OPTIMIZATION OF *IN-VITRO* REGENERATION OF PINEAPPLE

(*Ananas comosus* (L) Merr)

GODFREY ARON

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN CROP SCIENCE OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA

2020

ABSTRACT

The objectives of this study were to determine the optimum concentrations of 6-Benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) and Murashige and Skoog (MS) media state on *in vitro* multiplication of local pineapple cv. “Smooth Cayenne”. Experiments were carried out at Mikocheni Agricultural Research Institute from September 2016 to July 2017. In the first experiment, crown and ratoon derived explants were cultured in MS medium supplemented with BAP at 0, 2, 3, 4, 5, 5.5mg/L in combination with NAA at 0, 0.01, 0.05, 0.1 and 2mg/L while in the second experiment, ratoon and crown derived explants were cultured in MS semi-solid, solid and liquid media. MS semi-solid and solid media were prepared by adding 1.25 and 2.5g/L of phytagel as gelling agent. The pineapple explant were divided into four portions, planted in MS media with different BAP and NAA combinations and then incubated in a growth room at temperature of 26` ± 2 °C and photoperiod of 16h dark and 8h light per day. Data on number of plantlets per quarter explant and weight of plantlets at 16th week from the date of culture initiation were collected. Data were analyzed using Gen Stat Computer Software version 12th based on split plot in a completely randomized design, and treatment separation was performed based on Duncan Multiple Range Test at $p \leq 0.05$. Crown derived explants cultured in BAP at 5mg/L and NAA at 2mg/L produced the highest number of 16 plantlets per quarter explant each weighing 1.979g. Moreover, crown derived explants cultured in semi-solid MS media produced the largest number of plantlets of 16.33 plantlets per quarter explant each weighing 1.966g. It is recommended that tissue culture propagators should improve *in vitro* propagation of pineapple by culturing crown-derived explants in MS semi-solid media supplemented with BAP at

5mg/L and NAA at 2mg/L. Further studies are required to determine the effect of crown and ratoon-derived seedlings on field performance of pineapple.

DECLARATION

I, Godfrey Aron, hereby declare to the senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other University.

…….……..……. ……..……

Godfrey Aron Date

(MSc. Candidate)

…….……..……. ……..……

Prof. T. J. Msogoya Date

(1st Supervisor)

…….……..……. ……..……

Dr. M. S. Seth Date

(2nd Supervisor)

COPYRIGHT

ACKNOWLEDGEMENT

Special thanks to my entire family, my wife Nuru Mbuma, sons Kabole and Laurent and my daughters Mpeligwa and Matrida for their constant comfort to me during my studies. I extend my heartfelt gratitude to my employer, Tanzania Peoples Defence Forces for granting to me study leave as well as monthly salary which reduced stress while undertaking my studies.

Sincere thanks to my Supervisors Prof. Theodosy J. Msogoya of SUA for his tireless guidance, support and mentoring and Dr. Micah Songalael Seth of Mikocheni Agricultural Research Institute for his patient listening, encouragement and expert guidance throughout my research period.

It is pleasure to offer my appreciation to Dr. Ndunguru who were the officer in charge at Mikocheni Agricultural Research Institute (MARI) Dar es Salaam in Tanzania for his administrative role in providing me with logistic support to smoothen my research. I also express my sincere gratitude to laboratory staff at MARI Mr Kusirieli Lema, Ms Nana Amir, George Mmbwambo, Ms Justina Misungwi and Ms Lilian Mrema who cheerfully encouraged me during my study.

I thank the National Fund for Advancement of Science and Technology of the Swedish International Development Agency (NFAST-SIDA) through Tanzania Commission for Science and Technology (COSTECH) for funding this research through grant number CST/NFAST-SIDA/2015/22 to Mikocheni Agricultural Research Institute. Its support was indeed meaningful and valuable.

DEDICATION

Dedicated to almighty GOD who mysteriously kept me healthier throughout my MSc. studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ACORD	Association for Cooperative Operations Research and Development
BA	Banzyl Adenine
BAP	6-Benzylaminopurine
COSTECH	Tanzania Commission for Science and Technology
Cv	Cultivar
FAOSTAT	Food and Agriculture Organization Statistics
FAOTRADE	Food and Agriculture Organisation
g	Gram
IAA	Indole-3-Acetic Acid
IBA	Indole Butyric Acid
ISAAA-	International Service for the Acquisition of Agri-biotech Applications (ISAAA
Kn	Kinetine
MARI	Mikocheni Agricultural Research Institute
mg/L	Milligram per Litre
MS	Murashige and Skoog media formulation
NAA	Naphthalene Acetic Acid
NaOCl	Sodium hypoclorite
SC1	Subculture 1
SC2	Subculture 2
SC3	Subculture 3

TC	Tissue Culture
USDA	United States Department of Agriculture
µM	Micro molar

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Pineapple (*Ananas comosus* L. (Merr)) is a perennial tropical monocot herb of the family *Bromeliaceae* and is one of the commercially important tropical fruits constituting a major export item for some countries (Duval *et al*., 2001). Pineapple is the second most important fruit crop in the World after banana and contributes to over 20 % of the world production of fruits (Coveca 2002). Costa Rica, Brazil, Thailand, Philippines and Indonesia are the main pineapple producers in the world supplying 47% of the total output followed by China, India, Nigeria, Kenya, Mexico and Paraguay and which together provide most of the remaining pineapple fruit (Medina *et al*., 2005).

Pineapple is found in almost all the tropical and subtropical areas of the world and has become one of the leading tropical fruits. Pineapples is used for its high sugar content, attractive flavor and vitamins A and C. Presently, pineapples are consumed in form of fresh fruits, canned chunks and slices (Khan *et al*., 2004).

In spite of the profitability and marketability of the crop, many regions of Tanzania grow pineapples as an extra crop and not as a basic and strongly depended commercial crop as other countries do. Only a few areas in the country grow it and focus it as the basic commercial crop and grow it in a considerable huge farm per person or family.

A good example is Bagamoyo where people cultivate pineapples by acres and acres. However, these people grow the crop as their basic commercial crop, but there is no

report that indicates the exportation of pineapples from Tanzania. Many regions in the country have soil suitable for pineapple production not only as an extra crop but also as basic and more focused commercial crop, as done for coffee, tea, cashew nuts and cereal crops. Regions which have good soil for the fruit are Morogoro, Coast Region, Tanga, Lindi, Mbeya, Mwanza and Dar es Salaam. If well cultivated and sowed, qualitative seedling pineapple can improve not only individual income but also the community at large. Tanzania produces about 214,840 tonnes per year or 17.9 per cent of all fresh fruits produced in the country (FAOSTAT, 2013).

In 2010 Costa Rica exported 1,677,702 tonnes of the fruit followed by the Philippines, Belgium, The Netherlands and US dominates the importation of the fruit (FAOSTAT/COMTRADE, 2013).

Biologically, hormones in plants differ from most of those in animals by having pleiotropic effects in the sense that they are involved in the control of a wide range of developmental processes. Moreover, two or more hormones can interact synergistically or antagonistically in many circumstances (George *et al*, 2008).

