TISSUE CULTURE OF TWO NERICA RICE CULTIVARS

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DECLARATION

This is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to Mwiti Tapkey.

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LIST OF ABBREVIATIONS

BAP	6-benzylaminopurine
B5	Gamborg 1968 medium
IAA	Indole acetic acid
KARI	Kenya Agricultural Research Institute
KOH	Potassium Hydroxide
MS	Murashige and Skoog (1962) medium
NAA	1-Naphthaleneacetic acid
NaCl	Sodium chloride
NERICA	New Rice for Africa
N6	Chu 1978 medium
PEG	Polyethylene glycol
WARDA	West Africa Rice Development Association
2,4-D	2,4-Dichlorophenoxyacetic acid

ABSTRACT

In Kenya, rice is a highly important food crop and is considered to be the country's third staple food after maize and wheat. However, there has been a decline in rice production in recent years owing to the changes in climatic conditions, widespread soil degradation and susceptibility of the rice to fungal diseases such as *Magnaporthe grisea* commonly known as rice blast. To tackle this problem, the country is introducing NERICA (New Rice for Africa) developed by conventional breeding and tissue culture techniques that involved crossing the high yielding Asian rice (*Oryza sativa* sub species *japonica*) with the locally adapted African rice (*Oryza glaberrima*). NERICA requires less water to grow, matures early and produces higher annual yield per unit of land. The objective of this study was to develop an alternative propagation method using tissue culture that might be used for genetic improvement of the rice.

The study involved use of dehusked whole rice seeds sterilised using 1.5% sodium hypochlorite. Root and leaf explants were obtained from *in vitro* germinated plants. The explants were separately inoculated on callus induction medium consisting of N6 macronutrients, B5 micronutrients, N6 vitamins supplemented with 500 mg/l L-glutamine and 0.5 mg/l to 4 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D). Other hormonal combinations used on the N6 basal media for callus induction were 1 mg/l naphthalene acetic acid (NAA) or 1 mg/l 6-benzylaminopurine (BAP) combined with 2 mg/l 2,4-D. All the callus induction experiments were done in both darkness and in light (16 hours light and 8 hours dark) at 26±2° C. The friable calli obtained were subcultured onto to fresh media of the same composition for four weeks before being transferred to Murashige and Skoog (MS) medium with 0.5 mg/l to 3 mg/l BAP combined with 0.5 mg/l to 1 mg/l NAA or 0.2 mg/l IAA for regeneration. MS with 2.5 mg/l kinetin and 0.5 mg/l NAA was tested too. MS basal was used as a control experiment. The regenerated plantlets were planted in sterile soil in pots and covered with a transparent polythene bag to retain humidity.

Somatic embryogenic calli were produced from whole rice seeds at the optimal concentration of 2 mg/l 2,4-D in darkness between 8-14 days. Calli were obtained from root explants from N6 media supplemented with 2 mg/l 2,4-D and 1 mg/l NAA. No callus was produced from the leaf explants. Callus obtained from root explants gave root regeneration only in all the experiments which were on MS media containing 2.5 mg/l kinetin and 0.5 mg/l NAA, 0.5 mg/l BAP and 0.2 mg/l IAA, 2 mg/l BAP and 1 mg/l NAA and also 3 mg/l BAP and 0.5 mg/l NAA.

Embryogenic callus from rice seeds cultured on medium with 2 mg/l 2,4-D gave roots only when transferred to MS medium containing 2.5 mg/l kinetin and 0.5 mg/l NAA. That on medium with 0.5 mg/l BAP and 0.2 mg/l IAA gave shoot buds which did not elongate. Callus on medium with 2 mg/l BAP and 1 mg/l NAA gave plantlets which did not elongate and became necrotic in 2 weeks. Callus subcultured onto to medium supplemented with 3 mg/l BAP and 0.5 mg/l NAA formed roots while that transferred to MS basal medium produced plantlets in six to nine weeks with frequencies of 67% and 77% for NERICA 4 and NERICA 11 respectively.

The plantlets transferred to sterile soil survived for 12 days, therefore mature plants were not obtained. This study has demonstrated that NERICA 4 and 11 varieties can be regenerated *in vitro* from mature embryo explant callus and opens the possibility of future improvement of the crop through genetic engineering.

CHAPTER ONE

1.0 INTRODUCTION

Rice is one of the most important food crops in the world and feeds over half of the global population. It consists of the two cultivated species, namely the Asian rice (*Oryza sativa*) and the African rice (*Oryza glaberrima*). *O. glaberrima* is traditionally found in diverse West African agro ecosystems but it is largely abandoned in favour of high yielding *O. sativa* cultivars due to its poor agronomic performance (Linares, 2002). However, *O. sativa* cultivars are often not sufficiently adapted to various abiotic and biotic conditions in Africa.

In 1992, the Africa Rice Centre (formerly known as West Africa Rice Development Association-WARDA) started work on interspecific hybridization between *O. glaberrima* and *O. sativa* in an attempt to combine the useful traits of both species (Jones *et al.*, 1997). Because the two species do not naturally interbreed, a plant tissue culture technique called embryo-rescue was used to assure that crosses between the two species survive and grow to maturity. The new rice displayed heterosis, the phenomenon in which the progeny of two genetically different parents grow faster, yield more, or resist stresses better than either parent. Eighteen interspecific cultivars named new rice for Africa (NERICA), which are suitable for upland conditions, have been selected and disseminated to several African countries (AfricaRice, 2010).

NERICA cultivars have wide potential for cultivation in Africa and the introduction of additional traits by various breeding techniques will help increase yield and expand cultivation in Africa. Totipotency of somatic plant cells is the basis for micro propagation which is being extensively used for obtaining a large number of genetically identical plantlets. One of these breeding techniques is tissue culture that can allow for *in vitro* screening for improved genotypes. The tissue culture technique has been around for more than 30 years. It is seen as an important technology for the production of disease-free, high quality planting material and the rapid production of many uniform plants. Tissue culture technique produces plants that have particularly good flowers, fruits, or have other desirable traits and it quickly produces mature plants (Bhojwani and Razdan, 1996). It can also be used for multiple productions of vegetative species and in the regeneration of whole plants from plant cells that have been genetically modified. Tissue culture of NERICA rice has the

potential as a novel and powerful way to introduce desirable traits into the rice and to supplement traditional plant breeding techniques (Sinclair *et al.*, 2004).

In this study, two varieties of NERICA were used to try and develop a propagation system using tissue culture that can be utilized for further improvement of the crop. A tissue culture system for NERICA will also be useful for introduction of additional traits through genetic transformation.

1.1 Statement of the Problem and Justification

Many African countries are facing diminishing yields of all types of foods due to climate change, nutrient-scarce soils and pests and diseases while population is increasing, and Kenya is no exception. Rice production has declined in recent years and with rice being a major food crop in the country; it has contributed in the rise in the cost of living. One of the approaches Kenya is using to tackle this problem is embracing agricultural biotechnology including tissue culture. Tissue culture technology has thus far made it possible for farmers to have access to large quantities of superior clean planting material that are early maturing, access bigger bunch weights from conventional material and also access higher annual yield per unit of land previously realised with conventional material (Bhojwani and Razdan, 1996).

Studies done on rice have shown genetic improvement by providing and developing desirable characteristics such as improved high yield and controlling physical characteristics of the rice. For example, the National Crops Resources Research Institute at Namulonge in Uganda was able to improve three upland rice varieties to create drought-resistant cultivars i.e. NERICA 4 ‘Gold’, Nerica 10 and Suparica 1 ‘white’ using tissue culture (Kasozi, 2010). The new varieties have different physical characteristics compared to the parent crops. They have long aromatic grains and they perform very well in poor soils.

The tissue culture system will be useful in providing disease-free plants with guaranteed consistency and in checking and screening genetic stability of the different rice varieties produced.

In the process of performing the tissue culture, other novelty characteristics might also be discovered (Sano *et al.,* 1984). Hence development of tissue culture technique for NERICA will be of a great advantage to the farmers and economy of Kenya.

1.2 Research Hypothesis

H_A: The regeneration of NERICA rice through tissue culture technique can be achieved.

1.3 Objectives

1.3.1 General Objective

To develop a tissue culture system for two NERICA rice cultivars

1.3.2 Specific Objectives

1. To obtain callus from mature seeds and other explants of NERICA
2. To regenerate the callus into plantlets using auxins and cytokinins
3. To establish the regenerated plants in soil in the greenhouse

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of rice domestication

There are only two species of cultivated rice in the world: African rice, *Oryza glaberrima* and Asian rice, *Oryza sativa* (Linares, 2002). Native to sub-Saharan Africa, *O. glaberrima* is thought to have been domesticated from the wild ancestor *Oryza barthii* (formerly known as *Oryza brevilugata*) by people living in the floodplains of the Niger River some 3,500 years ago. On the other hand, the two sub species of *Oryza sativa* (*O. japonica* and *O indica*) are thought to have been domesticated in Asia (Huang *et al.*, 2012).

Oryza glaberrima has been found to have several useful traits. It has moderate to high level of resistance to blast (Silue and Notteghem, 1991), rice yellow mottle virus (Attere and Fatokun, 1983), rice gall midge, insects (Alam, 1988) and nematodes (Reversat and Destombes, 1995). It has also undergone improvement for over 3500 years for hardiness and drought resistance hence has good level of tolerance to abiotic stresses such as acidity, iron toxicity, drought, and weed competition (Sano *et al*., 1984). It also has profuse vegetative growth, which serves to smother weeds (Jones *et al*., 1994).
However, African rice has relatively low yields because it lodges or falls over when grain heads are full. Grains may also shatter, further reducing yield (Dingkuhn *et al.,* 1998).

Oryza sativa also has some useful traits. It has high yields, doesn't shatter and lodge. On the other hand *O. sativa* is "exotic" to Africa and therefore there are many areas where it cannot thrive because of the stresses of disease, pests, drought or soil problems (Jones, 2008). Asian rice also cannot compete with weeds due to their semi-dwarf phenotypes (Dingkuhn *et al.*, 1998).The yield advantage of *O. sativa* has, however, positively influenced its wide spread adoption thereby replacing *O. glaberrima* in most parts of Africa. The survival to date of *O. glaberrima* has majorly been due to the fact that it is more tolerant and/or resistant to most stresses found on the African continent.

Though farmers and scientists alike have for a long time acknowledged the fact that both species have distinct disadvantages and advantages over each other, the taxonomic divide have always posed a formidable challenge in cross breeding the two. For a long time, farmers

have had to choose whether it is *O. sativa* or *O. glaberrima* they will grow. Whichever choice made, farmers had to forego the benefits held by the other species as well as endure the disadvantages that come with the chosen species. The net effect has been lower rice productivity and higher rates of poverty and food insecurity, especially among the sub-Saharan Africa smallholder farmers who lack adequate capacity to counter any disadvantages inherent in the species they choose (IRRI, 2011).

2.2 Development of NERICA rice

New Rice for Africa ("NERICA") is an interspecific cultivar of rice developed by the Africa Rice Centre to improve the yield of African rice varieties. The NERICA Project was funded by the African Development Bank, the Japanese government and the United Nations Development Programme (Moseley *et al*., 2010).

For years, scientists dreamed of combining the ruggedness of the African rice species (*Oryza glaberrima*) with the productivity of the Asian species (*Oryza sativa*) (AfricaRice, 2010). But the two species are so different. Attempts to cross them failed since the resulting offspring were all sterile. In the 1990s, rice breeders from the Africa Rice centre (then known as the West Africa Rice Development Association-WARDA) turned to biotechnology in an attempt to overcome the infertility problem (AfricaRice, 2010). Key to the effort were gene banks that hold seeds of 1,500 African rice, which faced extinction since farmers had already abandoned them for higher yielding Asian varieties (De Vries and Toenniessen, 2001). Biotechnological tools such as genetic tagging, molecular marking and DNA profiling helped sort through thousands of genes and identify plants with desired traits that could be used to develop the NERICA rice. A total of 1721 rice accessions (1130 *O. glaberrima* and 591 *O. sativa* accessions) were evaluated and selected on the basis of morphological characters and agronomic traits for breeding (Jones *et al*., 1997).

Oryza glaberrima (CG 14) and three of the *O. sativa* (WAB 56-104, WAB 56-50 and WAB 181-18) accessions were then used to develop interspecific hybrids. After cross-fertilization of the two species, embryos were removed and grown on artificial tissue culture media using a process known as "embryo-rescue." Because the resultant plants are frequently almost sterile, they were backcrossed with the *O. sativa* parent whenever possible. Once the fertility of the progeny was improved (often after several cycles of back-crossing), anther culture was

used to double the gene complement of the male sex cells (anthers) and thus, produce true-breeding plants (AfricaRice, 2010).

A total of 18 varieties were named by WARDA's Variety Nomination Committee with the prefix NERICA followed by a number corresponding to their pedigree. Seven NERICA varieties (NERICA 1 to 7) were named in 2000, and a further 11 (NERICA 8 to 18) were named in March 2005 (Ndjiondjop *et al.,* 2005). The first generation of NERICA varieties 1 to 11, including the WAB450 progeny, was developed from crosses of the existing released variety CG 14 (*O. glaberrima*) and WAB56-104, which belongs to the subspecies *japonica* of *O. sativa* upland rice. Development of interspecific lines between *O. sativa* as female parent and *O. glaberrima* as male parent improved variety. On the other hand, NERICAs 12 to 18 are progeny of two series of crosses, using the same *O. glaberrima* (CG 14) parent but two different *O. sativa* parents (WAB56-50 and WAB181-18) (Ndjiondjop *et al.,* 2005). All the 18 NERICA varieties are suitable for the upland rice ecology of sub-Saharan Africa.

The new rice varieties, which are suited to drylands, were distributed and sown on more than 200,000 hectares during the last five years in several African countries, according to the Africa Rice Centre (Moseley *et al.,* 2010). The first of the NERICA varieties were available for field testing in the mid-90s. NERICA is now grown or under extension in Benin, Burkina Faso, Côte d'Ivoire, RD Congo, Congo-Brazzaville, Ethiopia, Guinea, Gambia, Gabon, Kenya, Madagascar, Malawi, Mali, Mozambique, Niger, Nigeria, Sierra Leone, Tanzania, Togo, and Uganda (AfricaRice, 2010). In Kenya, the varieties commonly grown are NERICA1, NERICA 4, NERICA10 and NERICA 11 (AfricaRice, 2010).

2.3 Characteristics and benefits of NERICA rice

Generally, some of NERICA's characteristics are that it has wide and droopy leaves, panicles or grain heads that are longer with 'forked' branches, and hold up to 400 grains, more tillers with strong stems to support and hold tightly the heavy grain heads and it yields as high as 2.5 tons per hectare at low inputs — and 5 tons or more with just a minimum increase in fertiliser usage (amounts to approximately 25% to 250% increase in production). NERICA also has 2% more body-building protein than their African or Asian parents (FAO, 2002).

