

**POTENTIAL USE OF DORMANT BUDS AS EXPLANTS AND CALLUS  
CULTURE FOR MICROPROPAGATION OF GINGER (*Zingiber officinale*  
Roscoe) .**

by

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**A Dissertation Submitted to the University of Zambia in Partial Fulfilment of the  
Requirement of the Degree of Master of Science in Crop Science (Agronomy).**

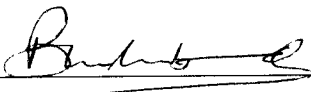
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## DECLARATION

I, **BRANCO MSUWILIWA LEVI MWENEBANDA**, do hereby declare that this dissertation represents my own work and that, to the best of my knowledge, it has not been previously submitted to this or any other university for the award of a degree.

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### APPROVAL

This dissertation of **BRANCO MSUWILIWA LEVI MWENEBANDA** is approved as fulfilling part of the requirements for the award of the degree of Master in Crop Science (Agronomy) by the University of Zambia.

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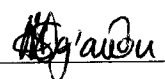
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## ABSTRACT

The study was carried out in the Tissue Culture laboratory of the Crop Science Department at the School of Agricultural Sciences of the University of Zambia from September, 2001 to May, 2002. *In vitro* regeneration and growth capability of two ginger varieties (China and MES 4) from Malawi was investigated on three different MS media: (1) 1.5 ppm BAP + 1.0 ppm NAA, (2) 3.0 ppm BAP + 1.5 ppm NAA and (3) 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub> using three different types of explants; (1) sprouted buds, (2) dormant modified buds which were dipped in 10.0ppm GA<sub>3</sub> for a day and (3) dormant unmodified buds. Two experiments were conducted; (1) direct explant culture in which the cultures were incubated for sixteen hours of light and eight hours of darkness and (2) callus culture in which the cultures were incubated in complete darkness for twenty-four hours. Varieties had no significant influence on most of the parameters evaluated. The type of media significantly influenced days to shooting and rooting and shoot height in light incubated experiment. The addition of GA<sub>3</sub> to media delayed shooting and rooting in light incubated experiment but produced taller plantlets compared to the other media in both experiments. In the light incubated experiment sprouted buds started shooting and rooting earlier than the others. These sprouted buds had lower contamination rate; had taller shoots and higher regeneration rate than the other explants. In the dark incubated experiment, explants did not have influence on contamination rate, roots per shoot and external environment survival rate but they significantly increased regeneration rate, shoots per explants and shoot height and reduced days to shooting and rooting. In both the experiments and for all the measured parameters there were no difference between dormant modified and dormant unmodified buds. The dark incubated explants did not yield callus as was expected but produced shoots just as those incubated in light. There were statistically significant differences between the dark and light incubated experiment for days to shooting and rooting and regeneration rate. Explants incubated in light started shooting and rooting earlier and had higher regeneration rate than those in dark. Dormant ginger buds have shown capacity to regenerate *in vitro* just like sprouted buds.

## **DEDICATION**

To my wife Ellidah, sons Grief and Lameck and daughters Faith and Jennifer for their patience during my two-year study period.

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## CHAPTER ONE

### 1.0 INTRODUCTION:

Ginger is the rhizome of *Zingiber officinale* Roscoe. It is a herbaceous perennial plant belonging to family Zingiberaceae and used as spice. The plant is a diploid with twenty-two ( $2n=22$ ) chromosomes. It is propagated by rhizome. The rhizome puts forth erect, leafy stems, 30-90 cm in height. The bases of the leaves sheathe the stem. Leaves are light - green, 15-20cm long, narrow, lanceolate and with a prominent midrib. Flowers are small, yellowish, speckled and each with a purple speckled lip and borne on a spike. The plants rarely produce seeds. The crop is ready for harvest after 5 - 9 months depending on variety and climate.

Ginger is believed to be native of South -Eastern Asia. It is grown from almost sea level up to an altitude of 1,500 metres (Anon. 2000). The spice can be grown over more diverse conditions than most other spices.

Ginger thrives on a variety of soils, provided drainage is adequate. However, a deep, well-drained friable loam soil rich in humus is ideal. Rainfall of 1500 mm to 1800 mm per annum is said to be ideal. The optimum temperature for ginger production ranges from 24°C to 32°C (Purseglove *et al.* 1987). The crop is cultivated in almost all the tropical and subtropical parts of India, Jamaica, Nigeria, Sierra Leone, Brazil, China, Japan, Indonesia and most parts of Southern Africa. India is the largest producer with the estimated annual production of 33,780 tonnes (Anon. 2000).

Ginger is an important commercial spice grown for its aromatic rhizomes which are used both as a spice and a medicine. It is used for culinary purposes in gingerbread, biscuits, cakes,

pudding, soups and pickles, production of ginger beer, ginger ale, and ginger wine. The ginger constituent, Zingerbain, is said to have antibacterial and anti-inflammatory effects in human (Conley, 1997). It is also used in medicine as a carminative and aromatic stimulant to the gastro-intestinal tract (Foster, 2000). In Malawi ginger is mainly used as an ingredient of curry powders, ginger bread, ginger biscuits, cake pudding, ginger ale, ginger beer and it is believed to be used as medicine by Malawians of Asian origin (Chizala *et al.* 1993 and Anon. 1999).

Ginger plants rarely produce seed and as such all planting materials are obtained from conventional propagation through rhizome pieces. However, conventional propagation has many limitations (Smith and Hamill, 1996, John *et al.*, 1997 and Yama *et al.* 2000). Diseases such as soft rot (*Pythium* spp.), bacterial wilt (*Pseudomonas solanacearum*), rhizome rot (*Fusarium oxysporum* Schlecht) and pests like Root-knot nematodes are transmitted through infected seed-rhizome (Bhagyalakshmi *et al.* 1994 and John *et al.* 1997). Smith *et al.* (1996) reported a 57 % yield loss when nematode infected 'seed' was used. Yama *et al.* (2000) stated that the limited supply of high quality rhizomes is compounded by fairly high levels of contamination during the initial explanting process with the major disease being bacterial wilt. The same workers also stated that ginger enters into dormancy in early winter and remain dormant until early spring, a situation that limits seed rhizome production to certain period of the year. They further stated that dormancy in ginger is induced by short days and high concentrations of abscisic acid (ABA) while breaking the dormancy required long days with high concentration of gibberellic acid (GA<sub>3</sub>).

Ginger is a vegetatively propagated crop and as such it is difficult to obtain sufficient seed-rhizome for a large area. The problem is made even worse by the fact that the same rhizomes are the products that are sold and consumed. John *et al.* (1997) reported that the multiplication rate under conventional methods is very low. It is in the range of 10-15 lateral buds from one

bud in 8-10 months. Yama *et al.* (2000) reported that large amount of seed rhizome was used with low multiplication rate.

The seedrate in ginger has been reported at 20 - 30% of the annual yield. A wide range of seedrates have been reported. John *et al.* (1997) reported seedrate of 900 - 1400kg/ha and 1500 - 1800kg/ha to 2250 - 2750 kg/ha. Purseglove *et al.* (1987) reported that rhizomes are cut into small pieces of 2.5 to 5cm long and weighing 28 -56 grammes, each having at least one good bud or growing point. In Malawi, seedrates of 1200 - 1800kg/ha have been reported. This represents 12 to 18% of current annual yield of about 10,000kg/ha fresh weight. Most farmers are not willing to keep this much ginger seed because they get a lot of money from the sale of the crop. However, the farmers pay much more money to buy the ginger planting materials.

The production of the crop in winter, under irrigation, is further complicated by the fact that ginger goes into dormancy soon after harvest in July/August until early summer in November/December. The lack of availability of planting materials for winter crop because of dormancy has made ginger production in winter impossible.

Rapid clonal propagation of ginger by direct organogenesis and callus culture in tissue culture provides a potential means for improving the supply of planting materials. *In vitro* multiplication of ginger has been reported to be successful by many authors working with the crop. However, the success and rate of *in vitro* multiplication has been reported to be highly dependent on varieties and type of media used. So far reports indicates that only sprouted buds have been used as source of explants and as source of calli. Nadgauda *et al.* (1980) reported that explants can only be collected in sprouting seasons which starts in early spring. Yama *et al.* (2000) reported that ginger micropropagation in tissue culture can be applied throughout the year. It is, therefore, postulated that even unsprouted dormant buds can be

used as explants. One of the method is the use of callus which could be induced from these dormant buds and used to produce ginger plantlets by organogenesis. Literature so far indicates that there has been no research on the use of unsprouted dormant buds as source of explants and as source of callus for ginger micropropagation.

Successful regeneration of unsprouted dormant buds through direct explant culture or callus culture to plantlets would increase ginger seed production and make it available throughout the year. This would increase area under ginger cultivation and make ginger amenable to irrigated production during winter.

The overall objective of the study was to explore ways of increasing supply of ginger planting materials through the use of *in vitro* multiplication techniques and thereby make it available to farmers throughout the year. The specific objectives were to:

1. Determine the suitability of dormant buds as source of explants in ginger micropropagation.
2. Compare the effectiveness of direct explants culture and callus culture in ginger micropropagation.
3. Develop micropropagation protocols for ginger varieties grown in Malawi.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Importance and production of ginger

The major producers and exporters of ginger in the world are India, China, Taiwan, Nigeria, Japan, Sierra Leone and Mauritius (Anon. 2000). In these countries the crop is an important source of foreign exchange (John *et al.*, 1997). Ezeh and Asumugha (1990) reported that India is the largest producer and exporter of ginger, followed by Nigeria, Sierra Leone, Jamaica, Singapore and Fiji in that order. Other countries that produce and export ginger include Bangladesh, China, Taiwan, Malaysia, Australia and the East Caribbean States. Ezeh and Asumugha (1990) reported that out of the 50,000 tonnes of ginger (dry weight) produced in the world, Nigeria produced 15,000 tonnes and was only surpassed by India. They also reported that Nigeria started producing ginger in 1927 and that in 1977 the country exported 3,000 tonnes which yielded US \$11.8 million. The same workers reported that ginger accounted for more than 3 % of total soft drink market in Nigeria. They stated that most of the production is done by peasant farmers with land holdings of 0.05 to 0.5 hectares. The total area under ginger in Nigeria was estimated at 10,000 hectares with average yield of 18,800 tonnes per hectare. Smith and Hamil (1996) reported that in South-Eastern Queensland, Australia, ginger is grown in an area of about 150 hectares and produced 5600 tonnes annually estimated at \$ A 13.5 million. It was estimated that ginger production in India was 33,780 metric tonnes with a third of this exported to Middle-East (Anon. 2000).

In Malawi, ginger is rated the third most important spice crop and it is superseded in importance only by Birds' eye chillies and paprika (Mbaso *et al.* 1998). However, ginger fetches more money per unit weight than both chillies and paprika. At the local market, in

1998, ginger was sold at about MK100.00/kg (US\$2.50/kg) fresh weight against MK40/kg (US\$1.0/kg) dry weight for both chillies and paprika (Mbaso *et al.* 1998).

Spices are potentially an important export commodity in Malawi. In 1986 and 1988 spice export worth MK898,000 (US\$ 449,000) and MK1,828,000 (US\$914,000) respectively, were realized in Malawi. Ginger, as third most popular spice contributed a great deal to this amount (Chizala *et al.* 1993).

Ginger is grown in almost all parts of Malawi by smallholder farmers. Kandoole (1990) reported that in the major spice producing areas of southern Malawi ginger was second most prevalent spice with 11.4% of spice producers. The rest of the farmers were engaged in bird's eye chillies production. It was reported that about ten percent of the farmers are engaged in ginger production in Lilongwe Agriculture Development Division (ADD) alone (Anon. 1999). It was speculated that there was a considerable number of farmers growing ginger in the remaining seven ADDs in the country. The annual production was estimated at about 180 metric tonnes (fresh weight) from a land area of about 15 hectares (Anon. 1999). The average yield has been estimated at 12.0 tonnes per hectare fresh weight. Yields in excess of 12.0 tonnes per hectare have been reported at research stations (Chizala *et al.* 1993). Almost all the ginger produced in the country is sold at local markets. Individual buyers buy from farmers' fields. It is believed that the traders process and export the ginger. The local demand for ginger in Malawi is so high that local production cannot cope with this demand (Chongwe, pers. comm.). The demand has further been increased by the decision of the major soft drink manufacturing company, Southern Bottlers, to buy ginger locally (Banda, pers. Comm.). However, increase in production is limited by insufficient supply of planting material of the crop. The problem of lack of adequate supply of ginger planting material has been recognized in the Malawi Agriculture and Natural Resources Research Masterplan of 1999 which states that seed production and good quality seed is a high priority for ginger research in Malawi

(Anon. 1999). The Lilongwe East Smallholder Spice Association (LESSA) also puts ginger seed multiplication as its first priority (Anon. 1999).