Tissue culture technology is potent and has opened extensive areas of research for micropropogation, secondary metabolite production and biodiversity conservation. Plant *in vitro* regeneration is a biotechnological tool that offers a tremendous potential solution for the propagation of endangered and superior genotypes of medicinal plants which could be released to their natural habitat or cultivated on a large scale for the pharmaceutical product of interest (Chandana *et al*., 2018)).

1.2 Problem Statement

Limited availability of good quality planting materials is one of the major limiting factors that affect large-scale production of pineapple in Tanzania. For instance, Majority of pineapple farmers in Tanzania consider shortage of high quality planting materials as the first constraint. Pineapple is traditionally propagated using suckers collected from field-grown plants, which are highly variable in size and age (USDA, 2013). The variability in field collected suckers affects pineapple productivity and fruit market quality. Thus, good quality planting material in large quantities is required for commercial cultivation of pineapples (Khan *et al.,* 2004). Tissue culture technology has the potential to address shortage of planting materials of elite plant genotypes (Almeida et al., 2002).The technology is a powerful for mass production of disease-free and uniform propagules within a short time (Nikumbhe *et al*., 2013; USDA, 2013; Khan *et al*., 2004).

Countrywise, there are limited reports on the effects of different combination of BAP and NAA on *in vitro* multiplication of Tanzanian local pineapple varieties using crown and ratoon suckers as explants. Murashige and Skoog (MS) growth media for *in vitro* pineapple culture are normally prepared to be in solid or liquid form. According to Almeida *et al*., (2002) the use of liquid MS medium during *in vitro* multiplication stage increased production of adventitious buds of pineapple cv. 'Perola' largely due to higher nutrient uptake. Similarly, higher *in vitro* regeneration rates of pineapple cv. 'Perolera' and 'Primavera' were obtained in liquid MS media (Guerra *et al*., 1999). The use of liquid media requires aeration of the culture using

bioreactor, which continuously pumps fresh oxygen into the media. Small-scale tissue culture laboratories cannot afford bioreactors technology but can alternatively use semi solid media or shake culture vessels to improve aerations (Aitcken-Christie *et al*., 1995). However, there are limited reports on the effects of semi-solid media on *in vitro* multiplication of Tanzania local pineapple varieties

1.3 Objectives

1.3.1 Overall Objective

The overall objective of the study is to increase production of pineapple planting materials through optimization of *in vitro* regeneration protocols.

1.3.2 Specific Objectives

1. To determine the optimum concentrations and the best combination levels of selected auxin and cytokinin for increased *in vitro* multiplication of pineapple.
2. To determine the suitable state of the MS media for *in vitro* proliferation of pineapple.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Production

Pineapple (*Ananas comosus* L. Merr) belongs to the family *Bromeliaceae* and is known as the queen of the fruit because of its excellent flavor and taste (Baruwa 2013). Its origin has been traced to Brazil and Paraguay in the Amazonic basin (USDA, 2013). Pineapple is drought tolerant and well adapted to tropical sandy soils with pH 4.5-6.5 (Ubi *et al.*, 2005). They can be grown in wet/dry areas of the tropics that do not support less water efficient crops. Pineapple is restricted to hot, tropical lowlands with temperatures above 25ºC.Worldwide pineapple production in 2011 constituted more than 19 million metric tons (FAOSTAT, 2013). Brazil dominates the production whereas Costa Rica dominates the world market with supply of pineapple cv. MD-2 (FAOSTAT/COMTRADE, 2013). Since 1960, pineapple production worldwide has risen to 400% .The introduction of pineapple cv.'Gold', developed and patented by French Del Monte in the 1990s, has further increased pineapple production by nearly 50% since 1998 (FAO, 2008).

Several studies have reported on the positive combined effect of BAP and NAA to increase cellular morphogenetic response and *in vitro* multiplication (Souza *et al*., 2003). For example, BAP at 2 mg/L and NAA at 2 mg/L in MS media produced 40 plantlets per axillary bud of pineapple cv. Smooth Cayenne, Red Spanish and Perolera (Dewald *et al*., 1988). Similarly, BAP at 1.5 mg/L and NAA at 0.5 mg/L produced 15,757 plantlets per basal leaf of pineapple cv. Smooth Cayenne within 7 months (Frioozabady and Gutterson, 2003). Unsurprisingly therefore, it is difficult to

predict how any hormone (or growth regulator or inhibitor) will affect any given plant system (George *et al*.,2008).

2.2 Propagation methods

2.2.1 Traditional methods

Pineapple produces very small and inviable seeds and thus the crop is vegetatively propagated using suckers collected from field grown crop (Sonejijr *et al*., 2002c; USDA, 2013). According to Roy (2000), there are four kinds of propagation material in pineapple plants namely crown, hapas (leaf axils), slips and ratoon suckers. Crowns take longer time of about 24 months from the date of planting to harvesting of fruits than ratoon and slips, which take about 18 and 20-22 months, respectively (Acland,1971). Hapas and slips are rare in nature, and therefore are not commonly used in pineapple propagation. All these planting materials are obtained in small amounts and usually infected with diseases, variable in size and age, and comprise of off-types (USDA, 2013). Consequence, *in vitro* multiplication technology was developed as an alternative method to improve the multiplication rate of pineapple (Zuraida, 2013).

2.2.2 In vitro propagation

Plant tissue culture is a rapid technique for production of many disease-free, uniform and high quality planting materials within a short time (Kumar and Naidu, 2006; ISAAA, 2006). Since all plantlets are developed from one initial explant, plants derived from tissue culture often flower and set fruit at the same time when planted in the field (Mhatre, 2007). *In vitro* propagation is implemented widely for large-

scale production of pineapple planting materials where the demand is higher (Mengesha *et al*., 2013). Pineapple crowns produce better *in vitro* derived shoots with good quality root systems and are therefore preferred as explant material in most *in vitro* propagation of pineapple (Mhatre, 2007).

In vitro micropropagation using MS media containing 6-benzylaminopurine (BAP) either alone or in combination with naphthalenic acetic acid (NAA) has been studied in several species of the genus *Ananas* (George *et al*., 1993; Borges *et al*., 2003; Fráguas *et al*., 2009). For instance, Firoozabady and Gutterson (2003) working with pineapple cv “Smooth Cayenne” obtained 15,757plantlets from 22 explants for the duration of 7 months when BAP at 1.5 mg/L and NAA at 0.5 were used as growth regulators in MS culture media. Also, Kiss *et al*., (1995) used BA at 4.5 mg/L and Kin at 5.3 mg/L to obtain 80,000 plantlets from 1 explant for the duration of 1 year. IAA at 1.5 mg/L and NAA at 2 mg/L, working with pineapple cv “Primavera” and cv “Perolera”, Almeida *et al*., (2002) obtained 161,080 plantlets per explants in 8 months. Benzylaminopurine concentration above 6 mg/L reduced the number of plantlets per explant as well as plantlet weight (Nelson *et al*., 2015). Similarly, Almeida *et al*., (1997) reported that 3.0 mg/l of BAP combined with 2.0 mg/l of IAA produced the best results for the production of pineapple plantlets.

