IN VITRO PROPAGATION OF GARLIC (*Allium sativum* L.)

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CERTIFICATE

*This is to certify that thesis entitled, "**IN VITRO PROPAGATION OF GARLIC (Allium sativum)**" 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 BIOTECHNOLOGY,** embodies the result of a piece of bona field research work carried out by **FABEEHA MUBARRAT, Registration no. 10-4043** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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.

Date: June, 2016

Place: Dhaka, Bangladesh

(Associate Prof. Homayra Huq)

Research Supervisor

LIST OF ABBREVIATIONS

Biol.	: Biological
EDTA	: Ethylene diamine tetra acetic acid
et al.	: And others
DMRT	: Duncan's Multiple Range Test
2,4-D	: 2,4-dichlorophcnoxy acetic acid
IAA	: Indole acetic acid
IBA	: Indole buteric acid
Int.	: International
2-ip	: 2- isopentyle adenine
J.	: Journal
KIN	: Kinetin
mg/L	: milligram per Liter
MS	: Murashige and Skoog
NAA	: Naphthalene acetic acid
PGR	: Plant growth regulator
Ppm	: Parts per million
Res.	: Research
Sci	: Science

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IN VITRO PROPAGATION OF GARLIC (*Allium sativum* L.)

ABSTRACT

The research work was conducted in the Laboratory of Biotechnology of the Department of Biotechnology, Sher-e-Bangla Agricultural University, from the period of July 2015 to June, 2016 to observe the response of different plant growth regulators on *in vitro* regeneration of garlic. The disease free, healthy and sterilized basal part of clove of 2-3 cm length were used as explants. Sterilization was done with 70% ethanol, 0.5% $HgCl_2$ along with few drops of Tween-20. Explants were placed in MS medium supplemented with the Kinetin (KIN), alone and in combination with 2, 4-Dichlorophenoxy acetic acid (2, 4-D). The highest shoot regeneration percentage (100%) was observed by 3.0 mg/L of KIN alone and by wide range of KIN in combination with 2, 4-D (i.e. all combination of 2.5, 3.0 mg/L KIN with 1.0, 1.5, 2.0, 2.5 mg/L 2, 4-D) . The highest shoot length (34.50 cm) was observed after 3 weeks of initiation in 3.0 mg/L KIN treatment. Combined doses of KIN and 2, 4-D showed 1 shoot after 3 weeks of initiation. Number of leaves did not exceed 3 and it was noticed through all combination of 2.0, 2.5, 3.0 mg/L KIN with 1.0, 1.5, 2.0, 2.5 mg/L of 2, 4-D. The highest root induction (100%) was observed by 2.5 mg/L KIN + (1.0, 1.5) mg/L 2, 4-D. It took minimum 3 days to induce root. The average and the highest length of root (3.5 cm and 2.93 cm respectively) was noticed by application of 3 mg/L KIN and 1.0 mg/L 2, 4-D. Maximum number of roots (10.67) were developed by 3.0 mg/L KIN+ 1.5 mg/L 2, 4-D. After transferring the plantlets in the field condition, 86% survival plants were recorded. Finally, *in vitro* regeneration of garlic was proved to be less time consuming. Moderate doses of hormones showed satisfactory responses in shoot proliferation and root induction which made the tool cost effective as well.

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Introduction

CHAPTER I

INTRODUCTION

Garlic (*Allium sativum* L.) belonging to the family Alliaceae is an important and widely cultivated culinary crop. It is considered to be originated in central Asia, especially Mediterranean region (Thompson and Kally, 1957). Then it was expanded to North Eastwards to Pamir Alai and Tien Shen regions of China via trade route. (Katyal and Chandra, 1996). Now-a-days China, Bangladesh, India, South Korea, Spain, Egypt, Thailand, Turkey are the leading producers of this crops.

Garlic is a fundamental component in many or most dishes of various regions, including eastern Asia, South Asia, Southeast Asia, the Middle East, northern Africa, southern Europe, and parts of South and Central America. The clove of garlic plant is the most commonly used part of the plant. With the exception of the single clove types, garlic bulbs are normally divided into numerous fleshy sections called cloves. Garlic cloves are used for consumption (raw or cooked) or for medicinal purposes. They have a characteristic pungent, spicy flavor that mellows and sweetens considerably with cooking.

Other parts of the garlic plant are also edible. The leaves and flowers (bulbils) on the head (spathe) are sometimes eaten. They are milder in flavor than the cloves, and are most often consumed while immature and still tender.

Bangladesh was ranked 4th in world garlic production in 2014. The annual production of garlic was 3,12, 000 million tons in 1,05005 acres of land in 2013-14 (FAO, 2014) .In year 2014-15,total 3,45,725 million tones of garlic was produced in 1,40,975 acres of land. Total yield per acres was 2452 kg/acres. Among the other spices, garlic ranked 2nd in terms production in 2014-15, It covered about 15 percent of the total area under spices cultivation, (BBS, 2014-15).

The per capita garlic consumption was set 3.34 kg for the fiscal year in 2014-2015. It has been estimated to rise up to 3.61 kg for the year 2016-17. The total domestic demand of garlic was 0.527 million tones in 2015. In 2020-21 it is assumed to be raised in 0.721 million tons (The financial express, Bangladesh, 2015).

Garlic is generally cultivated with traditional method. Production and consumption of garlic is increasing day by day in Bangladesh.

Garlic is an important spices crop, which is rich in carbohydrates, protein and phosphorus used against various conventional ayurvedic treatments. Garlic has numerous health benefits. Garlic contains a compound called allicin, which has potent medicinal properties. Garlic supplementation is known to boost the function of the immune system. Garlic has a significant impact on reducing blood pressure in people with high blood pressure. Garlic improves cholesterol levels, which may lower the risk of heart disease..Garlic contains antioxidants that may help prevent Alzheimer's disease and dementia.

Garlic is propagated exclusively vegetatively. Vegetative propagation of garlic is achieved through division of ground bulbs. This process has a low coefficient of multiplication due to sexual sterility and potential for transmission of viral diseases. Also due to difficulties of inducing flowering improvement of this crop through breeding program is very limited (Barandiran *et al, 1999*). Even the propagation rate of garlic in the field is very slow and it takes long time to develop a new variety for practical cultivation.

Many of elite garlic cultivars are susceptible to diseases caused by viruses, nematodes and fungi and suffer from insect and pests (Verbeek *et al.*,1995). Virus infection was shown to reduce the bulb yield by 20% to 60% and up to 80% in case of mixed infection. Depending on cultivar and stage of infection the virus infection is inevitable for vegetative propagation of garlic (Lot *et al*, 1998). Therefore *in vitro* propagation would be one of the key technologies for sustainable supply of this important plant source.

Plant tissue culture is a technique through which any plant part is cultured with the purpose of obtaining growth on a aseptic nutrient medium in controlled light and temperature. This technique is a tool for crop improvement provides an alternative means of plant propagation (Vasil, 1987). *In vitro* approaches have distinct advantage over alternative strategies for the conservation and the use of plant germplasm can offer some.

The *in vitro* technique can overcome the problems with viral infection, virus free garlic clones showed higher yield and improved quality (Nagakubo *et al.*, 1997). The tissue

culture techniques have got high potential for improvement of garlic in respect of yield and quality (Ali and Metwally, 1992).

Therefore, the low propagation rate and the continuous accumulation of deleterious viruses produced in the field have promoted the development of *in vitro* propagation of garlic (Nagakubo *et al.,* 1993). The propagation of garlic using *in vitro* technique has been studied by numerous workers (Ayabe *et al.*,1982). Improvement of shoot regeneration by adding various combinations of plant growth regulators to media have been reported (Myers, J. M. and P. W. Simon, 1998.) and encouraging results are shown that are helpful in obtaining a large quantity of regenerated shoots for commercial use.

Various explants such as shoot tip stem disc and root were successfully used to induce callus (Koch *et al*., 1995). Inspite of many efforts to develop of these *in vitro* techniques for garlic micro propagation, an efficient system for fast production of callus and subsequent regeneration of shoot is still lacking for this crop, which may overlook the potential combinations of certain plant growth regulators that are more suitable for shoot multiplication.

So, considering the above problems and prospects the present study was undertaken with following objectives:

- To establish an *in vitro* regeneration protocol
- To study the effect of growth regulator
- To identify the best concentration of hormone for *in vitro* shoot regeneration
- To assess the combined effect of two or more hormone on shoot regeneration potentiality
- To establish acclimatization ability of *in vitro* regenerated plantlet under natural environmental condition

Chapter II

Review of literature

CHAPTER II

REVIEW OF LITERATURE

A barrier to the improvement of garlic through traditional breeding is sexual sterility (Novak and Havranek, 1975; Novak, 1990). Conventional techniques of crop improvement are time consuming process. Therefore *in vitro* techniques are considered to be as alternative tool of unconventional method of garlic improvement (Novak *et al*, 1986; Novak, 1990). Related works already have conducted by different researchers around the world have been reviewed and some of the most relevant literatures are cited here under the following sub-headings.

2.1 Concept of garlic tissue culture

As a new, rapid and powerful tool for crop improvement techniques of plant tissue culture have been developed (Carlson,1975, Razdan and Cocking,1981) and received wide attention of modern scientists (D' Aamato, 1978;Skirvin, 1978; Larkin and Scowcroft, 1982) especially in the crop plants like garlic where conventional breeding is very complicated.

2.2 Explant

In vitro regeneration of garlic by means of tissue culture technique may be achieved through direct regeneration from root tips, clove and somatic embryogenesis.

Plantlet regeneration of garlic from stem tip (Kehr and Schaeffer, 1976; Abo El-Nil,1977; Novak 1980, 1983; Walkey et al.,1987). Leaf tissue (Havranek and Novak, 1973, Novak and havranek, 1975; Novak1980; Nagasawa and Finer, 1988; Rauber and Grunewaldt, 1988; Maggioni, *et al*, 1989), basal plate and receptacle, (Xue *et al*, 1991a, 1991b; Koch *et al*.1995) and root (Shuto *et al*. 1993), Myers and Simon, 1998) have been reported through callus culture.

Haque *et al*. (2000) performed an experiment where root tips of eight garlic (*Allium sativum L.)* genotypes were used for direct regeneration. Genotypic differences were evident in regeneration and bulblet formation among the cultivars used.

An experiment was performed by Myers and Simon (2001) on root segments form shoot dip-derived plantlets of three garlic clones for continuous friable callus production. The best production occurred on root segments initially cultured on MS medium with 4.5µM M 2,4-D.

Luciani *et al*. (2006) conducted an experiment to evaluate the effects of different explants (basal plates, meristems, immature umbels, and root tips) on callus induction and plants regeneration in garlic (*Allium sativum* L.*)*they found that meristems enhanced callus production and quality increased plant regeneration when culture on BDS (Basal Dunstan and Short, 1977) medium supplemented with 2,4-D.

Yasmin (2005) conducted an experiment to establish protocol to obtainin plantlets using root tip, basal plant and leaf dise explant of garlic. Ten garlic cultivar and different concentration and combinations of growth regulators were used. Auxin 2,4-D (2.0 mg/1) supplemented with MS medium exhibited highest callus induction ability using leaf discs explant. The highest performances (95%) in proliferation of calli were obtained at MS + picloram (2.0mg/1) +BPA (2.0mg/1) using root tip explant. MS + sucrose (30g/1)+BPA (2.0mg/1) and MS + NAA (2.0mg/1) + kinetin (2.0mg/1) showed the maximum shoot initiation and their subsequent regeneration.

Khan *et al.* (2004) used garlic root tips to regenerate shoots through callus culture. Higher percentage of callus was initiated from the combination of 5 mg /L Kinetin and 1.5 mg /L 2,4-D. Callus produced higher number of shoots in MS medium supplemented with 10 BAP.

Haque *et al*. (2003) performed an experiment to observe the callus induction, proliferation, organogenesis, and to produce regenerate plantlets under *in vitro* condition using root tip, basal discs and leaf discs. The highest callusing was recorded at 2,4-D (3 mg/1) + BAP (1.0mg/1) in MS medium. Highest performances in the proliferation of calli

were found at NIS +2,4-D (2.0 mg/L) + BAP (0.5 mg/1). Maximum number of regenerated plantlets was obtained at MS + NAA (2.0 mg /L) + BPA (1.0 mg/L).

