IN VITRO PROPAGATION OF GINGER (*Zingiber officinale)*

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Dedicated to my

BELOVED PARENTS

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IN VITRO PROPAGATION OF GINGER (*Zingiber officinale*)

ABSTRACT

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207 from the period of September 2013 to July 2014 to evaluate the effects of different plant growth regulators on *in vitro* propagation of ginger (*Zingiber officinale*). The healthy, disease free sprout was used as explants. Explants was inoculated in MS (Murashige and Skoog), media supplemented with the Kinetine (KIN), alone and in combination with GA_3 and Indolebutyricacid (IBA). The highest percent of shoot induction (90%) was observed on 3.0mg/L+1.5mg/L GA_3 and the minimum days to shoot induction (13.00 days) were achieved on KIN 3.0mg/L.The highest number of shoot (4.6), maximum number of leaf per explants (10.00) and average length of shoot (4.56 cm) were recorded in 3.0 mg/L KIN+ 1.5 mg/L GA_3.The highest percent of root induction 95% and the maximum (14.2) cm length of root induction were noticed on 2.5 mg/L IBA. Micropropagation was found very effective and promising method in the proliferation of ginger and can be used for large scale production of disease free and quality planting material.

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CERTIFICATE

*This is to certify that thesis entitled, "**IN VITRO PROPAGATION OF GINGER (Zingiber officinale)** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY,** embodies the result of a piece of bona fide research work carried out by **Rumana ferdous, Registration No. 08-2689** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma in any institute.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: December, 2014
Place: Dhaka, Bangladesh

……..
(Prof. Dr. Md. Ekramul Hoque)
Supervisor

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LIST OF ACRONYMS

AEZ	=	Agro-Ecological Zone
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
LAI	=	Leaf area index
ppm	=	Parts per million
et al.	=	And others
N	=	Nitrogen
TSP	=	Triple Super Phosphate
MP	=	Muriate of Potash
CRD	=	Completely Randomized Design
DAS	=	Days after sowing
ha^{-1}	=	Per hectare
gm	=	gram (s)
Kg	=	Kilogram
µg	=	Micro gram
SAU	=	Sher-e-Bangla Agricultural University
SRDI	=	Soil Resources and Development Institute

HI	=	Harvest Index
No.	=	Number
Wt.	=	Weight
LSD	=	Least Significant Difference
^{0}C	=	Degree Celsius
mm	=	millimeter
Max	=	Maximum
Min	=	Minimum
%	=	Percent
cv.	=	Cultivar
CV%	=	Percentage of coefficient of variance
Hr	=	Hour
T	=	Ton
viz.	=	Videlicet (namely)

CHAPTER I

INTRODUCTION

Ginger (*Zingiber officinale*) is an important herbaceous plant primarily used as a spice and in medicine at the global level. Underground rhizomes are utilized in herbal medicine, food and beverage and form an important raw material in pharmaceutical industries (Bhagyalaksmi and Singh, 1994). Ginger (*Zingiber officinale*) is a monocotyledon plant that belongs to the family of the Zingiberaceae, which consists of more than 1200 plant species in 53 genera. It include also other medical plants and well known spices like turmeric (*Curcuma longa*), lesser galangal (*Alpinia officinarum*), or cardamom (*Elettaria cardamomum var. minuscula*). The genus Zingiber was named after the Sanskrit word zindschi (hornshaped) by the English botanist William Roscoe (1753-1831) in a report published in 1807.

Ginger probably originates from South-East Asia. The ancient Greeks and Romans brought the rhizome to Southern Europe. Already in the 11th century it is mentioned in Anglo-Saxon veterinary pharmacopoeias and leech books, in the 13th century it was well known in all of Europe, and the Spanish established first plantations in the West Indies (mainly Jamaica) and in Mexico in the 16th century. Now-a-days ginger is cultivated in the tropical parts of the world, from Asia to Africa, and large parts of South and Central America, mainly in India, in southern China, Indonesia, Nepal, and Nigeria. The best quality is said to come from Jamaica (Wichtl, 2002).

It is commercially cultivated as an annual and it is being used as a spice and in medicine in Asia (Pandey *et al*., 1997). Ginger grows well on loam soil incorporated with humas but it does not stand under water logging condition (Purseglove, 1976).Under favorable conditions, the ginger plants continuously survive through rhizomes and the cultivated ginger is not known to set seeds (Ravindram *et al*., 2005). The axillary and terminal buds grow up as leaf stem which is known as aerial stem. Leaves are sheathing and arranged alternatively.

The structure of rhizome consists of two different zones which are separately by intermediate layers and a cambriam like layer presented is a significant trait in ginger.

Ginger is highly valuable plant having underground rhizome for its aromatic and medicinal properties. It contains high quantitative secondary metabolites, Oleoresin (Sakamura *et al*., 1986). The ginger rhizome contains 0.6 to 3.3 % essential oil, comprising more than 150 secondary metabolites. This includes bisabolane-type sesquiterpenes like α- zingiberene (about 30 %), β-sesquiphell and rene (15-20%), β-bisabolene (10-15%), arcurcumene, bicyclic sesquiterpenes like zingiberol (a mixture of β-eudesmol with *trans* and *cis* ring juncture) as main odoriphore, and monoterpenes, which is responsible for the 'lemony' aroma of ginger, dphellandrene as another important odoriphore, geraniol, borneol, α-terpineol, 1,8-cineol and its esters, and D-camphene (Kikuzaki and Nakatani, 1996).

Ginger is highly valued from the ancient period for its flavor and aroma. It has a carminative and stomachic properties and its essential oil has antifungal properties .Due to its pungent and spicy aroma, ginger is used in the manufacture of a number of food products like ginger bread, confectionary, ginger-ate, curry powders, certain curried meat, table sauces, carbonated drinks, ginger brandy, wine and beer in many western countries. In addition to common uses as a spice, ginger rhizomes have a long history of use as a medicine throughout the parts of Asia for its anty-inflammatory properties. It is also used in veterinary medicine and preparing ayurvedic, homeopathic and allopathic medicine . Due to diversified use of ginger, it has also great demand in the world market.

Several small scale clinical trials have shown beneficial effects of ginger or ginger extracts on osteoarthritis (Altman and Marcussen, 2001), gonarthritis, rheumatism and musculoskeletal disorders (Srivastava and Mustafa, 1992; Srivastava and Mustafa, 1989). *In vivo* experiments showed i) anti-

inflammatory effects on rat paw and skin edema for ginger extracts (Penna, 2003), ii) an inhibition of PMA-induced inflammation, epidermal ornithine decarboxylase activity, and skin tumor promotion in ICR mice for 6-gingerol, and iii) an inhibition of monosodium urate crystal-induced gout in Swiss albino mice equal to a ten fold lower dosage of indometacin for 6-shogaol.

In traditional medicine ginger is furthermore used against diabetes and dyslipidaemia. Efficacy in these indications has been confirmed in several animal models and a small placebo controlled clinical trial in humans. Thus, hyperlipidaemic patients showed a significant reduction in triglycerides, cholesterol, low density lipoprotein, and very low density lipoprotein and an increase in high density lipoprotein after consumption of 3 g ginger per day for 45 days. Body weight, glucose, insulin, total cholesterol, LDL cholesterol, triglycerides, free fatty acids and phospholipids in the serum of rats fed on a high-fat diet were markedly reduced by ginger (HDL cholesterol was unaffected) (Nammi *et al*. 2009).

Ginger is one of the important spice crops in Bangladesh. The potential yield of ginger is as high as 30-35 tons per hectare. Bangladesh produces about 72084 metric tons of ginger in an area of 28625 hectare of total land (BBS, 2013). Its yearly requirement is 122,000 metric tons. The country can produce only 40% of its requirement. The rest 60% demand for home consumption totally depends on import costing hard earned foreign exchange. Since ginger is vegetatively propagated through small pieces of rhizomes, large amount of total production is utilized as seed in the next season. Vegetative propagation of ginger has the high risk of spreading systemic infections. It is reported that a three-fold increase in the production of rhizomes could be possible by the effective control of the diseases (Balachandran *et al*., 1990).

Ginger is constrained severely can summarized as following: (1) ginger normally propagates by rhizome with a low proliferation rate, and the reproducing part (rhizome) is also the economically used part of the ginger

plant, which restricts the availability of ginger seeds needed for cultivation, (2) easily infected by soil-borne pathogens such as bacterial wilt (*Pseudomonas solanacearum*), soft rot (*Pythium aphanidermatum*) and nematodes (*Meloidogyne spp*.), which cause heavy losses in yield, (3) ginger rhizomes show variations and degeneration under long-term vegetative propagation and (4) normal breeding of ginger is a real problem due to poor flowering and seeds set.

In vitro approaches for the conservation and the use of plant germplasm can offer some distinct advantage over alternative strategies. Some of these are as follows: (1) There is the potential of virus elimination from contaminated tissue through meristem culture, (2) Clonal material can be produced where this is useful for the maintenance of elite genotypes, (3) Rapid multiplication may occur at any time where stocks are required using micro propagation procedures, (4) Germination of difficult or immature seed or embryo may be facilitated for breeding programmes, and (5) Distribution across the border may be safer, in terms of germplasm health status using *in vitro* cultures. Some more general positive advantages of *in vitro* techniques include the fact that storage space requirements are vastly reduced compared with field storage. Storage facilities may be established at any geographical location and cultures are not subject to environmental disturbances such as temperature fluctuation, cyclones, insect, pests, and pathogen (Rands *et al.*, 2010; Nandagopal *et al*., 2011)

In vitro culture techniques provide an alternative means of plant propagation and a tool for crop improvement (Vasil, 1987). Clonal multiplication of ginger through shoot multiplication has been reported (Hosoki & Sagawa, 1977; Wang, 1989; Balachandran *et al.,* 1990; Rout & Das, 1998). Micropropagation by using tissue culture technique can be a proper alternative to produce disease-free clones of ginger plant. Problems faced in ginger breeding have so far been the very low genetic variation in ginger plant. This is because ginger is

vegetatively propagated crop and hybridization is not effective since its floral biology has not been properly observed yet (Simmonds, 1986).

By producing huge quantity of export quality of ginger Bangladesh can enter into the export market easily. But the production of ginger is not satisfactory in Bangladesh. There is no predominant variety in Bangladesh. Farmer usually utilizes local varities. So there is a great requirement to increase the production of ginger. Only one or two types of ginger are normally cultivated all over the country. Genetic diversity is pre-requisite for any crop improvement. It is well known that ginger has very less genetic variability. So, mass propagation of uniform, healthy plants through tissue culture is the only viable technique for production of large numbers of clonal plants in a short time. Though several attempt was taken for last few decades to develop tissue culture systems of Ginger. But still the efficient regeneration protocols are requisite to develop a rapid, less expensive, efficient and easy method of micropropagation of Ginger. In this consequence, a new composition of growth regulators for rapid and efficient micropropagation of Ginger using sprout of ginger as explant has introduced. The present research focuses on the influence of Kinetine (KIN), Indolebutyricacid (IBA), Giberelic acid (GA_3), on rapid *in vitro* propagation of ginger plants grown in Bangladesh.

