PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF SEED PRIMING EFFECT ON *BRACHYPODIUM DISTACHYON* UNDER ABIOTIC STRESS

by
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PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF SEED PRIMING EFFECT ON *BRACHYPODIUM DISTACHYON* UNDER ABIOTIC STRESS

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ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF SEED PRIMING EFFECT ON *BRACHYPODIUM DISTACHYON* UNDER ABIOTIC STRESS

Plant breeders and scientists try to develop more precise solutions for the production of new varieties, which are more resistant to abiotic stress to supply the food and feed demand of the growing world population. Seed priming is a hydration technique by treating seeds with various chemicals to activate the metabolism of the plant prior to sowing. For many years, seed priming has been used to achieve synchronized germination and increased germination rate. However, in recent years, studies have focused on the effect of seed priming on the plant's defense mechanism against abiotic stress. *Brachypodium distachyon* is a model plant for important cereals such as barley, wheat and rye that have great importance as food and feed supply. In this study, *B. distachyon* (Bd-21 line) seeds were treated with distilled water for hydropriming, potassium nitrate (1, 2, 3 percent KNO_3) for osmopriming, and salicylic acid (0.25, 0.5, 0.75 mM SA) for hormonal priming to investigate the effect of seed priming on germination parameters and evaluate the physiological and biochemical changes caused by different priming methods in *B. distachyon* plants under salt and drought stress. The germination test showed that all priming methods had a positive effect on seed germination parameters, in particular, mean emergence time (MGT), germination index (GI), uncertainty of germination (U) and synchronization (Z). Morphological characteristics of plants such as biomass and plant height were positively affected by hydropriming and low concentrations of KNO_3. Relative water content (RWC) and chlorophyll concentration decreased while proline content and relative membrane permeability (RMP) increased under salt and drought stress. Antioxidant levels (catalase-CAT, peroxidase-POD and superoxide dismutase-SOD) showed extensive variation and significant differences among priming treatments. Overall, it can be concluded that osmopriming with 1 percent KNO_3 would be preferable for drought and salt stress tolerance in *B. distachyon*. To our knowledge, seed priming was performed for the first time in *B. distachyon* with this study. Therefore, the results of this study can further be used in omics studies to explore the molecular mechanisms underlying abiotic stress tolerance of *B. distachyon* and other cereal grains after seed priming.

ÖZET

ABİYOTİK STRES ALTINDAKİ *BRACHYPODIUM DISTACHYON*'DA TOHUM ÖN UYGULAMA ETKİSİNİN FİZYOLOJİK VE BİYOKİMYASAL ANALİZİ

Bitki ıslahçıları ve bilim insanları, büyüyen dünya nüfusunun gıda ve yem talebini karşılamak için abiyotik strese dayanıklı yeni çeşitler geliştirmeye çalışmaktadır. Tohum ön uygulaması (priming), bitkinin metabolizmasını aktive etmek için tohumun ekimden önce çeşitli kimyasallarla muamele edilmesi ile uygulanan bir hidrasyon tekniğidir. Priming senkronize çimlenmeyi sağlamak ve çimlenme oranını arttırmak içinyıllardır kullanılmaktadır. Bununla birlikte, son yıllarda yapılan araştırmalar, priming uygulamasının bitkinin abiyotik strese karşı savunma mekanizması üzerindeki etkisine odaklanmıştır. *Brachypodium distachyon*, buğday, arpa ve çavdar gibi gıda ve yem tedariğinde öneme sahip tahıllara model bir bitkidir. Bu çalışmada, *B. distachyon* (Bd-21 hattı) tohumları, hidropriming için distile su, osmopriming için potasyum nitrat (yüzde 1, 2, 3 KNO_3) ve hormonal priming için salisilik asit (0.25, 0.5, 0.75 mM SA) ile muamele edilmiştir. Bu sayede, priming uygulamasının çimlenme parametrelerine etkisinin incelenmesi ve priming metotlarının tuz ve kuraklık stresi altındaki *B. distachyon*'da sebep olduğu fizyolojik ve biyokimyasal değişimlerin değerlendirmesi amaçlanmıştır. Çimlenme testi, tüm priming uygulamalarının, özellikle ortalama çimlenme süresi (MGT), çimlenme endeksi (GI), çimlenme belirsizliği (U) ve senkronizasyon (Z) olmak üzere çimlenme parametrelerine olumlu etkisi olduğunu göstermiştir. Bitkilerin biyokütle ve bitki boyu gibi morfolojik özellikleri, hidropriming ve düşük konsantrasyonlu KNO_3 priming uygulamasından olumlu yönde etkilenmiştir. Tuz ve kuraklık stresi altında, prolin içeriği ve nispi membran geçirgenliği (RMP) artarken, nispi su içeriği (RWC) ve klorofil konsantrasyonu azalmıştır. Antioksidan seviyeleri (katalaz-CAT, peroksidaz-POD ve süperoksit dismutaz-SOD), priming uygulamaları arasında önemli farklılıklar göstermiştir. Genel olarak, yüzde 1 KNO_3 ile priming uygulamasının, tuz ve kuraklık stresi altındaki *B. distachyon*'da tolerans sağlamak için tercih edilebileceği sonucuna varılabilir. Bilgimiz dahilinde *B.distachyon*'da priming uygulaması ilk bu çalışmada yapılmıştır. Bu çalışmanın sonuçları, priming sonrası *B. distachyon* ve diğer tahıllarda abiyotik stres toleransının altında yatan moleküler mekanizmaları araştırmak için omik çalışmalarda kullanılabilir.

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LIST OF SYMBOLS/ABBREVIATIONS

A. thaliana	*Arabidopsis thaliana*
B. distachyon	*Brachypodium distachyon*
α	Alpha
β	Beta
C	Celsius
ε	Extinction coefficient
µmol	Micromole
µM	Micromolar
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ADP	Adenosine diphosphate
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
AVP	Arabidopsis vacuolar H^+-pyrophosphatase
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CAT	Catalase
CBF	C-Repeat binding factor
cDNA	Complementary deoxyribonucleic acid
Cl	Chloride
cm	Centimeter
DNA	Deoxyribonucleic acid
Cn_i,2	Combination of seed germinated in the i time
COR	Coronatine
CV_t	Coefficient of variation of germination time
DREB	Dehydration-responsiveelement-binding
ds	Decisiemens
DW	Dry weight

EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methane sulfonate
ERF	Ethylene response factor
EST	Expressed sequence taq
FAO	Food and Agriculture Organization of the United Nations
FISH	Florescence in situ hybridization
fi	Frequency of germination
FW	Fresh weight
g	Gram
G	Germination rate
GA_3	Gibberellic acid
GB	Glycine betaine
GI	Germination index
GR	Glycine reductase
h	Hour
H_2O_2	Hydrogen peroxide
ha	Hectare
HKT	High-affinity K^+ transporter
HR	Hypersensitive response
HSP	Heat shock protein
K^+	Potassium
KCl	Potassium chloride
kg	Kilogram
K_2HPO_4	Potassium phosphate dibasic
KH_2PO_4	Potassium phosphate monobasic
KNO_3	Potassium nitrate
LEA	Late embryogenesis abundant
m	Meter
M	Molar
MAPK	Mitogen activated protein kinase
Mbp	Million base pair
MET	Mean emergence time

$MgSO_4$	Magnesium sulfate
MGT	Mean germination time
mm	Millimeter
mM	Millimolar
MPa	Megapascal
MR	Mean germination rate
mg	Milligram
min	Minute
MKK	Mitogen activated protein kinase
ml	Milliliter
mM	Millimolar
mRNA	Messenger Ribonucleic acid
Mya	Million years ago
Na	Sodium
NaCl	Sodium chloride
nm	Nanometer
NO	Nitric oxide
OH^-	Hydroxyl
PAMP	Pathogen-associated molecular patterns
POD	Peroxidase
ppm	Parts per million
QTL	Quantitative Trait Locus
RMP	Relative membrane permeability
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
RWC	Relative water content
SA	Salicylic acid
SOD	Superoxide dismutase
SOS	Salt overly sensitive
TW	Turgor weight
U	Uncertainty of the germination process

UNESCO	United Nations Educational, Scientific and Cultural Organization
VRN	Vernalization
Z	Synchrony of the germination process
ZFP	Zinc finger protein
ZIP	Zinc-regulated transporters (Zrt), Iron-regulated transporter (Irt)-like Proteins

1. INTRODUCTION

Studying with complex organisms has always been a challenge in science. This problem was solved by introducing model systems. After the emergence of *Arabidopsis thaliana* as a model plant, studies in plant biotechnology have accelerated. However, since *Arabidopsis thaliana* is a dicot plant, it could not be an appropriate model plant for monocots such as wheat, rice and barley that, have great importance in agriculture. As a model plant, *Brachypodium distachyon* has highly suitable attributes for grass family (Poaceae) which contains cereal crops and forage grasses [1]. With its small size, simple growth conditions and small genome, *Brachypodium* has a great advantage over other model monocots such as maize and rice. Moreover, *Brachypodium* shows a strong variation for adaptation to stress conditions since it is not domesticated.

Environmental stress factors are one of the most important reasons for restricting agricultural production. Among the abiotic stress factors, drought and salinity are the most cautious stressors. Since plants cannot escape from these stress factors, they have developed highly complex response mechanisms. Many studies have emphasized the existence of a complex network in the cell in response to oxidative stress [2,3]. This network is attempted to be elucidated by scientists at the physiological, molecular and metabolic level.

Plant physiology consists of the various fundamental processes such as plant nutrition, hormones, photosynthesis, respiration, morphogenesis, stress physiology, germination, dormancy and phytochemistry (biochemistry of plants). Under abiotic stress, one of the first reactions of plants is thought to be stomatal conductance. Since this will affect the photosynthesis rate, it directly changes the energy level produced by the plant and thus its biomass. Water deficit also affects the water holding capacity of the plant and relative water content significantly decreases. In addition, the ion balance of plant will be disrupted by the excess ion in the soil, (i.e. the salinity of the soil) that the fluidity of the cell membrane is deteriorated. The greatest damage to the cell membrane is caused by reactive oxygen species (ROS). Plants activate antioxidant mechanisms to remove these reactive agents. Peroxidase, catalase and superoxide dismutase has a significant role in the cleavage of hydrogen peroxide [4].

Seed priming is a technique that prepares plants for future stress conditions by treating seed with various chemicals in order to activate and enhance the defense mechanism of the plant. There are many methods used for seed priming and they are named with the agents used in priming. For instance, hydropriming is a method of hydration with water. If salts or hormones are used, they are called osmopriming and hormonal priming, respectively. Since these chemicals affect the hydration of the seed, they prolonged the second phase of the germination. Phase II of germination is the phase in which DNA is repaired and metabolic activities are initiated. Thus, this situation is considered as the seeds that are primed may be more tolerant to stress in the future. In addition, seed priming allows for synchronized germination due to the extension of phase II. There are also many studies indicating that priming increases germination rate and decreases mean germination time [5]. However, it should not be ignored that priming agent, concentration, duration of priming and temperature significantly affect the efficiency of this method. These parameters depend on the genotype as well as the plant species.

In this study, *Brachypodium distachyon* seeds were treated with distilled water for hydropriming at 4°C and 20°C separately, potassium nitrate for osmopriming priming (1 percent, 2 percent, 3 percent KNO_3) and salicylic acid for hormonal priming (0.25, 0.5, 0.75 mM SA) at 20°C for 24 hours at dark. The objective of this study was to evaluate the seed priming effect on germination rate, seedling emergence, physiological and biochemical changes of *Brachypodium distachyon* (Bd-21 line) under drought and salt stress. Our results suggest that priming with KNO_3 and SA was an effective method for *Brachypodium distachyon* seeds. Germination percentage was not different in all priming methods including nonprimed seeds since all of them were germinated. However, osmopriming with KNO_3 and hormonal priming with SA significantly decreased the mean emergence time and ensure the synchronized germination of *Brachypodium distachyon* seeds. The highest GI values which best describe the germination percentage/speed relation were observed in higher concentrations of KNO_3 (2 percent and 3 percent KNO_3).

Plants were exposed to drought stress by water withholding for 12 days and salt stress by treating with 320 mM NaCl solution for 14 days at their vegetative stage. Plant height and chlorophyll content were measured during stress treatments. In non-stress conditions (control), all plants showed better performance on growth rate than nonprimed plants. Chlorophyll content decreased under both drought and salt stress conditions compared with

control. After harvest, the relative water content was measured and the lowest value in drought stress was observed in plants primed with 0.5 mM SA. Similarly, the permeability of the cell membrane was damaged in these plants under salt stress. The highest dry weight (biomass) from plants was observed under salt stress primed with 1 percent KNO_3, while under drought stress the biomass was high in plants primed with distilled water at 20°C. In all plants, proline content was significantly increased under both salt and drought stress. The highest proline concentration was observed in plants primed with 3 percent KNO_3 and 0.5 mM SA. In addition, the antioxidant mechanism was activated in *Brachypodium distachyon* under both salt and drought stress. Catalase activity increased in plants primed with 1 percent and 3 percent KNO_3 whereas peroxidase activity increased in plants primed with 0.5 mM SA under both salt and drought stress. Superoxide dismutase activity was significantly high in all primed plants under drought and salt stress (except plants primed with distilled water under salinity). Consequently, the effects caused by priming agents and concentrations vary widely under abiotic stress and control conditions when *Brachypodium* plants were evaluated at physiological and biochemical basis. Overall, it can be concluded that priming with 1 percent KNO_3 would be preferable for *Brachypodium distachyon* under salt and drought stress. Besides, lower concentrations of KNO_3 should be performed for future studies. To our knowledge, seed priming was performed for the first time in *Brachypodium distachyon*, therefore, this study is a comprehensive and innovative research that appraise the effect of seed priming on *Brachypodium distachyon*.

2. LITERATURE REVIEW

2.1. MODEL PLANTS

Model plants provide a great convenience for scientists to investigate the biological process of plants due to their short life cycles, small genomes, simple growth conditions, compact structure (small plant size) and suitability for genetic manipulation. In particular, developments in omics technologies and the completion of the whole genome sequence of *Arabidopsis thaliana* by `The *Arabidopsis* Genome Initiative' allowed scientific breakthroughs in plant biotechnology [6]. Today, *Zea mays* (maize), *Oryza sativa* (rice), *Nicotiana benthamiana* and *Brachypodium distachyon* have emerged as model plants with their desired attributes. The studies related to crops which supply the food and feed demand induce the need for the monocotyledonous (monocot) model plant [7].

2.1.1. *Brachypodium distachyon* as The Model Species for Monocots

Angiosperms, also known as flowering plants, are the most diverse vascular plant group spread all around the world. Based on their number of embryogenic leaves and cotyledons they are divided into two classes as; monocots and dicots. These plants have distinct features according to their morphological, physiological and evolutionary characteristics. The evolutionary separation between monocots and dicots started 140-150 Mya [8]. Monocots include grass family (*Poaceae)* members such as oat, wheat, barley and rice with their own specialized physiology, while dicots comprise the most common garden plants, trees, shrubs and legumes such as rose, tomato, sunflower and *Arabidopsis thaliana*. Although *A. thaliana* commonly used due to its desired attributes, it is not suitable for investigation of monocots either phylogenetically or physiologically [2,4].

Temperate crops and forage grasses such as wheat, barley and oat are economically important plants for monocots that play a significant role on human diet and animal feed. Besides, they have been used as a renewable energy source. However, due to their complex genomes (especially bread wheat), large physical size, poor genetic manipulation

efficiencies, special and difficult growth conditions, it is crucial to use model plants in agricultural studies especially in genomics research [1].

Brachypodium (from the Greek *brachys*"short" and *podion*"a little foot"), as a temperate wild type genus, naturally grows in Mediterranean and Middle-Eastern regions, north-east Africa and south-west Asia [9]. In particular, *B. distachyon*, also named as "purple false broom", has arisen as a model plant in 2001 for monocot plants [7]. *B. distachyon* is an annual grass that closely related to economically important grains (e.g. wheat, rye, barley). In fact, *Brachypodium* exhibits far greater synteny to the genomes of these major cereal grains (especially wheat) than rice or sorghum (Figure 2.1) [10]. It is estimated that *Brachypodium* is 10-15 Mya closer to wheat than rice by using available genomic and EST (expressed sequence tag) tools [11] and diverged from the Pooideae about 35–40 Mya [12].

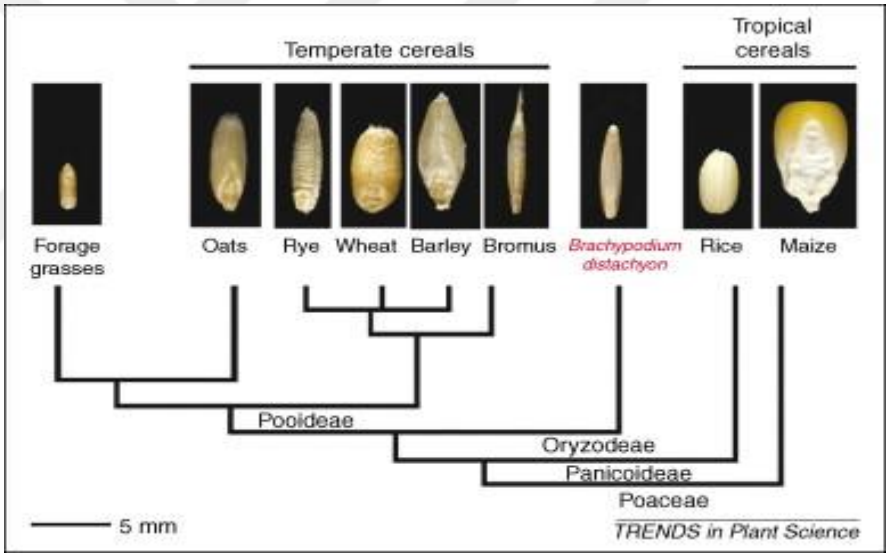

Figure 2.1. Phylogenetic relationships between cereals *and Brachypodium distachyon* [10].

Besides the advantage in phylogenetic position, *B. distachyon* has many characteristics which make it a suitable and attractive model for cereal and biofuel crop species (Figure 2.2). These desirable features include compact genome (272 Mbp), availability of whole genome sequence [13], 5 pair of chromosomes (2n=10) in diploid accessions, simple growth requirements, rapid life cycle (~12 weeks), small plant size (approximately 20 cm at maturity), self-fertility promoting inbreeding and efficient transformation protocols [14]. The variety of inbred accessions is essential to develop a new model system and agricultural research. In this context, several studies have been conducted for

characterization of inbred lines. The first karyological studies indicated that *B. distachyon* has three different ploidy levels (2n=10, 20, 30). However, according to the studies with fluorescence*in situ* hybridization (FISH), the 2n = 10 and 2n = 20 chromosome races are two different diploids with the chromosome number of x = 5 and x = 10, respectively, and the 2n = 30 race is their allotetraploid which naturally occurred by crossbreeding [8].

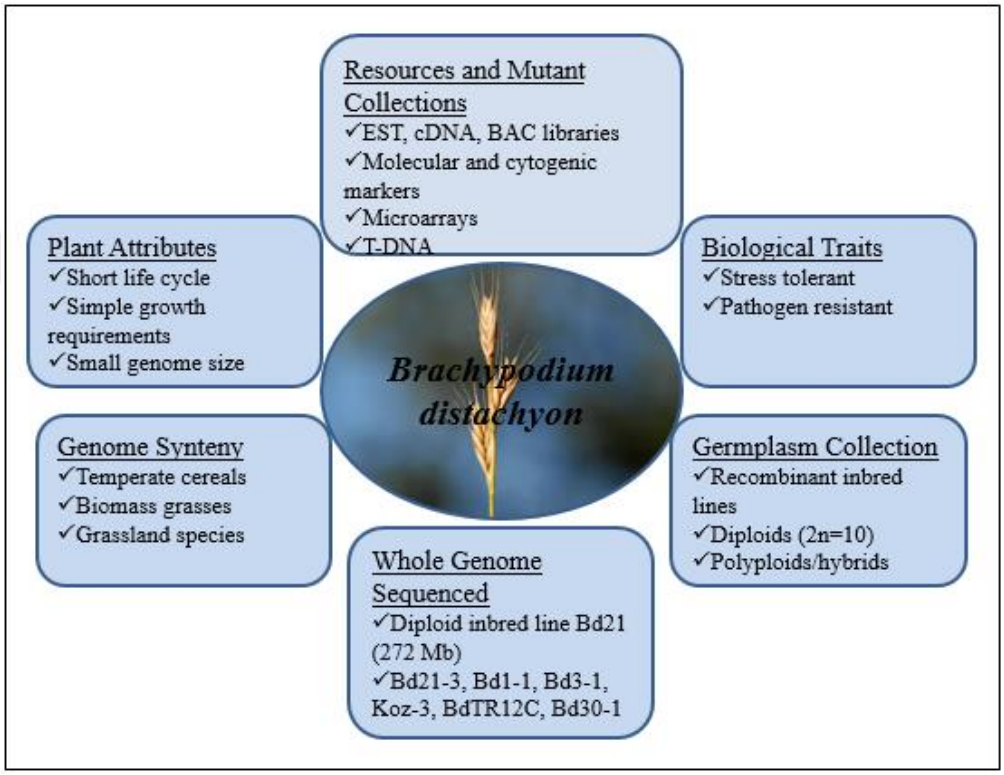

Figure 2.2. General attributes of *Brachypodium distachyon* [12].

The interest in *B. distachyon* as a new model plant has provided a great return to the studies related to the development of inbred lines, transformation techniques and genomic resources. Today, various functional genomics tools and resources are publicly available that contain BAC, EST libraries, a diverse collection of *B. distachyon* accessions, EMS mutants and T-DNA [15]. Well-established methodologies for *Agrobacterium*-mediated gene transfer and biolistics was performed and high transformation efficiency was achieved for *B. distachyon* [14,16,17]. Owing to all of these studies, *B. distachyon* has shown itself in different research fields such as investigation of vernalization and flowering by the regulation of three key genes VRN1, VRN2, FLOWERING LOTUS T (FT) [18,19], cell wall composition and saccharification for biofuel production via modifying lignin

content or lignin-hydrolyzing enzymes [20,21], root biology which is a recently focused field, important especially for osmotic stress studies [22].

2.1.2. *Brachypodium distachyon* versus Other Model Plants

Before the declaration of *B. distachyon* as a model plant, *A. thaliana* and rice were utilized as the major resources in plant research. In particular, *A. thaliana* has been accomplished as a model plant for the past few decades in plant studies. It has been a great contribution to databases and online tools, biotic and abiotic stress response studies, plant immunity, cell wall structure, cell biology, epigenetics, system biology and transgenic studies [23]. Indeed, it still plays a central role within model plants due to its high seed production, simple and fast growth, suitability for genetic manipulation [24]. However, as a eudicot, it is not an suitable model plant for cereals since *A. thaliana* does not share some important properties with grass family such as cell wall composition, grain production and plant development stages [25]. On the other hand, *Oryza sativa* (rice) with its compact simple genome (~441 Mbp) has been announced as another model plant for monocots [26]. However, it has special growth requirements. There are also some differences in agronomic traits of rice such as vernalization and dormancy mechanisms and resistance to pathogens or cold, among temperate and forage grasses [1,7]. Due to the complexity and large genomes of barley and wheat, they are not preferred in genomics studies. *Zea mays* (maize) with challenging cultivation conditions, big stature and laborious genetic transformation, it is not a favorable model plant [26]. Some genomic and physiological features of these plants are summarized in Table 2.1.

Table 2.1. Comparison of *Brachypodium distachyon* with other model plants [27].

Parameter	***Brachypodium distachyon***	***Arabidopsis thaliana***	***Triticum aestivum* (wheat)**	***Oryza sativa* (rice)**	***Zea mays* (maize)**	***Hordeum vulgare* (barley)**
Chromosome number	10 (2n)	10 (2n)	42 (2n)	24 (2n)	20 (2n)	14 (2n)
Genome size	272 Mb	164 Mb	16700 Mb	441 Mb	2400 Mb	5000 Mb
Growth requirements	Simple	Simple	Medium	Specialized	Specialized	Medium
Height (cm)	15-20	15-20	50-100	100-120	120-300	100-120
Life cycle (weeks)	8-12	8-12	12	20-30	8-15	15-20
Reproduction strategy	Self-fertilizing	Self-fertilizing	Self-fertilizing	Self-fertilizing	Cross-pollination	Self-fertilizing
Transformation	Highly efficient	Highly efficient	Inefficient	Highly efficient	Efficient but intense labor	Efficient but intense effort

From another perspective, since people adopted to the settled life, they began to domesticate plants for desired features. Domestication is an evolutionary process that alters some of the key characteristics of the plant by artificial selection. However, there are some consequences of plant domestication such as loss of genetic diversity and reproducibility [28]. The reduction in genetic diversity in some crop species through genetic erosion have been evaluated as 70-90 percent in wheat [29], 38 percent in maize [30] and 34 percent in soybean [31]. A wild type species, *B. distachyon,* is not subjected to this breeding process that it shows high genetic diversity. Therefore, it has a great advantage as a model organism compared to its close relatives, in particular for the study of agronomic traits.

2.2. STRESS FACTORS

The term "stress" in physiology and biology includes the environmental, biological and physical factors that make pressure on living organisms and elicit a response. Most of the time, stress is necessary for organisms to promote survival and adapt to changing environmental conditions. However, the stress concept in plants is quite different from animals and human beings. Plants, as sessile organisms, undergo some molecular and physiological changes to cope with stress. Related to that, change in gene expression and cellular metabolism occur under stress conditions, causing to a decrease in growth rate and product yield. The basic response mechanisms developed by plants against stress include ion homeostasis, osmoprotectant synthesis, antioxidant activation and synthesis, synthesis of polyamines, nitric oxide (NO) production and hormonal modifications [32]. For example, plants growing in arid areas produce abscisic acid (ABA) that regulates stomatal closure, and thus suppresses photosynthetic activity, respiration and cell growth [33,34]. In addition, plants produce toxic ions as much as reactive oxygen species (ROS) [35]. Plants have developed complex antioxidant defense mechanisms to overcome the damage of the ROS [36].

In the literature, minor changes in plant metabolism and pathways perceived also as stress [37]. However, the term of stress should not be used for simple and rapid rearrangement of metabolic fluxes. The stress concept in plants classified as eu-stress and dis-stress. Eu-stress activates and stimulates plant development, whereas dis-stress (also called distress) is a severe one that causes damage on cell metabolism, and thus has a negative effect on plant development. When plants are exposed to stress, they lose their vitality due to decline in metabolic activities such as accumulation of metabolites, photosynthesis and ion transport (alarm phase). After this stage, most of the plants activate their stress-coping mechanism in order to survive and acclimate to the environment (resistance stage). This leads not only to repair the previous damage on physiological functions but also to hardening of plants. If the stress is prolonged (long-term stress), the plant cannot cope with it and rapidly loses its vitality (exhaustion stage). The time of removal of stressors determines whether the plant will recover (regeneration phase) or die (Figure 2.3).

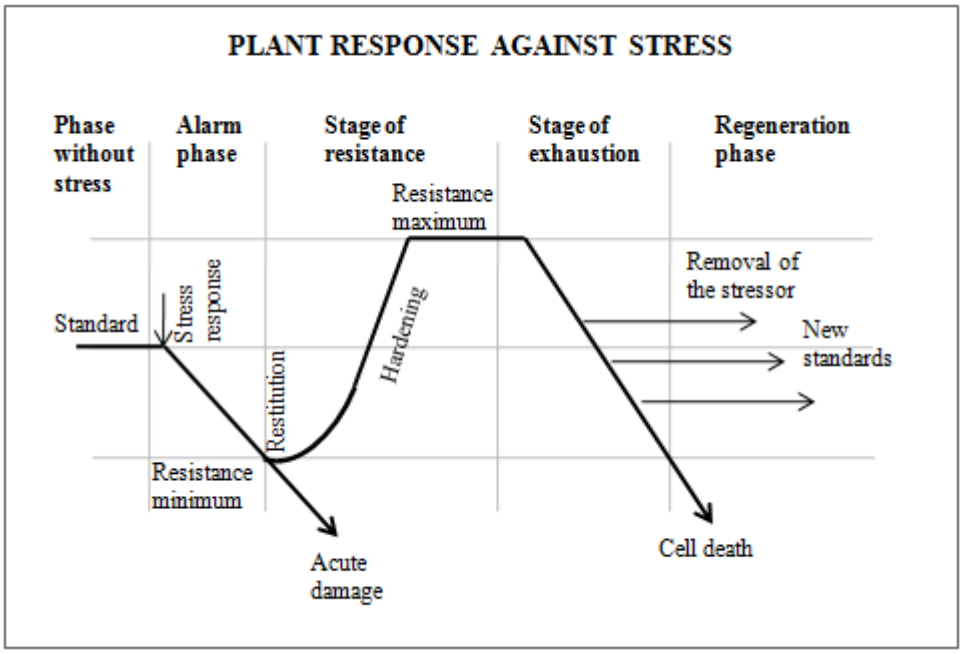

Figure 2.3. Phases of plant responses induced by stress exposure [37].

