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EVALUATION OF BIOCHEMICAL RESPONSES TO DROUGHT STRESS AS POSSIBLE SCREENING METHODS FOR DROUGHT TOLERANCE IN POTATO

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A thesis submitted to the faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Pretoria, 1998

DECLARATION

I declare that this dissertation is my own work. Technical assistance was available. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

A van der Mescht

(A. van der Mescht)

6 day of April 1999.

ABSTRACT

Potato is gaining in importance as high value nutritional crop in developing countries. Since the potato is more sensitive to drought than most other crop species, an understanding of how water stress affects growth, yield and development is of great economic and social importance. Although all potato cultivars are affected by drought, there are cultivar differences in susceptibility. Breeding and selection procedures are complicated by a poor understanding of this complex phenomenon as well as by the interaction between heat and drought stress in field studies. It is thus of importance to develop a laboratory screening method for early detection of drought tolerance in an attempt to shorten the testing period in breeding programmes.

Twelve potato cultivars (8 from South- Africa) with known growth periods and responses to drought in the field were grown in a glasshouse. Drought stress was induced three weeks after sprout emergence by the withholding of water. The physiological and biochemical assays evaluated as potential screening methods for drought tolerance included chlorophyll fluorescence, chlorophyll content, Cu/Zn superoxide dismutase activity, glutathione reductase activity, ascorbate peroxidase activity, free proline concentrations, polyamine titres and 2,3,5-triphenyltetrazolium chloride (TTC) reduction which measures cell viability.

Chlorophyll fluorescence parameters were measured at weekly intervals in drought stressed and well watered controls. The parameters calculated included the minimum fluorescence (Fo), the photochemical maximum (Fm), the maximum quantum efficiency of photosystem II (Fv/Fm), as well as the antenna efficiency of photosystem II (Fv'/Fm'). Additionally the levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll content and the ratio between chlorophyll *a* and *b* were determined. Results showed that cultivar differences following a drought stress could be measured using chlorophyll fluorescence parameters. Additionally there was a positive correlation between drought tolerance and chlorophyll fluorescence in cultivars with a short growth period. Superoxide dismutase, glutathione reductase and ascorbate peroxidase

activities were determined from freeze-dried leaf samples. Differences in glutathione reductase and ascorbate peroxidase activities could not be correlated with drought tolerance. The levels of glutathione reductase in stressed potato cultivars were consistently lower compared to control treatments. The levels of ascorbate peroxidase activity were generally higher in stressed plants compared to control plants. A correlation was found between yield loss under dryland conditions and superoxide dismutase activity. The ability of potatoes to maintain adequate levels of superoxide dismutase activity seemed more important than an increase in enzyme activity. As the metabolic rate is low in storage organs such as tubers we determined free proline concentrations in drought stressed potato leaves. The levels of free proline could not be correlated with drought tolerance. However, results showed that proline accumulation is a function of the growth period. Spermine titers after four weeks without water correlated with yield data under dryland conditions. The 2,3,5-triphenyl tetrazolium chloride viability assay was evaluated to estimate drought- and heat tolerance of leaves and tubers of the potato cultivars . Drought was simulated by floating leaf discs and tuber slices in 0.5M mannitol (-1.24MPa). After the drought acclimation treatment the leaf discs were subjected to a lethal drought stress by exposure to an osmotic potential of -2.48MPa. The viability of the leaf discs after the drought treatment was estimated by spectrophotometrically measuring the formazan concentration at 485 nm. As drought simulation in the laboratory can differ from field conditions due to the effect of heat stress, the cultivars were also evaluated for heat tolerance. A stress ranking was established which will enable breeders to distinguish between plant responses to heat and drought. Results from the physiological evaluations showed that the most promising physiological parameter to use as a screening method for drought tolerance is the levels of Cu/Zn superoxide dismutase activity. To confirm the efficacy of increased Cu/Zn superoxide dismutase levels to protect against drought the cytolsolic Cu/Zn superoxide dismutase gene from *Arabidopsis thaliana* was transferred to the cultivar Aviva. Results following the transformation experiments showed that the four transformed potato lines were more drought tolerant in comparison to the untransformed plants.

Dedicated to André and Waldo

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TABLE OF CONTENTS

Declaration i

Abstract ii

Dedication iv

Acknowledgements v

Table of Contents vi

List of Figures viii

List of Tables xi

List of publications from thesis xiv

List of abbreviations xvi

Chapter 1: Introduction

1.1 Description of the problem 1

1.2 Layout of thesis 4

1.3 The effects of drought on potato 8

1.4 Drought tolerant potatoes for South Africa?
A strategy for the development of a screening method 19

Chapter 2: Effect of drought on chlorophyll fluorescence

2.1 General introduction 26

2.2 Chlorophyll fluorescence as a measure of drought tolerance in potato 28

2.3 Addendum to Chapter 2 54

Chapter 3: Measurement of enzymes activity from the antioxidative system in response to drought stress

3.1 General introduction 58

3.2 Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase levels during drought stress in potato. 60

Chapter 4: The effect of drought on proline and polyamine levels

4.1 General introduction 74

4.2 Changes in free proline concentrations and polyamine levels during drought stress in potato. 77

Chapter 5: 2,3,5-Triphenyltetrazolium chloride reduction as a measure of drought tolerance

5.1 General introduction 91

5.2 The effect of drought- and heat stress on the viability of potato leaves on tubers. 93

Chapter 6: Potato transformation in an attempt to enhance drought tolerance

6.1 General introduction 112

6.2 Enhanced drought tolerance in transgenic potato expressing the *Arabidopsis thaliana* Cu/Zn superoxide dismutase gene 115

Chapter 7: Discussion 152

Appendix: Cultivar descriptions 160

LIST OF FIGURES

Chapter 1

Figure 1: Schematic presentation of the procedure to develop a screening method for drought tolerance). 22

Chapter 3

Figure 1: Regression lines of Cu/Zn superoxide dismutase activity in drought stressed and control potato cultivars. 66

Chapter 5

Figure 1: The viability of tuber slices (expressed a formazan production) subjected to heat stress was measured every 30 minutes at 485 nm for the cultivar Bravo. 98

Figure 2: The potato cultivar Aviva was treated as described in the legend to Figure 1. 99

Figure 3: The histogram summarises the drought response in potato leaves. The difference between the stress- and control treatments in leaves was calculated over time for twelve potato cultivars. 100

Figure 4: The histogram summarises the drought response in potato tubers. The difference between stress- and control treatments in tubers was calculated over time for 12 potato cultivars. ... 102

Figure 5: The histogram summarises the heat response in potato leaves. The histogram was calculated as described in the legend to Figure 3. 104

Figure 6: The histogram summarises the heat response in potato tubers. The histogram was calculated as described in the legend to Figure 4. 105

Chapter 6

Figure 1: Schematic representation of the ligation of the insert (800 bp), from the pcSODRH plasmid, with the vector (± 3,8 kb), from the pBI 221 plasmid, to produce the pBI 221 SOD plasmid. 121

Figure 2: Schematic representation of the ligation of the insert (1.6 kb), from the pBI 221 plasmid, with the vector (± 12 kb), from the pBI 121 plasmid, to produce the pBI 121 SOD plasmid. 122

Figure 3: A 1.2% agarose gel to check if the insert (SOD) had been spliced from the plasmid (pcSOD RH) and if the vector (pBI 221) had been successfully prepared for ligation and transformation. 129

Figure 4: A 0.8% agarose gels depicting the JAT Prep results of the 16 DH∝ colonies with *Amp* resistance obtained in the transformation studies of pBI 221 with an insert: vector ratio of 1:1. 131

Figure 5: A 0.8% agarose gel showing the *Pst I* digests of DNA isolated from selected DH5∝ colonies. 132

Figure 6: A 1.2% agarose gel to check if the insert (SOD) had been spliced from the plasmid (pBI 221 SOD) and if the vector (pBI 121) had been successfully prepared for ligation and transformation. 133

Figure 7: A 1.2% agarose gel showing the restriction enzyme digests to confirm the transformation of pBI 121 with SOD gene. .. 134

Figure 8: A Southern Blot, using the DIG labelling and detection kit as method of detection to confirm the transformation of pBI with the SOD gene. 135

Figure 9: A 1.2% agarose gel showing the results of the PCR products to confirm the successful transformation of the *Agrobacterium tumefacience* with the SOD gene via triparental mating. 136

Figure 10. Callus regeneration after 2 months from Aviva leaf explants dipped in either *A. tumefacience* LBA 4404 (pSOD) or *A. tumefacience* LBA 4404 (pBI121) for 5, 10 or 20 mins. .. 138

Figure 11: Agarose gel electrophoresis of A PCR analysis of DNA of putative transformed potato plants using SOD specific primers. 140

Figure 12: Agarose gel electrophoresis of A PCR analysis of DNA of putative transformed potato plants using nptII specific primers. 141

Figure 13: Autoradiograph of Southern blot of putative transformed potato plants. 142

Figure 14: The mean difference between the stress and control treatments in leaf discs presented in a histogram to summarises the drought response. 145

LIST OF TABLES

Chapter 2

Table 1: The drought response and growth period of 12 potato cultivars. 31

Table 2: A chlorophyll fluorescence study in drought-stressed and control potato cultivars using F_0 as a parameter. 34

Table 3: A chlorophyll fluorescence study in drought-stressed and control potato cultivars using Fm as a parameter. 37

Table 4: A chlorophyll fluorescence study in drought-stressed and control potato cultivar using Fv/Fm as a parameter. 38

Table 5: A chlorophyll fluorescence study in drought-stressed and control potato cultivars using Fv'/Fm' as a parameter. .. 39

Table 6: Chlorophyll a levels in drought stressed and control potato cultivars. 42

Table 7: Chlorophyll b levels in drought stressed and control potato cultivars. 44

Table 8: Total chlorophyll levels (a + b) in drought stressed and control potato cultivars. 45

Table 9: Drought tolerance as estimated by chlorophyll fluorescence and chlorophyll a and b levels. 47

Chapter 3

Table 1: Drought response and growth period of potato cultivars. Drought tolerance is defined in terms of yield reduction under dryland conditions. 63

Table 2: The mean values (n=6) of superoxide dismutase levels in drought stressed (two weeks without water) and non-stressed control potato plants. 67

Table 3: The mean values (n=6) of glutathione reductase activity in drought stressed (two weeks without water) and non-stressed control potato cultivars. 68

Table 4: The mean values (n=6) of ascorbate peroxidase activity in drought stressed (two weeks without water) and non-stressed control potato cultivars. 69

Chapter 4

Table 1: The drought response and growth period of potato cultivars. Drought tolerance is defined in terms of yield reduction under dryland conditions. 80

Table 2: Proline accumulation calculated at weekly intervals for 12 potato cultivars and expressed as a percentage of total proline accumulation. 82

Table 3: Spermine titres calculated at weekly intervals during drought stress in potato cultivars. 84

Chapter 5

Table 1: Summary of the cultivars evaluated for heat - and drought tolerance. 96

Table 2: Summary of the drought- and heat response in potato leaves and tubers. 101

Chapter 6

Table 1: Regeneration from Aviva leaf explants incubated on various antibiotic concentrations. 126

Table 2: Total number of Aviva leaf explants transformed with *A. tumefaciens* cultures after two months. 137

Table 3: Cu/Zn Superoxide dismutase levels during drought stress compared to control conditions in untransformed Aviva plants as well as four transformed Aviva lines. 144

Chapter 7

Table 1: Summary of the physiological reactions of 12 potato cultivars to drought stress 157

LIST OF PUBLICATIONS FROM THESIS

As a business unit manager ms. A. van der Mescht was responsible for the planning, organizing, technical aspects and funding of the project. Her duties also included training of junior scientists as can be seen in the list of co-authors on the publications. In Chapter 6 the tissue culture research was carried out by ms. M.M. Slabbert and ms. S. Murray. Ms. J.A. de Ronde provided technical advice on molecular aspects and technical assistance on physiological and molecular research. The part of the research presented in this dissertation was under supervision of dr. F.T. Rossouw.

Chapter 1

1.1 Van der Mescht, A. and Rossouw, F.T., (1997). Drought tolerant potatoes for South-Africa ? A strategy for the development of a screening method. *South African Journal of Science*. **93**: 257-258.

1.2 Van der Mescht, A. and Rossouw, F.T. (1998). The effects of drought on potato - A review. *Journal of the South African Society of Horticultural Science*. (In press).

Chapter 2

2.1 Van der Mescht, A., De Ronde, J.A. and Rossouw, F.T. (In preparation). Chlorophyll fluorescence as a measure of drought tolerance in potato. *South African Journal of Science*.

Chapter 3

3.1 Van der Mescht, A. De Ronde, J.A. and Rossouw, F.T. (1998).Cu/Zn Superoxide dismutase, glutathione reductase and ascorbate peroxidase levels during drought stress in potato. *South African Journal of Science*.(In press).

Chapter 4

4.1 Van der Mescht, A., De Ronde, J.A., Van der Merwe, T and Rossouw, F.T. (1998). Changes in free proline concentrations and polyamine levels during drought stress in potato. *South Africa Journal of Science*. **94**(In press).

Chapter 5

5.1 Van der Mescht, A., De Ronde, J.A. Van der Merwe, T., Daniels, C.L. and Rossouw, F.T. (Submitted). The effect of drought and heat stress on the viability of potato leaves and tubers. *South African Journal of Science.*

Chapter 6

6.1 Van der Mescht, A., De Ronde, J.A., Slabbert, M.M., Murray, S., Oelofse, D. and Rossouw, F.T. (In preparation). Enhanced drought tolerance in transgenic potato expressing the *Arabidopsis thaliana* Cu/Zn superoxide dismutase gene.

LIST OF ABBREVIATIONS

Amp	-	Ampicillin
ANOVA	-	analysis of variance
ATP	-	adenine triphosphate
bp	-	base pairs
chl	-	chloroplastic
cyt	-	cytoplasmic
CO_2	-	carbon dioxide
CV	-	coefficient of variation
CuZn	-	copper/zink
DNA	-	deoxyribonucleic acid
cDNA	-	complementary deoxyribonucleic acid
dATP	-	2'-Deoxy-adenosine-5'-triphosphate
dCTP	-	2'-Deoxy-cytidine-5'-triphosphate
dGTP	-	2'-Deoxy-guanosine-5'-triphosphate
dTTP	-	Thymidine-5'-triphosphate
EDTA	-	Ethylenediaminetetra acetic acid disodium salt
Fo	-	initial, constant or minimum fluorescence
Fm	-	maximum fluorescence
Fv	-	variable fluorescence
Fv/Fm	-	maximum quantum efficiency of PS II
$Fv'Fm'$	-	atenna efficiency of PS II
g	-	gram
GR	-	glutathione reductase
h	-	hour
H_20_2	-	hydrogen peroxide
HPLC	-	high pressure liquid chromatography
K	-	potassium
kb	-	kilo base p[illegible]s

KCl	-	potassium chloride
Kda	-	kilodalton
Km	-	kanamycin
LBA	-	Luria-Bertani agarose
LGP	-	long growth period
LSD	-	least significant difference
M	-	molar
mg	-	milligram
MGP	-	medium growth period
$MgCl_2$	-	magnesium chloride
min	-	minute
ml	-	millilitre
mM	-	milimolar
MS medium	-	growth medium of Murashige and Skoog
Mpa		Molar Pascal
NAA	-	naphthalene-acetic acid
N	-	nitrogen
NaCl	-	sodium chloride
NADP	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Nicotinamide adenine dinucleotide hydrogen phosphate
ng	-	nanogram
nm	-	nanometre
P	-	phosphorus
PCR	-	polymerase chain reaction
PSI	-	photosystem 1
PSII	-	photosytem 2
Q_A	-	primary quinone acceptor in PS II
Q_B	-	secondary quinone acceptor in PS II
qE	-	energy dependant quenching of chlorophyll

		fluorescence
qN	-	non-photochemical quenching
qP	-	photochemical quenching of chlorophyll fluorescence
1-qP	-	loss of PS II function
qQ	-	quenching coefficient
RIAT medium	-	*In vitro* regeneration medium for potato plantlets
rpm	-	revolutions per minute
SGP	-	short growth period
SOD	-	superoxide dismutase
t RNA	-	transfer ribonucleic acid
TTC	-	2,3,5- triphenyl tetrazolium chloride
UPOV	-	Union Internationale pour la Protection des Obtentions Vegetables. (International Union for the protection of new varieties of plants)
UV	-	Ultra violet
v/v	-	volume/volume
w/v	-	weight/volume
YM medium	-	selection medium for transformed *A. tumefacience*
µl	-	micro litre
µm	-	micrometer

CHAPTER 1

INTRODUCTION

1.1 Description of the problem

In South Africa large areas are subjected to poor rainfall distribution and/or low annual rainfall with the result that drought is a major limiting factor to crop production[1]. It is well known that the potato crop is rather sensitive to drought and that even short periods of drought can result in reduced yield and tuber quality[2]. The main reason for the potato's vulnerability to drought is the shallow rooting system of which the main portion (approximately 85%) is concentrated in the upper 0.3 m of the soil. Weisz *et al.* [4] suggested that not only limited soil water extraction but also physiological processes contribute to the potato's sensitivity to drought. It is thus reasonable to assume that cultivar variation in drought sensitivity is a function of physiological processes.

The physiological processes implicated most frequently in the literature in response to drought are photosynthesis (particularly differences in chlorophyll fluorescence), differences in concentration and activity of antioxidant enzymes (such as Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase), free proline accumulation, increases in polyamine levels and cell viability.

The primary effect of drought on all plants is stomatal closure and the subsequent impairment of carbon assimilation [5]. Chlorophyll fluorescence measurements give a quantitative assessment of inhibition or damage to electron transfer. This has been used to demonstrate that impairment of the photochemistry is a primary event following stresses of drought, high temperature and high light [5].

The misdirection of electrons in the photosystems results in the formation of

reactive oxygen species[6]. This oxidative injury caused by drought is curtailed to a limited degree by the production of antioxidants which prevent cell damage. The extent to which a plant is able to produce antioxidants to alleviate the deleterious effects of drought characterises its drought response profile. One of the enzymes considered important in oxidative injury repair is Cu/Zn superoxide dismutase. However, the mechanisms involved in the minimization of oxidative stress may play a secondary role during drought tolerance and direct correlations between increased concentrations of an enzyme and drought tolerance may be complex [7]. CuZn superoxide dismutase breaks down the superoxide radical to hydrogen peroxide and dioxygen[8]. Hydrogen peroxide which is toxic to the cells is removed by the action of other important enzymes in oxidative repair namely glutathione reductase, ascorbate peroxidase and dehydro ascorbate reductase. These reactions form part of the Halliwell - Asada pathway[6]. The levels of these enzymes and their activity during drought stress have been correlated with the plant's drought response [7].

The optimal activity of the enzymes discussed above is reduced by lowered pH. Proline accumulation is associated with the changes in cytoplasmic pH, reducing acidity [9]. In addition, proline inhibits protein denaturation caused by drought [9,10]. In general proline levels during drought are quantitative indicators of the drought response [11]. The biophysical properties of proline is discussed in detail in Chapter 4.

The synthesis of free proline and polyamines share a biochemical pathway at intermediates glutamic acid and L-ornithine[12,13]. It has been suggested that the role of polyamines is in maintaining the cation-anion balance in the plant cell. The polyamines are protonated at the physiological pH of cells, thus electrostatic binding of polyamines to negatively charged functional groups of membranes is favoured[14]. In binding to the negatively charged

phospholipid head groups on membranes, the polyamines influence the stability and permeability characteristics of these membranes, e.g. the loss of chlorophyll from thylakoid membranes is prevented by maintaining membrane integrity thus stabilizing the photosystem complexes during drought stress[15].

As a result of these interactions in physiological mechanisms during drought stress the aim of this work was to evaluate chlorophyll fluorescence, chlorophyll content, Cu/Zn superoxide dismutase levels, glutathione reductase levels, ascorbate peroxidase levels, free proline concentrations and polyamine titres as possible screening methods for drought tolerance in potato. 2,3,5-Triphenyltetrazolium chloride (TTC) reduction was added to the list as the TTC-assay measures the capability of plant tissue to carry out electron transport[16] and thus is a measure of cell viability.

Twelve potato cultivars differing in growth period and drought response were included in this study. Four cultivars were representative of a short (80-90 days), medium (90-100days) and long growth period (100-130 days from emergence to haulm die-back) respectively. In addition, two of the cultivars within each of the growth periods were drought tolerant and two were drought sensitive. Drought tolerant cultivars were selected on the basis of minimal yield reduction in field trials under dryland conditions when compared to irrigated trials [17,18]. The results of the field trials were confirmed for three cultivars in rain shelter trials [19]. This work was undertaken with the view to support the potato breeding programme for drought tolerance.

1.2 Layout of thesis

This thesis is divided into seven stand alone chapters, which contain the actual work done. Each chapter consists of an Introduction with its own reference list, a published or submitted journal article (with its own reference list) and an addendum which contains the Results which were not published (with its own reference list). In addition the general Introduction (Chapter One), contains three sections, an overall Introduction in which the rationale for all tests carried out is given, and two published papers, the first of which reviews comprehensively the potato's ability to cope with drought (Van der Mescht and Rossouw. The effects of drought on potato- A review. J. S. Afr. Soc. Hort. Sci., In press) and the second gives the workplan for the thesis (Van der Mescht and Rossouw,1997. Drought tolerant potatoes for South-Africa? A strategy for the development of a screening method. S. Afr. J. Sci. 93: 257-258).

There follows the Chapters on the research done, namely: Chapter Two, which contains a general Introduction titled, Effect of drought on chlorophyll fluorescence, one paper in preparation (Van der Mescht *et al.*, Chlorophyll fluorescence as a measure of drought tolerance in potato. S. Afr. J. Sci., In preparation) , and an addendum containing definitions of chlorophyll fluorescence parameters; Chapter Three contains a general Introduction titled, Measurement of enzyme activity from the antioxidative system in response to drought stress and one paper in press (Van der Mescht *et al.*, Cu/Zn Superoxide dismutase, glutathione reductase and ascorbate peroxidase levels during drought stress in potato. S. Afr. J. Sci., In press); Chapter Four contains a general Introduction titled: The effect of drought on proline and polyamine levels and one paper in press (Van der Mescht *et al.*,1998 Changes in free proline concentrations and polyamine levels in

potato leaves during drought stress. S. Afr. J. Sci., In press); Chapter Five contains a general Introduction titled, 2,3,5-Triphenyltetrazolium chloride reduction as a measure of drought tolerance, one submitted paper (Van der Mescht *et al.*,Submitted, A comparison of drought stress and heat stress in the leaves and tubers of 12 potato cultivars, S. Afr. J. Sci.); Chapter Six contains a general Introduction titled, Potato transformation in an attempt to enhance drought tolerance and one paper in preparation (Van der Mescht *et al.*, Enhanced drought tolerance in transgenic potato expressing the *A. thaliana* Cu/Zn superoxide dismutase gene. S. Afr. J. Sci.) and in the Discussion (Chapter Seven) the significant findings of each chapter are drawn together to make a coherent blue print for screening for drought tolerance in a potato breeding programme.

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1.3 The effects of drought on potato

Van der Mescht, A. and Rossouw, F.T. (1998)
Journal of the Southern African Society for Horticultural Science. (In press).

Abstract

The most important physiological stress to potato production in most areas of the world is drought. As the potato is more sensitive to drought than most other crop species, an understanding of how water stress affects growth, yield and development is of great economic importance. Drought affects the potato at the physiological, biochemical and molecular levels. The effects of drought on growth include: reduced plant size, fewer leaves, more senescent leaves, increased specific weight, reduced radiation-use efficiency, small misshapen tubers low in water content, secondary growth, growth cracking, greater N, P and K contents and a decrease in yield. Although all potato cultivars are affected by drought, there are cultivar differences in susceptibility. Breeding and selection procedures are complicated by a poor understanding of this complex phenomenon as well as by the interaction between heat and drought stress in field studies.

Introduction

Potato cultivars grown all over the world originated from a very restricted gene pool derived from a small number of tetraploid tubers from South America. The potato crop can be cultivated at high altitudes in tropical latitudes (e.g. the Andes, its probable place of origin), at mid-altitudes in semi-tropical latitudes and the lowlands of cool temperate regions[1]. The potato is a major food source, thus it is important to enhance the tolerance of the crop to environmental extremes such as heat, cold, flooding and drought. In many countries e.g. Greece and South Africa, a large part of the potato growing season coincides with the warm, dry summer period.

Drought has devastating effects on plant growth and development. Plant responses to drought vary between avoidance and tolerance[2]. These responses can be understood in different terms e.g. physiological, biochemical, genetic and morphological.

The effect of drought on growth and yield

The most important physiological stress to potato production in most areas of the world is drought. The potato is more sensitive to drought than most other crop species [3]. Stress periods as short as one day can cause primary effects such as closure of stomata and wilting. Morphological changes are exhibited when soil water content drops to only 70-85% of field capacity[4]. According to Weisz *et al.* [5] potato is more drought sensitive than other agronomic crops due partially to a shallow and perhaps less efficient root system. Mould and Rutherfoord [6] found that a soil water potential of -0,7 MPa was critical with regard to both yield and quality. In terms of transpiration, the drought sensitivity appears to be due to less effective soil water extraction. Weisz *et al.* [5] postulated that in addition to extracting less soil water, physiological processes related to leaf expansion must be contributing to the potato's hypersensitivity to drought.

According to Shimshi & Susnoschi [7] there is a linear relationship between the amount of soil water and the reduction in tuber yield when the water applied is less than the daily loss by evapotranspiration. Vayda [8] warns that this apparently simple relationship disguises a complex set of responses. Considering the complexities surrounding drought tolerance, a field screening technique to evaluate drought tolerance in potatoes was developed by Steyn *et al.* [9]. The drought tolerance and water use of different genotypes were accessed in the confined space of a rain shelter. Additionally Steyn [10] found that genotypic differences were more pronounced in spring trials in

comparison to autumn results. They concluded that the choice of genotypes is only influenced by the availability of water during spring and that the drought tolerant genotypes should then be used.

Photosynthetic efficiency is reduced at all stages of growth during water stress. However, drought has the most drastic effect on yield during the period of tuber initiation and bulking. Stolon and tuber initiation is blocked during the drought stress [11,12]. During drought, the number of tuber initiation events is reduced proportionally to the duration of the stress. Additionally, the bulking rate of tubers initiated prior to the onset of drought is decreased dramatically, resulting in loss of dry matter that is in turn proportional to both duration and severity of the stress[12,13]. Minhas & Bansal [14] have found that drought imposed at the stolon initiation stage reduced yield by 30-65% and increased the percentage of small tubers by 9-25%. However, yield trials measure past yield while the aim is to improve future yields under adverse environmental conditions. Thus, Steyn *et al.* [15] evaluated the use of a multiplicative interaction model to predict yield. They not only found the technique was suitable to determine the reaction of genotypes in a specific environment but they could also use the results to display the stability of a genotype in different environments.

During drought stress, the stomata close[16] causing a reduction in both transpiration and photosynthesis[17], which in turn inhibits growth, with tubers maturing earlier[18,19]. Inhibited growth during drought stress also results from a reduced canopy [20], limited light interception[9] and reduced light-use efficiency [21]. Inhibited growth is more pronounced when plants are exposed to both drought- and heat-stress [22]. During heat and drought stress tuber yields are low with a high percentage of small and malformed tubers [18,22]. Small tubers never reach commercial size as a result of delayed tuber initiation, shortage of assimilates for tuber growth and reduced time span

between tuber initiation and maturity [17,23]. Plants exposed to water stress generally have reduced plant size, fewer leaves, more senescent leaves, increased specific weight, reduced radiation use efficiency, small misshapen tubers low in water content, secondary growth, growth cracking and greater N, P and K contents [17]. In temperate regions, potato cultivars exhibit a differential tolerance to hollow heart. A correlation was found between susceptibility to hollow heart and transpiration [24].

Vayda [8] stated that even the relief of drought has adverse effects, for example, during drought conditions the basal portion of the potato ceases to grow. When adequate soil water is available, the apical end of the tuber resumes growth, causing malformation which reduces the marketable potential of the crop. Prolonged water stress during early tuber development results in depletion of starch at the basal end and browning during cooking. Rapid tuber growth often accompanies rehydration which causes growth cracks and hollow heart.

The effect of drought on the physiological, biochemical and molecular level

Although all potato cultivars are affected by drought, it is generally accepted that there are cultivar differences in susceptibility to water stress [16,18,25,26]. Cultivar responses may vary between the extremes of avoidance (by early tuber initiation and bulking) and tolerance reactions. The physiological basis for cultivar tolerance is unknown[8].

Little success has been achieved in breeding drought-tolerant potato cultivars through empirical methods. This is due to a poor understanding of the complex phenomenon of drought tolerance and a lack of reliable non-destructive screening techniques. Bansal *et al.* [27] have found that some cultivars are very sensitive to day length and are only tolerant to drought

when grown under short days.

One of the major effects of water stress is a decrease in photosynthetic efficiency. Unlike the effect of heat stress, fluorescence yield evaluations during drought reveals no changes in either photosystem II or photosystem I function[8]. However, chlorophyll *a* fluorescence measurements can give a quantitative assessment of inhibition or damage to electron transfer. Jefferies [28] evaluated the effects of drought on chlorophyll fluorescence in seven potato cultivars but he did not correlate his findings to drought tolerance. Results from Schapendonk *et al.* [29] indicated the inhibition of the Calvin-cycle enzymes. The degree of inhibition was greatest in drought sensitive cultivars.

Drought also affects the loss of water from the cytoplasm leading to electrolyte imbalance. Plants may respond by the accumulation of proline and betaine in an attempt to maintain the osmotic potential[30]. Results from Levy [31] have shown a possible correlation between low proline content and drought tolerance in potato tubers. Earlier work by Van der Mescht and De Ronde [32], which tested for proline incorporation into drought related proteins, showed that this amino acid was not utilized differently in proteins from drought stressed and control leaf samples. This suggests that proline interacts with the hydrophobic residues on the protein surface, causing an increase in protein stability[33].

The molecular responses of potato to drought stress have not been intensively investigated. According to Vayda [8] the level of expression of genes encoding dehydrin-like proteins has not been assessed in potato cultivars subjected to water stress. Van der Mescht *et al.* [34] have shown that drought dependent variation in *de novo* protein synthesis was greatest in a tolerant cultivar. Additionally, their findings showed a correlation between response to

drought in the field and leaf protein synthesis. In a subsequent publication Van der Mescht *et al.* [2] have shown that *de novo* protein synthesis was not only cultivar specific but also organ specific. The protein profiles during drought stress differed significantly between leaves and tubers. It was also investigated whether any of the drought-related polypeptides in a tolerant cultivar have a regulatory function. Protein - DNA binding studies suggest that the 38 kDa polypeptide's ability to bind DNA is drought and osmotic stress specific[35].

Drought and other physiological stresses cause oxidative injury. High antioxidant capacity or increased levels of antioxidants can prevent cell damage and may correlate with stress tolerance. Superoxide dismutase is a well described enzymatic antioxidant which breaks down the superoxide radical[36]. Transgenic potato lines harbouring either the *chl* or *cyt* superoxide dismutase genes from tomato showed elevated tolerance to paraquat[37].

Conclusions

The tolerance of potato to drought conditions has not been clearly identified at the genetic, physiological, morphological or molecular level. According to Vayda [8] this is due to the fact that sensitivity to drought operates at three levels namely: photosynthetic efficiency, initiation of stolon and tuber development, and carbon partitioning and growth deformities. Furthermore, field studies and cultivar assessment are complicated by the interaction between heat and drought stress, as water stress is often accompanied by heat stress. Vayda [8] concludes that the response of potato to drought and the genetics of tolerance are poorly understood except for the fact that it is a whole plant phenomenon and probably a polygenic characteristic.

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1.4 Drought tolerant potatoes for South Africa? A strategy for the development of a screening method.

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South African Journal of Science 93: 257 - 258.

Drought as a challenge

Drought is a major factor limiting crop production, thus drought continues to be a challenge to agricultural scientists. Drought is not only a challenge due to the economic importance of the problem, but also due to the complexity of factors affecting crop response to it[1].

Losses caused by extended drought can amount to millions of rands. Direct losses result from reduced yields while indirect losses include crops not planted, abandonment of land and land-use changes following the drought. Agricultural industries absorb primary losses but eventually the entire nation pays. This happens when the government makes relief grants to the agricultural sector which result in higher consumer prices due to a shortage of commodities[2].

The devastating effect of drought on the population made newspaper headlines in 1995[3,4,5,6]. According to the World Bank the world faces a growing water crisis as 80 countries representing 40 percent of the world's population are already experiencing chronic water shortages[3]. Additionally, British scientists at the University of East Anglia predicted a drought lasting a 100 years for Southern Africa[4]. They based their prediction on results from research on the impact of global warming. Global warming contributes to increased aridity which will exacerbate the problems of feeding people in dry areas[4]. This is especially true for the Northern Province of South Africa where low crop yields have left many people starving[5]. The Economist reported that the United Nations World Food Programme and the Food and Agriculture Organisation predict that Southern Africa will soon make a collective appeal for food aid due to crop failures. But drought is a double-edged threat to African farmers: hunger today versus food ... ...eat surpluses from rich

countries) will be followed by low prices for the local farmer's crops tomorrow[6].

