DEVELOPMENT OF A PLUM CHROMOSOME DOUBLING METHOD AND PROTEOMICS AND BIOCHEMICAL CHARACTERIZATION

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A thesis submitted in fulfilment of the requirements for the degree of Masters of Science in the Department of Biotechnology, University of the Western Cape.

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KEYWORDS

2D SDS-PAGE

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MALDI-TOF-MS

Marianna

Oryzalin

Plant Breeding

Polyploidy

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Reactive Oxygen Species

ABSTRACT

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Chromosome doubling has become an important tool in breeding programmes as it offers the ability of introducing novel traits into existing plants. Doubled haploid plants are highly valued by both consumers and breeders as these plants usually show larger flower, leaves and fruit, thus making them more marketable. Marianna open pollinated plum rootstocks' adaptability to different soil types and moisture conditions has been favoured in polyploidy studies as parental material in breeding programmes. The potential of the microtubule depolymerizing herbicide (oryzalin) for *in vitro* chromosome doubling were investigated by optimizing the concentration and incubation time of plant shoots to the antimitotic agent. Meristem tissues were treated for two time intervals (24 and 48 h) with five different concentrations of oryzalin (50, 75, 100, 150 or 200 µM) in liquid Murashige and Skoog (MS) medium. After treatment, plants were allowed to grow under a 16/8 h light/dark photoperiod at 24±2˚C for 4 weeks. One and two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS) was used to separate, visualise and identify differently expressed proteins. Furthermore, changes in ROS accumulation, photosynthetic pigmentation, lipid peroxidation and antioxidant enzyme activity (SOD, APX and GR) were investigated. Flow cytometry results revealed that treatment of plants with oryzalin concentrations ranging from 75 to 150 µM induced ploidy after 24 h exposure whereas, 200 µM produced mixoploids containing both tetraploid and octoploids plants after 24 h exposure. Longer incubations of 48

h were detrimental to plant tissues as complete mortality was observed in the higher concentration (100 to 200 µM) treatments. Mass spectrometry analysis identified 14 differentially expressed protein spots that were characterized into different functional categories. ROS accumulation, the extent of lipid peroxidation and antioxidant capacity were differentially regulated in response to oryzalin treatment whereas photosynthetic pigments were significantly enhanced. The results suggests that oryzalin-induced proteins may act as potential biomarkers to improve fruit characteristics in future breeding programs whereas antioxidant enzymes play an important role in scavenging ROS in plants to enhance their adaptability to different environmental conditions.

DECLARATION

I declare that the **DEVELOPMENT OF A PLUM CHROMOSOME DOUBLING METHOD AND PROTEOMICS AND BIOCHEMICAL CHARACTERIZATION** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged as complete references.

Thembeka Mabiya September 2015

Signed…….

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
APS	Ammonium persulphate
APX	Ascorbate peroxidase
ARC	Agricultural research council
CAT	Catalase
CBB	Coomassies brilliant blue
cm	Centimeters
Cd	Cadmium
CHAPS	3- [(3-chlolamidopropyl)dimethyl-ammonio]1- propanelsulphonate
Cu/Zn-SOD	Copper zinc superoxide dismutase
DAPI	4'-6-diamidino-2-phenylindole
DH	Doubled haploids
DMSO	Dimethyl sulphoxide
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol Clelan's reagent
EDTA	Ethylenediamine tetraacetic acid
FACS	Fluorescent activated cell sorter

Fe-SOD	Iron superoxide dismutase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Gluthione disulphide
G	Gram
g/l	Grams per litre
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
Hsp70	Heat shock protein 70
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IEF	Isoelectric focusing
kDa	Kilo daltons
KH_2PO_4	Dipotassium phosphate
KI	Potassium iodide
L	Litre
LC	Liquid chromatography
M	Molarity
MALDI-TOF	Matrix assisted laser desorption/ionisation

	time-of-flight
MDA	Malondialdehyde
mg/ml	Milligrams per millilitre
Mg	Milligrams
Min	Minutes
ml	Mili litre
mM	Milli molar
Mn-SOD	Manganese superoxide dismutase
MOWSE	Molecular weight search
mRNA	Messenger ribonucleic acid
MS/MS	Tandem mass spectrometry
MS medium	Murashige and Skoog basal medium
MS	Mass spectrometry
MudPIT	Multi-dimensional protein identification
MW	Molecular weight
n	Haploid
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium

NCBI	National centre for biotechnology information
nm	Nanometre
O_2	Singlet oxygen
O_2^-	Superoxide
OH	Hydroxide
OH^+	Hydrogen ion
PAGE	Polyacrylamide gel electrophoresis
pH	Potential of hydrogen
p*I*	Isoelectric point
PMF	Peptide mass fingerprint
PMSF	Phenyl methyl sulfonyl fluoride
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RuBP	Ribulose-1, 5-biphospahete
SDS	Sodium dodecyl sulfate
Sec	Seconds
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TEMED	*N, N, N', N'*- terramethylenediamine

Tris	Tris (hydroxymethyl) aminomethane
V	Voltage
Vh	Volt hours
v/v	Volume per volume
w/v	Weight per volume
µM	Micromolar
µg	Micrograms
µl	Micro litre
2n	Diploid
1D-PAGE	One Dimensional polyacrylamide gel electrophoresis
2D-PAGE	Two Dimensional polyacrylamide gel electrophoresis
%	Percentage
β	Beta

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CHAPTER 1

LITERATURE REVIEW

1.1 General introduction

The genus *Prunus* is a large group of deciduous and evergreen trees and shrubs in the subfamily *Prunoideae* belonging to *Rosaceae* family. These fruits are also commonly known as the stone fruits due to the hard endocarp or stone in the centre. The genus is divided into three subgenera Amygdalus (peaches and almonds), Prunophora (plums and apricots) and Cerasus (cherries). Stone fruits are valued for their delicious, edible fruits, spring blossoms and colourful foliage (Arismendi *et al.*, 2015).

Marianna is a diploid open pollinated plum rootstocks in the *Prunus* genus. This plum rootstock has been used as a model plant in most plant breeding research. This is due to the fact that it has been shown to be completely resistant to root-knot nematodes. It has also been shown that all the seedling selections of Marianna, which have been developed in California, retain the resistance of nematodes of the parent plant, suggesting that resistance may be a dominant characteristic (Uria, 1981). This rootstock has also been found to be adapted to different soil types and moisture conditions with moderate resistance to grown gall and crown rot (Southwick *et al.*, 1999).

Stone fruits have been widely used by breeders in polyploidy studies for producing and introducing new and improved cultivars into the market. This can be done by treating plants with antimitotic agents, however the treatment of plants with antimitotic agents can also influence expression of different proteins that may either be up or down regulated in plants. Polyploidy induction can also influence reactive oxygen species (ROS) caused by abiotic stress. In response to stressful conditions these plants will produce various antioxidant compounds to prevent cellular damage in order to protect cells and prevent disruption of metabolic processes (Yousurf *et al.*, 2012).

This review focuses on the use of antimitotic agent oryzalin to induce chromosome doubling, and the benefit of polyploid plants to breeders. It will also explore the protein influence and further the roles of antioxidative defence systems due to ROS accumulation.

1.2 Plant breeding

Plant breeding can be defined as the purposeful manipulation of plant species in order to create improved genotypes/phenotypes (Gosal and Chanal 2002) suited for the needs of farmers and consumers. The overall objective of plant breeding with agricultural crops is to improve yields, increase marketability, higher nutritional qualities and increased diversity among plants for genetic studies. This can be done either by traditional breeding or by means of genetic engineering. Integrating both traditional breeding and genetic engineering is vital for genetically improve plants and fruits. Both these techniques can be used to improve the success in biotechnology and breeding programmes. Each approach has different advantages and disadvantages. For instance traditional breeding creates new varieties while genetic engineering is used to improve existing varieties by changing specific attributes such as disease resistance (Janick *et al.,* 1996).

1.2.1 Traditional plant breeding

This method of breeding involves crossing of two closely related plants to combine the favourable characteristics from both parents and excluding the unwanted traits in the new seedling (Janick *et al*., 1996). The progeny will have a combination of both parental genes. The seedlings may closely resemble their parents, but never identical to either parent. Traditional breeding can be time consuming at times as breeders have to back-cross by looking at the progeny and select the ones with the most positive traits and exclude the least negative traits and then cross the selected progeny back to one of the original parents to transfer more positive traits (Conner *et al*., 1999). This process can take place over a number

of generations of which a number of years, until the progeny have all the desired traits and none of the negative traits.

1.2.2 Genetic engineering

Genetic modification is a method of changing the genes of plants to developed new and improved varieties. It is done for the same reasons as traditional breeding however instead of crossing two parents, specific genes associated with a desirable trait are selected and inserted into a new plant variety (Conner *et al*., 1999). Biotechnology tools such as genetic transformation can be used to insert genes of interest and exclude less desirable genes. An example of genetic engineering has been observed in the transformation of chokecherry (*Prunus virginiana L*) for improving the species by using *Agrobacterium tumfaciens* strain HA105 harbouring binary vector pB1121 carrying the neomycin phosphate gene (*nptII)* and β-glucoronidase (GUS) gene (*uid*A) (Dai *et al.*, 2007).

1.3 Biotechnology tools employed during breeding

Many interspecific and intergenetic crosses have not been successful due to incompatibility between genomes of different species, which can be caused by the difference in chromosome numbers. These differences would hinder the transfer of genes between species and prevent the formation of a viable zygote. The application of chromosome doubling have become an important technique in plant breeding programmes, to overcome interspecific crossing barriers (Lu and Bridget, 1997), induce ploidy and restoring fertility in sterile genotypes. Breeding of interspecific crosses is normally delayed due to the long dormancy periods of seeds, which may be caused by endogenous inhibitors, light requirements, low temperature and embryo maturity (Yeung *et al.*, 1981). Eliminating these factors will allow plants to germinate and grow quickly thereby shortening the breeding cycle (Fig. 1.1).

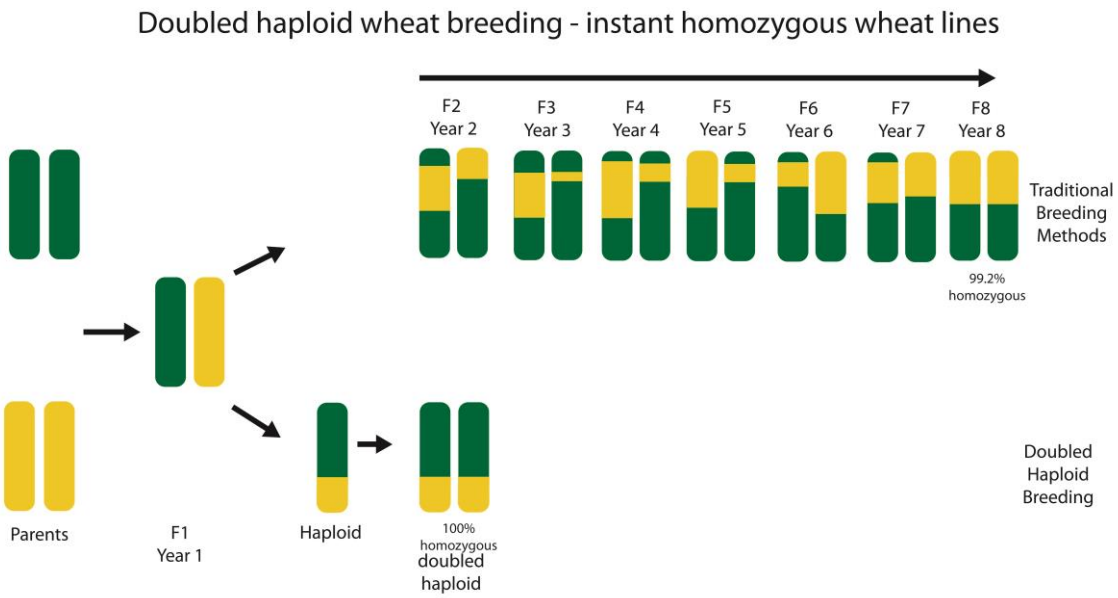

Figure 1.1 Chromosome doubling accelerates traditional breeding.
Traits from different parents are combined in F1 via crossing. With traditional breeding eight generations are required to produce homozygous lines. Haploids have only one allele of every gene, thus if converted back into diploids they can produce homozygous lines in a single step. (Figure adapted from Colodra wheat, 2013).

1.4 Antimitotic agents

Antimitotic agents are compounds obtained from nature that mimic biological agents. These agents arrest cells in mitosis by interfering with microtubule function at the metaphase plate thus inhibiting cell division. As a result the chromosomes are duplicated but not divided. The plants that develop from explants treated with antimitotic agents are called doubled haploids or polyploids (Goyal and Khan, 2009). The ability of these chemicals to induce polyploidy depends on the concentration of the chemical, the duration of exposure to the material and the species of plant being investigated. Antimitotic agents can be classified by their influence on tubulin polymerization and their binding site on tubulin. For instance toxoids, epothilones and discodermolide are a class of compounds that promote microtubule polymerization and stabilization of microtubules whereas vinca alkaloids, colchicine cryptophycins and estarmustine act on microtubules by inhibiting polymerization (Nagle *et al.*, 2006).

There are various antimitotic agents that are used in tissue culture plants for microtubule depolymerisation, these include pronamide and amiprosphomethy, but the most commonly used antimitotic agents are colchicine, oryzalin and trifluralin.

Colchicine is an alkaloid derived from the plant species *Colchicum autumnale* (Autum crocus). Colchicine acts by binding to soluble tubulin leading to the formation of a tubulin-colchicine complex (Molad, 2002). The application of colchicine to plants has several disadvantages such as its low affinity to plant microtubules. High concentrations are therefore required for chromosome doubling of plants. The high affinity of the alkaloid to mammalian microtubules makes colchicine toxic to humans. The drug shows a high mortality rate and produces mixoploids or chimeric plants.

Trifluralin is a yellowish-orange solid or crystal herbicide that is primarily used on grass to control broadleaf weeds and on crops (fruits, vegetables, soy and cotton), flowers and shrubs (Quesenberry *et al.*, 2010). Trifluralin acts in the same way as colchicine but has a stronger affinity to plant tubulin.

Oryzalin (3, 4-dinito-N^4, N^4-dipropylsulfanilimide) a dinitroaniline microtubule disrupting herbicide (Cox, 2001) derived from the toluidine chemical family from Dow AgroScience and is sold under many trade names including Dirimal, Ryzelan, and Surflan. It acts by inhibiting cell division in germinating weed by blocking cell division in the meristems. Oryzalin is an antimitotic agent that has a lower affinity to animal tubulin, low toxicity with a high affinity to plant tubulin. For this oryzalin is preferable to colchicine, which has a higher affinity to animal tubulin over plant tubulin (Wang *et al.*, 1991).

1.5 *In vitro* chromosome doubling

Haploid plants are plants with the gametophytic chromosome number (*n*) whereas doubled haploids (DH) are plants where the chromosomes have undergone spontaneous or induced

duplication. Doubled haploidy is the fastest route to the production of homozygous plants and these plants are valuable in breeding programmes and genetic studies of interspecific and intergenetic crosses. The application of DH is a convenient method in breeding programmes as it saves the time needed to produce pure breeding lines. Chromosome doubling has been used for a number of reasons in plants, these include restoring fertility in wide hybrids (Contreras *et al.*, 2007; Hardley and Openshaw, 1980), developing sterile cultivars, overcoming hybridization barriers (Kehr, 1996, Ranney, 2006), producing a range of genetic diversity among species and altering morphology of leaves, flower, fruits or the overal vigor of a plant.

