EFFECTS OF GLYPHOSATE ON *BRACHYPODIUM DISTACHYON*

by

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Submitted to the Graduate School of Engineering and Natural Sciences

in partial fulfillment of

the requirements for the degree of

Master of Science

Sabancı University

December 2010

EFFECTS OF GLYPHOSATE ON *BRACHYPODIUM DISTACHYON*

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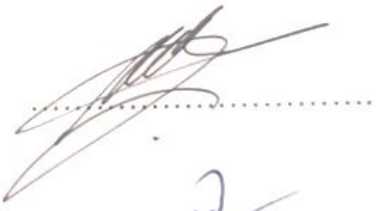

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DATE OF APPROVAL: 15.12.2010

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Biological Sciences and Bioengineering Master's Thesis, 2010

Thesis Supervisor: Hikmet Budak

Keywords: Glyphosate, *Brachypodium*, Lipid Peroxidation, Free Proline Content, Shikimate Accumulation

ABSTRACT

Glyphosate is a non-selective herbicide used widely as the most popular weed management tool, especially since the commercialization of glyphosate-resistant crops. Due to overuse, several weed species have evolved resistance towards glyphosate and this trend threatens the future of world food production. *Brachypodium distachyon* has been proposed as a model organism specifically for economically important crops such as wheat and barley. Thus, evaluating the effects of glyphosate on *Brachypodium* can supply the required information about the tolerance of glyphosate among such crops. In this study, lipid peroxidation, free proline content, shikimate accumulation and antioxidant enzyme activities have been investigated as biochemical markers of glyphosate damage applied at two different concentrations, 5% and 20% of the recommended field rate. Thirteen Turkish and two standard *Brachypodium* lines were screened part of this study. There were various levels of responsiveness among the lines, suggesting that resistance may arise in this species, as well, if they are exposed to continuous applications of glyphosate. Further physiological and molecular analyses are required for a more conclusive result on this subject.

BRACHYPODIUM DISTACHYON ÜZERİNDE GLYPHOSATE ETKİLERİ

Ayşegül Altıntaş

Biyolojik Bilimler ve Biyomühendislik Master Tezi, 2010

Tez Danışmanı: Hikmet Budak

Keywords: Glyphosate, *Brachypodium*, Lipid Peroxidation, Free Proline Content, Shikimate Accumulation

ÖZET

Özellikle glyphosate dayanıklılığı olan ekinlerin piyasaya sürülmesiyle birlikte, glyphosate yabani otların kontrolünde en yaygın kullanılan seçici olmayan herbisid haline almıştır. Aşırı kullanımı glyphosate dayanıklılığı olan yabani otların evrimleşmesine sebep olmakla birlikte, bu durum devam ederse dünya gıda üretimini tehdit edebilecek bir hal alabilir. *Brachypodium distachyon,* özellikle ekonomik önem taşıyan buğday ve arpa gibi ekinler için model organizma olarak öne sürülmüştür. Bu nedenle, *Brachypodium* üzerinde glyphosate etkilerini incelemek bu ekinlerin glyphosate toleransı hakkında gerekli bilgileri verebilir. Bu çalışmada, tavsiye edilen arazi uygulama oranının %5'i ve %20'si olmak üzere iki farklı konsantrasyondaki glyphosate uygulamasından kaynaklanan hasarları incelemek için lipid peroksidasyonu, serbest prolin içeriği, şikimat birikimi ve antioksidan enzim aktiviteleri araştırılmıştır. Onüç Türkiye kökenli ve iki standart *Brachypodium* türü bu çalışma kapsamında taranmıştır. Bu türler arasında farklı seviyelerde duyarlılık gözlemlenmiş olup eğer glyphosate devamlı olarak uygulanmaya devam edilirse benzer bir dayanıklılığın açığa çıkabileceğine işaret etmiştir. Bu konuda daha kesin sonuçlar elde etmek için daha dazla fizyolojik ve moleküler analizlere gerek duyulmaktadır.

"For my family and friends…"

Acknowledgement

I would like to express my gratefulness to my supervisor Hikmet Budak, for his guidance and support at every stage of this program. Thanks to his encouragement, I was able to complete this study in spite of a series of unfortunate events. His persistence has made sure that I did not give up on this project and on myself.

I would also like to thank Academic Support Program and Sabancı University for all the financial support I have received over the past 2,5 years.

I would like to thank each member of my thesis committee; Assoc. Prof. Devrim Gözüaçık, Assist. Prof. Murat Çokol, Assistant Prof. Alpay Taralp, and Assist. Prof. Javed Kolkar for their contribution during my thesis and presentation.

I am grateful for all the help I received from all my colleagues in Budak laboratory, and special thanks to Melda Kantar, Ani Akpinar and Duygu Kuzuoğlu for their support during this study. I would also like to acknowledge all the members of the Physiology laboratory for allowing me use their space and equipment throughout this study.

I wish to thank my dear friends Selda Kantar, Nurten Ukelgi, Bahar Açıksöz, Nazlı Keskin, Asli Çalık and Gökçen Gökçe for helping me go through a very trying time of my life.

I am eternally grateful to my family; my mother, my father and my brother, Arif Altıntaş, who believed in me even when I did not.

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1. Introduction

1.1. *Brachypodium distachyon* as a model organism

In molecular biology and genetics, model organisms are developed in order to establish information and techniques that may be applicable to phyllogenetically close species, whose genomes are more complicated. For this reason alone, *Brachypodium* attracts attention as a possible model for other economically important grass species, such as wheat and barley. Apart from the genetic similarities to cereals, *Brachypodium* has a rapid life cycle and a small genome, making it a suitable organism for research purposes.(Ozdemir et al., 2008)

Brachypodium distachyon has a small genome of about 355Mbp and less than 15% is highly repeated DNA. *Brachypodium* growth cycle ranges between 8 weeks to 18 weeks, depending on the specific light and temperature conditions. Previously, rice, *Oryza sativa*, was the closest model used in studying the genomics of weed species. However, the growth requirements of rice are more complicated and the life cycle is much longer when compared to *Brachypodium*. *Brachypodium* naturally exists in similar climates as wheat and barley, suggesting it is a lot more compatible as a model for these temperate cereals. Even though the rice genome has been sequenced earlier (2002), it is a tropical species and some of the physiological attributes may not be as close to temperate grasses as desired.(Draper et al., 2001; Goff et al., 2002)

Brachypodium is also ideal as a model grass considering the ease of tissue culture and the possibility of transformation by *Agrobacterium tumefaciens*. Calluses can be induced from mature and immature embryo, as well as, root and shoot tissue under optimized conditions.

Agro-transformation of calluses can be carried on with relative ease and transgenic embryo can be recovered without much difficulty.(Draper et al., 2001)

Figure 1: Brachypodium distachyon grown under greenhouse conditions. Inbred lines show differences in leaf erectness, plant height, seed size and other physical properties. (Filiz et al., 2009)

Just as most cereals are susceptible to pathogen attacks, a good model must have known pathogen interactions as well. *Brachypodium* has been shown to display blast-like symptoms, when exposed to *Magnaporthe grisea*, the causal agent of rice blast disease. Highly localized necrotic lesions were observed but the disease was stopped from spreading due to an innate resistance. Known resistance to this particular pathogen can be used as a means to study resistance pathways responsible for resistance in *Brachypodium*, whereas for rice the symptoms reach lethal levels. (Draper et al., 2001)

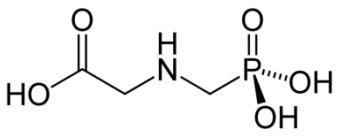
There are several inbred lines available for *Brachypodium* and depending on the specific geographic location they have been collected from, they can show very different traits. The inbred lines exhibit phenotypic diversity in such traits as plant stature, leaf characteristics, flowering time, seed size and yield to name a few. (Filiz et al., 2009)

1.2. Glyphosate, the most commonly used herbicide

Since its introduction to the market in 1970s, glyphosate has been the most commonly used herbicide due to the fact that it is effective, inexpensive and environmentally benign. It is a broad-spectrum herbicide and it is the only herbicide that inhibits the activity of 5-enolpyruvul-shikimate-3-phosphate synthase (EPSPS). Following foliar application, glyphosate is absorbed rapidly through the surface. Even though the uptake rate may vary among species, diffusion is the most common mode of transport for glyphosate. After absorption, glyphosate travels to its target tissues, meristems, young roots and leaves, storage organs and any other actively growing tissue, via the phloem of target plants. The efficacy of glyphosate is increased by excellent translocation, limited degradation and a slow mode of action. (Duke and Powles, 2008)

The popularity of glyphosate has promoted several developments in the genetically modified organism (GDO) research. Circa 1996, soybean, maize, cotton and canola were introduced as the first glyphosate-resistant crops (GRC), carrying a bacterial gene responsible for resistance to glyphosate. GRCs were rapidly adopted as the most efficient weed management choice making it possible to diminish the amount of manual labor required in agriculture. Chemical and physical properties of glyphosate (Table 1) allow it to be an efficient and relatively easy to use method for weed management. (Dill et al., 2008; Powles, 2008)

Table 1: Physical and chemical properties of glyphosate

Parameter	**Glyphosate**
Chemical Structure	
Chemical Name	*N*-(phosphonomethyl)glycine
Empirical Formula	$C_3H_8NO_5P$
Molar mass	169.08 g mol^{-1}
Water solubility	1.01 g/100 ml (20^oC)
Vapor pressure	$7.5x10^{-8}$ mmHg
pKa	pKa_1 0.8, pKa_2 3.0, pKa_3 6.0 and pKa_4 10.0
Half-life in water	DT_{50} 3-174 days
Half-life in soil	DT_{50} 5-91 days

1.3. Glyphosate mode of action

Glyphosate uniquely acts on shikimate pathway via the inhibition of the sixth enzyme, 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), on the pathway. EPSPS is essential for the synthesis of aromatic aminoacids as well as for the synthesis of several other aromatic compounds due to its key role in the production of chorismate, the precursor molecule for aromatic amino acids, phenylalanine, tyrosine and tryptophan. In the consecutive reactions converting shikimate-3-phosphate (S3P) to chorismate, EPSPS catalyzes the first step, the transfer of the enolpyruvyl moiety of phosphoenol pyruvate (PEP) to S3P. (Duke and Powles, 2008)

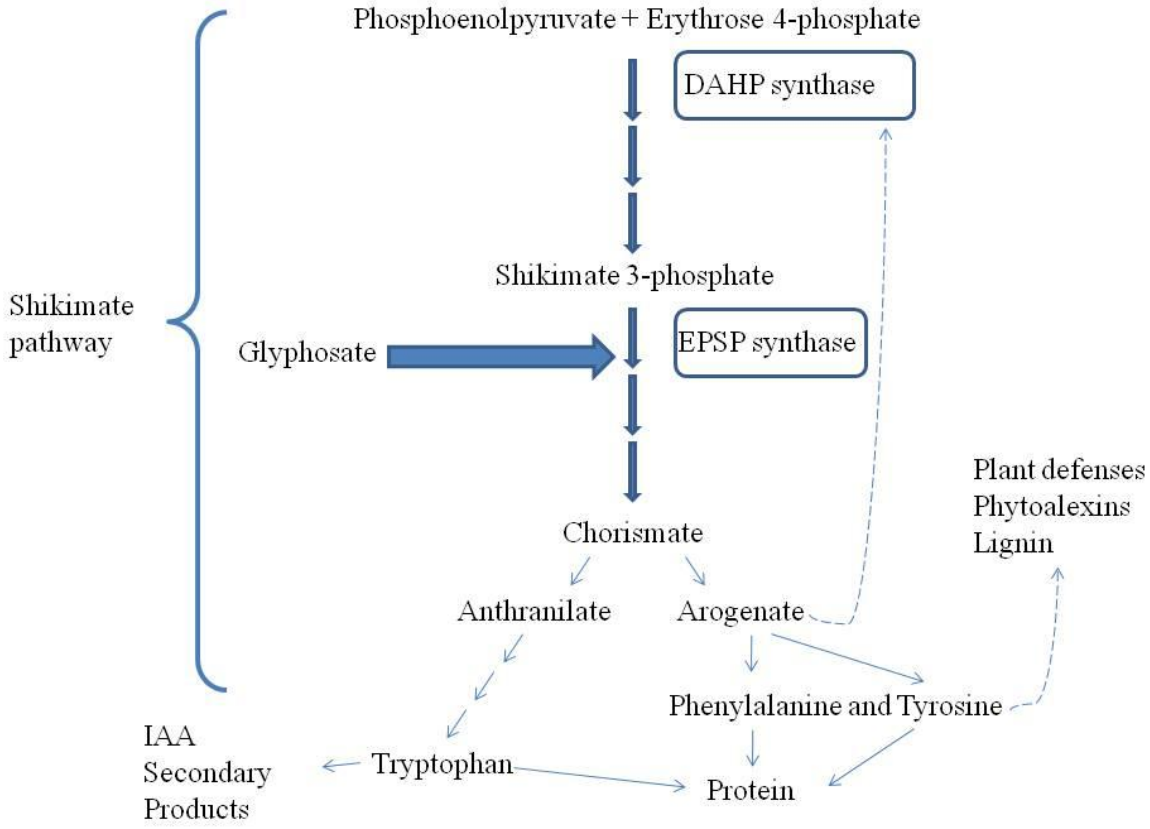


Figure 2: Shikimate pathway and glyphosate interaction. Glyphosate inhibits EPSPS and interrupts the downstream production of aromatic amino acids as well as the feedback mechanism. (Duke and Powles, 2008)

