IN VITRO REGENERATION OF GINGER

(*Zingiber officinale* Rosc.)

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DHAKA-1207

JUNE 2006

IN VITRO REGENERATION OF GINGER

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BY

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REGISTRATION NO. 04-01527

A Thesis
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 (MS)

IN

HORTICULTURE

SEMESTER : JANUARY- JUNE 2006

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DECLARATION

This is to certify that the thesis entitled, "***In vitro*** **Regeneration of Ginger"** 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 HORTICULTURE**, embodies the result of a piece of bonafide research work carried out by **ABU NASER MD. MAMUN, Registration No. 04-01527** under my supervision and my guidance. No part of the thesis has been submitted for any other degree in any institutes.

I further certify that any help or sources of information, received during the course of this investigation have been duly acknowledged.

Dated :
Dhaka, Bangladesh

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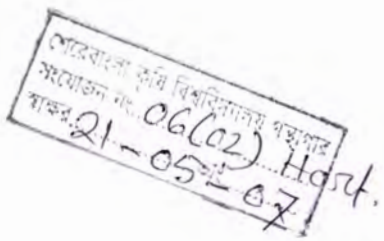

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TO MY

BELOVED PARENTS

LIST OF ABBREVIATION

ABBREVIATION	FULL NAME
BAP	= 6-benzylaminopurine
NAA	= 1-naphthylacetic acid
MS	= Murashige and Skoog
mg/L	= Milligram per litre
µM	= Micromole
2,4-D	= 2,4-dichlorophenoxyacetic acid
ppm	= Parts per million
EDTA	= Ethylenediaminetetraacetic acid

ACKNOWLEDGEMENTS

All praises are due to Allah, the almighty and creator for mercy and guidance in the completion of the thesis leading to MS degree and for guiding the author towards the right path of peace and success.

The author wishes to express deepest gratitude, profound appreciation and immense indebtness to his Supervisor Dr. Md. Al-Amin, Principal Scientific Officer and Head, Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur for his assistance in planning and valuable guidance during the execution of the study. He is also grateful to Dr. Al-Amin for his constructive instruction and heartiest cooperation during the preparation of the manuscript.

The author is indebted and grateful to his venerable Co-supervisor Dr. Md. Nazrul Islam, Chairman, Department of Horticulture and Postharvest Technology, SAU, Dhaka for his valuable assistance and advice in completing this thesis.

The author respectfully acknowledges to Md. Hasanuzzaman Akand, Assistant Professor, Department of Horticulture and Postharvest Technology, SAU, Dhaka.

The author is also grateful to BARI authority for provision of facilities and support needed to undertake this research work during the entire period of research work and thesis preparation.

The author expresses cordial thanks to Hasan, Reza and the Staff of Biotechnology Division, BARI, Joydebpur, Gazipur for their kind cooperation, encouragement and help in experiment setting and data collection through the entire period of research.

The author would like to express his best gratitude and feeling to his beloved parents, wife and relatives for their blessings, inspiration and cooperation in all phases for his academic life.

He is indebted to Md. Monowar Hossain, Typist for his assistance while preparing the manuscript.

The Author

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ABSTRACT

An experiment was conducted at the Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur on *In vitro* Regeneration of Ginger (*Zingiber officinale*). The experiment was undertaken to find out the suitable combination of plant growth regulators with appropriate concentration for successful *in vitro* propagation of ginger from shoot tip on MS media. The explant of one genotype of ginger was cultured in MS medium with different concentrations and combinations of NAA (0, 1.0, 2.0, 3.0, 4.0 mg/L) and BAP (0, 1.0, 2.0, 3.0, 4.0 mg/L) to observe the performance of regeneration. The rate of shoot multiplication was varied in different treatment combinations. The highest number of multiple shoots (7.10) was recorded in 1.0 mg/L NAA + 3.0 mg/L BAP. It was the lowest (2.42) in MS medium without growth regulators. The maximum number of roots (9.61) and vigorous roots was obtained in 1.0 mg/L NAA + 3.0 mg/L BAP. Days required for shoots initiation is also influenced by NAA and BAP with different combinations. The highest number of leaves (5.72) was found in 1.0 mg/L NAA + 4.0 mg/L BAP. The minimum time (20.67days) required for shoot initiation was obtained in 1.0 mg/L NAA + 3.0 mg/L BAP.

CHAPTER I

INTRODUCTION

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INTRODUCTION

Ginger (*Zingiber officinale*) belongs to the Zingiberaceae family is an important major spices in Bangladesh as well as in the world. It is a herbaceous perennial with underground rhizomes serial leafy shoots, leaves sheathy, alternately arranged linear with long and sessile flowers borne on a spike. Ginger is highly valued from the ancient period for its aroma and flavour. It has a carminative and stomachic properties and its essential oil has antifungal properties (Barthakur, 1992). In western countries, ginger is widely used in ginger bread, biscuits, cakes, puddings, soups and pickles. It is used in the production of ginger bear, gingerly and ginger wine. It can be used daily exclusive by all classes of people in the preparation of tasty curry to add typical flavour and pungency. It is also used in many Aurvedic medicines and now a days is being used as chewing ginger. Ginger is a native of tropical South East Asia. It is a plant of very ancient cultivation and as spice has long been used in Asia. It is one of the earliest spices know to Europe having been obtained by the Greek and Romans from the Arabian Traders. Later it was introduced into the West Indies, Africa and other warmer parts of the world. Ginger is cultivated in several parts of the world - the most important producing regions being India, Sierra Leone, Nigeria, Japan,

Nepal, Jamaica, Southern China, Pakistan, Taiwan, Australia, Mauritius, Malaysia and Indonesia. Among of these, Jamaica and India produce the best quality ginger, followed by West Africa. India and China are two prominent suppliers of ginger in the world Market (Singh and Singh, 1996).

In Bangladesh, there exists a large gap between the ginger production and requirement. The total production of ginger 38,000 MT in an area of 6882 hectare (BBS, 2003) which is approximately about 40% of our requirement, the rest is imported to meet up its demand for local consumption (BBS and Year Book of Agricultural Statistics of Bangladesh, 1997). Due to diversified use of ginger, it has also great demand in the world market. By producing huge quantity of export quality of ginger, Bangladesh can enter into the export market easily. It is also a crop of marginal lands and is being cultivated by many farm families who have only limited homestead areas. In fact, expansion of ginger production in the farmer's field is not satisfactory (Hasan *et al.*, 1997). Ginger grows well in northern part of Bangladesh such as Nilphamari, Dinajpur, Rangpur as well as other parts like Khulna, Rangamati, Bandarban, Khagrachari and Gazipur districts.

Ginger is exclusively vegetative propagated using rhizomes. Breeding of ginger has been severely hampered by poor flowering and seed set. Hence, most of the crop improvement programmes of this species are confined to evaluation and selection of naturally occurring clonal variations (Kackar *et al.*, 1993). Due to its sexual barrier, seed production is not possible in this manner. Therefore, it is difficult to create variation. As a result conventional breeding methods are inapplicable for the improvement of this crop. But, the genetic variability in the population is primarily necessary for any crop improvement. Plant breeders having been unsuccessful are forced to produce additional variation artificially. On the other hand, conventional crop improvement methods are no longer adequate to cope with the expanding population, decreasing land resources concomitant with increasing environment stress.