Popescu and Butnaru (2002) worked on variation in garlic (*Allium sativum* L.*)* calli formation and cytological behavior. Bulb explants (meristematic basal plates and bulb sections) of 3 garlic cultivars (Cenad, Inand and Mako) were cultured in MS medium supplemented with different combinations of 2,4-D, kinetin and IAA. Callogenesis was observed on the media supplemented with 2,4-D (2 mg/L). When meristematic basal plates were used as explants, the percentage of callogenesis was higher.

Sakar and Sawahel (1998) showed the origin of garlic explants govern their competence for plant regeneration. A two step procedure for *in vitro* regeneration of an Egyptian garlic culture was established using mature and immature cloves as explants. Proliferation of callus was highest on MS medium with B_5 vitamins supplemented with 2,4-D at 2 mg/L + kinetin at 0.5 mg/L. Callus regenerated plantlets on either basal MS medium or MS medium supplemented with kinetin at 1 mg/L, which was more effective than benzyladenine.

2.3 Plant growth regulators

According to Mahajan (2013), the average shoot length was maximum in the medium supplemented both with 0.5 mg/l Kinetin and 0.5 mg/l BAP (9.8±0.22 cm) while the MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l BAP gave lowest average shoot length of 2.3±0.42 cm.

Yanmaz, R. *et al* (2006) carried out a novel micro propagation method was developed for Tunceli garlic by root and shoot culture techniques. The different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) for root culture and NAA, indole-3-acetic acid (IAA) with benzyladenin (BA) (0, 0.1, 1.0 mgl-1) for shoot culture experiment were used in Murashige & Skoog (MS) medium which were supplemented with 30 g /L sucrose and 0.8 mg l-1 agar .The root tip culture was not found as a proper method for shoot proliferation, on the contrary, shoot culture was found effective on shoot formation. As an average, 1 or 2 shoots obtained per explant and lower doses of IAA and BA (0.1 mg /L, 0.1 mg /L) had an important effect on it.

Martin and Pietro (2004) carried out an efficient protocol for callus culture and shoot regeneration of garlic *(Allium sativum* L.*).* The influence of different callus induction media on the regeneration process in garlic was tested. The best results were obtained in induction media containing 13.3 PM benzyladenine + 0.14µM 2,4-D+10.7µ M NAA.

Mujica and Mogollon (2004) studied the effects of cytokinin of benzyladenine (BA), zeatin (Z), and isopentenyladenine (2ip) combined with sucrose on *in vitro* bulbs formation of garlic *(Allium sativum* L.*)*. The highest response was found on MS medium with 2.0 mg/L and high bulbs growth was obtained with high sucrose concentrations.

Du *et al*. (2004) studied virus elimination technology of garlic by shoot tip culture The effects of different culture media (MS, N6, and B5) and plant growth regulators (benzyladenine at 0.5,1 or 2 mg/L; zeatin at 0.5 or1.0mg/L; IAA at 0.2 mg/L, and NAA at 0.01, 0.021 0.05, 0.2, 0.4, 0.6, 0.8 or 1.0 mg/L) on garlic shoot tip development and propagation and root formation were studied. In MS medium supplemented with I mg benzyladenine and I mg FBA/liter, the size of the shoot tip was 0.2-0.6 mm with first leaf primordium for explants, and the rate of developed plantlets was 90%. The number of regenerated buds was highest when MS medium was supplemented with 0.5 mg benzyladenine and 0.2 mg IAA/liter. Culturing in the root formation medium (MS supplemented with 0.8 mg NAA/liter) resulted in 70% root induction, 85% virus elimination in the regenerated plantlets, and the establishment of a propagation system of virus free garlic.

Keller *et al*. (2003) performed an experiment on callus induction, ploidy level and plant regeneration *in vitro* garlic *(Album sativum* L.*)* cultures. Optimum callus induction from leaf explants of cv. Biancopiacentino was achieved on MS medium supplemented with 2 mg IAA, 0.5 mg 2,4-D and 0.1 mg kinetin per liter. Shoots were regeneration on MS medium containing 2 mg IAA and 4.5 mg kinetin per liter and rooting was induced on MS medium with 10g sucrose and 0.1 mg IBA per liter.

Zheng *et al*. (2003) reported an experiment on the development of an efficient cultivar-independent plant regeneration system from callus derived from both apical and non-apical root segments of garlic *(Allium sativum* L.*).* When the cytokinin 6-(gamma, gamma-dimethylallylamino) purine (0.1mg /L: 0.5(M) was present during callus

induction, shoot regeneration ranged from 30.10 to 47.60%. Shoot regeneration from callus induced on non-apical segments was higher, although not significant, compared to callus induction from apical root segments in the second series of experiments. All in all, an efficient callus induction and plant regeneration system was developed from both apical and non-apical segments taken along the entire length of the roots. This system has potential to be used for garlic transformation.

Kim *et al*. (2003) reported on high frequency of shoot multiplication and bulblet formation of garlic in liquid cultures. The highest multiplication rate was found when explants were cultured in bulbing medium (MS medium containing 0.1 mg /L NAA+11% ($W/_v$) sucrose supplemented with 10 µM JA. Darkness promoted the bulblet induction and growth compared to light conditions (16-h photoperiod of 50u mol). The dormancy of bulblets was broken by cold treatment at 4ºC for- 8 weeks.

Haque *et al*. (2003) carried out a study to develop a protocol of regeneration in Bangladesh local garlic using shoot and root meristems. The highest (95.55) regeneration from shoot meristem was found growth regulator free medium. 40% root meristem regenerated shoot on MS medium + 1 M (NAA) + 10M (BPA). Multiplication of shoots occurred on MS medium containing 0.5 M BAP.

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Robledo *et al* (2000) used root apices form *in vitro* cultured garlic cloves as axenic explants for organogenic callus production and plant regeneration were investigatd. Explants culture in media based on N6 or MS medium supplemented with 2,4-D alone or combined with kinetin. Shoots started to grow 3 weeks after culturing.

Sata *et al*. (2000) found an efficient novel method of direct somatic embryogenesis form basal tissue of garlic clove was developed by where the influence of plant growth regulators, basal medium and explant type on somatic embryo induction was examined. The best combination of growth regulator was 2,4-D and kinetin at 1.0mg/1 and 0.5mg/1 respectively

Myers and Simon (1999) conducted an experiment on regeneration of garlic callus as affected by variation, plant growth regulators and culture conditions over time. Treatment with 1.4 µM picogram and 13.3 µM BA stimulated the highest rate of shoot production.

Regeneration rate decreased as callus age increased, but healthy plantlets from callus culture up to 16-month old were produced for all clones. Regeneration of long-term garlic callus cultures could be useful for could propagation and transformation days. Final bulb weight, number of cloves/bulb and bulb yield 109.5%5 37.0% and 99.8% higher, respectively, in plants propagated tissue culture than in conventional plants.

Ayabe *et al*. (1998) mentioned that the restricted part of the undeveloped stem of the garlic clove, called the "stem disc", which is just under the basement of the immature foliage leaves, proved to be a very potent explant for the micro propagation of garlic. Twenty to thirty tissue-cultured shoots consistently were differentiated from a single clove during 1 month of culture on phytohormone-free Linsmaier and Skoog medium. In addition, more than 90% of the shoots formed bulblets *in vitro* during an additional 1 month of culture.

Gao *et al*. (2000) carried out experiment to determine the best medium composition and concentration of phytohormones with a view to accelerate the propagation of virus-free materials by .The results indicated that the best medium for inducing callus was MS+BA 0.2mg/1+NAA 0.5 mg/1.

Saker and Sawahel (1998) put forward on garlic explants and their competence for plant regeneration. Proliferation of embryogenic callus was highest on MS medium with B5 vitamins supplemented with 2,4-D at 2 mg/L + kinetin at 0.5 mg/L. Embryogenic callus regenerated plantlets on either basal MS medium or MS medium supplemented with kinetin at 1 mg/L, which was more effective than benzyl adenine. Various SDAPAGE protein profiles of the different embryogenic callus lines were observed, Cell lines IM1 and IM2, derived from immature clove explants showed the highest regeneration potentials.

Yun *et al*. (1998) reported on multiplication of shoots through shoot-tip culture of garlic. The most effective medium for induction and multiplication of shoots was MS medium supplemented with 0. mg IAA, mg Zip and 30 mg sucrose/liter. Bulbs lets were obtained on MS medium supplemented with 0.5 gm IAA, 0.1 mg Zip and 70 g sucrose/liter.

Barringer *et al*. (1996) studied on regeneration of *Allium* spp, *in vitro* by slicing the basal plant in USA. All the species of Allium produced more shoot when the basal plate was sliced than when it was left intact. More shoots were produced when 4.40µM benzyladenine was supplemented present in the MS medium than when it was absent.

Haque *et al*. (1998) developed a protocol for efficient plant regeneration in garlic. Different concentration of 2,4-D was investigated for callus induction. Callus inhibition of embryo formation was found better at concentration of 0.5 µM and > 1µM 2,4-D, respectively.

Haque *et al*. (1997) reported on high frequency shoot regeneration and plantlet formation from root tip of garlic in Japan. The influence of growth regulators basal media and age of root explants of garlic cultivar white Roppen on shoot initiation and proliferation was examined. The best growth regulator was NAA and 6-Benzyladenine at I and 10µM respectively inducing shoot initiation in 75% of explants. The frequency of shoot initiation on different basal media was similar. Explants root tips from plantlet taken15-18 days after sprouting showed the highest shoot initiation (95%). In contrast to MS medium, which produce more than 10 shoots per explant, *B5* medium produced smaller shoots, although the number was higher plantlets, after acclimatization in a growth cabinet, were successfully transplanted to the field and no phenotypic variation was observed among them.

Tapia (1996) worked on callus induction, organogenesis and *in vitro* plant regeneration of garlic. The effect of 2,4-D concentration was found genotype dependent. Maximum calli were observed on MS medium supplemented with 0.125gm 2,4-D/1. Organogenesis of callus was favored by 4.0 mg kinetin and 0.1 mg NAA/1.

Kudou *et al*. (1995) cultured garlic cv. Taiso explants *in vitro* on MS medium supplemented BA+NAA each at 0, 1 or 2 mg/L in all combinations. Callus formation occurred for all types of explants and was promoted by increasing BA and NAA concentrations.

Ma *et al*. (1994) worked with stem tips with 1-2 leaf primaordia which were cultured on 8 different media (M_1-M_8) consisting of either Ms medium supplemented with various

combinations and concentration of several growth regulators. The best callus induction and proliferation occurred on MS medium (MS medium supplemented with 0.5 mg NAA and 0.5 mg/1 kinetin).

Barandiaran *et al*. (1990) reported on method for callus culture and shoot regeneration of garlic *(Allium sativum* L*)*. Root de Cuenca was cultured on B5 induction media containing various combinations of benzyladenine. For regeneration, media containing various combination of benzyl adenine, kinetin, IAA and TIBA was used. The auxin 2,4-D frequently used in garlic tissue culture was detrimental when used at the levels described in the literature. The best result were obtained in induction media containing 13.3 µM benzyl adenine + 0.14 µM 2,4-D + 10.7 µM NAA.

Bhojwani,S. S. (1980) worked with shoot buds excised from dormant cloves of the New Zealand commercial garlic (*Allium sativum* L.) and a virus-free French cultivar 'Rose-de-Kakylis', proliferated both axillary and adventitious shoots on B-5 basal medium supplemented with 0.5 mg/L isopentenyladenine (2-ip) and 0.1 mg/L naphthalene acetic acid (NAA). An 8-fold increase in shoot number occurred every 6 weeks.

2.4 Establishment of *in vitro* garlic plantlets in rooting media

Metwally *et al.* (2014) observed *in vitro* regenerated garlic plantlets had roots per explants with 2-3 cm in length in average.He observed that regenerated garlic shoots formed a well developed root system within seven to eight days upon their cultures on MS medium without PGRs.