The reaction, proceeding via the unusual cleavage of C-O bond, leads to the formation of EPSP which will eventually be converted to chorismate. The reaction is considered as 'unusual' due to the C-O bond cleavage given that most PEP-utilizing enzymes act on P-O bonds. Being a phosphoenylpyruvate analog, one of the substrates of EPSPS, glyphosate competitively inhibits EPSPS versus PEP in a slowly reversible fashion, blocking aromatic aminoacid synthesis in glyphosate sensitive species. (Duke and Powles, 2008; Schonbrunn et al., 2001)

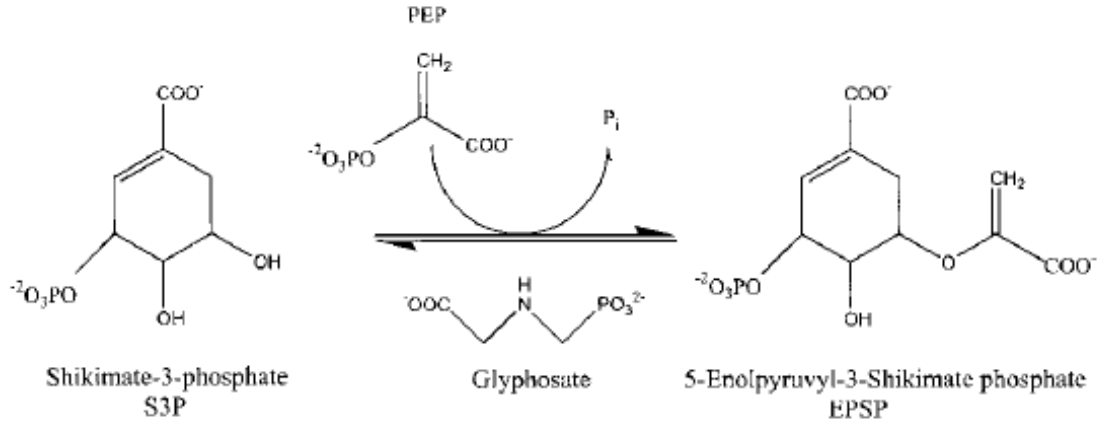


Figure 3: Glyphosate mode of action.(Schonbrunn et al., 2001)

Though the inhibition of EPSPS by glyphosate is a well-known fact, the actual mechanism leading to death of the plant is relatively controversial and appears to be species-dependent. Consistent with the slow appearance symptoms, it is generally accepted that glyphosate primarily halts protein synthesis via the inhibition of aromatic amino acids, resulting in the eventual death of the plant. However, evidence from some studies also suggest that accumulation of shikimate which cannot be converted to chorismate since EPSPS is inhibited may also play central role in glyphosate-mediated death. High levels of shikimate arrest the most of the carbon pool in cells and thus, block carbon flow to other essential pathways. As a result, plant suffers from carbon deficiency. Indeed, in sugarbeet, mode of action of glyphosate appears to proceed via interruption of carbon fixation, demonstrating the species-dependent aspect of glyphosate action. (Chen et al., 2001; Velini et al., 2008)

Whereas algae, higher plants, bacteria, fungi and some parasites are susceptible to glyphosate, the fact that mammals lack the shikimate pathway and uptake aromatic amino acids through their diet, makes glyphosate an attractive target for antimicrobial and anti-fungal purposes. (Schonbrunn et al., 2001).

1.4. Glyphosate resistant (GR) crops

Using a non-selective herbicide such as glyphosate involved different strategies in order to prevent damage to the crops. If even a small amount gets in contact with the leaves of a plant, it results in phytotoxic effects since glyphosate is potent and such contact should be avoided. For a while, glyphosate was applied on the fields before the annual crops, such as wheat, were planted in order to damage only the weeds on the field. However, a new set of weeds can grow in the same field afterwards and this required either a combination of methods or a clever strategy. Even though some gadgets were designed to shield the crops from exposure to glyphosate that was sprayed on the weeds in the vicinity, this was nowhere near solving the problem. (Duke and Powles, 2008)

In order to circumvent time limitations on glyphosate use, researchers searched for natural resistance against glyphosate. *Agrobacterium* was found to possess a naturally resistant form of the EPSPS gene, CP4. When CP4 was transformed into the genomes of certain crops, it resulted in high levels of resistance against glyphosate. Thus, glyphosate resistant soybean was produced with the addition of CP4 gene and showed upto 50-fold resistance to field rate of glyphosate. The glyphosate resistant maize was designed by site-directed mutagenesis to form GR forms of EPSPS. Even though transformation with other genes to encode GR forms of EPSPS and detoxification enzymes are proposed for future use, currently, most GR crops carry the CP4 gene isolated from *Agrobacterium.* (Padgette et al., 1996)

1.5. Herbicide resistance in weeds

Herbicide resistance usually refers to the condition that a plant survives the usual field dose of the specific herbicide. Glyphosate is a non-selective herbicide, suggesting that resistance does not exist naturally in any higher plants. However, different levels of tolerance may depend on the physiological characteristics of specific plants. Tolerance may vary among different lines of the same species, as well as among different species. Even though natural resistance is rare if not non-existent, evolved resistance is serious threat towards the popular use of glyphosate. In 1997, Bradshaw et al. declared that evolved resistance would be too slow a process in nature to even consider dangerous taking into account the complexity of the process of producing GR crops. However, such cases have occurred much more quickly than expected following several selections by extensive glyphosate use. (Reddy, 2001)

The number of papers reporting cases of resistance emerging in various species against glyphosate following continuous exposure increases every year. Powles (2010) likens the importance of glyphosate in global food production to the role of penicillin in fighting disease. Several documented cases of glyphosate resistance emphasize the rapidness of the evolution under the selective pressure of glyphosate overuse. For example, pigweed, *Amaranthus palmieri,* considerably affects cotton and soybean fields in southern United States. The resistance is caused by the amplification of EPSPS gene in the *A. palmieri* genome upto 160-fold in some cases. By increasing the copy number of EPSPS massively, this species has managed to surpass the inhibitive effects of glyphosate and the plants can continue normal metabolic function despite exposure to glyphosate at the usual rates. Other forms of resistance to glyphosate can occur as a mutation in the EPSPS gene preventing the glyphosate from bonding to and inhibiting this important enzyme in the shikimate pathway. Evolved resistance is a direct result of the reliance upon glyphosate as the sole weed control method in glyphosate resistant crop production. Any plant that can tolerate the applied dose of glyphosate possesses a vital advantage allowing it to spread wildly. If the evolved resistance is not managed quickly, glyphosate will seize its importance as the most commonly used herbicide and the investment in GR crops will result in huge economic losses. (Gaines et al., 2010; Laura D. Bradshaw, 1997; Powles, 2010)

The weed of interest for this study, *Brachypodium* was previously reported as resistant to triazines. Following several episodes of exposure in Lower Galilee, Israel, resistance towards triazines arose in *Brachypodium* and the rate of takeover was remarkable. Gressel et al. (1983) suggest that the resistance is maintained by a combination of enhanced metabolism and alterations at the target site. Considering that *Brachypodium* is a native plant in Turkey and it has, in the past, developed resistance to another herbicide, the possibility of glyphosate resistance would serve as a local threat, if not add to the global concerns. Local production of GR crops in Turkey would not be feasible if such a resistance would occur. So far, there are no reports of glyphosate-resistance in *Brachypodium* but this does not conclude that such an event may not occur in the near future if glyphosate remains the choice of weed management method. (Jonathan Gressel, 1983; Warwick, 1991)

1.6. Quantification of damage caused by glyphosate

1.6.1. Biochemical markers

When plants are exposed to environmental stresses, they activate various metabolic pathways in order to cope with the external conditions. Accumulation of sugars and compatible solutes is a well-known strategy for plants that are exposed to any kind of abiotic stress. Compatible solutes must be non-toxic molecules, which do not interfere with the steady-state metabolism. Some examples maybe listed as amino acids, glycine betaine, sugars or sugar alcohols. These solutes are usually localized to the cytoplasm at high concentrations and play a primary role of turgor maintenance. Other roles have been proposed for these solutes such as stabilization of cell structures and scavenging of reactive oxygen species. (Aprile et al., 2009)

Among all compatible solutes, proline is distributed around the cell most evenly and proline accumulation occurs in many other organisms in addition to plants. Although increased free proline content helps with osmotic pressure, it is possible that it can be instrumental in protecting the plasma membrane. Proline has been also proposed to function as a sink of energy and reducing power as well as a source for carbon and nitrogen. Plants may be accumulating proline as a way to scavenge the hydroxyl radical, which is produced excessively under stress conditions. (Ahmad and Hellebust, 1988; Mansour, 1998; Smirnoff and Cumbes, 1989; Verbruggen et al., 1996)

Two separate biosynthetic pathways are responsible for the accumulation of proline in plants: the ornithine-dependent pathway and the glutamate-dependent pathway. Under stress conditions, the glutamate-dependent pathway dominates proline production by the synthesis of L-proline from L-glutamic acid via Δ^1-pyrroline-5-carboxylate (P5C) catalyzed by P5C synthetase and P5C reductase. The second pathway has been indicated to function in specific growth stages in *Arabidopsis* and involves the transamination of ornithine. Apart from these two biosynthesis pathways, proline levels are controlled by the oxidation of L-proline by proline dehydrogenase to P5C, which is then converted to L-glutamic acid by P5C dehydrogenase. (Figure 4) (Bartels and Sunkar, 2005)

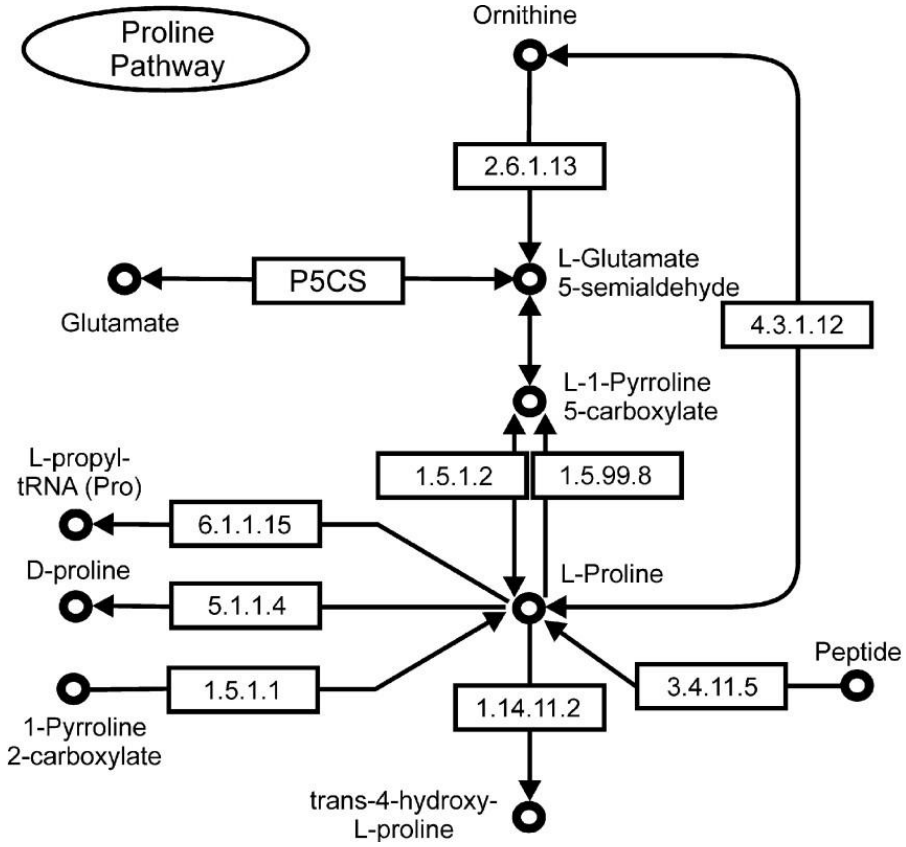


Figure 4: Proline biosynthesis explained in detail. (Aprile et al., 2009)

Roles of proline accumulation have been mostly verified in osmotic pressure studies by the overexpression of various enzymes in the proline biosynthesis pathways. These studies have resulted in increased proline concentrations and improved osmotic stress tolerance. These findings in osmotic stress studies have led scientists to draw conclusions about other biotic stress tolerance mechanisms. For this reason, free proline content is a well-established biochemical marker used for plant stress evaluations. In this study, we propose that *Brachypodium* accumulates proline in order to cope with the abiotic stress caused by the glyphosate application. (Laurent Jouve, 1993)