A lot of research has been done to investigate the factors affecting plant regeneration. The appropriate concentrations and combinations of plant growth regulators in the culture media are needed for large utilization in biotechnology. Great progress has been made in ginger for *in vitro* plant regeneration. Production of rapid clonal multiplication of ginger by shoot tip culture was reported by many workers (Jabeen 1987, Bhagyalakshmi and Singh 1988, Noguchi and Yanakawa 1988, Hosoki and Sagawa 1977, Pillai and Kumar 1982, Ilahi and Jabeen 1987). Arimura *et al.* (2000)

(2000) reported that 10-15 plantlets were regenerated from a single shoot tip after 30 days. In a study Him *et al.* (1997) reported that it is possible to obtain a multiplication rate of 7 x 10^5 plants per rhizome per year. Inden *et al.* (1988) reported that 7.5 x 10^5 or more plants can be produced from single shoot tip after one year. Tissue culture techniques can completely elimination of nematodes was reported by De Lange *et al.*, (1987). Pythium tolerant ginger can isolation by using culture filtrate as the selecting agent (Kulkarni *et al.*, 1984).

The Government is importing a considerable quantity of ginger from different countries every year. This is a great loss in hard-earned foreign currency of Bangladesh. The imported ginger is bigger in size and of high quality in comparison to that of our local product. As large volume is being imported, the timely availability is not properly ensured leading to price hike at different times. To overcome this situation, disease free planting materials should be produced and maintained, so that it can retain its good production capacity.

The traditional clonal propagation method appears unable to cope with the increased demand for disease free and healthy planting materials of ginger, an alternative system to the improvement of this crops is long been demanding. Plant tissue culture offers an efficient method for rapid propagation, production of pathogen free material and germplasm

preservation of plants. In tissue culture technique may play a key role in this regard.

Tissue culture proved an important tool in biotechnology because it offers plant materials of identical quantity with insurance against plant disease. To get disease free healthy plant materials it is very urgent to develop protocol for *in vitro* propagation of ginger.

Keeping the above fact in mind, the present study was undertaken with the following objectives :

(i) To develop a suitable regeneration protocal for ginger.

(ii) To determine the optimum combination of NAA and BAP on *in vitro* plant propagules for large-scale production.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Ginger is a homestead as well as field economic crop. Generally, *in vitro* propagation of ginger to obtain disease free plants depends on various factors, like composition on media, culture temperature, explant and environmental conditions (photoperiod humidity) etc. A good number of works has been done on tissue culture on ginger in different part of the world. Reports on *in vitro* culture of ginger are reviewed as follows :

Ginger was successfully micropropagated by inducing growth of axillary and adventitious shoots using buds (Hosoki and Sagawa, 1971; Nadgauda *et al.*, 1980), shoot tips (Pillai and Kumar, 1982; Sakamura *et al.*, 1986; Choi, 1991; Him and paez, 1997), meristems (Bhagyalakshmi and Sing, 1988), leaf pesudostems and decapitated crown sections (Ikeda and Tanal 1989; Pandey *et al.*, 1997) and stem discs and axillary buds (Nogouchi and Yamakawa a, b, 1998) *in vitro*.

In tissue culture local varieties of ginger (*Z. officinale*) and mango ginger (*Curcuma amada*) were successfully propagated using apical bud as explan on Murashige and Skoog (MS) medium augmented with different concentrations of BA and stimulated shoot elongation in MS medium containing 1.0mg benzyladenine per litre. The maximum number of

multiples was obtained within 15 days and they showed 97.5% rooting frequency (Jasrai *et al.*, 2000).

Ilahi and Jabeen (1987) observed that sterna cuttings taken from three months old plants, young buds, rhizome cuttings with shoot bud premordia and juvenile shoots were cultured on half-strength M.S. medium supplemented with different combinations of growth regulators. Calls could not be induced on the stem explant, but it was induced on the juvenin shoots which on subculturing to a medium with varying concentrations of 2,4-D and BAP, developed bud premordia. Callus induction was also observed on 2 week-old shoot buds which on subculturing, turned into green segmented pouch-like structures.

Malamug *et al.* (1991) have reported that the organogenic capability of soft callus of ginger (*Zingiber officinale*) is after three sub cultures.

In an experiment, Hoque *et al.* (1999) observed that optimum multiple shoot regeneration was obtain from shoot tip explants of ginger on MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP. Root initiation was found best on the half strength of MS medium 0.2 mg/L IBA. *In vitro* grown plantlets were successfully established under field condition.

Ikeda and Tanabe (1989) observed that leaf aerial pseudosterm cultured on solid medium (MS) supplemented with 11 mμM BAP in combination with 0.6 mμM NAA produced an average of 5 shoots and 15 roots.

Hosoki and Sagawa (1977) reported high contamination of cultures occurred when rhizomes or vegetative buds are used for micropropagation of ginger. But by using the leaf tissues this problem was eliminated almost completely.

The shoot apex cultured on MS medium developed into plantlets in 10 weeks. MS medium containing 0.5 ppm NAA+5 ppm BAP was best for *in vitro* regeneration (Choi and Kim *et al.*, 1991).

Callusin was the best on medium containing 0.5 ppm NAA, while shoot and root formation were best on medium containing 0.1 ppm NAA+1 ppm BA (Choi, 1991). Explants consisted of the base of the pscudostem or portions from the top middle or bottom with one leaf blade attached. All formed callus *in vitro* although response was best from the base or middle portion explants.

Babu (1992) observed that immature inflorescence (1 to 10 days old flower bads) of *Zingiber officinale* CV. Muran when cultured on modified MS medium supplemented with 10 mg BA and 0.2 mg 2, 4-D/1

produced vegetative buds. The vegetative buds later developed into plantlets directly without on intervening callus phase. About 38% of the buds produced multiple shoots ranging from 5 to 25. When individual flowers were cultured separately on the same medium plantlets developed from 45% of the ovaries. In 2% of the cultures the ovaries developed into fruits. The plantlets obtained from the individual flowers and immature flower buds produced profuse roots on MS medium containing with 1.0 mg /L NAA. Over 80% of these plantlets established easily in on soil.

In order to provide pathogen free, uniform planting material, micropropagation was investigated by Poonsapaya et al. (1993) as an alternative method to propagation from rhizomes. The use of antibiotics to supplement surface sterilization of shoot tips, increased the survival rate. The tips were cultured an MS medium supplemented with NAA at 0, 0.5 or 1.0 mg/L and BAP at 0, 1.0, 2.0 or 3.0 mg/L, and sucrose at 20.0 g/L. Shoot and bud production was the best in high concentration of BAP with low levels of NAA. The shoots rooted best when transferred to a medium supplemented with 10% activated charcoal, with or without NAA at 0.5 mg/L.

Choi (1991) observed that the optimal concentration of sucrose in the medium was 3% and rooting of shoots was enhanced by the addition of 2 g/L activated charcoal.

Plantlets with rhizomes *in vitro* from shoot tips of CV. Oshoga on modified Gamborg B, and Murashige and Skoog (MS) medium supplemented with NAA and BAP (Sakamura *et al.*, 1986).

De Lange *et al.* (1987) suggested that complete elimination of nematodes from ginger (*Zingiber officinale*) can be done by the potential use of tissue culture in ginger.

Ramchandran and Nair (1992) developed a protocol *in vitro* formation of root and rhizomes from anther explants of ginger (*Zingiber officinale* Rosc). Two of the anthers in the medium containing 1.5 mg/L, 4-D and 200 ml coconut milk/L and 0.6% sucrose formed callus and roots.

