AN INVESTIGATION ON THE CAUSE OF RECALCITRANCE TO GENETIC TRANSFORMATION IN SOYBEAN, *GLYCINE MAX* (L.) MERRILL

by

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DECLARATION

I declare that **AN INVESTIGATION ON THE CAUSE OF RECALCITRANCE TO GENETIC TRANSFORMATION IN SOYBEAN, *GLYCINE MAX* (L.) MERRILL** (Thesis) hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy has not previously been submitted by me for a degree at this or any other university; that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

Phetole Mangena

Date

DEDICATION

This research endeavour is gratefully dedicated to my late aunt; mmane Ngaletšane Violet Mangena who has been a great source of strength, inspiration and support. Thank you for being supportive, especially by supporting the growth and development of those who needed my immediate care and attention, when I couldn't be there for them. Thank you so much! You are and will always be dearly loved and missed. Modimo a go tšhegofatše kao safeleng.

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RESEARCH OUTPUTS

PUBLICATIONS:

1. **Published Book Chapter:** Mangena, P., Mokwala, P.W., Nikolova, R.V., 2017. Challenges of *In Vitro* and *In Vivo Agrobacterium*-Mediated Genetic Transformation in Soybean. In Soybean: The Basis of Yield, Biomass and Productivity by Minobu Kasai. Intech Open Science, Croatia, pp. 75–94. http://dx.doi.org/10.5772/66708.
2. **Published Book Chapter:** Mangena, P. The Role of Genotype, Culture Medium and *Agrobacterium* on Soybean Plantlets Regeneration During Genetic Transformation. In Transgenic Crops by Muhammad Sarwar Khan. InTech Open Science, London. ISBN 978-953-51-6452-4. DOI: 10.5772/intechopen.78773
3. **MDPI Journal-Agriculture:** Mangena, P., Mokwala, P.W. The Influence of Seed Viability on the Germination and *In-Vitro* Multiple Shoot Regeneration in Soybean (*Glycine max* L.). Volume 9 (2), Page 1-12. DOI: 10.3390/agriculture9020035
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CONFERENCES:

1. **Conference Paper:** Mangena, P., Mokwala, P.W., Nikolova, R.V., 2017. Effect of seed storage on seed viability, adventitious shoot induction and plant regeneration from cotyledonary explants of various soybean cultivars (*Glycine max* L.). Conference title: Protecting biodiversity through sustainability. Annual conference of South African Association of Botanists. Cape Town, South Africa. South African Journal of Botany, 103: 348. January 2017 (**Appendix 6**).
2. **Conference Paper:** Mangena, P., Mokwala, P.W., Nikolova R.V., 2016. The effect of aminoglycoside antibiotics on the induction of adventitious shoots in soybean. Conference: Biodiversity: Past, Present and Future. Joint conference of the South African Association of Botanists (SAAB), Free State Province, Bloemfontein, South Africa. South African Journal of Botany, 103: 327. January 2016 (**Appendix 7**).

ABSTRACT

Genetic transformation offers great opportunities for rapidly introducing, selecting or inducing desired characteristics in various leguminous plants for breeding purposes. But, this technique remains aloof for soybean improvement due to challenges such as genotype specificity, inefficient regeneration protocols and the rapid loss of viability in seeds required to develop explants. However, the rate of seed deterioration and its influence on *in-vitro* plant genetic transformation differs according to the age, storage duration and moisture content of the seeds used. The moisture status of the seeds is usually high during harvesting and deterioration (loss of viability) starts to occur when seeds are stored under ambient conditions for long periods. This seed deterioration also results in a phenomenon called “recalcitrance”, which is predominantly realised in soybean. In the present study, selected soybean genotypes were analysed for: **(i)** the efficiency of germination using seeds stored for 0, 3, 6 and 9-months under ambient conditions **(ii)** the effect of seed storage on *in-vitro* multiple shoot induction, **(iii)** the competency of the selected soybean genotypes on callus induction and *Agrobacterium*-mediated genetic transformation and **(iv)** the evaluation of protein profiles of the genotypes following co-cultivation of cotyledonary node explants with *A. tumefaciens*. The results obtained in this study showed that, seed stored for more than 3-months had reduced rates of germination, seedling development and *in-vitro* shoot multiplication. In particular, seed stored for 9-months showed a significant drop in seed germination, and less than 50% overall seed germination (Dundee-42%, LS678- 49%, TGx140-2F-44% and TGx1835-10E-48%) except for LS677 and Peking with 52 and 55%, respectively. The efficiency of multiple shoot induction also decreased with the prolonged seed storage, with all genotypes recording overall decline from about 96% to 40% regeneration efficiency over this period. The mean number of induced shoots decreased from more than 10.5 to 4.2 shoots per explant, for each genotype. The results obtained clearly indicated that efficient *in-vitro* shoot induction depended largely on seed storage duration, viability and significantly differed according to genotype. Following the evaluation for callus induction and *Agrobacterium*-mediated genetic transformation frequencies, the results indicated that the responses were genotype specific. This trend was consecutively observed in all soybean cultivars used (LS677, LS678, Dundee, Peking, TGx1740-2F and TGx1835-10E). Furthermore, the responses of the genotypes were also dependent on the culture media composition,

especially, plant growth regulators and antibiotics. Amongst the cultivars used, Peking demonstrated the highest callus induction capacity (more than 70%) on MS-A and the mean number of shoots induced (1.65) using cotyledonary explants co-cultivated with *Agrobacterium*. This was followed by LS677 (1.42 shoots), LS678 (1.40 shoots), Dundee (1.30 shoots), TGx1835-10E (0.80 shoots) and TGx1740-2F (0.75 shoots), respectively. These genotypes also demonstrated low yields of proteins, extracted using a TCA buffer, and separated by means of two-dimensional polyacrylamide gel electrophoresis. The one-dimensional and two-dimensional profiles of proteins extracted from explants infected with *Agrobacterium* differed significantly to those expressed without co-cultivation of cotyledonary nodes with bacteria. These observations suggested that, the infection and co-cultivation of explants with *Agrobacterium* may have caused the expression of new proteins. Newly expressed proteins could also be found to either promote or inhibit transgene integration and expression on the cotyledonary node explants transformed with *Agrobacterium tumefaciens* for trait improvement. This study has clearly demonstrated that soybean production is confronted with a myriad of stress factors, including seed storage and quality problems due to unfavourable storage duration and weather conditions, amongst others. Thus, soybean seeds used for germination, callus induction, multiple shoot induction and genetic improvement should be harvested at R8 stage after reaching physiological maturity (with 20-35% seed moisture content) to avoid any mechanical damage, shattering or loss of seed viability.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
6-BA	benzylaminopurine
B5	Gamborg's B5 medium
CHAPS	Cholamidopropyl-dimethylammonio-1-1propanesulfonate
CO_2	Carbon dioxide
˚C	Degree Celsius
2,4-D	2,4-Dichlorophenoxyaceic acid
DMA	Dimethylacrylamide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
F_0	Parental generation
F_1	First filial generation
GUS	β-Glucuronidase
HCl	Hydrochloric acid
IBM-SPSS	International Business Machine- Statistical Package for the Social Sciences
IEF	Isoelectric focussing
IBA	indole-3-butyric acid
GA_3	Gibberellic acid
g	Gram
KIN	Kinetin
kDa	Kilo Daltons
SDS	Sodium dodecyl sulfate
L	Litre
LS	Link Seed
µM	Micromolar
MES	Methylene-ethylene sulphate
mg	Milligrams
mm	Millimetres
ml	Millilitres
m^{-2}	Per square metres
mol	Mole

MS	Murashige and Skoog
1N	1 Normal
NaOH	Sodium hydroxide
NAA	α-Naphthalene acetic acid
OP	Optical density
Oc-1	*Oryza cystatin-1*
Rpm	Revolution per minute
%	Percent
P	Probability value
PDC	Programmed cell death
PGE	Percentage germination and emergence
PG wells	Polyacrylamide gel wells
PGRs	Plant growth regulators
PI	Proteinase inhibitor
POD	Pod number plant^{-1}
R	Reproductive stage
s^{-1}	Per second
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEI	Seedling establishment index
T-DNA	Transfer-Deoxyribonucleic acid
TEMED	N,N,N,N-tetramethylethane-1,2-diamine
TGx	Tropical *Glycine* cross
V	Vegetative stage
YEP	Yeast extract peptone
ZOOM-IPG	Zooming immobilised pH gradient

CHAPTER 1

PROBLEM STATEMENT AND GENERAL INTRODUCTION

1.1. PROBLEM STATEMENT

The modification of soybean through genetic engineering techniques such as; *Agrobacterium tumefacien*s-mediated transformation or particle bombardment has provided varieties with newly improved growth and yield characteristics. These techniques allow for precise and controlled addition of genes to the genomes of targeted plant species. Such genetically modified plants have new traits including, fungal resistance, nematode resistance, insect-pest resistance and improved tolerance to the adverse environmental conditions. Amongst some of the abiotic environmental stresses, drought is considered the most limiting factors in soybean growth and productivity.

However, genetic transformation in soybean still occurs at very low rates and most of the successfully applied protocols are genotype specific. This means that, a protocol successfully used to achieve transformation on one variety cannot be equally applied across all genotypes. This phenomenon is referred to as “recalcitrance” to genetic manipulation (Benson, 2000). Although significant progress has been made in the past for genetic transformation of specific genotypes of a few grain legumes and forage crops (as indicated by Anwar et al., 2010), recalcitrance remains a major obstacle for the improvement of many important crops, particularly in soybean. There is no adequate information that thoroughly explains why genetic transformation in soybean remains very inefficient, genotype specific and an inconsistent process.

The relationship between soybean recalcitrance to transformation, as well as to other growth influencing factors, such as; seed viability and seedling vigour is also not known. Seed viability refers to the ability of seeds to germinate into normal healthy seedlings, while seedling vigour refers to the production of viable seedlings which are amenable for regeneration under suitable conditions. These factors may also cause negative effects on soybean amenability to *in vitro* shoot multiplication and regeneration, as well as *Agrobacterium*-mediated genetic transformation.

Furthermore, it is not clear whether the infection of explants with *Agrobacterium* may cause changes in the protein profiles of soybean, which may ultimately influence the transformation process. These problems continue to limit transformation and have been ineffectively combatted through screening of amenable genotypes for transformation, as well as the optimisation via modification of tissue culture conditions and genetic engineering protocols. Finally, there is evidence showing that these challenges faced during genetic transformation of soybean and other crops, encourages the criticisms and scepticism around the use of genetically modified plants for medicinal and nutritional purposes.

1.2. GENERAL INTRODUCTION

Although the use of *in-vitro* tissue culture-based *Agrobacterium*-mediated genetic transformation has made notable progress in the genetic improvement of soybeans, its application for routine transformation still requires optimisation. According to Paz et al. (2006), *in-vitro* transformation protocols still require an efficient system for production of stable transgenic lines. This study investigated factors that cause the difficulty in the regeneration and production of genetically modified plants, focussing on soybeans.

1.2.1. The soybean

Soybean (*Glycine max* L.) is an annual seed leguminous crop plant that belongs to the family Fabaceae. It forms part of the order Rosales which includes crops and some ornamental plants (Mangena, 2015). This family is one of the largest families in the Angiosperms with over 600 genera and 12000 species, and is characterised by trifoliate leaves, dehiscent fruits, split valves and the plants are of determinate/ indeterminate growth forms (Taiz and Zeiger, 2002; Chavarria et al., 2017). Soybean constitutes a large number of varieties, in which many are bred and developed for both subsistent and commercial cultivation (Chavarria et al., 2017). They can produce over 50 fruit pods per plant with at least three (3) seeds per pod. It is currently considered to be the most important grain legume in Africa, particularly in the north-eastern regions (Adeyeye et al., 2014). Soybeans usually take about 90 days to mature, depending on the type of variety. However, early maturing cultivars could take less than 90 days (~75 days) while late maturing varieties can take up to 150 days to reach maturity stages (Shurtleff and Aoyagi, 2009).

The consumption of soybean as foods and feeds by humans or poultry is known for the benefit of regulating the rate and efficiency of animal growth. This increased use of soybean products necessitated the establishment of basic standard specifications. So far, it has been indicated that the protein dispensability index is the best tool to measure protein quality, with a range of 40 to 45% denoting acceptable quality (Dozier and Hess, 2011). According to the National Oilseed Processor Association, high quality soybean-based foods are generally preferred, and more standards had to be

made available to assist consumers and producers in the selection of the best soy products based on various physical and chemical properties (NOPA, 1999). Soybean is also high in essential amino acids and enzyme concentrates. Its processing has led to the production of high quality protein food ingredients formed by concentrated and isolated soy proteins with functional and nutritional applications such as bread, dietary supplements and infant formulas (Amanlou et al., 2012; Gandhi, 2009).

1.2.2. Status of soybean cultivation in South Africa

Globally, soybean is becoming the number one crop plant species widely cultivated with a world production of over 251.5 million metric tons (mt) projected for the 2017/18 production season (USDA, 2018). According to USDA (2018) the main soybean producing countries are the United States (117.21 mt), Brazil (114 mt), Argentina (57.8 mt), China (12.9 mt) and India (11.5 mt). South Africa's soybean yield is not as high as those mentioned above. Although, in South Africa, including other African countries, the yield projection for 2017/2018 were expected to decrease, following the drought caused by the El Niño-related conditions. The production levels ranging between 10000 to 225000 were recorded in South Africa from areas such as; Mpumalanga, Free State, Kwa-Zulu Natal, Limpopo, North West and Gauteng (DAFF, 2010). According to the Grain-SA 2016 report, overall soybean production in South Africa increased to 1 million tons in the 2015/2016 marketing season. This was due to the import substitution strategy aimed at investing towards soybean crushing capacity. The country is the largest importer of soybean oilcake in Sub-Saharan Africa, accounting for an average of 72% of soybean import demand in the region. Therefore, domestic soybean production were stimulated by the drive towards increasing the crushing capacity (Sihlobo and Kapuya, 2016). Despite environmental cues being a cause to limited soybean productions in South Africa, lower seed yields obtained are also due to the unavailability of improved stress resistant cultivars which are fully adapted to the African climate. This is so, because many soybean varieties are narrowly adapted to the African climate, and those genotypes always exhibit different rates of growth and productivity. In South Africa soybean production is confronted with drought stress, seed storage generated infections and seed quality problems due to unfavourable weather conditions.

1.2.3. *In-vitro* transformation of soybean

The goal of *in-vitro* plant tissue culture systems is to obtain many healthy and improved plants in a short period of time and at minimal costs. Although this is not easy to achieve, many protocols have been elaborated for the regeneration of widely adapted crops, such as cotton, maize, rice, canola, wheat and sorghum through this technique. Such protocols were reported by Pratap and Kumar (2011) as the key objects of genetic transformation that are used to initiate cultures suitable to facilitate alternative methods of transformation, like *in-vitro* mutagenesis or polyploidisation. All these techniques have played an active role in developing abiotic or biotic stress tolerant plants (Atif et al., 2013). Transgenic crops, including Roundup-Ready maize and soybean cultivars with resistance to active ingredient glyphosate of Roundup herbicide were developed. While genetic transformation has considerably expanded our understanding in modern genetics, it continues to generate more interesting knowledge. It is only now that most researchers fully appreciate the complexity and difficulties involved in this technique. This study attempts to make a significant contribution by providing new evidence that might reveal the critical factors that cause recalcitrance to genetic transformation in soybean. The information reported in this study explores reasons behind the unresponsiveness of different cultivars in plant tissue culture conditions for *in-vitro* regeneration, inefficient co-cultivation of cotyledonary explants with *Agrobacterium*, integration and expression of foreign DNA segments of interest via *Agrobacterium* based transformation. This study provides further information as to why *Agrobacterium*-mediated genetic transformation in soybean continue to be inefficient.

1.3. STUDY HYPOTHESES

The hypotheses in this study are outlined as follows:

i. Germination of different soybean seeds is negatively affected by the period of seed storage irrespective of the genotype.
ii. Soybean seeds stored under ambient conditions will deteriorates and this impacts negatively on germinability and *in vitro* regeneration of plantlets using explants derived from the germinated seeds.
iii. Soybean double cotyledonary node explants grown in MS basal culture medium without 0.5–4.0 mgL^{-1} of auxin-cytokinin in combination will not establish a successful callus culture.
iv. We hypothesise that, comparative analysis of different soybean genotypes can generate insights for the improvement of *in-vitro* based genetic transformation using *Agrobacterium tumefaciens* harbouring the pTF101.1 vector, with a *bar* gene conferring tolerance to glufosinate ammonium.
v. The infection of soybean explants with *Agrobacterium tumefaciens* during genetic transformation may lead to changes in the protein profiles of the infected tissues, which may promote or inhibit the frequency of transformation.

1.4. AIM AND OBJECTIVES OF THE STUDY

1.4.1. Aim:

The aim of the study was to investigate the cause of soybean recalcitrance to genetic transformation using the *in-vitro* plant tissue culture and proteomic analysis in several soybean genotypes.

1.4.2. Objectives of the study

The objectives of the study were to:

i. Determine the correlation between seed moisture content, seed viability and germination frequency of the soybean seeds stored under ambient conditions.
ii. Evaluate the effect of seed viability on *in-vitro* multiple shoot induction for soybean regeneration.
iii. Assess the competency of all genotypes for callus induction and *Agrobacterium*-mediated genetic transformation.
iv. Analyse changes in protein expression in the cotyledonary-node explants infected and co-cultured with *A. tumefaciens* carrying the pTF101.1 vector construct.
v. Identify key proteins associated with loss of genetic transformation frequency using a 2D gel electrophoretic method.

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CHAPTER 2

EFFECT OF SEED STORAGE DURATION ON SEED MOISTURE CONTENT, VIABILITY, GERMINATION AND SEEDLING DEVELOPMENT IN SOYBEAN

2.1. INTRODUCTION

2.1.1. Background to the problem

In order to increase production of soybean and greatly reap benefit from the high amount of proteins and oils stored in the seeds, new improved cultivars must be developed. However, the loss of viability and vigour by the seeds used as both harvest and propagules has major negative effects on growth and yields. This phenomenon called seed viability is displayed by most of the soybean genotypes. They fail to retain viability even during seed development (Shelar et al., 2008) and others start losing viability immediately after harvesting (Kandil et al., 2013). This shows that, the growth and yield production potential of this crop largely depends upon the quality of seeds the plant produces. Studies in the higher leguminous plants and grasses such as; *Cassia fistula*, *Leucaena leucocephala, Zea mays saccharata var. rugose*, have demonstrated the importance of good seed quality (Zhao et al., 2007; Msuya and Stefano, 2010; Okusanya et al., 2015; Valente et al., 2017).

The authors have emphasised how seed quality directly influences successful plant growth and its contribution on productivity levels. Similar observations were made by Afrakhteh et al. (2013) during the evaluation of growth and seedling characteristics of soybean cultivar Katool (JK) and Sari (DPX). Their findings also showed that the decrease in growth characteristics and seedling vigour are directly related to conditions that may result in decreased seed moisture content. Like many other pulses, soybean continues to be a recalcitrant crop, showing desiccation sensitivity which in most cases results in rapid loss of viability. Roos (1989) reported the increase in seed longevity by adjustment of seed moisture and temperature. By providing a stable relative humidity and maintaining the seeds under 30°C, he demonstrated a positive logarithmic relation between seed viability and storage conditions. Trigiano and Gray (2005) and Walters (1998) explained that, optimum seed moisture content

for obtaining the greatest viability also depends upon storage materials used. Seeds can be well stored in paper bags or tin containers under ambient conditions and later tested for viability using standard germination test. This test is being widely and reliably used by every seed testing laboratory to check the viability of the seeds. However, the challenge remains the significant variation observed by different laboratories when testing similar seed lots, as stated by Hampton (1999). These variations were reported by Pheneendranath (1980) and reviewed by Hampton (1999) for various soybean varieties. Multiple successes using the technique in soybean demonstrating high efficiency in determining seed viability and replicable results have been reported by Sheidaei et al. (2014), Usha and Dadlani (2015) and Bahry et al. (2017).

2.1.2. Motivation of the study

The aim of the study is to build a scientific basis on the correlation between soybean seed viability and storage of seeds under ambient conditions. The findings of this study provide insights regarding the effects that percentage seed moisture content has on soybean seed viability, germinability and seedling vigour. Unfortunately, the continuing deterioration observed in soybean seeds does not only affect growth and yields but, also hinders the improvement of the crop via modern techniques such as genetic engineering. The findings reported may lay a foundation in exploring the effect of vigour and seedling viability on genetic improvement via *Agrobacterium tumefaciens*-mediated genetic transformation.

The most widely and routinely used transformation system couples the use of cotyledonary node (coty-nodes) explants and *Agrobacterium tumefaciens*. The coty-nodes used as explants must be obtained from seedling sources developed from the germinated seeds. This system is however, rendered inefficient in soybean due to the lower frequencies of transformation so far recorded. Higher seed viability and seedling vigour appears to be a requisite for the successful optimisation of protocols used for genetic manipulation in this crop. Findings made in this study will therefore, assist in answering questions such as how to design a better protocol that can be used efficiently in genetic manipulation of soybean via plant tissue culture.

2.2. LITERATURE REVIEW

2.2.1. Seed germination process

Seed germination is defined as the protrusion of the seed radicle. In agronomic terms, germination is considered to have occurred once the primary root of the embryo has emerged from the cracking of the seed coat (AOSA, 2005). Seed germination was described by Hartmann et al. (2011) as the activation of metabolic machinery that leads to seedling emergence. The above definitions indicate the control of seed germination by several biochemical and physiological factors. These are regulations in the activation of enzymes, expression of plant hormones, cell division and cell differentiation etc. These factors have showed in many cases to be necessary for the further development of the embryo into a seedling. According to Bewley (1997) and Hartmann et al. (2011) germination does not only refer to the uptake of water by quiescent dry seeds and terminate with the elongation of the embryonic axes. But, it refers to the physiological process which ultimately provides starch reserves, fibre, oils and proteins. People depend on seed germination for almost all of their utilisation as supported by Bewley and Black (1994). Cultivation of crop species for above mentioned production relies primarily on successful seed germination.

2.2.1.1. Germination requirements

For germination to be achieved seeds must be viable, be of good quality and their physical structure free of any mechanical damage. In addition, the appropriate environmental conditions that include water, oxygen supply, proper temperature, and at times light must be met for successful germination (ISTA, 2003). For germination to be initiated, four (4) conditions namely; water, temperature, light and oxygen must be met, as described below.

i. Water availability

The uptake of water by seed parts, involves absorption through cell wall and protoplasmic macromolecules such as proteins and polysaccharides (Woodstock, 1988). This process is called imbibition, and if delayed it can lead to the suspension

and decrease in germination, especially in a number of species belonging to the Papilionaceae, Brassicaceae and Poaceae as indicated by Wierzbicka and Obidzinska (1998). In soybean and corn respectively, limited seed imbibition was reported by Helms et al. (1997) to reduce seedling emergence to 22 and 85%. This may reflect the different rates of water uptake by seeds or that more time is needed to break seed coat to allow imbibition. Different species follow different patterns for seed imbibition especially, when soil water potential is to be optimally sufficient for seed imbibition but, too low for radicle emergence from the testa.

ii. Proper temperature

Temperature is the most important environmental factor regulating the timing of germination (Edelstein et al., 1995). This factor affects both germination percentage and germination speed (Edwards, 1932). However, there is classification into temperature points namely; minimum, optimum and maximum temperature whereby preference varied according to species. The different species prefer different temperatures. Leguminous plants such as soybean germinate and grow well under a temperature range of 25–30°C. Temperatures that fall below minimum or above maximum to any species can cause injury to emerging seedlings or induce secondary dormancy on the seeds of that particular species (Finkelstein et al., 2008).

iii. Sufficient light

Light can be inhibitory to seed germination in some leguminous plant species (Smykal et al., 2014). In species like cowpea [*Vigna unguiculata* (L.) Walp.], germination is highly reduced by light (Motsa et al., 2015). Recent reports have demonstrated that light may act for both dormancy induction and help to break the dormancy to initiate germination. Hartmann et al. (2011) indicated that plants such as *Saintpaulia*, *Kalanchoe* and *Calceolaria* greatly depend on light for seed germination and seedling development. However, germination of *Allium cepa* and *Amaranthus cruentus* L. have showed to be inhibited by light and the effects are severe when the light is coupled with ethylene presence (Matilla, 2000). Amini et al. (2016) also reported the negative effect of light on germination in seeds of *Lepidium vesicarium*. Seeds that show sensitivity to light are usually smaller in size and their germination is improved by

sowing them too deep in the soil (Gutierrez et al., 2010). Plant species such as *Viscum album* and *Ficus aurea* presented a contrasting higher requirement for light. The seed of these species lost viability after a few weeks without light (Mishra, 2009). Among the seeds that require light, some are extremely sensitive while others require relatively high levels to trigger germination (Khan and Gulzar, 2002). In soybean, no literature seems to suggest that light has negative effects on poor performance in seed germination and seedling development.

iv. Adequate aeration

Exchange of gases between the germination substrate and the developing embryo was reported to be highly essential by Kaymakanova (2009). The aeration process or oxygen (O_2) uptake induces rapid germination that is uniform. The O_2 uptake by seeds was reported by Burris (1992) to be a good indicator of the germination rate. This follows the indication that seed respiration is directly proportional to the amount of metabolic activities carried out by the seeds during germination and it is therefore, a measure of seed vigour. Aeration has been observed to be limited by water logging during imbibition or imbibitional injury. Poor aeration has also been reported to decrease germination of many seeds, including seeds of soybean (Woodstock, 1988).

2.2.1.2. Resumption of seed metabolism

Seed germination is the resumption of growth of the embryonic plant inside the seed. The process requires a short duration of rehydration after which the seeds rapidly resume metabolic activities. Crowe and Crowe (1992) reported that, the structures, enzymes and respiratory processes necessary for the resumption of metabolic functioning of the embryo are reactivated during this period. During activation, seeds use proteins such as globulins and prolamins stored in membranes to generate and activate carboxypeptidases, aminopeptidases and proteinases to metabolically prepare for germination (Tiedemann et al., 2000). Gallardo et al. (2001) correlated the successful establishment of seedlings in *Arabidopsis* to proteins induction before radicle emergence and protrusion. In addition to enzymes, Miransari and Smith (2014) also indicated that other metabolites such as growth hormones produced by plants (for example; gibberellic acid and indole acetic acid) regulate seed germination. These

plant hormones regulate seed germination by controlling dormancy and the expression of genes which are only activated in their presence. The packaging of an embryo and the supply of nutrient reserves, hormones as well as the enzymatic machinery make seeds effective propagules for growth and reproduction (Finkelstein, 2004).

2.2.2. Seed germination tests

Examinations for seed germination are normally conducted to assess and predict the possible performance of the seeds in the field. Tests such as tetrazolium staining, standard germination test and volatile aldehyde test are used to discover whether the seeds do not germinate at all or uniformly or assess whether special environmental conditions are required in order for seed germination to occur (Aquila, 2008). These tests take into account the protrusion of the root radicle as a morphological marker for germination. According to the International Rules for Seed Testing (ISTA, 2003), seed tests do not only provide information about the germinability of the seeds to designated crop growers, but they can be applied for the evaluation of the quality of seeds as food or for research purposes. Furthermore, testing requires test methods that have been tested to achieve rapid, reliable and reproducible results. These sentiments have also been echoed by Hampton (1999), Mavi and Demir (2007) and Aquila (2008).

2.2.2.1. The standard germination test

The standard germination test remains the most widely used standard for evaluation of field emergence potential universally. This test is designed to measure the potential germination of the seeds. It is used to determine the maximum number of seeds which could germinate under optimum conditions. This method uses standardised ideal conditions in a laboratory, like the ISTA standards, to assess viability and germinative potential of seeds usually on wet paper towels. The identification of seed viability has been used as a component of seed physiology and standard germination to assess seed quality as first proposed by Franck in 1950. Although this test consistently indicated failure to obtain substantive results on seed deterioration, the standard test has succeeded in generating insights on the viability of the seeds (McDonald and Pheneendranath, 1978; Phaneendranath, 1980). Assessment of seed viability and seedling vigour in combination with the germination test can generate an accurate

estimate of seed longevity. Tests such as tetrazolium test, electrical conductivity, accelerated aging test and stress integrated germination test are reserved for seed viability examinations (Aquila, 2008). These vigour tests are continuously and constantly being evaluated for their efficacy. Studies by Kolasinska et al. (2000), Olasoji et al. (2011) and others have managed to distinguish seed viability levels via standard germination combined with other rapid seed vigour tests. Masuthi et al. (2015) also reported a success on seed germination from cluster bean seeds following different priming conditions. Seed germination was reported to have reached over 90.5% on average for the treated seeds and 83% in the control. The treated and non-treated seeds produced over 80 percent germination in the standard seed tests and field performance trials, demonstrating the efficiency of the standard germination test.

2.2.3. Soybean growth and development

2.2.3.1. Seed composition

Soybeans (*Glycine max* L. Merr.) are one of the most important sources of high quality plant proteins and oils worldwide. The high proportion of proteins (40%), lipids (20%), and water-soluble carbohydrates (10%) deposited in the endosperm play a crucial role in human and animal welfare (Bellalloui et al., 2010). In past years much attention has been paid to the fermented and unfermented soya products. Products such as soymilk, tofu and soy nuts which are high in proteins, lower in oil content and high in carbohydrates are being manufactured (Ghandi, 2009). Nutritionally, soybeans are generally low on sulphur containing amino acids (cysteine and methionine) but have relatively higher contents of lysine (Banaszkiewicz, 2011).

Nutritional composition of soybean products is affected by the amount of anti-nutritional factors like phytic acid, allergens and mycotoxins found in the processed products including soy flour, sauces and fermented products. In addition to the anti-nutritional factors; the variety or genotypes, extraction and processing methods and residual hulls present are some of the causes of decreased palatability in soybean foods (Banaszkiewicz, 2011). Ma et al. (2014) quantitatively analysed the nutritional protein profiles in different soybean products by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and did enzyme-linked

immunosorbent assay (ELISA) of the protein glycinin. The observations indicated that, genotype and environmental conditions have a significant effect on soybean composition. But, it still remains one of the most valuable and prominent oilseed crops in the world.

2.2.3.2. Soybean production in South Africa

Vulnerability of soybean growth to adverse environmental conditions negatively affects soybean production in many countries. In South Africa, like in the rest of the countries in the sub-Saharan region, soybean subsistence and commercial productions are inadequate. This is according to the South African National Seed Organisation's annual report (SANSOR, 2014). There are efforts to increase cultivation and production of soybean underway in South Africa. The progress so far achieved has been attributed to the use of biotechnologically improved genotypes and agricultural policies that now favour the introduction of genetically modified cultivars for stress tolerance (Aerni, 2005).

Although, there is continued expansion of soybean production nearly in all provinces of South Africa, there is still significant improvement required to reach internationally acceptable yields. In the case of Africa in particular, low yields are directly as a result of cultivation of low yielding varieties and the growth conditions. In addition, problems in infrastructural development and the unfamiliarity with the crop as discussed by Dlamini et al. (2014) exacerbate the slowly increasing yields problem.

2.2.3.3. The effect of soybean seed viability on yield

Loss of viability of sown or stored seeds is one of the major challenges that soybean producers face. Deterioration, which is loss of seed quality, occurs due to weathering, harvesting and storage conditions of the seeds. A number of factors play a role in causing seed deterioration during harvesting, storage and processing of soybean seeds as well. These processes negatively affects seed viability which has a greater impact on the level of germinability and seedling establishment. Afrakhteh et al. (2013) highlighted factors such as genotype, seed health, physical conditions of seed, and storage conditions as some of the causative agents of seed deterioration. The physical

conditions refer to the mechanical damage and fissures or tears that occur in the seed coat. The fissures allow for easy internal infestations by microorganisms. Seed viability directly influences the success of the crop and ultimately its yields.

2.2.3.3.1. Factors affecting soybean seed viability

According to Jepleting (2015), to achieve maximal seed germination and seedling vigour of a given cultivar effort must be made on controlling factors causing deterioration. Otherwise, the environmental conditions that seeds are subjected to before and after harvest will impact negatively on the seeds remaining viability for germination and seedling emergence. Some of those factors affecting seed viability are:

i. The genotype

Different genetic constitutions of cultivars have been known to affect seed viability and longevity. The negative effects of the genotypes are therefore, extended to germination, seedling development and subsequent growth of the plant under favourable conditions. Bilyeu et al. (2010) stated that the importance of seed genotype is often ignored compared to the environmental factors. Even though, better genotypes have been exploited for the selection and development of new superior varieties, Balesevic-Tubic et al. (2010) indicated that, some changes leading to loss of seed viability differ according to genotypes. These changes can include mutations or genetic variations are dependent on both seed and plant physiology and the expression of pre-determined genes which also differ according to genotype.

The genotypes possess genes that are responsible for the development of different seed sizes, variation in seed coats, seed colour and other characteristics of which seed quality and vigour rely upon (Jepleting, 2011). Poor seed vigour due to weak genotype has been reported by Qiu and Mosjidis (1993) in *Lespedeza cuneate* forage legume crop plant. Substantial variability was observed among the 54 genotypes used and poor seedling vigour caused failure in plant growth. Reduced seed vigour have also been reflected in many crop species such as maize (Tekrony and Hunter, 1994), lentils (Hojjat, 2011), and rice (Adebisi et al., 2004). High seed viability and effective

seedling establishment can contribute to successful plant growth and development. Some genotypes outperformed other genotypes for most seedling traits under field and growth chamber conditions (Qiu and Mosjidis, 1993). This shows that any seedling trait observed can be used to measure seed viability and seedling vigour according to genotype. This should of course, be accompanied by some of the vigour tests such as standard germination tests in general or the more specific techniques like the tetrazolium test for viability.

ii. Environmental factors

A good understanding of how soybeans grow and function under different environmental conditions will refine how improvement protocols are used to achieve high yields. Environmental constraints that include drought, chilling and pests limit agricultural production by limiting crops from growing up to their fullest potential (Ortiz, 1998). This problem can be resolved by the full-use and adoption of biotechnology, particularly the use of cultivars that show tolerance to these environmental factors. Abiotic (microorganisms etc.) and biotic (drought, salinity etc.) stress factors can cause excessive damage to the physiological and biochemical processes, thus negatively affecting growth and development of plants. Genetic engineering allows for the development of procedures which can be used to develop new varieties that confer resistance or have tolerance to the biotic or abiotic stress factors. Jewell et al. (2010) provided a review on the mechanisms that are targeted for manipulation to advance drought or chilling stress resistant crops. Ren et al. (2016) reported the use of *GmST1* transgene exhibiting strong tolerance to salt stress and increased sensitivity to abscisic acid (ABA) and decreased production of reactive oxygen species under stress in *Arabidopsis*. Similar reports have been documented for soybean, chickpea and peanuts to enhance abiotic stress tolerance (Reddy et al., 2012).

2.2.4. Effect of seed viability and moisture content on genetic improvement

Although modern technologies in breeding systems have allowed for the introduction of high yielding varieties, soybean production is still negligible due to lack of efficient genetic improvement systems (Paz et al., 2004). Good selection of soybean genotypes, particularly in the African region, that provide excellent sources for high

efficiency in and amenability to genetic improvement via *in vivo* or *in vitro* techniques is still needed. Currently, rapid loss of seed viability and genotype specificity still negatively affect genetic transformation of soybean. There is no reliable, reproducible and efficient *in vitro* or *in vivo* protocol that guarantees good results (Wang and Xu, 2008; Zia et al., 2011). Generally, loss of viability in soybean seeds may occur during harvest or storage and have a great impact on transformation. Loss of seed viability cause germination to decline slowly at first and then rapidly as seeds start to lose viability due to loss of seed moisture and prolonged storage. This has been described by Moyo et al. (2015) to be directly related to a reduction in the ability of the seeds to carry out all the physiological functions that allows seed transition to seedling stage.

Though this problem is not adequately researched, especially on its influences on the frequency of transformation in soybean (Homrich et al., 2012), both breeders and farmers face problems of seed quality deterioration. Maintaining the quality of seeds used as an explant source for genetic transformation is a prerequisite for success of gene transfer into host plants. The genetically modified seeds currently used also show poor seed viability and longevity (Wang et al., 2001). Susceptibility to bacterial, fungal and viral attacks is very high, especially in moist conditions (Afrakhteh et al., 2013). The use of broad spectrum fungicides, storage in cold conditions or even drying at 30°C to maintain a 3–7% moisture content of the seeds are the only means to preserve high seed viability. These techniques were reported by Hurburgh (2008) to control and increase soybean seed longevity.

2.3. PURPOSE OF THE STUDY

2.3.1. Hypothesis of the study

Germination of different soybean seeds is negatively affected by the period of seed storage under ambient conditions irrespective of the genotype.

2.3.2. Aim and objectives of the study

This study was aimed at investigating the effects that seed storage under ambient conditions has on seed viability and germination potential in various cultivars of soybean.

The objectives of this project were categorised as follows:

i. To evaluate the effects of storage under ambient conditions on soybean seed moisture percentage.
ii. To determine the effect of seed moisture loss on seed viability, seedling vigour and germination potential.
iii. To compare the effects that storage duration has on germination rates among various soybean cultivars.
iv. To assess the effects of loss of seed viability on seedling growth and characteristics.

2.4. MATERIALS AND METHODS

2.4.1. Soybean seeds

Soybean seeds (*Glycine max* L. Merr.) cultivar; Dundee, TGx 1740-2F and TGx 1835-10E were acquired from the Department of Plant Production, Soil Science and Agricultural Engineering. The LS 677, LS 678 seeds were purchased from Link Seeds South Africa and Peking was obtained from the department of Biodiversity, University of Limpopo. The seeds were replanted at the Amaloba nursery (University of Limpopo) and multiplied to produce fresh seed materials used for the experiments.

The vegetative and yield parameters such as; percentage germination and emergence, plant height, number of pods per plant, number of seeds and seed weight were evaluated. Seeds were harvested at maturity from each cultivar (Figure 2.1) and stored in paper bags for further analysis. Seeds were counted into sets of 100 seeds and separated into multiple batches. This was done for each cultivar harvest and weighed before being stored under ambient conditions, at room temperature and humidity (approximately 24±7°C, ≥62%). The seeds were kept for the entire duration in paper bags within a storage cupboard to protect them against excessive desiccation.

Figure 2.1. Soybean seed cultivars used in the experiments: (a) LS 677, (b) Peking, (c) TGx 1835-10E, (d) TGx 1740-2F, (e) Dundee, and (f) LS 678.

2.4.1.1. Seed moisture analysis

Seed moisture loss was determined by comparing the fresh seed weight with the weight of the seeds after storage. Moisture loss was measured before every seed sterilisation and standard germination test and was expressed as the mean percentage for each 100 seed lot. This was performed at initial and then after every three (3) months of seeds stored under ambient conditions. The equations below were used to calculate seed moisture content and seed viability index where the change in seed weight was obtained by reweighing the stored seeds.

$$\text{Seed moisture content (\%)} = \frac{\text{Weight of seed after storage}}{\text{Fresh 100 seed weight}} \times 100$$

For the determination of seed viability index, the formula below was used. V is the viability index, K_i is the initial 100 seed weight immediately after harvest, ΔSw is the change in seed weight measured after the duration of storage and *P* is the period of storage in days.

$$V = K_i - (1/\Delta Sw)\, P$$

2.4.2. Seed sterilisation and standard germination test

Soybean seeds were first re-weighed as described above and then surface sterilised using chlorine gas according to Paz et al. (2004) prior to use for standard germination tests. Petri dishes containing seed samples of known mass were placed in a desiccator jar in the fume hood. A 250 ml beaker containing 100 ml of bleach was placed into the jar with the seeds. An amount of 3.5 ml concentrated HCl was carefully added into the beaker with bleach. The jar was immediately closed, and the seeds surface sterilised with the liberated chlorine gas for sixteen hours (Figure 2.2).

Figure 2.2. Plant material disinfection protocol, showing seeds inside the desiccator jar and types of sterilant used for surface gas sterilisation.

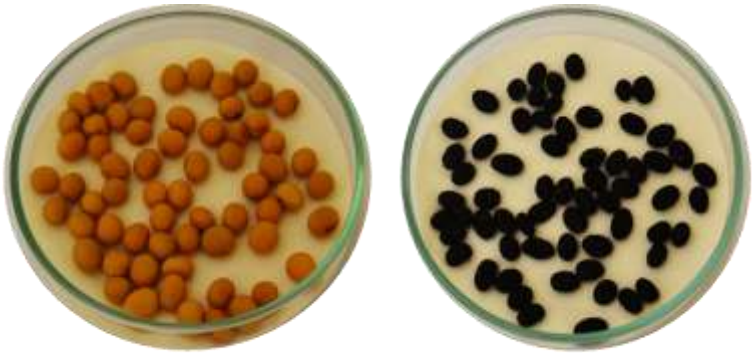

Figure 2.3. Examples of surface sterilised soybean seeds prepared for standard germination test.

Standard seed germination was evaluated following a procedure prescribed by the International Seed Testing Association (2003) with modifications. The disinfected soybean seeds were germinated by laying them on pre-sterilised damp filter papers in Petri dishes. The seeds were then covered with another single moistened filter paper and incubated in a growth room for seven days at 24±2°C temperature, 16h-photoperiod and 50–60 µmol $m^{-2}s^{-1}$ light intensity. A total of 200 seeds were used per cultivar, randomly separated into five (5) replicates containing 40 seeds each.

Seed germination was recorded as the splitting of the seed coat and the emergence of the radicle. The number of germinated seeds was recorded on a daily basis, with the first and final count made after 24 hours and 7 days of incubation respectively. All experiments for all cultivars were repeated at least three times. Standard germination tests were conducted at four different durations. The first germination test was carried out immediately after harvesting (initial) and consecutively after three (3) months, six

(6) months and nine (9) months of seed storage, under similar conditions. To calculate percentage germination of the soybean seeds, the equation below was used:

$$\text{Percentage germination} = \frac{\text{No. of total germinated seeds}}{\text{Total number of seeds tested}} \times 100$$

To determine whether the amount of distilled water used affected the viability of the seeds by causing imbibitional injury, evaluation on the amount of sterile distilled H_2O required to wet the filter papers was conducted. An amount of 5, 8, 11 and 15 ml of water was added to wet the filter papers used for germination. The suitable amount of sterile distilled water was then chosen for the rest of the experiments.

2.4.3. Seedling growth analysis

The germinated seeds were maintained in the growth room until the seven-day incubation period was completed and full protrusion of both the root radicle and epicotyls was achieved. After 7-days the seedlings were taken out of the Petri dishes and assessed. The morphology of seedlings developed from each cultivar was visually assessed for the number of abnormal and normal seedlings, evaluated as those exhibiting damage to essential structures such as the roots, hypocotyls, epicotyls and cotyledons.

Shoot length (considered as the length of the hypocotyl plus the epicotyl) and root length were measure using a millimetre ruler. For the assessment of seedling vigour, 15 seedlings were randomly selected from each replicate after germination and the recorded root and shoot length used to calculate seedling vigour index as described by Sheidaei et al. (2014). Results obtained from vigour calculations were used for classifying the various seed responses into vigour categories as: High, Low and Medium vigour seeds.

$$\text{Seedling vigour index} = \text{Mean radicle length} + \text{Mean shoot length} \times \text{Final percentage germination}$$

The mean percentage germination recorded per cultivar was also used to calculate the percentage viability of the seeds. The formula below was used to determine percentage viability of the seeds per cultivar. On the equation, viable seeds refers to those that germinated into normal seedlings as visually assessed.

$$\text{Percentage viability} = \frac{\text{No. of total viable seeds}}{\text{Total number of seed tested}} \times 100$$

2.4.4. Growth conditions

Soybean seeds placed on petri dishes for germination were incubated and maintained in a tissue culture growth room under 50–60 µmol.$m^{-2}s^{-1}$ light intensity with 10% humidity and at 24±2°C temperature.