Since glyphosate interrupts the shikimate pathway, a known consequence is the accumulation of shikimate in affected cells. Glyphosate interrupts the feedback mechanism as well as the downstream reactions, resulting in excess shikimate in the cytoplasm. When shikimate reaches high concentrations due to the sudden halt in the pathway, carbon sources are locked and the cell functions suffer from this situation. For this reason alone, shikimate

concentration is a good marker for measuring glyphosate related damages in plants. (Buehring et al., 2007)

In addition to specific alterations depending on the type of stress in question, lipid peroxidation can be a non-specific marker for the oxidative stress caused by extreme conditions of any kind. Malondialdehyde (MDA) occurs as a secondary product of the oxidation of polyunsaturated fatty acids and indicates the level of membrane damage caused by free radicals and can be used as an indicator of stress in most stress conditions.(Hodges et al., 1999)

1.6.2. Hydrogen Peroxide Scavenging Enzymes

A secondary effect of most abiotic stress conditions exhibits as the increase of reactive oxygen species (ROS), such as singlet oxygen, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide. These molecules are mostly produced in the chloroplast during the light reactions of photosynthesis. Mitochondria also contribute to the production of ROS but to a lesser extent compared to the chloroplasts. Once produced, ROS interrupt routine mechanisms locally at the chloroplasts, such as repair of photosystem II. In addition, stress-enhanced photorespiration and activities of NADPH increase H_2O_2 accumulation. Hydrogen peroxide can be quite detrimental to the cells by inactivating enzymes through the oxidation of thiol groups. Hydrogen peroxide can be converted into the highly reactive hydroxyl ion in the presence of transition metals, such as cuprous and ferrous ions, which may be freed under stress conditions. Hydroxyl ion extensively oxidizes proteins, lipids and nucleic acids once it is formed in the cells. (Halliwell, 1999)

Despite the previous negative reputation of toxicity, reactive oxygen intermediates (ROIs) are acknowledged for their role as signaling molecules under stress conditions. As the excessive secretion may be harmful to cellular balance, the concentration of ROIs must be tightly controlled at all times. Homeostasis is maintained by a delicate balance between ROI concentration and activities of scavenging enzymes, such as ascorbate peroxidase (APX), catalase (CAT), glutathione reductase and superoxide dismutase (SOD). Apart from these enzymes, cells possess an intricate array of metabolites that act as antioxidants, such as ascorbic acid, glutathione and caretenoids. (Mittler, 2002)

As signaling molecules, ROIs play different roles during biotic and abiotic stress responses. When a cell is under pathogen attack, ROI production is enhanced through the membrane bound NADPH oxidases. Specifically, hydrogen peroxide (H_2O_2) diffuses into the cells together with salicylic acid and nitric oxide (NO) in order to activate many plant defense mechanisms, including programmed cell death (PCD). For the activation of PCD, APX and CAT are downregulated by plant hormones so that the scavenging mechanisms can be instantly inhibited. Thus, ROIs are produced at a higher rate and are able to accumulate at high levels leading to the cell's inevitable death. (Delledonne et al., 2001; Mittler et al., 1999)

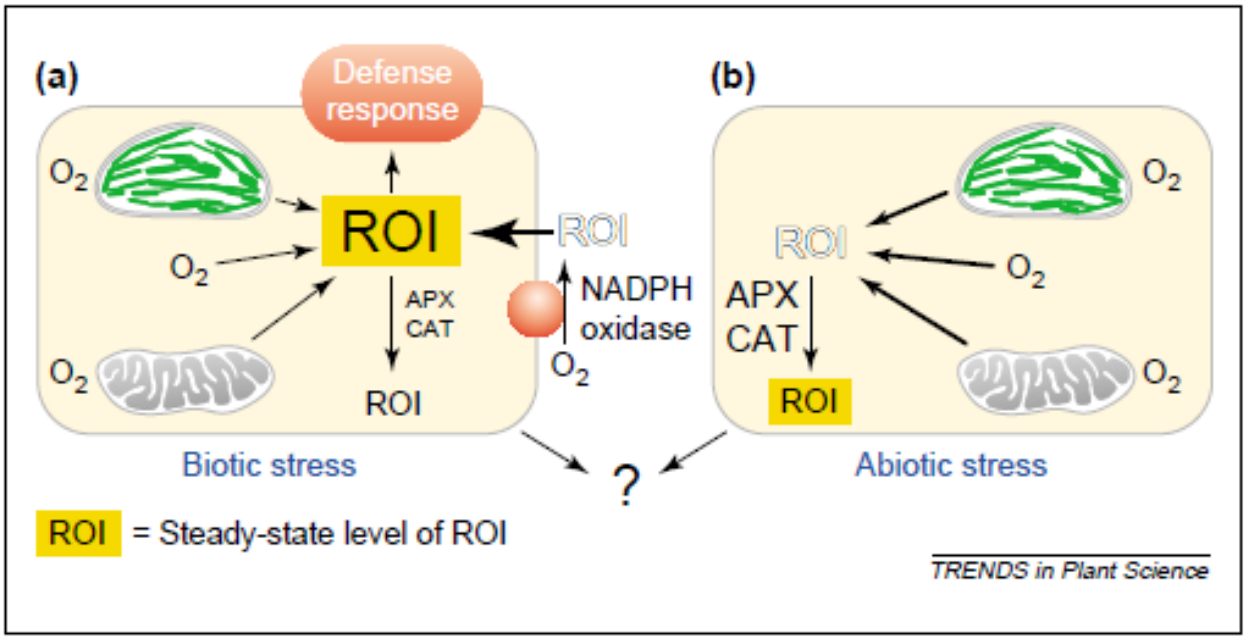


Figure 5: Plants regulate reactive oxygen intermediates differently under biotic and abiotic stress conditions.(Mittler, 2002)

Under abiotic stress conditions, plant cells employ a completely different strategy regarding the ROIs. Instead of letting them accumulate as in the PCD activation, cells induce ROI-scavenging enzymes, such as APX and CAT to keep the ROI concentration under control. Figure 5 illustrates this difference clearly by marking the sites of ROI production under each condition and the situation of the scavenging enzymes. However, there are a lot of details that are yet to be discovered about these strategies suggested by the question mark in the figure. (Mittler et al., 2004)

An interesting question arises when one considers the defense strategies that might occur under a combination of biotic and abiotic stresses. Several studies have demonstrated that higher levels of antioxidant enzymes such as CAT slow down the rate of PCD in the case of pathogen attacks and also, imply a lowered resistance towards pathogen infection.(Mittler et al., 1999; Polidoros et al., 2001)

2. Materials and Methods

2.1. Plant Growth

For this study, 45 pots have been prepared with equal volumes of soil and torf. Basal nutrients were added described in table 2 below.

Table 2: Summary of basal nutrients added to each pot.

	Molecular molar mass	Element molar mass	Nutrient	Weight of each pot	Number of Pots	mass	Solution Volume	each pot
$Ca(NO_3)_2$	236,15	28,0134	**N**	1,6	50	**134,9** g	500 ml	**10 Ml**
Fe-EDTA	100	13	**Fe**	1,6	50	**3,08** g	500 ml	**10 Ml**
KH2PO4	136,09	30,973761	**P**	1,6	50	**35,15** g	500 ml	**10 Ml**
K2SO4	174,27	32,065	**S**	1,6	50	**8,7** g	500 ml	**10 Ml**
$ZnSO_4$	287,54	65,409	**Zn**	1,6	50	**0,7034** g	500 ml	**10 Ml**

Pots =1.6 kg (soil + torf)

Into each pot, 4-7 seeds were planted into each pot depending on the number of the available seeds. Fifteen genotypes, BdTr1-13, Bd21 and Bd21-3, were selected for this experiment. The experimental setup is described below in table 3 and the study was conducted as Experiment 174 in the greenhouse records. Plants were watered daily. After germination, they were allowed to grow for 8 weeks for the purpose of retaining enough biomass for following assays.

Table 3: Experimental design

Genotype ID	5% glyphosate	20% glyphosate	Control
Bd Tr-1	21467	21468	21469
Bd Tr-2	21470	21471	21472
Bd Tr-3	21473	21474	21475
Bd Tr-4	21476	21477	21478
Bd Tr-5	21479	21480	21481
Bd Tr-6	21482	21483	21484
Bd Tr-7	21485	21486	21487
Bd Tr-8	21488	21489	21490
Bd Tr-9	21491	21492	21493
Bd Tr-10	21494	21495	21496
Bd Tr-11	21497	21498	21499
Bd Tr-12	21500	21501	21502
Bd Tr-13	21503	21504	21505
Bd 21	21506	21507	21508
Bd 21-3	21509	21510	21511

2.2. Glyphosate Application

Plants were allowed to flourish well into vegetative state before any glyphosate was applied. A single dose of glyphosate was applied and following the application, plants were exposed to direct sunlight to ensure absorption. Two different concentrations of glyphosate, 5% and 20% of the recommended field use, were applied foliarly to *Brachypodium*. (RoundUp, Monsanto)

2.3. Harvesting

In the couple of days following the application of glyphosate directly on the leaves of *Brachypodium*, it was visually evident that the plants were under stress. Most of the glyphosate-applied plants changed color quite rapidly and the samples were collected within 2 days.

2.4. Biochemical Markers

All the protocols used in this study are standard physiology protocols used by Cakmak Laboratory at Sabanci University. (Cakmak and Marschner, 1992)

2.4.1. Free Proline Content

Plants were harvested, weighed 70 mg, and ground into powder using tissue lyser. The tissue powder was incubated with 1ml 75% ethanol overnight with shaking and then centrifuged at 10,000*g*. An aliquot of each extract (100 µL) was incubated with 900 µL ninhydrin reagent (1% w/v ninhydrin, 60% v/v glacial acetic acid, 40% v/v H_2O) at 100 °C for 1 h. Toluene (3 mL) was added, followed by vortexing and incubation at 23 °C for 24 h. The absorbance was measured at 520 nm. The standard curve was prepared with L-Proline.

2.4.2. Lipid Peroxidation MDA levels

It is suggested that lipid peroxidation is a direct result of oxidative stress in cells, which can be caused by a number of different conditions. The MDA level, an index of lipid peroxidation, was measured spectrophotometrically as thiobarbituric acid reactive substances (TBARS) following a modified version of the protocol outlined by Hodges et al. 1999.

The original protocol required 0.5g fresh leaf material to be homogenized in 10 ml of 80% (v/v)ethanol using mortar and pestle. However, the brachypodium samples collected weighed much less than 0.5g and the protocol was scaled down 10:1 in order to complete the experiments with the available material. Following homogenization the samples are centrifuged at 3000g at 4C for 15 minutes, then the supernatant is divided into two new tubes as 250 ul aliquots. Into the first tube, 750 ul of a solution containing, 20% (w/v) trichloroacetic acid, TCA, and 0.01% (w/v) butylated hydroxytoluene, BHT is added. The second tube differs from the first tube as the solution to be added contains an extra component, 2-thiobarbituric acid, TBA. Both tubes are vortexed vigorously and incubated at 95C for 25 minutes. At the end of the incubation period, the samples are cooled down to room temperature and they are centrifuged at 3000g at 4C for 15 minutes. Using microcuvettes, the absorbance is measured at 532, 600 and 440 nm. The results are calculated using the formula below:

[(ABS 532+TBA) – (ABS 600+TBA)]-[(ABS 532-TBA)-(ABS 600-TBA)] = A

[(ABS 440+TBA)-(ABS 600+TBA)] x 0.0571 = B

MDA equivalents (nmol/ml) = (A-B/157 000)x 10^6

Once the measurement is completed, the raw data is analyzed to observe the changes in the MDA levels.

2.4.3. Shikimate Accumulation

Two-centimeter segments of *Brachypodium* leaves from all samples were submersed in 1 ml of 0.25 M hydrochloric acid and after one hour and a half of incubation at room temperature, the leaves lost most green color. From these extracts, 100 µl aliquots were mixed with 400 µl of 0.25 % periodate / 0.25 % meta-periodate and these mixtures were incubated at room temperature for one hour. The reaction was stopped by adding 400 µl of 0.6 M sodium hydroxide / 0.22 M sodium sulfite. Absorbance was measured at 380 nm using a spectrophotometer.

2.5. Antioxidant Enzyme Assays

2.5.1. Extraction

Shoot samples were stored at -80^{o}C till they were used for antioxidant enzyme analyses. From each sample, 0.08 grams were weighed and ground using TissueLyser and titanium beads. During this process, the samples were kept in liquid nitrogen to avoid defrosting and degradation. Onto the ground tissue, 2 ml of 50 mM K-P buffer, pH 7.6, was added and samples were centrifuged at 15000 g for 20 minutes. This extract was diluted 1:5 for use in Bradford and antioxidant enzyme assays.

2.5.2. Bradford Assay

Home-made Bradford Reagent was prepared by dissolving 0.1 g Commasie-Brilliant Blue (G250) in 50 ml absolute ethanol and adding 100 ml ortho phosphoric acid. The solution is filtrated once it is mixed with ddH_2O and 100 ml Glycerin is added before it can be refrigerated at least 24 hours before use.

