IN VITRO REGENERATION OF GRASS PEA (*Lathyrus sativus* L.) FROM COTYLEDONARY NODE EXPLANTS

MSc. THESIS

BY

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JIMMA UNIVERSITY

*IN VITRO*REGENERATIONOF GRASS PEA (*Lathyrus sativus* L.) FROM COTYLEDONARY NODE EXPLANTS

MSc. Thesis

Submitted to the School of Graduate Studies Jimma University College of Agriculture and Veterinary Medicine In Partial Fulfillment of the Requirements for the Degree of Masters of Science in Plant Biotechnology

By

Diriba Tesfaye

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DEDICATION

This thesis is dedicated to my beloved mother Werkitu Eda’a.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my confide work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillments of the requirements for MSc. degree in Plant Biotechnology at Jimma University and is deposited at the University Library to make available to borrowers under the rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

Diriba Tesfaye was born on April 27, 1987 in Agarfa twon, Bale zone, Oromia regional state. He attended his 1st cycle primary school at Asano primary school and 2nd cycle at Weib elementary and junior school from 1996 to 2003. He attended his secondary school at Agarfa senior secondary and preparatory school from 2004 to 2007. He joined Madawalbu University in November 2007 and graduated with BSc. degree in Plant Science in July, 2010. After graduation he was employed by Ministry of Education and served as an assistant graduate at Jigjiga University for about a year. He joined the school of graduate studies of Jimma University College of Agriculture and Veterinary Medicine in September 2011 to pursue his M.Sc. degree in Plant Biotechnology.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
CRD	Completely Randomized Design
DNMRT	Duncan's New Multiple Range test
EDTA	Ethylene Di-amine Tetra acetic Acid
IAA	Indole -3- Acetic Acid
IBA	Indole-3- Butyric Acid
Kn	kinetin
JUCAVM	Jimma University College of Agriculture and Veterinary Medicine
MS	Murashighe and Skoog
NAA	α-Naphthalene Acetic Acid
ODAP/BOAA	β-N-oxalyl-L-αβ -diaminopropionic acid
PGRs	Plant Growth Regulators
SAS	Statistical Analysis Software

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IN VITRO REGENERATION OF GRASS PEA (*Lathyrus sativus* L.) FROM COTYLEDONARY NODE EXPLANTS

ABSTRACT

Grass pea (Lathyrus sativus) have many valuable characteristics and its cultivation requires the least crop management but, its full potential has not been utilized because of the presence of the neurotoxic amino acid β-N-oxalyl-L-αβ -diaminopropionic acid (ODAP) which causes neurolathyrism in human beings on prolonged consumption. Conventional breeding practices and other approaches explored to date have not been successful in considerably reducing the toxin. Therefore, integration of in vitro techniques such as somatic hybridization, somaclonal variation and genetic transformation can contribute significantly to meet the challenge. Hence, the present study was carried out to evaluate the in vitro regeneration capacity of some grass pea genotypes and eventually to optimize protocol for in vitro propagation of the crop as in vitro regeneration capacity is prerequisite to alleviate the ODAP problem through in vitro techniques. In this study, three experiments were conducted: cotyledonary node shoot initiation of four genotypes (IVATLS-LS -B2, IVATLS-LS –B1, IVAT-LS- 690, and IVAT-LS-655),on MS media withthree concentrations of BAP (1, 2 and 3mg/l) + 0.1mg/lNAA were tested whereas for in vitroshoot multiplication, the combination effects of four levels of BAP (1, 2, 3, and 4 mg/l) and Kn(0,1,2 and 3mg/l) were used for the selected genotype (IVAT-LS-690 and for in vitro rooting, IBA and IAA alone with four concentrations each (0.1, 0.25, 0.5 and 1.0mg/l) were evaluated on half strength MS medium. Among the four genotypes tested for shoot initiation, shoot nitiation percentage was the highest (100%) for IVAT-LS-690,on MS medium augmented with 2 mg/l BAP +0.1mg/lNAA. With regards to the in vitro shoot multiplication of IVAT-LS-690, 3mg/l BAP+1mg/l Kn gave maximum shoot number per explant (11.5), and longest shoot (5.03cm). For in vitro rooting of this genotype, best result for percent of rooted shoot (86.66%), high number of roots per shoot (6) and longest root (4.9cm) were obtained from half MS medium supplemented with 0.5mg/l IBA and these rooted plantlets were acclimatized and established in soil. From this study it could be inferred that both genotype and BAP levels play crucial role for shoot regeneration capacity and the optimum hormonal combination for grass pea is genotype specific. MS media supplemented with BAP and Kn(3mg/l BAP+1mg/l) for shoot multiplication and half -MS medium supplemented with 0.5g/l IBA for rooting could be used for in vitro propagation of IVAT-LS-690 genotype. Therefore, this work could be used as a baseline for further studies on in vitro technique of grass pea aiming at meeting the challenge of ODAP.

1. INTRODUCTION

Grass pea (*Lathyrus sativus* L.) belongs to the Leguminosae family. It is one of the 150 species in the genus *Lathyrus* and diploid with a chromosome number of 2n =14 (Smartt, 1990). The crop is an annual climbing herbaceous with stems ranging from 20 to 90 cm in length and pinnately veined leaves with usually two leaflets. The flowers are solitary, auxiliary and are borne on long peduncles with corollas ranging from 12 to 24 mm in length and are reddish-purple, pink, and blue or white (Campbell and Clayton, 1997).

Several *Lathyrus* species and in particular *Lathyrus sativus* (Grass pea) have great agronomic potential as grain and forage legume, especially in drought conditions. Grass pea is rightly considered as one of the most promising sources of calories and protein for the vast and expanding populations of drought-prone and marginal areas of Asia and Africa (Girma, 2010). It is virtually the only species that can yield high protein food and feed under these conditions. It is superior in yield, protein value, nitrogen fixation, and drought, flood and salinity tolerance than other legume crops (Barik *et al.,* 2005).*Lathyrus* species have a considerable potential in crop rotation, improving soil physical conditions, reducing the amount of disease and weed populations, with the overall reduction of production costs (Vaz Patto *et al*., 2005). In addition, it is an efficient nitrogen fixer and improves soil fertility by adding around 67 kg/ha of nitrogen in a single season, thereby conferring yield and protein benefits for the subsequent non-legume crop (Wang *et al*., 2000).

In Ethiopia, grass pea is commonly cultivated as food and feed crop because of its resistance to drought, flood and moderate salinity (Wuletaw, 2003). It performs well under adverse agricultural conditions, and its many cultivars possess different attributes including the ability to resist both drought and flooding, high climatic adaptability and the ability to grow in cool climates and at high altitudes (Tiwari *et al*., 1996).

It is the fifth most important pulse crop in Ethiopia following faba bean (*Vicia faba*), field pea (*Pisum sativum*), haricot bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum*) (Dejene and

Lijalem, 2012). It occupies 8.7% of the total area and 7.6% of the total production of food legumes in Ethiopia (Wuletaw and Endashaw 2001). Despite, the problem caused by the neurotoxin b-N-oxalyl-L-a,b diaminopropionic acid (ODAP), the area for grass pea cultivation has increased recently(159,731 ha), exceeding the total area occupied by the highly expensive pulse crop, lentil (94 946 ha)(CSA, 2010)**.** This suggests that the crop is becoming more important than ever in the farming system. It's production is mainly concentrated in the North West Zone (58%), the Central Zone (16.3%), the North East (12%) and the Northern as well as the Southeast (12.9%) parts of Ethiopia (Wuletaw and Endashaw, 2001).