NERICA has profuse early vegetative growth giving rapid ground cover, followed by upright growth at reproductive stage. The profuse tillering is characteristic of *O. glaberrima*. The rapid ground cover enables the rice crop to smother, and therefore out-compete weeds. Upright growth, especially at reproductive stage is a characteristic of *O. sativa*; it enables the plant to support heavy seed heads through maturity up to harvest (Ndjiondjop *et al.,* 2005). In addition to the above mentioned beneficial traits, NERICA now has a number of advantageous traits from both the *O. sativa* and *O. glaberrima.*

Some of the important advantages exhibited by NERICA and which were acquired from *O. glaberrima* are that it is drought tolerant. It is also resistant to the African rice gall midge (the region's most devastating insect pest), the rice yellow mottle virus (a major disease in lowland rice) and the blast disease. NERICA grows well on infertile and acidic soils—which comprise 70% of West Africa's upland rice area, has early maturity (NERICA's typically mature in 90–100 days compared to typical improved upland *O. sativa* that mature in 120–140 days), allowing farmers to grow extra crops of vegetables or legumes and it has a taste, aroma and other grain qualities favoured by farmers.

In addition, NERICA has also some advantages inherited from *O. sativa*. It has non-shattering grains, it has secondary branches on panicles leading to more grains thus the high yield and it also has increased positive responsiveness to fertilization. Because of their success, NERICAs were quickly adopted by farmers in east, west, central and southern African countries (DANIDA, 2002).

2.4 Tissue culture technology in rice

A tissue culture system may provide genetic variations and a means of transformation of rice. Tissue culture technology has been applied in the recent past in the improvement of rice species in different ways. Several studies have been done on tissue culture of rice.

IR80482-64-3-3-3 rice variety, locally known to Mozambique farmers as Makassane, was developed by scientists at the International Rice Research Institute by back-crossing *O. sativa (L.)* and *O. sativa japonica* (IRRI, 2011). The rice then underwent tissue culture severally to develop this new hardy variety that has the advantage of being resistant to bacterial leaf blight and blast, it is tall enough to survive minor flooding while not too long to be prone to falling over, it needs no fertiliser and the yields were raised dramatically. Makassane was also

chosen by farmers and scientists as the best tasting locally selected long-grain variety (IRRI, 2011). Makassane was specifically developed to be grown in Mozambique.

Niroula *et al.,* (2005) investigated the effects of genotype and culture media compositions on callus induction and plant regeneration from matured rice grain culture, using tissue culture. Different callus induction media and six Asian rice genotypes were evaluated. It was concluded that application of a medium with 2.5 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) instead of 4 mg/l 1-Naphthaleneacetic acid (NAA) as well as higher levels of silver nitrate ($AgNO^3$), of up to 10 mg/l, was advantageous to accomplish overall efficiency of callus induction and plant regeneration from seeds of various rice genotypes. Also they concluded that different genotypes had different callus induction frequencies.

Joshi *et al.,* (2011) studied *in vitro* somatic embryogenesis in different *O. sativa* subspecies and were able to screen calli for drought tolerance using mature embryos of three aromatic (Pusa Basmati 1, Pant Sugandh Dhan 17, Taraori Basmati), one non aromatic (Narendra 359) and one indica (*Oryza sativa* L.) rice varieties as explants. Tissue culture for screening was done on Murashige and Skoog (MS) medium supplemented with different concentrations of polyethylene glycol (PEG)-6000 as chemical drought inducer. It was observed that PEG treated somaclones accumulated more proline, chlorophyll content and developed more tiller and height than normal somaclones. These levels of genetic polymorphism produced plantlets that were more drought resistant as compared to the parent plants (Joshi *et al.,* 2011).

Shanthi *et al.,* (2010) were able to study the performance of different *O. sativa* genotypes for salt tolerance under *in vitro* conditions. Genotypes exhibiting high and moderate salt tolerance as well as susceptible rice types were used in this study through embryo culture technique. Callus was initiated in Murashige and Skoog (MS) medium with 2 mg/l 2,4-Dichlorophenoxyacetic acid (2,4D) and 0.5 mg/l Kinetin with different concentration of sodium chloride (50 mM, 100 mM and 150 mM NaCl) added to create salt stress. Salt tolerant rice types developed more callus even in 150 mM NaCl concentrated media. From this study it was concluded that these somaclones could also be evaluated further in the natural field conditions to develop high yielding salt tolerant varieties or could be used as a donor for the development of salt tolerant varieties. This *in vitro* screening technique with different concentration of NaCl stress could also be used as a goof screening technique for salt tolerances.

2.4.1 Transformation

Ishizaki and Kumashiro (2007) showed genetic transformation of different NERICA rice types using *Agrobacterium*-mediated methods. Transgenic plants were regenerated using tissue culture. The objective of the study was to investigate the copy number of transgene, fertility and expression level of an introduced GUS gene in NERICA rice. Callus induction was done on N6 media (Chu, 1978) supplemented with 2 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and regeneration was done on Murashige and Skoog (1962) medium supplemented with 2.5 mg/l kinetin and 0.25 mg/l 1-Naphthaleneacetic acid (NAA). The transgenic NERICA rice produced were characterized with respect to fertility. Using expression level of an introduced GUS gene, they were able to show that the fertility of the produced rice using tissue culture was significantly higher than the original rice. This provided genetic variation and improvement to the rice.

Saika and Toki (2008) were able to develop, using tissue culture, a model system for rice cell culture transformation for the production of recombinant proteins using visual selection. *O. sativa* L. cv Nipponbare was used. Callus induction was done on N6 media (Chu, 1978) supplemented with 2 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and regeneration was done on Murashige and Skoog (1962) medium with no hormones. Correlation of the expression levels of transgene products when using visual selection i.e. green fluorescent protein (GFP) and an antibiotic resistance gene were done. Visual selection allows immediate identification of transgenic rice calli efficiently accumulating transgene products. It was concluded that there was no correlation between the two methods and that using visual selection was equally comparable to using an antibiotic resistance gene.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials

The two NERICA varieties used were NERICA 4 and NERICA 11. NERICA 4 is a pedigree of WAB 450-I-B-P-91-HB while NERICA 11 is a pedigree of WAB 450-16-2-BL2-DV1 (Ndjiondjop *et al.,* 2005). The NERICA seeds were obtained from Kenya Agricultural Research Institute (KARI) in Mwea division, Kirinyaga County of Kenya.

3.2 Media Preparation

Two different media were used, N6 medium and MS medium. Both media were supplemented with different hormones for the different experiments.

The N6 medium used contained N6 macronutrients (Chu, 1978), B5 micronutrients (Gamborg *et al.*, 1968), N6 vitamins (Chu, 1978), 30 g/l sucrose, 500 mg/l L-glutamine, 2 mg/l glycine and 0.3 g/l Fe-EDTA. The basal medium was supplemented with 0.3 g/l casein hydrolysate with 8 g/l of agar as solidifying agent, pH adjusted to 5.8 with 0.5 M KOH (Potassium hydroxide). The MS medium used contained MS (Murashige and Skoog, 1962) salts, 30 g/l sucrose with 8 g/l of agar as solidifying agent and pH adjusted to 5.8 with 0.5 M KOH.

The media were sterilised using an autoclave at 121 °C and 100 kilopascal (kPa) above atmospheric pressure for 15 minutes. The media were dispensed aseptically into sterilised bottles which were then sealed with aluminium foil to prevent desiccation and stored to cool. To provide dark conditions, the bottles were wrapped tightly with aluminium foil throughout the experiment period. The experiments done in the light were exposed in 16 hours of light and 8 hours of darkness.

3.3 Sterilisation and plating

Dehusked seeds (caryopses) of both varieties of NERICA rice namely 4 and 11 were surface sterilised first with 70% (v/v) ethanol for 1 minute and rinsed with sterile distilled water followed by immersion in 1.5% (v/v) sodium hypochlorite (40% (v/v) commercial bleach-JIK ^{TM}) for 30 minutes and rinsed three times with sterile distilled water.

Root and leaf explants were obtained by germinating sterile seeds on sterile N6 medium (Chu, 1978) containing no growth hormones. After 2 weeks, the roots and leaves were removed under sterile conditions and cut into 2 cm long explants for roots and 2 cm squares leaf disc explants for leaves.

3.4 Callus initiation and proliferation

N6 media supplemented with different concentrations of naphthalene acetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) hormones were used for callus induction. The minimum sample size used was 45 for the rice seeds, leaf and root explants. There were 3 replicates for each experiment each containing 15 samples. All experiments were done in both darkness and in light (16 hours in the light and 8 hours in darkness).

A total of seven experiments were done for the rice seed explant. Four experiments were done on N6 media supplemented with different concentrations of 2,4-D (0.5, 1.0, 2.0 and 4.0) mg/l. Experiment five was done on N6 medium supplemented with 2 mg/l 2,4-D plus 1 mg/l BAP while experiment six was done on N6 medium supplemented with 2 mg/l 2,4-D plus 1 mg/l NAA. Experiment seven was done on N6 medium without hormones.

A total of two experiments were done for the leaf and root explants. Experiment one was done on N6 medium supplemented with 2 mg/l 2,4-D and experiment two was done on N6 medium supplemented with 2 mg/l 2,4-D plus 1 mg/l NAA.

Once the callus developed, it was aseptically removed from the initiating explant and divided into smaller pieces of approximately 1 mm diameter. Friable calli were separated by rolling them onto the gelling agent each time the callus transfer took place. The calli were then incubated again onto fresh medium of the same composition at 26±2^{o}C for proliferation and growth.

3.5 Regeneration

MS medium supplemented with different hormone combinations of naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), indole acetic acid (IAA) and kinetin was used for regeneration of both calli obtained from rice seeds and root explants.

The minimum sample size used was 30 for the calli obtained from rice seeds. There were 3 replicates for each experiment each containing 10 explants. A total of seven experiments were done to evaluate regeneration of the calli obtained from rice seeds. Experiment one was done on MS medium supplemented with 2 mg/l BAP plus 1 mg/l NAA. After four weeks the calli were subcultured onto MS medium supplemented with 3 mg/l BAP plus 0.5 mg/l NAA. Experiment two was done on MS medium supplemented with 2 mg/l BAP plus 1 mg/l NAA and the calli were placed on fresh medium of the same composition every four weeks. Experiment three was done on MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA. After four weeks the calli were subcultured onto MS medium without hormones.

Experiment four was done on MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA and the calli were placed on fresh medium of the same composition every four weeks. Experiment five was done on MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA. After four weeks the calli were subcultured onto MS medium without hormones. Experiment six was done on MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA and the calli were placed on fresh medium of the same composition every four weeks. Experiment seven was done on MS medium without hormones and the calli were placed on fresh medium of the same composition every four weeks.

For the calli obtained from root explants, the minimum sample size used was 45. There were 3 replicates for each experiment. Three experiments were done for this type of calli. Experiment one was done on MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA and the calli were placed on fresh medium of the same composition every four weeks.

Experiment two was done on MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA and the calli were placed on fresh medium of the same composition every four weeks. Experiment three was done on MS medium without hormones and the calli were placed on fresh medium of the same composition every four weeks.

Calli used for regeneration was separated from initiating explant after four weeks and left to proliferate and grow on the same medium used for initiation for another four weeks. Calli obtained from rice seeds cultured on N6 medium supplemented with 2 mg/l 2,4-D and root explants cultured on 2 mg/l 2,4-D plus 1 mg/l NAA were separated into smaller portions measuring approximately 3 millimetre (mm) before being transferred aseptically onto MS medium supplemented with different hormones for regeneration. All the above regeneration experiments were done in the light for 16 hours and the dark for 8 hours at 26±2^{o}C.

3.6 Acclimatisation

The regenerated plantlets that were nine weeks old with sufficient root system were taken from the culture vessels without damaging roots. Adhered media around the roots was washed off by running tap water. The plantlets were then transplanted to plastic pots containing sterilised loam soil and were covered with perforated polythene bag and kept in the greenhouse to harden for 10 days. The plantlets were watered twice a day with sterile distilled water. After day 10 the polythene bag was removed and the potted plants were transferred to the green house.

3.7 Data analysis

Callus induction frequency was expressed in percentage form by calculating percentage of seeds and explants that formed calli against the total number of seeds and explants cultured. The regeneration frequency was expressed as a percentage of calli that developed into plantlets against the total number of calli inoculated. The data was then analysed using ANOVA to show significant difference according to the Least significant Difference test $p<0.05$. Documentation of results in form of photos of various explants and plantlets in petri dishes was done.

CHAPTER FOUR

4.0 RESULTS

4.1. Callus initiation from rice seeds

For NERICA 4 experiments done in the light (16 hours of light and 8 hours of darkness), seeds inoculated on N6 medium supplemented with 2 mg/l 2,4-D showed callus induction after 8-14 days. This 2,4-D concentration showed a significantly higher callus induction frequency of 98% ($p<0.05$) (Table 1). At 6 weeks the callus was white and compact (Figure 1).Very little callus was seen developing after 14-19 days, for the lower 2,4-D concentrations of 1 mg/l and 0.5 mg/l with an induction frequency of 29% and 20% respectively. The seeds inoculated on N6 medium containing high 2,4-D concentration of 4 mg/l showed germination by day 5 but there was no callus development even after 3 weeks. However, the shoots became necrotic by day 11. The N6 medium supplemented with 2 mg/l 2,4-D combined with 1 mg/l NAA or 1 mg/l BAP, showed callus induction within 15-21 days but low callus induction frequencies of 9% and 11% respectively (Table 1). The seeds of NERICA 4 inoculated on N6 medium without hormones germinated after 3 days. However, no callus formation took place even after a culture period of 2-3 weeks (Table1).

