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BAHÇEŞEHİR UNIVERSITY

TARGETED METABOLOMIC ANALYSIS OF CENTRAL CARBON METABOLISM ON MODEL PLANT *BRACHYPODIUM DISTACHYON* TO ELUCIDATE PHYSIOLOGICAL RESPONSE TO DROUGHT STRESS

Master's Thesis

ÖZGE TATLI

İSTANBUL, 2015

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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOENGINEERING

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Supervisor: Assist. Prof. Dr. Bahar SOĞUTMAZ ÖZDEMİR

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THE REPUBLIC OF TURKEY
BAHCESEHIR UNIVERSITY

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Assoc. Prof. Dr. Nafiz ARICA
Graduate School Director

I certify that this thesis meets all the requirements as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Serap AYDIN
Program Coordinator

This is to certify that we have read this thesis and we find it fully adequate in scope, quality and content, as a thesis for the degree of Master of Science.

Examining Committee Members	Signature
Thesis Supervisor Assist. Prof. Dr. Bahar SOĞUTMAZ ÖZDEMİR	________________________
Member Assoc. Prof. Dr. Serap AYDIN	________________________
Member Assist. Prof. Dr. İlkem Emrah NİKEREL	________________________

PREFACE

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ABSTRACT

TARGETED METABOLOMIC ANALYSIS OF CENTRAL CARBON METABOLISM ON MODEL PLANT *BRACHYPODIUM DISTACHYON* TO ELUCIDATE PHYSIOLOGICAL RESPONSE TO DROUGHT STRESS

Özge TATLI

Bioengineering

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Brachypodium distachyon which is native to the Mediterranean and Middle East Region, is a member of grass family (Poaceae) that also includes a number of cereals, providing the bulk of energy needed for human diet like wheat, barley, rye and oat. *B. distachyon*, has all desirable features to be a model organism, such as having a compact genome (272 Mb), 5 pair of chromosomes (2n=10), short generation time (~12 weeks), easy genetic transformation, self-fertility and simple growth requirements. The model plant properties and its close relationship to important crops, makes *Brachypodium* an attractive model plant for improvement of all temperate crop species, particularly the cereals. In recent years, in spite of an increasing interest in genomic and transcriptomic studies on *Brachypodium*, these are shown to be insufficient in understanding metabolic networks composed of biochemical reactions, mainly due to lack of correlation between mRNA and protein levels and the lack of enzymatic activity of proteins upon translation. Despite its importance of being the closest omic level to physiology, studies on metabolomics for *Brachypodium* are at their infancy. In order to elucidate the effect of drought stress on metabolism, the objective of this study is to set up quantitative metabolomics platform for *Brachypodium distachyon,* in particular, to optimize metabolite extraction protocols. For validation of extraction protocols, we used reporter metabolites (specifically ATP, glucose and overall starch) and focused on quantification of these, rather than overall,

high-coverage metabolome data. As such, we evaluated the effect of drought stress and genotypic differences based on these reporter metabolites in model plant *Brachypodium* using two different genotypes (each represented by 2 populations). Careful analysis of reporter metabolites revealed that there is no “one-protocol-fits-all”, meaning that there is a significant difference in metabolite recoveries upon different extraction protocols. Moreover, the extraction efficiency is also affected by different genotypes, which resultingly calls for optimization of protocols, tailor-made for a genotype. Quantitative analysis of reporter metabolites points to significant changes upon drought stress, yet the resulting change differs from one metabolism to another, i.e. energy metabolism responds differently than glucose and carbohydrate metabolism. ATP levels exhibited (ranging from $2.92\ 10^{-5} – 1.77\ 10^{-3}$ nmol/g FW (fresh weight)) a different trend between the extraction protocols. Glucose levels showed 1-4 fold increase upon drought stress. Starch levels in *Brachypodium* leaves, ranging between 0.02-2.7% FW, exhibited no significant or consistent change, as a response to drought stress. On the basis of these results, it can be concluded that the impact of drought stress on *Brachypodium* metabolism was significant and this study could aid for further metabolomics studies on drought stress response for the improvement of agriculturally important crops.

Key Words: *Brachypodium distachyon*, Metabolome analysis, Plant metabolite extraction, Drought stress, Model plant

ÖZET

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Özge TATLI

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Akdeniz ve Ortadoğu bölgesi yerel bitkisi olan *Brachypodium distachyon*, buğday, arpa, yulaf ve çavdar gibi beslenmede temel öneme sahip tahılların da içinde bulunduğu çim ailesinin (Poaceae) bir üyesidir. *B. distachyon*, kompakt bir genoma sahip olma (272 Mb), 5 çift kromozom (2n=10), kısa yaşam döngüsü (~12 hafta), kolay gen aktarımı, kendi kendine döllenmesi ve basit büyüme koşulları gibi özellikleri ile model organizma olmak için gereken bütün vasıflara sahiptir. Model bitki özellikleri ve önemli tahıllar ile yakın akrabalığı, *Brachypodium*'u tüm serin iklim tahıl türlerinin, özellikle de hububatların geliştirilmesi için dikkat çekici bir model bitki haline getirmektedir. Son yıllarda, *Brachypodium* üzerine birçok genomik ve transkriptomik çalışmalar yapılmasına rağmen, bu çalışmalar; mRNA ve protein seviyelerinin korelasyon göstermemesi, proteinlerin translasyon sonrası enzimatik olarak aktif olmaması gibi temel sebeplerle, biyokimyasal reaksiyonların oluşturduğu metabolik ağın anlaşılması konusunda yetersiz kalmaktadır. Fizyolojiye en yakın omik seviye olarak önemine rağmen, metabolomik alanındaki çalışmalar *Brachypodium* için nispeten başlangıç seviyesindedir. Bu çalışmanın amacı, kuraklık stresinin metabolizma üzerindeki etkisini aydınlatmak için *Brachypodium distachyon*'a uygun kantitatif metabolomik platformu oluşturmak, özellikle ekstraksiyon protokollerini optimize etmektir. Ekstraksiyon protokollerinin validasyonu için, raportör metabolitler (ATP, glikoz ve nişasta) kullanılmıştır ve yüksek kapsamlı metabolom verileri elde etmekten çok bu metabolitlerin kantitasyonu üzerine odaklanılmıştır.

Raportör metabolitler üzerinden, *Brachypodium distachyon*'da iki farklı genotip kullanılarak (her bir genotip 2 populasyon ile temsil edilir), kuraklık stresinin ve genotipik varyasyonun etkisi değerlendirilmiştir. Raportör metabolitlerin analizi ekstraksiyon protokollerine bağlı olarak verimin değiştiğini göstermiştir. Genotip farkı, protokollerin ekstraksiyon verimini önemli ölçüde etkileyerek, protokol optimizasyonunun ve genotipe spesifik ekstraksiyon yöntemlerinin gerekliliğini ortaya çıkarmıştır. Raportör metabolitlerin kantitatif analizi kuraklık stresine bağlı belirgin değişimlere işaret etmektedir. Ancak bu değişiklikler metabolizmalar arasında farklılık göstermektedir: enerji metabolizması glikoz ve karbonhidrat metabolizmasından farklı cevap vermektedir. ATP seviyeleri 2,92 10^{-5} - 1,77 10^{-3} nmol/g FW (yaş ağırlık) arasında değişmekte olup ekstraksiyon protokolleri arasında farklı bir eğilim bulunmuştur. Kuraklığa cevap olarak, glikoz seviyeleri 1-4 kat arasında artış göstermiştir. *Brachypodium* yapraklarındaki nişasta seviyeleri, 0.02-2,7% FW, kuraklık stresine cevap olarak belirgin ya da tutarlı bir değişiklik göstermemiştir. Elde edilen sonuçlar, kuraklığın *Brachypodium* metabolizması üzerinde belirgin bir etkisi olduğunu göstermiştir. Bu çalışma, tarımsal açıdan önemli tahılların geliştirilmesi için kuraklık stresine cevap üzerine ileride yapılacak olan metabolomik çalışmaları destekleyebilecek niteliktedir.

Anahtar Kelimeler: *Brachypodium distachyon*, Metabolom analizi, Bitki metabolit ekstraksiyonu, Kuraklık stresi, Model bitki

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ABBREVIATIONS

ATP	:	Adenosine tri-phosphate
CBF	:	C-repeat binding factor
CCM	:	Central Carbon Metabolism
ddH_2O	:	Double-distilled water
DNA	:	Deoxiribonucleic acid
E4P	:	Erytrose-4-phosphate
GC-MS	:	Gas Chromatography Mass Spectrometry
FAO	:	Food and Agricultural Organization
FT-ICR-MS	:	Ion Cyctron Resonance Mass Spectrometry
FW	:	Fresh Weight
GPS	:	Global positioning system
HPLC	:	High performance liquid chromatography
LC-MS	:	Liquid Chromatography Mass Spectrometry
Mbp	:	Megabase pair
MCF	:	Mitochondrial carrier family
MeOH	:	Methanol
mRNA	:	Messenger Ribonucleic Acid
miRNA	:	Micro RNA
MSTFA	:	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
NADH	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NBS-LLL	:	Nucleotide binding site leucine-rich repeat
NM	:	Nanometer
NMR	:	Nuclear magnetic resonance
PPM	:	Pulse per minute
RCA	:	Rubisco activase
RCF	:	Relative centrifugal force
RPM	:	Round per minute
RLU	:	Relative light unit
R5P	:	Ribose-5-phosphate

RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
TCA	:	Citric acid cycle
TF	:	Transcription factor
Xform	:	Chloroform

SYMBOLS

Fe	:	Iron
H_2O	:	Water
H_2O_2	:	Hydrogen Peroxide

1. INTRODUCTION

The world population is expected to exceed 9 billion by 2050 and global agricultural production needs to grow approximately by 70 percent in order to maintain the need for food supply (FAO 2009). Environmental disasters, climate change and other stress factors including cold, drought, heavy metals or salinity, with limited land resources available for agricultural expansion, significantly worsen the current situation. Improving plant adaptation to stress conditions is an urgent need to minimize yield loss of crops. Particularly, drought which can be described basically as water deficiency or incapable access to water is one of the greatest abiotic stress factors rapidly increasing and threatening agriculture on a global scale. In response to drought stress, plants activate a number of defense mechanisms to increase their drought tolerance. Complexity of stress tolerance limits the potential advances in this research area. Since model systems allow researchers to study complex processes, model plants have become valuable tools to study stress response.

Within the monocots, grasses that include cereal crops and forage grasses have major importance on human diet, as animal feed and capability of using as sustainable energy source. The major part of the human food supply is provided by the production of these cereals including rice, wheat, barley and oat. Hence, the agricultural and economical importance of grasses is immense and critical. *Brachypodium distachyon*, as member of grass family (Poaceae) has been proposed as a model plant for grasses since 2001 due to its well-suited model organism characteristics as well as its phylogenetic position.

Arabidopsis thaliana, as a dicot model plant, has mediated numerous scientific advances for almost 30 years; however, its applications are limited for monocot-specific researches because of the differences between monocots and dicots. So far, the studies on *Brachypodium* has shown that as a model plant it can fill the research gaps on monocots that *Arabidopsis* could not achieve.

Understanding the mechanisms of stress response is a complex process that needs all the outputs of multi-omics approaches. In order to figure out the response mechanisms of plants to drought stress, many studies have been performed on transcriptomic and proteomic levels. However, transcriptomic and proteomic approaches are insufficient in understanding the metabolite networks. Metabolomics links to the physiology with the integration of DNA, RNA, protein and metabolite analyses. Thus, this integrative information generates a more complete picture of the functional status of the living system.

Many investigations were carried out on abiotic stress response in plants at metabolomics level. However, a great major of these studies were performed on *Arabidopsis thaliana*. Although these studies mediated significant progress in understanding stress response mechanisms, monocot-specific mechanisms remained unclear. In *Brachypodium*, stress response studies are largely focused on genomic and transcriptomic research, but there is a lack of information about drought stress response studies at metabolomics level. In order to provide a complementary approach and bridge over physiology, it is required to get information on metabolomics level.

Briefly, metabolomics studies consist of two main stages: extraction of metabolites from cellular matrix and quantification of the extracted metabolites. For successful metabolome studies, metabolite extraction is a key step in providing useful and reliable data.

In our study, for evaluating alternative extraction protocols for metabolome analysis, for e.g. protocol validation, we used reporter metabolites (specifically ATP, glucose and overall starch) and focused on quantification of these, rather than overall, high-coverage metabolome data. As such, we evaluated the effect of drought stress and genotypic differences based on reporter metabolites including ATP, starch and glucose in model plant *Brachypodium distachyon* using two different genotypes (each represented by 2 populations).

Careful analysis of reporter metabolites revealed that there is no "one-protocol-fits-all", meaning that there is a significant difference in metabolite recoveries upon different extraction protocols. Moreover, the extraction efficiency is also affected by different genotypes, which resultingly calls for optimization of protocols, tailor-made for a genotype. Quantitative analysis of reporter metabolites points to significant changes upon drought stress, yet the resulting change differs from one metabolism to another, i.e. energy metabolism responds differently than glucose and carbohydrate metabolism.

In general, ATP levels (ranging from $2.92\ 10^{-5}$ – 1.77^{-3} nmol/g FW (fresh weight)) displayed a different trend between the extraction protocols. Glucose levels showed 1-4 fold increase upon drought stress. Starch levels in *Brachypodium* leaves, ranging between 0.02-2.7% FW, showed no significant or consistent change, as a response to drought stress. On the basis of these results, it can be concluded that the impact of drought stress on *Brachypodium* metabolism was significant and there is a necessity to develop tailor-made quenching and extraction methods for plant species, for further successful metabolome studies in plants.

The results obtained from this study will provide a basis for the construction of high-coverage metabolome platforms and potentially lead to more reliable metabolome studies in plants. Identification of novel drought-responsive metabolites based on reliable metabolome data and provision of integrated knowledge together with other 'omics' data might lead to further improvement of drought resistance in agriculturally important species.

2. LITERATURE REVIEW

2.1 MODEL ORGANISMS: WHY DO WE NEED THEM?

Biologists have searched for a tool to reduce the complexity of their particular biological area of interest for a better comprehension of basic biological principles. Therefore, they have fronted to relatively simple organisms for their scientific research. The need for model organisms in order to provide an improved understanding for the mechanisms underlying complex processes has been increased correlating with the increased interest on more specialized areas of biological studies (Vogel and Bragg 2009).

Model organisms differ from non-model organisms with some characteristics including being frequently small, hardy organisms with rapid life cycles and having small genomes as well as having simple growth requirements and ability to inbreed. The benefit and easy adoption of a model organism depend also on genome organization, exhibition of important biological traits, availability of whole genome sequence, efficient transformation procedure and mutagenesis strategies (Draper *et al*. 2001).

