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Optimization of Greenhouse Waterlogging Tolerance Screening and Low-Cost Seminal Roots Phenotyping Methods for Spring Wheat

Anjali Khadka
Msc in Plant Sciences – Plant Production Systems

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Abstract

Climate change projections predict that precipitation in Norway is likely to increase, and flooding and waterlogging scenarios will likely be more frequent in the future. Wheat is sensitive to waterlogging conditions, and there can be substantial yield loss due to waterlogging stress in wheat. However, there is a genetic variation in waterlogging tolerance in wheat. Screening for waterlogging tolerance in wheat has been done for many years, but the screening methodology varies with climate, soil, crop stage, and waterlogging event itself. Field screening for waterlogging tolerance in wheat is labor-intensive, time-consuming, and high cost.

Here, we developed and improved a methodology to screen waterlogging tolerance in wheat in the greenhouse, which can simulate field water logging conditions. Our greenhouse waterlogging methodology using starch (0.1% m/v) is promising and creates a highly reduced environment (below -500 mV) within four days of waterlogging. Using chlorosis as a trait for evaluating waterlogging tolerance, Best Linear Unbiased Predictors (BLUPs) of twenty spring wheat genotypes tested with this methodology showed a correlation of (R=0.44) with previously obtained field data for the same trait. The developed greenhouse waterlogging method using starch (0.1 % m/v) is cost-effective, time-efficient, and labor efficient compared to field screenings. Utilizing this method, screening for waterlogging tolerance in wheat can be done within one month of waterlogging. Chlorosis percentage and recovery scale are two phenotypic traits used for screening wheat genotypes in this method. This method is promising for efficiently screening diverse wheat populations for waterlogging tolerance within greenhouse settings, with potential application in other crops.

Notably, twenty spring wheat genotypes used to optimize this methodology were also collections of genotypes with contrasting haplotypes on *QTL6A.2* for chlorosis. These haplotypes have significant differences ($P < 0.05$) for chlorosis on field waterlogging and under this greenhouse waterlogging methodology. Follow-up experiments using this methodology would be recommended in further studies on validating this QTL.

Additionally, we established a cost-effective root phenotyping methodology using seed germination pouches with germination paper (dark blue grade 194) for phenotyping seminal root angle of wheat genotypes. Utilizing this method, contrasting haplotypes on *QTL6A.2* for chlorosis were tested for seminal root angle, and these haplotypes were found to have significant differences in seminal root angle. This result needs verification through further experiments. Identification of candidate genes on this locus would be recommended to understand the role of seminal root angle on waterlogging tolerance.

This work establishes a greenhouse-based waterlogging screening method as alternative or supplement to field screening. It shows promise for large-scale screening and QTL identification/validation for waterlogging tolerance of wheat population. Additionally, seminal root phenotyping method developed for assessing traits like seminal root angle, has potential applications in waterlogging tolerance screening as seminal root angle is proxy trait.

Keywords: waterlogging, tolerance, chlorosis, haplotypes, greenhouse, waterlogging, root phenotyping, seminal roots.

Abbreviations

BLUPs: Best Linear Unbiased Predictors

LOES: Low Oxygen Escape Syndrome

ABA: Abscisic acid

ET: Ethylene

DAW: Days after Waterlogging.

DASW: Days after Starch Waterlogging

GLO-Roots: Growth and Luminescence Observatory for Roots

RSA: Root System Architecture

CWD: Consecutive Wet Days

BP: Before Present

IPCC: Intergovernmental Panel on Climate Change

FAO: Food and Agriculture Organization

GWAS: Genome-Wide Association Studies

G*E: Genotype*Environment

QTL: Quantitative Trait Locus

CIMMYT: International Maize and Wheat Improvement Center

1 General Introduction

1.1 History of Wheat

Wheat is an important cereal crop in ancient agriculture (Zohary & Hopf, 2000) and is a pioneer cultivated crop, followed by rice and maize (Peng et al., 2011). Domestication of wheat as a cultivated crop happened about 12,000 years ago, as a significant part of the `Neolithic Revolution´ which shifted the human nomadic lifestyle of hunting and gathering to settled agriculture (Shewry, 2009). It is believed that diploid einkorn wheat *T. monococcum* L. (genome $A^m A^m$) is the oldest cultivated wheat crop domesticated from its wild progenitor *T. boeoticum* L. (genome A^bA^b) in the Fertile Crescent, Karacadag mountain range in southeastern Turkey (Heun et al., 1997).

It is believed that around 300,000-500,000 years before present (B.P.), wild einkorn wheat *T. urartu*, which is considered A genome progenitor of modern world durum and common wheat, must have hybridized with an unknown but close relative of goat grass *Aegilops speltoides* Taush. (genome SS). This unknown species is thought to be a B genome progenitor, which on hybridization with *T. urartu*, produces allotetraploid wild emmer wheat *T. dicoccoides* L., (genome A^uA^uBB) (Dvorak & Akhunov, 2005; Huang et al., 2002). Subconscious and gradual selection of wild emmer wheat by people converted it into cultivated emmer wheat *T. dicoccum* L. (2n = 4x = 28, genome A^uA^uBB). When cultivation of emmer extended from Fertile Crescent towards the natural habitat of wild goat grass *Ae. tauschii* Coss. (2n = 2x = 14, genome DD), probably near the southern and western side of the Caspian Sea as reported by Nesbitt and Samuel (1996) then there was the formation of allohexaploid *T. aestivum* L. sp. *spelta*., commonly known as early spelt (Kihara, 1944). The cultivated tetraploid emmer and allohexaploid spelt wheat were hulled (Rahman et al., 2020). Today's world durum wheat *Triticum turgidum* L. ssp. *durum* is a consequence of the domestication of wild emmer as domesticated emmer and then the continued evolution of this domesticated emmer as durum wheat landraces and through breeding approaches to form modern durum wheat cultivars from durum wheat landraces (Maccaferri et al., 2019). Studies also suggested that free-threshing today´s world common wheat *Triticum aestivum* L. (A^uA^uBBDD; 2n = 6x = 42) results from a natural mutation in hulled cultivated spelt wheat (McFadden, 1944; McFadden & Sears, 1946). However, its formation has contrasting thoughts as later studies suggested that cultivated spelt wheat is not an ancestral form of free-threshing common wheat (Dvorak et al., 2006). There is

still a puzzle about the formation of free-threshing hexaploid common wheat. Some studies postulated that the AABB sub-genome of hexaploid wheat was formed from free-threshing tetraploid wheat species. If this postulation holds, then *T. turgidum* L. spp. *dicoccoum* with hulled grains will not be its AABB genome progenitor. As domesticated free threshing was formed around 8500-9000 years ago, a possible AABB genome progenitor would be T. turgidum L. ssp. *parvicoccum*, but this species was completely extinct during the Roman period due to the popularity of T. turgidum L. spp. *durum.* The strong evidence of this free-threshing domesticated tetraploid that hybridizes with *Ae. tauschii* to form free-threshing hexaploid wheat will therefore remain uncertain. Also, no evidence exists of any hulled spelt-like types of hexaploid wheat formed due to hybridization between *T. turgidum* L. spp. *dicoccum* and Ae. tauschii. One possible condition is the simultaneous formation of free-threshing mutants along with hulled spelt-like hexaploid wheat, and free-threshing was preferred and selected. This free-threshing hexaploid wheat must have first settled in central Anatolia and then spread to Europe rapidly (Bogaard, 2016). The free-threshing trait is due to the dominant Q locus on the chromosome. Studies have already found that Q-phenotype will not be expressed as the result of suppression of the *Tg* gene found on chromosome 2D of Ae. tauschi (Kerber & Rowland, 1974). Understanding the elusive *Tg* homoeoalleles will be groundbreaking to elucidate the actual pathway of free-threshing hexaploid wheat in the future (Levy & Feldman, 2022).

Domestication led to the domestication syndrome, which involves the selection of genetic traits that made domesticated plants distinct from their wild progenitors. Two of the most important traits were non-shattering traits formed due to mutation at the Br. (brittle rachis) gene (Nalam et al., 2006) and the evolution of hulled grains into free-threshing naked grains through a dominant mutant at the Q locus, modifying the effect of the recessive *Tg* (tenacious glume) locus (Dubcovsky & Dvorak, 2007; Jantasuriyarat et al., 2004). Wheat has gone through two successful rounds of allo-polyploidization that first led to the formation of tetraploid wild emmer wheat and then the second led to the formation of allohexaploid wheat of today´s world. However, the exact formation of free-threshing hexaploid wheat remains a puzzle, and additional research is necessary to unravel its complex genetic history. Despite advancements in molecular understanding and the sequencing of the 16,000 Mbp wheat genome already been done, the precise picture of its progenitors remains a mystery to be solved (Levy & Feldman, 2022).

There are a few things that make wheat a major global crop of today. Thanks to its immense capacity to sustain many mutations (Akhunov et al., 2007) and rapid evolution to allopolyploidy (Bonjean & Angus, 2001). Wheat has a fascinating history that involves the spread of domesticated tetraploid wheat from its center of origin (Fertile Crescent) to Mediterranean climates, adapting to mild winters and warm rainless summers (Levy & Feldman, 2022). Similarly, the addition of the D genome through *Ae. tauschii*, an origin from central Asia, that not only brought in numerous resistant genes (Appels & Lagudah, 1990) and desirable yield qualities (Delorean et al., 2021) but also broadened the adaptation capacity of wheat to thrive through climates across the continental plateaus of Asia and also colder temperate regions in eastern, central, and northern Europe (Levy & Feldman, 2022). Consequently, wheat has been cultivated in diverse locations, spanning as far north as 67° N in Norway, Finland, and Russia and as far south as 45° S in Argentina (Bonjean & Angus, 2001; Zohary et al., 2012).

1.2 Wheat production, world population, and climate scenario

Wheat is an important cereal crop worldwide (Atkinson et al., 2015) as it accounts for 19% of daily calories and 21% of the protein intake of the global population (Tadesse et al., 2019). It is a vital source of minerals (P, K, Mg, Ca), dietary fiber, and bioactive substances (Shewry & Hey, 2015). Moreover, it is also a staple food crop for approximately 30% of the world's population (Liu et al., 2017). It, therefore, has a significant role in ensuring global food security (Ober et al., 2021). Global wheat production area has reached approx. 219.0 million hectares with a total annual production of approx. 770 million tons. China and India were the two largest wheat-producing countries, with annual production recorded 134.3 million tons and 107.6 million tons, respectively, in 2021 (FAO, 2021). Wheat production includes cultivated tetraploid, durum wheat, and hexaploid, common wheat, which cover 5% and 95% of global wheat production, respectively (de Sousa et al., 2021). Studies have reported that annual genetic gains in wheat yield have reached approx. 1% (Cobb et al., 2019; Tadesse et al., 2019). The human population is expected to increase by 25% and reach 10 billion over the next 30 years (Hickey et al., 2019). Also, projections indicate that demand for annual wheat production is expected to reach 1 billion tons by 2050 (Tadesse et al., 2019). It is evident that demand for wheat has risen every year in different regions of the world as a result of urbanization and dietary preferences for wheat-based foods, especially in regions like Africa (5.8%), Asia

(5.6%), and Australia (2.2%) (Shiferaw et al., 2013). To meet the projected demand for wheat, it is expected that the annual genetic gain of wheat yield must be increased to 1.7%. However, efforts to increase the speed of genetic gain to meet the projected demand for wheat are impeded by the impacts of global warming and climate change to a larger extent (Tadesse et al., 2019).

Changes in the frequency, intensity, and duration of floods, drought, and high-temperature events have already been evident in many parts of the world (Field et al., 2012; WMO, 2014). Reports further projected that these conditions will be common in the future. Such weather events in crop production areas reduce crop production and make the crop production system highly unstable. Climatic variability associated with climate change has a significant role in the interannual instability of crop production both at local and global levels (Lesk et al., 2016). This can be threatening in terms of local to global level food security (Tigchelaar et al., 2018). One intriguing fact is that extreme weather events of the exact nature do not co-occur in all regions of the world, and their impact may vary significantly. One of the best examples is the year 2018 A.D., when northern, central, and western Europe faced simultaneous extreme temperatures and dry conditions; low rainfall conditions from March to August represent months of a critical crop-growing period in these areas. This condition led to the highest yield reduction of the decade in various crop species, including wheat. Higher rainfall was experienced in southern parts of Europe, and higher than usual crop yield was recorded because of suitable spring conditions. Because of this Europe's overall crop production failure was fortunately compensated and saved Europe's disastrous crop failure in that year to a larger extent (Beillouin et al., 2020). Such unprecedented events, however, can be too frequent in central and northern Europe by 2050, projected by future climate projections (Barriopedro et al., 2011; Spinoni et al., 2018). Recent press release reports of the IPCC also have highlighted that there will be more than a 1.5 °C increase in global temperature in the next 20 years. It also mentioned water cycle will be changed, with intense rainfall along with flooding and extreme drought conditions in various regions of the world will be frequent. Increased precipitations are expected in higher latitudes. This increase in temperature is contributing factor to the change in the pattern of precipitation. A report by Trenberth et al. (2003), have mentioned that an increase in atmospheric temperature enhances the moisture-holding capacity of the atmosphere. This phenomenon is known as the Clausius-Clapeiron relationship, which explains that with each degree Celsius increase in temperature, there will be a 7% increase in precipitation intensity. Hence, there will be a likely chance of intense and frequent rainfall conditions. Boucher et al. (2013) mentioned that increased temperature has been shown to

increase heavy precipitation conditions compared to mean precipitation in various regions of the world. Willems (2013) have reported that there will be frequent intense and long-lasting precipitation resulting in increased frequency of flooding events in Europe and other part of the world along with global warming. As reported in many studies, unpredictable weather conditions brought about by climate change have shown that intense and frequent rainfall patterns resulting in waterlogging and flooding will be a likely scenario and will lead to more crop production failures than anticipated (Webber et al., 2018). Such conditions can be concerning for farmers as they will face a greater risk of unwanted large-scale yield failure, and this will threaten the stability of their production system.

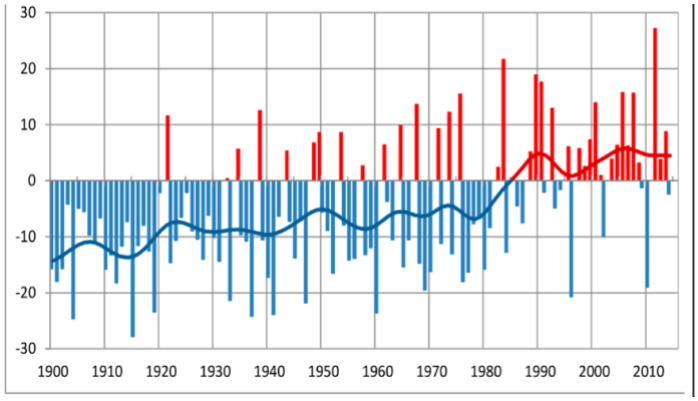
Wheat is a sensitive crop to waterlogging and waterlogging conditions due to high rainfall, and flooding has been one of the significant abiotic causes of reduction in wheat production (Zampieri et al., 2017). Around 15-20% of global wheat-growing areas are affected by waterlogging annually (Kaur et al., 2020). There are many reports of waterlogging effects in wheat production areas globally. Kulkarni et al. (2021) reported that the Indus River basins, which cover 96% and 26% of crop production area in Pakistan, have faced intense waterlogging conditions because of localized extreme rainfall patterns in recent years. Similarly, Yan et al. (2022) also mentioned that a considerable yield decline in wheat has occurred in China, a central wheat-producing area, due to flash flooding and waterlogging events. France, the fourth major wheat-producing area in the world, had prolonged precipitation in winter and spring in the year 2016A.DD. This condition resulted in waterlogging, reduced solar radiation, and crop diseases. Consequently, the country faced the biggest wheat yield failure in the decade (Ben-Ari et al., 2018). Similarly, other countries like the Netherlands, Belgium, Switzerland, Germany, and England also experienced significant wheat production decline due to increased waterlogging duration and area as the result of high winter and spring rainfall in the same year (Nóia Júnior et al., 2023). Waterlogging events can be more detrimental than expected in the future due to intense and frequent rainfall patterns (Liu et al., 2023). Major wheat production areas in Western Europe and southern Asia are more likely to be affected by waterlogging than drought (Zampieri et al., 2017). Also, Júnior et al. (2023) have reported a high probability of experiencing unprecedented extreme precipitation conditions in the spring season in the central and northern parts of Europe as well.

1.3 What is expected in Norwegian Agriculture by climate change climate projections?

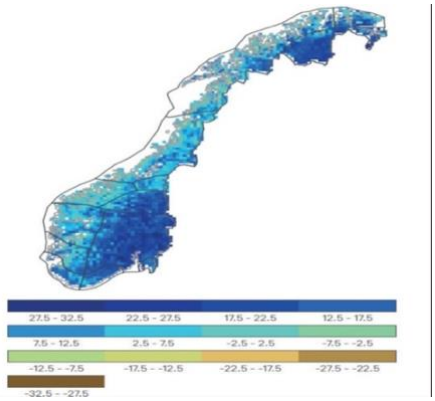
Many studies claim climate change will be beneficial to Norwegian agriculture. Climate projections have shown that Norway is one of the countries in Northern Europe with an overall positive impact of global temperature increase on crop production with an increase in the growing season, given that proper adoption measures to climate change are implied (Seehusen & Uhlen, 2019). The positive impact on crop yield will be higher in northern Norway because it is projected that the growing season will be increased by 1-4 weeks by 2050. It has also been predicted that there will be a 20-50% increase in the yield of potatoes, and it has also been expected that warming temperatures will open new avenues to grow legumes and perennial forage grasses in various parts of Norway in the future (Aalto et al., 2022). Very recent studies by Mróz et al. (2022) also found three days extension in the days to maturation of new wheat varieties because of increase average grain filling period by two days. In a current case study done by Telemark forskning in Vestfold and Telemark Counties, important wheat-growing regions in Norway, have reported that with rising summer temperatures, growth season will be increased by around one month by 2050, and there will be an increase in the number of growing degree days as well (Skaland, 2022). Such projections can, however, sometimes be superficial because climate change also indicates unexpected weather conditions like heat stress, drought, heavy rainfall, and flooding. These conditions can be extreme in amount, frequency, and intensity. Yield reductions caused by such extreme conditions can easily offset the advantage brought upon by increased temperature.

1.4 What about the waterlogging condition in Norway?

A.



B.



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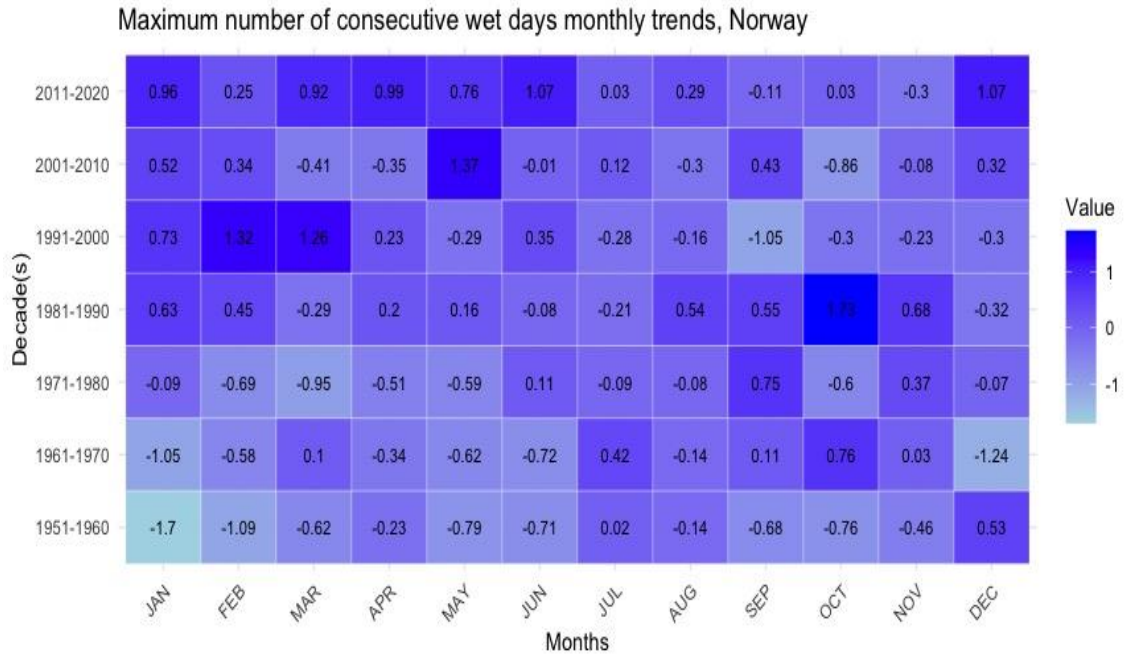


Figure 1. A) Relative deviation (%) of precipitation from 1900 to 2014 compared to mean precipitation from 1971-2000; B) Relative change in precipitation percentage between the period of 1971-2000 to 2071-2100 for March to May as measured in RCP4.5 model; C) Maximum number of consecutive wet days (CWD) monthly trends from 1950-2020 in Norway. Figure A and B source: Hanssen-Bauer, I. et al. (2017). Climate in Norway 2100. Available at https://www.miljodirektoratet.no/globalassets/publikasjoner/M741/M741.pdf. (accessed at 4.15.2023)

Figure C source: The World Bank Group (2021). Current Climate > Trends and Significant Change against Natural Variability. Available at: https://climateknowledgeportal.worldbank.org/country/norway/trends-variability-projections. (accessed at 5.21.2023)

It is projected that by 2100, the annual precipitation of Norway will be increased by 7-23% throughout the geographical region of Norway relative to the mean annual precipitation from

1961 to 1990. Moreover, climate projections also have anticipated that there will be frequent intense heavy rainfall in Norway in the coming years and flooding because heavy precipitation will occur at a higher rate. Days with heavy rain will be doubled as compared to now. The occurrence of a few hours of intense rainfall will be exceeded by more than 30% (Hanssen-Bauer et al., 2009). Annual precipitation has increased in Norway since the 1990s, mainly from the 1970s (Fig 1A). Similarly, it has been observed that spring precipitation (March-May) is more likely to increase in southeastern Norway, a central cereal-producing area of Norway (Fig 1B). The Consecutive Wet Days (CWD) index has been recently taken as a vital climatic indicator for analyzing precipitation changes. This index is the maximum number of consecutive wet days per period with daily precipitation exceeding 1mm (Zolina et al., 2010). This indicator is valuable for studying precipitation extremes (Huang et al., 2017) and forecasting floods.

A high value of CWD indicates a high chance of flooding, and an increasing trend of this index with time signifies that the occurrence of flooding events is likely to be increased (Li et al., 2017). If we look at the CWD monthly trends of Norway from 1950 – 2020 (Fig 1C), the maximum number of consecutive wet days has increased to nearly one day in March, April, and May, and in June, it exceeded one day. This period is significant as it coincides with the spring wheat growing season. The increase in the CWD value suggests a rising trend, which may lead to an increase in flooding events and, more importantly, prolonged waterlogging during the critical growth period of wheat. Consequently, waterlogging issues will likely become a prevalent scenario in wheat-growing areas. However, it is essential to note that shown CWD index represents the overall trend in Norway. To obtain a clearer understanding of the potential waterlogging scenario in the future, CWD studies specifically focused on the wheat growing period in the core wheat production areas. This will provide a more accurate and detailed picture of the likely waterlogging situation that may occur in wheat production areas of Norway. There is one recent study done by Skaland (2022) in Telemark and Vestfold county of Norway, which has reported that the number of wet days will be increased in the months of April-May, and the number of dry days will be increased in the months of August-September in the near future. They predicted that early summer (April-May) will be wetter and late summer (August) will be drier in the coming years. This indicates a potential scenario where waterlogging events may occur during the early growth stage of wheat, followed by drought conditions during the crop´s maturity phase. This sequence of excessive moisture during the initial stages and subsequent dryness can have significant consequences for wheat yield. They

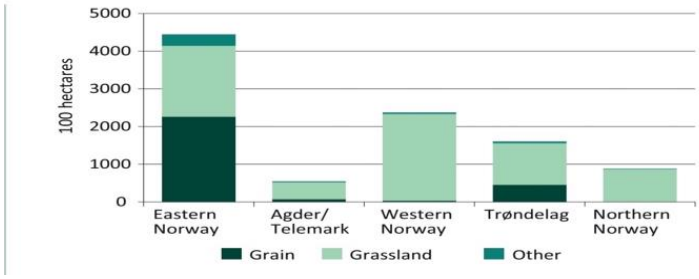
also further mentioned that precipitation patterns are highly unpredictable, but drought risk will be a likely scenario with increased temperature.

1.5 Wheat Production Scenario in Norway

Only 3.7% of the total land area is suitable for agricultural production in Norway, and only 30% is favorable for growing cereals. Additionally, cereals production areas are concentrated only in some parts of Norway. The southeastern part of Norway, mainly Akerhus, Ostfold, and Hedmark regions, cover 60% of the total cereals growing area. Similarly, Vestfold, Buskerud, and Oppland cover 22%, and Trondelag covers 16% of whole cereal growing areas. 95% of the total cereal growing area is limited to these regions. In the case of wheat, 70% of the total wheat growing area is limited to mainly three parts of Viken county, Akerhus, Ostfold, and Vestfold, where Ostfold is the largest wheat-growing region covering 35% of the total wheat growing area in Norway (Seehusen & Uhlen, 2019).

1.6 Will studies on waterlogging tolerance be important for future of Norwegian wheat production?

A.



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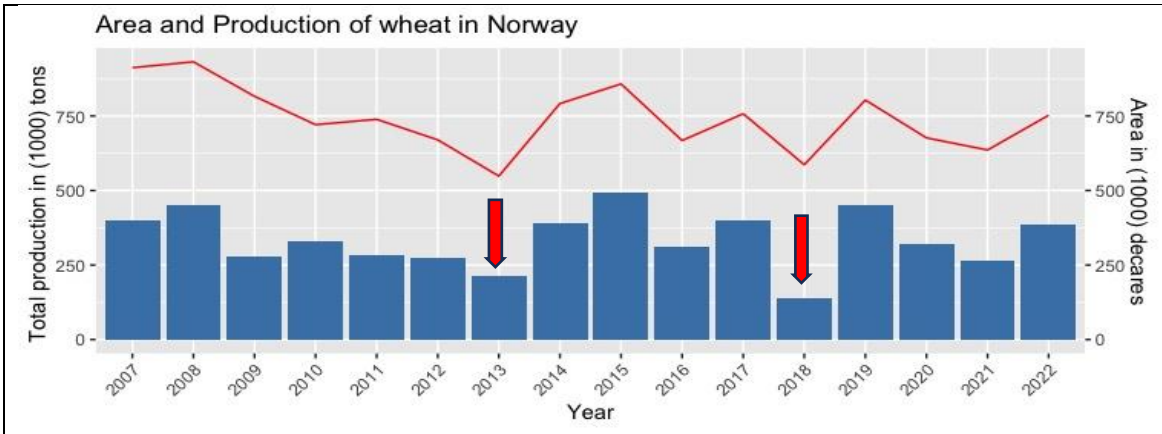


Figure 2. A) Distribution of main crops in various regions of Norway; B) Wheat production volumes (tons) and area (decares) from 2007 to 2022. Figure A was taken from Knutsen (2020); Data for Figure B was taken from Statistics Norway (2023). Cereals and oil seeds, area, and yields. Available at: (https://www.ssb.no/en/statbank/table/04607/)

Knutsen (2020) reported a 17% shrinkage in total cereal-growing land from 2001 to 2018. Cereal yield variability of 30-40% per year is primarily due to high variability in weather conditions (Seehusen & Uhlen, 2019). Looking at the trend of wheat production area and amount from 2007-2022, Norway (Fig 2B). The trend demonstrates a significant decrease of approximately 18% of wheat-growing land from 2002 to 2022. However, wheat productivity has increased by approx. 19% in 2022 compared to 2007. Thanks to the implementation of improved varieties and advanced agronomic practices. It is, however, worth noting that the productivity gain has not been consistent over the years, with specific years, such as 2018, as shown by the arrow in (Fig 2B). Wheat productivity decreased by 55% compared to the previous year. Such radical drop in crop production is primarily attributed to adverse weather patterns resulting from climate change. In 2018, extreme heat and drought prevailed throughout the growing season, significantly reducing wheat yield (Skaland, 2022). Similarly, 2013 was a typical waterlogging year in south-eastern Norway. There was much rain in May and June. Sowing of spring cereals was much delayed and the cereal crops also suffered from early season waterlogging. The decline in wheat area that year as shown by arrow in Fig 2B was partly due to the fact that many farmers that got delayed with the sowing of wheat and they switched to barley instead. Even many of those late-sown barley fields suffered from waterlogging during the crop establishment. This trend (Fig 2B) indicates the likelihood of unpredictable years deviating from predicted patterns, which could also apply to future waterlogging and flooding events. The potential increase in the frequency of unpredictable years threatens the stability of

the Norwegian wheat production system in the coming decades. To mitigate the possible yield reduction caused by these climate extremes, it is essential to develop and employ crop varieties resilient across multiple environmental conditions. In the case of waterlogging events, implementing adaptive agronomic measures such as drainage systems can further help reduce their impact. Nevertheless, such events do not occur annually, and their occurrence may deviate from predictions; it is prudent to be prepared with resilient crops. This highlights the importance of studying waterlogging tolerance in wheat varieties of Norway. Scanning of waterlogging tolerance in wheat varieties with Norwegian historical importance remains prime.

2. The Thesis

2.1 Background and motivation of the thesis

Field screening of waterlogging tolerance of spring wheat and barley central to Norway was done by Tove Kristina Sundgren in her Ph.D. thesis work (Sundgren, 2018). She conducted her research using wheat and barley population, comprising varieties, breeding lines, crossing parents, landraces, and other genotypes of historic importance to Norwegian wheat and barley breeding programs. This was by far the first study on waterlogging tolerance in wheat in the Norwegian context. She also identified a QTL on chromosome 6A (*QTL6A.2*) as highly significant for foliar chlorosis in spring wheat genotypes under waterlogging through a genome-wide association study (GWAS) (Sundgren, 2018). To investigate further, she constructed haplotypes on this locus using single nucleotide polymorphisms (SNPs) from two commercial SNP chips; Affymetrix 35K SNP array and Illumina 90K SNP array. Based on these SNP chips, three haplotype groups of genotypes named haplotypes 1, 2, and 3 were formed. Notably, she found a significant difference in mean chlorosis percentage between spring wheat genotypes belonging to two specific haplotype groups, haplotype 1 and haplotype 3, under waterlogging conditions. Genotypes under haplotype 1 were significantly more chlorotic than those under haplotype 3.

There are, however, many complexities associated with field-based screening methods. It is difficult to maintain even water table levels in experimental plots; otherwise, the level of waterlogging among them largely varies (Sundgren, 2018); keeping conditions homogenous on the field is complex as unexpected natural variations like other biotic and abiotic stress in a

field, change in temperature, pH, nutrients, and minerals occur in the soils. These various factors reduce the reproducibility of field waterlogging screening methods to a large extent. Moreover, field screening methods are time-consuming, labor, and cost intensive. Waterlogging screening methodology suitable for greenhouse or growth chamber conditions that can address large environmental variability of the field yet can simulate field waterlogging conditions can be used as a supplementary or alternative method for field screening methods.

It is worth mentioning that previous studies have also identified other large-effect QTL in the same genomic region of chromosome 6A. For example, *QRGA.UBO-6A.2* (Maccaferri et al., 2016), *qSRA-6A* (Alahmad et al., 2019), and *EPdwRGA-6A* (Alemu et al., 2021) were significantly associated with seminal root angle in different durum wheat populations. Foliar chlorosis is a stress symptom that is visually observed; they are the result of the effects of waterlogging on roots primarily, which are visually indiscernible. Given this overlapping genomic region on chromosome 6A, it can be assumed that *QTL6A.2* associated with foliar chlorosis under waterlogging might be associated with the difference in the seminal root angle of wheat genotypes.

2.2 Main Objectives

Based on the above motivations, the main objectives of this thesis were to:

1. Develop a suitable method for waterlogging tolerance screening inside the semi-controlled greenhouse as a viable alternative or supplement to the field screening method using contrasting haplotypes on *QTL6A.2* for chlorosis as screening material.

2. Develop a suitable phenotyping method for measuring seminal root angle and identify if there is any association of seminal root angle with waterlogging *QTL6A.2* for chlorosis.

3. Chapter A: Greenhouse screening of waterlogging tolerance in spring wheat of Norway

3.1 Introduction

3.1.1. Waterlogging: Definition, effects on soil, roots, and shoots:

Definition:

Waterlogging is defined as the condition when soil moisture exceeds field capacity, the amount of water that retains after excess water in response to gravitation has drained away, or the rate of flow has significantly reduced (Pampana et al., 2016). In ideal soil, soil: pore material is 50:50, and water: air volume in soil pore material is 50:50; when the soil pores become fully saturated with water, this condition is referred to as waterlogging. In such cases, a very thin layer of water above the soil surface may be present, or in some instances, waterlogging can occur even in the absence of a visible water layer above the soil surface due to the saturation of the soil pore material (Morales-Olmedo et al., 2015). Prolonged rainfall or irrigation, poor soil hydraulic conductivity or drainage, increased water table level, and lateral water flow are some causative factors of waterlogging (Liu et al., 2020).

Effects on soil:

Generally, the dissolved oxygen amount is 0.23 mol m^{-3} in cultivated soil, and in the case of waterlogged soil, it decreased to less than 0.05 mol m^{-3} (Pan et al., 2021). In average cultivated soil, O_2 is depleted by microorganisms and plants roots for respiration; however, good aeration maintains a good gaseous exchange with the atmosphere, i.e., O_2 inflow and CO_2 outflow; this exchange is rapid enough to restrict oxygen deficiency and accumulation of CO_2 to toxic level (Morales-Olmedo et al., 2015). Gaseous exchange in soil mainly occurs through diffusion and is influenced by factors such as soil texture, structure, pore size, distribution, and connectivity (Møldrup et al., 2001). When pore volume gets saturated by water, oxygen diffusion radically decreases as gas diffusion is 104 times slower in water than in air (Greenway et al., 2006). Moreover, when remaining dissolved oxygen is utilized by microbial activity and plant roots for respiration, it produces insufficient O_2 levels and toxic CO_2 concentrations in the soil. This situation results in hypoxia/anoxia in roots´ rhizosphere. Hypoxic conditions can happen as fast as a few hours, given that rapid consumption of dissolved oxygen by microorganisms and plants roots happens (Nickum et al., 2011). The oxygen concentration in normal cultivated soil is at least 10% (Goud et al., 2022), and a concentration below this indicates poor aeration and suboptimal condition for root growth (Morales-Olmedo et al., 2015). Another essential soil feature that changes during waterlogging is soil redox potential. It is commonly known as soil Eh. Soil Eh is a good indicator to find reduction levels in waterlogged soil measured in volts or millivolts (mV). Generally, in well-drained normal cultivated soil, soil Eh value ranged

between +300 to +500 mV (Macías & Camps Arbestain, 2010), while in waterlogged soil, it decreased down to +100 to -300 mV (Husson, 2013). Once trapped oxygen in waterlogged soils is finished, then anaerobic microorganisms start to capture oxygen from other compounds. They use other electron acceptors in place of oxygen. The first compound reduced is NO^{-3}; soil Eh during nitrate reduction remains within the +280 to +220 mV range. This also causes the depletion of nitrogen resources available to plants. Eh range falls lower once nitrate supply is emptied in soil and organisms reduced other compounds like MnO_2, $Fe(OH)_3$, SO_4^{2-} and CO_2, i.e., these compounds accept electrons and get converted to reduced elements. The reduction process occurs in chronological order of these compounds with a simultaneous decrease in Eh level (McBride, 1994). These elements get accumulated and reach phytotoxic levels. Some compounds like NO^{-3}, SO_4^{2-}, and K^+ might get leached to deeper profiles and become unavailable to plants. Waterlogging conditions not only create oxygen depletion, i.e., hypoxia followed by anoxia in soil but also lead to the accumulation of elements like manganese and iron to phytotoxic levels (Sundgren, 2018).