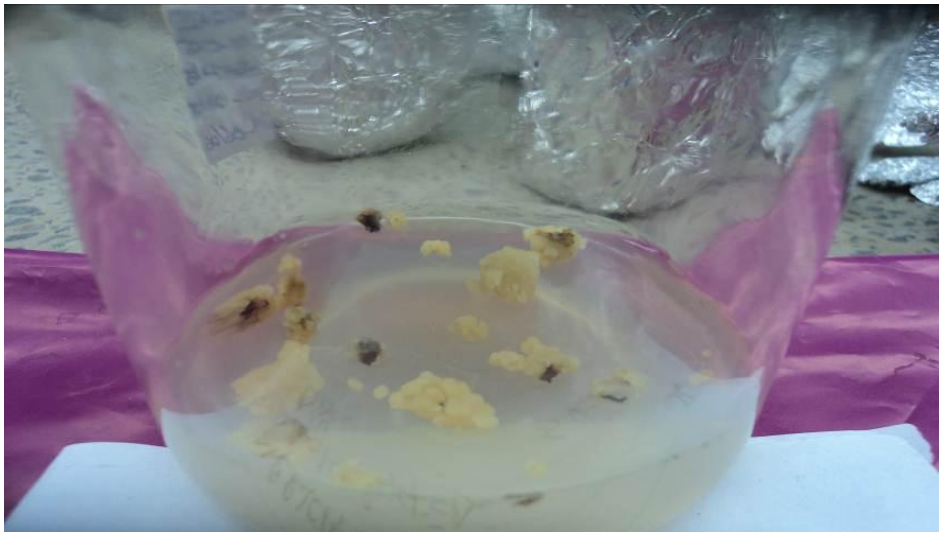

Figure 1: Six weeks old NERICA 4 calli (developed in the light) on N6 medium with 2 mg/l 2,4-D

Table 1: Development of callus derived from NERICA 4 seeds cultured on N6 media in the light

Hormones (mg/l)			No. of replicates	Total no. of seeds inoculated	% callus induction frequency	Mean± S.E %	Remarks
2,4-D	NAA	BAP					
0.5	0	0	3	45	20	20±6.7cd	There was slight callus development within 16-19 days
1.0	0	0	3	45	29	29±2.2d	Creamy callus seen within 14-19 days
2.0	0	0	3	45	98	98±2.3e	Creamy callus seen within 8-14 days
2.0	1.0	0	3	45	9	9±2.0ab	Compact callus seen within 15-21 days
2.0	0	1.0	3	45	11	11±5.9bc	Creamy callus developed within 15-19 days
4.0	0	0	3	45	0	0a	No callus produced. Short shoots were seen which became necrotic by day 11
0	0	0	3	45	0	0a	No callus was formed. Seeds germinated into plantlets within 8-14 days

Data analysis done using ANOVA n=45. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

For NERICA 4 experiments done in the dark, seeds inoculated on N6 medium supplemented with 2 mg/l 2,4-D showed callus induction after 6-12 days. This medium also showed a significantly higher callus induction frequency of 100% ($p<0.05$) (Table 2). At 6 weeks the callus was cream in colour and compact (Figure 2). Callus was seen developing after 13-19 days for the lower 2,4-D concentrations of either 1 mg/l or 0.5 mg/l with induction frequencies of 42% and 27% respectively. The seeds inoculated on N6 medium containing high 2,4-D concentration of 4 mg/l showed germination by day 4 but there was no callus development even after 3 weeks. The shoots became necrotic by day 11. The N6 medium supplemented with 2 mg/l 2,4-D combined with 1 mg/l NAA or 1 mg/l BAP, showed callus induction within 12-18 days and low callus induction frequencies of 16% and 22% respectively (Table 2).

Table 2: Development of callus derived from NERICA 4 seeds cultured on N6 media in the dark

Hormones (mg/l)			No. of replicates	Total no. of seeds inoculated	% callus induction frequency	Mean± S.E %	Remarks
2,4-D	NAA	BAP					
0.5	0	0	3	45	27	27±3.85b	A few seeds developed cream callus within15-19 days
1.0	0	0	3	45	42	42±4.5c	Creamy callus developed within 13-17 days
2.0	0	0	3	45	100	100±0d	Creamy callus seen within 6-12 days
2.0	1.0	0	3	45	16	16±2.22b	Compact callus seen within 12-17 days
2.0	0	1.0	3	45	22	22±8.01b	A few seeds developed creamy callus within13-18 days
4.0	0	0	3	45	0	0a	No callus produced. Short shoots were seen which became necrotic by day 11
0	0	0	3	45	0	0a	No callus was formed. Seeds germinated into plantlets within 6-13 days

Data analysis done using ANOVA n=45. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

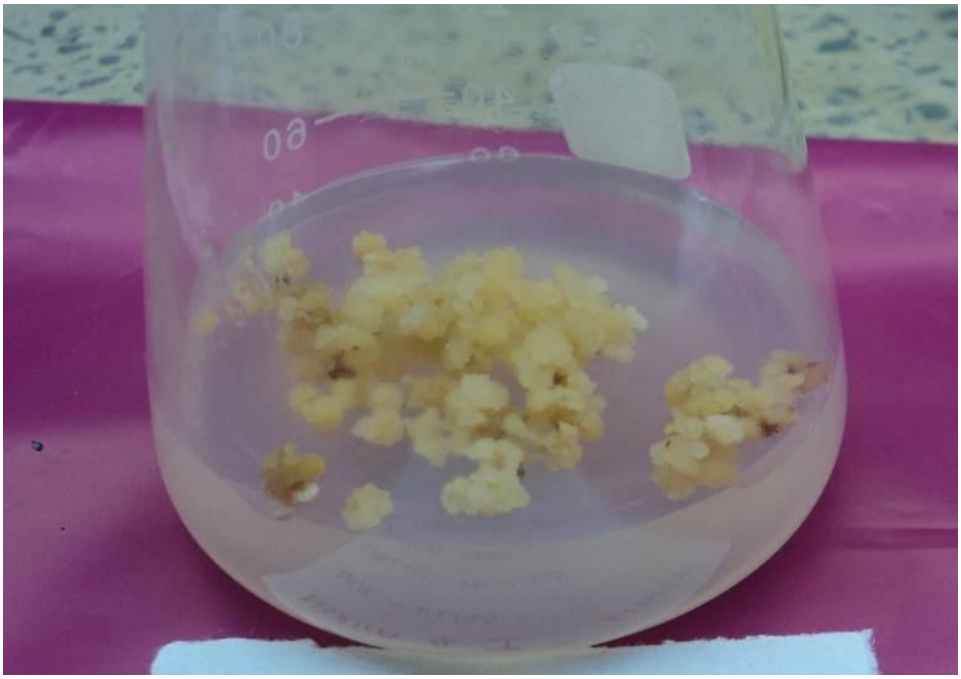

Figure 2: Six weeks old NERICA 4 calli (developed in the dark) on N6 medium with 2 mg/l 2,4-D

For NERICA 11 experiments done in the light (16 hours of light and 8 hours of darkness), seeds inoculated on N6 medium supplemented with 2 mg/l 2,4-D showed callus induction after 8-13 days. This concentration also recorded the highest callus induction frequency of 93% (Table 3). At 6 weeks the callus was white and compact (Figure 3). Significantly lower amounts of calli at (p<0.05) were seen developing after 14-19 days, for the lower 2,4-D concentrations of either 1 mg/l or 0.5 mg/l with induction frequencies of 29% and 16% respectively. The seeds inoculated on N6 medium containing high 2,4-D concentration of 4 mg/l showed germination by day 6 but there was no callus development even after 3 weeks. The shoots became necrotic by day 10. The N6 medium supplemented with 2 mg/l 2,4-D combined with 1 mg/l NAA or 1 mg/l BAP, showed callus induction within 15-19 days and low callus induction frequencies of 9% and 31% respectively (Table 3). The seeds of NERICA 11 inoculated on N6 medium without hormones germinated after 3 days. However, no callus formation took place even after a culture period of 2-3 weeks (Table 3).

Table 3: Development of callus derived from NERICA 11 seeds cultured on N6 media in the light

Hormones (mg/l)			No. of replicates	Total no. of seeds inoculated	% callus induction frequency	Mean± S.E %	Remarks
2,4-D	NAA	BAP					
0.5	0	0	3	45	16	16±2.2b	A few seeds developed creamy callus within 15-19 days
1.0	0	0	3	45	29	29±5.88c	Creamy callus seen within 14-19 days
2.0	0	0	3	45	93	93±3.85d	Creamy callus seen within 8-13 days
2.0	1.0	0	3	45	9	9±2.33ab	Compact callus seen within 14-18 days
2.0	0	1.0	3	45	31	31±5.8c	A few seeds developed creamy callus within 15-19 days
4.0	0	0	3	45	0	0a	No callus produced. Short shoots were seen which became necrotic by day 10
0	0	0	3	45	0	0a	No callus was formed. Seeds germinated into plantlets within 7-12 days

Data analysis done using ANOVA n=45. Values with different letters differ significantly according to the Least significant Difference test p<0.05 (Appendix B)

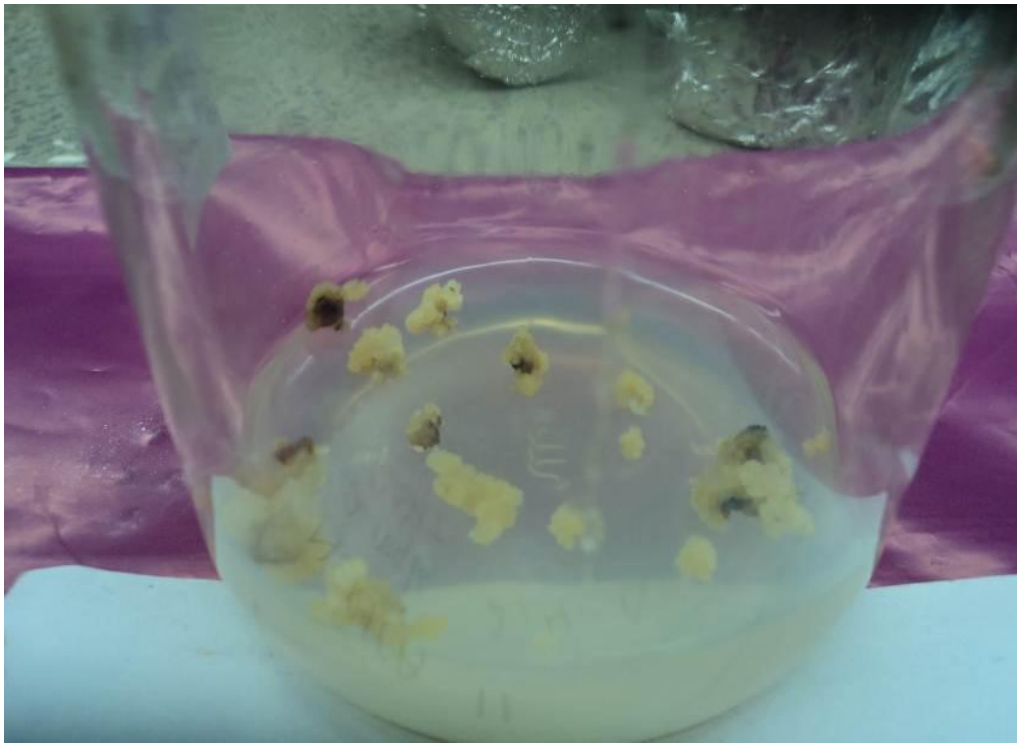

Figure 3: Six weeks old NERICA 11 calli (developed in the light) on N6 medium with 2 mg/l 2,4-D

For NERICA 11 experiments done in the dark, the medium with the highest induction frequency of 100% was N6 medium supplemented with 2 mg/l 2,4-D (Table 4). Callus induction on this medium was observed within 6-13 days. At 6 weeks the callus was cream in colour and compact (Figure 4). Rice seeds inoculated on N6 medium containing lower 2,4-D concentrations of either 1 mg/l or 0.5 mg/l showed significantly lower amounts of callus development at ($p<0.05$), within 14-18 days with induction frequencies of 31% and 22% respectively. Those seeds inoculated on N6 medium containing high 2,4-D concentration of 4 mg/l showed germination by day 5 but there was no callus development even after 3 weeks. However, the shoots exhibited retarded growth compared to the controls and they became necrotic by day 12. N6 medium supplemented with 2 mg/l 2,4-D combined with 1 mg/l NAA or 1 mg/l BAP, showed callus induction frequencies of 13% and 40% respectively within 13-17 days (Table 4).

Table 4: Development of callus derived from NERICA 11 seeds cultured on N6 media in the dark

Hormones (mg/l)			No. of replicates	Total no. of seeds inoculated	% callus induction frequency	Mean± S.E %	Remarks
2,4-D	NAA	BAP					
0.5	0	0	3	45	22	22±2.3bc	A few seeds developed cream callus within 14-17 days
1.0	0	0	3	45	31	31±5.88cd	Creamy callus seen within 14-18 days
2.0	0	0	3	45	100	100±0e	Creamy callus seen within 6-13 days
2.0	1.0	0	3	45	13	13±3.85b	Compact callus seen within 14-17 days
2.0	0	1.0	3	45	40	40±6.7d	Some seeds developed creamy callus within 13-16 days
4.0	0	0	3	45	0	0a	No callus produced. Shoots were seen which became necrotic by day 12
0	0	0	3	45	0	0a	No callus was formed. Seeds germinated into plantlets within 7-12 days

Data analysis done using ANOVA n=45. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

Figure 4: Six weeks old NERICA 11 calli (developed in the dark) on N6 medium with 2 mg/l 2,4-D

4.2 Callus initiation from root explants

NERICA 4 root explants inoculated on N6 medium supplemented with 2 mg/l 2,4-D under light (16 hours of light and 8 hours of darkness), showed no callus growth and the root explants turned brown in 6 weeks. Root explants inoculated on N6 medium supplemented with 2 mg/l 2,4-D and 1 mg/l NAA under light, showed callus induction within 5-6 weeks and an induction frequency of 70% (Table 5). The callus formed was watery and white in colour in appearance. NERICA 4 root explants inoculated on N6 medium supplemented with 2 mg/l 2,4-D and 1 mg/l NAA in the dark, showed callus development within 4-5 weeks with an induction frequency of 76%. Root explants inoculated on N6 medium supplemented with 2 mg/l 2,4-D in the dark, showed no callus growth and the root explants turned brown in 6 weeks (Table 5). The callus formed was compact and creamy in colour (Figure 5).

NERICA 11 root explants cultured in the light (16 hours of light and 8 hours of darkness) showed no callus growth on N6 medium supplemented with 2 mg/l 2,4-D only. The root explants became necrotic within 6 weeks. On the other hand, the root explants cultured in the light on N6 medium supplemented with 2 mg/l 2,4-D and 1 mg/l NAA showed callus development within 5-7 weeks with a callus induction frequency of 48% (Table 6). The callus formed was watery and white in colour in appearance (Figure 6). NERICA 11 experiments cultured in the dark showed that root explants inoculated on the same medium (N6 medium supplemented with 2 mg/l of 2,4-D and 1 mg/l NAA) exhibited callus formation within 4-5 weeks with an induction frequency of 56%. There was no callus induction on N6 medium supplemented with 2 mg/l 2,4-D only and the root explants became necrotic within 6 weeks (Table 6). The callus developed in the dark was compact and creamy in colour.