Figure 2.1: The most important genetic model organisms in use today. Clockwise from top left: yeast, fruit fly, *Arabidopsis*, mouse, roundworm, zebrafish

Reference: Model organisms [online] http://www.cubocube.com/dashboard.php?a=1179&b=1228&c=103 [Retrieve: 06.12.2014]

So far, model organisms like *Escherichia coli*, *Drosophila melanogaster* (fruit fly), *Mus musculus* (mice) or *Saccharomyces cerevisiae* (yeast) have contributed to numerous

scientific advances (Figure 2.1). Choosing an appropriate model organism is dependent on the question to be answered since each model organism has its own pros and cons. For example, yeast is a widely used model organism to study cell cycle because the cell cycle of yeast is similar to the cycle in humans and the proteins that are responsible for the regulation of cell cycle in yeast are homologous to those in humans. Similarly, while *Caenorhabditis elegans* (roundworm) and *Danio rerio* (zebrafish) are adopted for developmental biology, *Drosophila melanogaster* is an accepted model organism for genetics studies depending on their distinguishing properties (Vogel and Bragg 2009).

Since model systems allow researchers to study complex processes, especially due to their compact genome, model plants have become valuable tools to investigate broad problems in Plant Kingdom. In plant biology, common model species include *Arabidopsis thaliana*, *Oryza sativa* (rice) and *Brachypodium distachyon,* which represent well with desired attributes of a model organism. Among these model plant species, *Brachypodium distachyon* draw attention with sharing good synteny and orthology with crop species that provide food, fiber, feed and energy (Brkijacic *et al*. 2011) and proposed as a model plant, especially for monocotyledonous plants (monocots) by Draper *et al*. (2001).

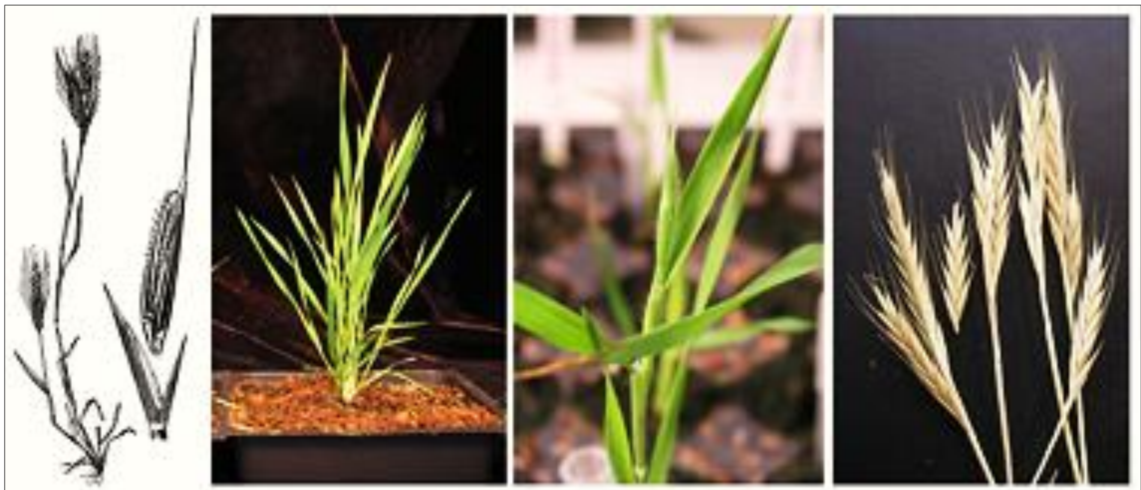
2.1.1 *Brachypodium distachyon*: Model for Temperate Grasses

Flowering plants are subdivided into two major groups including monocots and dicots. In evolutionary process, monocots and dicots were separated from each other 150 million years ago (Vogel and Bragg 2009). While grass family members such as wheat, oat and rye that possess one cotyledon have monocot-specific characteristics, sunflower and bean like plants with two cotyledons have dicot-specific characteristics. These two major groups differ from each other with seed, root and leaf structure and germination properties. *Arabidopsis thaliana,* as a dicotyledonous plant (dicot), has been widely used as a model organism in last 3 decades and mediated numerous scientific advances (Gutterson and Zhang 2004, Zhang *et al*. 2006, Zilberman *et al*. 2007, Century *et al*. 2008). However, though *Arabidopsis* is an excellent model species for dicots, its applications are limited for monocot-specific researches (Brkijacic *et al*. 2011). Due to the clear differences and divergence of some processes across monocots and dicots, a

closely related model plant is required since it may potentially reveal much more to understand mechanisms and processes in monocots, which are not conserved between these two subdivisions.

Within the monocots, grasses, which include temperate cereal crops like oat, rye, wheat and barley, and in particular forage grasses have major importance on human diet, as animal feed and are capable of using as sustainable energy source. Therefore, the agricultural and economical importance of grasses is immense and critical (Brkljacic *et al*. 2011, Ozdemir *et al*. 2008).

Figure 2.2: *Brachypodium distachyon*



Reference: Brachypodium Project [online] http://turboweed.org/Brachypodium.html [06.12.2014]

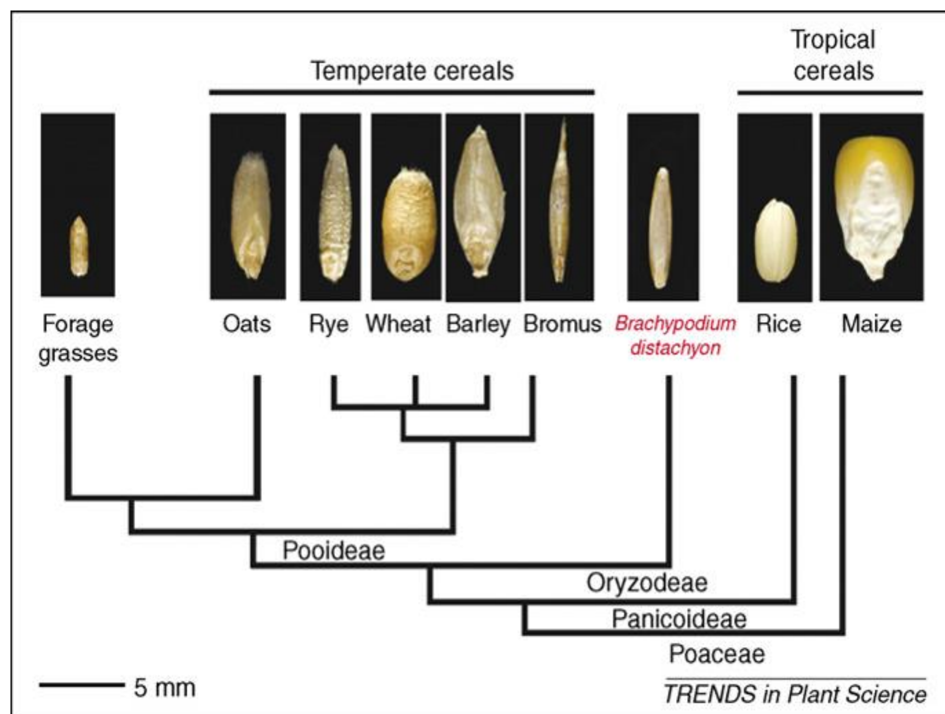
Brachypodium distachyon (*Brachypodium* hereafter, from the Greek *brachys* "short" and *podion* "a little foot") is an accepted model organism for temperate cereals (Figure 2.2). Its attributes are well suited to be a tractable model organism for grass species. *Brachypodium* has a compact genome (272 Mbp), easy genetic transformation, 5 pair of chromosomes (2n=10) in diploid accessions, short generation time (~12 weeks), small stature, lack of seed shattering, ability to self-pollinate and easily growing under simple environmental conditions (Draper *et al*. 2001, Ozdemir *et al*. 2008). Many applications like mapping studies, mutant analysis, and studies of natural diversity need the maintenance of large numbers of different genotypes. Availability of gene pool diversity, self-fertilization and lack of outcrossing of *Brachypodium* are useful attributes for

breeding and sustaining homozygous lines for these kinds of applications (Filiz *et al*. 2009a, Filiz *et al*. 2009b). Polyploid accessions of *Brachypodium*, like those in wheat, also may be useful for polyploidy studies. Moreover, it has one of the most compact genomes of grass family leading to be chosen as a candidate species among grasses for genome sequencing (Vogel and Bragg 2009). Today, whole genome sequence of *Brachypodium* is available (The international *Brachypodium* initiative 2010).

2.1.2 Phylogenetic Position and Geographical Distribution of *Brachypodium*

Brachypodium is a member of the subfamily Pooideae of the grasses (Poaceae), which also includes the grain crops barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereal* L.), and oat (*Avena sativa* L.). Close relationship of *Brachypodium* to these agriculturally and economically important cereals (Figure 2.3) makes it remarkable as a model organism for investigation of crop species, even more remarkable than rice (*Oryza sativa*) (Opanowicz *et al*. 2008).

Figure 2.3: Phylogenetic position of *Brachypodium distachyon*



Reference: Opanowicz, M., Vain, P., Draper, J., Parker, D., Doonan, J.H. 2008. *Brachypodium distachyon*, making hay with a wild grass, *Trends in Plant Science*, 13:172-177.

Similar to the ancestral range of small grain cereals, *Brachypodium* is native to the Mediterranean, the Middle East, South-West Asia and North-East Africa (Figure 2.4) (Opanowicz *et al*. 2008).

Figure 2.4: Geographical distribution of *Brachypodium*. The 2n=2x=10, 2n=2x=20, and 2n=4x=30, as red, yellow and blue dots, respectively.

Reference: Garvin, D.F. *et al*., 2008. Development of genetic and genomic research resources for *Brachypodium distachyon*, a new model system for grass crop research, Crop Science, 48:69–84.

Domestication of plants is an evolutionary process promoting phenotypic transition that enables the improvement of plant yield and productivity for human benefit. However, domestication of plants by human selection or selective breeding causes many traits to be exposed to genetic erosion due to loss of their ancestral genes and thus alters their physiological, developmental or morphological properties. *Brachypodium*, as a wild type species, can serve much about understanding biological basis underlying these traits, which may have been lost during domestication, compared to its domesticated relatives like wheat, oat and rye (Opanowicz *et al*. 2008, Bourguiba *et al*. 2012, Verelst *et al*. 2013, Tanackovic *et al*. 2014). In crops, gene variation is significantly reduced with the percentages of 34 in soybean, 38 in maize, 70-90 in wheat compared to their wild relatives (Bourguiba *et al*. 2012). *Brachypodium*, as a wild species, have the ability to show a strong variation for adaptation in terms of survival mechanism compared to domesticated cereals like wheat, oats and rye due to the absence of genetic erosion caused by

domestication process. Studies showed that *Brachypodium* shows high diversity in several agriculturally important traits and in this regard, it is a promising species to understand the mechanism underlying biological processes (Opanowicz *et al*. 2008, Bourguiba *et al*. 2012, Verelst *et al*. 2013).

2.1.3 Importance of *Brachypodium* Compared to Other Model Species

Plant biologists have widely adopted the small weedy species *Arabidopsis thaliana* as a generalized model plant. Due to its inherent biological properties *Arabidopsis* has risen up as an extremely powerful model system. However, *Arabidopsis* is not appropriate to deal with many aspects of cereal biology because of the 150-million-year biological distinctions between dicots and monocots. For instance, cell walls of grasses differ significantly from dicot cell walls in terms of types of the major structural polysaccharides, linkage form of these polysaccharides, and the abundance and importance of pectin, proteins and also phenolic compounds. Due to the distantly related phylogenetic position of *Arabidopsis* to Poaceae family, a grass model system is required for future investigations on cereals (Vogel and Bragg 2009). The differences between *Arabidopsis* and grass species in some areas such as mycorrhizal associations, grass plant architecture, grain characteristics, intercalary meristems, and developmental properties shows that *Arabidopsis* is not an appropriate model for the study of grasses. The immense importance of grasses as food, feed and, nowadays, as fuel, is a strong indicator of the need for the development of a truly tractable model system for the Grass Family (Koornneef and Meinkee 2010).

Rice has been promoted as another model plant for grass species. At a first glance, rice with its sequenced genome and large research community would seem to meet expectations instead of *Arabidopsis*. However, rice does not exhibit all relevant traits for temperate grasses. Although rice is an advantageous model for grass species with its sequenced genomes and large research network which accelerates the development of bioinformatics tools; its long life cycle and demanding growth requirements make it less preferable when compared to *Brachypodium* (Brkljacic *et al*. 2011). Besides, on closer inspection, rice is a poor choice for high-throughput genomic experiments in temperate

regions depending on the demanding growing conditions and large genome size. Because of the fact that rice is a semi-aquatic tropical grass, its practicality as a model for temperate grass species is limited especially in research studies when freezing resistance and vernalization are the target problems (Vogel and Bragg 2009). Depending on these issues and limitations of other model plant species, *Brachypodium* increasingly have gained favor as a model system for grass species, particularly the temperate grasses.

Table 2.1 Comparison of *Brachypodium* with other cereals and *Arabidopsis*

	Brachypodium distachyon	***Arabidopsis thaliana***	***Triticum aestivum***	***Zea mays***	***Oryza sativa***	***Hordeum vulgare***
Chromosome number	10(2n)	10(2n)	42(2n)	20(2n)	24(2n)	14(2n)
Genome size (1C)	272 Mb	164 Mb	16700 Mb	2400 Mb	441 Mb	5000 Mb
Reproductive strategy	Self-fertilizing	Self-fertilizing	Self-fertilizing	Cross-polination	Self-fertilizing	Self-fertilizing
Life cycle (weeks)	10-18	10-11	12 (spring)	10+	20-30	16+
Height at maturity (m)	0.3	0.2	Up to 1	Up to 2	1.2	Up to 1.2
Transformation	Facile	Facile	Possible	Facile	Facile	Facile
Growth requirements	Very simple	Very simple	Simple	Simple	Specialized	Simple

Reference: Opanowicz, M., Vain, P., Draper, J., Parker, D., Doonan, J.H. 2008. *Brachypodium distachyon*, making hay with a wild grass, *Trends in Plant Science*, 13:172-177.

