 | 0,48 | 0,05 | 1,84 | YES | Graminan | Levan |
| *Koeleria macrantha* | 436,5695399 | 1,40 | 0,18 | NO | 3,06 | 0,14 | 3,76 | 0,49 | YES | Neoseries | No fructan |
| *Lolium multiflorum* | 441,2380839 | 0,15 | 0,02 | NO | 2,71 | 0,04 | 3,89 | 0,79 | YES | Neoseries | Inulin |
| *Lolium perenne* | 1364,293608 | 0,30 | 0,03 | NO | 3,95 | 0,11 | 5,15 | 1,01 | YES | Neoseries | Inulin |
| *Lygeum spartum* | 24,13126478 | 0,75 | 0,03 | NO | 0,40 | 0,18 | 0,10 | 0,08 | NO | No fructan | No fructan |
| *Melica imperfecta* | 6,030194857 | 0,20 | 0,02 | NO | 0,06 | 0,04 | 0,03 | 0,02 | NO | No fructan | No fructan |
| *Melica nutans* | 175,3467817 | 8,19 | 0,04 | NO | 9,43 | 0,01 | 0,01 | 0,15 | PRI | 1-K | No fructan |
| *Melica transsilvanica* | 92,30015156 | 1,84 | 1,02 | YES | 0,15 | 0,02 | 0,04 | 0,01 | NO | No fructan | No fructan |
| *Nardus stricta* | 798,0627251 | 7,91 | 0,17 | NO | 10,74 | 0,05 | 17,46 | 4,88 | YES | Neoseries | Inulin |
| *Nassella hyalina* | 19,35975738 | 0,88 | 0,01 | NO | 0,17 | 0,01 | 0,08 | 0,02 | NO | No fructan | No fructan |
| *Nassella pubiflora* | 5,331654434 | 0,19 | 0,01 | NO | 0,04 | 0,00 | 0,04 | 0,01 | NO | No fructan | No fructan |
| *Nassella tenuissima* | 5,223540531 | 0,64 | 0,01 | NO | 0,10 | 0,01 | 0,08 | 0,02 | NO | No fructan | No fructan |
| *Oloptum miliaceum* | 20,88321181 | 0,34 | 0,01 | NO | 0,27 | 0,02 | 0,07 | 0,01 | NO | No fructan | No fructan |
| *Phleum pratense* | 862,2135442 | 1,37 | 0,39 | NO | 0,77 | 0,44 | 0,04 | 0,09 | YES | Levan | No fructan |
| *Poa alpina* | 1162,139382 | 0,51 | 0,10 | NO | 0,12 | 2,94 | 0,05 | 0,03 | YES | Levan | No fructan |
| *Poa bulbosa* | 11,06911396 | 0,17 | 0,01 | NO | 0,10 | 0,09 | 0,03 | 0,02 | NO | No fructan | No fructan |
| *Poa glauca* | 475,1038865 | 1,43 | 0,81 | NO | 0,14 | 2,03 | 0,07 | 0,02 | YES | Levan | No fructan |

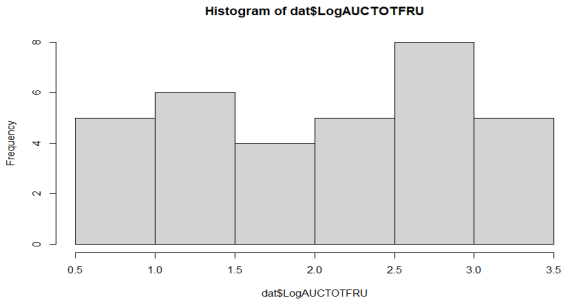
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Poa lanuginosa* | 20,67062288 | 0,08 | 0,01 | NO | 0,11 | 0,14 | 0,02 | 0,03 | NO | No fructan | No fructan |
| *Stipa capillata* | 16,85029748 | 0,85 | 0,61 | NO | 1,60 | 0,01 | 0,01 | 0,13 | PRI | 1-K | No fructan |
| *Trisetum flavescens* | 258,8956084 | 1,42 | 0,09 | NO | 2,04 | 0,05 | 2,86 | 0,45 | YES | Neoseries | No fructan |
| *Trisetum spicatum* | 428,1351174 | 2,30 | 2,20 | YES | 2,85 | 0,21 | 2,49 | 0,28 | YES | Neoseries | No fructan |
| *Triticum turgidum* | 543,8841466 | 1,85 | 0,05 | NO | 6,22 | 0,92 | 0,08 | 0,98 | YES | Graminan | Levan |

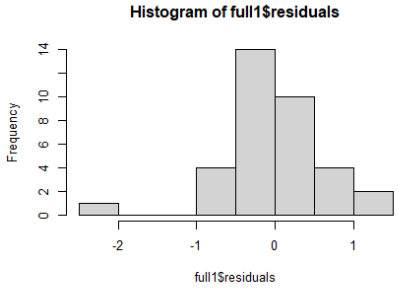
## 7.4 Graphs of the change in total fructan content over the sampling months