Nutritionally, seeds of grass pea contain 18.2–34.6% protein, 0.6% fat, 58.2% carbohydrate (about 35% starch), 1.5% sucrose, 6.8% pentosans, 3.6% phytin, 1.5% lignin, 6.69% albumin, 1.5% prolamineand 13.3% globulin, (Williams *et al*., 1994). According to Muehlbauer and Abebe, 1997, the essential amino acids found in grass pea seeds are (in grams per 16 grams of nitrogen): arginine7.85, histidine 2.51, leucine 6.57, isoleucine 2.34, tryptophan 0.40, and valine 4.68.

Although its cultivation requires the least crop management and the crops can provide a lot of benefit, its full potential has not been utilized because of the presence of the neurotoxic amino acid β-N-oxalyl-L-αβ -diaminopropionic acid (ODAP/BOAA) which causes neurolathyrism in human beings on prolonged consumption (Yigzaw *et al.*,2001) .

According to Dejene and Lijalem (2012), in Ethiopia different attempts were made to meet the challenges of ODAP.These include hybridization programmes aimed at toxin-free or low toxin content varieties, introductions of low-toxin or toxin-free varieties from international source, and collection, characterization and evaluation of local farmer's varieties (landraces) for low toxin content. All these approaches were mainly based on conventional breeding methods and have not been successful in developing a variety with low ODAP content.

Following the advent of modern approach for crop improvement different researches were done to solve the problem of ODAP. Zambre *et al*.,(2002)found high amounts of somaclonal variation for ODAP contents through *in vitro* selection techniques. An *in vitro* protocol for fast

production of advanced progenies significantly shortening generation cycles has also been developed in grass pea (*L. sativus*), over three generations per year can be obtained instead of the normal two allowing a faster progress in the *Lathyrus* improvement through assisting conventional breeding (Ochatt et al., 2002). Sachdev *et al*.,(1995)cloned a coding sequence with ODAP-metabolizing properties from a soil microbe which will be useful for genetic transformation. However, Vaz Patto *et al*.,(2006), reported that genetic transformation using *Agrobacterium tumefaciens* as a vector tried repeatedly has resulted in limited success due to the absence of *in vitro* plant regeneration protocol without an intermediate callus phase. Considering the absence of an adventitious plant production system in grass pea, an alternative callus-free regeneration could be achieved using cotyledonary node explants, which has already been exploited for *Agrobacterium*-mediated transformation of several grain legumes including kidney bean (Babaoglu *et al.*, 2000).

To develop grass peas varieties that have low ODAP content, *in vitro* techniques such as somaclonal variation have created new avenues that can create genotypes with low ODAP levels. However, to exploit the potential of somaclonal variation, testing the *in vitro* regeneration capacity of genotypes is crucial. Therefore, this work was done with the following objectives:

General Objective

- To evaluate the *in vitro* regeneration capacity of grass peas (*Lathyrus sativus* L.) genotypes

Specific Objectives

- To evaluate the *in vitro* regeneration capacity of cotyledonary node explants of four grass pea (*Lathyrus sativus)* genotypes
- To determine the suitable cytokininis (BA &KN) and the optimum concentration for *in vitro* shoot development of selected genotype
- To determine the effect of different auxins (IAA &IBA) and their concentration on *in vitro* rooting of selected genotype

2. LITERATURE REVIEW

2.1. Taxonomy, Botanical Description, Origin and Distribution

2.1.1. Taxonomy

Grass pea (*Lathyrus sativus* L.) is belonging to the family Leguminosae (Fabaceae), subfamily Papilionoideae, tribe Vicieae It is one of the 150 species in the genus *Lathyrus*, and it is a diploid with a chromosome number of 2n = 14(Campbell, 1997).Other economically important species include *Lathyrus cicera* and *L. tingitanus* for grain and *L. ochrus, L. latifolius* and *L. sylvestris* as forage species (Dejene and Lejalem, 2012).

2.1.2. Botanical Description

Lathyrus sativus is a much-branched straggling or climbing, herbaceous annual, with a well-developed taproot system, the rootlets of which are covered with small, cylindrical, branched nodules usually clustered together in dense groups. The stems are slender, 20-90 cm long, quadrangular with winged margins. Stipules are prominent, narrowly triangular to ovate with a basal appendage. The pinnate leaves are opposite, consisting of one or two pairs of linear-lanceolate leaflets, and a simple or much-branched tendril. Leaflets are entire, sessile, cuneate at the base and acuminate at the top (Davies *et al*., 1993).

The flowers are axillary, solitary, about 1.5 cm long, and may be bright blue, reddish purple, red, pink, or white. The peduncle is 3.0-5.0 cm long with two minute bracts. Flowers have a short and slender pediceland the crop is self pollinated with 5-10% out crossing.Pods are oblong, flat, slightly bulging over the seeds, about 2.5-4.5 cm in length, 0.6-1.0 cm in width and slightly curved. The dorsal part of the pod is 2-winged, shortly beaked and contains 3-5 small seeds. Seeds are 4-7 mm in diameter, angled and wedge-shaped. Color is white, brownish-grey or yellow, although spotted or mottled forms also exists (Defalco *et al*., 1991).

2.1.3. Origin and Distribution of Grass pea

The *Lathyrus* genus is believed to have originated in southwest and central Asia, with a significant subsequent spread to the east of the Mediterranean basin (Smartt J. 1990). However, the exact location was unknown as it was thought that the natural distribution had been completely obscured by cultivation. Even though southwest and central Asia, its presumed centre of origin now it is suggested that the crop was originated in the Balkan Peninsula. All Grass pea lines appear to divide into two geographical origins - one group derives from the Indian subcontinent, and another from the Mediterranean/European region, which typically has higher yields and larger seeds (Hanbury *et al.*, 2000). Morphologically, this crop resembles field pea, but its leaflets are long and grass-shaped rather than rounded, and it has a deep taproot system (Tiwari1996). This species is now widely distributed throughout Eurasia, North America, temperate South America and East Africa with a small amount being cultivated in Australia (Siddiqu et.al 996).

Grass pea grows in several tropical and subtropical areas of the world including Iraq, Iran, Afghanistan, Syria, Lebanon, India, Pakistan, Bangladesh, Ethiopia, Algeria, Egypt, Libya, Morocco, France, Spain, North America and temperate South America. The crop has different local names worldwide: Grass pea, Chickling pea, Indian vetch (UK and N. America), Almorta (Spain), Khesari or Batura (India), Alverjas (Venezuela), Gilban (Sudan), Guaya (Ethiopia), Matri (Pakistan), Gesette (France) and Pisello bretonne (Italy) (Campbell and Clayton 1997).