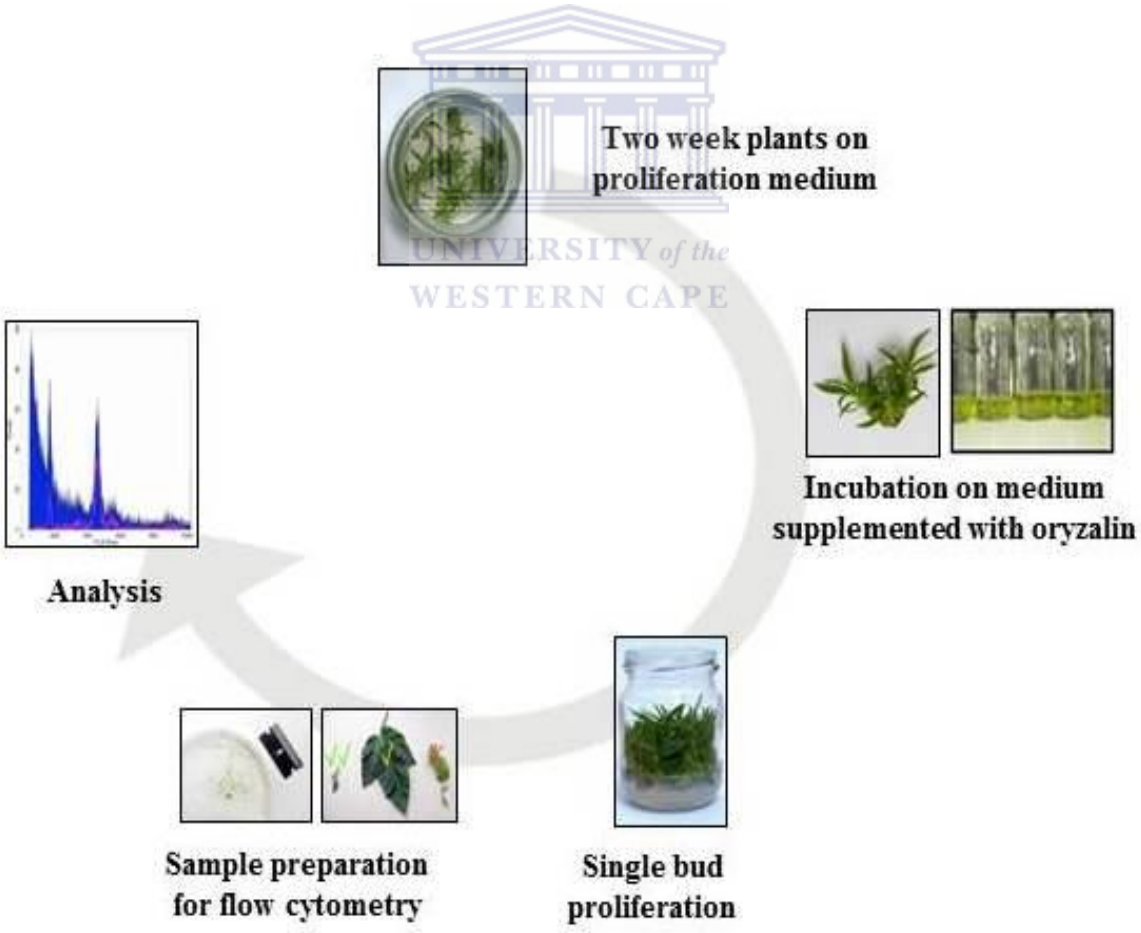

Figure 1.2 *In vitro* chromosome doubling.
Illustration of workflow of *in vitro* chromosome doubling from proliferation to sample preparation and analysis of obtained doubled haploids on flow cytometry.

1.6 Polyploidy

Polyploidy is the presence of more than two paired sets of chromosomes in an organism. Although rare in gymnosperms, polyploidy occurs in nearly all species of ferns and flowering plants (Soltis *et al.,* 2004). There is a difference of polyploids that arise within a single species forming mutations within chromosome numbers and those that arise due to the hybridization of two distinct species (Ramsey and Schemske, 1998). The former is known as autopolyploids and the latter as allopolyploids. Polyploid plants can either arise spontaneously or produced by artificial methods. Since the discovery of antimitotic agents, ploidy levels have been induced with high levels of success in agricultural and horticultural crops (Hancock, 1997).

1.7 Flow cytometry

Flow cytometry also referred to as the fluorescent activated cell sorter (FACS). The application of flow cytometry is a very popular method ranging from basic and applied research to industry (Dolezel *et al*., 2005). The flow cytometry allow for the individual measurement and counting of microscopic particles as they pass through a fluid stream in a light source. The scattered light or fluorescence will then be collected by an optical lens and passed through optical filter, which will pick up the fluorescent wavelength. Detectors will convert the light pulses to electric current pulses; amplify the signal and displaying the results as histograms (Shapiro, 2003). The use of flow cytometry has become the method of choice over the manual counting of chromosome numbers, due to its convenience, fast and reliability. Sample preparation only takes a few minutes and analysis is rapid. The manual counting of chromosomes by light microscope is an inexpensive, time consuming and tedious method. This chromosome counting has become second to FACS. The application of flow cytometry has been used in different fields which include medicine, immunology and pathology. In plants it has not only been used for determining chromosome numbers (Bakke,

2001) one of the most commonly use of it is for the determination of nuclear DNA content of unknown samples by comparing the position of the deoxyribonucleic acid (DNA) histogram of a known sample to a reference standard.

1.8 Plant proteomics

Proteomics can be defined as the study of protein expression, interaction, structure and function of proteins in an organism (Ndimba and Thomas, 2008). Proteomics describe the proteins expression in a genome from their identification of amino acid sequence to the determination of their relative amounts. Therefore, a proteome study in an organism of interest represents a comprehensive survey of all proteins expressed at a given time and at a defined physiological state (Aebersold and Man, 2003).

1.9 Significance of proteomics

Proteins were previously identified by means of transcriptomic, which is the analysis of messenger ribonucleic acid (mRNA) expression using microarray technology. Transcriptomic analysis does not give sufficient information about the expressed proteins due to the change in mRNA expression and the related protein product that does not always correlate (Thurston *et al.*, 2005). Proteomics offer the ability to identify a number of proteins expressed in the organism, function as well as dynamic range of cells and whole tissue. Proteomics can also reveal data such as post translational modifications, biological activity as well as protein abundance (Futcher *et al*., 1999; Panday and Mann, 2000).

1.10 Proteomic methods

Proteomics can be divided into gel-based and non-gel based proteomics (Monteoliva and Albar, 2004). Gel-based proteomics involves the separation of proteins by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE), whereas in non-gel based proteomic, methods such as multi-dimensional protein identification (MudPIT) and liquid

chromatography (LC)-MS are implemented. In this method, complex mixtures of proteins are enzymatically digested, than separated using high performance separation techniques prior to their identification by MS (McCormack *et al.*, 1997). The advantage of using non-gel proteomics methods is the technique being less affected by the size and solubility of the protein extract, and gel-based methods are favoured for their excellent protein resolving power and affordability (Blackstock and Weir, 1999). This section will focus on gel-based proteomics approach which was applied in this study.

In gel-based proteomics 1D-PAGE is used for the separation of proteins based on their molecular weight (MW) (Hames and Rickwood, 1990), whereas 2D-PAGE is used for the separation of proteins from complex mixtures. The protein samples are separated in two steps, the first dimension, which involves the separation of proteins based on their isoelectric point (*p*I), defined as the pH at which a molecule such as a protein has a net charge of zero and achieved by isoelectric focusing (IEF). The protein samples will be loaded onto pH gradient (IPG) strips and separated by IEF on the basis of their net charge. In the second dimension, proteins are separated based on their molecular MW by sodium dodecyl sulfate (SDS-PAGE), using a two-step equilibration buffer. The first equilibration buffer containing dithiothreitol (DTT), is used for the reduction of protein bonds and the second equilibration buffer containing iodoacetamide, used for the prevention of reformation of disulphide bonds of proteins (Weitermeier *et al*., 2005).

1.10.1 Protein staining

After the second dimension of protein separation, protein gels are then stained in order to visualise the protein spots on the gel. Protein spots can then be visualised by using different staining methods such as coomassie brilliant blue (CBB), silver or fluorescent stains (Gorg and Weiss, 2004). The most commonly used stain is CBB. There are two types of CBB

stains, R-250 and G-250. These stains are both compatible with MS, inexpensive and less sensitive than silver and fluorescent stains.

1.10.2 Mass spectrometry for protein identification

After staining the protein bands/spots of interests can be excised from the gel and proteolysed using trypsin followed by identification by means of peptide mass fingerprinting (PMF) using mass spectrometry (MS) (Patterson and Aebersold, 2003), matrix assisted laser desorption ionization-time of flight (MALDI-TOF) or protein sequencing using tandem mass spectrometry. The proteins are then identified through a database search using MASCOT search engine (Hood *et al.,* 2004). The software will search against a protein database such as Swiss-Prot or national centre for biotechnology information (NCBI), where the peptide masses are measured and correlated with known protein sequences from a database. This will give results about the possible identified proteins.

In contrast to MALDI-TOF MS for protein identification, tandem MS also known as MS/MS can isolate individual ions, fragments with the first MS and second analyser. This will give structural and sequence information. For instance for a peptide it will give the amino acid sequence of the fragment. These sequences will then be submitted to MASCOT and compared to known sequences in the database instead of using peptide masses for protein identification (Pandey and Mann, 2000; Dubey and Grover, 2001 and Mann *et al.,* 2001).

1.11 Reactive oxygen species as important signal in plants

Relative oxygen species (ROS) are produced in plant under normal conditions. When plants are exposed to environmental stresses they will purposely generate ROS signalling molecules to control stressful conditions such as pathogen defence programmed cell death and stomatal behaviour. The level of ROS production may increase causing damage to plant cell tissues and ultimately leading to plant death. There are several locations in plants were ROS are

produce, these include chloroplast, cell wall mitochondria and plasma membrane. ROS can be activated under a number of abiotic and biotic conditions (Chen *et al.*, 2002) such as salinity, drought, high temperatures, metal toxicity, pathogen, heavy metals and nutrient deficiency (Sharma and Dubey, 2007).

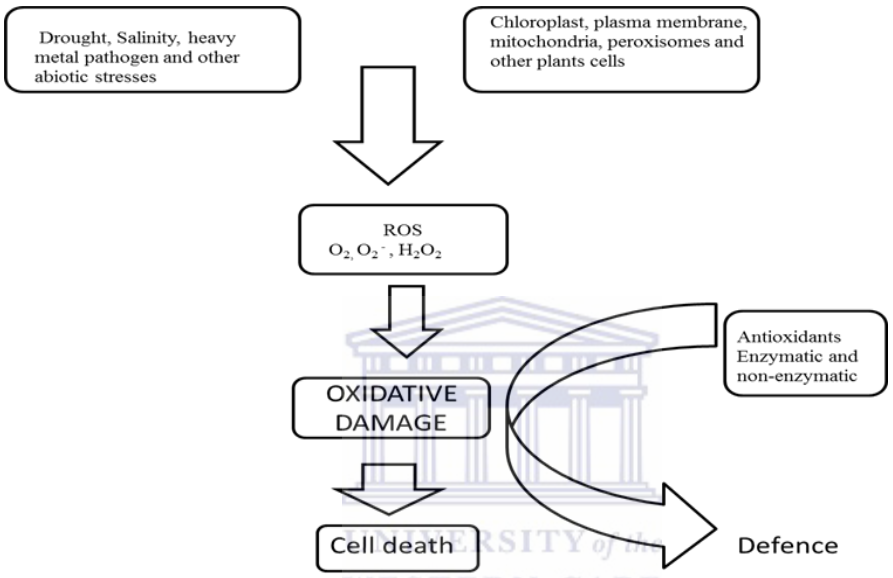

Figure 1.3 Abiotic stress induced ROS production and results in cell death.
Defence by antioxidant (enzymatic and non-enzymatic enzymes) will scavenge ROS thus preventing cell death. Modified figure adapted from Gill *et al.* (2010).

1.12 ROS scavenging mechanisms in plants

Plants use a mechanism known as the antioxidant system as a defence to regulate ROS levels according to their cellular needs at a particular time (Hossain *et al.*, 2011). The most common ROS include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) hydroxyl radical ($^{\bullet}OH$) and singlet oxygen (O_2^-); (Smirnoff, 1993; Sharma *et al*., 2012). ROS can both be harmful and beneficial to plant tissues. At high concentrations it can cause damage to biomolecules and at low concentrations it will act as a secondary messenger that mediated several responses in

plant cells. To protect themselves against toxic environments, plant cells and its organelles like chloroplast, mitochondria and peroxisomes will employ enzymatic and non-enzymatic antioxidant defence systems to scavenge ROS.

1.13 ROS scavenging enzymatic antioxidants

Antioxidants are produced in plants as a result of increasing levels of ROS accumulation and they will protect the plants cells against oxidative stress. Certain ROS can be very toxic to plants and must be detoxified in order for the plants to grow and survive (Gratão *et al.*, 2005). The antioxidant scavenging enzymes can be classified into enzymatic and non-enzymatic enzymes.

1.13.1 Enzymatic antioxidants

1.13.1.1 Superoxide dismutase (SOD)

Superoxide dismutase EC 1.15.1.1 is the most effective intracellular enzymatic antioxidant in all aerobic organisms. In response to environmental stresses, plants will produce increased levels of ROS. SOD provides the first line of defence in plant stress tolerance and it function by removing O_2 by catalysing its dismutation, O_2 and reducing it to H_2O_2 and O_2. SODs are classified into three known types based on the metal ion in their active site and they are found in different cellular compartments. Copper/zinc (Cu/ZnSOD) localised in the chloroplast of higher plants, manganese (Mn-SOD) found in the mitochondria of eukaryotic cells and peroxisomes and iron (Fe-SOD) if detected in plants are normally found in the chloroplast of higher plants (Gill *et al*., 2010; Ashraf, 2009).

1.13.1.2 Catalase (CAT)

Catalase EC 1.11.1.6 is an important scavenging enzyme that plays a role in the removal of H_2O_2 generated in peroxisomes using oxidases involved in β-oxidation of fatty acids,

photorespiration and purine catabolism. It has been reported by Ali *et al.* (2002) that apart from reaction of CAT with H_2O_2 it also reacts with methyl hydrogen peroxide (MeOOH).

1.13.1.3 Ascorbate peroxidase (APX)

APX plays an important role in scavenging ROS in higher plants and organism. APX scavenge H_2O_2 in water-water and ASH-GSH cycle. APX family consist of five different isoforms and has a higher affinity for H_2O_2 than CAT. Hsu and Kao (2007) have demonstrated that pre-treatment of *Oryza sativa* seedlings with H_2O_2 under non-heat shock conditions resulted in an increase APX activity and protected rice seedlings from Cadmium (Cd) stress.

1.13.1.4 Glutathione reductase (GR)

Glutathione reductase localised in the chloroplast althought small amount can also be found in the mitochondria and cytosol (Edwards *et al*., 1990). GR catalyses the reduction of GSH, a molecule involved in metabolic regulatory processes in plants where GR catalyses the nicotinamide adenine dinucleotide phosphate (NADPH) dependent reaction of disulphide bond of glutathione disulphide (GSSG). GR and glutathione (GSH) play a role in determining a plants tolerance to various environmental stresses.

1.14.1 Non-enzymatic antioxidants

1.14.1.1 Ascorbic acid

Ascorbic acid is an antioxidant that acts by preventing damaged caused by ROS in plants. It can be found in all plant tissue and occur in a higher rate in photosynthetic cells and meristems. It has been reported that ASH remain available in reduced form in leaves and chloroplast under normal conditions. ASH has been considered to be the most powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions (Gill *et al.*, 2010).

1.14.1.2 Glutathione

Glutathione is localised in various compartments in plant tissues like cytosol, endoplasmic reticulum, vacuole, mitochondria chloroplast as well as apoplast. It is found in its reduced form in plants (GSH) and plays a role in the physiological processes detoxification of xenobiotic and expression of stress responsive genes (Rausch *et al.*, 2005). GSH also plays roles in growth and development in plants as well as differentiation, cell death, senescence, pathogen resistance and enzymatic regulation. GSH is crucial for the maintenance of normal reduced cell state thereby counteracting the inhibitory effects of ROS induced oxidative stress.

AIMS OF THIS STUDY

With the above mentioned review, it shows that interspecific crosses may result in seedlings not germinating due to uneven number of chromosomes. The interest of breeders in doubling the chromosome numbers lies in the possibility in shortening the time needed to produce complete homozygous lines in comparison to conventional breeding. The hypothesis of the study was that the induction of chromosome doubling in diploid Marianna plum shoots with oryzalin would influence the proteome profile and mediated physiological and biochemical responses of the plant. In order to achieve this, the following aims were addressed:

(i) To develop an *in vitro* chromosome doubling method for Marianna plum shoots by treating shoots with different concentrations of antimitotic agent oryzalin for different incubation times.

(ii) To identify the differently expressed proteins in response to oryzalin treatment by means of 2D-SDS PAGE analysis coupled with mass spectrometry and,

(iii) To determine wether treatment of Marianna shoots with antimitotic agent would influence ROS accumulation, photosynthetic pigmentation and antioxidant capacity.