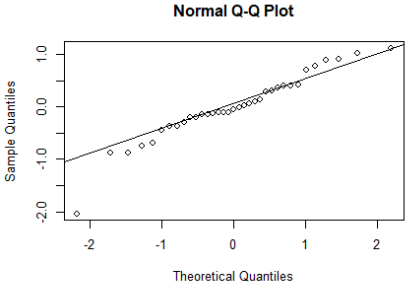


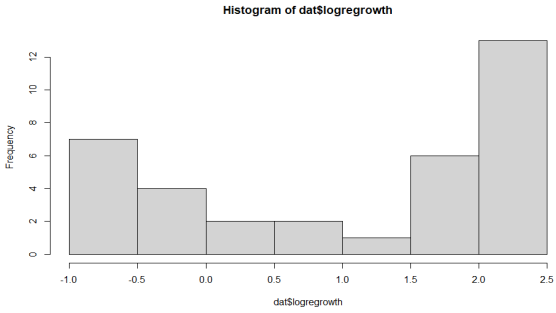
## 7.5 Diagnostics plot for PGLS

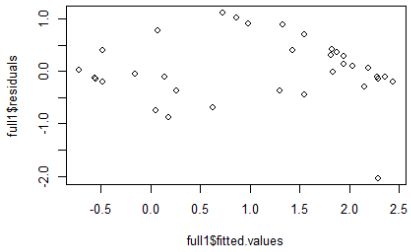
Regrowth vs fructan



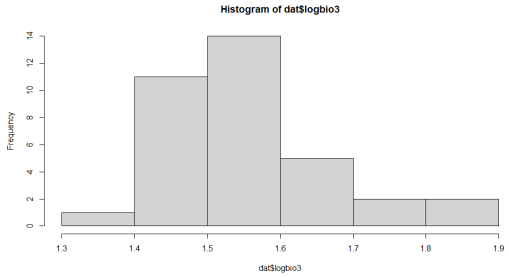



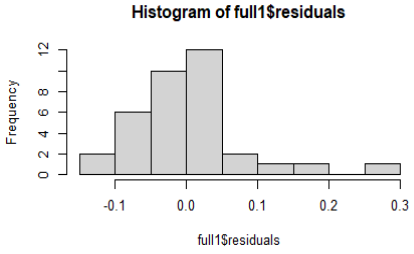



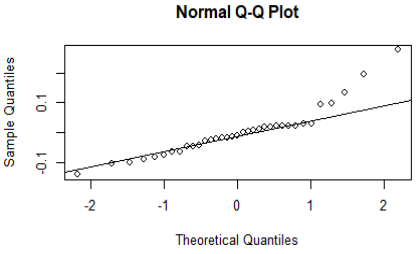



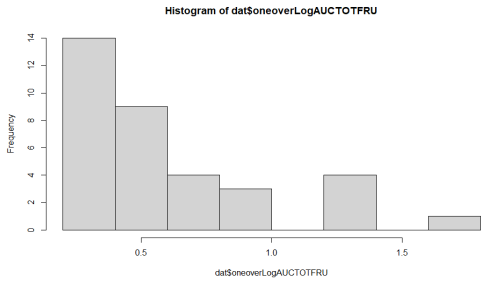



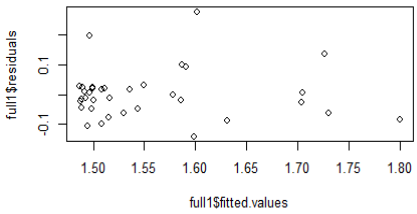

Fructan vs Isothermality



## 7.6 Correcting for Fru with Raf and Sta origin in the acid hydrolysis analysis.

The total fructan content in mg/g DW was calculated as:

$$x\frac{mg}{g\ DW}\ Glu\ PH + x\frac{mg}{g\ DW}\ Fru\ PH - (x\frac{mg}{g\ DW}\ Glu\ BH + x\frac{mg}{gDW}\ Fru\ BH + x\frac{mg}{gDW}\ Suc\ BH)$$

Using the µmol/g DW of Raf and Sta from the sugar analysis:

$$\frac{x\frac{\mu mol}{g\ DW} * 180{,}16\frac{g}{mol}}{1000} = x\frac{mg}{g\ DW}\ fructose\ from\ RFO\ origin$$

Thus,

$$Total\ fructan = x\frac{mg}{g\ DW}Glu\ PH + (x\frac{mg}{g\ DW}\ Fru\ PH - x\frac{mg}{g\ DW}\ fructose\ from\ RFO\ origin) - (x\frac{mg}{g\ DW}\ Glu\ BH + x\frac{mg}{gDW}\ Fru\ BH + x\frac{mg}{gDW}\ Suc\ BH)$$

The average DP for fructans was calculates as:

$$\frac{x\ \mu mol\ Fru\ PH}{x\ \mu mol\ Glc\ PH} + 1$$

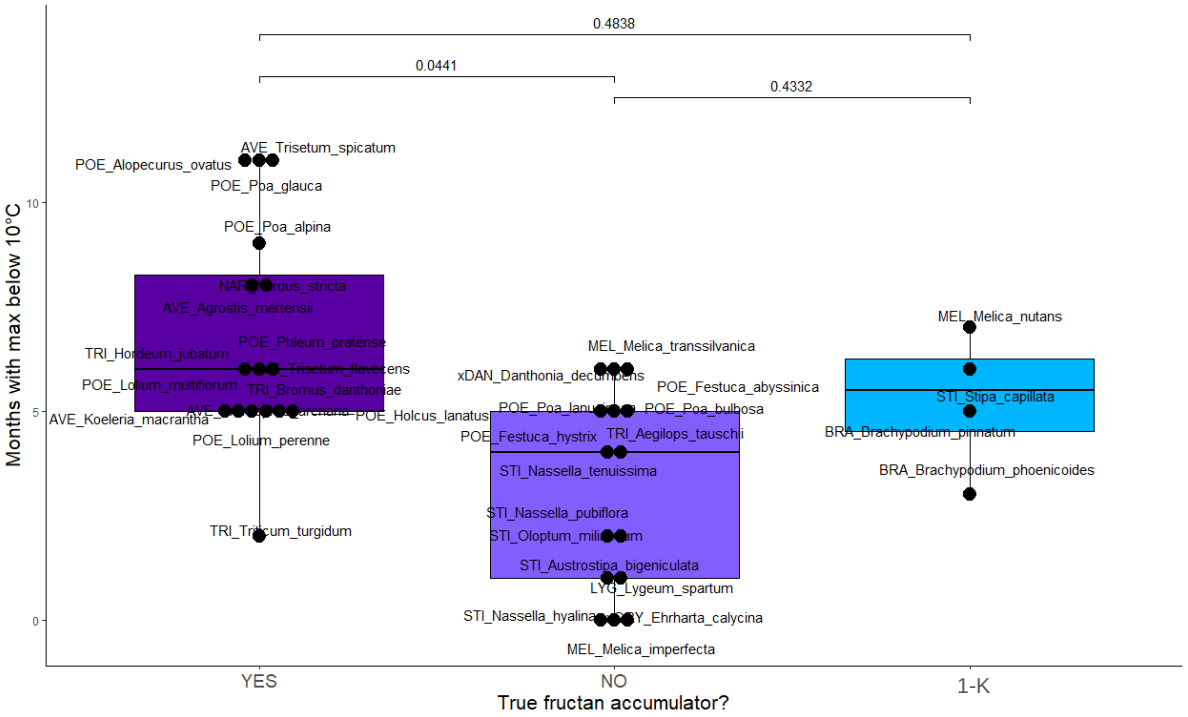
Using the µmol/g DW of Raf and Sta from the sugar analysis:

$$x\frac{\mu mol}{g\ DW} * x\ g\ DW = x\ \mu mol\ fructose\ from\ RFO\ origin$$

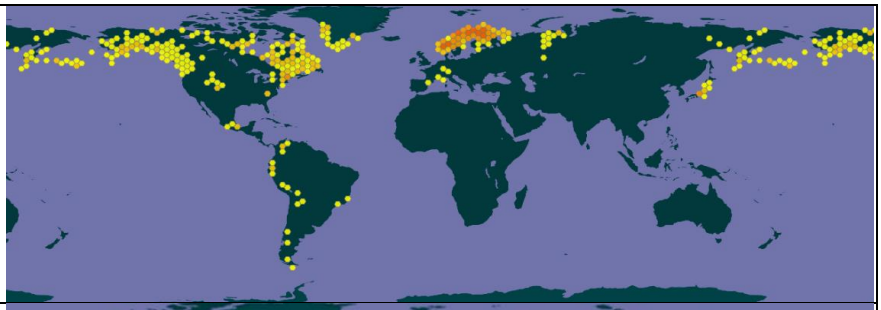
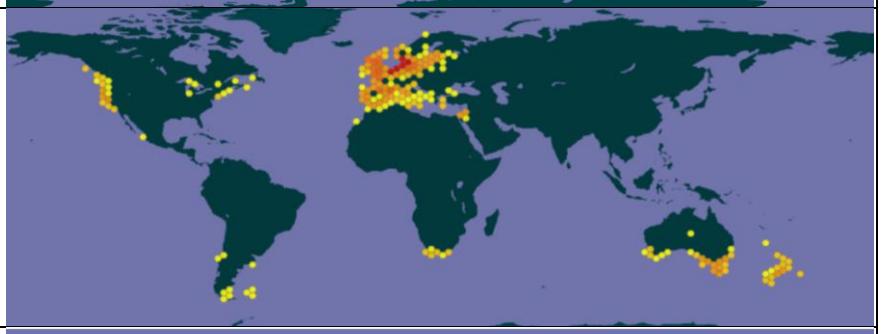
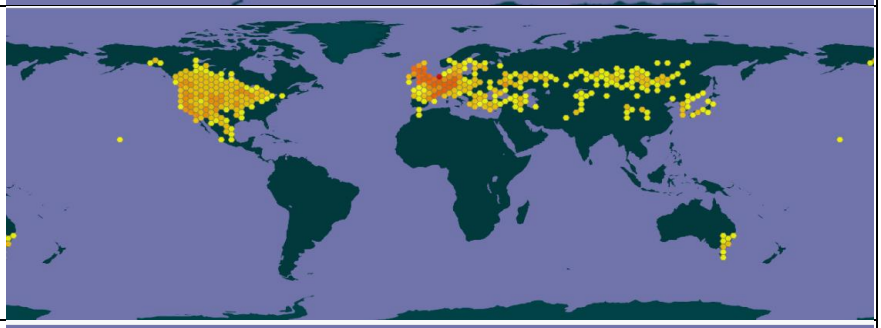
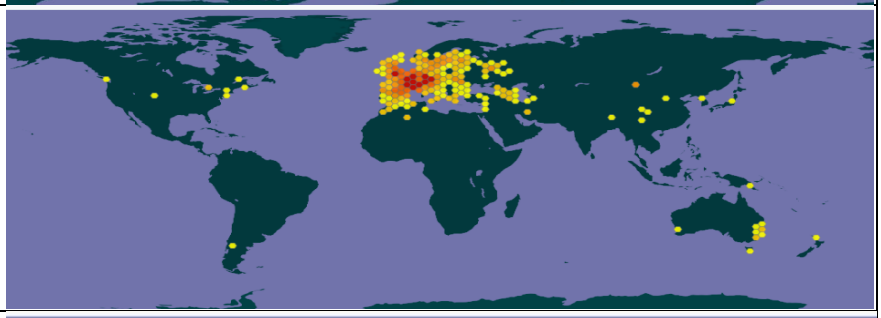
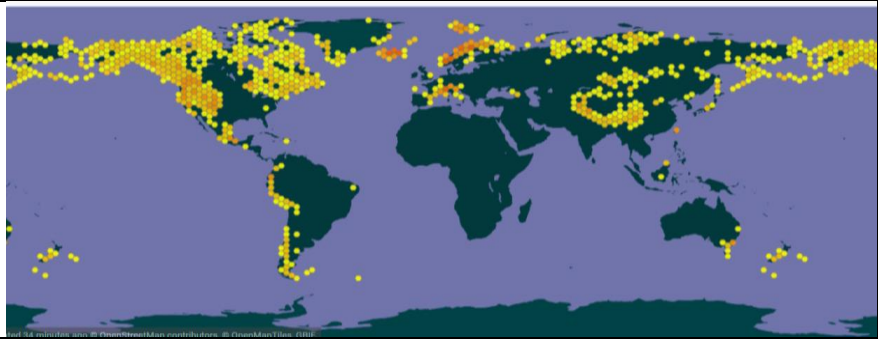
Thus,