Mahajan *et al.* (2013) found effective rooting of plantlets on MS media supplemented with 0.1mg/l NAA. He suggested that lower level of NAA (0.1 mg/l) resulted in elongated but fewer roots in *in vitro* regeneration. Maximum number of shoots were obtained in MS medium supplemented with 1.0 mg/l of Kinetin (5.0±0.55 shoots per explant).

2.5 *Ex vitro* survival of plantlets:

According to Mahajan, (2013), the rooted *in vitro* garlic plants transferred to small plastic cups resulted in 90 % survival rate after three weeks of transplantation.

Bekheet, S.A (2006) reported that high survival rate of garlic plants (90%) after using perlit and peatmoss in the rato 1:1 instead of sand and soil (2:1). The plantlets were adjusted after 2-3 weeks and were allowed to grow further for 7-8 days.

Khan *et al.* (2006) found about 75% of garlic plants survival in field condition after regenerating *in vitro*.

Chapter III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 Time and location of the experiment

The present research was carried out in the Laboratory of biotechnology of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, and Dhaka-1207 from the period of July 2015 to June, 2016.

3.2 Source of material

The planting materials (cloves) of garlic were collected from Agargaon bazar, Sher-e-Bangla Nager, Dhaka-1207. The healthy, disease free cloves of garlic were used as explants for the study of *in vitro* propagation.

3.2.1 Instruments and glassware

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. For the experiments the cate glasswares were used. Erlenmeyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders were oven dried (250^{o}C) used for media preparation. With the liquid detergent (Trix) the glassware were first rinsed and washed thoroughly with tap water, until the detergent was removed completely. Finally with distilled water they were rinsed and sterilized for 3-4 hours in oven at 16^{o}C-180^0C.

3.2.2 Culture medium

The selection of nutritional components and growth regulators plays vital role for success in tissue culture..For tissue culture media should contain essential for normal plant growth which include all major and minor elements, vitamins and growth regulators media composed of basal MS (Murashige and Skoog) medium supplemented with the plant growth regulators is ideal for explants for inoculation.

3.2.3 Plant growth regulators (PGRs)

To different media PGRs or hormones were added separately according to the requirements. Following 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, 3.0, 3.5 mg/L) for shoot induction
2. 2,4-D (1.0, 1.5, 2.0, 2.5 mg/L) was applied with KIN for shoot and root formation

3.3 The preparation of the stock solution of hormones

In order to prepare hormonal supplements, they were dissolved in proper solvent as shown against each of them below. 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
2,4-D	1 N NaOH

The stock solution of hormones was prepared by following a general procedure. In 10 ml of 70% ethyl alcohol or 1 (N) NaOH solvent 100 mg of solid hormone was placed in a small beaker and then dissolved in. By the addition of sterile distilled water using a measuring cylinder the volume was made up to 100 ml. The prepared hormone solution was then labeled and stored at 4±10C for use up to 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. In 1000 ml beaker 700 ml of sterile distilled water was poured.

Step-2. With the help of a hot plate magnetic stirrer 5 gm of readymade (Duchefa) MS media and 30 gm of sucrose was added and gently stirred to dissolve these ingredients completely.

Step-3. Either in single or in combinations different concentrations of hormonal supplements was added to the solution as required and mixed well.

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

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 for melting.

3.5 Steam heat sterilization of media (Autoclaving)

The culture medium was poured in 200 ml culture bottles and then autoclaving was done at a temperature of 121^{o}C for sterilization, for 30 minutes at 1.06 kg/cm2 (15 PSI) pressure. 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

Garlic clove were separated from compound bulb and pealed manually, washed thoroughly under running tap water and then with sterilized distilled water for several times. and air dried. Small and injured cloves were excluded. 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, cloves were immersed in 0.5% $HgCl_2$ within a beaker and added 3-4 drops of Tween-20 for 1 minute with constant hand agitation. Then cloves were washed 3-4 times with distilled water to make the material free from impurities and ready for inoculation in media.

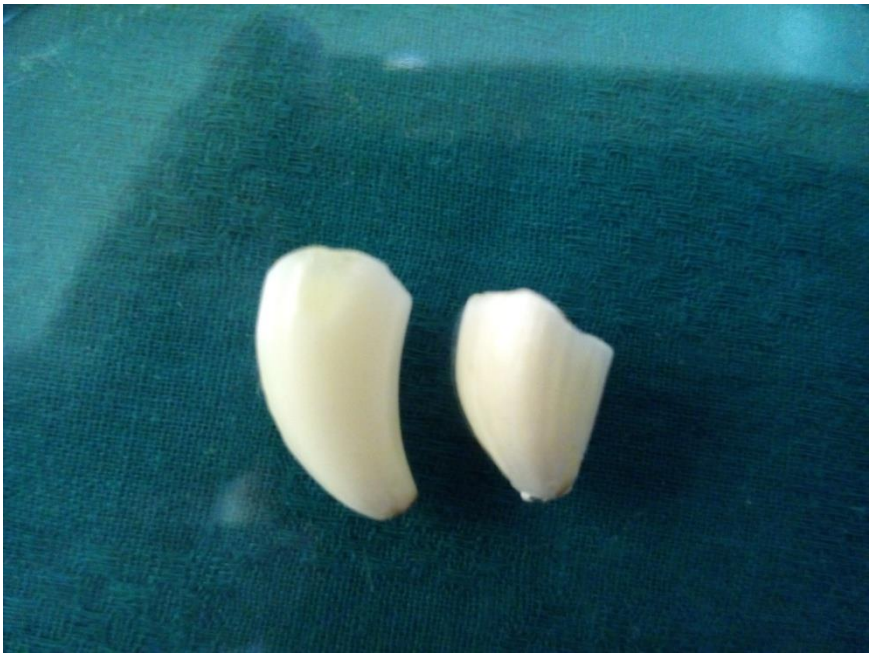


Plate 1: **Garlic explants (basal part of cloves); prepared for inoculation in MS media.**

3.7 Inoculation of culture

Within laminar airflow cabinet the sterilized explants were inoculated carefully. .Firstly the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with the same alcohol. In an autoclave all glassware, instruments and media were steam-sterilized. In a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner during the course of the work, instruments in use were placed. With 70% alcohol the hands and forearms were washed thoroughly with soap and water and repeatedly sprayed during the period of work. The mouth of all culture vial was flamed before and after positioning of the explants on the medium. Explants were transferred to large sterile glass petridish or glass plate with the help of sterile forceps under strict aseptic conditions for inoculation,. Sterile scalpel blade was used to make suitable size. Here the explants were further trimmed and extra outer cover was removed after cutting explants into suitable size (2-3 cm), explants are inoculated to culture bottles containing MS medium with plant growth regulator. Mouth of bottle is quickly flamed and capped tightly after vertically inoculating the explants in culture bottle. Afterwards proper labeling is done, mentioning, date of inoculation, PGR applied etc. the bottles was transferred to growth room.

3.8 Culture Room

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

3.9 Adventitious hoot regeneration

The explants were cultured on MS nutrient medium supplemented with different concentration of KIN (0, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L). Subculture was done with newly formed shoots after successful shoot proliferation. With the help of sterile forceps shoots were excised in aseptic condition and transferred to new In order to increase budding frequency MS media supplemented with same concentration of growth hormones. Throughout the entire culture period the observations on development pattern of shoots were made. Data recording was started after first weeks from inoculation.

3.10 Rooting of proliferated shoots

Newly formed shoots were transferred to rooting media from the culture vial. Two types of growth regulators (KIN and 2,4 D) 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 first week of inoculation.

3.11 Acclimatization

Acclimatization is a process by which *in vitro* regenerated plants are hardened to adapt to an in vivo environment.

Step-1: After 21 days of culture media, the plantlets were taken out from culture vial with the help of forceps . Utmost care is taken to prevent any damage to newly formed roots. To remove any traces of solidified agar media dipped in gentle warm water for acclimatization. With garden soil plastic pots were kept ready filled and compost in the proportion of 1:1 respectively. The plantlets were then transplanted in to the pots with special care after removing solidified agar media from newly formed roots,.

Step-2: After planting, the plantlets were with light intensity varied from 2000–3000 lux thoroughly watered and were kept at 25±2 °C. 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 transferred to shade house with less humidity and sunlight. The top of the pots were grew at room temperature and 70% RH for 14 days covered with transparent plastic sheet.

Step-4: Into different pot having bigger pot size, the plants were transferred to the soil after 3 weeks following depotting and potting. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary.

3.12 Treatments

Two sub experiments were conducted to fulfill the mentioned objectives. The experimental title and its treatment was given bellow

Sub-experiment 1. Effect of KIN on shoot proliferation
Treatments: Five levels of KIN (1.0, 1.5, 2.0, 2.5, 3.0 mg/L) and a control (0.0 mg/L) were used.

Sub-experiment 2. Combined effect of different concentrations of KIN and 2,4-D on shoot and root formation.
Treatments: Five levels of KIN (1.0, 1.5, 2.0, 2.5, 3.0, 3.5mg/L) were used with combination of four levels of 2,4-D(1.0,1.5, 2.0, 2.5 mg/L) a control (0.0 mg/L) were used.

3.13 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 1st and 3rd 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 1st week to 3rd week of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

- Percent of explants showing shoot induction
- Days for shoot induction
- No. of shoots per explants
- Average Length of shoot (cm)
- Length of the longest shoot
- No. of leaves per explants
- Length of leaves
- Percent of explants showing root induction
- Days for root induction
- No. of roots per explants
- Average length of root (cm)
- Length of the longest root (cm)
- Survival percentage

3.13.1 Calculation of days to shoots and roots induction

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

3.13.2 Calculation of number of shoots and roots per explants

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

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

3.13.3 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:

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

3.13.4 Calculation of number of leaf

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

3.13.5 Calculation of shoots and root length (cm)

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

3.14 Statistical analysis

The experiment was designed in a completely randomized design (CRD) with three replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique. Using MSTAT-C program differences among results through treatments means were compared by using Duncan's Multiple Range Test (DMRT) at 5% probability level.

Chapter IV
Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

Two separate experiments were conducted for the rapid micro propagation of the garlic. The objective of the present study was to develop a regeneration protocol of garlic. The results of these experiments are discussed in this chapter with Figure (1-3), Tables (1-17) and Plate (2-6). Analysis of variance have been presented in Appendices.

4.1 Sub-experiment 1. Shoot proliferation in garlic

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

4.1.1 Percent of explants showing shoot induction

Significant variation was found on percent of explants showing shoot induction imposing doses of KIN. Maximum (100%) shoot induction was in 3 mg/L KIN, whereas the lowest (50%) induction was found at control condition. (Table 1) Khan *et al.* (2004) found 10 mg/L BAP with highest shoot regeneration (56.80%) while working with proliferation from callus which is almost similar to the results of present study. Khan *et al.* (2013) observed that BAP helped in shoot regeneration from cultured callus. This result corroborated with the findings of Kudou *et al.* (1995) who reported that BAP was the most effective stimulator for shoot formation.

The result is presented in the following (**Table 1)**.

Table 1: Effect of KIN on the shoot initiation potentiality in garlic

KIN (mg/L)	Number of explants inoculated	Shoot initiation potentiality (%)
Control	25	50
1.0	25	88
1.5	25	90
2.0	25	95
2.5	25	98
3.0	25	100

4.1.2 Days to Shoot Induction

The shoot induction of the explants was found to be very fast when the hormone was used. Minimum 1 day was recorded for the 3 different concentrations- 2, 2.5 and 3 mg/L of KIN and the maximum 7 days in control.

Khan *et al* (2004) observed minimum 115.1 days for induction shoot by applying 10mg/L BAP while using cultured callus of garlic. Maximum 132.4 days was required for shoot induction by 20 mg/l BAP which was statistically similar with control treatment (130.1days).

The result is presented in the following (**Table 2)**.

