DROUGHT RESPONSE IN CULTIVATED POTATOES:

PHENOTYPIC BEHAVIOURS AND MOLECULAR ASPECTS OF SIGNALLING AND TUBERIZATION.

Robert okayo
Msc Plant Biotechnology
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Robert Okayo
810520617010
PBR 80424: Minor thesis plant breeding

Supervisors:
Dr. Gerard van der Linden
Ernest Aliche

Department of Plant breeding
Wageningen University and research Centre.
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Abstract:

Drought is one of the major constrains facing agricultural production worldwide and its impact is expected to increase in future. The world population is also expected to increase and this will require more safe, nutritious, and healthy foods. The cultivated potato *Solanum tuberosum* is one such a crop that has the potential of feeding the world. It has a potential of providing high calories in comparison to major cereals. However, it is highly susceptible to drought owing to its shallow root network that occupies the upper 0.3m of the soil layer. In this study, we investigated the response of 24 cultivars to drought. The phenotypic measurements were scored to determine the effect of drought on these cultivars. We found significant variation in response of the genotypes to drought, and that drought lowers the overall performance of the cultivars. Gene expression analysis was also done with *ABAOH* and *NCED* to get an insight on possible contrasts in drought response due to the role of ABA signalling in two cultivars, Albion and Premier. The expression analysis revealed that *NCED* and *ABAOH* were upregulated in mild stress and not in severe stress. This may indicate that signalling under severe drought stress may involve more molecular components than may not be explained by ABA signalling. Expression of the *StCDF* genes was also analysed to elucidate their role in tuberization signalling under drought stress. Studies have indicated that members of this mall family regulate the tuberization signal by supressing *CONSTANS (CO)* leading to the suppression of transcription of *StSP5G* and this allows the expression of the tuberization gene, *StSP6A*. The *StCDFs* gene expression analyses revealed that *StCDF-1, -2* and *-3* were upregulated under both mild stress and severe stress conditions in all cultivars tested, and this suggests that they may have a role in drought tolerance. *StCDF5* was downregulated in two cultivars, Mondial and Great Scot; these cultivars are known to shut down their tuber initiation on perception of drought. This may mean that this gene is involved in the regulation of tuber initiation shut down.

Acknowledgements:

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Lastly I would like to thank my fellow students for their continued support, stories and encouragement.

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Table of contents:

1 Introduction:

1.1 Drought

Abiotic factors have adversely hindered man's attempts to increase crops output to cater for his needs. Abiotic factors include drought, frost, salinity, flooding, extreme temperature and UV radiation (Bartels & Sunkar, 2005). The effects of abiotic factors are likely to increase in the coming years. Drought and salinity are the major abiotic factors and have a potential of reducing the production of major crops by half (Kawaguchi et al., 2003). Over the past years, global warming and more erratic rainfalls have been experienced not only in semi-arid regions but also in temperate regions globally. This therefore calls for an urgent and drastic action to develop strategies to cope with the changing climatic conditions. The steady increase in drought incidences globally is likely to affect small-scale farmers who lack the resources and strategies to mitigate the effects of drought. As a result they will experience unpredictability and unreliability in food production. The drought problem is further compounded with the increasing world population that will need more food to feed the increasing number of people. Plant scientists are therefore faced with the daunting task of developing cultivars that have an enhanced productivity under drought (Vörösmarty et al., 2000).

Plant under drought stress responds through complex mechanisms aimed at maintaining optimal water content in its tissues. These mechanisms can either be genetic, biochemical or metabolic employed within a single plant physiology to a larger ecological system to mitigate the negative effect of drought. Drought escape is one of the mechanisms, in which the plants complete their life cycle before the devastating effect of drought. Avoidance mechanisms through investment in roots can also be employed to enhance water acquisition capacity or closing of the stomata to conserve water. Plants can also improve the osmotic adjustment ability and increase cell wall elasticity to maintain tissue turgidity. Some plants alter the metabolic pathway while others shade off some organs such as the leaves to help conserve water (Xu et al., 2010). Plants can employ one or a combination of mechanisms to cope or overcome dehydration. These adaptive mechanisms have some drawbacks, such as drought escape can lead to reduction in total yield while closing of the stomata and leaf area reduction may reduce the photosynthetic capacity of the plant leading to reduced carbon assimilates and eventually low yield.

1.2 Potatoes:

Potato (*Solanum tuberosome.* L.) is one of the most important dicotyledonous sources of food. It belongs to the *Solanaceae* family and ranks fourth as the major food crop of the world exceeded only by the grasses: wheat, rice and maize (Monneveux et al., 2013) . The potatoes are grown for their tubers, which are rich in starch, low in fat and have protein contents as high as cereals. A medium sized fresh potato tubers can provide nearly a half of vitamin C needed by an adult (Rodríguez-Falcón et al., 2006). Currently more than half of the world potato cultivation is done in developing countries and most of the farmers are poor resource farmers in remote areas (Monneveux et al., 2013). Owing to potato's high dietary level, the crop has a potential for hunger reduction and improved nutrition in the world. Moreover, price shocks do not affect potato greatly since it is locally produced and traded, and thus its price depends on local production costs. The potato also has industrial application such as the use of starch in textile industry, paper making, glue and building materials (Monneveux et al., 2013).

The ploidy level of potatoes varies from diploid (2n=2x=24) to hexaploid (2n=6x=72) with the cultivated potatoes being tetraploid (2n=4x=48) (Hawkes, 1990). Its domestication originated from the Andean region of South America where it is estimated that it started around 700 years ago. The crop evolved over the years in the tropics, requiring a short day photoperiodic regime for tuber initiation (Kloosterman et al., 2013). Its optimal growth is achieved in relatively cool conditions and tuberization depends on temperature in addition to day length (Hooker, 1981). Under optimal condition, the potato has higher production per unit area and it has a potential of producing 9.52kg of yield or 5,600 kcal of energy per m^3 of water in comparison to 3,860 in maize, 2,300 in wheat and 2,000 kcal of dietary energy in rice. This makes the potato a water use-efficient crop in comparison to the major cereals (Vos & Groenwold, 1987) . However, potato is very sensitive to water stress in comparison to other crop plants. Therefore it is imperative to employ frequent irrigation systems to potato fields in stressful conditions to ensure or maintain yield. Potato sensitivity to water stress is a result of its shallow and often less extended root system compared most crops (Van Loon, 1981). The root system has a depth that ranges from 0.5 to 1.0m with the extensive root network of about 85% covering the top 0.3m of the soil. It has a relatively small root length per unit area that makes the potato plant absorb less available water from the soil compared to other crops (Gregory & Simmonds, 1992; Weisz et al., 1994). Tuber formation and bulking is therefore greatly affected under drought stress since this process requires optimal amounts of

water (Vos & Groenwold, 1987). The challenge of drought in potato cultivation is likely to increase in magnitude as more crops are being grown and the drought affected area increases. Farmers will therefore grapple with yield loss in the coming decades until remedy is found through maybe breeding for tolerant varieties or improved irrigation infrastructure in the developing countries. Drought tolerant is associated with many biological pathways making it a highly complex trait. Breeding for drought tolerant is therefore a complicated endeavour (Wang et al., 2003). Many physiological and morphological or agronomic characteristics are severely affected by drought and can be used as traits in drought tolerant breeding (Tourneux et al., 2003)

1.3 Gene expression in Potatoes under drought:

Potatoes just like any other plant, perceive stress when the conditions are suboptimal, and transduce the stress in form of signals. This leads to the expression of genes involved in repair mechanisms and/or the expression of transcriptional factors that regulate stress responsive genes (Bartels & Sunkar, 2005). Genomics studies have identified a number of genes in various organisms that are involved in regulation and activation of the drought linked transcripts. These proteins are grouped into two major clusters, functional proteins and regulatory proteins. Regulatory proteins are directly involved in the perception and transmission of drought signals, and are often transcription factors. Functional proteins include protection factors such as chaperones, LEA proteins and lipid transfer proteins. The functional group also include proteins involved in the biosynthesis of plant hormones such as ABA, Auxin and Jasmonic Acid (Shinozaki & Yamaguchi-Shinozaki, 2007). Some genes responsible for the control of signalling pathways studied in this project are *ABAOH*, *NCED* and members of the *CDF* gene family.

ABAOH and *NCED* are genes responsible for the maintaining the optimal balance of endogenous Abscisic acid (ABA) in plants. ABA is a critical plant hormone that controls growth and development process. It also contributes to the plant's adaptation response to adverse environmental stresses through integrating various stress signals and controlling the downstream expression of ABA responsive genes (Umezawa et al., 2006b). ABA is also referred to as stress hormone as it is responsible for stomatal closure under drought condition to conserve the intracellular water content (Tuteja, 2007). It has also been suggested that lack of ABA-biosynthesis genes leads to reduction in seed dormancy and causes wilting (McCarty, 1995).