Several varieties of ginger have been evaluated at research stations in Malawi for yield and quality. Varieties such as 'Bvumbwe'; Mkondezi Experiment Station (MES) '1', '2', '3', '4', '5', and '6'); 'Nigeria'; 'China'; 'USA'; 'Local' and many others have been recommended for production to the farming community. However, the high yielding varieties are Bvumbwe; MES 4, 5 and 6; China; Nigeria and USA (Chizala *et al.* 1993 and Mbaso *et al.* 1998 ).

## **2.2 Micropropagation of plants**

Micropropagation has been defined as the vegetative propagation of plants *in vitro* (Pierik, 1987). Taji (1996) stated that micropropagation involved production of plants from very small parts such as buds, nodes, leaves, leaf segments, root segments etc, grown aseptically (free from any micro-organisms) in a container where the environment and nutrition can be controlled. The resultant plants are usually genetically identical to parent plants. Micropropagation is one of the applications of plant tissue culture. Plant tissue culture has been defined as the science and art of growing isolated plant cells, tissue, organisms or whole plant on semi-solid or in liquid synthetic nutrient media under aseptic and controlled environmental conditions (John *et al.* 1997). A small plant part (bud, leaf segment, root segment, node e.t.c.) that forms the starting material for tissue culture is known as explant. Micropropagation can be through direct explant culture whereby development of shoots and roots (organogenesis) are initiated direct from the cultured explant and through callus culture whereby organogenesis and/or somatic embryo (embryogenesis) result from the callus which forms on the cut surfaces of explants. Pierik (1987) defined callus as the actively dividing non-organised tissues of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture. Callus culture is, therefore, the production of plants from callus produced in tissue culture. Pierik (1987) stated that any organ (roots, stems, buds, leaves, flowers e.t.c) can be used as starting material for callus induction.

Gomborg and Phillips (1999) outlines the following steps of micropropagation through direct explant culture:

- Initiation of sterile culture of the explant.
- Multiplication of shoots or other propagules from the explant.
- Development of roots on shoots to produce plantlets.
- Production of self-sufficient plants (hardening off or acclimation to growing in soil mixes).

The following is the procedure for callus culture as outlined by Dixon (1987) and Pierik (1987):

- Initiation of sterile culture of an explant on semi-solid medium with high concentration of growth regulators to induce callus from explant wounds.
- Incubation of the explant culture at 25°C in the dark for 3-8 weeks.
- Subculture the callus in another semi-solid medium with low concentration of growth regulators for organogenesis (shoots and roots formation).
- Production of self-sufficient plants (hardening off or acclimation to growing in soil mixes).

Micropropagation has several advantages in agriculture. Benson (1998) outlines the following advantages of micropropagation:

- It allows for more rapid division of desirable plants. One meristem can yield thousands of clones in a month.
- It eliminates a number of diseases (fungal, bacterial, and parasites) and reduces the incidence of several others (viral diseases). It allows for the transfer of plant germplasm with less risk of contamination.
- Saves space- It allows one to ship multiple shoots in a small container, each of which will rapidly grow to a new plant.

- Storage- One can maintain more varieties on a shelf than one can on an acre of land. Through *in vitro* storage large numbers of germplasm can be stored in very small vials. *In vitro* storage is safer than field storage.
- It is a fun and an exciting challenge to the scientists.

Vuylsteke and De Langhe (1984) reported that the feasibility of *in vitro* propagation depends on: (1) simplicity of culturing a plant part of the species (2) reproducibility or regeneration capability of the explants (3) potential for greatly increasing the multiplication rate. Therefore, *in vitro* propagation is not automatic and the ease of propagation may depend on plant species or variety.

Micropropagation or *in vitro* growth and development of a plant is determined by several factors (Pierik, 1987) such as:

- Genetic make up of the plant: different plant species and or varieties respond differently to *in vitro* propagation.
- Nutrients: water, macro and micro-elements, sugars. These will influence growth and development of plants in vitro.
- Physical growth factors such as light, temperature, pH, O<sub>2</sub> and CO<sub>2</sub> concentration.
- Some organic substances such as plant growth regulators (hormones) and vitamins.

Gamborg and Phillips (1999) stated that most plant species are capable of regeneration by organogenesis (development of organs such as shoots, roots etc). Pierik (1987) noted differences in cell division and regenerative capacity between plants within a single species. If a species regenerates organs easily *in vivo*, then almost the same can be expected *in vitro* although not always. Tisserate (1987) reported that any plant part can be successfully cultured *in vitro* and regenerate plantlets provided the explant is obtained at the optimum physiological

stage of development. Gamborg and Phillips (1999) stated that axillary buds are treated with hormones to break dormancy and produce shoot branches. Vuylsteke and de Langhe (1984) in the trial "feasibility of *in vitro* propagation of bananas and plantains" found no regeneration differences among dormant 'eyes', buds, peers, suckers and parent pseudostem. Pierik (1987) stated that use of callus in tissue culture enables mature cells in an explant from adult plant to revert to juvenile state and makes the cells easy to regenerate. They also reported that the starting material for callus induction can be either juvenile or adult. However, they reported, juvenile explants produce callus and organs (shoots and roots) much better than adult explants.

Gamborg and Phillips (1999) noted that a defined nutrient medium consists of inorganic salts, carbon source, vitamins and growth regulators. However, some modifications are made for specific purposes. They said that the most common media compositions are: Murashige and Skoog (1962), MS, and Linsmaier and Skoog (1965), LS. Pierik (1987) stated that agar (at 0.6 to 0.8%) is required. Carbon source (sucrose) of 1 to 5% is needed for most plant species. Temperature range of 20 - 28°C is ideal for most species. The optimum temperatures for *in vitro* growth and development is generally 3 - 4°C higher than *in vivo* (Pierik, 1987). Tisserate (1987) stated that intensity of light and photoperiod play an important role in morphogenesis of the culture. Pierik (1987) stated that high light intensity is necessary for promotion of photosynthetic leaf development. Plant growth regulators, auxins and cytokinins are the mostly used and these two are normally combined. Gibberellic acid (GA<sub>3</sub>) is used to reduce/break dormancy. Auxins which are important for root initiation are added in the concentrations of 0.01 - 10mg/l. Higher concentrations reduce rooting and enhance callusing. The commonest auxins used are 1-naphthaleneacetic acid (NAA) and indolebutylic acid (IBA). Others are 2,4-dichlorophenoxyacetic acid (2,4-D) and 3-indole-acetic acid (IAA). Cytokinins are important for shoot initiation and reduce apical dormancy. They are applied in the concentrations of 1- 10mg/l. These include kinetin and 6-benzylaminopurine (BAP). Others are zeatin and thidiazuron (TDZ). Lemos and Blake (1996 ) working with *Annona muricata* found that increasing BAP stimulated shoots production. These workers reported 5

and 4 shoots per explant in juvenile and mature explants respectively in six weeks. They also found 2.4 and 1.5 roots per shoot in juvenile and mature explant respectively. Gonzalez-Benito *et al.* (1996) working with Snapdragon reported that auxins did not improve rooting and that rooting took place in hormone-free media.

### **2.3 Callus induction**

Production of callus has been widely reported to be associated with high concentrations of plant growth regulators in general and auxins in particular. Larger explants have also been reported to favour callus production, possibly due to the larger wound area. Dixon (1987) reported that explants for callus initiation are aseptically transferred in semi-solid medium and incubated in dark at 25°C for 3 – 8 weeks and subcultured at 4 week interval. Gamborg and Phillips (1999) stated that different explants respond differently to callus production. They noted that explants from aseptic cultures responded faster and produced more callus. These workers also observed that organogenesis took longer in callus culture than in direct explants. Veronique and Korban (1996) reported that increasing concentrations of auxin, 2,4-D, produced more callus than cytokinins, BAP, TDZ and Zeatin. Concentration of BAP higher than 5.0 µM reduced callus initiation. They also observed that increasing glucose concentration in media increased callus production.

### **2.4 Somaclonal variations in micropropagated plants**

Skirvin *et al.* (1994) defined somaclonal variations as phenotypic variations observed among plants regenerated after passing through tissue or cell culture. These workers came up with names such as calliclones (variations from callus cultures) and protoclonal (variations from protoplasts). These are represented by somaclonal variations. Skirvin *et al.* (1994) stated that somaclonal variation can involve either single or multiple genes and can be due to alterations in DNA bases, genes, chromosomes, or entire set of chromosomes. These workers also reported that somaclonal variation occurs in all crops and that there were two types of

variations; genetic (heritable) which is stable through sexual cycle or repeated asexual propagation and epigenetic (non-heritable) which is unstable even when asexually propagated. Gamborg and Phillips (1999) reported that variability in tissue culture can either be permanent (genetic or heritable) or temporary (epigenetic or non-heritable). These workers suggested that the causes of this variability could be the result of carryover effects of the plant growth regulator treatments.

Pierik (1987) and Skirvin *et al.* (1994) outlined the following causes of somaclonal variations in tissue culture:

- Method of vegetative propagation; there are more variations when non-organised cells, single cells and calli are used than when direct explants are used .
- Different cultivars show different levels of variations.
- Plant growth regulators; 2, 4-D and NAA and strong synthetic cytokinins including BAP increase variations. Kinetin has less influence. High concentrations of the growth regulators increases variations.
- Type of tissue and starting material; preformed shoots such as buds, shoot-tips, axillary buds have less variations than leaves, stems, roots and protoplasts.
- Ploidy levels; polyploids are more variable than diploids.
- Number of subculturing; the more the number of subculturing the more the variabilities . This may be due to more exposure to growth regulators.
- Chimera plants; when these are used in micropropagation more variations occur.

Pierik (1987) reported that callus has both organized and non-organised cells making the callus heterogenous, the factor that contributed to genetic variations in callus cultured plants. Arene *et al.* (1993) stated that regenerating plants from callus might be attractive to plant

breeders as the variation generated by this system is known to be higher than that generated by other systems such as chemically induced mutagenesis. Skirvin *et al.* (1994) reported that callus is always associated with somaclonal variations. The same workers reported that the first tissue-culture derived cultivar was a calliclone of *Pelargonium* known as “Velvet Rose”. It appears here that plant breeders would like to regenerate plants from callus cultures so that they get variations which may lead to new superior cultivars while commercial tissue culture laboratories which produce plants for sale would avoid callus induction and use direct explant cultures so that they get true-to-type plants from the original cultivars.

High concentrations of plant growth regulators in culture media are associated with high rate of variation because these regulators induce callus which in turn influences variations. Arene *et al.* (1993) reported that auxins have more influence on callus induction than cytokinins. This also led to the conclusion that auxins influence variations more than cytokinins. Hoepfner *et al.* (1996) found that low concentrations of IBA, BAP and TDZ in MS media resulted in high regeneration and shooting with low off-type production. They also found that callus and adventitious explants such as leaves lead to high variations while direct explants culture and meristematic tissues reduce variations.

Long exposure to *in vitro* conditions have also been reported to increase somaclonal variations. Shepherd *et al.* (1996) looking at mitotic instability in bananas found that prolonged culture beyond five months and five subculture increased frequency of variations. Skirvin *et al.* (1994) reported that somaclonal variations could be detected through visual observations of morphological features, genetic markers (both biochemical and molecular markers, proteins and isoenzymes and DNA analysis respectively) and cytological (chromosome analysis). They also reported that based on literature and experience somaclonal variations is normally 1 to 3 percent.