Table 2: Effect of KIN on days for shoot induction in garlic

Treatment	Dose(mg/L)	Days for shoot induction
Control	0.0	6.67 a
KIN	1.0	3.00 b
	1.5	1.67 c
	2.0	1.00 d
	2.5	1.00 d
	3.0	1.00 d
LSD(0.05)		0.54
CV (%)		12.48

Values in the column are the means of thee replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

4.1.3 Number of shoots per explants

Different KIN concentrations did not have influence on the variation of number of shoots per explants. 1 shoot per explants was found at 0 to 3.5 mg /L concentration of KIN. The records were taken on weekly basis up to 3 weeks after initiation.

Mahajan *et al.* (2013) found 4.34±0.17 number of shoots per explant with 1mg/L BAP while using cloves of garlic as explants.

He observed that average number of shoots per explant was less when the MS medium was supplemented with BAP and Kinetin both as compared to medium supplemented with either BAP or Kinetin alone. Likewise, The MS medium supplemented with 1.0 mg/l Kinetin gave least number of average shoots per explant (1.5±0.10) by Qudah T.S (2011).

The result is presented in the following (**Table 3)**. Data was recorded at the end of each week after initiation.

Table 3: Effect of KIN on number of shoots per explants in garlic

Name of Treatment	Dose of Hormone(mg/L)	Number of Shoot		
		After 1 week	**After 2 week**	**After 3 week**
Control	0.0	1	1	1
KIN	1.0	1	1	1
	1.5	1	1	1
	2.0	1	1	1
	2.5	1	1	1
	3.0	1	1	1
	3.5	1	1	1

Values in the column are the means of three replicates.

4.1.4 Number of leaves per explants

On observing the number of leaves significant influence of hormones was found. Maximum 3 leaves were recorded with 2.0, 2.5 3.0 and 3.5 mg/L KIN after 3 weeks and in the case of lack of hormone result showed 1.67 leaves after 3 weeks of initiation.

The result is presented in the following **Table 4**.

Table 4: Effect of KIN on days for number of leaves explant in garlic

Name of treatment	Dose of Hormone (mg/L)	Number of Leaves per Plant		
		After 1 week	After 2 week	After 3 week
Control	0.0	0.00 d	1.33 c	1.67 c
KIN	1.0	1.00 c	2.33 b	2.67 b
	1.5	1.67 b	2.33 b	2.67 b
	2.0	2.00 ab	3.00 a	3.00 ab
	2.5	2.33 a	3.33 a	3.00 ab
	3.0	2.00 ab	3.00 a	3.00 ab
	3.5	2.00 ab	3.00 a	3.00 ab
LSD (0.05)		0.52	0.55	0.78
CV (%)		8.80	11.78	5.53

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

4.1.5 Average length of shoot (cm)

The maximum average length of shoot 33.43 cm was noticed from the 3.0 mg/L KIN which was statistically similar with 2.5 mg/L KIN (32.50 cm) and statistically different from rest of treatments; whereas the minimum (18.0 cm) in case of lack of hormone.

Mahajan *et al.* (2013) observed that BAP 1.0 mg/L gave average 6.9±0.22 cm of shoots from cloves of garlic after 28 days of initiation.

The result is presented in the following **Table 5** and **Figure 1**.

Table 5: Effect of kinetin for average length of shoots per explants

Name of Treatment	Dose of Hormone	Average Length of Shoot (cm)					
		After 1 Week		After 2 weeks		After 3 weeks	
Control	0.00	5.50	e	12.50	F	18.00	e
KIN	1.00	7.50	d	15.00	e	27.00	d
	1.50	8.50	c	18.00	d	28.33	c
	2.00	10.50	b	21.50	c	30.33	b
	2.50	12.50	a	22.70	b	31.33	b
	3.00	13.00	a	23.00	ab	33.43	a
	3.50	13.20	a	23.60	a	32.50	a
LSD (0.05)		0.83		0.64		1.15	
CV (%)		4.64		3.85		4.25	

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level DMRT.

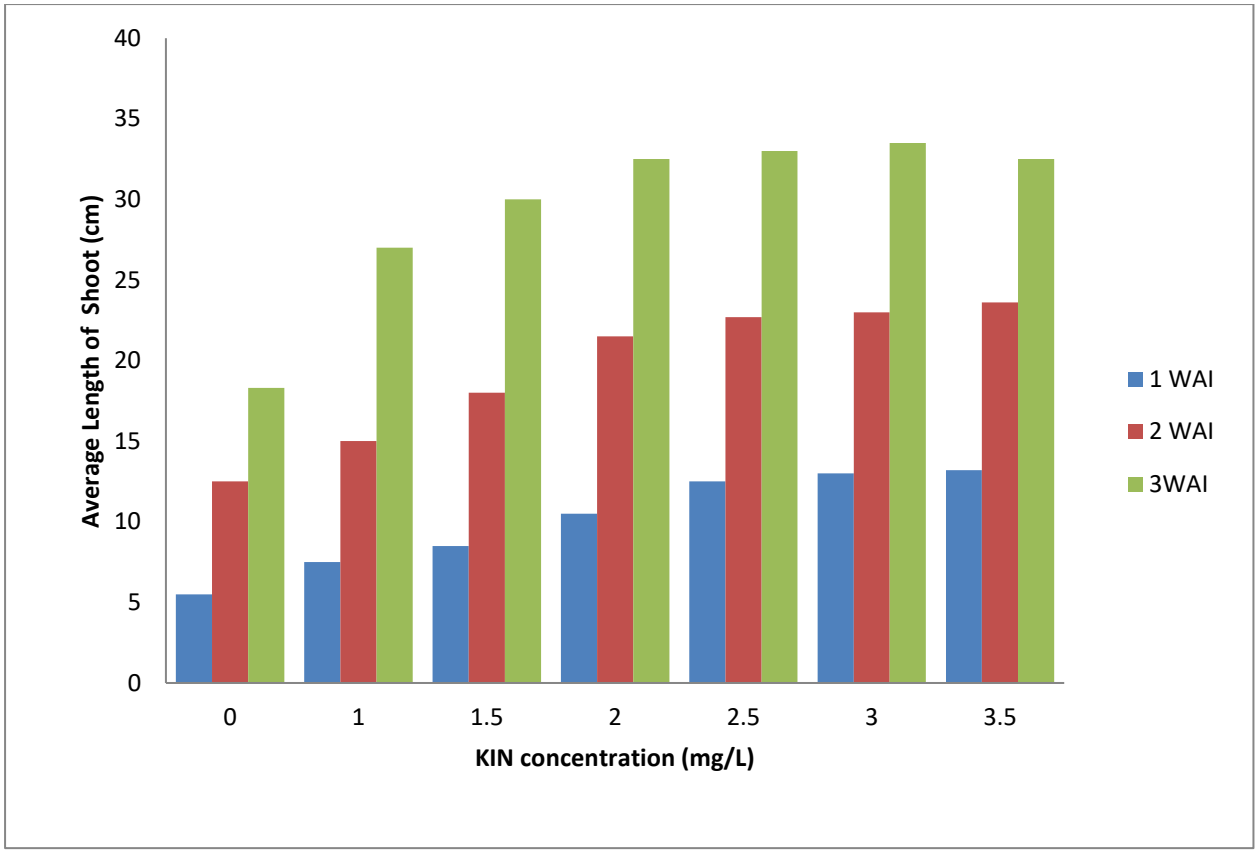


Figure 1: Effect of KIN on days for length of average shoot in garlic

4.1.6 Length of longest shoot (cm)

The maximum length of longest shoot 34.50 cm was noticed from the 3.00 mg/L KIN which was statistically similar with 3.5 mg/L KIN (33.70) and rest of others were found statistically different from these treatments. The minimum shoot length was 19.20 cm in control treatment.

The result is presented in **Table 6** and **figure 2** in the next page. Data was recorded at the end of each week after initiation.

Table 6: Effect of kinetin for days for length of longest shoot in garlic

Name of treatment	Dose of Hormone (mg/L)	Length of longest Shoot (cm)					
		After 1 Week		After 2 weeks		After 3 weeks	
Control	0.00	6.50	g	13.60	f	19.20	d
KIN	1.00	8.50	f	15.50	e	29.13	c
	1.50	9.50	e	18.50	d	30.50	c
	2.00	13.50	c	23.50	c	32.00	b
	2.50	14.87	b	25.30	a	33.20	ab
	3.00	15.70	a	24.60	b	34.50	a
	3.50	11.60	d	22.90	c	33.70	a

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

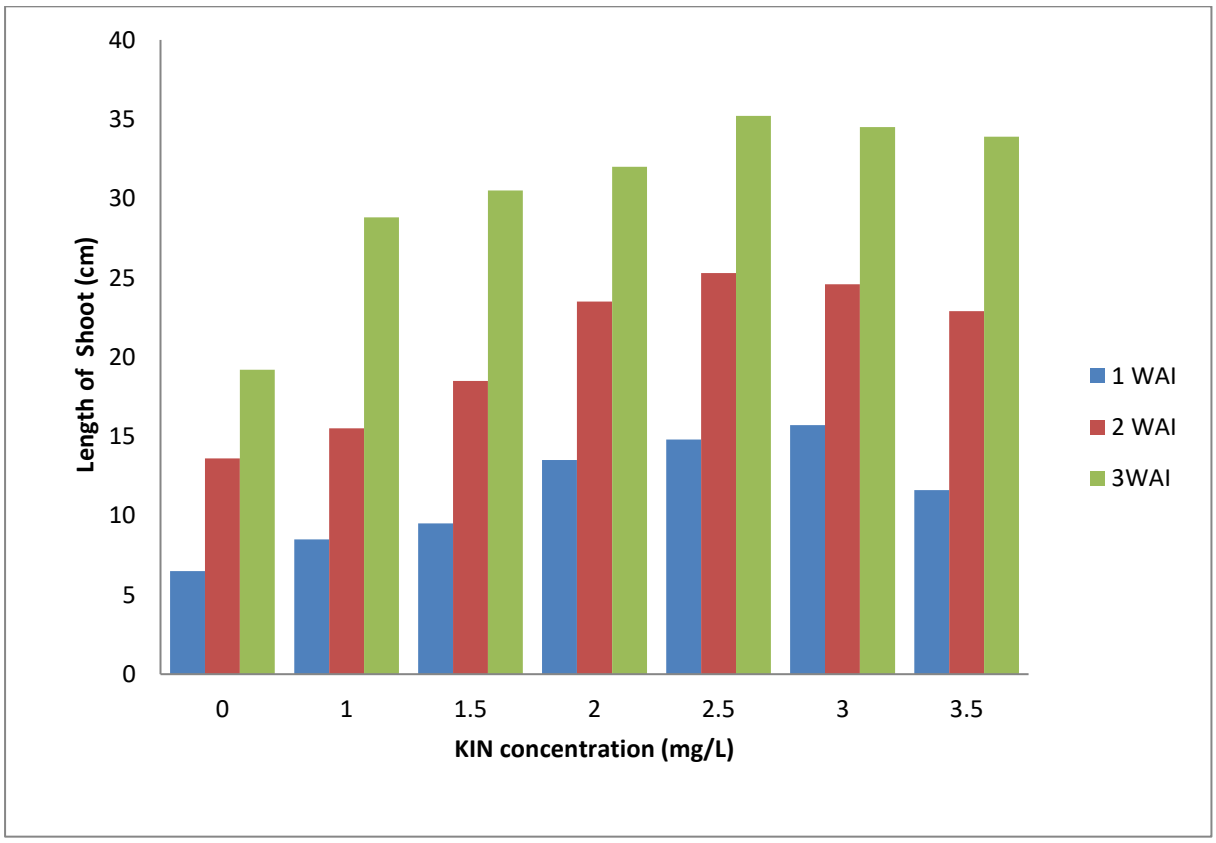


Figure 2: Effect of KIN on length of longest shoot in garlic.

4.2 Sub Experiment 2: The combine effect of KIN + 2,4-D on shoot proliferation and root initiation

In experiment 2,individual doses of KIN showing most potential performance was used as the basis of setting of different hormonal combination along with 2,4-D. Here the combined effect of different concentration of KIN + 2, 4 D also showed different results on different parameters assessed. These results are presented under following headings with Tables.