2.4.5. Statistical data analysis

All data obtained on seed germination and seedling vigour were analysed using analysis of variance, where the significance values were calculated at P≤0.05 using SPSS software version 24.

2.5. RESULTS AND DISCUSSION

2.5.1. Soybean seed planting

The main objective of this study was to assess the effect of moisture loss in soybean seeds as a result of storage and to determine the performance of the seeds during seed germination and seedling development. To ensure uniformity in the testing of the soybean seeds, the seeds were planted at the nursery to produce fresh seed materials and for multiplication purposes. This furthermore, provided a secure starting point to gain insights into the performance of the seeds and to obtain reproducible results during the experiments.

Figure 2.4. Planted soybeans under natural environmental conditions for four (4) months: (a) seedling, (b) soybean plant (V2), (c) plant begins flowering (R1) and (d) soybean plant with fruit pods (R6).

Figure 2.4 above, shows the growth of soybeans established through seeds at the nursery under natural field conditions. A system that assigns a V or R with a number was used to distinguish the vegetative (V) stage of plants depending on the number of expanded trifoliate leaves compared to the flowering (R) stage, as described by Pedersen and Lauer (2004). Even though poor seed germination was observed for

Peking, LS 678 and LS 677, growth of the successfully germinated seedlings was rapid, and the plants appeared normal (Table 2.1). The poor performance in germination and seedling development emphasise the need for distinguishing seeds of high viability to those that have low viability in order to achieve high yield benefits. The evaluation of seed viability and seedling vigour is the main focus of this study, and on field experiments this can be done by visually selecting seeds with less damaged seed coats for planting. Many reports, including that of Sheidaei et al. (2014) indicated that the level at which the quality of seeds is found at significantly affect the growth and traits of the plant.

Table 2.1. Percentage germination and emergence (PGE), plant height (PH), number of pods per plant (POD), average 100 seed weight (W100) and the number of seeds per pod (SEEDS).

Soybean cultivar used	PGE (%)	PH (cm)	POD	W100 (g)	SEEDS
Dundee	64^{a}	73.8^{a}	137.2^{a}	20.6^{a}	3.0^{a}
LS 677	30^{e}	74.5^{a}	233.4^{b}	22.6^{b}	3.0^{a}
LS 678	10^{f}	68.2^{b}	192.5^{c}	21.4^{c}	3.0^{a}
Peking	35^{d}	47.5^{c}	40.7^{d}	20.3^{d}	3.0^{a}
TGx 1740-2F	60^{b}	72.3^{a}	157.5^{e}	19.8^{e}	3.0^{a}
TGx 1835-10E	55^{c}	69.5^{b}	124.0^{f}	18.4^{f}	3.0^{a}

Values followed by the same letter are not different at 5% probability level according to t-test.

According to our results, LS cultivars (677 and 678) and Peking recorded the lowest germination percentage ranging between 10−35%. The highest germination percentage was obtained in cultivar Dundee followed by TGx 1740-2F and TGx 1835-10E respectively. However, LS 677 followed by LS 678 and TGx 1740-2F produced the highest number of fruit pods respectively. Furthermore, there was a direct positive correlation observed between the number of pods produced per plant and 100 seed fresh weight. The weights of these seeds was high, indicating the direct import of carbohydrates into seeds and the increase in size during seed filling as a result of good growth.

However, the observations made in seed filling and size were expected to vary since these characteristics could differ according to genotypes. Some cultivars may be low or even defective in importing sugars into their seeds, as reported by Ghassemi-

Golezani et al. (2015) in soybean cultivar Williams. No significant variations were observed among the cultivars on the total number of seeds produced per fruit pod, with overall average of three seeds per pod. This was mostly observed in soybean cultivar Peking than any other cultivar, which also produced the lowest number of pods per plant. As natural field conditions are seldom optimum, continuous replanting ensured production of enough number of seeds for the experiment.

2.5.2. Soybean seed moisture content analysis

Seed moisture and viability analysis measured by the loss in seed mass during storage revealed deterioration immediately after harvest. All seeds indicated the continuing decrease in moisture content for the entire duration of seed storage. According to Table 2.2, the mean moisture content of the seeds was high at 0-month and started showing a decline when measured after 3, 6 and 9 months consecutively. Soybean cultivar LS 677 and 678 presented almost the same amounts of mean loss of moisture content of about 27−29%, which was the highest obtained. They also gave 24.8 and 24.5 g of mean seed mass during the initial period, which showed slight differences. The observation suggests that, cultivars that are genetically similar in their traits are likely to retain or lose moisture almost at the same rate and period. In plants, variation in genotypes alone account for more than 70% of the variation in germination and seedling development (Shelar et al., 2008).

Table 2.2. Mean weights measured from seed lots of soybeans used for standard germination test and their change in percentage moisture content.

Cultivars used	(0-months)	3-months	6-months	9-months	Δ (%) moisture
Dundee	21.603^{b}	20.404^{a}	13.072^{c}	11.709^{b}	22.7^{c}
LS 677	24.842^{a}	18.099^{c}	16.493^{a}	10.741^{d}	29.4^{b}
LS 678	24.524^{a}	19.439^{b}	15.252^{b}	10.638^{e}	27.9^{a}
Peking	18.613^{d}	16.643^{e}	12.744^{c}	11.149^{c}	20.5^{a}
TGx 1835-10E	20.522^{c}	18.523^{d}	16.255^{a}	15.854^{a}	13.3^{d}
TGx 1740-2F	17.333^{e}	12.177^{f}	11.167^{d}	10.669^{f}	25.9^{d}

Mean percentage values which are followed by different letters are statistically different at 5% confidence interval according t-test.

Seeds of other cultivars; like Dundee, Peking, TGx 1835-10E and TGx 1740-2F also exhibited a significant loss of moisture. These cultivars presented changes in moisture content ranging between 20 to 26% by the end of the experiment, except for TGx 1835-10E. Soybean cultivar TGx 1835-10E produced the lowest change in moisture content among all seeds with 13.3% compared to 25.9% observed on its relative cultivar TGx 1740-2F. The two cultivars also varied significantly on their mean seed weights throughout the experiment. Unlike in LS cultivars, TGx 1740-2F and TGx 1835-10E had significantly varied percentage moisture content with about 12% difference amongst them. This could be attributed to the efficiency of seed parts hydration or imbibition even though the two cultivars are related.

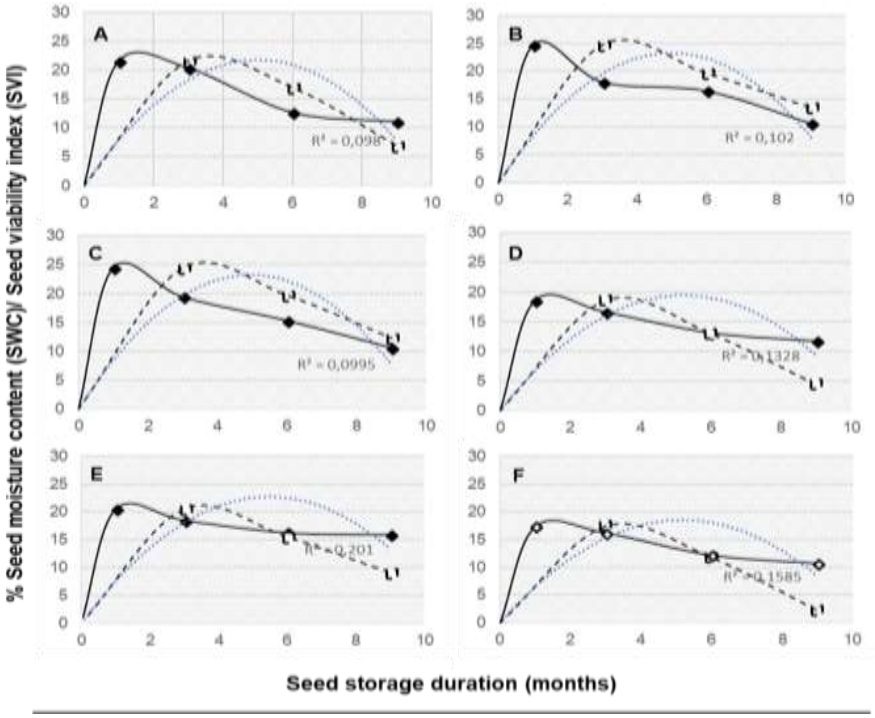

Figure 2.5. Seed moisture content (SMC) and viability (SVI) relations of the soybean seeds (A- Dundee, B- LS 677, C- LS 678, D- Peking, E- TGx 1835-10E and F- TGx 1740-2F) stored under ambient conditions. Solid line is the results of percentage moisture content of the seed during different storage periods. Dashed line is the results of viability indices determined from the change in seed weight. Round dots with R-squared coefficients shows the relations of moisture and viability in the seeds over time.

The observations also suggest that, the rate to which moisture is lost by seeds is controlled and determined by the storage conditions, as well as the storage materials. These may have caused the varied responses in the ability of the seeds to retain moisture. Furthermore, the results on seed viability calculated from the change in

moisture content of the presented seeds in Figure 2.5 indicate a positive correlation between seed viability and seed moisture content. Seed viability decreased with the loss or decrease in seed moisture as storage of the seeds was continued. These results suggest that, the level of seed viability is strongly influenced by the amount of water contained within the seeds. The strong correlation between moisture content of the seeds and viability was further demonstrated by the variation coefficient (R^2) that are also presented in Figure 2.5.

The results clearly indicated that there was a decrease in viability as moisture content decreased, where both may have a direct negative consequence on seed germination potential. These observations are supported by the Jepleting (2015) and Pinthus and Kimel (1978). These authors provided evidence showing that seed performance is affected by genotype and seed deterioration rates also vary according to storage conditions and the quality of the seeds, particularly during and after harvest. Additionally, Gleekia-kerkula (2012) reported lower moisture content of 8.03 and 8.49% on TGx 1903-7F and TGx 1904-6F respectively. He attributed these severe losses to storage conditions and the genotypes, which is in line with our observations for TGx 1835-10E.

2.5.3. Standard germination test of the seeds

In the evaluation of seed viability using standard germination test, variation in seed germination percentage was observed. In addition to this, differences in seedling development and seedling vigour were also observed. There was significant variation in germination according to genotypes, between Peking (black seeds) and the rest of the yellow seeds (Dundee, LS 677, LS 678, TGx 1740-2F and TGx 1835-10E) used in this study. All soybean cultivars with yellow seeds presented differences in terms of germination percentages and seedling morphology exhibited by germinated seeds as exemplified in Figure 2.6, 2.7 and 2.8. According to the results, germination percentage was high at 0 and after 3 months of seed storage. The germination percentage for all cultivars then started to decrease as storage of seeds continued for 6 and 9 months as demonstrated in Figure 2.6 below. The overall decrease in germination percentage may be an indication of seed deterioration due to the loss of seed moisture or viability as a result of storage, as shown in Figure 2.5.

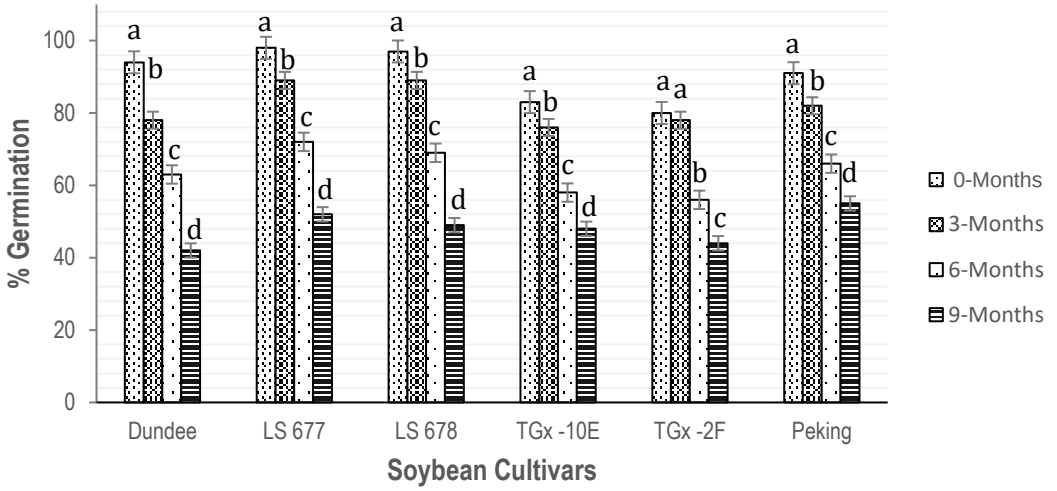

Figure 2.6. Germination percentages observed during standard germination test conducted initially at 0 and after 3, 6 and 9-months using seeds stored under room ambient conditions.

The viability of the seeds appeared compromised with the decrease in seed moisture content. These factors had a profound influence on the potential germinability of the seeds, at the same time negatively impacting on the quality of seedlings developed as indicated on Table 2.3. Soybean cultivar LS 677 and LS 678 (Figure 2.5) exhibited overall highest germination percentage among cultivars used within six months of seed storage. Their percentage germination was maintained at almost 50% for the entire experiment. This indicates good maintenance of maximum potential for germination and gradual deterioration on seed quality and germinability status of the LS cultivars. But gradual decrease in germination capacity as demonstrated for subsequent period also suggest that their viability is short lived as in the other tested cultivars.

These findings support observations by Shelar et al. (2008) who indicated that germination is very momentary in soybean compared to other oilseed crops. However, seeds of Peking indicated rather, an unusual response compared to the rest of the soybean seeds. The seeds produced over 80% germination in all replicates only when they were maintained in the growth room for over 7 days, whilst other cultivars showed minor signs of germination. Peking seeds demonstrated strong delay of germination, but seedling growth occurred normally when the seeds had managed to germinate, as indicated on Table 2.3. Despite the delay in germination, Peking seeds remained above 50% within 7-days of seed culture (Figure 2.6). The germination rate of Dundee

determined throughout the experiment was about 90% dropping down to 42% in subsequent experiments. This response was essentially the same as that of the Peking. In contrast, Dundee seeds germinated faster during the early periods, and dramatically decreased subsequently. Though seed germination percentage between TGx 1835-10E and TGx 1740-2F slightly varied (by less than 20%), probably due to germination and storage conditions their poor performance was consistently observed. Both cultivars achieved the highest germination percentage that was just above 80% in their first experiment. This was so, even though these cultivars produced the highest germination percentage in the field during replanting to obtain fresh seeds. The germination tests were carried out under suitable conditions, which showed little influence on the performance of the seeds.

2.5.3.1. Effect of moisture loss on seed germination

The effect of the amount of water used to hydrate the seeds was also examined by wetting the filter papers as described in 2.4.5. An amount of 11 ml dH_2O was sufficient for seed imbibition and establishment of seedling growth within 7 days of incubation. However, this rehydration of the seeds proved not to be sufficient to correct or replace the amount of moisture lost by the seeds, especially, after three months of storage when germination started to decrease significantly. When the seeds were rehydrated with 5.0 ml dH_2O, no promotive effects on seedling development were observed due to rapid drying as a result of water depletion and possible evaporation.

The observation showed that water is required for seed rehydration and plays a significant role in metabolic enzyme activation during this process. As Vertucci and Roos (1993) stated, water is essential for the chemical reactions within the seeds to induce successful germination and seedling growth. Seed moisture and very minimal hydration of dry seeds did have a negative effect on the germinability of the soybean seeds used. However, the seeds also showed sensitivity to excessive amounts of dH_2O, probably experiencing imbibitional injury. Generally, both very low and very high amounts of water have inhibitory effects on seed germination. The effects of low moisture content or dryness and imbibitional injury on seed germination and seedling formation were thoroughly reviewed by Woodstock (1988) and Rajjou et al. (2012). The reports indicated that, imbibitional injury may occur as a result of more water

entering the cells and this may negatively affect normal metabolic functioning of the seeds, particularly causing rupture of cells of the developing embryos and their supportive structures. Imbibitional injury may cause reversal of certain metabolic synthesis like the production of gibberellin and expression of alpha-amylases (Yu et al., 2015). They stated that the loss in moisture account for the recalcitrance of the seeds as a result of low seed viability due to rapid dehydration. Therefore, all observations made indicate that, adequate seed moisture and appropriate amount of water are required for rehydration by the embryogenic axis than any other seed part for successful soybean establishment (McDonald et al., 1988).

2.5.3.2. Effect of seed viability on germination

Combined analysis of seed moisture loss and viability shows that all soybean seeds showed a high viability index during the initial stage of the experiment and this outcome was changed by continued seed storage. The loss of seed viability due to the storage conditions as discussed by Malik and Yoti (2013) impact negatively on the production, and expansion of soybean as an oilseed legume crop. Other authors like Dadlani et al. (2010), Shaban (2013) and Shelar et al. (2008) attributed these germination decreases mainly to seed storage under very humid or high moisture conditions. They indicated that, these storage conditions may trigger increased respiration, heating and possible microbial infections which negatively affect the viability of the seeds. However, in this study, the seeds were stored at room temperature under ambient conditions.

All seeds indicated rapid deterioration by shrinking, almost immediately, losing seed moisture content instantly after harvesting (Table 2.3). The shrinking and loss of weight were a clear indication of the loss in seed viability. These changes had a significant influence on seed germination in all cultivars. Seed viability decreased as a result of prolonged seed storage as mentioned above and was accompanied by the decrease in percentage germination. Both the percentage of seed germination and normal seedlings were affected by the loss in seed viability. However, the delayed seed germination that was observed in the cultivar Peking provided somewhat contrasting results.

Table 2.3. Analysis of variance of data for seedling characteristics observed during the standard germination test after 7 days of incubation.

	Peking	LS 677	LS 678	Dundee	TGx -10E	TGx -2F
Initial						
Normal seedlings (%)	97.0[a]	97.5[a]	95.3[b]	92.8[c]	88.8[d]	84.5[d]
VI	12.3[c]	17.1[a]	12.9[b]	13.5[b]	9.10[d]	5.92[e]
SVI	1.70[b]	1.97[a]	1.93[a]	1.29[e]	1.55[c]	1.52[d]
Percentage vigour	100*	97.5*	95.0*	90.8*	85.8*	89.2*
(3) months						
Normal seedlings (%)	90.5[a]	94.5[a]	93.3[b]	90.8[c]	86.8[e]	82.2[e]
VI	5.16[a]	4.62[b]	4.386[c]	3.579[c]	2.516[d]	2.089[d]
SVI	0.677[a]	1.609[b]	1.488[b]	0.859[a]	0.304[c]	0.257[c]
Percentage vigour	93.7*	95*	97.5*	94.2*	74.2*[a]	68.3*[a]
(6) months						
Normal seedlings (%)	65.5[a]	70.8[b]	65.0[b]	60.1[b]	65.5[a]	71.0[b]
VI	2.96[a]	4.26[b]	4.34[b]	2.13[c]	3.61[d]	3.05[a]
SVI	0.36[a]	1.31[b]	1.32[b]	0.51[c]	0.36[a]	0.35[a]
Percentage vigour	68.3**	91.7*	80**	74.2**	88.4**	84.1**
(9) months						
Normal seedlings (%)	52.5[a]	59.2[b]	63.3[b]	55.5[a]	35.0[c]	44.2[d]
VI	2.82[a]	2.48[b]	3.77[c]	2.19[d]	1.98[d]	1.69[d]
SVI	0.31[a]	0.51[b]	0.78[c]	0.48[d]	0.21[e]	0.19[e]
Percentage vigour	85.8**	64.9*[a]	75**	64.1*[a]	58.3*[a]	60.3*[a]

Values along the rows with different letters are not significantly different at 5% probability level according to t-test. * Seeds are viable, ** Seeds viable with gradual decline in germinability and *[a] Seeds viable with rapid decline in germinability. VI- seed viability index. SVI- Seedling vigour index.

Rosenberg and Rinne (1986) explained the delayed germination as a result of the seeds' needs to undergo a change in moisture content as a prerequisite for better germination and seedling growth. Furthermore, the inability of the radicle to penetrate the seed coat could have affected the speed of germination in Peking as supported by Wierzbicka and Obidzinska (1998). However, our results clearly shows that the decrease in seed viability which is directly linked to the decrease in moisture at ≤40% immediately post harvesting, as observed in Peking and TGx 1835-10E have a detrimental effect on seed germination.

2.5.4. Effect of storage on seedling development

The results obtained indicate that seedling growth varied among cultivars as indicated on Figure 2.7 below. Significant differences in terms of the shoot/root lengths and seedling viability were observed as affected by both percentage seed moisture and the duration of storage. Production of normal seedlings ranged between 95–100% during the first month and decreased with the decline of other factors like moisture and

viability as storage progress for over 3-months. LS cultivars and Peking exhibited the overall highest seedling vigour and percentage viability (94%) as indicated in Table 2.3 and Figure 2.10. These cultivars were consistent with regard to the production of normal seedlings, irrespective of the delay in seed germination observed, particularly in Peking, during incubation as discussed above. Normal seedling growth was also observed in Dundee, TGx 1835-10E and TGx cultivars but percentage viability and normal seedling percentage dropped significantly with 55, 35 and 44% during the 9th month respectively. Stout seedlings with thicker hypocotyls and short epicotyls were observed in these cultivar LS 678 and LS 677, as well as Peking.

Figure 2.7. Seed germination during standard germination test of soybean cultivar Peking (a), TGx 1835-10E (b), LS 677 (c), LS 678 (d), TGx 1740-2F (e) and Dundee (f) after six months of seed storage.

Even though germination percentage reached 80% in some replicates, seedling development was dominated by abnormalities, especially in experiments conducted after 6 and 9 months of seed storage (Figure 2.7). Cracking of cotyledons, un-developing root radicles and highly reduced hypocotyls and epicotyls were some of the abnormal features (Figure 2.7). Most seedlings exhibited the difficulty in emerging from the seed coats. In contrast, Peking showed notable production of good seedlings even after 6-months of seed storage. Most of the seedlings appeared normal, with elongated hypocotyls as exemplified in Figure 2.7 a. However, the epicotyl growths were reduced due to the seed coats as well. More than 20% of seedling's cotyledons were still covered by the seed coats, which remained unbroken, limiting and negatively

affecting the growth of epicotyls as observed in TGx 1740-2F. As Figure 2.8 and 2.9 indicates the extent of primary root reduction and the presence/ absence of lateral roots in some of the soybean cultivars used, Dundee formed better roots during the first three months only whereas; both TGx 1835-10E and TGx 1740-2F showed overall poor root development (Figure 2.9). This was however, later followed by the continued reduced root formation recorded in more than 50% of the seedlings produced.

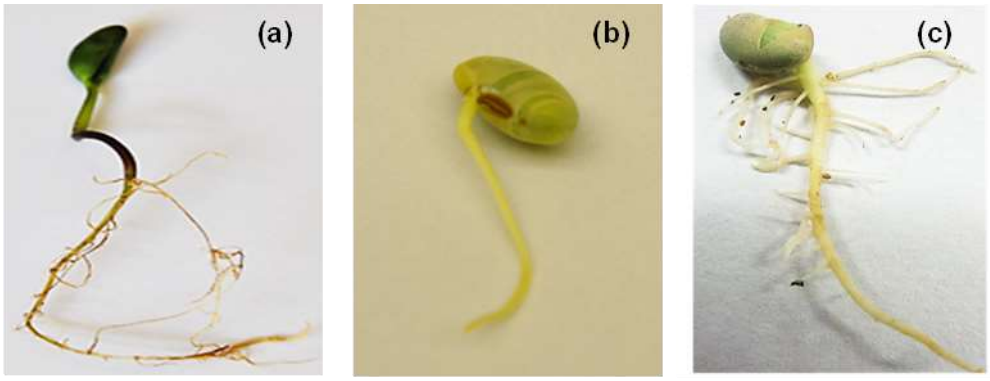

Figure 2.8. Seed germination establishment showing root development during standard germination test of soybean cultivar Peking (a), TGx 1835-10E (b), and LS 677 (c).

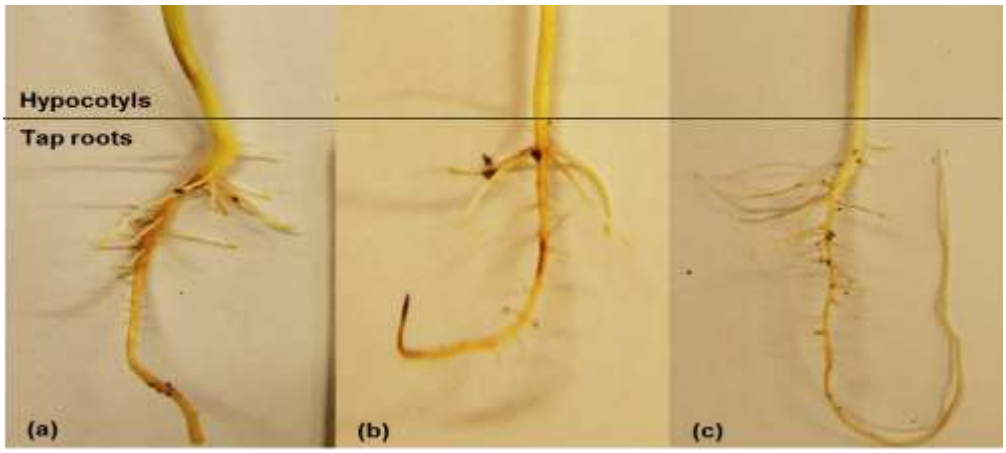

Figure 2.9. Growth and development of soybean root system. (a) Peking, (b) TGx 1740-2F and (c) LS 678 tap roots with lateral roots formation.

The presence of longer and shorter roots with lateral roots formed was also reported by Mut and Akay (2010). Their report indicated that the type of root morphology observed was due to seed size which had an effect on seedling viability. Rezapour et al. (2013) also reported similar observations when assessing the effect of seed size on germination of soybean cultivars Williams and TMS.

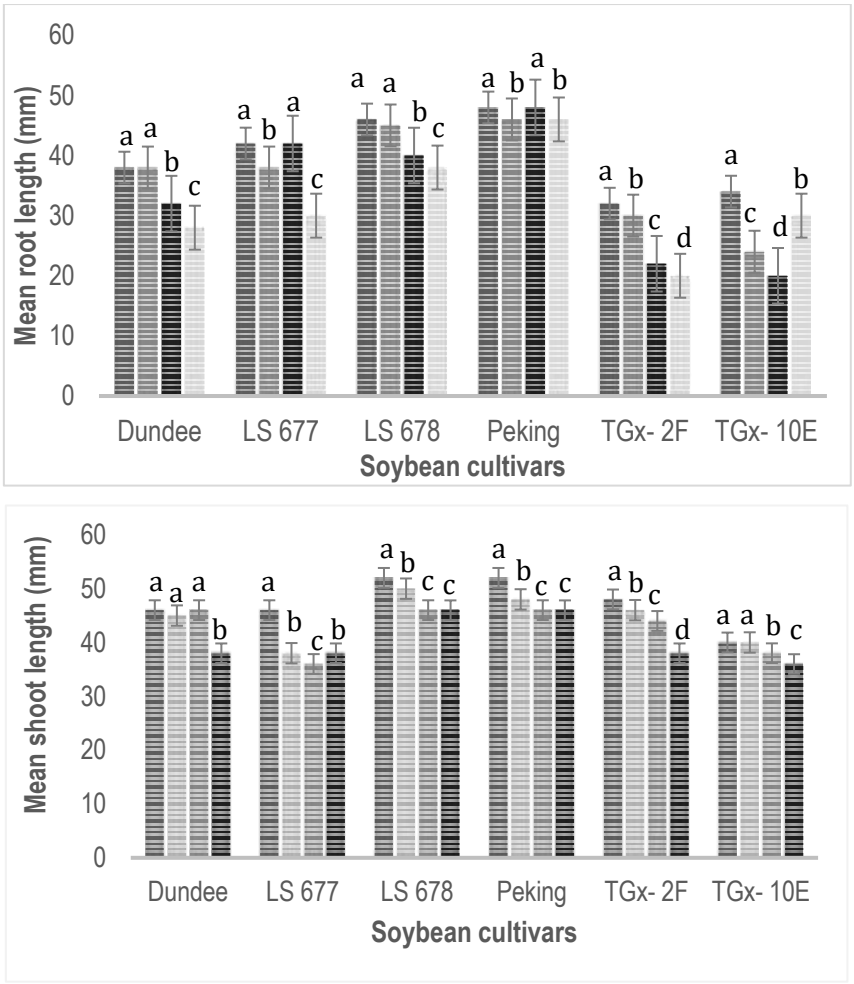

Figure 2.10. Differences in soybean seedling morphology in terms of root length and shoot length measured using a millimetre ruler. The bars on the charts are arranged in consecutive order starting from 0, 3-months, 6-months and 9-months of seed storage.

More than 80% of Peking seedlings produced long primary roots with lateral roots and the examples of the type of rooting observed is shown on Figure 2.8 (a). In LS cultivars, reduced lateral roots with elongated primary root were also observed. LS 678 formed long primary root with reduced lateral roots (Figure 2.8 c). Even though LS 678 performed best in terms of average root length initiation following Peking as indicated in Figure 2.9, seedlings with lateral roots were rather more predominant in LS 677 than LS 678. Less than 10% of the seedlings established in LS 678 managed to form lateral roots during the period of incubation compared to 40% obtained in LS 677. Lateral root initiation in LS 678 was only observed in one replicate shown on Figure 2.7 (f). The response may be due to the genotype or inhibition of growth processes such as respiration during incubation and the effect of light in the growth room. In general, root lengths (Figure 2.7 b) did not indicate any correlation between the decrease in seed moisture as a result of storage and the formation of strong normal seedlings. Cultivar

Peking and LS 677 including LS 678 exhibited the highest shoot length, thus achieving higher normal seedling percentages. This observation may be linked to the genotypes or have resulted from how this cultivar responded to the growth conditions. Shoot lengths ranged between 40–60 mm among all cultivars; except for TGx 1835-10E and TGx 1740-2F which produced 38 and 39 mm average shoot length after six and nine months respectively. However, the results also show clear inconsistencies among most of the cultivars, wherein the average shoot lengths do not decrease with the increase of the duration of seed storage as was observed for other parameters. All genotypes exhibited fluctuating mean values throughout the experiment.

In contrast, Peking presented a continuous slight decline in root length observed throughout the experiment as per vigour index (Figure 2.9). The differences observed among replicates were not statistically significant and may be attributed to the slow rate of seed germination observed rather than abnormalities with seedling growth characteristics. Dundee's shoot length was constant and remained at about 4.0 cm on average. The differences in shoot lengths between LS 677 and LS 678, as well as TGx 1740-2F and TGx 1835-10E were not significant. The results indicated that the shoot lengths varied among the cultivars themselves (between LS and TGx), perhaps, indicating variation according to genotype.

2.5.5. Implication of results on *in-vitro* improvement of soybean

The results show a significant interaction between the duration of storage and seed viability. In general, longer seed storage periods resulted in greater levels of germination and decreased vigour among cultivars. Higher germination rates and higher vigour indexes were mostly observed after three months of storage when the seeds had not morphologically or physiologically deteriorated (Table 2.2). Storage conditions and duration proved to have adverse effects on seedling viability and resultant morphology. However, the results and comparison of cultivar Peking to other cultivars showed that Peking was able to strongly retain germinability, and seedling growth integrity. Peking was followed by LS 677, LS 678, Dundee and then TGx 1740-2F and TGx1835-10E respectively. The observations made above suggest superior and inferior genetic differences that exist among cultivars. Therefore, these findings establish the need for careful screening and proper selection of cultivars to be used in

plant tissue culture, for the improvement of soybean. The efficiency of *in vitro* regeneration of soybean profoundly depends on good seedling establishment as an explant source. Moreover, the efficiency to improve and increase transformation frequencies in soybean heavily relies on seed vigour. Table 2.2 indicated potential genotypes for use in genetic improvement, to be selected based to their seed viability as well as seedling vigour. The selection of cultivars that maintain their high germinability and seedling vigour in addition to the genetic, structural and physical integrity will be beneficial in developing high yielding soybean cultivars with better traits and high seed quality.

2.5.6. Conclusions

Soybean seed quality rapidly deteriorates during storage and the problem can be noticed immediately after harvest. The continuous loss in seed moisture content due to lack of proper storage conditions affect seed longevity, and thus interferes with seed viability and the effects differ according to genotypes.

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CHAPTER 3

THE INFLUENCE OF SEED VIABILITY ON *IN-VITRO* SHOOT CULTURE IN SOYBEAN

3.1. INTRODUCTION

3.1.1. Background to the study

The loss in seed viability has proved to prevent preservation of the genetic integrity of stored seed samples in soybean (Mahmood et al., 2016). The problem of the loss of seed viability due to storage and storage conditions occurs not only in *Glycine max* but, even in other plants such as; *Galenia pubescens*, *Swertia chirayita*, and *Zea mays* L. (Govender et al., 2008; Pradhan and Badola, 2012; Mahmood et al., 2016). Therefore, the effect of seed storage on germination, seedling development, and *in vitro* shoot formation of the soybeans must be investigated. Factors such as seed quality determines the performance of soybean regeneration in tissue culture and the successive growth of the plant when acclimatised outside the aseptic culture conditions. The growth of soybean has shown to be negatively affected by both genetic and environmental factors, in addition to storage (Piper and Boote, 1999). The factors furthermore, have an impact on the chemical composition of the seeds (carbohydrate, oil, protein or fibre content), their uniformity, seed size, shape and colour (Filho et al., 2004). Subsequent to these, the highlighted effects on the soybean seeds affect *in-vitro* genetic improvement aimed at increasing yield of the crop.

3.1.2. Soybean improvement via *in-vitro* culture

The use of cotyledonary node explants (double or single coty-nodes) for *in-vitro* regeneration of soybean has been widely reported (Keneda et al., 1997; Paz et al., 2006; Raza et al., 2017; Mangena et al., 2017). Seed viability, including seedling vigour and seedling age, from which the explants are derived, and the concentrations of cytokinin added in the culture medium strongly influence *in-vitro* response. The studies mentioned above, including those in other leguminous crops such as, common bean and pigeonpea reported successful production of adventitious shoot formation

using seedlings as a source of explants (Dayal et al., 2003; Gatica-Arias et al., 2010). *In-vitro* shoot culture, however, has been reported to be negatively affected by the poor regeneration systems as a result of low seed viability. In circumventing this challenge, much still need to be done to ascertain and advance the role that tissue culture can play during plant regeneration. The inefficiencies that occur as a result of explant vigour, explant type, culture conditions, and poor genotype response which largely involve the regeneration system still present major challenges. Therefore, one of the main objectives of this study was to assess how different soybean genotypes may be influenced by the loss of seed viability as a result of seed storage. Thus, allowing for a clear elucidation of the relationship between seed longevity and the frequency of shoot induction from the predominantly used double coty-node explants in soybean.

3.1.3. Motivation to the study

Current research on soybean improvement still emphasises the importance of seed viability and seeding vigour as the predominant factor affecting *in-vitro* regeneration of soybean. However, the growing interest in yield improvement has focused attention on soybean seed composition and the use of tissue culture-based technology. Producing soybean seeds with desired yield traits and chemical composition has been reported by Poysa et al. (2002) as essential to meeting consumer needs and may be critical for profitability (Brown, 2006). FAO (2012) reported nutritional benefits of high soybean protein content in reducing cardiovascular diseases and lowering cholesterol levels. Pederson and Lauer (2002) reported on the increasing consumption of soybean seed-derived products, with associated increasing health benefits. Pederson and Lauer (2002) further showed that the increasing consumption of soybean products result in greater demand for soybean seeds, especially in meeting the populations' nutritional needs and ultimately, meeting the sustainable development goals of the United Nations (Park et al., 2011). As the world population continues to increase, it would be beneficial to continue assessing the effect of storage on soybean regeneration in order to help minimise yield losses and improve sustainable global production.

3.2. LITERATURE REVIEW

3.2.1. Domestication of soybean

Soybean [*Glycine max* (L.) Merrill] is one of the most important oilseed legumes used to produce several nutritional, pharmaceutical and industrial products. This plant belongs to the Fabaceae family and its history dates back to the 11th century BC, originating in the northeast region of China (Ishaq and Ehirim, 2014). Soybean is cultivated worldwide; in the tropics, sub-tropics and temperate regions mostly for commercial purposes. More laboratories have sought to improve and optimise the growth and yield characteristics of soybean. This has been carried out because soybean seeds contains large amounts of vegetable oil, proteins, carbohydrates and macro- or micro-nutrients, with oil-protein composition ranging between 10–40%. In addition to these, the seeds also contain a considerable amount of dietary fibres and vitamins as well as a low amount of antinutritional factors (Amanlou et al., 2012).

3.2.2. Cultivation of soybeans

Worldwide soybean major producers include the United State of America, Brazil, Argentina, China and India. These countries have been the biggest producers of soybean amounting to more than 219.8 million metric tons. About 228.87 million metric tons were projected to be produced by these countries for 2016/17, which is the highest production for the past three decades, according to USDA (2017). The United State has been the all-time biggest producer with 106.86 million tons followed by Brazil with 96.5 million metric tons for 2015/16 estimations (FAO, 2016). Countries such as the United States and China are fully benefiting from the commercial utilisation of this crop. These countries enjoy the high value and profits attained from soybean sub-products, meals and oils, as well as food and animal feedstocks. Soybean oil is considered the most important vegetable oil after palm oil. It accounts for about 25% of the global vegetable oil and fats consumption (Gandhi, 2009).

3.2.2.1. Productions in Africa

The cultivation areas and yield of soybean per hectare in Africa are still very low. This may be due to hot and dry conditions (Adeyeye et al., 2014), lack of high yielding varieties, and perhaps, the inadequate knowledge about plant growth and development, and their interaction with the environment (Chavarria et al., 2017). South Africa is still the leading producer of soybean in Africa. This country produced about 2.15 million metric tons followed by Nigeria, Zambia, and Uganda with 0.96, 1.94, and 0.60 million tons per hectare (USDA, 2017). These figures remain stagnant since production levels gradually increased. This ultimately resulted in 35% of the total harvested area devoted to annual and perennial oil crops in Africa, including soybean. These production levels are still very low compared to the high amounts of soybeans produced in countries like the United States, Brazil, Argentina, India and China (USDA, 2017).

Some of the smallest producers documented include Mozambique and Kenya with 2,200 and 1,230 metric tons respectively (FAO, 2012). There are a number of challenges that lead to low productions of soybean in the African regions. These includes; ineffective symbiosis between soybean and *Rhizobium* for nitrogen fixation, poor seed longevity of soybeans stored between growing seasons under humid conditions, and seed viability as well as drought stress, pests and diseases (Thoenes, 2017). The development of high yielding cultivars with better resistance to biotic and abiotic stress factors are the only potential to expand cultivation of soybean in Africa. This will in turn increase accessibility to high-protein soy food products for most of the malnourished African populations. Soybean agricultural expansion may even encourage major industrialisation of soy products, and the use of this crop for subsistent farming and medicinal purposes (Dixon and Summer, 2003).

3.2.3. Factors affecting soybean production

There are a myriad of factors that affect soybean growth and development, pod setting and maturation, as well as the process of seed filling. The growth and productivity of this crop is adversely affected by various biotic and abiotic stress factors, including physiological traits such as the genotype. However, the problem of climate change

caused by increasing atmospheric carbon dioxide (CO_2) is a leading contributor of the high heat and drought experienced in some regions, which currently affect crop production and chemical compositions of the seeds on a larger scale than any other factor (Bellaloui et al., 2017).

3.2.3.1. Effect of abiotic and biotic stress on soybean

Factors such as insect pests, viruses, drought and very high/ low temperatures have detrimental effects on soybean growth (Khan and Ahemad, 2011). Stress factors may lead to soybean plants falling over, flower abortion, and shattering of pods prior to harvesting (Blignaut and Taute, 2010). Huntingford et al. (2005) stated that soybean growth and development depends largely on the variability and intensity of these stresses. It has been predicted (Gregory et al., 2005) that these variations in climatic conditions may lead to drought or flooding or warmer or cooler temperatures which have a negative impact on crop yield thus, threatening food security. Similarly, biotic factors can prevent plants from achieving their maximum growth and reproductive potential as measured by vegetative growth and yield quantity (Mabulwana, 2013). Gregory et al. (2005) gave further insights on disease symptoms caused by pathogenic strains of *Cylindrocarpon*, *Fusarium*, *Phoma* and *Pythium* affecting legumes. Strains of the genera Pythium cause seedling mortality in cowpea. These soil-borne legume pathogens, including other widespread disease causing fungi induce root, stem and leaf rots in peas, soybean and alfalfa (Mangena, 2018). While considerable progress has been made in understanding the sensitivity of soybean to fluctuating growth conditions, evaluation of the genetic and physiological factors still need to be consolidated with the effects of already known abiotic and biotic factors (Jaleet et al., 2009).

3.2.3.2. Other factors affecting soybean improvement *in-vitro*

Since Hinchee et al. (1988) reported a successful production of transgenic soybean plants using *Agrobacterium*-mediated tissue culture based genetic transformation, many researchers attempted to optimise the conditions affecting soybean transformation efficiency. An improved transformation system will enhance soybean yield production by generating stress resistant varieties but, currently, the degree of

genetic improvement is still very low (Jia et al., 2015). The requisite for genetic transformation is an efficient and genotype independent regeneration system. Some reports showed that various soybean genotypes have different affinity for *Agrobacterium tumefaciens* strains (Meurer et al., 1998). The regeneration of transformed adventitious shoots from cotyledonary node explants has been previously reported (Meurer et al., 1998; Donaldson and Simmonds, 2000; Paz et al., 2004). Culture standardisation included suitable explant and explant source selection, efficient formation and recovery of shoots, and the adequate susceptibility of the explants to *Agrobacterium* infection. The optimisation of culture conditions has also been reported in other legumes such as *Cajanus cajan* (L.) by George and Eapen (1994), Mohan and Krishnamurthy (1998), and Dayal et al. (2003). All reported the successful development of an efficient plant regeneration protocol of pigeonpea for the production of transgenic plants. However, these studies clearly stated that, the establishment of a regeneration protocol that would provide a large number of transgenic plants for routine work is still a challenge and the already used protocols are genotype specific.

3.2.4. Health and economic benefits

Soybean is a leguminous grain crop with a very high health and nutritional value. This crop has the greatest potential for alleviating protein-based malnutrition and assist in life threatening conditions such as cancer, osteoporosis and cardiovascular diseases (Liu et al., 2007). One of the leading products of soybean, derived from the seeds is oil in addition to 40% proteins. This is a complex mixture of five fatty acids, namely; palmitic, stearic, monounsaturated oleic acid, polyunsaturated linoleic and linolenic acids, which have vast oxidative and chemical functionalities (Cahoon, 2003). Kinney (1997) estimated the oleic acid content of some transgenic soybean cultivars to be approximately 80% of the total oil seed content.

