Enhancing Drought and Osmotic Stress Tolerance by Overexpressing α-acetolactate decarboxylase and acetoin 2,3-butanediol dehydrogenase *In Planta*

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Declaration

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Abstract

The effect of certain rhizosphere colonising bacteria on plant growth has been extensively exploited in agriculture since the green revolution of 1950. The bacteria symbiotically colonise the rhizosphere, utilising exudates from the plant roots, while providing the plant with a number of beneficial actions such as increasing the nutrient availability to roots. These plant growth-promoting rhizobacteria (PGPR) have been well documented and researched since the green revolution began, however, many of the exact bacterial mechanisms responsible for plant growth promotion remain unknown. Previous research, aiming to elucidate such mechanisms, discovered that bacterial volatile organic compounds (VOCs) such as acetoin and 2,3-butanediol were able to increase growth of *Arabidopsis thaliana*. Subsequent research identified the genes responsible for the production of acetoin and 2,3-butanediol as α-acetolactate decarboxylase (*ALDC*) and 2,3-butanediol dehydrogenase (*BDH1*) respectively. These genes were previously successfully transformed into and expressed within *Arabidopsis* plants within our research group, leading to increased growth and disease tolerance.

In this study, *Arabidopsis thaliana* plants were transformed with the *ALDC* and *BDH1* genes using an *Agrobacterium*-mediated floral dip method in order to confirm the above-mentioned research, and to allow for the detection of the volatiles in the transgenic plants. However, due to a 240 base pair deletion within the *ALDC* gene, discovered in the T_2 generation, further research could not be performed using *Arabidopsis*. Sugarcane (*Saccharum officianarum*) was transformed with the *ALDC* and *BDH1* genes using a particle bombardment approach. Transgenic sugarcane plants were successfully genotyped, sequenced and assessed for transgene expression. The transgenic sugarcane was tested for increased growth, under both *in vitro* and *ex vitro* conditions, as well as for drought tolerance via an *ex vitro* pot trial. No significant differences were observed for the growth of the transgenic sugarcane *in vitro* compared to the untransformed control plants. The limited availability of transgenic material lead to difficulties in selecting plantlets that were of uniform size and root development for *in vitro* trials. This led to high variance in the data and inconsistent results within each transformed line. Larger quantities of transgenic material would have alleviated this issue by allowing for selection of plantlets at a uniform developmental stage. Neither were overall significant differences observed between the transformed and untransformed lines within the drought trial. Inconsistent conditions within the growth room where the drought trial was performed led to inconsistent drought pressures applied to the plants. In addition, a temperature spike during the trial led to the rapid onset of drought shock rather than the intended slower onset of drought stress. Untransformed sugarcane was also exposed to synthetic acetoin *in vitro*, with no significant differences in growth observed after the allowed growth period.

In general, this study was inconclusive. However, various aspects of the research were identified which could lead to more conclusive and consistent results. In addition, a method for directly confirming the production of VOCs *in planta* still needs to be established.

Samevatting

Die effek van sekere rizosfeerkoloniserende bakterieë op plantgroei word sedert die groen rewolusie van 1950 op groot skaal in die landbou benut. Die bakterieë koloniseer op ‘n simbiotiese wyse die risosfeer deur om van die uitskeidings van die plantwortels gebruik te maak, terwyl die plant 'n aantal voordelige aksies aangebied word, soos die beskikbaarheid van voedingstowwe aan wortels. Hierdie plantgroei bevorderende risoobakterieë (PGPR) is goed gedokumenteer en nagevors sedert die groen rewolusie begin het, maar baie van die presiese bakteriële meganismes wat verantwoordelik is vir die bevordering van plantgroei bly onbekend. Vorige navorsing, met die doel om sulke meganismes te belig, het ontdek dat vlugtige organiese verbindings (VOC's) soos acetoïne en 2,3-butandiol die groei van *Arabidopsis thaliana* kon verhoog. Daaropvolgende navorsing het die gene wat onderskeidelik verantwoordelik is vir die produksie van asetoïen en 2,3-butaandiol as α-asetolaktaat dekarboksilase (*ALDC*) en 2,3-butandiol dehidrogenase (*BDH1*) geïdentifiseer. Hierdie gene is voorheen suksesvol in *Arabidopsis*-plante getransfoormeer en tot uitdrukking gebring binne ons navorsingsgroep , wat gelei het tot groter groei en siektetoleransie.

In hierdie studie was *Arabidopsis thaliana*-plante getransformeer met die *ALDC* en *BDH1*-gene met behulp van 'n *Agrobacterium*-gemedieerde blommedipmetode om die bogenoemde navorsings resultate te bevestig, en die opsporing van vlugtige verbindings in die transgene plante moontlik te maak. Weens 'n 240 basispaar verwydering in die *ALDC* geen, wat in die T_2 generasie ontdek is, kon verdere navorsing nie met *Arabidopsis* uitgevoer word nie. Suikerriet (*Saccharum officianarum*) was getransformeer met die *ALDC* en *BDH1* gene met behulp van 'n deeltjie-bombardementbenadering. Transgeniese suikerrietplante is suksesvol genotipeer, gesekwenseer en beoordeel vir transgeenuitdrukking. Die transgeniese suikerriet was getoets vir verhoogde groei, onder *in vitro* en *ex vitro* toestande, sowel as vir droogteverdraagsaamheid deur middel van 'n *ex vitro* pot proef. Geen betekenisvolle verskille was waargeneem in die groei van die transgene suikerriet *in vitro* in vergelyking met die ontransformeerde kontroleplante nie. Die beperkte beskikbaarheid van transgene materiaal lei tot probleme met die seleksie van plantjies wat van eenvormige grootte en wortelontwikkeling was vir *in vitro* proewe. Dit het gelei tot 'n hoë variansie in die data en teenstrydige resultate binne elke getransformeerde lyn. Groter hoeveelhede transgene materiaal sou hierdie probleem verlig het deur die keuse van plantjies in 'n eenvormige ontwikkelingstadium. In die droogteverhoor was daar ook nie beduidende verskille tussen die getransformeerde en ongetransformeerde lyne gesien nie. Inkonsekwente toestande in die groeikamer waar die droogtetoets uitgevoer is, het gelei tot inkonsekwente droogtedruk op die plante. Daarbenewens het 'n temperatuurstyging tydens die proefneming tot die vinnige aanvang van droogteskok gelei, eerder as tot die beoogde stadiger aanvang van droogtestres.

Ontransformeerde suikerriet is ook *in vitro* aan sintetiese asetoïne blootgestel, met geen noemenswaardige verskille in groei waargeneem na die toegelate groeiperiode nie.

Oor die algemeen was hierdie studie onoortuigend. Verskeie aspekte van die navorsing is geïdentifiseer wat kan lei tot meer afdoende en konsekwente resultate. Daarbenewens moet 'n metode gevoer word om die produksie van VOC's *in planta* direk te bevestig.

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Abbreviations

%	Percentage
AB	*ALDC* + *BDH1*
ABA	Abscisic acid
ALDC	α-acetolactate decarboxylase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BDH1	2,3-butanediol dehydrogenase
bp	Base pair
CAF	Central Analytical Facility
CAT	Catalase
cDNA	Complementary DNA
cm	Centimeter
Col-0	Columbia-0
CTAB	Cetyl trimethylammonium bromide
DAB	3,3-diaminobenzidine
dd	Double distilled
DNA	Deoxyribonucleic acid
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EMS	Ethylmethane sulfonate
FNR	Ferredoxin-$NADP^+$ reductase
FW	Fresh weight
g/L	Grams per litre
GISH	Genomic *in situ* hybridization
h	Hour
HPS	High pressure sodium
IAA	Indole-3 acetic acid
Inc	Incorporated
IPB	Institute for Plant Biotechnology
ISR	Induced systemic resistance
kg	Kilograms
kPa	Kilopascals
L	Litre
LB	Luria-Bertani
LED	Light emitting diodes

M	Molar
mg/L	Milligrams per litre
MH	Metal halide
min	Minutes
mL	microlitre
mL	Millilitre
mm	Millimetre
mM	Millimolar
MS	Murashige and Skoog
MSC	Murashige and Skoog + casein
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NBT	4-nitroblue tetrazolium chloride
Neg	Negative
ng	Nanograms
nm	nanometre
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
photons/cm^2/s	Photons per centimetre squared per second
Pos	Positive
PVP	Polyvinylpyrrolidone
R	Rand
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
RWC	Relative water content
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SE	Standard error
sec	Second
seq	Sequence
SOD	Superoxide dismutase
T-DNA	Transfer deoxyribonucleic acid
TBE	Tris borate EDTA
TM	Trade marked
Tris	Trisaminomethane
TW	Turgid weight

USA	United States of America
v/v	Volume per volume
VOC	Volatile organic compounds
W	Watt
w/v	Weight per volume
WST	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium
ww	Without water
x*g*	Times gravity
µMol	Micromoles

1. General Introduction and Literature Review

Over millions of years, plants have evolved numerous mechanisms that allow them to adapt to environmental changes (Fowden *et al*., 1993). These adaptations allow plants to survive in sub-optimal conditions and eventually evolve to thrive in them. This has enabled the kingdom Plantae to colonize almost every environment on earth (Spalding, 1890). In the wild, this has obvious benefits. However, in agricultural systems the plants' anticipation of environmental fluctuations has a limiting effect on plant yield. In order to maintain the ability to adapt to environmental fluctuations, plants employ two primary strategies; the creation of nutrient reserves and self-limiting growth (Grime, 1977). Plants create nutrient reserves, which, during times of environmental stress, can be accessed to maintain metabolic activity. In agriculture, environmental stress is minimised so as to obtain maximum yields. Even so, crop plants continue to regulate their own growth in anticipation of harsher times. For example, fruit sizes of wild plants are optimized to meet the requirements for survival and population spread while minimizing the use of energy reserves (Spengler, 2019). This enables plants to use any available nutrients as efficiently as possible with the only goals being survival and reproduction. Under agricultural conditions, where growth conditions are optimized and nutrient supply is effectively unlimited, plants still retain their natural tendency to limit their own growth. This means that plants, even under perfect conditions, do not reach their theoretical maximum potential (Hartmann *et al*., 2011). It is this regulation that modern agriculture works to overcome through selective breeding and genetic manipulation. Over time, plant cultivation practices have led to the creation of numerous crop species whose survival strategies have been reduced in favour of more agronomically-favourable traits and which perform well under cultivated conditions. Improvements on these crop species continue to be developed, now with the advantages that biotechnological techniques bring to traditional crop improvement strategies.

With the world seeing a dramatic increase in its population to a predicted 9 billion by the year 2050 (Rouser and Ortiz-Ospina, 2018) there will be a dramatic increase in the demand for foodstuffs. The self-governed growth regulation by a plant's survival-focused physiology continues to limit production. As mentioned, traditional plant breeding has been used effectively to increase crop production by reducing physiological limitations as well as the impact of biotic and abiotic stresses. This is especially true since the onset of the green revolution where, for example, the introduction of short-stemmed (dwarfed) wheat cultivars allowed the plants to carry heavy heads without lodging, paving the way for dramatically increased yields (Phillips, 2013). Despite this, in many circumstances, supply simply cannot keep up with the growing demand for agricultural produce.

1.1. Rhizobacteria and Their Effect on Plant Growth

In the field, plants are exposed to a multitude of both harmful and beneficial microorganisms. Interactions between the plants and microorganisms are especially concentrated in the rhizosphere. The rhizosphere (Figure 1.1) consists of the endo-rhizosphere and ecto-rhizosphere, a thin layer of soil that surrounds plant roots, where root exudates are utilised by rhizosphere colonising organisms (Lynch, 1994). The size of the rhizosphere depends on a number of biological and abiotic factors, however, it generally ranges between 0.5 and 5 mm from the rhizoplane, the surface of the plant root (Kwon *et al*., 2010). Rhizosphere-colonising microorganisms, of which rhizobacteria are the most prolific, often have plant growth regulating properties (Bhattacharyya and Jha, 2012). Approximately 2% of rhizobacterial species have plant growth-promoting properties, including *Baccillus* and *Pseudomonas* spp. These rhizobacteria were termed plant growth-promoting rhizobacteria (PGPR) by Kloeper and Schroth (1981). PGPR are now narrowly defined as bacterial strains that are able to fulfil at least two of the following criteria: can aggressively colonize the rhizosphere, are plant growth stimulating and act as biocontrol agents (Vessey, 2003). These bacteria are able to form symbiotic relationships with the host plant root system, whereby they benefit plant growth, survival and reproduction, while utilising root exudates secreted by the host plant (Vessey, 2003). Since anywhere between 30 to 40% of carbon fixed by photosynthesis is estimated to be transferred to the rhizosphere (Badri and Vivanco, 2009), the large diversity of bacteria in the rhizosphere is not surprising.

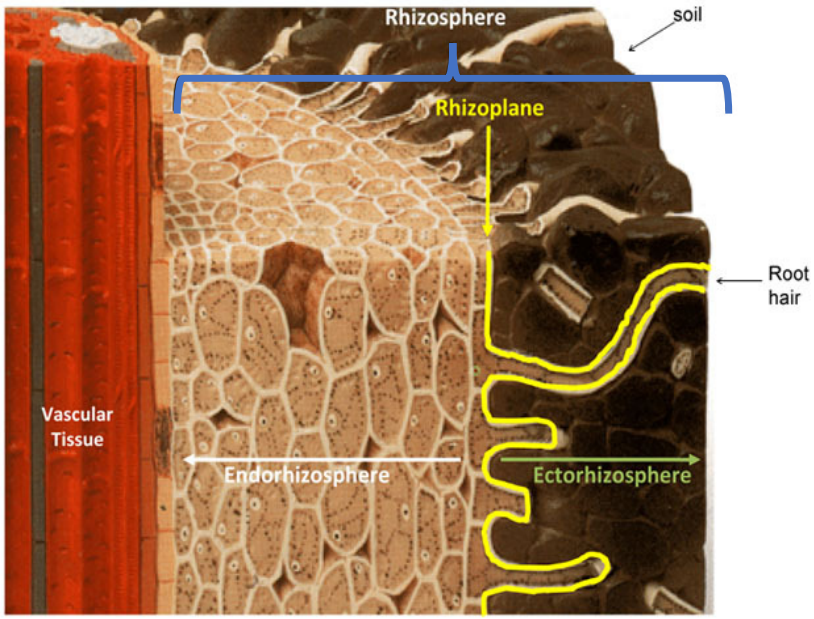

Figure 1.1: A sectional schematic of a plant root, demonstrating the position of the rhizosphere as well as the root structures involved in rhizosphere interactions. Modified from: https://www.nature.com/scitable/content/schematic-of-a-root-section-68110828/

PGPR are known to promote plant growth through direct and/or indirect methods (Podile and Kishore, 2006). Examples of indirect plant growth promotion would include pathogen exclusion and the solubilisation of inorganic nutrients (Vessey, 2003). Pathogen exclusion occurs when rhizobacteria colonize potential infection sites and outcompete pathogenic species, thereby excluding the pathogen from the infection site (Wood and Tveit, 1955). Bacteria that protect plants in this way are known as antagonistic bacteria, an example of which is *Pseudomonas flourescens* (Ganeshan and Kumar, 2006). Bacteria are also able to solubilise growth-limiting inorganic nutrients, such as iron and phosphate (Vessey, 2003). This is accomplished by producing various metabolites, such as iron-chelating siderophores and phosphate-releasing organic acids (Mehta and Nautiyal, 2001).

Certain PGPR species are able to directly influence a plant's growth via various bacterial metabolites. A number of these metabolites act as plant signalling molecules (Kai *et al*., 2010). Lumichrome, plant growth-stimulating hormones and plant-targeting volatile organic compounds (VOCs) are known to be produced by PGPR (Treadwell and Metzler, 1997; Loon, 2007). Various PGPR are able to influence plant growth through the use of plant growth-stimulating hormones such as auxin, cytokinins, ethylene and gibberellins (Loon, 2007). For example, Barbieri and Gallo (1993) demonstrated that auxin must be responsible for the increased growth of wheat when exposed to *Azospirillum brasilense,* as the mutant bacterial strain with altered indole-3-acetic acid production did not result in the same plant growth promotion. VOCs are molecules with a high vapour pressure, indicating that they have a fast evaporation rate at ambient temperatures (Herrmann, 2010). Having a high vapour pressure means that VOCs are readily translocated within the plant and in the atmosphere surrounding it above the soil line, thereby being able to quickly reach their biological receptor(s) or targeted machinery (Herrmann, 2010).

In addition to increasing plant growth, PGPR have also been shown to increase plant abiotic stress tolerance (Mayak *et al*., 2004). Deleterious plant responses to stressors such as heavy metals, drought and salt may be reduced when the plant is exposed to PGPR (Burd *et al*., 1998; Mayak *et al*., 2004). Very few direct mechanisms via which PGPR increase stress tolerance in plants have been elucidated. However, increased water stress tolerance is suggested to occur as a result of overall increased root growth and health of plants exposed to PGPR (Ngumbi and Kloepper, 2016). During drought conditions, plants will inhibit shoot growth to reduce evaporative losses and to divert energy to housekeeping functions such as osmotic regulation (Skirycz and Inzé, 2010). These responses, while beneficial to the plant's long term survival, may lead to a reduction in yield and become counterproductive in an agricultural setting (Ngumi and Kloepper, 2016). Treatment of plants with PGPR usually leads to an increase in shoot growth through various mechanisms.

Therefore, PGPR could maintain normal shoot growth of a plant under drought conditions (Vardharajula *et al*., 2011)

PGPR have also been reported as being able to activate an induced systemic resistance (ISR) to plant pathogens. ISR was first described by van Peer and colleagues (1991) in carnations that exhibited resistance against *Fusarium oxysporum* f.sp. *dianthi* when treated with *Pseudomonas* spp. strain WCS417. During testing, the antagonistic effect of strain WCS417 on the pathogen *Fusarium oxysporum* f.sp. *dianthi* was avoided by inoculating both bacteria to spatially separate plant parts. In this way, the WCS417 strain would have no direct effect on the pathogen. Thus, it was concluded that the observed resistance after inoculation of strain WS417 must have been plant-mediated. This plant-mediated resistance after inoculation of non-pathogenic microorganisms is distinct from systemic acquired resistance (SAR) which is triggered by the presence of a pathogen and peak expression is observed when the pathogen causes necrosis, in contrast to ISR-inducing bacteria which do not cause any visible symptoms (Benhamou *et al*., 1996; Kloepper *et al*., 1980). Both mechanisms, however, work by activating/priming the plant's innate disease defence machinery (Kuć, 1982).

In an agricultural setting, the use of PGPR is one way in which it is possible to reduce the plant's self-imposed physiological limitations mentioned earlier. PGPR have already been extensively exploited in agriculture since the onset of the green revolution in 1950 and are seen as an environmentally-friendly method of yield improvement (Kloepper and Schroth, 1981). The PGPR which are of importance to this study secrete plant growth-promoting substances (PGPS) in the form of VOCs, specifically acetoin and 2,3-butanediol.

1.2. Rhizobacterial Volatile Organic Compounds

As mentioned above, rhizobacteria are capable of releasing a multitude of VOCs, most of which remain uncharacterised. Those VOCs that have been studied display a range of effects on plant growth, from significantly increasing biomass to causing plant death (Blom *et al*., 2011). A summary of published research, focused only on rhizobacterial VOCs that led to an increase in plant growth, is presented in Table 1.1.

Table 1.1: Published rhizobacterial VOCs and their effect on specific plant species.

Rhizobacterial VOC or Equivalent Synthetic Compound	Recorded Plant Response	Plant Species	Reference
2,3-butanediol and acetoin	Shoot weight	*Arabidopsis*	Ryu *et al.,* 2003
Albuterol and 1,3-propanediol	Shoot weight	Tomato	Tahir *et al*., 2017
Dimethyl disulfide	Leaf surface area and sulfur uptake	*Nicotiana attenuata*	Meldau *et al*., 2013
Dimethylhexadecylamine	Chlorophyll content and iron uptake	Sorghum	Castulo-Rubio *et al*., 2015
3-Pentanol	Fruit production	Cucumber	Ryu and Song, 2013
Indole	Seed germination	Cabbage	Yu and Lee, 2013
Indole	Auxin production and root proliferation	*Arabidopsis*	Bailly *et al*., 2014
Indole	Auxin production	*Arabidopsis*	Bhattacharyya *et al*., 2015

Following a meta-analysis of all previous research up to 2011, Bailly and Weisskopf (2012) observed that plant growth inhibition was more frequently observed than growth promotion in response to bacterial VOCs. Furthermore, bacterial culturing conditions appear to play an important role in the effect on plant growth when the plants are exposed to the bacterial culture. Those plants exposed to bacterial cultures grown on Luria-Bertani (LB) media generally display deleterious effects when compared to those plants exposed to cultures grown on Murashige and Skoog (MS) media (Blom *et al*., 2011). This is understandable when the composition of each media type is studied. LB is an alkaline, complex medium, predominantly made up of hydrolysed proteins and has a higher agar concentration when compared to MS medium which is acidic, with a predominantly mineral composition and sucrose as a carbon source. The differing media compositions are thought to elicit the production of different VOC combinations, as well as differing rates of production and bacterial growth kinetics (Bailly and Weisskopf, 2012). Maximum VOC production is expected to occur during the stationary bacterial growth stage (Kai *et al*., 2010). Thus, if plants of the same physiological age are exposed to bacterial cultures grown on different media, the peak VOC production will occur during different plant developmental stages. Blom and colleagues (2011) demonstrated the varying effects 42 strains of the *Burkholderia* genus had on *A. thaliana* growth when the bacteria was grown on four different media types. Typically, these studies are performed using a centre-partitioned petri dish (I-plate). The central septum allows gaseous exchange between the two halves but physically separates the cultures and media. This technique enabled early studies to confirm that PGPR are able to promote growth without direct contact with the host plant's root system, confirming that biologically-active VOCs, with no relationship to classical plant hormones, can induce plant growth promotion (Ortíz-Castro *et al*., 2009). New, biologically-active VOCs are regularly being discovered as new PGPR strains are investigated. The effect of these VOCs on plants continues to be limited

to the physiological pathways that control plant growth (Bailly and Weisskopf, 2012). Our current understanding of how VOCs influence plant physiological pathways is limited and answers remain elusive.