4.2.1 Percent of explants showing shoot induction

Significant variations of different concentrations of KIN and 2, 4,-D showed regeneration potentialityin Garlic (Table 6). With 2.5 mg/L KIN + 1.0 mg/L 2,4-D; 2.5 mg/L KIN + 1.5 mg/L 2,4-D ; 2.5 mg/L KIN + 2.0 mg/L 2,4-D; 2.5 mg/L KIN + 2.5 mg/L 2,4-D ; 3.0 mg/L KIN + 1.0 mg/L 2,4-D; 3.0 mg/L KIN + 1.5 mg/L 2,4-D; 3.0 mg/L KIN + 2.0 mg/L, 2,4-D ;3.0 mg/L KIN + 2.5 mg/L 2,4-D. 100% shoot initiation was found. The lowest regeneration potentiality was seen in control treatment (60%).

Yanmaz *et al.* (2010) showed that lower auxin and cytokinin concentrations were effective for shoot induction. In medium, 0.1 mg/L BA and 0.1 mg/L. IAA were sufficient doses for Tunceli garlic.

The result is presented in **Table 7** in the next page.

Table 7: Effect of KIN and 2,4-D on percent of explants showing shoot induction

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explants inoculated	Percent of explants showing shoot induction
Control	0.0	20	60
KIN+2,4-D	1.0+1.0	20	87
	1.0+1.5	20	88
	1.0+2.0	20	93
	1.0+2.5	20	95
	1.5+1.0	20	95
	1.5+1.5	20	97
	1.5+2.0	20	98
	1.5+2.5	20	89
	2.0+1.0	20	87
	2.0+1.5	20	92
	2.0+2.0	20	94
	2.0+2.5	20	97
	2.5+1.0	20	100
	2.5+1.5	20	100
	2.5+2.0	20	100
	2.5+2.5	20	100
	3.0+1.0	20	100
	3.0+1.5	20	100
	3.0+2.0	20	100
	3.0+2.5	20	100

4.2.2 Days for shoot induction

Among different concentration of KIN+ 2,4-D variations were observed on days to shoot induction. In control the maximum (6.33) days were required to shoot induction .The minimum 1 days were recorded in case of 2.5 mg/L KIN + 2.5 mg/L 2,4-D ; 3.0 mg/L KIN + 1.0 mg/L 2,4-D; 3.0 mg/L KIN + 1.5 mg/L 2,4-D ; 3.0 mg/L KIN + 2.0 mg/L, 2,4-D ; 3.0 mg/L KIN + 2.5 mg/L 2,4-D mg/L which were statistically similar.

The result is presented in **Table 8** in the next page. Data was recorded at the end of each week.

Table 8: Effect of KIN +2,4-D on days for shoot induction

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explant inoculated	Days for shoot induction
control	0	20	6.33 a
KIN+2,4-D	1.0+1.0	20	4.00 c
	1.0+1.5	20	4.33 c
	1.0+2.0	20	4.33 c
	1.0+2.5	20	5.67 ab
	1.5+1.0	20	4.67 bc
	1.5+1.5	20	5.00 bc
	1.5+2.0	20	4.33 c
	1.5+2.5	20	2.33 d
	2.0+1.0	20	1.67 d
	2.0+1.5	20	1.33 d
	2.0+2.0	20	1.33 d
	2.0+2.5	20	1.00 d
	2.5+1.0	20	1.00 d
	2.5+1.5	20	1.00 d
	2.5+2.5	20	1.00 d
	3.0+1.0	20	1.00 d
	3.0+1.5	20	1.00 d
	3.0+2.0	20	1.00 d
	3.0+2.5	20	1.00 d
		LSD (0.05)	1.15
		CV (%)	6.98

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

4.2.3 Number of shoots per explants

After 3rd week of inoculation no significant variation was not observed on different concentration of KIN+2,4-D on the number of shoots per explant. One shoot per explant was observed in every treatment.

Mahajan *et al*. (2013) showed that maximum number of shoots were obtained in MS medium supplemented with 1.0 mg/l of Kinetin (5.0±0.55 shoots per explant).The MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l BAP gave least number of average shoots per explant (1.5±0.10).

Yanmaz R *et al* (2010) found generally one shoot was obtained per explant while working with shoot tips as explants with 0.1 mg /L IBA+0.5 mg/L IAA, 0.1 mg/L1BA+ 0.1 mg/L IAA, 0.0 mg/L NAA+ 0.1 mg/L BA and 0.0 mg /L IAA+0.1 mg /L BA combinations. He also found that average number of shoots per explant was less when the MS medium was supplemented with BAP and Kinetin both as compared to medium supplemented with either BAP or Kinetin alone.

The result is presented in **Table 9** in the next page.

Table 9: Effect of KIN and 2,4-D on number of shoots per explant

Name of the Phytohormone	Phytohormones concentration	Number of explant	Number of shoots		
			After 1 week	After 2 weeks	After 3 weeks
	Control		1	1	1
KIN+2,4-D	1.0+1.0	20	1	1	1
	1.0+1.5	20	1	1	1
	1.0+2.0	20	1	1	1
	1.0+2.5	20	1	1	1
	1.5+1.0	20	1	1	1
	1.5+1.5	20	1	1	1
	1.5+2.0	20	1	1	1
	1.5+2.5	20	1	1	1
	2.0+1.0	20	1	1	1
	2.0+1.5	20	1	1	1
	2.0+2.0	20	1	1	1
	2.0+2.5	20	1	1	1
	2.5+1.0	20	1	1	1
	2.5+1.5	20	1	1	1
	2.5+2.0	20	1	1	1
	2.5+2.5	20	1	1	1
	3.0+1.0	20	1	1	1
	3.0+1.5	20	1	1	1
	3.0+2.0	20	1	1	1
	3.0+2.5	20	1	1	1

Values in the column are the means of three replicates.

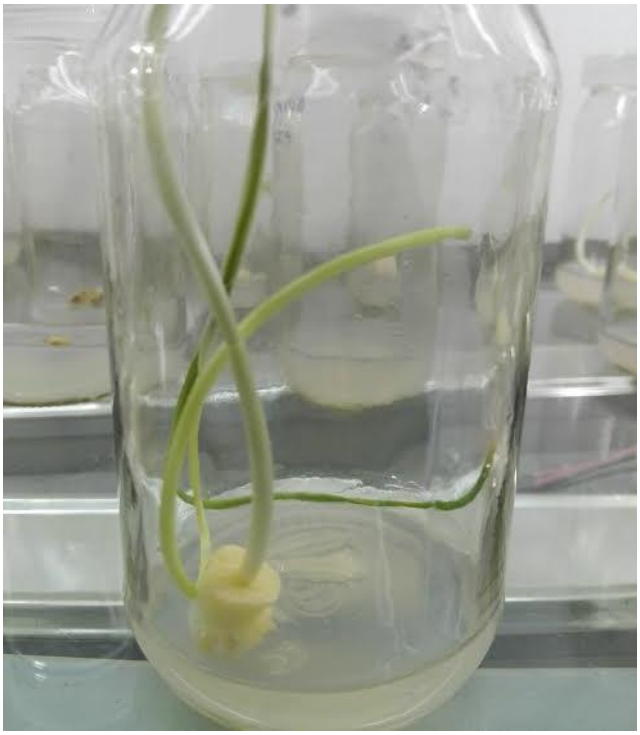


Plate 2: Effect of KIN and 2,4-D on number of shoots per explants in treatment 3.0 mg/L KIN + 1.0 mg/L 2,4-D.

4.2.4 Number of leaf per explants

The number of leaf per explants was not much significantly different according to the various concentrations of KIN+2, 4 D. The highest number of leaf per explants (3) was noticed on 2 mg/L KIN+ 1.5 mg/L ;2,4 D, 2 mg/L KIN+ 2 mg/L 2,4 D; 2 mg/L KIN+ 2.5 mg/L 2,4 D ;3 mg/L KIN+ 1mg/L 2,4 D; 3 mg/L KIN+1.5 mg/L 2,4 D;3 mg/L KIN+ 2.0mg/L 2,4 D; 3 mg/L KIN+2.5 mg/L 2,4 D , with statistical similarity, whereas the lowest (1) was found in control at 3 WAI.

The result is presented in **Table 10** in the next page.

Table 10: Effects of KIN and 2,4-D on number of leaves per explants

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explants Inoculated	Number of leaves per explants		
			After 1 Week	After 2 weeks	After 3 weeks
	Control		0 c	0.3333 c	1 d
KIN+2,4-D	1.0+1.0	20.00	0.33 b	1.33 b	1.33 c
	1.0+1.5	20.00	1.00 a	2.00 a	2.00 b
	1.0+2.0	20.00	1.00 a	2.00 a	2.00 b
	1.0+2.5	20.00	1.00 a	2.00 a	2.00 b
	1.5+1.0	20.00	1.00 a	2.00 a	2.00 b
	1.5+1.5	20.00	1.00 a	2.00 a	2.00 b
	1.5+2.0	20.00	1.00 a	2.00 a	2.00 b
	1.5+2.5	20.00	1.00 a	2.00 a	2.00 b
	2.0+1.0	20.00	1.00 a	2.00 a	2.00 b
	2.0+1.5	20.00	1.00 a	2.00 a	3.00 a
	2.0+2.0	20.00	1.00 a	2.00 a	3.00 a
	2.0+2.5	20.00	1.00 a	2.00 a	3.00 a
	2.5+1.0	20.00	1.00 a	2.00 a	3.00 a
	2.5+1.5	20.00	1.00 a	2.00 a	3.00 a
	2.5+2.0	20.00	1.00 a	2.00 a	3.00 a
	2.5+2.5	20.00	1.00 a	2.00 a	3.00 a
	3.0+1.0	20.00	1.00 a	2.00 a	3.00 a
	3.0+1.5	20.00	1.00 a	2.00 a	3.00 a
	3.0+2.0	20.00	1.00 a	2.00 a	3.00 a
	3.0+2.5	20.00	1.00 a	2.00 a	3.00 a
		LSD (0.05)	0.21	0.29	0.21
		CV (%)	13.68	9.19	5.15

Values in the column are the means of three replicates .Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

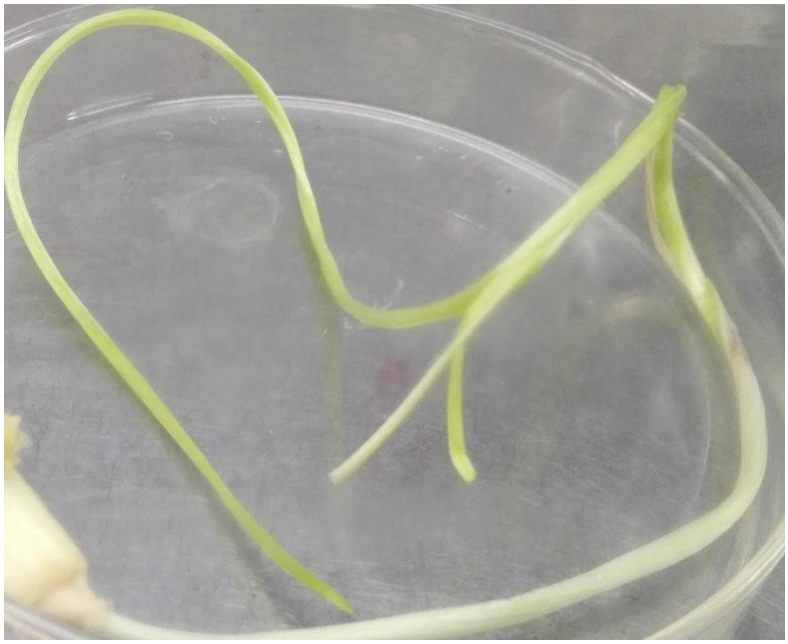


Plate 3: Effect of KIN and 2,4-D on number of leaves at 2.0mg/L KIN and 1.5 mg/L 2,4-D after 3WAI.