There are three main genes involved in the ABA synthesis from carotenoids in plants: *ZEP*, *NCED* and *AAO*. *ZEP* encodes zeaXanthin epoxidase that catalyses the epoxidation of zeaXanthin to produce epoxycarotenoid. *NCED* encodes 9-cis-epoxycaroteoid dioxygenase and it catalyses the cleavage reaction of epoxycarotenoids to generate the first C_{15} intermediate and *AAO* encodes abscisic aldehyde oxidase and it catalyses the conversion of ABA aldehyde to ABA in the final step in the ABA biosynthesis. Research has shown that *NCED* gene in cowpea (*VuNCED1*) is strongly induced in drought while *ZEP* gene in cowpea (*VuABA1*) was not induced. It also shows that the *NCED* is expressed prior to accumulation of ABA in drought incidences in maize, beans and tomatoes. *NCED* gene had been therefore suggested to play a critical role in the biosynthesis of ABA. The overexpression of this gene leads to higher accumulation of ABA and this further suggest a key role of this gene in the biosynthesis of ABA (Iuchi et al., 2001).

The catabolic reactions of ABA in the plant are crucial activities that regulate the level of endogenous ABA in plant. This reaction is classified into two parts: conjugation and hydroxylation (Umezawa et al., 2006a). ABA 8-hydroxylation catalyses the hydroxylation catabolic pathways and it is encoded by *cytochrome P450 CYP707A* family of genes. The expression of the hydroxylation genes in Arabidopsis was suggested to be regulated by ABA, dehydration and rehydration (Umezawa et al., 2006a).The hydroxylation pathways oxidize the methyl group at C−7′, C−8′, and C−9′ of the carbon ring structure but the main catabolic pathway is mainly thought to be associated with the hydroxylation in C-8 of the ring. The *cytochrome P450* catalyses the ABA 8-hydroxylation to 8'hydroxy ABA which is then isomerized naturally to phaseic acid (PA). The soluble reductase catabolizes the PA to generate dihydrophaseic acid (DPA) (Nambara & Marion-Poll, 2005). The inactivation of ABA occurs simultaneously during the course of ABA 8-hydroxylation. This process is very important in the maintenance of the endogenous levels of ABA especially when the plant needs to inactivate the ABA accumulated during the stress period.

Cycling Dof Factors (CDFs) are a set of DNA binding with One Finger (DOF) factors whose transcripts in Arabidopsis oscillate in light conditions. DOF proteins are plant specific transcription factors that consist of conserved 50 amino acids in the N-terminal region and a highly variable C-terminal region. The N-terminal domain binds specifically to the 5'-3'end sequence of the promoter of the targeted gene while the C-terminal contains specific protein-protein interaction domain and the regulatory elements. This complex structure of the DOF allows them to regulate many functions either as an enhancer or repressor of the expression of numerous plant genes. In so doing they are capable of controlling many biological

processes such as seed maturation and germination, tissue specific gene expression, light responses and plant hormone signalling (Corrales et al., 2014). Research done by (Yanagisawa & Sheen, 1998) showed that DOF in maize regulates the genes responsible for carbon fixation and nitrogen assimilation. The DOF genes are classified into four orthologous groups (A-D) based on the phylogenetic analysis. In Arabidopsis, the D group contains the *CDF1-5* and they play a critical role in the control of the photoperiodic flowering through the repression of transcriptional factor CONSTANS (*CO*) and FLOWERING LOCUS (*FT*) promoter (Kloosterman et al., 2013). This inhibition of flowering is alleviated through the action of protein complex consisting of FLAVIN-BINDING KELCH REPEAT F-BOX PORTEIN (FKF1) and GIGANTEA (*GI*) that accumulate in blue light perception. The higher accumulation of *FKF1-GI* complex degrades *CDF* protein levels and this lessens the inhibition effects to flowering (Corrales et al., 2014). In potatoes, a homologue of Arabidopsis *CDF* gene has been identified on chromosome 5 and has been referred to as *Solanum tuberosum CDF1* (*StCDF1*). This gene links with potato *StCO1*/2 and the potato homologue to *FT*, *StSP6A* that controls tuberization; and this *StCDF1* controls the plant maturity. *StCDF1* causes late maturity and allelic variation of the gene denoted as *StCDF1*.2 and *StCDF1*.3 lead to early maturing phenotype. Overexpression of *StCDF1*.2 and *StCDF1*.3 in the late maturing background leads to early tuberization and early senescence. However, there was no effect of overexpression on the flowering of potatoes contrary to Arabidopsis in which overexpression leads to late flowering. This indicates that there is distinct signal transduction pathway between the flowering and tuberization (Kloosterman et al., 2013). The experiment done by Corrales et al. (2014) found out that the expression of *Solanum lycopersicum CDF1*-5 genes follows the circadian rhythm that can be divided in two groups in *S. lycopersicum* under long day, *SlCDF1* and *SlCDF3* in contrast to *SlCDF2*, *SlCDF4* and *SlCDF5*. The expression analysis of *SlCDF1* and *SlCDF3* shows that the upregulation started by midnight to early morning, then climaxed at midday and thereafter decreased to lower levels by midnight. On the contrary, *SlCDF2*, *SlCDF4* and *SlCDF5* expression levels decreased in the early morning and minimal expression was maintained during the second part of the day and increased at the start of night and climaxed at the start of the day. In addition to photoperiod, *SlCDF1*-5 are also induced by different abiotic stresses such as salinity, drought, temperature and cold. In salt and osmotic stress conditions all the *SlCDF1*-5 were upregulated, in particular *SlCDF1* and *SlCDF4*. *SlCDF2*, *SlCDF4* and *SlCDF5* were upregulated at high temperatures of 30/35^{o}C while maximum expressions were observed in cold treatment for all except *SlCDF2*. Further analysis of *SlCDF1* and *SlCDF3* reveals that the two genes contribute to the increase in tolerance to both salt and drought. They

hypothesized that these genes could be involved as upstream regulators in drought and salt stress response pathways (Corrales et al., 2014).

1.4 Aims of the study:

This study was done with three main aims:

- To find out the impact of drought on 24 potatoes cultivars. This was done through phenotypic screening of cultivars grown in the greenhouse.
- To investigate the contrast in ABA signalling in drought in Albion and premier as a follow up study on Nasrin's findings. She found out that Albion is more sensitive to stress with extreme wilting which may point out to ABA deficiency. This was done through the expression analysis of *ABAOH* and *NCED* genes in Albion and Premiere.
- To study the tuberization signalling in potato through the generation of a transgene and in natural system. Long day Andigena plant was to be transformed with tuberization gene *StSP6A* under the control of inducible β-estradiol promoter. The process of generating a transgene is still on going as at the time of writing this report and the protocol is in appendix one. I therefore based this report on the natural system in tuberization signalling through the study of *StCDF* genes.

2 Materials and Methods:

2.1 Plant materials:

The potato plants were grown in a greenhouse at Wageningen University and Research Centre between 2nd August and 29th October 2014. The temperature regime was 24/16 ^{o}C day/night and this was controlled through ventilation on the roof. A total of 24 cultivars were used in the experiment (Table 1). There were six replicates arranged in a completely randomized block design to take care of the position and border effects. The tubers were pre-geminated and planted in black pots of 14cm diameter and in each pot one tuber was planted. The plants were fully watered for the first five weeks after planting, and then a mild stress treatment was applied to the three replicates (drought stress plots). The stress was maintained at 25% (vol/vol) water and this was measured daily using a water content meter.

Table 1: List of cultivars used in the experiment, showing origin and the maturity type.

Reference cultivars from previous experiments:			New Cultivars		
Cultivar	Origin	Maturity.	Cultivar	Origin	Maturity.
Eos		Late	Hansa	Germany	Intermediate.
Albion	Holland	Intermediate.	Seresta		Intermediate.
Daisy	France	Intermediate.	VR808,		Intermediate.
Great scot	United Kingdom	Intermediate.	Bartina,	Holland	Intermediate.
Mondial	Germany	Intermediate.	Kondor,	Netherlands	Intermediate.
Russent B.		Intermediate.	Sylvana,	France.	Intermediate.
Desiree	Holland	Intermediate.	Winston,	United	Early.
Nicola	Germany	Intermediate.	Charlotte	France	Early.
Spunta	Holland	Intermediate.	Mozart,		Early.
Premiere	Holland	Early.	VTN62-33-3,		Early.
Cherie	France	Early.	Lady Amarilla,		Early.
			Axion,		Late.
			Lady Rosetta,	Holland	Early.

2.2 Phenotyping:

Plant height, number of leaflets, leaves and stems measurements were taken at two different time points: at the start of drought period and at the end of the experiment. Plant height was

measured in centimetres from the soil surface to the terminal bud using a ruler. The stems were held upright while the measurement was being taken. Number of leaflets, leaves and stems were counted manually. The differences between the two time points were used for the analysis.

