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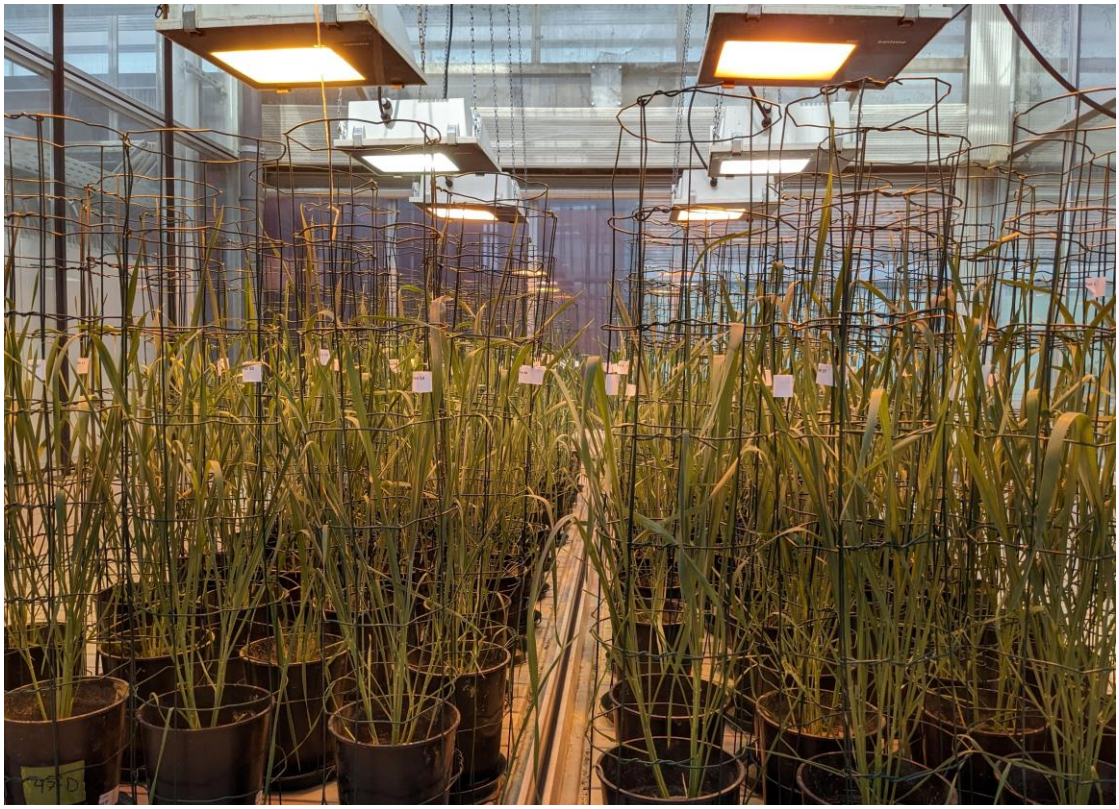

Winter wheat exudates

Improving wheats resilience to drought

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Master thesis, 30 hp
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Höstvetesutsöndringar
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Abstract

Wheat (*Triticum aestivum*) is a crop that dominates the diets of about 35% of the world's human population. But wheat yields can be severely affected by drought. Therefore, in this experiment, root exudation of winter wheat was compared to find out how exudation changes during drought stress. This was done by using control plants that were compared to plants that experienced 8 days of drought and subsequently, 3 days of rewetting. Moreover, it was explored which one of two wheat genotypes, Capo or Aristaro, is better adapted to drought by measuring plant physiology and if beneficial rhizosphere microorganisms could help alleviate drought in wheat. Exudations were analysed using a photometer. It was found that drought and rewetting treatment influenced shoot dry weight, shoot water content, relative chlorophyll, as well as exuded phenols, sugars and amino acids. Aristaro was found to be more drought tolerant, because Aristaro plants had higher and more stable shoot water content, lower non-photochemical quenching (NPQ(T)), and exuded more phenols and amino acids, which could help recruit plant-growth promoting rhizobacteria. However, Capo might be able to avoid drought through early maturity and can therefore be used in regions where drought occurs later in the year. The implications of this experiment are therefore useful for improving wheats resilience to drought and food security with use of microorganisms.

Key words: drought, PGPR, exudates, agriculture, food security

1. Introduction and Background

1.1 Background

Human population is expected to rise to over 9 billion by 2050, and with that, problems like larger food insecurity arise (Hakim et al. 2021). Wheat (*Triticum aestivum*) is a crop that dominates the diets of an estimated 35% of the world's population (El Sabagh et al. 2021; Grote et al. 2021). However, wheat yields can be severely affected by drought. In fact, drought is the most serious abiotic stressor affecting crop productivity (Xu et al. 2019; Cheng et al. 2021) and can reduce wheat grain yield by up to 70% (Ahmad et al. 2018).

Winter wheat (*Triticum aestivum*) yields in Eastern Austria are already mostly limited by drought stress (AGES 2022). As atmospheric temperature is predicted to rise further, more extended periods of drought can be expected (Gudmundsson and Seneviratne 2016; Khan et al. 2019). Every fourth month until the year 2100 could become drier than today, and in summer months June, July and August extreme dry periods could increase (ZAMG 2015).

Certain microorganisms might help alleviate drought for the plants (Hakim et al. 2021). Plants are thought to recruit these microorganisms through the exudation of specific root exudates, which are organic compounds that beneficial microorganisms can feed on or compounds that suppress the growth of harmful microbiota (Kang, Peng and Xu 2022; Bakker et al. 2013; Iannucci et al. 2021). The beneficial microorganisms in return help the plant by for example modulating hormone pools or increasing root surface area (Ahmad et al. 2022; Hakim et al. 2021; Herpell et al. 2023). However, it is not fully understood how the quantity and composition of winter wheat root exudates responds to drying and rewetting cycles and therefore also how this influences potential help by beneficial microorganisms.

1.2 How do plants respond to drought?

Drought stress in plants leads to damage to chlorophyll and the photosystem, as well as decreased biomass and crop yield losses (Khan et al. 2019; Camaille et al. 2021). This water shortage has effects on all developmental stages of the plant, but flowering and grain filling seem to be the most affected stages (Camaille et al. 2021). Roots continue to grow in search of water, while shoots are restricted (Ahmad et al. 2018). Drought also decreases cell turgor (the pressure of the cell against the cell wall) and water content in plant tissues (Camaille et al. 2021). This leads to a higher concentration of the cell's components, which can inhibit enzymatic activities, and decrease water flux from xylem to cells (Camaille et al. 2021). This in turn inhibits cell elongation and mitosis (cell division), and therefore, plant growth is restricted by drought (Camaille et al. 2021).

To limit water loss, plants will adjust their osmotic potential by accumulating soluble molecules, which helps keep water inside of the cell (Camaille et al. 2021). Osmo-protectants like sugars, the quaternary ammonium compound glycine betaine and amino acids, contribute the most to osmotic adjustment, especially total sugars (Ahmad et al. 2018). In wheat, the content of soluble sugars like glucose may increase up to 80% after a seven-day drought stress period (Camaille et al. 2021; Rorat 2006). Photosynthesis is also affected during drought stress; in fact, it changes the fastest during drought stress (Camaille et al. 2021). Stomata close because there is less water in the guard cells, lower humidity in the environment, or because of phytohormones like abscisic acid (Camaille et al. 2021). Diffusion of CO_2 is also affected: not only is stomatal conductance limited, but also mesophyll conductance can be limited (Camaille et al. 2021). This limits CO_2 influx, which decreases carbon assimilation and therefore, biomass

accumulation (Camaille et al. 2021). Osmotic adjustment can permit stomata to stay partially open and fix CO_2 (Ahmad et al. 2018).