Table 5: Callus development from NERICA root explants on N6 media in the light

Rice type	Hormones (mg/l)		No. of replicates	Total no. of explants inoculated	% callus induction frequency	Mean± S.E %	Remarks
	2,4-D	NAA					
NERICA 4	2.0	0	3	54	0	0a	No callus formed. The root explants turned brown after 6 weeks
NERICA 4	2.0	1.0	3	54	70	70±4.9c	Creamy calli with white patches was seen within 5-6 weeks
NERICA 11	2.0	0	3	54	0	0a	No callus formed. The root explants turned brown after 6 weeks
NERICA 11	2.0	1.0	3	54	48	48±3.7b	White calli seen within 5-7 weeks

Data analysis done using ANOVA n=54. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

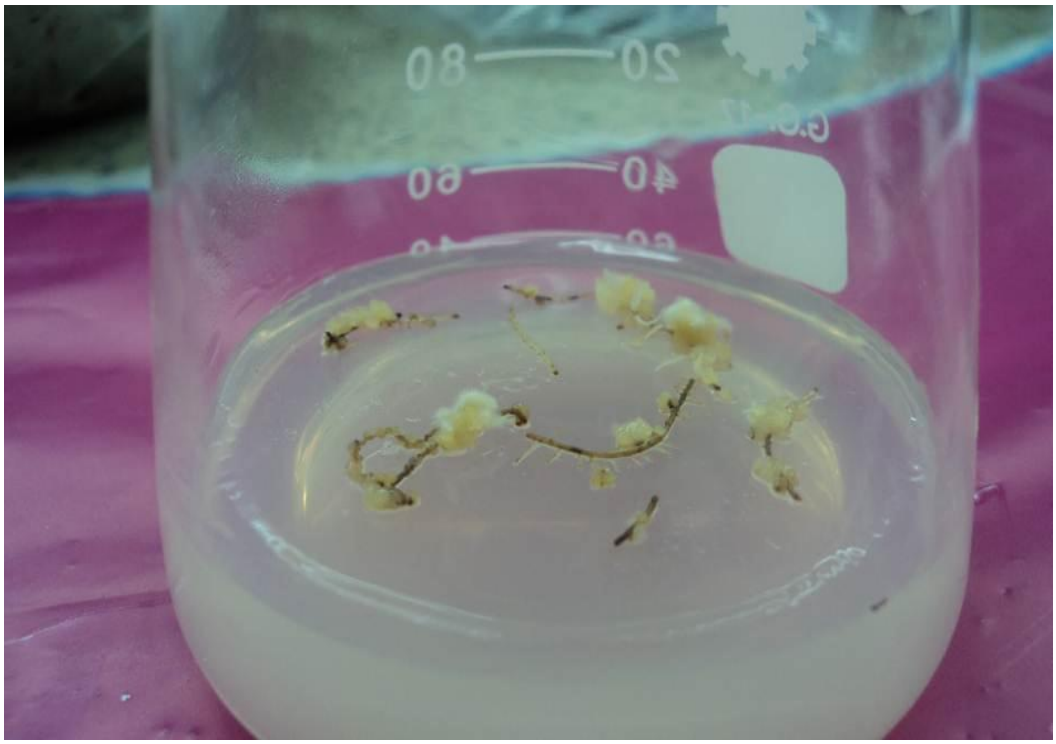

Figure 5: Nine weeks old NERICA 4 root explants calli developing in the dark on N6 medium with 2 mg/l 2,4-D and 1 mg/l NAA

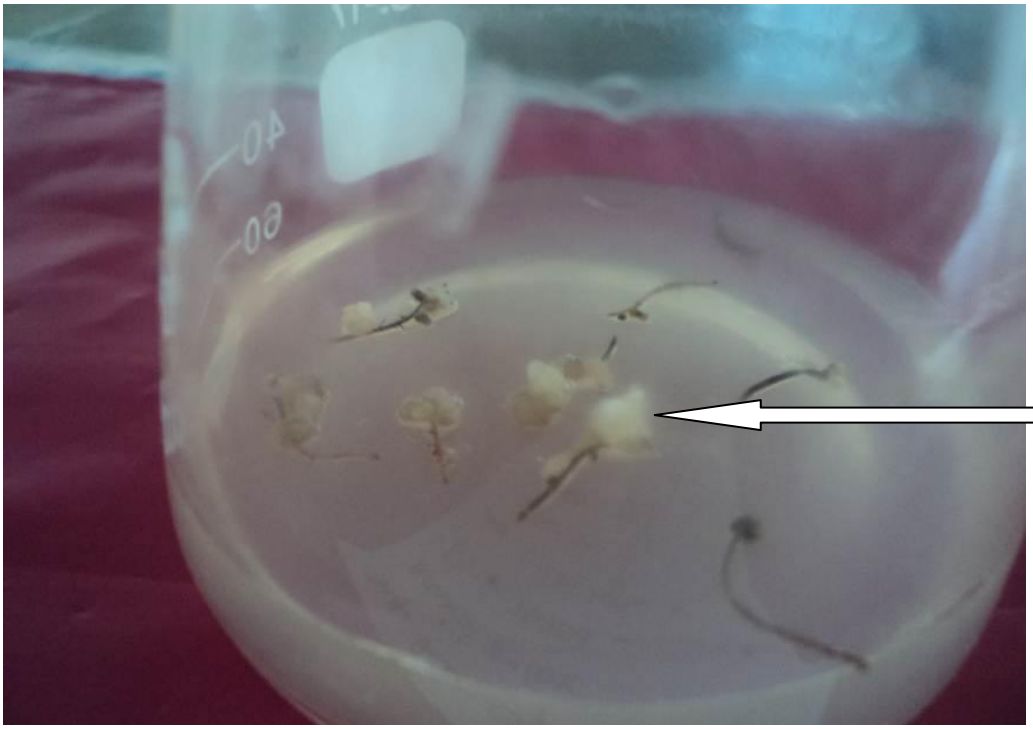

Figure 6: Nine weeks old NERICA 11 root explants calli developing in the light on N6 medium with 2 mg/l 2,4-D and 1 mg/l NAA

Table 6: Callus development from NERICA root explants on N6 media in the dark

Rice type	Hormones (mg/l)		No. of replicates	Total no. of explants inoculated	% callus induction frequency	Mean± S.E %	Remarks
	2,4-D	NAA					
NERICA 4	2.0	0	3	54	0	0a	No callus formed. The root explants turned brown after 6 weeks
NERICA 4	2.0	1.0	3	54	76	76±4.89c	Creamy calli with white patches was seen within 4-5 weeks
NERICA 11	2.0	0	3	54	0	0a	No callus formed. The root explants turned brown after 6 weeks
NERICA 11	2.0	1.0	3	54	56	56±3.2b	White calli seen within 4-5 weeks

Data analysis done using ANOVA n=54. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

4.3 Callus initiation from leaf explants

There was no callus development from leaf explants of both NERICA 4 and NERICA 11 cultured in the dark or in the light. The leaves were inoculated on N6 medium supplemented with 2 mg/l 2,4-D only and on N6 medium with 2 mg/l 2,4-D plus 1 mg/l NAA. The inoculated leaves started to show necrosis within 4 to 6 weeks (Figure 7).

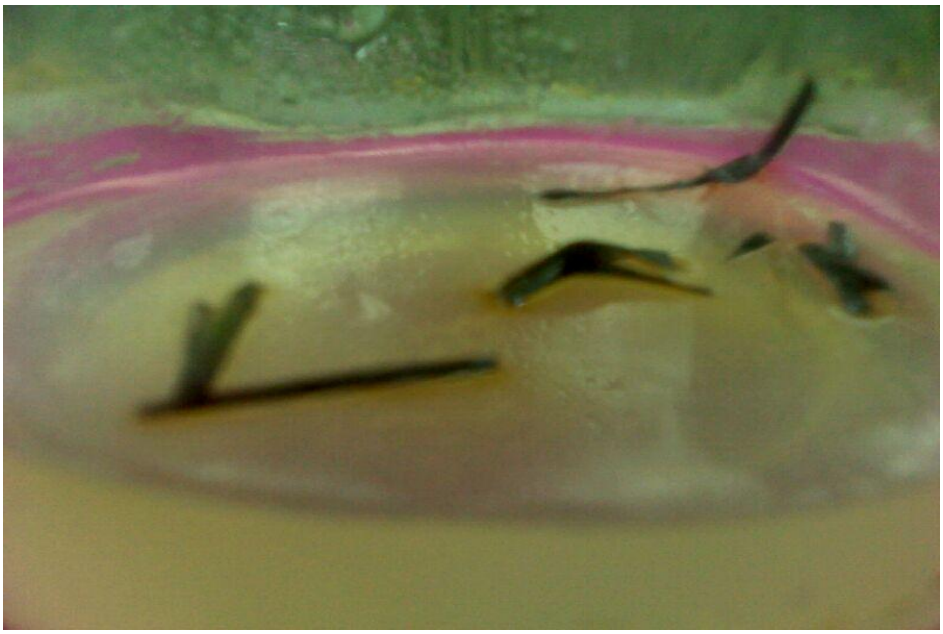

Figure 7: Six weeks old leaf explants of NERICA 11 inoculated on N6 media with 2 mg/l 2,4-D and 1 mg/l NAA showing necrosis

4.4 Regeneration of callus derived from rice seeds

4.4.1. Shoot and root initiation

NERICA 4 callus from rice seeds cultured on N6 medium with 2 mg/l 2, 4-D and was later subcultured onto to MS medium supplemented with 2 mg/l BAP plus 1 mg/l NAA developed green shoot-like growths by week 7 (Figure 8). However, the shoot buds failed to elongate even after being transferred to fresh medium of the same composition with 2 mg/l BAP plus 1 mg/l NAA every 4 weeks (Table 7). The callus browned after 11 weeks.

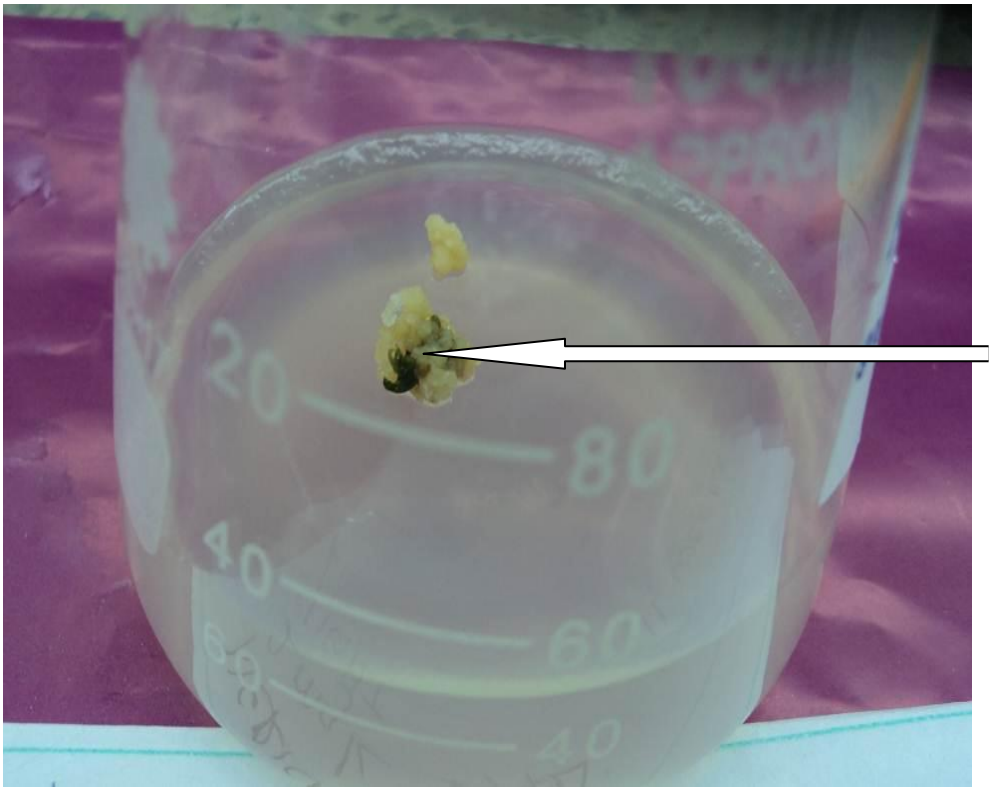

Figure 8: Seven weeks old NERICA 4 callus showing shoot initiation on MS medium with 2 mg/l BAP plus 1 mg/l NAA

NERICA 11 callus from rice seeds cultured on N6 medium with 2 mg/l 2,4-D and was later subcultured onto to MS medium supplemented with 2 mg/l BAP plus 1 mg/l NAA developed green shoot-like growths by week 8. However, the shoot buds failed to elongate even after being transferred to fresh medium of the same composition of 2 mg/l BAP plus 1 mg/l NAA every 4 weeks (Table 8). The callus browned in 11 weeks.

NERICA 4 callus from rice seeds cultured on N6 medium with 2 mg/l 2,4-D and was then subcultured onto MS medium supplemented with 2 mg/l BAP plus 1 mg/l NAA for 4 weeks and later subcultured onto MS medium containing 3 mg/l BAP plus 0.5 mg/l NAA developed root-like structures by week 6. The roots grew to approximately 2 cm by week 9. Unfortunately the callus and the roots became necrotic by week 13 even after change onto fresh MS medium containing 3 mg/l BAP plus 0.5 mg/l NAA every 4 weeks. No shoot regeneration was observed.

Table 7: Regeneration of callus derived from NERICA 4 seeds on MS media

Hormones (mg/l)				No. of replicates	Total no. of calli inoculated	Regeneration frequency %	Mean± S.E %	Remarks
BAP	NAA	KIN	IAA					
0	0	0	0	3	30	67	67±3.3b	Shoots and roots observed week 5 and week 6 respectively. Slight browning of callus was seen by week 6.
2.0	1.0	-	-	3	30	0	0a	Small green shoot-like and root-like growths were seen within 7-8 weeks but they did not elongate
3.0	0.5	-	-	3	30	0	0a	Root development by week 6 which grew longer by week 9. No shoots. Callus became necrotic by week 13
-	0.5	2.5	-	3	30	0	0a	Root development by week 6 that grew to long roots by week 9. No shoot development. Necrosis by week 11
0.5	-	-	0.2	3	30	0	0a	Small green shoot-like structures appeared in the 5th week but they became necrotic by 10th week

Data analysis done using ANOVA n=30. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

Table 8: Regeneration of callus derived from NERICA 11 seeds on MS media

Hormones (mg/l)				No. of replicates	Total no. of calli inoculated	Regeneration frequency %	Mean± S.E %	Remarks
BAP	NAA	KIN	IAA					
0	0	0	0	3	30	77	77±8.8b	Shoots and roots observed in week 4 and week 6 respectively. Slight browning of callus was seen by week 6.
2.0	1.0	-	-	3	30	0	0a	Small green shoot-like and root-like structures were seen after 7 weeks but they did not elongate.
3.0	0.5	-	-	3	30	0	0a	Root development by week 7 which grew longer by week 10. No shoots developed. Callus became necrotic by week 13
-	0.5	2.5	-	3	30	0	0a	Root development by week 5 that grew to long roots by week 8. No shoot development. Necrosis by week 11
0.5	-	-	0.2	3	30	0	0a	Small green shoot-like structures appeared in the 5th week but they became necrotic by 11th week.