Soybean oleic acid content was relatively high against 10.9–27.10% oil yield quantified in the six Sudanese *Cucurbita* cultivars (Mariod et al., 2009). Peanut, rapeseed mustard, sunflower, safflower, *Sesamum*, linseed, castor and cotton seeds are other predominant oil crops (Yadava et al., 2012). In comparison, extracted oils and proteins in soybean are the most used oils and proteins in medicines and pharmaceuticals,

industries, feed, manures and as components of all other soy-products. This makes soybean account for more than 67% of the world's oilseed production for economic and health benefits (USDA, 2017).

3.2.5. *In-vitro* tissue culture of soybean

The main objective of *in vitro* plant tissue culture systems is to successfully produce a large number of healthy plants in a short period of time, and at minimal expense. The establishment of this technique formed the basis for many plant improvement protocols, and the elimination of undesirable characteristics accompanying conventional breeding methods in most grain crops (Delgado-Sanchez et al., 2006). Although, regeneration protocols that could be routinely applied in genetic engineering techniques are still lacking, many *in vitro* protocols have been elaborated for the regeneration of important oilseed crops, such as, maize, cotton and canola (Qaim, 2009). Pratap and Kumar (2011) reported that these regeneration protocols are the key object of genetic improvement and are also used to initiate cultures suitable to facilitate alternative methods of transformation such as, *in vitro* mutagenesis.

3.2.6. Plant regeneration via tissue culture

The development and flexibility of tissue culture conditions such as; culture media and plant growth regulators, have enabled researchers to easily manipulate growth of plants *in vitro*. Optimal growth and morphogenesis of tissues of the same or different plant sources can be easily varied to achieve a desired tissue culture goal (Leva and Rinaldi, 2012). *In vitro* cultures allow regeneration via direct or indirect organogenesis of shoots or callus and through embryogenic culture. In organogesis, plants are produced via shoot formation and in embryogenesis, embryos are initiated (Trigiano and Gray, 2005). But, both paths require a series of nutrient formulations and appropriate growth conditions in order to be accomplished (Perez-Clemente and Gomez-Cadenas, 2012).

George (1993) reported axillary formation of shoots from shoot tips and lateral buds cultured on medium supplemented with cytokinin. The axillary shoots produced were sub-divided into shoot tips and nodal segments, serving as secondary explants for

further shoot proliferation or rooted as microcuttings. Texeira et al. (2011) evaluated the totipotency of ten soybean cultivars for development of somatic embryos from immature cotyledons. Both studies (George, 1993, and Texeira et al., 2011) used *in vitro* regeneration to enhance the genetic variability by inducing somaclonal variation of their experimental crops. These establishments are essential for increasing the number of superior crop genotypes. In soybean for example, production has been expanded through cultivation of transgenic cultivars that has been made possible by the development of tissue culture-based plant transformation protocols.

3.3. PURPOSE OF THE STUDY

3.3.1. Study hypothesis

Soybean seeds stored under ambient conditions will deteriorates and this impacts negatively on germinability and *in vitro* regeneration of plantlets using explants derived from the germinated seeds.

3.3.2. Aim and objectives of the study

The aim of this study was to assess the influence of seed storage on *in vitro* regeneration capacity of the various soybean cultivars.

The objectives were to:

i. Assess germinability of soybean seeds on Murashige and Skoog (MS) basal medium post seed storage for nine months under ambient conditions.
ii. Evaluate the frequency of shoot induction and regeneration on MS medium containing 2.00 mg/l 6-BA.
iii. Analyse and assess acclimatisation conditions for the *in vitro* regenerated soybean plantlets.
iv. Evaluate the growth and yield performance of the *in vitro* regenerated soybean cultivars adapted under greenhouse conditions.

3.4. MATERIALS AND METHODS

3.4.1. Soybean seeds

Glycine max (L.) seeds cultivar Dundee, LS 677, LS 678, TGx 1740-2F, TGx 1835-10E and Peking were harvested from healthy field grown plants cultivated at Amaloba Nursery, Turfloop in the University of Limpopo. The dry seeds were collected, counted into samples of 100 seeds each, weighed, and kept at room temperature with 40−55% humidity for *in vitro* shoot culture. Seed samples prepared were then analysed after every three (3) months for *in-vitro* seed germination and shoot regeneration.

3.4.2. Seed sterilisation for *in-vitro* germination

The seeds were washed a few times with soap in running tap water to remove dust and other detritus before surface sterilisation. The seeds were mopped dry with a paper towel. A total of 60 dry soybean seeds were placed in 90×15 mm sterile plastic petri dishes. The dishes were then transferred into a desiccator jar together with a 150 ml beaker containing a solution of 100 ml sodium hypochlorite (3.5%) mixed with 3.5 ml concentrated hydrochloric acid. The desiccator jar was placed in a fume hood and the seeds sterilised overnight for 16 hours.

3.4.3. Culture media preparation

Culture media were prepared using Murashige and Skoog salts added with vitamins, 3% sucrose, and 0.25% gelrite (Gellan gum) as indicated in Table 3.1 (Murashige and Skoog, 1962). The pH of the culture media was adjusted to 5.8 and autoclaved at 121°C for 20 min. The prepared media was dispensed into culture vessels when the temperature of the media dropped to 50–60°C. For seed germination; MS media was modified with 2.00 mgL^{-1} 6-benylaminopurine (6-BA). For the initiation of multiple shoots, MS media was supplemented with 6-BA and different concentrations of auxins (IBA and NAA) for *in-vitro* rooting. Shoot elongation was achieved on MS media without plant hormones.

Table 3.1. Modified MS basal culture medium based on Murashige and Skoog (1962)					
Macro elements	**Amount (mg/l)**	**Micro elements**	**Amount (mg/l)**	**Organic additives**	**Amount (mg/l)**
NH_4NO_3	1650	H_3BO_3	6.2	Glycine	2
KNO_3	1900	$MnSO_4$	22.3	Myoinositol	100
$CaCl_2.7H_2O$	440	$ZnSO_4.2H_2O$	0.25	Nicotinic acid	0.5
$MgSO_4.7H_2O$	370	KI	0.83	Thiamine HCl	0.5
FeEDTA	35	$Na_2MoO_4.2H_2O$	0.25	Pyridoxine	0.5
KH_2PO_4	170	$CuSO_4.5H_2O$	0.25	Sucrose	30000
		$CoSO_4.7H_2O$	0.03	Gelrite (Gellan gum)	2500

3.4.4. Aseptic seed germination

After 16-hour surface sterilisation, the decontaminated soybean seeds were taken out of the desiccator jar and placed open in the laminar flow hood to remove excess chlorine gas. The sterilised seeds were then germinated by inoculating them on a modified MS basal culture medium (Figure 3.1. a). The medium was supplemented with 2.00 mgL^{-1} 6-BA and experimental control did not contain 6-BA. A total of 15 replicates were prepared for each soybean cultivar and the experiments were repeated three times. Data on seed germination was recorded at 3-day intervals until the final count on the 10^{th} day. The germination of soybean seeds was recorded as the protrusion of the root radicle and seedling length was measured using a ruler. Seedling establishment index was determined using the following equation as described by Adebisi et al. (2013):

$$\text{Seedling establishment index} = \frac{\text{Seedling length} \times \text{Germination \%}}{100}$$

3.4.5. Explant excision and inoculation

The double cotyledonary nodes were prepared from the seeds germinated on modified MS medium using a scalpel (Figure 3.1. b). The 10-day old seedlings were transversely cut on the hypocotyl segment, 5 mm beneath the cotyledons. The epicotyls were also excised from the base, at the cotyledonary junction. The embryonic

region found at the junctions, section of the hypocotyl and the two cotyledons were excised to obtain the double cotyledonary node explants. A total of 30 double coty-nodes were prepared for each set of 15 replicates. Explants were then placed with their adaxial side down on the multiple shoots induction MS medium (Figure 3.1 c).

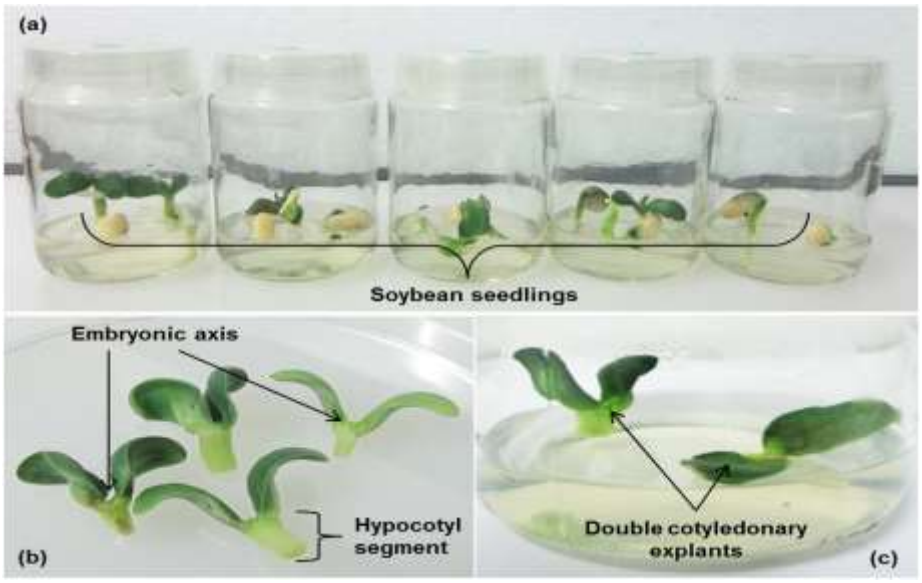

Figure 3.1. Soybean seed germination on modified MS basal medium: (a) seedling development *in-vitro*, (b) double cotyledonary node explants preparation, and (c) coty-node explants inoculated on MS culture medium supplemented with 2.00 mgL^{-1} 6-BA.

3.4.6. Multiple shoots induction

The cotyledonary node explants were incubated to initiate shoot growth in MS basal medium supplemented with 2.00 mgL^{-1} 6-BA. The shoot cultures were established and maintained in the growth room for three weeks. After 3 weeks, the dominating tall initiated shoots were excised-off the explants. The remaining shoot clumps with stunted multiple shoots and buds were sub-cultured on the same MS medium for further shoot growth. The explants remained intact, with cotyledons still attached to the junctions and hypocotyls in order to support further growth of stunted shoots. Multiple shoot induction was carried out under 24±2°C with 18-hour photoperiod (50–60 $\mu molm^{-2}s^{-1}$) in a tissue culture growth room for 21days.

3.4.7. Shoot elongation

After multiple shoot formation, the initiated shoots were excised from the explants and sub-cultured for shoot elongation. A full-strength MS basal medium was used for elongation and did not contain any plant growth regulators. The cultures were maintained in a tissue culture room under the same growth conditions as for shoot induction.

3.4.8. *In-vitro* rooting

The elongated shoots were then transferred to the rooting medium. The medium contained MS salts, vitamins, 3% sucrose and 0.25% gelrite. The MS basal medium was also amended with 2.70 mgL^{-1} indole-3-butyric acid and 2.30 mgL^{-1} 1-naphthaleneacetic acid.

3.4.9. Acclimatisation of rooted plantlets

After three weeks, rooted plantlets were rinsed with sterile distilled H_2O to wash off the gelrite medium and then transplanted to vessels containing sterile vermiculite. The plantlets were grown under 26°C temperature and 18-hour photoperiod for four weeks. After 4 weeks the plants (T_0) with at least two trifoliate leaves were transferred to the greenhouse.

3.4.10. Statistical analysis

Percentage seed germination and shoot induction responses such as the number of shoots and shoot lengths were monitored as growth parameters evaluated for every three (3) months for nine months. Data on germination was calculated in mean percentages, and the shoot numbers were calculated as the means of all replicates, compared using t-test and analysed using the analysis of variance in IBM SPSS Statistics 24.

3.5. RESULTS AND DISCUSSION

3.5.1. Effect of seed storage on germination

Soybean seeds of the different cultivars germinated on MS medium containing 2.00 mg/l 6-BA responded differently to the culture medium and period of seed storage. A significant variation in the germination percentages were observed among the different cultivars used during the period of incubation. Germination percentage reached 80% and above during the initial stages, for all cultivars except for TGx 1740-2F (with the highest germination percentage of 76%). In general, all soybean cultivars including TGx 1740-2F had higher germination percentages when seeds were used immediately after harvesting than the rest of the experiments conducted following seed storage (Figures 3.2 and 3.3).

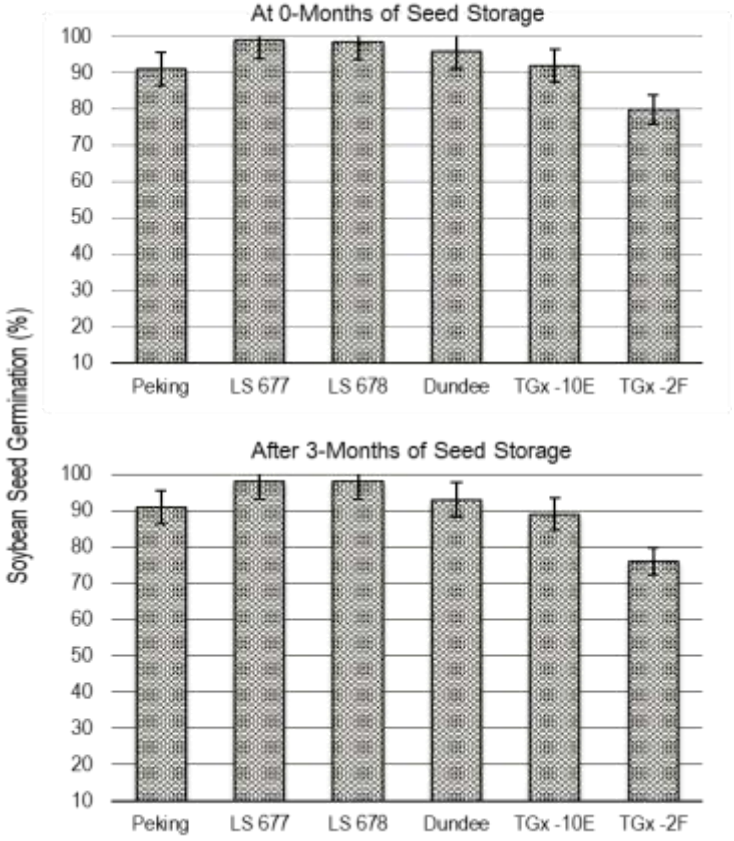

Figure 3.2. Soybean seed germination on modified MS basal medium immediately after harvest (0) and after 3-months of seed storage under ambient conditions.

Seed germination was at 80% and above, when seeds were cultured after harvesting and then started to decline. When soybean seeds were grown on the same medium

after three and six months (Figure 3.3), germination percentage showed a slight decline for cultivar LS 677, Dundee, and TGx 1835-10E. A further decrease was observed when the seeds were germinated after nine months of storage (Figure 3.3). The decrease in germination was observed in Dundee, LS 678, TGx 1740-2F and TGx 1835-10E during this period as indicated in Figure 3.3. Seeds of soybean cultivar TGx 1740-2F however, performed marginally higher than LS 678 and TGx 1835-10E during the last interval with 66, 62 and 55% germination rate respectively.

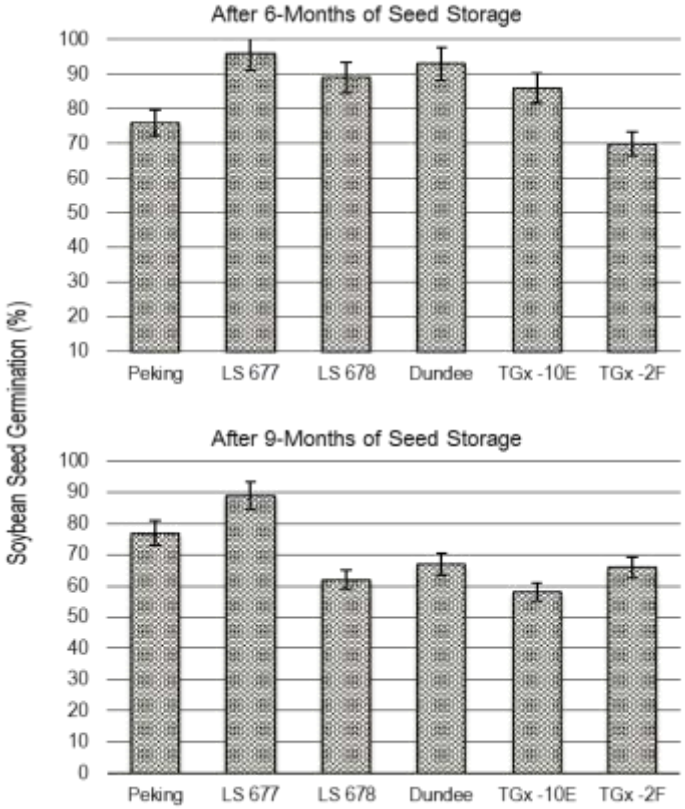

Figure 3.3. Soybean seed germination on modified MS basal medium following six (6) and nine (9) months after harvest and storage under ambient conditions.

Soybean cultivar LS 678 and TGx 1835-10E indicated a higher germination rate than TGx 1740-2F after the third and sixth month period of seed storage, until their decrease after 9-months. Cultivar LS 677 exhibited a significantly high germination percentage throughout the experiment. However, the percentage germination also indicated a decline with the increase in the period of seed storage. This trend was similarly observed in soybean cultivar Dundee, which was followed by the seeds of cultivar Peking. Overall, the performance of the soybean seeds illustrated a decline with respect to their mean seed germination over the different seed storage durations

(Table 3.2). The germination responses obtained in this study demonstrate observations similar to those made in Chapter 2. However, the seeds of soybean on MS medium started germinating only after 48 hours, while germination during standard germination test commenced within 24 hours of incubation. This indicates that seed imbibition on MS medium was delayed by 24 hours compared to imbibition on damp filter papers.

The observed differences in germination rates are in line with Dwivedi's (2014) report, which showed that germination time differs under different media. The findings of this report revealed that high germination percentage was recorded on culture medium containing phyto-hormones than any other kind of medium, such as damp paper towels or pre-treatment with fertilisers. Both Dwivedi (2014) and Singh et al. (2016) reported this, also indicating that seed storage had negative effects on seed vigour and germinability, with Singh further demonstrating that soybeans cannot be stored for more than a year in polythene bags without signs of seed deterioration. Retaining soybean seed quality during storage might be the only way to ensure better performance of the seeds when sown or grown as indicated by Afrakhteh et al. (2013). The above-mentioned reports are similar to the current results since the observations made also indicated a direct influence of seed storage to the germination rates achieved.

Although variations were assessed under *in-vitro* culture conditions, germination of seeds also appeared to be linked to plant genotype. Soybean cultivar LS 677 has consistently performed better, followed by LS 678 on two consecutive periods (0 and 3-months), then Dundee, Peking and the TGx cultivars respectively (Figure 3.2 and 3.3). This trend was similarly maintained in Chapter 2 as well. The relationship between percentage germination and the cultivar used has been reported by Khaliliaqdam et al. (2013). Their report among all tests suggested that soybean germination and emergence was dependent on the genotype used and seed quality maintained over time. Their germination tests revealed some interesting information among the cultivars where genotypes showing high electric conductivity (soybean cultivar DPX and Sahar) had higher germination percentages and that with less electric conductivity (cultivar Williams) had lower standard germination percentages.

3.5.2. Effect of seed storage on seedling development

The results obtained in this study (Table 3.2) showed a decrease in seedling establishment index (SEI) and a certain degree of seed deterioration probably during storage, before inoculation for germination on MS basal medium. The calculated seedling establishment indices decreased with the decline of seed germination as storage duration continued. This suggests a direct correlation between the mean germination percentage and SEI. Moreover, this observation implies that, the declining seed moisture content (as indicated in Chapter 2) may have caused a decline or slow pace of seedling development (Table 3.2). However, seedling morphology was not intensively evaluated since the presence of 2.00 mgL^{-1} 6-BA in the medium have an effect on seedling phenotypes and this response was shown to differ according to genotype.

In-vitro culture medium used allowed seeds to rehydrate and be able to attain adequate seed moisture required for germination and seedling development. Furthermore, the culture medium contained nutritive elements that supported seedling growth. The LS soybean cultivars (particularly; LS 677) remained high and produced better seedling growth, indicating possible long storability and resistance to deterioration compared to other cultivars used. However, cultivar Peking was found to be the poorest performer, perhaps due to the delay in germination, which has been strongly exhibited by this cultivar throughout the experiment.

Table 3.2. Correlation between mean germination percentage and germination index of soybean seeds cultured on MS medium supplemented with 2.00 mgL^{-1} 6-BA.

Soybean cultivar	Mean Germination Percentage (%)				Seedling Establishment Index (SEI)			
	Initial	3-months	6-months	9-months	Initial	3-months	6-months	9-months
LS 677	90.5^{a}	87.6^{a}	66.5^{a}	38.0^{a}	29.2^{a}	28.9^{a}	27.2^{a}	22.8^{a}
LS 678	86.5^{b}	83.3^{b}	62.2^{b}	28.2^{b}	28.4^{b}	21.5^{b}	14.8^{b}	14.5^{b}
Peking	55.5^{c}	82.3^{c}	50.7^{c}	41.8^{c}	28.0^{b}	16.2^{c}	8.7^{c}	12.2^{c}
Dundee	74.2^{d}	72.5^{b}	46.6^{d}	45.8^{d}	27.5^{c}	19.7^{d}	16.7^{d}	14.1^{b}
TGx 1835-10E	65.0^{e}	67.3^{b}	45.2^{d}	39.3^{b}	21.4^{d}	17.6^{e}	15.2^{b}	11.8^{c}
TGx 1740-2F	68.2^{f}	54.2^{c}	43.1^{b}	26.4^{d}	25.5^{e}	17.0^{e}	14.4^{b}	11.7^{c}

Values followed by the same letter are not statistically different at $p \leq 0.05$ according to t-test.

Daniel et al. (2009) emphasised that seeds deteriorate rapidly during storage and this may be reflected by the decline in physiological changes such as; seed viability and seedling vigour. Our findings are in line with Daniel's findings since the observed poor performance of the cultivars may have resulted from the loss of seed vigour during storage. Tang et al. (1999), Probert (2003) and Simic et al. (2006) reported similar results on the seeds evaluated in corn, legumes or grains, and yellow maize respectively. They all have reported a direct correlation between seed moisture content and seed vigour which had subsequent negative effects on seedling emergence and growth.

Seed moisture and storage conditions impact severely on seedling establishment and ultimately the entire growth of the plant. In *Ardisia crenata*, seed germination was reduced to 73%, accompanied by poor seedling development from seeds stored under dry conditions with less than 15% humidity. Dry conditions involved sowing the seeds in vermiculite-filled plastic boxes, stored outdoor under ambient low-temperature conditions without constant moistening with water (Tezuka et al., 2012). Adebisi et al. (2004) reported over 80% of seed germination and better seedling growth of soybean TGx1740-3F when seed moisture was well maintained in the seeds stored under room conditions (with ~50% humidity, at ~32°C) following chemical dressing with fungicides or insecticides.

However, significant deterioration of seeds during storage, which impacted negatively on germination and seedling growth, was also observed. The report by Adebisi et al. (2004) also suggested insect attack as another cause of seed deterioration in addition to the storage conditions. In contrast, the results obtained in this study were different from observations made by Adebisi et al. (2004) and Tezuka et al. (2012), attributing the variations and decline in seed germination as well as seedling development in *in-vitro* cultures used to the loss of seed viability that occurs during prolonged storage duration. Furthermore, physical evaluation of the germinating seeds did not indicate any evidence of attack by pests which may be attributed to the poor germination and seedling development observed in some of the soybean cultivars used.

3.5.3. Effect of culture medium on seedling establishment

Plant culture medium containing 6-BA has been successfully used by many researchers to grow soybean seeds during *in-vitro* germination. Yadav et al. (2010) reported double cotyledonary node explants derived from 3-day old seedlings germinated on MS medium containing Gamborg's B_5 vitamins, preconditioned with 2.0 mg/l 6-BA in mung bean (*Vigna radiata* L. Wikzek, cultivar ML-267). Involvement of 6-BA in the medium allowed for the development of suitably stout seedlings from which strong cotyledonary node explants can be derived from, for multiple shoot induction. Our previous studies illustrated how cytokinins, particularly 6-BA had an influence on seedling morphology (Mangena et al., 2015; Mangena et al., 2017). Findings in this study were in line with all other studies reported.

Figure 3.4. Soybean seedlings germinated on modified MS basal medium with 2.00 mgL^{-1} 6-BA (a), and without plant growth regulators (b) following 10-days incubation.

Figure 3.4 shows morphological differences that were observed during seed germination and seedling establishment. Soybean seedlings with thicker hypocotyls, reduced epicotyls, and inhibited root development were observed from MS basal medium containing 2.00 mgL^{-1} 6-BA (Figure 3.4 a). Well grown seedlings without deficiency symptoms were developed in all soybean cultivars, in the presence of 6-BA (2.00 mgL^{-1}). This descriptive evaluation of the seedling characteristics also conforms to observations made by Ma and Wu (2008) and Phat et al. (2015). The effect of 6-BA on seedling development for *in-vitro* regeneration in other crop species has also been frequently reported (Kim et al., 2001; Jeyakumar and Jayabalan, 2002; Dang and Wei, 2009).

3.5.4. *In-vitro* regeneration

Basal culture media modified with plant growth regulators (cytokinins and auxins) have been routinely used to initiate cell division and differentiation. The manipulation of these conditions play a crucial role for the optimisation of protocols used in efficient *in-vitro* regeneration, and tissue culture-based genetic improvement of crops, like soybean. Researchers continue to provide evidence suggesting that changes in hormonal compositions, both endogenously and exogenously have a greater effects on the entire regeneration process (Overvoorde et al., 2005; Teale et al., 2006; Phat et al., 2015). All studies have indicated that the physiological and biochemical processes during plant growth may be reinitiated, redirected and modified in order to achieve the critical changes required for crop improvement via *in-vitro* regeneration of plants.

3.5.4.1. Multiple shoots induction

On evaluating the efficiency of multiple shoot induction using plant growth regulators, we observed that 6-BA concentration (2.00 mgL^{-1}) used successfully initiated shoot proliferation amongst all the soybean genotypes used. The initial experiment which was followed by three months period of seed storage showed the highest number of shoots formed per explant (Table 3.3), where the shoot numbers differed significantly in the succeeding months. Figure 3.5 (a) demonstrates this induction of multiple shoots on the different cultivars used. In general, the frequency of shoot formation appeared to be influenced by the period of storage of the seeds used to develop the coty-node explants. The highest number of shoots, with a mean value of 13.73 shoots (maximum 16.77 shoots) per explant was observed in soybean cultivar Peking. This was slightly higher than the shoots induced after three months of seed storage and mean shoot number recorded in LS 677 (13.58), followed by LS 678 (13.38) and Dundee (10.52). Both LS 678 and LS 677 induced almost the same mean number of shoots in the first experiment, but slightly varied during and after the third experiment (Table 3.3). It should, however, be noted that the mean number of shoots obtained in soybean cultivar LS 677 (13.58 shoots) was never achieved in our previous studies using the same concentration of 6-BA with seeds stored three or more months under ambient conditions (Mangena et al., 2015).

Figure 3.5. Example of adventitious multiple shoots induced on double cotyledonary explants using MS medium supplemented with 2.00 mgL^{-1} 6-BA. (a) High number of proliferated shoots on explants from cultivar Peking, (b) multiple shoots on LS 677 and (c) adventitious shoots on LS 678 coty-node explants.

This observation was recorded during the 1st experiment as indicated on the Table (3.3), when multiple shoots were proliferated from the double coty-node explants derived from seedlings germinated using freshly harvested seeds. Therefore, the good quality seeds and adequate moisture content of the seeds (as described in Chapter 2) in this case may have had a profound effect on the number of shoots induced in cultivar LS 677. Furthermore, more shoots were also observed in all other soybeans, cultivar TGx 1835-10E and TGx 1740-27 with a mean of 10.57 and 9.15 shoots per explant in the initial experiment. In addition, these observations suggest that the decrease in shoot number during the 2nd, 3rd and 4th experiments may have resulted from a dramatic decline in seed viability, perhaps as a result of seed storage duration.

A number of researchers including; Kaneda et al. (1997), Benderradji et al. (2012), and Soto et al. (2013) reported about 6 shoots at most, per coty-node explant using 2.00 mgL^{-1} 6-BA. Shan et al. (2005) obtained maximum of 6 shoots as well per explant when explants were treated with 0.1 mg/l Thidiazuron (TDZ). The study by Shan et al. (2005) together with that of Keneda et al. (1997) furthermore suggesting that TDZ induced adventitious shoots more efficiently than 6-BA. Kaneda et al. (1997) additionally, argued that hypocotyl segments promoted more adventitious shoots than cotyledonary nodes. However, our results are in contrast with Kaneda and Shan's findings, clearly demonstrating that shoot multiplication is also increased by the quality

of seeds used to develop explants than the concentrations of PGRs used. This study clearly show that shoot induction can be negatively affected and influenced by a myriad of physiological and environmental factors emanating from storage conditions and periods. In addition, the culture medium and its composition, as well as the genotype or explant and explant sources can negatively affect shoot induction.

Table 3.3. Results of the induction of multiple shoots from the double cotyledonary nodes of soybean seedlings at different storage periods under ambient conditions.

Period	Soybean Cultivar	Min Shoots Induced	Mean Shoot No. ± SE	Max Shoots Induced	SD of the Mean	Coefficient of Variance
Initial	Peking	8.58[a]	13.73 ± 0.03[a]	16.77[a]	0.109[a]	19.3[a]
	LS 677	8.04[b]	13.58 ± 3.09[b]	14.03[b]	0.309[b]	18.1[b]
	LS 678	9.25[c]	13.38 ± 2.84[c]	13.50[c]	0.284[c]	18.3[b]
	Dundee	8.06[b]	10.52 ± 2.19[d]	10.97[d]	0.209[d]	21.0[c]
	TGx 1835-10E	5.09[d]	10.57 ± 1.47[d]	8.04[e]	0.474[e]	21.6[d]
	TGx 1740-2F	5.16[e]	9.15 ± 1.40[e]	7.14[f]	0.405[f]	19.6[a]
Three (3) Months	Peking	8.03[a]	13.04 ± 3.33[a]	16.07[a]	0.859[a]	24.3[a]
	LS 677	6.87[b]	13.00 ± 1.56[b]	13.73[b]	0.402[b]	12.0[b]
	LS 678	6.20[b]	9.73 ± 3.04[c]	12.40[c]	0.897[a]	28.3[c]
	Dundee	5.43[c]	9.87 ± 3.14[c]	10.87[d]	0.809[a]	28.9[c]
	TGx 1835-10E	4.40[d]	6.27 ± 3.04[d]	8.80[e]	0.784[c]	29.6[d]
	TGx 1740-2F	3.07[e]	5.93 ± 2.34[e]	6.13[f]	0.605[d]	39.5[e]
Six (6) Months	Peking	4.57[a]	9.10 ± 3.51[a]	15.13[a]	0.906[a]	38.6[a]
	LS 677	5.37[b]	9.37 ± 3.43[a]	10.73[b]	0.814[b]	36.6[b]
	LS 678	4.57[a]	8.44 ± 3.06[b]	9.13[c]	0.789[b]	36.3[b]
	Dundee	3.57[c]	8.85 ± 3.49[c]	7.13[d]	0.822[b]	39.4[c]
	TGx 1835-10E	4.20[b]	7.60 ± 2.52[d]	8.40[e]	0.652[c]	33.2[d]
	TGx 1740-2F	3.47[c]	4.23 ± 1.83[e]	6.93[d]	0.472[d]	82.1[e]
Nine (9) Months	Peking	3.10[a*]	7.77 ± 4.63[a]	6.27[a]	0.596[a]	59.5[a]
	LS 677	4.37[b*]	8.00 ± 4.79[a]	8.73[b]	0.599[a]	59.9[a]
	LS 678	3.30[a]	6.78 ± 4.34[b]	6.66[a]	0.641[b]	64.0[b]
	Dundee	1.83[c]	7.43 ± 4.64[c]	3.67[c]	0.624[b]	62.5[b]
	TGx 1835-10E	2.30[d]	5.53 ± 3.86[d]	4.60[d]	0.697[c]	69.8[c]
	TGx 1740-2F	1.97[c]	5.33 ± 3.22[d]	3.93[c]	0.604[b]	60.4[b]

*Means within columns followed by the same letter are not significant at p value less than 0.05 according to t-test. SD- standard deviation, SE- standard error, Min- minimum and Max- maximum.

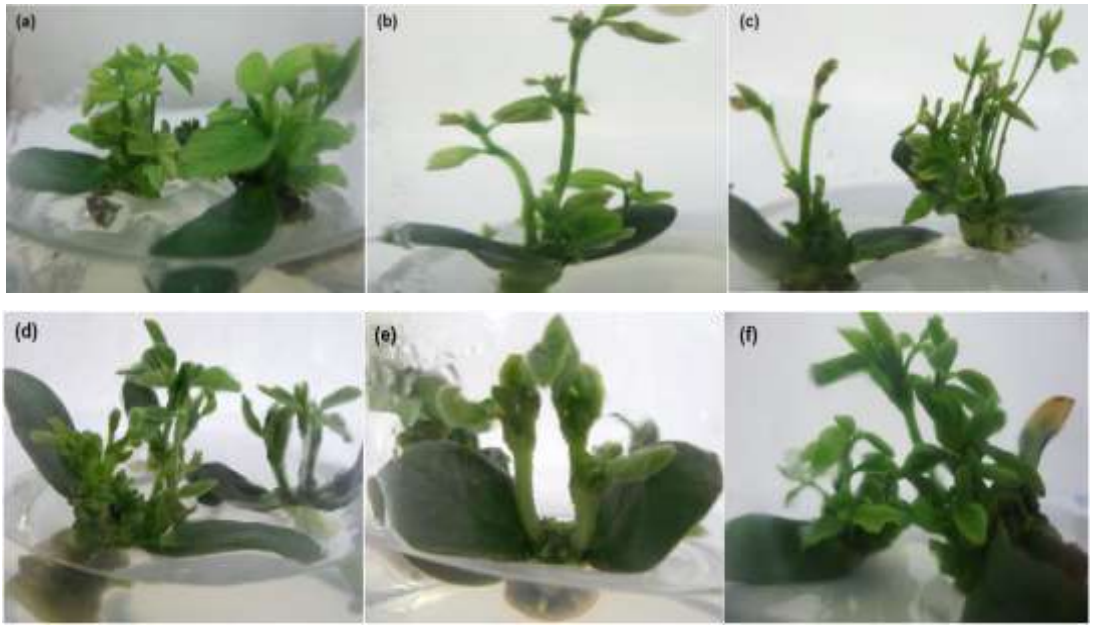

Figure 3.6. Development of adventitious shoots regenerated from various explants of soybean derived from 10-day old seedlings. (a) Shows shoots induced on Peking explants, (b) Dundee adventitious shoots, (c) elongated shoots on LS 678 coty-node explants, (d) TGx 1835-10E stunted shoots, (e) TGx 1740-2F fewer shoots, and (f) LS 677 multiple shoots.

Among the genotypes used; cultivar TGx 1740-2F in particular, illustrated very poor shoot initiation followed by TGx 1835-10E as shown on Table 3.3 and Figure 3.6 (d, e) following seed storage. From our observations, cultivar Peking, LS 677, LS 678, and Dundee achieved more than 90% regeneration efficiency (producing more than 10 shoots per explant) during the 1st experiment as indicated in Appendix 3.1 and Table 3.3. For TGx cultivars, the regeneration frequencies nonetheless, declined from 65% to 37.7 and 33.6% as well as 32.3 and 26% in the 3rd and 4th experiments for TGx 1835-10E and TGx 1740-2F respectively. The regeneration frequency of 65.5 and 60.5% was achieved in the first experiment for TGx 1835-10E and TGx 1740-2F respectively.

The mophology of the shoots was also affected (Figure 3.7). Abnormal shoots with signs of hyperhydricity were observed, as indicated in Figure (3.7. a and b). All shoots induced on explants derived from seeds stored for six to nine months exhibited highly stunted growths, with small compact calli cells formed around the hypocotyl bases of the explants (Figure 3.7. c). This was observed in all genotypes, particularly the TGx cultivars. Browning and yellowing of explants was also oberved. In some cases, the two axillary shoots directly arising from the cotyledonary junctions strongly inhibited further development of buds formed on explants (Figure 3.8). The excision of these

dominant shoots promoted vigorous growth of the buds into shoots as illustrated in Figure 3.8. However, the results obtained in this study also indicated that high shoot number was proliferated when axillary shoot excision was done during the initial period, when freshly harvested seeds were used, than after six and nine months of seed storage.

The responses of explants to *in-vitro* plant tissue culture have proved to be a highly complex phenomenon involving genetic mechanisms. Zeng et al. (2004) also shared the same view by indicating that the elite genotypes have a major influence by serving as a requirement for tissue culture establishment. However, many reports have indicated that the level of influence also varies according to genotype (Meurer et al., 1998; Paz et al., 2004; Jia et al., 2015). The varied shoot induction responses observed among the cultivars also indicate a strong influence of the genotypes on *in-vitro* regeneration. Genotype specificity of soybean, especially to regeneration during genetic transformation may continue to significantly limit the use of biotechnology in this crop's improvement until an effiecient genotype-independent protocol is introduced (Jia et al., 2015).

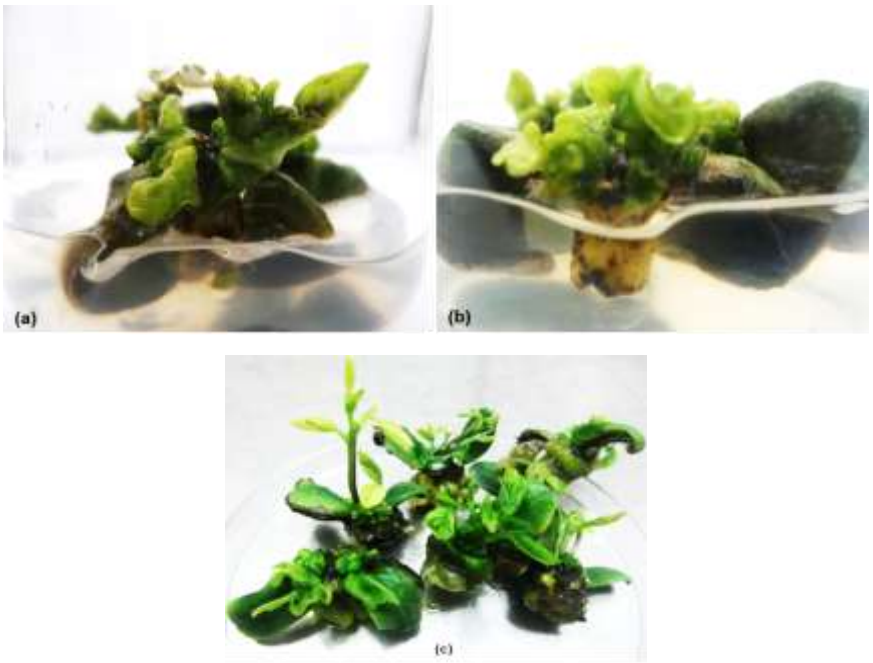

Figure 3.7. Examples of TGx 1740-2F (a and b), and TGx 1835-10E (c) double cotyledonary node explants showing multiple bud induction and reduced shoot growths.

Figure 3.8. Illustration of the suppression of soybean proliferative buds on double cotyledonary node explants, affected by dominant elongated shoots.

3.5.4.2. *In-vitro* elongation of induced shoots

The results obtained in this study did not reveal any relationship between seed storage and the efficiency of shoot elongation. Shoot elongation was observed on shoots and shoot clumps induced in all soybean cultivars used regardless of the shoots being formed immediately after harvest or after nine months of seed storage (Figure 3.9). Differences, however, were observed in the elongation frequency between individually sub-cultured shoots, and shoots grown as clumps. Shoot clumps in all cultivars generally required a prolonged period of incubation in tissue culture. At the same time, individually excised adventitious shoots required shorter periods of incubation and showed rapid elongation that took less than 3 weeks.

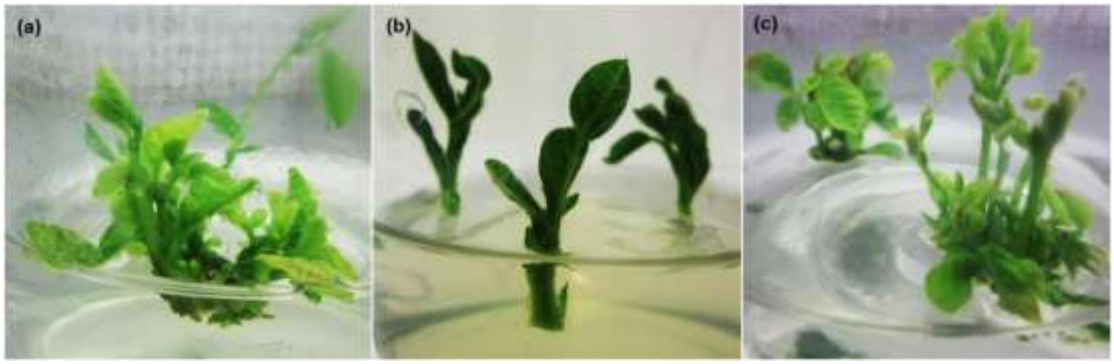

Figure 3.9. Soybean shoots derived from double cotyledonary node explants. (a) Shoot clumps subcultured on hormone free MS basal culture medium, (b) example of sub-cultured individual shoots, and (c) elongated shoots.

There was no correlation found between shoot elongation and seed storage duration or genotype as well as the moisture content of the seeds post shoot induction. The soybean cultivar TGx 1835-10E produced the highest mean shoot length of 4.85 cm

followed by Peking, LS 677, Dundee, LS 678 and TGx 1740-2F as indicated in Appendix 3.1, respectively. The average shoots lengths recorded was never consistent during all experiments but, did not appear to be genotype specific. The elongation potential of shoots which exhibited poor regeneration during shoot culture may have required some boosting with plant growth regulators as described by Ali et al. (2004). In cultivars such as Dundee and LS 677, shoots and shoot clumps transferred to MS basal medium without growth regulators initiated first the formation of adventitious roots instead of shoot elongation (Figure 3.10). This rooting resulted in gradual growth or elongation of stunted shoots and the formation of both small friable and compact callus cells, predominantly observed at the bases of excised shoots and clumps.

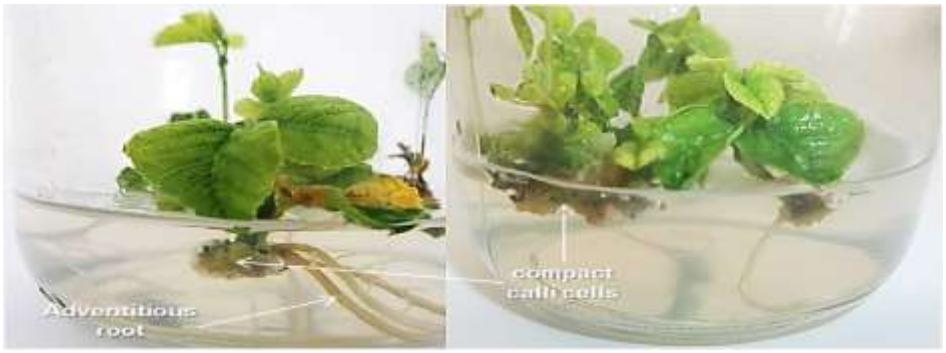

Figure 3.10. Examples of soybean shoots that induced roots instead of shoot elongation on MS culture medium without PGRs.