There is also photoinhibition: it occurs when the efficiency of photosynthesis decreases and radiation damage occurs, because of too much light (Camaille et al. 2021). As carbon assimilation is limited through stomatal closure, leaves absorb more light energy than can be used in photosynthesis (Camaille et al. 2021). Drought even leads to interruptions of protein synthesis, and proteins like Rubisco that are involved in photosynthesis, decrease in activity and content (Camaille et al. 2021). The integrity of chlorophyll molecules can also be affected, as chlorophyll can get photo-oxidised and affected by reactive oxygen species (ROS), due to over-reduction in the electron transport chain (Camaille et al. 2021). Higher levels of reactive oxygen species than in plants that don't encounter drought stress are produced, because more electrons leak from the photosynthetic electron transport chain directly to O_2 (Camaille et al. 2021). ROS are usually hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), singlet (O), or hydroxyl radicals (HO) (Camaille et al. 2021). These ROS can damage membranes and macromolecules. The plant reacts to this by increasing the abundance of antioxidant enzymes and an intensification of non-enzymatic antioxidant systems, such ascorbate, and the tripeptide glutathione (Camaille et al. 2021).

Proline is an amino acid that can increase 90% in concentration after a seven-day drought period (Camaille et al. 2021). Especially wheat accumulates proline more than other osmo-regulators, because during drought, proteins can collapse in the grain filling stage (Ahmad et al. 2018). Proline only slightly contributes to osmotic adjustment, and its main purpose is to protect cell functions, membranes and organs from damage due to free radicals accumulating during stress periods (Ahmad et al. 2018; Camaille et al. 2021).

1.3 How do plants stimulate microorganisms?

1.3.1 Introduction to root exudates

Roots are the main meeting point between plant and soil and affect greatly the efficiency with which plants can acquire water. How the root system develops in terms of root morphology and architecture, depends on genetics of the plant, soil type, nutrient, and water availability, and on soil and rhizosphere microbial communities (Iannucci et al. 2021). The soil volume influenced by root activity (the rhizosphere) contains much more microbial biomass than non-rooted soil because the plant roots release organic compounds that shape a carbon rich environment that microorganisms can feed on and that make microorganisms proliferate (Bakker et al. 2013, Iannucci et al. 2021). The plants release low-molecular weight exudates, such as carbohydrates, secondary metabolites, organic and amino acids to shape the rhizosphere and the microbial community to their benefit, and to mobilize limiting nutrients and detox heavy metals (Hakim et al. 2021; Bakker et al. 2013; Sun et al. 2021). Exudates are released especially at the tip of the root and lateral branches (Upadhyay et al. 2022), and are released at higher rates at the root tip than at mature regions (Jones et al. 2009).

The exudates differ between plant species, and so does the microbiome in the rhizosphere (REF). Exudates and microbiomes differ even within genotypes of the same plant species (Bakker et al. 2013; Vives-Peris et al. 2020). Furthermore, exudates are influenced by plant functioning and hence the age of the plant, herbivores, and roots of neighbouring plants (Vives-Peris et al. 2020). Exuded molecules also depend on soil and surrounding microbial community (Bakker et al. 2013; Upadhyay et al. 2022). Root exudates can be released passively or actively

by the plant (Huang et al. 2014), and plants secrete 5-21% of photosynthesis products as exudates (Upadhyay et al. 2022).

1.3.2 Beneficial microorganisms

A special group of soil life is called plant-growth-promoting-rhizobacteria (PGPR). They get recruited from the many microorganisms that are already living in the soil, which means that the soil itself is a major factor influencing the microbiome in the rhizosphere (Bakker et al. 2013). In contrast to plants, plant-growth-promoting-rhizobacteria (PGPRs) can shield themselves from drought by thickening their cell walls, going dormant and forming spores, as well as accumulating osmolytes and producing a biofilm with exopolysaccharides (EPS) (Hakim et al. 2021). The PGPR can also help plants mitigate negative drought effects through initiating a variety of physiological and anatomical adaptations in the plants (Camaille et al. 2021). The PGPR can:

- mobilize nutrients like phosphorus (P), zinc (Zn) and iron (Fe) (some PGPR also have the capacity to fix atmospheric nitrogen), that the plants can use for growth (Hakim et al. 2021),
- trigger the production of abscisic acid (ABA) by the plant (Hakim et al. 2021), which strengthens tolerance to drought (Hanaka et al. 2021). ABA is an abiotic stress response hormone and is involved in regulation of the stomatal closure and root water uptake (Ahmad et al. 2022; Begum et al. 2019; Hakim et al. 2021).
- produce indole-3-acetic acid (IAA), which leads to a higher level of this phytohormone in roots and shoots of the plant and improves plant root surface area and yield (Ahmad et al. 2022; Hakim et al. 2021; Hanaka et al. 2021).
- release additional cytokinins into the rhizosphere, which stimulates plant growth (Hakim et al. 2021), However, cytokinins can have negative impacts on drought tolerance as well (Camaille et al. 2021).
- help accumulate proline content, which protects the organs and cellular functions (Camaille et al. 2021, Hakim et al. 2021),
- lead to less oxidative stress for the plant (Camaille et al. 2021; Hakim et al. 2021),
- lower the plants internal level of "stress ethylene" that can otherwise lead to senescence and growth-arrest. The microbes do this by degrading the precursor of ethylene, the amino acid ACC, with ACC deaminase (Hakim et al. 2021; Herpell et al. 2023; Huang et al. 2014).
- form a biofilm that aggregates 2-3 times more soil around wheat roots under water stress leading to an increase in water use efficiency by the plant (Camaille et al. 2021; Hanaka et al. 2021),
- control pathogens (by producing antibiotic compounds) that could otherwise infect the plant (Hakim et al. 2021)
- change elasticity of the root cell membrane and increase the root surface area and length, which helps the plant to withstand drought (Ahmad et al. 2022).

It should be noted, that not only bacteria can be beneficial to the plant, but also arbuscular mycorrhizal fungi (AMF) can affect plant functioning (Tang et al. 2022). AMF can help in similar ways as PGPR (Cheng et al. 2021; Tang et al. 2022).

1.3.3 Specific root exudates analysed in this report and why they were chosen

The specific substances found in root exudates by wheat plants, are first and foremost sugars; they are the most common exudate because they attract PGPR as they are a carbon source for the microorganisms (Upadhyay et al. 2022; Huang et al. 2014). Moreover, amino acids are

released by the plant roots, as they can be recognized by the chemoreceptors of microorganisms (Upadhyay et al. 2022). Organic acids (malic, citric, oxalic, succinic, pyruvic acids and others) are also exudated, and for the microbes, they act as nutrients (Upadhyay et al. 2022; Iannucci et al. 2021). Exuded phenolic compounds and derivatives act as signals to the microbes (Huang et al. 2014). Furthermore, exudates with high molecular weight like proteins and complex carbohydrates are exuded actively (Upadhyay et al. 2022). Arabinogalactan proteins for example are important players mediating root-microbe interactions. These proteins are released as part of mucilage at the plant root tips (Huang et al. 2014). Interactions between plant and microbes are therefore mediated with root exudates, but also by other means of interaction (Upadhyay et al. 2022). Further studies are required to know the exact mechanisms behind PGPR recruitment (Upadhyay et al. 2022). For this study, it was decided to assess exuded dissolved organic carbon (DOC), phenolic compounds, total sugar, and amino acid exudation.

1.4 Experimental outlook

Exudation patterns of winter wheat (*Triticum aestivum*) were examined to find out how exudation changes during drought stress and rewetting, as well as if there are differences in exudation between genotypes. For this, exudates of two winter wheat genotypes (Capo and Aristo) were compared, during different stages of drought and rewetting. These two genotypes are supposedly well-adapted to drought (H. Grausgruber, *personal communication*), and Capo is the most cultivated variety of organic wheat in Austria (Probstdorfer Saatzucht 2023). During the drought phase, plants were kept at 20% of soil water holding capacity. During rewetting, the drought stress treated plants were rewatered to 50% soil water holding capacity. Dissolved organic carbon (DOC), phenolic compound, total sugar and amino acid exudation was compared, as well as shoot dry weight, shoot water content, non-photochemical quenching (NPQ(T)) and relative chlorophyll.