CHAPTER 2

MATERIALS AND METHODS

2.1 General chemicals and suppliers

Table 2.1 List of chemicals used in this study

Chemicals	Suppliers
β-Mercaptoethanol	Sigma
Acetic acid	KIMIX
Acetone	Merck
Agar	KIMIX
Agarose	White Scientific
6-benzylaminopurine	KIMIX
Bovine serum albumin	Roche
Bradford reagent	BIO-RAD
Bromophenol blue	Sigma
Carrier ampholytes	BIO-RAD
3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate CHAPS	Sigma
Coomassie brilliant blue	Sigma
4'-6-diamidino-2-phenylindole (DAPI)	PARTEC
De-streaking solution	GE Healthcare Bio-Science AB
Dithiothreitol	Fermentas
Dipotassium phosphate	Sigma
Ethylenediamine tetraacetic acid	Merck
Ethanol	KIMIX
Glacial acetic acid	Merck
Glucose	Merck

Glycerol	Merck
Glutathione peroxidase	Merck
Hydrocloric acid	Merck
Iodoacetamide	Sigma
Indole-3-butyric acid	Sigma
Isopropanol	KIMIX
Mineral oil	BIO-RAD and GE health care
Molecular weight marker	Fermentas
Murashige and Skoog Basal Medium	Sigma
Nitroblue tetrazolium	Sigma
Oryzalin	KIMIX
Potassium cyanide	Sigma
Potassium dihydrogen phosphate	Sigma
Potassium hydrogen pellets	Merck
Potassium phosphate dibasic	Sigma
Polyvinylpyrrolidone	Sigma
Riboflavin	Merck
Sodium chloride	Merck
Sodium dodecyl sulfate	BIO-RAD
Sodium hydroxide	Merck
Sucrose	Merck
Tetramethylethylenediamine	Sigma
Thiourea	Sigma
Tris-HCl	Aldrich
Trypsin	Promega
TWEEN® 20	Merck
Urea	Sigma

2.2 General stock solutions and buffers

80% acetone: 80% (v/v) acetone in distilled water.

0.5% agarose sealing solution: 0.5% (w/v) agarose prepared in 1X SDS-PAGE running buffer with a tint of bromophenol blue.

10% APS: 10% (w/v) APS in distilled water. The solution was freshly prepared before use.

APX buffer: 14.4 g Glycine; 3 g Tris; and 176 mg L-ascorbic acid.

Bradford reagent: 1 part BIO-RAD Protein Assay dye reagent concentrate diluted with 4 parts distilled water.

5 mg/ml BSA stock solution: 5 mg/ml BSA in urea buffer.

1.25% (w/v) CBB stock solution: 1.25% (w/v) CBB R-250 in distilled water.

CBB staining solution I: 50 ml of 1.25% (w/v) CBB stock solution, 10% (v/v) glacial acetic acid and 25% (v/v) propan-2-ol in distilled water.

CBB staining solution II: 6.25 ml of 1.25% (w/v) CBB stock solution, 10% (v/v) glacial acetic acid and 10% (v/v) propan-2-ol in distilled water.

CBB staining solution III: 6.25 ml of 1.25% (w/v) CBB stock solution and 10% (v/v) glacial acetic acid in distilled water.

De-staining solution: 10% (v/v) acetic acid and 1% (v/v) glycerol in distilled water.

50% DTT: 50% (w/v) DTT in urea buffer.

Equilibration base buffer I: 9 M urea, 0.375 M Tris-HCl (pH 8.8), 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT.

Equilibration base buffer II: 9 M urea, 0.375 M Tris-HCl (pH 8.8), 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) Iodoacetamide.

70% ethanol: 70% (v/v) ethanol in distilled water.

0.1 M HCl: 0.1 M HCl in distilled water.

0.2 M K_2HPO_4: 0.2 M K_2HPO_4 in distilled water.

1 M KH_2PO_4: 1 M KH_2PO_4 in distilled water.

5 M NaCl: 5 M NaCl in distilled water.

1 M NaOH: 1M NaOH in distilled water.

Plant sterilization solution: absolute commercial bleach containing 0.1% (v/v) Tween® 20.

SDS phenol extraction buffer: 0.1 M Tris-HCl pH 8.0, 2% SDS, 5% β mercaptoethanol, 30% sucrose and 1mM PMSF.

5X SDS sample loading buffer: 0.2 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.025% (w/v) bromophenol blue.

1X SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine containing 0.1% (w/v) SDS.

SOD Assay buffer: 13 mM Methionine, 2 µM Riboflavin, 50 mM EDTA and 100 µM of NBT.

6% TCA: 10% (w/v) TCA in distilled water.

10% TCA: 10% (w/v) TCA in acetone.

0.5 M Tris-HCl, pH 6.8: 0.5 M Tris in distilled water adjusted to pH 6.8 with concentrated HCl.

1.5 M Tris-HCl, pH 8.8: 1.5 M Tris in distilled water adjusted to pH 8.8 with concentrated HCl.

Urea buffer: 9 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). The solution was kept at -20ºC.

2.3 Plant tissue culture media and plant growth hormones

All plant tissue culture media and plant growth hormones were supplied by Sigma and KIMIX and prepared as follows:

1mg/ml BA: 1 mg 6-benzylaminopurine dissolved in 1 ml distilled water.

1mg/ml IBA: 1 mg Indole-3-butyric acid dissolved in 1 ml distilled water.

MS Solid medium: 4.4 g/l Murashige and Skoog basal salt with minimal organics, 3% sucrose, 1 mg/ml IBA, 0.1 mg/ml BA, and 0.7% agar adjusted to pH 5.6 with 1 M NaOH.

MS Liquid medium: 4.4 g/l MS basal salt, 3% sucrose, 1 mg/l BA, IBA 0.1 mg/l, kinetin 0.1 mg/l pH adjusted to 5.6.

Oryzalin: 1% dissolved in 1 M NaOH and made up to 0.1 L with distilled water.

The plant tissue culture media were autoclaved at 121˚C for 20 min using High Pressure Steam Autoclave HL-340 (Germany Industrial Corp, Taiwan).

2.4 Plant growth

Marianna plum rootstocks were obtained from the Horticultural department at the Agricultural Research Council (ARC) (Infruitec) in Stellenbosch, Western Cape. These rootstocks were grown till maturity at the ARC growth facility under 24±2˚C day/night temperatures with constant spraying with water after every 15 min intervals for 5 sec. The shoots produced from this rootstock were used for plant establishment in tissue culture (section 2.5).

2.5 Plant Tissue Culture

2.5.1 Plant establishment

Shoots were dissected from Marianna rootstocks grown at the ARC growth facility. Lateral buds from the shoots were excised, sterilized and placed onto culture media using a modified procedure described by Knauss (1976). Explants were placed aseptically onto MS culture medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.1 mg/ml kinetin, 0.1 mg/ml IBA. The pH of the media was adjusted to 5.6 before agar (0.7%) was added. The media was sterilized by autoclaving at 121˚C for 20 min and transferred to glass bottles in 15-20 ml volumes. Plants were then allowed to grow for 16/8 h light/dark photoperiod at 24±2˚C. The plants were sub-cultured every 3-4 weeks in fresh medium until

sufficient plants were produced. These plants were used for *in vitro* chromosome doubling as described in section 2.5.2.

2.5.2 Chromosome doubling

A stock solution of oryzalin was prepared in 1 M sodium hydroxide. The stock solution was filter-sterilized using a 0.22 µm filter and supplemented aseptically to autoclaved liquid MS medium at (50, 75, 100, 150 or 200 µM) concentration levels. Ten Marianna shoot cultures (2 cm in size) with one axillary bud each was cut and transferred to liquid MS medium containing different oryzalin concentrations. These plants were incubated in the dark at 24˚C for 24 or 48 h respectively with moderate agitation. After incubation the shoots were rinsed three times with autoclaved distilled water before they were transferred to solid MS proliferation medium to grow in standard 16/8 h light/dark photoperiod at ±24˚C for 4 weeks. These cultured shoots were used for downstream flow cytometry, proteomic and biochemical analysis.

2.6 Flow cytometry analysis

Four week old *in vitro* cultures were selected for flow cytometry analysis. Approximately, 0.5 cm^2 leaves of treated and control plants (grown in tissue culture) was chopped inside plastic petri dishes containing ice cold nucleic extraction buffer (400 µl) supplied with the DNA kit (Cystein UV Precise Partec Germany) and incubated for 2 min. After incubation the suspension was filtered through a SEFAR NYTAL filter and stained with 1.6 ml of 4, 6 – diaminidino-2-phenylindole (DAPI) solution. Prior to flow cytometry analysis, samples were kept in the dark for 30 sec to 1 min. The relative flourescent intensity of isolated nuclei (5000 per sample) was measured using a FACS BIO- RAD flow cytometer.

2.7 Profiling the proteome of Marianna shoots

2.7.1 Plant material

Four week old Marianna shoots were harvested from tissue culture and stored at -80˚C in 50 ml falcon tubes.

2.7.2 Protein isolation for proteomics analysis

The phenol-SDS method with slight modifications was used for protein extraction from shoots as described by Wang *et al.* (2005). Approximately 200 mg shoot material was ground in liquid nitrogen using a mortar and pestle. The ground material was then homogenised in 640 µl (1:1 ratio) phenol/SDS buffer. The mixture was centrifuged at 13, 200 rpm for 10 min at 4˚C. The upper phenol phase was precipitated with 3 volumes of 0.1 M ammonium acetate dissolved in 80% methanol at -20˚C for 2 h. The suspension was then centrifuged at 13, 200 rpm for 10 min at 4˚C. The supernatant was discarded and the pellet was washed once with 80% acetone and centrifuged at 13, 200 rpm for 10 min at 4˚C. The acetone was discarded and the pellet was allowed to air dry at room temperature. The air dried pellet was then re-suspended in 100 µl urea buffer (9 M urea, 2 M thiourea and 4% CHAPS) to obtain the soluble proteins, and stored at -20˚C until further use. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.7.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.7.3.1 Sample preparation and 1D-SDS PAGE analysis

Protein samples were pre-mixed with 5X SDS loading dye (0.2 M Tri-HCl pH 6.8, 0.5 M DTT, 10% SDS, 50% Glycerol and 0.025% bromophenol blue), boiled for 5 min at 95˚C and centrifuged at 13, 200 rpm for 1 min at room temperature prior to gel electrophoresis. The protein samples were separated on a 1D-SDS PAGE gel (Table 2.2) at 100 $V.cm^{-1}$, for 2 h at room temperature.

Table 2.2 Composition of 12% resolving and 5% stacking gel for 1D-SDS PAGE

Reagents	12% Resolving gel (ml)	5% Stacking gel (ml)
40% Acralymide (37.5.1)	3	0.025
Tris HCl pH 8.8	2.5	_
Tris HCl pH 6.8	_	0.25
10% (w/v) (SDS)	0.1	0.05
10% (w/v) (APS)	0.1	0.05
dH_2O	4.3	1.48
TEMED	0.004	0.002

2.7.4 Separation of proteins by two dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS PAGE)

2.7.4.1 Sample preparation for rehydration of IPG strips

Prior to 2D-SDS PAGE analysis, total soluble proteins extracted from plant shoots stored at -20˚C were thawed on ice. Protein samples at a final concentration of 200 µg were premixed with de-streaking solution (GE Healthcare Bio-Science AB) to a final total volume of 125 µl and centrifuged at 13, 200 rpm for 1 min at room temperature prior to rehydration. The foil of the linear (7 cm, pH range 3-10) immobilized protein gradient (IPG**)** strips was carefully removed and the IPG strip was incubated gel side down in the protein sample, avoiding the formation of air bubbles. The strips were then covered with mineral oil to avoid dehydration during the rehydration reaction. Samples were passively rehydrated overnight at room temperature.

2.7.4.3 Isoelectric Focusing (IEF)

After rehydration the IPG strips were rinsed with dH_2O to remove unabsorbed proteins by carefully blotting the strip with moist filter paper. The IPG strips were then placed gel side up

on the focusing platform of BIORAD machine. Pre-damped wicks with dH_2O were placed on each end of the strip to absorb excess salts and impurities during focusing. The IPG strips were then covered with mineral oil to avoid sample evaporation. Isoelectric focusing was performed at 20˚C using the parameters outlined in Table 2.3. After focusing, the strips were rinsed with dH_2O and equilibrated as described below prior to 2D-SDS-PAGE analysis.

Table 2.3 Isoelectric focusing programme for IPG strips

Step	Volts (V)	Duration (h)/Volts h (Vh)
1	250	0.15 h
2	100	1 h
3	500	12 000 Vh

2.7.4.4 Equilibration of IPG strips

The IPG strips were prepared for 2D-SDS PAGE analysis in a two-step equilibration reaction in order to solubilise focused proteins. The IPG strips were incubated (gel side up), in equilibration solution 1 [9 M urea, 2% (v/v) SDS, 0.375 M Tris-HCl, pH 8.8, 20% (v/v) glycerol and 2% (w/v) DTT] for 15 min with gentle agitation. After incubation, the strips were transferred to equilibration solution 2 [2.5% (w/v) iodoacetamide] and incubated for another 15 min with gentle agitation. The proteins were then separated on second dimension SDS-PAGE as described below.

2.7.4.5 Second dimensional SDS-PAGE

The proteins on the IPG strips were resolved on 12% SDS-PAGE prepared as described in Table 2.2 without the 5% stacking gel. The resolving gel was poured into the cast plate and each gel was overlaid with 100% isopropanol and allowed to polymerise for 1 h at room temperature. After polymerisation the isopropanol was poured out and the gel was rinsed

briefly with dH_2O and the gel surfaces were blotted dry with filter paper. Five microliter of pre-stained PAGE marker was loaded onto a small piece of filter paper, air dried and placed at the anode side of each IPG strip. The strips were rinsed briefly with dH_2O and blotted with moist filter paper before carefully loading on top of the resolving gels. Strips were then overlaid with cooled agarose sealing gel (section 2.2) to secure their position. Electrophoresis was conducted using PROTEAN® 3 Dodeca™ cell (BIO-RAD) at 100 $V.cm^{-1}$ at room temperature. After electrophoresis gels were stained with coomassie brilliant blue (CBB) as described in section 2.8.

2.8 Protein detection by staining with CBB

Proteins separated by 1D-SDS PAGE (section 2.7.3.1) or 2D-SDS PAGE (section 2.7.4.5) was detected in a three step CBB staining method. After electrophoresis gels were removed from the gel plate and immersed in CBB staining solution 1 (section 2.2) heated in microwave for 1 min and incubated on shaker for 1 h at room temperature. The staining solution was discarded and the staining process was repeated using CBB staining solution 2 (section 2.2) and finally CBB solution 3 (section 2.2) before de-staining the gels. This was done until the protein bands or protein spots were visible. The gels were then imaged using PharosFX™ Plus molecular imager scanner (BIO-RAD).

2.9 2D-SDS PAGE analysis and identification of protein spots

The 2D-SDS PAGE gels were imaged using the molecular imager PharosFX Plus system (BIO-RAD) and then analysed using PDQuest™ software. Three biological replicates for each group (control and treated samples) were analysed. Spots were manually edited using the group consensus tool to obtain spot expression consensus in all biological replicates per treatment. Differentially expressed proteins spots were either qualitative (absent/present), quantitative (fold expression change) or student's t-test at 95% significance spots. The

protein spots of interest were then manually picked with sterile pipette tips for mass spectrometry identification as described by An *et al.* (2014)

2.10 Biochemical analysis

2.10.1 Chlorophyll content analysis

Estimation of chlorophyll content was based on a modified method described by Hiscox and Israelstam (1980). Freshly harvested Marianna shoots were cut into pieces of approximately 0.5 cm^2. The shoots (100 mg per plant) was mixed with 0.5 ml of dimethylsulfoxide (DMSO) and incubated at 65°C for 3 h. The absorbance of an aliquot of the shoot-DMSO extract (200 µl) was read at 645 nm and 663 nm, with DMSO being used as a blank. The chlorophyll content was calculated according to the formula described by Arnon (1949).

2.10.2 Protein isolation for biochemical assays

Protein extracts were obtained from shoots, that were ground to a fine powder using liquid nitrogen and 100 mg of tissue was homogenized in 1 ml of buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000] for determination of antioxidant enzymatic activities, or 1 ml of 10% trichloroacetic acid (TCA) for H_2O_2 content, and lipid peroxidation levels. The homogenates were centrifuged at 12, 000 rpm for 15 min and the supernatants were used for the respective biochemical assays. Protein concentrations were determined as described by Bradford, (1976), using bovine serum albumin (BSA) as a standard.

2.10.3 Measurement of H_2O_2 content

H_2O_2 content was determined based on a method described by Velikova *et al.* (2000). The reaction mixture consist of 75 µl of the TCA extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. Samples were incubated at 25°C for 20 min and absorbance readings of samples were

measured at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance (A390 nm) of different H_2O_2 concentrations.

2.10.4 Measurement of lipid peroxidation

Products of lipid peroxidation (reflective of MDA content) were estimated as described by Buege and Aust (1978). For these measurements, 1 ml of TCA extract was mixed with 4 ml of 0.5% TBA (dissolved in 20% TCA). The mixture was heated for 30 min at 95°C and then cooled in an ice bath for 10 min. The specific absorbance of products was read at 532 nm and nonspecific background-absorbance at 600 nm was subtracted from the readings. The concentration of MDA was calculated using a molar extinction coefficient 155 mM^{-1} cm^{-1}.