## 2.5 Micropropagation of ginger.

Rapid clonal propagation of ginger in tissue culture has been reported by several authors working with the spice. Most authors have used different media for different varieties and the common explants and/or source of callus for organogenesis and embryogenesis has been sprouted buds

Hosoki and Sagawa (1977) got numerous adventitious shoots and roots using MS major elements, Ringe-Nitsch minor elements and vitamins, 2% sucrose, 1.0 ppm 6 - benzylaminopurine (BA) + 1.0 ppm 1- naphthaleneacetic acid (NAA). The cultures were incubated at  $27 \pm 2^{\circ}\text{C}$  under continuous light of about 2.1 klx (2100 lux). Nadgauda *et al.* (1980) using "Mahim" variety found that the medium reported by Hosoki and Sagawa (1977) was not suitable. For Mahim and local varieties, Nadgauda *et al.* (1980) found MS supplemented with  $0.88 \mu\text{M}$  BAP +  $0.46 \mu\text{M}$  Kinetin + 0.1mg/l cal. Pan. + 2% sucrose suitable. The cultures were incubated at  $25 \pm 2^{\circ}\text{C}$  and 16 hour light, 8 hour dark period. They stated that there should be two media for ginger propagation: Media I (semi-solid gelled with 0.5% agar) for initial culturing and shoot and root induction and Media II (liquid medium) for root induction. The same workers noted that media II may not be necessary if roots were formed on media I. Nadgauda *et al.* (1980) stated that unrooted shoots should be treated with  $28.58 \mu\text{M}$  IAA +  $24.60 \mu\text{M}$  IBA +  $26.85 \mu\text{M}$  NAA for 5 minutes and transferred directly to a sand: soil (1:1) mixtures in earthen pots. Relative humidity of  $85 \% \pm 5\%$  is required for acclimation. Nadgauda *et al.* (1980) also stated that explants should be collected only in the sprouting season. This means that unsprouted dormant buds cannot be used. However, Yama *et al.* (2000) reported that micropropagation of ginger could be applied throughout the year.

With MS media supplemented with  $11.0 \mu\text{M}$  BAP and  $0.6 \mu\text{M}$  NAA Ikeda and Tanade (1989) obtained 5 shoots per explant and 15.3 roots per shoot with pseudostem explants in seven

weeks. With BAP alone at 11.0 $\mu$ M BAP they obtained 10 shoots per explant and 16.3 roots per shoot with crown section explants in seven weeks. These workers also reported that high concentrations of NAA reduced both shoots and roots. Inden *et al.* (1988) using Japanese varieties of ginger with MS medium supplemented with 22 $\mu$ M BAP and 2.69  $\mu$ M NAA obtained 4 rooted shoots within 9 weeks. Balachandran *et al.*, (1990) obtained complete plantlets in 4 weeks when they used MS medium supplemented with 13.32 $\mu$ M BAP and gelled with 0.8% agar. Bhagyalakshmi *et al.* (1994) obtained plantlets using MS basal medium + 6% sucrose + 20% coconut milk + 100mg/l ascorbic acid +400g/l glutamine + 0.4 mg/l IBA + 0.8% agar. However, rapid shoot multiplication was obtained with 3% sucrose and 4-5mg/l BAP. The same workers also reported that micropropagated ginger matured two months later than the conventionally propagated ginger. Yield and quality was not affected by micropropagation. However, micropropagation of ginger had more advantages than conventional propagation. Sharma and Singh (1995) reported a simple and cost effective method of propagating ginger. They used potato extracts (200g potato/l) instead of MS. They omitted agar, used ordinary grade sugar instead of analytical grade. Tap water was used instead of distilled water. They managed to get plantlets. Mohammed and Quraishi (1999) reported maximum shoot multiplication of well developed roots and good plant height with MS salts and vitamins as basal medium, supplemented with 2mg/l BAP and 2mg/l NAA. The plants were obtained in 4 weeks.

Production of plants through callus culture has been reported in ginger. Nadgauda *et al.* (1980) obtained plantlets from callus culture of ginger. They macerated explants from shoot buds and cultured on medium of MS with 0.2 ppm BAP and 0.1 ppm kinetin plus 10 % coconut water ( CW) in dark to obtain callus. The callus was then subcultured in presence of light for organogenesis. Kackar *et al.* (1993) reported somatic embryogenesis in ginger. Callus from young leaf segments taken from *in vitro* shoot culture was subcultured in liquid medium with 2.0 ppm BAP to obtain somatic embryos.

Ginger micropropagation through callus culture results in genetic instability of the plantlets which leads to somaclonal variants. Rout *et al.* (1997) observed no variations in plantlets produced from bud explants. Shenoy and Vasil (1992) reported that there is high risk of genetic instability and somaclonal variations when callus is used in ginger micropropagation. Thus callus induction should be avoided when producing ginger plants for seed in tissue culture. However, callus could be very attractive to plant breeders if it leads to variations that plant breeders need for development of new cultivars, more especially in ginger because conventional breeding is impossible as the crop rarely sets seed.

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Equipment and materials

The research was carried out in the tissue culture laboratory of the Crop Science Department in the School of Agricultural Sciences of the University of Zambia from August, 2001 to May, 2002. The laboratory experiments were conducted over a period of twenty weeks.

Some of the equipment and materials which were used are as follows: an automatic autoclave of HA-series (HA-3D Hirayama, Japan); a steag laminar air flow cabinet (Prozesstechnik GmbH Carl-Benz-Strass 10, Germany); a Heraeus Incubator (BK 6160, Germany) and a Heraeus Heating oven (T6120, Germany).

Two ginger cultivars (China and Mkondezi Experiment Station No.4 abbreviated as MES 4. table 1) were grown at Mkondezi Agricultural Experiment Station, Nkhata-Bay district, at an altitude of 580 metres above sea level, 11.6 ° S 34.3 ° E in Malawi. These varieties were grown in the 2000/2001 growing season and harvested in May 2001. They were brought to Lusaka, Zambia in June 2001 and stored in cool dry sand in 20 litre buckets.

Table 1. Characteristics of ginger cultivars used in the experiment

<b>Cultivar</b>	<b>Yield Kg/ha</b>	<b>% crude protein</b>	<b>% Oil</b>	<b>% Crude fibre</b>	<b>% Ash</b>	<b>Country of origin</b>
China	6393	7.75	3.80	0.51	13.11	China
MES 4	6039	9.31	2.92	0.53	13.90	Uganda

Adapted from Mbaso *et al* (1998)

## **3.2 Treatments and experimental Design**

There were two sets of experiments in the study. The first experiment was direct explant culture while the second experiment was callus culture.

### **3.2.1 Direct Explant Culture.**

In this experiment two ginger varieties, China and MES 4, presented in table 2 were used.

Three types of explants were used in the experiment.

1. Sprouted buds.
2. Dormant modified buds.
3. Dormant unmodified buds.

The modification of dormant buds in (2) was achieved by dipping dormant buds in 10.0 mg/l (ppm) gibberellic acid (GA<sub>3</sub>) for 1 day before inoculation.

Three types of media were also used and these were:

1. MS + 1.5 mg/l (ppm) BAP + 1.0mg/l (ppm) NAA.
2. MS + 3.0mg/l ( ppm) BAP + 1.5mg/l (ppm) NAA.
3. MS + 3.0 mg/l (ppm) BAP + 1.5mg/l ( ppm) NAA + 0.2mg/l (ppm) GA<sub>3</sub>.

The materials in this experiment were incubated in light to encourage organogenesis.

### **3.2.2 Callus Culture.**

This second experiment consisted of the same treatments as those in first experiment but incubation was in dark at 25°C for callus induction (Table 2).

Since the study was done in a controlled environment, the experimental design used in both experiments was a completely randomized design (CRD). Ten cultures (replications) were used per treatment. The treatments were arranged as a 2 x 3 x 3 factorial experiment.

Table 2. Treatments and treatment combinations

Variety (V)	Type of media (M)	Type of explants (E)	Treatment combinations (Codes)
1. China (V <sub>1</sub> ) 2. MES 4 (V <sub>2</sub> )	1. MS + 1.5ppm BAP + 1.0ppm NAA (M <sub>1</sub> ) 2. MS + 3.0ppm BAP + 1.5ppm NAA (M <sub>2</sub> ) 3. MS + 3.0ppm BAP + 1.5ppm AA+0.2ppm GA <sub>3</sub> (M <sub>3</sub> )	1. Sprouted buds (E <sub>1</sub> ) 2. Dormant modified buds (E <sub>2</sub> ) 3. Dormant unmodified buds (E <sub>3</sub> )	1. V <sub>1</sub> M <sub>1</sub> E <sub>1</sub> 10. V <sub>2</sub> M <sub>1</sub> E <sub>1</sub>
			2. V <sub>1</sub> M <sub>1</sub> E <sub>2</sub> 11. V <sub>2</sub> M <sub>1</sub> E <sub>2</sub>
			3. V <sub>1</sub> M <sub>1</sub> E <sub>3</sub> 12. V <sub>2</sub> M <sub>1</sub> E <sub>3</sub>
			4. V <sub>1</sub> M <sub>2</sub> E <sub>1</sub> 13. V <sub>2</sub> M <sub>2</sub> E <sub>1</sub>
			5. V <sub>1</sub> M <sub>2</sub> E <sub>2</sub> 14. V <sub>2</sub> M <sub>2</sub> E <sub>2</sub>
			6. V <sub>1</sub> M <sub>2</sub> E <sub>3</sub> 15. V <sub>2</sub> M <sub>2</sub> E <sub>3</sub>
			7. V <sub>1</sub> M <sub>3</sub> E <sub>1</sub> 16. V <sub>2</sub> M <sub>3</sub> E <sub>1</sub>
			8. V <sub>1</sub> M <sub>3</sub> E <sub>2</sub> 17. V <sub>2</sub> M <sub>3</sub> E <sub>2</sub>
			9. V <sub>1</sub> M <sub>3</sub> E <sub>3</sub> 18. V <sub>2</sub> M <sub>3</sub> E <sub>3</sub>

### **3.3 Composition and preparation of media.**

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) (Appendices 1 and 2.) was used with different supplements of growth regulators namely, benzylaminopurine (BAP), naphthlaneacetic acid (NAA) and gibberellic acid (GA<sub>3</sub>) all obtained from Sigma Chemical Company in South Africa. These growth regulators were used in different concentrations. A 1.0 litre MS medium was prepared by, first, putting 400 ml of distilled water in the 1.0 litre bottle and then adding 30g sucrose, 100 ml of macronutrient stock solution, 1.0 ml micronutrient stock solution, 5.0 ml standard iron stock solution, 1.0 ml organic/ vitamin stock solution, 10 ml standard Myo-inositol stock solution, 15mg Benlate (a fungicide) to reduce internal contaminants, 100 mg citric acid and 50 mg ascorbic acid (to reduce blackening and browning of tissues) and different concentrations of BAP and NAA. Distilled water was then added to make up to the 1.0 litre. The pH of the solution was adjusted to 5.8 with 1.0M NaOH and then agar, 8.0 g was added.

The solution was boiled for one and half hours to dissolve the agar and then poured into pyrex, 50ml test tubes, about 20 ml in each test tube. The test tubes with the contents were autoclaved for 30 minutes at a temperature of 121°C. GA<sub>3</sub> is said to be degraded at temperatures of above 90° C and so the medium where GA<sub>3</sub> was added was autoclaved in the same 1.0 litre bottle. After autoclaving and while the medium was still in a liquid state at about 60°C GA<sub>3</sub> was added through filter-gun in the laminar flow cabinet (filter-sterilization). The media was then dispersed in test tubes.

### **3.4 Explant preparation**

Two months before the initiation of laboratory experiments ten dormant ginger rhizomes weighing about 1.0 kilogramme from each of the two cultivars was put in 1.0 litre glass jars and placed in full light in incubators. The jars were loosely closed to allow air circulation. This process was done in order to force

the rhizomes to sprout since this was the period the crop was dormant. The rhizomes started sprouting after six weeks.

Three days before inoculation, dormant buds about 1.0 – 2.0 cm in length were cut from the dormant rhizomes. These were put in 0.5 litre bottles and 10.0 ppm GA<sub>3</sub> was added such that all the buds were partially submerged in the solution. They remained in the solution for at least 24 hours so as to absorb the GA<sub>3</sub>. These formed the dormant modified buds used as one of the three types of explants. The following day the other types of explants, sprouted and dormant unmodified buds were prepared from sprouted and dormant rhizomes respectively.

