ORYZA CYSTATIN 1 BASED GENETIC TRANSFORMATION IN SOYBEAN FOR DROUGHT TOLERANCE

by

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[redacted]

DISSERTATION
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DECLARATION

I declare that ***ORYZA CYSTATIN 1*** **BASED GENETIC TRANSFORMATION IN SOYBEAN FOR DROUGHT TOLERANCE** (Dissertation) hereby submitted to the University of Limpopo, for the degree of Master of Science in Botany has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

_____ _____

Surname, Initials **Date**

DEDICATION

I dedicate this dissertation to my family and many friends. A special feeling of gratitude to my loving mother, Moyahabo and grandmother Marara Mangena whose words of encouragement and push for tenacity ring in my ears.

My friends Reconcile and Andries have never left my side and are very special. I also dedicate this dissertation to my church (ZCC) and colleagues who have supported me throughout the process. I will always appreciate all they have done, especially my supervisors for helping me develop my skills.

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ABSTRACT

Soybean is an important source of high quality protein and oil for both humans and animals, especially in protein formulations for pharmaceutical and nutriceutical use. This crop is adversely affected by both biotic and abiotic stresses impacting on its productivity. Soybean productivity can be improved via techniques such *Agrobacterium*-mediated genetic transformation. Soybean is recalcitrant and depends on suitable explants from which new shoots can be regenerated and be amenable for transformation. The goal of this study was to produce transgenic soybean plants that are tolerant to drought stress through *Agrobacterium tumefaciens*-mediated transformation. Multiple shoot induction on double and single coty-node explants, obtained from soybean seedlings derived from seeds germinated *in vitro* on Murashige and Skoog culture medium supplemented with cytokinins was studied. The effect of different concentrations of benzyladenine (1.57, 2.00 and 4.00 mg/l), and benzyladenine (2.00 mg/l) in combination with kinetin (1.00 mg/l) was tested. The results show that the double coty-node explants produce the highest number of shoots per explant, an average of 7.93 shoots on Murashige and Skoog medium supplemented with 2.00 mg/l benzyladenine. The lowest number being 1.87 shoots obtained from single coty-node explants cultured on Murashige and Skoog medium containing 4.00 mg/l benzyladenine. The single coty-node explants showed lower frequency (10–57%) of shoot induction when compared to the double coty-node explants (50–83%). The suitability of aminoglycoside antibiotics (hygromycin, tetracycline and rifampicin) for efficient elimination of *Agrobacterium tumefaciens* after co-cultivation was tested using a well agar diffusion assay. Co-culturing double coty-node explants with *Agrobacterium* containing pTF 101 vector carrying the *Oryza cystatin 1* gene resulted in 76.6, 63.3 and 60.0% shoot regeneration on Murashige and Skoog shoot induction media (shoot induction medium 1, shoot induction medium 2 and shoot induction medium 3) containing hygromycin, tetracycline and rifampicin at 500 mg/l respectively. These antibiotics showed the highest zones of inhibition against pTF 101 using the well agar diffusion assay. On the other hand, 85% plant regeneration was obtained during *in vivo* transformation following *Agrobacterium* injection into seedlings. These results imply that

both *in vitro* and *in vivo* protocols were suitable for transgenic shoot regeneration and plant establishment since all the plants continued surviving in the presence of 6.00 mg/l glufosinate-ammonium. Future work will focus on screening of transgenic plants using *beta-glucuronidase* and isolating the protein encoded by the *Oryza cystatin 1* gene to further confirm the generation of transformed plants carrying the gene of interest.

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ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
BA	Benzyladenine
C	Concentration
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
FABI	Forestry and Agricultural Biotechnology Institute
FAO	Food and Agriculture Organisation of the United Nations
GA_3	Gibberellic acid
GUS	ß-glucuronidase
Hyg	Hygromycin
IAA	Indole acetic acid
IBA	Indole butyric acid
Int	Intermediate
KIN	Kinetin
LS	Link Seed
MS	Murashige and Skoog
MES	Methyl ethyl sulfonate
NAA	Naphthalene acetic acid
NOPA	National Oilseeds Processors Association

Oc 1	*Oryza cystatin* 1
PGR	Plant growth regulator
P	Potency
PRB	Population Reference Bureau
Res	Resistance
Rif	Rifampicin
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SIM 1	Shoot induction medium 1
SIM 2	Shoot induction medium 2
SIM 3	Shoot induction medium 3
SPSS	Statistical Package for Social Sciences
Sus	Susceptible
Tet	Tetracycline
T-DNA	Transfer-Deoxyribonucleic acid
TDZ	Thidiazuron
WHO	World Health Organisation
YEP	Yeast extract peptone
ß	Beta
cm	Centimetre
°C	Degree Celsius

ha^{-1}	Per hectare
kDa	Kilo Daltons
kg	Kilogram
l	Litre
µ	Micro
mg	Milligram
mm	Millimetre
$m^{-2}s^{-1}$	Per square metre per second
mM	Millimolar
Ω	Omega
%	Percent
R^2	Square regression

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE IMPORTANCE OF SOYBEAN IN HUMAN LIVES

Soybean [*Glycine max* (L.) Merrill] is a leguminous plant of the Fabaceae family that grows in tropical, sub-tropical and temperate climates. This crop was first domesticated in the 11th century BC in northeast of China. It is believed to have been introduced to Africa in the 19th century by Chinese traders along the east coast of Africa (Ishaq and Ehirim, 2014). Most researchers have sought to improve and optimise soybean characteristics. This is carried out because it is the most important source of vegetable oil and protein in the world. Soybean consists of more than 36% protein, 30% carbohydrates, and an excellent amount of dietary fibre, vitamins and minerals. Soybean also consists of 20% oil, which makes it the most important crop for producing edible oil (Amanlou et al., 2012).

More than 216 million tons of soybeans were produced worldwide in 2007 (FAO, 2008). The above report reveals that 1.5 million tons were produced in Africa, where Nigeria is the largest producer of soybean in sub-Saharan Africa. However, low yields and shortage of biotic and abiotic stress tolerance varieties constrain the ability of other countries to increase production. Malnutrition, particularly protein deficiency is prevalent in many parts of Africa as animal proteins are too expensive for most populations. Many leguminous crops provide some proteins, but soybean is the only available crop that provides an affordable and high quality source of proteins (Jooyandeh, 2011).

1.2 GENETIC IMPROVEMENT OF SOYBEAN

Attempts to produce new breeding materials in soybean using a variety of transformation techniques have been pursued. But, this crop has proven to be highly recalcitrant or resistant to genetic transformation. Increases in soybean yield still rely on chemical applications (fertilisers, herbicides and pesticides) which generate various economic and ecological problems, particularly the problem of environmental pollution. The use of the Gram-negative bacterium, *Agrobacterium tumefaciens*, offers promise for efficient delivery of desired genes into this plant's genome. These soybean genetic

transformation attempts can now be carried out in *in vitro* and *in vivo* cultures. Improvements through *in vivo* and *in vitro* methods will overcome breeding limitations and lead to genetic modifications conferring tolerance to environmental stress, like drought resistance, pests and improving seed quality and overall yields.

1.2.1 *In vitro* soybean transformation

Earlier studies, such as those of Yan et al. (2000); Olhoft and Somers (2001); Paz et al. (2004) and that of Board and Kahlon (2011) are some of the reports showing that more research is still being focused or directed towards investigating the use of *in vitro* culture for soybean genetic transformation. This occurs despite the challenges faced during this approach such as the low rates of transformation; apparently due to factors including ineffective selection of transgenic plants, the low rates of transgenic shoot regeneration and problems associated with plant establishment. Furthermore, *in vitro* transformation system is prone to the problem of chimerism. However, *in vitro* regenerable cells in the axillary meristems of the cotyledonary node are still targeted for this purpose. This method was first reported by Hinchee et al. (1988) and is now successfully used in other legume transformation systems.

1.2.2 *In vivo* soybean transformation

In vivo (also known as *in planta*) transformation radically accelerates research in plant genetic transformation. This method also allows targeting the meristematic tissues or other tissues that can give rise to gametes or adventitious shoots. Very few reports are available for the transformation of soybeans using this method. A report by Zia et al. (2011) shows that plant tissues produced via *in vivo Agro*-injection transformations had the highest *GUS* expression. However, this higher *GUS* activity was observed mostly in seed pods than other parts of the plant. This study reported transformation efficiency up to 14.2% while in recent reports a maximum of 3.2% transformation efficiency was obtained during *in planta* ovary transformation and 0.97% by pollen tube pathway

transformation. All these methods successfully produced transformed plants without tissue culturing steps.

1.3 ANTIBACTERIAL ACTIVITY AND SELECTION SYSTEM

The antibiotics regularly used in the genetic transformation of plants can perform either of the two functions of selecting the transformed plants and the suppression or elimination of *A. tumefaciens* from *in vitro* culture. Usually after co-cultivation with the bacterial suspension, the cells of *A. tumefaciens* are undesirable in the culture and should be eliminated by adding antibiotics to the regeneration medium. The most frequently used antibiotics for these purposes are ß-lactam antibiotics: cefotaxime, carbenicillin, vancomycin and timentin. These antibiotics are typically used at higher concentrations ranging from 200 to 1000 mg/l. They act against bacteria by inhibiting the last synthesis stage of the bacterial cell wall (Wiebke et al., 2006). Our study evaluated aminoglycoside antibiotics for the suppression of *Agrobacterium* after explant infection, and we furthermore used glufosinate-ammonium as a selective agent for transformants. The bacterial vector contained a selection *bar* gene which confers resistance to the herbicide, and only allowed regeneration of the transformed plants. When applied, this herbicide inhibits the synthesis of aromatic amino acids such as glutamine, vitamins and many secondary metabolites. It is widely used because its expression has not yet shown any interference with the normal metabolism of transformed plants that has been reported (Gelvin, 2003).

1.4 SCOPE OF THE RESEARCH

This study explored the three aspects typically used for successful *Agrobacterium tumefaciens*-mediated genetic transformation of soybean.

i. In the first study, a precursor protocol for *in vitro* multiple shoot induction using double and single cotyledonary explants was developed. Chlorine gas surface sterilisation was used to decontaminate the soybean seeds. The sterile seeds

were inoculated for germination on MS medium containing plant growth regulators. The seedlings obtained were used to obtain cotyledonary explants used for multiple shoot induction in the medium similar to the one for germination.

ii. In the second study, evaluation of the effectiveness of aminoglycoside antibiotics against *A. tumefaciens* was carried out using the well agar diffusion assay. This study tested the concentrations of affordable and readily available aminoglycoside antibiotics that effectively inhibit *A. tumefaciens*. This was aimed at selecting antibiotics with good antibacterial activity, and without any signs of toxicity when used in *in vitro* transformation of soybean explants. This was carried out by applying different concentrations of antibiotic solutions in wells made on the YEP agar medium spread with the test Ω PKY and pTF 101 bacterial vectors.

iii. In the third study, soybean transformation via shoot regeneration and plant establishment both *in vivo* and *in vitro* were performed. During *in vitro* transformation, the developed precursor protocol for *in vitro* multiple shoots induction, together with the selected aminoglycoside antibiotics were applied for soybean transformation. A 2.00 mg/l BA concentration was selected for pre-treatment and germination of seeds to develop seedlings used as the explant source. The same concentration was used for shoot induction on double coty-node explants co-cultivated with *Agrobacterium* transformed with pTF 101 vector carrying the *oc 1* gene.

During *in vivo* transformation of soybean, the sterile seeds were imbibed in sterile water containing 2.00 mg/l BA and inoculated for germination in culture bottles containing moistened vermiculite as supporting medium. Seedlings obtained were infected with *Agrobacterium tumefaciens* strain Ω PKY suspension using an injector at the cotyledonary junction. The infected seedlings were incubated for 15 days in the growth room, and then their epicotyls were excised at the cotyledonary axil to initiate the transformed shoots.

1.5 PROBLEM STATEMENT

As indicated by the literature review, studies have been carried out to investigate the effect of different soybean genotypes, explant types, organic additive compounds and various trains of *Agrobacterium* in improving the rates of transformation. In this project, we focussed our research on modifying a full protocol to one that can be easily explored to improve transformation frequencies in recalcitrant soybeans. The overreaching hypothesis was that the improved *in vitro* shoot regeneration rates improve transformation frequencies of soybean with the *oc 1* gene to produce plants that are tolerant to drought stress.

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CHAPTER 2

IN VITRO MULTIPLE SHOOT INDUCTION IN SOYBEAN (*Glycine max* L.) CULTIVAR LS 677

2.1 INTRODUCTION

Soybean belongs to the family Fabaceae, included in the order Rosales which include crops and ornamental plants (Singh and Dashiell, 1987). This family is one of the three largest families of the Angiosperms, namely Poaceae, Fabaceae and Asteraceae, with 600 genera and 12,000 species. The family has many distinctive characteristics such as leaf arrangement, dehiscent fruits and split valves (Taiz and Zeiger, 2002). The scientific name of soybean is *Glycine max* (L.) Merrill. A number of cultivar varieties of this plant have been developed and the cultivar LS 677 has been used in this study. According to Rastogi (2007) a large number of specialists in diverse fields of plant biology are engaged in the production of varieties of the soybean for different purposes. This plant is manipulated using various methods for pharmaceutical, nutriceutical and industrial purposes and is currently being cultivated worldwide.

The methods of soybean cultivation during the distant past were based on conventional agriculture and relying only on conventional ways of propagation. The expansion of soybean cultivation is limited by the lack of varieties suitable for adverse environmental conditions such as drought and chilling. The available varieties are low yielding, susceptible to biotic stress and of late maturity. Advancements in soybean manipulations led to new developments integrating conventional breeding with modern technology, such as genetic transformation and *in vitro* tissue culture.

These methods facilitate rapid improvements that cannot be accelerated via breeding for efficient plant regeneration. Improvement in plant regeneration and transformation could generate new techniques by developing new efficient protocols for production of new cultivars with improved productivity. The expansion of soybean cultivation can be doubled by also doubling agricultural research (Alene and Coulibaly, 2009). These efforts serve to exploit the crop's economic potentiality quicker and easier. The need for soybean improvement is increasing, because of continuous increase in biotic and abiotic harmful factors. Several biotic stresses such as bacterial, fungal and viral attacks are among other factors adversely affecting soybean growth and productivity (Grossi-

de-Sa et al., 2006). These harmful factors cause stress, which is a constraint that is imposed on the normal metabolic patterns, causing injury, disease or aberrant physiology (Jaleet et al., 2009).

2.1.1 Motivation for the study

Conventional breeding is still very important for the genetic improvement of crops. However, breeding in soybean is difficult due to the fact that soybean is a self-pollinating crop, breeding is time consuming and the genetic variation among its varieties is narrow. These setbacks necessitate the use of rapid genetic modification methods to produce varieties with traits that are not available in the soybean gene pool. However, the use of genetic modification techniques such as *Agrobacterium*-mediated transformation relies on efficient regeneration systems, where the commonly used ones are somatic embryogenesis, and shoot organogenesis from cotyledonary nodes (Finer and McMullen, 1991). Although such systems have been previously reported (Zhang et al., 1999; Zeng et al., 2004; Popelka et al., 2006), a highly efficient repetitive regeneration system is still required to improve genetic transformation of soybean in order to obtain drought resistance plants.

The increase in soybean seed production will play a very important role of reducing food insecurity; since nearly 1.2 billion people live in a state of absolute poverty worldwide. African countries in particular have about 800 million people who are food insecure. Food insecurity and malnutrition lead to serious public health problems and lost human potential (Sharma et al., 2001). Considering soybean medicinal and food values, incorporation of a soybean diet into Africans' diet will reduce malnutrition in Africa. The possibility of introducing newly developed genetically modified drought tolerant soybeans will be helpful in stemming the tide of malnutrition.

2.1.2 Aim

This study was aimed at investigating the effect of different concentrations of cytokinins on *in vitro* seed germination and multiple shoot induction in soybean [*Glycine max* (L.) cultivar LS 677].

2.1.3 Objectives of the study were to:

i. Determine the effect of BA and BA in combination with KIN on *in vitro* seed germination and seedling development.
ii. Determine the effect of BA and BA in combination with KIN on multiple shoot induction using single and double coty-node explants.
iii. Establish a protocol for plant regeneration suitable for *Agrobacterium*-mediated transformation in soybean.

2.2 LITERATURE REVIEW

2.2.1 Legumes

Soybean [*Glycine max* (L.) Merrill] falls under a group of leguminous plants. The legume family (Fabaceae) is considered the third largest family of flowering plants with more than 1800 described species. This family includes herbs, shrubs, trees and vines that are distributed throughout the world, especially in the tropical rain forests (Sauer, 1993). The plants produce legumes, which are technically fruits. They are also known as pods, and are defined as more or less elongated dry fruits that are derived from single carpels that open or dehisces along one or two longitudinal sutures (Judd et al., 2002).

Somers et al. (2003) has indicated that leguminous plant species have been used for human consumption throughout history. They constitute the second most important group of food crops in terms of economic and nutritional importance as human food resources following cereals. Legumes are valued not only for their protein and

carbohydrate contents but also because of the oil content found in the seeds, such as soybean and peanut oilseeds (Nwokolo and Smartt, 1996; Somers et al., 2003). They make excellent and relatively inexpensive source of dietary proteins. They are favoured due to several desirable attributes that include their low sodium content; high potassium content; abundance of complex carbohydrates, ability to lower serum cholesterol in humans, high fibre content, low fat content (excluding the oilseeds), high concentration of polyunsaturated fatty acids (particularly essential fatty acids linoleic and linolenic), long shelf-life and the diversity of food that can be made from them (Messina, 1999; Venter and van-Eyssen, 2001; Montgomery, 2003; Shi et al., 2004; Trinidad et al., 2010).

In addition to these qualities, legumes contain several bioactive compounds whose beneficial effects in human health remain largely unreported. Although they are extensively used in Asia, the Middle East and Africa, some plant species remain underused in other parts of the world. One of the major factors which hinder their full utilisation is their growing conditions. In regions where they have been underused, the cultivars must be improved for wider adaptation of growing conditions in order to increase their utilisation. Furthermore, the environmental factors such as light intensity, temperature, and moisture and soil nitrogen must be thoroughly evaluated to determine their possible effect on legume growth and symbiotic N_2 fixation (Khan and Ahemad, 2011).

2.2.2 Soybean

2.2.2.1 Soybean cultivation

Glycine max (L.), commonly known as soybean is believed to be indigenous to Manchuria, China. The crop is considered one of the oldest cultivated crops, and was used by the Chinese as a source of food before 2500 BC (Lee et al., 2012). Soybean also forms part of the largest, diverse leguminous family ranging from herbaceous annuals to woody perennials that are essential components in natural and managed

ecosystems (Odu and Egbo, 2012). The world soybean production has recently been reported to be at 219.8 million metric tons (Gandhi, 2009). Limitations on soybean production are still observed, especially in African countries. The highest producer in Africa is Nigeria with 3.9 million metric tons followed by DR Congo, and Uganda with 1.8 and 1.7 million metric tons respectively. The lowest producers documented include Kenya (2200 metric tons), Tanzania (400 metric tons), and Mozambique (inadequate for marketed production) (FAO, 2012). In South Africa, soybean production currently ranges from 450 000 to 500 000 tons per annum. The largest producer is Mpumalanga with about 225 000 tons, followed by the Free State (110 000 tons), Kwa-Zulu Natal (75 000 tons), Limpopo (40 000 tons), North West (25 000 tons) and Gauteng (10 000 tons) respectively (DAFF, 2010). The limited production may also be due to the unavailability of cultivars that withstand biotic and abiotic stress, poor seed germination, fast plant development which lead to plants falling over, and shattering of pods prior to harvesting (Blignaut and Taute, 2010).

In Africa soybean is third in popularity after cowpea (*Vigna uniguiculata* L.) and common bean (*Phaseolus vulgaris* L.), mostly grown in the eastern and southern parts of Africa (Lupwayi et al., 2011). Like any other crop, their growth is also facing challenges of suffering from abiotic and biotic stress, which results in low yields, averaging from 594 to 742 kg/ha. But they have been found to have higher yields than soybean, and their adaptation to the changing climate does not show the same detrimental effects on their growth and development as does soybean (Mbagwu et al., 2011).

2.2.2.2 Importance of soybean

Soybean is one of the most important protein and oil crops in many countries. Soybean was improved because of its potential to enhance human and animal nutrition, and health (Ma and Wu, 2008). The seed was found to consist of approximately 8% plumule, 90% cotyledon, which has the highest amount of proteins and lipids, and 2% hypocotyledon. These compositions vary depending on the cultivar, growing season, geographical location, and the environmental stress (Martino et al., 2010). Isoflavones

are a subclass of plant flavonoid metabolites which are also found in soybean. These compounds were found to have health promoting effects against hormone related diseases such as cancer, osteoporosis, and cardiovascular diseases (Liu et al., 2007). Furthermore, the soybean has components that can also assist in the regulation of blood sugar, body fluids, adrenaline, kidney functions, liver functions and other aspects of human metabolism (Mbagwu et al., 2011).

2.2.2.3 Soybean utilisation

Soybean has been extensively cultivated for the production of food, feed and forage, fibre, industrial and medicinal compounds, and other end uses (Somers et al., 2003). Various reports show that the use of soybean for the medicinal and food industry has steadily increased (Zarkadas et al., 1997; Dixon and Summer, 2003; Jimoh and Kolapo, 2007). This extensive exploitation is primarily associated with their high nutritional quality, richness in amino acids and high protein content (Gandhi, 2009). Gandhi's report further indicated that, less than 10% of soybean produced is directly used for human consumption and the remaining for processing. Processing leads to production of high protein food ingredients such as protein concentrates and isolated soy proteins, which have functional and nutritional application in various types of bakery, dietary and infant formulas.

The nutritional value of these foods is often reduced by the high presence of antinutritional factors found in low quality raw soybeans. This makes the processing and the nutritional quality of soybean meals to be of utmost importance. Consumption of soybean meals by humans or poultry is known for the benefit of regulating the rate and efficiency of growth. The increased manufacture of soybean products necessitates 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 soybeans and soy based foods are generally preferred, and more standards must be made available to assist consumers

and producers in the selection of the best soybean products based on various physical and chemical properties (NOPA, 1999).

2.2.3 *In vitro* plant tissue culture

The goal of *in vitro* plant tissue culture systems is to obtain a large number of healthy plants in a short period of time and at a minimal expense. Although this is not easy to achieve, many protocols have been elaborated for the regeneration of important crops, such as soybean, maize and sorghum. Pratap and Kumar (2011) indicated that these protocols are the key object of genetic transformation, or can be used to initiate cultures suitable to facilitate alternative methods of transformation or *in vitro* mutagenesis. The use of *in vitro* culture is important because undesirable characteristics found in most crops pose a major challenge for plant breeders using conventional breeding methods (Delgado-Sanchez et al., 2006). These are challenges, such as susceptibility to drought, pests and diseases, as well as low nutritional qualities of crops (Ahmed et al., 2002).