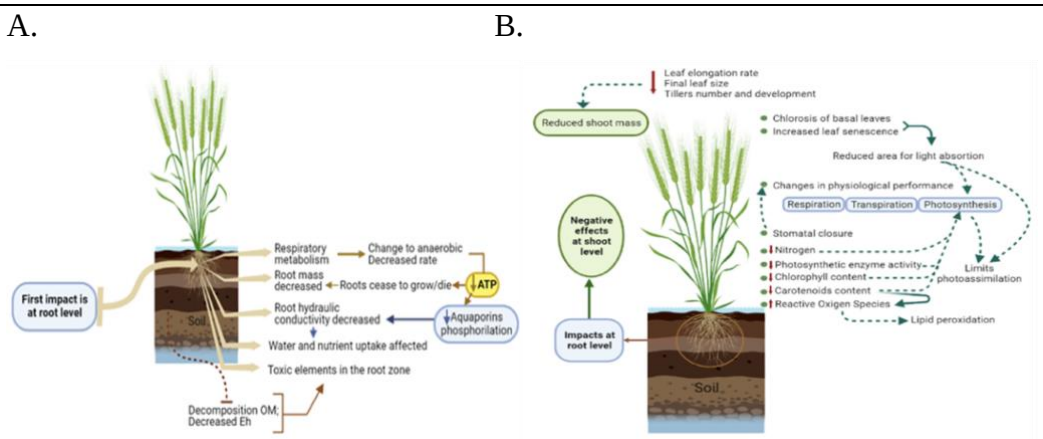


Figure 3. A) Effects of waterlogging on roots; B) Effects of waterlogging on shoots. Figures were taken from (Pais et al., 2022).

Effects on roots:

Reduced oxygen availability first affects roots by suppressing root respiration and reducing root activity (van Veen et al., 2014). As oxygen is an electron acceptor in the mitochondrial electron transport chain, reduced oxygen interferes with the electron transport chain and ATP production, restricting the mitochondrial respiration (Limami et al., 2014). The plant changes its aerobic respiration pathway to anaerobic respiration for energy production through glycolysis and ethanol fermentation (Baxter-Burrell et al., 2002). This pathway produces a very

less amount of ATP (Pan et al., 2021). Thus, energy production by plant tissues under waterlogging stress decreased by 65-97% compared to normal conditions (Gibbs & Greenway, 2003). Energy reduction on the root level impairs the phosphorylation of aquaporins, membrane proteins responsible for conducting water flow through cell membranes, thereby reduce roots´ hydraulic conductivity. This limits water uptake and causes internal water deficit (Malik et al., 2002). In case of prolonged waterlogging, toxic substances like lactic acid, alcohols, aldehydes, and other anaerobic metabolites get accumulated (Tamang et al., 2014) along with an upsurge of reactive oxygen species (ROS), primarily hydrogen peroxide (Zhang et al., 2017) leading to plant senescence and eventually cell death (Tamang et al., 2014). Increased ROS levels destroy cellular molecules, metabolites like proteins, lipids, pigments, PSII, DNA, etc. (Ashraf, 2009). Moreover, endogenously produced ethylene gets accumulated as oxidative breakdown or diffusion escape of ethylene gets restricted in waterlogging. As a result, excessive ethylene accumulation restricts the root extension (Herzog et al., 2016) and toxic substances like elemental manganese and iron get accumulated to toxic levels that cause root cells damage (Pan et al., 2021).

Effects on shoots:

Impaired root function caused by waterlogging stress affects the physiological response of shoots, specifically on the carbon fixation (Ploschuk et al., 2018). In response to water absorption deficit caused by poor root hydraulic conductivity, stomatal conductance decreases, stomatal resistance increases, and in an extreme case, leads to stomatal closure to prevent water loss. Such change in stomatal behavior causes a decrease in net CO_2 assimilation, which leads to a radical reduction in the net photosynthesis rate and transpiration rate (Ashraf et al., 2011), resulting in wilting of leaves and senescence (Arguello et al., 2016). Besides hindrance in photosynthetic activity caused by stomatal limitations, PSII damage by elevated reactive oxygen species (ROS) (Ashraf, 2012), leaf chlorosis caused by chlorophyll degradation, leaf senescence (Araki et al., 2012), reduction in leaf area, reduction in chlorophyll production, are also responsible for decreased photosynthetic rates in waterlogged plants. Impaired root and shoot growth combined with reduced phloem transport in hypoxic roots cause excess sugar accumulation in leaves initially (Herzog et al., 2016). This, in turn, reduces photosynthesis as a negative feedback mechanism. However, with prolonged hypoxia, accumulated sugars are utilized by plants to maintain cell membrane structures, and a continued decrease in photosynthesis, along with the utilization of stored sugar, leads to energy deprivation in plants (Sharma et al., 2019). Reduction in pigments like carotenoids, in turn, leads to elevated ROS

levels; this increases oxidative stress in plants by lipid peroxidation, reduces phospholipids phosphatidylcholin (PC) and phosphatidylethanolamin (PE) that result in lipid membrane disintegrity and energy deprivation in shoots of affected plants. Moreover, a reduction in glycolipids like monogalactosyldiacylglycerol (MGDG) and digalactosyldia-cylglycerol (DGDG) reduces the efficiency of photosynthetic electron flow, thereby reducing the photosynthesis (Xie et al., 2021).

3.1.2 Effects of waterlogging under different scenarios:

A study found organic acid toxicity caused by waterlogging, which restricts root growth, nutrient uptake, cell wall lignification, and root occlusions in rice (Colmer et al., 2019). Other studies reported toxic organic acids affected ions (K^+) flux in hypoxic roots of barley (Pang et al., 2007). Understanding of the effects of toxic organic acids in wheat is limited and further research is needed (Hossain & Uddin, 2011). In wheat, significant reduction of seminal root dry mass (Malik et al., 2001), death of seminal root apical meristem (Colmer & Greenway, 2010), termination of seminal root growth, growth of adventitious roots to restricted length were reported under hypoxic conditions. Shoot dry mass was decreased by 43% to 72% in 3 weeks old wheat seedlings subjected to 3-21 days of waterlogging in compared to seedlings under complete drainage under the same period (Malik et al., 2002). Another study found a 67% decrease in shoot dry mass in wheat plants subjected to hypoxic conditions. When subjected to waterlogging, there was as high as a 70% increase in chlorotic dry mass in common wheat (Pais et al., 2021). A study found a considerable decrease in chlorophyll content (15-33%) under 10 days of waterlogging at the leaf emergence stage in four wheat genotypes (Yadav et al., 2015), while another study found a decline of 41-61% in chlorophyll content when six wheat varieties were subjected to 28 days of waterlogging at tillering stage (Amrit et al., 2014).

Waterlogging at various growth stages reduces yield by reducing yield components like the number of spikes per plant, number of grains per spike, thousand kernel weight, and seeds number per spike. Waterlogging stress at stem elongation decreases kernels number per spike, kernel weight, spike weight, and eventually crop yield while waterlogging at the tillering stage of wheat under 21 days of waterlogging, yield decreased by 60% (Araki et al., 2012). Similarly, waterlogging at the seedling stage decreased root and tiller number, leaf size and area, and

photosynthetic capacity, decreasing overall plant growth and leading to significant yield loss (Shao et al., 2013).

The effects of waterlogging on plants have differential effects depending on various factors. It is because the severity of waterlogging depends on the developmental stage of plants (Davies et al., 2000; de San Celedonio et al., 2014), depth of waterlogging (Malik et al., 2001), and waterlogging duration (Jackson, 1979). Different studies have found that wheat growth stages are sensitive to waterlogging. Some studies have found the stage from stem-elongation to post-anthesis to be the most sensitive period of wheat to waterlogging (Araki et al., 2012; de San Celedonio et al., 2014). They also argued that the seedling stage might not be that sensitive regarding yield reduction as plants get enough time to recover through various adaptive mechanisms. While Setter and Waters (2003) reported seedling stage is the most sensitive stage to waterlogging compared to tillering and grain-filling stage. Tian et al. (2019) also reported the highest yield reduction in the seedling stage compared to the jointing and tasseling stage in maize.

Conversely, Pampana et al. (2016) reported no difference in waterlogging response when waterlogging was imposed on wheat plants at 3rd and 4th leaf stages, whereas Araki et al. (2012) mentioned waterlogging after anthesis is more detrimental to yield than waterlogging at jointing stage. In the case of barley, waterlogging during tillering cause a 25% yield reduction, whereas waterlogging during pre-flowering caused a 75% yield reduction, as de San Celedonio et al. (2014). In contrast to this study, Wu et al. (2015) reported that waterlogging at tillering and then the jointing stage has a detrimental effect on grain yield compared to the grain-filling stage in wheat. Authors instead argue that waterlogging at the grain filling stage alone might positively impact grain yield as delayed post-anthesis chlorophyll degradation due to waterlogging might be beneficial for plants as it increases time for translocating photosynthates to grain.

The severity of damage caused by waterlogging increases with an increase in the duration of waterlogging (Olgun et al., 2008). In the case of wheat, there are contrasting conclusions on the effects of waterlogging duration on yield. Melhuish et al. (1991) claimed that waterlogging of 1-2 days is enough to cause a detrimental impact on the grain yield of wheat (Malik et al., 2002) also found that waterlogging of only 3 days in the seedling stage is enough to cause a significant impact on wheat yield in the long run. However, Meyer and Barrs (1988), however, had previously reported that waterlogging for as long as four don´t cause yield reduction in

wheat. The contrasting findings on the impact of waterlogging duration might be attributed to other factors, as waterlogging effects vary with crop types and environmental conditions as studies use different wheat varieties under different environmental conditions. Many studies concluded prolonged waterlogging is detrimental to crop yield with increasing effect with duration of waterlogging (Ghobadi & Ghobadi, 2010; Tian et al., 2020; Zhang et al., 2016). There was a 67% decrease in carotenoid contents in wheat plants under twenty-one days of waterlogging compared to a 15% decline under seven days of waterlogging at tillering stage studied in wheat plants (Alizadeh-vaskasi et al., 2018). The same study also found higher carotenoid reduction (32-49%) under 14 days of waterlogging compared to 7 days at elongation period in the same wheat varieties. A study also found a higher yield reduction of maize in 6 days of waterlogging compared to 3 days (Ren et al., 2016b).

Four conditions describe the nature of waterlogging, they are: complete waterlogging, when the water level reaches the soil surface, which affects the root system completely, partial waterlogging, when water reach the soil surface, root systems are partially affected; partial submergence when water level covers some or half portion of plants and complete submergence when a whole portion of plants are entirely inside water. The depth of waterlogging determines the severity of waterlogging on plants. In a study when wheat was subjected to waterlogging at varying depths below the soil surface, i.e., 0, 10, and 20 cm, tillering reduction rate increased with increasing depth, i.e., 24, 45, and 62%, respectively, and adventitious root length reduction increased with increasing depth, i.e., 39, 58, and 73% respectively (Malik et al., 2001). They also found that there was a 50% increase in seminal root dry mass when waterlogging depth was decreased from the soil surface level to 10 cm below the soil surface.

The severity of waterlogging also depends upon concurrent environmental factors (e.g., temperature). Temperature is an important environmental factor in determining the severity of waterlogging. Waterlogging effects will have less effects on wheat at lower temperatures, as there will be slower oxygen depletion, slower shoot, and root activity, and slowing water and nutrient demand (Trought & Drew, 1982). Sometimes, conditions such as lower temperature in combination with low biological activity and mass flow of water, soil anoxia may not happen (Setter & Waters, 2003).

Soil types also determine waterlogging severity. A study found around 12% more yield reduction in winter wheat in clay soil waterlogging compared to sandy soil waterlogging. They thought it might be that, upon drainage, it took a longer time in clayey soil to return to the oxic condition compared to sandy soil; also, the denitrification rate is higher in clayey soil compared to sandy soil (Cannell et al., 1984). A recent study explained that soil with high clay and highly compacted soil with heavy machinery are more prone to waterlogging severity because of poor drainage (Ploschuk et al., 2018). This shows that waterlogging effects on plants depend on various factors and are very complex in nature.

3.1.3 Plant adaptation physiology to waterlogging:

Plants undergo various morphological and anatomical adaptations and waterlogging stress signaling mechanisms to cope with waterlogging stress. Formation of aerenchyma, an airy tissue that forms intercellular space which can help in gas exchange from non-waterlogged above-ground tissues to roots, is a typical morphological adaptation trait in various crops, including wheat (Colmer, 2003b). This tissue is not only involved in gaseous exchange but also in escaping CO_2 and toxic volatile substances from the waterlogged tissue of plants (Yamauchi et al., 2013). In some crops like rice, aerenchyma is formed constitutively, whereas crops like maize, barley, wheat, etc., form aerenchyma in response to waterlogging stress (Rajhi et al., 2011). Aerenchyma formation in wheat and barley response to waterlogging has been reported in many studies (Huang et al., 1997; Xu et al., 2022; Yamauchi et al., 2014). Barley genotypes with higher root cortical aerenchyma were found to have significantly higher yield under waterlogging stress than genotypes with lower cortical aerenchyma. Similarly, another important waterlogging adaptive mechanism is the development of adventitious roots. They are more resistant to waterlogging than seminal roots and produce more aerenchyma (Colmer & Greenway, 2011). Some studies found that adventitious roots grow upward towards the surface to get exposed to oxygen (Jia et al., 2021). Similarly, crops like deep water rice cultivars make radial oxygen loss barriers to prevent oxygen loss to intracellular spaces of rhizosphere (Colmer, 2003a), as an adaptive mechanism to waterlogging stress, while crops like wheat cannot form this barrier (Ejiri et al., 2021); however, some structural changes such as increased cortex-to-stele ratio, smaller surface area to volume, etc., have been reported in wheat as an adaptive response to waterlogging (Pedersen et al., 2021). Rapid elongation of internode (low oxygen escape syndrome (LOES), caused by ethylene-promoted gibberellins induction, is

reported in partially waterlogged rice (Kuroha et al., 2018). Studies also found deep water rice cultivars with the SUB1 gene undergo a quiescence strategy under complete submergence to suppress shoot elongation to reserve energy and utilize it after the water recedes (Colmer & Voesenek, 2009; Hattori et al., 2011). Furthermore, some rice cultivars under submergence produce a gas film in the leaf that promotes gas exchange and maintain aerobic respiration (Pedersen et al., 2009). An increase in pyruvate decarboxylase (PDC) or lactate dehydrogenase (LDH) enzyme for ethanol fermentation and energy generation is one of the examples of physiological adaptation to waterlogging (Pan et al., 2021). Through phytohormone signaling, waterlogged plants show adaptive mechanisms. For example, Ethylene and ROS are involved in programmed cell death which causes the degradation of root cortical cells that form tissue cavities and help in the formation of aerenchyma (Yamauchi et al., 2013). Reduction in abscisic acid (ABA) concentration in stem and AR primordia, as ABA inhibits aerenchyma development, is found in waterlogged plants, and reduced ABA and elevated ethylene is reported in deep-water rice cultivars (Yang & Choi, 2006). Significant reduction in ABA content was found in leaves and roots of waterlogging tolerant barley varieties under three weeks of waterlogging (Luan et al., 2018). Reactive oxygen species accumulation acts as a signaling pathway for the formation of aerenchyma and helps in ethanol fermentation by up-regulating ADH and PDC synthesis (Sumimoto, 2008). In tolerant wheat and barley varieties, enhanced ROS accumulation was found under waterlogging conditions compared to sensitive ones. Glutathione S-transferase (GST) enzyme production act as a regulating enzyme to control damage incurred by enhanced ROS accumulation, which was found in roots of waterlogging tolerant barley (Borrego-Benjumea et al., 2020).

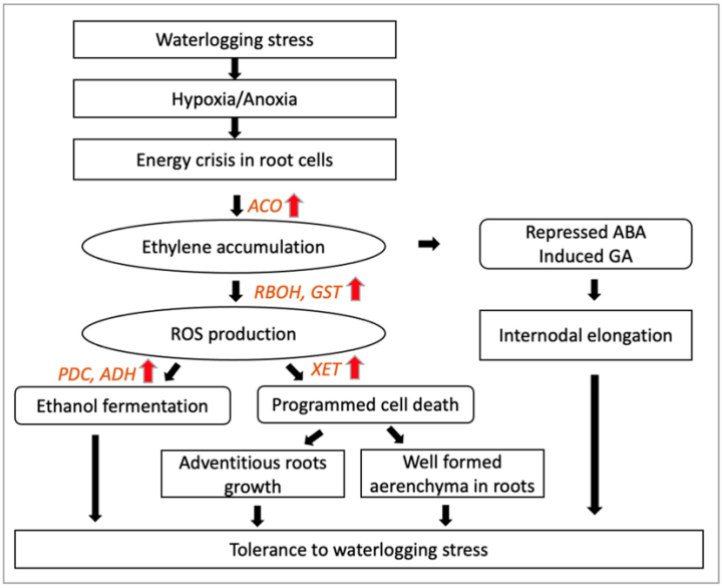


Figure 4. Diagram showing adaptive response mechanism in waterlogged plants. The figure was taken from (Tong et al., 2021)

3.1.4 Why are studies on waterlogging tolerance complex in nature?

Effects of waterlogging are responded to by plants either as stress symptom traits such as leaf chlorosis, reduced shoot biomass, reduced photosynthesis, early leaf senescence, decreased root growth, etc., (explained in "Effects of waterlogging under different scenarios") or are adaptive mechanisms traits such as aerenchyma formation, formation of ROL barrier, gas film in leaf, etc. (explained in "Plant adaptation physiology to waterlogging"). Both of these response traits, symptom or adaptive, vary with the type of crop species, its developmental stage, waterlogging conditions like duration and depth, soil environment, and other environmental factors (Ding et al., 2020). Considering all these factors, we can comprehend that a vast number of possible waterlogging environments can be created. Also, the interplay of various factors makes each waterlogging environment very complex. Studies have shown that there are strong genotype*environment interactions in waterlogging environments. Ducula-4, which was ranked as a tolerant wheat genotype by International Maize and Wheat Improvement Center (CIMMYT) (Villareal et al., 2001), was later tested in Australia by Setter et al. (2009). They found that specific genotype to be a sensitive one. Another study in western Australia also found inconsistent results on ranking 17 wheat genotypes under different locations (McDonald et al., 2006). A set of tolerant traits required for one waterlogging environment might be less beneficial in another waterlogging environment. This highlights the

importance of waterlogging studies on conditions and scenarios that can accurately represent the target environment to ensure accurate and applicable findings (Sundgren, 2018).

3.1.5 Waterlogging tolerance screening in wheat:

Wheat is considered a waterlogging-intolerant crop. However, it has been well-documented that waterlogging tolerance significantly differs among wheat genotypes (Gardner & Flood, 1993; McDonald et al., 2006; Musgrave & Ding, 1998). This provides an avenue for screening wheat genotypes for various waterlogging tolerance studies, such as documenting waterlogging tolerance of studied genotypes, identifying proxy waterlogging tolerant traits, identifying, and validating important quantitative trait locus (QTL) of waterlogging tolerance, selecting parental and progeny lines in waterlogging tolerance breeding programs.

Various methods have been developed to screen waterlogging tolerance in crops like wheat, barley, maize, rice, etc. They are field screening under artificial conditions or naturally waterlogged conditions, use of hydroponics systems with different nutrients media, controlled flooding conditions inside growth cabinets system, use of water-filled tanks or pots under semi-controlled greenhouse or glass house, use of lysimeters, etc. Some examples of various screening methods used in various crops are: field screening of spring wheat and barley under controlled waterlogging conditions used by Sundgren (2018) to document waterlogging tolerance of spring barley and wheat central to Nordic countries, Singh et al. (2018) also used field screening method to evaluate waterlogging tolerance of wheat breeding lines central to India in naturally waterlogged locations of India. McDonald et al. (2006) also used the field screening method to study waterlogging tolerance in wheat varieties in waterlogging-prone sites in Australia. Similarly, Setter et al. (2009) used waterlogging screening ponds under natural conditions using soils collected from waterlogged regions of western Australia to study waterlogging and elemental toxicities in wheat central to Australia. Hydroponics systems inside fully controlled growth cabinets were used by Bertholdsson (2013) to document proxy waterlogging adaptation traits in the seedling stage of the barley population central to Nordic regions. Arduini et al. (2019) also used a similar system to access waterlogging tolerance at the tillering stage of two commercial oats varieties central to Italy. This system was also reported to be used in other crops like soybean (Harrison et al., 2022) to study their waterlogging tolerance.

Similarly, Ploschuk et al. (2018) used individual pot methods to document the effect of waterlogging on the early and late stages of wheat, barley, and rapeseed. Yang et al. (2021) also used this method to study leaf water content in waterlogged wheat varieties in China. Zhou (2011) used individual pots inside a water-filled swim-ring-like container inside the greenhouse to screen barley genotypes for waterlogging tolerance in Australia. Similarly, water-filled tanks with experimental pots dipped inside were used in an experiment done by Jiang et al. (2022) to evaluate waterlogging tolerance in wheat cultivars central to China. A similar method had been employed to screen waterlogging tolerance in various crops like maize (Ren et al., 2016a) inside the greenhouse, and lentil (Lake et al., 2021) in outdoor settings. Zhou (2011) also used a soil-filled stainless-steel tank flooded with water kept inside the greenhouse to screen barley genotypes for waterlogging tolerance and identify associated QTLs. This method was further used by Broughton et al. (2015) to screen barley genotypes for studying waterlogging tolerance on them based on root porosity. Both studies were done in Australia on barley varieties central to Australia, Japan, and China, and soil material was collected from water-prone areas of Australia. Brisson et al. (2002) used lysimeters with attached oxygen meters to study the root response of wheat under waterlogging conditions in France. Growth cabinets under controlled flooding conditions were used by Byrne et al. (2022) to characterize tolerance properties of barley genotypes central to Ireland, water-filled tanks with experimental pots dipped inside them were used by Jiang et al. (2022) to evaluate waterlogging tolerance in wheat cultivars central to China, wide-mouth bottle with wet filter paper and filled with water for studying waterlogging tolerance of wheat varieties in the seed germination stage to identify QTL and putative genes associated with tolerance at the seedling stage by Pang et al. (2022). Similarly, cone-tainers inside water-filled plastic buckets were used to evaluate waterlogging tolerance of rough stalk bluegrass and tall fescue in Oregon, United States, by Liu, M. et al. (2017)

3.1.6 What are suitable phenotypic traits for the assessment of waterlogging tolerance?

Selection of sensitive and tolerant varieties from screening trials based on yield cannot be done as yield has very low heritability and depends on various factors (Arguello et al., 2016; Collaku & Harrison, 2005). The screening method for separating waterlogging tolerant and sensitive varieties needs reliable phenotyping trait(s). Such trait(s) must be easily quantified, have high heritability, and have a significant positive correlation with grain yield. Various traits have

been identified as reliable indicators for screening trails of waterlogging tolerance. Traits like root cortical aerenchyma , rate of germination index, plant height (Yu & Chen, 2013), rate of survival (Li et al., 2008), shoot biomass (Yu et al., 2014), foliar chlorosis and overall condition score (Sundgren, 2018), visual foliar chlorosis and survival rate (Zhou, 2011), chlorophyll fluorescence (Pang et al., 2004), the visual score of root color (Musgrave & Ding, 1998), number of tillers at maturity (Collaku & Harrison, 2002), shoot or root biomass (Ballesteros et al., 2015), Normalized Difference Vegetative Index (NDVI) (Arguello et al., 2016), etc. There are various phenotyping traits that are identified as reliable indicator(s) in waterlogging trials, but these traits are highly environment and crops specific. It might be because different crops use different response mechanisms under different levels of waterlogging stress. Because of this, it has been difficult to narrow down one or a few trait(s) that can be used as phenotyping trait(s) widely across different waterlogging environments. Therefore, the identification and use of reliable phenotypic trait(s) for screening waterlogging tolerance must be done for the target environment (Sundgren, 2018).

3.1.7 Is there any standard phenotyping method and phenotypic trait for screening waterlogging tolerance?

Despite numerous screening methods and phenotypic traits identified by researchers worldwide, standardization of specific waterlogging screening methods has been difficult to date. Screening methods and phenotypic traits are limited to the target environment. There are both advantages and disadvantages to various screening methods used. Field-based screening methods completely represent the natural environment in which plants face waterlogging stress. They are associated with high environmental variability.

Moreover, field waterlogging methods are time-consuming as it normally takes longer time for distinctive phenotypic traits to be developed; it demands severe stress to differentiate genotypic differences for waterlogging tolerance (Sundgren, 2018), is labor-intensive and highly costly as water sources must be ensured, and leveling of field is costly (Langan et al., 2022). Screening systems based on hydroponics using various growth media like agar can help understand anoxic stress to a greater level by removing confounding factors found in the field. However, these systems greatly underestimate soil and plant root interactions which are important factors influencing waterlogging stress and its severity (Pang et al., 2004). Screening systems based on controlled greenhouse/glasshouse in pots with soil substrate provide more controlled

conditions than the field yet represent closer conditions to the field than the hydroponics system. This screening system offers better control of other biotic and abiotic stress (Langan et al., 2022). Greenhouse systems can provide varying levels of environmental conditions ranging from basic tunnels only providing wind protection (Pang et al., 2004) to semi-controlled greenhouses with supplemental lighting to fully controlled greenhouse/growth cabinets with full control of lighting, temperature, and irrigation system (Luan et al., 2018) where individual pots, pots inside bucket/tanks, cone-tainers inside plastic buckets are kept for creating waterlogging conditions.

3.1.8 Assumptions of the Study:

Sundgren (2018) found that chlorosis percentage is one of the best yield-determining phenotypic predictors/indicators for measuring waterlogging tolerance in wheat. This phenotypic trait is measurable in an earlier stage, i.e., before tillering phase in wheat. Moreover, if this trait, combined with recovery, can be documented, an entire crop cycle may not be necessary to characterize tolerance levels in studied genotypes. In this study, we have assumed that it is feasible to replicate waterlogging conditions within a greenhouse, simulating the waterlogging conditions experienced in field settings. Here, we rely on the chlorosis percentage as a reliable phenotypic indicator in our screening approach to accurately assess the waterlogging tolerance of the studied wheat genotypes inside the greenhouse.

3.1.9 Justification of the Study:

Greenhouse waterlogging screening methodology has never been used to study waterlogging tolerance in Norwegian wheat. Replicating the absolute waterlogging field environment within a controlled setting like a greenhouse is challenging due to the intricate combination of soil physical, chemical, and biological factors present in natural soil environments. Nevertheless, a well-established and reliable method in a greenhouse can serve as a viable alternative or supplement to the field screening method. This approach is expected to offer cost-effectiveness, time efficiency, and reduced labor requirements compared to field screening while providing a representative controlled environment. Numerous studies have utilized greenhouses for screening waterlogging tolerance in various crops (Byrne et al., 2022; Broughton, 2015; Zhou, 2011). However, screening methodology may differ based on crop types and environmental conditions. Our screening method will be a tailored version of existing greenhouse screening

methods for waterlogging conditions and will, however, be unique and novel in the Norwegian context.

3.2 Experiment I: Design and testing of greenhouse waterlogging method

3.2.1 Specific objective:

- To develop waterlogging conditions for screening waterlogging tolerance in greenhouse.

3.2.2 Experimental site and Plant growth environment:

Experimental site: The experiment was conducted in greenhouse, SKP at Norwegian University of Life Science, Ås, Viken County, Norway.

Plant growth environment: Plants were grown at an average day /night temperature of 18/15 °C with relative humidity (R.H.) of 60% inside the semi-controlled greenhouse. Supplementary HPS lights were given to maintain 16 hours of daily photoperiod.

3.2.3 Plant material:

Eighteen genotypes of spring wheat were selected from the MASBASIS diversity panel. We selected our eighteen genotypes based on two main factors. The most important factor is that selected genotypes comprise a mixture of genotypes with contrasting haplotype groups for waterlogging QTL on chromosomes 6A.2, i.e., haplotype 1 and haplotype 3. Genotypes under haplotype group 1 are found to be significantly more chlorotic than genotypes under haplotype group 3 in Tove´s fieldwork. The next factor was these selected genotypes are genotypes of interest in long-term research of MASBASIS lines. Several of them were important, modern commercial cultivars. The seed source was seeds harvested from a MASBASIS field trial in 2019.

Table 1. Spring wheat genotypes selected for screening experiments with information on their 6A haplotype number and MASBASIS Line

S.N.	Genotypes name	6A Haplotype	MASBASIS Line
1.	Bastian	3	1003
2.	Bjarne	3	1005
3.	Runar	3	1020
4.	Zebra	3	1011
5.	Tjalve	3	1006
6.	Berserk	3	1016
7.	Polkka	3	1419
8.	Krabat	3	1174
9.	Bombona	1	1190
10.	Avle	1	1009
11.	Mirakel	1	1401
12.	Laban	1	1178
13.	GN07560	1	1405
14.	Vinjett	1	1116
15.	Berlock	1	1413
16.	Amulett	1	1189
17.	Cadenza	3	1443
18.	GN08554	1	1312

3.2.4 Soil:

Soil type used in the experiment was Sphagnum, i.e., white moss peat (Veksttrov, Norgo Ås, Lier, Norway). It has a PH of 5.0-6.0. It consists of 40% lump peat and 60% absorbent peat.

3.2.5 Experimental Design:

Greenhouse waterlogging experiment consists of two experimental designs:

A. Control Treatment Design.

B. Waterlogging treatment Design.

A. Control treatment design:

This comprises of RL98 – Ray Leach cone-tainer tray (Stuewe & Sons Inc, 31933 Rolland Dr, Tangent, OR, United States, Oregon), which has a dimension of (24 *12*6.75 inch) (Fig 5A). This cone-tainer tray consists of equally sized circular holes named cells. Each cell can perfectly fit 100 SC10R – ray leach super cell classic on it (Fig 5B) (Stuewe & Sons Inc, 31933 Rolland Dr, Tangent, OR, United States, Oregon). Each SC10R ray leach super cell classic has (diameter 1.5-inch, depth 8.25-inch, volume 10 cubic inch and volume 10 cubic inch/164 ml).

A.

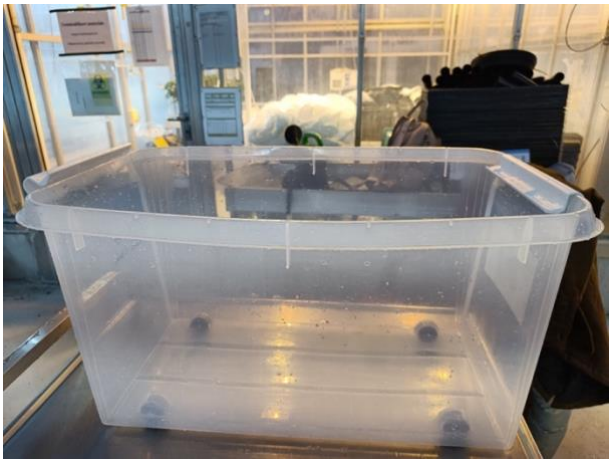
B.

Figure 5 A) Ray-leach cone-tainer tray; B) Ray leach-cone-tainer tray filled with SC10R – ray leach super cell classic, soil, and tag-sticks for genotypes identification

B. Waterlogging Treatment design:

Greenhouse waterlogging treatment was designed in Smart Store™ Classic 70 (Orthex Group, Espoo, Suomalaistentie 7, Finland) white plastic box (outer measurements 72 *40 * 38 cm, inner measurements 59.1 *30 * 33.2 cm, volume 70 L) (Fig 6A). Inside this plastic box above mentioned RL98 – Ray Leach cone-tainer tray with SC10R – ray leach super cell classics (details above) was fitted perfectly inside the box, and this whole set-up was considered as waterlogging treatment design (Fig 6B). This design was used only when the seedlings become ready for waterlogging treatment. This waterlogging treatment design was designed based on the method explained by Liu, M. et al. (2017).

A.



B.

Figure 6. A) White plastic box; B) Waterlogging treatment design complete set-up

3.2.6 Experimental design methodology:

Two equal-sized RL98 – Ray Leach cone-trainer trays were used, one for control and the other for waterlogging treatment. For simplicity, we named RL98 – Ray Leach cone-tainer trays as cone-tainers and SC10R – Ray Leach Super Cell Classics as cones. Here, outer rows of cells from all sides in this cone-tainers were kept as borders, as shown in the (Table 3 and Fig 7). The remaining inner rows of cells were divided vertically into three equal sections. Each section consists of (4*5 R XC cells) and was considered as replication. Each cone fitted inside cone-tainer tray cells was considered an experimental plot. So, plot-plot (P-P) distance was equal to cone-cone distance i.e., distance from mid-point of one cone to mid-point of next cone, which was 4.5 cm. Experiments were laid out in a Completely Randomized Block Design with three replications as shown in (Table 2). The seeding rate was 3 seeds/cone at a depth of 4 cm This was done for both cone-tainer trays.

Table 2. Eighteen wheat genotypes laid in Completely Randomized Block Design in a plastic cone-tainer tray. Number 1 to 18 denote genotypes number shown in Table 1

	Replication 1				Repication 2				Replication 3				
	Border												
Border													Border
	1	18	8	16	4	3	12	2	5	7	10	3	
	3	5	2	4	10	1	6	14	17	11	8	18	
	9	7	11	6	5	9	8	15	16	1	13	4	
	13	17	14	12	16	11	18	7	15	6	2	9	
	10	15			13	17			14	12			
	Border												

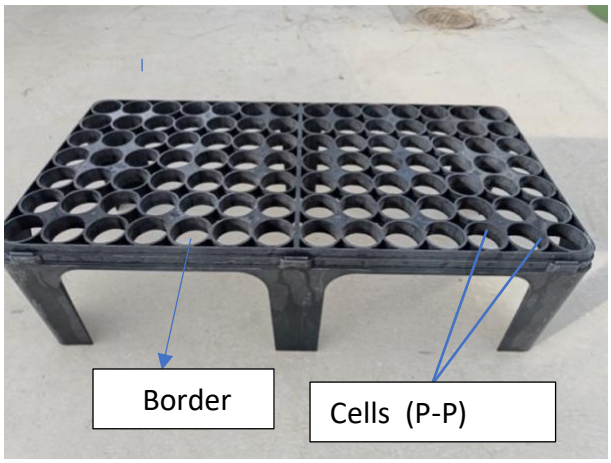


Figure 7. Cone-tainers showing border and cells

3.2.7 Treatment methodology:

For both control and waterlogging:

At first, cones were placed in both plastic cone-tainer trays (Fig 8A and 8B). After that, RL98covs -RL98 tray stainless steel cover (Stuewe & Sons Inc, 31933 Rolland Dr, Tangent, OR, United States, Oregon) with the dimension of (24 X 12 X3 inch) was placed over cone-tainer tray (Fig 8A). This RL98covs stainless steel cover was specifically designed by this company as a cover for the RL98 cone-tainer tray, to facilitate easy and fast filling up of soils into the cones. Then, cones were filled with Sphagnum (white moss peat) through RL98covs stainless steel cover manually. After that, seeds of selected wheat genotypes were seeded and covered with Sphagnum white moss peat soil from the top and watered using tap water. This was done in both cone-tainer trays. They were taken inside the greenhouse and left for germination and seedling growth to reach 3rd leaf stage. Up to this stage, both cone-tainer trays were treated equally and were given the same treatment and environment inside the greenhouse.