From a genetic point of view the minimization of yield loss due to drought conditions is a very elusive trait. Cultivars successful in one dry year may fail in another dry year as duration, timing and severity of drought varies from year to year. Furthermore drought is seldom the only abiotic stress, it interacts with other abiotic stresses such as heat and high salinity[1].

Potato yield is sensitive to abiotic stress

According to Vayda[7] potato yield is especially sensitive to drought and heat stress. The most important physiological stress which affects potato production worldwide is water deprivation stress. Potatoes are more sensitive to soil water reduction when compared to most other crop species. Very short periods of acute stress result in substantial reductions in total marketable yield[8]. Drought stress reduces photosynthetic efficiency at all stages of growth; but drought has the most drastic effect on yield during the period of tuber initiation and bulking[7,9]. Optimal water supply could increase the average potato yield in the world by 50%[10].

Although all potato cultivars are sensitive to drought there are differences in the degree of sensitivity. Strategies for the selection of drought tolerance in breeding programmes have been developed[7]. However, little success has been achieved through these empirical methods. This may be due to poor understanding of the complex phenomenon of drought resistance, lack of reliable non-destructive screening methods[11] and lack of data on the inheritance of stress tolerance in potato [10]

.

Richards[12] stated that it will never be possible to overcome the devastating effects of drought, that progress to improve yield during drought will be slow and that gains will be small. There are no well-documented examples of the release of drought tolerant cultivars bred on the basis of a physiological understanding of plant responses to drought. Thus the controlling factors influencing yield under drought should not be ignored as this may mean that important gains will be overlooked. We

propose a strategy to evaluate physiological processes as possible screening methods for drought tolerance in an attempt to improve yield under drought conditions.

Strategy

In general, like yield, quality and stress tolerance, there are no gene(s) for drought tolerance as such. Rather, there are genes for traits that contribute to drought tolerance[13]. The traits involved in drought tolerance may be used in the development of a screening method. This approach is similar to conventional breeding, except that the selection is based on certain traits contributing to drought tolerance rather than for drought tolerance itself (Figure 1). Firstly, potential traits contributing to drought tolerance must be identified. The potential traits to be evaluated as possible screening methods for drought tolerance include: drought-related protein synthesis[14], changes in free proline concentrations[15], maintenance of cell viability (2,3,5 Triphenyltetrazolium chloride assays[16]), stability of PSII function (chlorophyll fluorescence kinetics[17]), polyamine concentrations[18] and the levels of CuZn superoxide dismutase, glutathione reductase, peroxidase, ascorbate peroxidase and catalase[19]. These five enzymes are involved in the primary defence against reactive oxygen intermediates induced by the metabolic disturbance as a result of physiological stresses. Secondly, the screening method must be able to distinguish reliably between sensitive and tolerant cultivars. Thirdly, the value of the screening method must be proven by correlations with results obtained under field conditions. The ultimate test for any character consists of yield trials under target, in this case drought conditions[10]. Heritability studies can be combined with the studies on drought markers. The procedure to develop a screening method for drought tolerance is summarized in Figure 1.

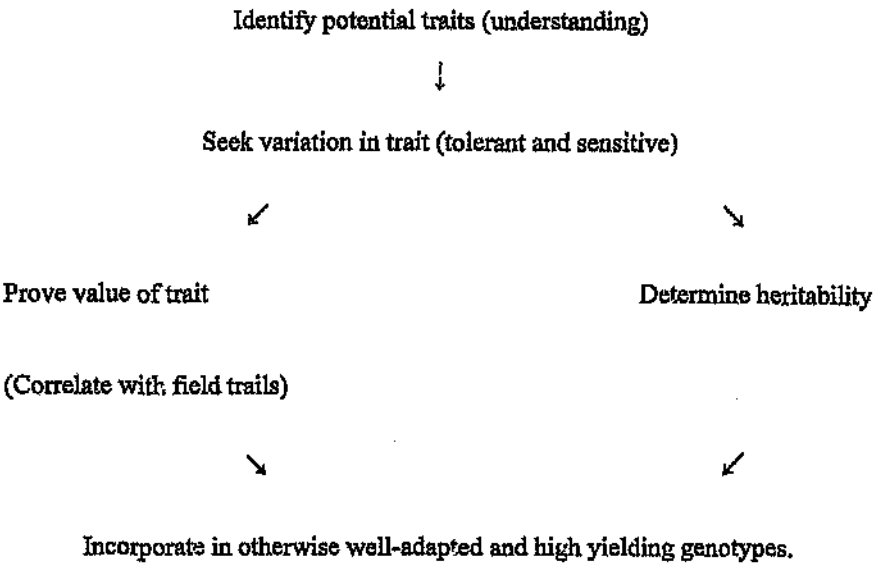


Figure 1: Schematic presentation of the procedure to develop a screening method for drought tolerance[13].

Conclusion

Although rapid and economical screening methods for drought tolerance have proved to be difficult to develop, such techniques offer a great promise for in assisting breeding programmes and in incorporating specific drought resistance traits into new varieties. In our laboratory, correlations between possible physiological screening methods and results obtained from field trials were shown. Results from 2,3,5 triphenyltetrazolium chloride reduction assays correlated to heat as well as drought tolerance in six cotton cultivars[20]. Free proline concentrations and results from field trials in tobacco cultivars showed a positive correlation. Furthermore a negative correlation between levels of superoxide dismutase during drought conditions and results from dryland trials indicated a drought avoidance mechanism in twelve potato cultivars[21].

A detailed knowledge of the biochemistry and physiology of the trait may also lead

to the identification of genes contributing to drought tolerance. To evaluate the contribution of these specific genes to drought tolerance, the drought - responsive gene of interest must either be transferred into a plant via genetic engineering or isogenic lines differing in this gene must be produced.

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CHAPTER 2
EFFECT OF DROUGHT ON CHLOROPHYLL FLUORESCENCE

2.1 General Introduction

Much information on photosynthetic processes can be obtained from chlorophyll fluorescence analysis. Chlorophyll *a* fluorescence is frequently used to assess the effects of environmental stress on the photosynthesis of plants. Stress situations will either affect the function of photosytem II or inhibit the carbon reduction cycle [1]. Besides stomatal closure, the primary effect of drought is the impairment of carbon assimilation [2]. Information on the carbon reduction cycle can be provided by chlorophyll fluorescence as the proton gradient and the redox state of the primary electron acceptor of photosystem II is influenced by the consumption of ATP and NADPH during carbon metabolism [3].

Chlorophyll fluorescence parameters were measured at weekly intervals in drought stressed and well watered controls. The parameters calculated included the minimum fluorescence (Fo), the photochemical maximum (Fm), the maximum quantum efficiency of photosytem II (Fv/Fm), as well as the antenna efficiency of photosystem II (Fv^{1}/Fm^{1}). Additionally the levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll content and the ratio between chlorophyll *a* and *b* were determined. In our present study the twelve potato cultivars fell into three groups with regard to their response to drought using chlorophyll fluorescence parameters : a tolerant group which gave tolerant reactions according to the literature for the fluorescence parameters (three cultivars), an intermediate group (five cultivars) and a sensitive group which gave sensitive reactions according to the literature for the fluorescence parameters (four cultivars). Additionally there was a positive correlation between drought tolerance in field trials and chlorophyll fluorescence parameters in cultivars with a short growth period (Chapter 2.2). Results

from chlorophyll *a* and *b* measurements were in agreement with chlorophyll fluorescence parameters as it could successfully distinguish between drought tolerance and drought sensitivity in cultivars with a short growth period but results were inconclusive for cultivars with a medium or long growth period.

The chlorophyll fluorescence parameters that could not be successfully correlated with drought tolerance under field conditions are given in Chapter 2.3.

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2.2 Chlorophyll fluorescence as measure of drought tolerance in potato

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Abstract

Drought is considered as one of the most serious constraints during potato production. During drought stress the rate of photosynthesis declines as a result of decreasing intercellular CO_2 concentrations. Much information on photosynthetic processes can be obtained from chlorophyll fluorescence. In the work presented here twelve potato cultivars differing in their drought response and representing three growth periods were subjected to drought by withholding water. Chlorophyll fluorescence parameters were measured at weekly intervals in drought stressed and well watered controls. Additionally the levels of chlorophyll *a*, chlorophyll *b*, total chlorophyll content and the ratio between chlorophyll *a* and *b* were determined. Results showed that cultivar differences following the drought stress could be measured using these chlorophyll fluorescence parameters. There was a positive correlation between drought tolerance and chlorophyll fluorescence (Fo, Fm and Fv/Fm) in cultivars with a short growth period. The drought tolerant cultivars Devlin and Avila correlated positively with all parameters measured while the drought sensitive cultivars Raritan and Vanderplank tested negative for all parameters measured. When the medium growers were evaluated it was found that the sensitive cultivar Sebago gave a sensitive phenotype with parameters Fo, Fm and total chlorophyll content; the sensitive cultivar Ono gave sensitive reactions with parameters Fv/Fm, chlorophyll *a* concentrations as well as chlorophyll *b* content. The drought tolerant cultivar Darius had no tolerant reactions while the drought tolerant cultivar Baraka had Fo and Fv/Fm indicating tolerance. When the cultivars with a long growth period were evaluated the sensitive cultivar Bravo tested sensitive with all parameters; the sensitive cultivar Kimberley Choice showed a sensitive phenotype for both chlorophyll *b* and total chlorophyll content. The drought tolerant cultivar Hoëvelder was tolerant when chlorophyll *a* and total chlorophyll was

measured while the tolerant cultivar, Late Harvest, showed a tolerant phenotype according to Fo, Fm and Fv/Fm measurements. From these results we can conclude that chlorophyll fluorescence can only be used as a measure of drought tolerance in potato cultivars with a short growth period.

Introduction

Drought poses severe constraints on potato production. Although many physiological aspects of drought tolerance in potato have been reported, such as free proline accumulation[1], transpiration rate[2], photosynthetic efficiency[3], betaine accumulation[4] and high antioxidant capacity[5], few of these have been shown to correlate with genetic variation for drought tolerance[6]. What we are attempting to do, is to determine whether potatoes physiological changes during drought stress are cultivar - specific so that they can be used as markers in breeding programmes. Photosynthesis is one of the physiological processes in plants which is severely affected by drought stress.

One of the primary responses during drought stress is the increased concentration of absicisic acid which causes the closure of stomatal guard cells to reduce water loss[7]. The rate of photosynthesis declines during drought stress as a result of decreased chloroplast activity, decreasing intercellular CO_2 concentrations and the subsequent impairment of carbon assimilation[8]. What is less well known is which chloroplast activity is the most severely affected by drought[9]. However, the solar energy, trapped by photosystem II and photosystem I, is converted to chemical energy through electron transport and carbon assimilation which in turn produce ATP and carbon skeletons for all major metabolic processes[10]. Chlorophyll fluorescence can provide information on the functioning of the carbon reduction cycle. The proton gradient and the redox state of the primary electron acceptor of photosystem II (light reaction) is affected by consumption of ATP and NADPH during carbon metabolism (dark reaction). Thus fluorescence yield is influenced by carbon assimilation. On the other hand there are reports on the effect of drought on thylakoid reactions. Havaux [11] showed that within the photosynthetic apparatus, photosystem II seems to be heat sensitive, while photosystem I activity, stromal enzymes or chloroplast envelope are

comparatively more thermostable. Changes in leaf water potential and osmotic potential influence the thermal tolerance of photosynthesis[12]. During increased temperatures or sensitivity (caused by drought) the photosystem II reaction centres are blocked followed by a dissociation of antennae pigment protein complexes[13] or a degradation of protein[14]. This results in a decrease in the amount of photosystem II centres bound to the photosynthetic membrane[14].

Chlorophyll *a* fluorescence measurements can give a quantitative assessment of inhibition or damage to electron transfer. The technique is rapid, sensitive, non-destructive, relatively cheap and able to detect injury even before visible symptoms appear[15]. Jefferies [16] evaluated the effects of drought on chlorophyll fluorescence in seven potato cultivars, but he did not correlate his findings to variation in drought tolerance or sensitivity.

The aim of this study was to evaluate the use of chlorophyll fluorescence as a screening technique for drought tolerance in potato. Cultivars were selected on the basis of their drought response in field trials as well as being representative of different growth periods. The growth period is measured from emergence to haulm die-back.

Materials and Methods

Plant Material

The trials were planted in a randomized block design with three replicates. Twelve potato cultivars with known drought tolerance or susceptibility and growth periods (Table 1) were grown in a greenhouse under conditions as previously described by van der Merwe, *et al.* [17]. Sprouted tubers were grown in plastic pots containing 2.5 kg soil mixture (loam: sand: vermiculite, 5:2:2) and 10 ml of commercial fertilizer (N:P:K, 2:3:2 with Zn). Plants were watered regularly (200 ml every day), for three weeks, after which cultivars were deprived of water to induce drought conditions while control plants were kept well watered. After drought stress was induced and the leaf on the third apical node was harvested weekly from drought stressed and

well watered control plants.

Chlorophyll fluorescence

Chlorophyll fluorescence measurements were taken weekly using a pulse - amplitude modulated fluorometer (PAM 101-103; H. Walz, Effeltrich, F.R.G.) attached to a strip chart recorder (Pederson). Dark adapted leaves, achieved by covering the leaf with a black plastic bag for 20 minutes, were subjected to various light intensities. The minimal fluorescence level, F_o, was taken as the first signal after the measuring light of 3 000 microeinsteins m^{-2} sec^{-2} was switched on. A saturation pulse of 5 000 microeinsteins m^{-2}. sec^{-2} was applied for 3 seconds to induce the maximal fluorescence level (Fm). This was followed by a light intensity of 1 000 microeinsteins m^{-2}.sec^{-2} until the fluorescence curve stabilized[18]. After the curve stabilized a second signal after the measuring light of 3 000 microeinsteins m^{-2} sec^{-2} was switched on was measured (Fo^1). The second saturation pulse yielded (Fm^1) followed by a second stabilization light intensity of 1 000 microeinstein m^{-2} sec^{-2}.

Table 1: The drought response and growth period of 12 potato cultivars. Drought tolerance is defined in terms of yield reduction under dryland conditions[19].

Cultivar	Growth period*	Drought response
Raritan	short	sensitive
Vanderplank	short	sensitive
Devlin	short	tolerant
Aviva	short	tolerant
Sebago	medium	sensitive
Ono	medium	sensitive
Darius	medium	tolerant
Baraka	medium	tolerant
Bravo	long	sensitive
Kimberley Choice	long	sensitive
Hoëvelder	long	tolerant
Late Harvest	long	tolerant

* Growth period is measured from emergence to haulm die-back.

Short growth period	:	± 80- 90 days
Medium growth period	:	± 90-100 days
Long growth period	:	± 100-130 days

Determination of chlorophyll a and b.

Chlorophyll a and b was extracted from 0.25 g freeze dried leaf material in 5 ml 80% acetone - chlorophyll extract. The concentrations of chlorophyll *a* and chlorophyll *b* were measured spectrophotometrically at 663 nm (chlorophyll a) and 645 nm (chlorophyll b) in ug ml^{-1} plant extract. The individual levels of both chlorophyll *a* and *b* were calculated using the coefficients and equations derived by Lichtenthaler and Wellburn [20] . The total chlorophyll *a* and *b* was also calculated.

Statistical analysis

The analysis of variance (ANOVA) was not calculated for the chlorophyll fluorescence parameters or the chlorophyll content as the coefficient of variation (CV) was generally higher than 30%. The high .lue of CV could be ascribed to the fact that the variances were not homogenic. Furthermore, the skewness of the chlorophyll fluorescence data varied between negative and positive. If the data were normally distributed, the skewness would be zero. High CV values are common when environmental stress components are measured. As such the variation within treatments exceeds the variation between treatments.

The least significant differences were calculated for chlorophyll content using statistic software (Statistica for Windows, Version 5).

Results

This study was carried out to determine whether chlorophyll fluorescence under simulated drought conditions correlated with potato response to drought in the field.

Minimal fluorescence (Fo)

According to Krause and Weis[21] lower Fo values may be an indication of drought tolerance. Thus, it is expected that the drought tolerant cultivars (Table 1) should have lower Fo values during drought stress when compared to the sensitive cultivars. In the results presented here the drought tolerant cultivars with a short growth period (Devlin and Aviva) had lower mean values in the stress treatment compared to the control treatment. An increase in Fo values indicating a sensitive reaction was observed after two weeks without water in the sensitive cultivars with a short growth period. (Raritan and Vanderplank) (Table 2). However, it should be noted that we are considering tendencies. Due to the nature of the data the differences are not significant. When the cultivars with a medium growth period were considered, results were inconsistent with two cultivars, Baraka and Sebago which are tolerant and sensitive respectively, reacting as expected while Ono and Darius gave opposite reactions to the field data. The cultivars with a long growth period reacted as expected with Late Harvest giving a tolerant reaction and Bravo and Kimberley choice showing sensitivity to drought. Hoëvelder was the exception, it is drought tolerant in the field but gave a sensitive reaction using Fo values (Table 1). A sensitive reaction would be higher Fo values during the stress treatment compared to the Fo values in the control treatment.

Table 2: Changes in the value of Fo with time after withholding water in stressed and control plants of different potato cultivars. three replicates were measured and were presented as mean ± standard deviation (cm X10) (cm x 10)

Cultivar	Treatment	Week 1	Week 2	Week 3	Week 4 ◊
Raritan	control	0.26 ± 0.01	0.37 ± 0.05	0.36 ± 0.01	-
	stress	0.30 ± 0.03	0.39 ± 0.17	0.35 ± 0.01	-
Vanderplank	control	0.25 ± 0.05	0.32 ± 0.06	0.33 ± 0.09	0.40 ± 0.10
	stress	0.27 ± 0.01	0.35 ± 0.08	0.28 ± 0.04	0.42 ± 0.12
Devlin	control	0.26 ± 0.01	0.34 ± 0.10	0.28 ± 0.01	0.39 ± 0.07
	stress	0.25 ± 0.03	0.33 ± 0.13	0.26 ± 0.04	0.34 ± 0.12
Aviva	control	0.27 ± 0.05	0.32 ± 0.11	0.31 ± 0.02	0.37 ± 0.07
	stress	0.25 ± 0.05	0.32 ± 0.11	0.27 ± 0.02	0.42 ± 0.10
Sebago	control	0.24 ± 0.01	0.34 ± 0.11	0.27 ± 0.07	0.41 ± 0.02
	stress	0.27 ± 0.02	0.41 ± 0.20	0.28 ± 0.04	0.42 ± 0.18
Ono	control	0.25 ± 0.02	0.38 ± 0.22	0.30 ± 0.01	0.41 ± 0.07
	stress	0.27 ± 0.02	0.28 ± 0.02	0.25 ± 0 01	0.44 ± 0.14
Darius	control	0.28 ± 0.03	0.29 ± 0.04	0.31 ± 0.03	0.35 ± 0.07
	stress	0.26 ± 0.05	0.46 ± 0.20	0.31 ± 0.08	0.42 ± 0.02
Baraka	control	0.26 ± 0.01	0.32 ± 0.07	0.30 ± 0.07	0.40 ± 0.04
	stress	0.28 ± 0.04	0.28 ± 0.01	0.30 ± 0.04	0.39 ± 0.04
Bravo	control	0.25 ± 0.04	0.31 ± 0.10	0.29 ± 0.01	0.31 ± 0.01
	stress	0.22 ± 0.03	0.31 ± 0.11	0.25 ± 0.01	0.41 ± 0.16
Kimberley Choice	control	0.25 ± 0.01	0.33 ± 0.10	0.26 ± 0.01	0.37 ± 0.02
	stress	0.24 ± 0.01	0.30 ± 0.08	0.34 ± 0.02	0.38 ± 0.07
Hoëvelder	control	0.29 ± 0.05	0.33 ± 0.07	0.28 ± 0.02	0.42 ± 0.07
	stress	0.23 ± 0.06	0.41 ± 0.24	0.31 ± 0.03	0.41 ± 0.07
Late Harvest	control	0.27 ± 0.02	0.29 ± 0.04	0.26 ± 0.01	0.40 ± 0.07
	stress	0.23 ± 0.01	0.27 ± 0.01	0.27 ± 0.01	0.40 ± 0.12

◊ Weeks without water

Photochemical maximum (Fm)

Sirvirason, Takeda and Senboku [15] showed that Fm is an indication of the ability of the plant to absorb light. The number of weeks after which maximum values are reached before the subsequent decline is of importance. Peak values of Fm were reached in two cultivars after two weeks without water (Raritan(SGP) and Sebago (MGP)) and in four cultivars (Devlin (SGP), Aviva (SGP), Late Harvest (LGP) and Kimberley Choice (LGP) after four weeks without water. The cultivars which reached peak values after two weeks without water were both sensitive to drought. The cultivars which reached peak values after four weeks without water included three drought tolerant cultivars (Devlin (SGP), Aviva (SGP) and Late Harvest (LGP) and one sensitive cultivar (Kimberley Choice (LGP)) (Table 3). From the results it was clear that Fm was an indication of drought tolerance for cultivars with a short growth period while results from cultivars with medium and long growth periods were inconclusive. However, it should be noted that we are considering tendencies. Due to the nature of the dat the differences are not significant.

Maximum quantum yield of photosystem II (Fv/Fm)

The ratio Fv/Fm is an indication of the maximum quantum yield of photosystem II. Brüggeman *et al.* [22] found that a ratio of 0.78 correlated with drought tolerance in tomato cultivars and a ratio of 0.67 correlated with drought sensitivity. In the present study with potato ratio Fv/Fm decreased to a ratio below 0.78 after two weeks without water in all of the twelve cultivars (Table 4). It was decided to use the tomato ratios on potatoes as both are Solanaceae.

In the work presented here, the Fv/Fm values were higher than 0.78 for six cultivars (Bravo (LGP), Late Harvest (LGP), Darius (MGP), Kimberley Choice (LGP), Hoëvelder (LGP) and Vanderplank (SGP)) after one week without water. Vanderplank (SGP), Bravo (LGP) and Kimberley Choice (LGP) are drought sensitive while Darius (MGP), Hoëvelder (LGP) and Late Harvest (LGP) are drought tolerant (Table 4). The Fv/Fm ratios were larger than 0.78 in all but two of the controls (Late Harvest and Kimberley Choice) after two weeks without water. In

general the Fv/Fm ratios were higher in the controls of the cultivars with a long growth period. Cultivars with a long growth period are also generally more drought tolerant compared to cultivars with a shorter grow period (A. Visser; personal communication). After three weeks without water an increase in Fv/Fm values above 0.78 was observed in five cultivars (Devlin (SGP), Aviva (SGP), Bravo (LGP), Late Harvest (LGP) and Ono (MGP)). Three of these cultivars (Devlin, Aviva and Late Harvest) were drought tolerant while two cultivars (Bravo and Ono) were drought sensitive (Table 4).

After four weeks without water four cultivars had values higher than 0.70. These cultivars included three drought tolerant cultivars (Devlin (SGP), Aviva (SGP) and Late Harvest (LGP)) and one drought sensitive cultivar (Kimberley Choice (LGP)). Additionally, after four weeks without water five cultivars had Fv/Fm ratio's lower than 0.67 indicating a sensitive reaction. These cultivars included two drought tolerant cultivars according to field trials (Darius (MGP) and Hoëvelder (LGP)) and three drought sensitive cultivars (Vanderplank (SGP), Ono (MGP) and Bravo (LGP))(Table 4). As a summary it was shown that the cultivars with a short growth period reacted as expected while the parameter was not successful in correlating laboratory results with field evaluations in medium and long growth period cultivars.

Antenna efficiency of photostem II (Fv'/Fm')

During increased temperatures or sensitivity (caused by drought) the photosystem II reaction centres are blocked followed by a dissociation of antennae pigment protein complexes[13]. This results in a decrease in the amount of photosystem II centres bound to the photosynthetic membrane.

Table 3: Changes in the value of Fm with time after withholding water in stressed and control plants of different potato cultivars. Three replicates were measured and were presented as mean ± standard deviation (cm x 10)

Cultivar	Treatment	Week 1	Week 2	Week 3	Week 4 ◊
Karitan	control	1.30 ± 0.04	1.44 ± 0.18	1.17 ± 0.18	
	stress	1.23 ± 0.03	1.38 ± 0.23	0.46 ± 0.01	
Vanderplank	control	1.25 ± 0.14	1.38 ± 0.15	1.49 ± 0.30	1.46 ± 0.23
	stress	1.28 ± 0.06	1.32 ± 0.10	1.34 ± 0.12	1.02 ± 0.08
Devlin	control	1.24 ± 0.15	1.42 ± 0.05	1.26 ± 0.10	1.32 ± 0.02
	stress	1.13 ± 0.07	1.23 ± 0.14	1.33 ± 0.22	1.34 ± 0.16
Aviva	control	1.25 ± 0.13	1.33 ± 0.17	1.59 ± 0.08	1.49 ± 0.06
	stress	1.17 ± 0.06	1.18 ± 0.11	1.32 ± 0.26	1.34 ± 0.04
Sebago	control	1.18 ± 0.20	1.43 ± 0.08	1.39 ± 0.03	1.45 ± 0.03
	stress	1.21 ± 0.04	1.33 ± 0.20	1.25 ± 0.05	1.29 ± 0.26
Ono	control	1.26 ± 0.07	1.47 ± 0.31	1.46 ± 0.03	1.38 ± 0.08
	stress	1.24 ± 0.09	1.21 ± 0.06	1.23 ± 0.08	1.32 ± 0.03
Darius	control	1.39 ± 0.13	1.30 ± 0.13	1.46 ± 0.12	1.23 ± 0.27
	stress	1.19 ± 0.09	1.38 ± 0.21	1.33 ± 0.04	1.05 ±0.24
Baraka	control	1.29 ± 0.07	1.43 ± 0.18	1.57 ± 0.09	1.41 ± 0.22
	stress	1.33 ± 0.19	1.21 ± 0.16	1.43 ± 0.12	1.21 ± 0.16
Bravo	control	1.36 ± 0.10	1.35 ± 0.07	1.36 ± 0.04	1.32 ± 0.12
	stress	1.10 ± 0.08	1.19 ± 0.14	1.16 ± 0.10	1.17 ± 0.06
Kimberley Choice	control	1.26 ± 0.04	1.51 ± 0.21	1.27 ± 0.03	1.47 ± 0.11
	stress	1.18 ± 0.08	1.26 ± 0.18	1.37 ± 0.11	1.37 ± 0.03
Hoëvelder	control	1.14 ± 0.23	1.46 ± 0.08	1.41 ± 0.08	1.54 ± 0.13
	stress	1.11 ± 0.15	1.31 ± 0.30	..41 ± 0.37	0.98 ± 0.11
Late Harvest	control	1.33 ± 0.04	1.33 ± 0.05	1.33 ± 0.02	1.49 ± 0.10
	stress	1.12 ± 0.10	1.19 ± 0.07	1.27 ± 0.11	1.37 ± 0.02

◊ Weeks without water

Table 4: Changes in maximum quantum yield of the primary photochemistry (Fv/Fm) after withholding water in stressed and control plants of different potato cultivars. Three replicates were measured and were presented as mean ± standard deviation (cm x10)

Cultivar	Treatment	Week 1	Week 2	Week 3	Week 4 ◊
Raritan	control	0.79 ± 0.05	0.73 ± 0.08	0.69 ± 0.03	
	stress	0.75 ± 0.02	0.72 ± 0.08	0.24 ± 0.01	
Vanderplank	control	0.79 ± 0.02	0.76 ± 0.03	0.78 ± 0.02	0.72 ± 0.02
	stress	0.78 ± 0.01	0.73 ± 0.05	0.79 ± 0.01	0.57 ± 0.15
Devlin	control	0.79 ± 0.02	0.75 ± 0.07	0.79 ± 0.06	0.73 ± 0.09
	stress	0.76 ± 0.03	0.73 ± 0.07	0.78 ± 0.01	0.70 ± 0.05
Aviva	control	0.79 ± 0.01	0.76 ± 0.05	0.80 ± 0.02	0.71 ± 0.07
	stress	0.76 ± 0.03	0.71 ± 0.12	0.79 ± 0.01	0.72 ± 0.06
Sebago	control	0.78 ± 0.03	0.76 ± 0.06	0.80 ± 0.01	0.71 ± 0.01
	stress	0.77 ± 0.02	0.70 ± 0.10	0.77 ± 0.02	0.68 ± 0.07
Ono	control	0.80 ± 0.02	0.75 ± 0.08	0.79 ± 0.05	0.70 ± 0.03
	stress	0.77 ± 0.02	0.76 ± 0.01	0.79 ± 0.10	0.66 ± 0.09
Darius	control	0.80 ± 0.01	0.77 ± 0.05	0.78 ± 0.06	0.70 ± 0.07
	stress	0.78 ± 0.03	0.66 ± 0.01	0.76 ± 0.05	0.56 ± 0.01
Baraka	control	0.797± 0.07	0.77 ± 0.04	0.80 ± 0.07	0.71 ± 0.01
	stress	0.78 ± 0.04	0.76 ± 0.04	0.79 ± 0.01	0.69 ± 0.08
Bravo	control	0.81 ± 0.02	0.77 ± 0.06	0.78 ± 0.01	0.76 ± 0.01
	stress	0.80 ± 0.02	0.74 ± 0.06	0.78 ± 0.07	0.64 ± 0.15
Kimberley Choice	control	0.80 ± 0.09	0.78 ± 0.03	0.79 ± 0.01	0.74 ± 0.05
	stress	0.79 ± 0.02	0.76 ± 0.03	0.74 ± 0.01	0.71 ± 0.06
Hoëvelder	control	0.79 ± 0.01	0.77 ± 0.03	0.798 ± 0.03	0.72 ± 0.02
	stress	0.78 ± 0.07	0.69 ± 0.01	0.77 ± 0.01	0.58 ± 0.02
Late Harvest	control	0.79 ± 0.01	0.78 ± 0.03	0.80 ± 0.01	0.72 ± 0.02
	stress	0.79 ± 0.03	0.77 ± 0.02	0.78 ± 0.08	0.70 ± 0.09

◊ Weeks without water

Table 5: Changes in the antenna efficiency of photosystem II after withholding water in stressed and control plants of different potato cultivars. Three replicates were measured and were presented as mean ± standard deviation (cm x 10)

Cultivar	Treatment	Week 1	Week 2	Week 3	Week 4 ◊
Raritan	control	1.32 ± 0.74	2.03 ± 0.20	4.11 ± 1.96	
	stress	1.79 ± 0.32	3.94 ± 0.70	4.00 ± 0.09	
Vanderplank	control	0.66 ± 0.54	2.25 ± 1.94	2.23 ± 1.07	2.36 ± 0.51
	stress	1.01 ± 0.75	1.81 ± 1.33	2.90 ± 0.42	1.25 ± 1.21
Devlin	control	0.99 ± 0.66	2.31 ± 1.03	1.85 ± 0.40	3.48 ± 0.32
	stress	1.10 ± 0.05	24.72 ± 9.23	4.55 ± 2.04	4.00 ± 0.70
Aviva	control	1.34 ± 0.97	1.31 ± 0.48	1.64 ± 0.30	2.82 ± 0.85
	stress	1.27 ± 0.93	3.38 ± 3.12	2.03 ± 0.89	9.50 ± 3.53
Sebago	control	0.87 ± 0.47	1.87 ± 0.86	2.72 ± 0.54	3.50 ± 0.12
	stress	1.31 ± 0.66	17.27 ± 8.33	1.25 ± 0.58	7.16 ± 0.83
Ono	control	1.15 ± 0.77	1.02 ± 0.17	1.93 ± 0.42	6.25 ± 0.30
	stress	1.26 ± 0.84	1.94 ± [illegible]	3.64 ± 0.90	39.00 ± 3.84
Darius	control	1.29 ± 0.57	1.41 ± 0.62	1.87 ± 0.17	2.63 ± 1.93
	stress	0.95 ± 0.66	1.89 ± 0.57	2.62 ± 0.88	58.50 ± 2.83
Baraka	control	1.54 ± 0.12	1.18 ± 0.90	1.60 ± 0.56	1.16 ± 0.62
	stress	1.18 ± 0.98	2.57 ± 2.02	1.67 ± 0.19	37.50 ± 5.96
Bravo	control	1.36 ± 0.88	1.76 ± 0.04	3.50 ± 0.70	4.31 ± 3.79
	stress	0.99 ± 0.68	1.78 ± 1.22	4.13 ± 0.33	9.66 ± 0.47
Kimberley Choice	control-	1.49 ± 0.07	1.19 ± 0.50	1.80 ± 0.67	2.55 ± 0.97
	stress	1.48 ± 0.53	1.18 ± 0.42	1.82 ± 0.60	4.58 ± 0.53
Hoëvelder	control	0.90 ± 0.71	2.15 ± 1.28	1.49 ± 0.22	2.03 ± 0.51
	stress	0.72 ± 0.67	3.70 ± 3.25	2.75 ± 0.35	7.50 ± 7.07
Late Harvest	control	1.14 ± 0.57	3.06 ± 3.45	1.74 ± 0.02	1.95 ± 0.40
	stress	1.03 ± 0.59	2.52 ± 0.14	1.51 ± 0.07	4.80 ± 0.52

◊ Weeks without water

The antenna efficiency of photosystem II (Fv^l/Fm^l) grouped the cultivars in three categories (Table 5): group I increased after two weeks and included the cultivars Bravo (LGP) and Raritan (SGP); group II increased after three weeks without water and included the cultivars Darius (MGP), Vanderplank (SGP), Hoëvelder (LGP), Kimberley Choice (LGP), Baraka (MGP) and Ono (MGP); group III showed an increase at week two, a decrease at week three and an increase again at week four without water and included the cultivars Devlin (SGP), Aviva (SGP), Late Harvest (LGP) and Sebago(MGP). Additionally the ratios varied from 0.6 to 58.5. From these results it is clear that the data is unreliable and a correlation with drought tolerance was not observed with this parameter (Table 5).