Chlorophyll fluorescence: This was evaluated using a hand held fluorometer following the manufacturer's instruction. Drought has been reported to reduce the rate of photosynthesis through inhibition of the photosystem II reaction centres. The rate of inhibition of the Photosystem II can be determined by the chlorophyll fluorescence (Krause & Weis, 1991). The hand held fluorometer measures the initial fluorescence denoted as (Fo), maximal fluorescence (Fm), variable fluorescence (Fv) and maximum quantum efficiency (Fv/Fm). The measurements were taken on the first fully expanded leaves (usually third from the apex) for the sink leaves. For the source leaves, the measurements were taken on the fifth leaves from the top. The leaves were dark-adapted for 1 minute prior to the measurements. Three measurements were taken on both sink and source leaves and the average scored.

Chlorophyll content was measured using SPAD-502 Chlorophyll meter (Minolta Co., Ltd. Japan), on sink and source leaves. This was done once towards the end of the experiment.

Stomatal conductance: This was done to estimate stomatal resistance to water vapour loss and gaseous exchange through the stomata. The measurement was done using a Decagon SC-1 Leaf Porometer The measurement was taken once on the lower side of the fully expanded leaf on both source and sink leaves and expressed as Mmol/m^2s. The temperature under which the measurement was taken was also recorded.

Shoot fresh weight: This was taken immediately after harvest. A weighing scale was used to measure the plants in grams. The dead materials were also included in the measurement, so the differences in foliage maturity among genotypes may be a confounding factor. Dry weight could not be ascertained because the potato regulations of NVWA did not allow material transfer from the greenhouse.

Tuber number and weight were also taken at the end of the experiment. The stolon tips with diameter greater than 1 cm was counted as tuber.

2.3 Statistical analysis

All statistics were done using GenStat 15th edition software. Analysis of variance was computed per trait. The normality of the data was first tested before the statistical analysis done.

2.4 Expression analysis

The RT-PCR was used to quantify the expression of genes under drought condition using SYBR Green detection. The genes investigated were *NCED*, *ABAOH*, *StCDF1*, *StCDF2*, *StCDF3*, *StCDF4*, and *StCDF5* under mild stress and severe stress. The cDNA used was synthesized by Jonathan Kalisvaart in 2013 from source leaves collected by Daniel Bustos in 2012 greenhouse experiment for the mild drought and 2013 greenhouse experiment of Ernest for the severe stress. They were stored at -20^{0}C. The cultivars, Albion, Premiere, Great Scot, Mondial (only the mild stress) and Eos were used in the analysis of *CDF* genes while Albion and Premiere were used in the analysis of *NCED* and *ABAOH* genes to investigate the contrast in ABA signalling in drought on the two cultivars.

The primers sequences for the expression analysis were obtained from (Kloosterman et al., 2013) publication. The primer sequence and properties are shown in appendix 2. The primer specificity was checked on NCBI website through primer blast against the mRNA of *Solanum tuberosum* and all were specific for the corresponding genes. The primers were kindly provided by Dr. Abelende Jose. They were diluted in MQ to an end concentration of 10 µM.

2.4.1 Performing the RT-PCR

The master mix was prepared for the genes of interest with the APRT as a reference gene to normalize the PCR. A total of 7 genes were investigated under mild stress and severe stress. The amount of the master mix per gene varied depending on the number of cultivars used in the analysis but the volume per biological replicate remained the same for all genes under investigation. The 96 and 384 well plates were used and the amount loaded per well was 10µl (Table 2). The plates were then loaded on Bio-RAD C100^{TM} Thermal cycler to detect the amplification and the programme was initiated (Table 3).

Table 2: The qPCR volume per technical replicate and the amount loaded per well.

Components	Volume per biological replicates	10µl reaction mix per well on
iQTM SYBR®Green mix.	11.3	5
FW Primer	0.675	0.3
RV Primer	0.675	0.3
MQ	5.4	2.7
cDNA	4.5	2.25

Table 3: Thermal cycler programme for the RT-PCR

Step	Temperature ^{o}C	Duration.
Initial denaturation	95	3 minutes
Denaturation	95	30 seconds
Annealing	60	30 seconds
Extension	72	60 seconds
Final extension	72	3 minutes
Hold	4	∞

2.4.2 Analysis of the expression data

The data from the qPCR machine were extracted and updated using the CFX Manager 3.0 software. The updated raw Ct expression values were exported into Excel and the differences between the Ct values of technical replicates were tabulated and analysed. The values that had a strong deviation from the average and the ones that were clearly wrong were discarded from the analysis. The relative change in gene expression was calculated using The $2^{-\Delta CT}$ Method as outline in Schmittgen and Livak (2008).

3 RESULTS:

The genotypes were subjected to mild stress to access the impact of dehydration on the plant performance and morphology. The 24 cultivars under investigation were replicated in six blocks randomly and two treatments applied to three replicates: control and mild stress. The control plants were watered daily while the mild stressed plants were watered to 25% soil water content after the start of the application of treatment. Different agronomical traits were measured during this experiment and these were: Stomatal conductance, chlorophyll content, chlorophyll fluorescence, plant height, number of leaflets, leaves and stems, shoot weight, tuber number and tuber weight. The overall results revealed that mild stress affected all the traits measured except chlorophyll fluorescence.

Table 4: The mean of traits under control and drought treatments and the analysis of variance.

				Two way Anova		
Trait		Control	Drought	Genotype(G)	Treatment(T)	G*T
Stomatal conductance	Source	147.7	108	0.051	<0.001	NS
	Sink	215.6	161.6	0.136	<.001	NS
Chlorophyll Content	Source	32.29	43.31	0.003	<.001	NS
	Sink	34.54	47.11	<.001	<.001	NS
Chlorophyll fluorescence	Source	0.7424	0.7591	0.004	0.008	NS
	Sink	0.7397	0.7331	0.046	0.438	NS
Plant Height		38.88	18.32	<.001	<.001	0.017
Leaf Number		1.1	5.6	<.001	0.051	NS
Leaflets		99.6	63.2	<.001	<.001	NS
No. of stems		-0.24	0.16	0.016	0.076	0.002
Shoot weights		38.14	30.59	<.001	<.001	NS

Tuber No.	9.71	5.64	<.001	<.001	0.023
Tuber weight	99.2	54.1	<.001	<.001	NS

With the exception of stomatal conductance in the sink leaves, the traits showed significant differences among genotypes. There were also significant differences between treatments in majority of the traits except for chlorophyll fluorescence at the sink, number of leaves, and stems. The interaction between the two factors (genotype and treatment) was only significant in plant height, number of stems and tuber number (Table 4).

3.1 Stomatal conductance:

There was high stomatal conductance in control plants in comparison to the drought treated plants for both sink and source leaves (Figure1 and 2). There was an overall reduction in stomatal conductance by approximately 26% with the source leaves having slightly higher reduction. This is in line with our expectation since the plant close their stomata in response to drought to reduce the water loss and this negatively impacts on the rate of photosynthesis.

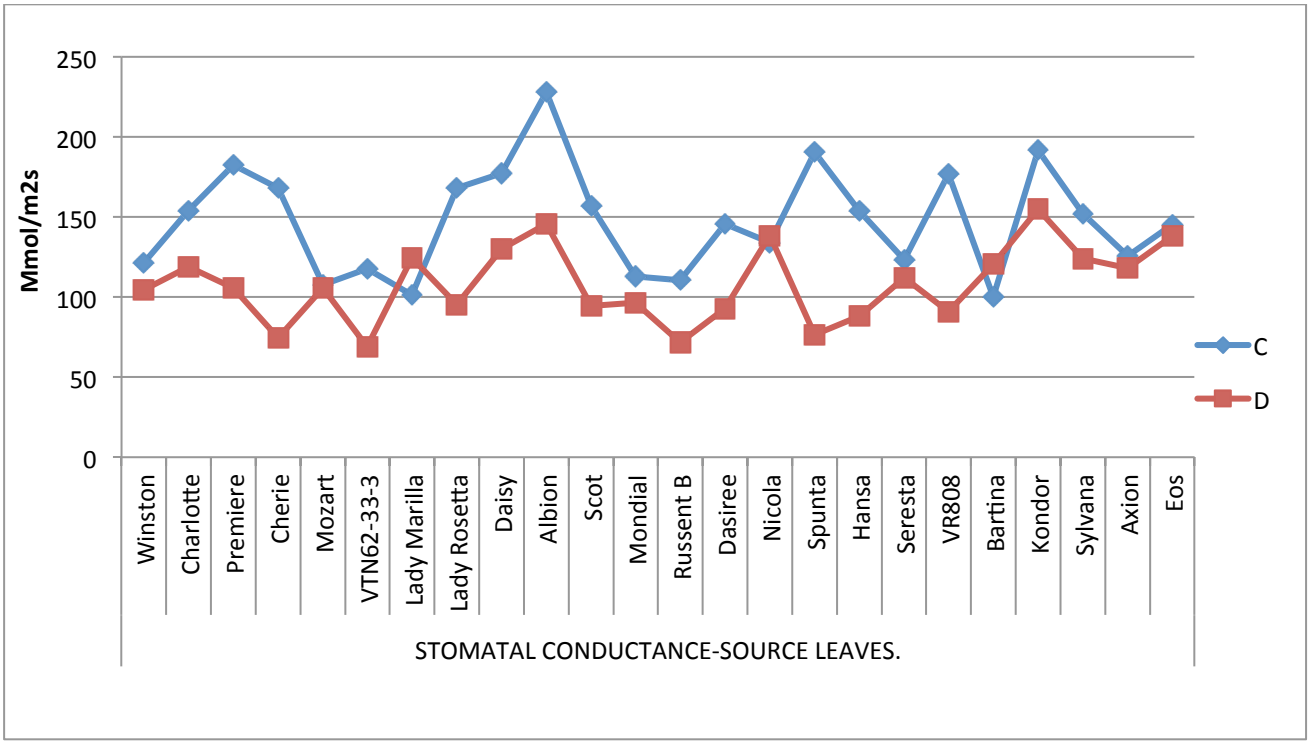


Figure 1: Stomatal conductance per plant per treatment at the source leaves.

