Physiological, agronomic and genomic evaluation of drought stress tolerance in potato (*Solanum tuberosum* L.)

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Resumen

Resumen

La patata (*Solanum tuberosum* L.) es uno de los cultivos más importantes a nivel mundial y contribuye de forma importante al suministro mundial de alimentos, produciéndose más de 370 millones de toneladas. El cultivo tiene, asimismo, una gran relevancia en la seguridad alimentaria, reducción de la pobreza y mejora de la nutrición humana. Debido a los efectos del cambio climático, las condiciones tienden a ser cada vez más extremas, siendo la disponibilidad de agua uno de los factores limitantes en la producción del cultivo, por lo que es importante poder seleccionar variedades que puedan mantener el rendimiento y hacer frente a las consecuencias del cambio climático. La mejora tradicional de patata es un proceso largo, costoso y con problemas como la naturaleza heterocigota y tetraploide. Durante las últimas décadas se han desarrollado tecnologías de secuenciación de nueva generación (NGS) que cada vez son más asequibles y permiten a los mejoradores identificar alelos específicos beneficiosos que son responsables de las variaciones deseables en los cultivos. Esta tesis intenta abordar el problema del efecto del estrés hídrico como consecuencia del cambio climático en el cultivo de la patata desde distintos puntos de vista.

Por un lado, se evaluó la respuesta diferencial de parámetros fisiológicos y de producción en seis variedades de patata bajo condiciones de déficit hídrico. Para ello se llevó a cabo un ensayo de invernadero con las variedades Ágata, Agria, Kennebec, Monalisa, Santé y Zorba. Se tomaron todas las medidas en la hoja más joven completamente expandida y en 4 tiempos distintos: antes del estrés (T0), a los 17 días (T1) y a los 24 días (T2) de retirarlas el riego y 5 días después de regarlas nuevamente (R1). Los parámetros fisiológicos evaluados fueron el contenido y la fluorescencia de la clorofila, contenido relativo de agua (RWC), apertura estomática, conductividad electrolítica y potencial hídrico. Tras el periodo de sequía se cortó la parte aérea de la mitad de las plantas para obtener la biomasa. Se estimaron los parámetros de producción como como número y peso medio de tubérculos, producción, materia seca y almidón. Los parámetros más afectados por el déficit hídrico fueron la apertura estomática y el potencial hídrico. La selección indirecta basada en parámetros asociados al estrés hídrico puede ser una herramienta útil en programas de selección en patata para la identificación de variedades más tolerantes.

Por otro lado, con el objetivo de comprender los mecanismos de respuesta al estrés hídrico a nivel de transcriptoma, se llevó a cabo un ensayo en el que se utilizaron las variedades Agria y Zorba para ver la diferencia de expresión de genes entre plantas control y plantas sometidas a estrés hídrico. Para ello, se secuenciaron mediante RNAseq, obteniendo alrededor de 50 millones de transcritos para cada variedad. Al comparar los transcritos significativos obtenidos de las plantas control con las de sequía de la variedad Agria, se vio que 931 genes estaban regulados "up", es decir, aumentaba su expresión, y 2077 genes estaban regulados "down", disminuyendo su expresión bajo condiciones de estrés. Cuando se compararon ambos tratamientos en las plantas de la variedad Zorba, se encontraron 735 genes regulados "up" y 923 genes regulados "down". Además, se analizaron los transcritos con el fin de averiguar sus funciones y destacar las vías metabólicas potencialmente relacionadas con la tolerancia a la sequía. Para ello se hizo una clasificación de ontología de genes (GO), las cuales describen la función molecular de los productos génicos, su rol en los procesos biológicos y su localización en componentes celulares. En este ensayo se ha visto que las principales ontologías a las que pertenecen los transcritos obtenidos pertenecen a componentes celulares, y más en concreto relacionados con la membrana y la pared celular. Estos resultados proporcionan una información valiosa relacionada con los mecanismos moleculares de tolerancia a estrés hídrico.

Por último, se evaluó un panel de 144 genotipos de patata durante dos años para observar la variación biológica de varios rasgos fisiológicos (contenido de clorofila, fluorescencia de clorofila, conductancia estomática, NDVI, área y perímetro foliar) y agronómicos (rendimiento, peso y número de tubérculos, contenido de almidón, materia seca y azúcares reductores). Estas mismas variedades se genotiparon mediante el chip "GGP V3 Potato array", obteniendo un total de 31190 marcadores SNP que tras el filtrado se quedaron en 18259. Se determinó la subestructura de la población utilizando el software STRUCTURE 2.3.4 y el análisis de mapeo asociativo se realizó utilizando el paquete estadístico GwasPoly. Se detectaron SNPs asociados tanto con los rasgos fenotípicos como con los agronómicos en varios cromosomas y se asociaron con funciones genéticas conocidas. Estos resultados podrían ser útiles para diseñar marcadores y utilizarlos en futuros programas de selección asistida relacionados con la tolerancia a la sequía en patata.

Abstract

Abstract

Potato (*Solanum tuberosum* L.) is one of the most important crops worldwide and contributes significantly to the global food supply, producing more than 370 million tons. The crop also plays an important role in food security, poverty reduction and improving human nutrition. Due to the effects of climate change, conditions tend to become increasingly extreme, with water availability being one of the limiting factors in crop production, so it is important to select varieties that can maintain yields and cope with the consequences of climate change. Traditional potato breeding is a long, costly process with problems such as heterozygous and tetraploid nature. Over the last few decades, next generation sequencing (NGS) technologies have been developed which are increasingly affordable and allow breeders to identify specific beneficial alleles that are responsible for desirable variations in crops. This thesis attempts to address the problem of the effect of water stress as a consequence of climate change on the potato crop from different perspectives.

On one hand, the differential responses of physiological and production parameters were evaluated in six potato varieties under water deficit conditions. For this purpose, a greenhouse trial was carried out with the varieties Agata, Agria, Kennebec, Monalisa, Sante and Zorba. All measurements were taken at the last fully expanded leaf and at 4 different times: before stress (T0), 17 days (T1) and 24 days (T2) after irrigation was withdrawn and 5 days after re-watering (R1). The physiological parameters evaluated were chlorophyll content and fluorescence, relative water content (RWC), stomatal conductance, electrolyte leakage and water potential. After the drought period, the aerial part of half of the plants was cut to obtain the biomass. Production parameters such as number and average weight of tubers, tuber yield, dry matter and starch contents were estimated. The parameters most affected by water deficit were stomatal aperture and water potential. Indirect selection based on parameters associated with water stress can be a useful tool in potato breeding programs for the identification of more tolerant varieties.

On the other hand, in order to understand the mechanisms of response to water stress at the transcriptome level, a trial was carried out using Agria and Zorba varieties to see the difference in expression between control plants and plants subjected to water stress. For this purpose, they were sequenced by RNAseq, obtaining about 50 million transcripts for each variety. When comparing the significant transcripts obtained from control plants with drought stressed plants of the Agria variety, it was found that 931 genes were up-regulated, increasing their expression, and 2077 genes were down-regulated, decreasing their expression under stress conditions. When both treatments were compared in Zorba plants, 735 genes were found to be up-regulated and 923 genes were found to be down-regulated. In addition, transcripts were analyzed in order to find out their functions and to highlight metabolic pathways potentially related to drought tolerance. For this purpose, a gene ontology (GO) classification was performed, which describes the molecular function of gene products, their role in biological processes and their localization in cellular components. This assay revealed that the main ontologies to which the obtained transcripts belonged were related to cellular components, and more specifically related to the cell membrane and cell wall. These results provide valuable information related to the molecular mechanisms of tolerance to water stress.

Finally, a panel of 144 potato genotypes was evaluated for two years to observe biological variation in several physiological (chlorophyll content, chlorophyll fluorescence, stomatal conductance, NDVI, leaf area and perimeter) and agronomic (yield, tuber weight and number, starch content, dry matter and reducing sugars) traits. These same varieties were genotyped using the "GGP V3 Potato array" chip, obtaining a total of 31190 SNP markers, which after filtering reduced to 18259. Population substructure was determined using STRUCTURE 2.3.4 software and association mapping analysis was performed using the GwasPoly statistical package. SNPs associated with both, phenotypic and agronomic traits were detected on several chromosomes and they were associated with known gene functions. These results could be useful for designing markers for use in future assisted selection programs related to drought tolerance in potato.

Chapter 1

General Introduction

Chapter 1: General introduction

1. General characteristics of the potato

1.1. Taxonomy and botany

The potato is an herbaceous crop belonging to the genus *Solanum* of the Solanaceae family, and the species *Solanum tuberosum* L. This species is subdivided into two subspecies: the subspecies *andigena* which is native to the Andes and is mainly cultivated in certain regions of Central and South America as it is adapted to short-day conditions, and the subspecies *tuberosum* which is widely cultivated worldwide and is adapted to long days (Hawkes, 1992).

S. tuberosum is a tuberous, erect-stemmed, deciduous plant, which loses its leaves and aerial stems in the cold season, although it behaves as a perennial as it can reproduce by its tubers (Garcia, 2014).

The potato has compound leaves, imparipinnate, with 3 or 4 pairs of oval leaflets ending in a single larger one. They have hairs on the surface, and their amount varies depending of the cultivar. The color of the leaves is usually green or yellowish-green, although sometimes they may have some purple pigmentation.

The stems are originated from the buds present on the tuber used as seed. They are cylindrical, thick and strong. Stems can reach a height between 0.5 and one meter depending on the cultivar and the availability of environmental resources, initially erect and with time progressively inclining towards the ground. Stem color can vary from green to red-purple pigmented with anthocyanins (Huaman, 1986).

The flowers are located at the end of the stem as inflorescences with a diameter of about 3 or 4 cm. The corolla is star-shaped with five petals that merge forming a tunnel. The color of the flowers is very diverse, varying from pale blue, violet or white.

The fruit is a small spherical berry, green or purplish in color, 1-3 cm in diameter, inside of which are the seeds. The size of the seeds is approximately 3 mm. They are flattened and covered with mucilage. In the case of potatoes, the seeds are not very important, as they usually reproduce through the tubers, and the seeds are usually only used for breeding.

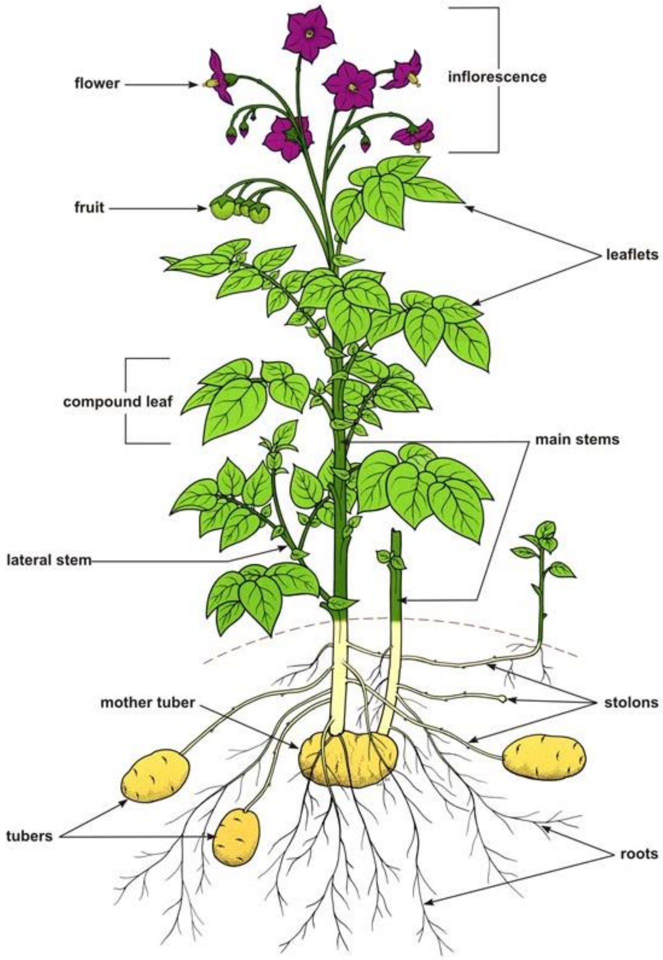


Figure 1.1. Potato plant morphology (International Potato Center, 2018).

The root system is fibrous and superficially branched, penetrating up to 0.8 m deep. The roots show a rapid growth from the first stages of development until the formation of tubers begins. Tubers are part of the underground stem, modified by the accumulation of reserves, acting as a nutrient storage organ destined to form a new plant. Tubers vary in shape and size depending on cultivar and soil type and are usually oval, round or elliptical. The color of the skin can be whitish, cream, yellow, orange, brown, pink, red, red-purple, or dark purple, and also the color of the flesh varies between these colors. On the surface of the flesh, there are some hollows, where the vegetative buds are located, which will give rise to new stems when they sprout (Zierer et al., 2021).

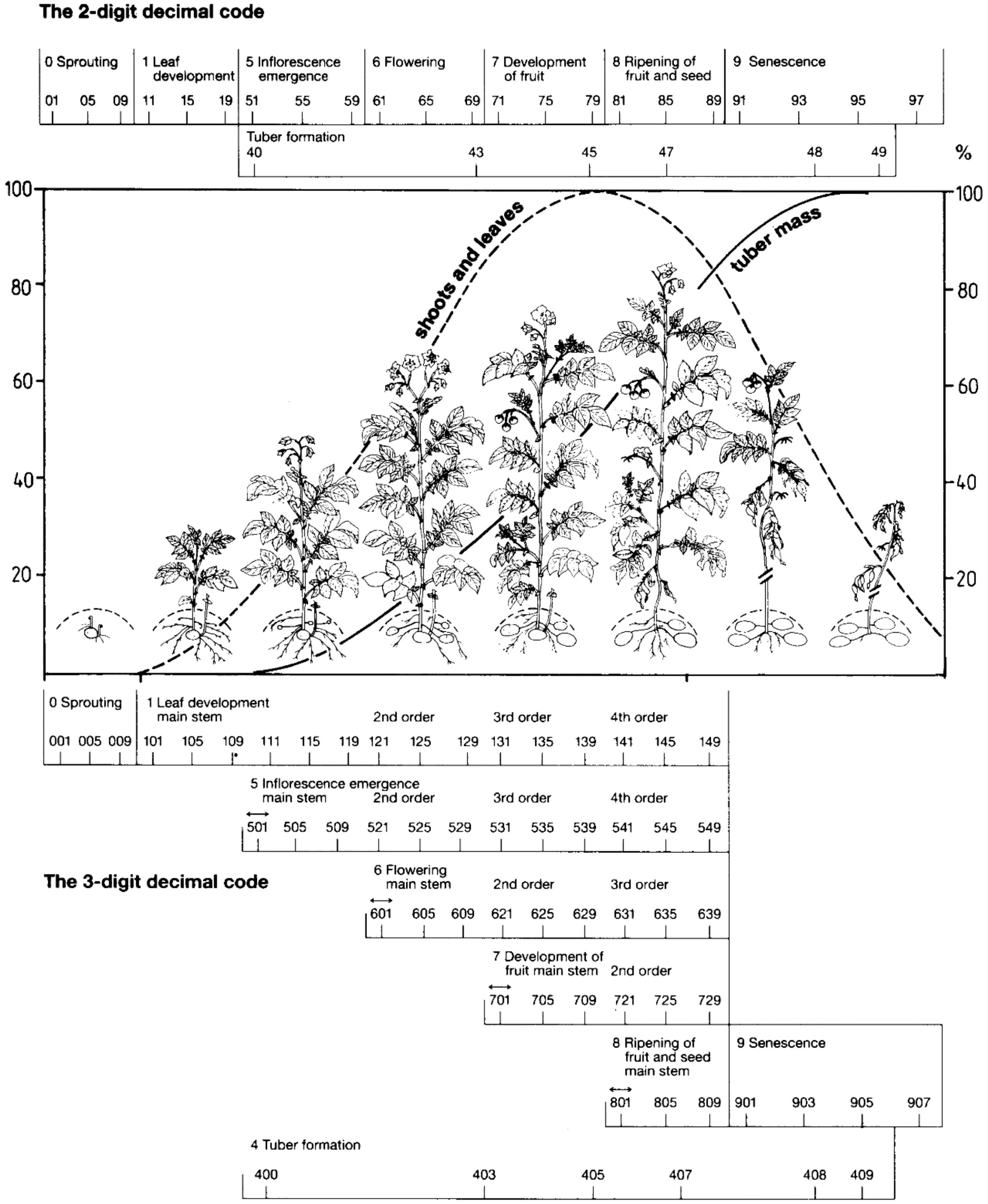


Figure 1.2. BBCH scale of potato vegetation stages (Hack et al., 1993).

According to Lieth, (1974) phenology is based on the study of biological events over time, the causes with respect to biotic and abiotic factors and the interrelationship between phases of the same or different species, although it is often associated with agricultural events such as planting, emergence, fruiting and harvesting. Factors influencing phenology vary according to species and include photoperiod, soil moisture, soil temperature, air temperature and solar illumination (Islam & Bala, 2008).

The growth and development of potato plants is divided into 10 stages according to Hack et al. (1993) and includes germination/sprouting, leaf development, lateral shoot formation, longitudinal growth of main shoots, development of harvestable vegetative parts, emergence of the flowering organ, flowering, fruit formation, fruit and seed maturation and senescence (Figure 1.2) (Kolbe & Stephan-Beckmann, 1997a, 1997b).

1.2. Origin of the potato crop

It is believed that potato cultivation began about 6000-10000 years ago in the Andean highlands and in the vicinity of Lake Titicaca located on the border of present day Peru and Bolivia by the inhabitants of this region. The genetic patterns of its distribution indicate that it probably started in the mountainous regions of southern Peru and northern Bolivia (Morales, 2007).

At that time, although the potato was small, bitter and rather indigestible, it was a staple food. It was consumed both boiled and roasted, although a common form of consumption was as "chuñu". “Chuñu" was obtained by dry-freezing the tubers during the cold nights in the Andean region, thus facilitating its consumption and preserving it for up to a year (De Jong, 2016).

The first species cultivated outside South America appeared in Spain, it is believed that the crop entered Europe via the Canary Islands in 1567 and later texts show that they were found in Seville in 1573 (Hawkes & Francisco-Ortega, 1993). There are two theories about the origin of the first potatoes to reach Europe. On one hand, there is the possible introduction from Chile (S. tuberosum group Chilotanum), as these have physiological similarities and have a photoperiod similar to the European varieties, which would have made it less difficult for them to adapt to the new conditions (Juzepczuk & Bukasov, 1929). On the other hand, there is the hypothesis of the introduction of Andean germplasm that constitutes an important group of native cultivars selected by Andean farmers, which is grown throughout the Andes at altitudes ranging from 2,000 to 4,000 meters above sea level, and forms tubers under short-day conditions (Hawkes, 1990).

The term Neo-tuberosum refers to cultivated potatoes grown under long-day conditions, developed by crossbreeding and selecting short-day adapted populations of S. tuberosum group Andigena (Spooner et al., 2014). Ghislain et al., 2009 found that the Neo-tuberosum germplasm is closely related to the Chilotanum group and less closely related to the Andigena group germplasm. Thus, these hypotheses suggest that both Andigena and Chilotanum group potato clones were initially introduced to Europe by multiple routes, although the Chilotanum group clones would have been preferred in the selection processes making them the predominant modern cultivars in Europe (Rodriguez, 2010).

After the arrival of the potato in Europe in the 16th century, it spread geographically throughout Europe slowly, as at first it was considered an ornamental plant of little interest (Garcia, 2014; Ruiz de Galarreta & Ríos, 2008). Thanks to the interest of botanists, curiosity and the possibility of using it as a medicine, the potato gradually became an interesting crop that also provided human food and livestock feed, starting to be cultivated in the Netherlands in an economic way around 1620 (Vos, 1992). The spread of the potato was responsible for an important part of the population increase between 1750 and 1850, as by that time it was already being cultivated in the fields of farmers in the vast North European lowlands, which extended from France to Russia via Belgium, the Netherlands, Germany, Denmark and Poland (De Jong, 2016).

1.3. Potato production and economic importance

Potato is the sixth most important crop worldwide, preceded by sugar cane, maize, rice, wheat and oil palm fruit, with 376 million tonnes produced in 2021 and 18.13 million hectares cultivated (FAOSTAT, 2022).

The main potato producer country is China (94,300,000 t), followed by India (54,230,000 t), Ukraine (21,356,320 t), United States of America (18,582,370 t) and

Russian Federation (18,295,535 t) (Figure 1.3). The total area under potato cultivation in 2021 is estimated at 18,132,694 ha, being China with 5,780,000 ha the largest cultivating country, followed by India (2,248,000 ha), Ukraine (1,283,000 ha), Russian Federation (1,142,111 ha) and Bangladesh (468,699 ha), ahead of the United States of America in sixth place with 378,670 ha (FAOSTAT, 2022).

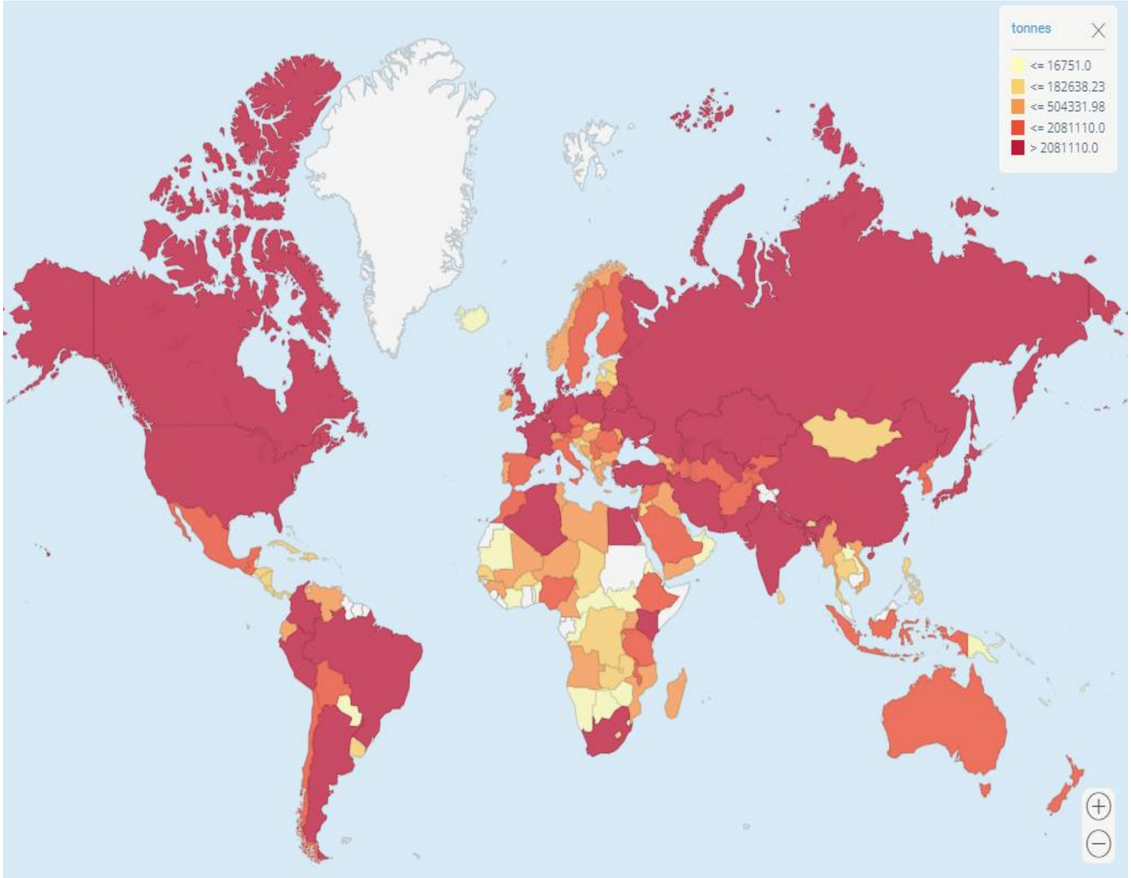


Figure 1.3. Map of the distribution of potato production worldwide (tonnes)(FAOSTAT, 2022).

The 27.3% of the world's total potato production comes fromEurope. Ukraine and the Russian Federation are the main producers as mentioned above, but other producers include Germany (11,312,100 t), France (8,987,220 t), Poland (7,081,460 t) and The Netherlands (6,675,590 t).

According to MAPA (2022), potato production in Spain reached 1,942,778 tonnes in 2022. The main producing regions were Castile and Leon (714,434 t), Galicia (381,734 t) and Andalusia (300,663 t) (Figure 1.4). In terms of the distribution of the cultivated area by Autonomous Communities, the two main producing areas in Spain were Galicia (18,702 ha) and Castile and Leon (16,421 ha).

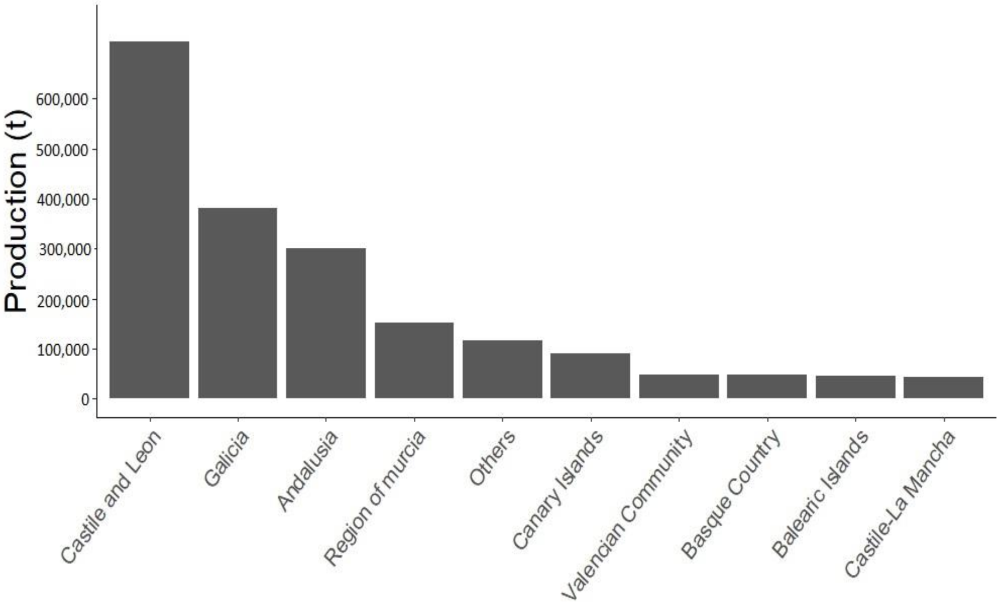


Figure 1.4. Main potato production areas in Spain in 2022 (MAPA, 2022).

Spanish potato production has decreased almost 7% compared to the previous season, which means a new historic minimum, falling below 2 million tonnes per year for the first time. These results are due to the spring rains in the eastern areas of the peninsula and the high temperatures recorded practically all over the country during the summer months, which have resulted in lower yields. Potato is considered a very efficient crop in terms of energy and nutrient production per unit area and has a similar nutrient and water consumption to cereals. In a future scenario, where more food will need to be produced in a smaller unit area, these characteristics will make the potato a very interesting crop (MAPA, 2022).

Most potato production is destined for human consumption, both in the form of fresh tubers and processed products. Marketed potatoes are divided into seven different product categories: fresh, frozen, French fries, dehydrated, starch, seed or other industrial categories. Before planting, growers will determine whether they are growing potatoes for one or more of the seven markets and accordingly make the selection of seed varieties, growing conditions or management practices (Bond, 2014).

2. Crop breeding

By the year 2022 we have reached 8 billion of global human population and over the next 30 years, it is expected that this number will increase by 25% to 10 billion. In this situation, plant breeders are under pressure to improve existing crops and develop new crops with higher yields, more nutrients and resistant to biotic and abiotic factors to cope with the huge food demand that the increasing population will bring (Hickey et al., 2019).

Crop production depends on both external and internal factors. External factors include biotic factors such as pathogens, pollination insects or symbiotic microorganisms and abiotic factors such as water availability, temperature, light or nutrients. Internal factors refer to genetic and epigenetic factors in plants, which determine the heritability of genes and their expression. It is very important to consider both sets of factors in breeding programs as yield depends on the most limiting factor at the time.

Next-generation sequencing (NGS) technologies have been developed over the last few decades and have allowed the assembly of a large number of reference genomes such as rice, maize, wheat or potato. In addition, the affordability of these new technologies allows breeders to identify specific beneficial alleles that are responsible for desirable variations in crops and to use DNA marker assisted selection (MAS) in breeding programs (Tian et al., 2021; Wallace et al., 2018).

2.1. Classical potato breeding

Salaman was the precursor of potato genetics about 100 years ago. His studies were based on the application of Mendel's newly discovered principles on stem traits, tuber shape, depth and color of eyes and immunity to Phytophthora infestans (Gebhardt, 2007). The polyploidy and tetrasomic inheritance of potato makes it difficult to apply Mendelian genetics of simple traits to this crop. In tetraploid species the alleles occur in four doses, simplex, duplex, triplex and quadruplex, so recessive alleles are quite difficult to discover and it is very hard to obtain pure lines due to severe inbreeding depression (Slater et al., 2014).

The conventional way to create new genetic variety is to perform targeted crosses between selected individuals to obtain progeny that segregate for the trait of interest. This strategy generates a very large population in which the breeder must identify genetically superior individuals through their phenotypic characteristics (Voss-Fels et al., 2019). Screening in a breeding program is often carried out in field trials or greenhouse trials. Testing is usually done over several years and at different locations to estimate the genetic potential of a genotype under a range of different conditions, although in most breeding programs only one location is used in the first screening phase (Bonierbale et al., 2020). The new population undergoes several selection cycles over years to reduce the population size, thus decreasing the number of varieties while the number of plants per variety evaluated increases (Slater et al., 2013). Finally, breeders usually register the most promising variety or varieties from their breeding program, those with clearly improved characteristics, and once registered they are available to interested farmers. This whole process can take 10-20 years, making traditional breeding programs very costly in terms of time and money (Voss-Fels et al., 2019).

2.2. Molecular potato breeding

In recent years, identification and characterization methods based on the use of molecular markers have been developed that overcome the limitations of traditional methods. The main advantages of such markers are that they are phenotypically neutral, have higher segregation or polymorphism, can be assessed from the early stages of seedling development, are independent of the season of the year in which the analysis is performed and are free of epistatic effects (Gebhardt, 2013; Sharma & Bryan, 2017).

All organisms undergo variations due to normal cellular changes or interactions with the environment, giving rise to genetic variations known as polymorphisms. For these variations to be useful to geneticists, they must be heritable and capable of being distinguished by the researcher. They may be visible phenotypic variations or genetic mutations identified by molecular techniques. These variations that occur in DNA can be of different types: substitution of a base, insertions or deletions of nucleotide sequences, inversion of a DNA fragment within a locus and rearrangement of DNA fragments around the locus of interest (Liu & Cordes, 2004).

Since the 1980s, the rise of PCR-based molecular markers has provided a new tool for the identification of traits of interest in potato related to resistance to pathogens such as Phytophtora infestans, viruses (PVY, PVX, PLRV) or nematodes, yield and quality traits, cold sweetening and nutritional quality (Hameed et al., 2018; Ramakrishnan et al., 2015). Marker-assisted selection can be used for germplasm characterization to identify and develop superior material in order to use them as parentals in breeding programs, early generation selection and choice of advanced clones (Bradshaw, 2017; Dale et al., 2016).

2.3. Molecular markers

Restriction fragment length polymorphism (RFLP) were the first markers used and have the advantage of allowing the genome to be studied in a very broad way since they include coding and non-coding DNA sequences (Becerra & Paredes, 2000). Restriction enzymes are used to cut the DNA molecule and identify character-linked regions, generating millions of DNA fragments that must be detected by radioactively labeled probes. They can be used as heterologous markers when homologs are not available, as they can be quite conserved between species and even genus (Gebhardt et al., 2001).

Markers based on amplified fragment length polymorphisms (AFLPs) replaced the previously mentioned RFLPs. These markers are obtained by combining restriction enzyme digestion and selective PCR amplification. Like RAPD, these markers can be used in any species to be studied since they do not require prior knowledge of the DNA sequence (Grover & Sharma, 2016). The main advantage is their high reproducibility, although they have some disadvantages such as being expensive, technically complex, laborious and sometimes produce uncertain results (Azofeifa-Delgado, 2006).