Chlorophyll a and b levels

The chlorophyll-protein complexes are important components of the photosynthetic apparatus, in which light energy is captured and transferred to reaction centres [23]. Two mechanisms are involved in the formation of chlorophyll-protein complexes namely the distribution of newly synthesized chlorophyll or by redistribution of chlorophyll. Chlorophyll *b* is biosynthetically derived from chlorophyll *a* and may play an important role in the reorganization of photosystems during adaption to light quality and intensity[24]. As a result the loss of chlorophyll *a* and/or *b* will have a negative effect on the efficiency of photosynthesis. In addition to chlorophyll fluorescence, the levels of chlorophyll *a* (Table 6), chlorophyll *b* (Table 7), total chlorophyll content (Table 8) and the ratio between chlorophyll *a* and *b* (Table 9) were determined.

The concentrations of chlorophyll *a* in samples after one week without water did not differ significantly to their respective controls with the exception of Vanderplank (SGP) where a significant increase ($P<0.05$) was observed. During the second week without water there were no significant differences between the stressed and control treatments. However, during the third week without water a significant decrease in chlorophyll *a* content was observed in the cultivars Vanderplank (SGP), Ono (MGP), Darius (MGP), Baraka (MGP), and Bravo (LGP) (Table 6). The levels of

chlorophyll *a* were significantly lower ($P<0.05$) in leaves after four weeks without water from the cultivars Vanderplank (SGP), Raritan (SGP), Ono (MGP), Darius (MGP), Baraka (MGP) and Bravo (LGP). From these only the two medium growers Darius and Baraka were drought tolerant (Table 6).

The level of chlorophyll *b* was variable during the stress period of four weeks. After one week without water a significant increase in chlorophyll *b* content compared to well-watered controls were observed in the cultivars Raritan (SGP) and Vanderplank (SGP) and a significant decrease was observed in the cultivars Sebago (MGP), Hoëvelder (LGP) and Late Harvest (LGP). After two weeks without water a significant decrease in chlorophyll *b* levels compared to well watered controls were observed in the cultivars Raritan (SGP), Devlin (SGP) and Baraka (MGP). In the third week without water the significant decrease ($P<0.05$) in chlorophyll *b* content was observed in six cultivars namely Vanderplank (SGP), Devlin (SGP), Aviva (SGP), Ono (MGP), Baraka (MGP) and Hoëvelder (LGP) (Table 7). After four weeks without water, the levels of chlorophyll *b* was significantly lower in all cultivars except Devlin (SGP), Aviva (SGP) and Sebago (MGP) (Table 7).

Table 6: Change in Chlorophyll *a* levels with time in drought stressed and control potato cultivars. Three replicates were measured ($\alpha = 0.05$)

Cultivar	Treatment	Week 1		Week 2		Week 3		Week 4	◊
Raritan	control	2.07	NS	1.80	NS	3.47	NS	2.60	*↓
	stress	2.94		1.41		2.37		0.45	
Vanderplank	control	2.02	*↑	2.61	NS	3.48	*↓	3.19	*↓
	stress	3.445		1.69		1.07		0.36	
Devlin	control	0.81	NS	1.14	NS	0.99	NS	0.69	NS
	stress	0.97		0.88		0.93		0.51	
Aviva	control	1.93	NS	2.75	NS	2.58	NS	2.27	NS
	stress	1.50		1.95		2.10		1.34	
Sebago	control	1.72	NS	1.73	NS	1.28	NS	1.10	NS
	stress	1.17		1.15		0.94		0.79	
Ono	control	0.13	NS	1.62	NS	0.19	*↓	1.67	*↓
	stress	0.36		1.62		0.34		1.23	
Darius	control	1.61	NS	2.57	NS	3.40	*↓	2.47	*↓
	stress	1.38		1.95		1.49		0.31	
Baraka	control	1.78	NS	2.22	NS	3.52	*↓	3.18	*↓
	stress	1.61		1.93		1.54		0.52	
Bravo	control	1.93	NS	2.83	NS	3.58	*↓	3.64	*↓
	stress	1.86		2.16		1.87		0.85	
Kimberley Choice	control	1.76	NS	2.20	NS	2.71	NS	2.06	NS
	stress	2.32		1.42		2.38		1.19	
Hoëvelder	control	2.63	NS	2.66	NS	3.07	NS	1.95	NS
	stress	1.91		1.75		2.10		1.77	
Late Harvest	control	2.55	NS	3.26	NS	3.10	NS	2.69	NS
	stress	1.42		2.40		2.27		1.59	

◊ Weeks without water

NS - Not significant

*↑ - Significant increase

*↓ - Significant decrease

The levels of total chlorophyll content ($a + b$) varied over time in the stress treatment compared to the well watered controls. After one week without water a significant increase in chlorophyll content was observed in the cultivars Raritan (SGP) and Vanderplank (SGP) while a significant decrease was observed in the cultivars Sebago (MGP) and Late Harvest (LGP). In the second week without water a significant decrease ($P<0.05$) in total chlorophyll content was observed in the cultivars Vanderplank (SGP) and Aviva (SGP). After three weeks without water a significant decrease in total chlorophyll content was observed in four cultivars namely Vanderplank (SGP), Darius (MGP), Baraka (MGP) and Bravo (LGP) and an significant increase was found in the cultivar Ono (MGP). After four weeks without water a significant decrease in total chlorophyll content was found in eight of the twelve cultivars (Raritan (SGP), Vanderplank (SGP), Sebago (MGP), Darius (MGP), Baraka (MGP), Bravo (LGP), Kimberley Choice (LGP) and Late Harvest (LGP) (Table 8). Total chlorophyll levels ($a + b$) after four weeks without water (Table 8) yielded similar results to chlorophyll b levels (Table 7). Total chlorophyll levels were significantly lower compared to control treatments in all cultivars with the exception of Devlin (SGP), Aviva (SGP), Ono (MGP) and Hoëvelder (LGP).

The ratio between chlorophyll a and b was mostly non significant ($P<0.5$) during the four weeks without water with few exceptions namely a significant increase in Bravo (LGP) after one week without water, Raritan (SGP) after two weeks without water and Sebago (MGP) after three weeks without water and a significant decrease in the chlorophyll a to b ratio in the cultivar Aviva (SGP) after four weeks without water (results not shown).

Results from chlorophyll a and b measurements were in agreement with chlorophyll fluorescence parameters as it could successfully distinguish between drought tolerance and drought sensitivity in cultivars with a short growth period but results were inconclusive for cultivars with a medium or long growth period (Table 9).

Table 7: Change in chlorophyll *b* levels with time in drought stressed and control potato cultivars. Three replicates were measured ($\alpha = 0.05$)

Cultivar	Treatment	Week 1		Week 2		Week 3		Week 4	◊
Raritan	control	0.67	* ↑	1.46	* ↓	2.09	NS	1.72	* ↓
	stress	1.78		0.57		1.19		0.17	
Vanderplank	control	0.84	* ↑	1.41	NS	2.15	* ↓	2.86	* ↓
	stress	2.24		0.83		0.51		0.21	
Devlin	control	1.17	NS	1.67	* ↓	2.33	* ↓	1.32	NS
	stress	1.25		0.84		1.16		0.58	
Aviva	control	0.70	NS	1.42	NS	1.45	* ↓	1.10	NS
	stress	0.72		0.76		0.05		0.79	
Sebago	control	1.28	* ↓	0.96	NS	1.21	NS	1.20	NS
	stress	0.09		0.98		0.27		0.38	
Ono	control	0.06	NS	1.11	NS	0.12	* ↓	0.99	* ↓
	stress	0.22		1.10		0.25		0.86	
Darius	control	0.66	NS	1.43	NS	1.18	NS	1.46	* ↓
	stress	0.95		0.81		0.73		0.18	
Baraka	control	1.07	NS	1.42	* ↓	2.10	* ↓	1.93	* ↓
	stress	0.69		0.63		0.57		0.23	
Bravo	control	0.65	NS	0.72	NS	1.73	NS	3.33	* ↓
	stress	0.41		0.32		0.87		0.19	
Kimberley Choice	control	0.74	NS	0.69	NS	1.34	NS	1.60	* ↓
	stress	1.05		1.10		1.00		0.69	
Hoëvelder	control	1.49	* ↓	1.23	NS	2.39	* ↓	2.13	* ↓
	stress	0.72		1.13		1.31		1.05	
Late Harvest	control	1.09	* ↓	1.69	NS	2.10	NS	1.37	* ↓
	stress	0.45		1.22		1.47		0.51	

◊ Weeks without water
NS - Not significant
* ↑ - Significant increase
* ↓ - Significant decrease

Table 8: Change in total chlorophyll levels ($a + b$) with in drought stressed and control potato cultivars. Three replicates were measured ($\propto = 0.05$)

Cultivar	Treatment	Week 1		Week 2		Week 3		Week 4	◊
Raritan	control	2.74	* ↑	3.26	NS	5.48	NS	4.32	* ↓
	stress	4.73		1.98		3.56		0.63	
Vanderplank	control	2.86	* ↑	4.03	* ↓	5.63	* ↓	6.05	* ↓
	stress	5.68		2.53		1.58		0.57	
Devlin	control	1.99	NS	2.82	NS	3.33	NS	2.02	NS
	stress	2.23		1.73		2.13		1.09	
Aviva	control	2.64	NS	4.18	* ↓	4.03	NS	3.37	NS
	stress	2.23		2.72		2.16		2.13	
Sebago	control	3.03	* ↓	2.43	NS	2.50	NS	2.30	* ↓
	stress	1.26		2.14		1.22		0.41	
Ono	control	0.20	NS	2.74	NS	0.31	*↑	2.66	NS
	stress	0.59		2.73		0.60		2.09	
Darius	control	2.28	NS	4.08	NS	4.59	* ↓	3.93	* ↓
	stress	2.33		2.76		2.22		0.49	
Baraka	control	2.85	NS	3.64	NS	5.62	* ↓	5.11	* ↓
	stress	2.30		2.57		2.12		0.75	
Bravo	control	2.59	NS	3.56	NS	5.31	* ↓	6.96	* ↓
	stress	2.28		2.48		2.74		1.04	
Kimberley Choice	control	2.51	NS	2.90	NS	4.06	NS	3.67	* ↓
	stress	3.37		2.52		3.38		1.88	
Hoëvelder	control	4.12	NS	3.90	NS	5.47	NS	4.09	NS
	stress	2.63		2.88		3.41		2.82	
Late Harvest	control	3.64	* ↓	4.96	NS	5.21	NS	4.06	* ↓
	stress	1.88		3.62		3.73		2.10	

◊ Weeks without water

NS - Not significant

*↑ - Significant increase

*↓ - Significant decrease

Discussion

Much information on photosynthetic processes can be obtained from chlorophyll fluorescence. The chlorophyll fluorescence parameters were used to provide an insight into damage occurring to electron transport of dark reactions. According to Jefferies [16] constant or minimum fluorescence (Fo) is induced by pre-illumination either with a weak modulated light or with far-red light (700-720 nm). Fo is an indication of fluorescence emission by excited antenna chlorophyll *a* molecules that occurred before the excitations have migrated to reaction centres. Fluorescence increases rapidly to Fm (maximum fluorescence) after illumination with actinic light. The fluorescence between Fo and Fm is termed variable fluorescence (Fv). Fv in turn consists of two phases namely: a rise to the point of inflection (I), followed by a slight dip (D) and another increase to Fm. Fv reflects the reduction of the primary electron acceptor Q_A. In the oxidised state Q_A quenches fluorescence which decreases gradually to a terminal level T. Quenching of Fv is dependent on re-oxidation of Q_A after establishment of carbon assimilation and also upon non-photochemical mechanisms of energy dissipation. Thus changes in Fo and Fv reflects on changes in primary photochemical events while changes in quenching of Fv includes effects on carbon assimilation.

Krause and Weis [21] showed that an increase in Fo (minimal fluorescence) may be an indication of permanent damage to photosystem II, thus lower Fo values may be an indication of drought tolerance. Additionally, a slight increase in Fo values may be a result of photoinhibition or broken chloroplasts. Jefferies [16] found a consistent reduction in Fo and Fv in drought stressed potato plants compared to irrigated plants. He suggested that drought reduced the amount of chlorophyll in leaf tissue. However, with the cultivars used in our trials the mean values of the control treatment were higher than that of the stress treatment in cultivars Baraka (MGP), Late Harvest (LGP), Ono (MGP), Devlin (SGP) and Aviva (SGP) (Table 2). This can be an indication that these cultivars adapt themselves to the stress period because of a tolerance to drought stress. The cultivar with the highest Fo for the stress treatment after two weeks

Table 9: Drought tolerance as estimated by chlorophyll fluorescence and chlorophyll *a* and *b* levels

Cultivar	Fo	Fm	Fv/Fm	Chl.*a*	Chl.*b*	Total Chl
Raritan	-	-	-	-	-	-
Vanderplank			-	-	-	-
Devlin	+	+	+	+	+	+
Aviva	+	+	+	+	+	+
Sebago	-	-		+	+	-
Ono	+	+	-	-	-	+
Darius	-	-	-	-	-	-
Baraka	+		+	-	-	-
Bravo		-	-	-	-	-
Kimberley Choice		+	+	+	-	-
Hoëvelder	-		-	+	-	+
Late Harvest	+	+	+	+	-	-

\+ = drought tolerant

\- = drought sensitive

Chl = chlorophyll

no entry means no response

without water was Darius (MGP) followed by Sebago (MGP), Hoëvelder (LGP) and Raritan (SGP). These cultivars showed permanent damage to their PS II after two weeks without water.

The higher the Fm (photochemical maximum) value, the more viable the plant as the photochemical maximum is an indication of the plant's ability to absorb light[15]. However, PS II was observed to be extremely robust to drought conditions and Fm values did not change significantly in tomatoes[11], *Ziziphus rotundifolia*[25], oak trees[26] and willow leaves[8,9]. Interestingly, Havaux [11] has shown that drought increased the tolerance of PS II photochemistry to heat or photoinhibitory light. In contrast the Fm (Table 3) value in this study reached peak values after four weeks without water in the tolerant cultivars Devlin (SGP), Aviva (SGP) and Late Harvest (LGP) as well as the drought sensitive cultivar Kimberley Choice (LGP). Raritan (SGP) and Sebago (MGP) reached their maximum after two weeks without water. Thus, with this parameter it can be concluded that cultivar Devlin (SGP), Aviva (SGP), Ono (MGP), Late Harvest (LGP) and Kimberley Choice (LGP) are tolerant to drought stress while Darius (MGP), Bravo (LGP), Raritan (SGP) and Sebago (MGP) are sensitive to drought stress.

It was previously found that a stress can affect the function of PS II and this reflects on the ratio (Fv/Fm) as a decrease. Brüggeman *et al.* [22] observed that a ratio of 0.78 suggest a tolerant tomato cultivar in contrast with a ratio of 0.67 which indicate sensitivity. As both tomatoes and potatoes are Solanaceae it was decided to use the ratios calculated for tomatoes on the results from potatoes. Fv/Fm values after one week without water was higher than 0.78 for the cultivars Bravo (LGP), Late Harvest (LGP), Darius (MGP), Kimberley Choice (LGP), Hoëvelder (LGP) and Vanderplank (SGP). After two weeks without water all the cultivar's Fv/Fm, decreased to a ratio below 0.78. In the third week without water some of the cultivars showed a higher value of Fv/Fm than in the second week, indicating a possible adaptation to the drought stress e.g. the cultivars: Devlin (SGP), Aviva (SGP), Bravo (LGP), Late Harvest (LGP) and Ono (MGP) showed a value higher than 0.78 (Table 4). In all the potato cultivars tested it was found that after four weeks without water, the

photochemical efficiency of PS II (Fv/Fm) of plants which had been stressed was lower than that of the control plants (Table 4). After four weeks without water only cultivars Vanderplank (SGP), Devlin (SGP), Aviva (SGP), Kimberley Choice (LGP) and Late Harvest (LGP) had Fv/Fm values higher than 0.70. Raritan (SGP) had a value of lower than 0.75 from the first week without water. Cultivars Darius (MGP), Hoëvelder (LGP) and Raritan (SGP) showed the lowest values over time. Kristjansdottir and Merker (1993)[27] compared Andean and European potato clones subjected to low temperatures using the Fv/Fm ratio's. They concluded that this parameter could be successfully incorporated into breeding programmes for cold tolerance on potato.

The antenna efficiency of PS II (Fv^l/Fm^l) grouped the cultivars in three categories (Table 5). A correlation with drought tolerance was not observed with this parameter.

In addition to chlorophyll fluorescence, the levels of chlorophyll *a* (Table 6), chlorophyll *b* (Table 7) total chlorophyll content (Table 8) and the ratio between chlorophyll *a* and *b* (results not shown) were determined. The results after four weeks without water were used as an indication of drought tolerance as this gave the best correlation to field trials. A significant decrease indicated drought sensitivity while non significant differences were an indication of drought tolerance. The results were summarized in Table 9. The week to week variations were very high as a result of light intensity in the glasshouses. The light intensity could not be controlled and cloudy wether influenced the results.

In conclusion it was found that with the chlorophyll fluorescence parameters used it could be shown that the cultivars Aviva (SGP), Devlin (SGP) and Late Harvest (SGP) were drought tolerant while Darius (MGP), Raritan (SGP), Vanderplank (SGP) and Bravo (LGP) were drought sensitive (Table 10). Intermediate reactions were observed for Sebago (MGP), Ono (MGP), Baraka (MGP), Kimberley Choice (LGP) and Hoëvelder (LGP). When the results of chlorophyll fluorescence as well as chlorophyll levels were compared to the drought tolerance as indicated in Table 9, a perfect correlation was found between drought tolerance and fluorescence results

for the cultivars with a short growth period. It is suggested to test a larger sample of cultivars with a short growth period to evaluate chlorophyll fluorescence as a screening parameter for drought tolerance.

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2.3 Addendum to chapter 2

The chlorophyll fluorescence parameters measured were as follow:

Fo - initial, constant or minimal fluorescence. Fo may be an indication of permanent damage to photosystem II (PS II), thus lower Fo values may be an indication of drought tolerance[1]. Additionally photoinhibition or broken chloroplasts may lead to an slight increase in Fo[2].

Fo^1 - The second minimal fluorescence measured after the fluorescence curve stabilized.

Fm - maximum fluorescence. The photochemical maximum is an indication of the plant's ability to absorb light, thus the higher Fm value, the more viable the plant[1,3].

Fm^1 - The second maximum fluorescence measured after the fluorescence curve stabilized.

Fv = Fm-Fo. - variable fluorescence[3].

Fv/Fm - photochemical efficiency of PS II[4]. The ratio Fv/Fm indicates the photochemical capacity of PS II and a decrease in this parameter is the most reliable sign of photoinhibition[5].

Q_A - primary quinone acceptor in PS II and

Q_B - secondary quinone acceptor in PS II[6].

qN - non-photochemical quenching of chlorophyll fluorescence.

qN $= 1 - (Fm^1 - Fo^1) / (Fm - Fo)$[7,8]

qP - photochemical quenching of chlorophyll fluorescence. A decrease in qP may be a consequence of limited dark reactions as inhibition of the Calvin cycle results in the accumulation of NADPH causing feedback - limitation of electron transport. The photochemical quenching also shows the proportion of open reaction centres, thus the ratio between oxidised quinone acceptors (Q_A) and the reduced quinone acceptors. $qP = (Fm^1 - Fs)(Fm^1-Fo^1)$[5,7,8].

1-qP - Photoinhibition occurs as a result of excessive excitation pressure on PS II and indicates empirically that plants start to lose PS II function when the steady state value of 1-qP was maintained above 0,43. This is an indication of loss of PS II function[9].

qE - energy dependent quenching caused by intrathylakoid acidification during light driven proton translocation accross the membrane. qE may also be viewed as a loss of the response of the membrane to the build up of a high increase in pH. This effect indicate the dynamic property of the thylakoid that regulates thermal energy dissipation in exess light[1,2].

qQ - As qQ and qE are strongly influenced by the utilization of reducing equivalents and ATP during photosynthesis, the quenching depends on metabolic activities. It is well known that the stress factor primarily inhibit the carbon reduction cycle and are therefor expected to become manifested by altered quenching coefficients, particularly of qQ (The inhibition of the carbon cycle would increase the proportion of reduced Q_A and thereby inhibit qQ).

$qQ = (Fm - Fv) / (Fm - Fo)$[1,10]

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CHAPTER 3

MEASUREMENT OF THE ENZYME ACTIVITY FROM THE ANTIOXIDATIVE SYSTEM IN RESPONSE TO DROUGHT STRESS

3.1 General Introduction

All organisms living in an aerobic atmosphere are confronted with the [illegible] of oxidation[1]. Although oxygen is a product of photosynthesis, a n[illegible] reactant in physiological processes as well as the terminal electron accept[illegible] light-independent mitochondrial respiration, it is also toxic[2]. The reac[illegible]s which occur during drought stress often result in the production of other chemical species e.g. hydroxy radicals, hydrogen peroxide and superoxide anions which have even higher oxidizing potential [1]. The ability of photosynthetic cells to tolerate these potentially toxic effects of oxidative damage depends upon the increased levels of antioxidants. Superoxide dismutase is a well described enzymatic antioxidant which breaks down the superoxide radical to hydrogen peroxide and dioxygen. The hydrogen peroxide is removed by glutathione reductase, dehydroascorbate reductase and ascorbate peroxidase through the Halliwell-Asada pathway[3]. Correlations between the simultaneous increase in two or more enzymes involved during the minimization of oxidative injury and known drought tolerance may enhance our understanding of drought tolerance. Malan, *et al*[4], found a correlation between drought tolerance in maize inbreds and CuZn superoxide dismutase and glutathione reductase activities. Increased activity of one enzyme alone did not confer drought tolerance.

In this study the activity of Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase was determined in twelve potato cultivars with known drought tolerance or susceptibility. Drought shock was induced three weeks after sprout emergence by the withholding of water. Results from the present study show that the changes in the activity of the enzymes measured were highly

significant during the second week of observation thus only these results were presented in chapter 3.2. It was shown that after two weeks without water the activity of glutathione reductase in stressed potato cultivars was consistently lower compared to control treatments. The levels of ascorbate peroxidase activity were generally higher in stressed plants compared to control plants. A negative correlation was observed between drought tolerance and increased Cu/Zn superoxide dismutase activity. It was concluded the ability of potatoes to maintain adequate levels of superoxide dismutase activity was more important than an increase in enzyme activity. In section 3.2 the results from the activity of Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxibase after one week, three weeks and four weeks without water were tabulated.

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3.2 Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase levels during drought stress in potato

Van der Mescht, A., De Ronde, J.A. and Rossouw, F.T. (1998)
South African Journal of Science (In press).

Abstract

Twelve potato cultivars with known drought tolerance or susceptibility were grown in a greenhouse under optimal conditions. Drought tolerance in potato is defined in terms of yield reduction under dryland conditions. Superoxide dismutase, glutathione reductase and ascorbate peroxidase activities were determined from freeze-dried leaf samples of the leaf on the third apical node. Differences in glutathione reductase and ascorbate peroxidase activities could not be correlated with drought tolerance. The activity of glutathione reductase in stressed potato cultivars were consistently lower compared to control treatments. The levels of ascorbate peroxidase activity were generally higher in stressed plants compared to control plants. A correlation was found between yield loss under dryland conditions and superoxide dismutase activity. The ability of potatoes to maintain adequate levels of superoxide dismutase activity seemed more important than an increase in enzyme activity.

Introduction

In South Africa, where drought is a severe problem, tolerance to drought stress of economically important crops is of great value. Since most plants can only survive a limited period of drought, an understanding of how drought affects their growth, metabolism, development and yield is essential[1].

The drought related responses in plants are of a complex nature and result from genomic re-organization and alterations in gene expression[2]. A central response during drought stress is the increased concentration of absicisic acid which stimulates the closure of stomatal guard cells to reduce water loss. The availability of CO_2 for photosynthesis is thus reduced, resulting in the misdirecting of electrons in the photosystems. This

process leads to the formation of reactive oxygen species[3]. Thus, as drought and other physiological stresses cause oxidative injury, high antioxidant capacity or increased levels of antioxidants can prevent cell damage and may correlate with stress tolerance. Superoxide dismutase (SOD) is a well described enzymatic antioxidant which breaks down the superoxide radical to hydrogen peroxide and dioxygen[4,5]. The hydrogen peroxide resulting from this reaction is potentially toxic to cells and is removed by glutathione reductase, dehydroascorbate reductase and ascorbate peroxidase through the Halliwell-Asada pathway. Glutathione reductase (GR) cooperates with SOD to remove superoxide radicals mainly in chloroplasts but also in the mitochondria and cytoplasm. According to Bowler [3] GR has a regulatory function due to the dependance of it's activity on the availability of NADPH. Additionally, this increase in GR enhances NADP availability and electrons can now be accepted from ferredoxin, thereby reducing superoxide formation. Peroxidases are also involved in reactions with a number of organic hydroperoxides. The reactions involve the acceptor molecules with simultaneous reduction of the peroxidic substrate namely ascorbate[6]. Ascorbate peroxidase activity is mainly found in the chloroplasts[3].

However, it is possible that mechanisms that reduce oxidative stress may play a secondary role during drought tolerance[3]. This may complicate a direct correlation between the increased concentrations of an enzyme and drought tolerance. Correlations between the simultaneous increase in two or more enzymes involved during the minimization of oxidative injury and known drought tolerance may enhance our understanding of drought tolerance. Malan, Greyling and Gressel [7], found a correlation between drought tolerance in maize inbreds and CuZn SOD and GR activities. Increased activity of one enzyme alone did not confer drought tolerance.

Due to the poor understanding of the complexity of drought tolerance and the lack of reliable non-destructive screening techniques, little success has been achieved in breeding tolerant potato cultivars[8]. Monk-Talbot *et al.*[9] found that qualitative and quantitative differences in superoxide dismutase exists between potato cultivars which vary in susceptibility to calcium-related disorders. Additionally, Perl *et al.* [10] found that transgenic potato lines harbouring either the *chl* or *cyt* SOD genes from tomato showed

elevated tolerance to paraquat. The aim of this study was to test whether there was a correlation between SOD, GR and ascorbate peroxidase concentrations and drought tolerance in potato cultivars in an attempt to screen for drought tolerance in a non-destructive way.

Materials and methods

Plant material

Twelve potato cultivars with known drought tolerance or susceptibility (Table 1) were grown in a glasshouse under conditions as previously described by Van der Mescht, *et al.* [11]. Drought stress was induced three weeks after sprout emergence by the withholding of water. The leaf on the third apical node was harvested weekly from drought stressed and non-stressed control plants. Leaf samples were freeze dried immediately after harvesting. The procedure continued for approximately 29 days at which time a lethal drought shock seemed to be induced. Six replicates were analysed.

Leaf water potential

Leaf water potential was monitored at weekly intervals of all twelve potato cultivars subjected to drought and the respective controls to monitor the drought stress. A pressure chamber, PMS-instrument, Oregon, U.S.A. was used for measurements[14]. Measurements were made between 8:30 and 10:00 in the morning.

Enzyme analysis

Enzyme extractions were performed as described by Malan *et al.* (1990)[7] with minor modifications. Leaf tissue (100 mg dry weight) was homogenized in 2.5 mℓ 0.1M potassium phosphate extraction buffer (pH 7.5) containing 0.1 mM EDTA, 200 mg polyvinylpyrrolidone and 1% w/v bovine serum albumin. The β-mercaptoethanol was omitted as this results in the inhibition of glutathione reductase activity[15]. Extracts were centrifuged at 13 000 x g for 30 minutes. SOD and GR activity in the supernatant were spectrophotometrically determined. The level of GR activity was measured by following the oxidation of NADPH spectrophotometrically at 340 nm[16]. SOD was calculated after measuring the inhibition of nitrate formation from hydroxyl ammonium chloride oxidation at 530 nm[17]. Enzyme activities was expressed as changes in absorbance min^{-1}

g^{-1} dry weight. Ascorbate peroxidase activity was measured at 265 nm[18]. The procedure is based on the rate of decrease in absorbance of ascorbate during ascorbate peroxidation.

Statistical analysis

The data was subjected to regression as well as variance analysis (ANOVA). The LSD (Bonferonni) was also determined. Regression analysis was on one autumn season. The experiment was repeated during the next autumn and the LSD (Bonferonni) was determined using data from both seasons.

Table 1. The drought response and growth period of potato cultivars. Drought tolerance is defined in terms of yield reduction under dryland conditions (A.F.Visser, personal communication, Nortjé and Visser [12] and Steyn *et al.*[13])

Cultivar	Growth period	Drought response
Raritan	short	sensitive
Vanderplank	short	sensitive
Devlin	short	tolerant
Aviva	short	tolerant
Sebago	medium	sensitive
Ono	medium	sensitive
Darius	medium	tolerant
Baraka	medium	tolerant
Bravo	long	sensitive
Kimberley Choice	long	sensitive
Hoëvelder	long	tolerant
Late Harvest	long	tolerant

Results

The leaf water potential did not differ significantly between cultivars in either the stress or control treatments. However, the leaf water potential was significantly lower in the stress treatment of all the cultivars compared to the control treatments (results not shown). The leaf water potential decreased from the mean -0.2 MPa in the controls to the mean value -1.2 MPa after one week without water, the mean value 1.6 MPa after two weeks without water and the mean value -1.9 MPa after three weeks without water.

Regression lines fitted curves to the activity of dismutase in potato plants over time during one season showed a parabolic curve. The activity of superoxide dismutase was maximal in the second week of observation (flowering stage). During the drought stress the activity of the enzyme followed a similar curve. In general, the area between the curves of stressed and control treatments was smaller in drought tolerant cultivars compared to drought sensitive cultivars (Figure 1). The statistical analysis (Table 2) during two seasons confirmed the observation that a significant increase in SOD activity at two weeks without water correlated with drought sensitivity.

The changes in enzyme activity were highly significant during the second week of observation thus only these results will be presented. The activity of SOD increased significantly in six cultivars of which five (Raritan, Ono, Sebago, Bravo and Kimberley Choice) were sensitive and one (Devlin) was tolerant to drought (Table 2). One of the sensitive cultivars (Raritan) had a short growth period, two of the sensitive cultivars had a medium growth period (Sebago and Ono) and two (Bravo and Kimberley Choice) had a long growth period. A significant decrease was observed for the tolerant cultivar Darius. (Table 2). The activity of GR in stressed potato cultivars was significantly less compared to control treatments in seven cultivars (Raritan, Devlin, Ono, Darius, Baraka, Kimberley Choice and Late Harvest). The changes in GR activity of cultivars subjected to drought compared to well watered control plants were non significant in the cultivars Vanderplank, Aviva and Bravo. The level of GR activity increased significantly in two cultivars (Sebago and Hoëvelder). Sebago is drought sensitive with a medium growth period while Hoëvelder is tolerant with a long growth period, (Table 3). There is no correlation between the GR activity and the drought response.The levels of ascorbate

peroxidase activity change significantly in nine out of the twelve cultivars. A significant increase in ascorbate peroxidase activity was observed in eight cultivars (Raritan, Vanderplank, Devlin, Ono, Darius Baraka, Bravo and Hoëvelder) and a significant decrease in one cultivar namely Aviva. Considering the eight cultivars with a significant increase in ascorbate peroxidase activity, four were sensitive and four were tolerant. Aviva is also drought tolerant (significant decrease). Non significant differences in enzyme activity was found for the cultivars Late Harvest, Kimberley Choice and Sebago (Table 4). A correlation between GR and/or ascorbate peroxidase activity and drought tolerance was not evident.