2.2.3.1 Culture conditions for plant regeneration

Development of tissue culture media has transformed the research approach in addressing problems facing crop improvement (Leva and Rinaldi, 2012). Optimal growth and morphogenesis of tissues in different plants can easily vary according to their nutritional requirements. Plant regeneration can be achieved via direct or indirect organogenesis of shoots or callus culture and through embryogenesis culture. In one process, plants are obtained through shoot formation, and in embryogenesis, embryos are initiated. But both paths require a series of nutrient formulations or appropriate culture conditions in order to be accomplished (Perez-Clemente and Gomez-Cadenas, 2012). Wang et al. (2001) indicated that when essential culture conditions are established, plants develop mechanisms to cope with and easily adapt to different types of stress. Furthermore, these culture conditions make it possible to develop tools, such as genetic transformation in crop improvement for sustainable agriculture (Sakhanokho and Kelley, 2009). Furthermore, these conditions make analysis of plants' responses

under different stressful conditions possible, than attempting to analyse in the field (Benderradji et al., 2012). Olhoft and Somers (2001) reported 91% infection of soybean cotyledonary-node explants using *A. tumefaciens* achieved by the addition of L-cystein in culture medium. In another related study, Paz et al. (2004) reported that suitable optimisation of culture conditions, such as co-cultivation and selection of transformed plants improves transformation frequencies of soybean.

2.2.3.2 Explants suitable for *in vitro* cultures

Success in the establishment of an efficient *in vitro* regeneration system is also dependent on the type of explant used. Soybean cotyledonary node explants with or without axillary buds obtained from 7 days old seedlings pre-treated with, thidiazuron (TDZ), (0.1–0.5 mg/l) or 0.5 mg/l BA have been reported to have increased regeneration efficiency (De-Carvalho et al., 2000; Popelka et al., 2006; Radhakrishnan et al., 2009). Franklin et al. (2004) reported 84 and 37% shoot regeneration from mature and immature cotyledons respectively, when TDZ (4.54 µM) was used in combination with BA (13.3 µM). Shan et al. (2005) recorded a high efficiency of shoot regeneration when cotyledonary node explants, derived from seedlings developed on MS medium containing 0.1–0.5 mg/l TDZ, were cultured on MS medium supplemented with 0.5 mg/l BA. The advantage of obtaining high shoot number using whole cotyledonary node explants over the traditional cotyledonary node explants for multiple shoot induction of four soybean cultivars (Jilin 35, Dongnong 42, Hefeng 25, and Hefeng 41), on media supplemented with cytokinins (BA and KIN) was also reported (Ma and Wu 2008).

2.2.4 Plant growth regulators

The first plant cell culture was made possible by the discovery of plant growth regulators, also known as plant hormones (Clifford et al., 1992). According to Saad and Elshahed (2012) Skoog and Miller revealed in 1957 that *in vitro* regeneration of shoots and roots in cultured cells can be manipulated by varying the concentrations of these hormones in the nutrient medium. The proportion of plant hormones, auxins to

cytokinins determines the type and extent of organogenesis in plant cell culture. Various reports, such as those of Malik and Saxena (1992) and Kaneda et al. (1997) demonstrated shoot regeneration attained through *in vitro* culture from mature cotyledonary explants when BA is combined with TDZ in culture.

The induction of a high number of shoots on culture media containing BA in combination with TDZ was attributed to TDZ activity, which has a cytokinin like structure (Franklin et al., 2004; Shan et al., 2005). Pre-treatment and maintenance of explants on media containing TDZ was suggested to cause abnormal changes in the growth and development of plants, such as hyperhydricity, short and compact shoots, difficulty in shoot elongation and rooting of the recovered shoots (Barclay and McDavid, 1998; Huetteman and Preece, 1993; Lu, 1993).

2.2.5 Aseptic conditions in plant tissue culture

To ensure success of tissue culture experiments, sterilisation of media, explants and instruments, including good working conditions must be attained. Soybean seeds or other plant parts must be thoroughly sterilised or made free of all microorganisms before being placed on the medium (inoculated). The methods of sterilisation include, physically destroying the micro-organisms by dry hot air, steam or irradiation (UV light or gamma irradiation), chemical sterilisation using sterilising chemicals such as ethylene oxide, alcohol, and sodium hypochlorite or by filtration (Pierik, 1997). The nutrient media are usually sterilised by autoclaving.

2.2.6 Surface gas sterilisation of explants

In principle there are four sources of contamination in plant tissue culture, which are the plant (internal or external infection), the nutrient medium, air and the research worker due to inaccurate work (Holford and Newbury, 1992). The most important of these sources is the plant itself. This requires the plant material to be well sterilised before being used *in vitro*. Surface sterilisation using chlorine gas usually results in excessive

dehydration and must be carried out with a minimum amount of time recommended depending on the type of explants used. Excessive chlorine gas might also damage the tissues. Therefore, it is necessary to remove all excess gas after sterilisation (Mariashibu et al., 2013).

2.2.7 Factors influencing sterility of culture

Factors influencing the rate of contamination in *in vitro* culture are directly related to the working conditions and the explants. For production of completely sterile cultures, factors that must be considered regarding the explants selection must include; the physiological or ontogenic age of the organ that is to serve as the explant source, season in which explants are obtained, size and the location of the explants. In addition to the above mentioned factors, the quality of the source plant and ultimately the goal of cell culture also need to be considered (Smith, 2000).

Generally, the greatest response is achieved when young tissues are used *in vitro* because they are easier to surface disinfect (Caula, 2005). The following factors can decrease contamination and improve response in culture:

i. Healthy plants selected from plants that are not under nutritional or water stress or exhibiting disease symptoms can assist in establishing virus free plants or plants without internal contaminants.
ii. Young tissue explants.
iii. Use of aseptically germinated seeds. Such seeds have a low rate of contamination (externally or internally) as compared to other explant source. Depending on what type of a response is desired from the cell culture, the choice of explant tissue will vary (Smith, 2000).

2.3 MATERIALS AND METHODS

2.3.1 Plant material

Soybean [*Glycine max* (L.) Merrill] seeds cultivar LS 677 was used in this study. The seeds were purchased from Link Seed South Africa (Link Seed SA).

2.3.2 Aseptic conditions

a) Sterilisation of instruments and culture media

All *in vitro* plant tissue culture experiments were performed in a specialised laboratory with designated preparation, inoculation and growth rooms for incubation of cultures under controlled conditions. The working areas were kept clean at all times to ensure sterile cultures and reproducible results. All aseptic procedures were performed in a laminar flow cabinet and the working area was disinfected with 70% ethanol before and during the performance of each experiment. All plant culture media, glassware (beakers, test tubes etc.), and instruments (scalpels and forceps) were sterilised by autoclaving for 20 minutes at 121°C. The instruments were again flame sterilised with 96% ethanol prior to use for excisions and inoculations during culture.

b) Seed sterilisation

The seeds were first washed with soapy water, rinsed with running tap water for 5–10 minutes and dried with a clean tissue paper. Washed seeds were placed in two 90 × 15 mm petri dishes and each Petri dish contained 90 seeds. Petri dishes with seeds were placed inside a desiccator jar with their lids half-open. Concentrated hydrochloric acid, 3.5 ml, was added drop-wise into a beaker containing domestic bleach placed in the desiccator jar to generate chlorine gas. The jar was closed and the seeds were then sterilised using the generated chlorine gas for 16 hours.

2.3.3 Plant tissue culture media

2.3.3.1 Culture media composition

a) Macro and micro- nutrient stock solutions

Murashige and Skoog (MS 1962) basal medium was used in this study. Macro and micro-nutrients, iron source, and vitamins were prepared as according to Pierik (1997). All macro and micro-nutrients, iron source and vitamins were prepared as stock solutions as presented on Table 2.1 and stored at 5°C.

b) Vitamin stock solutions

The vitamin stock solutions were prepared by dissolving the respective amounts of vitamin powders indicated in Table 2.1 in 100 ml distilled water, and stored at -20°C. All stock solutions were thawed and mixed well before use.

c) Plant growth regulator stock solutions

Stock solutions (mg/ml) of different plant growth regulators (PGRs) were prepared by first dissolving the respective amount of benzyl adenine (BA), kinetin (KIN), gibberrelic acid (GA_3), indole-3-acetic acid (IAA), and indole butyric acid (IBA) in few drops of 1N sodium hydroxide or dH_2O, after which distilled water was added up to the final volume of the stock solution. Due to thermo lability GA_3 was filter sterilised prior to addition to sterile culture media. The stock solutions were stored at -20°C.

Table 2.1 Chemical composition and preparation of basal MS medium according to Pierik (1997) at 20× concentration.

Murashige and Skoog culture medium		
	Concentration (mg/l stock solution)	Volume (ml) of stock solution per 1l culture medium
Macronutrients		
NH_4NO_3	33000	50
$(NH_4)_2SO_4$	---	
KNO_3	38000	
$CaCl_2.2H_2O$	8800	
$MgSO_4.7H_2O$	7400	
KH_2PO_4	3400	
$NaH_2PO_4.H_2O$	---	
Micronutrients (mg/500 ml)		
KI	16.6	5
H_3BO_3	124.0	
$MnSO_4.4H_2O$	446.0	
$ZnSO_4.7H_2O$	172.0	
$Na_2MoO_4.2H_2O$	5.0	
$CuSO_4.5H_2O$	0.5	
$CoCl_2.6H_2O$	0.5	
Iron source		
$FeSO_4.7H_2O$	556.0	5
$Na_2EDTA.2H_2O$	746.0	
Organic supplements		
Vitamins		
Glycine	2.0	5
Myo-inositol	100	
Nicotinic acid	0.5	
Pyridoxine	0.5	
Thiamine-HCl	0.1	
Sucrose	30	
Gelling agent		
Gelrite (%)	3.0	

2.3.3.2 Preparation of MS culture media

MS basal culture medium was prepared by pipetting the required volumes of stock solutions into 1 litre glass beaker filled with 500 ml distilled water. A 30 g/l mass of sucrose was dissolved into the medium, as a carbon source. Different concentrations and combination of PGR depending on the treatment of hormones required were also added into the basal media and the pH was adjusted to 5.8 using 0.5 M NaOH or 0.1 M HCl. The solidifying agent, gelrite (3 g/l), was dissolved into the medium using hot plate and magnetic stirrer. Aliquots of 40 ml of the prepared culture media were dispensed into 100 ml tissue culture bay jars after autoclaving.

2.3.4 *In vitro* seed germination and seedling development

The seeds were first washed with soapy water, rinsed with running tap water for 5–10 minutes and dried with a clean tissue paper. They were then sterilised using chlorine gas for 16 hours. The sterilised seeds were inoculated on MS media containing 3 % sucrose as a carbon source, 0.3 % gelrite as the solidifying agent and supplemented with different concentrations of BA (1.57, 2.00, 4.00 mg/l) alone and BA (2.00 mg/l) in combination with KIN (1.00 mg/l). Hormone free MS medium was used as a control. The cultures were incubated under controlled conditions to obtain seedlings with completely open cotyledons. A total of three seeds were inoculated per culture bottle (Fig. 2.1), and 30 replicates were made for each treatment. The experiment was repeated four times. Seed germination was recorded as the protrusion of the radicle.

2.3.5 Explant preparation and shoot induction

Two types of coty-node explants (single or double coty-nodes) were prepared from the seedlings. Double coty-node explants were obtained by excising out the epicotyls at the cotyledonary junctions and cutting off the hypocotyls 4–5 mm beneath the cotyledons. For single coty-node explants, some of the double coty-node explants were split in the middle of the hypocotyl-cotyledon junction. Explants were subcultured on MS media

supplemented with the same cytokinin concentrations/combination as the one used for germination and seedling development (Fig. 2.2).

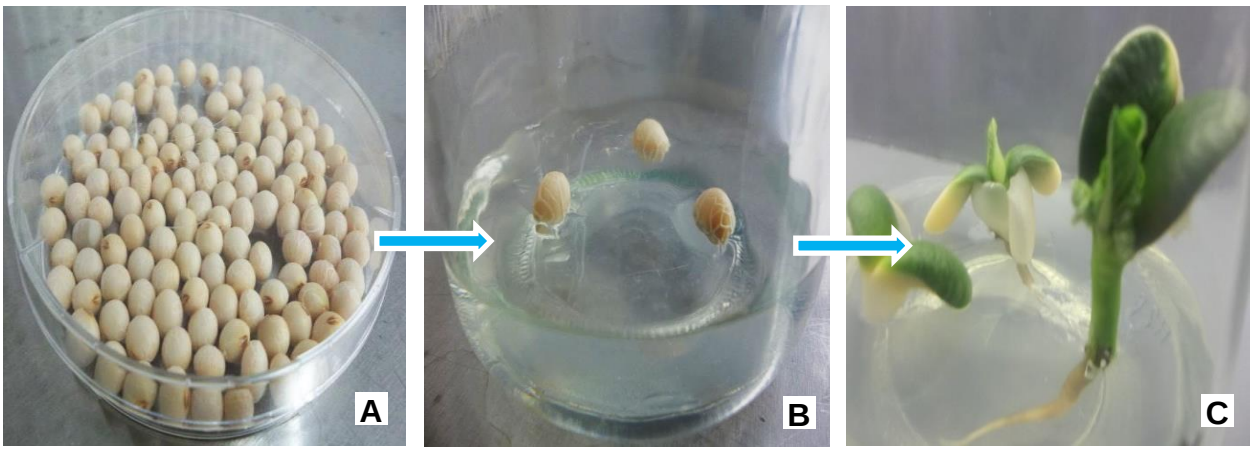

Figure 2.1 Sterilised soybean seeds inoculated on MS culture medium for germination and seedling development. (A) Sterilised soybean seeds, (B) seeds inoculated on MS medium and (C) 8 day old soybean seedlings.

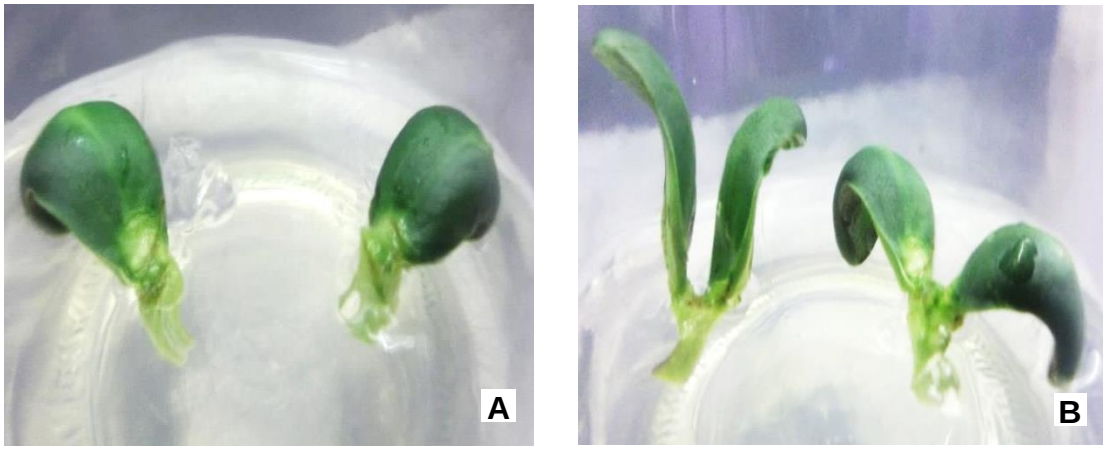

Figure 2.2 Examples of single (A) and double (B) coty-node explants with hypocotyls embedded on MS culture medium.

Hormone free MS medium was used as a control. Thirty explants per hormonal treatment were used and the experiment was repeated four times. The cultures were incubated under controlled growth conditions as indicated under 2.3.9. The number of induced shoots per explant was recorded weekly.

2.3.6 Shoot elongation

The *in vitro* developed multiple shoots from each explant were excised and cultured on MS medium supplemented with 0.5 mg/l BA and 0.6 mg/l GA_3 for 2–3 weeks.

2.3.7 Rooting of elongated shoots

Elongated shoots were rinsed in sterile distilled water and subcultured for rooting on MS medium containing 0.5 mg/l of indole-3-butyric acid (IBA) and 1.2 mg/l of indole acetic acid (IAA) and on a PGR-free MS medium.

2.3.8 Plant acclimatisation

In vitro rooted plantlets (40-60 mm in height) were transferred into 150 ml culture bottles quarter-filled with sterile vermiculate and covered with transparent plastic bags. The plastic bags were punctured and gradually opened to allow the plants to acclimatise. Plants were watered daily with distilled water depending on the moisture content and once a week with modified Hoagland nutrient solution (Epstein, 1972). Acclimatised plants were transplanted into plastic pots (10–15 cm), and maintained under controlled growth conditions as indicated at 2.3.9 below, until flowering and fruit pods maturity.

2.3.9 Growth conditions

All cultures were kept in a growth room at 24±2°C and 16:6 hour photoperiod of 50–60 $\mu molm^{-2}s^{-1}$ light intensity. *In vitro* produced plantlets were acclimatised under controlled conditions at 24±2°C and 16:6 hour photoperiod of 150–200 $\mu molm^{-2}s^{-1}$.

2.3.10 Data analysis

The mean number of shoots per explant was analysed using one-way ANOVA with SPSS 20 for Windows. Data on the frequency of shoot induction given in percentages were calculated as described by Ma and Wu (2008).

2.4 RESULTS AND DISCUSSION

2.4.1 The effect of cytokinins on seed germination and seedling development

Soybean seeds started to germinate within 3 days of incubation in all tested media containing PGRs (Fig. 2.3). Our results showed that the control seeds had a lower germination of 67.8% (Fig. 2.3) and produced thin (1–2 mm) elongated hypocotyls, epicotyls and primary root with lateral roots (Fig. 2.4 A), which were difficult to use for excision of explants.

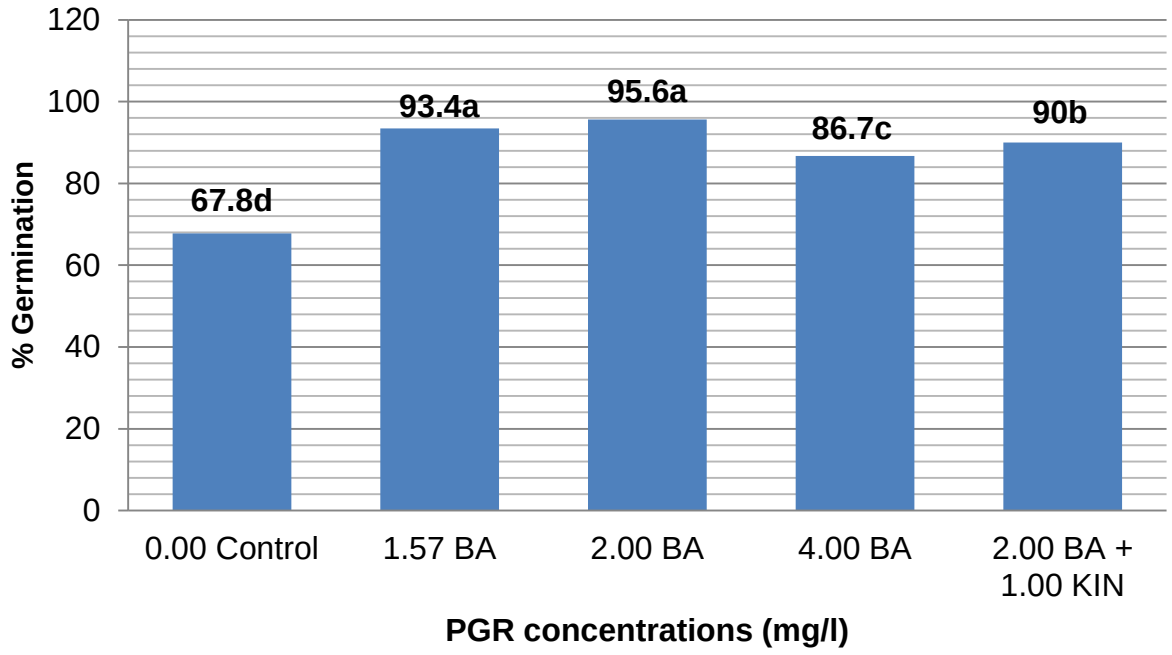

Figure 2.3 Percentage germination of seeds in the PGR free (control) and PGR containing MS medium. Percentages on the bar charts followed by the same letters are not significantly different at the 5% confidence level according to ANOVA.

Seedling growth and development differed significantly between treatments. Seedlings obtained from all PGR treatments showed high survival rate, thickening of hypocotyls, reduction of epicotyl length and primary roots with few or no secondary roots (Fig. 2.4 A–E). The thickest hypocotyls (4–5 mm diameter) were observed on seedlings grown on media containing 4.00 mg/l BA (Fig. 2.4 D), as compared to seedlings from the other hormonal treatments (3–4 mm diameter) in Figure 2.4 B, C and E. These growth responses can be possibly attributed to the exogenous application of cytokinins on germinated seeds as supported by Crosby et al. (1981).

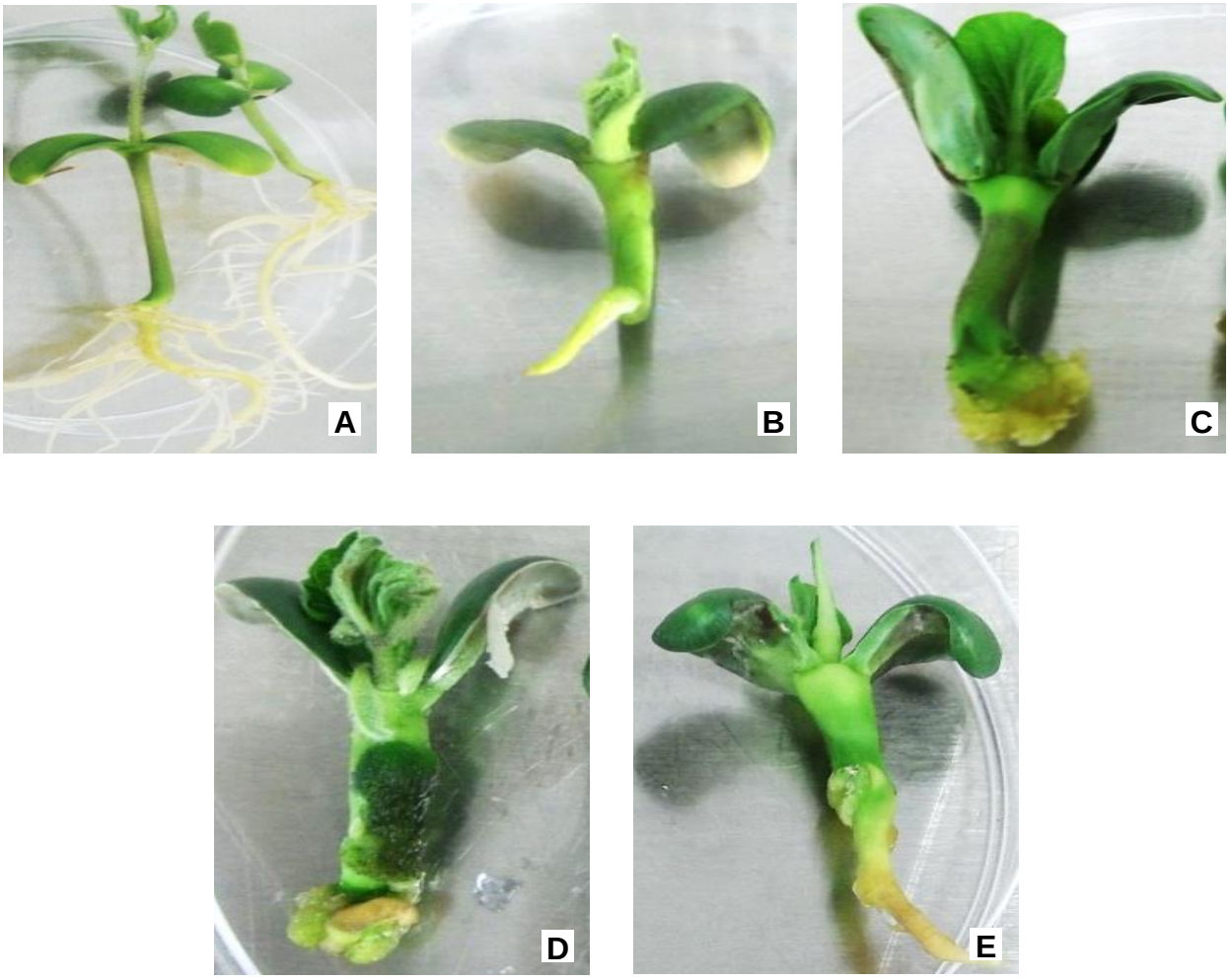

Figure 2.4 (A) Seedling developed on PGR-free MS culture medium with thin stems and vigorous root formation. Seedlings developed on MS media supplemented with 1.57 mg/l BA (B), 2.00 mg/l BA (C), 4.00 mg/l BA (D), 2.00 mg/l BA + 1.00 mg/l KIN (E) after 10 days of incubation showing thicker hypocotyls, with reduced roots and no secondary roots.

