IN VITRO REGENERATION OF TWO VARIETIES OF PIGEON PEA (*Cajanus cajan*) GROWN IN KENYA

By

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DECLARATION

This thesis is my original work and has not been submitted to any university for the award of any degree.

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DEDICATION

This work is dedicated to my parents, Samson and Rebecca Asande for their incredible support during my studies.

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LIST OF ABBREVIATIONS

BAP	6- benzyl amino purine
2, 4-D	2, 4-dichlorophenoxyacetic acid
IBA	Indole- 3- butyric acid
KARI	Kenya Agricultural Research Institute
kPa	kilopascal
Mg/l	Milligrams per liter
MS	Murashige and Skoog media
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulator
TDZ	Thidiazuron

ABSTRACT

Pigeon pea (*Cajanus cajan* (L) Millsp.) is an important multipurpose grain legume that is a good source of protein for populations living in the semi-arid tropics. Being a crop that is cultivated under rain-fed agricultural system, its production is threatened by several biotic and abiotic stresses. Attempts to address these problems through conventional breeding have achieved partial success due to narrow genetic variability among the cultivated species. In addition, breeding incompatibility problems associated with wild species warrant exploration of alternative approaches like gene transfer to introduce desirable traits. Development of *in vitro* regeneration protocols amenable to genetic transformation offer an attractive opportunity for improvement of pigeon pea. Therefore the aim of this study was to develop a protocol for *in vitro* regeneration of two pigeon pea (*Cajanus cajan*) varieties, KAT 60/8 and ICEAP 00557 grown in Kenya. Murashige and Skoog (1962) (MS) basal media supplemented with various auxin and cytokinin concentrations alone or in combinations and different explant types (embryos or leaves) were tested for callus initiation, induction of somatic embryos, shoot and root regeneration. For callus induction, MS medium supplemented with 0.5 – 4.0 mg/l 2, 4- dichlorophenoxyacetic acid (2, 4-D) and thidiazuron (TDZ) were tested. For shoot and root regeneration, 0.1 mg/l and 0.5 mg/l 6- benzyl amino purine (BAP) and 0.1- 1.0 mg/l Indole- 3- butyric acid (IBA) were tested respectively. Embryogenic calli were obtained on MS medium with 2 mg/l TDZ and 1 mg/l 2, 4- D. Six week old embryogenic calli were transferred to MS medium supplemented with 0.1 mg/l or 0.5 mg/l benzyl amino purine (BAP) or MS medium without hormones for shoot

regeneration. Regenerated shoots (>3 cm) were excised after approximately ten weeks and transferred to MS medium with indole-3-butyric acid (IBA), for regeneration of roots. Shoot regeneration (6.7%) was achieved with KAT 60/8 variety from leaf callus induced on 1 mg/l 2, 4- D for 4 weeks and sub cultured on regeneration medium with 0.5 mg/l BAP for five weeks. No regenerants were obtained from ICEAP 00557 embryo and leaf callus induced on 2, 4- D and sub cultured on regeneration medium with BAP. The highest frequency of regeneration from ICEAP 00557 was achieved with leaf explant on 0.5 mg/l TDZ giving 20.0%. On the other hand, a regeneration frequency of 16.67% was obtained with KAT 60/8 leaf explants on 2 mg/l TDZ. IBA at 0.5 mg/l gave the most profuse rooting. Rooted shoots were hardened in a mixture of soil and vermiculite (1:1) for 21 days after which they withered. From the results reported in this study, the best explants for *in vitro* regeneration of pigeon pea varieties KAT 60/8 and ICEAP 00557 are leaf discs.

The results obtained show that genotype, plant growth regulator and explant type have a great influence on the success of an *in vitro* regeneration system. Moreover, optimization of the protocols generated in this study will step up the prospects for mass production of pigeon pea and genetic manipulation.

CHAPTER ONE

1.0 INTRODUCTION

Pigeon pea (*Cajanus cajan* (L) Millsp.) is a drought tolerant pulse legume mainly grown for its grain in the semi-arid tropics of many developing countries (Nene and Sheila, 1990). The Indian sub-continent, central America, and eastern and southern Africa are the major pigeon pea producing regions in the world. It is produced as a vegetable or as an export grain crop in the east and south African countries (Shiferaw *et al.,* 2008). In Kenya, it is the third most important legume crop after beans and cowpeas, and is one of the fastest growing cash crops (Mergeai *et al.,* 2001; Shiferaw *et al.,* 2008). The dry grain is an important local pulse and export commodity for several African countries particularly Kenya, Malawi, Mozambique, Tanzania and Uganda (Minja *et al.,* 1999). Green pigeon pea is exported from Kenya to Europe (Snapp *et al.,* 2003) mainly from Machakos, Makueni, Kitui, Meru, and Mbeere.

Among the problems facing pigeon pea farming in Kenya is the access to high quality seeds and susceptibility to pests and diseases which hinder the utilization of improved varieties (Mergeai *et al.,* 2001; Shiferaw *et al.,* 2008). These challenges can be addressed through the use of biotechnology to develop disease and pest resistant varieties through tissue culture and genetic transformation and hence increase its production.

According to the economic review of agriculture 2007, 51% of Kenyan population lacked access to adequate food and this inaccessibility to food was closely linked to poverty levels which stood at 46%. This could also be attributed to the ever increasing

population which does not match the available food resources. The problem of food availability has been exacerbated by the effects of climate change and global warming. Due to climate change, drought cycles seem to have shortened from 5-7 years to 2-3 years and the impact is greatly felt in arid and semi arid areas of developing countries (Kiome, 2009). Therefore efforts to increase agricultural productivity by revamping development and application of agricultural technologies are of great necessity. These include improving and promoting indigenous crops that grow across a range of agro ecological zones such as the arid and semi arid lands. An example is pigeon pea, a legume that can be regarded as a food security crop and yet it is one of the orphaned crops in sub-Saharan African countries especially in Kenya (Kiome, 2009).

Plant tissue culture is a technique of *in vitro* cultivation of plant cells, tissues and organs under defined physical and chemical conditions and controlled environment (Loyola-Vargas, 2008). Two of the basic pathways used for tissue culture of different plant species are organogenesis and somatic embryogenesis. Organogenesis involves regeneration of adventitious organs directly (without callus) or indirectly (with an intervening callus phase) from the explant. Somatic embryogenesis is the development of embryos from somatic cells either with an intervening callus phase (indirect somatic embryogenesis) or without an intervening callus phase (direct somatic embryogenesis). Indirect regeneration often results in somaclonal variation making the strategy less desirable for large scale clonal propagation. Therefore, direct regeneration without callus phase is a reliable method for production of identical plants (Ritchie and Hodges, 1993; Kharabian and Darabi, 2005). Successful *in vitro* regeneration depends on the

control of morphogenesis which is influenced by the genotype, type of explant, composition of nutrient medium, hormone and culture environment (Bregitzar, 1992).

Tissue culture has a wide range of applications in basic and applied areas of research in plant biology which include; studies on cytology, nutrition, morphogenesis and pathology of cultured tissues, production of large numbers of identical plants, crossing of distantly related species by protoplast fusion and regeneration of the somatic hybrids, production of valuable compounds like secondary metabolites and recombinant proteins used in biopharmaceuticals, *in vitro* selection of stress tolerant plants, transfer of genes and regeneration of transformed plants and for the production of disease free plant materials (Svabova and Lebeda, 2005). Direct and indirect methods are used to introduce genes of importance into plants for instance, *Agrobacterium* mediated gene transfer and particle bombardment methods have been utilized in pigeon pea to produce transgenic plants (Thu *et al*., 2003).

1.1 Problem statement

Pigeon pea varieties KAT 60/8 (short duration variety) and ICEAP 00557(medium duration variety) are prone to pod sucking bugs and pod borers. These are the most significant pests in Kenya because they attack the crops in their growth stages. The pests (*Helicoverpa armigera)* bore holes on the pods, develop in them and feed on the seeds. The caterpillars of the legume pod borer *Maruca vitrata* feeds on leaves, flower buds and pods. The currently available option for controlling pigeon pea insect pests and diseases is by use of chemicals, which are expensive and not affordable to most small scale resource poor farmers (Mergeai *et al*., 2001; Villiers *et al*., 2008).

The pests have the capacity to develop resistance to pesticides and the habit of feeding inside the pods during most of its development makes it less vulnerable to insecticides. Its preference for the harvestable flowering parts of high value crops is responsible for huge agricultural losses and socio economic costs.

1.2 Justification

Drought is one of the most important environmental limitations to plant survival and productivity in the tropics. Pigeon pea remains one of the most drought tolerant legume crops. Its seeds and forage have over 20% protein and therefore its production should be promoted by incorporating agricultural biotechnology to address both biotic and abiotic stresses. Attempts to obtain pest resistant genotypes through conventional breeding have not been successful. This is mainly due to narrow genetic diversity and sexual incompatibility of the cultivated varieties with their wild relatives (Nene and Sheila, 1990; Varshney *et al.,* 2010). Genetic engineering approaches to introduce genes coding for insecticidal proteins is an attractive option for developing and breeding pest resistant genotypes (Kumar *et al.,* 1996 and Parde *et al.,* 2012).

Grain legumes are generally recalcitrant to tissue culture and transformation (Thu *et al*., 2003). However, through *in vitro* regeneration of pigeon peas somaclonal variants that are resistant to diseases like fusarium wilt and pod borers like *Helicoverpa armigera* can be obtained (Chintapalli *et al.,* 1997). Furthermore, *in vitro* regeneration is also a pre-requisite for genetic transformation of many crop species. The intent of this project is to develop an *in vitro* regeneration system of two commercial varieties of *Cajanus cajan*, KAT 60/8 and ICEAP 00557, grown in eastern Kenya using different explant types. The regeneration system will be of great importance since no information is

available on *in vitro* regeneration of KAT 60/8 and somatic embryogenesis of ICEAP 00557 pigeon pea varieties.

1.3 Objectives

1.3.1 Overall objective

To develop a reproducible *in vitro* regeneration protocol for two pigeon pea varieties grown in Kenya.

1.3.2 Specific objectives

1. To compare callus induction ability of selected pigeon pea explants namely, leaf disks and zygotic embryos.

2. To determine the effect of different concentrations of auxins and cytokinins on the regeneration of pigeon pea from calli and to investigate the conditions necessary for growth and acclimatization.

1.4 Research hypothesis

Pigeon pea (*Cajanus cajan* L. Millsp*)* varieties KAT 60/8 and ICEAP 00557 plants can not be regenerated *in vitro* from leaf disks and zygotic embryos.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cultivation of pigeon pea

Pigeon pea cultivation goes back to at least 3000 years, originating from India from where it was introduced to east Africa. Pigeon peas are widely cultivated in all tropical and semi-tropical regions (Screenivasu *et al.,* 1998; Thu *et al.,* 2003). Improved long (9 months), medium (6 months) and short (4 months) duration pigeon pea cultivars were developed and released in Kenya by the University of Nairobi (UoN), Kenya Agricultural Research Institute (KARI) and the International Crop Research Institute for Semi Arid Tropics (ICRISAT) (Kimani, 1991; Shiferaw *et al.,* 2008). In Kenya, farmers predominantly grow long duration varieties that take up to 11 months to mature in the field; these late maturing genotypes produce rather low yields of between 300 to 500 kg per ha (Omanga *et al*., 1996; Gwata and Shimelis, 2013). In addition, reports by FAOSTAT (2010) indicate that average pigeon pea production in Kenya is estimated to be 430 kg/ha.

In addition to the major pigeon pea growing Counties in Kenya which include Machakos, Makueni, Kitui and Meru, eight improved varieties were introduced in Karachuonyo and Nyakach areas of south west Kenya in 1997 due to low soil fertility and unreliable rainfall (Okoko *et al.,* 1997). The varieties introduced were KAT 60/8 and ICPL 87091 which are short duration varieties, ICEAP 00068 and ICP 6927 which are medium duration varieties, ICEAP 00053, ICEAP 00040, ICEAP 00020 and ICPL 9145 which are long duration varieties. Among the challenges faced was susceptibility

of the short duration varieties to pod borers (Okoko *et al.*, 1997). Other pigeon pea varieties grown in Kenya include: short duration varieties like NPP 670, ICPL 89091, Mbaazi-3, ICPL 87091, and medium duration varieties like ICEAP 00557, KAT 81/3/3, KAT 777 (Kimani, 1991; Mergeai *et al.*, 2001).

2.2 Economic importance

Pigeon peas is consumed as a food crop (dried peas, flour, or green vegetable peas) rich in mineral nutrients and it is also an excellent fodder because of its high nutritional value (Siambi *et al*., 1992). Peas are nutritionally significant, as they contain high levels of protein (17 to 28 % protein) and they also contain important amino acids such as methionine, lysine and tryptophan hence a key food supplement for resource poor farmers who consume mainly low protein cereals and root crops (Prabakharan *et al.*, 2011). In combination with cereals such as maize, pigeon peas make a well balanced human food. Peas are also a source of income for small scale farmers in the arid and semi-arid lands (ASALs) of Kenya.

2.3 Agronomy

Pigeon pea is a hardy crop and can be grown on a wide range of soils ranging from sandy loam to clay loam and even on marginal soils. It thrives well on well drained black cotton and red laterite soils with pH 5-7 (Valenzuela and Smith, 2002). It is rarely found above altitudes of 2000 m above sea level. The optimum temperatures for pigeon peas cultivation ranges from 25 to 38°C however it can tolerate temperatures of up to 45°C if the soil has adequate moisture and fertility. On the other hand it does not tolerate low temperatures and frost conditions. It is also sensitive to high salinity water logging and does not tolerate shallow soil (Choudhary *et al*., 2011).

Pigeon pea growth is optimum under rainfall conditions of between 600-1000 mm/year, whereas on deep, well-structured soil it will grow well where rainfall is of between 250 to 370 mm/year (Siambi *et al.*, 1992). They have a deep root system enabling them to grow well in the dry areas of Kenya. The bacterium *Rhizobium* that lives in the root nodules of the pigeon peas is able to fix nitrogen. Because of their capability to fix nitrogen they are important crop for green manure. After incorporation they can provide up to 40 kg nitrogen per hectare and hence improve soil fertility (Mapfumes *et al.*, 1993). Because of its extensive root system, they are able to take up nutrients and water from lower subsoil layers (Mapfumes *et al.,* 1993). Therefore, when intercropped they hardly compete with companion crops. This crop therefore grows and yields well under conditions of low rainfall and poor soils (Okoko *et al.,* 1997) and apart from being a source of food the woody stems are used as source of household fuel, thatch and fencing for subsistence farmers.