Standards were prepared using Albumin Fraction V diluted in 50 mM K-P Buffer to make 5 known concentrations, 0 mg, 100 mg, 200 mg, 400 mg, and 800 mg. Standard curve was

prepared by using 100 µl of the standard and 5 ml of Bradford Reagent measured at 595 nm spectrophotometrically. Using this standard curve, the amount of protein in each sample was estimated for later reference. It was important to measure the samples in one hour after Bradford assay was added, for coagulates form in the mixture due to the strong affinity of Commasie blue for proteins.

2.5.3. Ascorbate Peroxidase Activity Assay

Samples prepared according to the extraction method described above are used for this assay. Each sample (0.1 ml) was added to 0.7 ml of 50 mM K-P buffer, 0.1 ml 120 mM H_2O_2 in a plastic cuvette and the spectrophotometer was blanked before the addition of L- Ascorbic Acid at 290 nm. For consistency, each sample was observed in a kinetics assay rapidly after the addition of Ascorbic acid into the cuvette. The enzyme activity was observed for 12 seconds in this assay as activity seemed to disappear once this time frame was completed.

2.5.4. Catalase Activity Assay

The catalase activity assay is dependent on the known amount of H_2O_2 being added to the extracts in each case. For this assay, 0.1 ml of sample was added to 0.8 ml of K-P buffer in a plastic cuvette and spectrophotometer is blanked at 240 nm. The kinetics assay was started as soon as 0.1 ml of 100 mM H_2O_2 was added to the cuvette. The enzyme activity was observed for 12 seconds to follow the reaction to the end.

2.5.5. Glutathione Reductase Activity Assay

The glutathione reductase activity was measured by first blanking the spectrophotometer at 340 nm with plastic cuvettes containing 0.7 ml 50 mM K-P Buffere, 0.1 ml oxidized glutathione and 0.1 ml of sample. The kinetics assay was measured for 12 seconds after 0.1 ml of NADPH (0.8 mg / ml) was added to each sample.

2.5.6. Superoxide Dismutase Assay

For the superoxide dismutase assay, 2.9 ml of 50 mM K-P Buffer, 0.5 ml of Na_2CO_3, 0.5 ml of methionine, 0.5 ml of Nitro Blue Tetrazdium and 0.1 ml of sample were mixed in a glass bottle that can hold upto 5 ml. Into this mixture, 0.5 ml Riboflavin was added and they were incubated in the growth cabin for 10-15 minutes on top of a mirror for direct light exposure

from each angle. They were removed from direct light as soon as some blue tint appeared. The samples were read at 560 nm spectrophotometrically.

3. Results and Discussion

3.1. Biochemical Markers

3.1.1. Proline Accumulation

In order to quantify the damage caused by glyphosate on the overall cell metabolism, free proline concentrations were measured. The 20% glyphosate applied samples showed a much higher proline concentration than the 5% group, both were significantly higher than the control group. Some of the genotypes were affected more severely, whereas others appeared almost unharmed.

Figure 4 is a summary of the free proline contents for the genotypes that showed an increase in the level of free proline as the amount of glyphosate was increased. The other genotypes showed insignificant or no increase in the proline concentrations.

Each genotype exhibits different metabolic properties as it is evident from the starting values of the free proline concentrations for the control groups. Even though Bd Tr 9 appears to have the highest free proline after 20% glyphosate application, the starting value is almost twice as much as the other lines shown in the figure. We might compare Bd Tr 9 and Bd Tr 8 values, for a more accurate understanding and the data suggests that Bd Tr 8 does not accumulate as much free proline as Bd Tr 9. What is more interesting about Bd Tr 8 is that the proline content does not increase when the amount of glyphosate is quadrupled. This may be caused by the fact that Bd Tr 8 is highly susceptible to glyphosate damage and the proline accumulation method is not sufficient for this line to tolerate the stress conditions.

On the other hand, it is clear in figure 4 that Bd Tr 6 accumulates the highest amount of free proline compared to its control value and Bd 21-3 does not respond with as much proline to the same amount of glyphosate. The differences in the figure are mainly cause by the inert

variability among *Brachypodium* lines that were characterized previously according to several morphological, physiological markers.(Filiz et al., 2009)

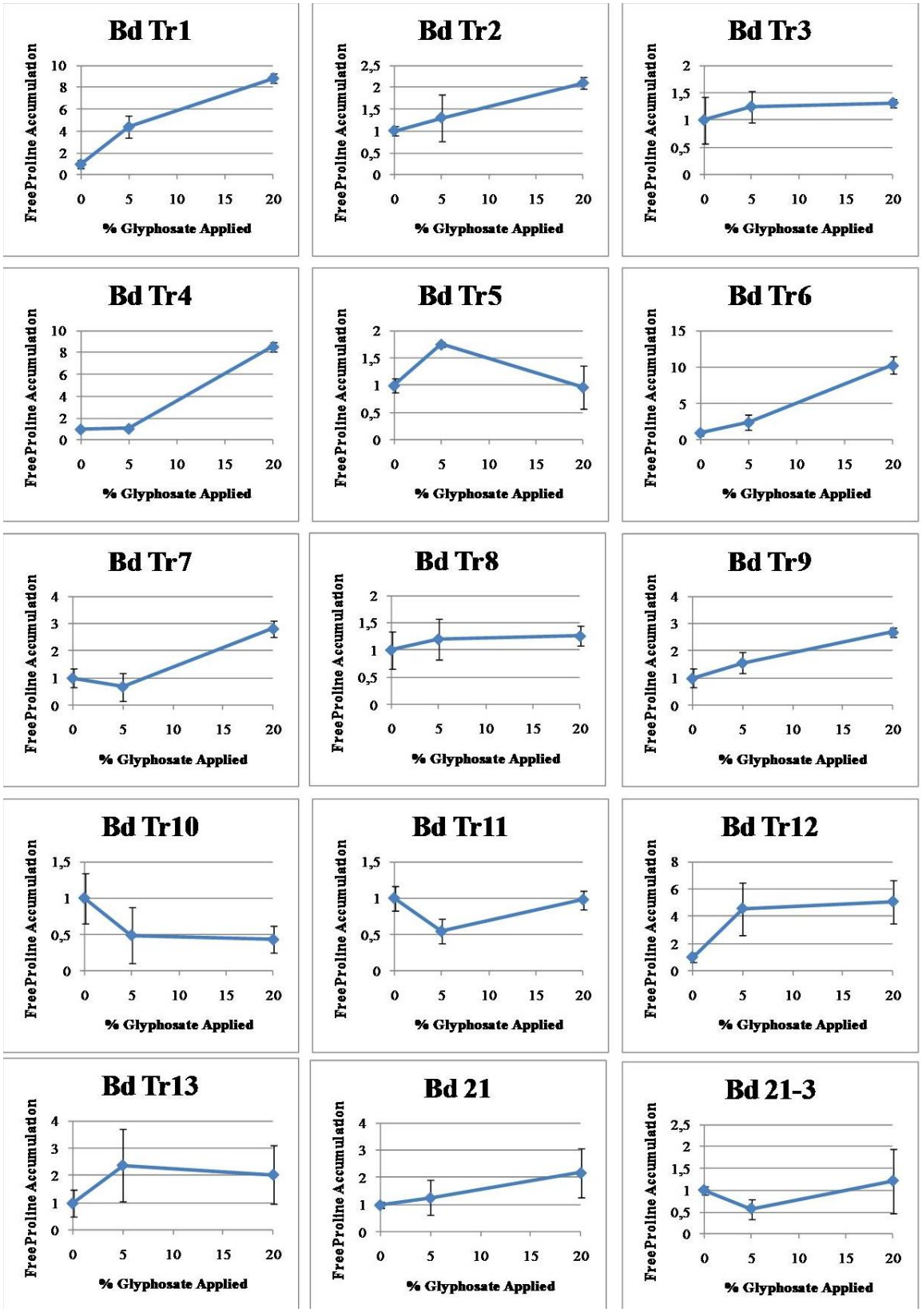


Figure 6: Proline accumulation at different levels of glyphosate application.

3.1.2. Lipid peroxidation

After glyphosate application, there was a rapid decline in plant health that was visible as wilting and color change in the leaves. Especially, the 20% applied group was severely damaged compared to the 5% group. Visual evidence was the first indicator of cell damage and MDA levels were used as an indicator in addition to free proline content.

Apart from one genotype, Bd 21, all *Brachypodium* samples showed a significant increase in the normalized MDA levels. Figure 5, below, is a summary of the lipid peroxidation results. Bd Tr 3 appears most affected by the 20% glyphosate application by a 2.5 fold increase in MDA levels. Two general trends can be observed in these graphs. The increase in the MDA levels for 5% shows the most variability. In some cases, it appears relatively small suggesting that 5% glyphosate application can be tolerated relatively well. In others, even 5% appears to be detrimental to the cell health, potentially indicating higher susceptibility to glyphosate.

The most interesting result comes from the Bd 21 line that shows a decrease in MDA levels, hinting a tolerance mechanism. In figure 4, the proline accumulation shows an increase and combined with figure 5, the cells are able to overcome the negative effects of glyphosate. Bd 21 line should be investigated further to see if the tolerance is the result of experimental error or this line has a specific method of coping with this potent herbicide.

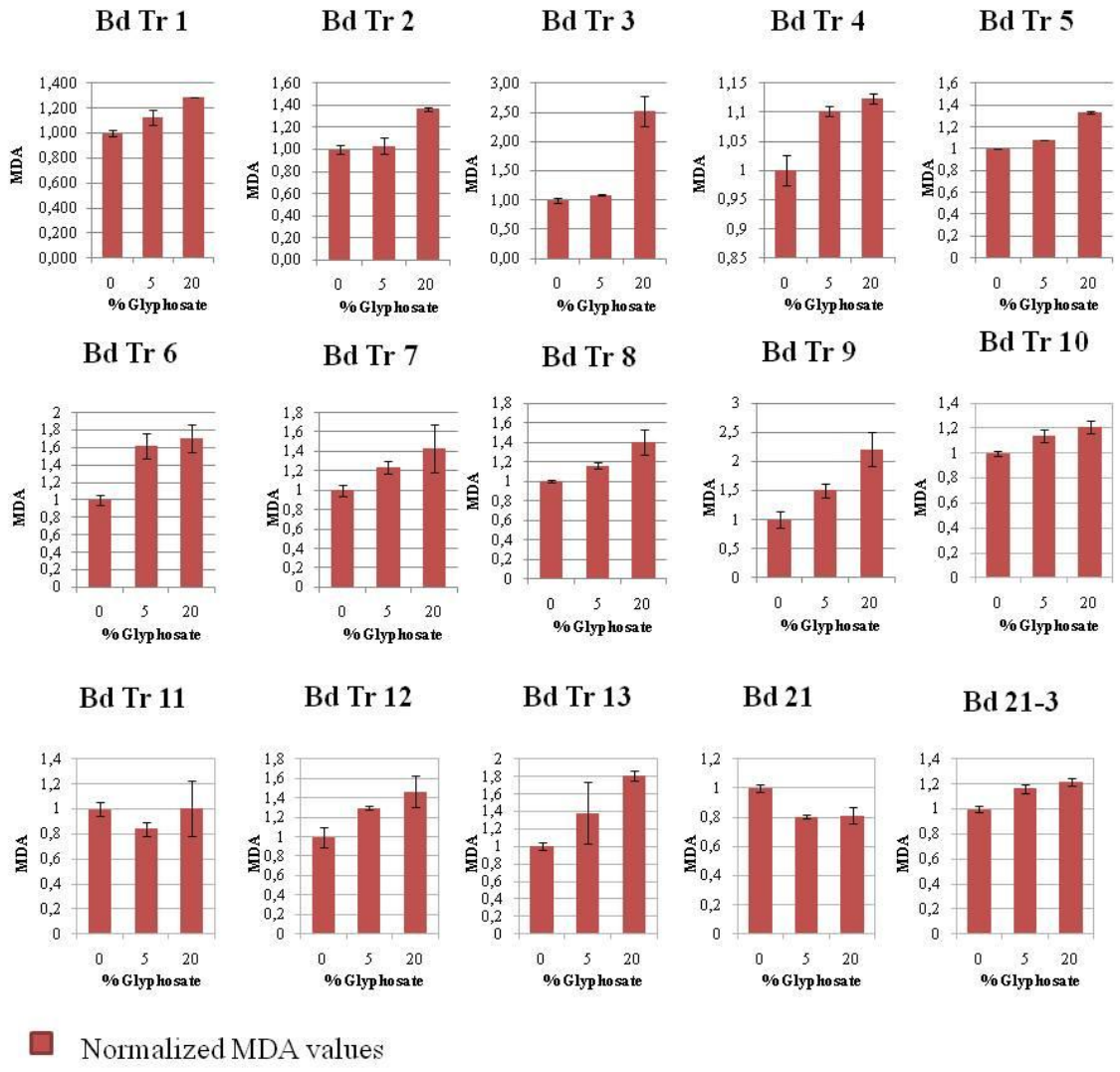


Figure 7: Lipid peroxidation results. Increased levels of glyphosate lead to higher damage in most genotypes apart from Bd 21.

