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ESTABLISHMENT OF AN EFFECTIVE IN VITRO REGENERATION PROTOCOL FOR QUALITY PROTEIN MAIZE IN GHANA

BY

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NOVEMBER, 2016

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DECLARATION

I, Shadrack Obeng Asamoah, hereby declare that this thesis, consists entirely of my own work produced from research undertaken under supervision and that no part of it has been published or presented for another degree elsewhere, except for the permissible excepts/references from other sources, which have been duly acknowledged.

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DEDICATION

This thesis is dedicated to my Lord and Saviour Jesus Christ through whose mercies I have come this far. I would also like to dedicate this thesis to Dr. Kwame Amoako Tuffuor, the initiator of Ghana School Feeding Programme (GSFP) and my supervisor, Dr. (Mrs) Antonia Y. Tetteh for their love, training and financial support.

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ABSTRACT

This study was aimed at establishing a consistent in vitro regeneration system for Quality Protein Maize (QPM) using mature embryos and root explants using two QPM genotypes from Ghana 'Obatanpa GH' and 'Etubi'. The effect of MS and N6 basal media supplemented with 2,4-D, 6-BA and CPPU on callus and embryogenic callus induction as well as plant regeneration were investigated. For primary callus induction, 4mg/L 2,4-D gave the highest frequency of 70.8% to 91.7% from mature embryo on both MS and N6 while in root explant, 3mg/L 2,4-D induced maximum frequency of 50.0% to 68.8% on both MS and N6. Increasing 2,4-D concentration from 0mg/L to 4mg/L on mature embryo and 0mg/L to 3mg/L on root explant resulted in increase in frequency of callus induction, respectively. Primary calli varied from white to creamy and opaque with compact or friable texture. For embryogenic callus induction using matured embryo, a combination of 1.0mg/L 2,4-D and 0.2mg/L 6-BA was required to achieve highest frequency of 62.5% to 91.7% whereas 2mg/L 2,4-D and 0.4µM CPPU produced maximum frequency of 62.5% to 70.8% whiles embryogenic calli development from matured embryo was high and dependent on

concentration of the growth regulators, development of embryogenic calli from root explants was low and inconsistent. A low and inconsistent frequency of embryogenic callus in root explant was obtained. Either 1mg/L or 2mg/L 2,4-D in combination with 0.2mg/L 6-BA produced 16.7% to 33.3% whereas at 3mg/L 2,4-D with 4 µM CPPU gave 29.1% to 37.5%. For shoot formation, 1mg/L 6-BA with 3mg/L IAA or 1mg/L 6-BA with 1.5mg/L IAA gave highest frequency of 53.3% to 73.3% and 40.0% to 66.7%, respectively, on mature embryoderived embryogenic calli. Root-derived embryogenic calli produced lower shoot regeneration frequency of 40.0% to 46.7% at 2mg/L 6-BA and 0.5mg/L IAA. For root development, 0.4mg/L 2,4-D with 2mg/L NAA gave highest frequency of 33.3% to 58.3% from mature embryo-derived embryogenic calli. Highest root frequency of 25.0% to 62.5% from root-derived embryogenic calli were achieved at 0.2mg/L 2,4-D with 1mg/L NAA.

Mature embryo explants developed comparatively higher plantlets than root explants. The two genotypes used as well as the basal media MS and N6 were not different with respect to primary callus induction, embryogenic callus induction, shoot and root regeneration.

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CHAPTER ONE

1.0 INTRODUCTION

Maize, a comparatively affordable cereal belongs to the grass family Poaceae. Since 2001, maize production has exceeded that of rice and wheat (FAOSTAT, 2013). Maize has become the most widely grown cereal crop in Africa especially Ghana since its introduction in the 16th century from Mexico, the centre of origin. As a source of human food and animal feed, maize provides more than 50% of the calories of the people in developing countries (Sinha, 2007; McCann, 2005), and 53% of the protein intake of local diets (Bressani, 1991). Maize is also a source of a raw material for many industries (Mejia, 2005), and currently a source of ethanol for biofuel production.

World demand for maize was estimated to be 868 million metric tons (mt) in 2013, however, supply that year was 863 million mt, representing a deficit of 5 million mt (International Grains Council, 2013). Current demand for maize is estimated to be 944 million mt/year (International Grains Council, 2013). World maize consumption is expected to rise to a record of 1,020 million mt in 2017/18, while production in the same period is projected at 1,016 million mt, indicating increase in demand not met by a commensurate production.

Until recently, average maize yield of 1.2 tons per hectare (t/ha) was recorded for subSaharan Africa, which is just below a quarter of the global average of 5.5t/ha, and about a sixth of the average yield in U.S of 7.8t/ha in 2006 (FAOSTAT, 2006). The recent maize yield is about 1.7 tons per hectare (International Grain Council, 2016).

In recent years, impressive advancements in maize productivity have been achieved through conventional breeding in West and Central Africa raising the productivity from a mean of

1.2t/ha to 1.7t/ha (International Grain Council, 2016). Despite these advancements, maize yield in West and Central Africa remains below the world average.

Key constraints to crop production globally include limited land and water resources, and abiotic and biotic stresses. In sub-Saharan Africa, the disparity in maize productivity is exacerbated by *Striga* infection, poor nutrient soils and drought. The key interventions to increasing yield of maize in sub-Saharan Africa has been through conventional breeding methods that requires many years to produce an improved cultivar. Conventional breeding is limited by wide crosses and linkage heave, and is laborious and expensive (DANIDA, 2002*)*.

Current advancements in biotechnology, involving molecular markers, polymerase chain reaction offers an alternate rapid means of improving crop yield. One of such methods is gene technology, which is often used as a complementary tool to conventional plant breeding.

A major component of the United Nation's Sustainable Developmental Goals (SDGs) is the application of biotechnology to alleviate food security problems in developing countries (Ayele *et al*., 2006; Motari *et al*., 2004; Acharya *et al*., 2003). Biotechnology for crop improvement encompasses gene discovery, plant transformation and regeneration. A prerequisite for plant transformation is the establishment of an efficient regeneration protocol, which depends on genotype (Huang and Wei, 2004; Tomes and Smith, 1985), type of explant, and a combination of growth regulators (Huang and Wei, 2004; Rakshit *et al*., 2010) and culture media (Bohorova *et al*., 1995; Machuka *et al*., 2008).

Maize varieties of temperate origin have been regenerated via subjecting to wide range of investigation in callus induction and somatic embryogenesis (Aguado Santacruz *et al*., 2007).

However, there are few reports on regeneration of tropical maize varieties through callus and embryogenic callus induction tissue culture technology (Demissie, 2008; Machuka *et al*., 2008; Machuka *et al*., 2011).

Various explants of maize have been examined for their totipotent ability to induce callus formation and regenerate whole new plant. These explants include immature embryos (Rakshit *et al*., 2010; Aguado-Santacruz *et al*., 2007; Ishida *et al*., 1996; Bohorova *et al*., 1995; Duncan *et al*., 1985; Green and Phillips, 1974), mature embryos (Huang and Wei, 2004), meristematic regions (Vladmir *et al*., 2006), leaf tissue (Ahmadabadi *et al*., 2007; Ray and Gosh, 1990; Conger *et al*., 1987), anthers (Barloy and Berkert, 1993; Ting *et al*., 1981), immature tassels (Songstad *et al*., 1992; Rhodes *et al*., 1986), tassel and ear meristems (Pareddy and Petolino, 1990), immature inflorescences (Pareddy and Petolino, 1990), and protoplasts (Moroczi *et al*., 1990), shoot apical meristem (Sairam *et al*., 2003; Zhang *et al*., 2002), shoot tips (O'Connor-Sanchez *et al*., 2002; Zhong *et al*., 1992), and in recent years, split seed explants (Al-Abed *et al*., 2006).

For efficient callus initiation from immature embryos of tropical and sub-tropical maize, Bohorova *et al.* (1995) used Chu's N6 media containing 3,6-dichloro-2-methoxybenzoic (Dicamba) and silver nitrate ($AgNO_3$).However, when Murashige and Skoog (MS) media was used, supplementation with 2,4-Dichlorophenoxyacetic acid (2,4-D) gave higher callus frequency than N6 and Dicamba suggesting that medium is playing a role in callus regeneration . For development of embryogenic callus in maize, combinations of 2,4-D and 6-benzyladenine (6-BA) were most efficient (Huang and Wei, 2004).

Furini and Jewell (1994) stated that callus induced from immature embryos with Dicamba formed more somatic embryos than with 2,4-D. However, Rooz (2002) successfully regenerated whole new plantlets from immature embryos cultured on MS medium supplemented with 0.1mg/L 2,4-D. Rakshit *et al*. (2010) used MS and N6 media in combination with 2,4-D and Dicamba to investigate induction of callus and regeneration of plant in elite Indian maize inbreds and found out that N6 medium supplemented with 1 mg/L Dicamba was optimal for inducing callus at highest frequency of 100%.

There have been successful regeneration protocols developed for Quality Protein Maize (QPM) for temperate genotypes (Al-Abed *et al*., 2006; Armstrong and Green, 1985) and few tropical varieties (Machuka *et al*., 2008). Aguado-Santacruz *et al*. (2007) used both N6 media supplemented with Dicamba, proline and MS media amended with 2,4-D and proline in their investigation on induction of calli from immature embryos of QPM *in vitro* and found out that N6 (15.4%) with Dicamba and proline and N6 (14.8%) with 2,4-D and proline were found to be most efficient formulation.

Neondo *et al.* (2016) successfully regenerated transformed plantlets from immature embryos of QPM on MS, LS and N6 media supplemented with 2,4-D and found N6 media effective for highest callus and embryogenic callus formation. Immature embryos have been considered as ideal explants for regeneration and recovery of transgenic maize lines (Huang and Wei, 2004; Green and Phillips, 1974). However, their major drawbacks are that they are strictly limited by suitable stage for culture (14 to 19 days) after pollination (DAP) (Oduor *et al*., 2006) and are highly genotype-dependent (Huang and Wei, 2004).

Even though, callus induced from immature embryo is more competent for genetic transformation of maize (Machuka *et al*., 2008; Aguado-Santacruz *et al.,* 2007; Huang and Wei, 2004; Bohorova *et al.,* 1995; Schläppi and Hohn, 1992). Huang and Wei, 2004 and Wang *et al*., 2008 have employed mature embryos for successful transformation and regeneration. Regenerated shoots from MS media amended with 2,4-D and 6-BA from mature maize embryos were successfully developed by Wang *et al*. (2009). For development of a successful regeneration protocol for transformation purpose from mature maize embryos, Ali *et al*. (2014) adopted Chu N6 media in addition with 2,4-D, kinetin, IBA and 6-BA.

Demissie (2008) produced plantlets from mature embryos of maize at frequencies of 43.3% to 75.7% on Linsmaier and Skoog (LS) media amended with 2,4-D and 6-BA. Huang and Wei (2004) were able to regenerate shoots at low frequency range of 38% to 43% from mature maize embryos on MS media amended with 2,4-D and 6-BA.

Somatic embryogenesis is a powerful system used for both transformation and multiplication of many plant species (Yuan *et al.*, 2011). There are two methods of developing somatic embryogenesis viz., direct and indirect methods. Direct method involves production of embryos directly from explants (Kohlenbach, 1978), whereas indirect method develops embryos via callus formation (von Arnold *et al.*, 2002). Green and Philips (1974) first developed whole new plant regeneration in maize from embryogenic calli using immature embryos of A188 inbred line as explants

Two types of embryogenic callus have been described in maize: Type I is compact and more easily obtained from mature embryos, while Type II is friable and has the capacity to regenerate plants over a longer period of time (Huang and Wei, 2004; Demissie *et al*., 2008; Ali *et al*.,

2014; Wang *et al*., 2009). For effective embryogenic callus formation, 2,4-D and 6benzyladenine (6-BA) combination was adopted (Huang and Wei 2004; Shohael *et al*., 2003).

In recent times, new cytokinin-like growth regulators such as thidiazuron, a herbicide, and CPPU (N-(2-chloro-4-pyridyl)-N'-phenyl urea) have proved to be effective in both primary and embryogenic callus formation as well as regeneration in citrus (Nakano *et al*., 1997; Nakajima *et al*., 2000; Tsuro *et al*., 2000). Fiore *et al.* (2002) used MS supplemented with 2,4-D and 4.0μM 4-CPPU for successful calli induction and regeneration of lemon stigma. However, the effect of MS and N6 supplemented with auxin and CPPU on maize genotypes is yet to be demonstrated. There is dearth of information on plant regeneration from root explants of maize. Lombardi *et al*. (2007) reported successful shoot regeneration from root explants of *Passiflora cincinnata* var. masta.

The importance of genetic transformation in crop enhancement programs in developing countries in Africa is minimal. The importance of strengthening food security in the continent calls for development of quicker methods besides conventional breeding to develop commercial cultivars with important agronomic traits such as high yield, biotic and abiotic stress tolerance, as well as improved nutritional qualities. In Ghana, the most widely cultivated and most consumed maize is 'Obatanpa GH', a Quality Protein Maize developed in 1992. A more recent QPM open-pollinated intermediate-maturing cultivar, 'Etubi', was released in 2007. Both cultivars were developed by Crops Research Institute of the Council for Scientific and Industrial Research, Ghana. There is dearth of information on regeneration protocols in these genotypes using any kind of explants.

This research sought to establish an effective *in vitro* regeneration protocol for QPM maize cultivars in Ghana. The specific objectives include:

1. To investigate conditions required for callus induction in mature maize embryos
2. To investigate conditions required for callus induction in maize roots
3. To determine the effect of growth regulators, 2,4-D, 6-BA, and CPPU on embryogenic callus formation in QPM
4. To develop a plant regeneration protocol for tropical QPM.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and domestication of maize

Maize crop is grown extensively all over the world in a range of agroecosystems. The crop has about 50 breeds in existence and consists of distinct colours, textures, grain shapes and sizes (IITA, 2010). Maize is thought to have been domesticated over 9,000 years ago in Central Mexico, maybe in the highlands between Oaxaca and Jalisco (Matsuoka *et al*., 2002). It arrived in Africa in the early 16th century via Portuguese trading and has since grown to be a major staple (Sinha, 2007).