3.5.4.3. *In-vitro* rooting of induced shoots

Healthy shoots with considerably thicker stems and reasonable number of leaves were essential to achieve complete root initiation by being tolerant to long periods of culture (Figure 3.11. a). Cultures that were kept on elongation MS medium for three weeks until the elongated shoots produced several leaves, were capable of inducing healthy adventitious roots. This was observed throughout the experiment (0, 3, 6 and 9-months) and did not differ according to genotypes. Adventitious shoots that were elongated to about 3 cm in length were considered eligible for rooting. The addition of 2.70 mgL^{-1} IBA in combination with 2.30 mgL^{-1} NAA proved to be very detrimental for the formation of adventitious roots on the *in-vitro* elongated shoots. In all cultivars used, shoots formed adventitious roots, and those that experienced difficulties in root formation required prolonged incubation on hormone free MS medium to be rooted. Both PGR containing, and hormone free MS culture medium efficiently achieved root

formation. Soybean cultivar LS 678 (Figure 3.11. c) and some cultures of TGx 1835-10 and 1740-2F showed some level of sensitivity to rooting culture medium containing growth regulators (IBA and NAA). All of the sub-cultured shoots induced callus and were better rooted on basal medium with PGRs for the entire experiment.

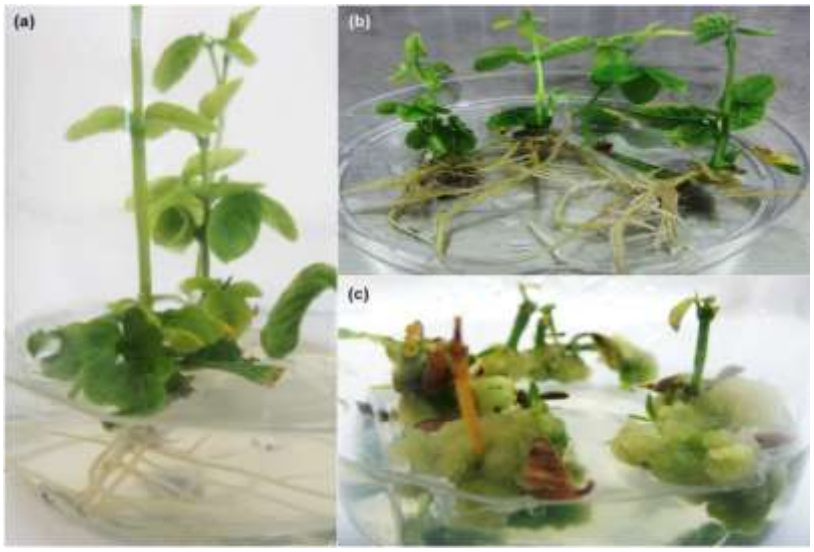

Figure 3.11. *In-vitro* elongated shoots on rooting MS culture medium without PGRs (a). (b) Rooted shoots ready for transplanting, and (c) Callus formation on some shoots sub-cultured for rooting on medium supplemented with 2.70 mgL^{-1} IBA and 2.30 mgL^{-1} NAA.

The LS 677, LS 678 and Peking archived the highest mean number of adventitious roots in all cultures (Appendix 3.1). Soybean cultivar LS 678 induced the highest mean number of roots during the initial and after 3-months periods of seed storage on hormone free MS medium (Appendix 3.1). Nonetheless, these variations were not significantly different and root formation was observed in all cultures, with at least two (2) adventitious roots on average, formed per explant. The successful rooting of *in vitro* derived shoots has been reported in most legumes. Eapen and George (1993) reported root formation from various explants of pigeonpea. Dayal et al. (2003) and Radhakrishnan and Ranjithakumari (2007) also reported *in-vitro* rooting in *Cajanus cajan* (L.) Millsp. leaf explants and soybean via half seed explants, respectively. All reports did not find any positive relationship between seed storage and root formation or correlation to the genotype of induced shoots, which is in line with what is reported in this study.

3.5.4.4. Acclimatisation of regenerated plantlets

After *in-vitro* rooting of shoots, the plantlets were acclimatised and well established on soil as indicated in Figure 3.12. To achieve this, hardening of plantlets in a growth room with 150–250 µmol $m^{-2}s^{-1}$ light intensity on a vermiculite medium was employed (Figure 3.12, b). In case of plantlets regenerated from explants showing low proliferation capacity and less vigorous development, rooted shoots were hardened for longer periods than those showing active growths. Plantlets from cultivars TGx 1740-2F and TGx 1835-10E were difficult to harden since they showed stress sensitivity to each transfer step of acclimatisation. This poor response was attributed to the inability of genotypes to cope with the change of environment from a heterotrophic to an autotrophic state, and this was also observed in soybean cultivar Dundee.

The transfer of plants to greenhouse conditions exposes them to high humidity, low light intensity and fluctuating temperatures. Although, most of the plantlets from these three cultivars efficiently developed adventitious roots, and shoots, most of them were incapable of overcoming hardening-related stress symptoms. Therefore, steps involved during hardening and acclimatisation may have caused a decline in the survival rates of normal rooted plantlets as indicated by Texeira et al. (2011). The conditions that plantlets are exposed to during acclimatisation differ with those in tissue culture growth rooms as reported by Mangena et al. (2017) and as indicated above. These variations in growth conditions are sometimes a big disadvantage in plant tissue culture, since they cause negative effects on the growth potential of the regenerated plantlets. This has been seen in many legumes, including soybeans during the *ex-vitro* stage as supported by Polisetty et al. (1997) and Paz et al. (2006).

The overall impression made by results obtained in this study is that, shoot regeneration depended largely on factors such as seed viability, seedling vigour and time used for seed storage before use for plant tissue culture. As a result, the best cultivar performance during acclimation was observed in soybean Peking, followed by LS 677 and LS 678, which showed high regeneration frequency over the entire period of seed storage. These genotypes were followed by soybean cultivar Dundee and TGx cultivars respectively. However, cultivar performance in this phase was not indicative

of good response by one cultivar over the other because variability showed to be linked to the genotype and the influence of storage on seed/explant vigour during storage periods. The possible negative or positive correlation found in this study between seed storage and successful soybean plant establishment is discussed further in 3.5.5.

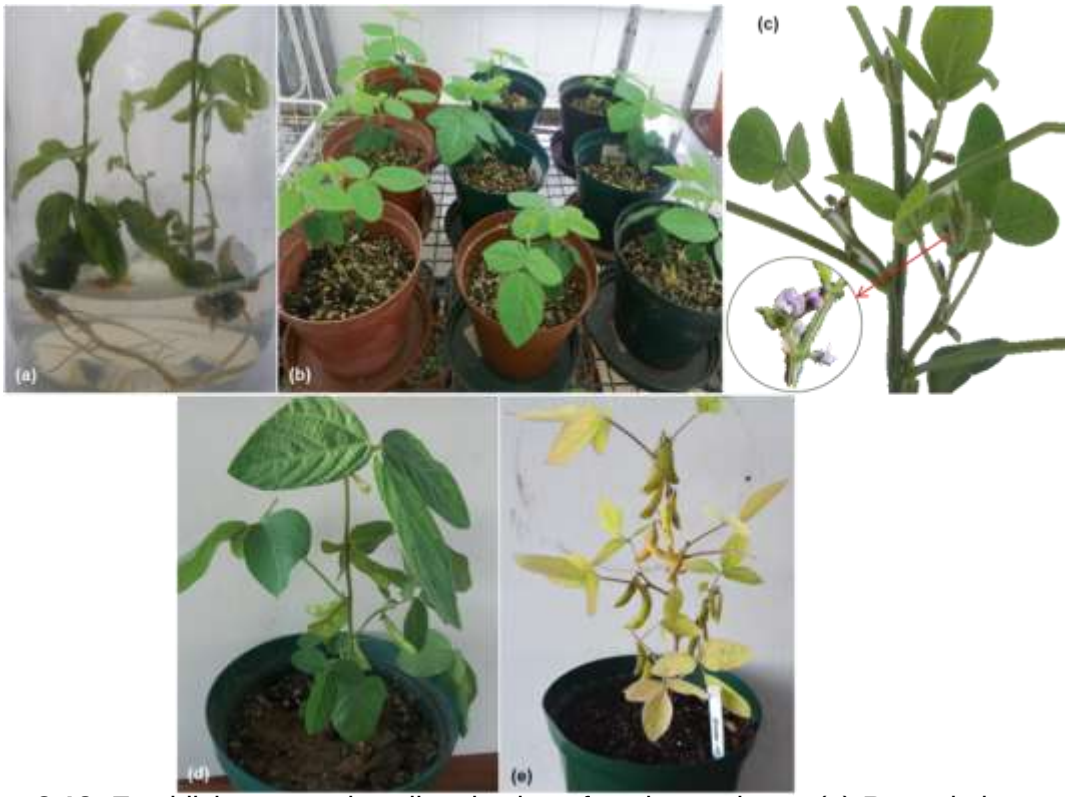

Figure 3.12. Establishment and acclimatisation of soybean plants. (a) Rooted shoots on MS rooting medium, (b) transplanted plantlets derived from rooted shoots formed, (c) soybean plant showing flowering and pod development, (d) fruit pods during maturation and pod filling, and (e) matured soybean pods.

3.5.4.5. Flowering and pod production

We noted that more than 50% of plants successfully regenerated from all genotypes flowered and subsequently fruited (Figure 3.12; c, d and e). The plantlets obtained from the hormone free medium during elongation and rooting however, achieved rapid growth and flowering than those established from PGRs containing medium. This is attributed to simultaneous axillary shoot initiation and development that has been easy to stimulate without the attempt to form multiple shoots. The already elongated axillary shoots were simply subcultured on MS medium for rooting and easily acclimatised, especially without the additional elongation/rooting requirements as seen on induced adventitious multiple shoots. Although not all cultivars showed flowering and fruiting,

82% of the successfully regenerated soybean plants produced flowers and fruit pods (Appendix 3.1). The plants that failed to flower demonstrated a less vigorous growth during acclimatisation. This occurred in spite of the plantlets reaching vegetative growths, of at least up to V3 stage, and then flower as well as pod abortions were observed. Soybean cultivar Dundee and TGx 1835-10E indicated poor flowering and fruit set of less than 50% on average (Appendix 3.1).

This response was consistent over the periods of seed storage and may have been due to genotypic response to inefficient flowering stimulus required by the plants as a result of the environmental growth conditions. The different genotypes might have required different day lengths in order to reach their full genetic potential for matured flowering and pod formation. Dan and Reichert (1998) reported 94% survival rates without sterility observed on soybean plants derived from BA (5.0–10 µM) under 16 h photoperiod for 4 weeks. However, the study clearly indicated that not all regenerants have been successfully grown to maturity as a result of cultivar variability. Comparatively, varied cultivar regeneration capacity among soybeans was also reported by Texeira et al. (2011) and Raza et al. (2017). Our findings are in agreement with these reports, indicating that flowering and fruiting in soybeans is partly dependent on the genotype and growth conditions, particularly day lengths and light intensity.

3.5.5. Effect of seed viability on shoot induction

Reports such as those of Olhoft and Somers (2001) and Paz et al. (2004) have established that the genetic improvement of recalcitrant crops such as, soybean is more reliant on an efficient regeneration protocol. However, the development of an efficient and efficacious regeneration protocol also depends largely on factors including, seed viability, storage conditions, and the genotype. Based on the regeneration frequencies (%) illustrated in Table 3.3 and Appendix 3.1, the cultivars illustrated the following: (i) the decrease in seed germination and seedling growth with respect to prolonged seed storage and (ii) the number of adventitious shoots formed on coty-node explants decreased as the period of seed storage was increased. The cultivar responses and their coefficient of variance varied significantly between the growth periods (Table 3.3). Furthermore, the correlation plots (Appendix 3.2) also

indicated a significant proportion of variance between germination index and the mean number of shoots proliferated. The results obtained showed a strong positive correlation as the period of storage was increased. In general, the mean number of shoots formed per explant decreased, with the decrease in germination and the increase in the period of seed storage. Therefore, to give a clear account of the effect of seed storage on subsequent *in-vitro* regeneration observed in this study, comparison of results obtained during the whole experiments were analysed.

3.5.5.1. Impact of seedling vigour on shoot regeneration

The problem of seed deterioration during seed storage has been reported, particularly, for field emergence (Egli et al., 2005; Wang et al., 2012). The prolonged seed storage, as well as the loss in seed moisture (as indicated in Chapter 2) had a negative impact on seedling vigour, which caused a decrease in explant vigour. However, this phenomenon is not yet accounted for in *in-vitro* tissue culture. In this study, we have observed that there is a strong relationship between seedling vigour, which had an influence in *in-vitro* shoot regeneration efficiency. Poor germinability of seeds also had negative effects on shoot induction (Appendix 3.2). However, there was synergy between seedling establishment on MS medium for the development of coty-node explants, and the number of shoots developed on those explants.

The probability plots in Appendix 3.2 and 3.3 confirmed these positive correlations in the number of shoots proliferated and the nature of seedlings (normal or abnormal) from which explants inducing shoots are derived from. Kaneda et al. (1997) and Shan et al. (2005) observed similar results when they proliferated shoots on explants derived from seedlings developed on MS medium containing PGRs. Shoot growth appeared to be improved, supported and directly linked with the quality, age, storage period and conditions of the seeds used to develop the explants. The results (Appendix 3.1 and 3.2) clearly indicates that, shoot induction was highest, during the initial period followed by three months, and the values decreased with prolonged seed storage.

3.5.5.2. Influence of explant vigour on shoot induction

Explant vigour also presented a strong correlation with the initiation of adventitious shoots from double cotyledonary node explants used (Appendix 3.3). Soybean cultivar Peking and LS 677 exhibited a reasonably high vigour index, for *in-vitro* shoot induction over nine months of storage. Even though, there was a decrease in explant vigour, growth of shoots obtained remained significantly high in all soybean cultivars. The lowest performers (cultivar TGx 1835-10E and TGx 1740-2F) also demonstrated some positive responses in terms of the mean number of shoots achievable per explant over the entire period. These observations suggest a more gradual effect that explant vigour have on the regeneration capacity of the plant. These observations appeared to be similar in all soybean cultivars and were supported by the vigour indices observed in all genotypes.

3.5.6. Conclusions

The efficiency of *in-vitro* seed germination and seedling development, and adventitious shoot formation is negatively affected by the decrease in seed viability. The regeneration efficiency of the soybean cultivars; LS 677, LS 678, Peking, and Dundee were found to be less affected while TGx cultivars (1835-10E and 1740-2F) were more negatively affected by seed storage. However, a significant number of shoots were induced in all soybean cultivars suggesting their suitability for further tests in *Agrobacterium*-mediated genetic transformation.

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CHAPTER 4

EVALUATION OF SELECTED SOYBEAN CULTIVARS FOR CALLUS INDUCTION EFFICIENCY USING DOUBLE COTY-NODE EXPLANTS

4.1. INTRODUCTION

4.1.1. Background to the study

Embryogenic tissue explants are normally cultured to produce callus and suspension cell cultures. These cultures have the potential to produce plantlets via somatic embryogenesis. They can as well be used to produce genetically transformed plants. Finer (2016) reported the method for generating transgenic soybeans by particle bombardment of somatic embryos proliferated from the cotyledons of immature seeds. Furutani and Hidaka (2004) induced and co-transformed embryogenic tissues from highly clustered globular callus tissues for plantlet regeneration from transgenic embryos. The establishment of callus cultures in soybeans have been achieved, especially using different seedling explants, at times serving as a critical starting point for other different types of cell cultures (Franklin and Dixon, 1995).

Transgenic plants from maize, pigeonpea and cassava have been produced from embryogenic callus cell cultures transformed widely via biolistic transfer of DNA coated particles. All these reports indicate that callus proliferation may be largely linked with the production of embryogenic tissue to produce transgenic plants. However, the use of this approach coupled with different modern transformation techniques still presents challenges. Amongst others, the occurrence of chimerism and genetic mosaic in callus tissues used for transgenic plantlet regeneration causes some of the problems encountered during *in vitro* tissue culture. The problems caused may include inefficient regeneration frequencies, genotype specificity and lack of reproducibility of the tissue culture protocol used.

4.1.2. Callus formation

Callus formation from double cotyledonary node explants involves the development of progressively more random planes of rapid cell divisions. The result is the formation of less specialised cells and disorganised cell structures. Sathyanarayana and Varghese (2007) described this mass of cells as a coherent but unorganised and amorphous tissue formed by the vigorous division of plant cells. These cells are often induced as a result of a wound response or through the treatment of explants with plant growth regulators. Callus tissues induced *in-vitro* often have similar characteristics like the ones that are naturally formed in response to tissue injury. Variations in terms of morphology, cellular structure and metabolic activities between callus cells derived from tissue culture and injured plant tissues also occur (Sathyanarayana and Varghese, 2007).

4.1.3. Motivation to the study

Many reports have focussed on the establishment of protocols to improve agronomic traits of soybeans. Both *in planta* and tissue culture-based techniques have been attempted for the realisation of the yield potential of this crop. Zia et al. (2011) reported a protocol that bypasses laboratory tissue culture steps. The study showed a procedure using *A. tumefaciens* containing EHA 105 with a pROKIILFYGUStnt plasmid, injected on NARC-4 and NARC-7 soybean pods at three developmental stages (pod formation, pod development and seed filling or maturation stage). This report and many others were aimed at improving soybean growth and yield, thus clearly indicating the magnitude of investigating the regenerability and transformation efficacy in this crop. Callus cultures have been used for the above-mentioned endeavours, including gathering molecular and physiological information to advance genetic transformation studies (Bourgaud et al., 2001). These cultures are still used as a form of micropropagation procedure and the initial stage/step for establishment of cell suspensions, protoplast culture, as well as direct and indirect somatic embryogenic cultures (Hartmann et al., 2011). Therefore, this chapter evaluated the totipotency of cotyledonary node tissues via callus initiation culture in all genotypes used in this study.

4.2. LITERATURE REVIEW

4.2.1. Callus culture

The callus culture refers to the growth of unorganised cell proliferation from isolated plant cells, tissues or organs in *in-vitro* tissue culture (Hartmann et al., 2011). Callus cells are produced from a single undifferentiated cell on a culture medium containing a combination of two or more growth promoting hormones; auxins and cytokinins. The plant growth regulators (PGRs) auxins and cytokinins are a group of natural and synthetic active substances that are used to stimulate growth of callus in plant tissue culture (Gaspar et al., 1996). These growth substances are being extensively used in both research and horticultural applications. The hormone auxin plays a crucial role in the production of callus, as well as root formation and the elongation of stems (Trigiano and Gray, 2005). However, cytokinins in callus culture are required to stimulate cell division in very low or higher concentrations since optimum amounts favour shoot induction. Cytokinin are routinely used in plant tissue culture to stimulate cell division leading to adventitious shoot formation (Franklin and Dixon, 1995).

4.2.2. Initiation and maintenance of callus culture

The success of callus culture initiation depends on the optimum culture conditions used for its establishment and maintenance. Murashige and Skoog (1962) basal culture medium is the most commonly used plant tissue culture medium for callus induction. This medium contains great amounts of salts due to its high content of potassium and nitrate and was based primarily on the mineral analysis of tobacco plant tissues. Gamborg's B5 medium (Gamborg et al., 1968) was established for soybean callus culture and contains low amounts of nitrate and ammonium salts compared to MS medium (Trigiano and Gray, 2005). B5 medium gained a widespread use in *in-vitro Agrobacterium*-mediated genetic transformation (Zhang et al., 1999; Olhoft and Somers, 2001; Paz et al., 2006). But, both B5 and MS are suitably optimised for use in the initiation of callus culture. Exogenously applied regulators such as 2.4-dichlorophenoxyacetic acid and naphthaleneacetic acid or kinetin are used for the initiation and development of calli cells (Pierik, 1997). The auxin-cytokinin ratio is very crucial for the morphogenesis of callus cells from the cultured tissues. According to

Trigiano and Gray (2005) the PGRs always exert morphogenic effects depending on their ratio and concentration used. Concentrations between 0.1–1.0 mgL^{-1} for cytokinins and 0.5–2.0 mgL^{-1} auxins are usually used for callus initiations and maintenance (Hartmann et al., 2011).

4.2.3. Applications of the callus culture

Callus culture establishment is viewed by Franklin and Dixon (1995) as a relatively routine procedure which serves as a prerequisite for a range of subsequent regeneration approaches. According to Ikeuchi et al. (2013), this technique can be applied for *in-vitro* regeneration, embryogenesis and other various research and industrial applications. For example, Tepe and Sokmen (2007) reported the use of callus culture from *Satureja hortensis* in the production of a secondary metabolite rosmarinic acid. The highest rosmarinic acid biomass yield was obtained from MS medium supplemented with 1.0 mgL^{-1} of indolebutyric acid (IBA) and 5.0 mgL^{-1} 6-benzylaminopurine (6-BA). In the case of polyploidisation to improve the horticultural value of flowering perennials, Nakono et al. (2008) reported the regeneration of tetraploid *Tricyrtia hirta* from a 1-year-old embryogenic callus culture. Other applications of *in-vitro* callus culture include suspension culture (Jain, 2001) and regeneration of transgenic plants from callus tissues of soybean (Liu et al., 2013).

4.2.4. Challenges in establishing callus culture

The use of callus culture is still largely challenging to achieve for many crop, medicinal and ornamental plant species. The culture limitations are specific to plant species or plant genotype to be cultured (Trigiano and Gray, 2005). Although, there is sufficient information in the literature to indicate which culture medium to use, for example, MS and B5 (Table 4.1). Only a few have been widely successful (Franklin and Dixon, 1995). The establishment of a callus culture does not only rely upon appropriately determined medium composition, but also on the genotype to be cultured. The problem of genotype specificity on callus-based regeneration protocols has also been a challenge, especially in genetic engineering. Niroula and Bimb (2009) reported a significant influence by callus culture on the subsequent recovery of regenerated plants from Nepalese rice genotypes. The cultures were prone to contamination and

required prolonged culture periods. Jain (2001) reported poor genotype stability in newly acquired agronomic traits of sugarcane, ryegrass, potato, maize and rice regenerants, also derived from callus cultures. Furthermore, the report indicated that in addition to this the traits may not be heritable, and the plants showed slower growth rates, and some had increased uncontrollable erectness. This implies that cell proliferation and re-differentiation during establishment of callus may lead to loss of transgene expression in successive generations or may cause serious epigenetic aberrations.

4.2.5. Soybean callus culture

Soybean plant tissues from leaves, stems, roots and seedlings have been used *in-vitro* to establish callus cultures. Zieg and Outka (1980) gave one of the initial reports on the formation of callus culture protocols from regenerated protoplasts in soybean. Optimisation of the regeneration protocol using half seed explants in the soybean organogenesis via callus culture was also reported by Radhakrishnan and Ranjithakumari (2008). The establishment of culture conditions that promoted indirect embryogenic growth of cells in the *in-vitro* culture of various plants made the development of callus cultures possible. Success of calli cultures relied on the ratios of auxin and cytokinin growth regulators as reported by Oswald et al. (1977). Radhakrishnan and Ranjithakumari (2007) initiated callus using half seed explants with 4.4–23.5 µM of cytokinin and 4.9–27.0 µM auxins. Kinetin (4.7–23.5 µM) and 6-BA (4.9–24.5 µM) as well as NAA (5.4–27.0 µM) and IBA (4.9–24.5 µM) were reported to proliferate callus initiation on MS media. These different combinations and varying concentrations were able to induce callus production in a wide range of crop species, including rice and maize (Kotchoni et al., 2012). Callus culture in soybean is considered advantagious over direct organogenesis when the culture is aimed at selecting and regenerating transgenic plants. Yang et al. (1991) successfully reported transgenic soybean calli with nodular characteristics induced by co-cultivating cotyledons derived from mature seeds of cultivar Kihoshu with strains of *Pseudomonas maltophilia*. However, continuing problems of efficient selection of transgenic calli from chimeras which fail to regenerate transgenic plants hinders the facilitation of callus culture in *in-vitro* genetic transformation of many genotypes (Radhakrishnan and Ranjithakumari, 2008).

4.3. PURPOSE OF THE STUDY

4.3.1. Study hypothesis

Soybean double cotyledonary node explants grown in MS basal culture medium without 0.5–4.0 mgL^{-1} of auxin-cytokinin in combination will not establish a successful callus culture.

4.3.2. Study aim and objectives

The main aim of this study was to assess the callogenesis efficiency, and the genetic transformation capacity of selected soybean genotypes during callus culture.

The main objectives were to:

i. Test the different suitable hormonal concentrations and combinations for callus initiation, preferably KIN, NAA and IBA.
ii. Establish an *in-vitro* callus cell culture protocol for soybean double cotyledonary node explants.
iii. Induce callus formation on double coty-node explants transformed with *A. tumefaciens*.

4.4. MATERIALS AND METHODS

4.4.1. Plant material and surface sterilisation

Chlorine gas surface sterilisation was used in this experiment to decontaminate the soybean seeds (*Glycine max* L.) genotypes: Dundee, LS 677, LS 678, TGx 1740-2F, TGx 1835-10E and Peking as indicated in section 3.4.4 (Chapter 3).

4.4.2. Seed germination and seedling development

The sterilised soybean seeds were germinated by inoculation (3 seeds per culture vessel) on MS basal culture medium. The medium was prepared by mixing the macro- and micro-nutrients as well as the iron source and vitamins. Then, the medium was supplemented with 30 gL^{-1} sucrose, 2.5 gL^{-1} gelrite and 2.00 mgL^{-1} 6-BA. The seed cultures were then incubated in a culture room at 24±2°C temperature with 16-hour photoperiod. The total number of germinated seeds, in which each cultivar consisted of 15 replicates, was recorded after 10 days.

4.4.3. Preparation of callus culture media

A full-strength MS basal culture media containing macronutrients, micronutrients and vitamins, 3% sucrose, as well as 0.25% gelrite were used in this study. The different concentrations and combinations of PGRs were added into the basal media according to Table 4.1. The PGRs were prepared as stock solutions by dissolving analytical reagent grade hormones in distilled water. For 1L of liquid MS medium, the required volumes of each stock solution were pipetted according to the table (Table 4.1).

Table 4.1. Concentrations and combinations of plant growth regulators used for establishment of callus culture for the selected soybean cultivars.

	Treatments	Kinetin (mgL^{-1})	Indole butyric acid (mgL^{-1})	α-Naphthalene acetic acid (mgL^{-1})
MS culture	**MS-**A	0.70	2.70	3.20
Media	**MS-**B	0.70	3.20	2.70
	MS-C	0.70	3.20	---
	MS-D	0.70	---	3.20
	MS-E	0.50	1.20	1.20
	Control	0.00	0.00	0.00
Callus induction on cotyledonary node explants infected with *Agrobacterium*				
Selected MS	**MS**-A*	0.70	2.70	3.20
medium	**MS**-B*	0.50	1.20	1.20

MS culture media with asterisks on the Table (4.1) refers to the concentrations of PGRs to be included that would have showed better proliferation of calli cells on the selected media (MS-A to MS-E) on un-infected cotyledonary explants, for use on explants infected with *A. tumefaciens*.

4.4.4. Explant preparation and inoculation for callus induction

The double cotyledonary node explants were prepared from the 10 days old soybean seedlings developed as described in 4.4.2. Double coty-node explants were prepared as described in Chapter 3 (3.4.5). The explants were then inoculated on MS media containing different combinations of plant growth regulators (Table 4.1). Calli were initiated by incubation in a growth room at 24±2°C temperature, 50–60 $\mu molm^{-2}s^{-1}$ light intensity and 16 h photoperiod for four weeks.

4.4.5. Callus induction on explants co-cultivated with *A. tumefaciens*

To test for the efficiency of callus initiation on double cotyledonary explants infected with *A. tumefaciens* containing the *oc-1* gene on pTF101.1 vector, the transformed explants were cultured on MS media A and E. The media selected induced the best callus initiation and development as indicated on 4.4.3 and 4.5.2 without co-cultivation with *Agrobacterium*. The procedures described below were followed:

4.4.5.1. *Agrobacterium* and infection of explants

Agrobacterium tumefaciens strain EHA 101 constituting construct pTF101.1 was used for the infection of double cotyledonary explants. The coty-node explants were derived from 10-day old soybean seedlings and prepared as described in Chapter 3 (3.4.5). The *Agrobacterium* was re-initiated, centrifuged (for 10 minutes at 4000 rpm) and the pellet resuspended in liquid infection medium (OD_{650} = 0.6 to 0.8) containing B5 major and minor salts, vitamins, iron source, 30 gL^{-1} sucrose, 0.59 gL^{-1} MES and the pH adjusted to 5.4. The infection medium was further supplemented with 1.67 mgL^{-1} 6-BA, 0.25 mgL^{-1} GA_3 and 40 mgL^{-1} acetosyringone as described by Paz et al. (2006). Incisions were made on cotyledonary junctions of the explants and prepared explants were then added in the infection medium with *Agrobacterium* and incubated at room temperature for 30 minutes with gentle shaking (at 250 rpm) on an orbital shaker (Orbishake-Labotec).

4.4.5.2. Co-cultivation of explants with *Agrobacterium*

After infection, the explants were then placed on co-cultivation medium containing B5 salts (major salts, minor salts, vitamins and iron source), 30 gL^{-1} sucrose, 3.9 gL^{-1} MES, 4.25 gL^{-1} Noble agar, at pH 5.4. Filter sterilised 0.25 mgL^{-1} GA_3, 400 mgL^{-1} cysteine, 154.2 mgL^{-1} dithiothrietol (DTT) and 40 mgL^{-1} acetosyringone were added into the medium after autoclaving. The cultures were then incubated for three days in a tissue culture growth room.

4.4.5.3. Callus initiation

Callus culture was established using infected explants on MS medium prepared as described above (4.4.3). The callus induction medium contained 0.70 mgL^{-1} KIN, 2.70 mgL^{-1} IBA and 3.20 mgL^{-1} NAA (MS-A) and the other MS medium (MS-E) contained 0.50 mgL^{-1} KIN, 1.20 mgL^{-1} IBA and 1.20 mgL^{-1} NAA. The media was further supplemented with the required amounts of filter sterilised ß-lactam antibiotics cefotaxime (100 mgL^{-1}) and vancomycin (50 mgL^{-1}) antibiotics, added onto the media after autoclaving. For the control, un-infected explants were prepared and subcultured on callus induction medium containing only the antibiotics.

4.4.6. Culture conditions

The prepared explants for callus initiation were incubated in a growth room under controlled temperature (24±2°C), 16 h photoperiod of light (60 $\mu molm^{-2}$ s^{-1}) and humidity level below 10% for 3 weeks for cell proliferation and callus induction. Cultures for the initiation of callus from the transformed explants were maintained under similar conditions described.

4.4.7. Data collection and analysis

Three independent experiments with 15 replicates containing two explants each were performed to evaluate the effect of selected PGRs on callus induction for both infected and uninfected coty-node explants. At the end of each experiment, the number of explants inducing callus were recorded. Parameters including callus texture, colour and tissue senescence were visually examined and recorded.

4.5. RESULTS AND DISCUSSION

4.5.1. Seed germination and seedling development

The aseptic seedling development was required in this study in order to efficiently establish a protocol for callus induction. Seed germination as measured by protrusion of the radicle was achievable from the 3rd day of incubation in a growth room. However, seed cultures were kept in the culture room for 10-days and it was found that all cultivars achieved more than 60% germination within this period (Figure 4.1). More than 50% seed germination was recorded in all soybean cultivars within five days except in cultivar Dundee and Peking. It was observed that, germination of Peking seeds was gradual but, later increased to 62% on average within the 10 days of incubation.

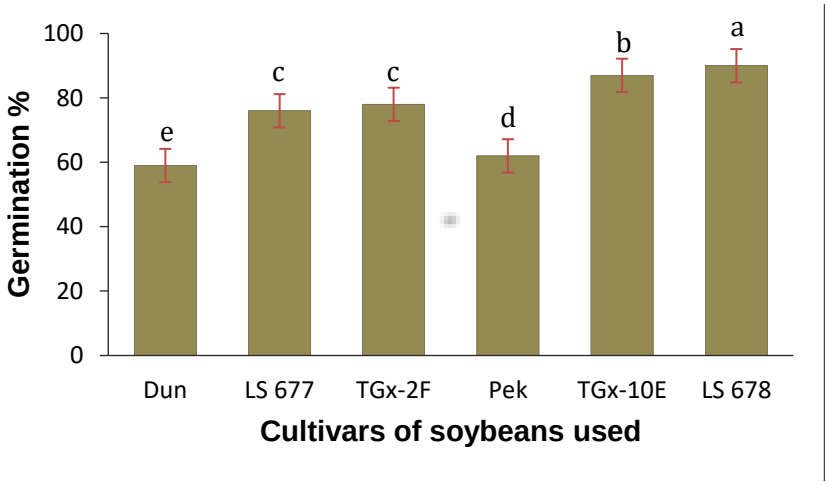

Figure 4.1. Percentage germination achieved within 10 days of incubation under tissue culture conditions.

The observations made in cultivar Peking, may be as a result of a slow seed imbibition than seed viability, because black seeds have strong seed coats compared to the yellow seeds. This observation is in accordance with the results obtained in Chapter 2 and 3 as well as according to Bahry et al. (2017). The seeds proved to be viable by ultimately achieving more than 60% overall germination percentage, which is more than the 50% germination rate prescribed by ISTA (2003). There are reports suggesting that, reduced seed viability may negatively affect seedling vigour required for successful initiation of callus cells (El-Kaaby et al., 2015; Radhakrishnan and Ranjithakumari, 2007).

El-Kaaby et al. (2015) reported the decrease in callus mean fresh weight due to the reduced seed viability. However, El-Kaaby's report attributes reduced germination frequency to surface sterilisation of the seeds conducted in 6% sodium hypochlorite (6%-NaOCl). The highest percentage seed germination was observed in LS 678 followed by TGx 1835-10E with 90 and 87% respectively (Figure 4.1). The results furthermore showed that, the performance of LS 677 and LS 678 was significantly different (76 and 90% respectively) compared to the TGx cultivars (1835-10E and 1740-2F) in which the differences in mean germination percentage was by a margin below 10%. In general, related genotypes are normally expected to give partly similar results, including percentage germination, as described by Bahry et al. (2017).

4.5.2. Callus induction on cotyledonary node explants

The results showed that, MS basal culture medium, double cotyledonary node explants and different kinds of plant growth regulators (KIN, NAA and IBA) were successfully used for callus induction. The MS medium used, together with the different hormonal combinations resulted in significantly high amounts of friable and compact callus obtained (Table 4.2). Culture medium composition of MS-A and MS-E, as illustrated on Table 4.2, both produced the largest amounts of callus compared to other hormonal combinations. The variations in callus induction capacity and callus phenotypes (green, brown or white callus) were also observed amongst the genotypes used. No callus induction was observed on medium used as a control without PGRs. Two types of callus cells were formed; friable and compact callus, and the results are described below:

4.5.2.1. The formation of callus on coty-node explants

At large, the results showed that callus was easily initiated from the double coty-node explants on the MS media used (Table 4.2). According to the results obtained, large masses of callus cells were induced on soybean cultivar LS 677, LS 678, Dundee, TGx 1740-2F, TGx 1835-10E and Peking, respectively. The callus induced had no deficiency symptoms or did not show any signs of oxidative browning. But most importantly, there was no inhibition of callus formation observed, in all cultivars in relation to poor explant response. Although, mild chlorosis on some of the explants

used were observed, especially following prolonged periods of incubation in a culture room for more than four weeks. It is important to note that, those explants showing chlorotic symptoms did not have any negative effects on callus development as indicated in Figure 4.5 (f). According to the results on Table 4.2, PGRs efficiently stimulated and sustained callus proliferation on the type of explants used. In a similar study, El-Kaaby et al. (2015) reported the use of explants derived from germinated seed to optimise MS-based callus culture protocol in chilli pepper. The report stated that callus was efficiently induced from all seedling explants cultured on MS media.

Other successes reported using mature and immature seedling explants include those of Radhakrishnan and Ranjithakumari (2008), Odutayo et al. (2005) and Mohajer et al. (2012) in legumes such as sainfoin and cowpea. Mohajer et al. (2012) also used leaf, stem and root explants of sainfoin to induce callus on medium supplemented with 6-BA (2.5 mgL^{-1}) and NAA (0.5 mgL^{-1}). The above reports showed that callus induction was achievable using seedling derived explants, after two to three weeks of incubation under different concentrations of 6-BA, IBA and NAA. In addition, it was widely noted that, different genotypes usually responded differently to the callus initiation conditions, especially for species like soybean.

Table 4.2. Effect of different MS culture media supplemented with growth regulators on soybean callus induction using double cotyledonary node explants

Culture media	Callus induction capacity (%)	Frequency of callus induction (%)	Callus characteristics and morphogenic response		
			Roots	Shoots	Callus type
MS-A	85.5 ± 0.29[a]	70.0 ± 0.88[a]	7.50[a]	10.5[a]	Friable
MS-B	55.0 ± 0.15[b]	30.0 ± 0.44[b]	16.5[b]	23.6[b]	Compact
MS-C	45.0 ± 0.33[c]	30.0 ± 1.00[b]	41.7[c]	62.5[c]	Compact
MS-D	50.0 ± 0.35[d]	25.5 ± 1.05[c]	35[d]	44.8[d]	F & C
MS-E	70.5 ± 0.24[e]	65.5 ± 0.73[d]	15.0[b]	17.5[e]	F & C
Control	0.00	0.00	95.5[e]	100.0[f]	—

Evaluation was carried out 4 weeks after callus initiation. Values were mean ± standard error. **Frequency of callus induction** was calculated as (total number of explants inducing callus without shoots or roots/ total culture explants) × 100. **Callus induction capacity** was calculated as (total explants inducing callus/ total explants cultured) × 100. F refers to friable and C to compact. Values accompanied by similar superscript letters were not significant at P≤ 0.05 confidence level according to t-test.

4.5.2.2. Influence of PGRs on callus induction

The difficulty in seed germination observed in some soybeans; especially cultivar Dundee and Peking did not negatively affect the induction of callus, as previously mentioned. However, the typical swelling and expansion of cotyledonary explants to form callus during incubation observed in all soybeans within 6–14 days could be ascribed to media compositions. An example of callus development during early stages is shown on Figure 4.2 (a and c) below, demonstrating swelling and callus formation on some of the coty-node explants. Two out of the five PGRs combinations (MS-A and MS-E) used induced callus with little or no morphogenic growth response of shoots and roots, and these two compositions were used for the growth of callus on *Agrobacterium* infected explants. More than 85% of the explants subcultured on these media initiated callus better, than when callus was formed on cut hypocotyl segments of the cotyledonary nodes as observed in MS-B, MS-C and MS-D (Figure 4.2, b and d).

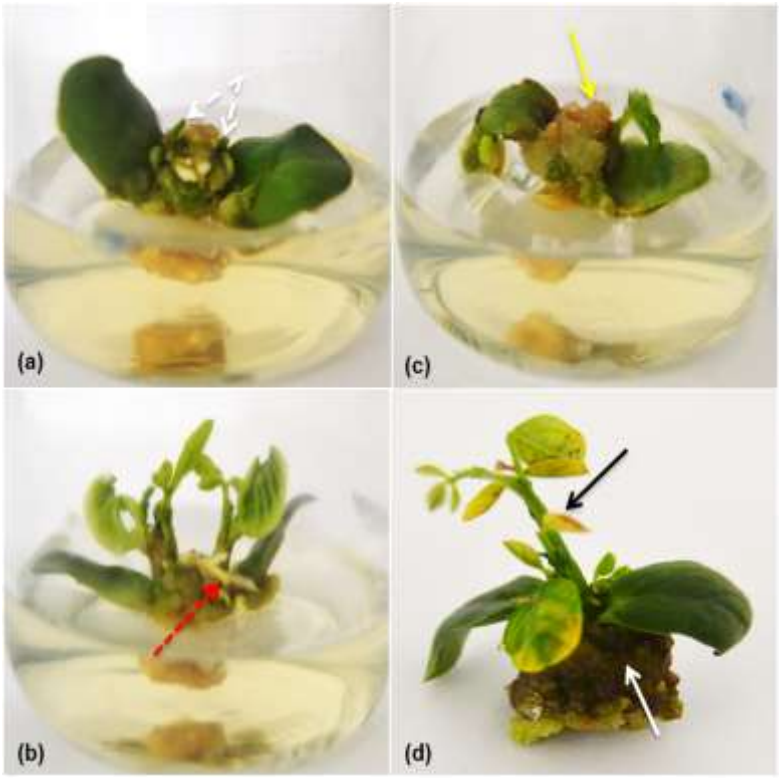

Figure 4.2. Early stages of callus initiation on MS medium supplemented with different hormonal combinations (KIN, IBA and NAA) and the formation of adventitious roots/shoots. (a) Swelling of coty-node during incubation for callus induction. (b) Calli cells formed on double cotyledonary node explants. (c) Root initiation on explants subcultured for callus initiation. (d) Shoot development on coty-nodes subcultured for callus initiation (brown callus is found underneath the cotyledons).

The results obtained in this study showed that, all soybean cultivars formed more callus on some but, very low callus percentage on other MS media combinations used. Explants inoculated on MS-A induced the most uniform and larger amounts of callus, followed by MS-E. But, MS-E medium contained relatively lower amounts of plant hormones (Table 4.1) compared to MS-A, and other media compositions (MS-B, MS-C and MS-D). The variations obtained on MS-E despite the lower concentration of PGRs used may be attributed to the proliferative capacity of auxin to cytokinin ratio used. The proliferation of callus in MS-B, MS-C and MS-D was significantly lower compared to explants subcultured on MS-A and MS-E. Some media compositions were generally not efficient in callus formation, like MS-C and D. These kinds of difficulties in the initiation and maintenance of callus cells was also reported by Ribas et al. (2011) when experimenting on embryogenic callus culture of *Coffea arabica* (L.). The report quantified the use of a semi-solid MS medium supplemented with 4.52 µM of 2.4-D in combination with 4.65 µM of Kinetin. The established embryogenic callus obtained was reported to exhibit a very slow pace of growth.

According to Trigiano and Gray (2005), MS medium used was originally developed for the regeneration of tobacco plants *in-vitro*. Currently, the medium serves as one of the most suitable and commonly used medium for *in-vitro* plantlets regeneration, including callus initiation. However, it may be difficult to induce callus in some of the genotypes simply because of the high salt content that the medium contains, even when using required amounts of auxins and cytokinins (high auxin - low cytokinin ratio). Patel and Patel (2013) reported efficient callus initiation on MS medium supplemented with 0.5–2.0 mgL^{-1} of 2.4 dichlorophenoxyacetic acid (2.4-D), 3-indoleacetic acid (IAA) and NAA. The study achieved callus induction in *Gymnema sylvestre* using leaf explants, cultured on medium with those amounts of 2.4-D, IAA and NAA. Other reports include those of Ahlawat et al. (2013) and Zang et al. (2016).

4.5.2.3. Organogenesis during callus culture

A small number of explants showed root and shoot organogenesis, particularly when subcultured on MS medium-B to D. Approximately 7.5% of explants formed roots from proliferated callus cells and 10.5% of explants formed shoots (Table 4.2). The total number of roots and shoots produced during callus culture are illustrated on Figure

4.3 (a and b). In contrast, a large number of explants formed clumps of friable (white) and compact (yellow to orange) callus (Figure 4.4, a and b) on media A and E. No cell senescence was observed on explants cultured on MS-A, while cell browning, and cell death were observed on some of the hypocotyl sections of the coty-node explants in MS-C and MS-D.