2. Materials and methods

For this experiment, 50 plants of genotype Capo, and 50 plants of Aristaro were kept in a single pot per plant. 10 plants per genotype were harvested at 4 timepoints, respectively. Furthermore, half of the plants were subjected to a drought and rewetting phase, to compare them with the other half of the plants, the controls. Figure 1 describes different ways of conducting such an experiment, and explains that the way this experiment was conducted, and was chosen for its reliability.

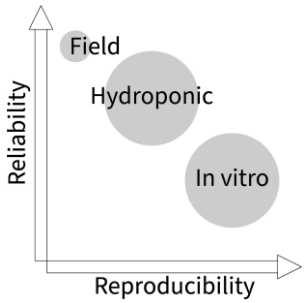


Figure 1: Image about different techniques for collection of root exudation. Drawing by the author was inspired by Vives-Peris et al. (2020). The image compares different ways of conducting a root exudation experiment. The experiment of this report can be put near the field circle in this figure, as it was conducted in a greenhouse with soil substrate.

2.1 Location, growth conditions and plant material

The experiment was conducted in a research greenhouse chamber (located at N: 48,3197°, E: 16,07039° (WGS 84)) at 180 m above sea level (Amt der NÖ Landesregierung-NÖ Atlas 2023) in Universitäts- und Forschungszentrum Tulln, Boku Standort Tulln, Konrad-Lorenz-Straße 24, 3430 Tulln an der Donau. The floor area of the chamber was 6m by 4m. The plants were kept between 10-26°Celsius, with an average temperature of 20°Celsius and 12h photoperiod in the greenhouse. Average relative humidity was 50%. There was additional lighting with HPS lamps. For measuring average radiation at pot level, a quantum sensor (by Spectrum Technologies, Inc.) was used. It measures photosynthetic active radiation (PAR) in µmol (photons) m^{-2} s^{-1}. PAR measured at the top of the plant canopy (without plant parts shading the sensor) was 470 µmol (photons) m^{-2} s^{-1}, with a standard deviation of 130 µmol m^{-2} s^{-1}. The intensity of natural radiation outside of the glasshouse was recorded every 12 minutes by external light sensors.

Seeds of two different winter wheat genotypes were used for this experiment. Capo is a variety established in 1989 by Probstdorfer Saatzucht (2023). It has a high tillering capacity and is intended for organic agriculture (Probstdorfer Saatzucht 2023). It is the most cultivated variety of organic wheat in Austria and is supposed to be especially drought tolerant (Probstdorfer Saatzucht 2023). Aristaro is a variety by Die Saat and is also intended for organic agriculture and drier areas (Die Saat Aristaro 2023). The plants were germinated in heat sterilized compost in multipots on 14.11.2022 at room temperature for 9 days and subsequently put into a vernalisation chamber at 4°C. 50 single plants per genotype were potted into one pot each on 19.1.2023. The pots were filled with soil described below.

2.2 Soil

Agricultural soil was sampled from an agricultural field close to Melk (Lower Austria). It was then transported to Boku in Tulln (Lower Austria), left drying at ambient conditions. Bigger

soil aggregates were smashed with a hammer into smaller pieces and then sieved through a 2mm sieve. This was done to ensure that the soil used in the experiment was as homogenous as possible. The agricultural soil´s pH was 6.2. It consisted of 1.32 g organic carbon per 100 g of soil, and 14.4 g sand, 56.2 g silt and 29.4 g clay per 100 g of soil (Duboc et al. 2022). The soil was then mixed with quartz sand, to support drainage during irrigation, the final mix was 40% agricultural soil with grain size diameter <2mm, 40% quartz sand, and 20% agricultural soil with diameter 2-4mm (percentages based on dry weight). Finally, pots that could hold a 2-litre volume were used and filled with 1.8 kg of this final substrate mixture and one plant each. Soil water holding capacity (WHC) was used to estimate how much water could be stored in the soil as it can reflect the soils' ability to provide water for plant growth (Zhang et al. 2021). At 100% water holding capacity, the final soil mix could hold 0.3 g water per g soil.

2.3 Experimental timeline

After having been potted on 19.1.2023, the plants were also fertilized until the start of the drought stress phase with a solution modified after Middleton and Toxopeus (1973). 10 plants per genotype respectively, were harvested after 50 days on 10.3.2023 (at 20% water holding capacity of the soil (WHC)), 14.3.2023 (at 20% WHC), 15.3.2023 (rewetted, at 50% WHC) and 17.3.2023 (rewetted, at 50% WHC). This timeline is also visible in figure 2 below. Figure 3 shows the soil water content throughout the days of the experiment. Control plants had on average 0.13 grams of water per g dry weight soil, while drought treated plants had a soil water content of 0.04 grams of water per g dry weight soil until day 8. After harvesting on day 8, the remaining drought treated plants were rewetted to the same WHC as control plants.

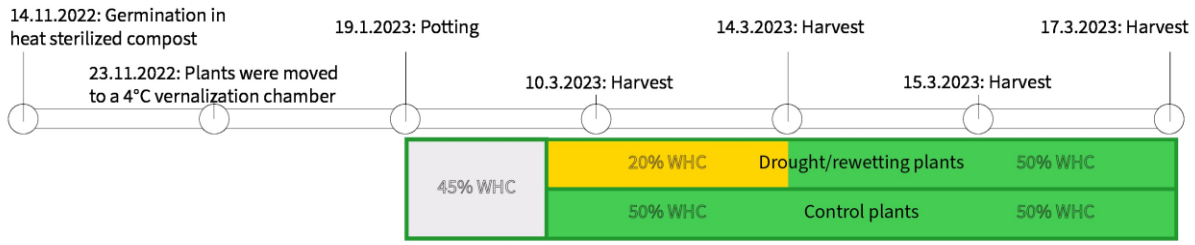


Figure 2: The chronological order of the experiment, visualized. WHC: water holding capacity of the soil that the plants were growing in. Yellow symbolizes the timeframe where drought/rewetting treated plants were held at 20% WHC, green symbolizes the timeframe where plants were held at 50% WHC. Drawing by author.

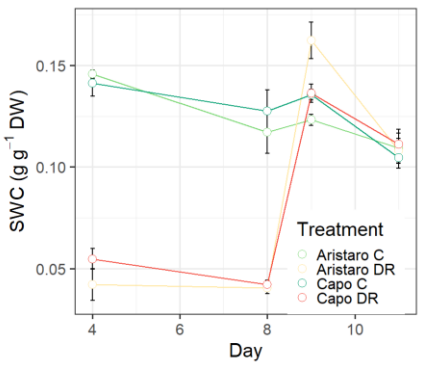


Figure 3: Soil water content (SWC) in g per g dry weight (DW) on days 4, 8, 9 and 11 of the experiment. Ari-C: Aristaro Control, Ari-DR: Aristaro drought stressed (day 4 and 8) and rewetted

(day 9 and 11), Cap-C: Capo control, Cap-DR: Capo drought stressed (day 4 and 8) and rewetted (day 9 and 11), n = 5, error bars indicate standard errors.

All plants were held at 45% WHC before splitting them into two groups for the experiment, and then during the experiment controls were held at 50% WHC. In the drought treatment, during the drying phases, the plants were held at 20% WHC, and then during rewetting at 50% like the controls. The controls were harvested on the same day as the drought/rewetting treatment group, but they did not encounter drought. The experiment was terminated with the last plants being harvested and sampled. This was at the beginning of the heading and flowering stage. For better comparability, a Zadoks growth scale is used, as it is a way of quantifying the developmental stage of a crop in an internationally recognised and standardised way by providing a thorough description of the plant (Government of Australia 2018; Begcy and Dresselhaus 2017). Aristaro had reached the Zadoks growth stage 50 at the end of the experiment, while Capo had already reached 59 (Zadoks et al. 1974). At each harvest, exudates like sugars, amino acids, phenols, and DOC were measured, and furthermore, non-photochemical quenching (NPQ(T)), relative chlorophyll, shoot dry weight and shoot water content were measured.