### **3.5 Explant sterilization**

A day before inoculation, all the explants were thoroughly washed with a commercial disinfectant, JIK (2 % m/v Sodium hypochlorite) and thereafter they were washed in running tap water for about 20-30 minutes. The washed explants were dipped in 0.7 % w/v (7g / litre of water) Mercuric (II) chloride for 5 minutes and rinsed with distilled sterilized water. The explants were, thereafter, steeped in 0.1 % w/v fungicide, Benlate (Benomyl) and kept in the solution overnight to reduce internal contamination in the explants. On the day of inoculation, all the sterilization was done in the laminar flow cabinet. The fungicide solution was poured out of the explants and then the materials were rinsed three times with distilled sterile water. The materials were then dipped in 85 % ethanol for 1 minute and thereafter, rinsed three times with distilled sterile water. The explants were again dipped in 0.7 % w/v mercuric (II) chloride for 5 minutes and thereafter rinsed three times with distilled sterile water. Distilled sterile water was then poured into the explants in petri dishes and these were ready for inoculation.

### **3.6 Inoculation in culture media**

All inoculations took place under the laminar flow cabinet. The tweezers and the scalpel that were used were first dipped in methylated spirit and heated on

to a burning spirit lamp before using them to cut explants. Explants were cut to small pieces of about 0.3- 0.5 cm. Scale leaves were removed to expose the meristematic tip or growing point. This explant was then placed in the test tube with medium with the cut surface slightly into the medium and the tip facing the test tube opening. After each and every inoculation, the tweezers and the scalpel were dipped in methylated spirit and heated on to a burning spirit lamp. Ten cultures (inoculants) were made for each of the eighteen treatments and for the two experiments, A and B. Thus one-hundred and eighty cultures were made per experiment.

### **3.7 Culture incubation and subculturing**

Cultures for direct explant culture (Experiment A), were put in growth racks and incubated at  $25 \pm 2$  ° C and daylength of 16 hours provided by white fluorescent tubes at light intensity of about 5.0 klux. Cultures for the callus culture (Experiment B), were incubated in dark at temperature of  $25 \pm 2$  ° C.

Cultures for callus culture experiment failed to develop callus as was expected and instead they developed multiple shoots just like the cultures from direct explant culture experiment. Subculturing (splitting the multiple shoots and re-inoculating individual shoot in different test tube with medium) was done three times for each treatment and experiment. The duration of one subculture was of about one week. The media used for subculturing was devoid of NAA so as to prevent excessive rooting which had been experienced in the initial cultures. However, NAA was later added to the media for the final subculture to initiate rooting.

### **3.8 Hardening-off and transfer to soil.**

Media for hardening-off were prepared at half-strength MS and solidified with 0.8 % agar but without plant growth regulators and fungicides. The rooted plantlets were transferred to this media in pyrex, 50 mls test tubes. The test

tubes were covered with aluminum foil on which holes had been made to expose the plantlets to the external environment. After one and half weeks in this media, the plantlets were transferred to black polyethylene sleeves with 1:1 sand: vermiculite mixture which had been sterilized by autoclaving at 121 ° C for one hour. The plantlets were covered with perforated white polyethylene pots to increase relative humidity. The first three days the plantlets were watered with MS solution where sugar had been excluded. After one and half weeks the plantlets were fully uncovered.

### **3.9 Data collection and statistical analysis**

The following data were collected: (1) Rate of contamination (%), which was recorded by counting the number of cultures infected for each treatment at shooting stage and after every subculturing. The percentage contamination was calculated as a proportion of the original cultures made or the total shoots at each subculture; (2) Days to shooting, the number of days taken from inoculation to the day the shoots were at least 10.0 mm tall; (3) Regeneration percentage, which was calculated from the number of cultures that regenerated out of the initial cultures; (4) Shoot height at first subculture, collected by taking shoot heights of at least five shoots per treatment at first subculturing; (5) Number of shoots per explant, the average number of shoots recorded for each explant in a treatment produced during experimentation period; (6) Days to rooting, the number of days it took a culture to give at least one root from the date of initial inoculation; (7) Number of roots per shoot, average number of roots recorded for each shoot in a treatment during experimentation period; (8) Percentage plantlets survival in external conditions, the percentage of the total plantlets planted in soil mixtures after hardening –off that survived and grew into independent plants.

Mstat-C computer programme was used for data analysis. All experimental data were subjected to analysis of variance (ANOVA) and Least Significant Difference, LSD, test was applied to separate means. A t-test was used to compare the influence of the two sets of experiments.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Direct explant culture

Results from the direct explant culture (experiment A) whose cultures were incubated in light are presented in Tables 3 (ANOVA table) to 7. In this experiment, means for the measured parameters were as follows: days to shooting (11.39), contamination rate (21.11%), regeneration rate (67.78%), shoots per explant (8.15), days to rooting (16.05), roots per shoot (11.20), height of shoots at first subculture (3.05 cm) and external environmental survival rate (93%).

Varieties significantly affected ( $P \leq 0.05$ ) the contamination rate. China variety had higher rate of contamination (23.89%) than MES<sub>4</sub> which had 18.33%. All the other measured parameters were not influenced by varieties (Table 3 and 4).

Culture media had no influence on contamination rate, regeneration rate, shoots per ex-plant, roots per shoot and external environmental survival rate. However, culture media significantly influenced the days to shooting ( $P \leq 0.05$ ), days to rooting and height of shoots at first subculture ( $P \leq 0.01$ ) (Table 3 and 4). Doubling the concentration of BAP and NAA without the addition of GA<sub>3</sub> reduced days shooting and rooting and height of shoots. The lower concentration of BAP and NAA had similar influence on days to shooting and days to rooting, as the media with GA<sub>3</sub>. Doubling the concentration of BAP and NAA in presence of GA<sub>3</sub> resulted in the tallest shoots (3.31 cm) compared to the same concentration of BAP and NAA without GA<sub>3</sub> (2.93 cm) and lower

concentration of BAP and NAA (3.12 cm). Though statistically not different, doubling the concentration of BAP and NAA without GA<sub>3</sub> yielded the lowest regeneration rate (65%) compared with 69.17% and 68.97% for the lower concentration of BAP and NAA and the media with GA<sub>3</sub> respectively (Table 3 and 4).

It would appear that at slightly lower level of significance than 5% ( $P > 0.05$ ) the culture media could be different in terms of regeneration rate.

Type of explants did not differ in their influences on shoots per ex-plants, roots per shoot and external environmental survival rate. However, explants were significantly different in contamination rate ( $P \leq 0.01$ ), days to shooting, regeneration rate, days to rooting and height of shoots at first subculture ( $P \leq 0.001$ ) (Tables 3 and 4).

Table 3. ANOVA table showing mean squares (MS) and F-values (F) of growth and development parameters of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in light<sup>z</sup>.

Source of Variation	df	Days to shooting <sup>y</sup>		% Contamination		% Regeneration		Shoots per explant		Days to Rooting <sup>x</sup>		Roots per shoot		Shoot height(cm) <sup>w</sup>		% Soil survival <sup>v</sup>	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Varieties (V)	1	0.44	0.35 <sup>NS</sup>	277.78	3.33*	44.44	0.28 <sup>NS</sup>	0.64	0.33 <sup>NS</sup>	0.21	0.21	0.12	0.05 <sup>NS</sup>	0.01	1.5 <sup>NS</sup>	0.75	0.35 <sup>NS</sup>
Media (M)	2	4.11	3.23 <sup>***</sup>	36.11	0.43 <sup>NS</sup>	69.44	0.36 <sup>NS</sup>	0.38	0.19 <sup>NS</sup>	6.02	7.75 <sup>***</sup>	1.36	0.31 <sup>NS</sup>	0.25	37.6 <sup>***</sup>	0.49	0.21 <sup>NS</sup>
V x M	2	18.11	14.17 <sup>***</sup>	36.11	0.43 <sup>NS</sup>	86.11	0.44 <sup>NS</sup>	0.24	0.12 <sup>NS</sup>	18.1	23.2 <sup>***</sup>	1.08	0.25 <sup>NS</sup>	0.04	6.13 <sup>***</sup>	0.35	0.14 <sup>NS</sup>
Explants (E)	2	208.36	163.07 <sup>***</sup>	436.11	5.23 <sup>**</sup>	2186.1	11.24 <sup>***</sup>	2.25	1.16 <sup>NS</sup>	294.8	379 <sup>***</sup>	2.53	0.58 <sup>NS</sup>	14.8	2223.5 <sup>***</sup>	2.36	1.26 <sup>NS</sup>
V x E	2	9.69	7.59 <sup>***</sup>	2.78	0.03 <sup>NS</sup>	2.78	0.02 <sup>NS</sup>	3.64	1.87 <sup>NS</sup>	6.33	8.14 <sup>***</sup>	0.08	0.02 <sup>NS</sup>	0.12	18.5 <sup>***</sup>	3.75	1.89 <sup>NS</sup>
M x E	4	6.15	4.82 <sup>***</sup>	40.28	0.48 <sup>NS</sup>	27.78	0.14 <sup>NS</sup>	1.64	0.84 <sup>NS</sup>	4.69	6.04 <sup>***</sup>	0.24	0.05 <sup>NS</sup>	0.08	12.63	1.74	0.85 <sup>NS</sup>
V x M x E	4	2.99	2.34 <sup>***</sup>	48.61	0.58 <sup>NS</sup>	19.44	0.10 <sup>NS</sup>	1.96	1.01 <sup>NS</sup>	2.17	2.79*	1.04	0.24 <sup>NS</sup>	0.05	7.00 <sup>***</sup>	2.06	1.12 <sup>NS</sup>
Error	18	1.28		83.33		194.44		1.94		0.78		4.39		0.007		2.04	
CV %		9.93		43.24		20.57		17.10		5.49		18.67		2.68		3.11	

NS, \*, \*\* and \*\*\* denotes not significant and significant at 5 %, 1 % and 0.1 % respectively.

z. Daylength of 16 hours at light intensity of 5.0 klux.

y. Number of days from explant inoculation to when the shoot was at least 10 mm tall..

x. Number of days from explant inoculation to when at least one root appears on the shoot.

w. Height of shoot at first subculture.

v. Percentage of plantlets surviving in external environmental conditions in 1 : 1 soil : vermiculite mixture.

CV % = Coefficient of variation

Table 4. The effects of varieties, culture media and explants growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in light<sup>1</sup>.

Treatments	Growth and development parameters									
	Days to shooting <sup>x</sup>	% Contamination	% Regeneration	Shoots per explant	Days to rooting <sup>w</sup>	Roots per shoot	Shoot height (cm) <sup>v</sup>	% Soil survival <sup>u</sup>		
<b>Varieties</b>										
China	11.28	23.89	68.89	8.02	16.00	11.10	3.07	91		
MES4	11.50	18.33	66.67	8.28	16.10	11.30	3.03	89		
SE±	0.266	2.15	3.29	0.33	0.21	0.49	0.02	4.37		
Sig.	NS	*	NS	NS	NS	NS	NS	NS		
<b>Media<sup>z</sup></b>										
M <sub>1</sub>	11.33ab	19.17	69.17	8.31	16.80a	11.92	3.12b	91		
M <sub>2</sub>	10.83b	21.67	65.00	8.18	15.33b	11.17	2.93c	90		
M <sub>3</sub>	12.00a	22.50	68.97	7.96	16.75a	11.58	3.31a	89		
SE±	0.33	2.64	4.03	0.40	0.36	0.60	0.023	5.35		
Lsd (0.05)	0.97	NS	NS	NS	0.76	NS	0.09	NS		
<b>Explants<sup>y</sup></b>										
E <sub>1</sub>	6.58b	14.17b	83.33a	8.65	10.33b	11.67	4.33a	91		
E <sub>2</sub>	14.00a	25.00a	59.17b	7.90	19.00a	11.25	2.42b	90		
E <sub>3</sub>	13.58a	24.17a	60.83b	8.10	18.83a	10.75	2.40b	89		
SE±	0.33	2.64	4.03	0.40	0.36	0.60	0.023	5.35		
Lsd (0.05)	0.97	7.83	11.96	NS	0.76	NS	0.09	NS		
CV %	9.93	43.24	20.57	17.10	5.49	18.67	2.68	3.11		

NS, \*; Not significant and significant at 5 % respectively.