The major limitations of *in vitro* propagation technology in developing countries include high cost of initial investment, lack of reliable electricity, expertise, occurrence of somacronal variations and high incidence of culture microbial contamination (Farahani, 2014). Microbial contamination is one of the major challenges facing plant *in vitro* propagation during different stages of culture

processes such as culture initiation and sub-culturing (Msogoya *et al*., 2003). The main aseptic procedures in laboratory involve media sterilization at 121°C for 15 minutes and explant treatment with 4.5% (v/v) laundry sodium hypochloride (NaOCl) for 15 minutes, dry heat sterilization of working tools at 180°C for 120 minutes, flaming of tools during working in 99% methylated spirit and disinfection of lamina flow bench and operators' hands with 70% methylated spirit (Maerere *et al*., 2003).

Growth media for plant *in vitro* propagation are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Basic media that are frequently used in plant *in vitro* propagation include Murashige and Skoog (MS) medium, Linsmaier and Skoog (LS) medium, Gamborg (B_5) medium and Nitsch and Nitsch (NN) medium (Abobkar *et al*., 2012). Murashige and Skoog (1962) medium is among the recommended plant growth media for *in vitro* propagation of pineapple. The ingredients of MS media include major salts, minor salts, vitamins and organic carbons (Maerere *et al*., 2007; George, 2008; Rinkesh, 2013).

Different hormones affect different plant processes (Small, 2015). For example, auxins such as indole acetic acid (IAA), indole butyric acid (IBA), 2, 4-D and NAA suppress the growth of axillary buds and apical dominance while stimulating root development during *in vitro* multiplication process. The effect of cytokinins on tissue or organ cultures can vary according to the particular compound used, the media state of culture, the variety of plant from which it was derived and age of explants (George., 2008 and Seth et al 2012). Generally, cytokinins such as BAP, zeatin,

kinetin, and gibberellins promote cell division, influence cell differentiation and multiplication (Small, 2015). Cytokinin application to a single site in the plant (e.g. to one leaf) causes the treated organ to become an active sink for amino acids, which then migrate to the organ from surrounding sites. The effect of cytokinins is most noticeable in tissue cultures where they are used, often together with auxins to stimulate cell division and control morphogenesis (George *et al*., 2008). Regeneration of shoots and roots from tissues and cells culture can be induced by increasing or decreasing the relative cytokinin-to-auxin ratio in the culture medium (Usman *et al.*, 2012).

Danso *et al*. (2008) tested the effect of BAP from 2.2 to 7.5 and NAA from 1 to 4 mg/L of NAA in liquid and solid media for cv. MD2.The highest number of plantlets in solid media were 29.3 using BAP at 5 mg/L and NAA at 2mg/L while in liquid media the highest number were 16.1 plantlets using BAP at 7.5 mg/L and NAA at 2 mg/L. *In vitro* multiplication of 7.5 and 9.0 buds per explant were produced from explants cultured on media supplemented with BAP alone at 4 and 6 mg /L, respectively (Nelson *et al*., 2015).

MS media are normally used as solid or in form of liquid whereby solidification of the media is achieved by adding gelling agent such agar, gerlite or phytagel (Bhojwani and Razdan, 2005). Liquid media has several advantages over solid media, which include increasing the number of *in vitro* shoots up to nine-fold in comparison to cultures in solid medium (Zuraida *et al*., 2011; Nikumbhe *et al.*, 2013). Moreover, liquid media is commercially cheaper than solid media as it

eliminates the cost of gelling agent. However, liquid media increases the incidence of culture contamination and vitrification or hyperhydricity (Bhojwani and Razdan, 2005).

In vitro shoots have a glass-like appearance (hyperhydricity) which means they are characterized by large intercellular spaces, less epicuticular wax, fewer stomata on leaves, chloroplasts with small granna and a lack of starch grains (Ivanova and Van Staden, 2010). As a result of hyperhydricity, *in vitro* derived shoots are difficult to acclimatize and establish in the field as they are very susceptible to environmental stress. Liquid media is also associated with an inefficient oxygen supply into the media, which limits *in vitro* multiplication unless oxygen is artificially supplied. The achievement of oxygen supply is made possible by using bioreactors where filtered oxygen is continuously pumped into the media (Aitcken-Christie *et al*. 1995).

The preparation of explants for tissue culture is performed under aseptic conditions in the laminar air flow cabinets (Almeida, 1994). Sodium hypochlorite (NaOCl) at 1% to 4% is usually used as sterilizing agent of explants (Nelson *et al.*, 2015). The sterile pineapple explants are placed onto the growth media in vessels, which are incubated and maintained in a growth room at a 16 hour photoperiod (40 $mmolm^{-2}$ s^{-1}) and of 27 ± 2ºC (Almeida *et al*., 2002). Subculture is usually carried out at an interval of four weeks to enhance proliferations.

Rooted *in vitro* plantlets are normally transplanted in soil-based mix media for acclimatization and hardening in a screen house at 75% relative humidity (Usman *et*

al., 2012; Mengesha *et al*., 2013). The recommended soil mix is comprised of coffee husk, soil and sand in a ratio of 1:2:1 (Mengesha *et al*., 2013). Pineapple *in vitro* plantlets require a long acclimatization period of 6.5 months until they reach an appropriate size for field transplanting due to their slow growth rate nature (Mengesha *et al.*, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Site

The research on *in-vitro* regeneration of pineapple was conducted at Mikocheni Agricultural Research Institute (MARI), Dar es Salaam, Tanzania.

3.2 Media preparation

All media types were prepared according to Murashige and Skoog (1962) and supplemented with appropriate concentrations of growth regulators. To get different media states the liquid MS media was amended with phytagel at 2.5g/l (100%) and 1.25g/l (50%) for solid and semi-sold MS media, respectively.

3.3 Source of explants and sterilization procedure

Crown and ratoon suckers of pineapple cv. ‘Smooth Cayenne’ were collected from field-grown crops in Matombo division in Morogoro Rural District and shipped to Mikocheni Agriculture Research Institute tissue culture Laboratory for downstream experiments.

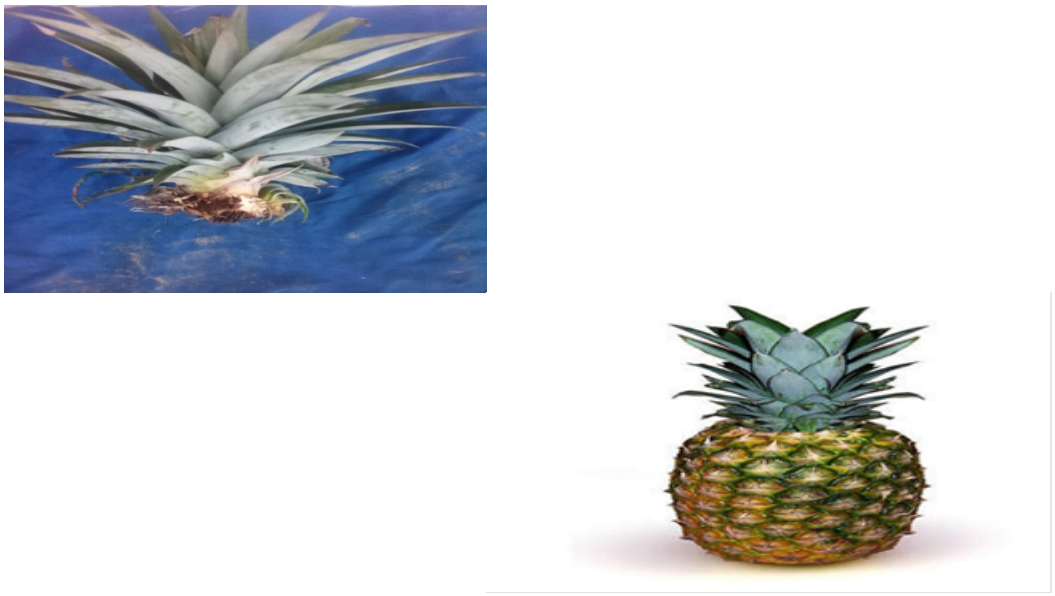

Figure 1: Pineapple explant sources: A - Ratoon sucker and B - pineapple fruit with a crown