2.10.5 Measuring APX and GR enzymatic activity

For all antioxidant enzyme activity assays, proteins were prepared using the homogenizing buffer described in section 2.6. APX (EC 1.11.1.11) enzymatic activities were measured using a modified method described by Asada (1984). The shoot extracts (extracts supplemented with ascorbate to a final concentration of 2 mM) were added to the assay buffer (50 mM K_2HPO_4, pH 7.0, 0.1 mM EDTA, and 50 mM ascorbate). The reaction was initiated with 1.2 mM H_2O_2 in a final reaction volume of 200 µl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of 2.8 mM^{-1} cm^{-1}.

GR activity was determined using a slightly modified method described by Esterbauer and Grill (1978) by following the rate of NADPH oxidation at 340 nm. The assay mixture contained: 0.2 mM NADPH, 0.5 mM GSSG, 1 mM EDTA in 100 mM K_2HPO_4 pH 7.8 and 50 µg of enzyme extract in a 200 µl reaction. GR activity was calculated based on the oxidation of NADPH in the reaction, using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

2.11 Statistical analysis

The data was analysed using the one-way analysis variance (ANOVA) and tested for significance by the Turkey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

CHAPTER 3

DEVELOPMENT OF *IN VITRO* METHODS FOR CHROMOSOME DOUBLING OF MARIANNA PLUM SHOOTS

3.1 Introduction

The stone fruit breeding programme in South Africa includes objectives for developing new *Prunus* rootstocks with specific adaptation to unfavourable local biotic and abiotic conditions. Good adaptation to replant sites, tolerance to drought and waterlogging, resistance to nematodes and other soil borne pathogens and graft compatibility are considered priority traits in selection criteria**.** Marianna (2*n*) is an open pollinated plum rootstock from parental linage, *Prunus cerasifera* x *P, munsoniana*. The rootstock is widely adapted to different soil types and moisture conditions with moderate resistance to crown gall and crown rot (Southwick *et al*., 1999). These traits have favoured the use of Marianna as a parent in breeding and polyploidy studies to develop new and improved cultivars in the South African breeding programme. Although polyploidy occur in nearly all flowering plants (Soltis *et al*., 2003) it can also be induced by means of antimitotic agents such as oryzalin. Oryzalin (3, 4-dinito-N^4, N^4-dipropylsulfanilimide) is a herbicide that have extensively been used to control weeds in orchards (Atland *et al.*, 2003). This dinitroaniline disrupting herbicide has been used in many polyploidy studies to increase the chromosome numbers in plants. It has also been shown to have a strong affinity to plant tubulin in comparison to colchicine that has a stronger affinity to animal tubulin (Pickens *et al.*, 2006). Oryzalin acts by disrupting mitosis in plants by inhibiting microtubule formation (Ramulu *et al.*, 1991) and thus results in doubled haploid plants. Breeding of interspecific crosses is normally delayed due to long dormancy periods of seeds. Polyploidy will allow plants to germinate and grow quickly, thereby shortening the breeding cycle (Contreras *et al.*, 2007). Furthermore, it may also

enhance the cytological, physiological and morphological attributes of the plant (Shi-Hai *et al.*, 2011), such as broader leaves, flowers, fruits and resistance to diseases. The purpose of this chapter was to induce chromosome doubling of diploid Marianna plum shoots by optimizing oryzalin concentrations and exposure times.

3.2 Different oryzalin concentrations and incubation times influencing plant survival rate

Marianna shoots were exposed to different concentrations of oryzalin for two time intervals (24 h and 48 h) in the dark and cultured for approximately four weeks. The survival rate for these explants were measured after four weeks. The exposure of explants for 24 h in different oryzalin concentrations showed a low but significant survival rate compared to a longer incubation period of 48 h (Fig. 3.1). The results showed that at treatment with 50 µM and 75 µM, there was some level of survival of explants at both incubations periods.

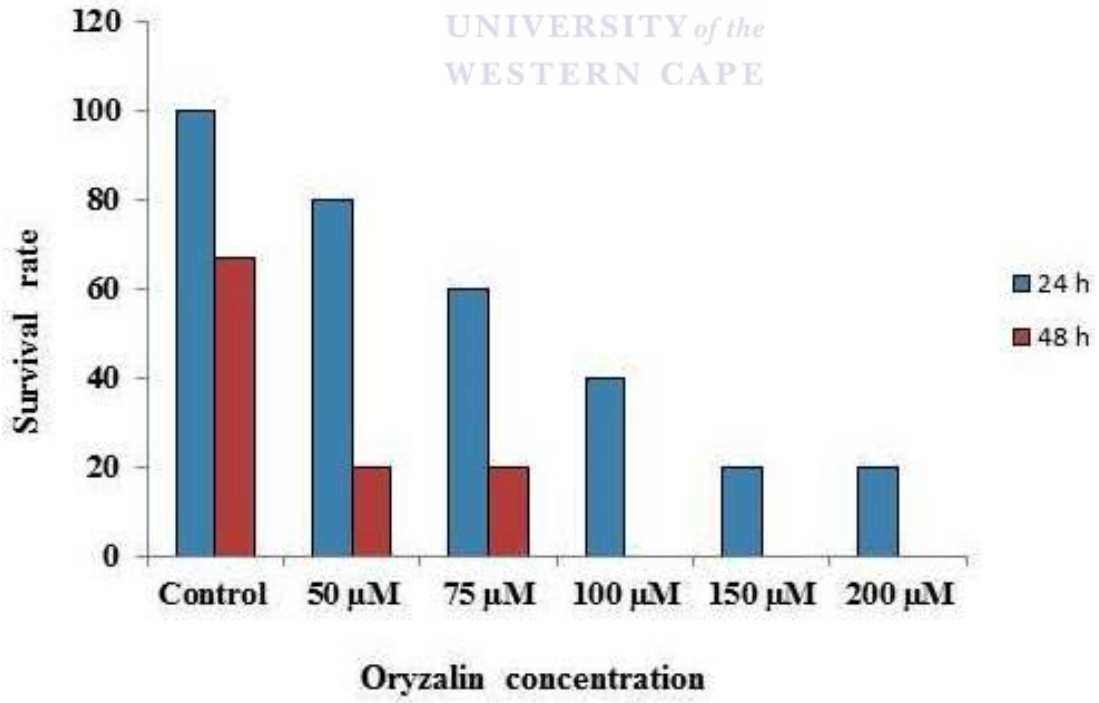

Figure 3.1 The effect of different oryzalin concentrations on plant survival at 24 and 48 h exposure.

A high mortality rate of 100% was observed in explants treated with 100 µM to 200 µM for 48 h (Fig. 3.1). The lowest possible concentration of oryzalin treatment at which explants survived was measured at 75 µM for both 24 h and 48 h incubations, albeit still low (Fig. 3.1). Given the high mortality rate or low survival rate observed for explants post 24 h exposure, down stream analysis (flow cytometry) was perfomed on explants exposed to different orazylin concentrations for 24 h.

3.3 Flow cytometry histogram of DAPI stained nuclei

Flow cytometry analysis was used to establish at which concentration of oryzalin treatment the ploidy levels of Marianna explants were increased. The results showed that no change in ploidy was observed when explants were exposed to 0 and 50 µM oryzalin for 24 h. This was indicative of the single peaks observed in the histogram (Fig. 3.2a-b).

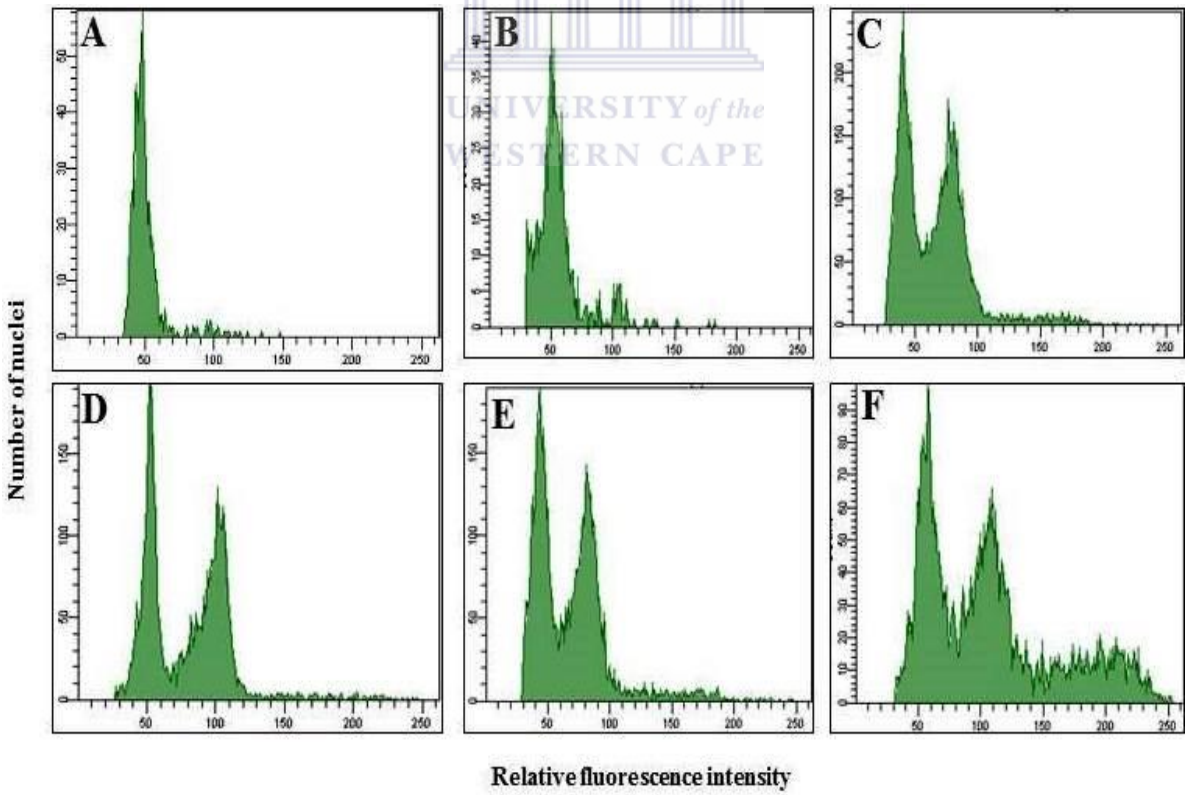

Figure 3.2 Flow cytometer histograms of DAPI stained nucleic composed of diploids, tetraploids and mixoploids.
A. Control; **B.** 50 µM; **C.** 75 µM; **D.** 100 µM; **E.** 150 µM and **F.** 200 µM.

An increase in oryzalin concentrations (75 µM to 150 µM) also increased the ploidy levels to produce tetraploids, 24 h after exposure (Fig. 3.2c-e). Exposure of explants to 200 µM oryzalin produced mixoploids or chimeric plants (2n+4n) which is a combination of both diploid and tetraploid plants (Fig. 3.2f). Based on the results obtained from this experiment all downstream analysis was perfomed on 75 µM oryzalin which is deemed to be the lowest possible concentration that induced ploidy levels in Marianna explants after 24 h exposure.

3.4 Oryzalin influence morphological characteristics of Marianna shoots

The exposure of Marianna plum shoots to different concentrations (75 µM to 150 µM) of oryzalin in tissue culture induced chromosome doubling. It also influenced the morphology of the induced polyploidy plants (Fig 3.3b).

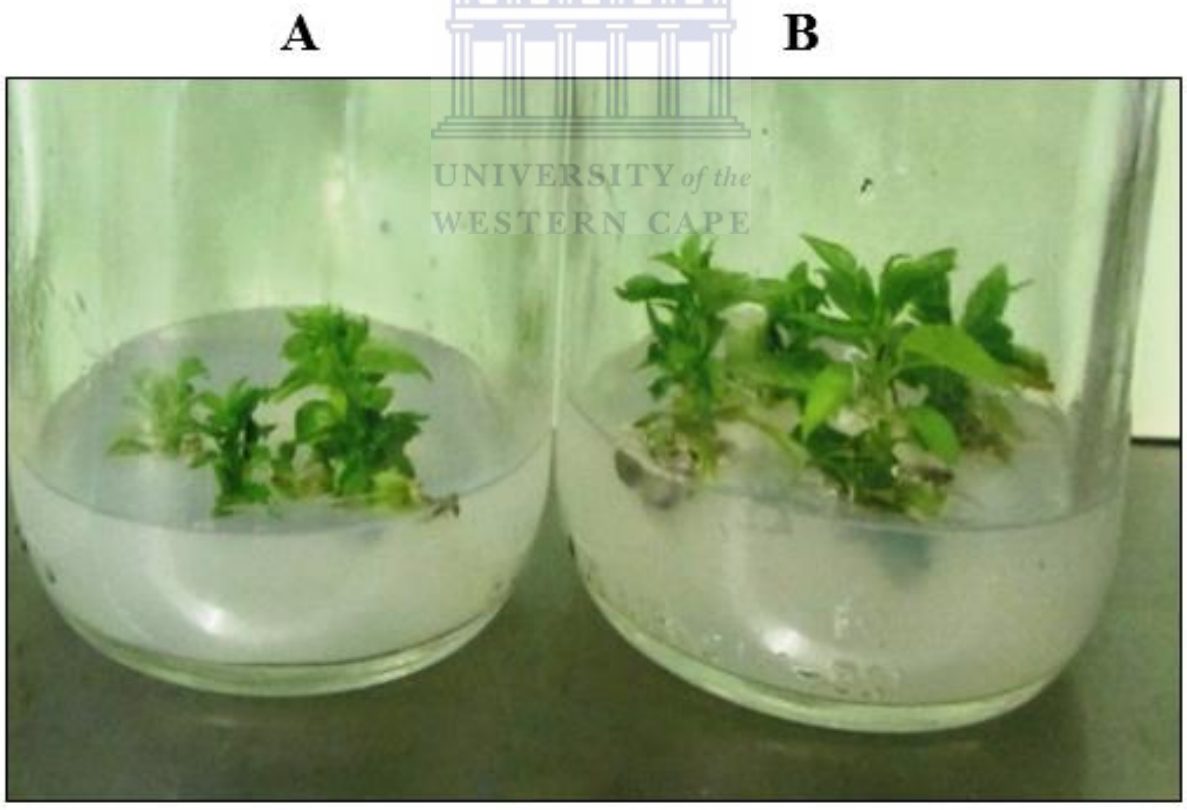

Figure 3.3 Morphological characteristics of control and treated plants in tissue culture.
Marianna control and treated shoots (75 µM) after 4 weeks *in vitro* on MS medium.
A. Control and **B**. Treated plants.

It was observed that plants exposed to 75 µM oryzalin (doubled haploids) for 24 h and grown in tissue culture for approximately 4 weeks had significantly larger leaves than the control plants (Fig. 3.3a-b). Similar results were obtained for other oryzalin concentrations that induced ploidy in Marianna shoots. There were no major difference in leaf colour and thickness between control and treated plants (Fig. 3.3). Plant length and weight was significantly influenced in response to oryzalin treatment at a final concentration of 75 µM. In both instances there was approximately 35% increase in height and weight when compared to the control plants (Fig. 3.4a-b).

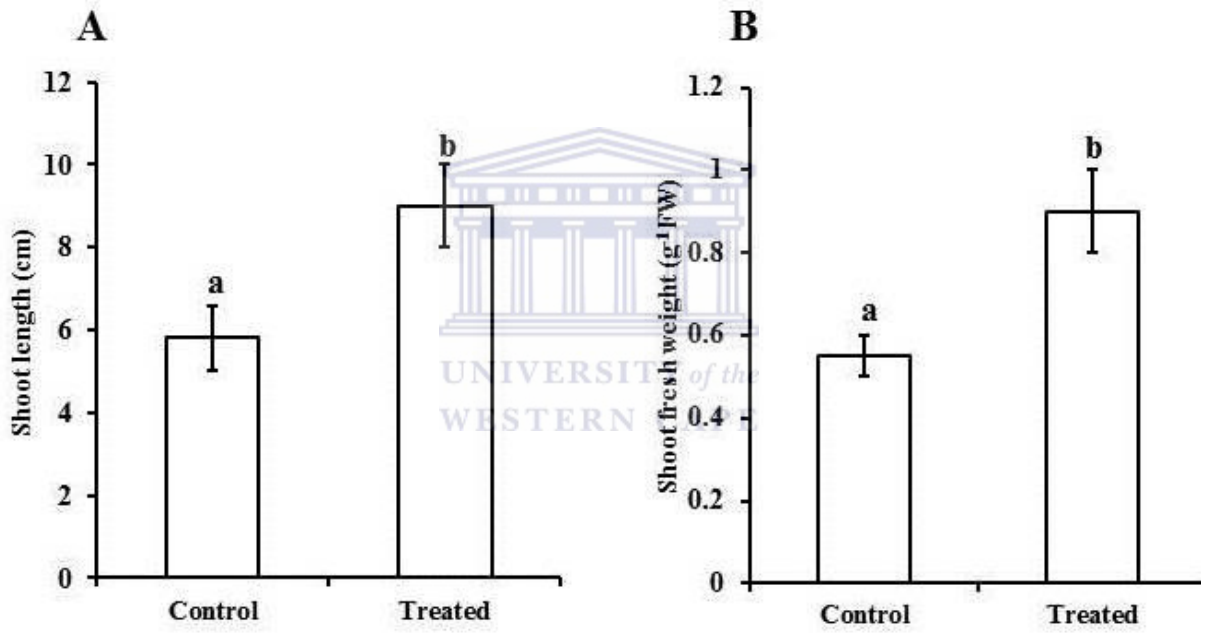

Figure 3.4 Oryzalin treatment influence growth parameters of Marianna plants.
Marianna plants treated with 75 µM oryzalin increased, **A**. shoot length and, **B**. fresh weight, compared to the control plants.