Similar to our findings, Malik and Saxena (1992) showed that pre-treatment of seeds with cytokinins resulted in development of stout seedlings from which multiple shoot could be stimulated. In the current study, the results show that thickened seedlings derived from seeds germinated on MS medium containing cytokinin, especially with 2.00 and 4 mg/l BA, (Fig. 2.4 C and D respectively) proved to be the best source of explants on which excisions can be made for shoot induction. Incisions can also be made on such seedlings for infection with bacteria during genetic transformation.

2.4.2 The effect of cytokinins on multiple shoot induction from single and double coty-node explants

Multiple shoots were directly induced from single and double coty-node explants cultured on media containing BA and BA in combination with KIN. The frequency of shoots formation was influenced by both explant type and the PGR concentrations and combination, as illustrated in Table 2.2 and Figure 2.5–2.6.

2.4.2.1 Multiple shoot induction on double coty-node explants

Explants from all PGR treatments (after 14 days of incubation) produced a significantly higher number of shoots from double coty-node explants as compared to the control, which did not contain PGRs. The highest number of shoots, with a mean of 7.93 shoots per explant, was obtained in the presence of 2.00 mg/l BA alone (Table 2.2). Although there was no major difference in the number of shoots induced by 1.57 mg/l (Fig. 2.5 A) and those induced by 2.00 mg/l BA (Fig. 2.5 B), shoots derived from 2.00 mg/l BA treatment were stronger and responded well or better to elongation and rooting (Fig. 2.7 A–B). Shan et al. (2005) obtained a maximum of 6 shoots per explant when explants were treated with 0.1 mg/l thidiazuron. Their explants were the cotyledonary nodes with the epicotyls, hypocotyls and cotyledons removed. The explants produced multiple bud tissues which could be further used to produce shoots or more multiple bud tissues. On the other hand, Ma and Wu (2008) reported a mean of 33 shoots per explant when whole cotyledonary node explant for soybean cultivar Hefeng 25 was pre-treated with

0.5 mg/l BA. However, in their case shoot buds were not allowed to develop on the explant but were excised and subcultured in a similar medium. Our observations showed that treatments with high concentration of BA (4 mg/l) shown in Figure 2.5 C or in combination with KIN (2mg/l BA+1mg/l KIN) in Figure 2.5 D, resulted in lower number of shoots (5–6) per explant accompanied by massive callus formation. Though this combination results in thick coty-node explants, it is not efficient in multiple shoot induction.

Table 2.2 Multiple shoot regeneration from single and double coty-node explants on MS media supplemented with different concentrations and combination of plant growth regulators after 21 days of culture.

Cytokinin treatments mg/l		**Mean number of shoots per coty-node explant**		**Frequency (%) of shoot induction per coty-node explant**	
BA	KIN	SCN	DCN	SCN	DCN
1.57	0.00	3.87[b]	7.00[a]	36.7[b]	83.3[a]
2.00	0.00	5.27[a]	7.93[a]	56.7[a]	80.0[b]
4.00	0.00	1.87[c]	5.00[c]	10.0[d]	50.0[d]
2.00	1.00	2.00[c]	6.27[b]	13.3[c]	73.3[c]
0.00	0.00	1.27[d]	2.13[d]	0.00[e]	0.00[e]

Values within columns followed by the same letters are not significantly different at the 5% confidence level according to ANOVA. Frequency of shoot induction = (No of explants with three or more shoots / total no of explants) × 100. Single coty-node (SCN). Double coty-node (DCN).

These results suggests that there was no synergism caused by the combination of BA with KIN for multiple shoot induction, as supported by Malik and Saxena (1992), where BA (80 µM) was combined with KIN (10 µM) which was ineffective in increasing shoot

induction to more than 6 shoots per explant. Wright et al. (1986) and Khalafalla and Hattori (1999), have shown that, when BA is combined with TDZ, shoot formation can be attributed to TDZ only rather than BA or by both. However, pre-treatment and maintenance of cultures on media containing TDZ may cause abnormal changes in the growth and development of plants. Such changes include hyperhydricity, formation of short and compact shoots, and the difficulty in shoot elongation and rooting of the recovered shoots (Lu, 1993; Huetteman and Preece, 1993).

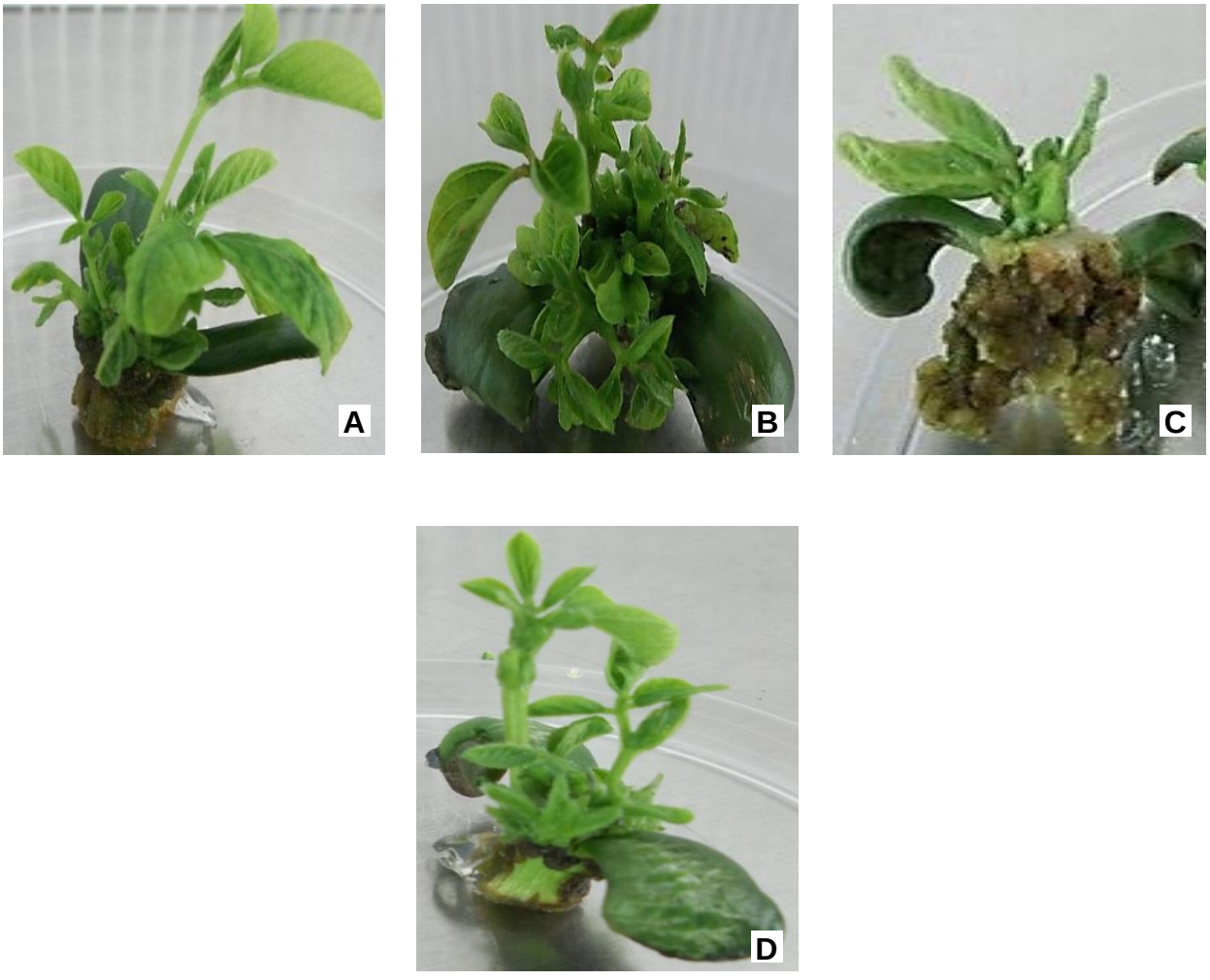

Figure 2.5 Double coty-node explants cultured on MS media containing 1.57 mg/l and 2.00 mg/l BA produced a high number of multiple shoots (A and B) while those cultured on media containing 4.00 mg/l BA and 2.00 mg/l BA in combination with 1.00 mg/l KIN produced few multiple shoots and lots of callus (C and D).

2.4.2.2 Multiple shoots induction on single coty-node explants

Similar to the double coty-node explants responses, the highest number of shoots obtained on single coty-node explants was on MS medium supplemented with 2 mg/l BA (Table 2.2). Callus initiation observed in the presence of a high BA concentration (4 mg/l) or BA in combination with KIN (2.00 mg/l BA and 1.00 mg/l KIN) was more pronounced on single coty-node explants (Fig. 2.6), as compared to the double coty-node explants. This suggests that morphogenic non-competent cells were induced to divide by wounding during explant preparation.

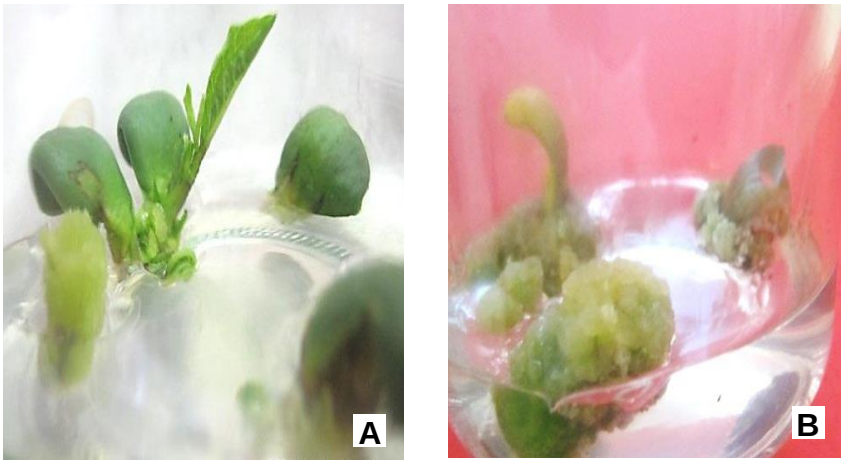

Figure 2.6 Multiple shoots and callus induction on single coty-node explants cultured on MS media. (A) Single coty-node explants showing induction of fewer shoots on MS medium supplemented with 2.00 mg/l BA. (B) Single coty-node explants showing callus initiation on MS medium containing 4.00 mg/l BA.

Ma and Wu (2008) also reported that explant damage during excision of cotyledonary nodes can easily cause dedifferentiation into callus if subcultured on media supplemented with high concentrations (>3 mg/l) of BA. The callus will compete with the axillary meristems for nutrient adsorption which will eventually have negative effects on shoot growth. The single coty-node explants showed lower frequency (10–57%) of shoot induction when compared to the double coty-node explants (50–83%). The high shoot regeneration frequency obtained on double coty-node explants may be due to the two meristematic regions present on the explants as compared to vertically split single

cotyledons as described by Zhang et al. (1999). Polisetty et al. (1997) also demonstrated that multiple shoots are not efficiently produced when only embryonic axes with axillary buds and cotyledons removed are used. The presence of the cotyledons appeared to support the survival and growth of shoots. On the other hand Franklin et al. (2004) reported high frequency of shoot induction from hypocotyl segments than from cotyledonary nodes on MS medium containing 9.04 µM TDZ in soybean CV Bonminori. The hypocotyl explants were reported to be competent in inducing a high number of shoots than the cotyledonary node explants. But the report suggested that explant responses to shoot induction depends on growth regulator concentration (preferably 9.04 µM TDZ) or genotype than on the explant type.

2.4.3 Shoot elongation and rooting

Our *in vitro* induced multiple shoots elongated and developed better when subcultured as clumps, than as excised individual shoots (Fig. 2.7 A). Addition of GA into the medium had no positive effect on plant growth since it resulted in weaker shoots as compared to shoots elongated on PGR-free MS medium. Rooting of elongated shoots also was more efficient on MS medium without PGRs than in the presence of auxin which induced callus formation at the base of the shoots (Fig. 2.7 B). Such observations were also shown by earlier reports such as those of Ko et al. (2003) and Kaneda et al. (1997) which reported the successful elongation and root initiation on culture medium without PGRs. In contrast, Islam et al. (2005) reported production of roots in *in vitro Cicer* root culture using 0.5–0.75 mg/l IBA. Ma and Wu (2008) achieved elongation and rooting in *in vitro* soybean culture using only 0.5 mg/l IBA.

2.4.4 Plant acclimatisation

Rooted plants were successfully acclimatised under controlled growth conditions over a period of 3–4 weeks attaining a 70% survival rate. The acclimatised plants grew to maturity and resembled normal seed-derived plants by flowering and production of seed pods (Fig. 2.7 C). Covering the plants with plastic bags reduced transpiration during the

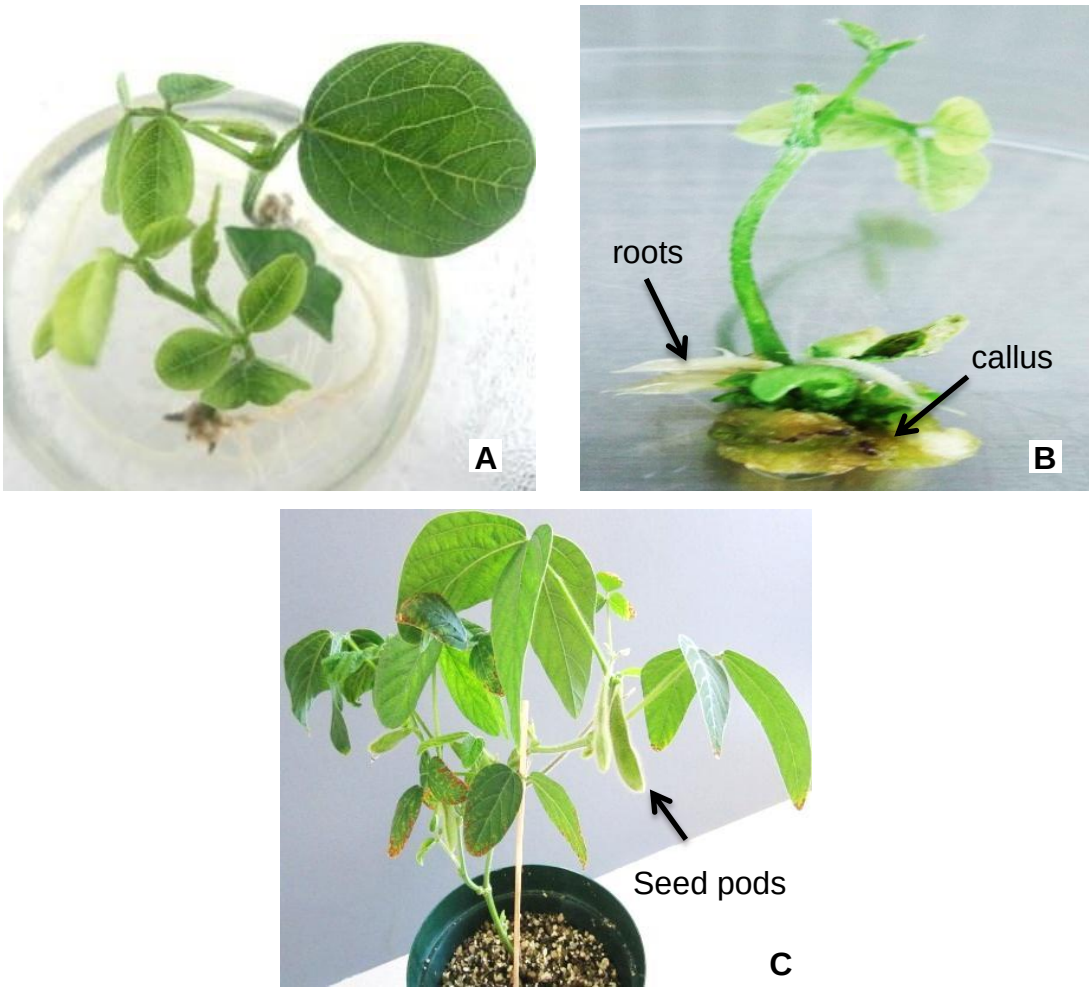

Figure 2.7 (A) Production of elongated and rooted shoots on MS medium without hormones after two weeks of culture. (B) Rooting on MS medium supplemented with IBA (0.5 mg/l) and IAA (1.2 mg/l) over 20 days which resulted into callus initiation and root growth inhibition. (C) Acclimatised plant showing production of seed pods.

acclimatisation period. Our results show that plants regenerated from culture media containing PGRs (1.57 and 2.00 mg/l BA) were the most responsive, with the production of seed pods. However, overall survival rate and growth of the plants were positively influenced by the BA (1.57 mg/l and 2.00 mg/l) concentrations used, except for a few small number of plants obtained from the MS medium containing PGRs (2.00 mg/l BA plus 1.00 mg/l KIN), and the control without hormones. The results further indicated that plants obtained from 2.00 mg/l BA in combination with 1.00 mg/l KIN had slow growth rate and development as compared to those obtained in 1.57 and 2.00 mg/l BA

respectively. These were followed by soybean plants obtained on MS medium containing 4.00 mg/l BA and the control, which exhibited severe deficiency symptoms. The response obtained in the combined concentrations (2.00 mg/l BA plus 1.00 mg/l KIN) may be due to the effect of auxin-cytokinin concentration levels on plant growth and development as described by Saad and Elshahed (2012).

2.5 CONCLUSION

The results of this study show that germination of soybean seeds on MS culture medium supplemented with BA in the range 2–4mg/l is important for establishing strong seedlings that can be used directly as a reliable explant source for shoot multiplication. Multiple shoot induction can be best achieved on MS medium in the presence of 2mg/l BA using double coty-node explants. Shoots regeneration by this approach is a promise to improve genetic transformation of soybean plants.

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CHAPTER 3

QUANTITATIVE DETERMINATION OF THE EFFECT OF SELECTED ANTIBIOTICS ON THE ELIMINATION OF *AGROBACTERIUM TUMEFACIENS*

3.1 INTRODUCTION

Agrobacterium tumefaciens is one of the most widely used Gram negative soil borne bacteria in plant genetic transformation. Genetic transformation using *A. tumefaciens* is a powerful tool in the improvement of many crops including grain legumes such as soybean. This process is considerably preferred over direct gene transfers (e.g. electroporation, microinjection or biolistic bombardment of cells with rapidly accelerated naked DNA molecules) because of relatively low complexity of intact transgenes integrated into host genome; ease of application; and low cost of the procedure.

Although this bacterium provides a method of choice for the improvement of most economically important plant species, most plant species and elite cultivars of particular species are highly recalcitrant to this method. In particular, the efficient genetic transformation of soybean, cotton, cereal grains, many legumes and tree species of horticultural and industrial importance remains a challenge (Gelvin, 2003). The use of supervirulent and binary vectors containing additional copies of *vir* genes has increased the transformation efficiencies in some species including cereals (Yukawa et al., 2008). This means that further alternative approaches to increase transformation frequencies of highly recalcitrant crops such as soybean might lie in manipulation of culture conditions, particularly the use of antibiotics.

Antibiotics play a very important role in controlling and eliminating the growth of *A. tumefaciens* after co-cultivation with explants, and for the selection of transformants. These are of utmost importance for efficient plant regeneration systems. But their optimisation in culture conditions has been neglected. Opabode (2006) indicated that cefotaxime (50 or 250 mg/l), which has been routinely used for most crops transformation was later found to greatly reduce transformation efficiency by 3-fold compared to carbenicillin (100 mg/l). In another report by Mamidala and Nanna (2009) cefotaxime and carbenicillin have both been shown to inhibit the regeneration of *Agrobacterium*-inoculated plant tissues in tomato. These negative effects limit the use of *A. tumefaciens* in exploiting its natural ability to introduce new genetic materials into plant cells. The choice of suppression and selection agents is vital for the successful elimination of the bacterium and selection of transformed cells from

non-transformed ones. Therefore, if thorough attention is given to the optimisation of the use of antibiotics during transformation, then this may accelerate the genetic improvement of soybean cultivars and other species.

3.1.1 Motivation for the study

The use of *A. tumefaciens* for plant transformation is a natural and simple way to achieve genomic changes in plants. *Agrobacterium* is the only known example of a natural inter-kingdom genetic exchange agent (Gelvin, 2003). Currently, the use of *A. tumefaciens* and *A. rhizogenes* for transformation serves as the common and feasible means of transferring genes of interest into different crop plants. Since McCormick et al. (1986) reported successful *Agrobacterium*-mediated transformation of tomato; many researchers have then reported transformation ranging from 6 to 40% efficiency in both monocotyledonous and dicotyledonous crop plants. However, attainment of high transformation efficiencies using this bacterium depends on the impact of antibiotic types and concentrations used during transformation, in addition to other factors such as explant type, culture medium and plant genotype (Lee et al., 2012).

Magdum (2013) reported that the lack of effective antibiotics and chemical washing agents that do not biochemically damage plant tissues during transformation are responsible for the unsuccessful transformation frequencies. Cefotaxime, carbenicillin and vancomycin are the most commonly used antibiotics in genetic transformation experiments. However, these antibiotics have showed a great negative effect on regeneration of transformed plantlets such as in tomato, chickpea and tobacco (Costa et al., 2000; Silva and Fukai, 2001; Eyidogan et al., 2009). Furthermore, the market cost of these antibiotics is very high. Highly efficient antibiotics and concentrations for effective control of *Agrobacterium*, and low phytotoxic effects caused by these antibiotics have not yet been well established.

3.1.2 Aim

This study was aimed at determining the concentrations of affordable and readily available antibiotics that can effectively inhibit *A. tumefaciens*.

3.1.3 Objectives:

i. Establish bacterial cultures of the two strains of *A. tumefaciens* (pTF 101 and Ω PKY) on yeast extract peptone (YEP) medium supplemented with various antibiotics as selective markers.
ii. Determine the inhibitory effect of different concentrations of six aminoglycoside test antibiotics on the two strains of *A. tumefaciens* using well diffusion assay.
iii. Determine the antibiotic potency by assessing the zones of bacterial inhibition on each agar plate.