The random amplified polymorphic DNA (RAPD) technique is based on the use of the PCR method to amplify DNA strands with a single primer of arbitrary sequence. Typically, a 10-base-long oligonucleotide is used that amplifies several loci at once, allowing multiple markers to be studied in a single PCR. Polymorphisms are detected as the presence or absence of a band of a given molecular weight, making it a dominant marker that does not allow differentiation between homozygotes and heterozygotes (Guimaraes et al., 2007). The advantages of RAPD markers are that results are obtained in a short period of time and they are relatively inexpensive.

Single sequence repeat markers, known as microsatellites or SSRs, are tandemly repeated motifs of two to six nucleotides. They are distributed throughout the genome and differences in the number of repeats can be seen by measuring the molecular weight of the resulting fragments by electrophoresis (Kordrostami & Rahimi, 2015). Microsatellite markers have many advantages such as being codominant, can be easily automated with fluorescent primers on an automated sequencer, and combine several markers at once without overlap. As disadvantage it can be mentioned that their development is very laborious since specific genomic regions must be identified and sequenced (Vieira et al., 2016).

Sequence characterized amplified region (SCAR) markers are generated from cloning and sequencing the RAPD fragments of interest. These markers can either maintain the dominant segregating behavior of the RAPDs from which they are derived or be converted to codominant markers. SCARs are more stable and give higher reliability than RAPDs (Kiran et al., 2010).

Cleaved amplified polymorphic sequence (CAPS) markers are also known as PCR- RFLP as they combine PCR techniques with classical RFLP methods. They are based on amplification with specific primers, digestion of the amplified fragments with restriction enzymes and separation of the digestion products on agarose gel (Shavrukov, 2016).

Table 1.1. Comparation of characteristics of the most popular molecular markers used in plants.

	RFLP	**AFLP**	**RAPD**	**SSR**	**SCAR**	**CAPS**	**SNP**
PCR	No	Yes	Yes	Yes	Yes	Yes	Yes
Restriction enzymes	Yes	No	No	No	No	Yes	No
Level of polymorphism	Medium	Medium	Medium	High	High	High	High
Abundance in the genome	Medium	High	High	Medium	Medium	Medium	Very high
Sequence information required	No	No	No	Yes	Yes	Yes	Yes
Inheritance	Co-dominant	Dominant	Dominant	Co-dominant	Co-dominant	Co-dominant	Co-dominant
Number of loci	One	Multiple	Multiple	Multiple	One	Multiple	Multiple
Automation	Low	High	High	High	High	High	High
Reproducibility	Low	High	Low	High	High	High	High
Cost	High	High	Low	High	Low	Low	Low

Single nucleotide polymorphism (SNP) markers are generally single base variations due to transitions, transversions, deletions or insertions. Every few base pairs, between 50 and 500, a SNP is usually found, which in some cases can be related to a trait of interest. SNP markers have multiple advantages, such as being the ones with the highest polymorphisms, they are codominant, and they are also capable of detecting polymorphisms that are difficult to detect with other markers (Vignal et al., 2002)..

2.3.1. *Next generation sequencing (NGS) technologies*

Thanks to the recent development of Next Generation Sequencing (NGS) platforms we can access much more genomic and transcriptomic data through faster, cheaper and more cost-effective practices. These techniques generate SNP markers and they offer flexibility, speed, cost-effectiveness, ease of data management, high throughput, abundant availability, robust and comparable genotype calls between different groups due to their biallelic status and potential for high levels of multiplexing (Nguyen et al., 2018).

Like RFLPs and AFLPs markers that are based on restriction enzymes, NGS also allows the use of these enzymes to reduce the genome representation (Davey et al., 2011). The two most commonly used restriction enzyme-based techniques are Genotyping-By-Sequencing (GBS) and restriction-site associated DNA sequencing (RAD-Seq). GBS technique makes use of restriction enzyme digestion, followed by adapter ligation, PCR and sequencing using an NGS platform (Poland & Rife, 2012). In this way genotyping and SNP discovery is done simultaneously, so that array biases are avoided and also no prior knowledge of the genome is required so it can be used in any species (Thomson, 2014). RADseq combines the use of restriction enzymes to cut DNA into fragments that are then identified to associate sequence reads to specific individuals. In this case, the presence or absence of sequence polymorphisms flanking the restriction enzymes is detected. If the reference genome is available, the sequences are aligned against it, although this is not necessary and this technique can be used even if the reference genome is not available (Davey & Blaxter, 2010).

The development of NGS technologies can also be applied in the field of plant transcriptomics by sequencing RNA molecules (Sharma et al., 2018). The RNAseq technique is based on obtaining sequences of expressed genes, for which complementary DNA molecules are first generated by reverse transcription of total RNA and then these sequences are assembled and, if available, mapped against a reference genome (Andrews & Luikart, 2014) (Figure 1.5). One of the advantages of RNAseq is that prior knowledge of the genome sequence is not necessary as the sequences obtained from sequencing themselves can be used to create de novo assemblies (Martin et al., 2013; Tiwari et al., 2017). Differentially expressed genes (DEGs) can be identified between two or more groups of samples to assess the behavior between those two groups by gene ontology (GO) annotation (Khamis et al., 2016). This technique will be presented in more detail in Chapter 4.

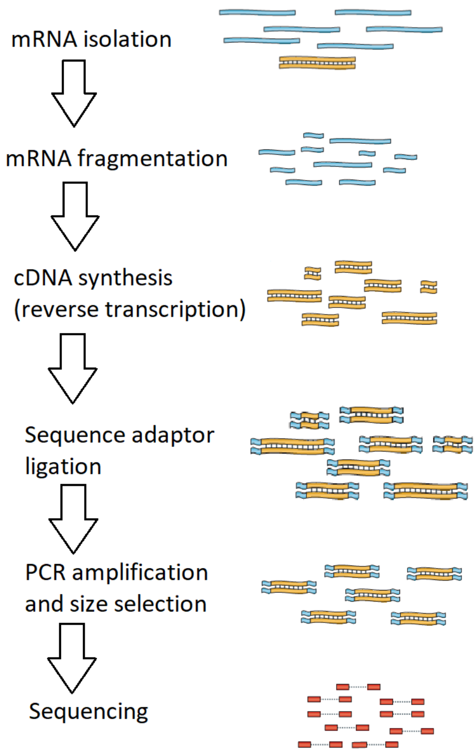


Figure 1.5. RNA sequencing experiment workflow (adapted from Martin & Wang, 2011).

The use of NGS technologies has enabled the development of high-density SNP genotyping platforms that are used for population genetic analysis (Patil et al., 2017). High-throughput SNP arrays are available for a wide range of plant species and are widely used in research and breeding of major crops (Varshney et al., 2014). In potato the first chip was developed due to the availability of the reference genome on which transcriptome data from six tetraploid cultivars were aligned (Felcher et al., 2012). This original Infinium 8303 SNP array has been expanded and new SNP arrays were created and widely used for germplasm characterization, linkage mapping, inbred line development, analyzing gene sequence diversity, and association mapping (Endelman et al., 2018; Naeem et al., 2021; Vos et al., 2015).

2.3.2. GWAS

Most of the important traits investigated in agriculture are quantitative, which means that they are influenced by more than one gene, by the environment and by gene-environment interactions and do not follow Mendelian inheritance (Gupta et al., 2014). Both, linkage mapping and association mapping, are techniques used to characterize the functional loci that influence the traits of interest. The main difference between the two approaches is based on the control over recombination. While in linkage mapping directed crosses are made to generate a mapping population, in association mapping the relationship between the population is not controlled, providing a higher mapping resolution (Myles et al., 2009).

Thanks to high-throughput marker genotyping, genome-wide association studies (GWAS) have investigated agriculturally important traits in many major crop species, such as corn, rice, wheat or potato (Bradshaw, 2022a; Saini et al., 2021; Shikha et al., 2021; Wang et al., 2020). GWAS studies involve populations that are genotyped and phenotyped to establish the genetic bases of relevant phenotypic variation, as shown in the figure 1.6 (Garreta et al., 2021). The genotypic data are usually SNP-type markers that have been obtained by genotyping-by-sequencing or array-based genotyping (Tibbs Cortes et al., 2021). In addition to having reliable genotypic data, it is also important to have good phenotyping data. Obtaining robust phenotypic data is a complicated task, since it is necessary to use a large number of genotypes, with their respective replicates, and usually assays are performed in different locations and over several years.

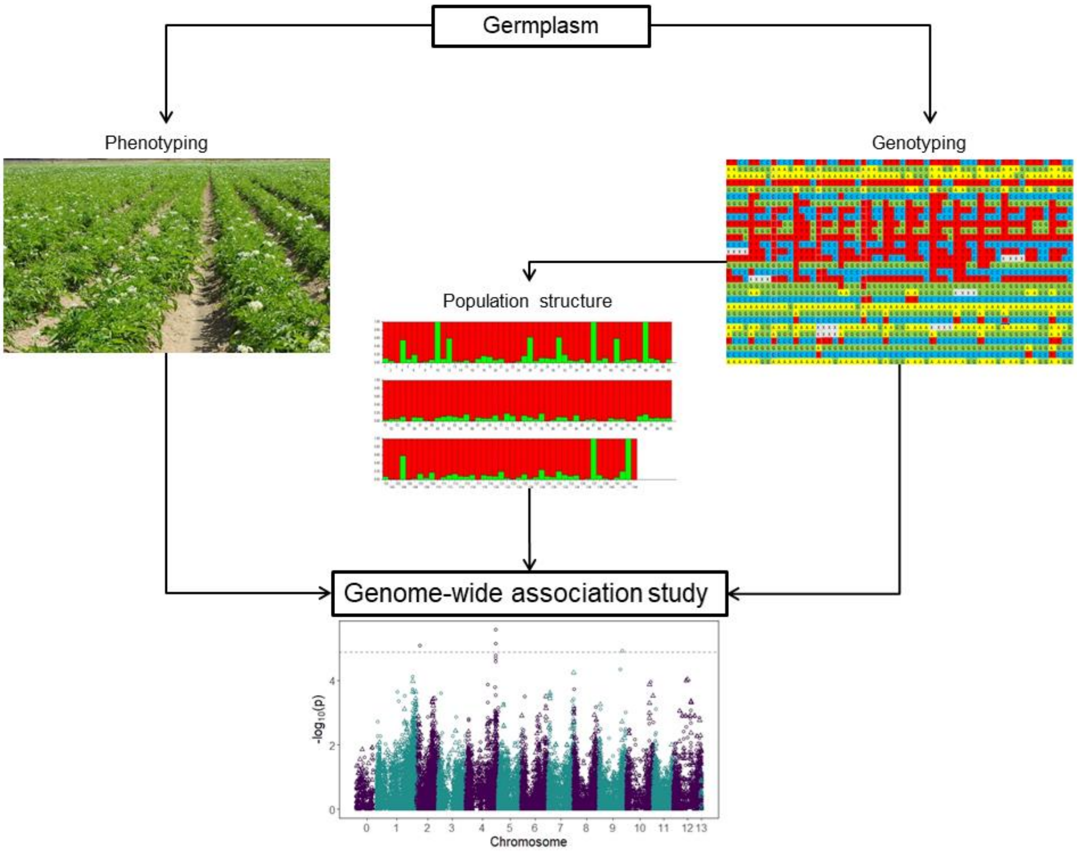


Figure 1.6. Schematic diagram of a typical methodology for a GWAS.

To study and correct the genetic relatedness of the mapping population, the marker data obtained from genotyping are used to estimate the degree of relatedness between individuals and assign them to subpopulations (Brachi et al., 2011; Zia et al., 2017). This estimation can be performed by STRUCTURE software or PCA, obtaining the Q matrix in which the proportion of each individual's variation that came from a particular subpopulation is estimated (Pritchard, 2007). However, in many cases correction based on the Q matrix alone is not always adequate to avoid false associations, and therefore it is useful to obtain the kinship (K) matrix that establishes the kinship between pairs of individuals and explains a proportion of the phenotypic variation (Xu et al., 2017). The mixed linear model (MLM) is the most widely used in GWAS analysis and uses the Q + K method in which the matrices mentioned previously are used. Compared to other methods, the best results are obtained using this approach, controlling type I and type II error rates (Viana et al., 2017).

Linkage disequilibrium (LD) describes the degree of non-random association of two alleles at different loci in a population. These loci may be next to each other on the same chromosome or they may be on different chromosomes (Flint-Garcia et al., 2003). The effectiveness of GWAS studies is highly dependent on the degree of LD between the genotyped markers and the QTL (Zia et al., 2017). The strength of the correlation between two markers depends on the distance between them, being stronger the closer the markers are (Myles et al., 2009). The rate at which LD decays with physical distance in the population determines the required marker density and the level of resolution at which a QTL can be mapped. If LD decays slowly within a region, a small number of markers are required to scan the genome and the mapping resolution will be low, whereas if LD decays rapidly, a relatively large number of markers will be required, but the mapping resolution will increase (Álvarez et al., 2014; Xu et al., 2017).

3. Drought stress in agriculture

According to IPCC reports (https://www.ipcc.ch/), climate change is mainly caused by emissions of greenhouse gases into the atmosphere, such as carbon dioxide, methane and nitrous oxide, as well as inadequate land use and management practices. The consequences of this climate change process include an increase in global warming and extreme, irreversible and widespread meteorological phenomena. During the last decades many drought episodes are being experienced in Europe, not only in the semi-arid regions of the Iberian Peninsula and the Mediterranean regions, but also in almost all the territory, from Western Europe to Eastern Europe (Grillakis, 2019). Figure 1.7 shows the spatial map representing the average duration of drought in months in Europe during the last century. Spain is experiencing frequent waves of very high temperatures, a generalized decrease in annual rainfall, numerous periods of drought and a greater number of torrential rains (Hervás-Gámez & Delgado-Ramos, 2019). In Spain there is a clear imbalance in water availability between the northern, central and southeastern areas and although the national average values show sufficient resources, there are numerous areas with water shortages due to this irregular distribution of resources (Estrela & Vargas, 2012).

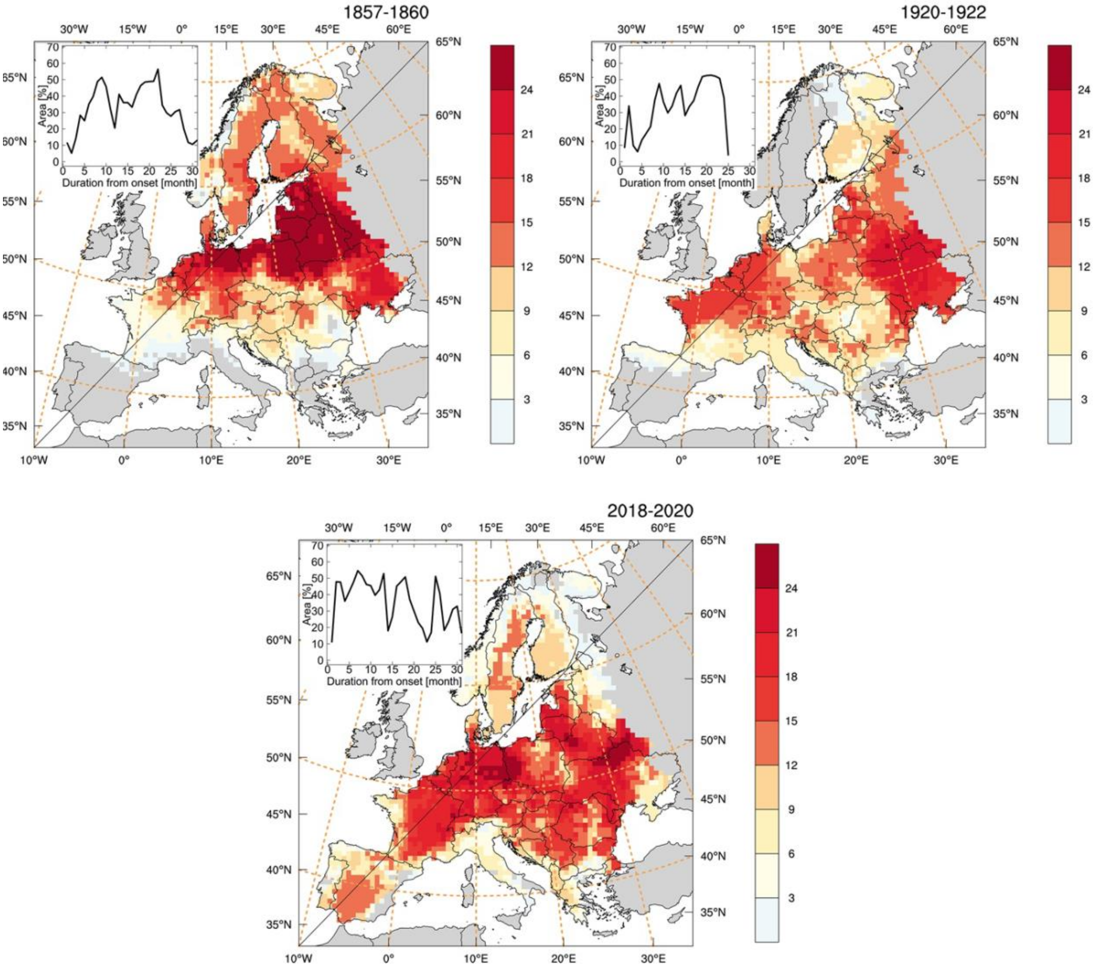


Figure 1.7. Spatial map representing the mean drought duration in months for the periods 1857-1860, 1920-1922 and 2018-2020 (Rakovec et al., 2022).

Agriculture is a sector that is highly vulnerable to climate change due to its large magnitude and its dependence on meteorological parameters, causing major economic repercussions. The yield of major crops in drought areas is expected to be reduced by more than 50% by 2050 and by almost 90% by 2100 with a consequent increase in prices that puts the food security of the planet at risk (Li et al., 2018; Malhi et al., 2021). Climate change represents a challenge for plant breeders, as they have to achieve further increases in crop yield and quality in this new scenario and will require an emphasis on tolerance to abiotic and biotic stresses, which will be intensified by climate change (Pareek et al., 2020).

In spite of climate change, there have always been regions on earth where climatic conditions are extreme, either in terms of temperature, water or salinity, and the wide diversity of plants that inhabit these areas leads us to believe that plants have defensive or adaptive mechanisms that have allowed them to evolve under these circumstances (Nemeskéri & Helyes, 2019). Regarding drought, it has been found that plants have three main methods of action: drought escape, drought avoidance and drought tolerance. The first of them is based on the ability of plants to modulate their vegetative and reproductive growth according to water availability, completing their biological cycle before the beginning of stress, favoring early varieties. Drought avoidance involves physiological and morphological changes that allow the maintenance of relatively high water content in plants, either by reducing water loss or by increasing water uptake. Finally, drought tolerance also involves morphological and physiological changes in plants, but in this case with the objective of withstanding low tissue water content (Basu et al., 2016; Boguszewska-Mańkowska et al., 2018).

Plants must combine biochemical, morphological, physiological and anatomical responses to adapt to drought stress and maintain crop yield. One of the main responses of plants to water stress is stomatal closure which can influence CO2 uptake and thus photosynthesis and plant growth since ion and water transport systems across membranes function to control turgor pressure changes in guard cells and stimulate stomatal closure (Osakabe et al., 2014). Plant roots also play an important role in water stress tolerance so it is necessary for plants to extend the root system, to increase the number of functional roots, and to increase the water absorption capacity of the root sheath to improve water uptake (Wu et al., 2022).

Evaluation of drought tolerant varieties is not easy as many factors are involved, including environmental and genetic factors, so non-destructive screening techniques that help evaluate plant responses to stress and allow breeders to select outstanding genotypes and growers to decide the timing of irrigation are of great interest (Nemeskéri & Helyes, 2019).

3.1. Drought stress in potato crop

Compared to other species, potato is very sensitive to water stress due to its shallow root system: about 85% of the root length is concentrated in the upper 0.3-0.4

m of the soil and about 50% of the available water in the soil must be in the root zone (Cantore et al., 2014; Ierna & Mauromicale, 2012). Plant response to water stress depends on the timing and duration of stress and susceptibility of the cultivar (Nasir & Toth, 2022). It has been found that the developmental stage of the plant is the most susceptible phase to water stress. It is at this early stage that stomatal closure occurs to prevent water loss and affecting photosynthesis, so the visible signs of stress in this period are usually a reduction in vegetative growth, plant height and leaf size and number (Deblonde & Ledent, 2001). When water stress occurs at the tuber formation stage, the consequences are a decrease in yield due to a reduction in the number and size of tubers, as well as their quality.

It is important to keep in mind that potato is an important crop in Mediterranean countries that are being severely affected by the consequences of climate change, so in these countries it is very important to optimize irrigation management and to develop tolerant varieties to water stress. In some areas such as southern Italy, Cyprus, Turkey or North Africa, planting has even started to be advanced to a winter-spring cycle (November to January) due to high temperatures and low rainfall (Cantore et al., 2014; Lombardo et al., 2020).

During the last few years, the response to drought in potato has attracted the interest of the scientific community and numerous studies have been carried out. Hill et al. (2021) in their publication explored the effects of water deficit on traits such as canopy, leaf area index (LAI) or dry matter, to identify phenotypes that may be associated with tolerance to water stress in potato. Furthermore, Zaki & Radwan (2022) conducted an in vitro and field trial with 21 commercial potato cultivars and found that a group of varieties behaved better under water stress conditions and also overexpressed genes related to stress tolerance. Despite the efforts to understand the mechanisms of drought tolerance, there is still work to do to identify and develop varieties that better adapt to the new climate change scenario.

Chapter 2

Objectives

Objectives

The general objective of this work was to better understand the response of potato (*Solanum tuberosum* L.) plants under water stress conditions from a physiological, agronomic and genomic point of view. This general objective is supported by several specific objectives corresponding to different chapters:

- Determination of the physiological response to water stress through a trial under controlled conditions in greenhouse with a small number of varieties of different vegetative cycles (Chapter 3).

- Analysis of the differential expression of genes related to water stress using RNASeq (Chapter 4).

- Agronomic and physiological evaluation under field conditions of a set of varieties subjected to water deficit in order to assess their response by estimating physiological and yield parameters (Chapter 5).

- Genome Wide Association Study (GWAS) to identify QTLs associated with traits related to water stress response (Chapter 5).

Chapter 3

Evaluation of physiological and yield parameters related to drought stress in potato under greenhouse conditions

The content of this chapter corresponds to the published article: Alvarez-Morezuelas A., Barandalla L., Ritter E., Lacuesta M., Ruiz de Galarreta J.I. 2022. Physiological response and yield components under greenhouse drought stress conditions in potato. Journal of Plant Physiology 278: 153790. https://doi.org/10.1016/j.jplph.2022.153790

Chapter 3: Evaluation of physiological and yield parameters related to drought stress in potato under greenhouse conditions

1. Abstract

Due to the effects of climate change, conditions tend to be increasingly extreme, with water availability being one of the main limiting factors in potato production. The objective of this study was to analyze the differential response of physiological and yield components in six potato varieties under water deficit conditions. For this purpose, a greenhouse trial was carried out with the varieties Agata, Agria, Kennebec, Monalisa, Sante and Zorba. The drought stress was applied in stressed plants 36 days after planting (DAP) by withholding water for 25 days. All measurements were taken at four different times: before stress (T0), 17 days (T1) and 24 days (T2) after stress and five days after re-watering. The physiological parameters evaluated were chlorophyll content and fluorescence, relative leaf water content, stomatal conductance, electrolytic leakage and water potential. After the drought period, the aerial part of half of the plants was cut to evaluate the produced biomass. At the end of the cycle yield components were determined. Stomatal conductance and water potential were the parameters that showed the highest differences between the two hydric conditions, and Monalisa was the variety with the best response in tuber production under stress conditions. Indirect selection based on parameters associated with water stress can be a useful tool in potato breeding programs for the identification of more tolerant varieties.

2. Introduction

Greenhouse gas emissions, mainly carbon dioxide (CO_2), have increased considerably in recent decades, leading to a significant climate change (IPCC 2014). Water availability is probably the most important limiting factor in crop production (Bates et al., 2008), affecting plant physiology and productivity and its negative effect will increase due to climatic change..

Potato (*Solanum tuberosum* L.) is one of the world's most important crops and provides an important contribution to the global food supply, producing more than 370 million tons of tubers and yielding 21.3 t/ha in 2019 (FAO, 2020). Potato plants use water relative efficiently, but due to its short and shallow root system, potato is considered a drought-sensitive crop and water deficit can cause significant yield and tuber quality losses (Anithakumari et al., 2012; Haverkort & Verhagen, 2008).

Drought has a strong effect on morphological and physiological aspects of potato plants, e.g. number and size of tubers, leaf area, photosynthetic rate, biomass or stomatal conductance (Rodríguez-Pérez et al., 2017; Schafleitner et al., 2007). Quality traits such as starch content or starch yield are also strongly influenced by the water status of the plant (Meise et al., 2019). However, physiological responses to drought depends on the variety, origin of seeds and physiological age of the tubers, but are also affected by the duration and severity of the stress (Monneveux et al., 2014).

Plants tolerance to water stress can be defined as the ability to maintain a certain level of physiological activity, which is achieved through the regulation of various genes and metabolic pathways which can reduce or repair the damage caused by stress (Zhang et al., 2018). Furthermore, from an agricultural point of view, tolerant crops are considered those that maintain yield also under drought conditions (Pinheiro & Chaves, 2011).

Breeding for drought tolerance using conventional methods is challenging because it represent a complex polygenic trait involved in multiple pathways. Moreover, in potato molecular breeding is more difficult, due to the lower efficiency of association mapping in tetraploids (Muthoni & Kabira, 2016). However, there is a wide genetic variability and a large pool of germplasm resources in native and wild potato species, allowing to find genotypes that are largely adapted to different environments based on genes for tolerance to different stresses (Anithakumari et al., 2012; Monneveux et al., 2013). One way to select varieties that are more tolerant to water stress is through indirect selection based on secondary traits associated with stress tolerance. These traits are easy to measure and have a high heritability (Obidiegwu et al., 2015; Rudack et al., 2017).

The identification of potato genotypes with tolerance to water stress could be a good strategy for mitigating the effects of climate change on the productivity of this crop. Thus, the aim of this study was to find traits of tolerance against water stress in six potato varieties analyzing the differential response of physiological and yield-related parameters under drought stress conditions in the greenhouse.

3. Materials and methods

3.1. Plant material and growth conditions

The potato varieties used in this assay were Agata, Agria, Kennebec, Monalisa, Sante and Zorba. They were selected for their different range of productivity and maturity, representing the commercial varieties currently grown in Spain.

The trial was conducted in a greenhouse under optimal conditions for potato such as 18-22ºC night/day, 80% of humidity and supplemented with artificial light to obtain 16h light/8h dark. Eight replications per cultivar and treatment were planted in 5 L pots with peat. Four of these eight replications were cut to estimate the produced biomass after the drought period, having four replicates at the end of the cycle. Each pot was watered weekly with 1 L of water to obtain water holding capacity. The drought stress was applied in eight randomly selected pots (D), 36 days after planting (DAP) by withholding water for 25 days. Control plants (C) were watered normally. After this drought period, all plants were watered again to obtain field capacity until the end of the cycle.

All the measurements were made on the last fully expanded leaf at 4 different times: before stress (T0, 36 DAP), at 17 day after stress (T1, 53 DAP), at 25 day after stress (T2, 60 DAP) and 5 days after re-watering (R1, 65 DAP). Plants were harvested at the end of their vegetative cycle.

3.2. Physiological parameters

Chlorophyll content (SPAD)

The chlorophyll content, which is closely related to the greenness of the plant (Adamsen et al., 1999), was measured in each genotype using a SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan). Measurements were taken on 3 leaves of each plant and in three randomly selected plants of each variety and treatment at each time.

Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a fluorimeter (FluorPen FP 100, Photon Systems Instruments, Drasov, Czech Republic) in the last fully expanded leaf in three randomly selected plants of each variety and treatment at the 4 different times. The photochemical efficiency of PSII was measured in dark adapted leaves (Fv/Fm) and in leaves exposed to light (Fv’/Fm’).

Relative Water Content (RWC)

The relative water content was measured on the last fully expanded leaf at T1, T2 and R1. Leaf discs of 2 cm Ø were taken from 3 randomly selected plants of each variety and treatment and immediately weighed to obtain the fresh weight (Wf) and then introduced into a 5 ml vial with deionized water. They were kept in dark for 24 hours at 4ºC and then re-weighed after dried gently with paper yo obtain turgid weight (Wt). The leaves were dried in an oven at 70°C for 24 hours and weighed to obtain the dry weight (Wd). The relative water content was calculated according to the formula (Barrs & Weatherley, 1962):

$$RWC\,(\%) = (Wf - Wd)/(Wt - Wd) * 100$$

Stomatal conductance (gs)

Stomatal conductance (gs, mmol $H_2O\ m^{-2}\ s^{-1}$) was measured using a porometer (Leaf Porometer, Decagon Devices, Pullman, Washington, EEUU) on the last fully expanded leaf of 3 randomly selected plants of each variety and treatment.

Electrolytic leakage (EL)

Electrolyte leakage was measured as in Mena-Petite et al. (2001). Leaf discs of 1 cm Ø were taken of 3 randomly selected plants for each variety and treatment. The leaf discs were introduced into a vial with 5 ml of deionized water and then initial conductance was measured (ECi) using a portable conductivity meter (CRISON CM 35, Barcelona, Spain). After 24 hours electrolytic conductance was determined again (ECf). Samples were autoclaved for 10 min at 121ºC and the total electrical conductivity (ECt) was measured. Electrolytic leakage was calculated according to the formula:

$$EL(\%) = \frac{(ECf - ECi)}{(ECt - ECi)} * 100$$

Water potential

Water potential of 3 replications of each variety and treatment was measured at predawn (Ψpd) and at midday (Ψleaf) along T0, T1, T2 and R1. For this measurement a Scholander chamber (Skye SKPM 1400) was used following the pressure-equilibrium technique (Scholander et al., 1965).

3.3. Agronomic traits

Aerial biomass

After the drought period, 4 plants of each genotype and treatment were cut to estimate the produced biomass. The aerial part of the plant was weighed to obtain the fresh weight and dried in an oven at 80ºC until the plants were completely dry and weighed again to obtain dry weight.

Tuber number and weight

The remaining four replicates were harvested at the end of the vegetative cycle for each variety. Fresh tuber weight was measured in 4 replications for each pot and the total number of tubers was counted.

Tuber dry matter and starch content

Dry matter content was measured in four replications of each variety and treatment. Tubers were weighed immediately after harvest (FW). After 72 hours at 80ºC, they were weighed again to obtain the dry weight (DW). The starch content was calculated with the following formula (Müller & Cervenkova, 1978):

$$\text{Starch} = \left(\frac{\text{DW}}{\text{FW}} * \ 100 - 6.0313\right) * 10$$

3.4. Statistical analysis

Statistical analysis was performed using the computing environment R (v 3.3.2). All reported experimental data were averages of three replicates of each variety and treatment for physiological traits and four replicates for agronomic traits, and were subjected to analysis of variance (ANOVA). Tukey's HSD test was used to determine the different significance levels among the factors. Pearson's correlation was used to determine the relationship between the parameters. Significant differences with respect to each control were considered according to Tukey's HSD test after ANOVA for each variety. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

4. Results

Different responses among varieties were observed along the drought period and subsequent recovery after watering and significant differences were found in different traits analyzed (Table 3.1 and Table 3.2), whereas only stomatal conductance, relative water content (RWC) and predawn (Ψpd) and midday (Ψleaf) water potential showed significant differences between treatments. On the other hand various significant interactions between variety and treatment (VxTr), variety and measuring time (VxTi), Tr xTi and even the triple interaction between the three factors were observed, depending on the measured trait. For RWC and water potential they were significant for all interactions. These findings indicate that the varieties perform different depending on water regime and measuring time.

Fv/Fm and Fv'/Fm' were estimated to evaluate the maintenance of photosynthetic activity under drought stress (Figure 3.1). All varieties showed a high value of Fv/Fm of 0.83 at T0, similar to unstressed plants (Björkman & Demmig, 1987). Both parameters didn't show significant differences between control and stressed plants, with exception of Monalisa variety which showed a decrease in Fv/Fm at the recovery time (Figure 3.1a) and a decrease in the Fv'/Fm' value 17 days after stress (T1)

(Figure 3.1b), being Agata and Zorba the ones that showed the lowest decline. All plants showed a slow decrease at R1 probably due to natural senescence of the plant.

Table 3.1. Analysis of variance of physiological variables in three replicates of six potato varieties (V) under irrigation and no irrigation conditions (Tr) at four different times (Ti). The evaluated parameters were chlorophyll content (SPAD), stomatal conductance (gs), chlorophyll fluorescence (Fv/Fm and Fv′/Fm′), relative water content (RWC), electrolytic leakage (EL%), predawn water potential (Ψpd) and midday water potential (Ψleaf).