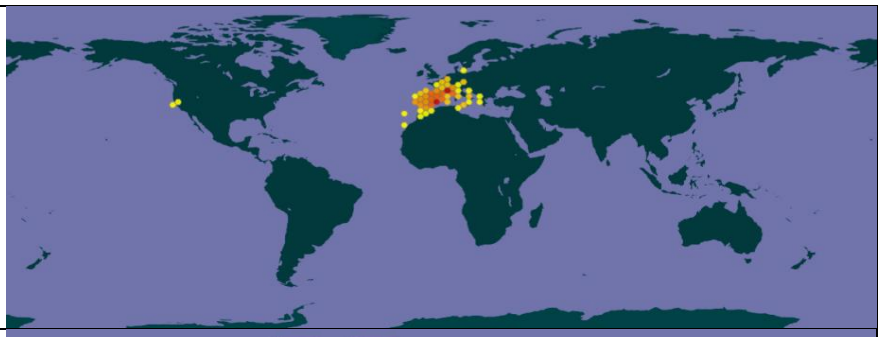
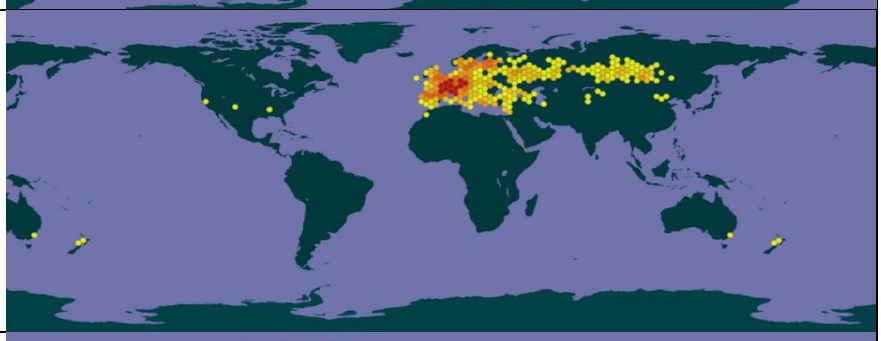
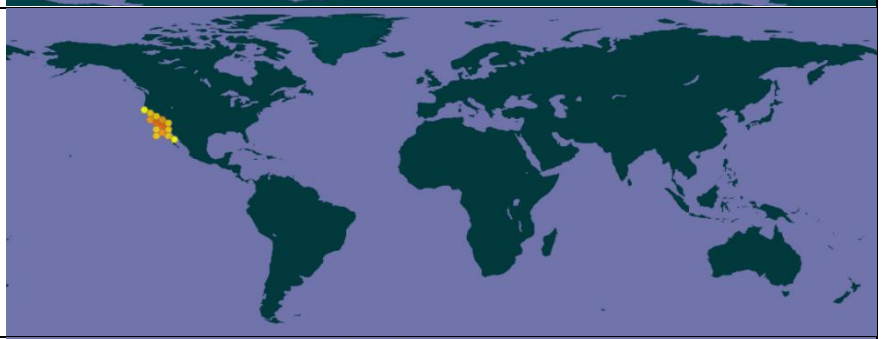
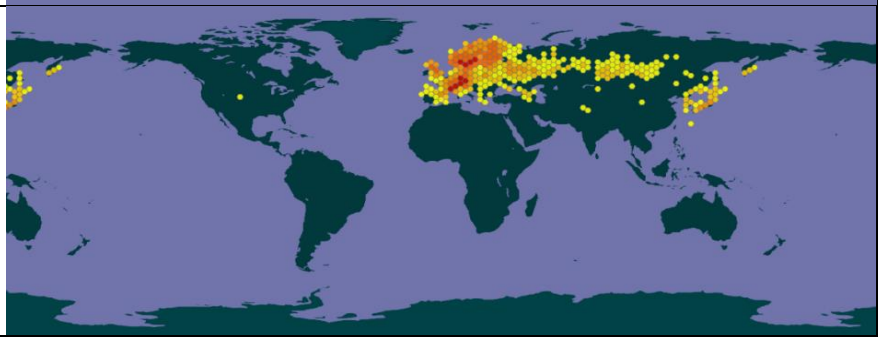
$$AVGDP\ Fructan = \frac{(x\ \mu mol\ Fru\ PH - x\ \mu mol\ fructose\ from\ RFO\ origin)}{x\ \mu mol\ Glc\ PH} + 1$$