Explants were prepared through a series of steps as follows:

1. Top apices of ratoon suckers and crowns were trimmed superficially to prevent the explants from destruction by heat during disinfection.
2. Explants were sterilized by washing them in tape water and detergents to remove debris from the field.
3. Explants were then passed into distilled water and liquid soap.

3.4 Surface sterilization of explants

a. Explants were surface sterilized in 2% NaOCl with 2-3 drops of Tween 20 for 10 minutes.

b. Explants were soaked into water bath at 60 ^{o}C for 10 minutes to eliminate fungal and bacterial microbes.

c. The remaining explant leaves were removed and each were divided equally into four parts under lamina flow cabinet (Figure 2).

3.5 *In vitro* manipulation of explants in the hood chamber

The remaining trimmed leaves were asceptically and carefully removed while leaving there contours intact without distorting the growing points (Fig. 2 A-D)

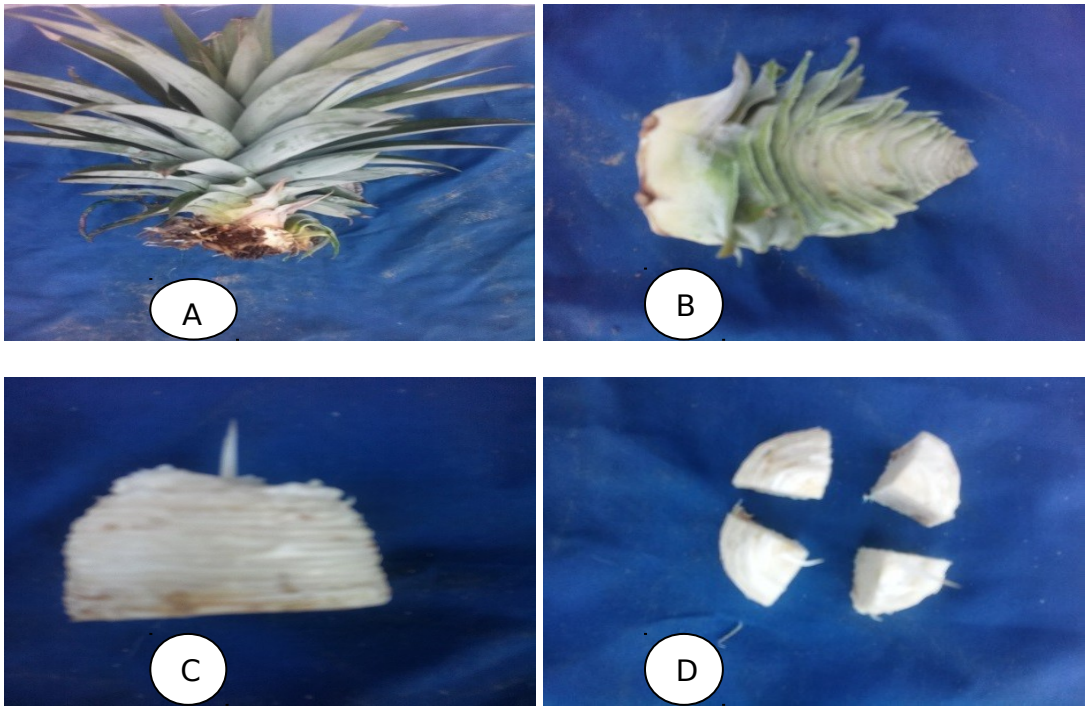

Figure 2: Pineapple explant preparation: A - Initial sucker, B - sucker with trimmed leaves, C – sucker with removed leaves and D – explant split into four pieces

d. Ex-plant pieces were soaked into 1% NaOCl for 2 minutes and then rinsed 3 times before incubating them into MS media.

e. Sterilized explants were incubated into MS media and placed in a growth room for proliferation at a temperature of 26^{o}C ± 2 and a photoperiod of 16 hours light and 8 hours dark.

Figure 3: Pineapple explant in culture vessels: Left - Explants in culture media and Right – sprouting explants

3.6 Experiment to determine the optimum concentrations and the best combination levels of selected auxin and cytokinin for increased *in vitro* multiplication of pineapple

3.6.1 Materials

Processed and surface sterilized explants were cultured/initiated on MS medium supplemented with either 0, 2, 3, 4, 5 or 5.5mg/l Benzyl aminopurine combined with either 0, 0.01, 0.05, 0.1 and 2mg/l Naphthalene acetic acid, vitamin, 30g/l sucrose and 2.5g/l or 1.25g/l phytagel.

3.6.2 Growth room management

The growth room was maintained at temperature of 26±2°C using an air conditioner and a photoperiod of 16 hours light (cool light fluorescent tubes 60-90 µm-^{2}s-^{1}) and 8 hours dark. During this period subcultures were carried out every after four weeks.

All pineapple cultures were maintained in the growth room for sixteen (16) weeks from the date of initial culture initiation.

3.6.3 Data management and analysis

3.6.3.1 Experimental design

All experiments were set as split plot in a complete randomized design. For assessment of the effect of growth regulators on the *in vitro* regeneration of pineapple the main factors were the explants sources (crown and ratoon sucker), while the sub factors were the growth regulators (BAP and NAA). In total there were 30 treatments replicated three times. Each replicate consisted 30 explants.

To determine the effect of media states on the performance of the *in vitro* grown pineapple the main factor was pineapple explants source (crown and ratoon suckers) and the sub-factors was the MS media state (Liquid, semi-solid and solid media). Each treatment was replicated three times and a replicate consisted of 30 explants.

3.6.3.2 Data collection

Data scoring was done 16 weeks from the date of culture initiation data considered were the number of shoots per explants and the fresh weight of plantlets. From each treatment five cultures were randomly picked and individual plantlets destructively separated from the cluster for counting, weighing and recording (Fig. 3).