The sink leaves had higher stomatal conductance in comparison to the source leaves. Albion had the highest stomatal conductance in the source leaves and its one of the highest in the sink leaves in control plants. Mozart had the same conductance in the source leaves under both treatments while in the sink leaves it had slightly higher than the control plants. Nicola had the same conductance for both treatments in source and sink leaves. Spunta had the highest difference in stomatal conductance (114mmol/m^2s) between the control and drought in source leaves. Cherie and Mondial had the highest differences in conductance of 134.03 Mmol/m^2 and 122.60 Mmol/m^2 respectively on the sink leaves. Cherie control plants germinated late and this could lead to differences in age of the leaves measured.

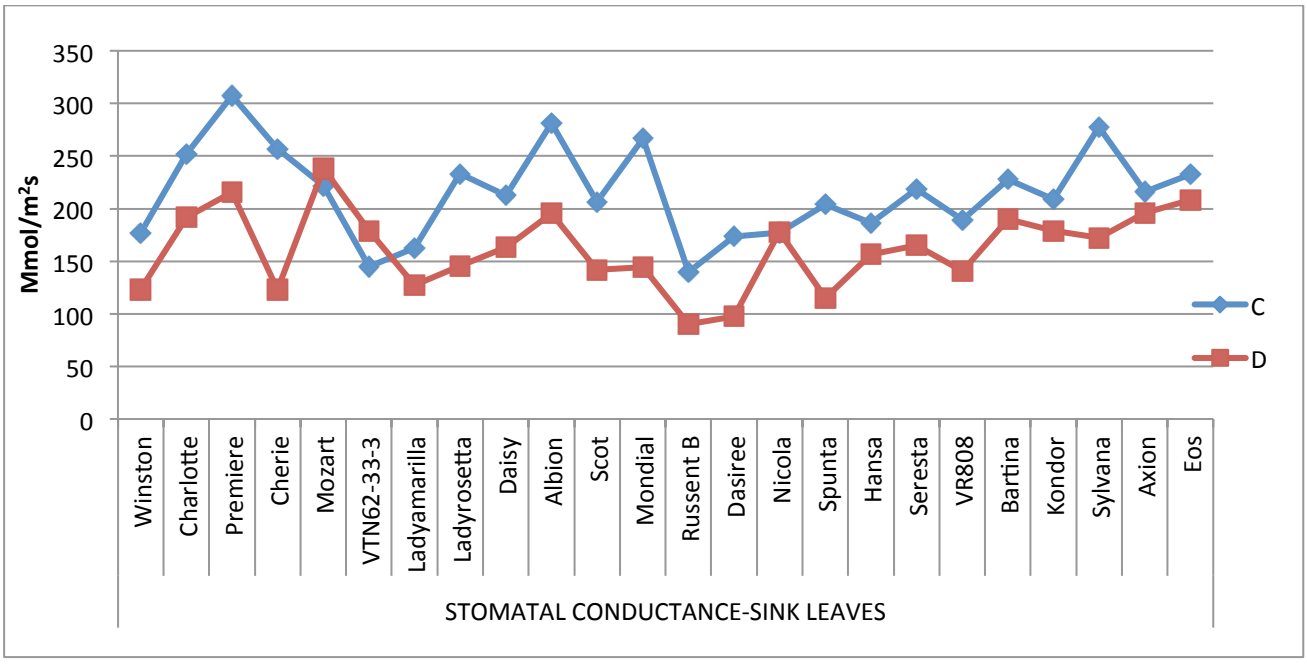


Figure 2: Stomatal conductance per plant per treatment at the sink leaves.

3.2 Chlorophyll content:

Chlorophyll content was on average higher in drought-treated plants by 35% in comparison to the control plants (Figure 3). There was highly significant difference between the genotype and treatment but there was no significant interaction of the two factors.

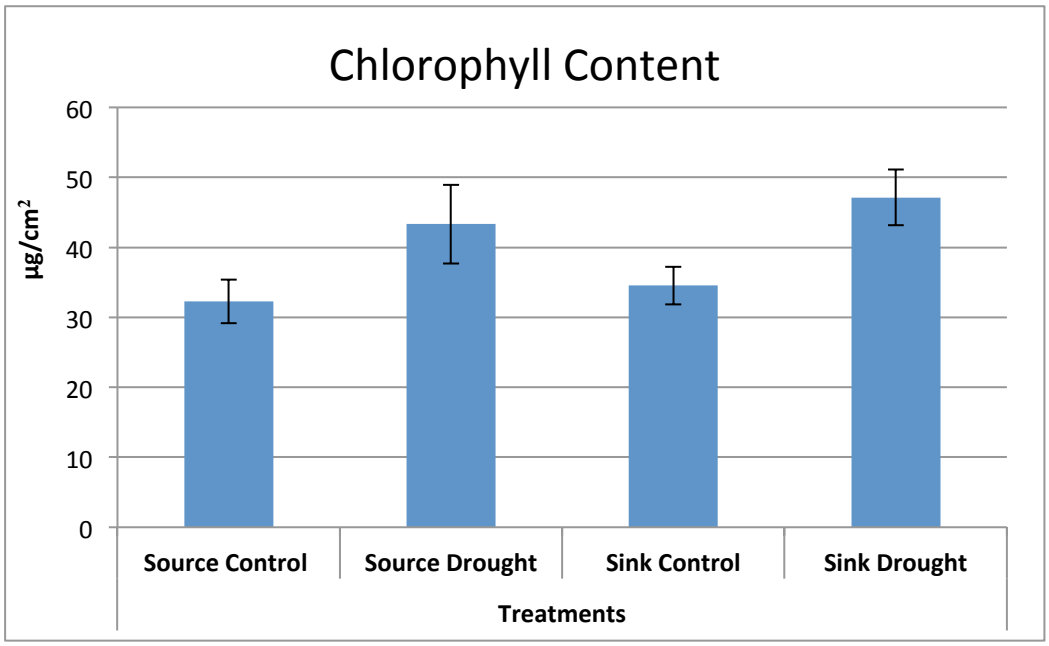


Figure 3: Average chlorophyll content per treatment in sink and source leaves.

3.3 Chlorophyll fluorescence:

Chlorophyll fluorescence analyzes the functional level of photosynthesis indirectly and its parameters are useful in estimating the effect of environmental stresses on the plant performance and quantify plant tolerance to drought stress (Li et al., 2006). Chlorophyll fluorescence is used to estimate the maximum photochemical efficiency of PSII, which is represented as a ratio Fv/Fm. It had been shown that photosystem II is a primary target of photo inhibition in plants under stress. This impacts the efficiency of photosynthesis in plants under stress. There was a slight change in chlorophyll fluorescence in the two treatments. The average value for chlorophyll fluorescence was 0.76 for both sink and source leaves. However, this data is not reliable since 1 minute dark adaptation was too short (Fig. 4).

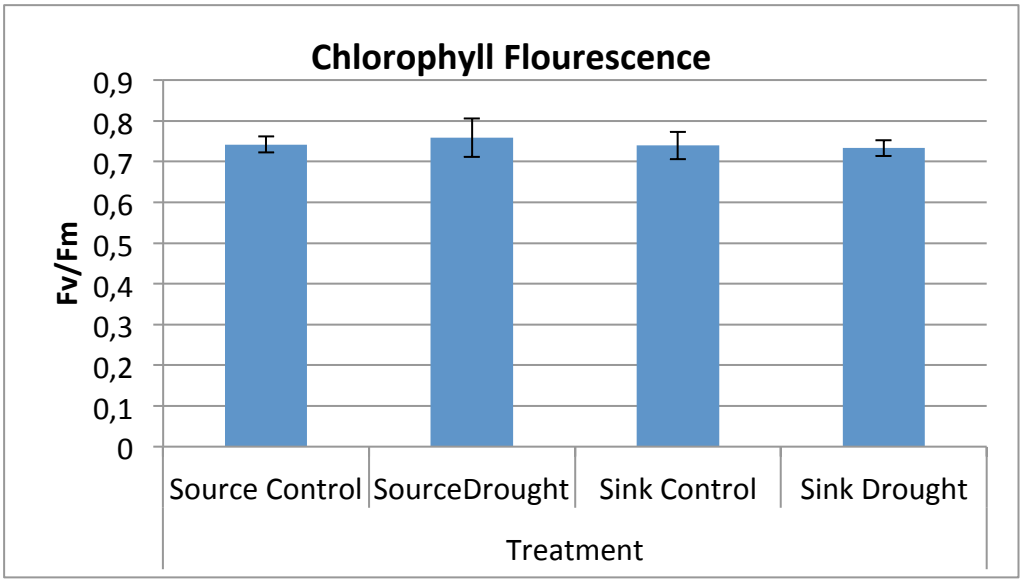


Figure 4: Average chlorophyll fluorescence per treatment in sink and source leaves.