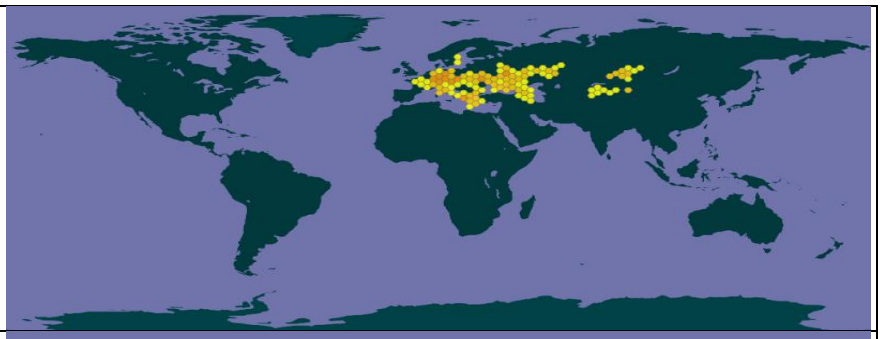
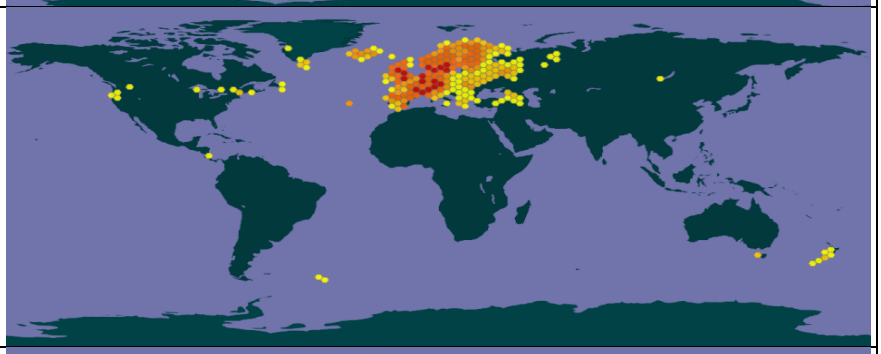
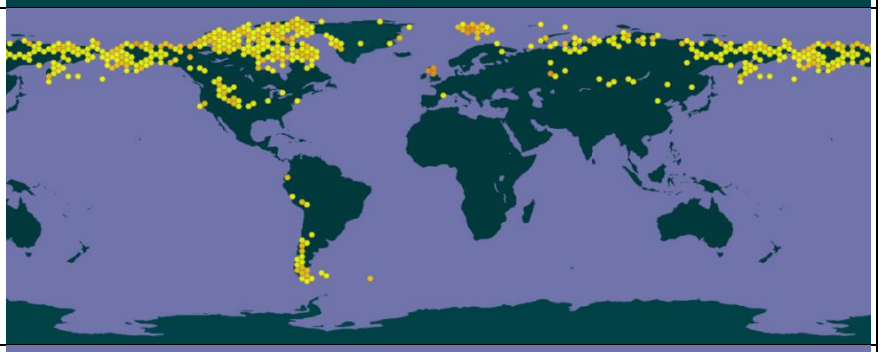
## 7.7 pAnova plot with species lables

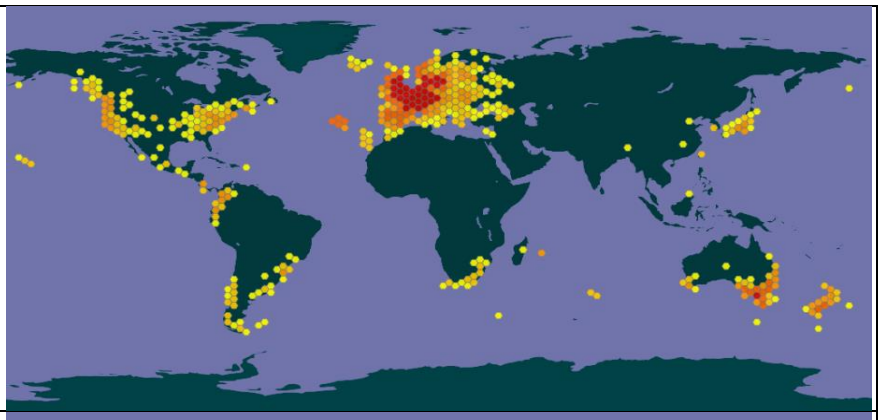
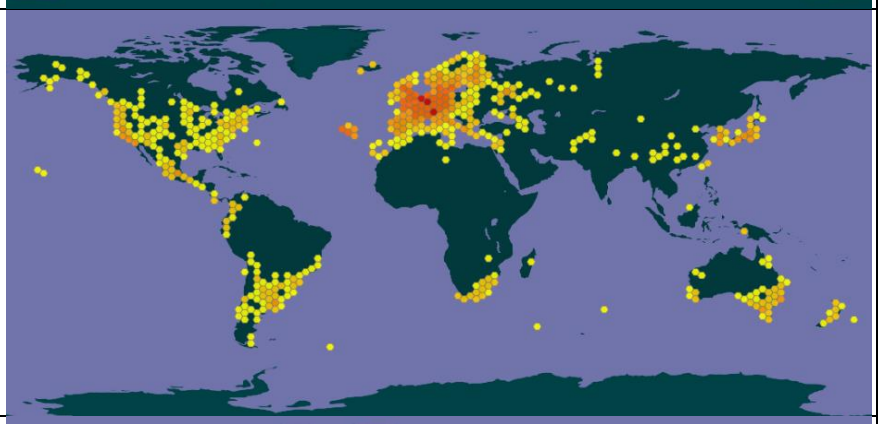
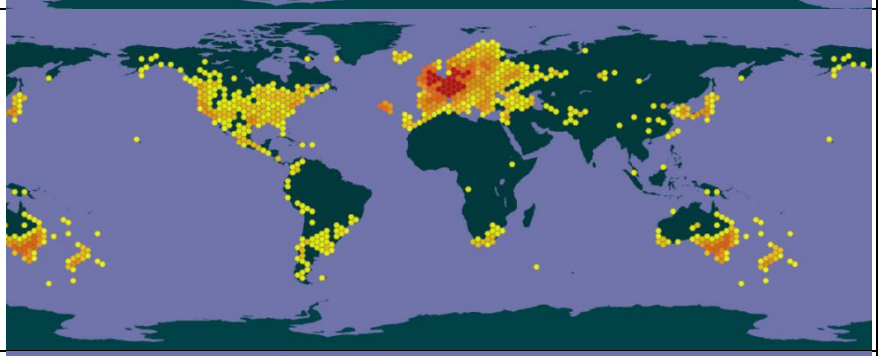
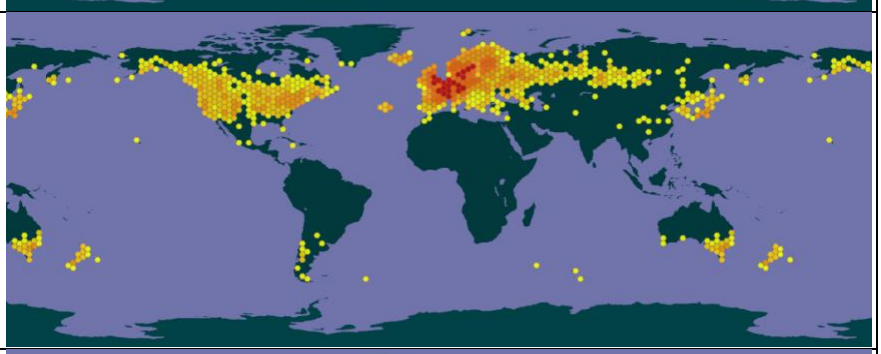
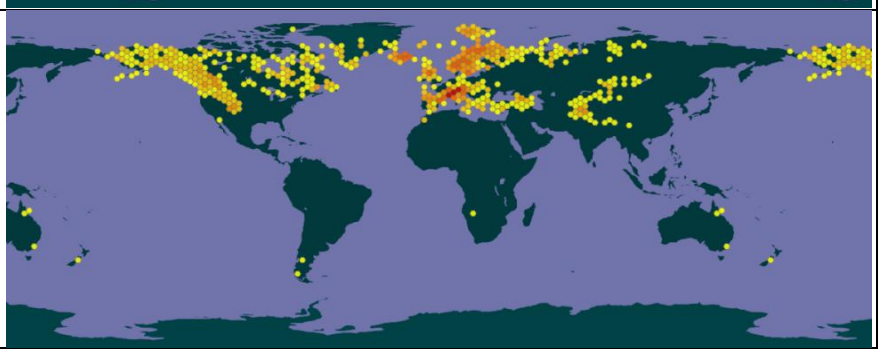