3.5 Discussion

The objective of this study was to to induce chromosome doubling of diploid Marianna plum shoots by optimising concentrations and exposure times of shoots to the antimitotic agent oryzalin. The results showed that treatment of Marianna shoots with oryzalin at concentrations ranging from (75 to 150 µM) for 24 h was effective for inducing ploidy in

plants. Oryzalin induced ploidy in Marianna plum shoots in the form of tetraploid and mixoploid or chimeric plants. As oryzalin concentrations increased there was also an increase in the ploidy level of plants observed after 24 h. Lower concentrations for shorter exposure to oryzalin produced a greater survival rate of plant shoots (Fig 3.1). This was observed when shoots were treated with (50 µM) for (24 h). However, no ploidy was induced and only diploids were detected at this low concentration (similar to control; Fig. 3.2a) which was indicative by the single peak detected in flow cytometry analysis (Fig. 3.2b). All the shoots that were treated with 200 µM oryzalin, only those incubated for 24 h survived (20%) whereas complete mortality was observed after 48 h exposure (Fig 3.1). As treatment duration increased, the percentage of diploids decreased. Longer exposure (48 h) of plant tissues to 200 µM showed a high mortality rate when compared to control plants. This phenomenon was also present even after 24 h incubation although not to the same extent.The control also showed a low survival rate after 48 h incubatin, in comparison to 24 h incubation, this can be the result of longer exposure of plants in the dark which influenced photosynthesis and resulted in low survival rate. The reduction in diploid shoots was concurrent with an increase in both mixoploids and increase shoot mortality (Fig. 3.2f). The occurance of mixoploids can be attributed to gradations in mitotic activity within the meristem tissues (Francis, 1997) such that some cells are cell cycling faster than others and thus more susceptible to inhibition by oryzalin. However, longer oryzalin exposure would ensure that a greater percentage of plants are exposed during cell cycling, thus unless each meristem tissue is doubled, the resulting shoot will be chimeric, displaying both tetraploid and octoploid plants. Treatment of Marianna shoots with oryzalin confirmed the outcome of a previous study done by Levin (1983) that polyploidy plants results in improved mophological characteristics. It is evident (Fig. 3.3b), that the leaves of polyploid plants appeared to be larger and broader then control plants. However, althought it showed improved morphology,

higher oryzalin concentrations resulted in a higher mortality rate of plants. Our results also showed that oryzalin treatment significantly influenced the growth parameters of treated shoots in comparison to control (Fig. 3.4a-b). The treated plants showed an increase in both shoot length and fresh weight in comparison to control.

In this study the percentage of tetraploid plants where higher than mixoploid plants. Treatment of Marianna shoots with oryzalin concentration of 75 µM for 24 h was the lowest concentration to induce ploidy in shoots and it was also the only concentration to show survival for both 24 and 48 h. We, therefore suggest the use of a lower oryzalin concentration (between 50 and 75 µM) after 24 h exposure to induce chromosome doubling and reduce plant mortality. The current and future studies are aimed at shortening the breeding cycle of plants by means of inducing chromosome doubling, and allow for better comparison of interspecific and intergenetic crosses, which will allow for a wider genetic diversity of plants.

CHAPTER 4

IDENTIFICATION OF ORYZALIN-INDUCED DIFFERENTIALLY EXPRESSED PROTEINS IN SHOOTS

4.1 Introduction

Exposure of plants to various herbicides greatly influence their growth and development, that poses a significant threat to the agricultural sector. Plants respond to various environmetal stress conditions by producing several genes that will be differentially regulated to survive and surpass these unfavorable conditions (Tuteja *et al.,* 2008). Chemical stress induces oxidative stress reponsive genes as a consequence of reactive oxygen species (ROS) accumulation that are toxic to plant cells. To eliminate ROS, plants under stress will produce antioxidant enzymes such as superoxide dismutases, ascorbate peroxidases and catalases (Wang *et al.,* 2003). ROS accumultion also greatly influence protein expression in plants. Different proteins are activated when plants are exposed to environmental stress conditions and the level of expression can be identified using proteomic analysis. Proteomics allow the study of structural, functional, abundance, and interactions of proteins at a given time point (Ghosh and Xu, 2014) This highthroughput method involves the analysis of proteins expressed in a genome by identifying the associated amino acids and determine their relative abundance. Proteomics is associated with various approches that include structural, the study of the three dimensional structure of proteins within a genome; cell map which is the protein-protein interatction while expressional proteomics is the study of changes in protein expression under specific physiological conditions and response to various stresses (Blackstock and Weir, 1999). The expressional proteomics approach can be used to identify novel or disease specific proteins in various organisms and establish their locations (Graves *et al.,* 2002). Proteomic analyses have been widely used in various crops species which

includes strawberry (*Fragaria ananassa),* rice (*Oryza sativa. L)* and sorghum *(Sorghum bicolor. L)* (Hjerno *et al*., 2006; Parker *et al.,* 2006; Ngara *et al.,* 2012). Despite these reported advances of proteomics in fruit and food crops, there is very little evidence of proteomic analysis on Marianna plum rootstocks. These plum rootstocks are widely adapted to different soil types and moisture conditions and it offers a great potential as being used as parental material in breeding programmes and ploidy studies. The measurement of protein expression in ploidy studies would provide insight into cellular activities that take place under chemical treatment conditions. Therefore, the aim of this chapter was to identify differentially expressed proteins in Marianna plum shoots exposed to the antimitotic agent oryzalin using 2D-SDS PAGE and MALDI-TOF analysis.

4.2 Separation and visualisation of Marianna shoot samples on 1D-SDS PAGE

Total soluble protein was extracted (section 2.7.2) from Marianna shoots and quantified by separation on 1D-SDS PAGE to evaluate the quality and loading quantities prior to 2D-SDS PAGE. For each sample, 20 µg of protein sample was separated and visualised on a CBB stained polyacrylamide gel (Fig. 4.1). The protein samples for treatment and control shoots showed similarity in terms of protein expression, abundance and banding patterns which suggest that protein loading was uniform. It also showed that protein extracts (Fig. 4.1) covered the MW range of between 15 to 100 kDa. Results showed that the intensities of the two bands in the treated sample (indicated by red arrows) were significantly higher than those observed in the control sample. Although a higher intensity profile was observed in these bands, it could be attributed to more than one protein separating as a single band, or the result of pipetting errors. As such, the use of 1D-SDS PAGE could not reliably be used to identify differentially expressed proteins, therefore protein separation in the second dimension, could aid in protein identification as it would produce individual spots.

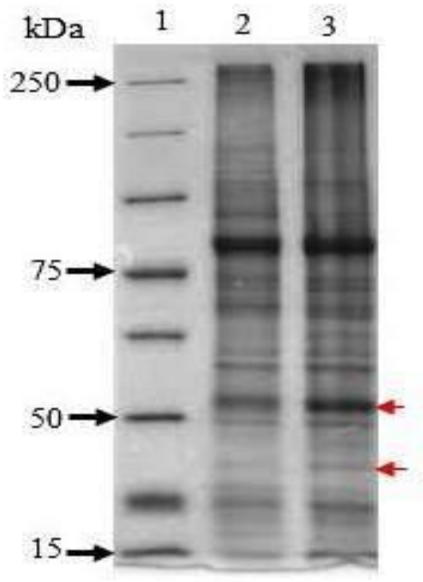

Figure 4.1 One dimensional gel electrophoresis of total soluble protein of Marianna shoots. Approximately 20 µg of phenol extracted proteins of shoots was separated on a 12% SDS-PAGE gel and stained with CBB. Lane 1 represents the molecular weight marker. Lane 2: control sample and Lane 3: oryzalin treated sample.

4.3 Proteomic analysis of Marianna shoots in response to oryzalin treatment

2D-SDS PAGE analysis was used for the detection and identification of proteins in Marianna shoot samples for control and treated plants. In 1D-SDS PAGE, proteins were separated based on their molecular weight whereas 2D-SDS PAGE resolves proteins according to their isoelectric point (p*I*) and molecular weight allowing for precise comparison between protein samples. Approximately 200 µg of shoot proteins (in triplicate) were resolved on 7 cm IPG strips of pH range 3-10 and separated on a 12% (v/v) 2D-SDS PAGE gels. The resolved proteins were stained with CBB, visualised and imaged as described in section (section 2.8). The identified protein spots from the 2D-SDS PAGE (control and treated samples) were quantified using the PDQuest™ advanced software and identified using MALDI-TOF MS/MS analysis (Fig. 4.2). The results in Fig. 4.2 shows the 2D-SDS PAGE protein profiles of Marianna shoots (control and treated) and the master gel with the selected proteins spots for mass spectrometry identification.

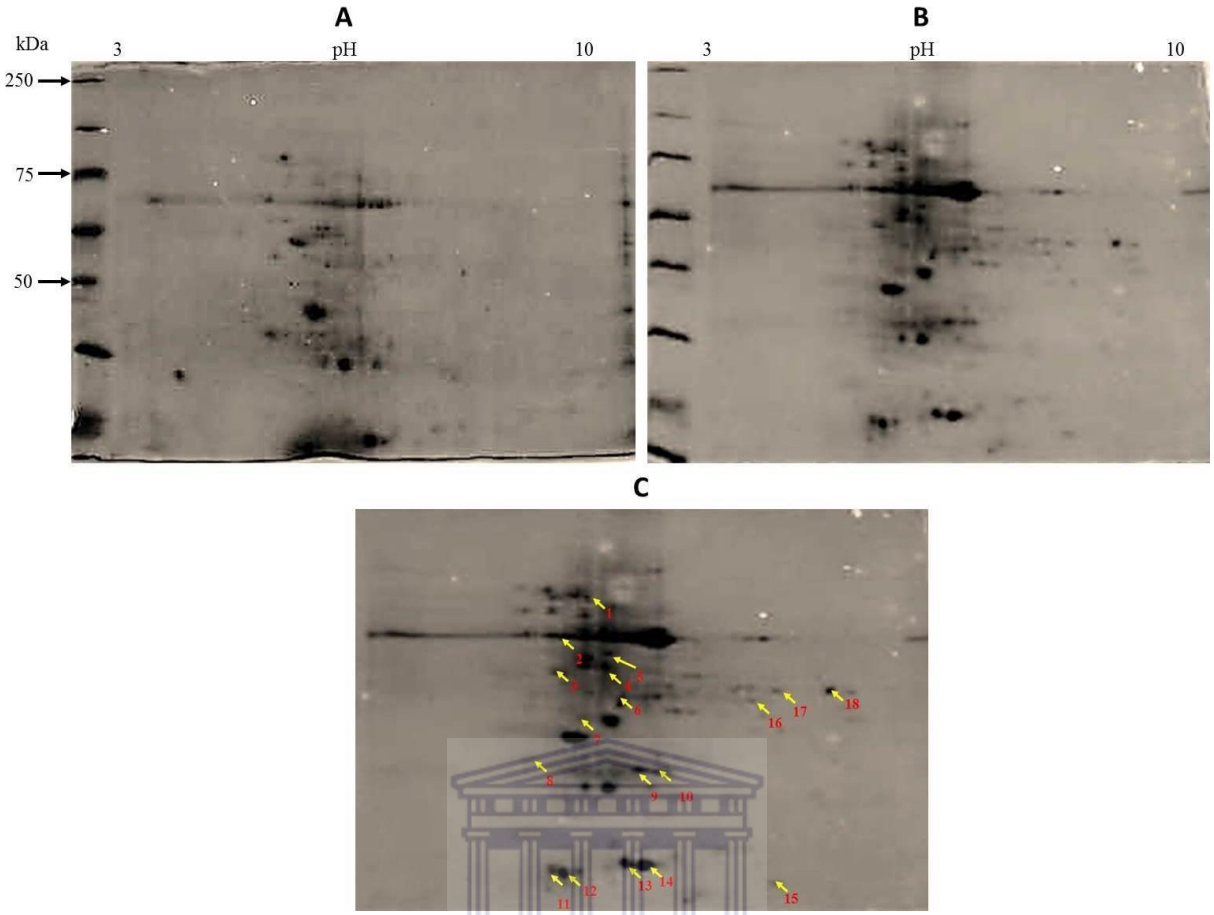

Figure 4.2 Proteome profiles of Marianna shoots in response to oryzalin treatment.
Phenol extraction of plant shoots of control and treated tissue. Protein extracts were separated in first dimension by IEF using pH range 3-10 on a 12% SDS PAGE. 200 µg of protein sample was loaded on each strip. Numbered spots (1-18) were selected for identification using MALDI-TOF MS analysis. **A.** Control, **B.** Treated and **C.** Master gel with selected spots.

The protein spots whose abundace changed with a ≥ 95 statistical confidence (student's t-test) were picked from CBB stained gels, trypsinised and analysed using MALDI-TOF MS analysis. Figure 4.3 show a zoomed representation of three differentialy expressed protein spots as determined by PDQuest™ software from well resolved and reproducible spots. Protein spots were either induced, un-induced or expressed following oryzalin treatment as stated in section 2.5.2. From the three representative spots two were significantly upregulated in response to oryzalin treatment (Fig. 4.3b-c), whereas the other one was completely downregulated (if present) in response to the treatment (Fig. 4.3a).

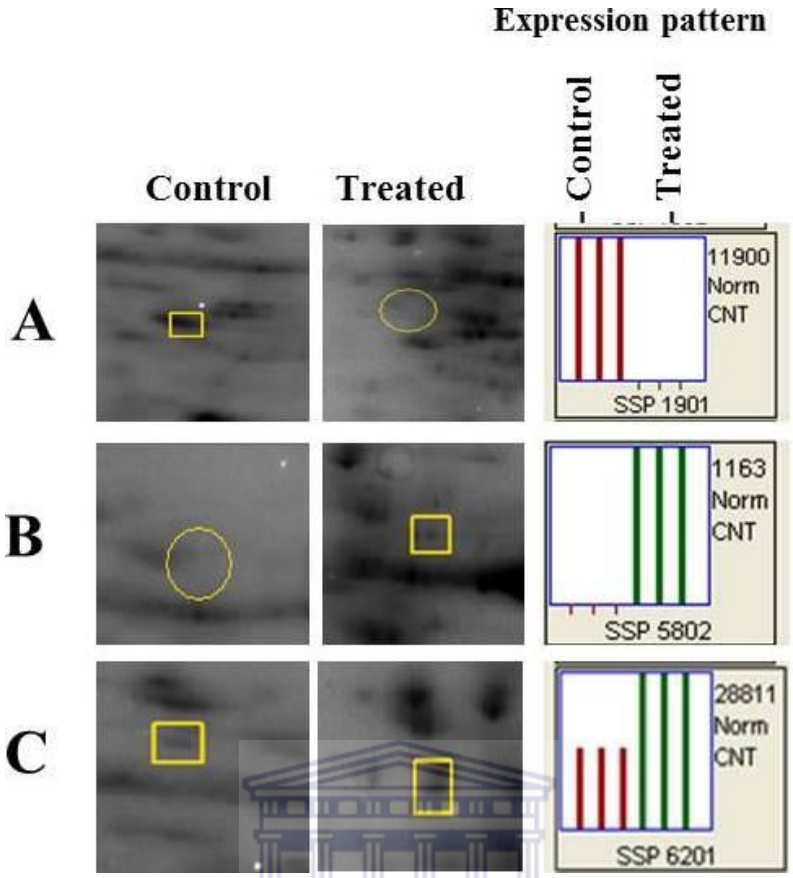

Figure 4.3 Zoomed in gel sections of representative spots showing differential expression patterns following oryzalin treatment.
Responsive spots were either; **A**. un-induced, **B**. induced or **C**. expressed following oryzalin treatment. The overall expression patterns of these representative spots amongst the three biological replicates used in the analysis are also shown in PDQuest™ software generated bar graphs.