2.2 STRESS FACTORS

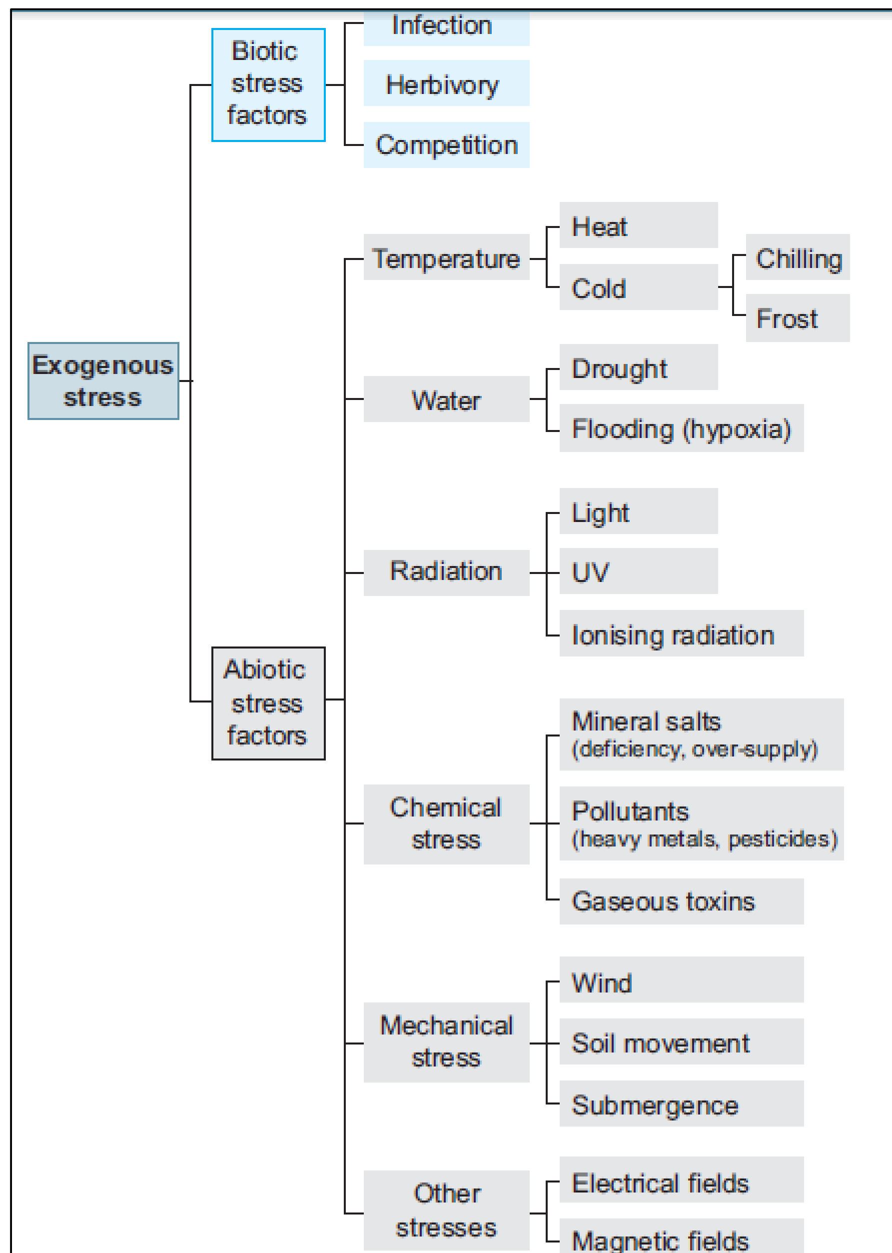
Grand challenges in plant science are increasing quality, yield and ability of adaptation to extreme environmental conditions. The global population is expected to exceed 9 billion by 2050 and in order to feed this population, agricultural production needs to grow globally by 70% (FAO 2009). In addition to the increase in world population, major part of cultivation areas in the world (96.5%) of cultivation areas in the world is exposed to different kinds of stress factors (Velthuizen *et al*. 2007). Abiotic stress factors including climate change, drought, salinity and heavy metals are major obstacles for increasing agricultural production.

Due to the complex nature of the biology of cells, any environmental stimulus can activate a number of pathways of cellular signaling and interactions which are evolved mechanisms in order to allow to appropriate stress responsive biological processes in the cell (Wrzaczek *et al*. 2011). As in all living organisms, there is a range of optimal environmental conditions for normal growth and development of plants. Optimal growth

and ‘physiological normal type’ that reveal the potential performance of plants are achieved under optimal quantity or intensity, like in greenhouse. However, optimal conditions almost never occur for plants and due to their sessile nature, plants are much more exposed to environmental changes which affect their growth and development negatively and prevent to maximize their physiologically achievable performance. Therefore, plants are forced to develop more efficient and complex strategies by evolutionary process than animals and humans in order to respond and adapt to conditions, which deviate from the optimal. These deviations that hinder normal growth and developmental process of plants are called ‘stress’ and they often cause losses in productivity. Inducers creating stress in plants are divided into two major groups (Figure 2.5) including abiotic stress factors (e.g. drought, salinity) and biotic stress factors such as pathogens (Schulze *et al*. 2005).

Due to the serious negative effect of stress, a better understanding of plant physiological responses to the abiotic and biotic stressors is required. Many scientific efforts have been focused on understanding stress response and identification of specific genes, proteins or metabolites that are responsible for tolerance phenotypes. Stress can be thought as an environmental stimulus or influence, which, deviate from the normal range of homeostatic control in the organism of interest. Exceeded stress tolerance activates a number of mechanisms at molecular, biochemical and physiological levels. Study of stress responses show that there is much crosstalk among signaling networks during specific stress response (Fraire-Velàzquez *et al*. 2011). Thus, plants may respond to stress perception by an initial global response and follow with specific stress responses. According to these studies, stress initially induces a number of signaling cascade reactions, which activate reactive oxygen species production, hormone accumulation (e.g. accumulation of abscisic acid, jasmonic acid, salicylic acid) and kinase reactions. This signaling mechanism leads to the expression of stress-responsive genes and creates the overall stress response of the plant. After the control of stress, organism establish a new physiological state and reestablish its homeostasis until the stress is terminated, returning plant to its original state or putting it to another physiological state. However, plants have to cope with this stress and respond to several stressors during almost whole year (Wrzaczek *et al*. 2011).

Figure 2.5: Exogenous stress factors



Reference: Schulze,E.D., Beck,E. and Müller-Hohenstei, K. 2005. Stress Physiology, *Plant ecology,* Germany pp: 8.

2.2.1 Biotic Stress Factors

The term 'biotic stress' refers to interactions with other organisms including infection of organism or mechanical damage to a particular part of the organism, caused by bacteria, viruses, fungi, nematodes, parasites, insects and weeds. These stress factors lead to severe

yield losses and therefore to economic problems. For instance, cereals are constantly endangered because of these biotic stress factors such that, only one of them, the hemibiotrophic fungus, which causes maize anthracnose characterized by necrotic tissue is responsible for annual losses of up to one billion dollars in the U.S (Frey *et al*. 2011).

2.2.2 Abiotic Stress Factors

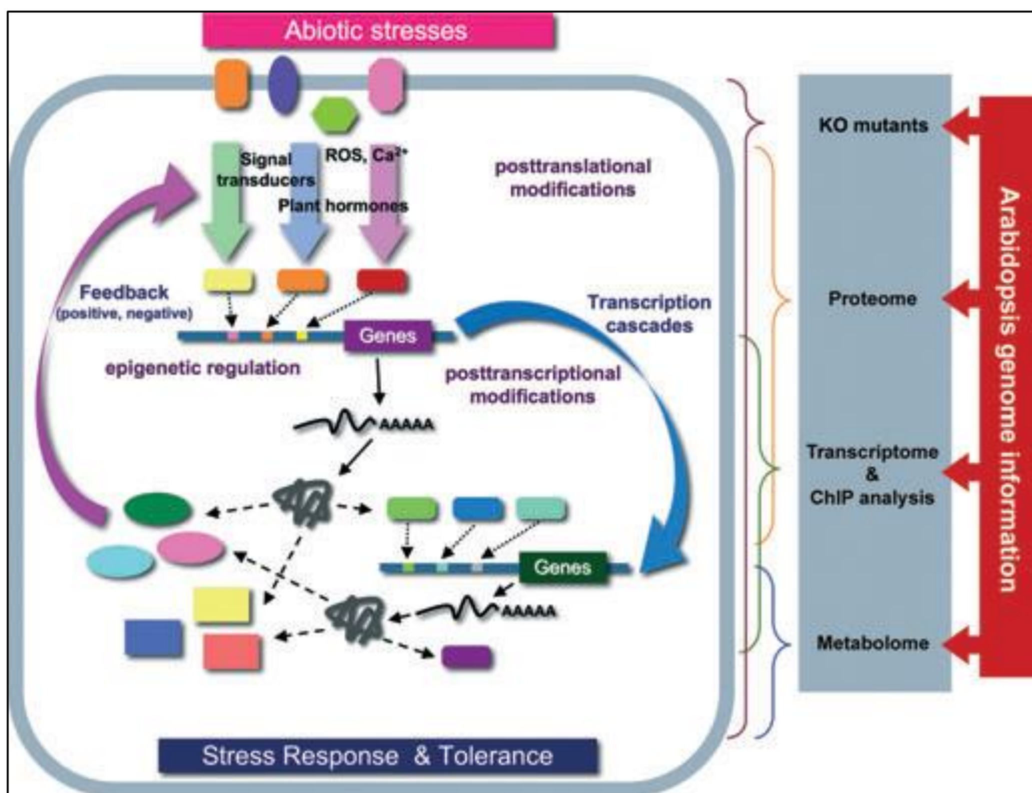
The term ‘abiotic stress’ includes several stressing factors caused by environmental conditions (non-living factors). Some of these factors can be categorized as low- and high-temperature, drought, high salinity, oxidative stress, heavy metals, changes in photoperiod, light intensity and quality, nutrient abundance and starvation, freezing and flooding, air and soil pollution. Intergovernmental Panel of Climate Change reports show that these stress factors will increasingly effect crop production and in the near future, understanding abiotic stress responses will be one of the urgent challenges in plant science (Hirayama and Shinozaki 2010, Wrzaczek *et al*. 2011).

Recent years have seen great progress for our understanding of abiotic stress adaptation mechanisms. Physiological and molecular analyses, which are performed specially in *Arabidopsis thaliana,* provided a general picture of abiotic stress responses in plant species. Depending on the diversity of abiotic stresses, it can be assumed that a strong specific component is required for every individual stress responses. On the other hand, a striking common component takes place in the general response to all type of abiotic stresses. In substance, all abiotic stress factors cause the induction of reactive oxygen species (ROS) production, however they are generated in different forms and in different subcellular compartments. Although ROSs are assumed as only damaging molecules that attack to vital cell components such as fatty acids, proteins, and nucleic acids in the cell, they primarily take place in stress response regulation mechanism acting as signaling molecules and also in the regulation of developmental processes (Genga *et al*. 2011, Wrzaczek *et al*. 2011).

2.2.3 Stress Response in Plant Metabolism

The process of metabolism involves the breakdown of complex organic constituents of the organism with the release of energy for use in various functioning mechanisms. Miscellaneous compounds that take part in or are formed by these metabolic reactions are called metabolites. A series of biochemical reactions occurring within a cell are referred as metabolic pathways (Yordanov *et al*. 2003).

Figure 2.6: Overall stress response mechanism



Reference: Hirayama, T. and Schinozaki, K. 2010. Research on plant abiotic stress responses in the post-genome era: past, present and future, *the Plant journal*, 61, 1041–1052.

The metabolome of a living system refers to the complete set of metabolites produced by the living system during its life cycle under all distinct environmental conditions. These metabolites, the end-products of gene expression and protein activity, are products which modulate cellular processes through several ways like feedback inhibition or as signal molecules. Metabolism and the metabolite content of cells, tissues and organs of an

organism are eventually affected by the changes in gene transcription and protein amount. Metabolomics facilitates direct information about how these changes in mRNA or protein are associated with the changes in phenotype with the integration of DNA, RNA, protein and metabolite analyses. Thus, this integrative information generates a more complete picture of the functional status of the living system (Dixon *et al.* 2006, Genga *et al*. 2011, Nikerel *et al*. 2012).

In plants, in response and adaptation to stress factors (Figure 2.6), the changes which occur in metabolome composition include the following: i) catalytic activity of enzymes which are responsible for the production of specific metabolites ii) production of abnormal compounds or production of compounds in abnormal level iii) regulation of metabolite concentration to provide homeostasis iv) synthesis of compounds involved in stress tolerance. Individual plant species contain tens of thousands different metabolites. These distinct metabolites produced by Plant Kingdom are estimated to be between 100000-200000 (Pichersky and Gang 2000, Camilla and Roessner 2013). The metabolic composition of plants is likely to be altered during different physiological and environmental conditions and can also reflect different genetic backgrounds. Plant metabolomic studies are important for understanding physiological effects of stress factors that are one of the most important agricultural issues. Recently, studies on metabolic changes occurring during stress response process of plants play an important role for metabolome analysis (Rizhsky *et al*. 2004, Valliyodan and Nguyen 2006). In stress response process, the main purpose is identification and quantification of metabolites involved in stress tolerance.

2.2.4 Drought Response

Plants are constantly exposed to drought stress that is one of the greatest abiotic stress factors (Figure 2.7), rapidly increasing and threatening agriculture on a global scale (Ding *et al*. 2013). Drought can be described basically as water deficiency or incapable access to water. In response to drought stress, plants activate a number of defense mechanisms to increase their drought tolerance and survival rate. Defense mechanisms for drought include numerous metabolic changes like osmotic regulation, antioxidant production,

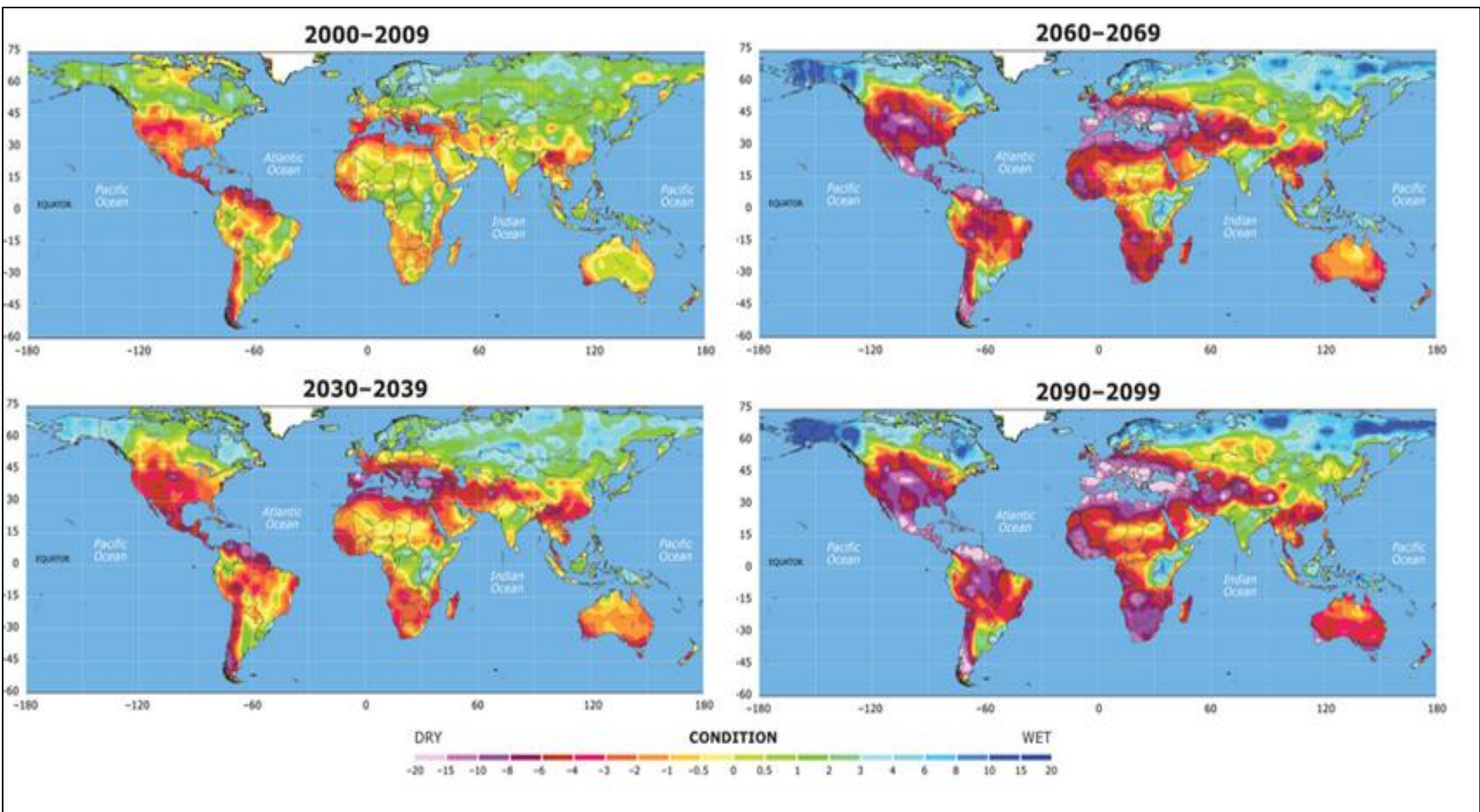
eliminating unwanted reaction products as well as morphological changes like reduced stomatal conductivity and leaf width (thus reduced rate of photosynthesis), developed root system (Bartels and Sunkar 2005, Ding *et al*. 2013).