Yield components.

Tuber number and weight decreased in response to drought with apparent variation of the yields between genotypes. There was significant genotype and treatment interaction for the tuber number but not for tuber weight.

Cultivar Albion recorded the lowest shoot weight in both control and drought treatments and this may mean that it had finished its life cycle before the rest. Most of the early maturing cultivars had higher shoot weight in the drought plants in comparison to the controls plants at harvesting time and this is contrary to the late maturing plants that had higher shoot weight in the control plants than the drought plants. The late maturing phenotype also had higher differences shoot weights between control and drought plants.

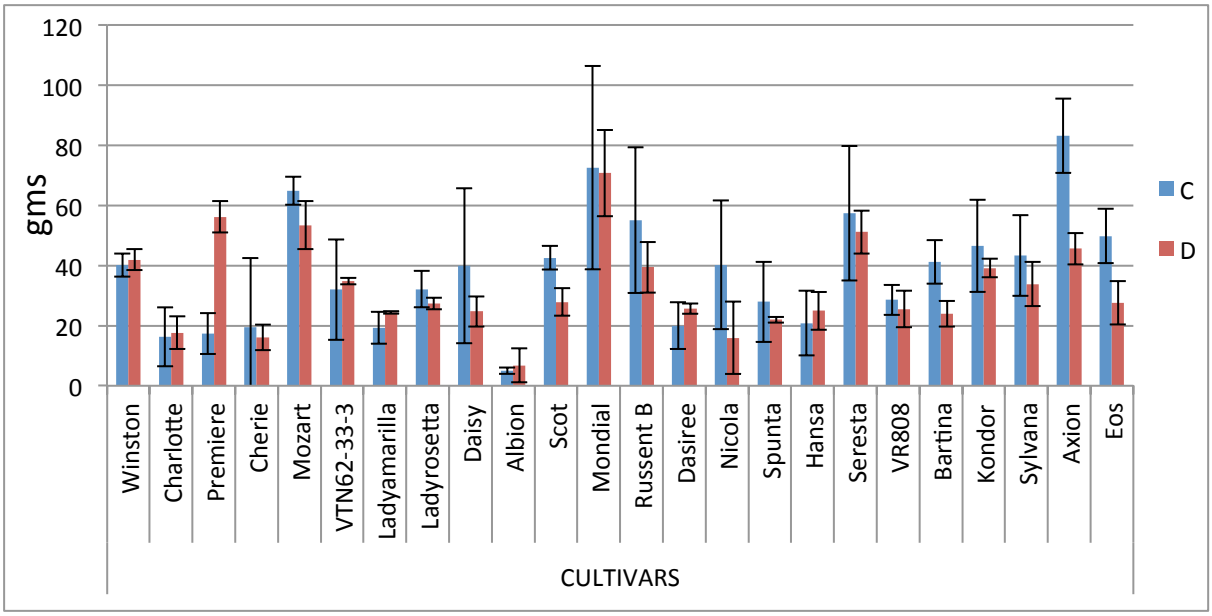


Figure 5: Shoot fresh weight per cultivar per treatment.

There was a notable reduction in tuber number in response to the drought with variations apparent among genotypes. Premiere did not demonstrate any change in tuber number and Albion had more tubers in drought stress and this may because they tuberize before the application of stress due to their early life cycle. There was a high percentage reduction in Mondial, Mozart and Sylvana (75, 71 and 67 percent, respectively).

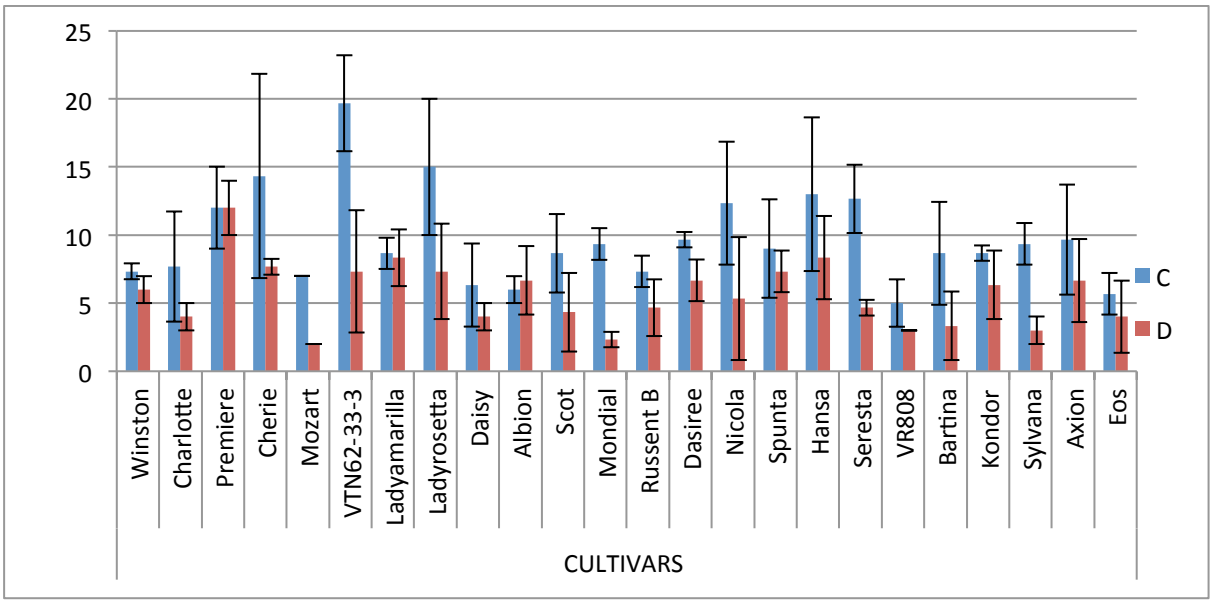


Figure 6: Tuber numbers per cultivar per treatment.

There was high tuber weight in control plants in comparison to drought treated plants with differences apparent among genotypes. The genotype Kondor did not demonstrate much

change in the tuber weight. The reductions in tuber weight in early maturing cultivars were less in comparison to the late maturing ones (Fig 7).