OBJECTIVES: Based on the above mentioned context and prospects, the present investigation, therefore, has been carried out with the following objectives:

1) Establishment of *in vitro* regeneration protocol of Ginger.

2) Assessment of combined effect of plant growth regulators for *in vitro* response.

3) To regenerate the plants that are genetically identical to the source material.

4) Acclimatization and cultivation of regenerated plantlets of Ginger.

CHAPTER II

REVIEW OF LITERATURE

Plant tissue culture forms the backbone of plant biotechnology, which is comprised of micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant tissue culture is a technique through which any plant part is cultured on a sterile nutrient medium in controlled light and temperature with the purpose of obtaining growth. The idea of plant tissue culture originated from the cell theory that was formulated by Schwann in 1839. Tissue culture techniques have for decades played a great role in the micropropagation of horticultural and ornamental plants. In fact, the first ever successful plant tissue culture was achieved in horticultural plants (Altman and Ziv, 1997). These techniques have been widely used in disease elimination and vegetative propagation (Husseyg, 1979). Nowadays, it is very common practice all over the world to explore different aspects about ginger using this technology. But unfortunately, it is very limited in Bangladesh. However, some related works already performed by different institutes home and abroad have been reviewed and some of the most relevant literatures are cited below.

2.1 Explant

Hossain *et al.* (2010) showed that callus culture of ginger was induced on MS medium from young leaf segments gave best shooting response.

Sultana *et al.* (2009) reported that three explants of two varieties of ginger viz., Suruchi and BARI ada-1 were cultured on MS medium supplemented with 0.5 mg/l Dicamba, 0.75 mg/l Dicamba and 1 mg/l 2, 4 D. Highest percentage of

callus induction (87.60%) was obtained from leaf explants of Suruchi and 0.5 Dicamba interactions. The interaction of Suruchi x leaf x 1.0 mg/l KIN + 1.0 mg/l BAP produced highest (87.60%) percentage of shoot. Root induction was best (85.40%) from Suruchi x leaf x MS +1 mg/l IBA interaction. The regenerated plantlets were successfully established into pot after proper hardening.

Kavyashree, (2009) developed an efficient *in vitro* multiplication protocol for *Zingiber officinale var. Varada* through direct regeneration of vegetative buds. Multiple shoots were induced from vegetative buds on LSBM fortified with BAP (17.76 µM) with 96% initiation response. The repeated sub culture resulted in rapid shoot at the average rate of 4-fold per culture.

Bhagyalaksmi *et al*. (2004) discovered that meristems of ginger with or without leaf primordial was induced to form shoots on three quarter strength MS medium containing 6% sucrose, coconut milk (CM) 20 %, ascorbic acid (AA) 100 mg/l, glutamine (GL) 200 mg/l, activated charcoal (AC) 250 mg/l, BAP 0.5 mg/l, IBA 0.4 mg/l and agar 0.8% showed best result.

Pandey *et al.* (1997) observed that the highest number of shoots was produced after 5 weeks of culturing (6-8 shoots) when explant pseudo stems of ginger cultured on Murashige and Skoog medium with 5 mg/l BA + 0.5 mg/l NAA.

Dogra *et al.* (1994) achieved *In vitro* propagation of *Zingiber officinale* using rhizome buds and they also observed that the buds produced multiple shoots when cultured on MS medium with 2.5 mg/l BA and 0.5 mg/l NAA. Mohammed and Quraishi, (1999) used shoot tips (3-5 mm) as explants where maximum number of shoots from single explants was 3.33 when medium contained 3.0 mg/l BAP. Maximum plant height during the same period was on medium containing 2.0 mg/l BA and shoot number was also relatively better.

Kanker *et al*. (1993) observed that the performance for percent callus induction was also influenced by the variety and explant. The highest (57.66%) callus induction was found from the leaf of Indian variety followed by leaf of Jongli variety (46.00%). In case of BARI ada-1 variety the lowest percentage (9.32%) of callus induction was observed in the root explants followed by root of Sherpuri (14.67%) and these values were observed significantly different. Highest percentage (35.74%) of shoot induction was observed at Jongli variety and the lowest shoot induction was performed by Sherpuri (16.27%)) Highest percentage (31.40%) of shoot induction was produced by sprouting buds followed by leaf (27.40%) and root (24.00%) explants respectively.

Ilahi and Jabeen, (1987) showed that stem cuttings from 3 month old plants, young buds, rhizome cuttings with shoot bud primordial and juvenile shoots were cultured on ½ MS medium supplemented with different combinations of growth hormones. Whereas callus could not be induced on the stem explants, callus was induced on the juvenile shoots which on sub culturing to medium with varying concentrations of 2, 4-D and BAP developed bud primordial. Young shoot buds along with a portion of rhizome when inoculated on MS medium containing 2,4-D and BAP each @ 0.5 mg/l ruptured and some callus was produced. Excised single shoot when transferred to a medium supplemented with 0.1 mg/l each of 2, 4-D and BAP started rooting within 2-3 days. Young shoots inoculated on MS medium supplemented with 0.5 mg/l both of 2, 4-D and BAP developed greenish white hard callus within 4 weeks. These calli when sub cultured on a media containing 0.1 mg/l of 2, 4-D and 0.5 mg/l BAP produced good callus growth within a week. Shoot buds were observed after 4 weeks which ultimately grew into small plantlets (10-15 plantlets in each culture). Good callus growth was observed within 4 weeks when 2 week old shoot buds were inoculated on a medium supplemented with BAP and 2, 4-D each @ 0.5mg/l.

2.2 Growth induction and development in ginger

Mohammed *et al*. (2011) showed augmentation of MS-medium with 4.5 mg/l BAP developed the highest number of shoots and leaves (8.0 and 15.50 respectively). Shoot lets were highly rooted on half strength of B5 medium supplemented with 1.0 mg/l NAA. The maximum percentage of acclimatization, hardening and rhizomes production of *in vitro* derived plants in greenhouse was 80–100%.

Hossian *et al.* (2010) carried an experiment with varieties of ginger viz., Fulbaria, Syedpuri, Chittagongi, Jangli, Indian, Chaina, Sherpuri and BARI ada-1. For callus induction, Indian (51.84%) and among the treatments, selected MS media supplemented with 1 mgl/L IAA + 3 mgl/L BAP showed the maximum number of callus (2.681). For shoot regeneration, MS media supplemented with 4 mgl/L BAP + 3 mgl/L KIN + 1 mgl/L IAA showed the best performance in ginger. Considering the combined effect of BAP, IAA, KIN and genotype, maximum regenerants (2.33) were found in Indian placed in selected media. Indian showed the best response (43.46%) on root initiation and it observed that half strength MS media supplemented with 2 mg/L IBA + 2 mg/L NAA was very effective. For the plant survival rate from sprout, leaf and root were about 86.67%, 80.00% and 85.71%, respectively.

Khatun *et al*. (2003) observed that the frequency of shoot proliferation was maximum at 2.5mg/l BAP and 0.5mg/l KIN and the number of shoot was 22-25 per explants of ginger. It took 26 days for shoot induction and 30 days for root induction. Multiplication rate in the treatment with BAP 0.5mg/l which showed 2 plantlets were lowest. Among the BAP-2,4 D formulations,

maximum multiplication was observed at BAP 1.0 and 0.5 mg/l 2,4 D where the number of shoots was 15. Numerous adventitious shoot primordia were observed near the basal portion of the shoot cluster.

Palai *et al.* (1997) observed that when *Zingiber officinale* cultivars cultured on Murashige and Skoog medium supplemented with increased concentration of BA from 6 to 8 mg/l, there was decreased multiplication of shoots. No shoots or roots were produced in the basic medium whereas an average of 5.6 shoots and 3 roots appeared in 1 ppm BAP. Some were auxiliary buds and others were adventitious buds at nodes.

Hosoki and Sagawa, (1977) showed about 30% of the explants did not produce shoots, however, an average of 5.7 shoots was produced when scale leaves were removed from these explants 2-3 months after culture. Only a few shoots appeared in 1 ppm BAP+ 1 ppm NAA medium although many roots were formed. Shoots were later transferred individually to 1 ppm BAP medium and shoots elongated and roots also formed within 2 months. Some plantlets produced additional adventitious shoots. Numerous plantlets with roots were produced by repeated sub culture of individual plantlets on 1 ppm BAP medium.

Wickson and Thimann, (1958) discovered that cytokinins could release the lateral buds from apical dominance. In the presence of cytokinins, the dormant buds of vegetative apex are stimulated to grow and elongate. Skoog and Miller (1957) reported that the cell division or cell differentiation was also associated with auxins and cytokinins.

2.3. *In vitro* microrhizome production in ginger

Zheng *et al*. (2008) studied the effect of kinetin (KIN), Gibberellic acid (GA) and naphthalene acetic acid (NAA) on increasing *in vitro* microrhizome production of ginger. Concentrations of GA, KIN and NAA of 1.33-2.35, 0.49-0.66 and 0.62 g/l respectively gave a microrhizome weight of over 0.25g/l. The optimal conditions for micro rhizome production were 80 g/l sucrose, 2X MS macro elements and 1X MS micro elements, with a photoperiod of 24L: 0D (light/dark). 100% survival could be achieved on transfer of the *in vitro* ginger plantlets with microrhizomes to soil. Increases in sucrose in the culture medium decreased the height of ginger plantlets significantly; at the same time, efficient propagation and rhizome weight of each plant increased significantly at sucrose concentrations below 80g. Propagation efficiency reached 6.2 sprouts per plant, with a rhizome weight per plant of 0.68 g, and individual rhizome weight of 0.11 g in medium containing 80g sucrose. With 110g sucrose in the culture medium, propagation efficiency, rhizome weight per plant, and individual rhizome weight were 4.2, 0.59 and 0.14 g, respectively. At sucrose concentrations above 110g, ginger plantlets were etiolated and died. Hence, a sucrose concentration of 80–110g in the culture medium was considered optimal for induction of rhizomes of ginger.