Winnaar *et al.* (1989) used sprouting buds as explant from turmeric rhizomes and cultured on a nutrient medium containing 1.0 mg/L BAP and 2% sucrose for one month, after they were transferred to fresh medium of the development of multiple shoots and roots occurred after 3-4 weeks. Further shoot growth and proliferation followed on subculture, up to 8 multiple shoots being obtained from a single explant.

Bhat *et al.,* (1994) conducted an experiments to induce rhizome formation on *in vitro* shoot cultures of *Zingiber officinale* using MS medium. Among the various factors, such as temp, photoperiod, sucrose, growth regulators and nutrient composition of the medium only sucrose (at 9% or 12% was found to be effective. Experiments involving substitution of sucrose with other sugars and varying the volume of the culture medium indicated that the greater availability of carbon energy source rather than the osmotic effect of sucrose was responsible for rhizome formation.

Ishida and Adachi (1997) studied the effect of phytohormones on the regeneration of plantlets of three cultivars of ginger (Indo. Taiwan and Oshuge) on MS medium containing 2.0 mg/L 2.4-1). They found stiky callus of indo and Oshuge produced adventitious shoots with frequencies of 41.7 and 33.3% respectively, on MS medium containing 0.1 mg NAA and 1.0 mg/L BAP. These shoots readily forms plantlets on MS medium containing 0.5 mg/L BAP.

In vitro propagation of ginger (*Zingiber officinale*) was achieved using rhizome buds of the elite lines SG 666 and SDR. The buds produced multiple shoot when cultured aseptically on MS medium supplemented with 2.5 mg/LBAP 0.5 mg/L NAA. The greatest number of roots was formed on medium supplemented with 1.0 mg/L NAA, plantlets were

successfully established in the field and were morphologically similar (Dogra *et al.*, 1994).

Huang (1995) developed a technique for *in vitro* propagation and preservation of ginger germplasm resources. In this technique plantlets with complete roof systems were regenerated directly from shoot tips (02-0.9 mm in length) of ginger on MS medium containing 2.0 mg/L BAP and 0.6 mg/L NAA. Adventitious shoots and roots were also regenerated from leaf sheaths on MS medium containing 1.0 mg/L BAP and 0.6 mg/L NAA. Auxiliary shoot proliferation was the main method of shoot multiplication on MS media, with the rate being approximately 6.0 multiplications per month.

Efficient plant regeneration was achieve, when embryogenic cultures were transferred to MS medium containing 8.9 ppm BAP (Kackar *et al.*, 1993). Embryonic callus cultures of ginger were induced on MS medium from young leaf segments taken from *in vitro* shoot cultures. The medium was supplemented with one of the auxins 2, 4-D, IAA, NAA or dicamba at various concentrations. Dicamba at 2.7 ppm was the most effective in inducing and maintaining embryogenic cultures.

In an experiment were conducted by Sharma and Singh (1995) to determine the effects of different medium with sucrose and also

investigate the effects of supplementing medium with 2.0mg/L of benzyl adenine (BA), omitting agar and replication distilled water with tap water on shoot and root production in ginger. The highest number of shoots/explant was produced on MS medium with ordinary sugar and no growth regulators, followed by MS medium with sucrose. The longest roots were obtained on potato extract with dextrose. Use of tap water and omission of agar also gave good results.

In vitro microrhizome of ginger (*Zingiber officinale*) was successfully produced from tissue culture derived shoots by transferring them to liquied MS medium supplements with 1.0 mg/L GA_3 and 0.05 mg/L NAA for shoot proliferation. After 4 weeks of incubation, the medium was replaced with microrhizome induction medium consisting of MS salts supplemented with 8 mg/L BA and 75 g/L sucrose. Microrhizome formation started after 20 days of incubation. After storage for months in moist sand at room temperature, 80% of the microrhizomes sprouted producing roots and shoots (Sharma and Singh, 1995).

Pandey *et al.* (1997) conducted an experiment of *in vitro* propagation of ginger to produce disease free *Zingiber officinale* plantlets through tissue culture using BAP, Twin forty and NAA. The highest number of shoots with an average of 5.33 shoots/pseudostem was produced after 5 weeks of

culture when 5.0 mg/L BAP in combination with 0.5 mg/L NAA was used into the medium. This medium also produced the longest shoots.

An efficient method was established by Devi *et al.* (1999) for the *in vitro* production of *Zingiber officinale*. The best medium for plantlet production from bud explants was Murashige and Skooge (MS) supplemented with BAP and Kinetin (both at 0.1 mg/L). Shoot production was favoured following transfer of plantlets to a medium containing BAP at 4 mg/L. Shoots produced roots when transferred to growth regulator free MS medium.

Sharma and Singh (1997) observed high frequency *in vitro* multiplication of disease free clones of ginger by culturing small, active buds on MS medium supplemented with 2.0 mg/L kn and 20.0 g/L sucrose. An average of 7 shoots/bud was obtained after 4 weeks of culture. A high multiplication rates well-developed plantlets (4 shoots/bud) with a 6.8 cm shoot length and a 7 cm root length was also obtained on MS medium containing 2.0mg/L kn, 2.0mg/L NAA and 20.0 g/L sucrose. The multiplication rate did not decrease even after 24 months of subculture on the same medium, *in vitro* derived plants performed well under field conditions, were morphologycally identical to the mother plants which were free of ginger yellows (*Fusarium oxysporum* f. sp. *zingiberi*).

In a study, Barthakur and Bordoloi (1992) conducted an experiment on *Curcum amanda* (mango ginger).. They observed that rhizome explants produced shoots and roots simultaneously when cultured on MS medium containing NAA (0.5 mg/L) NAA and BAP (4.0 mg/L). *In vitro* produced plants, on transfer to the field, had a survival percentage of 60-70%.

In tissue cultures, virus disease of ginger was controlled by heating to 50^0C for 5 minutes. Cultures were grown in MS medium with 2.0 mg/L BAP+ 0.2 mg/L IAA then in the same medium with half the concentration of BA and IAA, followed by the final transfer to a B_5 medium with 0.2 mg/L IAA. The cultivation of virus free stock to given high yields of 5 t/667 m^2 is discussed (Gao *et al.*, 1999).

Arimura *et al.* (2000) established an efficient method of *in vitro* propagation of ginger. An etiolated shoots in ginger CV. Atibaia was used for micropropagation. Basal portion of plantlets were grown at dark in Murashige and Skoog (MS) medium containing 0,5.0,10.0,or 20.0 µM NAA for 30 days to induce formation of etiolated shoots. Culture of shoots in MS supplemented with 25 µM Kinetin regenerated adventitious shoots along the nodes. These regenerated shoots were transferred to growth regulator free MS for 30-35 day to induce rooting. This scheme makes it possible to regenerated 10-15 planter from each initial explant.

Lin *et al.* (2001) observed that the optimum initial culture medium (MS medium) containing 2.0 mg/L benzyl adenine and 0.2 mg/L IAA for inducing calluses and buds, the bud induction rate was 23.3%. On the optimum subculture medium (MS medium) containing 0.1 mg/L Kn and 0.5 mg\L IAA the build multiplication factor was 6.3 per month. Roots differentiated easily in unspecialized MS medium.