2.4 *In vitro* culture of pigeon peas

Plant tissue culture is the growth of microbe free plant material in a controlled aseptic environment such as a sterilized nutrient medium in test tubes. It involves culture of plant parts, tissues and organs and is based on the fact that all plant cells retain their ability to use all their genes to develop into any tissue or a whole plant (Singh, 2003); commonly referred to as totipotency. Through micropropagation rapid multiplication of stock plant material to produce a large number of plants can be achieved, including those plants that do not produce seeds. Micropropagation is used to multiply novel plants, such as transgenics or plants bred through conventional methods.

2.4.1 Surface sterilization

Plants growing under field conditions are naturally contaminated hence the need for surface sterilization of explants before *in vitro* culture. Villiers *et al.,* (2008) reported surface sterilization of seeds from ICEAP 00020, ICEAP 00040, ICEAP 00053, ICEAP 00554, ICEAP 00557, ICPL 86012 and ICPL 88039 cultivars of *Cajanus cajan* in 30% (v/v) commercial bleach (equivalent to 1% NaOCl) for 30 minutes. Sterilization of pigeon peas seeds in 0.1% mercuric chloride for up to 10 minutes has also been reported (Kumar *et al*., 1998; Geetha *et al*., 1998; Mohan & Krishnamurthy 2002; Aboshama, 2011; Prabhakaran *et al.*, 2011; Prasad *et al*., 2011; Ugandhar *et al*., 2012). Subrahmanyam *et al*., (1988) reported surface sterilization of eight species of *Atylosia* and *Cajanus* seeds and flower buds in 10% Chlorox. Thu *et al.,* (2003) reported surface sterilization of ICPL 87 seeds in 70% ethanol before agitating in 10% sodium hypochlorite for 45min.

Clough and Bent (1998) used vapour- phase sterilization on *Arabidopsis thaliana* seeds. Chlorine fumes were released on adding concentrated hydrochloric acid (3 ml) into 100 ml of commercial bleach (3.8% sodium hypochlorite) in a beaker and it is the chlorine fumes that sterilized the seed explants.

2.4.2 Source of explant

For pigeon pea, choice and source of explant determine the response of a cultivar to tissue culture (Aboshama, 2011). There have been attempts to regenerate pigeon pea from various explants for instance leaves (Eapen and George, 1993; Geetha *et al*., 1998; Sing *et al*., 2002; Villiers *et al.,* 2008;) cotyledonary nodes (Mehta and Mohan, 1980; Kumar *et al*., 1984; Prakash *et al*., 1994; Geetha *et al.,* 1998; Sing *et al*., 2002), shoot

apices (Cheema and Bawa,1991; Sing *et al.*, 2002, 2004) epicotyls (Kumar *et al*., 1984; Naidu *et al.,* 1995; Geetha *et al*., 1998; Prabhakaran, 2011) cotyledons (Aboshama, 2011; Prabhakaran, 2011) and mature zygotic embryos (Ugandhar *et al*., 2012).

2.4.3 Callus culture and somatic embryogenesis

Callus induction has been achieved from epicotyls and cotyledon explants of pigeon pea using different concentrations and combinations of IAA (Indole acetic acid), kinetin and 2,4-D (2,4-dichlorophenoxy acetic acid) on Murashige and Skoog (1962) basal media. For example in a report by Prabhakaran *et al.,* (2011) on an unspecified cultivar of pigeon pea, 1 mg/l IAA in combination with a range of kinetin concentrations (0.5 -0.9 mg/l) was tested, whereas 0.6-1.0 mg/l 2, 4-D was tested alone. The highest percentage (95%) of callus formation was recorded on MS media augmented with IAA (1.0 mg/l) and Kinetin (0.9 mg/l) and 90% on MS medium with 2, 4-D (1 mg/l). Thu *et al.,* (2003) reported callus induction from surface sterilized ICPL 87 seeds and embryo axes on B5 medium containing 3% sucrose, 0.3% phytagel and various BAP concentrations (0, 0.2, 2, 5, 10 mg/l). Callus only developed on B5 medium with 10 mg/l BAP. In their experiments, no callus was recorded from embryo axes on hormone free MS medium, limited callus development was recorded on MS with 0.2 to 2 mg/l BAP, while 5 to 10 mg/l BAP gave callus that could not develop further. For mature seeds, callus only developed on B5 with 10 mg/l BAP after 20 days.

Kumar *et al.,* (1998) reported higher plant regeneration frequencies through somatic embryogenesis using Pusa 606 cotyledon and leaf explants. Embryogenic callus and somatic embryos were induced on MS medium using Thidiazuron (0.2-25.0 µM) alone. In their experiments MS with TDZ (10 µM) recorded the highest (73.7% for leaves and

45.3% for cotyledons) percentage of embryogenic calli. Browning of calli which did not regenerate even after frequent subcultures was also noted. This was the first report of successful *in vitro* regeneration through somatic embryogenesis. Aboshama (2011) described induction of somatic embryogenesis from mature cotyledons of pigeon pea cultivars, ICPL 87-118 and ICPL 151, cultured on MS with TDZ (0.5-4.0 mg/l) and 2,4D (0.5-4.0 mg/l). In his findings, TDZ or 2, 4-D at 2 mg/l gave the best response. He observed that genotype, type and concentration of PGR were of great influence to the frequency of somatic embryogenesis. Maturation treatment was crucial for increasing the frequency of normal germinated somatic embryos. This was been achieved by using abscisic acid and withdrawal of growth regulators (Aboshama, 2011; Kumar *et al*., 1998).

Somatic embryogenesis of haploid cultures and production of embryos up to globular stage using anther culture techniques has also been reported in pigeon pea (Bajaj *et al.,* 1980). In their experiments, pollen grains from T 21 cultivar anthers were cultured on MS with a combination of IAA (2 mg/l) and kinetin (4 mg/l).

2.4.4 Direct organogenesis

Direct organogenesis in seven pigeon pea cultivars namely ICEAP 00020, ICEAP 00040, ICEAP 00053, ICEAP 00554, ICEAP 00557, ICPL 86012 and ICPL 87091 has been achieved using the petiolar part of leaf explants cultured on MS shoot induction media augmented with 5 µM BAP (Benzyl amino purine) and 5 µM Kinetin. Shoot elongation was achieved using 0.58 µM gibberellic acid ($GA_{3)}$, while 11.4 µM IAA was used for rooting (Villiers *et al.,* 2008). Direct shoot bud differentiation and multiple shoot induction from cultivar LGG- 29 zygotic embryo explants have also been

achieved. In this case, MS containing 1.0 mg/l BAP and 1.0 mg/l NAA was the best (with 5 out of 8 explants producing an average of 7 shoots per explant) for maximum shoot bud differentiation. Elongation of shoot buds was done using 0.4 mg/l GA_3 after which maximum rooting (mean number of roots per shoot being 5.5± 0.03) was achieved on 1.0 mg/l indole - 3 - butyric acid (IBA) followed by NAA and IAA (Prasad *et al.,* 2011). In addition, 93.2% multiple shoot formation has also been reported using cotyledonary nodes of Hyderabad C pigeon pea seedlings exposed to 2 mg/l BAP in 4 weeks (Geetha *et al.,* 1998).

Krishna *et al.,* (2011) reported regeneration of shoot buds from embryonic axes of JKR 105 cultivar of pigeon pea on MS basal medium augmented either with BAP alone, kinetin alone and TDZ alone or combinations of BAP with NAA or BAP with IBA. In their experiments, greater regeneration of shoot buds (mean of 17.5) was recorded in 4 weeks on MS with 2.5 mg/l BAP followed by elongation in lower concentrations.

Root induction has been reported on MS and ½MS medium supplemented with 2% sucrose and 0.25, 0.5 and 2 mg/l IBA. Best rooting response was recorded on 0.5 mg/l IBA producing a mean root length of 5.9±0.3 cm (Krishna *et al*., 2011). However, some cultivars of *Cajanus cajan* for instance, VBN_1, VBN2, CO5 and SA_1 have demonstrated profuse rooting on the same medium supplemented with a higher (3%) concentration of sucrose (Franklin *et al*., 2000). This variation has been attributed to carbon source concentration (Krishna *et al*., 2011). MS medium fortified with 0.1, 0.2 and 0.5 mg/l IAA, 0.1, 0.2 and 0.5 mg/l NAA and 0.1, 0.2 and 0.5 mg/l IBA has been tested for root induction on shoots of Hyderabad C cultivar of *Cajanus cajan*. IAA and NAA at 0.2

mg/l gave better rooting over 0.1 and 0.5 mg/l while 0.5 mg/l of IBA gave the best rooting results of up to 92.4% (Geetha *et al.*, 1998).

2.4.5 Somaclonal variation

Somaclonal variation can be of great use in producing new crop varieties (Evans and Sharp, 1986). Depending on the culture conditions and the source of the explants, somaclonal variants of economic importance can be obtained. The origin and expression of various observed variations is very diverse. One of the more frequent variations is difference in chromosomal number e.g. aneuploidy, mixoploidy and polyploidy. Some of the causes of somaclonal variation include: lengthy culture periods, growth regulators used or nutritional stress. The result is change in the number of chromosomes or the position of the chromosomes (Singh, 2003).

Somaclonal variation for varietal improvement in pigeon pea has been exploited (Chintapalli *et al.*, 1997). Cotyledon explants were used and the progeny screened for variability. The issue culture produced different mutation events resulting in both dominant and recessive alleles. Significant variation in seed mass, plant height and damage due to *Helicoverpa armigera* were obtained when compared to the seed derived control populations. Results obtained in R1, R2 and R3 generations indicated a definite gene for white seed coat, reduced plant height, a possibility of additional genes for pest resistance and increased seed mass.

Saxena *et al.*, (2011) reported fifteen somaclones from cotyledonary explants of *Cajanus cajan* var. 'ICPL87. The mutant inbreds displayed considerable variation for days to maturity, plant height, seed size, seed colour and grain yield.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials

Seeds of commercial Kenyan pigeon pea (*Cajanus cajan)* varieties KAT 60/8 and ICEAP 00557 were obtained from Kenya Agricultural Research Institute (KARI), Katumani. They were sterilized and germinated aseptically on moist filter paper and hormone free MS in the tissue culture laboratory at the School of Biological Sciences, University of Nairobi. Zygotic embryos, cotyledonary nodes and leaves from germinated seedlings were used as explants.

3.2 Explant preparation

Mature seeds were washed under running tap water for 5 minutes and then rinsed with sterile distilled water. Three drops of commercial liquid soap (teepol®) and three drops of sodium hypochlorite (10%) were added and then swirled for 10 minutes before decanting and discarding the water. The seeds were rinsed in three changes of sterile distilled water before shaking in mercuric chloride (0.1%) for 5 minutes. They were then rinsed in four changes of sterile distilled water before germinating them aseptically. The surface sterilized seeds were aseptically placed on sterile moist filter paper and others placed on culture tubes containing autoclaved hormone-free MS media gelled with 8 g/l agar for germination. The seeds were either soaked overnight or germinated for 4 days on moist filter paper to obtain zygotic embryos and cotyledonary nodes explants, respectively. Another set of seeds were germinated on MS medium without hormones for 10 days to serve as a source of leaf explants. The above three sets

of seeds were maintained under white fluorescent light at 2000 Lux for 16h/day photoperiod and a temperature of 25± 2 ^{o}C.

Vapour sterilization with the use of chlorine fumes was also used in sterilizing the seeds (Clough and Bent 1998). This was achieved by adding concentrated hydrochloric acid (3 ml) into 150 ml of commercial bleach (3.8% v/v sodium hypochlorite) in a beaker. Prior to adding HCl, a beaker containing bleach and petri-dishes containing seeds were placed inside a desiccator surrounding the beaker. After addition of HCl the desiccator was immediately closed, placed inside a fume chamber and the seeds left to sterilize overnight.

3.3 Culture medium

The medium used was based on Murashige and Skoog, 1962 (MS) salts and vitamins. It was supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar, L- glutamine (200 mg/l), casein hydrolisate (500 mg/l), and silver nitrate (10 mg/l). The pH of the media was adjusted to 5.8 prior to autoclaving for 20 minutes at 121°C at 15 psi pressure. Medium of 50ml was dispensed into culture bottles and covered with aluminium foil before sterilization. For callus induction, thidiazuron (0.5, 1.0, 2.0 and 4.0 mg/l) and 2, 4-D (0.5, 1.0, 2.0 and 4.0 mg/l) were tested while benzyl amino purine (0.1 and 0.5 mg/l) was tested for shoot induction.

3.4 Experimental design

All experiments were set in a completely randomized design as described by Compton, (1994). The treatments had five replicates each consisting of six explants (30 explants

per treatment). Data on the frequencies of callus induction, shoot induction, somatic embryogenesis and number of roots per shoot were recorded.

3.5 Callus induction and somatic embryogenesis

All zygotic embryos used in the experiments were carefully detached from soaked seeds before chopping off the radical of the axis. Leaf margins were cautiously trimmed before slicing them into segments of approximately 5.0 mm^2 in size. The excised explants (zygotic embryos and leaf segments) were separately placed in culture bottles containing MS medium supplemented with 2, 4 - D (0.5, 1.0, 2.0 and 4 mg/l) and TDZ (0.5, 1.0, 2.0 and 4.0 mg/l). Cultures were incubated at 25±2°C with 16 hour photoperiod approximately (2000 Lux) using cool, white florescent light for 4 to 6 weeks.

3.6 Shoot regeneration

Embryogenic calli that formed on zygotic embryo and leaf explants were separated from the initial explants and transferred to MS with 0.1 and 0.5 mg/l BAP (6- Benzyl amino purine) and MS without hormones for shoot regeneration. After 8 weeks, the regenerated shoots were transferred into shoot elongation medium containing 0.4 mg/l gibberellic acid (Prasad *et al.,* 2011) or lower BAP concentration (Geetha *et al., 1998*).

3.7 Root induction and hardening of plantlets

In addition to the harvestable shoots regenerated in the experiments described above, cotyledonary nodes were inoculated onto MS medium fortified with a combination of BAP (0.2, 0.5, and 1.0 mg/l) and NAA (0.1 mg/l) for 14 to 28 days to regenerate supplementary shoots. The regenerated shoots were rooted in MS medium containing

0.1, 0.5, 1.0 mg/l indole-3-butyric acid (IBA) and MS medium without growth regulators.