In non-tolerant species, drought causes significant yield losses in plants. Therefore, improving drought stress tolerance is quite important but it is limited by the complexity of stress tolerance.

Physiological studies on drought stress have shown that many metabolites including sugars (e.g sucrose, glucose, trehalose, sorbitol), sugar alcohols (e.g. mannitol), aminoacids (e.g. proline), amines (e.g. glisine, betaine, polyamine) accumulate significantly in response to stress conditions and act as antioxidant, osmolite or low-molecular-weight chaperon molecules so that they stabilize the photosystem II complex (protein complex that takes place in photosynthesis) to regulate photosynthesis, protect enzyme and protein structure, maintain membrane integrity and scavenge the reactive oxygen species (Taji *et al*. 2002, Bartels and Sunkar 2005). The introduction of genes responsible for the synthesis of proline, betaines, and polyols into plants results in increased abiotic stress tolerance (Rathinasabapathi, 2000; Chen and Murata, 2002). Many other compounds are available for drought stress response. They may function like signaling molecules or chelating agents that bind toxic metals and ions as well as redesigning lipids to optimize membrane structure and fluidity.

However, non-tolerant plants in comparison to stress-resistant ones do not have the ability to synthesize these metabolites in a significant concentration. Thus, illumination of stress response mechanism, metabolic networks and biochemical composition is important to improve adaptation and resistance of plants to stress conditions (Seki *et al*. 2007).

Figure 2.7: Current Palmer Drought Severity Index [PDSI] 2000-2009. A reading of -4 or below is considered extreme drought.



Reference: Drought may threaten much of globe within decades [online] https://www2.ucar.edu/atmosnews/news/2904/climate-change-drought-may-threaten-much-globe-within-decades [Retrieve: 28.12.2014]

2.2.4 Stress Response Studies in Model Plants

As with all organisms, plants thrive within a range of environmental conditions that are optimal for their growth and development. They should adapt themselves each time to these conditions whenever they deviate from the optimal. Since these deviations cause losses in plant productivity, significant efforts have been made for the illumination of stress response mechanisms. Understanding the mechanism of stress response is a complex process that needs all the outputs of multi-omics approaches: genomics, transcriptomics, proteomics and metabolomics.

Many investigations have been carried out on abiotic stress response in plants at metabolomics level. However, a great major of these investigations has been performed on *Arabidopsis thaliana*. Studies with *Arabidopsis* on temperature stress can be classified as; low-temperature stress (Cook *et al*. 2004, Vannini *et al*. 2004, Hannah *et al*. 2006, Guy *et al*. 2008, Maruyama *et al*. 2009, Korn *et al*. 2010), high-temperature stress (Kaplan *et al*. 2004, Gray and Heath 2005, Larkindale *et al*. 2005) and moderate temperature stress (Morsy *et al*. 2007, Laura *et al*. 2010, Du *et al*. 2010) responses and these studies mediated significant progress in understanding temperature stress response mechanism. Also, a number of metabolomic studies have been performed in *Arabidopsis thaliana* on other abiotic stress factors that has crucial importance on plant productivity including drought stress (Rhizsky *et al*. 2004, Mattana *et al*. 2005, Bouchabke-Coussa *et al*. 2008, Lugan *et al*. 2009) and salt stress (Kim *et al*. 2007, Kempa *et al*. 2008, Kanani *et al*. 2010).

On the other hand, in *Brachypodium distachyon*, stress response studies are largely focused on genomic and transcriptomic research. This can be exemplified by the identification of stress responsive miRNAs (Wei *et al*. 2009, Zhang *et al*. 2009, Jeong *et al*. 2013), but stress response studies on transcriptome level in *Brachypodium* are not limited with only stress-related miRNAs. For example, putative transcription factors (TFs) were identified and stress-related TFs (Mochida *et al*. 2011, Tripathi *et al*. 2012) were predicted. In another study, molecular and functional characterization of cold-responsive C-repeat binding factors (CBF) (Ryu *et al*. 2014) was carried out in *Brachypodium*. Besides, superoxide dismutases, which are antioxidant proteins

catalyzing dismutation of free radicals to hydrogen peroxide and dioxygen, thus taking place in stress response mechanism were screened (Filiz *et al*. 2014). On proteome level, proteome and phosphoproteome changes induced by salt stress were carried out on *Brachypodium* leaves resulting in the identification of 60 differentially expressed unique proteins (Lv *et al*. 2014). It has been shown that beyond genome and proteome, 57 different accession of *Brachypodium* showed different physiological responses to drought stress (Luo *et al*. 2011).

The studies on particularly drought stress response in *Brachypodium* include, genomic and transcriptomic studies as in the case of other stress response studies. Expression of nucleotide binding site leucine rich repeat (NBS-LRR) genes in drought stress, which might potentially benefit to identify new target stress resistance genes for the improvement of disease-resistant crops were analyzed (Tan and Wu 2012). Drought-responsive miRNAs were identified (Budak and Akpinar 2011, Bertolini *et al*. 2013), the physiological and molecular effects of drought stress on leaves were studied (Verelst *et al*. 2013) and also the effect of altered expression of rubisco activase (RCA) on CO_2 fixation under drought and salt stress (Bayramov and Guliyev 2014) was examined. However there is no study on drought stress response at metabolomics level in *Brachypodium*. Metabolomics links to the physiology with the integration of DNA, RNA, protein and metabolite analyses. In order to provide a complementary approach and bridge over physiology, it is required to get information on metabolomics level.

2.3 CARBON METABOLISM

Cellular metabolism refers to the set of chemical reactions that occur in biological systems to maintain life and central carbon metabolism (CCM) is core part of the cellular metabolism Glycolysis and the tricarboxylic acid (TCA) cycle, known as CCM, are responsible for the production of accessible energy and primary metabolites of other metabolisms (Figure 2.8). These intermediate metabolites of CCM are involved in the synthesis of RNA/DNA, fatty acids, amino acids like molecules that are the main building blocks of the cell. Therefore, CCM is critical to plant growth and development.

Central carbon metabolism consisting of glycolysis, the oxidative pentose phosphate pathway and the TCA cycle is the “metabolic backbone” of the cell and ubiquitous throughout nature. The essentiality of central carbon metabolism depends on energy supply in heterotrophic cells and a wide range of other physiological functions. All of the reducing energy, ATP and crucial precursor molecules used in wide range of other cellular pathways are produced by these reactions. In central carbon metabolism, complex series of enzymatic steps are used to convert simple sugars into metabolic precursors. These produced precursors are crucial for generation of the entire biomass of the cell (Fernie *et al*. 2004).

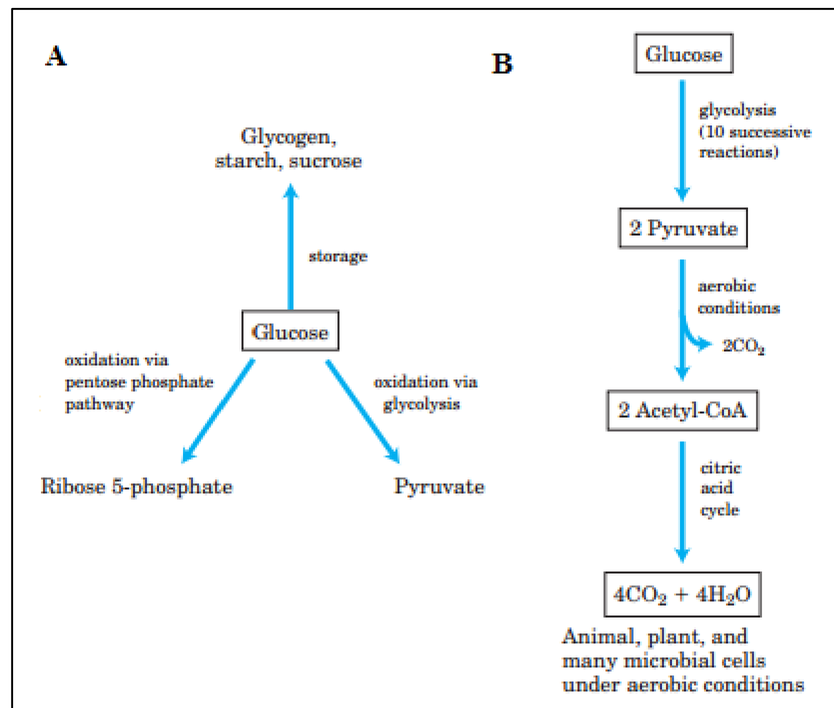
2.3.1 Glycolysis

Glycolysis is the pathway for the catabolism of glucose and can be defined basically as a metabolic pathway that converts glucose into pyruvate involving ten enzyme-catalyzed reactions. Every glucose molecules, which is converted to pyruvate, generates two molecules of ATP. In the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase, reducing energy is produced by glycolysis, in the form of NADH (reduced Nicotinamide Adenine Dinucleotide). Glycolytic reactions, which are catalyzed by the enzymes 3-phosphoglycerate kinase and pyruvate kinase, produce ATP. However, in most of the cells, these glycolytic reactions are only a minor source of ATP whereas major source of ATP of the cell is provided by mitochondrial metabolism (Rawn 1989, Fernie *et al*. 2004).

Glycolysis and the oxidative pentose phosphate pathways gives precursors for many biosynthetic pathways. For example, while aminoacid alanine is synthesized from pyruvate, aspartate is synthesized from oxaloacetate. Phosphoenolpyruvate (a glycolytic pathway intermediate) and erythrose 4-phosphate (a pentose phosphate pathway intermediate) are together the precursors for the amino acids phenylalanine, tyrosine, and tryptophan. These aminoacids are involved in the synthesis of many compounds including lignin and suberin, the phytohormone indoleacetic acid, and flavonoids. Five-carbon intermediates from the oxidative pentose phosphate pathway are precursors for the

synthesis of nucleotides. Dihydroxyacetone phosphate leads to the formation of glycerol backbone for lipid synthesis (Smith *et al*. 2009).

Figure 2.8: Glucose and pyruvate in CCM. A) Major pathways of glucose utilization. B) Catabolic fates of the pyruvate formed in glycolysis.



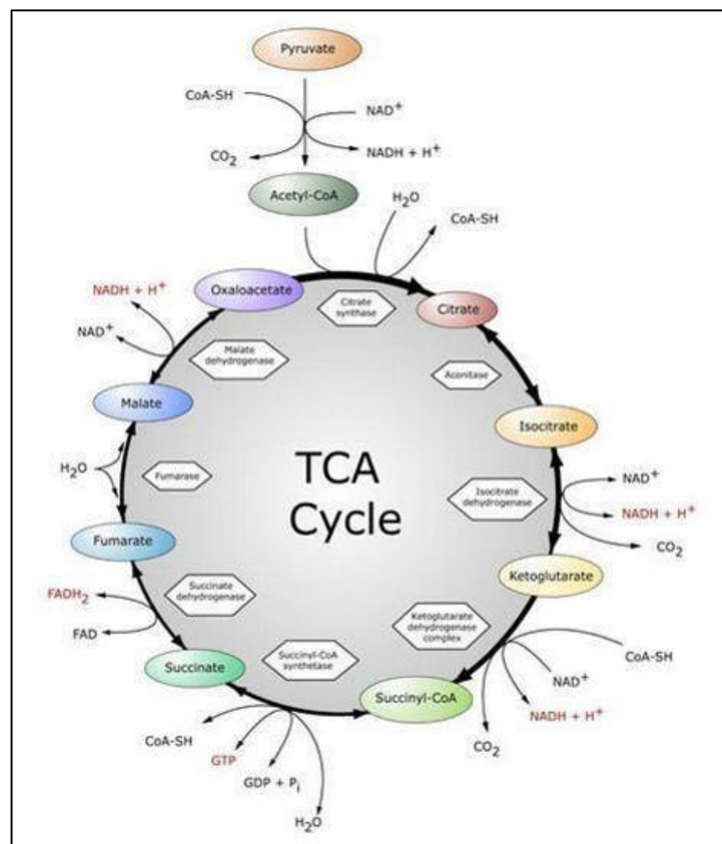
Reference: Nelson D.L., Cox, M.L. 2004. Glycolysis, Gluconeogenesis, and the Pentose Phosphate Pathway, *Lehninger, Principles of Biochemistry*, USA:W. H. Freeman, pp. 523-525.

2.3.2 Pentose Phosphate Pathway

Pentose phosphate pathway (PPP) which is also called “phosphogluconate pathway” or “hexose monophosphate shunt” is a biochemical pathway parallel to glycolysis that reduces NADPH and pentose sugars (5-carbon sugars). Although PPP involves oxidation of glucose, its primary role is anabolic rather than catabolic. The non-oxidative synthesis of pentose sugars follows the initial phase (the oxidative phase) where NADPH is produced. This biochemical pathway occurs in the cytosol in many organisms, however, in plants, most steps of PPP take place in plastids.

The primary results of the pathway are a) reducing molecules are generated in NADPH form which is used in reductive biosynthesis reactions within the cells such as fatty acid synthesis, b) ribose-5-phosphate (R5P) that is used in the synthesis reactions of nucleotide and nucleic acids is produced, c) erythrose-4-phosphate (E4P) that participate to aromatic amino acid synthesis is produced, d) aromatic amino acids, which are precursors for many biosynthetic pathways, like the lignin in wood, are produced (Kruger and Schaewen, 2003, (Smith *et al*. 2009).