A. B.

C. D.

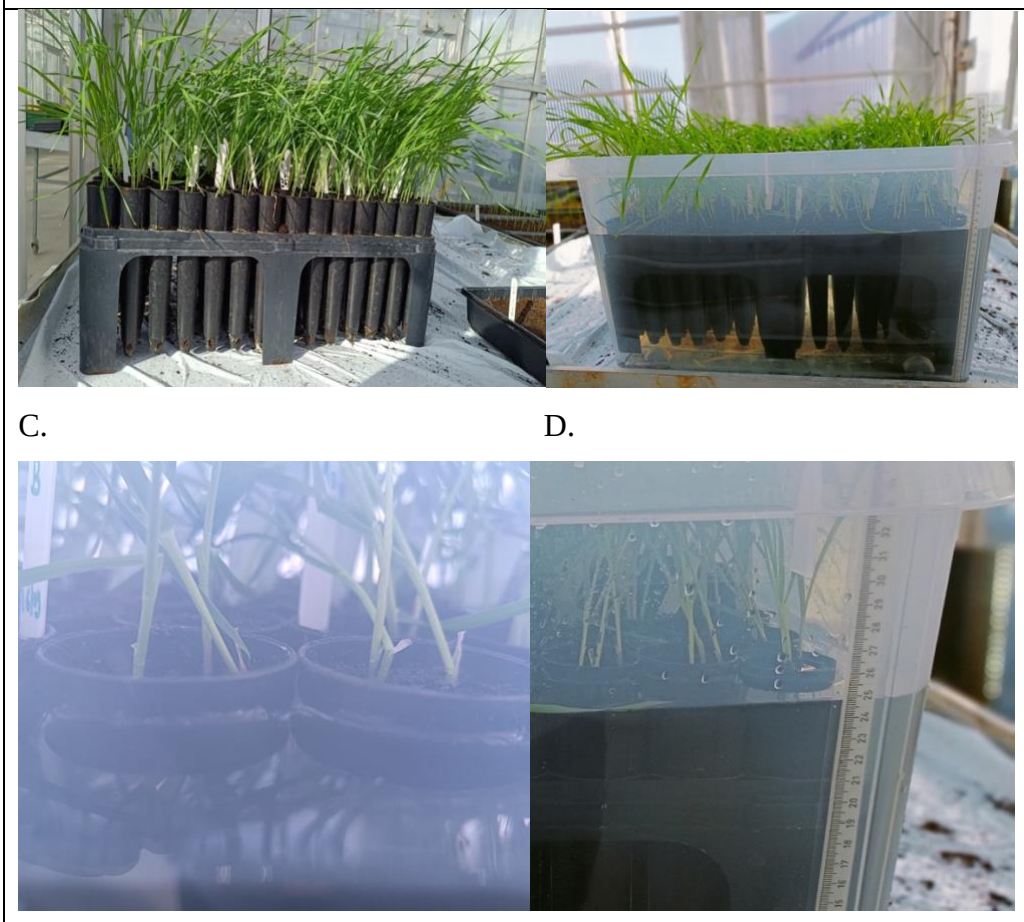
Figure 8. A) Stainless steel cover placing on cone-tainer for placing soil inside cones; B) Manual placement of soil and wheat seeds in cone-tainer; C) Wheat seeds placed at depth of 4 cm; D) Covering up cone containers with soil and placing them inside the greenhouse.

After the seedlings reached 3rd leaf stage, one cone-tainer tray (waterlogging treatment) was kept inside a white plastic box (above mentioned), and then, water was filled inside the white box carefully using a plastic pipe joined to the water tap. This experimental setup was designed based on the method explained by Liu, M. et al. (2017). The other cone-tainer tray (Control treatment) was kept in normal conditions inside the greenhouse (Fig 9B). Seedlings on control treatments were watered regularly with tap water throughout the experiment.

3.2.8 Waterlogging treatment and duration:

Waterlogging treatment was started 15 days after sowing. i.e., when all the genotypes reached 3rd leaf stage. The water level was kept at 25 cm, measured from the bottom of the bucket (Fig 9D). The level of water was measured using measuring tape. Additional water was added into the bucket to maintain the initial water level into the bucket after 6 days of waterlogging treatment, and this process was continued every 4-5 days throughout the experiment.

A. B.



C. D.

Figure 9. A) Control treatment cone-tainer; B) Waterlogging treatment inside plastic bucket (waterlogging set-up); C) Water level kept just below surface of soil. D. Water level inside waterlogging set-up measured using measuring tape.

3.2.9 Measurements:

Foliar chlorosis symptoms were measured when the difference in chlorosis symptoms among genotypes was distinguishable. Here, measurements were taken as a visual score of chlorosis

percentage in 10 cm (approximately) length of all leaves in a plot (cone) starting from tip to downward. This 10 cm length of leaves from the tip was only considered because there was a lot of shading effects in replication 2 as compared to replication 1 and 3. Also, this helped to remove confusion with chlorosis due to leaf senescence.

3.2.10 Statistical Analysis:

Software R studio (version 4.2.2) was used for analysis of all the data obtained. The phenotypic data obtained as chlorosis percentage was analyzed for Analysis of variance (ANOVA). ANOVA was done to see if there were differences in chlorosis percentage measurements for all the genotypes under study. Tukey HSD test was selected as a post-hoc test to assess the pairwise differences between group means of chlorosis percentage. Phenotypic data of chlorosis percentage were analyzed through linear mixed model $Y_{ij} = \beta_0 + v_i + u_{0j} + \varepsilon_{ij}$. Here, Y_{ij} is the observed value of the "Chlorosis percentage" variable for the i-th genotype and j-th replication. β_0 is the fixed intercept, which captures the overall mean chlorosis percentage across all genotypes and replications, v_i represents the random intercept for the i-th genotype, which denotes the deviation from the overall mean chlorosis specific to that genotype, u_{0j} represents the random intercept for the j-th replication, that covers the deviation from the overall mean chlorosis specific to that replication, ε_{ij} denotes the residual error term, which accounts for the random variation in the observed in chlorosis percentage. This linear mixed model was fitted using the lmer () function in the “lme4” package in R studio. This model considers genotypes and replication as random effects in relation to the dependent variable, chlorosis percentage. Estimated random effects also known as Best Linear Unbiased Predictions (BLUPs) for each level of the genotypes factor, were calculated using the ranef () function inside the “Lme4” package. Welch two sample t-test was done to compare the estimated genotypes mean of chlorosis percentage (BLUPs) between two haplotype groups.

3.2.11 Results:

A. B.

Figure 10. A) Control waterlogging plot (green); B) Symptoms of waterlogging treatment after 15 days of waterlogging.

Table 3. Results summary of ANOVA of chlorosis percentage of eighteen spring wheat genotypes.

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Genotypes	17	3914	230.24	3.343	0.00134 **
Replication	2	18	8.91	0.129	0.87911
Residuals	34	2342	68.87		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' 1*

Results of ANOVA showed that chlorosis percentage was highly significant ($p < 0.01$) among the genotypes under study (Table 3).

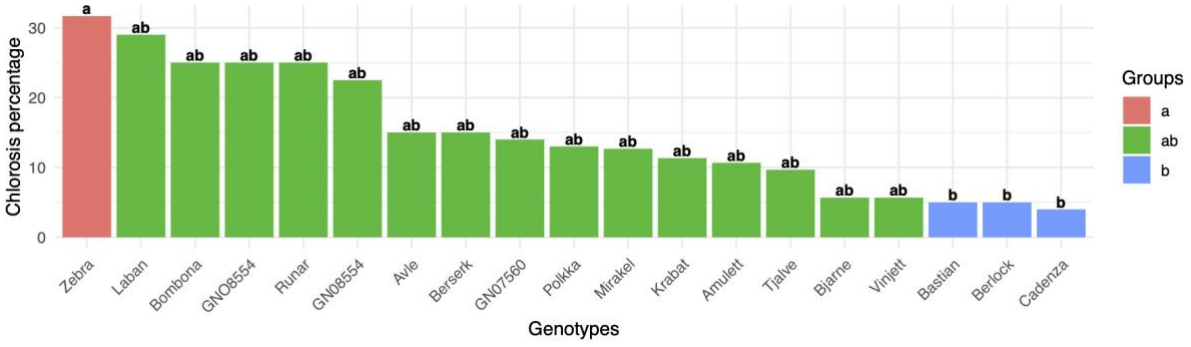


Figure 11. Results of Tukey HSD in bar diagram. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in mean chlorosis percentage among genotypes using Tukey HSD. Genotypes with at least one same letter indicate no significant difference.

Results of Tukey HSD showed that genotype Zebra was significantly chlorotic than, Bastian, Berlock, and Cadenza. The other genotypes are statistically at par with each other, and with Zebra, Bastian, Berlock and Cadenza (Fig 11).

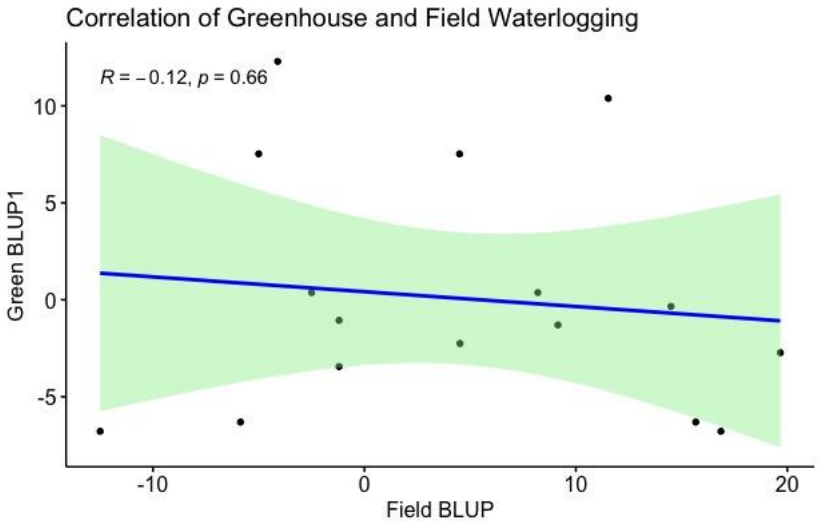


Figure 12. Pearson´s Product-Moment Correlation between the mean chlorosis percentage of eighteen genotypes in field waterlogging conditions in 2014 measured in BLUPs (Field BLUP) and the mean chlorosis percentage of the same eighteen genotypes in greenhouse conditions in 2022 A.D. measured in BLUPs (Green BLUP1) showing correlation coefficient (R) and P-value (P).

Results of Pearson´s Product-Moment Correlation showed that there was a very weak non-significant (R = -0.12) correlation between the field mean chlorosis percentage (Field BLUP) and greenhouse mean chlorosis percentage among genotypes (Green BLUP1) (Fig 12).

3.2.12 Discussion:

From the results of ANOVA, it was found that there was a significant difference among genotypes for chlorosis percentage. However, a very weak and negative correlation between field chlorosis and greenhouse waterlogging chlorosis (BLUPs) was obtained. One of the possible explanations behind the negative correlation might be that plants were at the senescence stage of their 1st and 2nd leaves at chlorosis assessment time. Despite taking the top 10 cm from the edge of the leaves for chlorosis assessment, chlorosis symptoms were confused with natural senescence in some genotypes. As a result, some genotypes which were found tolerant in the field might were given more chlorosis percentage scores than sensitive ones.

One important observation made through this experiment was that waterlogging stress we created through this methodology was insufficient. We kept the water level below the surface of the soil (top of the cone) initially. As the water level decreased, new water was added to the box periodically throughout the experiment to prevent plants from exposing to air. This potentially might have added extra oxygen to water and might have been available to plants aiding them to overcome the imposed stress. Wheat seedlings' recovery mechanism surpasses the stress given to them to a greater extent, because of which seedlings continued to grow and produce newer leaves and tillering, too. We observed in later days that initial chlorosis symptoms started to disappear as new greener leaves were formed in the canopy. We thought that the water level below the soil surface might be insufficient to create anticipated stress. To address this issue, we increased the water level above the soil surface periodically. Unfortunately, this adjustment was also not enough because the transpiration rate was higher, and the rate of decrease in water level was fast than we expected. Moreover, cones were not stable in cone-tainer trays and were floating. These floating cones were lifted from their normal position, which make the cone surface (soil surface) exposed to air. By 27 days of waterlogging, seedlings had already initiated tillering and developed numerous leaves. We could observe minimal chlorosis in newer-formed leaves. Unfortunately, the chlorosis symptoms developed were not enough to make a chlorosis assessment. Before symptoms are fully developed and become visually distinguishable among genotypes, newer green leaves were grown, and symptoms became visually indiscernible (Fig 13B). As a result of which, we could not make the next assessment of chlorosis. Moreover, we encountered the growth of unwanted algae as the next problem in our methodology (Fig 13B). These algae were responsible for making the experiment look unappealing and hindered the observation of chlorosis in plants. They also might have inadvertently benefitted stressed plants by aiding oxygen to them.

However, it is important to note that, as compared to plants in control plots, some amount of chlorosis was seen throughout the experiment in waterlogged plants. Plants in control plots were green, healthy and were developing faster than plants in waterlogging plots throughout the experiment. Booting and heading had already started in plants of control plots by 27 days of waterlogging (Fig 13A), but there was no single heading observed in waterlogged plants at this time. This observation proved the fact that even though enough chlorosis symptoms were not developed in waterlogged plants through this method, overall growth and development of waterlogged plants slowed down. This indicates that the methodology we used in this experiment was working. The major limitation of this experiment was that the stress produced

through this waterlogging methodology was not enough for plants to develop strong and measurable chlorosis symptoms as plants' adaptive mechanisms surpass the stress incurred.

A. B.

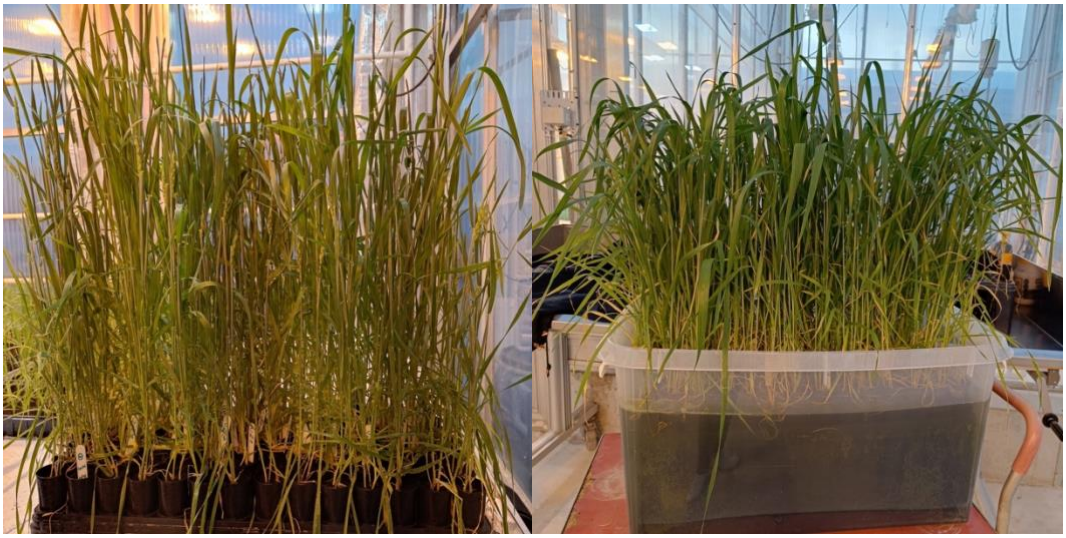


Figure 13. A) Plants under control plots with heading (yellow color was contrast of HPS light inside green house; B) Plants under waterlogging plots after 27 days of waterlogging.

3.2.13 Conclusion:

Through this experiment, a few critical limitations in developing the methodology for waterlogging stress conditions inside the greenhouse were identified. Sufficient water level inside the box for waterlogging might be one of the important improvements that can be made to create enough and consistent stress throughout the experiment. Cone stability can be another important improvement. This will prevent exposure of soil surface to air and prevent oxygen diffusion. It is imperative to consider these technical challenges and address them importantly in the next experiment to develop a reliable waterlogging stress methodology inside the greenhouse.

3.3 Experiment II: Improvement of greenhouse methodology

3.3.1 Background:

There was a need of improvement in waterlogging experimental design used in the previous experiment. We realize the importance of cones' stability inside the water level. For this, we improvised our waterlogging experimental design used in experiment I.

3.3.2 Specific Objective:

- To make cones stable inside the white plastic box in waterlogging set-up and achieve waterlogging conditions in the designed waterlogging method.

Before we conduct a real experiment. We conducted pre-testing of cones stabilizing materials as explained below.

Pre-testing of cones stabilizing materials:

Assumptions: We assumed that mixture of sphagnum moss peat soil with stones and sand can help stabilize cones inside water. As weight of stones and sand mixture will increase overall weight of materials inside cones and increase overall density (D ∝M). This will help cones to remain inside water for longer time.

Methodology of pre-testing:

layers of stones, then sphagnum moss peat soil, and then sand was filled inside the cone. Five cones were laid to deep inside the water level in the white box. The setup was left for testing for a few days.

Results: The tested cones were found stable inside the white plastic box.

Conclusions: We concluded that, mixture of sand, stones and soil inside cone will be perfect for stabilization of cones inside water-level for longer days.

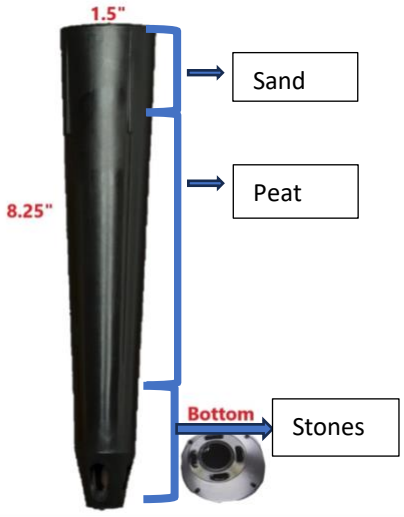


Figure 14. SC10R – Ray Leach Super Cell Classics showing layers of stones, peat and sand used for cone stabilization. The figure was modified from the original taken from (https://stuewe.com/product/ray-leach-super-cell-recycled/)

3.3.3 Experimental Site and Plant growth environment:

This experiment was conducted inside the greenhouse, SKP building of the Norwegian University of Life Sciences (NMBU).

Plant growth conditions: It was maintained the same as mentioned in experiment I. However, inside a semi-controlled greenhouse, they might be a reduction in temperature as the experiment was conducted in the months of November and December.

3.3.4 Plant material and soil:

The same as explained in experiment I apart from the modification with sand and stones as described above.

3.3.5 Experimental Design:

Experimental designs (both control and waterlogging treatments) were done the same as previously mentioned in Experiment I. The only difference was made in the inside materials of the cones. At first, a few small stones of approximately the same sizes were kept inside in the lowermost level of the cone, and then peat soil was kept up at the middle layer. After that, seeds were sowed at the rate of 3 seeds/cone. Here, the seeding depth was increased from 4 cm to 9 cm to maintain a layer of sand above it. Then, the uppermost layers were filled with sand (Fig 14). This modification was necessary to ensure the stability of cones inside the water level. These plastic cone-tainers trays were then taken inside the greenhouse for germination and seedling growth up to 3rd leaf stage (Fig 15).

Figure 15. Cones filled with layers of stones, peat, and sand for cone stabilization.

3.3.6 Waterlogging treatment and duration:

After seedlings of all the genotypes reached 3rd leaf stage, one of the cone-tainers trays was kept inside a white box the same as explained earlier in Experiment I. This was done 24 days after sowing. It took many days for waterlogging treatment in experiment II as compared to experiment I for all genotypes to reach to 3rd leaf stage. This was because the temperature in 2nd experiment was decreased in the winter season. After the cone-tainer tray was carefully kept inside the white box, then it was filled with tap water carefully with the help of a plastic pipe connected to tap water. This time, the water level was increased from 25 cm. to 28 cm measured from the bottom of the box (Fig 16). From the previous experiment, we realized adding up new water into the box time and again adds up new oxygen to plants and helped to adapt fast. However, the transpiration rate was so high in the plants inside the box. The rate of decrease in water level was higher than we expected. Eventually, we ended up adding an extra amount of water 15 days after waterlogging treatment. This was an important step at this stage because with decreased water level below the soil surface, plants would have an even better chance to adapt easily, and the experiment would have ended up as the previous experiment even though adding up new water at the mid of the experiment was not a good idea.

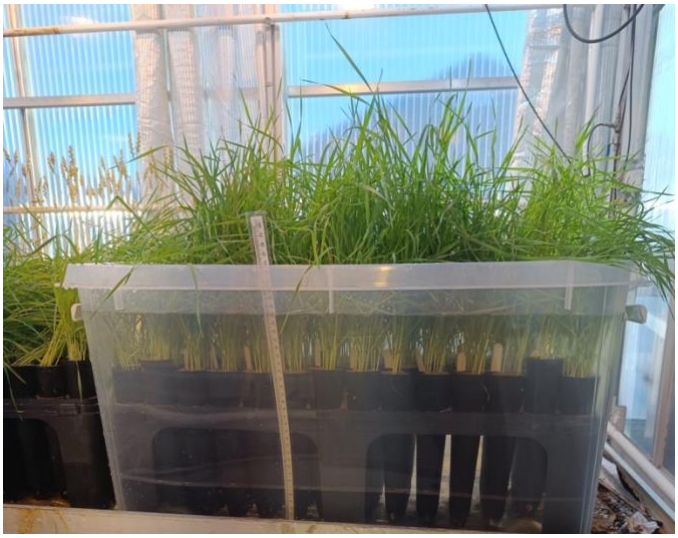


Figure 16: Waterlogging design showing cone-tainer dipped inside a white plastic box with the level of water inside the box shown by measuring tape.

Control treatment: For the control treatment, cone tainer tray continued to be kept as it was. Seedlings in the control treatment were watered regularly with tap water using a plastic pipe.

3.3.7 Chlorosis Assessment and Statistical Analysis:

Assessment of chlorosis and statistical analysis were done the same as previously explained in Experiment I.

3.3.8 Results:

Table 4. Results summary of ANOVA of chlorosis percentage of eighteen spring wheat genotypes taken on two different dates.

	January 1(17 DAW)	January 8 (24 DAW)
GDf	17	17
GSS	8124	8249
GMSS	477.9	485.3
G-F value	2.077	2.052
G-P value	0.0342 *	0.0365 *
RDf	2	2
RSS	3126	1072
RMSS	1562.9	536.2
R-F value	6.793	2.267
R-P value	0.0033 **	0.1191
EDf	34	34
ESS	7823	8041
EMSS	230.1	236.5

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '1*

GDf: Genotype Degree of freedom, GSS- Genotype Sum of Square, GMSS- Genotype Mean Sum of Square, G-F value, F-statistics value, G-P value, P-statistics value, RDf- Replication degree of freedom, RSS- Replication Sum of Square, RMSS- Replication Mean Sum of Square, R-F value, F statistics, R-P value, P statistics, EDf- Error Degree of freedom, ESS-Error Sum of Square, EMSS-Error Mean Sum of Square. DAW= Days after waterlogging.

Results of ANOVA showed significant differences ($p < 0.05$) among the genotypes for mean chlorosis percentage under study on both dates. i.e., 17 and 24 DAW, respectively (Table 4).

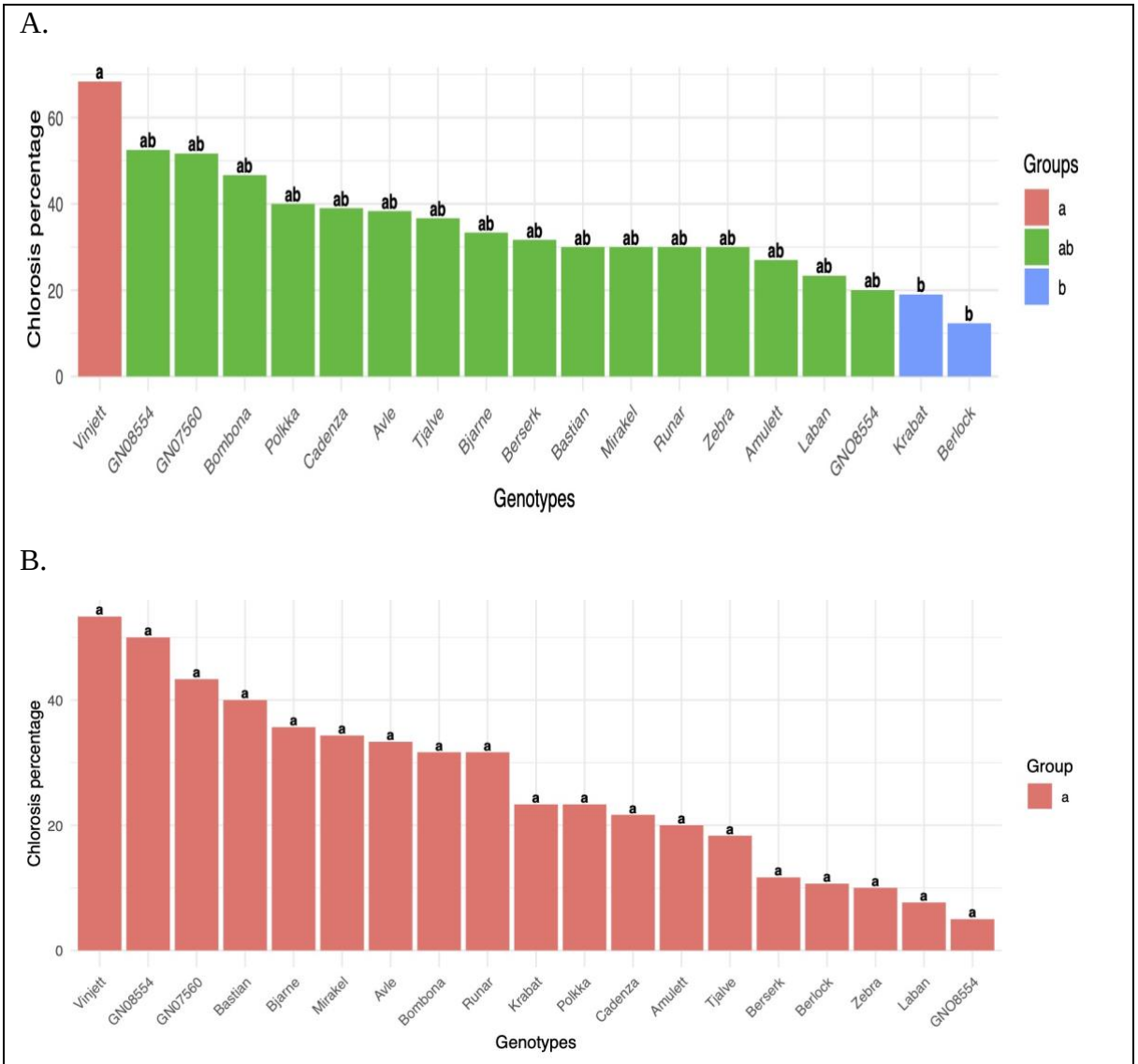


Figure 17. A) Results of Tukey HSD in bar plots taken on 17 DAW; B) Results of Tukey HSD in bar plots taken on 24 DAW. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in mean chlorosis percentage among genotypes using Tukey HSD. Genotypes with at least one same letter indicate no significant difference. DAW=Days After Waterlogging.

Results of Tukey HSD showed that Vinjett was significantly more chlorotic than Krabat and Berlock. The remaining other genotypes are statistically at par with each other, also with Vinjett, Krabat, and Berlock in data taken on 17 DAW (Fig 17A). While in the case of data taken on 24 DAW, even though ANOVA analysis showed a significant difference among genotypes for mean chlorosis percentage, results of the Tukey HSD test showed that there was no statistically significant difference among these genotypes for mean chlorosis percentage on that date (Fig 17).

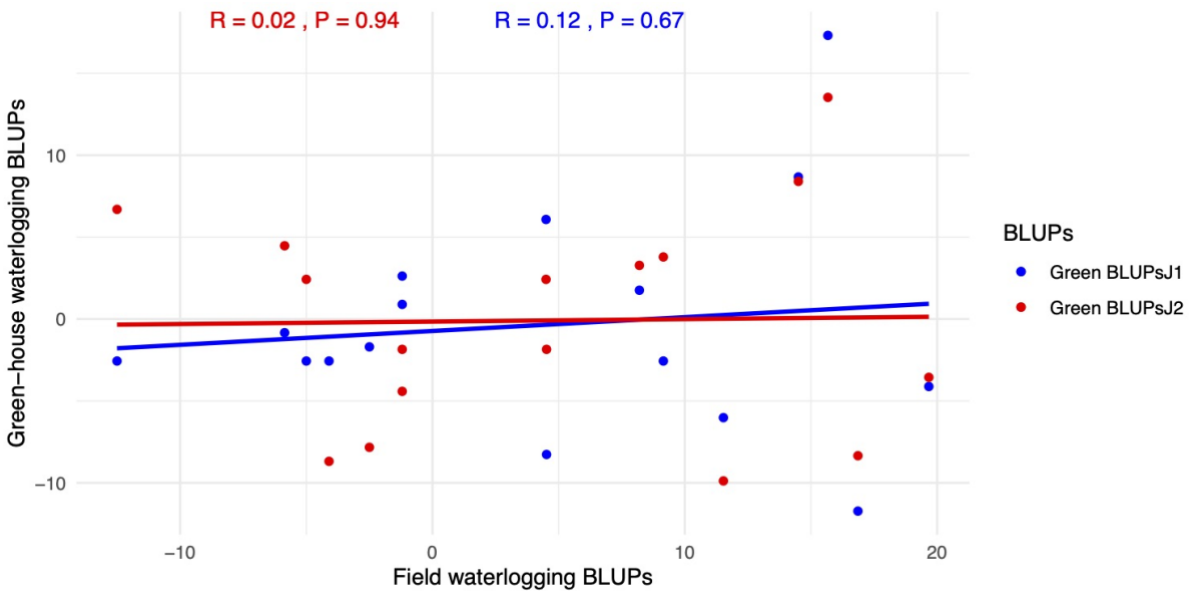
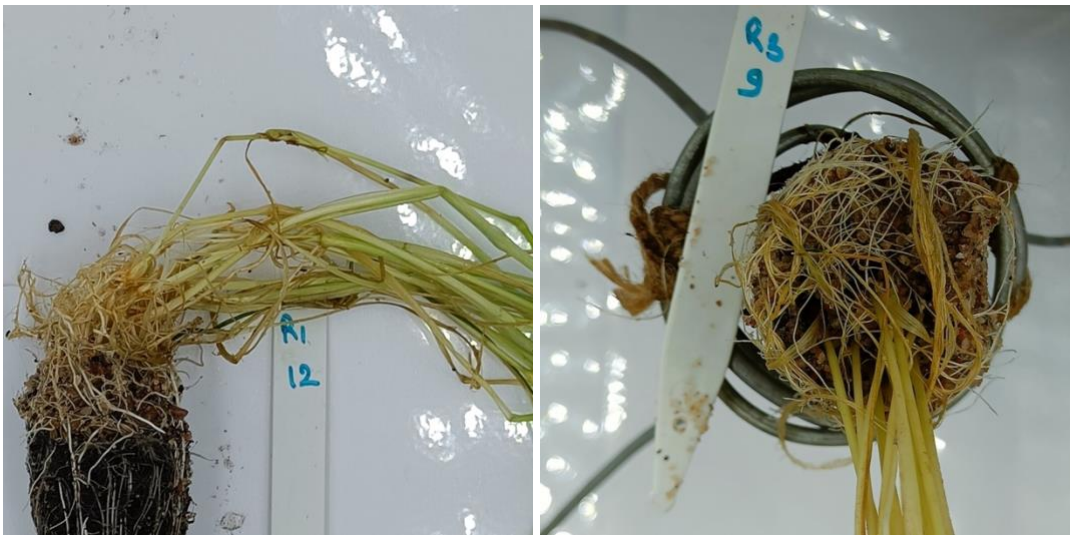


Figure 18. Pearson´s Product-Moment Correlation of Field waterlogging BLUPs and Greenhouse waterlogging BLUPs for chlorosis percentage taken on 17 DAW (Green BLUPsJ1) and 24DAW (Green BLUPsJ2) showing correlation coefficient (R) and P-value (P). DAW= Days After Waterlogging.

Results of Pearson´s Product-Moment Correlation showed that there was a very weak, non-significant (R< 0.5) correlation between the mean field chlorosis percentage of genotypes and the greenhouse mean chlorosis percentage of genotypes on both dates. The correlation was found to be weaker in 24 DAW than in 17 DAW i.e., R= 0.02 and R= 0.1, respectively (Fig 18).

3.3.9 Results on aerial-like roots:

A. **B.**



C. **D.**

E. **F.**

Figure 19. A) Laban; B) Bombona; C) Zebra; D) Tjalve; E) GN07560; F) Berserk. Figure A and B show profusely developed aerial-like roots by Laban and Bombona; Figure C and D show sparingly developed aerial-like roots by Tjalve and Zebra, and Figure E and F show no development of aerial-like roots by GN07560 and Berserk.

Table 5. Results summary of ANOVA of aerial-like roots scale of eighteen spring wheat genotypes.

	Df	Sum	Mean Sq	F value	Pr(>F)
Genotypes	17	345.5	20.324	13.72	5.94e-11 ***
Residuals	36	53.3	1.481		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Results of ANOVA showed that there was very highly significant difference ($p < 0.001$) among the genotypes for the development of aerial-like roots (Table 5).

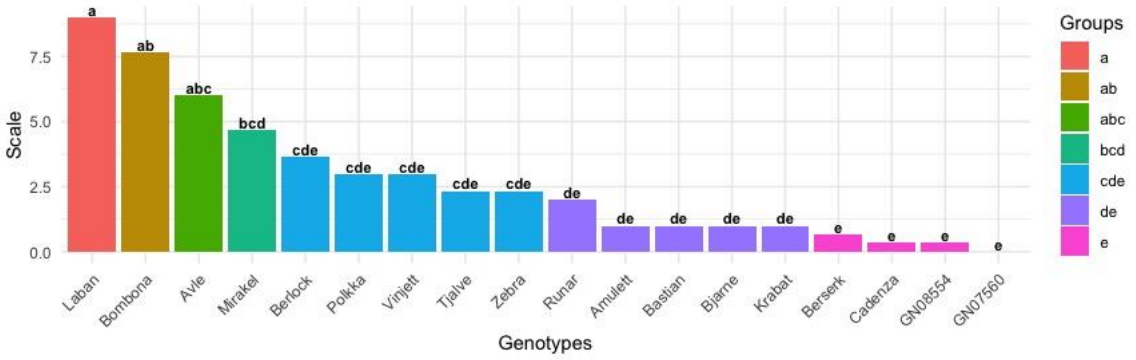


Figure 20. Results of Tukey HSD test of aerial-like roots development scale of eighteen spring wheat genotypes measured in scale. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in the average aerial roots development scale among genotypes using Tukey HSD. Genotypes with at least one same letter mean no significant difference.

Laban, Bombona, and Avle developed these aerial-like roots to a significantly higher degree than Runar, Amulett, Bastian, Bjarne, Krabat, Berserk, Cadenza, GNO8554 and GN07560 (Fig 20). Laban developed these roots statistically at par with Bombona and Avle and significantly higher than the rest of the genotypes.