3.2 LITERATURE REVIEW

3.2.1 The use of *Agrobacterium* in genetic transformation

Genetic transformation has been the most promising method for crop improvement. The transgenic plants obtained provide the required traits without the loss of genetic integrity (Kumar et al., 2011). To exploit this new approach, an efficient transformation protocol is required. *Agrobacterium*-mediated gene transfer in plants is a highly efficient transformation process and holds great potential for this purpose. However, this technique is governed by various factors including host plant, culture explants and culture conditions (Mehrotra and Goyal, 2012). *Agrobacterium* has been successfully used to transform various economically and horticulturally important monocots and dicot species. In dicot legumes such as soybean, regenerable cells in the axillary meristems of the cotyledonary-nodes have been frequently used (Zhang et al., 1999; Donaldson and Simmonds, 2000; Ko and Korban, 2004; Liu et al., 2008; Lee et al., 2012).

Olhoft and Somers (2001) reported the addition of L-cysteine in culture media in order to obtain two-fold increased transgenics. This report was followed by improvement in transformation efficiencies also using the cotyledonary-node method by Jaiwal et al. (2001); Paz et al. (2004) and Liu et al. (2009). In monocot crops, Hensel et al. (2009) recorded stable transgenic plants reaching 86% in barley, 10% in wheat, 4% in triticale, and 24% in maize. These reports were achieved following combined implementation of meaningful advances in both culture conditions and the use of hypervirulent *Agrobacterium* strains.

3.2.2 The effect of antibiotics on *in vitro* plant regeneration

Successful transformation using *Agrobacterium* depends not only on the efficiency of the plant regeneration system but also on the subsequent elimination of this bacterium from transformed tissues (Tang et al., 2000). If the *Agrobacterium* is not eliminated, it will overgrow the tissue and destroy it. Antibiotics such as; vancomycin, timentin, cefotaxime and carbenicillin are required for this purpose, and the level of the antibiotics needed to eliminate *Agrobacterium* may also affect plant regeneration (Shackelford and Chlan, 1996). Various reports have shown that antibiotics like cefotaxime and vancomycin are usually used alone or in combination in order to reduce bacterial growth, meanwhile being ineffective and damaging on soybean explants. Pollock et al. (1983) and Reed and Tranprasert (1995) provided a review(s) on the negative effects on plant regeneration caused by these antibiotics on species such as *Nicotiana plumbaginifolia*. The effects of these antibiotics have been studied on soybean. Kim et al. (2009) reported low shoot regeneration using cotyledonary node explants at concentrations of 50 and 100 mg L^{-1} of carbenicillin and timentin respectively. Wiebke et al. (2006) reported death of soybean embryogenic tissues when cefotaxime at 350 and 500 mg L^{-1} was used on cultivars Bragg and IAS5. Magdum (2013) also reported induction of necrosis and other phytotoxicity responses in pearl millet cereal grain crop.

3.2.3 Antibacterial activity

Accurate determination of bacterial susceptibility to antibiotics is essential for successful *in vitro Agrobacterium*-mediated genetic transformation (Bonev et al.,

2008). This kind of evaluation is necessary when novel antibiotics, rather than commonly used antibiotics such as cefotaxime, carbenicillin and vancomycin are to be tested for their effective elimination of *A. tumefaciens* during co-cultivation. One method commonly used for quantifying the ability of antibiotics to inhibit bacterial growth is agar diffusion assay as demonstrated by Shackelford and Chlan (1996); Tang et al. (2000); Estopa et al. (2001) and Alsheikh et al. (2002). This method can be performed using paper discs amended with antibiotics or can be inoculated on wells punched in the agar plate. Antibiotic diffusion from source (wells or discs) into the agar medium leads to inhibition of bacterial growth in the vicinity of the source, and to the formation of clear zones without bacterial growth.

3.2.3.1 Disc diffusion assay

The disc diffusion assay is a widely used method in the evaluation of antibiotic efficiency for the elimination of *A. tumefaciens* from transformation cultures (Holford and Newbury, 1992; Shackelford and Chlan, 1996; Cheng et al., 1998; Brasileiro and Aragao, 2001). However, Pollock et al. (1983) and Reed and Tanprasert (1995) have reported that low inhibitory effects are observed when the disc diffusion assay is used as compared to diffusion from wells. This could be due to the low diffusion rates occurring from the paper discs as compared to the liquid diffusion of antibiotics from the wells into agar.

3.2.3.2 Agar well diffusion assay

The “non-disc” assay involves the application of antibiotic solutions of different concentrations in wells punched into agar plates inoculated with the test bacterial strain. This method has proved to be efficient, and one that gives accurate results as compared to above mentioned protocols (Holder and Boyce, 1994). Bonev et al. (2008) further demonstrated that, factors affecting the accuracy and reproducibility of diffusion methods include thickness and uniformity of the agar. In “non-disc” assay, the choice of the well size and temperature of incubation are usually the factors that negatively affect the accuracy of results, and they can be easily optimised (Mishra et al., 2006). Comparative analysis of antimicrobial agents against bacteria relies on the ability of the antibiotics to diffuse freely through the solid nutrient medium.

According to Bonev et al. (2008), this assumption may be incorrect considering significant deviations that may occur when either discs or wells containing tested antibiotics are used. Similar observations with different bacterial inhibitory effects were made in other related approaches such as the E-tests (Epsilometer test) and the standardised single disc method (Lang and Garcia, 2004).

3.2.4 Major setbacks on the effectiveness of antibiotics against *Agrobacterium*

One of the problems of using antibiotics that are common and already known to be effective against *Agrobacterium* (such as cefotaxime, timentin and carbenicillin) is that, they are required in high concentrations of more than 500 mg/l in plant tissue culture. This requirement has led to the identification of the economic viability as one of the factors affecting progress in genetic transformation. Farzaneh et al. (2013) reported that high costs of carbenicillin and cefotaxime makes the genetic transformation procedure very costly, especially in optimisation of protocols used for recalcitrant crops, such as soybean. The report stated that Meropenem antibiotic is effective against *Agrobacterium* strains (LB A4404, C58 and AGL0), and it was also identified to be economically viable with less toxicity to plant tissue explants. The high costs or the expensiveness of these antibiotics reduce their accessibility, and provide a need for the search of alternative antibiotics that are cheap, or other stringent methods to prevent *Agrobacterium* survival during culture.

The infected explants often need to be subcultured a number of times on the medium containing antibiotics to maintain suppression of the *Agrobacterium* (Cheng et al., 1998). In addition to the problems mentioned above, bacterial resistance of *Agrobacterium tumefaciens* to the commonly used ß-lactam antibiotics also becomes a challenge. Problems of resistance of *Agrobacterium* to the antibacterial agents, market value, and the higher amounts (mg/l) of antibiotics required for *in vitro* tissue culture, shift the research focus towards finding other alternative protocols for *Agrobacterium* control. Hammerschlag et al. (1997) reported reduction in *A. tumefaciens* overgrowth using a 1 hour vacuum infiltration with an acidified medium, without having negative impact on shoot regeneration. Bent (2000); Supertana et al. (2005) and Liu et al. (2009) used *in planta* or *in vivo* transformation that avoid the use of tissue culture, and the need for antibiotics.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial culture

Agrobacterium tumefaciens bacterial culture, transformed with pTF 101 and ΩPKY plasmid vectors containing the *oc* 1 gene construct were used. These are derivatives of base vector pTF 101.1 from strain EHA 101. The transformed *Agrobacterium* cultures carrying plasmid vector pTF 101 and Ω PKY were grown on solid yeast extract peptone (YEP) medium containing 100 mg/l spectinomycin plus 50 mg/l kanamycin for pTF 101; and 100 mg/l rifampicin and 10 mg/l tetracycline for Ω PKY respectively, at 28°C. Bacterial inoculum was prepared by allowing 2 ml cultures of *Agrobacterium* to grow to saturation (for 16–24 hours) at 28°C in a shaker orbital incubator (145 rpm). The *Agrobacterium* was then collected by pelleting at 3,500 rpm for 10 minutes at 20°C and the pellet was resuspended in infection liquid medium.

3.3.2 Preparation of selective antibiotic stock solutions

Antibiotics were purchased as powder from Sigma-Aldrich and Rochelle chemicals. Six antibiotics were used: kanamycin monosulphate, spectinomycin dihydrochloride pentahydrate, rifampicin (purchased from Rochelle chemicals), tetracycline hydrochloride, streptomycin sulfate and hygromycin B (purchased from Sigma-Aldrich). Powders were accurately weighed and dissolved in the appropriate solvent (Table 3.1) to yield the required concentrations (mg/l). Stock solutions were prepared using the formula:

$$\left(\frac{1000}{P}\right) \times V \times C = W$$

Where P- is the potency of the antibiotic; V- volume in ml; C- final concentration of solution and W- weight of the antibiotics to be dissolved in V. The stock solutions were stored at -20°C

3.3.3 *Agrobacterium tumefaciens* elimination

The evaluation of the inhibitory effect of the above listed antibiotics for the elimination of *A. tumefaciens* by the agar well diffusion assay was performed as

described by Bell and Grundy (1968). Test antibiotics at concentrations: 150, 300 and 500 mg/l, and the combination of rifampicin (50 mg/l) plus tetracycline (10 mg/l), and kanamycin (50 mg/l) plus spectinomycin (100 mg/l) were pipetted into 5 mm diameter wells, punched on the agar medium. The applied antibiotics diffused from the wells on the basis of their ability to move through the matrix formed by the gelling of agar. Agar medium spread with *Agrobacterium* inoculated with dimethyl sulfoxide (DMSO) was used as a control. The antibacterial activity was determined by measuring and recording the zones of bacterial inhibition in millimetres (mm). The experiment was repeated four times, with three replicates made for each selected concentrations, and combinations of antibiotics. The average diameters obtained were compared with the theoretical zones of clearance to determine if the bacterium is susceptible, resistant or have intermediate response, with reference to Appendix 3.2.

3.3.3.1 Selective antibiotics

Antibiotics used as selective markers for *Agrobacterium* pTF 101 and Ω PKY were also tested for *Agrobacterium* growth inhibition. Kanamycin (50 mg/l) in combination with spectinomycin (100 mg/l) is used as selective marker antibiotics for pTF 101 and rifampicin (50 mg/l) in combination with tetracycline (10 mg/l) were used for selection in Ω PKY. These antibiotics [rifampicin (50 mg/l) plus tetracycline (10 mg/l) and kanamycin (50 mg/l) plus spectinomycin (100 mg/l)] were interchanged and pipetted in wells punched on the medium inoculated with pTF 101 and Ω PKY respectively.

3.3.3.2 Test antibiotics

The following broad-spectrum antibiotics: hygromycin, kanamycin, rifampicin, spectinomycin, streptomycin and tetracycline were used as described above. Three different concentrations of each antibiotic were prepared the same day the assay was conducted. These antibiotics were applied at the final concentrations of 150, 300 and 500 mg/l as shown in Table 3.1.

Table 3.1 Preparation of antibiotic solutions.

Antibiotic	**Solvent**	**Diluent**	**Concentration (mg/l)**		
			150	**300**	**500**
Hygromycin B	dH_2O	dH_2O	+	+	+
Kanamycin monosulphate	dH_2O	dH_2O	+	+	+
Rifampicin	DMSO	dH_2O	+	+	+
Spectinomycin dihydrochloride Pentahydrate	dH_2O	dH_2O	+	+	+
Streptomycin sulfate	dH_2O	dH_2O	+	+	+
Tetracycline hydrochloride	dH_2O	dH_2O	+	+	+

+ indicate that the designated concentration for the antibiotic has been used/ added into wells for the diffusion assay.

3.3.3.2.1 The effect of test antibiotics against pTF 101

YEP solid medium was prepared and 40 ml of the medium was distributed into 90 × 15 mm Petri dishes after autoclaving and then allowed to solidify. The plates were inoculated with the bacterial suspension of a strain of *A. tumefaciens* that had been previously grown on YEP liquid medium inoculated with appropriate selective filter sterilised antibiotics as indicated in 3.3.1. The bacterium was spread throughout the agar medium using a cotton swap. Two wells (of 8 mm depth and 5 mm diameter) were punched on the agar plates using a flame sterilised cork-borer and then, 30 µl of each solution of antibiotics to be tested were pipetted into the wells. The plates were then sealed with a parafilm and incubated at 28°C for 2 days in the Gallenkamp size 1 incubator. During incubation, the antibiotics diffused from the wells, from an area of high concentration to the area of low concentration. The diameters of the zone of bacterial growth inhibition were then measured in millimetres from the edges of the zone of clearance.

3.3.3.2.2 The effect of test antibiotics against Ω PKY

Agar diffusion assay using bacterial suspension of the strain Ω PKY of *A. tumefaciens* was performed exactly the same way as the assay for pTF 101, described above (3.3.3.2.1). Various volumes of antibiotics (30 µl) to be tested were pipetted into the wells (As shown on Table 3.1). The plates were also sealed with a parafilm and incubated at 28°C for 2 days in the Gallenkamp size 1 incubator. The antibiotics diffused from the holes, from an area of high concentration to the area of low concentration. The diameters of the zones of inhibition were also measured in millimetres from the edges of the zone of clearance and recorded.

3.4 RESULTS AND DISCUSSION

The inhibitory effects of the six test antibiotics, and the antibiotics used as selective markers (pTF 101: spectinomycin 100 mg/l and kanamycin 50 mg/l, and Ω PKY: tetracycline 10 mg/l and rifampicin 50 mg/l) on the hypervirulent *A. tumefaciens*, pTF 101 and Ω PKY strains are presented in Figure 3.1 and 3.2.

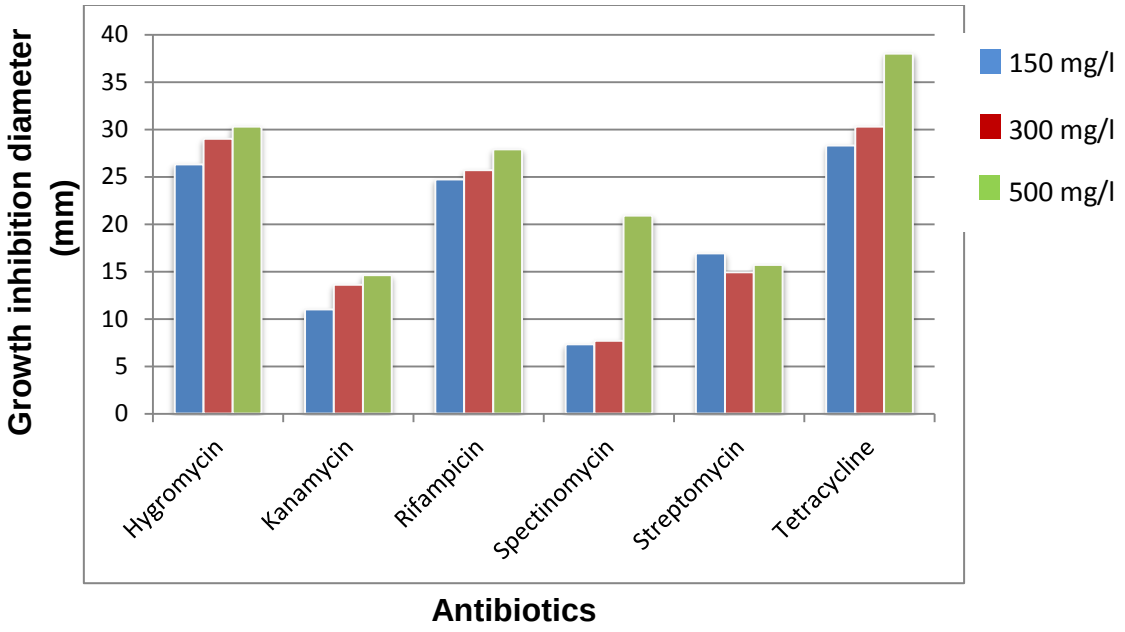

Figure 3.1 The inhibitory effect of the six test antibiotics on *A. tumefaciens* strain pTF 101.

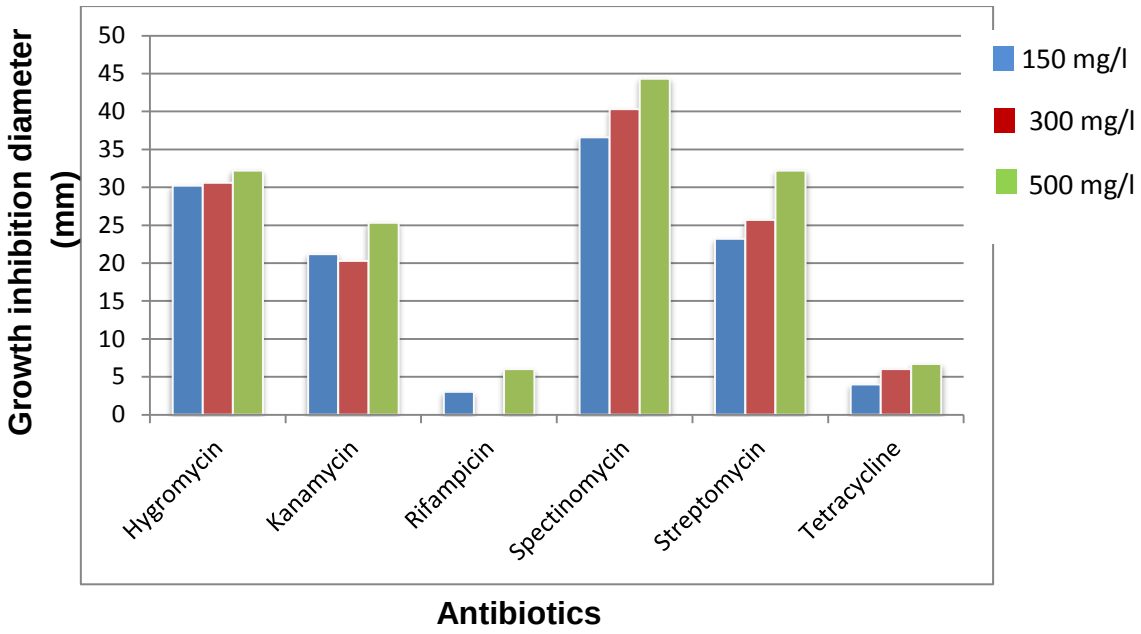

Figure 3.2 The inhibitory effect of the six test antibiotics on *A. tumefaciens* strain Ω PKY.

3.4.1. The effect of selective antibiotics against *A. tumefaciens*

Figure 3.3 shows growth inhibition in *Agrobacterium* pTF 101 on medium supplemented with selected concentration of rifampicin (50 mg/l) in combination with tetracycline (10 mg/l) normally used as selective markers for Ω PKY. The average diameter of the zone of inhibition was 18 mm. Based on the standard data presented in Appendix 3.2, this average diameter suggest intermediate susceptibility range on this bacterial strain as postulated by Pollack et al. (2009) and Wheat (2001).

Similar observations were made when *Agrobacterium* Ω PKY was treated with kanamycin in combination with spectinomycin used as selective markers for pTF 101 (Fig. 3.4). The average diameter of the zone of inhibition was 16 mm, which was smaller than one obtained in pTF 101 strain (18 mm) inoculated on medium supplemented with rifampicin and tetracycline (Fig. 3.3). The results show that Ω PKY strain showed low resistance to the combination of kanamycin-spectinomycin, as compared to tetracycline-rifampicin combination. The combinations of antibiotics used in this study as selective agents have not been reported before. This implies that specific concentrations of these combinations of antibiotics need to be screened for the purpose of eliminating *Agrobacterium* or for control of these strains during co-cultivation in plant genetic tranformation.

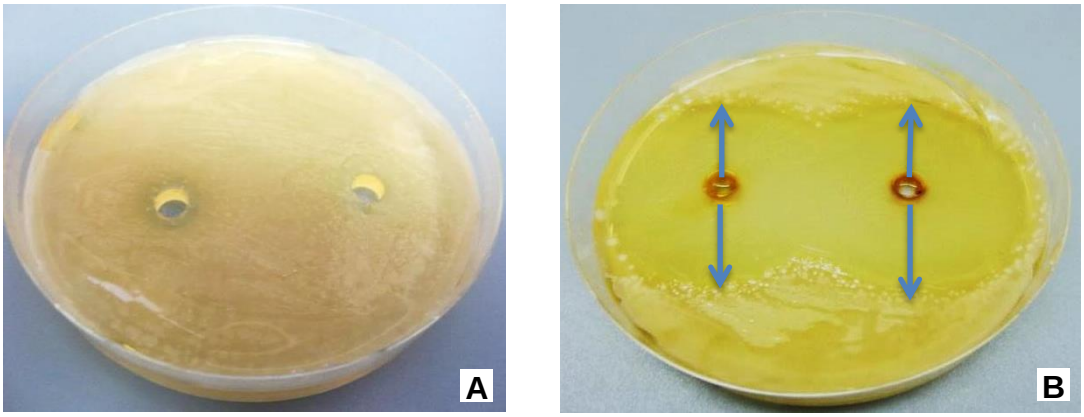

Figure 3.3 Inhibitory effect of selective marker antibiotics on *A. tumefaciens* pTF 101 strain. (A) Overgrowth of *A. tumefaciens* pTF 101 strain on agar plate, each well filled with mixture of spectinomycin (100 mg/l) and kanamycin (50 mg/l) selectable marker. (B) Inhibition of *A. tumefaciens* of pTF 101 strain by rifampicilin (50 mg/l) in combination with tetracycline (10 mg/l).

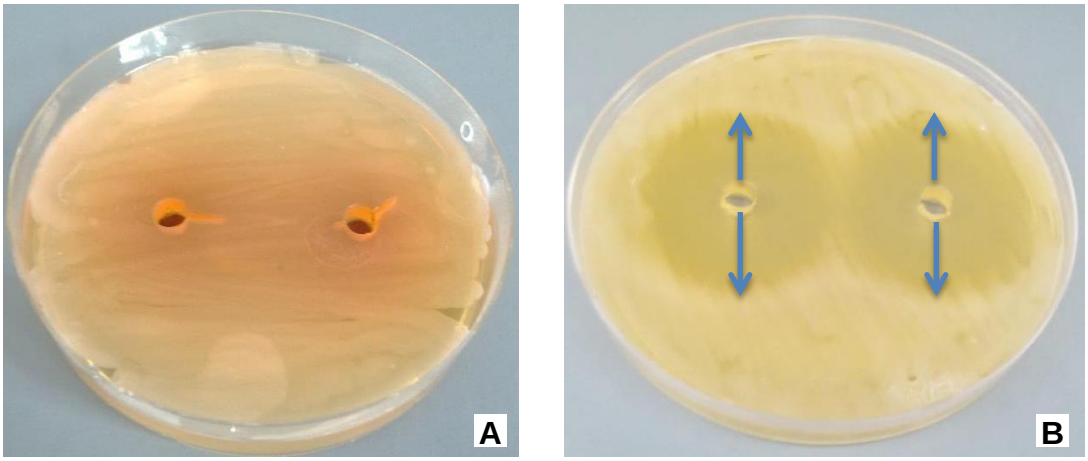

Figure 3.4 Inhibitory effect of selective marker antibiotics on the strain of *A. tumefaciens* Ω PKY strain. (A) Overgrowth of *A. tumefaciens* of Ω PKY strain on agar plate, each filled with rifampicin (50 mg/l) and tetracycline (10 mg/l), selactable marker. (B) Inhibition of *A. tumefaciens* of Ω PKY strain by spectinomycin (100 mg/l) in combination with kanamycin (50 mg/l).