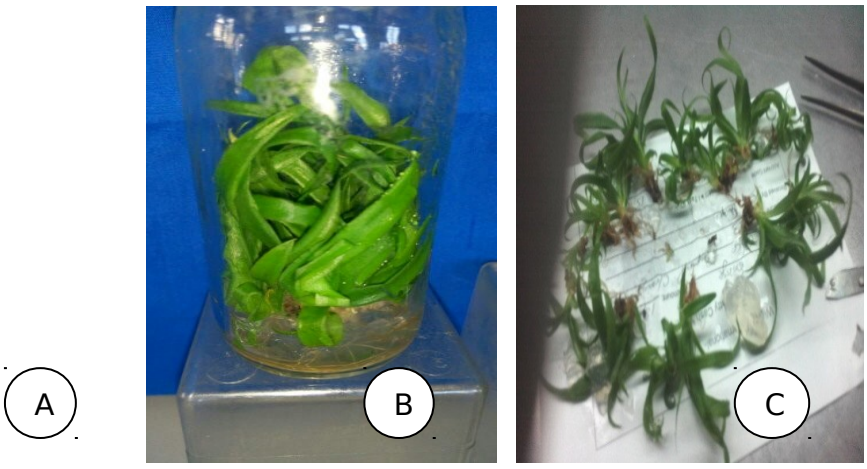

Figure 4: A – plantlets in cluster of culture, B - Plantlets split from the cluster and C – plantlet in analytical balance for weighing

3.6.3.3 Data Analysis

Analysis of variance (ANOVA) was performed using GenStat Computer Software version 12th based on statistical model for split plot design. Means separation were carried out based on Duncan Multiple Range Test (DMRT) at $P \leq 0.05$.

3.7 Experiment to determine the suitable state of the MS media for *in vitro* proliferation of pineapple

3.7.1 Materials

MS media and explants were prepared as stated above in sections 3.2 and 3.3. BAP concentration of 5mg/L were combined with NAA at 2mg/L and incorporated into MS media in various states (solid, liquid and semi-solid). Solid and semi-solid media were prepared by adding phytagel at 2.5 and 1.25 g/L into MS media, respectively. Liquid media were devoid of phytagel. BAP at 5 mg/L and NAA at 2 mg/L were added in each MS media.

3.7.2 Growth room management

The growth room was maintained at temperature of 26±2°C using an air conditioner and a photoperiod of 16 hours light (cool light fluorescent tubes 60-90 $\mu m^{-2}s^{-1}$) and 8 hours dark. During this period subcultures were carried out every after four weeks. All pineapple cultures were maintained in the growth room for sixteen (16) weeks from the date of initial culture initiation.

3.7.3 Data management and analysis

3.7.4 Experimental design

The experiment was set as split plot in a completely randomized design. Pineapple explant source were considered as main factor (crown and ratoon suckers) and the

sub-factor consisted of MS media state (liquid, semi-solid and solid media). Each treatment was replicated three times and a replicate consisted of 30 explants.

3.7.5 Data collection

Data scoring was done 16 weeks from the date of culture initiation data considered were the number of shoots per explants and the fresh weight of plantlets. From each treatment five cultures were randomly picked and individual plantlets destructively separated from the cluster for counting, weighing and recording (Fig. 3) in section 3.6.3.2 above.

3.7.6 Data Analysis

Analysis of variance (ANOVA) was performed using GenStat Computer Software version 12^{th} based on statistical model for split plot design. Means separation were carried out based on Duncan Multiple Range Test (DMRT) at $P \leq 0.05$ similar to section 3.6.3.3 above.

CHAPTER FOUR

4.0 RESULTS

4.1 The influence of growth regulators combinations on *in vitro* proliferation of pineapple

Benzylaminopurine and Naphthalene acetic acid combinations at different concentrations had very significant difference on number ($p \leq 0.001$) and weight of plantlets ($p \leq 0.001$) at 16 week from culture initiation (Table 1). For instance, BAP at 5 mg/L combined with NAA at 2 mg/L resulted in the highest *in vitro* proliferation with 12.3 plantlets per initial explant and biggest plantlets each weighing 1.919g.

Moreover, BAP at 5.5 mg/L in combination with NAA at 2 mg/L ranked the second with proliferation of 10.2 plantlets per quarter explant each weighing 1.824g.

Table 1: Influence of various BAP and NAA combinations on number and weight of plantlets at 16th week from culture initiation

BAP*NAA (mg/L)	Mean number of plantlets per quarter explant	Mean weight of plantlets (g)
0*0mg/L	4.5a-e	1.099a-c
0*0.01mg/L	3.833a-c	0.888a
0*0.05mg/L	5.833b-g	1.124b-d
0*0.1mg/L	5.833b-g	1.247c-f
0*2mg/L	6.167d-h	1.135c-f
2*0mg/L	4.667a-e	1.464ef
2*0.01mg/L	6c-h	1.498f
2*0.05mg/L	5.833b-g	1.363d-f
2*0.1mg/L	4.5a-e	1.374d-f
2*2mg/L	5.5b-f	1.298c-f
3*0mg/L	4.167a-d	1.372d-f
3*0.01mg/L	8g-i	1.382ef
3*0.05mg/L	7.5f-i	1.317c-f
3*0.1mg/L	7.833g-i	1.356d-f
3*2mg/L	6.667e-h	1.457ef
4*0mg/L	3.667ab	1.465ef
4*0.01mg/L	2.833a	1.014ab
4*0.05mg/L	5b-e	1.325c-f
4*0.1mg/L	3.667ab	1.296c-f
4*2mg/L	7.5f-i	1.282c-f
5*0mg/L	5.167b-e	1.284c-f
5*0.01mg/L	5.5b-f	1.212b-e
5*0.05mg/L	8.167hi	1.395ef
5*0.1mg/L	9.167ij	1.440ef
5*2mg/L	12.333k	1.919g
5.5*0mg/L	6.333d-h	1.449ef
5.5*0.01mg/L	6.167d-h	1.374d-f
5.5*0.05mg/L	6.167d-h	1.369d-f
5.5*0.1mg/L	5.333b-f	1.498f
5.5*2mg/L	10.167j	1.824g
Mean	6.1	1.357
P-value	<0.001	<0.001
SE (±)	0.650	0.1

C.V (%)	26.0	13.4

Means followed by the same letters(s) within the column are not significant at 5% level based on Duncan Multiple Range Test at $p < 0.05$. * = Significant ($p < 0.05$), ** = Highly significant at ($p < 0.01$) and ***= Very highly significant at ($p < 0.001$

4.2 Interaction influence of source of explant and growth regulator combinations on *in vitro* proliferation of pineapple

Results indicate that interaction of explant source and growth regulator combinations had a very high significant ($p < 0.001$) effect on the number of plantlets and high significant ($p < 0.002$) effect on the weight of plantlets 16 weeks from culture initiation (Table 2). Crown-derived explants performed better on MS media amended with BAP at 5 mg/L and NAA at 2 mg/L. The combination had the highest proliferation with 16 plantlets per quarter explants each weighing 1.859 g. Moreover, crown-derived explants in BAP at 5.5 mg/L and NAA at 2 mg/L ranked the second with 14 plantlets per initial explant each weighing 1.785 g.