The most important reason for the researchers to focus on the investigation of stress response mechanisms is that the world population is growing rapidly while agricultural productivity does not show sufficient increase to keep up with the food demand. Sustainability in the production of cereal products, which constitute a large part of the food consumption, has started to be interrupted due to climate change. The world population will reach 9.1 million in 2050. Considering that only 3.5 percent of agricultural areas are not affected by any stress factors, it will not be possible to meet the human food demand [38]. The main reason behind this situation is the environmental factors such as excessive heat and cold, floods, salinity, drought, radiation and lack of minerals that negatively affect the crop yield rather than lack of sufficient agricultural land. These abiotic stress factors may directly affect the plant in terms of physiological and morphological aspects, and may indirectly affect the macro-and micronutrients in the soil [39]. The biotic stress caused by the pathogens also affects the crop yield, though not as much as abiotic stress (Figure 2.4). For this reason, increasing the yield in arable lands and developing biotic and abiotic stress-resistant plant species have gained agricultural importance. Many scientific studies have been performed on the molecular regulation of stress response to elucidate their complex defense mechanisms [40].

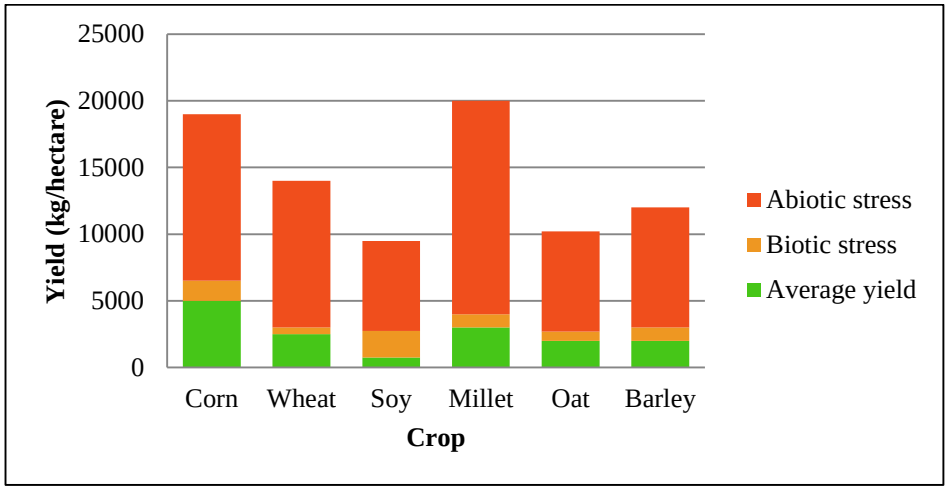

Figure 2.4. Yield losses caused by biotic and abiotic stress factors in economically important crops [41].

2.2.1. Biotic Stress Factors

The damage caused by organisms contains bacteria, fungi, viruses, parasites, weeds plants, insects and is known as biotic stress. Plants defend themselves by developing a variety of response mechanisms. With the break down of the cell wall, pathogens exposed to pathogen-associated molecular patterns (PAMPs) recognizing receptors that locate at extracellular surfaces. Receptors initiate the defense mechanism to block the infection. This immune system is namedPAMP-triggered immunity (PTI) [42]. Moreover, R-gene mediated resistance (major resistance genes) cause oxidative stress, which leads to the production of ROS. ROS production is required for a hypersensitive response (HR) which programme the cell death in order to inhibit the entrance of the pathogen to the nutrients and water in the plant cell [43].

Troubles caused by biotic stressors can be a great challenge for farmers and scientist due to yield loss, and thus, economic concern. Bacteria, fungi nematodes and viruses are considered as primary pathogens that are responsible for plant diseases. Fungi together with bacteria affect different parts of the plant by causing leaf spots and vascular wilts. Nematodes generally attack the plant's root system. It is difficult to diagnose nematode-related diseases because they show similar symptoms with nutrient deficiency. Viruses

damage plants by stunting, chlorosis and malformations, and they produce local lesions [44].

2.2.2. Abiotic Stress Factors

Abiotic stress is an environmental factor, which significantly inhibits the development and yield of the plant. Drought, salinity, flooding, high temperature, chilling, high radiation, heavy metals influence worldwide food security because of the climate change and disruption of the environment by human activities [45]. A study conducted by Boyer in 2008 demonstrated that economically important crops lost more than 50 percent of their yield because of abiotic stress in globally arable lands [41].

The pathways in plant response against abiotic stress includes many interactions and crosstalks [46]. Omics studies and system biology approach have been utilized to understand the regulatory elements in this complex mechanism. To date, reactive oxygen species (ROS) have been assumed as a key product which regulates transcription and enzyme activities [47]. ROS accumulating in different forms and different subcellular locations participate in cellular signaling with plant hormones. Hormones are another prominent regulators of plant stress response. Abscisic acid (ABA) has a critical function in environmental stress, especially in osmotic stress [48].

2.2.2.1. Drought Stress

Among abiotic stress, drought stress is the most common and restrictive factor of crop yield all around the world. The impact of the drought is not only observed on plants but also it decreases the organic carbon in soil and increases erosion and soil salinity [49]. In consequence of severe limitations of drought, the development of new cultivars that are resistant and exhibit high yield potential in these arid areas has become a major challenge in plant science. For this purpose, it is essential to evaluate the mechanism behind the plant resistance against drought stress.

Drought is defined as water deficit or unability of accession to water by the plant as a result of insufficient rainfall, water holding capacity of soil and water lost due to the

evaporation. When plants cannot get enough amount of water from the soil, they undergo some morphological, physiological and biochemical changes. First of all, the initial effect of drought is noticed on germination and seedling growth. The negative effect of drought stress on germination potential, seed vigor, seed weight, yield and biomass have been reported for some crops such as soybean [50], rice [51] and wheat [52]. Mitosis and cell elongation are impaired by poor water flow which results in the reduction of growth [53]. Reduced stomatal closure, turgor pressure and slow rate of photosynthesis are the main reasons for the limitation in leaf expansion and low biomass [54,55]. The very first physiological response to drought is stomatal closure to inhibit water loss because of the transpiration. Rubisco, the key enzyme for carbon fixation, undergoes down-regulation under stress conditions. Furthermore, increased formation of ROS such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), damages lipids and proteins in the cytoplasm and cell wall. Owing to the antioxidant defense mechanism which involves peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), plants can partially scavenge ROS [56]. Accumulation of osmoprotectants or osmolytes containing ammonium compounds, amino acids (proline) and soluble sugars allow the plant to adapt to the demanding conditions [57].

2.2.2.2. Salt Stress

Every year, around 1.5 million ha of irrigated lands and 8.5 million ha of rain-fed land are uninhabited because of salinization [58]. It is estimated that the total sodic soil is 434 million ha while saline soil is 397 million ha according to the FAO/UNESCO soil map of the world (1970-1980) [59]. Salinity is another abiotic stress decreasing crop yield. Soil salinity is observed especially in areas with lack of drainage. Improper use of irrigation, lack of adequate drainage, or high levels of salts in water and scanty rainfall are the reasons for soil salinity.

Salt stress can lead to some morphological, physiological and biochemical changes in plants, similar to drought stress (Figure 2.5). High salt concentration limits essential nutrients and water uptake by plant systems. This situation causes osmotic stress and afterward, ionic stress arises on plants. Under ionic stress, Na and Cl ions compete with nutrients potassium (K^+) and calcium (Ca^{2+}) [60]. After signal perception, the activation of

defense mechanism takes place via gene regulation. Synthesis of antioxidant compounds and osmoprotectants have a crucial function in plant tolerance against salt stress. In addition to that, molecular chaperons (e.g. HSP, LEA, COR) and transcription factors are the most significant regulators in gene expression. Transcription factors, including ZIP, NAC, WRKY, ERF and DREB families, control the expression of stress-responsive genes by binding to the cis-acting element. Overexpression of NAC increased both salt and drought resistance in *A. thaliana* [61], rice and wheat [62]. Upregulation of OsNAC5 and ZFP179 under salt stress controls the accumulation proline, soluble sugars and LEA proteins that have molecular chaperon-like activities [63].

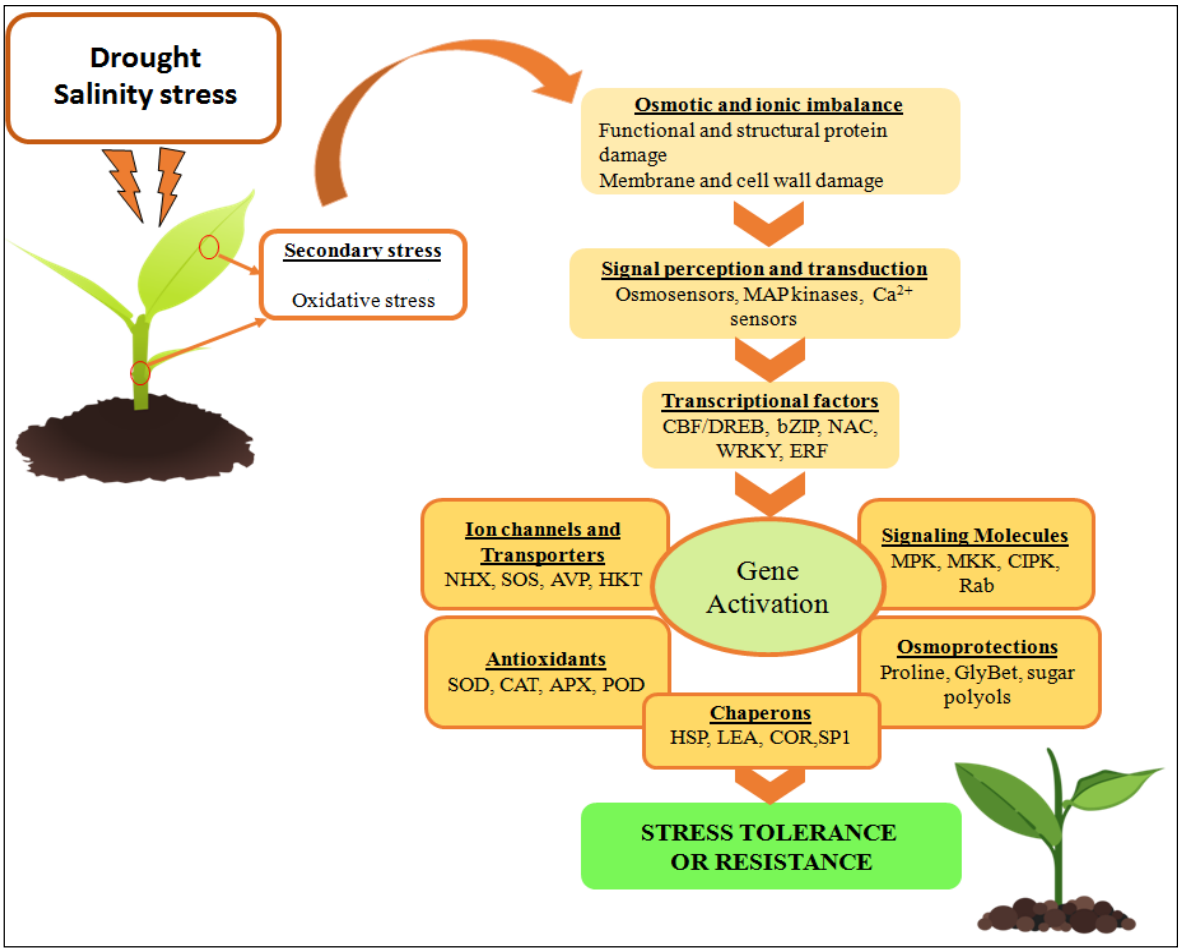

Figure 2.5. Plant response mechanism against drought and salinity stress [64].

2.3. PLANT STRESS RESPONSE: PHYSIOLOGICAL AND BIOCHEMICAL CHANGES

Plants are exposed to abiotic and biotic stress by the environment. In the evolutionary point of view, since plants cannot escape from stress conditions, they have to undergo some physiological and biochemical changes. These changes in cell or tissue basis are induced after signal perception followed by induction of multiple cellular signaling pathways that involves complex interactions or crosstalks. The ability to cope with stress and plant responses significantly depend on the level and duration of stress as well as plant species and its developmental stage [65].

The main effects of osmotic stress are reduction of turgor pressure and water potential, conformational changes in macromolecules and alteration in the cell sap [66]. When plants grow under water-limited conditions or cannot get enough water due to the ion imbalance of the soil, a serious decline occurs in leaf water content. In the 1980s, relative water content (RWC) was introduced as a remarkable measurement for determination of plant water status [67]. High RWC was found to be associated with avoidance from dehydration. Under normal conditions, the rate of RWC of a plant is about 90-95 percent, whereas for dry or wilting plants this rate is about 30 to 40 percent depending on plant species. The decrease in RWC was observed in many plants such as *B. distachyon* [27], wheat [68], rice [69] and barley [70].

Stomatal conductance is another reliable physiological indicator of stress tolerance. Plants tend to close their stomata in order to prevent water loss under water deficiency. However, stomatal closure limits the plant from absorbing CO_2, causing a decrease in the activity of photosynthesis. The diminution in photosynthetic activity is also related to the "non-stomatal" mechanisms that contain structural disruptions of chloroplasts and chlorophyll synthesis [71]. Photosynthetic pigments are important for absorbing light to produce energy and organic compounds. Chlorophyll (Chl) is a major component of chloroplast and it is directly proportional to the photosynthetic rate. The reduction of chlorophyll content is considered as a sign of stress as a result of pigment photo-oxidation and chlorophyll degradation. Under salt stress, reduction in photosynthetic pigments has been reported for different crops such as *Triticum aestivum* [72], *Phaseolus vulgaris* (common bean) [73], sunflower [74], sorghum [75] and *B. distachyon* [76]. Likewise salinity stress, drought

stress can cause damage to chlorophyll and deterioration of thylakoid membranes. In contrast, there are other reports which measured an increase in chlorophyll content or no significant effect under drought stress [77]. Under water deficit conditions, black gram (*Vigna mungo*) showed an increase in chlorophyll content, whereas a decrease in other cultivars [78]. These alterations may depend on the ratio between chlorophyll a (Chl a) and chlorophyll b (Chl b). Chl a and Chl b are two functional pigments that are found in the chloroplast. Chl a is a principal pigment that catches the light used in the reaction while Chl b is the accessory pigment that collects energy and passes it on to Chl a. During chlorophyll degradation, Chl b may be converted into Chl a. This situation may cause an increase in Chl content [79]. Carotenoids are other pigments, which are responsible for bright red, yellow and orange color in many fruits and vegetables. They have a crucial function in the plant immune system and protect the plant from photo damage. Since these pigments radiate at different nanometers (nm) (the maximum red absorption of Chl shifts from 660 to 665 nm; Chl b shifts from 642 to 652 nm; between 400 and 500 nm for carotenoids in the blue spectral range), they can be measured easily with a spectrometer [80].

One of the most substantial stress responses in plants is over-production of compatible organic solutes which protect the plant from stress via the cellular osmotic adjustment, maintaining membrane integrity, stabilization of proteins and removal of reactive oxygen species [81]. These highly soluble, non-toxic compatible solutes include sucrose, polyols, proline, quaternary ammonium compounds and trehalose [82]. Proline accumulates in the cytosol in response to drought and salinity. Proline accumulation was observed in some plants such as *B. distachyon* under salt and cold stress [83], wheat under drought stress [84], sugar beet [85] and *A. thaliana* [76] under salt stress. In a proteomic study of *B. distachyon* under drought stress, proline content accumulated more in shoots than roots [86]. Proline has been also recognized as a molecular chaperone since it stabilizes proteins and acts as a buffer to balance pH. Moreover, proline responsive elements such as PRE [87] and ACTCAT [88] are activated by proline via the expression of stress-responsive genes. In plants, glutamate is considered as the main precursor of proline. Glutamate (Glu) is converted to proline by two enzymes, pyrroline-5-carboxylate reductase (P5CR) and pyrroline-5-carboxylate synthase (P5CS) in cytosol and plastids. Proline is catalyzed into glutamate by Pro dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) in

mitochondria. During production and degradation of proline, pyrroline-5-carboxylate (P5C) occurs as an intermediate product. P5C is in equilibrium with Glu semialdehyde. However, overproduction of P5C may cause toxic effect on the cell because Glu semialdehyde reacts with cellular components [89]. Thus, overexpression of proline may be the precursor of programmed cell death. Studies showed that overexpression of P5CS in tobacco plants has shown increased proline accumulation under salt and drought stress [90], while overexpression of P5CR in tobacco plants has not been resulted in an increase in osmotolerance [91]. The mechanism behind proline accumulation is still unknown and needs to be elucidated.

The production of ROS is one of the earliest biochemical plant response that is stimulated by pathogens or environmental stressors. ROS causes great damage to cells due to the oxidizing DNA, proteins, lipids and enzymes. The increase of Ca^{2+} concentration in the cell leads to accelerated production of NADPH oxidases and they promote ROS generation in the cytosol, mitochondria and chloroplast [92]. Molecular oxygen (O_2) is unreactive in their nature. Nevertheless, O_2 is capable of creating dangerous reactive states (free radicals) such as superoxide radical (O^{-}_2), H_2O_2 or a hydroxyl radical (OH) [4].

ROS tragically damage cell by rising lipid peroxidation and protein degradation. Unsaturated lipids in the cell membrane are transformed into unstable lipid radicals in the presence of OH group. At the end of the reaction, lipid radicals converted malondialdehyde (MDA), which is a highly reactive organic compound. Therefore, MDA assumed as a convenient marker for lipid peroxidation [71]. These deteriorations in the cell membrane also affect the permeability of the cell, i.e. ion transfer. The already impaired ion balance of the plant under drought stress changes the fluidity of the cell membrane. The increase in electrolyte leakage has been regarded as one of the main reason for increased membrane permeability. About 50 percent or more electrical leakage is evaluated as disruption in membrane structure and damaged cell [93].

Plants evolve different mechanisms to remove ROS or avoid ROS production. Avoiding ROS production mechanism might occur in different ways; 1) anatomical adaptations via keeping the stomata in a special structure, 2) physiological adaptations such as CAM and C4 metabolism, and 3) activation of molecular mechanisms to suppress photosynthesis. Besides, the enzymatic or non-enzymatic antioxidant defense mechanism takes place for the removal of ROS. The non-enzymatic antioxidants, which include carotenoids, ascorbic

acid, and glutathione collaborate to keep the photosynthetic membrane integrity. The enzymatic mechanism contains ROS-scavenging enzymes such as SOD, CAT, POD and ascorbate peroxidase (APX). SOD converts O^{-}_{2} coming from photosystem I (PSI) to H_2O_2. CAT removes H_2O_2 by decomposing of hydrogen peroxide to oxygen and water. POD scavenges H_2O_2 by decomposition, accompanying the oxidation of phenolic as well as non-phenolic substrates (RH) (Figure 2.6).

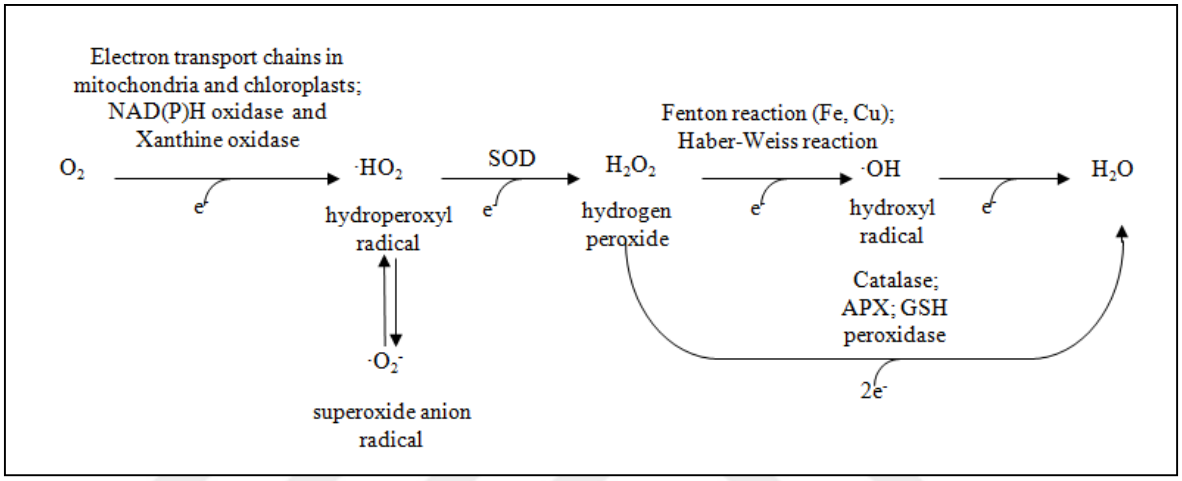

Figure 2.6. The enzymatic mechanism involved in ROS-scavenging [94].

SODs are metalloproteins which work with Zn, Mn, Cu and Fe acting as cofactors. There are three isoenzymes of SODs in plants having different structures and functions. Fe-SOD is present in chloroplast, mitochondria, peroxisomes and cytosol while Cu-SOD is found in cytosol and mitochondria. Mn-SOD is only found in mitochondria and peroxisomes. Fe-SODs and Mn-SODs are the most ancient types of SODs due to their occurrence in both prokaryotes and eukaryotes [95]. Previous studies confirmed that abiotic stress causes to increase in antioxidant activity while some of them showed no change or decrease. SOD activity is triggered under salt stress in *A. thaliana* [96] and *Nicotiana tabacum* [97]. In contrast, SOD activity decreased in *Oryza sativa* [98] and *Allium sativum* [99] under drought stress. In *B. distachyon*, antioxidant defense mechanism was activated by drought stress [100] and different light treatments in a controlled environment [101]. These contradictions showed that the antioxidant defense mechanism can be affected by different factors such as level and duration of stress exposure, stress type and type of plant species.

Therefore, the mechanism underlying the tolerance of plants to stress still needs to be elucidated.

2.4. SEED PRIMING

Seed invigoration or seed enhancement is a common technique, which adds remarkable value to the applied seed during germination and in field performance. It is an umbrella term that comprises many pre-sowing treatments of seed such as seed coating, thermal and hydration treatments. Seed priming, one of the seed hydration technique, is generally used interchangeably with seed invigoration.

Seed priming allows the plant to be prepared for future stress conditions by treating seeds with various chemicals in order to activate and enhance the defense mechanism of the plant [102]. The history of seed application dates back to hundreds of years. Roman naturalist Gaius Plinius Secundus was able to accelerate the germination by keeping the legume seeds in water before planting in the soil [103]. Evelyn (1664) reported that the temperature applied to the seed affected the germination [104], and a century later, Ingenhousz (1779) reported that light had an effect on the emergence of the seedling [105]. In a study by Strogonov (1964), for the first time, it was shown that plants from seeds treated with salt solutions could be more resistant to salt stress [106]. In this respect, the continuously developed seed applications have been used in field crops to increase germination and emergence rate, improve yield and stress adaptation, and obtain better allometric properties [106,107].

2.4.1. The Subcellular Basis of Seed Priming

Efficiency in seed germination is very important for agricultural sustainability. Rapid and uniform seedling emergence indicates synchronized germination. Germination process consists of three stages. In stage I, imbibition occurs by the seeds. Stage II is the phase in which physiological and metabolic activities initiated. During this phase; the DNA is repaired, new mRNAs and proteins are synthesized, the cell cycle is activated and the hormonal balance is modified. In stage III, radicle emergence is observed as a consequence of cell elongation [5]. Low-quality seeds require more time to germinate than the normal

seeds. Seed priming is a very common technique that enables controlled hydration of the seeds. The very first radicle emergence formed through cell expansion and seed hydration. Active cell division starts after radicle emergence. Flow cytometry analysis demonstrated that during priming, the cell cycle is arrested at the G_2 phase and cells are prepared for division [109]. Cell metabolism is active in this phase and the genetic repair mechanism is better than the nonprimed seeds. In seed priming, since cells do not receive enough water required for phase III as a result of the high osmotic potential, phase II of germination process extends. This situation allows for synchronized germination (Figure 2.7) [110].

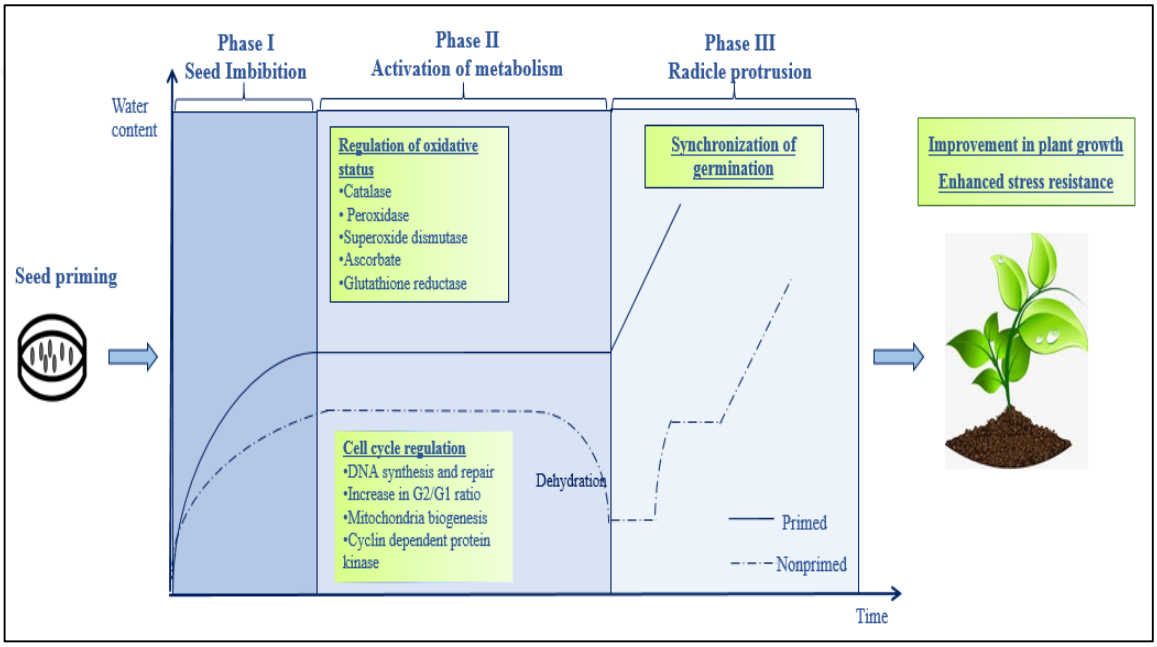

Figure 2.7. Germination phases of unprimed and primed seeds [5].

Studies on gene expression of *Brassica oleracea* via cDNA microarray demonstrated that genes involved in energy production and chemical defense mechanism were up-regulated in osmoprimed seeds [111]. In tomato, priming with polyethylene glycol (PEG) resulted in high ATP/ADP ratio. Also, increased ATP level was conserved for a 4-6 month when stored at 20°C [112]. Priming in pea and leek seeds increased the number of mitochondria. However, this improvement did not affect respiration levels. The association between priming and mitochondrial performance should be further examined [113].

Priming of seeds also improved the integrity of ribosomes. Treating *Brassica oleracea* seeds with PEG prior to sowing enhanced RNA levels of genes encoding elongation

factors and ribosomal subunits, and thus rRNA synthesis [111]. The proteomic analysis of sunflower seeds showed that CAT and SOD levels significantly increased during priming [114]. It is assumed that osmopriming induces oxidative stress on seeds that causes to the generation of ROS. These free-radical scavenging enzymes are quite important to protect cells from cell damage.

Seed dormancy is defined as a state that prevents seeds to germinate under unfavorable conditions such as low and high temperature and lack of water. Endo-β-mannase is the regulatory enzyme in breaking dormancy and weakening of endosperm in the presence of ethylene. Seed priming helps to reduce the inhibitory effect of stressful conditions during germination. Osmopriming with PEG in thermosensitive lettuce improved germination percentage in the absence of ethylene at 35°C [115]. Imbibition of lettuce cv. Dark Green Boston seeds with ACC, the precursor of ethylene), increased the activity of endo-β-mannase. This study suggested that priming was a promising strategy that could be effective on dormancy.

After seeds soaked in low water potential solutions, it is essential to dry seeds back to their original moisture content. There are two different methods for drying seeds as re-drying and surface drying. In surface drying, seeds are dried using filter paper at room temperature and sown, while in re-drying, seeds are dried back to original weight with forced air. The studies in which strategy is appropriate exuded that using surface drying is more effective for seedling emergence, α-amylase activity, shoot and root length [116].