Most of the genotypes in haplotype 1 developed these roots in higher amounts compared to genotypes in haplotype 3. Apart from GNO8554, and GN07560, genotypes in haplotype 1 developed these roots either profusely or sparingly. Similarly, genotypes in haplotype 3 developed sparingly to no aerial-like roots (Fig 20).

3.3.10 Discussion:

Improvements made in this experiment made cones stable in the cone-tainer trays. The water level was increased from 24 cm to 28 cm to maintain a sufficient water level inside the box. However, our anticipated water depth was not enough for our experimental setup. The rate of water level decrease was fast than we expected. Interestingly, we found some genotypes developed aerial-like roots, as shown in Fig 20, in search of oxygen. It was even more intriguing to learn that different wheat genotypes showed varying degrees of aerial root development. Some genotypes were not able to develop these roots, some developed them

sparingly, and some developed them profusely. These aerial-like roots might be adventitious roots that are developed to escape and come out of the water surface in search of oxygen. Jia et al. (2021) have mentioned that adventitious roots might come out of the soil surface in search of oxygen during waterlogging stress. But it was unknown whether the roots developed were adventitious roots or some other roots developed by wheat genotypes as adaptive mechanisms. Before the water level decreased by a substantial amount and before these aerial roots get exposed to air, we were compelled to add some extra amount of water after 17 days of waterlogging. This decision assumed that the prevention of exposure of these aerial roots to air outweighed the potential limitations of adding new oxygen imposed by additional water.

After 17 and 24 days of waterlogging, a chlorosis assessment was done. Results of ANOVA showed highly significant differences among the genotypes under study for chlorosis percentage on both days. Tukey HSD results of 17 DAW showed that a highly significant difference between genotype Vinjett and genotypes Krabat and Berlock while Tukey HSD results of 24 DAW showed no significant difference among the genotypes for chlorosis percentage. Results of the correlation of mean chlorosis percentage (BLUPs) in field experiments and greenhouse experiments among the studied genotypes showed consistently insignificant results in this experiment, too. (R= 0.1 and R = 0.02 on 17 and 24 DAW, respectively). There might be several factors behind it. At this point of time, we anticipated one of the reasons behind this consistently very weak correlation might be that field environment waterlogging stress cannot be reproduced inside the greenhouse for wheat. As in the field, a lot of various factors are responsible for affecting waterlogging stress. It also can be true that obtained correlation (R= 0.1) might be the highest possible correlation that can be obtained between greenhouse waterlogging and field waterlogging. Another reason might be that there might still be critical technical issues in the methodology we developed. Stress might still not be enough for plants to develop strong chlorotic phenotypes. The latter reason might be the viable reason because Sundgren (2018) in her field experiments reported that, in field conditions, chlorosis symptoms were visually indiscernible among wheat genotypes under less severe stress. This gave us a strong testament to believe that necessary stress was not created in our experimental setup. It was also clear from our experiment that waterlogged plants were continuing to develop newer leaves and tillering in this experiment, too. Normally, impeded growth and development of waterlogged stressed plants are expected.

Moreover, some important obstacles were noticed during the chlorosis assessment. There were a lot of shading effects on plants in Replication 2. This made the chlorosis assessment difficult and confusing. It was found imperative to address plant density issues to decrease the shading effect. Likewise, the water level must be increased from 28 cm. to above to prevent the need for additional water during the experiment. We also encountered a lot of algal growth inside the white box which might have added oxygen to wheat genotypes.

3.3.11 Conclusion:

From this experiment, we realized there were still some technical issues to be addressed in the methodology before drawing conclusions about the results of the correlation obtained in this experiment. It was realized that normal water waterlogging might not be enough for creating sufficient waterlogging stress inside the greenhouse. We might need some additional elements/substances to establish a strong waterlogging environment inside the greenhouse with this setup. Also, assessment of soil redox potential in these waterlogged soils was found crucial because it was uncertain if an adequate reducing environment is created in waterlogged cones used in our experiment. By doing this, a better picture of conditions inside waterlogged soils can be obtained. Moreover, plot-plot spacing (cone-cone) spacing is also strongly recommended to address as the shading effect has largely affected the chlorosis assessment. Additionally, we realized waterlogging at 3rd leaf stage of wheat plants might be relatively late, especially when these experiments include smaller cones. Even though this has no strong testament to making a difference in results as plants in control plants were growing healthy in these cones. This factor can still be considered, and seedlings of the younger stage can be used. It is found imperative to address these technical issues in the next experiment to develop a reliable waterlogging methodology inside the greenhouse.

3.4 Experiment III: Comparison of normal waterlogging with starch (0.1% m/v) waterlogging method to improve waterlogging methodology inside the greenhouse

3.4.1 Specific objective:

- To create severe stress conditions using starch (0.1% m/v) in the waterlogging method developed in Experiment II and compare the results with normal waterlogging.

3.4.2 Background:

From the above two experiments, it was clear that the normal waterlogging method established for greenhouse waterlogging could not produce adequate stress. Starch (0.1% m/v) waterlogging has been used by some studies previously, which showed that it can mimic waterlogging conditions by creating ample reducing environment (Byrne et al., 2022; Mano & Takeda, 2012). We decided to test this method under our greenhouse waterlogging settings and compare this method with our previously used (Experiment 2) normal waterlogging method.

3.4.3 Experimental Site:

This experiment was also conducted inside the greenhouse, SKP building of the Norwegian University of Life Sciences (NMBU).

3.4.4 Plant growth environment:

Plant growth condition was maintained as same explained in Experiment I. The experiment was done in the months of February and March 2023.

3.4.5 Plant material:

For this experiment, we removed genotypes, i.e., Cadenza and GN 08554, as their field chlorosis data from 2014 was not available in Tove´s thesis. We added four new genotypes (bold letters in Table 6) to this new experiment. Added genotypes were selected as they showed comparatively extreme phenotypes in field waterlogging. The screening plant materials used in this experiment are 20 spring wheat genotypes, where 11 of them are in haplotype group 3 and 9 of them in haplotype 1. Details of them are shown in Table 6.

Table 6. Twenty spring wheat genotypes with information on their 6A haplotype number and MASBASIS line.

S.N.	Genotypes name	6A Haplotype	MASBASIS Line
1.	Bastian	3	1003
2.	Bjarne	3	1005
3.	Runar	3	1020
4.	Zebra	3	1011
5.	Tjalve	3	1006
6.	Berserk	3	1016
7.	Polkka	3	1419
8.	Krabat	3	1174
9.	Bombona	1	1190
10.	Avle	1	1009
11.	Mirakel	1	1401
12.	Laban	1	1178
13.	GN07560	1	1405
14.	Vinjett	1	1116
15.	Berlock	1	1413
16.	Amulett	1	1189
17	**SW71237**	**1**	**1328**
18	**GN04528**	**3**	**1182**
19	**Dulus**	**3**	**1058**
20	**Filin**	**3**	**1050**

3.4.6 Experimental Design:

Two experimental designs were prepared for this experiment.

A. Control treatment design

B. Waterlogging treatment design.

A. Control treatment design:

In this experiment, a completely different control treatment design from the previous two experiments was prepared. Here, the same white box (used in the previous two experiments) was used. But three white boxes were taken instead of one. Each white box was considered as one replication. Three cone-tainers were used simultaneously for each white box. Unlike previous experiments, normal water waterlogging inside these white boxes was considered a control treatment.

B. Waterlogging treatment design:

In this design also, three white boxes along with three cone-tainers trays were used. Each white box with a cone-tainer tray fitted inside was considered a replication. Here, starch (0.1 % w/v) solution was used as a waterlogging treatment instead of normal water.

First, six white boxes used for both control and water-logging treatment were wrapped with black polythene (Fig 21). This was done to avoid algae growth inside the box. In previous experiments, algal growth was found as a problem. As algae were thought to provide oxygen to water-logged plants. Furthermore, it made the experimental site dirty and created obstacles during data collection.

Figure 21. White box wrapped with black polythene.

Preparation of 0.1 % w/v starch solution:

A total of 225 liters of water was first boiled in three Casseroles using induction heaters, as shown in Fig 22A in the batch. 0.1 % w/v of starch, i.e., 225 g of starch, was mixed in batch in boiled water as shown in Fig 22B, then stirred with the help of an aluminum stick Fig 22C and left to dissolve for 2-3 minutes. Here, three white boxes were filled with starch solution,

and the depth of starch waterlogging treatment measured from the bottom of the bucket was kept at 31 cm. For this, 75 liters of 0.1% w/v of starch solution were kept in each white box. These boxes with the hot starch solution were left for cooling down to normal room temperature overnight inside the greenhouse (Fig 22D).

A. B.

C. D.

Figure 22. A) Water boiled using casserole; B) Adding starch on boiling water; C) Light stirring of starch solution; D) Keeping starch solution inside white box overnight for cooling

3.4.7 Experimental design methodology:

For cone-tainer trays used in both control (normal water waterlogging) and treatment (starch solution waterlogging), outer rows of cells from all sides in this cone-tainer trays were kept as

borders same like in previous experiments. Remaining inner rows of cells in cone-tainer trays were used for laying 20 genotypes. Genotypes were laid in Completely Randomized Block Design throughout the cone-tainer trays. Here, one cone-tainer tray was considered as one replication. By doing this adjustment, the P-P distance was increased from 4 cm (in experiments I and II) to 12 cm (in experiment III). This was done to decrease plant density inside the box so that the shading effects of one replication to another can be decreased. For uniform placement of treatment genotypes in these cone-tainer trays, experimental plots were selected differently than the previous two experiments (Fig 23). These modifications maintain uniform P-P distance throughout the replication. The same adjustments were made for all six cone-tainer trays used in both the control and treatment designs.

Figure 23. Frontal (left) and overhead (right) view of genotypes laid in Completely Randomized Block Design in each replication (cone-tainer tray)

3.4.8 Treatment Methodology:

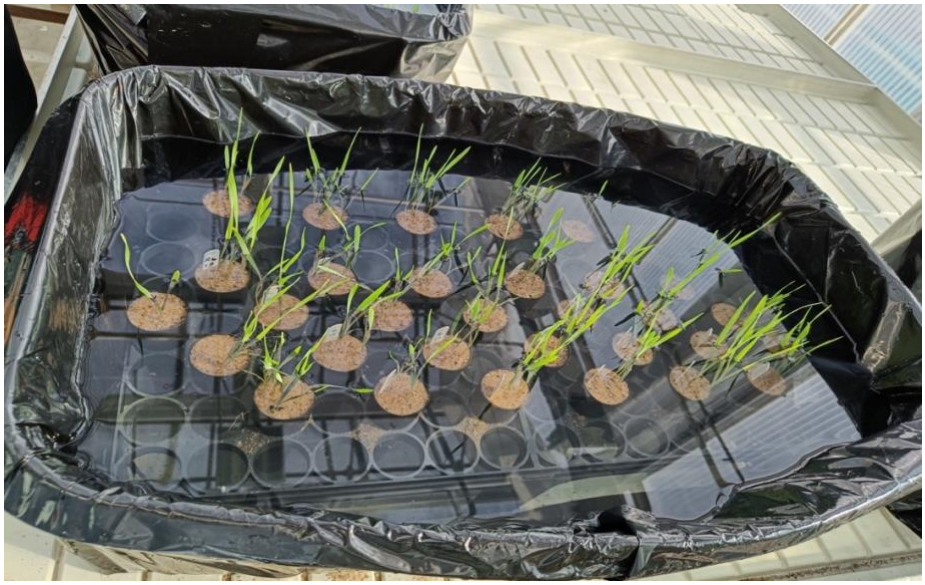
The same methods as explained in experiment II were followed before the start of waterlogging treatment. The only difference was that seedlings were subjected to waterlogging at 2nd leaf stage instead of 3rd leaf stage. When all the genotypes in cone-tainer trays reached 2nd leaf, these cone-tainer trays were subjected to waterlogging treatment for both normal (control) and starch solution (treatment) waterlogging. Waterlogging (both normal and starch) was started on March 2, 2023.

3.4.9 Waterlogging treatment and duration:

For control treatment plots/boxes, each box with cone-tainer trays was filled with normal water up to the depth of 31 cm measured from the bottom of the bucket (Fig 24C). The depth of water from the surface of the cone was maintained at 8.5 cm (Fig 24D).

For starch waterlogging treatment, each box with cone-tainer trays was filled with 0.1% w/v starch solution up to the depth of 31 cm measured from the bottom of the bucket, and the depth of water from the surface of the cone was maintained at 8.5 cm (Fig 24D). These whole setups were kept inside the greenhouse (Fig 24 B). Starch waterlogging was discontinued after 18 days of waterlogging.

A.



B

C. D.

E. F.

Figure 24. A) Overhead view of cone-tainer tray inside water inside white box wrapped with polythene; B) and C) Side view of one of the replications of normal and starch waterlogging respectively; D) Overall view waterlogging experiment of starch and normal waterlogging where each black box represents one replication. Three of them are waterlogged with normal water and remaining three with starch (0.1% m/v) waterlogging; E. Water level above soil surface maintained as 8.5 cm; F) Water level from bottom of white box kept at 31 cm.

3.4.10 Monitoring of soil redox potential:

In the previous two experiments, we could not assess either an ample reducing environment was created in our waterlogging experiment or not. From the previous two experiments, we realized the importance of accessing soil redox potential to make sure about reducing the environment created in water-logged conditions. So, for this experiment, we decided to monitor soil redox potential. Soil redox potential was measured using HI 3230, a platinum electrode that was connected to HI 8424, a millivoltmeter/pH meter (Hanna Instruments, Inc, Woonsocket, RI, USA).

3.4.11 Calibration and testing:

At first, the electrode was calibrated into Redox Buffer Solution (Mettler Toledo, Greifensee, Switzerland) that has standard Redox and pH values controlled at 25°C i.e., 220mv ± 5 mV/pH 7.00 ± 0.05 respectively. After that, redox values of starch and normal water were also noted (Fig 25). This was done to test the effectiveness of the electrode before using it in the real experiment.

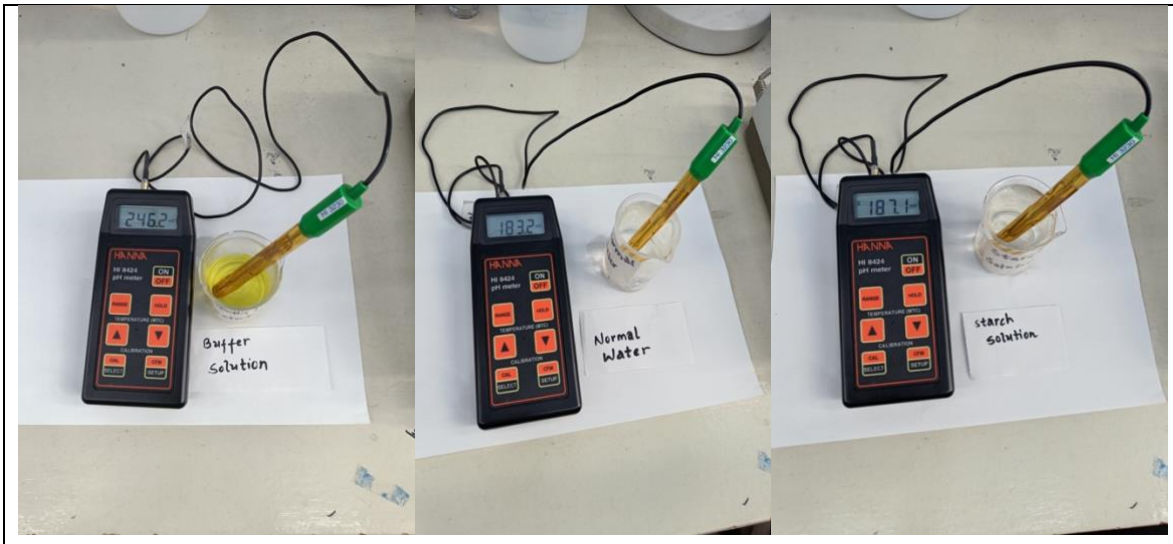


Figure 25. HI 3230, the platinum electrode connected to HI 8424 millivolt/pH meter through a BNC connector at one end and dipped into buffer solution (left), normal water (middle), and starch solution (right). Redox values (Eh) are displayed on the screen of the HI 8424 millivolt/pH meter. At room temperature, the standard Eh value of the buffer solution is 220mv ± 5 mV.

3.4.12 Measurement of soil redox potential:

To measure soil redox potential, HI 3220 electrode was connected to the BNC connector of the HI 8424 meter, which is a millivoltmeter/pH meter. Through selecting the appropriate range options on the millivoltmeter, it was set to display the measured values in millivolts (mV). This was important because redox potential is typically measured in millivolts (mV). In addition to redox potential, the HI 8424 meter can also show a measure of pH. After selection of the appropriate range options, corresponding values of the parameter measured by the used HI 3220 electrode can be made displayed on the screen of HI 8424 meter.

A. B.

Figure 26. A) HI 3230 platinum electrode dipped into waterlogged cones at a depth of 4 cm; B) HI 3230 platinum electrode joined to HI 8424 millivolt/pH meter through BNC connector.

After that, three random points (cones/plots) on each replication (box/cone-tainer tray) were taken. The HI 3230 electrode was dipped to 4-5 cm inside the cone, and redox values were noted when the hourglass symbol on HI8424 screen disappear. Average values on three points were calculated and considered as soil redox value of that replication for that day. This was done for all replications in both treatments. Monitoring of soil redox values was done every day from the start of the experiment waterlogging experiment to the end of the waterlogging experiment in both normal water (control) and starch solution waterlogging treatment.

Measurement of redox potential was discontinued in starch waterlogging treatment after the starch solution was completely removed from the boxes to study their recovery. It is because it was difficult to measure redox potential in normal soil as the electrode doesn´t work fine in a solid medium. For normal waterlogging treatment (control), monitoring of soil redox potential was continued to the end of the experiment.

3.4.13 Measurement of chlorosis:

In this experiment, the chlorosis percentage of the whole crop canopy was considered. Genotypes with necrosis along with chlorosis were given more percentage score of chlorosis.

3.4.14 Statistical Analysis:

Statistical analysis was done same as previously explained in experiment I.

3.4.15 Results:

Table 7. Results summary of ANOVA of chlorosis percentage of twenty spring wheat genotypes listed in Table 6 in Tove´s field waterlogging experiment in 2014

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotypes	19	5715	300.79	7.531	6.11e-08 ***
Replication	1	76	75.63	1.893	0.177
Residuals	39	1558	39.94		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Results of ANOVA showed that there was highly significant difference ($p < 0.001$) among genotypes for mean chlorosis percentage in field trial 2014 (Table 7).

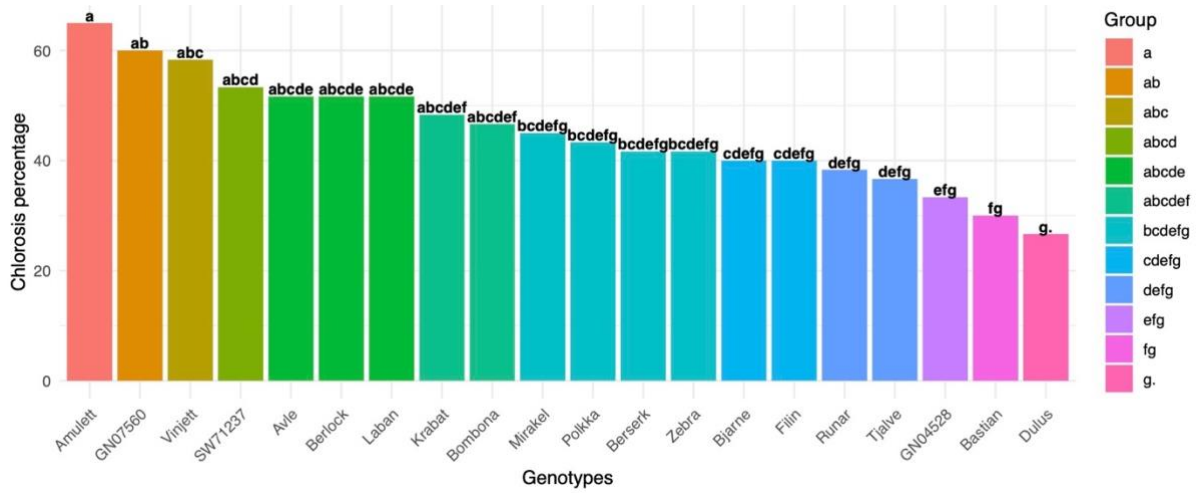
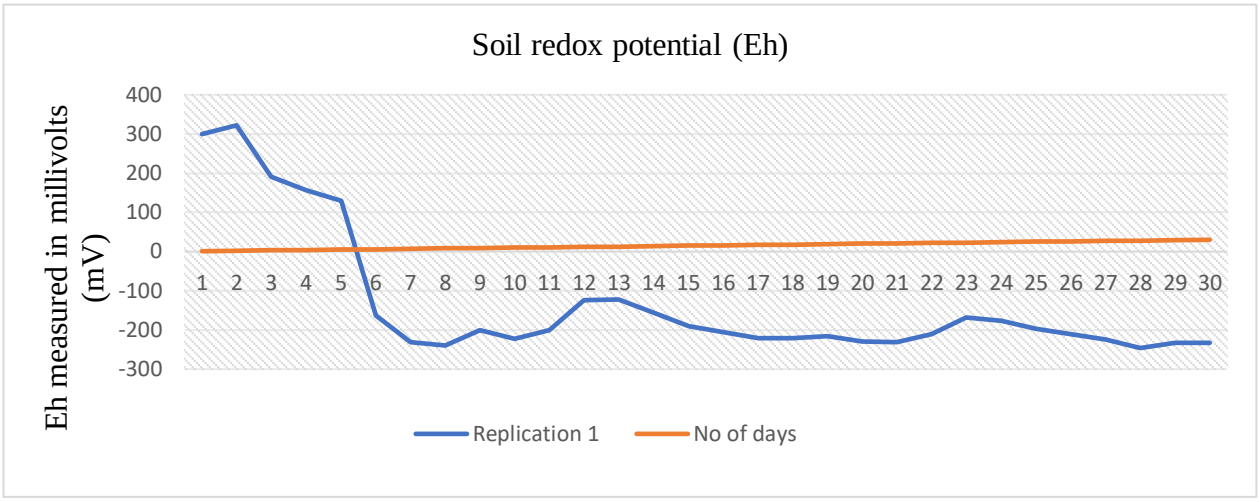


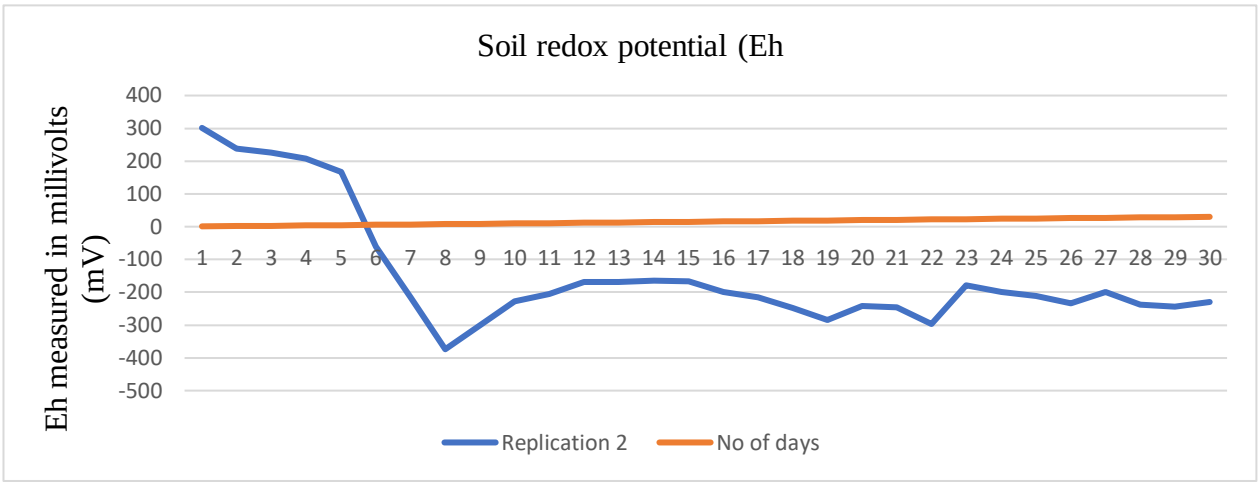
Figure 27. Results of Tukey HSD test of twenty spring wheat genotypes from a field waterlogging experiment of 2014. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in the mean chlorosis percentage among genotypes using Tukey HSD. Genotypes with at least one same letter indicate no significant difference.

Result of Tukey HSD showed that Amulett was significantly chlorotic than Mirakel, Polkka, Berserk, Zebra, Bjarne, Filin, Runar, Tjalve, GNO4528, Bastian and Dulus and statistically at par with other genotypes. Similarly, GNO7560 was significantly chlorotic to Bjarne, Filin, Runar, Tjalve, GNO4528, Bastian and Dulus and statistically at par with other genotypes. Vinjett was significantly more chlorotic than Runar, Tjalve, GN04528, Bastian, and Dulus and statistically at par with other genotypes. Likewise, SW71237 was significantly chlorotic to GN04528, Bastian, and Dulus and statistically at par with other genotypes. Dulus was significantly less chlorotic than to rest of the genotypes. Bastian was statistically at par with Dulus, GN04528, Tjalve, Runar, Filin, Bjarne, Zebra, Berserk, Polkka, Mirakel, Bomboma, and Krabat and significantly less chlorotic than other genotypes. GN04528 was statistically at par with Avle, Berlock, Laban, Krabat, Bombona, Mirakel, Polkka, Berserk, Zebra, Bjarne, Filin, Runar, Tjalve, Bastian, and Dulus and significantly less chlorotic than other genotypes (Fig 27).

A



B.



C.

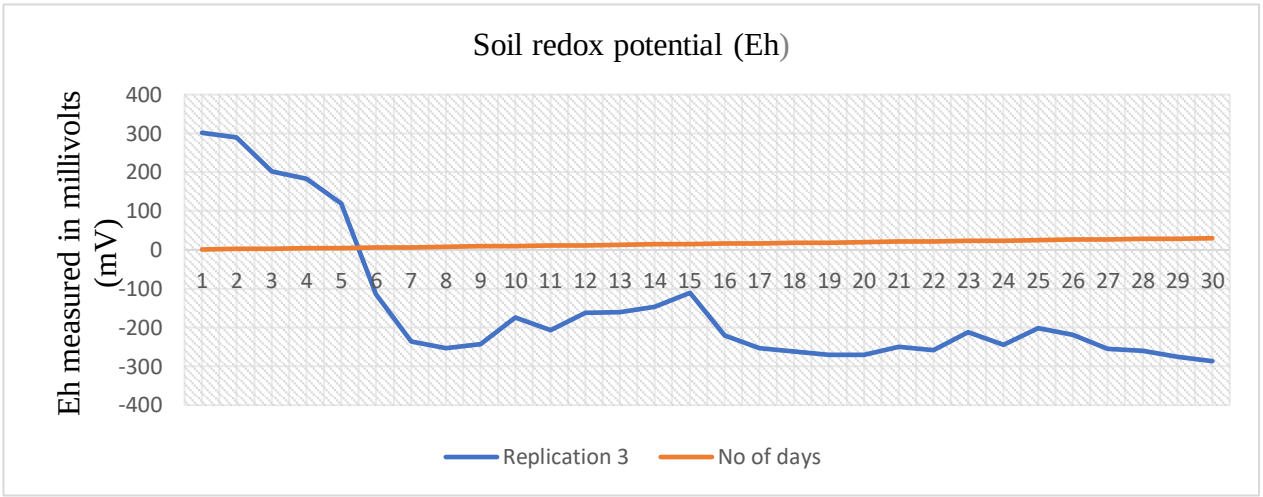
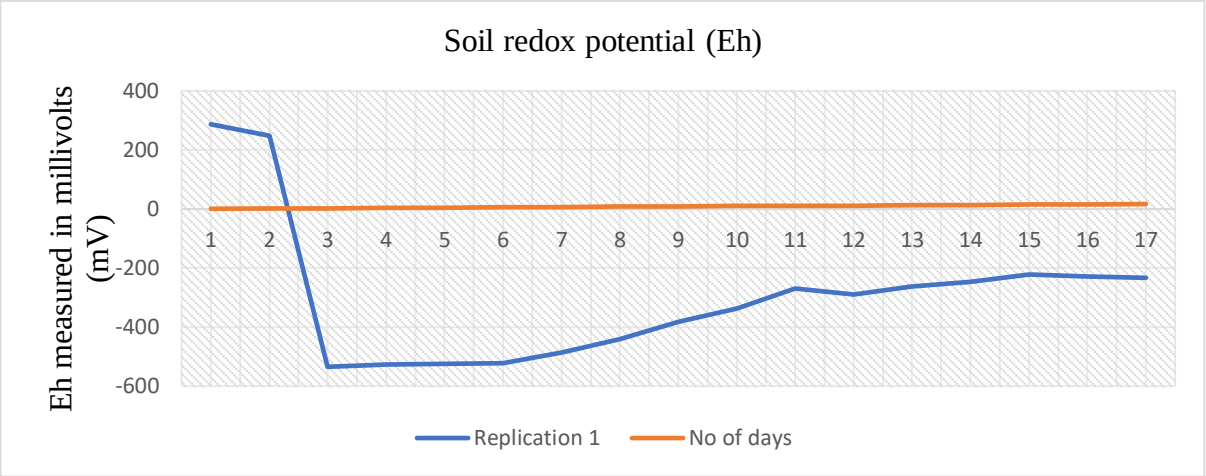
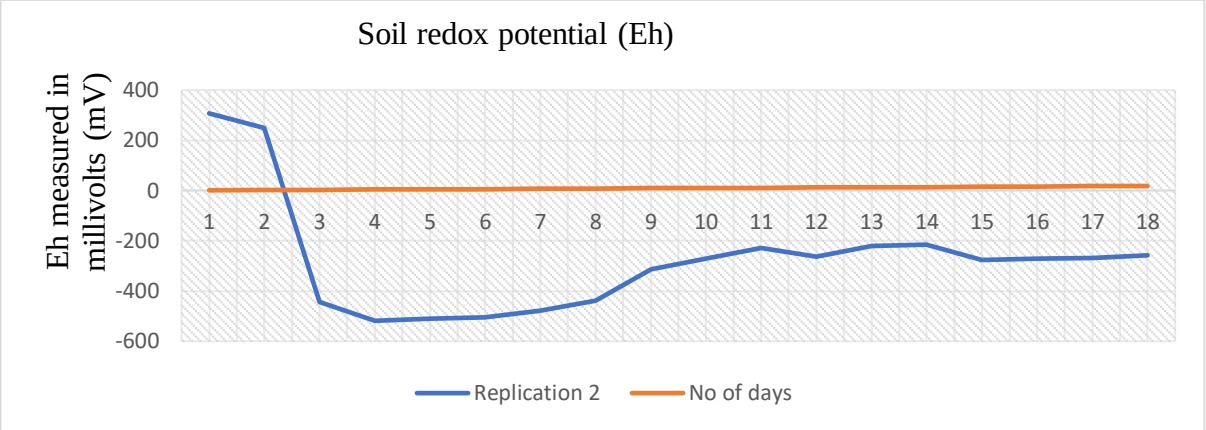


Figure 28. Measurements of Soil redox potential (Eh) up to 30 days of normal waterlogging across three replications.

A



B.



C.

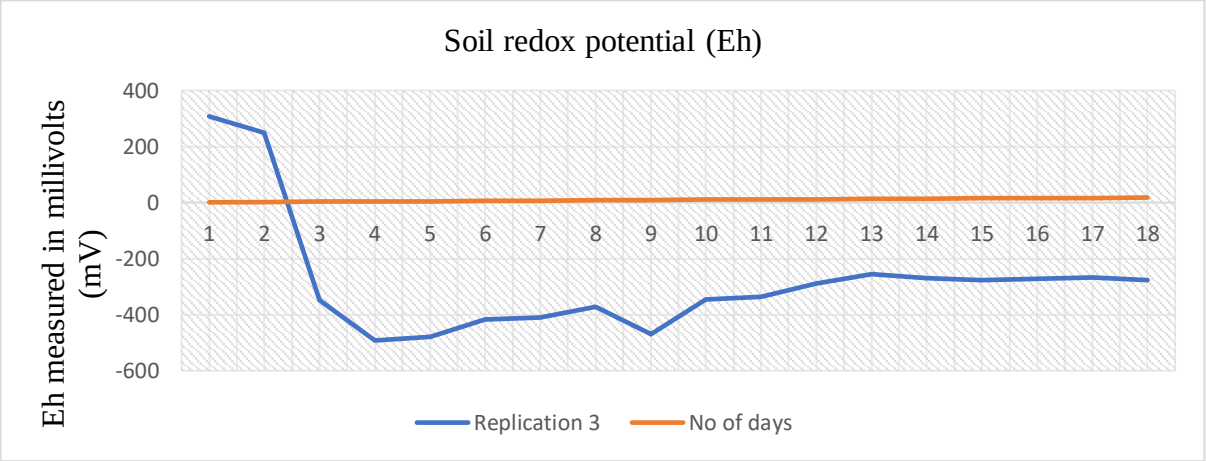


Figure 29. Measurements of Soil redox potential (Eh) up to 18 days of starch waterlogging across three replications.

In normal water waterlogging experiment, it took six days for the soil redox potential (Eh) values to drop below 0 mV in all replications. The Eh values continued to decrease and reached below -300 mV but did not drop below -400 mV in any replication. Throughout the entire experiment, in all replications, there was a persistent trend of the Eh values showing fluctuations, with values increasing and decreasing. However, the Eh values remained below -200 mV until the end of the experiment in all replications (Fig 28).

During the starch waterlogging experiment, the soil redox potential (Eh) rapidly decreased to below -400 mV within three days. On the fourth day, it reached even lower, below -500 mV, and continued to remain below -400 mV for the subsequent nine days of waterlogging. After that, Eh values showed a tendency to increase and displayed minor fluctuations, with values varying by small amounts. However, throughout the entire waterlogging period in all replications, the Eh consistently remained below -200 mV (Fig 29).

Figure 30. Starch (left) and normal (right) waterlogging plot at 12 days of waterlogging.

A. B. C. D.

Figure 31. Representative images of chlorosis induced by starch waterlogging. A) Genotype GN07560, most chlorotic genotype on 12 days of starch waterlogging; B) Genotype Tjalve, least chlorotic genotype on 12 days of starch waterlogging; C) Genotype Berserk, most chlorotic on 18 days of starch waterlogging; D) Genotype Zebra, least chlorotic on 18 days of starch waterlogging.

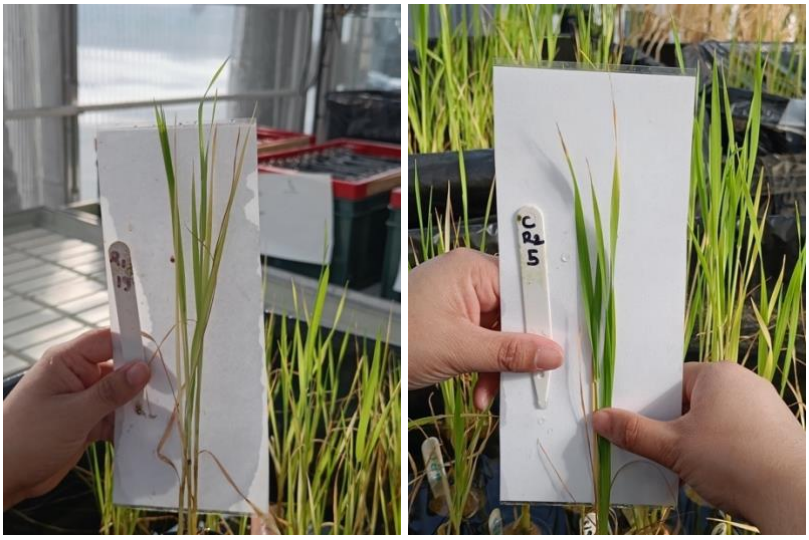


Figure 32. Genotype Dulus, most chlorotic genotype; Genotype Tjalve, least chlorotic genotype observed in 28 days of normal waterlogging.