2.1.4. Growth conditions

In general grass pea is a crop that grows well under the high temperatures of the subtropics as a winter crop. It is therefore best adapted to areas with arid or semiarid conditions. Because of the wide range adaptability of the crops the optimum temperature and rain fall is not well known so far (Abd El Moneim *et al.*, 2001). It is adapted to grow under drought stress conditions, useful throughout the arid regions of China,South Asia, Middle East and North Africa. Despite its tolerance to drought, it is tolerant to excessive rainfall and can be grown on land subject to flooding (Kaul *et al.*, 1986; Campbell *et al.*, 1994). In addition to these its capacity to withstand moderate salinity has been recognized (Campbell *et al.*, 1994; Hoque *et al.*,2006*)*.

2.2. Acreage of Grass pea production

Grass pea is amongst the principal grain legumes cultivated by smallholder farmers in Ethiopia. On average, it occupies 9% of the annual acreage of all pulses cultivated in the country. It is the fifth most important pulse following faba bean (*Vicia faba*), field pea (*Pisum sativum*), haricot bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum*). Despite the problem caused by the neurotoxin ODAP, the area for grass pea cultivation has increased (159 731 ha) exceeding the total area occupied by the highly expensive pulse crop, lentil (94 946 ha) (CSA 2010).

2.3. Importance of Grass pea

2.3.1. Human consumption

In Ethiopia, particularly in the northern regions, *teff*, wheat, barley, maize and sorghum, either singly or in combination, are used to produce fermented, sour pancake-like unleavened bread called *enjera*. *Lathyrus* grain is ground into *shiro* and is used in the preparation of *wott*, a sauce that is eaten together with the *enjera.* For snacks, cereals, legumes or their mixture are most often consumed roasted or boiled. Boiled grass pea (*nifro*) is consumed in most areas. *Kitta*, and unleavened bread made from grass pea, is consumed to a more limited extent, mainly at times of acute food shortages (Tekele-Haimanot *et al.* 1993).

2.3.2. Animal feed

The crop is an annual legume commonly grown for its grain, but also used for fodder or green manure. The vegetative types are utilized in the production of fodder or forage for animals. The young plants are used as a fodder for cattle or for grazing, as in Ethiopia.Normally, the fields are allowed to be strip-grazed by cattle before the crop is allowed to grow and then harvested for seed. *Lathyrus* has great potential as a fodder crop. Gowda and Kaul (1982) reported that in studies at Bari, Joydepur, fodder yields of 7-10 t/ha were obtained in intercropping with maize, without affecting the grain yield of the maize

2.3.3. Agronomic importance

There are around 187 species and subspecies in the *Lathyrus* genus. Grasspea is the only member of this genus that is widely cultivated as a food crop, while *L. odoratus* (sweet pea) is grown commercially for its flower morphology (Razdan *et al.*,2006). Grasspea performs well under adverse agricultural conditions, and its many cultivars possess different attributes including the ability to resist both drought and flooding, high climatic adaptability and the ability to grow in cool climates and at high altitudes (Tiwari and Campbell 1996). These crops also have the ability to adapt to saline, alkaline, clay or otherwise poor soils, and are hardy and easy to cultivate (Sinha *et al.,* 1983).In addition to nutritional benefits, Grasspea has an important role as a legume crop in crop rotations, reportedly adding around 67 kg/ ha of nitrogen to the soil in a single season and conferring yield and protein benefits on the subsequent non-legume crop (Wang *et al.,* 2000).

2.4. Limitations of Grass pea Productionand Effort Made

The main limitation of grass pea is the neurotoxin β-*N*-oxalyl-L-α,β-diaminopropanoic acid (ODAP). If the seeds are consumed as a major part of the diet for an extended period, irreversible crippling can occur. This will remain a major limiting factor in the production of this valuable crop until now. Historically, the main objectives of grass pea breeding in Ethiopia were to develop high-yielding varieties with <0.2% ODAP content, improve crop management practices and boost agronomic performance. These objectives have remained the anthem of all grass pea genetic improvement research in the country until now (Dejene and Lijalem, 2012). To meet these objectives, several research approaches have been proposed. Wuletaw *et al*., 1995 suggested the following research directions, hybridization programmes aimed at toxin-free or low toxin content varieties, adaptation tests of introductions with low-toxin or toxin-free varieties from international sources, collection, characterization and evaluation of local farmer's varieties.

In spite of the continued efforts made over the years within the context of the objectives for grass pea genetic improvement research in Ethiopia, the mission for grass pea varieties with low and stable ODAP content *via* conventional crop improvement has not been successful until now. The past 50 years of on-station and on-farm research was able to

produce only a single grass pea variety known as Wasie. This variety was officially released in 2006 for production in Ethiopia (MoARD 2008). According to studies conducted at Debre Zeit Agricultural Research Center, the ODAP content of this variety is 0.08%, which is five times lower than that of the local variety. However, the release of this variety has been controversial with the variety remaining still shelved because no efforts for its multiplication and dissemination to farmers have been made. Moreover, reports in the literature indicate that the ODAP content of Wasie has increased in subsequent field and laboratory tests. This is not any surprising because the trait is highly influenced by the environment (Dejene and Lijalem 2012).

2.5. *In vitro* Techniques for Genetic Improvement of Grass pea

2.5.1. *In vitro* Propagation

In vitro plant tissue culture involves regeneration of entire plants from pieces of plant tissues or explants because of tot potency, i.e. capability to give rise to new identical plants. It has various applications in plant improvement and disease eradication. The success of plant tissue culture depends on the type of plant material, i.e. dicotyledons regenerate better than monocotyledons, and gymnosperms have very limited regenerative capacity except when juvenile. There are even differences within single species where age, type and size of explants can influence plant responses. Nutritional media, environment and plant growth regulators also affect the success of plant tissue culture (Razdan, 2002).

In general Legumes are notoriously recalcitrant to tissue culture and the most prevalent mode of regeneration in grain legumes has been reported *via* direct organogenesis from cotyledonary node explants. Shoot apices, leaflets and embryo axes were also common regeneration pathways for direct organogenesis. Several reports indicated that most of the explants are responsive to cytokinins, especially BAP, Kn and TDZ. Regeneration *via* callus has been poor in many legumes though some legumes like soybean and pea shoots were recovered from callus tissue at a low frequency (Atika and Deepak, 2003).

According to Ochatt *et al.,*2001 the optimum hormonal combination for regeneration of grasspea *(Lathyrus sativus)* varieties was genotype specific. Some genotypes of grasspea responded best on auxin- free medium with 5.0 mg/l of BAP, while others performed

better with 0.1 mg/l of NAA + 5.0 mg/l of BAP or even only on 3.0 mg/l of BAP. Rooting was optimum on half strength hormone-free MS medium, though differences appeared within genotypes.

There are various reports of *in vitro* shoot formation of lentils (*Lens culinaris*) Khanam *et al.*, 1995 reported best result in multiple shoot regeneration on MS medium containing 0.5 mg/l of BAP + 0.5 mg/l of kinetin. On the other hand, Sarker *et al.*,2003 obtained healthy shoots with well-developed leaves on MS medium supplemented with 2mg/l of BAP + mg/l Kn, though various concentrations and combinations of these growth hormones initiated shoot formation. Previous reports indicated that root induction in lentils was achieved on a half strength MS medium containing 0.25 mg/l of IBA resulted in 25% rooting along with an average of 7.87 roots per shoot with a mean length of 7.13cm,Khawar and Ozcan (2002). Polanco and Rulz (1997) also suggested that cytokines combination to auxin has an inhibitory effect on rooting in lentils.