Figure 2.9: TCA cycle



Reference: Fates of pyruvate [online] https://biochemtastic.wordpress.com/2013/04/07/fates- of-pyruvate/ [Retrieved on 30.11.2014]

2.3.3 TCA (Tricarboxylic Acid) Cycle

TCA cycle is also called Krebs cycle or citric acid cycle. It is the process that the pyruvate produced in glycolysis is oxidized to high-energy phosphate compounds. Basically, a

two-carbon unit derived from pyruvate is condensed with a four-carbon unit. Oxidative decarboxylation of the resulting six-carbon unit then generates reducing energy in the form of NADH and $FADH_2$, produces CO_2, and regenerates the four-carbon unit to allow a further turn of the cycle.

In the cytosol, phosphoenolpyruvate (PEP) is converted to malate or pyruvate by TCA (tricarboxylic acid) cycle. These organic acids, malate and pyruvate, are then carried into the mitochondria, through specific members of the mitochondrial carrier family (MCF). In mitochondria, they are subsequently interconverted with a series of reaction, producing energy and reducing power (Figure 2.9) (Smith *et al*. 2009). Thus, the production of energy for cellular metabolism is achieved.

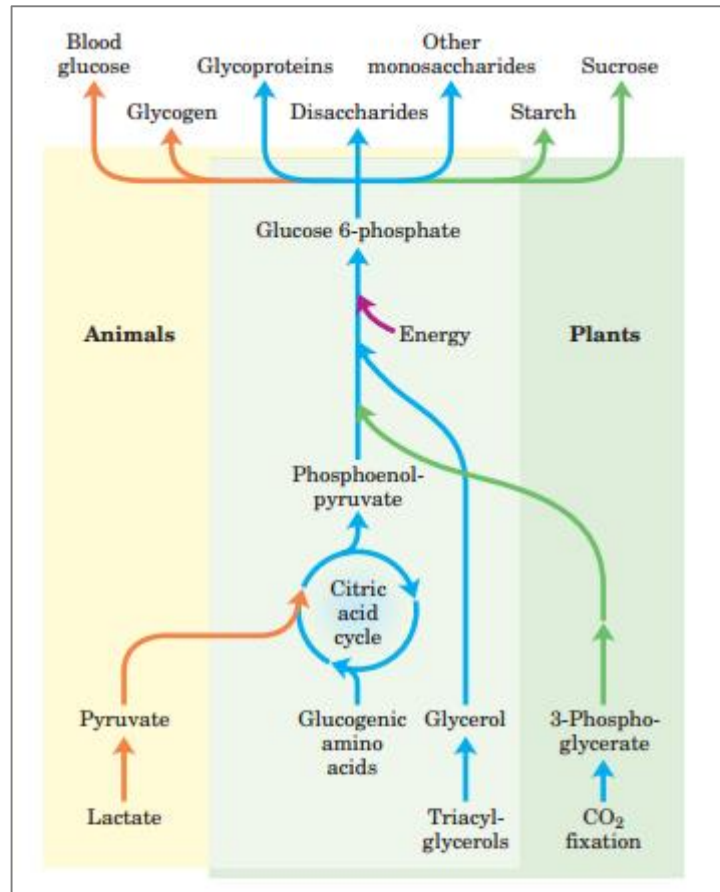
2.3.4 Carbohydrate Metabolism

Plants synthesize carbohydrates from carbon dioxide and water by photosynthesis and these carbohydrates store the absorbed energy internally (Figure 2.10). The most important carbohydrate, used as energy source, is glucose. Glucose is a monosaccharide and it is stored as starch in plants as primary carbohydrate, which is a polymer of glucose.

By glycolysis and oxidative pentose phosphate pathway, carbohydrate is converted into pyruvate and malate. These are catabolic pathways. They convert carbon in reduced form. This reduced form is taken from the atmosphere by photosynthesis (Dashty 2013).

Under certain physiological conditions, glycolysis can operate in reverse direction and forms hexose sugar from phosphoenolpyruvate. This is known as gluconeogenesis. In addition to these mechanisms, glyoxylate cycle is another way to form carbohydrates. Inıtially, acetyl CoA and oxaloacetate are condensed to form citrate. Then citrate is isomerized to isocitrate and it is cleaved into succinate and glyoxylate. Citric acid cycle and gluconeogenesis together can form carbohydrates from succinate which is a byproduct. (Brownleader *et al*. 1997).

Figure 2.10: Carbohydrate synthesis from simple sugars



Reference: Nelson D.L., Cox, M.L. 2004. Glycolysis, Gluconeogenesis, and the Pentose Phosphate Pathway, *Lehninger, Principles of Biochemistry*, USA:W. H. Freeman, pp. 523-525.

2.4 METABOLOMICS

The metabolome refers to the complete set of metabolites produced by the organism under all possible conditions. Metabolomics is an analytical approach that allows to comprehensively analyzing this large scale of metabolites, identifying and quantifying every individual metabolite. It contributes the understanding of the complex biochemical interactions in biological systems.

Metabolomics approaches are metabolite profiling, targeted metabolite analysis and metabolite fingerprinting. Targeted metabolite analysis focuses on the analysis of selected known metabolites providing quantitative information. It is carried out for a few metabolites that have a role in a particular pathway (i.e. phytohormones) and requires determining accurate concentrations of these metabolites.

Metabolite profiling provides the analysis of a large set of metabolites in a sample that are structurally related to each other in general like organic acids, carbohydrates. It allows for a semi-quantitative measurement, namely it aims to identify concentration levels comparatively instead of accurate concentrations.

Metabolic fingerprinting allows for rapid characterization of specific metabolic state of the biological system in a nonspecific manner to compare (Camilla and Roessner 2013). Platforms that are used for metabolome analysis are as follows; NMR (nuclear magnetic resonance), FT-ICR-MS (Fourier transform ion cyclotron resonance mass spectrometry), LC-MS (liquid chromatography-mass spectrometry) or GC-MS (gas chromatography-mass spectrometry) (Camilla and Roessner, 2013). With its high separation power and repeatability, GC-MS based metabolomics is accepted as a golden approach for metabolite profiling depending on the changes of environmental conditions. It provides a 'snapshot' for the current metabolic situation of the cell.

2.5 STATE OF ART

A great major of the studies in drought-stress response of plants were performed on genomics and transcriptomics. There is a lack of information about drought stress response studies at metabolomics level. In order to provide an integrated knowledge together with other 'omics' data and bridge over physiology, it is required to get information on metabolomics level. Since metabolomics studies consist of two main stages: extraction of metabolites from cellular matrix and quantification of the extracted metabolites, metabolite extraction is a key step in maintaining useful and reliable data for successful metabolome studies.

Efficient metabolite extraction protocols provide a basis for the construction of high-coverage metabolome platforms, thus lead to more reliable metabolome studies in plants. Therefore, in this study, reporter metabolites (specifically ATP, glucose and overall starch) were used for evaluation and validation of alternative extraction protocols for metabolome analysis, with a focus on quantification of these, rather than overall, high-coverage metabolome data. As such, the effect of drought stress and genotypic differences based on reporter metabolites including ATP, starch and glucose in model plant *Brachypodium distachyon* using two different genotypes (each represented by 2 populations) were evaluated.

3. MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Kits

All chemicals used in this research are listed in Table 3.1.

Table 3.1: List of chemicals

Chemical product	Trademark	Catalog No.
Chloroform (HPLC-grade)	Merck	102444.2500
Methanol (HPLC-grade)	Sigma-Aldrich	34860
Absolute Ethanol	Sigma-Aldrich	32205
Liquid nitrogen		
HPLC-grade water	Sigma-Aldrich	270733
Acetonitrile	Sigma-Aldrich	34967
Iodine solution	Sigma-Aldrich	318981

Reference: This table was prepared by Özge TATLI.

3.1.2 Equipments

All equipments used in this research are listed in Table 3.2.

Table 3.2: List of equipments

Equipment	Trademark
Luminoskan	Thermo, USA
Thermoshaker	Biosan, LATVIA
Centrifuge	Beckman Coulter, USA
Micropipettes	Eppendorf, GERMANY
Deepfreeze (-20)	Thermo, USA
Deepfreeze (-80)	Thermo, USA
Vortex mixer	Thermo, USA
Concentrator	Eppendorf, GERMANY

Spectrophotometer	Perkin Elmer, USA
HPLC	Agilent, USA

Reference: This table was prepared by Özge TATLI.

3.2 METHODS

3.2.1 Plant Material

Brachypodium distachyon seeds used in this study were kindly provided by Prof. Dr. Metin Tuna, Namık Kemal University, Department of Field Crops, Tekirdağ, Turkey. Two different genotypes of *Brachypodium*, each represented by 2 different populations, were used in this study.

Genotype 23 sampled from Şile/Ağva region at an elevation of 441 ft. According to GPS location, its coordinates are as follows: N41°05.347' (Latitude), E029°45.249' (Longitude) (Figure 3.1). Populations 12 and 13 of this genotype (labelled as 23-12 and, 23-13) were used as plant material. The other genotype, genotype 45, sampled from Kahta/Adıyaman region at an elevation of 665 ft. According to GPS location, its coordinates are as follows: N37°44'2.3" (Latitude), E38° 32' 0.2" (Longitude). Populations 6 and 11 of this genotype (labelled as 45-6, and 45-11) were used as plant material.

Figure 3.1*: Brachypodium distachyon;* Genotype 23 (left) and Genotype 45 (right), under normal conditions.

Reference: This photograph was taken by Özge TATLI.

3.2.2 *Brachypodium distachyon:* Growth Conditions and Stress Treatment

Brachypodium seeds were stratified at 4 ºC for 7-10 days in dark between moist filter papers in petri plates. After cold treatment, they were kept under light at room temperature for 5-7 days. Following germination, they were transferred to peat-soil mixture in plastic pots and grown under controlled environment (16/8 h light/dark photoperiod at 22/24 °C, relative humidity 60-70%, and a photosynthetic photon flux of 320 μmol m^{-2} s^{-1} at canopy height provided by fluorescent lamps) in the greenhouse (Ozdemir and Budak 2008, Filiz *et al.* 2009) until the vegetative plants were developed. Drought stress was given to plants by withholding water for 7-10 days until yellowing of the leaves were observed morphologically while the control groups were watered every day until sample collection.

Figure 3.2: *Brachypodium distachyon*; Genotype 23, normal conditions (left) and Genotype 45, drought conditions (right).

Reference: This photograph was taken by Özge TATLI.

3.2.3 Plant Metabolite Extraction

3.2.3.1 Methanol extraction

For sample collection, leaves were cut using scissors. Harvested plant material was frozen immediately in liquid nitrogen (-196°C) to stop all enzymatic processes and avoid degradation and modification of metabolites in the sample. Harvesting was performed at

the same time of the day for all samples to minimize biological variations due to daily changes of metabolism.

Extraction protocol includes quenching the metabolism by liquid nitrogen and extraction in two-steps involving methanol and water. After all insoluble compounds are removed (e.g. protein, starch, cell wall), supernatants are combined for large metabolome coverage.

For each sample, up to 30 mg (FW) frozen tissue was homogenized to a fine powder by grinding with a mortar and pestle using liquid nitrogen for the solvent to penetrate the tissue to extract metabolites effectively. Homogenized tissue was taken into a labelled 2-mL microcentrifuge tube.

Polar metabolites were extracted from homogenized samples according to the protocol of Roessner and Dias (2012) with some modifications. 500 µL methanol (GC-grade, >99.9 percent purity) was added into the homogenized samples in round-bottom shaped microcentrifuge tubes and vortexed. Samples were then shaken for 15 min at 70°C in Thermoshaker and centrifuged for 15 min at 14000xg. Supernatant was then transferred into a clean microcentrifuge tube and 500 µL double distilled water (ddH_20) was added onto the pellet. After centrifugation at 14000xg for 15 min, two supernatants were combined. Last step was repeated 2 more times to understand whether metabolites could be further collected. Combined supernatants collected from each step were labelled as sp1, sp12, sp123 and sp1234. For ATP assay, 30 µl aliquots were taken from each sample and remaining supernatants were dried under speed vacuum concentrator. Dried sample were used for glucose analysis in HPLC. All samples were stored in -80°C until used.

3.2.3.2 Methanol/Chloroform extraction

Preparation and homogenization of samples were performed as described in Section 3.2.2.1. Metabolites were extracted from homogenized samples according to protocol of Fiehn (2006).

1 mL cold extraction solvent consisting of chloroform:methanol:water (1:2.5:1 v/v/v, GC grade, >99.9 percent purity) was added to each homogenized sample. Samples were shaken for 5 min at 4°C and then centrifuged at 20.800 rcf for 2 min. Supernatants were collected and transferred to a clean microcentrifuge tube. 400 µL distilled water was added into each sample and vortexed for 10 seconds. Again samples were centrifuged at 20.800 rcf for 2 min. Upper phases (a mixture of methanol:water containing polar phase) are collected and then transferred into a clean microcentrifuge tube. For ATP assay, 30 µL aliquots were taken from each sample and remaining supernatants were dried under speed vacuum concentrator and stored in -80°C until used for glucose analysis in HPLC. Lower phases (a mixture of chloroform:methanol containing lipophilic phase) were dried under speed vacuum concentrator. Dried samples were stored in -80°C until used for the determination of starch level.

3.2.4 Assay for Determination of ATP Level

ATP was used as a reporter metabolite to evaluate the efficiency of metabolite extraction protocols, the effect of drought stress and response of different genotypes. ATP in each supernatant was quantitatively detected *via* luminometer (Luminoskan Ascent Thermo).

The quantitative detection of ATP is based on following reaction:

$$ATP + D\text{-}Luciferin + O_2 \rightarrow Oxyluciferin + AMP + Ppi + CO_2 + LIGHT$$

Since ATP is the limiting component in this reaction, the intensity of the emitted light is proportional to ATP concentration. Measurement of the light intensity *via* a luminometer provides direct quantitation of ATP (Lundin and Thore 1975).

For preparation, lyophilized reagent containing the enzyme was reconstituted using reconstitution buffer. A “blank”, containing rL/L Reagent and sample buffer (methanol:water mixture for this study) was run in the assay to determine the amount of “background” RLU that would be subtracted from the sample RLU. Sample RLU values should be corrected for possible sample buffer inhibition of light output when converting RLU to ATP mole. This is done by constructing an ATP standard curve. Therefore, a

standard curve was constructed from a series of ATP concentrations with ten times dilution. 5 μL of ATP Standard, diluted to the proper concentrations was added to a 96-well plate and assayed using rL/L Reagent according to the luminometer protocol. After the construction of ATP standard curve, each sample was measured via luminometer by adding 5 μL sample and 50 μL Reagent into the well of 96-well plate to determine the amount of ATP in the sample.

In order to rule out matrix effect, increasing concentrations of the analyte (ATP standard) was added to individual aliquots of the selected samples. Graphs for each sample were plotted according to determined RLU values to analyze the response of samples to matrix effect.