Ryu and colleagues (2003) published the earliest report on PGPR and VOCs. That study attempted to determine the mechanism behind the plant growth-promoting VOCs by testing the effects of the volatiles on various *Arabidopsis thaliana* mutant lines with impairments in well-characterised phytohormone biosynthetic and signal transduction pathways. Plant lines with mutations in ethylene (*ein2*) and cytokinin (*cre*) signalling showed either no or limited responses to VOC treatments, suggesting that the VOCs may be impacting on these signalling pathways. However, the specific mechanism(s) that enables or facilitates these interactions remains unknown. This conclusion was reinforced by a transcriptional study performed by Kwon and colleagues (2010) where ethylene biosynthesis, (*ACO2, ACS4, ACS12* and *SAM-2*) and the ethylene response (*CHIB*, *ERF1* and *GST1*) genes were upregulated in response to PGPR. Further investigation of the *Arabidopsis* proteome yielded similar results (Kwon *et al*., 2010). Other studies have also implicated the involvement of cytokinins, abscisic acid, and auxins in plant growth promotion in response to plant growth-promoting VOCs. Ortíz-Castro and Valencia-Canterolant (2008) noted that growth promotion by *Bacillus megaterium* was not observed in mutant plants with impaired histidine kinase cytokinin receptors. In plants, the abscisic acid (ABA) signalling pathway is closely tied to sugar-sensing (Smeekens, 2000). In a 2008 study by Zhang and colleagues, it was reported that *Arabidopsis* seedlings exposed to the *Bacillus subtilis* GBO3 strain showed an increase in photosynthetic activity and endogenous sugar concentrations, suggesting that the rhizobacteria were partially impairing sugar-sensing *in planta* in order to increase photosynthetic activity

1.3. Acetoin and 2,3-Butanediol

Acetoin and 2,3-butanediol, both volatile four-carbon alcohols (Ryu *et al*., 2003), can be produced naturally in a number of biochemical reactions by *Bacillus subtilis, Lactococcus lactis, Lecuconostoc mesenteroides* and many other microorganisms (Bassit *et al*., 1995; Schmit *et al*., 1997; Huang *et al*., 1999). Importantly, acetoin (Figure 1.2A) is an intermediate metabolite in the 2,3-butanediol (Figure 1.2B) production pathway (Figure 1.3). In bacteria, acetolactate synthase converts pyruvate to α-acetolactate. The α-acetolactate is then decarboxylated by α-acetolactate decarboxylase (ALDC, EC 4.1.1.5) to acetoin. At this point, acetoin can be removed from the cell or converted into 2,3-butanediol by 2,3-butanediol dehydrogenase (BDH1, EC 1.1.1.4) (Figure 1.3). In bacteria, acetoin is produced to avoid acidification, for the regulation of the nicotinamide adenine dinucleotide (NAD)/reduced NAD (NADH) ratio and as an energy source (Huang *et al*., 1999). In plants, there is

no evidence to suggest that acetoin is produced naturally. However, the precursor to acetoin, α-acetolactate is found in plastids as an intermediary step in the synthesis of branch-chain amino acids; valine, leucine and isoleucine (Chipman *et al*., 1998).

In the study by Ryu *et al.* (2003), seven common PGPR strains were studied to determine their effect on the growth of *A. thaliana* and to identify the PGPS they produced. Of the six strains, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a had significant effects on plant growth. Both bacterial strains led to an increase in the growth of *A. thaliana* when compared to water and *E. coli* DH5α controls. Ryu *et al*. (2003) identified acetoin and 2,3-butanediol as the VOCs contributing to this increased plant growth. The 2003 study also demonstrated that plants exposed to mutant strains of bacteria, defective in the production of 2,3-butanediol, resulted in significantly lower plant growth rates than those of plants exposed to the wild type bacterial strains. Furthermore, exogenous application of synthetic 2,3-butanediol similarly enhanced plant growth, confirming the study's findings.

In a follow-up study from 2004, Ryu *et al*. observed induced systemic resistance (ISR) in *Arabidopsis*, resulting from exposure to VOCs produced by *B. subtillis* GB03 and *B. amyloliquefaciens* IN937. In the study, disease severity caused by *Erwinia* (*Pectobacterium*) *carotovora* subsp. carotovora was shown to be significantly reduced after activation of ISR by exposure to the bacterial VOCs.

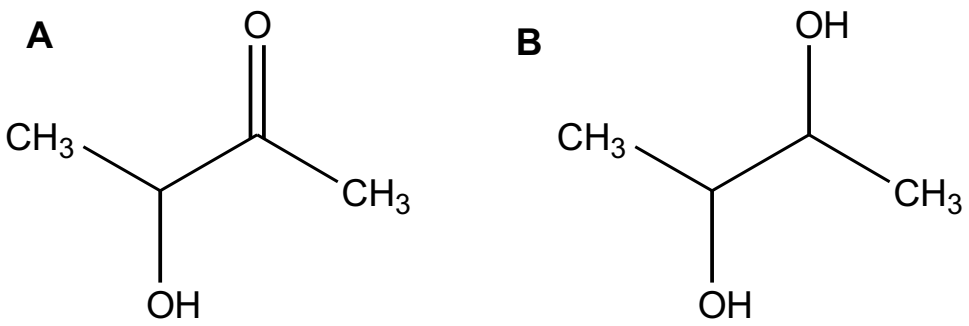

Figure 1.2: Chemical structures of A) acetoin, B) 2,3-butanediol (Chemical structures prepared on CS ChemDraw, PerkinElmer, United Kingdom).

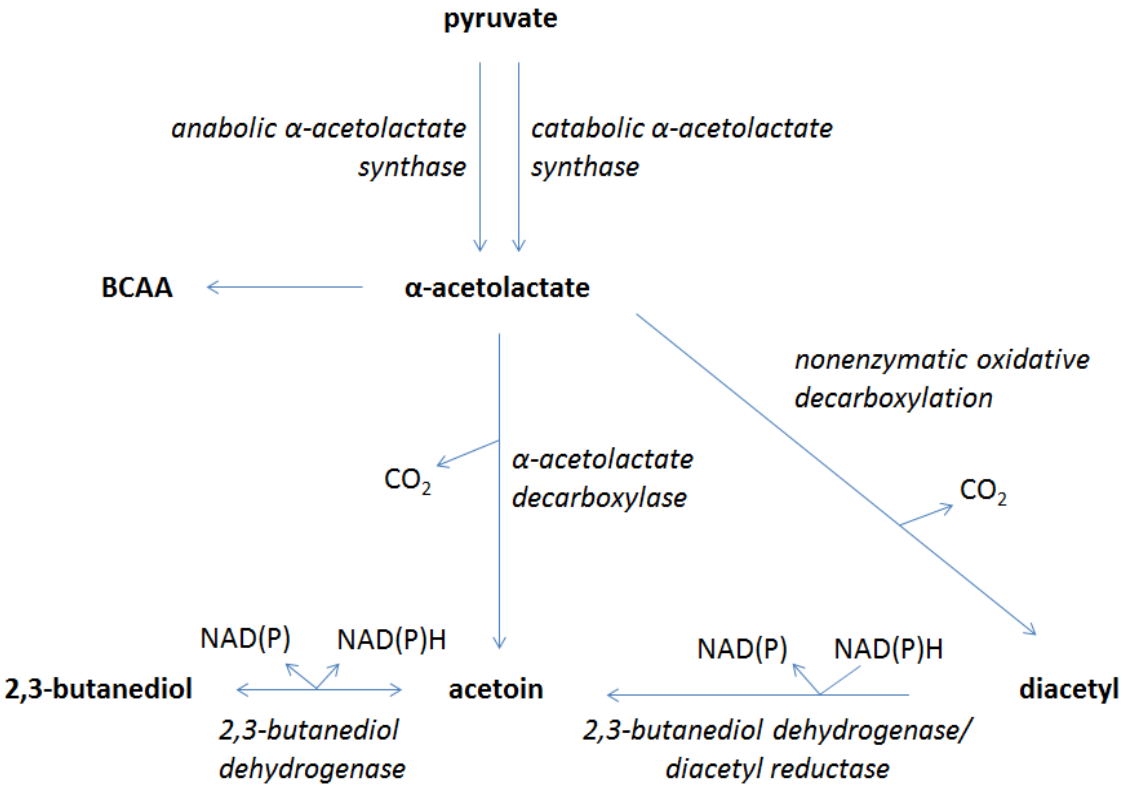

Figure 1.3: The biochemical pathway within bacteria for the production of 2,3-butanediol and acetoin from pyruvate (modified by Dempers, 2015 from Xu *et al*., 2011)

1.4. Previous Research within the Institute for Plant Biotechnology

In previous research within the Institute for Plant Biotechnology, Dempers (2015) cloned the *ALDC* (Annexure A) and *BDH1* (Annexure B) genes, from *Aspergillus niger* ATCC 1015 and *Saccharomyces cerevisiae* W303 respectively, and transformed these into *A. thaliana* Columbia-0, under the control of constitutive CaMV35S promoters. Since α-acetolactate is restricted to the chloroplast in plant cells (Miflin, 1974), both genes were modified to contain the ferredoxin-$NADP^+$ reductase (FNR) chloroplastic transit peptide sequence from *Spinacia oleracea* to direct the proteins to the chloroplast. Transgenic *Arabidopsis* plant lines expressing only *ALDC* as well as double transgenic lines expressing both genes showed significantly enhanced growth compared with wild type plants over a number of parameters measured, including leaf area and fresh and dry biomass (Dempers, 2015). Transgenic lines containing only the *BDH1* gene were indistinguishable from wild type plants, presumably since they did not produce acetoin as a substrate for the BDH1 enzyme (Dempers, 2015). Although it was shown that the transgenes were incorporated into the genomes of the transgenic lines and that these were expressed, it was not possible to detect either acetoin or

2,3-butanediol production *in planta* in any of the lines, using either liquid chromatography – mass spectrometry or gas chromatography – mass spectrometry methods. It was speculated that these lines may either utilise the VOCs immediately, or else that the VOCs are produced at levels below the detection limits of the instrumentation used in these analyses (Dempers, 2015). In a brief and unrepeated salinity trial, Dempers also observed a significant difference in growth between those lines transformed with the *ALDC* gene and wild type controls, where the transgenic plants far out-performed the respective controls. This result was not recorded in the Dempers (2015) dissertation as further and more comprehensive investigation was required. However, the apparent increased salinity tolerance was promising and held value due to the close link between salinity stress and water stress in plants (Bartels and Sunkar, 2007).

Interestingly, using synthetic acetoin and 2,3-butanediol, Dempers (2015) showed that *A. thaliana* Columbia-0 plants showed enhanced growth in response to acetoin only under long day length (14h:10 h day:night photoperiod) conditions, whilst no significant growth enhancement was observed under short day length (10 h:14 h photoperiod) conditions. In contrast to the results of Ryu *et al*. (2003), 2,3-butanediol did not enhance plant growth under any of the conditions tested.

With the aim of confirming the ISR observations by Ryu *et al*. (2004), Van der Merwe (2016) completed assessments on disease development on both wild type *Arabidopsis,* exposed to the synthetic acetoin and 2,3-butanediol compounds, and transgenic *Arabidopsis* that was transformed with the *ALDC* and *BDH1* genes. For both the synthetic volatile and transgenic plant assessments, a *Botrytis cinerea* spore suspension was spot-inoculated onto five alternate juvenile leaves on each plant. For the synthetic volatile assessment, this was performed 24 h post-incubation with acetoin and 2,3-butanediol. Additionally, during incubation with the pathogen, fresh acetoin and 2,3-butanediol were regularly added to maintain constant exposure by the plants to these volatiles. These plants were then compared to two controls. The first control was established by inoculating plants, not exposed to acetoin or 2,3-butanediol, while the second control consisted of plants exposed to the volatiles but not inoculated with *B. cinerea*. Disease development was assessed phenotypically by lesion diameter scoring as well as by semi-quantitative reverse transcriptase PCR (RT-sqPCR) for gene marker analysis of seven disease response genes linked to ISR and SAR. Through visual inspection, van der Merwe observed that lesion development was delayed for the acetoin-treated plants in comparison to both the water control and the 2,3-butanediol-treated plants. The RT-sqPCR analysis indicated that exposure to synthetic acetoin and 2,3-butanediol prior to infection lead to upregulation of the *PR2* gene, an indicator of SA-regulated SAR response. Only exposure to acetoin lead to the upregulation of genes associated with an ISR response. These results led van der Merwe to conclude that acetoin was responsible for triggering the ISR response, while 2,3-butanediol was able to trigger an SAR response.

The transgenic *Arabidopsis* plants inoculated with *B. cinerea* were compared to a negative control established by inoculating leaves with a suspension not containing fungal spores. Phenotypically the plants transformed with only the *ALDC* gene and those transformed with both the *ALDC* and *BDH1* genes showed slightly better resistance to infection than the wild type plants. However, further statistical analysis of lesion scores revealed no significant difference between treatments. The RT-sqPCR analysis showed upregulation of both ISR- and SAR-related genes in plants transformed with only the *ALDC* gene and inconclusive results for the other transgenic plants containing the *ALDC* and *BDH1* genes, and *BDH1* gene alone. In conclusion, van der Merwe noted that the expression of the *ALDC* gene alone was sufficient for induction of ISR in *Arabidopsis*.

1.5. Sugarcane in South Africa

The agronomic genus *Saccharum* (sugarcane) plays an important role in South Africa's economy. The crop belongs to the Poaceae family of grasses and is a C4 photosynthesizing perennial crop which is best suited to production in tropical to subtropical environments (Edwards and Huber, 2014). In South Africa, the crop is produced in Kwazulu-Natal, Mpumalanga and the Eastern Cape (Department of Agriculture, Forestry and Fisheries, 2014). The crop is processed and utilised for both human consumption and for the production of biofuels. Worth over fourteen billion Rand, South Africa's sugarcane industry ranks in the top fifteen of the 120 sugarcane-producing countries world-wide (https://sasa.org.za/, Accessed 01/07/2019). Approximately 85 000 individuals are employed directly by the industry, with 350 000 more employed indirectly. To put these statistics into perspective, this means that around 2% of the South African population is dependent on the sugarcane industry for a living (https://sasa.org.za/facts-and-figures/, accessed 01/06/2019).

With environmental conditions changing rapidly in South Africa, especially in the regions that grow sugarcane, there is a growing need for more resilient and climate-smart sugarcane cultivars. The recent drought, along with other economic factors, threatens to collapse the South African industry entirely (https://www.sacanegrowers.co.za/News/Article/24, accessed 02/06/2019). KwaZulu-Natal alone has suffered more than R2 billion worth of losses in the industry as a result of a three year long drought the region has been experiencing from late 2016. For a dryland production crop such as sugarcane, drought is one of the most important yield limiting environmental factors impacting the industry. Plant stress due to drought causes yield losses due to its negative impact on various physiological processes (Silva *et al*., 2007). Processes such as photosynthesis, respiration, stomatal conductance and electron transport are all yield-limiting factors affected by water stress (Zhang *et al*., 2001). The stages of sugarcane growth where water availability is most critical are during tillering,

grand growth and maturation, as 70-80% of the sugarcane yield is established during these phases (Ramesh, 2000). Assessing the physiological response of sugarcane to water stress during these growth stages is one way in which drought tolerant sugarcane lines can be established. Physiological indicators such as chlorophyll fluorescence, chlorophyll content, leaf temperature and relative water content have all successfully been used to indicate various degrees of water stress and tolerance in sugarcane (Silva *et al*., 2007).

A number of other factors, such as cheaper sugar imports, are also starting to impact on the economic sustainability of the local sugarcane industry (https://www.sacanegrowers.co.za/News/Article/26, accessed 19/08/2019). Other sugar producing countries, which are not experiencing the drought pressures that South Africa is, and are also free from the South African health promotion levy, Revenue Laws Act, 2017 - Act No. 14 (Obtained from: https://www.sars.gov.za/ClientSegments/Customs-Excise/Excise/Pages/Health%20Promotion%20Levy%20on%20Sugary%20Beverages.aspx), also known as the "Sugar Tax", are able to produce sucrose at a lower cost. In order to remain competitive with the international market, sugar and sugar-related product prices in South Africa are falling to levels that are not sustainable in the long term (https://mg.co.za/article/2017-09-22-00-sas-sugar-industry-under-assault, accessed 19/08/2019). Since the introduction of sugar taxation, the industry has lost approximately R1,3 billion in revenue. Furthermore, it has caused local demand for sugar by both manufacturers and consumers to fall, with demand for healthier sugar alternatives on the rise. The resultant sugar surplus of 200 000 tones to date was then exported for a R5000 loss in revenue per ton (https://www.sacanegrowers.co.za/News/Article/24, accessed 19/08/2019).

Both the environmental and economic stresses within the industry have created a growing demand for more drought-tolerant, higher yielding, sugarcane cultivars that will enable a more climate-smart sugarcane industry to evolve in South Africa. Alternative uses for sugarcane are also being explored, such as ethanol production and bagasse-based packaging materials.

1.6. The Sugarcane Genome

Sugarcane used in agriculture today is a polyploid, interspecific hybrid between *Saccharum officinarum* and *Saccharum spontaneum.* The genome is extremely complex, 2n=8x=80 (where n is the haploid chromosome number and x is the monoploid chromosome number of this polyploid organism), which sets it apart from other agricultural species (Zhang *et al*., 2018). D'Hont *et al.* (1996) and Cuadrado *et al.* (2004) have used genomic *in situ* hybridization (GISH) to determine that modern cultivars contain around 15-20% *S. spontaneum* and 75-80% *S. officinarum* chromosomes,

with less than 5% being recombinant or translocated chromosomes. In the past, this complexity has made genetic research on sugarcane prohibitively difficult. A comparative mapping study of modern sugarcane, performed by Aitken *et al.* (2014), confirmed a genome-wide collinearity with sorghum. Thus, since 2014, sorghum, with its less complex genome, has been used as a model organism for sugarcane (Garsmeur *et al*., 2018). Recently, however, the sugarcane genome has been sequenced (Garsmeur *et al*., 2018; Zhang *et al*., 2018). This has allowed rapid breakthroughs in gene annotation and the population genetics of modern sugarcane. With the now rapidly growing database of genetic information on sugarcane, steps can be taken to selectively improve its agronomic performance using modern biotechnological techniques.

1.7. Improving Sugarcane Using Biotechnological Techniques

Sugarcane production continues to grow throughout the world. However, with more demand every day for sugarcane-related products and not enough arable land to support its cultivation, supply cannot keep up with demand. This has led to a need for more efficient, higher yielding sugarcane cultivars. Unfortunately, due to genome complexity, the lack of genetic variation available, poor fertility and the extensive time required for elite cultivar breeding, it is largely accepted that traditional breeding can no longer effectively introduce new, favourable traits into commercial sugarcane cultivars (Mariotti, 2001; Lakshmanan *et al*., 2005). It is, however, possible to overcome the shortfall of traditional breeding by using biotechnological methods, namely, genetic engineering, marker-assisted selection and mutagenesis. These methods, which may also be used in a complementary manner, are able to dramatically increase the productiveness of a selective breeding program. The establishment of an effective biolistics-mediated transformation protocol by Bower and Birch (1992) was the first notable step forward towards an integrated molecular breeding program for sugarcane. The method established by Bower and Birch (1992) allows for the insertion of transgenes into the sugarcane genome, making it possible to introduce favourable agronomic traits that would otherwise have been impossible to introduce through traditional breeding methods. Marker-assisted selection then allows these transgenes to be quickly and effectively integrated into the sugarcane genome whilst maintaining all other agronomic traits of interest.

Genetic engineering for increased overall sugar yield has proved to be complex and resulted in limited success (Wu and Birch, 2007). Sugar yield in sugarcane is a complex trait, under the control of multiple biochemical pathways and genes. As such, it is expected that the altering or addition of single genes in such a complex genome will yield limited, if any, success. Predominantly, enzymes involved with sugar production and accumulation are targeted for manipulation, for example, the

transformation of sugarcane with sucrose 1-fructosyltransferase by Nicholson (2007), which lead to a small increase in sucrose content. To date, there has only been one commercial release of a transgenic sugarcane line in Indonesia (Noguera *et al*., 2015). This drought tolerant sugarcane line expresses the choline dehydrogenase (*betA*) enzyme which causes the accumulation of glycine betaine, a membrane protectant was developed by PT Perkebunan Nusantara, alongside the Indonesian Sugarcane Plantation Research Centre and the University of Jember.

Another important biotechnological method employed for the improvement of sugarcane is induced mutagenesis. This entails the random mutation of the sugarcane genome followed by extensive selection for beneficial traits (Haughn and Somerville, 1986). Mutagenesis is useful as it provides a method for altering genetic code while bypassing stringent regulations and restrictions involved with transgenic organisms. Mutations can be induced chemically or physically. Physical mutagenesis tends to yield high chromosome aberration frequencies, large scale deletions and lead to large sections of non-repairable DNA damage (Parry *et al*., 2009). On the other hand, chemically-induced mutagenesis tends more towards the induction of single nucleotide DNA base pair mutations. For this reason, chemical mutagenesis is more useful in crop improvement as it results in lower mortality rates and is easier to administer to samples (Koch *et al*., 2012). For example, Koch and colleagues (2012) used ethylmethane sulfonate (EMS) to create sugarcane with herbicide resistance to imazapyr.

While these biotechnological techniques have been around for a number of years, they have only recently been employed, to any degree of success, on sugarcane. More research is required to optimise these processes and techniques for sugarcane as our knowledge and understanding of its genome evolves.

2. Project Rationale and Aim

South Africa is presently suffering from a debilitating drought, which has already caused a significant decline in agricultural production and substantial losses to the South African economy. This research aims to pave the way for creating enhanced agricultural productivity and yield, using a novel strategy involving the transgenic expression of volatile growth-promoting compounds in sugarcane.

Enhanced growth alone would be beneficial to the agricultural industry, however, previous research has also suggested that these specific transgenic plants may be more tolerant to saline conditions (Dempers, 2015). Since salinity is closely linked to water availability and drought, this means that these plants may also have increased drought tolerance (Bartels and Sunkar, 2007). This research thus offers several possibilities for economic impact in the agricultural sector in South Africa. The novel strategy of using transgenes to express volatile growth enhancing compounds *in planta* could lead to the enhancement of crop yields via plants which grow larger and have enhanced resistance to both abiotic (e.g. drought and salinity) and biotic (pathogen) stress, with a reduced need for costly chemical inputs.

In this project, the specific aim was to enhance sugarcane growth and drought tolerance through the incorporation of transgenes α-acetolactate decarboxylase (*ALDC*) and 2,3-butanediol dehydrogenase (*BDH1*) into sugarcane NCo310. In order to achieve this aim, a number of key objectives needed to be fulfilled. Previous research findings on *ALDC* and *BDH1* transformed *Arabidopsis* needed to be confirmed, followed by the *in vitro* testing of untransformed sugarcane exposed to synthetic acetoin to asses growth responses in relation to those previously observed in *Arabidopsis*. Sugarcane was also transformed, by means of particle bombardment, with the *ALDC* and *BDH1* transgenes with subsequent screening for transgene incorporation and expression. The selected sugarcane transformants were then used in *in vitro* growth trials as well as *ex vitro* potted drought trials.

3. Methods and Materials

3.1. Generation and Testing of Transgenic *Arabidopsis thaliana*

3.1.1. Plant Growth and Culturing Conditions

Arabidopsis thaliana Columbia-0 (Col-0) seeds were sown onto pure peat jiffy-7® disks (Jiffy Products International BV, Netherlands) and cold stratified in the dark for 48 h at 4°C before being placed into the growth room. The growth room had a diurnal temperature range of 24 ± 4°C during the day to 19 ± 4°C at night. Plants where grown under LED tubes (Philips GreenPower TLED 20W HB) at 50 µMol photons/cm^2/s with a short day photoperiod (10:14 h day:night). Plants were watered by sub-irrigation and fertigated every 2 weeks with 9 g/L Phostrogen (Solabiol, United Kingdom). The short-day length photoperiod was used to maintain plants in the vegetative growth stage until flowering was desired. To initiate flowering, the plants were moved to a Snijders Labs (Tilburg, Netherlands) Economic Lux climate chamber. The chamber was maintained at day:night temperatures of 23°C:19°C and a 14:10 h day:night photoperiod to stimulate flowering. Osram, 6500 kelvin fluorescent tubes were used within the growth chamber to achieve a light intensity of at 50 µMol photons/cm^2/s.