Most of the studies reported for chickpea (*Cicer arietinum* L.) regeneration have been possible based on cotyledonary nodes or shoot apices derived from seedling explants. Using cotyledon explants, callus induction was noticed in all several concentrations of auxins (2,4-D, NAA, IAA) and cytokinins (BAP, Kn). Highest (95%) callus induction and maximum number of shoot buds were observed in MS + 3.0 Kn + 3.0 mg/l BAP (Huda *et al.*, 2003). According to Jayanand *et al.*,2003 IAA alone promoted root formation (1/2MS + 1 mg/l IAA). However, ½ MS + 1.0 mg/l IBA were most effective for rooting of shoots in chickpea.

In cowpea (*Vigna unguiculata*), shoot regeneration was possible on MS medium supplemented with higher concentration of BAP for initiation followed by 1.0 mg/l of BAP. But the regeneration efficiency varies among different genotypes. On the other hand, 1.0 mg/l of IAA or 0.05 mg/l of IBA were found to promote rooting though hormone-free MS medium was also sufficient (Machuka *et al.*, 2000).

2.5.2. Somaclonal Variation

The term ‘somaclone’ was coined to refer to plants derived from any form of cell culture, and the term ‘somaclonal variation’ was coined to refer to the genetic variation among such plant (Fourre, 2000). Somaclonal variation results from both pre-existing genetic variation within the explants and the variation induced during the tissue culture phase (Kaeppler *et al*, 2000). There are two types of somaclonal variation: heritable (genetic) and epigenetic. Heritable variation is stable through the sexual cycle or repeated asexual propagation; epigenetic variation may be unstable even when asexually propagated. Epigenetic variation is also known as developmental variation, and includes persistent changes in phenotype that involve the expression of particular genes (Mehta *et al*, 2000). The best known example of epigenetic variation is the loss of auxin, cytokinin, or vitamin requirements by callus (Predieri, 2001). Somaclonal variation provides a valuable source of genetic variation for the improvement of crops through the selection of novel variants, which may show resistance to disease, improved quality, or higher yield.

2.5.3. Embryo Rescue

Embryo rescue is one of the earliest and successful forms of *in vitro* culture techniques that is used to assist in the development of plant embryos that might not survive to become viable plants (Sage *et al*, 2010). Embryo rescue plays an important role in modern plant breeding, allowing the development of many interspecific and intergeneric food and ornamental plant crop hybrids. This technique nurtures the immature or weak embryo, thus allowing it the chance to survive. Plant embryos are multicellular structures that have the potential to develop into a new plant. The most widely used embryo rescue procedure is referred to as embryo culture, and involves excising plant embryos and placing them onto media culture (Mehetre et al, 2004). Embryo rescue is most often used to create interspecific and intergeneric crosses that would normally produce seeds which are aborted. Interspecific incompatibility in plants can occur for many reasons, but most often embryo abortion occurs(Cisneros et al,2010).In plant breeding, wide hybridization crosses can result in small shrunken seeds which indicate that fertilization has occurred, however the seed fails to develop. Many times, remote hybridizations will fail to undergo normal sexual reproduction, thus embryo rescue can assist in circumventing this problem.

2.5.4. Somatic Hybridization

Somatic hybridization is a process by which two different plant species fuse together to form hybrid and the hybrids produced is known as somatic hybrid.This technique involves the following steps; protoplast isolation, fusion of different protoplast, selection of hybrid cells, culture of hybrid cell and regeneration of hybrid plant (Ali *et al*, 2000). The production of somatic hybrid plants by protoplast fusion is a potentially useful method for the combination of genetic materials. Protoplast fusion can sometimes lead to the production of new genetic variants as a consequence of the recombination of nuclear and/or of cytoplasmic genomes(Furuta *et al*, 2004). Many intra- and interspecific and several intergeneric somatic hybrid plants have been reported so this techniques could be significant to improve grass pea limitation through wide hybrization with another crops.

2.5.5. Genetic Transformation

In Genetic engineering of grain legumes regeneration of shoots from the cotyledonary node explants after *Agrobacterium* infection is emerging as a rapid and relatively efficient method of transformation in a number of legume species including soybean (Olhoft *et al.,* 2002), *Lotus japonicus* (Oger *et al.,* 1996), barrel medic (*Medicago truncatula)*(Trieu and and *Trifolium repens* (Larkin *et al.,* 1996).

ForGrass pea *Agrobacterium*-mediated genetic transformation aimed at reduction of ODAP could bring forth a genetic improvement (Singh *et al.,* 2002). In view of a coding sequence with ODAP-metabolizing properties being already characterized and cloned from a soil microbe (Sachdev *et al.,* 1995), the remaining constraint for *Agrobacterium*-based transformation is an efficient *in vitro* plant regeneration protocol without an intermediate callus phase. Considering the absence of an adventitious plant production system in grass pea, an alternative callus-free regeneration could be achieved using cotyledonary node explants, which has already been exploited for *Agrobacterium*-mediated transformation of several grain legumes including kidney bean , pea (Babaoglu *et al.,* 2000), pigeon pea, and soybean (Zhang *et al.,* 1999).

3. MATERIALS AND METHODS

The study was conducted in the tissue culture laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) from November 2012 to November 2013.

3.1. Plant Materials

For these study four genotypes of grass pea, namely IVATLS-LS -B2, IVATLS-LS –B1, IVAT-LS- 690, and IVAT-LS-655 were obtained from Debrezeit Agricultural Research Center. These genotypes are characterized by better agronomic performance except their ODAP limitation

3.2. Explants Preparation and Sterilization

Dry seeds of each genotype were kept under running tap water for 30 min, in separate container, followed by an 8-minutes treatment with 5% tween – 20 and rinsed five to six times with double-distilled water. Then the seeds were surface-sterilized with a 70% ethanol for 30–45 s, followed by five rinses in autoclaved double-distilled water. The surface-sterilized seeds of each genotype were inoculated in separate screw-capped jars on basal media [Murashige and Skoog, 1962) free from PGR gelled with 0.8% (w/v) agar. The seeds were allowed to germinate at 26 ± 2^0C with 25 µmol m^{-2} s-1 photon flux density provided by cool white fluorescent tubes. Then from seven-day-old seedlings, cotyledons were excised and used for tissue culture studies.

3.3. Media Preparation

Murashige and Skoog (1962) nutrient medium was used along with the proper type and concentration of plant growth regulators throughoutexperiments. All macronutrients, micronutrients, Fe-Na-EDTA, FeSO4 mixture and vitamins were used. Stock solutions for each of the MS components were prepared separately by weighing a proper amount of powder of each component and completely dissolved in double distilled water. Then they were stored in refrigerator at 4^0C for a maximum of one month. The plant growth regulator stocks were prepared in 1mg/ml concentration. After weighing an accurate amount of BAP, Kn., IAA, and IBA ready- made powder, they were allowed to

completely dissolved in double distilled water using NaOH (for auxins) and HCl (for cytokinins) and stored at 4^0C in refrigerator.