Table 2: Interaction effect of explant source, BAP and NAA combinations on number and weight of plantlets at 16 week from culture initiation

BAP* NAA (mg/L) x Source	Mean number of plantlets per quarter explant	Mean weight of plantlets (g)
0*0mg/L x Crown	5.7b-i	1.295d-l
0*0.01mg/L x Crown	3.3a-c	0.973abcd
0*0.05mg/L x Crown	6.7d-j	1.147a-h
0*0.1mg/L x Crown	6.0c-j	0.973a-d
0*2mg/L x Crown	8.3h-l	1.460e-m
2*0mg/L x Crown	6.0c-j	1.468e-m
2*0.01mg/L x Crown	8.3h-l	1.658l-o
2*0.05mg/L x Crown	8.0g-k	1.259c-j
2*0.1mg/L x Crown	5.3b-i	1.374e-l
2*2mg/L x Crown	6.7d-j	1.270d-k
3*0mg/L x Crown	4.3a-f	1.372e-l
3*0.01mg/L x Crown	11.3l-p	1.369e-l
3*0.05mg/L x Crown	10.7k-o	1.307d-l
3*0.1mg/L x Crown	11.3l-p	1.275d-k
3*2mg/L x Crown	8.7i-m	1.274e-k
4*0mg/L x Crown	3.7a-d	1.120a-1
4*0.01mg/L x Crown	1.7a	0.829ab
4*0.05mg/L x Crown	6.3c-j	1.130a-g
4*0.1mg/L x Crown	4.7a-g	1.120a-l
4*2mg/L x Crown	11.0k-o	1.393e-l
5*0mg/L x Crown	7.0e-j	1.210c-i
5*0.01mg/L x Crown	7.3f-j	1.180c-h
5*0.05mg/L x Crown	11.7m-p	1.311d-l
5*0.1mg/L x Crown	11.7m-p	1.223l-o
5*2mg/L x Crown	16.0q	1.859m-o
5.5*0mg/L x Crown	9.0j-o	1.575h-n
5.5*0.01mg/L x Crown	8.7i-m	1.341d-l
5.5*0.05mg/L x Crown	8.3h-l	1.309d-l
5.5*0.1mg/L x Crown	6.7d-j	1.383e-l
5.5*2mg/L x Crown	14.0pq	1.785l-o
0*0mg/L x Ratoon	3.3a-c	0.904a-c
0*0.01 mg/L x Ratoon	5.7b-i	0.804a
0*0.05mg/L x Ratoon	5.0b-h	1.101a-e
0*0.1mg/L x Ratoon	5.7b-i	1.093a-e
0*2mg/L x Ratoon	4.0a-e	1.190c-h
2*0mg/L x Ratoon	3.3a-c	1.460e-m
2*0.01mg/L x Ratoon	3.7a-d	1.658l-o

BAP* NAA (mg/L) x Source	Mean number of plantlets per quarter explant	Mean weight of plantlets (g)
2*0.05mg/L x Ratoon	3.7a-d	1.467e-m
2*0.1mg/L x Ratoon	3.7a-d	1.375e-l
2*2mg/L x Ratoon	4.3a-f	1.327e-l
3*0mg/L x Ratoon	4.0a-e	1.372e-l
3*0.01mg/L x Ratoon	4.3a-f	1.327e-l
3*0.05mg/L x Ratoon	4.7a-g	1.395e-l
3*0.1mg/L x Ratoon	4.3a-f	1.438e-m
3*2mg/L x Ratoon	4.7a-g	1.641k-o
4*0mg/L x Ratoon	3.7a-d	1.465e-m
4*0.01mg/L x Ratoon	3.7a-d	1.521h-l
4*0.05mg/L x Ratoon	4.0a-e	1.521h-m
4*0.1mg/L x Ratoon	2.7ab	1.472f-m
4*2mg/L x Ratoon	4.0a-e	1.174b-h
5*0mg/L x Ratoon	3.3a-c	1.359e-l
5*0.01mg/L x Ratoon	3.7a-d	1.479f-m
5*0.05mg/L x Ratoon	4.7a-g	1.244c-j
5*0.1mg/L x Ratoon	6.7dj	1.657l-o
5*2mg/L x Ratoon	8.7i-m	1.979o
5.5*0mg/L x Ratoon	3.7a-d	1.575h-n
5.5*0.01mg/L x Ratoon	3.7a-d	1.407e-l
5.5*0.05mg/L x Ratoon	4.0a-e	1.429e-m
5.5*0.1mg/L x Ratoon	4.0a-e	1.613j-n
5.5*2mg/L x Ratoon	6.3c-j	1.864no
Mean	6.1	1
P-value	<.001	0.002
SE (±)	0.941	0.1
C.V (%)	26.0	13.4

Means followed by the same letters(s) within the column are not significant at 5% level based on Duncan Multiple Range Test at $p < 0.05$. * = Significant ($p < 0.05$), ** = Highly significant at ($p < 0.01$) and ***= Very highly significant at ($p < 0.001$

4.3 Effect of MS media state on *in vitro* proliferation of pineapple

The study revealed that MS media state had a significant ($p < 0.046$) effect on the number of plantlets per initial explant (Table 3). Semi-solid MS media resulted in

higher proliferation with 13.7 plantlets per initial explant compared to 8.3 plantlets in liquid MS media. However, semi-solid and solid MS media resulted in statistically the same number of plantlets per initial explant.

Table 3: Effect of MS media state on number and weight of plantlets on *in vitro* proliferation of pineapple

MS mdia state	Number of plantlets per quarter explant	Weight of plantlets (g)
Liquid	8.3a	1.437
Semisolid	13.7b	1.546
Solid	10.0ab	1.583
P-value	0.046	0.168
S.E (±)	1.27	0.099
C.V (%)	29.1	8.2

Means followed by the same letters(s) within the column are not significant at 5% level based on Duncan Multiple Range Test at $p < 0.05$. * = Significant ($p < 0.05$), ** = Highly significant at ($p < 0.01$) and ***= Very highly significant at ($p < 0.001$)

4.4 Interaction effect of source of explant and MS media state on *in vitro* proliferation of pineapple

There was a high significant difference (p = 009) between of the interaction of explant types and MS media state on weight of plantlets (Table 4). Crown-derived explants cultured in semi-solid MS media produced the vigorous plantlets each weighing 1.966g followed by crown-derived explants in solid MS media and ratoon-derived explants in semi-solid MS media each weighing 1.763g and 1.578g, respectively.

Table 4: Interaction effect of explant source and MS media state on *in vitro* proliferation of pineapple

Explant source*media state	Number of plantlets per quarter explant	Weight of plantlets (g)
Ratoon x Liquid MS media	6.7a	1.126a
Ratoon x Solid MS media	8.3a	1.295ab
Crown x Liquid MS media	10.0a	1.403bc
Ratoon x Semi-liquid MS media	11.0ab	1.578cd
Crown x Solid MS media	11.7ab	1.763de
Crown x Semi-solid MS media	16.3b	1.966f
P-value	0.817	0.009
S.E (±)	1.6	0.124
C.V (%)	29.1	8.2

Means followed by the same letters(s) within the column are not significant at 5% level based on Duncan Multiple Range Test at $p < 0.05$. * = Significant ($p < 0.05$), ** = Highly significant at ($p < 0.01$) and ***= Very highly significant at ($p < 0.001$

CHAPTER FIVE

5.0 DISCUSSION

5.1 Effects of growth regulators combinations on *in vitro* proliferation of pineapple

Growth regulators are important in *in vitro* propagation as they influence plant cell division, elongation and development. In so doing they influence plant growth and development.