Besides all of these advantages, stress memory is induced by seed priming via transcription factors, post-translational modifications and epigenetic changes. Priming causes moderate stress on seeds and this initial stress exposure leads to cross-tolerance and irreversible transition through radicle emergence. Even seeds are dried back to the original moisture content, primed seeds tend to adapt more efficiently than non-primed seeds for future stress conditions. This concept refers to "stress memory" [117].

Although the effect of seed priming on germination and stress tolerance is clearly observed, the mechanisms underlying stress tolerance, especially antioxidative activity, repairing processes and germination metabolism, still needs to be elucidated.

2.4.2. Seed Priming Methods

Several methods of seed priming have been classified as hydropriming, osmopriming, hormonal priming, biopriming and solid matrix priming.In priming application, the choice of the agent and its concentration, duration, temperature and storage conditions are quite important for the efficiency of the treatment. Optimization of these parameters is required for each cultivar since seed priming is not only species-specific but also genotype-dependent [118].

2.4.2.1. Hydropriming

Hydropriming is the most cost-effective and easy method among other priming strategies. It is simply applied by soaking the seeds into distilled water and re-drying to the original moisture content of the seed. No use of any other chemical makes this method easy and environmental-friendly. However, since the rate of water uptake depends on the seed tissue, in the literature, hydropriming referred to the uncontrolled treatment of the seed [119]. This unequal water uptake may lead to unsynchronized germination and seedling emergence. To surmount these limitations, it is highly important to develop well-defined hydropriming procedure in terms of duration, temperature and volume of the water. Nevertheless, it has been utilized in almost all studies related to seed priming. Hydroprimed seeds demonstrate higher germination rate, seedling emergence, yield and better performance under stress conditions on rice [119,120], wheat [122], maize [123], cotton [124], sunflower [125] and onion [126].

There are many studies struggling with stress resistance on various plant species. However, stress tolerance related to priming might change according to growth stages of plants. The improvement of stress tolerance is obvious after seedling emergence while it may be not clear at the adult stage [5]. For this reason, it is essential to evaluate how long the effect of priming on the plant continues and how it affects the next generations. To elucidate the effect of hydropriming on stress tolerance, Tall Wheatgrass (*Agropyron elongatum*) seeds were kept in distilled water for hydropriming and -1.2 MPa of PEG 6000 for 24 h for osmopriming under dark. The results indicated that hydropriming was a beneficial priming technique for enhancing seedling emergence and hydroprimed *Agropyron elongatum* seeds

showed better performance under drought stress [127]. The positive effect of hydropriming compared with osmopriming and hormonal priming was also investigated in *Brassica napus* under drought stress [128]. In another study, three different rice varieties were soaked in distilled water and different concentrations of NaCl solutions for enhancing salt and drought stress resistance. Priming with NaCl was found to be more effective than hydropriming for the improvement of stress tolerance and seedling vigor using physiological and biochemical analysis [129].

On-farm priming, another type of hydropriming, is preferred mostly by farmers who have low seed yield. The main difference of on-farm priming is that soaking of the seed in water followed by surface drying, and then immediately sowing. The duration of the treatment is the primary focus and avoiding damage of seed is the limit (also called safe limit). Therefore, this technique is known as "low-risk priming" [102].

2.4.2.2. Osmopriming

Osmopriming defined as soaking seeds in low water potential solutions that allow slow imbibition of water by seeds. Although the water potential of priming agents generally change in the range of – 1.0 down to -2.0 MPa [130], temperature and duration of priming treatment must be adjusted according to the cultivar or species. Osmopriming activates early phases of germination while it prevents radicle protrusion [131]. The most commonly used substances for osmopriming include sorbitol, mannitol, polyethylene glycol (PEG), glycerol, and inorganic salts such as NaCl, KNO_3, KCl, KH_2PO_4, K_3PO_4, $CaCl_2$ and $MgSO_4$. In the literature, priming with inorganic salts is also mentioned as "halo-priming" [132].

PEG as the most preferred priming agent was shown to improve germination rate, seedling emergence and tolerance to drought stress in sorghum [133], Chinese cabbage [134], rice[135], barley[136] whereas tolerance to salinity in wheat [137], *Brassica napus* [138] and *Medicago sativa* [139]. In maize, priming with NaCl and $CaCl_2$ showed better field performance under salinity and drought stress, respectively [139,140]. Priming studies with potassium salts also exhibited very promising results. In order to investigate the effect of priming duration and temperature, wheat seeds were soaked in different concentrations of

KCl and K_2HPO_4. The highest germination percentage was observed on 12 h duration and 20ºC, while the most suitable priming agent was determined as K_2HPO_4 [142].

Aside from its osmotic properties, KNO_3 might function as a nutritional supply. A combined application of KNO_3 with ethylene helped nitrate-deficient larnbsquarters (*Chenopodium album* L.) seeds to break dormancy [143]. Also, priming with 1 percent KNO_3 had positive effect on germination instead of 0, 0.25, 0.50, 1.50, and 2.00 percent KNO_3 in rice [144]. In order to investigate the effect of priming on plant height, tiller numbers, plant dry weight, grain yield, total chlorophyll and starch content, wheat seeds were soaked in GA_3 (150 ppm), KCl (1 percent), KNO_3 (3 percent) and cycocel (500 ppm) for 12 hours. KNO_3 (3 percent) was found to be significantly effective under salt stress [145]. In another study, wheat seeds were imbibed with distilled water and (5mM and 10 mM) NaCl and KNO_3 for 24 h to elucidate the soil characteristics (moisture content, pH and electrical conductivity), leaf area. The results demonstrated that distilled water application showed better effects under drought and heat stress, while 5 mM KNO_3 was more effective in salt stress. In two different studies conducted under drought and salt stress, maize seeds were primed with distilled water, KNO_3 and urine for 24 h at 23°C. Priming with KNO_3 enhanced germination rate, germination percentage, seedling and radical length, seedling to radical length ratio [146], total chlorophyll and carotenoid contents, proline amount, and activities of CAT, SOD and POD [147].

2.4.2.3. Hormonal Priming

Improved seed performance can also be achieved by using polyamines and phytohormones such as abscisic acid (ABA), gibberellins, auxins, ethylene, kinetin and salicylic acid (SA). Plant growth regulators have a significant impact on seed metabolism. For instance, ethylene is required for releasing dormancy, and gibberellic acid activates β-amylase for breakdown of starch stored in seed during germination [148]. Seed priming with ascorbic acid and gibberellic acid (GA_3) improved germination and emergence percentage, root and shoot growth and seedling dry weight in wheat [149].

Seed viability and seedling vigor index, root and plumule length, root fresh weight were investigated in primed Broad bean (*Vicia faba*) seeds with 0.0, 0.5, 1.0, and 3.0mM SA, and 0.5 mM SA treatment was found to be a more effective priming treatment in terms of

morphological parameters [150]. Proline and glycinebetaine (GB) content, enzyme activities, germination percentage, shoot and root lengths, fresh and dry weights were analyzed for lentil. Seeds soaked in the solutions of 100 mM NaCl, 0.5 mM SA and combination of 100 mM NaCl and 0.5 mM SA. The higher GB accumulation was observed in shoot rather than in root with SA and salt stress treatments. Also, it was shown that GB accumulation depended on the level of salt tolerance [151]. In order to elucidate the effect of seed priming with salicylic acid (SA) on wheat photosynthesis, wheat seeds were primed with SA (0, 400, 800, 1200, 1600, 2000 and 2400 μM). Gas exchange parameters were measured in three different growth stages such as tillering, heading and grain filling. The highest rate of photosynthesis, transpiration rate and stomatal conductance of plants were found in priming treatment with 1200 μM SA [152]. Similar positive effects of SA were found on wheat under drought stress [152–155], rice under chilling stress [157] and maize under lead stress [158].

Polyamines also protect plants against abiotic stress by acting as free-radical scavenging. In rice seedlings, chilling tolerance improved by using spermidine and 5-aminolevulinic acid as priming agent [159] and seed priming with 10 ppm putrescine solution showed the best effect on germination and early seedling emergence [160].

2.4.2.4. Biopriming

Biopriming is bacterial and fungal inoculation of seeds together with seed imbibition to enhance the plant productivity and resistance against stress conditions. In 1978, Kloepper and Schroth introduced the term plant growth promoting rhizobacteria (PGPR), for the first time [161]. Rhizobacteria, which is closely related to rhizosphere gives more beneficial result rather than film coating and pelleting to manage plant diseases and stress tolerance. PGPR inoculation positively affected the stress resistance and crop production on tomato [162], wheat [163], rice [164] and soybean [165]. Common bacterial antagonists are *Azotobacter chroococcum*, *Pseudomonasflorescens* and *Serratia polymuthica*, while common fungal antagonists are *Trichoderma harzianum*, *Trichoderma viride* and *Clonostachys rosea* [166].

2.4.2.5. Solid Matrix Priming

As an alternative to liquid priming, solid matrix priming (SMP) is applied by mixing seeds with water and solid material. In SMP, the water uptake is controlled, which means it allows the aeration and slow hydration of seeds [167]. In addition, SMP might be more cost-effective treatment compared with osmopriming and hormonal priming since it requires small volume of agents. There are many substances used for solid priming, which can be natural (e.g. vermiculite, charcoal, peatmoss, clay and sand) and artificial (e.g. Agro Lig and Micro Cell). Some chemical and physical properties such as water holding capacity, non-toxicity and low water solubility should be considered during the selection of the material to obtain successful results. Positive effects of SMP on germination rate, seed vigor and seedling growth under abiotic stress accomplished in bean [168] and onion [169]. Besides, the study on maize indicated that solid matrix priming with sand improved antioxidant activity of the plant under salt stress [170].

2.5. AIM OF THE STUDY

The aim of the study is to 1) investigate the effect of hydropriming (distilled water at 4°C and 20°C), osmopriming (1 percent, 2 percent, 3 percent KNO_3) and hormonal priming (0.25 mM, 0.5 mM, 0.75mM SA) at 20°C for 24 hours in dark on germination parameters including final germination percentage (G), mean germination time (MGT), germination index (GI), uncertainty of germination (U), synchronization of germination (Z) and mean emergence time (MET) of *B. distachyon*, and 2) evaluate the stress tolerance mechanism induced by different priming methods in *B. distachyon* plants under salt and drought stress via comparative morphological (plant height and biomass), physiological (chlorophyll content, RWC, RMP, proline content) and biochemical (antioxidant levels such as POD, CAT and SOD) analysis.

3. MATERIALS

3.1. PLANT MATERIAL

Brachypodium distachyon seeds (accession Bd-21) were provided from JGI's collection (DOE Joint Genome Institute, Walnut Creek, California).

3.2. CHEMICALS

Potassium nitrate (Sigma Aldrich, CAS#7757-79-1), Salicylic acid (Duchefa Biochemie, CAS#69-72-7), Sodium chloride (Sigma, CAS#7647-14-5), Bovine serum albumin (Sigma Aldrich, CAS#12657), Coomassie Brilliant Blue G-250 (Sigma Aldrich, CAS#6104-58-1), Methanol (Sigma Aldrich, CAS#67-56-1), 3 percent sulfosalicylic acid (Ricca Chemical, CAS#8115-32), Ninhydrin (Himedia, CAS#485-47-2), Acetic acid (Sigma Aldrich, CAS#64-19-7), Phosphoric acid (Isolab, CAS#7664-38-2), Toluene (Isolab, CAS#108-88-3), Acetone (Sigma Aldrich, CAS#67-64-1), Hydrogen peroxide 30 percent (Merck, K50671809 838), Potassium phosphate dibasic (Sigma, CAS#16788-57-1), Potassium phosphate monobasic (BioShop, CAS#7778-77-0), Ethylenediaminetetraacetic acid (Sigma, CAS#638192-6), Guaiacol (Himedia, CAS#90-05-1), Riboflavin B2 (Sigma Aldrich, CAS#83-88-5), Nitrotetrazolium blue chloride (Sigma Aldrich, CAS#N6876), Sodium carbonate (Sigma Aldrich, CAS#497-19-8), L-methionine (Sigma Aldrich, CAS#M9625).

3.3. GLASSWARE AND CONSUMABLES

Petri dishes 90 mm, Filter paper, Forceps, Micro spoon spatula 180 mm, Parafilm, Micropipettes (0.1-10 µl, 20-200 µl, 100-1000 µl), Micropipette tips (100 µl, 200 µl, 1000µl), Glass measuring cylinder (50 ml, 250 ml, 500 ml and 1000 ml), Foil Roll Aluminium, Plastic Viols, Glass bottles borosilicate (50 ml, 250 ml, 500 ml, 1000 ml and 2000 ml), Scissors, Liquid nitrogen tank, Porcelain mortar and pestle, Glass test tubes, 96 well plate flat bottom, Glass beaker (500 ml and 1000 ml), Hellma Suprasil quartz

absorption cuvettes, Polystyrene Spectrophotometer cuvette, Weighing dishes, Eppendorf tubes (1.5 ml and 2 ml), Microtube racks for 1.5 ml and 2 ml, Serological Pipettes Polysterylene (10 ml), Erlenmeyer's flasks (50ml), Vitrovent magenta boxes, Centrifuge Tubes Conical-Bottom (15 ml and 50 ml), Metal Test Tube Racks.

3.4. EQUIPMENTS

Climatic Chambers (Aralab, S#1799, 1877, 1778), Analytical Balance with 0.0001 and 0.001 precision (Shimadzu), Stereo Microscope (Zeiss Stemi Dv4), Chlorophyll meter (SPAD-502Plus), Conductivity meter (EcoSense EC30A Conductivity, TDS & Temperature Pen), Soil Survey Instrument (ZD-07 4 in 1 Soil Survey Instrument), Incubator (Memmert TUN55), Measuring Tape, Spectrophotometer (Thermo Scientific, Varioskan LUX multimode microplate reader), pH meter (Mettler Toledo, Sevencompact), Spectrophotometer (Genesys 10S UV-Vis), Fume Hood, Water bath (Grant subaqua 12 Plus S#QS1126010), Centrifuge (Eppendorf, S#5811AK563617), Centrifuge (Eppendorf, 5424, S#5424ZR734628), Shaker (Sartorius Stedim Biotech CERTOMATİS Orbital Shaker), Autoclave (Wisd, MeXterile 60), Magnetic Stirrer With Hot Plate (SciLogex MS-H280-Pro), Vortex Mixer (WiseMix, Wisd, VM-10).

4. METHODS

4.1. SEED PRIMING TREATMENTS OF *Brachypodium distachyon*

The experimental set-up was conducted with three different priming methods. Priming conditions were carried out according to Tiryaki et al. (2004), with modifications [171]. The first method was hydropriming. Bd21 seeds were soaked in distilled water and kept at 4°C (HP4) and 20°C (HP20). In osmopriming method, seeds were treated with 1 percent, 2 percent, 3 percent (w/v) KNO_3 solutions at 20°C. For hormonal priming method, seeds were treated with 0.25 mM, 0.5 mM, 0.75 mM salicylic acid (SA) solutions at 20°C. Nonprimed (NP) seeds served as control. All treatments were executed at dark for 24 h, in a petri dish (15 seeds per petri dish with three replicates for each priming treatment) on double layer filter paper using 4 ml priming agent (refer to Table 4.1 for experimental set-up and Figure 4.1 for the seeds in the petri dish during priming).

Table 4.1. Experimental set-up for seed priming applications.

Priming Treatment	NP	HP4	HP20	1% KNO_3	2% KNO_3	3% KNO_3	0.25 mMSA	0.5 mM SA	0.75 mM SA
									

NP: Nonprimed
HP4: Hydroprimed at 4°C
HP20: Hydroprimed at 20°C
Osmopriming (KNO_3) and hormonal priming (salicylic acid-SA) were applied at 20°C.
All seeds were primed for 24 hours at dark.
(9 priming applications X 3 replicates X 15 seeds per dish = 405 seeds)

Figure 4.1. Image of *B. distachyon* seeds during priming application.

After priming, seeds were rinsed with distilled water for 1 min and dried at room temperature for 4 h on filter paper for surface drying [116]. Seeds in each petri dish were weighed before priming to obtain original weight (W_0) and after priming to obtain saturated weight (W_1). Seed imbibition rate calculated as follows [172]:

$$\text{Imbibition rate (\%)} = \frac{W_1 - W_0}{W_0} \times 100 \tag{3.1}$$

4.2. GERMINATION TEST

For germination test, seeds were kept at 15°C at dark in a petri dish on double layer filter paper with 3ml of distilled water. The suboptimal temperature of 15°C instead of optimal condition (25°C) was chosen in order to visualize the effects of priming treatments on germination [114]. Seeds were considered as germinated when radicle length reached 2 mm. The number of germinated seeds was recorded daily until numbers got stabilized (approximately 6 days). Germinated seeds were removed into a freshly prepared petri dish [173].

Six different germination parameters were evaluated in this study. The equations 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7 were calculated as described by Ranal et al. (2009) [174] while the germination index (Equation 3.8) was calculated by the formula determined by Ajmal Khan and Ungar (1998) [175].

Germinability of *Brachypodium* seeds was evaluated by estimating germination percentage:

$$\text{Germination percentage (\%)} = \frac{\text{number of germinated seeds}}{\text{number of seeds sown}} \times 100 \tag{3.2}$$

Mean germination time (MGT) was calculated by the expression:

$$\text{MGT} = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i} \tag{3.3}$$

where n_i: number of seeds germinated in the i^{th} day, t_i; day from the start of the experiment to the i^{th} day; and k: last day of germination.

Uncertainty of the germination (U) was calculated by the expression:

$$U = -\sum_{i=1}^{k} \log_2 f_i \tag{3.4}$$

where k: number of germination days and f_i is the relative frequency of germination expressed by:

$$f_i = \frac{n_i}{\sum_{i=1}^{k} n_i} \tag{3.5}$$

Synchrony of the germination (Z) was calculated by the expression:

$$Z = \frac{\sum_{i=1}^{k} C_{n_i,2}}{C_{\sum n_i,2}} \tag{3.6}$$

where n_i: number of seeds germinated in the i^{th} day and $Cn_{i,2}$ is the combination of the seed germinated in the i^{th} day, two by two, expressed by:

$$C_{n_i,2} = n_i(n_i - 1)/2 \tag{3.7}$$

Germination index (GI) of seeds was calculated by the expression;

$$GI = \sum_{i=1}^{k} \frac{n_i}{t_i} \tag{3.8}$$

4.3. SEEDLING EMERGENCE AND GROWTH CONDITIONS OF *Brachypodium distachyon*

The germinated *B. distachyon* seeds were planted to peat-soil mixture (2:1) and grown for 3-4 weeks. The number of emerged seedlings was recorded daily. Mean emergence time (MET) was calculated according to the equation of Ellis and Roberts (1981) [176]:

$$\mathrm{MET} = \frac{\sum D_n}{\sum n} \tag{3.9}$$

where D_n is the number of days counted from the beginning of the emergence and n is the number of seedlings emerged on day D.

Approximately one-month-old plantlets were transferred into plastic pots, each pot contained 3 plantlets. The plants were grown under a controlled environment (16/8 h light/dark photoperiod at 25/22 °C, a photosynthetic photon flux of 320 μmol m-2 s-1 at canopy height provided by fluorescent lamps and relative humidity 60 percent-70 percent) in the greenhouse [14].

4.4. ABIOTIC STRESS TREATMENTS

For each stress treatment (drought and salinity) and control group, the seedlings were divided into 9 different priming applications under which there were 5 replications consisting of 45 plants (Table 4.2).

Table 4.2. Experimental set-up for abiotic stress treatments in the greenhouse.

Priming / Stress	NP	HP4	HP20	1% KNO_3	2% KNO_3	3% KNO_3	0.25 mM SA	0.5 mM SA	0.75 mM SA
Control									
Salinity									
Drought									

There were three plants in each pot (total 405 plants) and pooled for sample collection (except biomass analysis).
(9 priming applications X 5 replicates X 3 stress treatments = 135 pots)

4.4.1. Drought Treatment

The soil survey instrument is a device that measures the moisture level, temperature and pH of the soil with a probe (refer to Table 4.3 for soil moisture levels) [177]. The control group was continued to be irrigated on a daily basis at'wet+' level while drought stress group was withheld from water. The moisture content of the soil exposed to drought stress was measured with this device every day and the moisture level was monitored (Figure 4.2). The day on which the soil's moisture level decreased from 'Wet +' to 'Nor' level (in 4 days), it was considered as the first day of drought stress and the plants were exposed to stress for the next 12 days. Fresh leaf samples were collected at the end of the 12^{th} day of drought stress treatment and three plants in each pot were pooled [27].

Table 4.3. Moisture levels of soil measured by Soil Survey Instrument.

Soil Survey Instrument Levels	Stress Levels	Moisture Range (%)
Wet+	Well Watered	70-80
Wet	Light Drought	60-70
Nor	Moderate Drought	50-60
Dry	Severe Drought	35-50
Dry+	Extreme Drought	25-30

Figure 4.2. Soil moisture measurement of *Brachypodium distachyon* with Soil Survey Instrument in the greenhouse.

4.4.2. Salinity Treatment

For salinity stress, the experimental group was irrigated with 50 ml of 320 mM NaCl solution for 14 days whereas control group was irrigated with distilled water every day until sample collection.

The measurement of electrical conductivity (EC) was performed by a conductivity meter (EcoSense EC30A Conductivity, TDS & Temperature Pen) to determine the level of dissolved ions. At the end of salinity treatment, the soil in 3 pots of each priming groups was removed from roots and mixed. 25 ml of double distilled water was added onto 10 g of soil sample and shaken for 30 min at 175 rpm at 25°C. Samples were filtered with filter paper and EC was measured by using conductivity meter according to the manufacturer's instructions [178].

4.4.3. Biomass and Plant Height Measurements

During stress treatment, measurement of plant height was performed from top of the soil to the top of the main plant stem. Measurement was performed with 9 replicates using 3 pots (3 plants per pot) for each priming treatment.

Above-ground biomass was used to assess the organic matter levels of plants produced by photosynthesis. For biomass measurements, three plants from one pot were harvested separately from the ground surface. Samples were weighed for fresh weight (FW) and then dried in an incubator for 24 h, at 80°C to obtain the dry weight (DW). Biomass of plants was evaluated as DW, which is the total organic matter of the plant.

4.4.4. Relative Water Content Analysis

Relative water content (RWC) was measured to evaluate the water status of leaves according to Barrs and Weatherly (1962) [179]. Approximately, 500 mg of leaf tissue was collected from both unstressed (control) and drought stress-treated plants at the end of the 12^{th} day and immediately weighed to measure fresh weight (FW). Then leaves were soaked in distilled water in a petri dish and kept for 4 h at RT in dark. After hydration, leaves were

dried gently with filter paper and then weighed for turgid weight (TW). Samples were dried for 24 h at 80°C and weighed to obtain dry weight (DW). Three individual seedlings from one pot were pooled and three biological replicates were measured. The RWC was determined as follows:

$$\text{RWC}\ (\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100 \tag{3.10}$$

4.4.5. Determination of Relative Membrane Permeability

Relative membrane permeability (RMP) was indicated by the extent of electrolyte leakage of the cell membrane and its determination in our study was carried out according to Wang et al. (2014) with modifications [180]. 200 mg of fresh leaf samples were collected and cut into pieces as 1 cm in diameter. Leaf pieces were submerged into 20 ml distilled water and kept at 50°C for 2 h. The initial conductivity (EC_1) from each sample was measured with a conductivity meter (EcoSense EC30A Conductivity, TDS & Temperature Pen). Then, the samples were boiled for 10 min, cooled to room temperature and second conductivity (EC_2) was measured. The relative membrane permeability was calculated by the given formula:

$$\text{RMP}\ (\%) = \frac{\text{EC}_1 - \text{EC}_0}{\text{EC}_2 - \text{EC}_0} \times 100 \tag{3.11}$$

where EC_0 is the electrical conductivity of distilled water.

4.4.6. Chlorophyll Content Analysis

4.4.6.1. Utilization of Chlorophyll Meter

During stress treatment, the chlorophyll content of plants was measured daily by using chlorophyll meter (SPAD-502Plus). The chlorophyll meter measures the transmittance of infrared (940 nm) red (650 nm) radiation through the leaf. The same leaf a plant was

placed toward the emitting window of the instrument and SPAD value was recorded immediately. The calculation was done according to the given formula [181]:

$$\text{Chl content } (\mu g/cm^2) = 5{,}99 \times e^{0{,}0493 \times SV} \quad (3.12)$$

where SV is the SPAD value obtained from chlorophyll meter.

4.4.6.2. Acetone Extraction

Although measurement of chlorophyll content via SPAD values is an easy and non-destructive method, there are some disadvantages. Chlorophyll content may depend on several factors such as the distribution of chlorophyll, leaf thickness, anatomical trait and leaf water content at the measurement side. In this respect, it is necessary to measure chlorophyll content based on the absorption of light by acetone that contains chlorophyll extracts. In this study, chlorophyll content analysis was performed via both chlorophyll meter and acetone extraction, and methods compared as well. Exactly 50 mg of leaf tissue was homogenized with liquid nitrogen and added into 5 ml of ice-cold acetone (80 percent, v/v). Homogenate was centrifuged at 5000 rpm for 2 min and the supernatant was transferred to another falcon tube. Pellet was re-extracted with 5 ml of ice-cold acetone (80 percent, v/v) and centrifuged again. Supernatants were pooled in a falcon tube and final volume was adjusted to 12.5 ml with ice-cold acetone (80 percent, v/v). The absorbance was read with a spectrophotometer at 663, 645 and 480 nm for chl a, chl b and carotene, respectively. Chlorophyll content was calculated according to Arnon's equation [182]:

$$\text{Chl content (mg/ml)} = (20{,}2 \times A645) + (8{,}02 \times A663) \times \left(\frac{12{,}5}{1000 \times 0{,}05}\right) \quad (3.13)$$

$$\text{Carotene content}\left(\frac{\text{mg}}{\text{g FW}}\right) = A480 + (0{,}114 \times A663 - 0{,}638 \times A645) \quad (3.14)$$

where A480, A645 and A663 are absorbance values at 480, 645 and 663 nm, respectively.

4.4.7. Determination of Free Proline

Proline content was measured using a rapid colorimetric method, and the method was conducted according to Bates et al. (1973), with modifications [183]. Approximately 500 mg of leaf tissue was completely homogenized with 10 ml of 3 percent sulfosalicylic acid and shaken at 160 rpm at room temperature for 3 h in dark. The extract was centrifuged at 5000 rpm for 5 min. Acid-ninhydrin solution was prepared by warming 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid. Two ml of the supernatant was added into2 ml of acid-ninhydrin solution. Additionally, 2 ml of glacial acetic acid was added into the reaction mixture. The reaction mixture was incubated in boiling water for 1 h. The reaction was terminated with ice immediately. The red color was observed due to the reaction between proline and ninhydrin (Figure 4.3a). Proline in the reaction mixture was extracted by adding 4 ml of toluene and waited for separation of the solution into 2 phases. (Figure 4.3b). The absorbance of the chromophore-containing toluene (upper part) was read at 520 nm with a spectrophotometer. The standards were prepared with 0-80 μg/ml proline solution. The procedure was carried out as described above with standard solutions and purified proline was used to generate a standard curve for the quantification of sample concentrations (Figure 4.4). The proline content was calculated as follow:

$$\text{Proline } (\mu\text{mol proline/g FW}) = \frac{\frac{(\mu\text{g proline/ml}) \times \text{ml of toluene}}{115{,}5\ \mu\text{g}/\mu\text{mole}}}{\text{g of sample}/5} \tag{3.15}$$

where the value of 115,13 is the molecular weight of proline and the value of 5 is the dilution factor.

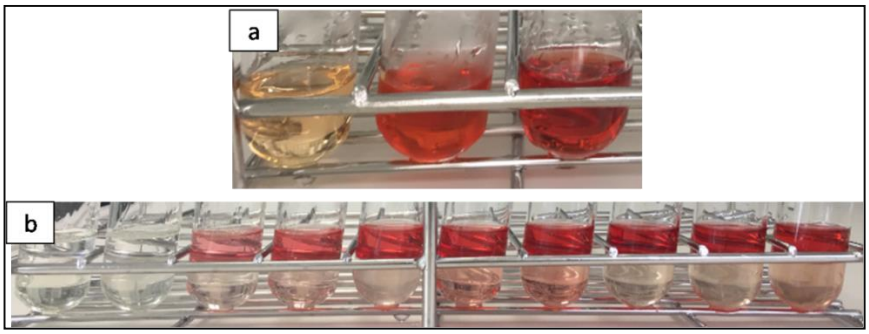

Figure 4.3. Observation of red color occurred by the proline-ninhydrin reaction. (a) Difference between colors in control, salinity and drought, (from left to right), (b) The separated toluene phase of proline standards with 0, 0.005, 0.01, 0.02, 0.03, 0.04, 0,05, 0.06, 0.07, 0.080 mg/ml of proline.