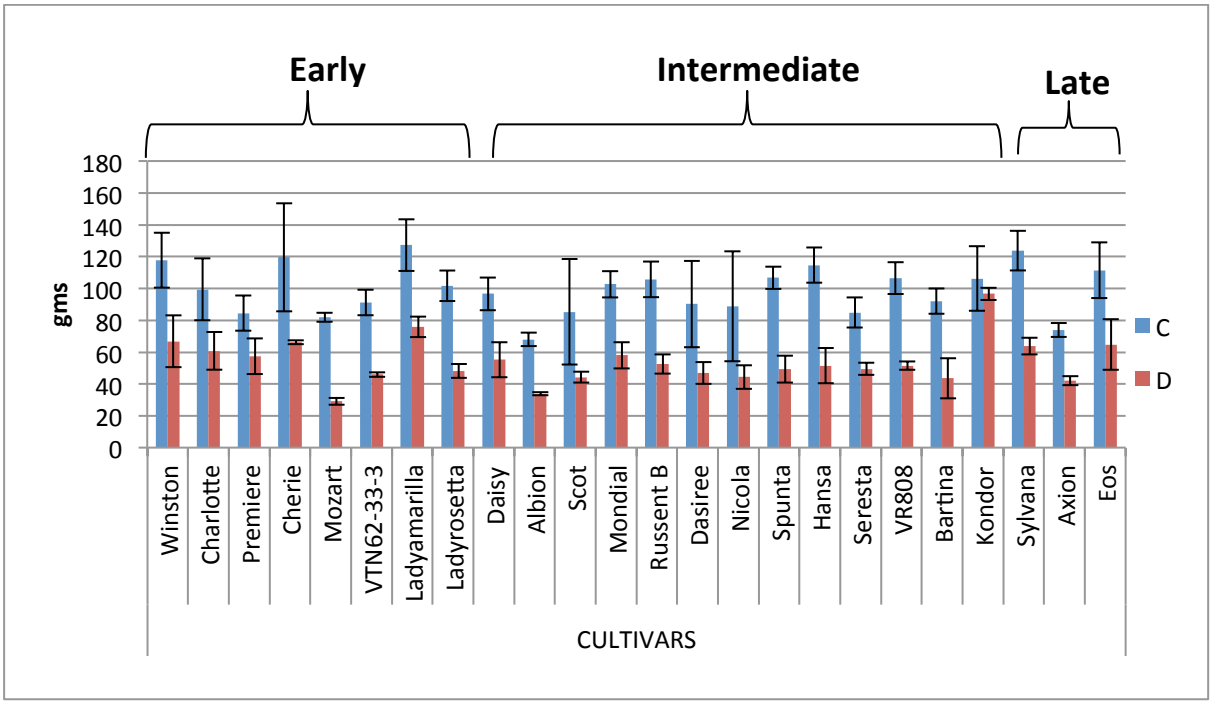


Figure 7: Tuber weight per cultivar per treatment

3.4 Expression analysis:

Expression analysis of *ABAOH* and *NCED* was done to investigate how the ABA synthesis in Albion may be affected in drought stress. This was done using Albion and Premiere, as a follow up study on Nasrin's results. Nasrin found out that Albion wilts faster in comparison to other genotypes in mild stress. In our experiment, our findings were in agreement with Nasrin's since we found Albion to be wilting under mild stress (Fig. 8). *NCED* and *ABAOH* genes are involved in the regulation of the amount of endogenous ABA in the plant under stress. The analysis was done for both the severe stress and the mild stress conditions. In mild stress there was up regulation of *ABAOH* in Albion and Premier while *NCED* was only upregulated in Premiere under mild stress and this up regulation was not significantly different from the control. Under severe stress both the genes were down-regulated in both genotypes (Fig 9).

Figure 8: Albion under mild stress

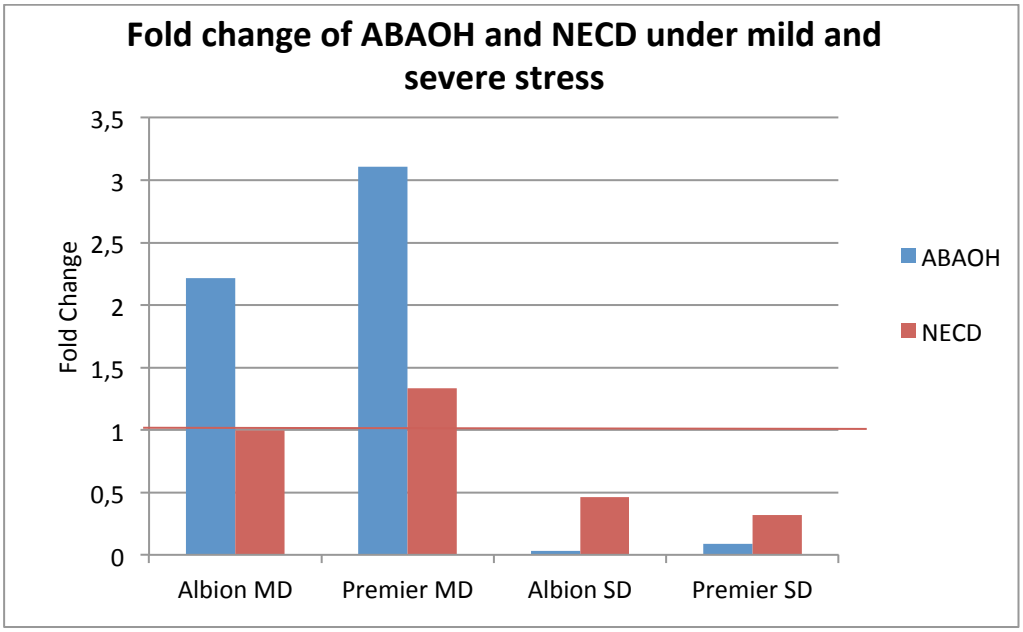
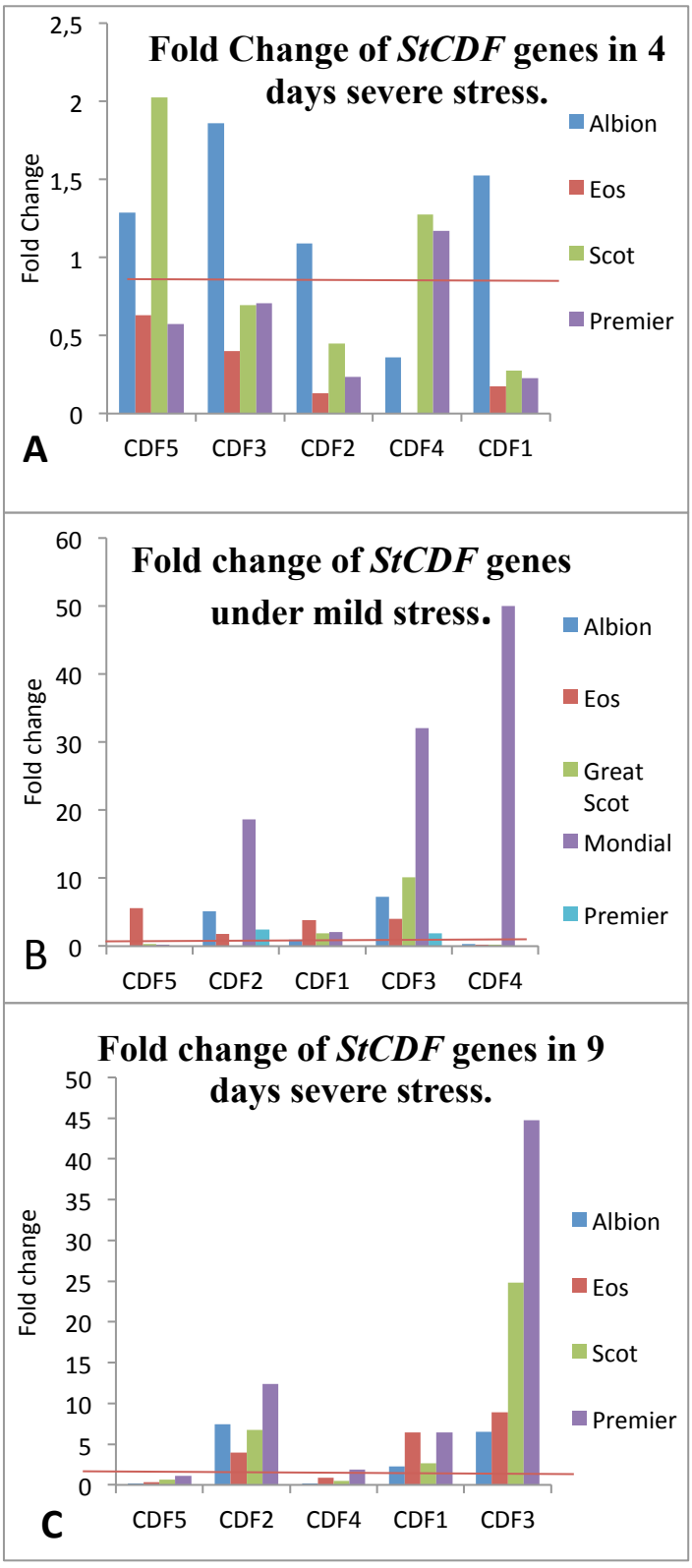


Figure 9: Fold change expression analysis for *ABAOH* and *NCED* genes under mild stress (MD) and severe stress (SD) in relation to the control. A threshold of 1 means up-regulation.

CDF expression analysis was measured on the leaves to find out if these genes are regulated by drought stress in potatoes and their roles in tuberization. The potato cultivars were subjected to mild stress, and 4 days and 9 days severe stress (no watering).



StCDF1, *StCDF2* and *StCDF3* were all up regulated in mild stress and 9 days severe stress giving a pattern in up regulation. At 4 days of severe stress, there was no particular pattern of up regulation. All genes were upregulated in Albion except *CDF4* at 4 days of severe stress. *CDF4* was upregulated in Great Scot and Premier while *CDF5* was up regulated in Scot and Albion (Fig 10A).

In mild stress, *StCDF1*, *StCDF2* and *StCDF3* were all up regulated in all cultivars analysed while *StCDF5* was up regulated in Eos and *StCDF4* was up regulated in Mondial only. There was insufficient cDNA for Premier in *StCDF*-1,-4 and -5 while Albion was insufficient in *StCDF*-4. Great Scot was not amplified in *StCDF*2 (Fig 10B).

In 9 days severe stress, *StCDF1*, *StCDF2* and *StCDF3* were all upregulated. Premier was slightly upregulated *StCDF4* (Fig10c).

Figure 10: Fold change expression analysis for *StCDF* genes under 4 days severe stress (A) mild stress (B) and 9 severe stress (C) in relation to the control. A threshold of 1 means up-regulation.

4 Discussion.

In this study, a total of 24 cultivars were screened for their response to mild drought stress. Mild water stress unfavourably affects the plant basic development, growth, carbon assimilation and plant productivity. To evaluate the effect of drought on these cultivars, growth and yield parameters were measured and analysed. There was reduction in the performance of cultivars in relation to drought as indicated by the traits measured.