Chirangini and Sharma,(2005) discovered that microrhizome were observed at the base of all *in vitro* derived shoots cultured on the media supplemented with a range of sucrose concentration (3-9%) within 8 weeks of incubation. The best response in terms of number (upto 6 micro rhizomes per culture tube) were obtained in the media supplemented with 7% sucrose and highest average fresh

weight of 0.81 g with 3-5 buds was observed in MS media supplemented with 5% sucrose .

Rout *et al*. (2001) declared that rhizome formation was initiated on MS media containing 4.44-8.88 µM BA, 2.85-8.57 µM IAA and 3% sucrose within 8 weeks of culture. The high percentage of rhizome formation was noted on MS medium containing 4.44 µM BA, 5.71 µM IAA and 3% sucrose. The medium having sucrose in combination with maltose or fructose caused a decline in rhizome formation as compared to sucrose alone. The results also show that the rhizome frequency was maximum in the medium containing 6-8% sucrose whereas medium supplemented with 1-2% sucrose did not produce rhizomes.

Sharma and Singh, (1995) showed that microrhizomes of ginger were successfully produced from tissue cultured derived shoots by transferring them to liquid MS medium supplemented with 1mg/l BAP, 2mg/l calcium panthothenate, 0.2 mg/l GA3 and 0.05 mg/l NAA for shoot proliferation. After 4 weeks of incubation, the medium was replaced with microrhizome induction medium (MS+ 8mg/l BAP+ 75g/l sucrose). Microrhizome formation started after 20 days of incubation in stationary cultures at 25+1oC in the dark. Microrhizomes with 1-4 buds and weight 73.8 to 459 mg each were harvested after 50-60 days. After storage for 2 months in moist sand at room temperature, 80% of the micro rhizomes sprouted producing roots and shoots.

Bhat *et al*. (1994) studied the *in vitro* induction of rhizomes in ginger and reported that among the various factors such as temperature, photoperiod, sucrose, growth regulators and nutrient composition of the medium tested for rhizome production, only sucrose (9% or 12%) was found to be effective.

Sharma *et al*. (1994) showed the shoot buds of ginger were successfully encapsulated in 4% sodium alginate gel. Encapsulated buds were germinated *in vitro* to form roots and shoots. *In vitro* germination from encapsulated buds ranged from 16.7% to 81.8% on different media after 5 weeks of incubation. Normal plantlets with an average shoot length of 2.3 cm and 1.7 cm root length were successfully transplanted in unsterilized soil without any hardening process.

Bapat and Rao, (1990) showed lginate matrix used for encapsulation has been reported to provide nutrients to the microhizomes.

Bapat and Rao, (1988) discussed the importance of the production of synthetic seeds or encapsulated organs as a novel delivery system for the multiplication and long term storage of elite genotypes of ginger.

Redenbaugh *et al*. (1986) discovered encapsulation of somatic embryos in alginate gel has been showed good result in microhizome of ginger

2.4. Growth regulator on micropropagation in ginger

Rout *et al*. (2001) observed the meristematic growth and multiplication were initiated on MS medium supplemented with different concentration of BA and Adenine sulphate (Ads) alone or in combinations. The media having kinetin alone or in combination with Ads showed a low rate of shoot multiplication and inhibited shoot elongation as compared to BA alone or BA+ Ads. The combination of kinetin plus IAA or NAA did not favour the multiplication of the shoot meristem of ginger. The medium containing BA+NAA produced multiple shoot which became yellowish within 4 week of culture. The medium having IAA or NAA alone had no effect on shoot multiplication or growth. Interestingly, BA in combination with Ads and IAA showed a great significant

number of multiple shoots as compared with BA+IAA or BA+Ads. About 92.2% of cultures showed multiple shoot with root hairs in a medium having 26.6µM BA, 8.57 µM IAA and 11 µM Ads. The increase in IAA concentration higher than 11.42 µM suppressed the rate of shoot multiplication and stunted growth. The maximum number of multiple shoots (32.4) was obtained in the medium containing 26.6 µM BA, 8.57 µM IAA and 1111.1 µM Ads 4week after culture initiation.

Palai *et al.* (1997) showed different concentration of growth regulators were used for bud break in two cultivars viz. Suruchi and Suprabha. The number of days required to bud break varied from 6-10. However, in most of the treatments, the percentage of explants showing bud break was more than 80-90 and 75-80 in Suruchi and Suprabha, respectively. In media devoid of growth regulators, the time required for bud sport was always more than 20 days. Among all the growth regulators tested, the media containing BA (2.0-4.0 mg/l) induced bud sport in about 6-7 days of culture. Kinetin was ineffective in stimulating bud sprout as compared to BA. The rate of shoot multiplication varied with the cultivars. The media containing BA (4.0-6.0 mg/l), IAA (1.0-1.5 mg/l) and Ads (100 mg/l) showed higher rate of shoot multiplication in both the cultivars. The percentage of cultures with multiple shoots varied from 52.8 to 86.8 and 58.6 to 72.4 in Suruchi and Suprabha respectively after 4 week of culture. The number of shoots/ explants was also different depending on the cultivar and growth regulators. Shoot bud multiplication decreased as the concentration of BA increased from 6.0-8.0 mg/l while IAA had intermediary effect. Number of shoot/ explants was higher in media combination of BA (6.0 mg/l) + IAA(1.5 mg/l)+ Ads (150mg/l) in Suruchi (31.2) and BA (6.0 mg/l) + IAA(1.5 mg/l)+ Ads (100mg/l) in Suprabha (32.8) .

CHAPTER III

MATERIALS AND METHODS

3.1 Time and location of the experiment:

The present research was carried out in Biotechnology Lab. of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2013 to July 2014.

3.2 Experimental materials:

3.2.1 Source of material

The planting materials of ginger (*Zingiber officinale*) were collected from Agargaon bazar, Sher-e-Bangla Nager, Dhaka-1207.

3.2.2 Plant material:

The healthy, disease free sprout of Ginger of 1-2cm length were used as explants for the study of *in vitro* propagation of ginger shown in plate 1

Plate 1: Explant of ginger.

3.2.3 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glassware

The Borosil glassware was used for all the experiments. Oven dried (250^0C) Erlenmeyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware's were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water, until the detergent was removed completely. Finally they were rinsed with distilled water and sterilized in oven at 160-180^0C for 3-4 hours.

3.2.5 Culture medium

The degree of success in tissue culture is mainly related to the choice of nutritional components and growth regulators. Presence of plant growth regulators plays a significant role in a successful regeneration of any plant species. Media for tissue culture should contain all major and minor elements, vitamins and growth regulators which are essential for normal plant growth. Explants were inoculated onto media composed of basal MS (Murashige and Skoog,1962) medium supplemented with the plant growth regulators. Composition of MS media have been shown in appendix I. Hormones were added separately to different media according to the requirements. To do so, stock solutions of hormones were prepared ahead of media preparation and stored at 4^0C temperature.

1. KIN (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) alone or in combination with GA_3 (0.25, 0.5, 0.75 , 1.00 and1.5 mg/L), were used for shoot proliferation.
2. IBA (0.5, 1.0, 1.50, 2.00 and 2.5 mg/L) was applied separately for root formation.
3. Sucrose (3%) was used as carbon source and media were solidified with agar (0.8%).
4. The pH was adjusted to pH 5.8 prior to autoclaving at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.3 The preparation of the stock solution of hormones:

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Generally, cytokinins were dissolved in few drops of acidic solutions (1N HCl) and Auxins were dissolved in few drops of basic solutions (1N NaOH).

Hormones (Solute)	**Solvents used**
KIN	1 N NaOH
IBA	70% ethyl alcohol

In present experiment, the stock solution of hormones was prepared by following procedure. 100 mg of solid hormone was placed in a small beaker and then dissolved in 10 ml of 70% ethyl alcohol or 1 (N) NaOH solvent. Finally the volume was made upto 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4 ± 1^0C for use upto two month. Growth regulators were purchased from Sigma, USA.

3.4 The preparation of culture media:

To prepare 1000 ml of culture media the following steps were followed:

Step-1. 700 ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. 5 gm of MS media and 30 gm of sucrose was added and gently stirrered to dissolve these ingredients completely with the help of a Hot Plate magnetic stirrer.

Step-3. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

Step-4. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting .

3.5 Steam heat sterilization of media (Autoclaving)

For sterilization, the culture medium was poured in 200 ml culture bottles and then autoclaving was done at a temperature of 121^0C for 30 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving, the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.6 Preparation of explants

The healthy diseases free sprouts were washed thoroughly under running tap water and then with sterilized distilled water for several times. Subsequently the explants were transferred to laminar airflow cabinet and kept in a 250 ml sterilized beaker. The beaker with explants was constantly shaken during sterilization. They were treated with 70% ethanol for 1-2 minute and rinsed with autoclave distilled water for 3-4 times. After treating with 70% ethanol, the explants were immersed in 0.1% $HgCl_2$ within a beaker and added 3-4 drops of Tween-20 for about 4-5 minutes with constant shaking in clockwise and anticlockwise direction. Then explants were washed 3-4 times with autoclaved distilled water to make the material free from chemical and ready for inoculation in culture media.

3.7 Inoculation of culture

The sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with the same alcohol. All glassware, instruments and media were steam-sterilized in an autoclave. During the course of the work, instruments in use were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. The worker's hands and forearms were washed thoroughly with soap and water and repeatedly sprayed with 70% alcohol during the period of work. The mouth of all culture vial was flamed before and after positioning of the explants on the medium.

For inoculation, explants were transferred to large sterile glass Petridis or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer cover was removed with sterile scalpel blade to make suitable size. After cutting explants into suitable size (1.5-2 cm), explants are transferred to culture bottles containing MS medium with plant growth regulator. After vertically inoculating the explants in culture bottle, the mouth of bottle is quickly flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

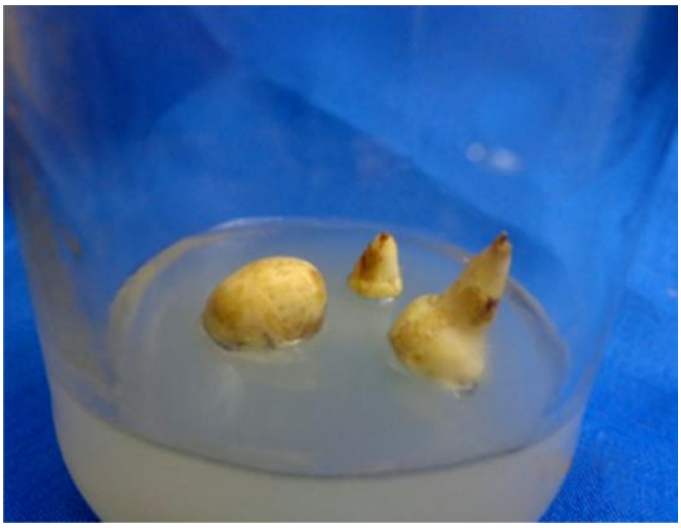


Plate 2. Inoculation of culture with the explants of Ginger.