3.8 Acclimatization of plantlets

Plantlets with sufficient roots were carefully uprooted from the medium in culture vessels and their roots washed thoroughly under running tap water to remove any sticking medium before transplanting into pots containing sterilized soil: vermiculite (1:1) mixture for 7 to 21 days. The pots were covered with perforated polythene bags for 10 days and watered with ¼ MS twice a day while being maintained in the green house. After 10 days, the polythene bags were removed and the plants exposed to the natural environment.

3.9 Data analysis

For callus induction, calculation of percentages of zygotic embryos and leaf disc explants that callused against the total number of embryos and leaf discs used in the inoculation process gave the frequencies of callus induction. Regeneration frequency was obtained by calculating the percentages of calli developing into shoots against the total number calli used in the inoculation process. Data on length and number of roots per plant were assessed on the 3rd week. Recorded data were transformed ($P'=\arcsin\sqrt{P}$) before subjection to analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test conducted to separate means when ANOVA was significant ($p<0.05$). Photographs were also taken to show different stages of development.

CHAPTER FOUR

4.0 RESULTS

4.1 Seed germination

Germination of the surface sterilized ICEAP 00557 and KAT 60/8 pigeon pea seeds inoculated on semi-solid hormone free MS medium (Plate 1) ranged between 95- 100 % within 4 - 7 days. In addition no bacterial or fungal contaminants were observed and hence explants obtained from the seedlings for subsequent experimentation were disease free.

Plate 1: Four day old pigeon pea seedlings germinated on semi-solid MS medium without growth regulators.

4.2 Callus and shoot induction

4.2.1 Callus induction from embryo explants

On MS basal medium, 16.7% and 20% of the zygotic embryo explants (with the root section chopped off) for KAT 60/8 and ICEAP 00557 respectively, developed callus after 14 days of culture (Table 1). The calli developed at the base of the embryos and exhibited growth as the embryos displayed normal elongation (Plate 2A). However,

83.3% of KAT 60/8 (Plate 2B) and 80% of ICEAP 00557 pigeon pea zygotic explants cultured on hormone free MS medium elongated without calli formation. The calli obtained was generally white and compact (Plate 2A).

Table 1: Effect of MS with 2, 4 - D or TDZ on callus induction from zygotic embryo explants of pigeon pea

Hormone (mg/l)		Total No. of explants	Callus induction	
			KAT 60/8	ICEAP 00557
2,4 – D	TDZ		Mean± S.E	Mean± S.E
-	-	30	16.7± 0.5**a**	20.0± 0.7**a**
0.5	-	30	100.0± 0.0**b**	100.0± 0.0**b**
1.0	-	30	100.0± 0.0**b**	90.0± 0.4**bc**
2.0	-	30	90.0± 0.3**c**	80.0± 0.6**c**
4.0	-	30	83.0± 0.5**c**	100.0± 0.0**b**
-	0.5	30	100.0± 0.0**b**	100.0± 0.0**b**
-	1.0	30	100.0± 0.0**b**	100.0± 0.0**b**
-	2.0	30	100.0± 0.0**b**	93.3± 0.4**b**
-	4.0	30	100.0± 0.0**b**	100.0± 0.0**b**
2,4-D	*F*		*22.60*	*21.41*
	d.f		*(4,20)*	*(4,20)*
	P		*<0.001*	*0.001*
TDZ	*F*		*79.14*	*34.35*
	df		*(4,20)*	*(4,20)*
	P		*<0.001*	*<0.001*

Data were transformed (P'=arcsine√P) prior to ANOVA n=30. Values with different letters differ significantly according to the Least Significant Difference test $p<0.05$.Values are means of data in their original scale.

All embryo explants of KAT 60/8 inoculated onto MS medium supplemented with 0.5 mg/l 2, 4 – D formed calli after 14 days of culture. A similar trend was also obtained on MS medium supplemented with 1.0 mg/l 2, 4 – D (Table 1). On media containing 2.0 and 4.0 mg/l 2, 4 – D, 90% and 83.3% of the embryo explants formed calli respectively, after 14 days of culture (Table 1). Callus induction on the zygotic embryos of KAT 60/8

was significantly (p<0.05) influenced by the concentration of 2, 4-D, where 0.5 and 1.0 mg/l had the highest (100%) calli frequencies (Table 1). Formation of callus from embryo explants was evident by the 3rd day (the base of the embryos begin to swell) of inoculation onto MS medium supplemented with 0.5- 4.0 mg/l of 2, 4 – D (Plate 2C& D). Embryo explants inoculated on MS medium augmented with 0.5 mg/l to 4.0 mg/l formed calli with a creamy appearance and the size varied among the concentrations.

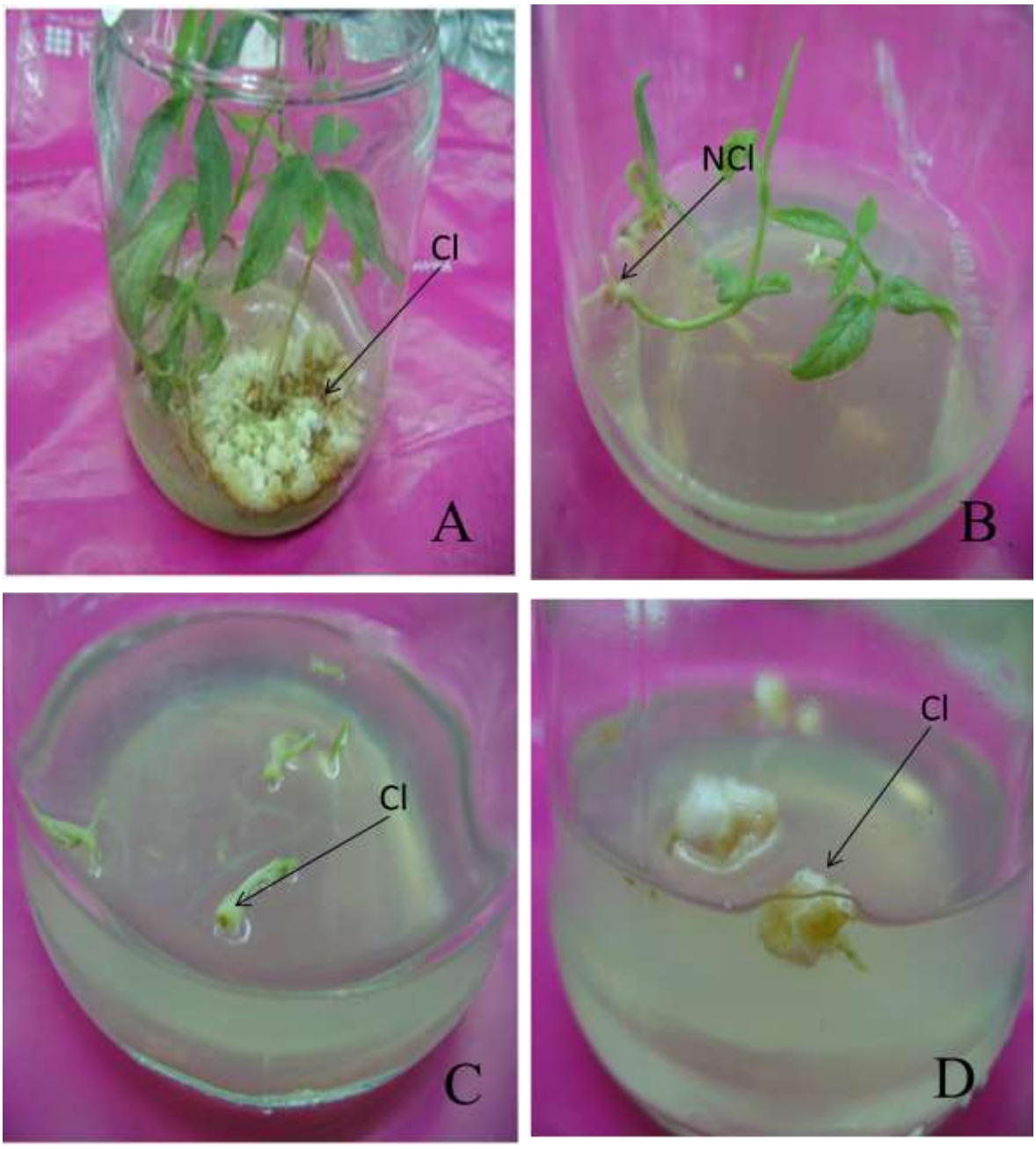

Plate 2: Effect of 2, 4 - D on calli induction from zygotic embryo explants of pigeon pea, KAT 60/8, on MS medium (A) Fifty six day old plant with callus (Cl) on MS basal medium; (B) Forty two day old plant on MS basal medium without callus (NCl); (C) Embryo explant with swollen base on 1 mg/l 2, 4 D after 3 days of culture: callus (Cl); (D) Embryo callus (Cl) on 1 mg/l 2, 4- D after 7 days of culture.

For ICEAP 00557, calli formation was generally visible on embryo explants at least after 3 days of culture irrespective of the 2, 4–D concentration. At 14 days after culture, 100% of the embryo explants inoculated into medium supplemented with 0.5 mg/l 2, 4–D formed calli, although relatively large calli were also present after 7 days of culture. For those cultured on 1.0 mg/l 2, 4–D, 90% of explants had formed calli at 14 days after culture, whereas a callus formation frequency of 80% was recorded for embryo explants on medium with 2.0 mg/l of 2, 4 - D and 100% for explants on MS medium with 4.0 mg/l of 2, 4 - D (Table 1). The calli formed on MS medium supplemented with 2, 4 - D at 0.5 - 4.0 mg/l were creamy in appearance. The concentration of 2, 4- D significantly (p<0.05) influenced induction of callus from ICEAP 00557 zygotic embryos though no generalized trends were observed.

For KAT 60/8 embryo explants inoculated onto MS medium supplemented with TDZ, irrespective of the concentration (0.5 – 4.0 mg/l), 100% of the explants formed calli between day 5 and day 21 after inoculation (Table 1). Therefore, TDZ concentrations did not significantly (p>0.05) influence calli induction frequencies. The calli formed were generally white and compact. The embryo explants inoculated onto medium augmented with TDZ at 0.5 mg/l developed callus at the base as from the 10^{th} day as the embryos showed normal elongation. On MS medium containing 1.0 to 4.0 mg/l TDZ, shoot elongation from zygotic embryos explants of KAT 60/8 was suppressed as the concentration increased (Plate 3).

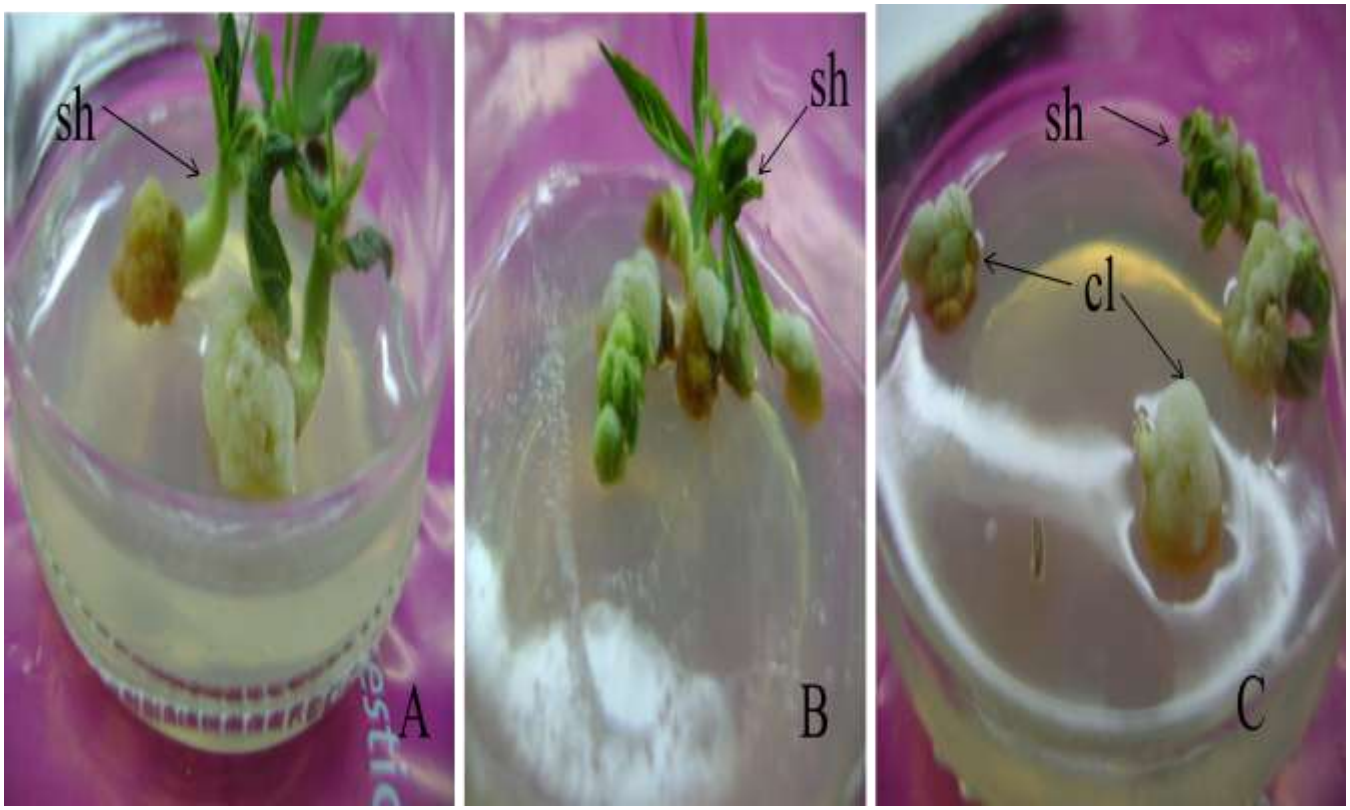

Plate 3: Influence of increasing TDZ concentrations on callus induction from KAT 60/8 pigeon pea zygotic embryo explants after 42 days of culture. (A) Elongating shoots (sh) on MS medium with 0.5 mg/l TDZ; (B) Relatively suppressed shoot (sh) elongation on MS medium with 1.0 mg/l TDZ; (C) Callus (Cl) on MS with 4.0 mg/l TDZ: shoots (sh).