4.2.5 Average length of shoot (cm)

With different concentration of KIN+2,4-D, significant influence was found on the average length of shoot (cm) .The highest average length of shoot 33.7 cmwas noticed with 3.0 KIN+ 1.5mg/L2,4-D, which is statistically similar with 2.0 mg/L KIN + 2.0 mg/L 2,4-D ;2.0 mg/L KIN + 2.5 mg/L 2,4-D ; 3 mg/l KIN + 1mg/L 2,4-D mg/l; 3 KIN + 2.0 mg/L 2,4-D ; 3 mg/l KIN + 2.5 mg/L 2,4-D whereas the minimum 11.3 cm average shoot length was found in control.

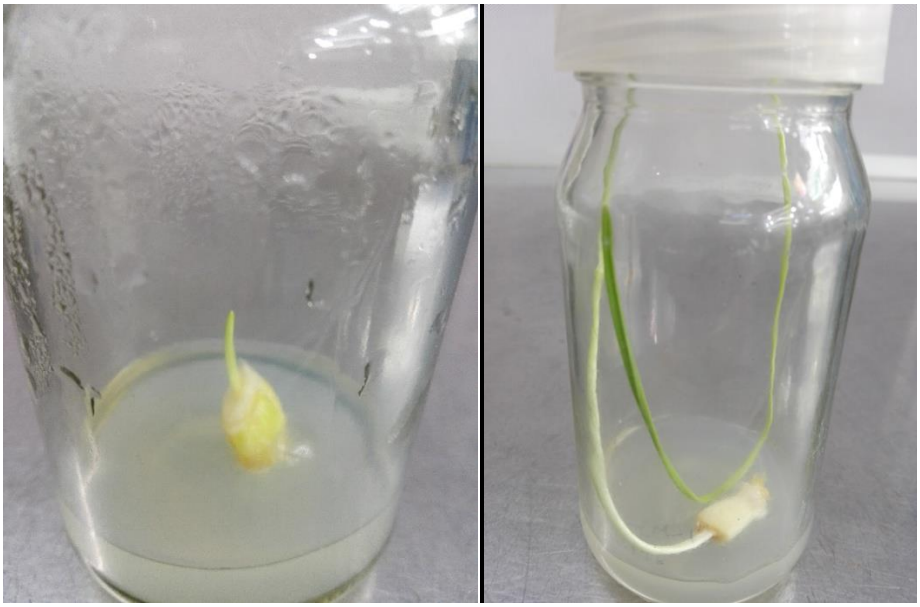
According to Mahajan, (2013) The average shoot length was maximum in the medium supplemented both with 0.5 mg/l Kinetin and 0.5 mg/l BAP (9.8±0.22 cm) while the MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l BAP gave lowest average shoot length of 2.3±0.42 cm.

The result is presented in **Table 11** in the next page.

Table 11: Effect of KIN and 2,4-D on average length of shoot

Name of the hormones	Phytohormones concentration	No. of explant	Highest Length of shoots		
			After 1 Week	After 2 weeks	After 3 weeks
	Control		4.067 i	9.1 k	11.33 g
KIN+2,4-D	1.0+1.0	20	11.3 h	19.7 j	25.53 ef
	1.0+1.5	20	12.5 g	22.7 i	24.4 f
	1.0+2.0	20	13.5 f	22.97 hi	25.33 ef
	1.0+2.5	20	14.3 e	23.7 ghi	24.87 f
	1.5+1.0	20	13.6 f	24.5 efgh	25.57 ef
	1.5+1.5	20	15.3 d	24 fghi	25.33 ef
	1.5+2.0	20	16.5 c	24.73 efg	26.07 ef
	1.5+2.5	20	16.6 c	25.6 bcde	26.67 de
	2.0+1.0	20	18.7 a	25.4 cdef	28.9 bc
	2.0+1.5	20	17.3 b	26.3 abcd	28.07 cd
	2.0+2.0	20	15.3 d	26.7 abc	29.83 b
	2.0+2.5	20	15.6 d	26.6 abc	29.8 b
	2.5+1.0	20	15.3 d	27.8 a	29.87 b
	2.5+1.5	20	15.6 d	27.03 ab	30.4 b
	2.5+2.0	20	15.7 d	27.33 a	32.57 a
	2.5+2.5	20	13.07 f	26.7 abc	32.73 a
	3.0+1.0	20	13.5 f	24.8 defg	33.47 a
	3.0+1.5	20	15.47 d	26.33 abcd	33.7 a
	3.0+2.0	20	13.53 f	24.6 efg	33.57 a
	3.0+2.5	20	12.47 g	23.77 ghi	32.73 a
		LSD (0.05)	0.5373	1.398	1.47
		CV (%)	2.29	3.49	3.17

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.



A B

C

Plate 4: Effect of 3.0 mg /L KIN+ 1.5 mg /L 2,4-D on average shoot length in garlic- (A) after 2 days of initiation ; (B) after a week of initiation; (C) after 3 weeks of initiation

4.2.6 Length of the longest shoot (cm)

Significant variation was found in the length of the longest shoot (cm) with different concentrations of KIN+2,4-D, With3.0 mg/L KIN +1.5 mg/L 2,4-D maximum length of longest shoot (31.13 cm) was noticed which was statistically dissimilar with other treatment. In control the minimum 12.17 cm shoot was observed.

The result is presented in the following **Table 12** in the next page**.**

Table 12: Effect of KIN and 2,4-D on highest length of shoot

Name of the hormones	Phytohormones concentration	Number of explant	Average Length of shoots		
			After 1 Week	After 2 weeks	After 3 weeks
	Control		3.9 g	7.067 k	12.17 l
KIN+2,4-D	1.0+1.0	20	10.8 f	18.9 j	23.63 ijk
	1.0+1.5	20	11.9 e	20.7 i	24.27 ghij
	1.0+2.0	20	12.3 e	22.3 h	22.67 k
	1.0+2.5	20	13.6 d	22.7 g	23 jk
	1.5+1.0	20	12 e	22.9 fg	23.77 hijk
	1.5+1.5	20	14.3 c	23 f	23.5 ijk
	1.5+2.0	20	15.5 b	24.4 cd	25.2 efgh
	1.5+2.5	20	15.6 b	23.6 e	24.6 fghi
	2.0+1.0	20	16.7 a	23.4 e	24.53 ghi
	2.0+1.5	20	16.3 a	24.3 d	25.77 efg
	2.0+2.0	20	14.3 c	24.7 ab	26.57 de
	2.0+2.5	20	14.6 c	24.6 bc	26.07 def
	2.5+1.0	20	14.3 c	24.8 ab	25.57 efg
	2.5+1.5	20	14.53 c	24.7 ab	27.23 cd
	2.5+2.0	20	14.63 c	24.6 bc	28.23 bc
	2.5+2.5	20	14.53 c	24.7 ab	29 b
	3.0+1.0	20	15.37 b	24.6 bc	28.87 b
	3.0+1.5	20	15.3 b	24.9 a	31.13 a
	3.0+2.0	20	15.6 b	24.8 ab	29.53 b
	3.0+2.5	20	15.3 b	24.7 ab	28.83 b
		LSD (0.05)	0.4271	0.2448	1.34
		CV (%)	4.87	5.64	3.19

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

4.2.7 Days for root induction

Application of different hormonal concentration showed variation on days to root induction. In media lacking growth regulator for induction of root maximum 7 days was required. With application of1.0 mg/L KIN+2.5 ml/L 2,4-D; 1.50 mg/L KIN+2.0 ml/L 2,4-D ; 1.5 mg/L KIN+2.5 ml/L 2,4-D ; 2.5 mg/L KIN+2.5 ml/L 2,4-D it took 7 days to induct root.

Metwally *et al.* (2014) observed that regenerated garlic shoots formed a well developed root system within seven to eight days upon their cultures on MS medium without PGRs.

The result is presented in **Table 13** in the next page. Data was recorded at the end of each week after initiation.

Table 13: Effect of KIN +2,4-D on days for root induction

Name of the Phytohormones	Concentration (mg/L)	Number of explants inoculated	Days for root induction
Control	0	20	9.67 a
KIN+2,4-D	1.0+1.0	20	4.00 ghi
	1.0+1.5	20	4.33 ghi
	1.0+2.0	20	5.67 efg
	1.0+2.5	20	7.67 bc
	1.5+1.0	20	3.67 hi
	1.5+1.5	20	4.33 ghi
	1.5+2.0	20	7.33 bcd
	1.5+2.5	20	8.33 ab
	2.0+1.0	20	4.00 hi
	2.0+1.5	20	6.00 def
	2.0+2.0	20	5.33 fgh
	2.0+2.5	20	6.00 def
	2.5+1.0	20	3.67 hi
	2.5+1.5	20	4.00 hi
	2.5+2.0	20	6.67 cdef
	2.5+2.5	20	7.00 bcde
	3.0+1.0	20	3.00 i
	3.0+1.5	20	3.00 i
	3.0+2.0	20	4.00 ghi
	3.0+2.5	20	5.33 fgh
		LSD (0.05)	1.45
		CV (%)	6.30

4.2.8 Percent of explants showing root induction

On percent of explants showing root induction there were variations among growth regulators applied. With 2.5 mg/L KIN + 1.0 mg/L 2,4-D; 2.5 mg/L KIN + 1.5 mg/L 2,4-D the highest percentage (100%) of root induction was recorded. The control treatment showed lowest percentage (87%) of root induction.

Keller and Senula (2012) Metwally *et al.* (2012) indicated that *in vitro* rooting of garlic is easily achieved on MS medium without PGR .However Tapia (1987) reported that garlic roots formed well on MS medium supplemented with KIN and IAA **.**

Kapoor *et al.* (2011) found evidence that MS medium without any growth of plant growth regulators is best for root induction in garlic. This finding showed similarity with that of Shoto *et al*. who reported that garlic callus initiates roots on hormone-free MS medium.

The result is presented in **Table 14** in the next page.

Table 14: Effect of KIN and 2,4 D on Percent of Plants Showing Root Induction.

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explant inoculated	Percent of explants showing root induction
KIN+2,4-D	1.0+1.0	20	87
	1.0+1.5	20	88
	1.0+2.0	20	93
	1.0+2.5	20	95
	1.5+1.0	20	95
	1.5+1.5	20	97
	1.5+2.0	20	98
	1.5+2.5	20	89
	2.0+1.0	20	87
	2.0+1.5	20	92
	2.0+2.0	20	94
	2.0+2.5	20	97
	2.5+1.0	20	100
	2.5+1.5	20	100
	2.5+2.0	20	98
	2.5+2.5	20	99
	3.0+1.0	20	95
	3.0+1.5	20	97
	3.0+2.0	20	99
	3.0+2.5	20	98

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

4.2.9 No. of roots per explants

The highest number of roots per explants (10.67) was found in 3.0 mg/L KIN +1.5 2,4-D at 3 WAI (Table 15), which was statistically different with other treatments. The lowest number of roots per explants was obtained in controlled treatment.

Metwally *et al.* (2014) observed *in vitro* regenerated garlic plantlets had 2-5 roots per explant.

Maximum numbers of shoots were obtained in MS medium supplemented with 1.0 mg/l of Kinetin (5.0 ± 0.55 shoots per explants).

Mahajan *et al.* (2013) found effective rooting of plantlets on MS media supplemented with 0.1mg/l NAA. He suggested that lower level of NAA (0.1 mg/l) resulted in elongated but fewer roots in *in vitro* regeneration.

The result is presented in the following **Table 15**. Data was recorded at the end of each week after initiation.