Table 8. Results summary of ANOVA of chlorosis percentage of eighteen spring wheat genotypes from starch waterlogging taken on different dates.

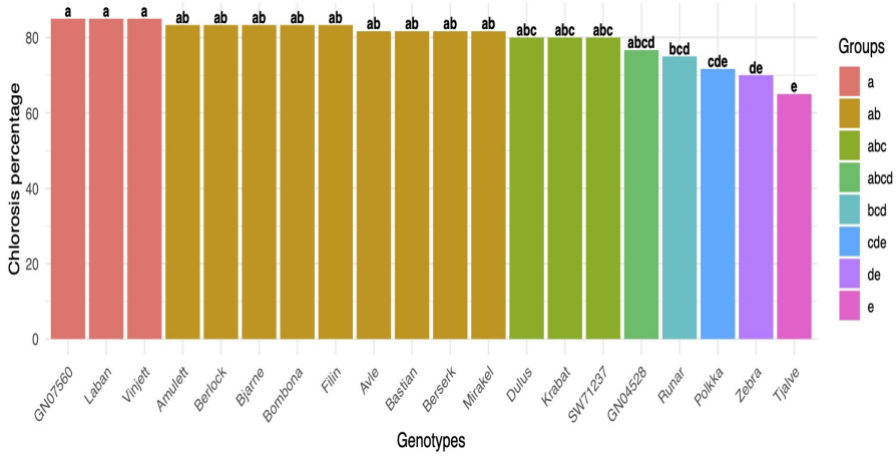
	March 9	March 11	March 14	March 18	March 20
GDf	19	19	19	19	19
GSS	1770.2	1301.4	1715.0	3817	3521
GMSS	93.17	68.49	90.26	200.88	185.33
G-F value	1.499	1.125	9.026	6.152	2.435
G-P value	0.1413	0.3670	6.51e-09 ***	1.06e-06 ***	0.00962 **
RDf	2	2	2	2	2
RSS	470.9	403.6	3.3	93	407
RMSS	235.47	201.82	1.67	46.25	203.75
R-F value	3.790	3.315	0.167	1.416	2.677
R-P value	0.0316 *	0.0471 *	0.847	0.255	0.08174
EDf	38	38	38	38	38
ESS	236.1	2313.7	380.0	1241	2893
EMSS	62.13	60.89	10.00	32.65	76.12

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '1*

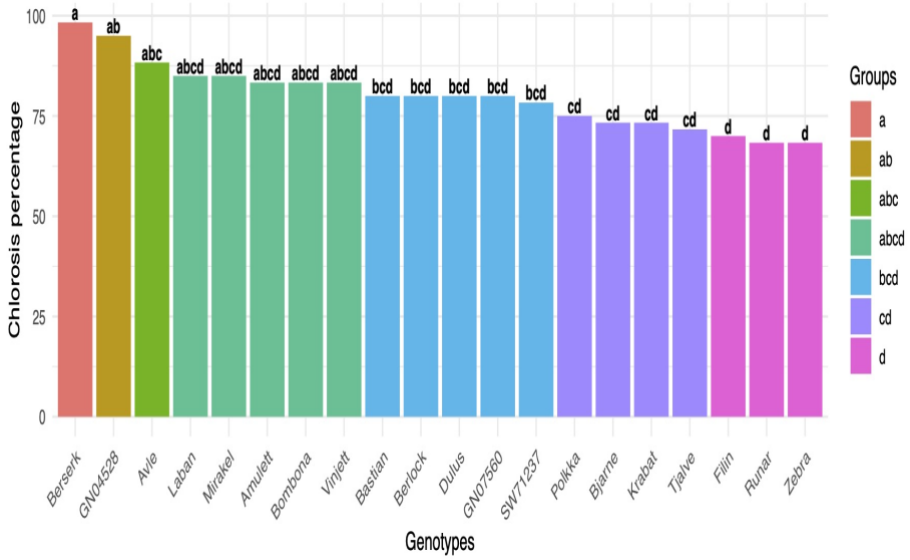
GDf: Genotype Degree of freedom, GSS- Genotype Sum of Square, GMSS- Genotype Mean Sum of Square, G-F value, F-statistics value, G-P value, P-statistics value, RDf- Replication degree of freedom, RSS- Replication Sum of Square, RMSS- Replication Mean Sum of Square, R-F value, F statistics, R-P value, P statistics, EDf- Error Degree of freedom, ESS-Error Sum of Square, EMSS-Error Mean Sum of Square. March 9, 11, 14, 18 and 20 indicate 7, 9, 12, 16 and 18 days after starch waterlogging (DASW).

Results of ANOVA showed a significant difference between genotypes under study for mean chlorosis percentage was found only after 12 days of starch waterlogging. There was a highly significant difference between genotypes under study for mean chlorosis percentage on March 14, i.e., 12 days of waterlogging, and March 18, i.e., 16 days of waterlogging ($p< 0.001$); Similarly, the high significant difference among genotypes for mean chlorosis percentage was found on March 20, i.e., 18 days after waterlogging. ($p< 0.01$) (Table 8).

A.


Chlorosis percentage
0
20
40
60
80
a a a ab ab ab ab ab ab ab ab ab abc abc abc abcd bcd cde de e
GN07560 Laban Vinjett Amulett Berlock Bjarne Bombona Filin Avle Bastian Berserk Mirakel Dulus Krabat SW71237 GN04528 Runar Polkka Zebra Tjalve
Genotypes
Groups
a
ab
abc
abcd
bcd
cde
de
e

B.


Chlorosis percentage
0
25
50
75
100
a ab abc abcd abcd abcd abcd abcd bcd bcd bcd bcd bcd cd cd cd cd d d d
Berserk GN04528 Avle Laban Mirakel Amulett Bombona Vinjett Bastian Berlock Dulus GN07560 SW71237 Polkka Bjarne Krabat Tjalve Filin Runar Zebra
Genotypes
Groups
a
ab
abc
abcd
bcd
cd
d

C.

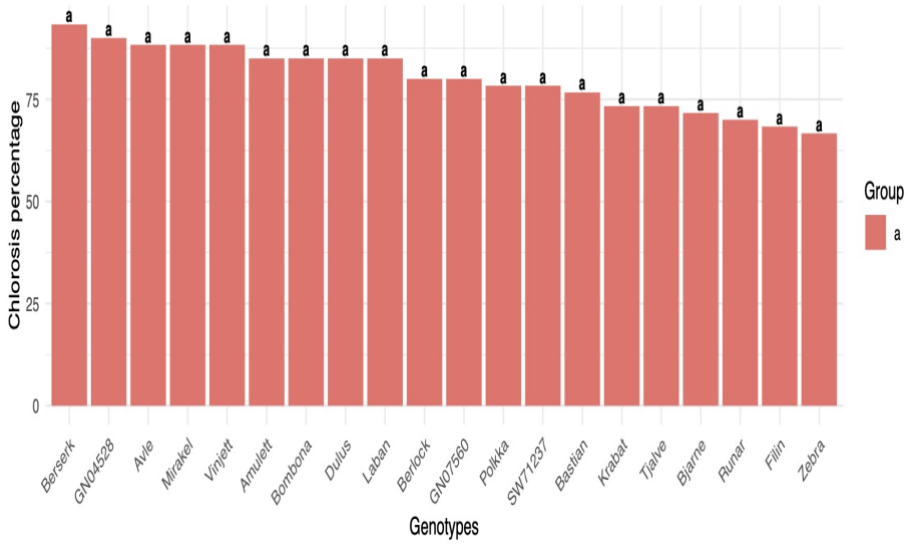

Chlorosis percentage
0
25
50
75
a a a a a a a a a a a a a a a a a a a a a a
Berserk GN04528 Avle Mirakel Vinjett Amulett Bombona Dulus Laban Berlock GN07560 Polkka SW71237 Bastian Krabat Tjalve Bjarne Runar Filin Zebra
Genotypes
Group
a

Figure 33. A) Results of Tukey HSD in bar plots on March 14; B) on March 18; C) on March 20. Genotypes are grouped according to their significant differences. Letters in top of bar diagram mark significant difference found in mean chlorosis percentage among genotypes using Tukey HSD. A, B and C denote results of data taken on March 14, 18 and 20 i.e., 12, 16 and 18 days of starch waterlogging respectively. Genotypes with at least one same letter indicate no significant difference.

Results of the Tukey HSD test showed that Laban, GN07560, and Vinjett were significantly more chlorotic than Runar, Polkaa, Zebra and Tjalve and were statistically at par with other genotypes while Tjalve was statistically at par with Polkaa and Zebra and significantly less chlorotic than other genotypes on data taken on March 14. i.e., 12 days of waterlogging (Fig 33A)

Similarly, Filis, Runar and Zebra were significantly less chlorotic than Berserk, GN04528, and Avle and statistically at par with other genotypes in data taken on March 18, i.e., 16 days of waterlogging. Berserk was significantly more chlorotic than Bastian, Berlock, Dulus, GN07560, SW71237, Polkka, Bjarne, Krabat, Tjalve, Filin, Runar and Zebra and statistically at par with other genotypes. Similarly, GN04528 was significantly more chlorotic than Polkka, Bjarne, Krabat, Tjalve, Filin, Runar and Zebra and statistically at par with other genotypes. (Fig 33B).

There was no statistically significant difference among genotypes for mean chlorosis percentage in data taken on March 20 i.e., 18 days of starch waterlogging (Fig 33C).

Results of Tukey HSD from three different dates showed that Zebra, Tjalve, and Runar were significantly less chlorotic than other genotypes under all three dates consistently, while Avle remained consistently more chlorotic under all dates. There was an inconsistent ranking of genotypes taken on three different dates. Surprisingly, Berserk and GN04528 tend to be more chlorotic in 16 and 18 days of starch waterlogging compared to 12 days of waterlogging, even though they belong to the less chlorotic haplotype (haplotype 3).

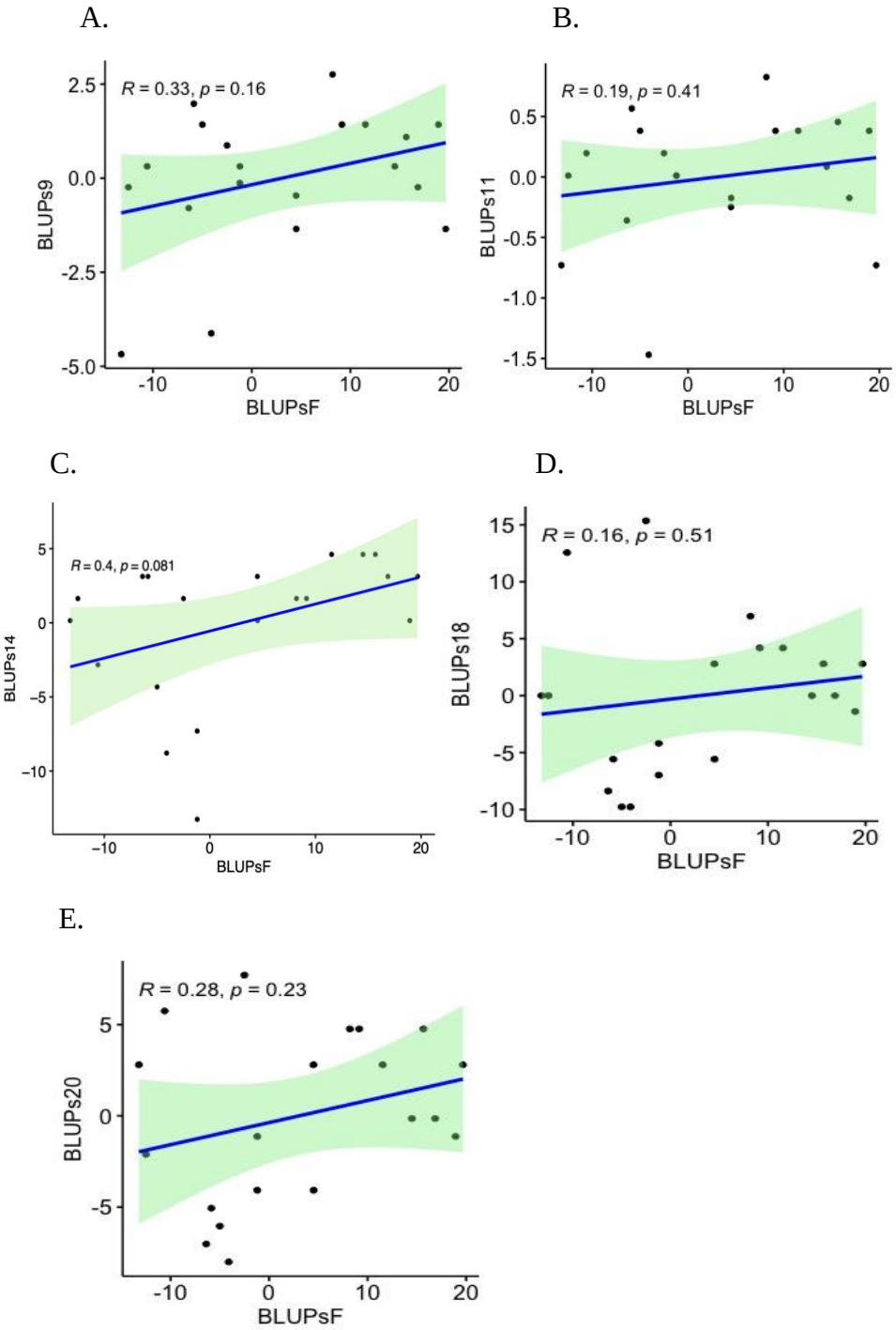
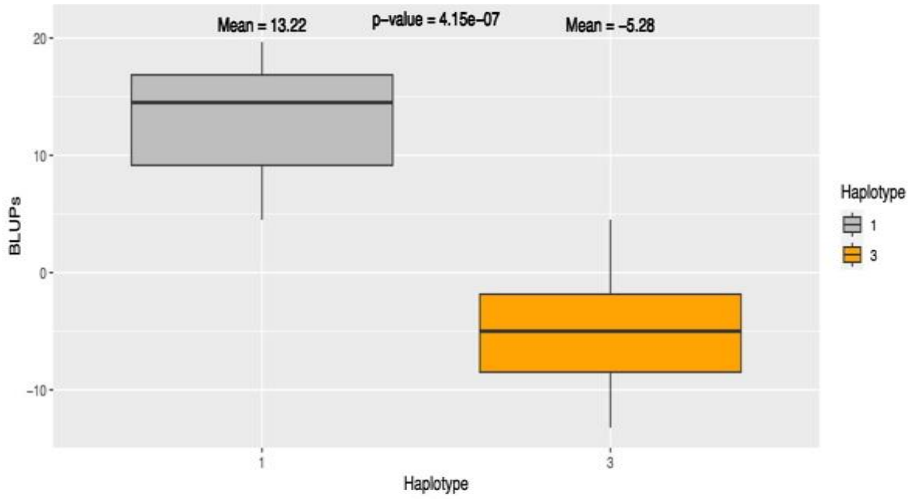


Figure 34. Pearson´s Product-Moment Correlation between the mean chlorosis percentage of twenty genotypes in field waterlogging conditions in 2014 calculated in BLUPs (BLUPsF) and mean chlorosis percentage of twenty genotypes in greenhouse starch waterlogging conditions in 2023 calculated in BLUPs (BLUPs9, BLUPs11, BLUPs14, BLUPs18, BLUPs20). A, B, C, D, and E indicate measurements on 7, 9,12, 16, and 18 days after starch waterlogging, respectively.

Results of Pearson´s Product-Moment Correlation showed that the mean chlorosis percentage of twenty genotypes in field waterlogging conditions in 2014 and the mean chlorosis

percentage of twenty genotypes in greenhouse starch waterlogging conditions at 2023A.D (BLUPs) in different dates showed different correlations. The correlation was found as (R= 0.4) on 12 days of starch waterlogging as shown in Fig 34C, followed by (R= 0.3) on 7 days of starch waterlogging as shown in Fig 34A, and then (R= 0.2) on 18 days of starch waterlogging (Fig 34E). A relatively poor correlation (R= 0.1) was obtained on 9 and 16 days of starch waterlogging (Fig 34B and 34D)

A.



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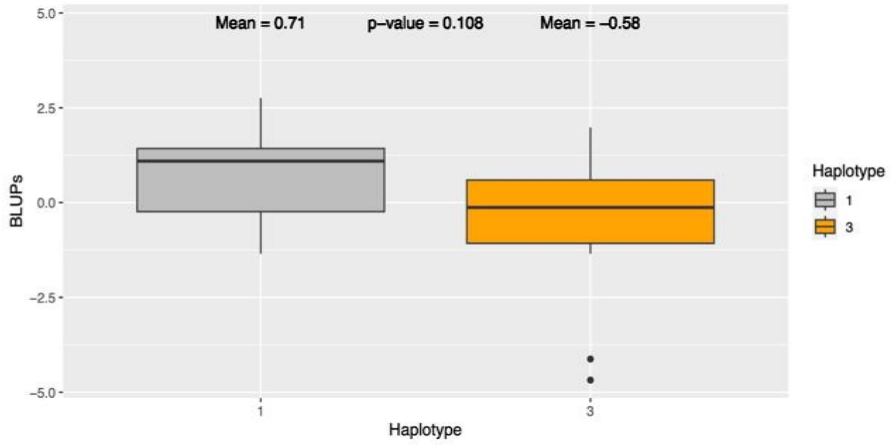
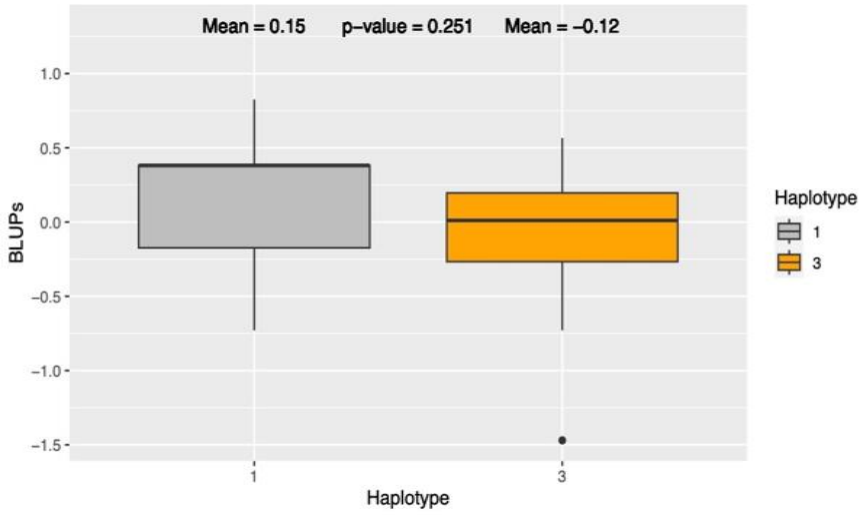
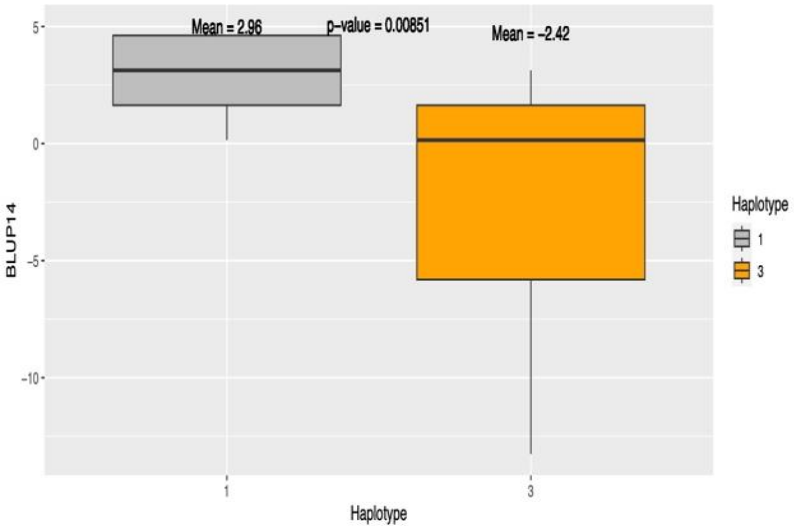


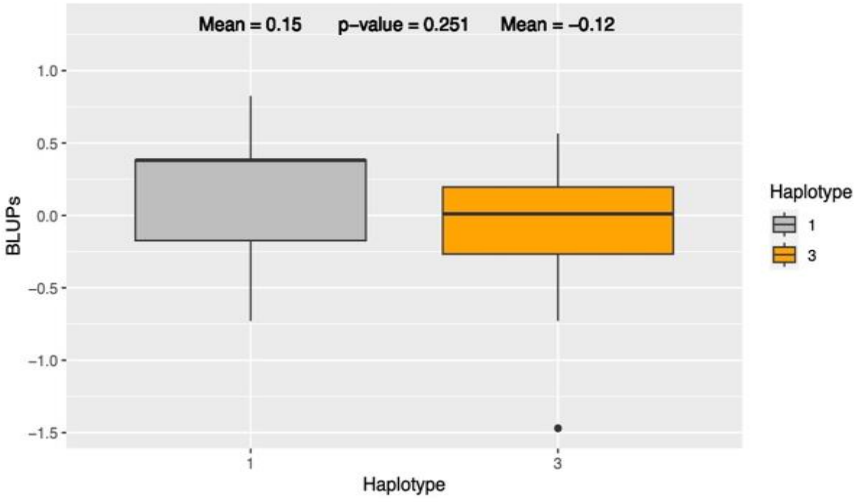
C.


Mean = 0.15
p−value = 0.251
Mean = −0.12
BLUPs
Haplotype
1
3

D.


Mean = 2.96
p−value = 0.00851
Mean = −2.42
BLUP14
Haplotype
1
3

E.


Mean = 0.15
p−value = 0.251
Mean = −0.12
BLUPs
Haplotype
1
3

F.

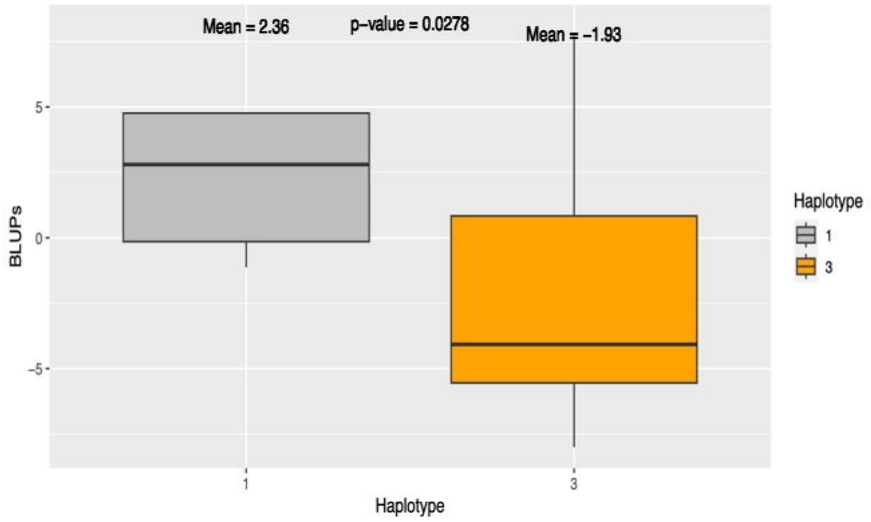


Figure 35. Results of Welch Two sample t-test among two QTL6A.2 haplotype groups of genotypes. A, B, C, D, E, and F denote data taken on field trials in 2014, 7, 9, 12, 16, and 18 days of starch waterlogging 2023 respectively. P-value at mid of two box plots in each figure indicates the significance level. P < 0.05 indicate a significant difference, and P >0.05 indicate no significant difference among the two haplotype groups for mean chlorosis percentage.

The results of Welch's two-sample t-test showed that there was significant difference for mean chlorosis percentage among two contrasting haplotypes in field waterlogging experiment (Fig 35A). In case of starch waterlogging significant difference among haplotype groups for mean chlorosis percentage was obtained 12 days after starch waterlogging and 18 days after starch waterlogging ($p < 0.05$) (Fig 35D and 35F). Interestingly, the analysis showed non-significant differences among the two contrasting haplotype groups on other dates (Fig 35B, 35C and 35E).

Table 9. Results summary of ANOVA of chlorosis percentage of eighteen spring wheat genotypes from normal waterlogging.

	March 18	March 24	March 30
GDf	19	19	19
GSS	8177	11476	5853
GMSS	430.4	604.0	308.07
G-F value	1.970	3.507	2.361
G-P value	0.0426 *	0.000706***	0.0148 *
RDf	2	2	2

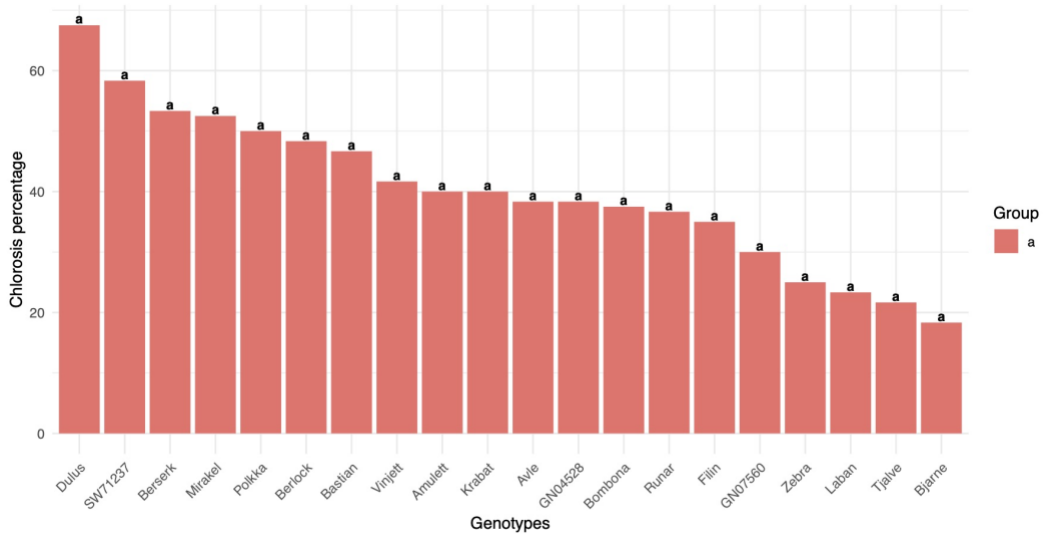
RSS	111	506	111
RMSS	55.7	252.9	55.29
R-F value	0.255	1.468	0.424
R-P value	0.7764	0.244560	0.6581
EDf	33	34	33
ESS	7209	5857	4306
EMSS	218.5	172.3	130.49

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '1*

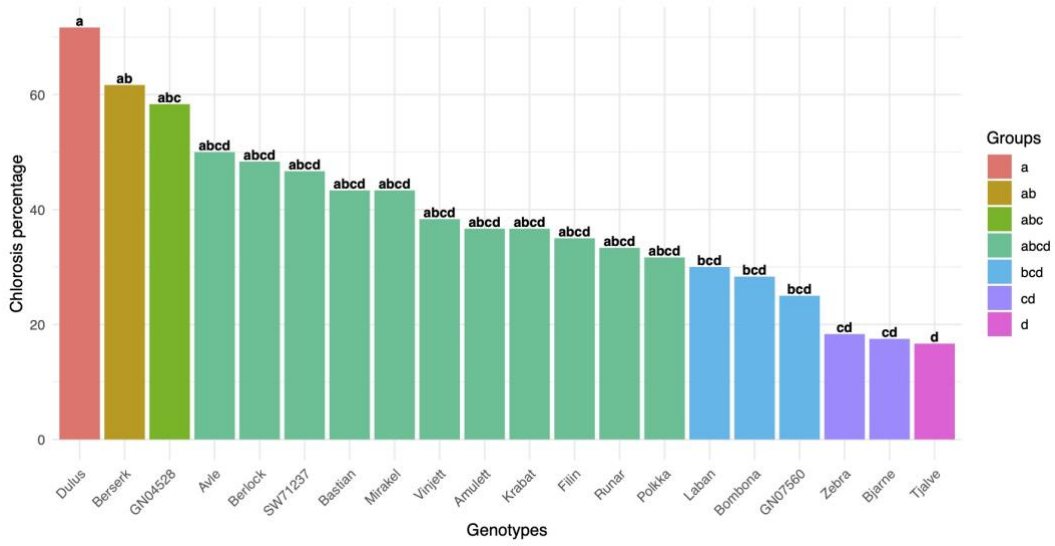
GDf: Genotype Degree of freedom, GSS- Genotype Sum of Square, GMSS- Genotype Mean Sum of Square, G-F value, F-statistics value, G-P value, P-statistics value, RDf- Replication degree of freedom, RSS- Replication Sum of Square, RMSS- Replication Mean Sum of Square, R-F value, F statistics, R-P value, P statistics, EDf- Error Degree of freedom, ESS-Error Sum of Square, EMSS-Error Mean Sum of Square. March 18, 24 and 30 indicate 16, 22 and 28 days of normal waterlogging.

Results of ANOVA showed that there was significant difference among genotypes for mean chlorosis percentage under normal waterlogging on 16 days ($p < 0.05$) of normal waterlogging while high significant difference on 22 days ($p < 0.001$) and significant difference in ($p < 0.05$) 28 days of normal waterlogging respectively (Table 9).

A.



B.



C.

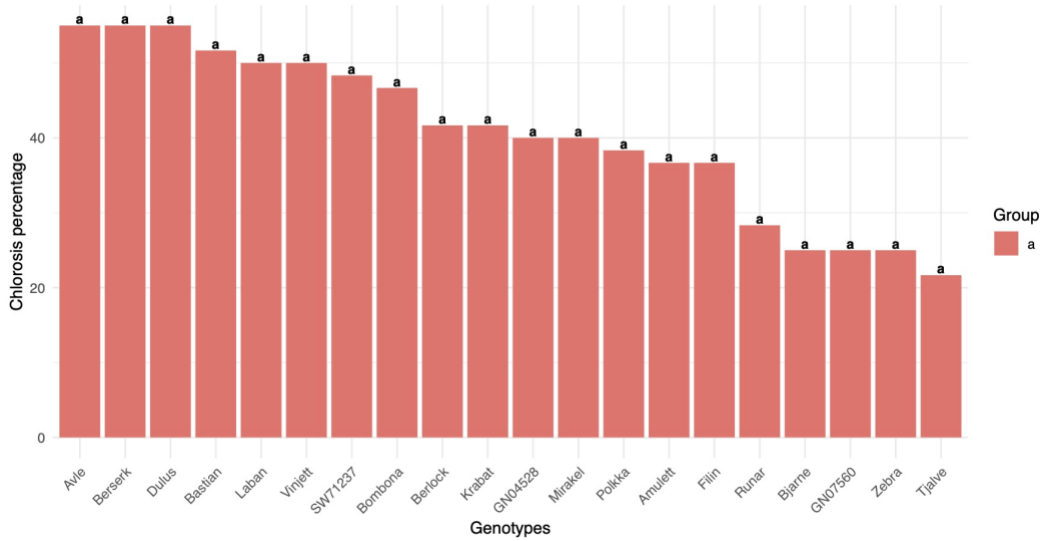


Figure 36. Results of Tukey HSD in bar plots. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in mean chlorosis percentage among genotypes using Tukey HSD. A, B, and C denote the results of data taken on 16, 22, and 28 days of normal waterlogging, respectively.

Results of Tukey HSD showed there was no significant difference among genotypes for mean chlorosis percentage on data taken on 16 and 28 days of waterlogging (Fig 36A and 36C). Significant difference among genotypes for mean chlorosis percentage was found only on 22 days of waterlogging (Fig 36B). Dulus was found to be significantly more chlorotic than Laban, Bombona, GN07580, Zebra, Bjarne, and Tjalve and statistically at par with other genotypes. Berserk was found to be significantly chlorotic to Zebra, Bjarne, and Tjalve and statistically at par with other genotypes. Dulus and Berserk consistently exhibited higher chlorosis levels,

whereas Tjalve, Zebra, Bjarne, and GN07560 consistently showed lower chlorosis levels across all three measurements. It is worth noting that the ranking of genotypes based on chlorosis percentage was not consistent across the three measurements (Fig 36A, 36B and 36C)

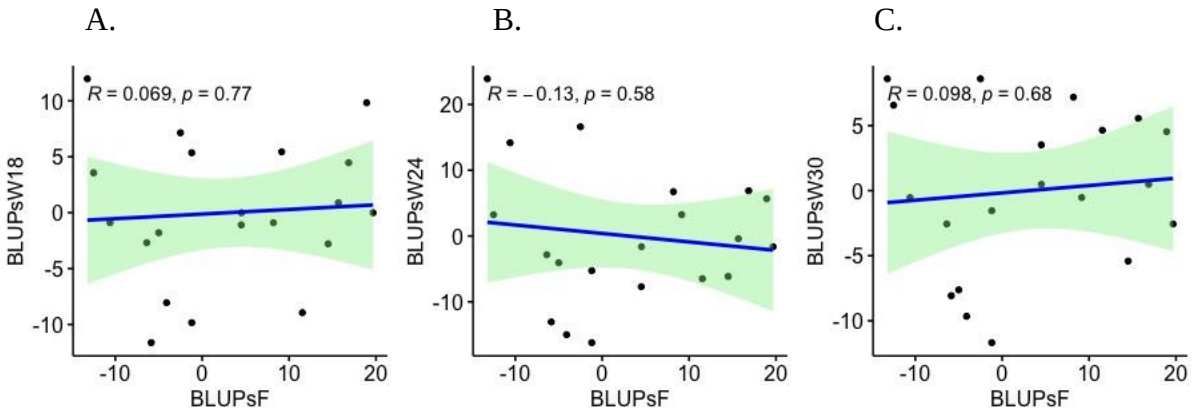
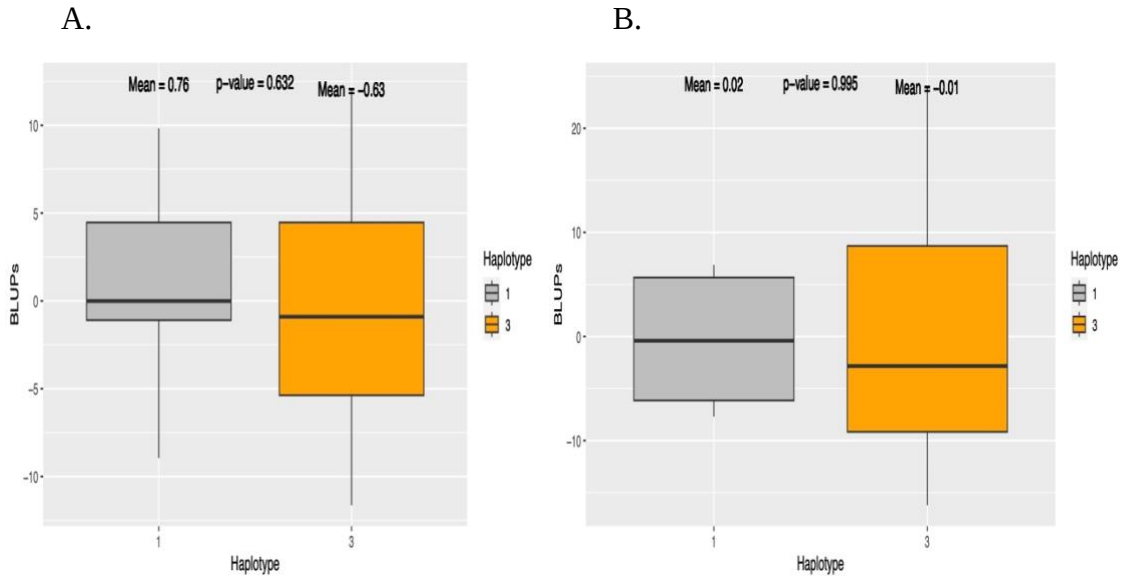


Figure 37. Pearson´s Product-Moment Correlation between the mean chlorosis percentage of twenty genotypes in field waterlogging conditions in 2014 calculated in BLUPs (BLUPsF) and the mean chlorosis percentage of twenty genotypes in greenhouse normal waterlogging conditions in 2023 measured in BLUPs (BLUPsW18, BLUPs24, BLUPs30). BLUPsW18, BLUPs24, and BLUPs30 indicate measurements taken on March 18, March 24, and March 30, 2023, i.e., 16 days, 22 days, and 28 days of waterlogging, respectively.