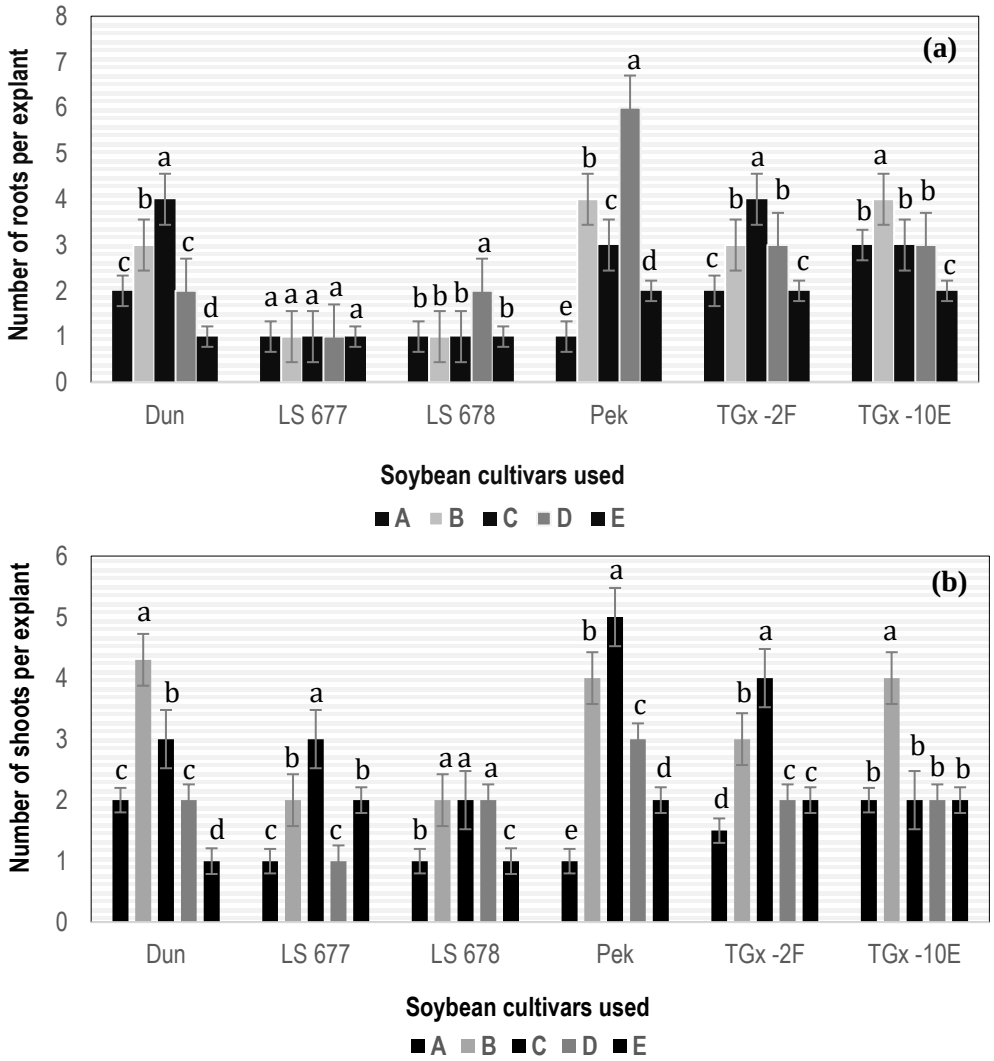

Figure 4.3. Effect of auxin-cytokinin ratio on root/shoot initiation in callus culture of soybean. (a) The average number of roots induced per explant on MS-A to MS-E media. (b) The overall mean number of shoots per explant on MS (A—E) culture medium.

Explants subcultured on MS-A, in some cases, induced clumps of compact pigmented callus (Figure 4.3, a). Similar observations were made in MS-E. However, the MS-E exhibited more of these embryogenic calluses with notable multiple buds, which were greener than callus observed in MS-A (Figure 4.3, c). Similar findings were made by Furutani and Hidaka (2004) when proliferating co-transformed buds from immature cotyledons in soybean on MSD40 solid medium. Similar results were also reported by

Texeira et al. (2011). Moreover, Cid et al. (1999) made similar observations when using seedling-derived hypocotyls, cotyledons, cotyledonary nodes and primary leaves for *Eucalyptus grandis* x *E. urophylla in-vitro* regeneration on MS (SP medium) medium containing 2.0 µM thidiazuron (TDZ).

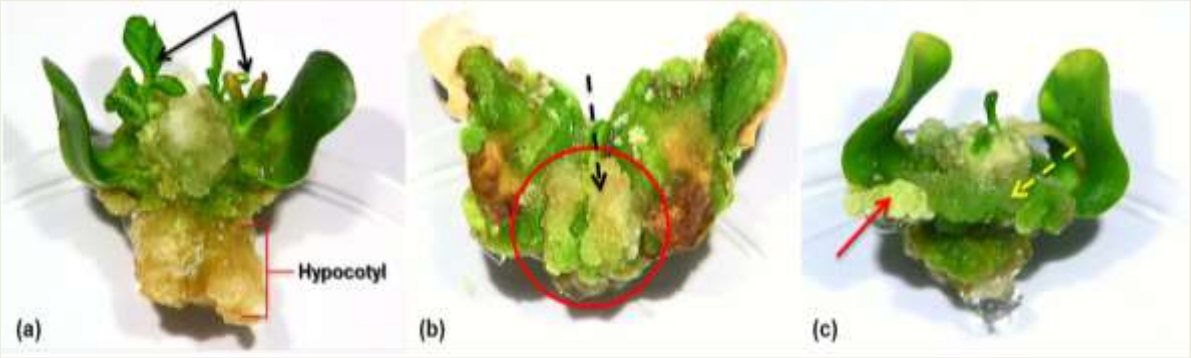

Figure 4.4. Morphogenesis on double coty-node explants subcultured for callus formation (a-black arrows) and swollen hypocotyls, friable callus (b-dotted black arrow), green pigmented callus (c- yellow dotted arrow) and light green callus induction. (b) Calli cells formed on double cotyledonary node explants. (c) Formation of embryogenic calli.

4.5.2.4. Genotype based response and variation

Since the main objective of this study was to assess the efficiency on high quality callus initiation across soybean genotypes and their cotyledonary explants. It is imperative to evaluate callus formation, callus with shoots/roots and all aspects of the callus phenotype (friable/compact) according to different cultivars. Even though all cultivars produced high quality callus on MS medium containing 0.7 mgL^{-1} KIN, 2.7 mgL^{-1} IBA and 3.2 mgL^{-1} NAA as indicated in Table 4.2, in relation to Table 4.1. The results observed on MS-A and MS-E formulations identified as superior for culture of high quality callus initiation on coty-node explants could still be linked to the soybean cultivars used.

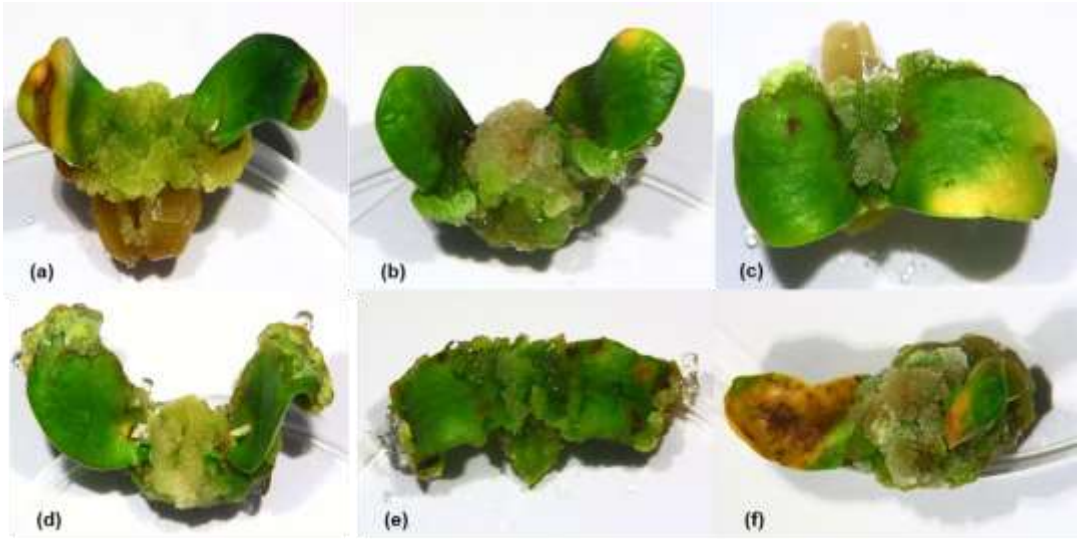

Figure 4.5. Callus developed on soybean double cotyledonary node explants on MS medium supplemented with 0.70 mgL^{-1} KIN, 2.70 mgL^{-1} IBA and 3.20 mg L^{-1} NAA. (a–f) Callus induced on LS 677, LS 678, Dundee, Peking, TGx 1740-2F and TGx 1835-10E respectively.

Callus initiation and cell multiplication for example; in soybean cultivar LS 678 and Peking responded rapidly to the culture than any other genotype. On the other hand, both MS-E and MS-A media induced lower percentages of explants with shoots and roots (15.0 and 17.5%, for MS-E and 7.5% for roots and 10.5% on shoot formation on MS-A) across all genotypes. The media induced the largest amount of callus in LS 677, LS 678, Dundee, TGx 1740-2F, TGx 1835-10E and Peking consecutively than any other media composition. Soybean cultivar TGx 1740-2F and Peking, as well as TGx 1835-10E (Figure 4.3, a-b) consistently exhibited a high number of roots and shoots. This may be as a result of genotype specificity than media composition or tissue plasticity.

TGx 1835-10E produced the largest amount of chlorophyll callus and exhibited high frequency of shoot and root organogenesis than TGx 1740-2F. Cultivar Peking on the other hand, produced clumps of callus cells on the cotyledon margins, junction and also exhibited some browning of the cells at the bases of the hypocotyls. Meanwhile, TGx 1835-10E presented clumps of both friable and compact callus, with browning of some cells on the cotyledonary junctions. According to these results, there were clear differences in the production of the callus cells in all cultivars and that was probably due to their genotypic characteristics.

4.5.3. Callus induction on co-cultured explants

The different cultivars (Dundee, LS 677, LS 678, TGx 1740-2F, TGx 1835-10E and Peking) were evaluated for their efficacy in callus proliferation from co-cultured double cotyledonary node explants. On this basis, the response of each cultivar to callus initiation and development was assessed following co-cultivation of explants with *A. tumefaciens* harbouring pTF 101.1 vector construct. According to the results obtained, the double coty-node explants induced less callus using infected explants with *Agrobacterium* and media composition.

4.5.3.1. Effect of antibiotics on callus initiation

Callus initiation was delayed in the MS media A and E containing cefotaxime and vancomycin antibiotics. This was confirmed when coty-node explants not co-cultivated with the bacterium were subcultured on MS medium containing antibiotics. This culture medium was used as a control. According to the observations made in all cultivars, the initial signs of callus initiation appeared after 3 weeks of culture. In contrast, callus induction was observed within 1-2 weeks, after culturing uninfected coty-nodes on medium without antibiotics (as discussed in 4.5.2). A slightly noticeable callus induction was later observed after 5 weeks of incubation. The callus formation capacity of explants subcultured on MS media containing antibiotics (Figure 4.6 a, b) differed significantly with the previously recorded callus initiation on media without antibiotics nor explant infection with *Agrobacterium* (Figure 4.6 c).

Although, traces of callus initiation and swelling of explant's hypocotyls and cotyledons was observed on media with antibiotics, initiated callus could not grow further, turning brown and drying just a week after. Zhang et al. (2001) made similar observations when investigating the effect of kanamycin on tissue culture and induction of somatic embryos in cotton. The report indicated reduction of callus formation at 10 mgL^{-1} kanamycin, with complete inhibition and death of tissues observed at 60 mgL^{-1}.

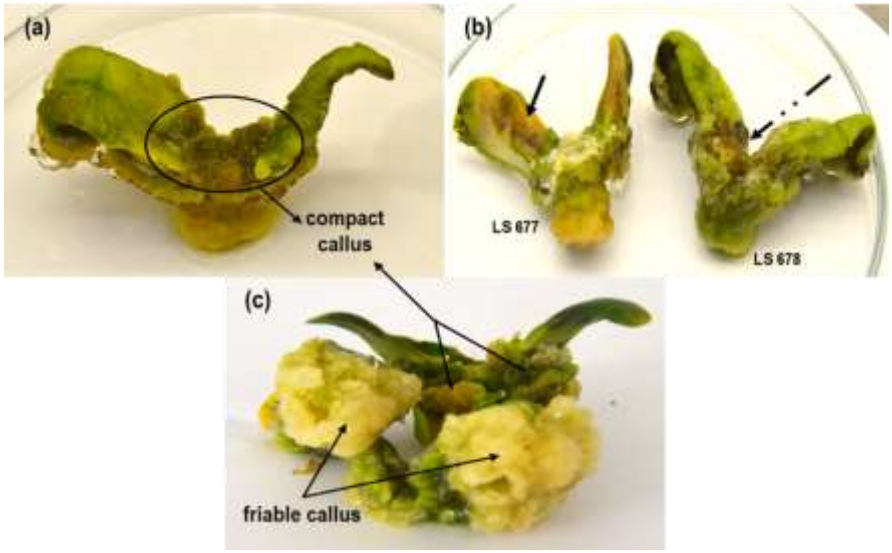

Figure 4.6. Effect of media composition on callus initiation and development. (a) Peking double coty-node explant co-cultured with *Agrobacterium* after 5 days of culture. (b) Un-infected coty-node explants taken from LS 677 and LS 678 after 3 weeks of culture on MS callus medium containing antibiotics. (c) Callus cells initiated on un-transformed explants obtained from MS medium without antibiotics. Solid arrow indicate chlorosis on cotyledons and dotted arrow show necrotic spots.

This study used cefotaxime (100 mgL^{-1}) and vancomycin (50 mgL^{-1}) which have been reported by Paz et al. (2006) to have prophylactic and proliferative effects in soybean culture. Grzebelus and Skop (2014) shared the same sentiments when assessing the effect of β-lactam antibiotics on *in-vitro* carrot protoplast cultures. They assessed three types of different β-lactam antibiotics (cefotaxime, carbenicillin and timentin) at five different concentrations (100, 200, 300, 400 or 500 mgL^{-1}), which were fairly higher than what is used in this study. The formation of very small callus has been observed in this study (Figure 4.7). The levels of explant competency appeared very poor compared to callus induction on media without antibiotics, for both proembryonic masses and large sized callus observed in the previous section above (4.5.2). These results show that media composition used or simply the addition of antibiotics had a profound effect on the proliferation and subsequent growth of callus cells.

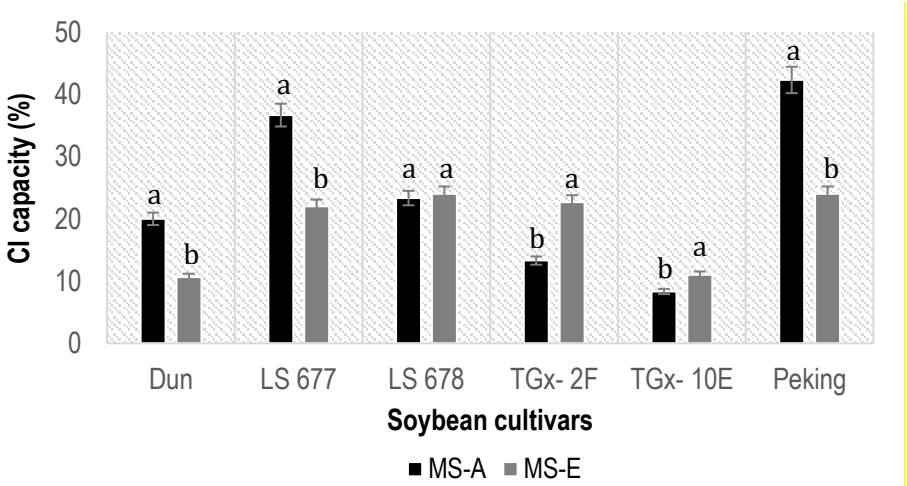

Figure 4.7. Callus induction (CI) capacity on double cotyledonary-node explants on MS-A and MS-B.

4.5.3.2. Influence of explant infection on callus induction

Callus induction capacity and explant survival was very poor on explants co-cultivated with *A. tumefaciens*. On all the media supplemented with antibiotics, callus initiation was inefficient and for those explants that produced microcalli, the callus turned brown and died within a few days. As a result, very little callus or no callus was obtained from the infected explants on media supplemented with antibiotics as indicated in Figure 4.8 and Table 4.3. In soybean cultivar Peking and LS 677, which had the highest callus induction capacity of 20.0 and 13.3% respectively, some of the survived explants produced shoot buds (Figure 4.8 a and b). Bud development in these explants also had a negative effect on the induction of callus as observed in the same Figure (4.8 a and b). Given the formation of very small callus cells (microcalli) observed on the infected explants, these results suggest that *Agrobacterium* had negative effects on the explant by reducing their proliferative capacity.

Table 4.3. Effect of the infection of double coty-node explants with *A. tumefaciens* during callus induction in soybean.

	Callus induction frequency	Callus type	Morphology/ size	Callus colour
MS medium composition-A				
Dundee	6.67[d]	compact	microcalli	brown
LS 677	13.33[b]	compact	microcalli	brown
LS 678	8.33[c]	compact	microcalli	brown
TGx 1740-2F	1.00[e]	compact	no callus	----
TGx 1835-10E	1.00[e]	compact	no callus	----
Peking	20.0[a]	slightly friable	microcalli	white/brown
MS medium composition-E				
Dundee	3.33[b]	compact	microcalli	brown
LS 677	----	----	----	----
LS 678	3.33[b]	compact	microcalli	brown
TGx 1740-2F	----	----	----	----
TGx 1835-10E	----	----	----	----
Peking	10.00[a]	compact	microcalli	brown

Evaluation was carried out after 4 weeks of culture. Percentage on callus induction frequency was calculated as (total number of explants inducing callus without shoots or roots/ total cultured explants) x 100%. All values within the column are the mean values calculated from 30 replicates per cultivar. The experiments were repeated at least twice.

The size and amounts of callus induced was small compared to what was observed in the last section (4.5.3). This indicates that, the levels of competency by meristematic tissues found at the cotyledonary junctions was also very poor, including the proembryonic masses and developmental responses. Initiated microcalli did not increase in size or grow further (Figure 4.8 d) compared to the callus induced on MS medium containing antibiotics (Figure 4.6) but lived for a few days before dying-off (Figure 4.8 c, e and f). This observation was also made by Zhang et al. (2001). However, in Zhang's report, the antibiotic used for callus initiation was kanamycin.

Severe tissue browning observed in this study may have been caused by the susceptibility of the different cultivars used, to *Agrobacterium*. Browning of tissues may have been influenced by the co-cultivation period as supported by Zhang and Finer (2016). Their report emphasised that, longer co-cultivation periods potentially increase transformation efficiencies by allowing more time for *A. tumefaciens* to interact with cells of the explants. But, this can be detrimental since the bacterium can overgrow the explants.

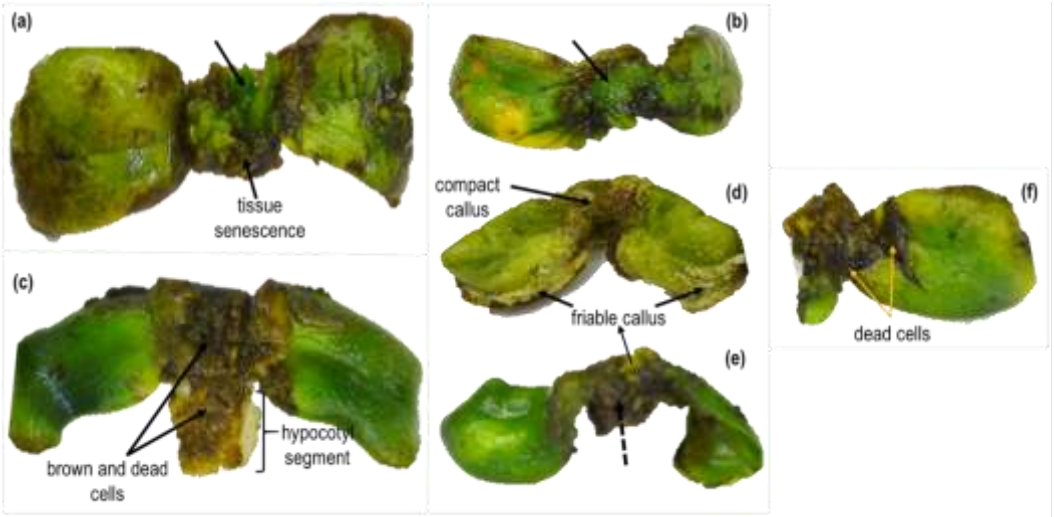

Figure 4.8. Double cotyledonary node explants showing different effects of *Agrobacterium* on callus induction in soybean. (a) and (b) coty-node explants of Peking and LS 677 showing multiple buds induced on callus induction medium. (c) Tissue browning and senescence on coty-node explants. (d) and (e) Compact and friable callus formed after few days of culture. (f) Cell death on infected coty-node explant.

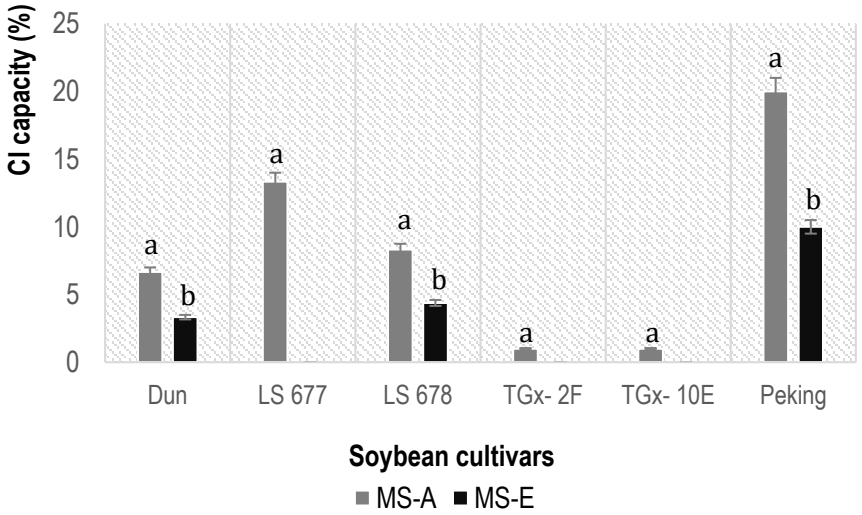

Figure 4.9. Callus induction (CI) capacity on double cotyledonary-node explants infected with *A. tumefaciens* on MS-A and MS-B.

Similar observations were made by Mangena (2015) using *A. tumefaciens* strains carrying the ΩPKY and pTF101.1. This study indicated that, the frequency of *Agrobacterium* contamination (known as overgrowth) differs according to strains. Therefore, confirming that, *Agrobacterium* carrying pTF101.1 or ΩPKY vector are some of the high-virulence bacterial strains (Zhang et al., 1999; Paz et al., 2006), which then make them difficult to control under *in-vitro* tissue culture conditions. This outcome presents a major challenge since the increase in antibiotic concentration to a higher amount than used in this study may as well affect the frequency of callus

induction. The percentage in callus induction capacity decreased from 50–85.5% on a media without antibiotics to 8.3–36.6% on media with antibiotics (Figure 4.7) or 0.0–20.0% in infected double coty-node explants (Figure 4.9). According to Hiei et al. (1994), the abrasive effects of the infection are influenced by inoculating explants in *Agrobacterium* suspension containing millions of bacterial cells. Thus, the large numbers of bacterial cells may overwhelm and cause lacerations on plant tissue. Furthermore, the presence of large number of cells of the pathogen can induce plant defence system which may limit transformation and regeneration from transformed cells (Zhang and Finer, 2016).

4.5.4. General implications of the results

The results indicate that, the best frequency of callus formation was obtained in both MS-A (containing 0.70 mgL^{-1} KIN, 2.70 mgL^{-1} IBA and 3.20 mgL^{-1} NAA) and MS-E (supplemented with 0.50 mgL^{-1} KIN, 1.20 mgL^{-1} IBA and 1.20 mgL^{-1} NAA). Previous findings by Barwale et al. (1986) and Franklin and Dixon (1995) demonstrated how PGRs direct the development of plant cells in a culture by essentially influencing the plasticity and totipotency of the explant tissues. These results support this view and are in line with many of these insights, especially in showing that, the combinations of auxins (IBA and NAA, 2.7–32 mg L^{-1}) and cytokinin (KIN, 0.7 mg L^{-1}) used were critical in determining the cell division and developmental patterns in all cultivars used in this study.

Plant tissue swelling and initiation of calli was observed within a week of culture. The induced callus culture grew into significantly large amounts of cells, while maintaining their morphogenic potential as shown by the number of roots and shoots formed, as indicated in Figure 4.3. In contrast, cells induced on MS media supplemented with antibiotics gradually lost their competency in further growth. All these implied that, callus initiation was relatively efficient on uninfected explants than those co-cultured with *Agrobacterium tumefaciens*.

The results furthermore, clearly demonstrated the effect that media composition has on callus initiation and development, as well as morphogenesis, including potential organogenesis in *in-vitro* culture of soybeans. Although, cotyledonary node explants

displayed rapid swelling responses, the frequency of calli production was very low with *A. tumefaciens*. The production of phenolic compounds produced by wounded cells, where wounding was brought during explant preparation, somehow worsened the problem. This observation suggests that the exposure of plant cells to *Agrobacterium* may have affected the cells' response. The bacterium in the inoculum evidently caused negative effects on explant response, even though this may be required for efficient and successful *Agrobacterium*-mediated genetic transformation.

4.5.5. Conclusions

The use of cotyledonary explants was successful for the establishment of callus culture. This type of explants has been more frequently and widely applied in many tissue culture protocols. But, the results obtained suggests that their efficiencies, especially in all methods used in this study relied primarily on the viability of explant, explant amenability to *Agrobacterium* inoculum and plant growth regulators. The findings in this study clearly demonstrate that more work need to focus on optimisation of tissue culture conditions, particularly in reducing the detrimental effects of *Agrobacterium* infection, if a high frequency, genotype independent protocol of soybean transformation still need to be developed.

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CHAPTER 5

EVALUATION OF SELECTED SOYBEAN CULTIVARS FOR *AGROBACTERIUM TUMEFACIENS*-MEDIATED GENETIC TRANSFORMATION

5.1. INTRODUCTION

5.1.1. Background to the study

It has been more than three (3) decades, since the introduction of genetically modified plants established through *in-vitro Agrobacterium*-mediated transformation. In soybean, the first successful transformation was reported by Hinchee et al. (1988), using cotyledonary explants with *Agrobacterium* pTiT37-SE harbouring pMON9749 for herbicide glyphosate tolerance. The success in this method depended upon several factors; which included tissue culture conditions, strains of *Agrobacterium* used, and the selected host plant genotypes aimed at receiving the transgenes. To date, this technique has succeeded in the production of high yielding transgenic plants, particularly for corn, chickpea, rice, cowpea, as well as a few new soybean cultivars (Ko et al., 2004; Raveendar and Ignacimuthu, 2010; Mehrotra et al., 2011; Patel et al., 2013).

Genetic transformation is now considered the most economic and highly effective method of genetic engineering that has been reported so far. The method holds the potential and promise to efficiently regenerate transgenic plants, especially for recalcitrant legume crops. Legumes like soybeans are some of the most important pulse crops and a good source of high quality proteins and oils, required for human consumption, health benefits and industrial processing. According to Wilson (2004), soybean contains 34–57% proteins, 8.3–28% oil content, 24–54% oleic and linoleic acid content, as well as 9–12% of carbohydrates. All of these constituents are mostly contained within the seeds and therefore, make soybean seeds, the only harvested part of this plant. Soybean seeds are also essential for providing plant materials used for the development of new and ideal transgenic plants with high chemical compositions and resistance to biotic/abiotic stress.

5.1.2. Motivation to the study

The vegetative and reproductive stages of soybean continuously show high sensitivity to biotic and abiotic stress constraints. Yield quality and quantity of this crop is severely affected by high temperatures, chilling, waterlogging and water deficit stress (Mangena et al., 2017). Soybean is known to grow in a wide range of soils, able to attain optimum growth even under harsh growth conditions. However, cultivation of this crop is now limited by the various abovementioned environmental constraints which are being exacerbated by climate change. To circumvent challenges posed by all stress factors; an efficient and rapid system of transformation that develops non-chimeric transgenic plants with resistance to these conditions must be advanced. Furthermore, genetic transformation in soybean has been reported by Paz et al. (2004), Zia et al. (2011) and Testroet et al. (2017) to still show the problem of genotype specificity in many established protocols. This implies that, a protocol developed for one cultivar may not be used for the transformation of other varieties.

The currently applied gene manipulation techniques such as DNA bombardment, sonication assisted *Agrobacterium*-mediated transformation and electroporation need to be optimised in order to achieve high transformation frequencies (Zia et al., 2011). However, *Agrobacterium*-mediated transformation remains a rapid, simple and cheap procedure for the genetic manipulation of many important crop species. As this technique is coupled with plant tissue culture; additives like L-cysteine, dithiothreitol and other thiol compounds, including the different co-cultivation periods, surfactants and selection regimes also require re-evaluation for the successful establishment of a genotype independent protocol.

As these conditions currently remain inadequate for successful genetic transformations, new high-yielding varieties that show resistance to diseases and tolerance to drought stress will not be possibly developed. It was therefore, essential for this study to evaluate the different soybean genotypes for *Agrobacterium*-mediated genetic transformation efficacy. Secondly, to assess the amenability of the cultivars used for both *Agrobacterium* strain and the optimised culture conditions used.

5.2. LITERATURE REVIEW

5.2.1. Plant transformation

Plant transformation is a modern technique aimed at modifying the genome of host plants to express the traits of interest. Wieczorek and Wright (2012) described this technology as a quicker and better method to select plant material with better adaptation to diverse ecological conditions. The technology can be used for manufacturing of larger seeds and fruits, production of secondary metabolites, medicine, biodiesel, feeds and fibre. However, this technique owes its success to the establishment of plant tissue culture by Gottlieb Haberlandt in his investigation on the culture of single cells in 1902 (Thorpe, 2007). Plant tissue culture is the axenic culture of tissues and organs under sterile *in-vitro* conditions. The development and improvement of plant tissue culture has led to the establishment of the various types of *in-vitro* tissue culture based transformation protocols. Callus culture, protoplast culture, nodal culture, meristem culture and other forms of axenic cultures are currently applied as the basis for the establishment of protocols used for the regeneration of transformed plants (Mineo, 1990; Birch, 1997).

5.2.1.1. Methods of plant transformation

Many different methods have been invented for the genetic transformation of forage and pulse crops. Microprojectile bombardment, electroporation and *Agrobacterium*-mediated transformation are amongst the methods used. According to Finer and Dhillon (2008) successful transgene expression has been predominantly achieved mainly through *Agrobacterium* or particle bombardment-mediated genetic transformation. The other transformation approaches are considered less widely used, highly expensive to carry out and inadequate for the successful transformation of many recalcitrant species (Paz et al., 2004).

Such methods include *in-planta* transformation carried out by infiltrating plant tissues with *Agrobacterium* inoculum, bypassing tissue culture conditions (Zia et al., 2011). The floral dip technique reported by Zhang et al. (2006) in the genetic transformation of *Arabidopsis thaliana* and *Medicago truncatula* using *A. tumefaciens* is one of the

few examples. Floral dip method was designed specifically for *Arabidopsis Agrobacterium*-mediated transformation, via transformation of the female gametophyte prior to flowering in order to generate independent transgenic seeds (Stewart, 2008). Other transformation frequencies were reported in tomato and sweet potato also using floral dip (Xing et al., 2008; Yasmeen et al., 2009) and the *in-vivo* transformation of *Oryza sativus* L. for resistance against fungal sheath blight disease caused by *Rhizoctonia solani* Kuhn (Naseri et al., 2012).

Electroporation-mediated transformation, silicon carbide-mediated transformation, liposome-mediated transformation and chloroplast-mediated transformation are some of the other techniques less widely adoapted in public research laboratories worldwide (Lee et al., 2013). Dhir et al. (1992) reported an efficient electroporation-mediated transformation of soybean cultivar Clerk 63 using calli derived protoplast culture. The successful transformation of soybean was also reported by Ghada et al. (2015) with 76.9, 64.0, 58.3 and 38.0% regeneration efficiency on cotyledonary explants, hypocotyls, epicotyls and leaf explants respectively, using *A. rhizogenes* strain R1000.

5.2.1.2. Optimisation and improvement of genetic transformation

A number of methods of plant transformation have been established in the past decades or over. However, there is rarely a stably routine transformation protocol that can be efficiently applied to all monocot and dicot species. Progress so far made, indicates progressive challenges in the genetic transformation technology and the problems are compounded by the lack of an efficient system of transformation. Mineo (1990) indicated that most of the scientific inquiries regarding genetic engineering are directed to tissue or organ response in tissue culture. This is so, because *Agrobacterium*-mediated transformation, as one of the most widely applied technique relies primarily on *in-vitro* tissue culture conditions.

Lee et al. (2012) stated that, the success of this approach critically depends upon the target tissue response under proper proliferative culture conditions. In addition to the use of tissue culture based protocols, Finer and Dhillon (2008) highlighted the need to optimise and increase delivery of the bacterial cells, induce *vir* genes and increase amenability of host plant tissue. For genetic transformation to hold the most promise

for future increases in efficiency of *Agrobacterium*-mediated transformation, great consideration must be given to pre-culture conditions, bacterial strains and the plant host tissues used.

5.2.2. Soybean transformation

Glycine max L. is one of the world's most widely researched pulse legume crop species. This crop attracted great interest to researchers across the globe due to its high amounts of oils, proteins, carbohydrates, dietary fibres, vitamins and minerals. For the last few decades, many laboratories have been researching about genetic traits aimed at improving the quantity and quality of soybean seeds (Lee et al., 2013). Verma et al. (2014) indicated that, efficient improvement of soybean traits is possible via genetic engineering, which offers new possibilities than conventional breeding methods. Conventional breeding modifies plant genetics by hybridizing plant types, taking several years to create new and better plant varieties (Tinker, 2008).

However, genetic transformation, which is a modern breeding technique, can manage to rapidly and effectively as well as efficiently combine unique growth characteristics derived from different plant varieties to produce one or more newly improved varieties. This technique is considered a better alternative than conventional breeding as it allows for exogenous genes transfer. The genes of interest from a lineage far from soybean can also be introduced into this crop. This possibility depends on a well-coordinated expression and complex interaction of genes or gene products by both host and inserted vector construct (Tinker, 2008) and only require to be optimised, particularly for recalcitrant crops such as soybean.

5.2.3. Traits of interest for transgenic soybeans

Many soybean cultivars normally present challenges in containing better traits in one or more of the following characteristics; yield (including seed size, seed number per pod, pods per branch), pest resistance, growth, flowering time, susceptibility to drought or water lodging, seed vigour, and seed dormancy. Tinker (2008) listed the composition and content of proteins, oils, secondary compounds and composition of fibre, minerals, vitamins and carbohydrate content as part of those very limited and

complex traits. All these characteristics are highly required since they have a greater value for human health and industrial use. The whole purpose of generating transgenic plants is to be able to manipulate and retain these traits by inserting genes of interest from other sources into the targeted soybean varieties (Korth, 2008). In soybean thus far, reasonable progress has been made in conferring characteristics such as the ability to survive herbicide treatment, insecticides, disease resistance and to withstand salinity stress. Rizwan et al. (2015) reported the development of sulfonylureas herbicide resistant soybean plants, and including other crops like canola, sunflower, wheat and corn. These transgenic plants were achieved through the use of seed mutagenesis. Virus disease and insect resistant soybean plants were also reported by Grossi-de-Sa et al. (2016). The development and optimisation of soybean transformation techniques have showed to allow the establishment of transgenic soybean plants carrying genes from many different sources, including microbes, insects and animals (Korth, 2008).

5.2.4. Transgenic plant analysis

In general, transgenic plant analysis is required to probe the transgene mostly using polymerase chain reaction. This method, including other methods of analysis is performed to determine transgene integration (Zale, 2008). According to Gelvin (2003) the expression of the transgenic construct within the target plant genome constitutes a single transformation event independent of other events. But, the challenge in transformation arises when the effects of the transgene cannot be identified among the non-transformed plants. Kumar and Fladung (2001) indicated that, the analysis of transgenic plants revealed a profound effect of DNA methylation on the expression of the gene of interest. Methylations, including other intrinsic and extrinsic factors were reported to reduce the stability of the transgene expression (Kumar and Fladung, 2001). These challenges contribute in the problem of recalcitrance in many agro-economic crops such as wheat, corn and soybean. They create the need for optimisation and simplification of plant tissue culture conditions which efficiently detect transgenic plants from non-transformed ones. Therefore, researchers and techniques need to ensure efficient means of achieving long term stable transgene expression which would allow easy detection and analysis in transformed plants (Kohli et al., 2010).

5.2.5. The impact of genetic transformation in agriculture

Plant transformation is a technique designed for use mostly in proprietary and commercial agriculture. The basic technique of plant genetic transformation was developed in the early 1980s and the first genetically modified crop was released in the mid-1990s (Qaim, 2009). This technique offers direct gene transfer across species boundaries, particularly in crop species proving difficult or impossible to genetically improve using conventional breeding methods (Gelvin, 2003). Traits so far achieved, especially those in use for agricultural purposes include better resistance to pests and diseases, tolerance to drought stress and other abiotic stresses.

Enhanced quality of nutrient content for food products and production of special substances used in pharmaceutical and industrial processing are additional benefits subsequent successful cultivation of crop plants. The above mentioned agricultural services could only be largely achieved when improvements in biotechnology, primarily for techniques such as *Agrobacterium*-mediated genetic transformation are successful. The accomplishments will benefit the agricultural sector by eliminating the use of agrochemicals, production of highly viable seeds for cultivation in most croplands and varieties that provide increased yields with minimum harvest losses (Wieczorek, 2003).

5.2.6. Perceptions in plant biotechnology

The use of transgenic plants has increased in recent years, especially cultivation of cultivars that are resistance against biotic stress. One of the biotechnological defences developed in crops, is the use of *Bt* crystal protein (CP) which is toxic against plant eating insects (Ibrahim and Shawer, 2014). But, as the use of commercial crops expressing different resistant genes including *Bt* toxins is increasing, resistance by targeted pest species is also emerging (Natarajan et al., 2013). These problems emerge as researchers attempt to maximise plant protection and lower production costs, while increasing yields. However, some of these challenges experienced by genetic engineering create a growing concern in the safety and guarantees of using genetically modified plants. Today, some of the concerns arise due to the fact that there are perceptions of unknown consequences to altering the natural state of

organisms through foreign gene expression (Phillips, 2008). Ambiguous reports of allergic reactions on people who consumed transgenic plants were also reported by Buchanan (2001). Other reported potential issues of concern include generation of antibiotic resistant bacteria, known as “superbugs”, mycotoxin contaminations, uncertainty on metabolic regulations and potential damage to the natural environment (Rani and Usha, 2013).

5.3. PURPOSE OF THE STUDY

5.3.1. Hypothesis of the study

We hypothesise that, comparative analysis of different soybean genotypes can generate insights for the improvement of *in-vitro* based genetic transformation using *Agrobacterium tumefaciens* harbouring pTF101.1 vector, with a *bar* gene conferring tolerance to glufosinate ammonium.

5.3.2. Aim and objectives of the study

The purpose of this study was to assess the efficiency of *in-vitro* plantlets regeneration using double cotyledonary-node explants following *Agrobacterium*-mediated genetic transformation in the selected soybean genotypes.

Four main specific objectives were outlined:

i. Evaluate shoot organogenesis on cotyledonary explants co-cultivated with *Agrobacterium tumefaciens*.
ii. Select soybean transformants from the non-transformed shoots using the herbicide glufosinate-ammonium as a selective agent.
iii. Quantify and compare the *in-vitro* regeneration and transformation frequencies of the six soybean cultivars using statistical means.

5.4. MATERIALS AND METHODS

5.4.1. Plant materials

Six cultivars of soybean (*Glycine max* L.) were used for *Agrobacterium*-mediated genetic transformation experiment, which included Dundee, LS 677, LS 678, TGx 1740-2F, TGx 1835-10E and Peking.

5.4.2. Culture media preparation

The basal media; Murashige and Skoog and Gamborg's B5 media were used in this study. The macro- and micro nutrients, iron source, vitamins and carbon source were prepared as described by Pierik (1997). The media for bacterial re-initiation, co-cultivation, explant infection, and shoot induction washing medium were prepared as described by Paz et al. (2006). *In-vitro* shoot regeneration, including shoot induction, elongation and rooting were initiated on MS medium supplemented with different concentrations of plant growth regulators (PGRs). Stock solutions (mg/mL) of different PGRs were prepared by dissolving respective amount of 6-benzylaminopurine (6-BA), kinetin (KIN), gibberellic acid (GA_3), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) in few drops of 1N sodium hydroxide (NaOH) or sterile distilled water.

5.4.3. Preparation of *Agrobacterium tumefaciens*

The binary plasmid pTF101.1 with *oc-1* gene and phosphinothricin acetyl transferase *bar* gene for glufosinate-ammonium tolerance transformed in *A. tumefaciens* strain EHA101 was used. The bacterium was grown from glycerol stock on solidified yeast peptone (YEP) medium containing 50 mgL^{-1} kanamycin and 100 mgL^{-1} spectinomycin. A 2 mL of *Agrobacterium* colony was inoculated into 50 mL liquid YEP medium containing antibiotics (same as above, without agar) and shaken at 28°C in an orbital-shaker (250 rpm). The culture was incubated at 28°C until the optical density (OD_{600}) reaches 0.8–1.0. *A. tumefaciens* culture was centrifuged for 10 min at 3500 rpm and pellet cells resuspended in infection medium prepared as described by Paz et al. (2006).

5.4.4. Seed sterilisation and germination

The soybean seeds were first rinsed to remove any detritus with soapy tap water, dried and placed in standard Petri dishes (100 mm × 15 mm). The seeds were surface gas sterilised with chlorine gas for 16 hours as described by Paz et al. (2006). Sterilised seeds were then inoculated on MS basal medium containing 3% sucrose as a carbon source, 0.25% gelrite as a solidifying agent and 4.0 mgL^{-1} 6-BA. Seed cultures were incubated for 10 days in a tissue culture growth room at 24±2°C under 50–60 $\mu mol\ m^{-2}s^{-1}$ light intensity and ~10% humidity.

5.4.5. Explant preparation, infection and co-cultivation of explants with *Agrobacterium*

The double cotyledonary node explants were prepared by excising out the epicotyls and cutting off the hypocotyls 4–5 mm beneath the cotyledons as described by Mangena et al. (2015). The explants were wounded by cutting 2–8 times on the cotyledonary junctions and then infected with *Agrobacterium* suspension in a shaker (110 rpm) for 30 min (Paz et al., 2006). Double coty-node explants uninfected with *Agrobacterium* were used as a control. Thereafter, about ten infected explants per Petri plate were co-cultured with *Agrobacterium* on co-cultivation medium overlaid with a filter paper. The co-cultivation plates were then incubated in a growth room under condition similar to those used for seed culture for 5 days.