Table 15: Effect of KIN and 2,4-D on number of root per explant

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explant Inoculated	Number of Roots Per Explants		
			After 1 Week	After 2 weeks	After 3 weeks
	Control		0 h	2.667 h	5 g
KIN+2,4-D	1.0+1.0	20.00	5.00 def	6.00 defg	8.00 def
	1.0+1.5	20.00	5.33 cdef	7.33 abcd	8.67 bcde
	1.0+2.0	20.00	5.33 cdef	5.33 fg	8.00 def
	1.0+2.5	20.00	5.33 cdef	4.67 g	6.67 f
	1.5+1.0	20.00	4.33 efg	6.67 bcdef	8.00 def
	1.5+1.5	20.00	5.33 cdef	6.33 cdef	8.67 bcde
	1.5+2.0	20.00	5.00 def	6.33 cdef	8.33 cde
	1.5+2.5	20.00	3.33 g	5.33 fg	7.67 def
	2.0+1.0	20.00	6.00 abcd	8.67 a	9.67 abc
	2.0+1.5	20.00	4.33 efg	7.33 abcd	8.67 bcde
	2.0+2.0	20.00	5.67 bcde	6.67 bcdef	8.00 def
	2.0+2.5	20.00	6.67 abc	5.67 efg	7.67 def
	2.5+1.0	20.00	5.00 def	8.00 ab	9.67 abc
	2.5+1.5	20.00	4.00 fg	7.00 bcde	8.33 cde
	2.5+2.0	20.00	6.33 abcd	8.00 ab	8.00 def
	2.5+2.5	20.00	6.33 abcd	7.00 bcde	7.33 ef
	3.0+1.0	20.00	5.67 bcde	7.00 bcde	10.00 ab
	3.0+1.5	20.00	7.00 ab	8.00 ab	10.67 a
	3.0+2.0	20.00	7.33 a	7.67 abc	9.00 bcd
	3.0+2.5	20.00	6.33 abcd	7.67 abc	9.00 bcd
		LSD (0.05)	1.37	1.39	1.26
		CV (%)	15.88	12.65	9.17

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

4.2.10 Average length of roots per explants (cm)

With application of 3.0 mg/L KIN and 1.5 mg/L 2,4-D the highest average length of root 2.93 cm was found after 3 weeks of initiation and was not statistically similar with other treatments. From growth hormone free culture media the minimum root length 1cm was reported after 3 weeks of initiation.

Metwally *et al* (2014) observed highest number of roots was observed on MS media containing 0.1mg/l NAA, after 5-7 days of culturing. In addition, root length was also affected by NAA in the medium. A lower level of NAA (0.1 mg/l) resulted in elongated but few roots.

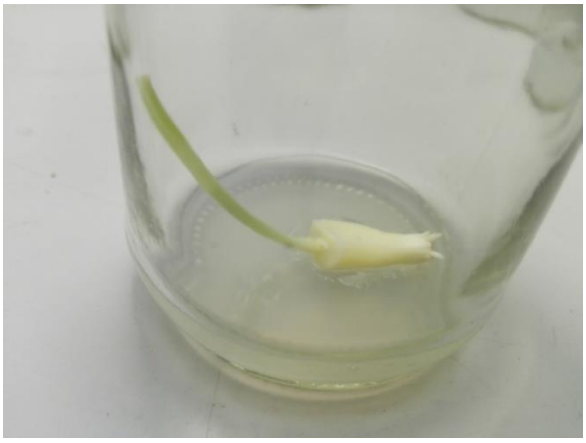
Data regarding the parameter length of longest roots in garlic is illustrated in following table. These data have been recorded after each week of initiation up to 3 weeks.

The result is presented in **Table 16** in the next page. Data was recorded at the end of each week after initiation.

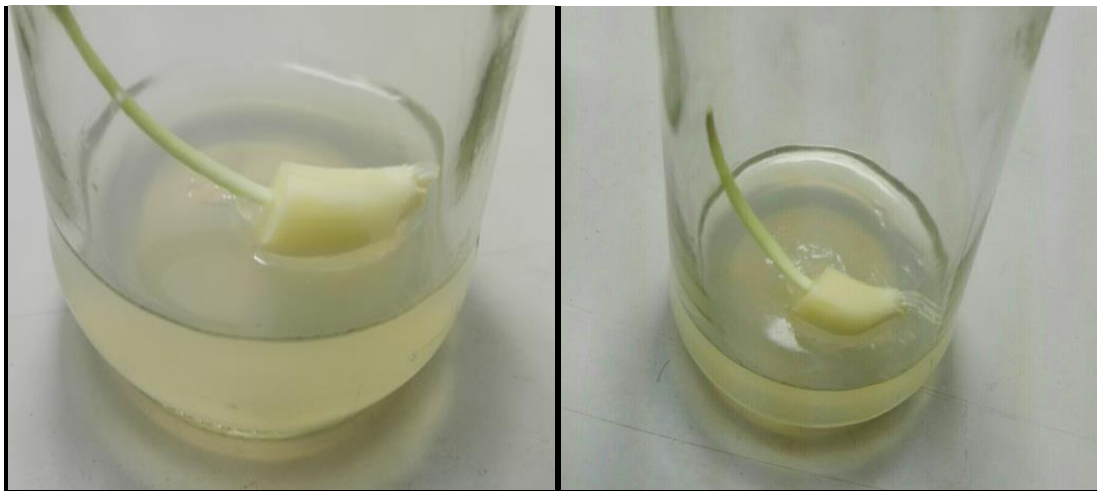
Table 16: Effect of KIN and 2,4-D on average length of root

Name of the Phytohormones	Phytohormones concentration (mg/L	Number of explant Inoculated	Highest Length of Root (cm)		
			After 1 week	After 2 weeks	After 3 weeks
	Control	20.0	0 i	0.333 f	1 g
KIN+2,4-D	1.0+1.0	20.00	1.00 h	1.50 e	2.10 def
	1.0+1.5	20.00	1.20 gh	1.80 d	1.90 f
	1.0+2.0	20.00	1.80 ab	1.87 cd	1.93 ef
	1.0+2.5	20.00	1.60 bcdef	2.10 bcd	2.10 def
	1.5+1.0	20.00	1.33 defgh	2.23 ab	2.53 abcd
	1.5+1.5	20.00	1.50 bcdefg	2.40 ab	2.43 abcde
	1.5+2.0	20.00	1.50 bcdefg	2.40 ab	2.47 abcd
	1.5+2.5	20.00	1.37 cdefg	2.30 ab	2.40 bcdef
	2.0+1.0	20.00	1.73 abc	2.47 a	2.60 abcd
	2.0+1.5	20.00	1.43 cdefg	2.20 abc	2.30 cdef
	2.0+2.0	20.00	1.43 cdefg	2.27 ab	2.30 cdef
	2.0+2.5	20.00	1.17 gh	2.20 abc	2.40 bcdef
	2.5+1.0	20.00	1.23 fgh	2.07 bcd	2.60 abcd
	2.5+1.5	20.00	1.30 efgh	2.40 ab	2.40 bcdef
	2.5+2.0	20.00	1.43 cdefg	2.27 ab	2.43 abcde
	2.5+2.5	20.00	1.67 abcde	2.23 ab	2.73 abc
	3.0+1.0	20.00	2.00 a	2.20 abc	2.83 ab
	3.0+1.5	20.00	2.00 a	2.10 bcd	2.93 a
	3.0+2.0	20.00	1.50 bcdefg	2.13 abcd	2.37 bcdef
	3.0+2.5	20.00	1.70 abcd	2.13 abcd	2.27 cdef
		LSD (0.05)	0.31	0.30	0.45
		CV (%)	13.1	8.61	11.59

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.



(A)



(B) (C)

Plate 5: Effect of 3.0 mg/L of KIN and1.5 mg/L of 2,4-D on number of roots and average length of root in garlic. Figure showing rooting (A) after 2 days, (B) 7 days and (C) 2 weeks.

4.2.11 Length of the longest root (cm)

By applying 3.0 mg/L KIN + 1.5 mg/L 2,4-D the longest root length (3.4 cm) was obtained. In control the minimum0.9 cm of length was found.

Metwally *et al.* (2014) observed *in vitro* regenerated garlic plantlets had roots per explants with 2-3 cm in length in average.

The result is presented in the following **Table 17**.

Table 17: Effect of KIN and 2,4-D on the longest length of root

Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explant Inoculated	Average Length of roots		
			After 1 week	After 2 weeks	After 3 weeks
	Control		0 H	0.33 j	0.90 h
KIN+2,4-D	1.0+1.0	20.00	1.07 G	1.80 i	2.20 g
	1.0+1.5	20.00	1.90 A	2.00 h	2.40 f
	1.0+2.0	20.00	1.30 F	2.40 fg	2.70 d
	1.0+2.5	20.00	1.60 cd	2.30 g	2.70 d
	1.5+1.0	20.00	1.00 G	2.60 de	2.70 d
	1.5+1.5	20.00	1.30 F	2.50 ef	2.60 de
	1.5+2.0	20.00	1.50 de	2.50 ef	2.70 d
	1.5+2.5	20.00	1.50 de	2.30 g	2.40 f
	2.0+1.0	20.00	1.70 bc	2.70 cd	3.30 b
	2.0+1.5	20.00	1.80 ab	2.60 de	2.70 d
	2.0+2.0	20.00	1.30 f	2.50 ef	2.70 d
	2.0+2.5	20.00	1.60 cd	2.50 ef	2.50 ef
	2.5+1.0	20.00	1.30 f	2.70 cd	2.90 c
	2.5+1.5	20.00	1.50 de	2.90 ab	3.00 c
	2.5+2.0	20.00	1.60 cd	2.80 bc	3.00 c
	2.5+2.5	20.00	1.40 ef	2.70 cd	3.20 b
	3.0+1.0	20.00	1.80 ab	3.00 a	3.30 b
	3.0+1.5	20.00	1.90 a	3.00 a	3.40 a
	3.0+2.0	20.00	1.50 de	3.00 a	3.00 c
	3.0+2.5	20.00	1.70 bc	2.80 bc	3.00 c
		LSD (0.05)	0.12	0.14	0.17
		CV (%)	4.80	3.42	3.74

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

4.2.12 Acclimatization of plantlets

The plantlets were taken for acclimatization after 21 days of culture.

In culture room 100% plantlets survived. After that the plantlets were shifted to shade house where humidity was 70% RH and sunlight was indirect.

Transparent plastic sheet were used to cover the top of the pots in the shade house. The plantlets grew for 7 days at room temperature with periodic watering (2 days interval), about 80% of the plantlets survived.

Afterwards the plantlets were planted to the soil in different pots of bigger size following potting and depotting. The plantlets were watered daily basis. Soil was mulched on its upper layer occasionally whenever necessary. In such atmosphere 86% plants were survived. Regenerated plants were found morphologically similar to the mother plant.

The result is presented in the following **Table 18**.

Table 18: Survival rate of *in vitro* regenerated plants of Garlic

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In growth chamber	20	7days	15	100
In shade house	20	7 days	12	80
In pot culture condition	20	3 days	13	86

Values in the column are the means of three replicates. Mean values, in a column with the same letters are not statistically different from each other at 5% level by DMRT.

According to Mahajan, (2013), the rooted *in vitro* plants transferred to small plastic cups resulted in 90 % survival rate after three weeks of transplantation.

Khan *et al.* (2014) found about 75% of survival in field condition after regenerating *in vitro*.

Plate 6: Acclimatization of regenerated plantlets of garlic.

Chapter V

Summary

CHAPTER V

SUMMARY

The present experiment was held in the Laboratory of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka during the July to November, 2017. The experiment was designed in a view to observe the effects of different growth regulators singly or in combinations on direct regeneration of garlic *in vitro* conditions. The basal part of clove was excised explant.

Significant variation was found on percent of explants showing shoot induction with imposing doses of KIN. The shoot induction of the explants was found to be very fast when the hormone was used. Minimum one day for shoot induction was recorded for the 3 different concentrations- 2, 2.5 and 3 mg/L of KIN and the maximum one was found 7 days in control. Also with different concentration of KIN+ 2,4-D, variations were observed on days to shoot induction. In control the maximum days were required to shoot induction .The minimum one days were recorded in case of 2.5 mg/L KIN + 2.5 mg/L 2,4-D and 3.0 mg/L KIN + (1.0-2.5) mg/L 2,4-D.

Different KIN concentrations did not have influence on the variation of number of shoots per explants. 1 shoot per explants was found at 0 to 3.5 mg /L concentration of KIN. The records were taken on weekly basis up to three weeks after initiation. Significant variation was not observed on different concentration of KIN+2,4-D on the number of shoots per explants either . One shoot was observed in every treatment applied.