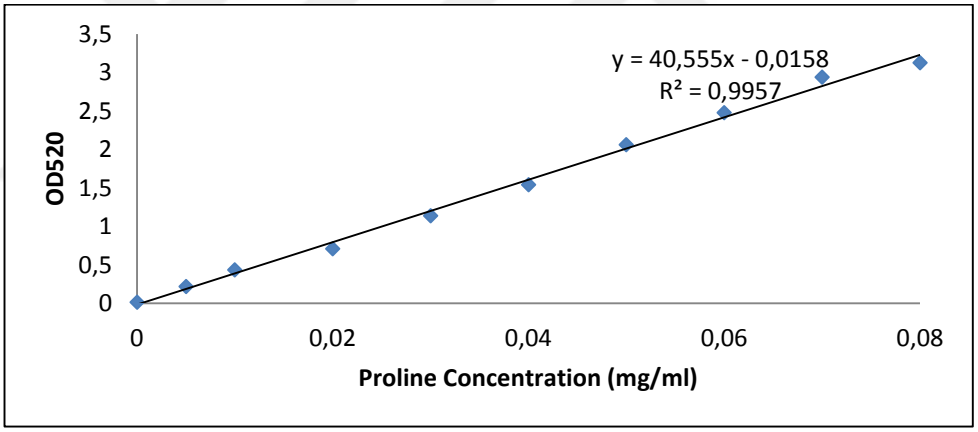

Figure 4.4. Construction of standard curve for proline content determination.

4.4.8. Enzyme Extraction and Antioxidant Enzyme Activity Assays

The crude enzyme was extracted according to Choudhary et al. (2012) with modifications [184]. 100 mM potassium phosphate (K-P) buffer was prepared by adding 100 mM KH_2PO_4 solution into 500 ml of 100 mM K_2HPO_4 solution until pH 7.6 and stored at 4°C. 500 mg of leaf tissue was grounded with liquid nitrogen and transferred into 50 ml falcon tube. 5 ml of K-P buffer including 0,1 mM Titriplex III (Na-EDTA) was added onto grounded leaf tissues. Homogenate was centrifuged at 4000 rpm for 15 min. The

supernatant was transferred into 2 ml eppendorf tube and centrifuged again at 12000 rpm for 10 min.

Crude protein concentration was determined according to Bradford (1986) using bovine serum albumin as standard [185]. The enzyme extract was stored at -80°C until used for antioxidant activity assay.

4.4.8.1. CAT Activity Assay

CAT activity was measured depending on the consumption of H_2O_2 (extinction coefficient of 36 $M^{-1}cm^{-1}$) into water and oxygen (Figure 4.5 and Figure 4.6).

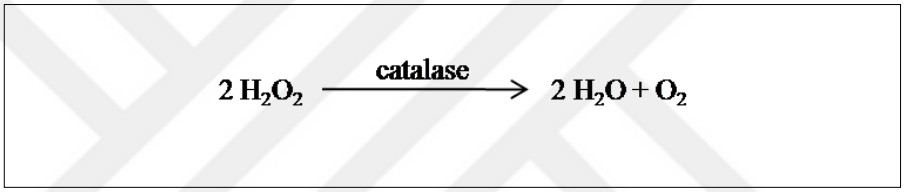

Figure 4.5. The enzymatic reaction of CAT [186].

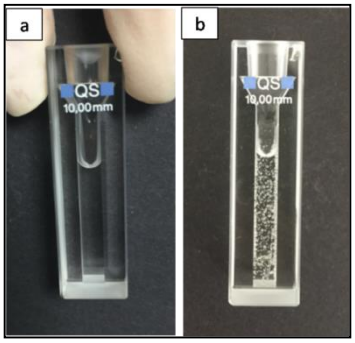

Figure 4.6. CAT activity assay via spectrophotometric method (a)K-P buffer and H_2O_2 solution without CAT enzyme, (b) Formation of O_2 in the presence of CAT enzyme.

The reaction mixture contained 800 µl of 100 mM K-P buffer (pH 7.6) including 0.1 mM Na-EDTA and 100µl of 100 mM H_2O_2. The reaction initiated by adding 100 µL of enzyme extract (1:4 dilution with100 mM K-P buffer, pH 7.6) and the rate of change in absorbance was read at 240 nm for 30 sec [187]. CAT activity was calculated as follow:

$$\text{CAT activity (U/min/mg protein)} = \frac{(\Delta A/\varepsilon) \times 1000 \text{ ml}/0{,}5 \text{ min}}{\text{mg protein /ml}} \quad (3.16)$$

where ΔA is the change in absorbance at 240 nm and ε is the extinction coefficient of H_2O_2.

4.4.8.2. POD Activity Assay

POD activity was measured based on the formation of tetraguaiacol (extinction coefficient of 26,6 $M^{-1}cm^{-1}$) by using guaiacol as a hydrogen donor as shown in Figure 4.7. POD activity was measured by a colorimetric method depending on the orange color of the solution formed by tetraguaiacol (Figure 4.8).

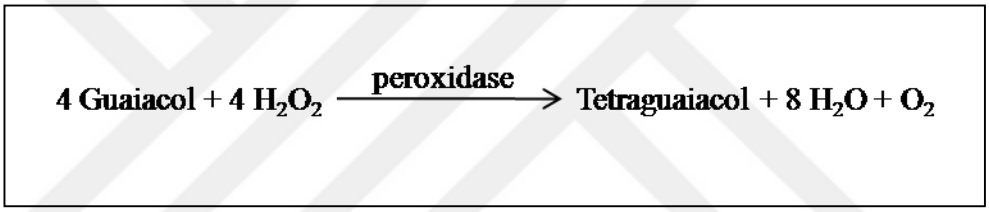

Figure 4.7. The enzymatic reaction of POD [188].

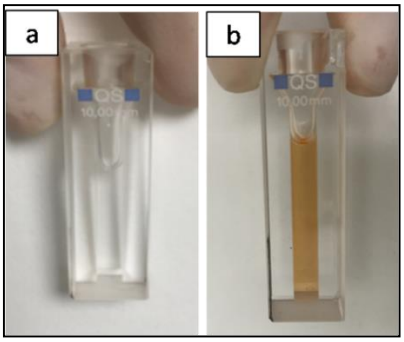

Figure 4.8. POD activity assay via spectrophotometric method. (a) K-P buffer, guaiacol and H_2O_2 solution without POD enzyme, (b) Formation of orange color by tetraguaiacol in the presence of POD enzyme.

The reaction mixture contained 1 ml of 100 mM K-P buffer (pH 7.6) including 0.1 mM Na-EDTA, 10 µl of 12 mM H_2O_2 and 17 µl of 20 mM guaiacol. The reaction was initiated

by adding 34 µl of enzyme extract and the rate of change in absorbance was read at 470 nm for 30 sec [189]. POD activity was calculated as follows:

$$\text{POD activity (U/min/mg protein)} = \frac{(\Delta A/\varepsilon) \times 1000 \text{ ml}/0{,}5 \text{ min}}{\text{mg protein /ml}} \qquad (3.17)$$

where ΔA is the change in absorbance at 470 nm and ε is the extinction coefficient tetraguaiacol.

4.4.8.3. SOD Activity Assay

SOD activity was determined by a spectrophotometric method based on the SOD-mediated inhibition rate of nitroblue tetrazolium reduction (NBT) to the blue formazan. In the reaction, NBT^+ is reduced to monoformazan (MF^+) by $O_2^{\cdot -}$ (Figure 4.9). The monoformazan converts to the end product diformazan by the same sequence of reaction. In the presence of SOD, it shifts the reaction to the left. Therefore, the light color at the end of the reaction indicates higher SOD activity. The reduction of NBT^+ is inducted by irradiated riboflavin and L-methionine [190].

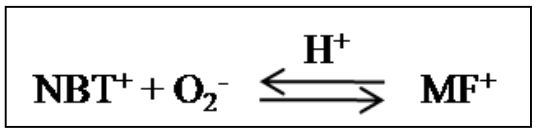

Figure 4.9. The enzymatic reaction of SOD [191].

The reaction mixture contained 2.9 ml of 100 mM K-P buffer (pH 7.6), 0.5 ml of 0.5 M Na_2CO_3 (pH 10.2), 0.5 ml of 1.2 M L-methionine, 0.5 ml of 74.6 mM NBT, 0.5 ml of 0.045 mM riboflavin which was prepared in a glass test tube at dark. The reaction was initiated by adding 100 µl of enzyme extract. Two different blanks were prepared without enzyme extract and one of them was kept at dark while the other was kept under light (Figure 4.10). All samples were incubated under light for 10 minutes. After incubation, the reaction stopped at dark and the absorbance was read at 560 nm with spectrophotometry [187].

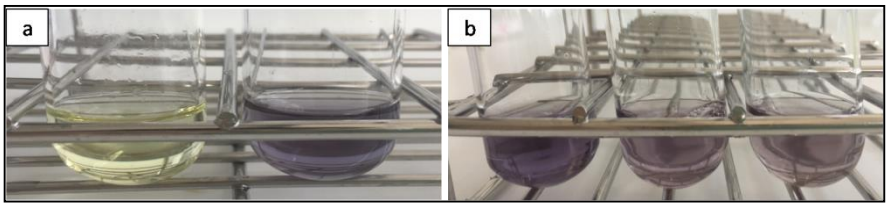

Figure 4.10. The color change by the formation of blue formazan. (a) Blank kept in dark and under light (from left to right), (b) Control, salinity and drought (from left to right).

SOD activity was calculated as follow:

$$\text{SOD activity (U/mg FW)} = \frac{\%\ \text{inhibition of NBT}/V_r/V_t}{\text{mg of fresh weight}} \quad (3.18)$$

where Vr is the volume used in the reaction, Vt is the total volume of the enzyme extract, FW is the fresh weight of leaf tissue.

4.4.9. Statistical Analysis

The results are expressed as mean±SE (n=3). Analysis of the data was performed with one-way ANOVA and Student' t-test by using MS Excel 2007.

5. RESULTS

5.1. EFFECT OF SEED PRIMING ON SEED IMBIBITION RATE

Seed hydration is one of the most critical factors that force the seed to germinate. Different priming agents and temperature affect the water uptake of the seed. In this study, priming with distilled water (at different temperatures), KNO_3 and SA had a significant effect on seed imbibition rate (Figure 5.1). The highest imbibition rate was observed in seeds treated with distilled water at 20°C, whereas the lowest imbibition rate was observed in seeds treated with distilled water at 4°C. In all KNO_3 and SA primed seeds (Hereinafter, seeds/plants primed with 1 percent KNO_3 refer to as 1 percent KNO_3 seeds/plants; seeds/plants primed with 2 percent KNO_3 refer to as 2 percent KNO_3 seeds/plants; seeds/plants primed with 3 percent KNO_3 refer to as 3 percent KNO_3 seeds/plants; seeds primed with 0.25 mM SA refer to as 0.25 mM SA seeds/plants; seeds primed with 0.5 mM SA refer to as 0.5 mM SA seeds/plants; seeds primed with 0.75 mM SA refer to as 0.75 mM SA seeds/plants), the imbibition rate was higher when compared with HP4 seeds and lower when compared with HP20 seeds. No significant difference was observed between 2 percent and 3 percent KNO_3 seeds and they were significantly lower than 1 percent KNO_3 seeds due to its high osmotic potential. There was no difference between 1 percent KNO_3 and SA seeds, while 2 percent and 3 percent KNO_3 seeds showed significantly lower imbibition rate than 0.5 mM SA application. No significant difference was observed in imbibition rate between SA concentrations.

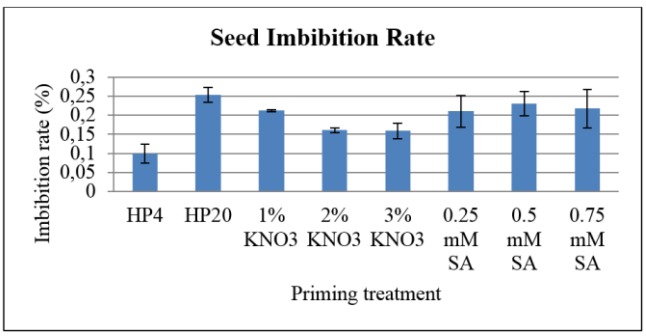

Figure 5.1. Effect of priming treatments on seed imbibition rate. Error bars represent the standard deviation of three biological replicates.

5.2. EFFECT OF SEED PRIMING ON GERMINATION AND SEEDLING EMERGENCE

After seed priming, seeds were exposed to germination test and they were observed daily by a stereo microscope (Figure 5.2).

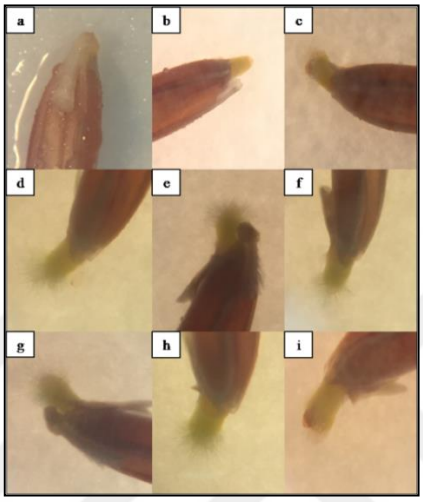

Figure 5.2. Stereo microscope images of *B. distachyon* radicles on the first day of the germination test (a) NP, (b) HP4, (c) HP20, (d) 1% KNO_3, (e) 2% KNO_3, (f) 3% KNO_3, (g) 0.25 mM SA, (h) 0.5 mM SA, (i) 0.75 mM SA priming treatments.

For germination test, germination percentage (G), mean germination time (MGT), germination index (GI), the uncertainty of germination (U) and synchronization of germination (Z) was calculated for all treatments. After germination, seeds were transferred into soil and mean emergence time (MET) of seedlings was also calculated (Table 5.1).

The final germination percentage was 100 percent in all treatments, which means all seeds were germinated. The Student's t-test showed that MGT was significantly different in all priming treatments in which high MGT indicated slow germination (H_0=Seed priming has no effect on germination parameters prior to sowing and stress treatment; physiological and biochemical changes under salt and drought stress, p=0,05. H_1= Seed priming significantly affects germination parameters prior to sowing and stress treatment; physiological and biochemical changes under salt and drought stress, $p \leq 0,05$). The lowest MGT value was observed in 2 percent KNO_3 seeds. HP4 seeds had a tendency to

germinate later with the highest MGT value among primed seeds. The lowest GI value was observed in NP seeds. There was no significant difference between KNO_3, SA and their concentrations. Priming with 2 percent and 3 percent of KNO_3 has significantly reduced the uncertainty of germination when NP used as control. The higher value of Z demonstrates better synchronization of germination. In this study, NP seeds showed the lowest Z value, however, it was not significantly different from HP4, HP20, 1 percent KNO_3 and 0.5 mM SAseeds according to the Student's t-test which means that priming with 2 percent, 3 percent KNO_3, 0.25 mM and 0.75 mM SA was ensured synchronized germination of *B. distachyon* seeds (refer to Appendix for estimated p-values).

Table 5.1. Seed priming effect on germination and seedling emergence. (G; germination percentage, MGT; mean germination time, GI; germination index, U; the uncertainty of germination, Z; synchrony of germination, MET; mean emergence time).

Treatment	**G**	**MGT**	**GI**	**U**	**Z**	**MET**
NP	100	3,78±0,367	4,13±0,341	1,37±0,364	0,38±0,077	1,44±0,102
HP4	100	3±0,200*	5,26±0,363**	1,31±0,196	0,44±0,115	1,04±0,077**
HP20	100	2,64±0,115**	6,01±0,299***	1,22±0,206	0,41±0,048	1,02±0,039**
1% KNO_3	100	2,36±0,115**	6,61±0,509**	0,84±0,241	0,57±0,160	1±0***
2% KNO_3	100	2,11±0,139***	7,22±0,346***	0,39±0,420*	0,81±0,214*	1±0***
3% KNO_3	100	2,27±0,038*	6,83±0,167***	0,82±0,099*	0,59±0,067*	1±0***
0.25 mM SA	100	2,36±0,077**	6,61±0,192***	0,93±0,078	0,52±0,055*	1±0***
0.5 mM SA	100	2,33±0,204**	6,78±0,536***	0,85±0,371	0,61±0,177	1,02±0,039**
0.75 mM SA	100	2,33±0,176**	6,79±0,192***	0,89±0,166	0,58±0,070*	1,02±0,039**

The data were expressed as mean±SE with three replicates
*significant at p≤0,05
** significant at p≤0,01
***significant at p≤0,001 which were determined by Student's *t*-test, compared with NP.

After germination test, seeds were transferred first to peat-soil mixture in the viols as shown in Figure 5.3. The number of the emergence of seedlings was recorded daily in order to calculate mean emergence time. Data obtained from *B. distachyon* seedlings demonstrate that MET was significantly affected by all priming treatments (Figure 5.4).The maximum time of emergence was observed in NP seedlings (p≤0,01 for HP4, HP20, 0.5mM SA and 0.75 mM SA; p≤0,001 for 1 percent, 2 percent, 3 percent KNO_3 and 0.25 mM SA

seedlings). The lowest MET value was noted in all KNO_3 and 0.25 mM SA primed seedlings.

Figure 5.3. *B. distachyon* seedlings in plastic viols. NP seedlings were shown as an example.

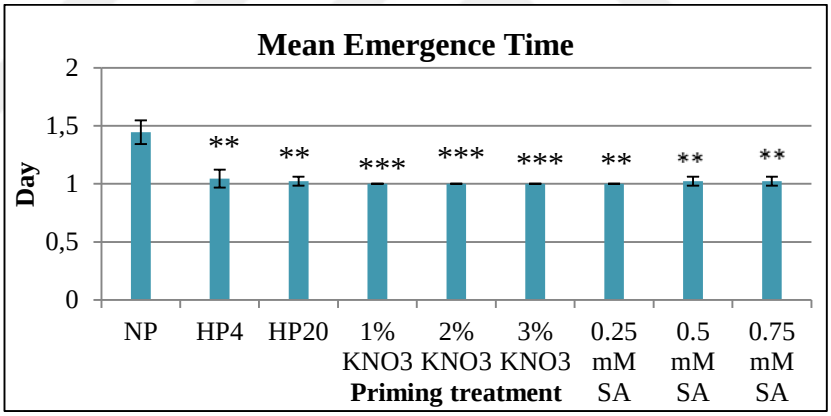

Figure 5.4. Effect of seed priming on mean emergence time (MET) of *B. distachyon* seedlings. Error bars represent the standard deviation of three biological replicates. (** significant at p≤0,01, ***significant at p≤0,001 which were determined by Student's t-test, compared with NP).

5.3. ABIOTIC STRESS TREATMENT ON *Brachypodium distachyon*

Plants were grown in the greenhouse to provide the same environment for the appropriate treatment of drought and salt stress and assess the seed priming effect on these abiotic stress. The moisture loss of soil was monitored by a soil survey instrument. The moisture

level of both control and salinity group did not change and remained 'wet+' level, while the moisture level of drought-stressed decreased to 'dry' and 'dry+' level. The reduction in soil water status in drought is clearly observed with respect to control and salinity (Figure 5.5).

Figure 5.5. Water status of soil in (a) untreated, (b) salt-treated and (c) drought-treated plants.

The electrical conductivity (EC) of the soil was measured to indicate whether the plants were exposed to salt stress or not. The EC values were significantly increased in all salt-treated soils compared with control as expected.

5.4. EFFECT OF SEED PRIMING ON *Brachypodium distachyon* MORPHOLOGY UNDER ABIOTIC STRESS

In this study, it was noticed that different priming agents, their concentrations and temperature affected *B. distachyon* morphology under salt and drought stress. Figure 5.6 represents the plants by comparing each priming treatment in itself under salt and drought stress. There was considerable phenotypic variation within stress treatments. Figure 5.7 represents the plants that express the variation between priming treatments under certain stress treatment.

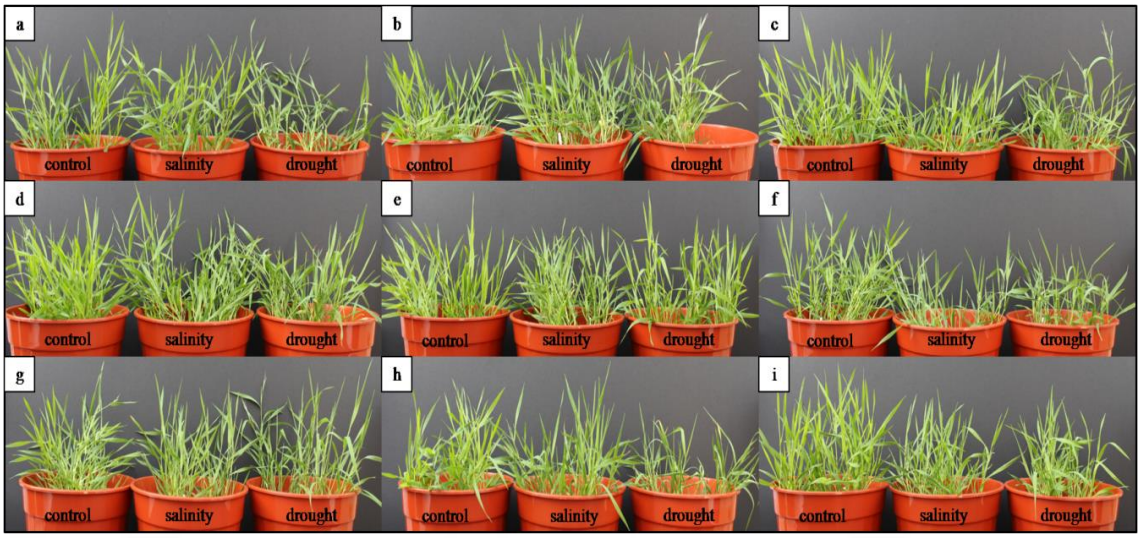

Figure 5.6. The effect of each priming treatment on *B. distachyon* at the end of the 14th day of the stress treatment.(a) NP, (b) HP4, (c) HP20, (d) 1% KNO_3, (e) 2% KNO_3, (f) 3% KNO_3, (g) 0.25 mM SA, (h) 0.5 mM SA, (i) 0.75 mM SA treatments.

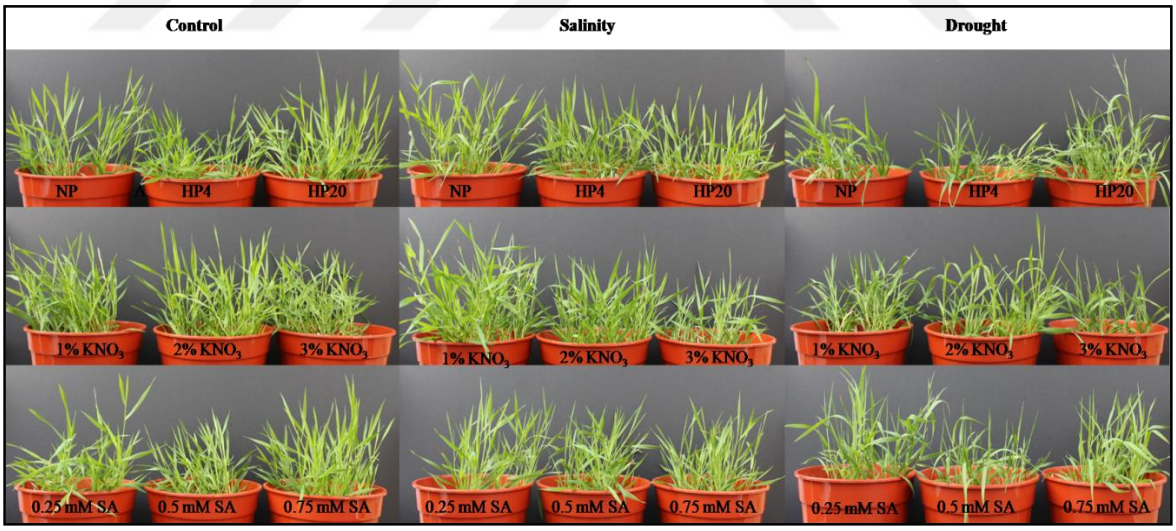

Figure 5.7. The variation between priming treatments on *B. distachyon* under certain stress treatment (control, salinity and drought).

5.4.1. Plant Height

The growth rates of plants were evaluated by measuring their height over time. If total plant height at the end of the stress treatment was considered, plant height significantly increased in NP plants under salinity and drought stress, while plant height decreased in 2 percent KNO_3 plants under salinity, compared with control (Table 5.2). The situation in NP plants thought to be due to the fact that the plant passed to the heading stage earlier than the other plants. On the other hand, it was important to point out the decrease in plant growth rate in the last days of salt and drought stress when compared with untreated plants.

Table 5.2. Plant height of *B. distachyon* at the first and last day of stress treatments.

Plant height (cm)						
Treatment	Control		Salinity		Drought	
	Day1	Day14	Day1	Day14	Day1	Day14
NP	19,05±1,494	24,17±1,665	22,94±1,602	28,5±3,513*	22,06±1,018	26,5±1,740*
HP4	18,78±1,251	24,17±0,833	19,11±1,000	23,06±0,918	19,94±0,822	24,22±1,347
HP20	20,5±0,726	26,11±1,347	21,28±0,694	24,67±0,866	21,22±1,134	26,78±0,855
1% KNO_3	21,56±1,873	26,72±1,855	23,78±1,110	28,22±1,058	22,06±1,171	26,06±0,948
2% KNO_3	21,72±0,948	27,11±0,255	20,11±1,828	23,33±2,088*	21,33±0,601	26,00±1,093
3% KNO_3	19,39±1,251	25,67±1,641	19,89±1,005	24,56±0,419	19,33±0,441	23,89±1,584
0.25 mM SA	20,16±0,601	27±1,093	20,28±0,192	26,06±2,071	21,78±1,437	28,06±2,124
0.5 mM SA	18,44±2,175	24,56±3,155	19,11±0,385	22,28±0,096	18,89±0,347	22,06±0,481
0.75 mM SA	18,67±1,000	23,28±0,694	19,22±0,948	22,11±1,206	19,22±0,509	23,06±1,347

The data were expressed as mean±SE with three replicates
*significant at p≤0,05which were determined by Student's *t*-test, compared with control

Among priming treatments, priming with HP20, 1 percent KNO_3, 2 percent KNO_3, 3 percent KNO_3 and 0.25 mM SA significantly increased plant height under optimal conditions (unstressed conditions), compared with NP and 0.75 mM SA plants. The lowest

growth rate was observed in plants which were primed with distilled water at 4°C, 0.5 mM and 0.75 mM SA (Figure 5.8).

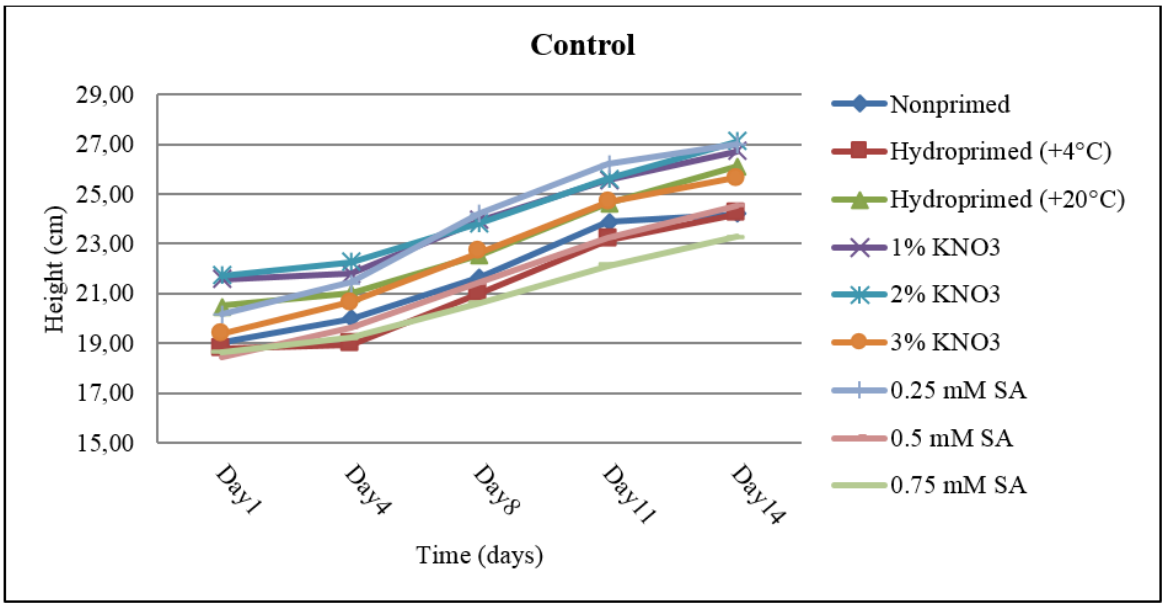

Figure 5.8. Seed priming effect on the plant height of untreated *B. distachyon.*

Under salt stress, maximum plant height was recorded in NP and 1 percent KNO_3 plants followed by 0.25 mM SA plants whereas the minimum height was measured in HP4, 0.5 mM and 0.75 mM SA plants, similar with the control group. Results showed that priming with 1 percent KNO_3 had a positive effect on plant growth under salt stress (Figure 5.9).