3.8 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled environment. The cultures were maintained at 25±2 °C with light intensity varied from 2000–3000 Lux (23 W white bulbs). White fluorescent lamps were used for growth of the culture. The photoperiod was generally 14 hours light and 10 hours dark having 70% relative humidity (RH).

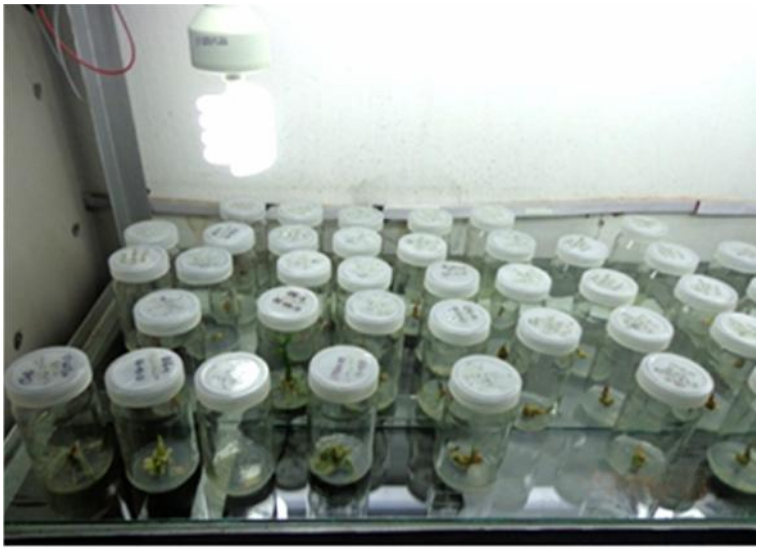


Plate 3. Incubation of inoculated culture vial.

3.9 Shoot proliferation

The explants were cultured on MS nutrient medium supplemented with different concentration of KIN alone or in combination of GA_3. Percentage of explants showing shoot proliferation, days for shoot induction, number of shoots per explants, average length of shoots, and number of leaves per explants were considered as parameter for evaluating this experiment. After successful shoot proliferation, subculture was done with newly form shoots. Shoots ware excised in aseptic condition with help of sterile scalpel blade and

sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency. The observations on development pattern of shoots were made throughout the entire culture period. Data recording was started after 3rd weeks from inoculation.

3.10 Rooting of multiple shoots

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. One type of growth regulators (IBA) was used in different concentration (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) along with MS media. The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 3rd week of inoculation.

3.11 Acclimatization

Acclimatization or "hardening-off' is a process by which *in vitro* propagated plants are made to adapt to an *in vivo* environment.

Step-1: After 35 days of culture on rooting media, the plantlets were taken out from culture vial with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in gentle warm water to remove any traces of solidified agar media for acclimatization. Plastic pots (6×6 cm) were kept ready filled with garden soil and compost in the proportion of 1:1 respectively. Immediately after removing solidified agar media from newly formed roots, the plantlets were then transplanted in to the pots with special care.

Step-2: After planting, the plantlets were thoroughly watered and were kept at 25±2 °C with light intensity varied from 2000–3000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

Step-3: Then the plants were shifted to shade house with less humidity and indirect sunlight. The top of the pots were covered with transparent plastic sheet and grew at room temperature and 70% RH for 14 days with periodic irrigation (2days intervals).

Step-4: After 3 weeks, the plants were transferred to the soil following depoting and poting into different pot having bigger pot size. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary.

3.12 Data recording:

The observations on development pattern of shoots and roots were made throughout the entire culture period. Five replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 3 and 6 weeks of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week starting from third week to fifth week of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Days for shoot induction
2. No. of shoots per explants
3. Average Length of shoot (cm)
4. Length of longest shoot
5. No. of leaf per explants
6. Days for root induction
7. No. of roots per explants
8. Average length of root (cm)
9. length of the longest root (cm))

3.12.1 Calculation of days to shoots and roots induction

Days to shoots and roots induction were calculated by counting the days from explants inoculation to the first induction of shoots/roots.

3.12.2 Calculation of number of shoots and roots per explants

Number of shoots and roots per explants was calculated by using the following formula,

$$\text{Number of shoots / roots per explants} = \frac{\text{Number of shoots / roots per explant}}{\text{Number of observation}}$$

3.12.3 Calculation of percent of shoots and roots induction from culture:

Number of shoots and roots were recorded and the percentage of shoot and root induction was calculated as:

$$\text{Percent (\%) of shoot induction} = \frac{\text{Number of explants induced shoot}}{\text{Number of explants incubated}} \times 100$$

The percentage of root induction was calculated as:

$$\text{Percent (\%) of root induction} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot incubated}} \times 100$$

3.12.4 Calculation of number of leaf

Numbers of leaves produced on the plantlet were counted and the mean was calculated.

3.12.5 Calculation of shoots and root length (cm)

Shoot and root length were measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the mean was calculated.

3.13 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with five replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment

means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using MSTAT-C (1990) program.

CHAPTER IV

RESULTS AND DISCUSSION

Three separate experiments were performed for the rapid micro propagation of the medicinally important spice Ginger. The overall objective of the present study is to develop a regeneration protocol of Ginger. The results of these experiments were presented and discussed in this chapter with Figure (1-6) and Tables (1-10). Analyses of variance in respect of all the parameters have been presented in Appendices (I-XII).

4.1 Sub-experiment 1. Multiple shoot proliferation in Ginger.

This experiment was conducted under laboratory condition to evaluate the effect of different plant growth regulator on multi shoot proliferation. The relative ratio of auxin to cytokinin has been used in the current investigation. The response of explants to different plant growth regulators in single or in combination varied significantly. The results are presented separately under different headings below.

4.1.1 Effect of KIN on multiple shoot proliferation

The result of the effect of different concentration of KIN have been presented under following headings with Figure 1-5 and Plate 4-7.

4.1.1.1 Percent of explants showing shoot induction

There was significant variation on percent of explants showing shoot induction at different concentrations of KIN. Maximum percentage (64%) of shoot induction was induced in treatment 3.0 mg/L KIN and minimum percentage (28%) was induced in control (Table 1).

Table 1. Effect of KIN on the shoot initiation potentiality in *Zingiber officinale*.

KIN (mg/L)	Number of explants inoculated	Shoot initiation potentiality (%)
1.0	25	40
1.5	25	44
2.0	25	52
2.5	25	56
3.0	25	**64**
0.0	25	28

4.1.1.2 Days for shoot induction

Significant variation was observed among different concentration of KIN on days to shoot induction. The maximum days (36.40 days) to shoot induction were recorded in control treatment and it was minimum 13 days (Figure 1) in 3.00 mg/L of KIN. Baksha, *et al.* (2005) noticed the lateral buds developed into shoots 10 - 15 days after inoculation on MS supplemented with 2 mg/L BAP + 0.5 mg/L NAA.

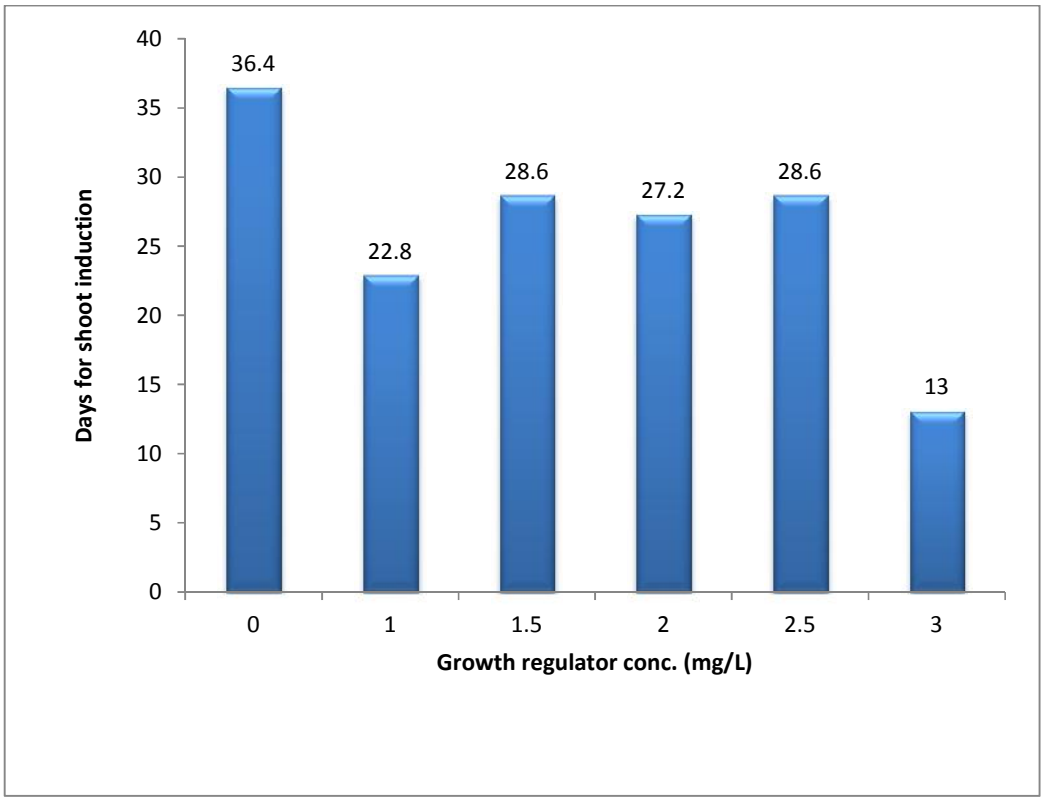


Figure 1: Effect of KIN on days for shoot induction in Ginger.

4.1.1.3 Number of shoots per explants

The influence of different concentration of KIN on the number of shoots per explants. Data were recorded after 3 and 6 weeks of culture on MS media. The results have been presented in Figure 2 and Plate 4. There was no significant variation at 3 weeks after inoculation (WAI) among different concentration of KIN. 3.00 mg/L KIN gave the highest number of shoots (5.6) whereas the lowest number of shoots (1.2) was found at 6 WAI with hormone free media (Figure 2). BAP variations affecting shoot proliferation were also reported by (Bhandari *et al.*, 2010; Gantait *et al.*, 2010). Baksha, *et al.* (2005) noticed 3.2

shoot per explant in media supplemented with 2.0mg/L BAP. As Qu *et al.* (2000) reported high cytokinin level present in the medium causing cytogenetic instability thus unsuitable for clonal propagation. Moreover, most of the shoots so formed were stunted.