On observing the number of leaves significant influence of hormones was found. The maximum three leaves were recorded with 2.0, 2.5 3.0 ,3.5mg/L KIN after 3 weeks and in case of lack of hormone showed 1.67 leaves after three weeks of initiation. But the number of leaf per explants was not much significantly different according to the various concentrations of KIN+2,4-D. The highest number of leaf per explants (3) was noticed

from 2 mg/L KIN+ (1.5-2.5) mg/L 2,4 D; 3 mg/L KIN+ (1-2.5) mg/L 2,4 D, all of these were statistically similar, whereas the lowest was found in control at 3 WAI.

The maximum average length of shoot 33.43 cm was noticed from the 3.00 mg/L KIN which was statistically similar with 2.5 mg/L KIN (32.50 cm) and statistically different from rest of other treatments; whereas the minimum (18.00 cm) in case of lack of hormone. With different concentration of KIN+2,4-D, significant influence was found on the average length of shoot (cm) .The highest average length of shoot 33.7 cm was noticed with 3.0 KIN+ 1.5mg/L 2,4-D, which is statistically similar with 2.0 mg/L KIN + (2.0-2.5) mg/L 2,4-D and 3 mg/l KIN + (1-2.5) mg/L 2,4-D. The minimum 11.3 cm average shoot length was found in control.

The maximum length of shoot 34.50 cm was noticed from the 3.00 mg/L KIN which was statistically similar with 3.5 mg/L KIN (33.70) and rest of others were found statistically different from these treatments. The minimum shoot length was 19.20 cm in control treatment. Significant variations was found on the length of longest shoot (cm) applying different concentration of KIN+2,4-D, With3.0 mg/L KIN +1.5 mg/L 2,4-D the length of longest shoot (31.13cm) was noticed which was statistically dissimilar with other treatment , in control the minimum 12.17 cm shoot was observed.

Significant variations of different concentrations of KIN and 2,4-D showed different regeneration potentiality in Garlic. With 2.5 mg/L KIN + (1.0-2.5) mg/L 2,4-D and 3.0 mg/L KIN + (1.0-2.5) mg/L 2,4-D, 100% shoot initiation was found. The lowest regeneration potentiality was seen in control treatment.

On percent of explants showing root induction there were variations among growth regulators applied. With 2.5 mg/L KIN + 1.0 mg/L 2,4-D; 2.5 mg/L KIN + 1.5 mg/L the highest percentage (100%) of root induction was recorded. The control treatment showed lowest percentage (87%). Application of different hormonal concentration showed variation on days to root induction. In media lack of growth regulator to induct root

maximum 7 days was required. With application of1.0 mg/L KIN+2.5 ml/L 2,4-D; 1.50 mg/L KIN+2.0 ml/L 2,4-D ; 1.5 mg/L KIN+2.5 ml/L 2,4-D ; 2.5 mg/L KIN+2.5 ml/L 2,4-D were it took 7 days to induct root.

By applying 3.0 mg/L KIN + 1.5 mg/L 2,4-D the maximum average root length (3.5 cm) was obtained. In control the minimum 0.9 cm of length was found.

With application of 3.0 mg/L KIN and 1.5 mg/L 2,4-D the longest root was 2.93 cm was found after 3 weeks of initiation and was not statistically similar with other treatments. From growth hormone free culture media the minimum root length 1cm was reported after 3 weeks of initiation

In culture room 100% plantlets survived. After shifting of the plantlets to shade house, about 80% of the plantlets survived. Afterwards when the plantlets were planted to the soil, 86% plants were survived. Regenerated plants were found morphologically similar to the mother plant.

The results from the experiment implies that use of combination of KIN and 2,4-D cause slightly better responses regarding parameters observed than use of KIN singly. The experiments showed high regeneration potentiality with satisfactory shooting and rooting with very short duration of time. The findings can be beneficial for regeneration of diseases free plantlets for conventional breeding and development of more precise *in vitro* regeneration protocol as well.

Chapter VI

Conclusion

CHAPTER VI
CONCLUSION

From the experiments following conclusions can be drawn:

i. An *in vitro* regeneration protocol has been developed in Garlic.

ii. Better response is observed by overall moderate higher dose (2.5-3.0 mg/L KIN) in case of shoot proliferation.

iii. Better response for rooting is observed by lower level of auxin (1-1.5 mg/L 2,4 D).

iv. Better performance for shoot and root formation in garlic was observed in the combined effect of KIN and 2,4 D doses than individual effect of KIN.

v. Multiple shooting was not observed satisfactorily by using basal part of clove as explants.

Chapter VII

Recommendations

CHAPTER VII

RECOMMENDATIONS

1. Further verifications regarding the different levels of concentrations of auxins and cytokinines should be performed to evaluate their influence on the speed of garlic proliferation.

2. Apart from the culture performed in this study, other cultures like meristem, shoot tip and root tip cultures can be performed to reflect in the best method.

3. Researches could be conducted on different genotypes of garlic to check if there is any genotypic influence or not.

4. Other influencing factors such as- ascorbic acid, activated charcoal and polyvinylpyrrolidone could be kept under considerations.

Chapter VIII

References

CHAPTER VIII

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Appendices

APPENDICES

Appendix I. Analysis of variance on days for shoot induction (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.444	0.222	2.50	0.1317
Factor A	5	74.944	14.989	168.625	0.000
Error	10	0.889	0.089		
CV (%)	12.48%				

Appendix II. Analysis of variance on number of leaves at 1 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.286	0.143	1.6364	
Factor A	6	11.810	1.968	22.5455	0.2353
Error	12	1.048	0.087		
CV (%)	8.80%				

Appendix III. Analysis of variance on number of leaves at 2 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	1.524	0.762	8.0	
Factor A	6	8.286	1.381	14.50	0.000
Error	12	1.143	0.095		
CV (%)	11.78%				

Appendix IV. Analysis of variance on number of leaves at 3 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.381	0.190	1.0000	
Factor A	6	6.571	1.095	5.7500	0.005
Error	12	2.286	0.190		
CV (%)	5.53%				

Appendix V. Analysis of variance on average length of shoot at 1 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.020	0.010	.0455	
Factor A	6	163.26	27.210	123.6818	0.000
Error	12	2.640	.220		
CV (%)	4.64%				

Appendix VI. Analysis of variance on average length of shoot at 2 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.046	0.023	0.1765	
Factor A	6	344.383	57.397	443.1400	0.000
Error	12	1.554	0.130		
CV (%)	3.85%				

Appendix VII. Analysis of variance on average length of shoot at 3 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	4.964	2.482	5.9532	0.0160
Factor A	6	491.883	81.980	196.6408	
Error	12	5.003	0.417		
CV (%)	4.25%				

Appendix VIII. Analysis of variance on length of the Longest Shoot at 1 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	1.032	0.516	3.9853	0.0471
Factor A	6	212.906	35.484	273.9597	
Error	12	1.554	0.130		
CV (%)	3.14%				

Appendix IX. Analysis of variance on length of the longest shoot at 2 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.14	0.070	0.4565	0.0000
Factor A	6	393.591	65.599	427.8163	
Error	12	1.840	0.153		
CV (%)	3.90%				

Appendix X. Analysis of variance on length of the longest shoot at 3 WAI (Effect of KIN)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.758	0.379	0.5555	
Factor A	6	495.326	82.554	120.9797	0.000
Error	12	8.189	0.682		
CV (%)	3.72%				

Appendix XI. Analysis of variance days for shoot induction (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	1.175	0.587	1.2052	0.3103
Factor A	20	214.603	10.730	22.0195	0.0000
Error	40	19.492	0.487		
CV (%)	6.98%				

Appendix XII. Analysis of variance on number of leaves per explants at 1WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.032	0.016	1.00	
Factor A	20	3.937	0.997	12.4	0.000
Error	40	0.635	0.016		
CV (%)	13.68%				

Appendix XIII. Analysis of variance on number of leaves per explants at 2WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.127	0.063	2.1053	0.1351
Factor A	20	8.889	0.444	14.7368	0.000
Error	40	1.206	0.030		
CV (%)	9.19%				

Appendix XIV. Analysis of variance on number of leaves per explants at 3WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.032	0.016	1.0000	
Factor A	20	24.889	1.244	78.4000	0.000
Error	40	0.635	0.016		
CV (%)	5.15%				

Appendix XV. Analysis of variance on number of average length of shoot 1 WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.021	0.011	0.1584	
Factor A	20	450.533	22.527	335.5427	0.0000
Error	40	2.685	0.067		
CV (%)	4.87%				

Appendix XVI. Analysis of variance on number of average length of shoot 2 WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.958	0.479	22.0767	0.0000
Factor A	20	929.193	46.460	2140.3710	0.0000
Error	40	0.868	0.022		
CV (%)	5.64%				

Appendix XVII. Analysis of variance on number of average length of shoot 3 WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	3.957	1.978	3.0007	0.0611
Factor A	20	901.557	45.078	68.3770	0.0000
Error	40	26.370	0.659		
CV (%)	3.19%				

Appendix XVIII. Analysis of variance on number of the longest length of shoot after 1 WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.675	0.338	3.1716	0.0527
Factor A	20	517.004	25.850	242.8335	0.0000
Error	40	4.258	0.106		
CV (%)	2.29%				

Appendix XIX. Analysis of variance on number of the longest length of shoot after 2 WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	5.711	2.855	3.9756	0.0266
Factor A	20	943.819	47.191	65.7045	0.0000
Error	40	28.729			
CV (%)	3.49%				

Appendix XX. Analysis of variance on number of the longest length of shoot after 3 WAI (Combined effect of KIN and 2,4-D)

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	4.086	2.043	2.5763	0.0886
Factor A	20	1522.886	76.144	96.0186	0.0000
Error	40	31.721	0.793		
CV (%)	3.17%				

Appendix XXI. Analysis of variance on Days for root induction

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	17.238	8.619	11.2074	0.0001
Factor A	20	204.857	10.243	13.3189	0.0000
Error	40	30.762	0.769		
CV (%)	6.11%				

Appendix XXII. Analysis of variance on number of roots per explants at 1WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	3.841	1.921	2.7945	0.0731
Factor A	20	145.556	7.278	10.5889	0.000
Error	40	27.492	0.687		
CV (%)	5.88%				

Appendix XXIII. Analysis of variance on number of roots per explants at 2WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	1.175	0.587	0.8343	
Factor A	20	113.270	5.663	8.0451	0.000
Error	40	28.159	0.704		
CV (%)	12.65%				

Appendix XXIV. Analysis of variance on number of roots per explants at 3WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	18.668	9.333	16.00	0.000
Factor A	20	88.00	4.40	7.54	0.000
Error	40	23.333	0.583		
CV (%)	9.17%				

Appendix XXV. Analysis of variance on the average length of root at 1 WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.667	0.333	2.9737	0.000
Factor A	20	2.336	0.467	4.1657	0.000
Error	40	1.121	0.112		
CV (%)	9.54%				

Appendix XXVI. Analysis of variance on the average length of root at 2 WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.521	0.261	1.8192	0.2119
Factor A	20	4.765	0.238	20.6618	0.0001
Error	40	1.432	0.0358		
CV (%)	9.5%				

Appendix XXVII. Analysis of variance on the average length of root at 3 WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.269	0.1345	0.9822	
Factor A	20	9.786	0.4893	6.6522	0.000
Error	40	3.007	0.0751		
CV (%)	5.6%				

Appendix XXVIII. Analysis of variance on the longest length of root at 1 WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.164	0.082	2.2812	0.1153
Factor A	20	10.414	0.521	14.5027	0.000
Error	40	1.436	0.036		
CV (%)	13.31%				

Appendix XXIX. Analysis of variance on the longest length of root at 2 WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.030	0.015	0.4623	
Factor A	20	12.548	0.627	19.6495	0.000
Error	40	1.277	0.032		
CV (%)	8.61%				

Appendix XXX . Analysis of variance on the longest length of root at 3 WAI

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	Probability
Replication	2	0.139	0.070	0.9522	
Factor A	20	9.737	0.487	6.6522	0.000
Error	40	2.927	0.073		
CV (%)	11.59%				

Appendix XXXI. Composition of Duchefa Biochemic MS (Murashige and Skoog, 1962) medium including vitamins

Components	**Concentrations (mg/L)**	**Concentrations (µM)**
Micro Elements		
$CoCl_2.6H_2O$	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		
$CaCl_2$	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		
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