2.2 Maize production

The present annual world production of maize on 159 million hectares is 944 million metric tons (mt) with the leading producer, the United States, producing 42% (International Grains Council, 2013). Africa produces 7% of the world production (FARA, 2009) and the biggest African producer is South Africa with 13 million mt, followed by Nigeria with nearly 9 million mt. Africa imports 28% of her required maize (International Grains Council, 2013). Remarkable improvement in maize production in sub-Saharan Africa has been achieved over the last 20 years, owing to widespread adoption of improved maize varieties, such as droughttolerant, disease resistant, high yielding and early maturing varieties (Babatunde *et al*., 2008). Despite these advancements, maize productivity remains as low as 1.7t/ha compared to at least 7t/ha in developed countries (IITA, 1997).

Production of maize in Ghana over the last 20 years was less than 1 million mt, however, since 2012, production has increased and remained at a little over 1 million mt (FAOSTAT, 2013). Table 2.1 shows statistics of maize production in Ghana from 2006 to 2012. Although

production of maize in Ghana has in recent years steadily increased by marginal amounts, Ghana remains a net importer of maize.

Table 2.1 Maize productions (tons) from 2006 to 2012 for the major maize growing regions in Ghana

Region	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**
Ashanti	164,226	169,383	182,848	186,830	253,374	173,735	210,000
Brong Ahafo	363,595	381,435	402,688	446,260	510,172	434,741	460,000
Central	166,847	176,222	225,214	226,420	195,394	202,362	202,500
Eastern	209,542	227,505	280,806	303,400	380,505	364,166	375,000
Greater Accra	2,134	2,775	2,762	3,310	3,584	4,461	3,500
Northern	98,157	88,037	131,857	155,500	202,316	192,604	195,000
Upper East	14,712	8,756	38,256	51,140	62,256	75,273	75,300
Volta	48,286	49,978	72,858	97,060	93,887	97,857	88,300
Western	73,210	75,406	77,553	79,010	74,191	56,134	70,000
Total	**1,140,709**	**1,179,497**	**1,414,842**	**1,548,930**	**1,775,679**	**1,601,333**	**1,679,600**

(Source: www.aphlis.net)

2.3 Maize consumption and uses

Worldwide consumption of maize in 2012/2013 was 868 million mt and a projection in 2014/2015 was estimated to be 940 million mt with Africa consuming 30% (IGC, 2013). The rate of consumption is expected to rise to a record high of 1,020 million mt in 2018/19 because of its new and diversified uses (IGC, 2013). Besides the primary use of maize as food and feed, it also finds uses as a major industrial crop for manufacturing of starch, sweeteners, and ethanol biofuel in recent times. Changes in dietary pattern of people of developing countries toward increased consumption of animal protein in recent years has also led to a rise in demand for maize, primarily as feed for livestock and poultry (IGC, 2013).

Moreover, new uses of maize for ethanol biofuel has contributed to its high demand.

To meet the demand of maize in developing countries, interventions in maize productivity, control of biotic and abiotic stress factors, and development and adoption of high-yielding and disease-resistant varieties are needed. Current maize production practices in sub-Saharan Africa are predominantly traditional and incorporate use of landraces and minimal application of agrochemicals. The development of new and improved varieties through conventional breeding may be enhanced by application of biotechnology techniques specified in the Sustainable Development Goals (Motari *et al.*, 2004; Acharya *et al.*, 2003; Ayele *et al.*, 2006).

2.4 Types of maize grain

Differences in maize grains arise due to differences in the chemical compounds stored in the kernel to give rise to high-protein, high-starch, and high-oil varieties. Based on the texture of the starch in the endosperm, maize may be classified into dent, flint, floury, waxy or pop types. Additionally, maize may be tropical or temperate depending on the temperature at which it thrives; it may be lowland, midaltitude, or highland maize on the basis of elevation of cultivation. Finally, differences in the colour of the maize grain bring some form of differences among maize kernels. Some may be white, yellow, purple, blue, or red.

2.5 Ghana maize cultivars

Various types of maize cultivars are grown in Ghana. Some are hybrids, synthetic cultivars, open-pollinated varieties (OPVs), and landraces. While majority of these are normal maize, such as Okomasa, Abeleehi, Dodzi and Dorke (white and dent), a few are Quality Protein Maize types (QPM), such as "Obatanpa GH' (white dent and flint), 'Mamaba' (white and dent), and 'Abontem' (yellow and dent).

2.6 Quality Protein Maize

Quality protein maize (QPM) is an improved form of normal maize via insertion of *opaque*-2 gene to increase synthesis of lysine and tryptophan (Mertz *et al*., 1964). It was further improved in the late 1990s at the International Maize and Wheat Improvement Center (CIMMYT) by Dr. Surinder Vasal and Dr. Evangelina Villegas (Kataki and Babu, 2003).

Dependence on conventional maize for infant feeding leads to protein energy malnutrition (kwashiorkor) and associated diseases such as Pellagra. Similarly, an exclusive conventional maize feed for monogastric animals results in poor growth and development. Consequently, QPM was developed to solve the problem of malnutrition associated with the consumption of conventional maize.

QPM development in Ghana was started in 1989 at Crop Research Institute (CRI), Fumesua. The popular varieties released are 'Obatanpa GH', 'Mamaba', 'Dadaba', 'CIDA-ba', 'Etubi', 'Akposoe', 'Golden Jubilee', 'Aziga' and 'EV DT-W 99 STR QPM Co', 'TZE-W Pop STR QPM C0', 'TZEE-W Pop STR QPM C0' (Asiedu *et al*., 2010). Since its development in 1992 in Ghana, 'Obatanpa GH' has remained the predominant maize grown and consumed in Ghana. It serves as a source of protein supplement and enhances growth and healthy development when fed to infants, livestock and poultry (Twumasi-Afriyie and Sallah, 1994; Twumasi-Afriyie *et al*., 1994). Many QPM varieties have been used as materials for biotechnology research encompassing tissue culture and plant regeneration protocols (Machuka *et al*., 2011; Aguado-Santacruz *et al*., 2007).

2.7 Maize *in vitro* culture

Maize *in vitro* culture is a biotechnological method of regenerating maize in vitro from an explant. To produce clones of maize plants that have been improved for a specific trait, such

as early-maturity, productive ears, short plants or some other desirable trait. *In vitro* culture is also employed for regeneration of whole maize plants from plant cells that have been genetically improved (Kranz and Lorz, 1993; Tomes, 1985; Hirochika *et al*., 1996).

The choice of explants, media formulation, growth regulators and culture environment were important elements that influenced the success of in vitro propagation of maize. Maize explants are simply tissues from any part of the plant intended for *in vitro* culture and may include mature embryos (Huang and Wei, 2004; Green and Phillips, 1974), immature embryos (Huang and Wei, 2004; Green and Phillips, 1974), anthers (Ting *et al.,* 1981), glumes (Suprasanna *et al.,*1986), immature inflorescence (Pareddy and Petolino, 1990).

Other explant types include immature tassels (Songstad *et al.,* 1992; Rhodes *et al.,* 1986), leaf segments (Conger *et al.,* 1987; Ray and Ghosh,1990), seedling segments (Santos *et al.,* 1984), shoot tips (O'Connor-Sanchez *et al.,*2002; Zhong *et al.,* 1992), shoot apical meristems (Zhang *et al.,* 2002), single and undifferentiated cells (Demissie, 2008) and split seed (AlAbed *et al*., 2006). Explants of various organs vary in their rates of regeneration. The most used explants are mature and immature maize embryos because they have high rates of cell division (Tahir *et al.,* 2011).

There have been successful regeneration of whole new plants from mature embryos of maize and closely related cereal crops (Al-Abed *et al.,* 2006; Huang and Wei, 2004; Ward and Jordan, 2001; Akula *et al.,* 1999; Ozgen *et al.,* 1998; Carvalho *et al.,* 1997; Rueb *et al.,* 1994; Wang, 1987; Green and Phillips, 1974). Using mature embryos from dry seeds as explant have numerous advantages over immature, including their ease of handling, availability throughout the year and in large quantity.

Green and Phillips (1974) were first to report on induction of callus from mature embryos of maize but however, they could not regenerate plantlets. Wang (1987) was successful in regenerating plantlets genotype-dependent from mature embryos of two maize inbreds, B73 and Mo17, at frequency range of 4 to 5%. Huang and Wei (2004) reported the highest shoot regeneration frequency range of 19.9% to 32.4% using mature embryos of maize

2.7.1 Culture media

Maize tissue culture medium is a liquid or gel formulated to support plant growth (Scholz *et al.,* 1995) and is generally made of either some or all of the following constituents: basal medium, vitamins, amino acids, micronutrients, sugar, antioxidants. Basal media is constituted from a combination of macronutrients including salts and hydrated salts of phosphorus (P), sulfur (S), nitrogen (N), calcium (Ca), potassium (K), and magnesium (Mg). Well-known types of basal media are the standard Chu N6 medium, Murashige and Skoog's (1962) basal salt (MS) medium, and Gamborg's B5.

The micronutrients include iron (Fe), copper (Cu), zinc (Zn), boron (Bo) manganese (Mn) and molybdenum (Mo). Vitamins including the B vitamin group such as riboflavin, thiamin (B1), pyridoxine (B6), nicotinic acid, as well as folic acid, p-aminobenzoic acid, biotin, ascorbic acid, myo-inositol, vitamin E (tocopherol) and pantothenic acid may be added. Amino acids of common use in plant regeneration include casein hydrolysate, otherwise known as casamino acids (a mixture of amino acids), L-glutamine, L-asparagine, L-tyrosine, L-glycine and L-proline. Sugars for in vitro culture may be maltose, sucrose, galactose, lactose, glucose and fructose. Other reagents that may be added as supplements include activated charcoal, silver nitrate and adenine. Addition of solidifying agents such as agar, phytagel or Gelzan produces a solid medium.

MS medium and N6 medium supplemented with 3% sucrose, 2.3g/L proline, 200mg/L casein hydrolysate was used by Rakshit *et al.,* (2009) to develop a protocol for primary callus initiation and whole new plant regeneration in elite Indian maize inbred lines. In investigating the influence of parental genotype on initiation of embryogenic callus from elite maize germplasm, Tomes and Smith (1985) used MS basal salt with 1mM asparagine and Chu N6 basal salt with 6mM proline as media and solidified them with Gelrite. Huang and Wei (2004) employed N6 basal salt and Gamborg B5 vitamins supplemented with 690mg/L proline, 2g/L glycine, 30g/L sucrose, 1g/L casein hydrolysate and 8g/L agar as media solidifying agent to regenerate plantlets from mature maize embryos.

2.7.2 Plant growth regulators

Plant growth regulators are added to with basal medium to promote cell division and elongation and to form shoot and root. Auxins and cytokinins are the growth regulators needed for shoo regeneration. Salkowski discovered the first auxin, indole-3-acetic acid (IAA) in 1885 and it was later quantified in 1928 by Went. Since then, many auxins have been discovered and synthesized for use *in vitro* culture of plants. Besides IAA, 2,4dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) are the auxins mostly used. Auxins promote cell elongation mostly in root formation, as well as the induction of callus.

Cytokinins are plant hormones that are derivatives of the purine adenine. Folke Skoog discovered cytokinins and its effects using coconut milk in the 1940s at the University of Wisconsin, Madison (Kieber, 2002). Commonly used cytokinins in culture media are adenine-type cytokinins such as 6-benzylaminopurine (also called benzyladenine 6-BAP or BA), zeatin, kinetin, and phenylurea-type cytokinins, such as N-(2-chloro-4-pyridyl)-Nphenylurea (4-CPPU), thidiazuron (Yao, 2010) and diphenylurea, which possesses embryogenic and

organogenic potentials (Fiore *et al*., 2002; Nakajima *et al*., 2000; Tsuro *et al*., 2000; Millan-Mendoza and Graham, 1999; Nakano *et al*., 1997).

Cytokinins promotes cell division and enhance shoot formation and axillary shoot proliferation (Muller and Sheen, 2008). The proportion and concentration of auxins and cytokinins added to the medium mainly affects the type of morphogenesis in plant tissue culture. High ratio of auxin to cytokinin promotes root initiation in plantlets whereas at low auxin to cytokinin ratio, adventitious shoot proliferation predominates (Smigocki and Owens, 1989).

Exogenous 2,4-D (Rooz, 2002; Kranz and Lorz, 1993) has been used for *in vitro* somatic embryo induction. It was proven in carrot, one of the most widely studied that, single cultured cells need 2,4-D to induce embryo formation (Komamine and Nomura, 1985). The growth regulators, 2,4-D, 6-BA and IBA were used Huang and Wei (2004) to develop callus and somatic embryos in their study to develop effective plant regeneration protocol from mature embryos of maize. Tomes and Smith (1985) in their study to find out the influence of parent genotype on induction of somatic embryos from elite maize (*Zea mays* L.), used 2,4-D to induce callus after 14 and 28 days. Rakshit *et al*., (2009) initiated callus and regenerated whole plants from elite Indian maize inbreds using MS andN6 amended with 2,4-D, BAP, IAA and NAA as growth regulators.

Yuan *et al.* (2009) reported that, MS amended with low concentration of 6-BA, NAA and IBA were suitable for root induction and growth of centipede grass. Liang and Keng (2008) cultured nodal segments of *Phyllantus niruri* (Euphorbiaceae) on MS amended combined with 1.0mg/L BA to produce shoot (6.6 shoots per explants) within four weeks. In alfalfa, callus induction

were initiated in the presence of NAA and a short 2,4-D shock was sufficient to induce embryo development under the subsequent hormone-free conditions (Dudits *et al*., 1991).