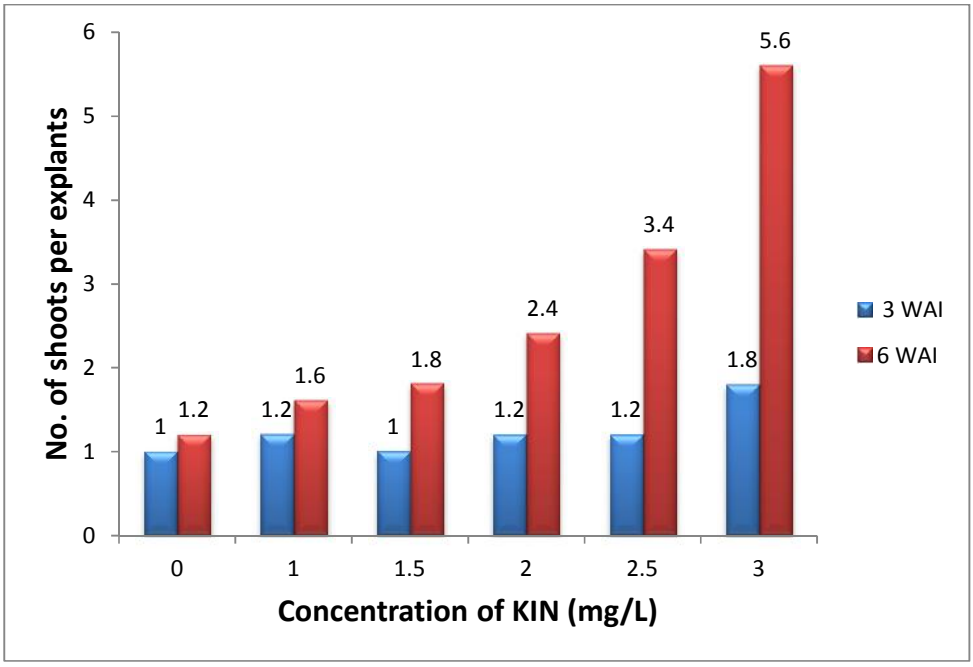
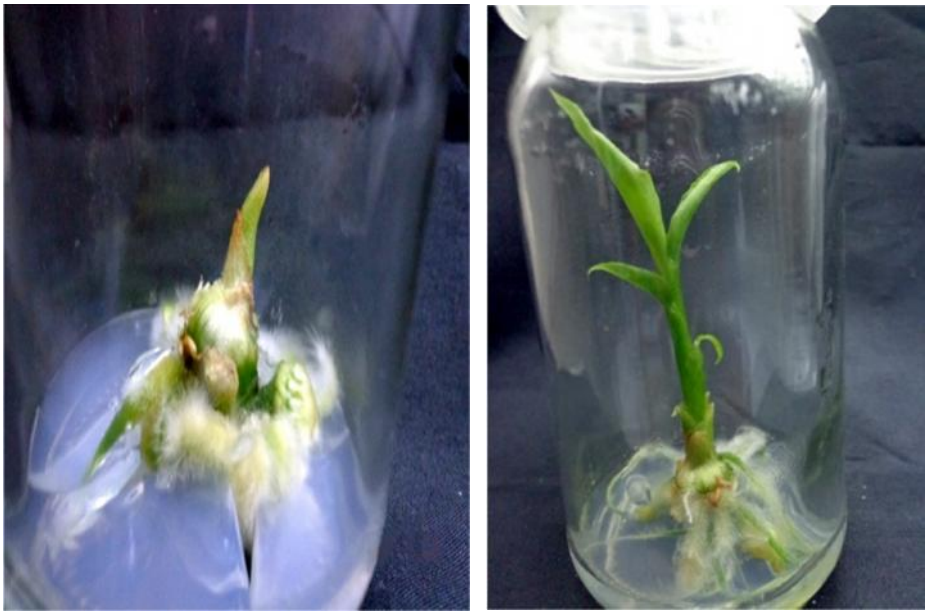


Figure 2: Effect of 3.0 mg/L KIN on the number of shoots per explants in Ginger.



A **B**

Plate 4: Effect of 3.0 mg/L KIN on the number of shoots per explants in Ginger (A) after 3 weeks (B) after 6 weeks of inoculation.

4.1.1.4 Number of leaf per explants

Significant influence was found on the number of leaf. The maximum 7.2 leaves were recorded with 3 mg/L KIN and the minimum 1.4 in case of lack of hormone (Figure 3 and Plate 5). It is observed that callus culture of ginger was induced on MS medium from young leaf segments gave best shooting response.

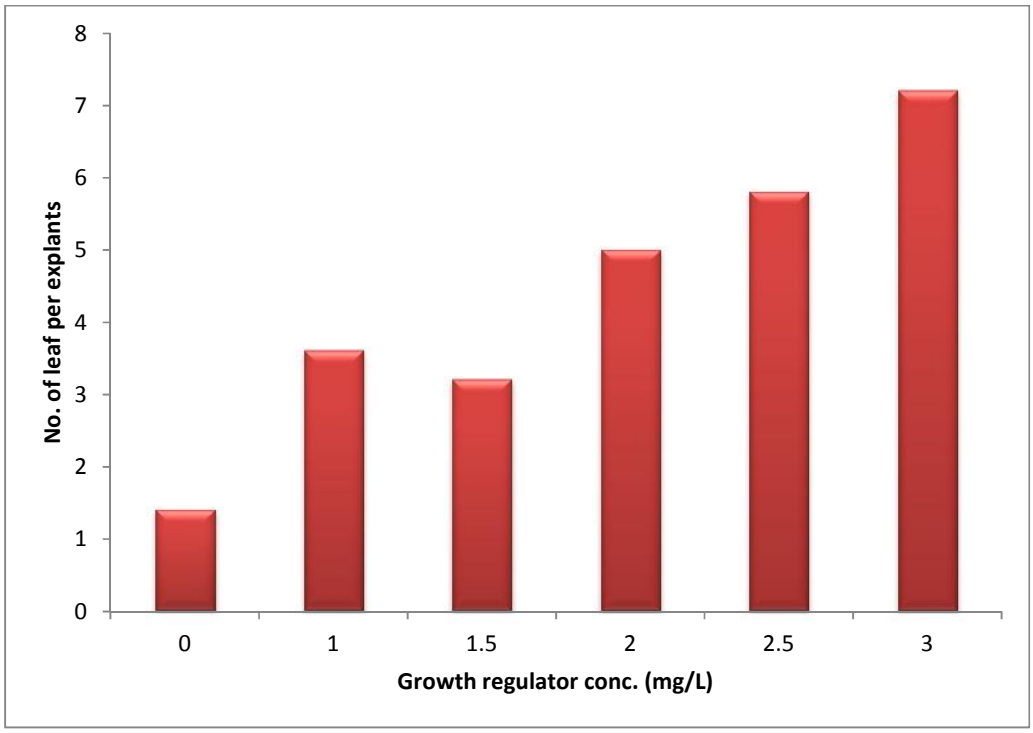


Figure 3: Effect of KIN on number of leaf per explants in Ginger.

Plate 5: Effect of 3.0mg/L KIN on the number of leafs per explants after 6 WAI

4.1.1.5 Average length of shoot (cm)

The results of average length of shoots have been presented in Figure 4. The maximum average length of shoot 2.68cm was noticed from the 2.5 mg/L KIN which was statistically similar with 2.00 mg/L KIN (2.6cm) and statistically different from rest of others whereas the minimum 1.12 cm in control

treatment. (Figure 4). Baksha, *et al.* (2005) noticed 2.5 cm length of shoot in 4.0 mg/L BAP.

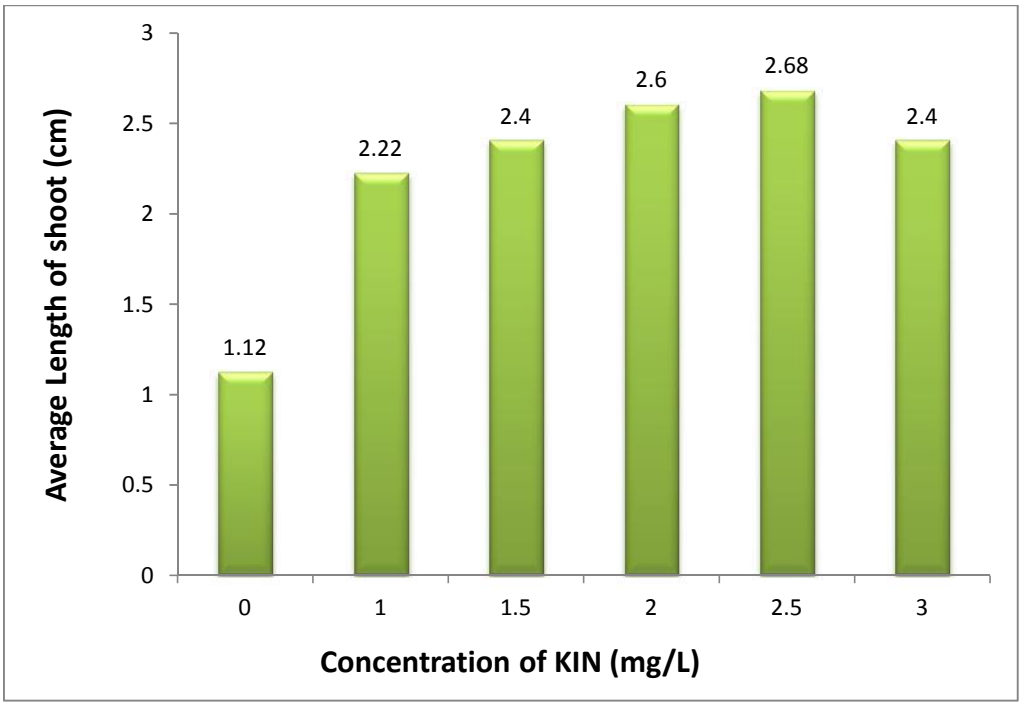


Figure 4: Effect of KIN alone on average length of shoot (cm) in Ginger.

4.1.1.6 Length of longest shoot (cm)

The results of length of longest shoots have been presented in Figure 5. The maximum length of longest shoot 3.66 cm was noticed from the 2.5 mg/L KIN which was statistically similar with 2.0 mg/L KIN (3.36cm) and statistically

different from rest of others whereas the minimum 1.5 cm in control (Figure 5). Baksha, *et al.* (2005) noticed 2.5 cm length of shoot in 4.0 mg/L BAP.