The culture media were prepared by taking the appropriate volumes of the stock solutions. Sucrose (30g/l) was added in the media as a carbon source. The volume was then adjusted and the appropriate type and concentrations of growth regulators were added on the basis of the requirement for either of shoot multiplication or rooting. Then the pH was adjusted to 5.8 ± 2 using either 1N NaOH or 1N HCl solution before 0.8 % w/v agar was added. It was then melted and dispensed into the culture vessels and finally autoclaved at a temperature of 121ºC with a pressure of 15 Psi for 20 minutes. Filter sterilization was used for IAA as it is less stable and heat labile for autoclaving.

3.4. Culturing and Culture Conditions

The cotyledonary node explants cultured on MS medium supplemented with different PGRs were maintained on shoot initiation medium. The culture vessels were kept in a growth chamber with 16 hours photoperiod at about 26 ± 2^0C with 25 µmol m^{-2} s-1 photon flux density provided by cool white fluorescent tubes and sub culturing was done every 15 days.

3.5. Treatments and Experimental Design

Three different experiments were carried out in this study; shoot initiation from cotyledonary node explants of different genotypes, shoot multiplication and rooting of one best initiated genotype.

3.5.1. Invitro Regeneration of Shoot from Cotyledonary node explants

In this experiment the *in vitro*initiation and shoot growthof cotyledonary nodes of four genotypes of grass pea was evaluated on MS medium supplemented with different concentrations of BAP with 0.1 mg/lNAA. Explants obtained from each genotype's cotyledonary node were cultured on MS media supplemented with different levels of BAP (0mg/l, 1.0mg/L, 2.0 mg /L and 3.0 mg/L) with 0.1mg/l NAA. The experiment arranged factorially (4X3) in completely randomized design (CRD) with 15 explants per treatment where three explants cultured per jar and five jar per treatment randomly placed on the

shelves under uniform light and it was replicated five times. Data was collected on number of explants developed into shoot, number of shoots developed per explants and shoot length (cm) after 10-15 days of culturing. A growth regulator free medium was used as control.

3.5.2 Effect of Different Concentrations of BAP and Kinetin on Shoot Multiplication of Genotype: IVAT-LS- 690

Under this experiment, MS medium containing different concentrations of BAP (1, 2, 3 and 4 mg/l) in combination with Kn (0, 1, 2 and 3 mg/l) was used and factorially arranged (4 x 4) in CRD, replicated five times, with 15 explants per treatment where three explants cultured per jar and randomly placed on shelves under uniform light. Data was collected on number of *explants* developed into shoot, shoots number per explants and shoots length (cm) after 15 days. A growth regulator free medium was used as a control for these experiments too.

3.5.3. Effect of Different Concentrations of IAA and IBA on *In Vitro* Rooting of Genotype: IVAT-LS- 690

In this experiment two auxins (IBA and IAA) and their different concentrations were tested for *in vitro* rooting. The micro-shoots having a size of 2-3 cm were taken from the shoot multiplication experiment and cultured on half- strength MS medium supplemented with IBA(0.0mg/l, 0.1mg/l, 0.25mg/l0.5mg/l and 1mg/l) and IAA(0.0mg/l, 0.1mg/l, 0.25mg/l 0.5mg/l and 1mg/l). The experiments were set individually for both auxins as a single factor experiments in CRD using five jars and three explants per and jars were placed on shelves under uniform light. Data was collected after 15 days on number of shoots induced roots, number of roots per shoot and root length(cm).

3.5.4. Acclimatization

Plantlets with well-developed roots were taken from culture tubes and after washing the roots in running tap water, they were transferred to plastic pots containing 1:2:1 ratio of top soil, sand and compost respectively and covered with polyethylene bags to maintain high humidity. The potted plantlets were kept in the culture room at 27 ± 1^0C and with a photon flux density of 50µmolm^{-2} s^{-1}. After a week, plantlets were transferred to larger

pots containing soil and compost (1:1) and kept under field condition for another 20 days and their percentage of survival were determined according to method used by Fratini *et al*., (2009).

3.6. Data Analysis

Data analysis was done using SAS statistical package, version 9.2 (SAS Institute Inc. 2008). Duncan's new multiple range test was used to separate the means for significant effect at 5 % probability.

4. RESULTS AND DISSCUTION

In this study in vitro shoot initiation of four grass pea genotypes and optimum concentration of cytokinis and auxins for in vitro shoot multiplication and rooting of one genotype (IVAT-LS-690) were studied. The results of the study are presented as follow;

4.1. Initiation Response of Cotyledonary Node Explants of Four Genotypes of Grass Pea (L. *sativus)*.

In this experiment shoot initiation of four genotypes cotyledonary nodes were studied and analysis of variance showed significant differences among the genotypes and BAP concentrations for all response variables; percentage of shoot initiation, average number of shoots per explants and average shoots length (Table 1).

Table 1: Shoot initiation of four genotypes on different concentrations of BAP with 0.1mg/l NAA

Genotypes	**Hormone Con. (mg/ l)** BAP	**%age of Shoot initiation**	**Mean No. of Shoots/explant**	**Mean Shoots length (in cm**
IVATLS-B2	1	33.33f	4.37f	3.64c
IVATLS-B1	1	53.33def	5.03e	3.65c
IVAT-LS-690	1	79.99abc	3.47g	4.63b
IVAT-LS-655	1	39.99ef	1.77h	3.58cd
IVATLS-B2	2	53.33edf	9.23c	4.50b
IVATLS-B1	2	53.33def	10.87ab	4.98a
IVAT-LS-690	2	100.00a	11.42a	4.98a
IVAT-LS-655	2	39.99ef	6.50d	4.63b
IVATLS-B2	3	39.99ef	10.93ab	3.35d
IVATLS-B1	3	59.99cde	11.2ab	3.57cd
IVAT-LS-690	3	73.33bcd	10.63b	4.51b
IVAT-LS-655	3	86.66ab	6.6d	4.74b
CV		7.03	5.61	4.17

At 1mg/l BAP,IVAT-LS-690attained the highest percentage of shoot development and maximum average shoot length,whereasIVATLS-B1showed maximum average number of shoots per explants. IVAT-LS-655 was less responsive as compared to the rest three at this concentration in terms of mean no. of shoots/explants. Best percentage of shoot initiation and maximum average shoots per explants was recorded from IVAT-LS-690at 2mg/l BAP while longest average shoot length was fromIVAT-LS-690andIVATLS-B1.At 3mg/l BAP best percentage of shoot initiation was obtained from IVAT-LS-655whileIVATLS-B1 andIVAT-LS-690givesmaximum average shoot per explants and longest average shoot length were obtained fromIVAT-LS-655 andIVAT-LS-690.

Different results were observed among genotypes as BAP levels increases from 1mg/l to 3mg/l. Percentage of shoot formation increases with increasing BAP levels from 1-3mg/l for genotypes IVATLS-B1 and IVAT-LS-655. However, for IVATLS-B2 and IVATLS-LS 690 it increase up to 2mg/l and then decreased. Number of shoots per explant increases for all genotypes except for IVAT-LS-690as BAP levels increases while shoots length decreases with increasing BAP levels except IVAT-LS-655.

The cotyledonary node explants failed to regenerate shoots on MS basal medium free of growth regulators indicating the importance of external BAP in grass pea cotyledonay node culture initiation (data not shown). But genotypes responses varied at each concentration of BAP. On top of this, high concentration of BAP induced multiple shoots but with stunted growth for all genotypes except IVAT-LS-655.This is because of an *in vitro* growth and morphogenesis of plants that are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (George, 1993).