Means followed by the same letters in a column and within the same treatments (varieties, media and explants) are not significantly different at 5 % level of significance.

z. Denotes media used ; M<sub>1</sub> (MS + 1.5 ppm BAP + 1.0 ppm NAA), M<sub>2</sub> (MS+3.0 ppm BAP + 1.5 ppm NAA) and M<sub>3</sub> (MS + 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub>).

y Denotes the explants used; E<sub>1</sub> (Sprouted buds), E<sub>2</sub> (Dormant modified by dipping in 10.0 ppm GA<sub>3</sub> for one day) and E<sub>3</sub> (Dormant unmodified buds).

x. Number of days from explant inoculation to when the shoot was at least 10 mm tall.

w. Number of days from explant inoculation to when at least one root appears on the shoot.

v. Height of shoot at first subculture.

u. Percentage of plantlets surviving in external environmental conditions in 1: 1 soil : vermiculite mixture.

t. Daylength of 16 hours at light intensity of 5.0 klux.

Sprouted explants reduced the time of shooting and rooting and also had a lower contamination rate. There was increased regeneration rate and rapid shoot growth with sprouted explants compared with the other type of explants. Modifying dormant explants by dipping them GA<sub>3</sub> had no advantage over dormant unmodified explants in all the parameters measured.

There were significant interactions of varieties and culture media (Variety x Media); varieties and explants (Variety x Explant) and culture media and explant (M x E) for shooting and rooting period and shoot growth ( $P \leq 0.001$ ) (Tables 5, 6 & 7). MES<sub>4</sub> variety on media with GA<sub>3</sub> and China variety on lower concentration of BAP/NAA media exhibited delayed shooting (Table 5). Explants regenerated earlier on medium with higher concentration of BAP/NAA without GA<sub>3</sub> compared to other media (Table 5). Similar to shooting, China variety on lower concentration of BAP/NAA media and MES<sub>4</sub> variety on media with GA<sub>3</sub> delayed rooting (Table 5). With both varieties, tallest shoots resulted on media with GA<sub>3</sub> (Table 5).

Sprouted explants from China variety took the lowest period to start shooting and rooting and had the tallest shoots (Table 6). Modified and unmodified explants of both varieties took long to start shooting and rooting and had short shoots (Table 6). Sprouted explants on lower concentration of BAP/NAA media had the lowest period of shooting and rooting and produced the tallest shoots (Table 7). Modified and unmodified explants took longer to start shooting and rooting and had shorter shoots on all the media (Table 7). The interaction of varieties, culture media and type of explants (Variety x Media x Explant) significantly ( $P \leq 0.001$ ) influenced height of shoots at first subculture (ANOVA table 3). Sprouted China explants on lower concentration of BAP/NAA media and media with GA<sub>3</sub> produced the tallest shoots (Data not shown). This interaction (Variety x Media x Explant) also influenced days to rooting ( $P \leq 0.05$ ). Sprouted explants of China variety on lower concentration of BAP/NAA media and media with GA<sub>3</sub> reduced the rooting period (Data not shown)

Table 5. The effects of varieties and culture media on growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in light<sup>v</sup>.

Varieties	Culture media <sup>z</sup>											
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	Mean	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	Mean	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	Mean
	Days to shooting <sup>y</sup>			Days to rooting <sup>x</sup>			Height of shoots (cm) <sup>w</sup>					
China	12.17	11.17	10.5	11.28	16.83	16.0	15.33	16.06	3.03	2.88	3.28	3.06
MES4	10.45	10.55	13.5	11.5	15.3	14.67	18.17	16.05	3.00	2.97	3.13	3.03
SE±				0.462				0.360				0.033
CV %				9.93				5.49				2.68
Sig.				***				***				***

\*\*\* denotes significant at 0.1% level of significance

z. Denotes media used ; M<sub>1</sub> (MS + 1.5 ppm BAP + 1.0 ppm NAA), M<sub>2</sub> (MS+3.0 ppm BAP + 1.5 ppm NAA) and M<sub>3</sub> (MS + 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub>).

y. Number of days from explant inoculation to when the shoot was at least 10 mm tall.

x. Number of days from explant inoculation to when at least one root appears on the shoot.

w. The height of shoots at first subculture.

v. Daylength of 16 hours at light intensity of 5.0 klux..

Table 6. The effects of varieties and explants on growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in light<sup>v</sup>

Varieties	Type of explants <sup>z</sup>						Mean					
	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>	Mean	E <sub>1</sub>	E <sub>2</sub>		E <sub>3</sub>	Mean			
	Days to shooting <sup>y</sup>											
China	5.83	13.5	14.5	11.28	9.83	18.67	19.67	16.00	4.42	2.32	2.47	3.07
MES4	7.33	14.5	12.67	11.5	10.83	19.33	18.00	16.10	4.25	2.52	2.33	3.03
SE±				0.462				0.360				0.033
CV %				9.93				5.49				2.68
Sig.				***				***				***
	Days to rooting <sup>x</sup>											
	Height of shoots (cm) <sup>w</sup>											

\*\*\* denotes significant at 0.1% level of significance

z. Denotes the explants used; E<sub>1</sub> (Sprouted buds), E<sub>2</sub> (Dormant modified by dipping in 10.0 ppm GA<sub>3</sub> for one day) and E<sub>3</sub> (Dormant unmodified buds).

y. Number of days from explant inoculation to when the shoot was at least 10 mm tall.

x. Number of days from explant inoculation to when at least one root appears on the shoot.

w. The height of shoots at first subculture.

v. Daylength of 16 hours at light intensity of 5.0 klux..

Table 7. The effects of culture media and explants on growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in light<sup>u</sup>.

Media <sup>z</sup>	Type of explants <sup>y</sup>											
	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>	Mean	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>	Mean	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>	Mean
	Days to shooting <sup>x</sup>				Days to rooting <sup>w</sup>				Height of shoots (cm) <sup>v</sup>			
M <sub>1</sub>	6.0	13.25	14.75	11.33	10.10	18.25	20.0	16.08	4.40	2.23	2.43	3.02
M <sub>2</sub>	7.25	13.3	12.0	10.83	10.50	18.5	17.0	15.33	4.08	2.45	2.25	3.93
M <sub>3</sub>	6.5	15.5	14.0	12.0	1	0.40	20.25	16.75	4.53	2.58	2.53	2.21
SE±				0.80				0.624				0.033
CV %				9.93				5.49				2.68
Sig.				***				***				***

\*\*\* denotes significant at 0.1% level of significance

z. Denotes media used ; M<sub>1</sub> (MS + 1.5 ppm BAP + 1.0 ppm NAA), M<sub>2</sub> (MS+3.0 ppm BAP + 1.5 ppm NAA) and M<sub>3</sub> (MS + 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub>).

y Denotes the explants used; E<sub>1</sub> (Sprouted buds), E<sub>2</sub> (Dormant modified by dipping in 10.0 ppm GA<sub>3</sub> for one day) and E<sub>3</sub> (Dormant unmodified buds).

x. Number of days from explant inoculation to when the shoot was at least 10 mm tall..

w. Number of days from explant inoculation to when at least one root appears on the shoot.

v. Height of shoot at first subculture.

u. Daylength of 16 hours at light intensity of 5.0 klux.

## 2 Callus culture

Results of this experiment whose cultures were incubated in the dark are presented in Tables 8 (ANOVA table) to 12. Most of the cultures in the experiment failed to produce callus and instead they produced shoots just like cultures incubated in light. Those cultures which directly resulted into shoots from the dark-incubated experiment were treated as those cultures from the light-incubated experiment.

The means for the measured parameters in this dark-incubated experiment were as follows: days to shooting (25.64), contamination rate (23.61%), regeneration rate (54.72), shoots per explant (8.82), days to rooting (29.56), roots per shoot (11.11), height of shoots at first subculture (3.54 cm) and external environmental survival rate (92.3%) (Tables 8 and 9).

Varieties had significant influence ( $P \leq 0.01$ ) on height of shoots at first subculture. All the other parameters were not influenced by the varieties.

Culture media exerted significant effects ( $P \leq 0.001$ ) on growth of shoots up to first subculture. Media with GA<sub>3</sub> produced the tallest shoots while media with lower concentration of BAP and NAA (Media 1) had the shortest shoots (Tables 8 and 9).

Explants significantly influenced days to shooting, regeneration rate, shoots per explant, days to rooting and height of shoots at first subculture ( $P \leq 0.001$ ). Sprouted explants shoot and rooted earlier than dormant modified and dormant unmodified explants. These sprouted explants also gave higher regeneration rate, shoots per explant and shoot height at first subculture (Tables 8 and 9). Modified dormant explants had no advantage over unmodified explants for all the parameters except for height of shoots (Tables 8 and 9). The modified explants resulted into taller shoots compared to shoots from unmodified explants.

Table 8. ANOVA table showing mean squares (MS) and F-values (F) of growth and development parameters of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in dark<sup>z</sup>.

Source of Variation	df	Days to shooting <sup>y</sup>		% Contamination		% Regeneration		Shoots per explant		Days to Rooting <sup>x</sup>		Roots per shoot		Shoot height (cm) <sup>w</sup>		% Soil survival <sup>v</sup>	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Varieties (V)	1	0.03	0.01 <sup>NS</sup>	2.78	0.04 <sup>NS</sup>	25.00	0.15 <sup>NS</sup>	0.004	0.012 <sup>NS</sup>	0.11	0.04 <sup>NS</sup>	0.11	0.05 <sup>NS</sup>	1.21	435.60 <sup>***</sup>	0.66	0.45 <sup>NS</sup>
Media (M)	2	0.53	0.17 <sup>NS</sup>	52.78	0.76 <sup>NS</sup>	144.4	0.88 <sup>NS</sup>	0.59	1.55 <sup>NS</sup>	0.44	0.16 <sup>NS</sup>	1.86	0.78 <sup>NS</sup>	0.99	355.60 <sup>***</sup>	0.59	0.31 <sup>NS</sup>
V x M	2	0.19	0.06 <sup>NS</sup>	52.78	0.76 <sup>NS</sup>	100.0	0.61 <sup>NS</sup>	5.81	15.31 <sup>***</sup>	0.44	0.16 <sup>NS</sup>	0.03	0.01 <sup>NS</sup>	0.82	296.40 <sup>***</sup>	0.45	0.24 <sup>NS</sup>
Explant (E)	2	193.9	62.9 <sup>***</sup>	52.78	0.76 <sup>NS</sup>	2636	16.08 <sup>***</sup>	4.34	11.44 <sup>***</sup>	187.1	66.0 <sup>***</sup>	8.03	3.36 <sup>NS</sup>	24.29	8745.70 <sup>***</sup>	2.26	1.36 <sup>NS</sup>
V x E	2	0.36	0.12 <sup>NS</sup>	36.11	0.52 <sup>NS</sup>	108.3	0.66 <sup>NS</sup>	7.31	19.26 <sup>***</sup>	0.78	0.27 <sup>NS</sup>	0.19	0.08 <sup>NS</sup>	0.10	36.30 <sup>***</sup>	3.65	1.79 <sup>NS</sup>
M x E	4	0.57	0.18 <sup>NS</sup>	86.11	1.24 <sup>NS</sup>	65.28	0.40 <sup>NS</sup>	6.51	17.15 <sup>***</sup>	0.44	0.16 <sup>NS</sup>	1.82	0.76 <sup>NS</sup>	0.08	30.25 <sup>***</sup>	1.64	0.75 <sup>NS</sup>
V x M x E	4	0.65	0.21 <sup>NS</sup>	311.1	4.48 <sup>**</sup>	20.83	0.13 <sup>NS</sup>	5.94	15.65 <sup>***</sup>	1.11	0.39 <sup>NS</sup>	5.74	2.40 <sup>NS</sup>	0.98	351.15 <sup>***</sup>	2.03	1.11 <sup>NS</sup>
Error	18	3.08		69.44		163.89		0.379		2.83		2.39		0.003		2.11	
CV %		6.85		35.29		23.39		6.98		5.70		13.91		1.49		3.21	

NS, \*, \*\* and \*\*\* denotes not significant and significant at 5 %, 1 % and 0.1 % respectively.

z.. Incubated in total darkness for 24 hours.

y. Number of days from explant inoculation to when the shoot was at least 10 mm tall..

x. Number of days from explant inoculation to when at least one root appears on the shoot.

w. Height of shoot at first subculture.

v. Percentage of plantlets surviving in external environmental conditions in 1: 1 soil : vermiculite mixture.