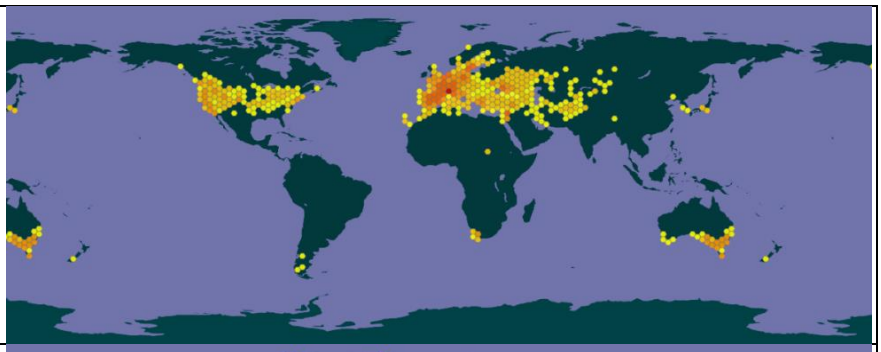
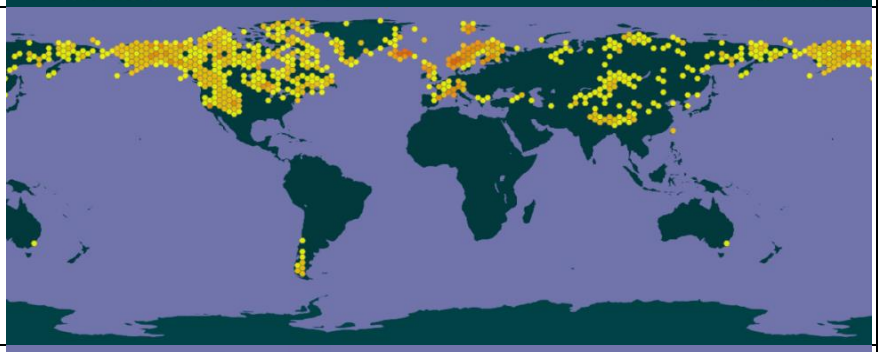
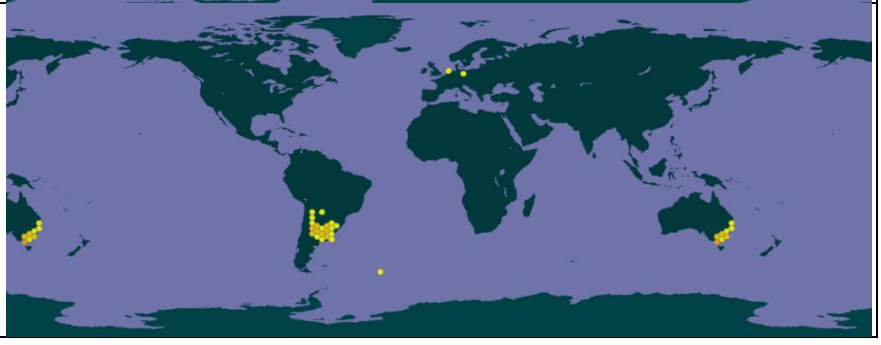
## 7.8 Distribution pattern of each species (from gbif)