These result is in agreement with Ochatt(2001) who reported that the optimum hormonal combination for regeneration response of grass pea is genotype specific; genotype LB responded best on auxin-free medium with 5.0 mg/l BAP, while colored-seeded genotypes

responded best with 0.01 mg/l NAA, plus 5.0 mg/l BAP for LIII, but 3.0 mg/l BAP for L12 genotypes.

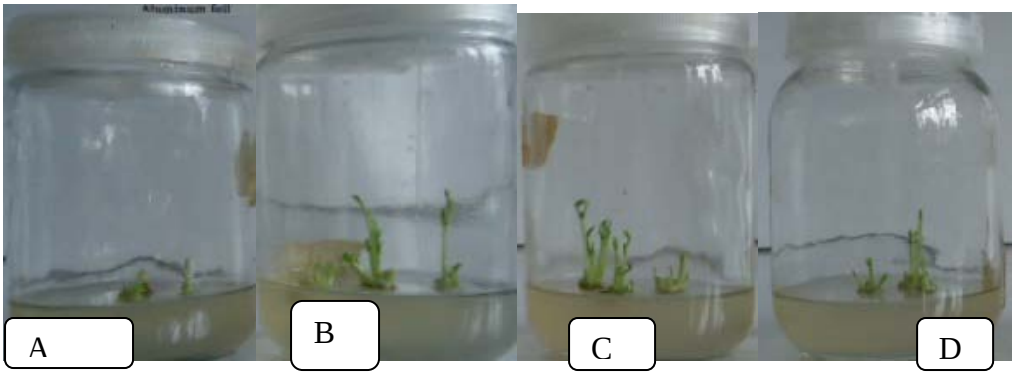

Figure 1**:** Response of cotyledonary node cultured on MS shoot initiation media after 8 days. (A) IVATLS-B2, (B) IVATLS-B1(C) IVAT-LS-690 and (D) IVAT-LS-655

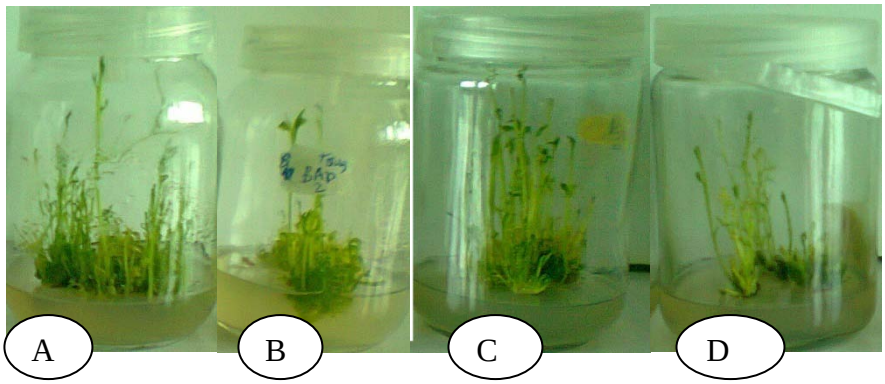

Figure 2: Shoot development of IVATLS-B2 (A), IVATLS-B1 (B), IVAT-LS-690 (C) and IVAT-LS-655 (D) on MS medium supplemented with different level of BAP after two week of culturing.

4.2. Effect of Two Cytokines (BAP and Kn) and Their Concentrations on Shoot Multiplication of IVAT-LS-690

In this study the combined effect of BAP and Kn concentrations on shoot multiplication of IVAT-LS-690 were compared. Nodal segments were taken from the plantlets of this genotype and cultured on MS medium containing different concentrations of BAP and Kn combinations. ANOVA revealed that statistically significant differences among the concentrations of BAP and Kn combination tested for all response variables ; percentage of culture regenerating shoots, average number of shoot per explants and average shoot length were computed (Table 2)

Table 2: The combined effects of BAP and Kn. on shoot multiplication of IVAT-LS-690

Hormone Con. (mg/ l) BAP	**Hormone Con. (mg/ l)** Kn.	**%ageof Shoot formation**	**Mean No. of Shoots/explant**	**Mean Shoots length (in cm)**
1	0	33.33d	1.00g	3.20f
1	1	33.33d	1.00g	3.20f
1	2	44.44dc	1.67f	3.49e
1	3	77.77abc	7.53d	4.38c
2	0	66.66bad	9.06c	4.43c
2	1	44.44cd	2.17f	3.52e
2	2	88.89ab	11.17a	5.00a
2	3	100.00a	11.17a	5.00a
3	0	58.89bcd	10.39b	4.96a
3	1	100.00a	11.50a	4.96a
3	2	100.00a	10.98a	4.77ab
3	3	55.55bcd	6.33e	4.14d
4	0	77.77abc	9.50c	4.60bc
4	1	55.55abd	9.50c	4.60bc
4	2	44.44cd	6.33e	4.90a
4	3	88.89ab	9.50c	4.60bc
CV		6.2	4.7	7.9

*****Means with different letters in a column are significantly different.

All the treatments of Kn and BAP except control showed shoot development .However, the best response was observed on a medium supplemented with 3 mg/l BAP+1mg/l Kn which resulted in the highest percentage of shoot regeneration (100%) and highest number of shoots (11.5) per explants with a mean shoot length of 4.96 cm (Table 2) though, the result from this treatment is not statistically different from that of a medium supplemented with 2mg/l BAP + 2mg/l Kn, 2mg/l BAP + 3mg/l Kn 3mg/l BAP + 2mg/l Kn. Statistically the second best result of this experiment was obtained from medium supplemented with 3 mg/l BAP + 0mg/l Kn.

These results are in agreement with the results of Sevimay,*et al.*,2005 who obtained best results from a medium supplemented with BAP and Kn. combinations. Debnath*et al*., 2010 also obtained best shooting in a medium supplemented with a combination of BAP and Kn. though NAA was used additionally. But Barik 2004 *et al.*,reportedmaximum number of shoots per explants (11.3) and longest shoot (4.9cm) on BAP alone at 2mg/l for grass pea species. On the other hand, Sarker *et al.* (2003) obtained healthy shoots with well developed leaves on MS medium supplemented with 2mg/l of BAP + mg/l Kn, though various concentrations and combinations of these growth hormones initiated shoot formation for lentils (*Lens culinaris) a* close species of grass pea.

In general , the results demonstrated that highest mean number of shoot per explants and longest average shoot was obtained from MS medium containing 3 mg/l BAP+1mg/l Kn. Even though, BAP alone at 3mg/l was almost similar to the second best result of BAP and Kn combination with better average shoot length but plantlets from BAP and Kn combination appeared more healthy and green (Fig.3). In addition to this, percentage of shoot development was lower on BAP alone.