	SPAD	**g*s***	**Fv/Fm**	**Fv′/Fm′**	**RWC**	**EL%**	**Ψpd**	**Ψleaf**
Variety (V)	18.33***	6.21***	12.82***	25.73***	8.86***	17.98***	8.04***	5.91***
Treatment(Tr)	0.16ns	133.06***	0.42ns	2.07ns	35.05***	2.03ns	160.86 ***	172.94***
Time (Ti)	2.07ns	50.02***	223.62***	170.63***	6.55**	12.10***	37.75***	79.82***
V x Tr	1.54ns	2.25	2.15	2.24	5.83***	1.03ns	2.64*	1.58ns
V x Ti	3.04***	9.51***	4.08***	2.36**	7.25***	8.19***	4.90***	4.86***
Tr x Ti	3.64*	23.07***	0.59ns	0.36ns	25.65***	1.86ns	33.06***	63.66***
V x Tr x Ti	0.66ns	1.25ns	2.47**	1.98*	4.51***	3.60***	2.04*	0.97ns

*, **,*** Significant at P=0.05, P= 0.01 and P=0.001, respectively, ns: not significant

Table 3.2. Analysis of variance of agronomic traits in four replicates of six potato varieties (V) under irrigation and no irrigation conditions (Tr). The evaluated parameters were tuber number, yield, tuber weight, dry biomass, dry matter and starch yield.

	Tuber number	**Yield**	**Tuber weight**	**Dry biomass**	**Dry matter**	**Starch yield**
Variety (V)	12.064***	6.256***	8.206***	5.641***	4.653**	6.257***
Treatment(Tr)	2.897ns	33.117***	8.498**	4.653*	9.157**	5.741*
V x Tr	7.683***	5.878***	2.706*	7.530***	3.543*	8.078***

*, **,*** Significant at P=0.05, P= 0.01 and P=0.001, respectively, ns: not significant

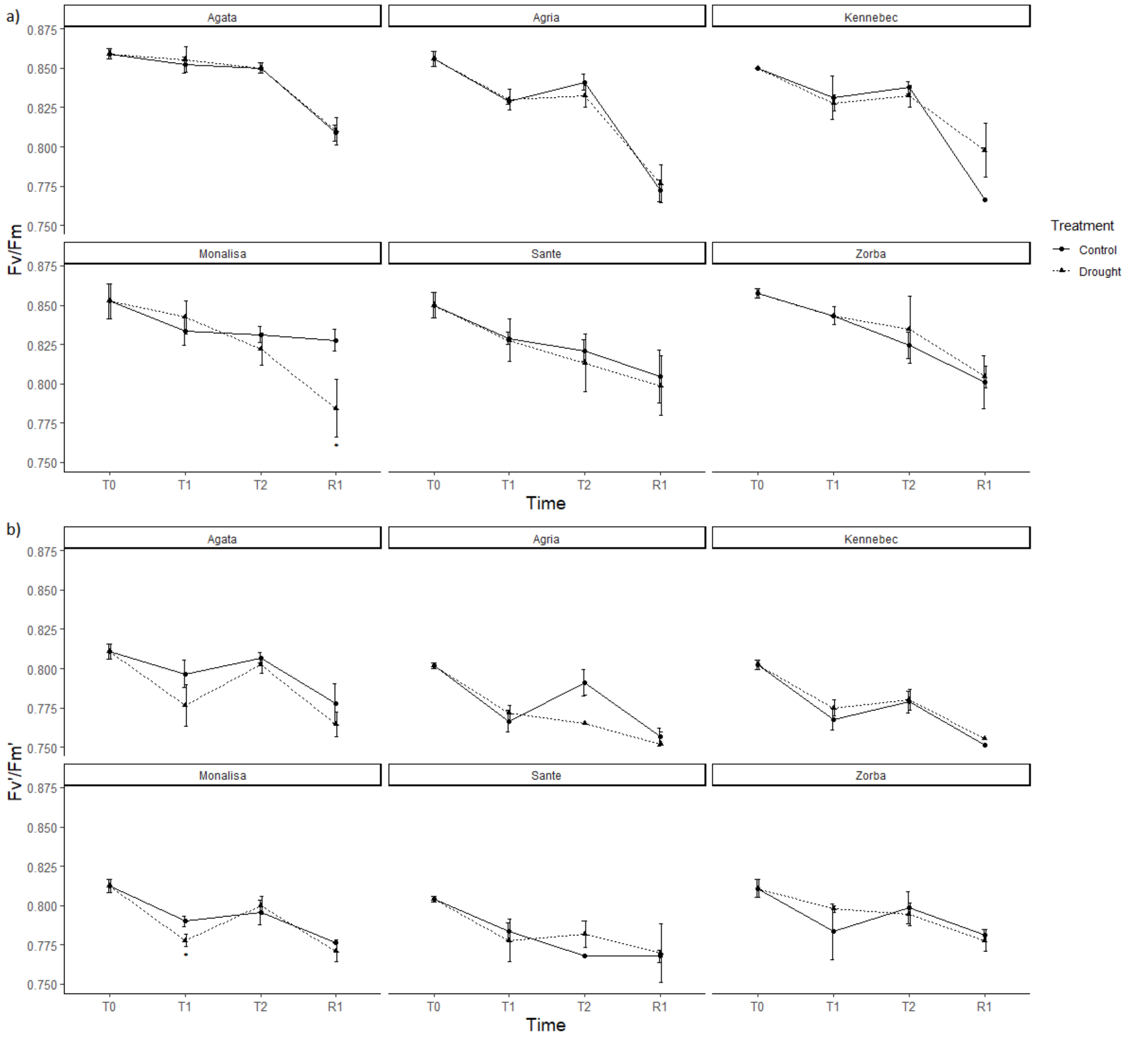


Figure 3.1. a) Photochemical efficiency of PSII measured in dark adapted leaves (Fv/Fm) and b) in leaves exposed to light (Fv'/Fm') in three replicates of six potato varieties at four different times in control and water stressed plants. Significant differences with respect to each control are represented by asterisks according to Tukey's HSD test after ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

In this study the chlorophyll content measured as SPAD units didn't show significant differences between control and stressed plants.

Agata showed the lowest RWC (50%) in control plants although no effect of drought was observed. In contrary, Monalisa showing the highest RWC of all varieties studied (70%) was affected at the beginning of the stress (T1), but recovered the water content after re-watering. The rest of varieties were able to maintain the RWC during the stress period (Figure 3.2). Table 3.3 shows that this parameter correlates positively with plant biomass under drought conditions in the stress period and negatively during recovery (Table 3.4). It also correlates significantly with stomatal conductance, another trait that is closely related to leaf water conditions.

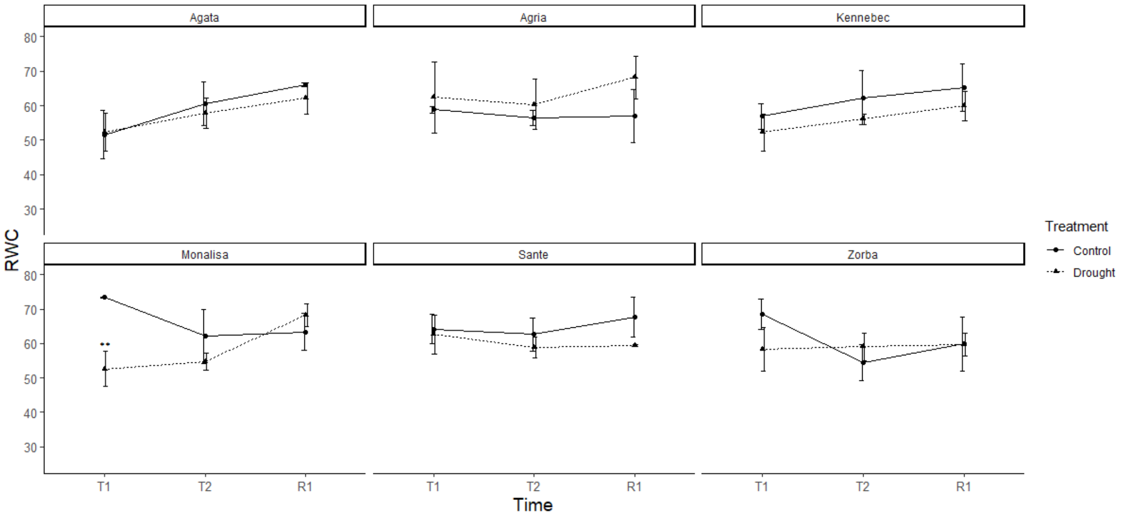


Figure 3.2. Relative water content (RWC) measured in three replicates of six potato varieties at three different times in control and water stressed plants. Significant differences with respect to each control are represented by asterisks according to Tukey's HSD test after ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Stomata closure is used by plants to reduce water loss through transpiration, maintaining an adequate leaf water level. All varieties were affected by water deficiency and closed their stomata earlier at T1. The highest stomatal closure was at T2, precisely the moment of the highest stress. Agria and Kennebec varieties closed their stomata more drastically at the first stage of stress and Monalisa showed lower stomata closure (Supplementary Figure 3.1). When plants were re-watered (R1), the stomata were opened again significantly in varieties Monalisa, Sante and Agria, getting levels quite similar to control plants, whereas Kennebec, Agata and Zorba revealed less recovery of stomata conductance after re-watering (Figure 3.3).

Table 3.3. Pearson's correlation coefficients between physiological parameters: [chlorophyll content (SPAD), stomatal conductance (gs), chlorophyll fluorescence (Fv/Fm and Fv′/Fm′), RWC, electrolytic leakage (EL%), predawn water potential (Ψpd) and midday water potential (Ψleaf)] and yield components (tuber number, total yield, tuber weight, dry biomass, tuber dry matter and starch yield) estimated under drought stress (below diagonal) and in control plant (above diagonal) at T2.

	SPAD	**gs**	**Fv/Fm**	**Fv′/Fm′**	**RWC**	**EL%**	**Ψpd**	**Ψleaf**	**Tuber number**	**Yield**	**Tuber weight**	**Dry biomass**	**Dry matter**	**Starch yield**
SPAD	1	-0.07ns	**-0.62****	-0.17ns	-0.18ns	0.01ns	0.06ns	0.38ns	0.23ns	0.30ns	-0.18ns	**0.54****	0.03ns	-0.18ns
gs	0.21ns	1	0.22ns	0.24ns	0.12ns	-0.36ns	-0.02ns	**-0.41***	0.37ns	-0.31ns	-0.40ns	0.04ns	-0.33ns	-0.35ns
Fv/Fm	-0.20ns	-0.13ns	1	**0.52****	0.15ns	0.24ns	-0.12ns	-0.30ns	-0.19ns	-0.32ns	0.06ns	-0.31ns	-0.02ns	0.10ns
Fv′/Fm′	-0.35ns	**0.48***	0.29ns	1	-0.30ns	0.26ns	-0.17ns	0.00ns	-0.04ns	**-0.43***	-0.28ns	**-0.48***	-0.32ns	-0.20ns
RWC	0.39ns	-0.12ns	-0.09ns	-0.15ns	1	-0.31ns	0.10ns	-0.29ns	0.23ns	0.24ns	0.06ns	-0.04ns	-0.14ns	-0.16ns
EL%	-0.01ns	**0.68*****	0.11ns	**0.55****	-0.40ns	1	-0.29ns	0.18ns	-0.23ns	**-0.41***	-0.02ns	-0.21ns	-0.14ns	-0.06ns
Ψpd	**-0.60****	-0.12ns	0.34ns	**0.51***	-0.02ns	0.04ns	1	0.12ns	0.33ns	0.16ns	-0.07ns	0.06ns	0.10ns	0.00ns
Ψleaf	0.23ns	0.04ns	0.22ns	0.07ns	0.13ns	-0.08ns	0.09ns	1	0.08ns	0.18ns	-0.06ns	0.06ns	0.14ns	0.12ns
Tuber number	-0.24ns	-0.07ns	-0.25ns	0.11ns	0.00ns	-0.19ns	0.38ns	0.13ns	1	-0.13ns	**-0.79*****	0.05ns	-0.35ns	**-0.42***
Yield	0.29ns	**0.48***	0.17ns	-0.08ns	-0.11ns	0.31ns	**-0.41***	-0.20ns	**-0.49***	1	**0.59****	0.36ns	**0.61****	**0.51***
Tuber weight	0.09ns	-0.25ns	0.02ns	-0.37ns	0.05ns	-0.19ns	-0.38ns	-0.14ns	**-0.79*****	0.38ns	1	0.11ns	**0.58****	**0.59****
Dry biomass	0.18ns	**-0.54****	0.02ns	**-0.42***	**0.46***	**-0.66*****	0.01ns	0.09ns	-0.01ns	-0.16ns	0.34ns	1	0.38ns	0.15ns
Dry matter	-0.06ns	-0.17ns	-0.29ns	-0.12ns	0.16ns	-0.33ns	0.06ns	-0.24ns	0.14ns	-0.28ns	-0.02ns	0.25ns	1	**0.75*****
Starch yield	0.27ns	-0.24ns	**-0.47***	-0.25ns	0.37ns	**-0.51***	0.01ns	0.10ns	0.22ns	-0.33ns	-0.06ns	**0.43***	**0.70*****	1

*, **,*** Significant at P=0.05, P= 0.01 and P=0.001, respectively., ns: not significant

Table 3.4. Pearson’s correlation coefficients between physiological parameters: [chlorophyll content (SPAD), stomatal conductance (gs), chlorophyll fluorescence (Fv/Fm and Fv’/Fm’), RWC, electrolytic leakage (EL%), predawn water potential (Ψpd) and midday water potential (Ψleaf)] and production parameters (tuber number, total yield, tuber weight, dry biomass, tuber dry matter and starch yield) estimated under drought stress (below diagonal) and in control plant (above diagonal) at R1.

	SPAD	**g*s***	**Fv/Fm**	**Fv’/Fm’**	**RWC**	**EL%**	**Ψpd**	**Ψleaf**	**Tuber number**	**Yield**	**Tuber weight**	**Dry biomass**	**Dry matter**	**Starch yield**
SPAD	1	**-0.42***	0.10ns	0.23ns	-0.06ns	0.29ns	-0.29ns	-0.23ns	-0.15ns	0.19ns	0.07ms	0.38ns	-0.12ns	-0.13ns
g*s*	-0.30ns	1	-0.37ns	**-0.50***	0.33ns	-0.34ns	0.28ns	0.20ns	-0.17ns	0.27ns	0.38ns	0.11ns	**0.44***	**0.42***
Fv/Fm	**0.56****	-0.33ns	1	**0.72*****	-0.24ns	-0.26ns	-0.21ns	-0.19ns	0.12ns	**-0.77*****	**-0.47***	-0.39ns	**-0.66*****	**-0.65*****
Fv’/Fm’	**0.51***	-0.13ns	**0.60****	1	-0.37ns	0.10ns	-0.27ns	-0.07ns	0.30ns	**-0.61****	**-0.59****	-0.17ns	**-0.69*****	**-0.75*****
RWC	-0.13ns	**0.53****	-0.33ns	0.03ns	1	0.09ns	0.05ns	0.26ns	**-0.42***	0.32ns	**0.51***	0.06ns	0.33ns	0.38ns
EL%	-0.01ns	-0.30ns	0.28ns	0.05ns	-0.33ns	1	-0.18ns	-0.02ns	0.22ns	0.38ns	0.03ns	0.16ns	0.03ns	-0.16ns
Ψpd	-0.24ns	**0.45***	**-0.54****	-0.35ns	**0.76*****	**-0.43***	1	-0.18ns	**-0.47***	-0.04ns	0.32ns	-0.30ns	0.14ns	0.21ns
Ψleaf	0.06ns	-0.35ns	**0.46***	-0.19ns	-0.28ns	0.17ns	-0.28ns	1	0.26ns	-0.10ns	-0.10ns	**0.42***	0.18ns	0.06ns
Tuber number	-0.03ns	-0.01ns	**0.49***	**0.45***	-0.09ns	0.13ns	-0.23ns	-0.01ns	1	-0.13ns	**-0.79*****	0.05ns	-0.35ns	**-0.42***
Yield	0.00ns	-0.01ns	-0.33ns	-0.32ns	0.37ns	-0.38ns	**0.49***	0.04ns	**-0.49***	1	**0.59****	0.36ns	**0.61****	**0.51***
Tuber weight	-0.15ns	-0.04ns	**-0.44***	**-0.56****	-0.10ns	-0.06ns	0.05ns	0.10ns	**-0.79*****	0.38ns	1	0.11ns	**0.58****	**0.59****
Dry biomass	-0.04ns	**-0.44***	0.12ns	-0.12ns	**-0.46***	**0.48***	-0.36ns	0.13ns	-0.01ns	-0.16ns	0.34ns	1	0.38ns	0.15ns
Dry matter	-0.16ns	-0.18ns	-0.17ns	0.17ns	-0.32ns	0.22ns	-0.39ns	-0.15ns	0.14ns	-0.28ns	-0.02ns	0.25ns	1	**0.75*****
Starch yield	-0.01ns	-0.07ns	-0.01ns	0.25ns	-0.39ns	0.33ns	-0.32ns	-0.38ns	0.22ns	-0.33ns	-0.06ns	**0.43***	**0.70*****	1

*, **,*** Significant at P=0.05, P= 0.01 and P=0.001, respectively., ns: not significant

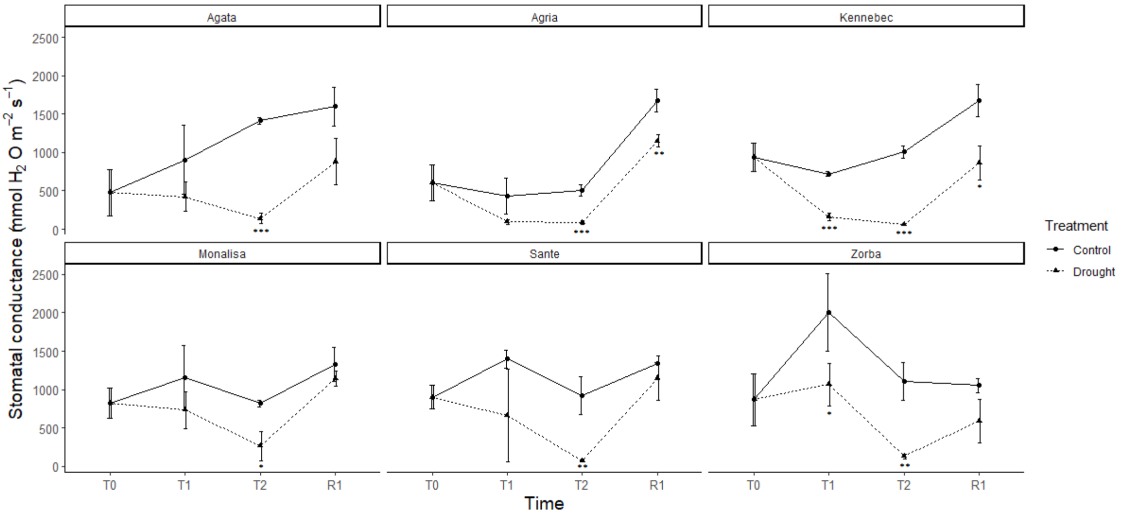


Figure 3.3. Stomatal conductance (mmol H_2O m^{-2} s^{-1}) measured in three replicates of six potato varieties at four different times in control and water stressed plants. Significant differences with respect to each control are represented by asterisks according to Tukey's HSD test after ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

Drought stress could affect also membrane integrity and permeability. Zorba was the only variety that didn't show significant differences at any time. The rest of varieties had more electrolyte leakage during stress, particularly Monalisa and Sante at T2, but they are able to recover to normal values after re-watering. The varieties Agata and Agria were less affected during the stress period, but after re-watering they did not recover the control values (Figure 3.4). The results show that electrolytic conductance increases under drought conditions, and that it is negatively correlated with plant biomass production (Table 3.3), although the alteration was reversible in four of the six varieties except in Agata and Agria, the ones with the lowest stomatal conductance (Figure 3.3).

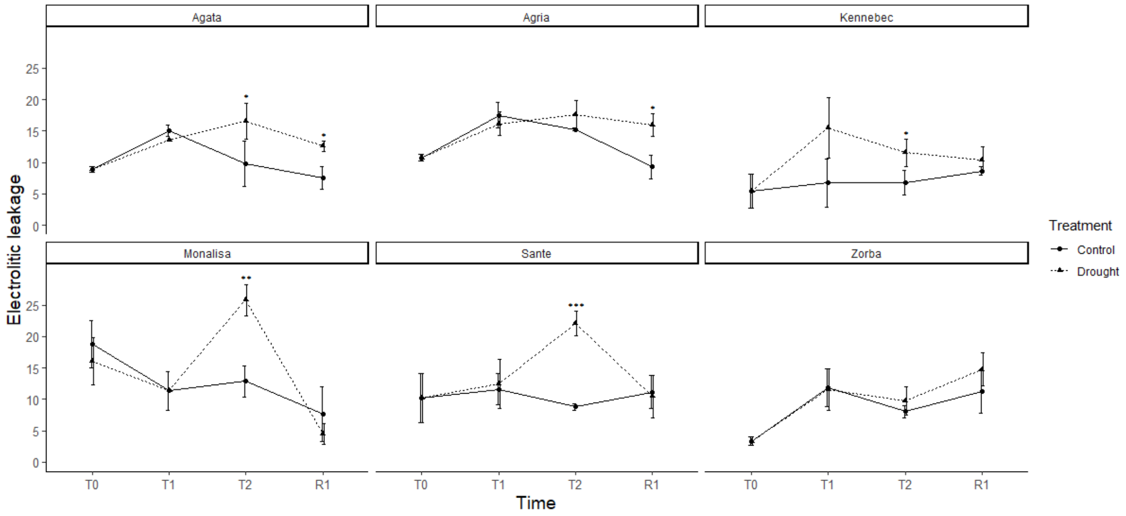


Figure 3.4. Percentage electrolytic leakage measured in three replicates of six potato varieties at four different times in control and water stressed plants. Significant differences with respect to each control are represented by asterisks according to Tukey's HSD test after ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

The water status of the plant was evaluated by the water potential measured at predawn and at midday (Figure 3.5). All varieties showed significant differences during the stress period in both measured parameters. Predawn water potential (Ψpd) showed a significant decrease at T2 in all varieties except in Agata, which only showed significant decrease at R1. Lower reduction in (Ψpd), was also observed in Sante, whereas the rest of varieties showed similar decrease in (Ψpd) at T2 (-0.75 MPa as average) indicating that these varieties were subjected to a similar soil moisture (Figure 3.5). None of the varieties recovered the control values after re-watering. Concerning water potential measured at midday (Ψleaf), the varieties that showed highest decrease were Kennebec, Monalisa and Santé reaching values below -1MPa. At R1, Kennebec recovered the control values, despite the fact that during stress the water potential of the stressed plants decreased considerably.

After the drought period (T2) most of the varieties, except Agata and Sante, showed values of Ψpd of approximately -0,8 MPa, (Figure 3.5) pointing out that these varieties were subjected to the same water stress. After re-watering none of the varieties analyzed were able to recover the control values of Ψpd, suggesting that the 5 days of re-watering were not enough to recover plants from the strong stress induced. The lower decrease of Ψpd observed in Agata at T2 could be related to the low stomatal conductance of this variety (Figure 3.3).

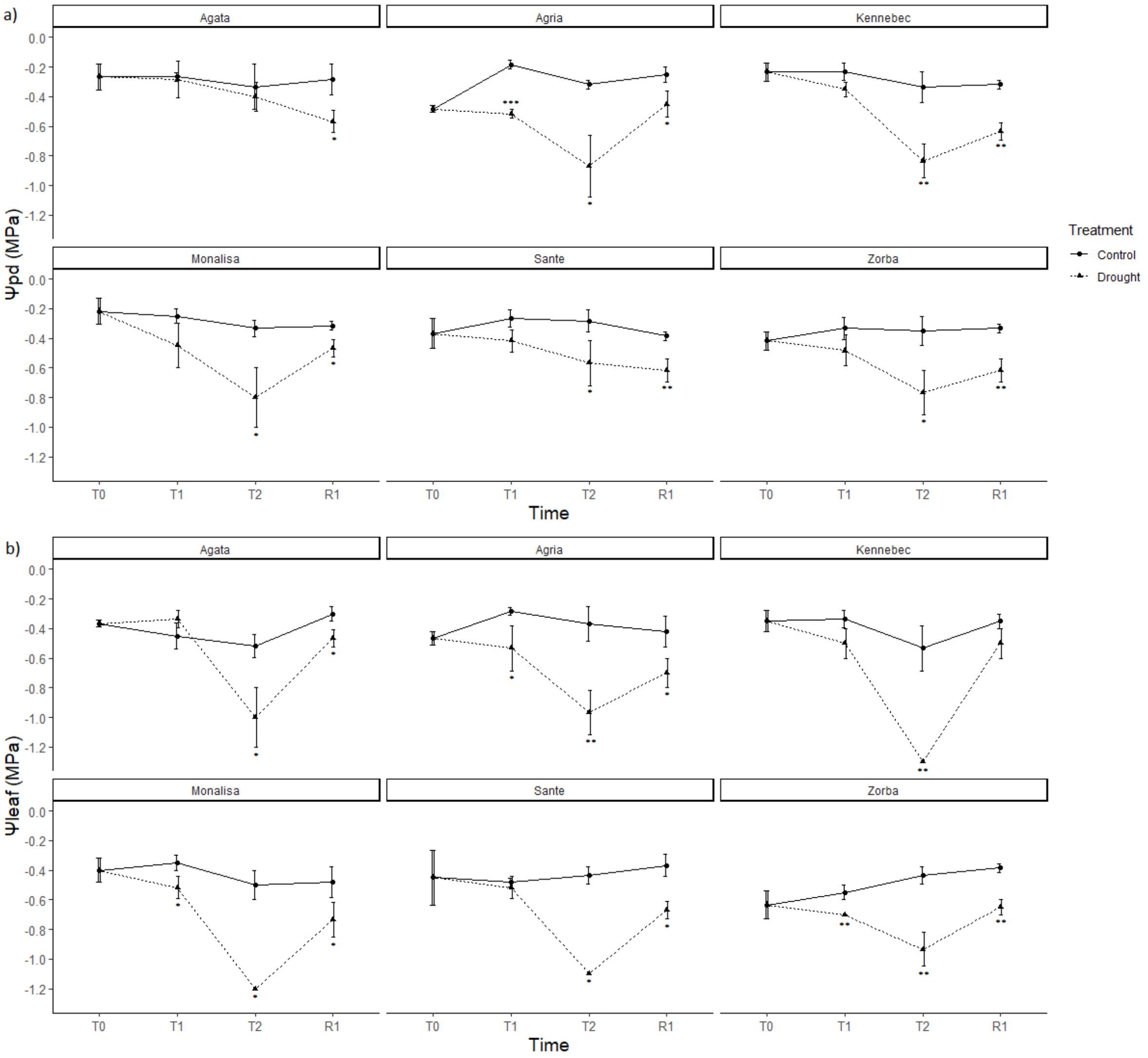


Figure 3.5. a) Predawn water potential [Ψpd (MPa)] and b) midday water potential [Ψleaf (MPa)] measured in three replicates of six potato varieties at four different times in control and water stressed plants. Significant differences with respect to each control are represented by asterisks according to Tukey's HSD test after ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001.

Although the similar response in Ψpd in most varieties, water potential at midday (Ψleaf), showed different response among them, showing a significant and fast response at T1 in Agria and Monalisa, although the lowest values were observed at T2 in Monalisa and Kennebec, reaching values near -1,2 MPa. Only Kennebec recovered the control values at R1, suggesting a better response to drought stress in this parameter although was not enough to avoid yield reduction (Figure 3.6).

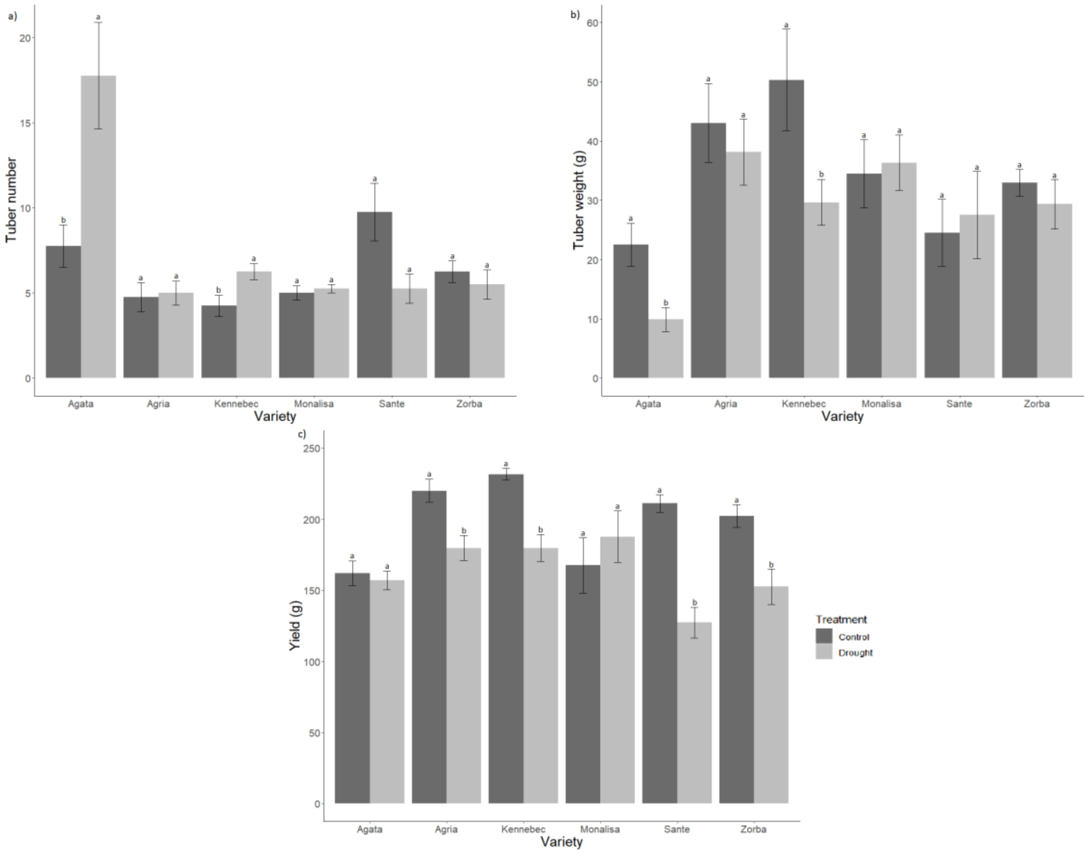


Figure 3.6. a) Tuber weight, b) tuber number and c) yield measured in four replicates in control and drought stressed plants. Different letters indicate significant differences between the two treatments according to Tukey HSD test ($p<0.05$).

With respect to biomass production evaluated at the end of the drought period, Kennebec and Zorba varieties produced the highest fresh biomass. However, significant difference between fresh biomass in control and stressed plants was only observed for Zorba and Agria. Monalisa was the variety with the smallest biomass production. No variety showed a significant difference between dry biomass in control and stressed plants (Supplementary Figure 3.2).

Production parameters were measured at the end of the vegetative cycle. For Agata no significant differences were found in yield, the tuber number was higher in drought stressed plants and average tuber weight was higher in control plants, leading to more but smaller tubers under drought conditions. Agria, Kennebec, Sante and Zorba varieties showed significant higher yield in control plants. In the case of Kennebec drought stressed plants produced a significantly higher number of tubers, but of significant lower tuber weight. Monalisa variety didn't show significant differences in tuber number, weight and yield and could be considered as tolerant to drought stress (Figure 3.6).

With respect to tuber dry matter and starch yield, Agata, Monalisa, Sante and Zorba did not show significant differences between stressed plants and control in either parameter, although a slight decrease could be observed in Agata, Agria and Monalisa. In contrary, Kennebec showed a significantly decrease in dry matter and starch yield in stressed plants and Agria revealed lower tuber dry matter in water stressed plants (Supplementary Figure 3.3).

Pearson's correlations were performed to evaluate the relationship between the different parameters during drought stress (T2) in watered and stressed plants (Table 3.3). In control plants it is important to note significant correlations between stomatal conductance and leaf water potential and between yield and Fv'/Fm' and EL%. In stressed plants more correlations between parameters can be detected (Table 3.3). Stomatal conductance was positively correlated with Fv'/Fm', EL% and yield, and negatively with dry biomass. Yield also correlated negatively with water potential.