Arimura *et al.* (2002) conducted an experiment to evaluate the effect of NAA (0.125, 0.25 and 0.5 mg/L) and benzyl adenine (0.5, 1.0 and 2.0 mg/L) on the development of ginger plantlets *in vitro*. The highest shoot and root ratio and fresh and dry weight were obtained from MS culture medium supplemented with 0.25 mg/L NAA+ 0.5 mg/L benzyl adenine.

In an experiment, Him and Paez (1997) observed good auxiliary and adventitious buds in MS medium containing MS salts, benzyl adenine (BA) at 2.0, 2.5 or 3.0 mg/L an NAA at 0.0 of 0.25 mg/L. Shoot multiplication and subsequent subculture were carried out using medium containing 3.0 mg/L BAP. Plant acclimatization was completed with the laboratory hardening under 85 $\mu Em^{-1}S^{-1}$ light for 7 days thee plant were transplanted into 9 substrates in humidified chambers in the shade. The percentage of established plants was better in the presence of sand only of sand in combination with other media. It was observed that it is possible to obtain a multiplication rate 70000 plants $rhizome^{-1}$ $year^{-1}$.

Kulkarni *et al.* (1984) reported isolation of pythium tolerant ginger by using culture filtrate as the selecting agent.

Plantlets with complete root systems were regenerated directly from shoot-tips (0.2-0.9 mm in length) of ginger (*Zingiber officinale)* on MS, medium containing 2.0 mg/L BA and 0.6 mg/L NAA (Huang *et al.*, 1995). Adventitious shoots and roots were also regenerated from leaf sheaths on MS (4) medium containing 1.0 mg/L BA and 0.6 mg/L NAA. Axillary shoot proliferation was the main method of shoot multiplication on MS (7) media with the rate being approximately 6 multiplications per month. Plantlets with 3 leaves were stored for 12 months on MS (7) medium supplemented with 3% mannitol cultured at 15^0C and continous white fluorescent light at an intensity of approximately 1200 lux without genetic variation occurring.

Faria and Illg (1995) observed that bud multiplication was promoted from rhizome axillary buds in MS culture medium by combining 10 μM/L BA with 5 μM/L IAA. Shoot formation occurred when buds were transferred to MS/2 medium containing only 10 μM/L BA at a rate of 15-20 new shoots every 30 days. Rooting was obtained when the shoots were placed in water or MS/2 medium with 5 μM/L NAA or IAA.

In an experiment, Dogra *et. al.* (1994) observed that *in vitro* propagation of *Z. officinale* was achieved using rhizome buds of the elite lines SG 666 and SDR. The buds produced multiple shoots when cultured aseptically on MS medium supplemented with 2.5 mg/L BA + 0.5 mg/L NAA. The greatest number of roots was formed on medium supplemented with 1.0 mg/L NAA. Plantlets were successfully established in the field and were morphologically similar.

Ravindran *et al.* (1994) National Research Centre for Spices (NRCS) have been taken a trial on evaluation of some ginger cultivars for various morphological, yield and quality characters revealed considerable variability for most of these traits. The NRCS has an *in vitro* gene bank for medium term storage of germplasm and is working on the use of cryopreservation for long-term storage. The NRCS is also working on the development of high yielding, disease resistant, high quality lines.

In an study, Balachandran *et al.* (1990) observed that rhizome buds excises from ginger were inoculated on MS medium with different combinations of benzyladenine and kinetin. For shoot multiplication 3.0 mg/L BAP was found to be optimum for this sppecies. Rhizome buds produced shoots and roots simultaneously and within 4 weeks complete plantlets were formed. These were successfully established in the field.

According to Kackar *et al.* (1993) stated that embrygenic callus cultures of ginger (*Zingiber officinale*) were cv. Eruttupetta were induced on MS medium from young leaf segment taken from *in vitro* shoot cultures. The medium was supplemented one of the auxins 2, 4-D, IAA, NAA or dicamba, at 2.7 µM was the most effective in inducing and maintaining embryogenic cultures. Efficient plant regeneration was achieved when embryogenic cultures were transferred to MS medium containing 8.9 µM BA. Histological studies revealed various stages of somatic embryogenesis characteristics of the monocot system. The *in-vitro* raised plants were successfully established in soil.

In a study, Babu *et al.* (1992) concluded when already differentiated tissues from leaf were used for plant regeneration, through an intermediary callus phase there may be a possibility of increase rate of somaclonal variation that can be exploited for crop improvement in ginger, especially since other conventional methods of creating varions are ineffective.

In a study, Arimura *et al.* (2000) observed the effect of NAA and BAP on ginger sprouting in solid and liquid medium. They found that NAA increased shoot length in both solid and liquid medium. NAA at 0.5 mg/L promoted the highest number of roots and the longest roots where BAP influenced the number of shoots will maximum response at 1.0 mg/L.

It was reported by Anonymous (1988) large scale multiplication through tissue culture has been achieved in ginger.

Ginger shoot multiplication was achieved by meristem culture on the Murashige and Skoog (MS) basal medium supplemented with 26.6 µM 6-BA, 8.57 µM IAA and 1111.1 µM adenine sulphat and % (W/V) sucrose. *In vitro* rhizome formation from in vitro raised shoots was achieved on MS medium supplemented with 4.44 µM BA, 5.71 µM IAA and 3-8% sucrose after 8 weeks of culture. Culture variations such as photoperiod, carbohydrate, nutrient composition and growth regulators were tasted for the maximum yield of rhizomes. Among the different photoperiods a 24-h photoperiod helped in the formation of more rhizomes as compared with other photoperiods (Rout *et al.*, 2001).

From the above review, it was evident that different plant growth regulators (PGRs) at different concentrations individually or in combinations influence *in vitro* culture of Ginger. However, very few investigations have yet been carried out on these factors.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present experiment was carried out in the Biotechnology Laboratory of the Bangladesh Agricultural Research Institute, Joydebpur, Gazipur during April 2005 to March 2006. Materials and methods used to conduct the present study have been presented in this chapter.

3.1 Experimental Materials

3.1.1 Plant materials

The shoot tips of ginger was used as plant materials in the present experiment. The collected rhizomes were preserved in sandy soil in a pot in the laboratory to get growing buds for using as explants.

3.1.2 Collection of plant materials

The plant materials used in the present investigation were collected from the local market of Gazipur district. The variety was designated on the market name as Gazipur local. It was used as source population for this study by using the shoot.

3.2 Culture media

MS medium was used with different hormonal supplements as culture medium for shoot initiation, multiplication and maintains and regeneration of roots from multiplied shoot for pathogen free plantlets

production. The composition of MS medium presented in Table-1 which is used in the present experiment. Hormones were added separately to different media according to the requirements. For the preparation of medium stock solutions were prepared at the beginning and stored at recommended temperature. The respective media were prepared from the stock solution during the study period. The detail procedures of preparation of medium have been furnished below.

3.2.1 Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions. The various constitutes of the medium were prepared into stock solutions for ready use to expedite the preparation of the medium. Separate stock solutions for macronutrients, micronutrients, irons, vitamins, growth regulators etc were prepared and used.

3.2.1.1 Stock solution A (Macro-nutrients)

Stock solution of macro-nutrients was prepared with 10 times the final strength of the medium in one litre of distilled water. Ten times weight of the salts required for one litre of medium were weighed accurate and dissolved thoroughly in 750 ml of distilled water and final volume was made up to one litre by further addition of distilled water. The stock solution was filtered through a Whatman No. 1 filter paper to remove all

the solid contaminants and solid particles like cellulose, dust, cotton etc. The stock solution was poured into a clean plastic container and stored in a refrigerator at 4°C for ready use.