3.2.5 Colorimetric Assay for Determination of Starch Content

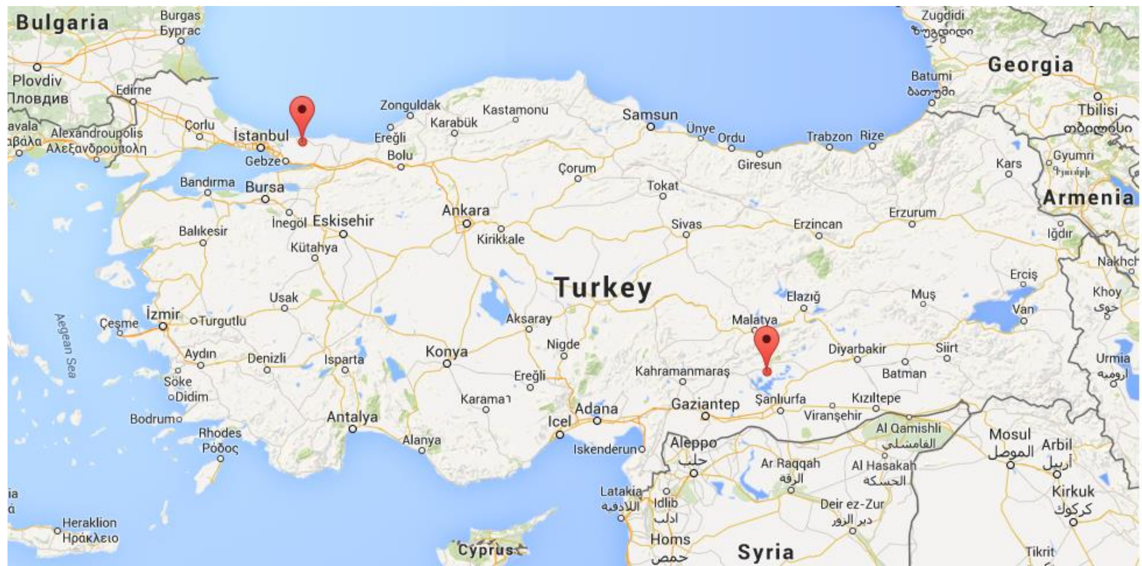
Starch was used as another reporter metabolite to evaluate the effect of drought stress and response of different genotypes. Vacuumed (dried) lower phases of the samples which were extracted by methanol:chloroform extraction were dissolved in 100 μL water to determine starch content. Starch calibration curve was prepared with a serial of dilutions with known starch concentrations (3:1.5:0.75:0 g/L) and after mixing with 1.5 mL iodine solution absorbances were read at 550 nm in spectrophotometer. Since starch contents of the samples stay below the limit of detection of the assay, increasing concentrations of starch solution (3:1.5:0.75 g/L) was added to individual aliquots (20 μL) of each sample to achieve detectable level. Absorbances were read. Then, a calibration curve was created for each sample and starch concentration in the sample was determined from the intercept of this calibration curve.

3.2.6 HPLC Analysis for Determination of Glucose Content

Samples which were dried and stored for determination of glucose content were dissolved in 200 μL acetonitrile:HPLC water mixture (1:1 v/v) for HPLC analysis. Similar to the determination of starch content, increasing concentrations of glucose standard (0:5:10:20 g/L) was added to individual aliquots of each sample. Glucose concentration analysis was

performed by Agilent 1100 High Performance Liquid Chromatography (HPLC) system with refractive index detector using the Zorbax Carbohydrate Analysis Column 4.6x250 mm (Agilent, USA). Conditions were set as 1400 mL/min flow rate, 30°C, eluent 75 (Acetonitrile):25 (HPLC water). A calibration curve was constructed for each sample. Glucose concentration in the sample was determined from the intercept of this calibration curve.

Figure 3.3: Location of Genotype 23 (Şile/Ağva) and 46 (Kahta/Adıyaman).



Reference: This image was prepared by Özge TATLI.

4. RESULTS AND DISCUSSION

4.1 OPTIMIZATION AND EVALUATION OF TWO DIFFERENT PLANT METABOLITE EXTRACTION METHODOLOGIES: ATP AS A REPORTER

The metabolome of an organism refers to the complete set of small-molecules chemicals and is a reflection of its metabolic state, hence gives information about the activated biological processes under particular conditions (Faijes *et al*. 2007). Metabolomics studies consist of two main stages: extraction of metabolites from cellular matrix and quantification of the extracted metabolites. For successful metabolome studies, metabolite extraction is a key step in providing useful and reliable data. Significant efforts have been made for the identification of optimal metabolite extraction procedure for different platforms and organisms (Tambellini *et al*. 2013). In evaluating alternative extraction protocols, for e.g. protocol validation, we used reporter metabolites (specifically ATP, glucose and overall starch) and focused on quantification of these, rather than overall, high-coverage metabolome data. The effect of drought stress and genotypic differences based on these reporter metabolites in model plant *Brachypodium distachyon* was evaluated.

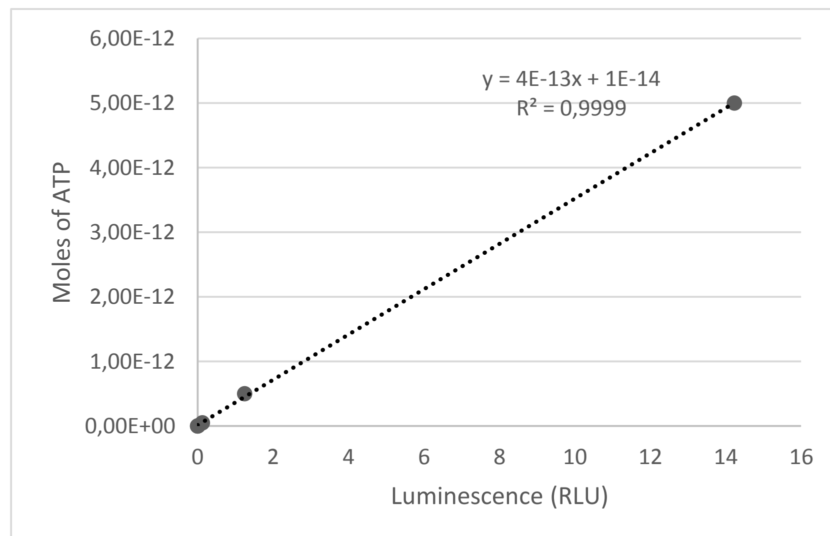
4.1.1 Optimization of Methanol Extraction Procedure

Methanol extraction (MeOH) procedure consists of two steps including methanol and water treatment (Section 3.2.2.1). After removing all insoluble molecules, both supernatants are combined for large metabolome recovery. Here, ATP levels collected in each recovery step were measured to understand whether the addition of these steps lead to obtain higher amounts of metabolites. Standard curve for ATP assay was constructed according to manufacturer's instructions (Promega ATP assay kit) (Figure 4.1).

Extraction of intracellular metabolites was carried out from two different *Brachypodium* genotypes (23-12 and 45-11) for protocol optimization. In Figure 4.2A, while 'sp1' refers to the first supernatant collected after methanol treatment, sp12, sp123 and sp1234 refer

to the combined supernatants with each additional recovery step using water. As shown in the figure, while scarce amount of ATP was obtained from the first supernatant (sp1), additional steps permitted to collect increased amount of ATP and saturation occurred with the last recovery step (sp1234). The pool, which was composed of first, second and third supernatants (sp123) showed a significant increase in the amount of ATP. There was approximately 10% increase in ATP levels with additional recovery steps. For both genotypes, there was a similar increasing trend for ATP levels depending on the recovery steps indicating that sp123 is required for larger amounts of metabolite collection and sp1234 might be preferably collected depending on the metabolite of interest. For example; since secondary metabolites are present in little amounts in the cell, it can be useful to apply all recovery steps to obtain larger amount of metabolite for such a study. Comparison of two different genotypes from different geographical regions of Turkey was also shown in Figure 4.2A. These two genotypes exhibited significantly different profiles for ATP levels. ATP level of genotype 23 was higher with respect to the genotype 45. Since these two genotypes were sampled from different geographical regions of Turkey (one from North and the other from South), this difference most likely arise from geographical adaptation of plants, supporting Luo *et al*. (2011) that found significant variation in response to drought stress, between different *Brachypodium* accessions.

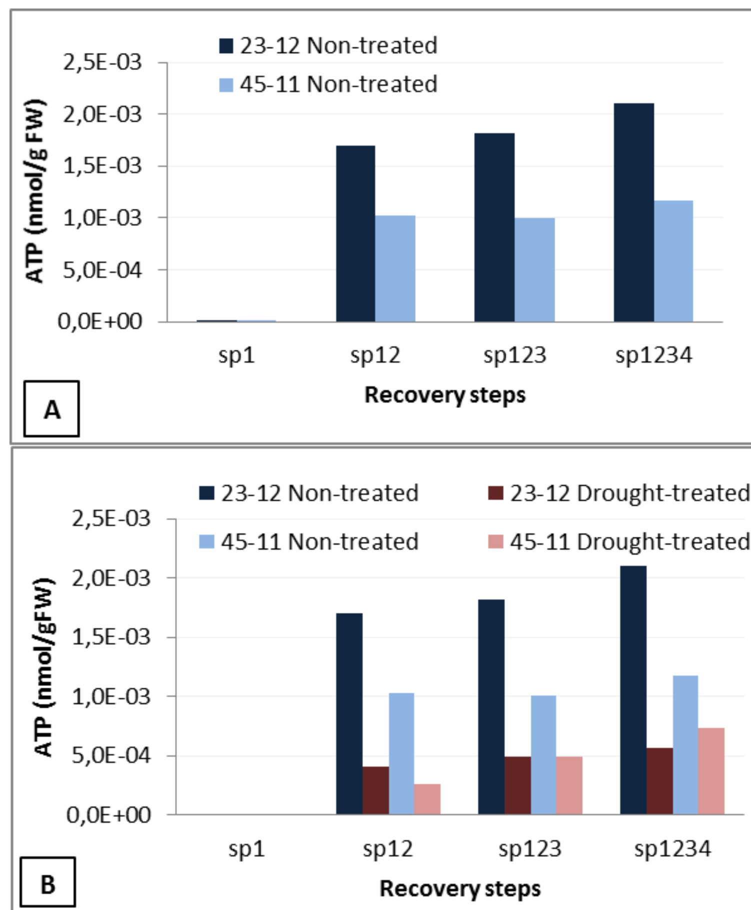
Figure 4.1 ATP standard curve



Reference: This graph was prepared by Özge TATLI.

ATP levels in drought-treated individuals of these two genotypes (Figure 4.2B) were compared. For both conditions, again there was a similar manner in increasing ATP levels depending on the additional recovery steps in methanol extraction procedure, indicating the necessity of at least third step. The decrease of ATP amount in drought-treated samples compared to those in untreated ones was further evaluated for different populations using two metabolite extraction protocols (Figure 4.2B).

Figure 4.2 ATP levels with additional recovery steps in Methanol extraction: A) Detected ATP levels in different genotypes. B) Detected ATP levels in both different genotypes and conditions.



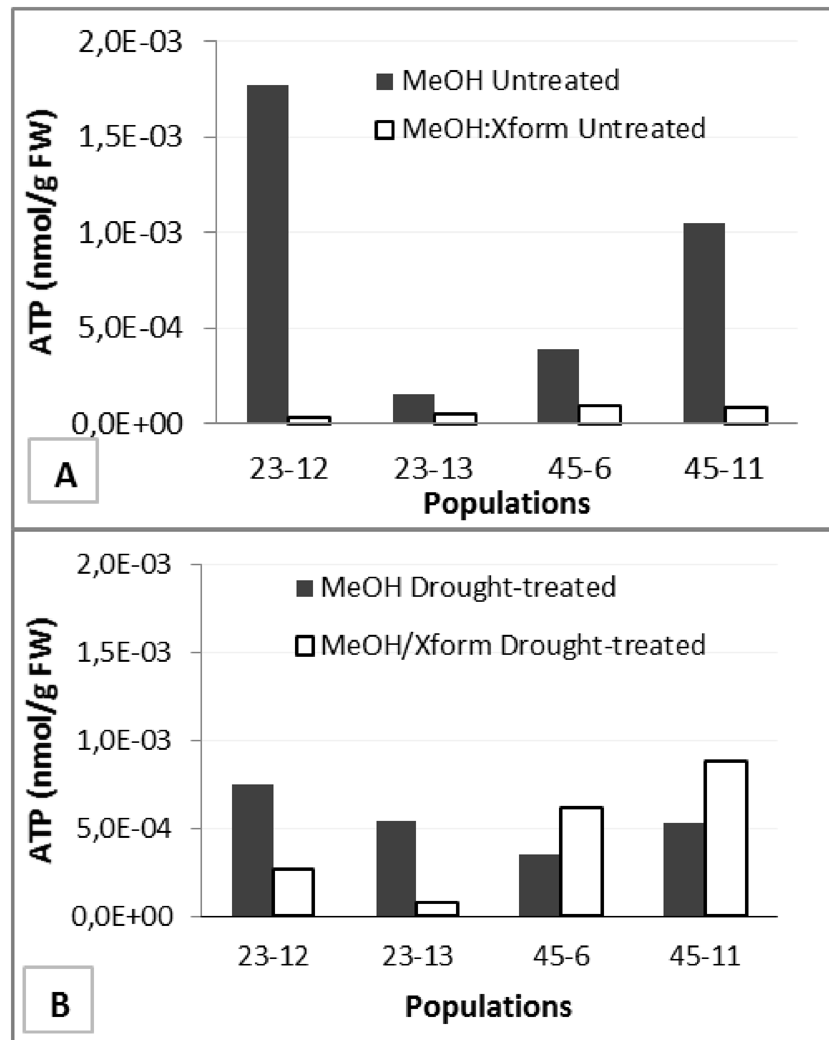
Reference: This graph was prepared by Özge TATLI.

4.2.2 Comparison of Methanol Extraction and Methanol:Chloroform Extraction

ATP levels in samples that were extracted by methanol (MeOH) and methanol:chloroform (MeOH:Xform) extraction procedures were shown in Figure 4.3 in order to evaluate two methods comparatively. Figure 4.3A represents the comparison of

ATP levels in non-treated samples whereas Figure 4.3B represents comparative levels of ATP in drought-treated samples. Interestingly, each method showed a different pattern for different populations. While methanol extraction procedure was more efficient to extract ATP from genotype 23, methanol:chloroform extraction was better for the extraction of ATP from genotype 45. We assume that morphological difference in leaves of genotypes (Filiz *et al*. 2009) may alter the effect of used chemicals for extraction since morphological variations arise due to the difference in organic content of leaf tissues. There is significant difference in metabolite recoveries upon different extraction protocols.

Figure 4.3: The comparison of ATP Levels according to the extraction method: A) untreated samples, B) drought-treated treated samples.