CV % = Coefficient of variation

Table 9. The effects of varieties, culture media and explants growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in dark<sup>t</sup>.

Treatments	Growth and development parameters									
	Days to shooting <sup>x</sup>	% Contamination	% Regeneration	Shoots per explant	Days to rooting <sup>w</sup>	Roots per shoot	Shoot height (cm) <sup>y</sup>	% Soil survival <sup>u</sup>		
<b>Varities</b>										
China	25.61	23.33	53.89	8.84	29.61	11.06	3.73	89		
MES4	25.67	23.89	55.56	8.80	29.50	11.17	3.36	91		
SE±	0.41	1.96	3.02	0.15	0.40	0.36	0.01	4.97		
Sig.	NS	NS	NS	NS	NS	NS	**	NS		
<b>Media<sup>z</sup></b>										
M <sub>1</sub>	25.83	25.80	55.81	8.94	29.60	11.25	3.32c	91		
M <sub>2</sub>	25.42	23.36	57.52	8.57	29.33	10.67	3.45b	90		
M <sub>3</sub>	25.67	21.67	50.83	8.96	29.73	11.42	3.87a	89		
SE±	0.51	2.41	3.70	0.18	0.49	0.45	0.02	6.09		
Lsd (0.05)	NS	NS	NS	NS	NS	NS	0.05	NS		
<b>Explants<sup>y</sup></b>										
E <sub>1</sub>	21.00b	21.60	71.67a	9.52a	25.00b	11.50	5.18a	91		
E <sub>2</sub>	28.08a	25.83	44.17b	8.47b	32.00a	10.25	2.83b	90		
E <sub>3</sub>	27.83a	23.40	48.33b	8.48b	31.67a	11.58	2.63c	89		
SE±	0.51	2.41	3.70	0.18	0.49	0.45	0.02	6.09		
Lsd (0.05)	1.51	NS	10.98	0.53	1.44	NS	0.05	NS		
CV %	6.85	35.29	23.39	6.98	5.70	13.91	1.49	3.21		

NS, \*\*; Not significant and significant at 5 % respectively.

Means followed by the same letters in a column and within the same treatments (varieties, media and explants) are not significantly different at 5 % level of significance.

z. Denotes media used ; M<sub>1</sub> (MS + 1.5 ppm BAP + 1.0 ppm NAA), M<sub>2</sub> (MS+3.0 ppm BAP + 1.5 ppm NAA) and M<sub>3</sub> (MS + 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub>).

y Denotes the explants used; E<sub>1</sub> (Sprouted buds), E<sub>2</sub> (Dormant modified by dipping in 10.0 ppm GA<sub>3</sub> for one day) and E<sub>3</sub> (Dormant unmodified buds).

x. Number of days from explant inoculation to when the shoot was at least 10 mm tall..

w. Number of days from explant inoculation to when at least one root appears on the shoot.

v. Height of shoot at first subculture.

u. Percentage of plantlets surviving in external environmental conditions in 1: 1 soil : vermiculite mixture.

t. Incubated in total darkness for 24 hours.

There were significant interactions of varieties and culture media (Variety x Media), varieties and explants (Variety x Explant) and culture media and explants (Media x Explant) for shoots per explant and shoot height ( $P \leq 0.001$ ) (Tables 10, 11 and 12). MES<sub>4</sub> on low concentration BAP/NAA media yielded the highest number of shoots per explant while China on low concentration BAP/NAA media gave the lowest shoots. China variety on media with GA<sub>3</sub> produced the tallest shoots while the shortest shoots resulted from MES<sub>4</sub> on low concentration BAP/NAA media (Table 10) Sprouted explants of China and MES<sub>4</sub> varieties produced the tallest shoots while Sprouted explants of China variety yielded the highest number of shoots (Table 11). Sprouted explants on high concentration of BAP/NAA media and modified explants on media with GA<sub>3</sub> yielded highest shoots per explants. Sprouted explants on media with GA<sub>3</sub> gave the tallest shoots (Table 12).

The interaction of varieties, culture media and explants (Variety x Media x Explant) was highly significant ( $P \leq 0.001$ ) for shoots per explant and height of shoots at first subculture (ANOVA table 8). Sprouted explants of China variety on high concentration of BAP/NAA media produced the highest number of shoots per explant (data not shown). The same sprouted China buds on media with GA<sub>3</sub> resulted in quick growth of shoots (data not shown).

Table 10. The effects of varieties and culture media on growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in dark<sup>x</sup>

Varieties	Culture media <sup>z</sup>							
	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	Mean	M <sub>1</sub>	M <sub>2</sub>	M <sub>3</sub>	Mean
	Shoots per explant			Shoot height (cm) <sup>y</sup>				
China	8.15	8.95	9.40	8.83	3.45	3.4	4.33	3.73
MES4	9.73	8.18	8.52	8.81	3.18	3.5	3.4	3.36
SE±				0.253				0.022
CV %				6.98				1.49
Sig.				***				***

\*\*\* denotes significant at 0.1% level of significance

aa. Denotes media used ; M<sub>1</sub> (MS + 1.5 ppm BAP + 1.0 ppm NAA), M<sub>2</sub> (MS+3.0 ppm BAP + 1.5 ppm NAA) and M<sub>3</sub> (MS + 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub>).

y. Height of shoot at first subculture.

x. Incubated in total darkness for 24 hours.

Table 11. The effects of varieties and explants on growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in dark<sup>x</sup>

Varieties	Type of explants <sup>z</sup>				Shoot height (cm) <sup>y</sup>	Mean		
	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>	Mean				
China	10.25	8.58	7.67	8.83	5.38	2.92	2.9	3.73
MES4	8.78	8.35	9.3	8.81	5.00	2.73	2.35	3.36
SE±				0.253				0.022
CV %				6.98				1.49
Sig.				***				***

\*\*\* denotes significant at 0.1% level of significance

z. Denotes the explants used; E<sub>1</sub> (Sprouted buds), E<sub>2</sub> (Dormant modified by dipping in 10.0 ppm GA<sub>3</sub> for one day) and E<sub>3</sub> (Dormant unmodified buds).

y. Height of shoot at first subculture.

x.. Incubated in total darkness for 24 hours

Table 12. The effects of culture media and explants on growth and development of two ginger varieties (*Zingiber officinale* Roscoe) under *in vitro* conditions and incubated in dark<sup>w</sup>.

Culture media <sup>z</sup>	Type of explants <sup>y</sup>					Mean	Shoot height (cm) <sup>x</sup>	Mean
	E <sub>1</sub>	E <sub>2</sub>	E <sub>3</sub>	E <sub>1</sub>	E <sub>2</sub>			
M <sub>1</sub>	9.55	8.28	9.00	8.94	5.10	2.55	2.30	3.32
M <sub>2</sub>	10.28	6.98	8.73	8.57	4.93	2.85	2.58	3.45
M <sub>3</sub>	8.73	10.15	8.00	8.96	5.53	3.08	3.00	3.87
SE±				0.31				0.026
CV %				6.98				1.49
Sig.				***				***

\*\*\* denotes significant at 0.1% level of significance

<sup>z</sup> Denotes media used ; M<sub>1</sub> (MS + 1.5 ppm BAP + 1.0 ppm NAA), M<sub>2</sub> (MS+3.0 ppm BAP + 1.5 ppm NAA) and M<sub>3</sub> (MS + 3.0 ppm BAP + 1.5 ppm NAA + 0.2 ppm GA<sub>3</sub>).

<sup>y</sup> Denotes the explants used; E<sub>1</sub> (Sprouted buds), E<sub>2</sub> (Dormant modified by dipping in 10.0 ppm GA<sub>3</sub> for one day) and E<sub>3</sub> (Dormant unmodified buds).

<sup>x</sup> Height of shoot at first subculture.

<sup>w</sup>. Incubated in total darkness for 24 hours.

### **4.3 Direct explant culture versus callus culture**

The influence of the light and the dark incubation were compared to determine the more efficient one. The results are presented in table 12.

Incubating cultures in light significantly reduced the number of days to shooting and rooting ( $P \leq 0.001$ ) and increased regeneration rate ( $P \leq 0.01$ ). Cultures incubated in light started shooting and rooting in 11.39 and 16.06 days, respectively. Those cultures incubated in dark took 25.61 and 29.56 days to start shooting and rooting respectively. There were 67.78% and 54.72% regeneration rates of cultures incubated in light and dark respectively. Though not significantly different, dark incubated cultures yielded taller shoots (3.57 cm) than the light incubated cultures (3.07 cm).

Table 13. Effects of light and dark incubation on growth and development of two ginger varieties (*Zingiber officinale Roscoe*) under *in vitro* conditions.

Type of Culture	Growth and development parameters									
	Days to Shooting <sup>x</sup>	Contamination %	Regeneration %	Shoots per explant	Days to Rooting <sup>w</sup>	Shoot height(cm) <sup>v</sup>	Roots per Shoot	Shoot height(cm) <sup>v</sup>	% Soil survival <sup>u</sup>	
Light <sup>z</sup>	11.39	21.11	67.78	8.18	16.06	11.22	3.07	11.22	89	
Dark <sup>y</sup>	25.61	23.61	54.72	8.84	29.56	11.11	3.57	11.11	91	
Mean	18.50	22.36	61.25	8.51	22.81	11.17	3.32	11.17	90	
Sd±	1.22	2.44	4.29	0.43	1.32	0.33	0.38	0.33	6.30	
Sig.	***	NS	**	NS	***	NS	NS	NS	NS	

NS, \*, \*\*, \*\*\*, not significant, significant at 5%, 1% and 0.1% respectively.

z. Daylength of 16 hours at light intensity of 5.0 klux.

y. Incubated in total darkness for 24 hours.

x. Number of days from explant inoculation to when the shoot was at least 10 mm tall..

w. Number of days from explant inoculation to when at least one root appears on the shoot.

v. Height of shoot at first subculture.

u. Percentage of plantlets surviving in external environmental conditions in 1: 1 soil : vermiculite mixture.

## CHAPTER FIVE

### 5.0 DISCUSSIONS

Explants in the current study started shooting, on average, in about nineteen days (about eleven and twenty-six days for light and dark incubation experiments respectively, table 13). This average period is above the one reported by Hosoki and Sagawa (1977) and John *et al.* (1997). Hosoki and Sagawa (1977) reported that ginger explants started shooting in fourteen days (two weeks) after inoculation. John *et al.* (1997) also reported that shooting of ginger explants started in 14 to 15 days. The workers quoted above used only sprouted explants which were incubated in light. The delay in shooting in this study could be due to inclusion of dormant explants and the dark incubation. Dormant explants and dark incubation delayed shooting in this study (Tables 4, 9 and 13). Contamination rate in the study was 22.36% (Table 13) and this is less than half the rate of 50% reported by Ikeda and Tanabe (1986). These workers sterilized the ginger explants with 70% Ethanol and 5% Sodium hypochlorite. The low contamination rate in the current study could be due to the use of strong sterilizing agents, 0.7% Mercuric chloride and 0.1% Benlate. Torres (1989) reported that ethanol and sodium hypochlorite were not effective in eliminating microorganisms from underground explants, which usually have internal contaminations. This worker recommended strong sterilizing agents for such explants. Ginger buds used as explants are from underground and as such use of strong sterilizing agents is a prerequisite in order to reduce contaminations.