For ICEAP 00557 zygotic embryos inoculated on TDZ containing MS medium, 100% calli induction frequencies were obtained on 0.5 and l.0 mg/l after 14 days of culture (Table 1). Formation of white compact callus was observed in both cases as from the 6^{th} and 5^{th} day, respectively. On medium with 2.0 mg/l TDZ, 93.3% callus development was recorded by the 6^{th} day while 100% callus formation was noted by the 5^{th} day on medium augmented with 4.0 mg/l of TDZ (Table 1). Callus formation on zygotic embryo explants on TDZ containing medium was not significantly (p>0.05) influenced by the various concentrations. Zygotic embryos of ICEAP 00557 cultured on MS medium containing 0.5 – 4 mg/l TDZ had repressed shoot elongation in addition to

callus formation at the base, as the concentration of TDZ increased (Plate 4).

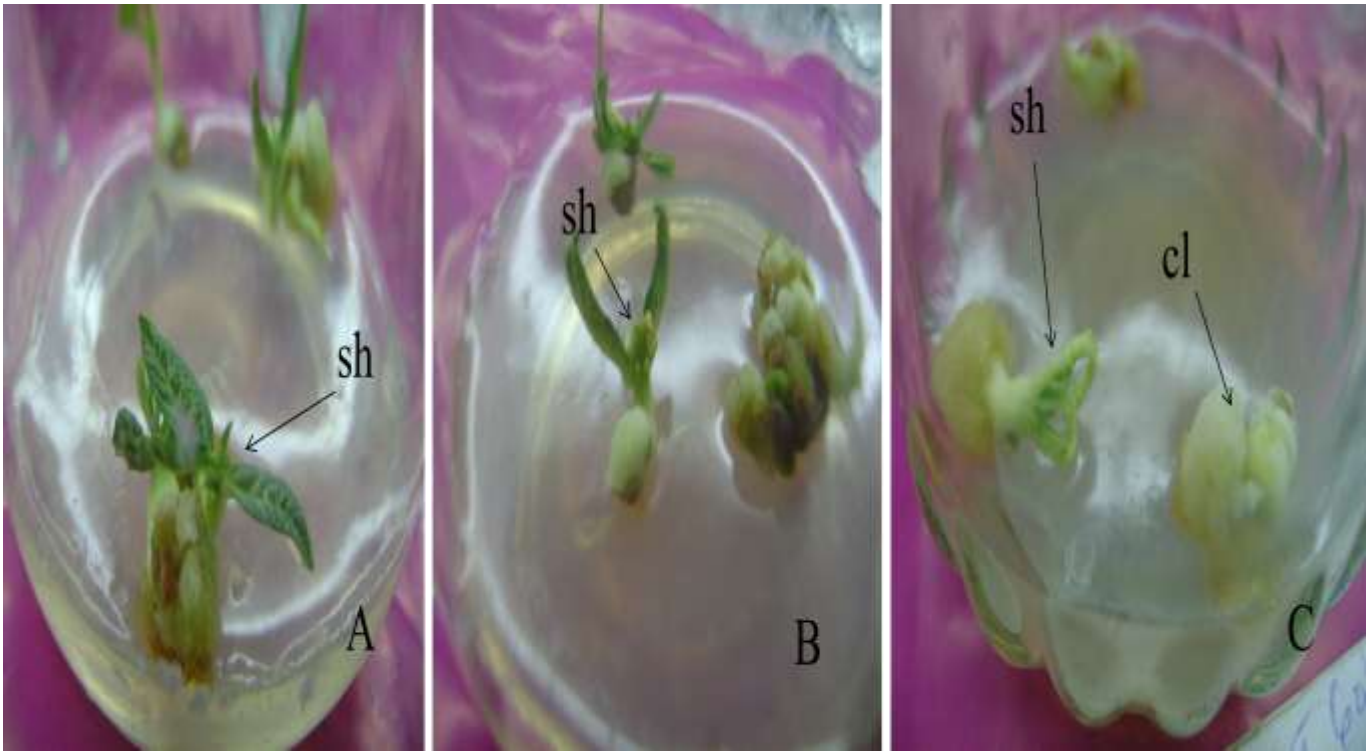

Plate 4: Influence of increasing TDZ concentrations on callus induction from ICEAP 00557 zygotic embryo explants after 42 days of culture. (A) Elongating shoots (sh) on MS medium with 0.5 mg/l TDZ; (B) Relatively suppressed shoots (sh) on MS medium with 1.0 mg/l TDZ; (C) Callus (cl) on MS medium with 4.0 mg/l TDZ: shoots (sh).

4.2.2 Callus induction from leaf explants

On MS medium without growth regulators (control) 13.33% of the leaf explants from KAT 60/8 and 6.7 % from ICEAP 00557 varieties formed calli. The leaf explants enlarged in size and developed white compact callus after 20- 42 days of culture (Plate 5A). A relatively high percentage (86.7 for KAT 60/8 and 93.3 for ICEAP 00557) of leaves cultured on growth regulator free MS medium increased in size but did not develop callus (Plate 5B). For KAT 60/8, the leaf explants cultured in treatments consisting of 2, 4 – D alone callused within 7 and 18 days, forming pale yellow calli (Plate 5C). For media containing 0.5 mg/l 2, 4-D, 93.3% of the explants formed pale

yellow calli while for media containing 1.0 mg/l to 4 mg/l 2, 4-D, all explants (100%) formed calli (Table 2). Induction of callus was not significantly (p>0.05) influenced by the concentration of 2, 4- D or TDZ.

Table 2: Effect of MS medium with 2, 4 – D or TDZ on callus induction from leaf explants of pigeon pea.

Hormone (mg/l)		Total No. of explants	Callus induction	
			KAT 60/8	ICEAP 00557
2,4 – D	TDZ		Mean± S.E	Mean± S.E
-	-	30	13.33± 0.7**a**	6.7± 0.3**a**
0.5	-	30	93.3± 0.2**b**	80.0± 0.6**c**
1.0	-	30	100.0± 0.0**b**	93.3± 0.2**b**
2.0	-	30	100.0± 0.0**b**	76.7± 0.4**c**
4.0	-	30	100.0± 0.0**b**	60.0± 0.5**d**
-	0.5	30	86.7± 9.1**b**	73.3± 0.5**b**
-	1.0	30	100.0± 0.0**b**	83.3± 0.6**c**
-	2.0	30	100.0± 0.0**b**	100.0± 0.0**d**
-	4.0	30	100.0± 0.0**b**	100.0± 0.0**d**
2,4-D	*F*		*48.67*	*9.415*
	d.f		*(4,20)*	*(4,20)*
	P		*<0.001*	*0.001*
TDZ	*F*		*32.464*	*17.757*
	Df		*(4,20)*	*(4,20)*
	P		*<0.001*	*<0.001*

Data were transformed (P'=rcsine√P) prior to ANOVA n=30. Values with different letters differ significantly according to the Least significant Difference test p<0.05.Values are means of data in their original scale.

Plate 5: Effect of 2, 4 – D or TDZ on KAT 60/8 leaf explants (A) Forty two day old leaf explants on MS basal medium with callus (cl); (B) Forty two day old enlarged pigeon pea leaf discs (El) on basal medium but without callus; (C) Fourteen day old leaf callus (cl) on 1 mg/l 2, 4 – D; (D) Twenty one day old leaf callus (cl) on 2 mg/l TDZ.

For ICEAP 00557 leaf explants, callus induction was significantly (p<0.05) influenced by the concentration of 2, 4- D with the highest calli induction frequency (93%) obtained on MS containing 1.0 mg/l 2, 4 – D after 10 days of culture (Table 2). The lowest frequency (60%) was obtained on media supplemented with 4.0 mg/l 2, 4-D after 12-21 days (Table 2). MS augmented with 2, 4 – D at 0.5 and 2.0 mg/l gave 80% callus

formation frequency from the 10th day and 76.67% of callus formation from the 9th day, respectively.

For KAT 60/8 leaf explants on TDZ, a relatively lower callus formation frequency (86.67%) was achieved on 0.5 mg/l TDZ and a frequency of 100 % was obtained for 1.0 mg/l to 4.0 mg/l TDZ (Table 2). Callus induced on media containing TDZ was generally white and compact (Plate 5D) and appeared from the cut margins within 10 to 21 days. Several shoot buds were also observed on the leaf surface, around the midrib and the petiolar parts of leaf explants cultured on 0.5 mg/l TDZ, after 28 days. Induction of callus was not significantly ($p>0.05$) influenced by the concentration of TDZ.

On the other hand, callus induction from ICEAP 00557 leaf explants on TDZ was significantly ($p<0.05$) influenced by the concentration of the cytokinin. Explants on MS with TDZ (0.5 mg/l) were swollen before forming white compact callus at the leaf margins between the 10th and 21st day. Shoot buds emerged directly from the leaf explant (Plate 6A) and in some cases the shoots emerged as early as 12 days after inoculation (Plate 6B and C). Leaf explants cultured on MS containing TDZ at 2.0 and 4.0 mg/l recorded 100% callus induction with leaf expansion noted before forming callus at the margins by the 12th and 10th day respectively. At 2.0 mg/l, regeneration of 12 shoot buds in total was observed while at 1.0 mg/l the explants expanded before forming callus as from the 10th day.

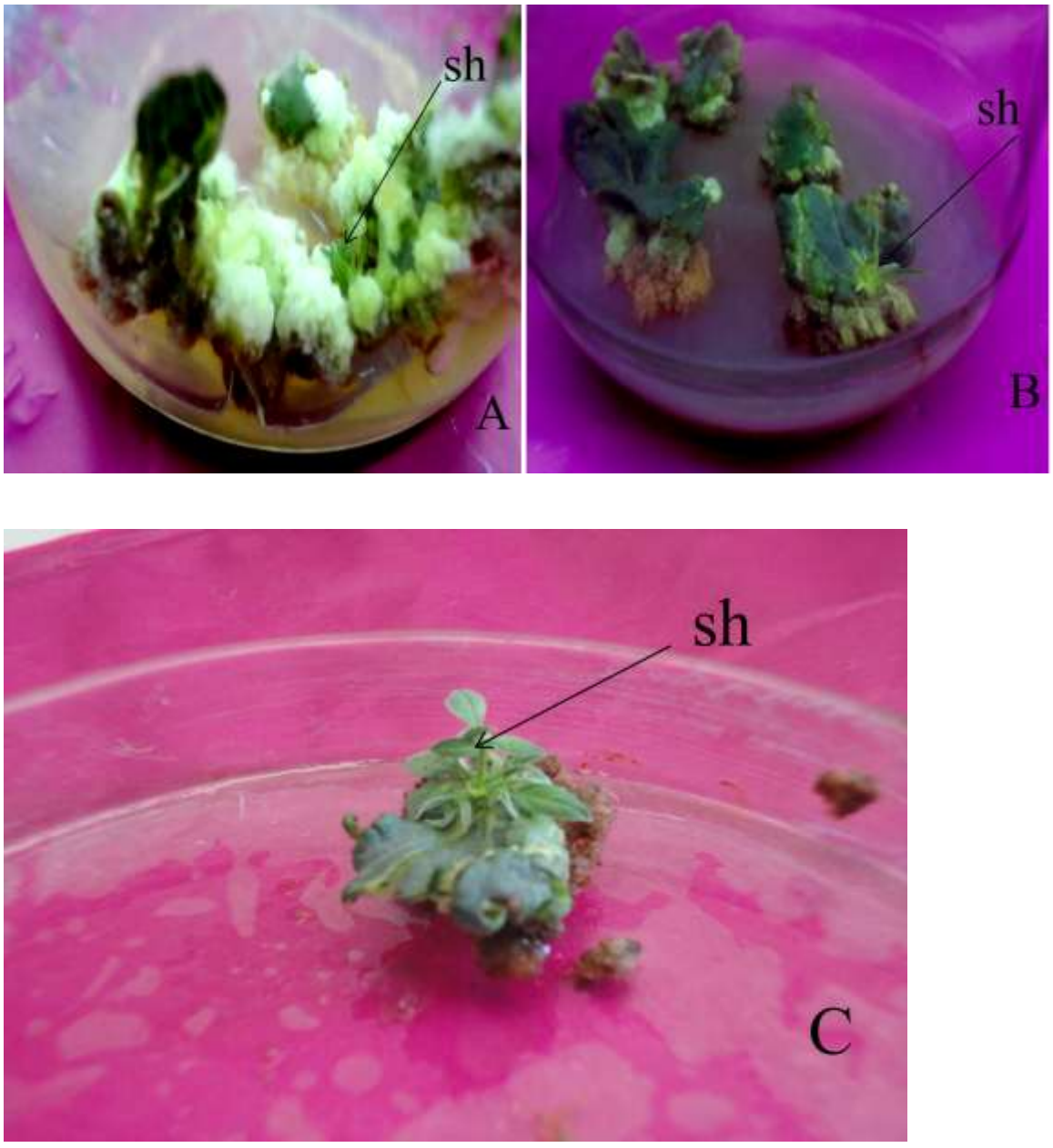

Plate 6: Effect of TDZ in MS medium on ICEAP 00557 leaf explants. (A) Forty two day old leaf callus on 0.5 mg/l TDZ showing callus and shoot (sh) induction; (B) Forty two day old leaf explant on 0.5 mg/l TDZ showing shoot (sh) regeneration; (C) Fifty six day old leaf explant on 0.5 mg/l TDZ showing shoot (sh) regeneration.

4.2.3 Response of 2, 4- D induced zygotic embryo calli on shoot regeneration medium

For KAT 60/8 variety, callus obtained from MS medium supplemented with 0.5, 1.0, 2.0 and 4.0 mg/l 2, 4 – D showed no regeneration (neither somatic embryogenesis nor organogenesis) when transferred to MS medium consisting of 0.1 or 0.5 mg/l BAP. Callus on hormone free MS medium (controls) exhibited rapid growth and turned brown within 11 to 14 days after subculture. Pieces of callus aseptically transferred to MS medium containing 0.1 mg/l BAP increased in size and turned cream to light green as from the 8th day. Gradual loss of the green coloration accompanied by brown patches spread on the surface of callus was observed from the 56th day (Plate 7A). Frequent subcultures only helped to expand and maintain the green colour of callus but no regeneration was observed. On MS medium supplemented with 0.5 mg/l BAP, the pieces of callus enlarged and turned cream to green by the 5th day and acquired a more intense green coloration than callus on medium with 0.1mg/l BAP, but later lost the coloration as from the 56th day. Discoloration was accompanied by brown patches on the surface of the callus (Plate 7B).