3.2.1.2 Stock solution B (Micro-nutrients)

It was made 100 times the final strength of the medium in one litre of distilled water as described earlier for the stock solution A. This stock solution was also filtered and stored in refrigerator at 4°C.

3.2.1.3 Stock solution C (Iron stock : Fe-EDTA)

It was made 100 times of the final strength of the medium in one litre distilled water. Here, two constituents, $FeSO_4.7H_2O$ and Na_2EDTA, were dissolved in 750 ml of distilled water in a conical flask by heating in a water bath until the salts dissolved completely and the final volume was made up to one litre by further addition of distilled water. This stock was also filtered and stored in refrigerator at 4°C.

3.2.1.4 Stock solution D (Vitamins and amino acids)

Each of the desired ingredients except myoinositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 400 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 9±1°C for later use. But the

myoinositol was made separately 100 folds (100x) the final strength of the medium in 1000 ml of distilled water. This stock solution was also filtered and stored in a refrigerator at 9±1°C.

3.2.1.5 Stock solution E (Growth regulators)

In adding to the nutrients, it is generally to add growth regulators (hormones) such as auxin (NAA) and cytokinin (BAP) to the medium to support good growth of tissues and organs. The following growth regulators were used in the present investigation.

Auxin : 1-naphthylacetic acid (NAA)

Cytokinin : 6-benzylaminopurine (BAP)

The growth regulators were dissolved in appropriate solvents as follows :

Growth regulator	Solvents
NAA	0.1 N NaOH
BAP	0.1 N NaOH

To prepare the stock solution of these hormones, 10 mg of the hormones was placed on a clean watch glass and then dissolved in 1 ml of respective solvent. The mixture was then washed with distilled water and collected in a 100 ml measuring cylinder and was made 100 ml with distilled water. The solution was then poured into a glass vial and stored at 0°C for a period up to two weeks.

Table 1. Constituents of Stock Solutions for MS Medium.

Constituents	Concentration (mg/L)
a) Macro-nutrients (10x)	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
b) Micro-nutrients (100x)	
$MnSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Iron source (10x)	
$FeSO4.7\ H_2O$	27.80
$Na_2EDTA.2H_2O$	37.30
d) Organic nutrients (100x)	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Myo-Inositol	100.00

3.2.2 Media Preparation

To Prepare one litre of MS medium, the following steps were followed :

i) By using of magnetic stirrer 30 gm of sucrose was dissolved in 500 ml of distilled water.

ii) 100 ml of stock solution of macro-nutrients (stock A), 5ml of stock solution of micro-nutrient (stock B), 10 ml of stock solution of Fe-EDTA (stock C), 50 ml of myoinositol and 10 ml of each of the stock solution of vitamins were added to the aforementioned 500 ml sucrose solution and mixed well.

iii) Different concentration of hormonal supplements as required were added either in single or in different combination to the solution and were mixed thoroughly by magnetic stirrer.

iv) Each hormonal stock contained 10 mg of the chemical, in 100 ml of the solution, to make one litre of medium addition of 10 ml of stock of any of the hormones, resulted in 1mg/L concentration of hormonal supplement. Similarly, for 2,4,6,8 mg/L concentration of the hormonal supplement 20, 40, 60, 80 ml of stock solution were added respectively to prepare one litre of medium.

v) Finally the whole solution was made one litre by addition of distilled water.

vi) The p^H of the medium was adjusted to 5.8 with digital p^H meter with the help of 0.1N NaOH or 0.1N HCl, whichever necessary.

vii) For the solidifying of the medium 8.0g of sigma brand Bacto agar was added to heated in a microwave oven for 10-12 minutes until the agar dissolved completely.

viii) The prepared method medium was dispensed in to culture tubes were plugged with plastic plugs or aluminum foil and marked with a glass marker to indicate specific hormonal supplement.

3.2.3 Sterilization

The culture vessels were plugged with aluminum foil and marked with different indication with the help of glass marker to indicate specific hormonal supplement. The culture vessels were then sterilized at 1.5 kg/cm^2 pressure at 121^0C for 20 minutes in an autoclave.

3.2.4 Sterilization of working instrument

All instruments such as culture vessel, pipettes, beakers, petridishes, glassware, plastic caps, forceps, spatulas, needles, scalpels and aluminum foil were sterilized in an autoclave at a temperature of 121^0C for 20 minutes at 1.5 kg/cm^2 pressure.

3.3 Culture condition

MS medium supplemented with different concentrations of 1-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) are used for shoot proliferation and multiplication. The p^H of all media and 1.5 kg/cm^2 pressure for 20 minutes culture vessels used were 25 x 100 mm flat bottomed closed with polypropylene cap containing 10 ml medium in each.

3.3.1 Explants preparation

The collected rhizomes were planted in soil in a pot. After 4 weeks new sprouting buds were collected and used as explants. The shoots then washed thoroughly under running tap water and Trix in plastic pot for 10 minutes to reduce the surface organisms. The roots and outer tissues of the shoot were removed with the help of a sharp stainless steel knife. A number of outer leaves were removed until the shoot measured about 1.0 to 1.5 cm in length and 1.5 cm in width at the base.

3.3.2 Surface sterilization of the explants

The explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) and a few drops of Tween for 15 minutes. Then the mercuric chloride solution was removed by washing with sterilized distilled water in a laminar air-flow cabinet. Finally, the explants were prepared by 5 x 10 mm portion containing one or more pairs of leaf primordia with 3-5 mm

of rhizomatous bases were cut by sharp sterilized knife were used as initial explants.

3.3.3 Shoot tips inoculation

All the operations from sterilization to transfer onto the media were performed under aseptic condition. The surface sterilized shoot tips were transferred immediately into the semisolid media and each culture tube containing 10 ml of MS medium supplemented with different concentration of hormones required as per treatments. Then the culture tube covered with aluminum foil and scaled with parafilm.

3.3.4 Subculture and shoot regeneration

Successful shoot formation became evident when small green leaves began to emerge. It is the sign of regeneration and these tiny leaves when developed in their actual shape they were transferred on different hormone supplemented for further proliferation and development. Subculture was carried out regularly at 30 days interval.

3.3.5 Culture incubation

All the cultures were kept at 25 ± 2^{0}C illuminated with 1.83 m florescent tubes. Broad spectrum of light with red wavelength were presented on the growth room. The room was illuminated 16^{th} hr photoperiod daily with a light intensity of 1500 lux. All the prepared cultures were kept in growth room on the shelves.

3.3.6 To ensure aseptic condition

All inoculations and aceptic manipulation were carried out in laminar air flow cabinet. The cabinet was switched on for half an hour before use and cleaned with 90% propanol to reduce the chances of contamination. The working instruments like scalpels, needles, forceps etc. were sterilized by clipping in 70% ethyl alcohol followed by flaming and cooling method inside the chamber. While not in use, these were kept immersed in alcohol. Hands were also made sterile by 70% ethyl alcohol. Surgical operations were taken care of as usual to obtained possible contamination free condition. Talking and sneezing while the culture material was being transfered or inoculated from one place to another were avoided. Cultured tissue was taken out of the test-tube and inner portion of the aluminum foil were flamed before closing it. The aluminum cap of the test-tubes containing the medium and the cultured test- tubes recapped with the sterilized aluminum foil in quick succession.