3.1.3. Shikimate Accumulation

Shikimate accumulation is the most direct marker for damage caused by glyphosate application. When a plant is exposed to glyphosate foliarly, even small amounts of the herbicide diffuse through the membrane and start affecting the shikimate pathway in a negative way. It is a well-known fact that glyphosate interacts directly with EPSP synthase as an inhibitor and interrupts aromatic amino acid synthesis. In addition, EPSPS plays a role in the feedback mechanism for the same pathway. When these two effects are combined, shikimate accumulation occurs rapidly.

In this study, *Brachypodium* shoot samples were collected within 2 days post glyphosate application and figure 6 suggests that a period of a few days is long enough to accumulate 4-70 fold shikimate depending on the specific *Brachypodium* line when plants are exposed to 20% of the recommended field rate for glyphosate. Even though the overall increase varies among different lines in the study, the general trend is a considerable increase in 20% group compared to the control group. When shikimate accumulates at the rates described in the figure, it is imaginable how the overall plant suffers. Protein production is halted and carbon sources are depleted as shikimate contains 7 carbon atoms per molecule.

On the other hand, the most interesting results were observed in the 5% group. The responsiveness to 5% showed great variability among groups. For example, Bd Tr 1 seems almost unaffected by 5% glyphosate whereas Bd Tr 5 acummulated almost as much shikimate as a response to 5% and 20% rates. This might suggest that for a line, such as Bd Tr5, 5% glyphosate is as lethal as 20% and for other lines, such as Bd Tr 1, 5% can be somewhat tolerable compared to 20% application. A different study might address the question of varying responses to 5% by trying a gradient using smaller concentrations of glyphosate.

When all three assays are considered, it is quite clear from this study that even 20% of the recommended value was lethal for the *Brachypodium* lines. However, in sublethal doses, such as 5 % of the recommended rate, the responses vary among the lines. The experiments should be repeated with different amounts and should include molecular analyses to be able to classify these differences. It is possible that there might be genetic or morphological differences among these lines that effect the free proline content, MDA levels and shikimate accumulation. Exploring the specific conditions that result in such differences would shed some light on the glyphosate tolerance mechanisms of weeds, such as *Brachypodium.*

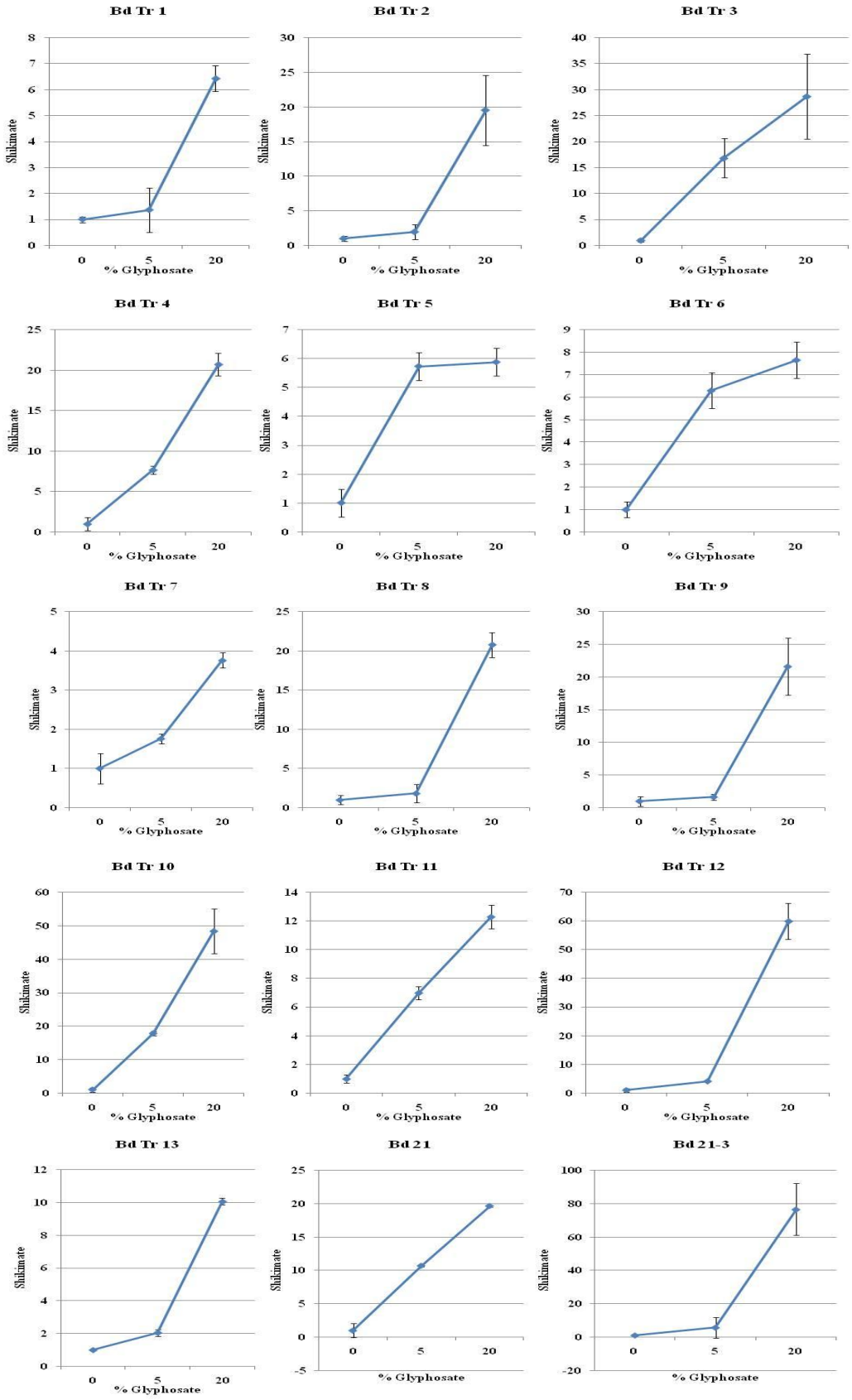


Figure 8: Shikimate values for each *Brachypodium* line. Increases show great variation among different genomes.

3.2. Antioxidant Enzymes

3.2.1. Bradford Assay

In order to calibrate the spectrophotometer, absorbance of 5 known standards of Albumin fraction V were measured at 595 nm. As these standards provided with a linear correlation, the calibration succeeded and the measurements were carried on for all the samples. (Figure 9)

Bradford assay was essential for the estimation of protein concentrations in the extracts that were used in the enzyme assays. Table 4 summarizes the protein content of each sample used in the following experiments. These values were used in all following calculations to normalize the activity values according to the total protein content of the extracts. Samples contained a wide range of protein even though the starting weight for each sample was 0.08 g. Physical disruption of the plant tissue during extraction may have caused these varying concentrations, despite all the other steps having been repeated uniformly.

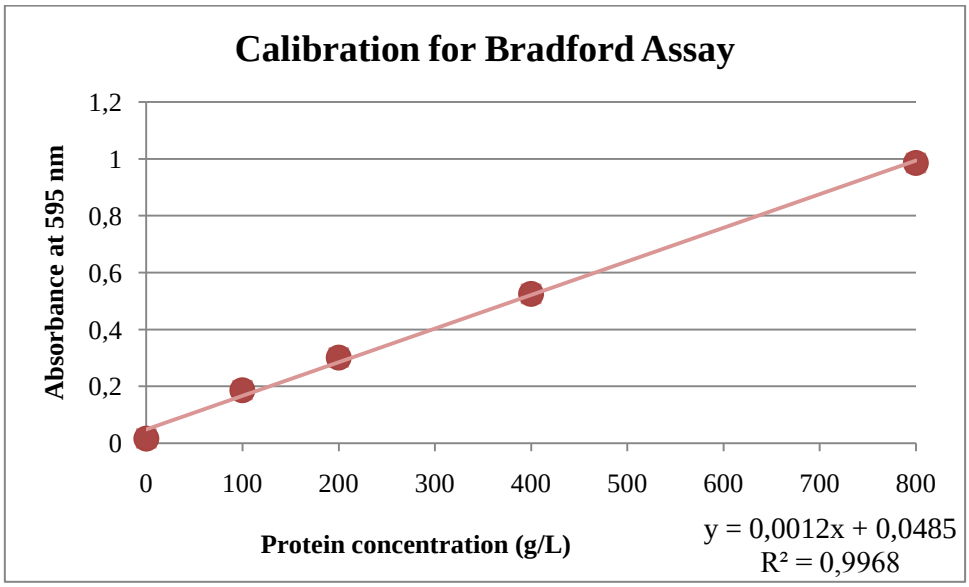


Figure 9: Standard curve prepared by Albumin Fraction V standards (0-800 g/L).

Table 4: Protein concentrations estimated by Bradford Assay

Sample	Concentration (g/L)	Sample	Concentration (g/L)	Sample	Concentration (g/L)
Bd Tr 1 C A	91.4	Bd Tr 6 C A	5.7	Bd Tr 11 C A	1.1
Bd Tr 1 C B	22.1	Bd Tr 6 C B	7.5	Bd Tr 11 C B	63.1
Bd Tr 1 C C	98.1	Bd Tr 6 C C	13.6	Bd Tr 11 C C	21.2
Bd Tr 1 5 A	53.5	Bd Tr 6 5 A	8.2	Bd Tr 11 5 A	21.9
Bd Tr 1 5 B	2.9	Bd Tr 6 5 B	5.8	Bd Tr 11 5 B	27.3
Bd Tr 1 5 C	88.6	Bd Tr 6 5 C	7.1	Bd Tr 11 5 C	123.8
Bd Tr 1 20 A	45.7	Bd Tr 6 20 A	57.2	Bd Tr 11 20 A	97.4
Bd Tr 1 20 B	55.1	Bd Tr 6 20 B	30.6	Bd Tr 11 20 B	53.1
Bd Tr 1 20 C	76.1	Bd Tr 6 20 C	8.7	Bd Tr 11 20 C	53.8
Bd Tr 2 C A	14.9	Bd Tr 7 C A	35.8	Bd Tr 12 C A	15.1
Bd Tr 2 C B	119.5	Bd Tr 7 C B	4	Bd Tr 12 C B	47.4
Bd Tr 2 C C	102.7	Bd Tr 7 C C	10.7	Bd Tr 12 C C	6
Bd Tr 2 5 A	60.5	Bd Tr 7 5 A	4.5	Bd Tr 12 5 A	6.2
Bd Tr 2 5 B	28.4	Bd Tr 7 5 B	10.9	Bd Tr 12 5 B	24.8
Bd Tr 2 5 C	82.8	Bd Tr 7 5 C	31.2	Bd Tr 12 5 C	25
Bd Tr 2 20 A	70.8	Bd Tr 7 20 A	0.4	Bd Tr 12 20 A	27.3
Bd Tr 2 20 B	73.5	Bd Tr 7 20 B	26.6	Bd Tr 12 20 B	67.1
Bd Tr 2 20 C	95.2	Bd Tr 7 20 C	42.8	Bd Tr 12 20 C	73
Bd Tr 3 C A	16.2	Bd Tr 8 C A	0.7	Bd Tr 13 C A	24.4
Bd Tr 3 C B	14.8	Bd Tr 8 C B	14.4	Bd Tr 13 C B	13.6
Bd Tr 3 C C	4.4	Bd Tr 8 C C	12.5	Bd Tr 13 C C	14.9
Bd Tr 3 5 A	25.4	Bd Tr 8 5 A	11.7	Bd Tr 13 5 A	6.6
Bd Tr 3 5 B	15.2	Bd Tr 8 5 B	3.3	Bd Tr 13 5 B	2.7
Bd Tr 3 5 C	28.5	Bd Tr 8 5 C	19.7	Bd Tr 13 5 C	24.4
Bd Tr 3 20 A	116	Bd Tr 8 20 A	132.3	Bd Tr 13 20 A	50.7
Bd Tr 3 20 B	88.8	Bd Tr 8 20 B	87.7	Bd Tr 13 20 B	35.5
Bd Tr 3 20 C	88.4	Bd Tr 8 20 C	55	Bd Tr 13 20 C	87.8
Bd Tr 4 C A	53.8	Bd Tr 9 C A	55.3	Bd 21 C A	34.6
Bd Tr 4 C B	2.3	Bd Tr 9 C B	35.5	Bd 21 C B	27.1
Bd Tr 4 C C	62.7	Bd Tr 9 C C	38.8	Bd 21 C C	26.9
Bd Tr 4 5 A	22.1	Bd Tr 9 5 A	20.8	Bd 21 5 A	33.4
Bd Tr 4 5 B	63.5	Bd Tr 9 5 B	21.5	Bd 21 5 B	20.5
Bd Tr 4 5 C	6.3	Bd Tr 9 5 C	5.4	Bd 21 5 C	25
Bd Tr 4 20 A	64.6	Bd Tr 9 20 A	53.1	Bd 21 20 A	48.5
Bd Tr 4 20 B	56.8	Bd Tr 9 20 B	67.3	Bd 21 20 B	38.4
Bd Tr 4 20 C	62.6	Bd Tr 9 20 C	71.4	Bd 21 20 C	9.8
Bd Tr 5 C A	40.2	Bd Tr 10 C A	4.7	Bd 21-3 C A	3.8
Bd Tr 5 C B	6.6	Bd Tr 10 C B	17.8	Bd 21-3 C B	9.4
Bd Tr 5 C C	17.2	Bd Tr 10 C C	14.9	Bd 21-3 C C	11.5
Bd Tr 5 5 A	31.3	Bd Tr 10 5 A	18.8	Bd 21-3 5 B	23.8
Bd Tr 5 5 B	10.3	Bd Tr 10 5 B	16.2	Bd 21-3 5 B	58.1
Bd Tr 5 5 C	97.2	Bd Tr 10 5 C	46.1	Bd 21-3 5 C	30.7
Bd Tr 5 20 A	63.8	Bd Tr 10 20 A	58.6	Bd 21-3 20 A	58.6
Bd Tr 5 20 B	72.4	Bd Tr 10 20 B	102.9	Bd 21-3 20 B	3.2
Bd Tr 5 20 C	73.2	Bd Tr 10 20 C	9.2	Bd 21-3 20 C	20

3.2.2. Ascorbate Peroxidase Activity

The first antioxidant enzyme measured in this activity was ascorbate peroxidase (APX) and the graphs included in Figure 10 show a general increase in the amount of APX in the plant extracts correlated with the amount of glyphosate applied. The increase ranges from 1.2 fold to 18 fold in the 20% group and the increase in less dramatic in the 5% group.