5.4.6. Transgenic shoot organogenesis

After 5 days of co-cultivation, the explants were thoroughly rinsed in liquid shoot induction washing medium containing 3.9 gL^{-1} methyl ester sulfonate (MES), pH 5.7, 1.11 mgL^{-1} 6-BA, 100 mgL^{-1} cefotaxime and 100 mgL^{-1} vancomycin. After washing, the explants were sub-cultured on shoot induction MS culture medium as indicated below:

4.4.6.1. Shoot induction using infected and uninfected explants

Agrobacterium infected cotyledonary explants that were co-cultured for 5-days were subcultured on MS medium containing 2.0 mgL^{-1} 6-BA. The medium was also supplemented with 8 mgL^{-1} glufosinate, 100 mgL^{-1} cefotaxime and 50 mgL^{-1} vancomycin. However, the uninfected cotyledonary explants used as control were separated into two sets. One set of explant cultures was established on the MS culture medium containing only 2.0 mgL^{-1} 6-BA without antibiotics and the herbicide.

Another set consisted of explants cultured on medium similar to the medium used for infected explants, with antibiotics and the herbicide. Shoot cultures were incubated at 24±2°C temperature under 50–60 µmol $m^{-2}s^{-1}$ light intensity for three weeks. After 3 weeks of culture, the primary axillary shoots initiated were excised off the explants and discarded. Explants were then transferred into a fresh shoot induction medium containing the same amount of 6-BA and glufosinate and maintained under the same culture conditions for 4 weeks.

5.4.7. *In-vitro* elongation and rooting

Adventitious transgenic and non-transgenic shoots that were 2.0–2.5 cm long or over, were subcultured for further elongation. The shoots were cultured on MS basal medium containing 0.55 mgL^{-1} GA_3. Elongated shoots were then inoculated for rooting on MS medium modified with 0.75 mgL^{-1} KIN, 3.25 mgL^{-1} IBA and 2.75 mgL^{-1} NAA. Both cultures were incubated under growth conditions similar to those described for shoot organogenesis (5.4.6).

5.4.8. Plant acclimatisation

Rooted plantlets were acclimatised as described by Mangena (2015). The *in-vitro* rooted plantlets were later transferred into 13 cm diameter plastic pots three-quarter filled with vermiculite mixed with Garden Tech (SA) potting soil at ratio 3:2. The plants were maintained under controlled growth conditions until V3 vegetative stage. After reaching this stage, the plants were then moved and kept in a glasshouse under natural conditions, until flowering and fruit pod maturity. The conditions were monitored but not controlled.

5.4.9. Culture growth conditions and statistical analysis

All *in-vitro* cultures were kept in a tissue culture growth room at 24±2°C, equipped with white fluorescence light of 50–60 µmol $m^{-2}s^{-1}$ and 16-hour photoperiod. For *in-vivo* acclimatisation, plantlets were maintained at the temperature similar to *in-vitro* cultures but, under 150–200 µmol$m^{-2}s^{-1}$ and 16-hour photoperiod. The experiments on germination, shoot induction, elongation, rooting and acclimatisation were conducted with 15 replicates containing 2 explants each. The experiments were repeated at least three times and the means and standard deviations of the results were calculated. The significance analysis of the experiment was performed using analysis of variance (ANOVA), IBM SPSS Statistics version 24.

5.5. RESULTS AND DISCUSSION

5.5.1. *In-vitro* germination of soybean seeds

Before the transformation experiments, the influence of 4.0 mgL^{-1} 6-BA on seed germination and seedling development was recorded. The selection of this 6-BA concentration for seedling establishment has been accounted for in a study by Mangena et al. (2015), especially the use of stout and thicker seedlings to prepare explants used for *in-vitro Agrobacterium*-mediated transformation. All cultivars recorded more than 60% seed germination and the results are illustrated on Figure 5.1. The results show variation among the genotypes, with cultivars such as LS 678 and LS 677, including Peking still recording the highest number of seeds that germinated within 7 days of culture.

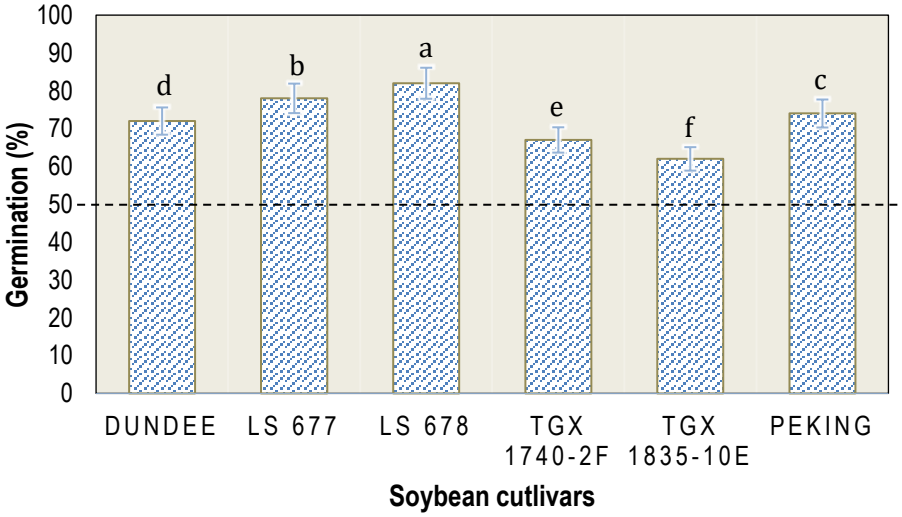

Figure 5.1. Overall mean germination percentages of seeds inoculated on MS medium supplemented with 4.0 mgL^{-1} 6-BA.

Soybeans such as; TGx 1740-2F and TGx 1835-10E still showed a poor and gradual germination rate, accompanied by a large number of abnormal seedlings with highly reduced hypocotyls. Furthermore, the presence of 4.0 mgL^{-1} 6-BA resulted in partial inhibition of radicle development as reported by Mangena et al. (2015). On the other hand, 6-BA had a profound effect on the thickening of stems, as well as the inhibition of the epicotyl growths. This kind of seedling morphology has been observed before and appeared necessary to produce seedlings required to prepare suitable explants for infection with *Agrobacterium* during transformation. On the other hand, differences

in the germination percentages significantly increased according to genotypes. LS and TGx cultivars presented lower variations amongst themselves. This trend was also observed in other studies as demonstrated by their standard deviations on Figure 5.1. The genotype Peking and LS produced higher germination percentage than the TGx cultivars, as similarly obtained in other studies.

5.5.2. *Agrobacterium*-mediated transformation

The development of an efficient protocol for *Agrobacterium*-mediated genetic transformation in soybean is important for improving the genetic pool of this crop. Soybean is considered recalcitrant to genetic manipulation, and its *in-vitro* based genetic transformation is characterised by four (4) main parameters. Those are; the explant type, culture media, *Agrobacterium* and plant genotype. The following results discusses the effects of parameters mentioned above on the optimisation of efficient soybean transformation procedure.

5.5.2.1. Effect of culture media on transformation

Shoot induction was achieved on the different MS media compositions used in this study. All explants showed adventitious shoot formation within 30-days of culture, under the tissue culture growth conditions used. According to the results, the highest number of shoots was obtained on MS medium containing 2.0 mgL^{-1} 6-BA. The other MS basal culture medium supplemented with 2.0 mgL^{-1} 6-BA in combination with antibiotics (cefotaxime-100 mgL^{-1} and vancomycin- 50 mgL^{-1}) was used successfully to induce multiple shoot buds. Both media described above, were used to subculture un-infected explants and were considered as controls. A small number of shoots was induced on media containing antibiotics than a medium without antibiotics, as indicated on Table 5.1 and 5.2.

Mostly, multiple buds and highly reduced shoots were observed on many explants cultured on MS medium containing the antibiotics (Figure 5.2 b and c). Intact and elongated adventitious shoots induced on this medium ranged between 0.6–1.9 shoots per explants as indicated on Table 5.1. Suppression of shoot initiation and growth clearly appears to be instigated by the presence of antibiotics in the medium.

These results confirm the observations made in the previous section on callus induction. The initiation and proliferation of callus was evidently inhibited by the presence of antibiotics in the culture medium. Similar findings were made by Yi et al. (2006), when assessing the effects of carbenicillin and cefotaxime on callus and somatic embryogenesis from adventitious roots of papaya. Extreme inhibition in callus growth and abnormal somatic embryos were obtained from the media supplemented with about 250–500 mgL^{-1} of carbenicillin and cefotaxime.

On the other hand, explants subcultured on MS basal medium with only 2.0 mgL^{-1} 6-BA, without antibiotics induced the highest significant number of shoots within two weeks of culture (Table 5.1 and Appendix 5.3). This medium even produced the highest number of shoots, which successfully elongated and rooted on MS medium containing different combinations of hormones (Figure 5.5).

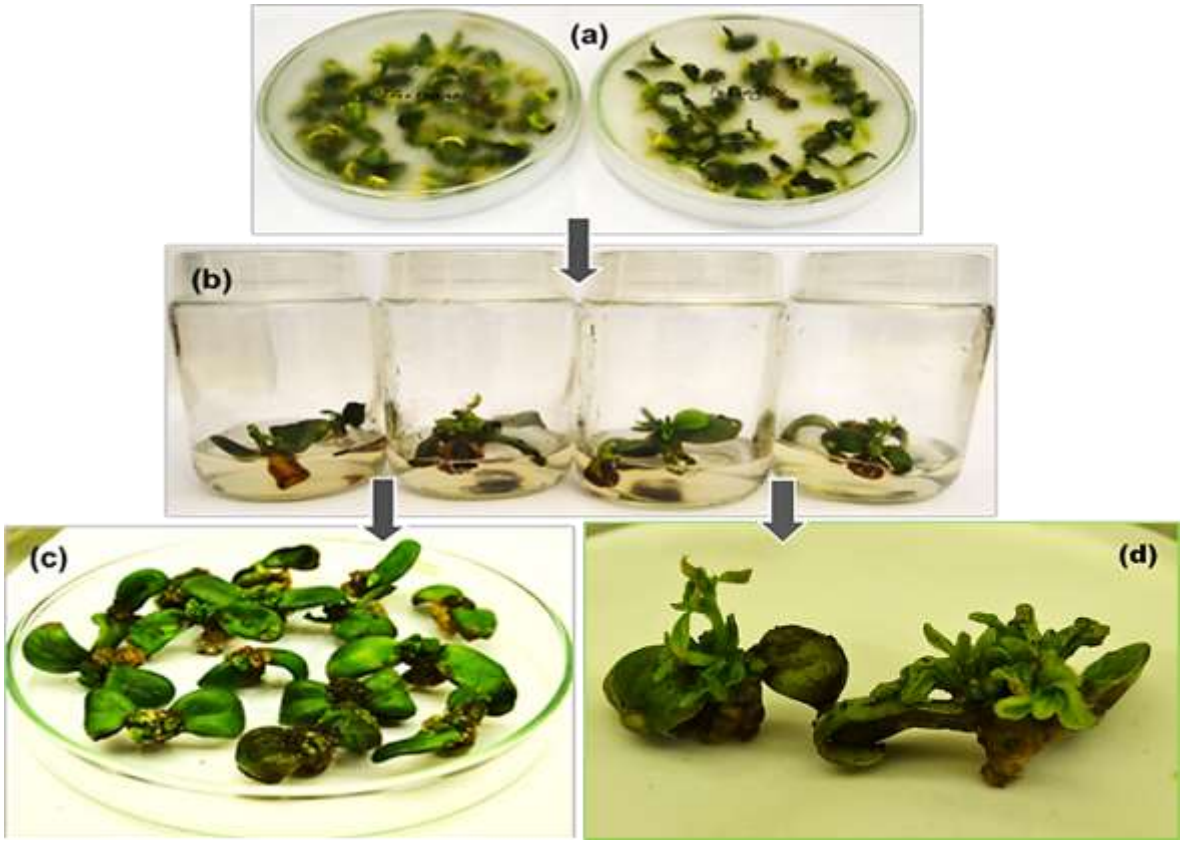

Figure 5.2. Examples of steps involved during shoot induction on coty-nodes transformed with *Agrobacterium* tumefaciens. (a) Double cotyledonary-node explants co-cultured with *Agrobacterium*. (b) Explants subcultured on shoot induction medium showing the development of shoots. (c) Explants showing shoot growth inhibition (suppressed buds), following subculturing for 30 days on MS medium with 2.0 mgL^{-1} 6-BA. (d) Vigorous shoot growth occurring on explants subcultured on MS medium supplemented with 2.0 mgL^{-1} 6-BA.

Generally, significant differences were observed among the basal media in both percentage of explants forming shoots (Table 5.2) and the mean number of shoots per explant (Table 5.1). Similar shoot induction dynamics were reported by El-Siddig et al. (2009) and Yan et al. (2000). The transformation trials in these reports revealed great variations in the regeneration frequency, which mostly depended on the explant type and the culture conditions, particularly the culture media composition.

Table 5.1. Comparison of the effect of MS basal culture media with or without antibiotics and *Agrobacterium* infection of double cotyledonary-node explants on average number of shoots induced per explant after 30 days of culture.

Culture Media without Antibiotics				Culture Media with Antibiotics			
Cultivar	**Mean shoot ± Std. Error**	**Std. Deviation**	**Variance**	**Cultivar**	**Mean shoot ± Std. Error**	**Std. Deviation**	**Variance**
Uninfected coty-node explants				Uninfected coty-node explants			
Dundee	3.850 ± 0.274	1.226	1.503^{f}	**Dundee**	1.350 ± 0.221	0.988	0.976^{a}
LS 677	4.450 ± 0.344	1.538	2.366^{d}	**LS 677**	1.750 ± 0.216	0.967	0.934^{a}
LS 678	4.650 ± 0.372	1.663	2.766^{c}	**LS 678**	1.950 ± 0.185	0.826	0.682^{c}
TGx 1740-2F	3.400 ± 0.336	1.501	2.253^{e}	**TGx 1740-2F**	1.150 ± 0.150	0.671	0.450^{d}
TGx 1835-10E	4.450 ± 0.394	1.762	3.103^{a}	**TGx 1835-10E**	0.750 ± 0.190	0.851	0.724^{b}
Peking	5.000 ± 0.384	1.717	2.947^{b}	**Peking**	1.350 ± 0.109	0.489	0.239^{e}
				Explants infected with *Agrobacterium*			
				Dundee	1.250 ± 0.270	1.209	1.461^{c}
				LS 677	1.400 ± 0.328	1.465	2.147^{b}
				LS 678	1.400 ± 0.328	1.465	2.147^{b}
				TGx 1740-2F	0.750 ± 0.216	0.967	0.934^{d}
				TGx 1835-10E	0.800 ± 0.156	0.696	0.484^{e}
				Peking	1.650 ± 0.335	1.496	2.239^{a}

Values with an asterisk superscript are significantly different at p value = 0.05 confidence level according to t-test. Values with similar superscript letters are not statistically significant at the given p value.

In this study, the results showed that morphogenetic processes are strongly influenced by the culture media, predominantly the presence of antibiotics in the medium. Niedz and Evens (2010) furthermore highlighted more culture media related factors that simultaneously affect transformation efficiency. Those were *Agrobacterium* culture (strain EHA105 containing a binary vector pBINGUSint) density and inoculation density, which negatively influenced the transformation of *Citrus* rootstock, variety US812.

5.5.2.2. Effect of double cotyledonary-node explants on transformation

The requirement of using suitable explants for soybean regeneration *in-vitro* is one of the main goals leading to successful genetic transformation. The observations made in this study showed that, the use of double cotyledonary-nodes as explants is still superior to the development of a simple and effective transformation protocol. These explants withstood the culture conditions used and provided a feasible means of shoot culture establishment. Double coty-nodes offer prolonged support for shoots and buds initiated on the cotyledonary junctions (Figure 5.2) with minimal deficiency symptoms. Furthermore, the explants showed no signs of susceptibility to culture medium, especially by not exhibiting any deficiencies.

Zhang et al. (2004) reported the sensitivity of explants such as; hypocotyl nodes and full or half-split immature cotyledons to antibiotics like kanamycin, and further established the concentration that causes explant death. However, in this study, the results displayed such a response, when the explant consists of two cotyledons still attached to the hypocotyl segments. The response by these explants was much better, especially subsequent to the infection of explants with *Agrobacterium* (Figure 5.2). Additionally, the shoots or buds appeared to be well and effectively supported by these explants.

Double cotyledonary explants can be compared with the use of single coty-node explants. Single coty-nodes prepared by longitudinally splitting the double cotyledonary-nodes at the cotyledonary junction into two single nodes. This was also reported by Trick et al. (1997), Yan et al. (2000) and Ko et al. (2006) during soybean transformation. Even if positive results were obtained, especially in terms of the ability of single coty-nodes to rapidly proliferate pre-existing axillary meristems and rapid cell division because of wounding, these explants are less preferred to double coty-nodes. Problems encountered as a result of severe wounding and *Agrobacterium* overgrowth still made the double cotyledonary explants preferable over the single coty-nodes.

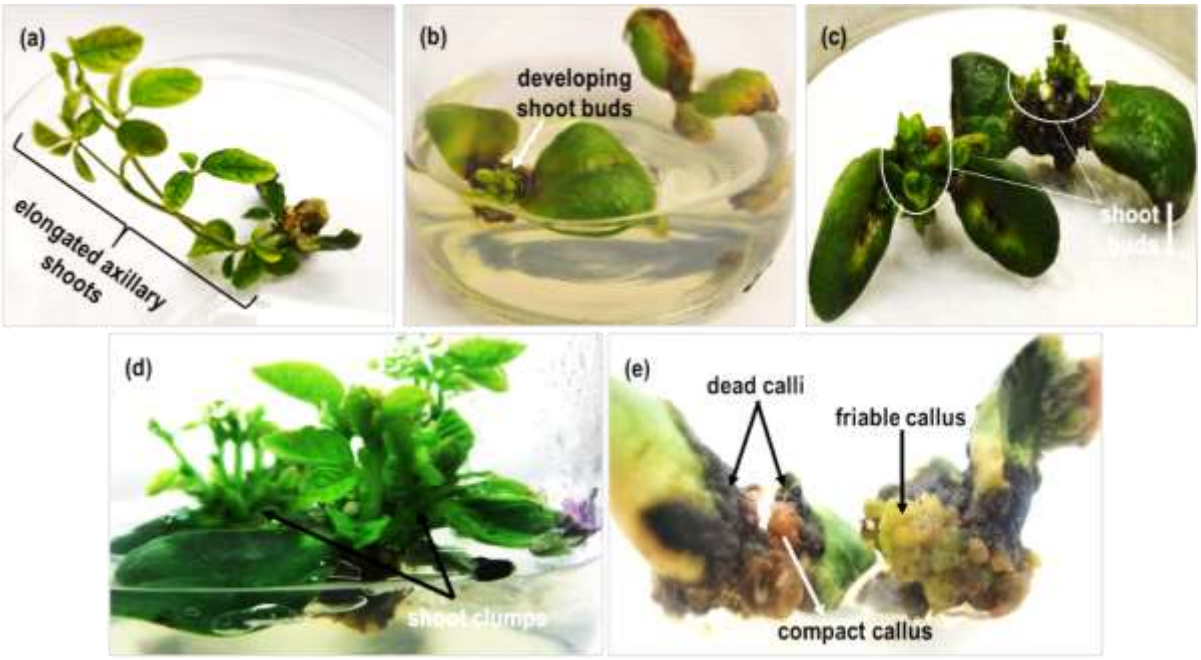

Figure 5.3. Adventitious shoot formation on un-infected cotyledonary-nodes and coty-nodes transformed with *A. tumefaciens*. (a) Example of axillary shoots induced on control MS medium without PGRs. (b) and (c) Shoot bud initiation on explants cultured on MS medium supplemented with 6-BA (2.0 mgL^{-1}), cefotaxime (100 mgL^{-1}) and vancomycin (50 mgL^{-1}). (d) Shoot growth on un-infected cotyledonary-nodes cultured on MS containing 2.0 mgL^{-1} 6-BA. (e) Callus formation and oxidation on tissue explants infected with *A. tumefaciens*.

In general, double coty-nodes are found to be better explants because they can be maintained in culture for longer period and provide full support for the developing axillary meristematic cells forming shoots during organogenesis (Mangena et al., 2017). Meanwhile, single coty-node are more suitable for *Agrobacterium*-mediated transformation through in-direct somatic embryogenesis or establishment of suspension cultures. Many researchers have used cotyledonary-nodes as the explants to obtain transgenic soybeans (Di et al., 1996; Li et al., 2017).

5.5.2.3. *In-vitro* formation of transformed shoots

Shoot proliferation was difficult to achieve from the meristematic regions of the *Agrobacterium* infected cotyledonary-node explants. Even though, shoots which developed from the pre-determined meristematic cells were removed from the cotyledonary-node explants.

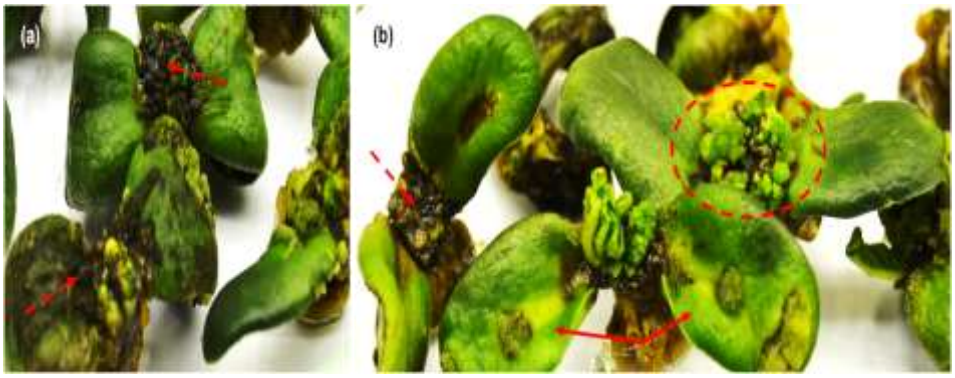

Figure 5.4. The response of double cotyledonary-node explants during *Agrobacterium*-mediated transformation on MS medium shoot induction medium after 30 days of culture.

Then, the explants were freshly re-cultured on new shoot induction MS medium, where poor shoot formation was still observed (Figure 5.4 and Appendix 5.1). Tissue browning, which led to blackening profoundly affected the established shoot culture (Figure 5.4). This had serious effects on the initiation and growth of shoots. Both friable and compact callus were also observed on explants exhibiting tissue blackening. The results showed that the rate of shoot induction and therefore, transformants regeneration was dramatically decreased in all cultures tested for *Agrobacterium*-mediated genetic transformation. No shoot elongation or *in-vitro* rooting was observed on explant transformed with *Agrobacterium*. All soybean genotypes, including Peking were affected by the transformation conditions.

Only 0.31% regeneration frequency was obtained in LS 677. Shoot induction frequency of all cultivars ranged between 0.0–20% as indicated on Table 5.2. The detrimental effects of browning on the number of shoots induced per explant, shoot growth and transformation efficiency were also reported by Li et al. (2017). This problem has been correspondingly reported to have affected the efficiency of many culture procedures. This includes, non-transformation procedures, which normally leads to failure of many *in-vitro* plantlets regeneration processes (Hartmann et al., 2013). There were significant variations observed between the results obtained from MS medium containing antibiotics and the response of infected explants. No much differences were recorded regarding the mean number of shoots induced per explant. But, there were differences in terms of genotype response as demonstrated by variance statistics in Appendix 5.1 and 5.2.

Table 5.2. The response of soybeans to modified MS culture media and infection with *Agrobacterium tumefaciens* carrying a pTF101.1 vector.

Source of Variation	Explants forming shoot buds (%)	Shoot induction frequency (%)	Regeneration frequency (%)
Culture medium with antibiotics			
Dundee	55.00^{c}	15.00^{d}	0.91^{c}
LS 677	60.00^{b}	30.05^{c}	2.70^{b}
LS 678	55.00^{c}	35.20^{b}	.
TGx 1740-2F	45.00^{d}	5.05^{e}	.
TGx 1835-10E	65.00^{a}	.	.
Peking	65.00^{a}	40.05^{a}	5.23^{a}
***Agrobacterium* infected explants**			
Dundee	75.5^{e}	10.0^{c}	.
LS 677	80.0^{d}	15.0^{b}	0.31^{a}
LS 678	90.5^{b}	20.0^{a}	.
TGx 1740-2F	85.0^{c}	0.0^{e}	.
TGx 1835-10E	55.5^{f}	5.00^{d}	.
Peking	100.0^{a}	0.00^{e}	.
Culture without antibiotics/infected explants			
Dundee	100.0^{a}	85.0^{c}	77.70^{c}
LS 677	100.0^{a}	95.6^{b}	80.03^{b}
LS 678	100.0^{a}	80.5^{d}	78.60^{c}
TGx 1740-2F	95.0^{b}	75.0^{e}	57.32^{e}
TGx 1835-10E	100.0^{a}	85.0^{c}	67.91^{d}
Peking	100.0^{a}	100.0^{a}	83.70^{a}

Values within columns designated by same alphabets are not statistically different at 1% confidence level. Values accompanied by similar superscript letters are significantly different at p-value less than 0.05%. **Explants forming buds (%)** was calculated from the mean number of shoots inducing shoot buds, **Shoot induction frequency (%)** was determined from the mean number of explants inducing more than three or more shoots per explant, and % **regeneration frequency** is calculated from the mean number of shoots per cultivar reaching rooting and acclimatisation stages.

Previous studies have indicated that genetic transformation in soybean is highly genotype-specific (Yan et al., 2000; Paz et al., 2004; Li et al., 2017). These reports support this study, by concurring with the findings. For example, genotypes such as; TGx 1740-2F and TGx 1835-10E showed consistent difficulties in achieving any significant response under the used culture conditions. Overall, the highest number of shoots induced per explants were obtained in cultivar Peking, LS 677, LS 678, Dundee, TGx 1740-2F and TGx 1835-10E, respectively. Presumably, the generation of reactive oxygen species upon the infection of *Agrobacterium*, which led to cell death

and tissue browning as indicated in Figure 5.4 and supported by Li et al. (2017) was also more pronounced on Dundee and TGx cultivars than LS genotypes and Peking. However, the use of appropriate concentrations of antioxidants such as the DTT could prevent cell necrosis and improve the transformation efficiencies in soybean.

5.5.2.4. *In-vitro* elongation and rooting

In the present study, normal shoot elongation and rooting was mainly achieved from shoots developed on MS basal medium without antibiotics, used as a control (Figure 5.5 a and d). Better shoot elongation of about 3–5 cm in height was achieved on MS medium without PGRs. Culture medium containing 0.55 mgL^{-1} GA_3 was tested but, resulted in thin and abnormally tall shoots. Induced shoots were then successfully rooted on the medium containing KIN (0.75 mgL^{-1}), IBA (3.25 mgL^{-1}) and NAA (2.75 mgL^{-1}).

This rooting medium produced mass of brown/white compact callus, particularly for cultivar LS 677 and Dundee as indicated on Figure 5.5 b and c respectively. All multiple buds and shoots formed on MS medium supplemented with antibiotics displayed serious difficulties in elongation and the formation of adventitious roots. The presence of antibiotics in the media may have caused the inhibition of shoots/buds elongation as well as for rooting of individual adventitious shoots. Consequently, shoot clumps obtained were not elongated *in-vitro*, especially when they were excised-off the cotyledonary explants.

Furthermore, shoot buds formed on infected explants also exhibited a similar response as observed on un-infected explants grown on media containing antibiotics. However, shoot growth inhibition and bacterial overgrowth caused death of many shoots during this culture. The adventitious shoots obtained were simply difficult to elongate because of the infection, exacerbated by the inefficiency of the antibiotics to eliminate the bacteria. Latent bacterial cells continued to contaminate the cultures, especially when subculturing for elongation and rooting was performed. It was clearly observed that, excising the shoots off the explants further exposed the shoots to the *Agrobacterium* overgrowth.

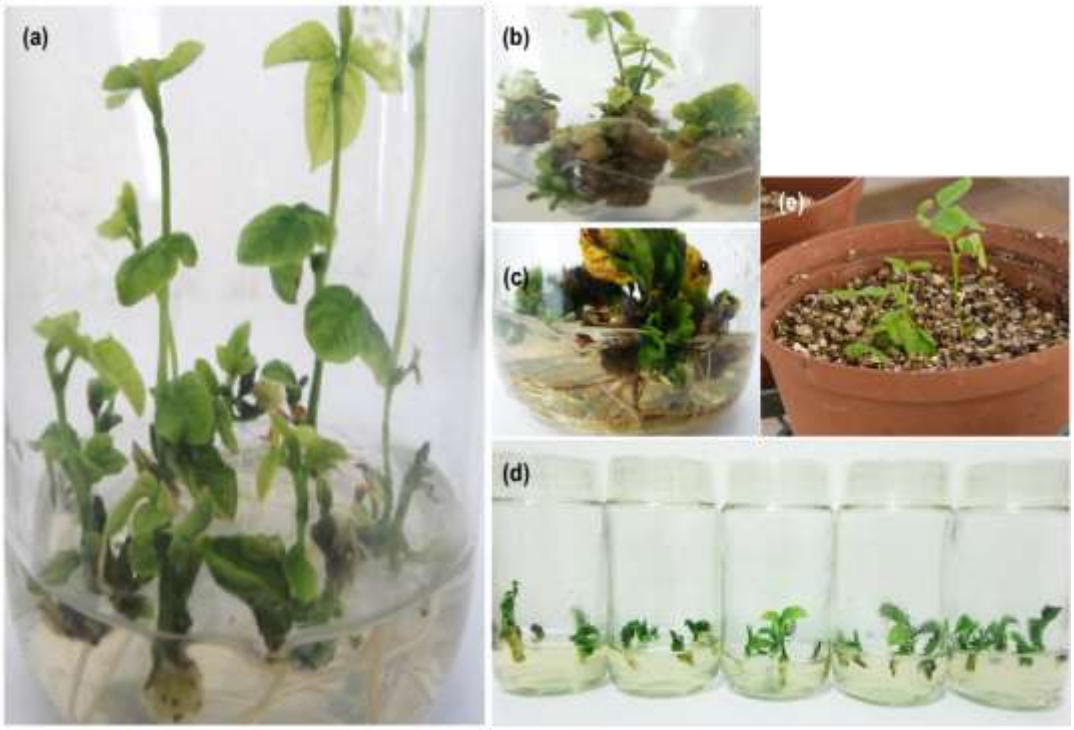

Figure 5.5. *In-vitro* elongation, rooting and acclimatisation. (a) Further elongation and rooting of shoots on hormone free medium. (b) and (c) Callus formation on medium containing growth regulators. (d) Stunted and partially elongated shoots cultured on hormone-free medium. (e) Hardening of *in-vitro* regenerated plantlets.

5.5.2.5. Hardening of *in-vitro* regenerated plantlets

No acclimatisation of plantlets was performed for any transformed plantlets except for the microplants obtained on MS medium without antibiotics or infection of explants with the *Agrobacterium*. The inefficiency of shoot culture to keep the adventitious shoots actively growing led to the complete failure of acclimatisation and development of a culture system that would be used for soybean transformation. The growth of shoots into plantlets to be acclimatised depended on the efficiency of the culture medium and most importantly, control of *Agrobacterium* growth without raising antibiotics to a level toxic to the tissue.

However, the successfully rooted plantlets were transplanted into 25 cm PC plastic pots and maintained in a moderate humidity growth room at 150–200 $\mu mol.m^{-2}s^{-1}$ at 16-hour photoperiod with 25±2°C temperature. The plantlets developed *in-vitro* had small thin leaves and reduced stems. They presented quick dehydration and needed to be rehydrated at least once every 4 days to avoid desiccation and death of plants. Pospisilova et al. (1999) indicated that, *in-vitro* regenerated plantlets can experience

sudden impairment as a result of the changes in environmental conditions. According to Chandra et al. (2010), these condition changes impact largely on the success of *in-vitro* based regeneration particularly, on the ability to transfer plants out of culture at low cost and with high survival rate.

5.5.3. Overall genotype response and implications of the results

The infection of the double cotyledonary-node explants with the inoculum inhibited shoot induction in all genotypes. Complete suppression of shoot growth from the initiated multiple buds was observed mostly in Dundee, TGx cultivars followed by LS cultivars and Peking. More than 70% of the cultured explants showed inhibition of shoot growth and shoot elongation, following incubation of *Agrobacterium* co-cultured explants for more than 30 days. In general, multiple bud initiation observed failed to grow into shoots because of the culture media and sensitivity of explant tissues to *Agrobacterium*.

These observations have been highlighted in most studies of transformation. Zaidi et al. (2006) reported 7.0 and 8.3% transformation efficiencies of *Oryza sativa* cultivar MDU 5, producing hygromycin-resistant calli using two binary vectors (pKHG4 and pIG121Hm) containing *hph* and *GUS* gene. The poor percentage of regenerated transgenic plantlets obtained was attributed to the ineffective and unrobust tissue culture systems. Furthermore, some of the stages like callus induction and regeneration was also found to be genotype specific. The efficiency of shoot induction achieved in this study also result in similar problems encountered during soybean regeneration of transgenic plantlets as reported by Zaidi et al. (2006), Zhang et al. (2014) and Wang et al. (2017).

The frequency of shoot induction in the controls (cultures established on MS medium without antibiotics and MS medium with antibiotics) of 86.9 and 20.9% was also consistent with several shoots per coty-node explant previously reported (Soto et al., 2013; Raza et al., 2017). These observations clearly indicate that, the regeneration of transgenic plants through the cotyledonary node method of soybean transformation in tissue culture still present challenges. The results further show poor reproducibility, less efficiency and genotype specificity. The variations clearly indicate that, the

cultivars used will variably express and transmit the transgenes if any event of transformation takes place. This was further exhibited by the different oxidation of tissues, chlorosis and wounded tissue necrosis. Soybean cultivar Peking and Dundee showed some resistance to production of phenolics and subsequent oxidation of tissues. Paz et al. (2004) reported effective use of agents that inhibit browning which include, but not limited to, cysteine, dithiothreitol (DTT) and sodium thiosulfate. A preferred cysteine in combination with DTT were used in all culture media. This may have given positive results in Peking and Dundee than in any other cultivar. According to the obtained results, optimisation of this method is still required, especially to increase the regeneration capacity of the soybean cultivars used.

5.5.4. Conclusions

This study indicates that, a high *Agrobacterium*-mediated genetic transformation efficiency in soybean may be improved by efficient control of *Agrobacterium* culture without inducing antibiotics toxicity. The shoot regeneration efficiency was significantly high using MS culture medium containing 2.0 mgL^{-1} 6-BA.

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CHAPTER 6

THE EVALUATION OF TOTAL PROTEIN PROFILES USING 1-D AND 2-D GEL ELECTROPHORESIS ON SOYBEAN COTY-NODE EXPLANTS CO-CULTURED WITH *AGROBACTERIUM*

6.1. INTRODUCTION

6.1.1. Background to the study

Soybean (*Glycine max* L.) is an important leguminous pulse crop grown for the production of oil and proteins. The legumes include cowpea, lentils, peas, peanuts and other pod producing plants that are cultivated commercially or privately as a food source. These plants have played a crucial role in the traditional diets of many countries including Brazil, China, India and regions in the Middle East and South America (Messina, 1999). In contrast, in many African and European countries soybean has a less significant role due to poor growth conditions. The growth and productivity of this crop has been adversely affected by biotic and abiotic stress factors (Mangena, 2015).

Even though it has the potential to become a major crop in less cultivated regions (Africa and Europe) because of its many uses (as feed, food etc.) plant modifications to increase yield are required (Sinclair et al., 2014). Genetic modification techniques such as *Agrobacterium*-mediated transformation, electro and chemical cell surface poration or direct protoplast-mediated DNA transfer need to be used to improve the agronomic traits of this crop in those regions. The limitations in genetic transformation protocols like genotype specificity, low transformation frequencies and lack of a routinely applied transformation protocol impacts negatively on soybean productivity (Zhang et al., 2001).

A rapid and highly efficient protocol for genetic transformation is a prerequisite, not only for yield trait improvement but also for gene function and molecular breeding studies of all legumes (Li et al., 2017). However, *Agrobacterium tumefaciens* is the most preferred method of genetic transformation in soybean and other legumes. The

technique is rapid, cheap to perform and easily optimised by improving the infection and regeneration efficiency, either *in vivo* or *in vitro*. Therefore, this study will investigate the amenability of genotypes by comparing variations through total protein profiling of explants following co-cultivation with *Agrobacterium*. This is carried out to further identify the protein profiles of transformed explants with the non-transformed explants, which may lead to the identification of specific proteins that may promote or inhibit this process.

6.1.2. Soybean proteins and analysis

The proteins found in soybean have been reported to lower blood cholesterol, reducing the risk of cancer and osteoporosis (Messina, 1999). Soybeans are very low in fats, therefore, being an excellent source of dietary fibre and a variety of micronutrients and phytochemicals. The essential amino acids and specific proteins such as isoflavones that this crop contains are required by the human body, particularly because the body cannot synthesise them. As a result of this, soybean is considered an important source of affordable proteins and vitamins.

Proteins found in soybean have received considerable attention for the aforementioned functions and their potential role in enhancing food products, as well as nutritional health. However, a promising approach to producing transgenic soybean plants is to study protein expression in the infection stage of transformation. In this case, proteins expressed resulting from induced wounding and infection by *Agrobacterium tumefaciens* can be identified using Sodium-dodecyl gel electrophoresis (SDS-PAGE). Proteins which are synthesised in infected explants, which may promote or suppress gene transfer and expression may be identified and the corresponding genes isolated.

6.1.3. Motivation to the study

Since the ability to genetically modify soybean plants via *Agrobacterium*-mediated transformation was established by Hinchee (1988), a large number of optimised protocols have been tested. A few number of soybean varieties have been successfully transformed, including other commercial grains such as maize, sorghum,

rice and wheat using this technique. Transgenic soybean plants achieved using this technique include transformation of cultivar A0949 with a Nos-NPT 11 gene and the *bar* gene in cultivar Jack Purple and Tianlong 1 (Chee et al., 1989; Li et al., 2017). Carotenogenic and herbicide tolerance genes have also been introduced in other legumes like mung bean and cowpea, including maize, rice and canola (Mundlara and Rashid, 2006; Lee et al., 2012). However, soybean like many legumes is still considered recalcitrant to *Agrobacterium*-mediated genetic transformation. The susceptibility of this crop to strains of *Agrobacterium* used and the efficient transfer and expression of genes of interest as well as the effective distinguishing of regeneration-competent cells and tissues to those that cannot regenerate is still a massive challenge. These constraints warrant a thorough investigation on the causal effects which makes this crop recalcitrant. The use of SDS-PAGE to probe specific proteins expression patterns as a result of explant infection may determine whether these proteins promote or suppress the regenerability of transformed tissues during transformation.

6.2. LITERATURE REVIEW

6.2.1. Importance of soybean

Soybean plays a critical role in world agriculture, with about 40% proteins, 20% oil and 30% carbohydrates contained within the seeds. This crop serves as the cheapest and most profitable form of oilseed worldwide for many producers, especially small holder poultry farmers (Tefera, 2011). The industrial processing of this crop to manufacture high protein rich feeds for livestock, pigs and fish farms is growing immensely. The use of soybean in the production of edible oil and biodiesel as a green alternative fuel is also expanding (Silva et al., 2010). In human nutrition and health, soybean meals have proved to reduce the cause of a number of acute and chronic conditions. Messina (1999) reported the improvement of body calcium retention lowering urinary calcium excretion after the use of soy-proteins compared to consumption of a mixture of animal proteins.

Soybeans were also found to contain low fats (approximately 5%), easily modulated trypsin inhibitors and other compounds considered as non-nutritive components. Some of these compounds like phytate were considered to reduce mineral bioavailability of beans but, it has been postulated that phytic acid also lowers the risk of colon and breast cancer (Vucenik et al., 1997). Soybean is considered an excellent source of iron, zinc and folate which serve as essential nutrients and reduce the risk of neural tube defects in humans and promote efficient uptake of vitamin C (Daly et al., 1995; Hunt et al., 1994). However, among all legumes, soybeans are unique because they are a concentrated source of isoflavones that naturally reduce the risk of cancer and heart disease (Messina, 1999).

6.2.2. Protein synthesis in soybean

Soybean like other plants contains a variety of proteins that include catalytic, transport, structural, storage and regulatory proteins. Regulatory proteins are those that regulate gene or protein expression and cell to cell recognition and signalling (Lesk, 2010). The storage proteins that are contained in the seeds, especially the cotyledons make-up 80% of the total proteins found in this crop. Both essential and semi-essential protein

building blocks (amino acids) including arginine, leucine, lysine, phenylalanine and tyrosine can be found (Le et al., 2007). Seeds of soybean contain larger amounts of abundant and usable stored proteins than any part of the root or shoot of the plant. The 7S and 11S globulins are most predominant storage proteins while 2S, 9S as well as 15S globulins are less abundant proteins (Taski-Ajdukovic et al., 2010). These proteins are synthesised as a result of plant growth and development involving the various vegetative and reproductive stages.

Other groups of proteins however, can also be induced when the plant is enduring stress (biotic or abiotic stress). Stewart and Bewley (1981) reported differential composition of integral membrane associated proteins due to chilling treatment in soybean cultivar Biloxi and Fiskeby. Recently, Brumm and Hurburgh (2002) and Zarkadas et al. (2007) reported that both genetic and environmental factors strongly influence the synthesis of protein compositions in soybean seeds. Drought and salinization in particular are the most challenging stress constraints worldwide. Different soybean genotypes have been genetically modified in the attempt to increase seed protein yield and oil content, in order to circumvent the negative effects caused by these growths limiting factors. But, the negative correlation that exists between protein content and seed yield of newly bred cultivars pose a secondary challenge thus limiting these applications (Taski-Ajdukovic et al., 2010).

6.2.3. Proteolytic enzymes in soybean

A major impediment to increasing soybean yield through large scale cultivation for both commercial and subsistence farming include sensitivity of this crop to the abiotic stress such as drought and expression of proteolytic enzymes. Even though, proteolytic enzymes (also known as proteases) are essential for the maintenance and survival of soybean plants, these enzymes can be damaging when present in higher concentrations or overexpressed (Habib and Khalid, 2007). According to Scott et al. (1992) proteases are responsible for the catalyses of hydrolytic cleavage of numerous specific peptide bonds, including cleavage for the assembly of 2S and 11S globulin storage proteins in the soybean cotyledons. These enzymes are also widely distributed nearly in all animals and microorganisms (Lawrence and Koundal, 2002; Valueva and Mosolov, 2004; Christeller, 2005).

The expression of proteases is natural but can be triggered by environmental stimuli as a regulatory defence mechanism. Their proteolytic activities serve as mediators of signal initiation during stress, termination of cellular processes such as cell or tissue senescence, hormonal inductions and many other regulations in biological systems (Habib and Khalid, 2007). Apoptosis or programmed cell death (PCD) in plants that comprises cytoplasmic shrinking, membrane blebbing, and nuclear condensation involves activation of protease enzymes. Several proteolytic enzymes such as cysteine, trypsin and aspartic proteases have also been thoroughly described (Christeller, 2005).