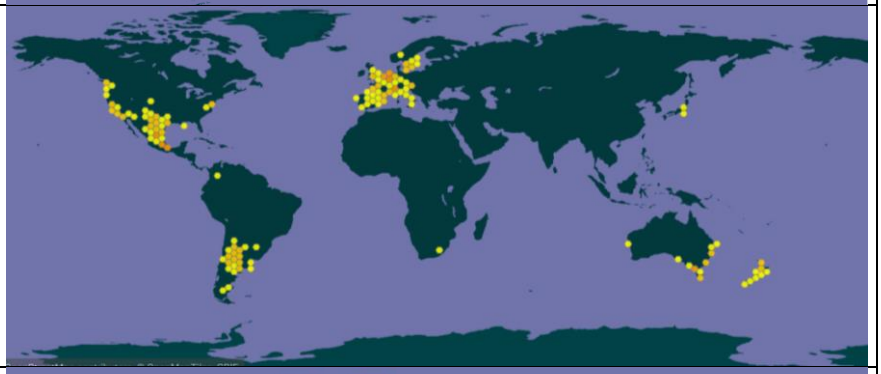
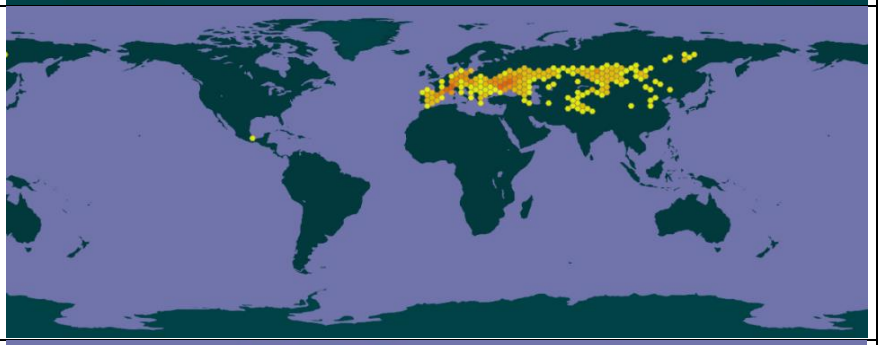
| | |
|---|---|
| AVE_Agrostis_mertensii |  |
| AVE_Ammophila_arenaria |  |
| AVE_Koeleria_macrantha |  |
| AVE_Trisetum_flavecens |  |
| AVE_Trisetum_spicatum |  |

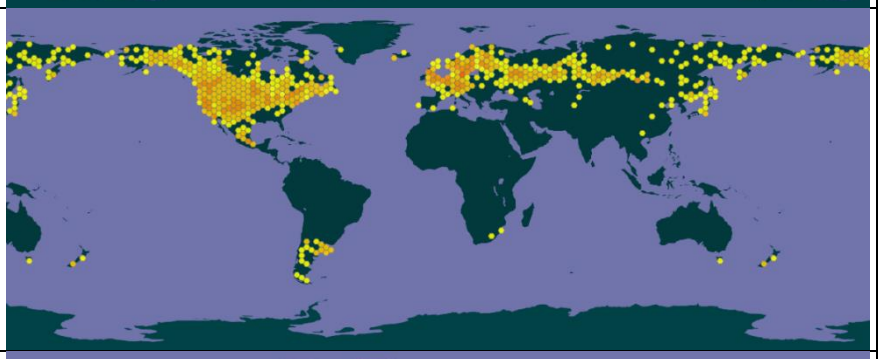
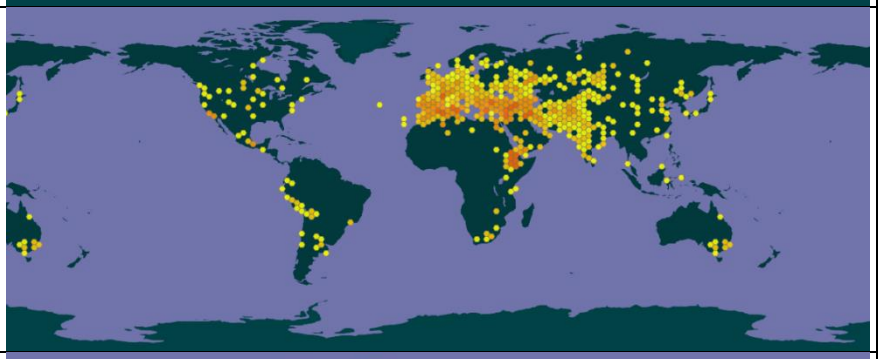
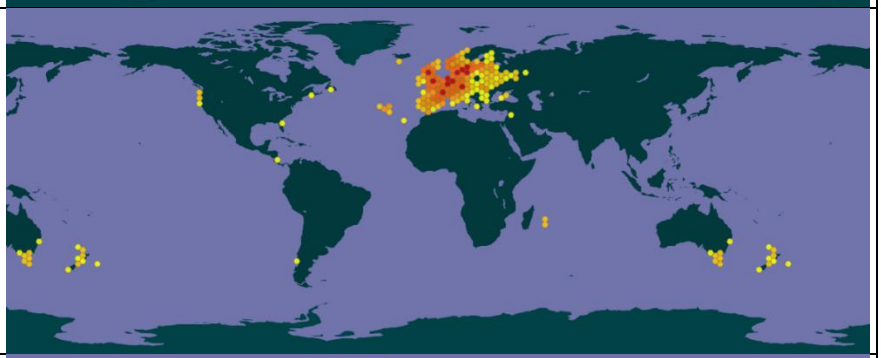
| | |
|---|---|
| BRA_Brachypodium_phoenicoides |  |
| BRA_Brachypodium_pinnatum |  |
| LYG_Lygeum_spartum |  |
| MEL_Melica_imperfecta |  |
| MEL_Melica_nutans |  |

| | |
|---|---|
| MEL_Melica_transsilvanica |  |
| NAR_Nardus_stricta |  |
| POE_Alopecurus_ovatus |  |
| POE_Festuca_abyssinica |  |
| POE_Festuca_hystrix |  |