2.4 Filtering and analysis of plant parameters

In the greenhouse, a MultispeQ V 2.0 (by PhotosynQ INC. East Lansing, MI 48823 USA) was used to measure non-photochemical quenching (NPQ(T)), and relative chlorophyll content on the wheat plants leaves. Those measurements were done on the day of harvest, but before harvesting. Counting from the top, the second leaf on the main shoot was measured, respectively.

At the respective harvest timepoints, the wheat plants roots were washed to remove the soil, then soaked 5 minutes in bacteriostatic solution (5 mg Micropure (Katadyn GmbH, Morenfelden-Walldorf, Germany) per litre of HQ water) to remove the exudates coming from broken roots. After that, the wheat plants roots were soaked for 2 hours in bacteriostatic solution (5 mg micropure per litre of HQ water) to avoid immediate consumption of exudates by microorganisms (Canarini et al. 2019). This was done under the greenhouse conditions described before. The sampling process is also explained visually in figure 4. To be noted is that not all soil could be removed from the plant base, as the plants was germinated in a small volume of compost which proved impossible to wash out.

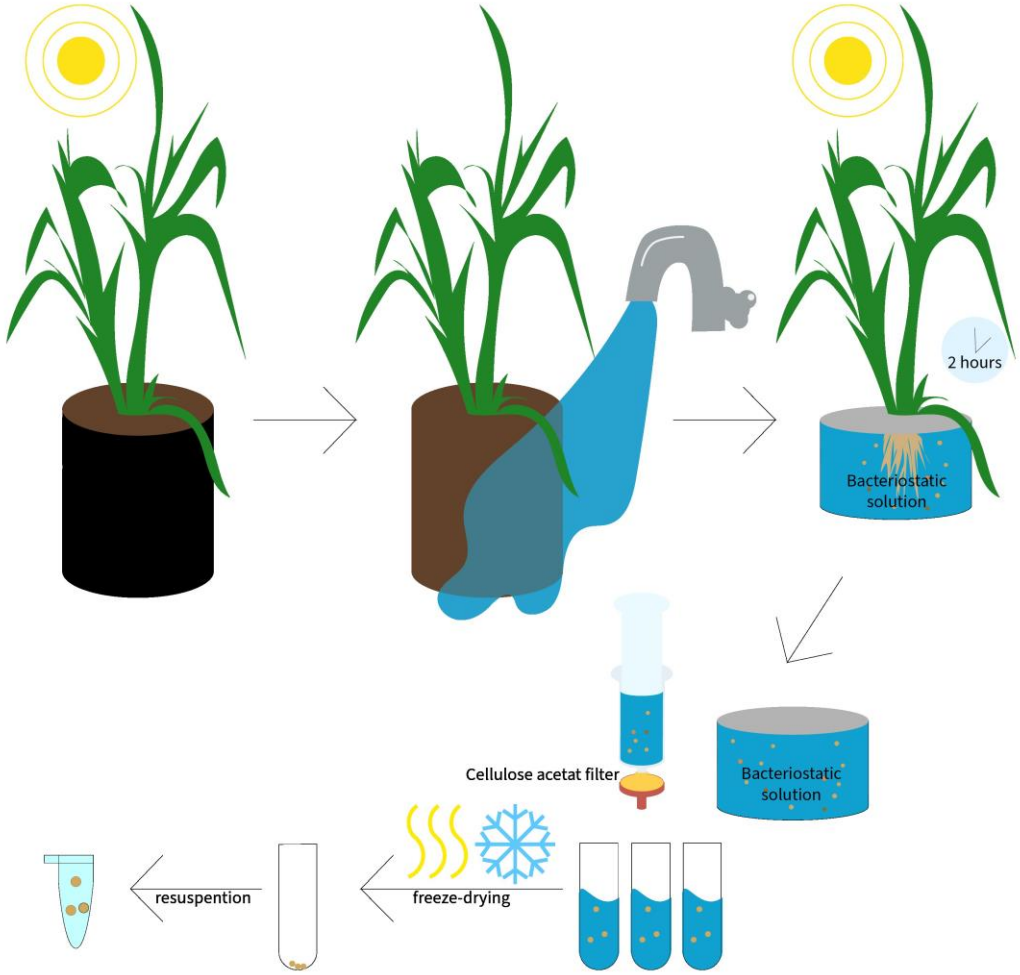


Figure 4: Process of harvesting root exudates. At first, the soil surrounding the roots is washed away, then plants are briefly soaked for 5 minutes in bacteriostatic solution to remove exudates from broken roots, and finally soaked for 2 hours in bacteriostatic solution to collect root exudates. Afterwards, the bacteriostatic solution containing the exudates is filtered, freeze-dried, and resuspended. Drawn by author.

The bacteriostatic solution containing the exudates was then filtered through Machery-Nagel Chromafil CA-20/25-S cellulose acetat 0.2 µm filters into three aliquots of 45 ml each and stored at -20° Celsius (figure 5). The first aliquot was later put into a -80°C freezer and then freeze-dried with a Christ alpha-1-4-LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH, An der Unteren Söse 50, 37520 Osterode am Harz). It was evaporated to dryness under a vacuum, and the residue was dissolved in 1 mL of HQ water and was used for further analysis.

Figure 5: Laboratory filtering with the cellulose acetat filters. Picture taken by author.

All analyses were conducted with 96-well plates and a TECAN Infinite M Nano+ Photometer (Tecan Trading AG, Switzerland). Samples were always pipetted twice into well plates, next to each other, to calculate the mean of the two wells. The absorbances were measured as follows: DOC analysis was conducted according to Oburger et al. (2022), where 250 µl of the raw exudate sample was pipetted onto a UV-Star Greiner 96 well plate, and absorbance was measured at 260nm. Carbohydrates were analysed following Hansen and Möller (1975), where carbohydrates are dehydrated with a concentrated H_2SO_4 to form a furfural which is then condensed with anthrone and forms a green colour complex. Phenolics were analysed according to Ainsworth and Gillespie (2007). This reaction works by transferring electrons from phenolic compounds, and in general other reducing substances, to form a blue complex. Finally, total free amino acids were analysed following Jones, Owen and Farrar (2002). This assay works by having free amino acids react with OPAME (o-phthaldialdehyde and 3-mercaptopropionic acid) which leads to a fluorescing solution, which can be measured at 340nm excitation and 450nm emission wavelength. For this amino acid assay specifically, root exudates were diluted 8-fold.

Fresh shoot and root weight was also recorded at harvest. Roots and shoots were separated from each other with scissors and then dried on paper tissue and weighted. Also, the dry shoot and root weight was recorded, after drying in the oven at 70°C. Fresh soil was taken from the plants pots at harvest at 4 different places (1 at the top and 3 from the mid-sides) and dried at 110° Celsius. Both fresh and dry soil weight were also recorded, to be able to calculate soil water content (fresh weight-dry weight)/dry weight).

2.5 Data analysis and methods with R

To calculate exudation from the measurement, first, the average absorbance was calculated from the measured double pipetted sample. Then, this value, minus the value of the vertical intercept, was divided by the incline to get the sample concentration. The vertical intercept and incline are values that got calculated before with help of the used standards, as they are of a known concentration, and combined with their measured absorbance values, they give a slope that can be used for calculating unknown sample concentrations from their respective sample absorbance. This raw sample concentration of exudation in mg/L was converted to µmol exudation/plant/hour. So, this was the raw mg exudation/L to µmol/L by using the molar weight, and then the exudation in µmol/L times 0.4 (sampling volume exudates in L), divided by 2 (because of two-hour sampling time- where the plants released exudates). For calculating shoot water content, dry weight was subducted from fresh weight, to calculate dry matter, and then subducted from 100%, to calculate the water content.