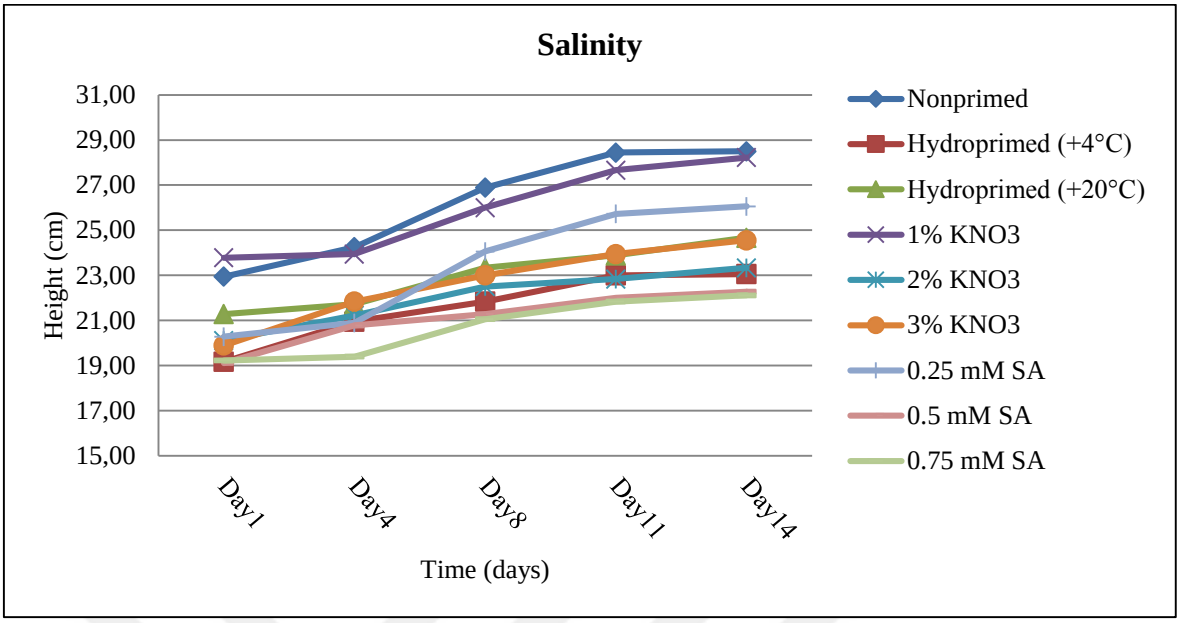

Figure 5.9. Effect of seed priming on the plant height of salt-treated *B. distachyon.*

Under drought stress, plant height in 0.5 mM and 0.75 mM SA plants was significantly lower than all plants. Contrastly, the maximum plant height was recorded in NP, HP20, 1 percent KNO_3 and 0.25 mM SA plants (Figure 5.10).

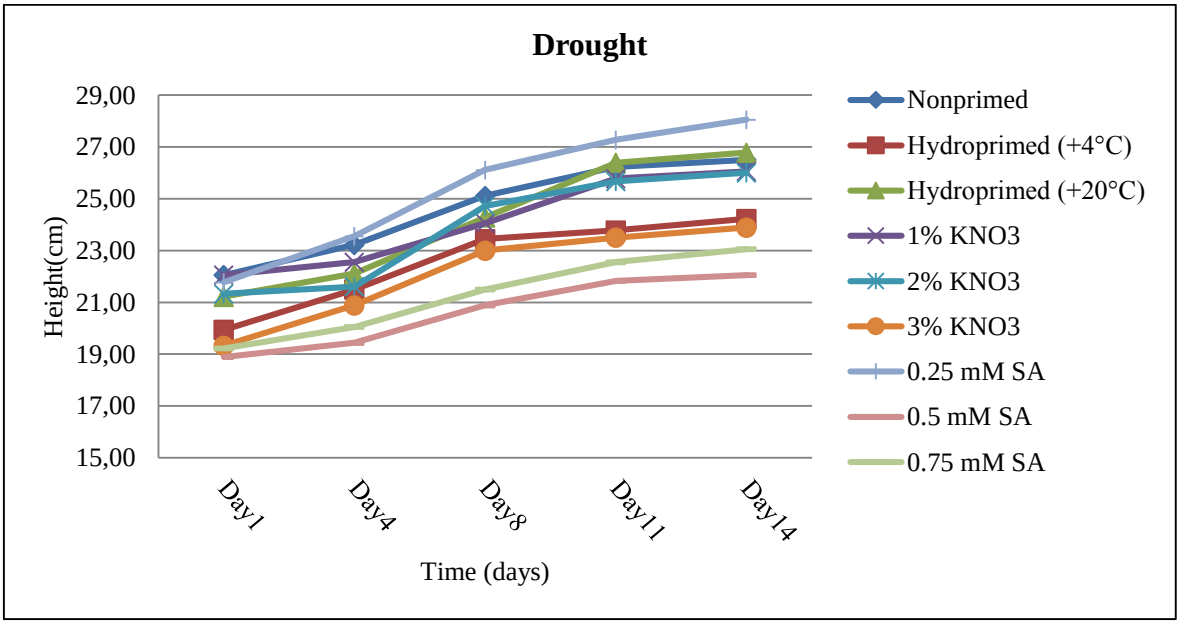

Figure 5.10. Effect of seed priming on the plant height of drought-treated *B. distachyon.*

5.4.2. Biomass

Above-ground biomass of *B. distachyon* was evaluated by measuring the dry weight of the plant (Table 5.3). Three plants in one pot were used as biological replicates. Due to the high standard deviation, significant results could not be observed. However, according to the Student's *t*-test, there was an increase in biomass in NP and HP4 plants under drought and salt stress, respectively ($p \leq 0,05$). Seed priming did not affect biomass under salt and drought stress compared with control. Nevertheless, the biomass of HP4 ($p \leq 0,01$), 1 percent KNO_3 ($p \leq 0,05$) and 0.75 mM SA ($p \leq 0,05$) plants were higher under the stress of salinity than the drought.

Table 5.3. Above-ground biomass of *B.distachyon* under salt and drought stress.

Biomass (mg)			
Treatment	Control	Salinity	Drought
NP	343,33±79,036	484,93±86,555	487,47±43,050*
HP4	390,33±97,659	601,1±7,940*	496,27±21,442
HP20	521,13±84,358	501,33±49,769	653,87±143,181
1% KNO_3	455,43±120,496	605,97±55,851	380,47±94,547
2% KNO_3	456,53±65,858	416,83±44,408	368,77±143,645
3% KNO_3	290,76±30,335	416,97±156,524	301,6±83,772
0.25 mM SA	320,4±83,600	328,7±25,417	386,4±47,373
0.5 mM SA	438,83±101,189	291,1±94,067	365,87±33,400
0.75 mM SA	369,53±112,734	420,7±119,984	402,3±57,610

The data were expressed as mean±SE with three replicates
*significant at $p \leq 0,05$which were determined by Student's *t*-test, compared with control

In the control group, an increase in biomass was observed in HP20 plants when compared with NP, 3 percent KNO_3 and 0.25 mM SA plants and also increase in 2 percent KNO_3 plants when compared with 0.25 mM SA plants. Seed priming did not affect above-ground biomass of *B. distachyon* under optimal conditions. Under salinity stress, seed priming effect on biomass varied. HP4 and 1 percent KNO_3 plants had the highest biomass within all priming treatments. There was no difference between SA treatments and they had the

lowest biomass. Under drought stress, 3 percent KNO_3, 0.25 mM and 0.5 mM SA plants had lower biomass compared with other priming treatments and NP plants. The highest biomass was recorded in HP4 and 1 percent KNO_3 plants (Figure 5.11 and Appendix).

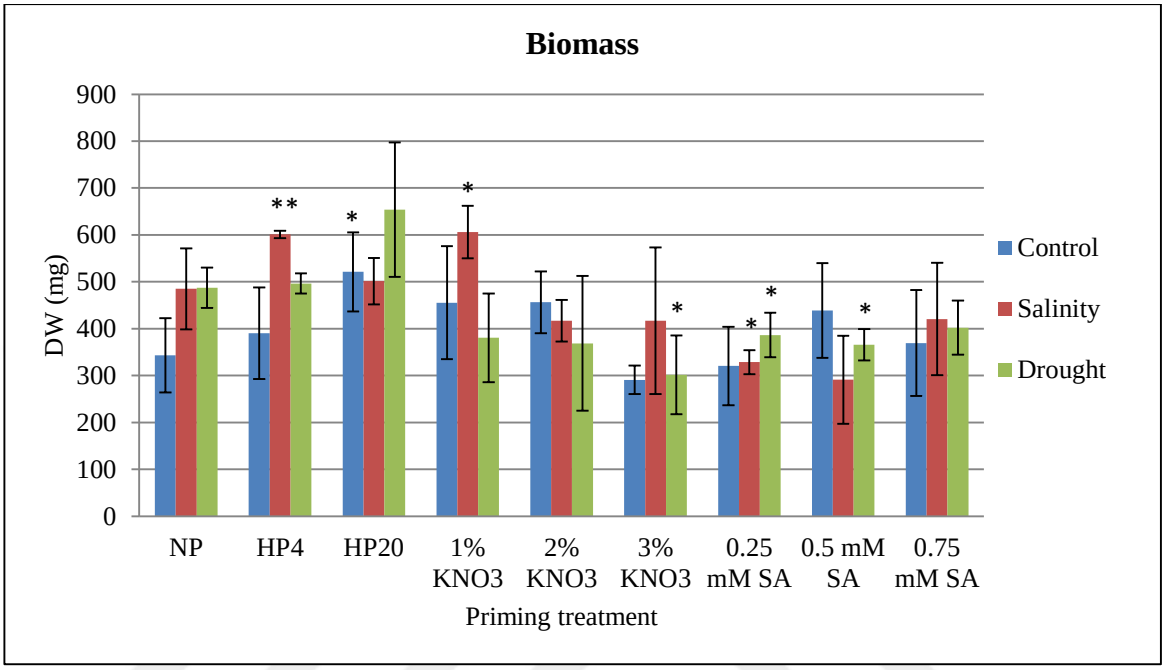

Figure 5.11. Effect of seed priming on above-ground biomass of *B. distachyon* under salt and drought stress. (* significant at p≤0,05, ** significant at p≤0,01 which were determined by Student's t-test, compared with NP).

5.5. PHYSIOLOGICAL ANALYSIS OF SEED PRIMING ON *Brachypodium distachyon* UNDER ABIOTIC STRESS

5.5.1. Relative Water Content

Relative water content (RWC) was used to evaluate plant water status based on cellular hydration and dehydration on *B. distachyon*. Since we could not obtain stable results in our previous studies on salt stress, this experiment was carried out on untreated (control) and drought-treated plants. RWC was significantly affected by drought stress on some priming treatments. RWC in NP, HP20 1 percent KNO_3 and 2 percent KNO_3 plants was not significantly changed in drought stress compared with control which means that these priming methods might allow plants to keep their water holding capacity. On the other

hand, RWC in HP4, 3 percent KNO_3, 0.5 mM SA, 0,75 mM SA ($p \leq 0,05$) and 0.25 mM SA ($p \leq 0,01$) plants were significantly decreased under drought stress (Table 5.4).

Table 5.4. Relative water content (RWC) measurement of untreated and drought-treated *B. distachyon.*

Treatment	**RWC (%) Control**	**RWC (%) Drought**
NP	87,59±2,069	88,20±1,292
HP4	93,04±2,539	78,11±2,787*
HP20	94,69±0,924	92,97±0,333
1% KNO_3	94,33±0,624	88,25±8,437
2% KNO_3	92,73±1,166	86,48±5,338
3% KNO_3	94,93±0,593	77,67±5,827*
0.25 mM SA	94,33±1,051	73,72±5,663**
0.5 mM SA	95,01±0,436	70,62±9,230*
0.75 mM SA	94,56±0,954	80,20±3,457*

The data were expressed as mean±SE with three replicates
*significant at $p \leq 0,05$
** significant at $p \leq 0,01$which were determined by Student's *t*-test, compared with control

If the effect of the priming was evaluated between the control groups, the RWC value of NP plants was quite low compared to all priming treatments. It indicated that seed priming with distilled water, KNO_3 and SA increased water holding capacity of *B. distachyon* under optimal conditions. The response of plants to drought conditions varies considerably between priming treatments. RWC value was significantly increased in HP20 plants while it decreased in HP4, 3 percent KNO_3 and SA treatments under drought stress when compared with NP plants. There was no difference between NP, 1 percent KNO_3 and 2 percent KNO_3 plants. The highest RWC values were obtained from HP20, 1 percent KNO_3 and 2 percent KNO_3 plants. The lowest RWC was recorded in 0.5 mM SA plants with approximately 25 percent decrease under drought stress. There was no difference between SA treatments (Figure 5.12 and Appendix). Therefore, seed priming with distilled water and low concentrations of KNO_3 at 20°C along with NP had a positive effect on the water holding capacity of *B. distachyon* under drought conditions.

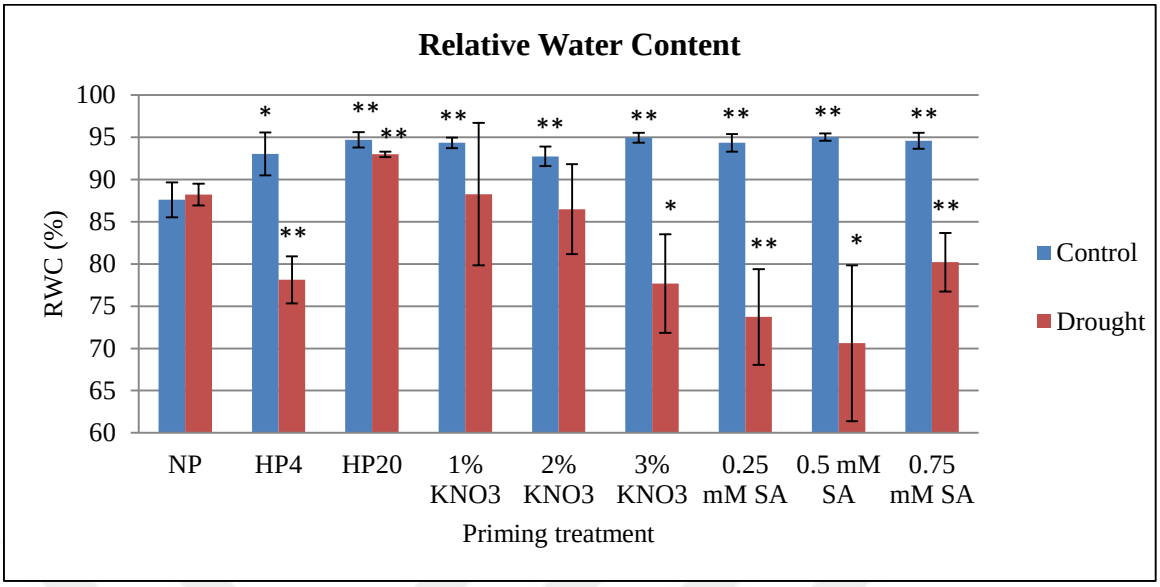

Figure 5.12. Seed priming effect on RWC of *B. distachyon* under drought stress. (* significant at p≤0,05, ** significant at p≤0,01 which were determined by Student's t-test, compared with NP).

5.5.2. Relative Membrane Permeability

Relative membrane permeability was evaluated based on the electrolyte leakage of leaf tissue. The increase in RMP value means further deterioration of the fluidity of the cell membrane. In this study, RMP was significantly increased in HP20, KNO_3 and SA priming treatments under salt stress and decreased in all KNO_3 and SA treatments except 0.5 mM SA under drought stress. NP and HP4 plants were not affected by both salt and drought stress (Table 5.5).

Table 5.5. Relative membrane permeability (RMP) measurement of untreated, salinity-treated and drought-treated *B. distachyon.*

Treatment	RMP (%) Control	RMP(%) Salinity	RMP (%) Drought
NP	76,72±11,546	77,99±8,943	73,42±1,418
HP4	78,97±12,679	72,37±5,240	85,63±6,796
HP20	88,87±5,748	112,59±13,404*	81,58±11,165
1% KNO_3	93,68±0,967	112,98±11,704*	86,06±0,495*
2% KNO_3	96,70±1,177	94,43±4,899	85,91±4,743*
3% KNO_3	108,39±1,712	128,46±4,017***	88,48±5,595**
0.25 mM SA	104,26±2,173	117,12±2,852**	90,08±2,127***
0.5 mM SA	101,95±5,212	125,05±1,957***	95,96±0,505***
0.75 mM SA	98,18±2,571	124,78±1,037***	88,79±4,071***

The data were expressed as mean±SE with three replicates
*significant at p≤0,05
** significant at p≤0,01
***significant at p≤0,001 which were determined by Student's *t*-test, compared with control

Between the control groups, the least damage in the cell membrane was observed in NP, HP4 and HP20 plants while the most damage was in 3 percent KNO_3 and 0.25 mM SA plants (Figure 5.13). For KNO_3 priming treatments, it was noted that the RMP value increased with higher concentrations. There was no difference between 0.5 mM SA and 0.75 mM SA plants. For salt-treated plants, there was a statistically significant increase of RMP value in all KNO_3 and SA treatments together with HP20 when compared with NP and HP4 plants due to the high ion imbalance. Priming with 2 percent KNO_3 had a positive effect on RMP between all KNO_3 and SA treatments. The highest RMP values were obtained from 3 percent KNO_3, 0.5 mM SA and 0.25 mM SA plants. In plants treated with SA, the lowest RMP value was recorded in 0.25 mM SA and there was no difference between 0.5mM SA and 0.75 mM SA plants. Consequently, NP, HP4 and 2 percent KNO_3 was the best treatments in terms of RMP value under salt stress. For drought-treated plants, priming treatments significantly increased RMP compared with NP plants, except HP20 plants. RMP value was higher in 0.5 mM SA plants with respect to 1 percent KNO_3, 2 percent KNO_3 and 0.25 mM SA plants while it was higher in 0.25 mM SA than 1 percent KNO_3 plants (Appendix). Therefore, lower concentrations should be preferred for osmopriming and hormonal priming under drought stress.

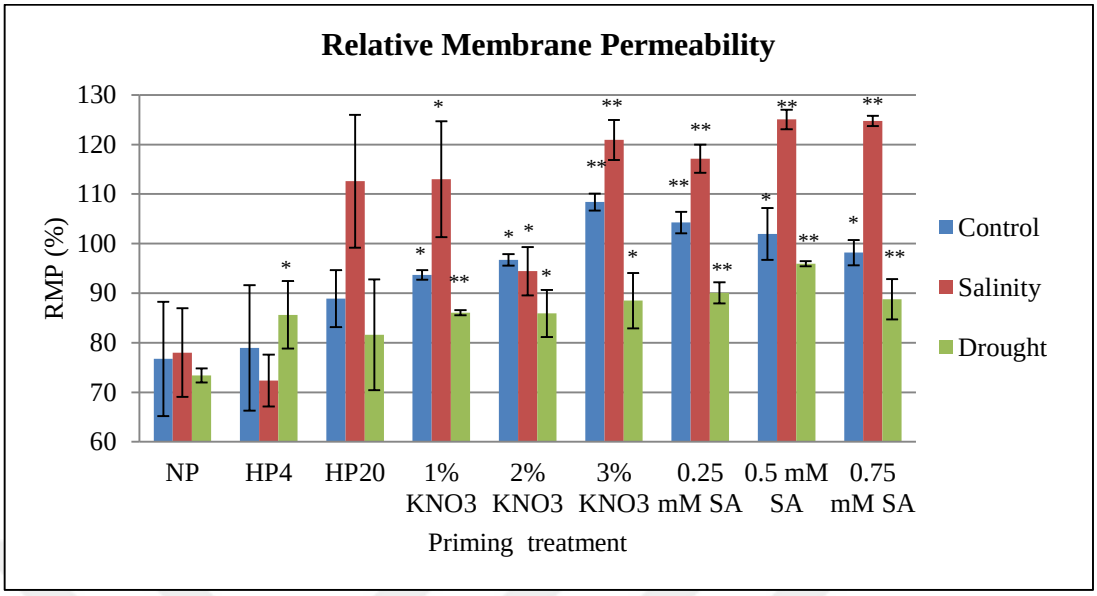

Figure 5.13. Effect of seed priming on RMP of *B. distachyon* under salt and drought stress. (* significant at p≤0,05, ** significant at p≤0,01 which were determined by Student's t-test, compared with NP).

5.5.3. Determination of Photosynthetic Pigments

5.5.3.1. Chlorophyll Content Measurement via Chlorophyll Meter

The chlorophyll (chl) content of plants was measured daily with a chl meter in order to evaluate the chl production behavior of the plant under abiotic stress and control conditions. The results of chl content via SPAD values are given in Table 5.6. During stress treatment, there was a fluctuation in chl content, and finally, it decreased at the end of the abiotic stress treatment in all plants including control. However, if the chl content at the last day of the stress treatment was considered, the higher chl content was measured in HP20 plants under salt (p≤0,05) and drought stress (p≤0,01) compared with control. In addition, priming with 1 percent KNO_3 had a positive effect on chl content under drought stress (p≤0,05). Between different SA concentrations, there was a significant reduction in 0.5 mM SA (p≤0,05) and 0.75 mM SA (p≤0,01) plants. Therefore, higher concentrations of SA might be an effective priming treatment for chl content in *B. distachyon* under salt stress.

Table 5.6. Chlorophyll content via SPAD values of *B. distachyon* at the first and last day of stress treatment.

Chlorophyll content of *B. distachyon* via SPAD values (mg/cm^2)						
Treatment	Control		Salinity		Drought	
	Day1	Day14	Day1	Day14	Day1	Day14
NP	65,65±4,429	42,01±3,334	61,29±8,578	44,60±8,922	63,84±4,343	49,14±2,723*
HP4	67,86±1,176	47,19±0,885	62,35±5,425	46,92±6,284	56,52±3,705	51,41±1,267**
HP20	53,38±3,943	39,84±0,300	59,86±5,534	46,75±3,902*	51,66±5,322	50,13±3,091**
1% KNO_3	55,79±5,495	41,89±2,269	58,44±1,534	41,28±2,349	59,66±6,985	46,88±0,582*
2% KNO_3	46,19±3,318	42,48±1,040	60,15±8,368	42,16±2,072	57,18±7,824	44,46±2,693
3% KNO_3	64,35±6,054	42,55±0,850	62,02±5,200	46,65±3,496	65,38±3,115	46,89±3,513
0.25 mM SA	51,74±1,028	42,22±1,785	56,09±6,853	41,65±5,798	57,07±5,240	42,99±3,551
0.5 mM SA	53,44±3,289	44,34±1,149	62,73±2,421	41,65±0,626*	53,68±5,972	44,91±5,494
0.75 mM SA	56,69±3,280	42,72±2,218	47,63±2,699	35,53±1,541**	61,60±4,740	42,58±2,093

The data were expressed as mean±SE with three replicates
*significant at p≤0,05
** significant at p≤0,01 which were determined by Student's *t*-test, compared with control

According to the Student's t-test, chl content in HP4 plants was higher than other priming treatments and NP plants under unstressed conditions. The lowest chl content was observed in HP20 plants (Figure 5.14). Under salt stress, the lowest chl value was observed in 0.75 mM SA with respect to HP4, HP20 and all KNO_3 priming treatments. The highest chl content was found in 3 percent KNO_3 plants under salt stress (Figure 5.15). Priming with 0.25 mM SA and 0.75 mM SA was significantly reduced chl content of *B. distachyon* under drought stress when compared with NP, HP4 and HP20. In addition, low concentrations of KNO_3 might be preferred for higher chl content under drought stress. There was no significant difference between KNO_3 and SA treatments except 1 percent KNO_3 (Figure 5.16 and Appendix).

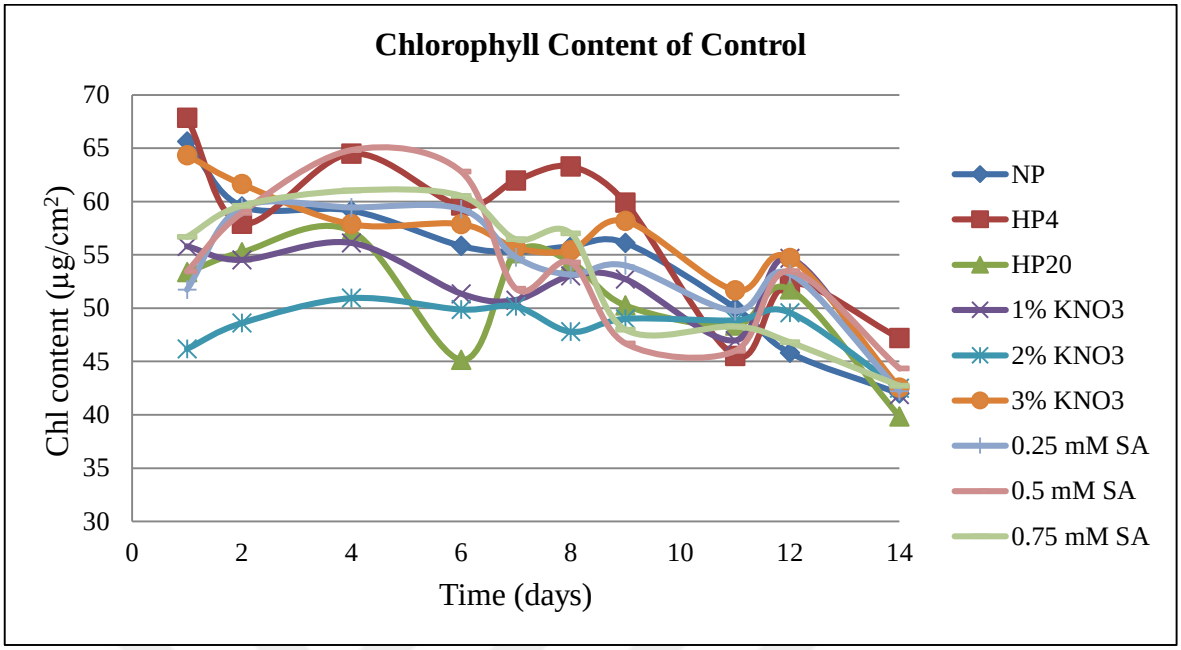

Figure 5.14. Seed priming effect on chlorophyll content via SPAD values of untreated *B. distachyon.*

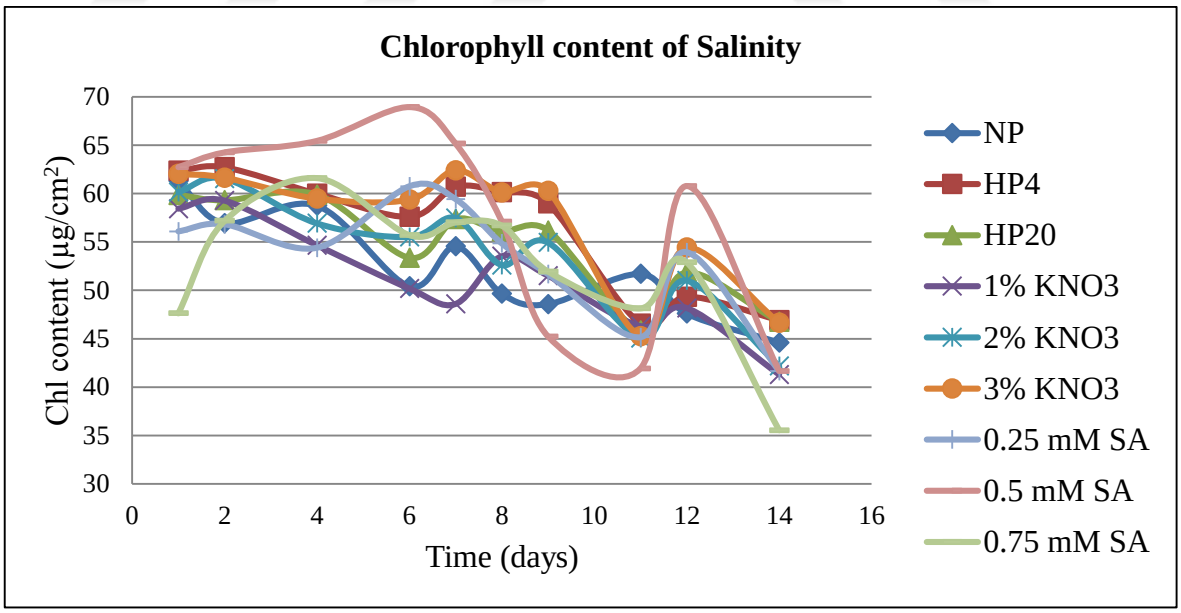

Figure 5.15. Seed priming effect on chlorophyll content via SPAD values of salt-treated *B. distachyon.*

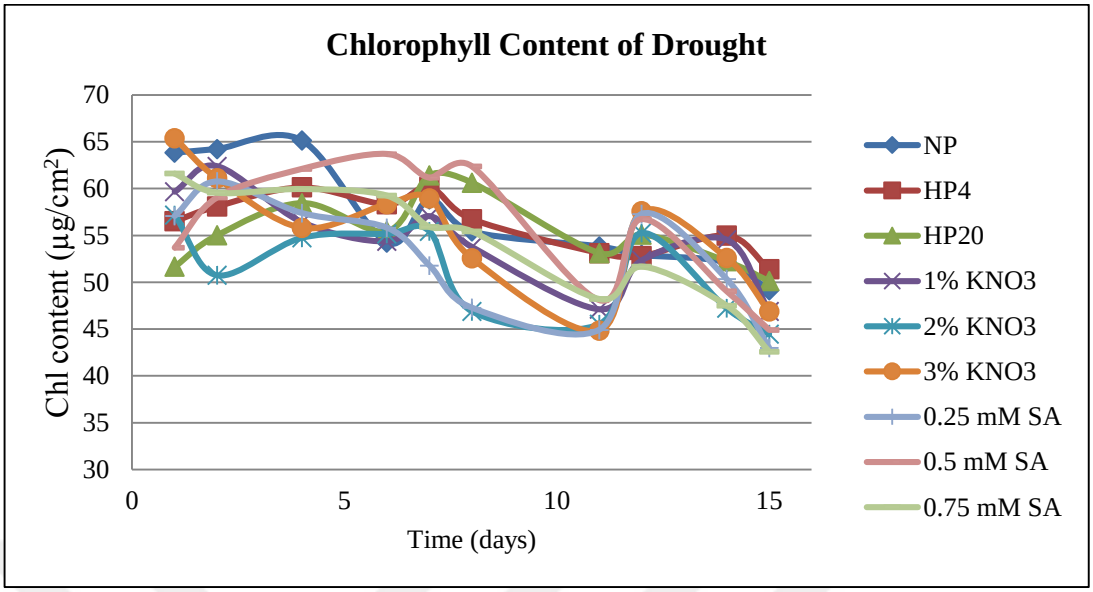

Figure 5.16. Seed priming effect on chlorophyll content via SPAD values of drought-treated *B. distachyon.*

5.5.3.2. Chlorophyll Content Measurement via Acetone Method

Chlorophyll content of the plant is measured with a chl meter and it can be monitored day by day during stress, but the results can be deceiving since the measurements can be influenced by the current water capacity of the leaf and distribution of chl. For this reason, chlorophyll content was also determined by using the acetone as a solvent via spectrophotometric methods. Statistical analysis showed that chl content of *B. distachyon* leaves did not change under abiotic stress when compared with control (Table 5.7).