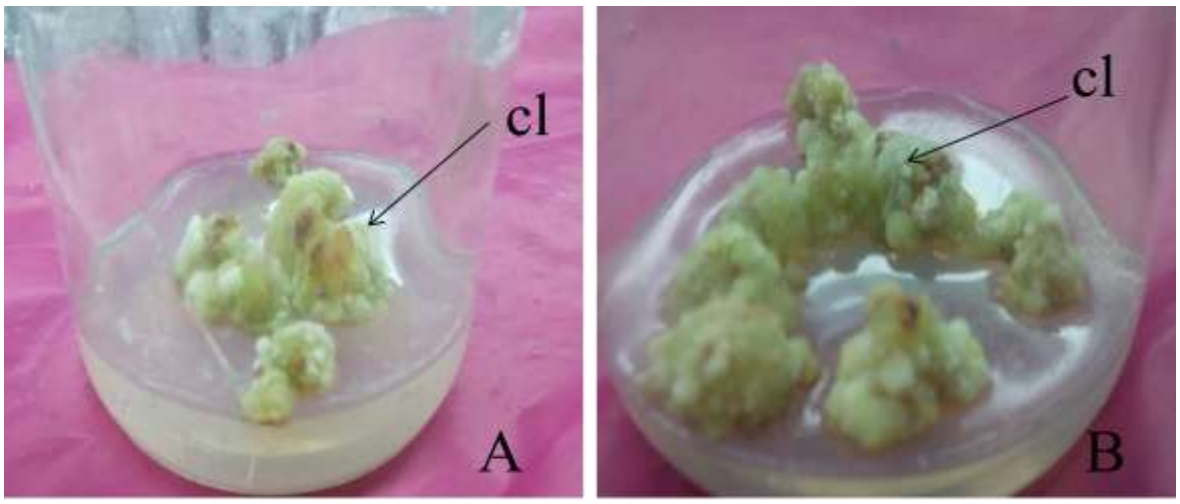

Plate 7: Callus on shoot regeneration medium (A) Fifty six day old embryo calli (cl) of KAT 60/8 on 0.1 mg/l BAP; (B) Fifty six day old embryo callus (cl) of KAT 60/8 on 0.5 mg/l BAP.

For ICEAP 00557 variety, pieces of callus obtained from embryo explants and maintained on MS medium with 0.5, 1.0, 2.0 and 4.0 mg/l 2, 4 – D did not produce any regenerants when aseptically transferred to regeneration medium composed of MS supplemented with 0.0 to 0.5 mg/l BAP, in spite of the frequent subcultures. Pieces of callus on MS medium without growth regulators exhibited rapid growth and remained cream for a period of 14 days after which they developed brown patches and later turned dark brown. On MS medium with 0.1mg/l BAP, the callus enlarged and developed green patches on the surface by the 10^{th} day. As from the 49^{th} day, the callus gradually lost the green colour and turned brown. Frequent sub cultures only helped to delay the browning. Callus maintained on MS medium with 0.5 mg/l BAP increased in size and turned green by the 5^{th} day. Like KAT 60/8, the green coloration was more intense than in MS medium supplemented with BAP at 0.1 mg/l, and by the 56^{th} day, the green colour slowly faded away.

4.2.4 Response of 2, 4 - D induced leaf-calli on regeneration medium

For KAT 60/8 variety, calli on MS medium without growth hormones only expanded in size and thereafter turned brown between the 14^{th} and 18^{th} day without forming shoots. No regeneration was achieved from leaf callus sub- cultured on MS medium with 0.1mg/l BAP. Instead the calli exhibited rapid growth and attained a cream to light green coloration by the 12^{th} day. Discoloration of callus was observed by the 56^{th} day followed closely by browning and eventual death. A shoot regeneration frequency of 6.7% was obtained for callus maintained on medium supplemented with 0.5 mg/l BAP. The regenerated shoots were visible on calli surface at 35 days of transfer to regeneration medium consisting of 0.5 mg/l BAP (Plate 8A). The shoots were exercised from the calli after 42 days and then elongated on a lower concentration (0.1 mg/l) of BAP for 28 days (Plate 8B).

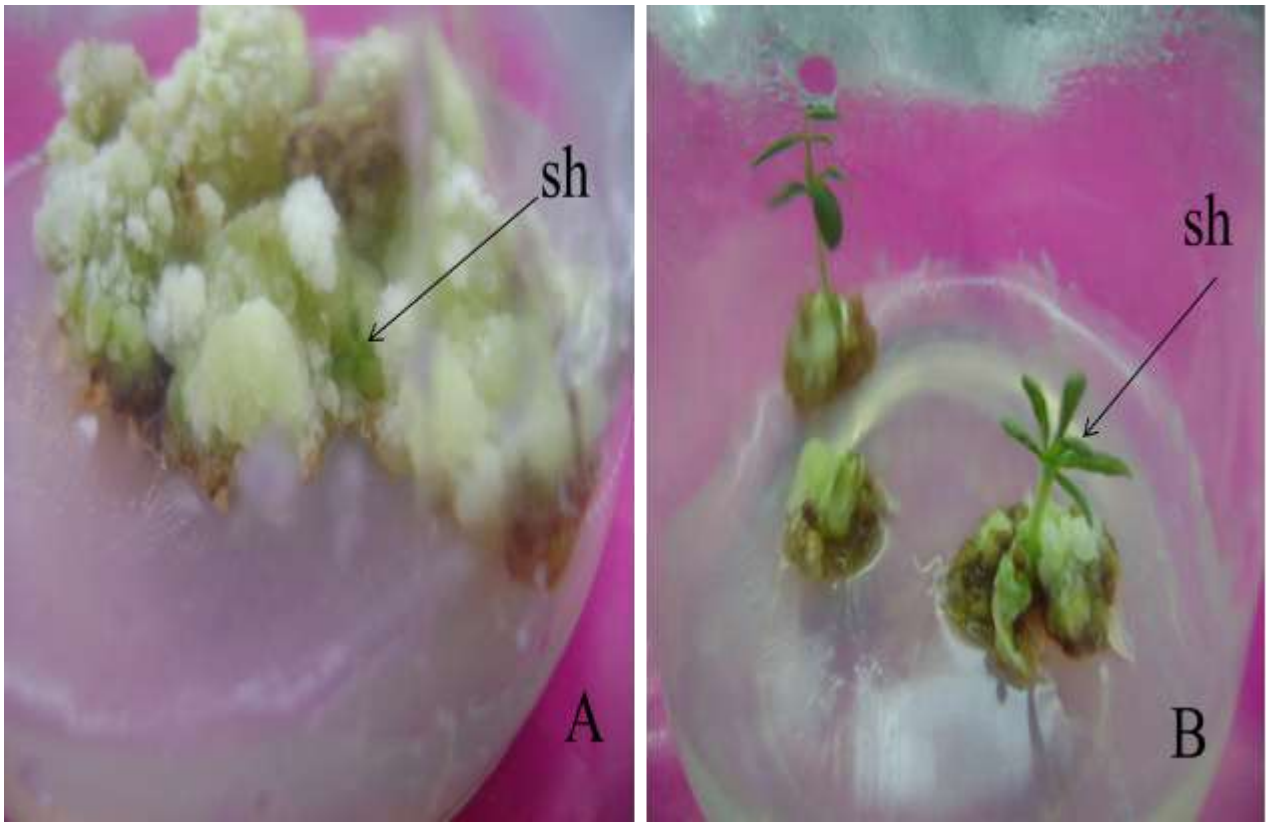

Plate 8: Effect of BAP on shoot regeneration of KAT 60/8 leaf-induced calli. (A) Emerging shoot (sh) from leaf callus on MS medium with 0.5 mg/l BAP at 35 days after sub culture; (B) Shoots (sh) elongated on MS medium with 0.1 mg/l BAP, at 28 days after sub culture.

No regeneration was recorded from leaf callus of ICEAP 00557 obtained from MS medium supplemented with 0.5 to 4.0 mg/l 2, 4 – D and maintained on regeneration medium consisting of MS basal or medium augmented with 0.5 mg/l BAP for 56 days. On growth regulator free medium, callus enlarged and began to develop brown patches scattered on the surface after 14 days. Sub-culturing delayed the browning. On MS medium with 0.1 mg/l BAP, calli expanded as they turned light green from the 8^{th} day and later developed brown patches as the green colour slowly faded away beginning the 56^{th} day. MS medium supplemented with BAP at 0.5 mg/l gave green callus by the 5^{th} day, although the callus slowly lost their green colour by the 49^{th} day.

4.2.5 Shoot regeneration from zygotic embryo callus induced on MS medium supplemented with TDZ

Callus induced from KAT 60/8 embryo explants on MS medium supplemented with 0.5 to 4.0 mg/l TDZ, and aseptically transferred to shoot regeneration medium consisting of MS augmented with 0.0 to 0.5 mg/l BAP generally produced shoot regenerants although at low frequencies (Table 3).

Table 3: Effect of BAP on shoot regeneration from calli initiated using zygotic embryo explants of pigeon pea on MS supplemented with TDZ.

Hormone (mg/l)			Shoot regeneration	
			KAT 60/8	ICEAP 00557
Conc. of BAP in regeneration medium	Conc. of TDZ in initiating medium	Total no. of pieces of callus	Mean ± S.E	Mean± S.E
0.0	0.5	30	0.00± 0.00**b**	0.00± 0.00**b**
0.0	1.0	30	0.03± 0.01**b**	0.00± 0.00**b**
0.0	2.0	30	0.07± 0.02**a**	0.00± 0.00**b**
0.0	4.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.1	0.5	30	0.00± 0.00**b**	0.00± 0.00**b**
0.1	1.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.1	2.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.1	4.0	30	0.03± 0.02**b**	0.10± 0.04**a**
0.5	0.5	30	0.00± 0.00**b**	0.00± 0.00**b**
0.5	1.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.5	2.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.5	4.0	30	0.15± 0.05**a**	0.00± 0.00**b**
F			*2.60*	*1*
d.f			*(11,48)*	*(11,48)*
P			*0.012*	*0.5*

Data were transformed (P'=arcsine√P) prior to ANOVA n=30. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$.Values are means of data in their original scale.

Callus obtained from MS medium with 0.5 mg/l TDZ and maintained on medium without growth regulators did not produce any regenerants. The white compact callus turned brown after 42 days of culture. A shoot regeneration frequency of 3.33% (Table 3) was obtained from callus induced on MS medium containing 1.0 mg/l TDZ for 42 days and later sub cultured on hormone free medium for 35 days (Plate 9 A). Calli induced on 2.0 mg/l TDZ for 42 days and later maintained on medium without growth regulators, regenerated shoots with a frequency of 6.67% after 35 days (Table 3).

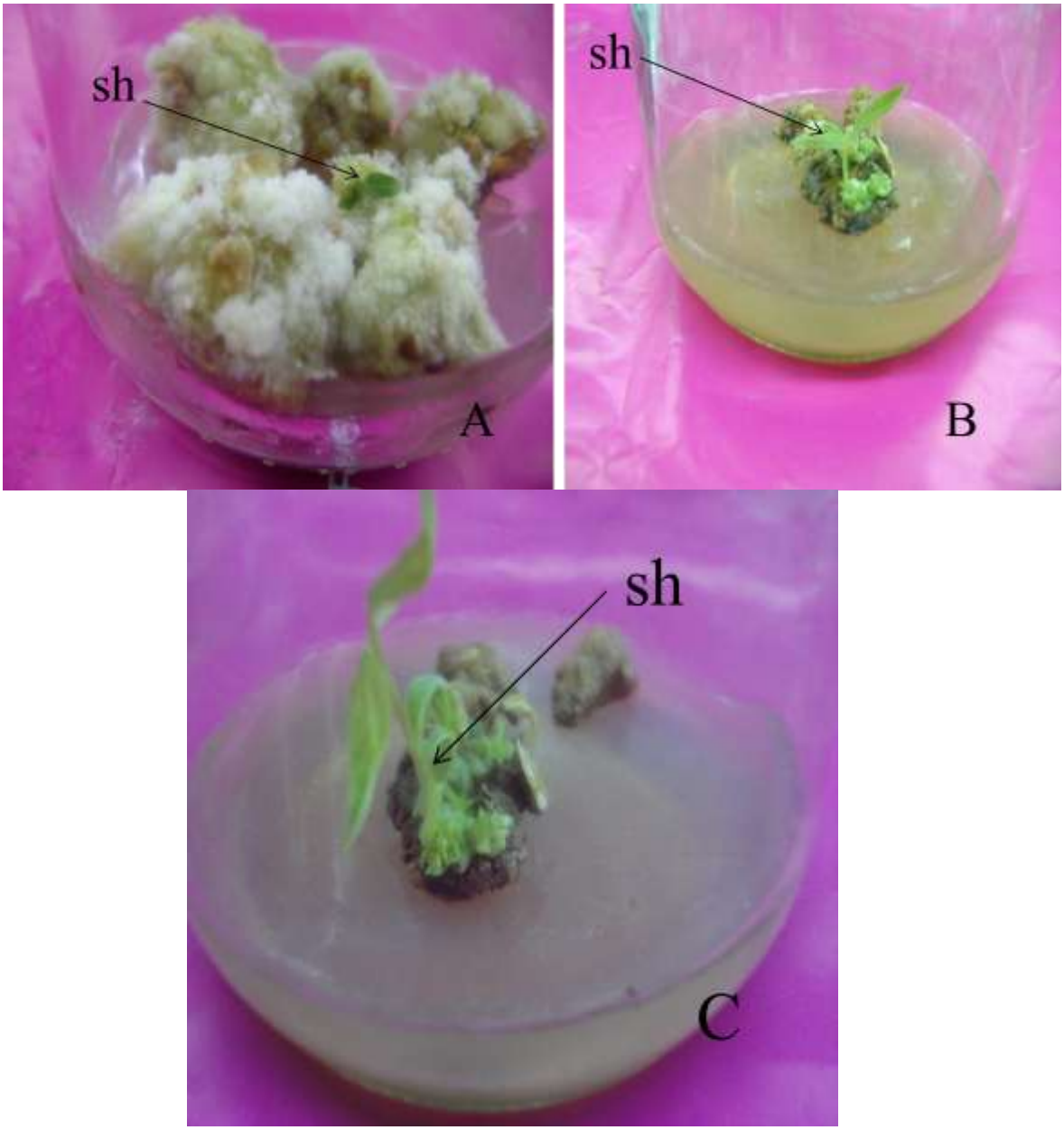

Plate 9: Shoot regeneration from zygotic embryo derived KAT 60/8 calli. (A) Thirty five day old embryo callus showing shoot (sh) regeneration on hormone free MS medium; (B) Forty nine day old callus displaying shoot (sh) regeneration on MS with 0.5 mg/l BAP; (C) Sixty three day old callus showing shoot (sh) regeneration on MS with 0.5 mg/l BAP.