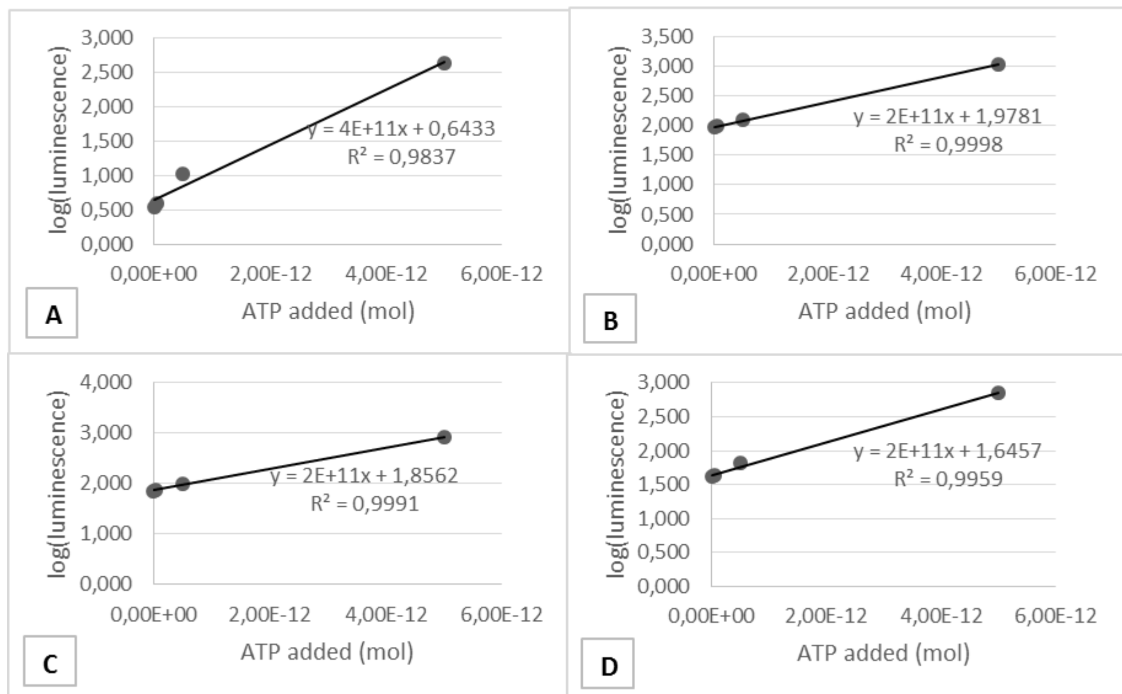


Reference: This graph was prepared by Özge TATLI

4.1.3 Evaluation of Matrix Effect

In biological analyses, components of matrixes in biological samples might influence the response of interested analytes and lead to inaccurate quantitation, known as 'matrix effect' (Chiu *et al*. 2010). Specifically for ATP levels, even if the proteins and any particles are quite effectively removed from the sample, still a lot of endogenous substances such as phospholipids are remaining, releasing phosphate molecules and possibly causing matrix effects. Standard additions of analyte of interest (in this case ATP) into each sample can be used to take the matrix effects into account (Shariati-Rad *et al*. 2013).

Figure 4.4 Calibration curves with spiked samples: A) Genotype 23-12, B) Genotype 23-13, C) Genotype 45-6, D) Genotype 45-11.



Reference: This tables were prepared by Özge TATLI.

Assuming a log-normal distribution of samples at low concentration, a calibration curve was constructed for each sample based on log-linear model Assuming a log-normal distribution of samples at low concentration with increasing concentrations of ATP standard added to individual aliquots of the sample (Figure 4.4). Results of standard

addition experiments confirmed that measurement of ATP levels within plant matrix is reliable and highly accurate.

4.2 DROUGHT- AND GENOTYPE-BASED COMPARISON OF EXTRACTION PROTOCOLS

Brachypodium, as a wild type cereal, has the potential to show wider variation compared to domesticated cereals. For instance, Schwartz et al. (2010) found a substantial variation in flowering time among *Brachypodium* accessions. This natural variation can be used potentially to uncover genes responsible for the regulation of flowering in grasses.

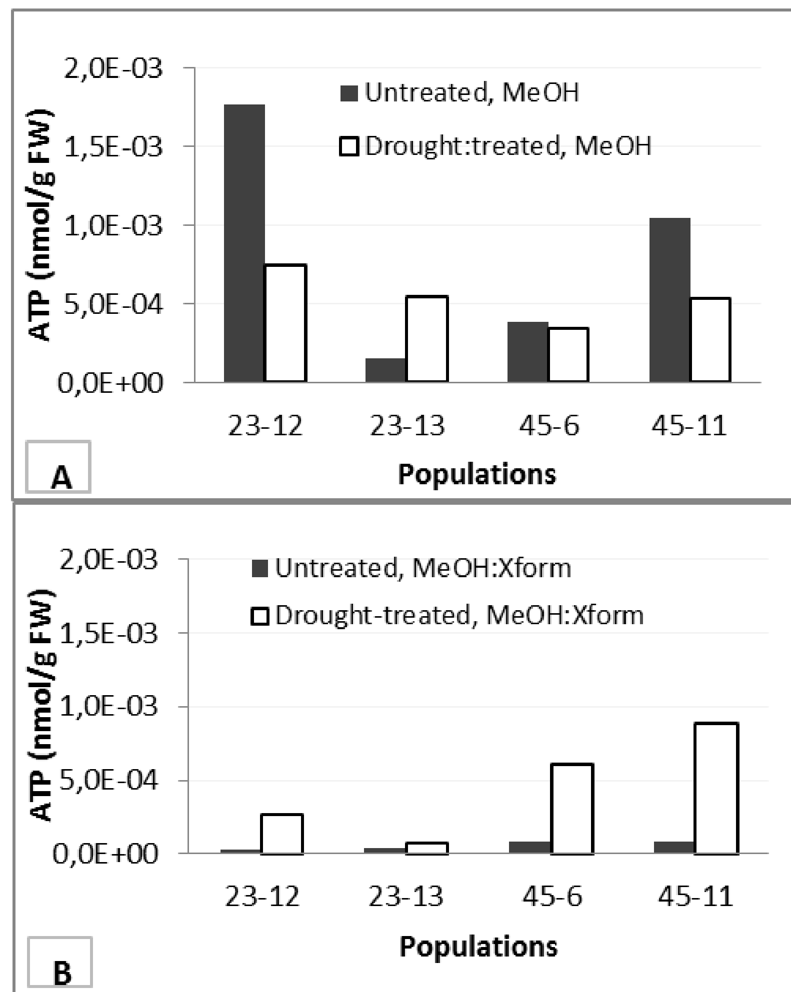
Drought tolerance is an important trait for sustainable crop production. Natural diversity in drought physiology has not been well-characterized in most of the plant species. However, Luo *et al*. (2011) showed that 57 different accession of *Brachypodium* represented different physiological responses to drought stress, such as chlorophyll fluorescence and leaf water content. They identified four groups of accessions differing in their sensitivities to drought stress.

4.2.1 ATP as a Reporter Metabolite

After assessing the efficiency of extraction protocols, in order to evaluate the effect of drought and genotypic differences based on reporter metabolites, starch, glucose and ATP levels were measured. A decrease, ranging between 10-30%, in ATP levels of drought-treated *Brachypodium* individuals, which were extracted with methanol extraction procedure, was observed (Figure 4.5A) and this indicated a pronounced effect of drought on energy metabolism. In the light of a previous study (Zagdańska 1995), it can be speculated that these reduction and energetic cost might depend on the slowdown of energy metabolism to decrease the rate of the energy-consuming pathways that do not carry primary importance for survival so that it can maintain integrity due to the water deficit conditions. However, the comparison of ATP levels between drought-treated and non-treated samples which were extracted with methanol:chloroform extraction did not represent a similar pattern with methanol extraction (Figure 4.5B). Moreover,

unexpectedly ATP levels in drought-treated samples were much higher than those of untreated ones, indicating that ATP levels (ranging from 2,92 10^{-5} - 1,77 10^{-3} nmol/g FW (fresh weight)) displayed a different trend between the extraction protocols.

Figure 4.5: Comparison of ATP levels based on genotype and corresponding response to drought treatment using: A) MeOH extraction, B) MeOH:Xform extraction.



Reference: These graphs were prepared by Özge TATLI.

4.2.2 Starch as Reporter Metabolite

Starch measurements are used as a proxy to storage metabolism. Starch concentrations were estimated via constructed log-linear model using absorbance values obtained from spectrophotometer (Figure 4.7) and comparative starch concentrations between two different conditions (drought and normal) were evaluated. The statistical analysis of

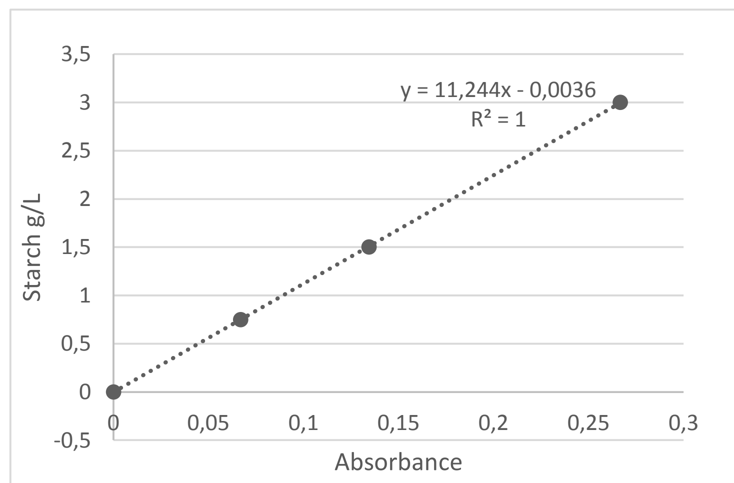
estimated starch concentrations showed that there was no significant effect of drought stress on starch content except one population. Population 23-12 showed significant decrease in starch content under drought conditions (Table 4.1). The reason could be to increase glucose production by hydrolysis of starch.

Table 4.1 Statistical analysis of the data represented in Figure 4.6.

Genotypes	Populations	p values for the test
23	12	**0.045***
	13	0.138^{NS}
45	6	0.332^{NS}
	11	0.137^{NS}

^{NS} Not significant
* Significant at p ≤ 0.050
Reference: This table was prepared by Özge TATLI.

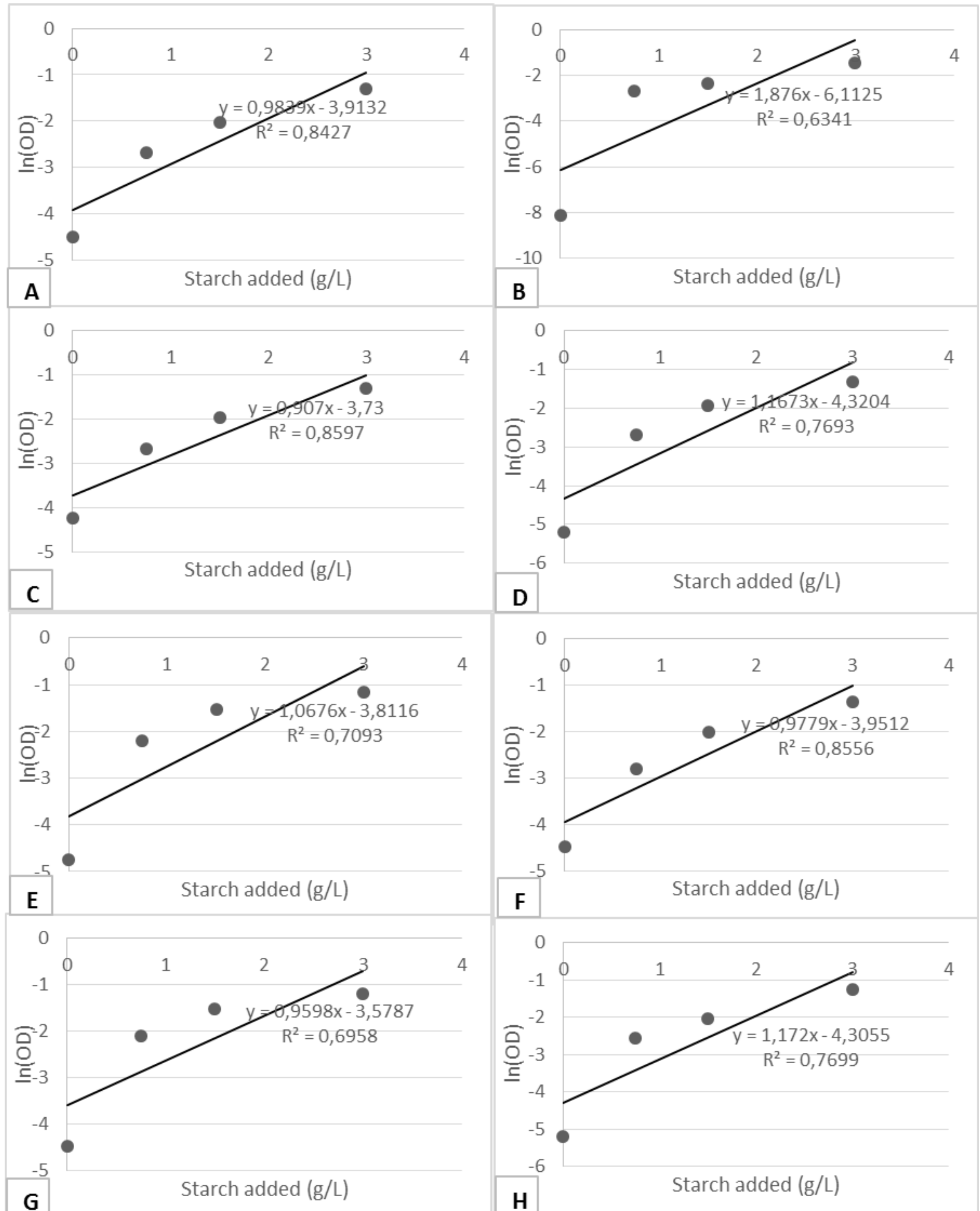
Figure 4.6: Calibration curve for starch assay.



Reference: This graph was prepared by Özge TATLI.

Percentages of starch in leaves under normal conditions ranged between 0.8-2.7% supporting recent study that was carried out on the deposition and characterization of starch in *Brachypodium distachyon* (Tanackovic *et al*. 2014).

Figure 4.7: Estimated starch contents from samples: A) Genotype 23-12, non-treated, B) Genotype 23-12, drought-treated, C) Genotype 23-13, non-treated, D) Genotype 23-13, drought-treated, E) Genotype 45-6, non-treated, F) Genotype 45-11, drought-treated, G) Genotype 45-11, non-treated, H) Genotype 45-11, drought-treated.



Reference: These graphs were prepared by Özge TATLI.