Pearson's correlation analyses were also performed on data obtained after re-watering plants (R1). Table 3.4 shows that water potential was the parameter most affected by other parameters in the drought period. This parameter was highly correlated with stomatal conductance, RWC and tuber yield and negatively correlated with Fv/Fm and electrolytic leakage.

5. Discussion

Water deficit is one of the principal causes affecting potato production. The effect of drought on plants is different depending on the variety; with some being more sensitive than others. The duration and intensity of drought are also factors affecting plant productivity (Sprenger et al., 2015). In this work, six commercial local varieties were chosen to assess the impact of water stress. This water stress treatment was gradually imposed to favor or facilitate the development of the drought resistance mechanisms by plants (Blum, 1996) and the response of physiological and yield responses, as well as for the ability to recover after re-watering of the six varieties were studied.

The predawn water potential (Ψpd) is a good indicator of the water status of the soil, while the leaf water potential indicates the water status of the plant (Bartlett et al., 2016; Esperón-Rodríguez et al., 2018). The predawn water potential has also a high influence on several metabolic processes (Martínez-Vilalta & Garcia-Forner, 2017) and Table 3.3 revealed that water potential correlated negatively with yield under drought conditions and positively during recovery (Table 3.4), indicating that plants were able to recover after re-watering.

One of the first mechanisms used by plants when exposed to water deficit is the stomata closure, preventing water loss through transpiration but also reducing the entry of CO_2 for photosynthesis into the leaves (J. Li et al., 2017; F. Liu et al., 2005). In our study, all varieties close significantly stomata under stress at T2, although some closed stomata more drastically than others being Agria and Kennebec the ones that closed stomata early at T1 of the drought period (Figure 3.3). At T2 all varieties showed a strong stomatal closure with Monalisa showing the lowest closure (Figure 3.3). In a study carried out by Demirel et al., (2020) they also analyzed the drought response of Agria variety and found also a significant decrease in stomatal conductance. This general response of the varieties concerning to stomatal closure would suggest that potato could be included in the group of isohydric plants (isohydric plant model or water savers), that avoid the water losses through a tight or better stomatal control of transpiration under water stress, acquiring more carbon (biomass) in relation to water transpired that is, an increased WUE. This is considered as an important component of drought resistance in different crops (Blum, 2009; Bota et al., 2016; Polania et al., 2016).

According to this, during drought plants showed a negative correlation between stomatal conductance and aerial dry biomass (Table 3.3), suggesting that even closing stomata, plants have been able to fix CO_2 into biomass that was not affected by drought in any of the varieties analyzed. It is important to mention, that Agata variety had a low stomatal conductance before applying the stress, and may be for this reason was able to tolerate better the drought stress without significant differences in tuber production. However, if the stress is too intense, stomata closure may not be sufficient, and the water status of the plant may still be affected (Drapal et al., 2017) as can be observed in Monalisa at T1, when the stomatal closure is not enough to avoid the reduction of RWC, although the further closure produced at T2, allowed to maintain RWC at control levels (Figure 3.2), and the recovery after stress.

A key factor of potato yield under water stress is the ability to maintain photosynthetic activity. The imbalance between CO_2 assimilation and light reactions can produce damages of PSII, the photosynthetic organ of the plants. The parameter Fv/Fm measured in dark adapted leaves is an estimator of the maximum quantum yield of PSII and its decrease indicates damage and inhibition of photosynthesis (Krause & Weis, 1991). Moreover, and the photochemical efficiency of PSII in light adapted leaves (Fv'/Fm') indicates the proportion of light absorbed by the antenna that reaches the reaction center and will be used to create reducing power for CO_2 assimilation (Miranda-Apodaca et al., 2015). No significant differences were observed in the photosynthetic activity of plants exposed to water stress (Figure 3.1), suggesting that the photosynthetic organ has not been damaged, as also seen in previous studies (Rodríguez-Pérez et al., 2017; Tourneux et al., 2003a; Aliche et al., 2020). Jefferies, (1994) demonstrated that excess light energy was dissipated by photorespiration and had no significant effect on the photosynthetic function. This could be due to the quick response of plants to water stress, closing their stomata to avoid water loss, adjusting the photochemical apparatus and preventing damage of the photosynthetic apparatus (Ahmadi et al., 2010; Rodríguez-Pérez et al., 2017), as can be seen in the positive correlation between both parameters during the stress period (Table 3.3). There was no correlation between Fv'/Fm' and yield but it was negatively correlated with biomass during drought in both, irrigated and stressed plants (Table 3.3), suggesting that biomass production was more affected by a reduction of CO_2 assimilations due to the stomatal closure than to a limitation of photochemical reactions. After re-watering, a negative correlation was observed between the two parameters related to damage of the photosynthetic organ and the total tuber production in control plants (Mane et al., 2008).

Chlorophyll content is also an important factor related to the photosynthetic rate. However, it is a controversial trait, since some authors mention that it tends to be reduced under water restriction, while others affirm that chlorophyll synthesis under stress conditions is a tolerance mechanism in plants (Farhad et al., 2011; Rolando et al., 2015; Rudack et al., 2017). In our study we didn't find significant differences between control and stressed plants as in the study of Demirel et al. (2020), but they also reported no significant differences of this parameter for Agria. This could be because plants were not sufficiently affected by the applied drought stress. The chlorophyll content correlated negatively with water potential (Table 3.3) during the drought period, since plants tend to reduce their chlorophyll content when leaves lose turgor as reported by Rolando et al. (2015).

Compared to other crops, potato leaves have a high relative water content (RWC) which makes this parameter one of the main indicators for defining the water status of the plant and RWC decreases in response to water stress. Anithakumari et al., (2012) reported that the relative water content could be used in large populations as an easy method which reflects the balance between the water supply and the transpiration rate of the plant. However, in our study, only Monalisa decreased its RWC significantly under drought conditions (Figure 3.2), probably because maintained their stomata more open during stress that the other varieties whereas the other varieties by closing stomata are able to maintain their RWC similar to control plants.

Membrane permeability is another parameter negatively affected by water stress, increasing permeability under drought conditions (Rodríguez-Pérez et al., 2017). Membrane permeability was measured as the percentage of electrolyte leakage of leaves (Figure 3.4). An increase in electrolyte leakage could reflect a loss of ability of biological membranes to regulate the transport of ions under drought stress. Although strong membrane damage could cause photosynthetic damage no effect on Fv/Fm nor on Fv´/Fm` was observed in this study (Figure 1.1). Table 3.3 shows that EL% correlates positively with Fv'/Fm' and stomata conductance in stressed plants, confirming than the under drought, the plants that keep their stomata more open and can be more susceptible to this stress.

Drought usually leads to a reduction in tuber weight and tuber number. These reductions parameters may vary depending on genotypes and the severity of the applied stress (Aliche et al., 2018). In addition, the stability in tuber production under drought stress is important, but also the overall tuber production should be relatively high (Romero et al., 2017). For example, Agata did not show significant differences in production under drought conditions, but had in general a low production. Agria, Kennebec, Sante and Zorba showed a decrease in tuber yield under drought conditions. In contrary, Monalisa did not showed differences in yield under drought conditions, but its production was high and similar to that of other varieties affected by drought (Figure 3.6).

In addition to tuber production another important aspect to consider is the quality of the tubers, such as dry matter content and starch yield (Drapal et al., 2017). In our study we observed that only the varieties Agria and Kennebec decreased significantly their tuber dry matter content, like in a study performed by Tourneux et al., (2003b). However, in another study presented by Lahlou et al., (2003) the tuber dry matter increased under drought stress. This may occur if the dry matter content is influenced by other factors such as radiation, temperature and cultivation techniques as mentioned by Tourneux et al. (2003b).

The present study revealed wide physiological and agronomic differences between the six studied potato genotypes. Agata suffered a delay in the stress as indicated by no changes in Ψpd until R1, but showed membrane alterations that were not recovered after re-watering as occurred to stomatal conductance and, in general had low production. Monalisa was the only variety that did not show significant differences in any of the production parameters, which indicates its good response to water stress. It was sensitive to the reduction of Ψpd, showed a rapid response and showed a rapid response decreasing Ψleaf and stomatal conductance and RWC at T1, but showed a rapid recovery at R1 getting values equal or near to control in stomatal conductance, Ψpd and Ψleaf, and RWC and electrolyte leakage.

As shown in the results, stomatal conductance and water potential, mainly Ψpd represent physiological parameters that are easy to measure and give a good and quick response to water stress, so that they could be used for the quick identification of stress and selection of tolerant varieties. Therefore, indirect selection based on parameters associated with water stress can be a useful tool in potato breeding programs for the identification of more water stress tolerant varieties with stable yields, at least under greenhouse water stress conditions. We have evaluated in our study only a limited number of potato varieties in detail. It will be necessary to extend these analyses to other varieties with respect to the most promising indirect parameters. Also a validation of yield and quality parameters in field trials will be necessary.

Chapter 4

Comparative RNAseq of two potato varieties for the identification of differential expression genes under water-stress.

The content of this chapter corresponds to the published article: Alvarez-Morezuelas A., Barandalla L., Ritter E., Ruiz de Galarreta J.I. 2022. Transcriptome analysis of two tetraploid potato varieties under water stress conditions. International Journal of Molecular Science 23, 19905. https://doi.org/10.3390/ijms232213905

Chapter 4: Comparative RNAseq of two potato varieties for the identification of differential expression genes under water-stress.

1. Abstract

Potato (*Solanum tuberosum* L.) is one of the most important crops worldwide, but due to its sensitivity to drought, its production can be affected by water availability. In this study, the va-rieties Agria and Zorba were used to determine the expression differences between control and water-stressed plants. For this purpose, they were sequenced by RNAseq, obtaining around 50 million transcripts for each variety and treatment. When comparing the significant transcripts obtained from control and drought-stressed plants of the Agria variety, we detected 931 genes that were upregulated and 2077 genes that were downregulated under stress conditions. When both treatments were compared in Zorba plants, 735 genes were found to be upregulated and 923 genes were found to be downregulated. Significantly more DEGs were found in the Agria variety, indicating a good stress response of this variety. "Abscisic acid and environmental stress- inducible protein TAS14- like" was the most overexpressed gene under drought conditions in both varieties, but expression differences were also found in numerous transcription factors and heat shock pro-teins. The principal GO term found was "cellular components", more specifically related to the cell membrane and the cell wall, but other metabolic pathways such as carbohydrate metabolism and osmotic adjustment were also identified. These results provide valuable information related to the molecular mechanisms of tolerance to water stress in order to establish the basis for breeding new, more tolerant varieties.

2. Introduction

Over the last few decades, there has been a global increase in temperatures, resulting in an increase in the frequency and severity of drought conditions (Aksoy et al., 2015). This situation is expected to worsen in the coming decades and will even require irrigation in traditionally rain-fed areas (Hill et al., 2021). Drought leads to important reductions in crop yields, particularly in potato, where water stress is the second cause of yield loss after pathogens (Dahal et al., 2019).

Potato crops have relatively high water efficiency, but at the same time have a high water requirement, which makes them very susceptible to water stress (Hill et al., 2021). This susceptibility is due to the fact that the crop has a shallow and sparse root system, only about 30–100 cm deep, leading to decreased yield under drought conditions. Breeding for drought tolerance is a critical issue to avoid yield losses (Boguszewska-Mańkowska et al., 2018). Lack of water affects the crop at all stages of development, from emergence to tuber filling, and has negative effects on parameters such as biomass, yield, tuber number and quality (Barra et al., 2019; S. Sharma et al., 2017).

Plants use different strategies to mitigate the effects of water stress by reducing water loss through stomata closure, increasing water absorption from the soil by developing their root system or accelerating their growing cycle. These mechanisms require molecular signaling processes which include transcription factors, protein kinases and stress-related proteins (Hu & Xiong, 2014).

To mitigate the impact of abiotic stresses on crops, both agronomic and genotypic solutions are needed. Water, soil and plant management have an important influence on the effects of water stress, but in many cases, these methods are not sufficient. It is necessary to combine these techniques with the breeding of varieties adapted to climatic conditions (George et al., 2017). Potato breeding is a complex and lengthy process, since the crop is autotetraploid, highly heterozygous and inbreeding-depressed (Patil et al., 2017). In recent years, new technologies have been developed for studying the genome which can accelerate this process. However, drought tolerance is a complex polygenic trait, which hampers the detection of differentially expressed genes (Saidi & Hajibarat, 2020). Transcriptome analysis has been performed in different crops such as wheat (Iquebal et al., 2019), grapevine (Haider et al., 2017), maize (Zenda et al., 2019) or rice (Chung et al., 2016) for exploring the molecular regulatory mechanisms of plants in response to water stress.

Classical plant breeding methods are based on a phenotypic approach, which is slow, labor-intensive and expensive. Thanks to the increased knowledge of the molecular mechanisms in plants, the transcriptome and the expression patterns of studied genes, plant breeding has been improving in recent years (Varshney et al., 2018). With the development of high-throughput sequencing technologies, a significant coverage of cDNA sequences from RNA samples can be obtained. By applying these sequencing technologies to different cDNA libraries from samples of interest, transcriptome overviews for profiling can be obtained (Lavin Trueba & Aransay, 2016).

The application of next-generation sequencing techniques such as RNAseq allows the generation of gene expression profiles to characterize stress response and provides information for the discovery of new genes and the analysis of metabolic pathways associated with the response to environmental stresses (Bykova et al., 2017; Patil et al., 2017). Absolute measurement of gene expression using RNAseq provides quantitative and qualitative information which is more accurate than other previously used techniques such as microarrays (Jain, 2012).

RNAseq assays in potato, targeting diseases such as *Phytophthora infestans* (Duan et al., 2020) or nematodes (Chandrasekar et al., 2022), salt stress (Li et al., 2020), anthocyanin accumulation in tubers (Ahn et al., 2022) and nitrogen response (Gálvez et al., 2016), were performed.

In this study, we used this new sequencing technique for analyzing in two varieties the genes that are overexpressed or repressed in order to understand the mechanisms of drought-stress response for accelerating the selection processes in potato breeding programs.

3. Materials and Methods

3.1. Plant Material and Growth Conditions

The potato varieties Agria and Zorba were used in this study. The trial was conducted in a greenhouse under optimal conditions for potato growing (18–22 °C, around 80% humidity) and a 16:8 h day–night light cycle. Eight tubers per variety and treatment were planted in 5 L pots with peat. Each pot was watered weekly with 1 L of water up to water holding capacity. The drought stress was applied 36 days after planting (DAP) and was maintained for 25 days without any water supply. Control plants were watered as normal [36]. After the drought period (61 DAP), samples for RNA extraction were collected from five plants of each variety and treatment. All leaf samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

3.2. RNA Extraction and cDNA Library Preparation for Sequencing

Total RNA of leaf tissues from five biological replicates from each cultivar and treatment (20 samples in total) were isolated using the innuPREP Plant RNA kit (Analytik Jena GmbH, Jena, Germany) according to the manufacturer's protocol. RNA quantification and quality of total RNA was measured using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). The construction of the cDNA libraries using TruSeq stranded mRNA (Illumina, San Diego, CA, United States) and sequencing on a Novaseq 6000 150PE platform were performed by Macrogen Inc. (Seoul, Korea). These RNAseq data can be accessed at NCBI through SRA with the accession number PRJNA897005 (https://www.ncbi.nlm.nih.gov/sra/PRJNA897005, accessed on 3 November 2022).

3.3. Transcriptome Analysis

Quality control of the raw reads in each of the 20 libraries was performed by calculating the overall reads' quality, total bases, total reads, GC (%) and basic statistics using fastqc. The trimming tool Trimmomatic (Bolger et al., 2019) was used to remove adapter sequences and bases with base quality lower than three from the ends. Using the sliding window method, bases of reads that do not qualify for window size 4, and mean quality 15, were also removed. Afterwards, reads with length shorter than 36bp were dropped.

In order to map cDNA fragments obtained from RNA sequencing, trimmed reads were mapped to the reference genome GCF_000226072 using HISAT2 (Kim et al., 2015). Known genes and transcripts were assembled with StringTie (Pertea et al., 2015) based on the reference genome model. After assembly, the abundances of gene/transcripts were calculated as read counts, and FPKM (fragment per kilobase of transcript per million mapped reads) values or RPKM (reads per kilobase of transcript per million mapped reads) were used as normalization values. For the differential expression analysis between control and drought-stressed plants for both varieties, DESeq2 software (Love et al., 2014) was applied, with q-value < 0.05 and fold change |log2| ≥ 2 as screening cutoffs.

3.4. GO Enrichment Analysis

GO (gene ontology) enrichment analysis of the DEGs was performed using the g:Profiler (Raudvere et al., 2019) tool. This tool performs statistical enrichment analysis to find over-representation of information from gene ontology terms, biological pathways, regulatory DNA elements, human disease gene annotations, and protein–protein interaction networks. The gene or gene product molecule associated with GO ID was summarized by parsing the ontology file and the annotation file for the GO graph structure.

3.5. Validation of Differentially Expressed Genes

In order to validate the RNAseq results, RT-qPCR was performed for twelve randomly selected DEGs; eight of these genes were common to both studied varieties and two others were variety-specific in each case. Specific primer pairs for the selected genes were designed with Primer3 software (Untergasser et al., 2012). Their sequences are available in supplementary Table 4.1. The β-tubulin gene was used as reference gene (Nicot et al., 2005). The RT-qPCR experiments were performed using a Roche LC480 II System (Roche Diagnostics Nederland BV, Almere, the Netherlands), with three technical and five biological replicates of the same samples used for RNAseq. Each PCR reaction contained 50 ng of cDNA, 100 nM of each primer and 1X PyroTaq EvaGreen qPCR Mix (CMB Cultek Molecular Bioline) and was adjusted with RNAsefree water to a final volume of 20 μL. The reactions were performed under the following conditions: 95 °C for 15 min, followed by 50 cycles at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. For calculating and calibrating the expression levels of target genes in different varieties, the $2^{-\Delta\Delta Ct}$ method was applied (Pfaffl, 2007).

4. Results

RNA quality control revealed that all the samples had 28S:18S ratios in a range of 1.8–2.0 and a mean RNA integrity number (RIN) > 7.0, which met the requirements for library construction and sequencing. To obtain a global view of the transcriptome of the potato drought tolerance, four libraries were generated and indexed using mRNA extracted from drought treatment and unstressed control leaves of two varieties. High-throughput RNA sequencing using Illumina technology was performed.

After sequencing and removing low-quality reads, a total of 54,042,913 and 56,473,479 reads were acquired from the drought treatment and control leaves for the Agria variety, and 53,513,496 in the drought treatment and 55,798,228 in control leaves for Zorba variety (Table 4.11). The GC content was around 43% in all cases, and the majority of reads (around 90%) could be mapped to the reference genome sequence (Table 4.11).

Table 4.1. Summary of sequencing data.

		Control	**Drought**
Agria	Raw reads	57,004,343	54,509,300
	Clean reads	56,473,479	54,042,913
	Total mapped	50,569,589 (89.54%)	48,485,736 (89.71%)
	GC content (%)	43.63	43.38
	Q30 (%)	96.08	96.18
Zorba	Raw reads	56,278,132	53,964,119
	Clean reads	55,798,228	53,513,496
	Total mapped	50,271,909 (90.09%)	48,158,218 (89.99%)
	GC content (%)	43.48	43.41
	Q30 (%)	96.12	96.14

When control plants were compared with drought ones in the Agria variety, a total of 3008 DEGs were identified, of which 931 were upregulated and 2077 were downregulated. When comparing both treatments in Zorba plants, 735 genes were found to be upregulated and 923 genes were downregulated. The number of up- and downregulated genes was based on ǀlog2 fold changeǀ ≥ 2 and adjusted *p*-value < 0.05 (Figure 4.1). In both varieties, most of the DEGs were downregulated, indicating that the transcriptomic response to drought was primarily downregulation in gene expression.

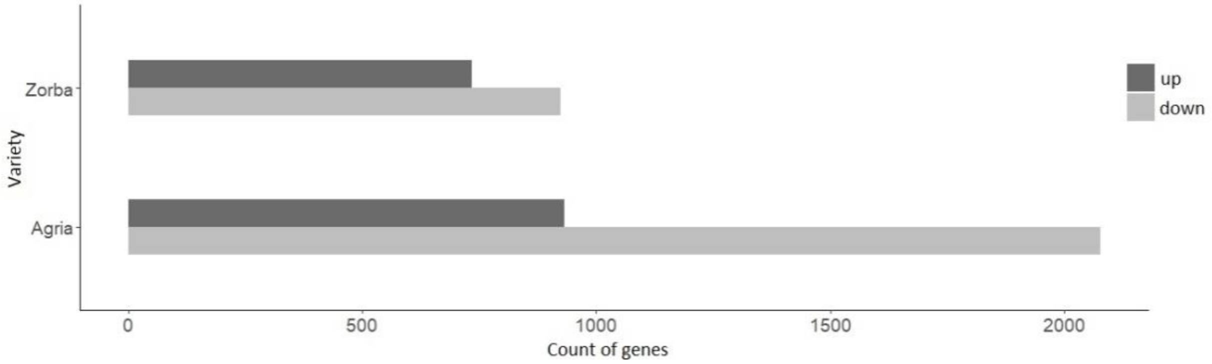


Figure 4.1. Count of genes up- and downregulated under drought stress in Agria and Zorba varieties.

When comparing the total number of DEGs, 1277 common DEGs were identified: 510 of these DEGs were upregulated, 755 were downregulated and 12 were oppositely regulated (Figure 4.2).

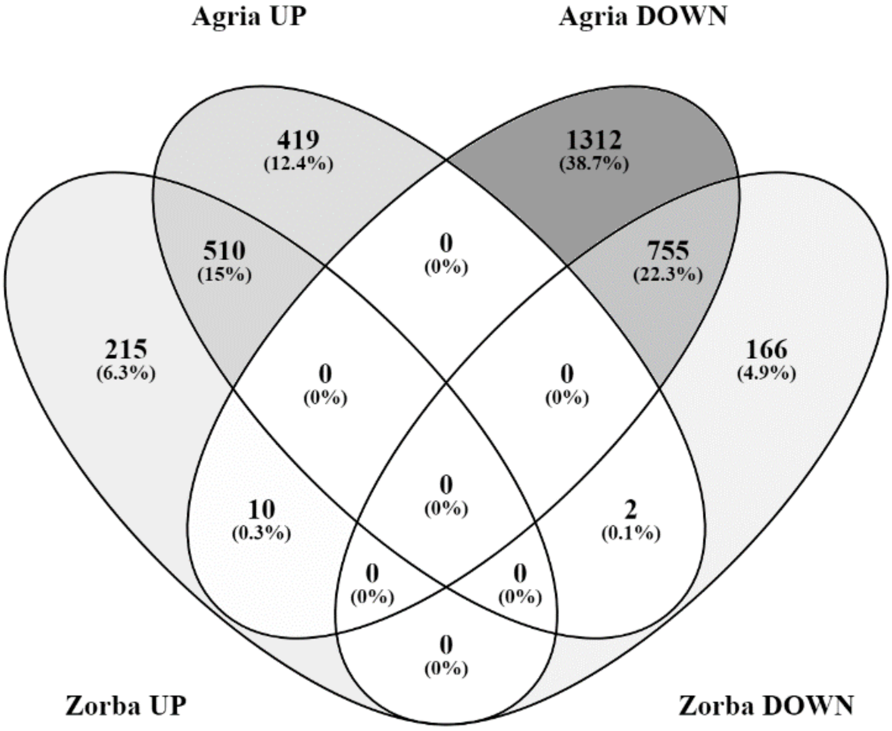


Figure 4.2. Venn diagram summarizing DEGs in the Agria and Zorba varieties in response to control and drought stress.

The top 10 upregulated genes and downregulated genes are detailed in Table 2 for the Agria variety. A calcium-binding protein, hyoscyamine 6-dioxygenase, L-ascorbate oxidase, GDSL esterase/lipase, abscisic stress-ripening protein and bZIP transcription factor, among others, differed significantly between control and stressed plants. When comparing the differentially expressed genes of the Zorba variety, we detected beta-hexosaminidase, glucan endo-1,3-beta-glucosidase, tropinone reductase, peroxidase 51, O-acyltransferase WSD1 and nonspecific lipid transfer protein differed significantly (Table 3). The most overexpressed gene in both varieties was "abscisic acid and environmental stress-inducible protein TAS14", with a log2 fold change of 3449.52 in the Agria variety and 692.09 in the Zorba variety. One of the genes that most decreased its expression under water-stress conditions was "36.4 kDa proline-rich protein", with a log2 fold change of −39.22 and −15.42, respectively.

Table 4.2. List of genes showing the highest significantly different expression in the comparison of Agria control and drought-stressed plants.

Gene Name	**Description**	**log2$^{\text{fold change}}$**	***p*-Value**
Downregulated genes			
LOC102603621	-	−126.51	4.56×10^{-7}
LOC102594756	36.4 kDa proline-rich protein-like	−39.22	5.85×10^{-6}
LOC102596805	fasciclin-like arabinogalactan protein 2	−37.45	3.65×10^{-8}
LOC102604005	putative calcium-binding protein CML19	−32.55	1.62×10^{-4}
LOC102602308	hyoscyamine 6-dioxygenase	−31.44	3.14×10^{-4}
LOC102582168	L-ascorbate oxidase-like	−30.82	5.63×10^{-7}
LOC102603929	probable xyloglucan endotransglucosylase/hydrolase 1	−30.46	1.54×10^{-4}
LOC102586959	probable WRKY transcription factor 53	−30.40	5.99×10^{-7}
LOC102583042	1-aminocyclopropane-1-carboxylate synthase-like	−28.26	1.62×10^{-7}
LOC102606325	GDSL esterase/lipase At5g33370-like	−27.13	3.40×10^{-8}
Upregulated genes			
LOC107057685	abscisic acid and environmental stress-inducible protein TAS14-like	3449.52	1.28×10^{-19}
LOC102583792	abscisic stress-ripening protein 2	149.50	2.89×10^{-13}
LOC102606049	fidgetin-like protein 1	140.15	6.90×10^{-10}
LOC102598218	translocator protein homolog	109.83	6.19×10^{-18}
LOC102590433	-	73.36	5.58×10^{-9}
LOC102606174	bZIP transcription factor 53-like	50.69	2.22×10^{-15}
LOC102598306	SNF1-related protein kinase regulatory subunit gamma-like PV42a	48.76	3.16×10^{-14}
LOC102592988	-	48.09	2.74×10^{-16}
LOC102584616	-	42.46	2.71×10^{-13}
LOC102591763	branched-chain-amino-acid aminotransferase 2, chloroplastic-like	39.31	1.50×10^{-8}

Table 4.3. List of genes showing highest significantly different expression in the comparison of Zorba control and drought-stressed plants.

Gene Name	Description	$\log2^{\text{fold change}}$	*p*-Value
Downregulated genes			
LOC102598924	protein PMR5	−19.17	8.26×10^{-18}
LOC102592481	-	−16.05	7.36×10^{-13}
LOC102594756	36.4 kDa proline-rich protein-like	−15.42	1.85×10^{-3}
LOC102599076	beta-hexosaminidase 3	−11.63	9.22×10^{-10}
LOC102587252	protein STRICTOSIDINE SYNTHASE-LIKE 11-like	−11.33	5.95×10^{-9}
LOC102605226	glucan endo-1,3-beta-glucosidase, basic isoform 1-like	−11.33	5.66×10^{-8}
LOC102605560	glucan endo-1,3-beta-glucosidase, basic isoform 1	−10.91	5.85×10^{-7}
LOC102590679	tropinone reductase homolog	−10.66	7.47×10^{-14}
LOC102594482	delta(7)-sterol-C5(6)-desaturase-like	−10.37	2.30×10^{-6}
LOC102592844	peroxidase 51	−10.31	1.63×10^{-15}
Upregulated genes			
LOC107057685	abscisic acid and environmental stress-inducible protein TAS14-like	692.09	1.47×10^{-12}
LOC102606049	fidgetin-like protein 1	177.36	2.71×10^{-10}
LOC102590433	-	140.98	8.29×10^{-11}
LOC102580665	O-acyltransferase WSD1-like	69.42	4.71×10^{-22}
LOC102577501	nonspecific lipid transfer protein a7	53.62	2.18×10^{-9}
LOC102596984	nonspecific lipid-transfer protein 2-like	50.35	1.77×10^{-8}
LOC102597309	nonspecific lipid-transfer protein 2-like	48.98	5.38×10^{-11}
LOC102598306	SNF1-related protein kinase regulatory subunit gamma-like PV42a	48.30	2.75×10^{-13}
LOC102582408	probable protein phosphatase 2C 51	37.80	5.86×10^{-10}
LOC102587411	MLO-like protein 6	36.71	7.14×10^{-6}

For obtaining an overview of the putative functions of genes that participate in drought-stress response, GO enrichment analysis was used to identify DEGs between irrigated and nonirrigated plants. The DEGs were classified into three categories: biological processes (BP), cellular components (CC) and molecular function (MF). For the biological process category, “DNA replication initiation” was the only GO term in both varieties (Figures 4.3 and 4.4). Eleven GO terms were found for “cellular components” in both varieties, and nine of them were identical: MCM complex, plant-type cell wall, cell wall, external encapsulating structure, plasma membrane, cell periphery, integral component of membrane, intrinsic component of membrane and membrane. Photosystem and apoplast-related DEGs were found in Agria (Figure 3), whereas THO complex and extracellular region-related DEGs were found in Zorba (Figure 4.4). Eleven GO terms were involved in “molecular function” in Agria and six GO terms in Zorba. Four of them were identical: hydrolase activity hydrolyzing O-glycosyl compounds, hydrolase activity acting on glycosyl bonds, oxidoreductase activity and catalytic activity. In both cases, genes related to catalytic activity were the most abundant, with a total of 703 unique DEGs in Agria and 413 unique DEGs in Zorba. Membrane-related genes also have a very important influence and were the most abundant in the cellular component category. In this case, 540 unique DEGs were found in Agria and 294 unique DEGs in Zorba.

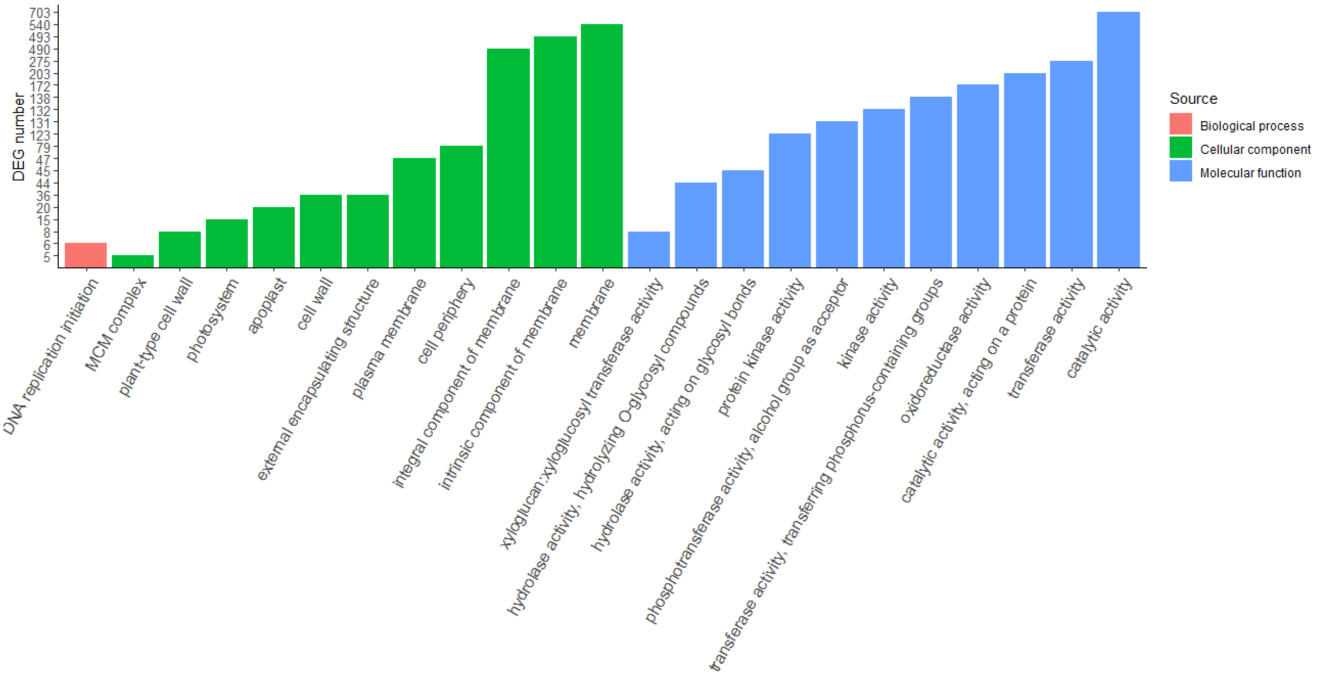


Figure 4.3. Gene ontology classification analysis of DEGs between control and drought-stressed potato leaves in Agria variety.