Data analysis done using ANOVA n=30. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

NERICA 11 callus was obtained from N6 medium with 2 mg/l 2,4-D. The callus subcultured onto MS medium supplemented with 2 mg/l BAP plus 1 mg/l NAA for 4 weeks then transferred to MS medium containing 3 mg/l BAP plus 0.5 mg/l NAA showed root-like structures by week 7 (Figure 9). The roots elongated to approximately 2.5 cm by week 10. Unfortunately the callus together with the roots became necrotic by week 13 even after change onto fresh MS medium containing 3 mg/l BAP plus 0.5 mg/l NAA every 4 weeks. No shoot regeneration was observed.

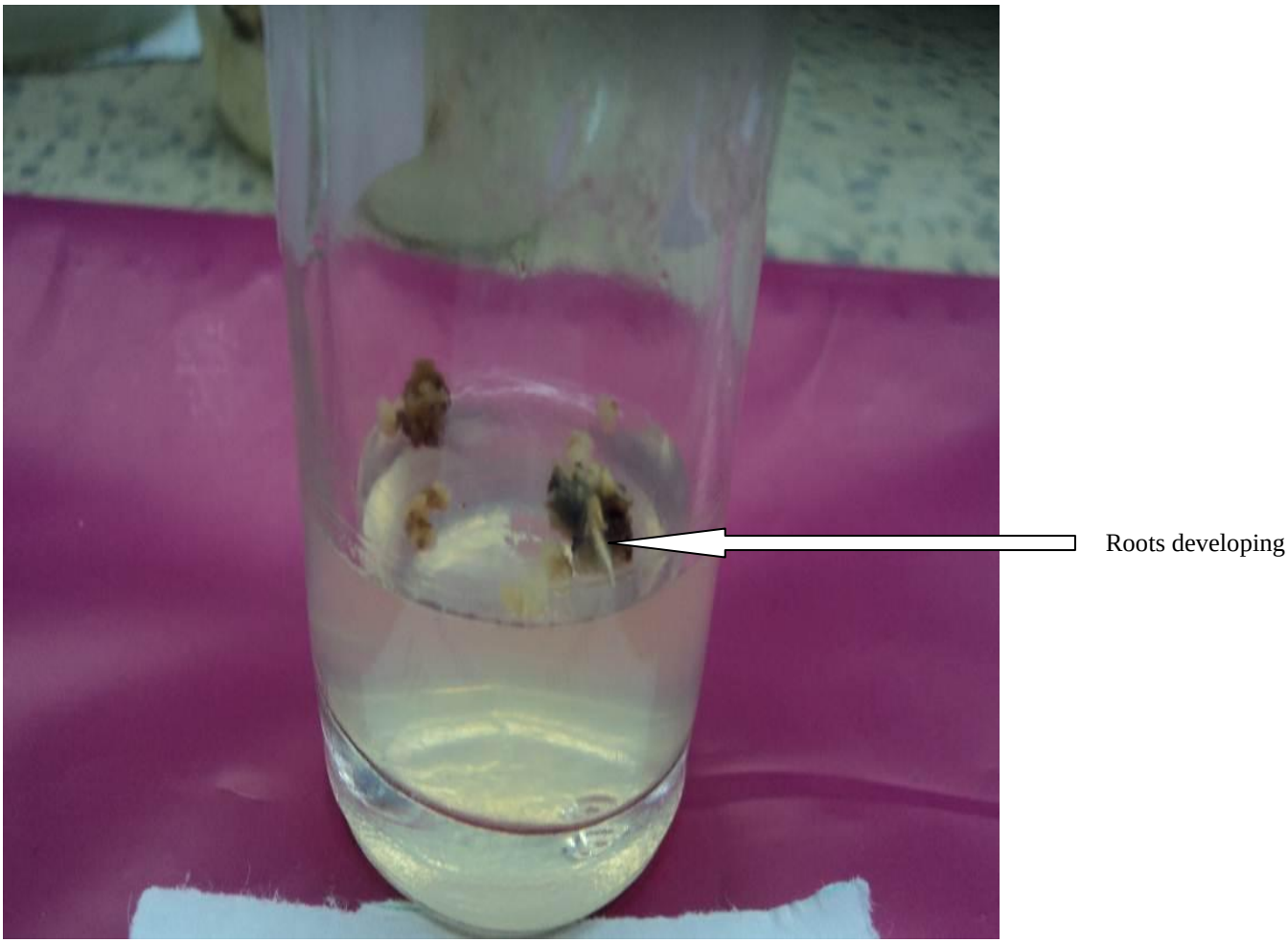

Figure 9: Seven weeks old NERICA 11 callus showing root development on MS medium with 3 mg/l BAP plus 0.5 mg/l NAA

NERICA 4 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA showed development of roots by week 6 that grew into long roots by week 9. The callus browned by week 11 but no shoot regeneration was observed even after transfer to fresh medium of the same composition every 4 weeks (Table 7). NERICA 4 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA and then transferred after 4 weeks to MS medium without hormones showed green shoot-like growths by week 6 (Figure 10). The shoot-like growths did not elongate but browned with the rest of the callus by week 11 even after transfer to fresh MS medium without hormones after every 4 weeks (Table 7).

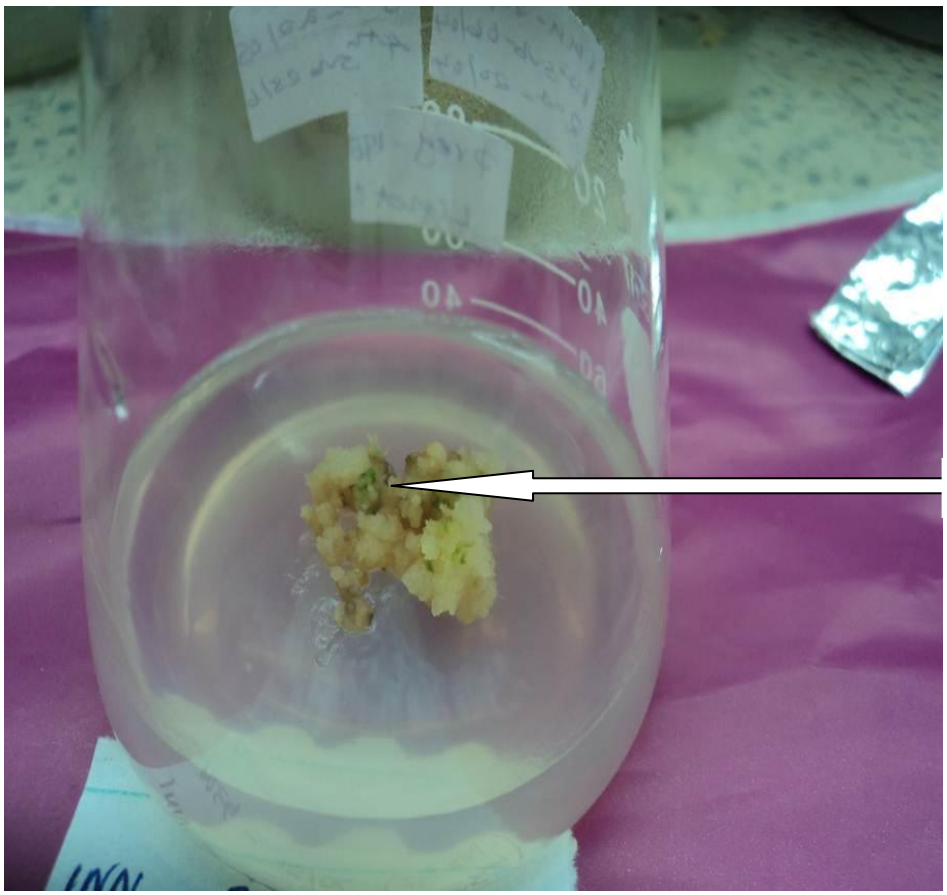

Figure 10: Six weeks NERICA 4 callus subcultured onto 2.5 mg/l kinetin plus 0.5 mg/l NAA showing shoot initiation on MS medium without hormones

NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA showed development of roots by week 5 (Figure 11) that grew into approximately 1 cm roots by week 8 (Figure 12). The callus browned by week 11 but no shoot buds developed even after transfer to fresh medium of the same composition every 4 weeks (Table 8). NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA for 4 weeks and then transferred to MS medium without hormones showed green growths by week 5. The shoot-like growths did not elongate but browned together with the callus by week 11 despite transfer to fresh MS medium without hormones after every 4 weeks (Table 8).

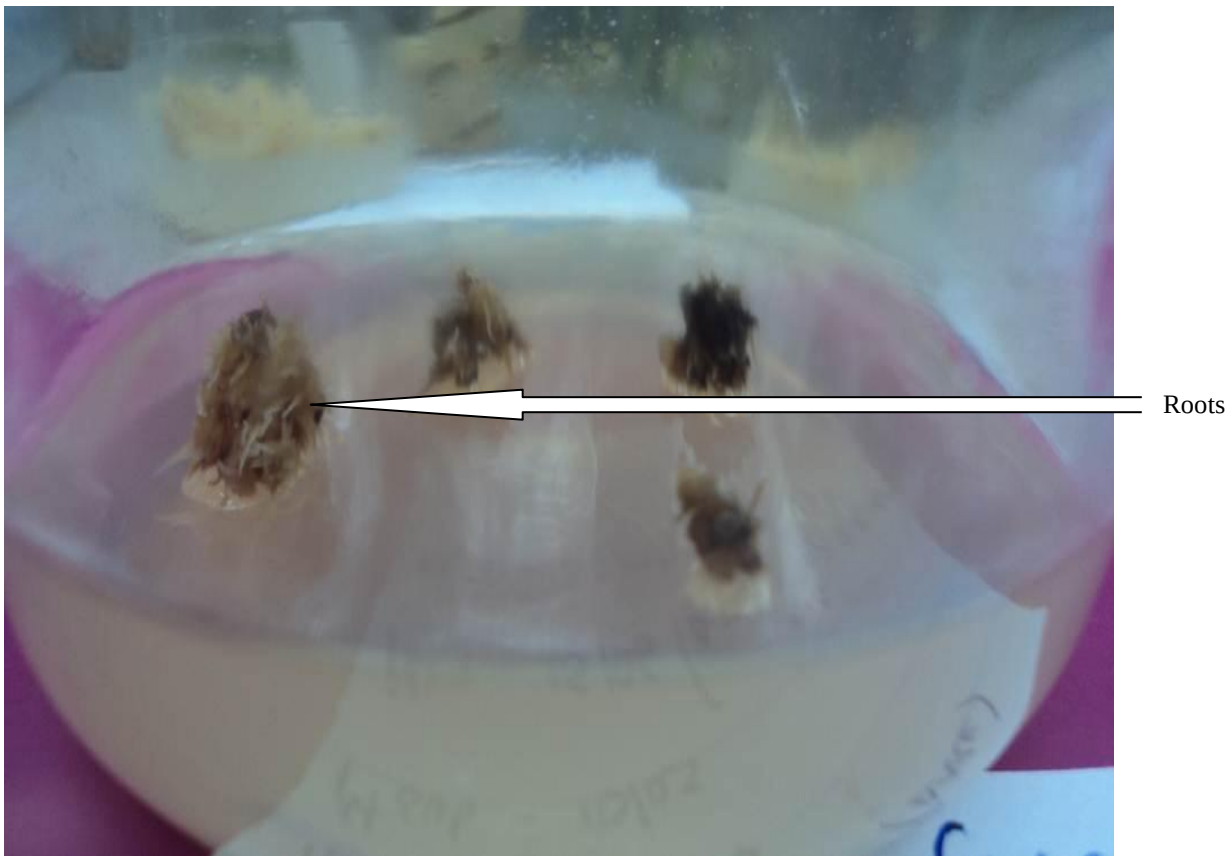

Figure 11: Five weeks old NERICA 11 callus showing root initiation on MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA.

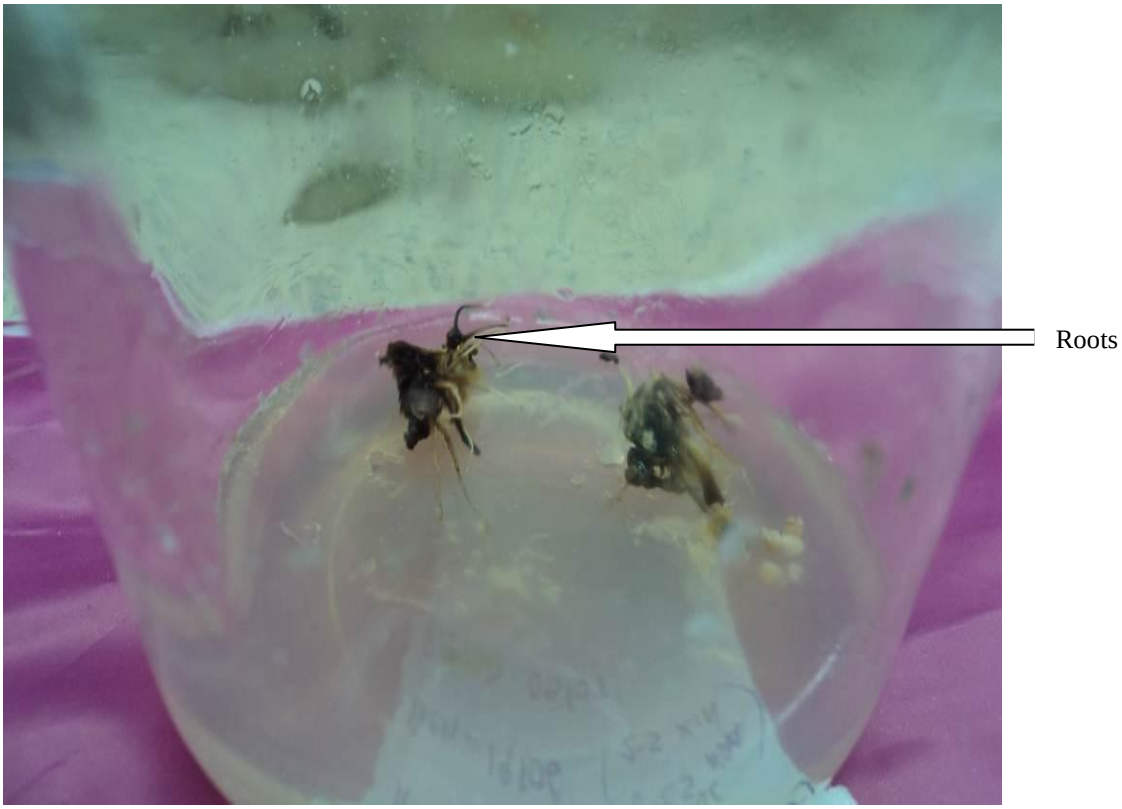

Figure 12: Eight weeks old NERICA 11 callus showing root development on MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA

NERICA 4 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with a lower concentration of hormones i.e. 0.5 mg/l BAP plus 0.2 mg/l IAA showed shoot buds (Figure 13) in about 5 weeks but they did not elongate. The callus with the shoot buds turned brown by week 10 even after transfer to fresh medium of the same composition every 4 weeks (Table 7). NERICA 4 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA for 4 weeks and then transferred to MS medium without hormones showed development of shoot buds by week 5 which grew in size and developed roots by week 6. But they did not elongate instead withered by week 8 and the callus turned brown by week 10 even after transfer to fresh MS medium without hormones every 4 weeks (Table 7).