When dealing with *in vitro* propagation it is important to balance the growth regulators as they may be both beneficial or detrimental to plants. For instance auxins have dual mechanism of action in that at lower concentration they promote growth whereas at high concentration they inhibit.

In vitro propagation is influenced by many factor such as type of explants, genotype of explants, type and concentration levels of growth regulators, and their interaction. Therefore for every plants thought for micropropagation it is important to optimize the protocol for efficient reproduciable and routine production of plantlets. The highest number of plantlets observed in this study was obtained when BAP at 5 mg/L was combined with NAA at 2 mg/L regardless of the type of explants used. This was therefore regarded as an optimum levels of the BAP and NAA concentration.

Dewald (1988) reported 40 plantlets per explant within 12 months from MS media with BAP at 2 mg/L and NAA at 2 mg/L. Usman *et al.,* (2012) reported that BAP at 1.12 mg/L and NAA at 0.56 mg/L is an optimal combination for *in vitro*

multiplication of pineapple with taller and vigorous plantlets. These differential responses of pineapple explants to BAP and NAA combination could be associated with BAP: NAA combination ratio, genetic makeup, types of explants and MS media used as reported by Souza *et al*., (2003) and George *et al*., (2008).

5.2 Effect of MS media state on *in vitro* proliferation of pineapple

Contrary to the previous studies, the current study produced a smaller number of plantlets per quarter explants in MS liquid media than those in semi-solid and solid media. This result contradicts with previous reports where the use of liquid MS medium increased *in vitro* production of pineapple cv. 'Perola', 'Perolera', 'Primavera' and ‘Maspine’ (Guerra *et al*., 1999; Almeida et al., 2002; Zuraida *et al.*, 2011; Nikumbhe *et al*., 2013). Usman *et al*., (2012) reported multiplication rates of 74.3 plantlets per explant in MS liquid compared to 47.3 plantlets per explant in MS solid media. This may be caused by availability of mechanical shakers or aerator machines in the incubator.

Improved oxygen supply is made possible by using bioreactors where filtered oxygen is continuously pumped into the media (Aitcken-Christie *et al*. 1995). Hence the lower *in vitro* multiplication of pineapple reported in MS liquid media in this study mighty be associated with poor oxygen supply into the media. In this study, oxygen was supplied into the media by shaking the culture vessels during the working time of eight hours per day. The use of MS semi-solid media is therefore recommended as the best option for enhancing *in vitro* proliferation of pineapple in the absence of bioreactor technology to supply oxygen into the media.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

From this study it can be concluded that this study successfully optimized *in vitro* regeneration system for pineapple. A routine regeneration system was optimized and made available for *in vitro* regeneration of pineapple.

Furthermore, the best *in vitro* regeneration of pineapple was achieved when crown-derived explants are cultured in semi-solid MS media. This is the first report where the best explant source together with the best MS media state coupled with the optimum concentration of BAP and NAA were reported on *in vitro* proliferation of pineapple “Smooth cayenne”.

6.2 Recommendation

For maximum multiplication of pineapple one requires suitable source of explants, proper media state amended with proper growth regulators at optimum concentration. Any plant sought for commercial multiplication requires optimization of regeneration system as *in vitro* propagation is genotypic dependent, and influenced by many factors such as media state and media formulations.

REFERENCES

Abobkar, I. M. S. and Ahmed, M. E. (2012). Plant Tissue Culture Media

Aclan, J.D., (1971). East African crops. An introduction to the production of field and plantation crops in Kenya, Tanzania and Uganda. Longman Group Limited. FAO, pp 142:143.

Aitken-Christie, J., Kozai, T., Takayama, S. (1995). Automation in plant tissue cultures – general introduction and overview. In: Aitken-Christie, J., Kozai, T., Smith MAL (eds) *Automation and environmental control in plant tissue culture*. Kluwer Academic Publ, Dordrecht, pp 1–18.

Almeida, W. A. B., Matos, A. P. de., and Souza, A. S. (1997).Effects of benzylaminopurine (BAP) on in vitro proliferation of pineapple (Ananascomosus (L.) Merr). *ActaHorticulturae*, 425:242-245.

Almeida, W. A. B., Santa, G. S., Rodrigez, P. M. and Costa, P. M. (2002).Optimization of protocol for the micropropagation of pineapple.

Almeida,W.A.B. (1994). de.Efeito da benzilaminopurinanasdiferentesfases da propagação*in vitro* do abacaxizeiro (Ananascomosus (L.) Merr.).83p. Dissertação (Mestrado) – Universidade Federal da Bahia, Escola de Agronomia, Cruz das Almas, 1994.

Baruwa, O.I. (2013). Profitability and constraints of pineapple production in Osunstate,Nigeria: *JHR* 2013,Vol 21(2):59-64

Bhojwani, S.S. and Razdan, M.K. (2005). Plant tissue culture: Theory and practice, a revised Edition. *South Africa Journal of Botany*. 72: 191

Borges, A.V., Djenidi, S.S., Lacroix, G., The´ate,J., Delille. B. and Frankignoulle, M., (2003).Atmospheric CO2 flux from mangrove surrounding waters, *Geophys.* 30(11), 1558, doi:10.1029/ 2003GL017143

Chandana B. C., Kumari Nagaveni H.C., Heena M.S., Shashikala S.K and Lakshmana D., (2018). Role of plant tissue culture in micropropagation, secondary metabolites production and conservation of some endangered medicinal crops. ~ 246~Journal of Pharmacognosy and Phytochemistry 2018; SP 3:246-251

Danso, K.E., Ayeh,K.O., Oduro,V., Amiteye,S.andAmoatey,H.M.(2008). Effect of 6-benzylaminopurine and naphthalene acetic acid on in vitro production of MD2 pineapple planting materials Ghana. *World Applied Sciences Journal*, 3(4): 614-619.

Dewald, M.G., Moore, G.A., Sherman, W.B., Evans, M.H., (1988) Production of pineapple *in vitro*, *Plant Cell Rep*. 7, 535-537.

FAOSTAT (2008). FAO Statistic Division.http://faostat.fao. org/site/339/ default.aspx.

FAOSTAT (2013) http://faostat.fao.org

FAOSTAT/COMTRADE(2013)http://faostat.fao.org/site/702/default.aspx

Firoozabady, E. and Gutterson, N. (2003). Cost-effective *in vitro* propagation methods for pineapple. *Plant Cell Rept*., 21: 844-850.*Fruits*, 56 : 415-421.