Although literature on application of CPPU in regeneration of maize is limited, there are reports of use of CPPU to initiate somatic embryogenesis in citrus. Callus induction, somatic embryogenesis and plant regeneration were achieved in lemon [*Citrus limon* (L.)] and sweet orange [*Citrus sinensis* (L.)] from cultures of stigma and style transverse thin cell layer explants on Murashige and Tucker medium (MT) with different combinations of 2,4-D and 4CPPU. A low frequency of regeneration from callus was obtained, ranging from 0% to 24.8% in somatic embryos and a much higher shoot regeneration of 53% to 75% (Fiore *et al*., 2002). Nookaraju *et al*. (2007) studied the influence of pre-bloom sprays of CPPU and 6-BA supplemented culture media on embryo recovery in six stenospermocarpic grapevine cultivars and the rate of embryo recovery ranged from 0.45% to 61%.

2.7.3 Factors affecting in vitro culture

When culturing explants in vitro, physical factors in the growth medium, such as pH, temperature, light (quality and duration), osmotic pressure and the gaseous environment have to be maintained within acceptable limits (Stewart, 2008). A combination of light and temperature regimes of 27 ºC to 28 ºC under a 16 h photoperiod with cool white fluorescent light ($40\mu molm^{-2}s^{-1}$ - $80\mu molm^{-2}s^{-1}$) at pH 5.8 were employed by Rakshit *et al.* (2009), Tomes and Smith (1985) and Huang and Wei (2004) to regenerate plants from maize.

2.7.4 Callus induction in maize

Callus is a mass of undifferentiated and unorganized cells obtained from tissues of plant for biotechnological uses (Momoko *et al.,* 2013). The specific auxin to cytokinin ratios in plant in

vitro culture medium give rise to an unorganized dividing and growing mass of callus cells (Green and Phillips, 1974). Induction and transformation of callus have been established as a powerful tool to study plant functional genomics, growth regulation, and plant cell morphogenesis (Kotchoni *et al*., 2012).

Callus cultures are often broadly classified as organogenic, embryogenic and nonembryogenic (Huang and Wei, 2004). Embryogenic calli are classified as Type I and Type II. Type I callus is often compact whereas Type II callus is mostly friable and fall apart easily (Demissie, 2008; Huang and Wei, 2004). Developed callus can go through direct organogenesis and/ or embryogenesis where entirely new plants are formed from the cells (Huang and Wei, 2004). Genetically identical clones of plants with desirable traits can be developed from callus tissues. Callus cells can be transformed by inserting gene of interest using biolistic bombardment known as gene gun or *Agrobacterium tumefaciens* (Schläppi and Hohn, 1992).

Green and Phillips (1974) reported successful initiation of callus from mature embryos of msize cultured on Linsmaier and Skoog medium supplemented with 2,4-D. Huang and Wei (2004) also developed embryogenic callus in maize using N6 in combination with 2,4-D and 6-BA in MS media. Aguado- Santacruz *et al*. (2007) formed efficient regenerable callus from immature maize embryos employing N6 medium supplemented with 2,4-D and Dicamba.

2.7.5 Somatic embryogenesis

Somatic embryogenesis is used as a model to understanding the physiological and biochemical activities that occur in plant developmental processes (Quiroz-Figueroa *et al.,* 2006). It is an asexual process whereby somatic cells are used to develop embryos similar to zygotic embryos (Feher, 2005). Somatic embryogenesis was first reported by Steward *et al.* (1958) using carrot

cell suspension cultures. Somatic embryogenesis may be direct when embryos are induced directly from explant tissue (Kohlenbach, 1978), or indirect when embryos are developed through callus intervention (Street and Withers, 1974). Somatic embryogenesis are used for clonal propagation, virus elimination, genetic transformation, regeneration of whole new plants from protoplasts, and development of synthetic seed.

George *et al.* (2008) reported that, in vitro culture under a high auxin concentration and low concentration of cytokinin induces somatic embryos. KrishnaRaj and Vasil (1995) reported that, a high concentration of exogenous auxin is required to maintain the embryogenic nature of most embryogenic monocot cultures. The most commonly used auxin for somatic embryo induction is 2,4-D (Reynolds, 1984) whereas cytokinin, when required, is mostly 6-BA (Huang and Wei, 2004). Komamine and Fujiwara (1980) also used zeatin to improve the frequency of somatic embryos to 90% in carrot cells. Cytokinins like CPPU performed well in inducing somatic embryos in citrus *limon* and sweet orange (Fiore *et al*., 2002).

There are several reports on successful somatic embryo induction in maize (Ali *et al*. (2014); Machuka *et al*. (2008); Usman *et al*. (2007); Huang and Wei (2004); Moi *et al*. (1993); Perez *et al*. (1993); Emons and Kieft (1991); Fransz and Schel (1991); Debjani and Ghosh (1990); Pareddy and Petolino (1990); Vasil *et al*. (1986); Armstrong and Green (1985) and Lu *et al*. (1983). Inspite of these numerous reports (Huang and Wei, 2004; Aguado Santacruz et al., 2007; Wang *et al*., 2009;), the success rate of induction of embryogenic callus reported is usually low to moderate because the optimum levels of growth regulators that induce highest embryogenic calli frequency has not been achieved. Ali *et al*. (2014) attained embryogenic callus frequency range of 2% to 8% for both mature and immature embryos of 'Sultan', 'Soneri', 'Golden' and 'Agaiti 85' maize genotypes. Machuka *et al*. (2008) also achieved Type

I embryogenic calli at a frequency of 17.6% and 52.9% in Type II from Kenyan maize inbreds and hybrids respectively. Usman *et al*. (2007) had an embryogenic frequency range of 45% to 72% from 87014 Z28-11 and POP48SRS5-57 inbreds. Huang and Wei, (2004) obtained frequency of embryogenic callus formation of 0.2% to 23.4% in maize varieties C8605 and 9046. Embryogenic callus production frequencies of 30% to 80% in maize genotypes IPBvar4 and Pi23 were reported by Perez *et al*. (1993).

2.7.6 In vitro regeneration of maize

Plant regeneration is a process of developing a whole new plant from tissue culture and it may be accomplished either via somatic embryogenesis or organogenesis. Plant regeneration has been successfully developed from calli from anthers (Ting *et al.,* 1981), glumes (Suprasanna *et al*., 1986), immature inflorescences (Pareddy and Petolino, 1990), immature tassels (Rhodes *et al*., 1986; Songstad *et al.,* 1992), leaf segments (Conger *et al.,* 1987; Ray and Ghosh, 1990), seedling segments (Santos *et al.,* 1984) and shoot apical meristems (Zhang *et al.,* 2002). Immature embryo-derived callus has been identified as most effective for plant regeneration than calli from other explant tissues (Aguado-Santacruz *et al*., 2007). Green and Phillips (1974) first identified the potential of mature embryo to induce callus but however, plantlets were not regenerated. Wang (1987) successfully developed genotype-dependent plantlets from mature embryos of these two maize inbreds B73 and Mo17 at a frequency of only 4% to 5%.

2.8 Application of maize tissue culture

The major application of maize tissue culture is for the development of transgenic plants. Different authors have reported effective recovery of genetically transformed maize plants from immature embryos using different techniques (Frame *et al*., 2002; Shou *et al*., 2004). Genetically transformed maize plants of tropical and sub-tropical genotypes were acquired by means of micro-projectile attack of calli from shoot tips of germinated mature embryos

containing embryogenic-like and organogenic structures (O'Connor-Sanchez *et al.,* 2002). Zhang *et al*. (2002) performed transformation of recalcitrant elite maize inbreds through micro-projectile bombardment of in vitro shoot meristematic cultures produced from germinated seedlings. *Agrobacterium*-mediated transformation of shoot meristem yielded transgenic maize plants through both callus initiation and direct organogenesis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant material

Two genotypes of Quality Protein Maize (QPM), 'Obatanpa GH' and 'Etubi' used were developed by Crops Research Institute (CRI), of the Council for Scientific and Industrial Research, Kumasi, Ghana. 'Obatanpa GH' is tropically adapted, open-pollinated, intermediate-maturing, white dent and flint variety. It was released in 1992 by CRI, Ghana, in partnership with the International Institute of Tropical Agriculture (IITA), Ibadan, International Maize and Wheat Improvement Centre (CIMMYT), Mexico, and the Sasakawa Global 2000 (Badu-Apraku *et al*., 2006). 'Etubi' is also tropically adapted, open-pollinated maize cultivar and intermediate-maturing, with white flint endosperm. It was also released by CRI, Ghana, in collaboration with IITA and CIMMYT in 2007 (Boateng *et al*., 2012).

3.2 Planting of donor explants

Seeds of each genotype were planted on two 15-hill row of dimensions 6 m × 1 m separated by 1 m alley. Spacing between hills in a row was 0.75 m. The recommended agronomic practices for pest and disease control were applied. Maize stem borers (*Busseola fusca, Sesamia calamistis*) and cutworms (*Agrotis spp.*) were controlled using Conpyrifos 48 % (1-1.5L/ha) and Cymethoate Super (1-1.5L/ha). Fertilizer equivalent to 120:60:40kg/ha of NP_2O_5-K_2O plus sulphate of ammonia (125kg/ha) was applied at 21 days after planting and at ear emergence. Post-emergence weeds were controlled by application of Atrazine (4.5L/ha) and hand weeding with a hoe. Plants were harvested 100 to 110 days after planting, ears were detached and transported to the laboratory for explant preparation.

3.3 Pretreatment and sterilization of explants

Mature seeds from the ears were aseptically detached and pre-treated with a fungicide solution containing 7g\L Funcidor (64% mancozeb, 8% metalaxyl) and bactericide (0.1g\L ampicillin and 3.65g\L chloramphenicol) (Fatmi and Schaad, 1988). The solution was prepared by dissolving 7.0g Funcidor in 1L distilled water. A 0.1g ampicillin and 3.65g chloramphenicol were dissolved in a small volume (3mL) of 70% ethanol and the supernatant after decantation was added to the solution. After thorough stirring, the ethanol was evaporated off by gentle heat treatment for 10 min. For pre-treatment by surface sterilization, seeds were soaked in the fungicide solution for 24h under a laminar flow hood. The soaked seeds were then rinsed three times with sterilized deionised water, immersed in 70% ethanol for 5min rinsed again for three times as before and then immersed in 20% sodium hypochlorite (7% chlorine) containing 2-3 drops of Tween 20 for 30min. Seeds were then rinsed three times with sterilized deionised water as before. The kernels were again surfacesterilized with 10% sodium hypochlorite (7% chlorine) for 15min and rinsed three times with sterilized deionised water. The surface

sterilized seeds were divided into two, one of which were excised for their plumule and the other cultured for their root fragments. All experiments were carried out in the laminar flow hood.

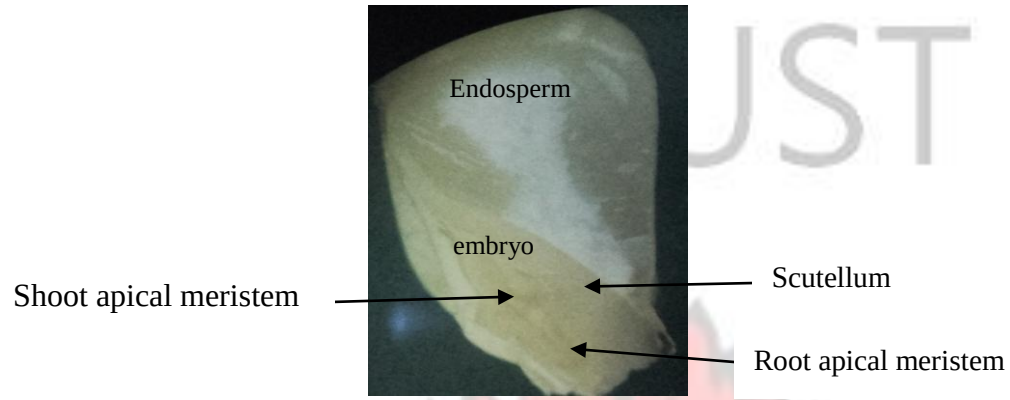


Figure 3.1 Longitudinal section of maize seed.

3.4 Media and callus induction

Two sets of media were tested viz., Murashige and Skoog (MS) (1962) with vitamins and Chu (N6) media supplemented with Gamborg (B5) vitamins (Chu *et al.*, 1975), 2.0mg/L glycine, 3g/L casein hydrolysate. The media were supplemented with 100mg/L myo-inositol, 2.3g/L proline, 500mg/L asparagine, and 30g/L sucrose. Both media were amended with 0, 1, 2, 3 or 4mg/L of 2,4-D (Table 3.1) at pH 5.7 ± 0.1. Three (3g/L) gelzan was added as a solidifying agent. The media were autoclaved at 121$_{o}$C at 15 psi for 20 to 25 min. and cooled to 65 ^{o}C. All reagents were purchased from Phytotechnology Laboratories.

About 22mL of media were poured into pre-labeled Petri plates and allowed to solidify in a laminar flow hood. Eight mature embryos and root fragments respectively were placed onmedia and sealed with laboratory muslin tape. In another set of experiments, eight root explants (0.5 to 1.0cm) were tested as before. Media without addition of 2,4-D served as control. The effects tested were media, maize variety, explant, and 2,4-D level. The design was

2×2×2×5 factorial experiment arranged in a completely randomized design with three replications. In all 40 treatments were studied. The Petri plates were arranged in the growth chamber (Sanyo Versatile Environmental Test Chamber MLR 351 H, SANYO) and maintained at 27± 1 ^{o}C for 21 days in the dark.

Growth of calli was examined on the 3rd, 7th and 14th day of culture. After 14 days of culture, the number of explants per plate that had developed callus were recorded and percentage callus induction calculated. In addition, the colour of callus as well as the texture were recorded. The primary calli were then transferred to the embryogenic medium for embryogenic calli formation.

3.5 Induction of embryogenic calli

Primary calli harvested from callus induction media were layered on MS and N6 media amended with 2,4-D and two types of cytokinins, 6-BA and CPPU for embryogenic callus formation. The total number of treatments were 288. The composition of the basal embryogenic media was same as the MS and N6 basal media of the induction media with the exception of plant growth regulators. Concentration of the growth regulators were varied for 2,4-D at 0, 1, 2, 3mg/L, 6-BA at 0, 0.2, 0.4mg/L, and finally CPPU at 0, 0.4, 4.0µM to investigate the effect of these growth regulators on formation of embryogenic callus and subsequent conversion into somatic embryos. The pH was adjusted to 5.7 ± 0.1. Gelzan was added to the medium at a concentration of 3 g/L and autoclaved at 121^{0}C at 15 psi for 20 to 25 min and cooled to 65 ^{o}C.