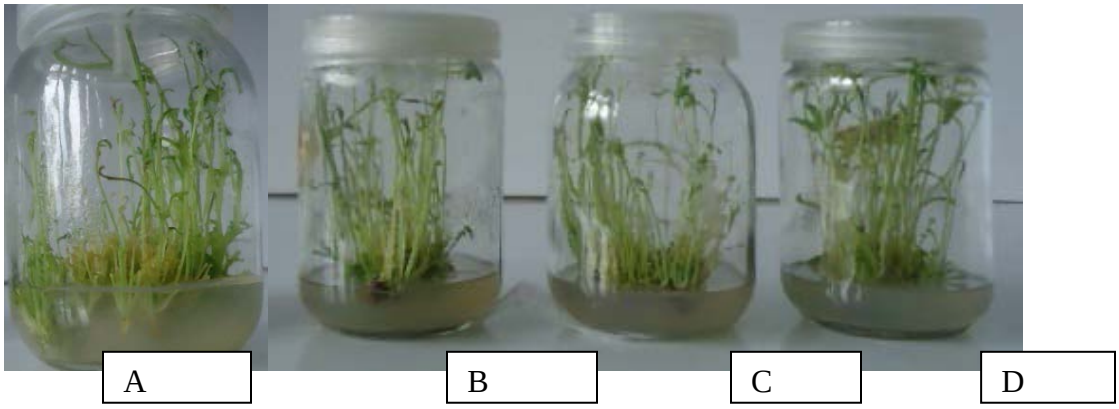

Figure 3: Shoot multiplication on MS medium supplemented with BAP and Kn combination. A= MS+3 mg/l BAP+0 mg/l Kn , B=MS+3 mg/l BAP+1.0 mg/l Kn, C= MS+ 2 mg/l Kn+2 mg/l BAP, D= MS+ 2 mg/l Kn+3 mg/l BAP

4.3. Effect of IAA and IBA Concentrations on *In Vitro* Rooting of IVAT-LS-690

For this experiment, micro shoots grown with BAP 3mg/l + Kn 1mg/l,that showed the best shoot number as well as shoot length were used for to rooting study. Half strength MS medium augmented with various concentrations of IAA and IBA individually were tried in two experiments to induce rooting.

Analysis of variance from this experiment showed significance difference among treatments (IAA and IBA concentration) for all response variables; percentage of rooting, number of root per shoot and average root length.

On half-strength MS medium supplemented with different levels of IAA the highest percentage of rooting (80%), maximum root number (5.6 roots per shoot) and longest average root length (4.9 cm) was obtained at 1 mg/l IAA, while shoots cultured on media devoid of auxins failed to form roots on both experiments. Even though, there was no statistically significance difference among 0.1,0.25 and 0.5 mg/l concentration; percentage of rooting, number of roots per shoot and average roots length were increased with increased concentration of IAA(Table 3).

Table 3: Effects of IAA concentration on *in vitro* rooting of genotype IVAT-LS-690

IAA Con. (mg/ l)	%age of rooting	Mean No. of roots/shoot	Mean No. of roots length (cm)
0.1	40.00b	1.20c	3.68b
0.25	46.66b	3.40b	3.97b
0.5	53.33b	3.50b	3.99b
1.0	80.00a	5.60a	4.90a
CV	9.17	11.54	5.38

*****Means with different letters in a column are significantly different.

On the other hand, from half-strength MS medium supplemented with different concentrations of IBA; highest percentage of rooting (86.66%), maximum root number (6 roots per shoot) and longest average root length (4.91 cm) were recorded on 0.5mg/l IBA (Table 4). Concentrations of auxins exceeding 0.5 mg/l showed a reduction in rooting response for IBA whereas rooting response increased with increasing concentration of IAA.

Table 4: Effect of IBA concentration on in vitro rooting of IVAT-LS-690

IBA Con. (mg/ l)	%age of rooting	Mean No. of roots/shoot	Mean No. of roots length (cm)
0.1	46.66b	1.20c	3.65c
0.25	40.00b	3.50b	3.99b
0.5	86.66a	6.00a	4.91a
1.0	53.33b	3.40b	3.91cb
CV	8.17	10.27	5.24

*****Means with different letters in a column are significantly different.

These results are similar to Ochatt *et al.* 2001, who observed high rooting percentage (90%) on half-strength MS medium supplemented with 1mg/l IAA) or 0.01 mg/l NAA but, in contrast to Dayal *et al*., 2003 who obtained best rooting on medium supplemented with 2.2 mg/l IAA. These

authors claimed that they observed 100% rooting percentage on this concentration. Similarly, Mohan and Krishnamurthy (1998) also noticed that 80-85 survival percentage on half-strength MS medium with IBA (0.5 mg/l).

The rooted shoots on half-MS media supplemented with IBA, showed fast elongation of roots with numerous thin laterals whereas only few elongation with callus like structure were observed on media supplemented with IAA (Fig 4&5).

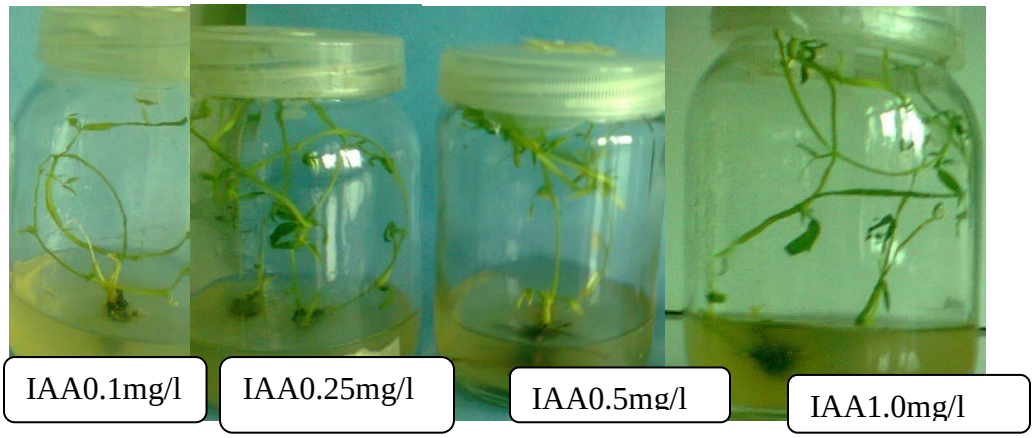

Figure 4: In vitro rooting of IVAT-LS-690 on different concentration of IAA

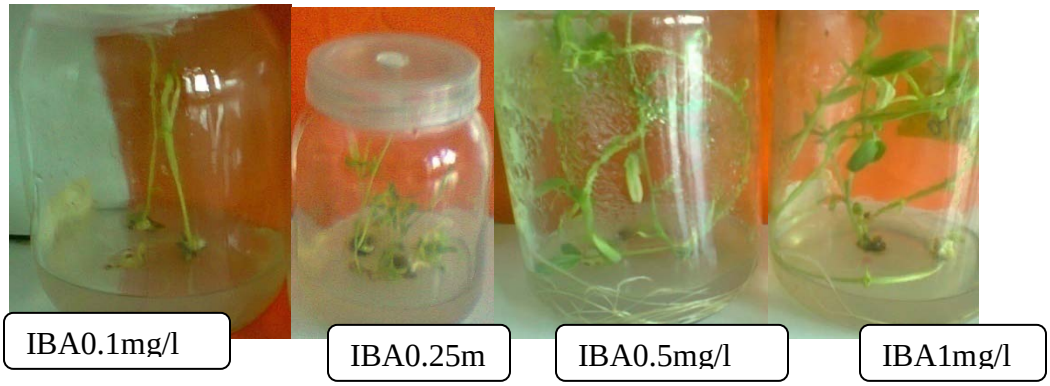

Figure 5:In vitro rooting of IVAT-LS-690 on different concentration of IBA

4.4. Acclimatization

Best rooted shoots were transferred into pots containing 1:2:1 ratio of top oil, sand and compost respectively. As recommended by Fratini *et al.*, 2009. The transferred plantlets showed 60% survival and looked healthy after three weeks of acclimatization. But among the survived plantlets some of them showed fast elongation with light green color (Fig 6). This may be due to low amount of light which led to etiolating. The low rate of survival could be due to the delicate nature of roots of this crop which make them susceptible to mechanical injury during transfer that decreases the survival rate of the plantlets.