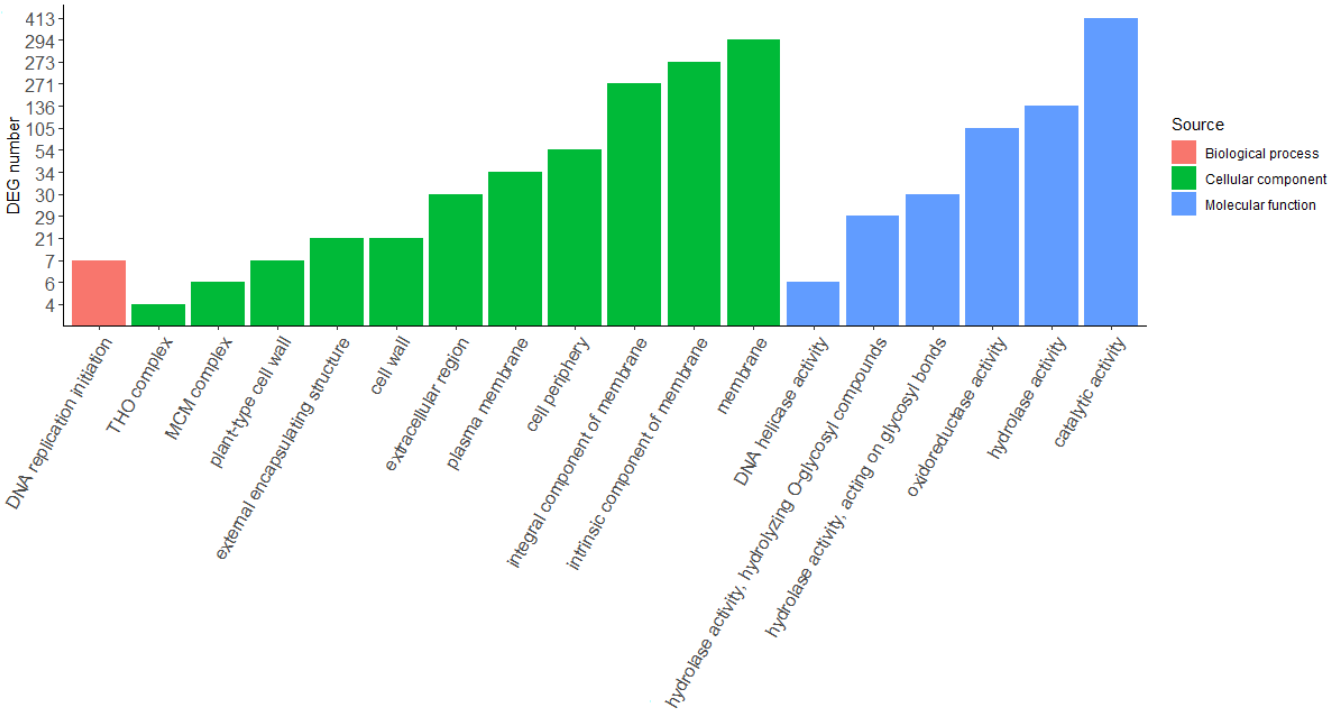


Figure 4.4. Gene ontology classification analysis of DEGs between control and drought-stressed potato leaves in Zorba variety.

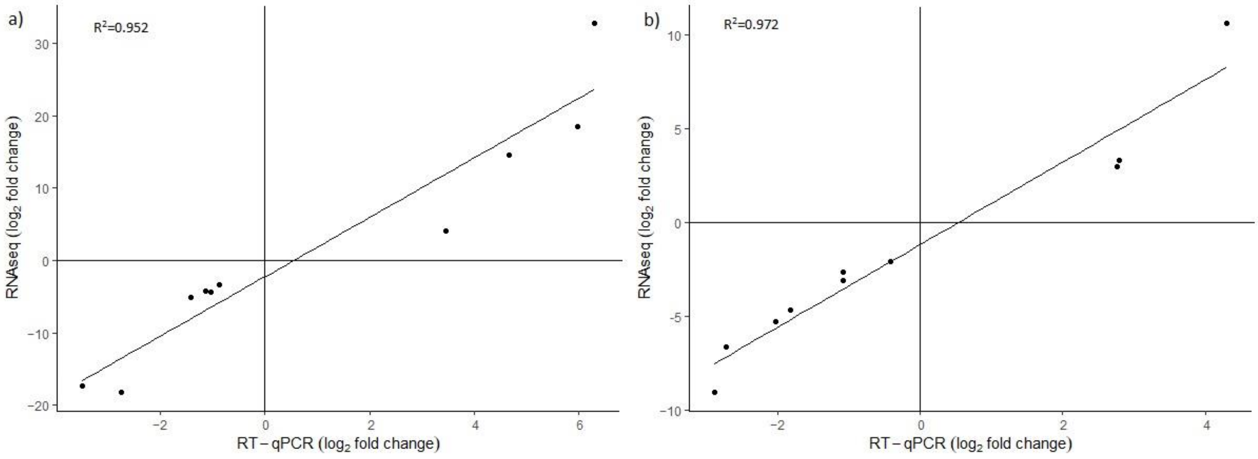


Figure 4.5. Validation of RNAseq results with RT-qPCR in (**a**) Agria variety and (**b**) Zorba variety.

Ten DEGs were randomly selected for RT-qPCR analysis to validate the results of RNA sequencing. The expression levels determined by RT-qPCR followed the same trends as in transcriptome sequencing. In the comparison of control and stressed plants, the correlation coefficients of gene expression trends in sequencing data and

RT-qPCR results were 0.952 for Agria and 0.972 for Zorba, indicating that our transcriptome sequencing data were highly reliable (Figure 4.5).

5. Discussion

We have applied in this study mRNA sequencing to evaluate transcriptome changes in potato leaves under water-stress conditions and unstressed control conditions in two genotypes. Using Illumina sequencing technology, we generated around 55 million transcripts in each library. The numbers of differentially expressed genes indicate that there was stress in leaves of the plants under drought stress, with a total of 3008 DEGs in the Agria variety and 1658 in the Zorba variety.

Drought tolerance is a complex trait and involves multiple mechanisms that can act in combination to avoid or tolerate periods of water deficit. Gene expression experiments comparing water-stressed and nonstressed potato plants have been performed in several studies (Gong et al., 2015; Moon et al., 2018; Sprenger et al., 2018). As mentioned by (Zhang et al., 2014), plants vary in the timing and speed of response to drought conditions, depending on their genetic background and ecotype, but some drought response genes, such as those involved in osmotic adjustment or cell signaling and communication, are conserved among plant taxa.

The response to water stress is a complex character that exhibits itself in different ways, as evidenced by the large amount of GO enrichment in stressed leaves. In this study, DEG enrichment was observed in the membrane-related category. The cell membrane is one of the main components affected by water stress and is affected in its composition of both phospholipids and proteins that help to maintain membrane integrity, preserve cellular compartments and activate phospholipid signaling pathways in response to stress (Pieczynski et al., 2018). In both varieties, we have observed a significant increase in membrane-related genes, such as MCM complex, plant-type cell wall, cell wall, external encapsulating structure, plasma membrane, cell periphery, integral component of membrane, intrinsic component of membrane and membrane, suggesting that these plants have activated water stress defense mechanisms to maintain intracellular water.

In our data, ABA-related genes were significantly expressed in both varieties under drought-stress conditions. Abscisic acid plays an important role in plant adaptation to environmental stresses such as water limitation. Genes involved in ABA biosynthesis, catabolism and signaling represent interesting candidate genes for the breeding of drought-tolerant crops. When plants are under water stress, one of the first responses is the expression of ABA-responsive genes with a consequent increase in this hormone. In their article, Krannich et al., 2015 mention some possible candidate genes related to abscisic acid biosynthesis, catabolism and signaling, such as abscisic acid receptor (PYL), protein phosphatase 2C (PP2C), or serine/threonine protein kinase (SnRK2). According to Chen et al., 2020, under drought stress, ABA regulates the signal pathway by inhibiting the phosphatase activity of PP2C protein through its receptor PYL protein family, and PP2C and SnRKs were upregulated after drought stress. In our study, we found that genes related to the PYL4 receptor were inhibited by water stress, while PP2C and SnRKs were overexpressed.

Transcription factors (TFs) are proteins that bind to specific DNA sequences in order to regulate gene expression through their activation or repression (Chacón-Cerdas et al., 2020). Gonçalves, 2016 has reported numerous studies where the expression of transcription factors is closely related to the response to various abiotic factors such as drought acting on signal transduction pathways. In our study, we also found numerous transcription factors that were differentially expressed under stress conditions such as ethylene-responsive transcription factors, bZIP, WRKY and bHLH. This is in agreement with previous findings identifying several transcription factors involved in plant responses to drought by (Ambrosone et al., 2017).

The expression of several stress-responsive genes is mediated by MYB family transcription factors, which are also involved in the ABA-dependent response, leading to the accumulation of ABA in cells (Lata et al., 2011). MYB transcription factors are formed by one, two or three imperfect helix-turn-helix repeats and are grouped into three families depending on the MYB domain arrangement. The most common in plants is the R2R3 type, which has been described in potato as 123 MYB-like TFs (http://planttfdb.cbi.pku.edu.cn, accessed on 22 August 2022). WRKY-type transcription factors play an important role in plant response to abiotic factors and are activators of ABA signaling (Manna et al., 2021). When we compared water-deficient plants with normally irrigated plants, we found that most genes related to WRKY transcription factors were downregulated under stress conditions.

According to Shin et al., 2011, AtHB-7 is involved in plant stress tolerance and is dramatically upregulated after drought-stress treatment. In our case, overexpression of this gene was also found in both varieties when comparing drought-stressed plants (LOC102589092 and LOC102585726). These authors also demonstrated the function of StMYB1R-1 as a transcription factor, and its overexpression in transgenic potato enhanced the expression of drought-regulated genes such as AtHB-7, RD28, ALDH22a1 and ERD1-like, and improved plant tolerance to drought stress.

Phosphorylation and dephosphorylation of proteins regulated by kinases and phosphatases, respectively, is an effective mechanism in numerous signal transduction pathways. For example, MAPKs and CDPKs are known for their roles in water-stress signaling pathways. At the end of the phosphorylation cascade, transcription factors are either activated or suppressed by kinases or phosphatases regulating gene expression (Joshi et al., 2016). In our study, we also observed that in terms of molecular functions, genes related to hydrolase, kinase and phosphotransferase activity are significantly responsive under water restriction.

In the GO annotations of stressed and irrigated plants, DEG enrichment was observed in the photosystem-related category in the Agria variety, while the Zorba variety was not significant. The DEGs found in that GO were downregulated, as in the study presented by Moon et al., 2018, demonstrating that under water-stress conditions, there is a decrease in photosynthesis, which helps to maintain the water status of the plant by inhibiting water uptake and plant growth. In addition, inhibition of photosynthesis-related genes leads to a decrease in stomatal conductance, reducing water loss through transpiration. These results are in agreement with the results of physiological data obtained for the same plants (Alvarez-Morezuelas et al., 2022). Potato leaf development is particularly sensitive to water stress. Drought first causes stomatal closure, reducing CO_2 uptake for photosynthesis, reducing plant growth and yield.

Heat shock proteins are chaperones that contribute to protein stability under stress (Tang et al., 2020). In our study, we observed differential expression of some heat shock proteins, particularly in Agria. Almost all of them were upregulated. Our results were similar to those obtained by Barra et al., 2019 which suggest that there is a role for heat shock proteins in the maintenance of cell function under stress. Additionally, Gong et al., 2015 reported an increased expression of heat shock proteins under water-stress conditions.

In both varieties, the most overexpressed gene under drought conditions was the "Abscisic acid and environmental stress-inducible protein TAS14-like". In the study carried out by van Muijen et al., 2016, it was also found that there was a significant correlation between TAS14 expression and early response to drought for recovering after stress. Increased TAS14 expression at the beginning of stress reduces the rate of photosynthesis, allowing rapid recovery of the plant's water status. TAS14 protein is induced by abscisic acid and could act as a biomarker to evaluate the level of water stress in potato (Aliche et al., 2022; Muñoz-Mayor et al., 2012).

6. Conclusions

In this study, we used the RNAseq technique to detect expression differences for better understanding the molecular mechanisms of tolerance to water stress in potato plants. Despite the difficulty of the study due to the fact that potatoes are tetraploid and highly heterozygous, numerous DEGs were found between stressed and control plants, showing that the plants have activated stress response mechanisms. The Agria variety showed significantly more differentially expressed genes, 931 upregulated and 2077 downregulated, compared to 735 upregulated and 923 downregulated genes in the Zorba variety, suggesting that Agria has a better response to lack of water.

We have identified the main DEGs and mechanisms regulating plant tolerance to water stress, such as some ABA-responsive genes, numerous transcription factors or heat shock proteins. We have also identified metabolic pathways involved in plant protective functions such as cell wall maintenance, carbohydrate metabolism or osmotic adjustment. These results suggest that plants have responded to stress as expected by activating stress-responsive metabolic pathways. These data provide the basis for the study of gene function and the mechanisms of regulation of tolerance to water stress. Due to the complexity of this trait in which numerous genes are involved, further studies are needed to assess the degree of contribution to tolerance of these genes and the identified metabolic pathways. One of the factors affecting drought response in plants is the duration and timing of stress application, so a comparison of DEGs at different times could be made in future studies.

Chapter 5

GWAS for traits related to water stress tolerance in potato

The content of this chapter corresponds to the published article: Alvarez-Morezuelas A., Barandalla L., Ritter E., Ruiz de Galarreta J.I. 2023. Genome-Wide Association Study of Agronomic and Drought Tolerance in Potato. Plants 12, 734.

https://doi.org/10.3390/plants12040734

Chapter 5: GWAS for traits related to water stress tolerance in potato

1. Abstract

Potato (*Solanum tuberosum* L.) is often considered a water sensitive crop and its production can be threatened by drought events, making water stress tolerance a trait of increasing interest. In this study, a panel of 144 tetraploid potato genotypes was evaluated for two consecutive years (2019 and 2020) to observe the variation of several physiological traits such as chlorophyll content and fluorescence, stomatal conductance, NDVI, and leaf area and circumference. Also agronomic parameters such as yield, tuber fresh weight, tuber number, starch content, dry matter and reducing sugars were determined. GGP V3 Potato array was used to genotype the population, obtaining a total of 18259 high-quality SNP markers. Marker-trait association was performed using GWASpoly package in R software and Q + K linear mixed models were considered. This approach allowed us to identify eighteen SNP markers significantly associated with the studied traits in both treatments and years, which were related to genes with known functions. Markers related to chlorophyll content and number of tubers under control and stress conditions, and related to stomatal conductance, NDVI, yield and reducing sugar content under water stress were identified. Although these markers were distributed throughout the genome, the SNPs associated with the traits under control conditions were found mainly on chromosome 11, while under stress conditions they were detected on chromosome 4. These results contribute to the knowledge of the mechanisms of potato tolerance to water stress and are useful for future marker-assisted selection programs.

2. Introduction

Climate change is causing negative effects on crop production, both through biotic stresses and abiotic stresses such as temperature stress, drought and salinity (Pareek et al., 2020). The impact of climate change on crop yield and quality will vary depending on the area and crop system (Malhi et al., 2021). In Spain, crops are mostly grown using artificial irrigation systems, which optimize the limited water available. However, the availability of water resources has been decreasing in recent years and in the future it will be necessary to increase the amount of irrigation or even to irrigate in rainfed areas. Therefore, it will be essential to cultivate more water-efficient materials (Daccache et al., 2012).

Potato (*Solanum tuberosum* L.) is one of the most important crops in the world with an annual production of 359 million tons of tubers (FAOSTAT, 2020). It is a highly valued crop as it can grow in a wide range of environments, is very versatile in terms of uses, is a short duration crop and 85% of its biomass is edible (Lutaladio & Castaldi, 2009; Nasir & Toth, 2022). Potatoes are relatively water efficient and compared to other crops produce more calories per unit of water used (Hill et al., 2021; Sun et al., 2015). However this crop has also a high water requirement and it is considered a drought sensitive crop. The drought susceptibility of potatoes is associated with their shallow and sparse root system, but canopy development and variety also play an important role in water stress tolerance (Nasir & Toth, 2022; Zarzyńska et al., 2017). Drought is one of the main factors limiting yield, particularly in susceptible crops such as potatoes. If potato crops are not adapted to water stress, a reduction of between 18% and 32% is estimated for the year 2050, although with adaptation there would be a reduction of between 9 and 18% for the period 2040-2069 (Hijmans, 2003). It is difficult to estimate the global yield loss due to water stress alone, as other abiotic stresses such as temperature, solar radiation or salinity are closely related. However, some studies have reported a decrease between 15% and 91% in potato yield under water stress conditions (Aliche et al., 2018; Gervais et al., 2021; Obidiegwu et al., 2015).

Potato breeding activities in recent years have focused on searching for regions of the genome related to tuber quality traits (Pandey et al., 2022; Schreiber et al., 2014), agronomical important traits (Y. Li et al., 2018), floral traits (Zia et al., 2020), root and stolon traits (Yousaf et al., 2021), nitrogen use efficiency (Nieto et al., 2021) and markers have been developed for applying in marker-assisted selection for resistance to some diseases such as common scab (Koizumi et al., 2021; Yuan et al., 2020) or *Phytophthora infestans* (Mosquera et al., 2016; Wang et al., 2020).

Drought tolerance is a complex trait which depends on several factors such as the duration of the stress, the severity of the drought and the developmental stage of the plant. Stress in early growth stage is considered the most harmful (Aliche et al., 2020; Plich et al., 2020). From a physiological point of view, survival or recovery is the major objective in plant stress tolerance, but from an agricultural point of view, crop yield is the trait that determines crop drought tolerance (Krannich et al., 2015). Yield decrease is mainly associated with inhibition of photosynthesis, decrease in stomatal conductance to prevent water loss through transpiration, and reduction of leaf area (Aliche et al., 2020; Dahal et al., 2019; Gervais et al., 2021; Pinheiro & Chaves, 2011).

Breeding for drought tolerance is challenging and absolutely essential under the expected climatic changes that could lead to more frequent periods of low water supply. Genetic basis of drought tolerance is complex, but there are tools such as the DroughtDB database which collects genes of interest for drought stress in plants and helps us to understand the mechanisms of tolerance (Alter et al., 2015). Although an enormous amount of knowledge has been gained about drought tolerance in recent years, we are still far from understanding all the underlying mechanisms and signaling pathways involved (Krannich et al., 2015). Water stress tolerance traits are polygenic and affected by several minor alleles. Therefore a deeper understanding of the loci and alleles involved is needed.

Traditional potato breeding has certain difficulties due to the heterozygous nature of tetraploid potatoes and furthermore allelic combinations and genetic effects become even more complex when dealing with quantitative polygenic traits such as water stress tolerance (Naeem et al., 2021). The collection of accurate phenotypic data for the traits of interest in the study population is a major challenge, as these assays should be multi-year and multi-environment and should have a sufficient number of genotypes population under study (Byrne et al., 2020).

The potato genome is comprised of 12 chromosomes and has an average size of approximately 840Mbp. For a few years now the complete genome sequence is available and allowed the development of Single Nucleotide Polymorphisms (SNP) arrays by the potato community (Xu et al., 2011). Several generations of SNP arrays were generated, building on the original Infinium 8303 SNP array (Felcher et al., 2012). In recent years advances in sequencing have been developed, sequencing costs have decreased and the number of reads has increased (Visser et al., 2014).

Association mapping, also known as linkage disequilibrium (LD) mapping is a powerful tool for the association of a phenotype with a genotype and the identification of causal genes/loci (Baldwin et al., 2011). One of the most attractive aspects of association mapping is, that it is not necessary to establish mapping families, and instead historical recombination events can be explored at the population level (Korte & Farlow, 2013; Zhu et al., 2008). The absence of biparental crosses for identifying QTL makes association mapping easier and less expensive(Myles et al., 2009).

In this study we have performed Genome Wide Association Studies (GWAS) with the aim of identifying QTLs associated with physiological and agronomic traits of interest for potato breeding under water stress and unstressed conditions, in order to accelerate the selection processes in potato breeding programs.

3. Materials and Methods

3.1. Plant material and location

A total of 144 tetraploid potato genotypes belonging to *Solanum tuberosum ssp tuberosum* were used in this study, representing a wide range of parents used in breeding programs. The field experiments were performed in the facilities of NEIKER research center in Spain (42°51'05.7"N, 2°37'13.2"W) during the years 2019 and 2020.

3.2. Experimental design

The trials were conducted from May to September in both years and the climatic conditions at the experimental field in terms of average maximum and minimum temperature; humidity and total precipitation are shown in table 5.1. The experimental design in each year included two blocks, irrigated (control) and non-irrigated treatments. In each block the genotypes were planted in a completely randomized experimental design with two replicates of 5 plants each, at a distance of

0.30 m between plants and 0.75 m between rows. The irrigation strategy in the case of the irrigated control field was based on the weekly replenishment of the accumulated water deficit from the third week of June onwards. For the estimation of the doses of each of the irrigations, a daily soil water balance was calculated using the FAO56 dual coefficient model (Allen et al., 2005) and the meteorological data recorded by the Arkaute weather station belonging to the EUSKALMET network.

Table 5.1. Maximum and minimum temperatures, humidity and precipitation in the experimental field for years 2019 and 2020.

Year 2019					
	14- 31 May	June	July	August	1-17 September
Max. temperature (ºC)	18.1	25.4	7.1	27.5	22.2
Min. temperature (ºC)	5.7	10.0	3.1	13.0	10.0
Humidity (%)	79.9	70.3	2.3	73.5	75.1
Precipitation (l/m^2)	33.9	17	2.1	24.7	30.5
Year 2020					
	26- 31 May	June	July	August	1-28 September
Max. temperature (ºC)	26.8	22.2	6.4	27.7	24.6
Min. temperature (ºC)	9.2	0.7	2.4	13.0	11.0
Humidity (%)	68.3	7.2	3.2	72.3	71
Precipitation (l/m^2)	0	5.8	8.8	31.4	33.5

3.3. Phenotypic data collection

Four physiological traits were measured in each genotype at two different dates, 50 days after planting (DAP) and 70 DAP. The chlorophyll content (CC) was measured using a SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan) in the last fully expanded leaf in three plants of each replicate and treatment. Photochemical efficiency of PSII was measured in leaves exposed to light (Fv'/Fm') using a fluorimeter (FluorPen FP 100, Photon Systems Instruments, Drasov, Czech Republic) also in the last fully expanded leaf in three plants of each replicate and treatment. Stomatal conductance (gs, mmol H2O m-2 s-1) was measured using a porometer (Leaf Porometer, Decagon Devices, Pullman, Washington, EEUU) in the last fully expanded leaf in one plant of each replicate and treatment. Normalized difference vegetation index (NDVI) was measured in each replicate using a Rapidscan (RapidScan CS-45, Holland Scientific, Lincoln, EEUU). Plants were scanned from 0.5 m above the crop canopy in five plants of each replicate along the row direction. Three leaves per replicate and variety were collected at 70DAP from each of the treatments to estimate leaf area and leaf circumference values using imageJ software.

Plants were harvested at 127 DAP in 2019 and 126 DAP in 2020 to allow late cultivars to complete their cycle. The whole experiment was harvested at once and total yield, tuber number per plant and average tuber fresh weight was assessed in each replicate. The five plants from each replicate were harvested together and the total value was divided by five to get yield and tuber number for each plant. Tuber weight was calculated as yield/tuber number. Dry matter content was measured in two replicates of each variety and treatment. Tubers were weighed immediately after harvest (FW). After 72 hours at 80ºC, they were weighed again to obtain the dry weight (DW). The starch content was calculated with the following formula (Müller & Cervenkova, 1978):

$$\mathrm{Starch} = \left(\frac{\mathrm{DW}}{\mathrm{FW}} * 100 - 6.0313\right) * 10$$

The determination of reducing sugars content present in the samples was assessed by spectrophotometry based on the reduction of dinitrosalicylic acid (Lindsay, 1973). Two replicates per variety and treatment were analyzed. The potatoes were peeled and mashed into a homogeneous juice. A total of 0.3 gr of the mixture was weighed and 1ml of distilled water and 2ml of dinitrosalicylic acid were added. Then the samples were heated at 100°C in a water bath with stirring for 10 min. Afterwards the samples were diluted with distilled water and the absorbance was measured in the UV-VIS spectrophotometer at 546 nm. The percentage of reducing sugars was calculated as follows:

$$\%\mathrm{reducing\ sugars} = (\mathrm{absorbance} - 0{,}00385) * 1{,}07893$$

Analysis of variance (ANOVA) was performed on the data of both years for each parameter using R software (R Core Team, 2017) and the mean values of all traits were used to calculate the marker-trait associations.

3.4. DNA extraction and Genotyping

Genomic DNA was extracted from 144 fresh potato leaves using innuPREP Plant DNA Kit (Analytik Jena, Germany) following the manufacturer's instructions. DNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Inc. USA). The extracted DNA was sent to Neogen (Scotland, UK) for genotyping with the GGPv3 Potato 35K array. The software Genome Studio (Illumina, San Diego, CA) was used for genotype calling, scoring four alleles per locus. The total set of markers obtained was filtered to ensure the quality of the SNPs, removing markers with a missing value rate higher than 10% and those with a minor allele frequency below 0.05.

3.5. Population structure, Linkage Disequilibrium and GWAS study

The population structure matrix (Q-matrix) was analyzed using K-values ranging from 1 to 10 for the entire population with 18,259 SNP markers with Structure v.2.4 software (Pritchard, 2007). Three independent analyses were performed for each K-value. In this analysis, the length of the burn-in period was 100,000 with 100,000 MCMC replications after burn-in. The optimal value of K was identified using a previously developed method based on delta K (ΔK) (Evanno et al., 2005) in Structure Harvester website (Earl & vonHoldt, 2012). The relationship between genotypes and the genetic diversity in the population was calculated from the SNP marker data using TASSEL software (Bradbury et al., 2007).

Linkage Disequilibrium was estimated for high quality SNPs after filtering using TASSEL software (Bradbury et al., 2007). The pairwise squared allele-frequency correlations (r^2) between SNP markers were calculated with sliding window of 50 SNPs. These results were plotted against physical distance and an internal trend line was drawn as a non-linear logarithmic regression curve to estimate LD decay using R (Remington et al., 2001).

Association mapping analysis was performed with the phenotype and genotype data using the statistical package GwasPoly (Rosyara et al., 2016) developed for R software (R Core Team, 2017). The mixed model was used to perform association analysis with correction for kinship (K) and for sub-populations (Q). To correct for multiple testing, we used the 5% Bonferroni threshold ($-\log_{10}(P) = 5.01$).

4. Results

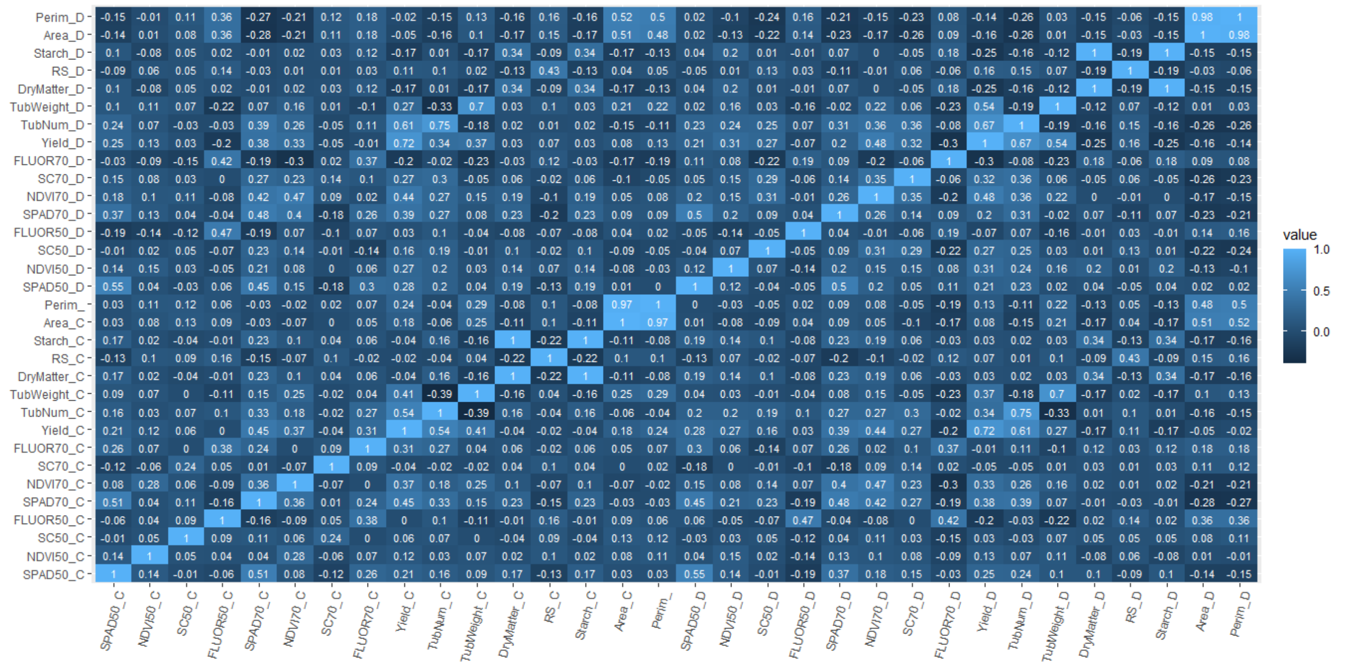
4.1. Phenotypic data analysis

Analysis of variance (ANOVA) showed highly significant differences for all traits between genotypes, between treatments and interactions between genotypes and treatments (GxT) in both years (Table 5.2). Descriptive statistics for the traits are provided in the Supplementary Table 5.1.

Table 5.2. Analysis of variance between genotypes (G) and treatments (T) in 144 tetraploid potato varieties.