4.3.3 Glucose as a Reporter Metabolite

Glucose levels were estimated via a constructed log-linear model using measured peak area -from HPLC measurements- of the samples extracted by methanol extraction (Figure 4.9) and methanol:chloroform extraction (Figure 4.10). Calibration curve used for calculations is represented in Figure 4.8. Comparative glucose concentrations between two different conditions (drought and normal) and two protocols were evaluated. The statistical analysis of estimated glucose levels showed that there was no significant effect of drought stress on glucose content of samples that were extracted by methanol extraction, except one population. Population 23-12 showed significant increase in glucose content under drought conditions (Table 4.2).

Figure 4.8: Calibration curve for HPLC analysis.

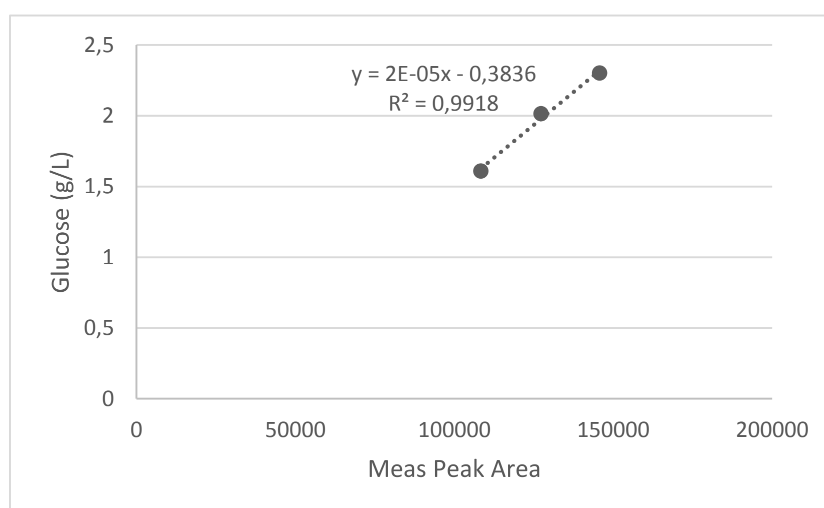
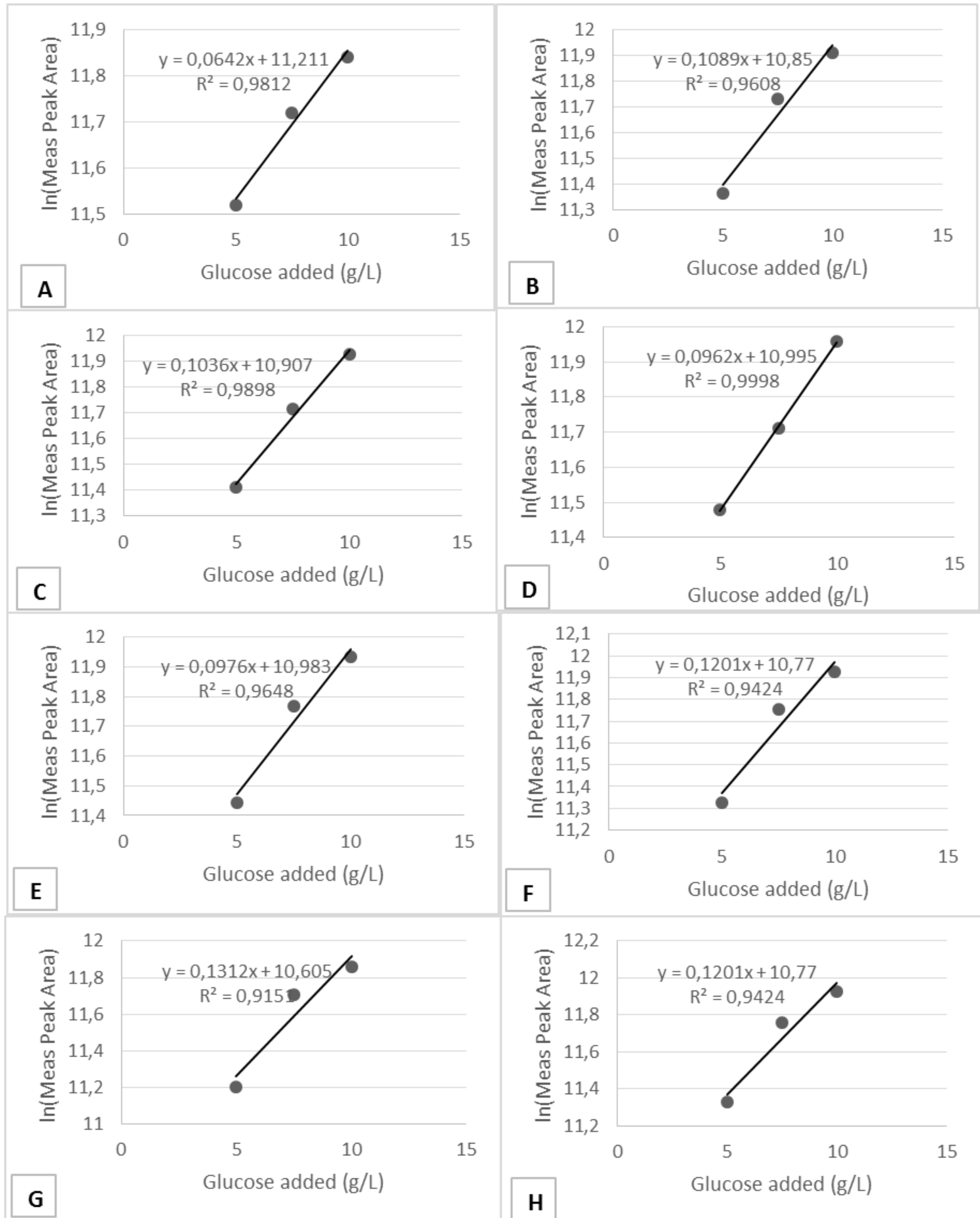


Table 4.2 Statistical analysis of the data represented in Figure 4.10 and 4.11.

Genotypes	Populations	p values for the t-test (MeOH)	p values for the t-test (MeOH:Xform)
23	12	**0.026***	**0.001***
	13	0.072^{NS}	0.317^{NS}
45	6	0.112^{NS}	**0.004***
	11	0.206^{NS}	0.076^{NS}

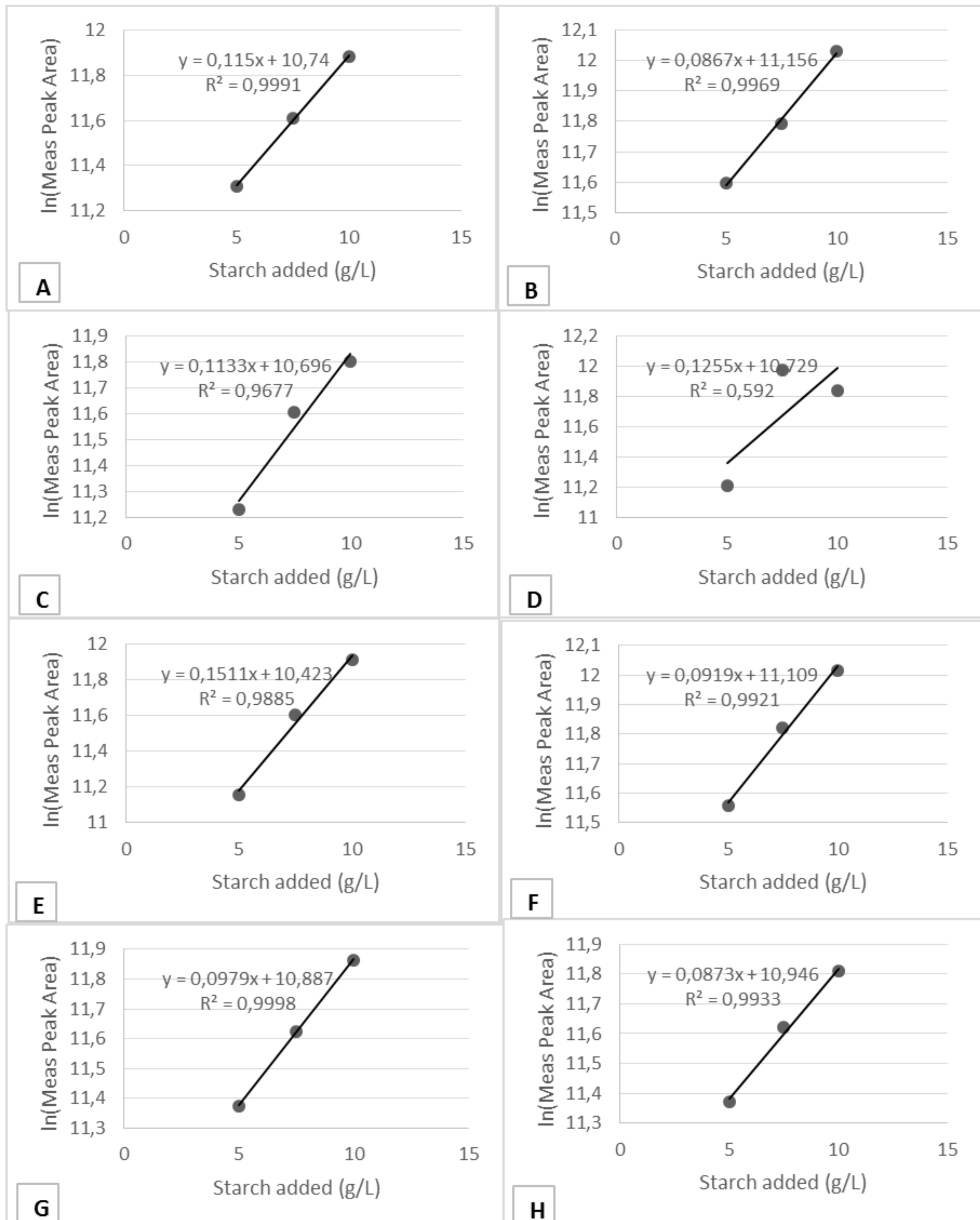
^{NS} Not significant
* Significant at $p \leq 0.050$
Reference: This table was prepared by Özge TATLI.

Figure 4.9: Estimated glucose concentrations from samples, extracted with MeOH extraction: A) Genotype 23-12, non-treated, B) Genotype 23-12, drought-treated C) Genotype 23-13, non-treated, D) Genotype 23-13, drought-treated, E) Genotype 45-6, non-treated, F) Genotype 45-11, drought-treated, G) Genotype 45-11, non-treated, H) Genotype 45-11, drought-treated.



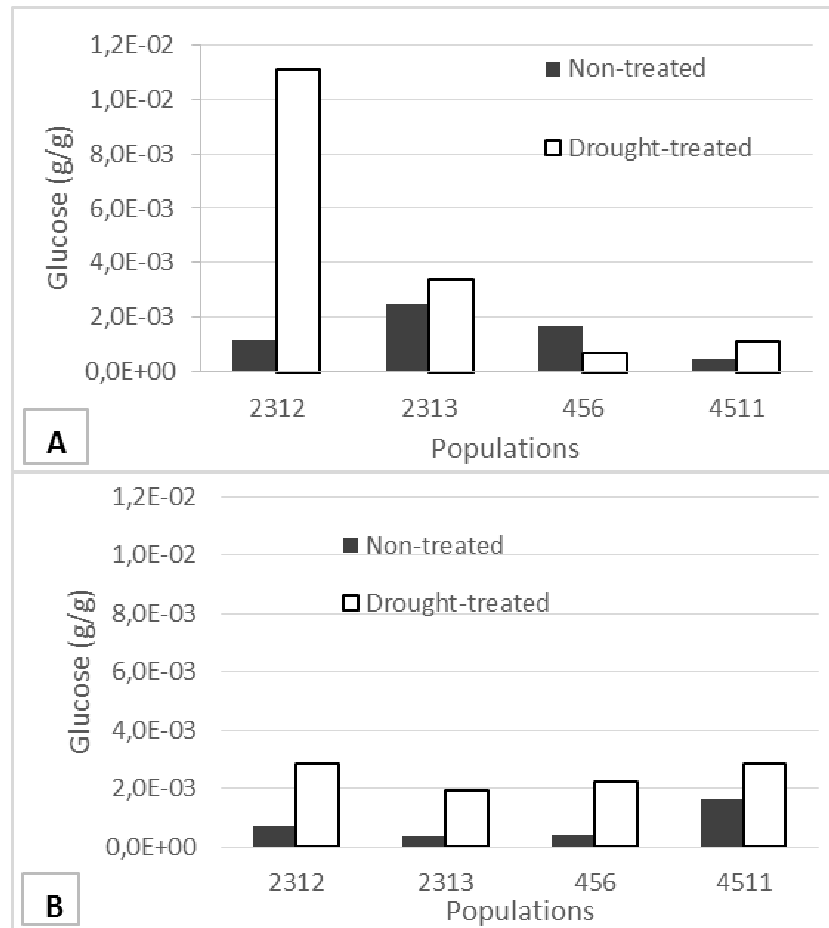
Reference: These graphs were prepared by Özge TATLI.

Figure 4.10: Estimated glucose concentrations from samples, extracted with MeOH:Xform extraction: A) Genotype 23-12, non-treated, B) Genotype 23-12, drought-treated, C) Genotype 23-13, non-treated, D) Genotype 23-13, drought-treated, E) Genotype 45-6, non-treated, F) Genotype 45-11, drought-treated, G) Genotype 45-11, non-treated, H) Genotype 45-11, drought-treated.



Reference: These graphs were prepared by Özge TATLI.

Figure 4.11: Comparison of glucose contents between drought-treated and untreated samples: A) MeOH extraction, B) Methanol:chloroform extraction.



Reference: These graphs were prepared by Özge TATLI.

The statistical analysis of estimated glucose levels of samples which were extracted by methanol:chloroform extraction, was performed. While populations 23-12 and 45-6 showed significant increase in glucose content (p=0.001, p=0.004 respectively) under drought conditions, populations 23-13 and 45-11 did not show a significant difference in glucose content depending on drought conditions (Table 4.2). Overall, glucose levels showed 1-4 fold increase (Figure 4.11). The results in this thesis are consistent with literature, where it has been shown that under drought conditions plants accumulate simple sugars such as glucose and sucrose, increasing invertase activity in their leaves (Tuteja 2010) and increase in glucose concentrations might be ~5 fold under drought stress (De Roover *et al*. 2000).

5. CONCLUSION

- Based on the different response of ATP levels according to extraction method and validation of ATP measurements with spiking experiments, it can be concluded that there is no one protocol that fits all species, even genotypes.

- In response to drought stress, the response level differs among genotypes highlighting the importance of natural variation in *Brachypodium*. There is no significant or consistent change determined in starch levels. However, there is consistently statistically significant increase in glucose levels upon drought stress.

- Taken overall results together, we saw that impact of drought stress on *Brachypodium* metabolism is significant and tailor-made protocols are needed in plant metabolomics.

- This study will provide a basis for the construction of high-coverage metabolome platforms and potentially lead to more reliable metabolome studies in plants and help for further metabolomics studies on drought stress response in order to improve agriculturally important crop species.

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