3.3.7 Preparation of pots

Sand, loamy soil, cowdung and mustard oil cake were used at different ratio for making potting mixture. Two sets of potting mixture were used for growing *in vitro* grown plantlest *in vivo* condition. 15 x 30 cm^2 pots were used for this purpose.

3.3.8 Weaning, acclimatization and establishment of plantlets

After taking out from the culture room, culture vessels were placed in a shaded room in the laboratory for 2 to 3 days with low light intensity and high temperature (about 30^0C to 35^0C) to minimize the shock encountered by plantlets during acclimatization. Plantlets of 25-30 days with well developed roots were removed from the culture vials with the help of forceps. The roots were washed thoroughly with tap water and transplanted into plastic pot containing sandy loam soil along with cowdung, sand and oil cake at different ratio. Then the plantlets grew freely in the ambient environment. The plants was watered with normal tap water applied in every alternate day. The plantlets were established after 25-30 days and transferred to a field of loamy soil enriched in well decomposed cowdung.

3.4 Treatments

The present experiment consisted of two factors:

A) 1-naphthylacetic acid (NAA)

B) 6-benzylaminopurine (BAP)

There were 5 levels of factor A (NAA - 0, 1.0, 2.0, 3.0, 4.0 mg/L) and B (BAP - 0, 1.0, 2.0, 3.0, 4.0 mg/L).

There were 25 (5x5) treatment combinations and they were as follows:

Explant	Treatment Combinations	
	NAA (mg/L)	BAP (mg/L)
Shoot Tip	0	0
	0	1
	0	2
	0	3
	0	4
	1	0
	1	1
	1	2
	1	3
	1	4
	2	0
	2	1
	2	2
	2	3
	2	4
	3	0
	3	1
	3	2
	3	3
	3	4
	4	0
	4	1
	4	2
	4	3
	4	4

3.5 Design

The experiment as laid in the Completely Randomized Design (CRD) with three replications.

3.6 Data recording

To investigate the effect of different treatments on shoot proliferation and rooting, the following parameters were recorded.

a) **Number of shoots per plantlet :** Number of shoots were counted at the interval of 5 days up to 30 days after explanting.

b) **Shoot length (cm) per plantlet :** Length of shoots were measured at the interval of 5 days up to 30 days after explanting.

c) **Number of roots per plantlet :** Number of roots were counted at an interval of 5 days up to 30 days after explanting.

d) **Number of leaves per plantlet :** Number of roots were counted at an interval of 10 days up to 30 days after explanting.

e) **Days to shoot initiation:** Number of days were counted from days of inoculation to days when the new shoots emerged.

3.7 Statistical Analysis

Data collected on different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The means for all the treatments were calculated and analysis of variance of all the characters. The significance of the difference between the pair of means was evaluated at 1% level of significance by Duncan's Multiple Range Test (DMRT, Gomez and Gomez, 1984) using MSTAT Computer programme.

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

In vitro regeneration of ginger was successfully done in the present study. The results obtained from the experiment has been presented and discussed in this chapter. The results of analysis of variance in respect of all the parameters have been presented in Appendices 1 to 5.

4.1 Effect on NAA and BAP

4.1.1 Number of shoots per plantlet

A significant difference was found on number of shoots developed per plantlet (Appendix1) at different concentrations of NAA and BAP. The results on the main effect of different supplements of NAA and BAP on multiple shoot regeneration presented in Table 2 & 3. Among the various concentrations of NAA, the maximum number of shoots (5.15) was found in MS medium supplemented with 1.0 mg/L NAA followed by 2.0 mg/L NAA (4.89). In BAP, the maximum number of shoots (5.62) was found in the treatment 3.0 mg/L BAP which is followed by 4.0 mg/L BAP (5.29).

Balachandran *et al.* (1990) observed that shoot multiplication in 3.0 mg/L BAP was found optimum for ginger. Shoots were successfully regenerated on medium with concentration of 3.0 mg/L BAP. Him and Paez *et al.* (1997) observed shoot multiplication and subsequent

subculture well carried out using medium containing 3.0 mg/L BAP. For shoot multiplication, concentration of 3.0 mg/L BAP appears to be optimum (Perveen *et al.*, 1997).

The combined effect of different supplements of NAA and BAP on number of shoots per plantlet presented in Table 4. The maximum number of shoots (7.10) per plantlet was obtained from the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP and significantly higher than other treatments. The lowest (2.42) was obtained from the control treatment.

Hoque *et al.* (1999) observed the similar result. He found that the best multiple shoot regeneration was obtained from the shoot tips of ginger on MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP. He also found *in vitro* grown plantlets were successfully established in field condition under this combination. Poonsapaya *et al.* (1993) obtained the shoot and bud production was the best in low levels of NAA with high concentration of BAP.

The results of present experiment similar with the findings of Hoque *et al.* (1999). He obtained the maximum number of shoot in ginger on MS medium supplemented with the combination of 1.0 mg/L NAA + 3.0 mg/L BAP.

4.1.2 Shoot length per plantlet

A significant difference was found on shoot length per plantlet at different concentrations of NAA and BAP. The results on the main effect of different supplements of NAA and BAP on shoot length per plantlet presented in Table 2 & 3. The highest length of shoot (8.69cm) was found in MS medium containing 1.0 mg/L NAA followed by 3.0 mg/L NAA (8.62cm). Among the treatments, 3.0 mg/L BAP produced the highest length of shoot (8.87 cm) which is followed by 4.0 mg/L BAP (8.48cm).

The combined effect of different combinations of NAA and BAP on shoot length per plantlet presented in Table 4. The highest length of shoot (10.24cm) was significantly observed in the treatment combination of 1.0 mg/L NAA + 3.0 mg/L BAP which is followed by 1.0 mg/L NAA + 4.0 mg/L BAP (9.83cm).

Arimura *et al.* (2000) found the effect of NAA and BAP on shoot length in both solid and liquid medium. He found that NAA increased shoot length in both solid and liquid medium. In another experiment, Jasrai *et al.* (2000) was successfully propagated through tissue culture with different concentrations of BAP and found stimulated shoot elongation in MS medium containing 1.0 mg/L BAP.

The result of current investigation is not fully supported by the mentioned auther so far. This variation might be due to the different concentrations of NAA (auxin) and BAP (cytokinin) and their association.

4.1.3 Number of roots per plantlet

Results on the main effect of different supplements of NAA and BAP on number of roots per plantlet presented in Table 2 & 3. The maximum number of roots (7.87) was significantly found in MS medium containing 1.0 mg/L NAA which is followed by 2.0 mg/L NAA (4.71). In BAP, 3.0 mg/L produced the maximum number of roots (5.67) per plantlet which is statistically identical with 4.0 mg/L BAP (5.63).

The combined effect of different combinations of NAA and BAP on number of roots per plantlet presented in Table 4. From this result, it was also noticed that the maximum number of roots (9.61) was significantly found in the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP which is followed by 1.0 mg/L NAA + 4.0 mg/L BAP (8.15).

Babu *et al.* (1992) observed that buds produced profuse roots in rooting MS medium supplemented with 1.0 mg/L NAA which is over 80% of these plantlets established easily in soil. The similar result also obtained by Dogra *et al.* (1994) in his experiment. He observed that the greatest number of roots was formed on medium supplemented with 1.0 mg/L

NAA where the plantlets were successfully established in the field and were morphologically similar.