Trait	F value					
	2019			2020		
	Genotype(G)	Treatment(T)	G x T	Genotype(G)	Treatment(T)	G x T
SPAD_50	4.83***	15.17***	1.39**	8.57***	5.51*	1.55***
NDVI_50	2.53***	411.95***	2.37***	2.26***	38.25***	1.52**
SC_50	3.89***	293.64***	3.95***	6.61***	275.80***	2.18***
FLUOR_50	7.72***	148.46***	2.79***	3.81***	3.81ns	3.56***
SPAD_70	6.58***	62.22***	2.20***	8.63***	7.04**	1.56***
NDVI_70	4.30***	360.85***	2.58***	2.89***	61.99***	1.75***
SC_70	4.49***	322.74***	3.76***	4.23***	140.85***	1.72***
FLUOR_70	6.21***	584.25***	3.10***	3.72***	252.39***	1.57***
Yield	19.63***	900.35***	5.20***	7.88***	627.35***	1.89***
TubNum	11.63***	352.54***	1.98***	6.25***	17.57***	1.54***
TubWeight	8.97***	859.01***	2.62***	6.24***	541.72***	1.95***
DryMatter	1453.69***	1200***	802.88***	1326.9***	1200***	530***
RS	51.10***	1200***	30.74***	32.97***	72.53***	18.85***
Starch	1497.04***	1200***	853.63***	1388.46***	1200***	558.55***
Area	5.58***	358.25***	2.56***	3.58***	178.29***	1.58***
Perim	6.24***	256.34***	2.14***	3.69***	189.32***	1.67***

*, **, *** Significant at P=0.05, P= 0.01 and P=0.001, respectively



	SPAD50_C	NDVI50_C	SC50_C	FLUOR50_C	SPAD70_C	NDVI70_C	SC70_C	FLUOR70_C	Yield_C	TubNum_C	TubWeight_C	DryMatter_C	RS_C	Starch_C	Area_C	Perim_	SPAD50_D	NDVI50_D	SC50_D	FLUOR50_D	SPAD70_D	NDVI70_D	SC70_D	FLUOR70_D	Yield_D	TubNum_D	TubWeight_D	DryMatter_D	RS_D	Starch_D	Area_D	Perim_D
Perim_D	-0.15	-0.01	0.11	0.36	-0.27	-0.21	0.12	0.18	-0.02	-0.15	0.13	-0.16	0.16	-0.16	0.52	0.5	0.02	-0.1	-0.24	0.16	-0.21	-0.15	-0.23	0.08	-0.14	-0.26	0.03	-0.15	-0.06	-0.15	0.98	1
Area_D	-0.14	0.01	0.08	0.36	-0.28	-0.21	0.11	0.18	-0.05	-0.16	0.1	-0.17	0.15	-0.17	0.51	0.48	0.02	-0.13	-0.22	0.14	-0.23	-0.17	-0.26	0.09	-0.16	-0.26	0.01	-0.15	-0.03	-0.15	1	0.98
Starch_D	0.1	-0.08	0.05	0.02	-0.01	0.02	0.03	0.12	-0.17	0.01	-0.17	0.34	-0.09	0.34	-0.17	-0.13	0.04	0.2	0.01	-0.01	0.07	0	-0.05	0.18	-0.25	-0.16	-0.12	1	-0.19	1	-0.15	-0.15
RS_D	-0.09	0.06	0.05	0.14	-0.03	0.01	0.01	0.03	0.11	0.1	0.02	-0.13	0.43	-0.13	0.04	0.05	-0.05	0.01	0.13	0.03	-0.11	-0.01	0.06	-0.06	0.16	0.15	0.07	-0.19	1	-0.19	-0.03	-0.06
DryMatter_D	0.1	-0.08	0.05	0.02	-0.01	0.02	0.03	0.12	-0.17	0.01	-0.17	0.34	-0.09	0.34	-0.17	-0.13	0.04	0.2	0.01	-0.01	0.07	0	-0.05	0.18	-0.25	-0.16	-0.12	1	-0.19	1	-0.15	-0.15
TubWeight_D	0.1	0.11	0.07	-0.22	0.07	0.16	0.01	-0.1	0.27	-0.33	0.7	0.03	0.1	0.03	0.21	0.22	0.02	0.16	0.03	-0.16	-0.02	0.22	0.06	-0.23	0.54	-0.19	1	-0.12	0.07	-0.12	0.01	0.03
TubNum_D	0.24	0.07	-0.03	-0.03	0.39	0.26	-0.05	0.11	0.61	0.75	-0.18	0.02	0.01	0.02	-0.15	-0.11	0.23	0.24	0.25	0.07	0.31	0.36	0.36	-0.08	0.67	1	-0.19	-0.16	0.15	-0.16	-0.26	-0.26
Yield_D	0.25	0.13	0.03	-0.2	0.38	0.33	-0.05	-0.01	0.72	0.34	0.37	0.03	0.07	0.03	0.08	0.13	0.21	0.31	0.27	-0.07	0.2	0.48	0.32	-0.3	1	0.67	0.54	-0.25	0.16	-0.25	-0.16	-0.14
FLUOR70_D	-0.03	-0.09	-0.15	0.42	-0.19	-0.3	0.02	0.37	-0.2	-0.02	-0.23	-0.03	0.12	-0.03	-0.17	-0.19	0.11	0.08	-0.22	0.19	0.09	-0.2	-0.06	1	-0.3	-0.08	-0.23	0.18	-0.06	0.18	0.09	0.08
SC70_D	0.15	0.08	0.03	0	0.27	0.23	0.14	0.1	0.27	0.3	-0.05	0.06	-0.02	0.06	-0.1	-0.05	0.05	0.15	0.29	-0.06	0.14	0.35	1	-0.06	0.32	0.36	0.06	-0.05	0.06	-0.05	-0.26	-0.23
NDVI70_D	0.18	0.1	0.11	-0.08	0.42	0.47	0.09	0.02	0.44	0.27	0.15	0.19	-0.1	0.19	0.05	0.08	0.2	0.15	0.31	-0.01	0.26	1	0.35	-0.2	0.48	0.36	0.22	0	-0.01	0	-0.17	-0.15
SPAD70_D	0.37	0.13	0.04	-0.04	0.48	0.4	-0.18	0.26	0.39	0.27	0.08	0.23	-0.2	0.23	0.09	0.09	0.5	0.2	0.09	0.04	1	0.26	0.14	0.09	0.2	0.31	-0.02	0.07	-0.11	0.07	-0.23	-0.21
FLUOR50_D	-0.19	-0.14	-0.12	0.47	-0.19	0.07	-0.1	0.07	0.03	0.1	-0.04	-0.08	-0.07	-0.08	0.04	0.02	-0.05	-0.14	-0.05	1	0.04	-0.01	-0.06	0.19	-0.07	0.07	-0.16	-0.01	0.03	-0.01	0.14	0.16
SC50_D	-0.01	0.02	0.05	-0.07	0.23	0.14	-0.01	-0.14	0.16	0.19	-0.01	0.1	-0.02	0.1	-0.09	-0.05	-0.04	0.07	1	-0.05	0.09	0.31	0.29	-0.22	0.27	0.25	0.03	0.01	0.13	0.01	-0.22	-0.24
NDVI50_D	0.14	0.15	0.03	-0.05	0.21	0.08	0	0.06	0.27	0.2	0.03	0.14	0.07	0.14	-0.08	-0.03	0.12	1	0.07	-0.14	0.2	0.15	0.15	0.08	0.31	0.24	0.16	0.2	0.01	0.2	-0.13	-0.1
SPAD50_D	0.55	0.04	-0.03	0.06	0.45	0.15	-0.18	0.3	0.28	0.2	0.04	0.19	-0.13	0.19	0.01	0	1	0.12	-0.04	-0.05	0.5	0.2	0.05	0.11	0.21	0.23	0.02	0.04	-0.05	0.04	0.02	0.02
Perim_	0.03	0.11	0.12	0.06	-0.03	-0.02	0.02	0.07	0.24	-0.04	0.29	-0.08	0.1	-0.08	0.97	1	0	-0.03	-0.05	0.02	0.09	0.08	-0.05	-0.19	0.13	-0.11	0.22	-0.13	0.05	-0.13	0.48	0.5
Area_C	0.03	0.08	0.13	0.09	-0.03	-0.07	0	0.05	0.18	-0.06	0.25	-0.11	0.1	-0.11	1	0.97	0.01	-0.08	-0.09	0.04	0.09	0.05	-0.1	-0.17	0.08	-0.15	0.21	-0.17	0.04	-0.17	0.51	0.52
Starch_C	0.17	0.02	-0.04	-0.01	0.23	0.1	0.04	0.06	-0.04	0.16	-0.16	1	-0.22	1	-0.11	-0.08	0.19	0.14	0.1	-0.08	0.23	0.19	0.06	-0.03	0.03	0.02	0.03	0.34	-0.13	0.34	-0.17	-0.16
RS_C	-0.13	0.1	0.09	0.16	-0.15	-0.07	0.1	-0.02	-0.02	-0.04	0.04	-0.22	1	-0.22	0.1	0.1	-0.13	0.07	-0.02	-0.07	-0.2	-0.1	-0.02	0.12	0.07	0.01	0.1	-0.09	0.43	-0.09	0.15	0.16
DryMatter_C	0.17	0.02	-0.04	-0.01	0.23	0.1	0.04	0.06	-0.04	0.16	-0.16	1	-0.22	1	-0.11	-0.08	0.19	0.14	0.1	-0.08	0.23	0.19	0.06	-0.03	0.03	0.02	0.03	0.34	-0.13	0.34	-0.17	-0.16
TubWeight_C	0.09	0.07	0	-0.11	0.15	0.25	-0.02	0.04	0.41	-0.39	1	-0.16	0.04	-0.16	0.25	0.29	0.04	0.03	-0.01	-0.04	0.08	0.15	-0.05	-0.23	0.37	-0.18	0.7	-0.17	0.02	-0.17	0.1	0.13
TubNum_C	0.16	0.03	0.07	0.1	0.33	0.18	-0.02	0.27	0.54	1	-0.39	0.16	-0.04	0.16	-0.06	-0.04	0.2	0.2	0.19	0.1	0.27	0.27	0.3	-0.02	0.34	0.75	-0.33	0.01	0.1	0.01	-0.16	-0.15
Yield_C	0.21	0.12	0.06	0	0.45	0.37	-0.04	0.31	1	0.54	0.41	-0.04	-0.02	-0.04	0.18	0.24	0.28	0.27	0.16	0.03	0.39	0.44	0.27	-0.2	0.72	0.61	0.27	-0.17	0.11	-0.17	-0.05	-0.02
FLUOR70_C	0.26	0.07	0	0.38	0.24	0	0.09	1	0.31	0.27	0.04	0.06	-0.02	0.06	0.05	0.07	0.3	0.06	-0.14	0.07	0.26	0.02	0.1	0.37	-0.01	0.11	-0.1	0.12	0.03	0.12	0.18	0.18
SC70_C	-0.12	-0.06	0.24	0.05	0.01	-0.07	1	0.09	-0.04	-0.02	-0.02	0.04	0.1	0.04	0	0.02	-0.18	0	-0.01	-0.1	-0.18	0.09	0.14	0.02	-0.05	-0.05	0.01	0.03	0.01	0.03	0.11	0.12
NDVI70_C	0.08	0.28	0.06	-0.09	0.36	1	-0.07	0	0.37	0.18	0.25	0.1	-0.07	0.1	-0.07	-0.02	0.15	0.08	0.14	0.07	0.4	0.47	0.23	-0.3	0.33	0.26	0.16	0.02	0.01	0.02	-0.21	-0.21
SPAD70_C	0.51	0.04	0.11	-0.16	1	0.36	0.01	0.24	0.45	0.33	0.15	0.23	-0.15	0.23	-0.03	-0.03	0.45	0.21	0.23	-0.19	0.48	0.42	0.27	-0.19	0.38	0.39	0.07	-0.01	-0.03	-0.01	-0.28	-0.27
FLUOR50_C	-0.06	0.04	0.09	1	-0.16	-0.09	0.05	0.38	0	0.1	-0.11	-0.01	0.16	-0.01	0.09	0.06	0.06	-0.05	-0.07	0.47	-0.04	-0.08	0	0.42	-0.2	-0.03	-0.22	0.02	0.14	0.02	0.36	0.36
SC50_C	-0.01	0.05	1	0.09	0.11	0.06	0.24	0	0.06	0.07	0	-0.04	0.09	-0.04	0.13	0.12	-0.03	0.03	0.05	-0.12	0.04	0.11	0.03	-0.15	0.03	-0.03	0.07	0.05	0.05	0.05	0.08	0.11
NDVI50_C	0.14	1	0.05	0.04	0.04	0.28	-0.06	0.07	0.12	0.03	0.07	0.02	0.1	0.02	0.08	0.11	0.04	0.15	0.02	-0.14	0.13	0.1	0.08	-0.09	0.13	0.07	0.11	-0.08	0.06	-0.08	0.01	-0.01
SPAD50_C	1	0.14	-0.01	-0.06	0.51	0.08	-0.12	0.26	0.21	0.16	0.09	0.17	-0.13	0.17	0.03	0.03	0.55	0.14	-0.01	-0.19	0.37	0.18	0.15	-0.03	0.25	0.24	0.1	0.1	-0.09	0.1	-0.14	-0.15

Figure 5.1. Pearson's correlations between different physiological and agronomical traits under control (indicated with postfix "_C") and water stress conditions (indicated with postfix "_D") in a panel of 144 potato varieties.

The correlation of physiological and yield-related variables between control and stressed samples was studied (Figure 5.1). Yield is one of the most important traits when looking for tolerance to abiotic stresses. We saw that the yields under control conditions and under water stress conditions were correlated with more or less the same traits, especially with number and weight of tubers under both control and drought conditions. Yield_C and Yield_D was also correlated with most of the physiological parameters and the highest correlations occurred 70 days after planting (DAP). All correlations were positive, except for FLUOR50, FLUOR70, dry matter and starch.

4.2. Population structure analysis and linkage disequilibrium

STRUCTURE software revealed that the study population was formed by two subpopulations of 133 and 11 genotypes respectively, since the obtained delta K value was 2 (Supplementary Figure 5.1a). The probabilistic assignment of each genotype to belong to one of the assigned groups was also performed for deriving the corresponding values of the Q matrix (Supplementary Figure 5.1b). These results indicate that there was genetic diversity in the population with different structural dimensions, which was considered also for the association analysis. A genetic distance matrix was performed between all genotypes to evaluate the genetic diversity and it was observed that the highest value between two varieties was 0.4, the minimum value was 0.26 and the mean value was 0.37.

Linkage disequilibrium (LD) decay was determined using the filtered SNP data. In our study the genetic distance between markers was calculated as the point of intersection between the half decay r^2 value of the genome and the smoothing spline regression model fitted to LD decay (Supplementary Figure 5.2).

4.3. Genome-wide association analysis

Table 5.3. Number of SNPs per chromosome before and after filtering and size of each chromosome. CH01 to CH12 refers to each of the 12 potato chromosomes, CH00 are control markers that are not associated with any chromosome and CH13 refers to the chloroplast.

Chromosome	Number of SNPs (total)	Number of SNPs (filtered)	Chromosome length (bps)
CH00	464	156	
CH01	3958	2486	88,663,952
CH02	3335	1914	48,614,681
CH03	2919	1637	62,190,286
CH04	2798	1611	72,208,621
CH05	2538	1520	52,070,158
CH06	2390	1461	59,532,096
CH07	2457	1407	56,760,843
CH08	2043	1234	56,938,457
CH09	2204	1296	61,540,751
CH10	1865	1061	59,756,223
CH11	2249	1361	45,475,667
CH12	1942	1098	61,165,649
CH13	28	17	155,312
Total	31190	18259	810,654,046

The total of 31,190 markers were filtered to ensure the quality of the SNPs, removing markers with a missing value rate higher than 10% and those with a minor allele frequency below 0.05, obtaining 18,259 SNP markers. These SNP markers provide a genome-wide coverage along the 12 chromosomes of tetraploid potatoes (Table 5.3).

The association mapping was performed with kinship correction to minimize false positive associations. The Q+K model was used with the 18,259 high-quality SNP markers and the panel of 144 accessions. The results of the Q-Q plots indicate that the observed $-\log_{10}(P)$ values are in accordance with the expected $-\log_{10}(P)$ values (Supplementary Figure 5.3).

The results of the association analysis are presented as marker-trait associations to get an overall impression of the effect of water stress in our population. In this study eighteen QTLs were identified above the Bonferroni threshold. Five of these QTLs were associated with two of the traits measured under control conditions, while the rest were associated with traits measured in plants under water stress.

Two SNP markers associated with chlorophyll content measured at 70 DAP were found, one on chromosome 6 (PotVar0039950) and the other on chromosome 11 (solcap_snp_c2_15287). Two other QTLs were also found on chromosome 11, which in this case were associated with the number of tubers in control plants (solcap_snp_c2_37217 and ST4.03ch11_2070850). The marker solcap_snp_c2_15676, located on chromosome 5, was also associated with this trait (Table 5.4, Figure 5.2).

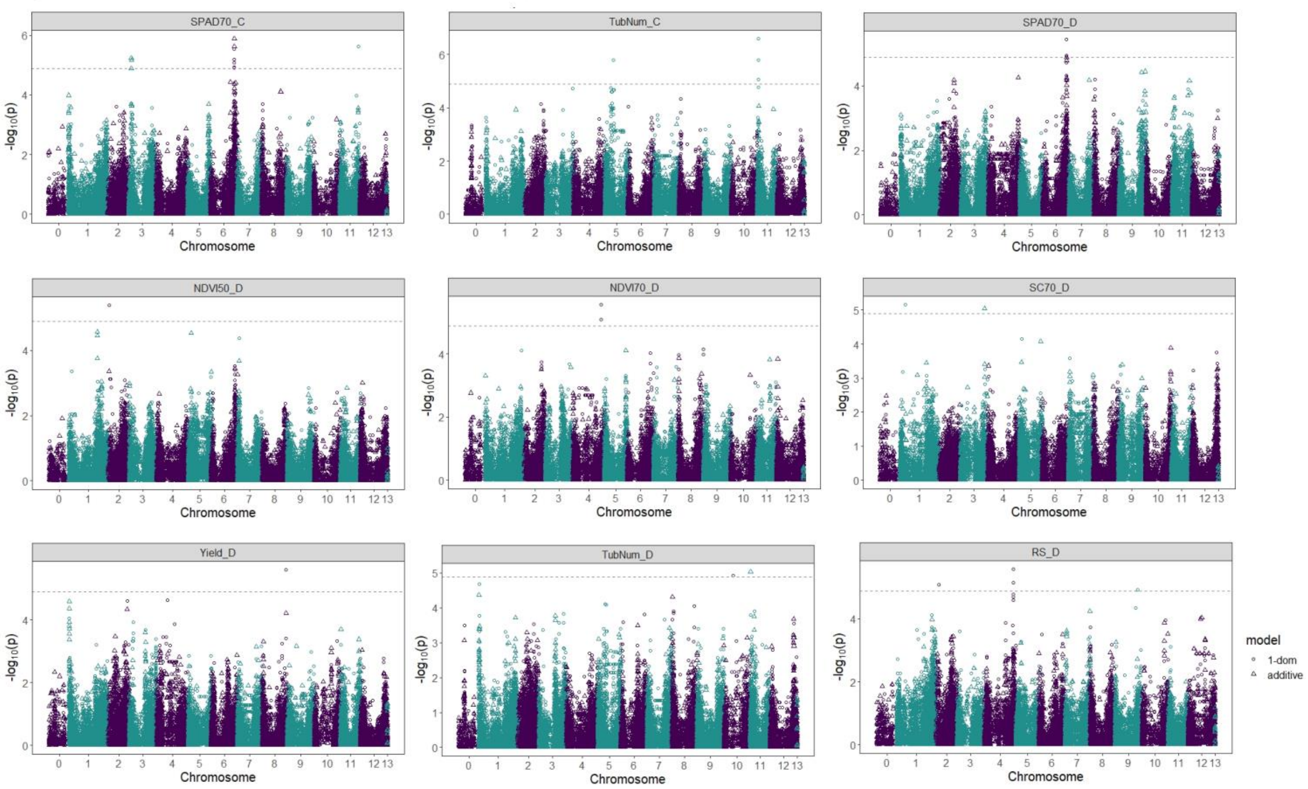


Figure 5.2. Manhattan plots for the traits with significant SNPs associated under control and drought stress conditions in 144 potato varieties.

If we observe the physiological parameters under drought conditions we can see that most of the associations occurred with measurements taken at 70 DAP. Two QTLs associated with Normalized Difference Vegetation Index (NDVI) were found, both on chromosome 4 (solcap_snp_c2_43735 and PotVar0113919), the marker solcap_snp_c2_45637 on chromosome 1 was also found to be associated with stomatal conductance and the marker PotVar0039950 on chromosome 6 was associated with leaf chlorophyll content. Although almost all associations were found in measurements taken at 70 DAP, the NDVI was also associated with one marker (solcap_snp_c1_6462) in the first stress phase, at 50DAP (Table 5.4, Figure 5.2).

Table 5.4. Significant SNPs associated with evaluated physiological and agronomical traits under control and drought stress conditions in 144 potato varieties.

Trait	Marker	Chrom	Position	Ref	Alt	Effect	R^2	p-value	FDR	Biological function
SPAD70_C	PotVar0039950	6	53985614	C	T	-2.07	0.0183	2.14x10^{-2}	0.0361	Radical SAM superfamily protein
SPAD70_C	solcap_snp_c2_15287	11	41743380	A	G	-4.20	0.0681	7.05x10^{-6}	0.0138	P-loop containing nucleoside triphosphate hydrolases superfamily protein
TubNum_C	solcap_snp_c2_15676	5	18718517	G	T	-25.38	0.0438	0.0003	0.0222	RNA-binding CRS1 / YhbY (CRM) domain-containing protein
TubNum_C	solcap_snp_c2_37217	11	1818959	A	G	-32.80	0.0006	0.0486	0.05	-
TubNum_C	ST4.03ch11_2070850	11	2070850	A	T	42.25	0.0153	0.0355	0.0416	Di-glucose binding protein with Kinesin motor domain
NDVI50_D	solcap_snp_c1_6462	2	2450782	G	T	0.03	0.0744	2.53x10^{-6}	0.0027	Plant protein with unknown function
SPAD70_D	PotVar0039950	6	53985614	C	T	-3.65	0.0712	4.25x10^{-6}	0.0083	Radical SAM superfamily protein
NDVI70_D	solcap_snp_c2_43735	4	64055406	A	G	-0.07	0.0095	0.0432	0.0444	GroES-like zinc-binding dehydrogenase family protein
NDVI70_D	PotVar0113919	4	64089292	A	G	-0.07	0.0049	0.0461	0.0472	Ascorbate peroxidase
SC70_D	solcap_snp_c2_45637	1	12022163	A	G	-182.03	0.0729	3.26x10^{-6}	0.0055	Hypothetical protein
Yield_D	solcap_snp_c2_26653	8	54286889	G	T	-0.75	0.071	4.37x10^{-6}	0.0111	Osmotin
TubNum_D	PotVar0064470	11	787325	G	T	-10.25	0.0614	2.03x10^{-5}	0.0166	Alternative oxidase family protein
TubNum_D	solcap_snp_c2_55085	10	20334943	A	G	27.36	0.0586	3.23x10^{-5}	0.0194	Transketolase
RS_D	solcap_snp_c1_3746	2	7050595	C	T	0.14	0.034	0.0016	0.025	Cofactor assembly of complex C
RS_D	solcap_snp_c2_25284	4	65872176	A	G	0.10	0.0041	0.0277	0.0388	Sucrose transporter
RS_D	solcap_snp_c2_55785	4	65970953	G	T	0.11	0.0952	0.01	0.0277	Leucine-rich receptor-like protein kinase family protein
RS_D	solcap_snp_c2_55783	4	65971150	A	G	0.11	0.0952	0.01	0.0305	Leucine-rich receptor-like protein kinase family protein
RS_D	solcap_snp_c2_55775	4	65972399	C	T	0.11	0.0952	0.01	0.0333	Leucine-rich receptor-like protein kinase family protein

One of the most important parameters when assessing stress tolerance is the maintenance of crop yield. In this case we saw that the marker solcap_snp_c2_26653, located on chromosome 8, was associated with yield under water stress conditions and that it is co-localized with the osmotin gene (Soltu.DM.08G027260.1). The markers PotVar0064470 and solcap_snp_c2_55085, located on chromosomes 10 and 11 respectively, were associated with tuber number under stress conditions. The trait for which the most associated QTLs were found was the content of reducing sugars under drought conditions, one of them (solcap_snp_c1_3746) was found on chromosome 2, while the other four were located on chromosome 4, and three of them (solcap_snp_c2_55785, solcap_snp_c2_55783, solcap_snp_c2_55775) co-localized with the same gene, leucine-rich receptor-like protein kinase family protein (Table 5.4, Figure 5.2).

5. Discussion

Thanks to new massive sequencing techniques and the development of chips such as the GGP Potato 35K array used in this study, we can obtain a global view of the genome and select regions and genes of interest related to the desired trait (Felcher et al., 2012; S. K. Sharma et al., 2018). The traits evaluated in this work have complex inheritance patterns that make the ability of existing mapping technologies to detect the underlying genetics even more difficult. Different studies have analysed the heritability of yield and its components under control and water stress conditions. These traits under control conditions have a fairly acceptable heritability of around 0.7 (G. Ozturk & Yildirim, 2014) but it is not very clear how water stress affects the heritability of these traits. In some studies it drops to 0.06 (Cabello et al., 2015) while in other studies this decrease was much lower (Rudack et al., 2017).

When analysing multiple testing exist the problem of having false positives, so it is important to adjust the p-value of each marker when performing the statistical analysis (Gupta et al., 2014). In our study we can observe that the FDR values are higher than the Bonferroni p-values. The Bonferroni correction is the most commonly used in association studies, but this method is very strict and can sometimes fail to identify important associations, so the FDR correction is usually used (Khlestkin et al., 2019; Wang et al., 2010).

In this study, QTLs related to chlorophyll content measured at 70DAP and tuber number under control conditions were identified. These two parameters also showed a significant positive correlation, indicating that the amount of chlorophyll in the leaves of the plants has an effect on the number of tubers.

When plants are under water stress, one of the tolerance mechanisms is the inhibition of photosynthesis, and as a consequence chlorophyll content decreases. Chlorophyll content was significantly associated with two SNPs, solcap_snp_c2_15287 and PotVar0039950. The solcap_snp_c2_15287 (Soltu.DM.11G023130) was co-localized with a gene encoding for a "P-loop containing nucleoside triphosphate hydrolases superfamily protein", which are a type of hydrolases that catalyse the hydrolysis of the beta-gamma phosphate bond of a bound nucleoside triphosphate (NTP) and the obtained energy from this reaction is used to make conformational changes in other molecules (Kumar et al., 2018). In an assay on water-stressed *Arabidopsis*, they found an association between two P-loop-containing nucleoside triphosphate genes and proline content, which is closely related to plant response to drought (Verslues et al., 2014). Another study in rice showed that a new DEAD-box helicase ATP-binding protein (OsABP), a kind of P-loop containing nucleoside triphosphate hydrolase, was upregulated in response to multiple abiotic stresses, including NaCl, dehydration, ABA and blue and red light (Macovei et al., 2012).

The PotVar0039950 marker was found to be associated with the SPAD70 trait under both, control and water stress conditions. This marker co-localizes with a "Radical SAM superfamily protein" gene (Soltu.DM.06G028800.1) and is located on chromosome 6. Radical SAM is a designation for a superfamily of enzymes that are involved in numerous processes, such as enzyme activation, post-transcriptional and post-translational modifications, lipid metabolism, or biosynthesis of antibiotics and natural products (Frey et al., 2008). In a previous study in *Sonneratia apetala* they found that the SAMS1 gene was related to this group of proteins and indicated that SAMS1 enhanced the plant's cold resistance by enhancing the biosynthesis of S-adenosyl-Lmethionine (SAM). In addition, SAMS1 is also involved in ethylene biosynthesis, which is closely related to the plant's response to drought stress (Shen et al., 2021).

Normalized Difference Vegetation Index was associated with PotVar0113919 marker which co-localized with the ascorbate peroxidase gene (Soltu.DM.04G030200.1). Ascorbate peroxidase (APX) is an enzyme essential for protecting chloroplasts and other parts of the cell from damage caused by reactive oxygen species and its production increases when plants are exposed to unfavorable environmental conditions (Asada, 1992). The expression of APX encoding genes is modulated by those environmental stimuli, such as drought (Caverzan et al., 2013). Other studies in cowpea and wheat showed in sensitive cultivars an increase of APX transcripts in response to water stress (D'Arcy-Lameta et al., 2006; Sečenji et al., 2010). Also in potato an increases in ascorbate peroxidase activity was observed under drought and heat stress treatments in three of the four tested varieties (Demirel et al., 2020b). In our study this gene is associated with NDVI70 under stress conditions, similar as in another study where ascorbate peroxidase concentrations were correlated with photosynthesis, Fv/Fm and chlorophyll parameters (Alhoshan et al., 2019).

The increase of the yield under water deficit was associated with solcap_snp_c2_26653 on chromosome 8 and is co-localized with the osmotin gene (Soltu.DM.08G027260.1). Osmotin is a multifunctional protein. Its overexpression induces abiotic stress tolerance, lowering the osmotic potential under stress (M. A. Bashir et al., 2020). Studies in cotton and tomato showed that the overexpression of the osmotin gene had a protective role and enhances drought stress tolerance (Goel et al., 2010; Parkhi et al., 2009). Increases in leaf expansion, chlorophyll and relative water content were observed due to overexpression of osmotin in transgenic sesame plants and were fully recovered after rewatering (Hakim et al., 2018).

Tuber numbers under stress conditions were associated with two SNP markers, PotVar0064470 and solcap_snp_c2_55085. PotVar0064470 was co-localized with an "Alternative oxidase family protein", gene (Soltu.DM.11G001000.2). Alternative oxidase (AOX) activity is important for maintaining photosynthetic electron transport under stress, and also helps plants to cope with excess energy under drought, by avoiding the over reduction of chloroplast electron carriers (Bartoli et al., 2005; Sunil et al., 2019). During severe or prolonged mild drought stress in N*icotiana tabacum*, the amount of AOX protein was important for maintaining the photosynthetic rate and improving growth during prolonged water deficit (Selinski et al., 2018). In our study the number of tubers was significantly correlated with photosynthesis-related parameters such as chlorophyll content or NDVI, which confirms the protective function of AOX. Also associated with tuber number was solcap_snp_c2_55085 which co-localized with "Transketolase" gene. Transketolase (TK) is an enzyme that participates in both, the pentose phosphate pathway in all organisms and the Calvin cycle of photosynthesis (Chapagain et al., 2018). In a study with wheat plants, the decrease in transketolase level suggested the suppression of the two pathways in the leaves of drought-stressed plants (H. Liu et al., 2015). However, in studies with transgenic rice co-overproduction of Rubisco and transketolase did not improve photosynthesis (Suzuki et al., 2017).

The content of reducing sugars under drought conditions was associated with five QTLs. One of these QTLs was solcap_snp_c2_25284 and co-localized with sucrose transporter (Soltu.DM.04G031670.1). Cellular accumulation of soluble sugars during drought stress influences the expression of sugar transporters (Kaur et al., 2021), which is in agreement with the results obtained in our study. In potato, some studies have also analyzed the export of sucrose from the source to the leaves, by analyzing the expression of genes related to sucrose transporters (SWEETs and SUTs), which are involved in stress response (Aliche et al., 2020; Chen et al., 2020). The markers solcap_snp_c2_55785, solcap_snp_c2_55783, and solcap_snp_c2_55775 were on chromosome 4 and are co-localized with the same gene, leucine-rich receptor-like protein kinase family protein (Soltu.DM.04G031690.1). Studies in rice showed that overexpression of LRK, which encodes a leucine-rich receptor-like kinase, increased drought tolerance (Kang et al., 2017; Ouyang et al., 2010). A potato gene, StLRPK1 encoding a protein belonging to leucine-rich repeat receptor-like kinases was identified and the results suggest that StLRPK1 may participate in the responses against environmental stresses in potato, which is in accordance with our results (Wu et al., 2009).

In this study we found markers associated with the evaluated physiological traits. Other authors have previously reported QTLs and genomic regions associated with chlorophyll content, chlorophyll fluorescence and NDVI in water stress assays in other populations, indicating that these results are robust (Anithakumari et al., 2012; Díaz et al., 2021; Khan et al., 2015). For yield-related parameters, we found markers related to yield, tuber number and reducing sugar content as in previous studies reporting QTLs associated with these traits (Massa et al., 2015; Rak et al., 2017; Tagliotti et al., 2021).

One additional, important aspect to consider is the validation of the significant SNP markers by expression analyses in control and water stress conditions using RT-qPCR in more sensitive and more tolerant genotypes. This aspect will be considered in a follow up publication.

Chapter 6

General discussion

Chapter 6: General discussion

Potato is one of the most important crops worldwide and has a great economic relevance, being even the basis of food in some countries and compared to grain crops it is a major source of nutrients. The genome sequence of potato was released and published in 2011 by the International Potato Genome Sequencing Consortium (PGSC) and the potato genome size is estimated to be 830Mb distributed in 12 chromosomes (Xu et al., 2011).

Potato breeding using conventional methods is time consuming and complicated because it is an autotetraploid crop and has very complex quantitative inheritance patterns. Despite the difficulty of breeding this crop, during the last decades different breeding programs have made efforts to obtain varieties with the traits of interest. Several studies have focused on breeding for diseases such as late blight (Paluchowska et al., 2022), viruses (Kaushik et al., 2013), nematodes (Sudha et al., 2016); quality traits such as cold sweetening (Kawchuk et al., 2008), fry color (Byrne et al., 2020), starch content (Gebhardt et al., 2005); and tolerance to abiotic stresses such as heat (Singh et al., 2020), drought (Tagliotti et al., 2021), salinity (Sanwal et al., 2022) and nitrogen use efficiency (Getahun et al., 2020). Therefore, the current challenge for potato breeders and biotechnologists is to design breeding programs that integrate marker-assisted selection for specific alleles, genetic selection for unspecified alleles, and phenotypic selection (Bradshaw, 2017).

Environmental stress represents the most limiting factor for agricultural productivity due to its detrimental effects on plant growth and yield. Drought is a difficult concept to define, since it can be described from different points of view. In general, it is considered that water stress occurs when the water available in the soil for plants decreases due to low humidity at specific period of time. On the other hand, a plant is considered to be under water stress when the transpiration rate of the leaves exceeds the water absorption by the roots (Salehi-Lisar & Bakhshayeshan-Agdam, 2016).