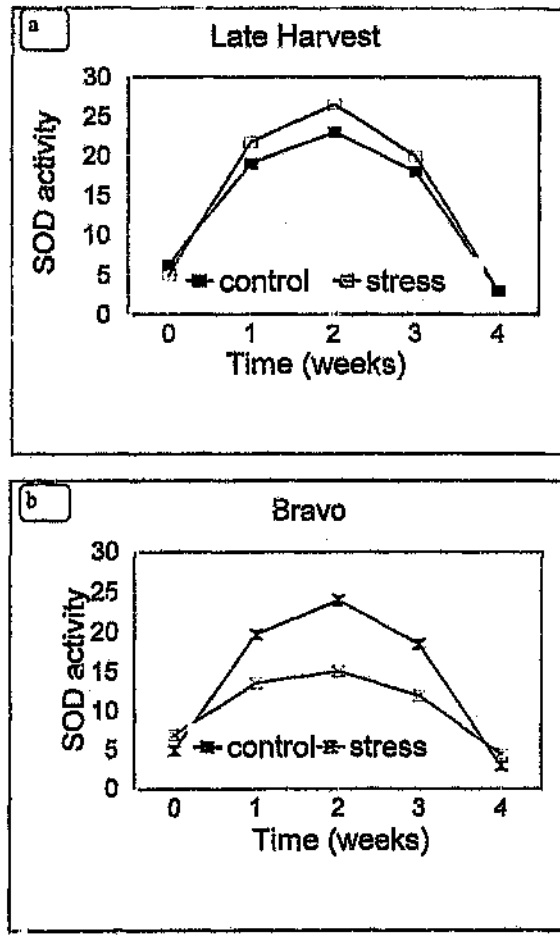


Figure 1: Regression lines of Cu/Zn superoxide dismutase (SOD) activity (nmol/g.dry weight) in drought stressed and control potato cultivars. Non significant differences after two weeks without water correlated with drought tolerance (a) while significant differences between the stress and control treatments after two weeks without water correlated with drought sensitivity (b). Enzyme activity was measured as units/gram dry weight.

Table 2. The mean values (n=6) of superoxide dismutase levels in drought stressed (two weeks without water) and non-stressed control potato plants. The LSD (Bonferroni) = 2.62. Enzyme activity was measured in nmol/gram dry weight.

Cultivar	Control treatment	Stress treatment	Significance ($\alpha \simeq 0.05$)
Raritan	20.83	36.21	significant increase
Vanderplank	32.48	34.22	non significant
Devlin	24.13	35.76	significant increase
Aviva	13.59	13.04	non significant
Sebago	29.37	35.77	significant increase
Ono	14.42	35.09	significant increase
Darius	35.08	25.96	significant decrease
Baraka	34.41	33.45	non significant
Bravo	15.95	34.77	significant increase
Kimberley Choice	27.42	36.21	significant increase
Hoëvelder	36.40	38.06	non significant
Late Harvest	37.28	37.58	non significant

Table 3: The mean values (n=6) of glutathione reductase activity in drought stressed (two weeks without water) and non-stressed control potato cultivars. The LSD (Bonferroni) = 1.14. Enzyme activity was measured in µmol NADPH consumed/minute/gram dry weight.

Cultivar	Control treatment	Stress treatment	Significance $\alpha = 0.05$
Raritan	17.84	15.20	significant decrease
Vanderplank	18.04	18.98	non significant
Devlin	20.28	18.25	significant decrease
Aviva	16.62	17.64	non significant
Sebago	17.13	21.25	significant increase
Ono	18.26	16.58	significant decrease
Darius	20.69	17.47	significant decrease
Baraka	20.31	17.16	significant decrease
Bravo	19.34	20.40	non significant
Kimberley Choice	21.02	16.99	significant decrease
Hoëvelder	16.89	19.69	significant increase
Late Harvest	18.67	16.88	significant decrease

Table 4: The mean values (n=6) of ascorbate peroxidase activity in drought stressed (two weeks without water) and non-stressed control potato cultivars. The LSD (Bonferroni) = 0.33. Enzyme activity was measured in nmol ascorbate oxidised/minute/gram dry weight.

Cultivar	Control treatment	Stress treatment	Significance $\alpha = 0.05$
Raritan	6.65	6.12	significant increase
Vanderplank	6.99	5.74	significant increase
Devlin	6.72	5.89	significant increase
Aviva	6.31	7.26	significant decrease
Sebago	5.47	5.34	non significant
Ono	7.99	7.05	significant increase
Darius	6.69	5.52	significant increase
Baraka	7.59	4.78	significant increase
Bravo	6.45	5.39	significant increase
Kimberley Choice	5.64	5.67	non significant
Hoëvelder	9.48	7.63	significant increase
Late Harvest	7.76	7.56	non significant

Discussion

The phenotypic expression of drought resistance is an interaction between morphological characteristics and physiological mechanisms. The physiological mechanisms involved during stress are usually due to different strategies which are a combination of mechanically - linked traits. These mechanisms vary between the extremes of avoidance and tolerance[19]. One of the possible tolerance mechanisms includes elevated activity of SOD, GR and peroxidises, especially ascorbate peroxidase, in an attempt to overcome oxidative stress. The possible benefits of elevated SOD, GR and ascorbate peroxidase activity during drought stress have been extensively described[3,5]. However, in most studies, changes in enzyme activities during increasing drought stress in sensitive and tolerant cultivars have been monitored without the comparison to a well-watered control over time. Malan *et al.* [7] stressed the importance to note that increased activity of only SOD or only GR is not sufficient to confer drought tolerance in maize and concluded that definite minimum threshold activities for both enzymes were needed for tolerance. Van Rensburg and Krüger [20] advocated the capacity to increase ascorbate peroxidase activity and GR activity as possible drought tolerance selection criteria in tobacco.

The leaf water potential did not differ significantly between cultivars. However, when drought stressed potato cultivars were compared to well watered control plants it was observed that the ability to maintain adequate activity or decreased activity of SOD correlated with drought tolerance as estimated in field trials. This may be due to the fact that drought tolerance is defined in terms of yield reduction, thus we are interested in plants avoiding drought by tuberization. The cultivars Vanderplank and Devlin showed opposite reactions. This may be explained by the interaction between heat tolerance and drought tolerance in Vanderplank. Drought tolerance was estimated in field trials where heat was not excluded. Vanderplank is either avoiding heat or is heat sensitive and may, thus, react as drought sensitive in field trials. The cultivar is possibly heat sensitive and drought tolerant. Yield may be enhanced in cooler areas. The cultivars Devlin and Darius are selections of the same cross (Kimberley choice x 890/20). A lethal stress was induced after three weeks without water. According to field trials these cultivars are drought tolerant confirming our opinion that drought avoidance via tuberization is more important than drought tolerance (vegetative growth during drought conditions).

According to Perl *et al.* [10] the SOD activity in plants should be adequate to reduce the toxic levels of superoxide. The resulting H_2O_2 should be scavenged by enzymes such as ascorbate peroxidase, GR or catalase to minimize the resulting toxic hydroxyl radical. However, it was observed that GR activity or ascorbate peroxidase activity is increased during drought stress in potato. The cultivar Sebago showed increased activity of GR and non-significant changes in ascorbate peroxidase activity, compared to the cultivar Aviva where a significant decrease in ascorbate peroxidase activity and non-significant changes in GR activity were observed. In potato the level of GR is not up-regulated during drought stress. This suggests that the levels of broad substrate peroxidase and catalase during drought stress should be investigated as well. Catalase activity is found mostly in peroxisomes and glyoxisomes where the H_2O_2 formed during photo- respiration is removed. Although its location is restricted it may play an important role in defence against oxidative stress as H_2O_2 readily diffuses across membranes. Peroxidises with a broad substrate specificity are important since they are found in the cell wall where they react with H_2O_2 to generate phenoxy compounds which polymerase to produce lignin[3].

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CHAPTER 4

THE EFFECT OF DROUGHT ON PROLINE AND POLYAMINE LEVELS

4.1 General Introduction

Maintaining water status is one of the challenges faced by plants exposed to drought. Water use efficiency is maximized throughout the life of a plant by developmental adaptions for example C_4 metabolism, reduced leaf area and thickened wax cuticles. On the other hand the immediate and sometimes catastrophic effect of drought conditions can be minimized by induced physiological responses. One of the most common induced responses in all organisms subjected to drought is the accumulation and/or production of compatible osmolytes such as sugars, certain amino acids and quaternary ammonium compounds. The immediate effect of water shortage is buffered as the accumulation of osmolytes lowers water potential and allows additional water to be taken up from the environment. The most widely distributed osmolyte is most probably the amino acid proline. In plants the role of proline is not only restricted to osmotic adjustment (if concentrated in the cytoplasm), proline synthesized during water deficit may serve as a nitrogen reserve and proline may help to stabilize protein tertiary structure as cells dehydrate[1].

Investigations by Levy [2] on free proline accumulation in drought stressed potato tubers showed ,paradoxically ,a correlation between low proline content and drought tolerance. Since the metabolic rate is low in storage organs such as tubers I reasoned that a metabolic more active tissue such as leaves would provide more information on free proline concentrations in potato. In Chapter 4.3 it was shown accordingly that increased levels of free proline did not correlate with drought tolerance. The results however, show that proline accumulation was a function of growth period. Drought tolerant cultivars with a short growth period accumulated most proline two weeks after water was Withheld, drought tolerant cultivars with a medium growth period accumulated most proline three weeks after water was

withheld and drought tolerant cultivars with a long growth period accumulated most proline four weeks after water was withheld.

There are increasing interest in the value of polyamines during abiotic stress as several of the physiological changes that are characteristic of plant senescence are also common to various types of environmental stress[3]. In response to drought, polyamines may partially replace calcium by binding to phospholipid components of the membrane thus maintaining membrane integrity[4]. Besford *et al.* [5] found a correlation between drought tolerance and putrescine accumulation in monocotyledons as well as increased concentrations of spermine and spermidine in drought tolerant dicotyledons during drought stress. The levels of putrescine, spermidine, spermine, diaminopropane and agmatine were determined during increasing drought stress. In chapter 4.2 it was shown that there is a correlation between spermine concentrations and drought tolerant in potato, after four weeks without water. Increased levels of spermidine and agmatine were not evident during drought stress (Chapter 4.3).

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4.2 Changes in free proline concentrations and polyamine concentrations in potato leaves during drought stress

Van der Mescht, A., De Ronde, J.A., Van der Merwe, T. and Rossouw, F.T. (1998) South African Journal of Science (In press)

Abstract

Investigations on free proline concentrations in stressed potato tubers showed, paradoxically, a correlation between low proline content and drought tolerance. As the metabolic rate is low in storage organs such as tubers we determined free proline concentrations in drought stressed potato leaves. Twelve potato cultivars with known drought tolerance or susceptibility were grown in a greenhouse under optimal conditions. Drought stress was induced three weeks after sprout emergence by withholding water. The leaf on the third apical node from drought- stressed and well-watered control plants was harvested and freeze- dried weekly. The levels of free proline could not be correlated with drought tolerance. However, results showed that proline accumulation was a function of the growth period. Spermine concentrations in the leaves after the plants were four weeks without water correlated with yield data under dryland conditions.

Introduction

The accumulation of proline has been reported in many organisms, from bacteria to higher plants in response to environmental stress. The adaptive value of proline accumulation to the plant is still debatable as the mechanism of action of the compound has not yet been fully elucidated [1]. Additionally the role of proline appears to vary in different species. This leads to conflicting opinions, such as that proline accumulation is beneficial to plants subjected to drought in contrast to the suggestion that proline accumulation is only an indication of damage caused during stress[2]. The possible physiological functions of proline accumulation during drought include the maintenance of osmotic potential in response to stress[3], as a nitrogen reserve in leaves able to recover after the stress[4], a storage compound for reduced nitrogen and carbon under stress situations[5], as a hydroxy-radical scavenger[6],

a protectant against denaturation of proteins[7], a means of controlling the cell pH thus reducing acidity, a role in the regulation of cellular redox potentials[6], and a signal of senescence[8].

The literature on the potential value of proline accumulation includes the research of Van Rensburg and Krüger [7] who showed a correlation between drought tolerance and the leaf water potential at which proline started to accumulate rapidly in tobacco. Proline accumulation was also positively correlated with drought tolerance in ten barley cultivars[9], with partial desiccation of celery somatic embryos[10], and with two maize cultivars[11]. Investigations by Levy [12] on stressed potato plants showed, paradoxically, a possible correlation between low proline content and drought tolerance in the tubers. Since the metabolic rate is low in storage organs such as tubers a more active tissue, such as potato leaves, should provide more information on free proline concentrations.
Furthermore, there is increasing interest in the value of polyamines during abiotic stress[13]. The syntheses of free proline and polyamines share a biochemical pathway at intermediates glutamic - acid and L-ornithine[14,15].It is suggested that the polyamines maintain the cation-anion balance in the plant cell. As putrescine, diaminopropane, spermidine and spermine are protonated at the physiological pH of cells, electrostatic binding of polyamines to nucleic acids, negatively charged functional groups of membranes and to proteins is favoured[16]. In binding to the negatively charged phospholipid groups on membranes, the polyamines influence the stability and permeability characteristics of these membranes. For example the loss of chlorophyll from thylakoid membranes is prevented by maintaining membrane integrity thus stabilizing the photosystem complexes during drought stress[17].

The electrostatic binding of polyamines to nucleic acids is well known. Spermine binds two phosphates on both of the DNA strands, imposing rigidity of structure. Alteration of the interaction between DNA and spermine results in the transformation from the functional B form of DNA to the nonfunctional Z form. Spermidine is an integral component of some t-RNAs implying a role in the transcription - translation sequence[18]. Its possible role may be as a second messenger. Additionally, it is reported that polyamines activate a nuclear protein kinase that phosphorylates non-histone proteins[18].

Besford *et al* [17] compared the accumulation of different polyamines during drought stress. They correlated putrescine accumulation in monocotyledons with drought tolerance, while spermine and spermidine accumulation were found in drought tolerant dicotyledons during drought stress.

The aim of the study reported here was to determine the levels of free proline as well as the concentrations of different polyamines during drought stress in potato leaves.

Materials and Methods

Plant material

Twelve potato cultivars with known drought tolerance or sensitivity (Table 1) were grown in a greenhouse under conditions as previously described by Van der Mescht, *et al.*[19]. Drought stress was induced three weeks after sprout emergence by withholding water. The leaf on the third apical node was harvested weekly from drought- stressed and well-watered control plants. Leaf samples were freeze-dried immediately after harvesting. The procedure continued for four weeks after which a lethal stress was imposed. Three replicates were measured for each cultivar at each time interval.

Free proline determination

The method of Bates, Waldren and Teare [20] was used with minor modifications. Freeze-dried leaf samples (0,1g) were homogenized in 10 ml of a 3 % sulphosalicylic acid solution. The homogenate was filtered through Whatman # 2 filter paper using a buchner funnel and vacuum pump. The reaction mixture containing 2 ml filtrate, 2 ml acid ninhydrin and 2 ml glacial acetic acid was incubated in test tubes for 1 hour at 100°C. The reaction was terminated on ice. Free proline was extracted by the addition of 4 ml toluene. The reaction mixture was mixed vigorously with a vortex for approximately 15 seconds and allowed to warm to room temperature. Absorbance of the chromophore containing toluene phase was read at 520 mm. The concentration of free proline was calculated from a standard curve using the following equation: [(µg proline/ml x ml toluene)/115,5 µg/µmole]/[(g sample)/5] = µmole proline/g dry weight.

Table 1. The drought response and growth period of potato cultivars. Drought tolerance is defined in terms of yield reduction under dryland conditions

Cultivar	Growth period*	Drought response
Raritan	short	sensitive
Vanderplank	short	sensitive
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Sebago	medium	sensitive
Ono	medium	sensitive
Darius	medium	tolerant
Baraka	medium	tolerant
Bravo	long	sensitive
Kimberley Choice	long	sensitive
Hoëvelder	long	tolerant
Late Harvest	long	tolerant

*Growth period is measured from emergence to haulm die-back.

Short growth period	± 80 - 90 days
Medium growth period	± 90 - 100 days
Long growth period	± 100 - 130 days

Polyamine analysis

The HPLC method, as developed by Flores and Galston [21] was used with minor modifications as the concentration of different polyamines can be determined at the same time. Polyamines were extracted from 0.1g freeze- dried leaves at 4°C. Samples were homogenized in 5ml of 5% perchloric acid and incubated for 1 hour on ice. After incubation, the samples were centrifuged at 14 000 rpm for 20 min at 4°C. An aliquot of 500μl of the supernatant was added to 1 ml 2M sodium hydroxide and 10μl benzoyl chloride. The mixture was incubated for 20 min at room temperature, 2 ml of saturated NaCl was added and the benzoylated polyamines were extracted in 2ml diethyl ether. The

mixture was centrifuged at 14 000 rpm at 4°C for 5 min and 1 ml of the ether phase was collected, evaporated to dryness and redissolved in 1 ml methanol. The samples were HPLC analysed at a flow rate of 1ml/minute. Separation was achieved using a step gradient elution in a methanol: water solvent system and a high- resolution reversed phase C18 column (250 mm x 4.6 mm in diameter) packed with 5μm size silica particles. The eluate was monitored by UV detection at an absorbance wave length of 254nm to determine the concentration of putrescine, diaminopropane, spermidine, spermine and agmatine. The peak values was calculated from a standard curve using standards from Sigma Aldrich.

Results

Free proline concentrations

Proline accumulation was measured at weekly intervals. As the variation within and between treatments were very high, the percentage accumulation was calculated as the difference between the stress and control treatments expressed as a percentage of total accumulation over time.

using the equation: (Sw-Cw)(St-Ct), where

Sw = mean value of stress treatment per week

Cw = mean value of control treatment per week

St = sum of mean values of stress treatments of four weeks

Ct = sum of mean values of control treatments of four weeks.

Results are presented in Table 2.

Cultivars sensitive to drought stress with a short growth period accumulated most proline later compared to the tolerant cultivars, e.g. Raritan after four weeks without water and Vanderplank after three weeks without water. The drought- sensitive cultivars with a medium growth period were inconsistent as Sebago accumulated most proline after two weeks and Ono only after four weeks. When drought- sensitive cultivars with a long growth period were considered, Kimberley Choice accumulated most proline after three weeks without water (one week earlier than tolerant cultivars).

Table 2. Proline accumulation calculated at weekly intervals for 12 potato cultivars and expressed as a percentage of total proline accumulation

Cultivar	Week			
	1	2	3	4
Raritan	10.0%	24.3%	15.0%	50.7%
Vanderplank	5.5%	30.3%	36.8%	27.4%
Devlin	7.1%	35.9%	25.7%	31.3%
Aviva	10.7%	34.5%	28.0%	26.8%
Sebago	14.3%	34.2%	21.4%	30.1%
Ono	3.3%	30.8%	32.7%	33.2%
Darius	11.9%	26.5%	37.0%	24.6%
Baraka	3.8%	25.1%	39.2%	31.9%
Bravo	8.9%	25.9%	28.4%	36.8%
Kimberley Choice	7.7%	27.8%	34.1%	30.4%
Hoëvelder	11.0%	28.1%	26.3%	34.6%
Late Harvest	14.0%	21.7%	29.5%	34.8%

Although Bravo is a sensitive cultivar, maximum proline was accumulated after four weeks. This is similar to tolerant cultivars with a long growth period.

From the results presented in Table 2, it is evident that proline accumulation is a function of growth period. Drought tolerant cultivars with a short growth period accumulated most proline two weeks after water was withheld, drought- tolerant cultivars with a medium growth period accumulated most proline after three weeks whereas drought- tolerant cultivars with a long growth period accumulated most proline after four weeks.

Polyamine concentration

The concentrations of putrescine, spermidine, spermine, diaminopropane and agmatine were determined during increasing drought stress. The analysis of variance (ANOVA) was not conducted on the polyamine levels as the coefficient of variation (CV) was generally

higher than 30%. The high value of CV could be ascribed to the fact that the variances were not homogenic. Furthermore, the skewness of the data varied between negative and positive. If the data were normally distributed, the skewness would be zero. High CV values are common when environmental stress components are measured.

Although putrescine and diaminopropane were compared with standards from Sigma Aldrich, these polyamines were not visualized in the samples under the HPLC conditions used in the analysis. The concentrations of spermine, spermidine and agmatine were cultivar- and age- dependent (results not shown). However, when the synthesis of spermine was evaluated at four weeks without water, a possible correlation with drought tolerance was observed (Table 3). Spermine was not detected under the HPLC conditions used in either the stress or control treatments of drought- sensitive cultivars with the exception of Ono. The detection limit was 0.05 nmol/g dry weight. In contrast to the sensitive cultivars spermine was detected in the stress and control treatments of the drought- tolerant cultivars with the exception of Darius for which spermine was detected only in the stress treatment.

Table 3: Spermine concentrations calculated at weekly intervals during drought stress in potato cultivars. Spermine concentrations were measured in nmol/g dry weight. Three replicates were measured and were presented as mean ± standard deviation.

Cultivar	Treatment	Week 1	Week 2	Week 3	Week 4*
Raritan	control stress	- -	0.05±0.01 0.06±0.02	- -	- -
Vanderplank	control stress	2.15±2.56 0.40±0.73	1.09±1.33 -	3.31±1.73 0.07±0.73	- -
Devlin	control stress	- 0.13±0.01	0.20±0.15 0.30±0.22	0.68±0.40 0.26±0.34	0.19±0.26 0.42±0.65
Aviva	control stress	1.67±2.44 0.09±0.04 -	0.050±.01 0.060±0.01	0.06±0.02 0.41±0.59	0.08±0.02 0.08±0.03
Sebago	control stress	- -	- -	- -	- -
Ono	control stress	0.90±0.86 0.58±0.22	0.69±0.11 0.60±0.49	0.76±0.21 0.83±0.25	0.70±0.13 0.72±0.10
Darius	control stress	- -	0.28±0.22 0.09±0.08	0.21±0.07 0.24±0.03	- 0.26±0.094
Baraka	control stress	0.24±0.33 0.05±0.02	0.19±0.16 0.54±0.69	0.09±0.04 0.07±0.01	0.61±0.83 0.26±0.05
Bravo	control stress	0.06±0.03 0.05±0.01	0.05±0.01 0.05±0.01	- -	- -
Kimberley Choice	control stress	- -	- -	- -	- -
Hoëvelder	control stress	0.42±0.22 0.58±0.31	1.19±1.50 0.60±1.17	0.63±1.13 2.00±2.34	0.57±0.06 0.59±0.06
Late Harvest	control stress	1.63±2.73 3.61±1.15	0.05±0.01 0.05±0.01	0.05±0.01 -	0.05±0.03 0.05±0.02

* Weeks without water

- Values below detection limit

Discussion

When the adaptive value of proline accumulation or polyamine levels needs to be estimated, two approaches could be followed. One is to use a single genotype and genetically manipulate proline/polyamine content or proline/polyamine synthesis. The changes in proline or polyamines could then be correlated with plant performance during stress. The second approach and the one followed in this study is to exploit genotypic differences in the rate of proline accumulation and polyamine levels in several genotypes[22]. The rate of proline accumulation as well as the spermine concentrations were similar to cultivar performance during drought stress.

The rate of proline accumulation was a function of maturity group (Table 2). The longer the growth period, the later proline accumulated in the drought- tolerant cultivars. Maximum proline formation occurred after two weeks without water in cultivars with a short growing period, after three weeks without water in cultivars with a medium growth period and after four weeks without water in cultivars with a long growth period. This is in agreement with the report of Singh, *et al.* [9] who showed that barley cultivars differ in the rate of proline accumulation during water stress. However, their results also showed that the quickest rates of proline accumulation were found in cultivars whose water status declined fastest during stress[9,10].

Plants growing in environments exposed to frequent and often severe drought may be adapted to survive in one of two ways. They may avoid drought by means of abbreviated life cycles, increased water absorption and/or by retarding water loss and tolerating drought[23]. It is possible that potatoes with a short growth period avoid drought by early tuberization. As drought tolerance is defined in terms of yield reduction, (yield reduction is greater in sensitive cultivars) these cultivars are termed drought tolerant. The cultivars able to sustain vegetative growth longer, take longer to accumulate maximum proline. Yield reductions are also greater in sensitive cultivars (Raritan and Vanderplank) than in tolerant cultivars (Devlin and Aviva). Thus in potato it seems that proline accumulation is an indication of injury to leaves rather than a tolerance mechanism.

The inconsistent timing of maximum proline accumulation in sensitive cultivars with a medium growth period may be explained because reactions to drought may be dominated by either avoidance or tolerance mechanisms. The sensitive cultivar Ono took longer than the tolerant cultivars to accumulate maximum proline. This may be explained in terms of the lack of an avoidance mechanism. The sensitive cultivar Sebago, however, showed maximum proline accumulation as early as two weeks without water. This may be an indication of an avoidance mechanisms and injury is shown early.

It is possible that drought tolerance mechanisms exist in cultivars with a long growth period. Maximum proline accumulation was shown after four weeks without water in tolerant cultivars. At this stage a lethal stress was induced in the leaves. A lethal stress is defined as the stage when plants will not recover when rewatered. The sensitive cultivar (Kimberley Choice) showed injury at an earlier stage. Kimberley Choice accumulated maximum proline after three weeks without water. As the tolerance mechanism is inferior to that in Hoëvelder and Late Harvest, injury, as indicated by maximum proline accumulation, was shown earlier. Although Bravo is sensitive to drought, injury (proline accumulation) was not shown earlier than in the tolerant cultivars.

The polyamine levels were evaluated in mature potato leaves. Although standard curves were obtained for putrescine, diaminopropane, spermine, spermidine and agmatine, the levels of putrescine and diaminopropane were probably too low to detect. This is in agreement with Pfosser, *et al.* [24] who stated that low polyamine titres were generally found in resting or mature tissue. Popappa and Millier [25] showed that polyamine concentrations were highest at early stages of development of strawberry fruit and later declined. The synthesis of spermine, spermidine and agmatine were cultivar and age dependent (results not shown), while the synthesis of spermine, after four weeks without water, showed a possible correlation with drought tolerance. Increased levels of polyamines were not evident during drought stress. Edrei, *et al.* [26] compared eight wheat varieties differing in drought and salt tolerance. Only the known salt tolerant variety responded with increased putrescine-levels during drought and salt stress. The spermidine concentration was slightly influenced by drought and salt stress whereas spermine concentration increased only under

salinity. When proline accumulation and polyamine concentrations were studied during osmotic adjustment of rape leaf discs, Aziz and Larher [27] found that the most abundant polyamine was spermidine. Additionally, the increase in polyamine levels occurred before the onset of proline accumulation during a moderate osmotic stress.

We recommend evaluating the role of polyamines during drought stress in potato using growing tissue such as root tips, and relative short time intervals. As free polyamines were determined in our study, it is also suggested that the polyamines conjugated to small molecules (trichloro acetic acid soluble conjugates) be evaluated as well as those bound to high molecular weight substances (trichloro acetic acid insoluble bound polyamines)[24].

Although adaptive metabolic responses to drought stress certainly exist, proline accumulation is probably not one of them for potato leaves. In this case proline accumulation is possibly an indication of injury rather than of tolerance as potatoes are probably avoiding drought by tuberization. However, this paper shows for the first time that proline accumulation is a function of growth period.

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CHAPTER 5

2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE REDUCTION AS A MEASUREMENT OF DROUGHT TOLERANCE

5.1 General Introduction

Viability during adverse environmental conditions can be tested using triphenyltetrazolium chloride (TTC) reduction under uniform laboratory conditions and in much less time than yield trials[1]. TTC-reduction measures the ability of individual cells to function physiologically[2]. The reduction of the tetrazolium salt occurs in the mitochondria where electrons from the electron transport chain are accepted by the tetrazolium salt via the dehydrogenase pathway[3]. Formazan is the reduced form of the tetrazolium salt and the red colour can be measured spectrophotometrically.

We have shown in a previous study on cotton [2] that acclimation to drought stress as measured by TTC-reduction could be used as a screening technique. Additionally, we have shown that formazan production was relatively lower in stressed leaves of drought sensitive cotton cultivars compared to the leaves of the unstressed control treatment. However, for tolerant cultivars the opposite reaction was observed where the formazan levels were higher in the stress treatment compared to the unstressed control treatment. Similar tendencies were found for drought as well as heat stress[3]. This is possible due to the fact that the sensitive clones had inefficient tolerance mechanisms to survive a moderate drought stress and the plant could not adapt to drought stress when a severe stress was applied. The tolerant reaction is the opposite of this. Cultivars had efficient tolerance mechanisms during a moderate stress and also survived better when a severe stress was applied. This resulted in higher formazan production in the stress treatment compared to the control treatment[2].

In the present study on potatoes (Chapter 5.2) results show that heat and drought tolerance is organ and cultivar specific. When these results were compared with data from rain shelter trials, a negative correlation was found for drought tolerance and interestingly, a positive correlation was found with heat tolerance. This observation confirms the our opinion that results from field trials are the interaction between drought- and heat-tolerance mechanisms. Additionally information concerning the correlation between heat and drought tolerance is of great economic value[2]. It may be used to determine the best locality for a specific cultivar as well as for cultivar improvement in a breeding programme. The correlation between heat- and drought tolerance is summarized in Table 2 (Chapter 5.2).

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5.2 A comparison of drought stress and heat stress in the leaves and tubers of 12 potato cultivars

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Abstract

Although potato yield is extremely sensitive to drought- and heat stress, there are variations in the degree to which cultivars are affected by these stresses. Strategies for the selection of tolerant cultivars in a breeding programme can therefore be developed. The 2,3,5-triphenyltetrazolium chloride viability assay was evaluated to estimate drought- and heat tolerance of leaves and tubers of 12 potato cultivars which differ in their response to drought. Drought was simulated by floating leaf discs and tuber slices in 0.5M mannitol (-1.24MPa). After the drought acclimation treatment the leaf discs were subjected to a lethal drought stress by exposure to an osmotic potential of -2.48MPa. The viability of the leaf discs after the drought treatment was estimated by spectrophotometrically measuring the formazan concentration at 485 nm. Lower absorbance values in the control treatment compared to the stress treatment, indicated a tolerant reaction. As drought simulation in the laboratory can differ from field conditions due to the effect of heat stress, the cultivars were also evaluated for heat tolerance. A stress index was established which will enable breeders to distinguish between plant responses to heat and drought.

Introduction

Potato yield is optimal under growing conditions with adequate light, water and cool temperatures. According to Vayda [1] potato yield is dramatically influenced by heat and drought. Total and marketable yield are substantially decreased by short periods of severe stress. There are however variations in the degree to which cultivars are affected by these stresses. Thus, strategies for the selection of tolerant cultivars in a breeding programme can be developed.

Several physiological methods have been developed to measure abiotic stress tolerance in crop plants. These methods include: regrowth[2], triphenyltetrazolium chloride reduction (TTC)[3], vital staining[4], protoplasmic streaming[5], plasmolysis[6], leakage of ions[7] and measurement of ultra violet absorbing compounds[8]. Ishikawa, et al. [2] compared a number of viability assays in an attempt to identify the best ones for estimating freezing, heat and salt tolerance in brome grass. They found that TTC reduction was the most convenient assay whereas regrowth, although time consuming and labour intensive, was the most sensitive and reliable assay. Although membrane injury as estimated by electrolyte leakage has been found to be unreliable in some cases[9], TTC tests and conductivity have been successfully used for evaluating heat and cold tolerance in potato. However, TTC reduction was found to be more sensitive than the conductivity test for evaluating heat tolerance[10]. Vratsanos and Rossouw [11] also showed a positive correlation between TTC viability assays and heat tolerance in three potato cultivars. De Ronde and Van der Mescht[12] expanded the TTC assay to measure drought stress in cotton. A positive correlation between drought tolerance and TTC reduction was shown in six cotton cultivars.

The TTC assay is based on the ability of viable cells to metabolically reduce tetrazolium salts into soluble formazans [13]. Earlier work of ours had shown that formazan production was relatively lower in stressed leaves of drought sensitive cotton cultivars compared to the leaves of the unstressed control treatment. However, for tolerant cultivars the opposite reaction was observed where the formazan levels were higher in the stress treatment compared to the unstressed control treatment. Similar tendencies were found for drought as well as heat stress[3]. This is possible due to the fact that the sensitive clones had inefficient tolerance mechanisms to survive a moderate drought stress and the plant could not adapt to drought stress when a severe stress was applied. The tolerant reaction is the opposite of this. Cultivars had efficient tolerance mechanisms during a moderate stress and also survived better when a severe stress was applied. This resulted in higher formazan production in the stress treatment compared to the control treatment. Thus, the method measures the ability of the plant tissue to adapt to increasing stress

conditions[3]. De Ronde and Van der Mescht[12] hypothesized that a lower formazan level in the control treatment compared to a stress treatment indicates a drought tolerant reaction.

Van der Mescht, De Ronde and Rossouw [14] showed that drought related protein synthesis is cultivar and organ specific in potato. It is thus important to evaluate the effect of drought stress on different organs. Additionally De Ronde and Van der Mescht [12] showed that drought and heat tolerance were negatively correlated in some cotton cultivars. The aim of this research was to evaluate TTC reduction as a metabolic indicator of drought - and heat tolerance in potato leaves and tubers.

Materials and Methods

Plant material

Twelve potato cultivars (Table 1) were grown in a glasshouse under conditions as previously described by Van der Mescht, *et al.*[15] Leaves from the third apical node were harvested four weeks after emergence. Fresh tubers were harvested for the TTC tests.