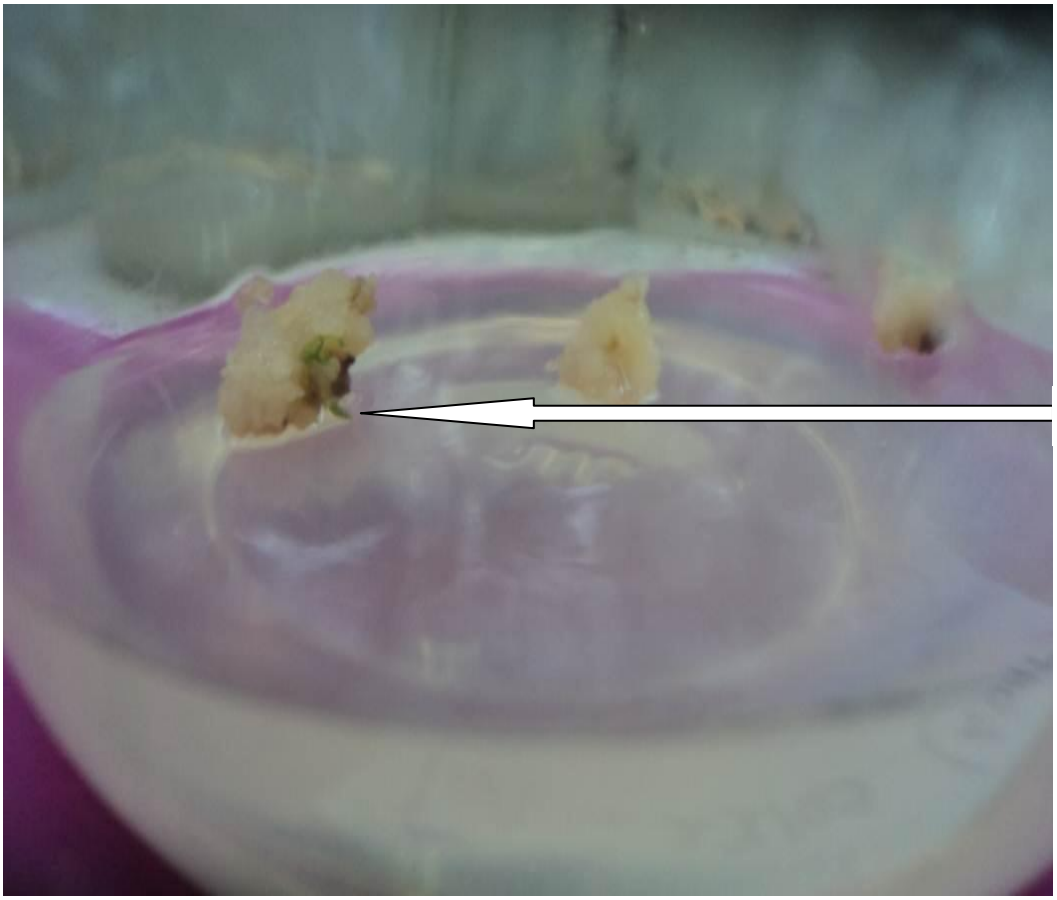

Figure 13: Five weeks old NERICA 4 callus showing shoot initiation on MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA

NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA showed shoot buds in about 5 weeks but they did not elongate. The callus turned brown by week 10 even after transfer to fresh medium of the same composition every 4weeks (Table 8). NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D and subcultured onto MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA and then transferred after 4 weeks to MS medium without hormones showed shoot bud development by week 5 which grew in size and developed roots by week 7 (Figure 14). But they did not elongate but instead withered by week 9 and callus turned brown by week 10 despite transfer to MS medium without hormones every 4 weeks (Table 8).

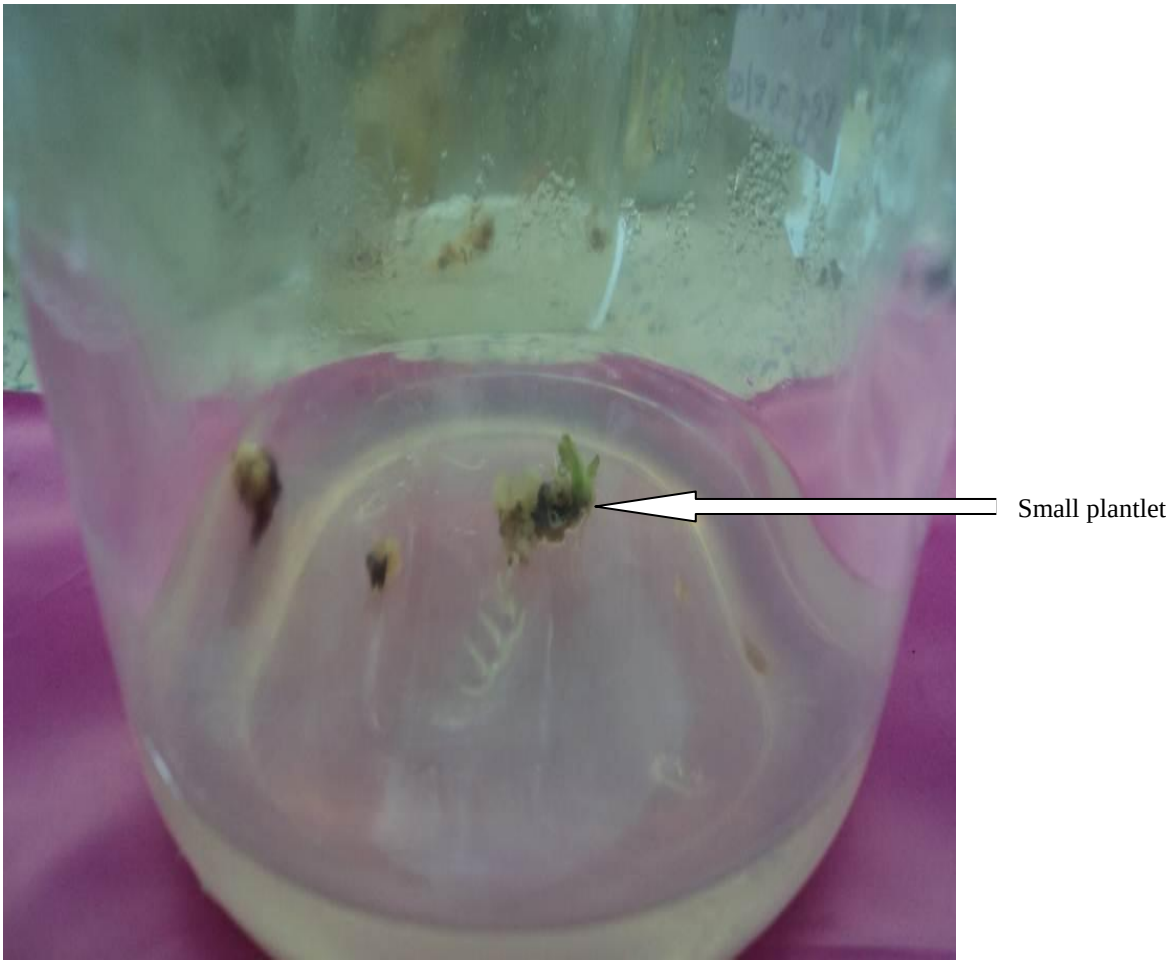

Figure 14: Seven weeks old NERICA 11 callus showing plantlet development on MS medium without hormones

4.4.2. Plantlet regeneration

NERICA 4 callus from rice seeds initiated on N6 medium with 2 mg/l 2,4-D and was later subcultured onto to MS medium without hormones developed shoot buds by week 5. Roots were seen in the 6th week (Figure 15). The callus showed browning at around this time but good growth of the shoots and roots was exhibited when transferred to fresh media of the same composition. There was development of more buds by week 8 with some developing into plantlets after a culture of 8-9 weeks (Figure 16). There were a total of 20 plantlets produced from the 3 replicates done which translated to a regeneration frequency of 67% (Table 7).

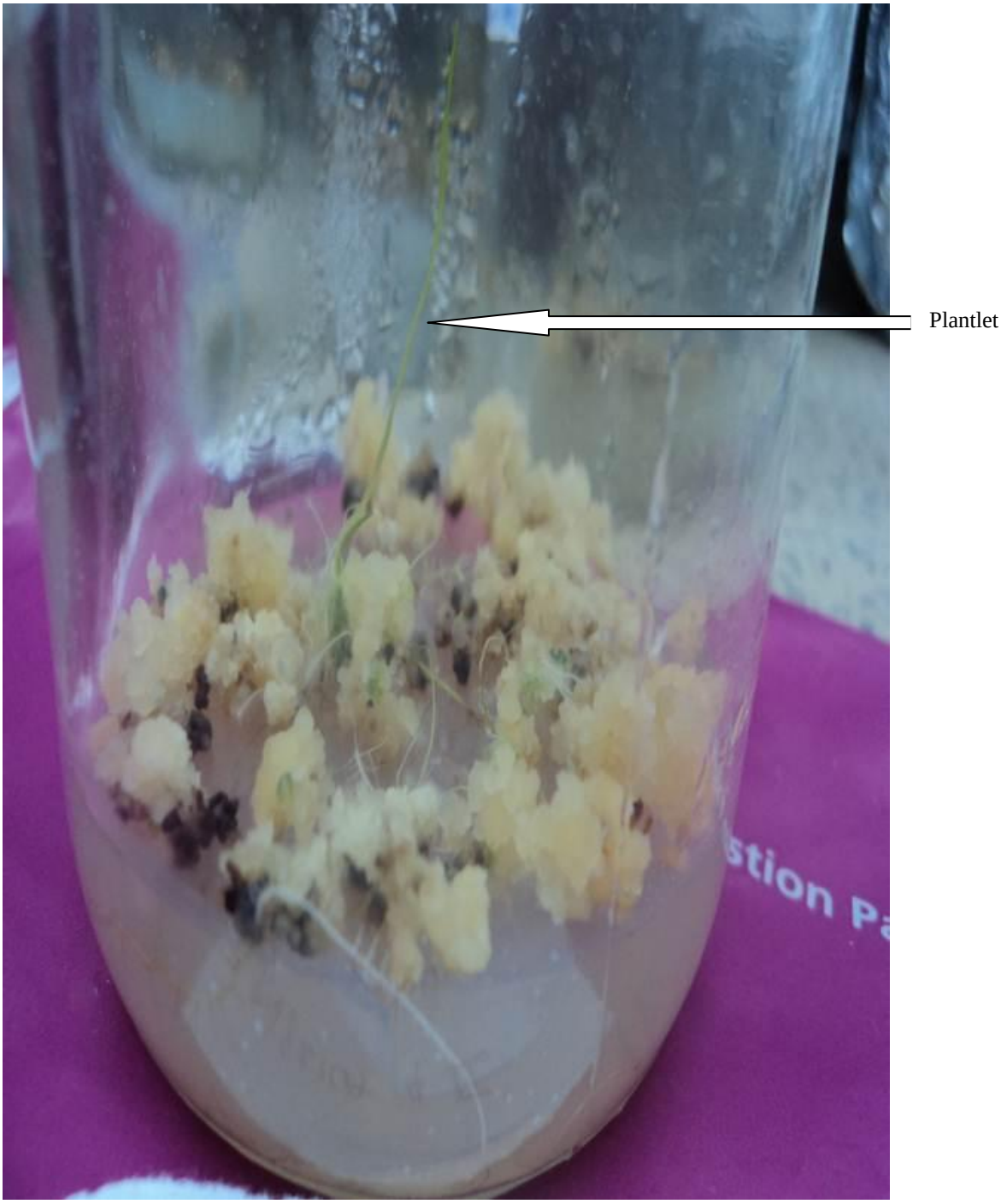

Figure 15: Six weeks old NERICA 4 callus showing plantlet development on MS medium without hormones

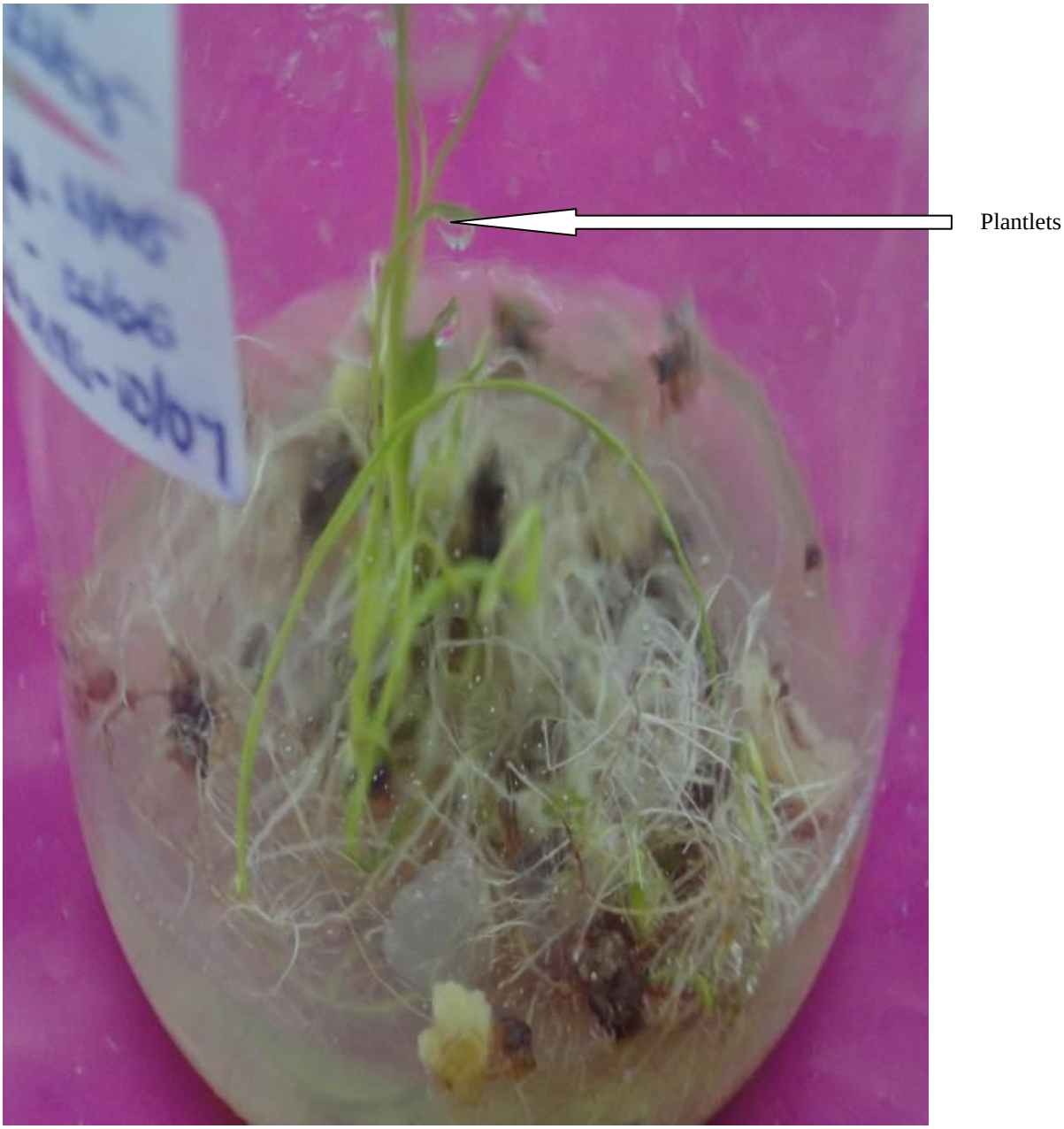

Figure 16: Nine weeks old NERICA 4 callus showing plantlets on MS medium without hormones