Sharma and Singh (1995) reported a regeneration rate of 16.7 to 81.8 % in ginger buds cultured on different media. In the current study an average of 61.25 % (67.78 % and 54.72 % for light and dark incubation respectively, table 13) regeneration was obtained and this falls within the reported range. Hosoki and Sagawa (1977) reported 5.7 rooted ginger shoots per initial explant in 2 to

3 months. Nadgauda *et al* (1980) obtained 3 to 5 ginger shoots per explant in 8 weeks while Ikeda and Tanabe (1986) obtained 5 shoots per explant in MS media supplemented with both BAP and NAA but they obtained 10 shoots when only BAP was added to MS media. The average of 8.51 shoots per explant (Table 13) achieved in the current study in 20 weeks look realistic considering the period. Rooting of *in vitro* derived shoots is normally determined by auxins in the media. Hoepfner *et al.* (1996) working with Red raspberry found that auxins, NAA, encouraged rooting while cytokinins, BAP, suppressed rooting. John *et al.* (1997) reported that in ginger most shoots started rooting in multiplication media at the same time shooting started. In this study the initial media was supplemented by both BAP and NAA and rooting started, on average, in about twenty-three days (16.02 and 29.56 days for light and dark incubation respectively, table 13) about 4 days after shooting. This finding is supported by the above reports. John *et al.* (1997) reported that root growth is better on liquid media than on solid media. Ikeda and Tanade (1986) found 15.3 to 16.3 roots per shoot on liquid media. The current study recorded 11.17 roots per shoot (Table 13) on solid media. This finding could be justified considering that solid media which has been reported above to be associated with low root growth was used.

Shoot height at first subculture is rarely reported in most studies. In this study shoot height was recorded in order to compare growth in light and dark before first subculturing. The average shoot height of 3.32 cm (3.07 and 3.57 cm for light and dark incubation respectively, table 13) recorded in the study might not be very good as it shows the shoots were very tall, a situation which could inhibit multiple shooting and give few shoots per explant.

It is believed that the survival of plantlets in the external environment depends on hardening-off procedures and handling of the plantlets after the controlled environment. Gamborg and Phillips (1999) reported more than 85 % plantlet survival in African violet. John *et al.*(1997) reported 80 % survival for

Cardamom, *Elettaria cardamomum* and 70-80 % survival in most forest trees. The survival of the plantlets in external environment in the present study was 90 %. The high survival rate achieved in the present study could be the result of proper hardening-off and caring of the plantlets after *in vitro* conditions.

The two varieties used in the study have different origins but they have very similar characteristics (Table 1). The similarities of the varieties in most characteristics could make one believe that the varieties were closely related and this relatedness could be the cause of lack of differences in most of the measured parameters. The differences in contamination rate in the light incubated experiment could be due to the differences in development and establishment of the microorganisms in the varieties during the period of cultivation and not necessarily due to varietal differences.

It is difficult to attribute the differences in height of shoots at first subculture in the dark incubated experiment to varietal differences because of the similarities of the varieties.

High concentration of BAP/NAA (3.0 ppm BAP + 1.5ppm NAA) without GA<sub>3</sub> reduced the period to shooting and rooting of ginger explants in the direct explant experiment (Table 4). The absence of GA<sub>3</sub> reduced shoot height in both the experiments (Tables 4 and 9). The addition of GA<sub>3</sub> to media in ginger micropropagation has rarely been reported. Lemos and Blake (1996) working with *Annona muricata* found that addition of GA<sub>3</sub> to media did not increase regenerative capability of the crop. This may explain why the addition of GA<sub>3</sub> to media in this study had no advantage over treatments with low BAP/NAA (1.5ppm BAP + 1.0ppm NAA) and those with high BAP/NAA without GA<sub>3</sub> for most measured parameters. However, it is difficult to explain the differences in the influence on days to shooting and rooting (Table 4) between media with GA<sub>3</sub> (3.0ppm BAP + 1.5ppm NAA + 0.2ppm GA<sub>3</sub>) and that of high BAP/NAA. The difference may suggest that GA<sub>3</sub> reduces the effectiveness of BAP and NAA in media.

Many workers have used different concentrations of BAP and NAA in MS media with success in ginger micropropagation. Hosoki and Sagawa (1977) used 1.0 ppm BAP/NAA and obtained well rooted shoots. Ikeda and Tanade (1986) obtained plantlets by using 2.5 ppm BAP and 0.1 ppm NAA. Inden *et al* (1988) used 5.0 ppm BAP and 0.5 ppm NAA and obtained plantlets in ginger. With these variations in media, it is not surprising to find no differences in influences between media with low BAP and NAA and that with high concentration on most measured parameters such as regeneration rate, shoots per explants, roots per shoot and many others.

In both experiments sprouted explants took shorter period to start shooting and rooting, had higher regeneration rate and taller shoots than both dormant modified and dormant unmodified explants (Tables 4 and 9). This trend is justified by findings from many studies (Pierik, 1987; Torres, 1989). Pierik (1987) reported that juvenile explants remain juvenile in tissue culture and generate very easily while adult explants remain adult and regenerate with repeated subculturing to fresh media. This explains reasons for dormant explants taking long to start shooting compared to sprouted buds. Pierik (1987) stated that the physiological state of explants can be modified by dipping in the plant growth regulators such as BAP and GA<sub>3</sub> to reduce dormancy and make them regenerate in tissue culture. Torres (1989) observed that dormant plant materials have scales that contain bud break inhibitors and removal of these scales and soaking buds in GA<sub>3</sub> assists in breaking dormancy. The regenerative ability of dormant explants achieved in the current study is justified by the above reported studies. In the present study, both dormant modified (dipped in GA<sub>3</sub>) and dormant unmodified explants regenerated. This could suggest that removing bud scales had more influence than dipping buds in GA<sub>3</sub>. Sprouted explants started shooting earlier than other explants and this might have contributed to the taller shoots for sprouted explants than the others. Actively growing shoot tips are reported to be free or have low content microorganisms (Gamborg and Phillips, 1999). This may be the reason for lower contamination

rate in sprouted explants compared to dormant explants that was observed in direct explant experiment. Lemos and Blake (1996) working with *Annona muricata* found that juvenile explants had more shoots per explants than mature ones. This may explain the reason for higher shoots per explant in sprouted explants compared to dormant explants in the dark incubated experiment.

Nadgauda *et al* (1980) recommended that for the different varieties, some modifications could be made to procedures and media used. Lemos and Blake (1996) working with *Annona muricata* acknowledged that the differences in response to different concentrations of BAP and NAA between juvenile and mature explants could be due to endogenous levels of cytokinins and auxins present in the original explants.

In the study, varieties behaved differently on the different media and explants (interaction of varieties and media and varieties and explants) for days to shooting and rooting and shoot height in the direct explant experiment and shoots per explant and shoot height at first subculture in the callus culture experiment. However, no one variety is superior over the other on all the media used for the parameters measured (Variety x Media) interactions are not consistent). This inconsistency in the interaction of varieties and media could be attributed to the suspected relatedness of the varieties as reported earlier in this paper. The interactions of varieties and explants (Variety x Explant) and media and explants ( Media x Explant) for days to shooting and rooting and shoot height in the direct explant experiment were consistent. The interactions of varieties and explants (Variety x Explant) and media and explants ( Media x Explant) for shoots per explant and shoot height at first subculture in the callus culture experiment were consistent. In both the experiments sprouted explants of both varieties and sprouted explants on all the media were superior to the other explants on the same varieties and media. The differences in response to different concentrations of BAP and NAA between juvenile and mature

explants could be due to endogenous levels of cytokinins and auxins present in the original materials (Lemos and Blake, 1996).

There was negligible to very little callusing in the callus culture experiment. The failure of callusing could be attributed to the small size of explants used. Gamborg and Phillips (1999) reported that callus formation increased by increasing size of explants and increasing wound site. In this experiment, small explants of 0.3cm to 0.5cm were used to reduce contamination. Explants taken from aseptic or *in vitro* culture derived plants have been reported to respond faster and produce more callus (Gamborg and Phillips 1999 and Kackar *et al.* 1993). Kackar *et al.* (1993) reported callus from young ginger leaf segments taken from *in vitro* shoot culture. In this experiment *in vivo* ginger explants were used and this might have reduced callusing. Callus production has also been reported to be associated with high concentration of plant growth regulators, especially auxins. Pierik (1987) reported that callus induction from explants required higher concentration of plant growth regulators than organogenesis from callus. Gonzalez-Benito *et al.* (1996) working with Snapdragon observed callus on MS media supplemented with 0.1 ppm NAA. Barandiaran *et al.* (1999) obtained 93.4 % callus in garlic, *Allium sativum*, on MS media supplemented with 3.0 ppm BAP + 2.0 ppm NAA + 0.03 ppm 2,4-D. In both the studies mentioned above the cultures were incubated in dark at 25 ° C for up to three months. The concentrations of plant growth regulators used in the present study fall within those reported. The cultures were also incubated in dark at 25 ° C for more than two months. The failure of callusing, as stated above, could be attributed to the small size of explants used and the use of *in vivo* explants. This callusing failure and the subsequent failure to obtain plantlets from this callus culture experiment prevented the determination of somaclonal variants from the two experiments in the study. Somaclonal variations in tissue culture have been strongly linked to callus induction.

Direct explant culture (light incubation) was superior over callus culture (dark incubation) in days to shooting and rooting and regeneration rate (Table 13). The two types of culture had similar influences on all the other parameters measured. Many authors have reported the importance of light intensity and photoperiod for morphogenesis *in vitro*. Pierik (1987) stated that dark period followed by a light period is necessary for shoot induction and that the length of darkness was species dependant. The worker further noted that explants established well under 0.5 to 1.0 klux illumination using 16 hour photoperiod. Hosoki and Sagawa (1977) obtained well established ginger plantlets under continuous light of 2.1 klux. Nadgauda *et al.*(1980) found that 16 hours of light and 8 hours of darkness were very ideal in ginger growing *in vitro*. These reported findings support the results found in the present study where light incubated explants started shooting and rooting much earlier than those incubated in dark. These light incubated cultures had higher regeneration rate than those in dark. Yama *et al.* (2000) found that photoperiod of 12 to 14 or more hours increased shoot induction in ginger while 8 to 11 hours reduced shoot induction. They further reported that long day length increased gibberellic acid (GA) which induced shooting while short day length increased abyscic acid (ABA) which reduced shooting. Light in the present study might have increased the action of endogenous GA that enabled quick shooting and increased regeneration. Rooting period was correlated to shooting period in both light and dark incubation.

The lack of differences in influences between the two cultures for external survival rate, shoots per explant and roots per shoot could be attributed to the fact that these were obtained after first subculture whereby all the cultures in the experiments were incubated in light. The vigorous sterilization process that was used in this study severely reduced contamination rate and this could be the cause for lack of differences in contamination rate between the two experiments.

Devlin and Withan (1983) reported that there was high concentration of auxins such as NAA in plants which are shaded than those in light and so plants which are shaded grow taller than those in light. These workers further noted that light causes inhibition of stem growth by lowering the level of endogenous GA in plants. With these findings one would have expected the dark incubated ginger shoots to be taller than those incubated in light in the present study. However, the addition of exogenous GA and NAA to the explants in both light and dark experiments offset the expected results. Devlin and Withan (1983) reported that the application of exogenous GA and auxins to plants growing in light increased stem growth.

## CHAPTER SIX

### 6.0 CONCLUSION

The findings from the study have shown that dormant ginger buds can regenerate *in vitro* just like sprouted buds. The findings also show that ginger explants can be incubated in both light and dark and regenerate and grow *in vitro*. However, light incubation has been found to be more efficient than darkness incubation in terms of regeneration rate and duration to shooting for both sprouted and dormant buds.

The two ginger varieties used in the study have shown to be similar in most of the *in vitro* growth and regeneration parameters. The culture media used also had similar regeneration rate and growth of ginger both in light and dark. The addition of GA<sub>3</sub> to media has shown to have no advantage over media without GA<sub>3</sub> in terms of regeneration ability of dormant ginger buds. However, addition of GA<sub>3</sub> has shown to increase shoot growth up to first subculture in both light and dark.

Modifying dormant buds by dipping in GA<sub>3</sub> has shown not to improve *in vitro* growth and regeneration of ginger over unmodified buds.

Dormant ginger buds can be used as explants in micropropagation during the dormant state of the crop. Both light and dark incubation of cultures can be used in ginger micropropagation. The two ginger varieties used in the study should be propagated using the same micropropagation protocols. A wide range of concentrations of BAP and NAA without GA<sub>3</sub> can be used for ginger micropropagation but the low concentration of BAP and NAA which was used in this study could be encouraged to reduce costs.

It is suggested that the callus culture experiment be repeated using larger ginger explants so as to induce callus which may improve the *in vitro* multiplication rate (more shoots per explants) and also create somaclonal variations from which plant breeders could select superior clones.