The correlation between field and greenhouse normal waterlogging mean chlorosis percentage (BLUPs) was found as high as (R= 0.09) on 28 days of waterlogging (Fig 37C) which is a very low correlation. A negative correlation was found on 22 days of waterlogging (R= -0.13) (Fig 37B) The results of the correlation across three measurements were insignificant (Fig 37).



C.

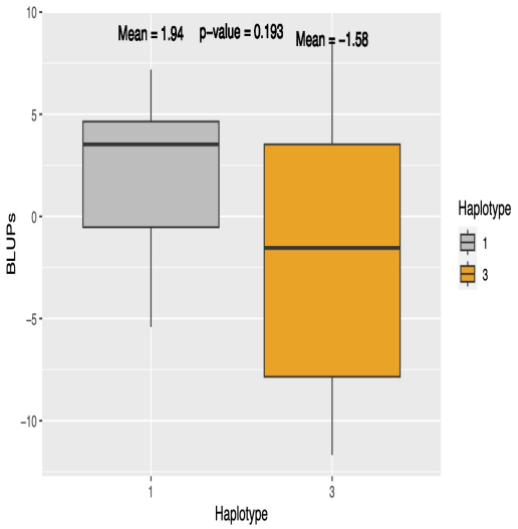


Figure 38. Results of Welch´s two-sample t-test among two QTL 6A.2 haplotype groups of genotypes on normal waterlogging. A, B, and C denote data taken on March 18, 24, and 30, i.e., 16, 22, and 28 days of normal waterlogging.

Results of Welch´s two-sample t-test showed there was no significant difference between the two haplotypes group for mean chlorosis percentage across all measurements ($p>0.05$) under normal waterlogging (Fig 38A, 38B and 38C).

Table 10. Summary of ANOVA table for regrowth studies on 14 and 21 days after removal of starch waterlogging.

	Regrowth on 14 days	Regrowth on 21 days
GDf	19	19
GSS	127.52	203.60
GMSS	6.711	10.716
G-F value	0.927	1.82
G-P value	0.557	0.146
RDf	2	2
RSS	18.23	5.63
RMSS	18.225	5.625
R-F value	2.517	0.779
R-P value	0.121	0.383
EDf	38	38
ESS	282.44	281.71

EMSS	7.242	7.223

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '1*

GDf: Genotype Degree of freedom, GSS- Genotype Sum of Square, GMSS- Genotype Mean Sum of Square, G-F value, F-statistics value, G-P value, P-statistics value, RDf- Replication degree of freedom, RSS- Replication Sum of Square, RMSS- Replication Mean Sum of Square, R-F value, F statistics, R-P value, P statistics, EDf- Error Degree of freedom, ESS-Error Sum of Square, EMSS-Error Mean Sum of Square.

A. B. C.

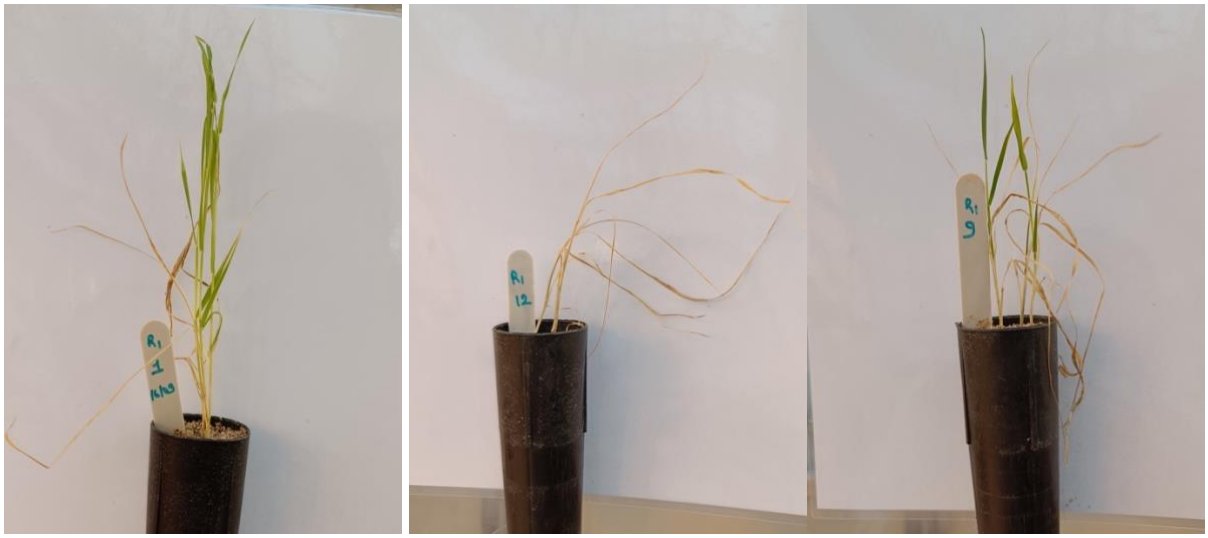
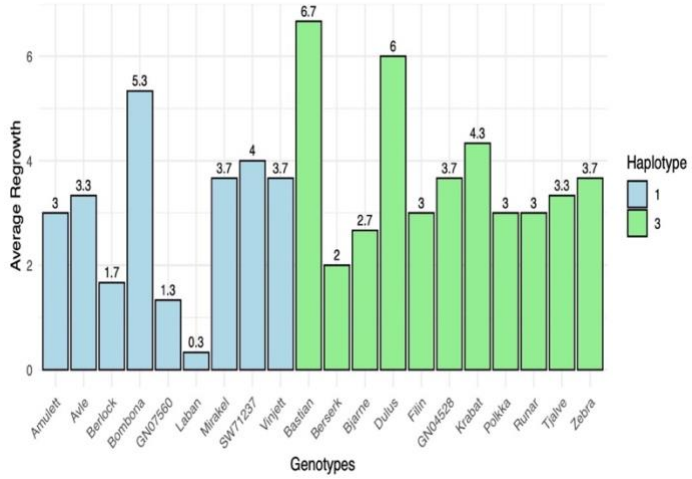


Figure 39. Representative figure of regrowth after 21 days of removal of starch waterlogging. A) Genotype Bastian with the largest regrowth scale; B) Genotype Laban with smallest regrowth scale; C. Genotype Bombona with relatively larger growth scale among genotypes from haplotype 1.

A.



B.

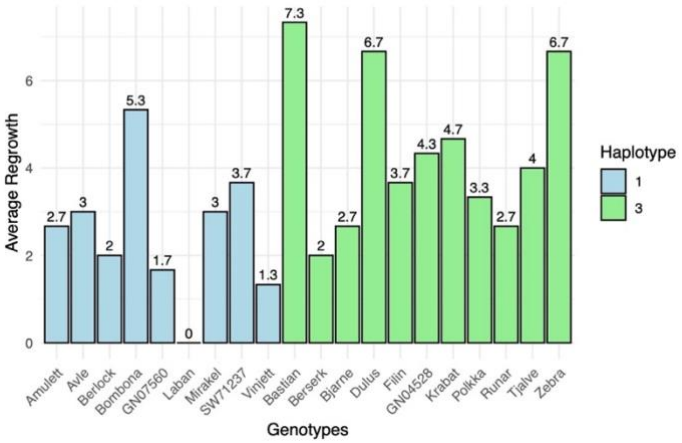


Figure 40. A) Average regrowth scale measured after 14 days; B) after 21 days of removal of starch waterlogging in twenty genotypes.

Results of ANOVA showed that there was no significant difference among studied genotypes for their average regrowth in fourteen days and twenty-one days after removal of starch waterlogging. The bar plot of the average regrowth of genotypes in fourteen days after removal of waterlogging showed that Bastain exhibited the highest regrowth value, followed by Dulus and Krabat, whereas Berserk had lowest regrowth value among genotypes under haplotype 3 (Fig 40A).

In the case of genotypes from Haplotype 1, Bombona displayed the highest regrowth value, followed by SW71237, while Laban had the lowest regrowth value. Remarkably, despite belonging to haplotype 1, Bombona´s regrowth was higher than many genotypes within haplotype 3 (Fig 40A). Similarly, the regrowth value of SW71237 and Vinjett was also higher than that of some of the genotypes within haplotype 3 (Fig 40A).

In twenty-one days after the removal of waterlogging, Bastian continued to have robust re-growth, followed by Dulus and Zebra, whereas Berserk continued to be poorest in regrowth, followed by Runar among genotypes under haplotype 3. Notably, regrowth increased in the case of Filis, GNO4528, Krabat, Polkka, Tjalve, and Zebra compared to the previous date, with Zebra exhibiting the highest regrowth among them. In the case of genotypes of haplotype 1, Bombona continued to be robust in regrowth, followed by SW71237. Laban was completely collapsed by this time, while regrowth of Vinjett decreased compared to the previous date (Fig 40B).

3.4.16 Discussion:

The results from soil redox potential (Eh) measurements indicate that the hypoxic effect induced by starch waterlogging is stronger and quicker compared to normal waterlogging. While it took three additional days for normal waterlogging to reach negative Eh values, plants in starch waterlogging were already experiencing high hypoxic conditions, with Eh values beyond -500 mV. The two different waterlogging methods in the greenhouse had varying effects on the plants, and they responded differently under these conditions.

In starch waterlogging conditions, symptoms of chlorosis began to appear within four days and were visually distinguishable within seven days of waterlogging. Apart from a few exceptions like Berserk and GN04528, almost all other genotypes showed almost similar chlorosis behavior as shown in field conditions across all measurements. On the other hand, it took ten days for plants in normal waterlogging to develop visible chlorosis, and it became visually distinguishable among genotypes only after 16 days of waterlogging; all genotypes in normal waterlogging conditions produced completely unfolded 3rd leaves within one week and started producing 4th leaves, while plants in starch waterlogging had just begun producing 3rd leaves by that time. The growth of plants in normal waterlogging conditions continued, with the production of newer leaves throughout the experiment. In contrast, plants in starch waterlogging experienced high growth reduction compared to plants under normal waterlogging.

The hypoxic condition under normal waterlogging condition was confirmed by soil redox values (Eh) measured in 3rd experiment as the Eh value reached as low as -300 mV, and it remained below -100 mV throughout the experiment. The measured Eh value is a strong testament to believe that a hypoxic condition was created in normal water, but plants took a longer time, i.e., two weeks to develop chlorosis level to be visually distinguishable among genotypes. Moreover, plants continued to grow and produce newer leaves and tillering, which is normally not expected in waterlogging-stressed plants. This could be due to the plants' faster adaptation to the hypoxic conditions conferred by normal waterlogging. Plants' response showed that this stress condition was comparatively smaller for plants even though chlorosis symptoms were evident. In plots with very less plant density (3 plants/plot), newer leaves made

chlorosis assessment difficult. Additionally, plants in this method don´t respond similarly to field conditions which makes the correlation between field and greenhouse waterlogging consistently poor across all measurements, it reached as high as (R=0.1), which is still a very low correlation and, in some cases, even negative correlation (R= -0.1). The ranking of genotypes across three different experiments was also inconsistent. Some genotypes like Dulus, Berserk, and GN04528 were more chlorotic, and genotypes like GN07560 were less chlorotic, which was completely opposite of their conditions in field waterlogging, while other genotypes behaved quite similarly to field conditions. Such differential behavior of some plants in two different waterlogging settings i.e. in field and greenhouse waterlogging conditions, can be because reducing conditions created through normal waterlogging in the greenhouse is a slow process and some genotypes might get enough time to sense waterlogging stress and convert their mechanisms favorable for their growth and become less chlorotic, those plants which could not sense waterlogging conditions unless it´s severe they seemed to be more chlorotic. This verifies that each genotype exhibited different responses to waterlogging conditions based on severity, and their pace of tolerance varied. The duration of waterlogging also played a crucial role in the tolerance mechanisms shown by some genotypes. Some genotypes initially displayed a stronger chlorotic phenotype but later became less chlorotic and more robust. For example, Bastian appeared to be more chlorotic in the initial phase but later showed reduced chlorosis and better regrowth.

In the case of starch waterlogging, soil redox values dropped significantly to below -400 mV within three days of waterlogging reached as low as > -500 mV and remained below -200 mV throughout the measurements. There was a fluctuation of Eh values throughout the experiment. As the Eh value is highly temperature dependent, and fluctuation in daily temperature also might have played role in the fluctuation of Eh. A similar trend was also found in another study done by Byrne et al. (2022). The actual reason behind the fluctuation of Eh values is not clearly understood. However, Eh values below -200 mV are strong evidence to believe that a hypoxic condition was created in the starch waterlogging method. However, there was an inconsistent ranking of genotypes across all measurements. This might be because of the genotype´s individual pace of tolerance mechanisms. Some genotypes though seemed to be chlorotic initially are more robust in later phases in this method as well e.g., Bastian. The correlation between field and starch waterlogging chlorosis decreased over time, likely due to prolonged exposure to severe waterlogging conditions. Correlation results were also not consistent across all measurements. Furthermore, significant differences between the two haplotype groups were

not observed across all measurements. This showed that the timing of assessment plays a crucial role in precisely capturing differential chlorosis among genotypes, especially under greenhouse conditions. As the severity of waterlogging conditions depends upon the duration of waterlogging, too longer duration of severe stress might be detrimental to tolerant genotypes too, such that differences in the tolerance level of tolerant and sensitive genotypes significantly reduced, and all genotypes might seem to be chlorotic, and this results in the false assessment of tolerance level. Early assessment can also be not true as some plants take a longer time to show their tolerance level even though initially, they are observed relatively more chlorotic than others. So, optimization of timing for chlorosis percentage assessment would be important while using methods like starch waterlogging that produce severe stress in a short time.

In the case of our study, two assessment timings were found to be better at simulating field conditions. One is the first assessment immediately after the development of the visually distinguishable chlorotic symptom, where the correlation observed was relatively higher (R=0.33), i.e., 7 days of starch waterlogging. The second assessment, 12 days of starch waterlogging, was found suitable time to as a stronger correlation (R=0.4) was observed. However, one must be careful when plants are developing new leaves, as that might obscure chlorosis percentage assessment. Timing can be relatively different while using different genotypes. Nevertheless, it is wise to access chlorosis a few days later of newly formed leaves. This consideration might not be necessary in plots with a higher number of plants but would be very important in plots with few (2-3 plants/plot) as it obscures the assessment to a larger extent and sometimes can be misleading as assessing chlorosis percentage is quite subjective. Correlation dropped down to (R=0.1) on 16 days and (R=0.2) on 18 days of waterlogging; one of the reasons behind this might be because of high chlorosis observed on Berserk and GN04528 as these genotypes were found to be unexpectedly more chlorotic compared to field conditions. Moreover, the duration of waterlogging also might have played a role in decreased correlation as plant tolerance decrease with increased duration of waterlogging.

Even though a reduced environment was created in both normal and starch waterlogging conditions, the differential response of plant genotypes under two different methods showed that very strong/ severe hypoxic conditions must be created within a short time inside greenhouse conditions to simulate waterlogging conditions as expected in the field. As, in the field, microorganisms rapidly utilize trapped oxygen, and oxygen depletion rapidly occurs but in normal greenhouse waterlogging, oxygen depletion takes time and by that time, some plants

can sense waterlogging conditions and adapt, this might be the reason they seem to be less chlorotic even though they were found more chlorotic under field waterlogging condition. But when stress is severe, then chlorotic phenotypes of tolerant and sensitive genotypes become more distinct and show fairly like the response they show in field conditions. This might be the reason plants under normal waterlogging behaved differently than field waterlogging conditions and plants under starch waterlogging behaved fairly like field waterlogging conditions.

Correlation obtained in starch waterlogging is relatively higher (R=0.4). Significant (R> 0.5) correlation might be difficult to obtain because there are various other environmental factors like temperature, field waterlogging conditions, other stress like mineral toxicities, different soil environment that affect waterlogging conditions, it´s severity and plants response to it. Simulating absolute field waterlogging conditions seems to be practically impossible. However, we strongly believe that given the stress conditions is strong in short period of time, differential tolerance among tested genotypes can be captured effectively which is likely to be same behavior observed in field.

Additionally, a significant difference among two contrasting haplotypes on *QTL6A.2* obtained in chlorosis in starch waterlogging make further clear that, this method has applicability to use as an alternative method to field waterlogging for validation of this QTL.

Sundgren (2018) concluded that chlorosis percentage might not be a strong indicator to distinguish between tolerant and sensitive genotypes if regrowth conditions are not considered. She found some genotypes that were more chlorotic and recovered well after the removal of waterlogging. In our study, we found no significant difference in regrowth conditions between the two haplotype groups. This might be because the waterlogging stress was applied for a longer duration, and the difference in regrowth ability between the genotypes decreased, or it could indicate that some genotypes, which were more chlorotic initially, had higher regrowth ability. Interestingly, Berserk consistently showed poor regrowth as well. The consistently poor results of this genotype across all experiments were intriguing. This finding is in line with Tove´s fieldwork results, where this specific genotype showed poor recovery (Sundgren, 2018) Understanding the response physiology of this genotype would be of great interest in future experiments. Even though we could not capture significant differences among genotypes for their regrowth ability, regrowth is an important parameter to be considered while assessing

tolerance level in studied genotypes. Regrowth analysis revealed that some genotypes, like Bombona which belong to more chlorotic group (Haplotype 1) had stronger regrowth than many of the genotypes belonging to less chlorotic group (Haplotype 3), which also confirm that regrowth is important parameter to be considered in screening trials.

3.4.17 Conclusion:

Based on our experiments and the results we obtained, we conclude that normal waterlogging produces a reducing environment slower than starch waterlogging. Plants behave differently under normal waterlogging than field waterlogging because of slow and less severe stress. While in starch waterlogging, a strong reducing environment is created in a short period of time, plants respond fairly like field waterlogging in fast severe stress. In the starch waterlogging method, a maximum of 12-14 days of waterlogging will be enough to precisely access the difference in chlorosis percentage among tested wheat genotypes, and additional 2-3 weeks of the recovery period will be enough to access their growth after recovery. Assessment of chlorosis along with recovery will give a better picture of tolerance levels in genotypes under waterlogging stress. Overall, the starch greenhouse waterlogging conditions is found to be a better simulating method of field waterlogging conditions, as it not only better replicates field conditions but is also more time-efficient compared to normal waterlogging.

4 Chapter B: Development of low-cost and easy seminal roots phenotyping method in greenhouse

4.1 Introduction

4.1.1 Why are studies on Root System Architecture important?

The first green revolution was introduced in the mid-1960s through the introgression of dwarfing genes *Rht-B1b* and *Rht-D1b* in commercial wheat cultivars by Dr. Norman E. Borlaug. Introduced dwarf wheat varieties were high yielding and highly responsive to chemical fertilizers and pesticides. This causes a substantial increase in wheat grain yield and significantly reduces famine in developing countries (Hedden, 2003). There was a 208% yield increase in wheat between 1960 and 2000 in developing countries (Prabhu, 2022). This gain in

wheat yield was attributed to optimal growing conditions. Soil degradation, unpredictable climate change, harmful impacts of fertilizers and pesticides, shrinkage of wheat production land, etc., have been evident in recent years. Moreover, the global human population is estimated to reach 10 billion by 2050 A.D., which demands annual genetic gain of wheat yield increase from 1% to 1.7% (Tadesse et al., 2019). This has pressed the need for an increase in wheat productivity under sub-optimal climate and soil conditions (Atkinson et al., 2019). Therefore, scientists today have brought up the concept of a second green revolution to develop wheat crops with enhanced water and nutrient uptake efficiency (Lynch, 2022). Roots are vital organs for nutrient and water absorption from soil and have a significant impact on shoot development and, consequently, yield. Therefore, improving root traits for crop improvement has been a critical target in the concept of the second green revolution (Malamy, 2005). Along with water and nutrient uptake, roots are essential organs to provide anchorage and mechanical support to crops, store metabolites, and have an essential role in plant–microbe interactions (Takahashi & Pradal, 2021). Roots are also the primary plant organs affected by abiotic stress like waterlogging, drought, nutrient deficiency, minerals toxicity, and salinity problems in plants (Chen et al., 2020). They are the first plant structures to sense and respond to these stressors preceding any impact on and response from above-ground plant parts (Rebored & Henriques 1991). Root System Architecture (RSA) largely determines the functions of roots. RSA is defined as the spatial configuration of roots along the soil profile (Lynch, 1995; Pandey & Bennett, 2019), and the shape of the RSA is determined by the length, branching, angle, and thickness of roots. Various traits characterize RSA, such as rooting depth, root growth angle, root length and density, root area and volume, root distribution, root hair, etc. (Germon et al., 2020). RSA plays a significant role when plants are subjected to sub-optimal conditions like drought, waterlogging, salinity, nutrient deficiency, etc. (Ludlow & Muchow, 1990; Paez-Garcia et al., 2015) in addition to the normal functioning of the root, and it directly affects grain yield (Smith & De Smet, 2012). RSA is highly plastic in response to these sub-optimal conditions and provides ample sources of natural variations among plants. This provides avenues to identify beneficial root traits to improve crop productions (Kano et al., 2011).

However, the phenotyping of roots to study RSA has largely lagged as roots are hidden inside the soil, firmly attached to it, and have complex structure (Delory et al., 2022). Moreover, studying root systems in a field without destroying large or few parts of roots has been complex for many years. Also, it has been practically impossible to define perfect RSA as RSA highly varies with environmental conditions. However, understanding RSA has been the continuous

effort of scientists for many years through various root phenotyping methods (McCormack et al., 2017). Various field-based and lab-based root phenotyping methodologies have been established and used. However, no method is established as a standard method as each method has its merits and demerits associated with it (McGrail et al., 2020).

4.1.2 Root phenotyping methods:

Various field-based methods, like trenching, soil core, mesh bag, shovelomics, monolith, etc., have been used to study root phenotype (Li et al., 2022). The trenching method is the earliest root phenotyping method that includes digging out the soil at a certain depth and then washing roots to study root traits used by Weaver et al. (1922). This method, however, is low throughout, labor intensive and time demanding, and unsuitable for large-scale root studies (Takahashi & Pradal, 2021). Similarly, the soil core method includes a soil core of a certain length (1-2 cm) that is taken out through cylinders of specific diameters, and then extracted soil is rinsed to collect root structures and study root distributions (Kücke et al., 1995). This method covers only a certain portion of the root, and estimation of the whole root system is not possible (Takahashi & Pradal, 2021). The mesh bag method involves digging out the soil at a certain depth and keeping it in it, growing plants by filling the soil, removing the mesh bag, and washing out roots to study root components. Through this method, root systems of an earlier stage of crop growth are possible to study; however, collecting mesh bag without destruction in the later phase of crop growth is difficult, which make this method suitable only for the earlier growth phase of crops (Steen, 1991). The monolith method involves the insertion of boxes or cylinders of larger diameter with the bottom end open. However, this method is labor-intensive and time-consuming as the insertion of the monolith is difficult (Wu & Guo, 2014). The Shovelomics method includes removing the first 20 cm of root materials through excavation, washing them out, and imaging them. This method made high throughput field phenotyping possible to a larger extent and has widely been used to study RSA of crops like rapeseed, cowpea, canola, common beans, etc. (Trachsel et al., 2011). This method, however, allows only partial roots studies; deep roots and lateral roots get missed out during excavation. These field-based methods have been improved recently. However, they all are destructive methods that cause the loss of certain root structures like root hairs or lateral roots. They are also time-demanding and labor-intensive (Li et al., 2022). Some non-destructive field-based methods like minirhizotron with attached video cameras (Johnson et al., 2001) have been

developed. This method, however, captures images of a certain portion of roots but cannot show the entire root system (Takahashi & Pradal, 2021).

Some soil-less root phenotyping methods inside controlled conditions have also been used, like the use of hydroponics, aeroponics, agar-gel-based method, pouch-wick method, etc. These methods are normally done inside controlled environments or labs. Aeroponics systems include using an incubator, water pump, and compressor, where nutrients and air pressure can be controlled (Carter, 1942). In a hydroponics system, plants are grown in a nutrient medium, where root studies are easy and non-destructive compared to soil, but this method is feasible only for the early stage of plant growth (Li et al., 2022). Pouch and wick systems include the study of root features based on germination paper, which is also only feasible for root studies of seedlings (Hund et al., 2009). Agar-gel based root phenotyping system includes two flat layers with agar-gel between their space, where seedlings are grown, and roots traits are captured by a flatbed scanner; this method is also only feasible to study root traits of seedlings because of its limited support capacity and limited nutrient capacity of agar-gel used inside it (Bengough et al., 2004). Soil-less root phenotyping methods are non-invasive, high throughput, time, and labor efficient. A major limitation of these systems is that they cannot completely represent the root systems of plants grown in actual fields and are suitable only at the seedling stage of plants (Kuijken et al., 2015).

There are some soil-based root phenotyping methods where plants are grown in containers, boxes, or rhizotrons with transparent sides, and root traits are captured through in-situ image acquisition. These methods better simulate field conditions than soil-less methods, but they limit actual RSA development because of the limited size of containers/boxes/ rhizotrons used. For example, Rhizopots can make in-situ observations of root morphology and the life span of root hairs. This method is high resolution but low throughput (Xiao et al., 2020). Another soil-based method developed is GROWSCREENRhizo, where plants are grown in rhizotrons of specific dimensions with transparent planes where root images are captured at the rate of 60 rhizotrons/hour (Pfeifera et al., 2012). There are other soil-based root phenotyping systems developed, like Growth and Luminescence Observatory for Roots (GLO-Roots) (Rellán-Álvarez et al., 2015), that grow plants in soil-filled vessels/rhizotrons which comprise luminescent reporters and imaging systems along with image analysis platform for RSA studies in-situ of model plant species *Arabidopsis thaliana* L., GLO-Bot (LaRue et al., 2022) which has a robotic platform that capture root growth from germination to adult stage through the

automated imaging system of *A. thaliana* L. in a soil-like environment. These two phenotyping methods capture root images throughout a plant's life and provide a large set of RSA trait data. Nevertheless, a significant demerit of these methods is that data understanding and analysis are very complex in nature (Li et al., 2022).

Many other new methods offer in situ non-destructive 3D root phenotyping like X-ray computed tomography (Hou et al., 2021), magnetic resonance imaging (Pflugfelder et al., 2022), ground penetrating radar (Zhang et al., 2019), electrical capacitance (Schierholt et al., 2019), electrical impedance tomography (Corona-Lopez et al., 2019), neutron tomography (Krzyzaniak et al., 2021) been developed in recent years. However, these phenotyping technologies require corresponding software to analyze captured data (Li et al., 2022). These phenotyping methods are beneficial as they can help visualize the development of a complete root system in natural soil conditions. For instance, these root phenotyping platforms and software associated with image analysis are in the infant stage and will require more time and research; they also must be low-cost for their large-scale utilization to screen RSA in large populations at high throughput and high-frequency (Li et al., 2022).

Root researchers can select root phenotyping methods based on the root traits of their interest. Some root traits are proxy. Based on studies on such proxy traits, researchers can make sound predictions on the overall root architecture of mature plants. Traits that can be easily phenotyped, have good heritability and are highly correlated to grain yield under given environmental conditions are useful indicators while phenotyping a large number of populations in root studies. For example, studies on seminal roots are done to compare the root systems of mature plants. Roots phenotyping methods either soilless methods like gel-based method (Bengough et al., 2004), germination paper-roll method (Watt et al., 2013), growth pouch method (Hund et al., 2009) or soil-based clear pot method (Richard et al., 2015), are found to be used by many root researchers to study seminal roots in crops like wheat (Bai et al., 2013), maize (Tuberosa, R. et al., 2002) as seminal roots can be easily phenotyped using these methods at earlier stage of crop growth. These phenotyping methods are proven to be high-throughput, with high reproducibility, low-cost, time-efficient method to study seminal root traits under controlled conditions (Alahmad et al., 2019).

4.1.3 Wheat Root System:

The root system of wheat comprises two types of roots: seminal and adventitious. Seminal roots, originating from the seed, are embryonic roots, while adventitious roots, also known as crown or nodal roots, emerge from the crown region of the plant (Klepper et al., 1984). Seminal roots comprise one primary root, two pairs of lateral seminal roots on each side, and also occasionally a sixth root known as the central root (Boudiar et al., 2020). The first pair of seminal lateral roots appear after 1-4 days of seed imbibition, while the second pair emerge after 5-9 days of seed imbibition (Hohn & Bektas, 2020). Seminal roots are the first ones to penetrate the soil profile (Watt et al., 2008) and appear prior to the emergence of the fourth leaf (Boudiar et al., 2020); therefore, they have a significant role in establishing RSA in wheat. They also remain active throughout the life cycle of plants and can delve deeper into soil profiles compared to nodal roots. Moreover, during stressful conditions like drought, nodal roots might not emerge, or their development might be impaired (Maccaferri et al., 2016; Sanguineti et al., 2007), but seminal roots play a vital role during such conditions as they can absorb water from deep soil profiles and make available to plants (Araki & Iijima, 2001).

Wheat has genetic variation in root architecture reported in many studies (Manschadi et al., 2006; Richard et al., 2015; Roselló et al., 2019; Ruiz et al., 2018). Among many traits that affect root system architecture (RSA), the angle made by roots when they emerge out of seed and penetrate the soil, i.e., seminal root angle, largely determines RSA of mature wheat plants (Wasson et al., 2012). Studies on genetic variability of wheat root traits have been done through studies of seminal root angle, as this root trait in wheat has proven to be representative of mature roots and has been considered as proxy in many studies (de Dorlodot et al., 2007; Fang et al., 2017; Hassouni et al., 2018; Tuberosa, Roberto et al., 2002) because it has high heritability, is expressed in early stage and can be screened in seedling stage using high throughput and cost-effective root phenotyping methods (Bengough et al., 2004; Richard et al., 2015).

4.2 Justification of the Study:

Seminal root angle is found to be associated with adult geometry of the RSA (Maccaferri et al., 2016; Manschadi et al., 2008; Rufo et al., 2020). Laboratory studies of seminal root angle done

at the seedling stage were found to have consistently correlated with crown root angle measured at the adult stage in the field in many studies (Hendel et al., 2021; Maccaferri et al., 2016; Rufo et al., 2020; Wasson et al., 2012). RSA traits such as root length, density, and root depth are major components that affect water absorption from deeper soil profiles. Adaptation of plants in drought conditions largely depends upon these traits, and their distribution in the soil profile is determined by seminal root growth angle (Borrell et al., 2014; Oyanagi, 1994). Narrow seminal root angle is found to be associated with roots at greater depth in mature roots of wheat reported in many studies (Bengough et al., 2004; Manschadi et al., 2008; Nakamoto & Oyanagi, 1994) and also in other crops like rice and sorghum (Mace et al., 2012; Uga et al., 2011). It has been beneficial during stress conditions like drought as plants can utilize residual moisture from deeper soil profiles. The advantages of narrow seminal root angles under drought stress have been reported (Acuña & Wade, 2012; Hamada et al., 2012; Manschadi et al., 2008; Reynolds et al., 2007). Similarly, wider seminal root angles are responsible for roots to be in shallower depth and help in nutrient uptake that is found in the shallower profile of soil (Hohn & Bektas, 2020; Miguel et al., 2015). There are various studies that have been done to understand the role of seminal root angle for drought tolerance and nutrient uptake, as mentioned above. But studies on seminal root angle have never been done particularly for the waterlogging stress tolerance to the best of my knowledge. It has, however, been mentioned in many studies that a wider seminal root angle is beneficial for wetter conditions as it promotes lateral root growth and allows plants to capture residual oxygen from wider space (Alahmad et al., 2019; Hohn & Bektas, 2020; Sanguineti et al., 2007). Alahmad et al. (2019) identified a major QTL on chromosome 6A for seminal root angle in durum wheat, overlapping with the QTL6A.2 region identified for chlorosis in Sundgren (2018). This investigation is by far the first work to investigate the association of seminal roots with waterlogging tolerance on common wheat to the best of my knowledge. To achieve this, we will analyze contrasting haplotypes in QTL6A.2 for chlorosis, which has not been extensively explored before. If we find significant differences among them for seminal root angle. This result could open new avenues to learn more about underlying genetic mechanisms for waterlogging tolerance in wheat in future studies.

4.3 Hypothesis:

Null hypothesis: Contrasting haplotypes of *QTL6A.2* for chlorosis under waterlogging has no difference in seminal root angle.
Alternative hypothesis: Contrasting haplotypes of *QTL6A.2* has a significant difference in seminal root angle.

4.4 Experiment I: Testing of seed germination paper

Part 1: Identification of suitable germination paper and sticking substances

4.4.1 Experimental site:

This experiment was done in the greenhouse, SKP building at Norwegian University of Life Sciences, Ås, Viken County, Norway.

Identification of appropriate seed germination paper was an important step in our study. For this, germination papers of various types were tested (Table 11). These papers were provided by Ahlstrom-Munksjö (Ahlstrom Germany GmbH), a paper company based in Germany.

The selection criteria for tested germination paper were:
1. It must be durable, i.e., must not degrade with time.
2. It must hold good moisture for seed germination, i.e., should not dry up.
3. It must harbor good root development of seeds laid on it.
4. It must give good contrast of root images in pictures.

Table 11. Various germination papers and their details:

Paper grade	Paper discription	Weight (g/m^2)	Thickness (mm)	Size (mm)
191	Filter board, blue, plain	700	1.35	210 x 297
193	Filter board, yellow, plain	160	0.32	210 x 297
194	Filter board, dark blue, plain	430	0.68	210 x 297

These germination papers were tested in seed germination growth pouches. The seed germination growth pouch is an assembly of various parts such as germination paper, laminated A4 paper, black plastic polythene, and butterfly clips (Fig 41). This growth pouch was prepared based on growth pouch systems reported by (Atkinson et al., 2015). Each part of the growth pouch was made separately and assembled (Fig 41).

Figure 41. Germination growth pouch with layers of polythene cover, germination paper, and laminated A4 paper stitched with butterfly clip.

Seed germination growth pouches were prepared for all three different grades of germination papers.