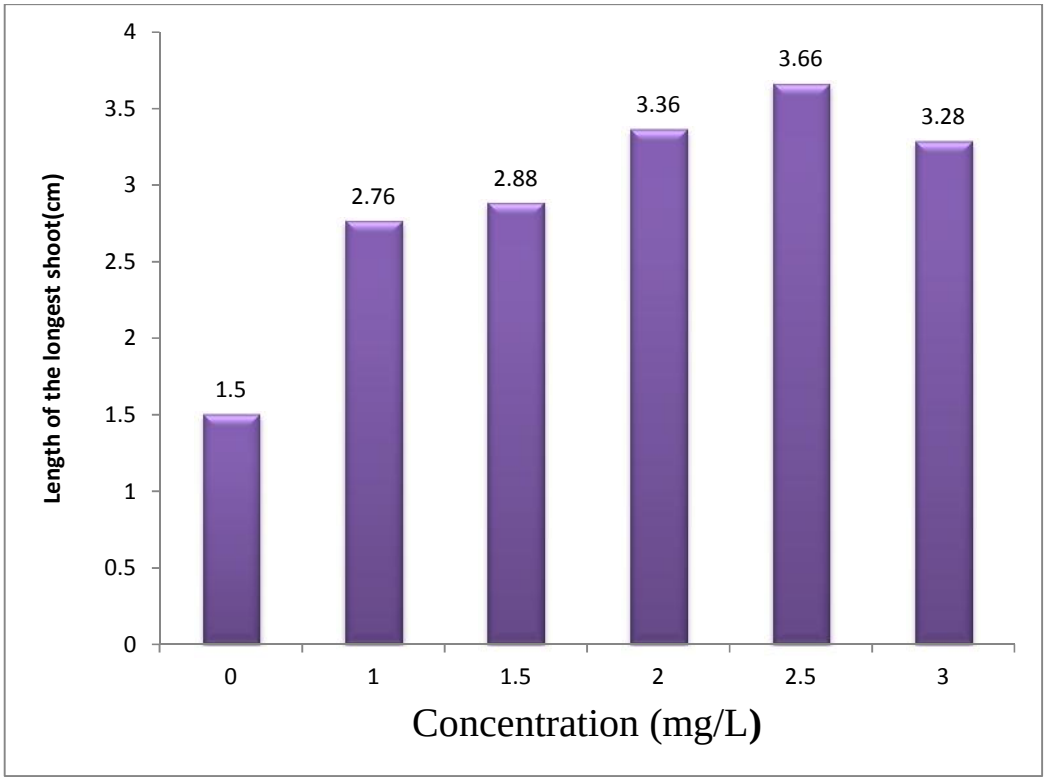


Figure 5: Effect of KIN alone on length of longest shoot in Ginger.

4.1.2 The combine effect of KIN + GA_3 on multiple shoot proliferation

The result of the combined effect of different concentration of KIN + GA_3 have been presented under following headings with Table 2-6.

4.1.2.1 Percent of explants showing shoot induction

The different concentrations of KIN and GA_3 showed significant variations on regeneration potentiality in Gingers (Table 2). The highest regeneration potentiality (90%) was observed in 3.0 mg/L KIN + 1.5 mg/L GA_3 and the lowest regeneration potentiality (28%) was observed in control (Table 2).

Table 2. Combined effect of KIN and GA_3 on percent of explant showing shoot induction

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explant inoculated	Percent of explants showing shoot induction
KIN+GA_3	1.0+0.25	20	42
	1.0+0.5	20	40
	1.0+0.75	20	43
	1.0+1.0	20	43
	1.0+1.5	20	44
	1.5+0.25	20	46
	1.5+0.5	20	48
	1.5+0.75	20	49
	1.5+1.0	20	44
	1.5+1.5	20	46
	2.0+0.25	20	47
	2.0+0.5	20	50
	2.0+0.75	20	52
	2.0+1.0	20	54
	2.0+1.5	20	55
	2.5+0.25	20	57
	2.5+0.5	20	59

	2.5+0.75	20	61
	2.5+1.0	20	60
	2.5+1.5	20	60
	3.0+0.25	20	70
	3.0+0.5	20	72
	3.0+0.75	20	80
	3.0+1.0	20	85
	3.0+1.5	20	**90**

4.1.2.2 Days for shoot induction

Variation was observed among different concentration of KIN+GA3 on days to shoot induction. The maximum days to shoot induction were recorded in control (36.40 days) and 3 mg/LKIN+0.75 mg/L GA3 required minimum 12.2 days (Table 3). Baksha, R. *et al.* (2005) noticed the lateral buds developed into shoots 10 - 15 days after inoculation on MS supplemented with 2 mg/L BAP + 0.5 mg/L NAA.

Table 3. Combined effect of KIN and GA3 on shoot proliferation potentiality

combination of growth regulator	Concentration	Days for shoot induction	Number of shoots per explants	
			3 WAI	6 WAI
Control	0	36.4 a	1 d	1.2 d
KIN+GA3	1.0+0.25	21.6 b	1.6 bcd	3 bc
	1.0+0.5	19 cdef	1.8 bcd	2.8 bc
	1.0+0.75	18.4 defg	2 bcd	3.2 bc
	1.0+1.0	17.4 fghi	1.4 cd	3 bc
	1.0+1.5	16.8 ghij	1.4 bcd	3.2 bc
	1.5+0.25	17.4 fghi	2 bcd	3.4 bc
	1.5+0.5	15.2 jk	2.4 bc	3.4 bc
	1.5+0.75	14.8 jk	1.8 bcd	3.6 bc
	1.5+1.0	16 hij	2 bcd	3 bc
	1.5+1.5	15.6 ijk	1.8 bcd	2.6 c
	2.0+0.25	14.8 jk	1.8 bcd	3.2 bc
	2.0+0.5	20.6 bc	1.6 bcd	3.6 bc
	2.0+0.75	18.6 defg	2.4 bc	3 bc

	2.0+1.0	20	bcd	1.8	bcd	3.8	bc
	2.0+1.5	19.8	bcde	1.8	bcd	3.6	bc
	2.5+0.25	17.6	fghi	1.8	bcd	3.6	bc
	2.5+0.5	18	efgh	2	bcd	3.6	bc
	2.5+0.75	16.8	ghij	2.6	b	3.8	bc
	2.5+1.0	16.8	ghij	1.8	bcd	3.2	bc
	2.5+1.5	17.4	fghi	1.8	bcd	3.8	bc
	3.0+0.25	14.8	jk	2.4	bc	3.8	bc
	3.0+0.5	15.2	jk	2.2	bc	4	b
	3.0+0.75	12.2	l	1.6	bcd	3	bc
	3.0+1.0	16.2	hij	2	bcd	4	b
	3.0+1.5	13.8	kl	4.6	a	6.4	a
	CV (%)	7.91		8.91		24.62	

*WAI=Weeks After Inoculation. Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

4.1.2.3 Number of shoots per explants

Data were recorded after 3 and 6 weeks of culture on MS media. There was significant influence of different concentration of KIN+GA3 on the number of shoots per explants after 3rd weeks of inoculation. The results have been presented in table 3. There was significant variation at 3WAI among different concentration of KIN+GA3. But 3.0 mg/LKIN+1.5 mg/L GA3 gave the highest number of shoots (4.6 and 6.4 at 3WAI and 6WAI) (Plate 6) respectively whereas the lowest number of shoots (1.00 and 1.20 at 3WAI and 6WAI) respectively was found with hormone free media . Kanker *et al*. (1993) observed that callus culture of ginger was induced on MS medium from young leaf segments gave best shooting response. Sultana *et al.* (2009) reported that percent shoot induction were highest (76.33%) by the interaction of Suruchi x 1.0 mg/L KIN +1.0 mg/l BAP. Mohammed and Quraishi (1999) observed that maximum number of shoots was achieved at 1.5 mg/L BAP.

Plate 6: Effect of 3.0mg/ L KIN + 1.5 mg/L GA_3 on the production of maximum number of shoot per explants.

4.1.2.4 Number of leaf per explants

The Number of leaf per explants was significantly different according to the various concentrations of KIN+GA_3. The results have been presented in Table 4. Significantly the highest number of leaf per explants (10.00) was noticed from 3.0 mg/L KIN+1.5 mg/L GA_3, whereas the lowest was 1.4 in control at 6WAI. Mohammed *et al*. (2011) reported augmentation of MS-medium with 4.5 mg/l BAP recorded the highest number leaves 15.50.

Table 4. Combined effect of KIN and GA_3 on number of leaf per explants potentiality.

Combination of growth regulator	Concentration	No. of leaf per explants
Control	0	1.40 g
KIN+GA3	1.0+0.25	3.60 f
	1.0+0.5	3.20 f
	1.0+0.75	4.00 f
	1.0+1.0	3.20 f
	1.0+1.5	3.60 f
	1.5+0.25	5.20 e
	1.5+0.5	6.00 bcde
	1.5+0.75	6.40 bcde
	1.5+1.0	6.40 bcde
	1.5+1.5	7.40 b

	2.0+0.25	5.40	de
	2.0+0.5	6.00	bcde
	2.0+0.75	5.80	cde
	2.0+1.0	6.00	bcde
	2.0+1.5	6.80	bcd
	2.5+0.25	6.80	bcd
	2.5+0.5	7.00	bc
	2.5+0.75	6.60	bcde
	2.5+1.0	6.20	bcde
	2.5+1.5	6.20	bcde
	3.0+0.25	6.00	bcde
	3.0+0.5	5.80	cde
	3.0+0.75	6.40	bcde
	3.0+1.0	6.40	bcde
	3.0+1.5	10.00	a
	CV (%)	16.64	

4.1.2.5 Average length of shoot (cm)

With different concentration of KIN+GA_3, significant influence was found on the average length of shoot (cm) the results have been presented in Table 5. The average length of shoot (4.56 cm) was noticed from the 3.00 mg/L KIN+1.50 mg/L GA_3, whereas the minimum 1.12 cm in control. Hashembadi, D, and Kaviani, B. (2010) obtained the best proliferation of shoot per explant (9.67) on medium supplemented with 0.5 mg/L BA + 0.5 mg/L NAA. Baksha, R. *et al.* (2005) noticed the highest average length of shoot (4.0 cm) on MS supplemented with 2 mg/L BAP + 0.5 mg/L NAA.