Shoots were regenerated at a frequency of 3.33% from 42 day old callus induced on MS medium with 4.0 mg/l TDZ and sub cultured on MS medium containing 0.1 mg/l BAP for 30 days (Table 3). Callus induced on MS medium with 2 mg/l TDZ and maintained on regeneration medium consisting of MS with 0.1 mg/l BAP for 30 days, gave numerous shoot buds. The rest of the callus developed green patches but later browned to death. On MS medium containing 0.5 mg/l BAP, shoots were regenerated after 42 days, at a frequency of 13.33% from embryo callus developed on medium with 4.0 mg/l TDZ (Plate 9B& C). Besides this relatively higher (13.33%) shoot regeneration frequency, were numerous shoot buds. Shoot regeneration was significantly influenced ($p<0.05$) by the concentration of BAP. The Least Significant Difference test revealed that mean shoot regeneration on growth regulator free MS medium and 0.5 mg/l BAP, from callus induced on 2 and 4 mg/l TDZ respectively were different from other BAP concentrations.

ICEAP 00557 embryo explants maintained on MS medium with 0.5 to 4.0 mg/l TDZ did not produce any regenerants when transferred to hormone free regeneration medium for 56 days (Table 3). Expansion of callus was followed by necrosis in 21 days. Non-harvestable shoots were recorded at a frequency of 10% for embryo callus obtained from MS medium augmented with 4.0 mg/l TDZ and transferred to medium supplemented with BAP at 0.1 mg/l. The remaining calli enlarged and later became necrotic after 28 days. On MS medium with 0.5 mg/l BAP actively dividing calli increased in size and developed green patches in 7 days after which they turned brown without regeneration. Shoot regeneration from ICEAP 00557 embryo explants was not significantly ($p>0.05$) influenced by the concentration of BAP.

4.2.6 Shoot regeneration from leaf discs cultured on MS supplemented with TDZ

For KAT 60/8 calli induced on medium containing 0.5 mg/l TDZ, a regeneration frequency of 10% was achieved on growth regulator-free medium (Table 4). In addition, non harvestable shoots were also regenerated on the same medium (hormone free) after 21 days of culture. The rest of the explants and calli from 0.5 mg/l TDZ became necrotic between the 21st and 35th day of transfer to regeneration medium. Leaf calli from MS medium containing 1.0 to 4.0 mg/l TDZ only produced a white compact calli that began to brown from the point of contact with the hormone free medium without producing any regenerants by day 21. Leaf discs cultured on MS medium with 0.5 mg/l TDZ then transferred to regeneration media consisting 0.1 mg/l BAP gave numerous shoot buds arising directly from the leaf surface after 28 days of culture. The leaf discs cultured on 1.0 mg/l TDZ did not produce any regenerants when transferred to medium containing 0.1 mg/l BAP, but developed white compact calli that turned dark brown after 21 days in culture. However shoots were obtained at a frequency of 16.7 % after 28 days for calli induced on 2.0 mg/l TDZ for 42 days before transfer to regeneration medium containing 0.1 mg/l BAP (Plate 10). Shoot regeneration was not significantly (p>0.05) influenced by the concentration of BAP (Table 4).

Plate 10: Effect of BAP concentration on regeneration of KAT 60/8 calli illustrated by a fifty six day old leaf with shoot (sh) regenerants on 0.1 mg/l BAP.

For ICEAP 00557, leaf explants with callus transferred after 42 days from MS medium with 0.5, 1.0, 2.0 and 4.0 mg/l TDZ to medium without growth regulators did not produce any shoot regenerants. Those cultured onto MS medium with 2.0 mg/l TDZ for 75 days before transfer to hormone free MS gave clusters of somatic embryos after 14 days. Transfer to 0.5 mg/l BAP increased the size of the embryos but no germination was attained.

A shoot regeneration frequency of 20% was achieved for calli obtained using leaf explants cultured on MS medium containing 0.5 mg/l TDZ for six weeks before transfer to regeneration media containing 0.1 mg/l BAP (Table 4). The shoots (approximately 15mm) appeared while the explants were still on MS medium with 0.5 mg/l TDZ. Leaf explants with callus induced on both 2.0 and 4.0 mg/l TDZ produced shoots at a frequency of 6.67 % (Table 4) after transfer to regeneration media containing 0.1 mg/l BAP for 28 days (Plate 11A& B). In addition to the shoots originating from leaf explants transferred from 2.0 mg/l TDZ to MS containing 0.1 mg/l BAP, there were somatic embryo clusters displaying globular and heart shaped stages of development (Plate 12).

All initial leaf explants and callus transferred to medium with 0.5 mg/l BAP did not produce any regenerants (Table 4). Instead, the calli became necrotic after 28 days of transfer to regeneration medium. Shoot regeneration was significantly ($p<0.05$) influenced by the concentration of BAP, while the least Significant Difference test revealed that only mean shoot regeneration at 0.1 mg/l BAP from callus induced on 0.5 mg/l TDZ was different from other BAP concentrations (Table 4).

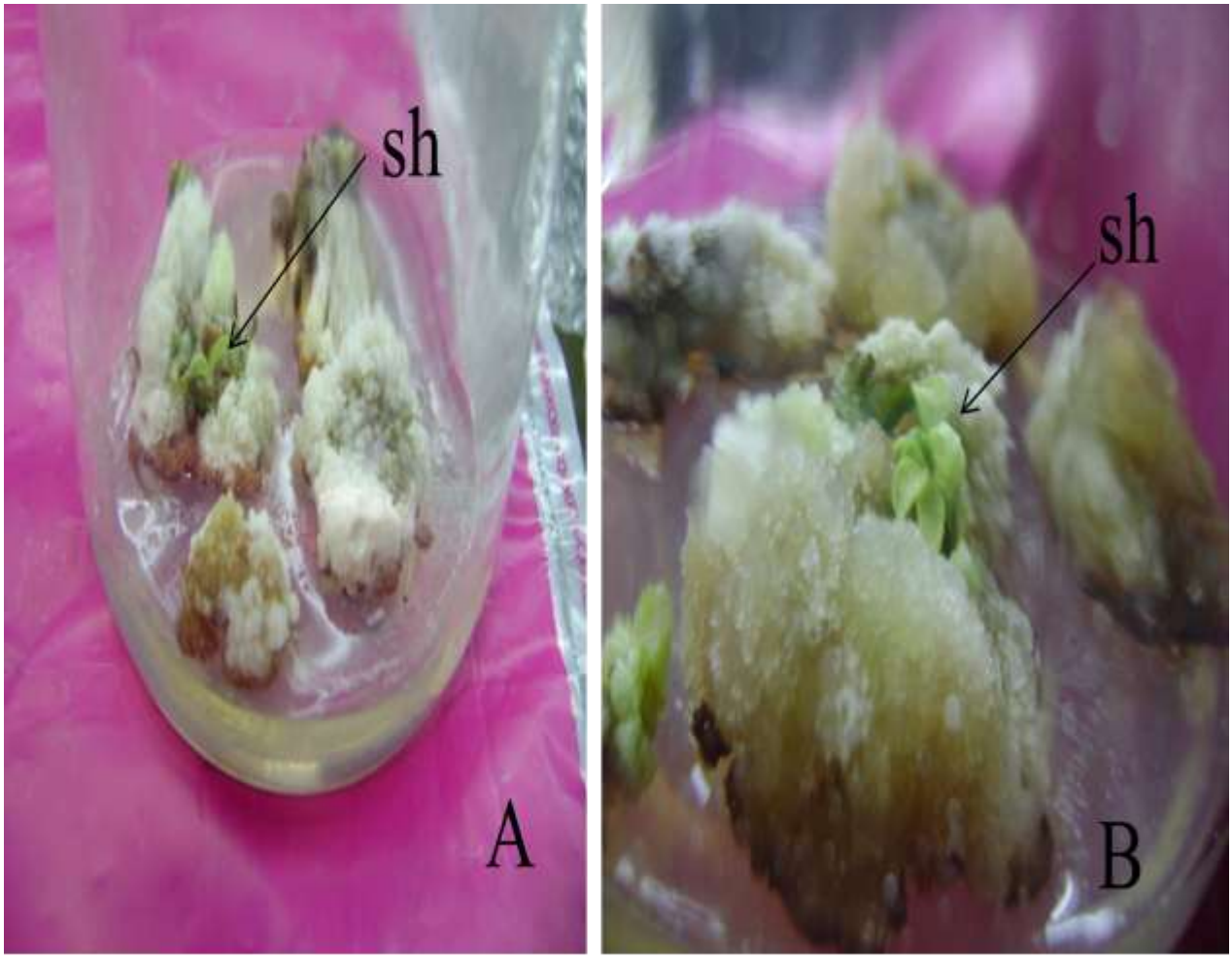

Plate 11: Effect of MS medium with 0.1 mg/l BAP on regeneration of pigeon pea var. ICEAP 00557 leaf explants. (A) Twenty eight day old cultures showing callus formation and shoot (sh) regeneration; (B) Forty two day old leaf callus with shoot (sh) regenerants.

Table 4: Effect of BAP on shoot regeneration from pigeon pea leaf calli induced on MS medium supplemented with TDZ.

Hormone (mg/l)			Shoot regeneration	
			KAT 60/8	ICEAP 00557
Conc. of BAP in regeneration medium	Conc. of TDZ in initiating medium	Total no. of callus pieces	Mean± S.E	Mean± S.E
0.0	0.5	30	0.10± 0.02**b**	0.00± 0.00**b**
0.0	1.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.0	2.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.0	4.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.1	0.5	30	0.00± 0.00**b**	0.20± 0.09**a**
0.1	1.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.1	2.0	30	0.17± 0.09**a**	0.07± 0.02**b**
0.1	4.0	30	0.00± 0.00**b**	0.07± 0.01**b**
0.5	0.5	30	0.00± 0.00**b**	0.00± 0.00**b**
0.5	1.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.5	2.0	30	0.00± 0.00**b**	0.00± 0.00**b**
0.5	4.0	30	0.00± 0.00**b**	0.00± 0.00**b**
F			*1.928*	*2.449*
d.f			*(11,48)*	*(11,48)*
P			*0.06*	*0.02*

Data were transformed (P'=arcsine√P) prior to ANOVA n=30. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$.Values are means of data in their original scale.

4.2.7 Shoot regeneration from cotyledonary explants

For KAT 60/8, cotyledonary nodes inoculated onto MS medium augmented with BAP and NAA at a ratio of 1.0:0.1 produced an average of 2.4 shoots per expant in 21 days. Those explants subjected to medium with BAP and NAA at a ratio of 0.5: 0.1 yielded a mean of 2.8 shoots per explant in 21 days while nodal explants inoculated onto medium with BAP and NAA at a ratio of 0.2: 0.1 produced an average of 1.7 shoots per explant.

For ICEAP 00557, cotyledonary nodes inoculated onto MS with BAP and NAA at a ratio of 1.0:0.1 produced a mean of 1.9 shoots per explant in 21 days. Explants on medium with BAP and NAA at a ratio of 0.5:0.1 produced a mean of 2.0 shoots per explant in 21 days while within the same duration nodal explants on medium with BAP and NAA at a ratio of 0.2:0.1 produced a mean of 2.2 shoots per explant.

4.3 Formation of somatic embryos from leaf callus

For ICEAP 00557, somatic embryos were obtained from leaf explants calli maintained on MS medium with 2 mg/l TDZ for 77 days. The clusters of somatic embryos appeared 28 days after the explants with callus were sub cultured onto growth regulator free medium (Plate 12A& B). Most of the calli were brown in spite of the frequent subcultures and addition of 10 mg/l silver nitrate in the medium. A microscopic view of the somatic embryos displayed different early stages of development ranging from globular to torpedo shaped (Plate 12 C). When the embryos were transferred to germination medium consisting of either MS or ½MS medium with or without 0.1 or 0.5 mg/l BAP, no germination was recorded.

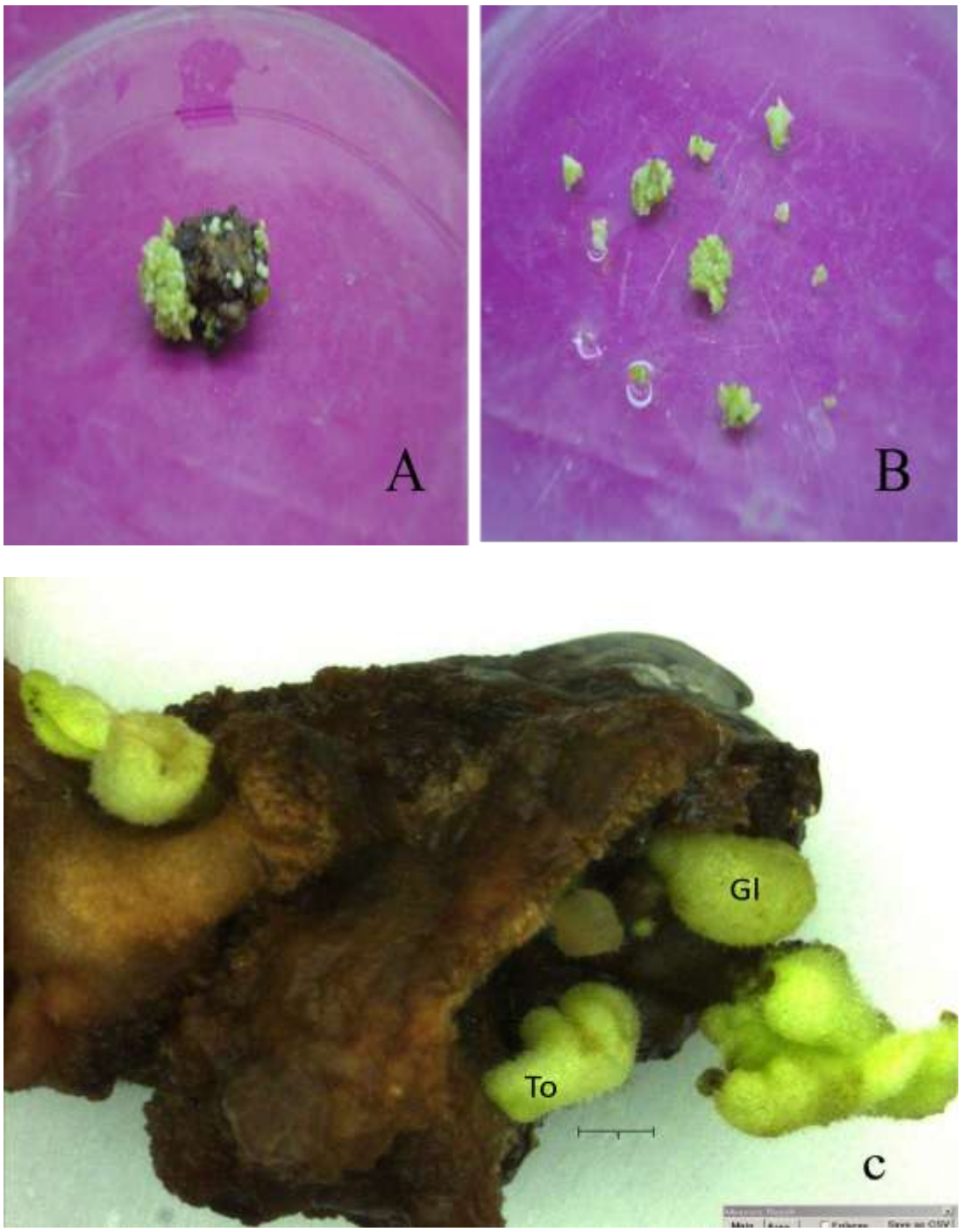

Plate 12: One hundred and twenty six day old ICEAP 00557 somatic embryos (A) Cluster of somatic embryos on leaf callus; (B) Isolated cluster and individual somatic embryos from leaf callus. (C) A magnified view of somatic embryos at different stages of development from leaf explant on medium without growth regulators: globular (Gl), Torpedo (To) (bar = 1 mm).