Fráguas, C. B., Dornelles, C. M. V., and Lima, G. P. P., (2009). *In vitro* bud induction and multiplication of cv. 'IAC Gomode-mel' pineapple fruit with benzyl amino purine and naphthalene acetic acid. *Ciência Rural*, v.39, n.6, p.1682- 1687,

George, E.F., (1993). Plant Propagation by Tissue Culture (Part 1, 2) (2nd edn.), Exegetics Ltd., England (1993), pp. 582-794

George, E.F., Hall.M.A, and De Klerk, G.J. (2008). Plant propagation by tissue culture. Springer, Dordrecht

Guerra, M. P., Vesco, L. L.dal., Pescador, R., Schuelter, A. R. and Nodari, R. O. (1999). Estabelecimento de um protocol regenerative paramicropropagacao do abacaxizeriro. *PesquisaAgropecuariaBrasileira*, 34 : 1557-1563.

Ivanova, M. and Staden, J.V. (2010). Natural ventilation effectively reduces hyperhydricity in shoot cultures of Aloe polyphyllaSchönland ex Pillans. *Plant Growth Regulation*, *Coverage*, v.60, n.2, p.143-150, Available from: .Accessed: Mar. Jun. 20, 2012.

Khan, S., Nasib, A. and Saeed, B.A., (2004). Employment of in vitro technology for large scale multiplication of pineapples (Ananascomosus).*Pakistan Journal of Botany*., 36: 611-615.

Kiss, E. and Kiss, G.(1995). A novel method for rapid micropropagation of pineapple. *Horticultural Science*, v.30, n.1, p.127-129.

Kumar, V. and Naidu, M.M., (2006). Development in coffee biotechnology – *in vitro* plant propagation and crop improvement. *Plant Cell Tiss. Organ*.

Maerere, A.P., Kusolwa, P.M., Msogoya, T.J. and Nsemwa, T.L.H.,(2003).Evaluation of the effective in vitro regeneration and multiplication potential of local banana cultivars in Tanzania.*In*: Proceedings of the Second Collaborative Research Workshop on Food Security, Morogoro, Tanzania, 28 - 30th May 2002, 169 – 174.

Mengesha,A., Ayenew, B. and Tadesse, T. (2013) "Acclimatization of *in vitro* Propagated Pineapple (*Ananascomosuss* (L.), var. Smooth cayenne) Plantlets to *ex Vitro* Condition in Ethiopia," *American Journal of Plant Sciences*, Vol. 4 No. 2, , pp. 317-323.

Mhatre, M. (2007) Micropropagation of pineapple, Ananas comosus (L.) merr. In: *Jain SM and H Haggman* (eds.)

Msogoya,T. J., Kanyagha,H., Mutigitu,J., Kulebelwa, M. and Mamiro, D. (2012). Identification and management of microbial contaminants of banana *in vitro* cultures Available from: www.m.elewa.org

Murashige, T., and Skoog, F. (1962).A revised medium for rapid growth and bio assays with tobacco tissue cultures. *PhysiologiaPlantarum*, 15: 473-497.

Nelson, B. J., Asare, P.A. and Arthur Junior, R., (2015). *In vitro* Growth and Multiplication of Pineapple under Different Duration of Sterilization and Different Concentrations of Benzylaminopurine and Sucrose. *Biotechnology,* 14(1), 35.

Nikhumbe, P. H., (2013) *In vitro* technology for propagation of pineapple (*Ananascomosus)* cv K. ewijas,Vol 10;172-74.

Rinkesh, J. (2013) Plant Tissue Culture Medium; MSc Thesis in Microbiology, Veer Normad South Gujarat Uni.Surat

Small, S. (2015). Plant Growth Factors: Plant Hormones. Colorado State University.*CMG Garden Notes Number* 145.

Soneji, J. R., Rao, P. S. and Mhatre, M. (2002a) Somaclonal variation in micropropagated dormant axillary buds of pineapple (Ananas comosus L., Merr.).*J. Hortic. Sci. Biotech*. 77, 28-32.

Soneji, J. R., Rao, P. S. and Mhatre, M. (2002b) *In vitro* regeneration from leaf explants of pineapple (Ananas comosus L., Merr.). J. *Plant Biochem. Biot.* 11: 117-119.

Soneji, J. R., Rao, P. S. and Mhatre, M. (2002c) Germination of synthetic seeds of pineapple (Ananas comosus L., Merr.).*Plant Cell Rep*. 20, 891-894

Souza, M. B., Kraus, E. J., Endres, L. and Mercier, H. (2003) Relationships between endogenous hormonal levels and axillary bud development of *Ananas comosus* nodal segments. *Plant Physiology and Biochemistry,* Volume 41(8):733-739.

Ubi, W., (2005). Preliminary trial of fertilizer types on pineapple (Ananas comosus) grown on coastal and sands of Cross River State, Nigeria. *Global J. Pure and applied sci.* 11(4):457-460.

USDA (2013). http://www.ers.usda.gov/media/1152682/fts-356.pdf

Usman, I. S. (2013). Development of an efficient protocol for micropropagation of pineapple (*Ananas comosus* L. var. smooth cayenne). *African Journal Agriculture Research*., 8: 2053-2056.

Zuraida, A. R., NurulShahnadz, A. H, Harteeni, A., Roowi, S., CheRadziah, C.M.Z.and Sreeramanan, S. (2011). A novel approach for rapid micropropagation of maspine pineapple (Ananas comosus L.) shoots using liquid shake culture system. *African Journal of Botany*, 10(19), 3859-3866.

`

APPENDICES

Appendix 1: The table for Analysis of variance of explants type, concentration levels of BAP and NAA, and their interactions as they influence the *in vitro* proliferation ability of pineapple

Number of plantlets at 16 week

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication	2	5.633	2.817	0.46	
Source (A)	1	590.422	590.422	96.70	0.010
Error (a)	2	12.211	6.106	2.41	
BAP_NAA (B)	29	732.800	25.269	9.96	<.001
Source x BAP_NAA (A x B)	29	297.578	10.261	4.05	<.001
Error (b)	116	294.156	2.536		
Total	**179**	**1932.800**			

Weight (g) at 16 week

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication	2	0.17948	0.08974	1.78	
Source (A)	1	0.18075	0.18075	3.59	0.199
Error (a)	2	0.10069	0.05034	1.53	
BAP_NAA (B)	29	6.82992	0.23551	7.14	<.001
Source x BAP_NAA (A x B)	29	2.10780	0.07268	2.20	0.002
Error (b)	116	3.82870	0.03301		
Total	**179**	**13.22734**			

Appendix 2: The table for Analysis of variance of the effect of media state, the type of explants and their interactions on proliferation of *in vitro* culture of pineapple

Number of plantlets at 16 week

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication	2	12.000	6.000	1.29	
Source (A)	1	72.000	72.000	15.43	0.059
Error (a)	2	9.333	4.667	0.48	
Media (B)	2	89.333	44.667	4.62	0.046
Source x Media (A x B)	2	4.000	2.000	0.21	0.817
Error (b)	8	77.333	9.667		
Total	**17**	**264.000**			

Weight of plantlet at 16 week

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Replication	2	0.11893	0.05946	2.02	
Source (A)	1	1.09964	1.09964	37.31	0.026
Error (a)	2	0.05895	0.02947	1.91	
Media (B)	2	0.06947	0.03474	2.25	0.168
Source x Media (A x B)	2	0.27377	0.13688	8.87	0.009
Error (b)	8	0.12350	0.01544		
Total	**17**	**1.74426**			