Barthakur and Bordoloi (1992) observed that rhizome explants produced shoots and roots simultaneously when cultured on MS medium containing 4 mg/L BAP which plantlets transfer to the field and had a survival percentage of 60-70%. Arimura *et al.* (2000) obtained NAA at 0.5 mg/L promoted the highest roots. The result of current investigation is not supported with the above findings due to the different concentrations of NAA and BAP and their associations.

4.1.4 Number of leaves per plantlet

Number of leaves per plantlet was significantly influenced at various concentrations of NAA and BAP. The results on the main effect of different supplements on number of leaves per plantlet presented in Table 2 & 3. Among the various supplements, the maximum number of leaves (4.79) was found in MS medium supplemented with 1.0 mg/L NAA which is statistically identical with 3.0 mg/L NAA (4.74). The highest number of leaves (4.72) was obtained from 3.0 mg/L BAP which is followed by 4.0 mg/L BAP (4.68).

A significant difference was found on the combined effect of different supplements of NAA and BAP on number of leaves per plantlet presented

in Table 4. The mean value significantly indicate that the maximum number of leaves (5.72) was found in MS medium supplemented with the treatment combination 1.0 mg/L NAA + 4.0 mg/L BAP which is followed by the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP (5.11).

4.1.5 Days required to shoot initiation

Different concentrations of NAA and BAP showed significant difference on number of days to shoot initiation (Appendix 5). The results on the main effect of different supplements of NAA and BAP on days required to shoot initiation presented in Table 2 & 3. Among the various concentrations of NAA, the minimum time (25.68 days) was required for shoot initiation from the treatment 1.0 mg/L NAA. In BAP, the minimum time (26.21 days) was required in 3.0 mg/L BAP.

The combined effect of different supplements of NAA and BAP on number of days required for shoot initiation presented in Table 4. The mean values indicate that the minimum time (20.67 days) for shoot initiation was recorded from the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP. The longest period (41.38 days) was required for shoot initiation from the control treatment.

Balachandran *et al.* (1990) observed that MS medium supplemented with 3.0 mg/L BAP produced shoots within 4 weeks complete plantlets were formed which were successfully established in the field. The result of current investigation is not supported by Pandey *et al.* (1997) where he found the highest number of shoots with an average of 5.33 shoots/pseudostem was produced after 5 weeks of culture when 5 mg/L BAP in combination with 0.5 mg/L NAA was used into the medium. This variation on days to shoot initiation observation may be due to the genotype, different concentrations of growth regulators and culture environments.

The number of days for shoot initiation is an important physiological traits and determining the vigour of the explant also the duration of the crop. Hoque *et al.* (1999) observed that the initiation of shoot with leaf primordial took place within 30 days but without leaf primordial only shoot initiated even within 20 days which is agree with the above report.

Table 2. Main effect of different concentrations of NAA in MS medium on the growth and development of plantlets in Ginger.

Concentration of NAA (mg/L)	No. of Shoots/ Plantlet	Shoot Length (cm.)	No. of Roots/ Plantlet	No. of Leaves/ Plantlet	Days to Shoot Initiation
0	4.03d	6.03c	3.40d	3.08d	35.15a
1	5.15a	8.69a	7.87a	4.79a	25.68c
2	4.89b	8.30b	4.71b	4.13c	28.39b
3	4.68c	8.62a	4.44c	4.74a	29.01b
4	4.60c	8.29b	4.69b	4.42b	29.48b

In a column, the figures having same letters do not differ significantly at 1% level of significance as DMRT.

Table 3. Main effect of different concentrations of BAP in MS medium on the growth and development of plantlets in Ginger.

Concentration of BAP (mg/L)	No. of Shoots/ Plantlet	Shoot Length (cm.)	No. of Roots/ Plantlet	No. of Leaves/ Plantlet	Days to Shoot Initiation
0	3.43e	6.49d	4.23c	3.25d	36.63a
1	4.18d	8.02c	4.77b	3.97c	30.22b
2	4.83c	8.08c	4.82b	4.53b	27.28c
3	5.62a	8.87a	5.67a	4.72a	26.21c
4	5.29b	8.48b	5.63a	4.68a	27.38c

In a column, the figures having same letters do not differ significantly at 1% level of significance as DMRT.

Table 4. Combined effect of different concentrations of NAA and BAP in MS medium on the growth and development of plantlets in Ginger.

Treatment Combination			No. of Shoots/ Plantlet	Shoots Length (cm)	No. of Roots/ Plantlet	No. of Leaves/ Plantlet	Days to Shoot Initiation
Explant	Hormone						
	NAA (mg/L)	BAP (mg/L)					
Shoot Tip	0	0	2.42i	3.26n	1.05p	1.46n	41.38a
	0	1	3.69h	5.61m	3.12o	2.65m	37.16ab
	0	2	4.88cde	7.34ij	3.59n	3.81jk	34.03c
	0	3	6.24b	7.09jk	4.22kl	4.43defg	33.47c
	0	4	4.90cde	6.85k	5.03fghi	4.50def	28.25de
	1	0	3.45h	6.37l	7.69c	3.78k	33.64c
	1	1	3.70h	8.90cd	6.60e	3.70k	26.16def
	1	2	5.16cd	8.15efg	7.32d	5.63a	24.03fg
	1	3	7.10a	10.24a	9.61a	5.11b	20.67g
	1	4	6.34b	9.83b	8.15b	5.72a	23.90fg
	2	0	4.66efg	7.65hi	4.55jk	4.43efg	36.25bc
	2	1	4.72def	9.01c	5.30f	4.55de	27.30def
	2	2	4.83cdef	7.38ij	3.89lmn	3.39l	24.79ef
	2	3	4.98cde	8.17efg	4.61j	4.20ghi	26.48def
	2	4	5.25c	9.30c	5.20fg	4.06hij	27.15def
	3	0	4.38fg	7.07jk	3.78mn	3.91ijk	36.56bc
	3	1	4.40fg	8.31ef	4.10lm	5.05b	29.40d
	3	2	4.66efg	8.96c	4.63j	4.69cde	26.37def
	3	3	4.93cde	7.85gh	4.81hij	5.13b	24.58ef
	3	4	5.07cde	9.29c	4.88ghij	4.87bc	28.13de
	4	0	4.22g	8.13fg	4.06lm	4.08hij	35.30bc
	4	1	4.41fg	8.24efg	4.73ij	3.90jk	30.06d
	4	2	4.62efg	8.55de	4.67ij	5.14b	27.19def
	4	3	4.84cdef	9.07c	5.12fgh	4.71cd	25.38ef
	4	4	4.92cde	9.12c	4.90ghij	4.25fgh	29.47d

In a column, the figures having same letters do not differ significantly at 1% level of significance as DMRT.

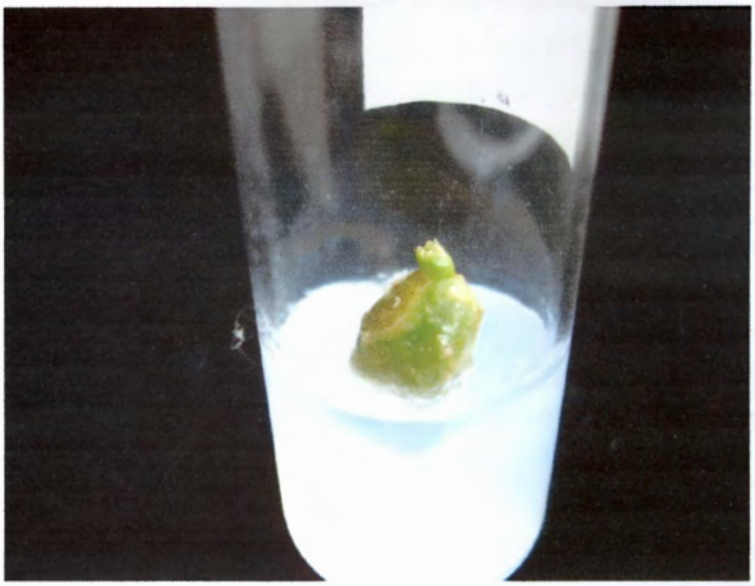

Plate 1. The initial explant cultured on MS medium supplemented with NAA and BAP.