After pouring the media into petri plates, eight primary calli were seeded on the embryogenic media and arranged in 2×2×2×3×3×4 factorial experiment in a completely randomized arrangement in three replications in growth chamber at 27 ± 1 ^{0}C in darkness for 24h and then transferred to 16h photoperiod with cool white fluorescent light (80µmolm^{-2}s^{-1}). Sub culturing

was done every two weeks onto fresh media. After four weeks of culture, embryogenic calli were counted per plate.

3.6 Plantlet regeneration

Embryogenic calli were transferred onto regeneration media with 8 calli per plate. The composition of the basal shoot regeneration media was the same as the callus induction medium except the plant growth regulators. In these media, IAA and 6-BA were tested at 0, 0.5, 1.0 or 1.5mg/L and 0, 1, 2 or 3mg/L, respectively. The pH of the shoot regeneration media was adjusted to 5.7 ± 0.1 before autoclaving as described above (see section 3.5). Each treatment was replicated three times in a completely random design. Cultures were maintained at 27 ± 1 ^{0}C in the dark for 24h under 16h photoperiod and light intensity (4080µmolm^{-2}s^{-1}).

After 2 weeks of culture, the percentages of shoots regenerated per petri dish were calculated. Regenerated shoot were then transferred to MS and N6 media supplemented with 2,4-D (0, 0.2 or 0.4mg/L) and NAA (0, 1 or 2mg/L) for root regeneration. Cultures were incubated as described above. Shoots which developed roots were counted and were acclimatized 2 weeks after culture. Only plantlets with well developed roots were acclimatized.

3.7 Data Analysis

Results on the number of days to callus formation, properties of the callus (colour and texture), number of primary calli producing embryogenic calli, as well as the number of embryogenic calli producing shoots and roots were collected at regular intervals. Means, standard deviations, range, and coefficient of variation were computed. Analysis of variance was computed and differences in means were separated by the least significant difference (LSD) test. All data analysis were carried out using SAS 9.3 (Statistical Analysis System, Cary, NC, 2011).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Effect of 2,4-D on callus induction from embryo and root explants.

The addition of 2,4-D is critical to initiate the formation of callus in mature embryos (Rakshit *et al*., 2010; Huang and Wei, 2004). Analysis of variance of results showed that, there were significant difference between 2,4-D treatments (Appendix 3). Lowest induction frequency (25% to 50%) occurred at 2,4-D of 0mg/L for both 'Obatanpa GH' and 'Etubi' embryos cultured on MS and N6 media (Table 4.1).

Increasing the concentration of 2,4-D in the medium to 4mg/L correspondingly increased the percentage of callus formation of 'Obatanpa GH' embryo on MS (83.33%) and N6 (70.83%) and 'Etubi' embryo on MS (91.67%) and N6 (79.17%) (Table 4.1). Bhaskaran and Smith (1990) have reviewed the role of plant growth regulators in cereal tissue culture. The requirement of 2,4-D for primary callus production in mature embryo have been inconsistent. Huang and Wei (2004) reported that requirement of 4.0mg/L 2,4-D for maximum callus production of 97.6%; 3mg/L 2,4-D for highest callus initiation of 80.0% to 86.0% (Wang *et al*., 2008); and finally, 2mg/L 2,4-D for highest callus induction of 40% to 70% (Green and Phillips, 1974) and 50% to 69% (Ali *et al*., 2014).

The inconsistent callus induction frequencies chiefly arise from variations in genotype (Choudhury *et al*., 2008). In this study, 4mg/L 2,4-D was optimal for the induction of maximum primary calli from mature embryos (91.7%) and it indicates that to achieve callus induction frequency of over 90% from mature maize embryos, 4mg/L 2,4-D must be used which buttresses that of Huang and Wei, 2004. The results of this study also showed that, callus formation in embryo of 'Obatanpa GH' began at a faster rate than 'Etubi' but slowed down with increase in 2,4-D concentration.

Root explants cultured on both MS and N6 control developed primary callus at a low frequency (16.67% to 37.50%) (Table 4.1). Analysis of variance showed that, there were significant difference between the various levels of 2,4-D (Appendix 3). Increasing the concentration of 2,4-D resulted in an increase in callus induction. A high 2,4-D concentration of 2mg/L was optimum for the induction of primary callus from 'Etubi' on MS (56.25%) and N6 (56.25%) (Table 4.1). The highest frequency of callus induction from 'Obatanpa GH' root explants was attained at 3mg/L 2,4-D on MS (50%) and N6 (68.75%) (Table 4.1). However, increasing the concentration of 2,4-D to 4mg/L resulted in a decline in callus formation.

Auxins, usually 2,4-D in the range of 1-3mg/L are essential for the establishment of callus from cereal embryos. Some reports are, 3mg/L 2,4-D for highest callus initiation of 80.0 % to 86.0 % (Wang *et al*., 2008); and 2mg/L 2,4-D for highest callus induction of 40% to 70% (Green and Phillips, 1974) and 50% to 69% (Ali *et al*., 2014). The results of this paper has showed that, it is also essential for callus induction from root explants of cereals especially maize since 2mg/L 2,4-D was optimal for 'Etubi' roots and 3mg/L 2,4-D was also optimal for

'Obatanpa GH' roots. Sen *et al*. (2014) reported the production of highest callus at 2mg/L 2,4-D from roots of *Achyranthes aspera* L.

This study indicated inconsistencies in callus induction frequencies and it chiefly arise from variations in genotype (Choudhury *et al*., 2008). Iantcheva *et al.* (2005) induced embryo and regenerated plantlets from root explants of *Medicago truncatula.* Lombardi *et al*. (2007) developed in vitro shoot from root explants of *Passiflora cincinnata* var. *masta*.

Analysis of variance of the results of this study showed a significant difference in the explants used to induce primary calli (Appendix 3). The frequency of inducing primary calli from mature embryos (91.67%) was better than root explants (68.75%) and that mature embryos which are readily available throughout the year can be used as an effective alternative explant source in maize transformation although researchers have been successful in transformation of maize with immature embryos (Huang and Wei, 2004; Al-Abed *et al*., 2006).

Analysis of variance in this results showed that media and maize varieties did not affect the induction of primary calli from both embryo and root explants of 'Obatanpa GH' and 'Etubi' (Appendix 3). This can be because of similarities in genetic makeup of the varieties and similarities in the composition of MS and N6.

Table 4.1 Effect of media and varying concentration of 2,4-D on callus induction from embryo and root explants in two maize varieties.

		MS		N6	
	2,4-D (mg/L)	'Obatanpa GH' (%)	'Etubi' (%)	'Obatanpa GH' (%)	'Etubi' (%)
embryo	0	50.00 ± 17.68^{abc}	25.00 ± 12.50^{a}	43.75 ± 26.5^{abc}	31.25 ± 8.84^{a}
	1	62.50 ± 12.50^{ab}	81.25 ± 8.84^{bc}	54.27 ± 7.22^{abc}	58.33 ± 7.22^{ab}
	2	66.67 ± 19.04^{abde}	54.27 ± 7.22^{ab}	50.00 ± 0.00^{abc}	75.00 ± 17.68^{b}
	3	70.83 ± 14.43^{adf}	75.0 ± 12.50^{bcf}	62.50 ± 12.50^{a}	62.50 ± 12.50^{ab}
	4	83.33 ± 14.43^{adf}	91.67 ± 14.43^{cf}	70.83 ± 7.22^{cd}	79.17 ± 7.22^{bdf}

roots	0	25.00±0.00^{bce}	37.50±17.68^{ae}	0.00	25.00±0.00^{ae}
	1	50.00±35.36^{acde}	41.67±31.46^{a}	33.33±26.02^{ae}	31.25±8.84^{a}
	2	16.67±7.22^{cg}	56.25±26.52^{ab}	29.17±19.09^{beg}	56.25±26.52^{ab}
	3	50.00±33.07^{be}	45.83±31.46^{adh}	68.75±26.52^{cf}	50.00±25.00^{ab}
	4	37.50±33.07^{bc}	50.00±17.68^{ab}	37.50±21.65^{abf}	31.25±8.84^{ac}

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

This study showed that, the swelling of scutellum and appearance small outgrowths of callus from mature embryos and root explants began on the third to fifth days after culture plating. These developed into callus mass within fourteen days of incubation. (Figure 4.1). Formation of calli within fourteen days was reported by Huang and Wei (2004) on culture of mature embryos of elite maize inbred lines on N6 media amended with 4mg/L 2,4-D and Wang *et al*. (2008) from mature embryos of maize inbred line 9046 on MS media, but in twenty-one days of culture.

Physical appearance of the calli was variable in that some calli from both embryo and root explants of 'Obatanpa GH' and 'Etubi' appeared compact while others were friable on both MS and N6 media. All calli were white to creamy and opaque and primary calli from mature embryo were bigger in size that calli from root (Figure 4.1). Earlier reports on colour appearance of primary calli from mature maize embryos of open-pollinated and inbred line varieties have been yellowish and soft on N6 media amended with 2mg/L 2,4-D (Huang and Wei, 2004) and white and soft on (Demissie *et al*., 2008). The calli developed from this study were more similar to the compact, opaque, white to pale yellow calli of immature embryos from Kenyan maize (Odour *et al*., 2006).

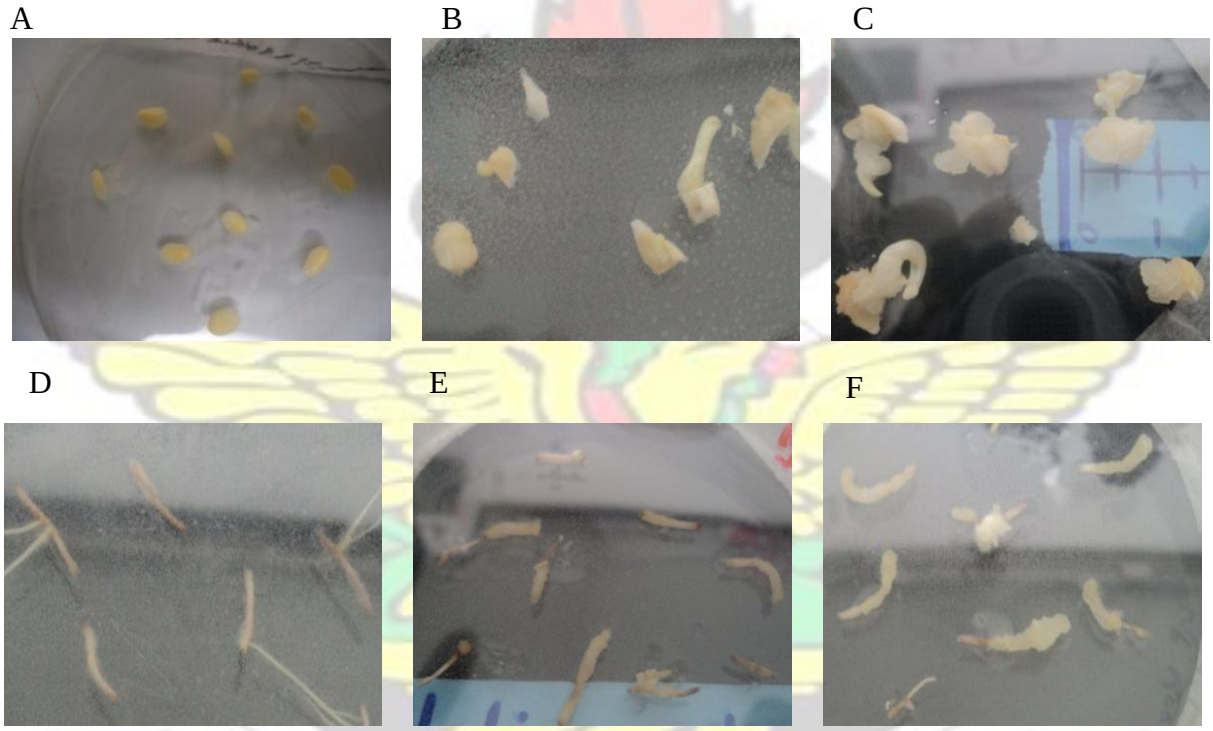


Figure 4.1 Callus development from embryo explants (A,B,C) and root explants (D,E,F) on MS and N6 supplemented with 3mg/L 2,4-D. (A) Day 3: swellings of mature embryos; (B) Day 7: Beginning of callus induction; (C) Day 14: large-sized callus; (D) Day 3: swellings of the roots; (E) Day 7: further swelling and callus induction at the tips; (F) Day 14: increase in size of callus.

4.2 Effect of growth regulators on embryogenic calli development

Primary calli were transferred to embryogenic medium consisting of MS basal salts or N6 supplemented with varying concentrations of 2,4-D and 6-BA or 2,4-D and CPPU. The results were presented in Table 4.2 A, and B. Microscopic images showed the development of globular structures indicating the initiation of somatic embryos (Figure 4.2) on MS or N6 independent of the concentration of 6-BA and CPPU in the basal media, cultivar and the type of explant used.

The embryogenic calli formed from both mature embryo and root of 'Obatanpa GH' and 'Etubi' on MS and N6 supplemented with 2,4-D and 6-BA or CPPU were principally identical in texture and colour in this study. In terms of calli texture, two types, compact (Type I embryogenic callus) and friable (Type II embryogenic callus) wereobtained from both explants on both media (Figure 4.2). With regard to colour, both calli were cream to yellow with faint pink to purple streak (Figure 4.2). The friable callus also appeared translucent and spongy. Huang and Wei (2004) gave a similar description of compact (Type I) and friable (Type II) light yellow or creamy embryogenic callus among mature embryos of
CIMMYT maize lines CML 111, CML 117, CML 124, and CML 125 genotypes.