6.2.4. The role of cysteine proteinases in soybean

Proteolytic enzymes called cysteine proteases catalyses the hydrolysis of various polypeptide substrates for the production and assembly of proteins that get remobilised or degraded (Du Pleesis, 2013). These endoproteases have a molecular mass of about 21–30 kDa and cleave the target protein on specific sites of thiolate groups (Grzonka et al., 2001). Cysteine proteases are well known for their key role in biochemical processes, implicated for the development and continuation of several diseases. Their role in disease formation, especially during PCD involves dismantling of organelles and the different macro molecules required for plant growth and development (Beers et al., 2000).

They are largely involved in translation and folding of storage proteins, protein remobilisation, signalling controls and to a lesser extent for morphogenesis. These enzymes catalyse cleavage of sulfhydryl groups which entails a cysteine and histidine residue. They play a crucial role during senescence caused by both biotic and abiotic stress, particularly, oxidative stress caused by exposure to drought resulting from higher temperatures (Guerrero et al., 1998). Cysteine protease accounts for about 90% of the hydrolytic degradation of storage proteins in plants, especially in soybean, wheat and maize seeds (Qi et al., 1992; Grzonka et al., 2007).

6.2.5. The role of cysteine proteinase inhibitors

The papain-like and caspase-like proteinases are two kinds of cysteine proteases involved in PDC caused by biotic and abiotic stress factors (Solomon et al., 1999). According to Martinez et al. (2007) chilling, salinity, drought and heat shock can induce the occurrence of cysteine proteases in the affected plant tissues. However, cysteine protease inhibitors (cysteine PI) such as *Oryza*cystatins (OC) originating from *Oryza sativa* L. (rice) mature seeds have inhibitory effects on cysteine proteases (Kondo et al., 1990). They include OC-I and OC-II cystatins, which are water soluble and confer drought resistance in host plants.

These *Oryza*cystatins (OC-I and OC-II) differ in the inhibitory efficiencies and show greater activities for papain and cathepsin, respectively (Birk, 2003). The interaction of cysteine protease inhibitors on plant protease activity render these enzymes inactive or less active and would require proteolytic processing to be activated. In this case, the cysteine PI functions to regulate the activity of corresponding proteases and plays a key regulatory role in activities involving these enzymes (Habib and Khalid, 2007). In addition to this, Qi et al. (2005) reported receptor clearance signalling and growth factor activities as part of their functions in many biological processes.

6.2.6. Separation and analysis of proteins

Protein analysis techniques have been used in plants to purify individual components and study them in isolation, particularly for the nutritional and trait improvement of crops. The use of a one or two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the popular tools utilised for protein analysis in plant extracts. This technique has demonstrated that plant cells contain a large and complex set of molecules forming chains of polypeptides (Lesk, 2010). High resolution one-dimensional and two-dimensional SDS-PAGE has been used to successfully isolate and separate individual amino acids even when the protein profile is influenced by exogenous factors. Zurfluh and Guilfoyle (1980) examined alterations in protein synthesis on soybean hypocotyls developed from seedlings pre-treated with 50 µM 2,4-dichlorophenoxyacetric acid (2,4-D). The report indicated that the spectrum of polypeptide synthesis on the hypocotyls appeared to be altered in response to 2,4-D

when compared to auxin-untreated seedlings. This technique has been used to assess changes caused by plant growth regulators (PGRs), *A. tumefaciens* infection and environmental stress factors on the patterns of polypeptide synthesis in various parts of plants, including elongated and basal sections of immature and mature soybean plants (Zurfluh and Guilfoyle, 1980; Le et al., 2007; Taski-Ajdukovic et al., 2010). Other applications include isolation and detection of proteases, lipids and phosphoproteins using this technique (Karpe and Hamsten, 1994; Du Plessis, 2013; Kinoshita et al., 2009).

6.2.7. Use of tissue culture-derived plant materials for protein analysis

The use of microshoots or callus cells to isolate and study protein changes has been a common practice in many research laboratories. The analysis of proteins in soybeans exogenously treated with ammonium on the number of ribosomal structures, expression of ribosomal genes and encoded proteins of small subunit rp56 and 18S rRNA in plant tissue derived from *in-vitro* culture. This study examined the possible optimum ratio of ammonium to nitrate in the nitrogen supply used by plant cells for protein synthesis. In soybean root nodules, *in-vitro* culture was also used to examine protein expression to assess uricase activity during nitrogen fixation (Larsen and Jochimsen, 1986). Similar results of total profiling of the protein content have been sufficiently and efficiently obtained on the initiated callus.

Stejskal and Griga (1995) reported the comparative analysis of protein composition studied in soybean somatic embryos that were induced on immature zygotic embryos and seeds using *in-vitro* cultures. Callus culture in particular, makes it possible to also study the peroxidase isozyme activity on both callus and somatic embryos, as reported by Stejskal and Griga (1995). These studies provide evidence of the efficient use of *in-vitro* callus cultures in assessing the biochemical physiology of plant tissues. Tissue culture propagated cells serve as important plant materials, which reproducibly respond to exogenous factors that may alter protein synthesis. Therefore, allowing direct analysis of proteins in the treated tissues without interference or contamination. The process is relatively efficient in soybean, since it is the only grain legume in which cell cultures can be reliably induced and used to study changes in protein patterns (Hartmann et al., 2011).

6.2.8. Protein analysis in transgenic plants

The synthesis of specific cell proteins and protein complexes is a multistep process occurring in different cellular compartments. This process is initiated and regulated by the expression of different genes and gene combinations naturally contained in the plant's genome (Leary and Huang, 2001). New genes of interest and gene combinations are being expressed in many crop plants using genetic engineering. This technique revolutionised plant breeding by introducing *in-vivo* and *in-vitro* protocols that rapidly and efficiently facilitate the production of genetically modified crops with improved agro-economic traits. The development of transgenic banana, cassava, coffee, maize, millet and cowpea include some of the improved crops, established through genetic transformation (Brink et al., 1998). However, the analyses of transgenic plants for proteins encoded by the expressed genes of interest have been evaluated using SDS-PAGE.

Expression of *Bacillus thuringiensis* (*Bt*) crystal protein in transgenic cotton, corn and potato has been analysed using this technique (Gould, 1998). The modification of crops with short sequences of genes from *Bt* expresses crystal proteins, conferring their resistance to pests. Others include glyphosate-resistant crops (Roundup Ready crops such as corn, alfalfa and canola) expressing genes deactivating key enzymes involved in amino acid synthesis and Roundup Ready or Herbicide-Tolerance (HT) soybean expressing the same gene. In *Carica papaya*, viral resistance of the ringspot virus is conferred by the expression of PRV coat proteins which is encoded by the coat PRV protein gene isolated from Papaya ringspot virus (PRV) (Trigiano and Gray, 2005; Tripathi et al., 2008; Bohn et al., 2013). All these involved analyses of the proteins expressed using polyacrylamide gel electrophoresis under different extraction and separation conditions. SDS-PAGE is still commonly used by many laboratories to comparatively analyse proteins expressed in different transgenic lines worldwide (Pickardt and Saalbach, 2000).

6.3. PURPOSE OF THE STUDY

6.3.1. Study hypothesis

The infection of soybean explants with *Agrobacterium tumefaciens* during genetic transformation may lead to variations in the protein profiles of the infected tissues.

6.3.2. Study aim and objectives

This study was aimed at investigating the variations in protein profiles of cotyledonary-node explants infected and co-cultivated with *A. tumefaciens* and compare the protein patterns expressed among the cultivars used during *Agrobacterium*-mediated transformation.

The study objectives were to:

i. Infect and co-cultivate the double cotyledonary-node explants with *Agrobacterium tumefaciens* carrying a pTF101.1 vector.
ii. Extract and solubilise the proteins from co-cultured explants using detergents and other components required for electrophoretic technique.
iii. Determine the protein profiles of infected coty-node explants using 2D SDS-PAGE.
iv. Visualise and quantitively analyse protein band patterns using Coomassie (Brilliant) Blue stain.

6.4. MATERIALS AND METHODS

6.4.1. Plant materials and sterilisation

Freshly harvested soybean seeds cultivar Dundee, LS 677, LS 678, Peking, TGx 1740-2F and TGx 1835-10E were used in this study. The seeds were acquired as indicated in Chapter 2. The seeds were surface sterilised using chlorine gas as described in Chapter 3.

6.4.2. *In-vitro* germination and explant preparation

Sterilised soybean seeds were inoculated on MS medium containing 4.0 mgL^{-1} 6-BA for germination. The germination cultures were kept in a tissue culture growth room at 24±2°C, 50–60 $\mu molm^{-2}s^{-1}$ light intensity and 16-hour photoperiod for 10 days. After 10 days, seedlings obtained were transversely cut on the hypocotyls, 4–6 mm beneath the cotyledons and their epicotyls excised-off at the junctions to obtain double cotyledonary node explants as indicated in Figure 3.1.

6.4.3. Co-cultivation and sample preparation

6.4.3.1. Explant co-cultivation with *Agrobacterium*

Soybean cotyledonary-node explants were immersed into an infection medium containing *Agrobacterium tumefaciens,* reinitiated and resuspended on infection medium as described by Paz et al. (2004). The medium contained Gamborg's B5 salts, vitamins and 30 gL^{-1} sucrose and was supplemented with filter sterilised GA_3 (0.25 mgL^{-1}), 6-BA (1.67 mgL^{-1}) and 40 mgL^{-1} acetosyringone, added in the medium after autoclaving. Cotyledonary-node explants were allowed to incubate at room temperature for 20 minutes with occasional gentle agitation on an Orbital Shaker (120 rpm).

After infection, the infected explants were transferred to agar plates containing co-cultivation medium prepared as described in section 5.4.5. Petri plates were then tightly wrapped with a parafilm and placed in a tissue culture growth room for 4 days.

After 4 days of co-cultivation, the co-cultured explants were removed from co-cultivation media and briefly washed with sterile distilled water. The cotyledonary node explants that were not infected with *Agrobacterium* were also incubated on B5 co-cultivation for 4 days and used as a control.

6.4.3.2. Plant tissue sample preparation

A total of three Petri dishes containing 30 double cotyledonary node explants were prepared for each cultivar. The *Agrobacterium* co-cultured explants and coty-nodes used as control were homogenised into a fine powder in liquid nitrogen using a mortar and pestle. Homogenised tissues were then transferred into 25 mL sterile centrifuge tubes and kept at −80°C until use for protein extraction.

6.4.4. Buffer preparations

The following buffers were prepared by adding the different buffer components according to the Bio-Rad Bulletin number 6040.

6.4.4.1. Lysis buffer

A total amount of 2 mL lysis buffer was freshly prepared per 200 mg sample, for protein sample preparation. Lysis buffer was prepared by mixing 1920 µL of protein solubilizer (prepared by mixing 8 M urea with 2% CHAPS), 24 µL protease inhibitor (100X), 20 µL DTT (2 M) and 36 µL of deionised water. The buffer was thoroughly mixed and stored at -4 °C until use.

6.4.4.2. Sample buffer

Sample buffer was prepared by mixing 0.5 M Tris-Cl (pH 6.8), 2.0 mL SDS (10%), 1.0 mL glycerol (10%) and 0.5 mL bromophenol blue (0.5%) with 4.8 mL deionised water. The mixture was stored at room temperature. SDS reducing buffer was prepared by adding 50 µL of 2-mercaptoethanol to 0.95 mL of sample buffer before use.

6.4.4.3. Resolving buffer

The resolving buffer was prepared by dissolving 18.2 g Tris base in 80 mL of water. Buffer pH was adjusted to 8.8 with 5M HCl, and final volume made to 100 mL using deionised water. The buffer was kept at 4°C until use.

6.4.4.4. Stacking gel buffer

The stacking gel buffer was prepared by dissolving 6.1 g Tris base in 80 mL deionised water. The buffer pH was adjusted to 6.8 using 5M HCl, and water was added to a final volume of 100 mL. The buffer was stored at 4°C until use.

6.4.4.5. Electrode buffer

Electrode buffer (5X), pH 8.3 was prepared by dissolving 9 g of Tris-base, 43.2 g of glycine and 3 g of SDS in deionised water. The final buffer volume was made up to 600 ml using deionised water.

6.4.5. Protein extraction and precipitation

A 1 mL of cold 10% (w/v) 2,2,2-trichloroacetic acid (TCA) and 0.07% (v/v) ß-mercaptoethanol acetone were added in 200 mg of ground coty-node tissues. The mixture was vortexed for 1 minute and then incubated at −20°C for 2 hours. After incubation, the mixture was centrifuged at 10,000 x g for 5 minutes at 4°C to remove chlorophylls. The supernatant was discarded, and the step repeated at least once. Pellet was resuspended in 1 mL cold acetone containing 0.07% (v/v) ß-mercaptoethanol by vortexing.

The mixture was then centrifuged at maximum speed for 20 minutes at 4°C in a microcentrifuge. The supernatant was discarded, and this step repeated until the pellet became colourless. Pellet was dried under vacuum for 2–5 minutes and then resuspended in 1900 µL freshly prepared lysis buffer. The mixture was sonicated on ice for 3–6 rounds of 15 seconds each at 20% power to solubilise the precipitated proteins. The lysate was incubated on a rotary shaker for 15 minutes at room

temperature. A 5 µL of 99% N,N dimethylacetamide (DMA) was added to the lysate and incubated on a rotary shaker for 30 minutes at room temperature. A 2 M dithiothreitol (DTT) was added to the lysate to quench any excess DMA. The lysate was centrifuged at 16,000 x g for 20 minutes at 4°C and the supernatant transferred to sterile centrifuge tube. The extracted proteins were stored at −80°C until use for gel electrophoresis.

6.4.6. Gel preparation

The 12% resolving gel was prepared by mixing deionised water (3.35 ml), 1.5 M Tris-HCl, pH 8.8 (2.5 ml), 4.0 mL acrylamide/bis concentrate (30% *T*, 2.7% *C*), 10% (w/v) SDS (0.1 ml), 10% (w/v) ammonium persulfate (50 µl) and 0.05% of TEMED (5 µl). The stacking gel was prepared by mixing deionised water (4 ml), 2.5 ml of 0.5 M Tris-HCl, pH 6.8, 1.3 ml of acrylamide/bis concentrate (30% *T,* 2.7% *C*), 100 µl of 10% (w/v) SDS, 100 µl of 10% (w/v) ammonium persulfate and 10 µL of TEMED.

6.4.7. Protein fractionation

Protein samples used in the fractionation were prepared by mixing 10 µl of extracted protein with 10 µl of 4X concentrated sample loading buffer prepared as described above. The protein mixture was boiled for 5 minutes to denature the proteins. About 20 µl of boiled protein samples were loaded into a well in the gel, together with one loaded well of 5 µl molecular weight marker. The tank was closed, connected to BIO-RAD power pack and electrophoresis carried out at 100 volts. The gel was removed from the plates after reaching the bottom of the resolving gel and placed in a staining container.

6.4.7.1. First dimension: Isoelectric focusing (IEF)

The fractionated protein samples were separated according to their charge using IEF. A total of 40 µg of concentrated protein extract was pipetted into the IPG Runner cassette system rehydrated using the buffer prepared as described on 6.4.4.2. The acidic end of IPG Runner strips (pH 3–10) were inserted into the slot with the gel facing towards the film. The strips were rehydrated at room temperature overnight and then

inserted into the mini cell chamber. Isoelectric focusing was then performed, using electric voltage of 175 to 2000 v over 45 minutes and 2000 v overnight. After the completion of IEF, the focused strips were run immediately on the SDS-PAGE analysis gels.

6.4.7.2. Second dimension: SDS-PAGE

To resolve the complex protein mixture into the number of individual protein spots, Bio-Rad strips were removed from the cassette and incubated for 15 minutes in 5 ml LDS sample buffer containing a reducing agent DTT for solubilisation of focussed proteins. The strips were then incubated for 15 minutes in 5 ml LDS buffer containing an alkylating agent (Iodoacetamide). The strips were overlaid on the gel with 400 µl of 0.5% agarose sealing solution, and electrophoresis performed at 200 V for 40–50 minutes.

6.4.7.3. Visualisation of proteins and spot detection

Coomassie Brilliant Blue R-250 was used for profiling of proteins in the gels. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in water: 40% methanol and 10% glacial acetic acid. The gels were shaken using rotary shaker at room temperature. Then later de-stained using de-staining solution prepared by mixing 40% methanol and 10% glacial acetic acid.

6.4.8. Image acquisition and analysis

The gels were digitised by imaging with a scanner and the image analysed with the PD-Quest software using 8.1 software package. Proteins spots were detected by the software based on the spots parameters chosen by the selection of the biggest, smallest and least intense spots. One master gel was selected to compare the differential identity of the expressed proteins.

6.5. RESULTS AND DISCUSSION

In this study, we compared the protein profiles of the six selected soybean cultivars (Dundee, LS 677, LS 678, Peking, TGx 1740-2F and TGx 1835-10E) to determine variations expressed due to *Agrobacterium* infection, compared to those that were not infected, used as a control. Sample preparation for this proteomic analysis using TCA/acetone was efficient in precipitating the proteins. In order to justify protein yield obtained, the evaluation of precipitated proteins was conducted first using 1-D gel electrophoresis and then 2-D gel electrophoresis.

6.5.1. Recovery of co-cultured cotyledonary explants

Seeds of the soybeans were successfully germinated on MS culture medium prepared as described in 6.4.2. The seeds produced 84, 90, 85, 76, 74, and 80% germination for Dundee, LS 677, LS 678, Peking, TGx 1740-2F and TGx 1835-10E, respectively. This positive response in germination allowed for the successful preparation of sufficient cotyledonary explants used for the infection and co-cultivation with *Agrobacterium*. The results showed that, both better germination and bacterial culture initiation promoted the efficient co-cultivation of explants with *Agrobacterium*. Additionally, the high germination rates are is in part owed to the freshly harvested seeds that were used for the experiments. After the infection with *Agrobacterium*, there were no necrotic and chlorotic symptoms observed on co-cultured explants following both the infection and the period of incubation used. These results were similar and consistent with observations made in Chapter 4 and 5 when using freshly harvested soybean seeds.

6.5.2. Protein precipitation by TCA/acetone method

All soybean genotypes gave high protein concentrations, except Peking which yielded slightly dilute proteins lysate from 2,2,2-trichloroacetic acid-acetone (TCA/acetone) precipitation. The results clearly indicated that, the extraction and precipitation by TCA/acetone method (Bio-Rad Bulletin 6040) was the best choice for the co-cultured cotyledonary tissues. Furthermore, the lysate did not indicate any significant differences in the overall mass or amount of precipitated proteins. These results

implied that there was no pellet loss from repeated centrifugation during purification and decontamination step. Rajalingam et al. (2009) reported the inconsistencies, including the loss of pellet and less effectiveness of TCA in precipitating complex proteins, particularly for plant tissue samples. However, the results obtained in this study suggest that high quality samples can be obtained, by the TCA/acetone protein extraction protocol. This was in line with observations made by Natarajan et al. (2005) and Xu et al. (2006) who emphasised that the simplicity of the TCA/acetone protocol allows for a dynamic range of protein accumulation and reproducibility of the protein separation.

The protein samples showed very minimal degradation and no contamination as indicated by Harder et al. (1999) and Sarnighausen and Reski (2008). These authors have reported that, protein extraction must reproducibly trap comprehensive repertoire of proteins without degradation or contamination. However, Da-Cruz and Martinou (2008) and Zhou et al. (2008) reported that, high quality samples may be difficult to obtain due to the wide array of physicochemical properties of proteins like size, charge and hydrophobicity, as further indicated by Rajalingam et al. (2009). A number of other several protocols have showed to trap the full proteome but, TCA/acetone protein precipitation method used in this study was also efficient for sample preparation using cotyledonary node explants.

6.5.3. Protein evaluation through 1-D analysis

6.5.3.1. Protein analysis on uninfected cotyledonary-node explants

The total protein profile of the six selected soybean cultivars were visualised on 1-D SDS-PAGE gels as shown in Figure 6.1. Protein lysates contained a combination of low and high molecular weight proteins that migrated at approximately 3 to 100 kDa. This was observed both in the control and *Agrobacterium* transformed coty-node explants. These bands were clearly visualised on SDS-PAGE when only 10 µL of extracted proteins were loaded in the gel wells.

Table 6.1. Protein range identified using one-dimensional gel electrophoresis in six selected soybean cultivars.

Soybean cultivar	Well number	*Agrobacterium* uninfected explants (kDa)	*Agrobacterium* infected explants (kDa)
Dundee	S1	20 – 99	10 – 100
LS 677	S2	12 – 100	10 – 120
LS 678	S3	12 – 120	5 – 120+
Peking	S4	10 – 60	10 – 100
TGx 1740-2F	S5	12 – 100	5 – 100+
TGx 1835-10E	S6	12 – 100	4 – 100+

Note: The molecular weight of proteins was estimated in kilodaltons (kDa) using E. coli unstained protein standard marker.

A 1-D analysis of the proteins in the control showed that the predominant polypeptides in the uninfected cotyledonary explants were in the size range between 20−99 kDa for soybean cultivar Dundee, 12−120 kDa for both LS soybeans, 10−60 kDa in Peking and 12−100 kDa for both TGx soybean cultivars (Table 6.1). The analysis of the gels further indicated that, Peking produced lower concentration of proteins than any other soybean cultivar used. Figure 6.1 shows that a comparison can be made amongst the genotypes, with LS and TGx cultivars, including Dundee producing the most intense bands, than the bands observed in Peking. Furthermore, the figure shows that although, TCA/Acetone extraction protocol used is considered the best procedure for protein purification, it may not have been the best choice for all genotypes, especially Peking. Protein concentration with this method was low in Peking, even after they were concentrated using Millipore sigma protein concentration tubes.

This observation may imply that the ratio of protein concentration to TCA/acetone precipitate was significantly low, suggesting that the homogenates yielded lower protein concentration in this cultivar. According to Islam et al. (2004) this could be a common problem in protein precipitation, and could be improved by additional grinding of the sample material to improve the solubility of the extracted proteins. But, since the results obtained in Chapter 2 showed that Peking seeds also had a high deterioration rate, which was accompanied by a delayed seed germination compared to other genotypes. The results obtained during SDS-PAGE may also imply that, seed deterioration in Peking is directly proportional to the yielded protein concentrations of the seeds.

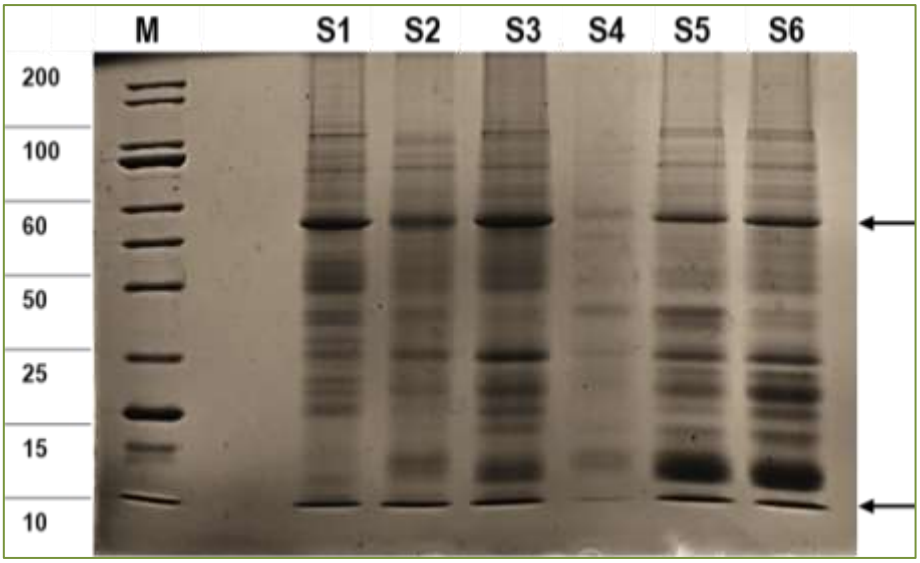

Figure 6.1. One-dimensional gel electrophoresis of uninfected cotyledonary node explants used as control from the six selected cultivars using SDS-PAGE (12%). **M**- molecular marker, **S1**- Dundee, **S2**- LS 677, **S3**- LS 678, **S4**- Peking, **S5**- TGx 1740-2F, **S6**- TGx 1835-10E.

This might be a clear indication that soybean seed deterioration commences immediately after harvesting as indicated in the previous chapters (2, 3 and 4). Protein bands obtained from both LS 677 and LS 678 were reproducibly resolved in Coomassie blue staining. This was followed by proteins extracted and precipitated in soybean cultivar Dundee, TGx 1835-10E and TGx1740-2F. However, in some instances, other replicates showed equally high intensity of protein bands in TGx 1835-10E and LS 678, followed by Dundee. Furthermore, soybeans that are genetically linked produced similar trend of protein profiles expressed. For example, protein bands observed in LS 677 (S2) and LS 678 (S3), as well as bands found in TGx 1740-2F (S5) and TGx 1835-10E (S6) are similar. This is a similar trend observed in Chapter 2, 3 ad 4 on the evaluation of germination, shoot induction and callus formation, which shows some level of genotype specificity.

6.5.3.2. Protein analysis on *Agrobacterium* infected explants

It was determined through visual inspection that there were more protein bands on gels ran with lysate samples from the *Agro*-infected double cotyledonary explants compared to the control (6.5.3.1). The level of expression of these proteins was also different, as indicated on Figure 6.2 compared to Figure 6.1. Nevertheless, the majority of the protein bands did show changes on gels of *Agro*-infected explants when they

were compared to the explants used as a control. These results show that some proteins were probably expressed in explants infected with *Agrobacterium*, that were not appearing on uninfected explants. Some intense bands were observed between 25 and 40 kDa in all cultivars. But, such similar protein bands were not observed in cultivar Peking. The molecular weights of those protein bands were approximated to be about 26 kDa and 40 kDa. Furthermore, all infected extracts showed a single band of approximately 5 kDa, which appeared to fall within a range of 4 – 9 kDa (Table 6.1). The results suggest a possible influence of this polypeptides response due to co-cultivation of explants with *A. tumefaciens*. The pattern expressed share similarities with other proteins described in other plant species as a result of stress or infection with *Agrobacterium*.

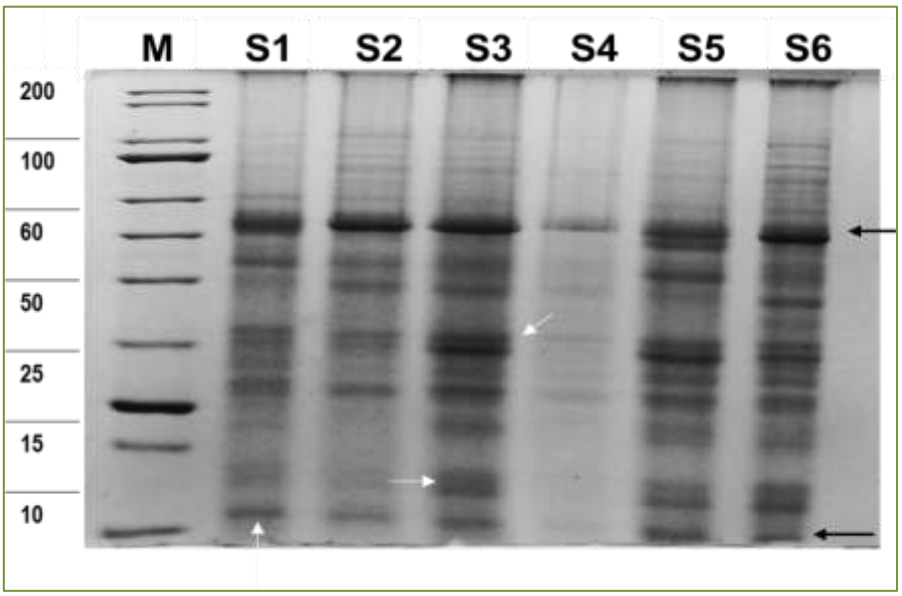

Figure 6.2. One-dimensional gel electrophoresis of *Agrobacterium* infected double cotyledonary node explants from the six selected cultivars using SDS-PAGE (12%). **M**-molecular marker, **S1**- Dundee, **S2**- LS 677, **S3**- LS 678, **S4**- Peking, **S5**- TGx 1740-2F, **S6**-TGx 1835-10E.

Kirova et al. (2005) reported differences in the expression of proteins as a result of drought stress response in soybean cultivar Hodgson. According to this report, the quantity of proteins expressed due to stress was high to some degree as compared to proteins produced in control plants. Thus, this study showed that soybean plants subjected to stress will cause an increase in the amount of soluble thermostable proteins. Similarly, the period of explant infection and co-cultivation with *Agrobacterium* could cause physiological stress that lead to the production of

additional stress-induced proteins or expression of transgene proteins. In case of this study, it could be the transcription of cystatin proteins encoded from the *Oc-1* gene contained within the pTF101.1 vector used. The period of co-cultivation plays an important role during integration of the T-DNA into plant chromosomes and stable expression of transferred genes during genetic transformation (Mangena et al., 2017).

6.5.4. Protein evaluation by 2-D gel analysis

The use of high density bacterial culture for infection process may give higher transformation rates, at the same time causing changes in the patterns of proteins expressed by plant tissues. By contrast, gels obtained from the control had different protein patterns compared to gels obtained from the *Agrobacterium* infected extracts (Figure 6.3 and 6.4, A-L). A comparison can be made between proteome in the control and *Agrobacterium* infected extracts. Although, proteins in all cultivars appeared to be most abundant at a range between 5 to 80 kDa. Some of these lower molecular weight proteins could be described as stress-related proteins. They probably have key regulatory roles in plant development and responses to *Agrobacterium* infection. Hashimoto et al. (2004) identified such proteins' involvement in many plant developmental programs, including phytohormone biosynthesis, biotic and abiotic stress plant responses.

Proteomic analysis of cotyledonary explants infected with *A. tumefaciens* identified more protein spots as indicated in Figure 6.3 (B, D, F, H) compared to the control without bacterial infection (Figure 6.3 A,C, E, G). A similar observation has been made in Figure 6.4 (J, L) of *Agro*-infected explants compared to Figure 6.4 (I, K). Although the majority of these differentially displayed protein spots ranged between 5 and 80 kDa of approximated molecular weight, only soybean LS 677 produced a clearly identifiable difference in protein expression (Figure 6.3 E, F).

This suggests that, the proteins may have been expressed in response to co-cultivation of explants with *A. tumefaciens* in comparison with the uninfected controls. Such observations can be further used to confirm possible transformation events. However, the low abundance and identification of protein spots observed in soybean such as Peking (Figure 6.4 G, H) was due to either the low abundant nature of proteins

in the rapidly deteriorating seeds, poor explant vigour or poor gel resolution generated through 2-D SDS-PAGE. Possible heavy posttranslational modifications (Imin et al., 2005) or limitations of isoelectric focusing may have as well contributed.

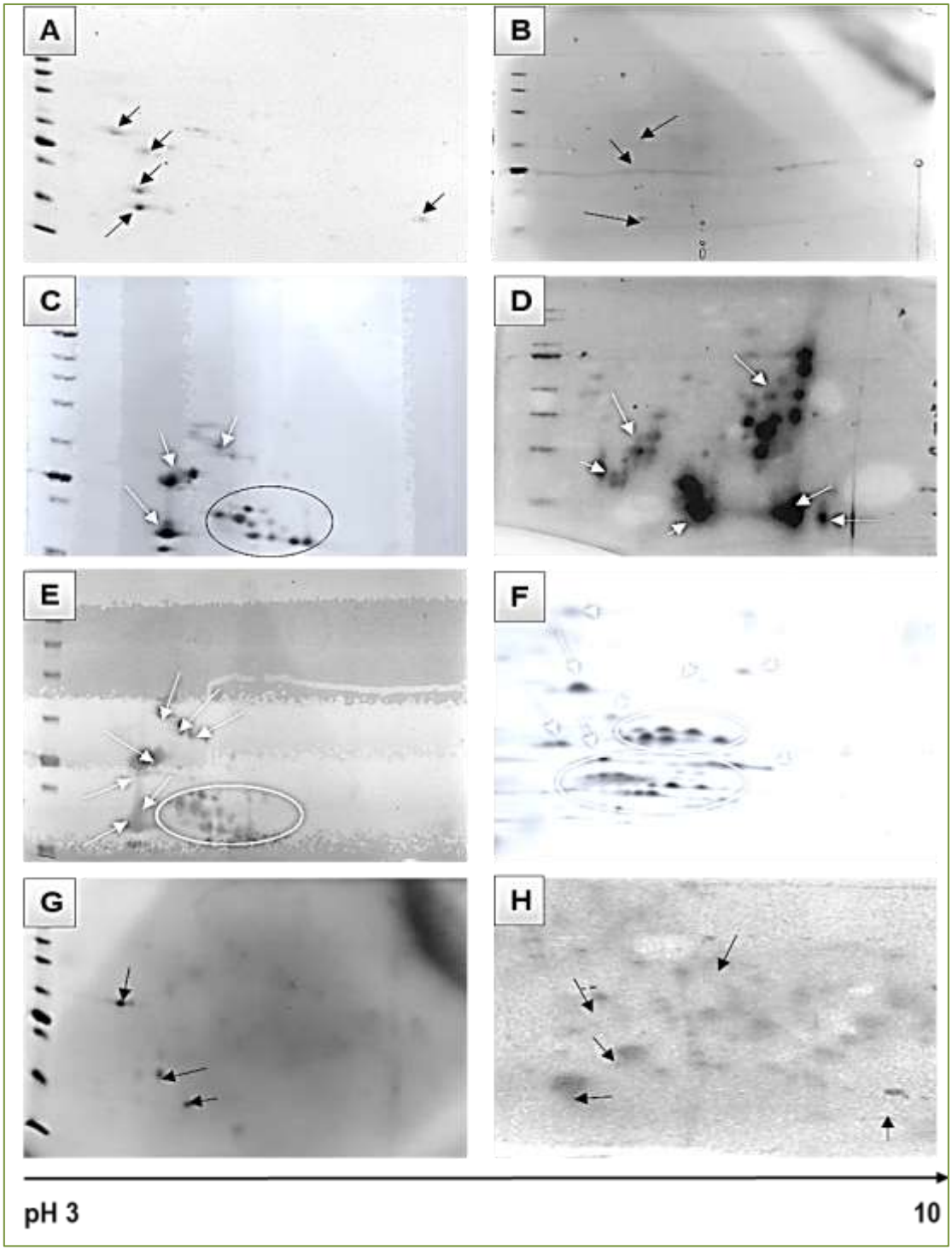

Figure 6.3. Two-dimensional gel electrophoresis of double cotyledonary node explants from the six selected cultivars using SDS-PAGE (12%). (a) Dundee- control, (b) Dundee- infected, (c) LS 677- control, (d) LS 677- Infected, (e) LS 679- control, (f) LS 679- infected, (g) Peking- control, (h) Peking- infected.

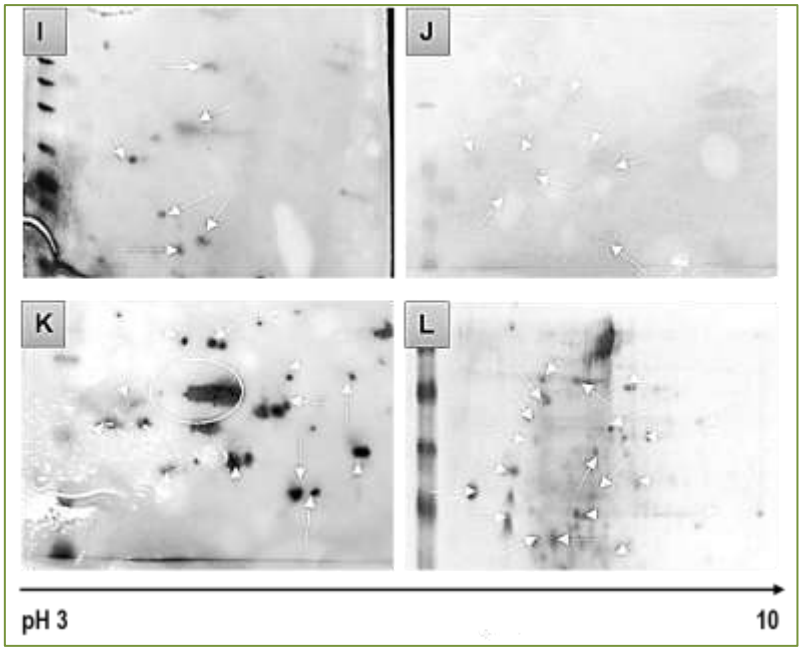

Figure 6.4. Two-dimensional gel electrophoresis of cotyledonary node explants from the six selected cultivars using SDS-PAGE (12%). (i) TGx 1740-2F- control, (j) TGx 1740-2F- infected, (k) TGx 1835-10E- control and (l) TGx 1835-10E- infected.

6.5.5. Overall implication of the study

Although the interaction between soybeans and *A. tumefaciens* have been well studied at physiological and morphogenic levels, not too much is known about the alteration of protein profile after co-cultivation. The advantage of using the SDS-PAGE method was to easily evaluate the variations in protein expression patterns between uninfected and *Agrobacterium* infected cotyledonary explants. This technique can also be used to evaluate transformation efficiency by detecting specific proteins encoded by the successful transgene integration and expression.

Theoretically, an increase in protein profiles and intensity of protein spots detected in all gels could indicate higher chances of stable transformation events. The results obtained indicate that, many proteins were expressed following infection and co-cultivation with *A. tumefaciens*. The proteins may be those that conveniently improve the transformation events or those that hinders the efficiency of transgene integration. It has been considered in many studies that *Agrobacterium tumefaciens* influence the regeneration and transformation of plants (Sunilkumar et al., 1999; Sunilkumar and Rathore, 2001; Surekha et al., 2007), but without thoroughly indicating the extent to

which this takes place (either negative or positive effects), and whether at molecular or physiological levels.

Although the use of this bacterium is considered a relatively easy and cheap method of genetic transformation for recalcitrant soybeans, its optimisation require insights on the kind of protein patterns it induces, for example, during and post co-cultivation period. Analysis and identification of these protein patterns will also assist in the selection of varieties which could be used in the breeding programmes, study the bacterium's influence on metabolomics or used to evaluate the level of resistance by plant tissues to bacterial induced tissue necrosis.

6.5.6. Conclusions

While one-dimensional sodium dodecyl sulphate gel electrophoresis revealed several different bands in the extracts infected with *A. tumefaciens*, the two-dimensional gel electrophoresis procedure also resolved more protein spots in *Agro*-infected extracts than in the control. This clearly indicate that, the infection and co-cultivation of double coty-node explants with *A. tumefaciens* causes the expression of proteins that either promote or suppress the efficiency of transgene integration and expression. Apart from the variations observed, the 2-D SDS-PAGE could serve as an important tool to identify and analyse different genotypes and for selecting the non-transformed from transformed soybean plants. The protein extraction and fractionation protocol should be improved to increase protein yields in soybean, particularly in cultivars such as Peking.

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CHAPTER 7

SUMMARY, GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSSIONS

7.1. SUMMARY

Retrospectively looking back, the introduction of genetically modified plants has simplified the exploitation of crops for many industrial applications. The GM plants have increased possibilities that many people benefit from, involving the direct use of crops for food, feed, production of dietary supplements, therapeutic proteins, beverages and the potential for manufacturing biodiesel. Although genetically modified organisms are considered controversial by some countries, transgenic plants are valuable in basic and applied research to better understand plant growth and development in response to biotic/abiotic stress. The detrimental effects of factors such as pests and drought stress impose a decrease in crop yields, as a result threatening food security, particularly in many developing countries.

Soybeans like the rest of the legumes and other grains are negatively affected by these stresses, especially drought stress. The limited genetic diversity of the soybean and its high sensitivity to drought stress continuously decreases yield, hindering the goal of meeting industrial and consumer demands. Generally, extensive modern breeding activities should focus on improving growth and yield characteristics of soybean in order to meet these demands. This study took a biotechnological approach by investigating the cause of recalcitrance to genetic transformation in soybean, which yielded very pertinent insights, summarised on Figure 7.1 below. The study conducted the following broad objectives:

i. Evaluated the impact of seed viability on germination and seedling development.
ii. Assessed the effect of seed viability on tissue culture based *in-vitro* shoot multiplication.
iii. Determined the proliferative competency of the selected soybean genotypes for callus culture establishment.

iv. Evaluated the efficiency of selected genotypes for *in-vitro Agrobacterium*-mediated genetic transformation.

v. Analysed the variations in protein profiles of the genotypes using 1-D and 2-D gel electrophoresis.

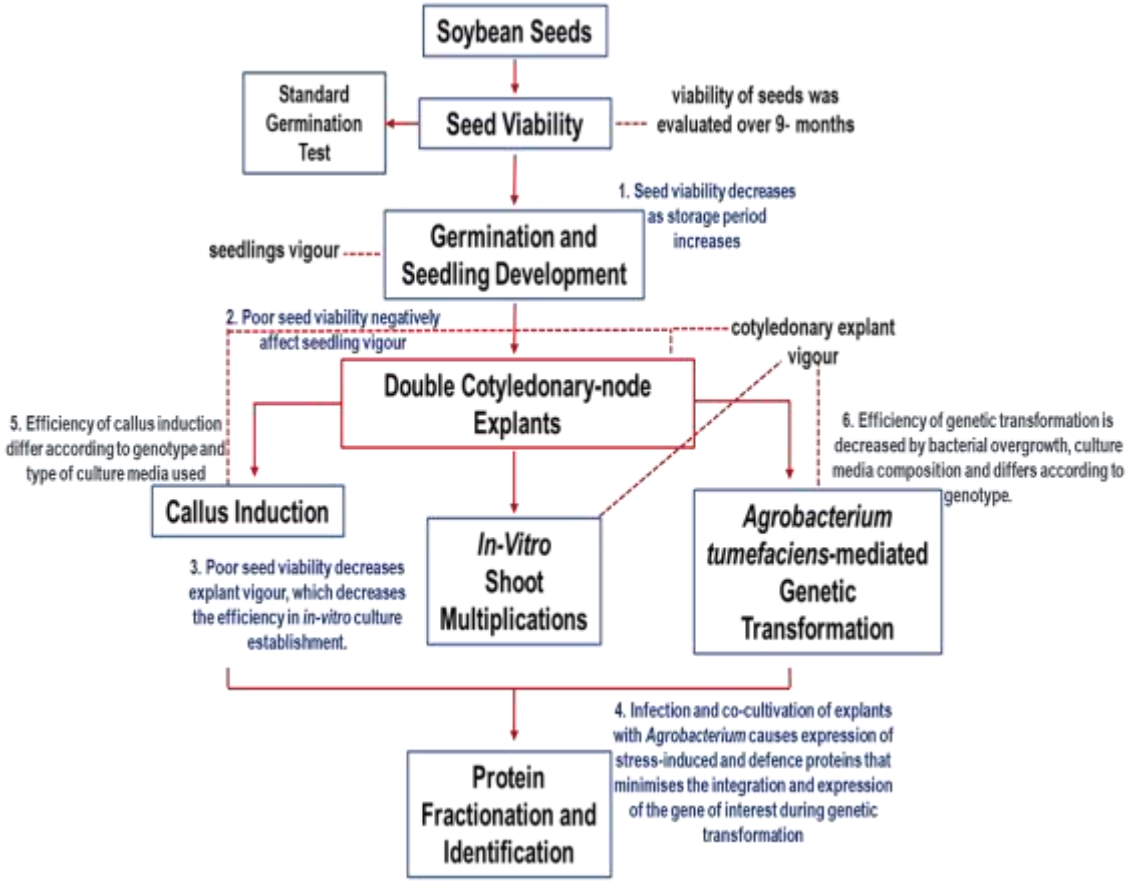

Figure 7.1. Schematic representation of the assessment of the recalcitrance to genetic transformation in soybean.