Induction of drought- and heat stress.

Leaf discs (7mm in diameter) or tuber slices (4mm in diameter with a 2mm width) of the control treatments were incubated in a sodium hydrogen maleate buffer pH 6,0 at 23°C for 3 hours[16]. The drought stress was induced by incubating the leaf discs or tuber slices in a sodium hydrogen maleate buffer containing 0.5 M mannitol (-1.24MPa) as an osmoticum[15]. After 3 hours the stress and control treatments were incubated in 1.0 M mannitol (-2.48MPa) and sampled over a period of 150 minutes. The moderate heat stress was induced by incubating the stress treatment at 37°C for 3 hours before the lethal stress was induced at 45°C[2].

TTC - assay

After the induction of the heat- or drought stress, leaf discs (7 mm in diameter) were submerged in 3 ml of 0.8% (w/v) TTC in 0.2 M sodium hydrogen maleate buffer at

pH 6.9. the discs were vacuum infiltrated for 5 minutes to enhance the penetration of the solution into the tissue. After an 20 hour incubation at 29°C in the dark, the discs were washed twice with distilled water. This was followed by the addition of 3 ml 95% ethanol and the samples were boiled until dry. The samples were resuspended in 3 ml 95% ethanol once room temperature was reached.

The formazan accumulation was measured spectrophotometrically at 485 mm^3.

Table 1: Summary of the cultivars evaluated for drought- and heat tolerance

Cultivar	*Growth period*
Raritan	short
Vanderplank	short
Devlin	short
Aviva	short
Sebago	medium
Ono	medium
Darius	medium
Baraka	medium
Bravo	long
Kimberley Choice	long
Hoëvelder	long
Late Harvest	long

Results

i) *Drought stress in leaves*

Viability was measured every 30 minutes over 3 hours after the lethal stress was induced. The formazan concentrations were higher in the stress treatment compared to the control treatment in the cultivars Darius and Ono indicating a tolerant reaction (Figure 1). In the sensitive reaction the formazan

concentrations over time were lower in the stress treatment compared to the control treatment (Figure 2). This was true for the cultivars Vanderplank, Bravo, Hoëvelder, Devlin, Kimberley Choice, Raritan, Sebago, Aviva, Baraka and Late Harvest. The area between the graphs was estimated as the difference between the mean of the stress treatment over time and the mean of the control treatment over time[3]. Results were presented in a histogram (Figure 3). The bigger the positive histogram the more tolerant the cultivar e.g. Darius and Ono in Figure 3. The more negative the histogram the more sensitive the cultivar e.g. Late Harvest in Figure 3. With this information a stress index was developed ranking the cultivars according to their viability during osmotic stress (Table 2).

ii) *Drought stress in tubers*

Tubers were subjected to osmotic stress as described for leaves. In contrast to the leaves all the cultivars showed a sensitive reaction when tubers were used. The stress index was calculated (Figure 4) ranking the cultivars form least sensitive to most sensitive. The cultivar Kimberley Choice was least sensitive while Aviva was most sensitive (Table 2). Additionally, the areas between the graphs were ten times larger compared to leaves. This is an indication of a hypersensitive reaction to drought in tubers.

iii) *Heat stress in leaves*

Leave discs were subjected to a moderate stress of 37°C for 3 hours before a lethal stress of 45°C was induced. Viability was measured every 30 minutes for 3 hours after induction of the lethal stress. Heat tolerant reactions were found in the cultivars Bravo, Baraka and Late Harvest while sensitive reactions were observed in the cultivars Kimberley Choice, Ono, Raritan, Devlin, Hoëvelder, Aviva, Darius, Sebago and Vanderplank (Figure 5). The stress index is calculated and indicated that Bravo was most heat tolerant and Vanderplank most heat sensitive (Table 2).

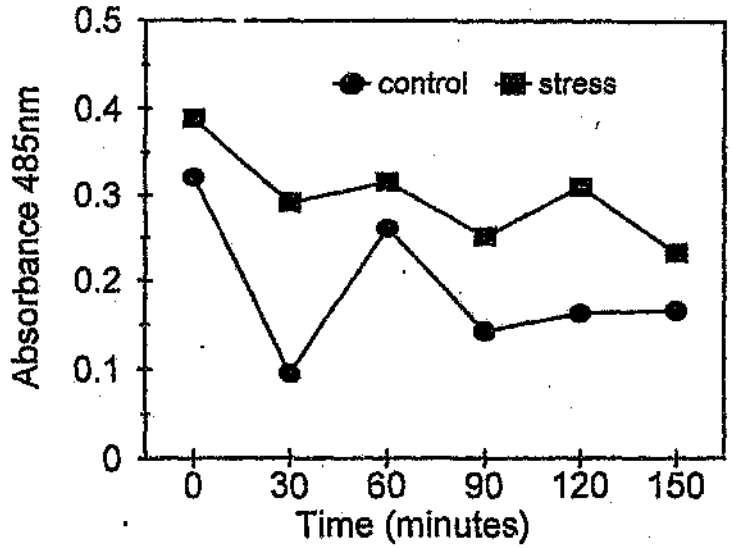


Figure 1: The viability of leaf discs (expressed as formazan production) subjected to drought stress was measured every 30 minutes at 485 nm for the cultivar Darius. The absorbance values of the treatment were higher compared to the control treatment indicating a tolerant reaction.

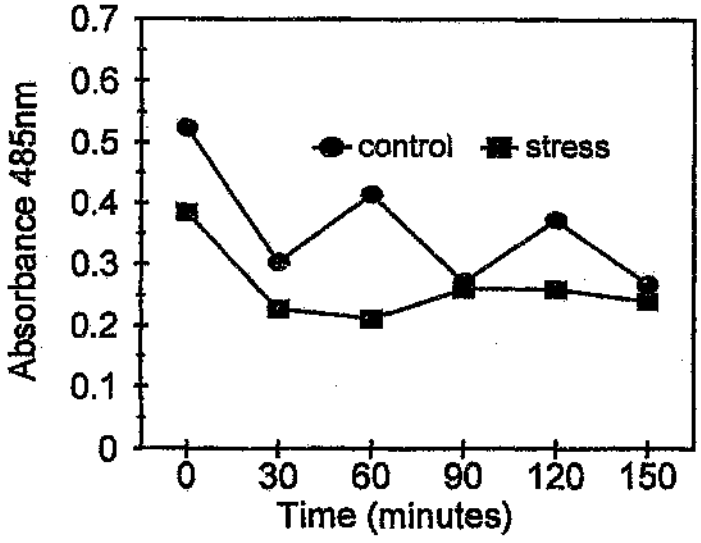


Figure 2: The potato cultivar Aviva was treated as described in the legend to Figure 1. The absorbance values of the stress treatment were lower compared to the control treatment indicating a sensitive reaction.

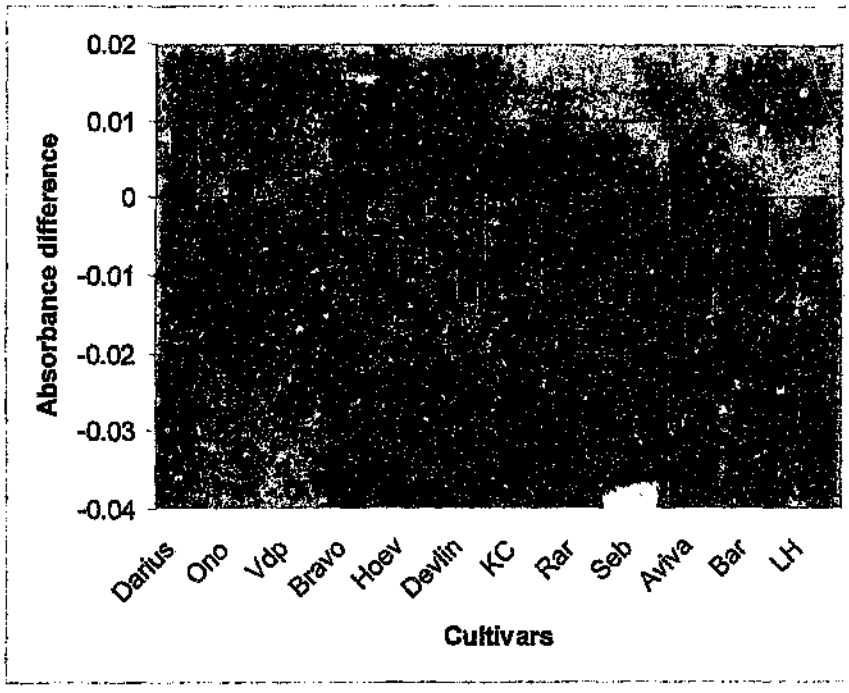


Figure 3: The difference between the stress and control treatments in leaves was calculated over time for twelve potato cultivars. The positive values indicate a drought tolerant reaction while the negative values indicate a sensitive *r* action to drought. Variation was estimated by the standard error of the mean.

Table 2: Summary of the drought- and heat response in potato leaves and tubers. Cultivars were ranked from the most viable to the least viable on a scale from 1-12.

Cultivar	Drought tolerance rating		Heat tolerance rating	
	Leaves	Tubers	Leaves	Tubers
Raritan	9	9	6	9
Vanderplank	3	3	12	2
Devlin	8	11	7	8
Aviva	10	12	9	12
Sebago	7	4	11	7
Ono	2	10	5	10
Darius	1	5	10	11
Baraka	11	6	2	6
Bravo	4	2	1	1
Kimberley Choice	6	1	4	5
Hoëvelder	5	7	8	3
Late Harvest	12	8	3	4

The cultivars were ranked according to the values of the difference between the mean of the stress treatments and the mean of the control treatments over time for each cultivar. The cultivar with the highest value was designated 1 and the cultivar with the lowest value 12. The standard deviation was very high as the measurements were taken over time thus, these results are not significantly different. As a result tendencies was used instead of statistical analysis (Prof. J.M.P. Geerthsen, personal communication).

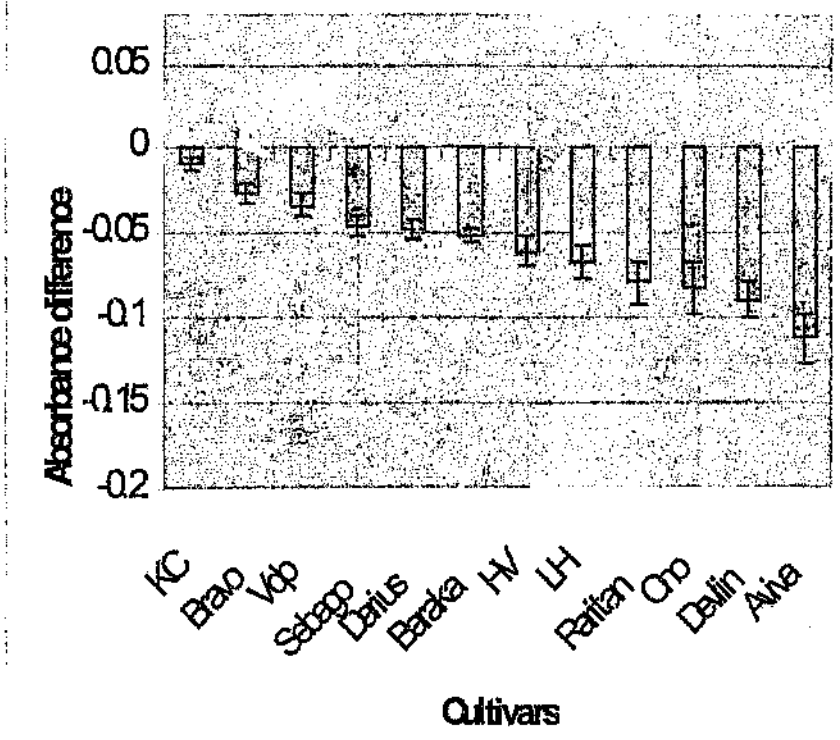


Figure 4: The difference between the stress and control treatments in tubers was calculated over time for 12 potato cultivars. The negative histogram indicate a sensitivity to drought in all cultivars.

iv) *Heat stress in tubers*

Potato tubers were subjected to heat stress as described for leaves. According to the histogram the cultivars; Bravo, Vanderplank, Hoëvelder, Late Harvest, Kimberley Choice, Baraka and Sebago showed tolerant reactions while cultivars Devlin, Raritan, Ono, Darius and Aviva showed sensitive reactions (Figure 6). A stress index with Bravo as most tolerant and Aviva as most sensitive was calculated and presented in Table 2. Similar to tubers and leaves subjected to osmotic stress the areas between the graphs of tubers subjected to heat stress were ten times higher than leaves subjected to heat stress.

Discussion

The TTC assay measures the capability of plant tissue to carry out electron transport. Additionally, inhibition of TTC-reduction is an indication of dehydrogenase inactivation resulting in a decrease in formazan production[17]. Results from leaves and tubers subjected to an osmotic stress of 0.5M mannitol (-1.24MPa) showed a sensitive response with the exception of leaves from the cultivars Darius and Ono. The difference in absorbance values between stress and control treatments in leaves subjected to osmotic stress is generally ten times higher compared to tubers subjected to the same stress (Figure 3 and 4). This may be an indication that potato leaves have a better ability to adapt to drought stress compared to tubers. Alternatively in the case of the drought treatment the difference may be in the relative accessibility of the tuber tissue, not covered with a waxy cuticle, to the treatment solution. A stress ranking was established by ranking the cultivars from the most viable to the least viable during osmotic stress. The difference between the ranking of leaves and tubers showed that the drought response was organ specific with the exeption of Raritan and Vanderplank (Table 2).

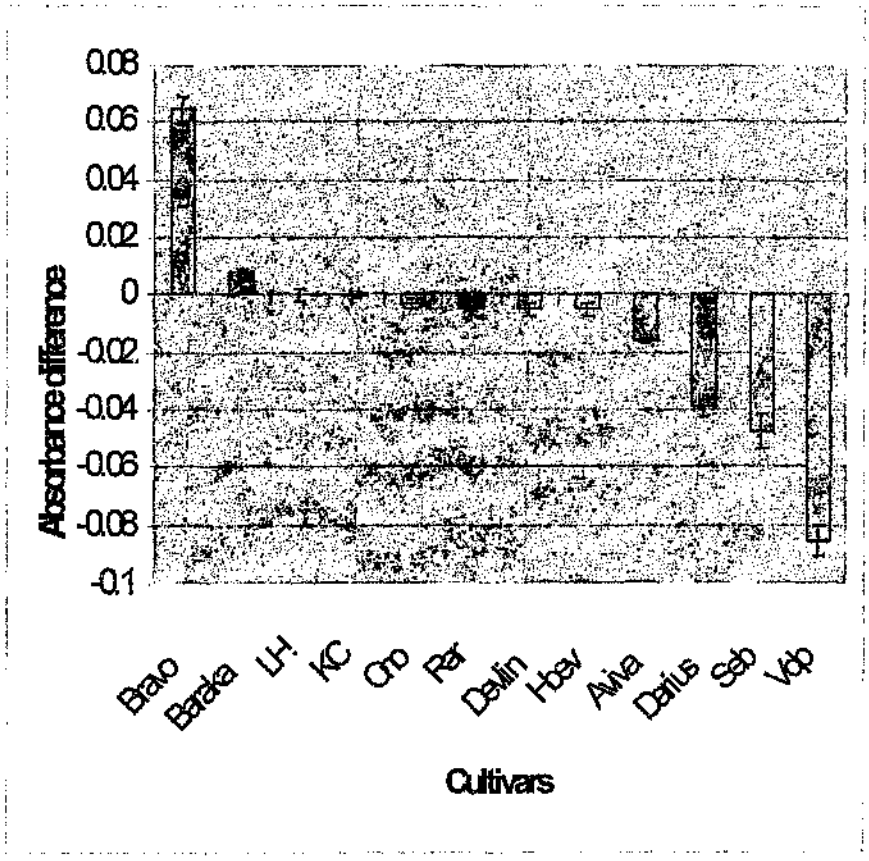


Figure 5: The histogram was calculated as described in the legend to Figure 3. The leaf discs were subjected to heat stress.

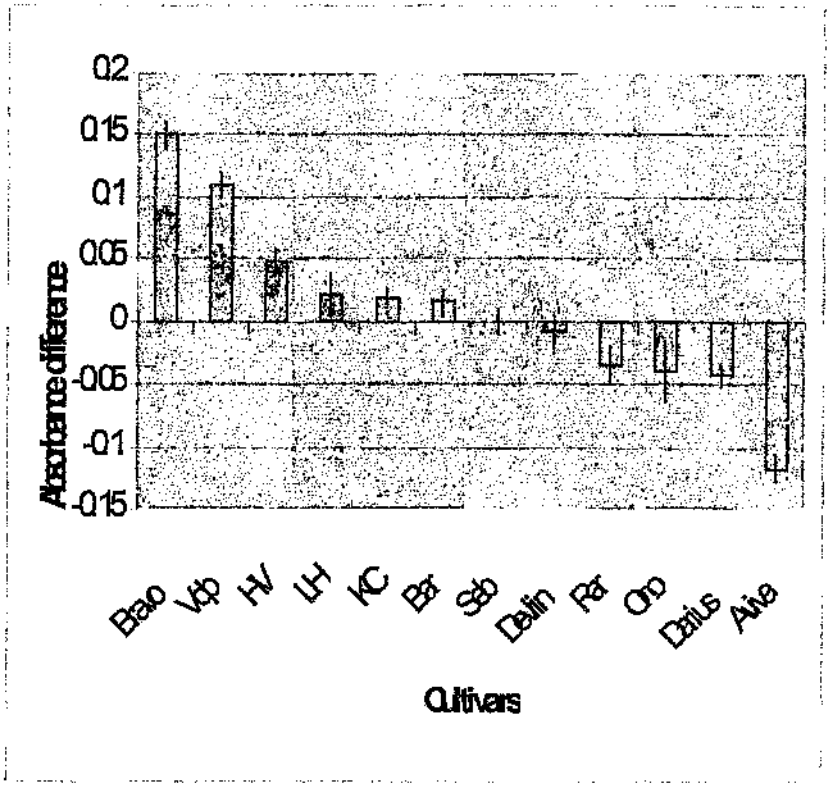


Figure 6: The histogram was calculated as described in the legend to Figure 4. The tuber slices were subjected to heat stress. The positive values indicate a heat tolerant reaction while the negative values indicate a heat sensitive reaction.

When leaves and tubers were evaluated for heat tolerance it was observed that potato leaves (Figure 5) were more heat sensitive compared to tubers (Figure 6). According to Van der Mescht, *et al.* [18] potato cultivars are selected for drought avoidance rather than tolerance as drought tolerance is defined in terms of yield reduction. Thus, we are interested in plants avoiding drought by tuberization. This may also be true for heat tolerance. The difference in absorbance values between stress and control treatment was lower in heat stressed leaves (lower than 0.06) compared to heat stressed tubers (lower than 0.15). These values are similar to the values found in drought- and heat-stressed *Eucalyptus grandis* clones [19] and cotton cultivars [12] . The only exeption was that these genotypes had more positive values compared to potato genotypes which had more negative values indicating sensitivity. The cultivar Bravo was most heat tolerant when both leaves and tubers were tested compared to Aviva which tested most sensitive for heat. According to the stress ranking (Table 2) the relative ranking between leaves and tubers were similar in the cultivars Devlin, Darius, Bravo, Kimberley Choice and Late Harvest. Tubers were more sensitive to heat than leaves for the cultivars Raritan, Aviva, Ono and Baraka while leaves were more heat sensitive compared to tubers in the cultivars Vanderplank, Sebago and Hoëvelder.

Information about the correlation between heat and drought tolerance is of great economic value. It may be used to determine the best locality for a specific cultivar as well as for cultivar improvement in a breeding programme. The data in Table 2 is also an indication of the correlation between heat and drought tolerance. When leaves were evaluated a negative correlation was found in the cultivars Vanderplank, Darius, Baraka and Late Harvest. The cultivars Vanderplank and Darius were relatively drought tolerant and heat sensitive while the cultivars Baraka and Late Harvest were relatively heat tolerant and drought sensitive. In the case of tubers it was found that the cultivar Darius was relatively drought tolerant and heat sensitive while Late Harvest and Hoëvelder were relatively heat tolerant and drought sensitive. Results of Li *et al.*[10] showed a negative correlation between heat and cold tolerance

in potato using TTC-reduction. They conclude that heat and cold resistance is mutually exclusive. It is possible that heat and drought tolerance is mutually exclusive in potato.

We conclude that heat and drought tolerance are organ and cultivar specific. In addition to our results on tubers that showed a sensitive reaction to drought, Shimshi and Susnoschi[20] found a linear relationship between reduction in tuber yield and soil moisture content during drought. Vayda [1] suggests that this relationship disguises a complex set of responses. A reduction in photosynthetic efficiency may play a major role in yield loss during water stress as it influences carbon assimilation[21]. Thus, it is suggested to use leaves in all subsequent experiments. According to Walter [22] stress responses during phenylpropanoid biosynthesis are preferentially expressed in order of heat shock, fungal elicitor and ultra violet light. Thus, it is possible that heat plays a dominant role when plants are subjected to both heat and drought stress. This is in agreement with the visual observations of breeders and this interaction complicated selection for drought tolerance (H.J. Vorster, personal communication).

Steyn *et al.* [23] subjected three of the cultivars (Vanderplank, Hoevelder and Late Harvest) used in this study, to differential water treatments in rain shelter trials during the autumn and spring seasons. Results showed that the effect of drought on yield was more detrimental in spring plantings compared to autumn plantings. According to Steyn *et al.* [23] the effect of water stress may be aggravated by higher temperatures during spring trials. When our data was compared to the data from the rainshelter trials a negative correlation was found between results from the TTC-reduction and the rainshelter trials. For example, Late Harvest was drought tolerant according to the results from the rain shelter trials but tested sensitive with the viability assay while Vanderplank was drought sensitive according to the results from the rainshelter trials and tolerant according to the TTC-reduction experiments. Hoëvelder showed sensitivity to drought but had a high yield potential according to the rainshelter trials but with the viability assay it tested more tolerant to Late Harvest. In field studies, cultivar assessment is complicated by the interaction

between heat and drought stress[1], thus the results from the rainshelter trials were compared to heat tolerance as estimated by TTC-reduction in potato leaves. The comparison between heat tolerance, as measured by TTC-reduction, and drought tolerance in rain shelter trials resulted in a positive correlation with Vanderplank and Hoëvelder testing sensitive and Late Harvest tolerant.

Viability as an indicator of heat-[24] and drought tolerance can be tested under uniform laboratory conditions and in much less time than yield trials, where drought tolerance and heat tolerance cannot be estimated separately. Additionally, osmotic substrates such as mannitol provide controlled water potentials and offer the opportunity to bypass many uncertainties in field studies [25].

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CHAPTER 6

POTATO TRANSFORMATION IN AN ATTEMPT TO ENHANCE DROUGHT TOLERANCE

6.1 General Introduction

Environmentally adverse conditions cause cellular damage by the enhanced production of reactive oxygen intermediates[1]. According to Herouart *et al.* the mechanisms involved in protecting plants against reactive oxygen species may be more efficient compared to other eukaryotes as plants not only consume oxygen during respiration but they also produce it during photosynthesis. Many studies suggest that a significant improvement of oxidative stress tolerance require overproduction of several enzymes. E.g. resistance to drought stress and paraquat correlated with high levels of superoxide dismutase and glutathione reductase in maize inbred lines[2], resistance against paraquat correlated with high levels of glutathione reductase, superoxide dismutase and ascorbate peroxidase in *Conyza bonariensis*[3] and paraquat tolerant lines of perennial ryegrass showed significantly higher levels of superoxide dismutase and catalase activity compared to sensitive lines[4]. Transgenic plants were generated to evaluate the effect of overproduction of reactive-oxygen intermediate scavenging enzymes. Contradictory results were found e.g. the initial experiments of Tepperman and Dunsmuir [5] showed that transgenic tobacco which overproduced a petunia chloroplastic Cu/Zn SOD was not more tolerant to paraquat compared to non-transformed plants. In contrast more resent of Sen Gupta *et al.* [6] have shown that the overproduction of pea chloroplastic Cu/Zn SOD in tobacco leaves improved tolerance to paraquat. High levels of Cu/Zn SOD activity (50 fold in transgenic tobacco)[5] did not confer tolerance to oxidative stress while a small increase in Cu/Zn SOD activity was able to provide resistance against paraquat in human and mouse cells. It is possible that glutathione reductase activity and ascorbate peroxidase activity were the limiting factors when Cu/Zn SOD activity was increased to very high levels.

In Chapter 3 it was shown that there was a correlation between increased Cu/Zn SOD activity during drought stress and drought susceptibility in potato cultivars. The results from the present study i n agreement with this observation (Chapter 6.2). Although there was a slight increase in enzyme activity in the four transformed lines during control conditions, the Cu/Zn SOD activity during drought stressed conditions showed either a non significant response or a significant decrease when compared to non-stressed plants. The transformed lines (SOD 1 and SOD 2) could withstand drought in the glasshouse for two weeks longer compared to the untransformed plants and one week longer than the transformed lines SOD 6 and SOD 7. These results were confirmed by 2,3,5-triphenyltetrazolium chloride reduction which showed that the four transformed potato lines were more tolerant compared to the non-transformed cultivar.

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6.2 Enhanced drought tolerance in transgenic potato expressing the *Arabidopsis thaliana* Cu/Zn superoxide dismutase gene.

Van der Mescht, A., De Ronde, J.A., Slabbert, M.M., Murray S., Oelofse, D. and Rossouw, F.T. (In preparation)

Abstract

All aerobic organisms must possess the means to protect themselves from the toxic effects of reduced oxygen species generated during normal cell metabolic activity or as a result of environmental stresses e.g. drought. Cells are protected from the deleterious effects of the free oxygen radicals by Cu/Zn superoxide dismutase (SOD) which catalases the initial step in detoxifying activated oxygen species. The potato cultivar Aviva was transformed with a cytosolic Cu/Zn superoxide dismutase gene from *A. thaliana* using *Agrobacterium* mediated gene transformation. Four transgenic potato lines were identified and evaluated for drought tolerance in the glasshouse. The transformed lines SOD 1 and SOD 2 could withstand drought in the glasshouse for two weeks longer than the untransformed plants and one week longer than the transformed lines SOD 6 and SOD7. These findings were confirmed by data from enzyme activity as well as 2,3,5-triphenyltetrazolium chloride reduction. Triphenyltetrazolium chloride reduction measures the efficiency of electron transport. Additionally, the inhibition of triphenyltetrozolium chloride reduction is an indication of dehydrogenase inactivation resulting in a decrease in formazan production. The transformed lines were more drought tolerant than the untransformed Aviva plants.

Introduction

All aerobic organisms must possess the means to protect themselves from the toxic effects of reduced oxygen species generated during normal cell metabolic activity or as a result of environmental stresses such as temperature extremes and/or drought. Drought in combination with high light intensities, ambient ozone, sulfur dioxide and

some pathogens exacerbates the effect of oxygen radicals[1]. Oxidative injury occurs when the capacity of cellular antioxidant systems is overwhelmed by oxygen-centred radicals and other oxidants generated within the cell[1]. The mitochondrial electron transport system as well as the electron transport chain of the photosynthetic apparatus within the chloroplasts are well-documented sources of superoxides radicals. Additionally singlet oxygen can be generated during the transfer of excitation energy from chlorophyll to oxygen. The resulting hydroxyl radicals are among the most reactive species known to chemistry, able to cause lipid peroxidation, the mutation of DNA and the denaturation of proteins. These molecular reactions in turn have some cellular effects such as membrane damage, loss of organelle function, reduced carbon fixation and electrolyte leakage. These cellular effects result in cell death[2,3].

Cells are protected from the deleterious effects of the free oxygen radicals by superoxide dismutase (SOD) which catalases the initial step in detoxifying activated oxygen species. The superoxide anion radicals are reduced to hydrogen peroxide and molecular oxygen[4]. A positive correlation between enzymes from the antioxidative system and drought tolerance was reported for maize[5], tobacco[6] and alfalfa [7]. As a result, superoxide dismutases (SOD) has become the object of intensive research in physiology, biochemistry and molecular as well as cell biology of plants. The superoxide dismutases are a divergent class of metalloenzymes which exists as distinct isozymes in different subcellular compartments. The manganese - SOD (Mn-SOD) usually is found within the mitochondrial matrix while the iron-SOD (Fe-SOD) occurs in plastids and the copper/zink- SOD (Cu/Zn - SOD) is localized in both the cytosol and plastids[8].

Correlations between elevated SOD activity and stress tolerance suggest that the regulation of SOD levels provides plants with a tolerant mechanism against oxygen toxicity, however, direct proof of this effect is lacking[9]. A true evaluation of the effects of changing SOD activity in plants might be obtained by genetic engineering[2]. The effect of overproduction of SOD-activity (increased copy number) or lack of

SOD-activity (antisense technology) during drought stress may enhance our understanding of the role of Cu/Zn SOD activity.

The first report of genetic manipulation of SOD in plants was described for tobacco and tomato. The regenerants overproduced a chloroplastic Cu/Zn SOD derived from petunia. There was no significant difference between either tobacco or tomato plants that produced elevated Cu/Zn SOD and the control plants. The authors concluded that the increased activity of SOD alone in the chloroplasts was not adequate to protect the cells against oxygen toxicity caused by ozone fumigation or the herbicide methyl viologen[10]. Different results were obtained when a chloroplastic Cu/Zn SOD from pea was introduced into tobacco and potato. The transgenic plants were more tolerant when subjected to methyl viologen (paraquat) and the membrane damage measured by electrolyte leakage[11]. Additionally tobacco plants that express a chimeric gene that encodes chloroplast-localized Cu/Zn from pea has been shown to be more tolerant to chilling and high light intensity[1], transgenic tobacco plants that over expressed mitochondrial M_n SOD as well as a chloroplast - targeted Mn SOD showed increased resistance to methyl viologen[12] and transgenic potato plants that expressed tomato Cu/Zn SOD's also have protection against methyl viologen toxicity[9]. Transgenic alfalfa (*Medicago sativa*) expressing Mn SOD were more drought tolerant compared to control plants. A three year field trial showed that yield and survival of transgenic plants were significantly improved, showing for the first time that increased tolerance of oxidative stress is also successful in adaption to field environments[7].

It was previously shown by Van der Mescht *et al.*[13] that the potato cultivar Aviva had only half the Cu/Zn SOD activity when compared to eleven other cultivars differing in growth period and drought tolerance. Aviva is a drought tolerant cultivar with a short growth period intended for the crisp market. The cultivar was bred at the ARC-Roodeplaat, Pretoria, South Africa. The aim of this study was to transform the cultivar Aviva with a Cu/Zn SOD gene in an attempt to enhance its drought tolerance.

Materials and Methods

Maintenance of in vitro plantlets

Aviva plantlets were obtained from the Potato gene bank (ARC-Roodeplaat) and were multiplied on MS medium (MS stocks 1-6, 20 mg/ml sucrose, 7.5 mg/ml agar, pH 5 8) in large bottles. Plant cultures were incubated in a growth room set at 26°C and a photoperiod of 16 hours light/8 hours dark.

Leaf disc regeneration

Leaves were excised from 4-5 week old *in vitro* plantlets, the apical and [illegible] were cut off, and the leaves were placed abaxial side down on the medium which had been poured into petri dishes. Twenty five leaves were used, leaf explants were subcultured onto fresh medium once a week and incubated in a growth room set at 26°C with a photoperiod of 16 hours light/8 hours dark.

The RIAT two-step regeneration procedure was used. Leaf explants were first incubated on RIAT medium (MS stocks 1-6, 20mg/ml sucrose, 2 µg/ml zeatin, 0.02 mg/l NAA; 0.02 µg/ml GA_3, 7.5 mg/ml agar, pH 5.8) until callus production could be seen on the cut edges of the leaf. Explants were subsequently transferred to RIAT medium with the auxin component (NAA) removed.

Kanamycin tolerance experiments

In order to determine the optimal kanamycin concentration for selection of transformed cells, Aviva leaf explants were incubated on regeneration media containing various levels of kanamycin (0 µg/ml; 25 µg/ml; 50 µg/ml; 75 µg/ml and 100 µg/ml). Regeneration on medium containing 250 µl/ml cefotaxime (the antibiotic added in order to control the *Agrobacterium tumefaciens* growth after transformation) was also evaluated. Results were taken after seven weeks.

Cloning of a Arabidopsis thaliana Cu/Zn SOD cDNA into transformation vectors

The plasmid pcSODRH consists of a 788 bp cDNA clone of a cytosolic Cu/Zn SOD from *A. thaliana* in the Eco R1 site of p Bluescript (SK^+). The insert consists of a full

coding sequence and 112 bp 5' and 206 bp 3' - untranslated region + 14 bases of poly A tail as previously described by Hinges and Slusarenko[14]. The pcSODRH was first restricted with *Sac* 1 and, after precipitation with *Eco* RV to yield the SOD insert (0.8kb). The pBI 221 plasmid (5.7 kb) was first restricted with the *Sac*1 restriction enzyme and then with *Sma* I to yield the pBI 221 vector (3.8kb) (Figure 1).