NERICA 11 callus from rice seeds initiated on N6 medium with 2 mg/l 2,4-D and was later subcultured onto to MS medium without hormones developed shoot buds by week 4 and there was development of roots by week 6 (Figure 17). The callus showed browning at around this time but good growth of the shoots and roots was exhibited when transferred to fresh media of the same composition. There was development of more buds by week 7 with some developing into plantlets after a culture of 8-9 weeks (Figure 18). There were a total of 23 plants produced from the 3 replicates done which translated to a regeneration frequency of 77% (Table 8).

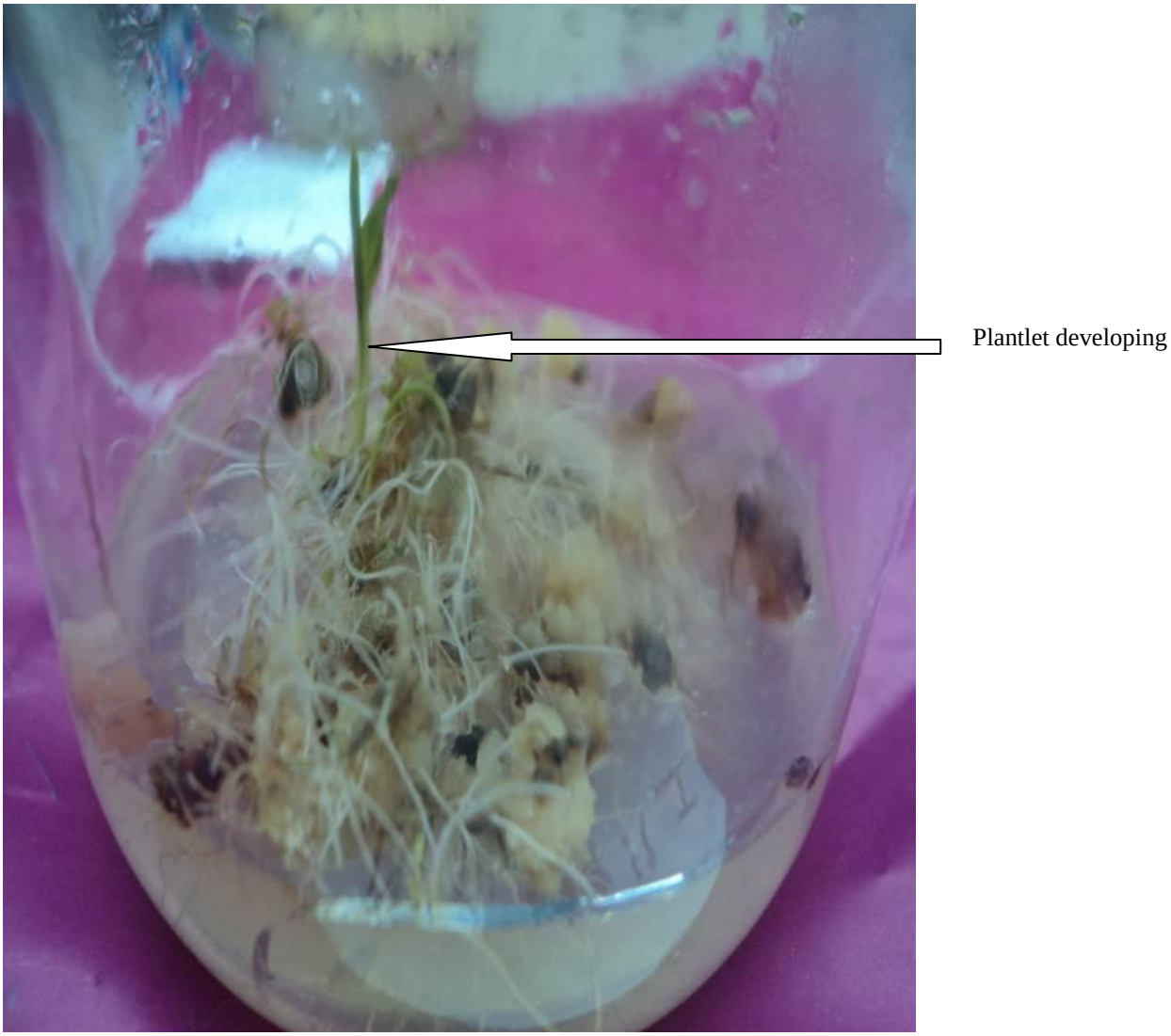

Figure 17: Six weeks old NERICA 11 callus showing plantlet development on MS medium without hormones

Figure 18: Nine weeks old NERICA 11 callus showing plantlet development on MS medium without hormones

4.5 Regeneration of callus derived from root explants

NERICA 4 callus obtained from root explants cultured on N6 medium with 2 mg/l 2,4-D plus 1 mg/l NAA and subcultured onto MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA showed root development by week 5. There was root proliferation by week 10. No shoot buds appeared. The root regeneration frequency was 100%. The callus appeared to brown after a period of 6 weeks despite being placed on fresh medium of the same composition every 4 weeks. Root development continued despite the browning of callus (Table 9).

Similar calli subcultured onto to MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA showed browning and darkening of callus by week 4 despite being placed on fresh medium of the same composition every 4 weeks. There was still some roots developing by week 6 in spite of the browning. The roots increased in size by week 10. No shoot buds were seen. The root regeneration frequency was significantly lower at 22% ($p<0.05$) (Table 9). NERICA 4 callus obtained from N6 medium with 2 mg/l 2,4-D plus 1 mg/l NAA and subcultured onto MS medium without hormones showed profuse root development by week 5. There was root proliferation by week 10. The callus showed browning by week 7 even after being transferred to fresh medium of the same composition every 4 weeks. Despite the browning of the callus, root development continued. No shoot buds were observed. The root regeneration frequency was 100% (Table 9).

Table 9: Root regeneration of callus from NERICA 4 root explants on MS media

Hormones (mg/l)				No. of replicates	Total no. of calli subcultured	Regeneration frequency %	Mean± S.E %	Remarks
BAP	NAA	KIN	IAA					
-	0.5	2.5	-	3	45	100	100±0b	white roots were seen by week 5 that developed into long roots by week 10
0.5	-	-	0.2	3	45	22	22±2.2a	The callus turned dark brown by week 4. Roots developed by week 6 and grew into long roots in 10 weeks
-	-	-	-	3	45	100	100±0b	Callus formed white roots in 5-10 weeks. Browning of callus by week 7

Data analysis done using ANOVA n=45. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D plus 1 mg/l NAA and subcultured onto MS medium supplemented with 2.5 mg/l kinetin plus 0.5 mg/l NAA showed root development by week 6. There was root proliferation by week 11. No shoot buds appeared. The root regeneration frequency was 96%. The callus appeared to brown after a period of 6 weeks despite being placed on fresh medium of the same composition every 4 weeks. Root development continued despite the browning of callus (Table 10).

NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D plus 1 mg/l NAA and subcultured onto MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA showed browning and darkening of callus by week 4 despite being placed on fresh medium of the same composition every 4 weeks. There was still some root development by week 6 despite the browning. There was no further root proliferation except for increase in size by week 10. No shoot buds were observed. The root regeneration frequency was significantly lower at 16% ($p<0.05$) (Table 10). NERICA 11 callus obtained from N6 medium with 2 mg/l 2,4-D plus 1 mg/l NAA and subcultured onto MS medium without hormones showed profuse root development by week 6 (Figure 19). There was root proliferation by week 10 (Figure 20). The callus showed browning by week 6 even after being transferred to fresh medium of the same composition every 4 weeks. Despite the browning of the callus, root development continued. No shoot buds were observed. Root regeneration frequency was 100% (Table 10).

Table 10: Root regeneration of callus from NERICA 11 root explants on MS media

Hormones (mg/l)				No. of replicates	Total no. of calli subcultured	Regeneration frequency %	Mean± S.E %	Remarks
BAP	NAA	KIN	IAA					
-	0.5	2.5	-	3	45	96	96±2.2b	white roots were seen by week 6 that developed into longer roots by week 11
0.5	-	-	0.2	3	45	16	16±2.2a	The callus turned dark brown by week 4. Roots developed by week 6 and grew into long roots in 10 weeks
-	-	-	-	3	45	100	100±0b	Callus was formed within 6-10 weeks. Browning of callus was seen by week 6

Data analysis done using ANOVA n=45. Values with different letters differ significantly according to the Least significant Difference test $p<0.05$ (Appendix B)

Figure 19: Six weeks old NERICA 11 callus showing root development on MS medium without hormones

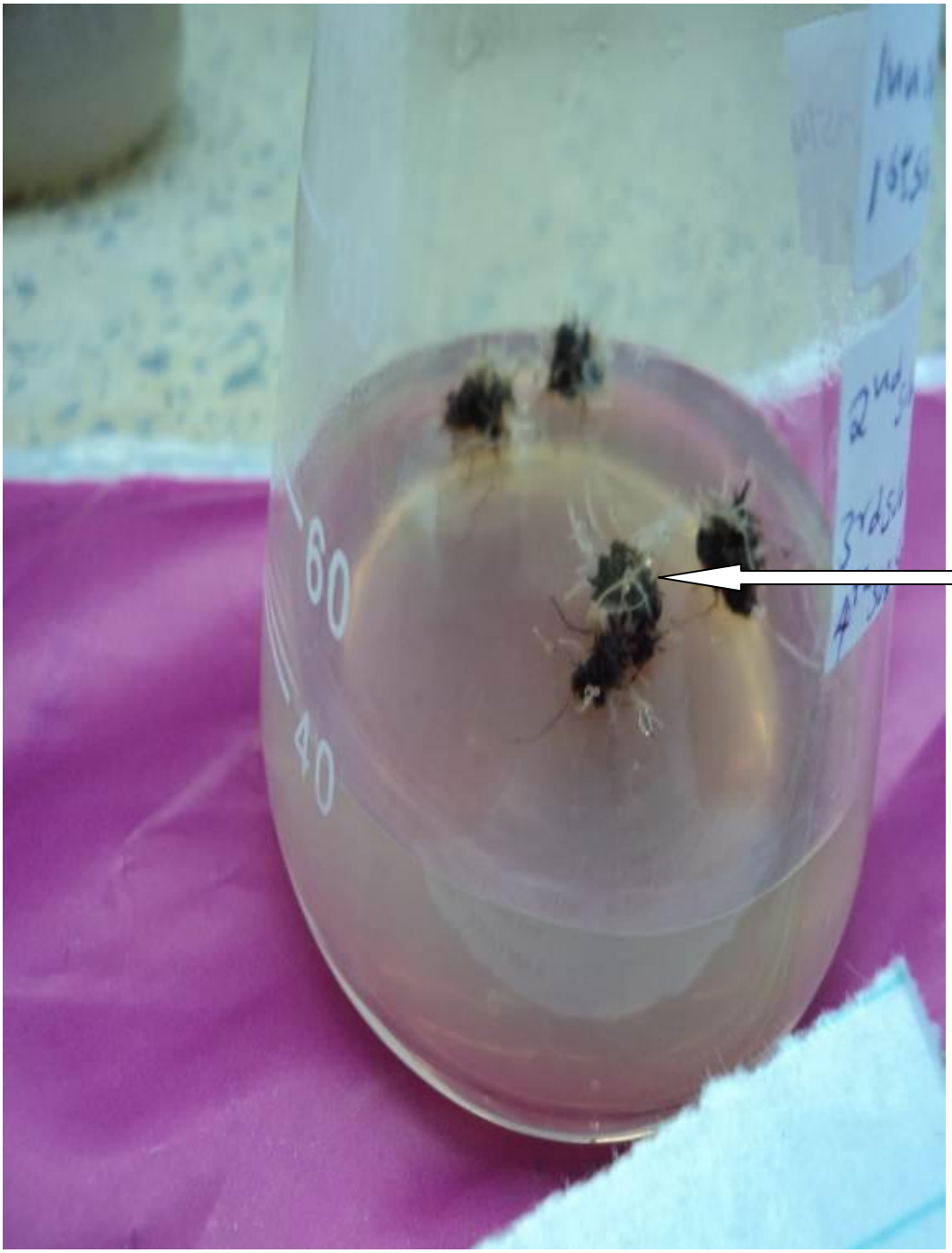

Figure 20: Ten weeks old NERICA 11 callus showing root development on MS medium without hormones

4.6 Hardening and acclimatisation of plantlets

The plantlets, which were of an approximate average height of 11 cm, when transplanted into autoclaved soil in pots covered with a polythene bag (Figure 21) for humidity retention (Figure 22) survived for 12 days. When the polythene bag was removed on the 10th day, the plantlets began to wither. By day 16, all the plants had turned yellow in colour (Figure 23). An attempt to leave the polythene bag for longer than 10 days produced the same result of withering.