Alimohammadi and Bagheriech-Najjar (2009) also emphasised the need for quantitative evaluation of other antibiotics like aminoglycosides that are not regularly used for the elimination of excess *A. tumefaciens*-mediated plant transformation. In other reports, such as in the transformation of tobacco, tomato and rice, Ogawa and Mii (2006) demonstrated the use of meropenem and moxalactam antibiotics for *Agrobacterium* elimination. These novel antibacterial agents (at concentration 0.20 to

400 mg/l) completely suppressed *Agrobacterium*, indicating their potency to replace the regularly used cefotaxime, carbenicillin and vancomycin that can be toxic to plant tissues at high concentrations.

3.4.2 The effect of broad-spectrum antibiotics on *A. tumefaciens*

The largest zones of inhibition (mm) were observed at 500 mg/l concentration, while the smallest were predominantly observed at 150 mg/l, as demonstrated on the bar charts on Fig. 3.1 and 3.2. The bacterial resistance to streptomycin and kanamycin antibiotics, used at 300–500 mg/l concentration was also observed in both strains.

3.4.2.1 Elimination of *Agrobacterium* strain pTF 101

Of the six antibiotics tested, tetracycline (500 mg/l) was found to be the most effective in eliminating *Agrobacterium* transformed with pTF 101 vector plasmid (Fig. 3.1). This concentration has shown consistent inhibition throughout with an average zone of 36 mm diameter. Then hygromycin (500 mg/l producing 30 mm zone diameter) was the next most effective antibiotic, followed by rifampicin at the same concentration (Appendix 3.1). Hygromycin and rifampicin were effective on controlling the growth of pTF 101 to the intermediate zone of inhibition (≥17 mm for rifampicin and ≥20 mm for hygromycin) according to Appendix 3.2. However, the other broad-specrum antibiotics had little effect in controlling this strain (Fig. 3.1).

3.4.2.2 Elimination of *Agrobacterium* strain Ω PKY

In contrast to pTF 101, the antibiotics spectinomycin, hygromycin and streptomycin showed larger zones of *Agrobacterium* inhibition respectively for Ω PKY with spectinomycin being the most effective antibiotic (Fig. 3.2). However, the other three broad spectrum antibiotics (tetracycline, rifampicin and kanamycin) had little or no clear zone of inhibition around the wells in all agar plates, including for the control (Appendix 3.1). These results further indicate that, the minimum concentration (150 mg/l) of the three most effective antibiotics used (against each strain) was also sufficient in inhibiting the bacterial growth arround the wells (23, 30, and 34 mm zone

of inhibition for streptomycin, hygromycin and spectinomycin respectively) of the agar plates. The zone of inhibition developed rapidly after 12 hours of incubation.
The observations made on both pTF 101 and Ω PKY implies that the conditions, such as the agar medium, especially its thickness and uniformity, used had no negative effects on the diffusion of the antibiotics, and thus the medium was suitable. This medium is commonly used for initiating *Agrobacterium* colonies in many studies (Zhang et al., 1999; Olhoft and Somers, 2001; Liu et al., 2004; Subramanyam et al., 2011). Han et al. (2007) indicated that it is more convinient to add measured quantities of the antibiotic solutions into wells, punched in the agar than using paper discs. This was done because an important factor influencing the diameter of the zone of inhibition, beside the concentration, is the absolute quantity of antibiotic present. This is supported by Lancini et al. (1995) who have indicated that by using wells, the quantity of antibiotic solution will provide uniformity of diffusion in the test. Our study is in line with these reports considering the degree of diffusion not being affected by the concentration of the antibiotics used, because the molecules diffuse freely from the wells. Both the higher concentrations of antibiotics, and most particularly, the type of antibiotics used have contributed to the attainement of these results.

In most reports, including those of Tang et al. (2000); Alsheikh et al. (2002) and Quisen et al. (2009), various antibiotics were evaluated for effective elimination of *A. tumefaciens*. Their results indicated that the type and the amount of antibiotics play a very important role in controlling the *Agrobacterium*. They have showed that the antibiotics suitable are cefotaxime, carbenicillin, and augmentin when used at concentration 250–2000 mg/l. This concentration range is higher than the one used in our study, which is 150–500 mg/l. Furthermore, according to Quisen et al. (2009) the most effective concentration of these antibiotics cannot be specified because their inhibitory effects differed according to the bacterial strain used. In contrast, it has been observed during this study that if tetracycline and spectinomycin are used at 500 mg/l concentration, the growth of *Agrobacterium* transformed with pTF 101 and ΩPKY is effectively inhibited.

3.4.2.3 General effectiveness of the antibiotics on the two *A. tumefaciens* strains

The effectiveness of the tested antibiotics is illustrated on Table 3.2 in reference to Appendix 3.2. As shown previously on Figure 3.1 and 3.2. The zones of inhibition obtained were large with respect to the three antibiotics [tetracycline (36 mm), hygromycin (30 mm) and rifampicin (30 mm) for pTF 101, and spectinomycin (44 mm), hygromycin (32 mm) and streptomycin (32 mm) for Ω PKY)], and all smaller with kanamycin (≤ 25 mm). The greatest difference in the zone sizes was observed between tetracycline and spectinomycin, which their correlation graphs are shown on Figure 3.5.

Table 3.2 Overall response of the six test antibiotics used at concentration 500 mg/l.

Antibiotic	***Agrobacterium* transformed with**	
	pTF 101	**Ω PKY**
Hygromycin	*Sus*	*Sus*
Kanamycin	*Res*	*Sus*
Rifampicin	*Sus*	*Res*
Spectinomycin	*Res*	*Sus*
Streptomycin	*Int*	*Sus*
Tetracycline	*Sus*	*Res*

Keys for the various responses used above are: *Sus*= Susceptibility, *Int*= Intermediate and *Res*= Resistance.

The difference in the zone of inhibition between tetracycline and spectinomycin antibiotics was up to 10 mm, when the mean diameters from other antibiotics were compared with them. However, small difference of up to 1–2 mm was observed between the two that showed the high zone of inhibition. The concentrations of tetracycline and spectinomycin were plotted against the diameter of the zones of inhibition. The slopes of the lines were calculated by the method of the least mean squares. However, when tetracycline plot was compared with the other broad-

spectrum antibiotic plots on pTF 101, the absolute diameters changed according to the type of antibiotic and concentration. The highest concentrations produced the highest zones of inhibition, and the three antibiotics (hygromycin, tetracycline and spectinomycin) were more inhibiting than the rest of the antibiotics used.

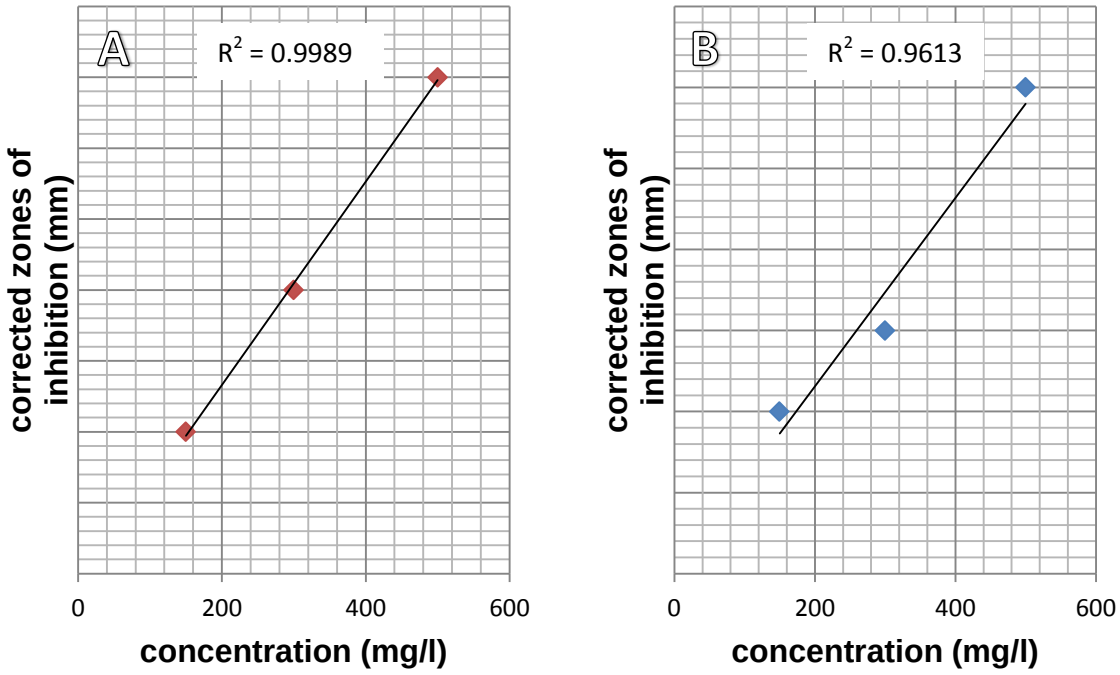

Figure 3.5 The standardised inhibition curves of *Agrobacterium* strains (A) pTF 101 and (B) Ω PKY by tetracycline hydrochloride and spectinomycin dihydrochloride pentahydrate at 150, 300 and 500 mg/l concentrations.

The constructed curves appear to be parallel. Similar observations were made in Ω PKY, with spectinomycin and other antibiotics. However, variations in slopes (of pTF 101 with tetracycline and Ω PKY with spectinomycin) show little difference from each other. This implies that the absolute or average zone sizes did not vary much from one another, and the curves also remain roughly parallel to one another. Their slopes (R^2) give a good correlation between antibiotic concentration and the diameter. Furthermore, the points on the plot indicate that there were no clear variations that may be related to the type of the agar used or its depth. These two standardised curves indicate that the two antibiotics were the most effective for eliminating *Agrobacterium*.

3.5 CONCLUSIONS

The agar diffusion assay results illustrate that tetracycline and spectinomycin have shown highly effective antibacterial activity respectively against *A. tumefaciens* containing vector pTF 101 and Ω PKY. These two antibiotics, in addition to hygromycin, rifampicin and streptomycin, will be tested in *in vitro* soybean transformation. The results obtained in this study further show that there is satisfactory inhibitory evidence in relation to the type and concentration of antibiotics to be tested for routine transformation purposes. There was a positive correlation between the three concentrations (150, 300 and 500 mg/l) of hygromycin used on both strains. These correlations suggest that further assays can be done to determine an appropriate concentration that may be used efficiently for antibacterial activity against *Agrobacterium.* The overall results shows that some of the antibiotics, not regularly used for the suppression of *Agrobacterium* may also be recommended and tested during *Agrobacterium*-mediated transformation.

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CHAPTER 4

IN VITRO AND *IN VIVO AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION IN SOYBEAN

4.1 INTRODUCTION

Soybean is one of the world's most important crops producing oils and proteins, with health and nutritional benefits. Various reports have established that most of the soybean components have beneficial nutritional and health effects as characterised by its potential to prevent diseases such as prostate cancer, heart disease and osteoporosis (Dixit et al., 2011; Sugano, 2006). However, the growth of this crop is adversely affected by environmental factors, and one of the main research focuses is to develop soybean plants resistant to drought, which will contribute to the increase in yield and nutritional quality of soybean.

Drought is considered the most damaging to soybean when compared to other tropical legumes such as *Vigna unguiculata*, and *Phaseolus vulgaris*. Drought causes a significant loss of yield in soybean as a result of provoking stunted growth, and promoting flower abortion. The impact of drought stress on soybean plant growth, and development can be circumvented by artificially transferring gene(s) that can confer tolerance to drought using transformed *Agrobacterium tumefaciens*. Such genes include the *oc 1* gene (*Oryza* cystatin 1) originating from rice (*Oryza sativa*). The *oc 1* gene confers resistance by inhibiting stress induced processes that cause premature senescence. However, an improved transformation protocol is required for efficient transfer and integration of this gene into soybeans as well as the production of fertile transgenic plants.

The production of stable transgenic soybeans continues to be a challenge as shown by its low rates or frequencies of transformation obtained. Reports such as those of Donaldson and Simmonds (2000); Paz et al. (2004) and Liu et al. (2008) have shown that, the low recovery rates of transgenic soybean are attributed to poor explant response, genotype, and inefficient regeneration protocols. *Agrobacterium*-mediated genetic transformation using immature cotyledons (Ko et al., 2003; Yan et al., 2000) and axillary meristematic tissues (Olhoft and Somers, 2001; Zeng et al., 2004) as explants obtained from soybean seedlings have been the predominantly tested methods due to the explants' morphogenetic potentiality. Earlier research by Somers et al. (2003), Paz et al. (2006) on soybean transformation attempted to improve *in vitro* shoot regeneration and transformation rates. However, genetic transformation of

soybean is still difficult to achieve because of the number of factors such as those mentioned above (Yan et al., 2000; Yi and Yu, 2006). *In vitro* soybean transformation has also been found to be more prone to the problem of somaclonal variation than *in vivo* transformations. On the other hand, non-tissue culture genetic transformation systems were reported to have worked successfully for *Arabidopsis* and cotton improvements, but not for recalcitrant legume crops such as soybean (Bent, 2000; Keshamma et al., 2008).

The lack of a feasible regeneration system for soybean plants via both *in vitro* and *in vivo* methods constantly results in very low and inefficient transformation frequencies that are being reported (Zhang et al. 1999; Paz et al. 2004; Asad and Arshad, 2011). This stimulates the search for an efficient transformation method that will produce fertile transgenic soybeans.

4.1.1 Motivation for the study

The current state of agricultural technology must be improved to meet the production challenges faced by the international community (Abah et al., 2010). *In vitro* regeneration and genetic transformation of plants needs to be adopted for the enhancement of medicinally or nutritionally important crop plants. Improvements can be achieved by optimisation of plant transformation systems that are used for genetic transformation of recalcitrant crops such as soybean. The World Health Organisation (WHO, 2005) has estimated that 20% of the world population primarily relies on home grown crops as their food source. This gives clear indication that crop plants are not only used for commercial purposes. Gandhi (2009) and Lee et al. (2012) also outlined the domestication of soybean for feed, food, forage, fibre, industrial and medicinal use in addition to the proprietary production of this crop.

Traditional meals produced by most Asian home growers of soybean include tofu, pehtze and sufu (Han et al., 2009). This shows the wide exploitation of soybeans in addition to industrial production of pharmaceutical products from their secondary metabolites. The pharmaceutical products manufactured include lecithin, tocopherols and saponins. Such abundant active metabolites are utilised to promote and maintain

human health. Tripathi and Tripathi (2003) have shown that approximately one quarter of prescribed drugs contain plant extracts or their active ingredients, obtained from genetically modified or transformed plants. The most popular is aspirin, derived from transformed species of *Salix* and *Spiraea*, and some of the valuable anti-cancer agents such as vinblastine are also derived from plant sources.

4.2 LITERATURE REVIEW

4.2.1 Importance of soybean as a food crop

The world population reached 6.6 billion in 2006, from 6 billion in 1999 as estimated by the Population Reference Bureau (PRB, 2006). The increase to the 8 billion mark has been projected for the year 2025. The highest increase in population is expected to occur in the developing countries (Sharma et al., 2001). In order for the countries to meet the needs of the increasing populations, FAO (1999) estimated that at least a 60% increase in food production must be achieved. The increase is required to accommodate the estimated population growth, to close nutrition gaps and also to meet dietary needs seen in developing countries. More than 800 million people in the world do not have enough food to eat.

Starvation causes 2400 deaths every day worldwide, of which three quarters are children under the age of five. About 33% of children under five in the developing countries have also experienced stunted growth. This report suggests that, there is a negative impact on overall health and intellectual development, as a result of undernourishment (UN, 2000). In a similar report (Sharma et al. 2001) further indicated that, nearly 70% of poor and food insecure people live in rural areas, especially areas found in Africa. This is in countries where the major cause of poverty and food insecurity as well as poor nutrition is attributed to low productivity in agriculture. Particularly in countries where agriculture is the driving force for broad-based economic growth and poverty alleviation (Wambugu, 1999).

4.2.2 Drought stress

Drought stress is defined as the absence of rainfall or irrigation for a period of time sufficient to deplete soil moisture and injure the plant (Kikuta, 2007). It results when loss of water exceeds the ability of roots to absorb water. In this case, plant water content will be reduced enough for plant not to sustain normal plant processes. This means that a decrease in water content will not support plant cell and tissue development (Yordanos et al., 2003). Drought has been found to be a major limitation to crop yields. It is said to be the most important environmental factor influencing crop yield losses, particularly in legume growing fields (De-Bruin and Pedersen, 2009).

Drought affects production in legumes by interfering with symbiotic fixation of atmospheric nitrogen (N_2) in all plants (Mundree et al., 2010). In addition, it causes yield losses as a result of the decrease in CO_2 assimilation and leaf area development (Hopkins, 1999). Sensitivity of soybean to drought or soil water deficit, even to modest water levels is very high. The basis for this sensitivity to water deficit has been shown to be associated with nodulation. Drought stress further increases soybean susceptibility to weeds, insects and diseases. As drought will continue to limit crop production, the prediction is that it will continue to be the major abiotic factor that is likely to affect crop yields globally (Lobato et al., 2008).

4.2.3 Plant tolerance to drought

Drought conditions that are constantly occurring in most parts of the world necessitate the development of plants that can grow during increasing environmental fluctuations (Bhat and Srinivasan, 2002). Xu and Chye (1999) provided evidence that an *oc 1* gene from rice can be expressed in response to stresses such as wounding, cold and drought, as well as programmed cell death. This gene can be transferred to other plant species, such as soybean to confer tolerance to drought stress by coding for enzyme inhibitors that will inhibit or supress protein enzymes induced due to drought stress (Balch et al., 1996). Cysteine proteases that are induced during drought stress can cause degradation of essential proteins, and thus resulting in death of the plant. The cysteine proteases production can be inhibited by the *oc 1*-

coded cysteine proteinase inhibitors. These inhibitors probably evolved from a cognate ancestral gene that is believed to have formed the cystatin superfamily which was classified on the basis of their molecular structure (Prins et al., 2008). They were characterised by their molecular weight (MW) that ranges from 10,000 to 20,000 Daltons as stefin or cystatins. They have a conserved amino acid sequence (shown below) or its homologues believed to be involved in the inhibition of cysteine proteinases. Almost all members of the cystatin superfamily are characterised by the presence of the following highly conserved segment: Glu ─ Val ─ Val ─ Ala ─ Gly (Habib and Fazilli, 2007).

4.2.3.1 Proteolytic enzymes

The biochemical degradation of cell proteins through hydrolysis of peptide bonds is caused by the action of proteolytic enzymes. They are a group of enzymes that break long chains of proteins into shorter fragments (peptides) and amino acids, and are also called proteinases. These enzymes are responsible for the protein metabolism required for the life cycle of plants. They were also found to be associated with the occurrence of cell death during senescence of organs and cell differentiation (Mashamba, 2010). The Proteolytic enzymes are activated under stress such as drought, and act on proteins in the cell resulting in premature cell death. The inhibition of these enzymes can lead to a delayed cell death. They involve two groups of enzymes, such as exoproteases and endoproteases, which act on the exterior and interior parts (respectively) of the protein substrate terminal structures or at the point where they break the peptide chain. They are grouped into aspartic, cysteine, metalloid and serine proteases according to their essential amino acid residues at the active sites. Their grouping or classification also depends on the pH optimum range of their activity, and the sequence homology of their amino acid residues (Laskowski and Kato, 1990).

4.2.3.2 Proteinase inhibitors

Proteinase inhibitors are enzymes that inhibit the activity of cysteine proteinases, (also known as cysteine inhibitors) that are found and widely distributed among plants. They are subdivided into three families (stefin, cystatin and kininogen) based

on their sequence homology; the presence and position of intra-chain disulfide bonds; and the molecular mass of the proteins (Turk and Bode, 1991). Stefin family consists of about 100 amino acid residues and has a molecular mass of about 11 kDa. They do not contain disulfide bonds or carbohydrate groups. Kininogens are composed of larger glycoproteins, and range from 60 to 120 kDa. Cystatins contain four conserved cysteine residues forming two disulfide bonds, and most of them are not glycosylated (Grzonka et al., 2001).

4.2.4 Improvement of soybean for yield increase

Emerging technologies rapidly adopted in agricultural research has increased soybean yields. World estimates already published (1980–2000) of annual gain in soybean yields have been attributed to genetically improved soybean that averaged at about 15 kg ha^{-1} to 30 kg ha^{-1} per year in both public and proprietary sectors (FAO, 2012). The estimated yields take into account the amount of soybeans produced that include both the quantities of the commodity sold in the market, and the quantities consumed or used by the producers. The harvesting losses, threshing losses, and unharvested portions of the crop are also considered (Seiter et al., 2004).

In order to achieve maximum yields, reducing plant stress caused by insect, pathogens, and drought is critical. Understanding how soybeans develop throughout their growing season and how genetic varieties determine the potential yields can provide insight into the selection of the best practices that lead to maximum yield (Pedersen, 2010). Therefore, the use of *in vitro* and *in vivo* tissue culture can assist in this regard, with the production and selection of high yield varieties that have better agronomic traits that match the ever-changing stresses in specific environments. As demand increases for soybean oils and proteins, the improvements of soybean quality, and production through genetic transformation and functional genomics becomes an important task throughout the world (Wang and Xu, 2008).

4.2.4.1 Increasing yields via conventional breeding

Conventional breeding strategies are still very important for the genetic improvement of crops. However, breeding of soybean remains a major challenge; due to the fact

that it is a self-pollinating crop and the genetic diversity of the currently used cultivars is quite narrow (Sudaric et al., 2010). Most of the currently used soybean genotypes have been derived from common ancestors, limiting breeding strategies to produce more genetically improved soybean cultivars. The challenge carrying out breeding is that, factors responsible for yield improvements are not clearly identified, and the desirable lines selected for future cultivars are based on yields screened for a very long period of years and across locations (Pedersen, 2010).

The challenge of not clearly identifying traits has led to failure to appropriately explain yields increases through cultivar development in terms of specific yield components. These have led to various studies, such as that of Cui and Yu (2005) reporting mixed or contradicting results. Their reports contradictorily demonstrate different individual factors being directly attributed to the yields of soybean currently obtained. De-Bruin and Pedersen (2009) compared old and new cultivars, and indicated that yield improvements are more strongly related to seed production m^{-2} canopy per year than seed size or seed mass. Other factors reported were seed pod number, plant size, and determinate and indeterminate growth of soybeans (Board and Kahlon, 2011).

4.2.4.2 Improvement via genetic transformation

Problems associated with conventional breeding (described above) brings the role of genetic transformation into play, considering its ability to circumvent the shortcomings of sexual reproduction, and rapid regeneration of fertile crops from sterile and vegetatively propagated crops (Sinclair et al., 2007). The most commonly used methods for soybean transformation are particle bombardment of shoot meristems and *Agrobacterium*-mediated transformation of cotyledonary nodes (Trick et al., 1997). According to reports (Finer and McMullen, 1991; Liu and Wei, 2005) so far published, these protocols have room for improvements since they have shown some limitations. Other techniques such as electroporation, silicon carbide fibres and liposome-mediated transformation are not optimised for soybean and are less efficient for this plant (Rech et al., 2008; Asad and Arshad, 2011).