After the ligation (1 insert : 1 vector), a transformation experiment was performed according to Chung and Miller [15]. The successful transformation of DH 5 ∝ yielded ampicillin resistant colonies. Plasmid DNA was extracted using the JAT preparation. Cells were pelleted by centrifugation for 2 minutes at 12 000 rpm. The pellet was resuspended in STE buffer containing 100 mM Nacl; 20 mM Tris (pH 7.5) and 10 mM EDTA, and an equal volume of phenol: chloroform (1:1) was added. The mixture were vortexed for 15 seconds and centrifuged for 5 minutes at 12 000 rpm. Transfer 40 µl of the upper, phase to an eppendorf tube and store at -20°C. (D. K. Berger, personal communication). Additionally a *Pst* I digestion of the uncut pBI 221 and transformed DH5 ∝ cells yielded two bands of 1.6 kb and 2.8 kb. This confirmed positively transformed DH5∝ cells with the SOD gene as the pBI SOD plasmid (4.6 kb) was first restricted with *Sac* I and after precipitation with *Hind* III to yield the SOD insert (1.6 kb). The pBI 121 plasmid was first restricted with the *Sac* I restriction enzyme and then with *Hind* III to yield the pBI 121 vector (10.3 kb). Ligation and transformation yielded the pBI 121 SOD vector. (Figure 2).

The method of Armitage[16] was used for the triparental mating procedure. Overnight cultures were established for *Agrobacterium tumefaciens* LBA 4404 (Rf^{100}) at 28°C, *E. coli* HB 101 (pRKK 2013)(Km^{100}) at 37°C and *E. coli* pBI 121 SOD (Km^{50}) at 37°C. For the triparental mating, 500µl *E. coli* and pB1 121 SOD were mixed in a two ml syringe . This mixture was dispensed onto a sterile filter which was placed on top of a LA plate without any antibiotic. The plates were incubated for 24-36 hours at 28°C. A streak from the lawn growth from the plates was taken and streaked onto Rf^{100}/Km^{50} plates. The plates were incubated for at least 48 hours at 28°C before the transformants were selected.

Growth of Agrobacterium tumefaciens

An aliquot of frozen *A. tumefaciens* LBA 4404 cells, into which the pSOD plasmid had been inserted by triparental mating was grown in YM medium (GIBCO BRL) supplemented with 50 μg/ml kanamycin and 100 μg/ml rifampicin on a shaker at 28°C for 36 hrs. Before plant transformation, the bacterial cells were pelleted by centrifugation (3300 rpm, 25 mins, 4°C) and resuspended in YM medium only.

Leaf disk transformation and regeneration

Potato leaf disks were pre-incubated on MS plates for 48 hr. Following this, leaves were immersed in *A. tumefaciens* cell suspensions for various time periods, blotted on sterile filter paper and replated onto the MS plates for two days of cocultivation, as had previously been determined for potatoes [17]. Following co-cultivation, explants were transferred to the RIAT two-step indirect regeneration medium. Leaf explants were first incubated on RIAT medium (MS stocks 1-6, 20 mg/ml sucrose, 2 μg/ml zeatin, 0.02 μg/ml NAA, 0.02 μg/ml GA_3, 7.5 mg/ml agar, pH 5.8) until callus production could be seen on the cut edges of the leaf. Explants were subsequently transferred to RIAT medium with the auxin component (NAA) removed (designated RIAT-).

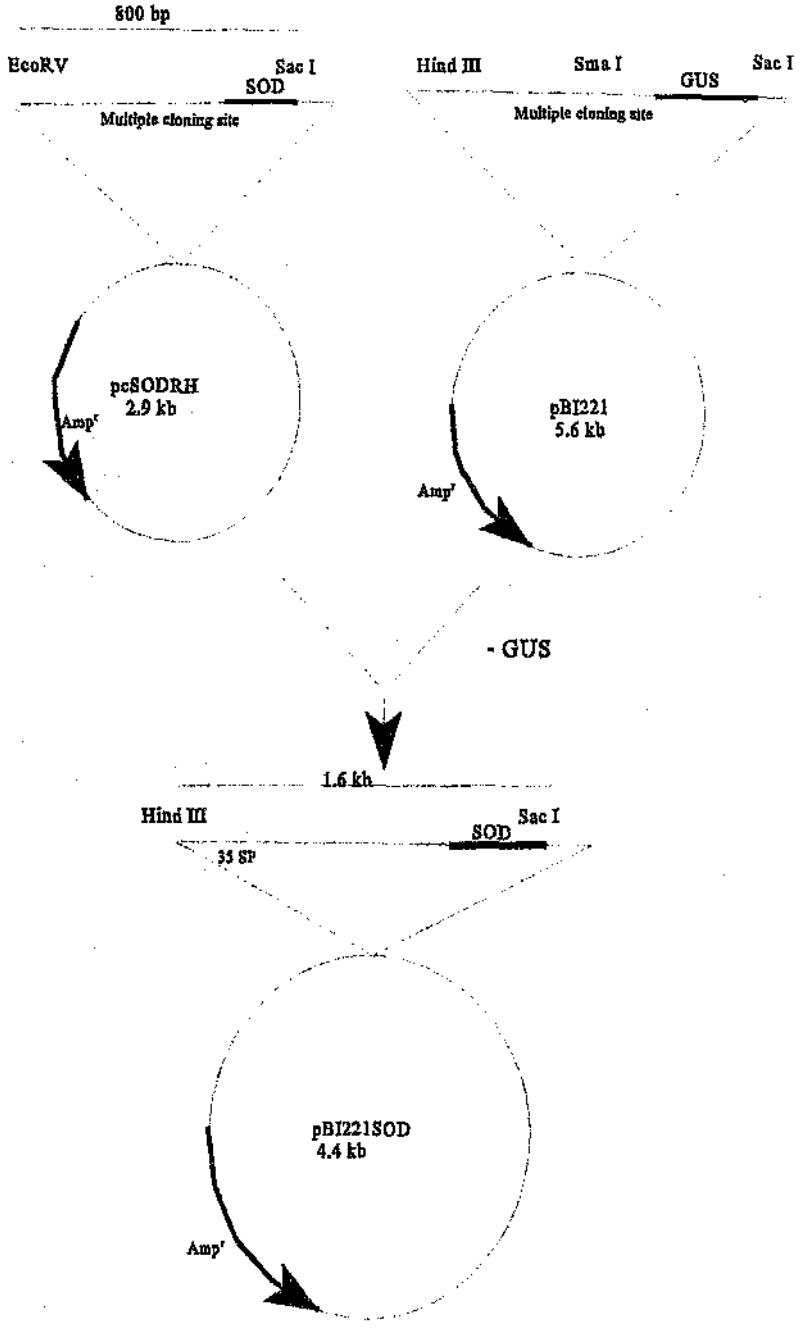


Figure 1: Schematic representation of the ligation of the insert (800 bp), from the pcSODRH plasmid, with the vector (± 3,8 kb), from the pBI 221 plasmid, to produce the pBI 221 SOD plasmid.

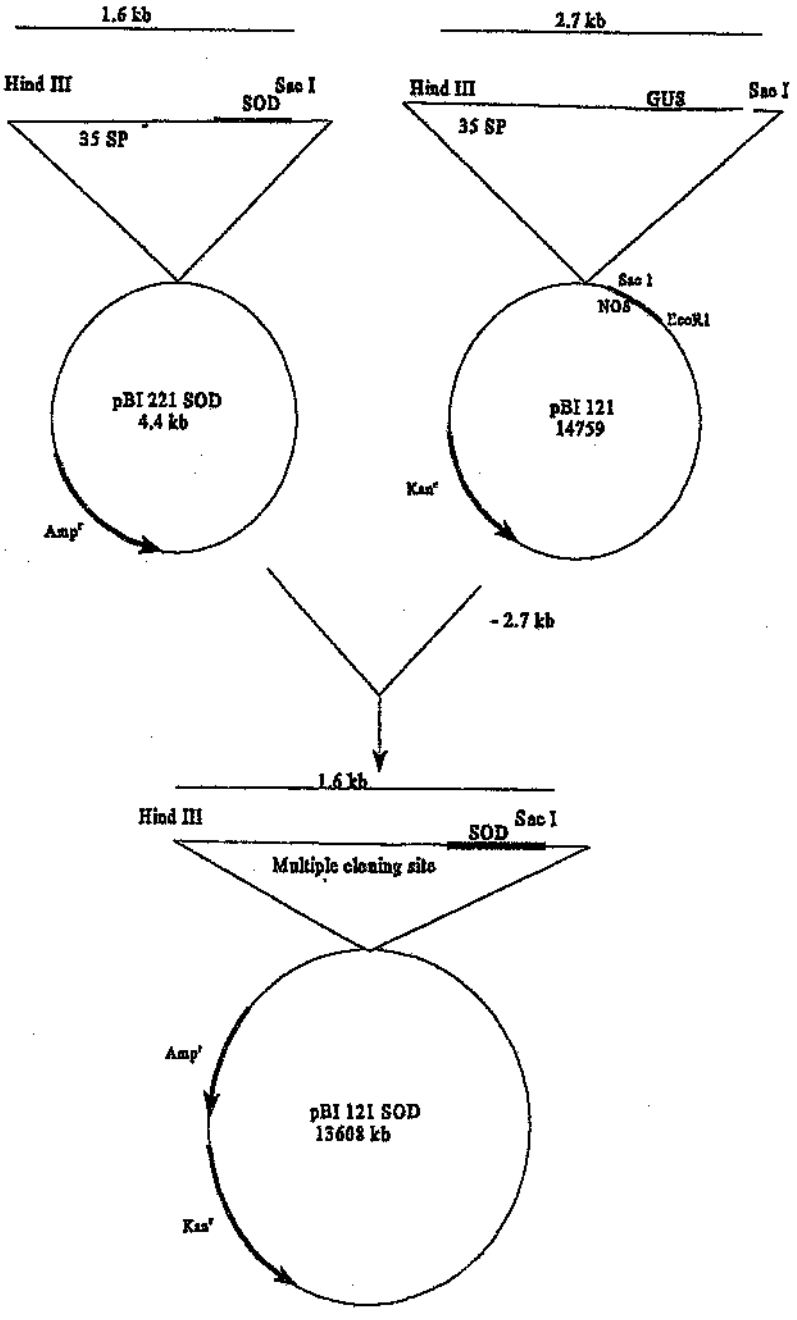


Figure 2: Schematic representation of the ligation of the insert (1.6kb), from the pBI221 plasmid, with the vector (± 12 kb), from the pBI 121 plasmid, to produce the pBI 121 SOD plasmid.

Molecular confirmation of transformed potatoes

DNA was extracted from seven putative transgenic tissue culture Aviva plantlets containing the SOD gene as well as a control Aviva plant. The method of Ish-Horowicz and Burke [18] was used for the isolation of the SOD plasmid DNA. The DNA was quantified with the use of a fluorometer. Electrophoresis of isolated DNA was performed on a 1% agarose gel.

In order to verify that transgenes had been integrated into the potato genome, PCR analysis was performed using specific primers. The SOD and nptII primers were used. The sequence of this primers were as follows:

nptII left: 5'GAGGCTATTCGGCTATGACTG3'

nptII right: 5'ATCGGGAGCGGCGATACCGTA3'

SOD left primer: 5'TTT GAA CAG CAG TGA GGG TG 3'

SOD right primer: 5'TTA GCC CTG GAG ACC AAT GA 3'

The PCR reactions were carried out in 10µl volumes containing the following final concentrations: 50mM KCl, 10mM Tris-HCL (pH 9.0), 0.1 % Triton X-100. 1.5 mM $MgCl_2$, 0.025mM each of dATP, dCTP, dGTP, dTTP, 0.5µM of each primer and 0.5U Taq polymerase. Standard amounts of DNA added were 40 ng plant DNA and 10ng pBI 121 plasmid DNA per reaction.

The PCR program used was designed as follows: 1) 35 cycles of stages 1 consists of dissociation of DNA strands at 94°C for 30 seconds, primer annealing at Tm for 30 seconds followed by primer elongation at 72°C for 45 seconds. 2) 1 cycle of stage 2 consists of dissociation of DNA strands at 94°C for 30 seconds, primer annealing at Tm for 30 seconds follows by primer elongation at 72°C for 5 minutes. The annealing temperature was assembled from the equation Tm=4(G+C)+(2(A+T)+/-5°C. The annealing temperatures used were 56°C and 64°C for the SOD and NPTII primers respectively. The PCR products were determined on a 1% agarose gel and visualized with ethidium bromide.

Southern blot analysis was performed with SOD as well as nptII probes which were DIG labelled according to the manufactures procedure using Boehringer Mannheim's DIG labelling and detection kit. The DNA was digested with *Hind* III and *Eco* RI.

Hardening off and drought stress of transformed plants

Four transgenic and one control (untransformed) tissue culture Aviva plantlets containing the SOD gene were hardened off [17]. The potatoes were grown in a glasshouse under conditions as previously described by Van der Mescht *et al.*. [19]. Drought shock was induced six weeks after introduction to the glasshouse by withholding water. The leaf on the third apical node was harvested weekly from drought stressed and non-stressed control plants. Three replicates were harvested. Leaf samples were freeze dried immediately after harvesting. The procedure continued for six weeks at which time a lethal drought shock was induced to the transgenic plants. The control (untransformed) plants died after four weeks without water.

Enzyme analysis

Cu/Zn SOD extractions were performed as described by Malan *et al.*[5] with minor modifications. Freeze dried leaf tissue (200 mg) was homogenized in 2.0 ml 0.1 M potassium phosphate extraction buffer (pH 7.5) containing 0.1 mM EDTA, 200 mg polyvinylpyrrolidone and 1% wv bovine serum albumin. Extracts were centrifuged at 13 000 x g for 30 minutes. Superoxide dismutase activity in the supernatant was spectrophotometrically determined by measuring the inhibition of nitrate formation from hydroxyl ammonium chloride oxidation at 530 nm[20].

2,3,5 - Triphenyltetrazolium chloride reduction

The accumulation of formazan was measured as described by Chen *et al.*[21] with minor modifications. Each sample consists of a leaf disc, 7 mm in diameter, with five repeats. The leaf discs, were subjected to a control treatment of three hours in 3 ml of 0.2 M sodium phosphate buffer and a moderate stress of three hours in 3 ml 0.5 M mannitol for acclimation, before incubation of both samples in 3 ml 1.0 M

mannitol solution. The experiment was performed at 29°C. The leaf discs were submerged in 3 ml of 0.8 % (w/v) TTC solution in 0.2 M sodium hydrogen maleate buffer, pH 6.9[22]. The discs were vacuum infiltrated for 5 minutes to ensure solution penetration into the tissue prior to a 18 hour incubation at 29°C in the dark. Subsequently, the discs were washed twice with distilled water followed by the addition of 3 ml 95% ethanol. The samples were boiled till dry and resuspended in 3 ml 95% ethanol when cooled. The reduction of TTC was estimated spectrophotometrically at 485 nm.

Results

Kanamycin tolerance experiments

In order to determine the optimal kanamycin concentration for selection of transformed cells, Aviva leaf explants were incubated on regeneration media containing various levels of kanamycin (0, 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml). Regeneration on medium containing 250 µg/ml cefotaxime (the antibiotic added in order to control the *A. tumefaciens* growth after transformation) was also evaluated. Results were taken after seven weeks, and are outlined on Table 1.

The cultivar showed callus regeneration after seven weeks, as was expected. The addition of cefotaxime to the regeneration medium did not appear to suppress regeneration as callus was formed. In the case of Aviva, cefotaxime stimulated shoot production, as four explants produced callus and shoots after seven weeks, whereas explants incubated on regeneration medium only produced callus. The addition of kanamycin to the medium killed the leaf explants, even at the lowest concentration tested (25 mg/l), so it was decided to use this concentration for selection of transformed cells in transformation experiments.

Table 1: Regeneration from Aviva leaf explants incubated on various antibiotic concentrations.

Treatment	# Explants	# Dead	# Callus only	# Callus + Shoots
R1AT + 0 mg/l Km	20	0	20	0
R1AT + 25 mg/l Km	20	20	0	0
R1AT + 50 mg/l Km	20	20	0	0
R1AT + 75 mg/l Km	20	20	0	0
R1AT +100 mg/l Km	20	20	0	0
R1AT + 250 mg/l Cx	20	0	16	4

Cloning of the Arabidopsis thaliana Cu/Zn SOD cDNA into transformation vectors

The plasmid pcSODRH consists of a 788 bp cDNA clone of a cytosolic Cu/Zn superoxide dismutase from *A. thaliana* in the *Eco* RI site of pBluescript (SK⁺). The insert consists of a full coding sequence and 112 bp 5'- and 206 bp 3'-untranslated region + 14 bases of poly A tail[14]. The isolated plasmid DNA was run on an 0.8% agarose gel to determine the purity of the plasmid DNA. The results showed the absence of contaminating substances and that the plasmid DNA had been successfully isolated in all cases.

The *Sac* 1 and *Hind* III digestions of the pcSODRH and pBI 221 plasmids, yielding the insert and the vector, respectively, are shown in Figure 3. A 1.2% agarose gel

was run to check whether the insert (SOD) had been spliced from the plasmid (pcSOD RH) and if the vector (pBI 221) had been successfully prepared for ligation and transformation. The pcSODRH was first restricted with *Sac* I and, after precipitation, with *Eco* RV to yield the SOD insert (0.8kb). The pBI 221 plasmid (5.7kb) was first restricted with the *Sac* I restriction enzyme and then with *Sma* I to yield the pBI 221 vector (3.8kb).

The transformation of insert: vector (1:1) yielded 16 DH5α colonies. This was expected since a successful ligation of insert with vector would yield *Amp* resistant DH5α colonies. The positive controls of the transformation experiment yielded 53 transformed DH5α colonies for pUC 18 and 46 transformed DH5α colonies for the uncut pBI 221. The positive controls contained the *Amp* resistance gene and thus the appearance of transformed DH5α colonies with *Amp* resistance was expected. The negative control yielded no transformed DH5α colonies. This was expected since no insert or vector was added to the transformation mixture. The lack of insert resulted in transformed DH5α cells having no *Amp* resistance.

The 16 DH5α colonies with *Amp* resistance obtained in the transformation studies with an insert: vector ratio of 1:1 were subjected to a JAT Prep to initially confirm the successful transformation of DH5α cells with the ligated insert and vector. Fourteen of the sixteen DH5α clones showed the same electrophoretic pattern (Figure 4). Two bands (one of 1.6 kb and one of 2.8 kb) were expected since the pBI 221 SOD plasmid contains two *Pst* 1 sites (Figures 5). These results, showed that 14 of the 16 DH5α clones were successfully transformed with the SOD gene (0.8kb). This represents a 88% transformation rate. These transformed DH5α cells were now named pBI 221 SOD (4.6kb). These clones were multiplied overnight in Luria-Bertani broth. Maxi-preparations were performed on these overnight cultures. Bead cultures were established from these overnight cultures and stored at -70°C until further use. A 1.2% agarose gel was run to check whether the insert (SOD) had been spliced from the plasmid (pBI 221 SOD) and if the vector (pBI 121) had been successfully prepared for ligation and transformation. The pBI 221 SOD plasmid

(4.6kb) was first restricted with *Sac* I and, after precipitation, with *Hind* III to yield the SOD insert (1.6kb). The pBI 121 plasmid was first restricted with the *Sac* I restriction enzyme and then with *Hind* III to yield the pBI 121 vector (10.3kb) (Figure 6).

Six colonies were selected at random and were subjected to a JAT Prep. However, no clear result was obtained from the results and it was decided to use restriction enzymes to partly confirm the transformation of pBI 121 with the SOD gene. The enzymes used for the confirmation of the transformation of pBI 121 with the SOD gene were *Eco* RI, *Bam* HI and *Pst* I. Two bands (one of 800 bp and one of ± 12.8 kb) were expected with the *Eco* RI restriction enzyme since the pBI 121 SOD plasmid contains two *Eco* RI sites. Two bands (one of 800 bp and one of ± 12.8 kb) were also expected with the *Bam* HI restriction enzyme since the pBI 121 SOD plasmid contains two *Bam* HI sites. Four bands (a 1 600 bp, a 1 947 bp, a 4 923 and a 5 138 bp) were expected with the *Pst* I restriction enzyme since the pBI 121 SOD plasmid contains four *Pst* I sites. However, an extra band of approximately 1000 bp was also observed. Thus, the restriction enzymes *Eco RI* and *Bam HI* yielded the expected results and confirmed that pBI 121 had been transformed with the SOD gene. However, the extra band observed with the *Pst I* digestion could not be explained and led to the decision to do a Southern Blot, using the DIG labelling and detection kit as method of detection, to confirm the transformation of pBI 121 with the SOD gene (Figure 7).

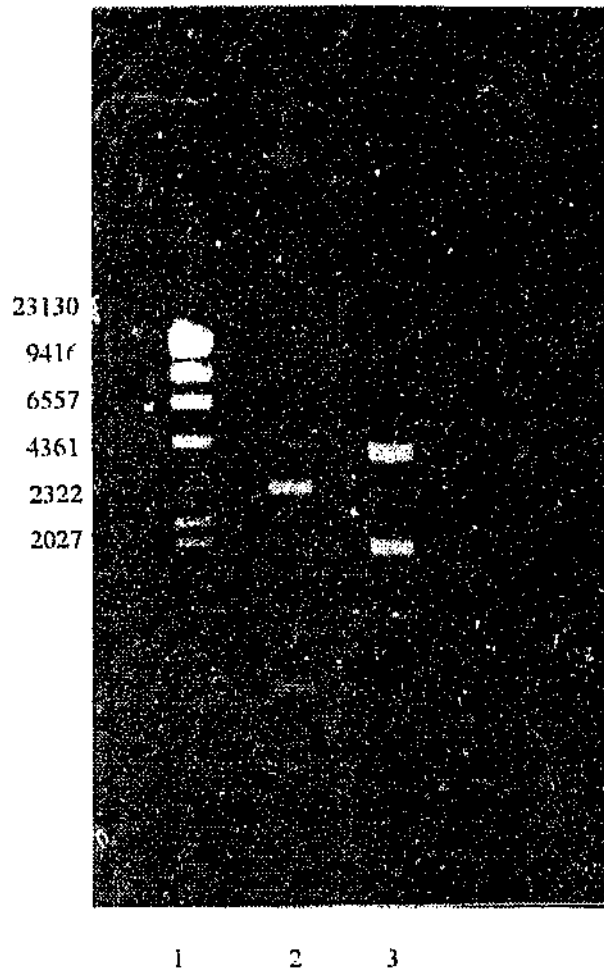


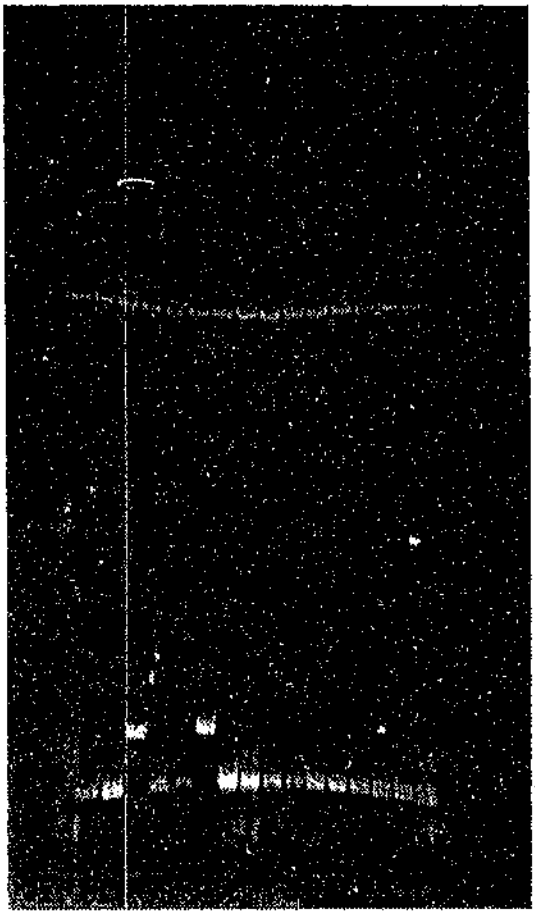
Figure 3: A 1.2% agarose gel to check if the insert (SOD) had been spliced from the plasmid (pcSOD RH) and if the vector (pBI 221) had been successfully prepared for ligation and transformation. The pcSODRH was first restricted with *Sac*I and, after precipitation, with *Eco* RV to yield the SOD insert (0.8kb). The pBI 221 plasmid (5.7kb) was first restricted with the *Sac* I restriction enzyme and then with *Sma* I to yield the pBI 221 vector (5.8kb). Lane 1: Molecular Weight Marker B; Lane 2: SOD insert (lower band); Lane 3: pBI 221 vector (upper band)

The Southern Blot would have to yield a band of 800 bp for the pcSODRH plasmid restricted with *Eco* RI, a band of 800 bp for the pBI 121 SOD plasmid restricted with *Eco* RI and *Bam* HI, and a band of 1.6 kb for the pBI 121 SOD plasmid restricted with *Pst* I. The results as expected were obtained and the transformed pBI 121 was now termed pBI 121 SOD (Figure 8).

The triparental mating experiment was conducted as described. All of the negative controls yielded no colonies while the positive controls yielded colonies as expected[17]. The DNA extracted from the triparental mating product, and various controls, was subjected to a PCR step using a SOD left primer and a SOD right primer. All the negative controls yielded no bands as expected. The *Agrobacterium tumefaciens* and all of the positive controls yielded the expected band. This indicated that the triparental mating had been successful (Figure 9).

Plant Transformation

In order to determine the optimum dip time for *A. tumefaciens*-mediated transformation of Aviva, leaf explants were dipped in *A. tumefaciens* LBA 4404 (pSOD) or *A. tumefaciens* LBA 4404 (pBI121) cell suspension for various periods (5, 10 and 20 mins). Callus production was scored after 2 months (Table 2), following which explants with callus were transferred to R1AT-. Three transformation experiments were initiated, and the total number of explants over the three experiments are outlined in Table 2. These results are also presented as graphs (Figure 10). Where an experiment had run for four months, shoot production from callus was recorded.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4: A 0.8% agarose gels depicting the JAT Prep results of the 16 DH∝ colonies with *Amp* resistance obtained in the transformation studies of pBI 221 with an insert: vector ratio of 1:1. Lane 1 + Lane 18: pBI 221 DNA; Lanes 2- 17 : DNA om the 16 DH5∝ colonies (possible pBI 221 SOD clones)

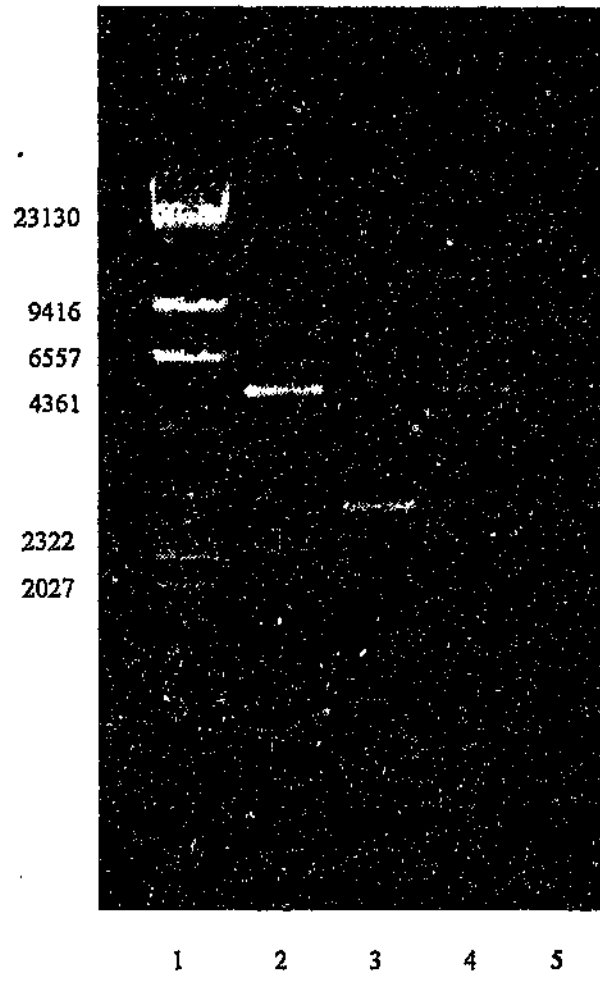


Figure 5: A 0.8% agarose gel showing the *Pst I* digests of DNA isolated from selected DH5∝ colonies with *Amp* resistance obtained in the transformation studies of pBI 221 with an insert: vector ratio of 1:1. Lane 1: Molecular Weight Marker II; Lane 2: pBI 221; Lane 3: possible pBI 221 SOD from lane 2 of the JAT Preparation results; Lane 4: possible pBI 221 SOD from lane 3 of the JAT Preparation results Lane 5: possible pBI 221 SOD from lane 7 of the JAT Preparation results

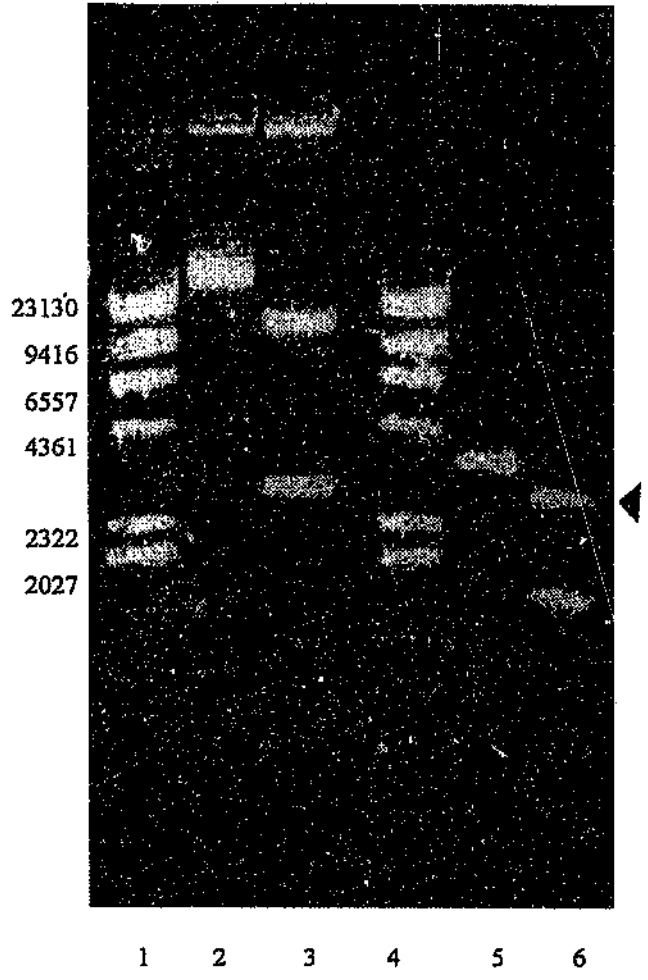


Figure 6: A 1.2% agarose gel to check if the insert (SOD) had been spliced from the plasmid (pBI 221 SOD) and if the vector (pBI 121) had been successfully prepared for ligation and transformation. The pBI 221 SOD plasmid (4.6kb) was first restricted with *Sac I* and, after precipitation, with *Hind* III to yield the SOD insert (1.6kb). The pBI 121 plasmid was first restricted with the *Sac* I restriction enzyme and then with *Hind* III to yield the pBI 121 vector (10.3kb). Lane 1: Molecular Weight Marker II; Lane 2: Uncut pBI 221 SOD; Lane 3: SOD Insert (lower band); Lane 4: Molecular Weight Marker II; Lane 5: Uncut pBI 121; Lane 6: pBI 121 Vector (upper band).