Table 5.7. Chlorophyll content of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

Chlorophyll Content via Acetone Method (mg/ml)			
	Control	Salinity	Drought
NP	2,48±0,702	2,55±0,124	2,23±0,491
HP4	2,32±0,149	2,44±0,198	2,06±0,193
HP20	2,20±0,457	2,52±0,696	2,14±0,250
1% KNO_3	2,55±0,604	3,04±1,372	3,42±1,076
2% KNO_3	1,94±0,057	2,29±0,421	2,63±1,002
3% KNO_3	2,01±0,038	1,98±0,085	2,12±0,252
0.25 mM SA	2,02±0,324	1,95±0,045	2,45±0,332
0.5 mM SA	2,40±0,261	1,99±0,413	2,17±0,079
0.75 mM SA	2,08±0,195	2,42±0,167	2,48±0,325

Among priming treatments, the only statistically significant decrease was observed in 3 percent KNO_3 and 0.25 mM plants under salinity compared with NP and HP4 plants and the lowest chlorophyll concentration was found in 0.5 mM SA plants. Priming treatments did not affect the total chl content of *B. distachyon* under optimal conditions compared with NP plants. The only difference was observed in HP4 plants that were higher than 2percent KNO_3 and 3 percent KNO_3 plants. Under drought stress, there was no significant difference in chl content between priming treatments and NP plants. The highest chl content was observed in 1 percent KNO_3. However, no significant difference was observed between 1 percent KNO_3 and other priming treatments due to the high standard deviation (Figure 4.17 and Appendix).

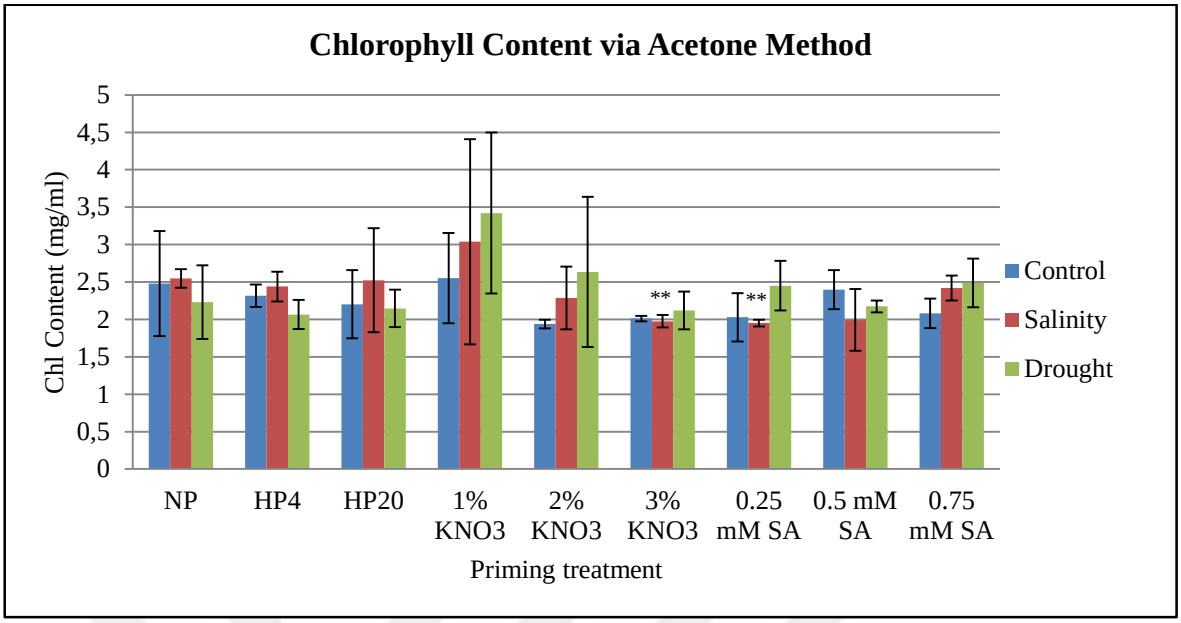

Figure 5.17. Seed priming effect on chl content of *B. distachyon* under salt and drought stress. (significant at p≤0,01, *** significant at p≤0,001which were determined by Student's t-test, compared with NP).

5.5.3.3. Carotene Content Measurement

The Student's t-test showed that seed priming with HP4, HP20, 1 percent KNO_3, 2 percent KNO_3 and also NP did not affect carotene content of *B. distachyon* under salt and drought stress. The only significant increase (p≤0,05) in carotene was observed in 0.75 mM SA plants under salt and drought stress (Table 5.8).

Table 5.8. Carotene concentration of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

Carotene Content (mg/g FW)			
	Control	Salinity	Drought
NP	0,43±0,069	0,41±0,054	0,36±0,039
HP4	0,38±0,027	0,39±0,070	0,35±0,065
HP20	0,37±0,049	0,39±0,046	0,42±0,069
1% KNO_3	0,43±0,079	0,47±0,095	0,43±0,084
2% KNO_3	0,40±0,017	0,53±0,202	0,40±0,030
3% KNO_3	0,40±0,027	0,41±0,038	0,38±0,093
0.25 mM SA	0,40±0,037	0,44±0,023	0,45±0,092
0.5 mM SA	0,43±0,015	0,42±0,066	0,41±0,021
0.75 mM SA	0,32±0,030	0,41±0,042*	0,43±0,055*

The data were expressed as mean±SE with three replicates
*significant at p≤0,05which were determined by Student's *t*-test, compared with control

Seed priming methods used in this study did not affect carotene content under optimal and abiotic stress conditions when compared with NP. However, in control group, carotene content in HP4 plants significantly lower than 0.5 mM SA and higher than 0.75 mM SA plants. The lowest carotene content was observed in 0.75 mM SA plants which are different from 2 percent KNO_3, 3 percent KNO_3, 0.25 mM SA and 0.5 mM SA plants. The statistical analysis showed that there was no significant difference between priming treatments among abiotic stress (Figure 5.18 and Appendix).

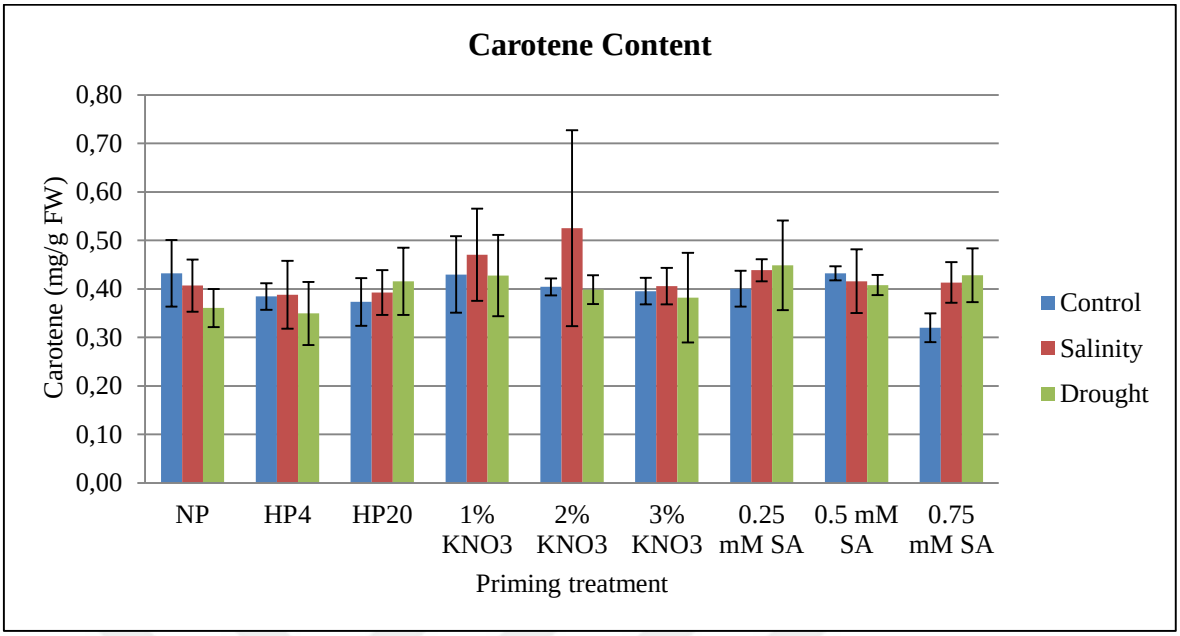

Figure 5.18. Seed priming effect on carotene content of *B. distachyon* under salt and drought stress.

5.5.4. Proline Content of *Brachypodium distachyon*

Proline accumulation determined by using the difference between absorbance values caused by the red color of the reaction of proline with ninhydrin. Leaf proline content was significantly influenced by drought and salt stress in all *B. distachyon* plants. The statistical analysis showed that under salt stress, proline accumulation was significantly high in NP, 3 percent KNO_3 and 0.75mM SA ($p \leq 0{,}001$); HP4, HP20, 1 percent KNO_3, 2 percent KNO_3, 0.25 mM SA and 0.5 mM SA ($p \leq 0{,}05$) plants under salt stress compared with control. Meanwhile, under drought stress, proline accumulation was higher in 0.25 mM SA ($p \leq 0{,}05$); NP, HP4, 0.5 mM SA, 0.75 mM SA ($p \leq 0{,}01$); 2 percent KNO_3 and 3 percent KNO_3 ($p \leq 0{,}001$) plants. There was no difference in HP20 and 1 percent KNO_3 plants under drought stress compared with control. Overall, priming with HP4, 3 percent KNO_3 and 0.5 mM SA had better performance for proline accumulation under salt and drought stress (Table 5.9).

Table 5.9. Proline concentrations of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

Proline concentration (µg/g FW)			
Treatment	Control	Salinity	Drought
NP	1,37±0,609	5,59±0,437***	20,32±6,647**
HP4	1,20±0,882	24,39±8,802*	28,81±8,152**
HP20	1,20±0,577	6,71±2,871*	4,82±3,182
1% KNO_3	2,61±0,378	6,77±1,575*	12,90±12,979
2% KNO_3	1,71±0,992	11,15±5,818*	10,59±1,169***
3% KNO_3	1,11±1,199	16,73±1,296***	30,03±2,257***
0.25 mM SA	2,27±0,649	10,81±3,610*	24,16±8,575*
0.5 mM SA	1,45±0,706	20,40±8,442*	30,42±8,997**
0.75 mM SA	1,30±0,805	11,92±1,121***	22,86±4,457**

The data were expressed as mean±SE with three replicates
*significant at p≤0,05
** significant at p≤0,01
***significant at p≤0,001 which were determined by Student's *t*-test, compared with control

Under unstressed conditions, only priming with 1 percent KNO_3 showed an increase in proline content when compared with NP. The highest proline accumulation was observed in 3 percent KNO_3, 0.5 mM and 0.75 mM SA plants compared with NP plants under salt stress. Proline content in HP4 plants was also significantly higher than HP20 and 1 percent KNO_3 plants. Proline concentration significantly reduced in 1 percent KNO_3 plants with respect to 3 percent KNO_3, 0.5 mM SA and 0.75 mM SA plants. There was no difference between 2 percent KNO_3 and SA plants. Under drought stress, it was found that there was no difference in proline content between priming treatments when compared with NP, except HP4. The lowest proline accumulation observed in HP20 and 2 percent KNO_3 plants (Figure 5.19 and Appendix).

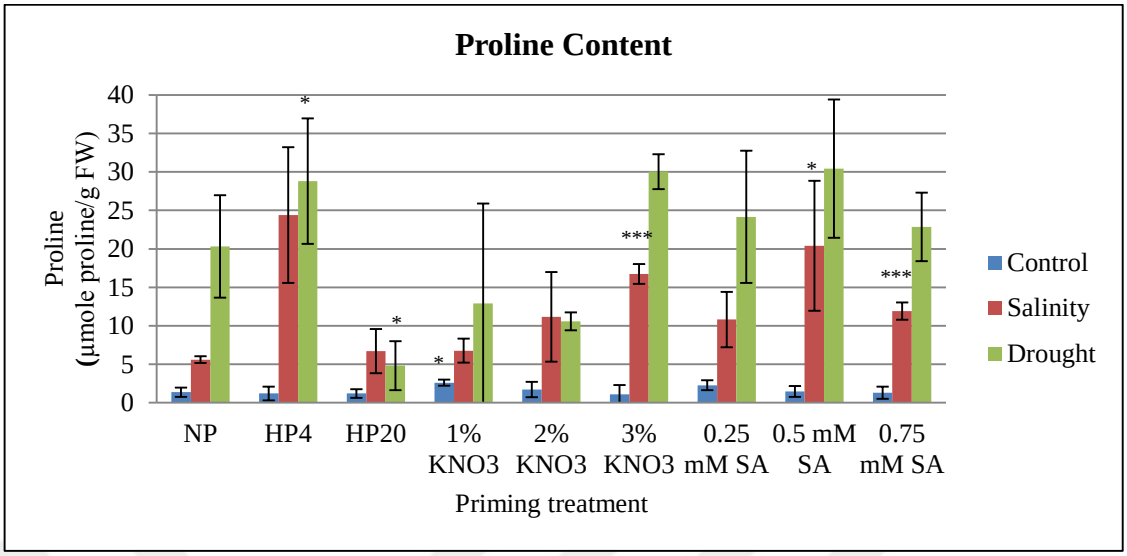

Figure 5.19. Seed priming effect on proline content of *B. distachyon* under salt and drought stress. (* significant at p≤0,05, *** significant at p≤0,001 which were determined by Student's t-test, compared with NP).

5.6. BIOCHEMICAL ANALYSIS OF SEED PRIMING EFFECT ON *Brachypodium distachyon* UNDER ABIOTIC STRESS

5.6.1. Enzyme Extraction and Protein Concentration

B. distachyon leaves were used to determine the antioxidant activity. Leaf tissue was homogenized with liquid nitrogen and crude enzyme extracted with K-P buffer. After enzyme extraction, the crude protein concentration was determined with Bradford assay. Under salt stress, seed priming with 0.25 mM SA revealed an increase in protein concentration (p≤0,05). Interestingly, protein concentration in HP4 plants reduced under salt stress when compared with control (p≤0,05). The protein concentration in NP, HP20 and 0.75 mM SA plants significantly increased under drought stress (p≤0,05) when compared with control (Table 5.10).

Table 5.10. The crude protein concentration of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

Crude protein concentration (µg/ml)			
Treatment	Control	Salinity	Drought
NP	1,08±0,234	1,51±0,209	1,59±0,175*
HP4	1,13±0,118	0,80±0,172*	1,31±0,025
HP20	0,80±0,137	1,03±0,095	1,07±0,035*
1% KNO_3	1,16±0,208	1,24±0,137	1,16±0,213
2% KNO_3	1,12±0,077	1,11±0,201	1,17±0,164
3% KNO_3	1,08±0,065	1,17±0,163	1,11±0,229
0.25 mM SA	0,87±0,049	1,03±0,085*	1,15±0,278
0.5 mM SA	1,08±0,124	1,26±0,097	1,29±0,222
0.75 mM SA	0,97±0,163	1,08±0,112	1,28±0,095*

The data were expressed as mean±SE with three replicates
*significant at p≤0,05which were determined by Student's *t*-test, compared with control

Under optimal conditions, seed priming did not affect the crude protein concentration of *B. distachyon*. Protein concentration in HP20 plants was significantly low when compared with HP4, 2 percent KNO_3, 3 percent KNO_3 and 0.5 mM SA plants. Also, protein concentration in HP4, 3 percent KNO_3 and 0.5 mM SA plantswas high compared with 0.25 mM SA plants. There was no difference in protein concentration between KNO_3 treatments. Under salt stress, it was found that the highest protein concentration observed in NP plants when compared with HP4, HP20, 0.25 mM SA and 0.75 mM SA plants. Meanwhile, the lowest protein concentration observed in HP4 plants with respect to 1 percent KNO_3, 3 percent KNO_3 and 0.5 mM SA plants. In addition, the protein concentration was significantly high in 0.5 mM SA than HP20 and 0.25 mM SA plants. Under drought stress, the Student's t-test showed that seed priming with KNO_3 and SA did not affect protein concentration. Similar to salinity, the highest protein concentration observed in NP plants when compared with most of the priming treatments except 0.25 mM SA and 0.5 mM SA treated plants. Furthermore, the protein concentration in HP4 plants was significantly higher than HP20 plants (Figure 5.20 and Appendix).

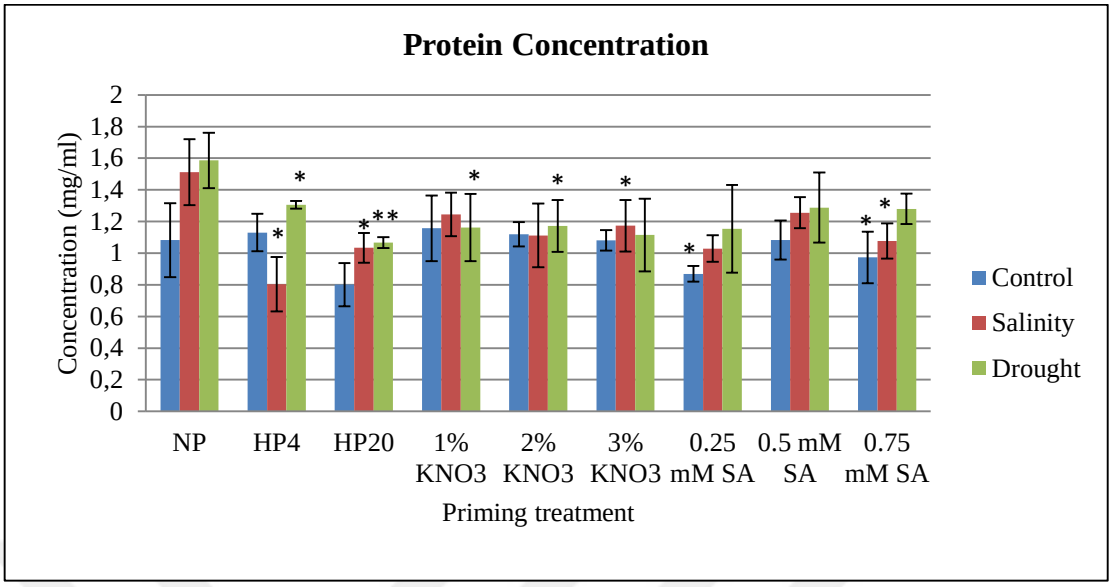

Figure 5.20. Seed priming effect on protein content of *B. distachyon* under salt and drought stress. (* significant at p≤0,05, ** significant at p≤0,01 which were determined by Student's t-test, compared with NP).

5.6.2. Catalase Activity Analysis

CAT activity in *B. distachyon* leaves was established for three biological replicates and depends on the rapid decomposition of H_2O_2 to H_2O and O_2 in the presence of CAT. The Student's t-test showed that there was a significant difference in HP20, 1 percent KNO_3, 3 percent KNO_3, 0.25 mM SA and 0.75 mM SA plants. CAT activity increased in HP20 (p≤0,001), 1 percent KNO_3 (p≤0,05), 3 percent KNO_3 (p≤0,05) plants under salt stress and 1 percent KNO_3 (p≤0,01), 0.75 mM SA (p≤0,001)plants under drought stress. Contrastly, CAT activity decreased in 3 percent KNO_3 (p≤0,01) and 0.25 mM SA (p≤0,05) plants under drought stress with respect to control group (Table 5.11).

Table 5.11. CAT activity of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

CAT activity (U/min/mg protein)			
Treatment	Control	Salinity	Drought
NP	0,98±0,054	0,88±0,191	0,98 ±0,092
HP4	1,24±0,463	1,14±0,499	1,12±0,171
HP20	1,05±0,129	1,58±0,137**	1,34±0,265
1% KNO_3	0,90±0,092	1,28±0,193*	2,58±0,504**
2% KNO_3	1,40±0,448	1,08±0,237	1,01±0,073
3% KNO_3	1,66±0,157	1,98±0,137*	1,15±0,075**
0.25 mM SA	1,82±0,563	1,78±0,477	0,94±0,127*
0.5 mM SA	1,46±0,541	0,85±0,347	1,58±0,858
0.75 mM SA	1,44±0,164	1,10±0,226	2,31±0,014***

The data were expressed as mean±SE with three replicates
*significant at p≤0,05
** significant at p≤0,01
***significant at p≤0,001 which were determined by Student's *t*-test, compared with control

The statistical analysis showed that CAT activity was higher in 3 percent KNO_3 and 0.75 mM SA plants compared with NP, HP20 and 1 percent KNO_3 plants under optimal conditions. The highest CAT activity in salt-treated plants was recorded in 3 percent KNO_3 plants when compared with all treatments except 0.25 mM SA plants. The highest CAT activity in drought-treated plants was observed in 1 percent KNO_3 and 0.75 mM SA plants which were significantly different than all treatments and NP plants, except 0.5 mM SA. It can be concluded that priming with a low concentration of KNO_3 and high concentration of SA significantly increased CAT activity under drought stress and priming with high concentration of KNO_3 increased CAT activity under salinity in *B. distachyon* (Figure 5.21 and Appendix).

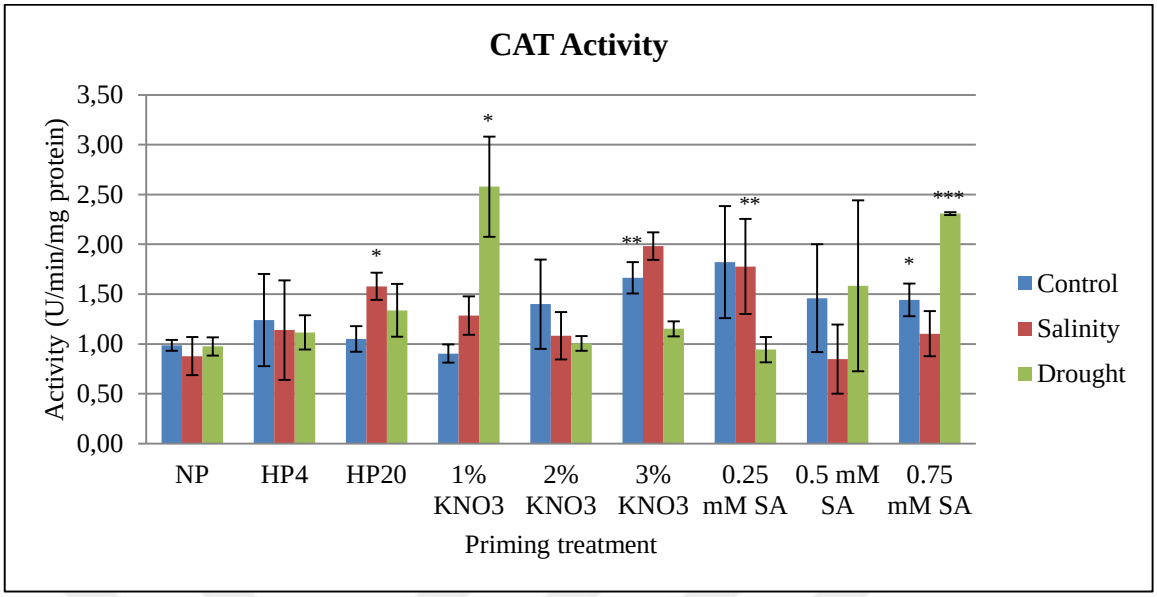

Figure 5.21. Seed priming effect on CAT activity of *B. distacyhon* under salt and drought stress. (* significant at p≤0,05, ** significant at p≤0,01 which were determined by Student's t-test, compared with NP).

5.6.3. Peroxidase Activity Analysis

In this study, guaiacol was used as a substrate for the determination of POD activity. At the end of the reaction, tetraguaiacol is formed as the final product that gives the orange color to the solution. The statistical analysis revealed that POD activity increased in HP4, 2 percent KNO_3 plants under salt stress; 1 percent KNO_3 under drought stress; 0.5 mM SA under both salt and drought stress when compared with control (p≤0,05, Table 5.12).

Table 5.12. POD activity of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

POD activity (U/min/mg protein)			
Treatment	Control	Salinity	Drought
NP	9,27±1,461	7,66±1,291	7,63±1,001
HP4	12,29±2,679	19,13±2,066*	12,90±0,594
HP20	8,90±1,286	11,25±4,057	10,03±1,187
1% KNO_3	12,14±0,891	11,89±0,483	13,95±1,683*
2% KNO_3	11,95±0,744	13,90±0,927*	10,57±1,175
3% KNO_3	12,43±0,738	12,56±0,824	15,26±2,716
0.25 mM SA	15,26±0,762	16,23±1,486	16,22±3,637
0.5 mM SA	10,64±0,813	14,02±1,663*	15,47±2,500*
0.75 mM SA	16,43±2,793	16,60±2,638	15,16±1,558

The data were expressed as mean±SE with three replicates
*significant at p≤0,05which were determined by Student's *t*-test, compared with control

POD activity significantly varied among priming treatments in plants grown under unstressed conditions. Priming with 3 percent KNO_3, 0.25 mM SA and 0.75 mM SA significantly increased POD activity when compared with NP plants. POD activity in HP20 plants was lower than all KNO_3 and SA plants except 0.5 mM SA. The highest POD activity was observed in 0.75 mM SA plants. Followed by 0.75 mm SA plants, the highest POD activity was found on plants primed with 0.25 mM SA. Priming with KNO_3 and SA significantly increased POD activity of *B. distachyon* under salt stress when compared with NP. Although the highest POD activity was observed in HP4 plants, it was not significantly different from other treatments except 1 percent KNO_3 and 3 percent KNO_3 plants due to the high standard deviation. Priming with 2 percent KNO_3, 0.25 mM SA and 0.75 mM SA significantly increased POD activity compared with 1 percent KNO_3plants under salt stress. Under drought stress, POD activity was higher than NP in all priming treatments. POD activity in HP20 and 2 percent KNO_3 plants was significantly lower than all priming treatments. There was no difference observed between the treatments of SA concentrations (Figure 5.22 and Appendix).