Aguado- Santacruz *et al*. (2007) developed white, compact callus and friable pale yellow or white clear embryogenic callus from immature embryos of QPM (CML-145, CML-176, and CML-186). The development of somatic embryos progress from the formation of globular structures through heart to torpedo shape to cotyledon has reported of this trend of somatic embryo formation in maize and other plants (Wang *et. al*., 2008). In this study, there was no such distinction but rather there were greenish structures which indicate the presence of

meristerm by which can develop into plants when transferred to a regeneration medium. The number of embryos developed varied depending on the concentration of both 2,4-D and 6BA.

4.2.1 Effect of 2,4-D and 6-BA on embryogenic calli development from embryo and root explants

Analysis of variance from this results showed significant difference between growth regulators, 2,4-D and 6-BA (Appendix 4). In the absence of both 2,4-D and 6-BA, no embryogenic calli were formed from primary calli induced by embryo. In the absence of 2,4D alone, 0.4mg/L 6-BA developed the highest embryogenic calli in both 'Obatanpa GH'

(29.2%) and 'Etubi' (25%) (Table 4.2A).

Increase in concentration of 2,4-D up to 4mg/L gave a significant ($P \leq 0.05$) rise in embryogenic calli induction response up to 66.7% to 91.7% (Table 4.2A). When the concentration of 2,4-D was increased to 1mg/L, 0.2mg/L 6-BA was optimal for embryogenic calli formation in both cultivars. However, the number of calli produced from 'Etubi' (91.7%) was higher although not significant from 'Obatanpa GH' (70.8%) on MS. On N6 medium, 87.5% embryogenic calli were developed in 'Obatanpa GH' with 1mg/L 2,4-D and 0.2mg/L 6-BA (Table 4.2A). Contrarily in Etubi, 2mg/L 2,4-D plus 0.2mg/L 6-BA was optimal resulting in the formation of 83% embryogenic calli (Table 4.2A).

When primary calli from root explants were cultured, embryogenic calli could not form on MS and N6 devoid of plant growth regulators. At growth regulator concentrations of 0mg/L 2,4-D with 0.4mg/L 6-BA, 1mg/L 2 4-D with 0.2mg/L 6-BA, 2mg/L 2,4-D with 0.2mg/L 6BA, significant ($P<0.05$) increases in embryogenic calli formation from 25.0% to a maximum of

33.3% were achieved on both MS and N6 (Table 4.2B). Maximum embryogenic callus induction in roots were achieved with 1mg/L 2,4-D and 0.2mg/L 6-BA on MS for both 'Obatanpa GH' and 'Etubi' and 2mg/L 2,4-D and 0.2mg/L 6-BA on N6 for both varieties (Table 4.2B).

However, in current study, increasing the concentration of 6-BA to 0.4mg/L at all levels of 2,4-D resulted in a decrease in the embryogenic callus formation frequency to an overall mean of 24.0% (Table 4.2A and B) indicating that 6-BA at higher concentration causes a decrease in embryogenic calli formation and that at a lower concentration enhances embryogenic calli formation. The presence of 2,4-D led to increased embryogenic calli development in both explants and maize cultivar. This shows that auxin 2,4-D is critical for cereal embryogenic calli formation from both mature embryo and roots explant (Vikrant and Rashid, 2002).

Auxin in the range of 1-4mg/L is essential for embryogenic calli development (Huang and Wei, 2004). Cytokinins in combination with auxin to induce somatic embryogenesis in callus culture has been reported for cereals (Bhaskaran and Smith, 1990; Gaspar et al., 1996). The findings from this study were similar to that of Huang and Wei (2004), who stated that adding lower concentrations of 6-BA improved the frequency of embryogenic callus induction In this study, addition of auxin 2,4-D level (1mg/L and 2mg/L) with low level of cytokinin, 6-BA (0.2mg/L) conforms with report by Huang and Wei (2004) that, 2.0mg/L 2,4-D and 0.2mg/L 6-BA combination was most efficient. Wang *et al*. (2008) stated that, addition of 6-BA was necessary for initiation of somatic embryos from embryogenic calli at all levels of 2,4-D combined with 0.1mg/L 6-BA.

Findings from the effect of 2,4-D and 6-BA on embryogenic calli induction shows clearly that primary calli from mature embryos were more responsive to embryogenic callus formation than root explants (Table 4.2A and B). Media and genotype did not affect the formation of embryogenic calli from primary calli of embryo explant or root explant (Appendix 4). The results from this study indicated that embryogenic calli formation is genotypeindependent.

Table 4.2A Embryogenic outgrowth on mature embryo explants from QPM maize genotypes cultured on media supplemented with 2,4-D and 6BA

Growth Regulators (mg/L)		Embryogenic calli development (%)			
		Murashige and Skoog basal medium		Chu N6 basal medium	
2,4-D	6-BA	Obatanpa GH	Etubi	Obatanpa GH	Etubi
0	0.0	0.0±0.0^{a}(0-0)	0.0±0.0^{a}(0-0)	0.0±0.00^{a}(0-0)	0.0±0.00^{a}(0-0)
	0.2	4.2±7.2^{a}(0-12.5)	12.5±12.5^{ab}(0-25)	25.0±0.0^{bc}(25-25)	20.8±7.2^{abc}(12.5-25.0)
	0.4	29.2±14.4^{bcd}(12.5-37.5)	25.0±0.0^{bc}(25-25)	16.7±7.2^{abc}(12.5-25)	8.3±14.4^{ab}(0-25)
1	0.0	0.0±0.00a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	4.2±7.2a(0-12.5)
	0.2	70.8±14.4fgh(62.5-87.5)	91.7±7.2h(87.5-100)	87.5±12.5h(75-100)	62.5±0.0fg(62.5-62.5)
	0.4	16.7±7.2abc(12.5-25)	75.0±0.0fgh(75-75)	25.0±0.0bc(25-25)	12.5±0.0ab (12.5-12.5)
2	0.0	12.5±21.7ab(0-37.5)	20.8±14.4abc(12.5-37.5)	12.5±0.0ab(12.5-12.5)	12.5±0.0ab (12.5-12.5)
	0.2	54.2±19.1bef(37.5-75)	25.0±12.5bc(12.5-37.5)	54.2±19.1ef(37.5-75)	83.3±14.4gh(75-100)
	0.4	29.2±7.2bcd(25-37.5)	50.0±12.5def(37.5-62.5)	4.2±7.2a(0-12.5)	37.5±12.5cde(25-50)
3	0.0	4.2±7.2a(0-12.5)	0.0±0.0a(0-0)	8.3±14.4ab(0-25)	0.0±0.0a(0-0)
	0.2	29.2±7.2bcd(25-37.5)	79.2±14.4gh(62.5-87.5)	12.5±12.5ab(0-25)	58.3±7.2ef(50-62.5)
	0.4	50.0±0.0def(50-50)	66.7±19.1fgh(50-87.5)	37.5±12.5cde(25-50)	50.0±0.0def(50-50)
4	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
	0.2	41.7±19.1de(25-62.5)	50.0±0.0def(50-50)	66.7±14.4fgh(50-75)	62.5±21.7fg(37.5-75)
	0.4	25.0±0.0bc(25-25)	25.0±12.5bc(12.5-37.5)	50.0±12.5def(37.5-62.5)	37.5±12.5cde(25-50)

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

Table 4.2B Embryogenic outgrowth on root explants from QPM maize genotypes cultured on media supplemented with 2,4-D and 6-BA

Growth Regulators (mg/L)		Embryogenic calli development (%)			
		Murashige and Skoog basal medium		Chu N6 basal medium	
2,4-D	6-BA	Obatanpa GH	Etubi	Obatanpa GH	Etubi
	0.0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
0	0.2	8.3±14.4ab(0-25)	12.5±12.5ab(0-25)	12.5±12.5ab(0-25)	18.8±8.8abc(12.5-25)
	0.4	4.2±7.2a(0-12.5)	8.3±7.2ab(0-12.5)	25.0±12.5bc(12.5-37.5)	12.5±12.5ab(0-25)
	0.0	0.0±0.0a(0-0)	4.2±7.2a(0-12.5)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
1	0.2	33.3±31.5cd(0-62.5)	25.0±25.0bc(0-50)	16.7±28.9abc(0-50)	29.2±26.0bcd(0-50)
	0.4	16.7±7.2abc(12.5-25)	16.7±19.0abc(0-37.5)	16.7±28.9abc(0-50)	16.7±19.1abc(0-37.5)
	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	4.2±7.2a(0-12.5)	0.0±0.0a(0-0)
2	0.2	16.7±14.4abc(0-25)	25.0±33.1bc(0-62.5)	25.0±25.0bc(0-50)	33.3±31.5cd(0-62.5)
	0.4	0.0±0.0a(0-0)	12.5±12.5ab(0-25)	16.7±7.2abc(12.5-25)	20.8±14.4abc(12.5-37.5)
	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	8.3±14.4ab(0-25)	4.2±7.2a(0-12.5)
3	0.2	8.3±7.22ab(0-12.5)	12.5±12.5ab(0-25)	12.5±21.7ab(0-37.5)	16.7±14.4abc(0-25)
	0.4	8.3±14.4ab(0-25)	4.2±7.2a(0.12.5)	16.7±28.9abc(0-50)	20.8±36.1abc(0-62.5)
	0.0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
4	0.2	0.0±0.0a	0.0±0.0a	12.5±12.5ab(0-25)	12.5±21.7ab(0-37.5)
	0.4	0.0±0.0a	0.0±0.0a	12.5±12.5ab(0-25)	12.5±21.7ab(0-37.5)

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

4.2.2 Effect of 2,4-D and CPPU on embryogenic callus induction

Analysis of variance from the results showed that, embryogenic calli formation were dependent on explants and growth regulators used (Appendix 5). In the absence of 2,4-D and CPPU, embryogenic calli could not formed on primary calli from embryo of both varieties on N6 but on MS media devoid of growth regulators, embryogenic calli of low frequency (4.2%) was formed on 'Obatanpa GH' whiles 'Etubi' showed no response (Table 4.3A).

Culturing maize embryos in the presence of CPPU alone produced low induction response in the range of 8.3% to 33.3% (Table 4.3A). Using 2,4-D alone on both MS and N6 developed embryogenic calli but at lowest frequency for 'Obatanpa GH' (8.3%) and 'Etubi' (12.5%) (Table 4.3A). This indicates that, for embryogenic calli formation, combination of auxins and cytokinins is essential requirements (Huang and Wei, 2004). CPPU at 0.4µM and varying 2,4-D from 0mg/L to 2mg/L resulted in 8 to 9 times increase in response in 'Etubi' (8.3% to 70.8%) on N6 whereas on MS it quadrupled (16.6% to 66.7%) (Table 4.3). In 'Obatanpa GH', response increased about five fold in both MS and N6 (12.5% to 62.5%). CPPU at 0.4µM and increasing 2,4-D beyond 2mg/L led to a sharp drop of 50% in embryogenic response across all MS and N6 cultures for both 'Obatanpa GH' and 'Etubi' (70.8% to 25.0%).

Growth regulator, 2,4-D at 2mg/L and CPPU to 4µM showed a further drop in response in Etubi on N6 (70.8% to 25.0%) and Obatanpa GH' on N6 (62.5% to 12.5%). A combination of 2mg/L 2,4-D and 0.4µM CPPU was optimum to produce highest embryogenic calli from Obatanpa GH (62.5%) and Etubi (66.7%) on MS and on from Obatanpa GH (62.5%) and Etubi (70.8%) on N6 (Table 4.3). However, 1mg/L 2,4-D and 4µM CPPU was also optimum for embryogenic calli induction from Obatanpa GH (62.5%) on MS. There was no significant

difference in embryogenic callus formation at this combination across all cultures of MS and N6 (Table 4.3A).

Cultures of primary calli from root explants with no plant regulators could not form embryogenic calli. This also shows that growth regulators are important for embryogenic calli formation (Wang *et al.,* 2008). Addition of CPPU alone at 0.4µM produced a significant increase ($P \leq 0.05$) in response up to 25.0% to 31.2% only on N6 for 'Obatanpa GH' and Etubi respectively while cultures on MS failed to develop embryogenic calli (Table 4.3B).

This indicated that although, media was not significant in embryogenic calli, addition of amino acids enhanced embryogenic calli on N6 (Neondo *et al*., 2016). This further dropped when CPPU level was increased to 4µM (25% to 4.17% for 'Obatanpa GH' and 31.2% to 16.6% for 'Etubi'). Consistent and significant increase in embryogenic callus were attained at 3mg/L 2,4-D and 4µM CPPU from 'Obatanpa GH' on MS (37.5%) and N6 (29.1%) and Etubi on MS (33.3%) (Table 4.3B). However, highest embryogenic calli was attained at 2mg/L 2,4-D and 4µM CPPU for Etubi (56.3%) on N6.

High levels of 2,4-D at 4mg/L and CPPU at 4µM was detrimental to embryogenic callus induction in response as low as between 6.3% to 12.5% was obtained (Table 4.3B). Based on maximum response of 70.8% in embryo and 56.3% in roots, it is evident that mature embryos performed better than roots in callus induction and must be considered for use in future transformation studies (Table 4.3B). The low performance of root explants in inducing embryogenic calli could be attributed to maturity of meristem, which does not respond well to embryogenic calli upon culturing.

Although researchers have been successful in transformation of maize with immature embryos, the difficulty in handling immature embryos requires search for other explants, hence further work is required to investigate the transformation ability of mature embryos. Several researchers have adopted mature embryos of maize as explants for successful in vitro culture (Wang *et al.*, 2008; Huang and Wei, 2004) and it is possibly one of the best explants for regeneration. Analysis of variance of the results showed that media and genotype were independent of the embryogenic calli formation (Appendix 5).

Although the response in roots was not as high as in mature embryos, it demonstrated a good potential in developing embryogenic calli and so further researches are required to find out the effect of other auxins such as IBA, NAA, Dicamba and other cytokinins as kinetin, coconut water and zeatin on embryogenic calli formation. Additionally, effect of additives as silver nitrate, activated charcoal and some amino acids on induction of embryogenic callus in maize roots would be worth investigating.