The experimental results have shown that, in general, seed viability decreases with the increase in storage duration of the seeds from the day they were harvested. This in turn has a negative influence on shoot and callus induction frequency of the explants. Additionally, the study furthermore showed that, the capacity to form callus and transgenic shoots from cotyledonary nodes transformed with *Agrobacterium* was also influenced by the components of the MS culture medium (antibiotics), explant type, *Agrobacterium* and the genotype. Comparative protein analysis between infected and uninfected soybean coty-nodes indicated varied protein profiles and significantly large amounts of proteins expressed according to genotypes and infection with *Agrobacterium*.

7.2. GENERAL DISCUSSION

As this study investigated the cause of recalcitrance in soybean transformation, the observations made are discussed below:

7.2.1. Seed storage under ambient conditions causes loss in seed moisture and viability

This study have shown the requirement to maintain high seed viability if an increased number of germinated seeds is to be achieved. The findings in Chapter 2 have demonstrated that, percentage germination and better seedling characteristics are directly proportional to seed viability, which in turn is influenced by seed moisture content. The observed loss of seed moisture, immediately after harvesting, directly caused negative effect on Peking seed germination followed by the TGx cultivars, Dundee and LS, respectively. As indicated in section 2.5.5 storage of seeds for over 6-months period have had a negative influence on seed viability, seedling vigour and resultant seedling morphology.

Germination generally declined as a result of seed storage duration in LS 677, LS 678, Dundee and Peking, followed by TGx 1735-10E and TGx 1740-2F, respectively. Other results and comparisons among cultivars on characteristics such as, moisture content, viability index and percentage of normal seedlings followed a similar trend, showing a gradual decline as seed storage duration progressed. These findings were in line with Adebisi et al. (2004)'s report who indicated that seed deterioration takes place under prolonged seed storage periods. These results suggest that; better seed germination and seedling development is achievable between 0-3 months after harvest for all seeds. However, seeds may deteriorate according to genotype, especially after this period.

7.2.2. The loss in seed viability had negative effects on multiple shoot induction

The observation made in Chapter 3 also suggest superior and inferior genetic difference in terms of the number of shoots induced per explant. The LS cultivars including Peking showed higher regeneration capacity than Dundee and TGx cultivars.

This study established that, the number of shoots initiated was different among cultivars used and the mean shoot number decreased as the period of seed storage was increased. Furthermore, high efficiency of *in-vitro* regeneration of selected soybean cultivars depended upon seed viability for the establishment of good explant source. Seedlings established between 0 and 3 months showed high vigour compared to seedlings germinated from 6-months onward. Therefore, there was a strong correlation observed between viability of cotyledonary explants developed from freshly harvested seeds and the initiation of adventitious shoots. This is a phenomenon that many researchers often ignore during *in-vitro* plant tissue cultures for subsequent *Agrobacterium*-mediated genetic transformation (Mangena and Mokwala, 2018).

7.2.3. The competency of genotypes and their cotyledonary explants on callus induction

Callus induction capacity on coty-node explants was important to gauge the totipotency and proliferative ability of tissues found on the cotyledonary junction. The infected and uninfected cotyledonary explants were used in this section (Chapter 4). The findings indicated a significant variation amongst the genotypes and culture medium composition. Even though, PGRs directly promoted callus development of plant cells from uninfected explants, as reported by Barwale et al. (1986) and Franklin and Dixon (1995), the medium did not give the same results when using infected explants. According to results obtained, the presence of antibiotics and *Agrobacterium* negatively influenced callus initiation. This is a clear indication of the dual role of antibiotics and *Agrobacterium* on the totipotence potential of cotyledonary tissues during *in vitro* regeneration culture. These factors were shown to negatively influence the competency and initiation of callus using cotyledonary explants. Similar observations were also made by Zhang et al. (2001) and Zhang and Finer (2016).

7.2.4. The evaluation of selected genotypes on *Agrobacterium*-mediated transformation

As indicated in section 5.5.3, co-cultivation of cotyledonary explants with *Agrobacterium* inhibited shoot formation. However, the suppression of shoot growth was found to differ among the cultivars used. The results demonstrated that, soybean

cultivar Peking, LS 677 and LS 678 were more resilience to bacterial overgrowth for a very short period of time, than TGx cultivars and Dundee which showed to be highly susceptible to bacterial overgrowth. Such observations have been highlighted in many transformation studies using different cultivars, and were thoroughly covered under the literature review in 5.2. It was clear that, the efficiency of *in-vitro* shoot induction was reduced following co-cultivation with *Agrobacterium* compared to results obtained from uninfected explants in Chapter 3. Evidently, induction of shoots was also negatively affected by the medium components, particularly the inclusion of antibiotics in the medium, as also indicated in Chapter 4. These observations clearly indicated that, the regeneration of transgenic plants in soybean still present some culture challenges. The poor regeneration frequencies of transgenic shoots obtained was attributed to the inefficient tissue culture protocol as also reported in other studies (Soto et al., 2013; Zhang et al., 2014; Zaidi et al., 2006; Raza et al., 2017; Wang et al., 2017).

7.2.5. Protein profiles of the genotypes analysed using 1-D and 2-D gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the protein profiles of selected soybean cultivars transformed with *Agrobacterium*. There were protein bands found in co-cultured cotyledonary explants that, were not found in the controls. This observation provided a novel molecular opportunity to reveal the mechanism behind genetic recalcitrance, and can be explored further in the future.

7.3. RECOMMENDATIONS

This study provided adequate evidence indicating that, soybean transformation will remain recalcitrant and genotype specific if seed quality and culture medium components are not thoroughly optimised. The results indicated that, the soybean genotypes used may efficiently express and transmit the transgenes if any event of transformation is to take place. This was clearly demonstrated by the varied responses obtained during callus and shoot proliferation cultures. Therefore, for the successful optimisation of a protocol routinely applied for the transformation of a wide range of soybean genotypes, the *Agrobacterium*-mediated genetic transformation procedure requires the following considerations:

i. The status or level of seed viability and seedling vigour must be taken into account during the development and establishment of any *in-vitro* regeneration and transformation procedure. This study has revealed that, seed viability and seedling vigour have far reaching implications on the amenability to transformation and regenerability of cotyledonary explants. The age of the seeds must be known, and the usage of freshly harvested seeds for in *in-vitro* plant tissue culture and transformation purposes is recommended.

ii. Re-evaluation of culture conditions, particularly decontamination and reduction of bacterial overgrowth. The amount and type of antibiotics used to eliminate the *Agrobacterium tumefaciens* should be thoroughly determined. Mangena (2015) reported successful induction of multiple shoots from cotyledonary-node explants infected with *Agrobacterium tumefaciens* using aminoglycoside antibiotics. In agreement, this study showed inhibition of multiple shoot formation by ß-lactam antibiotics, the commonly applied antibiotics in plant transformation. Therefore, a thorough evaluation of the type of antibiotics used (aminoglycosides/ß-lactams) must be conducted.

iii. Continued and effective use of additives, particularly antioxidants. Culture agents that inhibit tissue senescence which include, but not limited to ascorbic acid, cysteine, dithiothreitol and sodium thiosulfate are highly recommended to minimise tissue senescence that was observed in Chapter 4 and 5, where

cotyledonary explants were infected with *Agrobacterium*. The effectively optimised use of these antioxidants may give positive results in all selected soybean genotypes.

iv. In addition, *Agrobacterium tumefaciens* inoculum density must be thoroughly adjusted and optimised to avoid excess cell number and overgrowth. The minimum optimum density of 0.8 must be used. Explant infection by this bacterium on the cotyledonary junctions and bases of hypocotyls causes tissue decay in shoot culture. This effect occurs even though *Agrobacterium* growth in the medium has been effectively controlled. Explants need to be thoroughly flushed with an antibiotic solution before every subculture to reduce the number of bacterial cells on the wounded surfaces of the explants, causing bacterial overgrowths that subsequently kill the explants.

Other considerations could include, the use of affordable methods such as *in-planta Agro*-injection method of transformation to generate new genetically improved soybean plants. This technique was introduced by Chee et al. (1989) in genetic transformation of soybean and kidney bean by *Agro*-injecting seeds with a suspension of *Agrobacterium* strain EHA101 with pIG121 plasmid containing genes for neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT) and ß-glucuronidase (GUS). The soybean and kidney bean seeds *Agro*-infection yielded 12% transgenic soybean plants and 24% of transgenic kidney beans identified using NPTII amplified by polymerase chain reaction (PCR).

Generally, there are difficulties in the *in-vitro* regeneration and selection of transgenic plants during *Agrobacterium*-mediated genetic transformation. But, this technique is undoubtedly the best and affordable tool available for the transfer and expression of the gene of interest in host plant cells. The successful shoot and callus induction cultures observed in this study are some of the positives and progress made. However, the overall findings of this study clearly suggest that, more work still need to be done, especially focusing on the optimisation of tissue culture conditions and transformation protocol.

7.4. CONCLUSSION

Soybean is considered the golden crop by many people. This emanates from its wide range of uses and applications. Therefore, any biotechnological attempt to genetically improve soybean is necessary to break its recalcitrance and improve its growth under biotic or abiotic stress conditions. The use of seeds for the development of double cotyledonary-nodes used as explants remains superior for the establishment of cultures- callus and shoot cultures. This is so, because according to our findings, the efficiency of the cultures established relied primarily on (i) seed storage period of less than 3-months, (ii) high seed viability with over 70% seed germination, (iii) better seedling vigour with highly proliferative tissue explants, (iv) culture medium components (particularly the antibiotics), (v) *Agrobacterium* less than 0.8 OD and (vi) the genotype. These are factors that evidently cause recalcitrance in soybean to genetic transformation.

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APPENDICES

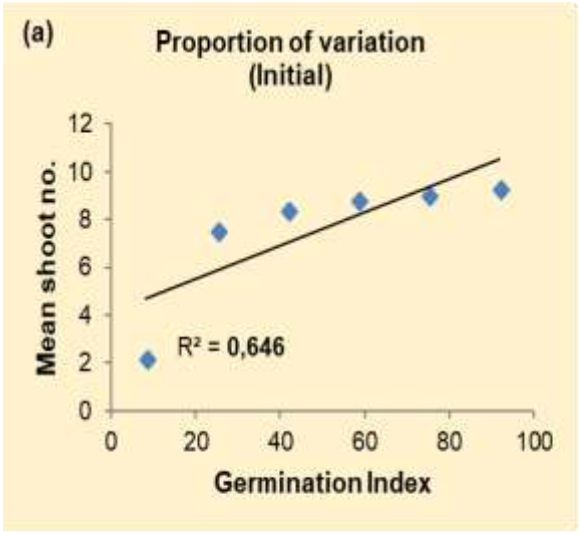
An Investigation on the Cause of Recalcitrance to Genetic Transformation in Soybean (*Glycine max* L. Merr.)

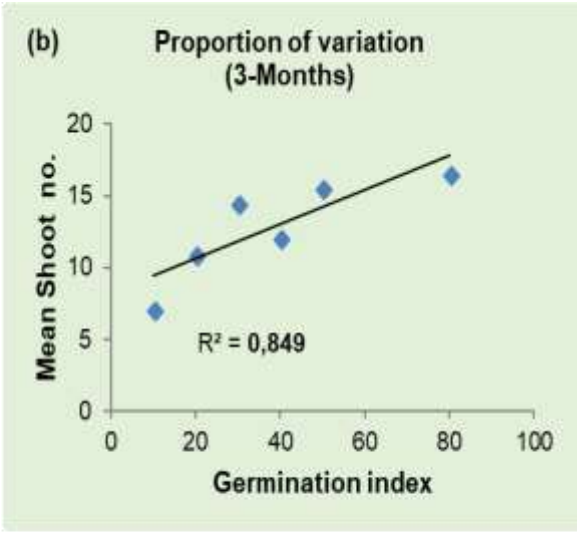
APPENDIX 3.1. Growth and yield performance of different *in-vitro* regenerated soybean plants following plantlets hardening.

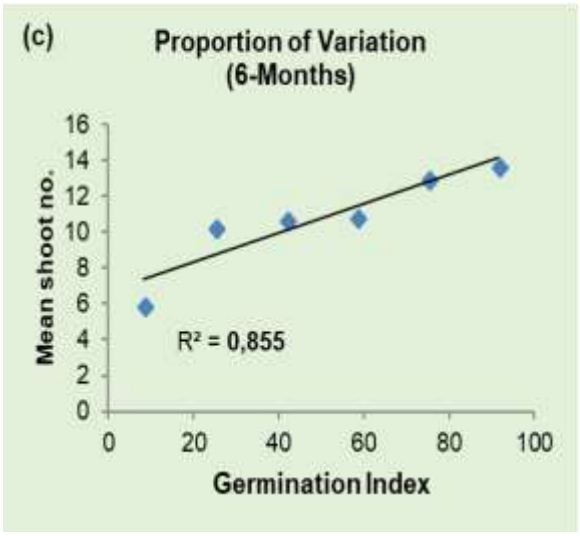
Growth and Yield Components	Initial						Three (3) Months						Six (6) Months						Nine (9) Months					
	PEK	LS 677	LS 678	DUN	TGx-10 E	TGx-2 F	PEK	LS 677	LS 678	DUN	TGx-10 E	TGx-2 F	PEK	LS 677	LS 678	DUN	TGx-10 E	TGx-2 F	PEK	LS 677	LS 678	DUN	TGx-10 E	TGx-2 F
In-vitro (*plant tissue culture*)																								
Explant vigour index	6.6^{a}	6.8^{b}	6.4^{c}	5.0^{d}	4.1^{e}	4.2^{e}	6.1^{a}	5.8^{b}	5.2^{c}	4.0^{d}	3.3^{e}	3.2^{e}	5.7^{b}	5.1^{c}	4.8^{c}	3.4^{e}	3.4^{e}	3.2^{e}	5.3^{c}	5.5^{b}	4.3^{d}	3.2^{e}	3.2^{e}	2.8^{f}
Average shoot height (cm)	4.5^{a}	4.3^{b}	3.9^{c}	2.2^{d}	3.1^{e}	3.1^{e}	4.7^{a}	4.8^{b}	3.5^{c}	2.8^{d}	3.2^{e}	2.9^{e}	3.2^{ae}	3.9^{c}	3.6^{c}	4.7^{b}	4.5^{b}	2.6^{e}	3.5^{e}	3.5^{e}	4.3^{ac}	2.9^{e}	4.8^{f}	3.8^{c}
Average root no.	2.8^{a}	4.4^{b}	2.6^{a}	2.9^{ab}	3.9^{d}	3.0^{e}	2.7^{a}	4.0^{b}	2.4^{a}	2.4^{ab}	3.4^{d}	3.0^{e}	3.5^{d}	4.2^{b}	4.5^{c}	2.3^{a}	3.1^{d}	3.3^{f}	3.1^{d}	3.0^{e}	4.1^{b}	3.5^{bd}	3.1^{e}	2.5^{a}
Average root length (cm)	3.9^{a}	4.4^{b}	2.6^{c}	2.9^{c}	3.9^{a}	3.2^{c}	3.7^{a}	4.0^{b}	2.4^{c}	2.4^{c}	3.4^{a}	3.2^{c}	3.5^{a}	4.2^{d}	4.5^{e}	2.3^{c}	3.8^{a}	3.3^{c}	3.9^{a}	3.3^{c}	4.1^{d}	3.5^{c}	3.1^{c}	2.7^{c}
Regeneration frequency (%)	93^{a}	94^{a}	92^{b}	83^{c}	74^{b}	77^{d}	92^{a}	93^{a}	92^{b}	80^{c}	53^{b}	50^{d}	87^{e}	86^{c}	80^{f}	71^{b}	37^{g}	33^{h}	73^{f}	67^{e}	66^{e}	57^{e}	32^{g}	26^{h}
Ex-vitro (post acclimatisation)																								
Plant height (cm)	36.0^{a}	37.5^{a}	31.5^{b}	41.2^{c}	31.6^{b}	28.1^{b}	35.0^{a}	37.2^{a}	32.5^{b}	40.2^{c}	30.2^{b}	28.0^{b}	35.2^{a}	36.4^{a}	36.3^{a}	39.6^{c}	34.4^{a}	34.8^{a}	38.5^{a}	36.2^{a}	44.3^{d}	48.4^{e}	38.2^{c}	30.8^{b}
Branch number plant^{-1}	3.80^{a}	4.20^{b}	3.50^{a}	4.20^{a}	2.53^{c}	3.50^{a}	3.20^{a}	4.82^{b}	3.33^{a}	3.24^{a}	2.63^{c}	3.00^{a}	3.37^{d}	2.75^{c}	3.45^{d}	3.80^{d}	3.45^{d}	3.71^{d}	4.04^{e}	3.10^{a}	3.00^{a}	4.25^{e}	4.05^{e}	3.63^{d}
Pod number plant^{-1}	30.2^{a}	21.6^{b}	19.7^{d}	13.6^{e}	10.90^{d}	15.1^{d}	26.2^{a}	19.6^{b}	9.7^{d}	11.6^{e}	8.90^{d}	10.1^{d}	18.3^{b}	11.4^{e}	10.2^{d}	13.4^{e}	6.2^{f}	8.8^{f}	22.6^{c}	13.7^{e}	11.9^{e}	6.2^{f}	8.33^{d}	9.83^{d}
Pod length (cm)	5.6^{a}	5.3^{b}	4.3^{c}	5.5^{a}	3.9^{c}	4.3^{c}	5.5^{a}	5.1^{b}	4.3^{c}	5.6^{a}	3.90^{c}	4.20^{c}	3.96^{c}	4.37^{c}	4.2^{c}	4.6^{a}	4.6^{a}	4.2^{c}	4.40^{c}	4.30^{c}	4.20^{c}	4.06^{c}	3.20^{d}	3.60^{f}
Pod weight (g)	0.92^{a}	0.70^{b}	0.72^{c}	0.45^{c}	0.33^{d}	0.39^{e}	0.91^{a}	0.50^{b}	0.45^{c}	0.44^{c}	0.30^{d}	0.36^{e}	0.44^{c}	0.47^{e}	0.57^{f}	0.31^{d}	0.55^{f}	0.49^{b}	0.68^{g}	0.42^{c}	0.43^{c}	0.34^{d}	0.41^{c}	0.32^{d}
Seed number pod^{-1}	2.67^{a}	3.33^{b}	3.01^{c}	3.00^{c}	3.00^{c}	3.01^{c}	2.67^{a}	3.33^{b}	3.01^{c}	3.00^{c}	3.00^{c}	3.01^{c}	2.00^{d}	2.33^{e}	2.30^{e}	2.33^{e}	2.00^{d}	2.00^{d}	2.33^{e}	3.33^{b}	3.00^{c}	2.33^{e}	2.00^{d}	2.33^{e}
Seed weight 100-seeds^{-1} (g)	8.74^{a}	11.6^{b}	11.2^{a}	10.25^{a}	12.8^{c}	11.99^{d}	9.88^{a}	11.4^{b}	10.2^{a}	9.95^{a}	12.3^{c}	8.99^{d}	7.99^{d}	10.3^{a}	10.1^{a}	9.43^{a}	9.89^{a}	7.40^{c}	7.39^{c}	11.3^{b}	9.93^{a}	9.46^{a}	8.61^{c}	7.22^{c}
Average plant flowering (%)	99.2^{a}	99.4^{a}	90.7^{b}	79.6^{c}	80.0^{d}	74.8^{e}	94.4^{b}	98.5^{a}	94.2^{b}	75.4^{d}	78.5^{c}	77.5^{c}	96.0^{b}	98.0^{a}	95.5^{b}	80.0^{d}	90.5^{c}	75.0^{e}	80.0^{b}	85.0^{a}	80.5^{b}	55.0^{e}	60.5^{d}	70.0^{c}
Average plant fruiting (%)	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	95.0^{b}	80.5^{c}	59.6^{e}	60.0^{d}	95.5^{b}	100^{a}	100^{a}	98.0^{b}	95.5^{c}	95.0^{c}	70.0^{d}	100^{a}	100^{a}	100^{a}	100^{a}	90.0^{b}	85.0^{c}

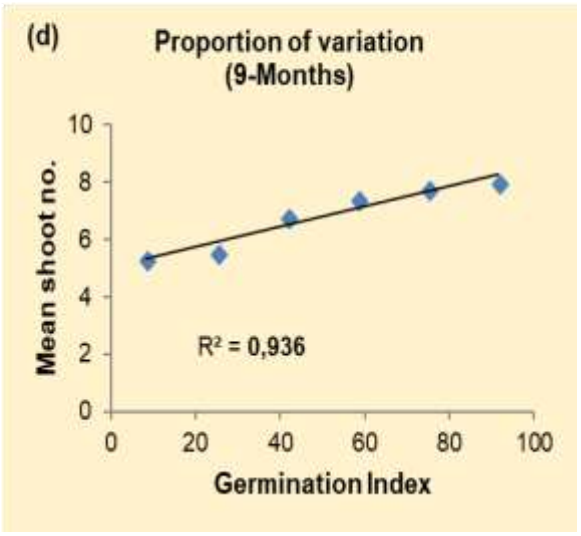
Regeneration frequency (%) was calculated from the number of explants that induced more than 5 shoots/ explant per cultivar. Percentage flowering was calculated from the number of plants producing flowers/ total number of plants successfully acclimatised. Percentage Fruiting was calculated from the number of plants producing more than 10 fruits/ total number of plants that produced flowers. Values followed by similar alphabets are not significantly different at p value less than 0.05.

Appendix 3.2. Probability plots of mean shoot number versus germination index.

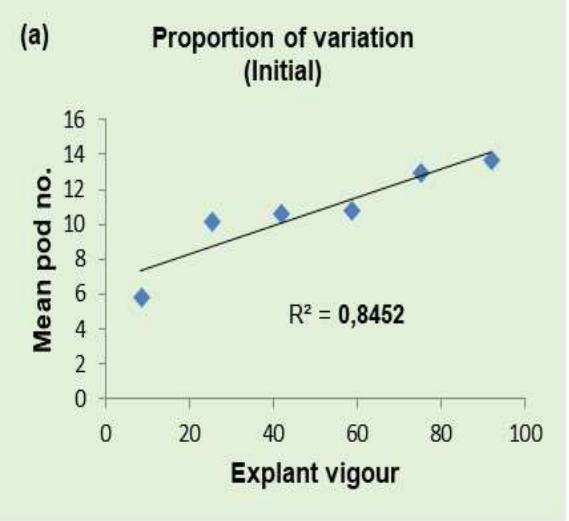


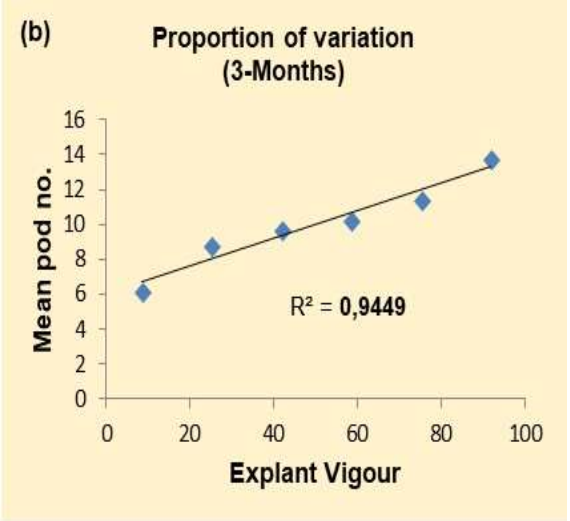


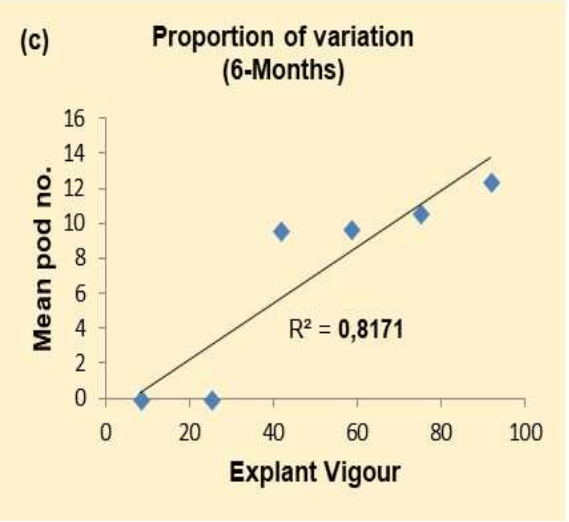


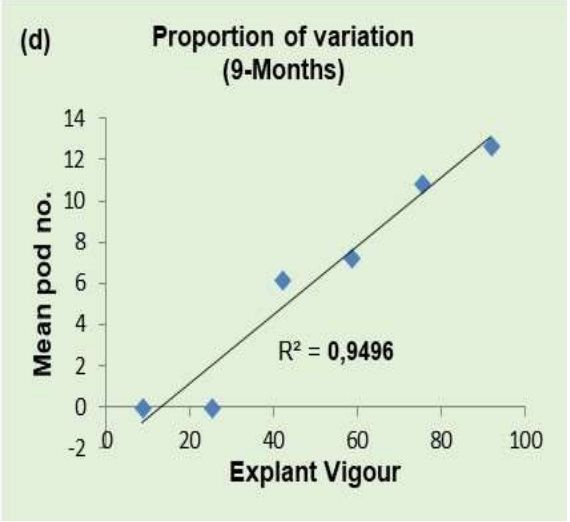


Appendix 3.3. Probability plots of explant vigour versus mean pod number.









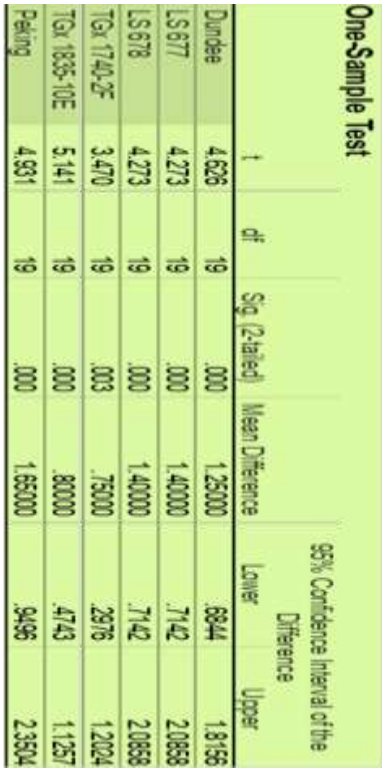
APPENDIX 5.1. Cultivar response to shoot induction during *Agrobacterium*-mediated genetic transformation using soybean double cotyledonary-node explants on MS basal culture medium supplemented with different concentrations of antibiotics and 2.0 mgL^{-1} 6-BA.

Descriptive Statistics

	N Statistic	Range Statistic	Minimum Statistic	Maximum Statistic	Sum Statistic	Mean Statistic	Mean Std. Error	Std. Deviation Statistic	Variance Statistic
Dundee	20	3.00	.00	3.00	25.00	1.2500	.27023	1.20852	1.461
LS 677	20	4.00	.00	4.00	28.00	1.4000	.32767	1.46539	2.147
LS 678	20	4.00	.00	4.00	28.00	1.4000	.32767	1.46539	2.147
TGx 1740-2F	20	3.00	.00	3.00	15.00	.7500	.21613	.96655	.934
TGx 1835-10E	20	2.00	.00	2.00	16.00	.8000	.15560	.69585	.484
Peking	20	4.00	.00	4.00	33.00	1.6500	.33462	1.49649	2.239
Valid N (list wise)	20								

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Dundee	Between Groups	15.226	4	3.807	4.559	.013
	Within Groups	12.524	15	.835		
	Total	27.750	19			
LS 677	Between Groups	20.086	4	5.021	3.636	.029
	Within Groups	20.714	15	1.381		
	Total	40.800	19			
LS 678	Between Groups	11.443	4	2.861	1.462	.263
	Within Groups	29.357	15	1.957		
	Total	40.800	19			
TGx 1740-2F	Between Groups	2.821	4	.705	.709	.598
	Within Groups	14.929	15	.995		
	Total	17.750	19			
TGx 1735-10E	Between Groups	3.486	4	.871	2.288	.108
	Within Groups	5.714	15	.381		
	Total	9.200	19			
Peking	Between Groups	11.050	2	5.525	2.982	.078
	Within Groups	31.500	17	1.853		
	Total	42.550	19			



One-Sample Test

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference Lower	Upper
Dundee	4.626	19	.000	1.25000	.6844	1.8156
LS 677	4.273	19	.000	1.40000	.7142	2.0858
LS 678	4.273	19	.000	1.40000	.7142	2.0858
TGx 1740-2F	3.470	19	.003	.75000	.2976	1.2024
TGx 1835-10E	5.141	19	.000	.80000	.4743	1.1257
Peking	4.931	19	.000	1.65000	.9496	2.3504

APPENDIX 5.2. Cultivar response to *in-vitro* shoot induction on MS basal culture medium containing 2.0 mgL^{-1} 6-BA and different concentrations of antibiotics using un-infected soybean double cotyledonary-node explants.

Descriptive Statistics

	N	Range	Minimum	Maximum	Sum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
Dundee	20	3.00	.00	3.00	27.00	1.3500	.22094	.98809	.976
LS 677	20	3.00	.00	3.00	35.00	1.7500	.21613	.96655	.934
LS 678	20	3.00	.00	3.00	39.00	1.9500	.18460	.82558	.682
TGx 1740-2F	20	2.00	.00	2.00	23.00	1.1500	.15000	.67082	.450
TGx 1835-10E	20	3.00	.00	3.00	15.00	.7500	.19022	.85070	.724
Peking	20	1.00	1.00	2.00	27.00	1.3500	.10942	.48936	.239
Valid N (list wise)	20								

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Dundee	Between Groups	.066	1	.066	.065	.802
	Within Groups	18.484	18	1.027		
	Total	18.550	19			
LS 677	Between Groups	.124	1	.124	.126	.726
	Within Groups	17.626	18	.979		
	Total	17.750	19			
LS 678	Between Groups	1.543	1	1.543	2.436	.136
	Within Groups	11.407	18	.634		
	Total	12.950	19			
TGx 1740-2F	Between Groups	.001	1	.001	.001	.973
	Within Groups	8.549	18	.475		
	Total	8.550	19			
TGx 1835-10E	Between Groups	3.091	1	3.091	5.219	.035
	Within Groups	10.659	18	.592		
	Total	13.750	19			
Peking	Between Groups	.175	3	.058	.213	.886
	Within Groups	4.375	16	.273		
	Total	4.550	19			

One-Sample Test

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Dundee	6.110	19	.000	1.35000	.8876	1.8124
LS 677	8.097	19	.000	1.75000	1.2976	2.2024
LS 678	10.563	19	.000	1.95000	1.5636	2.3364
TGx 1740-2F	7.667	19	.000	1.15000	.8360	1.4640
TGx 1835-10E	3.943	19	.001	.75000	.3519	1.1481
Peking	12.337	19	.000	1.35000	1.1210	1.5790

APPENDIX 5.3. Cultivar response to *in-vitro* shoot induction on MS basal culture medium supplemented with 2.0 mgL^{-1} 6-BA.

Descriptive Statistics

	N	Range	Minimum	Maximum	Sum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
Dundee	20	4.00	2.00	6.00	77.00	3.8500	.27410	1.22582	1.503
LS 677	20	6.00	2.00	8.00	89.00	4.4500	.34393	1.53811	2.366
LS 678	20	5.00	2.00	7.00	93.00	4.6500	.37187	1.66307	2.766
TGx 1740-2F	20	6.00	.00	6.00	68.00	3.4000	.33561	1.50088	2.253
TGx 1835-10E	20	6.00	1.00	7.00	89.00	4.4500	.39387	1.76143	3.103
Peking	20	6.00	3.00	9.00	100.00	5.0000	.38389	1.71679	2.947
Valid N (list wise)	20								

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Dundee	Between Groups	5.800	6	.967	.552	.760
	Within Groups	22.750	13	1.750		
	Total	28.550	19			
LS 677	Between Groups	7.700	6	1.283	.448	.834
	Within Groups	37.250	13	2.865		
	Total	44.950	19			
LS 678	Between Groups	11.950	6	1.992	.638	.699
	Within Groups	40.600	13	3.123		
	Total	52.550	19			
TGx 1740-2F	Between Groups	12.800	6	2.133	.924	.509
	Within Groups	30.000	13	2.308		
	Total	42.800	19			
TGx 1835-10E	Between Groups	14.100	6	2.350	.681	.668
	Within Groups	44.850	13	3.450		
	Total	58.950	19			
Peking	Between Groups	20.533	4	5.133	2.171	.122
	Within Groups	35.467	15	2.364		
	Total	56.000	19			

One-Sample Test

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Dundee	14.046	19	.000	3.85000	3.2763	4.4237
LS 677	12.939	19	.000	4.45000	3.7301	5.1699
LS 678	12.504	19	.000	4.65000	3.8717	5.4283
TGx 1740-2F	10.131	19	.000	3.40000	2.6976	4.1024
TGx 1835-10E	11.298	19	.000	4.45000	3.6256	5.2744
Peking	13.025	19	.000	5.00000	4.1965	5.8035

APPENDIX 6. Publication of the South African Association of Botanists (SAAB) conference proceedings, University of Western Cape, Cape Town, January 2017.

for several different wild populations. Clonal cultures of *S. frutescens* have also provided for an analysis of stress related responses with gamma-aminobutyric acid and canavanine being highest under extreme water deficit. With drought, photosynthetic rates per unit dry weight decreased from 0.21 μmol m^{-2} s^{-1} to 0.14 μmol m^{-2} s^{-1}. Sutherlandiosides and sutherlandins separate wild populations according to geographic localities and may be manipulated *in vitro*. Work on the *D. viscosa* has identified the extracts from this plant as an adjuvant therapy due to its apoptotic properties against breast cancer. Chemical signatures linked to terpenoid metabolism illustrate the impact of geoclimatic conditions on the extract's bioactivity.

doi:10.1016/j.sajb.2017.01.101

Bioactivity of selected South African medicinal plants used for the treatment of sexually transmitted diseases

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Sexually transmitted diseases have a major impact on sexual and reproductive health worldwide. Each year, the World Health Organization estimates 448 million new cases of curable STD's are diagnosed. Ethanol extracts of twelve South African medicinal plants used in the treatment of STD's and 3 flavonoids isolated from *Elaedendron transvaalense* were investigated for their antimicrobial activity against *Candida albicans, Gardnerella vaginalis, Neisseria gonorrhoeae* and *Oligella ureolytica*. The anti-inflammatory activities of the extracts and compounds were determined by measuring the inhibitory effect of the extracts and compounds on the pro-inflammatory enzyme lipoxygenase. The extracts and compounds were also investigated for their anti-HIV activities against recombinant HIV-1 enzyme using non-radioactive HIV-RT colorimetric assay. *Vachellia karroo* Hayne and *Rhoicissus tridentate* L.F. extracts showed good antimicrobial activity with MIC values ranging between 0.4 and 3.1 mg/ml. Extracts of *Jasminum fluminense* Vell, *Solanum tomentosum* L. var. and flavonoids 2 and 3 had good anti-inflammatory activity with IC_{50} less than the positive control, quercetin (IC_{50} = 48.86 ug/ml). *V. karroo* and flavonoid 3 exhibited moderate HIV-1 RT inhibition activity of 66.8 and 63.7% respectively. *Rhoicissus tridentata* and *Terminalia sericea* had the best RT inhibition activity (75.7 and 100%) compared to that of the positive control doxorubicin (96.5%) at 100 ug/ml concentration. The emergence of drug resistance in STD related microorganisms and potential side effects demand the discovery of newer drugs. The exploration of newer anti-microbial substances from natural sources may serve as promising alternatives. The observed activities may lead to new multi-target drugs against sexually transmitted diseases.

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Study on the effect of temperature and flower age on flowering, pollen performance and stigma receptivity of *Moringa oleifera* Lam.

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Moringa oleifera has become a household name in the current era of health and environmental concerns. The demand for the plant and its products have risen causing it to be grown in many parts of the world. However, the performance and behaviour of the moringa plant varies from area to area. Studies on flowering characteristics have partly been done for growing conditions in India where the plant originated but are lacking for other areas including Gauteng in South Africa. The effect of temperature and flower age on flowering, pollen performance and stigma receptivity were studied on moringa plants under Gauteng conditions at University of Pretoria. The exposure to the temperature treatments were done under in vivo and semi-in vitro environments. Stigma receptivity at different days of anthesis was studied to determine the most effective pollination day that achieves optimum fertilization of ovules. Pollen germination and tube lengths were found not to be significantly different ($p<0.05$) at temperature ranges in semi-in vitro experiments. There were significant differences ($p<0.05$) found in the number of pollen tubes reaching the ovary and in the number of ovules at different days of anthesis. The stigma was receptive from the day prior to anthesis to three days after anthesis, the optimum receptivity was on the day of anthesis. We recommended that for successful breeding experiments, pollinations have to be carried out on the day of anthesis during the spring flowering period.

doi:10.1016/j.sajb.2017.01.103

Effect of seed storage on seed viability, adventitious shoot induction and plant regeneration from cotyledonary explants of various soybean cultivars (*Glycine max* L.)

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Soybeans (*Glycine max* L.) are considered to be the most important and excellent source of protein, dietary fibre, and a variety of micronutrients and phytochemicals. However, to fully benefit from this variety of beneficial chemicals contained inside the seeds, *in vitro* protocols that enhance development of high yielding, disease free soybean plants must be established. Six soybean cultivars (LS 677, LS 678, Dundee, Peking, TGx 1740-2F, TGx 1835-10E) were evaluated for seed vigor, shoot proliferation and regeneration of adventitious shoots. The soybean cotyledonary nodes derived from seeds germinated for 10 d on basal MS medium containing 1.5 mg/l BA were used as explants. Results showed that *in vitro* seed germination and emergence declined significantly with the increase in the period of storage. The decline affected vigorously, proliferation and regeneration of shoots in TGx cultivars compared to LS varieties, Dundee and Peking. Adventitious shoot regeneration frequency of 38, 41.4, 76.6, 83.3, 86.2 and 96.0% was achieved using coty-nodes from TGx 1740-2F, TGx 1835-10E, Dundee, LS 678, LS 677 and Peking respectively.

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Evaluation of the antioxidant properties and phytochemical analysis of the selected SA medicinal plants

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APPENDIX 7. Publication of the South African Association of Botanists (SAAB) conference proceedings, University of Free State, Bloemfontein, January 2016.

chambers with 750 ppm CO_2 and ambient air (CO_2 concentration of 400 ppm). During this study phenological, chlorophyll *a* fluorescence measurements, as well as the dry matter partitioning of the sugarcane was determined. Differences in terms of the chlorophyll *a* fluorescence between the two varieties and between the two treatments were found. The photosynthetic efficiency of both varieties was affected, but each variety responded differently. The photosynthetic efficiency of NCo376 was immediately reduced, whereas N31 was only reduced two to three months after being exposed to the elevated CO_2 concentrations. These results suggested that elevated CO_2 concentrations do in fact have an effect on the photosynthetic efficiency of C_4 plants. However, there were no significant differences on the effect of elevated CO_2 on biomass production or sugar content. Fully capturing the benefits of rising CO_2 could assist the sugarcane industries in adapting to climate change.

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Quantifying the effects of elevated CO_2, surface O_3 and drought on canola

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The effect of climate change on agroecosystems is dependent on the combined effects of different climate change components (for example: increases in temperature, CO_2 and changes in precipitation) as well as other environmental factors such as increases in tropospheric O_3. This study aims to investigate the combined effects of elevated levels of CO_2, O_3 and water deficit conditions on the productivity of canola plants. Plants were fumigated in open-top chambers with elevated levels of CO_2 (750 ppm), O_3 (80 ppb and 120 ppb) and a combination of CO_2 and O_3. The fumigation of plants was performed under well-watered and water deficit conditions. The photosynthetic efficiency was investigated by means of chlorophyll *a* fluorescence. Significant differences ($P < 0.001$) were detected between water regimes and between treatments. Water stressed plants increased the photosynthetic efficiency of canola when exposed to O_3. Under well-watered conditions O_3 did manage to reduce the photosynthetic efficiency of the plants. Plants responded positively to elevated CO_2 and a combination of CO_2 and O_3, under both water regimes. These results suggest that elevated levels of CO_2 can reduce the damaging effects of O_3. However, it would seem that the degree of amelioration is cultivar dependant.

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The effect of aminoglycoside antibiotics on the induction of adventitious shoots in soybean

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The use of aminoglycoside antibiotics in the elimination of *Agrobacterium tumefaciens* during initiation of transformed adventitious shoots have been so far neglected. We report here the suitable type and amount of aminoglycosides that can be used to successfully induce shoots in soybean explants infected with *Agrobacterium* carrying the oc-*I* vector during shoot regeneration. The tissue explants used were double coty-nodes, with an adventitious shoot regeneration frequency of 76.6, 63.3 and 60.0% respectively when using hygromycin, tetracycline and rifampicin, each at 500 mg l^{-1}. These antibiotics have shown their suitability to inhibit the growth of *A. tumefaciens* during agar diffusion assays, and the efficient elimination of the bacterium following co-cultivation with soybean explants and subsequent shoot induction.

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Molecular phylogenetic study and character evolution in the palaeotropical genus *Emilia* (Cass.) Cass., Senecioneae (Asteraceae)

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Emilia is a widely distributed palaeotropical genus in the tribe Senecioneae (Asteraceae), comprising 117 species, mostly annuals. Although the taxonomic history of *Emilia* dates back as early as 1817, no phylogenetic study has been done to date. Bayesian and parsimony phylogenetic analyses were therefore performed on a good sample of *Emilia* species together with other closely related genera in the Senecioneae using nuclear ITS and plastid *trnL–trnF* sequence data to provide the foundation for a taxonomic revision of the genus. We thus address questions around the generic circumscription of *Emilia* including the status of similar genera *Emiliella* S. Moore and *Bafutia* C.D. Adams, assess Jeffrey's sectional classification of *Emilia*, and evaluate the distinctness of the morphologically similar species in the large-headed *Emilia coccinea sensu lato* complex. Key morphological characters were also used to investigate evolutionary trends in *Emilia*. The resultant phylogenies reveal *Emilia* to be polyphyletic, with *Bafutia* and *Emiliella* nested within it, and Jeffrey's sectional classification is not supported. Two of three doubtful species in the *E. coccinea* complex are genetically very similar, suggesting they may not be distinct species. Well-supported topological incongruences between nuclear and plastid phylogenies indicate that hybridization and/or introgression have played a role in the history of *Emilia*, as with many other senecionoid genera. A perennial life history is ancestral in *Emilia*, although most species are annual. Narrow leaves, discoid capitula, and non-yellow florets (e.g. purple, white, orange) have all arisen independently a number of times in *Emilia*.

doi:10.1016/j.sajb.2016.02.090

Influence of spacing and pruning on the growth and yield of *Artemisia annua* L.

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Spacing and pruning are key agronomic practices influencing plant growth and yield. This study was conducted at the Agricultural Research Council, Roodeplaat in order to determine the influence of spacing and pruning on the growth and yield of *Artemisia annua* L. The study was a 3 × 4 factorial laid out in a randomized complete