UNIVERSITY OF ZULULAND

IN VITRO MUTAGENESIS AND PRELIMINARY CHARACTERIZATION OF POTENTIAL DROUGHT TOLERANT SUGARCANE LINES

By

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DECLARATION

I, **Philani Justice Dlamini**, declare that this dissertation is entirely my own work. It has not been taken from the work of others except where I have appropriately acknowledged and referenced the original sources. This dissertation has never been submitted for any degree for examination in any university. I further acknowledge that I have read and understood the university's policies and rules applicable to postgraduate level, and I certify that I have, to the best of my knowledge and belief complied with their requirements. The final content of this dissertation remains entirely my responsibility.

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I certify that this statement is correct.

Dr. NR Ntuli

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ABSTRACT

Drought is a major cause of yield loss in agriculture and its impacts are expected to increase due to future increases in temperature. Traditional breeding in sugarcane is constrained by sugarcane's complex genome and an extensive breeding period. Tissue culture-based mutagenesis and selection is one of the most feasible and cost-effective tools for developing and selecting for drought stress-tolerant plants. Ethyl methanesulfonate (EMS) can increase genetic variability in the sugarcane genome resulting in the initiation of advantageous traits such as drought tolerance. Therefore, its potential application was investigated in this study. Eight-week-old sugarcane calli were exposed to 16 mM EMS for 0.5, 1, 2, and 3 hours to determine the optimal incubation period. To select for osmotic tolerance, calli were cultured on media containing 0, 150, 225 and 300 mM mannitol for eight weeks. Treatment of the callus with EMS for a minimum of 1 h resulted in a significant decrease in the average number of embryonic callus clump, callus fresh weight, and relative growth rate. Based on the embryo regeneration results, an incubation period of 1 h was optimum for inducing osmotic tolerance in sugarcane. The mannitol LD_{50} and LD_{90} concentrations for selection at embryo germination stage were determined at 224 and 407 mM mannitol, respectively. Under mannitol stress conditions, inhibition of callus growth and increase in abnormalities such as hyperhydric cells with an increase in water stress were observed. Six NCo376 putative osmotic tolerant mutants were isolated from the EMS-mutagenized callus and were *in vitro* characterized based on their physiological response to mannitol-induced stress. *Mut1* had the highest re-rooting (RR) ability under mannitol stress and *Mut4* had the highest relative water content (RWC). *Mut2* and *Mut6* demonstrated poor RR and RWC response and were characterized by high electrolyte leakage and H_2O_2 activity. Only *Mut1* and *Mut4* were the promising drought-tolerant mutants due to their better physiological response under mannitol stress. This study provides a preliminary *in vitro* screening method for osmotic stress tolerance based on sugarcane callus morphology at LD_{50} = 224 and LD_{90} = 407 mM mannitol, respectively and plantlet re-rooting at 332 mM mannitol. Future work will screen selected mutant lines in pots and under field conditions to confirm tolerance as *in vitro* screening may not completely eliminate escapes and may alter important agronomic traits.

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DEDICATION

To my mother, Mrs Thandukwazi Nelly Dlamini;

My daughter, Nokukhanya Melokuhle Thandokuhle Dlamini and

My late grandmother, Mrs Mphangile Eselina Ntshangase.

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CONFERENCE PRESENTATION

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ABBREVIATIONS

%	percentage
°C	degrees Celsius
2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	analysis of variance
CIM	callus induction media
DAB	3,3'-diaminobenzidine
DSM	osmotic tolerance selection media
DW	dry weight
EMS	ethyl methanesulfonate
FW	fresh weight
$g\ L^{-1}$	grams per litter
g	grams
h	hour(s)
H_2O_2	hydrogen peroxide
HSP70	heat shock protein 70
INTOL	index of tolerance
KCl	potassium chloride
KOH	potassium hydroxide
LD_{50}	50% lethal dose
LD_{90}	90% lethal dose
$mg\ g^{-1}$ FW	milligram per gram of fresh weight
mM	Millimolar
mm^2	square millimetre
MS media	Murashige and Skoog media
Mut	mutant(s)
PC	principal component
PCA	principal component analysis
PEG	polyethylene glycol
PEM	plantlet establishment media
R	correlation coefficient
R^2	coefficient of determination

RGR	relative growth rate
RM	rooting media
ROS	reactive oxidative species
RR	re-rooting ability
RWC	relative water content
spp.	species
WT	wild-type

CHAPTER 1

1 INTRODUCTION

Sugarcane (*Saccharum* s*pp.*) belongs to the Poaceae family (dos Santos and de Almeida Silva, 2015). Sugarcane is commercially produced for sucrose extraction (Zhou, 2013), accounting for more than 75% of sugar produced globally (Snyman, 2004). Moreover, sugarcane is also used as a source of biofuel, fertilizers, compost, and paper (Scortecci *et al.*, 2012). The industry, with estimated revenues of R12 billion in South Africa, is threatened by biotic and abiotic stresses such as drought, salinity, pests and diseases (Joubert, 2010; SASA, 2015). This affects approximately one million people that depend on sugarcane for a living; a significant percentage of the total agricultural labour force (Joubert, 2015).

Despite biotic and abiotic stress challenges, the sugarcane industry is also facing competition for water due to increases in demand by industry and the encroaching general population (dos Santos and de Almeida Silva, 2015). There is a competition between agriculture and industries when allocating water, where agriculture is often on the losing side because of the low economic return when compared with other industries (Acquaah, 2012). The use of supplementary irrigation results in an increase in the production costs (Singels *et al.*, 2016). This threatens the South African sugarcane industry as it has become cheaper to import sugarcane products from Brazil. All these reasons emphasize an urgent need to improve sugarcane varieties for water use efficiency and drought tolerance (Ferreira *et al.*, 2017).

Genetic variability is the main driving force for adaptive responses against drought and other stresses (Scortecci *et al.*, 2012). Conventional breeding has been primarily used to induce genetic variability in sugarcane for variety improvement (Zhou, 2013). However, its progress is limited by the sugarcane's complex genome (Butterfield *et al.,* 2001) and unfavourable South African climatic conditions for fertile pollen production (Zhou, 2013). Efforts have been made with the application of transgenic technologies such as insertion of drought and salinity tolerance genes, which include the transfer of heat shock proteins (HSPs) from *Erianthus* to sugarcane (Augustine *et al.*, 2015a). However, the genetic modification approach faces challenges that include regulatory

barriers for commercialisation, ethical and biosafety concerns and negative market perceptions of genetically-modified crops (Butterfield *et al.,* 2001). As a result, neither conventional breeding nor transgenic technologies provide an easy solution for sugarcane breeding.

Mutation breeding is one of the alternative approaches that improves genetic variability in sugarcane (Munsamy *et al.*, 2013). Genetic variation is induced by using chemical (Koch *et al.*, 2012) and physical (Suprasanna *et al.*, 2009) mutagens. Physical mutagens (irradiation) have been the most frequently used for crop development (Maluszynski *et al.*, 2000), but they are costly and require specialized equipment (FAO, 2016). Chemical mutagens such as ethyl methanesulfonate (EMS) have thus gained attention because they are relatively cheap and easy to use in comparison with physical mutagens (Kumar *et al.*, 2016). EMS induces single, randomly distributed nucleotide mutations in DNA (Koetle *et al.*, 2018)

In sugarcane, EMS mutagenesis is induced in callus tissues (Khalil *et al.*, 2018). This is followed by a high-throughput screening based on phenotyping of a large number of calli pieces (Manchanda *et al.*, 2018). The difficulty of callus screening lies with the loss of regenerative potential (Masoabi *et al.*, 2017) and high rate of escapes (Koch *et al.*, 2012; Rutherford *et al.,* 2017). Consequently, there is lack of correlation between tolerances of cells in culture versus at whole plant level (Snyman *et al.*, 2016). As a result, only about 16% of EMS-derived osmotic tolerant putative mutant are tolerant when screened *Ex vitro* in pots (Masoabi *et al.*, 2017). High humidity levels (~100%) and the length of the selection period may contribute to giving false positive results when screening under *in vitro* conditions for osmotic stress tolerance (Snyman *et al.*, 2016; Masoabi *et al.*, 2017).

The development of high throughput and efficient stress selection systems to identify potential mutants that display desirable phenotypes is required. Intensive work has been conducted to identify physiological traits that can be used as the standards to select for osmotic tolerance (Snyman *et al.*, 2016). However, mechanisms underlying plant defence responses to water deficit is difficult to understand because resistance to osmotic stress is a multigenic trait (Manners *et al.*, 2004). In addition, sugarcane physiological and biochemical responses to osmotic stress are not easy to evaluate at

the whole plant level because differentiated organs contain several cell types and ages that display contrasting sensitivities to osmotic stress (Lutts *et al.,* 2004). Consequently, the establishment of *in vitro* conditions to introduce genetic variability and selection for osmotic tolerance in sugarcane is vital. Moreover, the assessment of various physiological parameters as indicators of osmotic tolerance under *in vitro* conditions can contribute to developing efficient selection systems for osmotic tolerance in sugarcane to minimize escapes.

1.1 PROBLEM STATEMENT

Drought stress (osmotic stress) is a major cause of yield loss in sugarcane production, and the frequency of its occurrence is expected to increase in future because of temperature increases caused by climate change. Despite the advancements in conventional breeding and transformation technology, developing sugarcane for osmotic tolerance remains a major challenge. This is caused by the complexity of sugarcane genome and of the plant responses to osmotic stress, as well as the difficulty to identify and exploit physiological and morphological traits that can be used to select for osmotic-tolerant varieties suitable for commercial production. The efficient use of integrated biotechnological techniques such as *in vitro* culture and mutation breeding holds the advantage of inducing genetic variation and the screening of multiple mutant lines for osmotic stress tolerance. This also minimizes the number of years and space needed for conventional breeding.

1.2 AIM OF THE STUDY

The aim of this study was to utilize mutation breeding to produce osmotic-tolerant sugarcane and to study biochemical and physiological defence mechanisms displayed by the selected mutant lines under *in vitro* conditions.

1.3 OBJECTIVES OF THE STUDY

To address the aims of the study the following objectives have been set:

- To determine the effect of EMS incubation period callus growth and embryogenic proficiency in sugarcane.
- To determine mannitol lethal doses that inhibit callus growth by 50% (LD_{50}) and 90% (LD_{90}) for *in vitro* osmotic stress selection.
- To use plant physiological parameters to discriminate mutant lines at callus and plantlet level.

1.4 RESEARCH QUESTIONS

- What effect will an increase in EMS incubation period have on sugarcane callus?
- What are the different mannitol concentrations (LD_{50} and LD_{90}, respectively) that can be used to screen for osmotic tolerance in sugarcane mutants?
- How will the the EMS-treated callus and plantlets respond physiologically under osmotic stress?

1.5 HYPOTHESES

- Higher EMS incubation periods will inhibit callus growth and embryogenic proficiency.
- Higher mannitol concentrations will be more efficient for the selection of osmotic-tolerant mutant lines.
- EMS treatment will cause both callus and plantlets to display improved physiological responses under osmotic stress.

1.6 DISSERTATION OUTLINE

Chapter one contains the general introduction, aims, objective, research question, hypotheses as well as the structure of the dissertation.

Chapter two integrates a review of literature about the origin of the modern sugarcane cultivars and implications of its complex genome on breeding programs. It critically reviews the biological effects of osmotic stress on sugarcane growth in a way that bridges the signalling networks with physiological and genetical defence mechanisms. Special emphases are given to the technical challenges and prospects associated with the practical application of various breeding techniques used for sugarcane improvement. Chapters three, four and five each have the introduction, materials and methods, results, discussion and conclusion.

Chapter three, the effects of varying ethyl methanesulfonate incubation periods in order to generate mutant lines that are tolerant of mannitol-induced osmotic stress are tested. The lethal dose of mannitol was determined in order to screen embryonic calli tolerance to osmotic stress during shoot formation.

In **Chapter four,** a series of physiological parameters on *in vitro* selected plantlet lines (*Mut1*, *Mut2*, *Mut3*, *Mut4*, *Mut5* and *Mut6*), including water content, re-rooting, electrolyte leakage, and hydrogen peroxide activity, under mannitol-induced osmotic stress were used to confirm tolerance in the selected mutant lines.

Chapter five contain the general conclusions and recommendations for future work

CHAPTER 2
LITERATURE REVIEW

2.1 SUGARCANE TAXONOMY, DISTRIBUTION AND PRODUCTION

Sugarcane (*Saccharum* spp. hybrids) belongs genus *Saccharum*, in the grass family Poaceae or Gramineae (Moore *et al*., 2013). Sugarcane taxonomy is complicated due to its close relationship with other genera (Kumar and Singh, 2012). Sugarcane refers to six of the 37 species of tall perennial grasses that belong to the genus *Saccharum* (Mnisi and Dlamini, 2012). These hybrid species are *Saccharum barberi*, *S. edule*, *S. officinarum, S. spontaneum, S. sinense,* and *S. robustum. Saccharum* spp., with a centre of origin believed to be in India and Indo-Burma (Kumar and Singh, 2012).

More than 26.9 million hectares of land in the tropical and subtropical regions of the world are used for sugarcane production. Global production of sugarcane is estimated to be ~1.9 billion tons (70.9 tons per hectare) (FAOSTAT, 2015). Brazil is the leading sugarcane producing country that harvests nearly 618 million tons of sugarcane, a significant percentage of global fresh cane (dos Santos and de Almeida Silva, 2015). Brazil sugarcane production capacity is attributed to its geographic location with a predominant tropical climate (Moore *et al*., 2013). South Africa is ranked the 13th largest sugarcane producer of about 100 sugarcane producing countries (Mnisi and Dlamini, 2012; FAOSTAT, 2015). Sugarcane is predominantly produced in the KwaZulu-Natal province followed by Mpumalanga and Eastern Cape provinces (Joubert, 2015).

South Africa produces an average of 19.9 million tons of sugarcane annual and an average of 2.2 million tons of sugar is produced from it (SASA, 2015). The local demand for sugar is approximately 1.7 million tons per year (Singels *et al*., 2016). The sugarcane industry employs more than 2% of the South African population (SASA, 2015). A substantial amount of the population of KwaZulu-Natal and Mpumalanga provinces depends on the sugar industry for their income (SASA, 2012). In a total of 23 426 sugarcane growers in KwaZulu-Natal, 21 121 are small-scale farmers on the communal land and 1 305 are large-scale commercial farmers (Joubert, 2015).

2.2 SUGARCANE GENETICS

Parental species of sugarcane has a complex genome that originated from a large breeding pool of five closely related genera, namely; *Saccharum* species (*S. officinarum, S. spontaneum and S. robustum*), *Erianthus* species, *Miscanthus* species, *Narenga* species, and *Sclerostochya* species (Scortecci *et al.*, 2012). The *Saccharum* species ploidy level ranges from 5x to 16x (Manners *et al.*, 2004). The chromosome number of modern commercial cultivars ranges between 2n = 99 and 130 (Butterfield *et al.*, 2001). Consequently, the sugarcane genome is referred to as the '*Saccharum* complex' due to the inter-breeding group of ancestors that resulted in the unbalanced chromosomes of the modern commercial cultivars (Rutherford *et al.*, 2014).

Sugarcane parental species contain a diversity of genome content, structure and allelic variation (Manners *et al.*, 2004). The basic chromosome number of *S. officinarum* is 10 and of *S. spontaneum* is 8 (Grivet and Arruda, 2002). The chromosomal structural differences in the genomes of these two species (*S. officinarum* and *S. spontaneum*) cause meiotic instability and the production of aneuploid gametes (2n–n) (Rutherford *et al.*, 2014). As a result, some commercial cultivars have sterile seeds (Butterfield *et al.*, 2001) and narrow gene pool (Kumar and Singh, 2012), thus they are mainly propagated vegetatively. Sugarcane's high gene-copy number and complex genome have limited the understanding of how its genome functions and the prospect of annotating its genome (Manners *et al.*, 2004).

2.3 DROUGHT

Drought stress, signified by water deficit in relation to crop production, is divided into meteorological drought and agricultural drought (Chinnusamy and Zhu, 2009). Meteorological drought specifies precipitation deficit over the long term, whereas agricultural drought occurs when the amount of soil moisture is below the adequate level for growth and yields (Rai *et al.*, 2011). Drought results in either reduced or complete loss of yield because of water importance in plant physiology (Akıncı and Lösel, 2006). It accounts for over 50% of yield losses (Wani *et al.*, 2010) and its impact is expected to increase as temperatures increase due to climate change (Zingaretti *et al.*, 2012).

Irrigation is the primary means of addressing drought (Yamaguchi and Blumwald, 2005). About 60% of the sugarcane producing areas in South Africa require irrigation (Figure 2.1), where in countries such as Swaziland sugarcane cannot grow without irrigation (Zingaretti *et al.*, 2012). However, irrigation results in an increase in farm operational costs and water reservoirs dry out during long-term drought (Singels *et al.,* 2016). Other measures that are used to mitigate the economic effect of drought on farmers such as increasing recoverable value are not sustainable because they result in high prices in sugarcane products, and thus threatens the local industry (Singels *et al.,*2016).

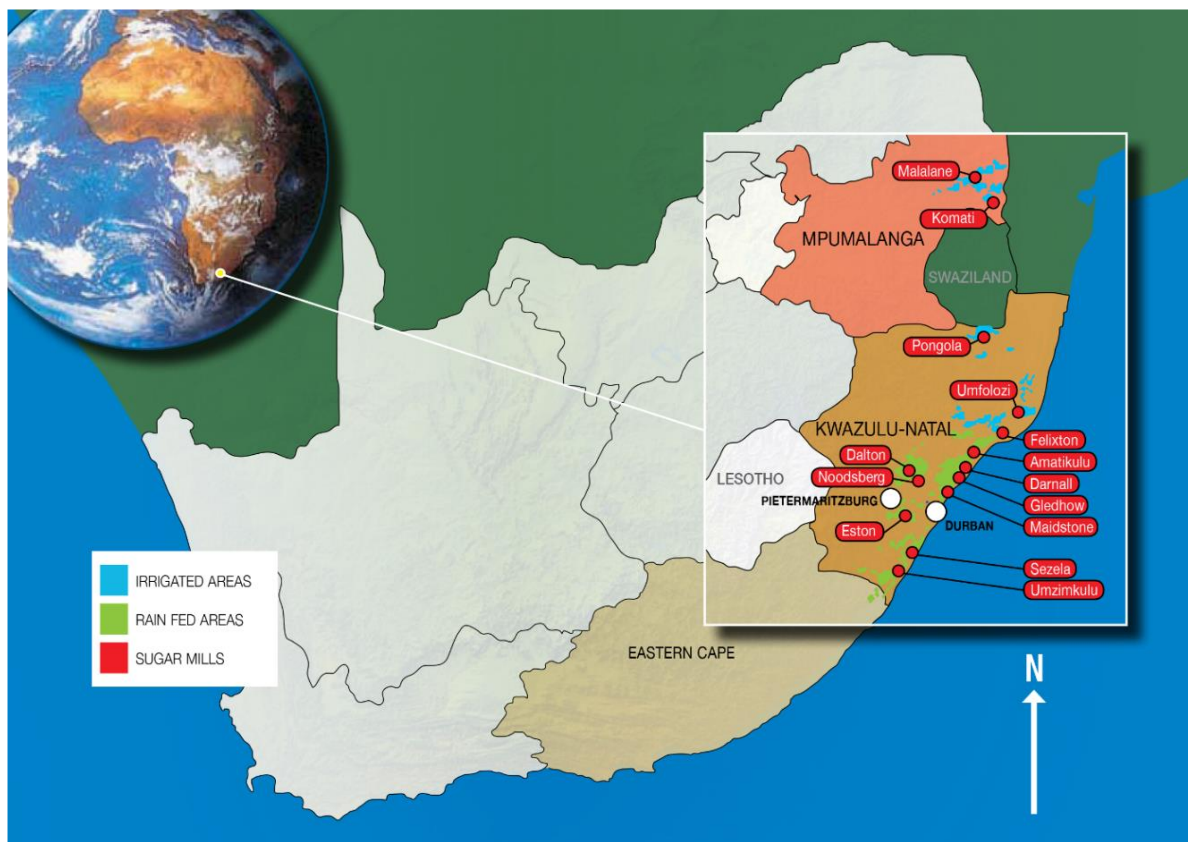

Figure 2.1 Map showing sugarcane growing regions in South Africa, rainfed indicated by green and irrigated indicated by blue (SASA, 2012).

Drought is often accompanied by an increase in soil salinity and excessive heat which results in evaporation (Li and Assmann, 2010) and upwelling of underground water that reduces the water table (Morris and Tai, 2004). High temperatures decrease water

availability for plant use through excessive evapotranspiration (de Carvalho *et al.*, 2015). Soil salinity reduces the amount of available water to the plant roots (Li and Assmann, 2010). In saline soils, drought may occur even under irrigation as high external salt concentration results in plant cell dehydration (Yamaguchi and Blumwald, 2005). It is estimated that by 2050 about 50% of arable land worldwide will become saline (Kan *et al.*, 2015). This will result from drought, excessive fertilizing (Yamaguchi and Blumwald, 2005) and irrigation with metal contaminated water (Peleg *et al.*, 2011).

2.3.1 Effect of drought stress on plant morphology

Severe water stress (drought) results in changes in plant morphology because of damages that occur in the cellular components or as a mechanism of defence (Murphy, 2011). Sugarcane primarily responds to drought by leaf rolling, which reduces the leaf surface area for light absorption and water loss (Inman-Bamber and Smith, 2005). This is followed by the cell wall shrinking and leaf senescence (Waraich *et al.,* 2011). Drought tolerant sugarcane cultivars have different stress tolerance/avoidance mechanisms, where cultivars N41 and N26 strive under mild droughts; N12 rolls its leaves to minimize its leaf surface area; and N19 closes leaf stomata and stop growing until soil moisture content is optimal again (Snyman *at al.,* 2016).

Sugarcane roots grow laterally in moist soils, but when the soils have low soil moisture content, roots grow deep and do not spread evenly (Waraich *et al.*, 2011). Yield is reduced because of root structural changes, caused by nutrients deficiencies as the primary function of lateral roots is to absorb nutrients (Yamaguchi and Blumwald, 2005). Furthermore, nutrients from other parts of the plant (leaves and stem) are re-allocated to the roots for growth thus the plant shoot development is affected (Inman-Bamber and Smith, 2005). Under *in vitro* conditions, the early root formation is an indication of sugarcane plants that are tolerant to herbicide stress (Koch *et al.*, 2012) and fungi stress (Mahlanza *et al.*, 2013). Again, drought tolerant genotypes are categorized by their ability to re-root when tested under *in vitro* osmotic stress conditions (Snyman *et al.,* 2016). Drought tolerant genotypes have a long root system compared with susceptible genotypes when tested under field conditions (Kumar *et al.*, 2017).

2.3.2 The drought stress on plant physiology

When the soil water is reduced for plant absorption, the osmotic potential in plant tissues is reduced (Rai *et al.*, 2011). As a result, plant physiological activities change to adapt (Kan *et al.*, 2015). These changes include the reduction in photosynthetic rate, inadequate nutrient transport and changes in hormones (Li and Assmann, 2010). The main contributing factors to the reduced of photosynthetic rate during drought include; (i) stomatal closure that aims to limit transpiration (Inman-Bamber and Smith, 2005); (ii) damages to chloroplast by reactive oxidative species (ROS) (Li and Assmann, 2010); and (iii) reduction in plant relative water content (RWC) (Kantar *et al.*, 2011).

In sugarcane, reduction on biochemical activities in the mesophyll and bundle sheath cells such as phosphoenolpyruvate carboxylase (PEPCase), RuBisCo, malic enzyme (NADP-ME), fructose-1, 6-bisphosphatase (FBPase) and pyruvate orthophosphate dikinase (PPDK) are amongst the reasons for photosynthesis reduction under severe water stress. This results in a reduction of sugar accumulation and plant biomass because photosynthesis is the key process underpinning sugar production in plants (Sage *et al.*, 2013).

2.4 PLANT DEFENCE MECHANISM TO DROUGHT

The physiological and morphological changes are driven by the genetics of the plant (Ciarmiello *et al.*, 2011). Drought causes more than 1000 changes in gene expression of *Arabidopsis* and rice. These changes are a survival strategy that is employed by plants to escape unfavourable conditions for growth. Reactive oxidative species (ROS), calcium, methyl jasmonate, trehalose and volatiles such as abscisic acid (ABA) are signals during drought stress that are genetically controlled (Geisler, 2010).

2.4.1 ABA reaction during drought stress

Hormones are important chemical messengers that are vital to plant growth and are fundamental components that regulate plant responses to drought stress (Huang *et al.*, 2008). The first signal released during drought stress is abscisic acid (ABA) which is synthesized in plant roots (Li and Assmann, 2010) and transported through the xylem

to the shoots (Peleg *et al.*, 2011). The common role of ABA is to regulate stomatal closure and opening during drought stress. Stomata primary function is to allow gaseous exchange, specifically carbon dioxide and oxygen (Murphy, 2011). However, during gaseous exchange plants also lose water in a form of vapour in a process known as transpiration (Huang *et al.*, 2008). Therefore, it is important during water limiting conditions for plants to reduce water losses by closing stomata (Sage *at al.,* 2013). In times of water stress plants regulate stomatal opening and closing by reserving signals from ABA (Geisler, 2010).

About two-thirds of gene expression during drought stress are ABA-regulated (Huang *et al.* (2008). ABA induces cytosolic calcium ions (Ca^{2+}) by activating membrane Ca^{2+} permeable channels (Geisler, 2010). This allows for ion exchange that results in the shrinking and swelling of guard cells, which drives the opening and closing of the stomata (Daszkowska-Golec and Szarejko, 2013). The three main key roles of ABA in plant growth during water stress include: (i) safeguarding the day-night interchanges of leaf growth rate; (ii) regulating stomatal movement to counteract the negative effect of transpiration; (iii) and improving plant tissue hydraulic conductivity that results in improved leaf growth (Sage *et al.*, 2013). The effect of other drought signals such as cytokinin, ethylene, and jasmonates are lesser than the activities of ABA during drought (Geisler, 2010).

2.4.2 Reactive oxygen species

Reactive oxidative species (ROS) are overproduced during drought (Geisler, 2010). In this case, ROS production is caused by over assimilation of carbon due to lack of reactants for photosynthesis (water), which results in the insufficient dissipation of electrons produced during the electron transport chain (Zingaretti *et al.*, 2011). The products of ROS are superoxide radicals (O^{-}_{2}), hydrogen peroxide (H_2O_2) and hydrogen radicals ($OH^{\cdot}$) (Rai *et al.,* 2011). Reduced yield, necrosis, and damage to cell components such as chlorophyll, lipids, proteins, and nucleic acids are likely to occur under severe drought due to the overproduction of ROS (Rai *et al.,* 2011; dos Santos and de Almeida Silva, 2015).

Despite the damage ROS can cause, ROS has also been described as a secondary messenger in response to most stressors (Geisler, 2010). H_2O_2 activates Ca^{2+} and potassium ions (K^+) on the plasma membrane of roots cells (Demidchik *et al.*, 2003). The role of Ca^{2+} is to regulate stomatal closure and opening during water limiting conditions (Li and Assmann, 2010). H_2O_2 also activate plant osmoprotectant such as small ubiquitin-related modifier (SUMO) during drought and heat stress (Hanumappa and Nguyen, 2010; Survila *et al.*, 2010).

Upper regulation of anti-oxidants counteracts the negative impact of ROS (Murphy, 2011). In sugarcane, superoxide dismutase (SOD) has been reported as the antioxidant enzyme with a well-defined role during drought stress. An increase of ROS results in an increase of SOD to counteract the impact of the ROS during drought stress (Rai *et al.,* 2011). Other antioxidant enzymes that are found in plants include ascorbate peroxidase (APX), catalase (CAT), glutathione-S-transferase (GST), polyamines, ascorbate (ASC) and phenolic compounds (Abbas *et al.,* 2014).

2.4.3 Drought stress-regulated genes and protein

During drought stress, several genes are regulated, either by being overexpressed (up-regulated) or suppressed (Geisler, 2010). A family of proteins known as heat shock proteins (HSP) play a central role in plant response to environmental stressors (Murphy, 2011). Heat shock protein 70 (HSP70) is up-regulated during drought, salinity, and heat (Geisler, 2010). Transgenic studies that utilize *Saccharum* spp. (C86032) show that HSP70 is overexpressed more than common drought-responsive genes such as dehydration responsive elements (Augustine *et al.*, 2015a). In addition, HSP70 overexpression enhances high membrane thermostability, relative water content, gas exchange parameters, chlorophyll content and photosynthetic efficiency (Augustine *et al.*, 2015a).

The common occurrence of heat and drought stress in the field limits HSP's activity (Li and Assmann, 2010). High temperatures often cause dysfunction and denaturing of functional protein including HSP during drought stress (Survila *et al.*, 2010; Wang *et al.*, 2014). Consequently, plants have developed multiple linked stress (drought and heat) response mechanisms that play complementary and sometimes overlapping

roles in protecting proteins and other plant organs from stress (Murphy, 2011). In such cases, denatured HSP's are functionally replaced by small ubiquitin-like modifiers (SUMO) (Larkindale *et al.*, 2005), which are up-regulated when HSP's are denatured (Hanumappa and Nguyen, 2010). Again, when plants are overexpressing HSP70, SUMO is suppressed (Larkindale *et al.*, 2005).

Another family of proteins known as late embryogenesis abundant (*LEA*) protein is encoded by the ubiquitin group of stress targeting genes (Survila *et al.*, 2010). *LEA* proteins are found in seeds and vegetative tissues (Survila *et al.*, 2010) and in sugarcane they are found in leaf tissues (Hanumappa and Nguyen, 2010). *LEA* proteins are characterized by their hydrophilic nature, which gathers and binds water molecules during drought (Sugiharto *et al.*, 2002). Dehydrin proteins enhance the plant's ability to hold water and ions within its cell surface, as result drought tolerant plants have high relative water content compared with susceptible plants (Augustine *et al.*, 2015a).

2.4.4 Proline as drought stress osmoprotectant

Amino acids play an important role in cell and plant survival during drought (Errabii *et al.,* 2006). At present, the most studied amino acid for abiotic stress tolerance is proline (Blum and Ebercon, 1976; Verbruggen and Hermans, 2008). Proline is increased by 100-fold under stress compared with normal growing conditions (Verbruggen and Hermans, 2008). Its accumulation is part of stress signalling regulating adaptive responses (Maggio *et al.*, 2002). However, proline accumulation also occurs as a complementary source of carbon and nitrogen during drought, where carbon availability becomes limited due to stomatal closure and consumption of NADPH by the Calvin Cycle (Sofo *et al.*, 2004). Therefore, its accumulation serves as a buffer to maintain cytosolic pH (Verbruggen and Hermans, 2008). Proline accumulation also lessens protein denaturation and it maintains cell redox levels (Maggio *et al.*, 2002).

Proline is used as a drought stress selection marker in some studies (Alvarez *et al.,* 2003; Ehsanpour and Fatahian, 2003). *In vitro* studies that characterize proline role in sugarcane during water stress indicate that it is a stress tolerance characteristic, where it is expressed in response to cell injury caused by ROS overaccumulation (Errabii *et al.,* 2007). However, the role of proline accumulation does not have a significant impact

on osmotic adjustment when compared with other plant adaptive responses (Mohamed *et al.*, 2000). In addition, external application of proline increases ROS production, reduces growth and induces a number of stress-responsive genes (Blum and Ebercon, 1976).

2.5 BREEDING FOR DROUGHT STRESS TOLERANCE

2.5.1 Conventional breeding

Conventional breeding has been used to improve variation and tolerance to environmental stress in sugarcane from as early as 1880 (Abbott, 1953). Conventional breeding is driven by Mendelian genetics, the random inheritance of parent’s traits to offspring. The inherited traits are determined by the genes present in the reproductive cells (gametes) of the parents united during fertilization (Hartley and Jones, 2005). To obtain progeny with desired characteristics, plant breeders often cross-pollinate parental lines with traits of interest (Zhou, 2013). After crossing, the offspring genetic and phenotypic variation results from genetic recombination, different chromosome number, mutation and the environment (Murphy, 2011). High yield/sucrose cultivars have been obtained through conventional breeding over the last 50 years (Zhou, 2013). In sugarcane there are more than two genes for each trait resulting in offspring's traits being highly unpredictable (Snyman, 2004), consequently resulting in long breeding and selection periods (Zhou, 2013).

Furthermore, most sugarcane genotypes flower poorly because of the meiotic instabilities (Butterfield *et al.*, 2001). While genotypes that are able to flower produce small and unusable seed (Poehlman and Sleper, 1995). Night time temperatures exceeding 20°C and 70% relative humidity are important for flower initiation (Moore *et al.*, 2013). In South Africa, the flowering period occurs in winter where the temperatures are below 20°C (Zhou, 2013), as a results some genotypes do not flower (Cheavegatti-Gianotto *et al.*, 2011). Flowering days also vary among different genotypes that are able to produce flowers. For these reasons, flowering is artificially stimulated in a controlled environment which is met by the use of glasshouse (Zhou, 2013). The glasshouse crossed hybrids are distributed to different growing regions for further selection processes (Ramburan, 2012). As result, conventional breeding is costly, time-

consuming, requiring a huge amount of space for a selection of progeny and has the disadvantage of introducing undesired traits by gene-linkage (Butterfield *et al.*, 2001).

2.5.2 Genetic engineering

Transgenes or genetic modification (GM) refers the transfer of the gene(s) of interest from one plant of the same or different species to another plant for improving traits of the recipient plant (Chen *et al.*, 1987). During this process, a known gene(s) that is responsible for the expression of the trait of interest is isolated, and the exogenous DNA is introduced into a target plant cell(s) (Augustine *et al.*, 2015a). The effectiveness of this method is attributed to the individual transfer of trait(s) of interest, resulting in faster crop improvement than most breeding approaches (Ribas *et al.*, 2011). In sugarcane, transgenic approaches commonly used include *Agrobacterium tumefaciens* facilitated transfer (Hernandez, 2000), cell electroporation (Arencibia *et al.*, 1995) and particle bombardment (Allsopp and Manners, 1997). Traits that have been improved using gene transfer in sugarcane include drought resistance (Augustine *et al.*, 2015a), disease resistance (Lakshmanan *et al.*, 2006), herbicide resistance (Gallo-Meagher and Irvine, 1996), pest resistance and an increase of sucrose content (Suprasanna *et al.*, 2011).

At present, there is no commercially available GM sugarcane in South Africa. The first commercial GM sugarcane cultivar was approved in 2017 in Brazil; it is tolerant to sugarcane borer pest (Phillips, 2017). This highlights the slow process of commercializing sugarcane transgenes attributed to public concerns for GM crops (Birch, 1997). Application of GM approach in sugarcane is also limited by gene silencing and limited information regards the heritability of the transgenes (Suprasanna *et al.*, 2011). In addition, monocotyledons have inefficient transformation system and low cell competence compared to dicotyledons (Snyman *et al.*, 2008).

The advancement of the clustered regularly interspaced short palindromic repeat (CRISPR) technology appears to be a promising alternative for sugarcane breeding (Doudna and Charpentier, 2014). Recently, application of CRISPR in sugarcane demonstrated effectiveness when modifying the cell wall characteristics for increased production of lignocellulosic ethanol (Jung and Altpeter, 2016). Advantages of

CRISPR/Cas9 are associated with its ability to add and eliminate gene(s) with high specificity (Svitashev *et al.*, 2016). For instance, a disease susceptible variety can be converted to a resistant genotype by eliminating the gene responsible for disease susceptibility (Mohan, 2016). However, as far as sugarcane is concerned it is unlikely that CRISPR/Cas9 can be effectively utilized for its improvement as its genome is not yet fully sequenced (Vicentini *et al.,* 2012). In addition, the allopolyploid nature of the sugarcane genome increases repeats resulting in irregularities when editing (Mohan, 2016).

2.6 TISSUE CULTURE AND MICROPROPAGATION

Tissue culture is a collective term used to describe different procedures used to maintain and grow plant cells (callus, cells, protoplasts) and organs (stems, roots, embryos) in aseptic *in vitro* conditions (Hartmann *et al.,* 2001). Cell theory of totipotency drove the early development of *in vitro* tissue culture (Vasil, 2008). Totipotency is the ability of the plant to develop into a whole plant level from a single cell or tissue (Kumar and Loh, 2012). The possible application of *in vitro* culture system in sugarcane was initiated by the production of roots from callus (Nickell, 1964), and later the development of a full sugarcane plantlet from callus tissue (Heinz and Mee, 1969).

In vitro plant cells can undergo dedifferentiation or redifferentiation to enter a new biological program that gives rise to somatic embryos. The establishment of sugarcane plants *in vitro* occurs through organogenesis and somatic embryogenesis routes, both of which have well-established protocols (Snyman *et al.,* 2011). Plants are regenerated either directly from explants with a minimum or absence of callus, or indirectly via a callus stage first (Figure 2.2). Numerous factors that affect somatic embryos have been studied in order to understand the basis of this process and manipulate it to develop and establish efficient plant regeneration protocols as a fundamental step for its biotechnological application (Loyola-Vargas *et al.* 2008).

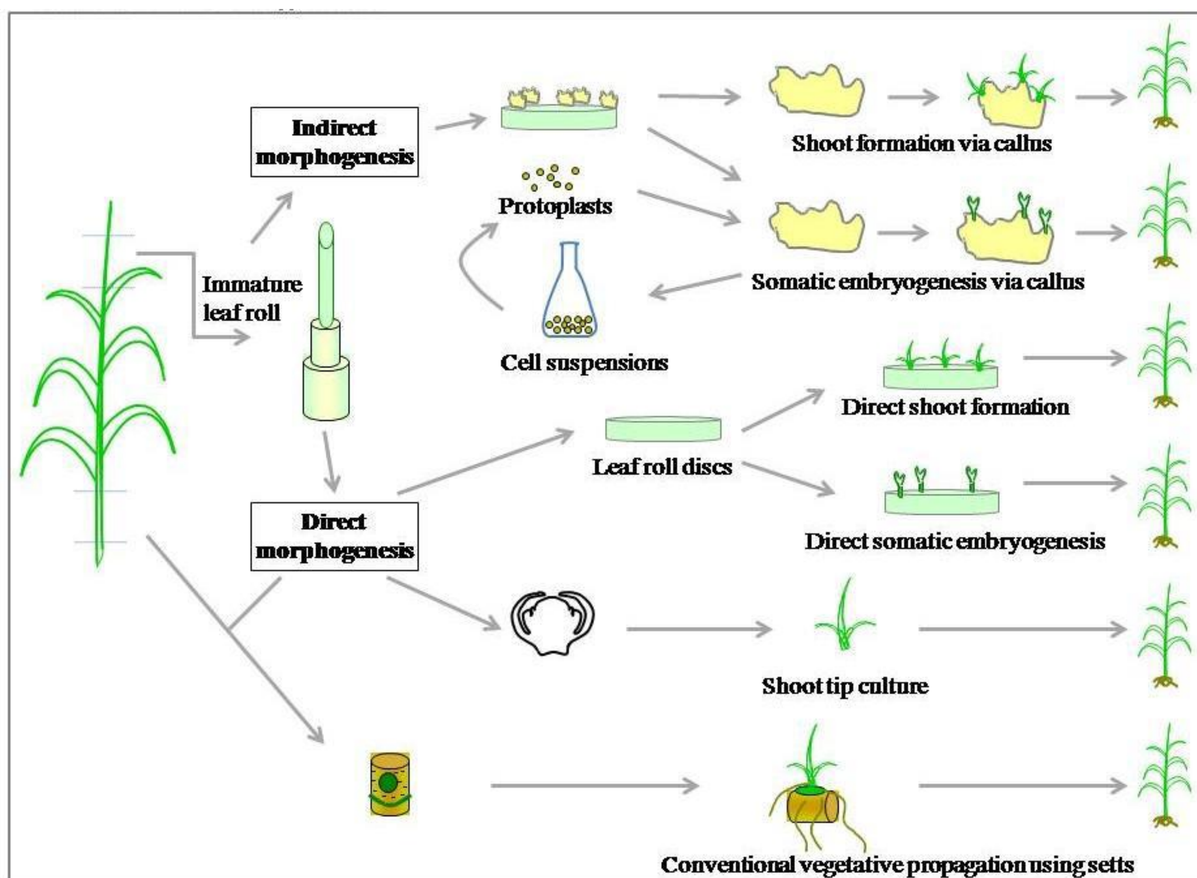

Figure 2.2 Schematic representation of various explant sources and routes that are used on sugarcane *in vitro* culture (Snyman, 2004).

A differentiated and specialized somatic plant cell or a group of somatic cells with specific functions must receive a stimulus from a set of plant growth regulators (PGRs), mainly auxins. This I followed by initiating the transduction to the nucleus where the specific regulatory and structural genes will be transcribed and subsequently will be translated into proteins involved in the differentiation that ultimately will lead to the formation of a new somatic embryo. These changes can be followed at morphological, ultrastructural, genetic, physiological, biochemical, and molecular levels.

2.6.1 Indirect morphogenesis approach

Indirect morphogenesis route refers to the culture of plant cells, tissues and organs, under controlled sterile laboratory conditions (Thorpe, 2007), that allows them to generate masses of undifferentiated cells called callus (Poehlman and Sleper, 1995).

The callus is attained by culturing cut pieces of plant organs on a suitable nutrient medium (Skoog and Miller,1957). A callus is sustained by a continuous sub-culturing of callus tissue to a culture medium (Vasil, 2008). Growth media and culture environmental conditions are manipulated to enhance cells' ability to regenerate into a whole plant (George, 1993). Plants are developed from specific regions of the callus in one of two routes; somatic embryogenesis or by shoot morphogenesis. This is followed by a stage known as organogenesis which is characterized by root development.

Embryogenic callus cultures, produced through the indirect embryogenesis are widely used as a substrate material to administer DNA transfer in sugarcane, specifically when using microprojectile bombardment (Poehlman and Sleper, 1995). The embryogenic callus is intensively used to induce somaclonal variation using mutagens and followed by *in vitro* selection approach for various traits (Koch *et al.*, 2012). In brief, application of indirect morphogenesis is achieved by (a) embryogenic callus initiation and establishment; (b) execution of specific treatments such as virus elimination, selection of variants, genetic transformation, and mutagenesis and regeneration of plants with the desired modifications.

Even though the indirect morphogenesis route has been successful, there are existing constraints. Developing and maintaining of callus cultures is labour-intensive, takes longer, there is variation in transformation efficiency and genotype-dependent responses (Snyman *et al.*, 2011). Some limitations have been observed under field conditions from plants produced through callus culture. There are variations in height, stalk diameter, stalk weight, pest susceptibility and lower sucrose content compared to conventionally propagated plants (Meyer and Snyman, 2011). The above mentioned phenotypic variations disappear between six to nine months (review by Snyman *et al.*, 2011). This variation is caused by DNA methylation as a consequence of *in vitro* stress (Zucchi *et al.*, 2002). Chimera segregation, somaclonal variation due to prolonged exposure to 2, 4-D, epigenetic transients, mutational variants and genetic fidelity also results to phenotypic variations in sugarcane (Larkin and Scowcroft, 1981; Raza *et al.*, 2012).

2.6.2 Direct morphogenic approach

Direct organogenesis involves the regeneration of shoots directly from either apical meristems or immature leaf discs (Snyman *et al.*, 2007) after exposure to at least one cytokinin (6-benzyladenine and kinetin) and an auxin (α-naphthalene acetic acid (NAA)), at a high cytokinin: auxin ratio. This is followed by the induction of rooting in response to auxin (3-indole butyric acid) or with the removal of growth regulators from the medium and supplementation with high sucrose contents. The effect of somaclonal variation is reduced due to the limited use of auxin (Snyman *et al.*, 2000). The most common application of direct morphogenic route is micropropagation (Table 2.1). Micropropagation allows mass replication of quality and disease-free plant material (Snyman *et al.*, 2007), even if it was obtained from diseased plant material (Ramgareeb *et al.*, 2010). Micropropagation is utilized for distribution of commercial seedcane in South Africa known as NovaCane® (Snyman *et al.*, 2018).

Table 2.1 Applications of direct and indirect morphogenesis routes in sugarcane.

Morphogenesis route	Application	References
Direct morphogenesis	Micropropagation	De Arauja Silva (2004)
		Lakshmanan *et al.* (2006)
	Genetic transformation	Taparia et al. (2012a)
		Mayavan *et al.* (2015)
	Germplasm presevation	Watt *et al.* (2009)
		Banasiak and Snyman (2017)
	Virus elemination	Ramgareeb *et al.* (2010)
	Trait selection	Snyman *et al.* (2016)
Indirect morphogenesis	Micropropagation	Behera and Sahoo (2009)
	Genetic transformation	Snyman *et al.* (2001)
		Taparia *et al.* (2012b)
	Germplasm preservation	Watt *et al.* (2009)
	Virus elimination	Ramgareeb *et al.* (2010)
	Mutation breeding	Khan *et al.* (2009)
		Mahlanza *et al.* (2017)

2.7 Mutation breeding

Plant spontaneous mutation is the main driving factor for variation and speciation (Hartley and Jones, 2005). However, spontaneous mutations are slow in nature and occur at random (Murphy, 2011). Mutagenesis provides a powerful technique to improve plant breeding and assist functional and genomic analyses of crop plants (FAO, 2016). Mutagenesis is a process whereby artificial changes are introduced in the plant DNA sequence and chromosome number using agents known as mutagens (Chopra, 2005). Mutagenic agents are of two groups, namely, physical and chemical mutagens (Acquaah, 2012). Mutagenesis can be performed with all types of plant material such as whole plant, usually seedlings, and *in vitro* cultured cells (Oladosu *et al.*, 2016).

Various researchers (Koch *et al.*, 2012; Suprasanna *et al.*, 2012) have been exploring the potential of induced mutation for its application in sugarcane improvement. This has given rise to the premeditated use of chemicals and physical mutagens to improve various traits in sugarcane (Suprasana *et al.,* 2009; Koch *et al.,* 2012). Mutations are classified based on the range of the DNA sequence affected by the mutational event. Small-scale mutation usually involves one or a few nucleotide changes. Small DNA changes include point mutations, deletions and insertions. Large-scale mutations affect the chromosomal structure (Oladosu *et al.*, 2016).

Mutagenesis is most effective when applied in actively dividing meristematic cells (Talebi *et al.*, 2012; Koch *et al.*, 2012). The most commonly used plant material to induce mutation are seeds (Oladosu *et al.*, 2016). However, vegetatively propagated plants such as sugarcane, mutagens are effectively induced from single cells and callus tissues (Suprasanna *et al.*, 2009; Mahlanza *et al.*, 2013). The efficiency of the mutagen on the target material is affected by the mutagen dosage, the pH of the solution, genotype, proper choice of the mutagen, incubation period and the type tissue used (Chopra, 2005; Hofmann *et al.*, 2004; Sega, 1984; Suprasanna *et al.*, 2009).

2.5.4.1 Physical mutagens

Initial attempts used to induce mutations physically in plants include heat treatment, centrifugation and ageing of seeds (Van Harten, 1998). Later, it was determined that the actual cause of mutation are sub-atomic particles such as alpha particles, beta particles, protons and neutrons. Physical agents that result in sub-atomic particles and neutrons include ionizing radiation, X-rays, gamma rays and thermal neutrons (Suprasanna *et al.*, 2009). Ionization radiation is one of the most commonly used physical mutagens. Its neutrons break DNA chemical bonds and result in the deletion or substitution of nucleotides. Physical mutagens account for about 70% of world commercial mutagenic crops (Maluszynski *et al.*, 2000). Application of physical mutagens required specialized equipment, as a result, they are costly. This has led to the adoption of chemical mutagens as an alternative (Oladosu *et al.*, 2016).

2.7.1 Chemical mutagens

Chemical-induced mutations were first reported on *Aspergillus* using mustard gas (1,5-dichloro-3-thiopentone) as a mutagen (Auerbach *et al.*, 1947). Several other chemicals such as alkylating agents, nitroso compounds, base analogues, azide and acridine dyes result in useful mutations in crops (Acquaah, 2012). Among these, alkylating agents such as ethyl methanesulfonate (EMS), ethyl-2-chloroethyl sulfide, 2-chloroethyl-dimethyl, ethyleneimine, diazomethane and N-Ethyl-N-nitrosourea are the most commonly used chemical mutagen group (Acquaah, 2012).

Analysis of DNA bases indicates that chemical mutagens are highly effective (Lodish *et al.*, 2000; Koetle *et al.*, 2018) because they are easier to administer and do not require sophisticated equipment (Koch *et al.*, 2012). However, their effects can also result in chromosomal aberrations in the form of deletions, inversions, and translocations (FAO, 2016). Some chemical mutagens do not alter the DNA sequence, instead, they result in the multiplication of chromosomes. The chemical mutagen, colchicine, does not result in changes to the DNA sequence but results in the multiplication of chromosomes (induced polyploidy). Colchicine treatment as a mutagen result in the improvement of drought-tolerant and agronomic traits in *Coccinia palmata* and *Lagenaria sphaerica* (Ntuli and Zobolo, 2008). Although chemical

mutagens result in more undesirable modifications than physical mutagens, but they are used extensively because they are easier to handle when compared with physical mutagens (Oladosu *et al*., 2016).

2.8 ETHYL METHANESULFONATE

Ethyl methanesulfonate (EMS) (Figure 2.3) is a monofunctional ethylating agent that catalyzes the reaction of the methanesulfonic anhydride with ethyl alcohol (Sega, 1984). Among the advantages of using EMS as a mutagen in crops, it is its ability to induce point mutations at high frequencies (Van Harten, 1998). Deletions, insertion of genes, chromosomal aberration frequencies are low in EMS treated plants and EMS is generally considered non-lethal at intermediate concentrations (Koch *et al*., 2012; Acquaah, 2012).

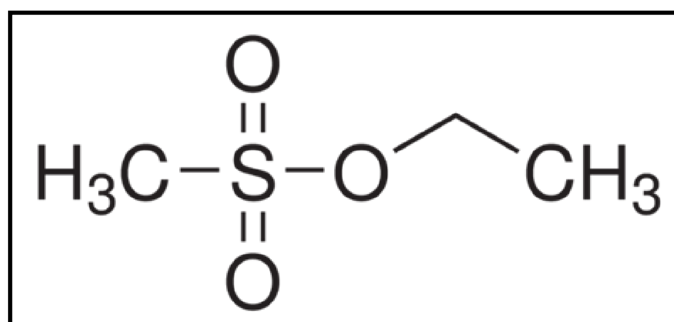

Figure 2. 3 A molecular structure of EMS (Sega, 1984).

2.8.1 Ethyl methanesulfonate point mutation

Point mutations are classified into transversions and transitions groups (Shirasawa *et al.,* 2016). A transversion is when purine is altered to a pyrimidine or a pyrimidine is altered to a purine. Transitions mutations it is when alteration of the nucleotide results to purine for a purine conversion and pyrimidine conversion for a pyrimidine (FAO, 2016). The effect of EMS on DNA often results in a higher percentage of GC to AT mutation (summarized in Figure 2.4) (Sega, 1984). Ethyl methanesulfonate changes the DNA nitrogen base, specifically *N*-7, O^6 *N*-3 and N^2 of guanine (Lodish *et al*., 2000). However, other than guanine, which accounts for about 79% of DNA nitrogenous base

changed by EMS; adenine, cytosine, and phosphate account about 7.4%, 0.6%, and 13% of mutations resulted by EMS, respectively (Sega 1984).

The structure and size of the genome do not affect the effectiveness of EMS mutagenesis. The *Arabidopsis* and *Zea mays* have a similar gene mutational density after EMS treatment even though the *Zea mays* genome size is 20-fold larger than that of *Arabidopsis* (Goll and Bestor, 2002). Among 4629-point mutations detected in tomato after EMS mutagenesis, 69.3% is cytosine/guanine to thymine/adenine transitions, 12.9% is thymine to adenine transversions (Shirasawa *et al.*, 2016). A similar trend in mutational frequency in sugarcane is reported despite its high gene-copy number and high chromosome imbalances. Consequently, EMS mutagenesis is ideal to induce random small-scale or points mutations at high frequencies in sugarcane. In addition to transition and transversion mutations, addition and deletion mutations account for about 0.3 and 1% of mutations results by EMS, respectively (Shirasawa *et al.*, 2016).

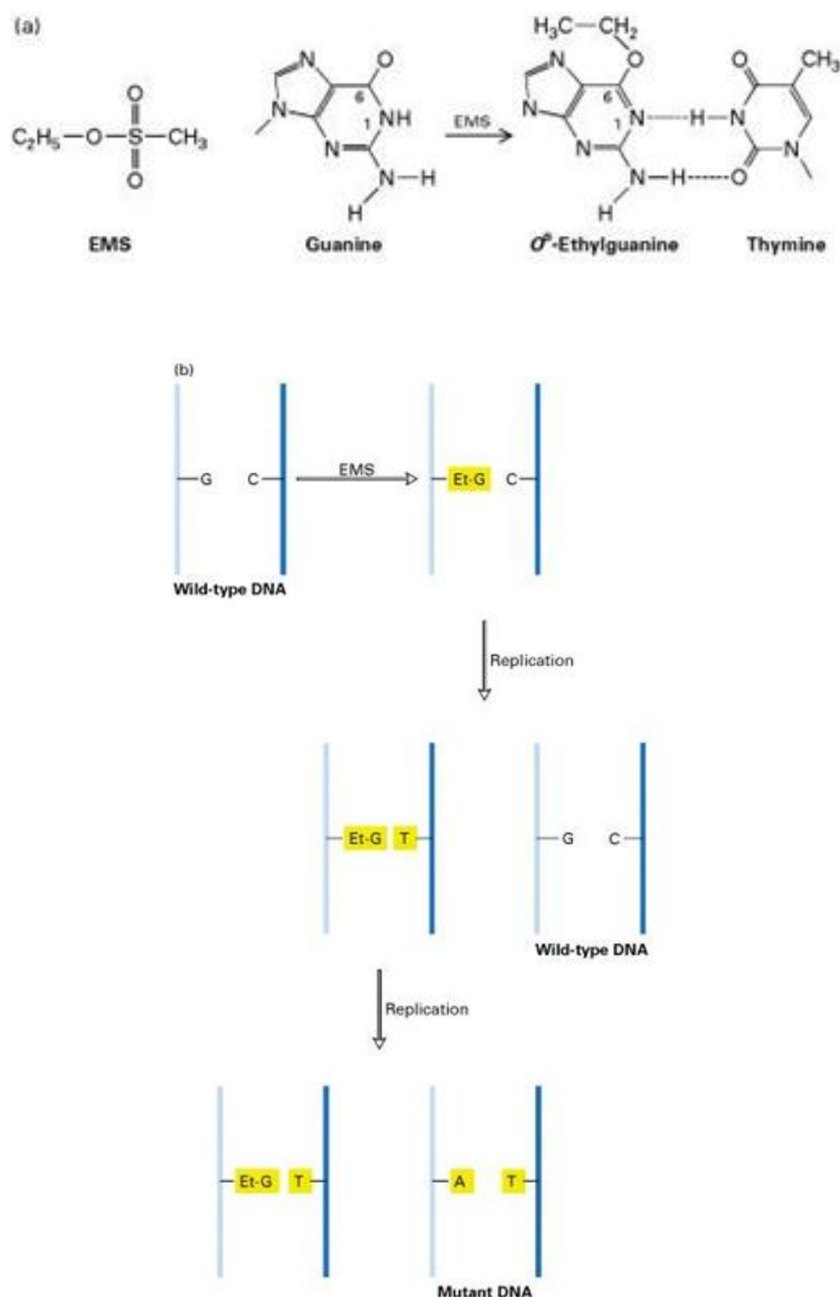

Figure 2.4 (a) The reaction of EMS and nitrogenous base guanine; the ethyl group of EMS reacts with O^6 to form *O-6*-ethylguanine. (b) The figure summarizing the complete mutation reaction resulted in EMS treatment. In brief; the resulted *O-6*-ethylguanine hydrogen bonds became non-compatible to cytosine and compatible with thymine. As a result, after DNA replication, thymine is incorporated. DNA repair mechanisms will find and excised the Et-G and match it to the inserted thymine to form an (A: T) base pair (Lodish *et al.*, 2000).

2.8.2 Ethyl methanesulfonate effect on gene function

Ethyl methanesulfonate is used to improve genetic responses to biotic and abiotic stress in various major crops including wheat, cauliflower, sugarcane, rice, potato and sweet potato (Table 2.2). The application of EMS in sugarcane results in the production of salt (Patade and Suprasanna, 2008), aluminium toxicity (Purnamaningsih and Hutami, 2016), drought (Masoabi *et al.,* 2017), herbicide (Koch *et al.,* 2012; Koetle *et al.,* 2018) and fungi (Mahlanza *et al.,* 2013) tolerant mutants.

Molecular characterization of EMS mutant sugarcane reveals that A-G/G-A transition results in S622N mutation on acetolactate synthase gene (*ALS*) which is responsible for herbicide tolerance. A number of missense mutations such as A to G change which results in asparagine to aspartic acid and G to A change result in serine to asparagine (occurring in six alleles) in sugarcane EMS mutants (Koetle *et al*., 2018). Missense mutations are required to improve sugarcane tolerance for environmental stress. An increase in environmental stress leads to an increase in some amino acids in response to the stress, where asparagine in young and mature sugarcane culm increases significantly under water stress (Iskandar *et al*., 2011). Asparagine acts as an osmoprotectant during sucrose accumulation in the culms (Ferreira *et al.,* 2017).

Genetic variation generated with EMS-induced mutagenesis is a random process, which makes it difficult to produce an intended DNA sequence (Shirasawa *et al.,* 2016). Abnormalities such as lack of chlorophyll, chimera and stunted growth are common on mutant produced from EMS (Masoabi *et al*., 2017). In the field, sugarcane EMS mutant lines do not vary on their agronomic traits such as a number of stalks per plot, stalk height or sucrose when compared with wild-type (Rutherford *et al.,* 2017). However, EMS results in loss-of-function of important traits such as pest resistance, where an increase in stalk borer is recorded on Mut1 when compared with wild type N12 variety under field conditions (Rutherford *et al*., 2017).

Table 2.2 Summary of the literature of *in vitro* developed crops for abiotic and biotic stress tolerance using various selection pressure selected under *in vitro* and/or field conditions.

Crop	Mutagen	Selection agent or trait	Selection stage	Reference
a) Drought tolerance				
Sugarcane	_	Polyethylene glycol; Mannitol	Callus	Lutts *et al.* (2004)
	_	Mannitol	Plantlet	Snyman *et al.* (2016)
	EMS	Polyethylene glycol	Callus	Masoabi *et al.* (2017)
Wheat	EMS	_	*F*	Khan *et al.* (2001)
	_	Mannitol	Callus	Begum *et al.* (2011)
Rice	_	Polyethylene glycol; Mannitol	Callus	Hassanein (2010)
	_	Polyethylene glycol	Callus	Wani *et al.* (2010)
	_	Mannitol	Callus	Ullah *et al.* (2014)
b) Salinity / heavy metal tolerance				
Sugarcane	_	NaCl	Callus	Errabii *et al.* (2007)
	_	NaCl	Callus; Plantlet	Ftwia *et al.* (1984)
	Irradiation	NaCl	Callus	Suprasanna *et al.* (2009)
	EMS	Al	Callus; Plantlet	Purnamaningsih and Hutami (2016)
Wheat	Proton radiation	HS (EC = 8 d. Sm^{-1})	*G*; *F*	González *et al.* (2009)
c) Agronomic traits; fungi and herbicide tolerance				
Sugarcane	EMS	*Fusarium sacchari* (PNG40)	Callus	Mahlanza *et al.* (2013)
	_	*Colletotrichum falcatum*	Callus	Sengar *et al.* (2009)
	EMS	Imazapyr herbicide	Callus	Koch *et al.* (2012)
	5-Azacytidine	Imazapyr herbicide; smut inoculum	Callus; Plantlet	Munsamy *et al.* (2013)
	Gamma rays	Height; cane yield; sucrose	*F*	Khan *et al.* (2009)

EMS – ethyl methanesulfonate; HS - hydroponic system; *G* – Greenhouse; *F* – Field selection; Al – Aluminium; NaCl – Sodium Chloride

2.9 SCREENING FOR DROUGHT TOLERANCE

During breeding programs, inferior genotypes are identified and eliminated from further consideration (Kimbeng *et al.*, 2009). Tested genotypes are considered superior if their performance exceeds the performance of control for the trait of interest (Zhou, 2013). Selections are conducted *in vitro* and/or *Ex vitro* to determine superior genotypes.

2.9.1 *In vitro* selection

In vitro mutagenesis and genetic transformation depend heavily on the *in vitro* selection (Snyman *et al.*, 2016). *In vitro* culture selection refers to the use of growth medium that contains selective agents to stress plant cells, tissues, organs and/or plants under *in vitro* conditions (Penna *et al.*, 2012) and regenerate plants with desirable characteristics (Rai *et al.*, 2011). An inhibitor or an antimetabolite is added to the culture media at the level that either kills or inhibits the growth of the mutated or wild type cells; calculated as the lethal dose (Penna *et al.*, 2012). This provides a controllable alternative for selection of the trait of interest, which offer an advantage of selecting within a short period. This approach does not require a large amount of space that is often demanded in the field (Suprasanna *et al.*, 2012).

Selection for drought tolerance at the whole plant level is constrained by the complexity of plant responses to water deficit (Zingaretti et al., 2012). At the whole plant level, it is difficult to measure a plants physiological response to stress as differentiated organs contain several cell types and ages that exhibit contrasting sensitivities to stress (Lutts et al., 2004). The use of an *in vitro* tissue culture system enables a feasible approach to study the cellular mechanisms of drought tolerance independent of organs (Errabii et al., 2007; Masoabi et al., 2017). The osmotic stress can be applied homogeneously to all cells, thus allowing the ability to control stress levels (Lutts et al., 2004). Iso-osmotic agents such as mannitol and polyethylene glycol are used to mimic drought stress that occurrs in natural settings (Iraki and Carpita, 1986).

Various *in vitro* screening approaches based on callus early traits have been developed for the detection of agronomic interesting mutants (Koch *et al.*, 2012). However, the accuracy of this approach is constrained by several factors including, epigenetic

changes and environmental conditions. *In vitro* culture environmental conditions include restricted gaseous exchange environment, high humidity, supply of carbon through culture media (sucrose), adequate supply of nutrient at regular rate, roots are exposed to light and nonexistence of competition and growth limiting factors such soil micro-organisms and vegetation (Miller and Cramer, 2004; Roycewicz and Malamy, 2012). As a result, plants selected *in vitro* are characterized by the high number of escapes or false positive results. Therefore, *in vitro* selected plants are further selected *Ex vitro* to confirm tolerance, eliminate escapes and for assessing other agronomic traits such as yield (Kangal *et al.*, 2018).

2.9.2 *Ex vitro* selection

Ex vitro selection entails the process that is undertaken after mutant lines with traits of interest have been identified under *in vitro* conditions, where they are further characterized to determine if they have all the expected, desirable agronomic characters. Such selections are primarily conducted under field environment. Interaction of genotype by environmental interaction (G X E) affects selection under field conditions (Sengwayo *et al.*, 2017). G X E interaction refers to the effect imposed by the environment on the yield of a genotype. Selection of genotypes is confounded by the environment of the year when the genotypes are grown and harvested. The genotype may yield well in one environment and poorly in the next (Kahram *et al.,* 2013). In nature, plants experience multiform nutrient distribution and must cope with dynamic environmental conditions.

In addition, normal cultural practice such as propagation, fertilizer application, weed management practice and labour are required. This approach is labour intensive, time-consuming and costly. Plant breeding programs have limited resource allocation and high costs are associated with variety development. As an alternative, quantitative data is used to select and estimate genetic values of important commercial traits such as disease tolerance and high sucrose content (Zhou, 2013).

CHAPTER 3
CHARACTERIZATION OF ETHYL METHANESULFONATE TREATED SUGARCANE CALLUS FOR *IN VITRO* OSMOTIC STRESS SELECTION

3.1 INTRODUCTION

Drought, heat and salinity stress are amongst the leading causes of yield loss in sugarcane (Azevedo *et al.*, 2011). Among these factors, drought is the main abiotic stress resulting in yield losses in sugarcane (Singels *et al.*, 2016). Its effects are expected to become widespread due to climate change (Ahuja *et al.*, 2010). The increasing incidence, duration and intensity of severe water deficit, has prompted many large sugarcane crop improvement programs to invest in water use-efficient and water stress tolerant varieties and water use-efficient crop productions systems. Drought tolerant sugarcane varieties are characterized by their ability to maintain growth even during water limiting conditions (Zingaretti *et al.*, 2012).

Genetic variability determines plants ability to adapt to changing environmental conditions (Zingaretti *et al.*, 2012). Conventional breeding is the most frequently used to introduce genetic variability for sugarcane improvement (Zhou, 2013). However, the conventional breeding application is limited by poor flowering in the South African context (Zhou, 2013) and the effect of genotype and environmental interaction that affects the expression of traits of interest when selecting under different agroecological conditions (Sengwayo *et al.*, 2017). Furthermore, sugarcane is allopolyploid (Amalraj and Balasundaram, 2006) as a result trait inheritance is unpredictable (Snyman, 2004).

As an alternative to conventional breeding, a well-developed *in vitro* culture system for sugarcane (Snyman *et al.*, 2011) is used concurrently with transgenic technologies (Augustine *et al.*, 2015a), somaclonal variation (Zambrano *et al.*, 2003) and *in vitro* mutagenesis (Masoabi *et al.*, 2017), to develop new traits. *In vitro* mutation breeding with the use of physical and chemical mutagens contributes significantly to crop improvement (Maluszynski *et al.*, 2000). Physical mutagenesis is the most regularly

used, however, its use is limited by the requirement for very specialized facilities that utilize irradiation (Patade *et al.*, 2008; Suprasanna *et al.*, 2009). Consequently, *in vitro* chemical mutagenesis is used as an alternative approach (Suprasanna *et al.*, 2012), with ethyl methanesulfonate (EMS) being the most frequently used chemical mutagen for sugarcane improvement (Mahlanza *et al.*, 2013). Ethyl methanesulfonate (EMS) is used to induce random point mutations in the sugarcane genome (Koetle *et al.*, 2018). This results in gain-of-function mutations, such as drought resistance (Masoabi *et al.*, 2017), or loss-of-function mutations, such as reduction of lignin in cell walls (Sattler *et al.*, 2014). Traits that have been improved in sugarcane using EMS include herbicide resistance (Koch *et al.*, 2012; Koetle *et al.*, 2018), fungi resistance (Mahlanza *et al.*, 2013), aluminium resistance (Purnamaningsih and Hutami, 2016) and osmotic tolerance (Masoabi *et al.*, 2017). In addition, *in vitro* selection reduces the amount of space, time and labour required for field selection (Snyman *et al.*, 2016).

The mutagenic effect of EMS depends on the incubation period, the concentration of the mutagen and the growth stage and age of the explant material (Sega, 1984; Purnamaningsih and Hutami, 2016). Actively dividing cells are required to induce mutations, thus callus tissue is a good substrate for EMS treatment (Hofmann *et al.*, 2004; Purnamaningsih and Hutami, 2016). Sugarcane has a complex genome, high chromosome imbalances and a narrow genetic basis (Grivet and Arruda, 2002). However, EMS seems to be the most viable mutagen of choice for sugarcane improvement as it results in random small-scale or points mutations at high frequencies (Koetle *et al.*, 2018), with low chromosomal aberration frequencies (Hassan *et al.*, 2004).

There is a need to determine the effect of EMS on sugarcane callus as well as the cellular response of EMS-treated callus tissues towards *in vitro* induced osmotic stress, to maximize the effectiveness of treatment for *in vitro* osmotic stress selection. Sugarcane cultivar NCo376 is one of the most widely cultivated cultivars in South Africa (Zhou, 2013). However, it is highly susceptible to drought stress (Inman-Bamber, 1982). Therefore, it was used in this study in order to gain information on the comparative effects of osmotic stress on cell necrosis, cell growth and cellular recovering abilities after EMS treatment. Furthermore, this work determined the

optimum incubation period and the mannitol lethal doses that inhibit shoot formation by 50% (LD_{50}) and 90% (LD_{90}) for *in vitro* osmotic stress selection.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Sugarcane cultivar NCo376 was collected from the University of Zululand farm (Orchard Unit). It was grown in pots in the greenhouse for six months (Figure 3.1) before apical meristems were cut just below the last internode and transferred to the laboratory. In the laboratory, leaves were carefully peeled off and the leafrolls were thoroughly washed with 70% ethanol to remove surface contamination.

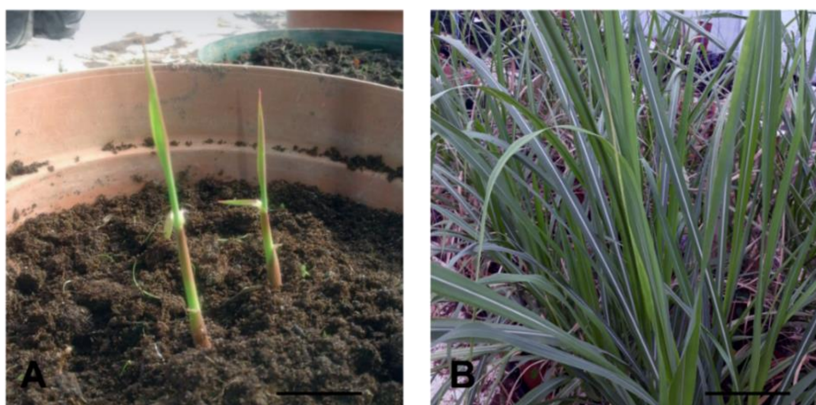

Figure 3.1 (A) Illustration of sett growing conditions in the greenhouse after two weeks (bar = 10 mm) and (B) six months after planting (bar = 10 mm).

3.2.2 Initiation of embryogenic callus

The callus was initiated according to Snyman (2004) with some modifications (Figure 3.2). The leafroll (Figure 3.2A) were placed in a petri dish containing half strength liquid Musharige Skoog (MS) media and sliced into 30 transverse sections (each approximately 3 mm thick; Figure 3.2B). Subsequently, 10 leaf disc were cultured on sterile callus induction media (CIM; Figure 3.2C). The CIM was composed of 4.4 g L^{-1} MS basal salts and vitamins (Sigma-Aldrich Johannesburg, South Africa), 3 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 20 g L^{-1} sucrose and 8 g L^{-1} agar. The media was adjusted to pH 5.8 using HCl or NaOH before autoclaving. Culture plates were sealed

with parafilm and they were incubated in a growth chamber (MRC LE-11) under darkness with the temperature maintained at 26 ± 1°C for eight weeks. Calli pieces were sub-cultured to a fresh CIM every two weeks. The detailed experimental procedure is summarised in Figure 3.3.

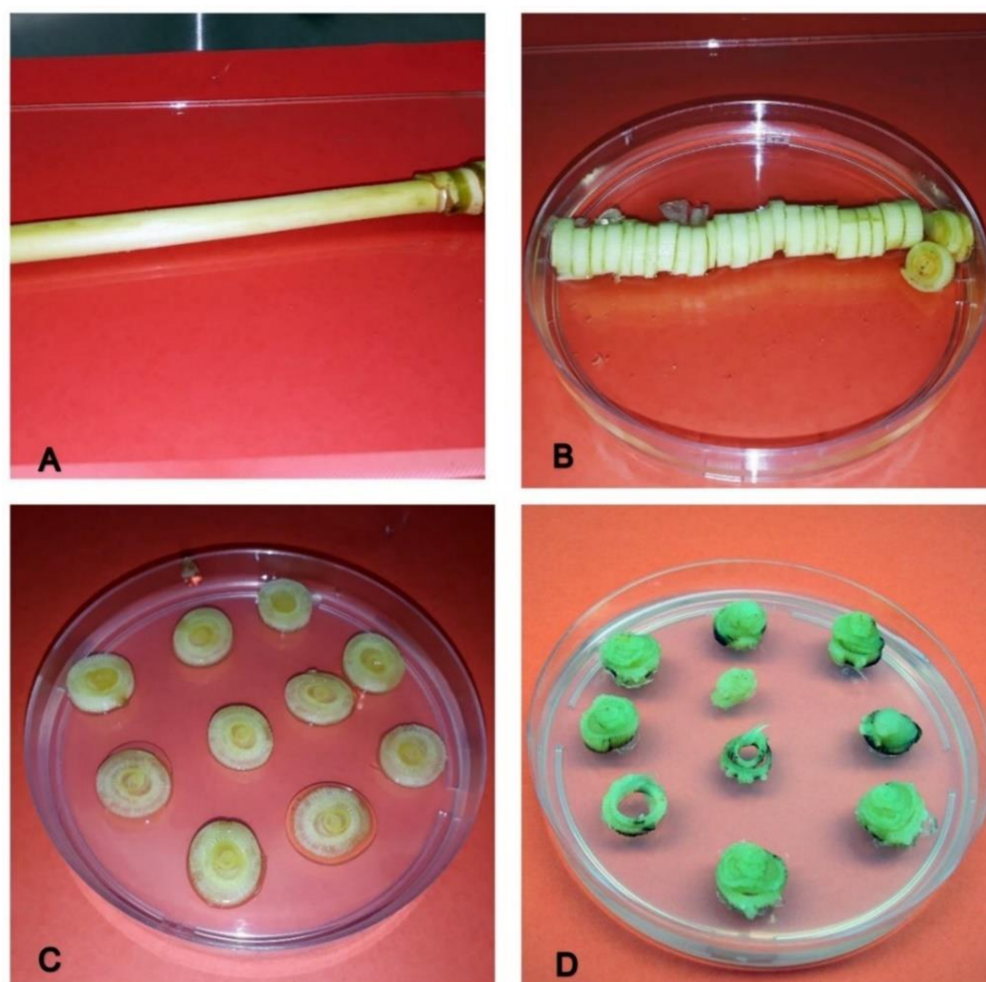

Figure 3.2 Illustration of callus initiation procedure: (A) sugarcane leaf roll (bar = 10 mm); (B) 3 mm leaf disks sliced on half-strength liquid MS media (bar = 10 mm); (C) leaf disks sections on 30 mL callus initiation media (bar = 10 mm) and (D) leaf disks with mucilaginous callus after four weeks in callus initiation medium (bar = 10 mm).

3.2.3 Mutagenic treatment of embryogenic callus

Under aseptically conditions (Figure 3.3A), embryonic callus pieces weighing 0.2 g (as illustrated in Figure 3.3B) were immersed in centrifuge tubes containing 10 mL of 16 mM ethyl methanesulfonate (EMS) solution (Figure 3.3D) composed of half strength liquid MS Medium (Koch *et al.*, 2012), for 0 h, 0.5 h, 1 h, 2 h and 3 h. Each Petri dish contained 25 calli pieces (Figure 3.3C) and each treatment was replicated three times (n= 3 x 25). After each incubation period, EMS treated calli pieces and controls were rinsed three times using half strength liquid MS media. Then air dried on an empty Petri dish for five minutes and cultured on CIM for a further four weeks in the darkness at 26±1°C.

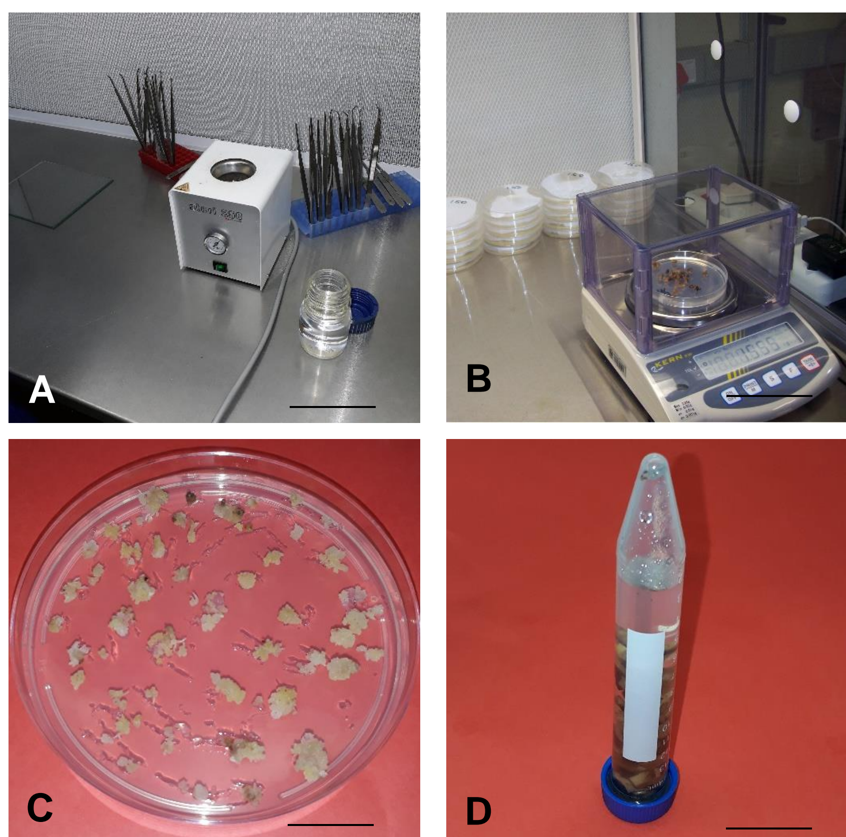

Figure 3.3 (A) Laminar flow culturing environments setting (bar = 10 mm); (B) callus fresh weight determination (bar = 10 mm); (C) eight weeks embryogenic calli (bar = 10 mm); (D) and calli immersed in centrifuge tubes containing 16 mM EMS mutagen solutions (bar = 10 mm).

Percentage change in callus fresh weight was determined by recording callus initial weight before exposure to EMS and final weight was recorded after four weeks' post-exposure to EMS. Three culture plates were used as replicates per treatment duration with each plate containing 25 calli samples. Changes in callus fresh weight were determined using the following formula: Fresh weight = 100 × [(callus final weight – callus initial weight) / callus initial weight]. Data regarding the size of callus and numbers of embryonic callus pieces where embryos germinated were recorded. Callus size was measured using the callus cover area determined by the isometric paper as described by Masoabi *et al.* (2017). Calli fresh weight (FW) was measured biweekly for twelve weeks to determine the effect of EMS on callus cumulative growth.

3.2.4 Osmotic stress selection conditions

Calli pieces were cultured in CIM containing varying strength of mannitol (Sigma-Aldrich, Johannesburg, South Africa) namely, 150, 225 and 300 mM for eight weeks in the darkness (26±1°C). Control treatments contained non-mutated callus cultured in CIM containing mannitol (negative control) and without mannitol (positive control). Calli pieces were sub-cultured every two weeks. After eight weeks of culturing in osmotic, calli pieces were sub-cultured onto CIM for two weeks to enable calli to recover.

Calli pieces relative growth rate (RGR) was quantified by measuring the callus growth (mm) in four directions, where each callus sample was dissected with two lines (Zharare *et al.* 2010). The lines were drawn through the centre of each callus sample to be measured and each radial line was labelled: R1; R2; R3 and R4 (Figure 3.5). Initial callus radial growth (mm) was recorded along each of the four radial lines and final callus radial growth (mm) was measured after eight weeks of exposure to the different concentrations of mannitol. Five replicates were used per treatment.

The sum of the four measurements was determined and the average was recorded as total growth of the callus. The RGR was determined using the formula: RGR = 100 x [(Final callus radial growth – initial callus radial growth) / initial callus radial growth]. Callus index of tolerance (INTOL) was determined using the modified formula of Shekhawa et al. (2009): INTOL = RGR treatment / RGR control.

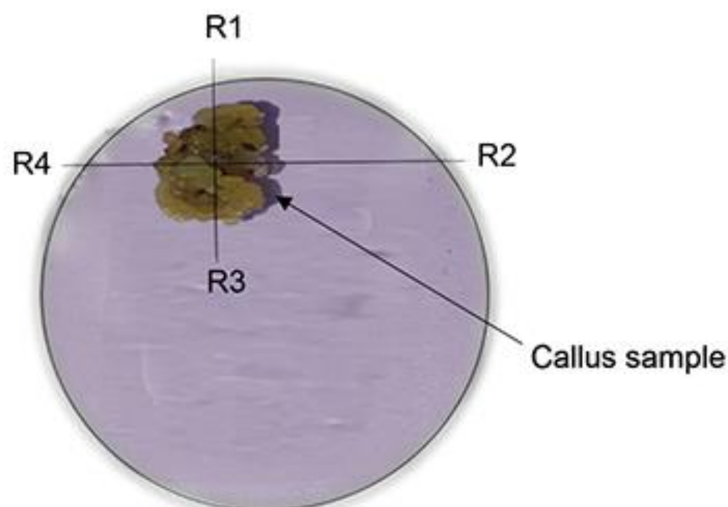

Figure 3.4 Illustration of the four axes along which the callus growth was measured.

Percentage of embryonic calli was recorded after two weeks of the recovery period. Whitish and yellowish calli pieces were selected as potential embryonic and dark brown calli pieces were considered non-embryogenic/necrotic (Taylor *et al.*, 1992). The control percentage of embryonic calli was used to calculate mannitol lethal dose that inhibits callus survival by 50% (LD_{50}) and 90% (LD_{90}) to determined optimum concentrations for *in vitro* selection using GraphPad Prism (v 7.04, GraphPad, San Diego, CA), using the formula: $y = mx + c$. The determined lethal doses were subsequently used during embryogenic germination to further eliminate escapes.

3.2.5 Embryo germination and selection conditions

Shoots were established by culturing the selected embryogenic calli on embryo germination media (EGM; CIM without 2, 4-D) supplemented with LD_{50} and LD_{90} mannitol. A growth chamber with cool white fluorescent lights (11120 LUX) and a photoperiod of sixteen hours of light and eight hours of darkness (16:8 h) was used. The temperature was maintained at 26 ± 1 °C and the cultures were sub-cultured to a fresh media biweekly for eight weeks. After eight weeks, calli with plantlets were transferred to EGM without mannitol stress to recover under the same growing

conditions for two weeks. The number of plantlets generated per callus and the percentage of stunted and albino plantlets were recorded.

3.2.6 Statistical analysis

ANOVA followed by comparisons test was used to determine variation across all treatments. The interaction between and within treatment groups was tested using a 5% level of significance (GraphPad Prism; ver. 7.04, GraphPad Software Inc., San Diego, CA).

3.2.7 Ethical statement

EMS solution and materials used during EMS mutagenesis were inactivated before incineration to minimize the carcinogenic effect of EMS. The EMS inactivating solution was composed by mixing an equal volume of 0.1M NaOH, 20% w/v and $Na_2S_2O_3$ (sodium thiosulfate). The EMS solution, tubes and gloves used during mutagenesis were soaked in an inactivation solution for 24 hours before incineration. All the experimental procedures were reviewed and approved by the University of Zululand Research Ethics Committee, Registration Number: UZREC 171110-030 PGM 2017/474.

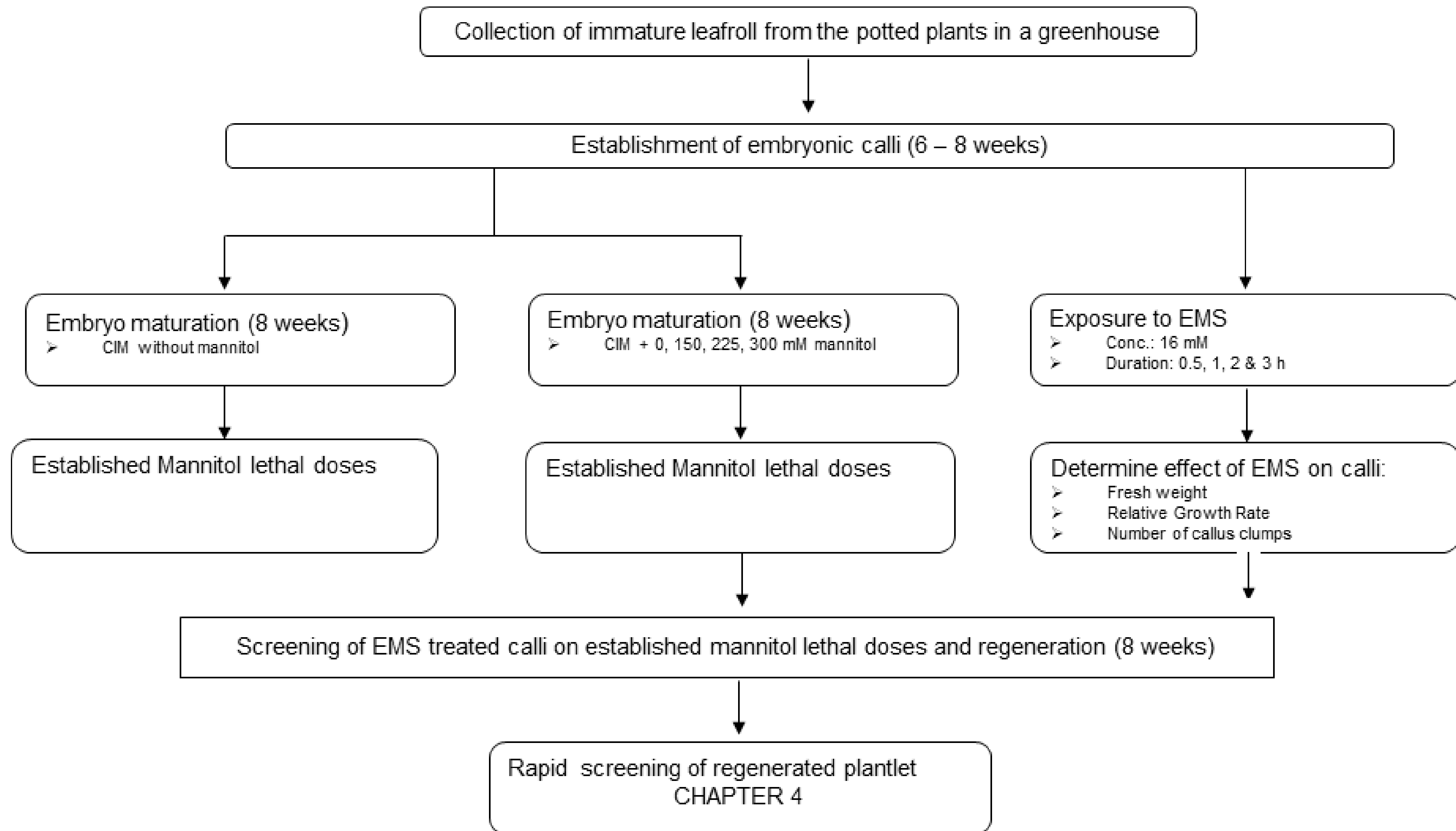

Figure 3.5 Experimental design for exposure of calli to ethyl methanesulphonate (EMS) and selection using mannitol. During regenerated, plantlets were exposed to the determined lethal doses.

3.3 RESULTS

This experiment aimed to determine the following: 1) the optimal ethyl methanesulfonate (EMS) incubation period of EMS on sugarcane callus; 2) the mannitol concentration that inhibits callus survival by 50% and 90%, which were subsequently used to screen during embryo germination stages; 3) the effect of mannitol induced osmotic stress on the regenerative potential of somatic embryos.

3.3.1 Effect of EMS on callus growth

An increase in EMS incubation period resulted in a significant decrease in the growth percentage of calli fresh weight ($P < 0.05$; Figure 3.6). A significant difference in calli fresh weight among treatments was detected at four weeks and it became more distinct on the eightieth and tenth week, respectively (Appendix 2). At four weeks, the callus that was not exposed to EMS recorded the highest increase in percentage fresh weight (13.38%), while calli exposed to EMS for 3 h resulted in the lowest increase (4.87%). At twelve weeks, callus fresh weight demonstrated different levels of sensitivity to EMS (Figure 3.7). The 0.5 h treatment was least sensitive to EMS and treatment of callus for a minimum of 1 h to 2 h showed the highest sensitivity. The fastest increase in fresh weight was recorded on a control treatment with a growth rate of 0.44 g $week^{-1}$, followed 0.5 h treatment (0.38 g $week^{-1}$). While the slowest growth was recorded from the 2 h treatment at a rate of 0.26 g $week^{-1}$. There were no significant changes in fresh weight of the 3 h treatment at week two and four.

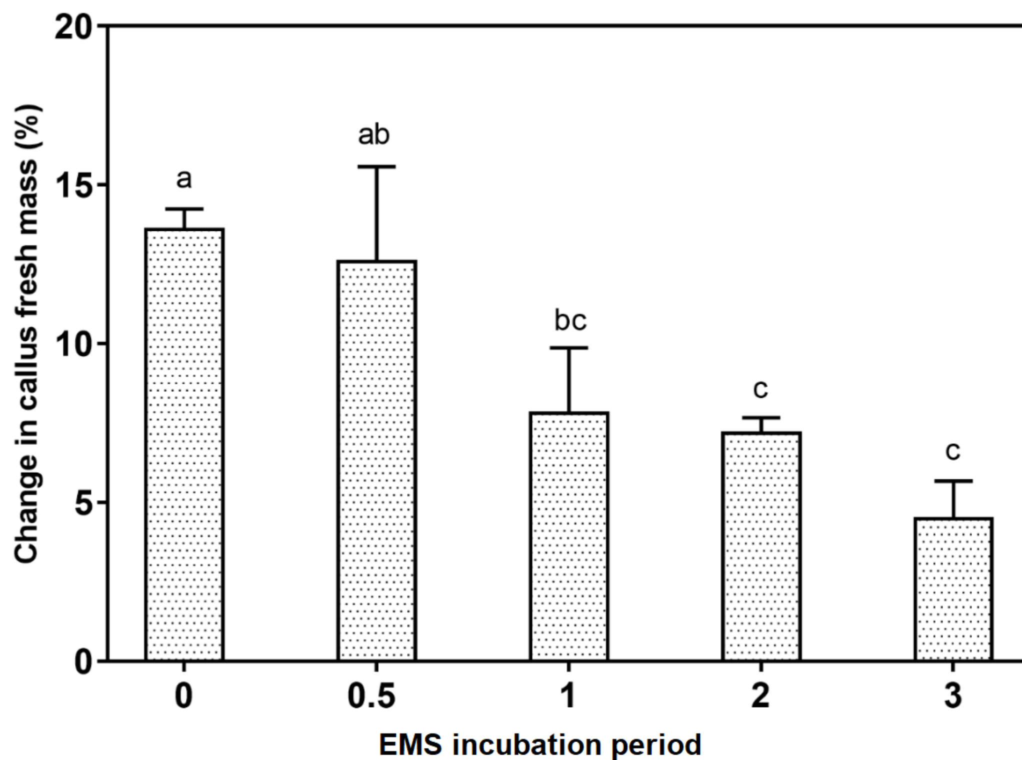

Figure 3.6 Percentage change in fresh mass at 4 weeks when callus was treated with 16 mm EMS for different time periods (0-3 hours). Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at $P \leq 0.05$.

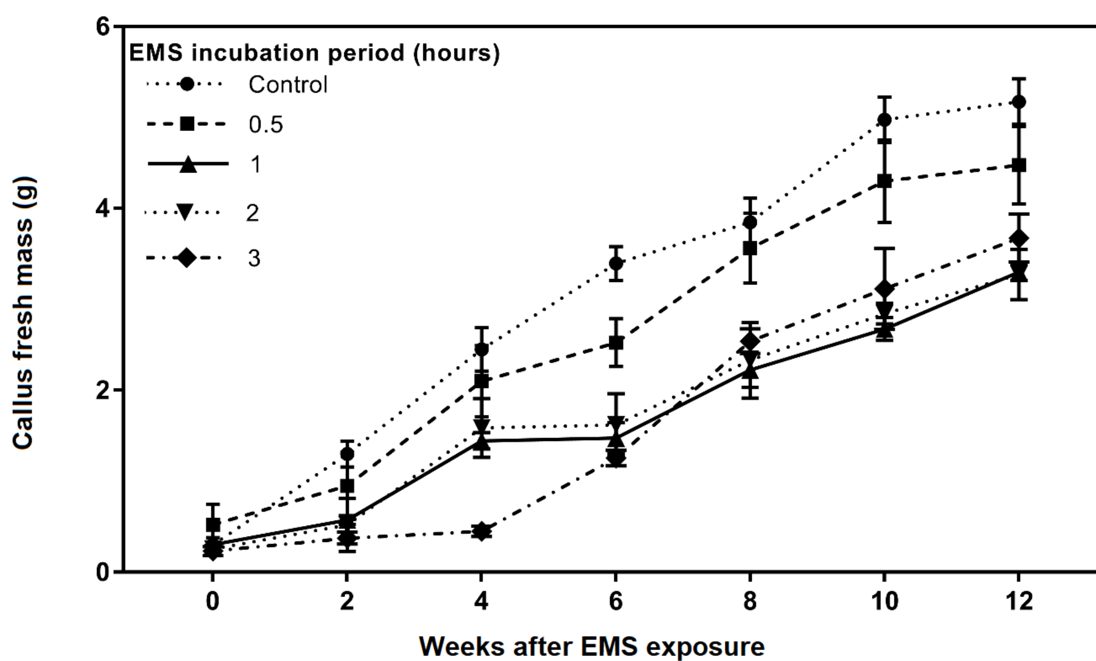

Figure 3.7 Change in fresh mass over 12 weeks when callus was treated with 16 mm EMS for different time periods (0-3 hours). Values mean ± SE, n = 15

Treatment of callus with EMS did not affect callus size. However, callus showed extensive growth when compared with the initial size of the callus piece at four weeks post exposure to EMS (Figure 3.8). The highest callus size (10.4 mm^2) was recorded on callus treated for half an hour whereas the 3 h treated callus recorded the lowest size (7.4 mm^2). Furthermore, an increase in EMS incubation period resulted in a decrease in the average number of embryonic callus (Figure 3.9). A significant decrease of the average number of embryonic callus was recorded on callus treated for three hours (40.22) when compared to the control (54.38: $P = 0.0386$) and the half an hour treatment (60.60; $P = 0.0142$), respectively. Pearson correlation coefficient (r) was used to determine if the was any relationship between callus size and the average number of embryonic callus. When the callus size increased, the average number of embryonic callus also increased. However, the Pearson correlation coefficient was weak showing that only about 13% ($R^2 = 0.13$; $P = 0.0826$) of data was related

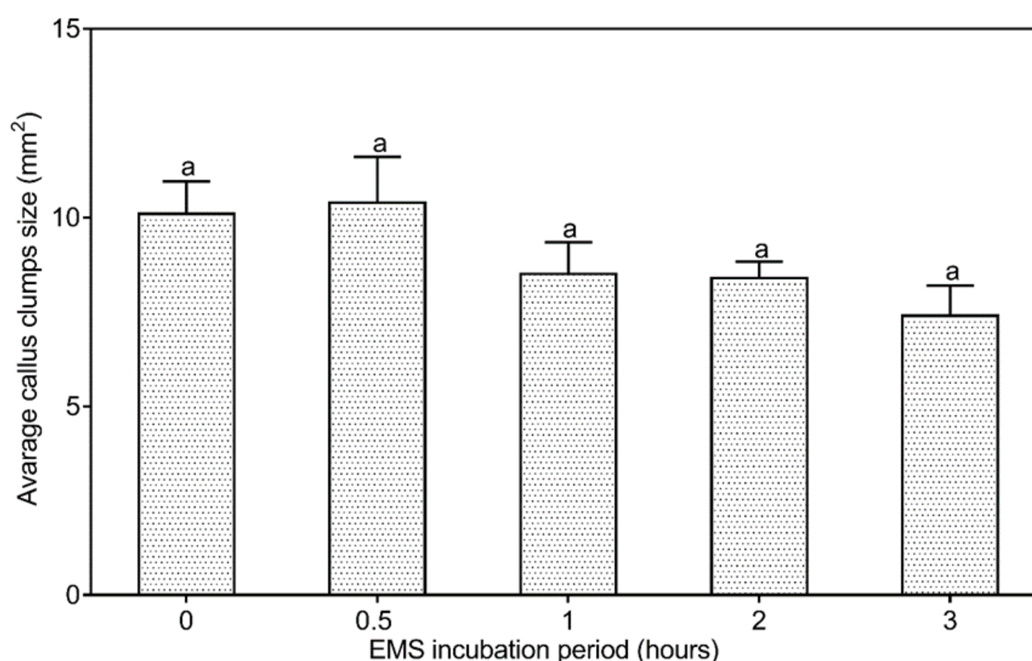

Figure 3.8 The avarage callus clump size at 4 weeks when callus was treated with 16 mm EMS for different time periods (0-3 hours). Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at $P < 0.05$.

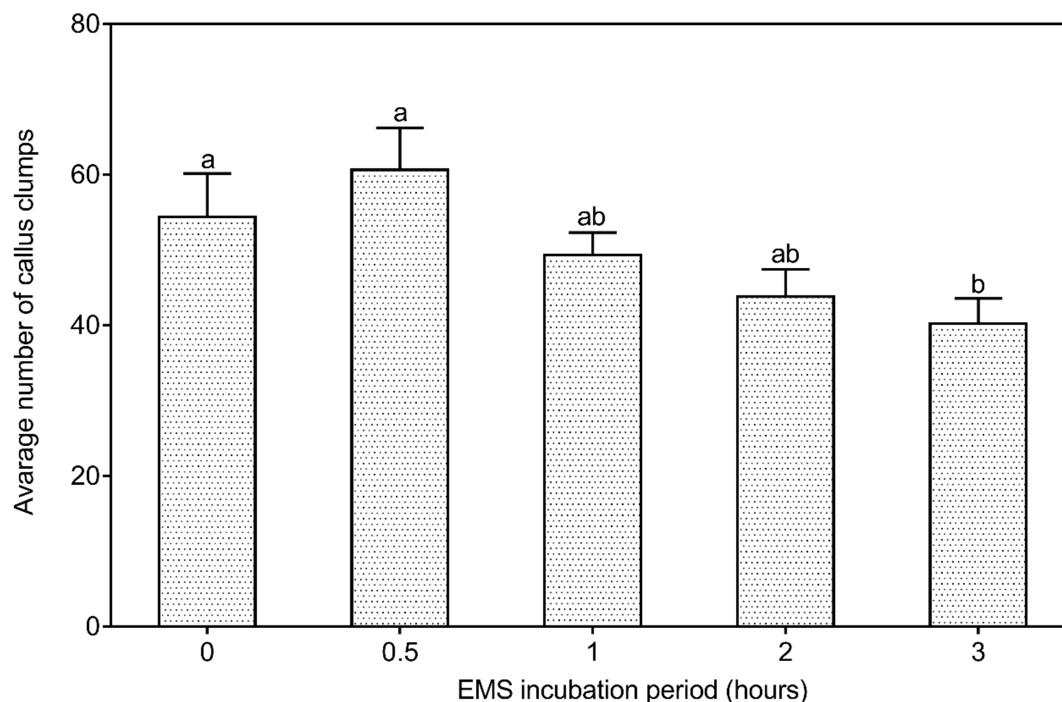

Figure 3.9 The avarage number of embryonic callus clumps at 4 weeks when callus was treated with 16 mm EMS for different time periods (0-3 hours). Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at $P < 0.05$.

3.3.2 Effect of osmotic stress in callus water content

When callus was exposed to EMS, the 1 h and 2 h treatments showed a significant reduction in callus water content with a mean decrease of 6.27% and 5.51% compared to the control, respectively (Figure 3.10). However, the water content of the callus treated for 0.5 h and 3 h with EMS did not differ from that of the control.

An increase in mannitol treatment resulted in a significant decrease in callus water content (Figure 3.11). However, within each stress treatment, callus water content progressively increased with an increase in EMS incubation period. For instance, on 150 mM mannitol treatment, the callus water content of 3 h treatment was increased by 22.01% in comparison with control ($P < 0.05$). When compared to the control, the 2 h and 3 h treatments (225 mM mannitol) resulted in 68.42% ($P < 0.001$) and 21.86% ($P < 0.001$) increase in callus water content, respectively. The largest decrease in callus

water content across all treatment was recorded from the control cultured on 300 mM mannitol. Even though calli treated with EMS for a long duration (2 h and 3 h treatments) recorded the largest callus water content under osmotic stress, they were phenotypically non-embryogenic. Non-embryonic calli contain larger and loosely held cell clusters with a soft texture and are semi-transparent with a yellow or grey colour.

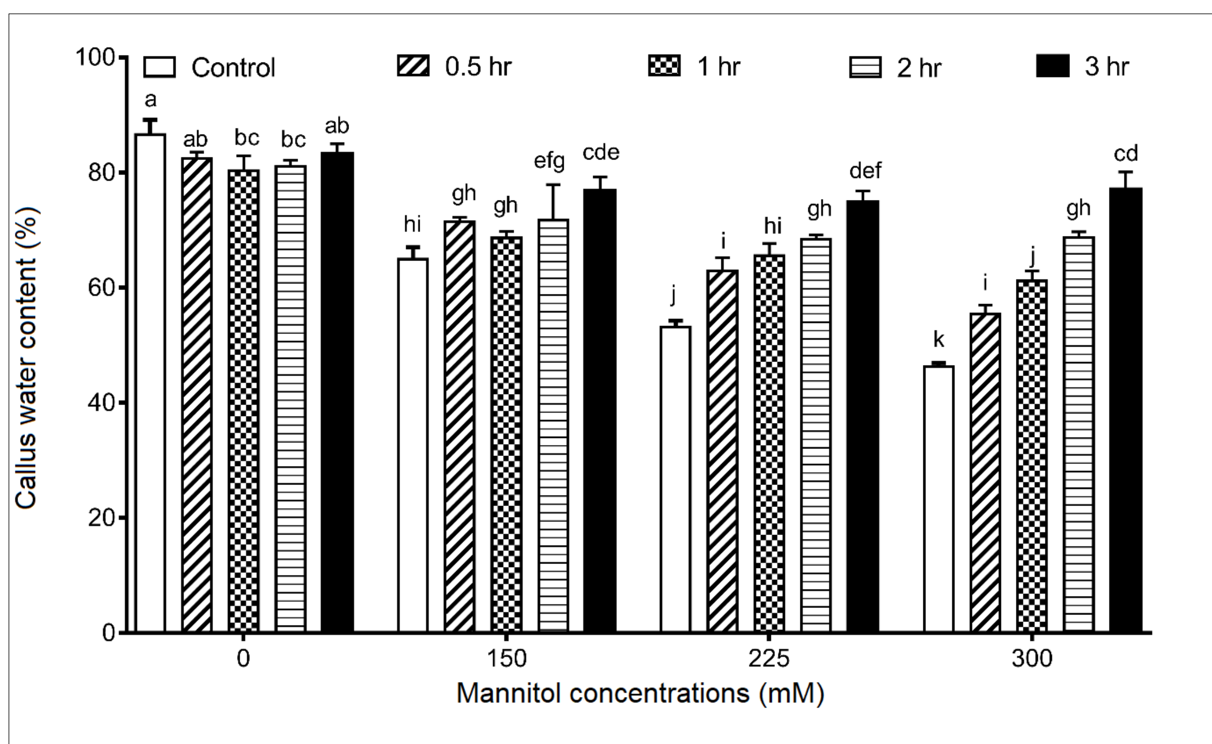

Figure 3.10 The effect of 0-300 mM mannitol stress on the water content of callus treated with 16 mM EMS for different time periods (0-3 hours) at 8 weeks. Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at $P < 0.05$.

3.3.3 Callus relative growth rate and index of tolerance

The relative growth rate after four weeks of exposure to EMS was significantly reduced by an increase in EMS incubation period. The 3 h treatment showed the largest reduction in relative growth rate with a mean decrease of 7.61% ($P = 0.0055$) compared with 1 h treatment and 20.71% ($P = 0.0079$) compared with the control. The 0.5 h and

1 h treatment did not differ significantly ($P > 0.05$) from each other in their relative growth rate (Figure 3.11).

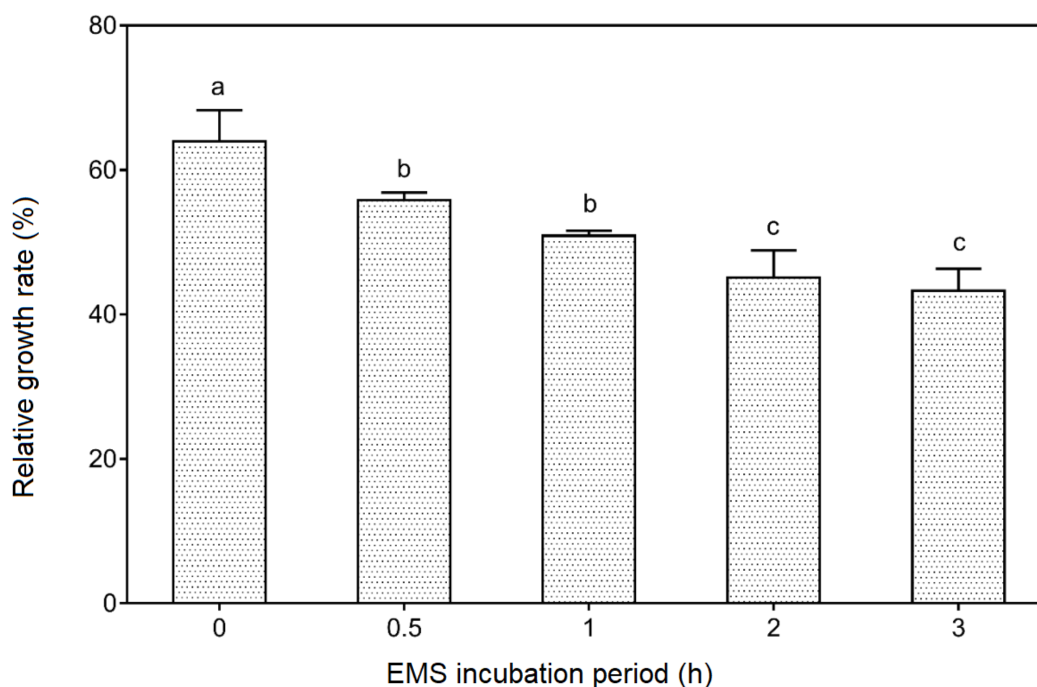

Figure 3.11 The relative growth rate at 4 weeks when callus was treated with 16 mm EMS for different time periods (0-3 hours). Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at $P \leq 0.05$ (n = 5).

At 12 weeks, a rapid increase in relative growth rate was observed in calli treated for a maximum of 1 h when compared with the control. An increase of 14.37% in relative growth rate was recorded for 1 h treatments when compared with control ($P = 0.0948$) and 10.92% ($P = 0.4534$) compared with the 0.5 h treatment (Figure 3.12). This was in contrast with the relative growth rate recorded at four weeks after EMS incubation.

An increase in mannitol-induced osmotic stress resulted in a reduction in calli relative growth rate. The control calli showed the largest reduction in relative growth rate with a reduction ranging between 27.60% to 78.8% under mannitol stress. In 150 mM mannitol treatments, relative growth rate of the 1 h treatment showed a significant increase of 28.30% ($P = 0.0020$) when compared with the control; and 21.50% ($P = 0.0467$) increase when compare with 0.5 h treatment. A similar growth trend was

observed at 225 mM mannitol, with calli treated for 1 h in EMS showing a significant increase in relative growth rate when compared with the control, 0.5 h and 3 h treatment respectively. In 300 mM mannitol, control calli showed a significant reduction in relative growth rate when compared with calli treated with EMS regardless of the incubation period. Furthermore, in 1 h treatment relative growth rate was decreased by 14.10% (*P* = 0.0790) compared with 2 h treatment and by 25.40% (*P* = 0.0168) compared with the 3 h treatment.

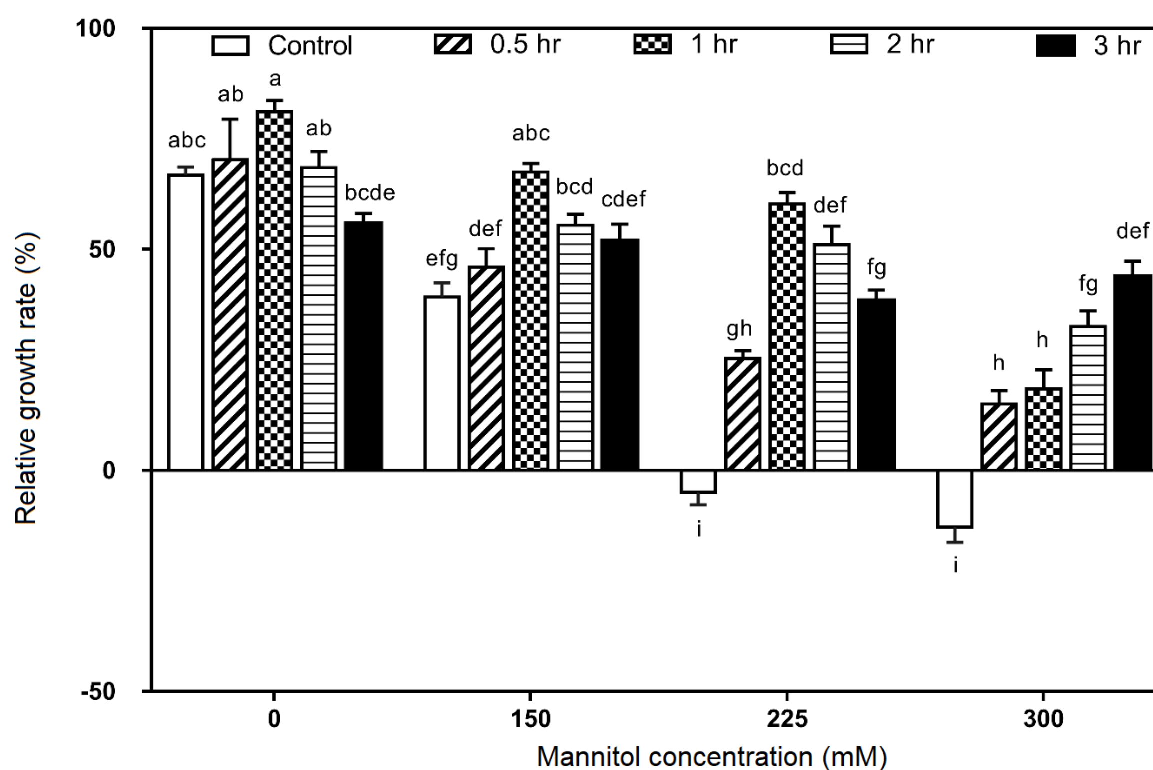

Figure 3.12 The effect of 0-300 mM mannitol stress on the relative growth rate of callus treated with 16 mM EMS for different time periods (0-3 hours) at 12 weeks. Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at $P < 0.05$.

An increased in EMS incubation period had an insignificant effect on calli index of tolerance (Figure 3.13). However, under mannitol stress, an increase in EMS incubation period resulted in a significant increase in calli index of tolerance. In 150 mM mannitol, a significant increase in the index of tolerance was recorded in 3 h treatment with a mean increase of 36.60% (*P* = 0.0312) compared with the control and by 27.60%

(*P* = 0.0279) compared to the 0.5 h treatment. In 225 and 300 mM mannitol, index of tolerance of callus incubated in EMS for a minimum of 1 h was significantly increased when compared with the control and 0.5 h treatment.

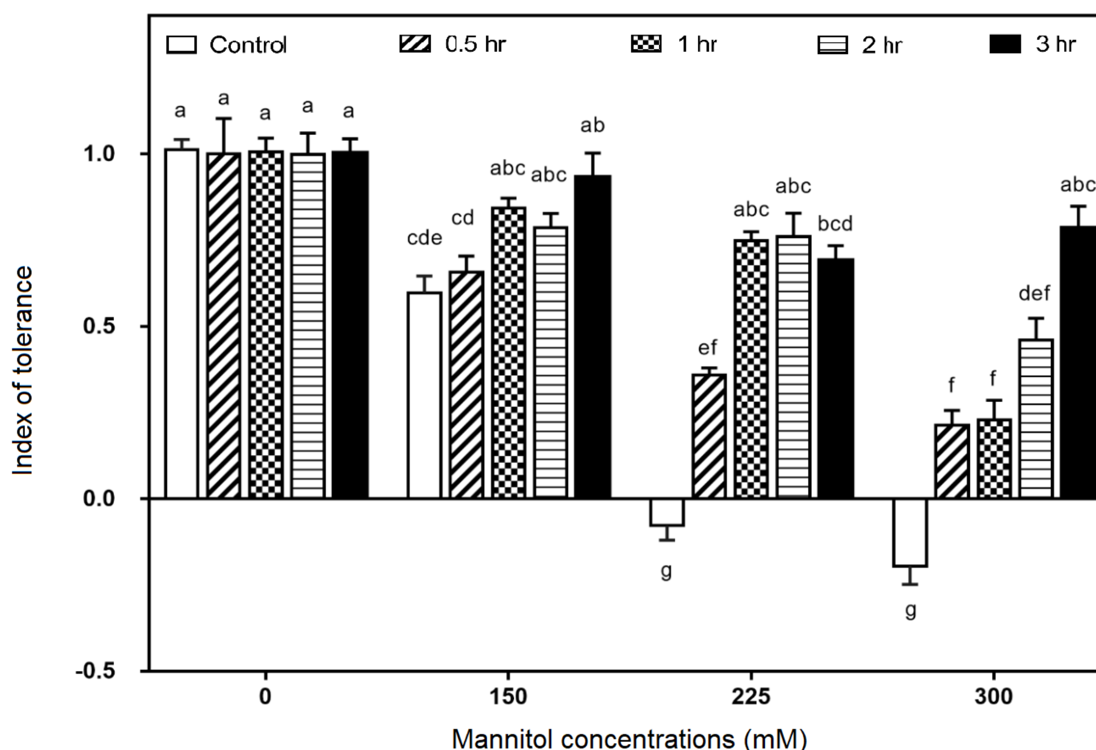

Figure 3.13 The index of tolerance of callus treated with 16 mM EMS for different time periods (0-3 hours) and subsequently exposed 0-300 mM mannitol induced stress for 8 weeks. Values mean ± SE, n = 15

Mean values with deferent letter(s) different significantly at *P* < 0.05.

3.3.4 Percentage embryonic calli

Percentage embryonic calli did not differ significantly with an increase in the EMS incubation period within each specific mannitol concentration (Figure 3.9). Calli started showing signs of necrosis within the first week after EMS treatment. The negative control calli (without mannitol and EMS) produced the highest amount (93%) of embryogenic calli (Figure 3.15; Figure 3.16A). The percentage of embryonic calli in the 3 h treatment group was significantly decreased by 34.62% compared to control (*P* = 0.0192). An increase in mannitol levels resulted in a progressive decrease in percent embryogenic calli (Figure 3.16). Callus appearing at least 50% brownish were classified

as non-embryonic (Figure 3.16B). Untreated calli recorded the lowest percentage embryogenicity (25.09%) when cultured on media containing 300 mM mannitol across all treatments. Under mannitol treatments, calli embryogenicity was not affected by EMS treatment ($P > 0.05$) but generally decreased with the severity of the mannitol-induced stress. After three weeks of exposure to mannitol, EMS treatment resulted in early root development in calli. This was intensified in the 225 mM and 300 mM mannitol treatments (Figure 3.16C). Two weeks after stress relief, EMS treated calli that appeared to be necrotic due to exposure to water stress started developing prolific yellowish callus pieces (Figure 3.16D).

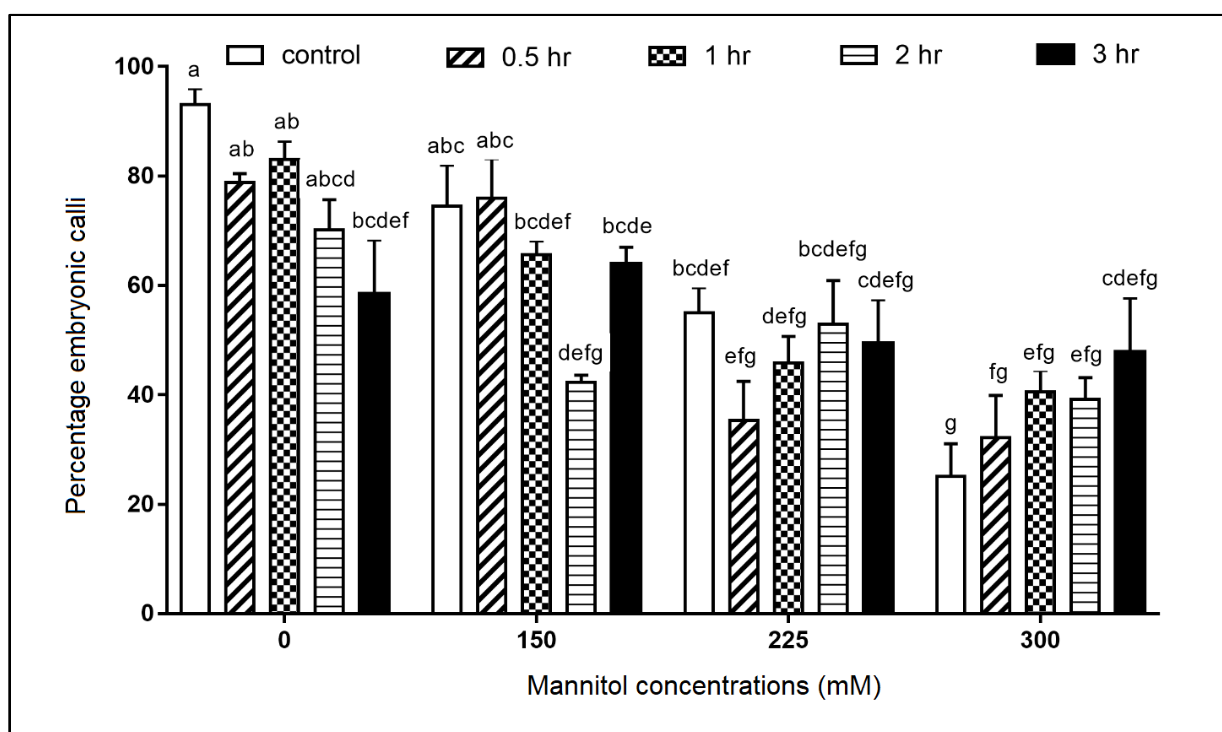

Figure 3.14 The effect of 0-300 mM mannitol stress on embryogenesis of callus treated with 16 mM EMS for different time periods (0-3 hours) at 12 weeks..

Mean values with deferent letter(s) different significantly at $P < 0.05$.

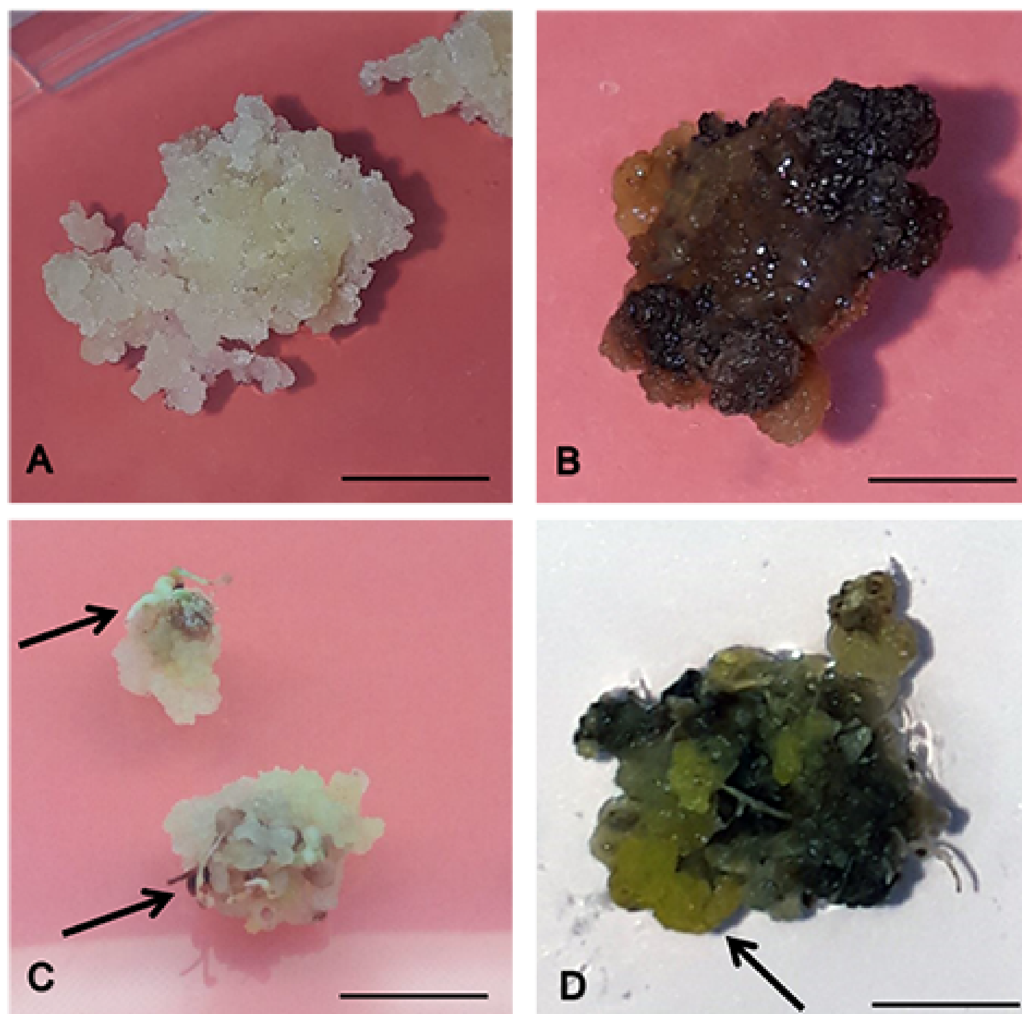

Figure 3.15 (A) Embryogenic untreated callus cultured in callus inducing medium (bar = 5 mm); (B) necrotic untreated callus cultured in 300 mM mannitol (bar = 5 mm); (C) early root development (arrows) on EMS mutated calli (bar = 5 mm) and (D) regeneration of yellowish callus pieces after two weeks recovery period (bar = 5 mm).

3.3.5 Determination of mannitol lethal doses

Mannitol concentrations lethal doses (LD) that inhibited callus survival by 50% and 90% was determined using calli that were not exposed to EMS (Figure 3.17). The mannitol lethal dose that constrains calli survival by 50% (LD_{50}) and 90% (LD_{90}), were LD_{50} = 225 mM and LD_{90} = 407 mM. There was a strong negative correlation between an increase in mannitol concentrations and embryonic calli percentage survival (R^2 = 0.92; P = 0.04), meaning that an increase in mannitol concentration results in a significant decrease in calli embryogenicity. This trend was also observed on EMS treated callus

regardless of the incubation period (Table 3.1). The determined lethal doses (LD_{50} = 225 mM and LD_{90} = 407 mM, respectively) were subsequently used for callus selection at embryo germination stage.

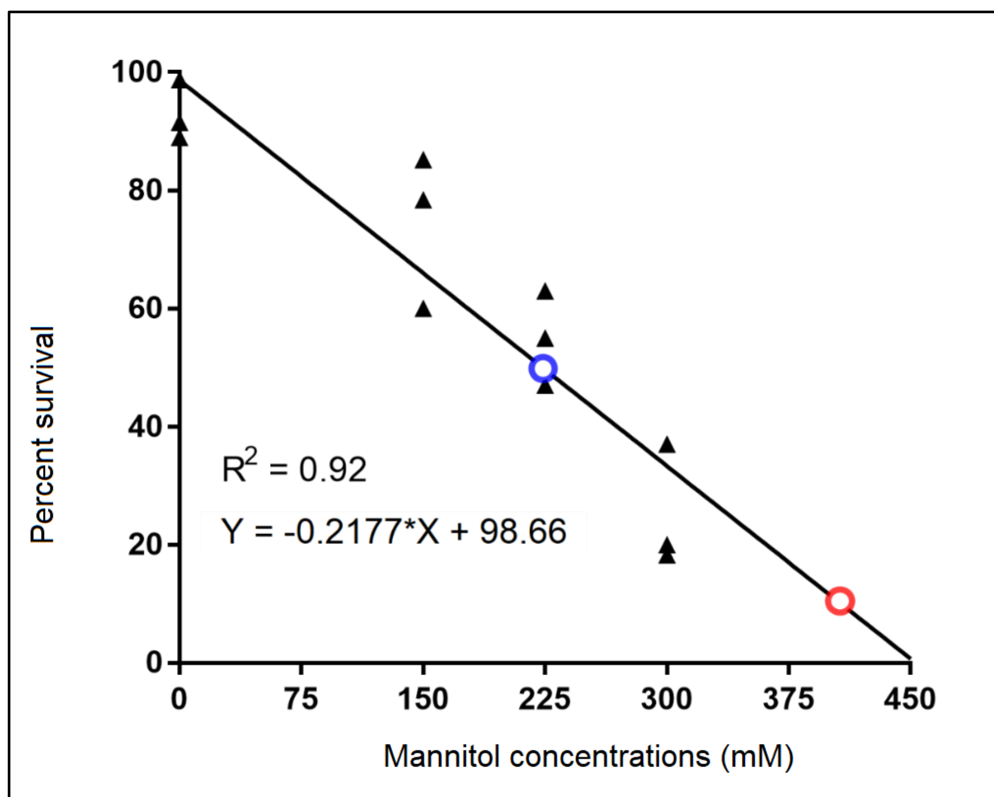

Figure 3.16 Linear regression analysis used to determine mannitol concentrations that inhibit survival by 50 (blue cycle) and 90% (red cycle) (LD_{50} and LD_{90}, respectively) for cultivar NCo376 using control callus.

3.3.6 Plantlet regeneration

When callus was transferred to a growth chamber with 16:8 h photoperiod for embryo regeneration, and calli started greening within the first week. However, the number of plantlets generated were recorded after eight weeks (Table 3.2). A significant reduction in embryo generation was observed in 2 h and 3 h treatments when compared to the control.

Table 3.1 Regression analysis of percent survival on mannitol lethal doses during selection in darkness

Incubation period (hours)	**Percent survival at LD_{50} = 225 mM**	**Percent survival at LD_{90} = 407 mM**	**Regression Model**	**Determination coefficient (R^2)**	**Level of significance of the regression model (*P*)**
0	50	10	Y = -0.2177*X + 98.66	0.92	0.0406
0.5	46	14	Y = -0.1729*X + 84.76	0.77	0.1228
1	51	23	Y = -0.1495*X + 84	0.97	0.0176
2	46	29	Y = -0.09194*X + 66.63	0.71	0.1601
3	53	45	Y = -0.04079*X + 61.71	0.47	0.3154

Table 3.2 Effect of ethyl methanesulfonate and mannitol-induced osmotic stress on sugarcane plantlet formation

Incubation period (h)	**Number of plantlets per callus cluster** Mannitol concentrations (mM)			**Percentage of phenotypically abnormal plantlets**	
	0	225	407	Stunted	Albino
Control	12.47 ± 1.92 [a]	6.10 ± 1.64 [a]	1.80 ± 1.79 [ab]	5.66 [b]	3.49 [b]
0.5	8.42 ± 1.42 [b]	3.73 ± 1.71 [b]	2.87 ± 1.06 [a]	12.51 [a]	1.98 [b]
1	13.07 ± 2.88 [a]	5.75 ± 2.11 [a]	2.27 ± 1.01 [a]	3.83 [b]	3.79 [b]
2	3.53 ± 2.72 [c]	1.93 ± 1.39 [c]	1.87 ± 1.06 [b]	14.17 [a]	25.97 [a]
3	2.90 ± 2.14 [c]	2.13 ± 1.60 [bc]	⁕	27.26 [a]	14.33 [a]

⁕ - no plantlets generated; Mean values with different letters within a column are significantly different at $P < 0.05$. Values mean ± SE, n = 15.

There was a significant reduction in plantlet generated on embryo germination medium supplemented with mannitol LD_{50} (225 mM) and LD_{90} (407 mM) compared with the medium without mannitol regardless of EMS incubation period. On 407 mM mannitol, no plantlets were developed from callus exposed to EMS for 3 h. However, some embryonic calli samples appeared phenotypically normal despite an inability to regenerate plantlets. An increase in incubation resulted in a significant increase in the number of stunted and albino plantlets (Figure 3.18F). Based on plantlets regeneration, the 1 h incubation period was considered an optimum incubation period for mutagenic

treatment of callus with EMS, as it did not result in a high percentage of abnormal shoots and under mannitol stress. Furthermore, the number of normal plantlets generated were not significantly reduced from that of the control.

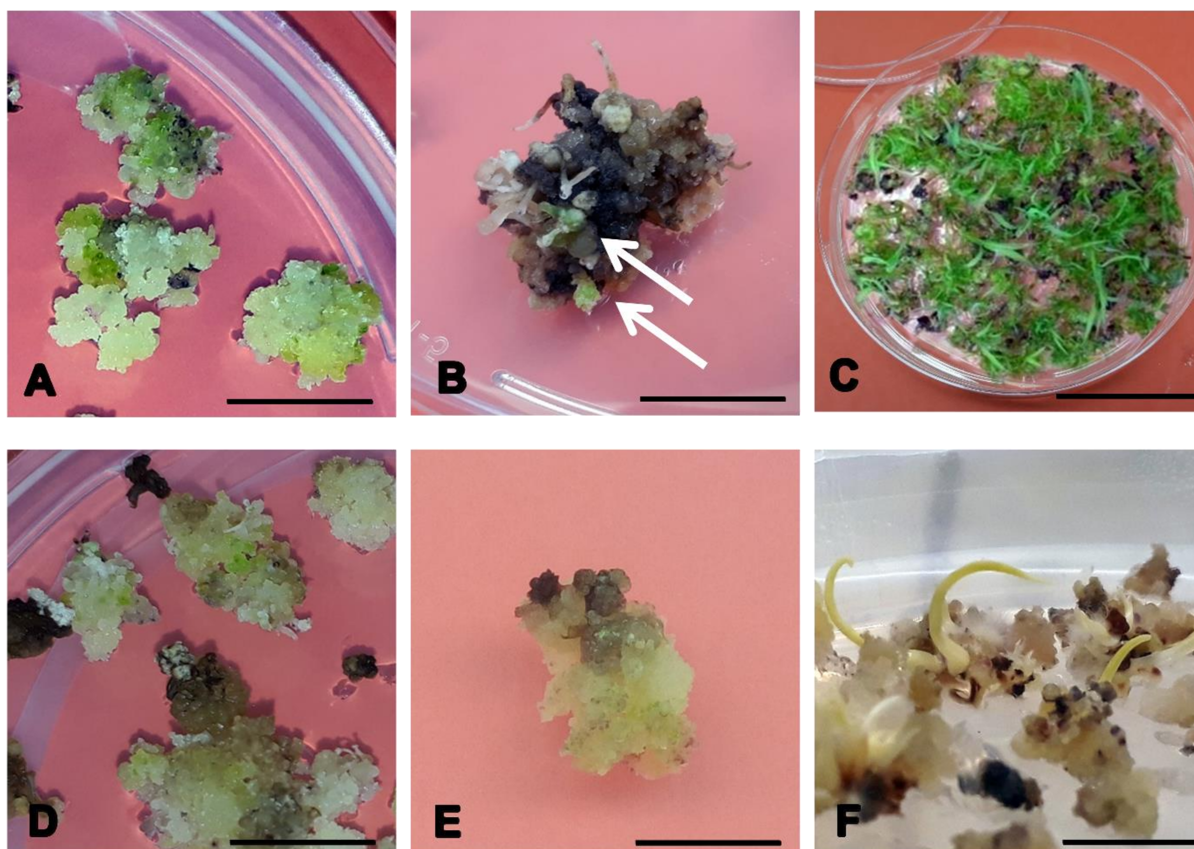

Figure 3.17 (A) Untreated callus after two weeks during plantlet formation on embryo germination medium (Bar = 5 mm); (B) control callus after two weeks on embryo germination medium supplemented with 407 mM mannitol (LD_{90}) (Bar = 2 mm); (C) plantlets after eight weeks on embryo germination medium (bar = 10 mm); (D) Callus treated for one hour after two weeks on embryo germination medium (Bar = 5 mm); (E) Callus treated for one hour on embryo germination medium supplemented with 407 mM mannitol (bar = 2 mm); (E) albino plantlets (bar = 5 mm).

3.4 DISCUSSION

Sugarcane breeding programs for osmotic stress tolerance is limited by a narrow gene pool and complexity of its genome. Ethyl methanesulfonate (EMS) mutagenesis has been successfully used to induce genetic variability (Koetle *et al.*, 2018) and to improve various traits in sugarcane (Kenganal *et al.*, 2008; Mahlanza *et al.*, 2013; Khalil *et al.*, 2018). The objective of this study was to induce mutations on sugarcane callus and subsequently select plants tolerant to mannitol to induce osmotic stress. The approach was to generate variation in embryogenic cells via somatic embryogenesis and mutagenesis using ethyl methanesulphonate (EMS) as described by Koch *et al.* (2012).

3.4.1 The effect of EMS on callus embryogenesis

To determine an optimum duration for inducing useful mutations, sugarcane embryogenic calli were incubated in EMS solution for various incubation periods. Incubating callus for 3 h in EMS solution decreased the callus fresh weight by 8.5% compared with the control (Figure 3.6). Furthermore, the callus size was marginally decreased with an increase in EMS incubation period (Figure 3.8). However, the average number of embryonic callus pieces was significantly decreased with the treatment of callus with EMS for 3 h (Figure 3.9). Exposure of callus to EMS have been previously reported to result in the decrease in the callus size and number of calli pieces (Masoabi *et al.*, 2017). A decrease in callus size, number of embryonic calli pieces and fresh weight (Figure 3.6; Figure 3.7) after exposure to EMS is due to the decrease of callus differentiation rate. An increase in EMS incubation period results in a decrease in callus relative differentiation rate (Khalil *et al.*, 2018). These results agree with the findings of Koch *et al.* (2012) and Purnamaningsih and Hutami (2016) who also reported that exposure of sugarcane callus to EMS resulted in a decrease in the callus fresh weight. EMS mutagenic effect is affected by the incubation period (Sega, 1984). Long exposure may facilitate absorption of the mutagen by the cells at an excessive level, resulting in denaturing of the proteins which is followed by cell death (Hofmann *et al.*, 2004; Gocke *et al.*, 2009).

The 0.5 h and 1 h treatment showed a rapid increase in relative growth rate as well as a high number of shoots per callus, respectively (Figure 3.13; Table 3.1). In contrast, calli exposed for 2 and 3 h to EMS showed a reduction in relative growth rate which was followed by a significant reduction in shoot formation and a significantly high number of phenotypically abnormal shoots. These suggest that calli relative growth rate might be an indication for calli potential to regenerate plantlets. Furthermore, an increase in EMS incubation period resulted in a significant decrease in the percentage of embryonic calli (Figure 3.15), consequently the calli's ability to regenerate plantlets (Table 3.1). A decrease in callus proliferation and plantlet regeneration with an increase of EMS concentrations and incubation period have been reported (Svetleva, 2005; Purnamaningsih and Hutami, 2016). Abnormal plantlet development such as chlorophyll deficiency and underdeveloped shoots after exposure to EMS are common and are documented in a number of studies (Kenganal *et al.*, 2008; Silué *et al.*, 2013). The inability of callus to develop plantlets after EMS exposure might be caused by chromosomal aberration or complete failure of the spindle mechanism as a result of DNA alkylation by EMS (Gnanamurthy and Dhanavel, 2014).

In this study, incubation of callus in 16 mM EMS for 0.5 h to 1 h was selected as an optimal treatment range for inducing useful mutations. An optimal concentration and treatment duration should not result in significant inhibition of plantlet regeneration or results in a high number of phenotypic abnormalities in plantlets (Novak *et al.*, 1990; Yadav *et al.*, 2016). Koch *et al.* (2012) and Masoabi *et al.* (2017), reported exposure of callus to 16 mM EMS for 4 h was optimal for producing herbicide and osmotic tolerant mutants. While Kenganal *et al.* (2008) suggested a concentration of 40 mM EMS for 2.5 h exposure of sugarcane callus to be sufficient for producing salt-tolerant lines. Purnamaningsih and Hutami (2016) recommended 0.3 - 0.5% v/v EMS concentration for incubation time of 0.5 to 1.5 h for aluminium tolerance plantlets. The variation in optimal concentrations and incubation period on this study and other researchers mentioned may be influenced by the difference in the cultivars used (Purnamaningsih and Hutami, 2016).

3.4.2 Callus water content

Mannitol induced osmotic stress resulted in a significant decrease in callus water content (Figure 3.10). In sugarcane, the callus water content decreases in the presence of mannitol-induced stress (Errabii *et al.*, 2007). Tissue water content is regarded as a good parameter to determine cellular water and metabolic status of the plant or cell (Lawlor and Cornic, 2002). For instance, Boaretto *et al.* (2014), reported that osmotic tolerant cultivars retain water under osmotic compared to susceptible cultivars. Hence, relative water content is used as the selection trait for osmotic stress tolerance (Augustine *et al.*, 2015a). In this study, treatment of callus with EMS for 2 and 3 h resulted in a significant increase in callus water content under mannitol-induced osmotic stress (between 150 mM and 300 mM). However, callus water content results are misleading when considering that a large proportion of calli treated for 2 and 3 h was non-embryogenic when phenotyping using Taylor *et al.* (1992) classification. Furthermore, the increase in the callus relative growth rate and index of tolerance on EMS treated callus when compared with the control might be due to excessive absorption of water by dead cells (Figure 3.12 and Figure 3.13). Suggesting that the use of index of tolerance in callus level for selection may be misleading to get potential resistant lines.

Calli tissues may appear phenotypically normal (architecture of dead tissue is preserved) even when they are no longer viable under *in vitro* conditions (Mahlanza *et al.*, 2013), which is characterized by excessive hydration and low lignification a condition known as hyperhydricity (Kacem *et al.*, 2017). Hyperhydric cells or tissues form a viscous liquid mass under high humidity levels (Rosskopf *et al.*, 2005). During culture, the plates are completely sealed with parafilm as a precaution for *in vitro* culture which results in high humidity levels (~100%) (Chen, 2004). Formation of liquid mass in plant tissues is also a consequence of defective cellulose biosynthesis process (Koiwa, 2010). Several *Arabidopsis* mutant lines that demonstrated abnormal root enlargement due to the accumulation of liquid contained defective cellulose. For *in vitro* studies, the callus water content may not be a clear indicator of cellular water status. Future work is required to validate this by conducting a cell viability test.

3.4.3 Mannitol *in vitro* screening

Screening for osmotic stress tolerance under field conditions is complicated due to the effect of genotype by environmental interaction and high cost due to the lengthy breeding period (Butterfield *et al*., 2001; Sengwayo *et al*., 2017). *In vitro* culture selection has given a cost-effective alternative approach for selection of stress-tolerant cell lines after *in vitro* mutagenesis (Masoabi *et al*., 2017). Plant tolerance to osmotic stress can be determined by applying the selecting agents such as sorbitol, polyethylene glycol and mannitol to the culture media (Rai *et al*., 2011). In this study, the mannitol lethal doses were investigated for the selection of osmotic stress tolerance cell lines of sugarcane. Mannitol lethal doses were determined using callus pieces that were not exposed to EMS cultured on callus inducing medium incorporated with mannitol ranging from 150 mM to 300 mM for eight weeks in the dark.

Callus relative growth rate, callus index of tolerance and embryogenicity were decreased with an increase of mannitol concentration during selection under darkness. The result of this study agrees with other studies that have reported a decrease in callus relative growth rate and embryogenicity with the severity of mannitol concentrations (Lutts *et al*., 2004; Errabii *et al*., 2007; Begum *et al*., 2011). In this study, mild and severe osmotic stress resulted in shrinkage of the control callus, demonstrated by negative relative growth rate and index of tolerance (Figure 3.11 and Figure 3.12). The cultivar (NCo376) used in this study is osmotic stress susceptible (Snyman *et al*., 2016). Callus obtained from osmotic susceptible cultivars been previously reported to shrink under high mannitol concentrations compared to osmotic tolerant cultivars (Lutts *et al*., 2004). Shrinkage of callus under water stress results because of the cells inability to maintain turgor pressure against the cell walls (Beck, 2012). High turgor pressure maintains plant form and facilitates cell expansion and growth (Koiwa, 2010). Water stress leads to decreases in cell solutes, cell volume as well as protein denaturing and total loss of water within the cell leading to dehydration and shrinking of the cells (Zingaretti *et al*., 2012). Reduction of relative growth rate in this study is potentially a survival strategy for long-term osmotic stress. Osmotic tolerant cells reduce their growth rate by reducing the number of mitotic and differentiating cells to maintain the growth cycle (Koiwa, 2010), resulting in a decrease in biochemical activities that require

water and thus ensuring the long-term survival of the plant cells (Zingaretti *et al.*, 2012; Ferreira *et al.*, 2017).

Evasion mechanisms are important traits in plants (Ferreira *et al.*, 2017). Water stress may be temporary and the ability of the plant to achieve its whole cycle is directly related to its ability to retain normal development after the water stress period (Bernacchia *et al.*, 1996). In this study, during the recovery period some callus that appeared to necrotic developed yellowish callus pieces (Figure 3.15D). However, the callus was unable to regenerate plantlets. Regeneration of prolific yellowish callus pieces (cell recovery) post water stress has been observed in other studies that used callus culture (Lutts *et al.*, 2004). Both EMS treatment and mannitol stress appeared to enhance the development of early root formation during selection under darkness (Figure 15C). Abnormal callus characterized by early root development has been reported on EMS mutated calli under stress conditions including herbicide (Koch *et al.*, 2012) and fungal infection (Mahlanza *et al.*, 2013). Early development of roots is a consequence of both the chemical mutagens and stress treatment. The callus that showed early root development was unable to develop into an embryo, consequently, early root formation of indirect organogenesis studies is undesired (Koch *et al.*, 2012).

During *in vitro* culture, the establishment of an optimum concentration of dehydration agent that affects cells, tissues, organs and the whole plant is important as it can decrease the chances of selecting false-positive tolerant lines (Daub, 1986). Therefore, in this work percentage embryogenicity data was used to determine the mannitol concentrations that inhibited embryonic calli by 50 and 90% (LD_{50} and LD_{90}, respectively) which was subsequently used to select during shoot formation. From the results, the LD_{50} and LD_{90} of for osmotic stress selection were calculated as 224 and 407mM mannitol, respectively. Mannitol lethal doses identified in this work are lower than those reported at whole plantlet level under *in vitro* conditions (Snyman *et al.*, 2016).

An increase in mannitol concentration resulted in a decrease in embryo formation. The highest embryo formation was recorded in the control treatment, followed by callus exposed LD_{50} and LD_{90}. A decrease in embryo formation with an increase of osmotic

stress in growth media has been reported on a number of studies using polyethylene glycol or mannitol as an osmoticum (Lutts *et al.*, 2004; Masoabi *et al.*, 2017). In LD_{90} callus treated for 1 h in EMS produced the highest number of plantlets per callus and lowest number of abnormal shoots when compared to callus treated for 2 h and 3 h. These findings suggest that treatment of callus for 1 h may have resulted in the successful induction of osmotic tolerance.

3.5 CONCLUSION

Mutated calli under osmotic stress had a higher relative growth rate, index of tolerance and recovery ability. Treatment of callus for 1 h was optimal for inducing useful mutations without inhibiting callus embryogenicity or resulting in a highly significant number of phenotypic abnormal plants. Six plantlets derived from sugarcane callus mutagenized with 16 mM EMS for 1 h and *in vitro* selected on 225 mM mannitol were selected for subsequent studies. These results suggest that determination and use of LD_{50} (and LD_{90}) as the stress thresholds, permits rapid selection of osmotic-tolerant callus lines at the same time reducing the amount of time and resources required to conduct timely and labour-intensive pot and field trials to select for osmotic stress tolerance. Accordingly, the establishment of plantlets under mannitol stress can be used to select for potential osmotic tolerant mutant lines. From this chapter, plantlets that appeared phenotypic normal were selected and transferred to plantlet establishment media for further characterization (Chapter four).

CHAPTER 4
APPLICATION OF PHYSIOLOGICAL INDICES TO SCREEN FOR DROUGHT TOLERANCE IN SIX SUGARCANE EMS MUTANTS

4.1 INTRODUCTION

During any breeding program, inferior genotypes are identified and eliminated for further consideration using phenotypic and other screening methods (Kimbeng *et al.*, 2009; Marcos *et al.*, 2018). Different selection methods have been developed to screen for drought-tolerant sugarcane genotypes under field conditions using various physiological traits (Shami and Singh, 2018). However, uncontrolled conditions, heterogeneity in the soil, huge amounts of plant material, time and labour required for the field experiment, are challenges associated with field selection (Zhou *et al.*, 2012; Yang *et al.*, 2017). As an alternative, selection has been conducted in a more controlled environment using rain-shelters (Eksteen *et al.*, 2014) or *in vitro* culture (Begum *et al.*, 2011). Timely and detailed measurements are required to discriminate between genotypes under rain-shelter or greenhouse environments (Eksteen *et al.*, 2014). Consequently, *in vitro* screening approach appears to be an attractive alternative to select lines produced from *in vitro* mutagenesis or genetic modification (Snyman *et al.*, 2016); and as an early screening method to characterize different genotypes (Kacem *et al.*, 2017). The *in vitro* screening approach allows the screening of large numbers of genotypes in a limited space over a short period of time (Kumar *et al.*, 2017).

Mass screening based on callus and plantlet early traits have been developed for the selection of agronomic interesting mutants, including mutants with tolerance to osmotic stress and herbicides (Koch *et al.*, 2012; Manchanda *et al.*, 2018). However, the selection at callus level is constrained by lack of correlation between tolerance of cells in culture versus the whole plant level (Mahlanza *et al.*, 2013; Rutherford *et al.*, 2017). For example, Masoabi *et al.* (2017) reported that only 16% of putative osmotic-tolerant mutants of cultivar NCo310 selected in cell culture were tolerant during *Ex vitro* pot experiments. While Khalil *et al.* (2018) reported that only 11% *in vitro* selected putative osmotic-tolerant mutants of cultivar ROC22 promised to be osmotic tolerant under field

conditions. Epigenetic changes during callus differentiation to form a whole plant level can contribute to lack of correlation between tolerance of cells in culture verses the whole plant level (Rai *et al.*, 2011) and each cell within the treated callus is affected and mutated differently.

Fast and reliable osmotic tolerance indicators such as chlorophyll content and stomatal conductance do not provide sufficient discriminatory power under *in vitro* conditions due to the size of the plantlet tissues and variation in environmental growth conditions (Zhu, 2002; Snyman *et al.*, 2016). Unlike field conditions, *in vitro* culture environmental conditions include restricted gaseous exchange environment, continuous supply of nutrients and high humidity levels (Hartmann *et al.*, 2001). Consequently, sugarcane physiological response under *in vitro* induced osmotic stress varies from that observed in *Ex vitro* conditions. For instance, the closing of leaf stomata as water preservation mechanisms is not likely to occur in the humid *in vitro* environment (Snyman *et al.*, 2016). Therefore, identifying selection traits for osmotic tolerant cultivars under *in vitro* conditions remain a major challenge (Ferreira *et al.*, 2017).

It is, therefore, necessary to determine various morphological and physiological traits that can provide enough discriminating power to select lines produced from *in vitro* culture to eliminate escapes. Sugarcane ability to re-root *in vitro* under mannitol and *Fusarium sacchari* stress have been suggested as good parameter to characterize genotypes and select tolerant lines at the whole plant level (Mahlanza *et al.*, 2013; Snyman *et al.*, 2016). A more stringent *in vitro* screen protocol is required to eliminate ‘escapes’ before mutant lines are evaluated for agronomic traits under field conditions (Rutherford *et al.*, 2017).

Therefore, the objective of this study was to use plant physiological parameters such as plantlet re-rooting, relative water content, relative electrolyte leakage and hydrogen peroxide activity to evaluate osmotic stress tolerance in six putative osmotic tolerant tolerant plantlets (*Mut1 – Mut6*); generated and selected for osmotic tolerance in Chapter three.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Six putatively osmotic-tolerant sugarcane mutant plantlets (*Mut1-Mut6*) generated from NCo376 by callus exposure to 16 mM EMS and selection on mannitol medium (Chapter 3) were characterized for osmotic-tolerance at whole plant level using a method described by Snyman *et al.* (2016). The experimental procedure for the establishment and screening of the mutant lines is summarized in Figure 4.1.

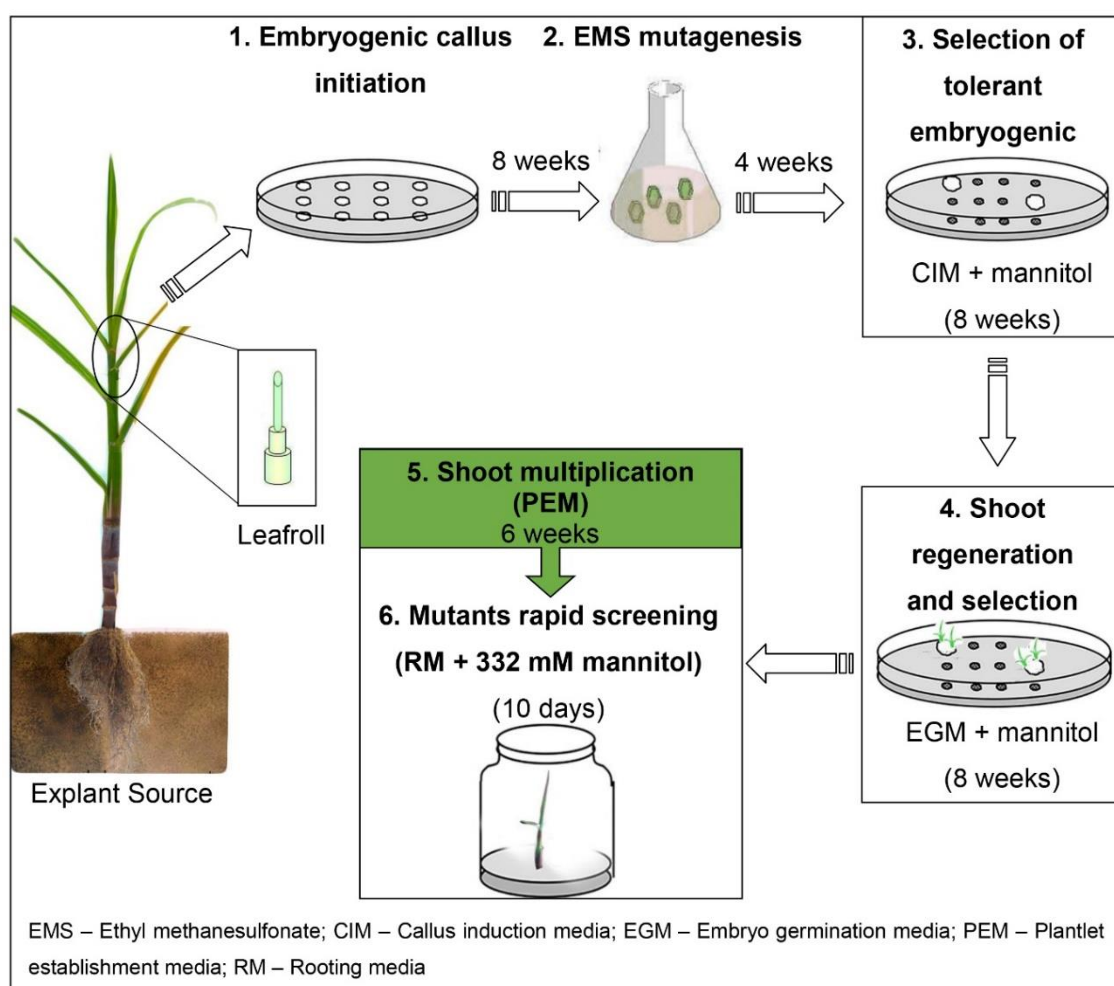

Figure 4.1 Summary of experimental procedures undertaken to induce mutation and confirm tolerance of putative EMS mutants to osmotic. Stage 1 to 4 illustrating screening in callus culture (Chapter 3) and stage 5 to 6 illustrating screening at whole plant level (Chapter 4).

4.2.2 Shoot multiplication

Individual rooted plantlets with a height greater than 20 mm were cultured in growth vessels containing between 40and 80 mL plantlet establishment media (PEM) for six weeks. The PEM was composed of 2.2 g L^{-1} MS basal salts and vitamins, 5 g L^{-1} sucrose, 8 g L^{-1} plant tissue culture agar and the pH 5.8 was corrected before autoclaving for 20 minutes (Koch *et al.*, 2012). Mutants were sub-cultured to a fresh PEM every two weeks and after six weeks' plantlets with a height greater than 70 mm were separated into individual shoots for subsequent use.

4.2.3 Root trimming and re-growth in medium with mannitol

To determine and rank the mutant osmotic stress, individual shoots were placed on sterilized glass (200 mm^2) and their roots were trimmed at length not greater than 1 mm. The shoots were placed on rooting medium composed of 4.4 g L^{-1} MS salts and vitamins, 1mg L^{-1} of 1-Naphthaleneacetic acid (NAA) 20 g L^{-1}, 332 mM mannitol and 8 g L^{-1} agar, pH was adjusted to 5.8 (Snyman *et al.*, 2016). NCo376 control was not exposed to mannitol stress. The plantlets were incubated in a plant growth chamber with a photoperiod of 16:8 (light/dark) for 10 days. The plant re-rooting percentage was recorded at 10 days after trimming.

4.2.4 Relative water content

The relative water content was recorded using the method described by Mullan and Pietragalla (2012). Leaves were randomly collected from plantlets after 10 days of mannitol stress. Leaf fresh weight was recorded immediately after removal from the plantlet and it was soaked in distilled water overnight at 4°C. Leaves were paper dried and turgid weight was recorded immediately. Leaves were then dried in an oven with temperatures set at 70°C for 24 hours and had their dry weight recorded. The relative water content was determined using the following formula: Relative water content = 100 x [(fresh weight – dry weight) / (turgid weight – dry weight)].

4.2.5 Electrolyte leakage

The electrolyte leakage was recorded as percent relative electrolyte leakage (REL) using a modified protocol described by Bajji *et al.* (2002). At room temperature, leaves with a length of 50 mm were placed in a 20 mL distilled water in a test tube. To remove solutes from the leaf surface and damaged cells, the test tube was shaken gently for three minutes and distilled water was replaced. Subsequently, electrolyte conductivity (EC1) was recorded using Thermo scientific Orion star A211 pH/conductivity meter (Life Technologies, Inc. Molecular Biology Products, Johannesburg, South Africa). The samples were incubated in 55°C water bath for 30 minutes and each sample electrolyte conductivity (EC2) was subsequently recorded. Electrical conductivity (EC3) was recorded after the samples were incubated at 100°C for an hour. Relative electrolyte leakage was calculated using the following formula, REL = 100 x [(EC2 – EC1) / EC3].

4.2.6 DAB staining

Hydrogen peroxide (H_2O_2) activity was determined using a 3,3'-diaminobenzidine (DAB) staining protocol (Daudi and O'Brien, 2012).

(a) Stock solution preparation

To make a 1% DAB stock solution, 0.1 g of 3, 3'-diaminobenzidine (DAB) was added in 10 mL distilled water and 10N HCl was added to lower the pH to 3.8. The solution was vortex until it turned to a light brown colour and it was placed at -20℃ until it was used. A 0.3% hydrogen peroxide stock solution was prepared by adding 100 µl of 30% H_2O_2 in 10 mL distilled water. The solution was stored aliquot at -20℃. A 0.01M phosphate buffer solution was composed of 1.20 g L^{-1} Na_2HPO_4 (anhydrous), 0.22 g L^{-1} NaH_2PO-4·H_20 and 8.5 g L^{-1} NaCl in distilled water. All reagents were mixed using magnetic stirrer until they were dissolved and the pH was adjusted to 7.2.

(b) Staining leaves with DAB solution

DAB staining was performed on leaves collected 10 days after culturing on RM containing 332 mM mannitol to determine H_2O_2 activity. Plantlet leaves were immersed in 10 mL DAB-Peroxidase substrate solution made of 500 µl of 1% DAB, and 500 µl of 0.3% H_2O_2 to 10 ml of PBS, pH 7.2 for overnight. Leaves were washed using 75% ethanol in hot water bath at 37°C for an hour. The presence of H_2O_2 in the leaves was visualized as brown deposits.

4.2.7 Statistical analysis

Statistical analyses were conducted using GenStat software (ver. 12.2; VNS International Ltd, USA). Duncan's multiple range test was used to determine significant differences at $P < 0.05$. The principal component analysis (PCA) and cluster analysis (CA) were used to discriminate and group mutants, respectively (XLSTAT 2018, Microsoft Excel plugin).

4.3 RESULTS

Selection under *in vitro* conditions has been conducted mainly on callus cultures with the disadvantages of loss of regenerative potential, lack of correlation between tolerances of cells in culture compared with whole plants. A preliminary protocol to *in vitro* screening for osmotic stress tolerance based on callus phenotype has been developed (Chapter 3). The main aim of the present chapter was to use plant physiological parameters such as plantlet re-rooting, relative water content, relative electrolyte leakage and hydrogen peroxide activity to eliminate escapes before the plantlets were evaluated *Ex vitro*.

4.3.1 Plantlet root regrowth

The average number of days for re-rooting ranged from 1.33 to 3.67 days (Table 4.1). Re-rooting of *Mut6 in vitro* plantlets took an average of 3.67 days, which was the longest duration for establishing new roots. The NCo376 control took the shortest duration (1.33 days) to initiate new roots, followed by *Mut1* with an average of 1.67 days.

Table 4.1 Average number of days for developing new roots and plant height to root length ratio (PH/R)

	RRD	**PH/R ratio**
NCo376	1.33	0.19
Mut1	1.67	0.18
Mut2	3.00	0.28
Mut3	2.67	0.28
Mut4	3.33	0.17
Mut5	3.00	0.14
Mut6	3.67	0.19

RRD – the average number of days for the development of new roots. PH/R ratio – Plant height to root length ratio

Some plantlets that were the fastest to re-rooting displayed a low plantlet height to root length ratio. However, they were inconsistencies with these findings, for instance, *Mut4* plantlet height to root length ratio was 0.17 and new root initiation took an average of 3.33 days. This was in contrast with *Mut1* results, where the plantlet height to root length ratio was 0.18 and an average number of days for new root initiation was 1.67.

All mutant lines, except *Mut1*, had a significant reduction in re-rooting ability when compared with the NCo376 (Figure 4.2). The *Mut2* plantlet showed an 11.53% (P = 0.0037) mean reduction in re-rooting ability when compared with NCo376 and 9.52% (P = 0.0471) mean reduction when compared with *Mut1*. The largest reduction in re-rooting was recorded *Mut3* with a mean decrease of 14.32% (P = 0.0039) compared with the NCo376 and 12.31% (P = 0.0090) compared to *Mut1*. The *Mut2* and *Mut6* did not differ significantly in their re-rooting ability (P = 0.5029).

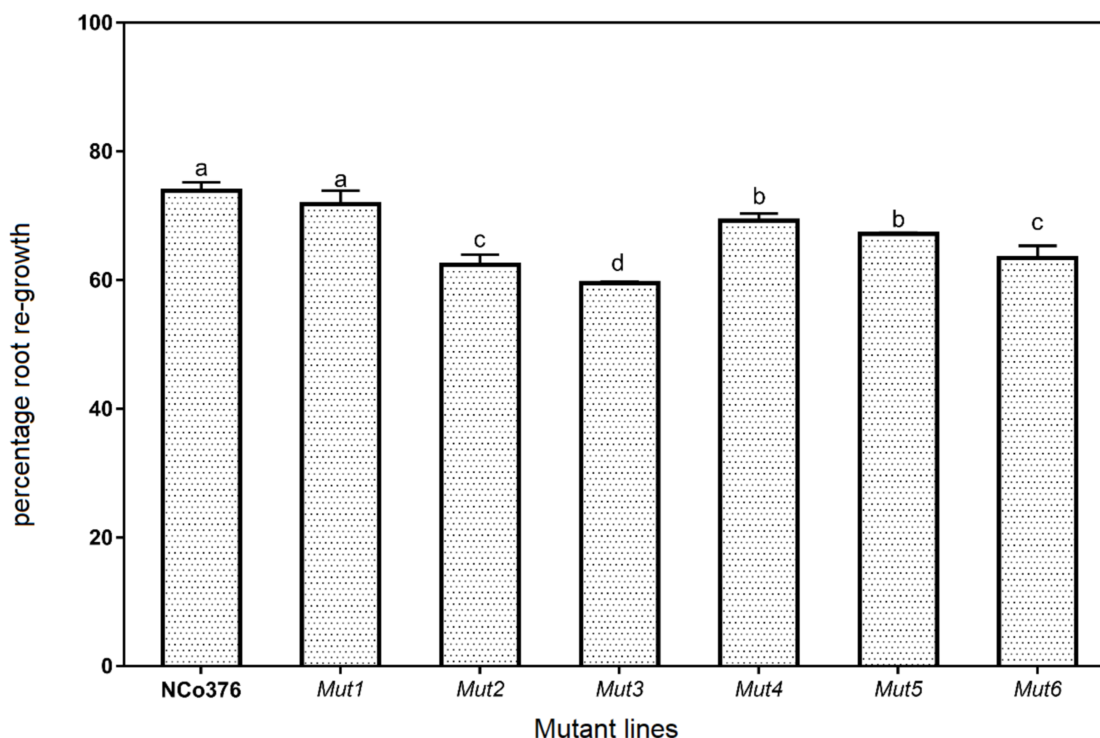

Figure 4.2 Re-rooting ability of different mutant lines after 10 days of exposure to 332 mM mannitol.

Mean values with deferent letter different significantly at $P < 0.05$.

4.3.2 Relative water content

Treatment of plantlets with mannitol stress resulted in a significant reduction in plantlet relative water content except for *Mut4* (Figure 4.3). The highest relative water content was recorded on *Mut4* (95.96%) and the lowest on *Mut2* (64.22%) plantlets. The *Mut2* plantlets showed the largest decrease in relative water content with a mean decrease of 31.74% ($P = 0.0016$) compared with *Mut4* and 25.61% ($P = 0.0186$) compared with the NCo376. A mean loss of 10.54% ($P = 0.0442$) of relative water content was recorded for *Mut1* plantlets when compared with the NCo376 and 16.67% ($P = 0.0113$) compared with the *Mut4* plantlets. The *Mut2*, *Mut3*, *Mut4* and *Mut6* plantlets did not differ significantly ($P > 0.05$) from each other in their relative water content.

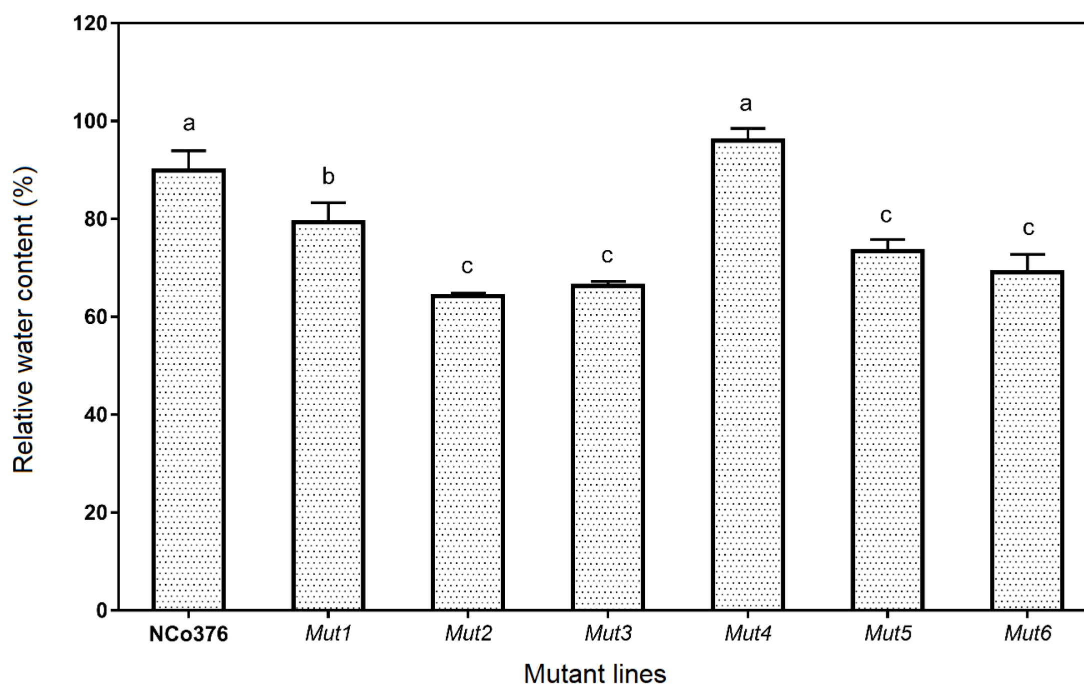

Figure 4.3 Relative water content of the different mutant lines after 10 days of exposure to 332 mM mannitol.

Mean values with deferent letter different significantly at $P < 0.05$.

4.3.3 Electrolyte leakage

Mannitol induced osmotic stress significantly increased the relative electrolyte leakage for all the mutants (Figure 5.4). *Mut6* showed the largest loss of relative electrolyte leakage with a mean difference of 59.73% ($P = 0.0043$) in comparison with NCo376. *Mut1*, *Mut3* and *Mut4* were not found to be significantly different ($P > 0.05$) and had intermediate relative electrolyte leakage of 56.15%, 60.24% and 58.31%, respectively. In comparison to NCo376, *Mut2*, *Mut3* and *Mut5* showed mean loss of 39.91% ($P = 0.0012$), 27.00% ($P = 0.0039$) and 36.01% ($P = 0.0149$) of relative electrolyte leakage, respectively.

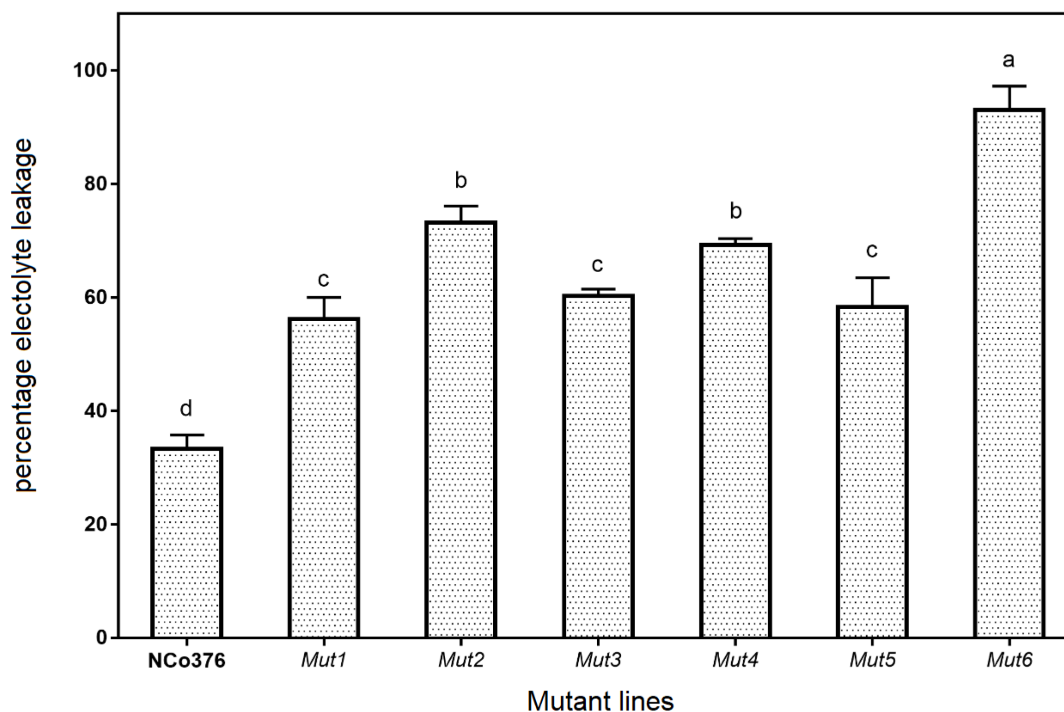

Figure 4.4 Relative electrolyte leakage of the different mutant lines after 10 days of exposure to 332 mM mannitol.

Mean values with deferent letter different significantly at $P < 0.05$.

4.3.4 DAP staining

Mannitol-induced osmotic stress enhanced H_2O_2 activity which is revealed as brown deposition on the leaves (Figure 5.5). *Mut1*, *Mut4*, and *Mut5* leaves appeared green under mannitol stress compared to *Mut2*, *Mut3* and *Mut6*, which appeared light green. *Mut2* and *Mut6* showed more intense brownish colour, indicating the highest potential of H_2O_2 accumulation among the tested mutants. *Mut1* and *Mut5* showed the least accumulation of H_2O_2.

Figure 4.5 DAB stained leaves to determine H_2O_2 activity after 10 days of exposure to mannitol-induced osmotic stress.

4.3.5 Principal component analysis

The principal component analysis was grouped into three quantitative traits which accounted for the variability among the six mutant lines (Table 4.2).

The first principal component (PC1) had an eigenvalue of 2.42 and accounted for 80.67% of the genetic variability observed. The contribution of each trait for variability for both the negative and positive loadings in the PC1 was 37.06% for the re-rooting ability, followed by relative electrolyte leakage (33.43%), and relative water content (29.51%), respectively (Table 6.1).

An interesting observation shown by PC1 was a strong negative correlation of electrolyte leakage with the re-rooting ability and relative water content. There was a highly significant positive correlation between re-rooting ability and relative water content, as indicated by the acute angle (Figure 4.6). The genotypes which have a high value of PC1 were expected to maintain growth under mannitol stress, consequential,

re-rooting ability and relative water content seem to be a good parameter to discriminate mutants for osmotic stress tolerance under *in vitro* conditions. Interestingly, *Mut1* re-rooting ability was higher than that of NCo376 even though NCo376 was cultured under normal conditions.

Table 4.2 Principal component analysis for quantitative traits

Variables	**PC1**
Eigenvalue	2.42
Variability (%)	80.67
Cumulative %	80.67
The contribution of each fact (%)	
RWC	29.51
RR	37.06
REL	33.43
Factor loading	
RWC	0.84
RR	0.94
REL	-0.89

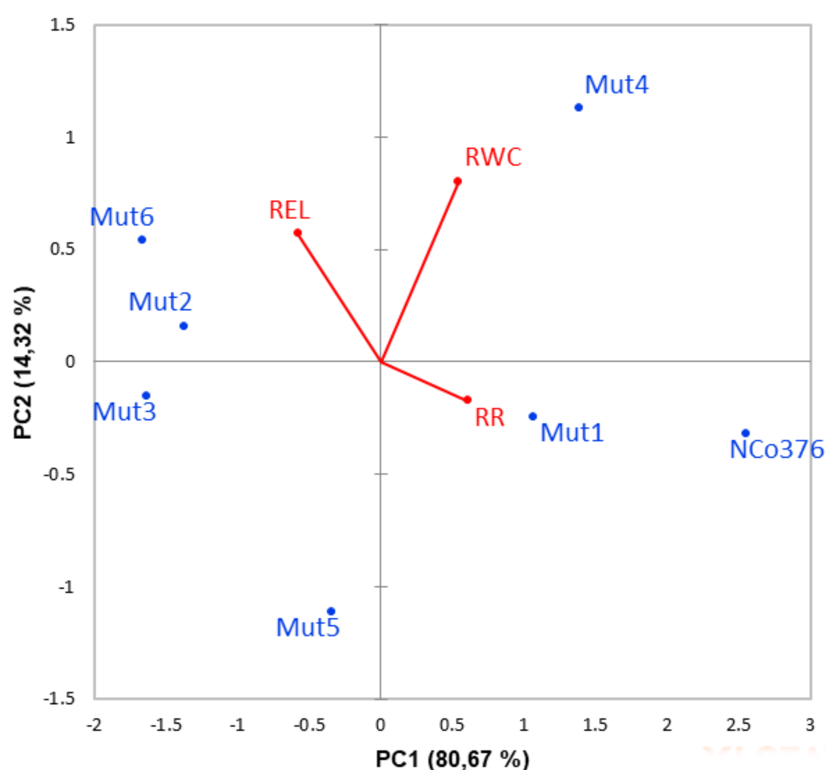

Figure 4.6 Principal component biplot showing a variation of the sugarcane mutant by physiological traits.

RR – re-rooting ability; RWC – relative water content; REL – relative electrolyte leakage.

4.3.6 Cluster analysis

The dendrogram (Figure 5.7) illustrated based on the physiological response data grouped the six mutants into three main clusters. Cluster one mostly consisted of mutants that showed higher relative water content and re-rooting ability under water stress, whereas cluster two mainly consisted of mutants that were characterized by higher relative electrolyte leakage. *Mut4* was more diverse than the other genotype but shares some similar characteristics with the *Mut1* and Nco376 in cluster one. The second cluster consisted only *Mut5* whereas the third cluster consisted of *Mut2, Mut3* and *Mut6.* In general, *Mut4* demonstrated the highest tolerance to the osmotic stress, followed by *Mut1*. *Mut5* moderate tolerance and *Mut3*, *Mut2* and *Mut6* were the least tolerant.

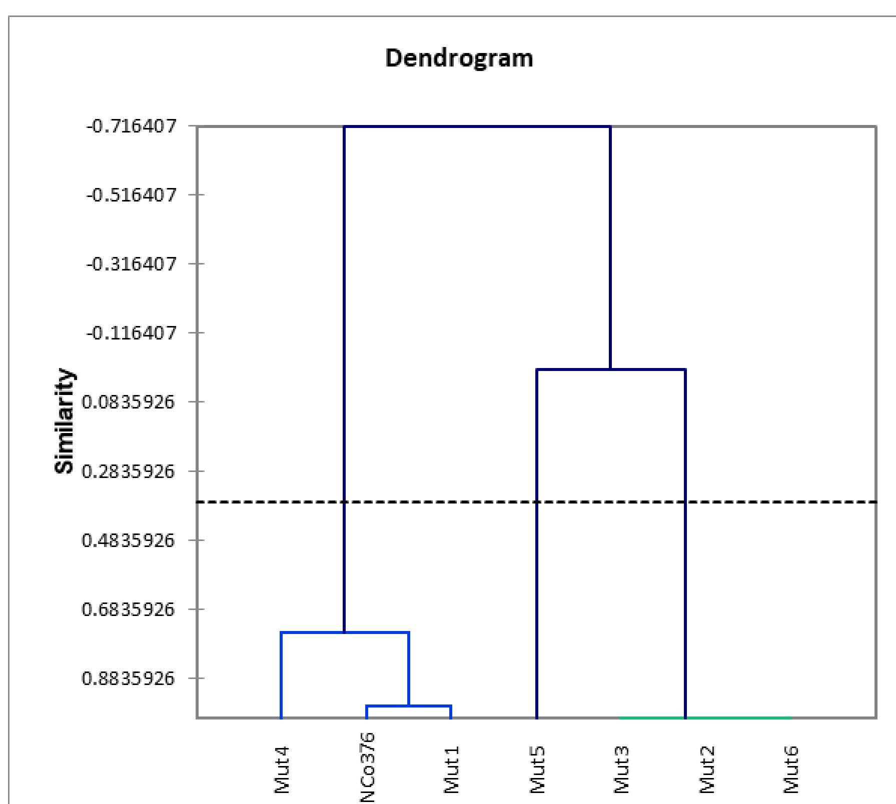

Figure 4.7 Hierarchical cluster analysis dendrogram displaying the six EMS mutants and NCo376.

4.4 DISCUSSION

Genetic improvement of sugarcane is crucial to create new varieties possessing traits of agronomic importance, such as high sucrose content, combined with resistance or tolerance to biotic and abiotic stresses. Conventional plant breeding in sugarcane is challenging due to long breeding period, polyploidy, low genetic variability and sterility (Thirugnanasambandam *et al.* 2018; Zhang *et al.*, 2018). Mutation breeding can complement conventional breeding for developing improved varieties of sugarcane. Therefore, it is essential to establish a high throughput selection and regeneration systems, which can be applicable for mass selection under *in vitro* conditions to minimize escape before further characterization under field conditions (Snyman *et al.*, 2016; Snyman *et al.*, 2019). Most of the *in vitro* selection protocols are dependent on selection at embryo germination level. This approach is constrained by a high number of escapes during when the selected lines are further evaluated under field conditions. Therefore, there is a need to optimize the selection protocol using a number of physiological and morphological parameters at the whole plant level to minimize escapes.

4.4.1 Physiological response to osmotic stress

Significant differences were observed in mutants' re-rooting ability, relative water content and relative electrolyte leakage under-mannitol water stress. The *Mut1* plantlets showed the highest (73.87%) re-rooting ability and the lowest re-rooting was recorded on *Mut3* (59.54%). *Mut1*, *Mut4* and *Mut5* showed a significant high re-rooting ability and relative water content when compared to *Mut2*, *Mut3* and *Mut6* (Figure 4.2). Lack of correlation intolerance exhibited in callus cultures versus the whole plant level is common in EMS mutants selected for osmotic tolerance (Masoabi *et al.*, 2017; Khalil *et al.*, 2018). Callus cultures are prone to epigenetic changes, consequently, tolerant callus in culture do not always exhibit tolerance at the whole plant level (Rai *et al.*, 2011). Even though screening at the embryo germination stage is more effective than at maturation, 'escapes' have been observed (Mahlanza *et al.*, 2013). 'Escapes' can be eliminated by assessing re-rooting ability in whole plant levels exposed to mannitol stress (Snyman *et al.*, 2016).

Plantlets that showed the highest re-rooting ability under water stress reduced their shoot growth. For instance, *Mut2* and *Mut3* showed a high plantlet height to re-rooting ability ratio under stress. However, *Mut1*, *Mut5* and *Mut6* showed a low plantlet height to re-rooting ability ratio under stress (Table 4.1). Different root and shoot characters have been reported in sugarcane under different soil moisture contents (Inman-Bamber and Smith, 2005; Marcos *et al.*, 2018). In moist soils, sugarcane roots show lateral growth and do not grow deep. However, in soils with low soil moisture content, shoot growth is reduced and roots grow deep (apical growth) (Laclau and Laclau, 2009). Root growth under water stress also differs among cultivars (Smith *et al.*, 2005; Snyman *et al.*, 2016). Osmotic tolerant cultivars such as N27 translocate nutrients from vegetative parts to the roots to maximize the rooting potential for water absorption (Robertson *et al.*, 1999). However, osmotic susceptible cultivars such as NCo376 display low root re-growth under *in vitro* mannitol stress (Snyman *et al.*, 2016) and low root biomass under field conditions (Inman-Bamber, 1982). Reducing shoot growth to save water is osmotic stress adaptive strategy used by plants (Farooq *et al.*, 2009).

Mannitol stress reduced mutated plantlets relative water content by between 10.54 and 31.74% when compared with untreated NCo376 (Figure 4.3). Reduction in relative water content due to water stress in sugarcane is well documented (Zingaretti *et al.*, 2012; Augustine *et al.*, 2015a). *Mut1* and *Mut4* maintained a higher relative water content when compared to *Mut2*, *Mut3*, *Mut5* and *Mut6*, respectively. relative water content is used as an osmotic tolerance stress marker in sugarcane (Augustine *et al.*, 2015b). Genotypes that are osmotic tolerant often have relatively high relative water content values compared to those in the susceptible group (Rampino *et al.*, 2006).

Osmotic stress leads to disturbance in plant metabolism and causes oxidative injuries which are characterized by an increase in relative electrolyte leakage and H_2O_2 (Bach-Pages and Preston, 2018). In this study, PCA showed that *Mut2*, *Mut3* and *Mut6* were highly characterized by relative electrolyte leakage and low relative water content and re-rooting ability (Figure 4.6). In these mutants, the average increase in relative electrolyte leakage ranged between 22.91 and 63.73%. The stress-induced relative electrolyte leakage is usually accompanied by accumulation of reactive oxygen species

and often results in programmed cell death (Demidchik *et al.*, 2014). Reactive oxidative species, specifically H_2O_2, are responsive to abiotic and biotic stress (Patade *et al.*, 2012) and in low concentrations, H_2O_2 can act as a secondary messenger for osmotic stress (Lima *et al.*, 2015). In contrast, excess H_2O_2 production and prolonged osmotic stress cause oxidative damages to plant macromolecules (Koiwa, 2010). In this study, DAB staining showed *Mut6* had high amounts of H_2O_2 activity, characterized by large brown patches and necrotic cells (Figure 4.5). High H_2O_2 activity results in damages in DNA molecules, proteins, cell membranes and eventually the plant dies (Boaretto *et al.*, 2014). In this study, analysis of H_2O_2 activity using DAB staining was constrained by the small size of the leaves of plantlet. Therefore, in future studies, alternative protocols such as utilizing enzyme activity-based analysis (Velikova *et al.*, 2000) will be used to improve the precision for assessment of H_2O_2 activity of *in vitro* plantlets.

4.4.2 Osmotic tolerant mutant lines

Principal component analysis (PCA) is used to identify superior genotypes for both stressed and non-stressed conditions (Shiri *et al.*, 2010). Based on the results given above, the most notable relationships illustrated by the PCA were a strong negative correlation between relative electrolyte leakage with relative water content and re-rooting ability. PCA demonstrated that plantlet re-rooting ability is a key marker to identify osmotic tolerant mutant lines under *in vitro* conditions, and this was in accordance with Snyman *et al.* (2016). Re-rooting ability contribution to PC1 was 37.06% (Table 4.2). Plantlet shoot growth did not appear to be a good indicator for determining osmotic-tolerant genotype under *in vitro* conditions due to an inconsistent response amongst the different mutant lines studied. According to Snyman *et al.* (2016), sugarcane leaves re-growth under mannitol stress are less susceptible to mannitol stress. This suggests that vegetative parts are not good parameters for assessing osmotic tolerance under *in vitro* conditions. PCA enables easy selection of tolerant genotypes when there are many traits to be used for selection (Farshadfar and Sutka, 2003; Bündig *et al.*, 2016). Nevertheless, some researchers have demonstrated that a minimum of three traits can be used in PCA to characterize cultivars for osmotic tolerance under *in vitro* conditions (Kacem *et al.*, 2017; Wu *et al.*, 2017).

Cluster analysis was performed in order to group mutants based on their performance under stress. *Mut3*, *Mut2* and *Mut6* are the least tolerant to mannitol stress and they could be considered ‘escapes’ from the callus selection. *Mut1* and *Mut4* plantlets showed a remarkable performance under stress conditions and they were grouped with NCo376 plantlets which were cultured under non-stress conditions. In general, these mutant lines can be ranked in following order, *Mut4* > *Mut1* > *Mut5* > *Mut3* = *Mut2* = *Mut6* (Figure 4.7). Field assessment will have to be conducted to assess the yield component of the mutants as EMS mutagenesis is random, and can alter the yield component characteristics and other agronomic traits. For instance, *Mut1* genotype that was *in vitro* selected by Koch *et al.* (2012) for herbicide resistance was found to be highly susceptible to *Eldana saccharina* when compared with its relative wild type N12 genotype (Koetle *et al.*, 2018). Interestingly, even though *in vitro* micropropagation can compromise traits such stalk diameter (Snyman *et al.*, 2018), some previous EMS mutant lines did not show variation in their important yield components such as the number of stalk/plots, stalk height, sucrose and fibre content (Rutherford *et al.*, 2017; Koetle *et al.*, 2018).

4.5 CONCLUSION

This study showed that the ability to re-root *in vitro* under mannitol stress is a good osmotic stress tolerance indicator and can be used to eliminate escapes from callus selection. *Mut1* and *Mut4* were characterized as putative osmotic tolerant mutant lines. Therefore, these mutants will have to be *in vitro* micropropagation to obtain enough replicates for field assessment.

To optimize the *in vitro* selection at the whole plant level, future work will have to incorporate proteomics analysis and marker-assisted selection to assess the status of important traits such as sucrose content. This will minimize the amount of time required to conducts rain-shelter and/or field selection to discriminate *in vitro* escapes and the time required to assess agronomic traits.

CHAPTER 5
GENERAL DISCUSSIONS AND CONCLUSIONS

5.1 GENERAL DISCUSSIONS

Drought stress is one of the main agronomic challenges that result in a reduction in sugarcane yield (Azevedo *et al.*, 2011). Conventional breeding has been used with limited success to improve sugarcane osmotic stress tolerance (Mastrangelo *et al.*, 2012). Conventional breeding is constrained by sugarcanes complex genome, low heritability and high genotype by environmental interaction influenced by differences arising from soil heterogeneity and other ecological factors (Zhou and Joshi, 2012; Sengwayo *et al.*, 2017). Furthermore, the application of transgenic technology is constrained by public concerns for genetically modified crops and legislation (Mastrangelo *et al.*, 2012). Sugarcane produced by means of *in vitro* mutagenesis, such as one utilized in this study, is more advantageous than conventional breeding and it is more acceptable than transgenic technologies. Therefore, in this study, investigations were conducted to determine the effect of varying ethyl methanesulfonate incubation periods in sugarcane callus and to determine optimum mannitol lethal doses to select at embryo germination stage. To induce osmotic stress and determine optimum lethal doses, calli were cultured on media containing 0, 150, 225 and 300 mM mannitol for eight weeks.

The results of this study indicated that incubation of calli for 2 and 3 h in EMS resulted in a significant reduction callus relative growth rate, fresh weight, dry weight and embryo regeneration. Furthermore, the 2 and 3 h treatment resulted in the severity of stunted and chlorophyll deficient embryos. Reactive oxidative species such as H_2O_2 increases programmed cell death. In this study, non-viable calli were visible as brownish necrotic cells on the callus surface, the number of which increased with an increase in EMS incubation period. The results suggest that exposing calli to 16 mM EMS for 1 h resulted in the induction of mutations without resulting in a significant number of abnormalities in the generated embryos.

Incubating calli on callus inducing medium that contained mannitol at embryo maturation and germination resulted in a high number of necrotic calli and significantly reduced embryo germination. The mannitol LD_{50} and LD_{90} concentrations for selection at the embryo germination stage were determined as 224 mM and 407 mM mannitol, respectively. Under mannitol stress conditions, inhibition of callus growth and increases in abnormalities such as hyperhydric cells might be due to the reduction of cell wall constituents.

In general, for plants selected at embryo germination stage, there is a lack of correlation between tolerances of cells in culture versus whole plant levels. Therefore, in this study to confirm tolerant plantlets were generated, they were tested at the whole plant level by assessing the plantlet root re-growth in media supplemented with 332 mM mannitol (Snyman *et al.*, 2016) for 10 days. Of the six NCo376 putative osmotic tolerant mutants that were isolated from the EMS-mutagenized callus; *Mut1* had the highest re-rooting ability under mannitol stress and *Mut4* had the highest relative water content. *Mut2* and *Mut6* demonstrated poor re-rooting ability and relative water content response and were characterized by high electrolyte leakage and H_2O_2 activity. Only *Mut1* and *Mut4* showed promising osmotic tolerance characteristics due to their better physiological response under mannitol stress.

5.2 CONCLUSION AND FUTURE WORK

In this study, we identified two mutants (*Mut1* and *Mut4*) which had enhanced osmotic stress tolerance. The mutants had high re-root growth and relative water content under osmotic stress. However, due to the project timeframe, mutants were not tested *Ex vitro*, therefore future work will include micropropagation and acclimatizing putative mutant to screen for tolerance *Ex vitro* using physiological analysis. Other analysis such as proteomics and gene expression will be done to improve the understanding of the effect of EMS in sugarcane osmotic stress tolerance. *In vitro* screening for osmotic stress tolerance is an effective method of selection, but whether the osmotic stress tolerance can be maintained in later generations of mutants still needs to be further investigated.

Mutants lines that show characteristic to be osmotic tolerant at the whole plant level will be acclimatized and exposed to drought stress under field conditions to determine the effect of drought stress to the relative water content, photosynthetic rate and membrane damages. In additions, genes that may have been affected by EMS mutagenesis and type of mutation will have to be identified to assess the observed enhanced drought stress tolerant on the mutant.

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APPENDIXES

Appendix 1 Growth regulators stock solutions preparation procedure and storage conditions

Concentrations of plant growth regulator dissolved to make a stock solution (4 ℃)	**Final concentration per litre of growth medium**
300 mg 2,4-Dichorophenoxyacetic acetic acid stock solution	
Dissolved in 5 mL auxin, dH_2O was added to make 10 mLfollowed by gentle agitation. The final volume was made up to 1000 mL	3 mL

10 mg Napthalene acetic acid stock solution	
Dissolved in 1 ml of 1 N NaOH. dH_2O was added to the final volume was made up to 10 ml	1 mL

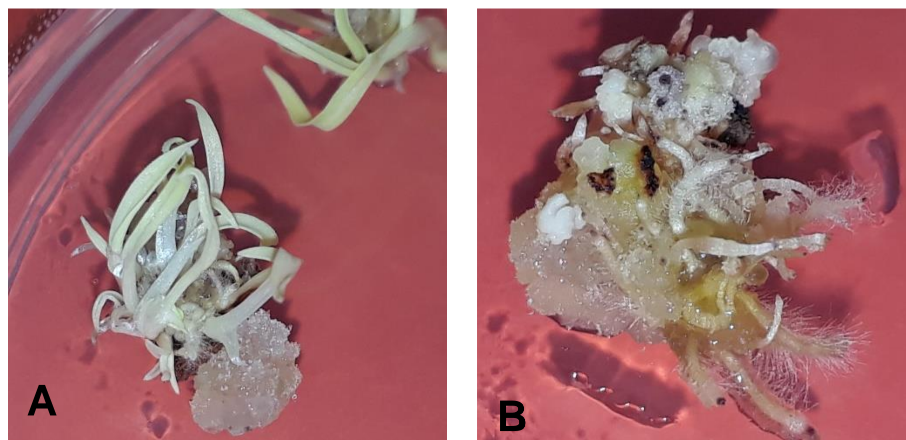
dH_2O – Distilled water

Appendix 2 Callus weekly average growth for all the treatments

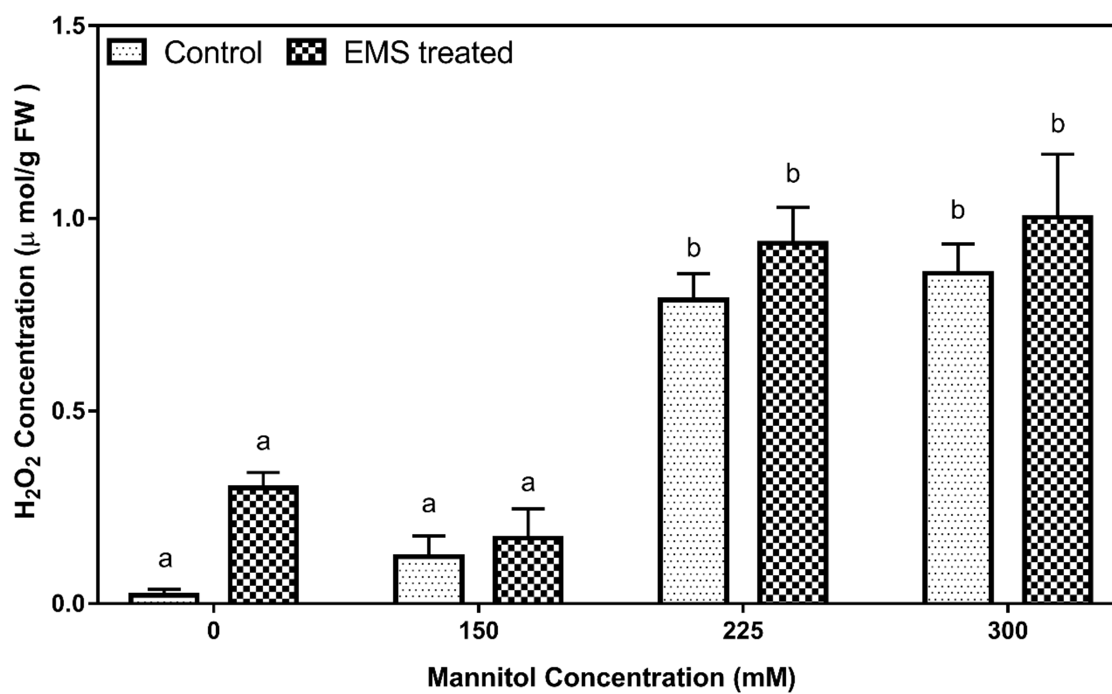
Week	Av. Mass (g)
0	0.32[a]
2	0.74[a]
4	1.61[b]
6	2.05[b]
8	2.90[c]
10	3.58[d]
12	3.98[d]

Mean values with different letters are significantly different at $P < 0.05$.

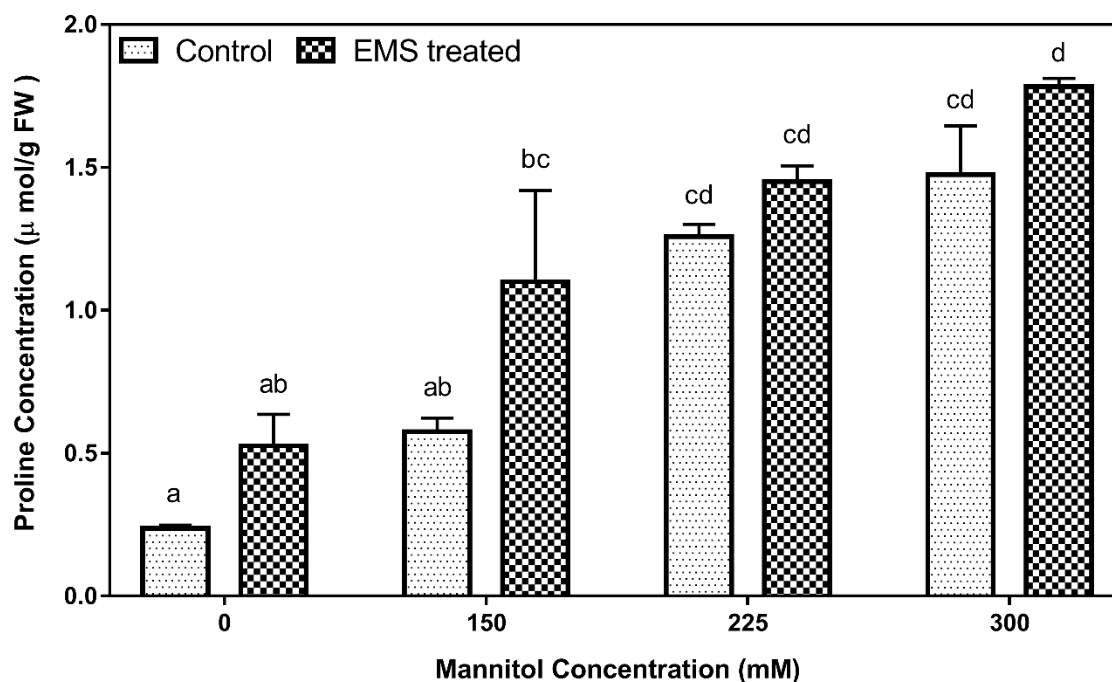
Appendix 3 Abnormalities during shoot formation on EMS treated calli. Albino shoots (A) and Callus that only developed roots (B).



Appendix 4 Hydrogen Peroxide activity in callus treated for one hour in EMS and control under different levels of osmotic stress



Appendix 5 Proline content in callus treated for one hour in EMS and control under different levels of osmotic stress



Appendix 6 Correlation coefficient *P* values

Variables	Mannitol	CLE	HCE	Lignin	Ash	CWC	DW
Mannitol	**0**	**0.003**	**< 0.0001**	**0.002**	**< 0.0001**	**< 0.0001**	**< 0.0001**
CLE	**0.003**	**0**	**0.001**	0.100	0.096	**0.003**	0.013
HCE	**< 0.0001**	**0.001**	**0**	**0.007**	**0.005**	**< 0.0001**	**< 0.0001**
Lignin	**0.002**	0.100	**0.007**	**0**	0.028	0.011	**0.003**
Ash	**< 0.0001**	0.096	**0.005**	0.028	**0**	**< 0.0001**	**< 0.0001**
CWC	**< 0.0001**	**0.003**	**< 0.0001**	0.011	**< 0.0001**	**0**	**< 0.0001**
DW	**< 0.0001**	0.013	**< 0.0001**	**0.003**	**< 0.0001**	**< 0.0001**	**0**

Appendix 7 Mutants physiological trait variation after 10 days of stress treatment

	RWC	**RR**	**A. DR**	**PH (cm)**	**PH/RR ratio**
NCo376	74.50	73.87	1.33	14.76	0.19
Mut1	64.62	71.85	1.67	13.35	0.18
Mut2	55.80	62.33	3.00	17.78	0.28
Mut3	63.02	59.54	2.67	15.18	0.28
Mut4	76.63	69.25	3.33	11.81	0.17
Mut5	73.22	67.17	3.00	9.93	0.14
Mut6	59.07	63.46	3.67	11.37	0.19

RWC – relative water content; RR – re-rooting; A. DR – Average number days to rerooting; PH – Plantlet height.

Appendix 8 PCA factor scores

	F1	F2	F3
NCo376	2.548	-0.319	-0.193
Mut1	1.065	-0.243	0.455
Mut2	-1.369	0.159	-0.071
Mut3	-1.628	-0.150	-0.678
Mut4	1.384	1.128	-0.178
Mut5	-0.338	-1.112	0.120
Mut6	-1.662	0.538	0.545

Appendix 9 PCA contribution of the observations (%)

	F1	F2	F3
NCo376	38.315	3.387	3.545
Mut1	6.697	1.959	19.680
Mut2	11.067	0.836	0.473
Mut3	15.636	0.752	43.698
Mut4	11.309	42.313	3.023
Mut5	0.676	41.133	1.375
Mut6	16.300	9.620	28.206

Appendix 10 PCA squared cosines of the observations

	F1	F2	F3
NCo376	**0.979**	0.015	0.006
Mut1	**0.810**	0.042	0.148
Mut2	**0.984**	0.013	0.003
Mut3	**0.846**	0.007	0.147
Mut4	**0.595**	0.395	0.010
Mut5	0.084	**0.906**	0.011
Mut6	**0.825**	0.086	0.089