4.4 Identification of proteins in Marianna shoots using MS/MS

Different expression of proteins between control and treated plants were assessed using PDQuest™ software. A total of 18 protein spots obtained from this analysis were selected for identification against the *Viridiplantae* (green plants) database using MALDI-TOF MS/MS analysis. Fourteen spots were positively identified and classified into different functional groups using bioinformatics analysis. These groups include stress responsive/detoxifying enzymes, cytoskeleton/amino acid, carbohydrate metabolism and energy related proteins (Fig. 4.4). The fourteen proteins identified are summarised in Table 4.1, which include the spot number, protein identities, plant species, matching peptides, MOWSE scores, expression, sequence coverage and theoretical MW/p*I* values.

Table 4.1 List of proteins identified by MALDI M/S and data base searching

Spot	Protein name and function	Plant species	Matching peptides	Score	Seq. Coverage %	Expression	Theoretical MW/pI
	Stress responsive/detoxifying proteins						
1b	Heat shock 70 kDA	*Solanum lycopersium*	11	526.35	23.6	Up	4.39
10	L-ascobic peroxidase	*Pisum sativum*	1	52.91	4.4	Down	5.44
11a	Major allergen	*Prunus ameniaca*	4	142.81	34.4	Up	4.72
	Carbohydrate metabolism						
5	Phosphoribulokinase	*Vitis sp*	1	30.84	13.5	Up	4.11
	Cytoskeleton/amino acid related						
3a	Actin	*Daucus sarola*	6	250.99	17.6	Down	5.35
4b	Glutamine synthetase	*Medicago sativa*	3	117.18	12.6	Up	6.31
	Energy						
2b	Chloropast envelop membrane	*Spinach oleracea*	10	522.04	24.2	Up	5.22
2b	Ribulose biosphosphate	*Nelumbo lutea*	3	90.58	8.6	Down	6.44
9c	Trisephosphate isomerase	*Hordeum vulgare*	4	193.05	19.4	Down	5.26
15	Nucleoside diphosphate kinase	*Spinacia oleracea*	1	32.32	7.8	Up	9.02
16	Malate dehydrogenase	*Psedotsuga menziesii*	2	121.89	100	Down	4.21
17c	Glyceraldehyde-3-phosphate	*Taxus baccata*	5	299.18	17.7	Up	6.46
18b	Fructose-biophosphate aldolase	*Spinach oleracea*	2	182.47	7.3	Up	5.92
	Unidentified protein						
11b	Unknown protein	*Psedotsuga menziesii*	1	40.59	100	Up	11

[a] Same protein in different spot. [b] Spots that produced multiple proteins. [c] Same protein in spot from different organism.

4.5 Protein distribution from functional categories

Positively identified proteins were classified into different functional categories. The majority of the Marianna shoot proteins were found to be related to energy proteins that showed a 50% occurrence (Fig. 4.4) and stress responsive/detoxifying proteins (21.4%). The high representations of these proteins represent their importance in contribution to protection of plants in stressful conditions. The lowest percentage of proteins identified in this study was associated with carbohydrate metabolism and unknown function which represent 7.1% identified proteins each (Fig. 4.4). The carbohydrate metabolism proteins play important roles in a plants development as well as the primary function of leaves.

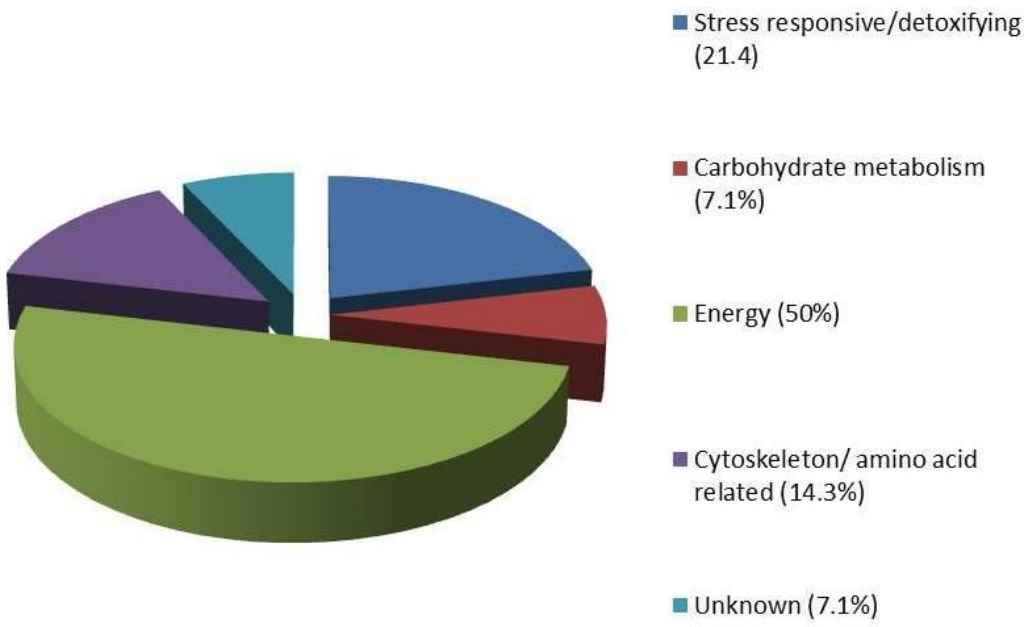

Figure 4.4 Functional characterization of Marianna shoot proteins in response to oryzalin treatment.
Numbers indicated in brackets represent the proteins within each functional category expressed as percentage of the 14 positively identified protein spots.

4.6 Discussion

The exposure of plant material to chemicals can influence the proteins being expressed in an organism. Proteins can either be up regulated or down regulated as a result. After treatment of Marianna shoots with oryzalin, proteins were resolved according to their molecular weights in 1D-SDS PAGE analysis. Following 1D-SDS PAGE analysis proteins were resolved in 2D-SDS PAGE according to their p*I* and molecular weight allowing for precise comparison between protein samples. The visualised proteins were then identified using mass spectrometry. From the CBB stained gels a total of 18 well resolved protein spots (Fig. 4.2c) were selected for mass spectrometry identification. The spots were excised from gels, trypsin digested and analysed using MALDI-TOF MS/MS analysis. Out of the 18 spots that were selected, 14 were positively identified. The unidentified protein spots (4) may possibly be the result of low quantities of proteins in the spots to produce a positive identification. MALDI-TOF analysis also produced an unknown protein in (spot 11); the identification of this protein

might be the result of unknown biological function of the protein to produce a positive identification. The unknown protein and malate dehydrogenase (spot 16) produced 100% sequence coverage in comparison to all other identified proteins spots, which produced sequence coverage of less than 40%. The sequence coverage represents the ratio of peptides from the protein spot of interest to match the sequence of proteins in the database (Perkin *et al*., 1999; Damodarana *et al*., 2007). A few protein spots (11-14) showed significant homology to a major allergen protein. The multiple spots for a single protein can be the result of several influences such as; dimeric and monomeric forms of proteins on the same gel, presence of proteolytic degradation by endogenous proteases, presence of different protein isoforms from multiple families as well as chemical modification of proteins during sample preparation (Albertin *et al.,* 2009). Some of the spots identified in this study were also shown to match different proteins. This was evident for spot 1 (Hsp70 and Chloroplast envelope) spot 4 (Actin and Gluthamine synthetase) and spot 18 (Fructose biophosphate aldolase and Glyceraldehyde-3-phosphate). Proteins were search against a large database of other green plants hence the different proteins from a single spot may be the result of all the proteomic data in the database. The positively identified proteins were subdivided and classified according to their biological functions into different categories as described by Ndimba *et al*. (2005). The stress related and detoxifying proteins (Hsp70 and Major allergen) which were up regulated in oryzalin treatment and were not present in the control sample, suggests that they were oryzalin responsive proteins. This phenomenon has previously been observed in a stress related study in rice plants that illustrate the abundance of significant proteins as a consequence of stress treatment only (Yan *et al.,* 2005). The proteins induced due to oryzalin treatment might possibly contribute towards a plants tolerance to chemical stress and plays an important role in a plants response to survive under stress conditions. From the classified protein spots, the majority of proteins were found to be related to energy proteins as can be

observed in (Fig. 4.5). The energy related proteins showed a 50% occurrence from all identified proteins. The importance of these proteins can be related to the ability of plants to drive photosynthesis by absorbing light energy to carry on metabolic activities in the plant cells. The other functional categories and proportion of proteins are stress responsive/detoxifying (21.4%), carbohydrate metabolism (7.1%) and cytoskeleton/amino acid related proteins (14.3%). These functional groups are putative until the functions of the proteins are determined experimentally. Future gene expression analysis (using quantitative real time PCR) is required to validate the expression profiles of these proteins identified.

CHAPTER 5

BIOCHEMICAL RESPONSES OF MARIANNA PLUM SHOOTS TO ORYZALIN TREATMENT

5.1 Introduction

The use of oryzalin as a mitotic inhibitor to induce polyploidy in plants have previous been described by Contreras *et al.* (2010) but no documented reports on its role in ROS accumulation currently exist. It is a well known fact that dinitroaniline herbicides like many other pollutants initiate the development of ROS in the cellular structures (Wu and von Tiedemann, 2002; Peixoto *et al.,* 2006; Song *et al.,* 2006). ROS such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydrogen radical (.OH) and hydrogen peroxide (H_2O_2) pose a severe threat to plant growth and development when produced in large amounts. In order to counter the deleterious effects caused by the over production of ROS, plants activate their endogenous defense mechanisms. These defense mechanisms include enzymatic antioxidants such superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Jiang *et al*., 2010). These ROS molecules are difficult to detect after their production (Dorta *et al.,* 2003; Radetski *et al.,* 2004); normally they are inferred from variations in the antioxidant activity (enzymatic and non-enzymatic) (Valavanidis *et al.,* 2006). The antioxidant defense can be regarded as biomarkers and the changes in their activities result in increased thiobarbituric acid reactive substances (TBARS) as a consequence of lipid peroxidation (Pang *et al.,* 2001; Wu and von Tiedemann, 2002).

This work aims to investigate the influence of oryzalin treatment on photosynthetic pigments, ROS accumulation and changes in antioxidant enzyme activties in Marianna shoots.

5.2 Oryzalin treatment influence photosynthetic pigments in Marianna shoots

Photosynthesis in plants is dependent on capturing light energy in the pigment chlorophyll, particularly chlorophyll a (Chl-a) and chlorophyll b (Chl-b). In this study, Marianna plants were grown and treated as described in section 2.5.2. The results showed that the total chlorophyll content were significantly increased in response to the antimitotic agent when compared to control plants (Fig. 5.1). The chlorophyll content in plants exposed to oryzalin showed a 20% increase in the total chlorophyll content compared to the control. A similar trend was observed when comparing changes in Chl-a in control and treated plants. However, no significant changes in Chl-b was observed between the control and treated plants.

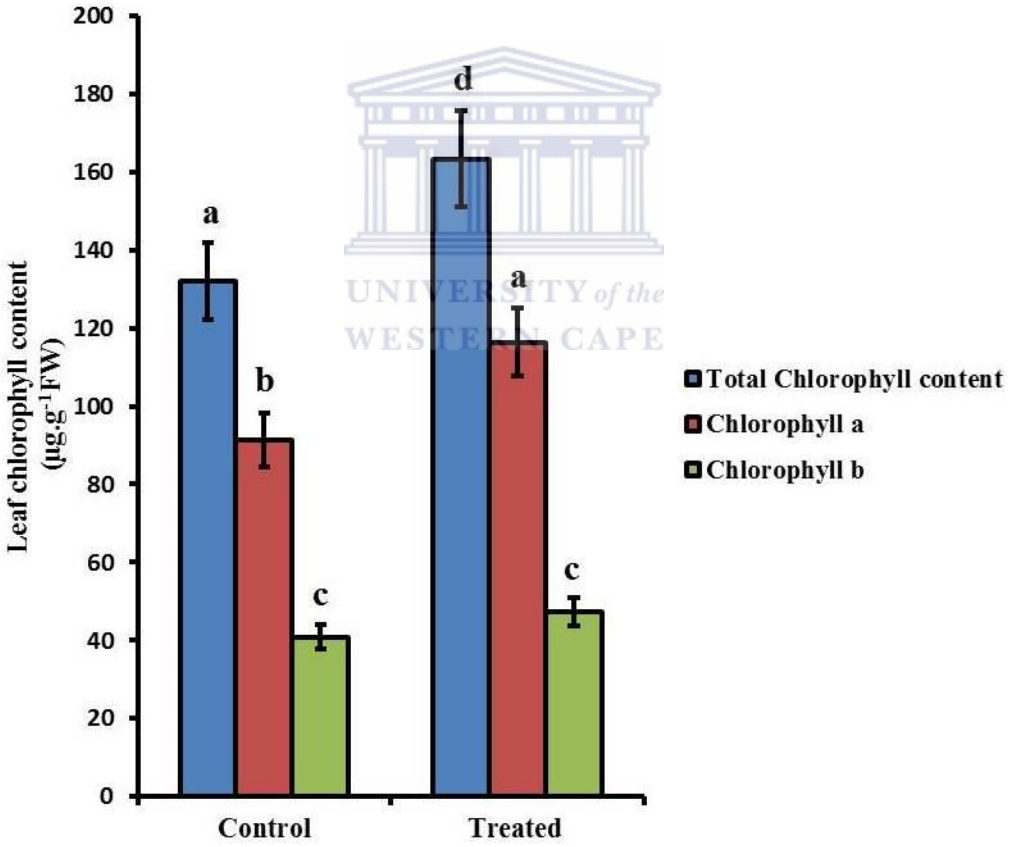

Figure 5.1 Oryzalin influence photosynthetic pigments in Marianna shoots.
Chlorophyll content measured in shoots of control and treated plants treated with 75 µM of oryzalin for 24 h. Error bars represents the mean (±SE) of the independent experiments from 10 plants per treatment. Means with different letters are significantly different from each other ($p < 0.05$).

5.3 Oryzalin influences H_2O_2 content and the extent of lipid peroxidation

Hydrogen peroxide content and the extent of lipid peroxidation (depicted as MDA levels) were measured as described in section 2.10.3 and 2.10.4 respectively. H_2O_2 content was significantly reduced by ± 133% in response to treatment with 75 µM oryzalin (Fig. 5.2a). On the otherhand, contrary to what was observed for H_2O_2 content, MDA levels were significantly increased by ± 53% in response to oryzalin treatment (Fig. 5.2b).

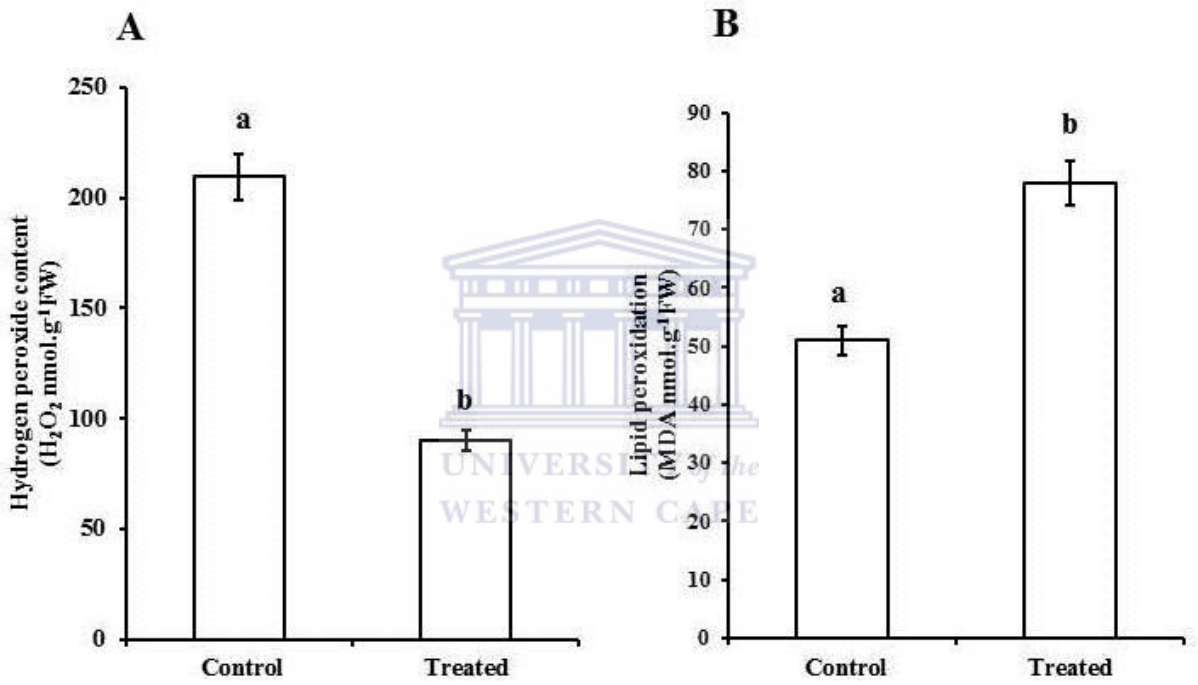

Figure 5.2 Oryzalin differentially regulate oxidative stress in Marianna shoots.
A. H_2O_2 content and **B**.Lipid peroxidation activity measured as (MDA). Error bars represents the mean (±SE) of the independent experiments from 10 shoots per treatment. Means with different letters are significantly different from each other ($p < 0.05$).