Plate 2. Initiation of multiple shoot derived from the plantlet cultured on MS medium supplemented with 2.0 mg/L NAA + 4.0 mg/L BAP after 30 days.

Plate 3. Showing multiple shoot derived from the plantlet cultured on MS medium supplemented with 2.0 mg/L NAA + 4.0 mg/L BAP after 30 days.

Plate 4. **Showing multiple shoot derived from the plantlet cultured on MS medium supplemented with 1.0 mg/L NAA + 4.0 mg/L BAP after 30 days.**

Plate 5. **Initiation of multiple shoot derived from the plantlet cultured on MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP after 30 days.**

Plate 6. **Showing of multiple shoot derived from the plantlet cultured on MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP after 30 days.**

Plate 7. **Effect of 1.0 mg/L NAA + 4.0 mg/L BAP on number of leaves per plantlet after 30 days.**

Plate 8. **Showing the maximum leaves in regenerated plantlet cultured on MS medium supplemented with 1.0 mg/L NAA + 4.0 mg/L BAP after 30 days.**

Plate 9. **Effect of 4.0 mg/L NAA + 2.0 mg/L BAP on number of leaves per plantlet after 30 days.**

Plate 10. **Showing the maximum number of roots in regenerated plantlets cultured on MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP after 30 days.**

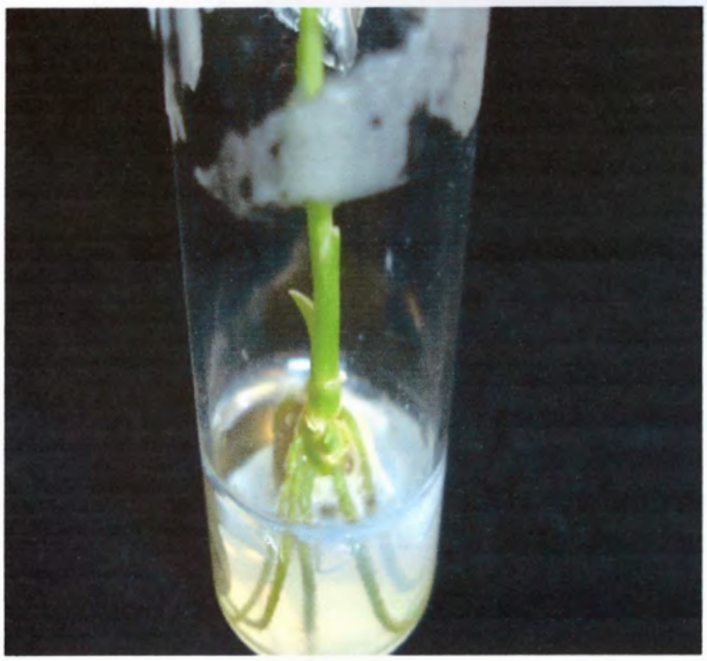

Plate 11. **Regenerated shoot with root cultured on MS medium supplemented with 1.0 mg/L NAA + 4.0 mg/L BAP after 30 days.**

Plate 12. ***Ex vitro*** **establishment of plantlets in the potting mixture.**

CHAPTER V

SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCLUSION

The experiment was conducted at the Biotechnology Laboratory, Bangladesh Agricultural Research Institute, Gazipur, during the period from April 2005 to March 2006. The study was undertaken to investigate the effect of different concentrations and combinations of NAA and BAP on *in vitro* shoot multiplication and rooting of ginger plantlets.

From this study, the main and combined effect of plant growth regulators showed significant difference on shoot initiation, multiplication, number of roots, number of leaves and time required (days). It was found that the maximum number of shoots (7.10) per plantlet was obtained from the treatment combination 1.0 gm/L NAA + 3.0 mg/L BAP which is significantly higher than other treatments. The lowest (2.42) was obtained from the control treatment. The highest length of shoot (10.24cm) was significantly observed in the treatment combination of 1.0 mg/L NAA + 3.0 mg/L BAP which is followed by 1.0 mg/L NAA + 4.0 mg/L BAP (9.83cm).

From this result, it was also noticed that the maximum number of roots (9.61) was significantly found in the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP followed by 1.0 mg/L NAA + 4.0 mg/L BAP

(8.15). A significant difference was found on the combined effect of different supplements of NAA and BAP on number of leaves per plantlet presented in Table 4. The maximum number of leaves (5.72) was significantly found in MS medium supplemented with the treatment combination 1.0 mg/L NAA + 4.0 mg/L BAP followed by the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP (5.11). The mean values indicate that the minimum time (20.67 days) for shoot initiation was recorded from the treatment combination 1.0 mg/L NAA + 3.0 mg/L BAP. The longest period (41.38 days) was required for shoot initiation from the control treatment.

The above findings of the present study suggested that MS medium supplemented with 1.0 mg/L NAA + 3.0 mg/L BAP may be the best for shoot initiation, multiplication and rooting of ginger and 1.0 mg/L NAA + 4.0 mg/L BAP the best for the maximum number of leaves.

The protocol developed through the present study may be useful for production of disease free and healthy planting materials of ginger for further use at farmer's level.

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APPENDICES

APPENDICES

Appendix 1. Analysis of variance of the data on number of shoots per plantlet.

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
NAA (A)	4	10.464	2.616	45.3069**
BAP (B)	4	46.499	11.625	201.3277**
A x B	16	45.509	2.844	49.2612**
Error	50	2.887	0.058	

** = Significance at 1% level of probability

Appendix 2. Analysis of variance of the data on shoots length (cm.) per plantlet.

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
NAA (A)	4	73.967	18.492	340.5660**
BAP (B)	4	49.210	12.302	226.5761**
A x B	16	33.505	2.094	38.5666**
Error	50	2.715	0.054	

** = Significance at 1% level of probability

Appendix 3. Analysis of variance of the data on number of roots per plantlet.

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
NAA (A)	4	169.491	42.373	992.7702**
BAP (B)	4	22.977	5.744	134.5817**
A x B	16	27.551	1.722	40.3438**
Error	50	2.134	0.043	

** = Significance at 1% level of probability

Appendix 4. Analysis of variance of the data on number of leaves per plantlet.

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
NAA (A)	4	29.109	7.277	3.22.6089**
BAP (B)	4	23.467	5.867	260.0771**
A x B	16	38.536	2.408	106.7715**
Error	50	1.128	0.023	

** = Significance at 1% level of probability

Appendix 5. Analysis of variance of the data on days to shoot initiation.

Sources of Variation	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
NAA (A)	4	719.686	179.922	44.4199**
BAP (B)	4	1072.954	268.239	66.2241**
A x B	16	171.147	10.697	2.6409**
Error	50	202.523	4.050	

** = Significance at 1% level of probability