Leaf explants of KAT 60/8 callused on MS medium with 2 mg/l TDZ for 91 days before transfer to 0.1 mg/l BAP. The somatic embryos appeared on medium containing 0.1 mg/l BAP after 28 days of culture. Secondary somatic embryos were also observed on the same medium (Plate 13). The embryos were transferred to hormone free MS, ½MS and MS medium with 0.1 mg/l BAP for 56 days but did not germinate.

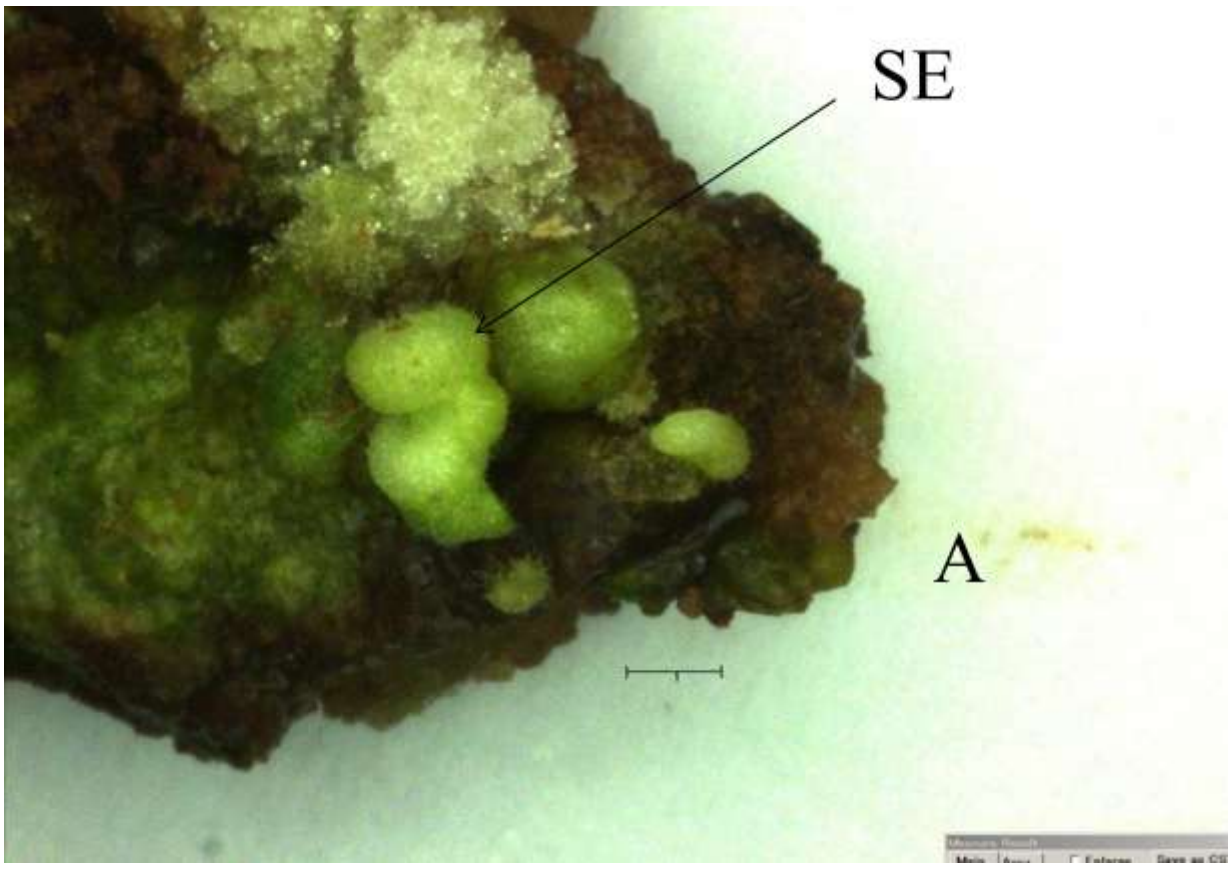

Plate 13: One hundred and nineteen day old somatic embryos from leaf explant of KAT 60/8 on MS with 0.1 mg/l BAP: secondary embryogenesis (SE) (bar = 1 mm).

4.4 Root regeneration

The shoots regenerated on MS medium with TDZ were elongated on 0.4 mg/l gibberellic acid (GA_3) and 100% were able to generate roots on MS with 0.1 -1.0 mg/l

IBA. However, 95% of the shoots subjected to rooting medium were obtained *in vitro* from cotyledonary nodes inoculated onto MS medium fortified with a combination of BAP (0.2, 0.5, and 1.0 mg/l) and NAA (0.1 mg/l) for 14 to 28 days.

Root development was achieved using MS medium supplemented with 0.1, 0.5 and1 mg/l IBA (Table 5). Shoots of KAT 60/8 planted in hormone free MS (control) displayed slow and scanty root development. However, the roots appeared normal. The roots were thin and began to appear between the 5^{th} and 12^{th} day and measured 5 cm to 9 cm long by the 18^{th} day. There were 1-2 roots per plant which had numerous lateral branches. Root induction from shoots of ICEAP 00557 on hormone free MS medium also produced 1 to 2 roots per plant by day 18. Root development started from the 5^{th} to 10^{th} day. The roots were thin and measured 4 to 10.5 cm long by the 18^{th} day. Besides, they were laterally branched (Plate 14A).

On MS medium supplemented with 0.1 mg/l IBA, KAT 60/8 plantlets had 1-3 roots per plant. The roots appeared by the 6^{th} day and measured 4 to 6 cm long by day 18.They appeared normal and were laterally branched. Laterally branched roots measuring 8 to 9cm long were also obtained from ICEAP 00557 shoots planted in MS medium with 0.1 mg/l IBA by the18^{th} day after culture. Between 1 and 7 roots per plant which appeared normal were recorded for ICEAP 00557 cultured on medium with 0.1 mg/l IBA (Plate 14B).

Profuse root induction was observed on MS medium augmented with 0.5 mg/l IBA for both KAT 60/8 and ICEAP 00557 shoots. For KAT 60/8 shoots, 2-11 roots appeared per plant by the 3^{rd} day. The roots measured 3 to 6 cm by day 18. They were thicker

than those induced on MS basal medium or supplemented with 0.1 mg/l IBA because of the callus on the surface of the roots and the relatively large diameter. They were laterally branched. By the 5th day, copious root development was noted from healthy shoots of ICEAP 00557 planted in MS medium with 0.5 mg/l IBA. The roots appeared at a frequency of 2 to 10 roots per plant by the 18th day. The laterally branched roots appeared abnormal (Plate 14C).

On MS medium at 1.0 mg/l IBA, 1 to 5 roots per KAT 60/8 plantlet were recorded. The roots measured 3 to 6 cm long by the 18th day and had callus on the surface. They were also laterally branched. ICEAP 00557 shoots on medium with 1.0 mg/l IBA had laterally branched roots possessing callus on the surface. The roots appeared by the 5th day of planting into the medium and by the 18th day, 0 to 7 roots per plant which measured 3 to 7cm long were recorded (Plate 14D).

Results reveal that for KAT 60/8, root regeneration was significantly (p<0.05) influenced by the concentration of IBA while the Least Significant Difference test showed that only mean root regeneration at 0.5 mg/l IBA was different from other IBA concentrations.(Table 5). On the other hand, induction of roots on ICEAP 00557 shoots was not significantly (p>0.05) influenced by the IBA concentrations.

Table 5. Effect of IBA on root induction of KAT 60/8 and ICEAP 00557 shoots.

PGR (mg/l)	Shoots Explants	Root induction KAT 60/8	ICEAP 00557
IBA		Mean± S.E	Mean± S.E
0	30	1.53± 0.37b	1.26± 0.62a
0.1	30	1.86± 0.65b	3.33±0.84 a
0.5	30	6.80± 0.44a	6.40±0.52 a
1.0	30	3.07± 0.56b	3.30± 0.34a
F		*6.38*	*2.69*
d.f		*(3,16)*	*(3,16)*
P		*0.005*	*0.081*

Data were transformed (P'=arcsine√P) prior to ANOVA n=30. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$.Values are means of data in their original scale.

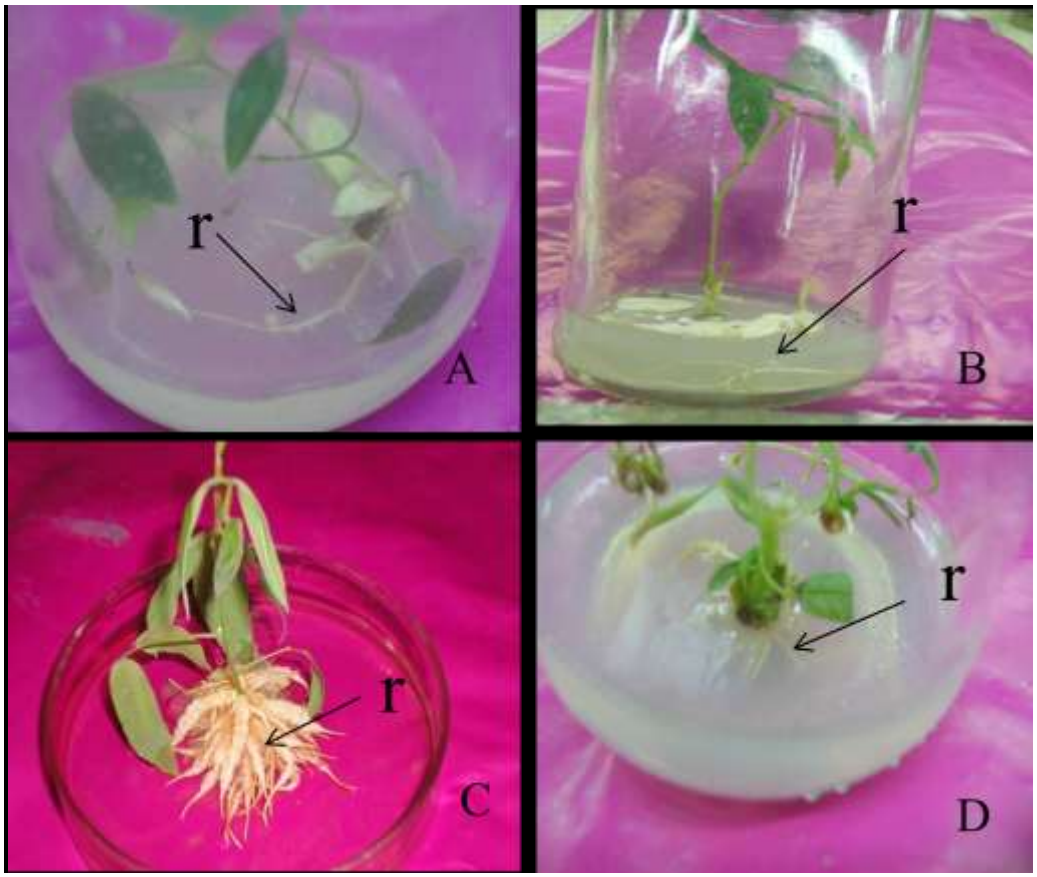

Plate 14: Root induction in pigeon pea var. ICEAP 00557 shoot explants on MS medium with IBA (A) Thirty five day old plantlet on hormone free MS medium: root (r); (B) Twenty one day old pigeon pea plantlet on 0.1 mg/l IBA: root (r); (C) Twenty one day old plantlet on 0.5 mg/l IBA: root (r); (D) Twenty one day old plantlet on 1.0 mg/l IBA: root (r).

4.5 Acclimatization of plantlets

Plantlets (30 plantlets for KAT 60/8 or ICEAP 00557) acclimatized on a mixture of soil and vermiculite (1:1) survived for 21 days (Plate 15). However, by day 6, a fungal infection at the soil line was observable. Despite the inclusion of the antifungal agent, bavistin (0.5% w/v), into the ¼ MS used for watering, the fungi could not be controlled.