For *in vitro* growth, *Arabidopsis thaliana* seeds were surface-sterilised by soaking in 2 mL of 70% (v/v) ethanol for 5 min, followed by 5 min in 2 mL of 1.75% (w/v) sodium hypochlorite containing 10 µL Tween-20, after which the seeds were rinsed twice in sterile ddH_2O. The surface-sterilised seeds were then spread onto 2.2 g/L (w/v) ½ Murashige and Skoog (MS) Basal Medium with vitamins (Murashige and Skoog, 1962), pH 5.7, solidified with 9.0 g/L Phyto Agar (Duchefa Biochemie, Netherlands). These plates were then placed at 4°C in the dark for 48 h for stratification, thereafter the plates were placed in a growth room at 25 ± 2°C with a 16 h:8 h light:dark photoperiod, using fluorescent tubes of light intensity 100 µMol photons/cm^2/s, for germination and growth.

3.1.2. Bacterial Growth Conditions

Escherichia coli DH5α cultures were used for plasmid multiplication and diagnostics. The *E. coli* cultures were grown at 37 ± 1°C in a temperature controlled incubator on Luria-Bertani (LB) media (10 g/L [w/v] peptone; 5 g/L [w/v] yeast extract; 10 g/L [w/v] sodium chloride) plates solidified with 15 g/L (w/v) bacto-agar or in liquid LB media broth. Liquid LB media cultures were continually shaken

at 250 rpm within the incubator. *Agrobacterium tumefaciens* GV3101 cultures were also grown on both solid and in liquid LB media containing 25 μg/mL (w/v) geneticin G418 and 50 μ/L (w/v) rifampicin. *Agrobacterium* cultures were kept at 28°C. Most cultures, for both *A. tumefaciens* and *E. coli*, were allowed to grow overnight.

3.1.3. Plasmid DNA Extraction from Transformed *E. coli* and *Agrobacterium* Cultures

Plasmid DNA was extracted from transformed bacterial cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison Wisconsin, USA) and performed as per the manufacturer's instructions.

3.1.4. Heat Shock Transformation of Chemically Competent *Agrobacterium*

Plasmid DNA was extracted from separate *E. coli* cultures containing the pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* plasmids using the plasmid extraction method mentioned above. 50 μL of previously prepared competent *Agrobacterium* GV3101 cells were mixed with 5 μL of 100 ng/μL plasmid DNA and incubated on ice for 30 min. The cell mixtures were heat shocked by incubating at 42°C for 45 sec followed immediately by incubation on ice again for two min. 250 μL of liquid LB media without any antibiotics was then added directly to the transformation mixture, followed by incubation at 28°C with shaking (250 rpm) for 45 min. During this incubation step solid LB media plates containing 25 μg/mL (w/v) geneticin and 50 μ/L (w/v) rifampicin as well as 50 mg/L (w/v) kanamycin for selection of pCambia2300::*FNR*:*ALDC* or 50 mg/L (w/v) hygromycin for selection of pCambia1300::*FNR*:*BDH1* containing bacteria, were removed from cold storage and incubated at 28°C. After incubation for 45 min, 50 μL of the relevant transformation mixture was plated out onto the incubated LB plates containing the appropriate antibiotics for selection.

3.1.5. *Agrobacterium*-Mediated Floral Inoculation Transformations

Arabidopsis thaliana Col-0 plants were transformed using a modified protocol for *Agrobacterium*-mediated floral inoculation transformations established by Narusaka *et al.* (2010). Separate 25 mL *Agrobacterium* cultures containing the pCambia2300::*FNR*:*ALDC* and the pCambia1300::*FNR*:*BDH1* plant transformation vectors (Dempers, 2015) were established in liquid LB media supplemented with 25 μg/mL (w/v) geneticin and 50 μ/L (w/v) rifampicin. 50 mg/L (w/v) kanamycin was added to select for both the pCambia2300::*FNR*:*ALDC* pCambia1300::*FNR*:*BDH1*

This was performed to ensure the growth of only bacteria containing the desired plasmids. After overnight incubation, 2 mL of each of the 25ml cultures were centrifuged at 12000x*g* for 2 min in a microcentrifuge and the supernatant discarded. The pelleted cells were then resuspended in 5% (w/v) sucrose containing 0.02% (v/v) Silwet L-77 to create the floral inoculation mixture. Immediately after resuspension, 2.5 μL of the mixture was used to inoculate each flower bud, by pipetting directly onto it. Care was taken to inoculate floral buds at the correct developmental stage, approximately 4 days pre-bud break. For the double transformation event, 1 mL of each culture was resuspended as mentioned above, before being combined into a single transformation mix. Care was taken to keep plants separated that were inoculated with the different plasmid strains. The separated plants were covered with a darkened humidity dome to maintain humidity and to exclude light for 24 h. Through the course of flowering, new buds were continually produced, these were also inoculated, along with the re-inoculation of the previous buds at seven-day intervals. During and after the inoculation period, the plants were grown in the Economic Lux (Snijders Labs) climate chamber under the conditions previously described. Mature siliques were collected and allowed to dry completely before the seeds were selected for positive transformants using appropriate antibiotics.

3.1.6. Selection for Transformed *A. thaliana* Lines

For selection of putatively transformed *A. thaliana plants*, surface-sterilised seeds were spread onto ½ MS media, prepared as described earlier, with the addition of appropriate antibiotics. To eliminate *Agrobacterium* from the seeds, 100 mg/L carbenicillin was used, along with 50 mg/L of kanamycin for selection of pCambia2300::*FNR*:*ALDC* putative transformants and 25 mg/L of hygromycin for the selection of pCambia1300::*FNR*:*BDH1* putative transformants. To select for double transformation events in seeds from plants transformed with both plasmids, a combination of 25 mg/L kanamycin and 25 mg/L hygromycin were used. After 14-21 d of growth on the selection media, putative transformants were easily identified at the two-leaf stage. Putative transformants displayed significantly increased growth when compared to non-transformants as well as remaining unbleached in the case of kanamycin selection and exhibiting a more prolific root development in the case of hygromycin selection. The putative transformants were then carefully removed and placed onto water-saturated |Jiffy-7® disks (Jiffy Products International BV, Netherlands), which were in turn placed into 1 L closed containers to maintain high humidity. The containers were slowly opened over a period of seven days, giving the plants time to fully adapt to conditions and to develop the protective cuticle layer. Once the plants had been fully hardened off, they were grown as described above. Once the plants were large enough, tissue samples were collected for DNA and RNA extractions and polymerase chain reaction (PCR) confirmation of transgene presence and expression.

3.1.7. DNA Extraction from *A. thaliana* Leaf Tissue

Crude genomic DNA extraction was performed using a modified protocol established by Lu (2011). Three small leaves were removed from the desired plants and ground in Edwards extraction buffer (200 mM Tris-HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% [w/v] SDS) in a microfuge tube using a sterilised plastic pestle. The homogenised sample was then vortexed and centrifuged at 14000 *x*g for 2 min. Next, 300 μl of the supernatant was removed and replaced with 300 μl of isopropanol, before the mixture was mixed and incubated at room temperature for 2 min. After incubation, the sample was spun at 15 000 *x*g for 5 min, the supernatant was discarded, and the pellet washed twice with 70% (v/v) ethanol. The pellet was left to dry before being resuspended in 100 μl ddH_2O. Genomic DNA was quantified using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific). Samples were stored at -20°C until needed.

3.1.8. Confirmation of Transgene Insertion and Expression Using PCR

Putatively transgenic *A. thaliana* plants were tested for transgene incorporation using transgene-specific primers (Table 3.1) in a PCR reaction. PCRs were performed using DNA samples extracted using the protocol described above. Amplification of specific DNA fragments was performed using the GoTaq™ DNA Polymerase kit (Promega). Amplification reactions were set up as detailed in Table 3.2. PCR reaction conditions (Table 3.3) were created using a T100™ Thermal Cycler (Bio-Rad). Amplified DNA fragments were separated and visualised by gel electrophoresis. A 12 μL aliquot of the PCR reaction was loaded onto a 1% (w/v) agarose gel (1% [w/v] agarose, 0.5% TBE buffer [5.4 g/L Tris, 2.75 g/L boric acid, 0.465 g/L EDTA; pH 8]) stained with 24 μL/L ethidium bromide (10 mg/mL) and electrophoresed, submerged in 0.5x (v/v) TBE buffer, at 110 volts for 45 min. The gel was then visualised under ultraviolet light by using a G:BOX gel documentation system (Syngene, SDI Group, Cambridge, UK)

Table 3.1: Primer sequences for the specific amplification of *ALDC* and *BDH1* transgenes.

Primer Name	Sequence	Product Size	T_m
ALDC_FW	5'-TATGGAGACATGGGTATCACA-3'	960 bp	70°C
ALDC_RV	5'-TAGTGAGAAGTGGGGACTCC-3'		74°C
BDH1_FW	5'-TTATGAGAGCTTTGGCATATTTCAAG-3'	1149 bp	67°C
BDH1_RV	5'-TTACTTCATTTCACCGTGATTGTTAG-3'		67°C

Table 3.2: Reagent mix used for the PCR amplification of transgenes *ALDC* and *BDH1.*

Component	Concentration	Volume
Green GoTaq™ Reaction Buffer	5 X	5.0 µl
dNTP Mix	10 mM	0.75 µl
Forward Primer	10 mM	0.75 µl
Reverse Primer	10 mM	0.75 µl
GoTaq™ DNA Polymerase	5 U/µl	0.1 µl
Template DNA	Variable	2.0 µl
Nuclease-free Water to		25 µl

Table 3.3: PCR reaction cycling conditions for the amplification of transgenes *ALDC* and *BDH1.*

PCR Step	Temperature	Time	No. of Cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	1 min	
Annealing	62°C	1 min	30
Extension	72°C	1 min	
Final Elongation	72°C	10 min	1

3.1.9. RNA Extraction and Complimentary DNA Synthesis from T2 Transgenic Plant Tissue

A modified cetyl trimethylammonium (CTAB) method was used for the extraction of total RNA from *A. thaliana* leaf tissue. Leaf tissue was cryogenically frozen in 1.5 mL microcentrifuge tubes and ground to a fine powder using an RNase-free plastic pestle. Ground samples were kept cryogenically frozen until all samples were prepared and ready for the extraction step. Once all tissue samples had been ground, 750 μL of extraction buffer (2% [w/v] CTAB; 2% [w/v] PVP; 100 mM Tris-HCl, pH 8.0; 25 mM EDTA; 2 M NaCl; 2% [v/v] β-mercaptoethanol) was added. The samples with extraction buffer were vortexed and incubated at 65°C for 30 min with occasional mixing by inversion. After incubation, the samples were centrifuged at 15 000 *x*g for 10 min in a microcentrifuge. An aliquot of 475 μl of the supernatant was transferred to a new microcentrifuge tube containing an equal amount of chloroform:isoamyl alcohol (24:1) and mixed by repetitive pipetting. Once again the samples were centrifuged at 15 000 *x*g and the chloroform:isoamyl alcohol extraction repeated. Finally, the supernatant was transferred to a clean microcentrifuge tube where 8 M LiCl was added to a concentration of 2 M and left to precipitate the RNA overnight at -20°C. After incubation, the samples were centrifuged at 15000 *x*g to pellet the RNA. The supernatant was decanted, and the pellet washed twice with 70% ethanol. The ethanol wash solution was removed, and the pellet allowed to air dry for 45 min before being resuspended in RNase-free water. RNA was quantified using a NanoDrop ND 1000 (Thermo Fisher Scientific) spectrophotometer before being treated with DNase (DNase 1, RNase-free Fermentas), according to the DNase manufacturer's instructions.

Complimentary DNA (cDNA) was synthesized from 1 μg DNase-treated total RNA, extracted as described above. Synthesis was performed according to the Maxima H minus first strand cDNA synthesis kit (Thermo Scientific) instructions, using oligo $(dT)_{18}$ primers as specified. The cDNA was then stored at -80°C until needed.

3.1.10. Sequencing of *ALDC* and *BDH1* Transgenes

All capillary electrophoresis sequencing and related reactions were performed by the Central Analytical Facility (CAF), Stellenbosch University. M13 primers and sequencing-specific primers (Table 3.4) were created for the sequencing of desired regions of the pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* plasmids (Dempers, 2015). The sequencing primers were designed to create overlapping fragments of +/- 400 bp in length of the transgenes. These fragments were then aligned using the Clustal Omega algorithm on Unipro UGENE (Unipro, Novosibirsk Russia). Alignments were compared to the original alignments performed by Dempers (2015) to the *ALDC* and *BDH1* reference sequences, An03g00490 and YAL060W*ALDC* respectively.

Table 3.4: Sequencing specific primers developed for capillary sequencing of pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* regions of interest.

Primer Name	Sequence	Annealing Temperature
ALDC_seq_F1	5'-ATGTATCTGCAACTGGGAAAAT-3'	58
ALDC_seq_F2	5'-GTCGAAGACGCGGATATAATT-3'	58
ALDC_seq_R1	5'-AGCGTGCCCTGTATATGTCT-3'	58
ALDC_seq_R2	5'-ATTAGAATGAACCGAAACCG-3'	58
BDH1_seq_F1	5'-GAATGTATCTGCAACTGGGAA-3'	58
BDH1_seq_F2	5'-GGTCCAATCTTCATGCCTAA-3'	58
BDH1_seq_F3	5'-GCAGTTCAGCCTTGGTTC T-3'	58
BDH1_seq_R1	5'-GTCTTCGACAACATAGCCGA-3'	58
BDH1_seq_R2	5'-ACC GGG ATA ATG TGA TGT TG-3'	58
BDH1_seq_R3	5'-CAG GTT TTT TAC AAC GTG CA-3'	58

3.2. *In Vitro* Growth Trial with Sugarcane Exposed to Synthetic Acetoin

Saccharum sp. Hybrid cv. NCo310 plantlets were regenerated from callus material. The selected callus tissue was placed onto MSC regeneration media (4.43 g/L Murashige and Skoog Basal Medium; 20 g/L sucrose; 0.5 g/L casein; 2.22 g/L gelrite; pH 6.0) within cell culture dishes. Once emergence of somatic embryos was observed and small shoots had appeared after approximately two weeks, the callus tissue with shoots was sub-cultured into larger sterile containers with fresh MSC media. After approximately six weeks of growth, with sub-culturing every second week, plantlets of the same size and with a similar degree of root development were selected and placed into separate Magenta jars for four days. For the growth trial, 80 mL of MSC media was poured into each of the 72 sterile containers and allowed to set. The same was done for 72 further sterile containers, this time with MC media (4.43 g/L MS, 0.5 g/L casein, 2.22 g/L gelrite; pH6.0). Four plantlets were individually weighed and spaced evenly with their positions recorded within each container. A 2.5 cm sterile petri dish was placed in the centre of each culture container (Figure 3.1). Four different treatment combinations were used in this trial, along with two controls. Two concentrations of acetoin were used, 200 ng and 2000 ng per tissue culture container. The acetoin dilutions were prepared fresh with ice-cold ddH_2O and kept on ice until required. The two treatments, 1 mL of 200 ng/mL and 1 mL of 2000 ng/mL of acetoin were applied to 24 containers each of both

MSC and MC media by pipetting the required volume directly into the small petri dish in the centre of the container. An equal volume of ddH_2O was used as a control and also applied to both MSC and MC media containers. After pipetting the treatment into each container, the container was immediately closed and sealed with two layers of parafilm to minimize the escape of the volatile acetoin. The containers were then placed in a Snijders Labs (Tilburg, Netherlands) Economic Lux climate chamber at 25°C with 14 h:10 h light:dark cycles using Osram, 6500 kelvin fluorescent tubes at 100 µMol photons/cm^2/s^1 for growth. After 30 days, the plantlets were carefully removed from the media, roots rinsed in ddH_2O, briefly blotted dry and weighed for calculation of relative growth.

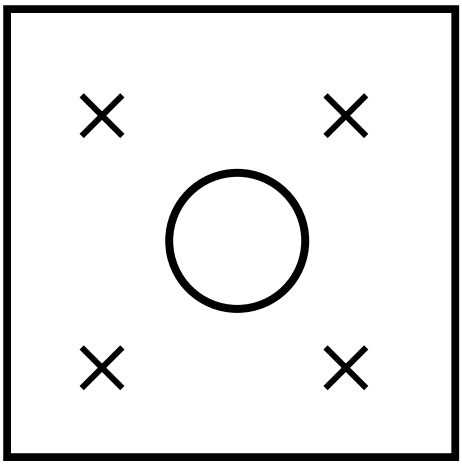

Figure 3.1: The layout in each container of plants (designated by an X) and the Petri dish (designated with an O) for the synthetic volatile growth trial.

3.3. Generation and Testing of Transgenic Sugarcane

3.3.1. Culturing Sugarcane Callus Tissue

Tissue for callus development was sourced from NCo310 sugarcane grown under glasshouse conditions on the Welgevallen Experimental Farm, Stellenbosch University, Stellenbosch. Mature stalk samples were cut and surface-sterilized before all leaf and old stalk material was removed from around the meristematic tissue. The remaining, axenic, meristematic tissue was cut into 2 mm thick disks and placed on MSC_3 media (4.43 g/L [w/v] MS basal medium; 20 g/L [w/v] sucrose; 0.5 g/L [w/v] casein; 3 mg/L [w/v] 2,4-dichlorophenoxyacetic acid; 2.22 g/L [w/v] gelrite; pH 6.0) for callus

induction. The meristematic tissue was left on the MSC_3 media, in the dark, at 26°C for callus induction. Regenerating callus was sub-cultured onto fresh MSC_3 media every two weeks.

3.3.2. Microprojectile Bombardment of Embryogenic Callus

Transformation of sugarcane was performed using a modified microprojectile bombardment protocol established by Bower and Birch (1992). Four days prior to bombardment, embryogenic callus samples were harvested and placed onto fresh MSC_3 media. Four hours prior to bombardment, the callus was transferred onto MSC_3Osm media (MSC_3 with the addition of 0.2 M sorbitol; 0.2 M mannitol). The callus was placed in a clump with a 2.5 cm diameter to maximise the microprojectile target area. During the 4 h waiting period, microprojectiles (0.7 micron tungsten particles; Bio-Rad, California, United States) were sterilised. Absolute ethanol was used to sterilise 5 mg of the tungsten microprojectile material, which was then washed in sterile ddH_2O to remove all ethanol. Once the wash steps were completed, the tungsten was suspended in 50 µl sterile ddH_2O. To the suspension, 5 µl of the pEmuKN (1 µg/µl [w/v]) helper plasmid containing the *nptII* (neomycin phosphotransferase) selectable marker gene was added, along with 10 µl of 1 µg/µl (w/v) transgene-containing plasmid (pUBI510::*FNR*:*ALDC* and/or pUBI510::*FNR*:*BDH1*)(van der Merwe, 2016). Next, 50 µl of filter-sterilised 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine was also added. The bombardment mixture was kept on ice throughout its preparation and until it was used for bombardment. A locally fabricated, 1000kPa helium gas projectile accelerator was used in conjunction with an 80 kPa vacuum chamber within which the target callus tissue was placed for bombardment. Callus tissue was bombarded with pUBI510::*FNR*:*ALDC* alone (*ALDC* lines) as well as with both pUBI510::*FNR*:*ALDC* and pUBI510::*FNR*:*BDH1* combined for double transformation (AB lines). Three hours post-bombardment, the callus was removed from the MSC_3Osm media and placed onto MSC_3 callus growth media. After 2 d on growth media, the callus was transferred to MSC_3 selection media containing 50 mg/L geneticin. Selection lasted approximately 8 weeks in the dark at 26°C, with sub-culturing onto fresh selection media every two weeks. Putative transgenic plantlets were regenerated from surviving callus clones on MSC media under 25 ± 2°C with a 16 h:8 h light:dark photoperiod, using fluorescent tubes of light intensity 100 µMol photons/cm^2/s^1. Putative transgenic clones were encouraged to develop shoots by regular sub-culturing into larger containers with a larger volume of MSC media.

3.3.3. Confirmation of Transgenic Sugarcane Lines

Confirmation of putative transformed sugarcane was accomplished through PCR amplification using the *ALDC* and *BDH1* gene-specific primers and the same PCR conditions as mentioned in section 3.1.8. Gene expression was also confirmed for transgenic sugarcane lines using cDNA synthesised from total RNA, extracted as previously described in section 3.1.8.

3.3.4. *In Vitro* Growth Trial of Transgenic Sugarcane

Plantlets from confirmed single transgenic lines (ALDC2, ALDC3, ALDC10) and double transformed transgenic lines (AB2, AB3, AB5) were grown and selected for this trial in the same way as for the wild type plantlets used for the synthetic acetoin growth trial (section 3.2). A total of 30 plantlets from each line were weighed before being divided into six sterile containers with 80 mL of MSC media. Untransformed NCo310 plants were used as the control, where 30 of these plantlets were placed into six containers, again with 80 mL of MSC media. All containers were then placed inside a Snijders Labs (Tilburg, Netherlands) Economic Lux climate chamber at 25°C with 14 h:10 h light:dark cycles and left to grow for 30 d. After the trial period, the plantlets were removed from the media, briefly blotted dry and weighed for calculation of relative growth.

3.4. Transgenic *ALDC* Sugarcane *Ex Vitro* Water Stress Trial

3.4.1. Selection and Preparation of *Ex Vitro* Plants

For the *ex vitro* study, three transgenic lines (ALDC2, ALDC3, ALDC10) were tested against wild type NCo310. For each line, 18 plants of similar size and physiological age were removed from tissue culture conditions and planted into 22 cm pots placed under glasshouse conditions with 25 ± 10°C daylight. The soil media used in the pots consisted of a ratio of 2:1:1 palm-peat:silica-sand:vermiculite, each pot was weighed to ensure that they contained the same total weight of water saturated media (3 kg). The potted plants were hardened off over a period of two weeks, where the humidity was slowly reduced to ambient levels, using incremental adjustments of humidity domes. Hygrotech Generic fertiliser (Hygrotech Stellenbosch) was applied every two weeks as the plants grew. When the first internode was visible just above soil level (approximately three months of growth), irrigation was removed, and the plants moved into a dedicated growth room where the water stress trial took place.