The need for growth regulators arises from the apical-basal axis and the radial axis that are laid down during the rudimentary stages of embryogenesis. These processes are under the control of auxins and cytokinins and so it is not surprising to find its differential response for both type and concentration of auxins and cytokinins. This report indicated that, CPPU has proven to induce embryogenic calli in maize with 2,4-D. Fiore *et al*. (2002) reported a positive role of CPPU in stimulating embryogenic callus formation and further inducing a different display of responses in the development of somatic embryos.

Within the context of this research, varieties and media used were not significant with the addition of 2,4-D levels with either 6-BA levels or CPPU levels meaning callus induction was

not dependent on genotype and media and so further research is required to validate the apparent genotype and media independent induction of embryogenic callus. The successful induction of embryogenic calli and formation of somatic embryos indicate the possibility of propagating genetically modified maize.

The production of Type I and Type II embryogenic callus on MS or N6 with 2,4-D, 6-BA and CPPU levels was interesting in that the hard and compact Type I embryogenic callus may not be appropriate for production of cell suspension because it would be hard to disperse. On the other hand, the friable ones would be amenable for preparation of cell suspension culture because disintegration would be simple. Using cell suspension culture, it would be easier to embark on protopolast culture and transformation. The research also revealed the formation of globular and vitreous structures of somatic embryos that were visible under a stereo microscope.

Successful development of somatic embryos offers opportunities to produce clean propagating materials as well as harvesting and desiccation of embryos for future planting as synthetic seeds. Somatic embryos are easy to use for plant transformation. Between the two cytokinins used, 6-BA produced better frequency of embryogenic callus than CPPU whereas embryogenic callus formed easily on mature embryos than on root explants.

The differential need for growth regulators in different explants in induction of both primary and embryogenic callus in mature embryos and root confirms that growth regulators perform differently in different explants. The need for growth regulators arises from the apical-basal axis and the radial axis that are laid down during the rudimentary stages of embryogenesis.

These processes are under the control of auxins and cytokinins and so it is not surprising to find its differential response for both type and concentration of auxins and cytokinins.

Previous reports on induction of embryogenic callus in maize show low to moderate success rate Ali *et al*. (2014) obtained embryogenic callus frequency range of 2% to 8% for both mature and immature embryos of 'Sultan', 'Soneri', 'Golden' and 'Agaiti 85' maize genotypes. Machuka *et al*. (2008) also achieved embryogenic calli frequency of 17.6% in Type I and 52.9% in Type II from Kenyan maize inbreds and hybrids. Huang and Wei, (2004) obtained frequency of embryogenic callus formation of 0.2 to 23.4% in maize varieties C8605 and 9046. In contrast, high values of 45% to 72% in maize genotypes 87014
Z28-11 and POP48SRS5-57 (Usman *et al*., 2007) and 30% to 80% in genotypes IPBvar4 and Pi23 (Perez *et al*., 1993) have been achieved.

In current study, despite some low and inconsistent responses, the success rate of embryogenic callus induction frequency achieved in mature maize embryos was 62.5% to
91.7% and in roots 25.00% to 56.30%.

Table 4.3A Embryogenic outgrowth on mature embryo explants from QPM maize genotypes cultured on media supplemented with 2,4-D and CPPU

Growth Regulators (mg/L)		Embryogenic calli development (%)			
		Murashige and Skoog basal medium		Chu N6 basal medium	
2,4-D	6-BA	Obatanpa GH	Etubi	Obatanpa GH	Etubi
0	0.0	4.2±7.2ab(0-12.5)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
	0.2	12.5±12.5abc(0-25)	33.3±14.4ef(25-50)	12.5±12.5abc(0-25)	8.3±7.2ab(0-12.5)
	0.4	20.8±14.4be (12.5-37.5)	20.8±14.4abe(12.5-37.5)	25±21.6bef(0-37.5)	8.3±7.2ab(0-12.5)
1	0.0	0.0±0.0a(0-0)	4.2±7.2ab(0-12.5)	0.0±0.0a(0-0)	12.5±21.6abc(0-37.5)
	0.2	29.2±14.4cef(12.5-37.5)	16.6±19.0abe(0-37.5)	20.8±14.4be(12.5-37.5)	20.8±7.2be(12.5-25)
	0.4	62.5±12.5d(50-75)	25.0±0.0bef(25-25)	29.1±19.1cef(12.5-50)	37.5±0.0efg(37.5-37.5)
2	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
	0.2	62.5±12.5d(50-75)	66.7±7.2d(62.5-75)	62.5±21.7d(50-87.5)	70.8±14.4d(62.5-87.5)
	0.4	20.8±14be(12.5-37.5)	50.0df(50-50)	12.5±12.5abc(0-25)	25.0±0.0bef(25-25)
3	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	8.3±7.2ab(0-12.5)	4.2±7.2ab(0-12.5)
	0.2	29.1±19.0cef(12.5-50)	0.0±0.0a(0-0)	37.5±0.0efg(37.5-37.5)	25.0±12.5bef(12.5-37.5)
	0.4	31.2±26.5cef(12.5-50)	33.3±19.1ef(12.5-50)	50±21.6df(37.5-75)	45.8±14.4f(37.5-62.5)
4	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0 a(0-0)
	0.2	43.8±26.5f(25-62.5)	31.2±8.8cef(25-37.5)	37.5±0.0efg(37.5-37.5)	37.5±0.0efg(37.5-37.5)
	0.4	37.5±0.0efg(37.5-37.5)	25.0bef(25-25)	50.0±21.7df(37.5-75)	37.5±12.5efg(25-50)

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

Table 4.3B Embryogenic outgrowth on root explants from QPM maize genotypes cultured on media supplemented with 2,4-D and CPPU

Growth Regulators (mg/L)		Embryogenic calli development (%)			
		Murashige and Skoog (MS) basal medium		Chu (N6) basal medium	
2,4-D	6-BA	Obatanpa GH	Etubi	Obatanpa GH	Etubi
	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
0	0.4	0.0±0.0a(0-0)	0.0±0.0a(0-0)	31.2±8.8cef(25-37.5)	25.0±17.6 bef(12.5-37.5)
	4	0.0±0.0a(0-0)	0.0±0.0a(0-0)	4.17±7.2ab(0-12.5)	16.6±19.1ab(0-37.5)
	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
1	0.4	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
	4	0.0±0.0a(0-0)	12.5±12.5abc(0-25)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
	0.0	4.2±7.2ab(0-12.5)	0.0±0.0a(0-0)	8.3±7.2ab(0-12.5)	4.2±7.2ab(0-12.5)
2	0.4	8.3±14.4ab(0-25)	4.17±7.22ab(0-12.5)	12.5±17.6abc(0-25)	37.5±17.6ef(25-50)
	4	0.0±0.0a(0-0)	20.8+7.2bf(12.5-25)	16.6±19.1abe(0-37.5)	56.3±26.5dg(37.5-75)
	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	4.2±7.2ab(0-12.5)	4.2±7.2ab(0-12.5)
3	0.4	8.3±7.2ab(0-12.5)	29.1±31.4cef(0-62.5)	25.0bef(25-25)	8.3±14.4ab(0-25)
4	37.5±25.0efg(0-75)	33.3±31.4ef(0-62.5)	29.1±26.0cef(0-50)	37.5±25.0efg(12.5-62.5)	
	0.0	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)	0.0±0.0a(0-0)
4	0.4	25bef(25-25)	12.5abc(12.5-12.5)	0.0±0.0a(0-0)	12.5±0.0abc(12.5-12.5)
	4	12.5abc(12.5-12.5)	12.5abc(12.5-12.5)	6.3±8.84ab(0-12.5)	6.3±8.84ab(0-12.5)

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

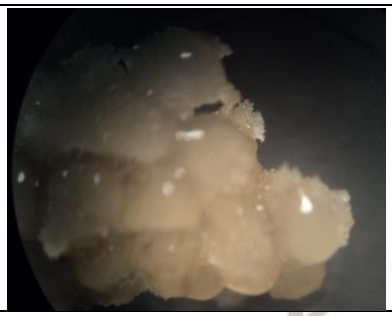
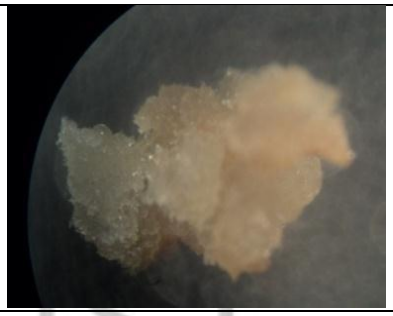
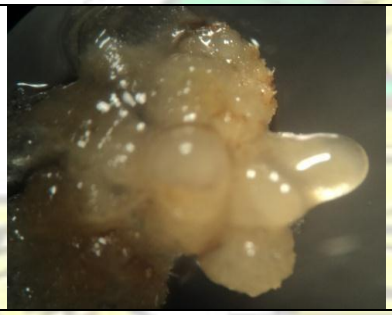
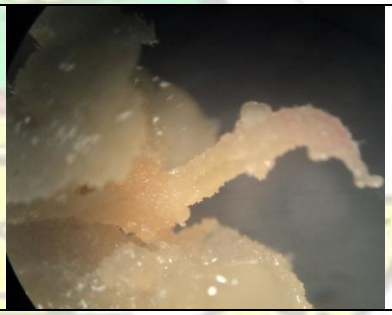
A 	B 
1mg/L 2,4-D+ 0.2mg/L 6-BA Embryos appear as globular structures	2mg/L 2,4-D+ 0.4µM CPPU (friable Type I embryogenic callus)
C 	D 
1mg/L 2,4-D+ 0.2mg/L 6-BA	2mg/L 2,4-D+ 0.4µM CPPU (compact Type II embryogenic callus)
E 	F 
2mg/L 2,4-D+ 0.4mg/L 6-BA	3mg/L 2,4-D+ 4µM CPPU
G 	H 
2mg/L 2,4-D+ 0.2mg/L 6-BA	2mg/L 2,4-D+ 4µM CPPU

Figure 4.2. Response of matured embryo and root explants cultured on

A. Mature embryos cultured on MS medium supplemented with 1mg/L 2,4-D and 2mg/L 6-BA
B. Mature embryos cultured on MS medium supplemented with 2mg/L 2,4-D and 0.4µM CPPU
C. Mature embryos cultured on N6 medium supplemented with 1mg/L 2,4-D and 0.2mg/L 6-BA
D. Mature embryos cultured on N6 medium supplemented with 2mg/L 2,4-D and 0.4µM CPPU
E. Root explants cultured on MS medium supplemented with 2mg/L 2,4-D and 0.4mg/L 6-BA
F. Root explants cultured on N6 medium supplemented with 3mg/L 2,4-D and 4µM CPPU

G. Root explants cultured on MS medium supplemented with 2mg/L 2,4-D and 0.2mg/L 6-BA H. Root explants cultured on MS medium supplemented with 2mg/L 2,4-D and 4µM CPPU

4.3 Effect of IAA and 6-BA on shoot regeneration from embryo and root explants The effects of two growth regulators, 6-BA and IAA was studied on shoot regeneration from embryo and root explants. Formation of shoots from embryogenic callus occurred within 14 days on MS and N6 regeneration media supplemented with IAA and varying concentrations of 6-BA (Table 4.4A). There was no response to shoot regeneration after culturing embryogenic calli from embryo explants of 'Obatanpa GH' on both MS and N6 media.

However, 'Etubi' embryo showed a low response (20%) in shoot regeneration on MS media whereas on N6, there was no shoot regeneration. When the media was devoid of IAA, low rates of shoot regeneration ranging from 6.7% to 20.0% on MS and 6.7% to 26.7% on N6 was observed (Table 4.4A). The results showed that, no shoot regeneration occurred in cultures devoid of 6-BA despite progressive addition of IAA up to 1.5mg/L making 6-BA an essential growth regulator for maize shoot regeneration. Increasing IAA to a concentration of 0.5mg/L resulted in a gradual increase in shoot regeneration with increasing concentration of 6-BA for both 'Obatanpa GH' and 'Etubi' on both MS and N6 (6.7% to 46.7%). When IAA was increased to 1.0mg/L, a similar response of increasing rate of shoot regeneration at increasing 6-BA concentration (10.0% to 73.3%) was observed.

When IAA was further increased to 1.5mg/L, a different response pattern was observed and it led to a decline in shoot regeneration (66.7% to 6.7%) on both MS and N6 for 'Obatanpa GH' and 'Etubi'. For shoot regeneration from embryo explants of 'Obatanpa GH', IAA of 1mg/L in combination with 6-BA at 3mg/L was optimum on MS (73.3%)and N6 (53.3%) and for

'Etubi', 1.5mg/L IAA combined with 1mg/L 6-BA also produced highest shoots on MS (53.3%) and N6 (66.7%) (Table 4.4A).

For root explant, shoots failed to form on all cultures of root-derived embryogenic callus in the absence of IAA and 6-BA (Table 4.4B). This means that IAA and 6-BA are basic requirements for shoot regeneration. Increasing IAA to 0.5mg/L resulted in very little response which peaked at a combination with 2mg/L 6-BA (46.7%) on both MS and N6 after which further additions of 6-BA to 3mg/L adversely affected shoot formation on N6 by dropping regeneration rate to a range of 6.7% to 13.3% (Table 4.4B).

Concentration of IAA above 0.5mg/L did not show any appreciable increase in shoot regeneration. The combination of IAA at 0.5mg/L and 6-BA at 2mg/L gave the highest shoot regeneration (46.7%) from Etubi on MS and N6 and Obatanpa on MS (46.7%) and N6 (40%) (Table 4.4B). There was no difference ($P \leq 0.05$) in shoot regeneration response between 'Obatanpa GH' and 'Etubi' (Table 4.4B). The results show that, the frequency of producing shoots from mature embryo was higher than from root explants and that, for shoot regeneration, mature embryo is the explant of choice rather than root.