After the preparation of the seed germination growth pouch, another important step was to identify appropriate substance to stick seeds laid on germination paper. For this, various sticking materials were tested along with the germination papers (Fig 42). Tested sticking substances were:

1. Paper clips
2. Double glue sticky tape
3. Single glue sticky tape

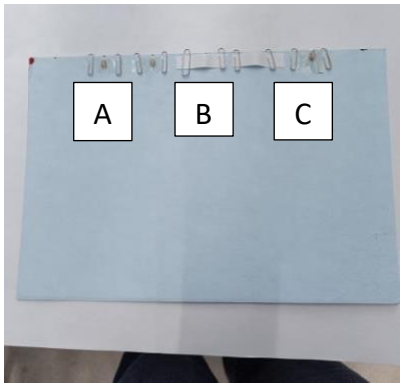


Figure 42. Sticking materials placed on germination paper to hold seeds on growth pouch. A =single glue plastic tape B= paper clip, C=double glue plastic tape.

The selection criteria for these tested sticking substances were:

1. It must stick seeds laid on germination paper firmly.
2. It must be durable.
3. It must not disturb the growth of roots.

Seed material: Wheat seeds from five genotypes of spring wheat were selected for the study of sticking substances and germination paper. Wheat genotypes taken for study are:

1. Bjarne
2. Chara
3. Chinese Spring
4. Laban
5. Sumai-3

4.4.2 Methodology:

At first, autoclaved petri-dish was taken. Filter paper (Whatman Grade 1, diameter 85mm) was kept inside petri-dish (Fig 43A) Then, distilled water was poured into a petri-dish to make the filter paper moist. Then, the seeds were placed in petri-dish (Fig 43B). Here, seven seeds from one genotype were placed in one petri-dish. Then, these petri-dishes were covered with aluminum foil and were allowed to germinate for 48 hours in darkness at room temperature.

A. B. C.

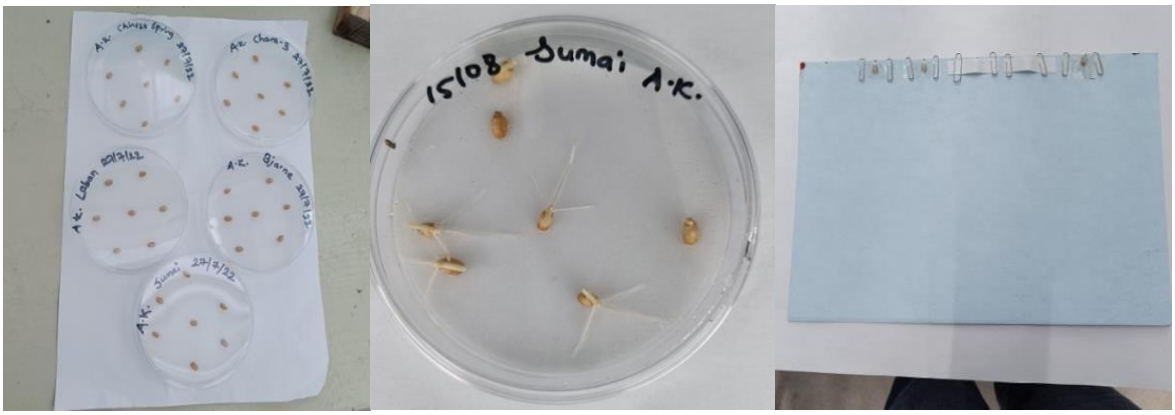


Figure 43. A) Placement of testing seed materials inside petri-dish; B) germinated seeds after 48 hours; C) Placement of seeds on germination paper using various sticking materials.

After that, pre-germinated seeds from the petri-dish were taken out and placed in germination paper with the help of stainless forceps. A careful selection of pre-germinated seeds from petri-dish was made. Poorly germinated ones were discarded. Here, each seed of five different genotypes was placed on the germination paper. The seed-seed distance was kept at 4.5 cm, and a 4 cm border space was left on each side. After that, sticking substances were used to stick pre-germinated seeds laid on germination paper (Fig 43C). All these testing sticking materials were used in all grades of germination papers. While fixing pre-germinated seeds with sticking substances, seeds were left open on their proximal and distal side to facilitate the growth of shoots and roots, respectively. After that, A4 paper was laminated with the help of lamination pouches and used as support for germination paper. The next step was to fix the size of polythene paper to use it as a cover for germination paper. Black polythene was cut in the shape and size of germination paper with the help of scissors. Then, holes were made at symmetric distances with seeds placed in germination paper, i.e., at each 4.5 cm distance. This was done to facilitate newly developed shoots to easily come out from the germination pouch to the air. This polythene covers germination paper from the frontal and back sides. After preparation of all parts of the seed germination growth pouch, they were assembled as shown in (Fig 41) with the help of butterfly clips (32 mm, Maped, Pringy, France).

After this, the seed germination growth assembly was designed. This growth assembly is an improvised form of seed growth assembly mentioned by Adeleke et al. (2020). The seed germination growth assembly consists of seed germination growth pouch, plastic tray (48*

38* 22 cm, Allibert, hoofddorp, Netherlands) with an aluminum frame on its top (Fig 44). Now, the aluminum frame as shown in Fig 44, was fixed at the top of this tray with the help of tape. This plastic tray assembly and growth pouch collectively was named seed germination growth assembly. Growth pouches were suspended on this seed growth assembly with the help of butterfly clips (Fig 44).

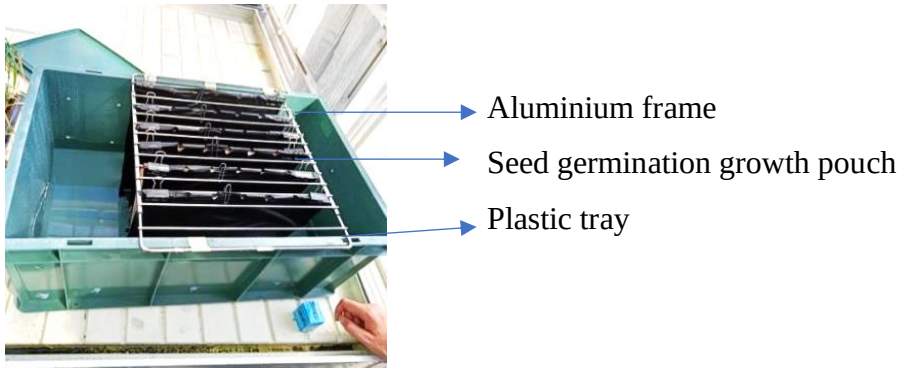


Figure 44. Seed germination growth assembly.

After that, the whole set-up was kept inside the greenhouse for ten days. The growth assembly was filled with fertilized water (50/50% solution mixture of YaraTera® CalcinitTM (14.4% NO_3, 1.1% NH_4, 19.0% Ca, Yara Norge AS, Oslo, Norway) and KristalonTM Indigo (7.5% NO_3, 1.0% NH_4, 4.9% P, 24.7% K, 4.2% Mg, 5.7% S, 0.027% B, 0.004% Cu, 0.2% Fe, 0.06% Mn, 0.004% Mo, 0.027% Zn, Yara Norge AS), EC 1.5 dS m^{-1}) such that the growth pouches suspended on it are submerged in fertilized water at least up to 3 cm. On the first day, all germination papers were sprinkled with water to make them moist. Then, after ten days, seed germination on various germination papers was observed, and root images were taken using a Fujifilm XF10 camera.

4.4.3 Results:

In our observations, we found that blue paper (grade 194) performed better than the other two germination papers used.

a. Dark Blue papers (grade 194) were found not degraded, but we could see black dots on yellow (grade 193) and light blue (grade 191) germination papers.
b. Additionally, we found better images of roots on dark blue paper as compared to other grade papers.

c. However, no difference in root germination was found in tested germination papers.

d. Paper clips rot with time (Fig 45). Similarly, the double-glued sticky tape did not stick properly with the germination paper. As they got stuck with polythene film from the other side, whereas single-glued sticky tapes were found to be firmly pasted with seed in germination paper. As well, these tapes did not disturb the growth of roots and shoots inside the growth assembly (Fig 45)

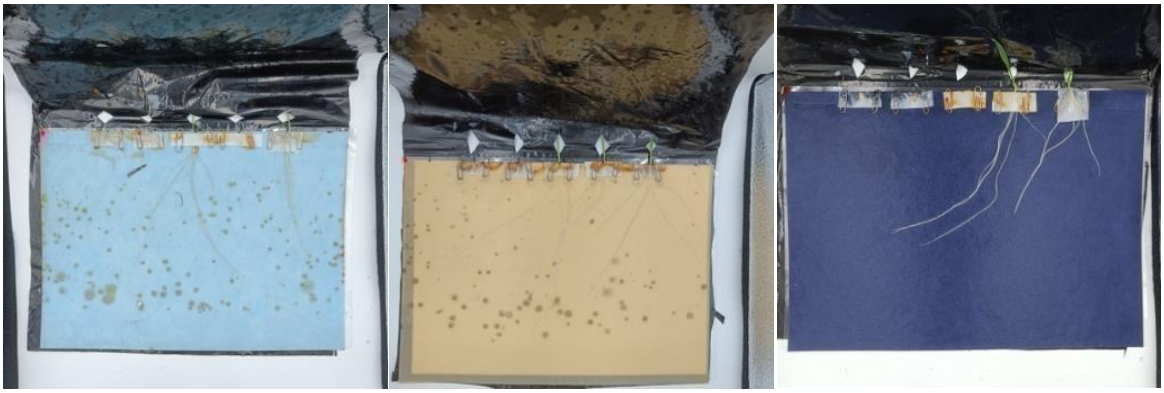


Figure 45: Germination of seed of wheat genotypes in light blue (grade 191), yellow (grade 193) and dark blue germination papers (grade 194), respectively.

4.4.4. Discussion:

Double-glued sticky tape and paper clips were found to be unsuitable for our seed germination growth assembly. They were not durable, and they could not stick seeds firmly against germination paper. We found that single-glued sticky tape was most suitable to use as a sticking substance for our further experiment. This tape not only fulfills the selection criteria of sticking substance but is also found to be suitable for visualizing roots and shoot development through it, which was not possible through paper clips as paper covers the seed part inside it (Fig 45). Additionally, for those seeds where paper clips and double-glued sticky tape were used, there was poor germination of seeds on all germination papers. Thus, they could be discarded in further experiments as sticking substances.

Similarly, yellow (grade 193) and light blue (grade 191) germination papers exhibit degradation with black dots and were found to be non-durable as compared to dark blue germination paper (grade 194). Yellow and light blue papers, nonetheless, harbor seed

germination. However, they might not be suitable germination papers as they degrade their quality in moisture with time, or they would have to be treated with a fungicide which causes extra cost if used. The dark blue (grade 194) germination paper was found superior in terms of durability, good germination, and better contrast of root images. This result is also supported by a previous study (Gioia et al., 2016)

4.4.5 Conclusions:

From this experiment, it is concluded that paper clips and double-glued plastic tapes were not suitable as sticking substances in germination paper. So, we discarded them and approve only single-glue plastic tape for sticking seeds against germination papers in our further experiments. Additionally, we found dark blue paper (grade 194) to be a promising germination paper in terms of our selection criteria.

Part 2: Suitability Assessment of Dark Blue (Grade 194) germination Paper and single glue sticking Substance

4.4.6 Background:

From previous experiment, we tested the suitability of sticking materials and germination papers to develop a suitable growth pouch for seminal root angle study. We found dark blue paper (grade 194) and single-glue plastic tape to be promising ones as germination paper and sticking materials, respectively. So, we decided to conduct one more experiment on germination paper with a higher number of replications to verify the suitability of dark blue paper (grade 194) and single-glued tape as part of our seed growth assembly.

4.4.7 Methods:

Same steps as the previous experiment were followed. Only single-glued plastic tapes were used as sticking material. Here, four dark blue (grade 194) and four light blue (grade 191) germination papers were used in this experiment. Yellow (grade 193) germination paper was not tested, as the paper company (mentioned earlier) sent limited numbers of it, and we were out of stock for this new experiment. We find it irrelevant to order those papers as they were found unpromising in the previous experiment, and shipping generally takes a longer time.

4.4.8 Results:

We found consistent results regarding paper quality. Light-color blue (grade 191) germination paper was found to be degraded in this experiment, too, whereas dark blue (grade 194) paper was found to be in good condition. The single-glued plastic tape worked well in this experiment as well for both germination paper grades, as shown in (Fig 46).

4.4.9 Discussion:

Based on our selection criteria, dark blue paper was found to be better than light blue paper, (Fig 46). Dark blue paper (grade 194) exhibited consistent durability and provided superior contrast to root images. These results further verify that dark blue (grade 194) is the most suitable germination paper. However, it is noteworthy that the light blue paper (grade 191) exhibited satisfactory seed germination results. Despite this, it is not recommended as a suitable germination paper as it degrades with moisture and doesn´t give a good contrast to root images. Similarly, single-glue sticky tape consistently gave good results in this experiment, too and found as suitable seed-sticking substance in germination paper.

Figure 46: germination of seeds using single-glue plastic tapes in dark blue and light blue papers, respectively.

4.4.10 Conclusion:

Based on our selection criteria and the results we got, we conclude that dark blue (grade 194) germination paper was the most suitable germination paper among those tested. We strongly recommend this paper be used for germination studies where root phenotyping is involved, as the roots are easily observable, and the contrast should be sufficient for downstream image analysis software, and the paper can withstand moist conditions over a longer period. We informed our results to the paper company (above mentioned) about suitability of dark blue (grade 194) paper among the tested germination papers for seminal root phenotyping studies through report.

We recommend single-glue plastic tape as the most suitable seed-sticking substance in germination paper among those tested. We were able to develop a suitable seed growth assembly needed for our study and decided to use this growth assembly as a model seed growth assembly for seminal root phenotyping in our next experiment. We claim this tailored seed growth assembly to be cheap, easy, and time efficient to build.

4.5 Experiment II: Establishing seminal root phenotyping methodology

4.5.1 Background:

From experiment I, the selection of suitable germination paper and seed-sticking substance was done, along with a suitable growth assembly model was established. The growth assembly model with the aluminum frame was not suitable for studies that involve more genotypes. A bigger size seed growth assembly was established, and this growth assembly is also an improvised form of the seed growth assembly model developed in experiment I and that mentioned by Adeleke et al. (2020).

4.5.2 Specific Objectives:

- To develop seminal root phenotyping method.
- To find if contrasting haplotypes on *QTL6A.2* have significant difference in seminal root angle.

4.5.3 Establishment of improvised seed growth assembly:

Construction of growth assembly frame: This comprised of plastic frame (48* 32* 22 cm). It was stitched with a wooden plank on its side. The wooden plank had holes on where an aluminum wire was woven (Fig 47). This growth assembly frame can accommodate eighteen growth pouches. It was laid on top of a plastic tray (as mentioned in the previous experiment) to give its final look (Fig 47). For the design of this customized seed growth assembly, we opted to utilize locally available materials such as plastic covers, wooden planks, and aluminum wires, which make this seed growth assembly to be low-cost and easy to design.

Figure 47: Front view of customized seed growth assembly without growth pouch and side view of it with growth pouch clipped on aluminum wire with butterfly clips. The plastic frame (red color) is placed on top of the plastic tray.

4.5.4 Experimental site:

This experiment was done inside the SKP at Norwegian University of Life Science, Ås, Viken County, Norway.

4.5.5 Root Growth environment:

Roots were grown at an average day /night temperature of 18/15 °C with relative humidity (R.H.) of 60%, 16 hours of daily photoperiod inside the semi-controlled greenhouse. Supplementary HPS lights were given to maintain the photoperiod. Experiment was conducted in the month of September 2022.

4.5.6 Selection of plant materials:

Eighteen genotypes of spring wheat were selected from the MASBASIS diversity panel. We selected our eighteen genotypes based on two main factors. The most important factor is that selected genotypes comprise a mixture of genotypes with contrasting haplotype groups for waterlogging QTL on chromosomes 6A.2, i.e., haplotype 1 and haplotype 3. Genotypes under haplotype group 1 are found to be significantly more chlorotic than genotypes under haplotype group 3 in Tove´s fieldwork. The next factor was these selected genotypes are genotypes of interest in long-term research of MASBASIS lines. Several of them were important, modern commercial cultivars. The seed source was seeds harvested from a MASBASIS field trial in 2019**.** We were interested to observe seminal root angle of these contrasting haplotypes That´s why we select them as plant material.

Table 12. Spring wheat genotypes selected for screening experiments with information on their 6A haplotype number and MASBASIS line number

S.N.	Genotypes name	6A Haplotype	MASBASIS Line
1.	Bastian	3	1003
2.	Bjarne	3	1005
3.	Runar	3	1020
4.	Zebra	3	1011
5.	Tjalve	3	1006
6.	Berserk	3	1016
7.	Polkka	3	1419
8.	Krabat	3	1174
9.	Bombona	1	1190
10.	Avle	1	1009
11.	Mirakel	1	1401

12.	Laban	1	1178
13.	GN07560	1	1405
14.	Vinjett	1	1116
15.	Berlock	1	1413
16.	Amulett	1	1189
17.	Cadenza	3	1443
18.	GN08554	1	1312

4.5.7 Preparation of growth pouches:

They comprised dark blue germination paper, single-glued plastic tape, laminated A4-paper, and polythene cover. Each growth pouch was considered as one plot. Here, four seeds were laid on germination paper. The seed-seed distance was kept at 5 cm with a border space of 3 cm on both sides ana a top space of 1 cm.

4.5.8 Experimental Design:

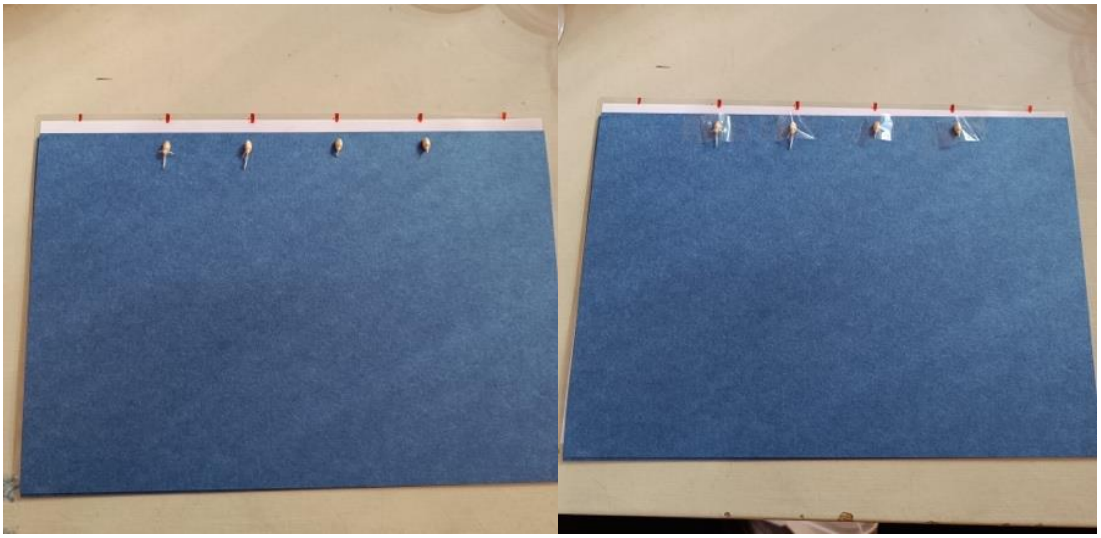
Three seed growth assemblies were used, and each growth assembly was considered as one replication. Eighteen growth pouches were laid in completely randomized block design, as shown in (Table 13) in each seed growth assembly.

Table 13. Eighteen wheat genotypes laid in Completely Randomized Block Design in plastic trays. Number 1 to 18 denote genotypes shown in Table 12

Replication 1	Replication 2	Replication 3
1	4	5
18	3	7
8	12	10
16	2	3
3	10	17
5	1	11
2	6	8
4	14	18
9	5	1
7	9	13
11	8	4
6	15	15
13	16	2
17	11	9
14	18	14
12	7	12
10	13	16
15	17	6

The methodology in this experiment was followed the same as mentioned in the previous experiment. All these three seed growth assemblies were kept inside the greenhouse for ten days.

A. B.



C. D. E.

Figure 48. A) Placement of germinated seed on germination paper; B) Sticking of single glue tape on germination paper to hold seeds on it; C) Seed germination growth assembly along with growth pouch: D) Three seed growth assemblies kept inside the greenhouse; E) Seedlings emerging from seed growth pouch.

4.5.9 Image acquisition and analysis:

Image acquisition set-up:

A square-shaped lightbox with attached white LEDs (40 *40 cm, Puluz, Schenzhen, China) (Fig 49A), was used. These white LEDs ensure even lighting inside the box. A tripod was fixed at a fixed height. from the bottom of the lightbox, and then the camera was mounted on the tripod facing to light-meter box at a fixed position and at a fixed height of 41.5 cm from the bottom of the photometer (Fig 49B). The camera used here was a standard Fujifilm XF10. This whole setup was kept fixed throughout the image acquisition process.

A. B.

C. D.

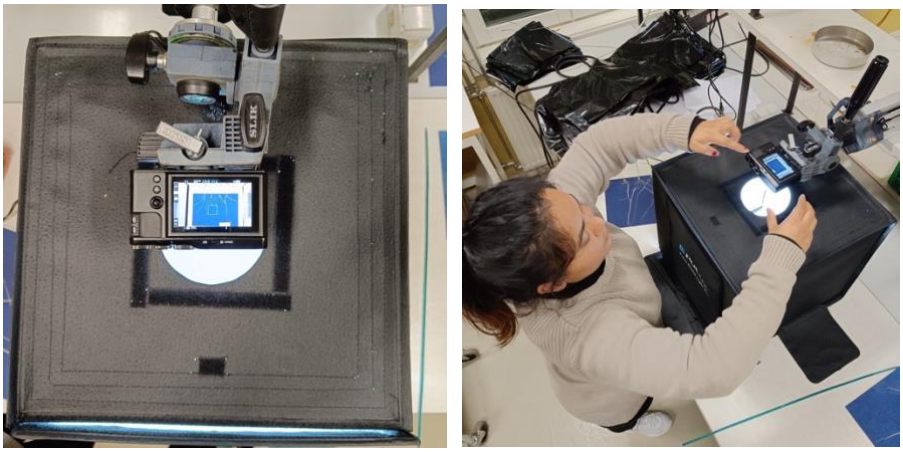


Figure 49. A) Light box; B) Image acquisition set up: Fuji Camera attached to the tripod at a fixed distance from the base of the lightbox; C) Frontal view of root images capture in image acquisition setup; D) Manual capture of root images in fixed image acquisition setup.

Image acquisition:

At first, germination papers were taken out from the germination growth assembly and carried to the image acquisition set-up. Germination papers were kept inside light box, and the light was turned on. Then images were taken from the camera mounted on the tripod (Fig 49C and D).

Image analysis: Analysis of the images taken from the Fuji camera was done by using ImageJ software (http://imagej.nih. gov/ij). Here, the angle between the outer pairs of seminal roots was measured at approximately 3 cm distance from the tip of the seed (Fig 50).

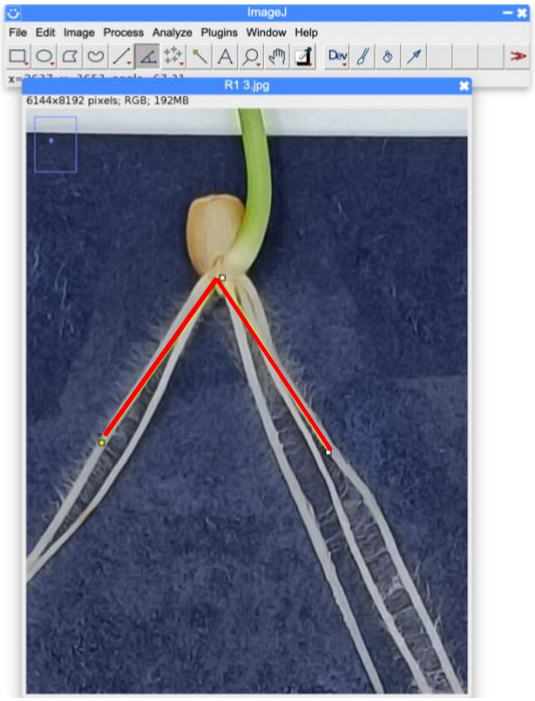


Figure 50. Angle between outer pairs of seminal roots measured from Image J software.

4.5.10 Statistical Analysis:

Software R studio (version 4.2.2) was used for analysis for all the data obtained. Analysis of variance (ANOVA) was done using this software to see differences in seminal root angle measurements for all the genotypes under study. Tukey HSD test was selected as a post-hoc test to assess the pairwise differences between group means of seminal root angle. Phenotypic data of seminal root angle were analyzed through linear mixed model $Y_{ij} = \beta_0 + v_i + u_{0j} + \varepsilon_{ij}$. Here, Y_{ij} is the observed value of the "Seminal Root angle" variable for the i-th genotype and j-th replication. β_0 is the fixed intercept, which captures the overall mean angle across all genotypes and replications; v_i represents the random intercept for the i-th genotype, which denotes the deviation from the overall mean angle specific to that genotyp; u_{0jj} represents the random intercept for the j-th replication, that covers the deviation from the overall mean angle specific to that replication, ε_{ij} denotes the residual error term, which accounts for the random variation in the observed seminal root angles. This linear mixed model was fitted using lmer () function in the “lme4” package in R studio. This model considers genotypes and replication as random effects in relation to the dependent variable, the seminal root angle. Estimated random effects also known as Best Linear Unbiased Predictions (BLUPs) for each level of the genotypes factor, is calculated using ranef () function inside the “Lme4” package. Welch two

sample t-test was done to compare the estimated genotypes mean of seminal root angle (BLUPs) between two Haplotype groups.

4.5.11 Results:

A. Amulett B. Vinjett

C. Bombona D. Berserk

E. Runar

Figure 51: Representative root images that show off directional growth of seminal roots in germination paper.

A. B.

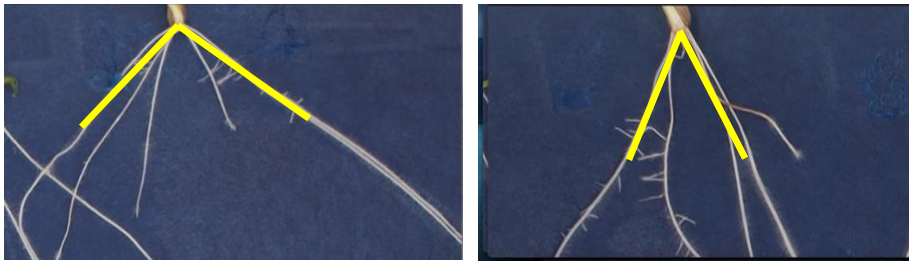


Figure 52. Seminal root angle of Avle (left) and Tjalve (Right)

Table 14. Results summary of ANOVA of seminal root angle of eighteen spring wheat genotypes.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotypes	17	2688.3	158.13	3.256	0.00165 **
Replication	2	51.5	25.76	0.530	0.59313
Residuals	34	1651.2	48.57		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

From the results of ANOVA, it was found that there was a highly significant difference (P< 0.01) among the wheat genotypes under study for seminal root angle (Table 14).

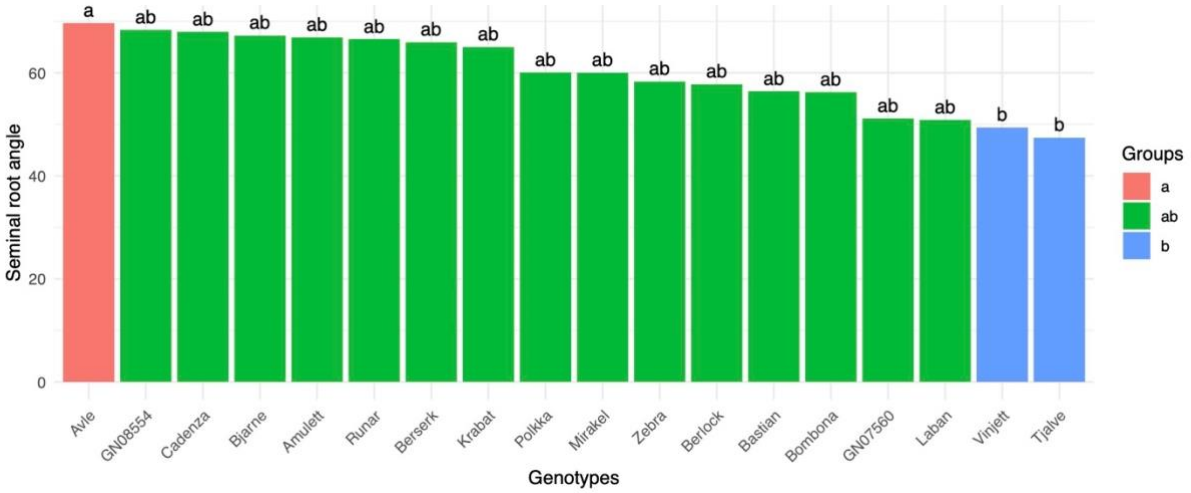


Figure 53: Results of Tukey HSD test of seminal root angle of eighteen spring wheat genotypes. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in mean seminal root angle among genotypes using Tukey HSD. Genotypes with at least the same letter indicate no significant difference.

Results from Tukey test showed that genotype Avle had significantly wider seminal root angle than genotype Tjalve and Vinjett (Fig 53). Other genotypes under study are statistically at par with Avle and Tjalve for seminal root angle (Fig 53).

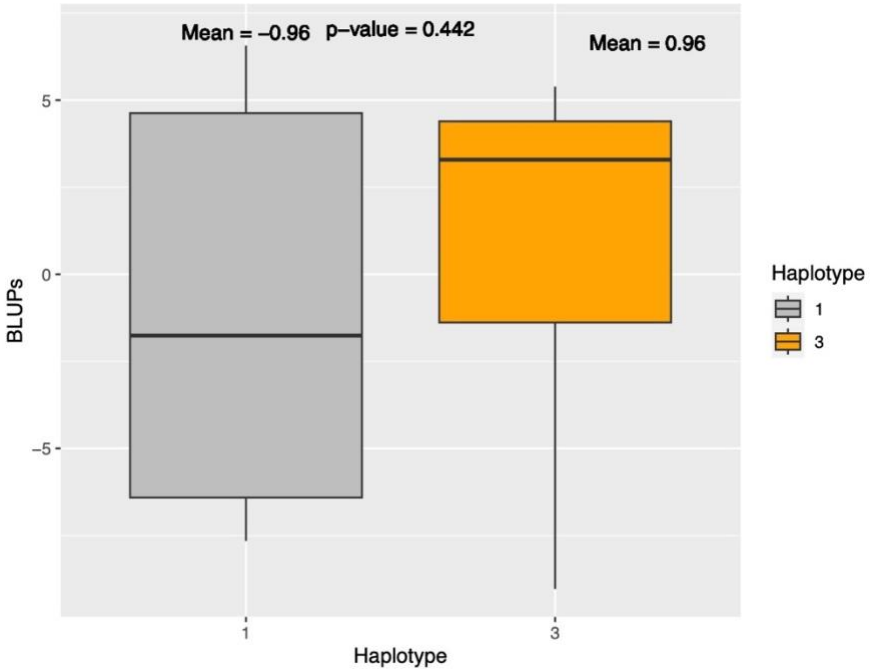


Figure 54. Results of Welch´s two-sample t-test among two contrasting haplotype groups on QTL6A.2 for chlorosis analyzed for their seminal root angle. p-value at the mid of two box plots in the figure indicates the significance level. P < 0.05 indicates a significant difference, and P >0.05 indicates no significant difference among the two haplotype groups for seminal root angle.

Results of Welch's two-sample t-test showed that there was no significant difference in seminal root angle among two haplotype groups on chromosome 6AL, i.e., Haplotype 1 and Haplotype 3 (p> 0.05) (Fig 54).

4.5.12 Discussion:

Generally, roots show positive gravitropism growing downwards in response to gravity, and negative phototropism, avoiding light sources. However, some roots in most of the genotypes showed atypical (hanging shape) growth on germination paper, as shown in Fig 51 in our experiment. Upon careful examination, we realized we had irregular-sized holes in the polythene cover. Such irregular-sized holes in the polyethylene cause non-uniform penetration of light in growing seeds, creating a heterogeneous growing environment among the roots. We

postulated that some roots might have, in response to excessive light entering through bigger-sized holes of polythene, made directional adjustments in search of darkness, and such off-type roots might have been formed while other roots maintained their normal growth behavior. However, the underlying reason behind such off-directional growth of roots remains largely unknown. Yet, we decided to adjust in the next experiment to make evenly sized holes diameter in polythene, assuming to avoid such off-directional root phenotypes. This adjustment aimed to ensure a more uniform and consistent environment for the roots during their growth. Most importantly, we realized the importance of using check lines to verify the methodology we used to be proven as reproducible and reliable.

4.5.13 Conclusion:

Considering the technical limitations encountered in the experiment, we decided to improvise our methodology one more time before we draw conclusions on seminal root angle differences among these haplotype groups.

4.6 Experiment III: Improving seminal root phenotyping methodology.

4.6.1 Background:

From experiment II, the necessity of adjustment in the seed germination growth pouch was realized. Uniform-sized holes were made in a polythene cover. Adjustment on seed-seed distance was made. Check varieties for verification of established methodology were ordered from Italy.

4.6.2 Specific Objectives:

- To obtain a reliable seminal root phenotyping methodology
- To see if contrasting haplotypes on *QTL6A.2* have significant difference in seminal root angle.

4.6.3 Experimental site:

This experiment was conducted at the SKP at Norwegian University of Life Science, Ås, Viken County, Norway.

4.6.4 Plant material:

Same spring wheat genotypes as used in the previous experiment were used. The only difference was that two spring wheat genotypes, Cadenza and GN08554, were replaced by two check varieties, Colosseo and Lloyd. Colosseo, an Italian durum wheat cultivar, and Lloyd, a US durum wheat cultivar, which were found to have wide and narrow seminal root angles in the laboratory's seedling stage, followed by Colosseo had a wide crown root angle, and Lloyd had a narrow crown root angle in their respective adult field stages as reported in (Maccaferri et al., 2016).

4.6.5 Roots Growth environment:

The same growth environment, as mentioned in Experiment II, was maintained inside the greenhouse. The only difference was that experiment was done in the month of March 2023, and the temperature outside might have fluctuated temperature inside as the greenhouse was semi-controlled.

4.6.6 Experimental Duration:

The experimental duration in this experiment was reduced to five days to reduce overlapping and off-directional growth of roots in germination paper.

4.6.7 Methodology:

The diameter of holes on the plastic cover used in the seed growth pouch was kept at 1 cm diameter to ensure even lighting in all seeds used in the experiment. The seed-seed distance was kept 6 cm whereas border space of 2 cm was kept on both sides, and 0.7 cm was kept as top-border. Besides these adjustments, the same methodology was used as in the previous experiment.

A. B. C.

Figure 55. A) Manual placement of seeds on germination paper with the help of forceps; B) Uniform holes of each 1 cm made at a symmetrical distance to seeds placement in germination paper; C) Seed germination growth pouch with a label mentioning genotype number and name pasted on polythene cover.

4.6.8 Image acquisition and image analysis, and data analysis:

All of them were done as same as mentioned in the previous experiment.