Advances in next-generation sequencing (NGS) technologies and the development of powerful computational pipelines have greatly reduced the cost of whole genome sequencing, enabling the discovery, sequencing and genotyping of thousands of markers. Thanks to NGS, numerous SNP markers can be detected in a single sequencing run easily and relatively economic. Moreover, in the case of potato, since the reference genome is available, this technology allows us to map the sequences obtained for identifying markers in specific regions of the genome (Ray & Satya, 2014). Thus, instead of selecting genotypes based on a phenotypic evaluation commonly used in traditional breeding programs, DNA markers can be used to select resistant clones in early generations and shorten the long selection process.

The response to water stress varies according to the variety, but also depends largely on the duration and timing of the stress. For this reason, it is important to evaluate the response of different varieties at different times of stress and their ability to recover when irrigated again, since in nature drought periods are not permanent, although they are becoming longer. The developmental stage of the plant at the time when the stress is applied may be the most significant factor determining yield responses among cultivars (Parkash & Singh, 2020).

In this context, the present work has shed new insights into the mechanisms used by potato plants in response to water deficit under controlled conditions at different times of stress in order to identify traits of interest that can be used to screen genotypes and select those with the best response to stress. In potato, water stress tolerance is a very complex trait involving numerous response genes, so rapid screening tools would be useful in the selection of more tolerant genotypes.

According to Handayani et al. (2019) the stay-green trait has been correlated with maintenance of photosynthetic activity, which results in high yield under drought conditions and is used as an indicator of drought tolerance in potatoes. Genotypes with efficient photosynthesis under low stomatal conductance are considered drought tolerant. Stomata closure is the first mechanism of plants to maintain water content. As we found in our study, this was true, and all varieties closed stomata significantly under stress conditions, although Agria and Kennebec closed stomata earlier.

Banik et al. (2016) proposed that the most commonly used indicators of resistance to drought stress are yield, leaf water content and leaf water loss, since leaf wilting is the most visual response to drought stress. In our study, only the variety Monalisa showed significant differences in RWC under water stress conditions, although this could be related to reduced leaf stomata closure to prevent water loss.

Under drought conditions, crop development is inhibited by the decrease in soil water potential, because plants need to reduce their osmotic potential below the soil water potential, leading to water deficit and low nutrient uptake (Wahab et al., 2022). A tolerance mechanism of plants to be able to extract water from the soil even under drought conditions is the accumulation of osmolites that decrease the leaf water potential (Ozturk et al., 2021). In our study, we found that predawn water potential Ψpd is a good indicator of soil water status and leaf water potential gives an idea of plant water status. All the varieties showed significant differences in both parameters under stress conditions and although all of them recovered their water potential, in no case the control values of Ψpd were recovered.

Drought tolerance is a complex trait, involving the interaction of morphological, physiological and biochemical parameters, although high yield potential under drought conditions is an obvious target for breeding strategies (Akhter Ansari et al., 2019). In addition to tuber yield, quality is an important trait for the potato industry and can be affected by water deficit. Some physiological disorders of tubers are closely related to low water supply in the crop. The most important effects are the reduction of dry matter and starch content in the tubers, with a consequent increase in the content of reducing sugars, caused by water deficit (Ávila-Valdés et al., 2020). In our study only two of the six varieties evaluated showed a decrease in dry matter and starch content under stress conditions, although this factor can be strongly influenced by other parameters such as radiation or temperature (Tourneux et al., 2003a).

The response to water stress is highly genotype dependent. At present, the effects of drought stress can be alleviated by selecting the most suitable potato genotype according to climate and improving agronomic practices (Nasir & Toth, 2022). In this study, we evaluated 6 varieties of different growing cycles to assess their behavior and found that Monalisa was the variety with the best response to stress, since it was the only one that did not show significant differences in any of the parameters related to yield.

Under field conditions, plants are often exposed to drought acclimation conditions as drought stress accumulates slowly over time. In contrast, under controlled environmental conditions, a rapid drought shock is often imposed that does not account for genotypic variation for acclimation and can result in very different responses and mask key response mechanisms (Banik et al., 2016). Therefore, it would be interesting to carry out a more exhaustive study to evaluate how these parameters are affected in other varieties and under other environmental conditions.

Since the RNA-seq technique was developed in the early 2000s, it has become a major tool used in molecular biology. Transcriptome analyses have been widely used to identify genes that are differentially expressed in response to different stresses or to analyze changes in plant gene expression at a specific time point and in a specific state (Wang et al., 2020). This technique allows researchers to identify interesting candidate genes involved in biological pathways and molecular mechanisms underlying the stress response (Imran et al., 2021).

During the previously mentioned trial in which six varieties were evaluated to identify physiological responses to water stress, RNA samples were collected from Agria and Zorba varieties to perform an RNAseq study and analyze differential gene expression between stressed and control plants. With this study, we were able to evaluate the response mechanisms of these plants to water stress and provide a basis for identifying the functional genes involved in the mechanism of susceptibility or tolerance in potato cultivars.

The response to water stress is a very complex trait that involves different genes and cellular mechanisms, as evidenced by the large number of gene ontology GO enrichment in the stressed leaves in this study. One of the categories in which we found differentially expressed genes (DEG) enrichment was related to photosynthesis. Photosystem-related DEGs were found to be expressed in a lower level under water stress conditions, indicating that under stress conditions there is a decrease in photosynthesis. Inhibition of the expression of photosynthesis-related genes causes stomata closure and reduces water loss through transpiration, helping to maintain the water status of the plant, which is in agreement with the results discussed previously.

According to Wang et al. 2020 abscisic acid (ABA), ethylene (ETH) and jasmonic acid (JA) signaling pathways play an important role in drought tolerance of potato (Wang 2020). Abscisic acid is a plant hormone that plays a key role in plant response to abiotic stresses. Under water deficit conditions, the biosynthesis of this hormone is increased, as well as its transport and accumulation in plant tissues (Bashir et al., 2019). In this work, it was found that ABA-related genes were significantly expressed under water stress conditions, and in particular the most overexpressed gene in both varieties was the "Abscisic acid and environmental stress-inducible protein TAS14-like".

According to Feki & Brini (2016) the plant response to water stress involves the activation of drought-inducible genes that encode for the production of osmolytes and proteins. They classify these proteins into two groups: on the one hand there are functional proteins including aquaporins, late embryogenesis abundant (LEA)- type proteins, heat shock proteins, detoxification enzymes and proteins involved in osmolyte biosynthesis; and on the other hand regulatory proteins including protein kinases and phosphatases, transcription factors and proteins involved in ABA perception and biosynthesis. Our results were in agreement with this theory since we found that under stress conditions there was a differential expression in genes related to transcription factors such as bZIP, WRKY or bHLH. We also observed that genes related to molecular functions such as hydrolases, kinases and phosphatases had a differential response and that some heat shock proteins were overexpressed under stress conditions, especially in Agria variety.

This work also presents the study carried out on a panel of 144 tetraploid potato varieties for the identification of QTLs associated with traits related to water stress response such as chlorophyll content and fluorescence, NDVI, stomatal conductance, leaf area and circumference, yield, tuber weight and number, dry matter, starch content and reducing sugars. These traits were evaluated in a field trial during the 2019 and 2020 seasons, in a plot with irrigation according to crop needs and another plot without irrigation. An analysis of variance of these traits and a correlation analysis were performed, which indicated that crop yield was strongly influenced by the physiological parameters evaluated. Good phenotyping is essential for association mapping to be relevant, although this is sometimes difficult as traditional phenotyping methods are based on subjective manual measurements and are sometimes laborious and time consuming. In recent years, the trend of high-throughput phenotyping has emerged as a solution to these drawbacks as it allows data to be obtained in more reliable, rapid and non-invasive ways (Xiao et al., 2022).

Drought tolerance is a complex trait because of its quantitative polygenic nature controlled by numerous genes of small effect and its low heritability. In addition, it is challenging to make an accurate assessment of tolerant cultivars because of the difficulty in massive screening of genes and traits and because of the high interactions between genotype and environment (dos Santos et al., 2022). The results obtained in this study demonstrate the quantitative nature of these characters since numerous genes were found to be associated with different traits under water stress conditions, both phenotypic traits such as chlorophyll content, stomatal conductance and NDVI, and agronomic and quality traits such as yield, number of tubers and reducing sugars content.

In addition, some of the traits evaluated such as yield are also polygenic in nature, and in these cases a very large reference population is required to estimate with high precision the individual effects of all loci controlling the trait (Caruana et al., 2019). In this study, 144 cultivars were used, which is a small population size that probably affected the detection of alleles in complex traits with low heritability (Kurawa et al., 2020). According to (Bradshaw, 2022b), over the last few years advances in sequencing technologies have led to a large increase in the number of markers but this has not been equally reflected in the increase in population size, so this is still a very limiting factor that researchers should pay attention to in order to improve GWAS studies.

Chapter 7

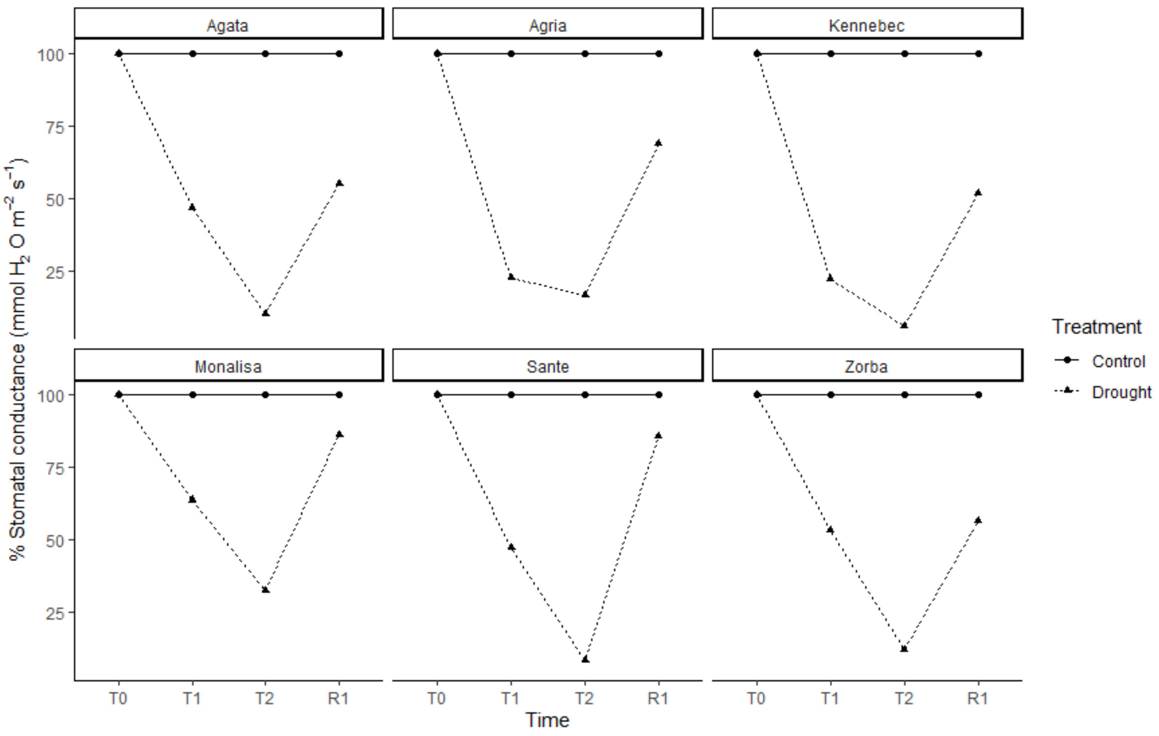
Conclusions

Conclusions

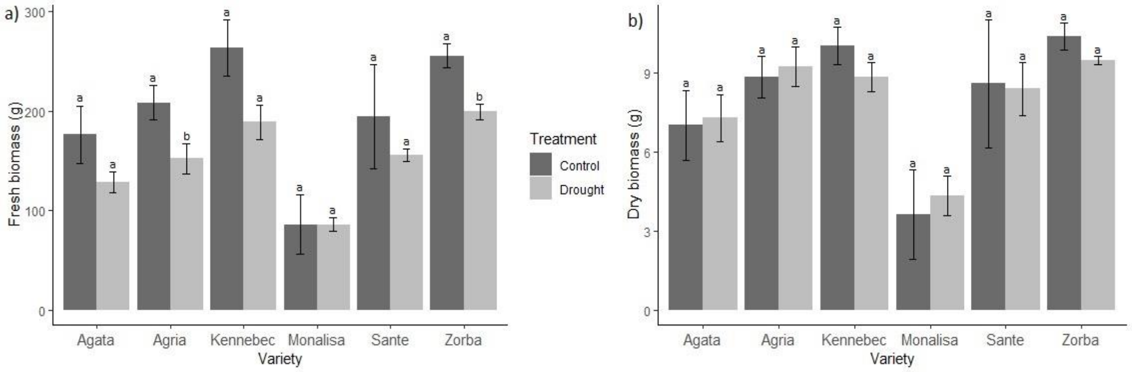
1. Stomatal conductance and water potential represent physiological parameters that are easy to measure and give a good and rapid response to water stress, so they could be used for rapid identification of stress and for selection of tolerant varieties.
2. The variety Monalisa did not show significant differences in any of the yield parameters, indicating its good response to water stress.
3. Indirect selection based on parameters associated with water stress can be a useful tool in potato breeding programs for the identification of more tolerant varieties.
4. The RNAseq technique is a useful tool for the detection of expression differences to better understand the molecular mechanisms of water stress tolerance in potato plants.
5. Numerous differentially expressed genes (DEG) were found between stressed and control plants and provide the basis for the study of gene function and regulatory mechanisms of water stress tolerance.
6. Agria variety showed significantly more DEGs than Zorba variety, suggesting that Agria has a better response to water stress. In both cases, more genes were downregulated than upregulated.
7. We have identified the main DEGs and mechanisms that regulate plant tolerance to water stress, such as some ABA-responsive genes, numerous transcription factors or heat shock proteins. We have also identified metabolic pathways involved in plant protective functions, such as cell wall maintenance, carbohydrate metabolism or osmotic adjustment.
8. Field trials were conducted under normal irrigation and non-irrigation conditions during 2019 and 2020 and significant differences were found in both years between genotypes and between treatments for all evaluated phenotypic traits. In addition, yield under both, control and water stress conditions, correlated with almost all physiological parameters.

9. From genotyping data we obtained 18259 good quality SNP markers and found that 18 were associated with some of the phenotypic traits evaluated under both, control and non-irrigated conditions.
10. Significant markers were found to be associated with physiological parameters SPAD70, NDVI50, NDVI70 and SC70 with agronomic parameters of yield, tuber number and reducing sugars content.
11. The SNPs that showed significant associations with phenotypic traits were co-localized with genes with functions related to water stress tolerance mechanisms, so they could be useful for designing markers to be used in future assisted selection programs related to drought tolerance in potato.

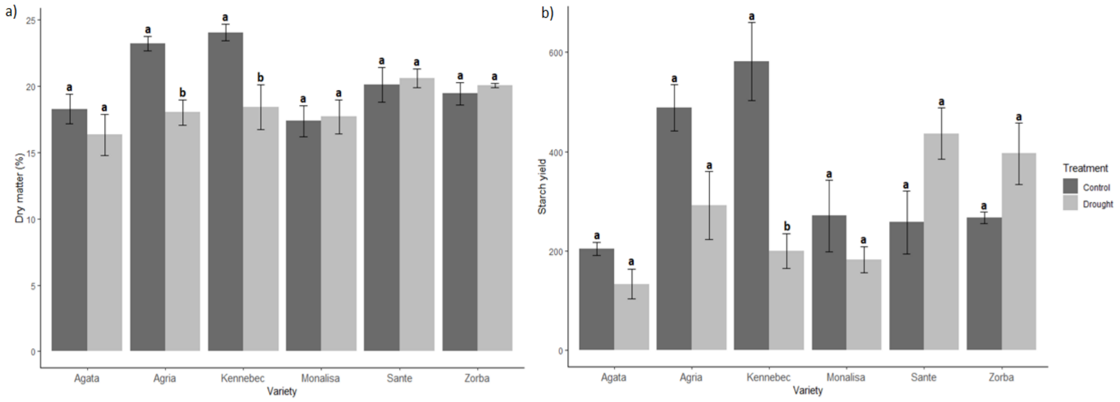
Supplementary material



Supplementary Figure 3.1. Reduction of stomatal conductance [%] in stressed plants measured in three replicates of six potato varieties at four different times.



Supplementary Figure 3.2. a) Fresh and b) dry biomass measured in four replicates in control and drought stressed plants at T2. Different letters indicate significant differences between the two different treatments according to Tukey HSD test (P<0.05).



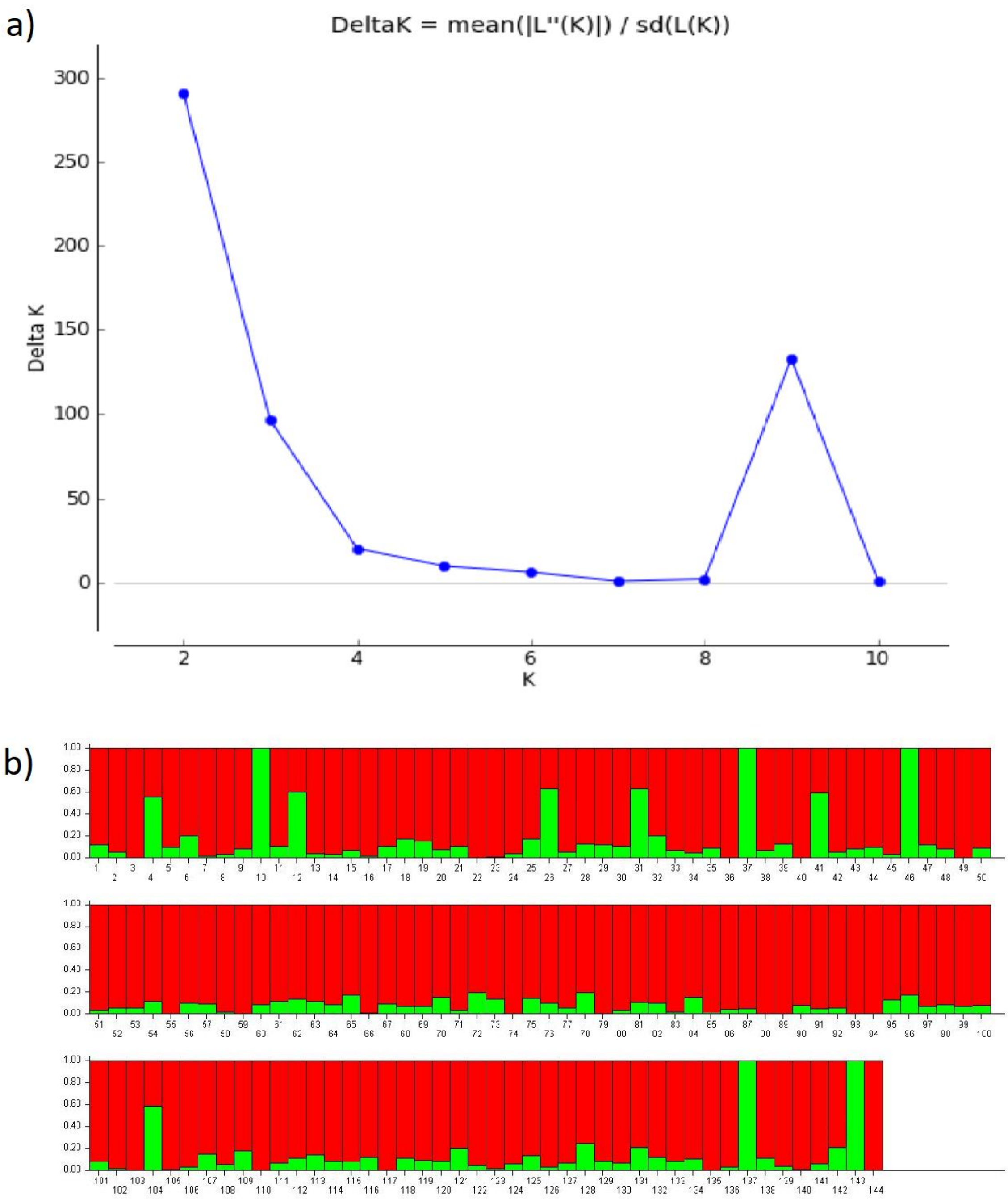
Supplementary Figure 3.3. a) Tuber dry matter and b) starch yield measured in four replicates in control and drought stressed plants. Different letters indicate significant differences between the two treatments according to Tukey test ($p<0.05$).

Supplementary Table 4.1. Sequences of primers used in RT-qPCR analysis.

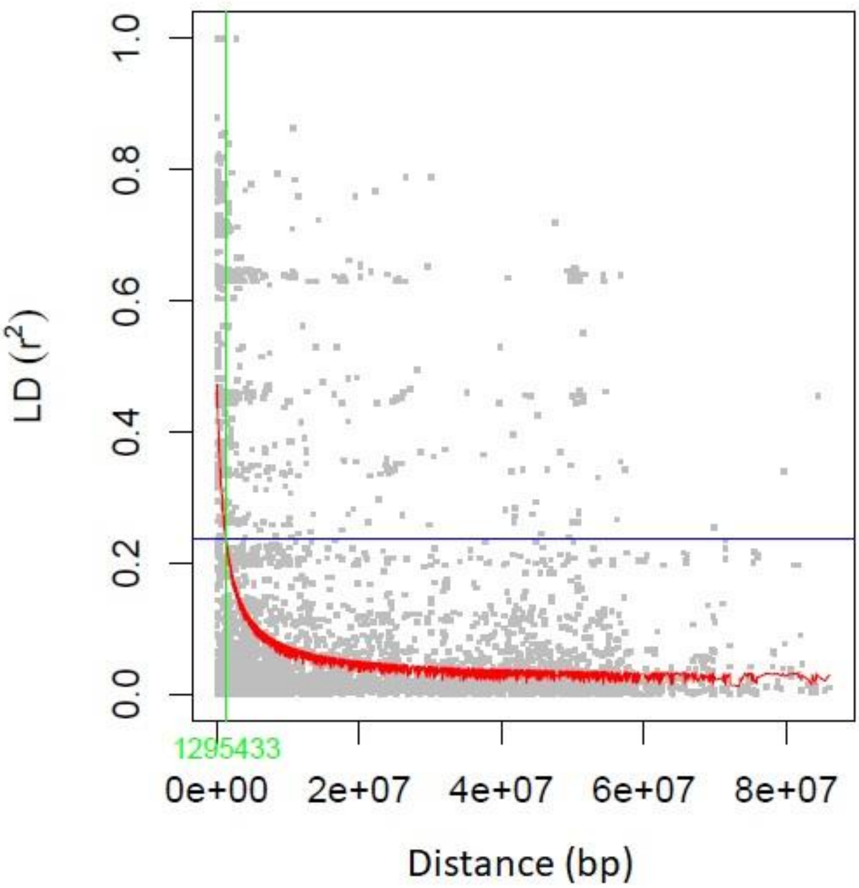
GENE ID	Primer sequence
LOC102594541	Forward: ACTGTGTGAGCAGATTCGGG Reverse: ATATCTAAGGCGCTGACGGC
LOC102584727	Forward: AATGGCTGGTGGGGGTGAT Reverse: CTCTGTGAGCCAAGTGCCAA
LOC102599586	Forward: GCCGACTGTTCTCTTCCACC Reverse: AGTTTGAATCCACTCCCTGCTG
LOC102606222	Forward: CGCAGCTAACAATCCGACCT Reverse: TAGTGGGCGTCATTTCGTCG
LOC102593738	Forward: ATCCTCATCCACAAAGAACACCAA Reverse: TGCTGCTCCTGCCAATCTGT
LOC102580220	Forward: AACAACAATAACTACATGCCGAGC Reverse: TTAGATGCAAGTGACAGGCCCAA
LOC107062634	Forward: GCTCCTGTGGTGTTTTTCAGGTAA Reverse: TCATATCCATCAGCAACTCGACC
LOC102606295	Forward: TGCTTTTGCTGGACTTTTAGGTATG Reverse: GCACGCTTCCACTTAGACCA
LOC102600114	Forward: TTTGCGTAAGTCCCCCGTC Reverse: CCTCTCTCGCTCAATATCCTTTTCA
LOC102598238	Forward: AGCAGTACCCTATTCCTCCTCG Reverse: CACAAATCTTTCTTGCCTGCCC
LOC102603161	Forward: TACTGGTGCTACTGGATTTCTTGC Reverse: GTCCTTTGCCACTGCCTCAT
LOC102601774	Forward: TTGGGTTCATCACTGGACTACAC Reverse: CTTTTGCAGGATCTACGAAGGGT
Tubulin	Forward: GGGAATAACTGGGCGAAAGGT Reverse: CCTCCACCAAGTGAGTGACAA

Supplementary Table 5.1. Mean, standard deviation and phenotypic variance of 144 tetraploid potato varieties.

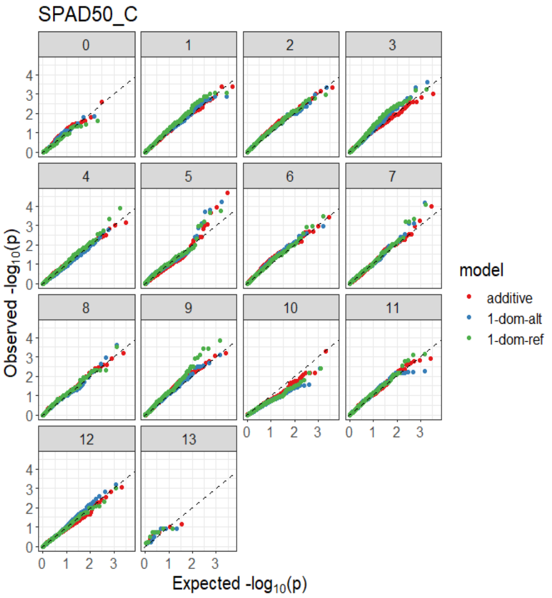
	2019			2020		
	Mean±SE	SD	σ^2	Mean±SE	SD	σ^2
SPAD50_C	45.13±0.349	4.18	17.49	42.00±0.347	4.16	17.34
NDVI50_C	0.88±0.001	0.01	0.0002	0.87±0.002	0.02	0.0004
SC50_C	424.07±15.427	185.13	34272.35	617.87±17.079	204.95	42003.42
FLUOR50_C	0.67±0.005	0.07	0.004	0.69±0.003	0.03	0.001
SPAD70_C	45.13±0.421	5.05	25.49	40.31±0.387	4.64	21.53
NDVI70_C	0.86±0.003	0.04	0.001	0.84±0.004	0.05	0.002
SC70_C	453.64±17.222	206.66	42708.85	501.71±14.696	176.35	31098.39
FLUOR70_C	0.69±0.004	0.05	0.002	0.72±0.003	0.04	0.001
Yield_C	5.56±0.169	2.03	4.10	4.51±0.146	1.75	3.05
TubNum_C	51.31±1.898	22.78	518.72	41.82±1.437	17.25	297.41
TubWeight_C	111.66±2.805	33.66	1133.08	114.30±2.988	35.86	1285.71
DryMatter_C	20.08±0.338	4.06	16.45	17.79±0.300	3.59	12.91
RS_C	0.20±0.013	0.16	0.02	0.16±0.010	0.12	0.01
Starch_C	13.11±0.347	4.17	17.37	10.76±0.308	3.69	13.64
Area_C	14.01±0.271	3.25	10.59	16.75±0.337	4.04	16.35
Perim_C	16.74±0.156	1.87	3.50	18.18±0.173	2.08	4.31
SPAD50_D	46.06±0.336	4.03	16.22	41.63±0.373	4.47	20.01
NDVI50_D	0.84±0.004	0.05	0.002	0.86±0.002	0.02	0.0006
SC50_D	283.44±12.079	144.94	21008.61	445.16±16.718	200.61	40246.29
FLUOR50_D	0.64±0.005	0.06	0.003	0.70±0.003	0.04	0.001
SPAD70_D	42.99±0.493	5.92	34.99	39.69±0.406	4.88	23.79
NDVI70_D	0.81±0.007	0.08	0.006	0.81±0.006	0.08	0.005
SC70_D	308.53±12.078	144.93	21005.9	362.38±17.309	207.71	43143.61
FLUOR70_D	0.62±0.005	0.06	0.003	0.68±0.003	0.04	0.001
Yield_D	2.13±0.079	0.95	0.904	2.66±0.098	1.18	1.38
TubNum_D	37.66±1.371	16.45	270.66	38.90±1.444	17.32	300.11
TubWeight_D	58.63±1.546	18.56	344.32	72.76±1.923	23.08	532.60
DryMatter_D	20.51±0.351	4.21	17.69	19.19±0.356	4.28	18.28
RS_D	0.16±0.007	0.09	0.007	0.16±0.008	0.10	0.009
Starch_D	13.55±0.360	4.32	18.68	12.20±0.366	4.39	19.3
Area_D	13.92±0.394	4.72	22.32	13.83±0.252	3.03	9.16
Perim_D	16.34±0.209	2.51	6.27	16.56±0.147	1.77	3.11



Supplementary Figure 5.1. a) Delta K values over 10 runs and b)bar plot displaying Q values obtained from STRUCTURE software in a population of 144 potato genotypes with a delta K=2.



Supplementary Figure 5.2. Linkage disequilibrium (LD) decay plot between r^2 and genetic distance.