4.1 Morphological parameters:

Water is essential for growth and it forms the largest component of plants cells and actively growing tissue such as leaves and root tips, can be approximately 90 percent water. Thus water deficit can inhibit or completely stop many physiological processes of the plant such as transpiration, photosynthesis, cell enlargement and enzymatic activities (Van Loon, 1981). This is in agreement with our findings since the drought treated plants had reduced biomass, number and weight of tubers, the emergence of new leaves, leaflets and stem were reduced in comparison to the control plants. This is as a result of effect of drought on the physiological processes in plants such as photosynthetic, leaf area expansion, partitioning of assimilates, tuber initiation, bulking and tuber growth (Van Loon, 1981). Cherie had a remarkable increase in stem length, leaves and leaflets; this may be because of its late emergence as a result of its dormancy.

4.2 Stomatal conductance:

Plants respond to drought stress through the closure of their stomata among other mechanisms. This response is mediated through ABA signalling. The closure of the stomata decreases the supply of carbon dioxide to the mesophyll cells impacting negatively on the photosynthetic rate. It is generally reported that potato plants close their stomata when water potential falls below -0.6MPa in an attempt to conserve the moisture (Monneveux et al., 2013). The stomatal conductance was highly reduced in drought treated plants and the reduction was higher in source leaves than in sink leaves in all cultivars. Stomatal activity plays a role in the carbon dioxide and oxygen exchange at vegetation and the ecosystem interface. Stomatal resistance will therefore lead to reduced CO_2 uptake and this limits photosynthetic rate. This may partly explain why we had lower yields in drought treated plants in comparison to the control plants. Drought did not affect stomatal conductance in Nicola in both sink and source leaves. This may mean that Nicola signalling for stomata

closure may have been impaired in drought but this can only be ascertained through molecular analysis of the regulatory network.

4.3 Chlorophyll content:

Chlorophyll is a molecule found in plants that absorbs sunlight and the plant utilizes it to synthesize carbohydrates from CO_2 and water in a process known as photosynthesis. In our experiment there was high chlorophyll content under drought in comparison to the control plants. This may be supported with Van Loon (1981) findings which states that so long as there is a continuous application of mild stress, the length of the vegetative period of potatoes may be considerably increased. In this stress environment, the plants stay green longer than in optimal condition and this may be because the plants had reduced expansion of leaves while they still make chlorophyll leading to higher chlorophyll per surface area. The sink leaves had higher chlorophyll content than the source leaves in all the cultivars. The plausible reason for this could be that the sink leaves were not fully expanded and thus the chlorophyll molecules are concentrated in smaller surface area than the source leaves.

4.4 Yield component.

The total tuber yield is the most important concern of potato breeders and farmers, and tuber initiation and bulking are most affected by drought (Steyn et al., 1998). There was a significant interaction between genotype and treatment in tuber numbers and this implies that genotypes respond differently to drought treatment for this trait. Some cultivars shut down completely their tuber initiation in the onset of drought while others continue the tuber transition in stolons. Cultivars such as Mondial, Morzart and Sylvana had relatively higher tuber number in optimal conditions but under drought they had higher percentage reduction on tuber numbers. Mondial is known to shut down its tuber initiation during drought and this characteristic may be shared with Mozart and Sylvana. The shutdown of tuber initiation may be responsible for large differences in tuber numbers in these cultivars. Premiere and Albion had the same tuber number in both treatments and this could indicate that drought did not affect the initiation of tubers in these cultivars. Premier and Albion are early maturing cultivars and thus they may have initiated most of their tubers before the onset of drought. Despite having the same tuber number, the control plants and drought plants in Premier and Albion had differences in tuber weights and this may imply that the control plants had enough photoasymilates to be stored in the tubers leading to higher tuber weight.

The correlation analysis revealed positive correlation between tuber numbers and leaflets. A higher number of leaflets which are large enough have a capacity to harvest carbon dioxide and light energy for photosynthesis and the plants will have relatively more energy to initiate more tubers. Stomatal conductance in source leaves was also correlated with tuber weight. Photosynthetic assimilates for tubers are likely to come mainly from the source leaves and so an increase in uptake of carbon dioxide at source leaves will lead to higher photosynthesis and thus to increased bulking.

4.5 Gene expression:

ABAOH and *NECD* expression analysis was done on Albion and Premier as a follow up study on Nasrin's findings. Nasrin found out that Albion invested less in foliage and wilt faster in mild stress. This was in line with our findings in which the Albion wilts in mild stress in comparison to the others. Accumulation of biomass in a plant depends on its ability to take up water and conserve it in its tissues. The plant roots tips perceive water scarcity and this triggers signalling to the leaves to control transpiration through regulation of the stomata aperture as a defence mechanism (Christmann et al., 2007). The stress response mechanisms are activated either directly resulting into the activation of the expression of stress responsive genes or through the expression of the transcription factor (Bartels & Sunkar, 2005). The phytohormone ABA is an endogenous messenger mainly involved in the control of adaptive response of plants to stress. The action of ABA can be signalled systematically to the guard cells for the induction of the stomata closure. It is generally known that most of the transcription factors regulate the ABA responsive gene expression while the stress responsive genes can be activated through the ABA-dependent or ABA-independent pathway (Tuteja, 2007). The ABA signalling pathway depends on the activity of a key gene in its biosynthesis, *NCED*, which is induced under drought in the vascular-tissue specific (Osakabe et al., 2014). The *ABAOH* gene regulates the amount of ABA during stress. In this study, both genes were not upregulated in severe stress while in mild stress there was up regulation only for *ABAOH* in both cultivars while *NCED* was only upregulated in Premier. This is in contrast with our expectation and previous findings from others that suggest that the level of ABA will increase with the severity of the stress (Lee & Luan, 2012). This may mean that endogenous ABA biosynthesis remain constant in Albion irrespective of the stress while in Premier, the ABA biosynthesis increased in mild stress. However, there was high fold increase in *ABAOH* under drought and this may lead to higher catabolic activity on the amount of the ABA synthesized and this may lower ABA in Albion and Premier with progress of drought severity. Another

plausible reason for the low ABA in severe stress could be that its sensitivity is affected under severe stress in these two cultivars.

CDF are a group of *DOF* genes that functions as transcription regulators that are involved in regulating plants responses to abiotic stresses such as drought and salinity and the control of flowering and tuberization signalling. In *Arabidopsis*, the *CDF* have been showed to bind the *CO* thereby supressing its transcription and consequently inhibiting flowering (Kloosterman et al., 2013). The plants perceive photoperiodic changes in the leaves and activate the transcriptional factor *CO* to express *FT* and its homologue *StSP5G* in potatoes. The *StSP5G* suppresses the activity of the tuberization gene *StSP6Á*. Drought can infringe on tuberization and flowering signalling through its effects on *CDFs* transcripts accumulation.

The expression analysis of the *StCDF* genes in mild stress and 9 days severe stress in this study showed a general pattern, in which *StCDF1*, *StCDF2* and *StCDF3* were upregulated in all cultivars. However, in 4 days severe stress there was upregulated of *StCDF1*, *StCDF2* and *StCDF3* only in Albion (Figure 10A, B and C). This may be because at 4 days of severe stress the plants may still be adjusting their physiology to stress. The increase in expression of these genes under drought stress may indicate their involvement in adaptation to drought in potatoes. The overexpression of *SlCDF1* and *SlCDF3* in *Arabidopsis* was found to induce higher accumulation levels of metabolites in the transgenics such as proline, glutamine, GABA, and sucrose, which plants normally accumulate under drought stress (Corrales, et al., 2014.). These metabolites are known to play a part in the osmotic adjustment, detoxification of ROS and intracellular pH regulation (Rajasekaran et al., 2000). Corrales, et al., (2014.) also found out that the *SlCD1* and *SlCD3* had higher expression of stress responsive genes such as *ERD10*, *COR15* and *RD29A* in a non-stress situation. This gives an indication that *CDF* genes may be involved in the upstream control of stress response pathway. It was also found that the overexpression of Arabidopsis *CDF3* gene led to the accumulation of metabolites that contributed to improved response to drought and salt stress (Corrales et al., 2014). Up regulation of *StCDF1*, *StCDF2* and *StCDF3* in this study \does not automatically imply that these contribute to plant abiotic stress response since many genes are up regulated in drought stress. However, these findings strongly suggest that indeed the *CDFs* have a contribution in plant response to abiotic stress. I would therefore suggest an experiment to explore further the function of *StCDF1*, *StCDF2* and *StCDF3* in potatoes in relation to abiotic stress.