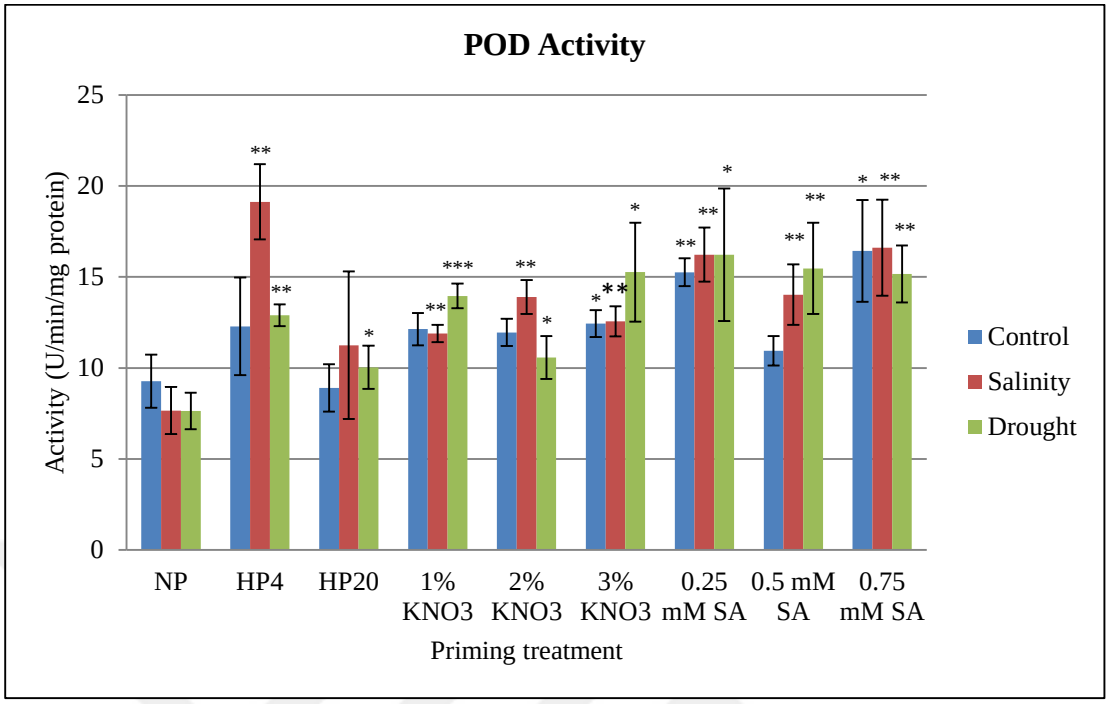

Figure 5.22. Seed priming effect on POD activity of *B. distachyon* under salt and drought stress. (* significant at p≤0,05, ** significant at p≤0,01, ***significant at p≤0,01 which were determined by Student's t-test, compared with NP).

5.6.4. Superoxide Dismutase Activity Analysis

SOD activity determined by a spectrophotometric method based on the SOD-mediated inhibition in the rate of nitroblue tetrazolium reduction to the blue formazan. The color change was higher in salt and drought stress that pointed out higher SOD activity. The statistical analysis revealed that SOD activity was higher in NP, 3 percent KNO_3, 0.25 mM SA, 0.75 mM SA ($p \leq 0.05$); 1 percent KNO_3, 2 percent KNO_3 and 0.5 mM SA ($p \leq 0,01$) plants under salt stress compared with control. In addition, SOD activity was higher in NP, HP4, HP20, 1 percent KNO_3, 2 percent KNO_3, 3 percent KNO_3 and 0.25 mM SA ($p \leq 0.05$); 0.75 mM SA ($p \leq 0.01$); 0.5 mM SA ($p \leq 0.001$) plants under drought stress compared with control. Although the highest activity was observed in NP plants, the most increase was in 0.5 mM SA plants under salt and drought stress (Table 5.13).

Table 5.13. SOD activity of *B. distachyon* under unstressed, salt-stressed and drought-stressed conditions against seed priming with different agents.

SOD activity (U/mg FW)			
Treatment	Control	Salinity	Drought
NP	0,40±0,023	0,45±0,013*	0,45±0,019*
HP4	0,36±0,038	0,41±0,005	0,45±0,010*
HP20	0,39±0,008	0,42±0,019	0,42±0,022*
1% KNO_3	0,34±0,020	0,42±0,011**	0,41±0,040*
2% KNO_3	0,36±0,018	0,41±0,005**	0,42±0,014*
3% KNO_3	0,35±0,041	0,42±0,003*	0,42±0,014*
0.25 mM SA	0,31±0,037	0,42±0,019*	0,37±0,025*
0.5 mM SA	0,25±0,012	0,37±0,029**	0,41±0,022***
0.75 mM SA	0,22±0,054	0,34±0,041*	0,41±0,067**

The data were expressed as mean±SE with three replicates
*significant at p≤0,05
** significant at p≤0,01
***significant at p≤0,001 which were determined by Student's *t*-test, compared with control

Under unstressed conditions, SOD activity was higher in NP plants compared with 1 percent KNO_3, and all SA primed plants. SOD activity was lower in 0.5 mM SA and 0.75 mM SA plants compared with all other priming treatments and NP. Also, priming with HP20 was increased SOD activity compared with 1 percent KNO_3, 2 percent KNO_3 and 0.25 mM SA under optimal growth conditions. There was no significant difference in SOD activity between different concentrations of SA. Under salt stress, the highest SOD activity was observed in NP plants compared with all priming treatments while the lowest activity was observed in 0.75 mM SA plants. In addition, priming with 3 percent KNO_3 showed higher activity than HP4 and 0.5 mM SA. Statistical analysis revealed that SOD activity was higher in NP, HP4, 2 percent KNO_3 and 3 percent KNO_3 than 0.25 mM SA plants under drought stress. Priming with distilled water at 4°C (HP4) showed the highest activity compared with 2 percent KNO_3, 3 percent KNO_3, 0.25 mM SA and 0.75 mM SA priming treatments. There was no difference between different concentrations of KNO_3 (Figure 5.23 and Appendix).

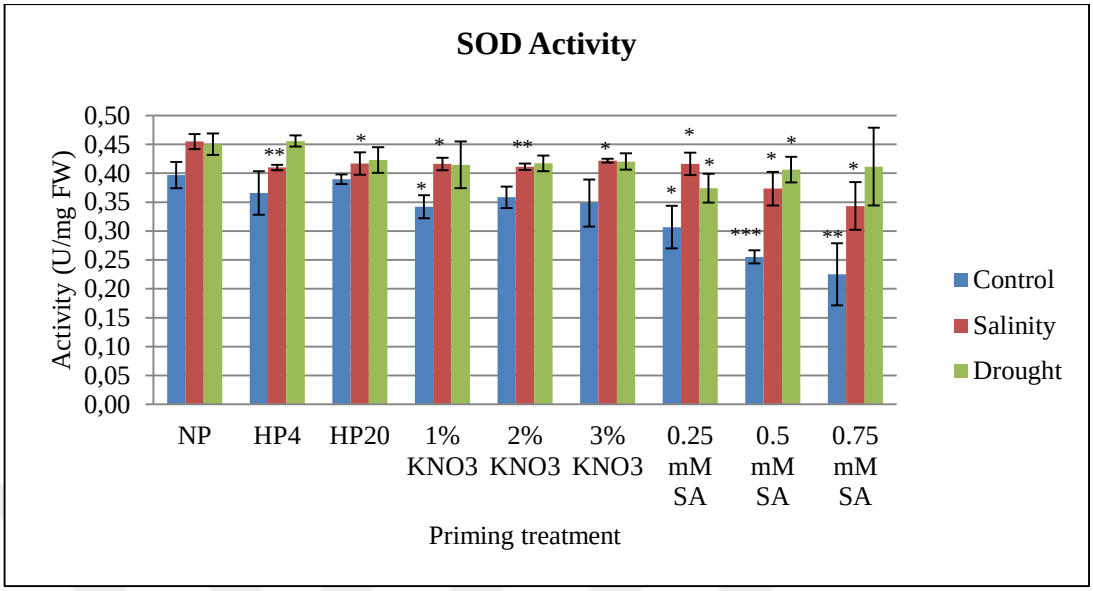

Figure 5.23. Seed priming effect on SOD activity of *B. distachyon* under salt and drought stress. (* significant at p≤0,05, ** significant at p≤0,01, ***significant at p≤0,01 which were determined by Student's t-test, compared with NP).

6. DISCUSSION

Seed priming is a simple technique used to increase seed germination and seedling emergence rate. This technique has been utilized for years to increase the germination percentage of normal and abnormal seeds. It was observed that plants grown from primed seeds were better adapted to stress conditions. Studies have shown that seed priming activated antioxidant defense mechanism during germination [190,191]. Thus, it is very significant to understand the mechanisms behind priming via physiological, biochemical, genomic and proteomic analysis. To our knowledge, seed priming was performed for the first time in *B. distachyon.* Therefore, it is still unknown how the treatment affects the germination and physiology of *B. distachyon* under abiotic stress. The present study evaluated the comparative performance of different priming methods on germination of *B. distachyon*. Moreover, physiological and biochemical alterations in *B. distachyon* were determined after the seed-primed plants were treated with drought and salinity stress.

Studies revealed that seed priming with distilled water, KNO_3 and SA enhance the germination rate and abiotic stress adaptation in wheat [151,177,193], rice [143,194] and maize [145,146]. Although there were many options in terms of priming agent, KNO_3 and SA were the most commonly used agents under abiotic stress. Therefore, three concentrations of KNO_3 and SA were chosen as priming agents for osmopriming and hormonal priming in this study. Seeds were treated at 20°C instead of optimal temperature (25°C) since it was observed in our preliminary studies that the temperature of 25°C increased fungal growth on *B. distachyon* seeds. Moreover, 25°C temperature speeds up the radical emergence that it is not easy to determine the effect of seed priming in terms of germination success when seeds are exposed to this temperature. On the other hand, priming temperature at 4°C was chosen due to the vernalization requirement of *B. distachyon*. Under normal conditions, *Brachypodium* seeds are kept at 4°C before sowing in soil. The decision of imbibition time in seed priming is very important. Seed priming with different imbibition times in two rice cultivars significantly changed the germination percentage when they were treated with low concentrations of KNO_3 (1 percent and 2 percent KNO_3) [144]. Since it is unknown which duration was effective in *B. distachyon*, priming duration was chosen as 24 hours that could be considered as one of the optimal condition for the duration of priming [196]. Since in our preliminary studies we obtained

effective results with imbibition time of 24 hours in seed priming, all experiments were carried out with this optimal imbibition time period.

Imbibition is diffusion of water by seeds. Seeds absorb water due to the protein, starch and cellulose [197]. The imbibition is a remarkable process for germination and seed growth since they depend on imbibition behavior. In this study, the highest imbibition rate was observed in seeds treated with distilled water at 20°C, whereas the lowest imbibition rate was observed in seeds treated with distilled water at 4°C as expected. With an increase in temperature, water absorption of tissues increases [198]. The difference in osmotic potential due to the increase in potassium concentrations adversely affected the imbibition rate of the seed.

Seed priming with KNO_3 and SA reduced the MGT of *B. distachyon* seeds. However, there was no difference between the concentrations of the selected priming agents. Therefore, higher or lower concentrations should also be considered for future studies. All primed and nonprimed seeds were germinated and germination percentage was found to be 100 percent. Thus, the GI was calculated to show that the application was successful. The best description of germination percentage/speed relationship can be expressed by GI analysis [199]. The GI value of all primed seeds was higher than the NP seeds but again there was no significant difference between the treatments and concentrations. The uncertainty of the germination (U) was reduced by priming with 2 percent and 3 percent KNO_3. Also priming with 2 percent, 3 percent KNO_3, 0.25 and 0.75 mM SA provided better synchronization of germination (Z) in *B. distachyon* seeds. This means that the choice of priming agents and concentrations affected germination parameters in a different manner.

After the germination test, seeds were transferred into soil. The seedling emergence was recorded day by day and MET was calculated. The highest value of MET which indicates slow emergence of seedlings was observed in NP seedlings. It means that better germination helps for improving the leaf emergence [200].

Drought stress was applied by withholding the plants from water. However, as soon as the plants withheld from water, the soil water content would not reduce to dry levels. Thus, the first day of stress was about four days later from withholding in our study. This was the time required for the soil moisture level to drop to 50-60 percent [177]. In another study in wheat, plants withheld from water for 5-7 d (soil relative water content around 35–40

percent) and severe drought stress was applied for 15 d (soil relative water content around 20–25 percent) [180]. Since the water retention time can vary according to the environment, season and the rate of water absorption of the plants. For salt stress, the plants were irrigated with 320 mM NaCl solution for 14 days. The choice of salt concentration was sufficient to expose the plants to moderate salt stress according to our previous studies and Demiral et al. (2017) [201]. At the end of the experiment, the electrical conductivity results showed that the salinity in the soil increased.

The effect of seed priming on the morphological characteristics of *B. distachyon* was also investigated in this study. The height of nonprimed plants was higher than primed plants under salt and drought stress because these plants had unexpectedly passed into the heading stage early, which can be investigated in a future study covering the effect of priming on different growth stages. Nonprimed plants produced offsprings earlier which was a sign of less tolerance to abiotic stress. Also, the highest plant height was observed in low concentrations of KNO_3 and SA primed plants under salt and drought conditions as well as optimal conditions which correlates with other studies in maize [202], wheat [203] and soybean [204]. Under drought stress, priming with distilled water at 20°C had a positive effect on plant height. Stimulation of cell elongation, division and enlargement ensure the increase in plant height [145].

Biomass is a renewable energy source that can be used for the generation of heat energy and electricity. It can be converted into biogas or biofuel. Therefore, it is important to grow plants that have high biomass for the industry [205]. On the other hand, biomass in dry weight (DW) is a measure of plant yield since it shows the organic content stored in the plant related to photosynthetic activity [206]. In this study, the high standard deviation was obtained for biomass measurement because three plants from one pot were not pooled and each plant assumed as one sample. Since they were whole organism, they differed in terms of morphology. Plants primed with 1 percent KNO_3 were found to have the highest biomass with the value of 605,97 mg under salinity and hydroprimed HP20 plants with the value of 653,87 mg under drought stress (Table 5.3). Similar results were found in studies with wheat [141,174]. Priming with distilled water at 4°C had high biomass under salt stress followed by 1 percent KNO_3. The effect of seed priming on the dry weight of plants was consistent with other studies but high standard deviation limited to observe the significant differences in the results. The lowest values were obtained from 0.5 mM SA

plants under salinity and 3 percent KNO_3 plants in drought with the value of 291,1 mg and 301,6 mg, respectively (Table 5.3). HP20 had better performance under unstressed and drought conditions. In other words, hydropriming at 4°C and osmopriming with 1 percent KNO_3 allowed the plant to adapt to saline conditions and HP20 to dry conditions. The low concentration of KNO_3 also had a positive effect on the plant height. Therefore, it could be evaluated that priming with 1 percent KNO_3 enhanced the morphological characteristics of *B. distachyon*. In our study, priming with SA did not affect the dry weight of plants statistically due to the high standard deviation, however the highest biomass value of was observed in 0.75 mM SA primed plants among SA treatments under both salt and drought stress. In another study with wheat indicated that priming with SA increased the biomass in wheat under water stress [207].

Relative water content was measured only for control and drought groups since inconsistent results were obtained from our previous results for salinity. Under optimal and drought conditions, the water holding capacity of the plant did not change in nonprimed plants (RWC value of 87,59 percent under unstressed and 88,20 percent under drought conditions, Table 5.4). However, RWC in these plants were lower than all primed plants. In other words, seed priming significantly increased the water holding capacity of plants grown under normal conditions and a similar result was obtained in a study with maize [193]. However, in the case of drought, priming with distilled water at 20°C, 1 percent and 2 percent KNO_3 positively affected the water holding capacity of *B. distachyon*. These plants also showed better performance in height measurements. Priming with 3 percent KNO_3 and all SA concentrations did not increase RWC of plants under drought stress. The lowest RWC was observed in plants primed with 0.5 mM SA.

Drought stress causes osmotic stress in plants while salinity causes both ionic and osmotic stress. Since RMP is an analysis that measures the electrical conductivity of the plant via ionic imbalance, it is not appropriate to compare the results of RMP between salt and drought stress. Due to the presence of NaCl in salt stress, the damage in salt stress appears to be more than the drought. Therefore, RMP analysis is a good indicator of the damage caused by NaCl in salt stress. In this study, the lowest damage was observed in nonprimed and hydroprimed plants. Among primed plants with KNO_3 and SA, the least damage was found on 2 percent KNO_3 plants with the value of 94,43 percent under salinity and 85,91 percent under drought stress. Followed by 2 percent KNO_3 plants, priming with 1 percent

KNO_3 had a positive effect on RMP of plants with the value of 112,98 percent and 86,06 percent under salt and drought stress, respectively (Table 5.5). This result provides similar findings with a study in rice [195]. The highest damage was observed in 3 percent KNO_3, 0.5 and 0.75 mM SA plants under salinity. The RWC values of these plants were also reduced by drought stress, which indicated that higher concentrations of KNO_3 and SA did not prevent the plant from oxidative stress.

Chlorophyll and carotenoids are crucial components of photosynthesis that has a key role in energy transduction. In the present study, chlorophyll content was measured in two ways; 1) SPAD values via chlorophyll meter, 2) total pigment content via acetone extraction. Firstly, the chlorophyll content of *B. distachyon* leaves was expressed as mg per leaf area. Under optimal, salt and drought conditions, chlorophyll content in all plants was reduced when compared with the first day of the stress treatment. Similar reduction was observed in another study with *B. distachyon* under salt and heat stress [208]. On the other hand, it increased in HP20 plants and decreased at 0.5 mM SA and 0.75 SA plants under salt stress based on the measurements at the end of the stress treatment. Under drought, NP, HP4, HP20 and 1 percent KNO_3 plants showed higher chlorophyll content than the control group. The highest chlorophyll content was observed in HP4 plants under both salt and drought stress. Although SPAD measurement is the best method for the expression of chlorophyll behavior during stress treatment [181], results may vary according to parameters such as water capacity or leaf thickness of the plant at the time of measurement. Therefore, different results can be obtained when the total chlorophyll content of the plant is measured. In the study conducted on the measurement of chlorophyll content of wheat under salt stress, SPAD values revealed that chlorophyll content increased under salt stress. Contrastly, it was observed that there was a decrease in chlorophyll content when whole plant pigment concentration was evaluated [209]. In our study, the highest chlorophyll concentration was observed in 1 percent KNO_3 plants under optimal and abiotic stress conditions by extraction of chlorophyll content with acetone. However, there was no difference between priming treatments and NP due to the high standard deviation. Nevertheless, lower KNO_3 concentration for priming application might be attempted for higher chlorophyll concentration for *B. distachyon*. It can be also concluded that seed priming with 1 percent KNO_3 tolerated the effect of abiotic stress in *B. distachyon* with respect to other treatments and control group.

Carotenoids are photosynthetic pigments that have a key role in photo-oxidative protection. They are the main precursors of vitamin A and give the yellow color to the grain. In a previous study with *B. distachyon*, it was detected that carotene content was increased when plants exposed to salt, drought and heat stress [208]. In the present study, abiotic stress treatment along with the priming treatment did not affect the carotene content of *B. distachyon*. The highest carotene content was observed in 1 percent KNO_3 and 2 percent KNO_3 plants under salinity, similar to the total chlorophyll content. However, there was no significant difference between priming treatments according to the Student's t-test.

Accumulation of free proline under water stress indicates that it regulates compatible solute and reduces water loss [210]. During the optimization of the protocol, it was found that the complete homogenization of the leaves with sulfosalicylic acid was crucial for proline extraction. Furthermore, more accurate results were obtained when the homogenate was kept at room temperature for 3 hours. In another study with *B. distachyon* plants, the proline content increased 2-fold at the end of the 48 hour drought stress treatment, while in our study it was observed that the proline content of *B. distachyon* under drought stress increased almost 30 fold in 3 percent KNO_3 plants under drought stress (Table 5.9) [86]. The increase in proline content of NP plants indicated that free proline determination could assess the stress response of *B. distachyon*. Among priming treatments, the highest proline accumulation was measured in plants primed with distilled water at 4°C, 3 percent KNO_3 and 0.5 mM SA. The RMP in these plants (except HP4) was also high and this increase could be due to the activation of pathways that are related to proline production. In addition, overexpression of proline in these plants may be related to programmed cell death and the damage observed in RMP may be the result of the toxic effect caused by P5C [89]. The concentrations where proline is beneficial or detrimental to plants should be investigated.

Antioxidants were shown to have the most important role in stress coping mechanisms for all living organisms. Although plants with the highest antioxidant value are known as fruits and vegetables, cereals also have a very high antioxidant capacity. However, due to the differences in polyphenol content and enzyme structure, extraction solvent and methodology have great importance for the studies [211]. The increased antioxidant level under abiotic stress in plants and higher ROS scavenging activity have a relation with enhanced stress tolerance of plants [212]. In recent years, scientists have been focused on

the capacity of the seed priming to enhance the plant's defense mechanism against abiotic stress conditions. In this study, we examined the seed priming effect on enzymatic antioxidants. CAT and POD are the most important antioxidants involved in the removal of H_2O_2 that gives the greatest damage to the proteins and cell membrane [213]. Both enzymes convert hydrogen peroxide to water, but peroxidase requires an organic compound (guaiacol is used in this study). This difference is thought to be due to structural differences in the active sites of two enzymes [214].

CAT activity was induced by seed priming with distilled water at 20°C, 1 percent KNO_3 and 3 percent KNO_3 under salinity and 1 percent KNO_3, 0.75 mM SA under drought stress. Priming with 3 percent $KNO_3$and 0.25 mM SA decreased CAT activity under drought stress. These results may suggest that seed priming with 3 percent KNO_3 activated a pathway involved in only salt stress [215]. Priming with 1 percent KNO_3 and 0.75 mM SA under drought stress increased CAT activity in *B. distachyon*. High standard deviation significantly influenced the analysis of CAT activity. This problem indicated the presence of undesirable molecules in the enzyme extract. The enzyme extract should be centrifuged at higher rpm in the subsequent studies. Nevertheless, seed priming in *B. distachyon* has the potential to improve CAT activity under abiotic stress.

Similar to CAT, accurate results could not be obtained in the POD activity due to the high standard deviation for some priming treatments. However, it is clearly observed that seed priming, especially priming with SA, increased POD activity of *B. distachyon* under optimal and abiotic stress conditions. The highest activity was found in plants primed with 0.25 mM SA and 0.75 mM SA in the control group. Under salt stress, POD activity was measured at maximum in plants primed with distilled water at 4°C (19,13 U/min/mg protein). Priming with distilled water and KNO_3 significantly enhanced POD activity under drought stress which correlates with the results obtained in a study with maize [147].

The induction of SOD activity is the consequence of O^{2-} generation under osmotic stress and SOD production act as a signal factor for other antioxidants [35]. In this study, SOD activity increased under salt and drought stress. Besides, hydropriming did not affect SOD level in salt stress. Seed priming might not affect the antioxidant level under optimal conditions, but it activates the defense mechanism of the plant in case of stress and thus makes the plant more tolerant than the control [216]. In this study, although the highest values are observed in plants growing from nonprimed seeds (the activity of 0,45 U/mg

FW under both salt and drought stress, Table 5.13), the results exhibited that priming with KNO_3 and SA activated the SOD production under salt and drought stress. Although SOD activity was low in unstressed conditions for primed plants, in the presence of stress conditions SOD activity was increased in primed plants more than NP plants.

When *B. distachyon* plants were examined on the biochemical basis, the activation of different antioxidant enzymes varied under salt and drought stress. The role of SOD in the cell is to convert free oxygen radicals into hydrogen peroxide, which can be reduced water by CAT and POD enzymes. However, it has been observed that although the activity of SOD increased in all priming treatments under abiotic stress, CAT and POD activity decreased in several priming treatments. Therefore, all of these antioxidants should be determined together in future studies.

7. CONCLUSION AND FUTURE PROSPECTS

- Priming was successfully applied to *B. distachyon* seeds and priming application provided rapid germination and seedling emergence.
- Priming with 2 percent KNO_3 and 3 percent KNO_3 significantly decreased the uncertainty of germination and ensure the synchronized germination.
- Priming with 1 percent KNO_3, 2 percent KNO_3 and 0.25 mM SA had a positive effect on the morphological characteristics of *B. distachyon* such as plant height and above-ground biomass.
- Under drought stress, nonprimed plants maintain their water holding capacity while RWC was significantly decreased in plants primed with 3 percent KNO_3 and all concentrations of SA. The highest RWC was observed in HP20, 1 percent KNO_3 and 2 percent KNO_3 under both control and drought-treated plants. Similar findings were recorded for RMP.
- Chlorophyll content was decreased in all plants by the induction of abiotic stress. The highest total chlorophyll content was measured in lower concentrations of KNO_3.
- Free proline accumulation enhanced by seed priming treatments under salt and drought stress. The highest proline concentration was measured in 3 percent KNO_3 and 0.5 mM SA due to the severe damage observed in RMP. Besides, the significant increase in proline content under abiotic stress showed that it was a valuable method to evaluate the plant response against stress.
- The lowest CAT activity was observed in nonprimed plants. Among primed plants, priming with 1 percent KNO_3 and 0.75 mM SA enhanced CAT activity under drought stress.
- Seed priming increased POD activity in control, salt and drought-treated plants. The highest POD activity was observed in plants primed with distilled water at 4°C under salt and 0.25 mM SA under drought stress.
- The SOD activity increased in all *B. distachyon* plants under abiotic stress conditions. The highest activity was observed in nonprimed plants under salinity stress and plants primed with distilled water at 4°C under drought stress.

- This study showed that seed priming affected the antioxidant defense mechanism in a different manner and the activity of various antioxidants could change under certain abiotic stress by seed priming.
- In general, seed priming with 1 percent KNO_3 enhanced the salt and drought tolerance of *B. distachyon* plants.

In conclusion, to the best of our knowledge, seed priming was performed for the first time in *B. distachyon*. The output of this study has a great potential to provide new insights for omics studies in *B. distachyon* and other cereals to investigate the mechanism behind abiotic stress tolerance related to seed priming.

In future studies;

- Genomic, transcriptomic, proteomic and metabolomic studies should be performed during germination and stress treatment to explore the molecular mechanism of seed priming in *B. distachyon*.
- Seed priming should be performed with lower concentrations of KNO_3. Seed priming with SA should be performed in a wide range of concentrations.The effect of priming duration and temperature should be examined more exhaustively.
- Time-dependent abiotic stress treatment should be performed and activation of stress-induced mechanism together with seed priming should be examined on different time points. Besides, the effect of seed priming on stress recovery performance should be investigated.
- The effects of seed priming should be examined in different growth stages of *B. distachyon* by considering 'priming memory'.The effect of seed priming on epigenetic changes should also be investigated to identify its impact on the progeny.
- The effect of seed priming on grain yield should be assessed for good field establishment.