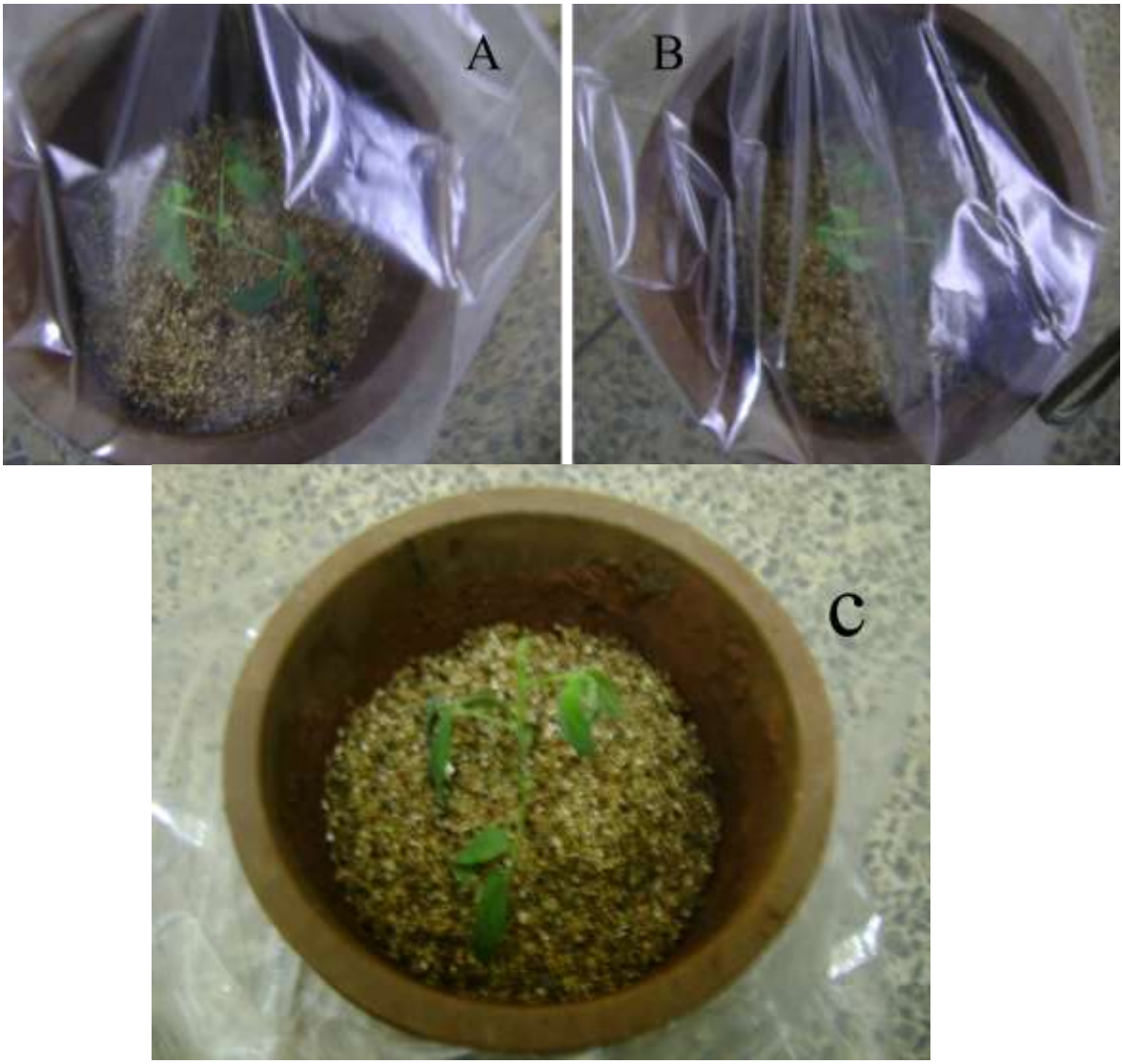

Plate 15: Response of pigeon pea plantlets on transfer to a mixture of vermiculite and soil. (A) Five day old plantlets of ICEAP 00557 in a mixture of vermiculite and soil; (B) Five day old plantlet of KAT 60/8 in a mixture of vermiculite and soil; (C) Fourteen day old plantlet of ICEAP 00557 in a mixture of vermiculite and soil.

The fungi spread along the plant height and as from the 10^{th} day of planting into the mixture of soil and vermiculite, the plantlet slowly withered to death.

CHAPTER FIVE

5.0 DISCUSSION

Callus induction from leaf and embryo explants of pigeon pea varieties KAT 60/8 and ICEAP 00557 on hormone free MS medium suggests that these two explant types had endogenous plant growth regulators (PGRs), probably auxins, at a level enough to induce calli in the absence of exogenous PGRs. However, lower callus induction frequencies (%) coupled with delay in callus initiation on hormone free MS medium, clearly indicates that the endogenous levels of the PGRs are fairly low. This observation is in agreement with the findings of Rao and Rao (1975) who reported presence of some endogenous levels of auxins in developing and growing seeds of an unspecified cultivar of *Cajanus cajan.* According to Chudasama and Thaker (2007), indole -3- acetic acid appears to be the most abundant endogenous auxin involved in plant growth and development. They investigated the changing levels of IAA in growing seeds of small and big seeded varieties of *Cajanus cajan* and suggested that IAA has a crucial role in seed growth.

Krishna *et al.,* (2011) in their experiments on *in vitro* regeneration through organogenesis and somatic embryogenesis using embryonic axis explants of JKR 105 variety of pigeon pea, suggested that subsidized shoot-bud formation on MS medium with BAP was due to the interaction of BAP with endogenous auxin concentrations which promoted calli regeneration. Therefore callusing of embryo and leaf explants of KAT 60/8 and ICEAP 00557 pigeon pea varieties on hormone free MS medium may be due to the endogenous levels of auxin present. Furthermore, interaction of exogenously applied IAA with cytokinins such as kinetin has been utilized for high frequency callus

induction (Prabhakaran *et al*., 2011). In contrast to the observations made in the experiments described herein this thesis, Thu *et al*., (2003) reported lack of callus induction from embryo axes isolated from sterilized seeds of ICPL 87 pigeon pea variety germinated on MS medium. The embryo axes and mature seeds were cultured on hormone free B5 medium supplemented with 3% sucrose and 0.3% phytagel. The embryos turned brown and died a few days after culture while the mature seeds produced normal seedlings on the medium.

Relatively high frequencies of callus formation (between 80 and 100%) were recorded for embryo explants of KAT 60/8 and ICEAP 00557 pigeon pea varieties cultured on MS medium with 2, 4- D. These results are similar to those reported by Prabhakaran *et al*., (2011) where 90% callus formation using MS medium containing 1.0 mg/l 2, 4-D, with epicotyls and cotyledons as explants of unspecified variety of pigeon pea were achieved; although they did not proceed to regeneration of plantlets from the calli.

Callus formation frequencies of 93.33% to 100% were recorded for leaf explants of pigeon pea KAT 60/8 on MS medium with 2, 4 – D, while relatively lower frequencies of 60- 93.33 % from leaf explants of pigeon pea ICEAP 00557 were noted in the same medium, with 1 mg/l 2,4 –D producing the highest percentage of callus . On the other hand, high frequencies (86% to 100%) of callus induction from embryo explants of the two pigeon pea varieties on MS medium containing different concentrations of TDZ were obtained. These are clear indications that callus induction for the two pigeon pea varieties is dependent on the genotype, explant type and hormone concentrations. This is in agreement with reports by Aboshama (2011) of ICPL 87-118 and ICPL 151 cultivars of *Cajanus cajan* indicating that the response of a plant to *in vitro* regeneration

is influenced by the genotype, type and concentration of growth regulator. In his experiments the effect of genotypes showed that ICPL 87- 118 yielded somatic embryos in addition to white compact calli that significantly increased more than ICPL 151. In addition to callus initiation, lower concentrations of TDZ e.g. 0.5 mg/l also induced direct shoot organogenesis. This is in line with reports by Aboshama (2011) in which prolonged culture of cotyledonary explants of pigeon pea variety ICPL 151 in medium containing TDZ gave a similar response.

Differences observed in the number and size of shoots from ICEAP 0057 and KAT 60/8 zygotic embryos inoculated onto MS with increasing concentrations of TDZ (0.5 to 4.0 mg/l) could be attributed to the effect of increased hormone accumulation in the explant. This observation has been attributed to the inability of the explant to degrade TDZ which is highly active and stable (Mok and Mok, 2001). Ugandhar *et al*., (2012) reported a significant decrease in the number of shoots from zygotic embryo explants of ICPL 87119, pigeon pea cultivar as the concentration of TDZ increased from 4.0 to 5.0 to 6.0 mg/l, while in lower concentration of TDZ (1.0 to 3.0 mg/l) the number of shoots increased.

Both 2, 4 – D and TDZ initiated callus development but callus obtained from MS medium fortified with 2, 4 - D did not regenerate to form shoots except from medium supplemented with 1.0 mg/l 2, 4 D giving a regeneration frequency of 6.67%. TDZ was superior to 2,4- D by producing more regenerants in form of somatic embryos, harvestable and non harvestable direct shoots which arose either from callus or directly from the surface of the explant.

Difficulties to regenerate plants from callus of both varieties obtained from the 0.5, 1.0, 2.0 and 4.0 mg/l 2, 4-D could be due to lack of morphogenic callus. This is contrary to reports by Brown *et al*., (1995) suggesting that 2, 4 –D was the most effective auxin for induction of somatic embryogenesis for many plants species. Eapen and George (1998) also demonstrated that 2,4- D is the most effective among the auxins (NAA, 2,4- D and picloram) tested for somatic embryogenesis in pea nut. On the other hand genotypic variation may have contributed to the failure to produce regenerants from calli induced using 2, 4-D, since somatic embryos have been reported in other genotypes (ICPL 87-118 and ICPL 151) using 2, 4- D or TDZ (Aboshama, 2011). Regeneration of somatic embryos from leaves of both varieties on 2.0 mg/l TDZ is a response usually initiated by suitable concentrations of high auxin or auxin/ cytokinin combinations (Aboshama, 2011). From the results obtained in the experiments reported herein in this thesis, it is evident that TDZ displayed an auxin-like activity by aiding in the production of embryogenic calli. This confirms reports by Aboshama (2011) that TDZ has both cytokinin and auxin like activity promoting cell division and differentiation in addition to inducing embryogenic competence in callus tissue.

Successful regeneration of pigeon pea, a recalcitrant legume, through somatic embryogenesis, was described for the first time by Kumar *et al*., (1998) from cotyledon and leaf explants of Pusa 606 cultivar following an earlier attempt by Nalini *et al*., (1996) who reported globular embryos that failed to produce plantlets from callus induced on immature leaflets of ICPL 87. The somatic embryos obtained in this study were similar to those described by Aboshama (2011) from ICPL 151 and Kumar *et al.*, (1998) using Pusa cultivars, although no germination was achieved on the different

media tested. The failure to regenerate the somatic embryos could be attributed to lack of maturation treatment with abscisic acid as reported by Aboshama (2011), Mohan and Krushnamurthy (2002). In their experiments, maturation treatment with abscisic acid increased the number of normal germinated somatic embryos significantly.

Regeneration of roots on MS medium without growth regulators in this study, is an indication of auxins produced in shoots and transported downwards to induce roots (Overvoorde *et al.,* 2010). Therefore, the shoots can form roots in the absence of exogenous auxins though in low frequencies. The delay (2 to 5 days longer) in emergence of the roots in growth regulator-free media could be attributed to low concentrations of the endogenous auxins. Hormone free MS liquid medium has been used for *in vitro* root elongation of Hyderabad C cultivar of *Cajanus cajan* (Geetha *et al.*, 1998). The addition of exogenous IBA accelerates the appearance of roots on pigeon pea shoots. Roots developed in MS medium containing IBA (0.1 mg/l) appeared normal like those of MS medium without hormones but developed faster (3 to 4 days earlier) than those in hormone free MS medium. Profuse rooting with callus on the surface of the roots obtained on MS medium supplemented with 0.5 mg/l IBA and 3% sucrose was similar to that reported on JKR 105 pigeon pea cultivar by Krishna *et al.*, (2011). In their rooting experiments, lower concentrations of sucrose (<1.5 %) in the medium caused leaf yellowing due to carbon deficiency. Prolific rooting has also been reported from some pigeon pea genotypes (VBN1, VBN2, SA1 and CO5) on the same medium by Franklin *et al.,* (2000). The micro shoots in their experiments rooted best on ½MS + 0.5 mg/ l IBA with a maximum 6-7 healthy roots with fast growth and secondary branching, followed by ½MS + 1.0 mg/l IBA. This is contrary to reports by

Prasad *et al*., (2011) who indicated maximum root induction frequency at 1.0 mg/l IBA for shoots of LGG-29 pigeon pea cultivar and this variation could be attributed to the effect of genotype.

While KAT 60/8 and ICEAP 00557 pigeon pea varieties recorded best rooting on MS medium with 0.5 mg/l IBA and lower frequencies on hormone free MS medium, Prasad *et al*., (2011) reported maximum root induction frequencies on MS medium supplemented with 1.0 mg/l IBA for pigeon pea var. LGG-29 and no root regeneration on hormone free MS medium and at 0.5 mg/l IBA. This clearly indicates that rooting ability varies in different cultivars. In contrast, Geetha *et al*., (1998) reported an optimum IBA concentration of 0.2 mg/l for root regeneration of Hyderabad C cultivar of pigeon pea beyond which the frequency decreased.

The withering recorded on acclimatized plantlets of KAT 60/8 and ICEAP 00557 pigeon pea cultivars could be attributed to the soil fungus spread along the plant height from the sixth day. The fast infestation of the fungus could be ascribed to a lot of moisture resulting from frequent watering.

This is the first account of *in vitro* regeneration of pigeon pea cultivar KAT 60/8 as there is no documented evidence of either direct or indirect regeneration. Direct regeneration of ICEAP 00557 pigeon pea variety has been reported but there is no documented evidence of regeneration through somatic embryogenesis.

5.1 Conclusion

- The study showed that for induction of embryogenic calli from leaf explants, MS medium with 2 mg/l thidiazuron (TDZ) was optimum.
- For regeneration of embryogenic calli into plantlets 0.1 -0.5 mg/l BAP was necessary.
- The study demonstrated that leaf explants respond better than mature zygotic embryos in generating embryogenic calli for both KAT 60/8 and ICEAP 00557 pigeon pea varieties.

5.2 Recommendation

This protocol can be useful for genetic transformation of pigeon pea KAT 60/8, a variety released by Kenya Agricultural Research Institute, Katumani which is highly affected by the pod sucking bugs leading to major yield losses. It can also be used to introduce other genes of stress resistance like diseases and drought. However, further research needs to be done to increase the frequency of shoots, germinate the somatic embryos and raise the plantlets to maturity.

Furthermore, optimization of media for *in vitro* regeneration of the varieties used in this study is an important component of an efficient protocol.

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