5.4 Antioxidant enzyme activity altared by oryzalin treatment

Plants seem to activate a complex antioxidative defense system in response to environmental changes that is displayed via the increase or decrease of enzymatic activities.

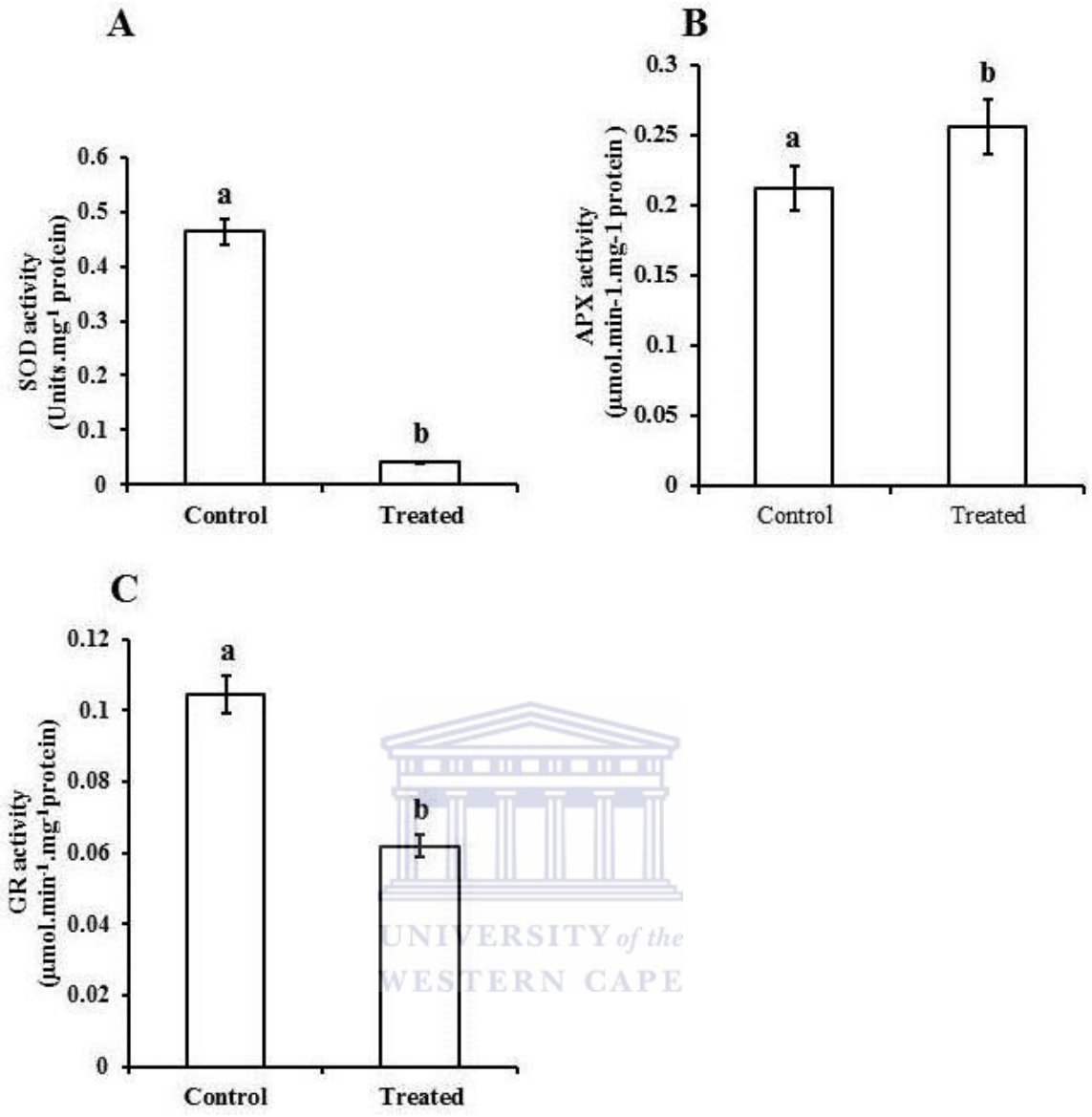

Figure 5.3 Marianna shoot antioxidant enzyme activities are influenced by oryzalin treatment. Plants were exposed to 75 µM oryzalin for 24 h in the dark. **A** SOD, **B** APX and **C** GR activity of shoots. Error bars represents the mean (±SE) of the independent experiments from 10 shoots per treatment. Means with different letters are significantly different from each other ($p < 0.05$).

The enzymatic activities of SOD, APX and GR of Marianna shoots were measured as described in section 2.10.5. The result showed that SOD and GR activity was significantly reduced in response to oryzalin treatment, whereas APX activity was upregulated. The decrease in SOD activity was approximately 91% whereas the reduction in GR activity was approximately 40% (Fig. 5.3a,c). The significant decrease in SOD activity in response to oryzalin indicate that oryzalin affect SOD activity and would thus result in a decreased

scavenging capacity of O_2^-. Similarly, the reduction in GR activity as a consequence of oryzalin treatment would therefore result in a reduced ability to metabolize H_2O_2 thus negatively influencing redox homeostasis and allow for increased lipid peroxidation. The increase in APX activity observed in response to oryzalin treatment suggest that oryzalin would increase the H_2O_2 scavenging capacity of APX

5.5 Discussion

The increase exposure of plants to unfavourable conditions will result in the increase accumulation of ROS. SOD is the first enzyme in the defence against ROS attack because it rapidly scavenges O_2^- to oxygen and H_2O_2. SODs are required at all times to provide protection in an organism. It is conceivable that high levels of oxidative stress may result in high SOD protein turnover, resulting in the requirement for new SOD enzyme synthesis to maintain SOD levels sufficient for effective protection (Scandalios, 1993). APX and GR are essential enzymes in the removal of H_2O_2 in order to maintain the redox state of ascorbate and glutathione (Foyer *et al.*, 1994). A significant decrease in SOD and GR activity in Marianna shoots was observed in response to oryzalin treatment (Fig 5.3.a, c), with a slight but significant increase observed for APX activity in the same treatment. These enzymes are important to regulated oxidative damage and maintain redox homeostasis. This reduction in SOD activity could be attributed to low O_2^- production in response to oryzalin treatment and thus not sufficient to activate stress responsive enzymes like SOD. This in turn would result in the low production of H_2O_2 (as a consequence of O_2^- scavenging) relative to the control as observed in Fig. 5.2a, coupled with an increase in APX activity. The increase in MDA content, an indicator of lipid peroxidation observed in Fig. 5.2b as a consequence of oryzalin treatment is in support of other findings that suggests that a reduction in GR activity (Fig.

5.3c) would increase the extent of lipid peroxidation (Fig. 5.2b) Our results suggest that antioxidant enzymes play a significant role and detoxifying ROS and limit the extent of lipid peroxidation. It has been suggested that enhanced ROS production would trigger defence enzymes to enable the plant to adapt to the ever changing environment (Esfandiari *et al.*, 2007). Plants can use the level steady state to monitor intracellular level of stress. The steady state must be regulated in order to prevent an oxidative burst by over accumulation of ROS, which could result in cellular damage (Carvalho, 2008). The result in Fig 5.1, supports the hypothesis that antioxidant enzymes modulates plants' tolerance to environmental changes as depicted by the significant increase in photosynthetic pigments of Marianna shoots in response to oryzalin treatment. We can therefore conclude that antioxidant enzymes play an important role in regulating plants' tolerance to various environmental conditions.

CHAPTER 6

GENERAL DISCUSSION

The main aim of the study was to develop an *in vitro* method for chromosome doubling for Marianna plum shoots and to determine the proteomics and biochemical responses to the antimitotic agent, oryzalin. The use of Marianna rootstocks was motivated by several factors which include the plants' tolerance to different soil types and moisture conditions and its moderate resistance to crown gall and crown rot diseases (Southwick *et al*., 1999). To our knowledge the proteomic and biochemical responses of Marianna plants to oryzalin have never been investigated, thus making this study a first of its kind. The results generated from this study would provide insight to how exposure to oryzalin will influence proteome turnover and antioxidant capacity of Marianna shoots. These results can also be used as a reference tool by researchers focusing on other stone fruit crops.

6.1 Development of plum chromosome doubling method

Marianna shoots were exposed to different oryzalin concentration (0, 50, 75, 100, 150 or 200 µM) for two time periods (24 or 48 h) to determine which concentration-incubation period combination would induce chromosome doubling. It was established using flow cytometry analysis that apart from 0 and 50 µM, chromosome doubling was observed in all other oryzalin concentrations after 24 h exposure. Flow cytometry analysis was used to establish chromosome doubling based on the following reasons: (i) this system has the potential to count a number of nuclei at a time; (ii) it is a rapid and reliable method for determining ploidy; (iii) it can also be used to determine the nuclear DNA content of an unknown sample by comparing with DNA of known ploidy. The advantage of using oryzalin in chromosome doubling is its simplicity and affinity to plant tubulin; however one limitation is its high frequency of producing chimeric plants (Chauvin *et al*., 2003). This method established for

chromosome doubling can be used as a reference in chromosome doubling techniques in breeding programmes for interspecific and intergenetic crosses.

6.2 Proteome analysis of Marianna shoots

Following chromosome doubling of Marianna shoots, proteomics analysis was used to identify differently expressed proteins in response to oryzalin. A total of 18 differently expressed proteins that were either up or down regulated following treatment of shoots were detected using 2D-SDS PAGE analysis. From the 18 differentially expressed proteins detected, 14 were identified using mass spectrometry analysis and characterised into different functional categories including stress responsive/detoxifying, energy, cytoskeleton/amino acid and carbohydrate related proteins based on bioinformatics analysis.

Stress responsive/detoxifying proteins

Four stress responsive and detoxifying proteins were identified in treated shoots, which include heat shock protein (Hsp70). The Hsp70 proteins were up regulated in response to oryzalin treatment. These proteins are involved in protein folding by binding to the polypeptide chains of other proteins. Hsp70 also act as molecular chaperones as their binding to other proteins to protect them from denaturing and degrading (Mulaudzi *at el.,* 2015). Hsp70s function in response to various stresses, this was evident in oryzalin treatment of Marianna shoots. Other proteins that were also classified into this category include L-ascorbate peroxidase (spot 10). This protein plays an important role in protecting plants against oxidative stress. Ascorbate peroxidase functions by utilizing the reducing power of ascorbic acid to eliminate harmful H_2O_2 in chloroplasts (Shigeoka *et al.,* 2002). Major allergen identified in spots (11, 12, 13 and 14) has pathogenic properties and is induced when plants are exposed to biotic stresses; this was evident in this study as this protein was up regulated in response to oryzalin treatment of shoots.

Energy proteins

Ribulose bisphosphate carboxylate (RuBisCO), is the most abandant protein on earth, this protein is responsible for the conversion of inorganic carbon, as carbon dioxde (CO_2) into organic compounds like phosphoglycerate (Kellogg and Juliana, 1997). The energy associated proteins are impotant in transformation of macromolecules and carbohydrates to provide energy for cellular processe required in plants. A number of protein spots were found to be associated with the energy related proteins, thus includes spots (1, 2, 5, 9, 15, 16, 17 and 18).

Cytoskeleton/amino acid biosynthesis

Cytoskeleton within the host cell prevents entry of pathogens to protect the plant. One of the proteins identified in this catergory include actin (spot 3), The proteins in this catergory function as the structural makeup for growth and development in cells. It is essential in cellular processes, such as cell division, cell motility and signal transduction (Hussey *et al.*, 2002). Glutamine synthetase (spot 4) was also identified in this category This protein plays an essential role as an enzyme for cellular nitrogen metabolism.

6.3 Biochemical responses of Marianna shoots to oryzalin

Various biochemical responses have been investigated in this study. These include the, photosynthetic pigmentation, ROS acccumulation and antioxidant capacity. The results showed that oryzalin treatment differentially regulated all biochemical parameters measured in this study. Total chlorophyll content was significantly increased and this was evident by the increase in Chl-a pigments, although no significant changes was observed for Chl-b. A similar result was observed for wheat cultivar in a study conducted by Nikolaeva, (2010). Oxidative stress markers such as H_2O_2 content and MDA levels showed opposite behaviour and this could be ascribed to the variation in antioxidant enzyme activity observed in Fig. 5.3.

Our results showed an increase in APX activity (Fig. 5.3b) in response to oryzalin treatment. However, this occurrence was not observed for SOD and GR activity (Fig. 5.3a,c). APX and GR are required to scavenge H_2O_2 produced mainly in the chloroplast and other cell organelles to maintain the redox state of the cell. APX utilizers the reducing power of ascorbic acid to eliminate harmful H_2O_2. Peroxidases are widely accepted as the 'stress enzyme'. Induction of peroxidase activity has been studied under a number of stressfull conditions including chilling, water and salinity (Prasad *et al.,* 1995). The increase in lipid peroxidation is directly linked to the reduction in GR activity. A reduction in GR activity coupled with an increase in lipid peroxidation has previously been described by Esfandiari (2007).

6.4 Conclusion and future prospects

The aim of this study was to develop an *in vitro* chromosome doubling method for Marianna plum shoots using the antimitotic agent, oryzalin. Furthermore, the study also explored the proteomic and biochemical responses of Marianna shoots in response to oryzalin. We hypothesized that the induction of chromosome doubling in Marianna plum shoots would mediate physiological, proteomic and biochemical responses.

In this study, it was established using flow cytometry analysis that 75 µM was lowest possible concentration of oryzalin that induced chromosome doubling in Marianna shoots after 24 h exposure. The other concentrations (0 and 50 µM) did not induce chromosome doubling (Fig. 3.2a-b) and/or the survival rate for these concentrations (100 – 200 µM) was too low (Fig. 3.1). Although a chromosome doubling method was established at 75 µM oryzalin after 24 h, the survival rate was 40% lower compared to the control (0 µM) and 20% lower compared to the 50 µM treatment. In order to reduce the mortality rate and still induce chromosome doubling of Marianna shoots an oryzalin concentration between 50-75 µM

should be tested. Additional research is required to determine the stability of these putative polyploidy plants. The application of this method will be useful for accelerating plant breeding and development of a greater genetic diversity amongst stone fruit species.

The identification of differentially expressed proteins in response to oryzalin could be used as biomarkers in future breeding programs. However, the expression levels of these proteins needs to be validated using quantitative real time PCR.

Furthermore, the antioxidant capacity of Marianna shoots were significantly influence in response to oryzalin treatment that in-turn altered ROS scavenging. These changes in enzymatic activities were shown to be important for the regulation of oxidative damage. However, it is imperative to identify individual isoforms of these antioxidant enzymes to establish their contribution to total enzymatic activity. This will allow for the identification of potential candidates that can be used in future breeding programs to establish cultivars with improved characteristics suitable for the export market.

This study in itself will contribute significantly to fruit tree breeding programs and will lay a proper foundation for the establishment of an improved chromosome doubling system in various stone fruits using herbicides such as oryzalin.

BIBLIOGRAPHY

1. Aebersold R and Mann M. 2003. Mass spectrometry based proteomics. *Nature.* 422:198-207.
2. Ahmad P, Jalle C.A, Salem M.A, Nabi G and Sharma S. 2010. Reactive oxygen species, antioxidant and signaling in plants. *Journal of Plant Biology*. 51:167-173.
3. Albertin W, Langella O, Joets J, Negroni L, Zivy M, Damerval C and Thiellement H. 2009. Comparative proteomics of leaf, stem, and root tissues of synthetic *Brassica napus. Proteomics.* 9:793-799.
4. Ali A.A and Alqurainy F. 2006. Activities of antioxidants in plants under environmental stress. In: N. Motohashi (Eds.). The Lutein-Prevention and Treatment for Diseases. pp. 187-256. Transworld Research Network, India.
5. An F, Fan J, Li QX, Li K, Zhu W, Wen F, Cavalho L.J.C and Chen S. 2014. Comparison of leaf proteomes of Cassava (*Manihot esculenta* Crantz) Cultivar NZ199 Diploid and autotetraploid genotypes. *PLos One*. 9:1-13.
6. Arimendi M.J, Almada R, Pimentel P, Bastias A, Salvatierra A, Rojas P, Hinrichsen P, Pinto M, Genove A.D, Travisany D Maass A and Sagredo B. 2015. Transcriptome sequencing of *Prunus* sp. Rootstocks to identify candidate genes involved in response to root hypoxia. *Tree Genetics and Genomes.* 11:11-16.
7. Arnon D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. *Plant Physiology*. 24:1-15.
8. Asada K. 1984. Chloroplasts: formation of active oxygen and its scavenging. *Methods in Enzymology.* 422-429.
9. Ashraf M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidants markers. *Biotechnology Advances.*27: 84-93.