SPAD50_C
0
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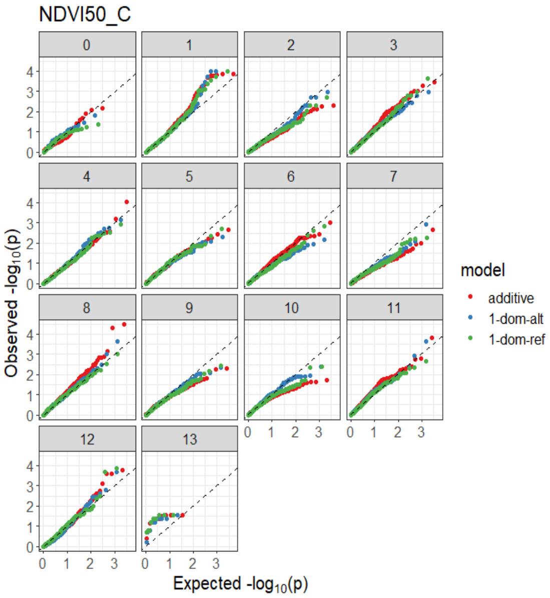
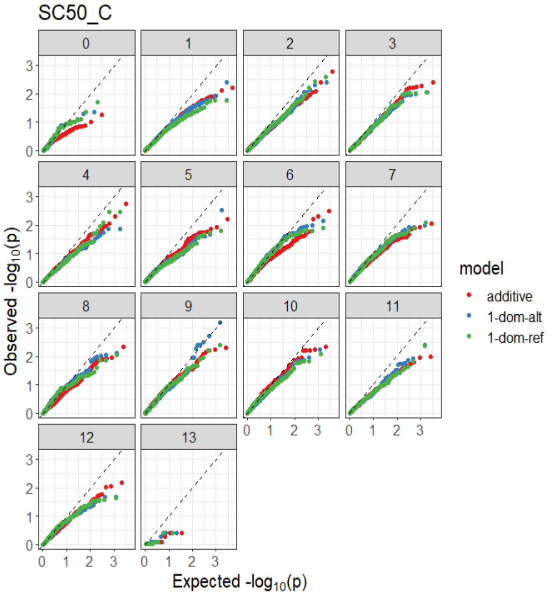
3
4
5
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


NDVI50_C
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


SC50_C
0
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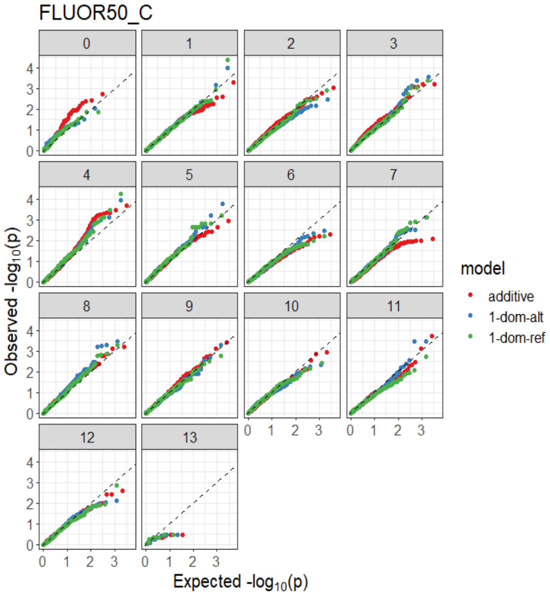
4
5
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


FLUOR50_C
0
1
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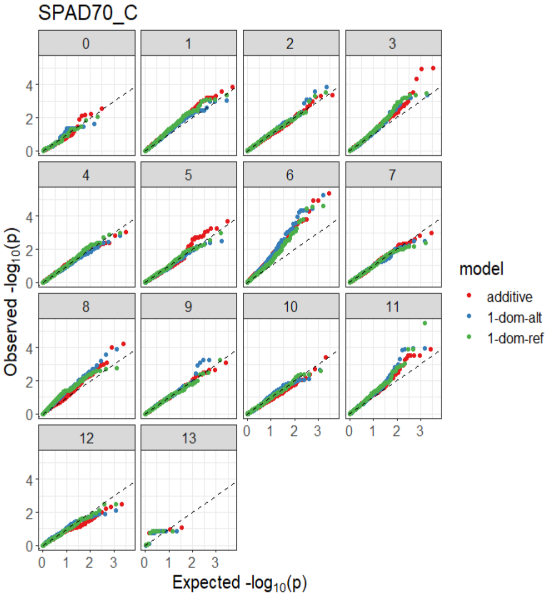
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


SPAD70_C
0
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4
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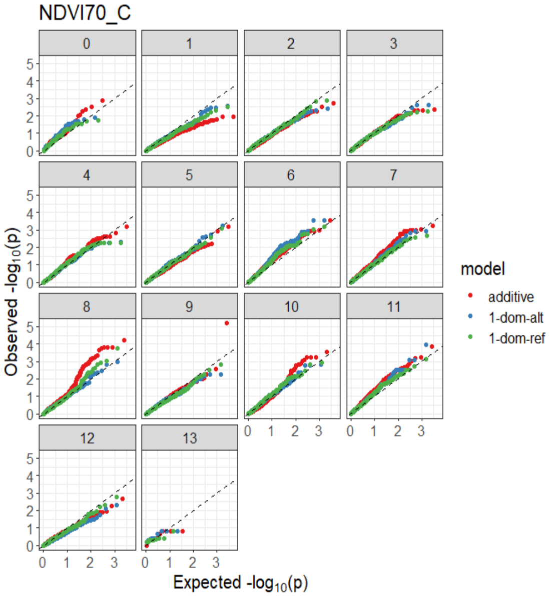
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


NDVI70_C
0
1
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3
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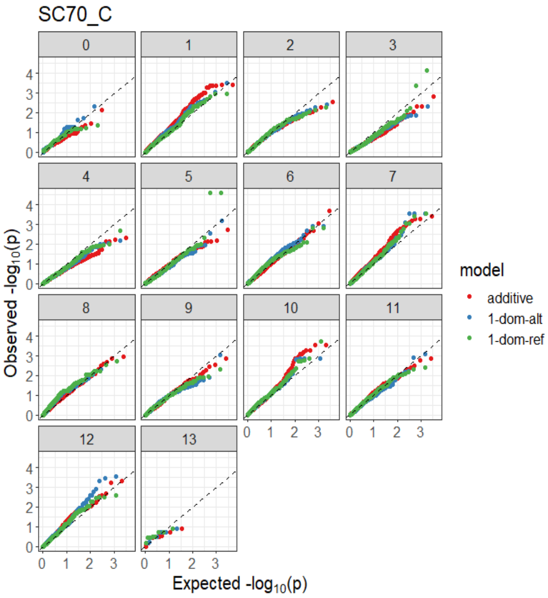
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


SC70_C
0
1
2
3
4
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6
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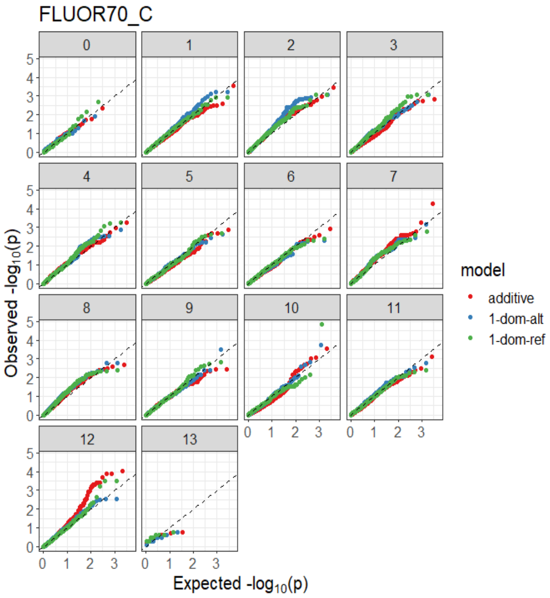
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


FLUOR70_C
0
1
2
3
4
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6
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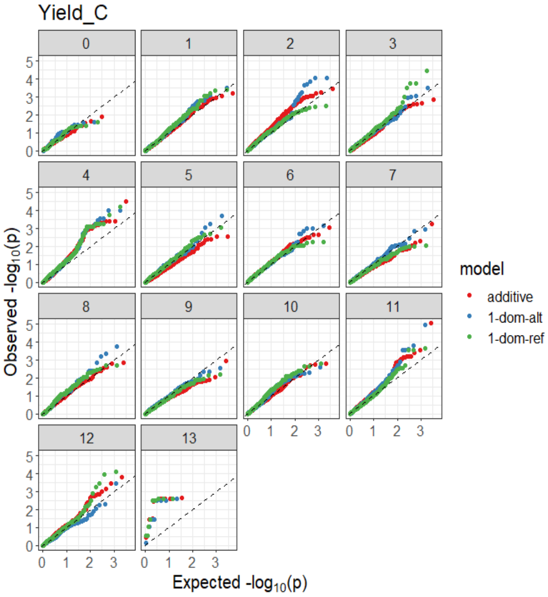
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


Yield_C
0
1
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7
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9
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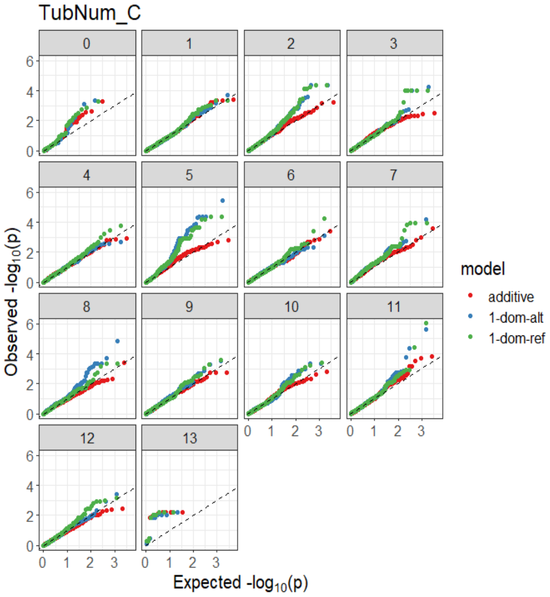
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


TubNum_C
0
1
2
3
4
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6
7
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9
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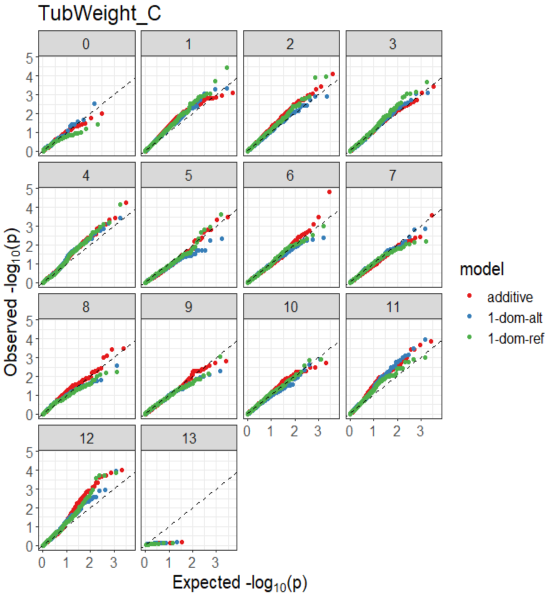
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


TubWeight_C
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4
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6
7
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9
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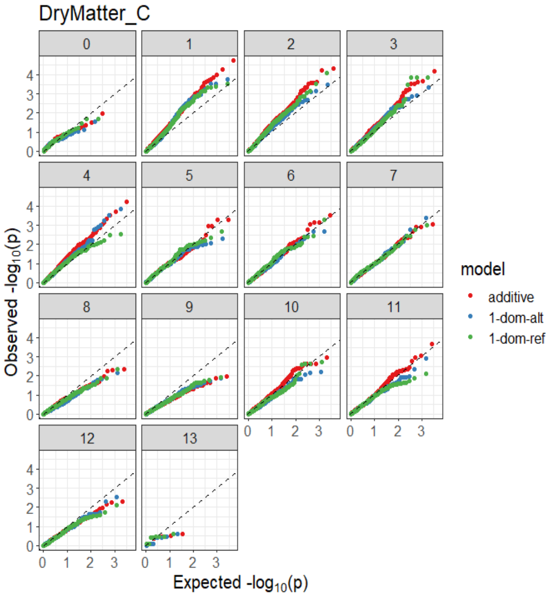
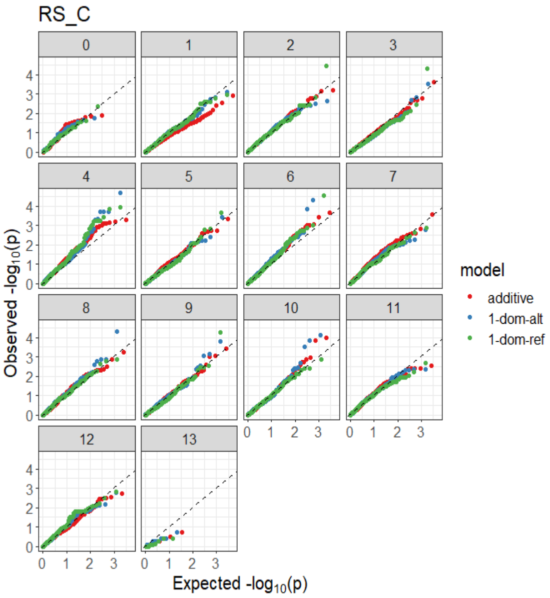
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


DryMatter_C
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Observed -log10(p)
Expected -log10(p)
model
additive
1-dom-alt
1-dom-ref


RS_C
0
1
2
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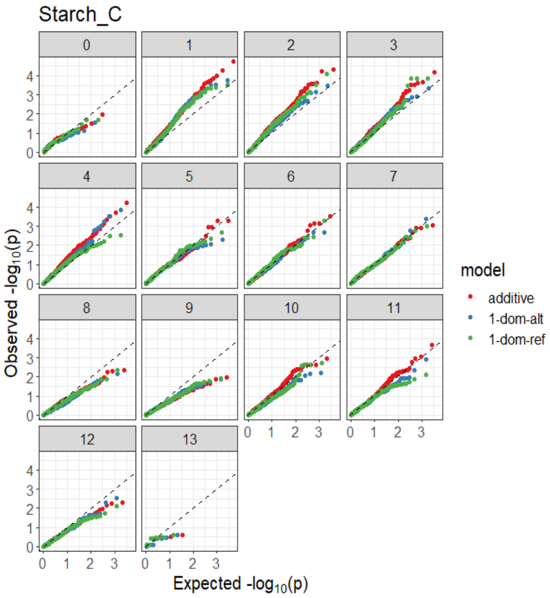
7
8
9
10
11
12
13
0 1 2 3 4
0 1 2 3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


Starch_C
0
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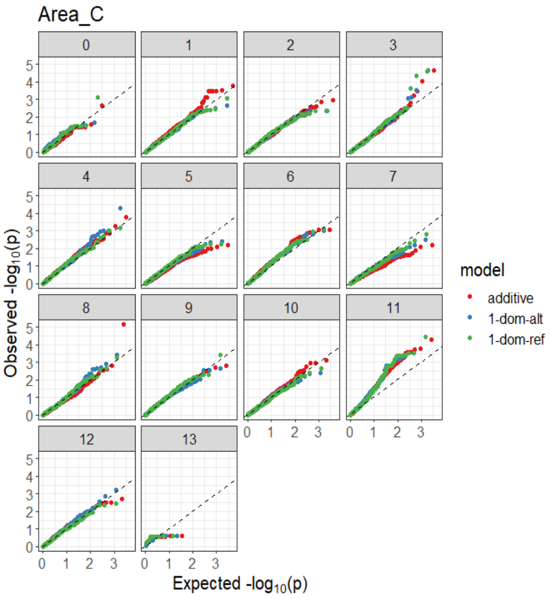
11
12
13
0 1 2 3 4
0 1 2 3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


Area_C
0
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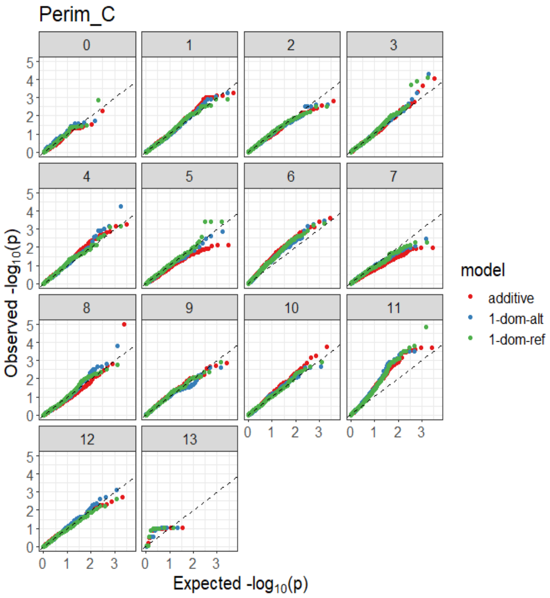
10
11
12
13
0 1 2 3 4 5
0 1 2 3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


Perim_C
0
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10
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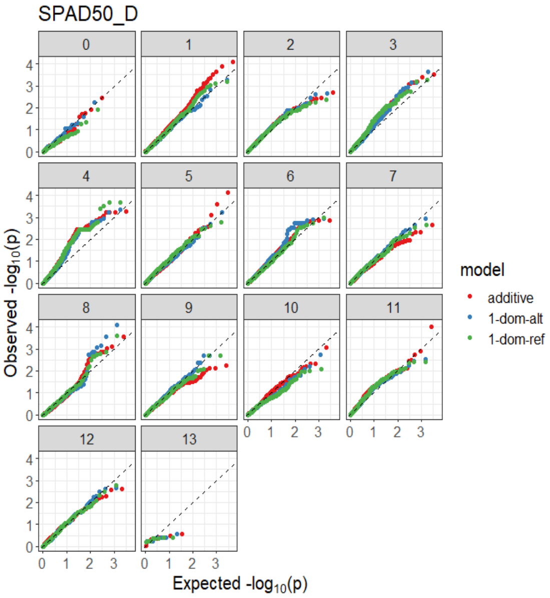
12
13
0 1 2 3 4 5
0 1 2 3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


SPAD50_D
0
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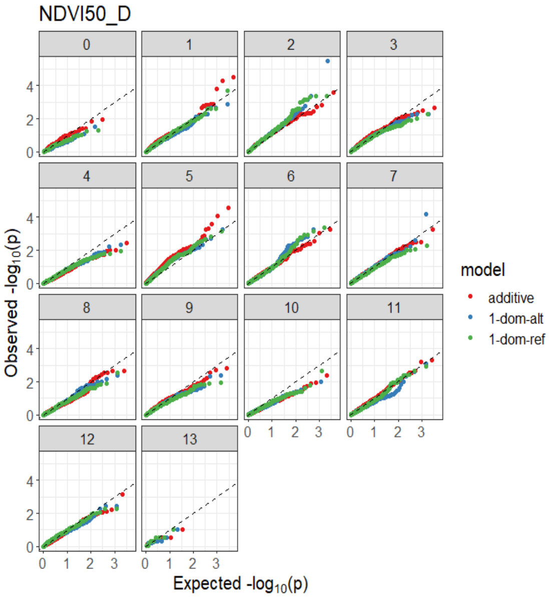
0 1 2 3 4
0 1 2 3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


NDVI50_D
0
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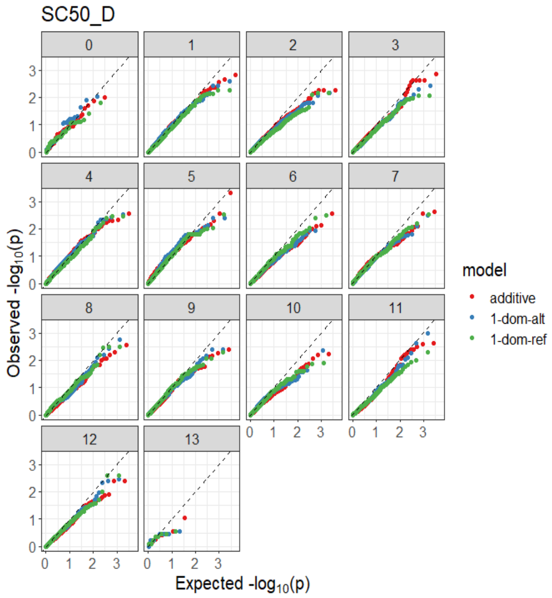
0 2 4
0 1 2 3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


SC50_D
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13
0
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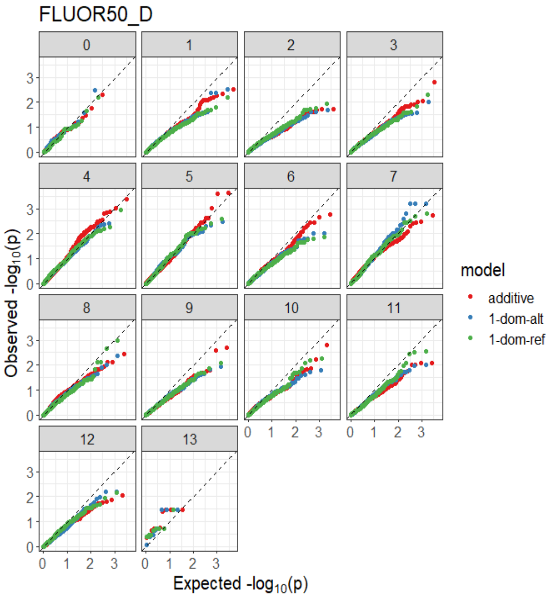
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


FLUOR50_D
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11
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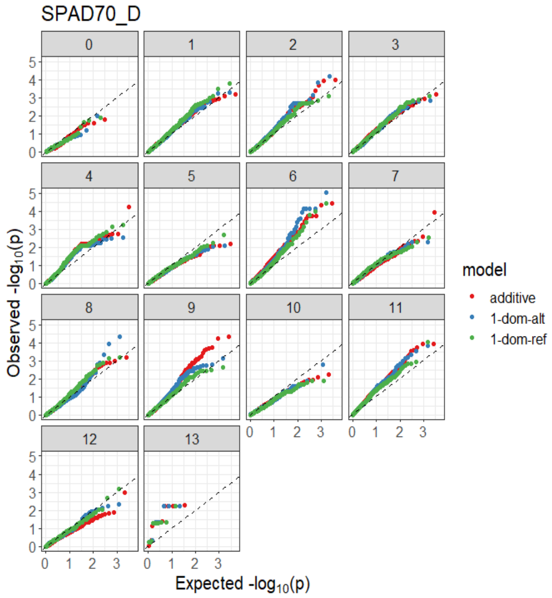
13
0
1
2
3
0
1
2
3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


SPAD70_D
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3
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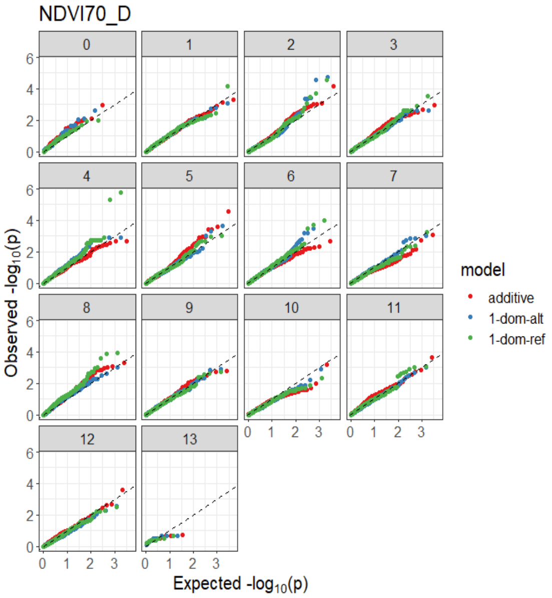
10
11
12
13
0
1
2
3
4
5
0
1
2
3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


NDVI70_D
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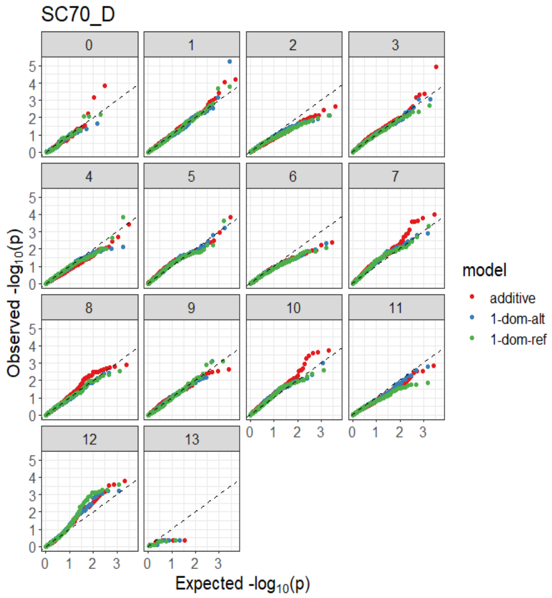
11
12
13
0
2
4
6
0
1
2
3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


SC70_D
0
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1
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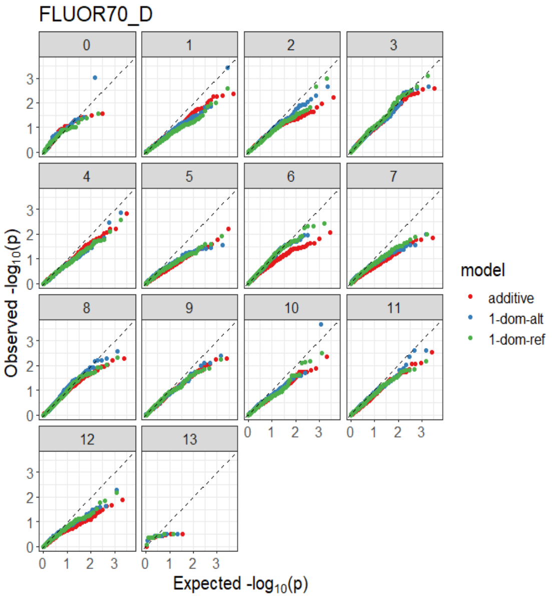
3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


FLUOR70_D
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12
13
0
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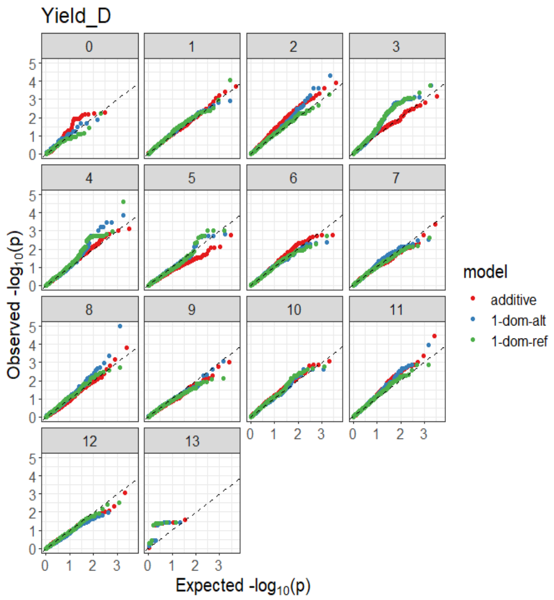
0
1
2
3
Observed -log$_{10}$(p)
Expected -log$_{10}$(p)
model
additive
1-dom-alt
1-dom-ref


Yield_D
0
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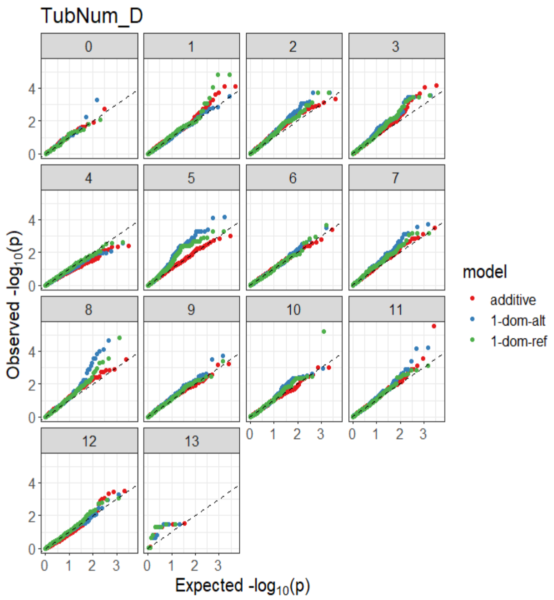
11
12
13
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
model
additive
1-dom-alt
1-dom-ref


TubNum_D
0
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2
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4
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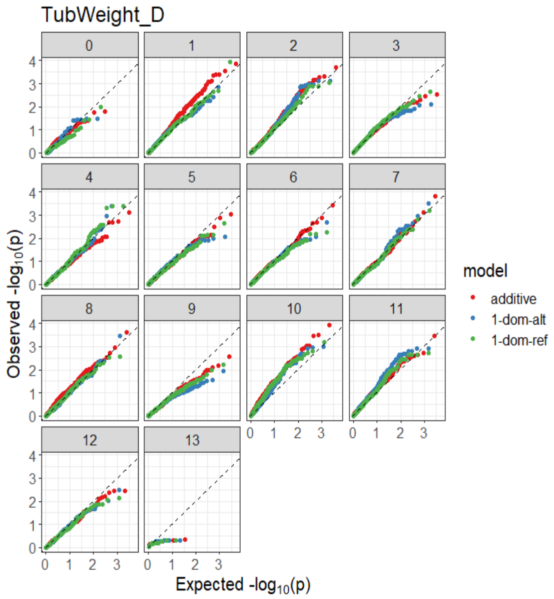
6
7
8
9
10
11
12
13
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
model
additive
1-dom-alt
1-dom-ref


TubWeight_D
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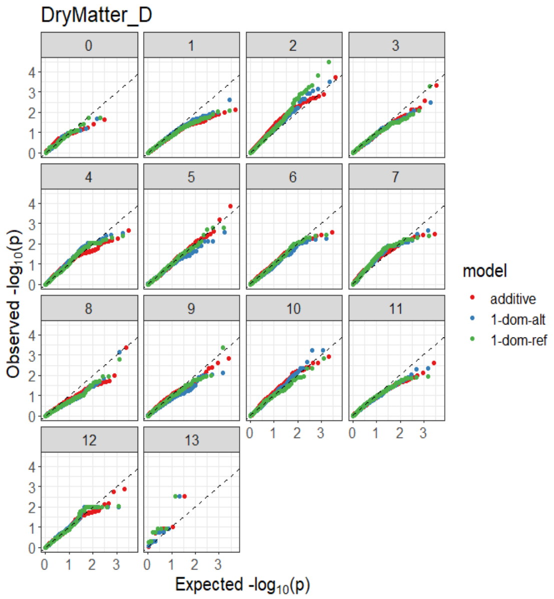
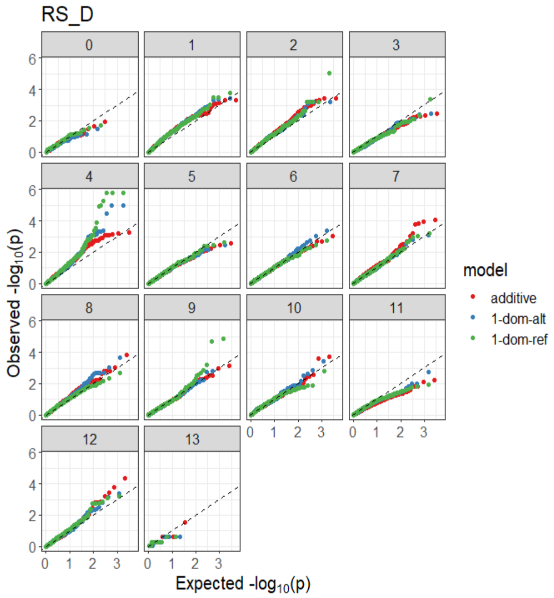
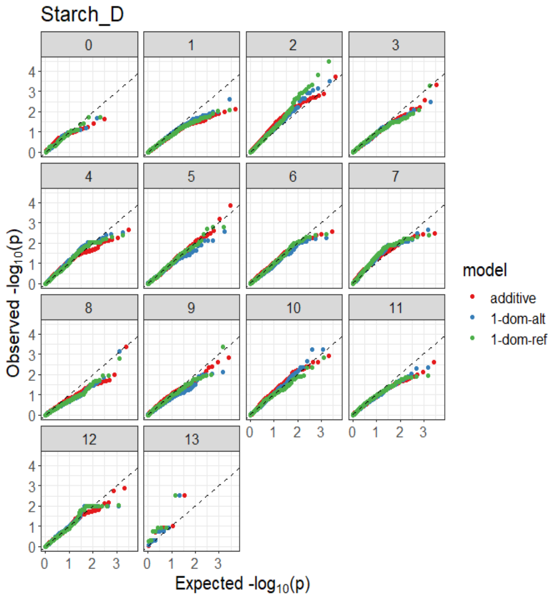
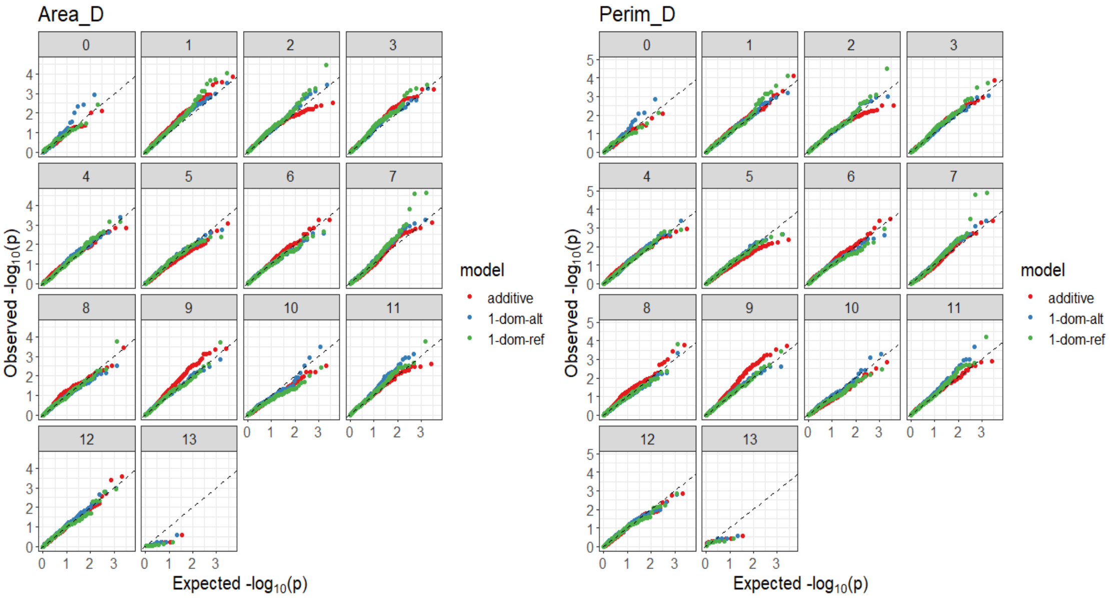
2
3
4
5
6
7
8
9
10
11
12
13
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
model
additive
1-dom-alt
1-dom-ref


DryMatter_D
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
model
additive
1-dom-alt
1-dom-ref


RS_D
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
model
additive
1-dom-alt
1-dom-ref


Starch_D
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Observed $-\log_{10}(p)$
Expected $-\log_{10}(p)$
model
additive
1-dom-alt
1-dom-ref



Supplementary Figure 5.3. Q-Q plots for all traits evaluated under control and drought stress conditions in 144 potato varieties. 1 to 12 refers to each of the 12 potato chromosomes, 0 are control markers that are not associated with any chromosome and 13 refers to the chloroplast.

References

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