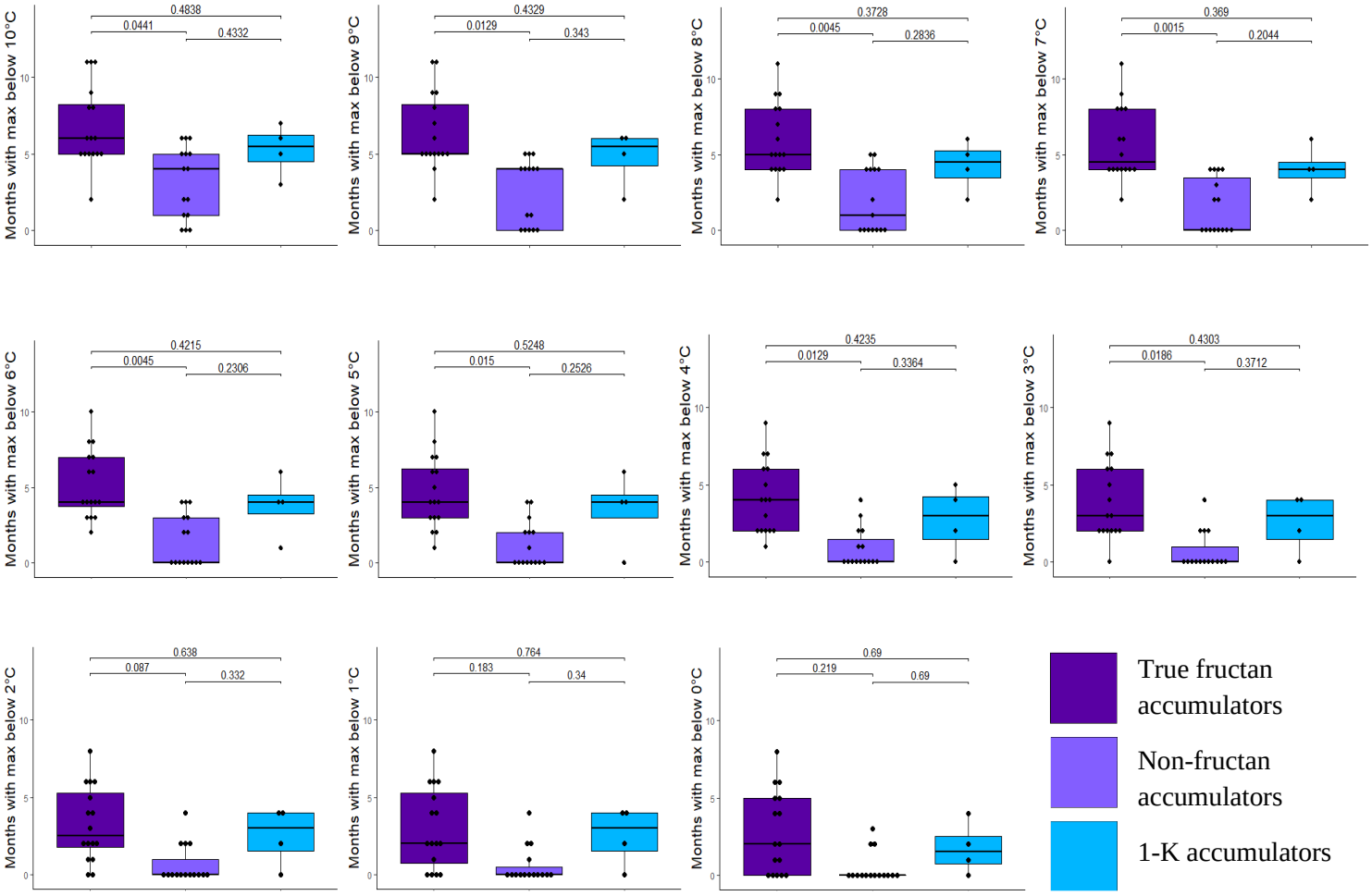
| | |
|---|---|
| POE_Holcus_lanatus |  |
| POE_Lolium_multiflorum |  |
| POE_Lolium_perenne |  |
| POE_Phleum_pratense |  |
| POE_Poa_alpina |  |

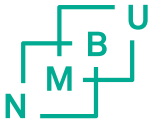
| | |
|---|---|
| POE_Poa_bulbosa |  |
| POE_Poa_glauca |  |
| POE_Poa_lanuginosa |  |
| STI_Austrostipa_bigeniculata |  |
| STI_Nassella_hyalina |  |

| | |
|---|---|
| STI_Nassella_pubiflora |  |
| STI_Nassella_tenuissima |  |
| STI_Oloptum_miliaceum |  |
| STI_Stipa_capillata |  |
| TRI_Aegilops_tauschii |  |

| | |
|---|---|
| TRI_Bromus_danthoniae |  |
| TRI_Hordeum_jubatum |  |
| TRI_Triticum_turgidum |  |
| xDAN_Danthonia_decumbens |  |
| xORY_Ehrharta_calycina |  |

## 7.9 pANOVA results for $T_{max}$ 10-0°C



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