Data analysis was conducted with R statistical software version 4.2.2 (R Core Team (2022)). A three-way ANOVA was conducted with shoot dry weight, shoot water content, non-photochemical quenching (NPQ(T)), relative chlorophyll and exuded dissolved organic carbon (DOC), phenols, sugars, and amino acids as dependent variables. Genotype, treatment, and sampling day were used as independent variables (figure 6 and 7). Tukey's Honest Significant Difference (HSD) was used afterwards to distinguish between significant pairwise differences using a 95% confidence interval. Significance level was 0.05, unless indicated otherwise. R packages used were tidyr (Wickham et al. 2023), readr (Wickham and Bryan 2023), Rmisc (Hope 2022), ggplot2 (Wickham 2016), dplyr (Wickham et al. 2023) and ggpubr (Kassambara 2023).

3. Results

3.1 Plant health parameters estimating impact of drought and genotype

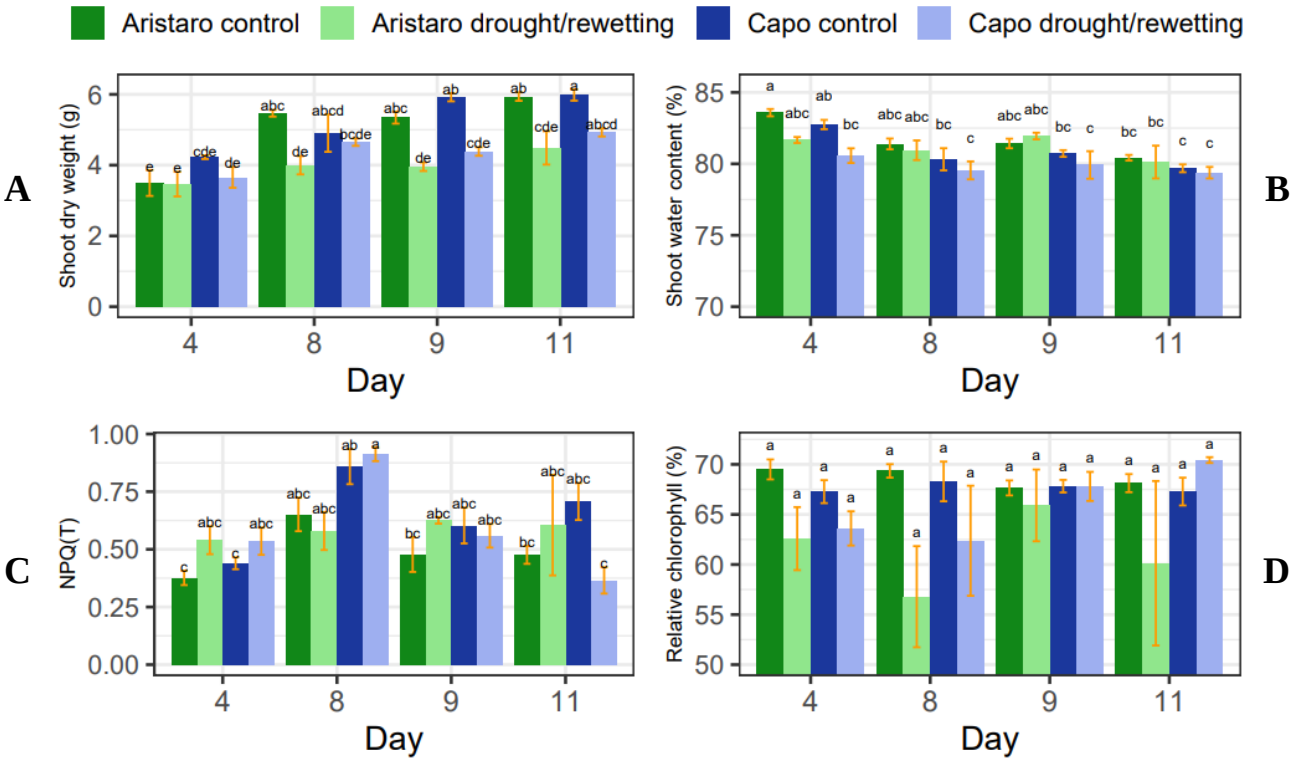


Figure 6: Drying and rewetting and genotype effects on days 4, 8 , 9 and 11 of the experiment. Day 4 and 8 were drought days, day 9 and 11 were rewetting days for the drought/rewetting treated plants. Figure shows shoot dry weight in grams (**A**), shoot water content in percent (**B**), non-photochemical quenching (NPQ(T)) which is a unitless measure of the part of incoming light that is dissipated as heat (**C**), and relative chlorophyll in percent (**D**). Distinct letters indicate significant differences between group means ($p < 0.05$, n = 5, 3-way ANOVA and Tukey HSD). Error bars shown represent standard error (± SE) of the mean.

Shoot dry weight increased from day 4 to 11 in both treatments ($p< 0.001$ for the effect of days) (figure 6A). However, in the drought affected group, shoot dry weight increased slower and therefore, the effect of drought lead to significantly less shoot dry weight ($p< 0.001$ for the effect of drought). Comparing biomasses on day 11, the end of the experiment, drought affected plants had, on average, 21% less biomass relative to controls on that day, from which Aristaro had 24% reduced shoot biomass compared to controls, while Capo had lost 18% compared to controls. In total, compared throughout the whole experiment, Capo lost 16%, while Aristaro lost 21% of biomass relative to their respective control group. Genotype also had a significant effect on shoot dry weight ($p= 0.018$): while Aristaro genotype plants had on average 4.52 g of dry weight, Capo had 4.83 g of dry weight.

Shoot water content was significantly lower in drought treated plants (p-value= 0.006) and as well as in the Capo genotype ($p= 0.0002$) (figure 6B). Upon drought, Capo also lost 1.3% of shoot water content, while Aristaro lost significantly less- 0.7% of shoot water content relative to their respective well-watered controls. Furthermore, there was also a significant effect of day of the experiment ($p< 0.001$).

Non-photochemical quenching (NPQ(T)) varied, and drought treatment did not significantly affect it (p= 0.679). However, NPQ(T) was significantly different between genotypes (p= 0.045). On day 8, the last day of drought, Capo had much higher NPQ(T) than on any other day (p< 0.001 for the effect of days) (figure 6C).

Relative chlorophyll differs significantly between treatments (p=0.007). The mean for drought stressed plants was 63.7%, while the mean for control plants was 68.2%, so drought stressed plants had 7% less relative chlorophyll than control plants in the measured leaves (figure 6D). Genotype differences were not significant.

Root dry weight in gram was also examined, however, no significant differences in genotype or treatment were found (genotype p= 0.225, treatment p=0.201, day p<0.001). Aristaro drought had on average 1.18 g of root dry weight, while Capo had on average 1.14 g of root dry weight. But these results must be treated with caution, as they are not representative, because the compost soil used for germination could not be washed out.