4.2.4.2.1 *Agrobacterium*-mediated genetic transformation of soybean

Agrobacterium-mediated genetic transformation is a tool which was developed in the early 1980s by Cheng et al. (1987) to transfer vectors with specific regions of DNA into plant cells. The method involves the use of *Agrobacterium* species: *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. The technique was first established by Hinchee et al. (1988), reporting the first recovery of transgenic soybean plants using cotyledonary node explants obtained from soybean cultivar Peking. Their experiment achieved only 6% of recovered shoots that were transgenic. *Agrobacterium*-mediated transformation serves as a method of choice for plant transformation because of the low copy numbers of DNA transferred, the defined integration of transgenes, and its capacity to integrate foreign genes into the transcriptionally active chromosomal regions of the host plant's genome. Furthermore, the method is simple and affordable, and has the potential to increase transformation efficiency of soybean since it is easy to optimise using organic supplements such as L-cysteine, dithiothrietol, and acetosyringone to reduce its genotype specificity to soybean cultivars (Zhang et al., 1999; Zeng et al., 2004; Yi and Yu, 2006).

4.2.4.2.2 *In vitro A. tumefaciens*-mediated transformation

The advancement of *in vitro* soybean transformation appears to be very gradual compared to some of the recent improvement in corn, and other cereal transformations (Cao et al. 2009). There are indications that, there is a need for improvement on transformation culture conditions to promote regeneration, and recovery of transformed plants before a high number of transgenic lines can be obtained (Olhoft and Somers, 2001). If these problems and low transformation frequencies are not controlled, they will severely limit the utilisation of *in vitro* tissue culture protocols for crop yield improvement, and genetic studies (Paz et al., 2006). Kumudini et al. (2008) compared new *in vitro* genetically modified soybean cultivars with older ones. The newly improved cultivars consistently maintained their yield advantage over the older cultivars when grown under shade and nitrogen (N) limiting conditions. Recently, Wang et al. (2010) demonstrated the use of new *in vitro* genetic transformation strategies to develop high yielding, and high quality soybean varieties.

All developments were aimed at transferring beneficial agronomic genes, and allow the regenerated plants to have desirable combinational properties for the purpose of increasing crop yield. *In vitro* research still illustrates the great difficulty in translating research at the basic level into improvement of crop yield. However, there are reports (Donaldson and Simmonds, 2000; Paz et al., 2004; Wang and Xu, 2008) that certainly showed progress, and have already contributed in the yield increase of soybeans. These successes provide a great insight in key elements that are required to achieve the great success in developing cultivars amenable for consistent increase in soybean yield (Richards, 2000).

4.2.4.2.3 *In vivo A. tumefaciens*-mediated transformation

Recent reports (Bent, 2000; Mello-Farias and Chaves, 2008) indicated that challenges encountered in soybean transformation are predominantly caused by the difficulties in plant regeneration, and low transformation efficiency in tissue culture. The development of an *in vivo* transformation (also known as *in planta* transformation) method that avoids tissue culture approach for achieving high transformation frequencies can be used as an alternative. This method may eliminate the constraints of genotype specificity; tissue culture derived genetic variations, and allows for the reduction of the number of infertile transgenic plants regenerated (Liu et al., 2009). A number of researches such as those of Trieu et al. (2000), Zhang et al. (2006) used *Agrobacterium*-mediated *in vivo* floral dip transformation, and successfully applied it in the model plant *Arabidopsis thaliana* and a legume plant *Medicago truncatula*.

Naseri et al. (2012) also reported the transfer of resistant genes which confers immunity to the fungal sheath blight disease caused by *Rhizoctonia solani* Kuhn on rice using *in vivo* method. Similar reports were documented on tomato (*Solanum lycopersicum* L.) and sweet potato (*Ipomoea batatas*), also using floral dip *Agrobacterium in vivo* transformation (Xing et al., 2008; Yasmeen et al., 2009). Ortiz (1998) indicated that improving and targeting specific genotypes for cropping systems may be facilitated by understanding specific gene-environment interactions with the help of molecular research. New soybean varieties with increased yield and seed quality can be obtained through multidisciplinary co-operation among breeders,

biotechnologists, and other plant scientists. This will widen the genetic diversity, preservation of lines, and the increase in crop production that can be achieved by an integration of biotechnology tools such as genetic transformation in conventional breeding (Manshardt, 2004).

4.3 PURPOSE OF THE STUDY

4.3.1 Aim

To evaluate the efficiency of *in vitro* and *in vivo Agrobacterium tumefaciens*-mediated genetic transformation on soybean for successful production of transgenic plants.

4.3.2 Objectives:

In vitro transformation of soybean

i. Germinate soybean seeds *in vitro* on MS basal medium supplemented with 4.00 mg/l BA to obtain seedlings for preparation of double coty-node explants.
ii. Infect and co-cultivate soybean explants with *A. tumefaciens* transformed with the *oc-1* gene.
iii. Carry out shoot induction from co-cultivated explants on MS medium containing 2.00 mg/l BA.
iv. Test the efficiency of selected antibiotics on the elimination of excess *A. tumefaciens* on *in vitro* culture.
v. Select the transformed shoots on MS medium containing 6.00 mg/l Glufosinate-ammonium salt.
vi. Elongate, root and acclimatise the regenerated transformed plants.

In vivo transformation of soybean

i. Germinate *in vivo* soybean seeds imbibed in a solution containing 2.00 mg/l BA to obtain seedlings for genetic transformation.
ii. Infect seedlings by injecting *A. tumefaciens* at the cotyledonary axil.

iii. Induce axillary shoot formation by excising off the epicotyls at the cotyledonary junction.
iv. Select transformed plants by watering the infected seedlings with Hoagland solution containing glufosinate as a selectable agent.

4.4 MATERIALS AND METHODS

4.4.1 Plant material for both *in vitro* and *in vivo* transformation

Soybean [*Glycine max* (L.) Merrill cultivar 677] seeds were used in this study. The seeds were purchased from Link Seed South Africa (Link Seed SA).

4.4.2 *In vitro* transformation of soybean

4.4.2.1 Aseptic conditions

All experiments for *in vitro* transformation were performed under aseptic conditions in a specialised laboratory for bacterial and plant tissue culture. All *in vitro* plant tissue culture experiments were performed in a specialised laboratory with designated preparation, inoculation and growth rooms for incubation of cultures under controlled conditions. The working areas were kept clean at all times to ensure sterile cultures and reproducible results. All aseptic procedures were performed in a laminar flow cabinet and the working area was disinfected with 70% ethanol before and during the performance of each experiment.

a) Sterilisation of instruments and culture media

All bacterial and plant culture media, glassware (beakers, test tubes etc.), and instruments (scalpels and forceps) were sterilised by autoclaving for 20 minutes at 121°C in the respective laboratories. The instruments were again flame sterilised with 96% ethanol prior to use for excisions and inoculations during culture.

b) Seed sterilisation

The seeds were first washed with soapy water, rinsed with running tap water for 5–10 minutes and dried with a clean tissue paper. Washed seeds were placed in two (90 × 15 mm) Petri dishes and each Petri dish contained 90 seeds. Petri dishes with seeds were placed inside a desiccator with their lids half-open. Concentrated hydrochloric acid (3.5 ml) was added drop-wise into the beaker containing domestic bleach placed in the desiccator to generate chlorine gas. The desiccator was closed and the seeds were then sterilised using the generated chlorine gas for 16 hours.

4.4.2.2 Bacterial culture

Agrobacterium tumefaciens bacterial culture, with vector pTF 101 vector containing the *oc 1* gene construct was used. This is a derivative of a base vector pTF 101.1 from strain EHA 101 obtained from the Forestry and Agricultural Biotechnology Institute (FABI). The strain was maintained on medium composed of yeast extract (5 g/l), peptone (10 g/l), NaCl (5 g/l), and Bacto-agar (12 g/l). The pH of the medium was adjusted to 7.0 with 0.1 M NaOH. Selective antibiotics kanamycin (50 mg/l) and spectinomycin (100 mg/l) were added to the medium after autoclaving.

4.4.2.3 Plant tissue culture media

4.4.2.3.1 Culture media composition

a) Macro and micro-nutrient stock solutions

Two basal media; Murashige and Skoog and Gamborg's B5 media were used in this experiment. The media were prepared as described by Pierik (1997) and Zhang et al. (1999). All macro and micro-nutrients were prepared as stock solutions as presented on Table 4.1 and stored at 5°C.

b) Vitamin stock solutions

The vitamin stock solutions were prepared by dissolving the respective amounts of vitamin powders indicated in Table 4.1 in 100 ml distilled water, and stored at -20°C. All stock solutions were thawed and mixed well before use.

c) Plant growth regulator stock solutions

Stock solutions (mg/ml) of different plant growth regulators (PGR) were prepared by first dissolving the respective amount of benzyl adenine (BA), gibberrelic acid (GA_3) and indole acetic acid (IAA) in few drops of 1N sodium hydroxide or dH_2O, after which distilled water was added up to the final volume of the stock solution. Due to thermo lability GA_3 was filter sterilised prior to addition to sterile culture media. The stock solutions were stored at -20°C.

4.4.2.3.2 Culture media preparation

All media were prepared by pipetting the required volumes of stock solutions (Table 4.1) into a 1liter glass beaker filled with 500 ml dH_2O. A 30g of sucrose was dissolved into the medium and the pH was adjusted to 5.8 using 0.5 M NaOH and the final volume of 1liter was made with distilled water. The basal media were supplemented as indicated on Appendix 4.1.

4.4.2.4 Soybean seed germination

Sterilised (as described in 4.4.2.1 b) soybean seeds (total of 40 seeds per experiment) were inoculated in culture bottles (3 per bottle) containing MS medium supplemented with 4 mg/l BA. Hormone free MS basal medium was used as a control. The culture bottles were incubated under controlled conditions as indicated in 4.4.2.12. Seed germination was recorded as the protrusion of the radicle on a daily basis for 14 days.

Table 4.1 Chemical compositions and preparation of basal MS and Gamborg's B5 media according to Pierik (1997) at 20 × dilution concentrations.

Nutrients	**Murashige and Skoog culture medium**		**Gamborg's B5 culture medium**	
	Concentration (mg/l stock solution)	Volume (ml) of stock solution/1l culture medium	Concentration (mg/l stock solution)	Volume (ml) of stock solution/1l culture medium
Macronutrients				
NH_4NO_3	33000	50	---	---
$(NH_4)_2SO_4$	---		134	10
KNO_3	38000		2500	10
$CaCl_2.2H_2O$	8800		150	10
$MgSO_4.7H_2O$	7400		250	10
KH_2PO_4	3400		---	---
$NaH_2PO_4.H_2O$	---		150	10
Micronutrients				
KI	16.6	5	15.0	1
H_3BO_3	124.0		60.0	
$MnSO_4.4H_2O$	446.0		200	
$ZnSO_4.7H_2O$	172.0		40.0	
$Na_2MoO_4.2H_2O$	5.0		5.0	
$CuSO_4.5H_2O$	0.5		0.5	
$CoCl_2.6H_2O$	0.5		0.5	
Iron source				
$FeSO_4.7H_2O$	556.0	5	556.0	10
$Na_2EDTA.2H_2O$	746.0		744.0	
Organic supplements				
Vitamins				
Glycine	2.0	5	---	1
Myo-inositol	100		100	
Nicotinic acid	0.5		5.0	
Pyridoxine	0.5		1.0	
Thiamine-HCl	0.1		10.0	
Sucrose	30		30	
Gelling agent				
Gelrite	3.0		---	
Noble agar	---		7.0	

4.4.2.5 Explant preparation

Uniform seedlings obtained from germination cultures were used to prepare double coty-node explants by aseptically excising off the epicotyls and cutting the hypocotyls approximately 6–8 mm beneath the cotyledons. Incisions were made on the adaxial surface of the cotyledons, at the embryogenic axil.

4.4.2.6 Infection of explants with *A. tumefaciens*

Pilot studies were done with the two strains (Ω PKY and pTF 101) and based on the study in chapter 3 pTF 101 was selected for *in vitro* transformation of soybean. The explants were immersed completely in liquid infection medium composed of B5 basal medium supplemented with GA_3 (0.25 mg/l), BA (2.00 mg/l) and acetosyringone (40 mg/l) (Appendix 4.1), and *A. tumefaciens* inoculum transformed with pTF 101 vector. The explants were incubated in the infection liquid medium for 30 minutes with occasional agitation.

4.4.2.7 Co-cultivation of infected explants

After infection, the explants were co-cultured with the *Agrobacterium* in 90×15 mm Petri dishes containing Gamborg's B5 co-cultivation medium, supplemented with 0.15 g/l MES (pH 5.4) and solidified with 4.25 g/l bactoagar before autoclaving. The co-cultivation plates were first overlaid with sterile Whatman no. 1 filter paper. The cotyledonary explants were transferred (10 per Petri dish) to the medium orientated in such a way that the adaxial surface of explants was touching the filter paper. The plates were wrapped with a parafilm and incubated under controlled conditions (4.4.2.12) for 4 days.

4.4.2.8 Shoot induction

Induction of transformed shoots from the infected double coty-node explants was carried out in two stages using SIM 1, SIM 2 and SIM 3 culture media (Appendix 4.1). Three shoot induction media (SIM 1–3) composed of MS basal medium were supplemented with 2.00 mg/l BA and various concentrations of antibiotics as

indicated on Appendix 4.1. The antibiotics and their respective concentrations used for the elimination of excess *A. tumefaciens* containing the vector pTF 101 and Ω PKY were based on the results obtained from the antibiotic sensitivity tests described in chapter 3.

a) Shoot induction stage I

Prior to inoculation for shoot induction, co-cultivated double coty-node explants were washed with shoot washing medium composed of B5 salts supplemented with the three different antibiotics as indicated on Appendix 4.1 for elimination of excess *A. tumefaciens*. Following washing explants (two per culture bottle) were inoculated by slightly embedding the hypocotyls on each of SIM 1, SIM 2 and SIM 3 media supplemented with 2 mg/l BA and various concentrations of antibiotics as indicated in Appendix 4.1. Explants cultured on MS medium without BA, antibiotics and glufosinate were used as a control. All cultures were incubated under controlled conditions (4.4.2.12) for 14 days.

b) Shoot induction stage II

After 14 days of culture, the initially developed primary shoots from stage I were cut-off and discarded. The explants were then subcultured on fresh SIM 1, SIM 2 and SIM 3 media prepared as described in 4.4.2.8 b, containing 2 mg/l BA and supplemented with 6 mg/l glufosinate-ammonium salt. The explants were oriented in such a way that the hypocotyls were slightly imbedded into media. The glufosinate-ammonium salt was used as a selective agent for identification of transformed shoots from nontransformed shoots which will not survive in the media. The cultures were incubated in the culture room for over 14 days as described in 4.4.2.12.

4.4.2.9 Shoot elongation

After four weeks of incubation the shoots were excised from the explants and subcultured on elongation MS medium containing 0.6 mg/l GA_3, and MS medium without GA_3. The medium was supplemented with 6 mg/l glufosinate-ammonium salt,

antibiotics (as for shoot induction media), and cultures were incubated as indicated in 4.4.2.12 for four weeks.

4.4.2.10 Rooting of elongated shoots

Elongated shoots were rooted on MS medium without plant growth regulators and supplemented with 6 mg/l glufosinate-ammonium salt. The cultures were maintained under growth conditions as in 4.4.2.12.

4.4.2.11 Plant acclimatisation

In vitro rooted plantlets (40-60 mm in height) were transferred into 150 ml culture bottles quarter-filled with sterile vermiculate and covered with transparent plastic bags. The plastic bags were punctured and gradually opened to allow the plants to acclimatise. Plants were watered daily with distilled water depending on the moisture content and once a week with modified Hoagland nutrient solution (Epstein, 1972). Acclimatised plants were transplanted into plastic pots (10–15 cm), and maintained under controlled growth conditions as indicated at 4.4.2.12 below, until flowering and fruit pods maturity.

4.4.2.12 Growth conditions

All *in vitro* cultures were kept in a growth room at 24±2°C and 16:8 hour photoperiod of 50–60 µmol $m^{-2}s^{-1}$ light intensity. The regenerated plants were acclimatised in a growth room at 24±2°C and 150–200 µmol $m^{-2}s^{-1}$ light intensity with 16:6 hour photoperiod.

4.4.2.13 Data analysis

The experiments were performed three times, with 15 replicates per treatment, and the data on seed germination and shoot induction collected on weekly basis was analysed using SPSS software version 21 package for ANOVA.

4.4.3 *In vivo* soybean transformation

4.4.3.1 Plant material and bacterial culture

Soybean seeds, cultivar LS 677 (as indicated at 4.3.1) were used in this study. The genetic transformation of soybean was carried out using a strain of *A. tumefaciens* which contained the Ω PKY vector.

4.4.3.2 Seed sterilisation, imbibition and sowing for germination

Sterile (as indicated in 4.4.2.1 b) soybean seeds were imbibed overnight for 12 hours in sterile dH_2O containing 2.00 mg/l BA. Seeds imbibed in sterile distilled water without hormones served as a control. The imbibed seeds were then inoculated for germination in culture bottles to minimise contamination, quarter-filled with sterile vermiculate (Figure 4.1), and then sown under controlled conditions (as described in 4.4.3.7) for 15 days. A total of 90 replicates (3 seeds per bottle) were used for each treatment, and the experiment was repeated three times. Seed germination was recorded as the protrusion of the epicotyl.

Figure 4.1 Soybean seeds (imbibed in 2 mg/l BA) sown in sterilised vermiculite for seed germination and seedling development.

4.4.3.3 Preparation of *A. tumefaciens* inoculum and plant infection

The *Agrobacterium* culture was grown on solid yeast extract peptone (YEP) medium containing 50 mg/l rifampicin and 10 mg/l tetracycline. Bacterial inoculum was prepared by allowing a 2 ml culture of *Agrobacterium tumefaciens* containing the Ω PKY vector construct to grow to saturation (for 16–24 hours) at 28°C in a shaker incubator (145 rpm). The *A. tumefaciens* was then collected by centrifugation at 3,500 rpm for 10 minutes at 20°C and the pellet was resuspended in infection liquid medium composed of 2.00 BA mg/l, 0.6 mg/l GA_3 and 40 mg/l acetosyringone. Ten days old soybean seedlings were infected with the *Agrobacterium* suspension. The infection was carried out by injecting the seedlings at the cotyledonary junction with the suspension of *A. tumefaciens* of the strain indicated above.

4.4.3.4 Shoot induction

Infected seedlings were immediately watered with an osmoticum (1.0 M NaCl) solution to prevent the “leak out” of the inoculum. They were then flushed-out with a half-strength Hoagland’s solution to remove excess osmoticum. New shoots were initiated by excising the epicotyls at the cotyledonary axil, after 10 days of incubation. The plants were further incubated in a growth room (4.4.3.7) for 15 days.

4.4.3.5 Screening of transformants

The plants with newly initiated shoots were watered weekly with Hoagland solution containing 6 mg/l glufosinate-ammonium salt selective agent for selection of transformants. Non-transformed plants were used as a control, and all plants were watered with Hoagland’s solution on a daily basis depending on the moisture content. The culture bottles were then covered with transparent plastic bags and maintained in the growth room at 24±2 °C, at a light intensity of 150–200 µmol $m^{-2}s^{-1}$ with 16:8 hour photoperiod as indicated at 4.4.3.7 for three weeks.

4.4.3.6 Plant acclimatisation

After three weeks of incubation, the plastic bags were gradually removed from the culture bottles to allow the plantlets to acclimatise. The plantlets from both the control and the treatments (80–120 mm height) were then transplanted into plastic pots (15 cm pots). Plants were further incubated in the same growth conditions as mentioned above, and watered with Hoagland's solution containing glufosinate-ammonium salt and sterile distilled water on a daily basis.

4.4.3.7 Culture conditions

All *in vivo* cultures for germination and seedling development were kept in a culture room at 24±2°C and 16:8 hour photoperiod of 50–60 µmol $m^{-2}s^{-1}$ light intensity. Plantlets were acclimatised under controlled conditions at 24±2°C and 16:8 hour photoperiod of 150–200 µmol$m^{-2}s^{-1}$.

4.4.3.8 Data analysis

All experiments were performed three times with 30 replicates per treatment, and data was statistically analysed using one-way analysis of variance (ANOVA) in SPSS program version 21.

4.5 RESULTS AND DISCUSSION

4.5.1 *In vitro* transformation of soybean

4.5.1.1 Seed germination

More than 80% seed germination was recorded within 10 days of culture. Seeds on PGR free medium reached a maximum germination of 77% after 13 days, while on BA (2.00 mg/l) containing medium germination reached a maximum of 95% on the 14^{th} day of incubation (Table 4.2). Soybean seedlings developed from seeds germinated on MS medium with BA had enlarged cotyledons, with thicker and shorter hypocotyls (4–5 mm), and without lateral roots. In contrast, control seedlings had thin

Table 4.2 Cumulative germination percentage of soybean seeds cultured on MS medium with and without 2 mg/l BA.

Day	Percentage germination (%)	
	MS control	MS + BA (2.00 mg/l)
1	0^{f}	0^{g}
2	0^{f}	0^{g}
3	0^{f}	43^{f}
4	23^{e}	46^{f}
5	23^{e}	46^{f}
6	35^{d}	56^{e}
7	48^{c}	68^{d}
8	48^{c}	68^{d}
9	65^{b}	75^{c}
10	66^{b}	82^{b}
11	76^{a}	86^{b}
12	76^{a}	93^{a}
13	77^{a}	93^{a}
14	77^{a}	95^{a}
15	77^{a}	95^{a}

Values within columns followed by the same letters are not significantly different at the 5% confidence level according to ANOVA.

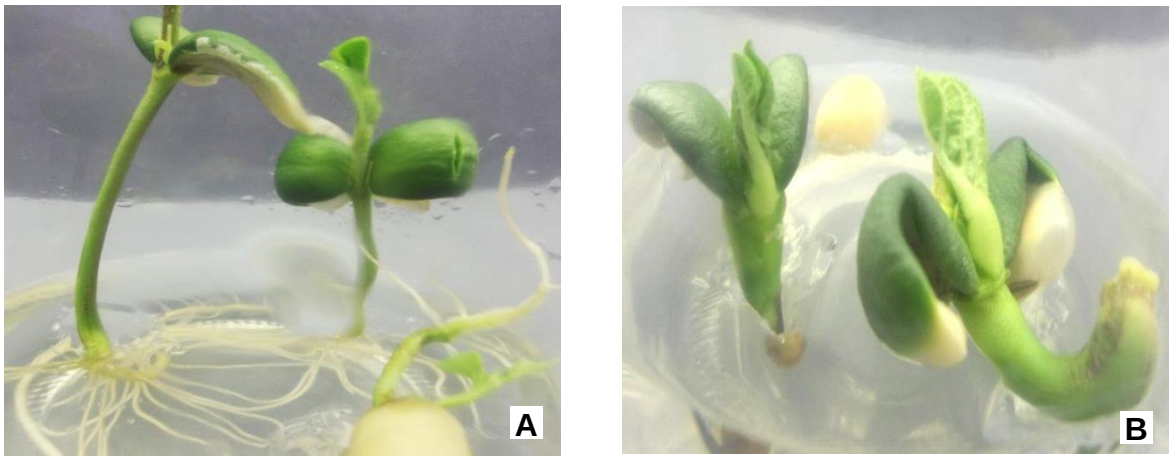

Figure 4.2 Seedlings developed on MS medium supplemented with 4.00 mg/l BA and without BA (control) after 15 days. (A) Seedlings in the control medium showing slender stems and long primary root with lateral roots. (B) Seedlings derived from seeds pre-treated with 4.00 mg/l BA producing thicker stems and reduced roots.

hypocotyls (1–2 mm in diameter) and with lateral or secondary roots (Fig 4.2). The *in vitro* germination of seeds on culture media containing hormones has been reported earlier in many other legumes such as cowpea, as well as chick pea and lentil (Malik and Saxena, 1992; Chaudhury et al., 2007). Their studies showed that 80–90% of seeds germinated, and maintained the structural integrity of the seedlings as compared to the control medium with typical slender bean seedlings. Addition of plant growth regulator to the germination medium was found to be beneficial in the production of explant sources suitable for shoot induction also as described by Raveendar et al. (2009).