Figure 21: NERICA 4 plantlet regenerated on MS medium without hormones transplanted in a pot covered with a polythene bag to retain moisture

Figure 22: Four day old Potted NERICA 11 plantlets regenerated on MS medium without hormones

Figure 23: Fourteen day old Potted NERICA 11 plantlets regenerated on MS media without hormones, exhibiting withering

CHAPTER FIVE

5.0 DISCUSSION

For callus induction from seed embryos, the N6 medium used was supplemented with various hormones and amino acid sources. For optimal callus induction, 2 mg/l 2,4-D inoculated in the dark was found to be the best for callus induction, callus proliferation and for producing callus that was embryogenic for both NERICA 4 and NERICA 11, with a callus induction frequency of 100% within 14 days. In this study, 0.5 mg/l and 1 mg/l 2,4-D produced low amounts of callus with callus induction frequencies ranging between 16-27% and 29-42% for NERICA 4 and NERICA 11 respectively while 4 mg/l 2,4-D produced no callus at all. The role of 2,4-D in cell division is to increase the rate of cell division and this attributes to the increased amount of callus (Revathi and Pillai, 2011). However, 2,4-D at high concentrations inhibits growth as observed in this study for experiments done on callus induction using 4 mg/l 2,4-D, where there was no callus development. High concentrations of 2,4-D can be used as a systemic herbicide after post emergence for the control of weeds on crops. Therefore, as observed in this study, the concentration of 2, 4- D significantly influenced induction of callus from both NERICA 4 and NERICA 11 rice seeds.

N6 medium supplemented with NAA or BAP did not add to the induction frequency of the callus of both NERICA 4 and NERICA 11 cultivars with callus induction frequencies ranging between 9-16% and 11-40% respectively. In the control experiments, seeds inoculated on N6 medium without hormones germinated after 3 days. No callus formation took place even after a culture period of 2-3 weeks. This showed that presence of auxin with or without a cytokinin, was necessary for callus induction in NERICA 4 and 11 rice. Similar results were obtained by Afolabi *et al*., (2008) who induced callus using 2 mg/l 2,4-D in NERICA cv. *Faro 55* while Niroula *et al*., (2005) were able to observe callus induction by using 2.5 mg/l 2,4-D and 0.5 mg/l kinetin in indica rice, *Oryza sativa*. Raghawa and Nabors (1984) reported the necessity of a cytokinin and an auxin for the production of callus using 1 mg/l of 2,4-D and 0.5 mg/l kinetin to induce callus in Asian rice, *O. sativa*. Bano *et al.* (2005) also obtained embryonic callus of *O. sativa* using 1 mg/l 2,4-D but in addition had 0.5 mg/l kinetin and 40 mg/l tryptophan.

In this study, N6 media supplemented with 500 mg/l of L-glutamine was used as the amino acid source together with 2 mg/l 2,4-D for the production of embryogenic callus. However,

Afolabi *et al*., (2008) observed embryonic callus of NERICA cv. *Faro 55* using 2 mg/l 2,4-D and 500 mg/l of proline and 500 mg/l glutamine. Bano *et al.* (2005) also obtained embryonic callus of *O. sativa* using 1 mg/l 2,4-D, and 0.5 mg/l kinetin and 40 mg/l tryptophan. Findings of this study show that embryogenic callus could still be obtained with L-glutamine alone in addition to 2,4-D. The study also shows that L-glutamine is an adequate substitute for proline or tryptophan and any one of these amino acids can be used.

Experiments done in the dark were more effective in callus induction compared to those done in the light. In the N6 media supplemented with 2 mg/l 2,4-D experiments, those done in the dark had a callus induction frequency of 100% for both NERICA 4 and NERICA 11 cultivars while those done in the light had induction frequencies of 98% and 93% for NERICA 4 and NERICA 11 respectively. The calli obtained in both experiments was compact and creamy and they showed to be embryogenic. Similarly, Biswas and Mandal, (2007) observed that callus induction of different genotypes of *O. sativa* were higher when done in the dark than in light with callus induction frequencies ranging between 85-98% and 80-90% respectively.

For leaf explants, there was no callus produced. This result shows that leaf explant cells have low totipotency hence failure to produce callus. There are no reports on successful callus induction in rice from leaf explants. For NERICA 4, root explants cultured on N6 medium supplemented with 2 mg/l 2,4-D plus 1 mg/l NAA had callus induction frequencies of 76% and 70% for experiments done in the dark and light respectively while NERICA 11 showed callus induction frequencies of 56% and 48% for experiments done in the dark and light respectively. This shows that induction of callus from root explants is more effective when done in the dark than in the light. On the other hand, N6 medium with 2 mg/l 2,4-D produced no callus at all. This shows that where as 2 mg/l 2,4-D only was effective for the induction of callus in rice seeds, it did not do the same for root explants. For production of callus in root explants, 1 mg/l NAA had to be added to the 2 mg/l 2,4-D.

Similarities observed on callus induction from root explants in both NERICA 4 and NERICA 11 include; the callus induction frequencies in both cultivars were highest in the medium supplemented with 2 mg/l 2, 4 D and 1 mg/l of NAA with no callus being produced in the media containing 2 mg/l 2, 4 D only. In a comparison between the experiments done in the dark and those done in the light, the ones in the dark had a slightly higher callus induction frequency than those done in the light. Some of the differences seen between the two varieties

during callus induction from root explants were that NERICA 4 had a higher callus induction frequency of 76% as compared to that of NERICA 11 of 56%. The two calli did have a difference in appearance. NERICA 4 calli was compact and yellowish in colour while the NERICA 11 calli was loose and white in colour (Figure 5 and Figure 6).

Only root regeneration was obtained from callus derived from root explants with no shoot development on MS medium for both NERICA varieties. Using callus induced on 2,4-D, MS medium containing 2.5 mg/l kinetin and 0.5 mg/l NAA showed high root regeneration frequencies of 100% and 96% for NERICA 4 and NERICA 11 respectively which could be attributed to presence of kinetin. The MS medium containing 0.5 mg/l BAP and 0.2 mg/l IAA exhibited very low root regeneration frequencies of 22% and 15% for NERICA 4 and NERICA 11 respectively. The optimal medium for root regeneration was MS medium without hormones which realised root regeneration frequencies of 100% for both NERICA varieties. Presently direct rice plantlet formation from root explant callus has not been published.

The results of root regeneration from callus derived from root explants for NERICA 4 and NERICA 11 had similarities that include, the root regeneration frequency in both cultivars was highest at 100% in MS medium without hormones followed by the MS medium containing 2.5 mg/l kinetin and 0.5 mg/l NAA with frequencies of 100% and 96% for NERICA 4 and NERICA 11 respectively, with very low root regeneration frequencies of 22% and 16% for NERICA 4 and NERICA 11 respectively being achieved in the MS medium containing 0.5 mg/l BAP and 0.2 mg/l IAA. Despite these similarities, NERICA 4 was seen to show root regeneration faster than NERICA 11 taking 5weeks and 6weeks respectively. Also generally NERICA 4 had higher root regeneration frequencies than NERICA 11.

The findings of this study showed regeneration of callus derived from seed embryos that were initially cultured on N6 medium with 2 mg/l 2,4-D to plantlets through somatic embryogenesis on MS medium without hormones. The regeneration frequencies were 67% and 77% for NERICA 4 and NERICA 11 respectively. This mode of regeneration by somatic embryogenesis is in agreement with a study done by Niroula *et al.,* (2005) who observed somatic embryogenesis of *Oryza sativa* using half MS medium with 2 mg/l BAP plus 1 mg/l NAA and 0.5 mg/l kinetin. Bano *et al.,* (2005) also observed somatic embryogenesis of *O.*

sativa using MS medium with 0.5 mg/l BAP plus 0.2 mg/l IAA. Afolabi *et al.*, (2008) also reported plant regeneration of NERICA cv. *Faro 55* by subculturing callus on pre-regeneration medium consisting of MS medium with 2 mg/l BAP and 1 mg/l NAA followed by a subculture on MS medium with 3 mg/l BAP and 0.5 mg/l NAA.

This study is the first study to show somatic embryogenesis of NERICA 4 and NERICA 11 using callus cultured on N6 medium with 2 mg/l 2,4-D to plantlets on MS medium without hormones. NERICA 4 regenerated within 5-6 weeks whilst NERICA 11 regenerated a bit earlier i.e. within 4-6 weeks. When fresh callus that was 4 weeks old was used for subculturing, plantlets were regenerated. Use of older callus of up to 3 months did not produce any plantlets. This is attributed to totipotency which reduces in old cells but is very active in young cells due to reduction of developmental plasticity Raghawa and Nabors (1984).

MS medium supplemented with 2 mg/l of BAP and 1 mg/l NAA as the pre-regeneration medium led to shoot initiation with no root formation. Transfer of callus to the regeneration medium consisting of MS medium supplemented with 3 mg/l BAP and 0.5 mg/l NAA after 4weeks led to no further growth of the shoot buds and the buds browned but there was root initiation. The results were similar for both NERICA 4 and NERICA 11 though the regeneration period was different with growth being seen in week 7 and week 8 respectively. Plant regeneration was not observed which negates to the result observed by Afolabi *et al*., (2008) who achieved regeneration of NERICA cv. *Faro 55* through somatic embryogenesis by using this protocol. The lack of similar results could be attributed to genetic variation between the NERICA varieties.

MS medium supplemented with 2.5 mg/l kinetin and 0.5 mg/l NAA produced root regeneration frequency of 100% for both NERICA 4 and NERICA 11. The hormones induced formation of roots on the callus but no shoot buds were formed. Transfer of callus to MS medium without hormones after 4 weeks led to shoot buds developing though the shoots did not elongate. These results are different to those of the study done by Ishizaki and Kumashiro (2007) where plantlets were attained by using MS medium without hormones after the callus had been subcultured onto MS media containing 2.5 mg/l kinetin and 0.5 mg/l NAA.

Shoot and root regeneration was observed when callus initiated on N6 medium with 2 mg/l 2,4-D was cultured on MS medium supplemented with 0.5 mg/l BAP and 0.2 mg/l IAA but did not elongate even after transfer to MS basal medium. The protocol was derived from the study done by Bano *et al.*, (2005) where *O. sativa* plantlets were obtained using the same medium. This variation could possibly be explained by genetic variations between *O. sativa* and NERICA rice species.

The regenerated plants only survived for 12 days when transferred to pots covered with polythene. The plants were watered twice a day with sterile distilled water. The failure to survive could be attributed to lack of sufficient moisture trapped in the polythene bag causing drying of the plants or due to overwatering of the plants. Another possible reason could be that the acclimatisation design used by placing the polythene bag too close to the plantlets is not suitable for NERICA rice. This is in contrast to the study by Singh *et al.*, (2010) where this acclimatisation design was used successfully for acclimatisation of aromatic indica rice varieties.

5.1 CONCLUSIONS

1) In this study, there was successful development of a protocol for regeneration of NERICA 4 and NERICA 11 plantlets from callus derived from mature seed caryopses through somatic embryogenesis.
2) This study showed seed embryos to be the best explants for somatic embryogenesis.
3) The study also showed N6 medium supplemented with 2 mg/l 2,4-D to be the most effective media for production of embryogenic callus.
4) The study showed MS media without hormones to be the best media for plantlet regeneration by somatic embryogenesis.

5.2 RECOMMENDATIONS

1) Further studies should involve successful acclimatisation and transfer of plants regenerated using this protocol to the greenhouse.
2) Further studies can be done to raise the plants to maturity and evaluate their suitability in terms of fertility and genetic variation.

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APPENDICES

Appendix A: Nutrient Media composition

Composition of MS, B5 and N6 media			
Ingredients	Amounts (mg/liter)		
	MS	N6	B5
Macronutrients			
KNO_3	1900	2830	2100
NH_4NO_3	1650	-	-
$CaCl_2.2H_2O$	440	166	150
$MgSO_4.7H_2O$	370	185	250
KH_2PO_4	170	400	-
$(NH_4)^2.SO_4$	-	463	134
$NaH_2PO_4.H_2O$	-	-	150
Micronutrients			
H_3BO_3	6.2	1.6	3
$MnSO_4.H_2O$	15.6	3.3	10
$ZnSO_4.7H_2O$	8.6	1.5	2
$NaMoO_4.2H_2O$	0.25	-	0.25
$CuSO_4.5H_2O$	0.025	-	0.025
$CoCl_2.6H_2O$	0.025	-	0.025
Kl	0.83	0.8	0.75
Iron Source			
$FeSO_4.7H_2O$	27.8	27.8	-
Na_2EDTA	37.3	37.3	-
EDTA Na ferric salt	-	-	43
Vitamins			
Thiamine-HCl	0.5	1	10
Pyridoxine-HCl	0.5	0.5	1
Nicotinic acid	0.05	0.5	1
Myo-Inositol	100	-	100
Glycine	-	2	-
Sucrose			
Sucrose	30	30	20

Agar			
Agar	8g	8g	8g
pH			
pH	5.8	5.8	5.5

From: Vasil I.K. 1984.

Appendix B: Analysis of Variance (ANOVA) Tables

Table 1: ANOVA for percentage callus induction of NERICA 4 seeds in the light

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21087.43	6	3514.57	87.42	<0.001
Within Groups	562.86	14	40.20		
Total	21650.29	20			

Table 2: ANOVA for percentage callus induction of NERICA 4 seeds in darkness

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21384.35	6	3564.06	80.20	<0.001
Within Groups	622.18	14	44.44		
Total	22006.53	20			

Table 3: ANOVA for percentage callus induction of NERICA 11 seeds in the light

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18958.74	6	3159.79	78.59	<0.001
Within Groups	562.86	14	40.20		
Total	19521.60	20			

Table 4: ANOVA for percentage callus induction of NERICA 11 seeds in darkness

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21413.91	6	3568.98	84.32	<0.001
Within Groups	592.56	14	42.33		
Total	22006.47	20			

Table 5: ANOVA for percentage callus induction of NERICA root explants in light

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11275.09	3	3758.36	132.82	<0.001
Within Groups	226.37	8	28.30		
Total	11501.46	11			

Table 6: ANOVA for percentage callus induction of NERICA root explants in dark

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13587.85	3	4529.28	176.20	<0.001
Within Groups	205.65	8	25.71		
Total	13793.50	11			

Table 7: ANOVA for percentage regeneration of NERICA 4 rice seeds

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10666.667	4	2666.667	400.00	<0.001
Within Groups	66.667	10	6.667		
Total	10733.333	14			

Table 8: ANOVA for percentage regeneration of NERICA 11 rice seeds

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14106.67	4	3526.667	75.57	<0.001
Within Groups	466.67	10	46.667		
Total	14573.33	14			

Table 9: ANOVA for percentage regeneration of NERICA 4 root explants

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12098.420	2	6049.210	1223.74	<0.001
Within Groups	29.659	6	4.943		
Total	12128.079	8			

Table 10: ANOVA for percentage regeneration of NERICA 11 root explants

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13551.012	2	6775.506	685.33	<0.001
Within Groups	59.319	6	9.886		
Total	13610.331	8			