A mobile tuberigen *StSP6A* has been showed to signal tuberization in potatoes. The *StCDF* on the other hand is involved in regulating tuberization signalling through its suppression of

StCO. If *CO* is expressed, it activates *StSP5G* which represses *StSP6A* thus blocking tuberization in potato. The suppression activity of *CDF* on *CO* depends on its accumulation and up regulation of *StCDFs* in drought will lead to suppression of *CO*. Consequently the *StSP5G* transcripts will be low thus alleviating the suppression of *StSP6A* leading to tuberization, while down regulation will lead to expression of *CO* and this will infringe in tuberization signalling.

The overexpression of *SlCDF3* in Arabidopsis was reported to delay flowering through the repression of *CO* (Corrales et al., 2014). The homolog of the *SlCDF3* in potato is *StCDF5*, which was down regulated under drought in our sensitive cultivars Mondial and Great Scot. These cultivars exhibit a tuberization shut down on perception of drought. The down regulation of *StCDF5* in these cultivars means that *CO* is expressed, and the expression of *CO* activates *StSP5G* which represses *StSP6A* thus blocking tuberization. This may suggest the involvement of *StCDF5* in tuber shut down in Mondial and Great Scot in the perception of drought.

5 Conclusion:

Drought affects the performance of the potato plants and thus the development of drought tolerant varieties is required for maintaining yield under climate changes and in drought stricken areas. The 24 cultivars showed reduced performance in drought

The investigation of the activity of ABA regulating genes in Albion and Premier did not give any contrast in the signalling. The low level of expression of *ABAOH* and *NCED* in severe stress may indicate that signalling under severe drought stress involve more molecular components than could be explained by ABA signalling.

The up regulation of *StCDF1*, *StCDF2* and *StCDF3* suggest that these genes could play a role in drought tolerance in potatoes. *StCDF5* in Mondial and Great Scot is likely gene that contributes in the suppression of the *StCO* thereby infringing into tuberization signalling in potatoes under drought.

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7 Appendix:

Appendix1: Transformation Protocol:

The Andegena plants were to be transformed with tuberization gene under an inducible promoter β-estradiol.

Starting materials:

TOPO Entry Vector with our gene of interest *SP6A* were available in the lab as glycerol stocks stored at -80^{0}c in the freezer. The destination vector pMDC7 containing β-estradiol inducible promoter was sourced from Ghent University in Belgium.

TOPO Entry vectors were inserted into chemically competent cells *Escherichia coli* DH5α strain through a 30 seconds heat shock in a water bath at 42^{0}c. The cells were then recovered in a SOC solution at 37^{0}c in a shaker at 200rpm for 1 hour. Then the cells were plated on 3% LB, agar containing 50ul/ml spectromycine and incubated overnight at 37^{o}c to allow the colonies to grow. The transformed grew on the plate and were picked and grown in an LB media containing spectromycine antibiotics at 37^{0}c in a shaker at 200rpm overnight. The Entry vectors were than isolated the following day using the QIAprep® Spin Miniprep kit by Qiagen. The quantity of the plasmid purified was measure using a Nanodrop and the plasmid stored in an Elusion Buffer (EB) at 4^{0}c.

The same procedure was repeated for the expression vector pMDC7.

LR REACTION:

The LR reaction was used to insert *SP6A* into an expression vector. The expression vector, pMDC7, had a spectromycine resistance gene. The components of the LR reaction consisted of 1.28µl of Entry vector, 2.42µl of destination vector and 4.3 µl of TE buffer to give 8µl total reaction mix. 2µl of LR Clonase II enzyme was added and vortexed briefly twice. The reaction was incubated at 25^{o}C for 1hour. The reaction was then stopped using 1µl of

Proteinase K solution and vortexed briefly. The LR reaction mix was incubated for 37^0c for 10 minutes.

The LR reaction mixture was transformed into chemically competent *E. coli* DH5α cells, incubated in Ice for 30 minutes and then heat shocked in water bath at 42^0c for 1 minute. Then 250µl of S.O.C. medium was added and the mixture incubated at 37^oC for 1hr with shaking at 200rpm. The transformed cells were plated overnight on selective plates containing a LB and 200µg/ml of spectromycine antibodies. Sterile toothpick was used to collect 3 colonies from the plate which were then incubated in LB media containing spectromycine antibodies with the same concentration as above and allowed to grow overnight. The three colonies grew and a colony PCR was performed to confirm the presence of our insert in the expression vector. The SP6A specific primers were used and the PCR reaction was set up as follows: an initial 2 minutes of denaturation at 95^0C; 29cycles at 95^0C for 30 sec, 55^0C for 30 seconds, 72^0c for 30 seconds; and final 9 minutes incubation at 72^0C. The PCR products were stained and separated on 1% agarose gel. The bands of approximately 500bp were found as expected on both colonies and this shows that our insert was indeed in our expression vector. To confirm the orientation of the insert, the purified plasmids were sent for sequencing by GATC Biotech using pMDC7 expression vector and reverse primers spanning the insert site. The returned insert sequence was blasted on NCBI nucleotide blast for *Solanum tuberosum* and perfect alignment was found. The backbone and the promoter were also checked and found to be in the right orientation.

Agrobacteria transformation:

The chemically competent *Agrobacterium tumefaciens* cells of *AGLO* were used for the transformation. The *AGLO* strain is resistance to spectromycin and rifarmpicin. Competence cells were sourced from -80^0c freezer and 50 µl of the competence cells were used. Then 2µl of expression vector with our insert in the correct orientation was added into the cells and mixed. The plasmids were purified and dissolved in MQ water to avoid the salt interference during electroporation. The resulting solution was transferred into precooled cuvette, placed in a Gene pulser and pulsed at a resistance of 200Ω, capacitance 25µF, voltage 1.4kV at a time constant between 4 and 5ms. 950 µl LB was immediately added the cuvette to recover the cells and the contents transferred into an eppendorf and incubated at 28^0c for 3 hours. The cells were then plated on LB supplemented with spectomycin and rifampicin and incubated at 30^0c for two days.

Appendix 2: Primer sets used in real-time RT-PCR gene expression analyses.

Gene	Primer sequence	Product length	TM	GC content.
NCED_F	TCGAAAACCCGGATGAACAAGTGA	120	62.78	45.83
NCED_R	AACCAGAAACTTTTGGCCATGGTTC	120	62.66	44.00
BAOH_F1	TTGCTGCACAAGATACAACAGCAAG	146	62.72	44.00
ABAOH_R1	TGTCCATGTCAACCCATGATTTTCT	146	60.99	40.00
StCDF1_R	GAGTGCCTTTTCCTCACTCG	130	58.30	50.00
StCDF1_F	TGCAGACTCGTCGATTGAAC	130	58.57	55.00
StCDF2_F	AGTTTCCGGATTCTTCTGGAG	121	57.38	47.62
StCDF2_R	TCCTCGTCATCATCCAGGTT	121	58.13	50.00
StCDF3_F	ATGTCTGAAGCAATTGCTATTAAG	142	55.82	33.33
StCDF3_R	GCTTGAGCTCTTGCTCGACT	142	60.39	55.00
StCDF4_F	CGGATTTCATCATCCAGCG	127	56.57	52.63
StCDF4_R	TCGGATCCCATTTGGTGTAT	127	56.29	45.00
StCDF5_F	CACAAGGCTGTTATGCATGG	99	57.43	50.00
StCDF5_R	CATAGGAACTGCAGCATTCC	99	56.56	50.00

Appendix 3: Correlations of different traits with yield indicators. The yellow shading represents positive correlations while the red shading represents negative correlations. White shading represents no correlation. The correlation analysis was divided into 3 parts. The overall for the whole experiment, Control for the control plants with the yield indicators and drought for only drought treated plants.

			Overrall					
Tuber_No	Correlations	0.1423	0.4102	0.1403	0.1115	-0.1582	-0.3516	-0.3595
	T-distribution	0.0971	<0.001	0.1019	0.1945	0.0649	<0.001	<0.001
Tuber_weight	Correlations	0.4788	0.3614	0.2408	0.2896	-0.3087	-0.4641	-0.6301
	T-distribution	<0.001	<0.001	0.0046	<0.001	<0.001	<0.001	<0.001
			Control					
Tuber_No	Correlations	-0.2048	0.4509	-0.0372	-0.1381	-0.1585	-0.0387	0.1
	T-distribution	0.0965	<0.001	0.7648	0.2652	0.2001	0.756	0.4207
Tuber_weight	Correlations	-0.0182	0.1655	-0.1004	-0.1586	-0.3834	-0.1209	-0.2137
	T-distribution	0.8838	0.1808	0.4188	0.2	0.0014	0.326	0.0825
			Drought.					
Tuber_No	Correlations	-0.3093	-0.0499	-0.0392	-0.0156	0.096	-0.1144	-0.0257
	T-distribution	0.0092	0.6817	0.7471	0.8981	0.4291	0.34	0.83
Tuber_weight	Correlations	-0.0877	0.0177	-0.0233	0.1885	0.0297	0.2173	0.1277
	T-distribution	0.4703	0.8845	0.8484	0.1181	0.8073	0.07	0.29
		HEIGHT	LEAFLETS	Poro_sink	Poro_source	Source_FVM	spad_Source	spad_sink