10. Atland J.E, Gilliam C.H and Wehtje G. 2003. Weed control in field nurseries. *HortTechnology*. 13:9-14.
11. Bakke A.C. 2001. The principals of flow cytometry. *Laboratory Medicine*. 32:207-131.
12. Blackstock W.P and Weir M.P. 1999. Proteomics: quantitative and physical mapping of cellular proteins. *Biotechnology*. 17:121-126.
13. Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 7291:248-254.
14. Buege J.A and Aust S.D. 1978. The thiobarbituric acid assay. *Methods Enzymol*. 52:306-307.
15. Carvalho M.H.C. 2008. Drought stress and reactive oxygen species. *Plant Signal and Behaviour*. 3:156-165.
16. Chahal G.S. and Gosal S.S. 2002. Principles and Procedures of Plant Breeding: Biotechnology and Conventional Approaches. pp. 604. Alpha Science, Pangbourne Harrow, United Kingdom.
17. Chauvin J.E, Souchet C, Dantec J.P and Ellisséche D. 2003. Chromosome doubling of 2x *Solanum* species by oryzalin: method development and comparison with spontaneous chromosome doubling *in vitro Plant Cell, Tissue and Organ Culture*. 73:65-73.
18. Chen T.H and Murata N. 2002. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Current Opinion in Plant Biology*. 5:250-257.
19. Conner A.J and Jacobs J.M.E. 1999. Genetic engineering of crops as potential source of genetic hazard in human diet. *Mutation Research*. 443:223-234.
20. Contreras R.N, Ranney T.G and Tallury S.P. 2007. Reproductive behaviour of Diploid and allotetraploid *Rhododendron* L.'Fragrant Affinity'. *HortScience*. 42:31-34.

21. Contreras R.N, Ruter J.M and Hanna W.W. 2010. An oryzalin-induced autoallooctoploid of *Hibiscus acetosella* Welw. ex Hiern. 'Pan-ama Red' (Malvaceae). *Journal of American Society for Horticultural Science*. 134:553-559.

22. Cox C. 2001. Oryzalin. *Journal of Pesticides*. 21:16-20.

23. Dai W, Magnusson V and Johnson C. 2007. *Agrobactrium* - Mediated transformation of Chokechrry (*Prunus virginiana*. L). *HortScience.* 42:140-142.

24. Damodarana S, Wood T.D, Nagarajanc P and Rabina R.A. 2007. Evaluating peptide mass Fingerprinting-based protein identification. *Genomics, Proteomics and Bioinformatics*. 5:152-157.

25. Doubled haploid wheat breeding. 2013. Available from: <http://www.coloradowheat.org>. [5 November 2013].

26. Dolezel J and Bartons J. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany*. 95:99-110.

27. Dorta, D.J, Leite, S, De Marco K.C, Prado I.M.R. and Rodrigues T. 2003. A proposed sequence of events for cadmium-induced mitochondrial impairment. *Journal of Inorganic Biochemistry*. 97:251-257.

28. Dubey H and Grover A. 2001. Current initiatives in proteomics research: The plant perspective. *Current Science.* 80:262-269.

29. Edwards E.A, Rawsthrne S and Mullineaux P.M. 1990. Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum*). *Planta.* 80:278-284.

30. Esfandiari E, Shekari F and Esfandiari M. 2007. The effect of salt stress on antioxidant enzymes activities and lipid peroxidation on the wheat seedling. *Notulae Botanicae Horti Agrobotanici.* 35:48-56.

31. Esterbauer H and Grill D. 1978. Seasonal variation of glutathione and glutathione reductasein needles of *Picea abies*. *Plant Physiology*. 61:119-121.
32. Foyer C.H and Mullineaux P. 1994. (Eds.). Causes of photooxidative stress and amelioration of defence systems in plants. Boca Raton, USA, CRC Press Inc.
33. Francis D. 1997. The stem cell concept applied to shoot meristems of higher plants. (Eds.). Stem cells. pp. 59-73. Academic press, San Diego.
34. Futcher B, Latter G.I. Monardo P, McLaughlin C.S and Garreles J.I. 1999. A sampling of the yeast proteome. *Molecular and Cell Biology*. 19:7357-7368.
35. Ghosh D and Xu J. 2014. Abiotic stress response in plant roots: a proteomics perspective. *Fronties in Plant Science*. 5:1-13.
36. Gill S.S and Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crops. *Plant Physiology and Biochemistry*. 48:909-930.
37. Gorg A and Weiss W. 2004. Protein profile comparison of microorganisms, cells and tissue using 2D gels. In D. W. Speicher (Eds.). Proteome analysis: interpreting the genome. Pp. 19-73. New York, Elsevier.
38. Goyal S and Khan S. 2009. A comparative study of chromosome aberrations in V*igna mungo* induced by ethylmethane sulphonate and hydrazine hydrated. *Thailand Journal of Agricultural Science.* 42:117-182.
39. Gratão P.L. Polle A and Lea P.J. 2005. Making the life of heavy metal-stressed plants a little easier. *Functional Plant Biology.* 32:481-494.
40. Graves P.R and Haystead T.A.J. 2002. Molecular biologist guide to proteomics. *American Society of Molecular Microbiology*. 66:39-63.
41. Hames B.D and Rickwood D. 1990. Gel electrophoresis of proteins: a practical approach. (2nd edition). New York: Oxford University Press. pp. 383-488.
42. Hancock J.F. 1997. The colchicine story. *HortScience.* 32:1011-1012.

43. Hardly H.H and Openshaw S.J. 1980. Interspecific and intergeneric hybridization.W.R. Fehr and H.H (Eds.). The American Society of Agronomy. pp. 131-142. The crop science of America, Inc.

44. Hiscox J.D and Israelstam G.F. 1980. Erratum: A method for the extraction of chlorophyll from leaf tissue without maceration. *Canadian Journal of Botany*. 58:403-407.

45. Hjerno K, Alm R, Canbäck B, Matthiesen R, Trajkovski K and Björk L. 2006. Down-regulation of the strawberry Bet v 1-homologous allergen in concert with the flavonoid biosynthesis pathway in colourless strawberry mutant. *Proteomics.* 6:1574-1587.

46. Hood B.L, Veenstra T.D and Conrads T.P. 2004. Mass spectrometry based proteomics. *International Congress Series.* 126:375-380.

47. Hossain M.A, Piyatida P, Da Silva T.J and Fujita M. 2011. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. *Journal of Botany*. 11:1-37.

48. Hsu Y.T and Kao K. 2007. Heat shock mediated H_2O_2 accumulation and protection against Cd toxicity in rice seedling. *Plant Soil*. 300:173-147.

49. Hussey P.J, Allwood E.G. and Smertenko A.P. 2002. Actin-binding proteins in the *Arabidopsis* genome database: properties of functionally distinct plant actin depolymerizing factors/cofilins. *Philosophical Transactions of the Royal Society of the London Series B-Biological Sciences.* 357:791-798.

50. Jannick J, Cummings J.N, Brown S.K and Hemmat M. 1996. *Apples In: Fruit Breeding. Tree and Tropical Fruits*, Vol 1*:* John Wiley and Sons. New York.

51. Jiang N, Luo X, Zeng J, Yang Z.R, Zheng L.N and Wang S.T. 2010. Lead toxicity induced growth and antioxidant response in *Luffa cylindriaca* seedlings. *International Journal of Agricultural Biology.* 12:205-210.

52. Kehr A.E. 1996. Polyploids in rhodododron breeding. *Journal of American Rhododedron Society.* 50:215-217.

53. Kellogg E. and Juliano N. 1997. The structure and function of RuBisCO and their implications for systematic studies. *American Journal of Botany.* 84:413-420.

54. Knauss J.F. 1976. A tissue culture method of producing *Dieffenbachia* Picta cv perfection free of fungi and bacteria. *Proceedings of the Florida State Horticultural Society.* 89:293-296.

55. Levin D.A. 1983. Polyploidy and novelty in flowering plants. *American Nature Journal*. 122:1-25.

56. Lu C and Bridgen M.P. 1997. Chromosome doubling and fertility study of *Alstroemeria aurea* x *A. caryoplyllae. Euphytica.* 94:75-81.

57. Mann M, Hendrickson R.C and Pandey A. 2001. Analysis of proteins and proteomes by mass spectrometry. *The Annual review of Biochemistry.* 70:437-473.

58. McCormack A.L, Schieltz D.M, Goode B and Yang S. 1997. Direct analysis and identification of proteins in mixtures by LC-MS/MS and database searching at the low-femtomole level. *Analytical Chemistry.* 69:767-776.

59. Molad Y. 2002. Update on Colchicine and its mechanism of action. *Current Rheumatology Reports.* 4:252-256.

60. Monteoliva L and Albar J.P. 2004. Differential proteomics: An overview of gel and non-gel based approaches. *Brief Functional Genomics Proteomics.* 3:220-239.

61. Mulaudzi-Musuku T, Mutepe R.D, Mukhoro O.C, Faro A and Ndimba B. 2015. Identification and characterization of heat-inducible Hsp70 gene from *Sorghum bicolor* which confers tolerance to thermal stress. *Cell Stress Chaperones*. 20:793-804.

62. Murashige T and Skoog F. 1962. A revised Medium for rapid growth and bio assays with tobacco cultures. *Physiologia Plantarum*. 15:473-497.

63. Nagle A, Hur W and Gray N.S. 2006. Antimitotic agents of natural origin. *Current Drug Targets*. 7:305-326.

64. Ndimba B.K., Chivasa S, Simon W.J and Slabas A.R. 2005. Identification of *Arabidopsis* salt and osmotic stress responsive proteins using two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics.* 5:4185-4196.

65. Ndimba B.K and Thomas L.A. 2008. Proteomics in South Africa: Current status, challenges and prospects. *Biotechnology Journal.* 3:1368-1374.

66. Ngara R, Ndimba R, Borch-Jensen J, Jensen O.N and Ndimba B. 2012. Identification and profiling of salinity stress-responsive proteins in *Sorghum bicolor* seedlings. *Journal of Proteomics.* 75:4139-4150.

67. Nikolaeva M.K, Maevskaya S.N, Shugaev A.G and Bukhov N.G. 2010. Cold-induced changes of enzymes, proline, carbohydrates, and chlorophyll in wheat. *Russian Journal of Plant Physiology.* 57:91-102.

68. Pandey A and Mann M. 2000. Proteomics study genes and genomes. *Nature.* 405:837-846.

69. Pang X, Wang D and Peng H.A. 2001. Effect of lead stress on the activity of antioxidant enzymes in wheat seedling. *Environmental Science and Technology*. 22: 108-111.

70. Parker R, Flowers T.J, Moore A.L and Harpham N.V. 2006. An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. *Journal of Experimental Botany*. 57:1109-1118.

71. Patterson S.D and Aebersold R.H. 2003. Proteomics: the first decade and beyond. *Nature Genetics.* 33:311-323.

72. Perkins D.N, Pappin D.J. Creasy D.M and Cottrell J.S. 1999. Probability-based protein identification by searching sequence database using mass spectrometry data. *Electrophoresis*. 20:3551-3567.

73. Quesenberry K.H, Dampier J.M, Lee Y.Y. Smith R.L and Acuna C.A. 2010. Doubling of the chromosome number of bahiagrass via tissue culture. *Euphytica.* 175:43-50.

74. Radetski C.M, Ferrari B, Cotelle S, Masfaraud J.F and Ferrard J.F. 2004. Evaluation of the genotoxic, mutagenic and oxidant stress potentials of municipal solid waste incinerator bottom ash leachates. *Science of the Total Environment.* 333:209-216.

75. Ramsey J and Schemske D.W. 1998. Pathways, mechanism, and rates of polyploidy formation in flowering plants. *Annual Review of Ecology and Systematics.* 29:467-501.

76. Ramulu K.S, Verhoeven H.A and Dijkhuis. 1991. Mitotic blocking, micronucleiation, and chromosome doubling by oryzalin amiprophos-methyl, and colochicine in potato. *Protoplasma*. 160:65-71.

77. Ranney T.G. 2006. Polyploidy: from evolution to new plant development. Combined Proc. *International Plant Propagators Society*. 56:137-142.

78. Rausch T and Wachter A. 2005. Sulfur metabolism: a versatile platform for launching defence operation. *Trends Plant Science.* 10:503-209.

79. Scandalio J.G. 1993. Oxygen stress and superoxide dismutase. *Plant Physiol*ogy. 101:7-12.

80. Shapiro H.M. 2003. Practical flow cytometry. Wiley-Liss, New Jersey.

81. Sharma P and Dubey R.S. 2007. 'Involvement of oxidative stress and roll of antioxidative defence system in growing rice seedlings exposed to toxic concentrations of aluminium'. *Plant Cell Reports.* 11:2027-2038.

82. Sharma P, Jha A.B, Dubey R.S and Pessarakli M. 2012. Reactive Oxygen Species, Oxidative damage, and antioxidantive defense mechanism in plants under stressful conditions. *Review Article.* 21:1-27.

83. Shi-Hai X, Xin-Bo G, Quan W, Qi-Fang P, Yue-Shen T, Pin L, Jing-Ya Z, Guo-Feng W and Ke-xuan T. 2011. Induction and flow cytometry identification of teraploids from seed derived explants through colchicine treatments in *Catharanthus roseus* (L.) G. Don. *Journal of Biomedical and Biotechnology*. 10:1-12.

84. Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y and Yoshimura K. 2002. Regulation and function of ascorbic peroxidase isoenzymes. *Journal of Experimental Botany.* 53:1305-1319.

85. Soltis D.E. 2003. The role of phylogenetic in comparative genetics. *Plant Physiology.* 132:1790-1800.

86. Southwick S.M, Yeager J.T, Osgood J, Norton M and Buchner R. 1999. Performance of new Marianna rootstocks in California for 'French' Prune. *HortTechnology*. 9:498-505.

87. Smirnoff N. 1993. The role of active oxygen in response of plants to water deficit and desiccation. *New Physiology*. 125:27-58.

88. Soltis D.E. Soltis P.S, Pires J.C, Kovarik A and Tate J.A. 2004. Recent and recurrent polyploidy in *Tragopogon* (Asteraceae). Cytogenic, genomic, and genetic comparison. *Biological Journal of the Linean Societry* (In press).

89. Thurston G, Regan S, Rampitsch C and Xing T. 2005. Proteomics and phosphoproteomics approaches to understand plant pathogen interactions. *Physiological and Molecular Plant Pathology*. 66:3-11.

90. Tuteja N and Sopory S.K. 2008. Chemical signalling under abiotic stress environment in plants. Plant Signalling and Behaviour. 3:525-536.

91. Uriu K. 1981. Soil and plant analysis and symptomology for diagnosis of mineral deficiencies and toxicities. (Eds.). Prune orchard management. Califonia Publication.
92. Valavanidis A, Vlahogianni T, Dassenakis M and Scoullo M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*. 64:178-189.
93. Velikova V, Yordanov I and Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science*. 151:59–66.
94. Wang Y.D.R, Duncan A.L, Rayburn J.F, Petolino A and Widholm J.M. 1991. The use of anti-microtubule herbicides for the production of doubled haploid plants from anther-derived maize callus. *Theoretical and Applied Genetics*. 8:205-211.
95. Wang W, Vinocur B and Altman A. 2003. Plant response to drought salinity and extreme temperature: towards genetic engineering for stress tolerance. *Planta*. 218:1-14.
96. Wang W, Vinani R, Scali M and Mauro C. 2005. A universal and rapid protocol for protein extraction from recalcitrant plant tissue for proteomic analysis. *Electronics.* 27:2782-2786.
97. Westermeie R and Marouga R. 2005. Protein detection methods in proteomics research. *Bioscience Reports*. 25:19-32.
98. Wu X.Y and Tiedemann T. 2002. Impact of fungicides on active oxygen species and antioxidant enzymes in spring barley (*Hordeum vulgare* L.) exposed to ozone. *Environmental Pollution Journal.* 116:37-47.
99. Yan S, Tang Z and Su W. 2005. Proteomic analysis of salt stress responsive proteins in rice root. *Proteomics.* 5:235-244.

100. Yeung E.C, Thorpe T.A and Jensen C.J. 1981. *In vitro* fertilization and embryo culture. *In* T.A Thorpe (Eds.). Plant tissue culture: Methods and Application in agriculture. pp. 253-271. New York. Academic press.

101. Yousuf P.Y, Hakeem K.U.R, Chandna R and Ahmad P. 2012. Role of glutathione reductase in plant abiotic stress. P. Ahmad and M.N.V Prasad (Eds.). Abiotic Stress response in Plants: Metabolism, productivity and Sustainability. pp. 149-157. Springer Science and Business Media.