The reaction of APX activity in response to increasing amounts of glyphosate can be categorized into three groups as can be seen in Figure 10. The first group shows an almost linear correlation between the amount of glyphosate applied and the measured APX activity. Bd Tr 8, Bd Tr 9, Bd 21 and Bd 21-3 belong to this group. In the second group($m_1<m_2$), including Bd Tr 2, Bd Tr5, Bd Tr 6, Bd Tr 12 and Bd Tr 13, the APX activity increases with a modest slope(m_1) first until 5% and the increase gets steeper(m_2) reaching 20%, suggesting these lines may tolerate glyphosate relatively well compared to the more detrimental effects of 20% glyphosate. The last group($m_1>m_2$) shows a rapid increase(m_1) in the APX levels until 5% and the slope of the graph is less steep for the second part(m_2). Lines belonging to this group appear to exhibit a more sensitive APX response compared to the second group at lower concentrations of glyphosate (5%). Bd Tr 3, Bd Tr 4, Bd Tr 7, Bd Tr 10 and Bd Tr 11 show the third kind of APX response. Bd Tr 1, however, seems to show almost no change in the APX activity among control, 5% and 20% glyphosate groups. Table 5 represents an overall categorization of APX responses.

In contrast to upto 18-fold increase observed in the APX activity assay, CAT, GR and SOD assays showed relatively minor changes. However, APX seemed to consistently increase in relation to the amount of glyphosate applied but CAT, GR and SOD assays showed varying responses.

Table 5: Summary of the APX responses.

Group 1 (linear)	**Group 2 ($m_1<m_2$)**	**Group 3 ($m_1>m_2$)**	**No Change**
Bd Tr 8	Bd Tr 2	Bd Tr 3	Bd Tr 1
Bd Tr 9	Bd Tr 5	Bd Tr 4	
Bd 21	Bd Tr 6	Bd Tr 7	
Bd 21-3	Bd Tr 12	Bd Tr 10	
	Bd Tr 13	Bd Tr 11	

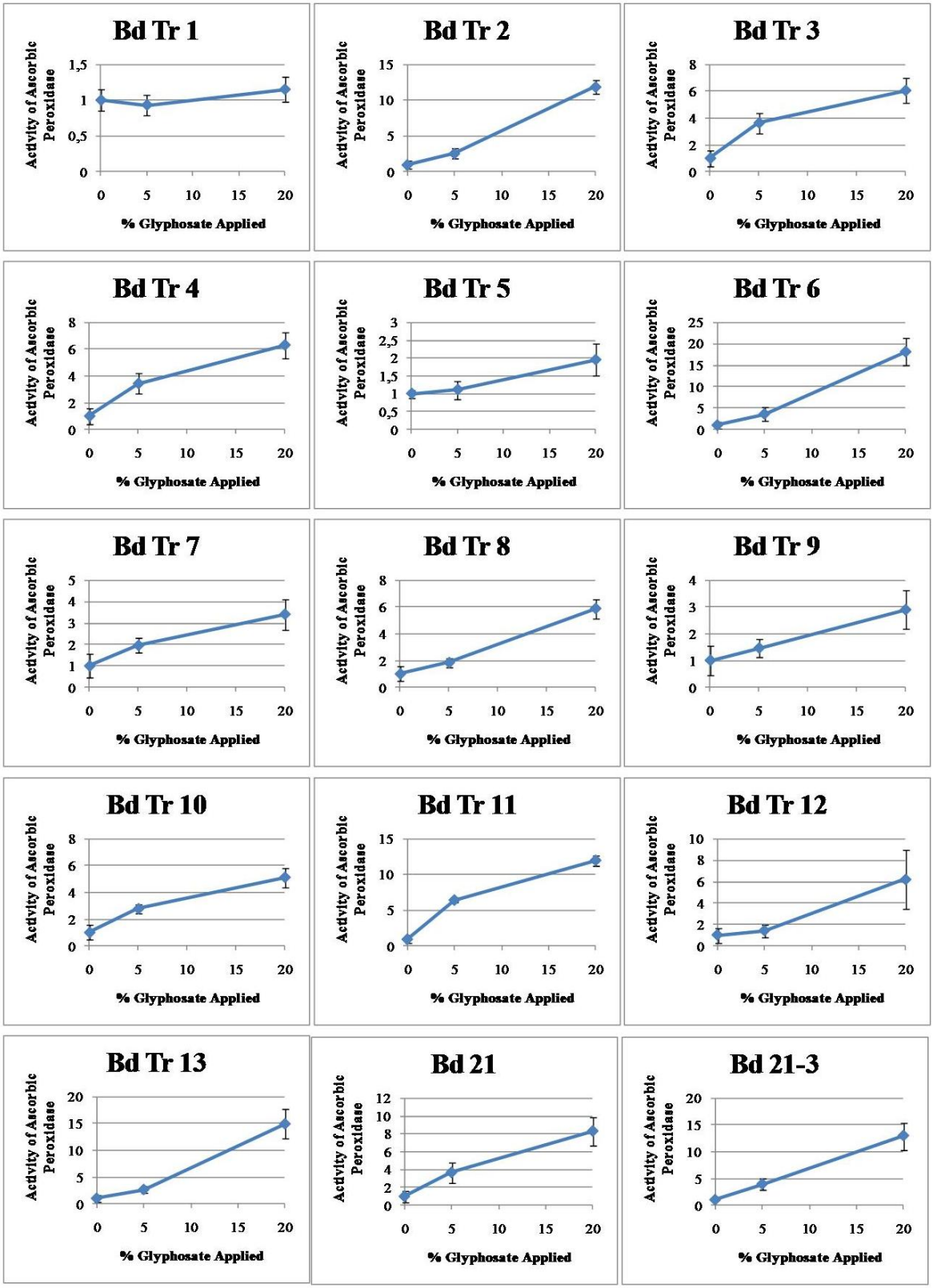


Figure 10: Ascorbic peroxidase activity summary.

3.2.3. Catalase Activity Assay

Catalase(CAT) activity assay results show a variety of patterns compared to the general increase in the APX data. Figure 11 shows the summary of CAT activity assay results for each of the fifteen lines used in the assay. If *Brachypodium* lines are grouped based on the CAT response to the increasing rate of glyphosate application, four general patterns arise.

First group displays a consistent increase in CAT activity and Bd Tr 2, Bd Tr 5, Bd Tr 8, Bd Tr 11, Bd 21 and Bd 21-3 exhibit this response. For the second group, including Bd Tr 7, Bd Tr 10 and Bd Tr 13, a stable decrease is observed. The third group exhibits first a decreased CAT activity for 5% glyphosate application and then back to the same amount of CAT as the control for 20% application. This group includes Bd Tr 1, Bd Tr 3, Bd Tr 9 and Bd Tr 12. Interestingly, the fourth pattern observed is the opposite, first an increase for 5% and a smaller increase or even a decrease in CAT activity for 20% application. Bd Tr 4 and Bd Tr 6 exhibit the fourth pattern.

On its own, the CAT activity assay appears to categorize the lines into four general groups, suggesting that depending on the line and the level of other antioxidant enzymes, CAT response are highly variable in relation to glyphosate dosage.

Table 6: Summary table for CAT response.

Group 1 (increase)	**Group 2 (decrease)**	**Group 3 (decrease, no change)**	**Group 4 (increase, decrease)**
Bd Tr 2	Bd Tr 7	Bd Tr 1	Bd Tr 4
Bd Tr 5	Bd Tr 10	Bd Tr 3	Bd Tr 6
Bd Tr 8	Bd Tr 13	Bd Tr 9	
Bd Tr 11		Bd Tr 12	
Bd 21			
Bd 21-3			

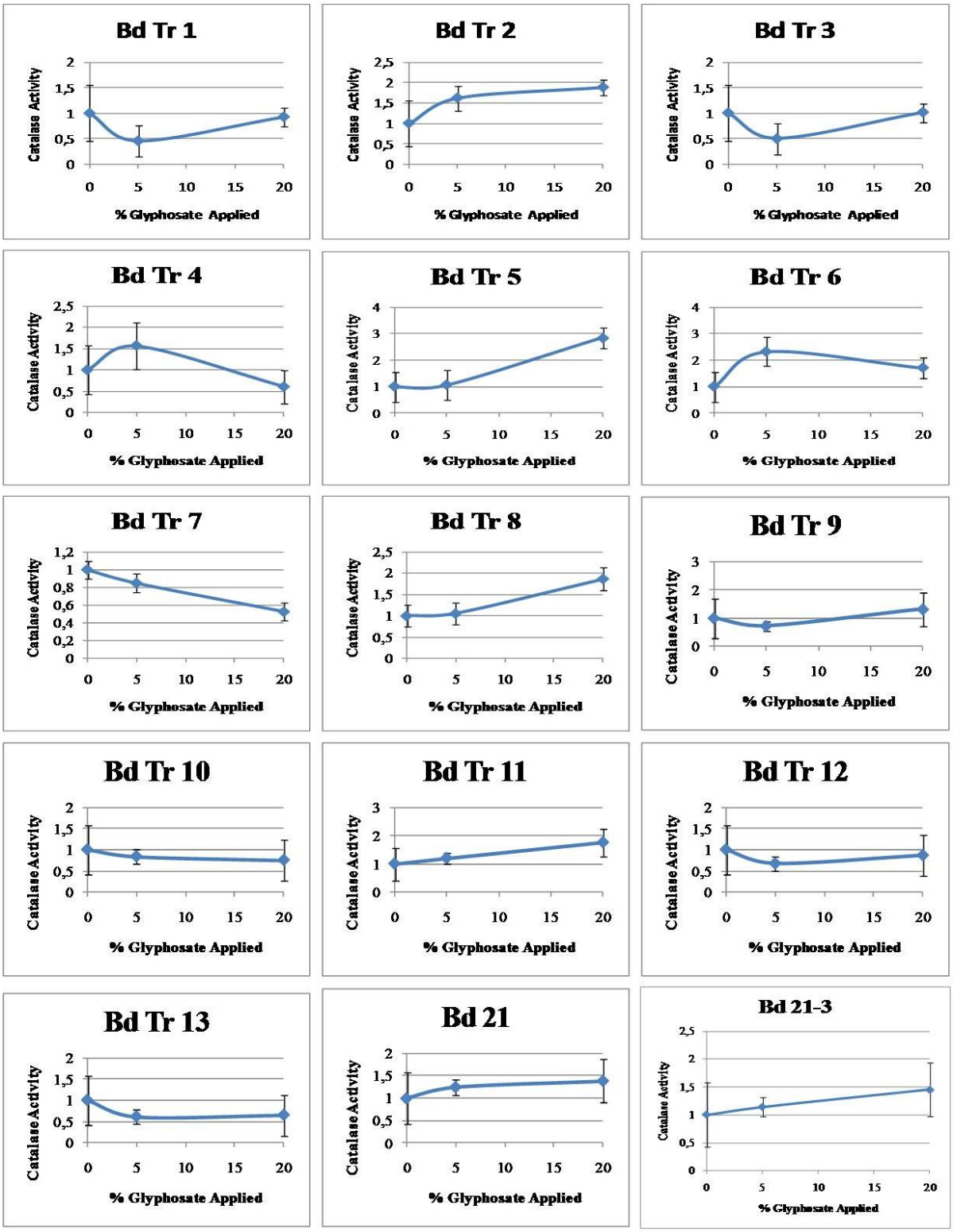


Figure 11: Catalase activity summary.