3.2 Exudation of dissolved organic carbon (DOC), phenols, carbohydrates (sugars) and amino acids

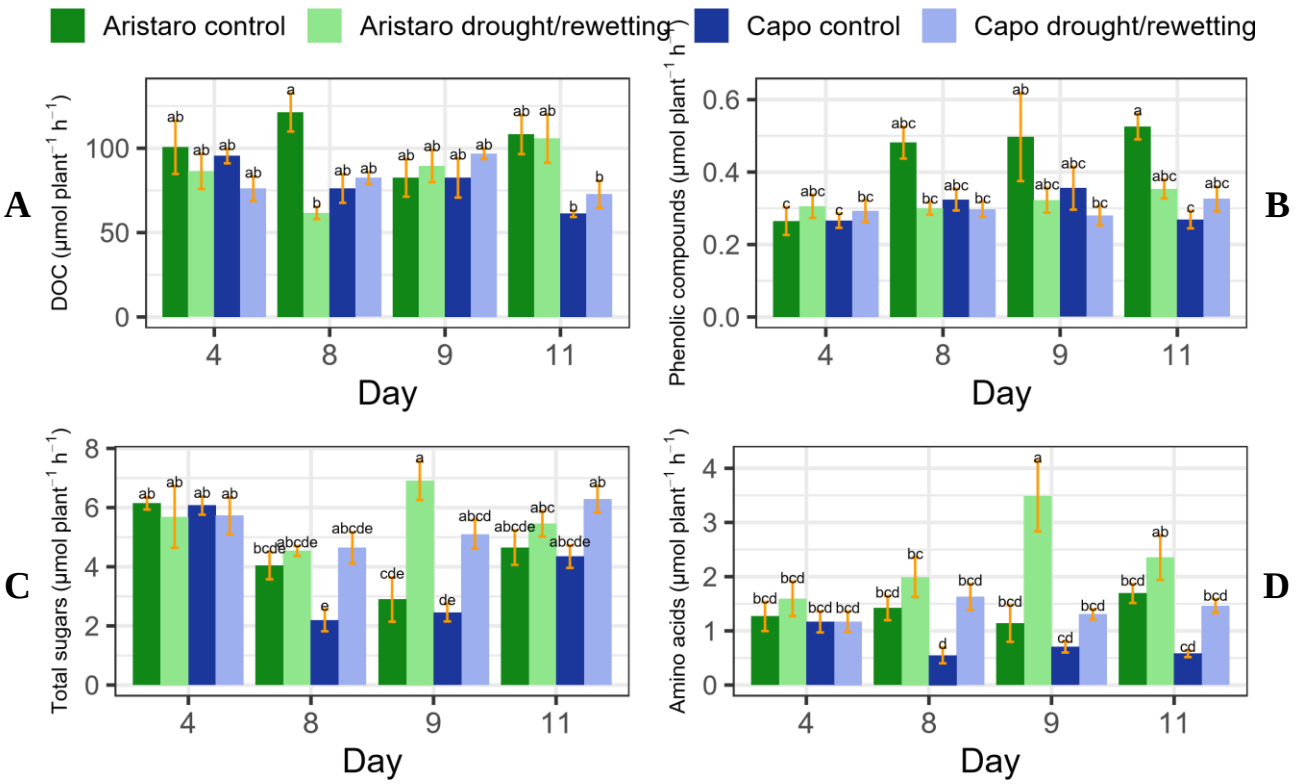


Figure 7: Drying and rewetting and genotype effects on days 4, 8 , 9 an 11 of the experiment. Day 4 and 8 were drought days, day 9 and 11 were rewetting days for the drought/rewetting treated plants. Figure shows exuded dissolved organic carbon (DOC) in µmol per plant per hour (**A**), exuded phenolic compounds in µmol per plant per hour (**B**), total exuded sugars in µmol per plant per hour (**C**) and exuded amino acids in µmol per plant per hour (**D**). Distinct letters indicate significant differences between group means (p < 0.05, n = 5, 3-way ANOVA and Tukey HSD). Error bars shown represent standard error (± SE) of the mean.

For DOC, treatment and day of the experiment were not significant, while genotype differed on especially the last day of the experiment, day 11 (figure 7A). P-value for genotype effect throughout the experiment was 0.004. However, due to a small amount of compost that was impossible to remove before the sampling of exudates, DOC values may not reliable.

For phenols, genotype ($p<0.001$), treatment ($p=0.005$) and day ($p= 0.026$) showed significant differences from each other in the ANOVA. While Capos phenols stayed at a similar level throughout the experiment, Aristaro exuded more and more phenols, especially in the control group (figure 7B). Aristaros phenol levels increased continuously towards the last day of the experiment.

Total sugars were affected by treatment ($p<0.001$) and day of the experiment ($p<0.001$) (figure 7C). Control plants had a low exudation on day 8 and 9, while for the drought/recovery plants, the lowest sugar exudation was recorded on day 8, the last day of drought. Day 9 and 11 sugar exudations were very similar for drought/recovery plants. In general, drought/recovery plants exuded more sugars than control plants (figure 8).

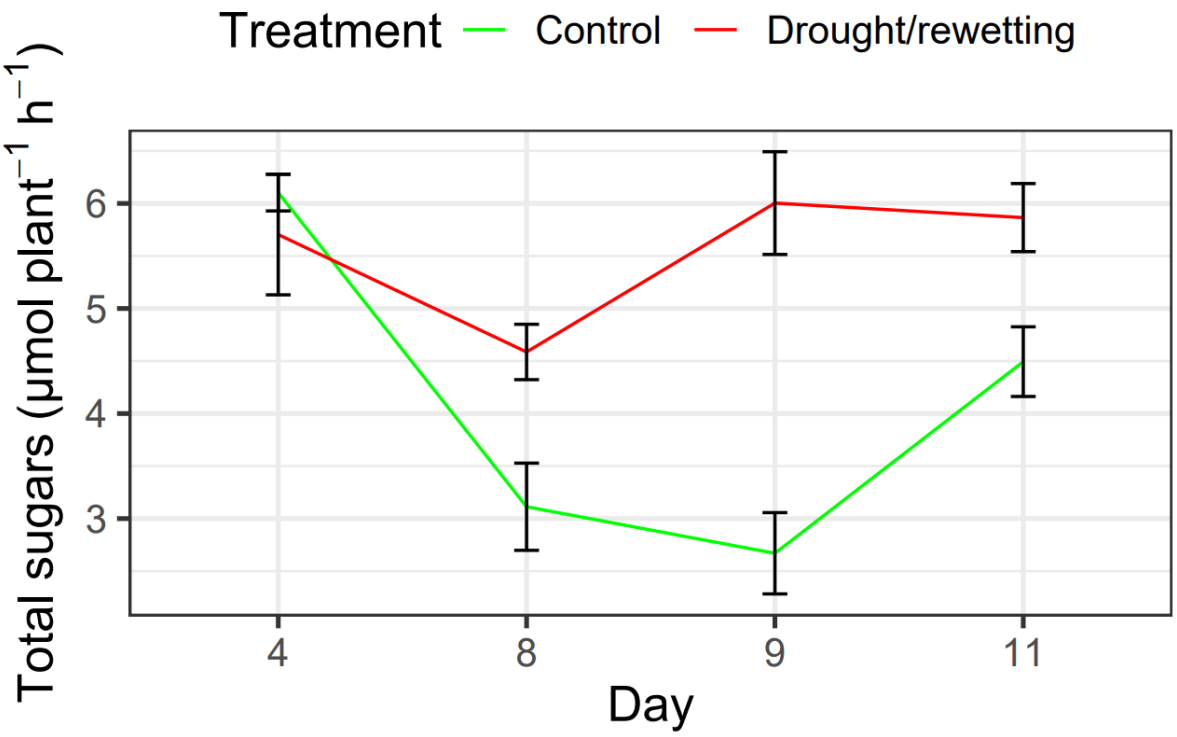
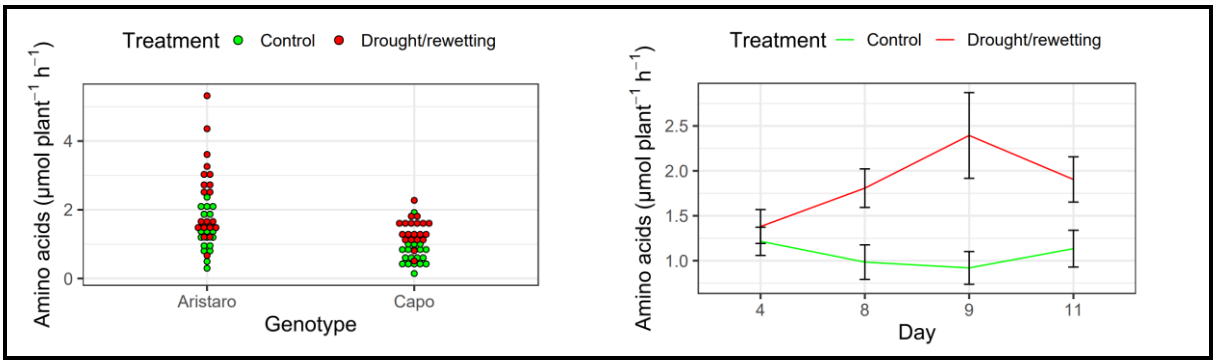


Figure 8: Means of total sugar exudation in µmol per plant per hour comparing control plants (in green) and drought/rewetting plants (in red). Day 4 and 8 were drought days, day 9 and 11 were rewetting days for the drought/rewetting treated plants. Error bars represent standard errors (± SE) of the mean.