The findings of the current research support the report of Smigocki and Owen (1989) that addition of low level of auxin to high level of cytokinin leads to shoot formation and that low level of IAA combined with high level of 6-BA promoted successful shoot regeneration. Gorji *et al.* (2011) and Rakshit *et al*. (2007) successfully regenerated shoots at highest frequency range of 63.7% to 81.7% and 63.6% to 81.8%, respectively from immature embryo on 0.5mg/L IAA and 1mg/L 6-BA, respectively.

The differences in levels of endogenous hormones in mature embryo and root may be responsible for the differential shoot regeneration response (Bahgat *et al*., 2008). Gorji *et al*. (2011) and Duncan *et al*. (1985) reported induction of shoots using 6-BA alone in both MS and N6 media.

Table 4.4A Plant regeneration from embryogenic callus of embryo explants cultured on MS and N6 supplemented with IAA and 6-BA

Growth Regulators (mg/L)		Shoot Regeneration (%)			
IAA	6-BA	MS media		N6 media	
		Obatanpa GH	Etubi	Obatanpa GH	Etubi
0	0.0±0.0a	20.0±0.0b	0.0±0.0a	0.0±0.0a	
1	20.0±0.0b	0.0±0.0a	6.7±11.5a	20.0±0.0b	
0	2	13.3±11.5ab	6.7±11.5a	20.0±0.0b	20.0±20.0b
	3	13.3±11.5ab	6.7±11.5a	20.0±0.0b	26.7±11.5bc
	0	0.0±0.0a	0.0±0.0a	6.7±11.5a	0.0±0.0a
	1	13.3±11.5ab	13.3±11.5ab	0.0±0.0a	0.0±0a
0.5	2	33.3±11.4c	26. 7±11.5bc	40.0±0.0cd	46.7±11.5cd
	3	46.7±11.4cd	40.0±0.0c	40.0±0.0cd	40.0±0.0cd
	0	6.7±11.5a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	1	10.0±14.1a	20.0±0.0b	20.0±0.0b	13.3±11.5ab
1	2	26.7±11.5bc	20.0±0.0b	26.7±11.5bc	20.0±0.0b
	3	73.3±11.5f	40.0±20.0cd	53.3±11.5de	40.0±20.0cd
	0	0.0±0.0a	0.0±0.0a	6.7±11.5a	0. 0±0.0a
	1	40.0±20.0cd	53.3±11.5de	20.0±0.0b	66.7±11.5ef
1.5	2	0.0±0.0a	0.0±0a	26.7±11.5bc	20.0±0.0b
	3	6.7±11.5a	6.7±11.5a	13.3±11.5ab	0.0±0.0a

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

Table 4.4B Plant regeneration from embryogenic callus of root explants cultured on MS and N6 supplemented with IAA and 6-BA

Growth Regulators (mg/L)		Shoot Regeneration (%)			
IAA	6-BA	MS media		N6 media	
		Obatanpa GH	Etubi	Obatanpa GH	Etubi
	0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	1	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
0	2	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	3	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
0		6.7±11.5a	6.7±11.5a	0.0±0.0a	0.0±0.0a
1		0.0±0.0a	6.7±11.5a	0.0±0.0a	6.7±11.5a
0.5	2	46.7±11.5cd	46.7±11.5cd	40.0±0.0cd	46.7±11.5cd
	3	26.7±11.5bc	26.7±11.5bc	13.3±11.5ab	6.7±11.5a
0		0.0±0.0a	6.7±11.5a	0.0±0.0a	0.0±0.0a
1		13.3±11.5ab	0.0±0.0a	0.0±0.0a	0.0±0.0a
1	2	6.7±11.5a	13.3±11.5ab	0.0±0.0a	26.7±11.5b
	3	26.7±11.5bc	20.0±0b	20.0±0.0b	6.7±11.5a
0		0.0±0.0a	6.7±11.5a	0.0±0.0a	6.7±11.5a
1		0.0±0.0a	6.7±11.5a	20.0±0.0b	6.7±11.5a
1.5	2	0.0±0.0a	6. 7±11.5a	0.0±0.0a	6.7±11.5a
	3	0.0±0.0a	0.0±0.0a	0.0±0.0a	13.3±11.5ab

Means in a column followed by different letters are significantly different at P≤0.05 using LSD.

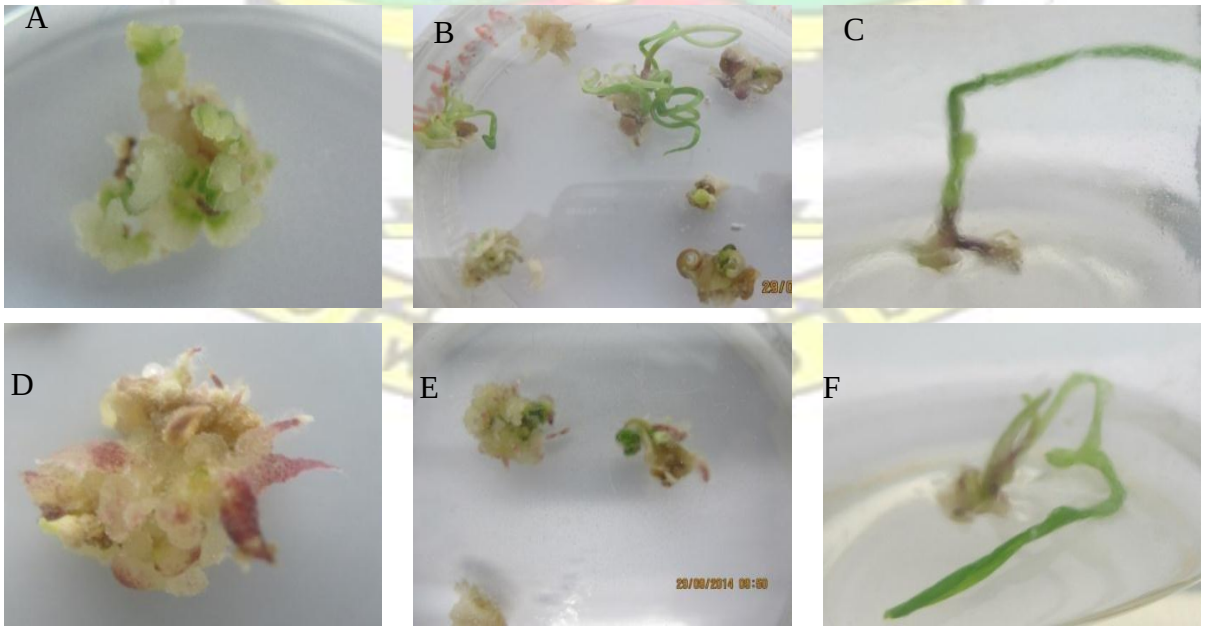


Figure 4.3 Regeneration of shoots from mature embryo and root embryogenic callus of 'Obatanpa GH' and 'Etubi' cultured on MS or N6 supplemented with IAA and 6-BA. (A) Initiation of shoot on MS medium supplemented with 1mg/L IAA and 3mg/L 6-BA (B) Elongation of shoots (C) Developed shoot from mature embryo explant; (D) Embryogenic callus of root explants on N6 medium supplemented with 0.5mg/L IAA and 2mg/L 6-BA (E) Initiation and enlongation of shoots(F) Developed shoots from root explant

4.4 Root regeneration from embryo and root explants

Shoots regenerated from mature embryo-derived and root-derived embryogenic calli were cultured on MS and N6 to test the influence of 2,4-D and NAA on root development. For embryo-derived embryogenic calli, roots were observed on all cultures devoid of 2,4-D and NAA (Table 4.5). When both 2,4-D and NAA were absent, the frequency of root regeneration ranged from 8.3% to 41.7% on both MS and N6 for 'Obatanpa GH' and 'Etubi'.

When NAA was at 0mg/L and 2,4-D concentration was at 0.4mg/L, there was root formation on all N6 but not MS (Table 4.5). The highest growth was attained at 0.2mg/L 2,4-D supplemented with 0mg/L NAA for 'Obatanpa GH' on both MS (58.3%) and N6 (50%) and at 0.4mg/L 2,4-D in combination with 2mg/L NAA for 'Etubi' on MS (50%) and N6 (58.3%) (Table 4.5).

Unlike root development from mature embryo-derived embryogenic calli, root-derived embryogenic calli did not form roots in the absence of both 2,4-D and NAA (Table 4.5) indicating that growth regulators were essential for root regeneration from root explants. Increasing NAA alone led to an increase in root regeneration and that MS and N6 supplemented with 2mg/L NAA developed maximum root frequency at a range between 25% to 50% although not significant from the response at 1mg/L NAA (12.5% to 25%) (Table 4.5).

This demonstrates that NAA is essential for root formation. The results showed that, for maximum root formation from root explants of 'Obantanpa GH', MS (50%) and N6 (50%) both supplemented with 2mg/L NAA alone was optimum. For Etubi root explants, MS and N6 with 0.2mg/L 2,4-D and 1mg/L NAA produced the highest frequency of roots.

Performance of mature embryo-derived and root-derived embryogenic calli to root regeneration were the same. To the best of my knowledge, there is no report on tissue culture that have employed 2,4-D and NAA combination to successfully regenerate roots. In an attempt to regenerate roots using 2,4-D and NAA, Islam *et al*. (2005) reported callus instead.

Several studies have used 2,4-D alone and NAA alone at various concentrations to develop roots (Gorji *et al*., 2011; Manivannan *et al*., 2010 and Rakshit *et al*., 2007) and found NAA to significantly promote root development. Satyavani e*t al*. (2013) developed maximum roots from cultures of seaside heliotrope (*Heliotropium crassavicum*) using only 2,4-D at a concentration of 2.5mg/L.

Spontaneous formation of roots devoid of plant growth regulators was unexpected, however growth regulators enhanced the rate of root formation and promoted diffuse root formation. The growth regulators, NAA alone and 2,4-D alone or in combination have proven to be best for root regeneration. Root development was not affected by explant and media type. Root regeneration was also genotype independent. The shoot and root regeneration frequency were unexpectedly lower than that reported in the literature. It may be necessary to test the effects of adjuvants such as silver nitrate or activated charcoal in shoots and roots regeneration in maize.

This is the first report on tissue culture of maize roots and it has shown promising results for primary callus induction, embryogenesis, shoot and root regeneration. Plantlets with

welldeveloped roots of up to 3-5cm are shown in Figure 4.4. The plantlets were rinsed with autoclaved distilled water to clean media and then planted on sterilized soil in the screen house to enhance growth.

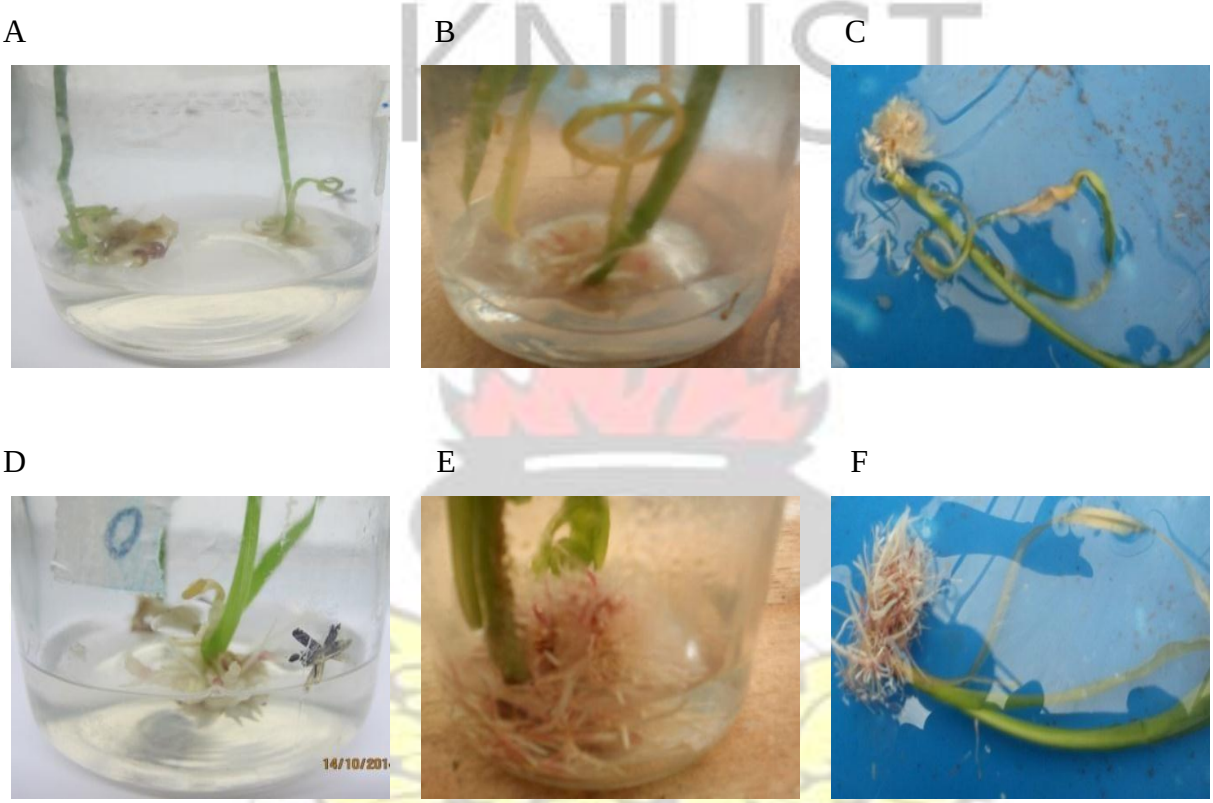


Figure 4.4 Root regeneration on mature embryo-derived and root-derived embryogenic calli. (A) Regenerated shoot from mature embryo cultured on MS supplemented with 0.4mg/L 2,4D and 2mg/L NAA. (B) Root regenerated on shoots from mature embryo; (C) Young seedling cleaned and ready for transfer into soil; (D) Regenerated shoot from root explants cultured on MS supplemented with 0.2mg/L 2,4-D and 1.0mg/L NAA; (E) Root formed on shoot regenerated from root explants; (F) Young seedling with profuse roots cleaned for transfer into soil.