3.2.4. Glutathione Reductase Activity Assay

As with the CAT activity assay, the varying patterns for glutathione reductase (GR) activity, are summarized in Table **7**, below. Graphs representing the change in GR activity relative to the amount of glyphosate applied can be found in **Figure 12: Glutathione reductase activity summary.**Figure 12.

Similar patterns arose in GR and CAT activity assays. Group one shows increased GR activity with increasing glyphosate doses. Bd Tr 7, Bd Tr 9, Bd Tr 10, Bd Tr 11 and Bd 21-3 can be included in this group due to their consistently increasing trends. In the second group, the behavior of GR is the opposite, consistently decreasing. As the amount of glyphosate is increased first to 5% and then to 20%, a decrease is observed for Bd Tr 2, Bd Tr 5, Bd Tr 8 and Bd Tr 13. The third and the fourth groups show changing behavior based on the concentration of glyphosate. Group three include Bd Tr 3, Bd Tr 4, Bd Tr 6 and Bd Tr 12, all exhibiting a decrease at the 5% rate and no change for the 20% rate. For group four, on the other hand, the amount of GR increases at the 5% rate and decreases at the 20% rate of glyphosate application. Bd Tr 1 and Bd 21 belong to the fourth group.

Table 7: Summary table for GR response.

Group 1 (increase)	**Group 2 (decrease)**	**Group 3 (decrease, no change)**	**Group 4 (increase, decrease)**
Bd Tr 7 Bd Tr 9 Bd Tr 10 Bd Tr 11 Bd 21-3	Bd Tr 2 Bd Tr 5 Bd Tr 8 Bd Tr 13	Bd Tr 3 Bd Tr 4 Bd Tr 6 Bd Tr 12	Bd Tr 1 Bd 21

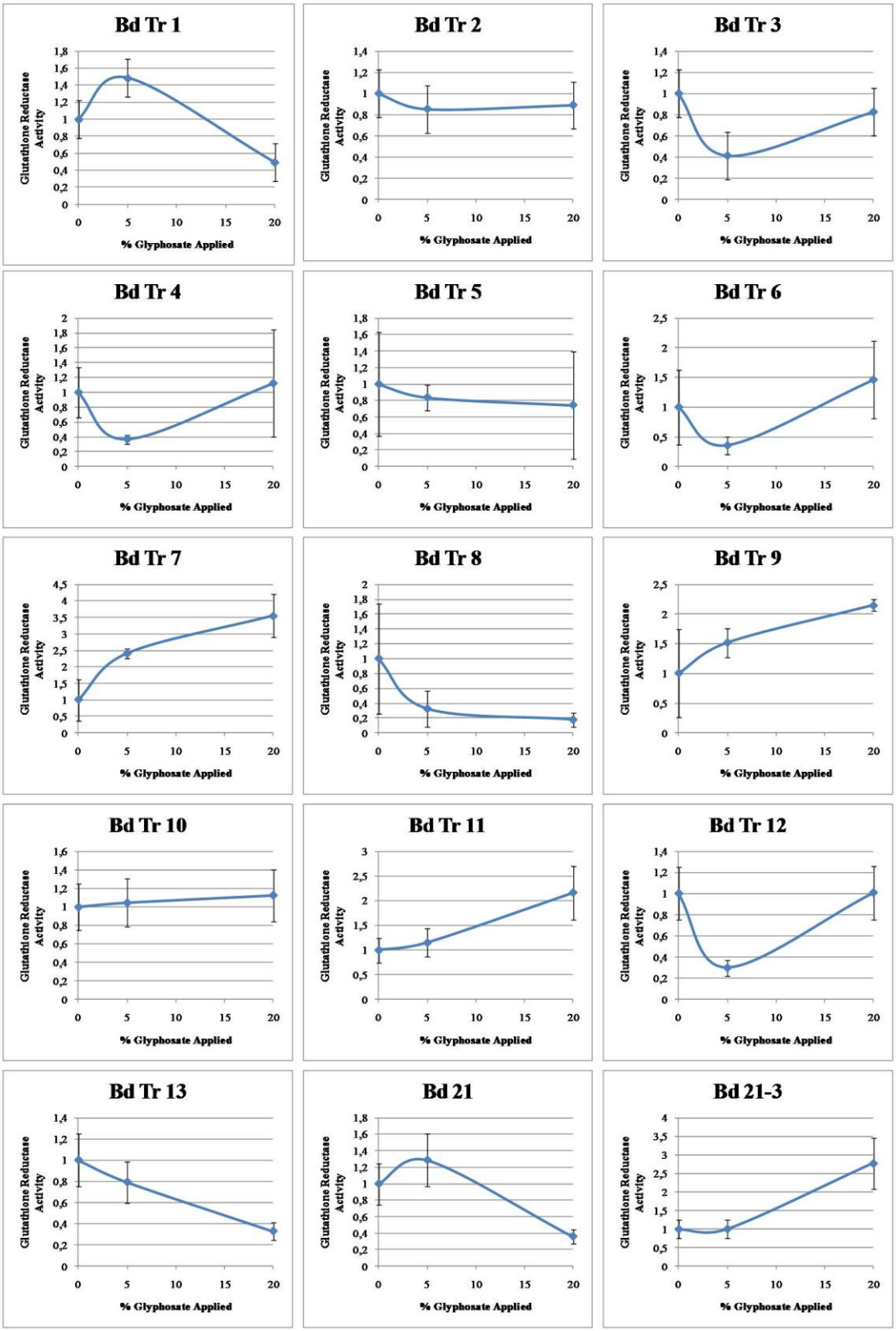


Figure 12: Glutathione reductase activity summary.

3.2.5. Superoxide Dismutase Activity Assay

Among all antioxidant enzyme assays included in the study, superoxide dismutase (SOD) activity assay appears to show the least change between lines and according to changing amounts of glyphosate. Most lines showed a slight decrease in SOD activity as the glyphosate amount was increased to 5% or 20% of the field application rate. However, the most interesting change was observed for Bd Tr 3, which showed an increase in GR activity at the 5% rate and no change for the 20% rate.

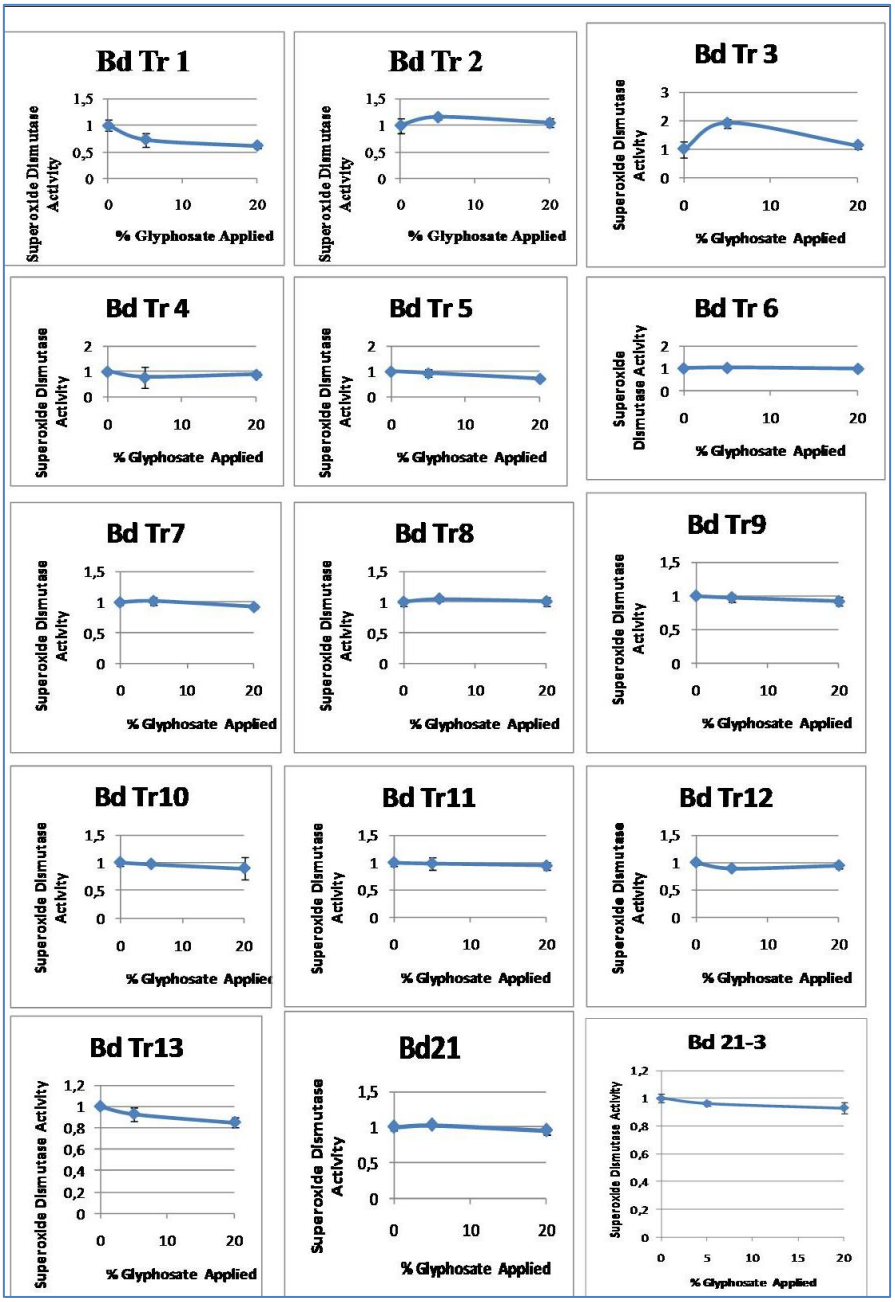


Figure 13: Superoxide dismutase activity summary

3.3. Discussion

For this study, two different rates(5% and 20% of field recommendation) of glyphosate were applied to fifteen different *Brachypodium* lines with three replicates. Samples were collected two days after glyphosate application. In order to compare the different reactions of these fifteen lines, physiological analyses were performed, including free proline content, lipid peroxidation, shikimate accumulation and antioxidant enzyme assays.

The overall comparison must have a multidimensional perspective, since these lines originated from different locations, thirteen from different geographical areas of Turkey and two standard lines originating from Israel. Each line varies genetically and morphologically from the other lines in the study. Given the diversity of the subjects in the study it was expected to receive such a complicated outcome.

The biochemical analyses were selected for their established use as stress markers in a range of abiotic stresses, apart from the shikimate accumulation assay, which is specific to the glyphosate stress. As glyphosate blocks the shikimate pathway, the consistent increase in the shikimate concentration proves that these plants were exposed to glyphosate, specifically. Shikimate accumulation increased with the amount of glyphosate applied for each line, without exception, suggesting that the variability among other results is caused by inert differences among different lines.

As for the other biochemical markers used in this study, they are all regulated under stress conditions via crossing pathways, resulting in interrelated observations. For example, under abiotic stress conditions antioxidant enzyme levels increase to prevent further damage caused by reactive oxygen species(ROS). Following this logic, we may conclude that if antioxidant enzyme levels increase, then the cells have activated oxidative defense mechanisms. If the

mechanism is successful in disarming ROS, then the cell will receive less damage. Lipid peroxidation assay is used as a marker of damage found in the cell membranes. The amount of damage will differ depending on not only the amount of glyphosate applied but also the amount of antioxidant activity within the cell.

Activities antioxidant enzymes are interdependent and compartmentalized within the cell. The concentrations and activities measured show different trends among each other due to the differentially active roles these enzymes play in the cell. Antioxidant enzymes are also supported by antioxidant metabolites, such as NO and salicylic acid that play a role as secondary messengers in many pathways. Given the network of various stress pathways, it is possible to anticipate the diversity of results that are presented in this study. Table 18 is summary of all the results obtained in this study, presented in a summary form.

Table 8: Combined Summary of all analyses.