3.4.2. Water stress Conditions and Experimental Layout

Water stress conditions were simulated inside a large growth room, under 14 h:10 h light:dark artificial lighting (combination of metal halide and high pressure sodium bulbs) and strong airflow. The plants were arranged according to a randomized plot design. At the start point of the experiment, all pots were manually watered until saturation, after which all watering was stopped until the end of the drought trial. Soil moisture was recorded in duplicate, on a randomly selected sample of three potted plants per line, on days 0, 3, 6, 9, 12 and 15, without water (ww) using a ProCheck soil moisture probe (Decagon Devices Inc, Pullman, USA). For each pot measured, the probe was inserted twice, to a depth of 8 cm each time. To ensure the random selection of pots for all data collection, pots were designated a number in order of appearance within the randomised plot design. Using a randomized selector in Excel, three numbers were selected per line, with the three corresponding pots used for non-destructive data measurements.

3.4.3. Recording Stomatal Conductance and Chlorophyll Fluorescence

Non-destructive analysis of stomatal conductance and chlorophyll fluorescence were also performed on the same randomly selected plants used for soil moisture measurement, as described above. Stomatal conductance was measured in triplicate, on the first visible dewlap leaf, using an SC-1 Leaf Porometer (Decagon Devices Inc, Pullman, USA) as per the manufacturer's recommendations. Measurements were taken from the midpoint of the bottom, middle and top third of the dewlap leaf. Stomatal conductance was expressed as the evapotranspiration rate in $mmol/m^2/s^1$. Chlorophyll fluorescence was also measured in triplicate, on the dewlap leaf, using an OS30p+ Chlorophyll Flourimeter (Opti-Sciences, Hudson, USA). As per the manufacturer's instructions, the area of leaf where the chlorophyll fluorescence was to be recorded was dark adapted for at least 30 min before the measurement was taken. This was ensured by using aluminium foil to cover the areas to be measured. Chlorophyll fluorescence was recorded as the normalised ratio of F_v/F_m where F_v represents the difference between maximum fluorescence and minimum fluorescence and F_m represents the maximum fluorescence.

3.4.4. Measurement of Relative Water Content (RWC)

Relative water content was calculated from samples of dewlap leaf tissue harvested from three randomly selected plants per line, on days 0, 7, 14 and 17 ww. Leaf samples of approximately 1.5 cm were cut from three sections (tip, middle and base) of each dewlap leaf. Each sample was immediately weighed to determine the fresh weight (FW) and placed in ddH_2O for 3 h. Next, the leaf

samples were blotted dry and weighed again to yield the full turgid weight (TW). The leaf samples were then individually bagged and placed in a drying oven at 80°C for seven days. The weight of the dried leaf samples (DW) was then recorded. RWC of each leaf sample was calculated using the formula laid out by González and González-Vilar (2001):

$$(FW-DW) / (TW-DW)*100$$

3.4.5. Reactive Oxygen Species (ROS) Assay

Levels of the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and the superoxide (O_2^-) anion were assessed visually through histochemical staining. Visual assessment was performed using 3,3-diaminobenzidine (DAB), and 4-nitroblue tetrazolium chloride (NBT) staining according to a modified protocol outlined by Kumar *et al.* (2014). H_2O_2 oxidises DAB in the presence of peroxidases to form a brown, insoluble precipitate, while O_2^- reacts with NBT to form formazan, which appears as a dark blue stain. The stains were performed in duplicate, using 1 cm sections of the dewlap leaf from each of the three randomly selected sample plants from each line, on days 0, 7, 14 and 17. The sections of leaf were placed into separate wells of a 12-well assay plate containing DAB stain solution (1.25 mg/mL DAB, dissolved in ddH_2O, pH 3.8; 1 μl/mL Tween-20) and NBT stain solution (3 mg/mL NBT dissolved in 10 mM potassium phosphate buffer pH 7;10 mM NaN_3; 1 μl/mL Tween-20). The leaf sections were kept submerged in the stain solution and placed under a vacuum of 80 kPa for 12 h. The leaf sections were then removed and rinsed in ddH_2O before undergoing bleaching by heating the leaf sections in 1:1:2 glycerol:acetic acid:ethanol bleaching solution until all chlorophyll had been removed. The stained leaf sections were then visualised and photographed with the aid of a backlit viewing box.

3.4.6. Catalase Assay

Catalase was extracted using a standard extraction buffer (0.1M potassium phosphate [pH7]; 1% [w/v] PVP; 0.1 mM EDTA) with the colorimetric catalase assay being performed using the Merck, CAT100 Catalase kit (Darmstadt, Germany). In order to standardise the extractions by sample weight, 10 μl of the extraction buffer was used per mg of leaf tissue. Catalase activity (Δ nmol/min/mg FW) was calculated using a standard concentration curve for the absorbance of the red quinoneimine dye versus the amount of H_2O_2 at standard concentrations of 0 mM, 1.25 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM (Annexure C). For the assay, only the dewlap leaf from each of the three randomly selected sample plants from each line, on days 0, 7, 14 and 17 was used for analysis. The leaf

material of each plant was ground to a fine powder under cryogenic conditions using a Retsch Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) before being used, as per the manufacturer's recommendations, for the determination of catalase activity. Absorbance was measured at 520 nm using a VersaMax ELISA Microplate Reader (Molecular Devices LLC, San Jose, California).

3.4.7. Superoxide Dismutase (SOD) Assay

SOD was extracted using a standard extraction buffer (0.1 M potassium phosphate, [pH 7]; 1% [w/v] PVP; 0.1 mM EDTA) and its activity determined using the colorimetric SOD determination kit by Merck (Darmstadt, Germany) which utilises a highly water soluble salt, WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium) which upon reduction by superoxide, forms a formazan dye. In order to standardise the extractions by sample weight, 10 µl of the extraction buffer was used per mg of leaf tissue. For the calculation of SOD activity, a standard concentration curve of 0.05-10 U/mL SOD was used (Annexure D), along with blanking reactions for each sample (Table 3.5). Plant leaf powder (prepared as above) was used, as per the manufacturer's recommendation, for each reaction. Absorbance at 450nm was measured using a VersaMax ELISA Microplate Reader (Molecular Devices LLC, San Jose, California). SOD activity was calculated from the absorbance readings using the formula:

$$\text{SOD activity (inhibition rate \%)} = ([A1-A3]-[AS-A2] / [A1-A3]) \text{ X } 100$$

where A1, A2 and A3 are the absorbance values of blanks 1, 2 and 3 which consist of differing combinations of reagents outlined in Table 3.5, and AS represents the absorbance of the sample.

Table 3.5: Differing reagent combinations used to create blanking solutions 1, 2 and 3 for the determination of SOD activity through a colorimetric assay.

	Sample	**Blank 1**	**Blank 2**	**Blank 3**
Sample Solution	X		X	
ddH_2O		X		X
WST Solution	X	X	X	X
Enzyme Solution	X	X		
Dilution Buffer			X	X

3.4.8. Chlorophyll Content

Chlorophyll content was determined using a protocol modified from Hiscox and Israelstam (1979). Frozen leaf tissue powder, prepared as described above, was used for chlorophyll extraction using 90% (v/v) acetone, samples were then vortexed until the powdered leaf material appeared colourless. Chlorophyll extractions were standardised for sample weight by adding 30 μl of 90% (v/v) acetone per mg sample. Absorbances of the chlorophyll extracts were measured at 645 nm and 663 nm using a VersaMax ELISA Microplate Reader (Molecular Devices LLC, San Jose, California). Chlorophyll content (μg/mg FW) was calculated using formulae first described by Arnon (1949):

$$\text{Chlorophyll A} = 12.7*A663 - 2.69*A645$$

$$\text{Chlorophyll B} = 2.90*A663 - 4.68*A645$$

And

$$\text{Total Chlorophyll} = 20.2*A645 + 8.02*A663$$

where A663 and A664 are the absorbance values for each sample at 663 nm and 645 nm respectively.

3.4.9. Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 8 for Macintosh (GraphPad Software, La Jolla, USA). Statistical significance ($p \leq 0.05$) was assessed using a one-way analysis of variance (ANOVA) where relevant. Where necessary this was followed by a two-way ANOVA with Brown-Forsythe and Welch multiple comparisons test and unpaired t test with Welch's correction where applicable (Brown and Forsythe, 1974; Welch, 1951).

4. Results and Discussion

4.1. Confirmation of Vector Sequences in Transgenic Bacterial Stocks

Glycerol stocks of *E. coli,* separately transformed with the pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* vectors, were previously prepared and stored at -80°C by Dempers (2015). Confirming the identity and sequences of the cloned transgenes was an important first step before attempting the transformation of *Arabidopsis* using these previously created vectors. Single-colony *E. coli* cultures were prepared from these glycerol stocks and used to confirm transgene presence via the use of transgene-specific primers (Table 3.1) via PCR (Figure 4.1). The sequence of interest within each vector was determined through capillary electrophoresis sequencing using sequencing primers (Table 3.4) designed to provide complete coverage over the length of the transgenes. The resultant sequences were aligned and used to obtain consensus sequences which were checked against the reference sequences An03g00490 and YAL060W*ALDC* for the *ALDC* gene (Figure 4.2) and *BDH1* gene (Figure 4.3) respectively.

PCR results with the transgene-specific primers confirmed the presence of both the *ALDC* and *BDH1* genes within the respective pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* vectors. Sequencing of the genes confirmed these findings, with both transgenes displaying complete sequence identity to their respective reference sequences.

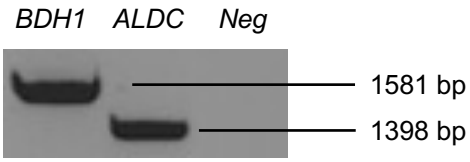

Figure 4.1: PCR results illustrating the presence of transgenes *ALDC* and *BDH1* within *E. coli* containing the pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* vectors.

```
Aspergillus niger    ATGGAGACATGGGTATCACAAGATATTTCGGCTGCGCGTTATCGGCGTGTTGCGTCAGTT 60
Concensus Seq (ALDC) ATGGAGACATGGGTATCACAAGATATTTCGGCTGCGCGTTATCGGCGTGTTGCGTCAGTT 60

Aspergillus niger    GATCTTGAGGGCGCTATCTTCTTCCGCTCCCTGATTGGTGCAGTGTTGGTACCTAAAAAG 120
Concensus Seq (ALDC) GATCTTGAGGGCGCTATCTTCTTCCGCTCCCTGATTGGTGCAGTGTTGGTACCTAAAAAG 120

Aspergillus niger    CCCAAAGGGCAAGCTGAAGTTTGTCGTATCTGTTTTAAGCAGGGACGAACCCATACAATA 180
Concensus Seq (ALDC) CCCAAAGGGCAAGCTGAAGTTTGTCGTATCTGTTTTAAGCAGGGACGAACCCATACAATA 180

Aspergillus niger    GCAATATCGTCCTCAGTTGATCCTCAATTCACCGCCTCCGTTATTGCTCCAAATACCAAC 240
Concensus Seq (ALDC) GCAATATCGTCCTCAGTTGATCCTCAATTCACCGCCTCCGTTATTGCTCCAAATACCAAC 240

Aspergillus niger    ATGGCAAACTGCATATACCAATACTCCGTGCTGGGAGCGCTCATGGACGGCATCTGTCAA 300
Concensus Seq (ALDC) ATGGCAAACTGCATATACCAATACTCCGTGCTGGGAGCGCTCATGGACGGCATCTGTCAA 300

Aspergillus niger    GATGGAACCACGGCGCAAAACATTCTCAAACATGGTGACCATGGCATCGGCACCATGCGC 360
Concensus Seq (ALDC) GATGGAACCACGGCGCAAAACATTCTCAAACATGGTGACCATGGCATCGGCACCATGCGC 360

Aspergillus niger    GGTCTCAACGGCGAACTAGTGATCATCGACGGGGTGGCATATCACTTCCCAGCGGACGGA 420
Concensus Seq (ALDC) GGTCTCAACGGCGAACTAGTGATCATCGACGGGGTGGCATATCACTTCCCAGCGGACGGA 420

Aspergillus niger    CCCCTACGCCCTGTCGAAGACGCGGATATAATTCCATACGCAATGGCTACTAAGTTCCAG 480
Concensus Seq (ALDC) CCCCTACGCCCTGTCGAAGACGCGGATATAATTCCATACGCAATGGCTACTAAGTTCCAG 480

Aspergillus niger    CCCACGCTGACCAGCCATGTTCCCTGCACCTCCATGTCGTCTCTATTCGACACGCTGTCA 540
Concensus Seq (ALDC) CCCACGCTGACCAGCCATGTTCCCTGCACCTCCATGTCGTCTCTATTCGACACGCTGTCA 540

Aspergillus niger    CCTGTATTTCCGGGTGAGCAGAACGTCTTCCTCTCGATTCGACTCGTTGCCTCCTTCTCG 600
Concensus Seq (ALDC) CCTGTATTTCCGGGTGAGCAGAACGTCTTCCTCTCGATTCGACTCGTTGCCTCCTTCTCG 600

Aspergillus niger    CGGGTCGTCTTTCGCGTGATTCCAGCCCAGTCAAACCCACGGGAAACCCTTTTAGATCTG 660
Concensus Seq (ALDC) CGGGTCGTCTTTCGCGTGATTCCAGCCCAGTCAAACCCACGGGAAACCCTTTTAGATCTG 660

Aspergillus niger    GCGAAAAGGCAGGAGATCCGAGAATGCAGACATATACAGGGCACGCTGTTCGGATTTTGG 720
Concensus Seq (ALDC) GCGAAAAGGCAGGAGATCCGAGAATGCAGACATATACAGGGCACGCTGTTCGGATTTTGG 720

Aspergillus niger    TCACCCAAGTATACCAGTGGGCTTAGCGTACCGGGATTCCACCTGCATTTGCTGTCCACG 780
Concensus Seq (ALDC) TCACCCAAGTATACCAGTGGGCTTAGCGTACCGGGATTCCACCTGCATTTGCTGTCCACG 780

Aspergillus niger    GATCGTACTTTGGGCGGCCATGTTATGGATTTTGATGCAGAGGATGGGCAGCTGGGTGCA 840
Concensus Seq (ALDC) GATCGTACTTTGGGCGGCCATGTTATGGATTTTGATGCAGAGGATGGGCAGCTGGGTGCA 840

Aspergillus niger    GCAGTGGTGAGAAATTATCAGGTGGAACTTCCTGATTCGGAGGAATTTCGCGAGGCACCC 900
Concensus Seq (ALDC) GCAGTGGTGAGAAATTATCAGGTGGAACTTCCTGATTCGGAGGAATTTCGCGAGGCACCC 900

Aspergillus niger    TTGAACTGCGTGAAGGAGCAAGAGCTGCACACTGCTGAGGGAGTCCCCACTTCTCACTAA 960
Concensus Seq (ALDC) TTGAACTGCGTGAAGGAGCAAGAGCTGCACACTGCTGAGGGAGTCCCCACTTCTCACTAA 960
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Figure 4.2: Sequence alignment between the *Aspergillus niger ALDC* reference gene An03g00490 (Labelled *Aspergillus niger*) and a consensus sequence generated from raw sequence data obtained from the region of interest within the pCambia2300::*FNR*:*ALDC* plasmid.

```
Saccharomyces cerevi ATGAGAGCTTTGGCATATTTCAAGAAGGGTGATATTCACTTCACTAATGATATCCCTAGG 60
Consensus Seq (BDH1) ATGAGAGCTTTGGCATATTTCAAGAAGGGTGATATTCACTTCACTAATGATATCCCTAGG 60

Saccharomyces cerevi CCAGAAATCCAAACCGACGATGAGGTTATTATCGACGTCTCTTGGTGTGGGATTTGTGGC 120
Consensus Seq (BDH1) CCAGAAATCCAAACCGACGATGAGGTTATTATCGACGTCTCTTGGTGTGGGATTTGTGGC 120

Saccharomyces cerevi TCGGATCTTCACGAGTACTTGGATGGTCCAATCTTCATGCCTAAAGATGGAGAGTGCCAT 180
Consensus Seq (BDH1) TCGGATCTTCACGAGTACTTGGATGGTCCAATCTTCATGCCTAAAGATGGAGAGTGCCAT 180

Saccharomyces cerevi AAATTATCCAACGCTGCTTTACCTCTGGCAATGGGCCATGAGATGTCAGGAATTGTTTCC 240
Consensus Seq (BDH1) AAATTATCCAACGCTGCTTTACCTCTGGCAATGGGCCATGAGATGTCAGGAATTGTTTCC 240

Saccharomyces cerevi AAGGTTGGTCCTAAAGTGACAAAGGTGAAGGTTGGCGACCACGTGGTCGTTGATGCTGCC 300
Consensus Seq (BDH1) AAGGTTGGTCCTAAAGTGACAAAGGTGAAGGTTGGCGACCACGTGGTCGTTGATGCTGCC 300

Saccharomyces cerevi AGCAGTTGTGCGGACCTGCATTGCTGGCCACACTCCAAATTTTACAATTCCAAACCATGT 360
Consensus Seq (BDH1) AGCAGTTGTGCGGACCTGCATTGCTGGCCACACTCCAAATTTTACAATTCCAAACCATGT 360

Saccharomyces cerevi GATGCTTGTCAGAGGGGCAGTGAAAATCTATGTACCCACGCCGGTTTTGTAGGACTAGGT 420
Consensus Seq (BDH1) GATGCTTGTCAGAGGGGCAGTGAAAATCTATGTACCCACGCCGGTTTTGTAGGACTAGGT 420

Saccharomyces cerevi GTGATCAGTGGTGGCTTTGCTGAACAAGTCGTAGTCTCTCAACATCACATTATCCCGGTT 480
Consensus Seq (BDH1) GTGATCAGTGGTGGCTTTGCTGAACAAGTCGTAGTCTCTCAACATCACATTATCCCGGTT 480

Saccharomyces cerevi CCAAAGGAAATTCCTCTAGATGTGGCTGCTTTAGTTGAGCCTCTTTCTGTCACCTGGCAT 540
Consensus Seq (BDH1) CCAAAGGAAATTCCTCTAGATGTGGCTGCTTTAGTTGAGCCTCTTTCTGTCACCTGGCAT 540

Saccharomyces cerevi GCTGTTAAGATTTCTGGTTTCAAAAAAGGCAGTTCAGCCTTGGTTCTTGGTGCAGGTCCC 600
Consensus Seq (BDH1) GCTGTTAAGATTTCTGGTTTCAAAAAAGGCAGTTCAGCCTTGGTTCTTGGTGCAGGTCCC 600

Saccharomyces cerevi ATTGGGTTGTGTACCATTTTGGTACTTAAGGGAATGGGGGCTAGTAAAATTGTAGTGTCT 660
Consensus Seq (BDH1) ATTGGGTTGTGTACCATTTTGGTACTTAAGGGAATGGGGGCTAGTAAAATTGTAGTGTCT 660

Saccharomyces cerevi GAAATTGCAGAGAGAAGAATAGAAATGGCCAAGAAACTGGGCGTTGAGGTGTTCAATCCC 720
Consensus Seq (BDH1) GAAATTGCAGAGAGAAGAATAGAAATGGCCAAGAAACTGGGCGTTGAGGTGTTCAATCCC 720

Saccharomyces cerevi TCCAAGCACGGTCATAAATCTATAGAGATACTACGTGGTTTGACCAAGAGCCATGATGGG 780
Consensus Seq (BDH1) TCCAAGCACGGTCATAAATCTATAGAGATACTACGTGGTTTGACCAAGAGCCATGATGGG 780

Saccharomyces cerevi TTTGATTACAGTTATGATTGTTCTGGTATTCAAGTTACTTTCGAAACCTCTTTGAAGGCA 840
Consensus Seq (BDH1) TTTGATTACAGTTATGATTGTTCTGGTATTCAAGTTACTTTCGAAACCTCTTTGAAGGCA 840

Saccharomyces cerevi TTAACATTCAAGGGGACAGCCACCAACATTGCAGTTTGGGGTCCAAAACCTGTCCCATTC 900
Consensus Seq (BDH1) TTAACATTCAAGGGGACAGCCACCAACATTGCAGTTTGGGGTCCAAAACCTGTCCCATTC 900

Saccharomyces cerevi CAACCAATGGATGTGACTCTCCAAGAGAAAGTTATGACTGGTTCGATCGGCTATGTTGTC 960
Consensus Seq (BDH1) CAACCAATGGATGTGACTCTCCAAGAGAAAGTTATGACTGGTTCGATCGGCTATGTTGTC 960

Saccharomyces cerevi GAAGACTTCGAAGAAGTTGTTCGTGCCATCCACAACGGAGACATCGCCATGGAAGATTGT 1020
Consensus Seq (BDH1) GAAGACTTCGAAGAAGTTGTTCGTGCCATCCACAACGGAGACATCGCCATGGAAGATTGT 1020

Saccharomyces cerevi AAGCAACTAATCACTGGTAAGCAAAGGATTGAGGACGGTTGGGAAAAGGGATTCCAAGAG 1080
Consensus Seq (BDH1) AAGCAACTAATCACTGGTAAGCAAAGGATTGAGGACGGTTGGGAAAAGGGATTCCAAGAG 1080

Saccharomyces cerevi TTGATGGATCACAAGGAATCCAACGTTAAGATTCTATTGACGCCTAACAATCACGGTGAA 1140
Consensus Seq (BDH1) TTGATGGATCACAAGGAATCCAACGTTAAGATTCTATTGACGCCTAACAATCACGGTGAA 1140