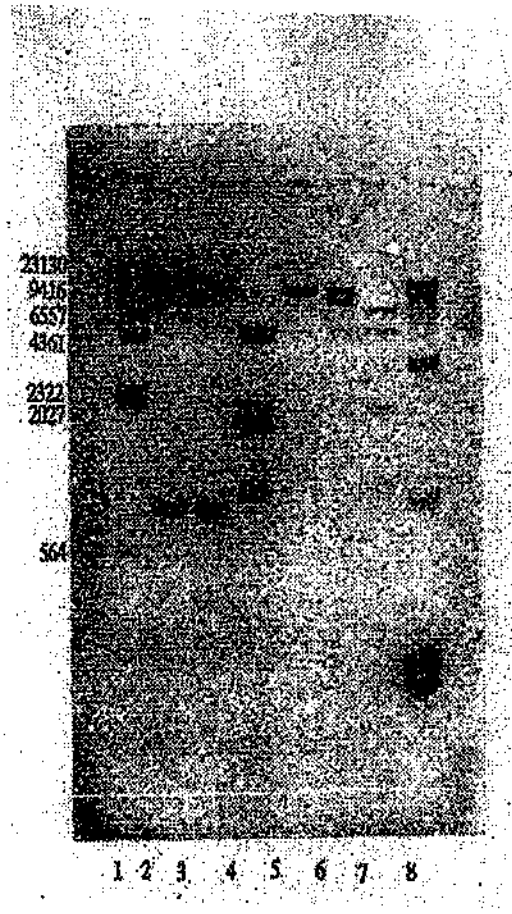


Figure 7: A 1.2% agarose gel showing the restriction enzyme digests to confirm the transformation of pBI 121 with SOD gene. The enzymes used were *Eco* RI, *Bam* HI and *Pst* I. Lane 1: Molecular Weight Marker II; Lane 2: *Bam* HI restriction of pBI 121 SOD; Lane 3: *Eco* RI restriction of pBI 121 SOD; Lane 4: *Pst* I restriction of pBI 121 SOD; Lane 5: *Bam* HI restriction of pBI 121; Lane 6: *Eco RI* restriction of pBI 121; Lane 7: *Pst* I restriction of pBI 121; Lane 8: *Eco* RI restriction of pcSODRH

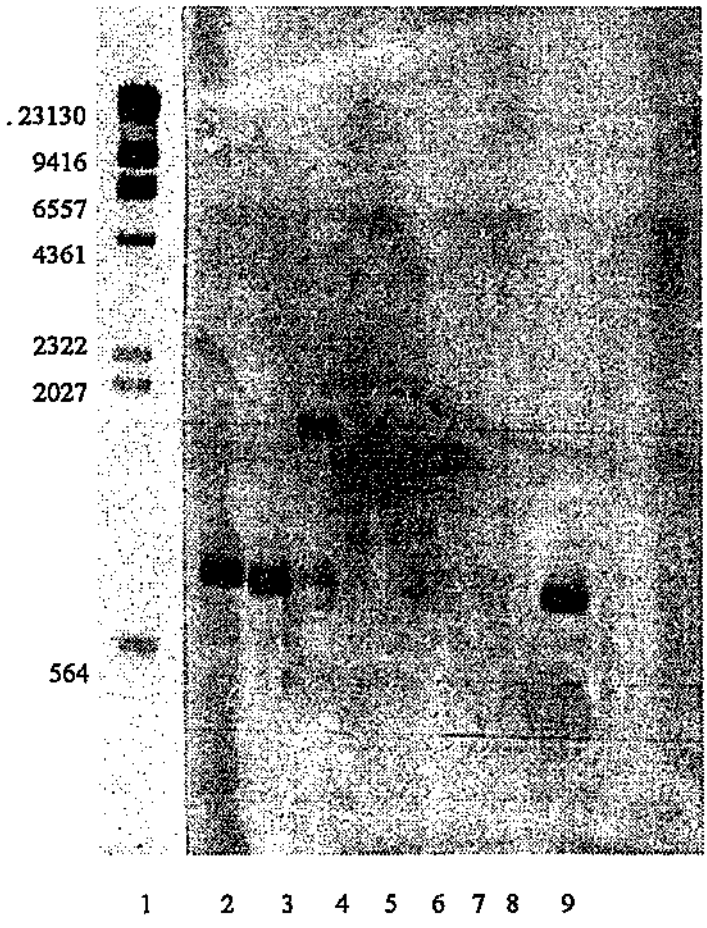


Figure 8: A Southern Blot, using the DIG labelling and detection kit as method of detection to confirm the transformation of pB1 with SOD gene. Lane 1:Molecular Weight Marker II; Lane 2: *Bam* HI restriction of pBI 121 SOD; Lane 3 : *Eco* RI restriction of pBI 121 SOD; Lane 4: *Pst* I restriction of pBI 121 SOD; Lane 5: *Bam* HI restriction of pBI 121Lane 6: *Eco* RI restriction of pBI 121; Lane 7: *Pst* I restriction of pBI 121; Lane 8: Open; Lane 9: Eco RI restriction of pcSODRH

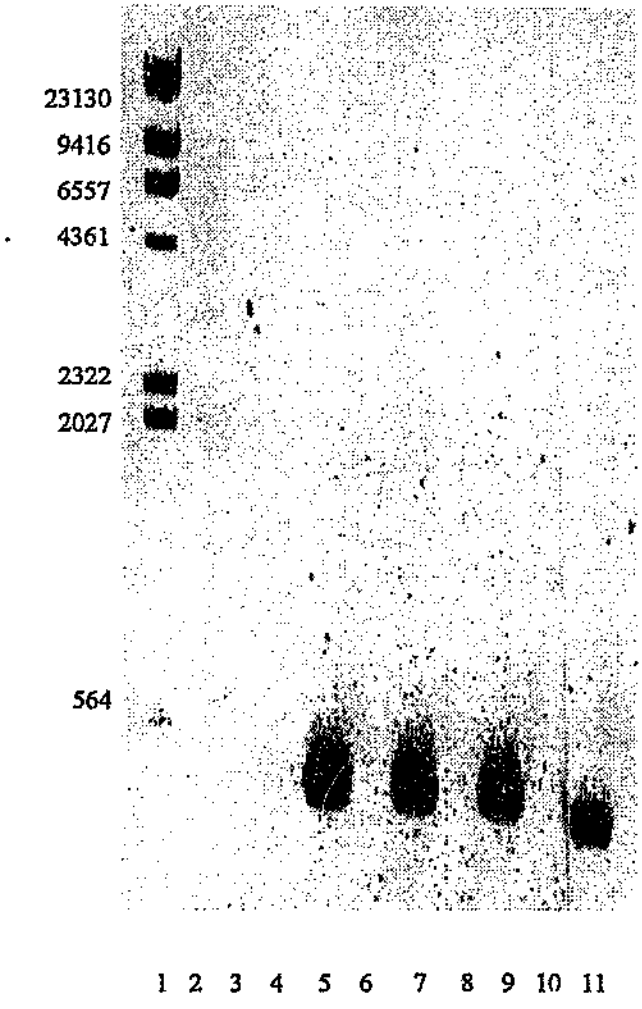


Figure 9: A 1.2% agarose gel showing the results of the PCR products to confirm the successful transformation of the *Agrobacterium tumefaciens* with the SOD gene via triparental mating. Lane 1: Molecular Weight Marker II; Lane 2: Bluescript; Lane 3: Bluescript; Lane 4: Bluescript; Lane 5: pcSODRH; Lane 6: pBI 221; Lane 7: pBI 221 SOD; Lane 8: pB 121;Lane 9: pBI 121 SOD; Lane 10: water; Lane 11: *A. tumefaciens* with the SOD insert.

Table 2: Total number of Aviva leaf explants transformed with A. *tumefaciens* cultures after two months.

TREATMENT	*A.tumefaciens*	DIP TIME	#EXPLANTS	#DEAD	#CONTAM	#CALLUS
R1AT	-	-	60	14	10	36
R1AT+K+C	-	-	50	50	0	0
R1AT+K+C	pSOD	5 mins	40	20	0	20
R1AT+K+C	pSOD	10 mins	40	19	10	11
R1AT+K+C	pSOD	20 mins	40	17	5	18
R1AT+K+C	pBI121	5 mins	50	43	0	9
R1AT+K+C	pBI121	10 mins	50	39	0	11
R1AT+K+C	pBI121	20 mins	45	20	20	5

The positive regeneration controls showed callus formation after two months (60% for Aviva), whereas all the negative control explants (incubated on regeneration medium supplemented with kanamycin and cefotaxime) died, as was expected.

Molecular confirmation of transformed potatoes

The expression of the SOD gene in the plants was studied by means of PCR analysis as well as Southern blot analysis. A polymerase chain reaction involves synthesizing multiple copies of A gene or A region of DNA. This is the result of oligonucleotide primers which bind to the opposite strands. Each cycle in the reaction involves denaturing the DNA, annealing the primers and extending them across the templates via DNA polymerase.

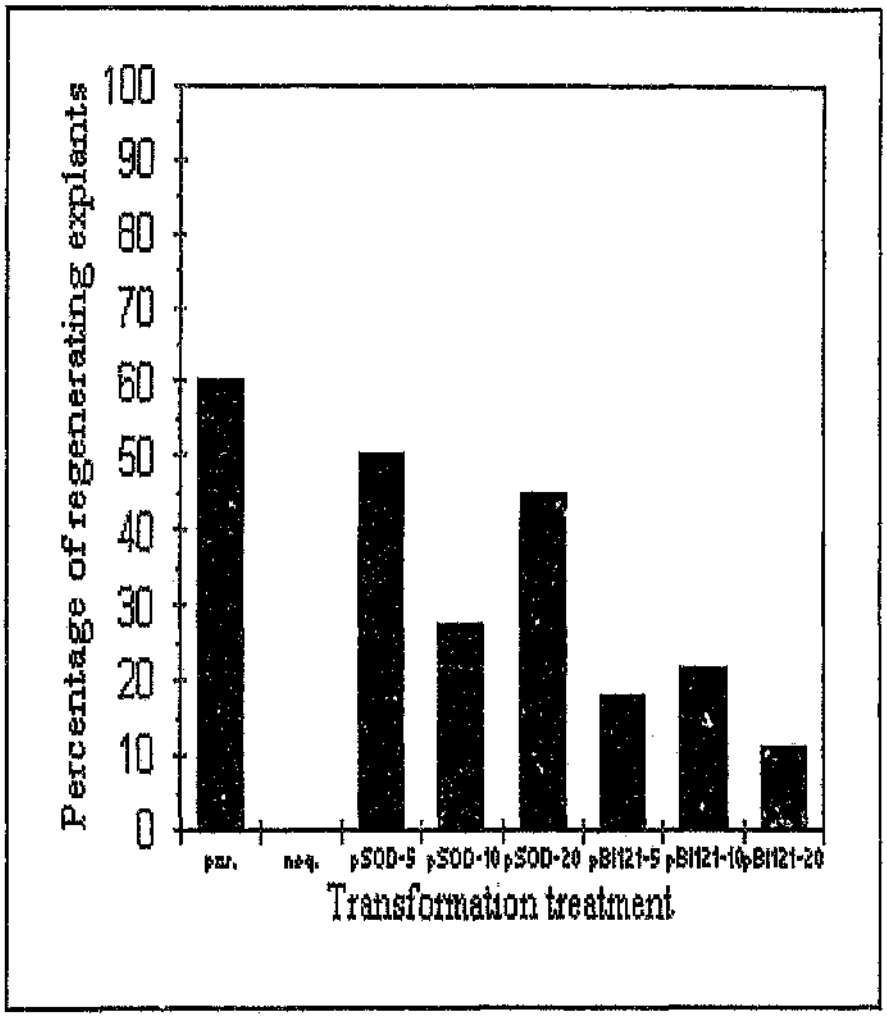


Figure 10: Callus regeneration after 2 months from Aviva leaf explants dipped in either *A. tumefaciens* LBA 4404 (pSOD) or *A. tumefaciens* LBA 4404 (pBI121) for 5, 10 or 20 mins.

Each newly synthesized DNA segment now becomes a template for the next round, resulting in exponential amplification of the original target DNA.

The PCR results with the SOD primers (Figure 11) indicated that the SOD primers are not specific enough, as the SOD *Arabidopsis* gene has a high homology with native potato SOD. The PCR fragment representing the SOD gene yielded in al the putative transformants except no 3, but unfortunately in a lesser extent in the control plant as well. All the negative controls yielded no bands as expected. Because of hese results it was decided to do a PCR with the nptII primers as the construct contains a kanamycin marker gene. The PCR with the ntpII primer result in a positive PCR fragment representing the nptII gene in the putative transformants no SOD1, SOD2, SOD5 and SOD7 (Figure 12). All the negative controls yielded no bands as expected.

Southern blot analysis was performed with the digested DNA. The Southern blot probed with the SOD DIG labelled probed resulted in binding with al the putatives as well as the control plant (results not shown). The Southern blot probed with the nptII DIG labelled probe resulted in binding of the putative transformants no SOD1, SOD2, SOD6 and SOD7 (Figure 13). All the negative controls yielded no bands as expected. This indicated that the SOD gene is definitely transformed into the putative transformant tissue culture plants SOD1, SOD 2, SOD 6 and SOD 7. It is possible that the other putative transformants are transformed with the SOD gene but discarded the nptII gene. In the future we will only use the plants which gave a positive reaction with both the tests.

Lethal drought stress

Transformed and untransformed potato plants were grown in the glasshouse under 23°C/16 ° C (day/night) temperatures. Four weeks after emergence water was withheld and the time taken to re ch lethal drought stress was recorded. Lethal drought stress was defined as the time (in wee:s) after which the plants did not

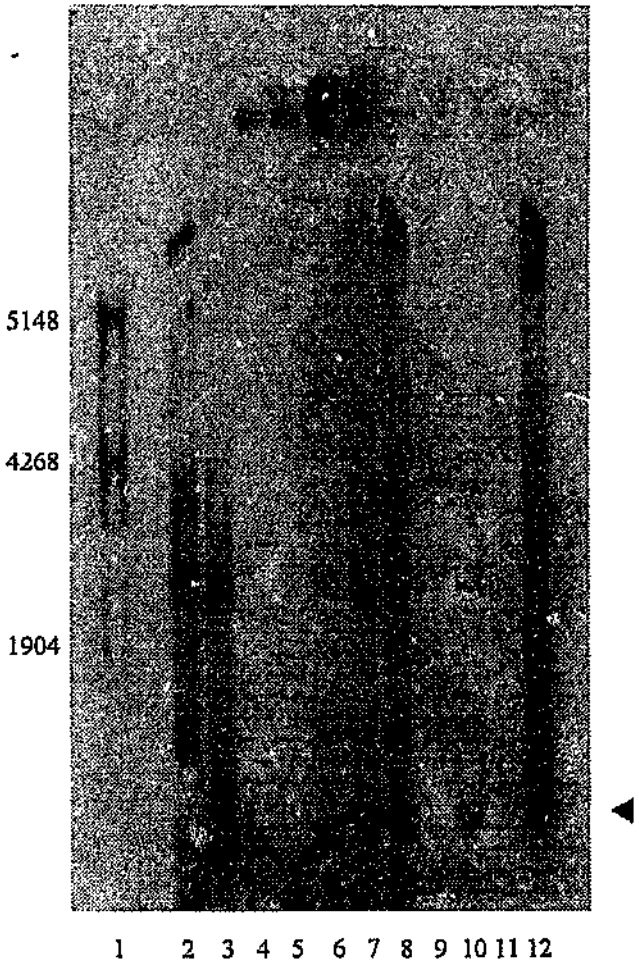


Figure 11: Agarose gel electrophoresis of A PCR analysis of DNA of putative transformed potato plants using SOD specific primers. Lane 1: molecular weight marker III; Lane 2-8: putative transformed plants; Lane 9: control plant; Lane 10: negative control plasmid; Lane 11: water ; Lane 12: pB1121 SOD positive control plasmid.

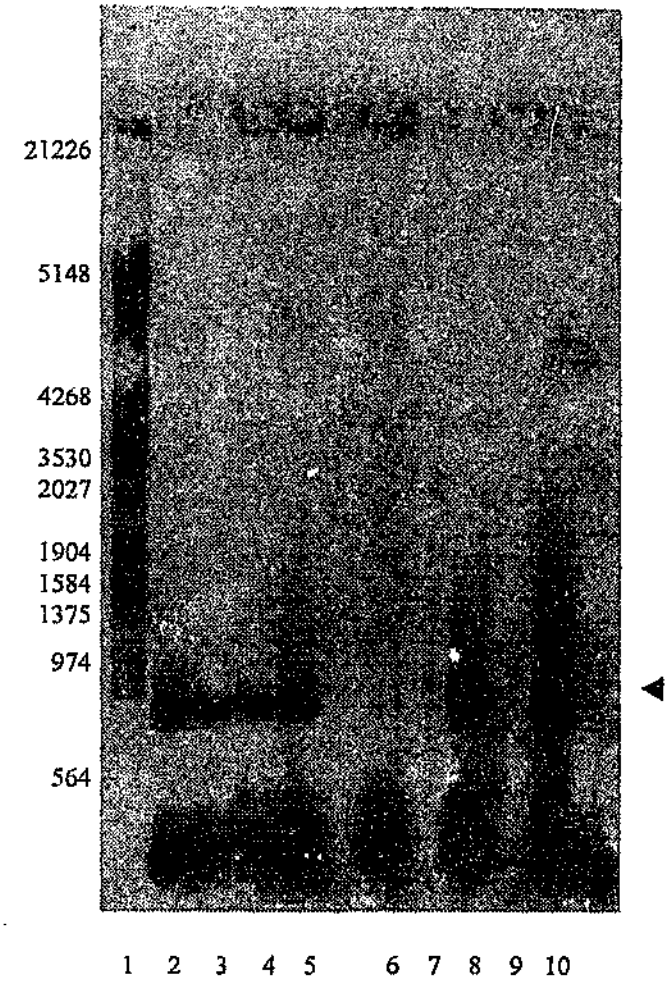


Figure 12: Agarose gel electrophoresis of a PCR analysis of DNA of putative transformed potato plants using nptII specific primers. Lane 1: molecular weight marker III; Lane 2-5: putative transformed plants; Lane 6: control plant; Lane 7: negative control plasmid; Lane 8: positive control plasmid; Lane 9: water; Lane 10: positive control plasmid.

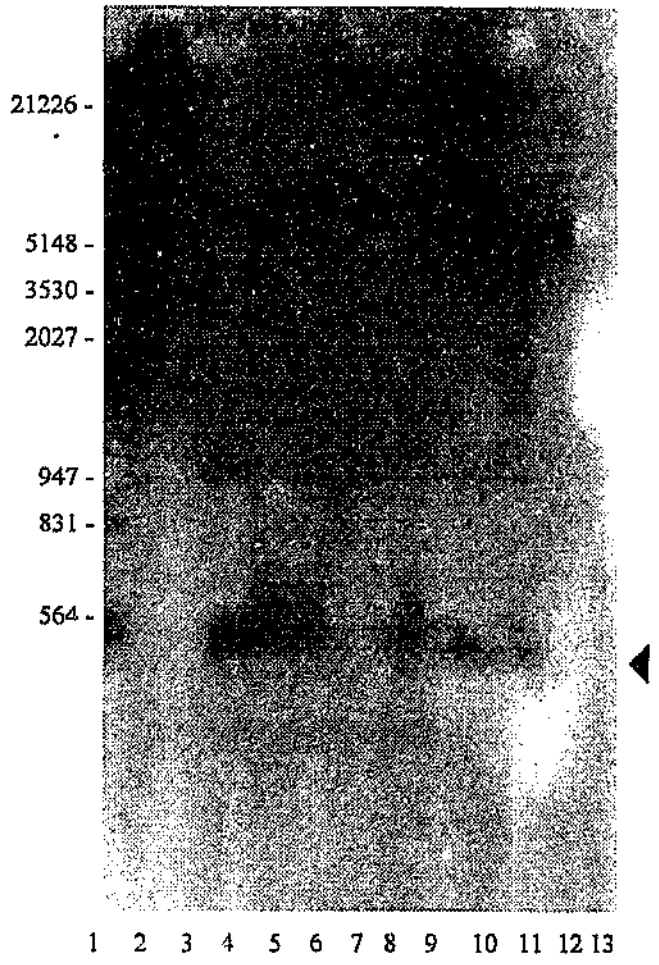


Figure 13: Autoradiograph of Southern blot of putative transformed potato plants, showing detection by autoradiograph of hybridization between the DIG labelled probe (nptII) and DNA extracted from putative transformed plant tissue. Lane 1: molecular weight markerIII ; Lane 2: positive control plasmid; Lanes 3-4: untransformed control plants; Lanes 5-12: putative transformed plants; Lane 13: negative control plasmid.

recover if rewatered. The time taken to reach lethal drought stress varied from four weeks for the untransformed Aviva plants to five weeks for the transformed lines SOD6 and SOD7, and six weeks for the transformed lines SOD1 and SOD2.

Cu/Zn Superoxide dismutase activity

The Cu/Zn superoxide dismutase activity was measured in leaves which were harvested at weekly intervals during six weeks that water was withheld. After two weeks without water a significant decrease in Cu/Zn SOD activity was observed in the transformed lines SOD 2 and SOD 7, after three weeks without water a significant decrease in Cu/Zn SOD activity was observed in the Aviva plants and the transformed line SOD 7, after four as well as five weeks without water a decrease in Cu/Zn SOD activity was observed in SOD1 and SOD2 while SOD6 and SOD7 showed non significant differences after four weeks without water (Table 3). The data are different from those in Chapter 3.2 as enzyme active was measured during the spring planting season while Chapter 3 consists of autumn results.

2,3,5-Triphenyltetrazolium chloride reduction

Viability as estimated by 2,3,5 triphenyltetrazolium chloride (TTC) reduction, was measured, as formazan production, every 30 minutes over a period of 3 hours after the lethal stress was induced. The formazan concentrations (reduced form of the tetrazolium salt) were higher in the stress treatment compared to the control treatment in three of the transformed plants namely, SOD 2, SOD 6 and SOD 7 indicating a tolerant reaction. In the sensitive reaction the formazan concentrations over time were lower in the stress treatment compared to the control treatment e.g. the untransformed Aviva cultivar and the transformed plant SOD 1. The area between the graphs was estimated as the difference between the mean of the stress treatment over time and the mean of the control treatment over time [22] and the results were presented in a histogram (Figure 14). The more negative the histogram the more sensitive the cultivar e.g. untransformed Aviva plants in (Figure 14). With this information it was clear that all the transformed potatoes (SOD1, SOD 2, SOD 6 and SOD 7) were more drought- tolerant compared to the untransformed Aviva plants.

Table 3: Cu/Zn Superoxide dismutase activity during drought stress compared to control conditions in untransformed Aviva plants as well as four transformed Aviva lines. Enzyme activity was measured in nmol/gram dry weight ($P<0.05$).

Cultivar	Treatment	Week 1*		Week 2		Week 3		Week 4		Week 5	
Aviva	control	1.981	NS	1.619	NS	2.070	*↓	lethal stress			
)	stress	1.711		1.502		1.673					
SOD 1	control	2.179	NS	2.066	NS	2.003	NS	2.179	*↓	2.024	*↓
	stress	2.170		2.117		1.161		1.782		1.265	
SOD 2	control	2.135	NS	2.370	*↓	1.963	NS	2.534	*↓	2.179	*↓
	stress	2.153		1.908		1.725		1.773		1.916	
SOD 6	control	2.345	NS	2.198	NS	2.216	NS	2.281	NS	lethal stress	
	stress	2.117		2.034		2.324		2.435			
SOD 7	control	2.948	NS	2.884	*↓	2.525	*↓	2.613	NS	lethal stress	
	stress	2.904		2.542		2.234		2.543			

* = Weeks without water; *↓ = Significant decrease; NS = not significant

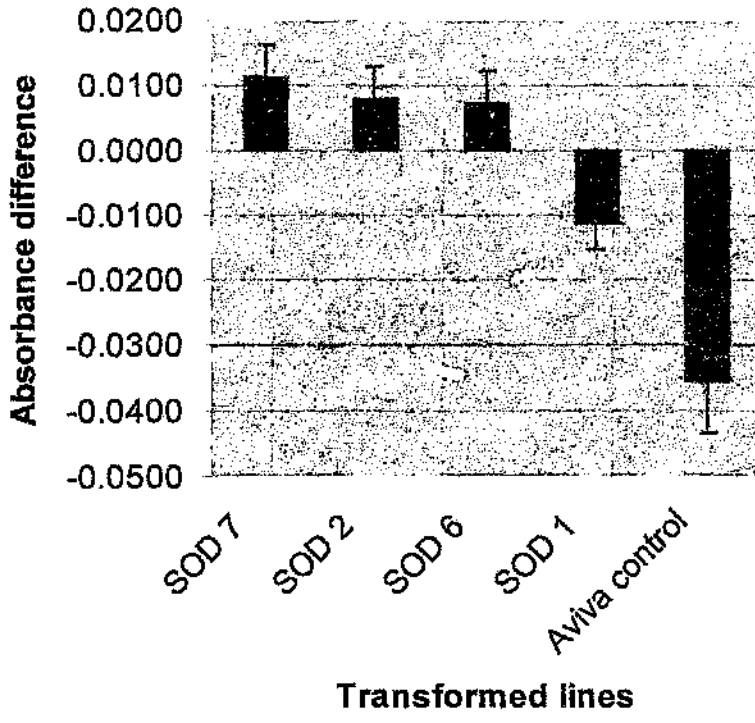


Figure 14: The mean difference between the stress and control treatments in leaf discs presented in a histogram. The positive values indicate a drought tolerant reaction while the negative values indicate a sensitive reaction to drought when measured by TTC-reduction. The standard deviation was very high as the measurements were taken over time, thus these results are not significantly different. As a result tendencies was used instead of statistical analysis.

Discussion

Much of the injury to plants caused by environmental stress is associated with oxidative stress at the cellular level[11]. Perl *et al.* [9] hypothesized that transgenic plants with constitutively elevated levels of SOD's should be more tolerant to photo-oxidative damage. However, the beneficial effects of SOD overproduction was mainly obtained by using methyl viologen (paraquat) as a stress factor. This approach is only an indication of the biological importance of SOD as paraquat is a superoxide generating redox cyder, while environmental stress conditions have much move pleiotropic effects on plants[23].

Van der Mescht *et al.* [13] had shown a correlation between increased Cu/Zn SOD activity during drought stress and drought sensitivity. The results from the present study are in agreement with this observation. Although there was a slight increase in enzyme activity in the four transformed lines when they were watered, the Cu/Zn SOD activity under drought stressed conditions showed either a non significant response or a significant decrease when compared to non-stressed plants. Additionally it was found that the transformed lines (SOD 1 and SOD 2) could withstand drought in glasshouse for two weeks longer than the untransformed plants and one week longer than the transformed lines SOD 6 and SOD7. High levels of Cu/Zn SOD activity (50 fold in transgenic tobacco according to Tepperman *et al.* [10] did not confer tolerance to oxidative stress while a small increase in Cu/Zn SOD activity was able to provide resistance against methyl viologen in human and mouse cells. It is possible that glutathione reductase activity and ascorbate peroxidase activity were the limiting factors when the SOD activity was increased to very high levels. In our present study we were not able to determine the copy number of the Cu/Zn SOD gene. This may be due to the high homology between the *A. thaliana* and the potato genes as well as the tetraploid nature of the potato. However, under control conditions SOD6 and SOD7 had significantly higher Cu/Zn SOD activity compared to Aviva, SOD1 and SOD2. Interestingly, it was the transformed lines that did not differ significantly from the control plants which survived the longest.

Triphenyltetrazolium chloride reduction gives an indication of viability. Additionally, the inhibition of triphenyltetrazolium chloride reduction is an indication of dehydrogenase inactivation resulting in a decrease in formazan production[21]. We have shown that during these aeration experiments, a higher formazan concentration in stressed plants compared to control plants over time represents a tolerant reaction in cotton while a lower formazan production in the stressed treatment compared to the control treatment was an indication of sensitivity [22]. Results from leaves subjected to an osmotic stress of 0.5 M mannitol (-1.24 Mpa) showed a tolerant response with the exception of the transformed plant SOD1 and the untransformed Aviva plants. Although plant SOD 1 tested sensitive it was still more tolerant than the untransformed control plants. A statistical analysis was not performed as the standard deviation was very high as the measurements were taken over time. In this case the tendencies (tolerant vs sensitive) were of importance (Prof. J.M.P. Geerthsen personal communication). We conclude that the plants transformed with the Cu/Zn SOD gene were more tolerant to drought compared to untransformed plants.

The TTC results were compared to the glasshouse results, but a direct correlation was not obvious. According to the TTC results SOD2 , SOD6 and SOD7 were drought tolerant while SOD1 (although less sensitive) and Aviva were drought sensitive. On the other hand, according to results from the glasshouse SOD1 and SOD2 were more drought tolerant than SOD6 and SOD7 which in turn were more tolerant than Aviva. A correlation between the two experiments may be complicated by the intensity of the drought stress. In the glasshouse water was withheld from the plants, resulting in increasing drought stress over time while TTC-reduction was measured at only one water regime which represents a mild drought stress (chapter 5). However, we conclude that the transformed lines were able to withstand drought longer before a lethal stress was induced compared to untransformed cultivar.

In view of the current data it seems reasonable to conclude that SOD plays a significant role in protecting living cells against the toxicity and mutagenicity of

active oxygen species by virtue of their capacity to scavenge the superoxide radical. Whether SOD has other biological functions remains an open question[3]. Additionally, little is currently known as how the genome perceives oxidative insult and mobilizes a response to it. Such information is interesting in and of itself, but it is also essential in any future attempt to raise tolerance to environmental oxidative stress in organisms and to reduce cellular damage by active oxygen. To understand these mechanisms it is necessary to identify the responsive genes and to understand their structure, regulation and expression[3].

According to Scandalios [3] future research should include the identification and characterization of cis-acting elements and trans-acting factors involved in SOD gene regulation and expression. This research will provide some depth in our understanding of the entire signal transduction pathway during oxidative stress and will enhance our efforts towards engineering organisms to better cope with oxidative insult.

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CHAPTER 7

DISCUSSION

The most important physiological stress to potato production in most areas of the world is drought[1]. As the potato is more sensitive to drought than most other crop species an understanding of the effect of drought on the physiology of the crop is of great economic importance[2]. However, little success has been achieved in breeding drought-tolerant potato cultivars through empirical methods. This is due to a poor understanding of drought tolerance and a lack of reliable non-destructive screening techniques[1]. Furthermore, field studies and selection for yield is complicated by the interaction between heat and drought stress as water stress is often accompanied by heat stress. An additional negative feature with regard to selection for yield is it's low heritability in hot and dry environments[3]. In this thesis I attempted to evaluate the efficiency of five physiological measurements to determine drought tolerance in potato.

According to Ludlow[4], there are no genes fi . drought tolerance as such, but there are genes for traits that contribute to drought tolerance. Thus, the traits involved in drought tolerance offer the opportunity to develop a screening method. The first step in the development of a screening method is the understanding of the functions of the physiological mechanisms involved in drought tolerance as well as the links between different physiological processes[5]. As a result of these interactions between physiological mechanisms during drought stress it was decided to evaluate chlorophyll fluorescence, chlorophyll content, Cu/Zn superoxide dismutase levels, glutathione reductase levels, ascorbate peroxidase levels, free proline concentrations and polyamine titers as possible screening methods for drought tolerance in potato. 2,3,5-Triphenyltetrazolium chloride (TTC) reduction was added to the list as the TTC-assay measures the capability of plant tissue to carry out electron transport[6].

The second phase in the development of a screening method is to test whether the technique(s) can reliably distinguish between sensitive and tolerant cultivars. Twelve potato cultivars were selected which differed in growth period as well as drought tolerance. Additionally, the cultivars were selected as the extremes of tolerance and sensitivity within the three growth periods. The outcome of our investigations on chlorophyll fluorescence and chlorophyll content lead us to group the cultivars into three groups namely a tolerant group which tested tolerant for all fluorescence parameters (Aviva, Devlin and Late Harvest), an intermediate group (Sebago, Ono, Baraka, Kimberley Choice and Hoëvelder) and a sensitive group which tested sensitive for all fluorescence parameters (Raritan, Vanderplank, Darius and Bravo). However, there was a positive correlation between drought tolerance in field trials and chlorophyll fluorescence parameters in cultivars with a short growth period (chapter 2).

The activity of Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase were evaluated during drought stress in the twelve potato cultivars. Differences in glutathione reductase and ascorbate peroxidase activities could not be correlated with drought tolerance. The levels of glutathione reductase in stressed potato cultivars were consistently lower compared to control treatments. The levels of ascorbate peroxidase activity were generally higher in stressed plants compared to control plants. When drought stressed plants were compared to well watered control plants we observed that the ability to maintain adequate or decreased concentrations of Cu/Zn superoxide dismutase correlated with drought tolerance under field conditions. This may be due to the fact that drought tolerance is defined in terms of yield reduction and that we are selecting for plants avoiding drought by tuberization rather than for tolerance (Chapter 3). With this parameter we were able to group the cultivars in a tolerant and sensitive group as expected. The tolerant group included the cultivars Vanderplank, Aviva, Darius, Baraka, Hoëvelder and Late Harvest while the sensitive group included Raritan, Devlin, Sebago, Ono, Bravo and Kimberley Choice. In contrast to the results from chlorophyll fluorescence and chlorophyll content, the cultivars with a medium and long growth period correlated with the

breeder's opinion of drought tolerance in the field.

Optimal enzyme activity may be reduced by a decrease in cell pH. Additionally Handa, Handa, Hasegawa and Bressan [6] proposed that proline accumulation could be associated with a change in cytoplasmic pH. This is in agreement with other reports on the physiological functions of proline namely a protectant against denaturation of proteins [7] and controlling the cell pH thus reducing the acidity[8]. In the present study it was found that proline accumulation is a function of growth period. Drought tolerant cultivars with a short growth period accumulated the highest levels of proline two weeks after water was withhold, cultivars with a medium growth period accumulated most proline three weeks without water while cultivars with a long growth period accumulated most proline after four weeks without water. It is possible that potatoes with a short growth period avoid drought by early tuberization. As drought is defined in terms of yield reduction, these cultivars are termed drought tolerant. The cultivars able to sustain vegetative growth longer, take longer to accumulate maximum proline (Chapter 4). The time of maximum free proline accumulation correlated perfectly with the breeders's opinion of drought tolerance but the timing of maximum free proline accumulation was inconsistent when the sensitive cultivars was concerned.