	Glyphosate	Free Proline Content	Lipid Peroxidation	Shikimate Accumulation	APX Activity	CAT Activity	GR Activity	SOD Activity
Bd Tr 1	5%	4,42	1,13	1,36	0,93	0,46	1,48	0,73
	20%	8,84	1,29	6,43	1,15	0,93	0,49	0,62
Bd Tr 2	5%	1,30	1,03	1,97	2,58	1,62	0,85	1,16
	20%	2,10	1,37	19,52	11,89	1,89	0,89	1,05
Bd Tr 3	5%	1,25	1,09	16,82	3,64	0,50	0,41	1,90
	20%	1,32	2,53	28,71	6,07	1,01	0,83	1,13
Bd Tr 4	5%	1,06	1,10	7,66	3,43	1,57	0,37	0,77
	20%	8,54	1,12	20,68	6,30	0,61	1,12	0,88
Bd Tr 5	5%	1,76	1,08	5,73	1,11	1,06	0,84	0,93
	20%	0,97	1,33	5,88	1,96	2,85	0,74	0,71
Bd Tr 6	5%	2,43	1,62	6,29	3,63	2,33	0,35	1,02
	20%	10,26	1,71	7,65	18,19	1,71	1,46	0,98
Bd Tr 7	5%	0,68	1,23	1,76	1,95	0,85	2,41	1,02
	20%	2,82	1,43	3,76	3,41	0,53	3,55	0,92
Bd Tr 8	5%	1,20	1,16	1,82	1,85	1,05	0,32	1,05
	20%	1,26	1,40	20,74	5,90	1,87	0,18	1,01
Bd Tr 9	5%	1,57	1,50	1,65	1,46	0,72	1,52	0,97
	20%	2,68	2,20	21,59	2,89	1,32	2,15	0,92
Bd Tr 10	5%	0,49	1,14	17,81	2,77	0,84	1,04	0,97
	20%	0,43	1,21	48,35	5,13	0,76	1,12	0,90
Bd Tr 11	5%	0,55	0,84	6,99	6,46	1,20	1,15	0,98
	20%	0,98	1,01	12,29	12,00	1,77	2,16	0,94
Bd Tr 12	5%	4,56	1,30	4,12	1,41	0,67	0,30	0,89
	20%	5,07	1,46	59,93	6,24	0,87	1,01	0,94
Bd Tr 13	5%	2,38	1,38	2,05	2,65	0,61	0,79	0,93
	20%	2,04	1,81	10,06	14,94	0,65	0,33	0,85
Bd 21	5%	1,26	0,80	10,69	3,67	1,25	1,29	1,03
	20%	2,17	0,81	19,61	8,29	1,39	0,36	0,95
Bd 21-3	5%	0,57	1,16	5,65	3,94	1,14	1,01	0,96
	20%	1,22	1,22	76,61	12,94	1,45	2,77	0,93

In order to examine possible correlation, data sets were plotted against each other. **Error! Reference source not found.** shows each graph with potential linear equations. Linear equations are included on each graph that best describe the relation, with their corresponding R^2 values. Most plots appear to be randomly distributed, according to the R^2 values for each of the graphs. However, a few show relatively consistent trends for the given variables. Table 9 summarizes potential correlations obtained from the paired analyses.

Table 9: Summary of the correlations suggested by the scatterplots. (R^2>0.05)

Variables		Correlation
Lipid peroxidation	Proline	+
APX	Proline	+
SOD	Proline	-
Shikimate	Lipid peroxidation	+
APX	Lipid peroxidation	+
APX	Shikimate	+
CAT	APX	+

Even though some of these trends are expected, the correlations are far from statistically significant. An overall conclusion that might sum up the whole study would be difficult to draw with the obtained results. Further repeats and additional analyses are required to enrich the picture and possibly tie all the findings together.

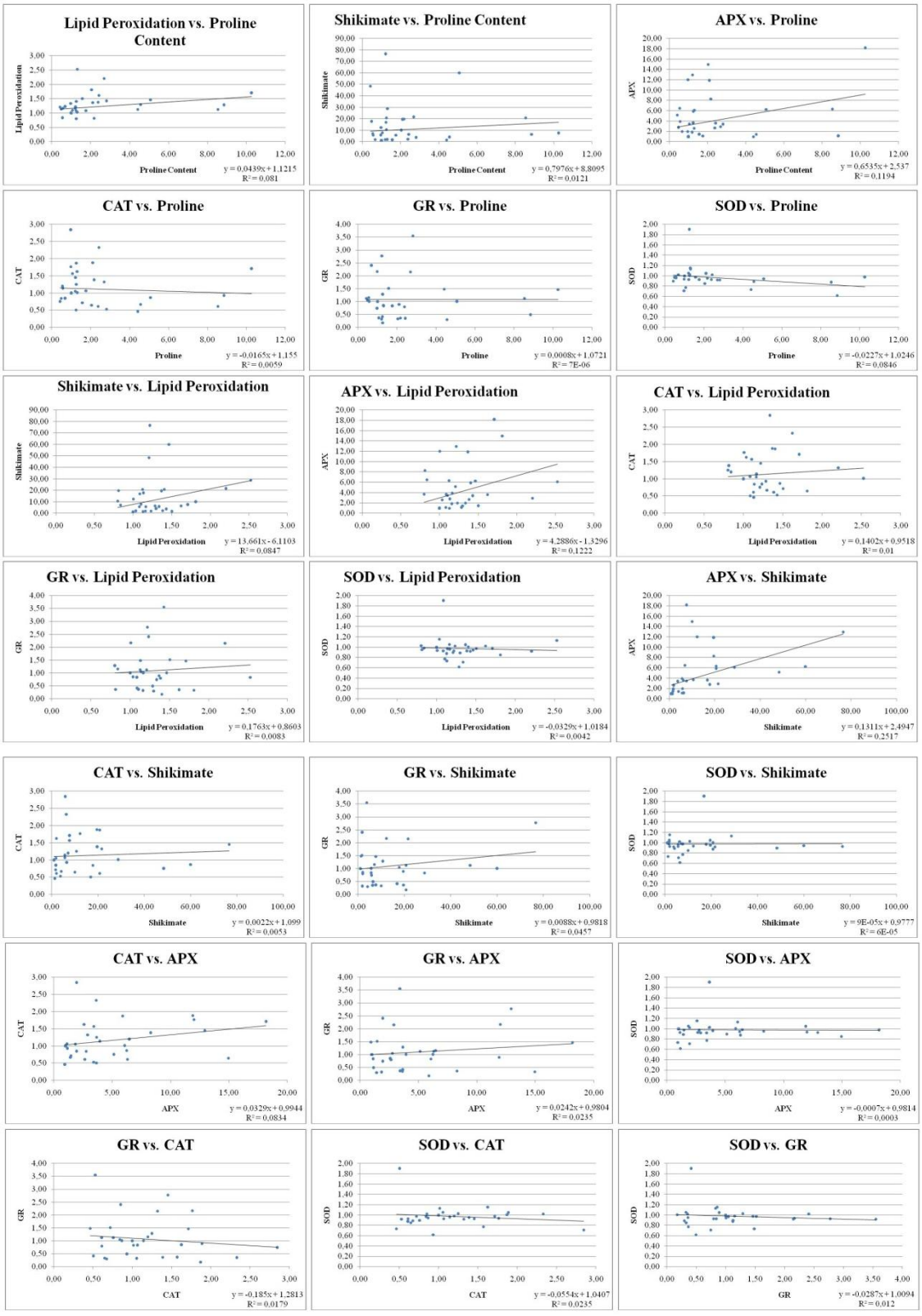


Figure 14: Scatterplot analyses of all data

Figure 15 displays the antioxidant enzyme data superimposed in order to give an overall idea of the interdepence of these four enzymes, APX, CAT, GR and SOD, which function in the same antioxidant defense mechanisms. For most of the lines, the increase in APX is relatively higher in comparison to the changes for the other three enzymes. It becomes difficult to formulate the relationship from these graphs. However, we can easily observe that the changes in the activity of each enzyme vary greatly among the fifteen lines used in this study.

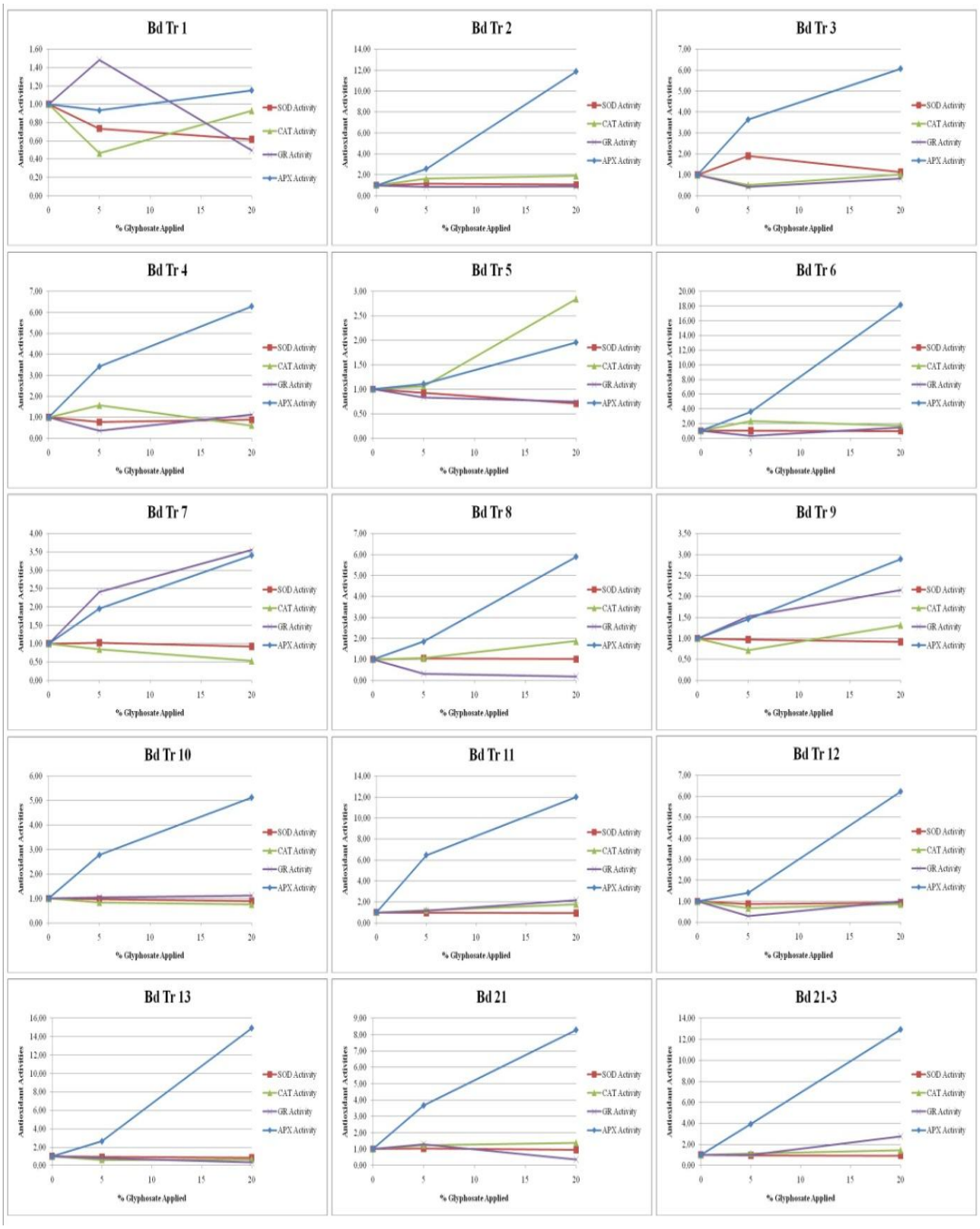


Figure 15: Antioxidant Enzyme Analyses Combined.

4. Conclusion

In conclusion, this study has investigated the effects of glyphosate on *Brachypodium* using four different parameters, lipid peroxidation, free proline content, shikimate accumulation and antioxidant enzyme activity. The results leaves more to be investigated in order to understand the differences in these parameters among different lines. Further studies should include the isolation of EPSPS, APX, CAT, GR and SOD genes from these lines to compare sequences and realtime analyses to see if these genes have been amplified. It is quite possible to observe that different lines have slightly different versions of these enzymes resulting in the variety of responses observed in this study.

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Appendix A: Chemical List

All chemicals and standard solutions were supplied by Merck (Germany), SIGMA (USA), Fluka (Switzerland), Applichem (Germany) and Riedel de Häen (Germany).

Appendix B: Equipment list

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
Balance:	Sartorius, BP 221 S, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Centrifuge:	Beckman Coultier ™ Microfuge® 18 Centrifuge, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Deep-freeze:	-80ºC, Thermo Electron Corporation, USA
	-20ºC, Bosch, TURKEY
Deionized water:	Millipore, MilliQ Academic, FRANCE
Heating block:	Bioblock Scientific, FRANCE
	Bio TDB-100 Dry Block Heating Thermostat, HVD Life
	Sciences, AUSTRIA
Ice machine:	Scotsman Inc., AF20, USA
Magnetic stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY
	VELP Scientifica, Microstirrer, ITALY
Micropipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
pH meter:	WTW, pH540 GLP Multical®, GERMANY
	HANNA, pH213 microprocessor pH meter, GERMANY
Refrigerator:	+4º, Bosch, TURKEY

Shaker:	Excella E24 Shaker Series, New Brunswick Sci., USA
	GFL, Shaker 3011, USA
	Innova™ 4330, New Brunswick Sci., USA
Spectrophotometer:	BIO-RAD, SmartSpec™ 3000, USA
	VARIAN, Cary 300 Bio Uvi-visible spec., AUSTRALIA
Tissue lyser:	Qiagen Retsch, USA
	MJ Research, PTC-100, USA
	TECHNE, TC 512, UK
Water bath:	TECHNE, Refrigerated Bath RB-5A, UK

JULABO, TW 20, USA