Saccharomyces cerevi ATGAAGTAA---------------------------------------------------- 1149
Consensus Seq (BDH1) ATGAAGTAA---------------------------------------------------- 1149
```

Figure 4.3: Sequence alignment between the *Saccharomyces cerevisiae* reference gene *YAL060WALDC* (Labelled *Saccharomyces cerevisiae*) and a consensus sequence generated from raw sequence data obtained from the region of interest within the pCambia1300::*FNR*:*BDH1* plasmid.

4.2. Transformation and Confirmation of Putative *Arabidopsis* Transformants

Arabidopsis plants were transformed using *Agrobacterium*-mediated floral inoculations. Each floral bud was inoculated at least twice in order to maximise the potential for transformation. Transformations were performed using the pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* plasmids separately, as well as in combination in order to obtain double transformants containing both genes. After maturation of siliques, seeds were harvested, surface-sterilized and placed on growth media containing the relevant antibiotics for selection of putative transformants. Putative transformants were removed from *in vitro* selection after reaching an adequate size and placed into *ex vitro* growth conditions where the plants were allowed to grow before tissue samples were taken for genotyping. The putatively transgenic T_1 plants were genotyped using a vector-specific M13 forward primer and gene-specific reverse primer to confirm integration of the transgene constructs into the plant genome. For the single transformation *FNR*:*ALDC* event, four putative transformants were visually identified during *in vitro* selection, of which all four were confirmed via PCR as containing the *FNR*:*ALDC* construct (Figure 4.4). For the single transformation *FNR*:*BDH1* event, again four plants were visually identified as being putative transformants. For this transformation event, only three of the four putative transformants were confirmed as *FNR*:*BDH1* transformants (Figure 4.5). Three putative transformants were successfully identified for the double transformation event with both *FNR*:*ALDC* and *FNR*:*BDH1* constructs, of which only two were confirmed as being true double transformants (Figure 4.6), with the third containing only the *BDH1* transgene. At this point, it was deemed unnecessary to repeat the above process using the forward gene specific primer as the amplification achieved using the reverse gene specific primer and forward M13 primer was sufficient for initial identity confirmation. Further sequencing was to be performed on the T_2 plants only to save cost.

For the purposes of this study, three independent, transgenic lines for each of the three transformation events was considered adequate for experimental use. The exception being made for the double transformant lines, of which only two lines were to be used. All experimentation was to be performed on T_2 plants, making a large T_2 seed yield important. Ordinarily transgenic *Arabidopsis* experimentation is performed using T_3 plants. This is due to the higher likelihood of obtaining transgenic plants homozygous for the transgene and higher seed yields per line. In this study, the use of T_3 plants was avoided due to the observed gene silencing of *ALDC* and *BDH1* genes by Dempers (2015). Considering this, T_1 plants were placed in ideal growth conditions in large pots and high light to encourage maximum seed production. The result of this was at least 1 mL of seed was collected from each of the T_1 lines and deemed sufficient for all experimentation.

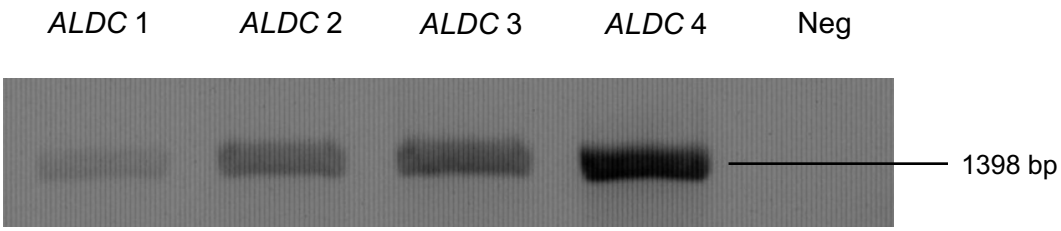

Figure 4.4: PCR results illustrating the presence of the *FNR*:*ALDC* construct within all four putative transformants along with a negative control (Neg) obtained from untransformed *Arabidopsis*.

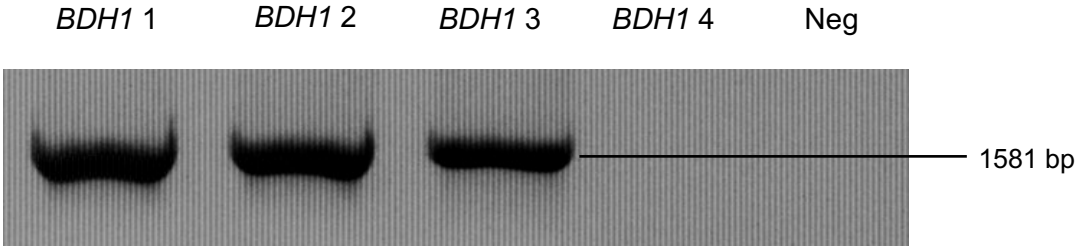

Figure 4.5: PCR results illustrating the presence of the *FNR*:*BDH1* construct within three of the four putative *BDH1* transformants along with a negative control (Neg) obtained from untransformed *Arabidopsis*.

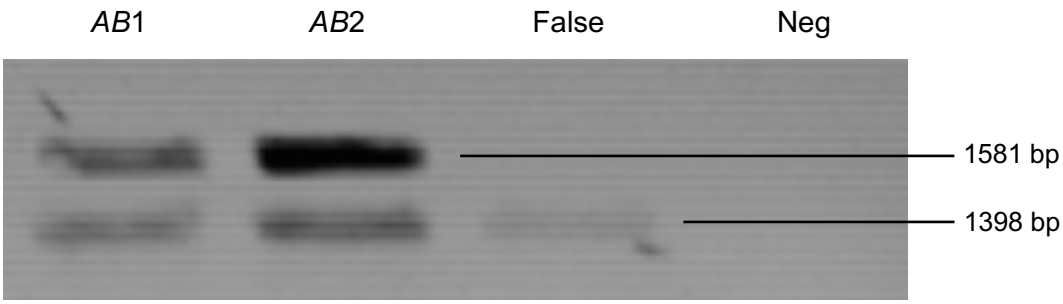

Figure 4.6: PCR results illustrating the presence of both the *FNR*:*ALDC* and *FNR*:*BDH1* constructs in two of the three identified putative double transformants. Also visualised is the falsely identified putative double transformant (False) and the negative control (Neg) obtained from untransformed *Arabidopsis*.

4.3. Testing for Transgene Presence and Expression in T2 *Arabidopsis* Lines

Genotyped T_1 plants were grown under conditions to maximise flowering and seed yield. Once the siliques had matured, T_2 seed was carefully collected from each T_1 line, with small amounts of seed from each line being surface-sterilised and germinated for genotyping and expression analysis. For both genotyping and expression analysis of the T_2 generation, the M13 forward primer previously used for the T_1 generation, was replaced with a gene-specific forward primer. Using this primer pair for *ALDC*, no amplification was achieved for any line previously confirmed, in the T_1 generation, as containing the *FNR*:*ALDC* construct. Only the *BDH1* gene amplified out in the single transformant *BDH1* lines and for the two double transformant lines (Figure 4.7). Following these results, the same primer pair was used in an attempt to amplify out the *ALDC* gene from DNA extracted previously from T_1 transformants. The same result was observed, with no amplification of the *ALDC* gene. Amplification was only achieved when using the *ALDC*_seq_F2 primer, situated further downstream of the *ALDC* gene-specific primer. No amplification could be achieved using any primer specific to the first ± 300 base pairs of the *ALDC* gene, suggesting that this region of the gene was not present in any of the *Arabidopsis* lines transformed with the *ALDC* gene. This was later confirmed by sequencing the area of interest (Figure 4.8), which showed that 240 bp were missing from the start of the *ALDC* gene (highlighted in red, Figure 4.8), at the end of the FNR leader sequence. The cause of the DNA deletion is unknown. Gene shearing due to freeze thaw cycles was considered, however, this is unlikely to have occurred in plasmid DNA and would have resulted in the linearization of the plasmid, rendering it non-compatible for transformation into *Agrobacterium*. Truncation from the T-DNA left border during ligation into the *Arabidopsis* genome was also considered. However, this hypothesis was also dispelled due to the confirmed presence of the *FNR* gene between the left border and the missing genetic material as well as the degradation of linearized plasmids when taken up by bacterial cells. The missing genetic material rendered all *Arabidopsis* lines transformed with the *ALDC* gene of no use for experimentation. Due to time constraints, repeated transformation of *Arabidopsis* plants could not be performed. This research line was abandoned in favour of an increased focus on transgenic sugarcane, transformed with the same *ALDC* and *BDH1* genes.

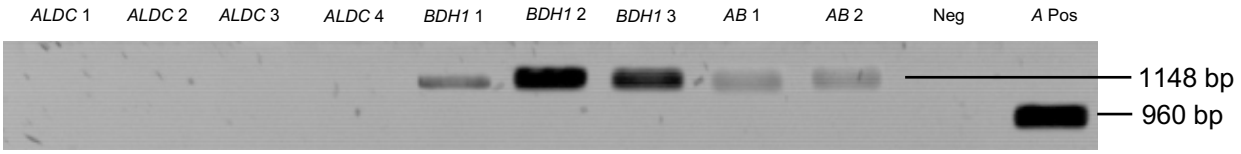

Figure 4.7: PCR results using gene specific primers for each previously confirmed T_1 transgenic lines (*ALDC* and *BDH1* single transformants with *AB* double transformants) as well as a positive control for the *ALDC* gene obtained from confirmed *E. coli* transformants (A Pos).

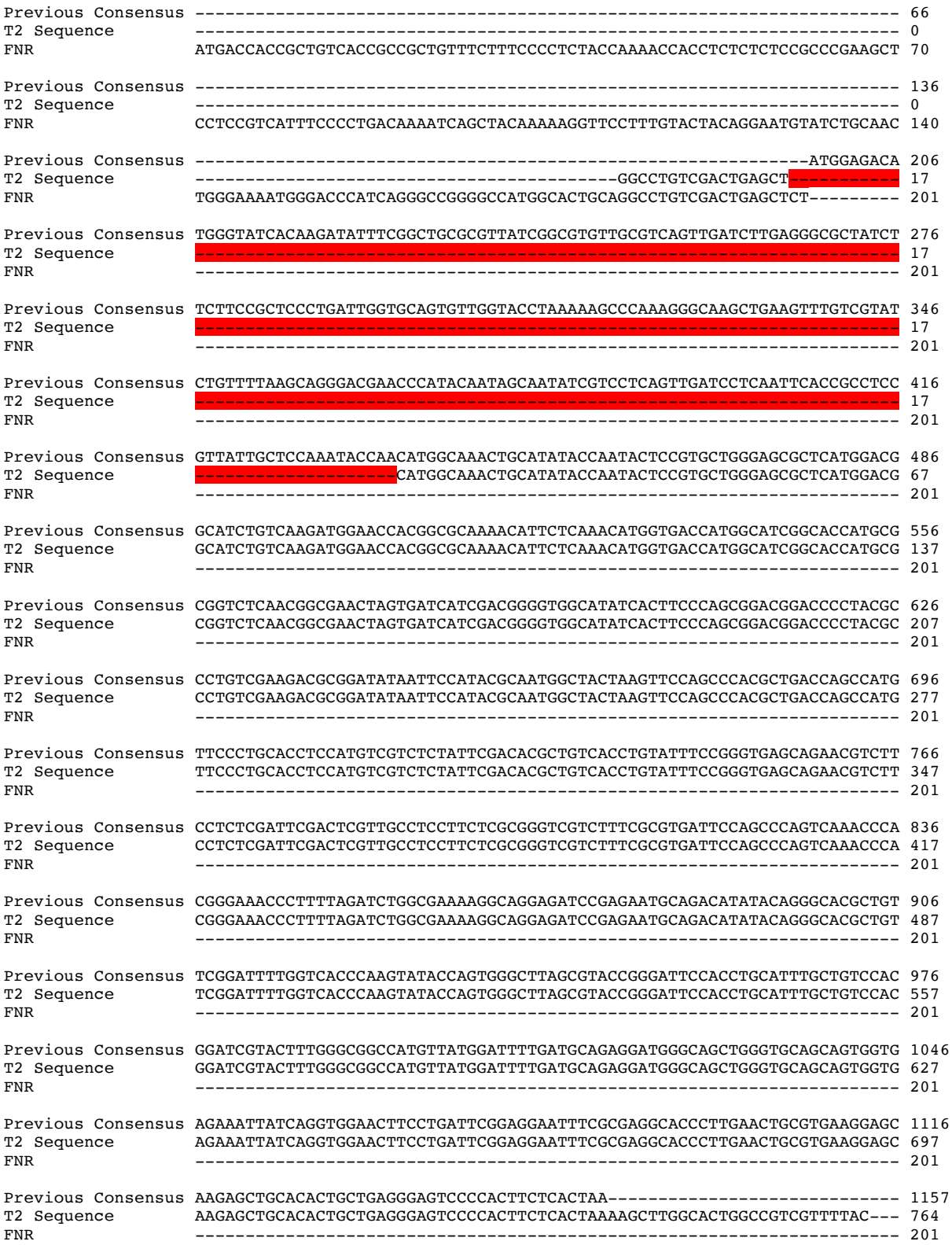

Figure 4.8: Multiple sequence alignment between the previously sequenced *ALDC* gene from pCambia2300::*FNR*:*ALDC*, *ferredoxin NADP*$^{+}$ *reductase* (*FNR*) and partial T2 *Arabidopsis ALDC* sequence. Highlighted in red, is the missing 240 bp sequence fragment from all T2 *Arabidopsis* lines containing the *ALDC* gene.

4.4. *In vitro* Growth Trial with Sugarcane Exposed to Synthetic Acetoin

In the previous study by Dempers (2015), it was reported that exposure to acetoin but not 2,3-butanediol lead to a significant increase in growth of *Arabidopsis*. Furthermore, a significant increase in *Arabidopsis* growth was only noted when plants were exposed to acetoin under long day-length conditions (Dempers, 2015). Dempers noted that acetoin has been shown to regulate a number of genes also involved with day length sensing in plants (Wang *et al*., 2003) and this could explain the increased growth under long day length conditions. As a result of these findings, and the fact that *in vitro* sugarcane culture conditions involve a long day-length, only the volatile compound acetoin was used to test for growth promotion in wild type NCo310 plantlets. The two different treatment concentrations of acetoin (200 ng and 2000 ng) were calculated according to the optimum concentrations established by Dempers and relative to the increase in volume of the *in vitro* plant culture containers used for sugarcane. Sucrose is typically added to plant culture media to counteract the effects of low carbon dioxide concentrations within *in vitro* containers, as well as suboptimal levels of available light (Eckstein and Zie, 2012). This exogenous sucrose can further limit photosynthesis in the plantlets (Van Huylenbroeck and Debergh, 1996). However, it has been shown that bacterial volatiles may at least partly influence plant growth through the regulation of photosynthesis (Sharifi and Ryu, 2018), thus adding sucrose to the growth trial media could potentially reduce the effect of acetoin on plant growth. Consequently, both the acetoin treatments as well as the water negative control were applied to plants on media both with and without sucrose.

The results of this growth trial (Figure 4.9) were inconclusive as no significant differences in relative growth were observed between acetoin treatments on either media containing or devoid of sucrose. However, there was, as expected, a significant difference in relative growth between plants grown with and without sucrose, which could provide a possible explanation as to why no significant growth promotion was observed with the addition of acetoin *in vitro*. As mentioned before, it is likely that increased plant growth due to acetoin exposure involves photosynthesis (Sharifi and Ryu, 2018). This was also hypothesised by Dempers (2015) when considering results of *in vitro* growth trials using *Arabidopsis* exposed to acetoin. In the case of Dempers' trial, however, a significant increase in growth was observed among *Arabidopsis* plants exposed to acetoin and grown on culture media not containing sucrose. The difference between *Arabidopsis* and sugarcane is that the slower and smaller *Arabidopsis* plants utilise less carbon dioxide within *in vitro* conditions, in addition to requiring significantly less light for photosynthesis. Thus, it is likely that, for *Arabidopsis*, photosynthesis is not limited to the same extent by utilizing culture media which does not contain sucrose. In other words, when exposed to acetoin *in vitro*, *Arabidopsis* plants grown without sucrose may still be able to display an upregulation in photosynthesis without being limited by carbon dioxide or light availability.

To resolve this, when performing growth trials using sugarcane, larger *in vitro* containers could be used, or containers opened and resealed at least once during the trial to increase CO_2 content within the culture containers. However, if culture containers were opened during the trial, VOC treatments would need to be reapplied in order to maintain plant exposure to VOCs. Higher output horticultural lighting could also be used to provide sufficient light for high light intensity-dependent species such as sugarcane.

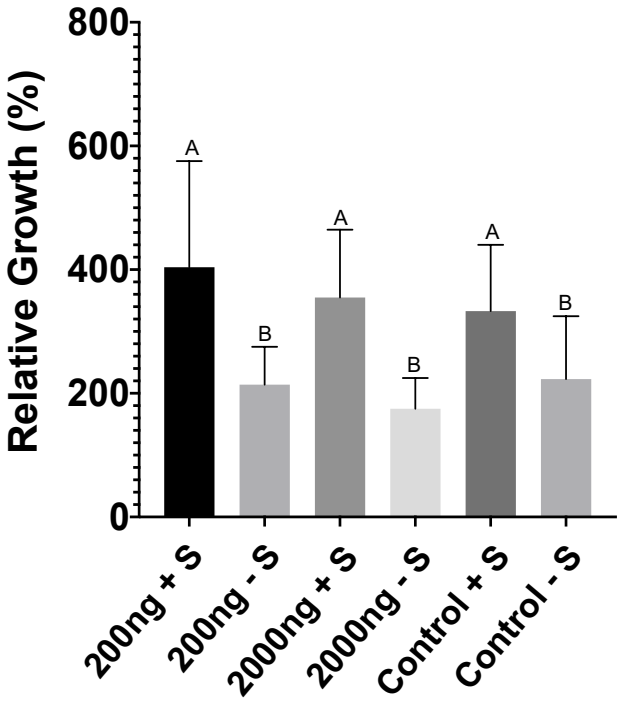

Figure 4.9: The relative growth as a percentage increase from original plant weight of each treatment (200ng and 2000ng) of acetoin applied to sugarcane plantlets grown in both MS media containing sucrose (+ S) and MS media without sucrose (- S). The values represent the mean ± SE (n = 24, each of the 24 replicates consisted of an average of four plants). Statistical significance ($p \leq 0.05$) was determined by t-test and indicated when letters differ.

4.5. Genotyping and Expression Analysis of Putative Sugarcane Transformants

Biolistic transformation of sugarcane requires co-bombardment of the expression vector, containing the transgene, as well as a helper plasmid, pEmuKN, containing a *nptII* selectable marker for putative transformant selection. Due to the nature of this indirect selection method, it was expected that a larger number of putative transformants would need to be screened to ensure that transformants containing the transgene would be identified. This was especially true for lines expected to contain both the pUBI510::*FNR*:*ALDC* and the pUBI510::*FNR*:*BDH1* plasmids, as for successful selection to occur, all three plasmids must be integrated into the genome of the plant line. In order to establish a sufficiently large number of putative transformants, three rounds of microprojectile bombardment were performed on 30 embryogenic callus samples per round. For each round of 30 samples, 10 were used for bombardment of each of the transgene combinations. From the 90 total bombarded samples, 15 putative transformants were found for pUBI510::*FNR*:*ALDC* (*A*) and nine for the double transformant pUBI510::*FNR*:*ALDC* + pUBI510::*FNR*:*BDH1* (*AB*). For the 15 putative *A* transformants, five were confirmed to express the *ALDC* gene (Figure 4.10). Unfortunately, two of these lines (*ALDC*1.1 and *ALDC*2.1) were lost in culture due to a temperature spike within the growth chamber, leaving three lines, namely *ALDC*2.2, *ALDC*3.2 and *ALDC*10. For the double transformation, four lines, *AB*2.1, *AB*3.2, *AB*4.2 and *AB*5.1, were confirmed as expressing both the *ALDC* and *BDH1* genes (Figure 4.11 and 4.12 respectively).

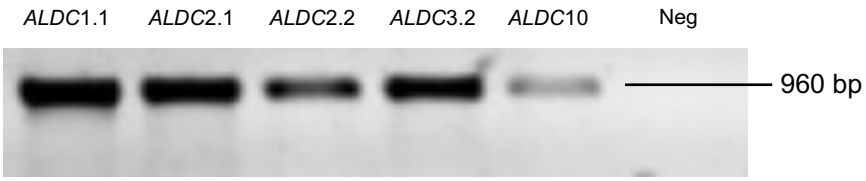

Figure 4.10: PCR results showing *ALDC* gene amplification from the cDNA of five putative transformant sugarcane lines, *ALDC*1.1, *ALDC*2.1, *ALDC*2.2, *ALDC*3.2 and *ALDC*10, transformed with the pUBI510::*FNR*:*ALDC* plasmid. Also displayed is a negative control using untransformed NCo310 sugarcane.

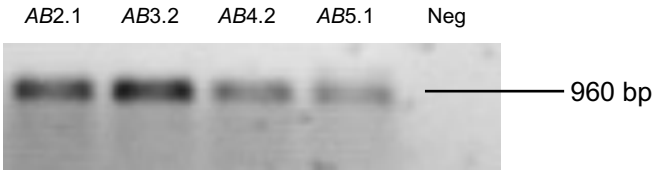

Figure 4.11: PCR results showing *ALDC* gene amplification, using gene specific primers, from the cDNA of five putative transformant sugarcane lines, *AB*2.1, *AB*3.2, *AB*4.2 and *AB*5.1, transformed with both the pUBI510::*FNR*:*ALDC* and pUBI510::*FNR*:*BDH1* plasmids. Also displayed is a negative control using untransformed NCo310 sugarcane.

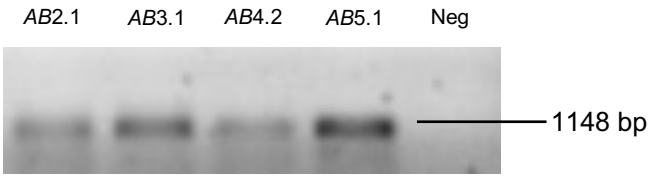

Figure 4.12: PCR results showing *BDH1* gene amplification, using gene specific primers, from the cDNA of five putative transformant sugarcane lines, *AB*2.1, *AB*3.2, *AB*4.2 and *AB*5.1, transformed with both the pUBI510::*FNR*:*ALDC* and pUBI510::*FNR*:*BDH1* plasmids. Also displayed is a negative control using untransformed NCo310 sugarcane.

4.6. *In Vitro* Growth Trial of Transgenic Sugarcane Lines

Previously confirmed transgenic sugarcane lines: *ALDC*2.2, *ALDC*3.2, *ALDC*10, *AB*2.1, *AB*3.2 and *AB*5.1 were selected for *in vitro* growth trials. Care was taken, when initiating the trial, to select 30 plantlets for each line that were as similar as possible in size, weight and root development. After 30 days, the trial was terminated and individual plantlets weighed, working quickly to avoid water loss. Using these fresh weights, the relative growth was calculated and displayed as a percentage increase from the original weight of the plant (Figure 4.13). Inconsistent results, leading to a high standard deviation, were observed, especially from the NCo310 plants to which the transgenic lines were compared. The consequently large standard deviation meant that there were no statistically significant differences in growth between any of the transgenic lines when compared to the NCo310 control. The reason for variation between plantlets within lines is mostly due to the nature of sugarcane culture and propagation from callus material. Callus material of the same age within different *in vitro* containers tends to develop at slightly different rates. As plantlets are regenerated, these slight differences are exaggerated, making it difficult to select plantlets that are of the same size, weight and level of root development. In future, this trial should be performed using a much larger number of replicates to account for such variability among plantlets. Additionally, as in the previous trial using synthetic acetoin (Section 4.4), culture conditions should be optimized to allow for the *in vitro* culture of sugarcane without the addition of sucrose to the culture media, in order to allow for plant growth to be more heavily influenced by photosynthetic factors that may be limited due to the addition of a carbon source to the media (Van Huylenbroeck and Debergh, 1996).

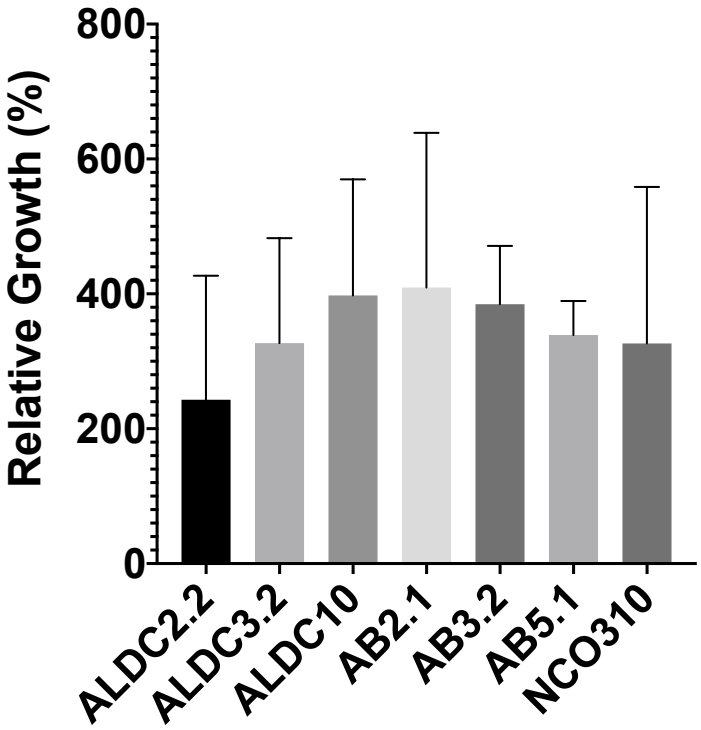

Figure 4.13: The relative growth as a percentage increase from original plant weight after 30 days for each transgenic line *ALDC*2.2, *ALDC*3.2, *ALDC*10, *AB*2.1, *AB*3.2 and *AB*5.1 compared to the NCo310 hybrid plant line as a control. The values represent the mean ± SE (n = 6, each of the six replicates consisted of an average of five plants). No statistical significance ($p \leq 0.05$) was determined by t-test between any of the transgenic lines and the untransformed NCo310 control.

4.7. Transgenic *ALDC* Sugarcane *Ex Vitro* Water Stress Trial

The transgenic lines *ALDC2, ALDC*3 and *ALDC*10 were selected for the water stress trial. Following *in vitro* selection, 18 plantlets, of approximately 7 cm, from each line were transferred to 22 cm pots and placed under humidity domes for gradual hardening off. After hardening off, the plants were placed in a green house, watered twice a week and fertilized every two weeks. At this point, care was taken to ensure no pests or diseases spread to the plants. This was accomplished through regular visual inspections and the use of Plant Protector (Reg. No. / Nr. L 8781 N-AR 1163; Efekto, Isando South Africa), a systemic broad-spectrum pesticide. Applications of pesticide were stopped three weeks before commencement of the drought trial. Once the plants reached a height of approximately 1 m, they were transferred to the growth room, arranged in a randomised block design and watered for the last time. Artificial lighting was used within the growth chamber, consisting of a mix of metal halide and high-pressure sodium bulbs. During the course of the trial, on days 0, 3, 6, 9, 12, and 15 of the drought trial, non-destructive measurements of soil moisture, stomatal

conductance and chlorophyll fluorescence were taken. For destructive measurements, plant tissue was harvested on days 0, 7, 14, and 17. Harvested tissue was used later to determine relative water and chlorophyll content, as well as the presence of ROS and to quantify catalase and superoxide dismutase. On these days, photographs were also taken of the same plants used for the harvesting of tissue. Visually, there were no differences in phenotype between plant lines as the drought trial progressed, with any differences between plants appearing to be random (Figure 4.14). Initially, the trial was intended to last 21 days but was limited to 17 due to a sudden spike in temperature within the growth chamber from 26°C to 39°C at the time of observation, on day 16 of the trial. This temperature spike caused partial tissue desiccation of all 12 plants still remaining in the trial. The cause of the temperature spike within the chamber remains unknown. Additional stress may have been caused by the infestation of *Saccharicoccus sacchari*, sugarcane mealybug (Figure 4.15 A), discovered on day 14 of the drought trial, which spread from an existing infestation on other plants in the growth room (Figure 4.15 B). The infestation was treated by manually removing any visible *S. sacchari* with forceps and a detergent, as well as mass-releasing *Coccinellidae* spp. (ladybirds) as a biocontrol agent. Due to time constraints, the drought trial could not be repeated. A repeat of the drought trial would be needed to confirm the results seen here. However, it is unlikely that any significant difference would be observed between repetitions if the trial was performed with the same methodology as before. *Ex vitro* pot trials display large variation between plants due to growth conditions and age of the plants. With the performed trial, the number of biological replicates was limited by resources and space available. If repeated, a greater number of biological replicates, grown in a more controlled environment, would be beneficial for the detection of smaller significant changes in recorded data.

Figure 4.14: *Ex vitro* drought trial progression, depicted by a representative sample of plants arranged in order of left to right by column; *ALDC*2, *ALDC*3, *ALDC*10, NCo310 and top to bottom by row; Day 0, Day 7, Day 14, Day 17.

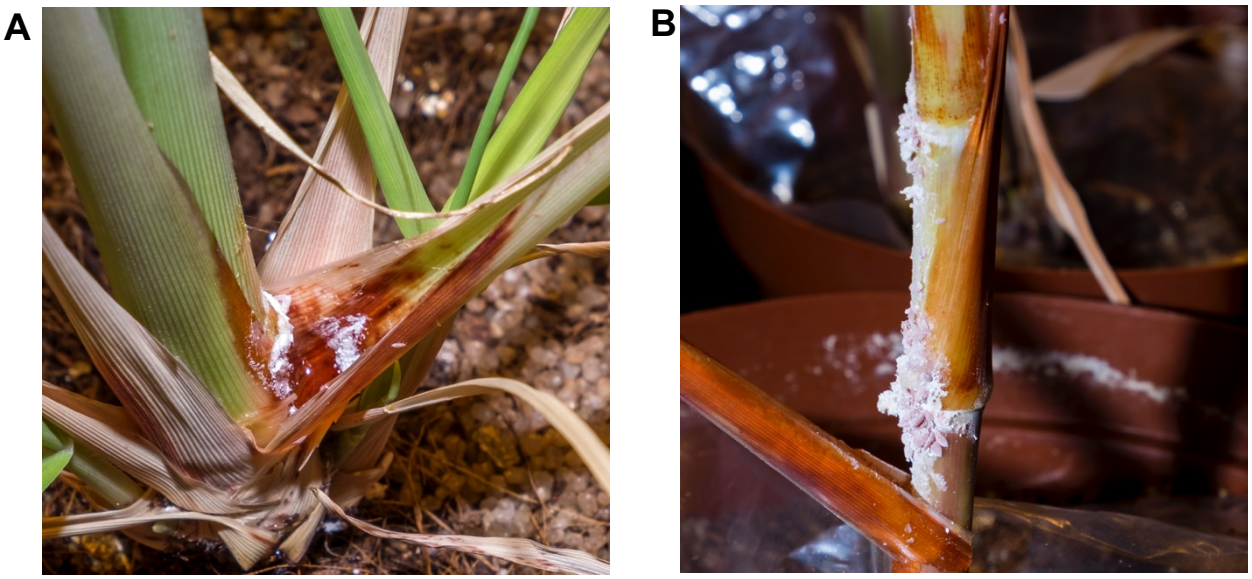

Figure 4.15: A young *Saccharicoccus sacchari* (sugarcane mealybug) colony (**A**) on the base of a transgenic *ALDC*3 sugarcane plant, which originated from one of many older colonies (**B**) found on sugarcane plants already present in the growth chamber.

4.7.1 Soil Moisture Content and Leaf Relative Water Content

Soil moisture content was measured using a ProCheck soil moisture probe (Decagon Devices Inc, Pullman, USA). Measurements were taken in duplicate along a perpendicular cross-section of the centre of each pot. The duplicate measurements were averaged for each pot and used to show the rate of water content decrease throughout the drought trial (Figure 4.16). A steady decrease in soil moisture was observed, with a decrease in rate after day nine. This result was expected as the lower the moisture content in soil, the more slowly it evaporates (Han and Zhou, 2013). Some variation in moisture content was observed between pots, possibly due to inconsistent airflow around individual pots within the growth chamber.

Relative water content (RWC) of leaves was also recorded. This is a destructive measurement and as such, data was gathered on days 0, 7, 14, and 17, with three tissue samples taken per dewlap leaf. The fresh, turgid and dried weight of the tissue samples were used to calculate RWC according the formula established by González and González-Vilar (2001). Whilst soil moisture is dependent mainly on environmental factors and helps quantify abiotic water stress, RWC indicates the degree of drought pressure within the plant at a cellular level. On the cellular level, RWC is an indication of the equilibrium level between the quantity of water consumed by the plant versus the quantity lost

through transpiration (Schonfeld *et al*., 1988). Both soil moisture and RWC are closely linked, however, RWC is the more powerful indicator of water stress within the plant (Arjenaki *et al*., 2010). As such, this study utilised soil moisture measurements to track the progression of abiotic stress levels during the trial, while RWC was used to indicate water stress within the plant. Results from the RWC measurements indicate a general increase in water stress pressure from day 14, with *ALDC*2 and *ALDC*10 on day 14 being significantly different from the NCo310 control (Figure 4.17). However, this trend did not continue throughout the data, indicating that the results may be misleading. Variation in the data also increased as the drought trial progressed. This was consistent with other results in this trial. Again, inconsistent environmental conditions may have contributed to the variation observed. It should also be noted that *S. sacchari* are sap-feeding insects which could lead to increased osmotic stress within certain plants depending on *S. sacchari* population size. Further examination of literature has suggested that the methodology utilised for rehydrating sample tissues could have also introduced errors. By placing leaf tissue directly into water for rehydration, excess apoplastic water ingress may occur (Arndt *et al*., 2015). To avoid this, sections of sugarcane leaf should be cut, avoiding the mid-vein, and placed basal side down in a sealed tube containing enough water to only cover the bottom 10% of the leaf. This revised method should limit water ingress to symplastic pathways, ensuring true measurements of turgid tissue weight can be recorded.

Overall, the results of soil moisture content and leaf relative water content proved inconclusive. The data showed no convincing differentiation between any of the transgenic lines and untransformed control plants. Additional biological replicates as well as a more controlled water stress environment would improve the experimental design, allowing for accurate determination of differences between lines.

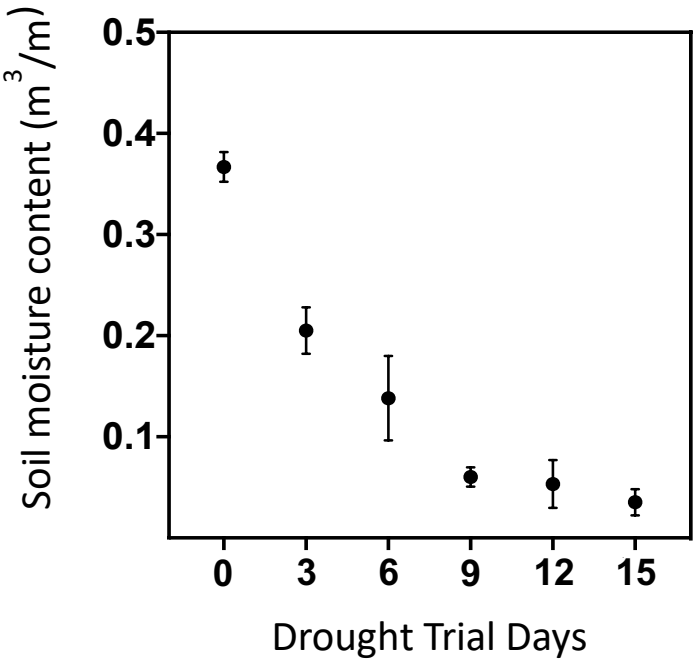

Figure 4.16: Average water content (m^2/m) of all randomly selected pots on days 0, 3, 6, 9, 12 and 15 of the *ex vitro* drought trial with variance between pots on each day displayed. The values represent the mean ± SE (n = 16). The data set was used to indicate a general trend, as such no assessment of statistical significance was performed.

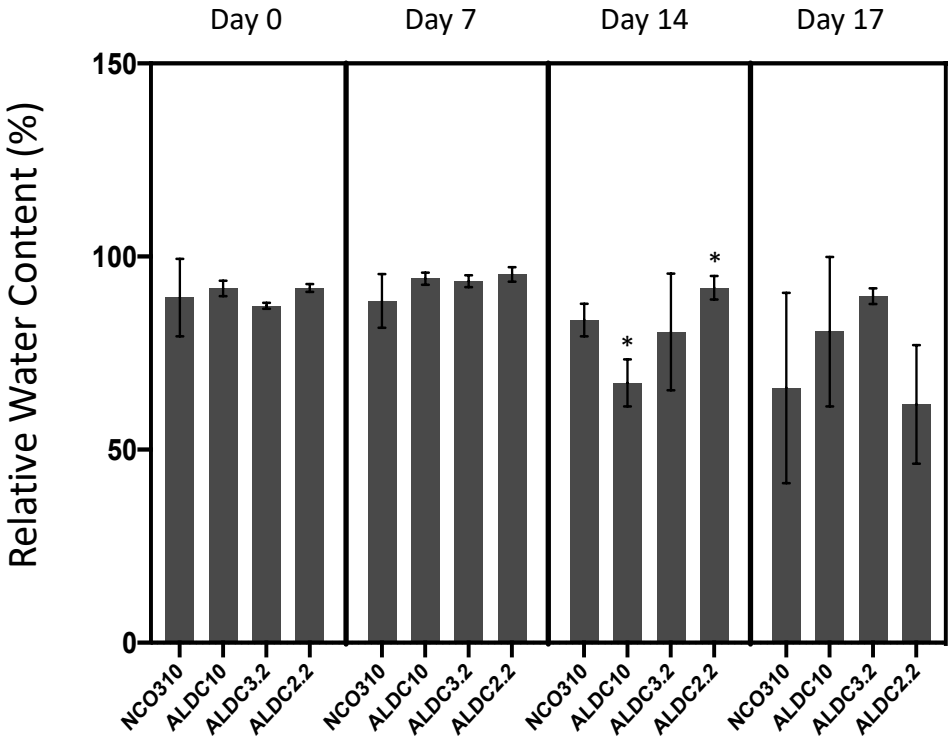

Figure 4.17: Relative water content (%) of each transformant line as well as untransformed NCo310 control plants on days 0, 7, 14 and 17 of the *ex vitro* water stress trial. The values represent the mean ± SE (n = 6, three plants were sampled per interval with two samples taken per plant). Statistical significance ($p \leq 0.05$), indicated with an *, was determined by t-test between the transgenic lines and the untransformed NCo310 control.

4.7.2. Stomatal Conductance, Chlorophyll Fluorescence and Chlorophyll Content

Stomatal conductance and chlorophyll fluorescence were non-destructive measures recorded on days 0, 3, 6, 9, 12 and 15 of the drought trial. This was accomplished using the SC-1 Leaf Porometer (Decagon Devices Inc, Pullman, USA) and OS30p+ Chlorophyll Fluorimeter (Opti-sciences, Hudson, USA). Readings were taken from three points on the dewlap leaf of each plant. Chlorophyll content is a destructive assay and was recorded from plant tissue harvested on days 0, 7 14 and 17.

Stomatal conductance, chlorophyll fluorescence and chlorophyll content are all indicators of photosynthetic activity. In the initial phases of drought, photosynthetic rate is limited by drought induced stomatal closure, with stomatal conductance being an indirect measure of stomatal activity (Cornic and Massacci, 1996). Stomatal closure leads to a reduction in CO_2 content within the leaf, which in turn leads to a reduction in photosynthetic activity (Wang *et al*., 2018). Total chlorophyll content was also measured for each plant. There is some debate as to whether a higher chlorophyll content relates directly to increased photosynthetic activity during drought conditions (Blum, 1998), however, significant research indicates that it may contribute to crop yield (Borrel *et al*., 1999). The expected pattern of these measures is that as drought pressures increase, photosynthetic activity would slow down. Differing rates in the drop of photosynthetic activity between plant lines can indicate varying degrees of drought tolerance. The results from stomatal conductance measurements displayed considerable variation between readings. This was primarily caused by the steady-state method of measurement employed by the leaf porometer itself. The device operates by clamping a sensor chamber onto the leaf surface and detecting a relative humidity gradient established within the flux path, between two points within the sensor chamber. Once a stable flux gradient is established, the device is able to calculate the leaf diffusion conductance. This system relies on establishing a reliable seal between the leaf surface and the sensor chamber. The morphology of the sugarcane leaves used for measurements in this trial meant that a reliable seal could not be achieved. Sugarcane leaves possess a prominent mid-vein which stopped the sensor chamber from fully sealing to the leaf surface (Figure 4.18). The incompatible sensor chamber lead to erroneous readings with any observed significant difference appearing to be random in occurrence (Figure 4.19). More accurate measures of stomatal conductance could be measured with an improved device, such as one that uses a mass flow method or by using significantly older plants which have a greater distance between leaf edge and the mid vein. Due to time constraints as well as budgetary concerns, older plants could not be used, nor could alternative sensors be purchased.

Chlorophyll fluorescence was a more reliable measurement with little variation in the data observed up to day nine of the drought trial (Figure 4.20). After this point, a slight but significant decrease in chlorophyll fluorescence was observed overall with some plant lines varying largely from the mean.

This is likely as an indirect result of the inconsistent conditions within the growth chamber. During the growth trial, it was observed that the dewlap leaves of some plants yellowed rapidly and at different points during the later stages of the drought trial. This could have been what introduced variation between plants within the same line and may have skewed the data. No single line consistently displayed a significant difference to the NCo310 control. During the trial, water stress appears to have accumulated rapidly after day nine. From day nine, plant leaves began to yellow and desiccate suddenly without displaying a gradual accumulation of water stress as expected. It is possible that the water stress method employed caused a more acute drought shock rather than a prolonged drought stress as initially planned (Farooq *et al*, 2009).

The chlorophyll content provided similar results to chlorophyll fluorescence, but with larger variation between samples (Figure 4.21). Only two statistically significant results were obtained, which appeared random and not to follow any pattern throughout the drought trial. One of the major impacting factors on chlorophyll content is light quality and specific spectrum. Light, which is predominantly in the blue spectrum, encourages the accumulation of chlorophyll A over chlorophyll B, while light in the red spectrum results in less overall chlorophyll within the leaf (Fan *et al*., 2013). The drought trial growth chamber used artificial lighting, namely, a mixture of high-pressure sodium (HPS) and metal halide (MH) bulbs. HPS bulbs emit a light that is further towards the red spectrum while MH bulbs emit light further towards the blue spectrum. When used simultaneously they provide a complete photosynthetic spectrum. However, it was observed throughout the day that different areas of the growth chamber predominantly received light from either HPS or MH bulbs. This inconsistent lighting may have contributed to the high degree of variation in chlorophyll content between randomly selected plants of the same line. For a true reflection of chlorophyll content, diffuse and consistent, full spectrum lighting should be utilised.

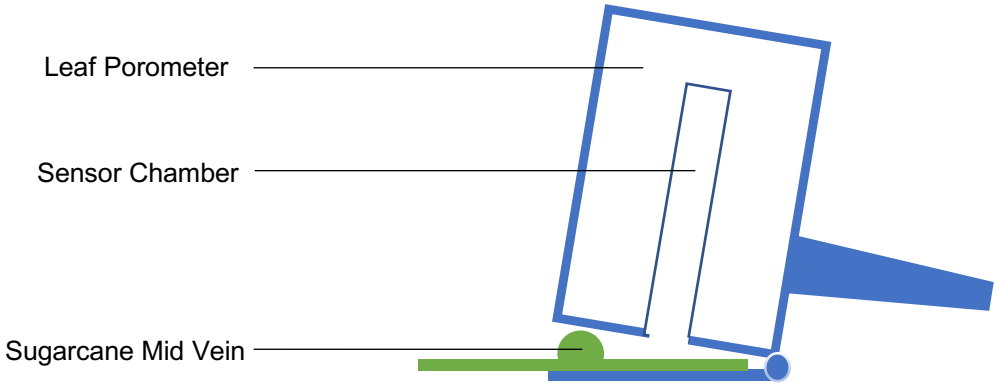

Figure 4.18: Leaf porometer sensor unit, as used on sugarcane leaves for stomatal conductance measurement. The figure indicates the prominent sugarcane mid vein and the orientation of sensor chamber in relation to it, resulting in an incomplete seal between the chamber and the leaf being achieved.

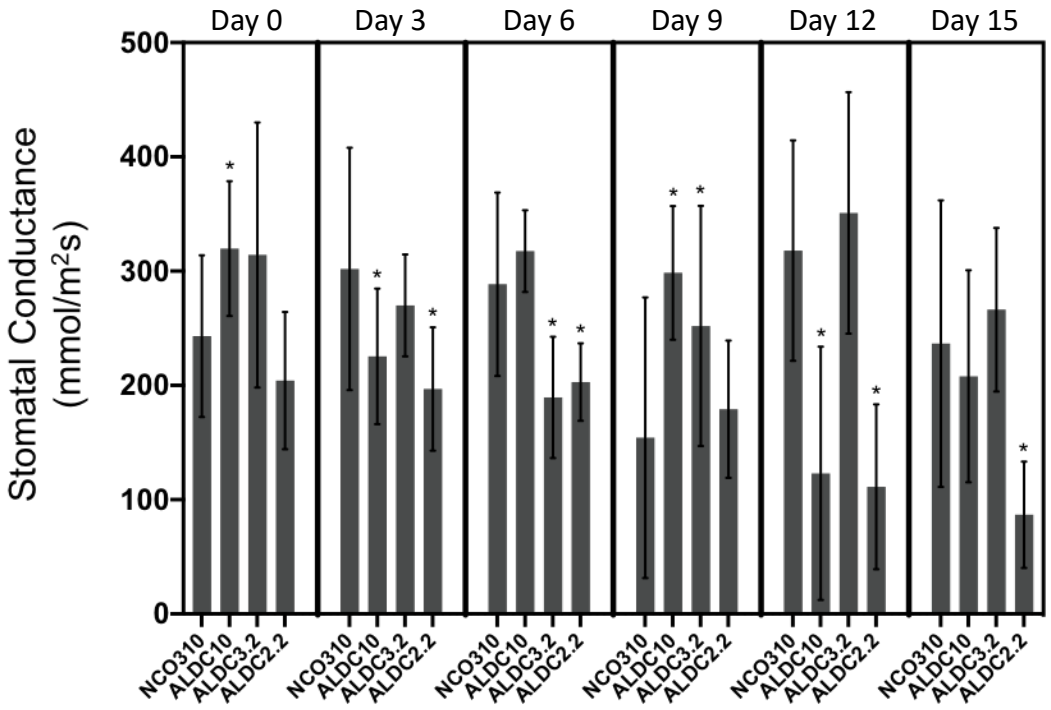

Figure 4.19: Stomatal conductance (mmol/m^2s) of randomly selected plants from each line, including NCo310 untransformed control, on days 0, 3, 6, 9, 12 and 15 of the drought trial. The values represent the mean ± SE (n = 12, four plants were sampled per interval with three samples taken per plant). Statistical significance ($p \leq 0.05$), indicated with an *, was determined by t-test between the transgenic lines and the untransformed NCo310 control.

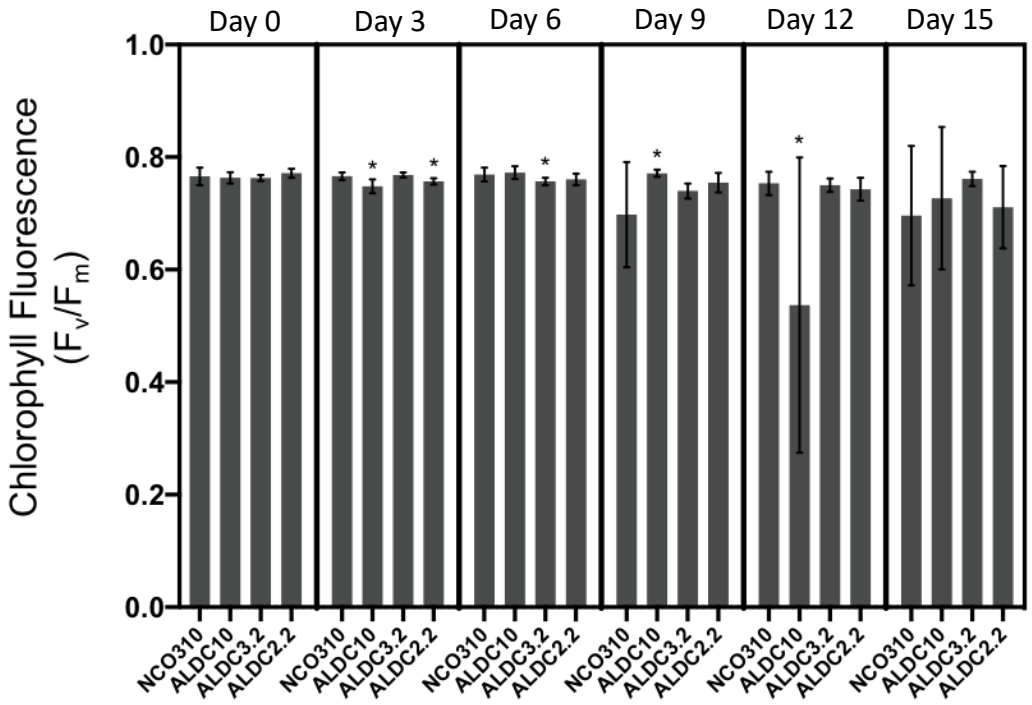

Figure 4.20: Chlorophyll fluorescence (F_v/F_m) of randomly selected plants from each line on days 0, 3, 6, 9, 12 and 15 of the drought trial. The values represent the mean ± SE (n = 12, four plants were sampled per interval with three samples taken per plant). Statistical significance ($p \leq 0.05$), indicated with an *, was determined by t-test between the transgenic lines and the untransformed NCo310 control.

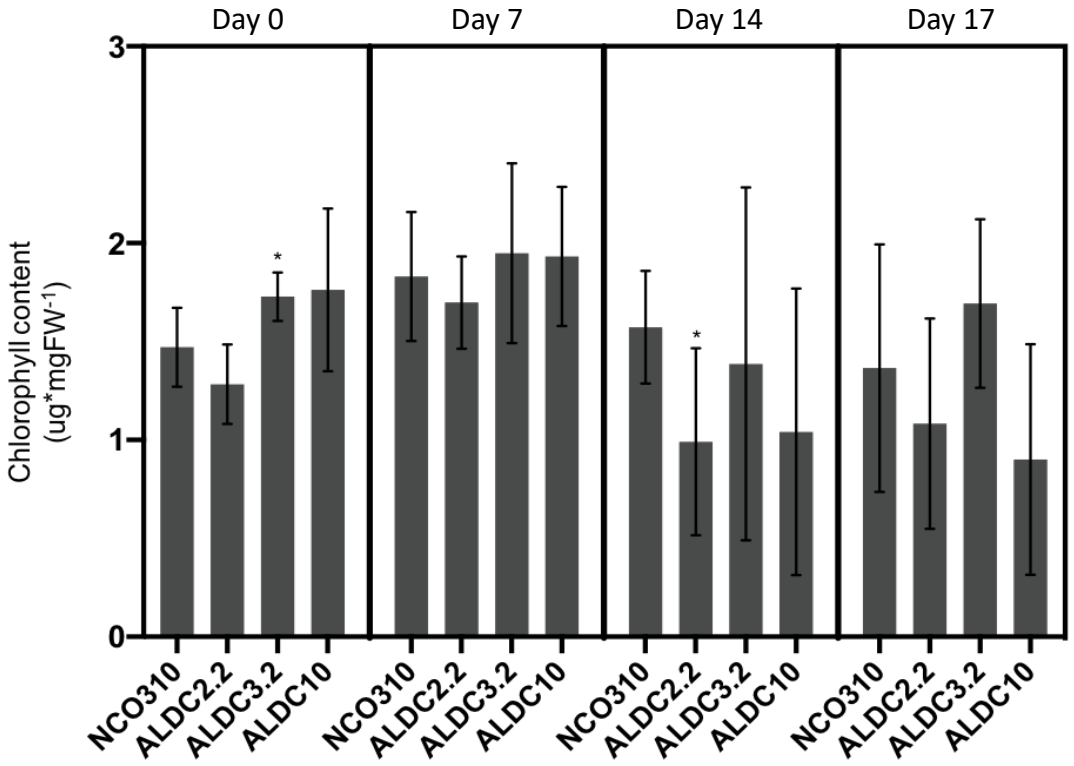

Figure 4.21: Total chlorophyll content ($\mu g*mgFW^{-1}$) of tissue samples harvested from randomly selected plants from each line on days 0, 7, 14 and 17 of the drought trial. The values represent the mean ± SE (n = 12, four plants were sampled per interval with three samples taken per plant). Statistical significance ($p \leq 0.05$), indicated with an *, was determined by t-test between the transgenic lines and the untransformed NCo310 control.

4.7.3. Accumulation of Reactive Oxygen Species (ROS)

Both hydrogen peroxide (H_2O_2) and superoxide (O_2^-) reactive oxygen species were assessed visually through histochemical staining methods. Hydrogen peroxide oxidises 3,3-diaminobenzidine (DAB) in the presence of peroxidases to form a brown precipitate which was visualised and recorded photographically (Figure 4.22). Superoxide reacts with 4-nitroblue tetrazolium chloride (NBT) to form formazan, a dark blue stain which was also visualised and recorded photographically (Figure 4.23). When assessing the stained leaf material, it is important to note that the obvious staining along where the leaf sample was cut is not as a result of ROS accumulation due to water stress, but rather a wounding response from damaged plant cells. Therefore, only staining at the centre of the leaf sample should be considered. Visual assessment of the stains yielded no obvious patterns,

especially for hydrogen peroxide which displayed little to no oxidation of DAB in any of the leaf samples. Blue formazan was only present in a few leaf samples from different lines. The staining procedure indicated no obvious difference between plant lines throughout the drought trial.

ROS are highly reactive compounds that can cause oxidative damage to plant cells. Hydrogen peroxide and superoxide are especially toxic due to their ability to initiate reactions that result in hydroxyl radicals and lipid peroxidases (Garg and Manchanda, 2009). Hydrogen peroxide and superoxide accumulate in the cells of plants that are exposed to stress (Sweetlove *et al*., 2002). The degree of ROS accumulation can be used to quantify the amount of stress a plant is exposed to in relation to other plants exposed to the same stresses. This makes it potentially useful for assessing plant drought tolerance levels. No obvious pattern of accumulation could be discerned from the qualitative assessments of ROS accumulation that were performed. The lack of visual ROS accumulation may be as a result of the rapid onset of drought pressures within the growth chamber. In future, a slower onset of drought pressures may result in a more obvious accumulation of ROS. It may also be helpful to rather perform a direct quantitative assessment of ROS, which may prove more sensitive than the colorimetric method used (Nadeem *et al*., 2019).

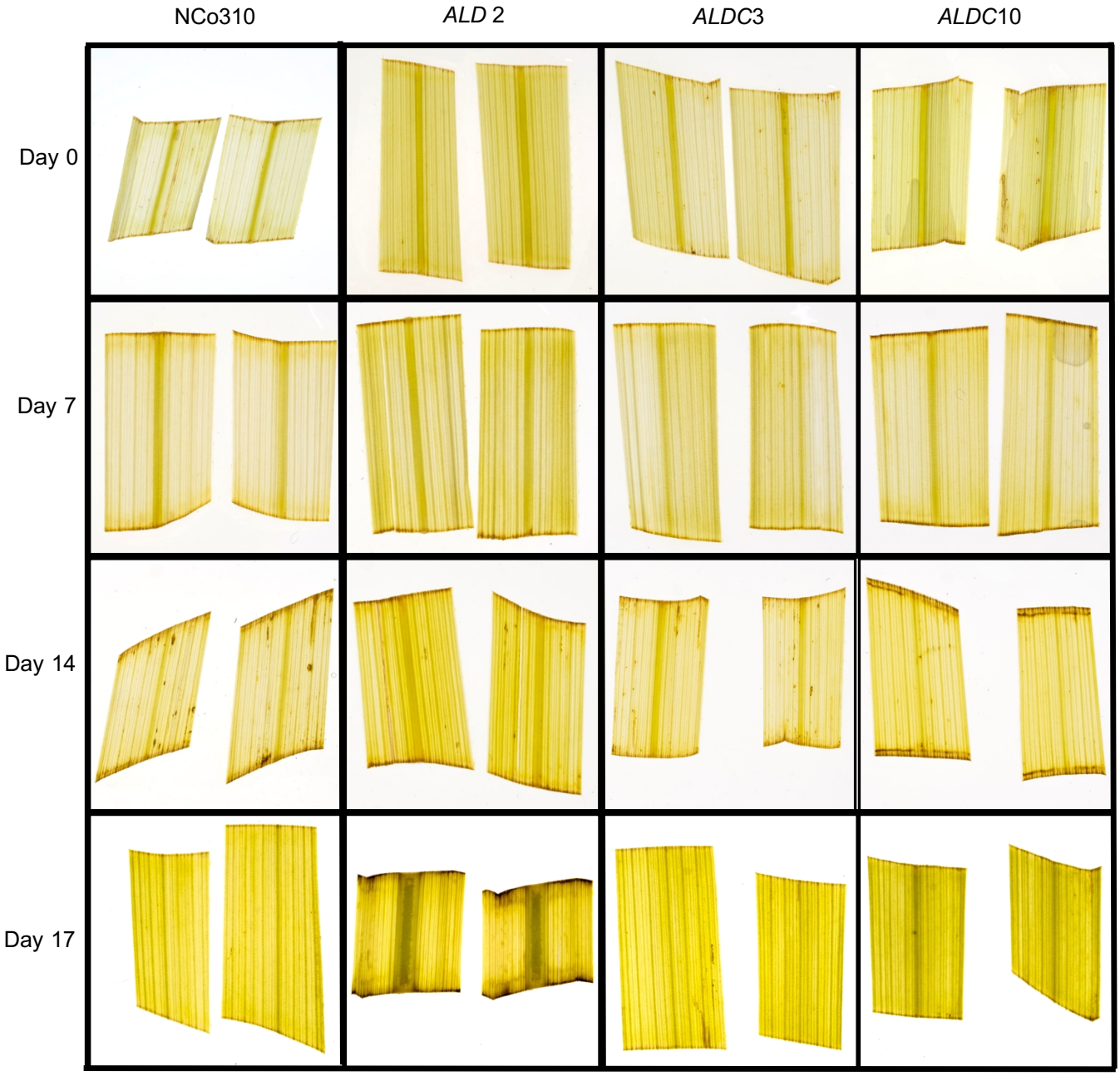

Figure 4.22: Sugarcane leaf samples stained with 3,3-diaminobenzidine to display hydrogen peroxide reactive oxygen species accumulation during the drought trial. Samples arranged in order of left to right; NCo310, *ALDC*2, *ALDC*3 and *ALDC*10 and top to bottom; Day 0, Day 7, Day 14 and Day 17 of the drought trial.

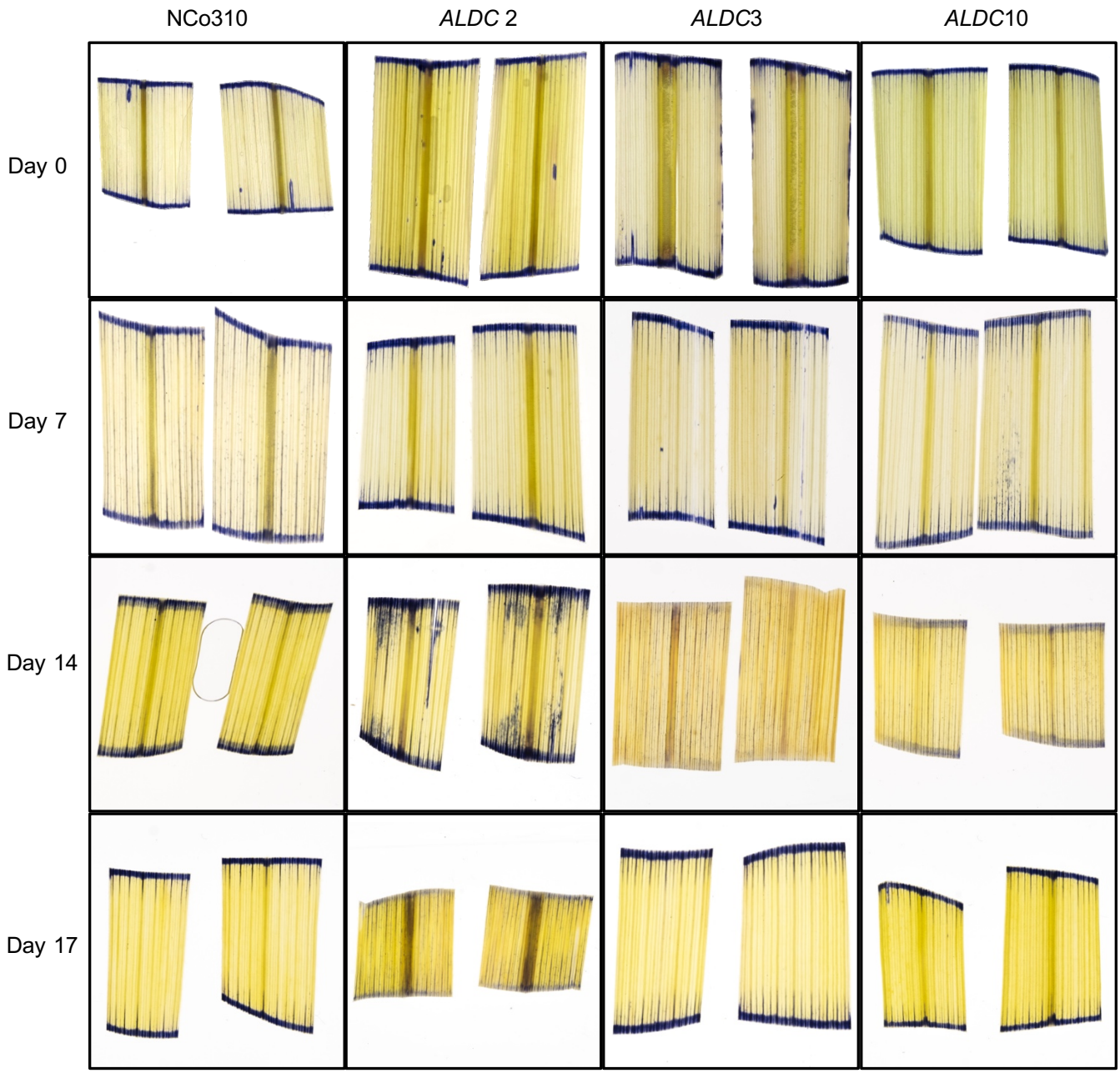

Figure 4.23: Sugarcane leaf samples stained with 4-nitroblue tetrazolium chloride to display superoxide reactive oxygen species accumulation during the drought trial. Samples arranged in order of left to right; NCo310, *ALDC*2, *ALDC*3 and *ALDC*10 and top to bottom; Day 0, Day 7, Day 14 and Day 17 of the drought trial.

4.7.4. Antioxidant Accumulation

The accumulation of antioxidants, catalase and superoxide dismutase (SOD), were assessed using colorimetric assay kits after extraction from sugarcane leaf tissue. Catalase activity (nmoles/min/mgFW) was calculated with the aid of a standard curve created using known concentrations of hydrogen peroxide. SOD activity (% inhibition of WST) was calculated directly from the absorbance values obtained from the colorimetric assay. No significant differences between transgenic lines and NCo310 control were observed for catalase activity until day 17, where lines *ALDC*3 and *ALDC*10 both displayed significantly higher activities than the control (Figure 4.24). For SOD activity, all three transgenic lines displayed significantly higher % inhibition of WST than the untransformed control in tissue harvested on day 0 (Figure 4.25). This trend did not continue throughout the drought trial, with only *ALDC*3 and *ALDC*10 displaying marginally, but statistically significantly, higher SOD activities on day 14.

Antioxidant accumulation within leaf tissue relates directly to the accumulation of ROS. Catalase acts against the build-up of hydrogen peroxide while SOD catalyses superoxide, preventing its accumulation (Alscher *et al*., 1993). Superoxide is present in various subcellular compartments wherever there is an electron transport chain, making it one of the most abundant ROS (Hippeli and Elstner, 1996). It has also been shown that phospholipid membranes are impermeable to superoxide molecules, meaning that SOD must be present in all subcellular compartments (Takahashi and Asada 1983). These factors make assessing SOD activity a potentially valuable indicator of a plant's ability to adapt to water stress. However, the complexity of SOD detoxification of ROS means it is difficult to use as a stand-alone indicator of drought tolerance, especially in *ex vitro* trials with relatively few biological replications. In the case of this study, inconsistent environmental conditions throughout the drought trial and physiological variability among plants led to results with significant differences in only two instances for catalase and five for SOD. There was no apparent correlation between respective ROS accumulation and antioxidant activities within leaf tissue samples. This being said, on day 0, a pattern was evident where the % inhibition of WST by SOD was significantly higher in the three transgenic plant lines than in the untransformed control. However, this result was not consistent with any other results of the drought trial and would need further investigation. In future, the link between osmotic stress and water stress should be considered as the addition of an *in vitro*, osmotic stress trial could prove valuable. *In vitro* environmental conditions are more consistent and logistically, it is possible to include a much larger number of biological replications. This, combined with an *ex vitro* drought trial, might provide a more complete picture with regards to plant stress and its various indicators, enabling a more accurate comparison of transgenic sugarcane lines to untransformed control plants.

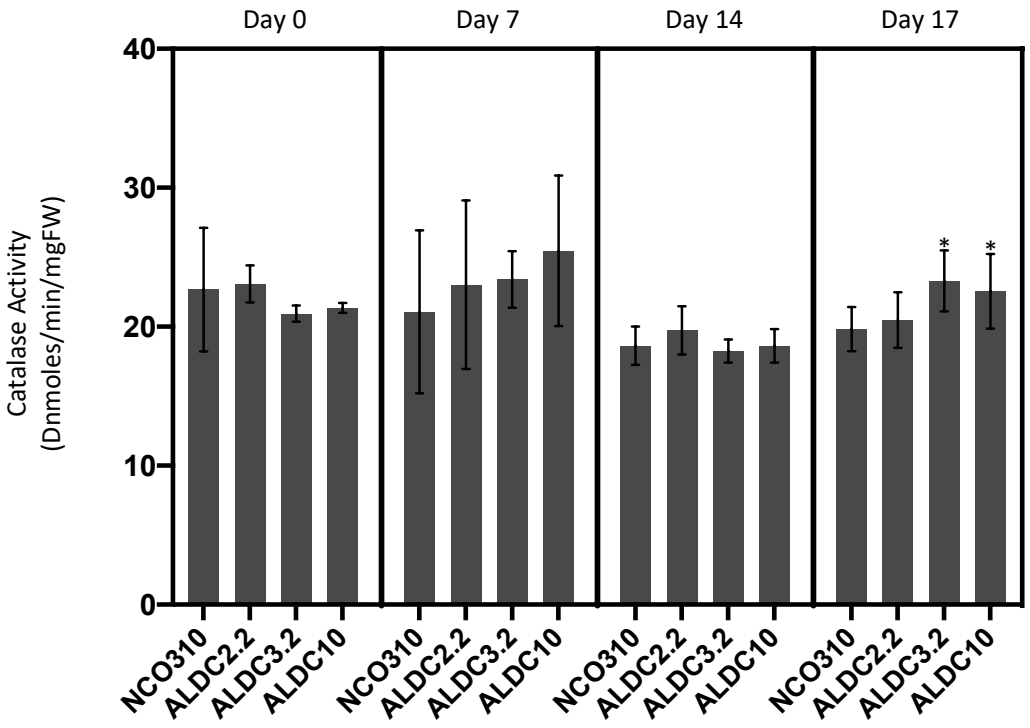

Figure 4.24: Catalase activity (nmoles/min/mgFW) in dewlap leaf samples extracted from each sugarcane plant line on days 0, 7, 14 and 17 of the drought trial and statistically compared to samples from the NCo310 untransformed control. The values represent the mean ± SE (n = 12, four plants were sampled per interval with three samples taken per plant). Statistical significance ($p \leq 0.05$), indicated with an *, was determined by t-test between the transgenic lines and the untransformed NCo310 control.

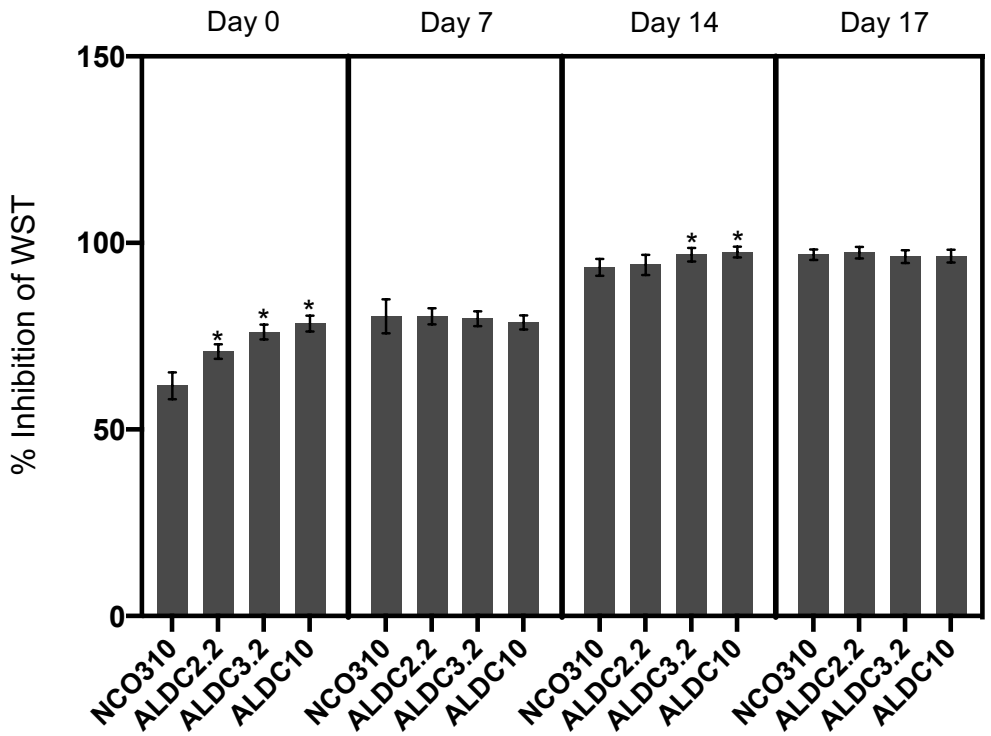

Figure 4.25: SOD activity (% inhibition of WST) in dewlap leaf samples extracted from each sugarcane plant line on days 0, 7, 14 and 17 of the drought trial and statistically compared to samples from the NCo310 untransformed control. The values represent the mean ± SE (n = 12, four plants were sampled per interval with three samples taken per plant). Statistical significance ($p \leq 0.05$), indicated with an *, was determined by t-test between the transgenic lines and the untransformed NCo310 control.

4.7.5. Results Summary and Further Discussion

In summary, the *Arabidopsis* section of this project was unable to proceed due the observed 240 bp deletion in the pCambia2300::*FNR*:*ALDC* construct used for plant transformations. The cause of this deletion remains unknown, with none of the possible hypotheses explaining it fully.

The focus of this project was therefore redirected towards sugarcane, where plants were exposed to synthetic acetoin as well as being transformed with the *ALDC* and *BDH1* transgenes. An *in vitro* growth trial using synthetic acetoin (Section 4.4) yielded results with no significant differences between the treated and control plantlets. This was partly due to a high degree of variation observed among individual NCo310 plantlets, as well as the required use of sucrose as a carbon source *in vitro*, limiting photosynthetic activity. Both the *in vitro* growth trial (section 4.6) and *ex vitro* drought trial (section 4.7), using transgenic sugarcane plants, yielded inconclusive results due to large

variation between plants, limited plant material and inconsistent environmental conditions.

Further insight into the results above can be gained from the hypothesis that plant growth in response to acetoin and other VOCs is partly linked to the regulation of photosynthesis (Sharifi and Ryu, 2018). A possibility, not previously discussed, is that acetoin influences plant responses differently in C_3 photosynthesising plants compared to the C_4 photosynthesising sugarcane. The only growth trial performed on C_4 photosynthesizing plants in response to VOCs used sorghum (Castulo-Rubio *et al*., 2015). The specific VOC used in that trial was dimethylhexadecylamine, which was observed to increase plant biomass and chlorophyll content. To date, no published works exist involving acetoin as a plant growth-promoting VOC on a C_4 photosynthesizing plant. It is possible that acetoin upregulates plant growth using biochemical pathways unique to C_3 photosynthesizing plants only.

5. Conclusions and future perspectives

This study was performed to determine the viability of producing acetoin and 2,3-butanediol *in planta*, via the incorporation and expression of the transgenes *ALDC* and *BDH1* in the plant genome. *Arabidopsis* was transformed using the pCambia2300::*FNR*:*ALDC* and pCambia1300::*FNR*:*BDH1* constructs. This line of research was abandoned following the discovery of a 240 bp deletion within the *ALDC* gene used for the transformation of *Arabidopsis*. The cause of this deletion remains unknown; however, it was deduced to have occurred between the transformation of *Agrobacterium* and the transformation of T_0 *Arabidopsis* flower buds. Following this setback, focus shifted to the transformation and testing of sugarcane plants using the same *ALDC* and *BDH1* genes. *ALDC* and *BDH1* single transformants as well as *AB* double transformants were successfully created and showed to be expressing the respective genes. *In vitro* trials comparing the relative growth of the transgenic sugarcane lines to the growth of an untransformed control line yielded no significant difference due to high variation between individual plants within each line. Sugarcane grown from callus material is highly variable with regards to developmental stage, especially for root development. This meant that with the given quantity of plant material to select from, it became difficult to select plants for the trial of uniform size and root development. For future trials, a much larger stock of plants generated from callus material would be needed in order to establish a growth trial consisting of plants of all the same size and developmental stage. The same can be said for the *in vitro* trial performed, comparing the growth of untransformed sugarcane plants, treated with different concentrations of synthetic acetoin to an untreated control. Additionally, in this trial, the growth of sugarcane plants, when treated with acetoin, was compared when grown on media both containing and without sucrose. It was clear that plants grown on media without the addition of sucrose performed worse than those grown on media containing sucrose. The reason for this is that under conditions unfavourable to photosynthesis, sucrose acts as the carbon source for the plant's growth. This is important because growth promotion due to VOCs has been linked to an upregulation of photosynthesis. For this reason, future experimentation would benefit from the optimisation of sugarcane *in vitro* growth in media not containing sucrose, thereby focusing any observed growth differences to the plants' physiology and any possible upregulation of photosynthesis. In addition to *in vitro* growth trials, *ex vitro* potted drought trials were performed over 17 days, comparing the performance of transgenic *ALDC* plant lines to untransformed controls. Again, no significant overall differences were observed between lines during the trial. This was due to high variation between individual plants, caused by inconsistent lighting, temperature and airflow within the growth room.

For future consideration, alternative methods of exposing plants to synthetic volatiles should be considered, such as the option depicted in annexure E. The device was developed during this study and would allow plants to be exposed to a consistent concentration of VOC without the need to

repeatedly open the culture vessel to replace the VOC treatment. In addition, the direct detection of VOCs produced *in planta* would be required to publish these works. Previous attempts to do so using gas chromatography mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) proved unsuccessful. A solution to this could be the expression of *ALDC* and *BDH1* genes within an analogous bacterial expression system where the detection of VOCs in liquid bacterial culture has been optimised already.

In conclusion, this study was unable to show any significant difference in the growth or drought tolerance of sugarcane expressing the *ALDC* transgene. Sugarcane in general is not well suited for the experimentation performed in this study. Consideration should be given to other C_4 photosynthesising plants for use in future investigation. Additionally, environmental conditions within which plant growth and performance is measured needs to be properly optimised. Due to the extended time frame needed to perform repeated sugarcane growth and drought trials, growth conditions could not be repeated under adjusted, optimised conditions. This leaves large gaps in the research that need to be filled in future with additional attention payed to the specific aspects mentioned above. This study has effectively paved the way for completion and subsequent publication of future studies on acetoin, 2,3-butanediol and their positive effect on plant growth, disease resistance and drought tolerance.

6. References

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Annexures

Annexure A

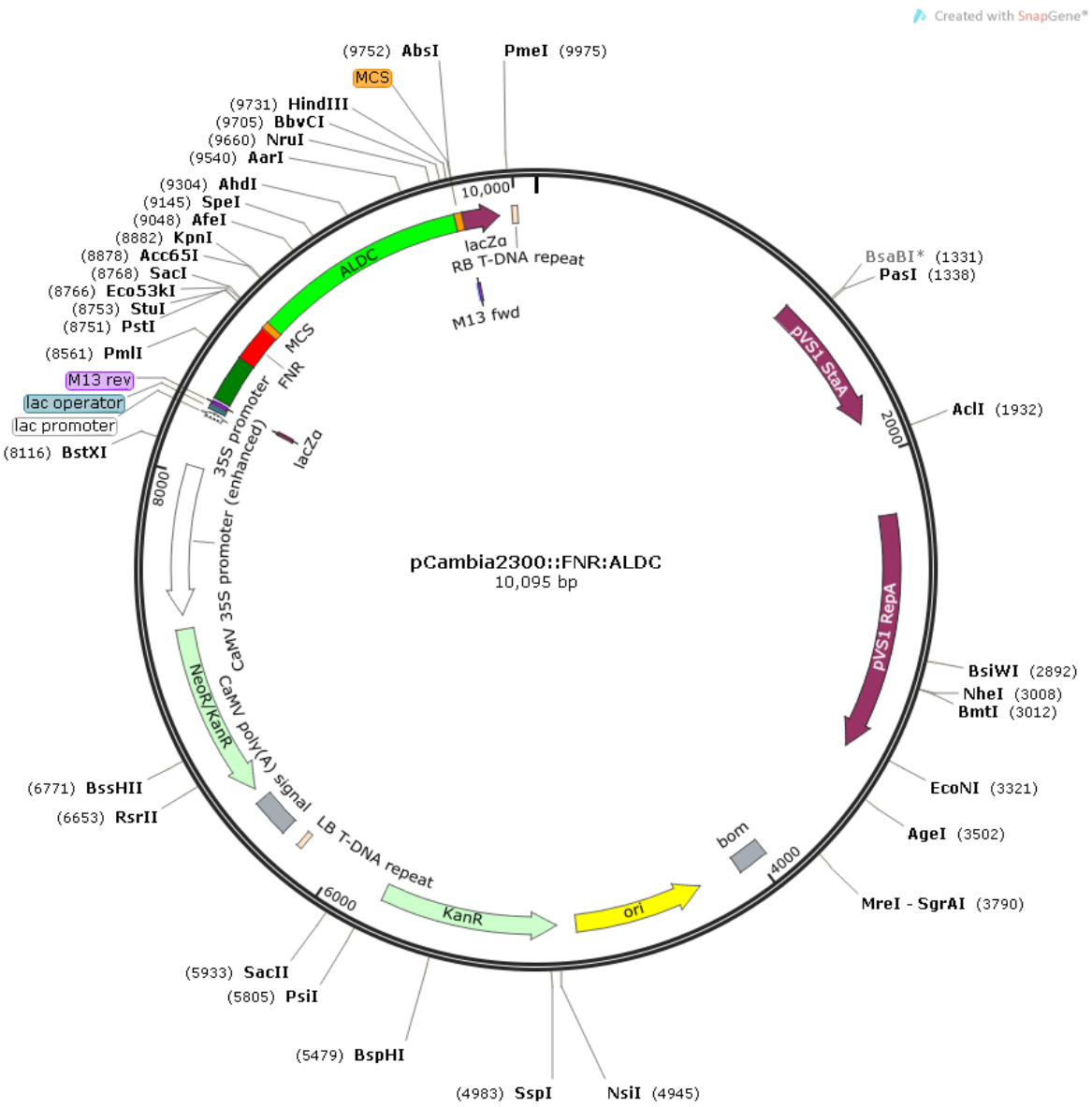


Figure 1.4.1: A plasmid map of the pCambia2300::*FNR*:*ALDC* plant transformation vector generated by Dempers (2015). The standard pCAMBIA2300 vector was modified by the addition of a CaMV35S promoter, ferredoxin-NADP$^+$ reductase (*FNR*) transit peptide and the α-acetolactate decarboxylase (*ALDC*) gene.

Annexure B

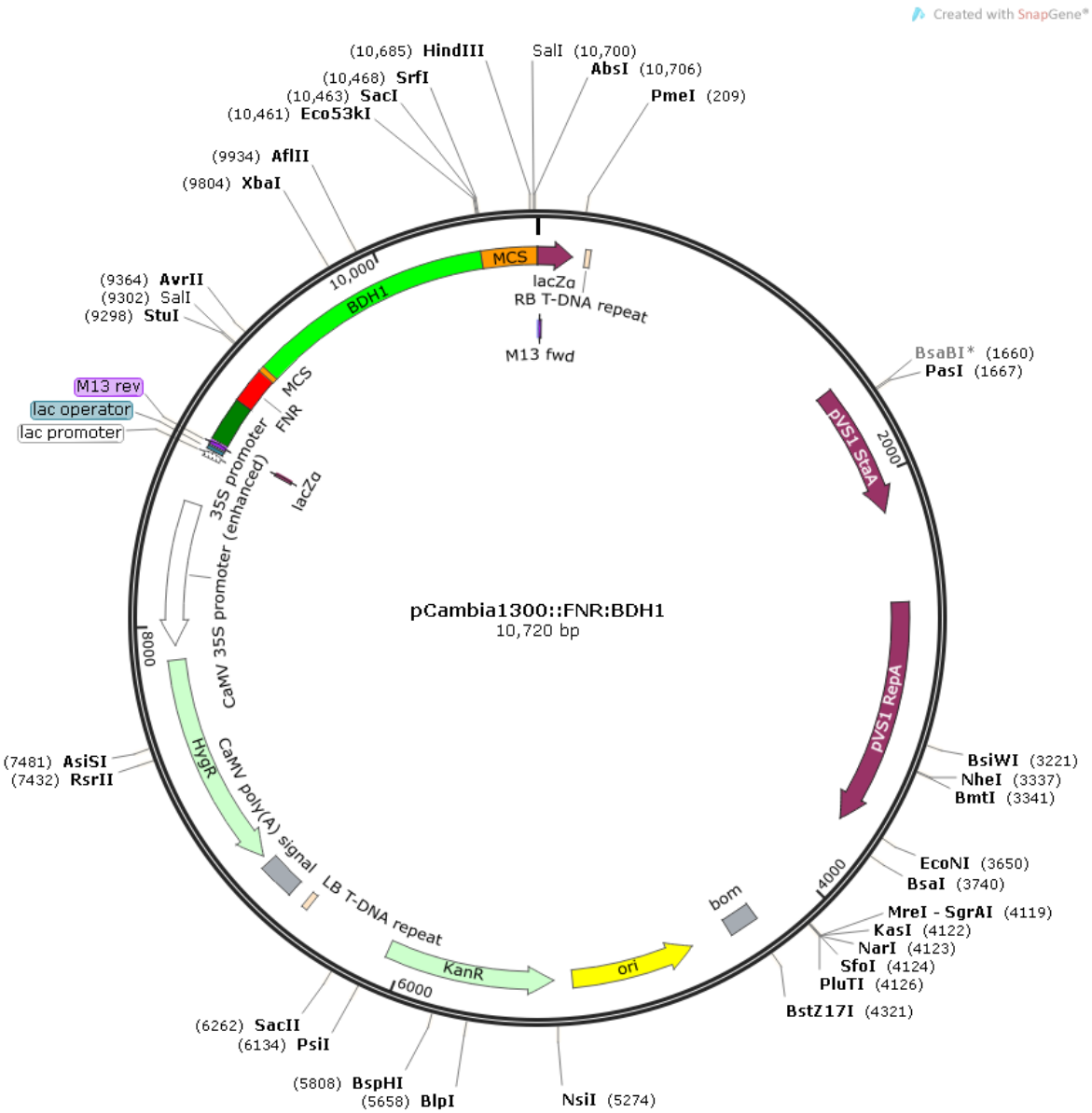
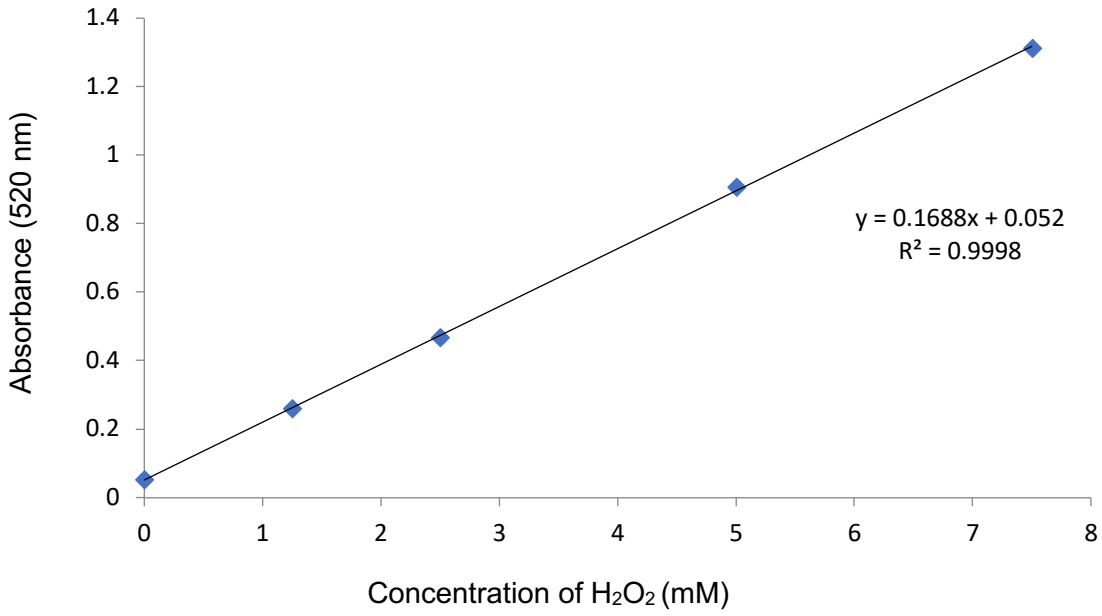


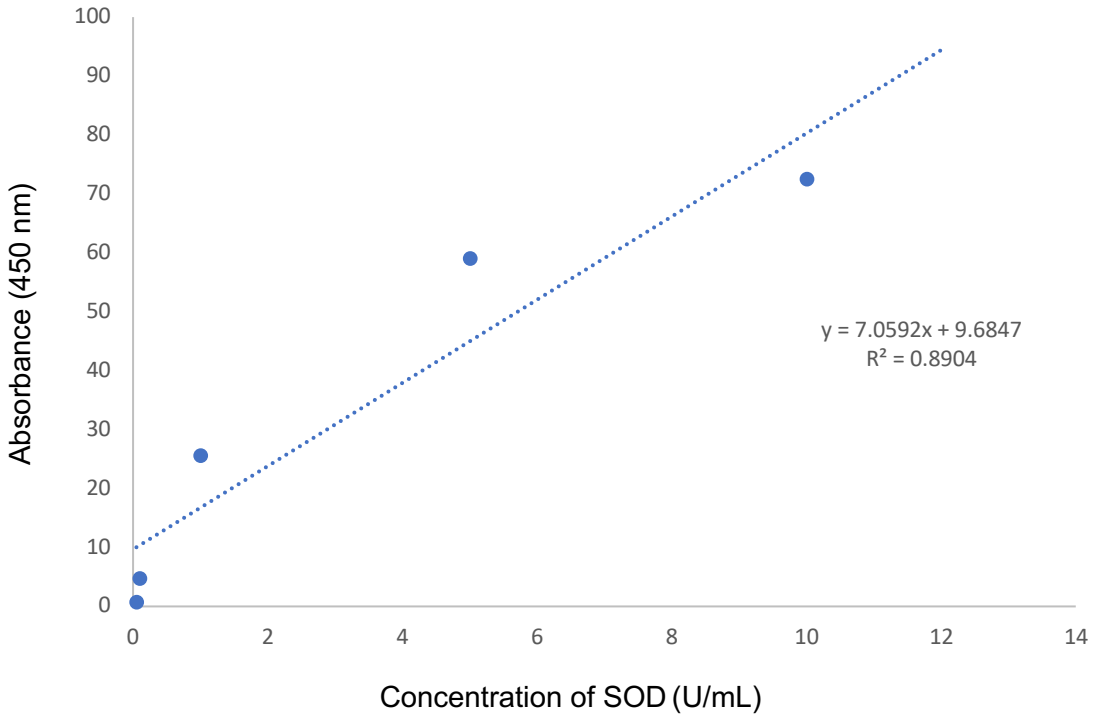
Figure 1.4.2: Plasmid map of the pCambia1300::*FNR*:*BDH1* plant transformation vector (Dempers, 2015). The standard pCAMBIA1300 vector was modified by the addition of a CaMV35S promoter, ferredoxin-$NADP^+$ reductase (*FNR*) transit peptide and the α-acetolactate decarboxylase (*BDH1*) gene.

Annexure C



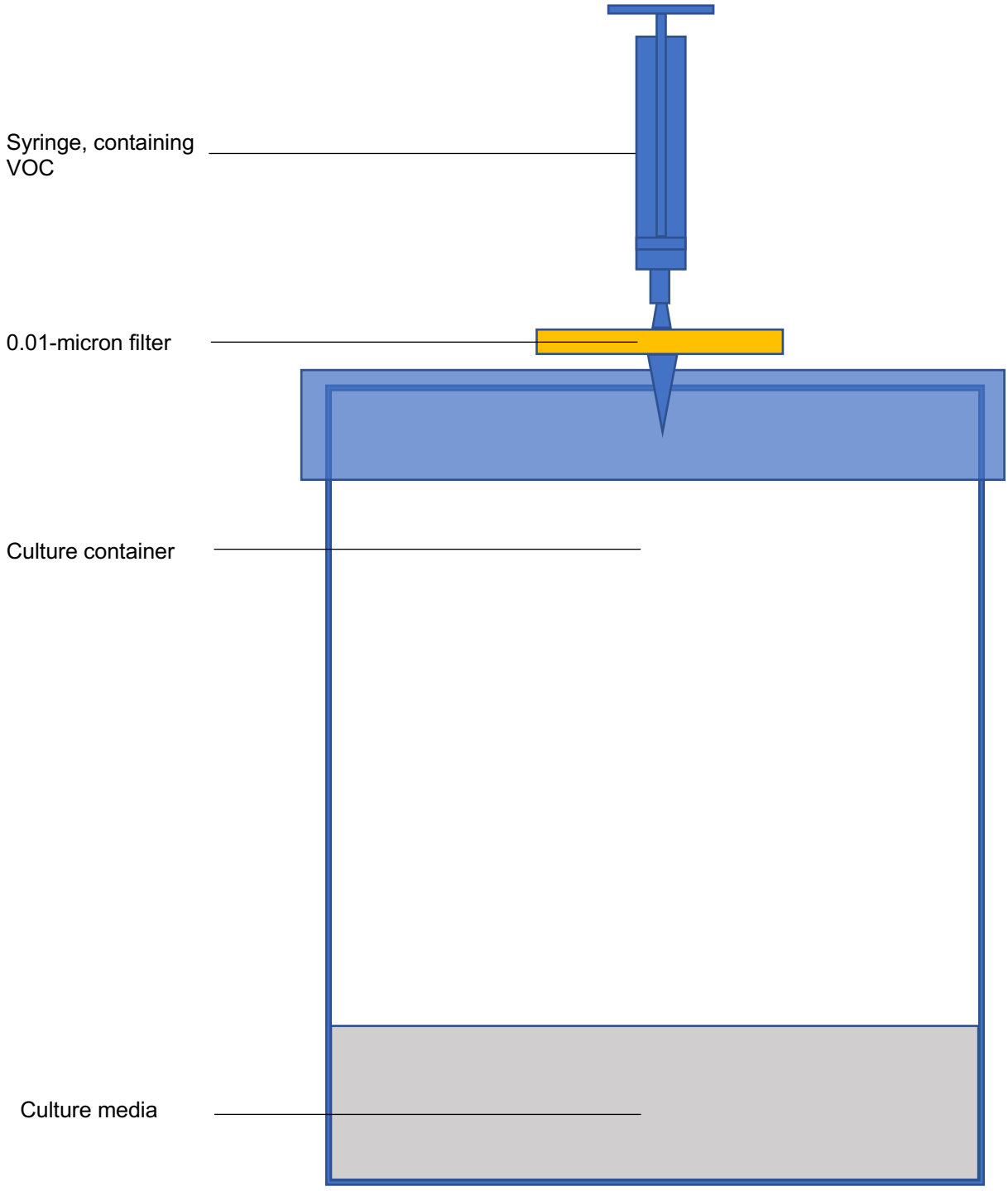
Annexure C: Catalase activity standard curve obtained for the absorbance of the red quinoneimine dye versus the amount of H_2O_2 at standard concentrations of 0 mM, 1.25 mM, 2.5 mM, 5 mM and 7.5 mM

Annexure D



Annexure D: SOD standard concentration curve of 0.05-10 U/mL SOD used to calculate SOD Activity as an inhibition rate (%) of WST dye formation.

Annexure E



Annexure E: A proposed solution to exposing plants *in vitro* to a continuous and constant supply of VOCs without having to repeatedly open and close the culture containers.