Table 4.5 Root regeneration from regenerated shoots on MS and N6 media

Explant	Growth Regulators(mg/L)		Root regeneration			
	2,4-D	NAA	MS media		N6 media	
			Obatanpa GH	Etubi	Obatanpa GH	Etubi
		0	25.0±0.0abc	41.7±14.4c	25.0±25.0abc	16.7±28.9ab
0	1	8.3±14.4a	16.7±14.4ab	16.7±14.4ab	16.7±14.4ab	
		2	41.7±28.9c	16.7±14.4ab	41.7±14.4c	8.3±14.4a
		0	58.3±14.4cd	33.3±14.4bc	50.0±25.0cd	50.0±0.0cd
	0.2	1	33.3±28.9bc	25.0±25abc	33.3±28.9bc	25.0±25abc
		2	0.0±0.0a	8.3±14.4a	0.0±0.0a	16.6±14.4ab
		0	0.0±0.0a	0.0±0.0a	25.0±0.0abc	8.3±14.4a
	0.4	1	12.5±17.7ab	0.0±0.0a	0.0±0.0a	0.0±0.0a
		2	50.0±25.0cd	50.0±25.0cd	33.3±38.2bc	58.3±28.9cd
		0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
0	1	25.0±0.0abc	12.5±17.7ab	25.0±0.0abc	25.0±0.0abc	
		2	50.0±35.4cd	37.5±17.7c	50.0±35.4cd	25.0±35.4abc
		0	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
	0.2	1	37.5±17.7c	62.5±17.7d	25.0±0.0abc	50.0±0.0cd
		2	12.5±17.7ab	25.0±0.0abc	12.5±17.7ab	0.0±0.0a
0	0.0±0.0a		0.0±0.0a	0.0±0.0a	0.0±0.0a	
	0.4	1	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
		2	0.0±0.0a	0.0±0.0a	12.5±17.7ab	12.5±17.7ab

Means within a parameter which have common superscripts are not different at P=0.001; Oba-'Obatanpa GH'.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The current low yield of maize (about 1.7tons/ha) (International Grain Council, 2013) requires that modern biotechnology methods such as genetic transformation should be used to improve the yield. However, genetic transformation depends on development of totipotent tissue. In this study, totipotent callus tissue were developed.

(i) Mature embryos cultured on MS or N6 media supplemented with 4mg/L 2,4-D and cultured in a growth chamber at a temperature of 27 ± 1 ^{o}C for 21 days in the dark resulted in highest primary callus induction.

(ii) Mature embryos were better in producing primary callus than root explants.

(iii) Maize roots required 3mg/L 2,4-D primary for callus induction.

(iv) For embryogenic callus induction, 0.2mg/L 6-BA and 1mg/L or 2mg/L 2,4-D was required for mature embryos and root

(v) 2mg/L 2,4-D and 0.4µM CPPU resulted in the highest formation of embryogenic calli from mature embryo-derived primary callus; 3mg/L 2,4-D and 4µM CPPU was required for embryogenic callus induction in roots, or combination produced consistent and highest frequency of embryogenic callus.

(vi) Frequency of embryogenic callus was higher in 6-BA than CPPU making 6-BA a better choice.

(vii) For highest shoot regeneration from embryo-derived embryogenic callus, 1mg/L IAA and 3.0mg/L 6-BA or 1.5mg/L IAA and 1mg/L 6-BA was required.

(viii) However, root-derived embryogenic callus required 0.5mg/L IAA and 2mg/L 6-BA for optimal shoot regeneration.

(ix) For root regeneration from mature embryo-derived embryogenic calli, 0.4mg/L 2,4-D and 2mg/L NAA produced maximum root frequency.

(x) Root-derived embryogenic calli developed highest root frequency with 0.2mg/L 2,4-D and 1mg/L NAA.

5.2 Recommendations

The following recommendations are provided:

- The effect of proline and asparagine, as well as silver nitrate on regeneration should be investigated.
- Other maize explants, such as leaf, anthers, shoot tips and ovules should be investigated.
- Similarly, the effect of Dicamba on primary and embryogenic callus induction should also be investigated.

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APPENDICES

Appendix 1 Composition of MS and N6 basal media

Constituent	MS medium (mg/L)	Chu's N6 medium (mg/L)
KNO_3	1900	2830
NH_4NO_3	1650	-
KH_2PO_4	170	400
$(NH_4)_2SO_4$	-	463
$MgSO_4.7H_2O$	370	185
$CaCl_2.2H_2O$	440	166
KI	0.83	0.80
H_3BO_3	6.2	1.6
$MnSO_4.H_2O$	-	4.4
$MnSO_4.4H_2O$	22.3	-
$ZnSO_4.7H_2O$	8.6	1.5
$Na_2MoO_4.2H_2O$	0.25	-
$CuSO_4.5H_2O$	0.025	-
$CoCl_2.6H_2O$	0.025	-
$FeSO_4.7H_2O$	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3

Source: Murashige and Skoog (1962) and Chu et al. (1975).

Appendix 2

2.1 Preparation of 10 mL 6-BA stock solution

A 0.125 M concentration of 6-BA was prepared by dissolving 0.282 g of 6-BA in 10 mL autoclaved distilled water. Stirring was done until a uniform mixture was achieved.

2.2 Preparation of 10 mL CPPU stock solution

A 0.0398 M concentration of CPPU was prepared by dissolving 0.1 g of CPPU in 10 mL autoclaved distilled water. Stirring was done until a uniform mixture was attained.

2.3 Preparation of 1000 mL 70 % ethanol

A 70 % ethanol was prepared by topping up a 700mL absolute ethanol in a 1000 mL measuring cylinder with 300 mL autoclaved distilled water.

2.4 Preparation of 1000 mL 20 % sodium hypochlorite (7 % available chlorine)

A 200 mL sodium hypochlorite (7 % available chlorine) was measured in a 1000 mL measuring cylinder and topped up with autoclaved distilled water of 800 mL to the brim.

2.5 Preparation of 1000 mL 10 % sodium hypochlorite (7 % available chlorine) A 10 % sodium hypochlorite was prepared by measuring 100 mL sodium hypochlorite (7 % available chlorine) and adding autoclaved distilled water to a 1000 mL

2.6 Preparation of 1 L fungicide solution

A fungicide solution was prepared by dissolving antibiotics ampicillin (0.365 %) and chloramphenicol (0.017 %) in a small volume of ethanol. After decantation, the supernatant

was added to a solution of 7 g Shavit in 1 L autoclaved distilled water. Ethanol was evaporated off by gentle heat treatment for 10 minutes

2.7 Explant sterilization protocol

1. Explant was submerged in fungicide solution and agitated gently for 24 hours after which were rinsed with autoclaved distilled water for 3 times
2. Explants were subjected to further sterilization with 70 % ethanol and agitated gently for 5 minutes following rinsing with autoclaved distilled water for 3 times.
3. Explant was treated with 20 % sodium hypochlorite and 2 drops of tween 20 added and agitated gently for 30 minutes following rinsing with autoclaved distilled water for 3 times.
4. Explant was further treated with 10 % sodium hypochlorite and shaken gently for 15 minutes after which seeds are rinsed with autoclaved distilled water for 3 times.
5. These activities were done aseptic under a laminar flow hood.

Appendix 3 Analysis of variance of variety, media, explants, 2,4-D and their interaction effects on primary callus formation

Source of variation	df[1]	Mean square	Source of variation	df	Mean square
Variety	1	260.03	Media*2,4-D	4	309.21
Media	1	510.76	Explant*2,4-D	4	720.92
Explant	1	9,916.68***	Variety*Media*Explant	1	275.90
2,4-D	4	1,511.66**	Variety*Media*2,4-D	4	108.73
Variety* Media	1	7.74	Variety*Explant*2,4-D	4	467.29
Variety*Explant	1	46.80	Media*Explant*2,4-D	4	128.47
Variety*2,4-D	4	481.23	Variety*Media*Explant*2,4D	3	261.34
Media*Explant	1	113.09			

[1]Degree of freedom; ***P<0.001; **P<0.01

Appendix 4 Analysis of variance of embryogenic callus response with 2,4-D and 6-BA

Source of variation	[1]df	Mean square	Source of variation	df	Mean square
Variety	1	1180.56	Variety*BA	2	314.03
Media	1	377.23	Media*BA	2	246.38
Explant	1	25,259.08***	Variety*Media*BA	2	180.10
2,4-D	4	2,316.20***	Explant*BA	2	3466.52***
Variety* Media	1	311.17	Variety*Explant*BA	2	179.44
Variety*Explants	1	423.67	Media*Explants*BA	2	1579.91**
Variety*2,4-D	4	320.02	Variety*Media*Explant*BA		78.99
Media*Explant	1	1,301.21	2,4-D*BA	8	1519.03***
Media*2,4-D	4	791.93**	Variety*2,4-D* 6-BA	8	418.95
Explant*2,4-D	4	1,326.99***	Media*2,4-D*6-BA	8	467.78
Variety*Media*Explant	1	117.96	Variety*Media*2,4-D*6-BA	8	193.93
Variety*Media*2,4-D	4	331.12	Explant*2,4-D*6-BA	7	844.90***
Variety*Explant*2,4-D	4	250.98	Variety*Explant*2,4-D*6-BA	7	342.36
Media*Explant*2,4-D	3	159.22	Media*Explant*2,4-D*6-BA	6	191.63
Variety*Media*Explant* 2,4-D	3	855.93**	Variety*Media*Explants*2,4D*6-BA	5	414.12
6-BA	2	24,779.54***			

[1]Degree of freedom; ***P<0.001; **P<0.01

Appendix 5

Analysis of variance of embryogenic callus response in maize mature embryo and roots with 2,4-D and CPPU

Source of variation	df	Mean square	Source of variation	df	Mean square
Variety	1	146.77	Variety*CPPU	2	41.27
Media	1	155.16	Media*CPPU	2	27.85
Explant	1	9,762.07***	Variety*Media*CPPU	2	80.19
2,4-D	4	1,922.48***	Explant*CPPU	2	2,641.66***
Variety* Media	1	14.85	Variety*Explant*CPPU	2	415.42
Variety*Explants	1	93.77	Media*Explants*CPPU	2	367.12
Variety*2,4-D	4	415.14	Variety*Media*Explant*CPPU	2	192.43
Media*Explant	1	278.12	2,4-D*CPPU	8	976.41***
Media*2,4-D	4	177.10	Variety*2,4-D*CPPU	8	203.43
Explant*2,4-D	4	483.50	Media*2,4-D*CPPU	8	196.05
Variety*Media*Explant	1	166.87	Variety*Media*2,4-D*CPPU	8	217.19
Variety*Media*2,4-D	4	260.59	Explant*2,4-D*CPPU	7	1,006.93***
Variety*explant*2,4-D	4	57.86	Variety*Explant*2,4-D*CPPU	7	146.18
Media*explant*2,4-D	4	666.68	Media*Explant*2,4-D*CPPU	6	300.72
Variety*Media*Explant*2,4-D	4	377.61	Variety*Media*Explants*2,4-D*CPPU	5	30.30
CPPU	2	13,854.22***			

***P<0.001; **P<0.01

Appendix 6

Analysis of variance for shoot regeneration

Source of Variation	df	Mean Square	Source of Variation	df	Mean Square
Variety	1	12.8704	Variety*BA	3	304.013
Media	1	1.23498	Media*BA	3	342.985
Variety*Media	1	160.297	Variety*Media*BA	3	41.445
Explant	1	10,204.7***	Explant*BA	3	1,222.75***
Variety*Explant	1	372.818	Variety*Explant*BA	3	380.154
Media*Explant	1	277.218	Media*Explant*BA	3	233.934
Variety*Media*Explant	1	58.0741	Variety*Media*Explant*BA	3	91.1716
IAA	3	2,871.85***	IAA*BA	9	3,355.35***
Variety*IAA	3	300.31	Variety*IAA*BA	9	150.237
Media*IAA	3	351.904	Media*IAA*BA	9	118.26
Variety*Media*IAA	3	50.8358	Variety*Media*IAA*BA	9	56.3397
Explant*IAA	3	531.767**	Explant*IAA*BA	9	772.615***
Variety*Explants*IAA	3	132.08	Variety*Explant*IAA*BA	9	207.807
Media*Explant*IAA	3	16.9902	Media*Explant*IAA*BA	9	211.521
Variety*Media*Explant*IAA	3	116.783	Variety*Media*Explant*IAA*BA	6	306.336
BA	3	4,384.67***			

***P<0.001; **P<0.01

Appendix 7

Analysis of variance for root regeneration.

Source of Variation	df	Mean Square	Source of Variation	df	Mean Square
Variety	1	117.154	Variety*NAA	2	109.235
Media	1	1.04315	Media*NAA	2	88.3085
Variety*Media	1	5.67936	Variety*Media*NAA	2	42.8856
Explant	1	2148.91	Explant*NAA	2	2,417.17**
Variety*Explant	1	81.8147	Variety*Explants*NAA	2	184.072
Media*Explant	1	27.6753	Media*Explant*NAA	2	67.381
Variety*Media*Explant	1	27.6753	Variety*Media*Explant*NAA	2	150.179
2,4-D	2	2,175.39**	2,4-D*NAA	4	3,971.98***
Variety*2,4-D	2	433.874	Variety*2,4-D*NAA	4	597.473
Media*2,4-D	2	309.883	Media*2,4-D*NAA	4	393.303
Variety*Media*2,4-D	2	99.9021	Variety*Media*2,4-D*NAA	4	235.329
Explant*2,4-D	2	625.475	Explant*2,4-D*NAA	4	2,692.95***
Variety*Explant*2,4-D	2	265.896	Variety*Explant*2,4-D*NAA	2	357.804
Media*Explant*2,4-D	2	387.22	Media*Explant*2,4-D*NAA	2	39.2628
Variety*Media*Explant*2,4-D	2	133.013	Variety*Media*Explant*2,4-D*NAA	2	15.4247
NAA	2	1,811.05			

***P<0.001;**P<0.01

Appendix 8