Table 5. Combined effect of KIN and GA_3 on the average length of shoot in Ginger.

combination of growth regulator	Concentration (Mg/L)	Average Length of shoot (cm)
Control	0	1.12 f
KIN+GA3	1.0+0.25	3.06 Bcd
	1.0+0.5	2.86 e
	1.0+0.75	2.98 cde
	1.0+1.0	2.92 de
	1.0+1.5	3.02 bcde
	1.5+0.25	3.2 b
	1.5+0.5	2.98 cde
	1.5+0.75	3.02 bcde
	1.5+1.0	3.04 bcde
	1.5+1.5	3.06 bcd
	2.0+0.25	3.08 bcd
	2.0+0.5	3.1 bcd
	2.0+0.75	3.14 bc
	2.0+1.0	3.08 bcd
	2.0+1.5	3.04 bcde
	2.5+0.25	3.06 bcd
	2.5+0.5	2.96 cde
	2.5+0.75	3.04 bcde
	2.5+1.0	3 cde
	2.5+1.5	3.08 bcd

	3.0+0.25	2.98	cde
	3.0+0.5	2.94	de
	3.0+0.75	2.98	cde
	3.0+1.0	3.08	bcd
	3.0+1.5	4.56	a
	CV (%)	4.06	

Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

4.1.2.6 Length of longest shoot (cm)

With different concentration of KIN+GA3, significant influence was found on the length of longest shoot (cm) the results have been presented in table 6 and Plate 7. The length of longest shoot (4.80 cm) was noticed from the 3.00 mg/L KIN+1.50 mg/L GA_3, whereas the minimum 1.50 cm in control. Hashembadi, and Kaviani, (2010) obtained the best proliferation of shoot per explant (9.67) on medium supplemented with 0.5 mg/L BA + 0.5 mg/L NAA. Baksha, R. *et al.* (2005) noticed the highest average length of shoot (4.0 cm) on MS supplemented with 2 mg/L BAP + 0.5 mg/L NAA.

Plate 7: Combined effect of 3.0mg/L KIN + 1.5mg/L GA_3 on the length of longest shoot per explants.

Table 6. Combined effect of KIN and GA_3 on the Length of longest shoot in Ginger.

combination of growth regulator	Concentration	Length of the longest shoot(cm)
Control	0	1.50 m
KIN+GA_3	1.0+0.25	3.42 l
	1.0+0.5	3.74 fghij
	1.0+0.75	3.96 bcdef
	1.0+1.0	3.42 kl
	1.0+1.5	3.66 hij
	1.5+0.25	3.76 fghij
	1.5+0.5	3.68 hij
	1.5+0.75	3.64 hijk
	1.5+1.0	3.86 cdefgh
	1.5+1.5	4.02 bcde
	2.0+0.25	3.98 bcdef
	2.0+0.5	3.80 efghi
	2.0+0.75	3.74 fghij
	2.0+1.0	4.14 b
	2.0+1.5	3.84 cdefghi
	2.5+0.25	3.80 defghi
	2.5+0.5	3.96 bcdef
	2.5+0.75	4.06 bc
	2.5+1.0	3.92 bcdefg
	2.5+1.5	4.04 bcd
	3.0+0.25	3.96 bcdef
	3.0+0.5	3.70 ghij

	3.0+0.75	3.60 ijkl
	3.0+1.0	3.54 jkl
	3.0+1.5	4.80 a
	CV (%)	4.26

4.2 Sub-experiment 2. Root induction in Ginger.

To develop root in the regenerated shoots, they were excised and transferred to rooting media supplemented with IBA. The results of experiment have been presented under different headings utilizing Figure 6 and Table 7.

4.2.1 Percent of explants showing root induction

There were comparable variations among growth regulators on percent of explants showing root induction. The results have been shown in (Table 7). The highest percentage (95%) of root induction was recorded with 2.5 mg/L IBA. The lowest percentage (30%) of root induction was recorded in control. The percent rooting and root initiation were better in MS medium with higher concentration of IBA as compared to lower concentration of IBA in the current study.

Table 7. Effect of plant growth regulator IBA on percent of explants showing root induction in *Zingiber officinale*

Name of the hormone	Hormone concentration (mg/L)	% explants showing root induction
Control	0.0	30
IBA	0.5	70

	1.0	75
	1.5	80
	2.0	85
	2.5	**95**

4.2.2 Days for root induction

Hormonal concentration has significant level of variation on days to root induction. The maximum 20.6 days to root induction was required in media lack of growth regulator. Minimum 14.4 days was required in 2.5 mg/L IBA (Figure 6). Baksha, *et al.* (2005) noticed that roots began to emerge from the tenth day of in the medium with 0.5 mg/L of NAA.

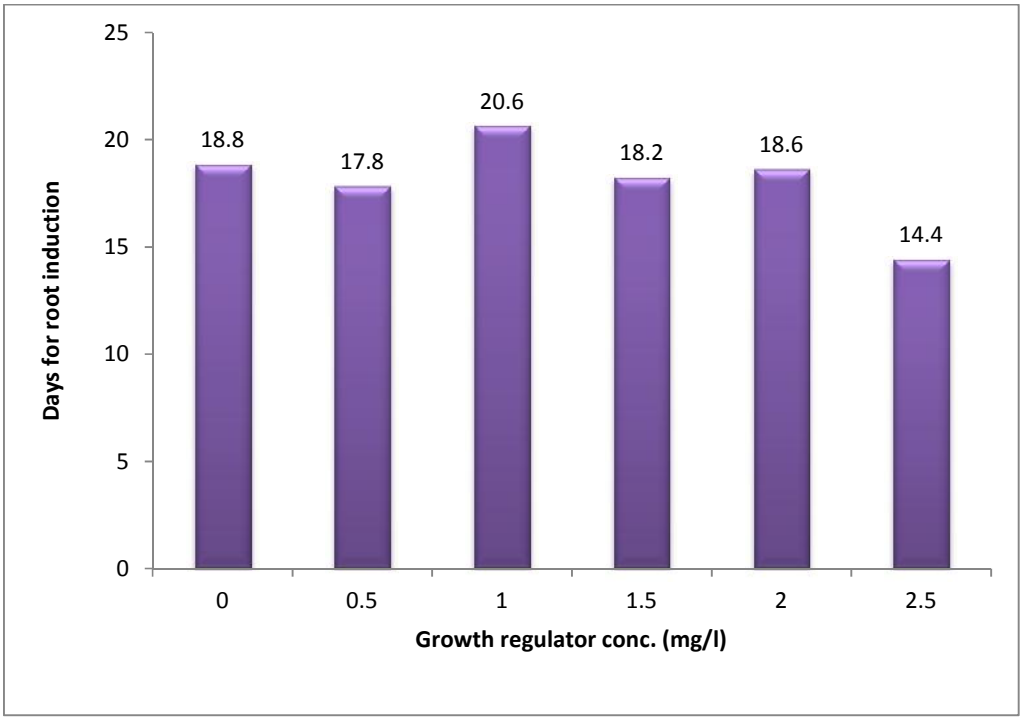


Figure 6: Effects of IBA on days for root induction

4.2.3 No. of roots per explants

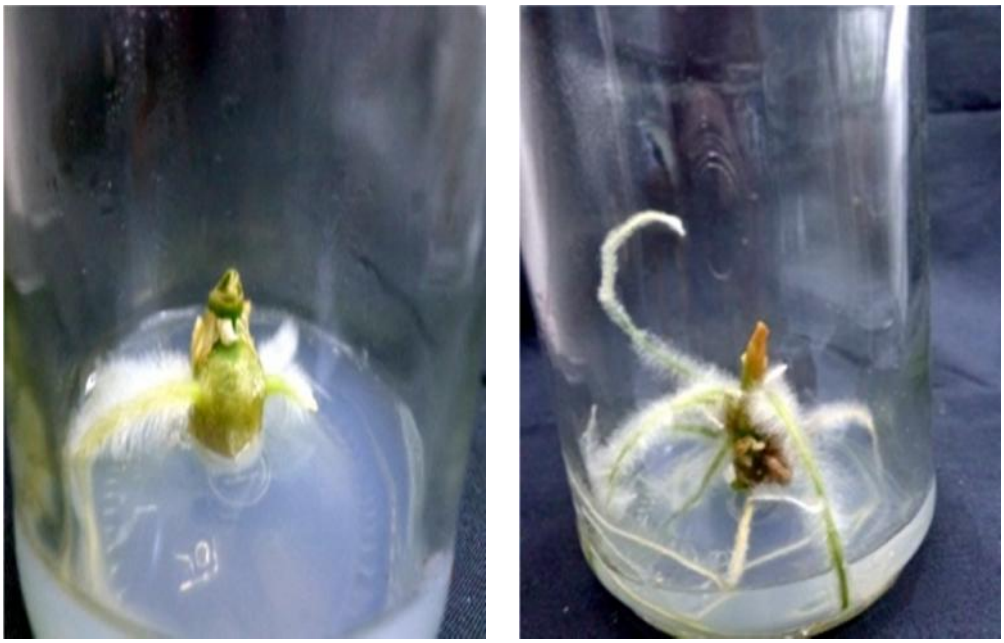
Significant variations were observed during data recording at 3WAI and 6WAI. Roots regenerated in the medium containing IBA were thin and long during initial stage of development (Plate 8).In case of IBA, the highest number of roots (5.8) per explants was recorded in 2.5 mg/L IBA at 3WAI, which was statistically different with 2 mg/L IBA (3.8). But the highest number of roots (10.0) was found at 6 WAI was in the treatment 2.5 mg/L IBA (Plate -8b). The minimum number of roots (0.6, and 0.6 at 3 WAI and 6 WAI) respectively were obtained in control (Table 8). Dwivedi, *et al.* (2014) found 10 roots in medium with IBA (0.5 mg/ L) in 8 weeks of time. Bhandari, A.K. *et al.* (2010) reported 2.6 roots in IBA 0.2 mg/L after 15 days of culture. Baksha, *et al.* (2005) noticed 3.2 roots per explants in IBA 1.5 mg/L.

Table 8. Effect of IBA on number of roots *in Ginger*.

Treatments	Concentration of IBA (mg/L)	Days for root induction	Number of roots per explants	
			3WAI	6WAI
Control	0	18.8 b	0.6 c	0.6 d
IBA	0.5	17.8 b	3.00 b	4.6 c
	1.0	20.6 a	3.6 b	5.8 b
	1.5	18.2 b	3.8 b	5.8 b

	2.0	18.6 b	3.4 b	5.6 b
	2.5	14.4 c	5.8 a	10 a
	CV (%)	39.51	25.49	15.31

*WAI=Weeks After Inoculation. Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.