## CHAPTER SEVEN

### 7.0 REFERENCES

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## APPENDICES

### Appendix 1: Composition of MS medium.

Components	Quantity mg/l
<b>A. Macronutrients</b>	
NH <sub>4</sub> NO <sub>3</sub>	1650.0
KNO <sub>3</sub>	1900.0
CaCl <sub>2</sub> x2H <sub>2</sub> O	440.0
MgSO <sub>4</sub> x 7H <sub>2</sub> O	370.0
KH <sub>2</sub> PO <sub>4</sub>	170.0
<b>B. Micronutrients</b>	
H <sub>3</sub> BO <sub>3</sub>	6.20
MnSO <sub>4</sub> xH <sub>2</sub> O	22.30
ZnSO <sub>4</sub> x7H <sub>2</sub> O	8.60
KI	0.83
NaMoO <sub>4</sub> x2H <sub>2</sub> O	0.25
CuSO <sub>4</sub> x5H <sub>2</sub> O	0.025
CoCl <sub>2</sub> x6H <sub>2</sub> O	0.025
FeSO <sub>4</sub> x7H <sub>2</sub> O	27.80
Na <sub>2</sub> EDTA x6H <sub>2</sub> O	37.30
<b>D. Carbon source</b>	
Sucrose	30g/l
<b>C. Vitamins and other supplements</b>	
Inositol	100.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Glycine	2.0
pH	5.8

Source: Murashige and Skoog (1962).

Appendix 2: preparation of MS stock solutions and MS medium.

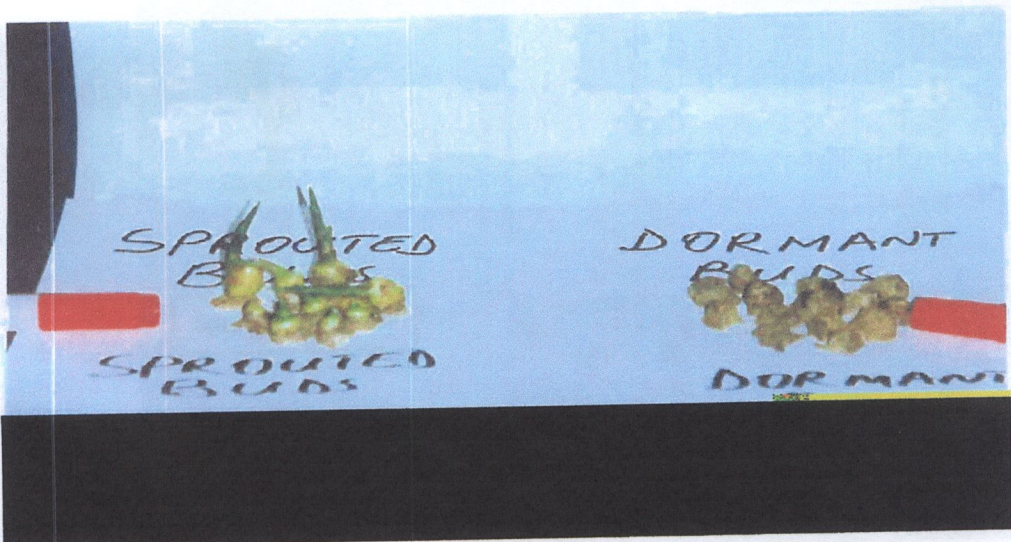
		Volume of stock per litre of medium			Storage of stock solution
		1 litre	2 litre	5 litre	
<b>MS Macro stock (conc. 10 x)</b>					
	<b>g/l</b>				
NH <sub>4</sub> NO <sub>3</sub>	16.5				
KNO <sub>3</sub>	19				
CaCl <sub>2</sub> x2H <sub>2</sub> O	4.4				
MgSO <sub>4</sub> x 7H <sub>2</sub> O	3.7				
KH <sub>2</sub> PO <sub>4</sub>	1.7	100 ml	200 ml	500 ml	+ 4°C
<b>MS Micro stock (conc. 1000 x)</b>					
	<b>mg/100 ml</b>				
H <sub>3</sub> BO <sub>3</sub>	620				
MnSO <sub>4</sub> xH <sub>2</sub> O	1690				
ZnSO <sub>4</sub> x7H <sub>2</sub> O	860				
KI	83				
NaMoO <sub>4</sub> x2H <sub>2</sub> O	25				
CuSO <sub>4</sub> x5H <sub>2</sub> O	2.5				
CoCl <sub>2</sub> x6H <sub>2</sub> O	2.5	1 ml	2 ml	5 ml	+ 4°C
<b>Iron stock (conc. 200 x)</b>					
	<b>g/l</b>				
FeSO <sub>4</sub> x7H <sub>2</sub> O	5.57				
Na <sub>2</sub> EDTA x2H <sub>2</sub> O	8.17	5 ml	10 ml	25 ml	+ 4°C
<b>MS Organic stock (conc. 1000 x)</b>					
	<b>mg/100ml</b>				
Nicotinic acid	50				
Pyridoxine-HCl	50				
Thiamine-HCl	10				
Glycine	200	1 ml	2 ml	5 ml	+ 4oC
<b>Myo-Inositol stock (conc. 100 x)</b>					
	<b>mg/100 ml</b>				
Myo-Inositol	1000	10 ml	20 ml	50 ml	+ 4°C
Sucrose (added as solid)		30 g	60 g	150 g	
PH		5.8	5.8	5.8	

Source: Murashige and Skoog (1962).

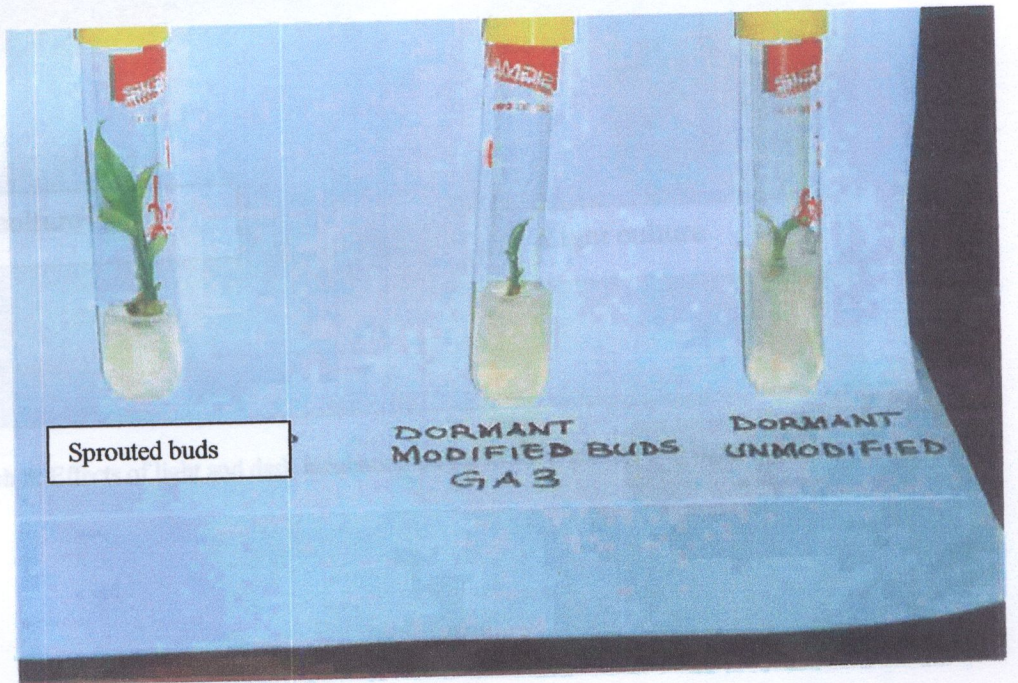
Appendix 3 : Molecular weights, Solubility and Conversion of Concentrations [  $\mu\text{M}$  to  $\text{mg/L}$  (ppm) ] of Plant growth Regulators ( PGRS).

PGRs	Mol. Wt.	Soluble in	Concentration (ppm)						
			0.1	0.2	0.5	1.0	2.0	5.0	10.0
<b>ABA</b>	264.3	NaOH	0.38	0.76	1.89	3.78	7.00	18.92	37.84
<b>Adenine</b>	135.1	NaOH	0.74	1.48	3.70	7.40	14.80	37.00	74.00
<b>Ads</b>	184.2	H <sub>2</sub> O	0.54	1.09	2.71	5.43	10.86	27.14	54.29
<b>BA/BAP</b>	225.3	NaOH	0.44	0.88	2.22	4.44	8.88	22.20	44.40
<b>2ip</b>	203.2	NaOH	0.49	0.98	2.46	4.92	9.84	24.61	46.21
		Ethanol							
<b>2,4-D</b>	221.0	NaOH	0.45	0.90	2.26	4.52	10.95	22.62	45.25
<b>GA3</b>	246.4	Ethanol	0.29	0.58	1.44	2.89	5.77	14.53	28.89
		Ethanol							
<b>IAA</b>	175.2	NaOH	0.57	1.14	2.85	5.70	11.40	28.50	57.00
		Ethanol							
<b>IBA</b>	203.2	NaOH	0.49	0.98	2.46	4.90	9.80	24.60	49.00
		Ethanol							
<b>IPA</b>	189.2	NaOH	0.53	1.06	2.64	5.29	10.57	26.43	52.85
<b>Kinetin</b>	215.2	NaOH	0.46	0.92	2.32	4.65	9.29	23.23	46.47
<b>NAA</b>	186.2	NaOH	0.54	1.07	2.69	5.37	10.74	26.85	53.71

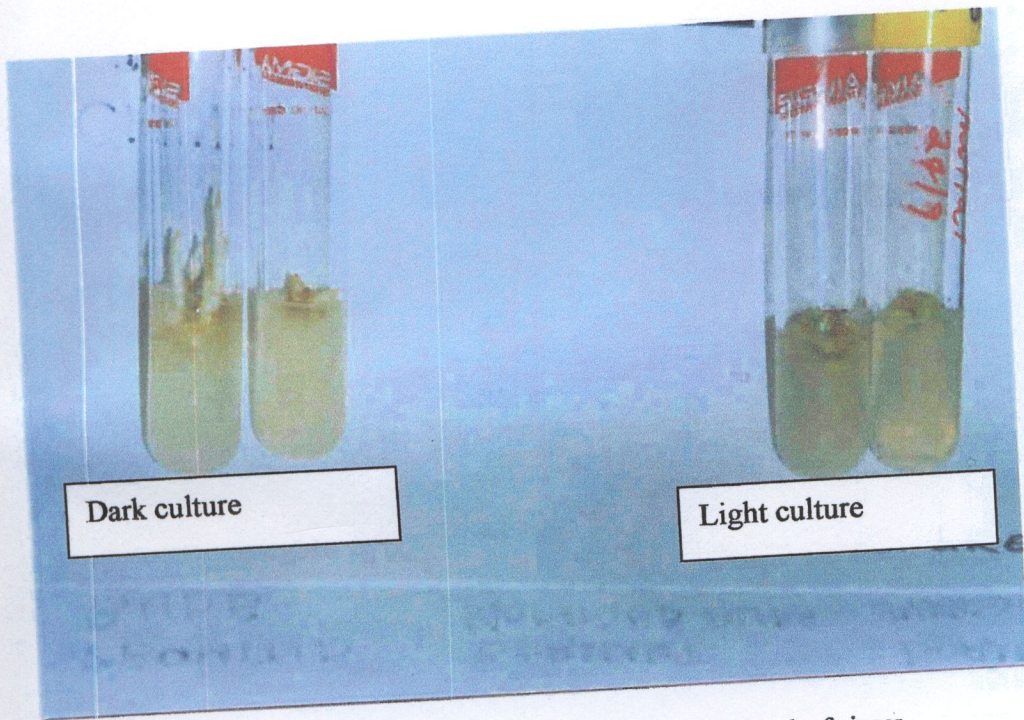
Source: John *et al.* (1997)



Photograph 1: Sprouted buds (left) and dormant buds (right)



Photograph 2: Effects of explants on the *in vitro* growth of ginger



Photograph 3: Effects of light and dark incubation on the *in vitro* growth of ginger