Amino acids differed between genotypes ($p<0.001$) and treatment ($p<0.001$) plants. Capo's exudation of amino acids was lower than Aristaros, in both drought/recovery plants and control plants (figure 7D).
For drought/recovery plants amino acid exudation peaked on day 9 and went down again on day 11 but was consistently higher than control plant exudation, however, upon statistical analysis, the effect of time was not significant. Control plant exudation of amino acids did not change much throughout the experiment (figure 9A and B).

A



B

Figure 9: Amino acid exudation in µmol per plant per hour of control plants and drought/rewetting plants by genotype, visualised as single measurements (every dot represents the amino acid exudation of one plant) (**A**). The second plot shows also amino acid exudation in µmol per plant per hour of control plants and drought/rewetting plants throughout the days (**B**). Day 4 and 8 were drought days, day 9 and 11 were rewetting days for the drought/rewetting treated plants. Error bars represent standard errors (± SE) of the mean.

4. Discussion

I looked at the exudation patterns of two genotypes of winter wheat (*Triticum aestivum*) to test if exudation changes during drought stress and rewetting, as well as to test if there are differences between genotypes. For this, two genotypes (Aristaro and Capo) were compared, that are both drought tolerant varieties. They were measured during different stages of drought (drought effects were measured on day 4 and 8, rewetting effects were measured on day 9 and 11 of the experiment) and compared to control treated plants held at constant 50% water holding capacity. During the drought phase, drought treated plants were kept at 20% of soil water holding capacity. During rewetting, the drought stress treated plants were rewatered to 50% soil water holding capacity (see figure 2 and 3).

4.1. Differences in Capo´s and Aristaro´s physiology, as well as in their adaptation to drought

Capo and Aristaro are genotypes that are well adapted to water deficit, and have similar genetic makeup, as Capo makes up some part of parent generation of Aristaro (H. Grausgruber, *personal communication*). In this experiment, in total, Aristaro had less shoot biomass, but higher and more stable shoot water content compared to Capo. Relative chlorophyll did not differ between genotypes, while NPQ(T) values varied and were higher in the Capo genotype. So, despite their shared genetic background, Capo and Aristaro exhibited different responses under drought and rewetting, suggesting different strategies in response to water stress. The sections below explore these differences, explaining the adaptive mechanisms employed by each genotype.

Zhang et al. (2022) found that wheat biomass performance during the drought stress acted opposite to yield. Plants that lost much biomass (30%) during a 20-day drought stress period showed yield loss only at around 10%, compared to plants that lost less biomass (25%), which lost 23% of yield. Therefore, a plant with more biomass seems to be a less accurate indicator of final yield (Zhang et al. 2022). In this experiment, shoot biomass was higher in Capo, and it was influenced by the water stress treatment. On day 11, Aristaro had 24% reduced shoot biomass compared to controls, while Capo had only lost 18%, which could indicate that Capo follows a different strategy than Aristaro. Early maturity is used by wheat to escape drought stress (Rijal et al. 2021). Shortening of the flowering time is considered the most effective way to escape drought as it makes it possible for the plant to mature before terminal drought stress (Rijal et al. 2021). However, one of the drawbacks of this strategy is that it limits grain yield (Rijal et al. 2021).

Keeping turgor constant is seen as a beneficial defence strategy against drought damages (Ahmad et al. 2018), and relative water content correlates well with drought tolerance and might be a better sign of water stress than other parameters of the plant (Datta et al. 2011). If water content drops, the inner structures of the chloroplast and chlorophyll content are negatively affected (Ahmad et al. 2018). Drought stress can reduce relative water content of wheat, which closes stomata and reduces photosynthesis (Ahmad et al. 2018). Relative water content is also often higher in plants which are adapted to drought (Datta et al. 2011), and water stressed plants have less relative water content than non- stressed plants (Bipin et al. 2021) and the adapted plants seem to be able to keep a higher photosynthetic rate and have a higher total grain yield per plant (Ahmad et al. 2018; Rijal et al. 2021).This is why relative water content is useful in selecting drought tolerant wheat genotypes (Rijal et al. 2021). Aristaro kept the shoot water content more stable and in general also had a higher shoot water content than Capo. Maintaining

higher content of water in the tissues can also be interpreted as drought avoidance of the plant, and it can be facilitated by controlling stomatal transpiration and deeper rooting in the soil (Rijal et al. 2021). Comparatively, however, Aristaro did not have significantly deeper or more rooting (but on average Aristaro had 0.04 g more root dry weight). However, due to the compost that was stuck onto the roots, root analysis is not reliable, and another experiment would have to be performed to evaluate the roots.

Non-photochemical quenching (NPQ(T)) showed significant differences in genotype, and on day 8, Capo drought treated had the highest NPQ(T) value of the whole experiment. NPQ(T) is an emergency safety measure used by the plants to get rid of excess energy thermally using carotenoids to avoid damage (Grieco et al. 2020). This is done because excess energy without sufficient levels of CO_2 inside the plant will cause too high levels of reactive oxygen species (ROS) and in this way, cell damage (Grieco et al. 2020). Therefore, the NPQ(T) mechanism is a protective measure that leads to downregulation of photosynthesis (Grieco et al. 2020). However, treatment did not influence NPQ(T), but this could also be because the drought stress phase was not long enough. In Grieco et al. (2020), NPQ values only increased in response to drought treatment from day 13 of drought stress onwards, which was a longer drought than in our experiment. However, it is unclear why the Capo genotype, in control plants as well as in drought treated plants, had higher NPQ(T) than Aristaro.

Capo and Aristaro did not have significantly different levels of relative chlorophyll, however drought stress treated plants had significantly less chlorophyll than control plants (on average 7% less). Chlorophyll content is positively correlated with crop yield (Ahmad et al. 2018). Relative chlorophyll is usually lower in water stressed plants (Ahmad et al. 2018) as was also observed in this experiment. This is because the chlorophyll in drought stressed plants gets photo-oxidised by elevated levels of reactive oxygen species (ROS) that have been generated by over-reduction in the electron transport chain (Camaille et al. 2021). Over-reduction is caused by excessive absorption of light and less CO_2 intake, which is caused by the closed stomata to reduce water loss during drought, because the electrons now attach to oxygen instead (Camaille et al. 2021).

To conclude, drought affected Capo and Aristaro differently, and they may use different strategies to cope with drought. Taken together, Aristaro may be slightly better adapted to drought, even though Aristaro had less shoot biomass than Capo and Aristaro also suffered a relatively higher loss of shoot biomass compared to its controls. However, in regions where drought appears later in the year, Capo may be a better choice, because of its fast development. For a definitive answer, yield in different drought scenarios should be compared.

4.2 Exudation of Capo and Aristaro

I could illustrate differences between Capo´s and Aristaro´s exudation patterns, and drought specific changes in exudation. DOC analysis showed that the two genotypes exuded different amounts of DOC, with Aristaro exuding more than Capo. Results for exudated dissolved organic carbon (DOC) however, must be treated with caution, as they could have been influenced by compost soils, which was impossible to remove from the plant's roots prior to sampling.

Aristaro also exuded more phenols than Capo, and while Capos phenolic exudation stayed rather stable throughout the experiment, Aristaro increased exudation of phenols with time, in both controls and drought treated plants. Aristaro control had the most phenolic exudation,

while drought treated plants exuded significantly less phenols. Phenol exudation could stem for example from salicylic acid, used by the plant during drought stress to scavenge ROS and is also responsible for stomatal regulation, growth, flower induction, defence against pathogens and ethylene biosynthesis (Ahmad et al. 2018; Huang et al. 2014).