4.5.1.2 *Agrobacterium tumefaciens*-mediated transformation

The results in chapter 2 have shown that the double coty-nodes are suitable explants in multiple shoot induction, and can be used for subsequent *Agrobacterium*-mediated transformation. In general, the double coty-node explants infected with *Agrobacterium* during co-cultivation showed considerable swelling, with little formation of callus within the first few days of incubation. Necrotic tissues were observed within two weeks of culture in some of the double coty-node explants, except in the control. The results of average number of shoots induced on different shoot induction media (SIM 1–3) supplemented with the selected antibiotics for elimination of *A. tumefaciens* overgrowth, and glufosinate as a selectable marker of regenerated shoots are shown on Table 4.3.

4.5.1.2.1 Explants co-cultivation with *Agrobacterium tumefaciens*

A. tumefaciens with pTF 101, was selected as a vector for *in vitro* transformation of soybean due to better re-initiation capacity than the vector Ω PKY. The *Agrobacterium* containing pTF 101 vector did not show any signs of resistance to the antibiotics used. Reports such as that of Hammerschlag et al. (1997) indicate that strain selection is of particular importance in *Agrobacterium*-mediated transformation, since the efficiency of transformation is also dependent on the strain of *A. tumefaciens* used. Furthermore, in another study by Maheswaran et al. (1992) emphasis was placed on the influence of hypervirulent strains for infection of recalcitrant plant species, and dependence on plant genotypes. The report

suggested that strain LBA 4404 is the strain of choice for apple transformation studies, because in contrast to strains derived from EHA 101 it could be effectively eliminated from explants using low concentrations of antibiotics.

4.5.1.2.2 Shoot induction on infected coty-node explants

Based on the study in Chapter 2, the double coty-node explants were found to be more responsive to *in vitro* multiple shoot induction as compared to single coty-node explants, resulting in highest frequencies of shoot regeneration (Table 2.2). Furthermore, the same results have shown that 2.00 mg/l BA hormonal concentration was the best for multiple shoot induction as compared to results on chapter 2.

Table 4.3 Shoot induction in media containing different antibiotic concentrations.

Culture media	Antibiotics	Concentration (mg/l)	Frequency of regeneration (%)	Mean no. of shoots/ explant
SIM 1	Hyg	500	76.6[a]	4.86[a]
SIM 2	Tet	500	63.3[b]	7.27[b]
SIM 3	Rif Tet	10 50	60.0[b]	3.80[a]
Control	0	0	0	1.3

Values within columns followed by the same letters are not significantly different at the 5% confidence level according to ANOVA. Antibiotics: hygromycin (Hyg), tetracycline (Tet) and rifampicin (Rif) are shown. Frequency of regeneration percentage= (no. of explants with 3 or more shoots/ total no. of explants) × 100.

The multiple shoots obtained from double coty-node explants infected with *A. tumefaciens* showed active growth during early days of culture regardless of explant damage done during preparation, explant inoculation and infection by the bacterium. This was in line with the results obtained by Somers et al. (2003); Paz et al. (2004) and Liu et al. (2004) who reported on the advantage of using cotyledonary explants during *Agrobacterium*-mediated soybean transformation. They clearly indicated that

the meristematic cells in the existing axillary meristems found on the coty-node junction promote shoot proliferation following co-cultivation. BA (2.00 mg/l) was used in all treatments (SIM 1, SIM 2 and SIM 3) for adventitious shoots initiation. The percentage of double coty-node explants regenerating shoots ranged from 60 to 76.6% in all antibiotic treatments (Table 4.3). The lowest mean number of adventitious shoots was observed in the control due to the lack of exogenous plant regulators. The highest mean shoot number per explant (7.27) was recorded on MS medium (SIM 2) supplemented with tetracycline (500 mg/l), and it was significantly higher than the mean shoot numbers obtained on SIM 1 and SIM 3, and the control (Table 4.3). SIM 2 medium was effective in multiple shoot initiation without any production of excessive callus (Fig. 4.3 B). Necrosis and chlorosis were not observed on most of the double coty-node explants cultured on these three shoot induction media.

Pollock et al. (1983)'s report identified aminoglycoside antibiotics as the ones that are ineffective in *in vitro* cultures of *Nicotiana plumbaginifolia* and usually have severe toxic effects causing early senescence to plant tissues. This study was supported by various reports, including those of Tang et al. (2000) and Alsheikh et al. (2002), which used ß-lactam antibiotics as the bactericides of choice for efficient elimination of *Agrobacterium* during transformation as compared to the aminoglycoside antibiotics. Our results show that the aminoglycoside antibiotics used in this study had no severe effect on the survival of plant tissue explants as shown on Figure 4.3 A, B, and C. Chlorotic and necrotic symptoms were also observed, mostly on the margins of the leaves of the regenerated shoots (Fig. 4.3 A). These symptoms can be attributed to the exhaustion of nutrients, and pH fluctuation (Tang et al., 2000).

The excess bacterial growth of *A. tumefaciens* was completely inhibited by the aminoglycoside antibiotics hygromycin, tetracycline and rifampicin in combination with tetracycline during 3 weeks of culture. Prolonged incubation (over a period of 3 weeks) of explants on MS medium containing tetracycline and hygromycin (500 mg/l) did not affect the growth of shoots (SIM 2 and 1). This was in contrast to the report by Habiba et al. (2002) that a continued exposure of infected explants to higher (>5 µg/ml) aminoglycoside antibiotic concentrations was toxic although a short exposure was tolerated.

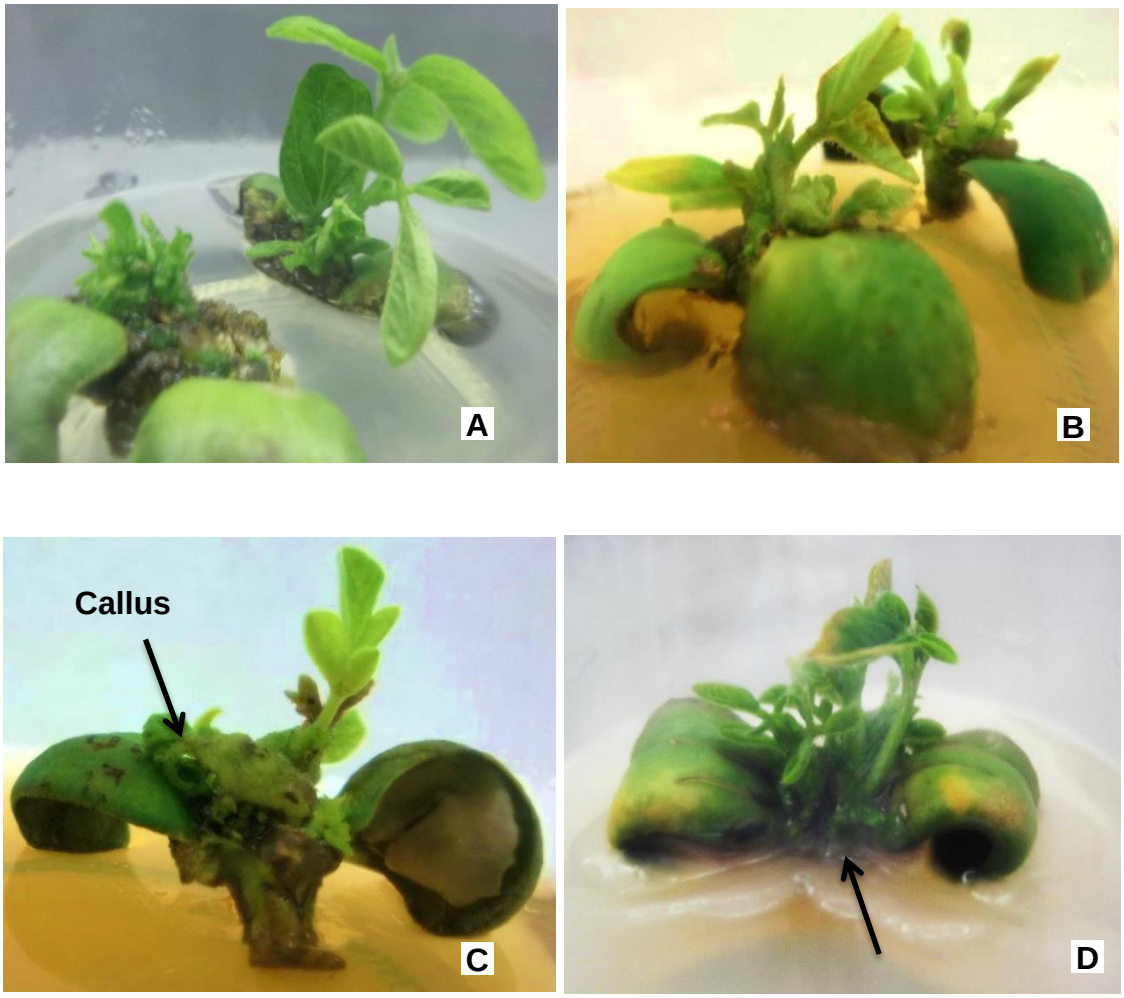

Figure 4.3 Multiple shoot induction on double coty-node explants infected with *A. tumefaciens* pTF 101 strain. Multiple shoots induced on SIM 1 (A), SIM 2 (B), and SIM 3 (C) shoot induction media. (D) Example of *A. tumefaciens* overgrowing a tissue explant causing cell death, and inhibition of shoot growth.

An average of 4.86 shoots was obtained on culture medium SIM 1(Table 4.3). This medium was supplemented with 500 mg/l hygromycin and produced healthy shoots, as the plants did not show any deficiency symptoms during culture (Fig. 4.3 A). Even if hygromycin is one of the aminoglycoside antibiotics not regularly used, production of healthy shoots in this medium implies that its unique structural and functional properties play a very important role when compared to other aminoglycosides used in plant tissue culture. Falkiner (1990) reported that the antibiotics which act specifically on bacterial cell walls would be more suitable to control infection in plant tissue culture and achieve high shoot multiplication. The report referred to ß-lactam antibiotics such as cefotaxime, vancomycin, ticarcillin and carbenicillin which target bacterial cell walls and are typically used in *Agrobacterium*-mediated soybean

transformation. However, our results show that the highest shoot multiplication (up to 80% frequency) can still be achieved when other aminoglycoside antibiotics such as hygromycin (SIM 1) are used, particularly at concentrations that do not inhibit shoot growth. On the basis of these results, tetracycline and hygromycin were found to be satisfactory in suppressing excessive *Agrobacterium* growth in culture when used alone at a 500 mg/l concentration as compared to the combination of rifampicin and tetracycline. Small number of shoots (3.8, Table 4.3), accompanied by callus formation and also stunted growth were observed on the SIM 3 (Fig. 4.3 C).

If *A. tumefaciens* is not eliminated from the plant culture medium by the antibiotics used, after the co-cultivation, it will overgrow the tissue and destroy it. Our results show that *Agrobacterium* was effectively inhibited by the antibiotics used in all shoot induction media used. Figure 4.3 D shows an example of failure of ß-lactam antibiotics to eliminate *Agrobacterium* overgrowth on MS basal medium supplemented with 2.00 mg/l BA after one week of culture, preventing shoot growth and inhibiting shoot elongation. Necrosis that occur at the base of the cut hypocotyls prevent the supply of nutrients to the developing shoots resulting in nutrient deficiency symptoms observed first on the older leaves and then later on the young leaves. The necrotic spots appeared localised at the margins of the leaves, until it covered the entire leaf. All shoots that were excised from contaminated explants did not survive when cultured on a fresh medium.

4.5.1.3 Elongation of shoots

Multiple shoots induced on the double coty-node explants have shown some sensitivity towards the media composition when they were excised-off the explants, and sub-cultured for elongation. Elongation of shoots occurred rapidly when the induced shoots and clumps were subcultured on elongation medium with their cotyledons still attached to them (Fig. 4.4 A). The presence of coty-nodes appeared to have been continuously supporting the elongating shoots, as described by Kaneda et al. (1997). On the other hand, the elongated shoots became chlorotic within 2 weeks of incubation, and the already elongated shoots appeared to be suppressing elongation of other small adventitious shoots. There were no differences in terms of morphology between all elongated shoots. Most of the shoots (about 60%) in all

treatments subcultured for elongation except the control did not survive till rooting. Those that have survived showed the formation of light green callus at the base of their cut surfaces (Fig. 4.4 B).

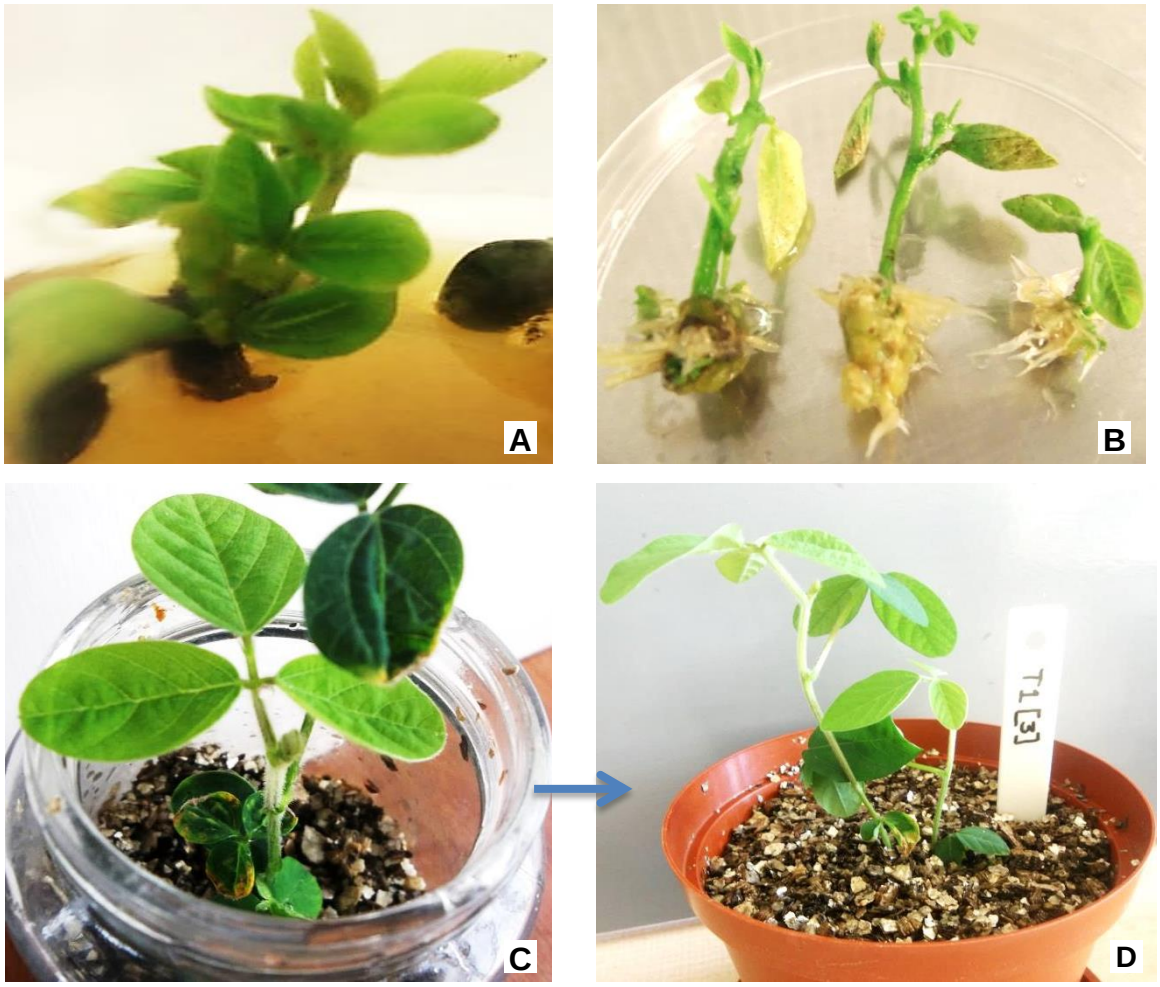

Figure 4.4 Plant establishment (A) coty-node explants with shoots subcultured on PGR free MS medium for elongation exhibiting rapid elongation. (B) Rooted shoots obtained from PGR free medium with some callus at the base. (C) Acclimatised regenerated plant in the culture bottle with the plastic bag removed. (D) An 8 weeks old acclimatised plant transplanted into plastic pot.

4.5.1.4 *In vitro* rooting of elongated shoots

More than 50% of the shoots produced roots within 2 weeks of culture on the hormone free MS basal medium. Short primary roots without lateral roots were observed within 2 weeks of culture on MS medium without PGRs. Sub-culturing of soybean shoots from elongation media that contained different antibiotics (Appendix

4.1) did not induce any notable morphological differences in the adventitious root phenotypes developed on PGR free medium supplemented with 6 mg/l glufosinate. Shorter and thicker roots were developed on shoots from all shoot cultures.

Shoots from the control gave normal root development consisting of the primary root with lateral roots. This suggest that the reduced root morphology in all treatments was observed probably as a result of the increase in endogenous level of BA resulting from the exogenous BA (2.00 mg/l) applied during shoot induction culture as supported by Polisetty et al. (1997). The adventitious root formation occurred on all shoots without the presence of auxins, such as IAA and IBA in the culture medium.

4.5.1.5 Acclimatisation of transformed plants

The transformed plants were successfully acclimatised in a growth room under controlled conditions. The survival rate of the regenerated plants was 60%. These regenerated plants resembled the normal seed-derived plants. However, the morphological parameters in terms of plant height, number of leaves, and stem diameters were observed to be different from those of plants derived from the control MS medium without growth regulators. Regenerated plants had thicker stem diameter (4–6 mm) as compared to the slender stems (3–4 mm) seen in the control. The increased stem diameter may be due to the BA (2.00 mg/l) hormone that was used during shoot induction, as supported by Leite et al. (2003). The regenerated plants showed vigorous growth within the first two weeks of transplanting into pots but, no flowering or seeds production was observed.

4.5.1.6 Conclusion

Aminoglycoside antibiotics tested were efficient as they appeared to be effective, and non-toxic to our soybean plant tissue explants. Our results show that aminoglycoside antibiotics (hygromycin and tetracycline) can be considered for *Agrobacterium*-mediated transformation of soybean and other legumes.

4.5.2 *In vivo* transformation of soybean

4.5.2.1 Germination of imbibed seeds

Higher germination percentages (87 and 97%) were obtained for both the control and pre-treated (2.00 mg/l BA) seeds respectively for the *in vivo* germination experiment (Fig. 4.5). This can be attributed to the effective sterilisation and imbibition of seeds before sowing into the sterile vermiculite for seed germination.

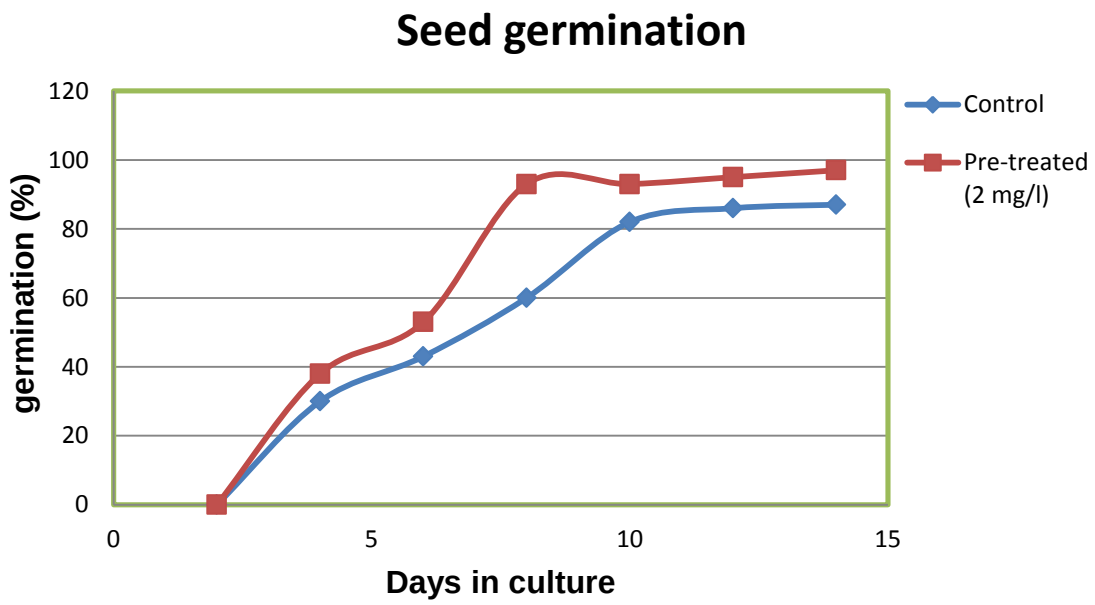

Figure 4.5 Percentage germination of soybean seeds in sterile vermiculite over 15 days of incubation.

McDonald et al. (1998) reported that seed imbibition is the most critical stage in successful soybean plant establishment. This study reported on seed imbibition with emphasis on absorption of water by the seed parts (seed coat, embryonic axis, cotyledons and the whole seed). However, our study shows that higher seed germination can be attained by imbibing soybean seeds in water containing 2.00 mg/l BA than in dH_2O only. Pre-treatment of seeds with BA (2.00 mg/l) also resulted into strong and stout seedlings with increased stem diameters (3–4 mm) and broad leaf areas similar to the seedlings developed from *in vitro* seed germination cultures (4.5.1.1).

4.5.2.2 Infection of seedlings with *Agrobacterium*

Only the seedlings developed from BA pre-treated were injected with the bacterial suspension carrying the Ω PKY vector. Seedling's health was not severely affected by the wounding or the infection. The wounded tissues appeared necrotic which could have resulted due to tissue damage and release of phenolic compounds causing oxidative browning and subsequent death of tissues. Olhoft et al. (2001) reported that less oxidised tissues could improve the interaction between *Agrobacterium* and plant cells. Further studies such as that of Paz et al. (2004) also indicated that browning of wounded tissues can be prevented by the application of antioxidants such as cysteine and dithiothreitol (DTT) that are predominantly used in the *in vitro* plant transformation during co-cultivation. However, no deaths of infected plants were observed as a result of wounding. This can be attributed to the effect of BA in delaying tissue senescence (Laloue et al., 2012).