Fratini *etal.*, 2009 reported 67% survival and Barik *et al*., 2004 reported 78% survival on their works but in our case only 60% were survived. Time period difference, genotype and direct transfer of rooted plantlets into the pot could be the possible reasons for the difference.

Figure 6**:** Acclimatized plantlets in pots under normal field condition.

5. SUMMARY AND CONCULUSION

Grass pea is a grain legume commonly cultivated in Ethiopia as grain legume and fodder because of its better performance under adverse agricultural conditions. The crop has so many valuable characteristics and its cultivation requires the least crop managements. But its full potential has not been utilized because of the presence of the neurotoxic amino acid β-N-oxalyl-L-αβ - diaminopropionic acid (ODAP/BOAA) which causes neurolathyrism in human beings. Conventional breeding practices and other agronomic approaches explored to date have not been successful in substantially reducing the toxin. Therefore, integration of *in vitro* techniques such as somatic hybridization, somaclonal variation and genetic transformation to conventional breeding methods can be significant investigates to meet the challenge. Hence, the present study was carried out to evaluate the *in vitro* regeneration capacity of some grass peas (*Lathyrus sativus* L.) genotypes; since testing *in vitro* regeneration capacity and protocol optimization is basic prerequisite for these modern approaches and need to be done first.

In vitro regeneration capacity of four grass pea genotypes (VATLS-LS -B2, IVATLS-LS –B1, IVAT-LS- 690, and IVAT-LS-655) was evaluated on three concentrations of BAP (1mg/l, 2mg/l and 3mg/l) in combination with 0.1mg/l NAA and the best responded genotype was selected based on its performance on shoot initiation media to determine the effect of different cytokinines and auxins concentrations for *in vitro* shoot multiplication and rooting.

The results revealed that both genotype and plant growth regulators concentrations have significance effect on regeneration capacity of cotyledonary nodes of the genotypes. Each genotype responded differently on the same concentration of PGL and among them IVAT LS 690 showed superiority performance over the others and was selected for subsequent experiments.

For *in vitro* shoot multiplication of genotype IVAT LS 690 among different concentrations of BAP and Kn combinations tested, best shoot multiplication was obtained on MS medium supplemented with 3 mg/l BAP+1mg/l Kn even though it was not statistically different from the

other treatments, i.e.,2mg/l BAP + 2mg/l Kn, 2mg/l BAP + 3mg/l Kn and 3mg/l BAP + 2mg/l Kn.

Among the different concentrations tested for the *in vitro* rooting of this genotype, the best rooting was obtained on half-strength MS medium supplemented with 1mg/l IAA and 0.5mg/l of IBA.

Based on the result of present study the following conclusions can be drawn;

- Different results were obtained from the four genotypes tested for *in vitro* shoot proliferation response and IVAT-LS-690 on 2mg/l BAP with 0.1NAA was found to be the best among the tested genotypes indicating that both genotype and PGL concentration play a crucial role on *in vitro* shoot regeneration capacity of cotyledonary nodes.

- For *in vitro* shoot multiplication of IVAT-LS-690, MS medium supplemented with 3 mg/l BAP +1mg/lKn gave the highest number of shoots per explants on a medium containing 2 mg/l BAP + 1mg/lKn which created a synergetic effect and interact with its endogenous counterpart to induce best shooting.

- Of the two auxins concentrations tested for in *vitro* rooting of IVAT-LS-690, half-strength MS medium supplemented with 0.5mg/l IBA gave the highest root number per shoot while 1mg/l for IAA

Based on the results of the present study, we suggest the following:

- Both genotype and PGL concentration play a crucial role on *in vitro* shoot regeneration capacity of cotyledonary node. Therefore, there is need to use different concentrations of PGL for different genotypes. Based on this 2mg/l BAP+0.1NAA could be used for shoot initiation of IVAT-LS-690, IVATLS-B2 and IVATLS-B1 while 3mg/l BAP+0.1mg/l NAA forIVAT-LS-655.

- MS medium supplemented with 3 mg/l BAP+1mg/l Kn could be used for shoot multiplication of IVAT-LS-690 while half -strength MS medium supplemented with 1mg/l IAA or 0.5mg/l IBA could be used for in vitro rooting.

- The optimized protocol could be used as a baseline for further studies of *in vitro* regeneration of grass pea

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7. APPENDIX

Appendix Table 1: Mean squares for Shoot growth parameters of four genotypes on different concentration of BAP with 0.1mg/l NAA

Source of variation	*D f*	*%age of shoot formation*	*Mean no. of shoots/explants*	*Mean No. of shoot length*
BAP	*2*	*0.32**	*241.79***	*4.61***
Genotypes	*3*	*1.39***	*50.93***	*2.10***
*BAP*Genotypes*	*6*	*0.38***	*3.54***	*0.90***
CV		*7.20*	*5.62*	*4.17*

**- highly significant at p<0.01, DF=degree of freedom, CV = Coefficient of variation, ns =non-significant, BAP=

6-Benzylaminopurine, NAA= α-Naphthalene Acetic Acid

Appendix Table 2:Mean squares for shoot growth parameters as effected by Kn. and BAP combinations on IVAT-LS-690

Source of variation	D f	%age of shoot formation	Mean no. of shoots/explants	Mean No. of shoot length
BAP	3	3373.48**	26.45**	3.84**
Kn	3	935.28^{ns}	18.31**	1.53*
BAP*KN	9	1314.43	29.5**	1.21*
CV		6.2	4.7	7.9

* Significant at $p<0.05$ **- highly significant at $p<0.01$, Pr = probability value, DF=degree of freedom, CV = Coefficient of variation, ns =non-significant, BAP =6-Benzylaminopurine, Kn. = kinetin

Appendix Table 3: Mean squares for root growth parameters as affected by IAA and IBA concentration on IVAT-LS-690

		On 4 different concentrations of IAA			**On 4 different concentrations of IBA**				
Source of variation	Df	%age of shoot formation	Mean no. of shoots/explants	No. of shoot length	Source of variation	Df	%age of shoot formation	Mean no. of shoots/explants	Mean No. of shoot length
IAA	3	0.6072*	16.14**	1.432**	IBA	3	0.481*	19.245**	1.514**
Replication	4	0.0401^{ns}	0.0954^{ns}	0.0320^{ns}	Replication	4	0.0823^{ns}	0.1062^{ns}	0.053^{ns}
CV		9.17	11.5	5.3			8.71	10.	5.2

* Significant at $p<0.05$ **- highly significant at $p<0.01$, Pr = probability value, DF=degree of freedom, CV = Coefficient of variation, ns =non-significant, IAA = Indole -3- Acetic Acid, IBA= Indole-3- Butyric Acid