Nutrient deficiency (P, Fe) also induces exudation of phytosiderophores and phenolic compounds into the soil around the roots to facilitate desorption, and the phenolics may also hinder competing microbes from accessing the P (Chai and Schachtman 2022). However, it is not likely that there was nutrient deficiency in our experiment that caused the enhanced exudation of phenols in Aristaro, because all plants were fertilized until the start of the experiment, and Capo did not show the same pattern of phenol exudation.

Phenolics like tannins could also act as substrates or signals to many different soil microbes and shape the rhizosphere microbiome and create plant-microbe symbiosis (Badri et al. 2013; Madal et al. 2010; Ulrich et al. 2022). As exudates, phenolics and amino acids have a bigger impact on the composition of the microbiome than sugars (Martin et al. 2018). Plants exudating less phenolics can therefore have decreased abundance of rhizosphere microbes (Huang et al. 2014). In this experiment, this could mean that Aristaro might have a different and more numerous microbial communities than Capo and might form more symbiotic relationships with PGPR. To confirm this, the microbial community of both genotypes would have to be examined further.

Total sugar exudation varied between days and treatments throughout the experiment. Sugar exudation by the plants that experienced drought was higher than by control plants. Similar results were found in Ulrich et al. (2022). Despite reduced photosynthesis, plants were found to invest carbon into root exudates (Ulrich et al. 2022). Increased total sugar exudation in drought stressed plants could be used by the plants to recruit PGPRs but it could also be attributed to increased levels of total sugar inside the plant, due to sugars function as osmoprotectant (Ahmad et al. 2018; Camaille et al. 2021; Upadhyay et al. 2022). This uncertainty is because it is unsure if plants exudate carbon actively or passively (Ulrich et al. 2022). Exudated soluble sugars like glucose have been shown to increase by 80% in stressed wheat after seven days of drought (Camaille et al. 2021; Rorat 2006). While drought treated plants may not have photosynthesized much to begin with on days 4 and 8, due to closed stomata, they still exuded sugars, but less on day 8. Glucose produced during photosynthesis can be exuded within minutes or hours but the exact time frame is unclear (Canarini et al. 2019). Zang et al. (2014) found that drought stress increases the residence time of recently fixed C in the leaves and postpones further transfer to soil, which may be a reason for the depression on day 8, but this hypothesis contradicts the supposed increase in sugar exudation found in Camaille et al. (2021). However, controls also experienced a depression in total sugar exudation on day 8 and 9 (figure 8). Due to the weather, light levels (in figure S1) could have inhibited photosynthesis on day 7 and 8 of the experiment, because on the day before and on the second sampling day itself (day 8), there was much less light available than on the other days. There is research indicating that light level influences exudation (Martin et al. 2018). As there was not much light, the plants (both control and drought treated) may not have been able to photosynthesize and produce and exude sugars.

After rewatering, on day 9, sugar exudation peaked in the now rewetted plants. This may be an attempt by the plants to stimulate rhizosphere nitrification to facilitate compensatory growth (Wang et al. 2020). The peak could also be interpreted as flushing out or balancing the sugar that was previously needed for the osmotic potential, however, little research exploring this

hypothesis is currently available. The depression in exudation by both drought and control plants may be a consequence of lower light absorption on day 7 and 8.

Amino acid exudation varied between genotype and treatment, with Aristaro exuding more amino acids. Drought treated plants also exuded more amino acids than controls. A similar pattern to the total sugar exudation was recorded (figure 9B). Drought treated plants exudation of amino acids peaked on day 9, the first rewatering day. Again, this may be seen as an attempt to compensate for growth reductions and recruit PGPR (Ahmad et al. 2022, Wang et al. 2020). When plants secrete more amino acids, there is a higher abundance of rhizobacteria on the roots (Shaposhnikov et al. 2023). Additionally, microorganisms are also able to change composition and amount of exudation of amino acids and sugars (Shapashnikov et al. 2023). This would suggest that Aristaro has a different microbiome compared to Capo, however, a genotype difference in the amount of sugar exudation was not observed.

Also responsible for the rise in amino acid exudation could be the amino acid proline, which is used in the biosynthesis of proteins (Rijal et al. 2021). Proline increases with drought stress, and the maximum increase has been found in wheat plants leaves (Ahmad et al. 2018). In the plant, proline contributes to osmotic adjustment but mainly protects cell functions, organs and membranes against ROS and may also be used as an energy source under stress conditions (Ahmad et al. 2018, Rubia et al. 2020). Moreover, proline can have a chemotactic effect (Rubia et al. 2020, Webb et al. 2014). Wheat plants accumulate proline in higher extent than other osmoregulators (Rijal et al. 2021). Wheat genotypes, that accumulate more proline under drought stress, seem to be better adapted to drought (Rijal et al. 2021). Genotypes with a better tolerance to abiotic stressors also release more proline into the rhizosphere (Vives-Peris et al. 2017). Therefore, proline has been found to be a good indicator of drought resistance in plants (Rijal et al. 2021).

Tryptophan could also be responsible for the higher amino acid exudation in drought treated plants. Studies have found that roots exuding tryptophan has led to bacterial IAA synthesis which benefitted plant growth (Liu et al. 2016; Upadhyay et al. 2022). This is because indole-3-acetic acid (IAA) is an auxin and a product of amino acid L-tryptophan (Upadhyay et al. 2022). Glycine-betaine, which contributes to osmotic adjustment, could also have influenced the higher amino acid exudation (Camaille et al. 2021). Aristaro exuded much more amino acids than Capo, which could indicate that Aristaro is better suited for drought affected areas (Rijal et al. 2021), and it could also indicate, again, that Aristaro has a distinct microbiome composition (Martin et al. 2018).

4.3 Conclusions

The present experiment shows that there are differences between drought influenced and control plants. Significant differences between drought/rewetting plants and control plants were found in shoot dry weight, shoot water content, relative chlorophyll and in phenol, total sugar, and amino acid exudation. Furthermore, genotype differences between Capo and Aristaro have been observed in shoot dry weight, shoot water content, NPQ(T), phenol and amino acid exudation. Because Aristaro had a higher and more stable shoot water content, lower NPQ(T) and exuded more phenols and amino acids, these results suggest that Aristaro is better suited to drought conditions and may possess a different root microbiome. However, Capo may be used in situations where drought conditions occur later in the year, as Capo reaches maturity earlier, which could help this genotype to avoid drought altogether.

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Supplements

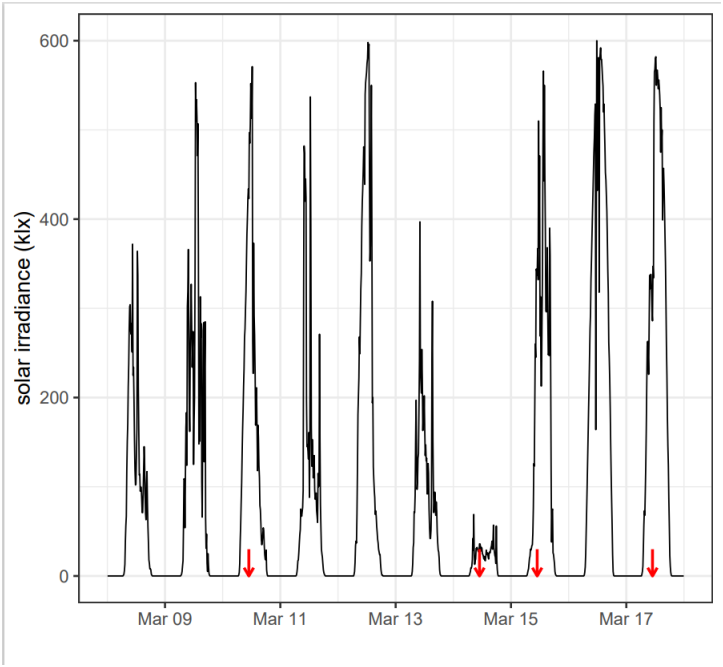

Figure S1: Solar irridiance data from the greenhouse in kilolux per day. Red arrows show sampling days 4, 8, 9 and 11.