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APPENDIX A: TABLES OF ESTIMATED P-VALUES FOR MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS VIA STUDENT'S T-TEST

Table A.1. Estimated p-values among priming treatments for MGT of *B. distachyon* seeds.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mMSA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0161	0,5000							
HP20	0,0037	0,0323	0,5000						
1% KNO_3	0,0021	0,0087	0,0561	0,5000					
2% KNO_3	0,0009	0,0016	0,0046	0,0805	0,5000				
3% KNO_3	0,0011	0,0019	0,0066	0,2561	0,0775	0,5000			
0.25 mM SA	0,0014	0,0032	0,0172	0,5000	0,0280	0,1026	0,5000		
0.5 mMSA	0,0030	0,0153	0,0848	0,4594	0,1490	0,3591	0,4521	0,5000	
0.75 mM SA	0,0015	0,0043	0,0244	0,4410	0,0581	0,2409	0,4075	0,5000	0,5000

Table A.2. Estimated p-values among priming treatments for GI of *B. distachyon* seeds.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mMSA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0085	0,5000							
HP20	0,0010	0,0253	0,5000						
1% KNO_3	0,0011	0,0100	0,0766	0,5000					
2% KNO_3	0,0002	0,0012	0,0051	0,0807	0,5000				
3% KNO_3	0,0001	0,0012	0,0070	0,2560	0,0778	0,5000			
0.25 mM SA	0,0002	0,0024	0,0215	0,5000	0,0280	0,1024	0,5000		
0.5 mMSA	0,0010	0,0077	0,0482	0,3580	0,1476	0,4360	0,3193	0,5000	
0.75 mM SA	0,0002	0,0015	0,0101	0,3119	0,0624	0,3623	0,1741	0,4999	0,5000

Table A.3. Estimated p-values among priming treatments for U of *B. distachyon* seeds.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mMSA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3999	0,5000							
HP20	0,2839	0,3149	0,5000						
1% KNO_3	0,0521	0,0305	0,0539	0,5000					
2% KNO_3	0,0191	0,0135	0,0188	0,0922	0,5000				
3% KNO_3	0,0328	0,0094	0,0197	0,4525	0,0801	0,5000			
0.25 mM SA	0,0530	0,0173	0,0398	0,3038	0,0491	0,1187	0,5000		
0.5 mMSA	0,0793	0,0669	0,1032	0,4868	0,1153	0,4525	0,3787	0,5000	
0.75 mM SA	0,0544	0,0252	0,0502	0,3849	0,0636	0,2769	0,4009	0,4303	0,5000

Table A.4. Estimated p-values among priming treatments for Z of *B. distachyon* seeds.

Z	**NP**	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mMSA**	**0.75 mM SA**
NP	0,5								
HP4	0,2464	0,5000							
HP20	0,2885	0,3560	0,5000						
1% KNO_3	0,0732	0,1687	0,0943	0,5000					
2% KNO_3	0,0152	0,0286	0,0171	0,0918	0,5000				
3% KNO_3	0,0130	0,0684	0,0113	0,4291	0,0762	0,5000			
0.25 mM SA	0,0356	0,1890	0,0367	0,3150	0,0398	0,1175	0,5000		
0.5 mMSA	0,0584	0,1275	0,0732	0,3978	0,1307	0,4350	0,2264	0,5000	
0.75 mM SA	0,0161	0,0811	0,0150	0,4646	0,0706	0,4366	0,1532	0,4039	0,5000

Table A.5. Estimated p-values among priming treatments for plant height of unstressed *B. distachyon* plants.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0681	0,5000							
HP20	0,0166	0,0503	0,5000						
1% KNO_3	0,0170	0,0477	0,3343	0,5000					
2% KNO_3	0,0083	0,0153	0,3323	0,4483	0,5000				
3% KNO_3	0,0302	0,1155	0,3677	0,2509	0,2311	0,5000			
0.25 mM SA	0,0068	0,0117	0,2125	0,4172	0,3114	0,1533	0,5000		
0.5 mM SA	0,1553	0,4233	0,2381	0,1817	0,1762	0,3086	0,1368	0,5000	
0.75 mM SA	0,1730	0,1143	0,0159	0,0198	0,0042	0,0405	0,0038	0,2655	0,5000

Table A.6. Estimated p-values among priming treatments for plant height of salt-stressed *B. distachyon* plants.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0342	0,5000							
HP20	0,0807	0,0458	0,5000						
1% KNO_3	0,4951	0,0015	0,0054	0,5000					
2% KNO_3	0,0528	0,4216	0,1824	0,0112	0,5000				
3% KNO_3	0,0724	0,0308	0,4256	0,0025	0,0651	0,5000			
0.25 mM SA	0,2020	0,0417	0,1721	0,0909	0,1051	0,0264	0,5000		
0.5 mM SA	0,0211	0,1091	0,0045	0,0003	0,0023	0,0638	0,0044	0,5000	
0.75 mM SA	0,0229	0,1706	0,0203	0,0014	0,0212	0,2629	0,0131	0,1463	0,5000

Table A.7. Estimated p-values among priming treatments for plant height of drought-stressed *B. distachyon* plants.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0737	0,5000							
HP20	0,4081	0,0251	0,5000						
1% KNO_3	0,3587	0,0631	0,1913	0,5000					
2% KNO_3	0,3475	0,0753	0,1933	0,4751	0,5000				
3% KNO_3	0,0635	0,3975	0,0249	0,0559	0,0651	0,5000			
0.25 mM SA	0,1910	0,0288	0,1942	0,1053	0,1051	0,0264	0,5000		
0.5 mM SA	0,0065	0,0293	0,0006	0,0014	0,0023	0,0638	0,0044	0,5000	
0.75 mM SA	0,0267	0,1743	0,0078	0,0172	0,0212	0,2629	0,0131	0,1463	0,5000

Table A.8. Estimated p-values among priming treatments for biomass of unstressed *B. distachyon* plants.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0037	0,5000							
HP20	0,1351	0,0244	0,5000						
1% KNO_3	0,0154	0,4544	0,0595	0,5000					
2% KNO_3	0,3074	0,0022	0,0738	0,0100	0,5000				
3% KNO_3	0,4223	0,0860	0,2539	0,0915	0,4996	0,5000			
0.25 mM SA	0,0180	0,0001	0,0060	0,0015	0,0358	0,1404	0,5000		
0.5 mM SA	0,0556	0,0049	0,0246	0,0076	0,0813	0,1853	0,3213	0,5000	
0.75 mM SA	0,0591	0,0001	0,4753	0,0306	0,0257	0,1169	0,3890	0,2411	0,5000

Table A.9. Estimated p-values among priming treatments for biomass of salt-stressed *B. distachyon* plants.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,4043	0,5000							
HP20	0,0953	0,0993	0,5000						
1% KNO_3	0,1095	0,0832	0,0437	0,5000					
2% KNO_3	0,1628	0,1411	0,0589	0,4640	0,5000				
3% KNO_3	0,0246	0,0167	0,0199	0,2136	0,2992	0,5000			
0.25 mM SA	0,0447	0,0202	0,0331	0,4703	0,4385	0,1404	0,5000		
0.5 mM SA	0,0172	0,0048	0,0252	0,4235	0,4896	0,1853	0,3213	0,5000	
0.75 mM SA	0,0846	0,0483	0,0412	0,3971	0,3873	0,1169	0,3890	0,2411	0,5000

Table A.10. Estimated p-values among priming treatments for biomass of drought-stressed *B. distachyon* plants.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3125	0,5000							
HP20	0,0476	0,1125	0,5000						
1% KNO_3	0,1665	0,2923	0,2809	0,5000					
2% KNO_3	0,0973	0,2355	0,2207	0,4958	0,5000				
3% KNO_3	0,2147	0,1204	0,0110	0,0671	0,0159	0,5000			
0.25 mM SA	0,3960	0,2424	0,0376	0,1314	0,0724	0,3310	0,5000		
0.5 mM SA	0,1761	0,3255	0,2134	0,4443	0,4229	0,0593	0,1355	0,5000	
0.75 mM SA	0,4006	0,4268	0,1013	0,2512	0,1997	0,1970	0,3233	0,2765	0,5000

Table A.11. Estimated p-values among priming treatments for RWC measurements in unstressed *B. distachyon plants*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5								
HP4	0,0224	0,5							
HP20	0,0027	0,1751	0,5						
1% KNO_3	0,0028	0,2206	0,3034	0,5					
2% KNO_3	0,0099	0,4287	0,0425	0,0523	0,5				
3% KNO_3	0,0020	0,1382	0,3588	0,1448	0,0216	0,5			
0.25 mM SA	0,0036	0,2302	0,3419	0,4973	0,0758	0,2184	0,5		
0.5 mM SA	0,0018	0,1276	0,3054	0,0972	0,0168	0,4315	0,1798	0,5	
0.75 mM SA	0,0030	0,1926	0,4401	0,3691	0,0513	0,2992	0,3961	0,2501	0,5

Table A.12. Estimated p-values among priming treatments for RWC measurements in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0024	0,5000							
HP20	0,0017	0,0004	0,5000						
1% KNO_3	0,4961	0,0596	0,1942	0,5000					
2% KNO_3	0,3077	0,0369	0,0518	0,3867	0,5000				
3% KNO_3	0,0189	0,4554	0,0052	0,0742	0,0628	0,5000			
0.25 mM SA	0,0062	0,1474	0,0021	0,0342	0,0235	0,2238	0,5000		
0.5 mM SA	0,0154	0,1248	0,0069	0,0355	0,0308	0,1630	0,3229	0,5000	
0.75 mM SA	0,0099	0,2311	0,0016	0,1003	0,0811	0,2767	0,0832	0,0839	0,5000

Table A.13. Estimated p-values among priming treatments for RMP measurements in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,4251	0,5000							
HP20	0,0891	0,1503	0,5000						
1% KNO_3	0,0322	0,0582	0,1131	0,5000					
2% KNO_3	0,0203	0,0391	0,0409	0,0132	0,5000				
3% KNO_3	0,0047	0,0114	0,0024	0,0001	0,0003	0,5000			
0.25 mM SA	0,0077	0,0174	0,0061	0,0008	0,0030	0,0304	0,5000		
0.5 mM SA	0,0130	0,0294	0,0216	0,0270	0,0820	0,0559	0,2591	0,5000	
0.75 mM SA	0,0453	0,0852	0,0650	0,0304	0,2131	0,0060	0,0318	0,2136	0,5000

Table A.14. Estimated p-values among priming treatments for RMP measurements in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,2131	0,5000							
HP20	0,0617	0,0130	0,5000						
1% KNO_3	0,0193	0,0027	0,4477	0,5000					
2% KNO_3	0,0351	0,0030	0,0702	0,0323	0,5000				
3% KNO_3	0,0014	0,0001	0,1334	0,0481	0,0004	0,5000			
0.25 mM SA	0,0024	0,0001	0,4152	0,2921	0,0011	0,0081	0,5000		
0.5 mM SA	0,0012	0,0000	0,1842	0,0765	0,0003	0,1282	0,0083	0,5000	
0.75 mM SA	0,0011	0,0000	0,1880	0,0785	0,0002	0,0993	0,0060	0,2136	0,5000

Table A.15. Estimated p-values among priming treatments for RMP measurements in drought-treated *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0485	0,5000							
HP20	0,2064	0,3188	0,5000						
1% KNO_3	0,0042	0,4692	0,3142	0,5000					
2% KNO_3	0,0204	0,4783	0,2864	0,4846	0,5000				
3% KNO_3	0,0252	0,1604	0,1418	0,1804	0,1437	0,5000			
0.25 mM SA	0,0008	0,1929	0,1383	0,0415	0,1388	0,2849	0,5000		
0.5 mM SA	0,0020	0,0686	0,1063	0,0069	0,0345	0,3027	0,0118	0,5000	
0.75 mM SA	0,0080	0,2641	0,1779	0,2197	0,2350	0,2570	0,3871	0,0528	0,5

Table A.16. Estimated p-values among priming treatments for chlorophyll content with SPAD values in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0300	0,5000							
HP20	0,1620	0,0001	0,5000						
1% KNO_3	0,4809	0,0098	0,0975	0,5000					
2% KNO_3	0,4131	0,0020	0,0067	0,3514	0,5000				
3% KNO_3	0,3997	0,0014	0,0032	0,3313	0,4676	0,5000			
0.25 mM SA	0,4639	0,0062	0,0424	0,4265	0,4185	0,3938	0,5000		
0.5 mM SA	0,1583	0,0136	0,0014	0,0855	0,0534	0,0480	0,0795	0,5000	
0.75 mM SA	0,3866	0,0158	0,0447	0,3368	0,4367	0,4528	0,3877	0,1627	0,5000

Table A.17. Estimated p-values among priming treatments for chlorophyll content with SPAD values in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3658	0,5000							
HP20	0,3606	0,4855	0,5000						
1% KNO_3	0,2836	0,1097	0,0530	0,5000					
2% KNO_3	0,3344	0,1406	0,0732	0,3261	0,5000				
3% KNO_3	0,3645	0,4763	0,4879	0,0458	0,0640	0,5000			
0.25 mM SA	0,3281	0,1730	0,1373	0,4621	0,4461	0,1348	0,5000		
0.5 mM SA	0,2992	0,1110	0,0445	0,4035	0,3511	0,0355	0,5000	0,5000	
0.75 mM SA	0,0789	0,0191	0,0049	0,0120	0,0056	0,0036	0,0761	0,1627	0,5000

Table A.18. Estimated p-values among priming treatments for chlorophyll content with SPAD values in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,1302	0,5000							
HP20	0,3491	0,2718	0,5000						
1% KNO_3	0,1166	0,0025	0,0741	0,5000					
2% KNO_3	0,0509	0,0078	0,0374	0,1013	0,5000				
3% KNO_3	0,2152	0,0521	0,1483	0,4986	0,1979	0,5000			
0.25 mM SA	0,0381	0,0090	0,0292	0,0673	0,2996	0,1241	0,5000		
0.5 mM SA	0,1494	0,0584	0,1126	0,2853	0,4520	0,3138	0,3190	0,5000	
0.75 mM SA	0,0149	0,0017	0,0124	0,0133	0,1971	0,0711	0,4356	0,2650	0,5000

Table A.19. Estimated p-values among priming treatments for chlorophyll content by acetone method in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3818	0,5000							
HP20	0,3319	0,3770	0,5000						
1% KNO_3	0,4595	0,3112	0,2754	0,5000					
2% KNO_3	0,1688	0,0143	0,2315	0,1131	0,5000				
3% KNO_3	0,1993	0,0242	0,2935	0,1376	0,1024	0,5000			
0.25 mM SA	0,2271	0,1579	0,3408	0,1703	0,3606	0,4734	0,5000		
0.5 mM SA	0,4423	0,3604	0,3135	0,3795	0,0357	0,0532	0,1381	0,5000	
0.75 mM SA	0,2411	0,1237	0,3740	0,1772	0,1879	0,3215	0,4249	0,1206	0,5000

Table A.20. Estimated p-values among priming treatments for chlorophyll content by acetone method in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,2769	0,5000							
HP20	0,4829	0,4389	0,5000						
1% KNO_3	0,3200	0,2871	0,3303	0,5000					
2% KNO_3	0,2252	0,3336	0,3512	0,2501	0,5000				
3% KNO_3	0,0029	0,0192	0,1659	0,1679	0,1815	0,5000			
0.25 mM SA	0,0016	0,0137	0,1553	0,1627	0,1621	0,3671	0,5000		
0.5 mM SA	0,0719	0,1199	0,2031	0,1801	0,2593	0,4794	0,4476	0,5000	
0.75 mM SA	0,2189	0,4592	0,4237	0,2805	0,3502	0,0143	0,0093	0,1234	0,5000

Table A.21. Estimated p-values among priming treatments for chlorophyll content by acetone method in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3408	0,5000							
HP20	0,4192	0,3695	0,5000						
1% KNO_3	0,1138	0,0772	0,0890	0,5000					
2% KNO_3	0,3177	0,2373	0,2699	0,2459	0,5000				
3% KNO_3	0,3955	0,4103	0,4618	0,0856	0,2600	0,5000			
0.25 mM SA	0,3142	0,1151	0,1798	0,1448	0,4082	0,1627	0,5000		
0.5 mM SA	0,4390	0,2538	0,4452	0,0886	0,2757	0,3951	0,1578	0,5000	
0.75 mM SA	0,2849	0,0946	0,1518	0,1528	0,4266	0,1370	0,4566	0,1269	0,5000

Table A.22. Estimated p-values among priming treatments for carotene content in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,2071	0,5000							
HP20	0,1892	0,3936	0,5000						
1% KNO_3	0,4874	0,2434	0,2185	0,5000					
2% KNO_3	0,3038	0,2192	0,2222	0,3395	0,5000				
3% KNO_3	0,2604	0,3559	0,3020	0,2960	0,3591	0,5000			
0.25 mM SA	0,2980	0,3261	0,2820	0,3296	0,4502	0,4426	0,5000		
0.5 mM SA	0,4997	0,0478	0,0888	0,4838	0,0787	0,0842	0,1616	0,5000	
0.75 mM SA	0,0507	0,0432	0,1297	0,0695	0,0129	0,0286	0,0374	0,0043	0,5000

Table A.23. Estimated p-values among priming treatments for carotene content in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3901	0,5000							
HP20	0,3950	0,4724	0,5000						
1% KNO_3	0,2274	0,1898	0,1777	0,5000					
2% KNO_3	0,2539	0,2314	0,2288	0,3861	0,5000				
3% KNO_3	0,4912	0,3854	0,3852	0,2100	0,2472	0,5000			
0.25 mM SA	0,2411	0,1939	0,1371	0,3339	0,3037	0,1742	0,5000		
0.5 mM SA	0,4426	0,3514	0,3503	0,2704	0,2738	0,4283	0,3346	0,5000	
0.75 mM SA	0,4487	0,3423	0,3304	0,2397	0,2612	0,4280	0,2469	0,4821	0,5000

Table A.24. Estimated p-values among priming treatments for carotene content in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,4218	0,5000							
HP20	0,1927	0,1903	0,5000						
1% KNO_3	0,1832	0,1787	0,4429	0,5000					
2% KNO_3	0,2183	0,2474	0,4076	0,3705	0,5000				
3% KNO_3	0,3897	0,3521	0,3513	0,3174	0,4312	0,5000			
0.25 mM SA	0,1419	0,1412	0,3535	0,4110	0,3030	0,2559	0,5000		
0.5 mM SA	0,1040	0,1461	0,4440	0,3830	0,3835	0,3596	0,2882	0,5000	
0.75 mM SA	0,1163	0,1310	0,4256	0,4960	0,3138	0,2890	0,4008	0,3266	0,5000

Table A.25. Estimated p-values among priming treatments for proline content in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,2346	0,5000							
HP20	0,3914	0,1771	0,5000						
1% KNO_3	0,0356	0,2023	0,0222	0,5000					
2% KNO_3	0,3540	0,3924	0,2833	0,1471	0,5000				
3% KNO_3	0,3980	0,2279	0,4650	0,0835	0,3090	0,5000			
0.25 mM SA	0,1132	0,3618	0,0776	0,2797	0,2677	0,1473	0,5000		
0.5 mM SA	0,4558	0,2727	0,3575	0,0551	0,3917	0,3733	0,1463	0,5000	
0.75 mM SA	0,4618	0,2335	0,4455	0,0529	0,3389	0,4307	0,1272	0,4259	0,5000

Table A.26. Estimated p-values among priming treatments for proline content in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0196	0,5000							
HP20	0,3072	0,0270	0,5000						
1% KNO_3	0,1821	0,0247	0,4899	0,5000					
2% KNO_3	0,1243	0,0753	0,1937	0,1809	0,5000				
3% KNO_3	0,0002	0,1452	0,0054	0,0012	0,1281	0,5000			
0.25 mM SA	0,0559	0,0569	0,1382	0,1100	0,4738	0,0473	0,5000		
0.5 mM SA	0,0342	0,3337	0,0478	0,0441	0,1357	0,2883	0,1070	0,5000	
0.75 mM SA	0,0009	0,0589	0,0375	0,0098	0,4319	0,0083	0,3503	0,1160	0,5000

Table A.27. Estimated p-values among priming treatments for proline content in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,1587	0,5000							
HP20	0,0205	0,0089	0,5000						
1% KNO_3	0,2558	0,1081	0,2203	0,5000					
2% KNO_3	0,0555	0,0176	0,0369	0,4070	0,5000				
3% KNO_3	0,0610	0,4237	0,0004	0,0699	0,0002	0,5000			
0.25 mM SA	0,3215	0,3041	0,0202	0,1820	0,0454	0,2009	0,5000		
0.5 mM SA	0,1353	0,4299	0,0096	0,0958	0,0182	0,4778	0,2576	0,5000	
0.75 mM SA	0,3383	0,2083	0,0048	0,1813	0,0098	0,0561	0,4293	0,1734	0,5000

Table A.28. Estimated p-values among priming treatments for crude protein concentration in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5								
HP4	0,4037	0,5000							
HP20	0,1084	0,0308	0,5000						
1% KNO_3	0,3762	0,4413	0,0567	0,5000					
2% KNO_3	0,4210	0,4577	0,0228	0,4106	0,5000				
3% KNO_3	0,4959	0,3129	0,0299	0,3222	0,1066	0,5000			
0.25 mM SA	0,1384	0,0222	0,2722	0,0647	0,0541	0,0107	0,5000		
0.5 mM SA	0,4970	0,3591	0,0482	0,3450	0,3999	0,4872	0,0424	0,5000	
0.75 mM SA	0,3084	0,1649	0,1587	0,1899	0,1335	0,2175	0,2186	0,2431	0,5000

Table A.29. Estimated p-values among priming treatments for crude protein concentration in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0104	0,5000							
HP20	0,0210	0,0867	0,5000						
1% KNO_3	0,1026	0,0236	0,0736	0,5000					
2% KNO_3	0,0613	0,0879	0,3233	0,2407	0,5000				
3% KNO_3	0,0725	0,0463	0,1774	0,3294	0,3766	0,5000			
0.25 mM SA	0,0194	0,0860	0,4812	0,0656	0,3111	0,1652	0,5000		
0.5 mM SA	0,0955	0,0159	0,0407	0,4659	0,2063	0,2859	0,0339	0,5000	
0.75 mM SA	0,0302	0,0662	0,3469	0,1256	0,4222	0,2656	0,3258	0,0817	0,5000

Table A.30. Estimated p-values among priming treatments for crude protein concentration in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0442	0,5000							
HP20	0,0073	0,0007	0,5000						
1% KNO_3	0,0475	0,1975	0,2845	0,5000					
2% KNO_3	0,0353	0,1569	0,2137	0,4808	0,5000				
3% KNO_3	0,0409	0,1529	0,3923	0,4215	0,3954	0,5000			
0.25 mM SA	0,0679	0,2414	0,3417	0,4881	0,4711	0,4430	0,5000		
0.5 mM SA	0,1052	0,4575	0,1184	0,2963	0,2910	0,2428	0,3107	0,5000	
0.75 mM SA	0,0478	0,3647	0,0205	0,2561	0,2309	0,1997	0,2876	0,4828	0,5000

Table A.31. Estimated p-values among priming treatments for CAT activity in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,2946	0,5000							
HP20	0,3175	0,3027	0,5000						
1% KNO_3	0,2231	0,1846	0,1298	0,5000					
2% KNO_3	0,1938	0,3728	0,1749	0,1000	0,5000				
3% KNO_3	0,0098	0,1441	0,0064	0,0020	0,2373	0,5000			
0.25 mM SA	0,1017	0,1617	0,0662	0,0426	0,2269	0,3615	0,5000		
0.5 mM SA	0,2048	0,3438	0,1790	0,1129	0,4552	0,3170	0,2738	0,5000	
0.75 mM SA	0,0300	0,2961	0,0283	0,0077	0,4517	0,1201	0,2067	0,4850	0,5000

Table A.32. Estimated p-values among priming treatments for CAT activity in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3114	0,5000							
HP20	0,0173	0,1479	0,5000						
1% KNO_3	0,0856	0,3606	0,0764	0,5000					
2% KNO_3	0,2448	0,4454	0,0313	0,2015	0,5000				
3% KNO_3	0,0051	0,0413	0,0213	0,0070	0,0049	0,5000			
0.25 mM SA	0,0724	0,1307	0,3013	0,1234	0,0695	0,2955	0,5000		
0.5 mM SA	0,4688	0,2681	0,0252	0,0979	0,2382	0,0063	0,0450	0,5000	
0.75 mM SA	0,2188	0,4646	0,0318	0,2182	0,4670	0,0047	0,0726	0,2176	0,5000

Table A.33. Estimated p-values among priming treatments for CAT activity in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,2355	0,5000							
HP20	0,1230	0,1885	0,5000						
1% KNO_3	0,0205	0,0089	0,0185	0,5000					
2% KNO_3	0,3855	0,2254	0,0818	0,0060	0,5000				
3% KNO_3	0,0832	0,3988	0,1977	0,0084	0,0601	0,5000			
0.25 mM SA	0,4145	0,1585	0,0656	0,0056	0,2902	0,0582	0,5000		
0.5 mM SA	0,2477	0,2457	0,3588	0,1154	0,1981	0,2587	0,1777	0,5000	
0.75 mM SA	0,0002	0,0003	0,0033	0,2466	0,0000	0,0000	0,0001	0,1491	0,5000

Table A.34. Estimated p-values among priming treatments for POD activity in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,1710	0,5000							
HP20	0,4165	0,0915	0,5000						
1% KNO_3	0,0630	0,4716	0,0219	0,5000					
2% KNO_3	0,0642	0,4360	0,0224	0,4157	0,5000				
3% KNO_3	0,0453	0,4727	0,0143	0,3684	0,2757	0,5000			
0.25 mM SA	0,0095	0,1028	0,0020	0,0098	0,0059	0,0098	0,5000		
0.5 mM SA	0,1471	0,2674	0,0660	0,1175	0,1333	0,0640	0,0027	0,5000	
0.75 mM SA	0,0408	0,1024	0,0129	0,0535	0,0467	0,0609	0,2993	0,0280	0,5000

Table A.35. Estimated p-values among priming treatments for POD activity in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0013	0,5000							
HP20	0,1495	0,0353	0,5000						
1% KNO_3	0,0061	0,0043	0,4170	0,5000					
2% KNO_3	0,0026	0,0155	0,2091	0,0266	0,5000				
3% KNO_3	0,0053	0,0070	0,3385	0,1896	0,1006	0,5000			
0.25 mM SA	0,0018	0,0911	0,0892	0,0086	0,0668	0,0190	0,5000		
0.5 mM SA	0,0064	0,0265	0,2103	0,0782	0,4647	0,1631	0,1178	0,5000	
0.75 mM SA	0,0063	0,1732	0,0964	0,0340	0,1220	0,0538	0,4350	0,1541	0,5000

Table A.36. Estimated p-values among priming treatments for POD activity in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0015	0,5000							
HP20	0,0472	0,0190	0,5000						
1% KNO_3	0,0009	0,0861	0,0077	0,5000					
2% KNO_3	0,0273	0,0335	0,3358	0,0122	0,5000				
3% KNO_3	0,0102	0,1476	0,0336	0,2732	0,0442	0,5000			
0.25 mM SA	0,0161	0,1353	0,0420	0,2176	0,0523	0,3895	0,5000		
0.5 mM SA	0,0073	0,1147	0,0249	0,2277	0,0331	0,4699	0,4105	0,5000	
0.75 mM SA	0,0023	0,0633	0,0104	0,1863	0,0146	0,4835	0,3619	0,4450	0,5000

Table A.37. Estimated p-values among priming treatments for SOD activity in unstressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,1886	0,5000							
HP20	0,3450	0,2186	0,5000						
1% KNO_3	0,0311	0,2350	0,0178	0,5000					
2% KNO_3	0,0690	0,4071	0,0483	0,2181	0,5000				
3% KNO_3	0,1088	0,3414	0,1184	0,4210	0,3860	0,5000			
0.25 mM SA	0,0211	0,0940	0,0181	0,1510	0,0753	0,1708	0,5000		
0.5 mM SA	0,0007	0,0083	0,0001	0,0030	0,0013	0,0178	0,0656	0,5000	
0.75 mM SA	0,0071	0,0194	0,0065	0,0226	0,0148	0,0304	0,0755	0,2411	0,5000

Table A.38. Estimated p-values among priming treatments for SOD activity in salt-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,0053	0,5000							
HP20	0,0413	0,3298	0,5000						
1% KNO_3	0,0163	0,2587	0,4828	0,5000					
2% KNO_3	0,0062	0,4079	0,3610	0,3074	0,5000				
3% KNO_3	0,0135	0,0209	0,3601	0,2475	0,0376	0,5000			
0.25 mM SA	0,0397	0,3397	0,4910	0,4938	0,3716	0,3480	0,5000		
0.5 mM SA	0,0112	0,0763	0,0768	0,0619	0,0717	0,0389	0,0782	0,5000	
0.75 mM SA	0,0109	0,0427	0,0425	0,0368	0,0409	0,0273	0,0430	0,2239	0,5000

Table A.39. Estimated p-values among priming treatments for SOD activity in drought-stressed *B. distachyon*.

	NP	**HP4**	**HP20**	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**	**0.25 mM SA**	**0.5 mM SA**	**0.75 mM SA**
NP	0,5000								
HP4	0,3658	0,5000							
HP20	0,1265	0,0645	0,5000						
1% KNO_3	0,1757	0,1071	0,4532	0,5000					
2% KNO_3	0,0563	0,0157	0,3879	0,3515	0,5000				
3% KNO_3	0,0716	0,0217	0,4471	0,4012	0,4203	0,5000			
0.25 mM SA	0,0128	0,0062	0,0539	0,0577	0,0489	0,0425	0,5000		
0.5 mM SA	0,0493	0,0224	0,2508	0,2362	0,2947	0,2502	0,1202	0,5000	
0.75 mM SA	0,0540	0,0083	0,4563	0,4056	0,3779	0,4770	0,0319	0,2160	0,5000