A B

Plate 8: Effect of 2.5mg/L IBA on the number of Root A) 3 WAI and B) 6 WAI

4.2.4 Average length of roots per explants (cm)

Average length of roots per explants (cm) was greatly regulated by the different concentration of IBA. The maximum average root length (10.00 cm) was obtained from 2.5 mg/L IBA (Table 9). The minimum 1.26 cm average length of roots per explants (cm) was in control. Jafari and Hamidoghli (2009) explained that concentration of 2 mg /L IBA has given a bigger number of roots and the maximum root length. Dwivedi, N.K. *et al.* (2014) found length 6 cm were obtained in medium with IBA 0.5 mg/ L in 8 weeks of time. Daneshvar, M.H. *et al.* (2013) noticed 6.32 cm root length in 1.0 mg/L IBA. Hashembadi, D, and Kaviani, B. (2010) obtained the maximum (8.75 cm) roots were achieved on medium supplemented with 1 mg/L IBA + 1 mg/L NAA. Baksha, R. *et al.* (2005) noticed the highest average length of 3.5 cm with 0.5 mg/L NAA and 2.2 cm in IBA 1.5 mg/L.

Table 9. Effect of IBA on average length of roots per explants (cm) in Ginger

Name of the hormon	Concentration of IBA (mg/L)	Average Length of root	Length of the longest root
Control	0	1.26 d	1.94 e
IBA	0.5	4.22 c	5.82 d

	1.o	4.12 c	6.30 cd
	1.5	5.28 b	7.04 c
	2.0	5.48 b	8.30 b
	2.5	10.00 a	14.20 a
	CV (%)	10.56	10.47

Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

4.2.5 Length of longest roots (cm)

Different treatments of IBA play a vital role on length of longest roots (cm). The length of longest root was 14.2 cm with 2.5 mg/L IBA (Table 9 and plate 9). The least root length (1.94 cm) was reported from growth hormone free culture media. Hashemabadi and Kaviani (2010) were found the maximum 4.3 mm diameter of root on medium supplemented with 1 mg/L IBA + 1 mg/L NAA. Jafari and Hamidoghli (2009) explained that concentration of 2 mg /L IBA has given a bigger number of roots and the maximum root length. Dwivedi, N.K. *et al.* (2014) found length 6 cm were obtained in medium with IBA 0.5 mg/ L in 8 weeks of time. Daneshvar, M.H. *et al.* (2013) noticed 6.32 cm root length in 1.0 mg/L IBA. Hashembadi, D, and Kaviani, B. (2010) obtained the maximum (8.75 cm) roots were achieved on medium supplemented with 1 mg/L IBA + 1 mg/L NAA. Baksha, *et al.* (2005) noticed the highest average length of 3.5 cm with 0.5 mg/L NAA and 2.2 cm in IBA 1.5 mg/L.

Plate 9: Effect of 2.5mg/L IBA length of longest roots (cm)

4.3 Experiment 3. Acclimatization of plantlets

The results of acclimatization or "hardening-off' have been presented in Table 10 and Plate 10. After 35 days of culture on rooting media, the plantlets were taken for acclimatization.

Table 10. Survial rate of *in vitro* regenerated plants of Ginger

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)

In growth chamber	15	7days	15	100
In shade house	15	14 days	12	80
In pot culture condition	15	30 days	13	86

The results of acclimatization showed that the 100% of plantlets were survived to culture room (Table 10 and Plate 10). Then the plantlets were shifted to shade house with less humidity (70% RH) and indirect sunlight (Plate 10). In the shade house, the top of the pots were covered with transparent plastic sheet and grew at room temperature for 14 days with periodic irrigation (2 days interval). In these conditions, the 80% of the plantlets showed survival (Table 10). After 3 weeks, the plantlets were transferred to the soil following depoting and poting into different pots of bigger size. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary (Plate 10 and pic B). In open atmosphere, survival rate was 86% (Table 10). It was also revealed that regenerated plants were morphologically similar to the mother plant.

Dwivedi, N.K. *et al.* (2014) found that the plantlets were regenerated in green house, *in-vitro* produced plants were successfully established in soil, with almost 90% survival.

Plate A: In culture room

Plate B: In shade house

Plate C: In field condition

Plate 10: Acclimatization of ginger plantlets (Plate A, B, C)

CHAPTER V

SUMMARY AND CONCULSION

The present research was carried out in Biotechnology Lab. of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of september 2013 to July 2014 to evaluate the effect of different plant growth regulator on multi shoot proliferation and root formation along with acclimatization for *in vivo* survival.

The healthy, disease free shoot tips of 1-2cm length were used as explants for the study for *in vitro* regeneration. The major findings have been presented below.

Maximum shoot induction(90%) was found at 3.0 mg/L KIN + 1.5 mg/L GA_3 and minimum (28%) in control. Root induction was maximum (95%) at 2.5 mg/L IBA.

Significant variation was observed among different concentration of KIN on days to shoot induction. The maximum days to shoot induction were recorded in control (36.40 days). With different concentration of KIN, significant influence was found on the number of leaf. The maximum 7.2 leaves were recorded with 3 mg/L KIN. The maximum average length of shoot 2.68 was noticed from the 2.5 mg/L KIN.

Significant variation was observed in combination of two hormones on days to shoot induction. The maximum days to shoot induction were recorded in control (36.40 days) and 3 mg/LKIN+0.75 mg/L GA3 required minimum 12.2 days.

The highest number of shoot (4.6) were recorded in 3.0 mg/L KIN+ 1.5 mg/L GA_3 and the lowest number of shoot (1.00 and 1.20 at 3WAI and 6WAI) respectively was found with hormone free media.

The number of leaf per explants was significantly different according to the various concentrations of KIN+GA_3 supplemented. The maximum number of leaf per explants (10.00) was noticed from 3.0 mg/L KIN+1.5 mg/L GA_3, whereas the minimum 1.4 in control. The average length of shoot (4.56 cm) was noticed from the 3.00 mg/L KIN+1.50 mg/L GA_3, whereas the minimum 1.12 cm in control. The length of longest shoot (4.80 cm) was noticed from the 3.00 mg/L KIN+1.50 mg/L GA_3.

The maximum 20.6 days to root induction was required in media devoid of growth regulator. Atleast 14.4 days was required in case of 2.5 mg/L IBA.

The highest number of roots (5.8) per explants was recorded in 2.5 mg/L IBA at 3WAI. Average length of roots per explants was greatly regulated by the different concentration of IBA. The maximum average root length (10.00 cm) was obtained from 2.5 mg/L IBA. The length of longest root was 14.2 cm with 2.5 mg/L IBA.

Regenerated plantlets showed 100% survival during in cuture room conditions and 80% in shade house stage of hardening and 86% in open atmosphere. Regenerated plants were found to be morphologically similar to the mother plant. Findings of the present study showed that micropropagation is effective method in the proliferation of *Zingiber officinale* and this experiment can be a useful tool for proliferation of *Zingiber officinale*.

CONCLUSION

Following conclusions can be made from the present study:

i. A regeneration protocol has been developed in Ginger.

ii. Overall moderate higher dose (3.0 mg/L KIN) showed better response *in vitro* regeneration in Ginger.

iii. Combined effect of KIN and GA_3 doses seems to be better than individual effect of KIN, for shoot formation and IBA doses showed better performance for root formation in ginger.

CHAPTER VI

RECOMMENDATIONS

Following recommendations could be addressed based on the present experiment:

1) Different combinations of cytokinin and auxin need to be verified for regeneration of ginger.

2) Rather than a sprouts, meristem as an explants and callus culture could be practiced.

3) To uncover the influence of genotype if any, research should be carried on with different types of genotype of Ginger.

4) Precise and detailed investigation on influence of other factors such as different elicitors, antioxidants on *in vitro* regeneration should be considered.

5) Further study may be conducted incorporating higher dose of growth regulators.

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APPENDICES

Appendix I. Composition of MS (Murashige and Skoog, 1962) medium including vitamins

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**mg/L**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**mg/L**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L

Appendix II. Analysis of variance on days to shoot induction

Source	Degrees of Freedom	Sum of squares	Mean Square	F -value	Probability
Replication	4	12.723	3.181	1.6156	0.1762
Treatment	25	2407.508	96.3	48.914	0
Error	100	196.877	1.969		
CV (%)	7.91				

Appendix III. Analysis of variance on no. of shoots per explants at 3 WAI

Source	Degrees of Freedom	Sum of squares	Mean Square	F- value	Probability
Replication	4	2.815	0.704	1.1892	0.3202
Treatment	25	50.931	2.037	3.4422	0
Error	100	59.185	0.592		
CV (%)	8.91				

Appendix IV. Analysis of variance on no. of shoots per explants at 6 WAI

Source	Degrees of Freedom	Sum of squares	Mean Square	F- value	Probability
Replication	4	6.108	1.527	2.16	0.079
Treatment	25	86.769	3.471	4.9097	0
Error	100	70.692	0.707		
CV (%)	24.62				

Appendix V. Analysis of variance on the number of leaves

Source	Degrees of Freedom	Sum of squares	Mean Square	F- value	Probability
Replication	4	3.723	0.931	1.0402	0.3904
Treatment	25	360.869	14.435	16.1324	0
Error	100	89.477	0.895		
CV (%)	16.64				

Appendix VI. Analysis of variance on Average length of shoot

Source	Degrees of Freedom	Sum of squares	Mean Square	F- value	Probability
Replication	4	0.11	0.028	1.8413	0.1268
Treatment	25	30.534	1.221	81.5501	0
Error	100	1.498	0.015		
CV (%)	4.06				

Appendix VII. Analysis of variance on length of longest shoot

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Replication	4	0.159	0.04	1.5538	0.1926
Treatment	25	35.609	1.424	55.7022	0
Error	100	2.557	0.026		
CV (%)	4.26				

Appendix VIII. Analysis of variance on Days for root induction

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Replication	4	198.867	49.717	0.9757	
Treatment	5	103.867	20.773	0.4077	
Error	20	1019.133	50.957		
CV (%)	39.51				

Appendix IX. Analysis of variance on No. of roots per explants 3 WAI

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Replication	4	0.467	0.117	0.1584	
Treatment	5	69.767	13.953	18.9412	0
Error	20	14.733	0.737		
CV (%)	25.49				

Appendix X. Analysis of variance on no. of roots per explants 6 WAI

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Replication	4	5.533	1.383	2.0244	0.1296
Treatment	5	226	45.2	66.1463	0
Error	20	13.667	0.683		
CV (%)	15.31				

Appendix XI. Analysis of variance on Average length of roots per explants

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Replication	4	4.039	1.01	3.5394	0.0244
Treatment	5	203.288	40.658	142.5249	0
Error	20	5.705	0.285		
CV (%)	10.56				

Appendix XII. Analysis of variance on Length of longest roots

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Replication	4	6.437	1.609	2.7803	0.055
Treatment	5	402.955	80.591	139.246	0
Error	20	11.575	0.579		
CV (%)	10.47				