4.5.2.3 Shoot induction

Shoot induction was considerably easy *in vivo* than *in vitro*. Both axillary shoots were induced in some of the plants at the axillary meristematic regions while mostly; single shoots were induced at that region. The data is summarised on Table 4.4. Shoots were initiated after removing the epicotyls from axillary meristems at the cotyledonary junction (Fig. 4.6). The shoots were formed at the cotyledonary junction where the epicotyls were removed. Sairam et al. (2003) obtained similar results on soybean when multiple buds were initiated from cotyledonary nodes. Seed pre-treatment with BA (2.00 mg/l) had positive influence on the morphology of the regenerated plants.

Plants regenerated from the pre-treated seeds had thicker stems (3–5 mm), a high number of axillary branches (3–4) and larger number of leaves (3–4 trifoliate leaves) as compare to the control which had slender stems (2–3 mm) and small number (1–2) of axillary branch and 1 or 2 trifoliate leaves (Fig. 4.7). According to Dybing and Reese (2008) pre-treatment of soybean seeds with 2 mM BA lead to flowering, fruit set and increased seed yield with more than 84% pod set. In contrast, production of flowers, fruit pods and seeds that were observed on all transformed plants in our study. This may be due to the 16 hour photoperiod used which may have been a

prolonged light period (16 hr of light followed by 8 hr of darkness) that caused inhibitory effect on flowering. A considerable difference in root morphology between the non-pre-treated seeds (control) and plants derived from BA pre-treated seeds was also observed. The control was characterised by vigorous root growth of the primary roots with many branching or lateral roots (Fig. 4.8 B), in contrast to plants from pre-treated seeds with stunted root growth of the main root and fewer lateral roots.

Table 4.4 *In vivo* soybean plant growth parameters of transformed and control plants.

VARIABLES	SOYBEAN GROWTH	
	Control	**Treatment (2.00 mg/l BA)**
Regeneration %	0^{b}	85^{a}
Mean shoot number	1.2^{c}	1.7^{c}
Average shoot length (mm)	155^{e}	145^{d}
Average root length (mm)	120^{g}	80^{f}
Shoot diameter range (mm)	$2–3^{i}$	$3–5^{h}$

Regeneration % is number of plants continuing growth following irrigation with Hoagland solution containing 6.00 mg/l glufosinate-ammonium. Values followed by the same letters are not significantly different at the 5% confidence level according to ANOVA.

Tian-fu and Jin-ling (1995) also found that soybean require relatively short day light periods (usually 8–10 hours) and a continuous dark period of about 14–16 hour for flower production. They reported that soybeans are highly susceptible to photoperiods and flower abortion can be caused by long-day photoperiod. Cho et al. (2000) reported almost similar root morphology, with the integration and expression of the DNA in soybean genome after transformation with *A. rhizogenes*. The report stated that infected plants showed stunted root growth with reduction in both root initiation and root development. The observed root phenotype can be attributed to BA seed pre-treatment as supported by Li et al. (1992) and Coenen et al. (2003).

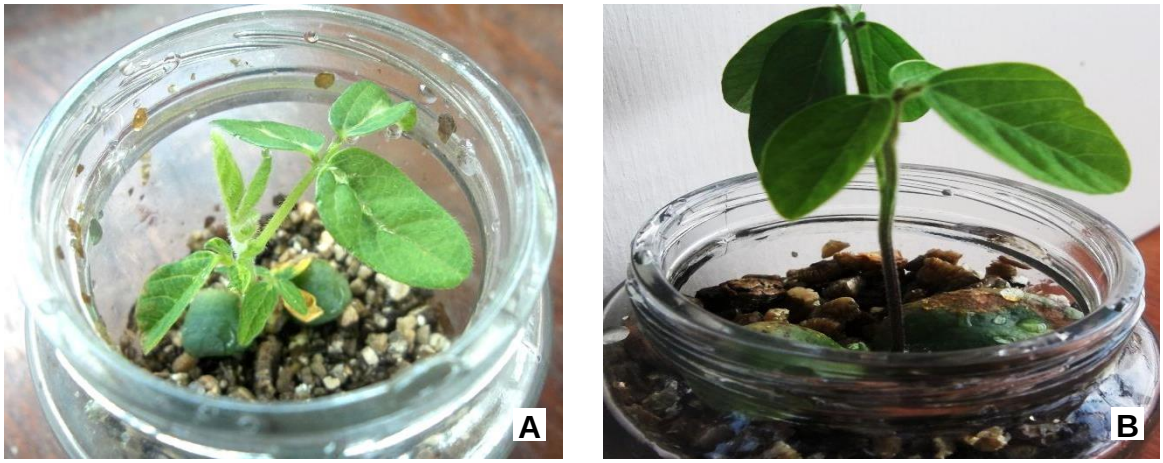

Figure 4.6 Examples of shoots induced after the removal of seedlings epicotyls. (A) Shoots induced on BA (2.00 mg/l) pre-treated seeds showing vigorous shoot growth. (B) Control with only 2 trifoliate leaves.

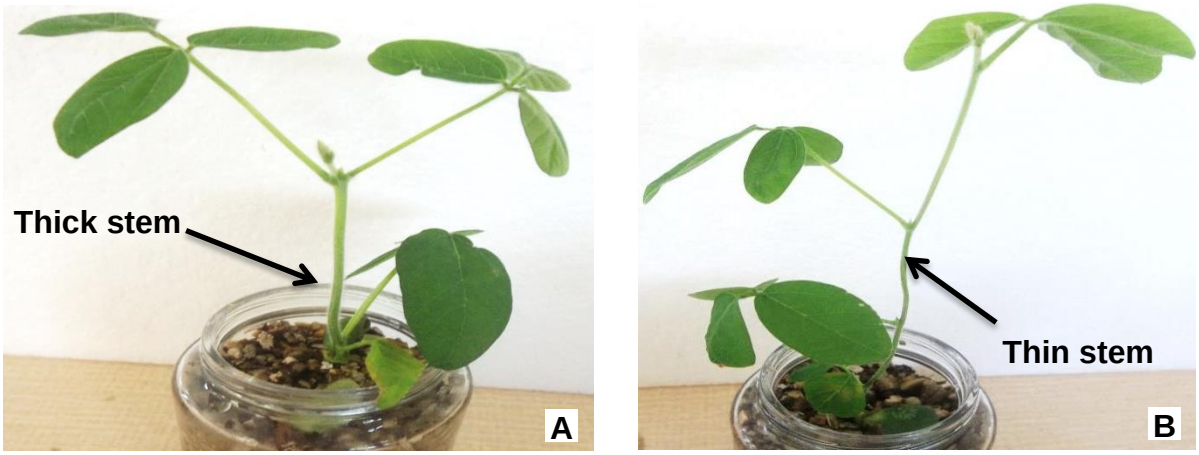

Figure 4.7 Effect of BA (2.00 mg/l) on soybean plant stems development. (A) Plant derived from BA pre-treatment, (B) control, without BA.

4.5.2.4 Screening and identification of transformed plants

The herbicide glufosinate was used as a selective agent for identification of transformed plants from non-transformed plants. A 6.00 mg/l concentration was used as recommended by Zhang et al. (1999) to allow for selection of transgenic plants. The results show that a total of 153 infected plants survived continuous application of glufosinate. Data on regeneration percentage following employing the herbicide is shown on Table 4.4. Healthy plants with no undesirable phenotypes such as severe chlorosis and necrosis were obtained in plants treated transformed with the

Agrobacterium as compared to the control plants. The non-transformed control plants showed more sensitivity to glufosinate than the transformed plants. These results suggest the usefulness of *in vivo* transformation and easy regeneration of plants with glufosinate as a selective agent. Reports by Murugananthan et al. (2007), Montaque et al. (2007) and others indicated that this herbicide can be used successfully as a selective agent for the recovery of transformed plants. No unusual phenotypic changes were observed in all plants. When comparing these results with other agents, such as kanamycin-based selection. Kanamycin selection system has been reported to be unsatisfactory because of the production of phenotypically abnormal plants (Bean et al., 1997, Montaque et al., 2007).

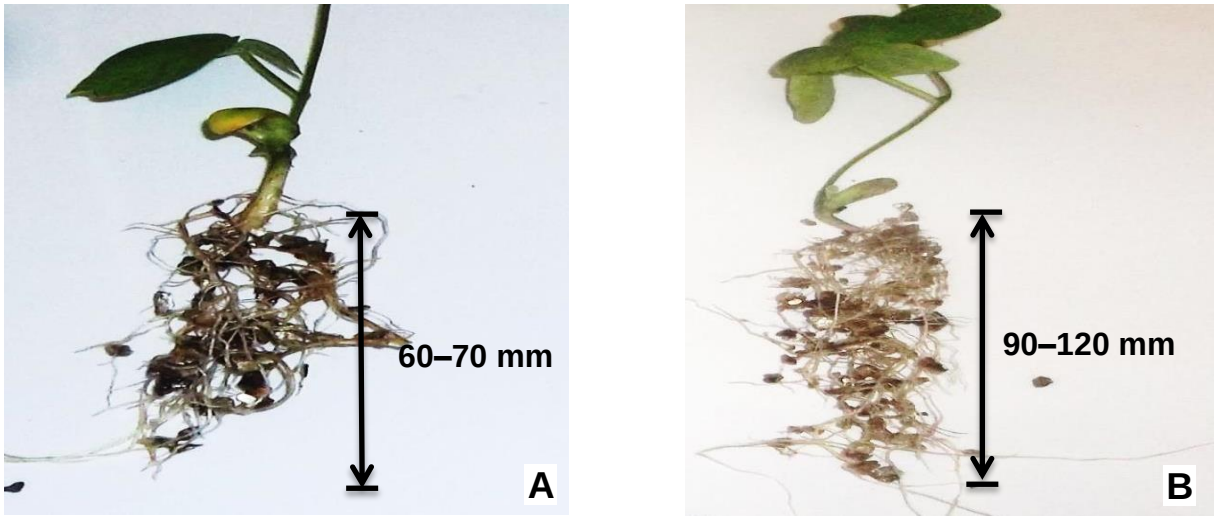

Figure 4.8 Root morphology of seven week-old soybean plant. (A) Stunted root growth on infected BA (2.00 mg/l) pre-treated plant. (B) Plant with long roots derived from non-pre-treated seeds.

4.5.2.5 Acclimatisation of transformed plants

All regenerated plants exhibited a healthy phenotype even after the removal of covering plastics during acclimatisation. From all the plants that survived irrigation with selective Hoagland's nutrient medium, no flower formation was observed (Fig. 4.9). The survival rate of 70% was obtained, which was higher than in *in vitro* transformation.

Figure 4.9 Acclimatisation of regenerated plants under controlled conditions. (A) Transformed plants covered with plastic bags for hardening. (B) The regenerated plant transplanted into 15 cm (150 mm^2) pot after removal of the plastic bag.

Transformed plants that survived glufosinate treatment showed no further growth, and formation of new branches. The trifoliate leaves also continued to appear normal without severe deficiency symptoms, but no further branching was recorded. Plants produced new young leaves at the shoot tips which died and fallen-off before any further development. Similar morphological characteristics were reported by Zia et al. (2011) during *in vivo* agro-injection of soybean pods in transformation of soybean seed embryos. Bermnier and Claire (2005) also reported retarded growth of the transformed plants, but in contrast, they had early flowering which later also resulted in flower abortion.

4.5.2.6 Conclusion

The findings show that infection of seedlings by injection with *Agrobacterium* can be used to obtain shoots which were able to survive continuous application of glufosinate-ammonium as a selective agent thus indicating the possibility of transformation. However, more studies need to be under taken to confirm the integration and expression of the *oc 1* gene into these plants.

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CHAPTER 5

GENERAL CONCLUSIONS, RECOMMENDATIONS AND FUTURE RESEARCH

5.1 GENERAL CONCLUSIONS

This study was set out to produce drought tolerant transgenic soybean plants by improving *Agrobacterium*-mediated genetic transformation. To accomplish this goal it became necessary to reach some prerequisite goals. Firstly, we investigated the effect of BA *in vitro* on multiple shoot induction in order to develop a precursor protocol for shoot regeneration required for soybean transformation. Double and single coty-node explants, obtained from stout seedlings derived from seeds pre-treated with cytokinins were used in this study.

The results have shown that both explants are suitable for incisions during genetic transformation, and produce a high number of shoots that can be regenerated, preferably from the double coty-node explants subcultured on MS medium supplemented with 2.00 mg/l BA. The *in vitro* test for the effectiveness of selected aminoglycoside antibiotics was performed for elimination of *Agrobacterium* strains pTF 101 and Ω PKY. The antibacterial activity assay indicated that the aminoglycoside antibiotics can be strongly recommended for *in vitro* elimination of *Agrobacterium* in soybean transformation. The typically used ß-lactam, cefotaxime or carbenicillin, antibiotics are not readily available, affordable and have been recently reported to be ineffective against some strains of *Agrobacterium tumefaciens* as indicated in our literature survey. Of the six antibiotics tested, tetracycline, spectinomycin, hygromycin and rifampicin have shown to exhibit the highest zones of inhibition on both *A. tumefaciens* strains respectively and therefore considered suitable to be used in transformation cultures. Rapid growth of *A. tumefaciens* pTF 101 as compared to Ω PKY, and complete lack of resistance to antibiotics of this strain showed that pTF 101 has a potential for *in vitro* transformation experiments. This study showed that these antibiotics at the concentration range of 150–500 mg/l effectively eliminate *Agrobacterium* growth.

In vitro genetic transformation of soybean using double coty-node explants infected with *Agrobacterium* pTF 101 on MS medium supplemented with 2.00 mg/l BA and the above mentioned antibiotics was a success. The precursor protocol developed in chapter 2 and the use of antibiotics selected based on the study described in chapter

3 led to an improved method for soybean transformation. Up to 76.6% shoot regeneration frequency was attained, with a minimum of 3.8 shoots induced per explant. The regenerated shoots were transformed since they have survived in the presence of glufosinate. Only transformed plants with the *bar* gene conferring resistance to glufosinate would survive on medium supplemented with glufosinate. On the other hand, *in vivo* transformation using Ω PKY showed positive results.

The method produced successful results for the *in vivo* regeneration of soybean in the absence of tissue culture conditions. This was carried out by injecting *Agrobacterium* on the seedlings developed from pre-treated seeds, at their cotyledonary junctions. The produced plantlets were taken as transformants since they have survived continuous application of glufosinate-ammonium used to screen transformed from non-transformed plants. This was achieved through the addition of glufosinate in Hoagland solution used for the irrigation of infected plants.

5.2 RECOMMENDATIONS

The following recommendations are offered for related research in the field of plant genetic transformation, especially for recalcitrant plant species such as soybean.

i. Given the nature of genetic transformation in soybean, other soybean cultivars should be tested using the above protocol to check if they will have similar trend of response thereby increasing the transformation rates.

ii. While the currently reported *in vitro* transformation protocols lead to low regeneration frequencies accompanied by the problem of chimerism. It would be advantageous to conduct a study which integrates *in vivo* with *in vitro* protocols, since, *in vivo* protocols have so far no evidence of generating chimeras as compared with *in vitro* methods reported in the literature.

iii. Given that the *in vitro* transformation study relied on plantlet survival under continuous application of glufosinate for the selection of genetically transformed plants. Determination of transgenic soybean plants using techniques such as ß-*glucuronidase* (*GUS*) expression and the identification

of the protein encoded by the transferred *oc 1* gene through sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) would be helpful.

iv. Intensive research on the optimal concentrations of aminoglycoside antibiotics that are effective on eliminating *Agrobacterium tumefaciens* would be of value for *in vitro* transformation of soybean and other legumes.

5.3 FUTURE RESEARCH

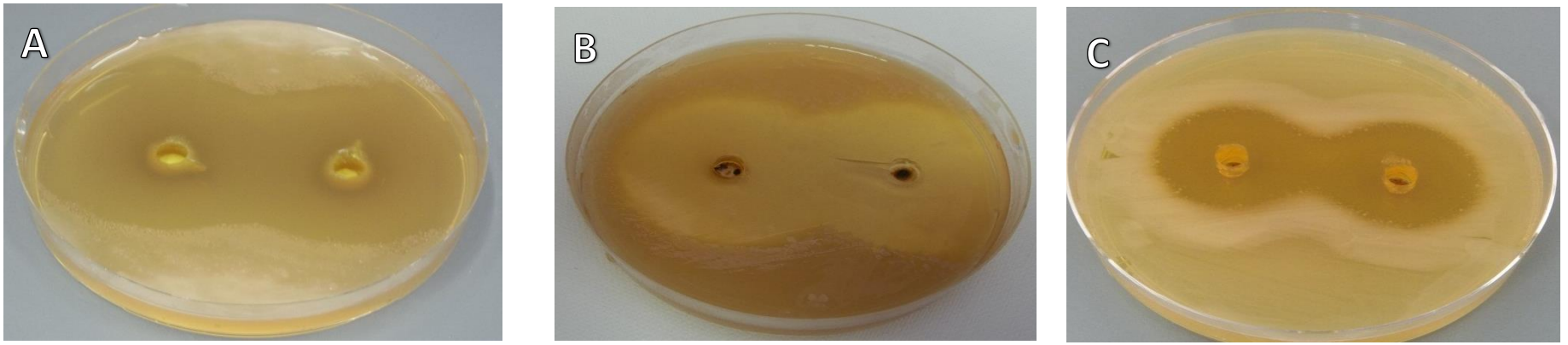
The most recently improved methods of soybean transformation still lead to low genetic transformation rates as compared to other plants. However, the results obtained in this study provide a guide to future research. A new approach for efficient soybean transformation could be the use of 10 day old seedlings which still possess meristems at their cotyledonary junctions than the typically used young embryos and embryogenic callus. These methods require long tissue culture periods, which need or consume large amounts of chemicals.

The callus cultures obtained are prone to chimerism, whereas those highlighted in this study (such as *in vivo* transformation) minimise these problems. In addition, the antibacterial activity using aminoglycosides on pTF and Ω PKY can be thoroughly assessed. Factors such as the agar medium used, the rate of diffusion and the depth or width of the punched wells (as compared to the use of discs), can also be investigated for their effect in evaluating antibiotics for the elimination of *A. tumefaciens*.

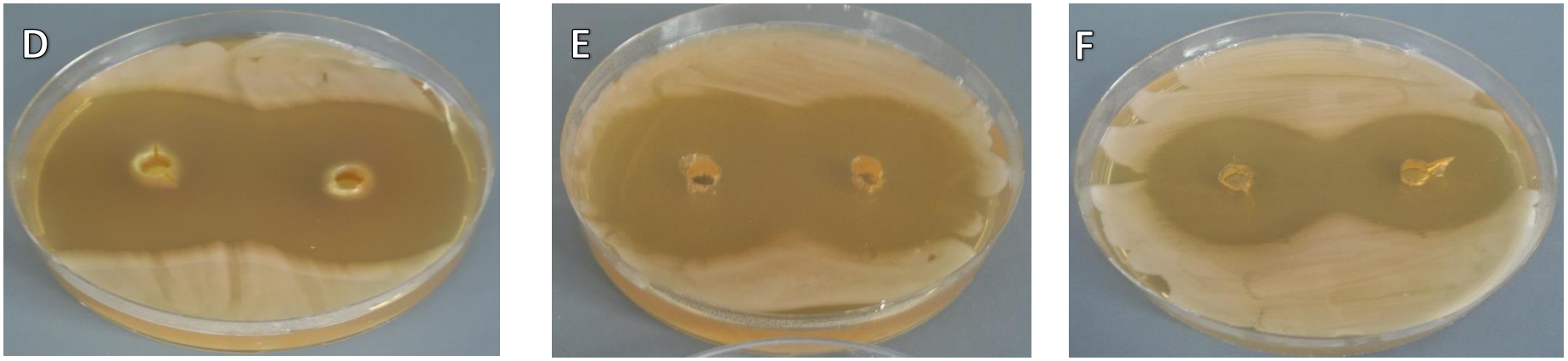
In section 4.3 and 4.4 we saw a high number of shoots or plantlets being obtained from *in vitro* and *in vivo* cultures. The technique of obtaining and establishing regenerated plants can be drastically accelerated by further optimising culture conditions. Successful *in vivo* and *in vitro* transformation at high frequencies accompanied by direct determination of transformed plants using non-selectable markers or reporter genes such as *GUS* expression can be achieved. The second generation (T_1) and other generations ($T_{1–4}$) of the first transformed plants (T_0) can then be analysed through other techniques such as SDS-PAGE to isolate the protein expressed by the gene of interest after being determined by *GUS* expression.

APPENDICES

Appendix 3.1 Comparison of antibacterial effect of the different antibiotics at the concentration (500 mg/l) exhibiting the largest zone of inhibition on agar plate inoculated with *A. tumefaciens* (strain pTF 101 and Ω PKY). A- Tetracycline, B- Hygromycin, C- rifampicin, D- spectinomycin, E- hygromycin and F- streptomycin. The results were obtained after 48 hours.



***Agrobacterium* strain pTF 101**



***Agrobacterium* strain Ω PKY**

Appendix 3.2 Theoretical zone of clearing for the six selected antibiotics

Antibiotic	Potency (unit/mg)	Inhibition zone diameter to nearest mm		
		Resistant	Intermediate	Susceptible
Kanamycin	750	≤ 13	14–17	≥ 18
Streptomycin	777	≤ 11	12–14	≥ 15
Tetracycline	996	≤ 14	15–18	≥19
Rifampicin	960	≤ 16	17–19	≥ 20
Spectinomycin	603	≤ 18	18–20	≥ 20
Hygromycin	979	≤ 20	20–25	≥ 25

Compiled with the aid of data obtained from Pollack et al (2009) and Wheat (2001).

Appendix 4.1 Culture media for genetic transformation of soybean

Media	Basal Media	Plant growth regulators (mgL^{-1})			Organic supplements (mgL^{-1})					Antibiotics/ herbicide (mgL^{-1})				Solidifying agents (mg)
		BA	IAA	GA_3	Aceto-syringone	Cys-teine	Dithiothrietol	Timentin	Methyl-ethane sulfonate	Hygromycin B	Tetracycline	Rifampicin	Glufosinate	
Infection Medium	B5	2.00	---	0.6	40	---	---	---	78	---	---	---	---	----
Co-cultivation medium	B5	2.00	---	0.6	40	400	154.2	---	78	---	---	---	---	Agar 2050
Shoot induction washing medium	B5	1.11	---	---	---	---	---	100	118	200	100	100	---	----
Shoot induction medium I	MS	2.00	---	---	---	---	---	50	118	SIM 1	SIM 2	SIM 3	---	Gelrite 3000
										500	500	10 Rif 50 Tet		
Shoot induction medium II	MS	2.00	---	---	---	---	---	50	118	SIM 1	SIM 2	SIM 3	6.0	Gelrite 3000
										500	500	10 Rif 50 Tet		
Elongation medium	MS	---	0.1	0.6	---	---	---	---	---	200	100	100	6.0	Gelrite 3000
Rooting medium	MS	---	---	---	---	---	---	---	---	---	---	---	6.0	Gelrite 3000