4.6.9 Results:

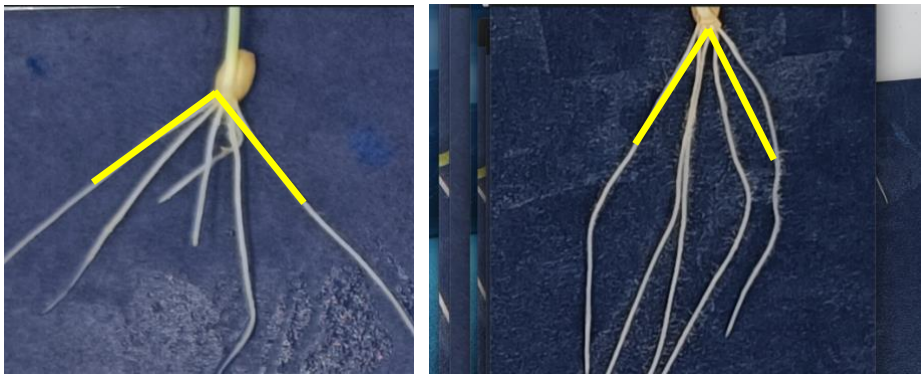


Figure 56. Seminal root angle of Colosseo (left) and Lloyd (right)

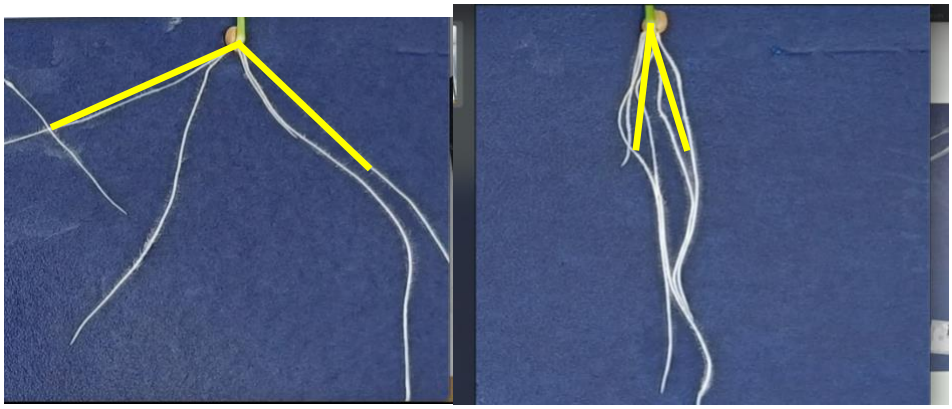


Figure 57. Seminal root angle of Bombona (left) and Berserk (right)

Table 15. Results summary of ANOVA of seminal root angle of eighteen spring wheat genotypes.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotypes	17	27656	1626.8	12.734	<2e-16 ***
Replication	2	71	35.3	0.276	0.759
Residuals	113	14436	127.8		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Results of ANOVA showed that there was a highly significant difference among the genotypes under study for seminal root angle ($p < 0.001$) (Table 15).

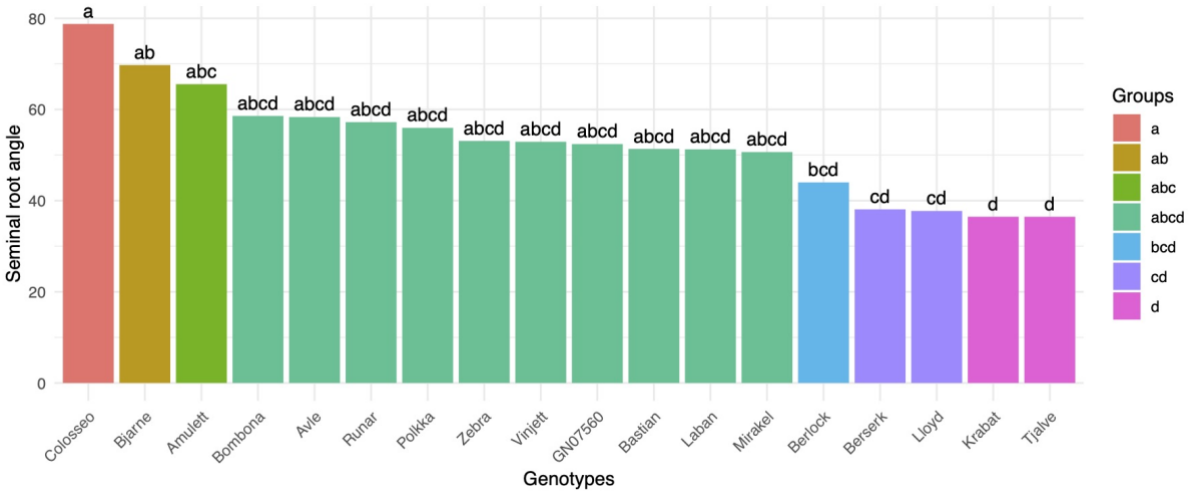


Figure 58. Results summary of Tukey HSD test of seminal root angle of eighteen spring wheat genotypes. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in mean seminal root angle among genotypes using Tukey HSD. Genotypes with at least of same letter indicate no significant difference.

Results of Tukey HSD showed that Colosseo had a significantly wider seminal root angle than Berserk, Berlock, Lloyd, Krabat and Tjalve and was statistically at par with other genotypes. Similarly, Bjarne had a significantly wider root angle than Berserk, Lloyd, Krabat and Tjalve whereas Amulett had a significantly wider root angle than Krabat and Tjalve. They are statistically at par with rest of the genotypes (Fig 58).

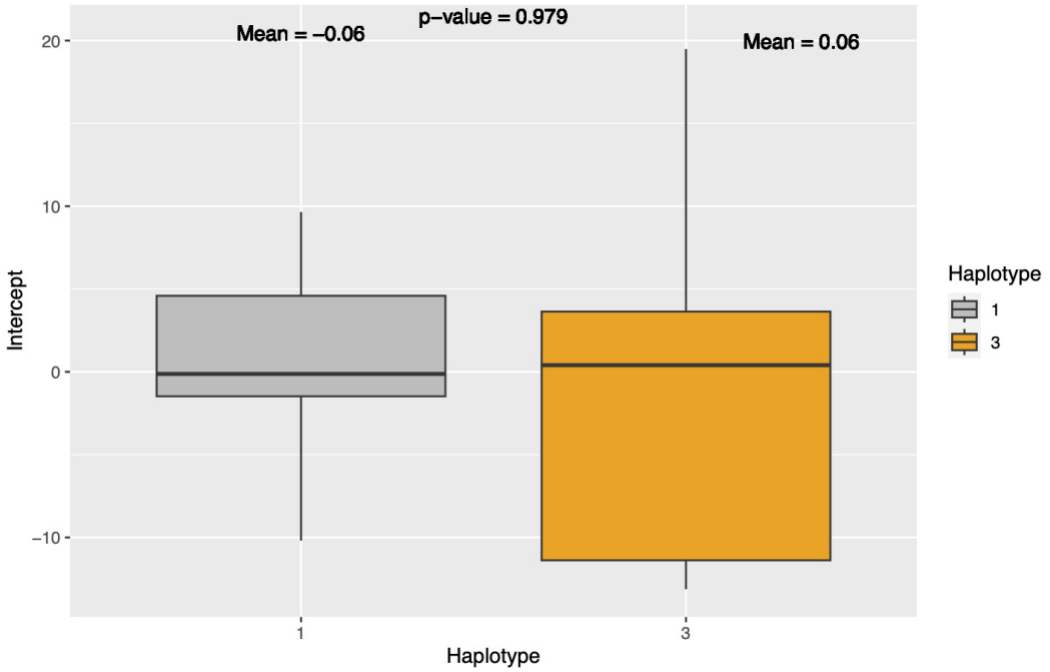


Figure 59. Results of Welch´s two-sample t-test among two contrasting haplotype groups on QTL6A.2 for chlorosis analyzed for their seminal root angle. P-value at mid of two box plots in each figure indicate the significance level. P < 0.05 indicate a significant difference, and P >0.05 indicate no significant difference among the two haplotype groups for seminal root angle.

From the results of the Welch Two sample t-test, it was found that there was no significant difference among the two haplotype groups for seminal root angle (p> 0.05) (Fig 59).

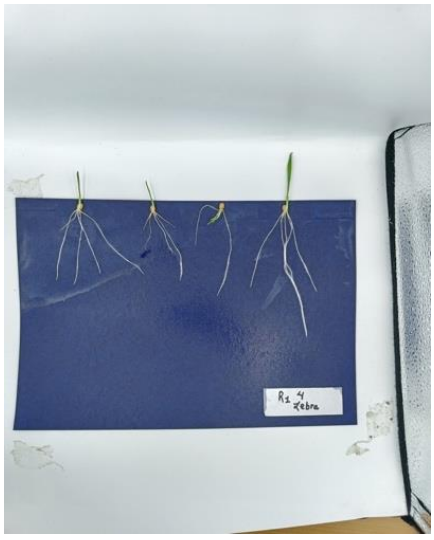
4.6.10 Adjustment on measurement of seminal root angle using image J software:

- Off-directional, very short, and overlapped phenotypes were discarded.

A.

B.

C.



D.

E.

.

Figure 60. Some representative images of off-directional and short phenotypes that were discarded in new measurement.

4.6.11 Results:

Table 16. Results summary of ANOVA of seminal root angle of eighteen spring wheat genotypes.

	Df	SumSq	MeanSq	F value	Pr(>F)
Genotypes	17	14050	826.5	5.368	4.69e-08 ***
Replication	2	820	409.8	2.662	0.0753 .
Residuals	91	14010	154.0		

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

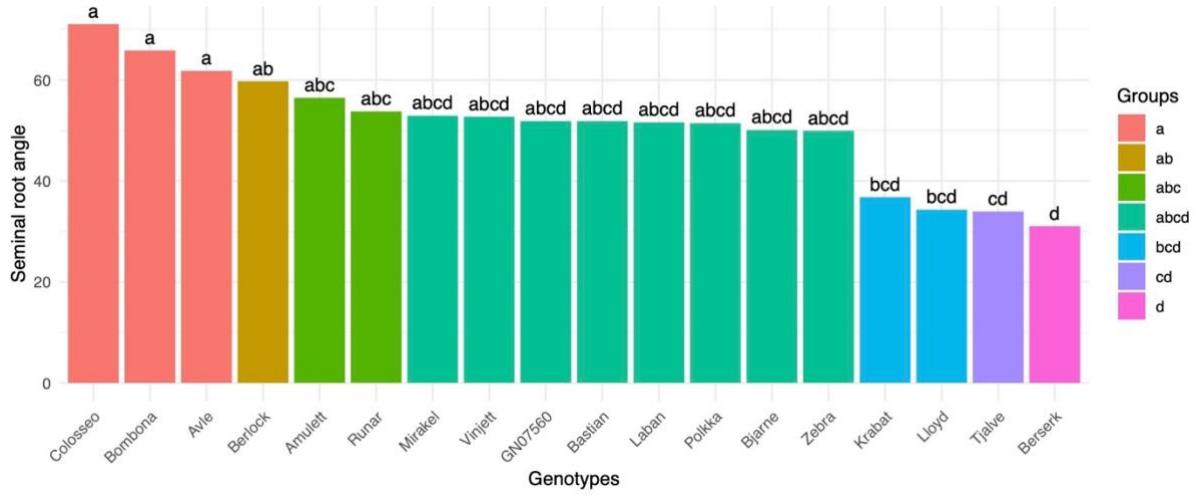


Figure 61. Results summary of Tukey HSD test of seminal root angle of eighteen spring wheat genotypes. Genotypes are grouped according to their significant differences. Letters at the top of the bar diagram mark significant differences found in mean seminal root angle among genotypes using Tukey HSD. Genotypes with at least of same letter indicate no significant difference.

Results of ANOVA showed that there was highly significant difference among genotypes for seminal root angle ($p < 0.001$) (Table 16).

Results of Tukey HSD showed, Colosseo, Bombona, and Avle had significantly wider seminal root angle than Krabat, Lloyd, Tjalve, and Berserk and were statistically at par with other genotypes. Additionally, Berlock had significantly wider seminal root angle than Tjalve and Berserk and Amulett had significantly wider seminal root angle than Berserk. They both were statistically at par with rest of the genotypes. Similarly, Berserk had a significantly narrower

seminal root angle than rest of the genotypes but was statistically at par with Krabat, Lloyd and Tjalve, Mirakel, Vinjett, GNO7560, Bastian, Laban, Polkka, Bjarne, Zebra (Fig 61).

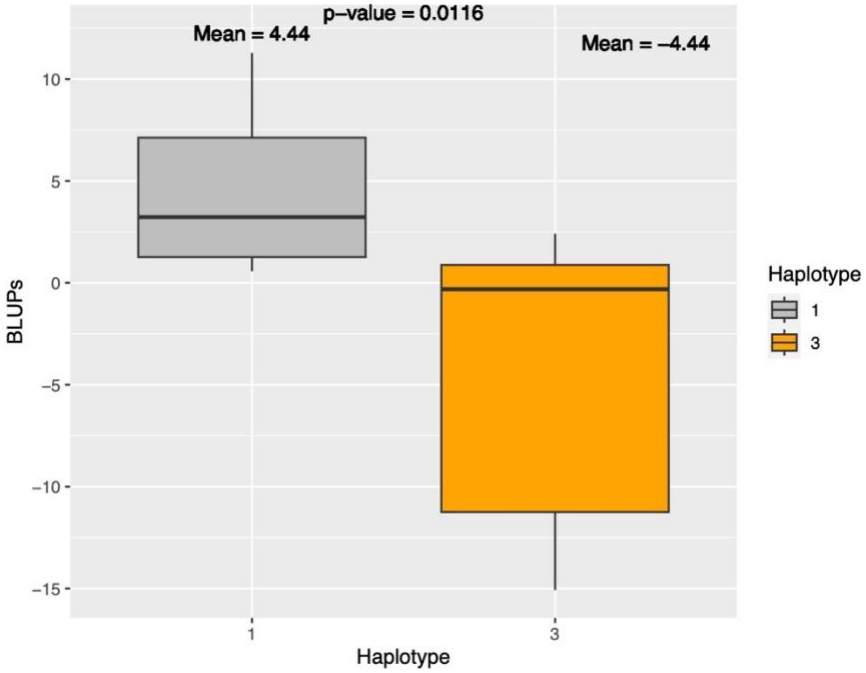


Figure 62. Results of Welch´s two-sample t-test among two contrasting haplotype groups on QTL6A.2 for chlorosis analyzed for their seminal root angle. P-value at mid of two box plots in figure indicates the significance level. P < 0.05 indicate a significant difference, and P >0.05 indicate no significant difference among the two haplotype groups for seminal root angle.

Results of Welch two sample t-test showed that there was significant difference among two haplotypes groups for their seminal root angle. Genotypes under haplotype 3 were found to have significantly narrow seminal root angle than haplotype 1 ($p < 0.05$) (Fig 62).

Table 17. Change in average seminal angle (%) and ranking of genotypes with new measurement

Genotypes	Old angle	New angle	Difference	Change (Absolute value)	Change in (%) (Absolute value)	Rank old	Rank New
Colosseo	78,75	71,06	7,69	0,097	9,76	1	1
Bjarne	**69,74**	**50,103**	**19,63**	**0,28**	**28,15**	**2**	**13**
Amulett	65,55	56,47	9,07	0,13	13,84	3	5
Bombona	58,57	65,82	7,24	-0,12	12,37	4	2
Avle	58,34	61,79	3,45	-0,05	5,91	5	3

Runar	57,22	53,76	3,45	0,06	6,033	6	6
Polkka	**55,96**	**51,44**	**4,52**	**0,08**	**8,081**	**7**	**12**
Zebra	**53,101**	**49,94**	**3,15**	**0,05**	**5,95**	**8**	**14**
Vinjett	52,91	52,74	0,17	0,003	0,32	9	8
GN07560	52,41	51,86	0,55	0,01	1,06	10	9
Bastian	51,34	51,83	0,49	0,009	0,96	11	10
Laban	51,25	51,58	0,32	0,006	0,63	12	11
Mirakel	**50,66**	**52,91**	**2,24**	**0,044**	**4,43**	**13**	**7**
Berlock	**44,00**	**59,73**	**15,72**	**0,357**	**35,73**	**14**	**4**
Berserk	38,08	31,03	7,05	0,18	18,51	15	18
Lloyd	37,74	34,31	3,42	0,09	9,074	16	16
Krabat	36,46	36,80	-0,34	0,009	0,936	17	15
Tjalve	36,46	33,95	2,51	0,068	6,88	18	17

Under new approach of measurement, genotypes Bjarne, Polkka, Zebra, Mirakel and Berlock had distinct change in their ranking (marked as bold in Table 17). Highest percentage of change in average seminal root angle was found in Berlock followed by Bjarne (Table 17).

Table 18. Comparison of seminal root angle measurement of check varieties (Maccaferri et al., 2016) used in our experiment

Genotypes	Seedling (S)	Field (F)	M1	M2	S-F	S-M1	S-M2
Colosseo	107.1	67.1	78.75	71.067	37.36%.	26.47%.	33.66%
Lloyd	76.0	53.9	37.74	34.31	29.08%.	50.34%.	54.79%.
Difference in genotypes (%)	29.03%.	19.66%.	52.03%	51.77%.			

The seedling seminal root angle is donated as Seedling (S) and Field crown root angle is denoted as Field (F) (Maccaferri et al., 2016). M1 and M2 denote 1st and 2nd measurement of seminal root angle in experiment III.

4.6.12 Discussion:

Including check varieties in the new experiment ensured that the customized seminal root phenotyping methodology developed in our lab was reliable. Colosseo consistently had a wider

seminal root angle, and Lloyd had a narrower seminal root angle, as it was obtained by a group of scientists in Italy who worked on seminal root angle using these cultivars in the seedling stage inside the laboratory and also in field (Maccaferri et al., 2016). They found that lab measurement of the seminal root angle for Colosseo and Lloyd at the seedling stage was wider than the crown root angle measured in the actual field at the adult stage (Table 18). But the difference between root angles in both genotypes was consistent in the seedling stage in the lab and adult crown root angle as there was only a 10% change in their difference in adult crown root angle compared to the seedling stage. They also verified that seminal root angle can represent crown root angle and overall root system architecture in the adult stage through this experiment. The seminal root angle measured in their laboratory for Colosseo and Lloyd at the seedling stage was wider than in our experiment. The difference in seminal root angle between Colosseo and Lloyd was also found higher in our experiment compared to what they found in their experiment (Table 18). This can usually happen, as different set-ups and phenotyping methods were used in our experiment compared to them. But interestingly, Colosseo was found consistently significantly wider than Llyod in both experiments. Similarly, both of our measurement approaches found Colosseo and Lloyd to have consistently significant differences for seminal root angle. Thus, this result provides ample evidence to support the fact that this developed customized phenotyping methodology for seminal root angle is working.

Fixing plastic holes symmetric to seed placement along with a fixed plastic hole diameter of 1 cm helped off-type hanging seminal root phenotypes to be reduced to a large extent in the new experiment. However, off-directional phenotypes, as shown in Fig 60, were still evident in 2nd experiment as well. We acknowledge two important factors to be considered using this methodology in further experiments. Space between two seeds placed in germination paper using this phenotyping method might have interfered with the vertical growth of seminal root phenotypes. In some cases, roots were out of space, as shown in Fig 60; this made measurement through Image J software confusing. Results become interesting when such confused off-directional phenotypes, along with overlapped and short root phenotypes, were removed, as results changed significantly. Removing such phenotypes on measurement made a significant change in the ranking of some genotypes like Bjarne, Polkka, Zebra, Mirakel, Berlock. Even though there was a slight variation in their average seminal root angle among the two measurements (Table 17). The highest percentage of change in average seminal root angle was found in Berlock followed by Bjarne. Genotypes like Colosseo, Bombona, Avle and Tjalve, Krabat, Lloyd and Berserk were found to have wider and narrower seminal root angle

respectively in both measurements. The difference in results obtained through two different approaches of measurements, along with consistent results of two check varieties in our improved phenotyping methodology, gave strong testament that significant difference in seminal root angle found between these contrasting haplotypes on *QTL6A.2* for chlorosis in later measurement deserve further experiment to verify this association. However, some adjustments in our phenotyping methodology would be necessary.

4.6.13 Conclusion:

There are two major technical limitations realized through several of these seminal root phenotyping experiments done in greenhouse. Spacing between seeds on germination paper was found to influence off-directional phenotypes (Fig 60). To address this, it is suggested to reduce the number of seeds per germination paper, ideally to one seed or a maximum of two seeds. Some genotypes exhibit faster root growth, and in such cases, the availability of space can interfere with their growth (Fig 60). However, this consideration may not be applicable to all genotypes, as the behavior of roots varies between different genotypes, and space may not significantly interfere with their growth. These off-directional phenotypes can also be attributed to plastic paper used as cover material in growth pouch. Some literature mentioned that plastic cover interferes with the roots, gets stuck with roots, and it takes a lot of time to remove it while analyzing root images in imaging software (Adeleke et al., 2020; Dupuy et al., 2017). It also might have interfered with seed directional growth as plastic remains completely intact with germination paper in the growth pouch, and slight disturbance during manual placement of growth pouch in growth assembly might have disturbed seeds' original direction in germination paper in our experiment too. It would be better to provide free space and undisturbed conditions for seeds to precisely capture root growth along the vertical plane in germination paper. Our tailored seminal root phenotyping method of placing 4 seeds/ germination paper would have been a cost-effective, easy, and fast phenotyping method if was found reliable, but adjustment of this method would be highly suggested because many off-directional phenotypes made confusion to observers while assessing seminal root angle in image software. Therefore, it is recommended to use one seed or a maximum of two seeds per germination paper and replace the plastic cover with an acrylic plate (Adeleke et al., 2020) in further experiments. The significant results obtained in this experiment are hoped not to be a

random coincidence, and further experiments are strongly advised to verify the obtained results.

5 General discussion

Waterlogging tolerance can be defined as a plant´s ability to maintain yield despite waterlogging stress relative to non-waterlogged conditions (Setter & Waters, 2003). Direct selection based on yield under waterlogging stress has very low heritability and remains to be ineffective (Collaku & Harrison, 2005). Effects of waterlogging conditions are expressed by plants in terms of stress symptoms like chlorosis, reduced leaf area, biomass, height, etc., at varying degrees. These stress symptoms have been used as indicators of waterlogging tolerance, among which chlorosis percentage is one (Zhou et al., 2007). Chlorosis percentage is relatively easy to observe and record, expressed in the early stage, and is highly correlated to yield. However, it requires severe waterlogging conditions and diverse screening populations otherwise can be a poor indicator of waterlogging tolerance, as differentiation of chlorosis among genotypes is challenging under less severe conditions (Sundgren, 2018). This is an important finding in our study, too, which is proven through several experiments previously discussed. Unless severe waterlogging conditions were created, effective chlorosis assessment was found to be consistently difficult.

5.1 Can starch waterlogging mimic field waterlogging?

Starch (0.1% m/v) waterlogging can mimic stronger reduced conditions compared to normal waterlogging as it has experimentally been proved by Miricescu et al. (2021) that under starch waterlogging, up-regulation of hypoxia-response genes, i.e., alcohol dehydrogenase *(HvADH1)* and hemoglobin *(HvHB)* are much higher compared to normal waterlogging conditions in barley. Our finding also found that under greenhouse waterlogging conditions, normal waterlogging conditions are comparatively slow processes to induce reduced conditions inside greenhouse waterlogging plots than starch waterlogging conditions. The reduction trend in soil redox values (Eh) recorded in our experiment showed that a strong reducing environment (below -500 mV) within 4 days in starch waterlogging treatment, while it took 6-7 days in normal waterlogging for Eh values to reach below 0 mV. Even though this Eh value of soil redox achieved in normal waterlogging is enough to indicate reduced conditions (below -100 mV), it was still a slow process for developing chlorosis symptoms visually

distinguishable in plants under greenhouse conditions. This finding is in line with the results found by Miricescu et al. (2021). Chlorosis symptoms developed much later in normal waterlogging compared to starch waterlogging and were outweighed by new green leaves formed in plants. This might not be the case in the field, but inside the greenhouse, plots are very small, and new leaves in the canopy can make chlorosis assessment challenging or misleading. Our findings strongly indicate that severe stress symptoms must be achieved in a short time. It was verified through our experiments that plants' response under starch waterlogging had a relatively stronger correlation (R= 0.4) than under normal waterlogging (R= 0.1) to plant response under field waterlogging. We can conclude that starch waterlogging (0.1% m/v) is an alternative to normal waterlogging to better simulate field waterlogging conditions. However, one must be careful about the duration of starch waterlogging. Pinpointing the right duration of starch waterlogging would be necessary as the longer the duration, the smaller will be the gap between tolerant and sensitive genotypes. Moreover, the very long duration might be ineffective as it can kill all the plants under study.

This starch methodology might not be necessary when other traits, like root aerenchyma formation, etc., are studied under waterlogging conditions, as some studies have successfully used normal waterlogging methods to study such traits (Liu, M. et al., 2017). Similarly, even with the normal waterlogging method, extending waterlogging duration can help precisely capture differentiation among tolerant and sensitive genotypes, which was achieved in an experiment done by Zhou (2011) in barley. He concluded that waterlogging duration, as long as nine weeks, can help distinctly distinguish tolerant and sensitive genotypes in barley, and he also found consistent results across two years, along with a large effect QTL explaining 50% of phenotypic variation was also identified. In the case of our normal waterlogging methodology, rather all the genotypes tend to be yellow, the distinct chlorotic difference was difficult to achieve in waterlogging duration as long as 4 weeks, and we ended up the experiment. It would, however, be interesting to examine if the results obtained by Zhou (2011) would be achievable also in wheat under normal waterlogging conditions through an extension of the waterlogging period as long as nine weeks or more.

5.2 Is chlorosis a reliable indicator of waterlogging tolerance in wheat?

Another important finding was that the assessment of chlorosis percentage solely is not enough for assessing waterlogging tolerance. Assessment of regrowth would be important, as

genotypes show differential regrowth ability. So, it is also a necessary indicator along with chlorosis percentage while assessing waterlogging tolerance in such screening trials. Having said that, assessment of chlorosis percentage along with growth recovery cannot be fully trusted as the sole selection criteria for waterlogging tolerance in breeding programs as studies reported that wheat genotypes with good green leaf area under waterlogging were found to be sterile and crop yield is an important trait in any crop breeding program (Van Ginkel et al., 1992). So, chlorosis percentage is only one of many indicators that determine waterlogging tolerance in waterlogging tolerance breeding programs. However, the assessment of chlorosis percentage along with regrowth on early breeding programs either in the field or greenhouse screening trails with thousands of lines under study can be reliable indicators to select promising tolerant varieties among the studied.

5.3 Could we establish a method /growth assay to phenotype seminal root angle on germination paper?

As described in the background of the thesis, the QTL region on 6A, associated with differences in chlorosis in MASBASIS, overlaps with a QTL for seminal root angle in durum wheat, and we wanted to investigate if root angle and chlorosis were also connected in MASBASIS. Therefore, a second main objective of this thesis work was to establish a method for screening seminal root angle in wheat. Seminal root angle phenotyping methodology developed in our experiment to study seminal roots of wheat in the early stage is also found to be a promising low-cost, and easy method. This methodology, however, needs some adjustments, as mentioned earlier, and is recommended to be used in further experiments with a large number of screening populations to validate whether our initial findings are reproducible or not.

5.4 Was seminal root angle associated with chlorosis and the 6A QTL?

Significant differences in seminal root angle among the contrasting haplotypes on *QTL6A.2* for chlorosis were observed in our study. This finding is quite intriguing because there are various understandings of seminal roots with respect to waterlogging conditions. Studies mentioned that seminal roots of wheat tend to cease their growth or even may die under hypoxic conditions (Malik et al., 2001; Thomson et al., 1990). However, this depends upon the severity of waterlogging events because under less severe waterlogging conditions, plants have the ability

to regrowth of seminal roots, and this varies among genotypes of wheat (Sundgren et al., 2018). Moreover, aerenchyma in seminal roots longer than 100 mm can hardly convey oxygen to the roots' apex (Thomson et al., 1990). These notions make the significance of seminal roots on waterlogging tolerance to be quite delusive. The physiological advantage of seminal roots, specifically on waterlogging tolerance, also seems to be unclear. One reason would have been that, as seminal roots define RSA, wider seminal roots help promote lateral roots on wider space that help capture residual oxygen from wider space. However, in our experiment, we found that less chlorotic groups (Haplotype 3) were the ones with narrow seminal root angle, and more chlorotic groups (Haplotype 1) had wider seminal root angle. So, this reason doesn´t apply to waterlogging tolerance conditions shown by less chlorotic groups of wheat genotypes in our study. Some studies have mentioned that adventitious roots comes out of the water surface for oxygen intake as an adaptive mechanism (Jia et al., 2021). It could have been that genotypes with narrow seminal roots develop profuse adventitious roots that come out of the water surface and help in oxygen intake. But, in our experiment, we are not sure whether those surface roots developed in experiment 2, are adventitious roots or not. Even if they were adventitious roots, it doesn´t hold true in our study as those less chlorotic genotypes were the ones that develop very few or no of these roots. It seems that less chlorotic genotypes either were less stressed due to other inherent root traits helping them in waterlogging tolerance, and they need not necessarily produce those roots. This indicates there is some other mechanism in roots yet to be explored that help those less chlorotic genotypes for higher tolerance to waterlogging. One potential reason can be that those genotypes which have narrow seminal root angles also have narrow stele size of seminal roots as narrow stele size lower oxygen demand through enhanced longitudinal oxygen diffusion (Sundgren et al., 2018), however extent to which seminal roots remain functionally active during waterlogging conditions is quite unclear. Another reason can be that those genotypes with narrow seminal roots develop roots to greater depth and utilize nitrogen resources that are leached in the deeper soil profile, and high nitrogen conditions help plants in waterlogging stress tolerance, and they might have shown less chlorotic symptoms. An experiment done by Trought and Drew (1980), found that wheat seedlings pre-treated with nitrogen retain a considerable amount of green shoot biomass and root biomass under fifteen days of waterlogging compared to untreated ones. But nitrogen leaching must not be an issue in greenhouse waterlogging conditions. So, this must not be a plausible reason in our case. It is understandable that chlorosis percentage is a response symptom developed in waterlogging stressed plants outweighing various adaptive mechanisms of plants rather than a single adaptive mechanism. There must be various underlying genes that

collectively are responsible for conferring chlorosis symptoms in plants, and these genes might share linkage with genes that are responsible for seminal root angle. Further studies for the functional characterization of such genes are important to study the underlying genetic mechanisms of chlorosis percentage under waterlogging and seminal root angle in wheat. Before that, validation to our obtained results through further experiments would be necessary.

Our findings on seminal root angle seems to be contrasting with the normal notions that are mentioned in various literature, as discussed in the previous paragraph. But root system architecture is highly context-dependent. Root ideotypes explained for one type of environment do not necessarily need to be true for all conditions of the environment. Studied wheat genotypes are genotypes with a history of their development in Nordic environments that comparatively have been developed under less rainfall or less wetter conditions. Selection and development of these genotypes under Nordic regions might have indirectly selected those genotypes with narrow root architecture compared to those developed under high rainfall areas. Fortunately, these genotypes also confer a considerable degree of waterlogging tolerance, which is proved by a field experiment done by Sundgren (2018) and in our greenhouse experiment. It is, in fact, beneficial to realize that those genotypes with narrow seminal root angles that confer narrow and deep root system architecture are less chlorotic /more tolerant under waterlogging conditions because genotypes with deep root system architecture are beneficial under drought conditions, too.

6. Suggestions for future experiments

Developed greenhouse waterlogging methodology using plastic boxes along with cone-tainers with small dimensions might be demanding while screening for a large number of populations for waterlogging tolerance. It is, however, possible to increase the dimension of plastic boxes and use bigger size pots instead of cone-tainers inside plastic boxes. It is advisable to decrease the depth of starch waterlogging used in this method due to the energy and labor required to heat necessary volumes of water to dissolve the starch. We used an 8.5 cm depth of starch waterlogging measured from the soil surface, this depth is expected to be unnecessarily higher depth than needed. A depth of 3-5 cm from the soil surface would be enough to create ample reducing environment.

In the case of the seminal roots phenotyping method developed it is advisable to use two or one seed per germination paper to effectively capture seminal root growth in germination paper.

Polythene cover can be replaced by acrylic plates, which make the experiment easier and undisturbed. Image J software to assess seminal root angle is found to be easy software. One can access 8-10 roots per minute using this software. It is, however, advisable to be careful while assessing seminal root angle, as off-type and very short root phenotypes must be discarded to get valuable measurements.

7 Future Implications

The developed greenhouse waterlogging method is a valuable methodology for efficiently screening a large number of wheat genotypes for waterlogging tolerance. It's cost-effective compared to field trials and requires minimal resources like plastic boxes or pots. This method can be used throughout the year, making it particularly beneficial in regions with short wheat-growing seasons. It saves significant time by allowing preliminary selection of promising genotypes from large populations during off-seasons, which can then be further evaluated in subsequent field trials during the main season. This also saves the high amount of resources needed for field screening by narrowing down promising genotypes from large population. This methodology can also be applicable to other crops for screening where chlorosis percentage indicates waterlogging tolerance, although some modification of the setup might be needed to accommodate the specific crop. Additionally, two contrasting haplotypes on *QTL6A.2* for chlorosis were found to have significant differences in mean chlorosis percentage in this methodology, too. This finding is important as this methodology also can be used as an alternative method to field screening for further validation of this QTL.

Seminal root phenotyping using the seed germination growth assembly is a fast, low-cost, and easy method. The significant difference in seminal root angle in contrasting haplotypes on *QTL6A.2* for chlorosis, if found to be validated through further experiments using larger populations, will be very important findings for breeders and researchers. As seminal root traits are proxy, screening for waterlogging tolerance can be done through phenotyping of seminal root angle. This screening methodology can be an even more low-cost and easy screening method compared to the starch waterlogging method. Our findings indicated that the haplotype associated with less chlorotic/more tolerant genotypes corresponded to the haplotype for narrow seminal root angle. Breeders are trying to optimize root ideotypes suitable for varying environmental conditions, and root traits beneficial under different environmental settings like drought and waterlogging hold substantial appeal for selection in breeding programs because

selection for drought tolerance would inadvertently select for waterlogging tolerance and vice-versa. This is highly beneficial in wheat improvement programs targeting climate resiliency.

8 Conclusion:

Greenhouse screening of waterlogging tolerance can be a faster and more cost-effective alternative to field trials, given a sufficiently high correlation between the results from field and greenhouse screenings. In the present study, we developed a method to mimic waterlogging conditions with a 0.1% (m/v) starch solution. The relevance of the method was tested by screening starch-waterlogged wheat seedlings for leaf chlorosis, and the results showed good correlation (R= upto 0.4) to chlorosis, assessed in waterlogging field trials (Sundgren, 2018). Additionally, the effect of the *QTL6A.2* haplotype on chlorosis, identified in the field trials, was also significant in the starch-waterlogged plants. These results strongly imply that starch (0.1% m/v) waterlogging is found to be a promising method, although we recommend that the methodology must be further verified through further experiments to test its repeatability and consistency. If consistent results are achieved, this methodology can be a good alternative to field waterlogging trials.

Similarly, germination paper-based seed growth assembly developed as seminal root phenotyping methodology in our experiments also found to be promising. Results obtained for seminal root angle difference in contrasting haplotype on *QTL6A.2* needs urgent verification through further experiments, and if this result is found consistent, then it will open new avenues to understand the underlying genetic mechanism of seminal root angle for waterlogging tolerance. To conclude, Norwegian spring wheat cultivars, which are less chlorotic under waterlogging stress, are the ones that are likely to have a narrow seminal root angle, which infers root growth to a deeper soil profile, which is, in turn beneficial trait for drought conditions. However, both findings are highly recommended for further investigations for validation.

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Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway