Technique establishment for *in vitro* selection of drought tolerant sugarcane genotypes.

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As the candidate's supervisor I have/have not approved this dissertation for submission

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Abstract

There is a need to have constant supply of sugarcane varieties adapted to different South African regions. However, the genetic improvement and selection of sugarcane cultivars with superior traits, e.g. drought tolerance, are difficult due to its complex polyploid and aneuploid genome. Biotechnology approaches are being investigated for the selection and/production of drought tolerant cultivars. Towards this end, the aim of this study was to establish: 1) the best source of meristematic explant for initiation and mass propagation of *in vitro* shoots; 2) *in vitro* conditions to screen and select for drought tolerance; and 3) physiological parameters as indicators of drought tolerance *in vitro*.

Sugarcane stalks and shoots from single-budded setts of NCo376 were used. From the former, 1.3 cm-long meristems were isolated and used for shoot induction, shoot multiplication and rooting. The single-budded setts (approx. 50 mm) were first germinated in 20 ml sterile water or sterile moist paper, resulting in 100% and 60% sett contamination, respectively. With 1 mg l^{-1} methylene blue (MB) there was 30% sett contamination, whilst 1 mg l^{-1} MB in combination with 1 ml l^{-1} Previcur® or 1 ml l^{-1} BRAVO® resulted in 40% and 7% contamination, respectively. The uncontaminated germinated shoots (approx. 1 - 2 cm) were excised after 10 days in culture and used as the other source of meristems. Meristems from both sources were multiplied and rooted *in vitro* and their plantlet yield was 60 shoots/sugarcane stalk meristem and 10 shoots/meristem from *in vitro*-germinated sett.

NCo376 and N41 varieties were used to determine the effect of mannitol (204, 326, 448 and 569 mM) on *in vitro* plantlet shoot and root re-growth. For both, increased mannitol in the media delayed shoot and root re-growth, with NCo376 being affected first. Stress was more significant on root than on shoot re-growth. For NCo376 plantlets, there were significant differences in root re-growth between 0 and 204 mM and the other tested treatments. For N41 plantlets, % root re-growth at day 10 on 569 mM mannitol was significantly higher than that at the other treatments. At 4 – 10 days, % shoot re-growth of NCo376 on 0 and 204 mM mannitol was greater than that at 326, 448 and 569 mM mannitol. Similar results were observed with N41 plantlets.

The LD_{50} and LD_{90} for mannitol were 332 and 606 mM for NCo376, and 851 and 1493 mM for N41. There was no differences between the effects of polyethylene glycol-6000 (PEG-6000) and mannitol on root re-growth at the same osmotic potential. However, PEG-6000-containing cultures required to be aerated. As at 87 mM PEG-6000, NCo376 plantlets showed 50% root re-growth compared to 10% in non-aerated cultures, mannitol was used in subsequent investigations.

Mannitol concentrations equivalent to LD_{50} and LD_{90} for NCo376 and N41 were used to screen N12, N36, N19 and N26 varieties. Based on the results obtained, the varieties were ranked on their tolerance to mannitol stress: N41 > N26 > N36 > N12 > N19 > NCo376.

Leaf electrolyte leakage, leaf chlorophyll content measured with Soil Plant Analysis Development (SPAD) measurements, and histochemical detection of hydrogen peroxide (H_2O_2) (with nitroblue tetrazolium) and superoxide anion ($O_2^{-\bullet}$) (with 3, 3'-diaminobenzidine) production were evaluated as indicators of stress using N41, N26, N19 and NCo376 on 332, 606 or 851 mM mannitol. N19 and NCo376 plantlets on 332 mM mannitol showed a higher % electrolyte leakage at day 5 (70%) than at day 10 (40 – 50%) of culture than N41 and N26 plantlets. A slight decrease in chlorophyll content was recorded at day 10 of culture in 332 and 851 mM mannitol, with no differences between NCo376 and N19, and N41 and N26. NCo376 and N19 accumulated more H_2O_2 than N41 and N26. $O_2^{-\bullet}$ accumulation was also greater in NCo376 and N19 than in N41 and N26. All these parameters detected stress at lower levels of mannitol (332 and 606 mM), but not at 851 mM. It was concluded that mannitol stress *in vitro* (332 – 606 mM), in combination with the physiological assays allow for the discrimination of *in vitro* osmotic stress among sugarcane varieties. Further work is necessary before recommendations can be made regarding the use of the other stress biomarkers.

FACULTY OF SCIENCE AND AGRICLTURE

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PREFACE

The experimental work described in this thesis was conducted at the University of KwaZulu-Natal, School of Life Sciences and at the South African Sugar Research Institute, Biotechnology Department, under the supervision of Professor M. P. Watt and Dr S. J. Snyman.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged.

Table of contents

List of Tables

List of Figures

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Dedicated to the sweet memories of my loving mother, R.S Vilakazi,

the inspiration behind my research

List of abbreviations

%	percent
°C	degrees Celsius
µM	micromolar
µm m^{-2} s^{-1}	micromole per meter per second
2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
APX	ascorbate peroxide
AQP	aquaporin
ASC	ascorbate
ATHK	histidine kinase receptor
BAP/BA	benzylaminopurine
bar	phosphinotricin acetyl transferase
bZIP	basic leucine-zipper protein
CAT	catalase
CDPKs	calcium-dependent protein kinases
cm	centimeter
CrylAb	Bt endotoxin
DAB	3,3- diaminobenzidine
DHAR	dehydroascorbate reductase
DMF	N,N-dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DREB	dehydration-responsive element-binding
EMS	methanesulphonic acid ethyl ester
g l^{-1}	grams per liter
GA_3	gibberelic acid
GM	genetically modified

GR	glutathione reductase
h	hour
H_2O_2	hydrogen peroxide
HSP	Heat Shock Protein
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ITP	inositol triphosphate
Kinetin	6-furfuryaminopurine
KZN	KwaZulu Natal
LD_{50}	50% lethal dose
LD_{90}	90% lethal dose
LEA	Late Embryogenesis Abundant protein
LSD	Least Significant Difference
MAPKs	mitogen-activated protein kinases
MB	methylene blue
MDHAR	monodehydro-ascorbate reductase
mg l^{-1}	milligrams per liter
ml	millimeter
ml l^{-1}	milliliter per liter
mm	millimeter
mM	millimolar
MMS	methylemethanesulphonate
MPa	megaPascal
MS	Murashige and Skoog (1962)
MT	moist towel
NAA	naphthaleneacetic acid
NADP-ME	NADP malic enzyme
NBT	nitroblue tetrazolium

npt	neomycin phosphotransferase
$O_2^{-\bullet}$	superoxide anion
$OH^{\bullet}$	hydroxyl radical
P	Previcur®
PEG-6000	polyethylene glycol-6000
PEPCK	phosphoenolpyruvate carboxylase
PEPCK	phosphoenolpyruvate carboxykinase
PGRs	plant growth regulators
PLD	phospholipase D
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
Rubisco	ribulose-1,5-bisphophate carboxylase
RWC	relative water content
SACU	Southern African Customs Union
SASA	South African Sugarcane Association
SASRI	South African Sugarcane Research Institute
SCMV	Sugarcane Mosaic Virus
SCYLV	Sugarcane Yellow Leaf Virus
sHsp	small Heat Shock Protein
SOD	superoxide dismutase
TDZ	thiadiazuron
TFs	Transcription Factors
Ti	tumour-inducing plasmid
UV light	ultraviolet light
W	water only
wk	week

1. Introduction and rationale for the study

Sugarcane (*Saccharum* spp. hybrids), a perennial grass which belongs to the Poaceae family (Daniels and Roach, 1987) is one of the top ten most planted crops in the world (Bower and Birch, 1992; Selman-Housein et al., 2000; Suprassanna et al., 2011; Pervaiz et al., 2013). It is grown mainly in tropical and sub-tropical regions in the world and the South African sugar industry is an eminent contributor as one of the world's major cost manufacturers of high quality sugar (www.sasa.org.za). In South Africa, the sugar industry contributes to employment, particularly in rural areas, by supporting small scale farmers (SASA, 2013). For such a sustainable sugar industry, there is need to produce novel genotypes that are able to grow in different regions (Kern, 2002; James, 2008; Snyman et al., 2011).

Sugarcane cultivars with desired traits, such as disease or stress tolerance, increased yield and improved ratooning ability, have been produced through breeding (Sengar et al., 2011; Snyman et al., 2011). However, sugarcane breeding is limited by many challenges, such as disease transmission, complex polyploid and aneuploid genome, and it takes 8 – 10 years to produce and release a novel and improved cultivar (Butterfield and Thomas, 1996; Butterfield et al., 2001; Snyman et al., 2011). Hence, new advances in biotechnology need to be explored in order to complement conventional breeding.

Sugarcane production via conventional methods involves vegetative propagation through setts, which is a lengthy process (8 – 15 years), that is threatened by the possible transmission of diseases from one generation to the next (Basnayake et al., 2011; Snyman et al., 2011). *In vitro* propagation can be used for precise breeding aimed at improving varieties with specific traits, as well as to accelerate the production of these varieties (George, 1993; Snyman et al., 2011). *In vitro* culture and manipulation make use of a single explant and through the exogenous application of plant growth regulators (PGRs) exploits the cell's totipotency in order to produce multiple genetically identical plants (George, 1993; Bhojwani and Radzani, 1996). *In vitro* culture methods are well established for sugarcane (Chen et al., 1988; Gamborg and Phillips, 1995; Snyman et al., 1996; 2000; Aftab and Iqbal, 1999; Snyman et al., 2001; Anold et al., 2002; Baksha et al., 2002; Ali et al., 2008; Patade et al., 2008; Behera and Sahoo, 2009; Khan et al., 2009; Basnayake et al., 2011; Joshi et al., 2013; de Arauja Silva, 2014) and have various applications, such as genetic transformation (Snyman et al., 1996, 2001), pathogen eradication (Irvine and Benda, 1985; Ramgareeb et al., 2010; Shahid et al., 2014), mutagenesis for production of novel genotypes (Khan and Khan, 2010; Koch et al., 2012, Mahlanza et al., 2013; Munsamy et al., 2013) and germplasm conservation (Watt et al., 2009).

The generation of genetic variation used for the selection of desirable traits is an important step in any plant breeding program (Roane, 1973; Lebeda and Svabova, 2010), as well as in the biotechnological methods mentioned above. The selection of stress tolerance traits through conventional breeding is performed in the field, where lines with desirable agronomic traits are crossed with those with stress tolerance (Ming et al., 2006). However, this process is both time-consuming and labourious (Jain, 2001; Patade et al., 2008). In contrast, *in vitro* selection is a method which allows rapid screening and selection of numerous genotypes performed in a limited space and monitored conditions without influences by biotic and abiotic factors which could negatively affect selection (Chaleff et al., 1983; Hajari et al., 2014). It has been shown to be a critical step in selection of genetically engineered and mutagenic events and it may be a way to discriminate amongst genotypes produced by traditional breeding (Bower and Birch, 1992; Chowdhury and Vasil, 1992; Arencibia et al., 1998; Ali et al., 2008; Patade et al., 2008; Wagih et al., 2004; Khamrit et al., 2012; van der Vyver et al., 2013).

Several studies have reported that *in vitro* culture alone (Sengar et al., 2009) or in combination with mutagenesis (Avinash et al., 2012; Koch et al., 2012; Mahlanza et al., 2013), induced with biological or physicochemical agents (Errabii et al., 2006; Shomeili et al., 2011; Gomez-Luciano et al., 2012; Rao and Jabeen et al., 2013), can be utilised to enhance mutants and genetic variability, as a prospective source of new commercial cultivars (Orbovic et al., 2008). The most commonly used approach for selection of stress tolerant genotypes is the *in vitro* selection pressure, which in the event of selecting for drought tolerant genotypes, involves the culture of explants on medium supplemented with either sorbitol (Albiski et al., 2012), mannitol (Cha-um and Kirdmanee, 2009; Cha-um et al., 2010) or polyethylene glycol (PEG) (Biswas et al., 2002; Patade et al., 2011; 2012; Soliman and Hendawy, 2013) of higher molecular weights. In sugarcane, *in vitro* selection strategies incorporating specified selection agents have been used to obtain plants with improved tolerance to salt (Patade et al., 2008), herbicide (Koch et al., 2012), pest (Mahlanza et al., 2013) and drought (Errabii et al., 2006; Rao and Jabeen, 2013). Imperative to the development of new drought tolerant commercial cultivars is the knowledge and comprehension of how plants tolerate water deficit and distinguishing tolerance to water stress (Nepomuceno et al., 2001). In sugarcane, genetic advancement programs aimed at developing drought tolerant cultivars necessitate the identification of important physiological mechanisms that can be used as selection criteria (Smit and Singels, 2006; de Almeida Silva et al., 2011). The present study focused on this issue under *in vitro* conditions.

In nature, crops are exposed to hostile environmental stresses, such as drought, that not only affect growth and development (Zingaretti et al., 2012), but also cause a homeostasis disturbance that require crops to alter their metabolism for protection against stress (Zingaretti et al., 2012; Lakshmanan and Robinson, 2014). Upon exposure to water deficit, a plant's initial response is a reduction in growth (Inman-Bamber et al., 2008; Wilkinson and Davies, 2010) which leads to a decrease in the rate of photosynthesis (Azevedo et al., 2011), which ultimately affects processes such as stomatal conductance, respiration, foliar temperature and radiation capture (Silva et al., 2007). Furthermore, drought-induced oxidative stress may occur as a result of a low carbon assimilation rate, which consequently leads to the overproduction of reactive oxygen species (ROS) (Edreva, 2005), such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•) and superoxide ($O_2^{-\bullet}$), which have detrimental effects on cellular components (Arora et al., 2002; Apel and Hirt, 2004). Sugarcane varieties differ in their response to drought stress, and it is feasible to identify more tolerant ones, which is essential, especially in areas that experience long periods of water deficit (de Almeida Silva et al., 2011). These physiological parameters are known to be responsive to water stress (de Almeida Silva et al., 2011) and have been shown to be useful as indicators for stress in field (de Almeida Silva et al., 2011; Cha-um et al., 2012; de Almeida Silva et al., 2014), pot trials (Zhao et al., 2010; dos Santos et al., 2014) and *in vitro* studies (Cha-um and Kirdmanee, 2008; Patade et al., 2011; Cha-um et al., 2012; Patade et al., 2012).

Consequently, the aims of the present study were to: 1) establish *in vitro* conditions to screen and select consequently, for drought tolerance; and 2) assess various physiological parameters as indicators of drought tolerance under *in vitro* conditions. In order to undertake the studies above, it was necessary to produce *in vitro* plantlets. The *in vitro* direct organogenesis standard culture procedures method (Ramgareeb et al., 2010) was used. However, this method not only requires extra care, it is also time-consuming and labourious since the meristems are obtained from sugarcane stalks. For this reason, the third aim of the present study was to establish the best source of meristematic explant for culture initiation and mass propagation of *in vitro* shoots.

2. Literature review

2.1 Background and economic importance of sugarcane

Grown mainly in tropical and sub-tropical parts of the globe, sugarcane is a tall perennial grass ranked among the ten most planted crops in the world (Bower and Birch, 1992; Selman-Housein et al, 2000; Suprasanna et al., 2011; Pervaiz et al., 2013). This grass species has culms equally bunched in stools of 5 – 10 inch or more (Pervaiz et al, 2013), and are juicy, with high sucrose content (Cheavegatti-Gianotto et al., 2011; Mnisi and Dlamini, 2012). This tropical crop thrives in soils with adequate drainage, that are rich in organic matter, medium to heavy and slightly alkaline (Anon, 2003). Being the most important source used for sweetening globally, sugarcane accounts for approximately 80% of the world's sugar production (Raza et al., 2012).

Sugarcane plays a crucial role in the economy of many developing countries (Bower and Birch, 1992; Allsopp and Manners, 1997). Although sugarcane-growing regions are mainly in the tropics, the crop is cultivated on approximately 23.8 million hectares in over ninety countries globally (Grivet and Arruda, 2001). There are some 45 million sugarcane farmers globally; their dependents and a vast number of agricultural laborers are involved in the cultivation, harvesting and supportive activities associated with cane farming, with 7.5% of these being rural farmers (Mnisi and Dlamini, 2012).

The South African sugar industry is not only a prominent contributor to the national economy in areas such as KwaZulu-Natal (KZN), Mpumalanga and the Eastern Cape, but is also amongst the world's leading cost capitalistic producers of high quality sugar (www.sasa.org.za). It is a versatile industry, merging the production of raw and refined sugar syrups, a range of by-products and specialized sugars. With an annual estimated average direct income of ZAR8 billion (SASA, 2013), the sugar industry contributes to employment, in particular to sustainable development, and in rural areas through supporting small scale farmers. It produces approximately 2.2 billion tons of sugar in each season, with 60% being marketed in the Southern African Customs Union (SACU), and the rest exported to markets in various regions such as Asia, Africa and the Middle East (SASA, 2013).

Sugarcane belongs to the genus *Saccharum* L., which is a division of the Poaceae family (Selman-Housein et al., 2000). There are generally six recognised species in the *Saccharum* genus, with *S. spontaneum* and *S. robustum* found in the wild, and *S. officinarum*, *S. barberi*, *S. sinense* and *S. edule* found primarily in cultivation (Daniels and Roach, 1987; Tarimo and Takamura, 1998; Jackson, 2005). *S. officinarum* clones are known as the 'noble' canes and were the main supporters of the world's commercial production prior to the exploitation of interspecific hybridization (Jackson, 2005). *S. barberi* and *S. sinense* are ancient land races

in India and China, respectively, and are distinguished by an aborted inflorescence (Jackson, 2005). Leading to the end of the 19th century, sugarcane clones of *S. officinarum*, a species which was domesticated from the wild species *S. robustum*, were the most cultivated cultivars (Grivet and Arruda, 2001; Lakshmanan et al., 2005). By crossing *S. officinarum* (2n = 80) with *S. spontaneum* (2n = 40 – 128), a wild and vigorous relative, and then backcrossing the hybrids to *S. officinarum*, initial sugarcane breeders were able to increase yield, disease resistance and better ratooning ability (Berding and Roach, 1987; Daniels and Roach, 1987; D'Hont et al., 1998; Grivet and Arruda, 2001; Lakshmanan et al., 2005).

The majority of the currently cultivated sugarcane varieties are derived from the interspecific hybridization of *S. officinarum* and *S. spontaneum* produced in the 1800s (Altpeter and Oraby, 2010; Snyman et al., 2011). The resulting progeny obtained from the early crosses were then backcrossed with *S. officinarum* and, since nobilisation is qualified by asymmetric chromosome transmission (Berding and Roach, 1987), current-day hybrids possess a limited gene pool and complex polyploid, aneuploid genomes (Butterfield et al., 2001). This complex cytology causes challenges in sugarcane breeding programs for the betterment of traits, since it is difficult to predict the resulting features of hybrids incurred by cross pollination of members of the *Saccharum* genus (Barnes, 1964; Jackson, 2005; D'Hont et al., 2008).

In order for the sugarcane industry to remain profitable, there is a need to have a constant supply of varieties that are able to grow in different regions of the world (Kern, 2002; James, 2008; Snyman et al., 2011). However, this is restricted by the complex genome, narrow gene pool, lengthy breeding and selection time, and the difficulty in predicting the outcome of crosses and lengthy vegetative propagation via stem cuttings (Ming et al., 1998; Brumbley et al., 2008; Snyman et al., 2011). In South Africa, the main challenges facing the industries are specific pests and diseases and low annual rainfall levels (Snyman et al., 2008). Therefore, the commercial varieties that are produced in South Africa should be able to withstand those (Snyman et al., 2011).

2.2 Sugarcane breeding

Presently, at the South African Sugarcane Research Institute (SASRI), and in other parts of the world, there are integrated breeding programs established for the development of new sugarcane varieties, and these programs make use of the germplasm of existing commercial and ancestral lines (Nair et al., 1999; Najarajan et al., 2000; Botha, 2007; Snyman et al., 2011). This involves extensive crossing of selected cultivars through cross pollination (Selman-Housein, 2000). It is achieved by aligning the arrows of the male and female clone in isolation to facilitate natural cross pollination. Manual dusting of the pollen onto the

flowering arrow of the female clone can also be employed (Sleper and Poehlman, 2006). Breeding sugarcane in this way is a lengthy process and generally takes between 12-15 years (Barba et al., 1978; Pathak et al., 2009).

Upon the production and selection of new genotypes through breeding programmes, they are vegetatively propagated by nodal cuttings, such as billets, seedcanes or setts (30 – 40 cm in height) to be used in clonal plantations (Snyman et al., 2011). Although, this is relatively a simple process, it has two main shortcomings, *viz*. slow propagation and the risk of diseases being transferred (Snyman et al., 2008; Snyman et al., 2011). The number of buds on a mature stalk restricts the multiplication rate, and propagation rates are eight – to tenfold in one annual growth cycle (Dookun et al., 1996; Snyman et al., 2008). Furthermore, the effectiveness of such propagation may be limited by the transmission of disease from the seedcane to the resultant crop in the event that phytosanitation measures are not rigorously followed (Snyman et al., 2011). Disease transmission occurs because in sugarcane, a ratooning monoculture, the infection is carried on through rootstocks (Lee, 1987; Victoria et al., 1999; Flynn et al., 2005). The removal of pathogens is a labourious process involving hot water treatment of sugarcane stalks, which fails to eradicate viral particles (Victoria et al., 1999; Flynn et al., 2005).

Some of the above-mentioned problems can be overcome with *in vitro* culture. According to Snyman et al. (2006), the use of such cultures can increase the vegetative propagation potential of sugarcane by 20 - 35 times. Furthermore, meristem-derived tissue culture plants are pathogen-free (Ramgareeb et al., 2010; Sengar et al., 2011; Snyman et al., 2011).

2.3 *In vitro* culture systems

Micropropagation is the outcome of *in vitro* culture manipulations that lead to the mass propagation of one genotype, usually possessing superior traits (Bhojwani and Radzan, 1996, George et al., 2008). This process is made possible by totipotency, which is the ability of a single cell to dedifferentiate, divide and differentiate into tissues, organs and plants. This is usually achieved *in vitro* through the exogenous application of PGRs (George, 1993; Bhojwani and Radzan, 1996, George et al., 2008). Numerous *in vitro* techniques used for the mass propagation of healthy sugarcane plantlets have been established (Table 1) and are required in present-day efforts to improve sugarcane germplasm via genetic engineering and induced mutagenesis (Rutherford et al., 2014). In addition, such protocols extend strategies to deal with the propagation and storage restrictions and narrow genetic diversity currently curbing sugarcane industries globally (Snyman et al., 2011).

There are two main morphogenic routes leading to the regeneration of whole plants *in vitro*, *viz*. somatic embryogenesis and organogenesis (Phillips, 2004; George et al., 2008). Both

routes can take place either directly from the explant or indirectly subsequent to an intervening callus stage (Gamborg and Phillips, 1995). A limited number of plant species have the ability to regenerate through both organogenic and somatic embryogenic pathways, including sugarcane (Table 1), as discussed below.

2.3.1 Organogenesis

Organogenesis involves the formation of shoots or roots from plant tissues (George et al., 2008). Direct or indirect organogenesis relies both on the manipulation of the medium components and on the plasticity of the plant tissue (George et al., 2008; Slater et al., 2008). The auxin:cytokinin ratio contained in the medium determines the developmental route of regeneration (George et al., 2008; Slater et al., 2008). In most cases during direct organogenesis, shoot multiplication occurs under the influence of both auxins and cytokinins, whilst root formation is influenced by the presence of auxin alone (George, 1993; Mamun et al., 2004; Khan et al., 2006; Ali et al., 2008; George et al., 2008).

According to Sugiyama (1999), organogenesis occurs in three distinct stages which are dependent on the balanced application of PGRs. During the first stage, cells adopt 'competence', wherein they are able to respond to plant regulator signals. In the second stage, the dedifferentiated cells are channelled and determined for particular organ formation in response to exogenous application of PGRs. During the third stage, exogenous supply of PGRs is ceased and morphogenesis proceeds independently.

In sugarcane, a combination of auxin and cytokinin has been employed to achieve shoot multiplication (Table 2), of which the commonly used cytokinin is benzylaminopurine (BAP) (Lee, 1987; Gambley et al., 1993; Huang et al., 2003; Ali et al., 2008). The optimal BAP concentration varies among different varieties and ranges between 0.5 to 2 mg l^{-1} (Mamun et al., 2004; Ali et al., 2008; Khan et al., 2008; Pathak et al., 2009), with 1.5 mg l^{-1} being commonly used (Mamun et al., 2004, Khan et al., 2006; Ali et al., 2008). A combination of BAP and 6-furfuryaminopurine (kinetin) can be used to improve shoot multiplication, and the optimum concentration also differs amongst varieties (0.1 to 0.5 mg l^{-1}) (Lee, 1987; Khan et al., 2006; Ali et al., 2008; Pathak et al., 2009). To induce direct organogenic pathways, auxins used to achieve shoot multiplication include naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA), whilst auxins commonly used for root formation are indole-3-butyric acid (IBA) and NAA.

Table 1: Examples of studies utilizing different morphogenic routes of sugarcane micropropagation and their applications.

Morphogenic route	Application	Reference
Direct somatic embryogenesis	Micropropagation	Aftab and Iqbal (1999) Snyman et al. (2000) de Arauja Silva (2014)
	Genetic transformation	Snyman et al. (2006) Taparia et al. (2012)
	Minimal growth and storage/ germplasm preservation	Watt et al. (2009)
Indirect somatic embryogenesis	Micropropagation Cryopreservation	Ho and Vasil (1983) Chanprame et al. (1993)
	Genetic transformation	Snyman et al. (1996), (2001)
	Mutagenesis	Khan and Khan (2010) Koch et al. (2012) Mahlanza et al. (2013) Munsamy et al. (2013)
Direct organogenesis	Pathogen eradication	Irvine and Benda (1985)
	Micropropagation	Baksha et al. (2002) Ali et al. (2008) Joshi et al. (2013)
	Virus elimination	Ramgareeb et al. (2010)
Indirect organogenesis	Micropropagation	Behera and Sahoo (2009) Khan et al. (2009) Dibax et al. (2012)
	Virus elimination	Ramgareeb et al. (2010) Shahid et al. (2014)

Most researchers use Murashige and Skoog (1962) (MS) as the nutrient formulation in sugarcane organogenesis protocols (Ho and Vasil, 1983; Lorenzo et al., 2001; Mamun et al., 2004; Behera and Sahoo, 2009; Ramgareeb et al., 2010), and sucrose is the most common carbon source (Lorenzo et al., 2001; Ali et al., 2008; Ramgareeb et al., 2010). Occasionally, coconut milk (Lee, 1987), myo-inisitol (Lee, 1987; Lorenzo et al., 2001) or coconut water (Ho and Vail, 1983, Mamun et al., 2004), arginine and thiamine (Lee, 1987) have been used as supplements in the media.

Table 2: Examples of various protocols developed for plantlet regeneration through the use of culture systems. PGRs 2,4-D = 2,4-dichlorophenoxyacetic acid, IBA = indole-3-butyric acid, BA or BAP = 6-benzylaminopurine, GA_3 = gibberellic acid, NAA = 1-napththylacetic acid, kinetin = N-6-furfuryladenanine and TDZ = thiadiazuron.

Morphogenesis route	Explant	PGR	Reference
Direct organogenesis	Single bud	BAP, Kinetin	Lee (1987)
	Meristem	BAP	Lorenzo et al. (2006)
	Apical meristem	BAP, IAA, IBA, Kinetin	Khan et al. (2006)
	Spindle fibres	BAP, IBA, Kinetin, NAA	Pathak et al. (2009)
	Apical meristem	BA, Kinetin, NAA	Ramgareeb et al. (2010)
Indirect organogenesis	Young meristem	2.4-D, BAP, IAA, IBA, Kinetin, NAA	Behera and Sahoo (2009)
	Meristem	IBA, IAA, Kinetin	Khan et al. (2009)
	Meristem	2.4-D, BA	Ramgareeb et al. (2010)
Direct somatic embryogenesis	Immature leaf roll	2.4-D, NAA	Nadar and Heinz (1997)
	Midrib segments	2.4-D, BAP, NAA	Franklin et al. (2006)
	Immature leaf roll	2.4-D	Snyman et al. (2001)
Indirect somatic embryogenesis	Spindle tissue	2.4-D	Barba et al. (1978)
	Immature leaf segments	2.4-D, BAP, Kinetin, NAA, zeatin	Falco et al. (1996)
	Mature seeds	2.4-D, IBA, picloram, TDZ	Chengalrayan et al. (2005)
	Shoot apical meristem, spindle leaves, pith parenchyma	2.4-D, BAP, Kinetin	Ali et al. (2007)

2.3.2 Somatic embryogenesis

Somatic embryogenesis involves the formation of embryos from somatic cells (Anold et al., 2002; George et al., 2008). In this developmental pathway, embryos develop from single, thin-walled, richly cytoplasmic cells with numerous starch grains and vacuoles (Ho and Vasil, 1983; Guiderdoni and Demarly, 1988). The embryos resemble their zygotic counterparts in both developmental stages and structure (George, 1993; Bhojwani and Radzan, 1996; Snyman et al., 2011). Similar to zygotic embryo development, somatic embryo formation is characterized by the formation of cells into: i) globular, ii) heart-shaped, and iii) torpedo-shaped phases in dicotyledons (Terzi and Loschiavo, 1990; Zimmerman, 1993; Litz and Gray, 1995; Dodeman et al., 1997); or i) globular, ii) scutellar, and iii) coleoptilar in monocotyledons (Gray et al., 1995).

According to Jimenez (2005), somatic embryogenesis has two phases, induction and expression. In the former, the cellular state (physiology, metabolism and gene expression) of somatic cells is reorganised and they acquire embryogenic features. This is influenced by alterations in culture conditions (e.g. culture composition, medium, PGRs, osmotic potential and/or carbohydrate source) and leads the cells to reach the expression stage, wherein their embryogenic competence is displayed and they differentiate into somatic embryos.

Plant regeneration through somatic embryogenesis takes place via: i) initiation of embryogenic culture through culturing the explant on medium containing PGRs, primarily auxin but occasionally also cytokinin; ii) proliferation of embryogenic cultures cultured on either liquid or semi-solid medium with PGRs, similar to initiation; iii) prematuration of somatic embryos cultured on medium without PGRs, where proliferation is inhibited and somatic embryo formation and development is stimulated; iv) maturation of somatic embryos on medium supplemented with ABA and/or diminished osmotic potential; and v) regeneration of plantlets by culturing on medium lacking PGRs (Anold et al., 2002).

Ho and Vasil (1983) reported that calli formed from sugarcane explants during somatic embryogenesis were not consistent in appearance and possessed morphogenically different regions. The different calli were classified as: i) type 1 embryogenic, white and nodular in appearance; ii) type 2, yellow, soft and friable and may be embryogenic; and iii) type 3 mucilaginous, shiny and non-embryogenic calli. In contrast, Taylor et al. (1992) categorised type 1 callus as semi-transparent with loose, large and elongated cells, type 2 as greyish-yellow mucilaginous calli, type 3 as compact, nodular calli with yellowish-white cells, and type 4 as friable, yellow calli. In this description, type 1 and 2 calli were considered non-embryogenic, whilst type 3 as embryogenic and type 4 as organogenic.

In sugarcane, the initiation of callus production requires the exogenous application of auxins to promote cell division and supress cell differentiation (George et al., 1993; Bhojwani and Radzani, 1996; Deo et al., 2010). Embryo formation in culture occurs under high levels of auxin, while auxin removal promotes embryo maturation and plantlet development (George et al., 1993; Bhojwani and Radzani, 1996; Khalil, 2002; Snyman et al., 2006). The most frequently reported medium that promotes callus induction in sugarcane consists of MS with vitamins, sucrose and 2.4-D (Table 2; Chen et al., 1988; Snyman et al., 2001; Khalil, 2002; Basnayake et al., 2011; Snyman et al., 2011). In sugarcane, the most commonly used auxin for optimal callus induction is 2.4-D at 3 – 4 mg l^{-1}, even though this may differ among varieties (Khalil, 2002; Basnayake et al., 2011), but picloram has also been used (Table 2).

In order to regenerate plants via somatic embryogenesis, an array of physical and chemical treatments should be employed with appropriate timing (Anold et al., 2002; George et al., 2008). The frequency and morphological quality of the somatic embryos can be optimized through the manipulation of various culture treatments such as the PGR source and concentration, nutrient medium composition (e.g. carbohydrate sources and concentrations, inorganic versus organic nitrogen sources), choice of explant, culture conditions (e.g. quality and quantity of light, liquid or semi-solid medium, temperature, pH and gaseous environment) and osmotic potential (Phillips, 2004; George et al., 2008).

2.3.3 Somaclonal variation and induced mutagenesis

Early on in culture studies, it was noted that not all the plants regenerated from tissue culture were exactly identical to their parent plants, and a few expressed some variability in their agronomic traits (Larkin and Scowcroft, 1981). Hawaiian researchers Heinz and Mee (1969), who were the first to describe the regeneration of sugarcane plantlets from callus, brought attention to the fact that some of the progeny were not true-to-type clones. Numerous alterations within the regenerated plantlets were observed, including changes in morphology (such as slower growth, denser tillering, more upright), elevated chromosome number (from 2n = 106 – 107 to 2n = 117 – 124), and varying electrophoretic forms of esterases in contrast to the parental genotype. Comparing chromosome numbers between somaclones and the parental clones revealed that differences in the magnitude of variation were a result of genetic dissimilarities (Rutherford et al., 2014). Two studies were set out to investigate yield facets, [(i.e. sugar content and Brix (the percentage of total soluble solids present in cane juice, including sucrose and non-sucrose constituents of sugarcane)] of *in-vitro*-regenerated plants (Krishnamurthi, 1977; Liu and Chen, 1978). In the study by Liu and Chen (1978), somaclones and parent clones were compared with regards to their fibre and sucrose content, sensitivity to mildew and Fiji disease resistance ratings. Some of the observed results included improved sucrose yields and resistance to both Fiji disease and

downy mildew. It was noted that many traits could be changed simultaneously (Liu and Chen, 1978) when an elaborate morphological analysis was taken, comparing measurements of curvature of leaf blade, stem colour, auricle length, bud shape and presence or absence of leaf hair. Sugar analyses from more than two successive crops showed increases between 2 and 12% in comparison to the donor clones. Chromosome analyses revealed that the genetic variation existed even amongst the same plant (Liu and Chen, 1978).

In 1981, Larkin and Scowcroft coined the term somaclonal variation to depict the genetic variability rendered in plants exposed to tissue culture cycles. Subsequent to the attention given to somaclonal variation research, it was soon acknowledged that somaclonal variation existed for nearly all phenotypic features in many species (Jayasankar, 2005), not only in sugarcane. The manipulation of culture condition, particularly the use of PGRs, which influence cell division (Gould and King, 1984), exclusive proliferation of certain cell types (Ghosh and Gadgil, 1979) and the extent of unsystematic growth (Karp, 1992) lead to the breakdown of standard cellular operation mechanisms, which in turn is responsible for the genetic unstableness in some *in-vitro*-generated plants. Different mechanisms have been reported to lead to somaclonal variation. These include: i) point mutations stimulated by exposing plant cells to medium supplemented with chemicals; ii) alteration of chromosome number and structure; iii) change of mitochondrial DNA; iv) DNA methylation; v) epigenetic variation caused by micro-environment of tissue culture conditions; and vi) alterations in plastid DNA (Jain, 1998; Miguel and Marum, 2011). In the event where the aim is to achieve true-to-type clonal propagation, somaclonal variation must be avoided. Nevertheless, it is useful as a source of genetic variation and can be beneficial to the industry aimed at producing genotypes with traits of interest (Rutherford et al., 2014).

Undoubtedly, unprompted mutations have played a crucial role in the past when it comes to the breeding of novel crop cultivars. All the same, their competency in the grand scale of breeding programs (which are aimed at rapidly releasing varieties adjusted to meet the fast-changing biotic and abiotic stresses) is rather limited because of their low rate of occurrence (Rutherford et al., 2014). Various mutagens, utilized to yield genetic variation, differ in terms of the type of mutations generated, and the ratio of each type of mutation that can be achieved (Rutherford et al., 2014). Stadler (1928) demonstrated that heritable mutations in barley can be induced through ionising radiation. The combination of such radiation and specific chemicals was later shown to be capable of inducing advantageous alterations in the genomes of various crops (Constantin, 1984; Ahloowalia and Maluszynski, 2001). From this approach, came several methods directed at improving mutation breeding supported by the potential alteration of genes through plant parts being exposed to chemical or physical

mutagens (Rutherford et al., 2014). Rao et al. (1966) reported that the use of such procedures in sugarcane, led to the release of a red rot-resistant mutant. Several studies have reported on the use of mutagens to obtain sugarcane mutants with increased yield, disease and pest resistance, and abiotic stress tolerance (Table 3).

Ionising radiation is employed for the production of mutations by transferring energy, through excitation or ionisation, to sites among or in proximity with the genetic material, thus enhancing the chemical reactivity of the concerned sites (Rutherford et al., 2014). Different types of radiation are used, including high-energy forms (e.g. ultra-violet (UV) light, X-rays, *y*-rays, and fast neutrons. Point mutations are known to occur as a result of exposure to UV light, leading to the formation of pyrimidine dimers (TT and CC), whilst X-and *y*- rays lead to point mutations and minute deletions when breaks in phosphodiester bonds occur (Rutherford et al., 2014). According to Sikora et al. (2011), fast electrons induce chromosome loss, translocation and large chromosome omissions. Most of the research done on sugarcane has been aimed at determining the most effective exposure time and the dose that results in the highest mutation percentage, without being harmful or fatal (Rutherford et al., 2014).

Chemical mutagens can affect the DNA directly or indirectly by producing mutations which result in base-pair transposition, generally GC to AT (Rutherford et al., 2014). For instance, ethylmethanesulphonate (methanesulphonic acid ethyl ester, EMS), an alkalating agent, haphazardly reacts with thymine and guanine residues, adding an ethyl group and results in them being discerned as cytosine and adenine, respectively, during DNA replication (Van Harten, 1998; Jander et al., 2003). Sodium azide, a mutagenic agent that has been widely used for inducing mutations in seeds, acts as a point mutagen during DNA replication (Al-Qurainy and Khan, 2009) upon its conversion to azidoalanine (Owais et al., 1983). This mutagen induces base substitutions (GC to AT) (Koch et al., 1994). The use of other mutagens, such as hydrogen fluoride, methylemethanesulphonate (MMS) and N-methyl-N-nitrosourea, has been common in other plants, but to a lesser extent in sugarcane (Parry et al., 2009).

The chemically-induced mutation method is considered to be relatively simple, regardless of the toxicity associated with chemical mutagens (Rutherford et al., 2014). *In vitro* mutagenesis through chemical mutagens in sugarcane is a recently researched area, with the first report by Ali et al. in 2007. Those authors investigated how embryogenic calli is affected by the presence of sodium azide. Their results revealed that the largest number of genetic variants was achieved by using 4.0 mg l^{-1} sodium azide, but all the regenerated plantlets died. Subsequently, EMS has been the preferred chemical mutagen for *in vitro*

chemically-induced mutagenesis of sugarcane calli (Sadal and Hoveize, 2012; Koch et al., 2012; Mahlanza et al., 2013).

Table 3: Examples of traits in sugarcane mutants obtained with gamma irradiation and chemical mutagens.

Mutagen	Trait	Reference
Gamma rays	Red rot resistance	Ali et al. (2007)
	Salt tolerance	Patade et al. (2008)
	Increased yield	Khan et al. (2007), (2010)
	Brown rust resistance	Oloriz et al. (2011)
Ethyl methanesulfonate	Red rot disease	Khairwal et al. (1984)
	Salt tolerance	Kenganal et al. (2008)
	Imazapyr tolerance	Koch et al. (2012)
	Fusarium sacchari tolerance	Mahlanza et al. (2013)
Sodium azide	*Colletotridium falcatum* resistance	Ali et al. (2007)
Sodium nitrite	Drought tolerance	Wagih et al. (2004)

2.4 Strategies used to induce stress tolerance in sugarcane

2.4.1 Conventional breeding strategies

Conventional plant breeding involves the recombination of genes of interest from crop species and their relatives through sexual hybridization to develop cultivars with superior traits, such as increased yield, tolerance to pests, diseases, drought, salinity and herbicides. New cultivars with desired traits such as high yield, disease tolerance and better ratooning ability have been created through sugarcane breeding (Sengar et al., 2011; Snyman et al., 2011). However, due to the previously discussed multiple challenges associated with breeding sugarcane, this approach takes 8 – 10 years to develop and release a novel and improved sugarcane cultivar (Gururaj, 2001; Snyman et al., 2011). Therefore, there is need for new advances in plant biotechnology in order to complement conventional breeding in areas such as: i) commercial cultivation engineered with new genes, ii) molecular pathogen diagnostics towards improving exchange between *Saccharum* germplasm and related

genera (Patade and Suprasanna 2008); iii) plant tissue culture for propagation and molecular breeding (Patade and Suprasanna 2008; Snyman et al., 2011); iv) recognition of newly developed varieties (Khan et al., 2009); and v) ranking of different traits amongst the varieties (Gurujaj, 2001).

2.4.2 Genetic modification

Genetic modification is defined as the targeted transfer of a gene or genes from one plant to another of the same or different species, or from another organism to develop a plant with traits of interest. Naturally, the plant would acquire the genes through crossing or natural recombination, but in genetic modification the genes are obtained artificially. These newly developed plants are referred to as transgenic or genetically modified (GM). The genetic manipulation of sugarcane, like other crops, is used as a tool to quicken the production of plants with superior and improved agronomic attributes that would have otherwise taken longer or not been possible if produced conventionally (Bower and Birch, 1992; Sengar et al., 2011). Several steps are involved in genetic modification, such as distinguishing of the gene of interest, cloning the gene into a suitable plasmid vector, introduction of the vector into a plant, and the expression of the gene encoding a polypeptide (Wang et al., 1988; Christou et al., 1989; Bower and Birch 1992; Becker et al., 2000).

Several transformation approaches have been developed for gene introduction in sugarcane leaf discs and calli. Microprojectile bombardment (Bower and Birch, 1992; Bower et al., 1996; Lakshmanan et al., 2005; Rivera et al., 2012), electroporation or polyethylene glycol (PEG) treatments (Arencibia et al., 1995; Rivera et al., 2012) and *Agrobacterium*-mediated genetic transformation (Arencibia et al., 1998) are the most frequently-used techniques. Some of the novel characteristics that have been achieved in sugarcane through these techniques include virus resistance, herbicide tolerance, altered sucrose enzyme regulation, and insect resistance (Table 4).

Microprojectile bombardment involves bombarding the target tissue with microprojectiles coated with foreign DNA (Birch and Franks, 1991; Hansen and Wright, 1999). There are essentially two methods of microprojectile bombardment, the particle inflow gun and the gene gun (Birch and Franks, 1991; Hansen and Wright, 1999, Deo et al., 2010). The former has DNA-coated microprojectiles that are accelerated into the target tissue (Birch and Franks, 1991; Newell, 2000; Deo et al., 2010). The gene gun makes use of micro-and macro- carriers (Birch and Franks, 1991; Deo et al., 2010), wherein a gas (generally helium) accelerates the macro-carriers containing DNA-coated micro-carriers into the target tissue (Birch and Franks, 1991; Newell, 2000; Deo et al., 2010).

Electroporation involves inducing membrane permeabilisation, allowing for a local drive force for both molecular and ionic transport via the pores (Arencibia et al., 1998). In this method, the application of an electric field allows for the temporary formation of pores (Newell, 2000; Rakoczy-Trojanowsha, 2002), through which the genes of interest can insert themselves in the plant cell. This is a fast and user-friendly method with negligible cell toxicity (Newell, 2000; Rakoczy-Trojanowsha, 2002). Polyethylene glycol treatment permits for the adhering and precipitation of exogenous DNA with genes of interest to the membrane of the target plant cell (Chen et al., 1987; Newell, 2000; Aftab and Iqbal, 2001). In this case, the cell can take up the precipitated DNA since endocytosis is induced during the treatment (Newell, 2000).

Agrobacterium-mediated gene transfer makes use of a disarmed pathogenic bacterium that introduces a plasmid with the gene of interest into the target organism (Arencibia et al., 1998). When the gram-negative *Agrobacterium tumefaciens* bacterium infects plants (De Cleene and Deley, 1976; Hansen and Wright, 1999, Newell, 2000), it leads to tumour formation known as gall tumour. The bacterium consists of a tumour-inducing (Ti) plasmid that incorporates itself in the DNA of the host cell and stimulates the formation of the tumour (de la Riva et al., 1998; Zupan et al., 2000). The T-DNA, the portion of the Ti plasmid that is integrated into the plant's DNA, has two types of oncogenic genes coding for the manufacturing of cytokinins and auxins responsible for tumour formation, and genes coding for opine (food source for the bacterium) formation (de la Riva et al., 1998; Hansen and Wright, 1999; Zupan et al., 2000). The Ti plasmid also has virulence genes that aid in the integration of the T-DNA into the host's DNA (de la Riva et al., 1998; Hansen and Wright, 1999; Zupan et al., 2000).

Transformation studies exploit the Ti plasmid, wherein the gene of interest is added within the T-DNA limits, and the oncogenes are removed and substituted with genes that permit for selection of the transformed cells (e.g. maker genes) (de la Riva et al., 1998; Hansen and Wright, 1999; Zupan et al., 2000). *Agrobacterium*-mediated genetic transformation has been successfully used in both monocotyledonous and dicotyledonous plants (Arencibia et al., 1998; Elliot et al., 1998, Anderson and Birch, 2012).

Table 4: Examples of superior traits obtained in sugarcane using genetic transformation methods (modified from Watt et al., 2010). *bar* = phosphinotricin acetyl transferase, *npt* = Neomycin phosphotransferase, CrylAb = Bt endotoxin, SCMV = Sugarcane Mosaic Virus, SCYLV = Sugarcane Yellow Leaf Virus.

Trait	Transformation method	Gene of interest	Reference
Herbicide tolerance			
Glufosinate ammonium	Microprojectile *Agrobacterium*	*bar* *npt II*	Chowdhury and Vasil (1992) Enrique-Obregon et al. (1998)
Glyphosate (roundup)	*Agrobacterium*	*cp4 epsps*	Snyman et al. (2001)
Sulfonylurea	Microprojectile	*als*	van der Vyver et al. (2013)
Disease tolerance			
Fiji leaf gall	Microprojectile	SCMV-CP	Joyce and McQualter (1998)
Sugarcane mosaic virus	Microprojectile	*npt II*	Sooknandan et al. (2003)
Fungal damage resistance	Microprojectile	FVS9-ORFI	McQualter et al. (2004a)
Sugarcane yellow leaf virus	Microprojectile	SCYLV-CP CrylAc Chinitase	Rangel et al. (2003) Gilbert et al. (2009) Khamrit et al. (2012)
Antibiotic resistance	Microprojectile	*npt-II*	Bower and Birch (1992)
Metabolic engineering			
Increased sugar content	*Agrobacterium*	UBI/PFP	Arencibia et al. (1998)
p- Hydroxybenzoid acid	Microprojectile	als	McQualter et al. (2004b)

2.5 Use of *in vitro* culture systems in selecting and screening for stress tolerance

The development of efficient approaches for the selection of traits of interest is a crucial step in plant breeding programmes (Roane, 1973; Novak and Brunner, 1992; Lebeda and Svabova, 2010). Conventionally, the selection of desirable traits is done in the field. However, this is both time-consuming and labourious (Novak and Brunner, 1992; Jain, 2001; Patade et al., 2008). Alternatively, *in vitro* selection can be employed, wherein the rapid selection of several clones of different varieties is done in a limited space and controlled conditions void of biotic and abiotic influences that could negatively interfere with selection (Chaleff, 1983; Jain, 2001; Hajari et al., 2014). It should be noted that *in vitro* conditions are different from those in field or pot trials. Some of the specific conditions *in vitro* include a

confined gaseous exchange environment, elevated humidity, supply of sucrose in the culture media, ample supply and homogenous dispersion of nutrients, exposure of roots to light, and nonexistence of soil micro- fauna or flora (Miller and Cramer, 2004; Roycewicz and Malamy, 2012). In contrast, in nature plants experience multiform nutrient distribution and have to cope with dynamic environmental conditions (Forde, 2002; Roycewicz and Malamy, 2012). The plant's ability to uptake and transport nutrients will therefore be constrained by these differences (Hajari et al., 2014). Despite the benefit of using *in vitro* systems in rapid screening of plants without environmental factors interfering, the findings must be interpreted with these shortcomings in mind (Roycewicz and Malamy, 2012).

Nevertheless, various researchers have exploited the use of *in vitro* cultures to select traits of interest amongst varieties, wherein plant cells, tissues or organs are cultured on media with the appropriate selecting agent (Rai et al., 2011; Perez-Clemente and Gomez-Cadenas, 2012). Suprasanna et al. (2009) stated that *in vitro* application of a selection pressure requires the concentration of the selecting agent that inhibits the growth of cells to be established and integrated into the selection medium in which the explants are cultured.

The development of new tolerant commercial cultivars is mainly dependent on comprehending how plants tolerate water deficit and identifying mechanisms of water stress tolerance (Nepomuceno et al., 2001). Developing drought tolerant cultivars has been one of the chief objectives of genetic advancement programs involving sugarcane (Inman-Bamber and Smith, 2005). These programs require the recognition of significant physiological mechanisms that can be used as selection criteria (Smit and Singels, 2006). Sugarcane varieties respond differently to drought stress, and the more tolerant ones can be identified (de Almeida Silva et al., 2011). This identification is crucial, specifically in areas prone to experiencing long periods of water deficit (de Almeida Silva et al., 2011).

2.6 Plant adaptive and protective responses to drought stress

Plants are exposed to hostile environmental conditions, and drought is the major abiotic stress that can compromise both the plant's growth and development (Zingaretti et al., 2012). Drought can be defined as a multidimensional stress (Yordanov et al., 2000) and a period of below normal precipitation that contributes to limits on plant productivity in an agricultural or natural system (Boyer, 1982; Kramer and Boyer, 1995). In the field, plants are often vulnerable to a number of environmental stresses (e.g. combined heat, high irradiance and water at dry and hot summer periods), which more often than not, concurrently affect the plant (Yordanov et al., 2000). Drought also restricts the areas suitable for agriculture. For any crop, water is essential during vegetative growth in order to reach maximum yield, and drought during this stage can drastically decrease productivity (Zingaretti et al., 2012).

According to Lakshmanan and Robinson (2014), plants have developed mechanisms of adaptation, avoidance, acclimation or a combination of these in order to survive and thrive under stressful abiotic conditions. Adaptation is associated with permanent genetic modifications of the plant's structure and function. In contrast, acclimation is when plants elicit temporary physiological and morphological alteration when exposed to periodic stresses. Acclimation is usually short-termed and irreversible since it is not affiliated with permanent genetic changes. On the other hand, avoidance is a protective method that may involve stress-avoiding structural traits, such as possessing deep roots in order to avoid the demand to extract water from uppermost soil which could be relatively dry. The fourth method, acclimation through phenotypic plasticity, allow plants to respond to a broad scope of environmental fluctuations. Therefore, the ability of plants to balance adaptation and phenotypic plasticity is crucial from an agricultural view as it will determine crop productivity under stressful conditions.

According to Zingaretti et al. (2012), during vegetative growth of any crop, the requirement of water is essential for maximum yield, and drought events during this phase can potentially limit productivity. Sugarcane is one of the crops that produce a higher amount of biomass per unit of cultivated area and its water requirements vary at each developmental stage, thus water requirement is higher during tillering and development of culms than during the maturation stage. Under water deficit conditions, sugarcane experiences a cellular homeostasis disturbance and is required to alter metabolism to protect itself against stress.

2.6.1 Molecular responses

Molecular biology along with genetic breeding programs have been beneficial tools in discovering genetic variability by reducing the time and increasing the efficiency (Zingaretti et al., 2012). Both the recognition and characterization of genes affiliated with drought tolerance brings cognition about the perception of the stress and plant responses to these unfavourable conditions (Zingaretti et al., 2012). Drought is initially perceived by the roots (Zingaretti et al., 2012), leading to changes in the expression pattern including genes whose products play a role in early responses such as signal transduction, transcription and translation factors, as well as late response genes such as water transport, oxidative stress, osmotic balance and damage repair (Ramanjulu and Bartels, 2002; Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001). Detection in low soil water content emits a signal to leaves activating stomatal closure (Taiz and Zeiger, 2006).

The plant hormone abscisic acid (ABA) is suggested to be involved in signalling between plant tissues (Schachtman and Goodger, 2008), as well as signalling the expression of certain stress-responsive genes (Shinozaki and Yamaguchi-Shinozaki, 2000). These

drought stress-responsive genes are divided into two categories: genes encoding functional proteins or genes encoding regulatory proteins. The functional protein group includes proteins such as transporters, chaperones, water channel, detoxification enzymes or proteases, whereas the regulatory group are proteins related to signalling and transcription factors (Shinozaki and Yamaguchi-Shinozaki, 2000).

Gene expression in plants is controlled at different levels and a substantial number of drought-induced genes are regulated at the transcriptional level (Xoconostle-Cazares et al., 2011). Transcriptome analyses that make use of RNA microarrays have demonstrated that ABA-dependent and independent signal transduction pathways function in drought-stressed plants (Shinozaki and Yamaguchi-Shinozaki, 2000). Several transcription factors (TFs) have been recognised as being induced under drought stress through the use of bioinformatics (Abe et al., 1997; Bartels and Sunkar, 2005; Ashraf et al., 2008). TFs can be divided into six family categories: bZIP (Basic leucine-zipper protein), MYB/MYC, Zinc-finger protein (Abe et al., 1997; Rodriguez-Uribe and O' Connell, 2006), AP2/EFF (APETALA2/ethylene-response factor) (Marcotte et al., 1989; Abe et al., 1997), CDT-1 (Furini et al., 1997) and NAC families (Yamaguchi-Shinozaki and Shinozaki, 1994; Bartels and Sunkar, 2005; Umezawa et al., 2006; Stockinger et al., 1997; Liu et al., 1998). With the aid of genomic analyses in the identification of TFs, transgenic plants expressing transcriptional activators have been developed for the output of drought tolerant plants (Lam and Meisel, 1999).

2.6.2 Physiological and biochemical responses

In nature, terrestrial plants have developed genetically encoded strategies to deal with water deficit stress (Monneveux and Belhassen 1996). One of these impressive strategies involves the accumulation of water to either escape from or delay the stress (Xoconostlen-Cazares et al., 2011). The plant's initial response to water deficit is a decrease in plant growth (Inman-Bamber et al. 2008; Wilkinson and Davies 2010) and then photosynthesis rates decrease due to the decline in plant water potential (Azevedo et al., 2011).

At the physiological level, changes can be observed in a number of processes, such as photosynthesis, respiration, stomatal conductance, radiation capture and foliar temperature, which ultimately affect crop productivity (Qing et al., 2001; Silva et al., 2007). Drought tolerant plants, such as resurrection plants, are able to cope with the stress by decreasing their metabolic activities, which are later resumed upon relief from the stress (Chandler and Bartels, 1999; Bartels, 2005). Some plants can sustain their biological activities at reduced water potentials, although with restrained development (Ramanjulu and Bartels, 2002).

Plant and cell water balance is influenced by transpiration to the environment and water absorption from the soil (Lawlor and Cornic, 2002). In events where transpiration surpasses

absorption, cell turgor decreases as relative water content (RWC) along with cell volume, while the cellular solute concentration increases, leading to a fall in both osmotic potential (π) and water potential (ψ) (Lawlor and Cornic, 2002). When RWC and cell turgor are low, growth and stomatal conductance for water is decreased (Lawlor and Cornic, 2002). Relative water content is a good measure of the association between cellular water status and metabolism, and a rapid and easily measured indicator of cellular water status (Lawlor and Cornic, 2002). In sugarcane, water content has been observed to decrease in the presence of stress conditions (Errabii et al., 2007). This is mostly seen in drought susceptible genotypes. For instance, Boaretto et al. (2014) reported that a drought tolerant cultivar retained water under water deficit, thus sustaining a higher leaf RWC compared to a drought susceptible cultivar. Drought stress tolerance is often affiliated with the sustenance of high RWCs in sugarcane cultivars (Landell et al., 2005; Cia et al., 2012; Silva et al., 2007).

Leaf chlorophyll concentration is determined through the use of organic extracting solvents including methanol (Cenkci et al., 2010), acetone (Liu et al., 2008; Efeoglu et al., 2009), N, N-dimethylformamide (DMF) (Cubas et al., 2008), dimethylsulphoxide (DMSO) (Netto et al., 2005), and subsequent chlorophyll content is measured using a spectrophotometer. Such *in vitro* evaluations are labourious, costly and time-consuming. Hence, more rapid and affordable alternative methods are useful in estimation of leaf chlorophyll content (Jangpromma et al., 2010). Li et al. (2006) showed that there are indirect and much faster techniques of measuring photosynthetic activity, such as the chlorophyll *a* fluorescence technique, which particularly measures the maximum photochemical efficiency of photosystem II (which can be evaluated via the variable-to-maximum chlorophyll *a* fluorescence ratio, [*F*v/*F*m] and estimated chlorophyll content (via SPAD unit). This method has been found to be just as efficient as the time-consuming gas exchange techniques in distinguishing differences among drought tolerant and susceptible barley genotypes (Li et al., 2006). In sugarcane, the portable fluorometer has been used to elicit the relationship between drought tolerance and chlorophyll content (Luo et al., 2004; Molinari et al., 2007; Silva et al., 2007; Shomeili et al., 2011; Cha-um et al., 2012).

In efforts to limit water loss to air, other plants utilise abscisic acid-mediated regulation of stomatal closure (Xoconostlen-Cazares et al., 2011). Being highly specialised cells, stomata are usually responsible for significant water loss through leaf transpiration (Xoconostlen-Cazares et al., 2011). During drought stress, stomata can close (Xiong et al., 2006; Xoconostlen-Cazares et al., 2011), resulting in the accumulation of gases, such as carbon dioxide and oxygen, which change photosynthesis (Bohnert and Sheveleva, 1998; Waseem et al., 2011). Consequently, an energy imbalance occurs resulting in a net flow of electrons

towards oxygen that can give rise to ROS and oxidative processes (Levine, 1999), as discussed below.

Preliminary work showed that in sugarcane there is decreased activity of enzymes such as ribulose-1,5-bisphosphate carboxylase (Rubisco), NADP malic enzyme (NADP-ME), phosphoenolpyruvate carboxylase (PEPC), PPDK, associated with the decline in leaf water potential (Du et al., 1998). Nevertheless, the available literature on the response of PEPC to drought stress is inconsistent, with some studies reporting a minute increase in activity (Saliendra et al., 1996) or no alterations in its activity (Vu and Allen, 2009).

Being a C_4 plant, sugarcane has developed a system for carbon dioxide fixation for use in sugar production with minimal water loss (Xoconostlen-Cazares et al., 2011). It makes use of a carbon dioxide concentrating mechanism which allows for, amidst other advantages, a reduction in photorespiration and an increased water use efficiency (Ghannoum, 2008). Studies suggest that sugarcane uses two different forms of C_4 metabolism, recognised by the decarboxylation enzymes utilized: (NADP-ME) and phosphoenolpyruvate carboxykinase (PEPCK), with PEPCK decarboxylation prevailing over NADP-ME (Calsa and Figueira, 2007; Granum et al., 2009). Those authors suggested that the elevated expression of PEPCK-encoding genes might be affiliated with water deficit since the samples were collected during the drier maturing season.

Through the nocturnal assimilation of carbon dioxide, sugarcane can effectively channel carbon dioxide to RuBisco (Xoconostlen-Cazares et al., 2011). These crops have developed a specific leaf anatomy, where chloroplast is present in bundle sheath cells, besides mesophyll cells as in C_3 metabolism (Xoconostlen-Cazares et al., 2011). Alternately, sugarcane does not undergo direct fixation in the Calvin cycle, but rather converts carbon dioxide to a 4-C organic acid capable of generating carbon dioxide in the chloroplasts of the bundle sheath cells, which then produce carbohydrates by the traditional C_3 pathway (Xoconostlen-Cazares et al., 2011). According to McNaughton (1991) and Zhu et al. (2008), during this process, stomata are opened at night, enabling the plant to colonise hot environments, as they are known to have a far less water expense compared to other plants.

Upon exposure to stress and in attempts to cope with the stress, a plant's adaptive responses are mainly focused oin maintaining water potential in important tissues (Xoconostlen-Cazares et al., 2011). In response to the stress, organelle function and cell integrity are limited when the membrane and endomembrane system drastically alter their temperament (Gigon et al., 2004). The cell wall, which is normally a physical barrier, also provides some protection, albeit limited because of its deforming features (Murphy and Ortega, 1995). During dehydration, the cell wall offers mechanical protection, but due to its

permeability, desiccation can occur if a strong enough stress is applied (Verslues et al., 2006).

a) Drought-induced oxidative stress

Under drought adversities, plants usually have a lower carbon assimilation rate, which results in an inadequate sink for electrons generated in the electron transport chain, therefore leading to an overproduction of ROS (Edreva, 2005) such as hydroxyl radicals (OH•), superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) (Inze and Montagu, 1995). ROS can react with various biomolecules, changing or blocking their biological functions, leading to damage to cellular components, such as enzymes, membrane lipids, nucleic acids, carbohydrates and to the photosystem II complex (Guan et al., 2000; Arora et al., 2002; Apel and Hirt, 2004). Hydroxyl radicals have the ability to instantly react with DNA, proteins and lipids, leading to cell damage (Inze and Montagu, 1995). Hydrogen peroxide and superoxide are able to demobilise different macromolecules, but their toxicity results when they are converted into hydroxyl radicals, a reaction catalysed by transition metals (Inze and Montagu, 1995). Different metabolic processes can also use ROS in a profitable way; for instance, H_2O_2 and $O_2^{-\bullet}$ are associated with the formation of lignin in the cell walls (Inze and Montagu, 1995). The leakage of electrolytes, due to membrane integrity disruption, has been used as a criterion to select for stress tolerance crops (Martin et al., 1987). Also, H_2O_2 and $O_2^{-\bullet}$ histochemical detection has been used to screen for tolerant cultivars (Kumar et al., 2013).

b) Drought-induced defense mechanisms

Plants make use of non-enzymatic and enzymatic protective mechanisms that scavenge ROS. Oxidative stress, the injuries caused by ROS, constitutes one of the chief damage factors in plants exposed to various abiotic factors such as drought (Kwon et al., 2002). Studies showed that ABA and ROS are affiliated with abiotic stress sensing associated with faster activation of defense mechanisms in tolerant than in sensitive poplar species (Chen and Polle, 2011). In attempt to reduce the toxic effects of ROS, plants utilize highly regulated enzymatic and non-enzymatic mechanisms to uphold a balance between the synthesis and quenching of ROS to maintain cell homeostasis (Guan et al., 2000; Sairam and Tyagi, 2004). An antioxidant can be considered to be any compound capable of quenching ROS without being converted into a harmful radical (Dedemo et al., 2013). According to Noctor and Foyer (1998), antioxidants and antioxidant enzymes disturb cascades of uncontrolled oxidation. Consequently, plants are capable of combating oxidative stress through ROS-scavenging systems such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and low molecular weight antioxidant compounds including glutathione,

polyamines, ascorbate (ASC) and phenolic compounds. Furthermore, enzymes such as dehydroascorbate reductase (DHAR), monodehydro-ascorbate reductase (MDHAR) and glutathione reductase (GR), which are required for the regeneration of glutathione and ascorbate are also implicated (Dedemo et al., 2013).

The interaction between drought stress and responses involving the antioxidant systems in sugarcane is poorly understood, for the most part because the complexity of the sugarcane genome forecloses a thorough perception of the genetic, physiological, and biochemical issues underlying this process (Azevedo et al., 2011). A recent report by Ribeiro et al. (2013) revealed how complex this can be in sugarcane, wherein whilst drought events caused reductions in leaf gas exchange in three different genotypes, photosynthesis in one of the studied genotypes was linked to both non-stomatal and stomatal restrictions, while in the other two genotypes photosynthesis was restricted only stomatally.

In point of fact, when most plant species are subjected to water deficit, the ROS-scavenging system is set in play (Kar, 2011), and this varies with the severity of the stress and the time length of exposure, tissue/organ involved and the developmental stage (Dourado et al., 2013). To a certain level, ROS production during stress may function as a signal to induce acclamatory or defense responses through transduction pathways, which may use hydrogen peroxide as a secondary messenger (Cruz de Carvalho, 2008; Miller et al., 2010). Over a certain level, however, ROS may have harmful effects in plant cells. Thus defences against ROS, involving enzymatic and non-enzymatic antioxidants working in an effective detoxification system, are triggered (Gratao et al., 2005; Impa et al., 2012). The non-enzymatic system involves alkaloids, flavonoids, phenolic compounds, carotenoids and tocopherols (Gratao et al., 2005). The enzymatic response includes an array of enzymes such as ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and many more (Mittler, 2002; Gratao et al., 2005; Gallego et al., 2012).

Only recently more attention has been given to the understanding of the antioxidant defense system in plants subjected to drought and water deficit conditions (Ghane et al., 2012; Doupis et al., 2013; Kaur et al., 2013). In sugarcane, the presence of antioxidant enzymes is increased under drought conditions (Patade et al., 2011; Patade et al., 2012; Hemapraba et al., 2013).

c) Drought sensing and signal transduction at a biochemical level

Although there is still obscurity about the sensor(s) for drought stress, it is generally accepted that the organ with such ability is the root system (Xoconostle-Cazares et al., 2011). This stress response is known to be mediated by the endogenous plant regulator ABA (Raghavendra et al., 2010). There are suggestions that the response to drought could

be elicited by a redox imbalance or possibly the change in the integrity of cell membranes (Kacperska, 2004). Research done on *Arabidopsis* showed that two histidine kinase receptors (ATHK) are induced during early drought stress (Urao et al., 1999). In addition, the *Arabidopsis* leucine rich protein RPK1 (Hong et al., 1997) and SPK1 from *Phaseolus vulgaris* (Montalvo-Hernandez et al., 2008) have been described as the most ample transcript under drought stress. In *Arabidopsis*, SPK1 is induced by ABA (Osakabe et al., 2005). Drought stress conditions stimulate high levels of ABA, coupled with potent alterations in gene expression and adaptive physiological responses (Christmann et al., 2007), and it is believed that ABA plays a fundamental role in early plant responses to drought stress. The discovery of the ABA receptor RCARs/PYR1/PYLs, known to deactivate type 2C protein phosphatases (ABI1 and ABI) (Kang et al., 2010), gives insights into both ABA-dependent gene expression and ion channels.

d) Drought-induced osmotic adjustment and protein synthesis

A frequently noted biochemical adaptation is osmotic adjustment, which is due to the accumulation of newly-produced metabolites (Yancey et al., 1982; Bartels and Sunkar, 2005). These are useful if water supply is to be limited at a later stage, because their hydrophilic and highly soluble nature enables them to produce a solvation surface that binds and captures water molecules (Xoconostlen-Cazares et al., 2011). Examples of these molecules include sugars, sugar alcohols, amino acids and glycine-betaine, which are non-toxic even at high concentrations, ensuring non-interference with cellular metabolism (Xoconostlen-Cazares et al., 2011).

Other workers have suggested that osmolytes may have additional functions other than maintaining turgidity, such as dealing with oxidative stress by quenching ROS (Chen and Murata, 2002; Bartels and Sunkar, 2005). During drought stress, sugars that accumulate are hypothesized to be involved in stabilizing membranes and forestalling membrane fusion together with other macromolecules such as Late Embryogenesis Abundant (LEA) proteins (Xoconostlen-Cazares et al., 2011). The disaccharide trehalose accumulates during drought stress and is associated with the development of embryos and flowers, as well as the regulation of both carbon metabolism and photosynthesis (Phillips et al., 2002). Glycine betaine has been suggested to be an osmoprotectant ensuring plant organs water equilibrium maintenance (Chen and Murata, 2002).

Proline accumulation is suggested to play a crucial role in plant stress tolerance (Verbruggen and Herman, 2008). According to Peng et al. (1996) and Hare and Cress (1997), proline acts as a compatible osmolyte and a store for carbon and nitrogen. Early *in vitro* studies showed that proline functions as a ROS scavenger during the onset of oxidative stress induced by

abiotic factors such as drought (Smirnoff and Cumbes, 1989; Hong et al., 2000). Proline has also been reported to stabilize protein structure, buffer cytosolic pH and balance plant cell redox status. The accumulation of proline during osmotic stress, which is due to its increased synthesis and diminished degradation, is part of stress signalling regulating adaptive responses (Maggio et al., 2002). In sugarcane, it is suggested that proline is a stress resistance marker (Alvarez et al., 2003; Ehsanpour and Fatahian, 2003). However, Errabii et al. (2007) suggested that proline accumulation in sugarcane is merely an indication of injury rather than a stress tolerance characteristic. In fact, this has been reported in other species under stress conditions (Cano et al., 1996; Garcia et al., 1997; Tonon et al., 2004). Some workers pointed out that from a quantitative point of view, the role of proline in osmotic adjustment is insignificant in cultivars under salt and mannitol stress (Mohamed et al., 2000; Benlloch-Gonzalez et al., 2005).

Different kinases known to be induced by stress (Conley et al., 1997) include MAPKs (Jonak et al., 1996), which are also activated by other abiotic stresses, indicating that reversible phosphorylation plays a vital role in drought signalling (Mizoguchi et al., 1996). ABA-mediated stomatal closure stimulates the increase in intra-or extracellular Ca_2^+ concentration, which in turn induces calcium-dependent protein kinases (CDPKs) (Schroeder et al., 2001, Ramanjulu and Bartels, 2002). According to Hirayama et al. (1995), the signal molecule inositol triphosphate (ITP) is induced by drought, salinity and cold stress in *Arabidopsis*, with the ABA sensing-related phospholipase D (PLD) regulating stomatal opening and ion channel activity (Sang et al., 2001).

Another mechanism regulating plant response(s) to drought stress is translational control (Xoconostle-Cazares et al., 2011). The proteins synthesized during translation have primary functions in membrane and protein protection (Xoconostle-Cazares et al., 2011). Proteins of this nature are involved in attaining water and ions, as well as maintaining homeostasis in basal cell functions (Xoconostle-Cazares et al., 2011). The LEA protein family, a group highly elevated in plant embryos, is classified as one of the above-mentioned proteins (Dure et al., 1981; Galau et al., 1986). Drought and osmotic stresses induce LEA proteins that are expressed at basal levels (Ingram and Bartels, 1996; Berrera-Figueroa et al., 2007). Five LEA protein groups have been identified according to their structural domains; these include group 3 and 5 which form dimers with a coiled-coil shape responsible for ion coordination during stress (Dure et al., 1981). Aquaporins (AQPs) are membrane-spanning proteins (can be found in the tonoplast or plasma membrane) (Johansson et al., 2000) involved in water uptake and allocation through the formation of water pores (Xoconostle-Cazares et al., 2011). Certain stress tolerant plant varieties have been shown to have differential aggregation of AQP (Montalvo-Hernandez et al., 2008).

Heat Shock Proteins (HSPs), molecular chaperones broadly distributed in nature and also highly present during stress, aid in protein folding and assembly as well as the remotion and disposition of non-functional proteins (Wang et al., 2003). Workers such as Alamillo et al. (1995) and Campalans et al. (2001) found that HSPs are induced by abiotic stress such as drought and salinity, and *in vivo* studies propose that these proteins preclude thermal aggregation (Lee et al., 1995), thus helping in post-abiotic stress cell recovery. The classification of these proteins is based on their molecular weights: the Hsp70 family (family Dnak), the Hsp100 family, the chaperonins (GroEL and Hsp60), the Hsp90 family and the small Hsp (sHsp) family (Wang et al., 2004). The chaperone protein highly induced under drought stress, cyclophilin, has systemic attributes allowing for involvement in protein folding, and the overexpression of cyclophilin-encoding genes bestows tolerance to multiple abiotic stresses (Gottschalk et al., 2008; Sekhar et al., 2010). Some of these responses have been detected *in vitro* (Alamillo et al., 1995; Alvarez et al., 2003; Errabii et al., 2007; Cha-um and Kirdmanee, 2008; Cha-um et al., 2010; Haslbeck and Buchner, 2015).

3 Materials and Methods

3.1 Collection and preparation of plant material

Sugarcane plant stalks of variety NCo376 were collected from the South African Sugar Research Institute (SASRI) in Mount Edgecombe, KwaZulu-Natal, South Africa, and used for plant regeneration via *in vitro* direct organogenesis. SASRI supplied *in vitro* shoots of varieties N41, N12, N19, N36 and N26. Shoots from all these varieties were multiplied, rooted and used to test the effect of mannitol and PEG-6000 stress on *in vitro* plant responses. The SASRI also supplied field-grown sugarcane stems of varieties NCo376 that were used for *in vitro* sett germination.

3.2 *In vitro* sett germination

Sugarcane stems of variety NCo376 were cut into single-budded setts, washed under running tap water and detergent (Care®), then surface sterilized with 70 % (v/v) ethanol, 1% sodium hypochloride and placed in 20 ml sterile water in culture bottles (1 sett/60 mm culture bottle). To eradicate culture contamination during sett germination, the anti-fungal agents 1 ml l^{-1} BRAVO® 500 (500 g l^{-1} chlorotalonil, Syngenta Ireland Ltd), 1 ml l^{-1} Previcur® N (722 g l^{-1} propamocarb hydrochloride, Bayer CropScience Inc.) and anti-bacterial methylene blue (1 mg l^{-1}) were tested. The setts were set out to germinate for 10 days in a growth room and the number of shoot-producing setts was recorded. Furthermore, to investigate if the anti-bacterial or anti-fungal agents delayed shoot production by the setts, the shoots produced were recorded in one of two categories, *viz*. short (1 – 2 cm in length) or long (> 2 cm in length) shoots.

3.3 *In vitro* direct organogenesis standard culture procedures

3.3.1 Stage 1: Apical meristem initiation

Sugarcane stalks of NCo376 were used to obtain the meristem for mass production of *in vitro* shoots. The outer leaf sheaths were removed, and the stalks were inverted and surface sterilized with 70% (v/v) ethanol. Under the laminar flow, the innermost leaf sheath was removed with a sterile blade and the stalk cut (approx. 30 – 40 mm in length) in liquid media [full strength MS salts and vitamins (Murashige and Skoog, 1962), 0.1 mg l^{-1} BAP, 0.015 mg l^{-1} Kinetin, 1.0 mg l^{-1} methylene blue and 20 g l^{-1} sucrose]. With a scalpel blade, the leaf sheaths were further removed with the aid of a light dissecting microscope (Zeiss, Germany). This was done until the conical-shaped meristem (approx. 1.3 cm) was reached. The meristem was removed using a sterile blade and cultured on semi-solid shoot induction media (1 meristem/60 mm Petri dish with 20 ml medium) containing full strength MS salts and vitamins (Murashige and Skoog [1962], 0.1 mg l^{-1} BAP, 0.015 mg l^{-1} Kinetin, 3.5 g l^{-1}

activated charcoal, 1.0 mg l^{-1} methylene blue, 8 g l^{-1} agar and 20 g l^{-1} sucrose, and incubated in the dark at 26 ± 1°C for a week.

After 1 week, the meristems were transferred onto semi-solid shoot induction medium (1 meristem/60 mm Petri dish with 20 ml medium) lacking activated charcoal [full strength MS salts and vitamins (Murashige and Skoog [1962], 0.1 mg l^{-1} BAP, 0.015 mg l^{-1} kinetin, 1.0 mg l^{-1} methylene blue, 8 g l^{-1} agar and 20 g l^{-1} sucrose]. For the first 4 days after culture, the cultures (1 meristem/60 mm Petri dish with 20 ml medium) were kept at low light intensity (by covering cultures with paper towel) in the growth room, after which they were moved to normal light exposure in the same Petri dishes at 16 h light, 200 µm m^{-2} s^{-1} photon flux density/8 h dark photoperiod and 26°C (day)/23°C (night) for 2 weeks.

3.3.2 Stage 2: Shoot multiplication

Once the meristems reached approx. 1 cm in length, they were subcultured onto liquid shoot multiplication medium (1 meristem/60 mm culture bottle with 20 ml medium) containing full strength MS salts and vitamins, 0.1 mg l^{-1} BAP, 0.015 mg l^{-1} kinetin, 1.0 mg l^{-1} methylene blue, and 20 g l^{-1} sucrose for 2 weeks. Once developed (approx. 1 cm in height), the shoots were maintained in the culture bottles (1 shoot/60 mm culture bottle with 20 ml medium) containing liquid shoot multiplication medium and were then subcultured into Magenta vessels (1 - 2 clumps/90 mm Magenta vessel with 60 ml medium) every 2 - 3 weeks onto fresh liquid shoot multiplication medium.

3.3.3: Rooting

Once multiplied for 2 – 3 weeks, individual shoots (approx. 1 cm in height), were separated from the shoot clumps and placed on liquid rooting medium (1 shoot/culture tube with 5 ml medium) containing full strength MS salts and vitamins (Murashige and Skoog [1962], 1.0 mg l^{-1} IBA, 1.0 mg l^{-1} methylene blue and 30 g l^{-1} sucrose) for 3 - 4 weeks.

3.4 Experimental design

The approach followed in this study is shown in Figure 1. Meristems (approx. 1.3 cm) and *in vitro* plantlets were obtained as described in 3.2 and 3.3, respectively. The established plantlets were used to investigate their tolerance and/sensitivity to osmotic stress provided as mannitol or PEG-6000. Parameters such as plantlet root and shoot re-growth were determined as indicators of mannitol stress tolerance, and root re-growth was found to be the best stress indicator than shoot regrowth, hence it was the chosen parameter for subsequent investigations. Additionally, the effect of mannitol or PEG-6000 as a drought stress agent on *in vitro* plantlet root re-growth was also investigated. Subsequently, mannitol LD_{50} and LD_{90} concentrations were established and used to determine the equivalent PEG-

6000 concentrations at the same osmotic potential. Mannitol and PEG-6000 induced similar effects on plantlet root re-growth, but because initial results showed that mannitol required no aeration of the cultures and was easily dissolved in the medium, it was the stress agent of choice for subsequent investigations. The established LD_{50} and LD_{90} mannitol concentrations (concentrations inhibiting root re-growth by 50 and 90%, respectively) were then used to screen different sugarcane varieties for tolerance and/sensitivity to mannitol stress. The leaf chlorophyll content (via SPAD meter measurements), leaf electrolyte leakage and leaf accumulation of H_2O_2 and $O_2^{-\bullet}$ were assessed as indicators of stress tolerance and/sensitivity at the established LD_{50} and LD_{90} mannitol concentrations.

3.5 Effect of mannitol stress on *in vitro* plant responses of varieties NCo376 and N41

3.5.1 *In vitro*-induced drought stress using mannitol

The effect of mannitol on NCo376 and N41 plantlets' responses was investigated. Towards this, the plantlets' roots, shoots or both were trimmed using a sterile blade in a laminar flow and the plantlets exposed to rooting medium (section 3.3.3) supplemented with different concentrations of mannitol (0, 204, 326, 448 and 569 mM). Each mannitol treatment had 15 replicates for both varieties. Plantlet root and shoot re-growth were recorded at 2 days intervals for a maximum of 10 days. Based on the results obtained, plantlet root re-growth was selected for subsequent investigations. To determine if the effect of mannitol was permanent, the plantlets were cultured on rooting medium lacking mannitol (recovery medium containing Murashige and Skoog (1962), 1.0 mg l^{-1} IBA, 1.0 mg l^{-1} methylene blue and 30 g l^{-1} sucrose) for a period of 14 days.

3.5.2 LD_{50} and LD_{90} determination

The plantlets' root re-growth at day 10 in the presence of the different concentrations of mannitol in the medium was used to obtain the LD_{50} and LD_{90} (concentrations inhibiting root re-growth by 50 and 90%, respectively) from a linear regression analysis of mannitol concentration vs. plantlet root re-growth (Graph Pad Prism 5.0, Graph Pad software Inc., San Diego, CA, USA), using the formula: $y = mx + c$, at the regression coefficient of ≥ 80%.

3.5.3 Comparison of mannitol and PEG-6000 effects on plantlet root re-growth of varieties NCo376 and N41

To investigate if mannitol and PEG-6000 induced similar effects on *in vitro* plantlet root re-growth, sugarcane cultivars NCo376 and N41 were subjected to mannitol and PEG-6000 concentrations of the same osmotic potential and plantlet root re-growth recorded at day 10. Cultures on PEG-6000 were placed in one of two conditions, *viz*. static (on the shelf) or on a shelf shaker (100 rpm) to determine the need for aeration. Plantlet root re-growth was

recorded after 10 days. Based on the results obtained, mannitol was the chosen stress agent for subsequent investigations.

3.6 *In vitro* screening of sugarcane varieties for responses to mannitol stress

LD_{50} and LD_{90} mannitol concentrations for varieties NCo376 and N41 (332 and 851 mM, respectively) and (606 and 1493 mM, respectively) were used to screen varieties N12, N29, N26 and N36 in order to discriminate mannitol stress tolerance and/sensitivity amongst them. Plantlet root re-growth was recorded after 10 days of culture in medium containing mannitol.

3.7 Evaluation of physiological parameters as indicators of drought stress

Based on the results obtained from the work described in section 3.5, only four sugarcane varieties were chosen for this part of the investigation, *viz*. NCo376, N19, N26 and N41. Plantlet root re-growth was observed at 0, 332 and 851 mM mannitol for 10 days. Estimated chlorophyll content, detection of hydrogen peroxide and superoxide and electrolyte leakage were determined as described below.

3.7.1 Leaf chlorophyll content (via SPAD-502)

The leaf chlorophyll content of the tested varieties was determined using a SPAD-502 Plus Minolta (Spectrum Technologies, Inc.). Measurements were taken at days 5 and 10 of exposure to the LD_{50} and LD_{90} mannitol concentrations on the middle section of leaf 3 of all the varieties on 5 replicates per leaf. Five replicates were used for each variety per mannitol concentration (332 and 851 mM).

3.7.2 Histochemical detection of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$)

At days 5 and 10 of mannitol stress (332 and 851 mM), the leaf accumulation of H_2O_2 and $O_2^{-\bullet}$ was detected histochemically by staining with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively (Kumar et al., 2013). For the detection of H_2O_2, the plantlets' entire leaves were immersed in a solution of DAB (1 mg ml^{-1}, pH 3.8) overnight, after which chlorophyll was extracted by boiling the leaves in 70% (v/v) ethanol for 10 minutes. The presence of a reddish-brown colour denoted H_2O_2 content and was photographed. For the detection of $O_2^{-\bullet}$, the plantlets' leaves were floated in 50 mM sodium phosphate (pH 7.5) with 0.2% NBT. A dark blue insoluble formazan compound denoted the presence of $O_2^{-\bullet}$ and was photographed. This was repeated 3 times with a total of 5 leaves per mannitol concentration.

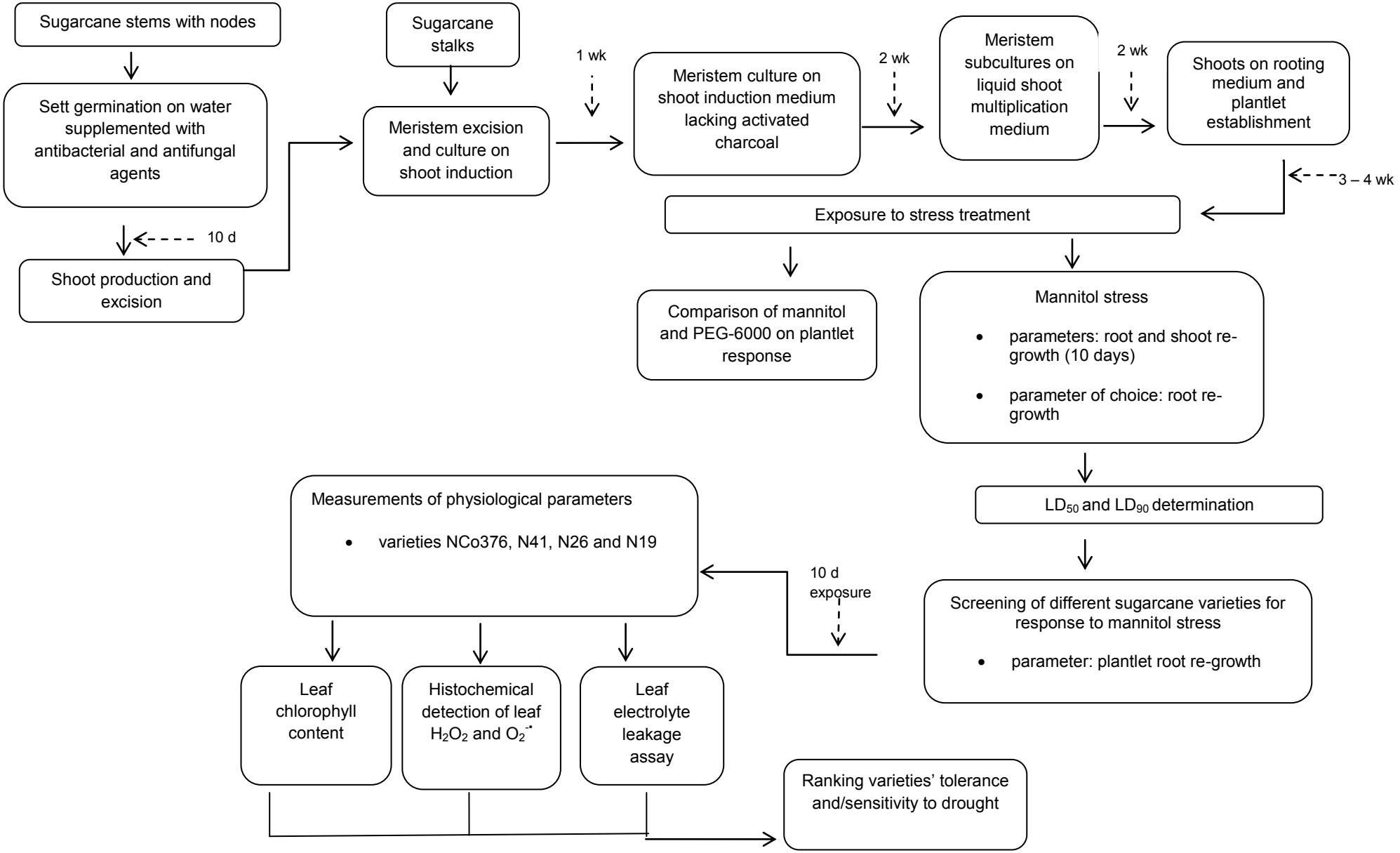


Figure 1: A summary of the experimental design and stages of direct organogenesis standard culture procedures adapted to establish *in vitro* shoot multiplication protocol.

3.7.3 Electrolyte leakage assay

The electrolyte leakage of NCo376, N19, N26 and N41 was evaluated at days 5 and 10 of mannitol stress exposure. Entire leaves were excised, weighed, immersed in 20 ml deionised water and the electrolyte conductivity (EC1) was measured using the CM100-2 Conductivity Meter (Reid and Associates, Durban, South Africa). The samples were then incubated at 55°C in a water bath for 30 minutes, and the electrolyte conductivity (EC2) of the solution was measured. Thereafter, the samples were incubated in a water bath at 100°C for an hour, and the electrical conductivity (EC3) of the solution was measured. Five replicates were used for each variety per mannitol concentration (332 and 851 mM). The relative electrolyte leakage was expressed as a percentage of the total conductivity calculated with the following equation:

$$\text{Relative electrolyte leakage (\%)} = [\{EC2 - EC1) / EC3] \times 100$$

3.8 Environmental conditions

All cultures except those maintained in the dark during shoot induction, were incubated at 16 h light, 200 µm m^{-2} s^{-1} photon flux density/8 h dark photoperiod and 26 °C (day)/23 °C (night). Shoot induction medium was at pH 4.5, whilst shoot multiplication and rooting medium were at pH 5.3 and were autoclaved at 121°C for 20 minutes.

3.9 Photography

All photographs were taken using a Nikon E4500 camera.

3.10 Data collection and statistical analysis

The statistical program Graph Pad Prism version 5.0 was used for all analyses. Data presented as percentages were subjected to arcsine transformation prior to further statistical analyses. All data were tested for normality using D'Agostino-Pearson test ($p < 0.05$) and analysed using Analysis of Variance (ANOVA) followed by the Least Significant Difference (LSD) test to distinguish significantly different means ($p < 0.05$).

4. RESULTS

4.1 Establishment of a protocol for mass propagation of *in vitro* shoots using sugarcane variety NCo376

This investigation served to establish the best source of meristematic explant for culture initiation and mass propagation of *in vitro* shoots.

4.1.1 Direct organogenesis using apical meristems explants from stalks of sugarcane variety NCo376

A direct organogenesis protocol for *Saccharum* cultivar NCo376 using apical meristems as explants (Ramgareeb et al., 2010) was used. Leaf rolls were surface-sterilized and 1.3 cm long meristems (Figure 2A) excised and established on semi-solid shoot induction medium with activated charcoal (Figure 2B) and incubated in the dark for 1 week. As a precaution, after 3 days, the meristems were moved to an undisturbed area of the medium in the same Petri dish to avoid phenolic suppression of shoot formation, although the phenolics were not detected as the medium was blackened by the presence of activated charcoal. After 1 wk in the dark, the cultures were transferred to semi-solid shoot induction medium lacking activated charcoal and placed in a photoperiod growth room for 2 weeks. During this stage, the outer leaf covering the meristem turned brown and was dissected away, facilitating the development of new shoots (Figure 2C). The newly-formed shoots were transferred to culture bottles (1 shoot/60 mm culture bottle) with 4 ml liquid shoot multiplication medium (Figure 2D - 1E). After 2 weeks, shoots were subcultured on Magenta vessels with fresh liquid multiplication media (Figure 2F), where approx. 60±0.93 shoots/explant were produced.

4.1.2 Direct organogenesis using apical meristems explants from shoots of *in vitro*-germinated setts of sugarcane variety NCo376

Isolation of 1 mm long meristems from sugarcane stalks (Ramgareeb et al., 2010) is a labourious process, and since they are very small, they can be easily damaged. Hence, another source of meristem explants was investigated: the shoots of setts germinated *in vitro*. Field-grown sugarcane stems (Figure 3A), cut into single-budded setts (approx. 50 mm, Figure 3B) were placed in culture bottles (1 sett/60 mm culture bottle) with sterile water (Figure 3C) and incubated in a photoperiod growth room. Individual shoots germinated from the setts after 10 days in culture (Figure 3D).

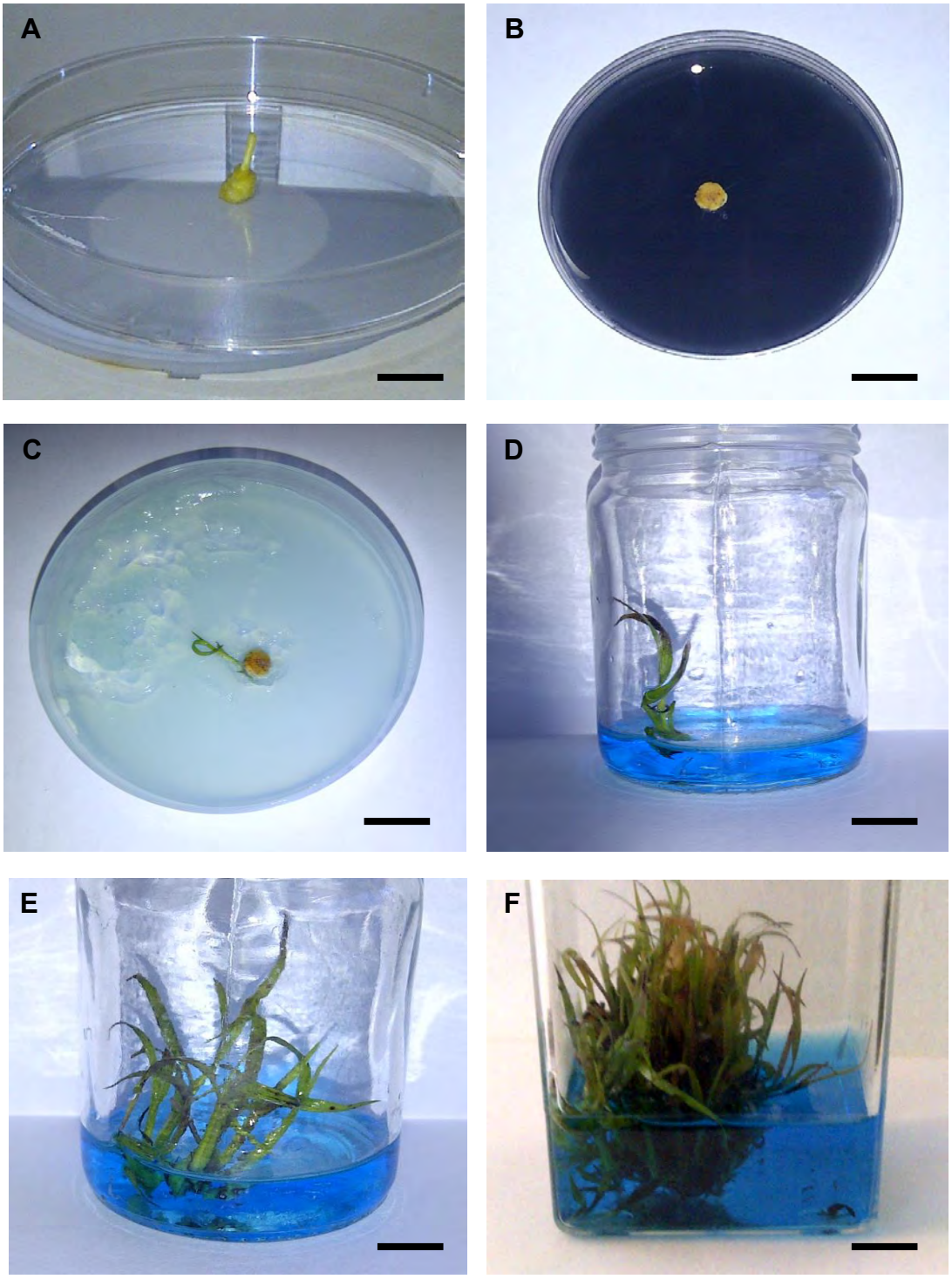


Figure 2: Different stages of direct organogenesis of variety NCo376 from an apical meristem explant. (A) 1 cm long meristem obtained from a sugarcane stalk, bar = 1.3 cm; (B) Meristem on semi-solid shoot induction medium containing activated charcoal, bar = 6.1 cm; (C) Meristem with developing shoot on shoot induction medium lacking activated charcoal, bar = 1 cm; (D) Shoot developed from meristem on liquid shoot multiplication medium, bar = 1.1 cm; (E) small clump of developing shoots on shoot multiplication medium, bar = 0.9 cm; (F) larger shoot clump on multiplication medium, bar = 1.6 cm.

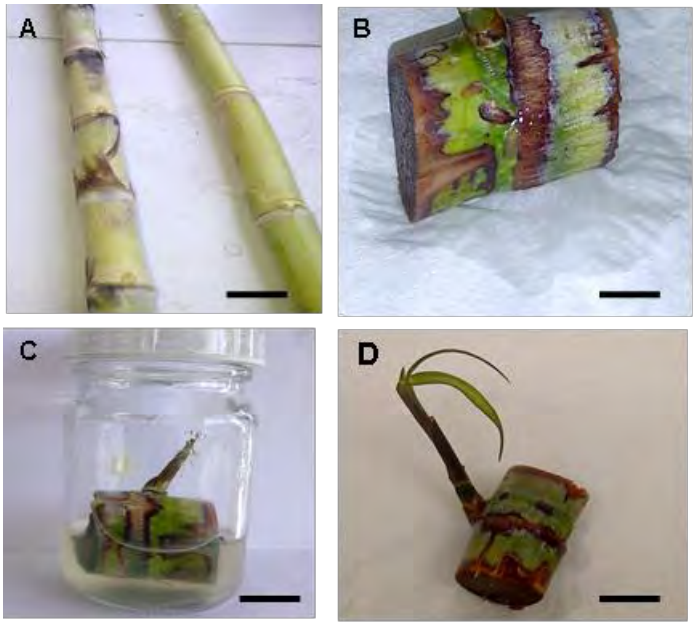


Figure 3: Stages of shoot production from (A) sugarcane stems with nodes, bar = 2.5 cm. (B) Individual single-budded sett, bar = 1.2 cm cultured on (C) 20 ml sterile water, bar = 1.5 cm. After 10 days, (D) individual shoot was produced, bar = 3.3 cm, from which meristem explant was excised.

As a significantly high % (60 - 100) of the germinated setts maintained in water only and in moist paper towel were found to be contaminated (Figure 4A), the need for antibacterial (methylene blue) and antifungal (BRAVO® and Previcur®) agents was investigated. Contamination of setts was significantly lower in treatments supplemented with methylene blue alone or combined with BRAVO® or Previcur® than in the control treatment (water only). However, no significant differences were detected amongst the treatments (Figure 4A). The inclusion of methylene blue alone or in combination with BRAVO® or Previcur® did not decrease shoot production (Figure 4B), when compared with the water only and moist paper towel treatments, as there was no significant difference amongst all the treatments.

To determine if methylene blue+BRAVO® delayed shoot production, the shoots produced were recorded in one of two categories, *viz*. short (1 – 2 cm in length) or long (> 2 cm in length) shoots. No significant differences in production of short shoots were detected amongst all the treatments (Figure 5A). The water+methylene blue treatment resulted in a significantly higher production of long shoots from the water only treatment, but not from the other treatments (Figure 5B). These results, therefore, indicate that the treatments did not delay shoot production.

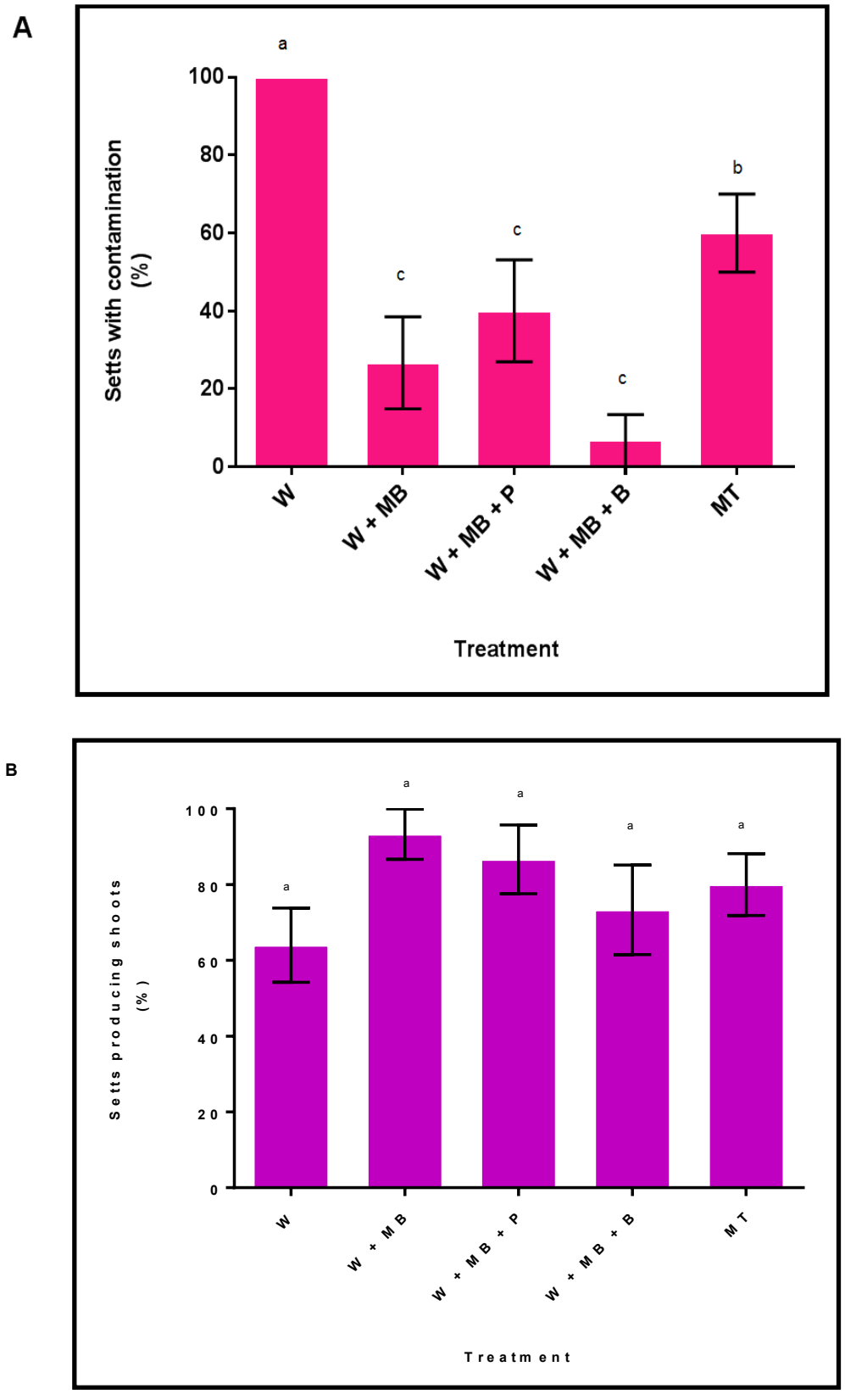


Figure 4: Effect of anti-bacterial and anti-fungal agents on the % setts with (A) contamination and (B) % setts producing shoots (B) 10 days after in different treatments. n = 25, mean ± SE. a – e denote statistically significant differences amongst the mean of the treatment groups: W = water only, W + MB = water + methylene blue, W + MB + P = water + methylene blue + Previcur®, W + MB + B = water + methylene blue + BRAVO®, MT = moist paper towel. Data were statistically analysed with ANOVA, $p < 0.05$.

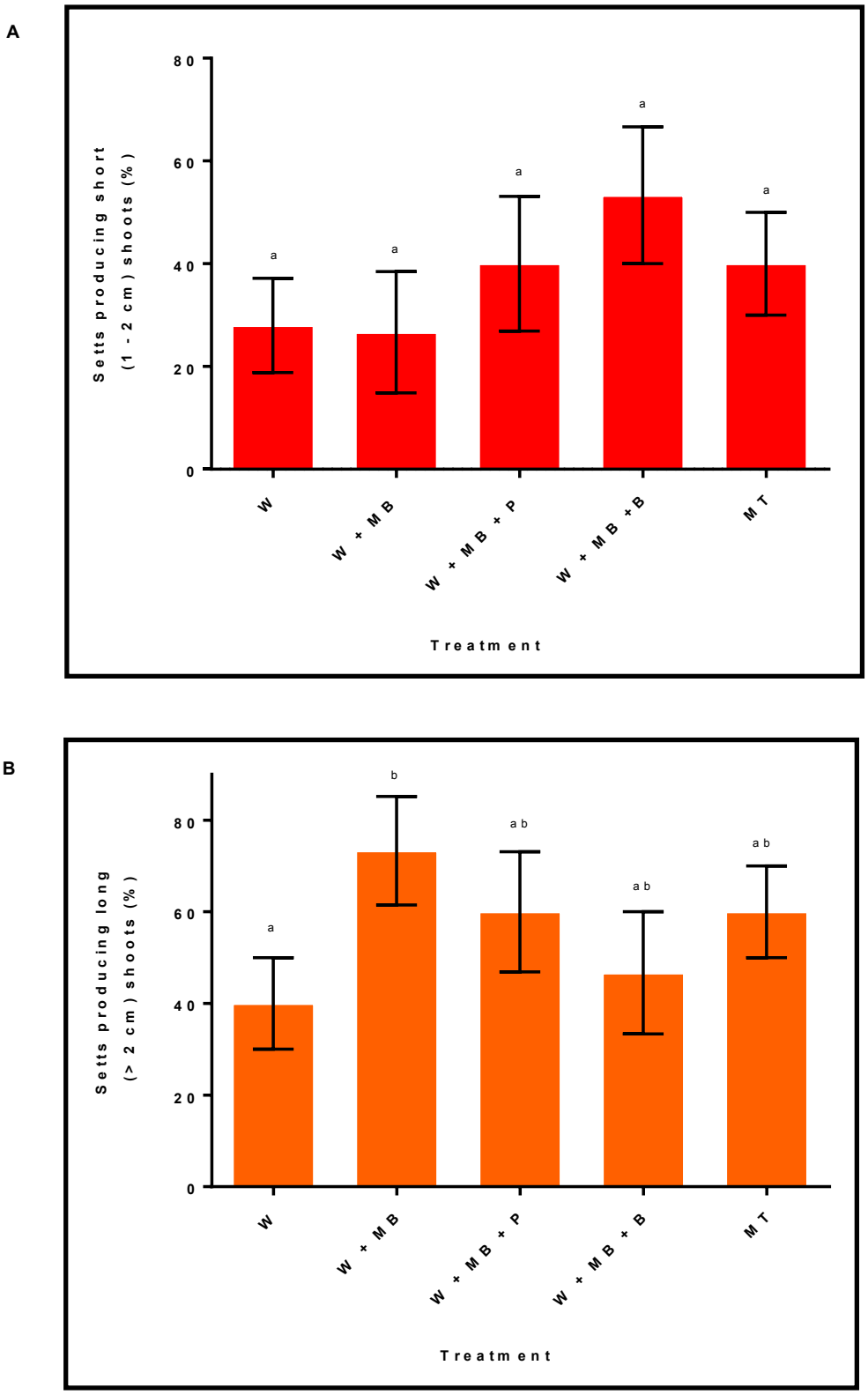


Figure 5: Effect of anti-bacterial and anti-fungal agents on the production of (A) short (1 - 2 cm in height) and (B) long (> 2 cm in height) shoots from individual single-budded sugarcane setts 10 days after germination in different treatments. n = 25, mean ± SE. a - b denote statistically significant differences amongst treatment groups: W = water only, W + MB = water + methylene blue, W + MB + P = water + methylene blue + Previcur®, W + MB + B = water + methylene blue + BRAVO®, MT = moist paper towel. Data were statistically analysed with ANOVA, $p < 0.05$.

Once isolated from the source, i.e. from stalks in protocol 1 and from shoots of *in vitro*-germinated setts in protocol 2, the explants were subjected to shoot induction and multiplication. After 2 - 3 weeks on shoot multiplication medium, a non-contaminated explant from the stalk yielded approx. 60±0.93 shoots, while a non-contaminated explant from the shoot of *in vitro*-germinated sett yielded approx. 10±0.86 shoots (Table 5). The low number of shoots produced with the latter protocol may have been due to endogenous sett contamination which only appeared during shoot multiplication. Even though the addition of methylene blue+BRAVO® decreased contamination during sett germination, there was 90% sett contamination when the shoots that developed from such setts were placed in shoot multiplication medium. Therefore, protocol 1 proved to be the most efficient protocol for the mass propagation of NCo376 *in vitro* shoots.

Table 5: A summary of the yield of shoots produced from each of the tested protocols used to source meristems of NCo376. Stage 1 involved the germination of setts *in vitro* and stage 2 involved meristem excision, shoot induction and multiplication (Section 4.1.1) In protocol 1, the meristems were obtained from the apex of sugarcane stalks, and in protocol 2 they were obtained from shoots of *in vitro*-germinated setts. In both protocols, semi-solid medium was used for shoot induction and once the meristems reached 1 cm in height, they were transferred to liquid shoot multiplication medium. n = 25.

Protocol	Source of meristem	Contaminated setts (%)		Number of shoots produced/meristem
		Stage 1	Stage 2	
1	Sugarcane stalk	N/A	0	±60
2	Shoot of *in vitro*-germinated sett	7	90	±10

4.2 Effect of different mannitol concentrations on shoot and root re-growth of NCo376 and N41 plantlets

To investigate the plants' responses to mannitol, the roots or shoots were trimmed prior to subjecting the plantlets to the stress (0, 204, 326, 448, 569 mM) for 10 days. Both shoot and root re-growth were determined.

For both varieties, the plantlets turned brown after 2 – 3 days in culture, but this did not hinder either shoot (visible as new green shoots) or root re-growth (visible as new root radicle). When NCo376 plantlets were subjected to 0 or 204 mM mannitol, root re-growth was 20% after 2 days, and it increased with time in culture up to day 8, when ±83% root re-

growth was observed (Figure 6). There were no significant differences in % plantlet root re-growth from day 4 to day 10 when NCo376 plantlets were cultured on 0 and on 204 mM mannitol (Table 6). At 326 mM mannitol, root re-growth was delayed from day 2 to day 4, whilst at 448 and 569 mM mannitol root re-growth was delayed from day 2 to day 6. At 326, 448 and 569 mM mannitol, the % plantlet root re-growth recorded were 10 - 35% after 8 days in culture. There was no significant difference in root re-growth over time between NCo376 plantlets cultured on 448 and 569 mM mannitol.

When N41 plantlets were subjected to 0, 204 or 326 mM mannitol, root re-growth increased to 30, 20 and 20%, respectively and increased with time in culture up to day 8, where 80 - 100% root re-growth was observed (Figure 6). There was no significant difference in root re-growth from day 4 to day 10 when N41 plantlets were cultured on 326, 448 and 569 mM mannitol (Table 6). At 448 and 569 mM mannitol, root re-growth was delayed from day 2 to day 4 and at these concentrations, the highest % plantlet root re-growth recorded was between 60 - 75% after 8 days in culture. There was no significant difference in % plantlet root re-growth between days 4, 6, 8 and 10 when N41 plantlets were subjected to 326, 448 and 569 mM mannitol.

Tukey's multiple comparison test showed which mannitol concentrations resulted in significantly different root re-growth within the sampling days (Table 7). For NCo376, there were no significant differences in % plantlet root re-growth of plantlets subjected to 0 or 204 mM mannitol at days 4, 6, 8 and 10 (Table 7). For NCo376 plantlets, there were significant differences between these two treatments (0 and 204 mM) and the other tested treatments (326, 448 and 569 mM mannitol) with respect to plantlet root re-growth. Similar results were observed for N41. For N41 plantlets, significantly different % plantlet root re-growth was observed at day 10 of culture on 569 mM mannitol as compared with the other tested treatments (0, 204, 326 and 448 mM mannitol). With NCo376 plantlets, 50% root re-growth was observed after 4, 5 and 4 days of culture in mannitol of 0, 204, and 326 mM, respectively. Plantlet root re-growth reached 50% in N41 after days 2 and 4 of culture in 0 and 204 mM mannitol, respectively.

As observed for root re-growth, increased mannitol concentration in the culture medium delayed plantlet shoot re-growth by 2 days, except in the case of 204 mM. For example, at 326, 448 and 569 mM mannitol, shoot re-growth was delayed from day 2 to day 4 for NCo376 and N41 plantlets (Figure 7). No significant differences in shoot re-growth were observed when NCo376 plantlets were subjected to 569 mM mannitol over time. Similar results were obtained for N41, where mannitol at 326, 448 and 569 mM resulted in non-significantly different plantlet shoot re-growth over time. For NCo376, significant differences in shoot re-growth of plantlets were observed across all tested mannitol concentrations over

time except at 569 mM mannitol (Figure 7, Table 7). At 0, 204 and 326 mM mannitol, culture days 2 and 4 showed significant differences in % plantlet shoot re-growth when compared with culture days 6, 8 and 10. With NCo376 plantlets, 50% shoot re-growth was observed after 4 days of culture in 0 and 204 mM mannitol. Plantlet root re-growth reached 50% in N41 after days 2 of culture in 0, 204 and 326 mM mannitol, whilst 50% shoot re-growth was observed after day 4 of culture in 448 and 569 mM mannitol.

Table 6: Statistical analysis of the % plantlet root re-growth results in Figure 6 for varieties NCo376 and N41. A Two-way ANOVA (Tukey's multiple comparison test) was performed using GraphPad Prism statistical package. These results compared which mannitol concentration resulted in statistical significantly different % plantlet root re-growth over time. n = 15.

	NCo376					**N41**				
	Mannitol (mM)					**Mannitol (mM)**				
	0	204	326	448	569	0	204	326	448	569
Days										
2	*	#	+	Δ	¤	*	#	+	Δ	¤
4	*	#	+	Δ	¤	**	#	+	Δ	¤
6	**	##	++	Δ	¤	**	##	+	Δ	¤
8	**	##	++	ΔΔ	¤	**	##	+	Δ	¤
10	**	##	++	ΔΔ	¤	**	##	+	Δ	¤

The number of *, #, +, Δ and ¤ denote statistical significant differences ($p < 0.05$) in % plantlet shoot re-growth at a particular mannitol concentration across all tested days.

When shoot re-growth was compared at each day across the tested mannitol concentrations, significant differences were detected. For NCo376, % plantlet shoot re-growth at days 4, 6, 8 and 10 was significantly higher in plantlets cultured on 0 and 204 mM mannitol than those cultured on 326, 448 and 569 mM mannitol at those days (Table 7). Similar results were observed with N41 plantlets. The above results indicated that the negative effect of mannitol was more significant on plantlet root than on shoot re-growth. Hence, plantlet root re-growth was the chosen parameter for subsequent investigations.

To determine if the negative effect of mannitol was permanent on plantlet root re-growth after exposure to mannitol, NCo376 and N41 plantlets were cultured on rooting medium lacking mannitol (recovery medium). The negative effect of mannitol on the ability of the plantlets to exhibit root re-growth was found to be permanent (Table 8).

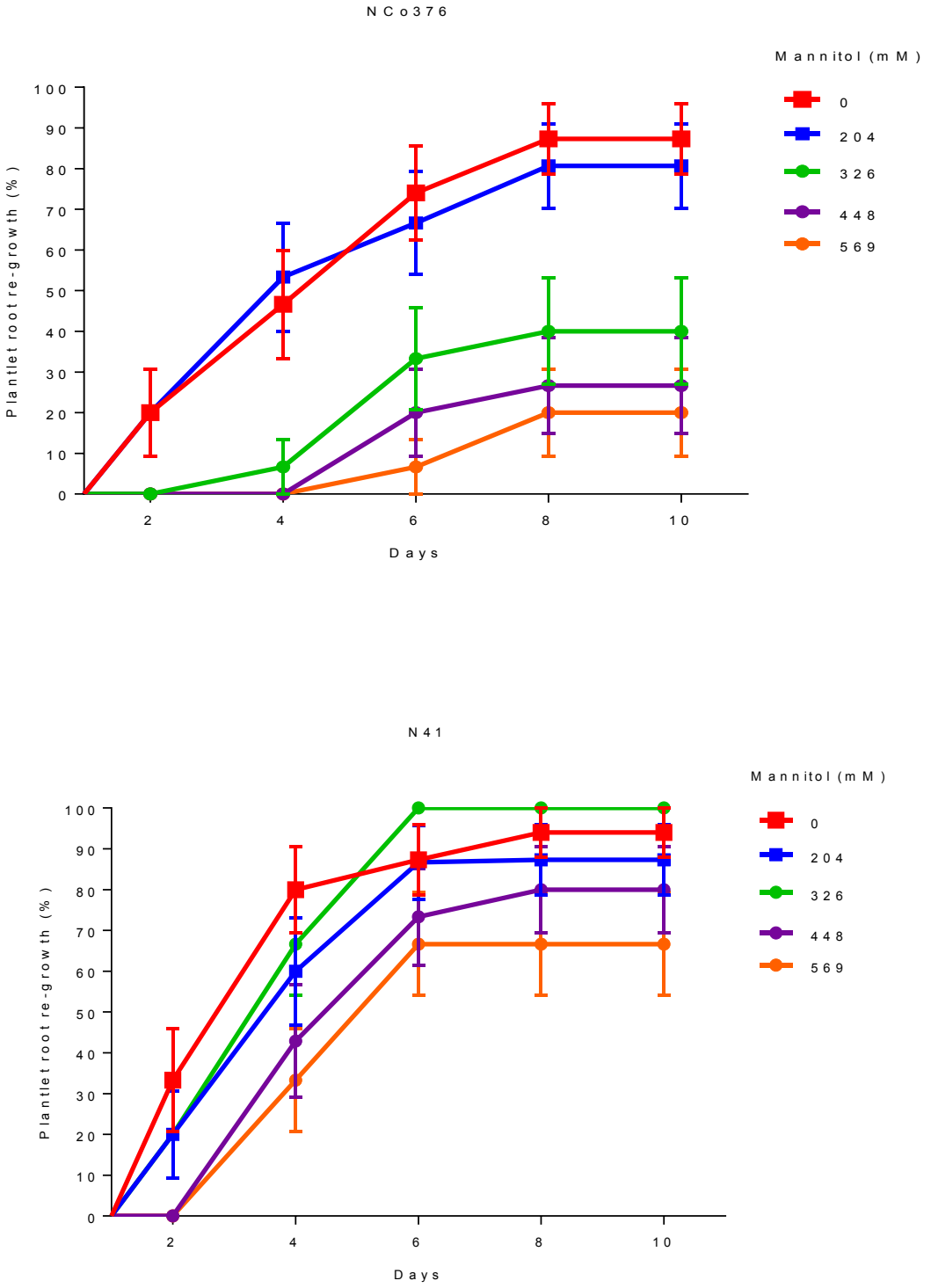


Figure 6: Effect of different mannitol concentrations on root re-growth of NCo376 and N41 plantlets at days 2, 4, 6, 8 and 10. All media contained full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA and 30 g l^{-1} sucrose and varying concentrations of mannitol. n = 15, mean ± SE. The experiment was repeated three times for each of the tested mannitol concentrations.

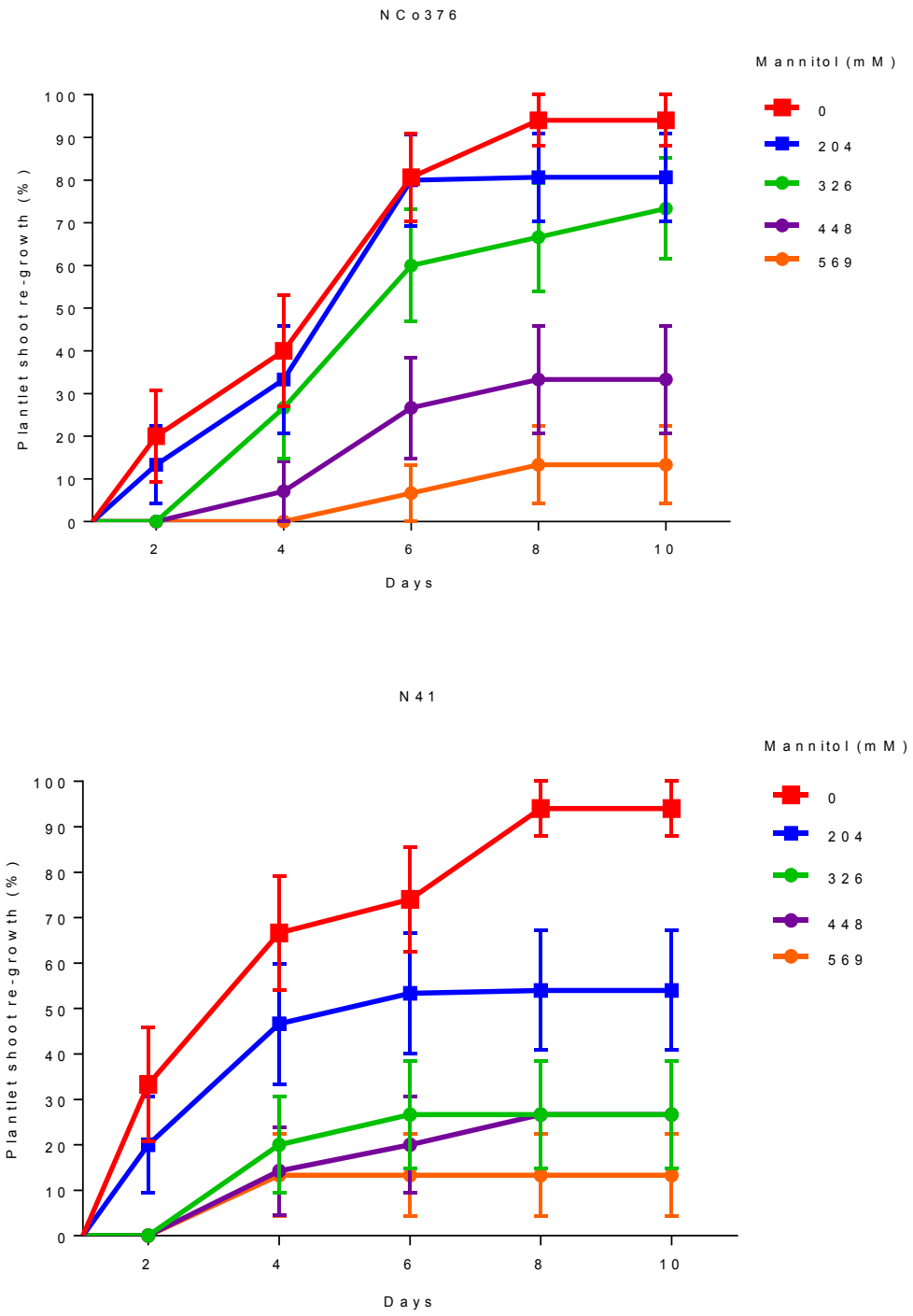


Figure 7: Effect of different mannitol concentrations on shoot re-growth of NCo376 and N41 plantlets at days 2, 4, 6, 8 and 10. All media contained full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA and 30 g l^{-1} sucrose and varying concentrations of mannitol. n = 15, mean ± SE. The experiment was repeated three times for each of the tested mannitol concentrations.

Table 7: Statistical analysis of the % plantlet shoot re-growth results in Figure 7 for varieties NCo376 and N41. A Two-way ANOVA (Tukey's multiple comparison test) was performed using GraphPad Prism statistical package. These results compared which mannitol concentration resulted in significantly different % plantlet shoot re-growth within the tested days. n = 15.

	NCo376					**N41**				
	Mannitol (mM)					**Mannitol (mM)**				
	0	204	326	448	569	0	204	326	448	569
Days										
2	*	*	*	*	*	*	*	*	*	*
4	#	#	# #	# #	# #	#	#	# #	# #	# #
6	+	+	+ +	+ +	+ +	+	+	+ +	+ +	+ +
8	Δ	Δ	ΔΔ	ΔΔ	ΔΔ	Δ	Δ	ΔΔ	ΔΔ	ΔΔ
10	¤	¤	¤¤	¤¤	¤¤	¤	¤	¤	¤	¤¤

The number of *, #, +, Δ and ¤ denote statistical significant differences ($p < 0.05$) in % plantlet root re-growth at different mannitol concentrations within the sampling days.

Table 8: Percentage plantlet root re-growth of plantlets exposed to different mannitol concentrations for 10 days (stress medium) and after 14 days in recovery medium. For both varieties, stress medium contained full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and varying concentrations of mannitol. Subsequently, the cultures were subcultured on recovery medium [full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose]. n = 15. The experiment was repeated three times. a – b denote statistical significant differences ($p < 0.05$) in % plantlet root re-growth.

Mannitol (mM)	**NCo376 root re-growth (%)**		**N41 root re-growth (%)**	
	Stress	**Recovery**	**Stress**	**Recovery**
0	93±13.6^{a}	93±13.6^{a}	100±0.0^{a}	100±0.0^{a}
204	87±13.6^{a}	87±13.6^{a}	93±13.6^{a}	93±13.6^{a}
326	40±19.6^{b}	40±19.6^{b}	86±16.0^{a}	86±16.0^{a}
448	27±17.7^{b}	27±17.7^{b}	80±16.0^{a}	80±16.0^{a}
569	20±16.0^{b}	20±16.0^{b}	73±18.9^{a}	73±18.9^{a}

4.3 Comparison of the use of mannitol and PEG-6000 as drought stress agents on *in vitro* plantlets of NCo376 and N41.

Mannitol and PEG (molecular weights of 4000 to 8000) are the most frequently used agents to induce osmotic stress (Mexal et al., 1975; Rai et al., 2011). Hence, it was important to investigate if they induced similar effects *in vitro*. Towards this, the mannitol concentrations resulting in 50% (LD_{50}) and 90% (LD_{90}) inhibition of root re-growth at day 10 were calculated for both varieties (Figure 8). From these, the equivalent PEG-6000 concentrations were determined based on their osmotic potentials (Table 9) and medium supplemented with different PEG-6000 concentrations were used to assess *in vitro* plantlet responses.

Plant injury in culture has been reported in the presence of PEG, mainly due to the considerable reduction of oxygen availability (Mexal et al., 1975; Verslues et al., 1998; Munns et al., 2010; El Siddig et al., 2013). For this reason, before comparing PEG and mannitol effects, the need for aeration was investigated by placing the cultures containing PEG in the medium on a shelf shaker (100 rev/min), and their root re-growth was compared with that of static cultures (on the shelf). For NCo376, at each of the concentration tested (87 and 250 mM PEG-6000), aeration resulted in significantly higher % plantlet root re-growth, e.g. at 87 mM root re-growth was 50% compared with 10% in the static cultures. Similar results were obtained with N41, e.g. at 350 mM, aeration resulted in 55% root re-growth compared with 10% in the static cultures (Figure 9). These results indicate that aeration of PEG cultures is essential to obtain significant root re-growth for both NCo376 and N41.

The calculations in Figure 8 and 9 were used to investigate if the determined LD_{50} and LD_{90} of mannitol and the equivalent PEG-6000 concentrations resulted in similar % plantlet showing root re-growth. For both varieties, LD_{50} and LD_{90} of mannitol and the equivalent PEG-6000 resulted in non-significantly different % plantlet root re-growth at the same osmotic potential (Table 10). For instance, at - 0.82 MPa, mannitol and PEG-6000 resulted in 50% root re-growth for NCo376, and at - 2.10 MPa, mannitol resulted in ±50% root re-growth whilst PEG-6000 resulted in ±55% root re-growth.

These results indicate that, at the same osmotic potential, mannitol and PEG-6000 resulted in similar root re-growth. Since PEG-6000 requires more labour, such as aeration of cultures, and is difficult to dissolve in the medium, mannitol was the stress agent of choice for subsequent screening of other varieties.

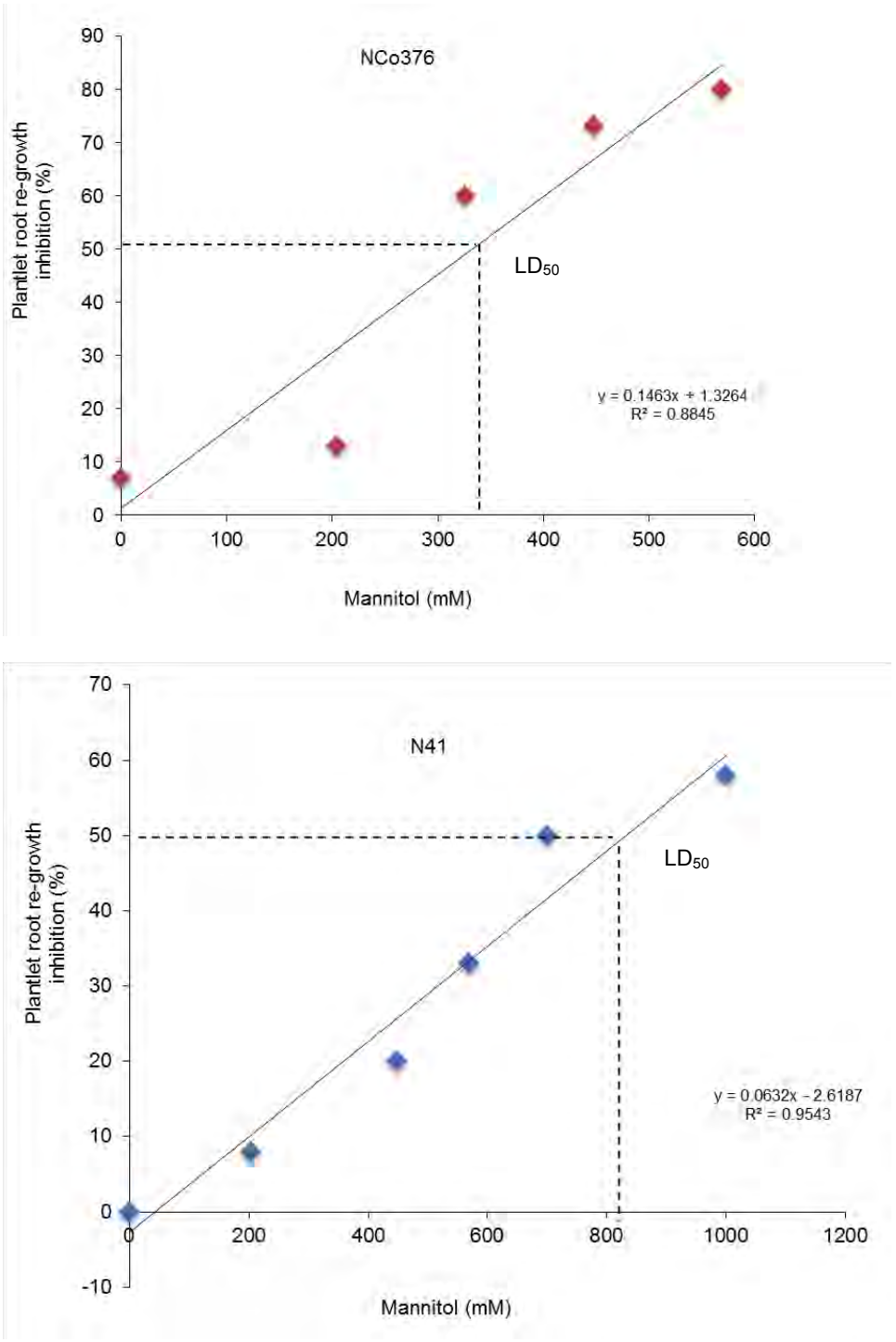


Figure 8: Determination of 50% lethal dose (LD_{50}) of mannitol for % plantlet root re-growth. This was calculated from the data obtained in Figure 6 at day 10. For both varieties, plantlets were cultured on stress medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and varying concentrations of mannitol for 10 days. n = 15. The experiment was repeated three times.

Table 9: LD_{50} and LD_{90} mannitol concentrations for inhibition of plantlet root re-growth and equivalent PEG-6000 concentrations for NCo376 and N41 used to screen other varieties for their drought tolerance status.

Variety	Osmotic potential (MPa)	Mannitol (mM)	PEG-6000 (mM)
NCo376			
LD_{50}	- 0.82	332	87
LD_{90}	-1.50	606	250
N41			
LD_{50}	- 2.10	851	350
LD_{90}	- 3.69	1493	615

Table 10: Root re-growth of plantlets at the mannitol and PEG-6000 concentrations that resulted in the LD_{50} and LD_{90}. For all varieties, plantlets were cultured on medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and the different concentrations of mannitol and PEG-6000. Plantlet root re-growth was recorded after 10 days to compare the effect of mannitol and PEG-6000 at the same osmotic potential. n = 15. The investigation was repeated three times. a – b denote statistical significant differences ($p < 0.05$) in % plantlet root re-growth.

Variety	Osmotic potential (MPa)	Osmoticum (mM)	Plantlet root re-growth (%)
NCo376	- 0.82	Mannitol (332)	50[a]
		PEG-6000 (87)	50[a]
	- 1.50	Mannitol (606)	10[a]
		PEG-6000 (250)	10[a]
N41	- 2.10	Mannitol (851)	50[a]
		PEG-6000 (350)	55[a]
	- 3.69	Mannitol (1493)	90[b]
		PEG-6000 (615)	85[b]

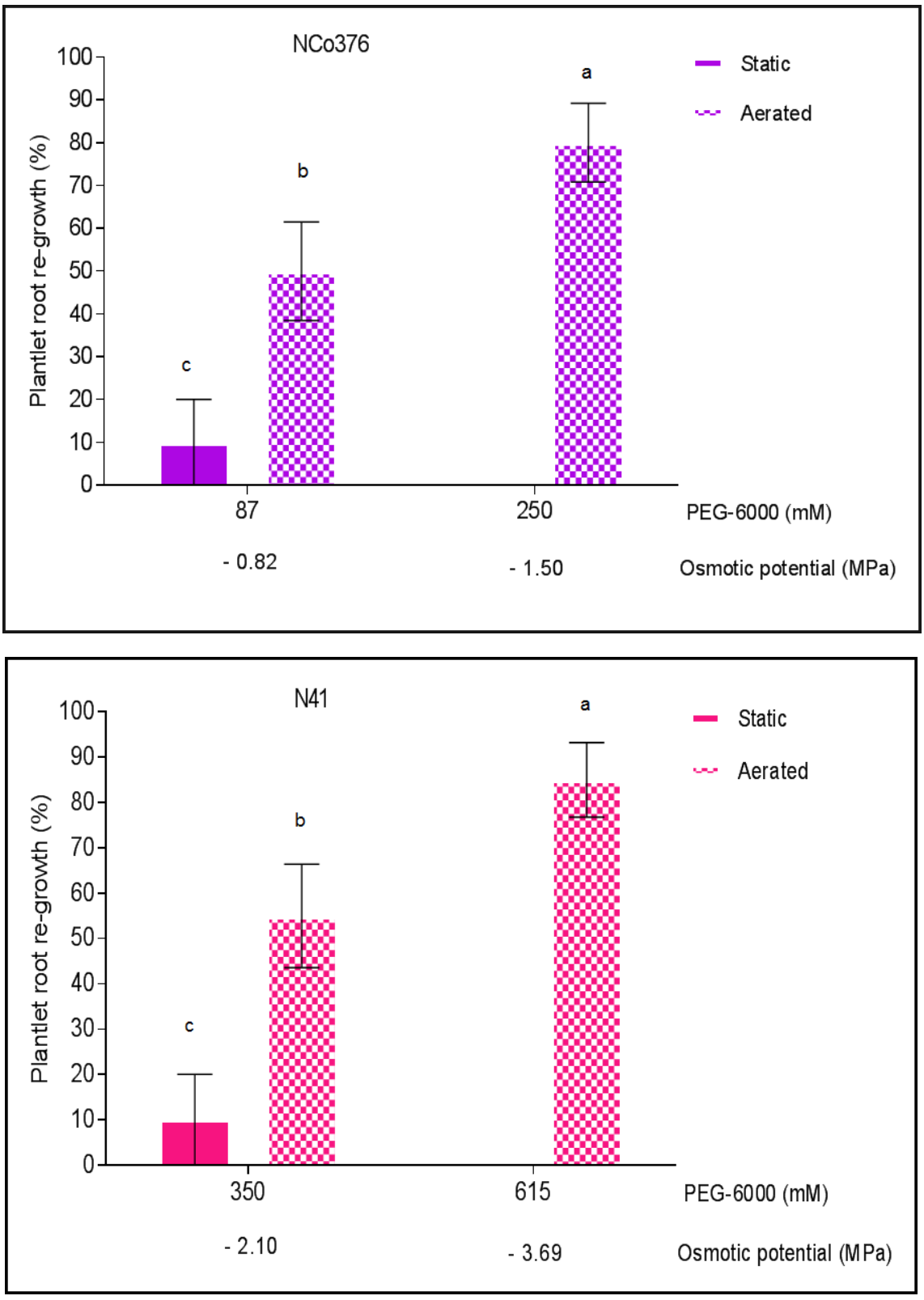


Figure 9: The effect of PEG-6000 on the % plantlet root re-growth when the cultures were static or aerated. For both varieties, plantlets were cultured on medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and the different concentrations of PEG-6000 calculated from the LD_{50} and LD_{90} mannitol concentrations for NCo376 and N41. n = 20, mean ± SE, repeated three times. a – c denote statistical significant differences in % plantlet root re-growth for static vs. shaker cultures at each PEG-6000 concentration. * denotes statistical significant differences in % plantlet root re-growth for either static or aerated cultures at the two PEG-6000 concentrations. Data were analysed with ANOVA, $p < 0.05$.

4.4 Screening of different sugarcane varieties for responses to mannitol stress.

The determined concentrations of mannitol that resulted in LD_{50} and LD_{90} for NCo376 and N41 (Table 9) were used to rank different varieties with respect to their sensitivity/tolerance to mannitol. As previously mentioned (Section 4.2), % plantlet root re-growth was the chosen parameter for screening for mannitol stress amongst varieties. However, only the 332, 606 and 851 concentrations were used to screen the different varieties, as 1493 mM mannitol did not dissolve completely in the culture media. The tested varieties were N12, N19, N26 and N36.

At 332 mM mannitol, there were no significant differences in % plantlet root re-growth amongst plantlets of NCo376, N12 and N19, and also amongst N26, N36 and N41 (Figure 10). At 606 mM mannitol, there were no differences in this parameter between N41 and N26, but it was significantly higher than the values obtained for N36, N12, N19 and NCo376 plantlets. Furthermore, root re-growth of N19 and NCo376 plantlets were significantly lower than the other varieties. No significant differences were detected in root re-growth amongst all the varieties at 851 mM mannitol. For N36, 332 mM mannitol resulted in significantly different % plantlet root re-growth than at 606 and 851 mM. For NCo376 and N19, mannitol at 332 mM resulted % plantlet with root re-growth which was significantly different from that at 606 and 851 mM mannitol. For N41 and N26, mannitol at 851 mM resulted in significantly lower % plantlet root re-growth than at 332 and 606 mM.

The above results indicate that the different sugarcane varieties responded differently to mannitol stress *in vitro* and these responses were used to deduce a probable ranking system (Table 11) with regards to the varieties' sensitivity or tolerance to mannitol stress. When compared with NCo376, varieties N12 and N19 had similar *in vitro* responses to 332 mM mannitol stress, whilst the *in vitro* response to mannitol of N26, N36 were similar to that of N41 at that concentration. At 606 mM mannitol, N41 and N26 demonstrated more tolerance to the stress, followed by N36 and N12, and N19 and NCo376 being the least tolerant to mannitol at that concentration. At the above-mentioned mannitol concentrations, N41 and N26 showed more tolerant to the stress than the other tested varieties. However, mannitol at 851 mM resulted in similar *in vitro* responses amongst all the tested varieties, suggesting that at this mannitol concentration accurate screening for stress tolerance was may not be feasible.

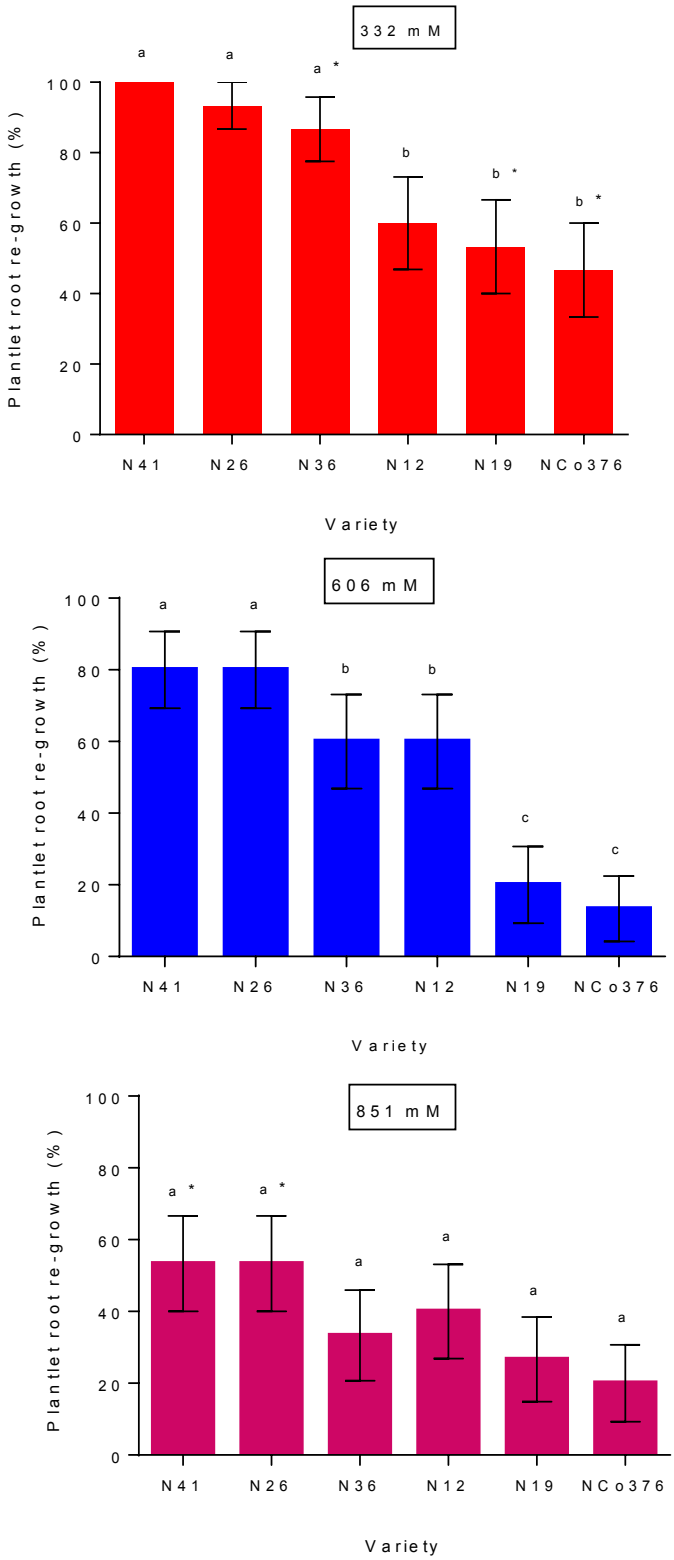


Figure 10: Percentage plantlet root re-growth of different varieties subjected to 332 and 606 mM mannitol which were determined to be the LD_{50} and LD_{90} for NCo376 *in vitro*, respectively, and 851 which is the LD_{50} for N41 at day 10. All plantlets were cultured on medium containing full strength MS salts and vitamins, 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and the different concentrations of mannitol. n = 15. Tukey's multiple comparisons test was performed to compare significantly different % plantlet root re-growth of the tested varieties within and across the mannitol concentrations. a – c denote statistically different % plantlet root re-growth of the varieties within each mannitol concentration. * denotes statistically different % plantlet root re-growth of each variety across the mannitol concentrations. Data was analysed using ANOVA, $p < 0.05$.

Table 11: Proposed ranking system of different varieties based on their *in vitro* responses to mannitol concentrations at the LD_{50} and LD_{90} established for NCo376 and N41 (Figure 10).

Mannitol (mM)	Probable ranking system
332	N41 = N26 = N36 > N12 = N19 = NCo376
606	N41 = N26 > N36 = N12 > N19 = NCo376
851	N41 = N26 = N36 = N12 = N19 = NCo376

4.5 Physiological parameters used to assess the impact of *in vitro* mannitol (drought) stress on sugarcane varieties.

The objective was to develop a quick and reliable *in vitro* method to screen for *in vitro* drought tolerance among different sugarcane varieties. In addition to the response of root re-growth to mannitol stress presented previously, other parameters were investigated with the aim to find other drought tolerance indicators. Towards this, four varieties were chosen, i.e. NCo376 and N19 which were found to be least tolerant to mannitol stress, and N41 and N26 which were found to be most tolerant to mannitol stress, under mannitol stress *in vitro* (Figure 10 and Table11). For all the varieties, culturing at 851 mM mannitol resulted in senescence of the leaflets and therefore the assessment of the below assays at this concentration of mannitol did not give a conclusive indication of tolerance to mannitol.

4.5.1 Electrolyte leakage assay.

At day 5 and day 10 of culture in 332 and 851 Mm mannitol (Figure 8), the leaflets of the tested varieties were assayed for their electrolyte leakage. At 332 mM mannitol no significant differences in % electrolyte leakage were detected between NCo376 and N19 and also between N26 and N41 (Figure 11) at both tested days, and electrolyte leakage of NCo376 and N19 were significantly higher than those of N26 and N41 plantlets. At 0 and 851 mM mannitol, no significant differences in electrolyte leakage were detected amongst all of the varieties. At day 5 and day 10, mannitol at 332 mM resulted in significantly higher electrolyte leakage than 0 and 851 mM for both NCo376 and N19 at both day 5 and day 10. All the tested varieties at both day 5 and day 10 showed very little leakage (< 40%) without mannitol, but more at day 5 than day 10. Mannitol at 0 and 851 mM resulted in similar electrolyte leakage amongst the tested varieties at both day 5 and day 10.

4.5.2 Leaf chlorophyll content measured with SPAD-502.

The leaf 'greenness' or relative chlorophyll content of different varieties was determined by taking SPAD meter measurements after day 5 and 10 of culture in medium containing 332 and 851 mM mannitol. At both days 5 and 10, N41 and N26 had a higher SPAD index compared with those of NCo376 and N19 plantlets when cultured on all medium containing the various mannitol concentrations. At 0 mM mannitol, at both day 5 and day 10, there were significant differences in the SPAD index amongst varieties, with NCo376 and N19 behaving similarly (Figure 12).

4.5.3 Histochemical detection of superoxide and hydrogen peroxide.

At day 5 and day 10 of culture in 332 and 851 Mm mannitol, the oldest leaflet of each of the plantlets from all varieties were assayed for the accumulation of superoxide ($O_2^{-\bullet}$) anion and hydrogen peroxide (H_2O_2) through staining with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively. Ten days of culture in mannitol media proved to be stressful to the varieties, as there was minimal staining and most of the leaflets had senesced and the stain was not picked up by the leaflets of any of the varieties. Staining using DAB resulted in more H_2O_2 accumulation (brown colour was more intense) in NCo376 and N19 than in N26 and N41 (Figure 13). Similar results were obtained with NBT staining, where $O_2^{-\bullet}$ accumulated to a greater extent (greater intensity of the blue colour) in NCo376 and N19, than in N26 and N41.

4.5.4 Ranking different sugarcane varieties based on physiological parameters.

The results obtained from the electrolyte leakage assay, leaf chlorophyll content via SPAD and the histochemical detection of superoxide and hydrogen peroxide may provide some indication as to the sensitivity or tolerance of the tested varieties to the mannitol stress when performed at day 5 of exposure to stress as 10 days of culture proved to be too stressful. Based on the obtained results, the tested varieties classified as either sensitive or tolerant to mannitol stress at 332 and 851 mM (Table 12). The ranking of the tested varieties revealed that N41 = N26 > N19 = NCo376, and they match the rankings previously obtained under mannitol stress (Section 4.4, Table 11).

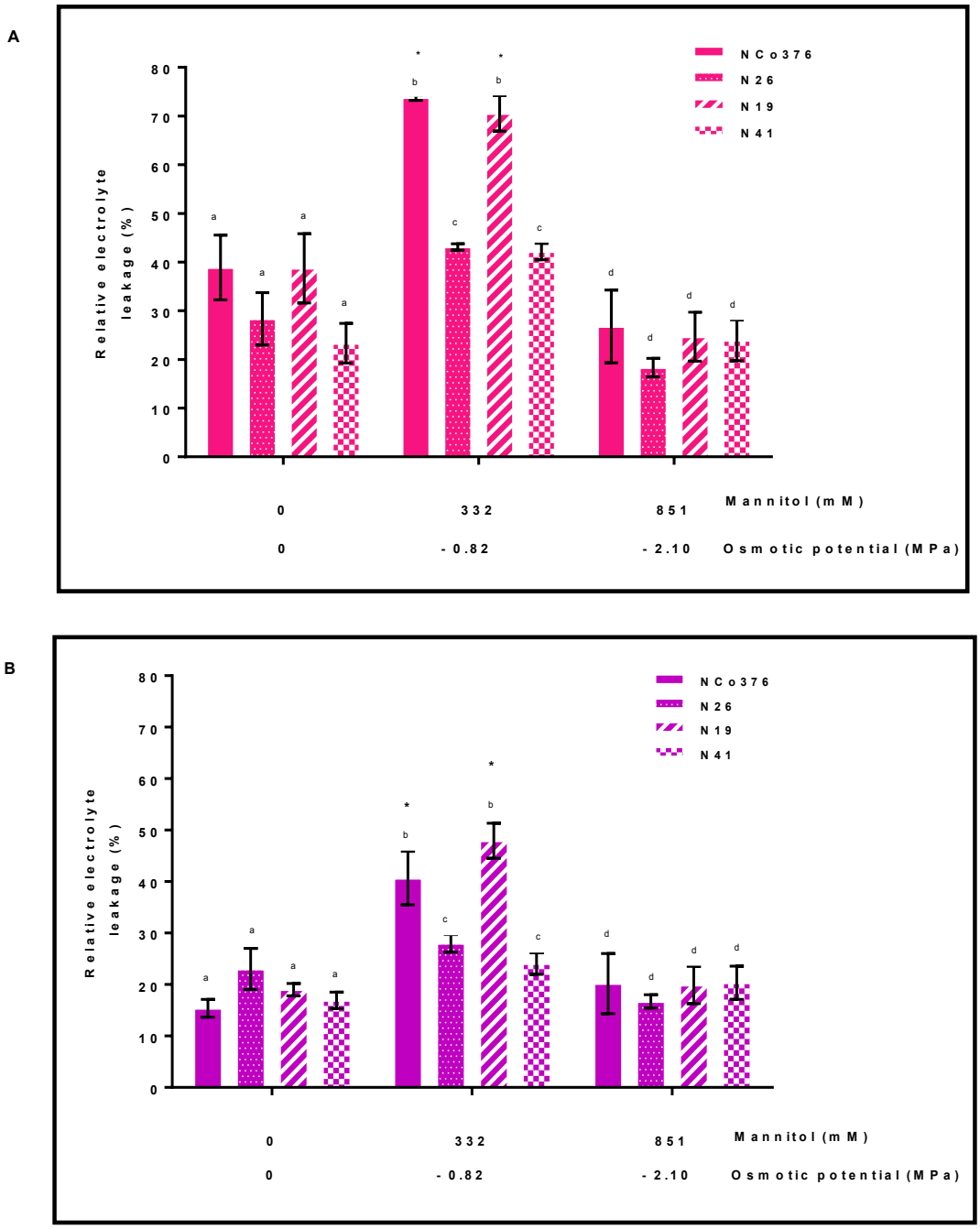


Figure 11: Percentage relative electrolyte leakage of four varieties subjected to different mannitol concentrations for 5 (A) and 10 (B) days. For all varieties, plantlets were cultured on medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and the different concentrations of mannitol. n = 15, mean ± SE. a – d denote statistical significant differences in % relative electrolyte leakage of the varieties within a particular mannitol concentration. * denotes statistical significant differences in % relative electrolyte leakage of each variety across all tested mannitol concentrations. Data were statistically analysed using ANOVA, $p < 0.05$.

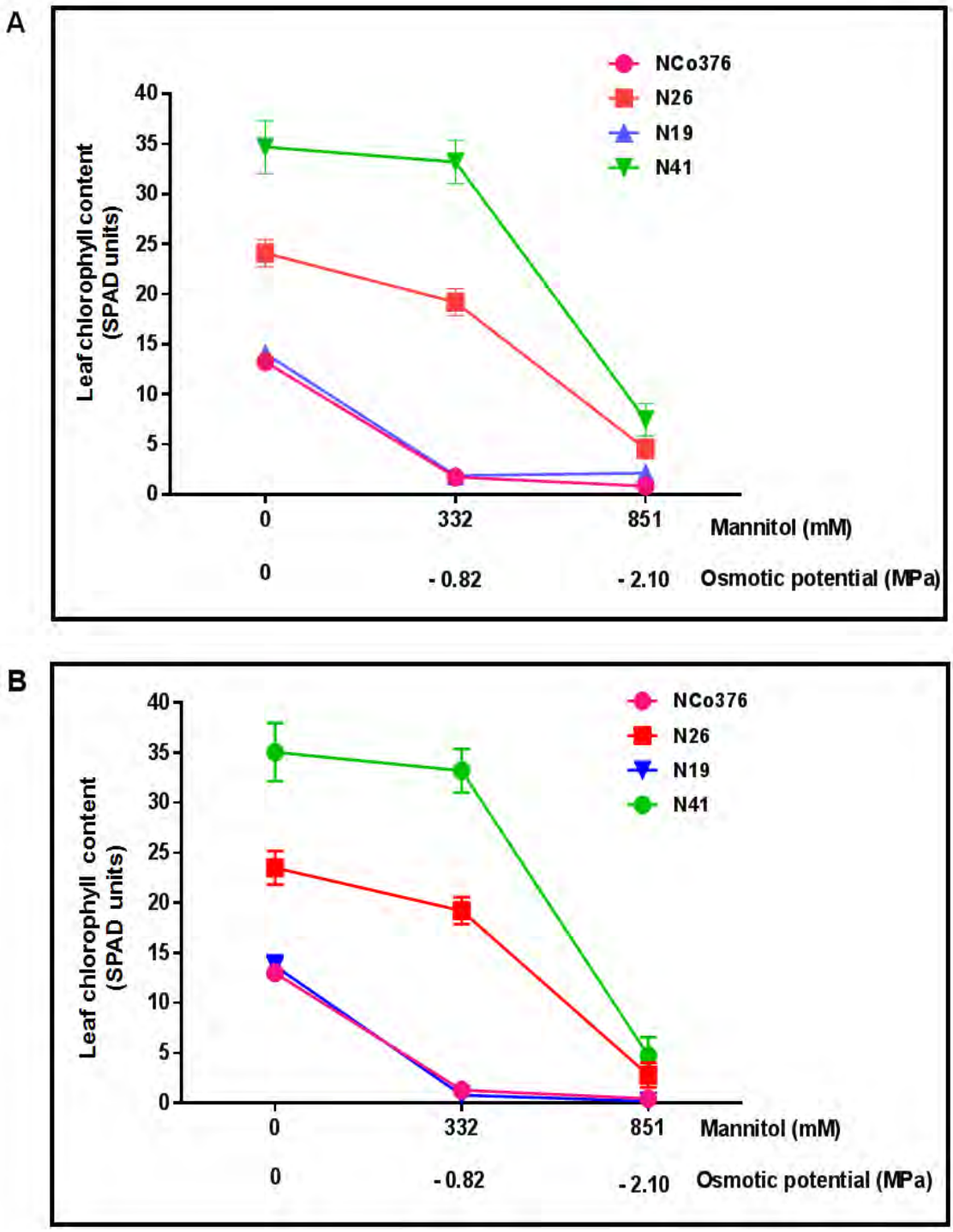
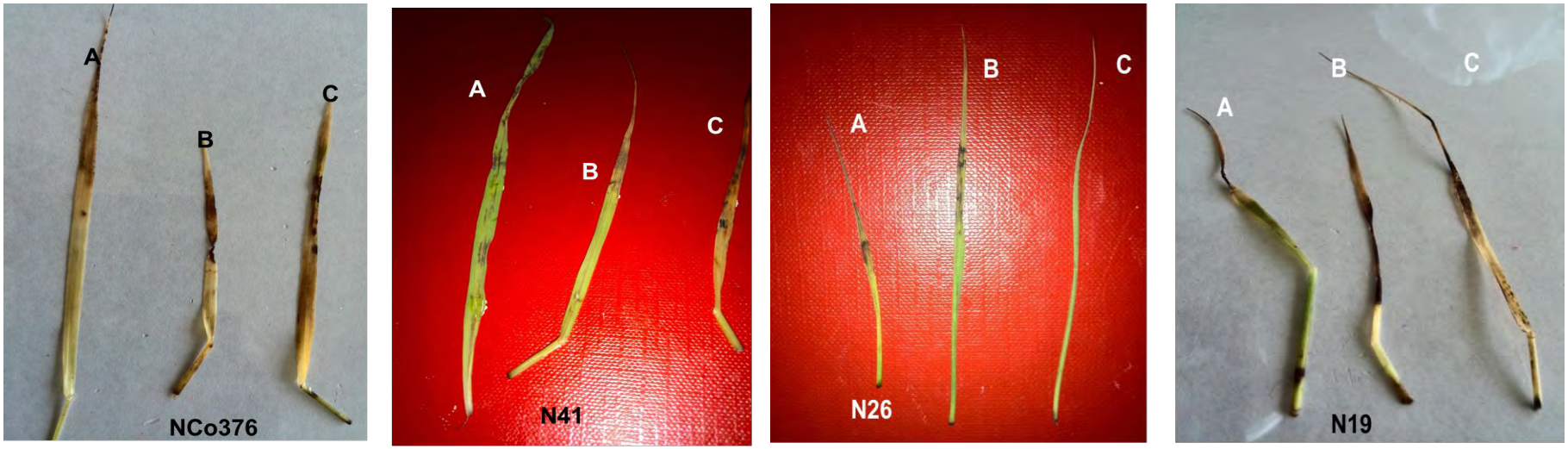


Figure 12: Leaf chlorophyll content of four varieties subjected to different mannitol concentrations for 5 (A) and 10 (B) days. For all varieties, plantlets were cultured on medium containing full strength MS salts and vitamins (Murashige and Skoog, 1962), 1.0 mg l^{-1} IBA, 30 g l^{-1} sucrose and the different concentrations of mannitol. n = 15, mean ± SE. Data were statistically analysed using ANOVA, $p < 0.05$.

DAB stain



NBT stain

Figure 13: Superoxide and hydrogen peroxide accumulation in the leaves of sugarcane varieties subjected to 0 (A), 332 (B) and 851 mM (C) mannitol for 5 days.

Table 12: Discrimination of tolerance and/ sensitivity to mannitol stress at 332 and 851 mM amongst varieties NCo376, N19, N41 and N26 based on the tested physiological parameters at day 5 of stress exposure. The % plantlet root re-growth was obtained from Figure 13. n = 5. a – b denote statistically significant different % plantlet root re-growth of the varieties within each mannitol concentration.

	Mannitol (mM)							
	332				**851**			
Variety	**Plantlet root re-growth (%)**	**SPAD meter**	**Electrolyte leakage**	**H_2O_2 and $O_2^{-\bullet}$ accumulation**	**Plantlet root re-growth (%)**	**SPAD meter**	**Electrolyte leakage**	**H_2O_2 and $O_2^{-\bullet}$ accumulation**
N41	100[a]	++	++	++	50[a]	+	+	+
N26	90[a]	++	++	++	50[a]	+	+	+
N19	57[b]	+	+	+	30[a]	+	+	+
NCo376	50[b]	+	+	+	25[a]	+	+	+

+ denotes sensitivity of variety, whilst ++ denotes tolerance to mannitol stress based on each of the tested physiological parameters.

5. Discussion

5.1 Determining the best source of meristematic tissue explant for mass propagation of *in vitro* shoots.

In sugarcane, the production and selection of new genotypes through breeding programs is followed by clonal propagation using nodal cutting known as billets, setts or seedcane, (Snyman et al., 2011). As previously described, although this process is relatively simple, there are two main disadvantages associated with it, *viz*. the likelihood of disease transmission and slow multiplication rate. According to Dookun et al. (1996) and Snyman et al. (2008), the multiplication rate is restricted by the number of buds present on a well-developed stalk, leading to propagation rates of eight-to tenfold in one growth cycle annually. Furthermore, the possible presence and transfer of systemic pathogens from the parent seedcane to the resultant crop may limit the effectiveness of such propagation if phytosanitation protocols are not strictly upheld (Snyman et al., 2011). Since sugarcane is a ratooning monoculture, this perpetuates pathogen infection through the rootstock (Lee, 1987; Victoria et al., 1999; Flynn et al., 2005). Pathogens can be removed conventionally through hot water treatment of sugarcane stalk, which is not only labour-intensive, but does not eradicate viral pathogens (Victoria et al., 1999; Flynn et al., 2005).

The use of *in vitro* techniques to mass propagate healthy sugarcane plantlets via direct organogenesis is therefore commonly used as an alternative to, or in support of the conventional methods. At SASRI, the explant for such propagation is usually the apical meristem culture from sugarcane stalks grown in the field (Snyman et al., 2011). As sugarcane stalks are field-grown, this increases the chance of disease transmission, and the excision of meristem from such explants requires skilled personnel and care. To overcome this limitation, Ramgareeb et al. (2010) used single-budded setts grown in the greenhouse as a source of meristems, and reported that this process takes 6 - 8 weeks to produce shoots from which the meristem could be excised (Ramgareeb et al., 2010).

In the present study, attempts were made to reduce the period it takes to obtain shoots from single-budded setts using the method of Ramgareeb et al. (2010), by germinating the setts *in vitro*. Comparisons of total shoot yield from the two sources of meristematic tissue explant, i.e. from stalk germinated in the field and shoots of *in vitro*-germinated sett, revealed that the stalk was the best source to use to obtain meristematic tissue explant (Table 5). This was because the meristems obtained from the shoots of *in vitro*-germinated setts produced less shoots as a result of endogenous contamination that became apparent once transferred to shoot multiplication medium (Table 5).

As this present study demonstrated that the length of time for sett germination was reduced from 6 - 8 weeks to 10 days under *in vitro* conditions, this approach seems to have potential to reduce the required time for sett germination. However, further work needs to be done to eradicate the persistent endogenous contamination.

5.2 Establishment of a protocol to screen for drought stress tolerance in sugarcane varieties *in vitro*.

The exploitation of effective approaches for selection of traits of interest is significant in crop breeding programs (Lebeda and Svabova, 2010). As previously mentioned, the selection of such traits is conventionally performed under field conditions, which is labour-intensive and time-consuming. If *in vitro* selection techniques could be employed in trait selection, this would have the advantage of rapid screening and selection of numerous clones of different varieties in a limited space, void of interfering environmental factors. In sugarcane, presuming there is a correlation between cellular and *in vivo* whole plant responses, *in vitro* culture can be employed to select drought tolerant cultivars (Mohamed et al., 2000). *In vitro* selection for cells with increased tolerance to drought stress has been reported in rice (Biswas et al., 2002) by assessing seed germination, % callus induction, callus health and % embryogenic callus production upon culture on 5, 10 and 15 g l^{-1} PEG-6000. Also, working with rice, Cha-um et al. (2010) used mannitol to investigate its effect on proline content and chlorophyll *a* fluorescence. Albiski et al. (2012) exposed *in vitro* potato plants to assess the sorbitol tolerance and measured parameters such as root length, leaf area, dry weights and stem thickness. In sugarcane, Errabii et al. (2006) assessed mannitol tolerance of calli and used proline accumulation, relative growth rate and callus water content as selection criteria. Cha-um and Kirdmanee (2009) also investigated drought tolerance in sugarcane through studying the effect of mannitol on photosynthetic rate, proline content and chlorophyll *a* fluorescence. In the above-mentioned studies, the authors suggest that these approaches could be applied in identification of crops with improved stress tolerance. However, it is worth noting some of the possible drawbacks associated with such *in vitro* selection, *viz*. loss of regeneration power during selection, epigenetic adaptation, and the lack of affiliation between the mechanisms of tolerance observed in culture and those of the whole plant (Tal, 1994; Rai et al., 2011).

The use of lethal doses of a selecting agent in screening for abiotic stress tolerance has been demonstrated by, amongst others, Koch et al. (2012), who screened and selected for herbicide imazapyr tolerance in embryogenic sugarcane calli cultured on plantlet regeneration medium supplemented with 0.042 (LD_{50}) and 0.08 (LD_{90}) µM imazapyr. In the present study, such an approach allowed for discrimination of different varieties with respect to their responses to mannitol stress (Figure 10). For example, For NCo376 and N19,

mannitol at 332 mM resulted in 50 – 58% plantlet root re-growth, which was significantly higher than that at 606 and 851 mM mannitol. For N41 and N26, mannitol at 851 mM resulted in significantly lower % plantlet root re-growth than at 332 and 606 mM.

In the present study, one of the objectives was to determine which growth parameter to use to discriminate *in vitro* responses to osmotic stress among sugarcane varieties through induced mannitol stress (204, 326, 448 and 569 mM). For this investigation, NCo376 and N41 plantlets with trimmed shoots, trimmed roots or both were used. The delay (by 2 days) of both root (at 36, 448 and 569 mM for NCo376 and 448 and 569 for N41) and shoot (326, 448 and 569 mM for NCo376 and N41) re-growth when cultured on mannitol indicated that mannitol stress had a negative effect on plantlet re-growth at those concentrations. In both growth responses, NCo376 was affected first, suggesting it may be more sensitive to mannitol stress (326, 448 and 569 mM) than N41. Significant differences in both varieties were observed in root rather than shoot re-growth across mannitol treatments (332, 448 and 569 mM) over time (Figure 6 and 7), indicating that the former was more responsive than the latter to mannitol stress at those concentrations. Hence, plantlet root re-growth was deemed the better indicator to screen and discriminate mannitol stress tolerance and/sensitivity amongst sugarcane varieties. Similar results were obtained by Govindaraj et al. (2010) who reported that root length was more affected in PEG-induced drought conditions than shoot length of pearl millet genotypes.

The level of mannitol stress tolerance was defined by the mannitol concentration required to inhibit NCo376 and N41 plantlets' root re-growth by 50 and 90% (Figure 8) using a linear regression analysis. The decrease in plantlet root re-growth observed with a gradual increase in mannitol concentration revealed that the LD_{50} and LD_{90} mannitol were 332 and 606 mM for NCo376, and 851 and 1493 mM for N41, respectively. These values were used to screen varieties N12, N19, N26 and N36 for mannitol stress tolerance/sensitivity using plantlet root re-growth as an indicator of the stress. In conclusion, the tested varieties had different % plantlet root re-growth (Figure 10), indicating that sugarcane varieties responded differently to the same mannitol stress conditions in culture. Since continuation of root growth in sugarcane under drought conditions is regarded as an adaptive mechanism that facilitates water uptake by the roots (Patade et al., 2011), the tested varieties exposed to mannitol at 332, 606 and 851 mM which exhibited high % plantlet root re-growth can be considered as tolerant to mannitol stress and able to thrive under such *in vitro* conditions.

Under *in vitro* conditions, drought stress has been induced through the use of mannitol (Ochatt and Power, 1989; Gangopadhyay et al., 1997; Samantaray et al., 1999; Sabba et al., 1990; Mohamed et al., 2000; Errabii et al., 2006) and PEG (Smith et al., 1985; Fallon and Phillips, 1989; Santos-Diaz and Ochoa-Alejo, 1994; Purushotham et al., 1998). According to

those authors, using these compounds is advantageous since they are non-ionic, non-penetrating osmotica that lower the water potential of the medium without being phytotoxic or taken up by the plant cells. The main drawback with using PEG is that it reduces oxygen levels and diffusion to root systems (Mexal et al., 1975). PEG decreases stirring of the culture medium, thus reducing oxygen movement to the roots, which then become oxygen deficient (Mexal et al., 1975; Verslues et al., 1998). As reported earlier by Verslues et al. (1998), when PEG-containing solutions were supplied with bubbles rather than air, there was a decrease in root growth. However, the oxygen unavailability was not overcome by vigorous bubbling with air and gentle aeration was suggested (Verslues et al., 1998).

In the present study, the decreased % plantlet root re-growth in non-aerated cultures supplemented with PEG-6000 suggested a marked decline in oxygen supply to the plantlets, resulting in plant death. This could be a result of the pronounced viscosity of the PEG-containing culture medium. The significantly high % plantlet root re-growth in aerated PEG-6000-containing cultures indicated that the availability of oxygen to the root system was sufficient enough to facilitate root re-growth under such *in vitro* conditions. In contrast, even with aeration, Verslues et al. (1998) reported marked oxygen deficiency in maize seedlings cultured in PEG solutions. Those authors suggested that oxygen must be supplemented in such solutions in order to avoid hypoxia. Mannitol and PEG-6000 at the LD_{50} and LD_{90} concentrations resulted in similar plantlet root re-growth, suggesting that either one could be used as an osmotic agent *in vitro*. Therefore, in this study, mannitol was used.

When the tested varieties were exposed to mannitol stress (332, 606 and 851 mM), they responded differently with respect to % plantlet root re-growth (Figure 10). Varieties N41, N26 and N36 were observed to be tolerant to mannitol stress, as they demonstrated higher % plantlet root re-growth compared with varieties NCo376, N19 and N12, which were observed to be sensitive to mannitol stress, especially at 332 and 606 mM.

In addition to plantlet root re-growth, other assays were performed to investigate possible selection criteria for mannitol stress tolerance at 332, and 851 mM mannitol previously established as the LD_{50} and LD_{90} concentrations for NCo376, respectively. The assays undertaken were the estimation of leaf chlorophyll content, leaf electrolyte leakage measurements and leaf accumulation of H_2O_2 and $O_2^{-\bullet}$ in the *in vitro* leaves of varieties NCo376, N19, N26 and N41. A comparison between the results obtained from the plantlet root re-growth and the above-mentioned assays was done in order to assess the credibility of these stress biomarkers.

The membrane integrity of the four varieties was evaluated through leaf electrolyte leakage measurements after culture on 0, 332 and 851 mM mannitol for 5 and 10 days (Figure 11).

The higher the electrical conductivity of the cell sap, the greater the extent of membrane permeability (Patade et al., 2012), i.e. the high levels of electrolyte leakage obtained for NCo376 and N19 as compared with those for N41 and N26, suggested that these varieties were more sensitive to the mannitol stress than N41 and N26. For example, at 332 mM mannitol, a higher electrolyte leakage was observed at day 5 of mannitol stress, with 75% for NCO376 and 70% for N19, whilst N41 and N26 showed a significantly lower electrolyte leakage, with 40 and 41%, respectively (Figure 11). Thus, their membrane integrity was likely to have been impaired, since cellular membranes are usually the first targets of stresses such as drought (Levitt, 1972). Similar results were obtained by Martin et al. (1987), who stated that minimized leakage of membranes was correlated with drought tolerance. Similarly, Almeselmani et al. (2011) observed in durum wheat genotypes, that the membrane integrity of drought tolerant genotypes was conserved compared with susceptible ones. For all the tested varieties in the present study, mannitol at 0 and 851 mM resulted in similar electrolyte leakage for both days 5 and 10. This was probably because plantlets cultured in 851 mM mannitol showed prominent and similar senescence patterns at both tested days. Based on the obtained results for plantlet root re-growth (Figure 10), NCo376 and N19 were suggested to be more drought susceptible than N41 and N26. These results correspond with those of the electrolyte leakage assay.

Another of the assays tested for detection of mannitol stress was the estimation of chlorophyll content through SPAD meter measurements. Chlorophyll degradation is affiliated with the effect of water stress conditions in sugarcane (Silva et al., 2007; de Almeida Silva al., 2011) and in the present study this was observed when plantlets were grown on mannitol (332 and 851 mM) (Figure 12). However, varieties NCo376 and N19 showed greater decline in leaf chlorophyll than N41 and N26, suggesting their sensitivity to mannitol stress at those concentrations. These results relate well to the responses of the varieties to plantlet root re-growth and electrolyte leakage. Although there are presently no reports on SPAD meter measurements of *in vitro* grown sugarcane, field work by de Almeida Silva et al. (2011) on sugarcane cultivars exposed to well-watered and drought irrigation regimes revealed that drought conditions had a negative effect on the SPAD unit, with drought susceptible cultivars showing remarkably lower SPAD unit values than drought tolerant ones at both day 4 and 90 after the start of irrigation treatments. It must be pointed out, however, that it was difficult to perform SPAD meter measurements on *in vitro* plants because of the small size of the leaves make it a lengthy and labour-intensive process. Hence, this approach is not recommended.

The accumulation of H_2O_2 and $O_2^{-\bullet}$ in the leaflets of varieties NCo376, N19, N41 and N26 was histochemically detected (Figure 13). Mannitol at 851 mM did not provide a good

indication of stress tolerance and/sensitivity because the leaves had senesced. However, at 332 mM mannitol, the results revealed greater accumulation of these ROS in varieties NCo376 and N19 than in N41 and N26, suggesting that the latter possessed more efficient adaptive or protective mechanisms to cope with the mannitol stress (332 and 851 mM). Similar results were observed by Kumar et al. (2013), where H_2O_2 and $O_2^{-\bullet}$ increased in fifteen-day old *Brassica juncea* seedlings after a 3 day exposure to mannitol stress (200 mM) *in vitro*.

From the results of leaf chlorophyll content, electrolyte leakage, and H_2O_2 and $O_2^{-\bullet}$ accumulation discussed above, varieties NCo376 and N19 were observed to be more sensitive to mannitol stress at 332 mM, whilst N41 and N26 were less sensitive at the same concentration (Figure 11, 12 and 13). In conclusion, based on the results obtained for the plantlet root re-growth, it is proposed that the *in vitro* drought tolerance status of the tested varieties can be ranked, with N41 = N26 = N36 > N12 = N19 = NCo376, N41 = N26 > N36 = N12 > N19 = NCo376 and N41 = N26 = N36 = N12 = N19 = NCo376 at 332, 606 and 851 mM mannitol, respectively.

5.3 Concluding remarks

a) Best source for meristematic tissue explant and mass propagation of *in vitro* shoots.

In terms of yield, meristems (approx. 1.3 cm in length) isolated directly from the stalk apex were the best source of meristematic tissue explant for *in vitro* mass propagation of NCo376 shoots. However, meristems isolated from shoots of *in vitro*-germinated setts can be used provided that sett germination is done in sterile water supplemented with 1 ml l^{-1} BRAVO® in combination with 1.0 mg l^{-1} methylene blue. The lower yield (10 shoots/meristem) of the explant obtained from the shoot of *in vitro*-germinated sett compared with the 60 shoots/meristem of the explant from stalks can be counteracted by optimization of decontamination protocol focused in eradicating pathogen manifestation during both shoot induction and multiplication when meristems are isolated from the former source. This approach should be pursued because it allows for rapid germination of setts in a controlled environment compared with the lengthy process of germinating setts in the greenhouse.

b) Establishment of mannitol tolerance screening protocol

The present study encompassed initial investigations into obtaining a quick and simple method to screen and select for drought tolerance amongst and within varieties through the employment of *in vitro* selection pressures. The approaches used involved the determination of the best morphological parameter to determine mannitol stress tolerance, which was found to be root re-growth. The LD_{50} and LD_{90} levels of mannitol for *in vitro* root re-growth were determined for a variety deemed drought tolerant (N41) and another deemed drought sensitive (NCo376) based on field trials (Snyman. pers. comm.). The established *in vitro* LD_{50} and LD_{90} mannitol concentrations were then used to screen for tolerance/sensitivity to mannitol amongst other varieties (N12, N19, N26 and N36). Based on this, the suggested rankings were N41 = N26 = N36 > N12 = N19 = NCo376, N41 = N26 > N36 = N12 > N19 = NCo376 and N41 = N26 = N36 = N12 = N19 = NCo376 at 332, 606 and 851 mM mannitol, respectively.

The measurement of leaf chlorophyll content, leaf electrolyte leakage and ROS accumulation in the leaves were also determined at the same LD_{50} and LD_{90} levels of mannitol. Of the physiological parameters tested, leaf ROS accumulation and electrolyte leakage are the recommended assays, because they are relatively easy to perform on *in vitro* plants as opposed to the leaf estimation of chlorophyll content of *in vitro* plants. Rankings based on these physiological assays (N41 = N26 > NCo376 = N19) were found to be similar to those obtained with plantlet root re-growth (N41 = N26 = N36 > N12 = N19 = NCo376) when mannitol stress did not exceed 332 mM. Furthermore, the results of the present study correspond with field rankings of varieties N41 (drought tolerant) and NCo376 (drought susceptible), as per SASRI rankings (Snyman. pers. comm.).

In addition, the following recommendations should be considered when employing the screening approach described above:

1) Mannitol stress exceeding 606 mM may result in plant injury and consequently death, thus making screening for tolerance, particularly the measurement of leaf chlorophyll content, leakage of electrolytes as a result of membrane impairment and the accumulation of ROS such as H_2O_2 and $O_2^{-\bullet}$, beyond this level, difficult.
2) The length of mannitol stress exposure is critical during *in vitro* screening and selecting for tolerance, and this should be performed at no later than day 5 after subjecting plantlets to mannitol stress.

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