Furthermore the synthesis of free proline and polyamines share a biochemical pathway at intermediates glutamic acid and L-ornithine[9,10]. It is suggested that the role of polyamines is in maintaining the cation-anion balance in the plant cell. Additionally polyamines are involved in free radical scavenging[11]. Drolet *et al.* [12] have shown that both chemically and enzymatically generated superoxide radicals were scavenged by polyamines. The polyamines were not simply inhibiting enzyme activity as they also had the capacity to scavenge superoxide radicals which were generated photochemically. The authors concluded that the actual mechanisms by which polyamines act as free radical scavengers have not been revolved. In our present study we found that the most abundant polyamine was spermine. The synthesis of spermine, agmatine and spermidine were cultivar and age dependent,

however, the synthesis of spermine, after four weeks without water, showed a correlation with drought tolerance in potato. Our results also showed that the cultivars could again be divided into a tolerant (Devlin, Aviva, Ono, Darius, Baraka, Hoëvelder and Late Harvest) and a sensitive group (Raritan, Vanderplank, Sebago, Bravo and Kimberley Choice). In this case we were able to correctly identify drought tolerant cultivars with a short and long growth period.

Our results on cell viability confirmed the general opinion that the potato crop is more sensitive to drought compared to other crops as a drought tolerant reaction was only observed in the leaves of two (Darius and Ono) cultivars. However, we were able to rank the cultivars from most tolerant to most sensitive for drought as well as heat. The value of the TTC-assay is thus in the ability to rank the cultivars and also in determining the correlation between drought- and heat-tolerance.

The third phase during the development of a screening method includes the comparison of the physiological results (screening techniques) with results obtained under field conditions. From the results discussed in chapters 2-5 it was evident that the most promising physiological parameters to use as a screening methods for drought tolerance is the levels of Cu/Zn superoxide dismutase activity, free proline accumulation and spermine levels . Both Cu/Zn superoxide dismutase and polyamines act as free radical scavengers during oxidative stress, while proline not only act as a protectant against denaturation of proteins but also reduces cell acidity. Due to the complex phenomenon of drought tolerance, a single test for drought tolerance could not be identified. However, we suggest to use these three parameters in future when screening for drought tolerance.

The final test of the value of the antioxidative system was to evaluate the contribution of the Cu/Zn superoxide dismutase gene to drought tolerance by the transformation of a well-adapted and high yielding genotype. In chapter 2 we had shown that the

potato cultivar Aviva had only half the Cu/Zn superoxide dismutase activity when comp red to eleven other cultivars differing in growth period and drought tolerance. Aviva is a drought tolerant cultivar with a short gr h period. Additionally we have shown in chapter 3 that there is a negative correlation between increased Cu/Zn superoxide dismutase activity and drought sensitivity. Thus we intended to enhance the Cu/Zn SOD activity in Aviva which gave the expected negative correlation but had only half the activity compared to the other cultivars. Although there was a slight increase in enzyme activity in four transformed lines during unstressed conditions, the enzyme activity during drought stress resulted in a significant decrease or a non significant response when compared to non-stressed plants. The four transformed lines survived drought conditions in the glasshouse longer than the untransformed plants. This result was co by the results from the 2,3,5-triphenyltetrazolium chloride reduction assays which showed that the four transformed lines were more drought tolerant than the untransformed Aviva plants.

Future research will include the multiplication of the transformed lines on field plots. The transformed lines will then be evaluated for drought stress using line source experiments, Cu/Zn SOD activity, spermine levels and free proline accumulation. Application for field testing of transformed potato lines were forwarded to the South African comity for genetic experimentation (SAGENE).

Table 1: Summary of the physiological reactions of 12 potato cultivars to drought stress

\+ tolerant reaction

\- sensitive reaction

0 no reaction

	Cu/Zn SOD	Free proline	Spermine	Fo	Fv/Fm	Fm	Chl *a*	Chl *b*	Total Chl	TTC
Raritan	-	-	-	-	-	-	-	-	-	-
Vanderplank	+	-	-	0	-	0	-	-	-	-
Devlin	-	+	+	+	+	+	+	+	+	-
Aviva	+	+	+	+	+	+	+	+	+	-
Sebago	-	-	-	-	0	-	+	+	-	-
Ono	-	-	+	+	-	+	-	-	+	+
Darius	+	+	+	-	-	-	-	-	-	+
Baraka	+	+	+	+	+	0	-	-	-	-
Bravo	-	+	-	0	-	-	-	-	-	-
Kimberley Choice		-	-	0	+	+	+	-	-	-
Hoëvelder	+	+	+	-	-	0	+	-	+	-
Late Harvest	+	+	+	+	+	+	+	-	-	-

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APPENDIX

1. Cultivar descriptions according to Union Internationale pour la Protection des Obtentions Vegetables. (International Union for the protection of new varieties of plants) (UPOV).

1.1 **RARITAN** - Cultivar description under South African conditions is not available

1.2 **VANDERPLANK**

	Characteristics			Note
1.2.1	Lightsprout	:	size	medium
1.2.2	Lightsprout	:	Shape	conical
1.2.3	Lightsprout	:	anthocyanin of base	red-violet
1.2.4	Lightsprout	:	intensity of anthocyanin colouration of base	weak
1.2.5	Lightsprout	:	pubescence of base	medium
1.2.6	Lightsprout	:	size of tip	medium
1.2.7	Lightsprout	:	habit of tip	medium
1.2.8	Lightsprout	:	intensity of anthocyanin colouration of tip	medium
1.2.9	Lightsprout	:	pubescence of tip	medium
1.2.10	Lightsprout	:	number of roottips	unknown
1.2.11	Lightsprout	:	protrusion of lenticels	mediu m
1.2.12	Lightsprout	:	length of lateral shoots	short
1.2.13	Plant	:	height	medium
1.2.14	Plant	:	type	stem
1.2.15	Plant	:	growth habit	semi-erect

1.2.16	Stem	:	thickness of main stem	thick
1.2.17	Stem	:	extension of anthocyanin	medium-strong
1.2.18	Leaf	:	size	large
	Leaf	:	length	unknown
	Leaf	:	width	unknown
1.2.19	Leaf	:	silhouette	open
1.2.20	Leaf	:	intensity of green colour	dark
1.2.21	Leaf	:	extension of anthocyanin colouration of midrib	medium
1.2.22	Leaflet	:	size	large
	Leaflet	:	length	unknown
1.2.23	Leaflet	:	width	broad
1.2.24	Leaflet	:	frequency of coalescence	high
1.2.25	Leaflet	:	waviness of margin	medium-strong
1.2.26	Leaflet	:	depth of veins	deep
1.2.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.2.28	Leaflet	:	glossiness of the upperside	dull
1.2.29	Leaflet	:	(midrif) frequency of secondary leaflets	high
1.2.30	Terminal leaflet:		frequency of secondary leaflets	high
1.2.31	Lateral leaflet	:	frequency of secondary leaflets	high
1.2.32	lateral leaflet	:	size of secondary leaflets	large
1.2.33	Inflorescence	:	size	medium
1.2.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.2.35	Plant	:	frequency of flowers	high
1.2.36	Flower	:	anthocyanin colouration of bud	absent
1.2.37	flower corolla	:	size	medium
1.2.38	flower corolla	:	colour of inner side	white

1.2.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.2.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.2.41	flower corolla	:	size of white tips in coloured flower	not applicable
1.2.42	Plant	:	frequency of fruits	few
1.2.43	Plant	:	time of maturity	unknown
1.2.44	Tuber	:	shape	oval
1,2.45	Tuber	:	depth of eyes	shallow
1.2.46	Tuber	:	smoothness of skin	medium
1.2.47	Tuber	:	colour of skin	yellow
1.2.48	Tuber	:	colour of base of eye	yellow
1.2.49	Tuber	:	colour of flesh	cream
1.2.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	absent
1.3	**DEVLIN**			
1.3.1	Lightsprout	:	size	medium-large
1.3.2	Lightsprout	:	Shape	conical
1.3.3	Lightsprout	:	anthocyanin of base	red-violet
1.3.4	Lightsprout	:	intensity of anthocyanin colouration of base	medium
1.3.5	Lightsprout	:	pubescence of base	medium
1.3.6	Lightsprout	:	size of tip	medium
1.3.7	Lightsprout	:	habit of tip	medium-open
1.3.8	Lightsprout	:	intensity of anthocyanin colouration of tip	weak
1.3.9	Lightsprout	:	pubescence of tip	strong

1.3.10	Lightsprout	:	number of roottips	unknown
1.3.11	Lightsprout	:	protrusion of lenticels	strong
1.3.12	Lightsprout	:	length of lateral shoots	short
1.3.13	Plant	:	height	$\bar{x}$ = 47,6 cm
				medium
1.3.14	Plant	:	type	intermediate
1.3.15	Plant	:	growth habit	erect
1.3.16	Stem	:	thickness of main stem	medium-
				thick
1.3.17	Stem	:	extension of anthocyanin	absent
1.3.18	Leaf	:	size	large
	Leaf	:	length	$\bar{x}$ = 29,5 cm
	Leaf	:	width	$\bar{x}$ = 22,4 cm
1.3.19	Leaf	:	silhouette	medium
1.3.20	Leaf	:	intensity of green colour	light
1.3.21	Leaf	:	extension of anthocyanin colouration	
			of midrib	absent
1.3.22	Leaflet	:	size	large
	Pinna	:	length	$\bar{x}$ = 12,1 cm
1.3.23	Leaflet	:	width	$\bar{x}$ = 6,9 cm
				broad
1.3.24	Leaflet	:	frequency of coalescence	low
1.3.25	Leaflet	:	waviness of margin	weak
1.3.26	Leaflet	:	depth of veins	shallow
1.3.27	Leaflet	:	anthocyanin pigmetration of blade of	
			young leaflets at apical rosette	absent
1.3.28	Leaflet	:	glossiness of the upperside	dull
1.3.29	Leaflet	:	(midrif) frequency of secondary leaflets	low
1.3.30	Terminal leaflet	:	frequency of secondary leaflets	low
1.3.31	Lateral leaflet	:	frequency of secondary leaflets	low

1.3.32	lateral leaflet	:	size of secondary leaflets	small
1.3.33	Inflorescence	:	size	medium
1.3.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.3.35	Plant	:	frequency of flowers	medium
1.3.36	Flower	:	anthocyanin colouration of bud	absent
1.3.37	flower corolla	:	size	medium
1.3.38	flower corolla	:	colour of inner side	white
1.3.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	weak
1.3.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.3.41	flower corolla	:	size of white tips in coloured flower	small
1.3.42	Plant	:	frequency of fruits	none
1.3.43	Plant	:	time of maturity	unknown
1.3.44	Tuber	:	shape	short-oval
1.3.45	Tuber	:	depth of eyes	shallow
1.3.46	Tuber	:	smoothness of skin	smooth
1.3.47	Tuber	:	colour of skin	yellow
1.3.48	Tuber	:	colour of base of eye	yellow
1.3.49	Tuber	:	colour of flesh	cream
1.3.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	absent
1.4	**AVIVA**			
1.4.1	Lightsprout	:	size	large
1.4.2	Lightsprout	:	Shape	broad cylindrical
1.4.3	Lightsprout	:	anthocyanin of base	red-violet
1.4.4	Lightsprout	:	intensity of anthocyanin colouration of base	medium

1.4.5	Lightsprout	:	pubescence of base	strong
1.4.6	Lightsprout	:	size of tip	large
1.4.7	Lightsprout	:	habit of tip	open
1.4.8	Lightsprout	:	intensity of anthocyanin colouration of tip	weak
1.4.9	Lightsprout	:	pubescence of tip	strong
1.4.10	Lightsprout	:	number of roottips	unknown
1.4.11	Lightsprout	:	protrusion of lenticels	medium
1.4.12	Lightsprout	:	length of lateral shoots	long
1.4.13	Plant	:	height	tall $\bar{x}$ = 85.6 cm
1.4.14	Plant	:	type	intermediate
1.4.15	Plant	:	growth habit	semi-erect
1.4.16	Stem	:	thickness of main stem	thick
1.4.17	Stem	:	extension of anthocyanin	absent
1.4.18	Leaf	:	size	medium-large
	Leaf	:	length	$\bar{x}$ = 31,6 cm
	Leaf	:	width	$\bar{x}$ = 20,9 cm
1.4.19	Leaf	:	silhouette	medium
1.4.20	Leaf	:	intensity of green colour	medium
1.4.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.4.22	Leaflet	:	size	large
	Pinna	:	length	$\bar{x}$ = 10,02 cm
1.4.23	Leaflet	:	width	$\bar{x}$ = 6,2 cm broad
1.4.24	Leaflet	:	frequency of coalescence	medium
1.4.25	Leaflet	:	waviness of margin	weak
1.4.26	Leaflet	:	depth of veins	medium

1.4.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.4.28	Leaflet	:	glossiness of the upperside	dull
1.4.29	Leaflet	:	(midrif) frequency of secondary leaflets	high
1.4.30	Terminal leaflet:		frequency of secondary leaflets	high
1.4.31	Lateral leaflet	:	frequency of secondary leaflets	medium-high
1.4.32	lateral leaflet	:	size of secondary leaflets	large
1.4.33	Inflorescence	:	size	large
1.4.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.4.35	Plant	:	frequency of flowers	high
1.4.36	Flower	:	anthocyanin colouration of bud	absent
1.4.37	flower corolla	:	size	medium
1.4.38	flower corolla	:	colour of inner side	white
1.4.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.4.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.4.41	flower corolla	:	size of white tips in coloured flower	not applicable
1.4.42	Plant	:	frequency of fruits	unknown
1.4.43	Plant	:	time of maturity	unknown
1.4.44	Tuber	:	shape	short-oval
1.4.45	Tuber	:	depth of eyes	shallow
1.4.46	Tuber	:	smoothness of skin	smooth
1.4.47	Tuber	:	colour of skin	yellow
1.4.48	Tuber	:	colour of base of eye	yellow
1.4.49	Tuber	:	colour of flesh	white
1.4.50	Yellow skinned varieties only			

	Tuber	:	anthocyanin colouration of skin in reaction to light	absent
1.5	SEBAGO			
1.5.1	Lightsprout	:	size	large
1.5.2	Lightsprout	:	Shape	broad cylindrical
1.5.3	Lightsprout	:	anthocyanin of base	red-violet
1.5.4	Lightsprout	:	intensity of anthocyanin colouration of base	medium
1.5.5	Lightsprout	:	pubescence of base	strong
1.5.6	Lightsprout	:	size of tip	large
1.5.7	Lightsprout	:	habit of tip	open
1.5.8	Lightsprout	:	intensity of anthocyanin colouration of tip	weak
1.5.9	Lightsprout	:	pubescence of tip	strong
1.5.10	Lightsprout	:	number of roottips	unknown
1.5.11	Lightsprout	:	protrusion of lenticels	medium
1.5.12	Lightsprout	:	length of lateral shoots	long
1.5.13	Plant	:	height	tall $\bar{x}$ = 85.6 cm
1.5.14	Plant	:	type	intermediate
1.5.15	Plant	:	growth habit	semi-erect
1.5.16	Stem	:	thickness of main stem	thick
1.5.17	Stem	:	extension of anthocyanin	absent
1.5.18	Leaf	:	size	medium-large
	Leaf	:	length	$\bar{x}$ = 31,6 cm
	Leaf	:	width	$\bar{x}$ = 20,9 cm
1.5.19	Leaf	:	silhouette	medium
1.5.20	Leaf	:	intensity of green colour	medium

1.5.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.5.22	Leaflet	:	size	large
	Pinna	:	length	$\bar{x}$ = 10,02 cm
1.5.23	Leaflet	:	width	$\bar{x}$ = 6,2 cm broad
1.5.24	Leaflet	:	frequency of coalescence	medium
1.5.25	Leaflet	:	waviness of margin	weak
1.5.26	Leaflet	:	depth of veins	medium
1.5.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.5.28	Leaflet	:	glossiness of the upperside	dull
1.5.29	Leaflet	:	(midrif) frequency of secondary leaflets	high
1.5.30	Terminal leaflet:		frequency of secondary leaflets	high
1.5.31	Lateral leaflet	:	frequency of secondary leaflets	medium-high
1.5.32	lateral leaflet	:	size of secondary leaflets	large
1.5.33	Inflorescence	:	size	large
1.5.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.5.35	Plant	:	frequency of flowers	high
1.5.36	Flower	:	anthocyanin colouration of bud	absent
1.5.37	flower corolla	:	size	medium
1.5.38	flower corolla	:	colour of inner side	white
1.5.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.5.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent

1.5.41	flower corolla	:	size of white tips in coloured flower	not applicable
1.5.42	Plant	:	frequency of fruits	unknown
1.5.43	Plant	:	time of maturity	unknown
1.5.44	Tuber	:	shape	short-oval
1.5.45	Tuber	:	depth of eyes	shallow
1.5.46	Tuber	:	smoothness of skin	smooth
1.5.47	Tuber	:	colour of skin	yellow
1.5.48	Tuber	:	colour of base of eye	yellow
1.5.49	Tuber	:	colour of flesh	white
1.5.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	absent

1.6. **ONO**

Cultivar description under South African conditions is not available.

1.7 **DARIUS**

1.7.1	Lightsprout	:	size	medium
1.7.2	Lightsprout	:	Shape	spheral
1.7.3	Lightsprout	:	anthocyanin of base	red-violet
1.7.4	Lightsprout	:	intensity of anthocyanin colouration of base	medium
1.7.5	Lightsprout	:	pubescence of base	strong
1.7.6	Lightsprout	:	size of tip	medium
1.7.7	Lightsprout	:	habit of tip	medium
1.7.8	Lightsprout	:	intensity of anthocyanin colouration of tip	weak
1.7.9	Lightsprout	:	pubescence of tip	medium
1.7.10	Lightsprout	:	number of roottips	unknown
1.7.11	Lightsprout	:	protrusion of lenticels	strong
1.7.12	Lightsprout	:	length of lateral shoots	short
1.7.13	Plant	:	height	tall $\bar{x}$ = 66.2 cm medium

1.7.14	Plant	:	type	leaf type
1.7.15	Plant	:	growth habit	semi-erect
1.7.16	Stem	:	thickness of main stem	thick
1.7.17	Stem	:	extension of anthocyanin	absent
1.7.18	Leaf		size	large
	Leaf	:	length	$\bar{x}$ = 34,3cm
	Leaf	:	width	$\bar{x}$ = 21,1cm
1.7.19	Leaf	:	silhouette	medium
1.7.20	Leaf	:	intensity of green colour	medium
1.7.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.7.22	Leaflet	:	size	large
	Pinna	:	length	$\bar{x}$ = 10,0 cm
1.7.23	Leaflet	:	width	$\bar{x}$ = 6,2 cm broad
1.7.24	Leaflet	:	frequency of coalescence	low
1.7.25	Leaflet	:	waviness of margin	weak
1.7.26	Leaflet	:	depth of veins	shallow
1.7.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.7.28	Leaflet	:	glossiness of the upperside	medium
1.7.29	Leaflet	:	(midrif) frequency of secondary leaflets	medium
1.7.30	Terminal leaflet:		frequency of secondary leaflets	medium
1.7.31	Lateral leaflet	:	frequency of secondary leaflets	low
1.7.32	lateral leaflet	:	size of secondary leaflets	small
1.7.33	Inflorescence	:	size	medium
1.7.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.7.35	Plant	:	frequency of flowers	high
1.7.36	Flower	:	anthocyanin colouration of bud	weak
1.7.37	flower corolla	:	size	medium

1.7.38	flower corolla	:	colour of inner side	white
1.7.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.7.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.7.41	flower corolla	:	size of white tips in coloured flower	not applicable
1.7.42	Plant	:	frequency of fruits	unknown
1.7.43	Plant	:	time of maturity	unknown
1.7.44	Tuber	:	shape	oval
1.7.45	Tuber	:	depth of eyes	shallow
1.7.46	Tuber	:	smoothness of skin	medium
1.7.47	Tuber	:	colour of skin	yellow
1.7.48	Tuber	:	colour of base of eye	yellow
1.7.49	Tuber	:	colour of flesh	light yellow
1.7.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	weak

1.8 **BARAKA**

Cultivar description under South African conditions is not available.

1.9 **BRAVO**

1.9.1	Lightsprout	:	size	medium
1.9.2	Lightsprout	:	Shape	narrow cylindrical
1.9.3	Lightsprout	:	anthocyanin of base	red-violet
1.9.4	Lightsprout	:	intensity of anthocyanin colouration of base	strong
1.9.5	Lightsprout	:	pubescence of base	strong
1.9.6	Lightsprout	:	size of tip	medium

1.9.7	Lightsprout	:	habit of tip	medium
1.9.8	Lightsprout	:	intensity of anthocyanin colouration of tip	medium
1.9.9	Lightsprout	:	pubescence of tip	strong
1.9.10	Lightsprout	:	number of roottips	unknown
1.9.11	Lightsprout	:	protrusion of lenticels	strong
1.9.12	Lightsprout	:	length of lateral shoots	short
1.9.13	Plant	:	height	$\bar{x}$ = 82,9 cm tall
1.9.14	Plant	:	type	intermediate
1.9.15	Plant	:	growth habit	semi-erect
1.9.16	Stem	:	thickness of main stem	thick
1.9.17	Stem	:	extension of anthocyanin	absent
1.9.18	Leaf	:	size	medium
	Leaf	:	length	$\bar{x}$ = 30,0 cm
	Leaf	:	width	$\bar{x}$ = 17,3 cm
1.9.19	Leaf	:	silhouette	medium
1.9.20	Leaf	:	intensity of green colour	medium
1.9.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.9.22	Leaflet	:	size	medium
	Pinna	:	length	$\bar{x}$ = 8,2 cm
1.9.23	Leaflet	:	width	$\bar{x}$ = 5,4 cm medium
1.9.24	Leaflet	:	frequency of coalescence	low
1.9.25	Leaflet	:	waviness of margin	strong
1.9.26	Leaflet	:	depth of veins	deep
1.9.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.9.28	Leaflet	:	glossiness of the upperside	medium

1.9.29	Leaflet	:	(midrif) frequency of secondary leaflets	high
1.9.30	Terminal leaflet:		frequency of secondary leaflets	high
1.9.31	Lateral leaflet	:	frequency of secondary leaflets	medium
1.9.32	lateral leaflet	:	size of secondary leaflets	medium
1.9.33	Inflorescence	:	size	large
1.9.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.9.35	Plant	:	frequency of flowers	high
1.9.36	Flower	:	anthocyanin colouration of bud	medium
1.9.37	flower corolla	:	size	large
1.9.38	flower corolla	:	colour of inner side	white
1.9.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.9.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.9.41	flower corolla	:	size of white tips in coloured flower	not applicable
1.9.42	Plant	:	frequency of fruits	unknown
1.9.43	Plant	:	time of maturity	unknown
1.9.44	Tuber	:	shape	round
1.9.45	Tuber	:	depth of eyes	shallow
1.9.46	Tuber	:	smoothness of skin	rough
1.9.47	Tuber	:	colour of skin	yellow
1.9.48	Tuber	:	colour of base of eye	yellow
1.9.49	Tuber	:	colour of flesh	white
1.9.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	strong
	KIMBERLEY CHOICE			
1.10.1	Lightsprout	:	size	large

1.10.2	Lightsprout	:	Shape	broad cylindrical
1.10.3	Lightsprout	:	anthocyanin of base	red-violet
1.10.4	Lightsprout	:	intensity of anthocyanin colouration of base	weak
1.10.5	Lightsprout	:	pubescence of base	strong
1.10.6	Lightsprout	:	size of tip	large
1.10.7	Lightsprout	:	habit of tip	open
1.10.8	Lightsprout	:	intensity of anthocyanin colouration of tip	medium
1.10.9	Lightsprout	:	pubescence of tip	medium
1.10.10	Lightsprout	:	number of roottips	unknown
1.10.11	Lightsprout	:	protrusion of lenticels	medium
1.10.12	Lightsprout	:	length of lateral shoots	short
1.10.13	Plant	:	height	$\bar{x}$ = 50,1 cm tall
1.10.14	Plant	:	type	stem
1.10.15	Plant	:	growth habit	spreading
1.10.16	Stem	:	thickness of main stem	thick
1.10.17	Stem	:	extension of anthocyanin	absent
1.10.18	Leaf	:	size	large
	Leaf	:	length	$\bar{x}$ = 28,0 cm
	Leaf	:	width	$\bar{x}$ = 17,0 cm
1.10.19	Leaf	:	silhouette	open
1.10.20	Leaf	:	intensity of green colour	light
1.10.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.10.22	Leaflet	:	size	large
	Pinna	:	length	$\bar{x}$ = 10,0 cm
1.10.23	Leaflet	:	width	$\bar{x}$ = 5,5 cm

				broad
1.10.24	Leaflet	:	frequency of coalescence	low
1.10.25	Leaflet	:	waviness of margin	weak
1.10.26	Leaflet	:	depth of veins	shallow
1.10.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.10.28	Leaflet	:	glossiness of the upperside	dull-medium
1.10.29	Leaflet	:	(midrif) frequency of secondary leaflets	medium
1.10.30	Terminal leaflet:		frequency of secondary leaflets	medium
1.10.31	Lateral leaflet	:	frequency of secondary leaflets	low
1.10.32	lateral leaflet	:	size of secondary leaflets	small
1.10.33	Inflorescence	:	size	medium-large
1.10.34	Inflorescence	:	anthocyanin colouration of peduncle	absent
1.10.35	Plant	:	frequency of flowers	high
1.10.36	Flower	:	anthocyanin colouration of bud	absent
1.10.37	flower corolla	:	size	medium
1.10.38	flower corolla	:	colour of inner side	white
1.10.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.10.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.10.41	flower corolla	:	size of white tips in coloured flower	not applicable
1.10.42	Plant	:	frequency of fruits	few
1.10.43	Plant	:	time of maturity	unknown
1.10.44	Tuber	:	shape	oval
1.10.45	Tuber	:	depth of eyes	shallow
1.10.46	Tuber	:	smoothness of skin	medium

1.10.47	Tuber	:	colour of skin	yellow
1.10.48	Tuber	:	colour of base of eye	red
1.10.49	Tuber	:	colour of flesh	cream
1.10.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	absent

1.11 **HOËVELDER**

1.11.1	Lightsprout	:	size	medium
1.11.2	Lightsprout	:	Shape	conical
1.11.3	Lightsprout	:	anthocyanin of base	red-violet
1.11.4	Lightsprout	:	intensity of anthocyanin colouration of base	weak
1.11.5	Lightsprout	:	pubescence of base	weak
1.11.6	Lightsprout	:	size of tip	small
1.11.7	Lightsprout	:	habit of tip	closed
1.11.8	Lightsprout	:	intensity of anthocyanin colouration of tip	weak
1.11.9	Lightsprout	:	pubescence of tip	absent
1.11.10	Lightsprout	:	number of roottips	unknown
1.11.11	Lightsprout	:	protrusion of lenticels	strong
1.11.12	Lightsprout	:	length of lateral shoots	short
1.11.13	Plant	:	height	medium
1.11.14	Plant	:	type	$\bar{x}$ = 56 cm leaf type
1.11.15	Plant	:	growth habit	erect
1.11.16	Stem	:	thickness of main stem	medium
1.11.17	Stem	:	extension of anthocyanin	weak
1.11.18	Leaf	:	size	medium
	Leaf	:	length	$\bar{x}$ = 20 cm
	Leaf	:	width	$\bar{x}$ = 16 cm

1.11.19	Leaf	:	silhouette	medium
1.11.20	Leaf	:	intensity of green colour	medium - dark
1.11.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.11.22	Leaflet	:	size	large
	Pinna	:	length	$\bar{x}$ = 12 cm
1.11.23	Leaflet	:	width	$\bar{x}$ = 6 cm
1.11.24	Leaflet	:	frequency of coalescence	medium
1.11.25	Leaflet	:	waviness of margin	medium
1.11.26	Leaflet	:	depth of veins	deep
1.11.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.11.28	Leaflet	:	glossiness of the upperside	glossy
1.11.29	Leaflet	:	(midrif) frequency of secondary leaflets	low
1.11.30	Terminal leaflet:		frequency of secondary leaflets	medium
1.11.31	Lateral leaflet:		frequency of secondary leaflets	low
1.11.32	lateral leaflet	:	size of secondary leaflets	medium
1.11.33	Inflorescence	:	size	medium
1.11.34	Inflorescence	:	anthocyanin colouration of peduncle	weak
1.11.35	Plant	:	frequency of flowers	high
1.11.36	Flower	:	anthocyanin colouration of bud	medium
1.11.37	flower corolla	:	size	medium
1.11.38	flower corolla	:	colour of inner side	red violet
1.11.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	medium
1.11.40	flower corolla	:	anthocyanin colouration of outer side in white flower	not applicable

1.11.41	flower corolla	:	size of white tips in coloured flower	medium
1.11.42	Plant	:	frequency of fruits	unknown
1.11.43	Plant	:	time of maturity	unknown
1.11.44	Tuber	:	shape	oval
1.11.45	Tuber	:	depth of eyes	deep
1.11.46	Tuber	:	smoothness of skin	medium
1.11.47	Tuber	:	colour of skin	yellow
1.11.48	Tuber		of base of eye	yellow
1.11.49	Tuber			cream
1.11.50	Yellow skinned variet...			
	Tuber	:	anth... ...ration of skin in reaction to lig...	absent

1.12 LATE HARVEST

1.12.1	Lightsprout	:	size	medium
1.12.2	Lightsprout	:	Shape	narrow cylindrical
1.12.3	Lightsprout	:	anthocyanin of base	red-violet
1.12.4	Lightsprout	:	intensity of anthocyanin colouration of base	strong
1.12.5	Lightsprout	:	pubescence of base	strong
1.12.6	Lightsprout	:	size of tip	medium
1.12.7	Lightsprout	:	habit of tip	medium
1.12.8	Lightsprout	:	intensity of anthocyanin colouration of tip	strong
1.12.9	Lightsprout	:	pubescence of tip	strong
1.12.10	Lightsprout	:	number of roottips	unknown
1.12.11	Lightsprout	:	protrusion of lenticels	mediu m
1.12.12	Lightsprout	:	length of lateral shoots	medium
1.12.13	Plant	:	height	medium

1.12.14	Plant	:	type	intermediate-leaf type
1.12.15	Plant	:	growth habit	erect
1.12.16	Stem	:	thickness of main stem	medium
1.12.17	Stem	:	extension of anthocyanin	absent
1.12.18	Leaf	:	size	medium
	Leaf	:	length	unknown
	Leaf	:	width	unknown
1.12.19	Leaf	:	silhouette	medium
1.12.20	Leaf	:	intensity of green colour	medium
1.12.21	Leaf	:	extension of anthocyanin colouration of midrib	absent
1.12.22	Leaflet	:	size	medium
	Pinna	:	length	unknown
	Leaflet	:	width	medium
1.12.24	Leaflet	:	frequency of coalescence	high
1.12.25	Leaflet	:	waviness of margin	weak
1.12.26	Leaflet	:	depth of veins	medium-deep
1.12.27	Leaflet	:	anthocyanin pigmetration of blade of young leaflets at apical rosette	absent
1.12.28	Leaflet	:	glossiness of the upperside	medium
1.12.29	Leaflet	:	(midrif) frequency of secondary leaflets	high
1.12.30	Terminal leaflet:		frequency of secondary leaflets	high
1.12.31	Lateral leaflet:		frequency of secondary leaflets	high
1.12.32	lateral leaflet:		size of secondary leaflets	medium
1.12.33	Inflorescence:		size	medium
1.12.34	Inflorescence:		anthocyanin colouration of peduncle	absent
1.12.35	Plant	:	frequency of flowers	high
1.12.36	Flower	:	anthocyanin colouration of bud	absent
1.12.37	flower corolla		: size	medium

1.12.38	flower corolla	:	colour of inner side	white
1.12.39	flower corolla	:	intensity of anthocyanin colouration of inner side in coloured flower	not applicable
1.12.40	flower corolla	:	anthocyanin colouration of outer side in white flower	absent
1.12.41	flower corolla	:	size of white tips in coloured flower	small
1.12.42	Plant	:	frequency of fruits	unknown
1.12.43	Plant	:	time of maturity	unknown
1.12.44	Tuber	:	shape	oval
1.12.45	Tuber	:	depth of eyes	medium
1.12.46	Tuber	:	smoothness of skin	medium
1.12.47	Tuber	:	colour of skin	yellow
1.12.48	Tuber	:	colour of base of eye	yellow
1.12.49	Tuber	:	colour of flesh	cream
1.12.50	Yellow skinned varieties only			
	Tuber	:	anthocyanin colouration of skin in reaction to light	absent

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